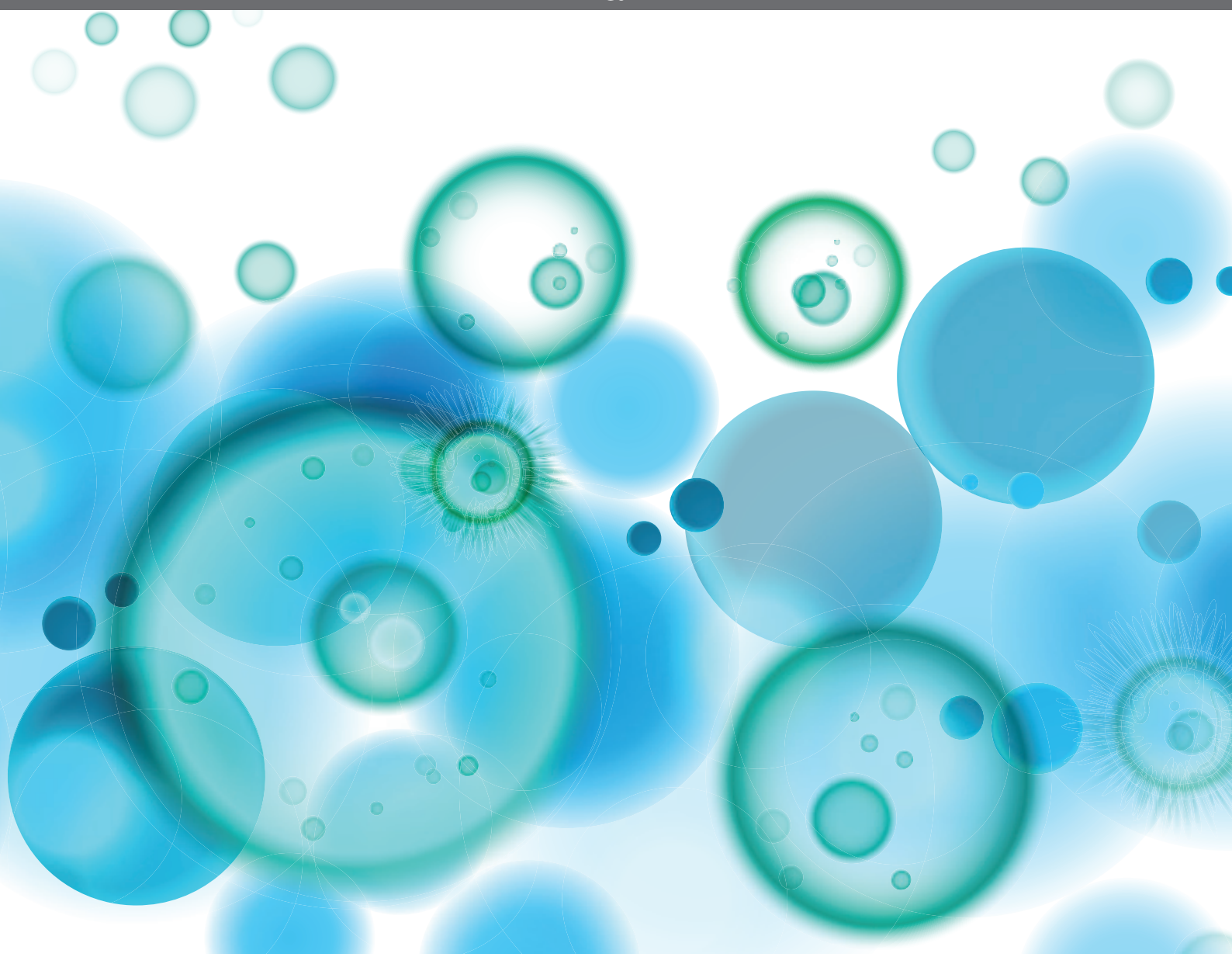
EMERGING INSIGHTS IN CONTROLLING AUTOIMMUNITY

EDITED BY: Zahava Vadasz, Elias Toubi, Dimitrios Petrou Bogdanos, Carlo Perricone and Ariel Miller

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EMERGING INSIGHTS IN CONTROLLING AUTOIMMUNITY

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S100a9 Protects Male Lupus-Prone NZBWF1 Mice From Disease Development

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Systemic lupus erythematosus (SLE) is an autoimmune disorder disproportionally affecting women. A similar sex difference exists in the murine New Zealand Black/White hybrid model (NZBWF1) of SLE with all females, but only 30-40% of males, developing disease within the first year of life. Myeloid-derived suppressor cells (MDSCs) are prominent in NZBWF1 males and while depletion of these cells in males, but not females, promotes disease development, the mechanism of suppression remains unknown. S100a9, expressed by neutrophils and MDSCs, has previously been shown to exert immunosuppressive functions in cancer and inflammation. Here we investigated if S100a9 exerts immunosuppressive functions in NZBWF1 male and female mice. *S100a9*$^{+/+}$, *S100a9*$^{+/-}$ and *S100a9*$^{-/-}$ NZBWF1 mice were followed for disease development for up to 8 months of age. Serum autoantibody levels, splenomegaly, lymphocyte activation, glomerulonephritis and proteinuria were measured longitudinally or at the time of harvest. In accordance with an immunosuppressive function of MDSCs in male mice, *S100a9*-deficient male NZBWF1 mice developed accelerated autoimmunity as indicated by increased numbers of differentiated effector B and T cells, elevated serum autoantibody levels, increased immune-complex deposition and renal inflammation, and accelerated development of proteinuria. In contrast, female mice showed either no response to S100a9-deficiency or even a slight reduction in disease symptoms. Furthermore, male, but not female, *S100a9*$^{-/-}$ NZBWF1 mice displayed an elevated type I interferon-induced gene signature, suggesting that S100a9 may dampen a pathogenic type I interferon signal in male mice. Taken together, S100a9 exerts an immunosuppressive function in male NZBWF1 mice effectively moderating lupus-like disease development *via* inhibition of type I interferon production, lymphocyte activation, autoantibody production and the development of renal disease.

Keywords: lupus, MDSC, S100A9, autoantibody, type I interferon, sex specific effects, mouse model, proteinuria

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease presenting with variable manifestations (1). Common indicators of SLE are elevated serum antinuclear autoantibodies (ANAs) and immune cell infiltration of affected organs such as the skin, heart, joints, or kidneys. The disease presents with a strong female predominance especially during child-bearing years, which has prompted much interest into the role of sex hormones (2–5). As such, numerous animal studies have shown that androgens exert a protective effect, while estrogens exacerbate disease development, the latter likely *via* direct effects on lymphocyte development and tolerance (6–12). Few studies however have looked into the mechanism driving immunoprotective effects of testosterone [reviewed in (13)]. Interestingly, immunosuppressive cells such as regulatory T and B cells, M2 macrophages and myeloid-derived suppressor cells (MDSCs) appear to be regulated by testosterone either directly during development or functionally during an immune response (14–20), suggesting that the presence of testosterone could lead to dysregulation of these cell populations and thus immune protection.

MDSCs have been ascribed a role in multiple immune disorders, but are best known for their role in cancer (21–25). The cells have a remarkable ability to suppress T cell responses by several methods including the production of reactive oxygen- and nitrogen-species (ROS, NOS), release of immunosuppressive cytokines (e.g. TGFβ, IL-10), production of indolamine 2',3'-dioxygenase (IDO), and the production of S100A8/S100A9 heterodimers (also known as calprotectin) (26–29). While most of these effector mechanisms are shared with many other cell subsets, S100A8 and S100A9 proteins are exclusively expressed by myeloid cells constituting up to 45% of all cytosolic proteins in human neutrophils (30–32). Levels of heterodimers of S100A8 and S100A9 (S100A8/A9) are often elevated during inflammation, although whether the complex exerts immune stimulatory or immunoprotective properties depends on the underlying pathology (24, 25, 33–39). For example, *S100a9*$^{-/-}$ mice showed increased susceptibility to respiratory infection and increased renal fibrosis and damage supporting an immunoprotective or immune-resolution role (33, 34). Oppositely, S100a9-deficiency promoted reduced disease in animal models of arthritis and Alzheimer's disease (24, 36). Importantly, none of these studies compared the response to S100a9-deficiency between males and females. In SLE patients (97% female cohort), serum levels of S100a9 have been found to be elevated and associated with SLEDAI scores and active bacterial infections (40). As for most studies, it remains unknown whether elevated S100a9 levels reflect proinflammatory or inflammation-resolution properties.

The (New Zealand black × New Zealand white)F1 (NZBWF1) mouse model develops a lupus-like disease characterized by hyperactive B cells, abnormal autoantibody production, glomerulonephritis, IgG immune complex (IC) deposition in the kidney glomeruli with complement fixation, and eventual renal failure resembling human SLE (41). Importantly, the model displays a similar female predominance with 100% of female NZBWF1 mice, but only 30-40% of male NZBWF1 mice, developing disease within one year. We and others previously reported an immunoprotective role for MDSC-like cells in male and young, prepubescent female NZBWF1 mice (14, 15, 42). While ROS/NOS has been identified as a mechanism of suppression in young females (14), the mechanism used by MDSCs in male NZBWF1 mice has not been identified. Based on studies suggesting a role for S100a9 in MDSC-driven immunosuppression in cancer (25), we hypothesized that MDSCs utilized S100a9 to inhibit immune activation and disease progression in NZBWF1 lupus-prone males. We report here that MDSC-like cells from *S100a9*$^{-/-}$ NZBWF1 mice failed to suppress B cell differentiation *in vitro*, suggesting that S100a9 is required for the immunosuppressive function of MDSC-like cells in these mice. Furthermore, male, but not female, *S100a9*$^{-/-}$ NZBWF1 mice showed increased disease development including elevated splenomegaly, hyperactive B and T cells, and accelerated renal disease. Interestingly, disease development in S100a9-deficient male mice was associated with elevated levels of type I interferon-stimulated gene transcripts and accumulation of IFNα-producing low density granulocytes (LDGs), suggesting that S100a9 limits type I interferon production in male lupus-prone mice hereby protecting the mice from disease development.

MATERIALS AND METHODS

Animals and Cells

S100a9-deficient C57Bl/6 mice were obtained from Dr. Thomas Vogl (Westfälische Wilhelms-Universität Münster). S100a9-deficiency was backcrossed onto the NZB/BinJ and NZW/LacZ backgrounds for at least 8 generations. Crosses between NZB.*S100a9*$^{+/-}$ and NZW.*S100a9*$^{+/-}$ mice were performed to generate *S100a9*$^{+/+}$, *S100a9*$^{+/-}$ and *S100a9*$^{-/-}$ NZBWF1 mice. For all analyses, littermates were used for comparison. Proteinuria was obtained from mice monthly starting at 3-4 months of age using dipsticks (Roche Biotech). Readings are presented on a scale from 0-4 (0, trace, 1, 2, 3, 4) corresponding with albumin levels of 0-400mg/dL urine as described by the manufacturer (Roche). When a mouse showed levels at or above 250 mg/dL (≥ 3), it was retested one week later. If consistently high, the mouse was euthanized for immediate analysis. Mice that did not develop proteinuria were euthanized at 8 months of age. For immunizations, *S100a9*$^{+/+}$, *S100a9*$^{+/-}$ and *S100a9*$^{-/-}$ male and female NZBWF1 mice were immunized i.p. with 20μg (4-Hydroxy-3-nitrophenylacetyl)$_{27}$ conjugated chicken γ-globulin (NP_{27}-CGG) in complete Freund's adjuvant (day 0). Immunized mice were bled on days -1, 7, 14, 21 and 28, and all animals were euthanized thereafter. Mice were maintained in the Biological Research Unit at the Lerner Research Institute, in accordance with Cleveland Clinic Foundation Animal Research Committee guidelines and all procedures were approved by the Institutional Animal Care and Use Committee of the Lerner Research Institute of the Cleveland Clinic Foundation and conducted in compliance with guidelines issued by the National Institutes of Health. For *in vitro* studies Gr1^{high}CD11b^{+}, Gr1^{low}CD11b^{+}, B220^{high}CD19^{+}CD138^{-} cells (*in vitro* differentiation assay) and Ly6C^{high}CD11b^{+}SSC^{low}, Ly6C^{low}CD11b^{+}SSC^{low} cells (real-time RT-PCR) were isolated by fluorescence activated cell sorting

(FACS) on a FACSAria I (BD Biosciences, San Jose, CA). All sorted cells were confirmed negative for CD3 and CD11c expression.

Flow Cytometry

Spleen single cell suspensions were prepared by gently separating single cells between the frosted areas of two microscopy slides and red blood cells were lysed using 1x ACK buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM EDTA, pH 7.3). Single cells were incubated with unlabeled anti-CD16/32 antibodies in 1x phosphate buffered saline (PBS) for 20 min. after which fluorescently labeled antibodies specific for CD3, CD4, CD8, CD11b, CD11c, CD19, CD21/35, CD23, CD25, CD38, CD44, CD62L, CD69, B220, GL-7, Gr1, IgD, IgM, SiglecH (all from eBiosciences Inc., San Diego, CA), CD138 (BD Pharmingen, San Jose, CA), were added in combinations and samples were incubated for an additional 30 minutes. Flow cytometry was performed on a LSR Fortessa (BD Biosciences) and all analyses were performed using FlowJo version 9.7.5 or later (Tree Star Inc., San Carlos, CA).

In Vitro B Cell Differentiation

$B220^+CD19^{high}CD138^-$ B cells from *S100a9*$^{+/+}$ NZBWF1 male mice were plated (2 x 10^5/well) with 5x10^4 flow sorted $Gr1^{high}CD11b^+$ cells from either S100a9$^{+/+}$ or S100a9$^{-/-}$ NZBWF1 male mice in the presence or absence of recombinant IFNαA (500 units/ml, PBL InterferonSource, Piscataway, NJ) and recombinant CD40L (10μg/ml, EBiosciences) as previously described (14). After 72 hrs, 25,000 cells per well were transferred to ELISPOT plates (EMDMillipore, Burlington, MA) pre-coated with anti-Ig antibody (Southern Biotech, Birmingham, AL) for additional 20 hrs of culture. Plates were developed using HRP-conjugated anti-IgG and anti-IgM specific secondary antibodies (KPL, Milford, MA and Southern Biotech, respectively) and AEC Substrate set (BD Biosciences). The numbers of IgM and IgG-secreting cells were detected on an ELISPOT reader (CTL Immunospot, New York, NY) and number of spots per 10^5 B cells were calculated.

Immunohistochemistry

For histological analyses, kidneys were harvested at the end of the study (8 months of age) or earlier if mice presented with severe proteinuria. One half kidney was fixed in 10% formalin for at least 24 hours, transferred to 80% ethanol and embedded in paraffin. Five μm sections were cut and stained with hematoxylin/eosin (Newcomer Supply, Middleton, WI) or Masson's Trichrome (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's guidelines. Whole sections were scored in a blinded fashion by a renal pathologist (JN) at the Cleveland Clinic. Renal score: kidneys were evaluated on a scale of 0-5 for each of the following characteristics: mesangial hypercellularity, endocapillary hypercellularity, extracapillary proliferation (crescents), immune deposits, tubular atrophy, tubular casts, tubular dilation and interstitial fibrosis and inflammation for a maximum score of 40. 0 = absent, 1 = 1-5%, 2 = 6-10%, 3 = 11-20%, 4 = 21-50%, 5 > 50%. Glomerular area was calculated by measuring the area of 5-15 individual glomeruli per section for each mouse using the Keyence BZ-X analysis software (Keyence, Osaka, Osaka, Japan) and the Keyence BZ-X700 All-in-one microscope (Keyence, Osaka, Osaka, Japan). For identification of Mac2, S100a8 and S100a9, sections were blocked in 2% fetal bovine serum (FBS) in Hank's Balanced Salt Solution (HBSS) for 30 min. Primary rat anti-mouse MAC2 (clone 125401)(BioLegend), rat anti-mouse S100a8 (clone MAB3059) or mouse S100A9 clone AF2065) (both from (R & D Systems, Minneapolis, MN) antibodies were added at 1:1500, 1:250 and 1:250 dilution, respectively, and sections were left to incubate for 1 hour in a humidified chamber at room temperature. After washing with 1x PBS, slides were applied rat-on-mouse horse radish peroxidase (HRP) polymer (#RT517) (Biocare medical, Pacheco, CA) for 20 minutes at room temperature, washed with 1x PBS, after which 3,3'-diaminobenzidine (DAB) substrate was added for 1 minute. Slides were counterstained with hematoxylin 7211 (Thermo Fisher Scientific) for 1 minute, treated with Clear Rite™ and mounted for microscopy. All light microscopy images were taken on an Eclipse 55i Nikon microscope equipped with a 12 megapixel DS-Ri1 Digital Camera.

Immunofluorescence Staining

Half kidneys and 2mm cross sections of spleens were isolated and immediately frozen in OCT™. Five μm sections were cut and sections were stained for the presence of IgG and complement C'3, or B220 and GL7, respectively. Briefly, sections were fixed with cold acetone and blocked with unlabeled anti-mouse CD16/CD32 (1:200, EBiosciences) in 10% non-immune goat serum (Invitrogen). Texas-red conjugated anti-mouse IgG (1:500, Southern Biotech), FITC-conjugated anti-mouse C'3 (1:500, ICL), FITC-conjugated anti-B220 antibodies (EBiosciences), biotinylated anti-GL7 antibodies (eBioscience), and Alexa Fluor 568–conjugated streptavidin (Invitrogen) were added as indicated for each staining combination and sections were incubated overnight at room temperature. The next day, sections were washed and mounted with 70% glycerol. Imaging was done on a Keyence BZ-X700 All-in-one microscope (Keyence, Osaka, Japan) and images were quantified using the Keyence BZ-X analysis software (Keyence, Osaka, Japan). Colocalization of IgG/C'3 in renal samples is displayed by the color yellow.

Hep2-Assay

The Hep2 assay was performed according to the manufacturer's guidelines (Bio-Rad, Hercules, CA). Positive and negative controls provided with the manufacturers' kit were added (1:64 dilution) to two wells on each slide. Serum from *S100a9*$^{+/+}$, *S100a9*$^{+/-}$ and *S100a9*$^{-/-}$ mice were diluted 1:10 in 1x phosphate buffered saline and added to remaining wells. Images were obtained on a Leica Leitz fluorescence microscope and processed using Image-Pro Plus software.

ELISA

Serum anti-chromatin and anti-histone IgG levels were determined as previously described using serum diluted 1:300 in serum diluent (43). Briefly, microtiter plates (Immulon 2HD, Thermo Fisher Scientific) were coated with purified chromatin or total histones overnight at 4°C, blocked in 5% gelatin/PBS for >2 hours, and incubated with diluted serum samples for 2 hours. Secondary horseradish peroxidase–conjugated anti-mouse IgG antibodies

(Invitrogen, Calsbad, CA) were added for 1.5 hours, and the plates were developed using 10 mg/ml 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid (ABTS) in McIlwain's buffer (0.09*M* Na2HPO4, 0.06*M* citric acid, pH 4.6) or a 3,3′,5,5′-Tetramethylbenzidine (TMB) Substrate Kit (Thermo Fisher Scientific). Anti-dsDNA IgG levels were determined using serum diluted 1:100 according to the manufacturer's guidelines (Alpha Diagnostics, Santa Monica, CA). Total serum IgG and total serum IgM levels were determined on serum diluted 1:100,000 as previously described (43). Serum S100a8/a9 heterodimer was detected using the mouse S100a8/S100a9 ELISA kit on serum diluted 2-4 times (MyBiosource, CA, USA). IgG1 specific to 4-Hydroxy-3-nitrophenylacetyl-chicken gamma globulin (NP-CGG) was determined as previously described using serum diluted 1:10,000 (42). Serum levels of soluble RAGE (sRAGE) and HMGB1 were performed on 1:10 diluted serum from 4 month old mice according to the manufacturer's instructions (MyBiosource). Serum BAFF levels were determined on 1:3-1:12 diluted serum samples according to the manufacturer's instructions (R&D Systems). All samples were run in duplicate. All ELISA plates were read on a Victor^{TM} plate reader (Perkin Elmer) at 405nm or 450 nm. When possible, concentrations were calculated based on standard curves provided with the individual kits. All analyses were done using GraphPad Prism v. 5.02 (San Diego, CA).

Real Time RT-PCR

For detection of *S100a4*, *S100a8* and *S100a9* transcript levels Gr1^{high}CD11b^{+} cells were flow sorted from male and female 4 week old NZBWF1 mice. RNA was isolated using the Micro RNeasy kit (Qiagen, Germantown, MD) and cDNA was prepared using qScript cDNA Supermix (QuantaBio, Beverly, MA). Transcript levels were determined in a SyBr-green based assay (PerfecCTa ® SYBR ® Green Fastmix ® ROX (QuantaBio)) using the following primers: *S100a4* Forward 5'-ctactgaccagggagctg c-3', *S100a4* Reverse 5'-tgttgctgtccaagttgctca-3', *S100a8* Forward 5'-gagtgtcctcagtttgtgcagaa-3', *S100a8* Reverse 5'-tgagatgccacacccacttttt-3', *S100a9* Forward 5'-gaagcacagttggcaaccttt-3', *S100a9* Reverse 5'-caggtcctccatgatgtcat-3', *β2M* Forward 5'-tcagtcgcggtcgcttc-3', *β2M* Reverse 5'-caagcaccagaaagactagggtc-3'. For detection of *Irf7*, *Isg15*, *Ifi202*, and *Ifna* total splenocytes from 8 months old *S100a9*$^{+/+}$ and *S100a9*$^{-/-}$ NZBWF1 male and female mice were isolated, RNA extracted and cDNA prepared as described above. βeta 2-microglubulin (*β2M*) was used as the internal control gene and the following gene specific primers were used: *Irf7* Forward 5'-gcgtaccctggaagcatt tc-3', *Irf7* Reverse 5'-gcacagcggaagttggtct-3', *Isg15* Forward 5'-ggtgtccgtgactaactccat-3', *Isg15* Reverse 5'- tggaaagggtaagaccgt cct-3', *Ifi202* Forward 5'-caagcctctcctggacctaa-3', *Ifi202* Reverse 5'-ctaggatgccactgctgttg-3', *Pan-Ifna* Forward 5'-cttccacaggatcactgtgta cct-3', *Pan-Ifna* Reverse 5'-ttctgctctgaccacctccc-3'.

Statistical Analyses

GraphPad Prism was used for all statistical analyses. One-way ANOVA tests were done for all group comparisons. One-way ANOVA results are provided in figure legends. The Kruskal-Wallis test followed by Dunn's multiple comparisons test was done for one-time point proteinuria scores (non-Gaussian distribution). Longitudinal proteinuria data were evaluated using a log rank test. Student's t-test with Welch's correction was done for pair-wise comparisons. $p < 0.05$ was considered statistically significant for all analyses.

RESULTS

S100a9 mRNA Expression Is Elevated in Gr1^{high}CD11b^{+} Cells From Male NZBWF1 Mice

Gr1^{high}CD11b^{+} MDSC-like cells exert immunosuppressive functions and are increased in male NZBWF1 mice as compared to female littermates (14). We have previously shown that *female* Gr1^{high}CD11b^{+} cells from 4 week old NZBWF1 mice utilized ROS/NOS production to inhibit cytokine-driven B cell differentiation, but failed to identify the mechanism used by *male* NZBWF1-derived cells (14). S100a8/a9 heterodimers are produced predominantly by neutrophils (30, 31) and have previously been associated with immunosuppression (25). We isolated Gr1^{high}CD11b^{+} and Gr1^{low}CD11b^{+} cells from 9 week old male and female NZBWF1 lupus-prone mice and determined levels of *S100a4*, *S100a8*, and *S100a9* mRNA. *S100a9* mRNA levels were significantly elevated in male Gr1^{high}CD11b^{+} cells as compared to female-derived cells ($p < 0.05$) (**Figure 1A**). A similar trend was seen in Gr1^{low}CD11b^{+} cells ($p = 0.08$). Levels of *S100a8* mRNA were also elevated in male Gr1^{high}CD11b^{+} cells as compared to female-derived cells ($p = 0.08$) (**Figure 1B**), while levels of *S100a4* mRNA were unchanged between males and females (**Figure 1C**).

To investigate whether the previously reported mechanism of suppression by male Gr1^{high}CD11b^{+} cells depended on a secreted factor, we cultured flow sorted NZBWF1 male CD19^{+}CD138^{-} B cells with supernatants from overnight cultured flow-sorted male Gr1^{high}CD11b^{+} cells in the presence of differentiation-inducing cytokines (CD40L and IFNα). B cell differentiation was effectively blocked in a dose-dependent manner (**Figure 1D**), suggesting that the inhibitory factor was indeed secreted by Gr1^{high}CD11b^{+} cells. As S100a8/S100a9 heterodimers can be secreted by neutrophils, we evaluated levels of S100a8/S100a9 heterodimers in serum, spleen, and bone marrow samples. S100a8/S100a9 heterodimers were slightly increased in male NZBWF1 mice at all sites ($p = 0.07$) (**Figure 1E**), prompting us to further investigate a possible role for S100a9 in Gr1^{high}CD11b^{+} MDSC-like cell-mediated immunosuppression during lupus-like disease development *via* the generation of S100a9-deficient NZBWF1 (see **Supplemental Figure 1**). Gr1^{high}CD11b^{+} cells were sort purified from *S100a9*$^{+/+}$ *and S100a9*$^{-/-}$ male NZBWF1 mice and tested for their ability to suppress B cell differentiation *in vitro* as previously described (14). Supporting an immunosuppressive role, S100a9-sufficient, but not S100a9-deficient, male Gr1^{high}CD11b^{+} cells inhibited B cell differentiation *in vitro* (**Figure 1F**).

Elevated Antibody Responses in Male *S100a9*$^{-/-}$ NZBWF1 Mice

To determine the *in vivo* effect of S100a9-deficiency, *S100a9*$^{+/+}$, *S100a9*$^{+/-}$ and *S100a9*$^{-/-}$ NZBWF1 mice were immunized with NP-CGG in CFA, and serum evaluated for the levels of NP-

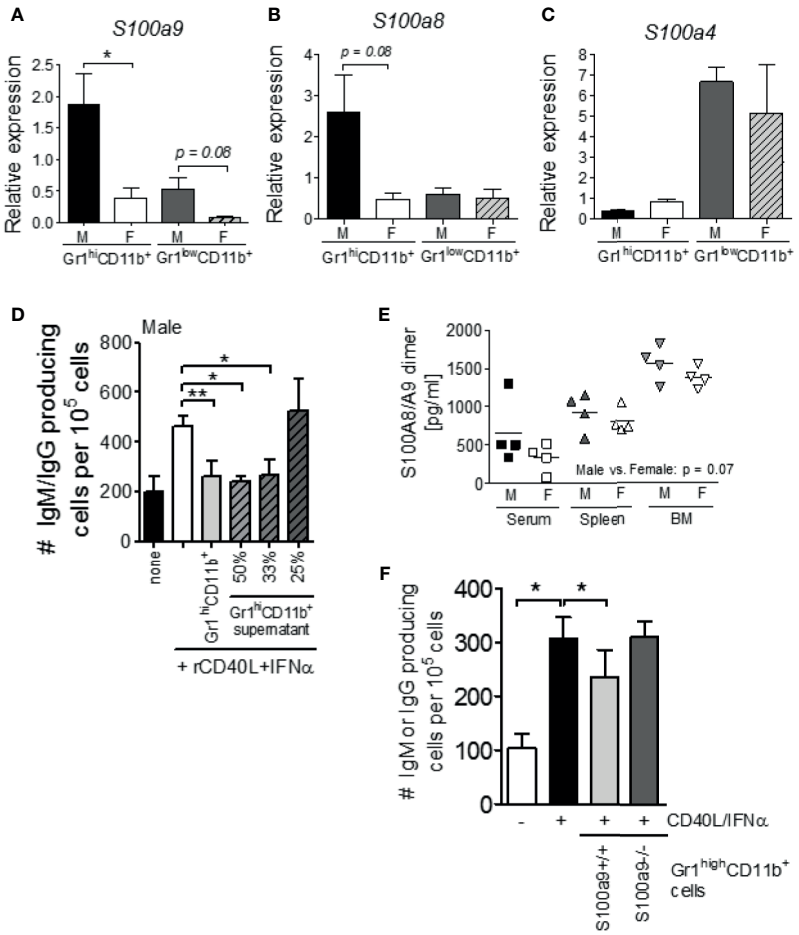


FIGURE 1 | *S100a9* expression is elevated within Gr1^{high}CD11b^{+} cells of male lupus-prone mice and regulates B cell differentiation *in vitro* and antibody production *in vivo*. Gr1^{high}CD11b^{+} and Gr1^{low}CD11b^{+} cells were isolated from 9 week old male and female NZBWF1 lupus-prone mice by flow cytometry and levels of *S100a9* **(A)**, *S100a8* **(B)**, and *S100a4* **(C)** mRNA were determined by RT-PCR analysis (n = 4). Graphs show mean +/- SEM. **(D)** Supernatants from overnight cultured Gr1^{high}CD11b^{+} cells from 4 wk old NZBWF1 male mice suppress IFNα/CD40L-driven B cell differentiation. Shown is the average (± SEM) of 4 independent experiments. **(E)** S100A8/A9 heterodimer levels in serum, spleen and bone marrow from 9 wk old NZBWF1 mice. Each symbol represents one mouse. Male vs. female: p = 0.07; two-way ANOVA. **(F)** Gr1^{high}CD11b^{+} cells from 9 wk old *S100a9*-deficienct male NZBWF1 mice fail to suppress cytokine-driven B cell differentiation *in vitro*. IgM/IgG-secreting cells were enumerated by ELISPOT and are presented as number of cells per 10^5 plated B cells. Data shown represent the mean ± SEM of 4 independent assays. *P < 0.05; **P < 0.01, Student's unpaired *t* test.

specific IgG_1 levels. Supporting an immunosuppressive role *in vivo*, Male *S100a9*$^{-/-}$ and *S100a9*$^{+/-}$ mice displayed significantly increased anti-NP IgG_1 levels as compared to male *S100a9*$^{+/+}$ mice ($p < 0.01$; **Figure 2A**). In contrast, immunization of *S100a9*$^{+/+}$, *S100a9*$^{+/-}$ and *S100a9*$^{-/-}$ female NZBWF1 mice resulted in similar levels of NP-specific antibodies between the strains (**Figure 2B**), suggesting a sex-specific effect of S100a9 in NZBWF1 lupus-prone mice. Four weeks post immunization, animals were euthanized and levels of splenic germinal center (GC) B cells and T follicular helper (Tfh) cells were analyzed. Further supporting a male-specific effect, percentages of GC B cells were significantly elevated in male, but not female, *S100a9*$^{-/-}$ NZBWF1 mice (**Figure 2C**). In contrast, the frequencies of Tfh cells were unchanged between all the groups, likely reflecting the advanced time point of evaluation (data not shown).

Male *S100a9*$^{-/-}$ NZBWF1 Mice Develop Splenomegaly And Present With Elevated Anti-Nuclear Autoantibodies

Splenomegaly is a well-established indicator of mouse lupus-like disease. Male *S100a9*$^{-/-}$ and *S100a9*$^{+/-}$ NZBWF1 mice displayed significantly larger spleens than male *S100a9*$^{+/+}$ mice ($p < 0.05$, **Figure 3A**). In contrast, no significant difference was observed between S100A9-sufficient and S100A9-deficient female mice. A similar trend was observed for total splenocyte count, although this measure only reached statistical significance when comparing *S100a9*$^{-/-}$ and *S100a9*$^{+/+}$ male mice ($p < 0.05$, **Figure 3B**). As expected, S100a9-sufficient female mice exhibited significantly larger spleens than S100a9-sufficient male mice ($p < 0.05$-0.01, **Figures 3A, B**) and *S100a9*$^{-/-}$ females ($p < 0.05$), suggesting that S100a9 might play a

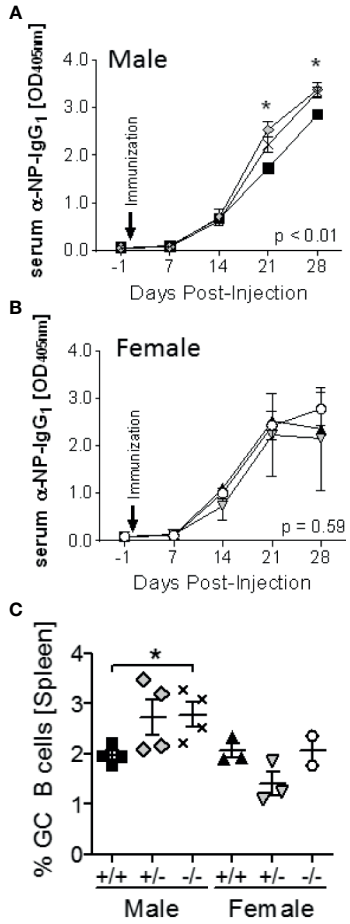


FIGURE 2 | Enhanced response to T-dependent Ag immunization in male *S100a9*$^{-/-}$ NZBWF1 mice. Male **(A)** *S100a9*$^{+/+}$ (black squares), *S100a9*$^{+/-}$ (grey diamonds), *S100a9*$^{-/-}$ (symbol X), and female **(B)** *S100a9*$^{+/+}$ (black triangles, up), *S100a9*$^{+/-}$ (grey triangles, down), *S100a9*$^{-/-}$ (open circles) NZBWF1 mice (n = 3-4) were immunized on day 0 with NP_{27}-CGG in CFA. Levels of NP-specific IgG1 antibodies were determined on days -1, 7, 14, 21 and 28. *p < 0.05, two-way ANOVA. **(C)** GC B cells were identified by flow cytometry four weeks post immunization. *p < 0.05, Student's unpaired t test with Welch's correction. One-way ANOVA: p < 0.05.

different role in disease progression in older female NZBWF1 mice.

We hypothesized that S100a9-deficient male, but not female, NZBWF1 mice would present with elevated levels of serum anti-nuclear autoantibodies; a hallmark of lupus-like disease in this animal model. Cohorts of male and female *S100a9*$^{+/+}$, *S100a9*$^{+/-}$, *and S100a9*$^{-/-}$ NZBWF1 mice were followed for up to 8 months of age or until severe renal disease was present (n = 5-11). Serum was obtained monthly and tested for total IgG, total IgM, and anti-nuclear autoantibodies by ELISA. At 4-5 months of age, serum IgM levels were significantly increased in all female mice as compared to male mice (p < 0.05), but there was no major difference between mice of similar sex (**Figure 3C**). In contrast, serum total IgG was significantly elevated in male *S100a9*$^{-/-}$ NZBWF1 mice as compared to *S100a9*$^{+/-}$ and *S100a9*$^{+/+}$ male littermates (p < 0.05, **Figure 3D**), while there was no difference between female mice. We also observed a modest elevation in serum anti-histones IgG and serum anti-dsDNA IgG levels and a significant increase in anti-chromatin IgG levels in male *S100a9*$^{-/-}$ mice (p < 0.05), with concentrations approaching those measured in the female cohorts (**Figures 3E–G**). Interestingly, there was a trend towards less serum anti-chromatin IgG in female *S100a9*$^{-/-}$ mice as compared with female *S100a9*$^{+/+}$ mice (**Figure 3F**, p = 0.07). Further analysis of the nature of serum autoantibodies from *S100a9*$^{+/+}$, *S100a9*$^{+/-}$ and *S100a9*$^{-/-}$ male and female NZBWF1 mice supported stronger and more wide-spread recognition of both cytoplasmic and nuclear antigens by serum antibodies from male *S100a9*$^{-/-}$ mice as compared with antibodies from male *S100a9*$^{+/+}$ mice, but no difference between female *S100a9*$^{+/+}$, *S100a9*$^{+/-}$ and *S100a9*$^{-/-}$ mice (**Figure 3H**).

S100a9$^{-/-}$ Male NZBWF1 Mice Develop Spontaneous B Cell Hyper-Activation

Lupus-like disease in NZBWF1 mice is characterized by hyperactive B cells, abnormal germinal center (GC) formation, and an accumulation of memory B cells and plasma cells (44, 45). Despite the difference in spleen size, there was no difference in the percentages of total $B220^+$ cells and follicular mature B cells ($CD23^{high}CD21^{low}IgM^{low}$) in the spleens of male and female S100a9-deficient and -sufficient NZBWF1 mice (**Figures 4A, B**). In alignment with previous findings (46), female *S100a9*$^{+/+}$ NZBWF1 mice displayed reduced percentages of MZ B cells ($B220^+CD21^{high}CD23^{low}$) as compared with *S100a9*$^{+/+}$ male mice, however a similar pattern was not observed in male *S100a9*$^{-/-}$ mice (**Figure 4C**). As expected, the percentage of GC B cells was increased in female *S100a9*$^{+/+}$ mice as compared to male *S100a9*$^{+/+}$ mice (p = 0.057), but there was no significant difference in the percentages of GC B cells between S100a9-sufficient and -deficient male mice (**Figure 4D**), despite a significant increase in GC area (p < 0.05) (**Figures 4G, H**). Female *S100a9*$^{-/-}$ mice showed a decreased frequency of GC B cells as compared with female *S100a9*$^{+/+}$ mice (p < 0.05) (**Figure 4D**), a pattern that was mimicked by smaller size GCs in female S100a9$^{-/-}$ mice (**Figures 4E, F**). Finally, frequencies of memory B cells and plasma cells (PCs) were elevated in male *S100a9*$^{-/-}$ mice as compared with male *S100a9*$^{+/+}$ mice (p = 0.08 and p = 0.05, respectively) and approached levels observed in *S100a9*$^{+/+}$ female mice (**Figures 4G, H**). Taken together, several B cell subsets, plasma cells and GCs from male *S100a9*$^{-/-}$ NZBWF1 mice acquired an intra-splenic balance approaching that observed in female *S100a9*$^{+/+}$ NZBWF1 mice, supporting a role for S100a9 in the regulation of B cell differentiation in male NZBWF1 mice.

S100A9-Deficiency Drives Spontaneous T Cell Activation and Differentiation in Male NZBWF1 Mice

It has previously been described that B cell hyper-activation and autoantibody production in female NZBWF1 mice depends on T

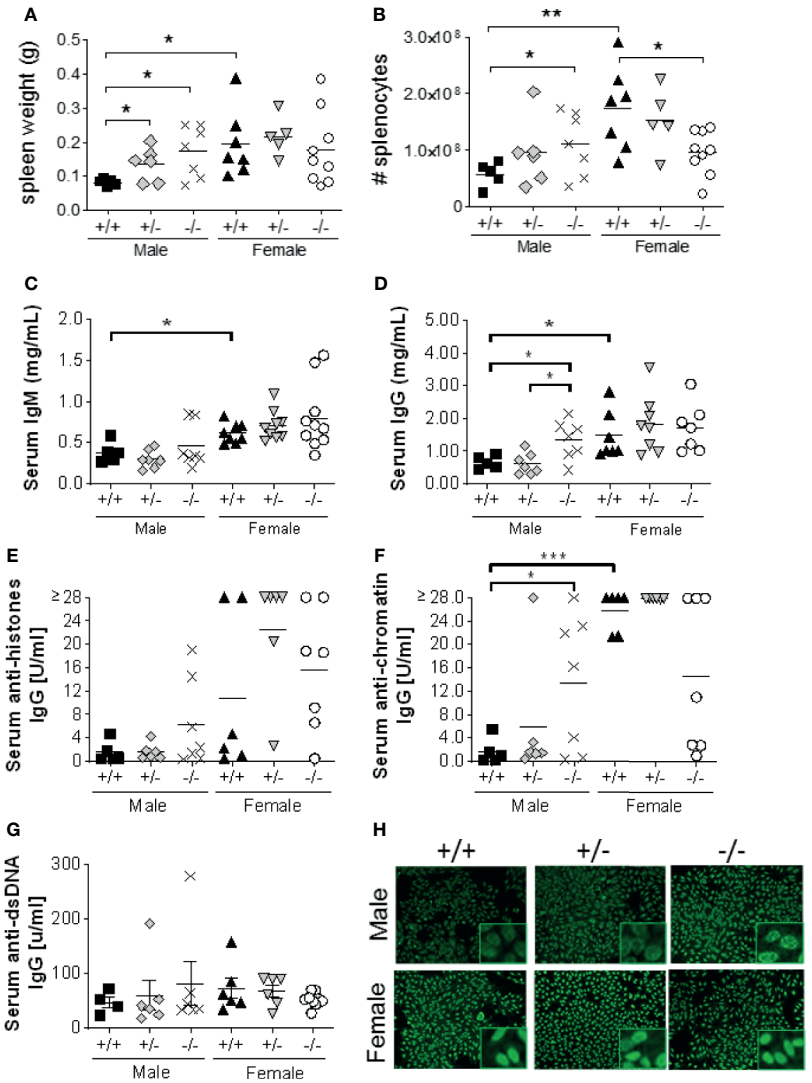


FIGURE 3 | *S100a9*$^{-/-}$ male BWF1 mice develop splenomegaly and elevated anti-nuclear autoantibodies. Spleens were harvested from eight month old BWF1 lupus-prone mice. Spleen weight **(A)** and splenocyte count **(B)** were measured (n = 5-11). Each symbol represents one individual mouse, horizontal lines represent mean values. C-G) Serum was obtained from five months old BWF1 lupus-prone mice and serum autoantibody levels were determined by ELISA: serum IgM **(C)**, serum IgG **(D)**, anti-histone IgG **(E)**, anti-chromatin IgG **(F)**, anti-dsDNA **(G)**. Each symbol represents one individual mouse (n = 5-11). **(H)** Hep2 Assay depicting nuclear and perinuclear antibody staining. Representative pictures shown from 5-11 mice/group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, Student's unpaired t test with Welch's correction. One-way ANOVA: $p = 0.21$ **(A)**, $p < 0.05$ **(B)**, $p < 0.01$ **(C)**, $p < 0.01$ **(D)**, $p < 0.01$ **(E)**, $p < 0.0001$ **(F)**, $p = 0.87$ **(G)**.

cell help (47). We determined the percentages of T helper cell subsets in S100a9-sufficient and -deficient, male and female mice. There was no statistically significant difference in the percentages of total CD4$^+$ T cells among the cohorts of male and female mice (**Figure 5A**). The expression of CD25 and CD69, was significantly elevated on female *S100a9*$^{+/+}$ CD4$^+$ cells as compared to male *S100a9*$^{+/+}$ CD4$^+$ cells ($p<0.01$), while male *S100a9*$^{-/-}$ NZBWF1 mice displayed elevated percentages of CD25$^+$CD4$^+$ cells, but not CD69$^+$CD4$^+$ cells, as compared with male *S100a9*$^{+/+}$ NZBWF1 mice ($p < 0.05$) (**Figures 5B, C**). Interestingly, we again observed a partial normalization in the levels of CD69$^+$CD4$^+$ T cells in *S100a9*$^{-/-}$ female mice as these approached levels in *S100a9*$^{+/+}$ males, but no significant change in the frequency of CD25$^+$CD4$^+$ T cells between female mice (**Figures 5B, C**).

Finally, male *S100a9*$^{+/+}$ NZBWF1 mice expressed high levels of naïve CD4$^+$ T cells (CD62L^{high}CD44^{low}) and low levels of effector/memory CD4$^+$ T cells (CD62L^{low}CD44^{high}), while the balance was shifted in both male *S100a9*$^{-/-}$ and female *S100a9*$^{+/+}$ NZBWF1 mice ($p = 0.05$ and $p < 0.01$, respectively) (**Figures 5D–F**). Thus, male *S100a9*$^{-/-}$ NZBWF1 mice presented with a hyperactive T cell phenotype similar to that

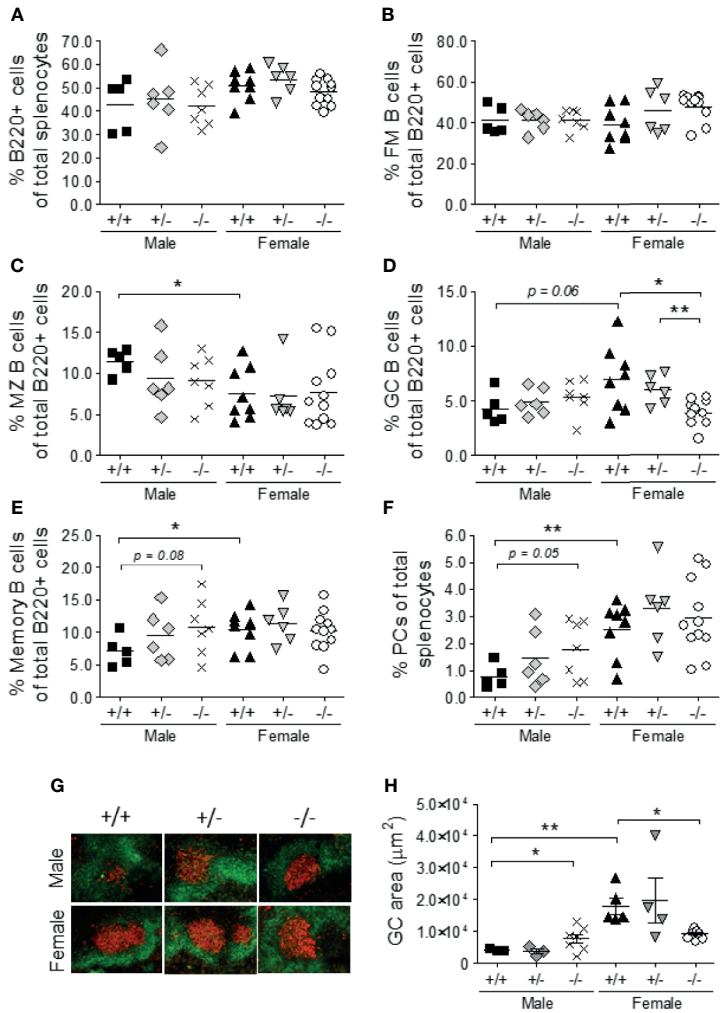


FIGURE 4 | *S100a9*-deficiency results in increased spontaneous germinal reactions in male BWF1 mice. Spleens were harvested from eight month old BWF1 lupus-prone mice and percentages of B cell subsets were determined by flow cytometry: **(A)** total B220+ cells, **(B)** Follicular mature ($B220^{+}CD23^{high}CD21^{low}IgM^{low}$) B cells, **(C)** Marginal Zone B cells ($B220^{+}CD21^{high}CD23^{low}$ IgM^{high}), **(D)** Germinal center B cells ($B220^{+}GL7^{+}CD38^{low}IgM^{low}$), **(E)** memory B cells ($B220^{+}$ $CD38^{high}GL7^{-}IgM^{-}$) and **(F)** Plasma cells ($B220^{-/low}CD138^{+}IgM^{-}IgD^{-}$). G) Frozen spleen sections were stained for B cells (B220-FITC) and germinal centers (GL-7-TexasRed). Representative pictures are shown. H) Germinal center areas from **(G)** were determined using the Keyence BZ-X analysis software. **(A–F, H)** Each symbol represents one individual mouse (n = 5-11). *p < 0.05; **p < 0.01, Student's unpaired t test with Welch's correction. One-way ANOVA: p = 0.13 **(A)**, p = 0.14 **(B)**, p = 0.33 **(C)**, p < 0.01 **(D)**, p = 0.36 **(E)**, p < 0.01 **(F)**, p < 0.01 **(H)**.

of female $S100a9^{+/+}$ NZBWF1 mice, further supporting an immunosuppressive role for S100a9 in male NZBWF1 mice.

S100a9$^{-/-}$ Male NZBWF1 Mice Develop Enhanced Glomerulonephritis and Renal Damage

Lupus-like renal disease in female NZBWF1 mice is characterized by the development of glomerulonephritis, the deposition of IgG-immune complexes (IgG-IC) in the glomeruli, complement fixation, and renal damage (44, 48). Kidneys were harvested from all mice at the end of the study and evaluated by immunostainings. Male $S100a9^{+/+}$ mice appeared relatively healthy at eight months of age with small glomeruli and minimal inflammation (**Figure 6A,** panels a, g). In contrast, $S100a9^{+/-}$ and $S100a9^{-/-}$ male mice displayed a progressive worsening as characterized by increased mesangial proliferation (black open-head arrows) and interstitial fibrosis (**Figure 6A,** panels b, c), increased collagen deposits (closed-head blue arrows), tubular atrophy, and visible proteinaceous material (black asterisk) (**Figure 6A,** panels h, i). Irrespective of their genotype, all females displayed significantly more damaged kidneys with significantly increased mesangial cell proliferation, interstitial fibrosis, and tubular casts than their male counterparts (**Figure 6A,** panels d-f and j-m).

Kidneys were further evaluated for the presence of IgG-IC deposition and complement factor C'3 fixation by

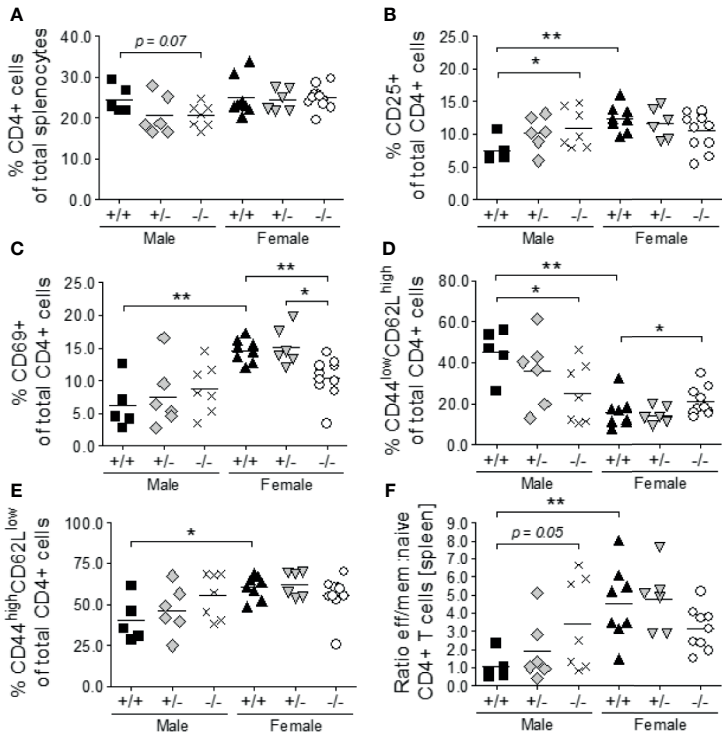


FIGURE 5 | *S100a9*-deficiency promotes the activation and differentiation of CD4+ T naïve cells to effector memory cells in *S100a9*$^{-/-}$ male BWF1 mice. Spleens were harvested from eight month old BWF1 lupus-prone mice and percentages of T lymphocytes and their subsets were determined by flow cytometry: total CD4^{+} cells **(A)**, CD25^{+} CD4^{+} cells **(B)**, CD69^{+} CD4^{+} cells **(C)**, naïve (CD44^{low}CD62L^{high}) CD4^{+} cells **(D)**, effector memory (CD44^{high}CD62L^{low}) CD4^{+} cells **(E)**, and the ratio between naïve and effector memory cells **(F)**. Each symbol represents one individual mouse. *p < 0.05; **p < 0.01, Student's unpaired *t* test with Welch's correction. One-way ANOVA: p < 0.05 **(A)**, p < 0.05 **(B)**, p < 0.0001 **(C)**, < 0.0001 **(D)**, p < 0.05 **(E)**, p < 0.01 **(F)**.

immunostaining. *S100a9*$^{-/-}$ males displayed increased levels of IgG-IC deposition (red color), increased C'3 fixation to the glomeruli (green color), and expanded areas of co-localization with C'3 (yellow color) as compared with *S100a9*$^{+/+}$ males (**Figure 6B,** panels n-p). In contrast, all female mice showed comprehensive overlapping IgG-IC deposition and complement C'3 fixation (**Figure 6B,** panels q-s, yellow color). Quantification of IgG-IC deposition showed statistically more depositions in *S100a9*$^{-/-}$ males and *S100a9*$^{+/+}$ females as compared with *S100a9*$^{+/+}$ males ($p < 0.05$ and $p < 0.01$, respectively) (**Figure 6C**). As renal damage progresses to renal failure in female NZBWF1 mice, proteinuria levels were measured monthly in all animals starting at 3-4 months of age to monitor the onset of renal failure. At 8 months of age, levels of proteinuria were significantly increased in male *S100a9*$^{-/-}$ and *S100a9*$^{+/-}$ NZBWF1 mice as compared to *S100a9*$^{+/+}$ male littermates ($p < 0.05$), while there was no statistical difference between the female mice (**Figure 6D**). The difference among male mice was not apparent at earlier time points (**Supplemental Figure 2A**). There was also no difference in the average time for disease to develop among female mice: *S100a9*$^{+/+}$: t = 6.8 months (n = 5), *S100a9*$^{+/-}$: t = 6.8 months (n = 5), and *S100a9*$^{-/-}$: t = 6.875 months (n = 8) months, indicating that S100a9-deficiency did not accelerate disease development in the females (**Supplemental Figure 2A**). Finally, renal damage was supported by an elevated renal score in female *S100a9*$^{+/+}$ mice ($p < 0.01$), and a trend towards a higher score in male *S100a9*$^{-/-}$ mice ($p = 0.08$). There was no statistically significant difference among the groups of female mice (**Figure 6E**). Given the presence of areas of fibrosis, we analyzed kidney sections for levels of pro-inflammatory Mac2^{+} macrophages. Numbers of Mac2^{+} macrophages within glomeruli increased slightly in both *S100a9*$^{+/-}$ and *S100a9*$^{-/-}$ male mice as compared with *S100a9*$^{+/+}$ male mice, albeit significant differences were not observed (**Supplemental Figures 2B, C**). It should be noted that a significant increase in tubular Mac2 expression was similarly observed in S100a9-deficient mice (males and females), although the significance of this finding remains unknown.

S100a9-Deficiency Does Not Alter Levels of HMGB1 and RAGE

While S100a9-deficiency drives disease development in male NZBWF1 mice, several of the humoral readouts tested suggested an immunostimulatory function of S100a9 in female animals. S100a9 has been shown to bind to multiple inflammatory molecules including high-mobility group box 1 (HMGB1) and receptor for advanced glycation end products (RAGE). We therefore tested levels of serum HMGB1 and soluble RAGE in male and female *S100a9*$^{+/+}$, *S100a9*$^{+/-}$ and *S100a9*$^{-/-}$ NZBWF1 mice at 4-5 months of age. Neither

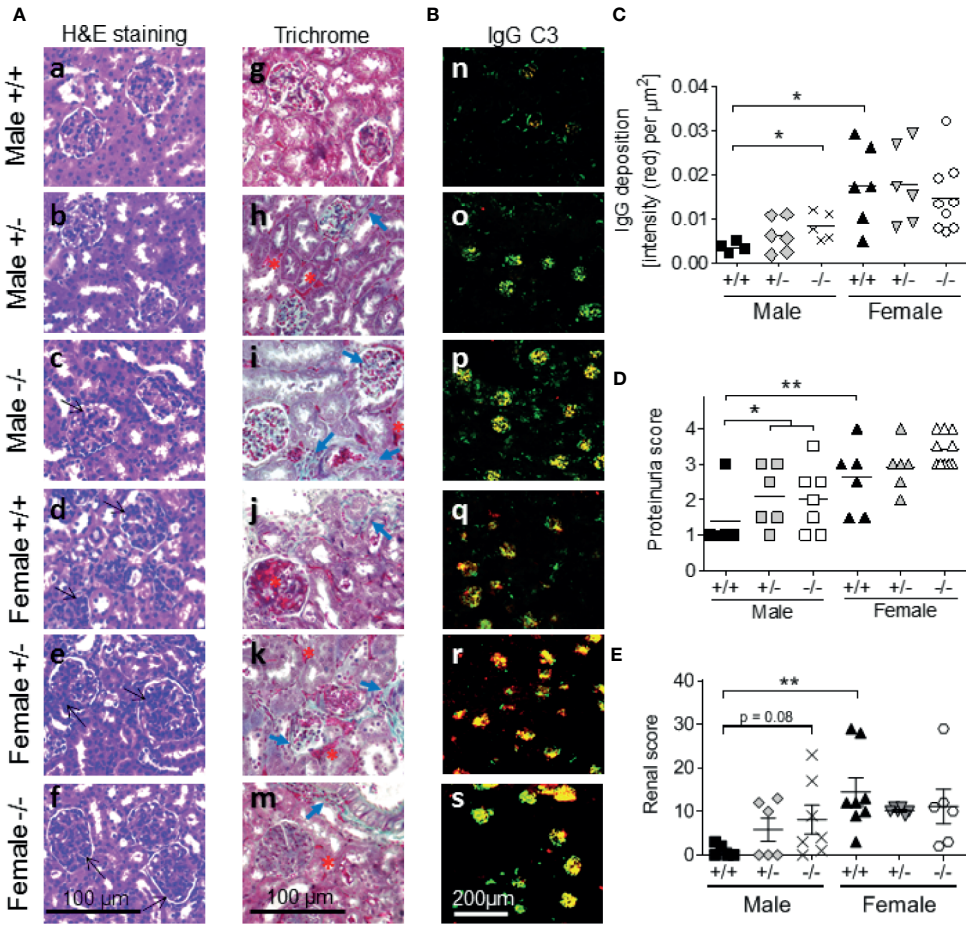


FIGURE 6 | S100a9-deficiency promotes proteinuria and renal damage in male BWF1 mice. Kidneys were harvested from eight month old BWF1 lupus-prone mice (*n* = 5-11) and stained. **(A)** H&E (a-f) and Masson's Trichrome (g-m) were performed to assess glomerulonephritis, mesangial proliferation (black open-head arrows), interstitial inflammation, tubular atrophy, collagen deposits (blue closed-head arrows), and cast formation (red asterisk). All images were taken at similar settings and magnifications. **(B, C)** Deposition of IgG-immune complexes (red) and fixation of complement factor 3 (green) were determined by immunofluorescent staining of kidneys (**B**, n-s). Colocalization of IgG and C'3 can be seen as yellow. IgG deposition was quantified **(C)**. **(D)** Proteinuria readings at the time of harvest. **(E)** Renal histology scores. Each symbol represents one individual mouse. *p < 0.05; **p < 0.01, Student's unpaired *t* test with Welch's correction **(C, D)**, Student's paired t-test **(E)**. One-way ANOVA: p < 0.01 **(C)**, p < 0.01 **(D)** and p < 0.01 **(E)**.

molecule was affected by expression of S100a9 and neither differed between male and female mice (**Supplemental Figure 3**), suggesting that these pathways were not accountable for the different outcome of S100a9-deficiency in males and females.

S100a9-Deficiency Drives Neutrophil Accumulation in Spleens of Male NZBWF1 Mice, but No Change in BAFF Levels

Further understanding the mechanism behind lupus-like disease development in S100a9-deficient male NZBWF1 mice is imperative for the identification of *de novo* molecular or cellular therapeutic targets. Previously, a population of B cell activating factor (BAFF)-producing B helper neutrophils (N_{BH}) was described located in the marginal zone in secondary lymphoid organs in response to inflammation, either induced or chronic as in SLE (49). We therefore tested levels of mature neutrophils ($Gr1^{high}CD11b^{+}SSC^{high}$) in spleens of S100a9-deficient and –sufficient, male and female NZBWF1 mice along with systemic levels of BAFF (**Figures 7A, B**). While levels of $Gr1^{high}CD11b^{+}SSC^{high}$ cells were ~ 3 fold higher in $S100a9^{-/-}$ male NZBWF1 mice than in $S100a9^{+/+}$ males and all female mice, we found no differences in levels of serum BAFF, suggesting that another mechanism may be responsible for driving lupus-like disease in male S100a9-deficient mice.

S100a9-Deficiency Results in Elevated ISG Signature in Male NZBWF1 Mice

It has been suggested that S100a9, granulocytic MDSCs ($Gr1^{high}CD11b^{+}$) and the interferon-related factor 7 (IRF7) exist in an auto-regulatory loop (50). Since IRF7 is essential for type I interferon production and type I interferons are known to drive

lupus-like disease in NZBWF1 mice (51, 52), we tested transcript levels of *Irf7* and interferon-stimulated genes *Ifi202* and *Isg15* in a small subset of S100a9-sufficient and -deficient male and female NZBWF1 mice. Interestingly, all three genes were significantly upregulated in *S100a9*$^{-/-}$ male NZBWF1 mice as compared to *S100a9*$^{+/+}$ male NZBWF1 mice ($p < 0.05$-0.01) (**Figures 8A–C**). In contrast, neither gene was upregulated in *S100a9*$^{-/-}$ female NZBWF1 mice as compared with *S100a9*$^{+/+}$ females. Both *Irf7* and *Isg15* displayed slightly increased levels in WT females as compared to WT males. In lupus, type I interferons have been shown to be produced by either SiglecH^{+} plasmacytoid dendritic cells (pDCs) or low density granulocytes (LDGs) (53, 54). To evaluate if levels of these cell subsets were affected by S100a9-deficiency and could account for the suspected increased levels of type I interferons, we analyzed splenic frequencies of both cell subsets. We found no differences in the levels of SigH^{+} pDCs between *S100a9*$^{+/+}$ and *S100a9*$^{-/-}$ mice of either sex, despite elevated levels of total pDCs in female *S100a9*$^{+/+}$ mice as compared with male *S100a9*$^{+/+}$ mice (**Figures 8D, E**). In contrast, levels of total Gr1^{+}CD11b^{+}SSC^{low} LDGs were significantly elevated in male S100a9-deficient NZBWF1 mice ($p < 0.05$), but not in female S100a9-deficient mice ($p = 0.12$) (**Figure 8F**). Further analysis of LDGs in NZBWF1 mice identified elevated *Ifna* transcripts in both Ly6C^{high} (Gr1^{low}) and Ly6C^{low} (Gr1^{high}) LDG subsets, although differences only reached statistical significance in the Ly6C^{high}CD11b^{+}SSC^{low} population (**Supplemental Figure 4**).

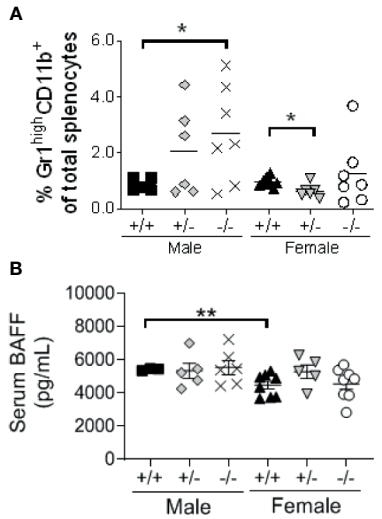


FIGURE 7 | Male S100a9-deficient BWF1 mice express elevated levels of mature neutrophils, but unaltered levels of BAFF. **(A)** Levels of Gr1^{high}CC11b^{+}SSC^{high} mature neutrophils was measured in spleens of male and female *S100a9*$^{+/+}$, *S100a9*$^{+/-}$ and *S100a9*$^{-/-}$ mice by flow cytometry. **(B)** Serum BAFF levels were measured by ELISA on serum from 4 month old mice. Each dot represent one mouse. Shown is Mean ± SEM. *$p < 0.05$; **$p < 0.01$. One-way ANOVA: $p < 0.05$ **(A)**, $p = 0.14$ **(B)**.

DISCUSSION

Lupus, along with many other autoimmune disorders, is more prevalent in females than in males. While this discrepancy has been extensively investigated from the point of view that the immune

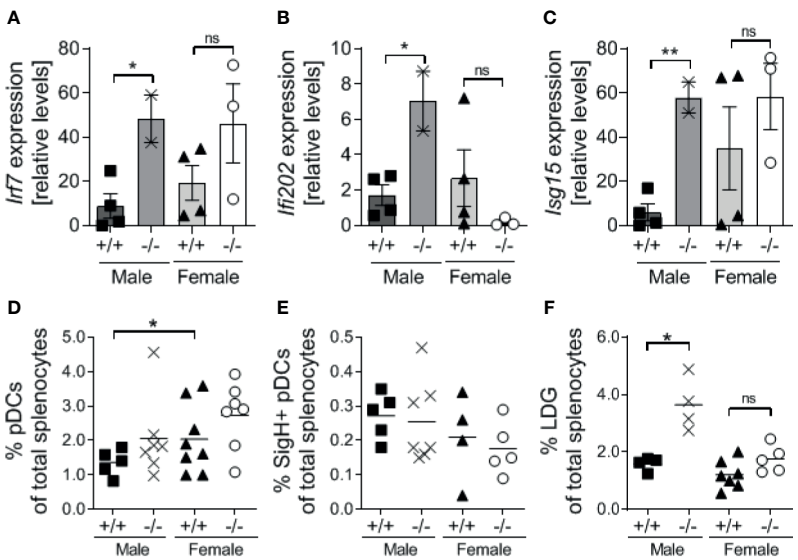


FIGURE 8 | Male S100a9-deficient BWF1 mice express elevated expression of interferon-stimulated genes and increased levels of low-density granulocytes. **(A–C)** Real-time RT-PCR was performed on total splenocytes obtained from a subset of *S100a9*$^{+/+}$ and *S100a9*$^{-/-}$ male and female BWF1 mice at the time of harvest (≥ 7 months of age) and levels of *Ifi202* **(A)**, *Irf7* **(B)**, and *Isg15* **(C)** were determined. Levels of total pDCs **(D)**, SigH^{+} pDCs **(E)**, and Low-density granulocytes (LDGs) **(F)**, were determined by flow cytometry. Each dot represent one mouse. Shown is Mean ± SEM. *$p < 0.05$; **$p < 0.01$, ns, not statistically significant. One-way ANOVA: $p < 0.05$ **(A)**, $p = 0.08$ **(B)**, $p = 0.07$ **(C)**, $p = 0.09$ **(D)**, $p = 0.67$ **(E)**, $p = 0.0001$ **(F)**.

system of females is hyperactive and therefore more likely to drive autoimmunity, only a few studies have evaluated why otherwise genetically predisposed males fail to develop disease. We previously reported that lupus-prone male NZBWF1 mice exhibited elevated levels of immunosuppressive neutrophil-like cells ($Gr1^{high}CD11b^{+}$ granulocytic MDSCs) and that depletion of these cells stimulated ANA production and IgG-IC deposition within the kidney glomeruli of lupus-prone male NZBWF1 mice (14). While we showed that the corresponding female MDSCs were immunosuppressive *via* the production of ROS/NOS only before puberty (at 4 weeks of age), male MDSCs remained immunosuppressive until at least 16 weeks of age (14). Here we show that MDSCs from male NZBWF1 mice utilize in part S100a9 as a mechanism of suppression, as cells from $S100a9^{-/-}$ male NZBWF1 mice were unable to inhibit cytokine-driven B cell differentiation *in vitro*, control antibody responses to T-dependent NP-CGG antigen, and most importantly, control lupus-like disease development. As such, B and T cell hyperactivation, ANA production, IgG-IC deposition and complement C'3 fixation in kidney glomeruli, as well as renal damage, were all significantly enhanced in S100a9-deficient NZBWF1 male mice, but largely unaffected female S100a9-deficient NZBWF1 mice.

We previously suggested that the inflammatory milieu in lupus-prone female NZBWF1 mice may drive the maturation of MDSCs into pro-inflammatory neutrophils and/or macrophages, as previously shown (55). Subsequent studies by others showed that MDSC-like cells from female NZBWF1 mice were indeed effectively eliminated in a process involving IFNα, IFNγ, IL-6 and ROS production (15, 49, 56). Oppositely, depletion of neutrophils (including MDSCs) resulted in increased IFNγ production by NK cells (57). In lupus, both IFNα and IFNγ have been suggested as drivers of disease. For example, blocking or elimination of the IFNγ receptor was previously shown to significantly reduce disease development in female NZBWF1 mice (58), showing that IFNγ contributes to disease. Interestingly, NK cell driven IFNγ production is induced by pDC-derived IFNα, and inhibition of IFNα or the IFNα/β-receptor has similarly been shown to ameliorate lupus-like disease (52, 59). Finally, MDSCs were shown to be upregulated in the absence of Irf7 in a tumor model (50). Irf7 is induced downstream of both Toll-like receptor 7 (TLR7), TLR9, and type I interferon receptor ligation, all key molecules in mouse lupus pathogenesis. Thus, it is likely that in the presence of high levels of IFNα, as seen in some lupus patients and many mouse models of lupus, Irf7 is upregulated and MDSCs downregulated. Interestingly, Irf7 binding sites have been identified in the S100a9 promoter (50), further supporting an autoregulatory loop between S100a9, MDSCs and Irf7. In our study, we found that *Irf7* mRNA was upregulated in male *S100a9*$^{-/-}$ NZBWF1 mice as compared to their S100a9-sufficient littermates. We suggest that *Irf7* mRNA is upregulated due to elevated levels of IFNα, which is supported by the concomitant upregulation of two other ISGs: *Isg15* and *Ifi202*, and the accumulation of *Ifna*-expressing LDGs in $S100a9^{-/-}$ NZBWF1 males. Thus, in this system, a lack of S100a9 interferes with the function of MDSCs, which in turn may lead to an accumulation of IFNα-producing LDGs, driving T and B cell activation, autoantibody production and end-organ inflammation and damage.

Surprisingly, female S100a9-deficient NZBWF1 mice displayed some evidence for reduced humoral disease as determined by reduced levels of activated T and B cells, reduced GC formation and diminished splenomegaly, although no differences in renal parameters were observed. In that regard, S100a9 has been found to possess both pro- and anti-inflammatory properties under different conditions. For example, some studies indicate that S100a8/a9 possess pro-inflammatory functions consistent with the complex being a TLR4 ligand (60, 61), while other evidence has suggested an immunoprotective role (33, 34, 39, 62). The two conclusions are not mutually exclusive and may depend on one of several variables such as the timing of investigation, whether inflammation is infectious or sterile, which cytokines are produced, which organ system is involved, and the sex of the animal. More studies are needed to determine if the latter is indeed involved, as the sex of the animals studied was not consistently reported in several of these studies, and no study compared the response in males and females. Both lupus patients and female NZBWF1 mice express elevated levels of several pro-inflammatory cytokines including IFNα, TNFα, IFNγ that may act to differentiate immunosuppressive MDSCs into proinflammatory MDSCs. In fact, we observed previously that $Ly6C^{high}CD11b^{+}$ MDSCs became immunostimulatory in female NZBWF1 mice as the mice aged (14). Here we observed elevated levels of $Gr1^{high}CD11b^{+}SSC^{high}$ neutrophilic cells in male S100a9-deficient mice, suggesting that these cells may be involved in the pathogenesis. Others have found that a subset of B helper neutrophils capable of producing BAFF was significantly increased in autoimmune mice and people with autoimmune diseases (49) however, we found no evidence for differential levels of BAFF in S100a9-sufficient and –deficient NBWF1 mice, and thus a potential pathogenic mechanism by mature neutrophils in S100a9-deficient male mice remains unknown. Finally, it should also be mentioned that the conformation of S100a9-contaning multimers can affect the function of the complex. As such, it has been shown that S100a8/S100a9 heterodimers can drive an inflammatory immune response *via* binding to TLR4/Mdm2 receptors, while $(S100a8/a9)_2$ tetramers are unable to bind TLR4 and thus unable to drive inflammation (63). Whether S100a9-containing complexes are different in male and female NZBWF1 mice, and how such complex formation is regulated, remains to be fully determined.

Besides transcriptional regulation as discussed above, post-translational modifications have been implicated in the function of S100a8 and/or S100a9. Post-translational modifications, including S-nitrosylation, S-gluthathionylation and phosphorylation, have been proposed to be differentially associated with pro- and anti-inflammatory functions of S100a9 (64–66). Recent studies have identified dysregulated miR-146a-5p and miR-155-5p miRNAs as drivers of phosphorylated S100a8/a9 heterodimers and the production of proinflammatory cytokines in female rheumatoid arthritis patients (67). Both of these microRNAs have also been found to be dysregulated in SLE patients (68, 69). No study has yet evaluated if this observation correlates with the presence of phosphorylated, proinflammatory S100a9 in lupus, although phosphorylated S100a9 has been found upregulated in SLE

patients (70) and to exert pro-inflammatory functions (64). Oppositely, S-nitrosylated S100a8 (and S100a9 to a lesser extend) has been proposed to inhibit leukocyte-endothelial cell interactions and leukocyte extravasation due to regulation of CD11b expression (66). Interestingly, S-nitrosylation is estrogen-dependent in endothelial cells (71), although whether estrogens have a similar effect on S100a8 and S100a9 in neutrophils remains unknown. Finally, the long-non-coding RNA Hotairm1 was found to drive the immunosuppressive function of MDSCs *via* its binding to S100a9 which functionally blocked secretion and led to an accumulation of the protein in the nucleus (72). Further studies are needed to identify the subcellular localization and post-translational modification status of S100a9 (and S100a8) in male and female lupus-prone NZBWF1 mice and SLE patients.

In summary, S100a9 is expressed and secreted by myeloid cells, including MDSCs, and exerts an immunosuppressive role in male, but not female, lupus-prone NZBWF1 mice. S100a9-expressing MDSCs functionally suppress B cell differentiation *in vitro*, B and T cell activation *in vivo*, and spontaneous autoantibody production. Interestingly, S100a9 may play a different role in female mice, as S100a9-deficiency results in slightly reduced disease patterns. Thus, as future studies evaluate the effect(s) of therapeutically targeting S100a9 or S100a8/S100a9 in various diseases, studying responses in both males and females will be of utmost importance to elucidate if such therapy will be equally effective in both sexes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Cleveland Clinic Foundation Animal Research Committee guidelines and the Institutional Animal Care and Use Committee of the Lerner Research Institute of the Cleveland Clinic Foundation.

AUTHOR CONTRIBUTIONS

LD developed the mouse model, helped with mouse harvests, ran flow cytometry, did immunization assays, and ran the NP-ELISA. AA helped with mouse harvests, ran S100a8/a9 ELISA, and performed immunofluorescence staining on kidney tissue. AD performed Hep2G assay, dsDNA ELISA, ISH imaging, and helped write the manuscript. MP did anti-chromatin, anti-histone, IgG, and IgM ELISA, helped with mouse harvests and flow cytometry, and analyzed immunostainings of the kidneys. EK performed HMGB1, RAGE, and BAFF ELISA, ISG RT-PCR, performed and analyzed all GC immunofluorescence staining on spleen tissue, did select flow analyses, and helped revise the manuscript. ND performed trichrome staining, and S100a8 and S100a9 immunohistochemistry. AW helped with ISG RT-PCR. AK did all B cell differentiation assays. NS performed S100a4/a8/a9 RT-PCR on sorted cells. LL did flow analyses. TV generated S100a9-deficient mice and helped revise the manuscript. TJ designed the study, supervised all analyses, mentored students, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.681503/full#supplementary-material

REFERENCES

1. Kotzin BL, West SG. Systemic Lupus Erythrematosus. In: R Rich, WT Shearer, BL Kotzin, JHW Schroeder, editors. *Clinical Immunology. Principles and Practice*, *2nd edn*. London, UK: Mosby International Limited (2001). pp. 60.61–60.24.
2. Gubbels Bupp MR, Jorgensen TN. Androgen-Induced Immunosuppression. *Front Immunol* (2018) 9:794. doi: 10.3389/fimmu.2018.00794
3. Gordon C, Wallace DJ, Shinada S, Kalunian KC, Forbess L, Braunstein GD, et al. Testosterone Patches in the Management of Patients with Mild/Moderate Systemic Lupus Erythematosus. *Rheumatol (Oxford)* (2008) 47:334–8. doi: 10.1093/rheumatology/kem342
4. Lahita RG, Cheng CY, Monder C, Bardin CW. Experience With 19-Nortestosterone in the Therapy of Systemic Lupus Erythematosus: Worsened Disease After Treatment With 19-Nortestosterone in Men and Lack of Improvement in Women. *J Rheumatol* (1992) 19:547–55.
5. van Vollenhoven RF. Dehydroepiandrosterone for the Treatment of Systemic Lupus Erythematosus. *Expert Opin Pharmacother* (2002) 3:23–31. doi: 10.1517/14656566.3.1.23
6. Roubinian JR, Papoian R, Talal N. Androgenic Hormones Modulate Autoantibody Responses and Improve Survival in Murine Lupus. *J Clin Invest* (1977) 59:1066–70. doi: 10.1172/JCI108729
7. >Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. Effect of Castration and Sex Hormone Treatment on Survival, Anti- Nucleic Acid Antibodies, and Glomerulonephritis in NZB/NZW F1 Mice. *J Exp Med* (1978) 147:1568–83. doi: 10.1084/jem.147.6.1568
8. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. Delayed Androgen Treatment Prolongs Survival in Murine Lupus. *J Clin Invest* (1979) 63:902–11. doi: 10.1172/JCI109390
9. Gubbels Bupp MR, Jorgensen TN, Kotzin BL. Identification of Candidate Genes That Influence Sex Hormone-Dependent Disease Phenotypes in Mouse Lupus. *Genes Immun* (2008) 9:47–56. doi: 10.1038/sj.gene.6364447

10. Bynote KK, Hackenberg JM, Korach KS, Lubahn DB, Lane PH, Gould KA. Estrogen Receptor-Alpha Deficiency Attenuates Autoimmune Disease in (NZB x NZW)F1 Mice. *Genes Immun* (2008) 9:137–52. doi: 10.1038/sj.gene.6364458
11. Dai R, McReynolds S, Leroith T, Heid B, Liang Z, Ahmed SA. Sex Differences in the Expression of Lupus-Associated miRNAs in Splenocytes From Lupus-Prone NZB/WF1 Mice. *Biol Sex Differ* (2013) 4:19. doi: 10.1186/2042-6410-4-19
12. Keisler LW, Vom Saal FS, Keisler DH, Walker SE. Hormonal Manipulation of the Prenatal Environment Alters Reproductive Morphology and Increases Longevity in Autoimmune NZB/W Mice. *Biol Reprod* (1991) 44:707–16. doi: 10.1095/biolreprod44.4.707
13. Jones JM, Jørgensen TN. Androgen-Mediated Anti-inflammatory Cellular Processes as Therapeutic Targets in Lupus. *Front Immunol* (2020) 11:1271:1271. doi: 10.3389/fimmu.2020.01271
14. Trigunaite A, Khan A, Der E, Song A, Varikuti S, Jorgensen TN. Gr1(high) CD11b(+) Cells Suppress B Cell Differentiation and Lupus-Like Disease in Lupus-Prone Male Mice. *Arthritis Rheum* (2013) 65:2392–402. doi: 10.1002/art.38048
15. Bird AK, Chang M, Barnard J, Goldman BI, Meednu N, Rangel-Moreno J, et al. Neutrophils Slow Disease Progression in Murine Lupus via Modulation of Autoreactive Germinal Centers. *J Immunol* (2017) 199:458–66. doi: 10.4049/jimmunol.1700354
16. Walecki M, Eisel F, Klug J, Baal N, Paradowska-Dogan A, Wahle E, et al. Androgen Receptor Modulates Foxp3 Expression in CD4+CD25+Foxp3+ Regulatory T-Cells. *Mol Biol Cell* (2015) 26:2845–57. doi: 10.1091/mbc.E14-08-1323
17. Frisancho-Kiss S, Coronado MJ, Frisancho JA, Lau VM, Rose NR, Klein SL, et al. Gonadectomy of Male BALB/c Mice Increases Tim-3(+) Alternatively Activated M2 Macrophages, Tim-3(+) T cells, Th2 Cells and Treg in the Heart During Acute Coxsackievirus-Induced Myocarditis. *Brain Behav Immun* (2009) 23:649–57. doi: 10.1016/j.bbi.2008.12.002
18. Lapierre P, Béland K, Martin C, Alvarez F Jr., Alvarez F. Forkhead Box p3+ Regulatory T Cell Underlies Male Resistance to Experimental Type 2 Autoimmune Hepatitis. *Hepatology* (2010) 51:1789–98. doi: 10.1002/hep.23536
19. Ma W, Zhang J, Guo L, Wang Y, Lu S, Wang Z, et al. Suppressed Androgen Receptor Expression Promotes M2 Macrophage Reprogramming Through the STAT3/SOCS3 pathway. *Excli J* (2019) 18:21–9.
20. Zhu W, Zhao Z, Chou F, Zuo L, Liu T, Yeh S, et al. Loss of the androgen Receptor Suppresses Intrarenal Calcium Oxalate Crystals Deposition via Altering Macrophage Recruitment/M2 Polarization with Change of the miR-185-5p/CSF-1 Signals. *Cell Death Dis* (2019) 10:275. doi: 10.1038/s41419-019-1358-y
21. Gabrilovich DI, Nagaraj S. Myeloid-Derived Suppressor Cells as Regulators of the Immune System. *Nat Rev Immunol* (2009) 9:162–74. doi: 10.1038/nri2506
22. Aochi S, Tsuji K, Sakaguchi M, Huh N, Tsuda T, Yamanishi K, et al. Markedly Elevated Serum Levels of Calcium-Binding S100A8/A9 Proteins in Psoriatic Arthritis are Due to Activated Monocytes/Macrophages. *J Am Acad Dermatol* (2011) 64:879–87. doi: 10.1016/j.jaad.2010.02.049
23. Boso M, Emanuele E, Minoretti P, Arra M, Politi P, Ucelli di NS, et al. Alterations of Circulating Endogenous Secretory RAGE and S100A9 Levels Indicating Dysfunction of the AGE-RAGE Axis in Autism. *Neurosci Lett* (2006) 410:169–73. doi: 10.1016/j.neulet.2006.08.092
24. Cesaro A, Anceriz N, Plante A, Page N, Tardif MR, Tessier PA. An inflammation Loop Orchestrated by S100A9 and Calprotectin is Critical for Development of Arthritis. *PLoS. One* (2012) 7:e45478. doi: 10.1371/journal.pone.0045478
25. Cheng P, Corzo CA, Luetteke N, Yu B, Nagaraj S, Bui MM, et al. Inhibition of Dendritic Cell Differentiation and Accumulation of Myeloid-Derived Suppressor Cells in Cancer is Regulated by S100A9 Protein. *J Exp Med* (2008) 205:2235–49. doi: 10.1084/jem.20080132
26. Jitschin R, Braun M, Buttner M, Dettmer-Wilde K, Bricks J, Berger J, et al. CLL-Cells Induce IDOhi CD14+HLA-DRlo Myeloid-Derived Suppressor Cells That Inhibit T-Cell Responses and Promote TRegs. *Blood* (2014) 124:750–60. doi: 10.1182/blood-2013-12-546416
27. Moline-Velazquez V, Cuervo H, Vila-Del Sol V, Ortega MC, Clemente D. & de, C.F. Myeloid-Derived Suppressor Cells Limit the Inflammation by Promoting T Lymphocyte Apoptosis in the Spinal Cord of a Murine Model of Multiple Sclerosis. *Brain Pathol* (2011) 21:678–91. doi: 10.1111/j.1750-3639.2011.00495.x
28. Ochoa AC, Zea AH, Hernandez C, Rodriguez PC. Arginase, Prostaglandins, and Myeloid-Derived Suppressor Cells in Renal Cell Carcinoma. *Clin Cancer Res* (2007) 13:721s–6s. doi: 10.1158/1078-0432.CCR-06-2197
29. Tacke RS, Lee HC, Goh C, Courtney J, Polyak SJ, Rosen HR, et al. Myeloid Suppressor Cells Induced by Hepatitis C Virus Suppress T-Cell Responses Through the Production of Reactive Oxygen Species. *Hepatology* (2012) 55:343–53. doi: 10.1002/hep.24700
30. Stroncek DF, Shankar RA, Skubitz KM. The Subcellular Distribution of Myeloid-Related Protein 8 (MRP8) and MRP14 in Human Neutrophils. *J Transl Med* (2005) 3:36. doi: 10.1186/1479-5876-3-36
31. Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, Two Abundant Ca(2+)-Binding Proteins of Neutrophils and Monocytes. *J Leukoc Biol* (1993) 53:197–204.
32. Hobbs JA, May R, Tanousis K, McNeill E, Mathies M, Gebhardt C, et al. Myeloid Cell Function in MRP-14 (S100A9) Null Mice. *Mol Cell Biol* (2003) 23:2564–76. doi: 10.1128/mcb.23.7.2564-2576.2003
33. De FK, Neill DR, Mathies M, Bangert M, McNeill E, Kadioglu A, et al. A New Protective Role for S100A9 in Regulation of Neutrophil Recruitment During Invasive Pneumococcal Pneumonia. *FASEB J* (2014) 28:3600–8. doi: 10.1096/fj.13-247460
34. Dessing MC, Tammaro A, Pulskens WP, Teske GJ, Butter LM, Claessen N, et al. The Calcium-Binding Protein Complex S100A8/A9 Has a Crucial Role in Controlling Macrophage-Mediated Renal Repair Following Ischemia/Reperfusion. *Kidney Int* (2015) 87:85–94. doi: 10.1038/ki.2014.216
35. Hiratsuka S, Watanabe A, Aburatani H, Maru Y. Tumour-Mediated Upregulation of Chemoattractants and Recruitment of Myeloid Cells Predetermines Lung Metastasis. *Nat Cell Biol* (2006) 8:1369–75. doi: 10.1038/ncb1507
36. Kim HJ, Chang KA, Ha TY, Kim J, Ha S, Shin KY, et al. S100A9 Knockout Decreases the Memory Impairment and Neuropathology in Crossbreed Mice of Tg2576 and S100A9 Knockout Mice Model. *PLoS. One* (2014) 9:e88924. doi: 10.1371/journal.pone.0088924
37. Zhang X, Wei L, Wang J, Qin Z, Wang J, Lu Y, et al. Suppression Colitis and Colitis-Associated Colon Cancer by Anti-S100a9 Antibody in Mice. *Front Immunol* (2017) 8:1774. doi: 10.3389/fimmu.2017.01774
38. Dai J, Kumbhare A, Youssef D, McCall CE, El Gazzar M. Intracellular S100A9 Promotes Myeloid-Derived Suppressor Cells During Late Sepsis. *Front Immunol* (2017) 8:1565. doi: 10.3389/fimmu.2017.01565
39. Hiroshima Y, Hsu K, Tedla N, Wong SW, Chow S, Kawaguchi N, et al. S100A8/A9 and S100A9 Reduce Acute Lung Injury. *Immunol Cell Biol* (2017) 95:461–72. doi: 10.1038/icb.2017.2
40. Soyfoo MS, Roth J, Vogl T, Pochet R, Decaux G. Phagocyte-specific S100A8/A9 Protein Levels During Disease Exacerbations and Infections in Systemic Lupus Erythematosus. *J Rheumatol* (2009) 36:2190–4. doi: 10.3899/jrheum.081302
41. Burnet M, Holmes MC. Genetic Investigations of Autoimmune Disease in Mice. *Nature* (1965) 207:368–71. doi: 10.1038/207368a0
42. Der E, Dimo J, Trigunaite A, Jones J, Jorgensen TN. Gr1+ Cells Suppress T-Dependent Antibody Responses in (NZB x NZW)F1 Male Mice Through Inhibition of T Follicular Helper Cells and Germinal Center Formation. *J Immunol* (2014) 192:1570–6. doi: 10.4049/jimmunol.1302479
43. Jorgensen TN, Roper E, Thurman JM, Marrack P, Kotzin BL. Type I interferon Signaling is Involved in the Spontaneous Development of Lupus-Like Disease in B6.Nba2 and (B6.Nba2 x NZW)F(1) Mice. *Genes Immun* (2007) 8:653–62. doi: 10.1038/sj.gene.6364430
44. Dubois EL, Horowitz RE, Demopoulos HB, Teplitz R. NZB/NZW Mice as a Model of Systemic Lupus Erythematosus. *JAMA* (1966) 195:285–9.
45. Cohen PL, Ziff M. Abnormal Polyclonal B Cell Activation in NZB/NZW F1 Mice. *J Immunol* (1977) 119:1534–7.
46. Atencio S, Amano H, Izui S, Kotzin BL. Separation of the New Zealand Black Genetic Contribution to Lupus from New Zealand Black Determined Expansions of Marginal Zone B and B1a Cells. *J Immunol* (2004) 172:4159–66. doi: 10.4049/jimmunol.172.7.4159
47. Mathis KW, Taylor EB, Ryan MJ. Anti-CD3 Antibody Therapy Attenuates the Progression of Hypertension in Female Mice with Systemic Lupus

Erythematosus. *Pharmacol Res* (2017) 120:252–7. doi: 10.1016/j.phrs.2017.04.005
48. Mellors RC. Autoimmune and Immunoproliferative Diseases of NZB/Bl mice and hybrids. *Int Rev Exp Pathol* (1966) 5:217–52.
49. Puga I, Cols M, Barra CM, He B, Cassis L, Gentile M, et al. B Cell-Helper Neutrophils Stimulate the Diversification and Production of Immunoglobulin in the Marginal Zone of the Spleen. *Nat Immunol* (2011) 13:170–80. doi: 10.1038/ni.2194
50. Yang Q, Li X, Chen H, Cao Y, Xiao Q, He Y, et al. IRF7 Regulates the Development of Granulocytic Myeloid-Derived Suppressor Cells Through S100A9 Transrepression in Cancer. *Oncogene* (2017) 36:2969–80. doi: 10.1038/onc.2016.448
51. Liu Z, Bethunaickan R, Huang W, Lodhi U, Solano I, Madaio MP, et al. Interferon-Alpha Accelerates Murine Systemic Lupus Erythematosus in a T Cell-Dependent Manner. *Arthritis Rheum* (2011) 63:219–29. doi: 10.1002/art.30087
52. Mathian A, Weinberg A, Gallegos M, Banchereau J, Koutouzov S. IFN-Alpha Induces Early Lethal Lupus in Preautoimmune (New Zealand Black x New Zealand White) F1 but Not in BALB/c mice. *J Immunol* (2005) 174:2499–506. doi: 10.4049/jimmunol.174.5.2499
53. Denny MF, Yalavarthi S, Zhao W, Thacker SG, Anderson M, Sandy AR, et al. A Distinct Subset of Proinflammatory Neutrophils Isolated From Patients With Systemic Lupus Erythematosus Induces Vascular Damage and Synthesizes Type I IFNs. *J Immunol* (2010) 184:3284–97. doi: 10.4049/jimmunol.0902199
54. Blasius A, Vermi W, Krug A, Facchetti F, Cella M, Colonna M. A Cell-Surface Molecule Selectively Expressed on Murine Natural Interferon-Producing Cells That Blocks Secretion of Interferon-Alpha. *Blood* (2004) 103:4201–6. doi: 10.1182/blood-2003-09-3108
55. Trigunaite A, Dimo J, Jorgensen TN. Suppressive Effects of Androgens on the Immune System. *Cell Immunol* (2015) 294:87–94. doi: 10.1016/j.cellimm.2015.02.004
56. Vlachou K, Fanouriakis A, Glymenaki M, Ioannou M, Mastorodemos V, Verginis P, et al. Defects Within the Myeloid Derived Supressor Cells (MDSCx) Compartment May Facilitate Aberrant Immune Responses in Systemic Lupus Erythematosus (SLE). *Ann Rheum Dis* (2012) 71(suppl. 1): A84–5. doi: 10.1136/annrheumdis-2011-201238.29
57. Huang X, Li J, Dorta-Estremera S, Di Domizio J, Anthony SM, Watowich SS, et al. Neutrophils Regulate Humoral Autoimmunity by Restricting Interferon-γ Production via the Generation of Reactive Oxygen Species. *Cell Rep* (2015) 12:1120–32. doi: 10.1016/j.celrep.2015.07.021
58. Haas C, Ryffel B, Le Hir M. IFN-Gamma Receptor Deletion Prevents Autoantibody Production and Glomerulonephritis in Lupus-Prone (NZB x NZW)F1 Mice. *J Immunol* (1998) 160:3713–8.
59. Santiago-Raber ML, Baccala R, Haraldsson KM, Choubey D, Stewart TA, Kono DH, et al. Type-I Interferon Receptor Deficiency Reduces Lupus-like Disease in NZB Mice. *J Exp Med* (2003) 197(6):777–88. doi: 10.1084/jem.20021996
60. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, et al. Mrp8 and Mrp14 are Endogenous Activators of Toll-Like Receptor 4, Promoting Lethal, Endotoxin-Induced Shock. *Nat Med* (2007) 13:1042–9. doi: 10.1038/nm1638
61. Wache C, Klein M, Ostergaard C, Angele B, Häcker H, Pfister HW, et al. Myeloid-Related Protein 14 Promotes Inflammation and Injury in Meningitis. *J Infect Dis* (2015) 212:247–57. doi: 10.1093/infdis/jiv028
62. Huang M, Wu R, Chen L, Peng Q, Li S, Zhang Y, et al. S100A9 Regulates MDSCs-Mediated Immune Suppression via the RAGE and TLR4 Signaling Pathways in Colorectal Carcinoma. *Front Immunol* (2019) 10:2243. doi: 10.3389/fimmu.2019.02243
63. Vogl T, Stratis A, Wixler V, Völler T, Thurainayagam S, Jorch SK, et al. Autoinhibitory Regulation of S100A8/S100A9 Alarmin Activity Locally Restricts Sterile Inflammation. *J Clin Invest* (2018) 128:1852–66. doi: 10.1172/JCI89867
64. Schenten V, Plançon S, Jung N, Hann J, Bueb JL, Bréchard S, et al. Secretion of the Phosphorylated Form of S100A9 from Neutrophils Is Essential for the Proinflammatory Functions of Extracellular S100A8/A9. *Front Immunol* (2018) 9:447. doi: 10.3389/fimmu.2018.00447
65. Lim SY, Raftery MJ, Goyette J, Geczy CL. S-Glutathionylation Regulates Inflammatory Activities of S100A9. *J Biol Chem* (2010) 285:14377–88. doi: 10.1074/jbc.M109.075242
66. Lim SY, Raftery M, Cai H, Hsu K, Yan WX, Hseih HL, et al. S-Nitrosylated S100A8: Novel Anti-Inflammatory Properties. *J Immunol* (2008) 181:5627–36. doi: 10.4049/jimmunol.181.8.5627
67. Jung N, Schenten V, Bueb JL, Tolle F, Bréchard S. miRNAs Regulate Cytokine Secretion Induced by Phosphorylated S100A8/A9 in Neutrophils. *Int J Mol Sci* (2019) 20:5699. doi: 10.3390/ijms20225699
68. Chen JQ, Papp G, Póliska S, Szabó K, Tarr T, Bálint BL, et al. MicroRNA Expression profiles Identify Disease-Specific Alterations in Systemic Lupus Erythematosus and Primary Sjögren's Syndrome. *PloS One* (2017) 12: e0174585. doi: 10.1371/journal.pone.0174585
69. Su YJ, Lin IC, Wang L, Lu CH, Huang YL, Kuo HC. Next Generation Sequencing Identifies miRNA-Based Biomarker Panel for Lupus Nephritis. *Oncotarget* (2018) 9:27911–9. doi: 10.18632/oncotarget.25575
70. Pavon EJ, Garcia-Rodriguez S, Zumaquero E, Perandres-Lopez R, Rosal-Vela A, Lario A, et al. Increased Expression and Phosphorylation of the Two S100A9 Isoforms in Mononuclear Cells From Patients With Systemic Lupus Erythematosus: A Proteomic Signature for Circulating Low-Density Granulocytes. *J Proteomics* (2012) 75:1778–91. doi: 10.1016/j.jprot.2011.12.020
71. Chakrabarti S, Lekontseva O, Peters A, Davidge ST. 17beta-Estradiol Induces Protein S-Nitrosylation in the Endothelium. *Cardiovasc Res* (2010) 85:796–805. doi: 10.1093/cvr/cvp368
72. Alkhateeb T, Bah I, Kumbhare A, Youssef D, Yao ZQ, McCall CE, et al. Long Non-Coding RNA Hotairm1 Promotes S100A9 Support of MDSC Expansion during Sepsis. *J Clin Cell Immunol* (2020) 11(6):600.

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REVIEW
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Role of Th22 Cells in the Pathogenesis of Autoimmune Diseases

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Upon antigenic stimulation, naïve CD4$^+$T cells differentiate into different subsets and secrete various cytokines to exert biological effects. Th22 cells, a newly identified CD4$^+$T cell subset, are distinct from the Th1, Th2 and Th17 subsets. Th22 cells secrete certain cytokines such as IL-22, IL-13 and TNF-α, but not others, such as IL-17, IL-4, or interferon-γ (IFN-γ), and they express chemokine receptors CCR4, CCR6 and CCR10. Th22 cells were initially found to play a role in skin inflammatory diseases, but recent studies have demonstrated their involvement in the development of various autoimmune diseases. Here, we review research advances in the origin, characteristics and effector mechanisms of Th22 cells, with an emphasis on the role of Th22 cells and their main effector cytokine IL-22 in the pathogenesis of autoimmune diseases. The findings presented here may facilitate the development of new therapeutic strategies for targeting these diseases.

Keywords: Th22 cells, IL-22, rheumatoid arthritis, systemic lupus erythematosus, psoriasis, immune thrombocytopenia, autoimmune diseases, autoimmune hepatitis

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INTRODUCTION

CD4$^+$ T cells mainly include Th1, Th2, Th17, Th9, Th22, follicular helper T (Tfh) cells and regulatory T (Treg) cells (1–4), and they were initially divided into two categories: Th1 and Th2 cells (5). This classification does not explain the pathogenesis of certain diseases; for example, neutralizing or eliminating IL-12 or Th1 cells and the cytokine IFN-γ could not prevent or alleviate experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA); however, the discovery of Th17 cells explains this paradox. Th17 cells do not express IL-4 or IFN-γ but secrete IL-17 at a high level (6, 7). Previous studies have shown that both Th17 and Th1 cells secrete IL-22 (8, 9). A recent report identified that a group of special T cell subsets that could secrete IL-22 and IL-13 but not IL-17 and IFN-γ (10). Mouse T lymphoma cells stimulated with IL-9 expressed a cytokine very similar to the secondary structure of IL-10, and it is named interleukin-10-related T cell-derived inducible factor (IL-TIF) (11). Further studies identified a new sequence from human T cells that encodes 23% amino acids homologous to IL-10 and is 87% similar to IL-TIF, and it is named IL-22 (12). The expression of IL-22 has been suggested to be associated with

Th1 (8, 13) and Th17 cells (9, 14). However, Th22 cell clones in the induced environment of Th1, Th2, Th17 and Treg cells do not secrete their characteristic cytokines but can secrete IL-22, which demonstrates that Th22 cells represent an independent T cell subset. It is named Th22 because of its high level of IL-22 secretion (15). Plank et al. performed a whole gene chip analysis and found differences between Th22 and Th17 cells, and they determined that Th22 cells were an independent cell lineage (16).

Th22 cells can differentiate into Th1 or Th2 cells under appropriate conditions. In an *in vivo* IFN-γ-rich inflammatory microenvironment or *in vitro* Th1-promoting conditions, Th22 cells have obvious plasticity. In an *in vitro* Th2 culture environment, Th22 cells showed increased secretion of IL-13 (16). The conditions for the transformation between Th22 and Th17 cells have not been clarified (17). Since other cells, such as Th17 cells, also secrete IL-22, the identification of Th22 cells is particularly important. Mousset et al. suggested that when using flow cytometry to identify Th22 cells, Th22 cells can be identified by integrating cell surface markers ($CCR4^+$, $CCR6^+$ and $CCR10^+$), combining cell transcription factors (AhR^+ and/or $STAT3^+$) and/or cytokine staining ($IL\text{-}22^+$), and requiring $IFN\gamma^-$, $IL\text{-}4^-$, $IL\text{-}9^-$, $IL\text{-}10^-$ and $IL\text{-}17^-$ (18). Here, we review the characteristics and effector mechanisms of Th22 cells, with an emphasis on the role of Th22 cells and their main effector cytokine IL-22 in the pathogenesis of autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), psoriasis,multiple sclerosis (MS), immune thrombocytopenia (ITP), immunoglobulin A nephropathy (IgAN), autoimmune hepatitis (AIH), autoimmune thyroid diseases (AITD), myasthenia gravis (MG), and systemic sclerosis (SSc).

CHARACTERISTICS OF TH22 CELLS

Th22 cells secrete IL-22, IL-13, IL-26, TNF-α and granzyme B but not IL-17, IFN-γ or IL-4 (16). Activation of the transcription factor aryl hydrocarbon receptor (AhR) significantly promotes the differentiation of naïve $CD4^+$T cells into Th22 cells (19). The expression of the signature cytokines and transcription factors of Th1, Th2 and Th17 cells was absent in Th22 cells (10, 15, 19). Th22 cells are abundant in human skin and play important roles in epidermal wound healing (15). These cells are tissue homing $CD4^+$T cells and exhibit anti-inflammatory, antibacterial and antiviral activities. Emerging evidence has shown the critical roles of Th22 cells in allergies, autoimmune diseases, intestinal diseases and tumors (20–22).

IL-22 is the main effector molecule of Th22 cells and belongs to the IL-10 family, and it acts by binding to IL-22 receptors which are composed of IL-22R1 (main high affinity chain) and IL-10R2 (helper receptor chain) subunits (23). IL-22R1 is mainly expressed in nonhematopoietic organs, such as the skin, lung, intestine and pancreas, but not in lymphoid organs including thymus, bone marrow and spleen (24). IL-22 has little effects on immune cells, and mainly acts on mucosal barriers of skin, respiratory system and digestive system. IL-22 binding protein (IL-22BP) or IL-22RA2, the soluble receptor of IL-22, is expressed in various tissues around the lung, colon and breast. IL-22BP blocks the binding of IL-22 to IL-22R and is the receptor antagonist of IL-22 (25). IL-22 can also be produced by lymphoid cells, including Th17 cells, innate lymphocytes (ILCs), dermis γδ T cells, Tc17 cells, nonlymphatic macrophages (26), neutrophils (27–29) and even fibroblasts of RA patients (28, 30–33). IL-22 in the intestine is mainly produced by ILCs. ILCs migrate from mucosal associated lymphoid tissue to the lamina propria after stimulation (12). γδT cells are the primary source of IL-22 in the skin, intestinal tract, lung, reproductive tract and other epithelial tissues, where they respond quickly to exotic pathogens at an early stage (34, 35). Moreover, IL-22 can induce the production of different antimicrobial proteins by keratinocytes, intestinal epithelial cells,bronchial epithelial cells and other different parts of the human body (36). In addition, IL-22 can directly act on endothelial cells through the activation of STAT3 and ERK pathways,stimulate endothelial cell proliferation and migration, and stimulate angiogenesis (37). IL-22 can also act on colonic subepithelial myofibroblasts (SEMFs) to produce inflammatory mediators such as chemokines, inflammatory cytokines and matrix metalloproteinases (MMPs) (38).

The binding of IL-22 to IL-22R activates downstream signal transduction (**Figure 1**) (39). IL-22 transmits phosphorylation signals downstream through Janus kinase (JAK) 1 and tyrosine kinase (TYK) 2, including the mitogen-activated protein kinase (MAPK) pathway (p38 kinase, ERK1/2, MEK1/2 and JNK), STAT3, STAT1 and STAT5 (39–41). Similar to other members of the IL-10 family, IL-22 phosphorylates STAT3 mainly at Ser727 and Tyr705 (23, 42, 43). IL-22 is also unique in that it induces the phosphorylation of serine residues in addition to tyrosine residues, and activates the ERK1/2 pathway (40), whereas IL-10 induces the phosphorylation of tyrosine residues on STAT3. This difference may be caused by the difference inreceptor R1. The binding of SRC homologous phosphatase 2 (Shp2) to Tyr-251 phosphorylation residues and the activation of IL-22R1 on Tyr-301 are necessary for the activation of STAT3 (39, 44). Moreover, the phosphorylation of STAT3 is essential for IL-22 to exert its effects on epithelial cells (45). IL-22 also induces the expression of suppressor cytokine signaling1 and 3 (SOCS1/3), which in turn inhibits the activity of STAT3 (39). Activation of STAT1 and/or STAT5 can be observed in tumors (46–48). IL-22 can also activate the PI3K-Akt-mTOR pathway, which is essential for the migration of hepatocytes and colonic epithelial cells (39, 49). The activation of Akt is important for the proliferation of human fibroblast-like synoviocytes and epidermal keratinocytes (50). IL-22 induces osteoclast formation in RA by p38MAPK/NF-κB and JAK2/STAT3 signaling (51).

DIFFERENTIATION OF TH22 CELLS

The differentiation of Th22 cells is regulated by many factors, which are different from other $CD4^+$T cell subsets including Th1, Th17 and Tfh cells (**Figures 2A, B**). Both IL-6 and TNF-α can induce the differentiation of Th22 cells, IL-6 alone drives the differentiation of naïve $CD4^+$T cells into Th22 cells.

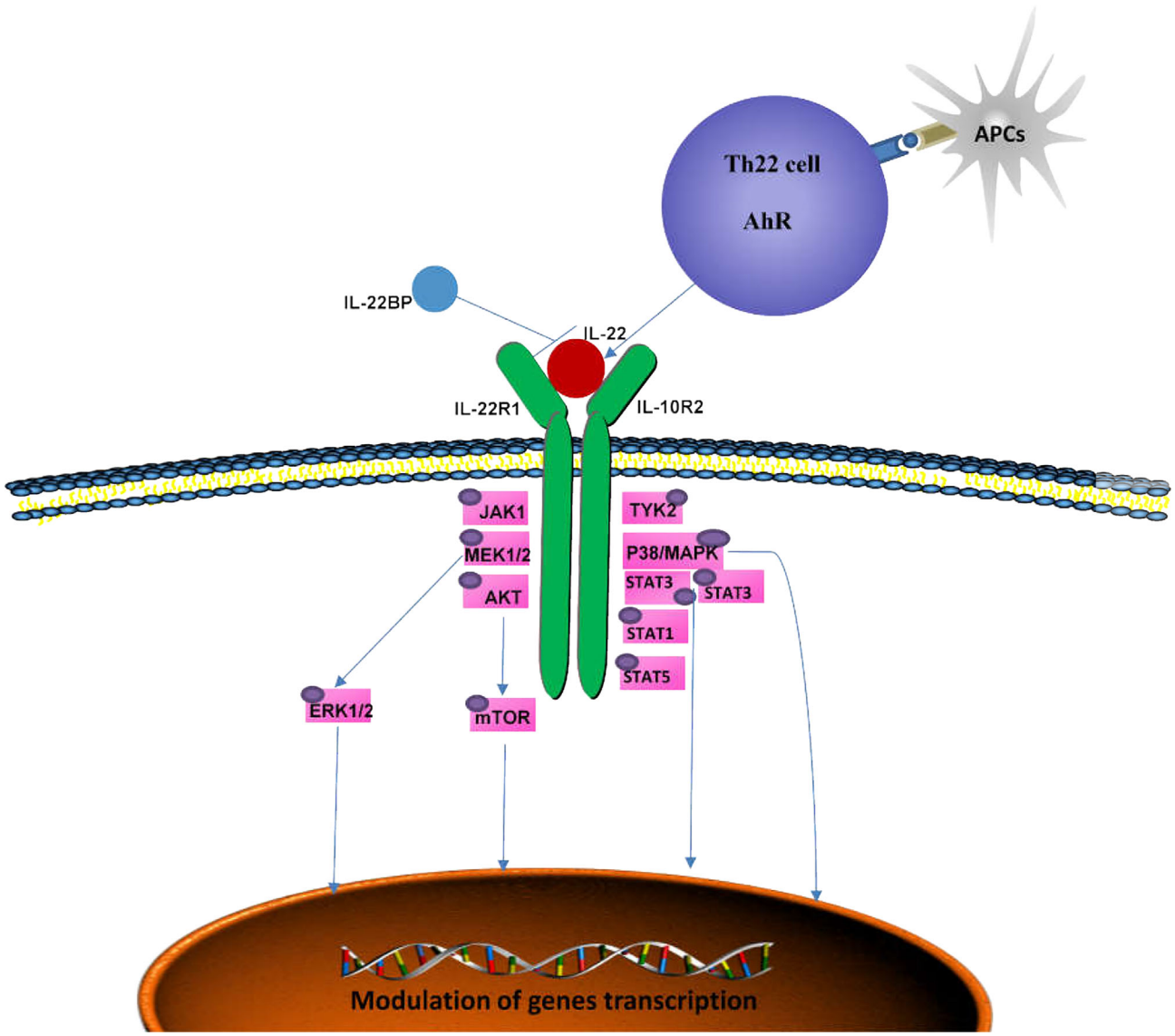


FIGURE 1 | Signaling pathways mediated by IL-22. The IL-22 receptor complex consists of IL-22R1 and IL-10R2. By binding to its receptor, IL-22 activates TYK2 and JAK1and triggers multiple intracellular pathways, including p38MAPK, MEK1/2, ERK1/2 and AKT, by phosphorylating serine and tyrosine in STAT3, STAT1 and STAT5, which can ultimately lead to immune homeostasis. IL-22BP is a soluble receptor antagonist that specifically neutralizes the activity of IL-22. AhR, aryl hydrocarbon receptor; APCs, antigen-presenting cells; JAK1, Jenus kinase 1; TYK2, tyrosine kinase 2; MAPK, mitogen-activated protein kinase; IL-22BP, IL-22 binding protein.

TNF-α further promotes the differentiation of Th22 cells induced by IL-6, while a high dose of TGF-β inhibits the differentiation of Th22 cells (10). In the presence of anti-IL-4/IFN-γ, IL-17A secretion still occurs when combined with IL-6, IL-23, IL-1β and 6-formylindolo (3, 2-b) carbazole (FICZ). The TGF-βR inhibitor (galunisertib) effectively inhibits the production of IL-17Abutdoes not affect the secretion of IL-22. Under this culture condition, the levels of IL-13 and granzyme B also increase significantly. Under this culture condition,the levels of granzyme B and IL-13 also increased significantly (16). IL-21 alone or in combination with IL-1β or IL-23 can induce Th22 cell differentiation and IL-22 expression (52). Many reports show that differentiated Th22 cells can be identified by integrating cell surface markers ($CCR4^+$, $CCR6^+$ and $CCR10^+$) or its correlated intracellular cytokines including $IL\text{-}22^+$, $IL\text{-}17^-$ and $IFN\text{-}\gamma^-$ **(Figure 3)** (15–19).

In addition,plasmacytoid-like dendritic cells (pDCs) could induce Th22 cells more strongly than conventional dendritic cells (cDCs), and both of them release high concentrations of TNF-α and IL-6 after activation. Blocking TNF-α and IL-6 inhibited 70% of Th22 cells in culture, which indicates that DCs may promote Th22 differentiation in both direct and indirect ways (10). Foreign antigens, such as microorganisms, can also activate DCs. After stimulation by the serotypes of

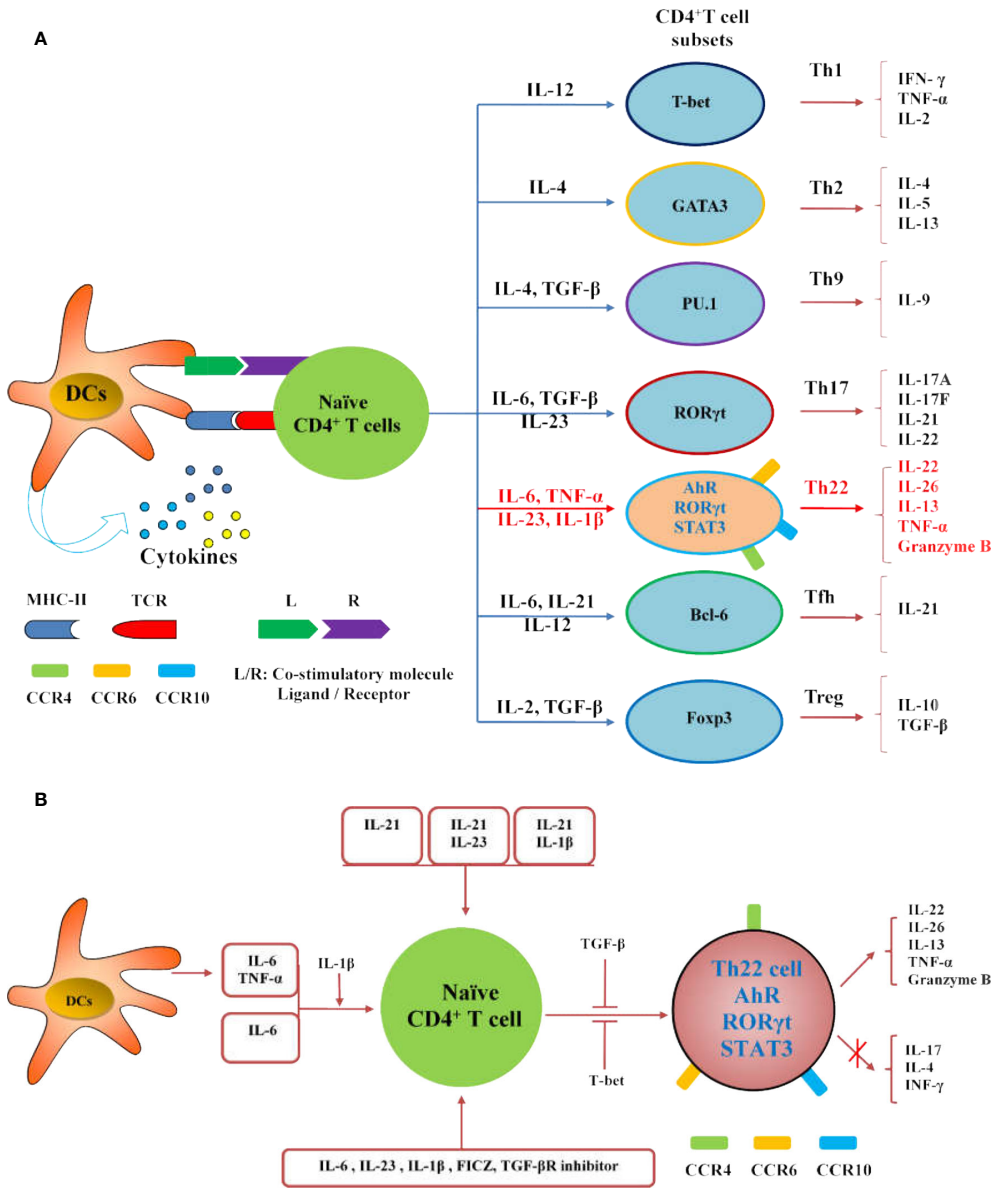


FIGURE 2 | Regulation of Th22 cells differentiation. **(A)** The diagram illustrates the differentiation of Th cell subsets from naïve $CD4^+$ T cells. **(B)** IL-21, IL-21 combined with IL-23 or IL-1β can induce the differentiation and IL-22 expression of Th22 cells; IL-6 and TNF-α secreted by DCs or external IL-6 or IL-6 and TNF- α can promote the differentiation of naïve $CD4^+$T cells into Th22 cells, and IL-1β promotes the differentiation; IL-6, IL-23, IL-1β, FICZ and TGF-βR inhibitor can promote the differentiation of naïve $CD4^+$T cells into Th22 cells. Both TGF-β and T-bet inhibit the expression of IL-22 in Th22 cells.

A. actinomycetemcomitans, the levels of AhR and IL-22 in T lymphocytes were increased,and the levels of TNF-α and IL-6 in DCs were significantly increased. This type of actinomycete can trigger the polarization of Th22 cells, which may be an important part of the subgingival biofilm (53). Langerhans cells (LCs) from the human epidermis and dermis can also induce naïve $CD4^+$T cells and peripheral blood T cells to differentiate into Th22 cells, and the effect of LCs on the epidermis is stronger than that of dermal DCs (54). Endogenous TLR4 ligands stimulate keratinocytes to secrete IL-23 which activates DCs and induces the differentiation of Th22 cells and IL-22 production (55). Activated B cells and initial T cells significantly inhibit the expression of RORγt and the production of IL-17, but significantly increase the differentiation of Th22 cells and the production of IL-22 by cultured Th17 cells *in vitro*. Further *in vivo* experiments showed that MRL/lpr lupus mice treated with activated B cells exhibited reduced levels of anti-dsDNA antibody and urinary protein. Meanwhile, Th17 cell differentiation was inhibited and Th22 cell differentiation was enhanced in these mice (56).

AhR is an important transcription factor in the differentiation of Th22 cells. However, the expression of IL-22 in naïve $CD4^+$T

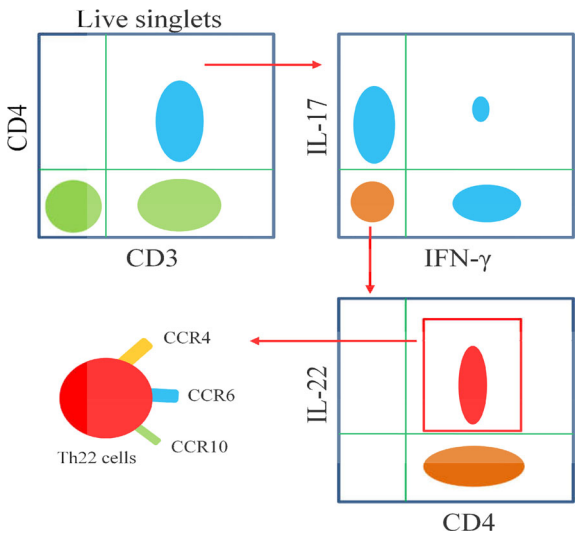


FIGURE 3 | Schematic diagram illustrates the flow cytometric analysis of Th22 cells. Th22 cells are identified as $CD3^+CD4^+IL\text{-}17^-IFN\text{-}\gamma^-IL\text{-}22^+$, as well as high expressions of CCR4,CCR6 and CCR10.

cells was reduced in the absence of RORc (γt) but increased in Tbx21-deficient cells. Therefore, RORγt is considered a positive regulator of Th22, while T-bet is considered a negative regulator (16). Runt-related transcription factor 3 (RUNX3) is a runt-domain family transcription factor. The number of Th22 cells decreased significantly with the inhibition of RUNX3 (57). Recent studies have shown that miR-31 mimic transfection can increase the levels of AhR and IL-22 and promote the differentiation of Th22 cells in coronary heart disease. Overexpression of miR-31 promotes the differentiation of Th22 cells by inhibiting the BTB domain and CNC homolog-2 (Bach2) pathway (58). Medroxyprogesterone acetate (MPA) can also enhance the Th22 cell response and decrease the expression of Th1 and Th17 cell signature genes by activating AhR signaling, thus affecting susceptibility to inflammatory diseases and infectious diseases (59).

TH22 CELLS IN AUTOIMMUNE DISEASES

Recent studies have shown that Th22 cells play a regulatory role in the initiation and development of many diseases, such as kidney disease (60), cardiovascular disease (61), tumors (62) and infectious diseases (63). IL-22, the main effector of Th22 cells, also exerts different functions in different autoimmune diseases. Cytokines of the IL-10 family mainly act on interstitial cells and tissue epithelial cells, which can promote the proliferation and repair of tissues and organs, protect the integrity of the barrier, and play a patrolling role (23). These cytokines have both proinflammatory and anti-inflammatory functions. Here, the roles of Th22/IL-22 in the pathogenesis of autoimmune diseases are reviewed (**Table 1**).

RA

RA is a common autoimmune disease that is characterized by uncontrolled joint inflammation, bone erosion and cartilage damage. The levels of Th22 cells, Th17 cells and IL-22 in

TABLE 1 | Dual role of Th22/IL-22 in autoimmune diseases.

Disease	Mechanism of Th22 cells	Th22 frequency	IL-22 serum level	References
Pathogenic				
RA	*Promote osteoclast differentiation, induce osteoclast formation by p38MAPK/NF-κB and JAK2/STAT3 signaling*	↑	↑	(20, 64, 65)
SLE	*Positively correlated with Th17 cells, correlated with the disease activity index and the severity index*	↑	↑	(66–68)
		↓	↓	(69–71)
Ps	*Activate keratinocyte overproliferation, induce dermatitis and acanthosis by activating the STAT3-mediated IL-23 pathway*	↑	↑	(72–74)
MS	*Activate the NF-κB pathway, inhibit Foxp3 expression,promote oligodendrocyte apoptosis*	↑	↑	(75, 76)
ITP	*Positively correlated with Th1/Th17/Tfh cells*	↑	↑	(77–79)
IgAN	*Activate STAT3 and JAK signaling pathways, regulate renal fibrosis through the ERK,AKT and p38 signaling pathways*	↑	↑	(80, 81)
AIH	*Th1/Th17/Th22 imbalance with Treg*	↑	↑	(82)*[A]*
AITD	*Secrete proinflammatory cytokines, such as IL-22 and IL-6*	↑	↑	(83–86)
SSc	*Express massive fibroblast growth factor, promote the response of skin fibroblasts to TNF-α*	↑	↑	(87–89)
AS	*Positively correlate with Th17 cells*	↑	↑	(20)
		—	↑	(90)
Vasculitis	*Involved in GCA B cell proliferation, differentiation and arterial remodeling*	*	↑	(91)
Protective				
MS	*Severity of EAE was reduced in IL-22BP deficient mice*	*	↑	(92)*[A]*
MG	*IL-22 level was negatively correlated with serum anti-ACHR antibody level*	*	↓	(93)
		—	*	(94)

"↑" represents an increase compared to the healthy control; "↓" represents a decrease compared to the healthy control; "—" indicates similar to the healthy control; "" indicates not mentioned; "[A]" indicates animal model; non an notated references include human studies.*
RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; Ps, psoriasis; MS, multiple sclerosis; ITP, immune thrombocytopenia; IgAN, immunoglobulin A nephropathy; AIH, autoimmune hepatitis; AITD, autoimmune thyroid diseases; SSc, systemic sclerosis; AS, ankylosing spondylitis; MG, myasthenia gravis.

patients with RA were significantly higher than those in healthy controls. The numbers of Th22 cells were positively correlated with the IL-22 levels. Moreover, the percentages of Th22 cells and Th17 cells were positively correlated with the disease activity score in 28 joints (DAS28) scores and C-reactive protein (CRP) levels in RA patients (20, 64, 65). Not only did the levels of IL-17 and IL-22 in plasma increase, but the levels of IL-17 and IL-22 in the subchondral bone marrow of patients with RA were also significantly higher than those in plasma. The percentage of Th1, Th17 and Th22 cells and the levels of IL-17 and IL-22 in bone marrow were also positively correlated with DAS28 (95). The frequency of Th22 and Th17 cells in peripheral blood and the levels of IL-22, IL-17 and IFN- γ in plasma were decreased in RA patients who received effective treatment with methotrexate and leflunomide, although significant changes were not observed in patients with nonresponsive RA (96).

However,there are different views on whether Th22/IL-22 is involved in T cell-mediated synovitis. Researchers have found that the disease severities of wild-type mice and IL-22-deficient mice are comparable to those of T cell-mediated arthritis,Th17 cells have a strong effect on synovial fibroblasts while targeting Th17 cells and IL-17A but not Th22/IL-22 has been suggested as a focus treatment for T cell-mediated synovial inflammation (97). Recently, IL-22 neutralization was shown to inhibit osteoclast formation. Th22 cells promote osteoclast differentiation by producing IL-22 and play an important role in bone destruction in patients with RA (98). High levels of IL-22 in synovial tissue induce the proliferation of synovial fibroblasts and produce chemokines to enhance the inflammatory response of synovial tissue (29). IL-22 also induces osteoclast formation by inducing the p38MAPK/NF-κB and JAK2/STAT3 signaling pathways in synovial fibroblasts. Moreover, Th22 cells migrate to synovial tissues, which might be associated with the high expression of C-C chemokine ligand 28 (CCL28) in RA patients (98). IL-22 induces osteoclast formation by p38MAPK/NF-κB and JAK2/STAT3 signaling in RA development (51). In an animal model of CIA, blocking chemokine receptors was able to effectively inhibit the progression of RA. After treatment with the selective CXCR3 antagonist AMG487, the percentage of Treg cells in CIA mice was increased while the percentages of Th1, Th17 and Th22 cells were decreased. The expression of T-bet, IL-17A, IL-22 and RORγt was down-regulated, and the expression of Foxp3 was up-regulated. Chemokine receptor antagonists have been suggested as an effective strategy for the treatment of RA (99). Current studies support the hypothesis that Th22/IL-22 plays a pathogenic role in RA pathogenesis, although this mechanism requires further study. Blocking IL-22 may serve as a novel effective therapeutic method for the treatment of the disease.

SLE

SLE is another common autoimmune disease that is characterized by increased autoantibodies and immune disorders leading to tissue and organ damage. The role of Th22 cells in SLE is still controversial. Zhao et al. found that the plasma levels of IL-17A and IL-22 in patients with SLE were higher than those in healthy controls, and they showed that Th17 and IL-22 levels were positively correlated with the SLE disease activity index (SLEDAI), indicating that IL-22 and IL-22$^+$CD4$^+$ T cells play an important role in the pathogenesis of SLE (66). In 2014, the team further found that the levels of IL-22 and IL-22$^+$CD4$^+$ T cell before and after immunosuppressant and glucocorticoid (GC) treatment did not differ compared with the healthy controls (100). In 2017, this group also found that the levels of CCR6$^+$ T cells, CCR6$^+$Th22 cells and plasma IL-22 increased in SLE patients. The percentage of Th22 cells was positively correlated with the area of lupus erythematosus and the severity index (RCLASI) of the skin. The percentage of Th22 cells in SLE patients with renal damage was positively correlated with ESR, suggesting that CCR6$^+$ Th22 cells may be a therapeutic target for SLE treatment (67). Defects in TGF-β1 signaling in patients with active SLE are also associated with the over-production of IL-22 (101). Yang et al. found that the IL-22 levels increased in MRL/LPR mice while treatment with anti-IL-22 monoclonal antibody significantly decreased the urinary protein, urea nitrogen and serum creatinine in these mice (68). Moreover, Th22 cells might be a better predictor of SLE tissue involvement than Th17 cells (102). Other studies have shown that the level of IL-22 is decreased in patients with SLE (69–71) and significantly lower in patients with primary SLE (70). Urinary IL-22 mRNA levels are decreased in SLE patients with proliferative glomerulonephritis. IL-22 mRNA can also be used to evaluate the activity of lupus nephritis (71). In Chinese SLE patients, IL-22 gene polymorphisms may increase susceptibility to SLE by reducing the expression of IL-22 (103). In addition, recent studies have shown that activated B cells suppress the development of lupus by promoting Th22 cell differentiation and inhibiting Th17 (56). These results show that both Th22 cells and IL-22 levels are related to SLE; however, the exact mechanism is still unclear. Moreover,these studies show that IL-22 levels differ at different stages of SLE (70) and indicate its involvement in tissue inflammation and damage to different organs (102). The currently available results suggest the complexity of IL-22 and the heterogeneity of SLE, which needs to be further explored.

PSORIASIS

Psoriasis is a chronic inflammatory autoimmune disease mediated by T cells, and it is characterized by the abnormal proliferation of keratinocytes. IL-22 is considered an activator of keratinocyte over-proliferation (104, 105). The levels of Th22 cells and plasma IL-22 in patients with psoriasis are increased and positively correlated with the severity of the disease (72–74). High levels of IL-22 can induce the expression of antimicrobial proteins (AMPs), antimicrobial peptides such as S100A7, S100A8, S100A9 and β-defensin, and neutrophil chemoattractants CXCL8, CXCL5 and CXCL1 in the epidermis (106, 107). It can also inhibit keratinocyte differentiation, interfere with the normal skin healing process, and induce the production of MMPs, which is conducive to extracellular tissue degradation (108, 109). IL-22 also induces dermatitis and acanthosis by activating the STAT3-mediated IL-23 pathway

(9, 110). In addition, IL-22 neutralizing antibody treatment decreased antimicrobial peptide levels and inhibited disease development in a psoriasis mice model, suggesting the therapeutic potential of IL-22 inhibitors in psoriasis (111).

Psoriasis is characterized by recurrent lesions in the same anatomic area. New lesions appear in areas that have healed after successful treatment because even if the psoriatic plaque disappears, tissue resident memory cells (TRMs) are still present in the skin (105, 112). In the active stage of psoriasis, the expression of IFN-γ, IL-17A and IL-22 in $CD4^+$ and $CD8^+$ T cells in the epidermis is increased. IL-22 is mainly produced by $CD4^+$ epidermal T cells, where it activates keratinocytes and leads to acanthosis. IL-17A is mainly produced by epidermal $CD8^+$ T cells, and it drives keratinocytes to participate in the recruitment of neutrophils and produces chemokines and proinflammatory cytokines (113). TNF-α promotes the differentiation of Th22 cells, while TNF-α blockade has achieved satisfactory results in the treatment of psoriasis patients. However, Th22 cells in the healed skin epidermis still produce IL-22 after 6 years of remission. Therefore, Th22 cells play an important role in the memory of psoriatic relapse (113). Previous findings suggest that IL-22 in the psoriatic epidermis is produced by $IL22^+IL\text{-}17^+$ Th17 cells. Recent studies show that there is no significant correlation between the expression of IL-17A and IL-22 in psoriasis, which does not support the existence of double-secreted IL-17A/IL-22 Th17 cells (114).

MS

MS is an autoimmune disease of the central nervous system (CNS), and it is characterized by the loss of the axonal myelin sheath and inflammation of the CNS, showing clinical symptoms such as muscle spasm and paralysis. Proinflammatory cytokines such as IL-22, IL-17, TNF-α and IFN-γ are involved in MS pathogenesis through multiple signaling pathways. The proportion of Th22 cells (75, 76) and the level of IL-22 in the serum of patients with MS increased (75, 76, 115–121), and the level of IL-22 in recurrent stage was significantly higher than that in progressive stage and remission stage (115, 116, 119, 122). The level of IL-22 in cerebrospinal fluid is also increased, and IL-22 increases the survival rate of brain astrocytes. The IL-22 receptor subunit IL-22R is mainly expressed on astrocytes (119), suggesting that astrocytes may play an important role in IL-22-mediated pathological changes in MS. IL-22 also inhibits the expression of Foxp3 by activating the NF-κB pathway and promotes the expression of Fas in oligodendrocytes, which leads to apoptosis of oligodendrocytes (123). Large-scale studies of 5019 MS patients in Norway and Sweden by Beyenet et al. identified IL-22RA2 as a risk gene for MS (124). The high expression of T-bet and CCR6 in Th22 cells of MS patients suggests that Th22 cells may migrate to the central nervous system. The infiltration of Th22 cells leads to an increase in T cell infiltration and contributes to the destruction of the blood-brain barrier (76, 125). In addition, the resistance to IFN-β therapy in MS patients may be related to the low expression of IFN receptor 1 on the surface of Th22 cells, and similar findings have been observed in the MS animal model of EAE (115).

A study in 2014 suggested that IL-22 played a protective role in CNS inflammation (126). The severity of inflammation was positively correlated with the level of IL-22BP in the cerebrospinal fluid of patients with MS. IL-22BP has a pathogenic effect on EAE in both mice and rats, and IL-22BP is theoretically an antagonist of IL-22. IL-22BP has been proposed to reduce IFN-γ produced by brain-derived T cells in lymph nodes (92). Previous studies showed that the histopathological features of EAE in IL-22-deficient mice were comparable to those in wild-type mice (14). Hannes et al. conducted EAE experiments on wild-type mice, IL-22 deficient mice, IL-22BP deficient mice, and IL-22 and IL-22BP double deficient mice and concluded that the loss of control of IL-22 signal in IL-22BP deficient mice reduced the severity of EAE, which supported the protective effect of IL-22 in MS; thus, they suggested that IL-22BP could be used as a new target for MS (92).

ITP

ITP is an autoimmune disease that is characterized by increased destruction and decreased production of platelets (127). The frequency of Th22 cells and the level of plasma IL-22 in patients with ITP were significantly higher than those in the control group (77, 78, 128). The increase in plasma IL-22 levels in ITP patients was reported to be associated with the dysregulation of Th1 and Th22 cells (128). Furthermore, Th22 cells in ITP patients were positively correlated with Th1 and Th17 cells (77, 79), and patients treated with high-dose dexamethasone (HD-DXM) exhibited significantly decreased frequencies of Th1 and Th22 cells and plasma concentrations of IL-22 (78). The level of IL-22 was also increased in pediatric ITP patients compared with healthy populations (129). A recent study showed that the frequencies of Th22, Th17, Tfh and Th1 cells in the bone marrow of ITP patients were significantly higher than those in the control group; moreover, the frequency of Th22 cells in bone marrow was significantly higher than that in peripheral blood (130). Notably, ITP patients with negative autoantibodies showed a higher percentage of Th22 cells than patients with positive detection of autoantibodies (77). In summary, Th22 plays a proinflammatory effect in ITP and acts synergistically with Th1/Th17 and Tfh cells. Th22 cells play an important role in the pathophysiological process of ITP patients. Therefore, blocking IL-22 may serve as a potential therapeutic target for treating ITP patients.

IGAN

IgAN is the most common primary glomerular disease characterized by inflammatory cell infiltration and IgA deposition in the mesangial area of the glomerulus, which is an important cause of renal failure (131). Although the exact pathogenesis is still unclear, the involvement of T cells has been confirmed. IgAN has a higher proportion of circulating Th2, Th17, Th22, Tfh and γδ T cells, but a lower proportion of Treg and Th1 cells (80). Peng et al. also showed that Th17 and Th22 cell frequencies in peripheral blood and plasma IL-22 levels were significantly increased in patients with IgAN, and Th22 cells were positively correlated with Th17 cells and plasma IL-22 levels. In addition, compared with IgAN patients without

proteinuria, IgAN patients with proteinuria showed a higher percentage of Th22 cells (81). Th22 cells in patients with IgAN are higher than those in healthy controls, and the percentage of increase is positively correlated with the degree of kidney disease in patients with IgAN. Moreover,tonsillitis aggravates the over-expression of Th22 cells and the chemokines CCL20, CCL22 and CCL27 and aggravates kidney damage in IgAN (132). Xiao et al. confirmed that streptococcal infection can increase the chemotaxis of Th22 cells and aggravate kidney inflammation (133). Chronic inflammation is a common sign of chronic fibrosis,and fibrosis is one of the common pathological changes of IgAN. IL-22 binds to kidney IL-22R1 to activate STAT3, JAK and other signaling pathways, and regulate renal fibrosis through the ERK, Akt, and p38MAPK signaling pathways. Therefore, Th22 cells promote renal fibrosis in IgAN (134, 135). Treatment is mainly reflected in the reduced chemotaxis of Th22 cells. After treatment with cordyceps (CS), dexamethasone and losartan, the frequency of Th22 cells in the IgAN mouse model decreased and the expression of CCR10, CCL27 and IL-22 was also significantly reduced (60, 136). The above treatments all regulate the chemotaxis of Th22 cells to inhibit inflammation and improve renal function in patients. Acteoside (the main component of Rehmannia glutinosa) can also inhibit Th22 cell proliferation and inhibit Th22 cell chemotactic factors to regulate Th22 cell chemotaxis (137). In addition, the IL-22R1 gene polymorphism is genetically associated with the development of childhood IgA nephropathy (138).

AIH

AIH is a chronic autoimmune inflammatory liver disease that is characterized by high autoantibodies, high liver enzyme levels, and liver damage (139). Hepatocytes are one of the target cells of IL-22. IL-22 has dual effects on hepatocytes: protecting hepatocytes and inducing acute phase proteins. IL-22 acts on liver progenitor cells (LPCs), which is beneficial for liver reconstruction after injury. In human and mouse chronic HBV-infected livers, IL-22 promotes hepatocyte proliferation through the STAT3 pathway (140, 141). Patients with drug-induced liver injury (DILI) have increased intrahepatic and peripheral Th22 cells and IL-22 levels, and the liver IL-22 level is positively correlated with regeneration. Th22/IL-22 has a hepatoprotective effect in DILI (142). Studies have indicated that the increase in Th22/IL-22 is related to the severity of hepatitis B virus-related chronic liver failure (HBV-ACLF) and suggested that Th22/IL-22 can be used as a biomarker for the prognosis of HBV-ACLF (143). Few studies have focused on the pathogenesis of Th22/IL-22 in AIH. Studies have shown that compared with healthy controls, the serum levels of IL-6,IL-10, IL-17F, IL-21, IL-23 and TNF-α in AIH are significantly increased while those of IL-22 and IL-17A are not. According to the grouping of immunoserological markers, the cytokines of type 2 AIH patients are characterized by elevated levels of IL-21 and IL-22 (144). The mouse experimental autoimmune hepatitis (EAH) model and AIH patients present reduced serum IL-10 and Treg levels. The number of Th22, Th17 and Th1 cells and the number of corresponding cytokines IL-22, IL-17A and IFN-γ were all reduced. Moreover, the number of Tregs was negatively correlated with the number of Th22, Th17 and Th1 cells and cytokine levels. More interestingly, the serum IL-22 and IL-17A levels were positively correlated with liver injury in patients with AIH, suggesting that the imbalance between Th1/Th17/Th22 and Treg cells may be involved in the process of AIH (82).

AITD

AITD mainly includes Graves' disease (GD) and Hashimoto's thyroiditis (HT). GD manifests as hyperthyroidism caused by the overproduction of thyroid hormone, while HT manifests as hypothyroidism (145). T cell dysfunction and/or corresponding cytokine abnormalities cause the destruction of immune tolerance, which leads to abnormal immune responses in AITD. Research on Th22/IL-22 in AITD is also limited. The study by Peng et al. showed that the percentage of Th17 and Th22 cells and plasma IL-17 and IL-22 in GD patients were increased and positively correlated with serum TSAb levels (83, 84). GD patients not only have higher Th22 cell frequencies and serum IL-22 levels than healthy people but also have higher IL-22 mRNA and AhR expression, whereas HT patients do not show an increase in Th22/IL-22 (146). However, different results have also been presented. Bai et al. found that the circulating Th22 cell level of HT patients was significantly higher than that of the healthy control group and the GD patient group, and was positively correlated with the serum IL-22 level and thyroid peroxidase antibody (TPOAb) titer. Under TNF-α and IL-6 stimulation,the T lymphocytes of HT patients showed an enhanced ability to differentiate into Th22 cells *in vitro* (85). Ruggeri et al. also believed that serum IL-22 levels in untreated, newly diagnosed HT patients were higher than those in healthy controls (86). The level of Th22/IL-22 in AITD patients is elevated,and Th22 cells may participate in the pathogenesis of AITD by secreting IL-22, IL-6 and other proinflammatory cytokines; however, the exact mechanism remains to be further confirmed.

MG

MG is an autoimmune disease that produces anti-acetylcholine receptor (ACHR) autoantibodies and neuromuscular transmission disorders and manifests as skeletal muscle fatigue and weakness (147). Thymectomy (TE) represents one of the treatment methods. The frequency of Th22 cells in patients treated with TE was not significantly different from that of the healthy controls. After TE surgery, the frequency of Th22 cells was significantly reduced (93). Studies have also shown that the levels of IL-17 mRNA in PBMC and IL-17 concentrations in serum increase while levels of IL-22 mRNA and serum IL-22 decrease in MG patients. In addition, the level of serum IL-22 is negatively correlated with the level of serum anti-ACHR antibody, suggesting that IL-22 plays a protective role in MG (94). A recent study showed that the levels of IL-22 in the PBMCs of MG patients did not differ from those in the control group (148). Thus, further research is required to clarify the specific role of Th22/IL-22 in MG.

OTHER AUTOIMMUNE DISEASES

SSc is an autoimmune connective tissue disease with skin and visceral organ fibrosis due to excessive deposition of extracellular matrix and vascular lesions (149). The increased frequency of circulating Th22 cells is positively associated with pulmonary interstitial disease in SSc patients (87). Moreover, the excessive production of IL-22 in injured skin was independent of IL-17 (88). Other studies have shown that IL-22 can enhance the response of fibroblasts to TNF-α, promote the inflammatory phenotype of fibroblasts, and enhance the ability of TNF-activated keratinocytes to stimulate fibroblasts (89). Th22 cells express massive amounts of fibroblast growth factor, suggesting that targeting the IL-22 signaling pathway may be effective for preventing fibrogenesis (150). Th22/IL-22 may be related to skin and visceral fibrosis in patients with SSc. Primary Sjogren's syndrome (pSS) is a chronic autoimmune disease that is characterized by lymphocyte infiltration in lacrimal and salivary glands, and it also presents elevated IL-22 levels (43, 151, 152). IL-22 plays a proinflammatory role in pSS pathogenesis and promotes salivary gland inflammation at an early stage (152, 153). IL-22 is predominantly secreted by Th17 and $NKp44^+$ NK cells in pSS patients (152). Studies have shown that the frequency of Th22 cells in the peripheral blood of ankylosing spondylitis (AS) patients is increased (20, 154), while other studies have shown that there is no difference in $IL\text{-}22^+CD4^+$ and $IL\text{-}22^+CD8^+$ T cells between AS patients and healthy controls; however, the secretion of IL-22 by circulating mucosal-associated invariant T (MAIT) cells is increased in AS patients (90). A number of types of vasculitis have been identified, and several studies have focused on Th22/IL-22 in vasculitis. A study by Zerbini et al. showed that the levels of IL-22 and IL-22R1 were higher in giant cell arteritis (GCA) patients who were confirmed to be positive for temporal artery biopsy (TAB) than in TAB-negative patients and normal controls. IL-22 is expressed in spindle cells and infiltrating immune cells, while IL-22R1 is expressed in endothelial cells; moreover, IL-22 is involved in B cell proliferation, differentiation and arterial remodeling of GCA (91).

THERAPEUTIC TARGETING OF IL-22

Previous investigations have shown a critical role of IL-22 during the pathogenesis of autoimmune diseases. Preclinical studies indicate that IL-22 may serve as a promising therapeutic target for treating autoimmune diseases. Th22 cells promote osteoclast differentiation while neutralization of IL-22 inhibits osteoclast formation, suggesting that blocking IL-22 could be effective in suppressing bone destruction in RA patients (98). Inhibition of IL-22 by neutralization antibodies has been shown to reduce the expression of chemotactic factors, decrease antimicrobial and hyperproliferative responses of keratinocytes, and prevent the development of imiquimod-induced psoriasis from skin inflammation (111). It has been reported that treatment with cordyceps sinensis, dexamethasone and losartan improves kidney functions associated with a reduction of Th22 cells in mice with IgA nephropathy, suggesting that these drugs may exert their protective effects through modulating Th22 cells (60, 136).

Currently,the safety, tolerability and therapeutic effects of Fezakinumab (ILV-094), a human monoclonal antibody that directly binds to IL-22, have been examined in atopic dermatitis patients in severalclinical studies (155). A small scale randomized, double-blind, phase 2a clinical trial involving 60 patients with moderate-to-severe atopic dermatitis has shown that Fezakinumab is well-tolerated with sustained clinical improvements after last drug dosing. Fezakinumab treatment has shown significant clinical improvements *versus* placebo in patients with severe disease as reflected by significant reductions of SCORing of Atopic Dermatitis (SCORAD) scores and Investigator Global Assessment (156). Furthermore, transcriptomic and immunohistochemistry analyses reveal that Fezakinumab has profound effects on multiple inflammatory pathways in these patients (157). Fezakinumab broadly decreases immune activation in skin tissues and reduces overall inflammatory burden and epidermal pathologic characteristics. The treatment effects of Fezakinumab are particularly evident in patients with high IL-22 baseline expression, suggesting that a precision medicine-based approach might be needed for improving therapeutic outcomes in patients with atopic dermatitis.

It has been revealed that IL-22 exerts protective roles in certain diseases. A phase II study (NCT02406651) is undergoing to investigate the therapeutic effects of recombinant IL-22 for GVHD after bone marrow transplantation (158). Other potential strategies including the modulation of chemotaxis of Th22 cells, administration of AhR agonists to enhance IL-22 expression,and application of IL-22BP are under investigation. Available results suggest that Th22 cells are involved in autoimmune pathogenesis through multiple effector functions, including the production of various cytokines, such as IL-22, IL-13 and IL-26. Moreover, Th22 cells may have close interactions with Th1, Th2 and Th17 cells during disease progression. Further investigations on the safety, tolerability and therapeutic effects of agents targeting Th22/IL22 pathway are needed for the effective treatment of autoimmune diseases.

CONCLUSION

Th22 cells and IL-22play diverse roles in the development of autoimmune diseases and have both proinflammatory and anti-inflammatory functions. Th22/IL-22 plays a pathogenic role in most autoimmune diseases, while IL-22 has been shown to have a protective effect in many other diseases involving skin and mucosal barrier. Thus, the function of IL-22 varies depending on the cellular source, types of inflammatory response, the affected tissue (mucosa or solid organ), and the concentration and duration of IL-22 itself in local environment. Emerging evidence supports the notion that Th22 cells may serve as therapeutic targets for autoimmune diseases. However, further studies are needed to elucidate the mechanisms of Th22 cells in disease pathogenesis and validate the therapeutic potential of targeting Th22 cells for the treatment of autoimmune diseases.

AUTHOR CONTRIBUTIONS

QJ and DC drafted the manuscript and designed the figures. GY and FX reviewed the manuscript structure and ideas. DC, JX, SW, and LL conceived the topic and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Wang W, Sung N, Gilman-Sachs A, Kwak-Kim J. T Helper (Th) Cell Profiles in Pregnancy and Recurrent Pregnancy Losses: Th1/Th2/Th9/Th17/Th22/Tfh Cells. *Front Immunol* (2020) 11:2025. doi: 10.3389/fimmu.2020.02025
2. Ruterbusch M, Pruner KB, Shehata L, Pepper M. In Vivo CD4(+) T Cell Differentiation and Function: Revisiting the Th1/Th2 Paradigm. *Annu Rev Immunol* (2020) 38:705–25. doi: 10.1146/annurev-immunol-103019-085803
3. Chatzileontiadou DSM, Sloane H, Nguyen AT, Gras S, Grant EJ. The Many Faces of CD4(+) T Cells: Immunological and Structural Characteristics. *Int J Mol Sci* (2020) 22(1):73. doi: 10.3390/ijms22010073
4. Xiao F, Han M, Rui K, Ai X, Tian J, Zhang W, et al. New Insights Into Follicular Helper T Cell Response and Regulation in Autoimmune Pathogenesis. *Cell Mol Immunol* (2021) 18(6):1610–2. doi: 10.1038/s41423-021-00688-7
5. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two Types of Murine Helper T Cell Clone. I. Definition According to Profiles of Lymphokine Activities and Secreted Proteins. *J Immunol* (1986) 136 (7):2348–57.
6. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A Distinct Lineage of CD4 T Cells Regulates Tissue Inflammation by Producing Interleukin 17. *Nat Immunol* (2005) 6(11):1133–41. doi: 10.1038/ni1261
7. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-Producing CD4+ Effector T Cells Develop *via* a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* (2005) 6(11):1123–32. doi: 10.1038/ni1254
8. Gurney AL. IL-22, a Th1 Cytokine That Targets the Pancreas and Select Other Peripheral Tissues. *Int Immunopharmacol* (2004) 4(5):669–77. doi: 10.1016/j.intimp.2004.01.016
9. Zheng Y, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, et al. Interleukin-22, a T(H)17 Cytokine,Mediates IL-23-Induced Dermal Inflammation and Acanthosis. *Nature* (2007) 445(7128):648–51. doi: 10.1038/nature05505
10. Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of Interleukin 22 But Not Interleukin 17 by a Subset of Human Skin-Homing Memory T Cells. *Nat Immunol* (2009) 10(8):857–63. doi: 10.1038/ni.1767
11. Dumoutier L, Louahed J, Renauld JC. Cloning and Characterization of IL-10-Related T Cell-Derived Inducible Factor (IL-TIF), a Novel Cytokine Structurally Related to IL-10 and Inducible by IL-9. *J Immunol* (2000) 164 (4):1814–9. doi: 10.4049/jimmunol.164.4.1814
12. Xie MH, Aggarwal S, Ho WH, Foster J, Zhang Z, Stinson J, et al. Interleukin (IL)-22,a Novel Human Cytokine That Signals Through the Interferon Receptor-Related Proteins CRF2-4 and IL-22r. *J Biol Chem* (2000) 275 (40):31335–9. doi: 10.1074/jbc.M005304200
13. Wolk K, Sabat R. Interleukin-22: A Novel T- and NK-Cell Derived Cytokine That Regulates the Biology of Tissue Cells. *Cytokine Growth Factor Rev* (2006) 17(5):367–80. doi: 10.1016/j.cytogfr.2006.09.001
14. Kreymborg K, Etzensperger R, Dumoutier L, Haak S, Rebollo A, Buch T, et al. IL-22 is Expressed by Th17 Cells in an IL-23-Dependent Fashion, But Not Required for the Development of Autoimmune Encephalomyelitis. *J Immunol* (2007) 179(12):8098–104. doi: 10.4049/jimmunol.179.12.8098
15. Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, et al. Th22 Cells Represent a Distinct Human T Cell Subset Involved in Epidermal Immunity and Remodeling. *J Clin Invest* (2009) 119(12):3573–85. doi: 10.1172/JCI40202
16. Plank MW, Kaiko GE, Maltby S, Weaver J, Tay HL, Shen W, et al. Th22 Cells Form a Distinct Th Lineage From Th17 Cells In Vitro With Unique Transcriptional Properties and Tbet-Dependent Th1 Plasticity. *J Immunol* (2017) 198(5):2182–90. doi: 10.4049/jimmunol.1601480
17. Yang P, Qian FY, Zhang MF, Xu AL, Wang X, Jiang BP, et al. Th17 Cell Pathogenicity and Plasticity in Rheumatoid Arthritis. *J Leukocyte Biol* (2019) 106(6):1233–40. doi: 10.1002/jlb.4ru0619-197r
18. Mousset CM, Hobo W, Woestenenk R, Preijers F, Dolstra H, van der Waart AB. Comprehensive Phenotyping of T Cells Using Flow Cytometry. *Cytometry A* (2019) 95(6):647–54. doi: 10.1002/cyto.a.23724
19. Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a Human Helper T Cell Population That has Abundant Production of Interleukin 22 and is Distinct From T(H)-17,T(H)1 and T(H)2 Cells. *Nat Immunol* (2009) 10(8):864–71. doi: 10.1038/ni.1770
20. Zhang L, Li YG, Li YH, Qi L, Liu XG, Yuan CZ, et al. Increased Frequencies of Th22 Cells as Well as Th17 Cells in the Peripheral Blood of Patients With Ankylosing Spondylitis and Rheumatoid Arthritis. *PloS One* (2012) 7(4): e31000. doi: 10.1371/journal.pone.0031000
21. Gittler JK, Shemer A, Suarez-Farinas M, Fuentes-Duculan J, Gulewicz KJ, Wang CQ, et al. Progressive Activation of T(H)2/T(H)22 Cytokines and Selective Epidermal Proteins Characterizes Acute and Chronic Atopic Dermatitis. *J Allergy Clin Immunol* (2012) 130(6):1344–54. doi: 10.1016/j.jaci.2012.07.012
22. Nograles KE, Zaba LC, Shemer A, Fuentes-Duculan J, Cardinale I, Kikuchi T, et al. IL-22-Producing "T22" T Cells Account for Upregulated IL-22 in Atopic Dermatitis Despite Reduced IL-17-Producing TH17 T Cells. *J Allergy Clin Immunol* (2009) 123(6):1244–52.e2. doi: 10.1016/j.jaci.2009.03.041
23. Ouyang W, O'Garra A. IL-10 Family Cytokines IL-10 and IL-22: From Basic Science to Clinical Translation. *Immunity* (2019) 50(4):871–91. doi: 10.1016/j.immuni.2019.03.020
24. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 Increases the Innate Immunity of Tissues. *Immunity* (2004) 21(2):241–54. doi: 10.1016/j.immuni.2004.07.007
25. Xu W, Presnell SR, Parrish-Novak J, Kindsvogel W, Jaspers S, Chen Z, et al. A Soluble Class II Cytokine Receptor,IL-22RA2,is a Naturally Occurring IL-22 Antagonist. *Proc Natl Acad Sci USA* (2001) 98(17):9511–6. doi: 10.1073/pnas.171303198
26. Wolk K, Kunz S, Asadullah K, Sabat R. Cutting Edge: Immune Cells as Sources and Targets of the IL-10 Family Members? *J Immunol* (2002) 168 (11):5397–402. doi: 10.4049/jimmunol.168.11.5397
27. Hansson M, Silverpil E, Linden A, Glader P. Interleukin-22 Produced by Alveolar Macrophages During Activation of the Innate Immune Response. *Inflammation Res* (2013) 62(6):561–9. doi: 10.1007/s00011-013-0608-1

28. Zindl CL, Lai JF, Lee YK, Maynard CL, Harbour SN, Ouyang W, et al. IL-22-Producing Neutrophils Contribute to Antimicrobial Defense and Restitution of Colonic Epithelial Integrity During Colitis. *Proc Natl Acad Sci USA* (2013) 110(31):12768–73. doi: 10.1073/pnas.1300318110
29. Ikeuchi H, Kuroiwa T, Hiramatsu N, Kaneko Y, Hiromura K, Ueki K, et al. Expression of Interleukin-22 in Rheumatoid Arthritis: Potential Role as a Proinflammatory Cytokine. *Arthritis Rheum* (2005) 52(4):1037–46. doi: 10.1002/art.20965
30. Simonian PL, Wehrmann F, Roark CL, Born WK, O'Brien RL, Fontenot AP. γδ T Cells Protect Against Lung Fibrosis *via* IL-22. *J Exp Med* (2010) 207 (10):2239–53. doi: 10.1084/jem.20100061
31. Dudakov JA, Hanash AM, van den Brink MR. Interleukin-22: Immunobiology and Pathology. *Annu Rev Immunol* (2015) 33:747–85. doi: 10.1146/annurev-immunol-032414-112123
32. Cochez PM, Michiels C, Hendrickx E, Van Belle AB, Lemaire MM, Dauguet N, et al. AhR Modulates the IL-22-Producing Cell Proliferation/Recruitment in Imiquimod-Induced Psoriasis Mouse Model. *Eur J Immunol* (2016) 46 (6):1449–59. doi: 10.1002/eji.201546070
33. Mashiko S, Bouguermouh S, Rubio M, Baba N, Bissonnette R, Sarfati M. Human Mast Cells are Major IL-22 Producers in Patients With Psoriasis and Atopic Dermatitis. *J Allergy Clin Immunol* (2015) 136(2):351–9.e1. doi: 10.1016/j.jaci.2015.01.033
34. Steinbach S, Vordermeier HM, Jones GJ. CD4+ and γδ T Cells are the Main Producers of IL-22 and IL-17A in Lymphocytes From Mycobacterium Bovis-Infected Cattle. *Sci Rep* (2016) 6:29990. doi: 10.1038/srep29990
35. Lu Z, Liu R, Huang E, Chu Y. MicroRNAs: New Regulators of IL-22. *Cell Immunol* (2016) 304-305:1–8. doi: 10.1016/j.cellimm.2016.05.003
36. Jia L, Wu C. The Biology and Functions of Th22 Cells. *Adv Exp Med Biol* (2014) 841:209–30. doi: 10.1007/978-94-017-9487-9_8
37. Protopsaltis NJ, Liang W, Nudleman E, Ferrara N. Interleukin-22 Promotes Tumor Angiogenesis. *Angiogenesis* (2019) 22(2):311–23. doi: 10.1007/s10456-018-9658-x
38. Andoh A, Zhang Z, Inatomi O, Fujino S, Deguchi Y, Araki Y, et al. Interleukin-22,a Member of the IL-10 Subfamily,Induces Inflammatory Responses in Colonic Subepithelial Myofibroblasts. *Gastroenterology* (2005) 129(3):969–84. doi: 10.1053/j.gastro.2005.06.071
39. Lejeune D, Dumoutier L, Constantinescu S, Kruijer W, Schuringa JJ, Renauld JC, et al. Interleukin-22 (IL-22) Activates the JAK/STATand P38 MAP Kinase Pathways in a Rat Hepatoma Cell Line. Pathways that are shared with and distinct from IL-10. *J Biol Chem* (2002) 277(37):33676–82. doi: 10.1074/jbc.M204204200
40. Sonnenberg GF, Fouser LA, Artis D. Functional Biology of the IL-22-IL-22R Pathway in Regulating Immunity and Inflammation at Barrier Surfaces. *Adv Immunol* (2010) 107:1–29. doi: 10.1016/B978-0-12-381300-8.00001-0
41. Zhu J, Zhou M, Zhao X, Mu M, Cheng M. Blueberry,Combined With Probiotics,Alleviates non-Alcoholic Fatty Liver Disease *via* IL-22-mediated JAK1/STAT3/BAX signaling. *Food Funct* (2018) 9(12):6298–306. doi: 10.1039/c8fo01227j
42. He H, Guttman-Yassky E. JAK Inhibitors for Atopic Dermatitis: An Update. *Am J Clin Dermatol* (2019) 20(2):181–92. doi: 10.1007/s40257-018-0413-2
43. Ciccia F, Guggino G, Rizzo A, Bombardieri M, Raimondo S, Carubbi F, et al. Interleukin (IL)-22 Receptor 1 is Over-Expressed in Primary Sjogren's Syndrome and Sjogren-Associated non-Hodgkin Lymphomas and is Regulated by IL-18. *Clin Exp Immunol* (2015) 181(2):219–29. doi: 10.1111/cei.12643
44. Che Y, Su Z, Xia L. Effects of IL-22 on Cardiovascular Diseases. *Int Immunopharmacol* (2020) 81:106277. doi: 10.1016/j.intimp.2020.106277
45. Pickert G, Neufert C, Leppkes M, Zheng Y, Wittkopf N, Warntjen M, et al. STAT3 Links IL-22 Signaling in Intestinal Epithelial Cells to Mucosal Wound Healing. *J Exp Med* (2009) 206(7):1465–72. doi: 10.1084/jem.20082683
46. Nagalakshmi ML, Rascle A, Zurawski S, Menon S, de Waal Malefyt R. Interleukin-22 Activates STAT3 and Induces IL-10 by Colon Epithelial Cells. *Int Immunopharmacol* (2004) 4(5):679–91. doi: 10.1016/j.intimp.2004.01.008
47. Boniface K, Bernard FX, Garcia M, Gurney AL, Lecron JC, Morel F. IL-22 Inhibits Epidermal Differentiation and Induces Proinflammatory Gene Expression and Migration of Human Keratinocytes. *J Immunol* (2005) 174 (6):3695–702. doi: 10.4049/jimmunol.174.6.3695
48. Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, et al. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *Am J Physiol Gastrointest Liver Physiol* (2006) 290(4):G827–38. doi: 10.1152/ajpgi.00513.2005
49. Dumoutier L, de Meester C, Tavernier J, Renauld JC. New Activation Modus of STAT3: A Tyrosine-Less Region of the Interleukin-22 Receptor Recruits STAT3 by Interacting With Its Coiled-Coil Domain. *J Biol Chem* (2009) 284 (39):26377–84. doi: 10.1074/jbc.M109.007955
50. Naher L, Kiyoshima T, Kobayashi I, Wada H, Nagata K, Fujiwara H, et al. STAT3 Signal Transduction Through Interleukin-22 in Oral Squamous Cell Carcinoma. *Int J Oncol* (2012) 41(5):1577–86. doi: 10.3892/ijo.2012.1594
51. Wen H, Liu Y, Li J, Wei D, Liu D, Zhao F. Inhibitory Effect and Mechanism of 1,25-Dihydroxy Vitamin D3 on RANKL Expression in Fibroblast-Like Synoviocytes and Osteoclast-Like Cell Formation Induced by IL-22 in Rheumatoid Arthritis. *Clin Exp Rheumatol* (2018) 36(5):798–805.
52. Yeste A, Mascanfroni ID, Nadeau M, Burns EJ, Tukpah AM, Santiago A, et al. IL-21 Induces IL-22 Production in CD4+ T Cells. *Nat Commun* (2014) 5:3753. doi: 10.1038/ncomms4753
53. Diaz-Zuniga J, Melgar-Rodriguez S, Monasterio G, Pujol M, Rojas L, Alvarez C, et al. Differential Human Th22-Lymphocyte Response Triggered by Aggregatibacter Actinomycetemcomitans Serotypes. *Arch Oral Biol* (2017) 78:26–33. doi: 10.1016/j.archoralbio.2017.02.008
54. Fujita H, Nograles KE, Kikuchi T, Gonzalez J, Carucci JA, Krueger JG. Human Langerhans Cells Induce Distinct IL-22-Producing CD4+ T Cells Lacking IL-17 Production. *Proc Natl Acad Sci United States America* (2009) 106(51):21795–800. doi: 10.1073/pnas.0911472106
55. Yoon J, Leyva-Castillo JM, Wang G, Galand C, Oyoshi MK, Kumar L, et al. IL-23 Induced in Keratinocytes by Endogenous TLR4 Ligands Polarizes Dendritic Cells to Drive IL-22 Responses to Skin Immunization. *J Exp Med* (2016) 213(10):2147–66. doi: 10.1084/jem.20150376
56. Yang J, Yang X, Wang L, Li M. B Cells Control Lupus Autoimmunity by Inhibiting Th17 and Promoting Th22 Cells. *Cell Death Dis* (2020) 11(3):164. doi: 10.1038/s41419-020-2362-y
57. Fu D, Song X, Hu H, Sun M, Li Z, Tian Z. Downregulation of RUNX3 Moderates the Frequency of Th17 and Th22 Cells in Patients With Psoriasis. *Mol Med Rep* (2016) 13(6):4606–12. doi: 10.3892/mmr.2016.5108
58. Huang R, Chen X, Long Y, Chen R. MiR-31 Promotes Th22 Differentiation Through Targeting Bach2 in Coronary Heart Disease. *Biosci Rep* (2019) 39 (9):BSR20190986. doi: 10.1042/BSR20190986
59. Piccinni MP, Lombardelli L, Logiodice F, Kullolli O, Maggi E, Barkley MS. Medroxyprogesterone Acetate Decreases Th1,Th17,and Increases Th22 Responses *via* AHR Signaling Which Could Affect Susceptibility to Infections and Inflammatory Disease. *Front Immunol* (2019) 10:642. doi: 10.3389/fimmu.2019.00642
60. Xiao C, Xiao P, Li X, Li X, Li H, Chen Y, et al. Cordyceps Sinensis may Inhibit Th22 Cell Chemotaxis to Improve Kidney Function in lgA Nephropathy. *Am J Transl Res* (2018) 10(3):857–65.
61. Huang Y, Xu T, Li J. Th22 Cell is a Gradually Proved Potential Biomarker for Acute Coronary Syndrome. *Mediators Inflammation* (2014) 2014:813926. doi: 10.1155/2014/813926
62. Huang YH, Cao YF, Jiang ZY, Zhang S, Gao F. Th22 Cell Accumulation is Associated With Colorectal Cancer Development. *World J Gastroenterol* (2015) 21(14):4216–24. doi: 10.3748/wjg.v21.i14.4216
63. Cui D, Zhong F, Lin J, Wu Y, Long Q, Yang X, et al. Changes of Circulating Th22 Cells in Children With Hand, Foot, and Mouth Disease Caused by Enterovirus 71 Infection. *Oncotarget* (2017) 8(17):29370–82. doi: 10.18632/oncotarget.14083
64. Zhang L, Li JM, Liu XG, Ma DX, Hu NW, Li YG, et al. Elevated Th22 Cells Correlated With Th17 Cells in Patients With Rheumatoid Arthritis. *J Clin Immunol* (2011) 31(4):606–14. doi: 10.1007/s10875-011-9540-8
65. Zhao L, Jiang Z, Jiang Y, Ma N, Zhang Y, Feng L, et al. IL-22+ CD4+ T Cells in Patients With Rheumatoid Arthritis. *Int J Rheum Dis* (2013) 16(5):518–26. doi: 10.1111/1756-185x.12099
66. Zhao L, Jiang Z, Jiang Y, Ma N, Wang K, Zhang Y, et al. IL-22+CD4+ T-Cells in Patients With Active Systemic Lupus Erythematosus. *Exp Biol Med (Maywood)* (2013) 238(2):193–9. doi: 10.1177/1535370213477597
67. Zhong W, Jiang Y, Ma H, Wu J, Jiang Z, Zhao L. Elevated Levels of CCR6(+) T Helper 22 Cells Correlate With Skin and Renal Impairment in Systemic Lupus Erythematosus. *Sci Rep* (2017) 7(1):12962. doi: 10.1038/s41598-017-13344-w

68. Yang X, Weng Q, Hu L, Yang L, Wang X, Xiang X, et al. Increased Interleukin-22 Levels in Lupus Nephritis and Its Associated With Disease Severity: A Study in Both Patients and Lupus-Like Mice Model. *Clin Exp Rheumatol* (2019) 37(3):400–7.
69. Pan HF, Zhao XF, Yuan H, Zhang WH, Li XP, Wang GH, et al. Decreased Serum IL-22 Levels in Patients With Systemic Lupus Erythematosus. *Clin Chim Acta* (2009) 401(1-2):179–80. doi: 10.1016/j.cca.2008.11.009
70. Lin J, Yue LH, Chen WQ. Decreased Plasma IL-22 Levels and Correlations With IL-22-Producing T Helper Cells in Patients With New-Onset Systemic Lupus Erythematosus. *Scand J Immunol* (2014) 79(2):131–6. doi: 10.1111/sji.12135
71. Luk CC, Tam LS, Kwan BC, Wong PC, Ma TK, Chow KM, et al. Intrarenal and Urinary Th9 and Th22 Cytokine Gene Expression in Lupus Nephritis. *J Rheumatol* (2015) 42(7):1150–5. doi: 10.3899/jrheum.140954
72. Luan L, Ding Y, Han S, Zhang Z, Liu X. An Increased Proportion of Circulating Th22 and Tc22 Cells in Psoriasis. *Cell Immunol* (2014) 290 (2):196–200. doi: 10.1016/j.cellimm.2014.06.007
73. Kagami S, Rizzo HL, Lee JJ, Koguchi Y, Blauvelt A. Circulating Th17,Th22, and Th1 Cells are Increased in Psoriasis. *J Invest Dermatol* (2010) 130 (5):1373–83. doi: 10.1038/jid.2009.399
74. Michalak-Stoma A, Bartosińska J, Kowal M, Juszkiewicz-Borowiec M, Gerkowicz A, Chodorowska G. Serum Levels of Selected Th17 and Th22 Cytokines in Psoriatic Patients. *Dis Markers* (2013) 35(6):625–31. doi: 10.1155/2013/856056
75. Xu W, Li R, Dai Y, Wu A, Wang H, Cheng C, et al. IL-22 Secreting CD4+ T Cells in the Patients With Neuromyelitis Optica and Multiple Sclerosis. *J Neuroimmunol* (2013) 261(1-2):87–91. doi: 10.1016/j.jneuroim.2013.04.021
76. Rolla S, Bardina V, De Mercanti S, Quaglino P, De Palma R, Gned D, et al. Th22 Cells are Expanded in Multiple Sclerosis and Are Resistant to IFN-β. *J Leukocyte Biol* (2014) 96(6):1155–64. doi: 10.1189/jlb.5A0813-463RR
77. Hu Y, Li H, Zhang L, Shan B, Xu X, Li H, et al. Elevated Profiles of Th22 Cells and Correlations With Th17 Cells in Patients With Immune Thrombocytopenia. *Hum Immunol* (2012) 73(6):629–35. doi: 10.1016/j.humimm.2012.04.015
78. Cao J, Chen C, Li L, Ling-yu Z, Zhen-yu L, Zhi-ling Y, et al. Effects of High-Dose Dexamethasone on Regulating Interleukin-22 Production and Correcting Th1 and Th22 Polarization in Immune Thrombocytopenia. *J Clin Immunol* (2012) 32(3):523–9. doi: 10.1007/s10875-012-9649-4
79. Zhan FX, Li J, Fang M, Ding J, Wang Q. Importance of Th22 Cell Disequilibrium in Immune Thrombocytopenic Purpura. *Med Sci Monit* (2018) 24:8767–72. doi: 10.12659/msm.912528
80. Ruszkowski J, Lisowska KA, Pindel M, Heleniak Z, Dębska-Ślizień A. Witkowski JM. T Cells in IgA Nephropathy: Role in Pathogenesis, Clinical Significance and Potential Therapeutic Target. *Clin Exp Nephrol* (2019) 23 (3):291–303. doi: 10.1007/s10157-018-1665-0
81. Peng Z, Tian J, Cui X, Xian W, Sun H, Li E, et al. Increased Number of Th22 Cells and Correlation With Th17 Cells in Peripheral Blood of Patients With IgA Nephropathy. *Hum Immunol* (2013) 74(12):1586–91. doi: 10.1016/j.humimm.2013.08.001
82. Liang M, Liwen Z, Yun Z, Yanbo D, Jianping C. The Imbalance Between Foxp3(+)Tregs and Th1/Th17/Th22 Cells in Patients With Newly Diagnosed Autoimmune Hepatitis. *J Immunol Res* (2018) 2018:3753081. doi: 10.1155/2018/3753081
83. Peng D, Xu B, Wang Y, Guo H, Jiang Y. A High Frequency of Circulating Th22 and Th17 Cells in Patients With New Onset Graves' Disease. *PloS One* (2013) 8(7):e68446. doi: 10.1371/journal.pone.0068446
84. Vitales-Noyola M, Ramos-Levi AM, Martínez-Hernández R, Serrano-Somavilla A, Sampedro-Nuñez M, González-Amaro R, et al. Pathogenic Th17 and Th22 Cells Are Increased in Patients With Autoimmune Thyroid Disorders. *Endocrine* (2017) 57(3):409–17. doi: 10.1007/s12020-017-1361-y
85. Bai X, Sun J, Wang W, Shan Z, Zheng H, Li Y, et al. Increased Differentiation of Th22 Cells in Hashimoto's Thyroiditis. *Endocr J* (2014) 61(12):1181–90. doi: 10.1507/endocrj.EJ14-0265
86. Ruggeri RM, Minciullo P, Saitta S, Giovinazzo S, Certo R, Campennì A, et al. Serum Interleukin-22 (IL-22) Is Increased in the Early Stage of Hashimoto's Thyroiditis Compared to Non-Autoimmune Thyroid Disease and Healthy Controls. *Hormones (Athens)* (2014) 13(3):338–44. doi: 10.14310/horm.2002.1483
87. Truchetet ME, Brembilla NC, Montanari E, Allanore Y, Chizzolini C. Increased Frequency of Circulating Th22 in Addition to Th17 and Th2 Lymphocytes in Systemic Sclerosis: Association With Interstitial Lung Disease. *Arthrit Res Ther* (2011) 13(5):R166. doi: 10.1186/ar3486
88. Mathian A, Parizot C, Dorgham K, Trad S, Arnaud L, Larsen M, et al. Activated and Resting Regulatory T Cell Exhaustion Concurs With High Levels of Interleukin-22 Expression in Systemic Sclerosis Lesions. *Ann Rheum Dis* (2012) 71(7):1227–34. doi: 10.1136/annrheumdis-2011-200709
89. Brembilla NC, Dufour AM, Alvarez M, Hugues S, Montanari E, Truchetet ME, et al. IL-22 Capacitates Dermal Fibroblast Responses to TNF in Scleroderma. *Ann Rheum Dis* (2016) 75(9):1697–705. doi: 10.1136/annrheumdis-2015-207477
90. Toussirot É, Laheurte C, Gaugler B, Gabriel D, Saas P. Increased IL-22- and IL-17a-Producing Mucosal-Associated Invariant T Cells in the Peripheral Blood of Patients With Ankylosing Spondylitis. *Front Immunol* (2018) 9:1610. doi: 10.3389/fimmu.2018.01610
91. Zerbini A, Muratore F, Boiardi L, Ciccia F, Bonacini M, Belloni L, et al. Increased Expression of Interleukin-22 in Patients With Giant Cell Arteritis. *Rheumatol (Oxford)* (2018) 57(1):64–72. doi: 10.1093/rheumatology/kex334
92. Lindahl H, Guerreiro-Cacais AO, Bedri SK, Linnerbauer M, Lindén M, Abdelmagid N, et al. IL-22 Binding Protein Promotes the Disease Process in Multiple Sclerosis. *J Immunol* (2019) 203(4):888–98. doi: 10.4049/jimmunol.1900400
93. Robat-Jazi B, Hosseini M, Shaygannejad V, Nafissi S, Rezaei A, Mansourain M, et al. High Frequency of Tc22 and Th22 Cells in Myasthenia Gravis Patients and Their Significant Reduction After Thymectomy. *Neuroimmunomodulation* (2018) 25(2):80–8. doi: 10.1159/000490855
94. Zheng S, Dou C, Xin N, Wang J, Wang J, Li P, et al. Expression of Interleukin-22 in Myasthenia Gravis. *Scand J Immunol* (2013) 78(1):98–107. doi: 10.1111/sji.12057
95. Li S, Yin H, Zhang K, Wang T, Yang Y, Liu X, et al. Effector T Helper Cell Populations Are Elevated in the Bone Marrow of Rheumatoid Arthritis Patients and Correlate With Disease Severity. *Sci Rep* (2017) 7(1):4776. doi: 10.1038/s41598-017-05014-8
96. Zhong W, Zhao L, Liu T, Jiang Z. IL-22-Producing CD4+T Cells in the Treatment Response of Rheumatoid Arthritis to Combination Therapy With Methotrexate and Leflunomide. *Sci Rep* (2017) 7:41143. doi: 10.1038/srep41143
97. van Hamburg JP, Corneth OB, Paulissen SM, Davelaar N, Asmawidjaja PS, Mus AM, et al. IL-17/Th17 Mediated Synovial Inflammation is IL-22 Independent. *Ann Rheum Dis* (2013) 72(10):1700–7. doi: 10.1136/annrheumdis-2012-202373
98. Miyazaki Y, Nakayamada S, Kubo S, Nakano K, Iwata S, Miyagawa I, et al. Th22 Cells Promote Osteoclast Differentiation *via* Production of IL-22 in Rheumatoid Arthritis. *Front Immunol* (2018) 9:2901. doi: 10.3389/fimmu.2018.02901
99. Bakheet SA, Ansari MA, Nadeem A, Attia SM, Alhoshani AR, Gul G, et al. CXCR3 Antagonist AMG487 Suppresses Rheumatoid Arthritis Pathogenesis and Progression by Shifting the Th17/Treg Cell Balance. *Cell Signal* (2019) 64:109395. doi: 10.1016/j.cellsig.2019.109395
100. Zhao L, Ma H, Jiang Z, Jiang Y, Ma N. Immunoregulation Therapy Changes the Frequency of Interleukin (IL)-22+ CD4+ T Cells in Systemic Lupus Erythematosus Patients. *Clin Exp Immunol* (2014) 177(1):212–8. doi: 10.1111/cei.12330
101. Rekik R, Smiti Khanfir M, Larbi T, Zamali I, Beldi-Ferchiou A, Kammoun O, et al. Impaired TGF-β Signaling in Patients With Active Systemic Lupus Erythematosus is Associated With an Overexpression of IL-22. *Cytokine* (2018) 108:182–9. doi: 10.1016/j.cyto.2018.04.011
102. Yang XY, Wang HY, Zhao XY, Wang LJ, Lv QH, Wang QQ. Th22, But Not Th17 Might be a Good Index to Predict the Tissue Involvement of Systemic Lupus Erythematosus. *J Clin Immunol* (2013) 33(4):767–74. doi: 10.1007/s10875-013-9878-1
103. Wang R, Zeng YL, Qin HM, Lu YL, Huang HT, Lei M, et al. Association of Interleukin 22 Gene Polymorphisms and Serum IL-22 Level With Risk of Systemic Lupus Erythematosus in a Chinese Population. *Clin Exp Immunol* (2018) 193(2):143–51. doi: 10.1111/cei.13133
104. Farber DL, Yudanin NA, Restifo NP. Human Memory T Cells: Generation, Compartmentalization and Homeostasis. *Nat Rev Immunol* (2014) 14(1):24–35. doi: 10.1038/nri3567

105. Owczarczyk Saczonek A, Krajewska-Włodarczyk M, Kasprowicz-Furmańczyk M, Placek W. Immunological Memory of Psoriatic Lesions. *Int J Mol Sci* (2020) 21(2):625. doi: 10.3390/ijms21020625
106. Guilloteau K, Paris I, Pedretti N, Boniface K, Juchaux F, Huguier V, et al. Skin Inflammation Induced by the Synergistic Action of IL-17a,IL-22IL-1 {Alpha},and TNF-{Alpha} Recapitulates Some Features of Psoriasis. *J Immunol* (2010) 184(9):5263–70. doi: 10.4049/jimmunol.0902464
107. Guttman-Yassky E, Nograles KE, Krueger JG. Contrasting Pathogenesis of Atopic Dermatitis and Psoriasis–Part II: Immune Cell Subsets and Therapeutic Concepts. *J Allergy Clin Immunol* (2011) 127(6):1420–32. doi: 10.1016/j.jaci.2011.01.054
108. Yang X, Zheng SG. Interleukin-22: A Likely Target for Treatment of Autoimmune Diseases. *Autoimmun Rev* (2014) 13(6):615–20. doi: 10.1016/j.autrev.2013.11.008
109. Georgescu SR, Tampa M, Caruntu C, Sarbu MI, Mitran CI, Mitran MI, et al. Advances in Understanding the Immunological Pathways in Psoriasis. *Int J Mol Sci* (2019) 20(3):739. doi: 10.3390/ijms20030739
110. Zhang N, Pan HF, Ye DQ. Th22 in Inflammatory and Autoimmune Disease: Prospects for Therapeutic Intervention. *Mol Cell Biochem* (2011) 353(1-2):41–6. doi: 10.1007/s11010-011-0772-y
111. Van Belle AB, de Heusch M, Lemaire MM, Hendrickx E, Warnier G, Dunussi-Joannopoulos K, et al. IL-22 is Required for Imiquimod-Induced Psoriasiform Skin Inflammation in Mice. *J Immunol* (2012) 188(1):462–9. doi: 10.4049/jimmunol.1102224
112. Diani M, Altomare G. Reali E. T Helper Cell Subsets in Clinical Manifestations of Psoriasis. *J Immunol Res* (2016) 2016:7692024. doi: 10.1155/2016/7692024
113. Cheuk S, Wikén M, Blomqvist L, Nylén S, Talme T, Ståhle M, et al. Epidermal Th22 and Tc17 Cells Form a Localized Disease Memory in Clinically Healed Psoriasis. *J Immunol* (2014) 192(7):3111–20. doi: 10.4049/jimmunol.1302313
114. Le ST, Merleev AA, Luxardi G, Shimoda M, Adamopoulos IE, Tsoi LC, et al. 2d Visualization of the Psoriasis Transcriptome Fails to Support the Existence of Dual-Secreting IL-17a/IL-22 Th17 T Cells. *Front Immunol* (2019) 10:589. doi: 10.3389/fimmu.2019.00589
115. Almolda B, Costa M, Montoya M, González B, Castellano B. Increase in Th17 and T-Reg Lymphocytes and Decrease of IL22 Correlate With the Recovery Phase of Acute EAE in Rat. *PloS One* (2011) 6(11):e27473. doi: 10.1371/journal.pone.0027473
116. Muls N, Nasr Z, Dang HA, Sindic C, van Pesch V. IL-22,GM-CSF and IL-17 in Peripheral CD4+ T Cell Subpopulations During Multiple Sclerosis Relapses and Remission. Impact of Corticosteroid Therapy. *PloS One* (2017) 12(3):e0173780. doi: 10.1371/journal.pone.0173780
117. Wing AC, Hygino J, Ferreira TB, Kasahara TM, Barros PO, Sacramento PM, et al. Interleukin-17- and Interleukin-22-Secreting Myelin-Specific CD4(+) T Cells Resistant to Corticoids are Related With Active Brain Lesions in Multiple Sclerosis Patients. *Immunology* (2016) 147(2):212–20. doi: 10.1111/imm.12552
118. Alvarenga-Filho H, Salles M, Hygino J, Ferreira TB, Sacramento PM, Monteiro C, et al. Fatigue Favors *in vitro* Th1 and Th17-like cell expansion and reduces corticoid sensitivity in MS patients. *J Neuroimmunol* (2017) 303:81–9. doi: 10.1016/j.jneuroim.2016.12.013
119. Perriard G, Mathias A, Enz L, Canales M, Schluep M, Gentner M, et al. Interleukin-22 is Increased in Multiple Sclerosis Patients and Targets Astrocytes. *J Neuroinflamm* (2015) 12:119. doi: 10.1186/s12974-015-0335-3
120. Bai Z, Chen D, Wang L, Zhao Y, Liu T, Yu Y, et al. Cerebrospinal Fluid and Blood Cytokines as Biomarkers for Multiple Sclerosis: A Systematic Review and Meta-Analysis of 226 Studies With 13,526 Multiple Sclerosis Patients. *Front Neurosci* (2019) 13:1026. doi: 10.3389/fnins.2019.01026
121. Magliozzi R, Howell OW, Nicholas R, Cruciani C, Castellaro M, Romualdi C, et al. Inflammatory Intrathecal Profiles and Cortical Damage in Multiple Sclerosis. *Ann Neurol* (2018) 83(4):739–55. doi: 10.1002/ana.25197
122. Jaime-Pérez JC, Turrubiates-Hernández GA, López-Silva LJ, Salazar-Riojas R, Gómez-Almaguer D. Early Changes in IL-21, IL-22, CCL2, and CCL4 Serum Cytokines After Outpatient Autologous Transplantation for Multiple Sclerosis: A Proof of Concept Study. *Clin Transplant* (2020) 34(12):e14114. doi: 10.1111/ctr.14114
123. Zhen J, Yuan J, Fu Y, Zhu R, Wang M, Chang H, et al. IL-22 Promotes Fas Expression in Oligodendrocytes and Inhibits FOXP3 Expression in T Cells by Activating the NF-κb Pathway in Multiple Sclerosis. *Mol Immunol* (2017) 82:84–93. doi: 10.1016/j.molimm.2016.12.020
124. Beyeen AD, Adzemovic MZ, Ockinger J, Stridh P, Becanovic K, Laaksonen H, et al. IL-22RA2 Associates With Multiple Sclerosis and Macrophage Effector Mechanisms in Experimental Neuroinflammation. *J Immunol* (2010) 185(11):6883–90. doi: 10.4049/jimmunol.1001392
125. Shabgah AG, Navashenaq JG, Shabgah OG, Mohammadi H, Sahebkar A. Interleukin-22 in Human Inflammatory Diseases and Viral Infections. *Autoimmun Rev* (2017) 16(12):1209–18. doi: 10.1016/j.autrev.2017.10.004
126. Laaksonen H, Guerreiro-Cacais AO, Adzemovic MZ, Parsa R, Zeitelhofer M, Jagodic M, et al. The Multiple Sclerosis Risk Gene IL22RA2 Contributes to a More Severe Murine Autoimmune Neuroinflammation. *Genes Immun* (2014) 15(7):457–65. doi: 10.1038/gene.2014.36
127. Kostic M, Zivkovic N, Cvetanovic A, Marjanović G. CD4(+) T Cell Phenotypes in the Pathogenesis of Immune Thrombocytopenia. *Cell Immunol* (2020) 351:104096. doi: 10.1016/j.cellimm.2020.104096
128. Cao J, Chen C, Zeng L, Li L, Li X, Li Z, et al. Elevated Plasma IL-22 Levels Correlated With Th1 and Th22 Cells in Patients With Immune Thrombocytopenia. *Clin Immunol* (2011) 141(1):121–3. doi: 10.1016/j.clim.2011.05.003
129. Jernås M, Hou Y, Strömberg Célind F, Shao L, Wang Q, Ju X, et al. Altered Cytokine Levels in Pediatric ITP. *Platelets* (2015) 26(6):589–92. doi: 10.3109/09537104.2014.974526
130. Wang Q, Li J, Yu TS, Liu Y, Li K, Liu S, et al. Disrupted Balance of CD4(+) T-Cell Subsets in Bone Marrow of Patients With Primary Immune Thrombocytopenia. *Int J Biol Sci* (2019) 15(13):2798–814. doi: 10.7150/ijbs.33779
131. Lai KN, Tang SC, Schena FP, Novak J, Tomino Y, Fogo AB, et al. IgA Nephropathy. *Nat Rev Dis Primers* (2016) 2:16001. doi: 10.1038/nrdp.2016.1
132. Gan L, Zhu M, Li X, Chen C, Meng T, Pu J, et al. Tonsillitis Exacerbates Renal Injury in IgA Nephropathy Through Promoting Th22 Cells Chemotaxis. *Int Urol Nephrol* (2018) 50(7):1285–92. doi: 10.1007/s11255-018-1792-2
133. Xiao C, Xiao P, Li X, Huang G, Li H, Chen Y. Streptococcus may Aggravate Inflammatory Damage in Chronic Nephritis *via* the chemotaxis of Th22 cells. *Am J Transl Res* (2019) 11(12):7432–40.
134. Weathington NM, Snavely CA, Chen BB, Zhao J, Zhao Y, Mallampalli RK. Glycogen Synthase Kinase-3β Stabilizes the Interleukin (IL)-22 Receptor From Proteasomal Degradation in Murine Lung Epithelia. *J Biol Chem* (2014) 289(25):17610–9. doi: 10.1074/jbc.M114.551747
135. Gan L, Zhou Q, Li X, Chen C, Meng T, Pu J, et al. Intrinsic Renal Cells Induce Lymphocytosis of Th22 Cells From IgA Nephropathy Patients Through B7-CTLA-4 and CCL-CCR Pathways. *Mol Cell Biochem* (2018) 441(1-2):191–9. doi: 10.1007/s11010-017-3185-8
136. Xiao C, Zhou Q, Li X, Li H, Zhong Y, Meng T, et al. Losartan and Dexamethasone may Inhibit Chemotaxis to Reduce the Infiltration of Th22 Cells in IgA Nephropathy. *Int Immunopharmacol* (2017) 42:203–8. doi: 10.1016/j.intimp.2016.11.025
137. Gan L, Li X, Zhu M, Chen C, Luo H, Zhou Q. Acteoside Relieves Mesangial Cell Injury by Regulating Th22 Cell Chemotaxis and Proliferation in IgA Nephropathy. *Ren Fail* (2018) 40(1):364–70. doi: 10.1080/0886022x.2018.1450762
138. Suh JS, Cho SH, Chung JH, Moon A, Park YK. Cho BS. A Polymorphism of Interleukin-22 Receptor Alpha-1 Is Associated With the Development of Childhood IgA Nephropathy. *J Interferon Cytokine Res* (2013) 33(10):571–7. doi: 10.1089/jir.2012.0097
139. Seki E, Brenner DA. Recent Advancement of Molecular Mechanisms of Liver Fibrosis. *J Hepatobiliary Pancreat Sci* (2015) 22(7):512–8. doi: 10.1002/jhbp.245
140. Radaeva S, Sun R, Pan HN, Hong F, Gao B. Interleukin 22 (IL-22) Plays a Protective Role in T Cell-Mediated Murine Hepatitis: IL-22 Is a Survival Factor for Hepatocytes *via* STAT3 activation. *Hepatology* (2004) 39(5):1332–42. doi: 10.1002/hep.20184
141. Feng D. Interleukin-22, Liver Progenitor Cells,and Liver Cancer. *Hepatology* (2014) 60(1):427–8. doi: 10.1002/hep.26899

142. Lai R, Xiang X, Mo R, Bao R, Wang P, Guo S, et al. Protective Effect of Th22 Cells and Intrahepatic IL-22 in Drug Induced Hepatocellular Injury. *J Hepatol* (2015) 63(1):148–55. doi: 10.1016/j.jhep.2015.02.004
143. Mo R, Wang P, Lai R, Li F, Liu Y, Jiang S, et al. Persistently Elevated Circulating Th22 Reversely Correlates With Prognosis in HBV-Related Acute-on-Chronic Liver Failure. *J Gastroenterol Hepatol* (2017) 32(3):677–86. doi: 10.1111/jgh.13537
144. Thomas-Dupont P, Remes-Troche JM, Izaguirre-Hernández IY, Sánchez-Vargas LA, Maldonado-Rentería Mde J, Hernández-Flores KG, et al. Elevated circulating levels of IL-21 and IL-22 define a cytokine signature profile in type 2 autoimmune hepatitis patients. *Ann Hepatol* (2016) 15(4):550–8.
145. Li Q, Wang B, Mu K, Zhang JA. The Pathogenesis of Thyroid Autoimmune Diseases: New T Lymphocytes - Cytokines Circuits Beyond the Th1-Th2 Paradigm. *J Cell Physiol* (2019) 234(3):2204–16. doi: 10.1002/jcp.27180
146. Song RH, Yu ZY, Qin Q, Wang X, Muhali FS, Shi LF, et al. Different Levels of Circulating Th22 Cell and its Related Molecules in Graves' Disease and Hashimoto's Thyroiditis. *Int J Clin Exp Pathol* (2014) 7(7):4024–31.
147. Binks S, Vincent A, Palace J. Myasthenia Gravis: A Clinical-Immunological Update. *J Neurol* (2016) 263(4):826–34. doi: 10.1007/s00415-015-7963-5
148. Wang L, Zhang Y, Zhu M, Feng J, Han J, Zhu J, et al. Effects of Follicular Helper T Cells and Inflammatory Cytokines on Myasthenia Gravis. *Curr Mol Med* (2019) 19(10):739–45. doi: 10.2174/1566524019666190827162615
149. Liu M, Wu W, Sun X, Yang J, Xu J, Fu W, et al. New Insights Into CD4(+) T Cell Abnormalities in Systemic Sclerosis. *Cytokine Growth Factor Rev* (2016) 28:31–6. doi: 10.1016/j.cytogfr.2015.12.002
150. Zhang M. Zhang S. T Cells in Fibrosis and Fibrotic Diseases. *Front Immunol* (2020) 11:1142. doi: 10.3389/fimmu.2020.01142
151. Furuzawa-Carballeda J, Sánchez-Guerrero J, Betanzos JL, Enriquez AB, Avila-Casado C, Llorente L, et al. Differential Cytokine Expression and Regulatory Cells in Patients With Primary and Secondary Sjögren's Syndrome. *Scand J Immunol* (2014) 80(6):432–40. doi: 10.1111/sji.12224
152. Ciccia F, Guggino G, Rizzo A, Ferrante A, Raimondo S, Giardina A, et al. Potential Involvement of IL-22 and IL-22-Producing Cells in the Inflamed Salivary Glands of Patients With Sjogren's Syndrome. *Ann Rheum Dis* (2012) 71(2):295–301. doi: 10.1136/ard.2011.154013
153. Ciccia F, Guggino G, Giardina A, Ferrante A, Carrubbi F, Giacomelli R, et al. The Role of Innate and Lymphoid IL-22-Producing Cells in the Immunopathology of Primary Sjögren's Syndrome. *Expert Rev Clin Immunol* (2014) 10(4):533–41. doi: 10.1586/1744666x.2014.884461
154. Shen H, Goodall JC, Hill Gaston JS. Frequency and Phenotype of Peripheral Blood Th17 Cells in Ankylosing Spondylitis and Rheumatoid Arthritis. *Arthritis Rheum* (2009) 60(6):1647–56. doi: 10.1002/art.24568
155. Uppal SK, Kearns DG, Chat VS, Han G, Wu JJ. Review and Analysis of Biologic Therapies Currently in Phase II and Phase III Clinical Trials for Atopic Dermatitis. *J Dermatolog Treat* (2020) 1–11. doi: 10.1080/09546634.2020.1775775
156. Guttman-Yassky E, Brunner PM, Neumann AU, Khattri S, Pavel AB, Malik K, et al. Efficacy and Safety of Fezakinumab (an IL-22 Monoclonal Antibody) in Adults With Moderate-to-Severe Atopic Dermatitis Inadequately Controlled by Conventional Treatments: A Randomized, Double-Blind, Phase 2a Trial. *J Am Acad Dermatol* (2018) 78(5):872–81.e6. doi: 10.1016/j.jaad.2018.01.016
157. Brunner PM, Pavel AB, Khattri S, Leonard A, Malik K, Rose S, et al. Baseline IL-22 Expression in Patients With Atopic Dermatitis Stratifies Tissue Responses to Fezakinumab. *J Allergy Clin Immunol* (2019) 143(1):142–54. doi: 10.1016/j.jaci.2018.07.028
158. Perusina Lanfranca M, Lin Y, Fang J, Zou W, Frankel T. Biological and Pathological Activities of Interleukin-22. *J Mol Med (Berl)* (2016) 94(5):523–34. doi: 10.1007/s00109-016-1391-6

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REVIEW
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Revisiting the Antigen-Presenting Function of β Cells in T1D Pathogenesis

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Type 1 diabetes (T1D) is characterized by the unresolved autoimmune inflammation and islet β cell destruction. The islet resident antigen-presenting cells (APCs) including dendritic cells and macrophages uptake and process the β cell-derived antigens to prime the autoreactive diabetogenic T cells. Upon activation, those autoreactive T cells produce copious amount of IFN-γ, TNF-α and IL-1β to induce β cell stress and death. Autoimmune attack and β cell damage intertwine together to push forward this self-destructive program, leading to T1D onset. However, β cells are far beyond a passive participant during the course of T1D development. Herein in this review, we summarized how β cells are actively involved in the initiation of autoimmune responses in T1D setting. Specifically, β cells produce modified neoantigens under stressed condition, which is coupled with upregulated expression of MHC I/II and co-stimulatory molecules as well as other immune modules, that are essential properties normally exhibited by the professional APCs. At the cellular level, this subset of APC-like β cells dynamically interacts with plasmacytoid dendritic cells (pDCs) and manifests potency to activate autoreactive CD4 and CD8 T cells, by which β cells initiate early autoimmune responses predisposing to T1D development. Overall, the antigen-presenting function of β cells helps to explain the tissue specificity of T1D and highlights the active roles of structural cells played in the pathogenesis of various immune related disorders.

Keywords: β cell, antigen presentation, autoimmune diabetes, innate immunity, crosstalk

INTRODUCTION

Since the early 1970s, type 1 diabetes (T1D) has been defined as an autoimmune disorder resulting from the intolerance to pancreatic β cell derived auto-antigens (1, 2), and subsequent studies have consistently demonstrated that islet β cell dysfunction and immune cell autoreactive response contribute to disease progression, while the exact mechanisms largely remain unknown. In the canonical paradigm, the damaged β cells expose self-antigens to resident or patrolling antigen presenting cells (APC) to initiate the immune process (3, 4). Indeed, polymorphisms within the class II major histocompatibility complex (MHC II) (e.g., I-A^{g7} in non-obese diabetic (NOD) mice

and HLA-DQ8 in human counterparts), strongly correlate with T1D propensity. Such MHC haplotype generates the anchoring site of MHC molecules that favors the binding of self-peptide, thereby facilitating the subsequent APC-T cell interaction (4). Other genetic predisposing factors further exacerbate the immune reactivity against β cells (5, 6). Given many of the identified autoantigens are not confined to islet and the autoreactive T cells constantly patrol in the peripheral circulation, it is, therefore, hard to explain the tissue specificity of T1D.

Accumulating evidence reveals that structural cells are not mere passive participants in immune related disorders. They can actively produce cytokines, chemokines, and even the MHC II molecules, a hallmark that is traditionally considered as a privilege of professional APCs. The emerging concept of "structural cell immunology" blurs the boundary between immune cells and non-immune cells, extends our understanding of the immune response initiation, and provides a chance to re-scrutinize the established disease pathogenesis (7). This concept is extensively corroborated in the tumor microenvironment (TME), where non-hematopoietic cells such as tumor cells, fibroblasts and other mesenchymal cells dynamically shape the anti-tumor immune response. This is also the case in other immune engaged disorders. For example, obese mice have an obvious increase in average adipocyte size, and the larger adipocytes express higher level of MHC II than the smaller ones, which directly activate T cells during the course of obesity (8–10). Endothelial cells (ECs) express Toll-like receptor 4 (TLR4) and receptors for TNF-α and IL-1β, and therefore, LPS stimulation activates ECs to produce pro-inflammatory cytokines and chemokines, which then recruit immune cells and propagate the immune response (10). Notably, under certain conditions, ECs express both MHC I and MHC II and directly present antigens to T cells by acting as APC like cells (11). In rheumatoid arthritis (RA), the fibroblast-like synoviocytes (FLS) in the inflamed synovium share similar intrinsic properties as the follicular dendritic cells (FDCs), which help to attenuate apoptosis of germinal center (GC) B lymphocytes and exacerbate disease progression (12). These previously unappreciated immune functions of non-immune cells give us insights that abnormality of structural and/or stromal cells may well interpret the etiological origin of autoimmune diseases.

It was noted that cytokines such as IFN-γ, TNF-α and IL-1β following autoimmune attacks elicit ER and oxidative stress to cause β cell damage. The impaired β cells, however, are able to secret chemokines to motivate more immune cells and the damage associated molecular patterns (DAMPs) to push forward this vicious cycle of crosstalk (13). Nonetheless, two critical questions are yet to be elucidated: what happened to β cells even before an obvious autoimmune strike? Would β cells be both victims and culprits in early T1D pathogenesis? In fact, β cells have the ability to express MHC and costimulatory molecules to prime the adaptive immune response. Based on such intriguing findings, we herein intend to summarize the characteristics of APC-like β cells and to sort out factors that endow β cell with the antigen-presenting function. We would also discuss how APC-like β cells regulate T1D development and highlight the potential intervention strategies against T1D in clinical settings.

PRESENCE OF APC-LIKE β CELLS IN THE ISLETS

Antigenic peptide MHC complex (pMHC) provides the first signal for antigen presentation. Most islet antigens have been identified by HLA binding/tetramers, including insulin, proinsulin, islet antigen 2 (IA-2) and glutamic acid decarboxylase 65 (GAD65) (14–17). Other antigens such as islet amyloid polypeptide (IAPP) and glucose-regulated protein 78 (GRP78) have been distinguished through analysis of antigen-specific T cells (18). MHC class I, expressed essentially in all nucleated cells, presents intracellular peptides onto β cell surface, which directly leads to the activation of $CD8^+$ T cells, an early feature of T1D development (19). CD4 T cells which specifically recognize peptide MHC class II complex, exert effector function and help B cells in autoantibody generation, which is essential to drive prolonged islet inflammation (20). Initially, most studies have considered that MHC class II is exclusively expressed in local professional APCs such as dendritic cells and macrophages (21). Intriguingly, several studies suggested that a proportion of β cells from type 1 diabetic patients or NOD mice also express MHC class II molecules (22–24). Similarly, RNA-Seq and immunohistological analysis demonstrated that β cells from recent-onset type 1 diabetic donors express MHC class II and its transcriptional regulator class II major histocompatibility complex trans-activator (CIITA) protein, which was hardly detectable in the islet cells of non-diabetic donors (25). Moreover, the $I\text{-}Ag^7$ expressed β cells isolated from islets of diabetic NOD mice could independently induce proliferation of $CD4^+$ T cells *in vitro* (26). In this case, β cells may serve as the APC-like cells in presenting autoantigen to activate islet-infiltrating $CD4^+$ T cells.

Other than acquisition of MHC molecule, a set of second signals are also required to act as APCs. For instance, co-stimulatory molecules and cell-adhesion molecules, key components in the formation of immunological synapse, are necessary for the optimal activation of antigen-specific T cells. Although no evidence shows the expression of B7-1/B7-2 (CD80/CD86) on human pancreatic β cells, transgenic overexpression of B7-1 on NOD pancreatic islet accelerates the progression of type 1 diabetes (27, 28). Clustering of T cells with APCs is primarily mediated by the interaction between lymphocyte function-associated-1 (LFA-1) on the surface of lymphocytes and the intercellular adhesion molecule-1 (ICAM-1) on the APC cells. ICAM-1 was not expressed on the surface of normal human islet cells, but it can be detected following a 72h induction by IFN-γ or TNF-α (29). Collectively, these lines of evidence indicate that islet β cells display essential phenotypic characteristics that are normally possessed by the classical APCs, which supports the idea that β cells actively engage in the initiation of autoimmune response and is responsible for their own demise in T1D pathogenesis.

POTENTIAL TRIGGERS FOR THE FORMATION OF APC-LIKE β CELLS

In β cell, generation of neoepitopes is associated with initial loss of immune tolerance, evidenced by local infiltration of effector T

cells and the emergence of autoantibodies. Post-translational modification (PTM) affects protein properties, which is generally involved in normal physiological process including establishment of immune tolerance during the thymic and peripheral selection (30). In some cases, however, abnormal PTM process might alter protein structure to create novel β cell-specific epitopes that are not tolerated by the immune system. For example, citrulline modified GAD65 in β cells elicits T cell response in T1D patients (15), and similar modification of GRP78 was observed in the pancreatic islet of NOD mice (31). This kind of changes in PTM can be induced by reactive oxygen species (32) and inflammatory cytokines, suggesting the relevance between β cellular stress and neoepitope formation (33, 34). Endoplasmic reticulum (ER) is necessary for β cell functionality since it undertakes biosynthesis of proinsulin. Under physiological condition, up to 20% of proinsulin fails to achieve the valid conformation, so the ER associated degradation (ERAD) and the unfolded protein response (UPR) signaling are critical for maintaining ER homeostasis. However, upon exposure to stressful microenvironments such as proinflammatory cytokines and ROS, proinsulin is more susceptible to undergo misfolding, which induces over-active UPR to cause ER stress (35). This pathological process could result in abnormal PTM of other proteins or direct generation of neoepitopes, which are then processed and presented by β cells and/or APCs (36). The analysis of NOD islet revealed that β cells deficient in IRE1α, a key component of ER stress, declines the expression of autoantigen and MHC class I complex (37). The hybrid insulin peptides (HIPs), another type of autoantigen in T1D, have been identified by sequencing epitopes from β cells in diabetic NOD mice. The fused peptide is produced through combination of proinsulin peptides with other peptides in β cell secretory granules, leading to a chimeric antigen with enhanced MHC binding affinity and the ability to break the immune tolerance (38). Unfortunately, the understanding of HIP formation remains at the phenomenal levels thus far, the underlying mechanisms are yet to be elucidated. Additional processes such as alternative RNA splicing also contributes to generation of neoepitopes. For example, mRNA splice peptide SCG5009, the product generated from the *Secretogranin V* gene, was processed by HLA molecules and presented to T cells in the pancreas to initiate T1D (39). In line with those observations, DNA and protein methylation events elicited by inflammatory cytokines or other stress signals have been found related to abnormal β cell activity and insulin expression (40).

Although the detailed mechanism largely remains elusive, the generation of neoantigens is apparently a consequence of β cell stress. The stress response can either stem from intrinsic cell abnormalities or external cytokine stimulations, which induce or exaggerate β cell dysfunction. The interferon (IFN) family contains IFN-α/β (type I) and IFN-γ (type II), both of which are strongly associated with T1D pathogenesis (41–43). IFN-α is a critical cytokine produced by the immune system against foreign virus or tissue damage (43, 44), while IFN-γ is secreted by T cells or NK cells with high potency in propagating islet inflammation (45). It is known that pancreatic IFN-α upregulates the expression of MHC I and costimulatory molecules on the pancreatic β cells in T1D patients, leading to autoantigen presentation and activation of cytotoxic $CD8^+$ T cells, which is considered as the early event of T1D (46, 47). Importantly, IFN-α alone, or IFN-γ plus TNF-α could induce MHC II gene expression in human islet coupled with the expression of the CIITA isoform (48, 49). Therefore, the IFN family members possess the ability to up-regulate the expression of MHC and molecules relevant to antigen presentation on the surface of islet β cells. As a result, external inflammatory cytokine stimulation and/or intrinsic β cell dysfunction induce neoantigen generation along with the expression of MHC and co-stimulatory molecules, which eventually endow β cells with antigen-presenting property (**Table 1**).

DYNAMIC CROSSTALK BETWEEN APC-LIKE β CELLS AND IMMUNE CELLS

It is believed that viral infection (Coxsackievirus in particular) is related to T1D pathogenesis, at least in part, through stimulating the production of type I IFN both in β cells and plasmacytoid dendritic cells (pDCs). Compared to β cells, pDCs have the

TABLE 1 | Key features of APC-like islet β cells.

Key features	Induced by	Effect	Reference
Neo-autoantigen generation			
Post-translationally modified peptides	ROS, ER stress	Abnormal modification of GAD65, GRP78 etc.; generation of neoepitopes	(15, 31, 33–35)
Hybrid insulin peptides	Islet inflammation	Producing chimeric antigen with enhanced MHC binding affinity; breaking immune tolerance	(38)
RNA splicing products	Inflammatory cytokines	Generation of non-self epitopes; activation of pancreas-infiltrating $CD8^+$ T lymphocytes	(39)
MHC molecule expression			
MHC-I	IFN-α	Presentation of intracellular peptides and activation of $CD8^+$ T cells	(19)
MHC-II	IFN-α; IFN-γ plus TNF-α	Activation of islet-infiltrating $CD4^+$ T cells	(22–24)
Co-stimulatory molecule expression			
B7-1	–	Providing the co-stimulatory signal for T cell activation	(27)
ICAM-1	IFN-γ, TNF-α	Facilitating the interaction between antigen presenting cell and T cell	(29)

ability to produce large amounts of IFN-α and IFN-β, which in turn act on β cells to induce the APC-like phenotype (50). IFN-α producing pDCs have been detected in the blood of T1D patients at their first diagnosis (51). In consistent with this observation, IFN-α therapy in patients with hepatitis C virus infection or leukemia is associated with an increased risk for developing T1D (32, 52). However, some studies also revealed that pDCs exhibit a negative regulatory role in T1D setting, as NOD mice without pDCs manifest exacerbated insulitis (53). Such contradiction implies a dual role for pDCs in T1D pathogenesis, which depends on the microenvironment created by β cells and other cell types. Indeed, pDCs present antigens acquired exogenously in a tolerogenic manner, thereby attenuating $CD8^+$ T cell proliferation (54). However, upon the presence of β cell damage, innate immune cells such as B-1a cells produce double-stranded DNA specific IgGs, and neutrophils generate DNA-binding cathelicidin-related antimicrobial peptide (CRAMP), both of which can activate pDCs to secrete IFN-α in the pancreatic islets (55). Therefore, pDCs not only sense the exogenous viral infection for immune defense, but also alarm the endogenous tissue damage signal to transform β cells into APC-like cells.

Accumulated evidence indicates that both $CD4^+$ and $CD8^+$ T cells are implicated in β cell destruction (56–58). Islet reactive T cells are initially primed and activated in the draining lymph nodes, after which they get entrance into the islet *via* pancreatic vasculature, leading to massive damage of β cells (59, 60). $CD8^+$ T cell is a heterogenous population consisting of $CD8^+$ $CD28^-$ T suppressor subset, which exhibits defect in pathologic immune responses (61, 62), and the cytotoxic subset, which is paramount in inducing β cell death. Insulin peptides are the typical autoantigens for type 1 diabetic patients. It was found that peptide derived from insulin B chain is processed by the proteasomes, and then translocated into the endoplasmic reticulum *via* the peptide transporter TAP1, by which they bind to HLA-A2 onto β cell surface to serve as the major target for cytotoxic $CD8^+$ T cell recognition (63). A recent study suggested that the circulating islet-reactive $CD8^+$ T cells are predominantly naïve and largely overlapped between T1D and healthy subjects (39), in which HLA I peptidomics and transcriptomic analysis were combined to identify the epitopes presented by β cells in T1D patients and healthy donors. It was interestingly noted that antigens processed by β cells through multiple pathways for HLA-A2 restricted presentation is crucial to activate circulating $CD8^+$ T cells (39).

Although the MHC II expressed β cells are capable of activating $CD4^+$ T cells, the detailed mechanisms, however, are not yet to be elucidated. Autophagy is an intracellular system that delivers damaged organelles and cytosolic proteins to lysosomes for degradation (64). Autophagy dependent processes participate in restricted antigen presentation through lysosome contained proteases, and promote MHC II presentation of peptides from intracellular source (65). For instance, studies found that $CD4^+$ T cells could specifically recognize citrullinated self-peptide presented by APCs, and autophagy plays a central role in the presentation of the post-translationally modified intercellular protein (66). It is therefore possible that β cells achieve the MHC II restricted cross-presentation of intracellular self-antigen in a manner similar as autophagy, which demands further investigations.

B cells are also central to T1D development. According to the functional specification, B cells are classified into three major subpopulations: the innate-like B1 cells (with $CD5^{high}$ for B1a and $CD5^{low}$ for B1b), the adaptive B2 cells (commonly noted B cells) and the regulatory B cell subset (Breg, marked by IL-10 production). As mentioned above, B1a cells are involved in T1D initiation by producing anti-dsDNA antibody upon sensing the β cell debris. However, this process is antigen non-specific and the antigen specific responses mediated by B2 cells may play an even greater role. Despite the presence of Breg subset, B cells are predominantly harmful as evidenced by the beneficial effect of total B cell depletion agents (e.g., anti-CD20 and anti-BAFF) (67, 68). B cells promote T1D progression by producing autoantibodies at the early phase and by presenting antigen to diabetogenic $CD4^+$ and $CD8^+$ T cells at the later phase. It is intriguing to note that the insulin reactive B cells are present in both T1D-prone NOD mice and T1D-resistant C57BL/6 mice (69). How the anergic state of B cells being breached in NOD mice and T1D patients is a big scientific question to answer. Cambier et al. revealed that BCR affinity, permissive pancreatic niche and abnormality in tolerance-regulating genes are essential for B cell mediated T1D pathogenesis (70). Moreover, the apparent transience of anergic B cell loss may well suggest that the loss of B cell anergy is a consequence of environmental insults, such as infection, injury and/or diet change (71). Thus, the APC-like β cells could aid in B cell escape of tolerance, resulting in an overt autoreactive B cell response.

SUMMARY AND PERSPECTIVE

Traditionally, professional APCs are considered to be the spotlight in T1D initiation. A population of $CD11c^+$ $CD103^+$ DCs relying on a transcriptional factor, the basic leucine zipper transcription factor AFT-like 3 (BATF3), has been identified in the murine islet. Those DCs are thought responsible for taking up and presenting antigens derived from secretory β cell granules and exogenous denatured proteins (72). Their presence in NOD mice is around 3- to 4-week of age, by then $CD4^+$ T cells simultaneously enter into the islet. $CD103^+$ DCs are also capable of cross-presenting and loading extracellularly acquired antigens onto MHC I to activate the autoreactive $CD8^+$ T cells (73, 74). Another important APC population is macrophage. Islet macrophages, originating from precursors from yolk sac, fetal liver and bone marrow, reside in the pancreas since embryonic development (75, 76). Lower phagocytotic activity of NOD macrophages compared to that of BALB/c (a mouse strain that is not prone to T1D) is suggested to impede the defective clearance of cell debris generated during the physiological β mass turnover, which then predisposes to T1D initiation (77). Studies in 3-week old NOD, NOD.$Rag1^{-/-}$ and B6.g7 mice revealed that islet macrophages are in an activated state with highly expressed MHC II, TNF and IL-1β, which are

comparable to macrophages in the barrier surface such as lung and intestinal tract (78). Single cell analysis of gene expression profile indicated that macrophages in NOD islet are more inflamed as manifested by the upregulated interferon signature genes including *Cxcl2*, *Cxcl9* and *Ccl5* (78). Therefore, depletion of islet macrophages with CSF-1 receptor monoclonal antibody impairs the presentation of insulin epitopes from destroyed islet cells and delays the entrance of DCs and $CD4^+$ T cells, thereby preventing T1D development in NOD mice (76).

Undoubtedly, the pancreatic islet niche is crucial for T1D development. For instance, vascular endothelial growth factor (VEGF), which was found highly enriched in the serum of systemic lupus erythematosus patients (79), is also abundant in T1D blood. However, circulating plasma levels of VEGF do not correlate to metabolic control in long-standing T1D and the levels are not affected by the presence of microvascular complications (80). In contrast, locally increased VEGF promotes islet vascular remodeling and facilitates lymphocytic infiltration (81). APC-like β cell is another intriguing concept that extends our understanding of the etiology underlying organ specific autoimmunity. Given the fact that autoreactive T cells are indeed also present in healthy individuals (82), antigen presentation that lowers the threshold for aberrant T cell activation thus becomes critical in the initiation of autoimmune diseases including T1D. First, stressed β cells themselves actively present antigens to effector T cells. Second, dysfunctional β cells secret granules containing modified neoantigens and/or denatured proteins to be taken up by the adjacent professional APCs (83, 84). Third, the dying β cells release large amounts of autoantigens together with DAMPs (e.g., HMGB1 as an alarmin) to substantially activate the surrounding professional APCs. The general idea is that T1D is an autoimmune disorder which both begins and ends up with β cell death. Physiological β cell turnover is transient and self-controlled, while pathological β cell death leads to unrestrained inflammatory response probably due to the defective clearance by phagocytes and the occurrence of secondary necrosis (85). Furthermore, many T1D susceptible genes are expressed in β cells and make them vulnerable to death upon insults such as viral infection and inflammatory cytokine stimulation (86). Taken as a whole, this may reflect that the antigen presentation process in T1D setting is more diverse and intricate than what we previously thought, and the microenvironment shaped by structural cells, immune cells and their extensive cross-talk is pivotal for T1D development (**Figure 1**).

It is noteworthy that the ectopic expression of immune modules in β cells is not confined to the antigen-presenting function. For instance, it has been discovered that inflammatory cytokines stimulate human β cells to express negative-regulatory costimulatory molecules, B7-H4 and PD-L1, which could then serve as a counterbalance for derailed T cell response (87, 88). Moreover, islet cells are able to produce chemokines and cytokines such as CCL2, CCL22, IL-6 and so on to exhibit either pro-inflammatory or anti-inflammatory effect (89–91). Another good example is the TLR family proteins, in which TLR3/9 are related to the anti-viral response, while TLR2/4 activation induces MyD88 dependent transcription of inflammatory mediators and Erk dependent cell cycle arrest of β cells (92, 93). Particularly, current

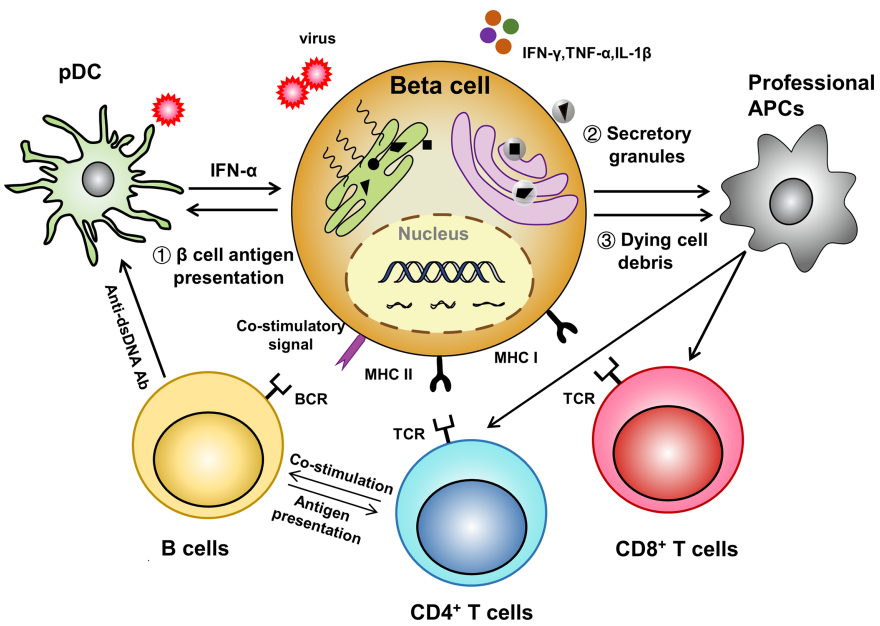


FIGURE 1 | A β cell centered view of antigen-presentation. (1) Stressed APC-like β cells directly present antigens to activate adaptive immune cells; (2) Dysfunctional β cells secret autoantigen containing granules which are subsequently up-taken and processed by professional APCs; (3) Dying β cells themselves and the released pro-inflammatory molecules further amplify the antigen presenting process.

immunotherapies were predominantly designed to suppress the functionality of autoreactive immune cells along with tolerance induction, thereby holding back the overdriven immune responses. In particular, vaccination strategies based on β cell derived autoantigens have shown therapeutic efficacy by inducing antigen specific immune tolerance. This could be achieved through oral, intranasal or parenteral (subcutaneous and intramuscular) administration of each single autoantigen such as insulin or GAD65 (94, 95). Alternatively, multiple antigenic epitopes could be integrated into one polypeptide by means of the plasmid DNA platform (96, 97). The discovery of β cells to act as a part-time APC provides novel insights that manipulation of the expression pattern of immune modules in β cells holds the potential in early T1D treatment and prevention of organ rejection following transplantation of genetically engineered, immune-evasive islets.

In summary, herein we provided a β cell centered view in T1D pathogenesis (**Figure 1**). Evidence derived from current studies suggested an important role of structural cells in the initiation of T1D development, which could also be the case in other autoimmune disorders. However, additional studies would be necessary to translate those discoveries into clinical settings for prevention and treatment of type 1 diabetes.

AUTHOR CONTRIBUTIONS

YL, FS, and T-TY wrote the manuscript. F-XW, C-LY, J-HL, S-JR, and FX gave us valuable suggestions and made critical revisions. SZ and C-YW conceptualized and supervised the preparation of this manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Bottazzo GF, Florin-Christensen A, Doniach D. Islet-Cell Antibodies in Diabetes Mellitus With Autoimmune Polyendocrine Deficiencies. *Lancet (London England)* (1974) 2(7892):1279–83. doi: 10.1016/s0140-6736(74)90140-8
2. Nerup J, Platz P, Andersen OO, Christy M, Lyngsoe J, Poulsen JE, et al. HL-A Antigens and Diabetes Mellitus. *Lancet (London England)* (1974) 2(7885):864–6. doi: 10.1016/s0140-6736(74)91201-x
3. Eizirik DL, Colli ML, Ortis F. The Role of Inflammation in Insulitis and Beta-Cell Loss in Type 1 Diabetes. *Nat Rev Endocrinol* (2009) 5(4):219–26. doi: 10.1038/nrendo.2009.21
4. Mathis D, Vence L, Benoist C. Beta-Cell Death During Progression to Diabetes. *Nature* (2001) 414(6865):792–8. doi: 10.1038/414792a
5. Colli ML, Moore F, Gurzov EN, Ortis F, Eizirik DL. MDA5 and PTPN2, Two Candidate Genes for Type 1 Diabetes, Modify Pancreatic Beta-Cell Responses to the Viral by-Product Double-Stranded RNA. *Hum Mol Genet* (2010) 19(1):135–46. doi: 10.1093/hmg/ddp474
6. Eizirik DL, Sammeth M, Bouckenooghe T, Bottu G, Sisino G, Igoillo-Esteve M, et al. The Human Pancreatic Islet Transcriptome: Expression of Candidate Genes for Type 1 Diabetes and the Impact of Pro-Inflammatory Cytokines. *PloS Genet* (2012) 8(3):e1002552. doi: 10.1371/journal.pgen.1002552
7. Krausgruber T, Fortelny N, Fife-Gernedl V, Senekowitsch M, Schuster LC, Lercher A, et al. Structural Cells Are Key Regulators of Organ-Specific Immune Responses. *Nature* (2020) 583(7815):296–302. doi: 10.1038/s41586-020-2424-4
8. Deng T, Lyon CJ, Minze LJ, Lin J, Zou J, Liu JZ, et al. Class II Major Histocompatibility Complex Plays an Essential Role in Obesity-Induced Adipose Inflammation. *Cell Metab* (2013) 17(3):411–22. doi: 10.1016/j.cmet.2013.02.009
9. Xiao L, Yang X, Lin Y, Li S, Jiang J, Qian S, et al. Large Adipocytes Function as Antigen-Presenting Cells to Activate CD4(+) T Cells *via* Upregulating MHCII in Obesity. *Int J Obes (2005)* (2016) 40(1):112–20. doi: 10.1038/ijo.2015.145
10. Bell E. Endothelial Cells as Sentinels. *Nat Rev Immunol* (2009) 9(8):532–3. doi: 10.1038/nri2608
11. Mai J, Virtue A, Shen J, Wang H, Yang XF. An Evolving New Paradigm: Endothelial Cells–Conditional Innate Immune Cells. *J Hematol Oncol* (2013) 6:61. doi: 10.1186/1756-8722-6-61
12. Lindhout E, van Eijk M, van Pel M, Lindeman J, Dinant HJ, de Groot C. Fibroblast-Like Synoviocytes From Rheumatoid Arthritis Patients Have Intrinsic Properties of Follicular Dendritic Cells. *J Immunol (Baltimore Md: 1950)* (1999) 162(10):5949–56.
13. Kim TK, Lee MS. Innate Immune Receptors in Type 1 Diabetes: The Relationship to Cell Death-Associated Inflammation. *Biochem Soc Trans* (2020) 48(3):1213–25. doi: 10.1042/bst20200131
14. Marre ML, McGinty JW, Chow IT, DeNicola ME, Beck NW, Kent SC, et al. Modifying Enzymes Are Elicited by ER Stress, Generating Epitopes That Are Selectively Recognized by CD4(+) T Cells in Patients With Type 1 Diabetes. *Diabetes* (2018) 67(7):1356–68. doi: 10.2337/db17-1166
15. McGinty JW, Chow IT, Greenbaum C, Odegard J, Kwok WW, James EA. Recognition of Posttranslationally Modified GAD65 Epitopes in Subjects With Type 1 Diabetes. *Diabetes* (2014) 63(9):3033–40. doi: 10.2337/db13-1952
16. van Lummel M, Duinkerken G, van Veelen PA, de Ru A, Cordfunke R, Zaldumbide A, et al. Posttranslational Modification of HLA-DQ Binding Islet Autoantigens in Type 1 Diabetes. *Diabetes* (2014) 63(1):237–47. doi: 10.2337/db12-1214
17. Kracht MJ, van Lummel M, Nikolic T, Joosten AM, Laban S, van der Slik AR, et al. Autoimmunity Against a Defective Ribosomal Insulin Gene Product in Type 1 Diabetes. *Nat Med* (2017) 23(4):501–7. doi: 10.1038/nm.4289
18. Babon JA, DeNicola ME, Blodgett DM, Crèvecoeur I, Buttrick TS, Maehr R, et al. Analysis of Self-Antigen Specificity of Islet-Infiltrating T Cells From Human Donors With Type 1 Diabetes. *Nat Med* (2016) 22(12):1482–7. doi: 10.1038/nm.4203
19. Bending D, Zaccone P, Cooke A. Inflammation and Type One Diabetes. *Int Immunol* (2012) 24(6):339–46. doi: 10.1093/intimm/dxs049
20. Formby B, Hosszufalusi N, Chan E, Miller N, Teruya M, Takei S, et al. Quantitative and Functional Analyses of Spleen and *in Situ* Islet Immune Cells Before and After Diabetes Onset in the NOD Mouse. *Autoimmunity* (1992) 12(2):95–102. doi: 10.3109/08916939209150315
21. Roche PA, Furuta K. The Ins and Outs of MHC Class II-Mediated Antigen Processing and Presentation. *Nat Rev Immunol* (2015) 15(4):203–16. doi: 10.1038/nri3818
22. Bottazzo GF, Dean BM, McNally JM, MacKay EH, Swift PG, Gamble DR. In Situ Characterization of Autoimmune Phenomena and Expression of HLA Molecules in the Pancreas in Diabetic Insulitis. *New Engl J Med* (1985) 313(6):353–60. doi: 10.1056/nejm198508083130604
23. Foulis AK, Farquharson MA. Aberrant Expression of HLA-DR Antigens by Insulin-Containing Beta-Cells in Recent-Onset Type I Diabetes Mellitus. *Diabetes* (1986) 35(11):1215–24. doi: 10.2337/diab.35.11.1215

24. Soldevila G, Doshi M, James R, Lake SP, Sutton R, Gray D, et al. DP, DQ Induction in Human Islet Beta Cells by the Cytokine Combination IFN-Gamma + TNF-Alpha. *Autoimmunity* (1990) 6(4):307–17. doi: 10.3109/08916939008998422
25. Russell MA, Redick SD, Blodgett DM, Richardson SJ, Leete P, Krogvold L, et al. HLA Class II Antigen Processing and Presentation Pathway Components Demonstrated by Transcriptome and Protein Analyses of Islet β-Cells From Donors With Type 1 Diabetes. *Diabetes* (2019) 68(5):988–1001. doi: 10.2337/db18-0686
26. Zhao Y, Scott NA, Quah HS, Krishnamurthy B, Bond F, Loudovaris T, et al. Mouse Pancreatic Beta Cells Express MHC Class II and Stimulate CD4(+) T Cells to Proliferate. *Eur J Immunol* (2015) 45(9):2494–503. doi: 10.1002/eji.201445378
27. Imagawa A, Hanafusa T, Itoh N, Miyagawa J, Nakajima H, Namba M, et al. Islet-Infiltrating T Lymphocytes in Insulin-Dependent Diabetic Patients Express CD80 (B7-1) and CD86 (B7-2). *J Autoimmun* (1996) 9(3):391–6. doi: 10.1006/jaut.1996.0053
28. Wong S, Guerder S, Visintin I, Reich EP, Swenson KE, Flavell RA, et al. Expression of the Co-Stimulator Molecule B7-1 in Pancreatic Beta-Cells Accelerates Diabetes in the NOD Mouse. *Diabetes* (1995) 44(3):326–9. doi: 10.2337/diab.44.3.326
29. Campbell IL, Cutri A, Wilkinson D, Boyd AW, Harrison LC. Intercellular Adhesion Molecule 1 Is Induced on Isolated Endocrine Islet Cells by Cytokines But Not by Reovirus Infection. *Proc Natl Acad Sci USA* (1989) 86 (11):4282–6. doi: 10.1073/pnas.86.11.4282
30. James EA, Pietropaolo M, Mamula MJ. Immune Recognition of β-Cells: Neoepitopes as Key Players in the Loss of Tolerance. *Diabetes* (2018) 67 (6):1035–42. doi: 10.2337/dbi17-0030
31. Rondas D, Crèvecoeur I, D'Hertog W, Ferreira GB, Staes A, Garg AD, et al. Citrullinated Glucose-Regulated Protein 78 Is an Autoantigen in Type 1 Diabetes. *Diabetes* (2015) 64(2):573–86. doi: 10.2337/db14-0621
32. Guerci AP, Guerci B, Lévy-Marchal C, Ongagna J, Ziegler O, Candiloros H, et al. Onset of Insulin-Dependent Diabetes Mellitus After Interferon-Alfa Therapy for Hairy Cell Leukaemia. *Lancet (London England)* (1994) 343 (8906):1167–8. doi: 10.1016/s0140-6736(94)90276-3
33. Yang ML, Doyle HA, Clarke SG, Herold KC, Mamula MJ. Oxidative Modifications in Tissue Pathology and Autoimmune Disease. *Antioxid Redox Signaling* (2018) 29(14):1415–31. doi: 10.1089/ars.2017.7382
34. de Jong VM, Abreu JR, Verrijn Stuart AA, van der Slik AR, Verhaeghen K, Engelse MA, et al. Alternative Splicing and Differential Expression of the Islet Autoantigen IGRP Between Pancreas and Thymus Contributes to Immunogenicity of Pancreatic Islets But Not Diabetogenicity in Humans. *Diabetologia* (2013) 56(12):2651–8. doi: 10.1007/s00125-013-3034-6
35. Walter P, Ron D. The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation. *Sci (New York NY)* (2011) 334(6059):1081–6. doi: 10.1126/science.1209038
36. Sun J, Cui J, He Q, Chen Z, Arvan P, Liu M. Proinsulin Misfolding and Endoplasmic Reticulum Stress During the Development and Progression of Diabetes. *Mol Aspects Med* (2015) 42:105–18. doi: 10.1016/j.mam.2015.01.001
37. Lee H, Lee YS, Harenda Q, Pietrzak S, Oktay HZ, Schreiber S, et al. Beta Cell Dedifferentiation Induced by IRE1α Deletion Prevents Type 1 Diabetes. *Cell Metab* (2020) 31(4):822–36.e5. doi: 10.1016/j.cmet.2020.03.002
38. Delong T, Wiles TA, Baker RL, Bradley B, Barbour G, Reisdorph R, et al. Pathogenic CD4 T Cells in Type 1 Diabetes Recognize Epitopes Formed by Peptide Fusion. *Sci (New York NY)* (2016) 351(6274):711–4. doi: 10.1126/science.aad2791
39. Gonzalez-Duque S, Azoury ME, Colli ML, Afonso G, Turatsinze JV, Nigi L, et al. Conventional and Neo-Antigenic Peptides Presented by β Cells Are Targeted by Circulating Naïve CD8+ T Cells in Type 1 Diabetic and Healthy Donors. *Cell Metab* (2018) 28(6):946–60.e6. doi: 10.1016/j.cmet.2018.07.007
40. Rui J, Deng S, Lebastchi J, Clark PL, Usmani-Brown S, Herold KC. Methylation of Insulin DNA in Response to Proinflammatory Cytokines During the Progression of Autoimmune Diabetes in NOD Mice. *Diabetologia* (2016) 59(5):1021–9. doi: 10.1007/s00125-016-3897-4
41. Devendra D, Eisenbarth GS. Interferon Alpha–a Potential Link in the Pathogenesis of Viral-Induced Type 1 Diabetes and Autoimmunity. *Clin Immunol (Orlando Fla)* (2004) 111(3):225–33. doi: 10.1016/j.clim.2004.01.008
42. Foulis AK. Interferon-Alpha and IDDM: Comment. *Diabetologia* (1996) 39 (1):127. doi: 10.1007/bf00400427
43. Stewart TA. Neutralizing Interferon Alpha as a Therapeutic Approach to Autoimmune Diseases. *Cytokine Growth factor Rev* (2003) 14(2):139–54. doi: 10.1016/s1359-6101(02)00088-6
44. Virus Interference: I. The Interferon. By Alick Isaacs and Jean Lindenmann, 1957. *CA: Cancer J Clin* (1988) 38(5):280–90. doi: 10.3322/canjclin.38.5.280
45. Yi Z, Li L, Garland A, He Q, Wang H, Katz JD, et al. IFN-γ Receptor Deficiency Prevents Diabetes Induction by Diabetogenic CD4+, But Not CD8+, T Cells. *Eur J Immunol* (2012) 42(8):2010–8. doi: 10.1002/eji.201142374
46. Marroqui L, Dos Santos RS, Op de Beeck A, Coomans de Brachène A, Marselli L, Marchetti P, et al. Interferon-α Mediates Human Beta Cell HLA Class I Overexpression, Endoplasmic Reticulum Stress and Apoptosis, Three Hallmarks of Early Human Type 1 Diabetes. *Diabetologia* (2017) 60(4):656–67. doi: 10.1007/s00125-016-4201-3
47. Lombardi A, Tsomos E, Hammerstad SS, Tomer Y. Interferon Alpha: The Key Trigger of Type 1 Diabetes. *J Autoimmun* (2018) 94:7–15. doi: 10.1016/j.jaut.2018.08.003
48. Pujol-Borrell R, Todd I, Doshi M, Bottazzo GF, Sutton R, Gray D, et al. HLA Class II Induction in Human Islet Cells by Interferon-Gamma Plus Tumour Necrosis Factor or Lymphotoxin. *Nature* (1987) 326(6110):304–6. doi: 10.1038/326304a0
49. Kim KA, Kim S, Chang I, Kim GS, Min YK, Lee MK, et al. IFN Gamma/TNF Alpha Synergism in MHC Class II Induction: Effect of Nicotinamide on MHC Class II Expression But Not on Islet-Cell Apoptosis. *Diabetologia* (2002) 45 (3):385–93. doi: 10.1007/s00125-001-0755-8
50. Reizis B, Bunin A, Ghosh HS, Lewis KL, Sisirak V. Plasmacytoid Dendritic Cells: Recent Progress and Open Questions. *Annu Rev Immunol* (2011) 29:163–83. doi: 10.1146/annurev-immunol-031210-101345
51. Allen JS, Pang K, Skowera A, Ellis R, Rackham C, Lozanoska-Ochser B, et al. Plasmacytoid Dendritic Cells are Proportionally Expanded at Diagnosis of Type 1 Diabetes and Enhance Islet Autoantigen Presentation to T-Cells Through Immune Complex Capture. *Diabetes* (2009) 58(1):138–45. doi: 10.2337/db08-0964
52. Fabris P, Floreani A, Tositti G, Vergani D, De Lalla F, Betterle C. Type 1 Diabetes Mellitus in Patients With Chronic Hepatitis C Before and After Interferon Therapy. *Aliment Pharmacol Ther* (2003) 18(6):549–58. doi: 10.1046/j.1365-2036.2003.01681.x
53. Saxena V, Ondr JK, Magnusen AF, Munn DH, Katz JD. The Countervailing Actions of Myeloid and Plasmacytoid Dendritic Cells Control Autoimmune Diabetes in the Nonobese Diabetic Mouse. *J Immunol (Baltimore Md: 1950)* (2007) 179(8):5041–53. doi: 10.4049/jimmunol.179.8.5041
54. Mukhopadhaya A, Hanafusa T, Jarchum I, Chen YG, Iwai Y, Serreze DV, et al. Selective Delivery of Beta Cell Antigen to Dendritic Cells *In Vivo* Leads to Deletion and Tolerance of Autoreactive CD8+ T Cells in NOD Mice. *Proc Natl Acad Sci USA* (2008) 105(17):6374–9. doi: 10.1073/pnas.0802644105
55. Diana J, Simoni Y, Furio L, Beaudoin L, Agerberth B, Barrat F, et al. Crosstalk Between Neutrophils, B-1a Cells and Plasmacytoid Dendritic Cells Initiates Autoimmune Diabetes. *Nat Med* (2013) 19(1):65–73. doi: 10.1038/nm.3042
56. Behrens GM, Li M, Davey GM, Allison J, Flavell RA, Carbone FR, et al. Helper Requirements for Generation of Effector CTL to Islet Beta Cell Antigens. *J Immunol (Baltimore Md: 1950)* (2004) 172(9):5420–6. doi: 10.4049/jimmunol.172.9.5420
57. Yagi H, Matsumoto M, Kunimoto K, Kawaguchi J, Makino S, Harada M. Analysis of the Roles of CD4+ and CD8+ T Cells in Autoimmune Diabetes of NOD Mice Using Transfer to NOD Athymic Nude Mice. *Eur J Immunol* (1992) 22(9):2387–93. doi: 10.1002/eji.1830220931
58. Christianson SW, Shultz LD, Leiter EH. Adoptive Transfer of Diabetes Into Immunodeficient NOD-Scid/Scid Mice. Relative Contributions of CD4+ and CD8+ T-Cells From Diabetic Versus Prediabetic NOD.NON-Thy-1a Donors. *Diabetes* (1993) 42(1):44–55. doi: 10.2337/diab.42.1.44
59. Gagnerault MC, Luan JJ, Lotton C, Lepault F. Pancreatic Lymph Nodes are Required for Priming of Beta Cell Reactive T Cells in NOD Mice. *J Exp Med* (2002) 196(3):369–77. doi: 10.1084/jem.20011353
60. Levisetti MG, Suri A, Frederick K, Unanue ER. Absence of Lymph Nodes in NOD Mice Treated With Lymphotoxin-Beta Receptor Immunoglobulin Protects From Diabetes. *Diabetes* (2004) 53(12):3115–9. doi: 10.2337/diabetes.53.12.3115
61. Filaci G, Rizzi M, Setti M, Fenoglio D, Fravega M, Basso M, et al. Non-Antigen-Specific CD8(+) T Suppressor Lymphocytes in Diseases Characterized by Chronic Immune Responses and Inflammation. *Ann New York Acad Sci* (2005) 1050:115–23. doi: 10.1196/annals.1313.013
62. Mikulkova Z, Praksova P, Stourac P, Bednarik J, Strajtova L, Pacasova R, et al. Numerical Defects in CD8+CD28- T-Suppressor Lymphocyte Population in Patients With Type 1 Diabetes Mellitus and Multiple Sclerosis. *Cell Immunol* (2010) 262(2):75–9. doi: 10.1016/j.cellimm.2010.02.002

63. Pinkse GG, Tysma OH, Bergen CA, Kester MG, Ossendorp F, van Veelen PA, et al. Autoreactive CD8 T Cells Associated With Beta Cell Destruction in Type 1 Diabetes. *Proc Natl Acad Sci USA* (2005) 102(51):18425–30. doi: 10.1073/pnas.0508621102
64. Valečka J, Almeida CR, Su B, Pierre P, Gatti E. Autophagy and MHC-Restricted Antigen Presentation. *Mol Immunol* (2018) 99:163–70. doi: 10.1016/j.molimm.2018.05.009
65. Dengjel J, Schoor O, Fischer R, Reich M, Kraus M, Müller M, et al. Autophagy Promotes MHC Class II Presentation of Peptides From Intracellular Source Proteins. *Proc Natl Acad Sci United States America* (2005) 102(22):7922–7. doi: 10.1073/pnas.0501190102
66. Ireland JM, Unanue ER. Processing of Proteins in Autophagy Vesicles of Antigen-Presenting Cells Generates Citrullinated Peptides Recognized by the Immune System. *Autophagy* (2012) 8(3):429–30. doi: 10.4161/auto.19261
67. Wang Q, Racine JJ, Ratiu JJ, Wang S, Ettinger R, Wasserfall C, et al. Transient BAFF Blockade Inhibits Type 1 Diabetes Development in Nonobese Diabetic Mice by Enriching Immunoregulatory B Lymphocytes Sensitive to Deletion by Anti-CD20 Cotherapy. *J Immunol (Baltimore Md: 1950)* (2017) 199 (11):3757–70. doi: 10.4049/jimmunol.1700822
68. Linsley PS, Greenbaum CJ, Rosasco M, Presnell S, Herold KC, Dufort MJ. Elevated T Cell Levels in Peripheral Blood Predict Poor Clinical Response Following Rituximab Treatment in New-Onset Type 1 Diabetes. *Genes Immun* (2019) 20(4):293–307. doi: 10.1038/s41435-018-0032-1
69. Hinman RM, Cambier JC. Role of B Lymphocytes in the Pathogenesis of Type 1 Diabetes. *Curr Diabetes Rep* (2014) 14(11):543. doi: 10.1007/s11892-014-0543-8
70. Packard TA, Smith MJ, Conrad FJ, Johnson SA, Getahun A, Lindsay RS, et al. B Cell Receptor Affinity for Insulin Dictates Autoantigen Acquisition and B Cell Functionality in Autoimmune Diabetes. *J Clin Med* (2016) 5(11):1–20. doi: 10.3390/jcm5110098
71. Smith MJ, Packard TA, O'Neill SK, Henry Dunand CJ, Huang M, Fitzgerald-Miller L, et al. Loss of Anergic B Cells in Prediabetic and New-Onset Type 1 Diabetic Patients. *Diabetes* (2015) 64(5):1703–12. doi: 10.2337/db13-1798
72. Unanue ER, Ferris ST, Carrero JA. The Role of Islet Antigen Presenting Cells and the Presentation of Insulin in the Initiation of Autoimmune Diabetes in the NOD Mouse. *Immunol Rev* (2016) 272(1):183–201. doi: 10.1111/imr.12430
73. Edelson BT, Kc W, Juang R, Kohyama M, Benoit LA, Klekotka PA, et al. Peripheral CD103+ Dendritic Cells Form a Unified Subset Developmentally Related to CD8alpha+ Conventional Dendritic Cells. *J Exp Med* (2010) 207 (4):823–36. doi: 10.1084/jem.20091627
74. Ferris ST, Carrero JA, Unanue ER. Antigen Presentation Events During the Initiation of Autoimmune Diabetes in the NOD Mouse. *J Autoimmun* (2016) 71:19–25. doi: 10.1016/j.jaut.2016.03.007
75. Calderon B, Carrero JA, Ferris ST, Sojka DK, Moore L, Epelman S, et al. The Pancreas Anatomy Conditions the Origin and Properties of Resident Macrophages. *J Exp Med* (2015) 212(10):1497–512. doi: 10.1084/jem.20150496
76. Carrero JA, McCarthy DP, Ferris ST, Wan X, Hu H, Zinselmeyer BH, et al. Resident Macrophages of Pancreatic Islets Have a Seminal Role in the Initiation of Autoimmune Diabetes of NOD Mice. *Proc Natl Acad Sci USA* (2017) 114(48): E10418–e27. doi: 10.1073/pnas.1713543114
77. Marée AF, Komba M, Finegood DT, Edelstein-Keshet L. A Quantitative Comparison of Rates of Phagocytosis and Digestion of Apoptotic Cells by Macrophages From Normal (BALB/c) and Diabetes-Prone (NOD) Mice. *J Appl Physiol (Bethesda Md: 1985)* (2008) 104(1):157–69. doi: 10.1152/japplphysiol.00514.2007
78. Ferris ST, Zakharov PN, Wan X, Calderon B, Artyomov MN, Unanue ER, et al. The Islet-Resident Macrophage is in an Inflammatory State and Senses Microbial Products in Blood. *J Exp Med* (2017) 214(8):2369–85. doi: 10.1084/jem.20170074
79. Ciprandi G, Murdaca G, Colombo BM, De Amici M, Marseglia GL. Serum Vascular Endothelial Growth Factor in Allergic Rhinitis and Systemic Lupus Erythematosus. *Hum Immunol* (2008) 69(8):510–2. doi: 10.1016/j.humimm.2008.05.010
80. Singh K, Sandler S, Espes D. The Increased Circulating Plasma Levels of Vascular Endothelial Growth Factor in Patients With Type 1 Diabetes Do Not Correlate to Metabolic Control. *J Diabetes Res* (2017) 2017:6192896. doi: 10.1155/2017/6192896
81. Villalta SA, Lang J, Kubeck S, Kabre B, Szot GL, Calderon B, et al. Inhibition of VEGFR-2 Reverses Type 1 Diabetes in NOD Mice by Abrogating Insulitis and Restoring Islet Function. *Diabetes* (2013) 62(8):2870–8. doi: 10.2337/db12-1619
82. Danke NA, Koelle DM, Yee C, Beheray S, Kwok WW. Autoreactive T Cells in Healthy Individuals. *J Immunol (Baltimore Md: 1950)* (2004) 172(10):5967–72. doi: 10.4049/jimmunol.172.10.5967
83. Calderon B, Carrero JA, Miller MJ, Unanue ER. Cellular and Molecular Events in the Localization of Diabetogenic T Cells to Islets of Langerhans. *Proc Natl Acad Sci USA* (2011) 108(4):1561–6. doi: 10.1073/pnas.1018973108
84. Vomund AN, Zinselmeyer BH, Hughes J, Calderon B, Valderrama C, Ferris ST, et al. Beta Cells Transfer Vesicles Containing Insulin to Phagocytes for Presentation to T Cells. *Proc Natl Acad Sci USA* (2015) 112(40):E5496–502. doi: 10.1073/pnas.1515954112
85. Lee MS, Kim KA, Kim HS. Role of Pancreatic β-Cell Death and Cell Death-Associated Inflammation in Diabetes. *Curr Mol Med* (2012) 12(10):1297–310. doi: 10.2174/156652412803833553
86. Størling J , Pociot F. Type 1 Diabetes Candidate Genes Linked to Pancreatic Islet Cell Inflammation and Beta-Cell Apoptosis. *Genes* (2017) 8(2):1–12. doi: 10.3390/genes8020072
87. Colli ML, Hill JLE, Marroquí L, Chaffey J, Dos Santos RS, Leete P, et al. PDL1 Is Expressed in the Islets of People With Type 1 Diabetes and Is Up-Regulated by Interferons-α and-γ *via* IRF1 Induction. *EBioMedicine* (2018) 36:367–75. doi: 10.1016/j.ebiom.2018.09.040
88. Ou D, Wang X, Metzger DL, Ao Z, Pozzilli P, James RFL, et al. Suppression of Human T-Cell Responses to β-Cells by Activation of B7-H4 Pathway. *Cell Transplant* (2006) 15(5):399–410. doi: 10.3727/000000006783981837
89. Bischoff L, Alvarez S, Dai DL, Soukhatcheva G, Orban PC, Verchere CB. Cellular Mechanisms of CCL22-Mediated Attenuation of Autoimmune Diabetes. *J Immunol (Baltimore Md: 1950)* (2015) 194(7):3054–64. doi: 10.4049/jimmunol.1400567
90. Campbell IL, Cutri A, Wilson A, Harrison LC. Evidence for IL-6 Production by and Effects on the Pancreatic Beta-Cell. *J Immunol (Baltimore Md: 1950)* (1989) 143(4):1188–91.
91. Van Belle TL, Pagni PP, Liao J, Sachithanantham S, Dave A, Bel Hani A, et al. Beta-Cell Specific Production of IL6 in Conjunction With a Mainly Intracellular But Not Mainly Surface Viral Protein Causes Diabetes. *J Autoimmun* (2014) 55:24–32. doi: 10.1016/j.jaut.2014.02.002
92. Eguchi K, Manabe I, Oishi-Tanaka Y, Ohsugi M, Kono N, Ogata F, et al. Saturated Fatty Acid and TLR Signaling Link β Cell Dysfunction and Islet Inflammation. *Cell Metab* (2012) 15(4):518–33. doi: 10.1016/j.cmet.2012.01.023
93. Ji Y, Sun S, Shrestha N, Darragh LB, Shirakawa J, Xing Y, et al. Toll-Like Receptors TLR2 and TLR4 Block the Replication of Pancreatic β Cells in Diet-Induced Obesity. *Nat Immunol* (2019) 20(6):677–86. doi: 10.1038/s41590-019-0396-z
94. Harrison LC, Wentworth JM, Zhang Y, Bandala-Sanchez E, Böhmer RM, Neale AM, et al. Antigen-Based Vaccination and Prevention of Type 1 Diabetes. *Curr Diabetes Rep* (2013) 13(5):616–23. doi: 10.1007/s11892-013-0415-7
95. Wang H, Yang J, Jin L, Feng J, Lu Y, Sun Y, et al. Immunotherapy of Autoimmune Diabetes by Nasal Administration of Tandem Glutamic Acid Decarboxylase 65 Peptides. *Immunol Invest* (2009) 38(8):690–703. doi: 10.3109/08820130903124770
96. Postigo-Fernandez J, Creusot RJ. A Multi-Epitope DNA Vaccine Enables a Broad Engagement of Diabetogenic T Cells for Tolerance in Type 1 Diabetes. *J Autoimmun* (2019) 98:13–23. doi: 10.1016/j.jaut.2018.11.003
97. Gottlieb P, Utz PJ, Robinson W, Steinman L. Clinical Optimization of Antigen Specific Modulation of Type 1 Diabetes With the Plasmid DNA Platform. *Clin Immunol (Orlando Fla)* (2013) 149(3):297–306. doi: 10.1016/j.clim.2013.08.010

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Depression and Obesity in Patients With Psoriasis and Psoriatic Arthritis: Is IL-17-Mediated Immune Dysregulation the Connecting Link?

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Patients with psoriasis are frequently obese and experience anxiety or suffer from depressive disorders. The immunopathogenesis of psoriasis and indeed psoriatic arthritis is largely based on the pivotal role of IL-17/IL-23 axis, to an extent that currently monoclonal antibodies selectively inhibiting IL-17 or IL-23 are routinely used for the treatment of psoriatic diseases. Emerging data, demonstrating a decisive role for IL-17 and IL-17 producing cell subsets, such as Th17 in the induction and progression of obesity and depression has led authors to suggest that psoriatic disease, obesity and anxiety/depression may indeed be interconnected manifestation of a state of immunedysregulation, the linked being IL-17 and its related cells. We discuss this hypothetical link in depth taking into account the beneficial effects anti-IL17 and anti-IL-17 receptor inhibitors in treating psoriatic disease and the on-going debate as to whether these biologics may exert a direct or indirect effect in ameliorating concomitant obesity and depressive disorders, which are frequently noted in the same patient.

Keywords: depression, IL-17, IL-23, immunity, obesity, psoriasis, psoriatic disease

INTRODUCTION

Depression is a frequent comorbidity of various autoimmune rheumatic and immune-mediated diseases, including psoriasis (Ps) and psoriatic arthritis (PsA) (1). More then a third of patients with these diseases experience anxiety, depression, and even suicidal ideation and behaviors (SIB), which is not always immediately relevant with the activity of the underlying disease (2–4). In fact, the rate of depression can be higher in patients with PsA than in those with Ps (5). The cause–effect bond between depression and Ps (as well as PsA) is puzzling (6). Remission of the disease, following successful treatment, especially with biologics has a positive effect on depression and anxiety, but not always. Chronic inflammation has been considered a favorable trigger of depression and chronic medical illness (7). The immunobiological basis of depression's development has started to emerge following studies demonstrating a fine imbalance between pro-inflammatory and anti-inflammatory cytokines (8–10). IL-17 has emerged as a master cytokine for the

immunopathogenesis of psoriatic disease, leading to the development of the plethora of agents specifically inhibiting its deleterious effect (11–15).

The direct or indirect effect of such biologics in fighting depression in patients with immune-mediated or autoimmune diseases such as Ps and PsA remains elusive (16). The lack of information resulting from inconsequential clinical data will become less elusive in the coming years as monoclonal antibodies (mAbs), such as those specifically inhibiting IL-17 and IL-17 receptors, are currently approved treatments for psoriatic disease patients and data are continuously analyzed and published (17). The impact of mAbs specifically inhibiting that; cytokine in depression, anxiety and even suicidal ideation and behaviors (SIB) will also become more evident, as animal models of depression become more sophisticated resembling more and more the human disease. The question arising from the clinical use of the approved drugs is whether anti-IL17 treatment is efficacious in combating depression, irrespectively of the disease patients are suffering from (Ps, PsA or other spondylarthropathies) and whether the magnitude of IL-17 inhibition can predict the performance as per depression scale.

So far, it has become apparent that patients suffering from depression have elevated levels of circulating IL-17 in their serum and that the percentage of Th17 cells, the T cell predominantly producing this cytokine, are also increased in people with depressive disorders (18). What is not clear and remains a matter of debate is whether this increase is an epiphenomenon resulting from the disease or whether the increase *per se* is playing a decisive role in the development and progression of neuroimmune depressive disorders in isolation or in combination with other non-immunologically relevant mechanisms (19). Research clinical trials using IL-17 or IL-17R blockers for the treatment of depressive disorders have not been initiated so far. It is worthy to mention data from two clinical trials, based on biologic therapy, and in particular that using infliximab which is a chimeric IgG1 mAb that blocks TNF-α for the treatment of refractory depression (20, 21). None of the two achieved its primary outcome, and infliximab did not appear to reduce symptoms of depression compared to placebo, though a favourable outcome has been reported in those patients with inflammatory indices (21).

It has also been well documented that people with depression are more frequently obese compared to those without and that obese people, because of their internal and external stigma, are experiencing more frequent depression. Again, it is not clear what is the pathophysiologal impact of IL-17 in the direct induction of obesity. A provocative hypothesis is taking into consideration that an impairment of immunoregulatory mechanisms, characterized by the functional inability of the immune system to promote the expression and production of IL-17 and other suppressory cytokines, is directly linked to the overexpression of IL-17 for cellular subsets such as—but not limited to—Th17.

If IL-17 is critical for the immunedysregulation noted in patients with depression, it should be expected that patients with that disorder at early stages of the disease will have well documented increased expression of IL-17, and that over time, serum IL-17 levels as well as the levels of Th17 and other IL-17-producing cells will become significantly amplified, especially in those patients who become more depressed and more obese because of the underlying disease. Prospective studies reporting on that are currently missing, but experimental data in animal models are rather informative and provide a wealth of information, which can lead to a better understanding of the complex nature and close interplay between immunedysregulation in psoriatic disease and its impact on depression and obesity.

We and others have shown that is IL-17 per se, as well as IL-17 axis, is pivotal for the development and progression of Ps and PsA (12, 13, 22–27). More recently, the imperative role of Il-17 in the induction and maintenance of ankylosing spondylitis and other spondylarthropathies has been revealed (28).

IL-17 SELECTIVE BIOLOGICS FOR THE TREATMENT OF PS AND PSA

Four biologic therapies targeting either IL-17 or IL-17R have been approved for the treatment of Ps. Secukinumab, a fully human IgG1κ mAb (29) and ixekizumab, a humanized IgG4 mAb (30, 31) selectively bind and neutralize IL-17A. Brodalumab is a fully human IgG2 mAb that binds and inactivates the IL-17A receptor leading to the inhibition of either IL-17A, IL-17C, IL-17E and IL-17F (32). Finally bimekizumab, a humanized IgG1 mAb, which selectively neutralizes IL-17A and IL-17F, is approved for the treatment of Ps but is also efficacious in PsA (33).

IL-17 LEVELS AND TH17 CELL SUBSETS IN PATIENTS WITH DEPRESSION

The neurological and psychiatric disease related implication of Th17 and Il-17 mediated cell damage is the focus of intense research and has just been started emerging. Th17 cells have been considered likely inducer of brain damage (34). Th17 induce neuronal cell death and promote neuronal toxicity in experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis (35) and IL-17 mRNA is overexpressed in active MS brain lesions (36), while IL-17 production from central nervous system resident T lymphocytes and glial cells are associated with disease-activity (37). CD8+ T cells producing IL-17 are elevated during disease-relapses compared to disease-remission (38). Work in mice has clear demonstrated that though not directly, IL-17 plays an important role in MS, as mice deficient for IL-17A/F escape from disease's appearance. Immunome data demonstrate that paediatric patients with drug refractory epilepsy are characterized by an IL-17 inducing CD4 and CD8 cell subset profile, which likely contributes to epileptogenesis (39) while autism spectrum disorders are characterized by an imbalance between proinflammatory Th17 and suppressory T regulatory

cells (Tregs) (40). Th17 cells are increased in patients with stable schizophrenia (41).

Depression is associated with the elevation of proinflammatory cytokines among which IL-17 appears to be one of those found elevated in patients with major depressive disorders (42, 43). Accumulated evidence suggest that the cytokine milieu noted in patients with depression, as well as that well-characterized in animal models of depressive disorders, underlined the important role of IL-1β, TNF-α and IL-6 (44–46). A recent meta-analysis investigating children and adolescents with depression has found that IL-6 predicts the future development of depression and conversely that the establishment of depression is a significant predictor of IL-6 increase (47). That effect appears to be influenced by other factors like gender or stressful life events. A longitudinal cross-lagged twin study has provided compelling evidence supporting the imperative role of IL-6 increase as an independent risk factor of depression rather than an epiphenomenal consequence of disease's establishment (48). In animal models of depression, IL-6 (and/or IL-1β appears to be instrumental for the development of chronic stress and depression-like behaviors (49). Also, numerous studies have shown that patients with major depression have elevated levels of TNF-α and that fluctuation of this pro-inflammatory cytokine (as well as that of IL-6) influences the mood behavior of the affected individuals (for review see (50). Furthermore, some data suggest that antipsychotic drugs exert an anti-inflammatory effect and decrease TNF-α and IL-6 levels in murine models of the disease (51). Intriguingly, however, a recent systematic review, meta-analysis and meta-regression including 38 eligible studies representing 58,256 failed to identify a prospective association of depression with TNF-α and a small association with IL-6 (45). Also, as we mentioned previously, data from a limited number of clinical trials have failed to identify a beneficial effect of anti-TNF-α biologics in patients with mood disorders (20, 21), while such drugs are successfully used for the treatment of psoriatic disease, other autoimmune rheumatic diseases and several immune-mediated inflammatory diseases.

Experimental work has been redirected to other newly identified pro-inflammatory cytokines, including IL-17. IL-17A mediated disruption of the blood-brain barrier by Th17 cells is well documented, and treatment of mice with IL-17A neutralizing antibodies prevents such a disruption (52). Though limited, data from studies in patients with depressive disorders are also noteworthy (**Table 1**). Interestingly, a serological study has found higher IL-17 levels in blood samples in 41 patients with major depressive disorder compared to those noted in 40 healthy age-matched controls with no history of malignancies or autoimmune diseases (53). However, lack of an association between blood levels of IL-17A and depression has also been reported, as anti-depressants appear not to exert an effect on IL-17 levels (57). The levels of IL-17 gene expression among 190 patients with depression were higher compared to 100 healthy individuals, while the mean mRNA expression of the immunoregulatory Foxp3 was considerably reduced in patients suffering from depressive disorders compared to the control group (54). A study in 40 patients with major depressive disorders and 30 healthy controls has found an imbalance of Th17/Treg ratio compared to healthy controls documented by a significant increase in peripheral blood mononuclear cell Th17 cell numbers, and a decrease in T regulatory cells (55). The same study also reported higher mRNA expression levels of retinoic acid-related orphan receptor-γt (RORγt), which is the specific transcription factor of Th17 cell and increased IL-17 serum levels in depressed patients compared to healthy controls (55). Another study found that women with major depressive disorder have increased Th17 and increased serum IL-17 compared to controls (56).

Considerable evidence in mice supports the notion that Th17 cells endorse susceptibility to depression-like behaviors (58). The percentage of Th17 cells is significantly increased (up to a 3-fold) in the brain of mice demonstrating depressive-like behavior compared to mice that do not have such behavior (59). Of interest, at the experimental level oral administration of BALB/C to mice sensitized by ovalbumin with the tricyclic-antidepressant desipramine diminished symptoms of allergic rhinitis symptoms in mice, up regulated CD4+CD25+Foxp3+ Treg cells and

TABLE 1 | A summary of human studies reporting on the role of IL-17 in depression.

Type of Study	Major findings	Comments	Reference
Serological study in patients with major depressive disorder	Patients with major depressive disorder have higher serum IL-17 compared to those found in age-matched healthy controls	The number of samples was limited to reach safely conclusions.	(53)
Molecular study in patients with depression	Cumulative mRNA levels of IL-17 gene expression in peripheral blood samples were significantly higher in 190 patients with depression compared to 100 healthy individuals	Sample size was sufficient enough but this study was not prospective and the effect of anti-depressants was not evaluated	(54)
Immunocellular study in patients with major depressive disorders & Serological study	Th17/Treg ratio of peripheral blood mononuclear cells was increased in 40 patients with major depressive disorders compared to 30 healthy controlsSerum levels of IL-17 measured by ELISA are increased in patients with major depressive disorders compared to 30 healthy controls	Interesting study showing an increase of Th17 cells and a decrease of Treg cell subsets	(55)
Cellular and serological study in patients with depressive disorder	Percentages of Th17 cells and serum levels of circulating IL-17 were increased in 30 patients with major depressive disorder compared to 30 sex-, age-, body mass index, ethnicity- and smoking status-matched healthy controls	The study included BMI, smoking-status and demographically matched healthy controls, which make the data more reliable.As for the previous studies the study in not prospective and the kinetics of cell subsets and circulating levels of IL-17 are not available	(56)

reduced CD4+IL-17+ Th17 cells, which were significantly increased in mice not receiving this antidepressant. Finally, *in vitro* addition of serotonin and treatment of MDD patients with selective serotonin reuptake inhibitors (SSRIs) reduced the production of Th17/Tc17-related cytokines by CD4+= and CD8+ T cells (60). Treatment of depressed mice with anti-IL-17 mAb inhibits glial differentiation and ameliorates anxiety and depression behaviors (61). In the human setting, CD4+CD25+ Tregs are found decreased in patients with major depression (62) (**Figure 1**).

IL-17 and Depression in Psoriatic Disease

A population-based cohort study found that patients with Ps who have elevated levels of IL-17A also have increased risk for depression and anxiety disorders. In a murine imiquimod model of psoriatic disease, administration of IL-17A was associated with acceleration of depression-like symptoms, while treatment with an anti-IL17A antibody diminished depression-like symptomatology (63). A recent case-based study reported three patients with moderate-to-severe Ps and comorbid depression, who were successfully treated with brodalumab (PASI 100 after treatment with brodalumab); in two of those, depressive symptoms either improved or resolved suggesting that the use of brodalumab is able to improve both skin lesions and depression (64). These results rather contradict those suggesting a potential link between SIB and brodalumab use, as there are data from the clinical Phase 3 Studies (AMAGINE-2) which report three events of suicide attempt that occurred in only one individual out of 486 participants (Patient-yr = 379.7) who received a constant dose of brodalumab 210 mg Q2W (65), which makes dermatologists rather hesitant to prescribe that mAb (65, 66), despite the lack of concrete causality (67). The exact mechanism by which brodalumab may indeed exert or participates in suicidal behavior has not been studied in the affected individual or in any other setting. The distinctive mechanism of action of brodalumab involves the broader blockade of IL-17 isoforms binding IL-17RA. If this action can provide a mechanistical explanation of the assumed suicidal ideation induction remains elusive. Studies of the effect of anti-IL17 RA or other anti-IL17 neutralizing antibodies in animal models of suicidal behavior (68) may shed a light on this provocative topic.

Depression and Suicide Ideation Following Anti-IL17 mAb Treatments in Ps and PsA

A recent two-year US pharmacovigilance report on brodalumab usage collecting data from 2,677 patients with an estimated exposure of 1,656 patient-years reported just 25 reports of depression but no suicide attempts (69). Data on the safety of ixekizumab in adult patients with plaque Ps, PsA and axial spondylarthritis from 21 clinical trials of 8,228 patients with an ixekizumab exposure of 20,895.9 patient-years have reported depression during ixekizumab treatment occurred in 203 patients with Ps, 37 patients with PsA and 13 patients with axial spondylarthritis. The cumulative data over the total

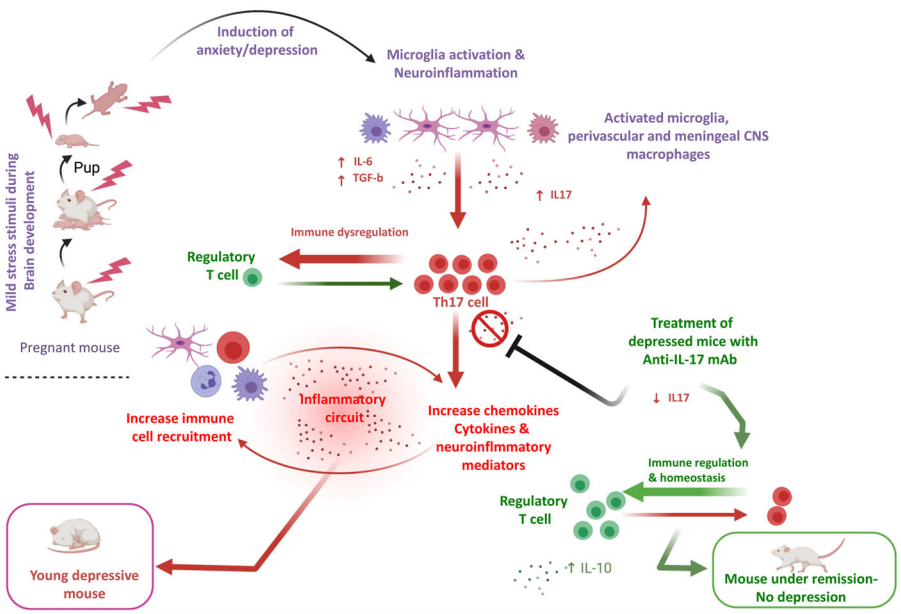


FIGURE 1 | An-IL-17-mediated hypothesis of depressive disorder in an experimental model. In a young adult depression mouse model exposed to cumulative mild stress (CPMS) characterized by microglial activation, IL-17 in brain and blood, as well Th-17 cells are elevated. A hypothesis based on the assumption that microglia activation is pivotal for the increase of pro-inflammatory cytokines such as IL-16 and TGF-b, which polarize CD4+ T cells towards Th-17 is formulated. Experimental data suggest that anti-IL-17 mAb treatment, diminishes IL-17 induction and Th-17 differentiation and ameliorates anxiety and depression-like behaviors (61) (prepared with BioRender).

exposure period have shown incidence rates of reported depression to be low (≤2.2 per 100 PY across indications) and decreased across the treatment periods. Of importance, the same study found that suicidal behavior/self-injury was present in 17 patients with Ps, one patient with PsA and two patients with axial spondylarthritis (70). A very recent *post hoc* Analysis of the Italian SUPREME multi-centre (50 sites) study has found that anxiety was resolved in 67 and 71% of Ps patients at weeks 16 and 48, respectively and that depression symptoms were improved in 81.3 and 70.6% of patients at weeks 16 and 48, respectively (71).

IL-17, Obesity and Depression in Psoriatic Disease

Currently, it is projected that by 2030, 20% of the world's population will be obese and 38% will be overweight (72). New piece of evidence suggests that the spatial prevalence of comorbid obesity/depression is not a random phenomenon and indeed common denominators at the cellular and immunophysiological level may account for mutual interactions between obesity and depression (73). Obesity is related to a higher grade of inflammation and this may hold true for depression too. Metabolic manifestations seen in obese people such as cardiovascular diseases and diabetes have been attributed at least in part in obesity-related chronic inflammation stemming from adipose tissue (74). Adipose tissue induces IL-6, which is an important mediator for CD4+ T cell polarization to Th17 cells (75). Blood concentrations of IL-17 (as well as IL-23) appear to be elevated in obese (BMI: 30–48 kg/m^2) compared to slim women (BMI: 18–25 kg/m^2) (76) (**Table 2**).

Of relevance, Ps and obesity are interconnected on the basis of various common denominators (82). For example, significant weight loss can improve psoriatic lesions and attributed to disease-remission. In addition, obesity is associated with higher incidence and prevalence of Ps. Finally, work in animals has shown that BMI increase participates in the development of Ps, while clinical studies have shown that increase in body weight participate in the relapse of psoriatic lesions and/or the *de novo* appearance of the disease (82). On the basis of that it becomes apparent that Ps, obesity and depression are interlinked and that IL-17, which significantly contributes to disease development may account for the induction and continuation of all three denominators in the same patient (**Figure 2**).

Intriguingly, recent data has shown that ixekizumab was efficacious in the treatment of moderate-to-severe Ps irrespectively of body weight (83). In most research clinical trials, anti-IL-17 inhibitors did not exert any effect on body weight increase. In AMAGINE 1, a phase 3 trial of brodalumab, treatment with this mAb showed higher rates of disease skin improvement (PASI 75 and PASI 90) at weeks 12 and 52 in normal weight patients compared to that noted in obese psoriatic patients (66, 84). Notably, IL-17 inhibitors are very effective independently from body weight; however, they tend to present better clearance rates in normal weight patients.

However, a recent study failed no correlation between body mass index and IL-17 expression in 95 patients with depression (85) and secukinumab-induced skin remission in patients with Ps does not reduce body weight after 12 or 24 weeks of treatment (86). A phase 4 randomized, multi-centre, open label, parallel group, active comparator-controlled study with a duration of 28 weeks and a 28 week extension phase (MEDABOLYX, NCT03440736) recruits patients in an attempt to assess whether secukinumab with lifestyle intervention can improve both skin symptomatology and cardiometabolic status compared to secukinumab alone. Though, the design of the study is on immediately addressing the emerging issue as to whether the IL-17 blocker can improve body weight/BMI, the results of the study could be informative and are highly anticipated.

Several studies, including those conducted by our group, have revealed a negative correlation between IL-17-producing cells and IL-10-producing or other regulatory cell subsets in patients with Ps and PsA, but it is not clear whether these negative correlations are largely depicted in patients stratified in accordance to the presence of obesity (or depression) (22, 87,

TABLE 2 | A summary of representative studies reporting on the role of IL-17 in obesity.

Type of Study	Major findings	Comments	Reference
Serological study in obese women	Circulated serum levels of IL-17 (as well as IL-23) were elevated in 20 obese women obese women compared to 20 lean women	Sample size is too small	(76)
Serological study in obese men and women	Plasma IL-17 levels were higher in 42 volunteers with a BMI >35 compared to those of 34 volunteers with normal BMIs. IL-17 levels were significantly higher in men with a BMI >35 than women with a BMI >35. IL-17 was elevated in those with a BMI >35 that had type 2 diabetes versus those without type 2 diabetes	Relatively small size study limited to plasma sample tests	(77)
Serological study in women with psoriasis and metabolic syndrome	IL-17 levels were higher in women with psoriasis and metabolic syndrome compared to those without	Small cohort tested	(78)
Serological study in patients with metabolic syndrome undergoing nonlinear resistance training	No association was found between IL-17 levels and metabolic syndrome variables and levels of IL-17A were not affected by training	Main cohort and control group small in size (in total 22 individuals)	(79)
Serological study in obese patients undergoing bariatric surgery	Plasma levels of IL-17A in 18 patients significantly decreased 6 months post-operatively	Small size tested	(80)
Serological study in obese and non-obese women participating in a randomized double blind placebo controlled trial investigating the effect of vitamin A	The mean concentration of circulating IL-17 was decreased after vitamin A supplementation in obese as well as non-obese women	One of the very first studies to demonstrate that vitamin A has an effect on IL-17 levels in obese women	(81)

88). Of relevance, secukinumab appears to exert an intriguing obliterating effect on disease-related autoantibodies in patients with Ps and PsA (89).

In the last decades, extensive piece of information underline that cutaneous Ps and PsA patients are at higher risk of developing obesity and cardiovascular disease. A major player for that increase, particularly in the industrialized countries, has been attributed to the adaptation of a western lifestyle with less physical activity and diet with high fat and carbohydrate, as well as excessive sodium consumption favor the development, all of which contribute to overweight and obesity (90). Depression is also a comorbidity, which patients with psoriatic disease frequently need to deal with. Whether these comorbidities contribute *via* an immunobiologically sound mechanism in disease development, relapses or progression or they are just epiphenomena, is a matter of heated debate. Needless to mention, that a significant impact on the resolution or improvement of anxiety and depression related symptoms, as well as on controlling weight, it is likely due to the positive effect the disease remission may exert in patients with Ps and PsA.

CONCLUSION

In conclusion, data from experimental models, and to a lesser extent from clinical studies in depression, obesity and psoriatic

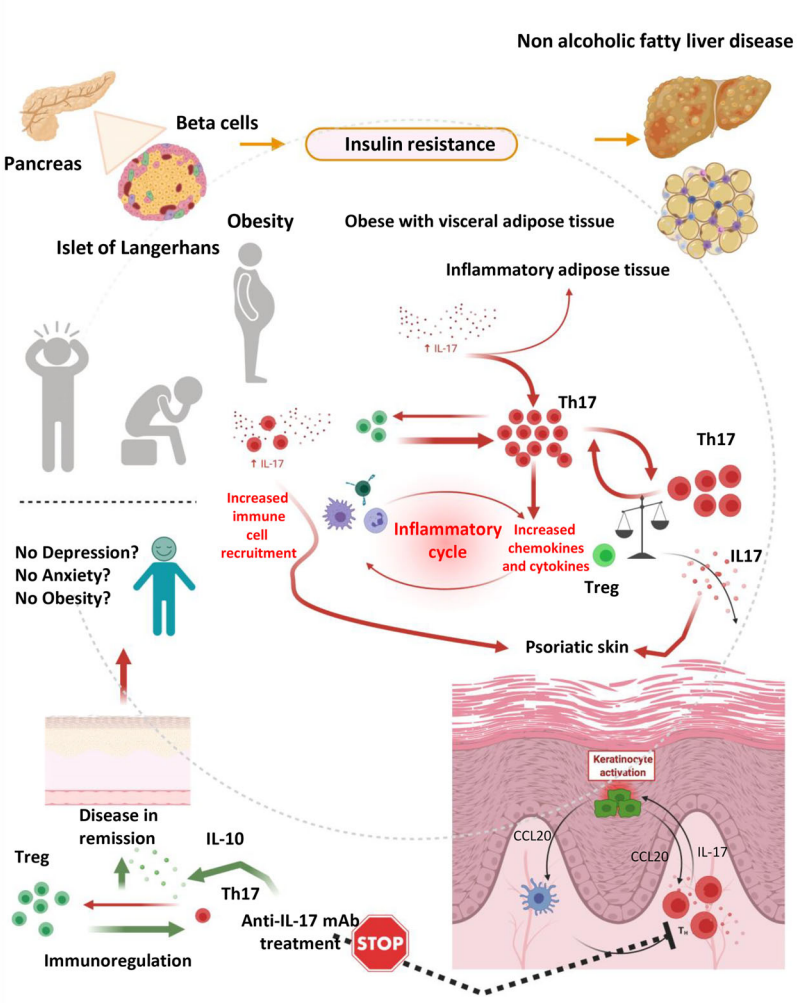


FIGURE 2 | The hypothesis of immunedysregulation in obese depressed patients with psoriatic disease. An inflammatory response mediated by IL-17 producing cells such as Th17 is playing a pivotal role in the development of obesity, depression and psoriasis, in isolation or in combination. Immundysregulation manifested as increased expression of IL-17 and IL-17 producing cell subsets and diminished expression of regulatory cells (Tregs) and related cytokines (such as IL-10) is driving complex reactions leading to the development and progression of psoriatic disease and concomitant anxiety, depression and obesity. Those manifestations can partially be attributed to the inflammatory milieu, which fosters consistent inflammation, keratinocyte activation, cellular damage and skin destruction, as well as interconnected depressive disorders and weight gain. This vicious circle is stopped by IL-17 selective inhibitors, which can lead to the remission of skin lesions (and in the remission of arthritogenic features in the case of psoriatic arthritis or axial spondylarthritis) (prepared with BioRender).

disease, provide the impetus for the understanding for the complex nature of the immunopathogenesis of these manifestations, and suggest that IL-17 could be a common denominator cross-linking some of involved features at the cellular and molecular level in susceptible individuals. However, these data must be treated with caution, as no clear cut evidence for a beneficial role of anti-IL-17 treatment (other than that related to skin lesions and arthritis) in managing anxiety, depression is currently provided, and data by no means are conclusive. Also, a direct effect of anti-IL17 inhibitors in obesity is not clearly documented and this is rather troubling for those promoting the hypothesis suggesting that anti-IL17 neutralizing antibodies may indeed have a clinical impact in fighting obesity. In the clinical setting, body weight does not appear to influence the efficacious effect of anti-IL17 inhibitors in patients with psoriatic disease and this cannot be underestimated. Circumstantial evidence supporting the opposite effect is also weak, raising the expectation of more well controlled, prospective studies investigating these issues in the near future. Limited data on male patients with PsA, have shown that secukinumab treatment is associated with a decrease in resestin and chemerin (but not adiponectin) at 6 months post-treatment compared to baseline. Such a decrease on adipokines was not noted in women with PsA. It must also be emphasized that, as per anti-TNF-α biologics it is not clear to what extent and under which circumstances anti-IL-17 biologics can cross the blood brain barrier, suggesting that their presumed ability to exert any anti-depressant effect would derive mainly from peripheral inhibition of IL-17 rather than a direct effect of those biologics on the brain (50). Nevertheless, the role played in by the IL-17 axis in the development of depression (and obesity) which are frequently regarded as comorbidities of psoriatic disease needs urgent attention and further exploration, mainly because it will assist efforts to better understand whether immunodysregulation involving IL-17 is indeed involved in the induction of either obesity or depression (or both).

AUTHOR CONTRIBUTIONS

EZ and DB had the original idea and scripted considerable part of original draft. AD and VS had performed extensive literature search and scripted part of the manuscript. ED scripted part of the original draft and edited the manuscript. DB prepared the artwork DB and EZ had edited the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Haugeberg G, Lund Nilsen TI, Kavanaugh A, Thomsen RS, Gulati AM, Hoff M. Physical and Psychosocial Burden of Psoriatic Arthritis: Longitudinal Data From a Population-Based Study in Norway. *Arthritis Care Res (Hoboken)* (2021) 73:138–45. doi: 10.1002/acr.24412
2. Kurd SK, Troxel AB, Crits-Christoph P, Gelfand JM. The Risk of Depression, Anxiety, and Suicidality in Patients With Psoriasis: A Population-Based Cohort Study. *Arch Dermatol* (2010) 146:891–5. doi: 10.1001/archdermatol.2010.186
3. Parisi R, Webb RT, Kleyn CE, Carr MJ, Kapur N, Griffiths CEM, et al. Psychiatric Morbidity and Suicidal Behaviour in Psoriasis: A Primary Care Cohort Study. *Br J Dermatol* (2019) 180:108–15. doi: 10.1111/bjd.17004
4. Wu JJ, Penfold RB, Primatesta P, Fox TK, Stewart C, Reddy SP, et al. The Risk of Depression, Suicidal Ideation and Suicide Attempt in Patients With Psoriasis, Psoriatic Arthritis or Ankylosing Spondylitis. *J Eur Acad Dermatol Venereol* (2017) 31:1168–75. doi: 10.1111/jdv.14175
5. McDonough E, Ayearst R, Eder L, Chandran V, Rosen CF, Thavaneswaran A, et al. Depression and Anxiety in Psoriatic Disease: Prevalence and Associated Factors. *J Rheum* (2014) 41:887–96. doi: 10.3899/jrheum.130797
6. Mathew AJ, Chandran V. Depression in Psoriatic Arthritis: Dimensional Aspects and Link With Systemic Inflammation. *Rheum Ther* (2020) 7:287–300. doi: 10.1007/s40744-020-00207-6
7. Kramer NE, Cosgrove VE, Dunlap K, Subramaniapillai M, McIntyre RS, Suppes T. A Clinical Model for Identifying an Inflammatory Phenotype in Mood Disorders. *J Psychiatr Res* (2019) 113:148–58. doi: 10.1016/j.jpsychires.2019.02.005
8. Koo J, Marangell LB, Nakamura M, Armstrong A, Jeon C, Bhutani T, et al. Depression and Suicidality in Psoriasis: Review of the Literature Including the Cytokine Theory of Depression. *J Eur Acad Dermatol Venereol* (2017) 31:1999–2009. doi: 10.1111/jdv.14460
9. Liu CS, Adibfar A, Herrmann N, Gallagher D, Lanctot KL. Evidence for Inflammation-Associated Depression. *Curr Top Behav Neurosci* (2017) 31:3–30. doi: 10.1007/7854_2016_2
10. Raison CL, Capuron L, Miller AH. Cytokines Sing the Blues: Inflammation and the Pathogenesis of Depression. *Trends Immunol* (2006) 27:24–31. doi: 10.1016/j.it.2005.11.006
11. Patel DD, Lee DM, Kolbinger F, Antoni C. Effect of IL-17A Blockade With Secukinumab in Autoimmune Diseases. *Ann Rheum Dis* (2013) 72(Suppl 2): ii116–23. doi: 10.1136/annrheumdis-2012-202371
12. Sakkas LI, Bogdanos DP. Are Psoriasis and Psoriatic Arthritis the Same Disease? The Il-23/IL-17 Axis Data. *Autoimmun Rev* (2017) 16:10–5. doi: 10.1016/j.autrev.2016.09.015
13. Sakkas LI, Zafiriou E, Bogdanos DP. Mini Review: New Treatments in Psoriatic Arthritis. Focus on the IL-23/17 Axis. *Front Pharmacol* (2019) 10:872. doi: 10.3389/fphar.2019.00872
14. Brembilla NC, Senra L, Boehncke WH. The IL-17 Family of Cytokines in Psoriasis: Il-17A and Beyond. *Front Immunol* (2018) 9:1682. doi: 10.3389/fimmu.2018.01682
15. Fasching P, Stradner M, Graninger W, Dejaco C, Fessler J. Therapeutic Potential of Targeting the Th17/Treg Axis in Autoimmune Disorders. *Molecules* (2017) 22(1):134. doi: 10.3390/molecules22010134
16. Kaushik SB, Lebwohl MG. Psoriasis: Which Therapy for Which Patient: Psoriasis Comorbidities and Preferred Systemic Agents. *J Am Acad Dermatol* (2019) 80:27–40. doi: 10.1016/j.jaad.2018.06.057
17. Gossec L, Baraliakos X, Kerschbaumer A, de Wit M, McInnes I, Dougados M, et al. EULAR Recommendations for the Management of Psoriatic Arthritis With Pharmacological Therapies: 2019 Update. *Ann Rheum Dis* (2020) 79:700–12. doi: 10.1136/annrheumdis-2020-217163
18. Osborne LM, Brar A, Klein SL. The Role of Th17 Cells in the Pathophysiology of Pregnancy and Perinatal Mood and Anxiety Disorders. *Brain Behav Immun* (2019) 76:7–16. doi: 10.1016/j.bbi.2018.11.015

19. Kowalczyk M, Szemraj J, Blizniewska K, Maes M, Berk M, Su KP, et al. An Immune Gate of Depression - Early Neuroimmune Development in the Formation of the Underlying Depressive Disorder. *Pharmacol Rep* (2019) 71:1299–307. doi: 10.1016/j.pharep.2019.05.022
20. Raison CL, Rutherford RE, Woolwine BJ, Shuo C, Schettler P, Drake DF, et al. A Randomized Controlled Trial of the Tumor Necrosis Factor Antagonist Infliximab for Treatment-Resistant Depression: The Role of Baseline Inflammatory Biomarkers. *JAMA Psychiatry* (2013) 70:31–41. doi: 10.1001/2013.jamapsychiatry.4
21. McIntyre RS, Subramaniapillai M, Lee Y, Pan Z, Carmona NE, Shekotikhina M, et al. Efficacy of Adjunctive Infliximab vs Placebo in the Treatment of Adults With Bipolar I/Ii Depression: A Randomized Clinical Trial. *JAMA Psychiatry* (2019) 76:783–90. doi: 10.1001/jamapsychiatry.2019.0779
22. Mavropoulos A, Varna A, Zafiriou E, Liaskos C, Alexiou I, Roussaki-Schulze A, et al. Il-10 Producing Bregs Are Impaired in Psoriatic Arthritis and Psoriasis and Inversely Correlate With IL-17- and Ifngamma-Producing T Cells. *Clin Immunol* (2017) 184:33–41. doi: 10.1016/j.clim.2017.04.010
23. Kelepouri D, Mavropoulos A, Bogdanos DP, Sakkas LI. The Role of Flavonoids in Inhibiting Th17 Responses in Inflammatory Arthritis. *J Immunol Res* (2018) 2018:9324357. doi: 10.1155/2018/9324357
24. Zaba LC, Fuentes-Duculan J, Eungdamrong NJ, Abello MV, Novitskaya I, Pierson KC, et al. Psoriasis Is Characterized by Accumulation of Immunostimulatory and Th1/Th17 Cell-Polarizing Myeloid Dendritic Cells. *J Invest Dermatol* (2009) 129:79–88. doi: 10.1038/jid.2008.194
25. Ghoreschi K, Laurence A, Yang XP, Hirahara K, O'Shea JJ. T Helper 17 Cell Heterogeneity and Pathogenicity in Autoimmune Disease. *Trends Immunol* (2011) 32:395–401. doi: 10.1016/j.it.2011.06.007
26. Krueger JG, Brunner PM. Interleukin-17 Alters the Biology of Many Cell Types Involved in the Genesis of Psoriasis, Systemic Inflammation and Associated Comorbidities. *Exp Dermatol* (2018) 27:115–23. doi: 10.1111/exd.13467
27. Cai Y, Shen X, Ding C, Qi C, Li K, Li X, et al. Pivotal Role of Dermal IL-17-producing Gammadelta T Cells in Skin Inflammation. *Immunity* (2011) 35:596–610. doi: 10.1016/j.immuni.2011.08.001
28. Yin Y, Wang M, Liu M, Zhou E, Ren T, Chang X, et al. Efficacy and Safety of IL-17 Inhibitors for the Treatment of Ankylosing Spondylitis: A Systematic Review and Meta-Analysis. *Arthritis Res Ther* (2020) 22:111. doi: 10.1186/s13075-020-02208-w
29. Langley RG, Elewski BE, Lebwohl M, Reich K, Griffiths CE, Papp K, et al. Secukinumab in Plaque Psoriasis–Results of Two Phase 3 Trials. *N Engl J Med* (2014) 371:326–38. doi: 10.1056/NEJMoa1314258
30. Griffiths CE, Reich K, Lebwohl M, van de Kerkhof P, Paul C, Menter A, et al. Uncover and U.-. Investigators, Comparison of Ixekizumab With Etanercept or Placebo in Moderate-to-Severe Psoriasis (UNCOVER-2 and UNCOVER-3): Results From Two Phase 3 Randomised Trials. *Lancet* (2015) 386:541–51. doi: 10.1016/S0140-6736(15)60125-8
31. Mease PJ, van der Heijde D, Ritchlin CT, Okada M, Cuchacovich RS, Shuler CL, et al. Ixekizumab, an Interleukin-17A Specific Monoclonal Antibody, for the Treatment of Biologic-Naive Patients With Active Psoriatic Arthritis: Results From the 24-Week Randomised, Double-Blind, Placebo-Controlled and Active (Adalimumab)-Controlled Period of the Phase III Trial SPIRIT-P1. *Ann Rheum Dis* (2017) 76:79–87. doi: 10.1136/annrheumdis-2016-209709
32. Roman M, Chiu MW. Spotlight on Brodalumab in the Treatment of Moderate-to-Severe Plaque Psoriasis: Design, Development, and Potential Place in Therapy. *Drug Des Devel Ther* (2017) 11:2065–75. doi: 10.2147/DDDT.S113683
33. Papp KA, Merola JF, Gottlieb AB, Griffiths CEM, Cross N, Peterson L, et al. Dual Neutralization of Both Interleukin 17A and Interleukin 17F With Bimekizumab in Patients With Psoriasis: Results From BE ABLE 1, a 12-Week Randomized, Double-Blinded, Placebo-Controlled Phase 2b Trial. *J Am Acad Dermatol* (2018) 79:277–286 e10. doi: 10.1016/j.jaad.2018.03.037
34. Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM. Differential Regulation of Central Nervous System Autoimmunity by T(H)1 and T(H)17 Cells. *Nat Med* (2008) 14:337–42. doi: 10.1038/nm1715
35. Siffrin V, Radbruch H, Glumm R, Niesner R, Paterka M, Herz J, et al. In Vivo Imaging of Partially Reversible th17 Cell-Induced Neuronal Dysfunction in the Course of Encephalomyelitis. *Immunity* (2010) 33:424–36. doi: 10.1016/j.immuni.2010.08.018
36. Lock C, Hermans G, Pedotti R, Brendolan A, Schadt E, Garren H, et al. Gene-Microarray Analysis of Multiple Sclerosis Lesions Yields New Targets Validated in Autoimmune Encephalomyelitis. *Nat Med* (2002) 8:500–8. doi: 10.1038/nm0502-500
37. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. Interleukin-17 Production in Central Nervous System-Infiltrating T Cells and Glial Cells is Associated With Active Disease in Multiple Sclerosis. *Am J Pathol* (2008) 172:146–55. doi: 10.2353/ajpath.2008.070690
38. Wang HH, Dai YQ, Qiu W, Lu ZQ, Peng FH, Wang YG, et al. Interleukin-17-Secreting T Cells in Neuromyelitis Optica and Multiple Sclerosis During Relapse. *J Clin Neurosci* (2011) 18:1313–7. doi: 10.1016/j.jocn.2011.01.031
39. Kumar P, Shih DCW, Lim A, Paleja B, Ling S, Li Yun L, et al. Pro-Inflammatory, IL-17 Pathways Dominate the Architecture of the Immunome in Pediatric Refractory Epilepsy. *JCI Insight* (2019) 4(8): e126337 (pages 1–12). doi: 10.1172/jci.insight.126337
40. Moaaz M, Youssry S, Elfatatry A, El Rahman MA. Th17/Treg Cells Imbalance and Their Related Cytokines (IL-17, IL-10 and TGF-β) in Children With Autism Spectrum Disorder. *J Neuroimmunol* (2019) 337:577071. doi: 10.1016/j.jneuroim.2019.577071
41. Borovcanin MM, Minic Janicijevic S, Jovanovic IP, Gajovic NM, Jurisevic MM, Arsenijevic NN. Type 17 Immune Response Facilitates Progression of Inflammation and Correlates With Cognition in Stable Schizophrenia. *Diagn (Basel)* (2020) 10(11):926 (pages 1–12). doi: 10.3390/diagnostics10110926
42. Beurel E, Lowell JA. Th17 Cells in Depression. *Brain Behav Immun* (2018) 69:28–34. doi: 10.1016/j.bbi.2017.08.001
43. Tsuboi H, Sakakibara H, Minamida Y, Tsujiguchi H, Matsunaga M, Hara A, et al. Elevated Levels of Serum Il-17A in Community-Dwelling Women With Higher Depressive Symptoms. *Behav Sci (Basel)* (2018) 8(11):102 (page 1–7). doi: 10.3390/bs8110102
44. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, et al. A Meta-Analysis of Cytokines in Major Depression. *Biol Psychiatry* (2010) 67:446–57. doi: 10.1016/j.biopsych.2009.09.033
45. Mac Giollabhui N, Ng TH, Ellman LM, Alloy LB. The Longitudinal Associations of Inflammatory Biomarkers and Depression Revisited: Systematic Review, Meta-Analysis, and Meta-Regression. *Mol Psychiatry* (2020). doi: 10.1038/s41380-020-00867-4
46. Miller AH, Raison CL. The Role of Inflammation in Depression: From Evolutionary Imperative to Modern Treatment Target. *Nat Rev Immunol* (2016) 16:22–34. doi: 10.1038/nri.2015.5
47. Colasanto M, Madigan S, Korczak DJ. Depression and Inflammation Among Children and Adolescents: A Meta-Analysis. *J Affect Disord* (2020) 277:940–8. doi: 10.1016/j.jad.2020.09.025
48. Huang M, Su S, Goldberg J, Miller AH, Levantsevych OM, Shallenberger L, et al. Longitudinal Association of Inflammation With Depressive Symptoms: A 7-Year Cross-Lagged Twin Difference Study. *Brain Behav Immun* (2019) 75:200–7. doi: 10.1016/j.bbi.2018.10.007
49. Sukoff Rizzo SJ, Neal SJ, Hughes ZA, Beyna M, Rosenzweig-Lipson S, Moss SJ, et al. Evidence for Sustained Elevation of IL-6 in the CNS as a Key Contributor of Depressive-Like Phenotypes. *Transl Psychiatry* (2012) 2:e199. doi: 10.1038/tp.2012.120
50. Uzzan S, Azab AN. Anti-TNF-Alpha Compounds as a Treatment for Depression. *Molecules* (2021) 26(8):2368 (pages 2–9). doi: 10.3390/molecules26082368
51. Sugino H, Futamura T, Mitsumoto Y, Maeda K, Marunaka Y. Atypical Antipsychotics Suppress Production of Proinflammatory Cytokines and Up-Regulate Interleukin-10 in Lipopolysaccharide-Treated Mice. *Prog Neuropsychopharmacol Biol Psychiatry* (2009) 33:303–7. doi: 10.1016/j.pnpbp.2008.12.006
52. Huppert J, Closhen D, Croxford A, White R, Kulig P, Pietrowski E, et al. Cellular Mechanisms of IL-17-Induced Blood-Brain Barrier Disruption. *FASEB J* (2010) 24:1023–34. doi: 10.1096/fj.09-141978
53. Davami MH, Baharlou R, Ahmadi Vasmehjani A, Ghanizadeh A, Keshtkar M, Dezhkam I, et al. Elevated IL-17 and TGF-beta Serum Levels: A Positive Correlation Between T-Helper 17 Cell-Related Pro-Inflammatory Responses With Major Depressive Disorder. *Basic Clin Neurosci* (2016) 7:137–42. doi: 10.15412/J.BCN.03070207
54. Galecka M, Blizniewska-Kowalska K, Orzechowska A, Szemraj J, Maes M, Berk M, et al. Inflammatory Versus Anti-inflammatory Profiles in Major

Depressive Disorders-The Role of IL-17, Il-21, IL-23, Il-35 and Foxp3. *J Pers Med* (2021) 11(2):66 (pages 1–14). doi: 10.3390/jpm11020066
55. Chen Y, Jiang T, Chen P, Ouyang J, Xu G, Zeng Z, et al. Emerging Tendency Towards Autoimmune Process in Major Depressive Patients: A Novel Insight From Th17 Cells. *Psychiatry Res* (2011) 188:224–30. doi: 10.1016/j.psychres.2010.10.029
56. Alvarez-Mon MA, Gomez-Lahoz AM, Orozco A, Lahera G, Diaz D, Ortega MA, et al. Expansion of CD4 T Lymphocytes Expressing Interleukin 17 and Tumor Necrosis Factor in Patients With Major Depressive Disorder. *J Pers Med* (2021) 11(3):220 (pages 1–16). doi: 10.3390/jpm11030220
57. Kim JW, Kim YK, Hwang JA, Yoon HK, Ko YH, Han C, et al. Plasma Levels of IL-23 and IL-17 Before and After Antidepressant Treatment in Patients With Major Depressive Disorder. *Psychiatry Investig* (2013) 10:294–9. doi: 10.4306/pi.2013.10.3.294
58. Beurel E, Lowell JA, Jope RS. Distinct Characteristics of Hippocampal Pathogenic TH17 Cells in a Mouse Model of Depression. *Brain Behav Immun* (2018) 73:180–91. doi: 10.1016/j.bbi.2018.04.012
59. Beurel E, Harrington LE, Jope RS. Inflammatory T Helper 17 Cells Promote Depression-Like Behavior in Mice. *Biol Psychiatry* (2013) 73:622–30. doi: 10.1016/j.biopsych.2012.09.021
60. Sales MC, Kasahara TM, Sacramento PM, Rossi AD, Cafasso M, Oyamada HAA, et al. Selective Serotonin Reuptake Inhibitor Attenuates the Hyperresponsiveness of TLR2(+) and TLR4(+) Th17/Tc17-like Cells in Multiple Sclerosis Patients With Major Depression. *Immunology* (2021) 162:290–305. doi: 10.1111/imm.13281
61. Kim J, Suh YH, Chang KA. Interleukin-17 Induced by Cumulative Mild Stress Promoted Depression-Like Behaviors in Young Adult Mice. *Mol Brain* (2021) 14:11. doi: 10.1186/s13041-020-00726-x
62. Li Y, Xiao B, Qiu W, Yang L, Hu B, Tian X, et al. Altered Expression of CD4 (+)CD25(+) Regulatory T Cells and its 5-HT(1a) Receptor in Patients With Major Depression Disorder. *J Affect Disord* (2010) 124:68–75. doi: 10.1016/j.jad.2009.10.018
63. Nadeem A, Ahmad SF, Al-Harbi NO, Fardan AS, El-Sherbeeny AM, Ibrahim KE, et al. Il-17A Causes Depression-Like Symptoms *Via* NFkappaB and p38MAPK Signaling Pathways in Mice: Implications for Psoriasis Associated Depression. *Cytokine* (2017) 97:14–24. doi: 10.1016/j.cyto.2017.05.018
64. Rivera-Oyola R, Stanger R, Litchman GH, Thibodeaux Q, Koo J, Fried R, et al. The Use of Brodalumab in Three Patients With Psoriasis and Psychiatric Comorbidities. *J Clin Aesthet Dermatol* (2020) 13:44–8.
65. Lebwohl M, Strober B, Menter A, Gordon K, Weglowska J, Puig L, et al. Phase 3 Studies Comparing Brodalumab With Ustekinumab in Psoriasis. *N Engl J Med* (2015) 373:1318–28. doi: 10.1056/NEJMoa1503824
66. Papp KA, Reich K, Paul C, Blauvelt A, Baran W, Bolduc C, et al. A Prospective Phase III, Randomized, Double-Blind, Placebo-Controlled Study of Brodalumab in Patients With Moderate-to-Severe Plaque Psoriasis. *Br J Dermatol* (2016) 175:273–86. doi: 10.1111/bjd.14493
67. Lebwohl MG, Papp KA, Marangell LB, Koo J, Blauvelt A, Gooderham M, et al. Psychiatric Adverse Events During Treatment With Brodalumab: Analysis of Psoriasis Clinical Trials. *J Am Acad Dermatol* (2018) 78:81–89 e5. doi: 10.1016/j.jaad.2017.08.024
68. Gould TD, Georgiou P, Brenner LA, Brundin L, Can A, Courtet P, et al. Postolache, Animal Models to Improve Our Understanding and Treatment of Suicidal Behavior. *Transl Psychiatry* (2017) 7:e1092. doi: 10.1038/tp.2017.50
69. Lebwohl M, Leonardi C, Wu JJ, Armstrong A, Rawnsley N, Merchant M, et al. Two-Year US Pharmacovigilance Report on Brodalumab. *Dermatol Ther (Heidelb)* (2021) 11:173–80. doi: 10.1007/s13555-020-00472-x
70. Genovese MC, Mysler E, Tomita T, Papp KA, Salvarani C, Schwartzman S, et al. Safety of Ixekizumab in Adult Patients With Plaque Psoriasis, Psoriatic Arthritis and Axial Spondyloarthritis: Data From 21 Clinical Trials. *Rheumatol (Oxford)* (2020) 59:3834–44. doi: 10.1093/rheumatology/keaa189
71. Talamonti M, Malara G, Natalini Y, Bardazzi F, Conti A, Chiricozzi A, et al. Secukinumab Improves Patient Perception of Anxiety and Depression in Patients With Moderate to Severe Psoriasis: A Post Hoc Analysis of the SUPREME Study. *Acta Derm Venereol* (2021) 101:adv00422. doi: 10.2340/00015555-3712
72. Kelly T, Yang W, Chen CS, Reynolds K, He J. Global Burden of Obesity in 2005 and Projections to 2030. *Int J Obes (Lond)* (2008) 32:1431–7. doi: 10.1038/ijo.2008.102
73. Chauvet-Gelinier JC, Roussot A, Cottenet J, Brindisi MC, Petit JM, Bonin B, et al. Depression and Obesity, Data From a National Administrative Database Study: Geographic Evidence for an Epidemiological Overlap. *PLoS One* (2019) 14:e0210507. doi: 10.1371/journal.pone.0210507
74. Nishimura S, Manabe I, Nagai R. Adipose Tissue Inflammation in Obesity and Metabolic Syndrome. *Discov Med* (2009) 8:55–60.
75. Ruddy MJ, Wong GC, Liu XK, Yamamoto H, Kasayama S, Kirkwood KL, et al. Functional Cooperation Between Interleukin-17 and Tumor Necrosis Factor-Alpha Is Mediated by CCAAT/enhancer-binding Protein Family Members. *J Biol Chem* (2004) 279:2559–67. doi: 10.1074/jbc.M308809200
76. Sumarac-Dumanovic M, Stevanovic D, Ljubic A, Jorga J, Simic M, Stamenkovic-Pejkovic D, et al. Increased Activity of Interleukin-23/interleukin-17 Proinflammatory Axis in Obese Women. *Int J Obes (Lond)* (2009) 33:151–6. doi: 10.1038/ijo.2008.216
77. Elisia I, Lam V, Cho B, Hay M, Li MY, Kapeluto J, et al. Exploratory Examination of Inflammation State, Immune Response and Blood Cell Composition in a Human Obese Cohort to Identify Potential Markers Predicting Cancer Risk. *PLoS One* (2020) 15:e0228633. doi: 10.1371/journal.pone.0228633
78. Pirowska M, Obtulowicz A, Lipko-Godlewska S, Gozdzialska A, Podolec K, Wojas-Pelc A. The Level of Proinflammatory Cytokines: Interleukins 12, 23, 17 and Tumor Necrosis Factor Alpha in Patients With Metabolic Syndrome Accompanying Severe Psoriasis and Psoriatic Arthritis. *Postepy Dermatol Alergol* (2018) 35:360–6. doi: 10.5114/ada.2018.77665
79. Nikseresht M. Comparison of Serum Cytokine Levels in Men Who are Obese or Men Who are Lean: Effects of Nonlinear Periodized Resistance Training and Obesity. *J Strength Cond Res* (2018) 32:1787–95. doi: 10.1519/JSC.0000000000002039
80. Villarreal-Calderon JR, Castillo EC, Cuellar-Tamez RX, Garcia-Garza M, Elizondo-Montemayor L, Garcia-Rivas G. Reduced Th1 Response is Associated With Lower Glycolytic Activity in Activated Peripheral Blood Mononuclear Cells After Metabolic and Bariatric Surgery. *J Endocrinol Invest* (2021). doi: 10.1007/s40618-021-01587-4
81. Farhangi MA, Saboor-Yaraghi AA, Keshavarz SA. Vitamin A Supplementation Reduces the Th17-Treg - Related Cytokines in Obese and non-Obese Women. *Arch Endocrinol Metab* (2016) 60:29–35. doi: 10.1590/2359-3997000000125
82. Paroutoglou K, Papadavid E, Christodoulatos GS, Dalamaga M. Deciphering the Association Between Psoriasis and Obesity: Current Evidence and Treatment Considerations. *Curr Obes Rep* (2020) 9:165–78. doi: 10.1007/s13679-020-00380-3
83. Kaushik SB, Lebwohl MG. Psoriasis: Which Therapy for Which Patient: Focus on Special Populations and Chronic Infections. *J Am Acad Dermatol* (2019) 80:43–53. doi: 10.1016/j.jaad.2018.06.056
84. Sbidian E, Chaimani A, Garcia-Doval I, Do G, Hua C, Mazaud C, et al. Systemic Pharmacological Treatments for Chronic Plaque Psoriasis: A Network Meta-Analysis. *Cochrane Database Syst Rev* (2017) 12:CD011535. doi: 10.1002/14651858.CD011535.pub2
85. Blizniewska-Kowalska K, Szewczyk B, Galecka M, Su KP, Maes M, Szemraj J, et al. Is Interleukin 17 (Il-17) Expression A Common Point in the Pathogenesis of Depression and Obesity? *J Clin Med* (2020) 9(12):4018 (pages 1–15). doi: 10.3390/jcm9124018
86. Wang HN, Huang YH. Changes in Metabolic Parameters in Psoriatic Patients Treated With Secukinumab. *Ther Adv Chronic Dis* (2020) 11:2040622320944777. doi: 10.1177/2040622320944777
87. Liu Y, Zhang C, Li B, Yu C, Bai X, Xiao C, et al. A Novel Role of IL-17A in Contributing to the Impaired Suppressive Function of Tregs in Psoriasis. *J Dermatol Sci* (2021) 101:84–92. doi: 10.1016/j.jdermsci.2020.09.002
88. Bovenschen HJ, van de Kerkhof PC, van Erp PE, Woestenenk R, Joosten I, Koenen HJ. Foxp3+ Regulatory T Cells of Psoriasis Patients Easily Differentiate Into IL-17A-producing Cells and Are Found in Lesional Skin. *J Invest Dermatol* (2011) 131:1853–60. doi: 10.1038/jid.2011.139
89. Patrikiou E, Liaskos C, Mavropoulos A, Ntavari N, Gkoutzourelas A, Simopoulou T, et al. Autoantibodies Against Specific Nuclear Antigens are Present in Psoriatic Disease and Are Diminished by Secukinumab. *Clin Chim Acta* (2020) 510:400–7. doi: 10.1016/j.cca.2020.07.037

90. Zachariae C, Skov L. Obesity as a Risk Factor for Psoriasis. *J Eur Acad Dermatol Venereol* (2020) 34:915–6. doi: 10.1111/jdv.16434

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Effects of Tocilizumab Therapy on Circulating B Cells and T Helper Cells in Patients With Neuromyelitis Optica Spectrum Disorder

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Tocilizumab, a humanized anti-IL-6 receptor monoclonal antibody, showed its therapeutic efficacy on neuromyelitis optica spectrum disorder (NMOSD). To assess the immunological effects of this drug on B cells, follicular T helper (Tfh) cells, and peripheral T helper (Tph) cells in patients with NMOSD, peripheral B cell and Tfh cell phenotypes were evaluated in 26 patients with NMOSD before and after tocilizumab treatment by nine-color flow cytometry, as well as the expression of costimulatory and co-inhibitory molecules on B cells. Results showed that the frequency of $CD27^{+}IgD^{-}$ switched memory B cells, $CD27^{-}IgD^{-}$ double-negative B cells, and $CD27^{high}CD38^{high}$ antibody-secreting cells was increased in patients with NMOSD. Tocilizumab treatment led to a significant shift of B cells to naïve B cells from memory B cells after 3 months. Three markers on B cells associated with T-cell activation (i.e., CD86 CD69, and HLA-DR) were downregulated after tocilizumab treatment. The frequencies of total Tfh and Tph cells were decreased, whereas that of follicular regulatory T cells tended to increase. Intrinsic increased PD-L1 and PD-L2 expression was characteristic of B cells in patients with NMOSD. Tocilizumab selectively restored PD-L1 on B-cell subsets. These results provided evidence that tocilizumab enhanced B- and T-cell homoeostasis by regulating B-cell differentiation and inhibiting lymphocyte activation in patients with NMOSD.

Keywords: NMOSD, tocilizumab, B cells, T helper cells, PD-1, PD-L

Abbreviations: AQP4, aquaporin-4; ASCs, antibody-secreting cells; DN B cells, double negative B cells; HCs, healthy controls; MFI, mean fluorescence intensity; NMOSD, Neuromyelitis optica spectrum disorder; PBMCs, peripheral blood mononuclear cells; PD-1, programmed cell death 1; PD-L, programmed cell death 1 ligand; SWM B cells, switched memory B cells; Tfh cells, follicular T helper cells; Tfr cells, follicular regulatory T cells; Tph cells, peripheral T helper cells; USM B cells, unswitched memory B cells.

INTRODUCTION

Neuromyelitis optica spectrum disorder (NMOSD) is an autoimmune inflammatory disease of the central nervous system involving pathogenic autoantibodies against aquaporin-4 (AQP4-IgG) (1, 2). Imbalances in B- and T-cell homoeostasis have been investigated. Among these, autoreactive B-cell subsets and loss of anergic maintenance may affect disease activity (3–5). The function of B cells is involved in antigen presentation, pro-inflammatory and anti-inflammatory cytokine production and immunoglobulin production. Circulating B cell subtypes include naïve B cells ($CD19^+CD27^-IgD^+$) and 3 kinds of memory B cells, namely unswitched memory B cells (USW, $CD19^+CD27^+IgD^+IgM^+$), switched memory B (SWM, $CD19^+CD27^+IgD^-IgM^-$), double-negative (DN, $CD19^+CD27^-IgD^-$) (3). Follicular T helper (Tfh) cells are critical in promoting B-cell autoimmunity and autoantibody production (6). In addition, peripheral helper T (Tph) cells was first defined with the markers of $CD4^+PD\text{-}1^{high}CXCR5^-$. These cells expressed indispensable cytokines that enable B-cell help, including IL-21, CXCL13, ICOS, and MAF. Like Tfh cells, Tph cells can also induce plasma cell differentiation *in vitro* through IL-21 secretion and SLAMF5 interaction. Different from Tfh cells, Tph cells have unique expression of chemokine receptors (such as CCR2, CX3CR1, and CCR5) that direct migration to inflamed sites (7). However, the dynamics of Tph cells in NMOSD remain unclear. Specifically, AQP4-specific T cells are expanded in patients with NMOSD and exhibit pro-inflammatory Th17 polarization (8, 9).

Co-inhibitory signals characteristic of programmed cell death 1 (PD-1) are expressed on T cells as feedback for activated responses (10, 11). Engagement of PD-1 ligand 1 (PD-L1) and 2 (PD-L2) also plays important regulatory roles in immune responses (12). However, little is known about the kinetics of PD-1, PD-L1, and PD-L2 expression on B cells from patients with NMOSD.

Pleiotropic pro-inflammatory interleukin (IL)-6 drives disease activity and may contribute to abnormalities in B and T cells (13). Therapeutic agents targeting the IL-6 axis are effective in settings of acute and chronic inflammation (14). Recently, we reported a phase 2 randomized controlled trial showing that tocilizumab, a humanized anti-IL-6 receptor monoclonal antibody (mAb), significantly reduced the risk of relapse compared to azathioprine in NMOSD. Tocilizumab also decreases serum AQP4-IgG titers in patients with highly active NMOSD (15). However, the underlying effects of tocilizumab treatment on circulating B and T cells are unclear. Accordingly, in this study, we performed a detailed analysis of immunological phenotypes in B and T cells before and after tocilizumab treatment in patients with NMOSD.

MATERIAL AND METHODS

Recruitment and Enrollment of Patients and Controls

Patients with NMOSD were diagnosed according to 2015 International Panel for Neuromyelitis Optica Diagnosis criteria. Patients who received tocilizumab treatment at Tianjin Medical University General Hospital between November 2017 and May 2019 were enrolled. The inclusion criteria in this study were as follows: those who received routine infusion of tocilizumab at a dose of 8 mg/kg every 4 weeks for at least 3 months. At study entry, patients were permitted to continue the baseline treatment of corticosteroids. The exclusion criteria were as follows: 1) patients who were concomitantly treated with oral immunosuppressants, such as azathioprine, mycophenolate mofetil, and tacrolimus; and 2) patients who received rituximab treatment for less than 6 months from initial tocilizumab infusion. We also included 20 age- and sex-matched healthy controls (HCs) who had no malignancy or autoimmune disorder and did not receive immunosuppressive therapy. The Institutional Review Board of Tianjin Medical University General Hospital reviewed and approved this study. Each participant provided written informed consent.

Flow Cytometry Analysis

All patients who received routine tocilizumab treatment were registered for immunophenotyping analysis. Peripheral blood was collected at baseline, 1 month, and 3 months after initiation of treatment. All fresh samples were immediately processed. Briefly, peripheral blood mononuclear cells (PBMCs) were prepared by density-gradient centrifugation, resuspended in phosphate-buffered saline (PBS)/3% human IgG (Bax International Inc., Vienna, Austria) to block Fc receptors and prevent nonspecific antibody binding, and then incubated for 15 min at 4°C in the dark. Subsequently, the cells were washed with PBS containing 1% bovine serum albumin. Background fluorescence was assessed using appropriate isotype- and fluorochrome-matched control mAbs.

To analyze the frequency of B-cell subpopulations, PBMCs were stained with allophycocyanin (APC) anti-human CD19 antibodies (clone HIB19), phycoerythrin (PE)/cyanine7 anti-human IgD antibodies (clone IA6-2), Alexa Fluor 488 anti-human CD38 antibodies (clone HIT2), Brilliant Violet 421 anti-human CD27 antibodies (clone M-T271), Alexa Fluor 488 anti-human HLA-DR antibodies (clone L243), APC anti-human CD86 antibodies (clone IT2.2), Brilliant Violet 421 anti-human CD69 antibodies (clone FN50), PE anti-human CD279 (PD-1) antibodies (clone EH12.2H7), APC anti-human CD274 (B7-H1, PD-L1) antibodies (clone 29E.2A3), and PE anti-human CD273 (B7-DC, PD-L2) antibodies (clone 24F.10C12). To characterize T-cell phenotypes, PBMCs were stained with fluorescein isothiocyanate anti-human CD4 antibodies (clone A161A1), Brilliant Violet 421 anti-human CD183 (CXCR3) antibodies (clone G025H7), PE/cyanine7 anti-human CD185 (CXCR5) antibodies (clone J252D4), APC/cyanine7 anti-human CD196 (CCR6) antibodies (clone G034E3), PE anti-human CD25 antibodies (clone BC96), and Brilliant Violet 421 anti-human CD127 (IL-7Rα) antibodies (clone A019D5). All the antibodies were purchased from Biolegend (San Diego, CA, USA).

Prior to the measurements, 1 μL of 300 nM 4′,6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA, USA) was added to exclude dead cells. The stained samples were assessed by flow

cytometry using a FACS Aria III flow cytometer (BD Biosciences, San Jose, CA, USA). The results were analyzed using FlowJo software (version 10).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism v8 and R software. Continuous and ordinal variables are presented with medians (interquartile ranges [IQRs]) or means (standard deviations [SDs] or standard errors of the means [SEMs]). Shapiro-Wilk tests were used to determine whether the variables had a normal distribution. For comparisons between patients and HCs, unpaired, two-tailed Student's *t* tests or Mann-Whitney *U* tests were used. Paired Student's *t* tests (parametric) or Wilcoxon matched pairs tests (nonparametric) were used to compare baseline and post-treatment results in patients with NMOSD. For multiple comparisons, the Bonferroni-Dunn adjustment was used for analysis of repeated-measures. *P* values less than 0.05 were considered significant.

RESULTS

Baseline Characteristics

The baseline characteristics of patients with NMOSD and HCs are described in **Table 1**. The median age of patients with NMOSD was 49.5 (IQR: 36.5–58.0) years. Additionally, 25 (96.3%) patients were women, and 24 (92.3%) patients were AQP4-IgG seropositive. The median duration of NMOSD was 2.59 (IQR: 0.63–4.10) years, and the median Expanded Disability Status Scale at baseline was 2.5 (IQR: 2.0–3.9). At baseline, 20 (76.9%) patients were being treated with oral corticosteroids (10mg/day, QD). They were permitted to receive corticosteroids for the first 12 weeks; thereafter, tocilizumab was used as monotherapy.

Relapse was defined as new onset of neurological symptoms or worsening of existing neurological symptoms with an objective change on neurological examination that persisted for more than 24 h, with signs and symptoms attributable solely to NMOSD, and preceded by at least 30 days of clinical stability. By the end of the study, three (11%) of 26 patients in the tocilizumab group had a relapse.

Tocilizumab Treatment Restored the Distribution of B-Cell Subsets

As shown in **Figure 1**, compared with HCs, patients with NMOSD exhibited significantly higher proportions of $CD27^+IgD^-$ switched memory (SWM) B cells (HC *versus* NMOSD:21.54% ± 1.69% *versus* 31.16% ± 3.53%, respectively; $P = 0.0291$), $CD27^-IgD^-$ double negative (DN) B cells (HC *versus* NMOSD: 5.41% ± 0.76% *versus* 8.80% ± 1.16%, respectively; $P = 0.0193$), and $CD27^{high}CD38^{high}$ antibody-secreting cells (ASCs) (HC *versus* NMOSD: 0.98% ± 0.12% *versus* 2.29% ± 0.30%, respectively; $P = 0.0003$), but statistical analysis showed no significant differences between HCs and NMOSD patients in the proportion of $CD27^-IgD^+$ naïve B cells (HC *versus* NMOSD: 62.80% ± 2.83% *versus* 57.05% ± 4.35%, respectively; $P = 0.2742$) and $CD27^+IgD^+$ unswitched memory (USM) B cells (HC *versus* NMOSD: 12.23% ± 1.03% *versus* 10.24% ± 1.14%, respectively; $P = 0.2015$). That is to say, the distribution of B-cell subsets was different from that of HCs, with an elevated proportion of DN, SWM cells, and ASCs.

There were no significant differences in the proportions of total B cells in lymphocyte cells at 1 or 3 months after tocilizumab treatment compared with those at baseline (1 month *versus* 0 month: 9.98% ± 1.19% *versus* 10.19% ± 1.32%, respectively, $P = 0.8027$; 3 months *versus* 0 month: 10.14% ± 1.03% *versus* 10.19% ± 1.32%, respectively, $P = 0.9332$) (**Figures 2A, B**). When come to the B cell subtypes, it was found that the frequencies of DN (1 month *versus* 0 month: 6.86% ± 0.93% *versus* 9.86% ± 1.21%, respectively, $P = 0.0091$) and SWM B cells (1 month *versus* 0 month: 24.58% ± 2.83% *versus* 33.74% ± 3.52%, respectively, $P = 0.0011$) were significantly decreased compared with those at the baseline after 1 month of tocilizumab treatment in patients with NMOSD. In contrast, the frequency of naïve B cells was significantly increased (1 month *versus* 0 month: 65.91% ± 3.92% *versus* 52.54% ± 4.25%, $P = 0.0107$). We did not find a significant decrease in the frequencies of ASCs (1 month *versus* 0 month: 2.28% ± 0.33% *versus* 3.06% ± 0.46%, $P = 0.0863$) or unswitched memory (USM) B cells (1 month *versus* 0 month: 5.38% ± 0.72% *versus* 6.32% ± 0.86%, $P = 0.1147$). After 3 months of treatment, tocilizumab led to a significant shift to a more naïve B-cell phenotype from mature memory B cells,

TABLE 1 | Demographics of participants in the study.

	HCs (n=20)	NMOSD (n=26)
Female, n (%)	19 (95.0)	25 (96.3)
Age at baseline, median (IQR)	43.0 (39.3-51.8)	49.5 (36.5-58.0)
Age at onset, median (IQR)	—	45.5 (34.0-57.3)
Disease duration(y), median (IQR)	—	2.59 (0.63-4.10)
ARR at baseline, median (IQR)	—	0.28 (0.00-0.64)
EDSS score, median (IQR)	—	2.5 (2.0-3.9)
AQP4-ab positive, n (%)	—	24 (92.3)
Preventive medications at baseline, n (%)		
No treatment	—	6 (23.1)
oral corticosteroids		20 (76.9)

EDSS, Expanded Disability Status Scale; HC, healthy control; NMOSD, neuromyelitis optica spectrum disorders; IQR, interquartile range; Treatment was defined as use of high-dose intravenous steroids, plasma exchange (PE) or intravenous immunoglobulin (IVIG) at relapse stage; Values indicate median (interquartile range) or number (percent).

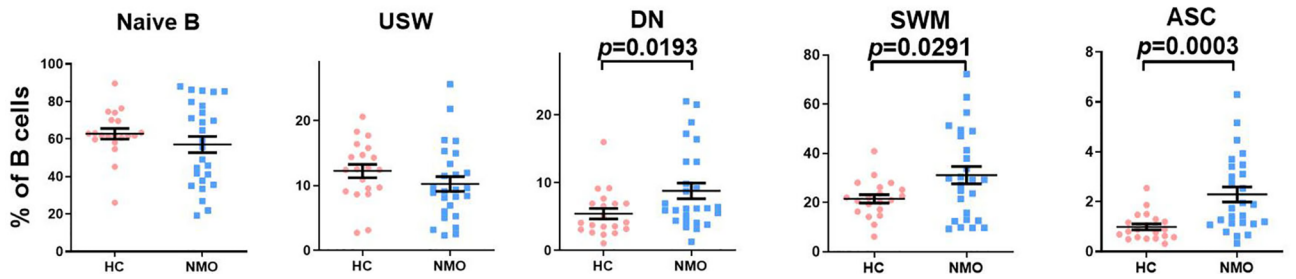


FIGURE 1 | The comparison of B cell subsets distribution in healthy controls and patients with NMOSD. Proportions of $CD27^{-}IgD^{+}$ naïve B cell, $CD27^{+}IgD^{+}$ unswitched memory B (USW), $CD27^{-}IgD^{-}$ double negative B cell (DN), $CD27^{+}IgD^{-}$ switched memory B (SWM) cells and $CD27^{high}CD38^{high}$ autoantibodies secreted cells (ASC) among total B cells in patients with NMOSD and healthy controls (HC). Unpaired, two-tailed Student's t tests were used. HC: n=20; NMOSD: n=26.

as demonstrated by an increase in naïve B cell numbers (3 month *versus* 0 month: 70.43% ± 3.76% *versus* 52.54% ± 4.25%, P = 0.0005) and decreases in SWM (3 month *versus* 0 month: 20.71% ± 2.10% *versus* 33.74% ± 3.52%, P = 0.0008) and DN B cell numbers (3 month *versus* 0 month: 5.62% ± 0.80% *versus* 9.86% ± 1.21%, P = 0.0003) compared with those at baseline, After 3 months of treatment, the proportion of abnormally elevated ASCs also decreased (3 month *versus* 0 month:1.47% ± 0.26% *versus* 3.06% ± 0.46%, P = 0.0406) (**Figures 2C-F**). Although not significant, tocilizumab tended to decrease the proportions of USM B cells at 3 months (3 month *versus* 0 month:5.90% ± 0.60% *versus* 6.32% ± 0.86%, P = 0.0791). We did not find any significant differences between the proportions of naïve B cells (1 month *versus* 3 month: 65.91% ± 3.92% *versus*

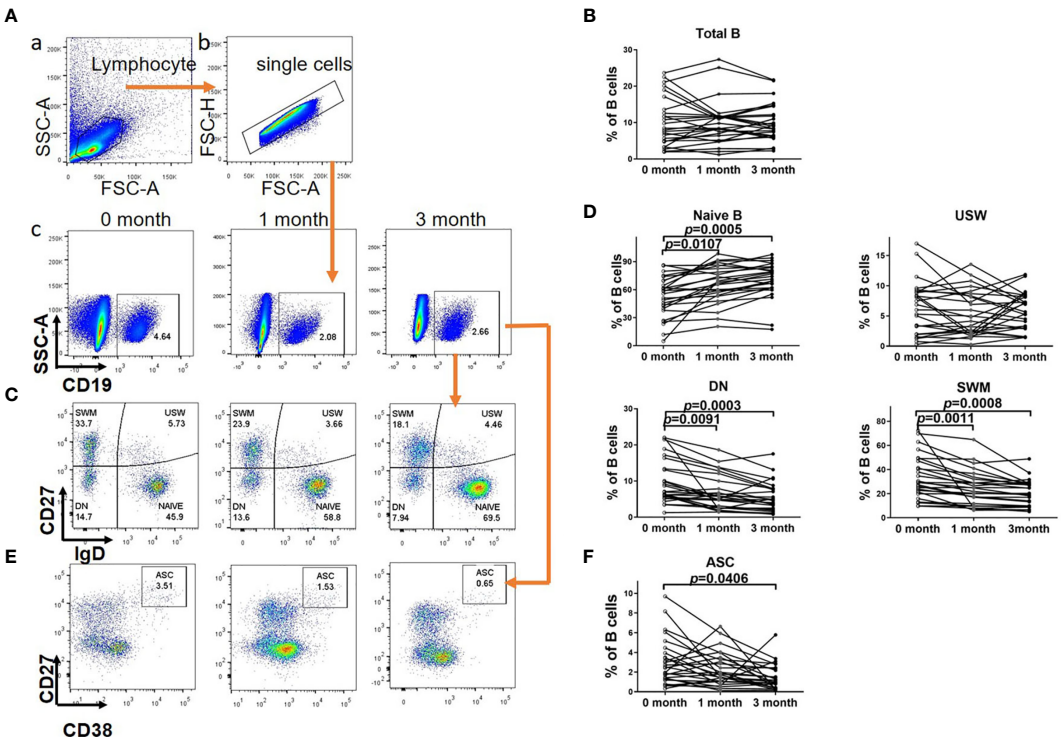


FIGURE 2 | B cell subsets distribution in NMOSD patients before and after tocilizumab. **(A)** Gating strategy for B subsets. (a) Sideward scatter-A (SSC-A) and Forward scatter-A (FSC-A) were used for lymphocytes gating. (b) FSC-H and FSC-A were used to gate single cells. (c) B cells was charactered by $CD19^{+}$. **(B)** Quantitative analysis of proportion of total B cells. The effect of tocilizumab treatment on these circulating B cell subsets was also assessed in NMOSD patients at baseline and after 1 month/3 months of treatment. **(C, E)** Representative flow cytometry of $CD19^{+}CD27^{-}IgD^{+}$ (naive), $CD19^{+}CD27^{-}IgD^{-}$ (double-negative, DN), $CD19^{+}CD27^{+}IgD^{+}$ (unswitched memory, USW), $CD19^{+}CD27^{+}IgD^{-}$ (switched memory, SWM) and $CD19^{+}CD27^{high}$ $CD38^{high}$ ASC (Autoantibodies secreted cells) from NMOSD patients before and after tocilizumab. **(D, F)** Quantitative analysis of proportion of B cell subsets and ASC. Significance was tested by repeated measures of analysis of variance and the Bonferroni–Dunn adjustment for multiple comparisons. Paired, two-tailed Student's *t* tests (if the distribution is normal) or Wilcoxon matched pairs tests (If it does not conform to the normal distribution) were used to compare baseline and post-treatment results, n=26.

70.43% ± 3.76%, *P*=0.0943), DN B cells(1 month *versus* 3 month: 6.86% ± 0.93% *versus* 5.62% ± 0.80%, *P*=0.0610), SWM B cells (1 month *versus* 3 month: 24.58% ± 2.83% *versus* 20.71% ± 2.10%, *P*=0.1023), USM B cells(1 month *versus* 3 month: 5.38% ± 0.72% *versus* 5.90% ± 0.60%, *P*=0.4315) and ASC (1 month *versus* 3 month: 2.28% ± 0.33% *versus* 1.47% ± 0.26%, *P*=0.0989) at 1 month of tocilizumab treatment and those at 3 months.

In a word, tocilizumab treatment restored the distribution of B-cell subsets, to be detailed, the elevated proportion of DN, SWM cells and ASCs was partial recovered, otherwise the proportion of naïve B cells increased.

Tfh, Peripheral T Helper, and Follicular Regulatory T Cells Were Differentially Affected in Tocilizumab-Treated Patients With NMOSD

First, we examined the proportions of Tph ($CD4^+CXCR5^-PD\text{-}1^{high}$) and Tfh ($CD4^+CXCR5^+PD\text{-}1^{high}$) cells in the peripheral blood of patients with NMOSD at baseline. We found that the proportions of both phenotypes were significantly higher in the patients with NMOSD than those in HCs (Tfh cells: 0.76% ± 0.11% *versus* 2.69% ± 0.23%, respectively, *P*= 0.0008; Tph cells: 3.08% ± 0.45% *versus* 6.59% ± 0.43%, respectively, *P* = 0.0009). Moreover, proportions of the Tfh cell subsets Tfh1 (0.12% ± 0.04% *versus* 1.23% ± 0.14%, respectively; *P* = 0.0006), Tfh17 (0.05% ± 0.01% *versus* 0.41% ± 0.05%, respectively; *P* = 0.0003), and Tfh2 (0.52% ± 0.06% *versus* 0.79% ± 0.10%, respectively; *P* = 0.0212) were significantly increased in patients with NMOSD. Additionally, proportions of the Tph cell subsets Tph1 (0.27% ± 0.08% *versus* 2.12% ± 0.22%, respectively; *P* = 0.0004) and Tph17 (0.22% ± 0.03% *versus* 0.80% ± 0.12%, respectively; *P* = 0.0002) were increased, whereas the proportion of Tph2 cells (2.58% ± 0.43% *versus* 3.25% ± 0.29%, respectively; *P* = 0.1836) was not significantly decreased (**Figures 3A, B**).

No significant differences were found in the proportions of $CD4^+CD45RA^-CXCR5^+CD25^+CD127^-$ Tfr cells in patients with NMOSD compared to those in HCs (5.49% ± 0.41% *versus* 5.77% ± 0.39%, respectively; *P* = 0.6273; **Figure 4I**).

Next, we assessed the effects of tocilizumab on Tfh, Tph, and Tfr cells. After tocilizumab treatment, there were significantly lower proportions of total Tfh cells at 1 month (0 month *versus* 1 month: 5.10% ± 0.70% *versus* 3.86% ± 0.57%, *P* = 0.0044) and 3 months (0 month *versus* 3 months: 5.10% ± 0.70% *versus* 3.18% ± 0.45%, *P* = 0.0009) compared to those before treatment (**Figures 4A, B**). Tocilizumab treatment tended to reduce the number of total Tph cells at 1 month (0 month *versus* 1 month: 6.61% ± 0.46% *versus* 5.61% ± 0.45%, *P* = 0.1884), and a significant decrease in the number of Tph cells was observed after 3 months (0 month *versus* 3 months: 6.61% ± 0.46% *versus* 5.38% ± 0.42%, *P* = 0.0106; **Figure 4C**). In addition, among the Tfh cell subsets, we observed a tendency for reduction in the proportion of Tfh1 cells (0 month *versus* 1 month: 3.54% ± 0.22% *versus* 3.15% ± 0.24%, *P* = 0.0929) and Tfh17 cells (0 month *versus* 1 month: 2.83% ± 0.19% *versus* 2.56% ± 0.18%, *P* = 0.1049)

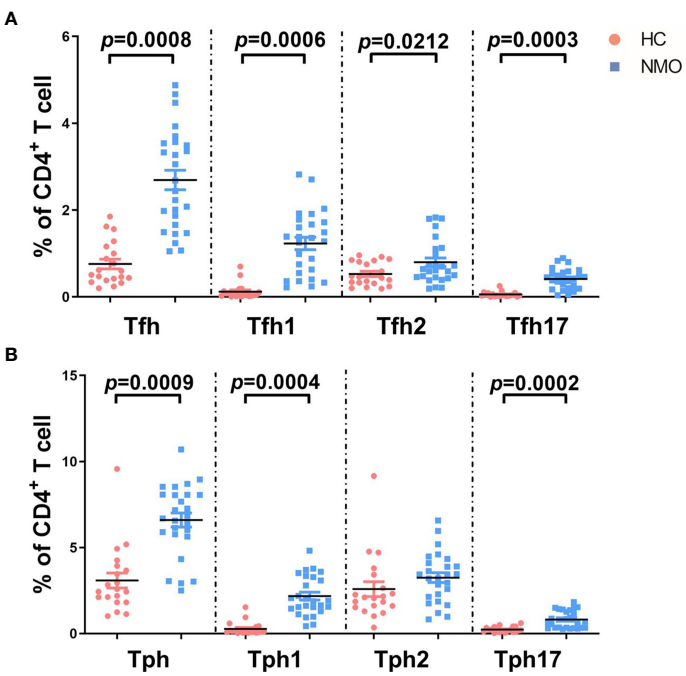


FIGURE 3 | The comparison of T cell subsets distribution in healthy controls and patients with NMOSD. **(A)** Proportions of T follicular helper (Tfh: $CD4^+CXCR5^+PD\text{-}1^{high}$) and subsets among total $CD4^+$ cells in patients with NMOSD and healthy controls (HC); **(B)** Proportions of peripheral T helper (Tph: $CD4^+CXCR5^-PD\text{-}1^{high}$) and subsets. Tfh1 ($CXCR3^+CCR6^-$), Tfh2 ($CXCR3^-CCR6^-$), and Tfh17 ($CXCR3^-CCR6^+$), Tph1 ($CXCR3^+CCR6^-$), Tph2 ($CXCR3^-CCR6^-$), and Tph17 ($CXCR3^-CCR6^+$). Unpaired, two-tailed Student's t tests were used. HC: n=20; NMOSD: n=26.

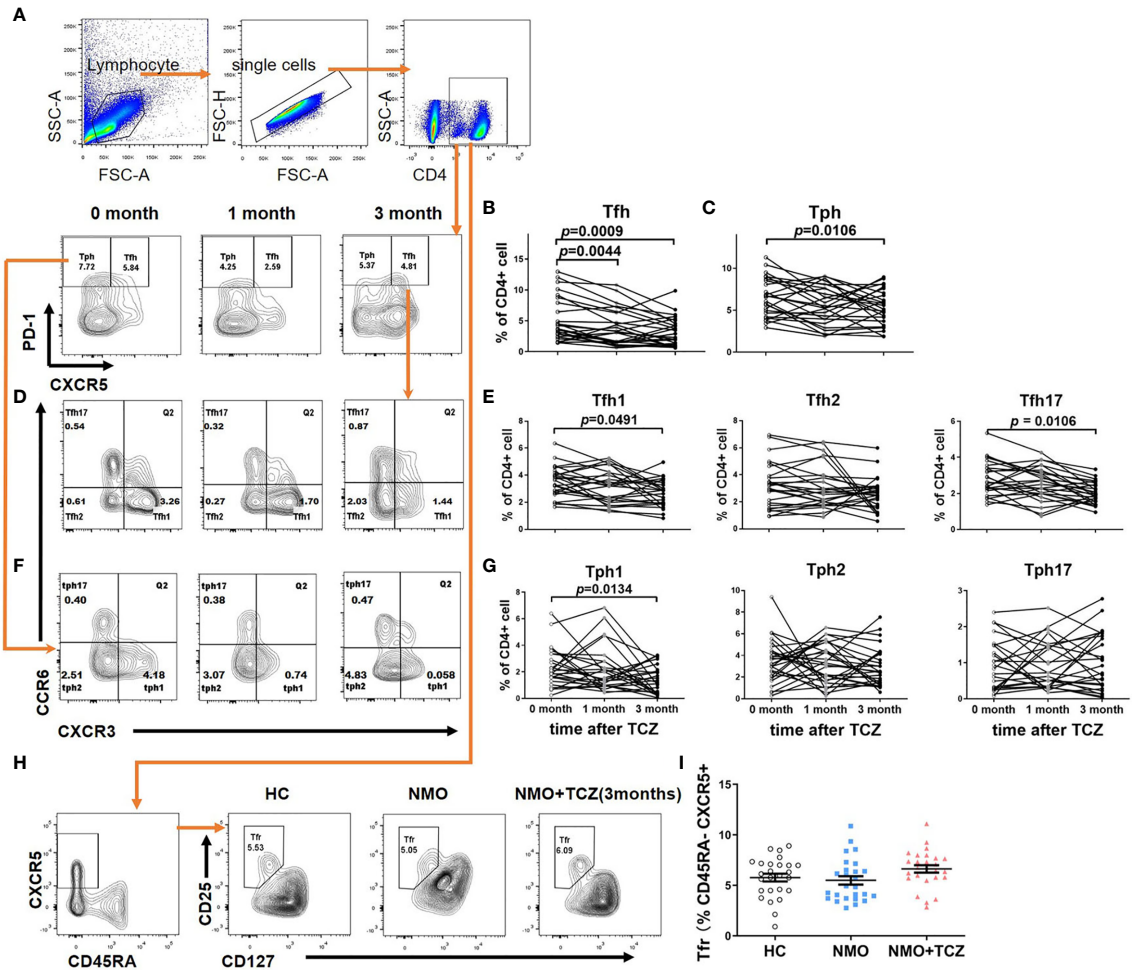


FIGURE 4 | T cells subsets distribution in NMOSD patients before and after tocilizumab. **(A, D, F)** showed gating strategy for subsets of T Follicular Helper Cell (Tfh) & peripheral T helper cells (Tph) and representative flow cytometry plot at baseline and after treatment for 1month or 3 months. **(B, C, E, G)** Quantitative analysis of proportion of total Tfh and its subsets: Tfh1 ($CD4^+CXCR5^+PD\text{-}1^{high}CXCR3^+CCR6^-$), Tfh2 ($CD4^+CXCR5^+PD\text{-}1^{high}CXCR3^-CCR6^-$) and Tfh17 ($CD4^+CXCR5^+PD\text{-}1^{high}CXCR3^-CCR6^+$), total Tph and its subsets: Tph1 ($CD4^+CXCR5^-PD\text{-}1^{high}CXCR3^+CCR6^-$), Tph2 ($CD4^+CXCR5^-PD\text{-}1^{high}CXCR3^-CCR6^-$), and Tph17 ($CD4^+CXCR5^-PD\text{-}1^{high}CXCR3^-CCR6^+$) at baseline and after treatment for 1month or 3 months (n=19). **(H)** Gating strategy for T follicular Regulatory cells (Tfr) and flow cytometry plot of a control and a representative NMOSD patient (before and after tocilizumab 3 months). **(I)** Quantitative analysis of proportion of Tfr in healthy controls and NMOSD patient before and after tocilizumab 3 months (n=26). Differences were considered statistically significant for $P<0.05$. Paired, two-tailed Student's *t* tests (if the distribution is normal) or Wilcoxon matched pairs tests (if it does not conform to the normal distribution) were used to compare baseline and post-treatment results, n=26.

at 1 month, and a significant decrease in the number of Tfh1 cells (0 month *versus* 3 month: 3.54% ± 0.22% *versus* 2.68% ± 0.20%, $P = 0.0491$) and Tfh17 cells (0 month *versus* 3 months: 2.83% ± 0.19% *versus* 2.03% ± 0.12%, $P = 0.0106$) at 3 months. There was no significant decrease in the proportion of Tph1 at 1 month of tocilizumab treatment (0 month *versus* 1 month: 2.34% ± 0.28% *versus* 2.16% ± 0.33%, $P = 0.6356$) until 3 months (0 month *versus* 3 months: 2.34% ± 0.28% *versus* 1.35% ± 0.21%, $P = 0.0134$). We did not find any significant changes in the frequencies of Tfh2 cells, Tph2 cells or Tph17 cell subsets after tocilizumab treatment (**Figures 4D–G**). The proportion of Tfr cells tended to increase; however, the difference was not statistically significant (0 month *versus* 3 months:5.77% ± 0.39% *versus* 6.63% ± 0.37%, respectively; $P = 0.1143$; **Figures 4H, I**).

To sum up, the frequencies of total Tfh and Tph cells were decreased, whereas that of follicular regulatory T cells tended to increase. The subtypes of Tfh1, Tfh17 and Tph1 cells decreased significantly.

Tocilizumab Inhibited the Activation of B Cells in Patients With NMOSD

Next, we studied the expression of the costimulatory molecules CD86 and CD69 as markers of activated B cells that modulate T-cell signaling. No significant changes in the mean fluorescence intensity (MFI) of CD86 or CD69 were observed in $CD19^+$ B cells after tocilizumab treatment for 1 month (not shown); however, the MFI of CD86 was decreased after 3 months (488.2 ± 30.47 *versus* 392.3 ± 32.62, respectively; $P = 0.0205$)

and that of CD69 decreased at 3 months (366.5 ± 25.87 *versus* 291.6 ± 22.50, respectively; P = 0.0387; **Figures 5A–F**). There were no significant differences in the expression of BAFF-R (2384 ± 124.9 *versus* 2336 ± 96.75, respectively; P = 0.7620) before and after treatment with tocilizumab (**Figures 5G, H**). In contrast, HLA-DR expression decreased significantly after 3 months of tocilizumab treatment (19017 ± 1174 *versus* 14550 ± 1033, respectively; P = 0.0125). In short, three markers on B cells associated with T-cell activation (i.e., CD86 CD69, and HLA-DR) were downregulated after tocilizumab treatment.

Effects of Tocilizumab Treatment on the Expression of PD-1 and Its Ligands in Peripheral B Cell Subsets

First, we compared the expression of PD-1 and its ligands PD-L1 and PD-L2 on B-cell subsets in HCs and patients with NMOSD. SWM B cells and ASCs exhibited significantly higher PD-1 expression in patients with NMOSD than that in HCs (NMOSD *versus* HCs: 197.7 ± 8.93 *versus* 113.2 ± 6.64, respectively; P<0.0001 and 323.5 ± 20.07 *versus* 200.1 ± 14.50 respectively; P < 0.0001). There were no significant differences in the expression of PD-1 on naïve B cells (NMOSD *versus* HCs): (148.5 ± 8.43 *versus* 130.5 ± 8.07, respectively; P=0.1378), DN B cells (147.9 ± 8.52 *versus* 130.3 ± 7.01, respectively; P=0.1201), and USW B cells (266.9 ± 14.47 *versus* 241.9 ± 15.80, respectively; P=0.2318) (**Figures 6A, B**). Intriguingly, we found that all B-cell subsets expressed higher levels of PD-L1 and PD-L2 in patients with NMOSD compared with HCs, to be detailed, the comparison of PD-L1 and PD-L2 levels (HCs *versus* NMOSD) on B cell subsets is that: naïve B (PD-L1: 346.8 ± 15.41 *versus* 401.6 ± 19.99, P=0.0449; PD-L2:126.7 ± 7.68 *versus* 170.0 ± 10.93, respectively; P=0.0038); DN B cells (PD-L1: 397.2 ± 20.57 *versus* 512.3 ± 29.88, P=0.0028; PD-L2:142.4 ± 8.59 *versus* 195.9 ± 15.94, respectively; P=0.0054); USW B cells (PD-L1: 692.6 ± 43.27 *versus* 908.7 ± 55.48, P=0.0054; PD-L2:203.1 ± 14.41 *versus* 267.9 ± 20.82, respectively; P=0.0098); SWM B cells (PD-L1: 432.5 ± 35.63 *versus* 592.9 ± 32.93, P=0.0026; PD-L2:140.0 ± 9.06 *versus* 200.8 ± 14.14, respectively; P=0.0068) and ASCs (PD-L1: 526.6 ± 30.41 *versus* 695.0 ± 45.53, P=0.0037; PD-L2:302.7 ± 18.76 *versus* 441.6 ± 32.68, respectively; P=0.0007) (**Figures 6C–F**).

Next, we compared the expression of PD-1 and its ligands on B-cell subsets in the patients with NMOSD after 3 months of treatment with those in HCs. SWM B cells and ASCs exhibited significantly higher PD-1 expression in patients with NMOSD after treatment than that in HCs (191.0 ± 10.96 *versus* 113.2 ± 6.64, respectively; P<0.0001 and 297.6 ± 20.43 *versus* 200.1 ± 14.50 respectively; P = 0.0008). There were no significant differences in the expression of PD-1 on naïve B cells (HCs *versus* NMOSD+TCZ: 130.5 ± 8.07 *versus* 147.2 ± 6.71, P=0.1431), DN B cells (147.9 ± 8.52 *versus* 130.3 ± 7.01, respectively; P=0.2120), and USW B cells (266.9 ± 14.47 *versus* 241.9 ± 15.80, respectively; P=0.4582). Statistically, tocilizumab did not significantly affect the expression of PD-L1 or PD-L2 on all B-cell subsets compared with the baseline except in naïve B cells (PD-L2:126.7 ± 7.68 *versus* 177.1 ± 10.55, respectively; P=0.0028), although a decreasing tendency was observed in several B-cell subsets. To be detailed, the comparison of PD-L1 and PD-L2 levels (HCs *versus* NMOSD+TCZ) on B cell subsets is that: naïve B (PD-L1: 346.8 ± 15.41 *versus* 372.0 ± 15.39, P=0.2630); DN B cells (PD-L1: 397.2 ± 20.57 *versus* 441.1 ± 24.45, P=0.1936; PD-L2:142.4 ± 8.59 *versus* 158.8 ± 10.47, respectively; P=0.2523); USW B cells (PD-L1: 692.6 ± 43.27 *versus* 794.8 ± 54.56, P=0.1680; PD-L2: 203.1 ± 14.41 *versus*

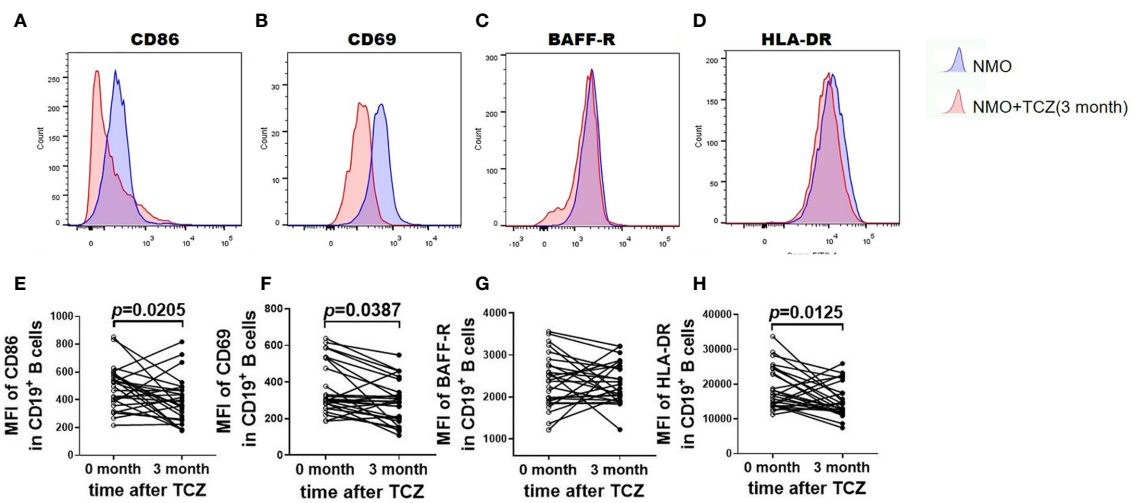


FIGURE 5 | Tocilizumab alters the expression level of activation marker. **(A–D)** was the representative flow cytometric histogram after surface staining activation marker on total B cells ($CD19^+$) from NMOSD patients before and after tocilizumab treatment. **(E–H)** was the quantitative analysis of the mean fluorescence intensity (MFI) of CD86, CD69, BAFF-R, HLA-DR in $CD19^+$cells before and after tocilizumab treatment. Difference was considered statistically significant for P < 0.05. Paired, two-tailed Student's t tests were used, n=26.

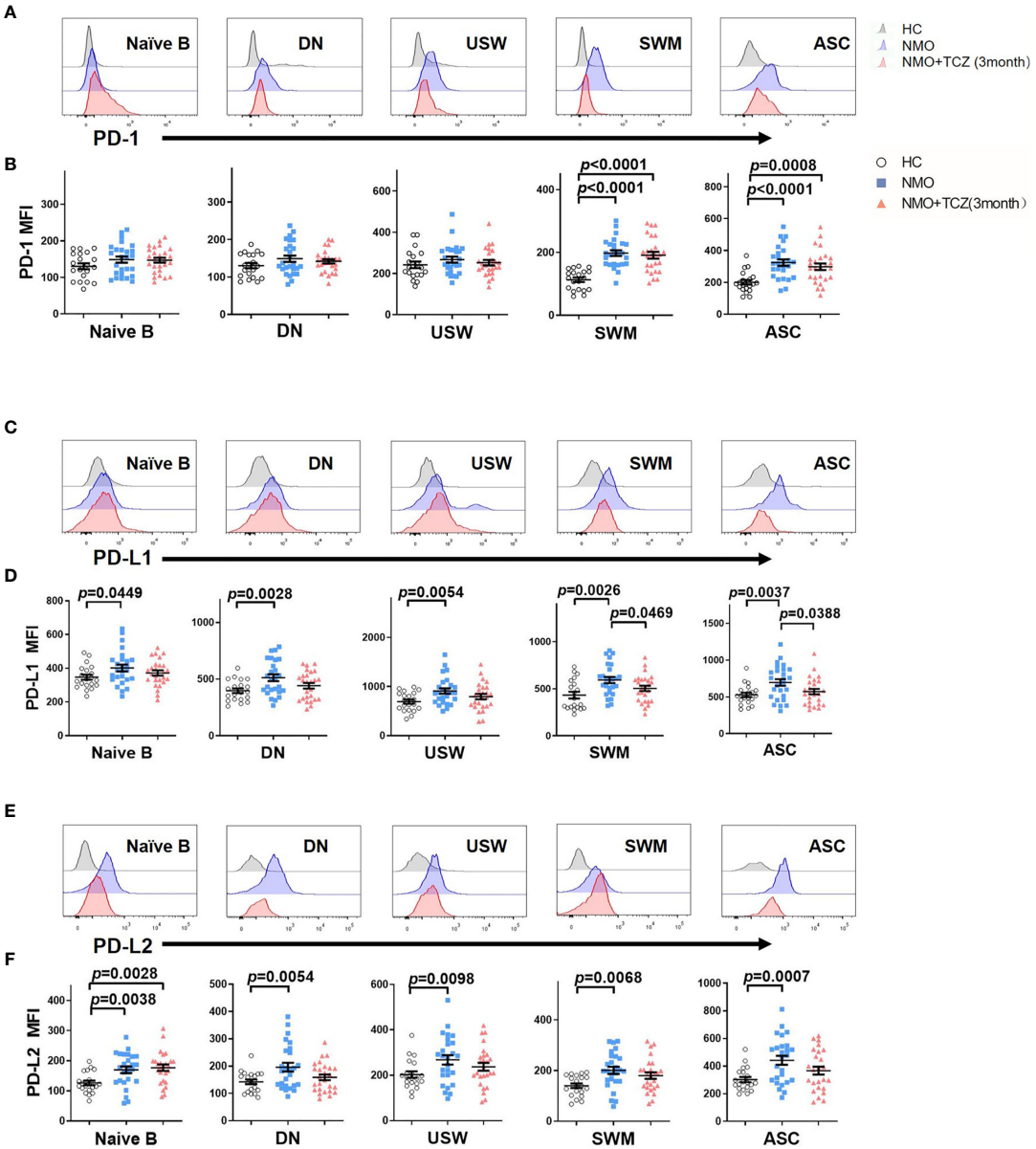


FIGURE 6 | The expression of PD-1 and its corresponding ligands PD-L1 and PD-L2 on B cells subsets from healthy control (HC) and NMOSD patients before and after treatment. **(A, C, E)** Representative histograms of PD-1, PD-L1, PD-L2 in several B cells subsets from a healthy control and NMOSD patients before and after treatment. **(B, D, F)** was the quantitative analysis of mean fluorescence intensity (MFI) of PD-1, PD-L1&PD-L2 in several B cells subsets. Unpaired, two-tailed Student's *t* tests (if the distribution is normal) or Mann-Whitney *U* tests (if it does not conform to the normal distribution) were used, n=26.

237.4 ± 17.43, respectively; *P*=0.1150); SWM B cells (PD-L1: 432.5 ± 35.63 *versus* 503.7 ± 28.82, *P*=0.1026; PD-L2:140.0 ± 9.06 *versus* 179.9 ± 12.96, respectively; *P*=0.0634) and ASCs (PD-L1: 526.6 ± 30.41 *versus* 571.4 ± 36.32, *P*=0.3676; PD-L2: 302.7 ± 18.76 *versus* 367.1 ± 28.63, respectively; *P*=0.0667).

Finally, we compared the expression of PD-1 and its ligands on B-cell subsets in the patients with NMOSD before and after 3 months of treatment. Statistical analysis shows that the expression of PD-L1 on SWM cells and ASCs after treatment was significantly lower than those before treatment (592.9 ± 32.93 *versus* 503.7 ± 28.82, *P*=0.0469 and 695.0 ± 45.53 *versus* 571.4 ± 36.32, *P*=0.0388 respectively) (**Figure 6B**). Additionally, there was no significant differences in the expression of PD-1 and its ligands on B-cell subsets (**Figures 6D, F**).

As a whole, tocilizumab-treated patients showed little changes in PD-1 expression among all B-cell subsets compared with the baseline. By contrast, treatment with tocilizumab does have a tendency to reduce PD-L1 and PD-L2 expression on B-cell

subsets. For instance, the expression of PD-L1 on SWM cells and ASCs after treatment was significantly lower than those before treatment (**Figures 6D, F**).

DISCUSSION

The differential effects of disease-modifying drugs (DMDs) on peripheral blood B-cell subsets and differentiation-related Th cells have been investigated in multiple sclerosis (16–18). However, few studies have reported the effects of DMDs in NMOSD.

In this study, we found that *in vivo* blockade of the IL-6 receptor decreased lymphocyte activation, altered B- and T-cell homoeostasis in patients with NMOSD, and promoted the normalization of abnormal B- and T-cell subsets observed at baseline. Specifically, tocilizumab treatment led to an increase in the number of naïve B cells and decreases in the number of memory B cells and ASC B cells. Similarly, the frequencies and absolute numbers of abnormally reduced Tfh cells increased.

Decreases in the number of memory B cells were consistent with those reported with tocilizumab in patients with rheumatoid arthritis in a previous study (19). Unlike in rheumatoid arthritis, however, we also observed a significant decrease in the number of ASCs, probably because of the more pronounced abnormalities of these cells in patients with NMOSD. Naïve B cells are also dysregulated in patients with NMOSD, as reflected by the lower frequency of naïve B cells. Remarkably, the frequency of naïve B cells was normalized in response to tocilizumab, and the changes in naïve B cells were attributed to the recovery of the reduced number of memory B cells, ASCs, and DN B cells and, to a lesser degree, to a decline in the number of USW B cells. Second, it is known that IL-6 plays an important role in the terminal differentiation of B cells. Thus, IL-6 receptor blockade may lead to a failure of differentiation from naïve B cells to memory B cells.

Double-negative B cells are thought to be mature antigen-experienced B cells with developmental marker expression profiles similar to those of post-switch memory B cells (20). The proportion of DN B cells is increased in patients with multiple sclerosis, resulting in increased production of pro-inflammatory and cytotoxic cytokines following ex-vivo stimulation (21, 22). Consistent with a previous study (23), we also observed expanded DN B cells in patients with NMOSD, suggesting the possible involvement of DN B cells in the pathogenesis of NMOSD (19, 24). In this study, tocilizumab treatment effectively reduced the numbers of DN B cells and may facilitate the inhibition of inflammation in NMOSD. DN B cells were further divided into two subgroups in a previous study on systemic lupus erythematosus (SLE), DN1 cells ($CXCR5^+$ $CD19^+$ IgD^-CD27^-) and DN2 cells ($CXCR5^-$ $CD19^+IgD^-$ $CD27^-$). DN2 cells express T-bet and CD11c, and their activation is mediated by hyper-responsiveness to TLR7 and leads to the generation of autoreactive ASCs (25). The deeper characterization of these cells, *via* immunophenotyping, has the potential to identify novel SLE phenotypes that associate with disease-activity (26). Moreover, the frequency of DN B cells in rheumatic arthritis is inversely correlated to a subsequent good response (27). Additional larger scale studies are needed to determine whether DN B cells may serve as predictors of treatment response to tocilizumab.

In this research, blockade of the IL-6 receptor restores abnormally elevated SWM B cells and ASCs seen at baseline. The decrease of SWM B cells and ASCs may explain the reduction of the pathogenic AQP4-IgG titers found in our previous clinical trial (15), which showed that among AQP4-IgG positive patients, tocilizumab decreased sera AQP4-IgG titers significantly by 50% at the end of the study compared with those at baseline.

In NMOSD, the balance in Tfh cells and related subsets is impaired, making these cells a potential therapeutic target. Consistent with previous studies, we found an upregulation of Tfh cells, particularly Tfh1 cells, in NMOSD patients compared with HCs (6, 28, 29), while 3 months of treatment with tocilizumab reduced the frequency of Tfh cells, and we found that Tfh cell differentiation was associated with IL-6 and plasmablast formation. In line with our findings, specific targeting of IL-6 using tocilizumab therapy in patients with rheumatoid arthritis can significantly reduce circulating Tfh cell numbers, which are correlated with reduced plasmablast formation (30). In this study, we observed similar results in tocilizumab-treated patients with NMOSD, indicating the therapeutic efficacy of tocilizumab in these patients.

Tph cells may play a role in promoting B-cell responses and antibody production within pathologically inflamed non-lymphoid tissues. The possible mechanism lies in that CXCL13 and IL-21 produced by Tph cells may recruit both Tfh and B cells and thus promote local autoantibody production and perhaps modulate other B cell functions such as cytokine production (31). We found that Tph cells were increased in patients with NMOSD. Tocilizumab treatment resulted in reduction of the number of peripheral blood Tph cells. Further research is needed to investigate if this can offer a potential strategy for therapeutic targeting of tissue T cell-B cell interactions.

The role of IL-6 in autoimmune diseases helps to understand the mechanism of the effect of tocilizumab on B- and T-cell subsets. For B cells, IL-6 can promote the terminal differentiation of B cells (32) and contribute to survival of plasma cells (33). IL-6 receptor blockade by tocilizumab is expected to inhibit the differentiation of B cells and survival of plasma cells, as both of them express the IL-6 receptor. For T cells, IL-6 is known to promote early Tfh cell differentiation by transiently inducing B cell lymphoma 6 (BCL-6) expression in $CD4^+$ T cells (34). Further, Tfh cells can promote germinal center formation through the production of IL-21, which sustains BCL-6 expression on B cells and promotes B cell activation, class-switch recombination, and plasma cell differentiation (35). Tph cell is a recently discovered Th cell subset, which can also promote B cell differentiation by secreting IL-21 (36). In general, pro-inflammatory IL-6 promotes the formation of germinal center B cells and Tfh/Tph cells in cooperation with IL-21.

The PD-1 pathway is one of the most important immune checkpoints and is indispensable for maintaining the homeostasis and tolerance of the immune system. The

expression of PD-1 family members on B cells in NMOSD has not yet been investigated in detail. Herein, we found that PD-1, PD-L1, and PD-L2 expression was increased on ASCs and memory B cells in NMOSD patient compared to that in healthy controls, consistent with previous studies in systemic lupus erythematosus (37). Because all B cells are pro-inflammatory in NMOSD, upregulation of PD-1 and its ligands on B cells may on account of cellular activation. Additionally, PD-L1-deficient B cells in an experimental autoimmune encephalomyelitis mouse model exhibit aggravated autoimmunity (38), suggesting that the PD-1 pathway may have roles in the ablation of autoimmunity. In our cohort, tocilizumab treatment selectively downregulated PD-L1 expression on ASCs and memory B cells in patients with NMOSD; this treatment could block or control autoimmune responses and help restore homeostasis. Although B cells expressing PD-1 may diminish after anti-CD19 or anti-CD20 mAb treatment, the effects of tocilizumab on PD-1-related co-inhibitory signaling are persistent. This indicates that the mechanisms of tocilizumab differ from those of rituximab or inebilizumab regarding inhibition of disease activity. Little is known about the expression and kinetics of PD-1, PD-L1, and PD-L2 by B lymphocytes from NMOSD patients. Not only B cells that express PD-L1 and PD-L2 and T cells expressing PD-1 interact *via* this pathway, but also B cell-expressed PD-1, that could mediate B-B or B-T interactions. We postulate that the PD-1 system on B cells acts as a feedback of neuroinflammation in NMOSD. The elevated PD-1 system on B cells may play a protective role in controlling autoimmunity in active NMOSD. When autoimmune inflammation is suppressed by tocilizumab treatment, the PD-1 system tends to restore.

This study has several limitations. First, the follow-up period was relatively short, and the long-term effects on B-cell subsets after tocilizumab treatment were not evaluated. Second, the sample size of patients with tocilizumab treatment was extremely small. Additionally, 3 patients in this study experienced attacks during follow-ups, but the reason is not still clear. High-throughput single-cell sequencing may be warranted to explore heterogeneity of cellular immune function in patients who didn't respond well to tocilizumab. Finally, it is a limitation that the mechanisms of the effects of tocilizumab on Tfh/Tph cell inhibition are not investigated in our study. Further evidence will be warranted on the mechanistic study.

CONCLUSIONS

In summary, we showed that tocilizumab inhibited the activation of mature B cells and Tfh cells but maintained co-inhibitory PD-1 expression on B cells in the peripheral blood of patients with NMOSD. These findings provide important insights into the effects of tocilizumab on the immune mechanism of NMOSD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Review Board of Tianjin Medical University General Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CZ and YL designed the study, collected, analyzed and interpreted the data and drafted and revised the manuscript. YL, PZ, and CD performed the research. YL and HZ did statistical analysis. T-XZ, MY, ZH, DJ, and GY collected and analyzed the data. F-DS revised the manuscript critically for intellectual content. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Wingerchuk D, Lennon V, Lucchinetti C, Pittock S, Weinshenker B. The Spectrum of Neuromyelitis Optica. *Lancet Neurol* (2007) 6:805–15. doi: 10.1016/S1474-4422(07)70216-8
2. Wingerchuk D, Banwell B, Bennett J, Cabre P, Carroll W, Chitnis T, et al. International Consensus Diagnostic Criteria for Neuromyelitis Optica Spectrum Disorders. *Neurology* (2015) 85:177–89. doi: 10.1212/WNL.0000000000001729
3. Bennett J, O'Connor K, Bar-Or A, Zamvil S, Hemmer B, Tedder T, et al. B Lymphocytes in Neuromyelitis Optica. *Neurol Neuroimmunol Neuroinflamm* (2015) 2:e104. doi: 10.1212/NXI.0000000000000104
4. Chihara N, Aranami T, Sato W, Miyazaki Y, Miyake S, Okamoto T, et al. Interleukin 6 Signaling Promotes Anti-Aquaporin 4 Autoantibody Production From Plasmablasts in Neuromyelitis Optica. *Proc Natl Acad Sci USA* (2011) 108:3701–6. doi: 10.1073/pnas.1017385108
5. Wilson R, Makuch M, Kienzler AK, Varley J, Taylor J, Woodhall M, et al. Condition-Dependent Generation of Aquaporin-4 Antibodies From Circulating B Cells in Neuromyelitis Optica. *Brain* (2018) 141:1063–74. doi: 10.1093/brain/awy010
6. Li Y, Zhang F, Qi Y, Chang G, Fu Y, Su L, et al. Association of Circulating Follicular Helper T Cells With Disease Course of NMO Spectrum Disorders. *J Neuroimmunol* (2015) 278:239–46. doi: 10.1016/j.jneuroim.2014.11.011
7. Rao DA, Gurish MF, Marshall JL, Slowikowski K, Fonseka CY, Liu Y, et al. Pathologically Expanded Peripheral T Helper Cell Subset Drives B Cells in Rheumatoid Arthritis. *Nature* (2017) 542:110–4. doi: 10.1038/nature20810
8. Agasing A, Wu Q, Khatri B, Borisow N, Ruprecht K, Brandt A, et al. Transcriptomics and Proteomics Reveal a Cooperation Between Interferon

and T-Helper 17 Cells in Neuromyelitis Optica. *Nat Commun* (2020) 11:2856. doi: 10.1038/s41467-020-16625-7
9. Varrin-Doyer M, Spencer C, Schulze-Topphoff U, Nelson P, Stroud R, Cree B, et al. Aquaporin 4-Specific T Cells in Neuromyelitis Optica Exhibit a Th17 Bias and Recognize Clostridium ABC Transporter. *Ann Neurol* (2012) 72:53–64. doi: 10.1002/ana.23651
10. Shi J, Hou S, Fang Q, Liu X, Liu X, Qi H. PD-1 Controls Follicular T Helper Cell Positioning and Function. *Immunity* (2018) 49:264–74.e264. doi: 10.1016/j.immuni.2018.06.012
11. Salama A, Chitnis T, Imitola J, Ansari M, Akiba H, Tushima F, et al. Critical Role of the Programmed Death-1 (PD-1) Pathway in Regulation of Experimental Autoimmune Encephalomyelitis. *J Exp Med* (2003) 198:71–8. doi: 10.1084/jem.20022119
12. Khan A, Hams E, Floudas A, Sparwasser T, Weaver C, Fallon P. PD-L1hi B Cells Are Critical Regulators of Humoral Immunity. *Nat Commun* (2015) 6:5997. doi: 10.1038/ncomms6997
13. Murakami M, Kamimura D, Hirano T. Pleiotropy and Specificity: Insights From the Interleukin 6 Family of Cytokines. *Immunity* (2019) 50:812–31. doi: 10.1016/j.immuni.2019.03.027
14. Kang S, Tanaka T, Narazaki M, Kishimoto T. Targeting Interleukin-6 Signaling in Clinic. *Immunity* (2019) 50:1007–23. doi: 10.1016/j.immuni.2019.03.026
15. Zhang C, Zhang M, Qiu W, Ma H, Zhang X, Zhu Z, et al. Safety and Efficacy of Tocilizumab *Versus* Azathioprine in Highly Relapsing Neuromyelitis Optica Spectrum Disorder (TANGO): An Open-Label, Multicentre, Randomised, Phase 2 Trial. *Lancet Neurol* (2020) 19:391–401. doi: 10.1016/S1474-4422(20)30070-3
16. Haas J, Bekeredjian-Ding I, Milkova M, Balint B, Schwarz A, Korporal M, et al. B Cells Undergo Unique Compartmentalized Redistribution in Multiple Sclerosis. *J Autoimmun* (2011) 37:289–99. doi: 10.1016/j.jaut.2011.08.003
17. Kemmerer C, Pernpeintner V, Ruschil C, Abdelhak A, Scholl M, Ziemann U, et al. Differential Effects of Disease Modifying Drugs on Peripheral Blood B Cell Subsets: A Cross Sectional Study in Multiple Sclerosis Patients Treated With Interferon-β, Glatiramer Acetate, Dimethyl Fumarate, Fingolimod or Natalizumab. *PloS One* (2020) 15:e0235449. doi: 10.1371/journal.pone.0235449
18. Kowarik M, Astling D, Lepennetier G, Ritchie A, Hemmer B, Owens G, et al. Differential Effects of Fingolimod and Natalizumab on B Cell Repertoires in Multiple Sclerosis Patients. *Neurotherapeutics* (2021) 18:364–77. doi: 10.1007/s13311-020-00975-7
19. Moura RA, Quaresma C, Vieira AR, Goncalves MJ, Polido-Pereira J, Romao VC, et al. B-Cell Phenotype and IgD-CD27- Memory B Cells are Affected by TNF-Inhibitors and Tocilizumab Treatment in Rheumatoid Arthritis. *PloS One* (2017) 12:e0182927. doi: 10.1371/journal.pone.0182927
20. Jacobi A, Reiter K, Mackay M, Aranow C, Hiepe F, Radbruch A, et al. Activated Memory B Cell Subsets Correlate With Disease Activity in Systemic Lupus Erythematosus: Delineation by Expression of CD27, IgD, and CD95. *Arthritis Rheum* (2008) 58:1762–73. doi: 10.1002/art.23498
21. Claes N, Fraussen J, Vanheusden M, Hellings N, Stinissen P, Van Wijmeersch B, et al. Age-Associated B Cells With Proinflammatory Characteristics Are Expanded in a Proportion of Multiple Sclerosis Patients. *J Immunol (Baltimore Md 1950)* (2016) 197:4576–83. doi: 10.4049/jimmunol.1502448
22. Fraussen J, Marquez S, Takata K, Beckers L, Montes Diaz G, Zografou C, et al. Phenotypic and Ig Repertoire Analyses Indicate a Common Origin of IgDCD27 Double Negative B Cells in Healthy Individuals and Multiple Sclerosis Patients. *J Immunol (Baltimore Md 1950)* (2019) 203:1650–64. doi: 10.4049/jimmunol.1801236
23. Kowarik M, Astling D, Gasperi C, Wemlinger S, Schumann H, Dzieciatkowska M, et al. CNS Aquaporin-4-Specific B Cells Connect With Multiple B-cell Compartments in Neuromyelitis Optica Spectrum Disorder. *Ann Clin Trans Neurol* (2017) 4:369–80. doi: 10.1002/acn3.418
24. Nakayamada S, Kubo S, Yoshikawa M, Miyazaki Y, Yunoue N, Iwata S, et al. Differential Effects of Biological DMARDs on Peripheral Immune Cell Phenotypes in Patients With Rheumatoid Arthritis. *Rheumatology (Oxf Engl)* (2018) 57:164–74. doi: 10.1093/rheumatology/kex012
25. Jenks SA, Cashman KS, Zumaquero E, Marigorta UM, Patel AV, Wang X, et al. Distinct Effector B Cells Induced by Unregulated Toll-Like Receptor 7 Contribute to Pathogenic Responses in Systemic Lupus Erythematosus. *Immunity* (2018) 49:725–39.e726. doi: 10.1016/j.immuni.2018.08.015
26. Sachinidis A, Xanthopoulos K, Garyfallos A. Age-Associated B Cells (ABCs) in the Prognosis, Diagnosis and Therapy of Systemic Lupus Erythematosus (SLE). *Mediterr J Rheumatol* (2020) 31:311–8. doi: 10.31138/mjr.31.3.311
27. Mahmood Z, Muhammad K, Schmalzing M, Roll P, Dörner T, Tony H. CD27-IgD- Memory B Cells are Modulated by *In Vivo* Interleukin-6 Receptor (IL-6R) Blockade in Rheumatoid Arthritis. *Arthritis Res Ther* (2015) 17:61. doi: 10.1186/s13075-015-0580-y
28. Nicolas P, Ruiz A, Cobo-Calvo A, Fiard G, Giraudon P, Vukusic S, et al. The Balance in T Follicular Helper Cell Subsets Is Altered in Neuromyelitis Optica Spectrum Disorder Patients and Restored by Rituximab. *Front Immunol* (2019) 10:2686. doi: 10.3389/fimmu.2019.02686
29. Fan X, Jiang Y, Han J, Liu J, Wei Y, Jiang X, et al. Circulating Memory T Follicular Helper Cells in Patients With Neuromyelitis Optica/Neuromyelitis Optica Spectrum Disorders. *Mediators Inflamm* (2016) 2016:3678152. doi: 10.1155/2016/3678152
30. Chavele K, Merry E, Ehrenstein M. Cutting Edge: Circulating Plasmablasts Induce the Differentiation of Human T Follicular Helper Cells Via IL-6 Production. *J Immunol (Baltimore Md 1950)* (2015) 194:2482–5. doi: 10.4049/jimmunol.1401190
31. Shen P, Fillatreau S. Antibody-Independent Functions of B Cells: A Focus on Cytokines. *Nat Rev Immunol* (2015) 15:441–51. doi: 10.1038/nri3857
32. Roldan E, Brieva JA. Terminal Differentiation of Human Bone Marrow Cells Capable of Spontaneous and High-Rate Immunoglobulin Secretion: Role of Bone Marrow Stromal Cells and Interleukin 6. *Eur J Immunol* (1991) 21:2671–7. doi: 10.1002/eji.1830211105
33. Cassese G, Arce S, Hauser AE, Lehnert K, Moewes B, Mostarac M, et al. Plasma Cell Survival is Mediated by Synergistic Effects of Cytokines and Adhesion-Dependent Signals. *J Immunol* (2003) 171:1684–90. doi: 10.4049/jimmunol.171.4.1684
34. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 Mediates the Development of T Follicular Helper Cells. *Science* (2009) 325:1001–5. doi: 10.1126/science.1176676
35. Rawlings DJ, Metzler G, Wray-Dutra M, Jackson SW. Altered B Cell Signalling in Autoimmunity. *Nat Rev Immunol* (2017) 17:421–36. doi: 10.1038/nri.2017.24
36. Makiyama A, Chiba A, Noto D, Murayama G, Yamaji K, Tamura N, et al. Expanded Circulating Peripheral Helper T Cells in Systemic Lupus Erythematosus: Association With Disease Activity and B Cell Differentiation. *Rheumatology (Oxford)* (2019) 58:1861–9. doi: 10.1093/rheumatology/kez077
37. Stefanski A, Wiedemann A, Reiter K, Hiepe F, Lino A, Dörner T. Enhanced Programmed Death 1 and Diminished Programmed Death Ligand 1 Up-Regulation Capacity of Post-Activated Lupus B Cells. *Arthritis Rheumatol (Hoboken NJ)* (2019) 71:1539–44. doi: 10.1002/art.40897
38. Bodhankar S, Galipeau D, Vandenbark A, Offner H. PD-1 Interaction With PD-L1 But Not PD-L2 on B-Cells Mediates Protective Effects of Estrogen Against EAE. *J Clin Cell Immunol* (2013) 4:143. doi: 10.4172/2155-9899.1000143

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Effects of Vitamin D and Dexamethasone on Lymphocyte Proportions and Their Associations With Serum Concentrations of 25-Hydroxyvitamin D_3 *In Vitro* in Patients With Multiple Sclerosis or Neuromyelitis Optica Spectrum Disorder

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Background: Clear associations have been found between vitamin D deficiency and several autoimmune diseases including multiple sclerosis (MS). However, the benefits of vitamin D supplementation on disease management remain a matter of debate.

Objective and Methods: Patients with MS (*N*=12) and neuromyelitis optica spectrum disorder (NMOSD; *N*=12) were enrolled along with 15 healthy controls. Changes in lymphocyte subset proportions during stimulation of their peripheral blood mononuclear cells (PBMCs) with the active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and correlations with serum concentrations of the vitamin D precursor 25-hydroxyvitamin D_3 (serum $25(OH)D_3$) were explored. The impact of $1,25(OH)_2D_3$ stimulation on the expression of vitamin-D-responsive genes in immune cells was also investigated.

Results: In both MS and NMOSD, stimulation of PBMCs with $1,25(OH)_2D_3$ followed by steroid suppressed the proliferation of total lymphocytes and T cells. The ratio of $CD19^+CD27^+$ memory B cells (Bmem) to all B cells after stimulation with $1,25(OH)_2D_3$ was negatively correlated with serum $25(OH)D_3$ in MS (Spearman's ρ=–0.594, p=0.042), but positively correlated in NMOSD (Pearson's r = 0.739, p=0.006). However, there was no relationship between the ratio of Bmem to $CD19^+CD24^+CD38^+$ regulatory B cells and

serum 25(OH)D_3 in either MS or NMOSD. In addition, the level of 1,25(OH)$_2D_3$-induced *CYP24A1* mRNA expression in PBMCs was significantly and negatively correlated with serum 25(OH)D_3 (for ΔC_T, r=0.744, p=0.014) in MS.

Conclusion: These findings suggest a beneficial impact of stimulation of PBMCs with vitamin D followed by steroid on the T-cell population. The association between patient serum 25(OH)D_3 and the proportion of Bmem under immune-cell stimulation differed between MS and NMOSD. Further investigations are warranted with larger patient populations.

Keywords: vitamin D, lymphocytes, memory B cells, *CYP24A1*, multiple sclerosis, neuromyelitis optica spectrum disorder

INTRODUCTION

1,25-Dihydroxyvitamin D_3 (1,25(OH)$_2D_3$), the active form of vitamin D, is receiving increasing attention due to its role as a regulator of the immune system (1). Immune cells, such as macrophages, dendritic cells, and activated lymphocytes, express both the vitamin D receptor (VDR) and 1α-hydroxylase (CYP27B1), the key enzyme that catalyzes the bioactivation of 1,25(OH)$_2D_3$ from its precursor 25-hydroxyvitamin D_3 (25(OH)D_3) (2–4). Vitamin D suppresses the production of proinflammatory cytokines such as interferon-γ, interleukin (IL)-2, and IL-17, enhances the secretion of anti-inflammatory cytokines such as IL-4 and IL-10, and shifts the balance toward immune responses mediated by T helper (Th) type 2 cells and regulatory T cells (3, 5). In addition, vitamin D interferes with B-cell proliferation and differentiation into memory B cells (Bmem) and antibody-secreting plasma cells (6, 7). From this perspective, the large amount of data linking decreased vitamin D levels to an abnormal immune response, such as increased autoimmunity, is of great concern (8–13). Low vitamin D levels have been correlated with disease activity or disability in various autoimmune disorders, including systemic lupus erythematosus (SLE), Sjögren's syndrome, multiple sclerosis (MS), and neuromyelitis optica spectrum disorder (NMOSD) (8, 9, 11, 12).

MS and NMOSD are autoimmune inflammatory demyelinating diseases that affect the central nervous system (CNS). Autoreactive T cells in the periphery and T cell–B cell collaboration contribute to the pathogenesis of CNS autoimmunity. CNS antigen-specific T cells, specifically $CD4^+$ Th1 cells and Th17 cells, and antibodies against the aquaporin-4 water channel (AQP4) are believed to play key roles in the development of MS and NMOSD, respectively (14, 15). In the periphery, B cells, and especially Bmem, may serve as antigen-presenting cells, and activate and differentiate the autoreactive T cells into the Th17 lineage by producing cytokines such as IL-6 and IL-21 (14, 16). In addition, Th17 and T follicular helper cells produce the cytokines IL-17a and IL-6, which promote granulocyte activation, and B-cell differentiation and antibody production (17). Vitamin D may exert an immunomodulating effect by suppressing the inflammatory autoimmune response. However, the benefit of vitamin D in terms of therapeutic applications is not clear for either MS or NMOSD (11, 18, 19).

This study investigated the effects of vitamin D on immune cells through peripheral blood mononuclear cell (PBMC) stimulation in patients with MS or NMOSD, and in healthy controls (HCs). The associations between serum 25(OH)D_3 concentration (hereafter serum 25(OH)D_3) and the proportions of lymphocyte subsets following stimulation with 1,25(OH)$_2D_3$ were also evaluated, and 1,25(OH)$_2D_3$-induced changes in mRNA expression of the genes encoding VDR (*VDR*), the vitamin-D-activating and vitamin-D-degrading enzymes 1α-hydroxylase (*CYP27B1*) and 24-hydroxylase (*CYP24A1*), respectively, and IL-10 (*IL-10*) were explored.

METHODS

Subjects

Patients with MS (20) (N=12) and AQP4-antibody-positive NMOSD (15) (N=12) were enrolled at Samsung Medical Center in Seoul, South Korea between November 2016 and August 2018. A total of 20 ml of peripheral venous blood samples were taken during remission in all patients. The same amount of venous blood was also obtained from 15 HCs who did not have a history of acute or chronic disease and had not been taking any medication during the previous 3 months. Total serum 25(OH)D_3 was measured using commercially available enzyme-linked immunosorbent assay kits (Eagle Biosciences, NH, USA) according to the manufacturer's instructions. The study was approved by the Institutional Review Board of Samsung Medical Center and written informed consents were obtained from all subjects.

PBMC Preparation and Culture

Whole blood was collected into lithium heparin tubes and PBMCs were then separated using density-gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare Biosciences, Pittsburgh, PA, USA). The isolated PBMCs were suspended in fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA) and 10% dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO, USA) at a concentration of 1×10^7 cells/ml, and then stored in liquid nitrogen until required for the stimulation experiments.

The PBMCs were cultured with RPMI (Roswell Park Memorial Institute) 1640 medium containing L-glutamine supplemented with 10% certified inactivated FBS and 50 units

of penicillin/streptomycin (Life Technologies). They were then incubated with a nonspecific stimulation mixture of 10 ng/ml lipopolysaccharide (LPS; Sigma ldrich), 100 ng/ml CD40 ligand (CD40L; Enzo Biochem, New York, NY, USA), and 5 nM cytosine-phosphate-guanosine-oligodeoxynucleotides (CpG-ODN 2006; Invivogen, USA), either alone (LPS+CD40L+CpG-ODN) or in combination with steroid (dexamethasone, 10 nM, Sigma Aldrich), $1,25(OH)_2D_3$ (1 μM, Sigma Aldrich), or both steroid and $1,25(OH)_2D_3$. When used, $1,25(OH)_2D_3$ was added 24 hours prior to LPS+CD40L+CpG-ODN. The cells were incubated for 72 hours, after which they were analyzed with flow cytometry.

Immunophenotyping by Flow Cytometry

The percentages of total lymphocytes and T cells, B cells, regulatory B cells (Breg), and Bmem in the PBMC samples were determined by flow cytometric analysis of the surface markers CD3, CD19, CD24, CD38, and CD27. In this study, $CD19^+CD24^+CD38^+$ cells were defined as Breg and $CD19^+CD27^+$ cells as Bmem (21). PBMCs were incubated with the following surface marker antibodies (all from BD Biosciences, San Jose, CA, USA) for 30 min at 4°C in the dark: anti-CD19-APC-Cy7 (clone SJ25C1), anti-CD3-PerCPCy5.5 (clone SK7), anti-CD24-FITC (Clone ML5), anti-CD38-PE (clone HIT), anti-CD27-PE (clone M-T271). They were then washed twice with phosphate-buffered saline (PBS), suspended in PBS, and analyzed using FACS Canto II flow cytometry with FACS DIVA software (version 6.1.3, BD Biosciences).

Real-Time Polymerase Chain Reaction

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription at 37°C for 30 min, followed by reverse transcriptase (RT) inactivation at 95°C for 5 min using the Fast Advanced RT buffer and Enzyme mix (Thermo Fisher Scientific, Waltham, MA, USA). Each gene-expression assay consisted of a 6-carboxy-fluorescein (FAM)-dye-labeled TaqMan MGB (minor groove binder) probe and two polymerase chain reaction (PCR) primers (TaqMan human FAM assays). The target genes were *VDR* (Hs00172113_m1), *CYP24A1* (Hs00167999_m1), *CYP27B1* (Hs00168017_m1), *IL-10* (Hs00961622_m1), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs02786624_g1). All of these genes were obtained from Thermo Fisher Scientific. *GAPDH*, which is present in all genomes, is commonly used as an endogenous control (i.e., a housekeeping gene) for analyzing the relative levels of gene expression. Real-time PCR was carried out using the TaqMan Fast advanced Master Mix (Thermo Fisher Scientific). Uracil *N*-glycosylase (UNG) was used to prevent the possible reamplification of carryover PCR products. The thermal cycle conditions were 50°C for 2 min (AMPerase UNG activation), 95°C for 20 sec (Taq activation), and then PCR for 40 cycles of 95°C for 1 sec and 60°C for 20 sec. Relative expression levels of DNA were calculated automatically using QuantStudio 6 Pro Real-Time PCR System software (Thermo Fisher Scientific). Cycle threshold (C_T) values were defined as the thresholds required for reference amplification. The average C_T was calculated for each target gene ($C_{T,\ \text{target gene}}$) and for *GAPDH* ($C_{T,\ GAPDH}$); the difference ($\Delta C_T$) was calculated for each target gene using the equation $\Delta C_T = C_{T,\ \text{target gene}} - C_{T,\ GAPDH}$.

Statistical Analysis

Lymphocyte subset percentages in PBMC samples were compared between the MS, NMOSD, and HC groups using one-way analysis of variance (ANOVA) or Kruskal–Wallis tests. Two-way ANOVA or Friedman's test was used to compare the proportions of specific lymphocytes and ΔC_T values for *VDR*, *CYP24A1*, *CYP27B1*, and *IL-10* mRNA expression among the four stimulation groups (LPS+CD40L+CpG-ODN alone or in combination with 1,25 $(OH)_2D_3$, steroid, or both) for each of the three study groups. Bonferroni's correction for multiple comparisons was performed. Correlations between the study participants' serum $25(OH)D_3$ and the percentage of each lymphocyte subset or ΔC_T values for mRNA expression were evaluated using Pearson's correlation or Spearman's correlation. SPSS (version 20, SPSS, Chicago, IL, USA) and Prism (version 8.4.3, GraphPad, La Jolla, CA, USA) were used for statistical analysis and data presentation. The criterion for statistical significance was $p<0.05$.

RESULTS

The characteristics of the study participants are summarized by group in **Table 1**. Patients with NMOSD were significantly older than HCs (mean age, 44 *vs.* 32 years; $p=0.013$). Vitamin D supplementation was reported by 25% (3/12) of the MS patients and 33% (4/12) of the NMOSD patients, but none of the HCs. Serum $25(OH)D_3$ was higher in the NMOSD group than in their HC counterparts ($p=0.030$).

In PBMC samples, the proportion of total lymphocytes was significantly lower in both the MS and NMOSD groups than in the HC group ($p=0.020$ and 0.005, respectively). The frequency of Breg among B cells was lower and the Bmem/Breg ratio was significantly higher in the NMOSD group than in the MS group ($p=0.026$ and 0.028, respectively); there were no significant differences in these parameters between the MS and HC groups or between the NMOSD and HC groups (**Figure 1**). There were no differences in the percentages of T cells, B cells, and Bmem between any of the study groups (HC *vs.* MS *vs.* NMOSD).

Changes in the Proportion of Lymphocytes in Response to Stimulation With $1,25(OH)_2D_3$

Nonspecific stimulation with LPS+CD40L+CpG-ODN resulted in a significant increase in total lymphocyte percentage ($p=0.014$) and significant reductions in the Bmem percentage and Bmem/Breg ratio ($p=0.010$ and 0.028, respectively) in the NMOSD group, while the T-cell percentage decreased in the MS group ($p=0.017$) (**Supplementary Figure 1**).

Stimulation of HC PBMCs with $1,25(OH)_2D_3$ significantly reduced the percentage of total lymphocytes ($p=0.027$),

TABLE 1 | Demographics and serum concentrations of $25(OH)D_3$ in study subjects.

	HCs (*N* = 15)	MS (*N* = 12)	NMOSD (*N* = 12)	*p* value
Age	32.3 ± 5.2	36.8 ± 9.8	44.1 ± 14.2	0.016[a]
Female, *N* (%)	8 (53.3)	9 (75.0)	11 (91.7)	0.085
Disease duration, months	N/A	27.5 (4.55–1.75)	53.5 (21.75–115.75)	0.236
$25(OH)D_3$, mg/dl	18.1 ± 5.8	20.9 ± 11.7	31.4 ± 18.5	0.028[a]
On taking $1,25(OH)_2D_3$, *N* (%)	0	3 (25.0)	4 (33.3)	0.060
ARR	N/A	1.0 (0.638–4.0)	0.7 (0.4–1.1)	0.148
EDSS	N/A	1.0 (0.625–2.875)	3.0 (1.25–3.0)	0.078
Use of drugs, *N* (%)	N/A	9 (75.0)[b]	11(91.7)[c]	0.590

N, number; ARR, annualized relapse rate; EDSS, Expanded Disability Status Scale; HCs, healthy controls; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; Values are presented as either mean ± SD or median (IQR) unless otherwise indicated.

[a]*significant only between HCs and NMOSD.*

[b]*interferon β-1b (n=4), interferon β-1a (n=4), and teriflunomide (n=1).*

[c]*azathioprine (n=5), mycophenolate mofetil (n=3), hydroxychloroquine (n=2), and methotrexate (n=1).*

regardless of subsequent stimulation with steroid (**Figure 2A**). $1,25(OH)_2D_3$ also caused a reduction in the percentage of total lymphocytes in PBMCs from the MS and NMOSD groups, but only when steroid was added after $1,25(OH)_2D_3$ ($p<0.05$ *vs.* steroid or $1,25(OH)_2D_3$ alone for both patient groups).

The proportion of T cells among total lymphocytes was also significantly reduced by stimulation of HC PBMCs with $1,25(OH)_2D_3$ ($p=0.012$), but that proportion increased upon subsequent stimulation with steroid. In the MS and NMOSD groups, the percentage of T cells increased after PBMC stimulation with steroid ($p=0.012$ and 0.158, respectively), but not if they were first stimulated with $1,25(OH)_2D_3$ (**Figure 2B**). $1,25(OH)_2D_3$ stimulation did not alter the proportions of B cells or Breg, or the Bmem/Breg ratio in any of the three study groups (**Figures 2C, D, F**). However, stimulation with $1,25(OH)_2D_3$ followed by steroid significantly increased the proportion of Bmem in PBMC samples compared with $1,25(OH)_2D_3$ stimulation alone in the NMOSD group (**Figure 2E**).

Associations Between Serum $25(OH)D_3$ and Percentage of Bmem

Prior to PBMC stimulation, there were no correlations between the Bmem/B cell ratio and serum $25(OH)D_3$ in any of the study groups. However, after nonspecific PBMC stimulation with LPS+CD40L+CpG-ODN, there was a negative correlation between the Bmem/B cell ratio and serum $25(OH)D_3$ in the HC group (Spearman's $\rho=0.529$, $p=0.043$), and a positive correlation (Pearson's $r = 0.731$, $p=0.007$) in the NMOSD group (**Figures 3A, C**). Additional stimulation with $1,25(OH)_2D_3$ did not alter these correlations for either group. In the MS group,

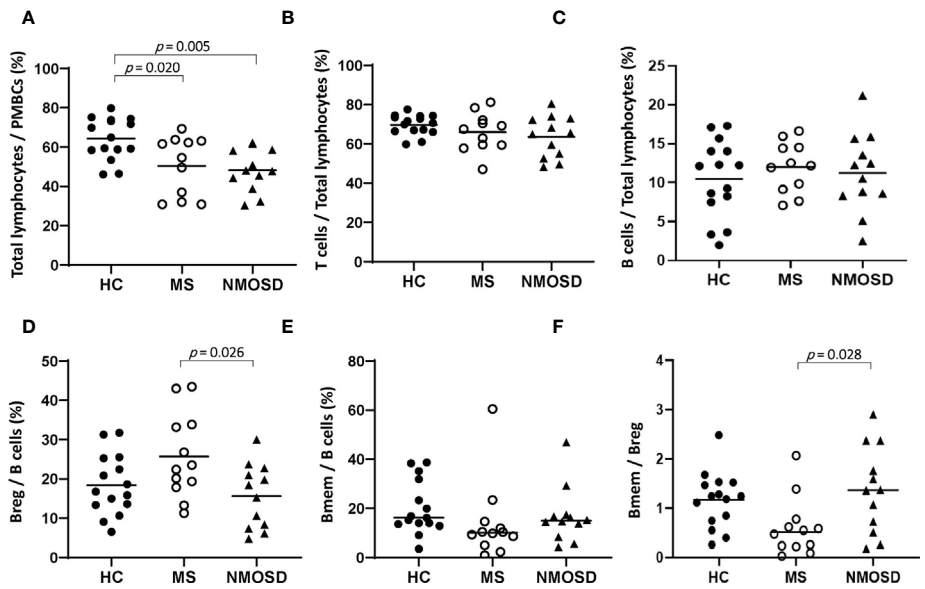


FIGURE 1 | The proportions of lymphocytes in PBMC samples from HCs and patients with MS or NMOSD. The percentage of total lymphocytes was lower in MS and NMOSD than in HCs. The Breg/B cell ratio was higher and the Bmem/Breg ratio was lower in patients with MS than in those with NMOSD. The data are presented as scatter plots in which the horizontal line indicates the mean **(A–D)** or median **(E, F)**. Only statistically significant *p* values ($p<0.05$) are presented. Bmem, $CD19^+CD27^+$ memory B cell; Breg, $CD19^+CD24^+CD38^+$ regulatory B cell; HC, healthy control; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; PBMC, peripheral blood mononuclear cell.

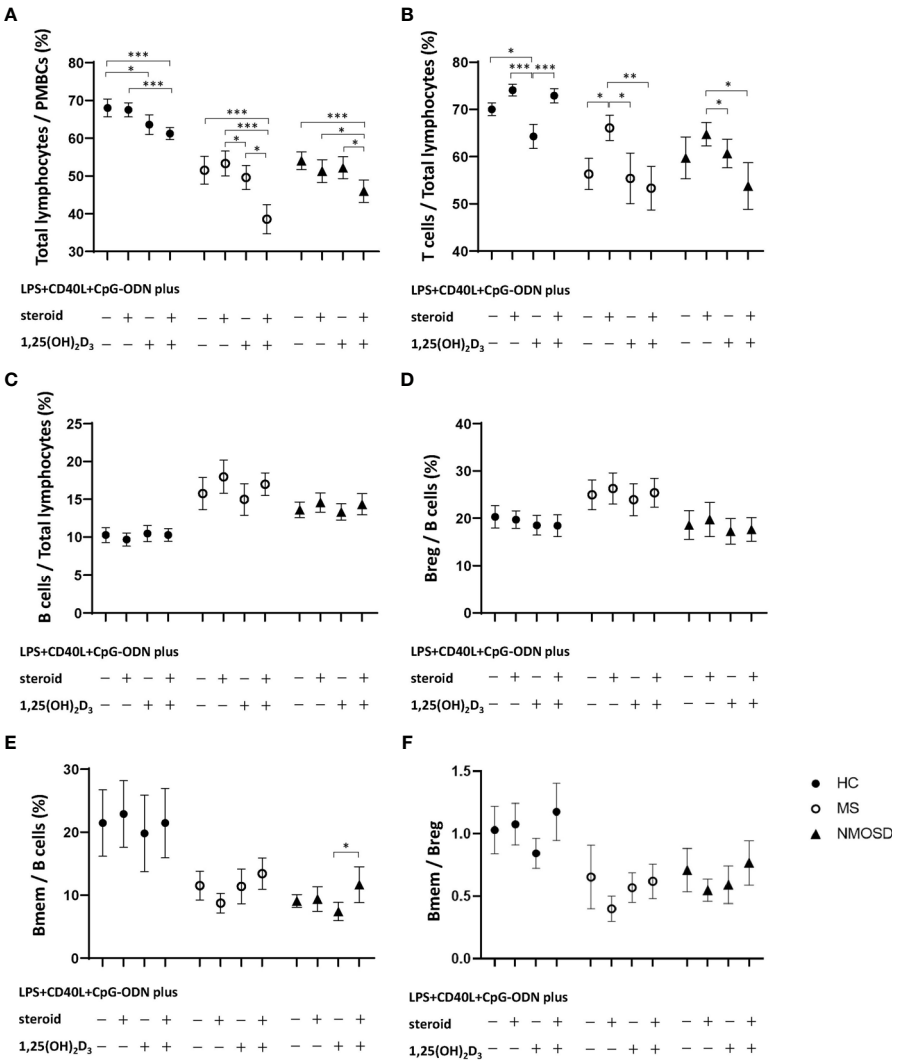


FIGURE 2 | Changes in the proportion of lymphocytes in PBMC samples in response to nonspecific stimulation with LPS+CD40L+CpG-ODN with or without 1,25 $(OH)_2D_3$, and/or steroid (dexamethasone) in HCs and in patients with MS or NMOSD. Stimulation with $1,25(OH)_2D_3$ significantly reduced the percentages of **(A)** total lymphocytes and **(B)** T cells in HCs. However, stimulation with $1,25(OH)_2D_3$ followed by steroid caused significant decreases in the percentages of **(A)** total lymphocytes and **(B)** T cells in the MS and NMOSD groups compared with those achieved by stimulation with steroid treatment alone. $1,25(OH)_2D_3$ stimulation did not alter the proportions of **(C)** B cells or **(D)** Breg, or **(F)** the Bmem/Breg ratio in any of the three study groups. Stimulation with $1,25(OH)_2D_3$ followed by steroid significantly increased the proportion of **(E)** Bmem in PBMC samples compared with $1,25(OH)_2D_3$ stimulation alone in the NMOSD group. Data are mean and standard-error values. Statistically significant differences are indicated by horizontal bars: $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; Bmem, $CD19^+CD27^+$ memory B cell; Breg, $CD19^+CD24^+CD38^+$ regulatory B cell; CD40L, CD40 ligand; CpG-ODN, cytosine phosphate guanosine oligodeoxynucleotides; HC, healthy control; LPS, lipopolysaccharide; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; PBMC, peripheral blood mononuclear cell.

an association between Bmem/B cell ratio and serum $25(OH)D_3$ was found only following stimulation with $1,25(OH)_2D_3$, which yielded a negative correlation (ρ=–0.594, p=0.042; **Figure 3B**). There was no correlation between the Breg/B cell ratio in PBMC samples and serum $25(OH)D_3$ in any of the groups, regardless of the stimulation conditions. A negative association was found between the Bmem/Breg ratio and serum $25(OH)D_3$ after stimulation of PBMCs with $1,25(OH)_2D_3$ only in the HC group (ρ=–0.583, p=0.023).

Changes in Expression Levels of *VDR, CYP27B1, CYP24A1*, and *IL-10* Genes in Response to $1,25(OH)_2D_3$

$1,25(OH)_2D_3$-induced changes in the PBMC mRNA expression levels of *VDR*, *IL-10*, *CYP27B1*, and *CYP24A1* are shown in **Figure 4**. PBMCs from 15 HCs, 11 MS patients, and 13 NMOSD patients were included (**Supplementary Table 1**). PBMC stimulation with LPS+CD40L+CpG-ODN increased *CYP27B1* mRNA expression, but the addition of $1,25(OH)_2D_3$ suppressed

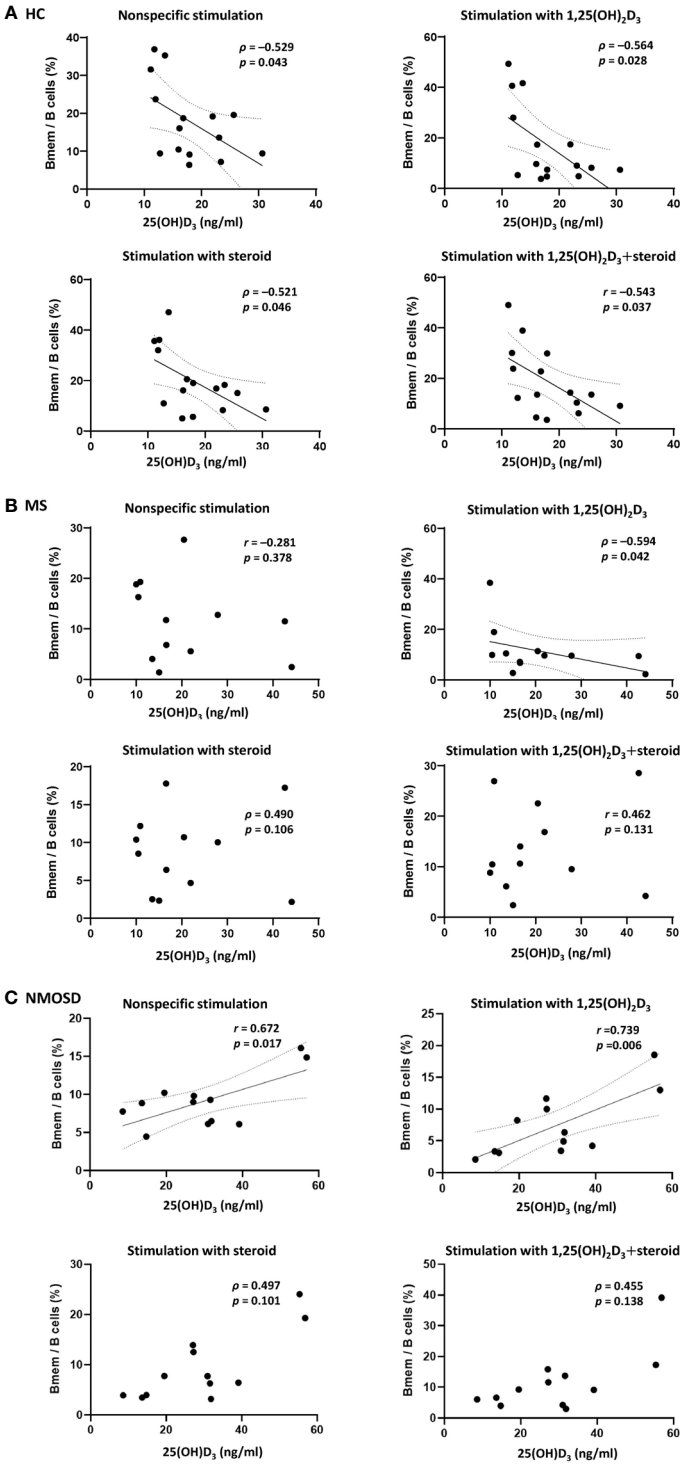


FIGURE 3 | Correlations between the proportion of Bmem in samples of PBMCs and serum 25(OH)D_3 in HCs and in patients with MS or NMOSD. A negative correlation between the percentage of Bmem and serum 25(OH)D_3 was found **(A)** in HCs regardless of the type of stimulation (i.e., steroid or 1,25$(OH)_2D_3$), and **(B)** in patients with MS only after stimulation of PBMCs with 1,25$(OH)_2D_3$. **(C)** In NMOSD, significant positive correlations were found after PBMC stimulation, with or without 1,25$(OH)_2D_3$. Linear regression lines indicate significant correlations. 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; Bmem, $CD19^+CD27^+$ memory B cell; HCs, healthy controls; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; serum 25(OH)D_3, serum concentration of 1,25-dihydroxyvitamin D_3.

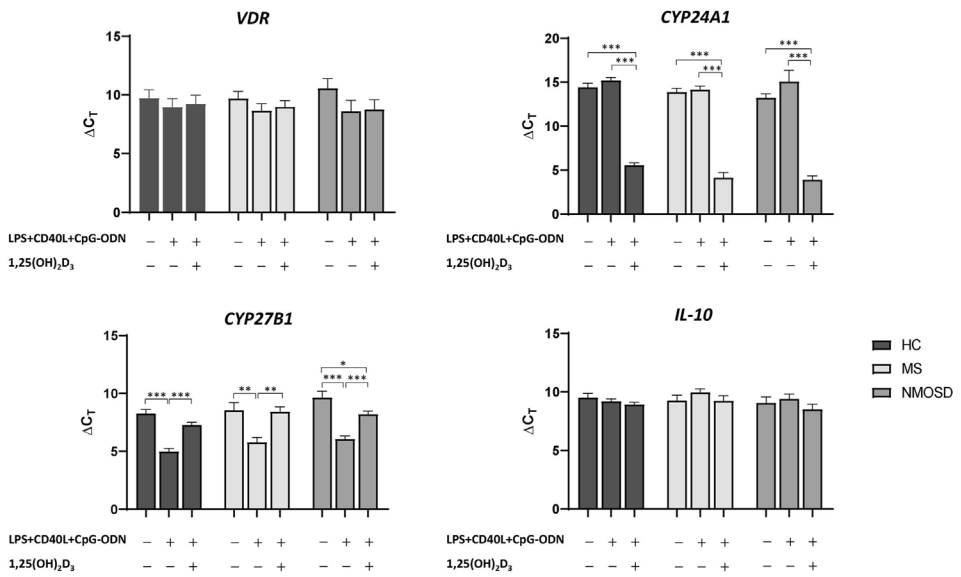


FIGURE 4 | Changes in the mRNA expression of *VDR*, *CYP27B1*, *CYP24A1*, and *IL-10* in PBMCs in response to stimulation with LPS+CD40L+CpG-ODN (nonspecific) with or without $1,25(OH)_2D_3$. In HCs and patients with MS or NMOSD, stimulation with $1,25(OH)_2D_3$ increased the mRNA expression of *CYP24A1* and decreased that of *CYP27B1* over those observed in samples stimulated with LPS+CD40L+CpG-ODN alone. *p<0.05, **p<0.01, ***p<0.001. $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CD40L, CD40 ligand; CpG-ODN, cytosine phosphate guanosine oligodeoxynucleotides; HC, healthy control; LPS, lipopolysaccharide; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; PBMC, peripheral blood mononuclear cell.

it. PBMC *CYP24A1* expression was also induced by stimulation with $1,25(OH)_2D_3$. These findings were observed in PBMCs from all study groups and were statistically significant. However, the expression levels of *VDR* and *IL-10* mRNA were unaffected by stimulation of PBMCs, with or without $1,25(OH)_2D_3$.

In patients with MS, the level of $1,25(OH)_2D_3$-induced *CYP24A1* mRNA expression in PBMCs was significantly and negatively correlated with serum $25(OH)D_3$ (for ΔC_T, r=0.744, p=0.014); however, no significant association was found for *VDR*, *CYP27B1*, and *IL-10* mRNA expression (**Figure 5**). In HCs, the level of $1,25(OH)_2D_3$-induced *IL-10* mRNA expression was positively correlated with serum $25(OH)D_3$ (for ΔC_T, r=0.590, p=0.026). In patients with NMOSD, there was no correlation between mRNA expression of *VDR*, *CYP27B1*, *CYP24A1*, or *IL-10* and serum $25(OH)D_3$, irrespective of stimulation with $1,25(OH)_2D_3$.

DISCUSSION

The findings of this study demonstrate that $1,25(OH)_2D_3$ exerted an inhibitory effect on the proliferation of lymphocytes, and especially T cells. However, in patients with MS and NMOSD, this $1,25(OH)_2D_3$-induced suppression of lymphocytes (total and T cells) was obvious upon subsequent stimulation with steroid. In addition, statistically significant associations in the opposite direction were found between serum $25(OH)D_3$ and the Bmem/B cell ratio after PBMC stimulation in patients with MS and NMOSD; it should be noted that there was no correlation between the Bmem/Breg ratio and serum $25(OH)D_3$. Moreover, the expression of *CYP24A1*, the gene encoding a $1,25(OH)_2D_3$-catabolizing enzyme, was less expressed in response to $1,25(OH)_2D_3$ in MS patients with higher serum $25(OH)D_3$.

Stimulation with $1,25(OH)_2D_3$ followed by steroid decreased the percentage of total lymphocytes and T cells in PBMCs from

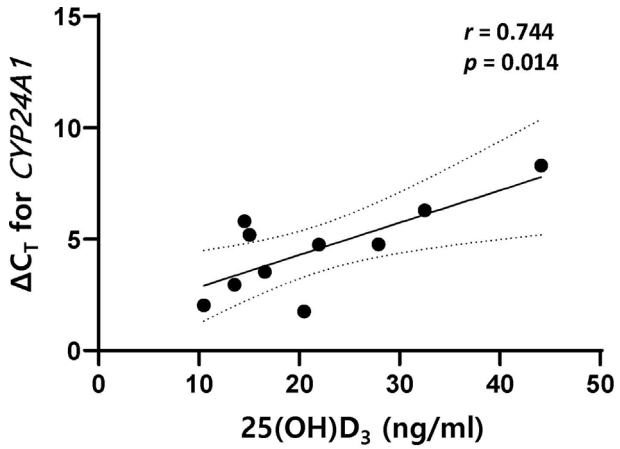


FIGURE 5 | There was a positive correlation between ΔC_T for *CYP24A1* and serum $25(OH)D_3$ in patients with MS. The mRNA expression level of *CYP24A1* after stimulation with $1,25(OH)_2D_3$ was lower in MS patients with higher serum $25(OH)D_3$. $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; ΔC_T, difference in cycle threshold between that for the target gene and that for *GAPDH*; MS, multiple sclerosis; serum $25(OH)D_3$, serum concentration of 1,25-dihydroxyvitamin D_3.

patients with MS and NMOSD. This finding could be explained by the known immune regulatory role of vitamin D. The active form of vitamin D, $1,25(OH)_2D_3$, induces monocyte proliferation, which contributes to the innate immune response and attenuates the cytotoxic activity and proliferation of $CD4^+$ and $CD8^+$ T cells by reducing proinflammatory cytokine production (1). $1,25(OH)_2D_3$ could enhance the anti-inflammatory effects of corticosteroids on monocytes and T cells *via* the induction of glucocorticoid receptor binding of steroids both *in vitro* and *in vivo* (22, 23). Combined treatment with steroid and $1,25(OH)_2D_3$ may thus have therapeutic potential in patients with MS or NMOSD by decreasing the T-cell-mediated autoimmune processes. $1,25(OH)_2D_3$ also exerts potent direct effects on B-cell responses, inhibiting their proliferation and differentiation into class-switched Bmem and plasma cells (6). However, there were no significant changes in the proportion of Breg and B cells in response to stimulation of PBMCs with $1,25(OH)_2D_3$. Little is known about the effect of glucocorticoid on B cells. Dexamethasone could induce apoptosis of B cells at all stages (24), and especially immature B cells, and could stimulate T-cell-dependent immunoglobulin production by enhancing the differentiation of B cells into mature plasma cells without proliferation (25). In NMOSD, it was reported that the frequencies of mature Bmem increased after 2 weeks of high-dose steroid treatment (26). In the present study, the proportion of Bmem in PBMCs from NMOSD patients, which were initially reduced by stimulation with $1,25(OH)_2D_3$, subsequently increased after stimulation with steroid, although the frequencies of Bmem did not differ between cells incubated with either $1,25(OH)_2D_3$ or steroid alone. This may indicate that steroid-induced Bmem proliferation could be augmented by $1,25(OH)_2D_3$ (22, 23). However, these findings were not observed in either HCs or MS patients; thus, further investigation is warranted.

It was particularly interesting that significant associations were observed between serum $25(OH)D_3$ and Bmem frequency in PBMCs stimulated with LPS+CD40L+CpG-ODN, with the correlation being negative in HCs and positive in NMOSD. In MS patients, the PBMC Bmem frequency was negatively correlated with serum $25(OH)D_3$ after preincubation with $1,25(OH)_2D_3$. Clinical data regarding the relationship between Bmem and serum $25(OH)D_3$ in autoimmune diseases are scarce. In SLE, a significant negative association was identified between serum $25(OH)D_3$ and Bmem, but not total B cells or plasmablasts (27). MS patients with a low vitamin D status exhibited decreased cerebrospinal fluid levels of vitamin D and greater intrathecal accumulation of class-switched Bmem and antibody-secreting plasma cells (7). Since Bmem are considered to be a source of proinflammatory cytokines, which are responsible for pathogenic effects during autoimmune processes (28, 29), it seems to be contradictory that NMOSD patients with higher serum $25(OH)D_3$ would have higher Bmem frequencies. However, the immune balance represented by the Bmem/Breg ratio was not correlated with the vitamin D level in NMOSD. After PBMC stimulation, the proportion of Bmem was significantly decreased in NMOSD, unlike in MS. The significance of a lower proliferation of non-Bmem in NMOSD patients with higher serum $25(OH)D_3$ remains to be established. The present findings also suggest that the suppressive effect of vitamin D on Bmem is stronger in MS patients with higher serum $25(OH)D_3$. A recent meta-analysis suggested that vitamin D supplementation has a therapeutic role in the treatment of MS; however, there is uncertainty about the most appropriate dose and factors influencing the immune regulatory roles of $1,25(OH)_2D_3$ (30). The therapeutic potential of vitamin D intake in patients with MS and NMOSD needs to be investigated further.

The biological activity of vitamin D is determined by the combination of levels of VDR expression and the activities of the metabolizing enzymes 1α-hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1) (1). $1,25(OH)_2D_3$ acts mainly *via* VDR-mediated regulation of gene expression, and VDR transcription is induced by $1,25(OH)_2D_3$ itself (31). IL-10 expression in activated B cells is enhanced by $1,25(OH)_2D_3$ more than threefold, primarily through the recruitment of VDR to the promoter of IL-10 (32). However, the mRNA expression levels of *VDR* and *IL-10* in PBMCs were not significantly influenced by the presence of $1,25(OH)_2D_3$ in the present study. This may be attributable to upregulated *VDR* expression by inflammatory signals offsetting the effects of $1,25(OH)_2D_3$ on immune cells (3), or mRNA measurement times that were inappropriate for allowing the detection of any effect (33). In stimulated PBMCs, feedback regulation of vitamin D metabolism by $1,25(OH)_2D_3$ was observed in HCs, and in patients with MS and NMOSD. The presence of CYP27B1 has been demonstrated in immune cells, enabling them to produce locally active $1,25(OH)_2D_3$ from $25(OH)D_3$ (1). CYP24A1 expression is also induced by $1,25(OH)_2D_3$, creating a self-regulatory feedback loop and enabling $1,25(OH)_2D_3$ to fulfill its role in maintaining immune balance (31). It is noteworthy that in response to stimulation with $1,25(OH)_2D_3$, *CYP24A1* mRNA expression was lower in MS patients with higher serum $25(OH)D_3$. This suggests that serum $25(OH)D_3$ is associated with impaired regulation of CYP24A1 activity. There are few previous reports on the associations between genetic polymorphisms in vitamin-D-regulated genes such as *CYP24A1*, vitamin D status, and MS risk (34, 35). Further studies with larger samples are needed to reveal the mechanism underlying the impaired regulation of vitamin D hydroxylation in MS.

This study had several limitations. The small number of samples in each study group must be considered when interpreting these data, which reduced the statistical power and may have introduced unintended bias. In addition, the enrolled patients were all in remission with heterogenous disease duration and had been taking immunosuppressive or disease-modifying drugs, which could affect immune cell function. Moreover, we did not obtain age-matched HCs compared with the NMOSD group, which could affect the composition and quality of the lymphocyte pool.

In conclusion, the findings of this study suggest that vitamin D plus steroid has a therapeutic benefit on T cells in MS and NMOSD, and that differential transcriptional activities of the CYP24A1 gene could exist that affect serum $25(OH)D_3$ in MS. In addition, vitamin D may have different inhibitory effects on

Bmem that are dependent upon serum 25(OH)D_3 in MS and NMOSD. The positive association between $CD19^+CD27^+$ B-cell frequency and serum 25(OH)D_3 in NMOSD after immune-cell stimulation must also be further explored to establish whether vitamin D does have beneficial effects in this autoimmune disease, or if this was simply an accidental correlation. Further large-scale studies could help to elucidate the immunoregulatory mechanism of vitamin D supplementation in patients with MS and NMOSD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The study was approved by the Institutional Review Board of Samsung Medical Center and written informed consents were obtained from all subjects.

AUTHOR CONTRIBUTIONS

Study concept and design: EBC and J-HM. Acquisition of data: EBC, JHS, SK, JK, JMS, BK, and J-HM. Analysis and interpretation of the data: EBC, BK, and J-HM. Draft and revision of the manuscript for content: EBC and J-HM. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.677041/full#supplementary-material

Supplementary Figure 1 | Changes in the proportion of lymphocytes in samples of PBMCs in response to stimulation with LPS+CD40L+CpG-ODN (nonspecific) in HCs and in patients with MS or NMOSD. A significant increase in total lymphocyte percentage was noted in the NMOSD group **(A)**. Relative B-cell proliferation compared with T cells was found in the MS and NMOSD groups **(B, C)**. The Bmem/Breg ratio was significantly reduced in HCs and patients with NMOSD, but not in those with MS **(D–F)**. Bmem, $CD19^+CD27^+$ memory B cell; Breg, $CD19^+CD24^+CD38^+$ regulatory B cell; CD40L, CD40 ligand; CpG-ODN, cytosine phosphate guanosine oligodeoxynucleotides; HC, healthy control; LPS, lipopolysaccharide; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; PBMC, peripheral blood mononuclear cell.

REFERENCES

1. Mora JR, Iwata M, Von Andrian UH. Vitamin Effects on the Immune System: Vitamins A and D Take Centre Stage. *Nat Rev Immunol* (2008) 8(9):685–98. doi: 10.1038/nri2378
2. Jeffery LE, Wood AM, Qureshi OS, Hou TZ, Gardner D, Briggs Z, et al. Availability of 25-Hydroxyvitamin D3 to APCs Controls the Balance Between Regulatory and Inflammatory T Cell Responses. *J Immunol* (2012) 189 (11):5155–64. doi: 10.4049/jimmunol.1200786
3. Kongsbak M, Levring TB, Geisler C, Von Essen MR. The Vitamin D Receptor and T Cell Function. *Front Immunol* (2013) 4:148. doi: 10.3389/fimmu.2013.00148
4. Rolf L, Muris AH, Hupperts R, Damoiseaux J. Vitamin D Effects on B Cell Function in Autoimmunity. *Ann New York Acad Sci* (2014) 1317(1):84–91. doi: 10.1111/nyas.12440
5. Royal IIIW, Mia Y, Li H, Naunton K. Peripheral Blood Regulatory T Cell Measurements Correlate With Serum Vitamin D Levels in Patients With Multiple Sclerosis. *J Neuroimmunol* (2009) 213(1–2):135–41. doi: 10.1016/j.jneuroim.2009.05.012
6. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory Effects of 1, 25-Dihydroxyvitamin D3 on Human B Cell Differentiation. *J Immunol* (2007) 179(3):1634–47. doi: 10.4049/jimmunol.179.3.1634
7. Haas J, Schwarz A, Korporal-Kuhnke M, Faller S, Jarius S, Wildemann B. Hypovitaminosis D Upscales B-Cell Immunoreactivity in Multiple Sclerosis. *J Neuroimmunol* (2016) 294:18–26. doi: 10.1016/j.jneuroim.2016.03.011
8. Garcia-Carrasco M, Jiménez-Herrera EA, Gálvez-Romero JL, de Lara LV, Mendoza-Pinto C, Etchegaray-Morales I, et al. Vitamin D and Sjögren Syndrome. *Autoimmun Rev* (2017) 16(6):587–93. doi: 10.1016/j.autrev.2017.04.004
9. Iruretagoyena M, Hirigoyen D, Naves R, Burgos PI. Immune Response Modulation by Vitamin D: Role in Systemic Lupus Erythematosus. *Front Immunol* (2015) 6:513. doi: 10.3389/fimmu.2015.00513
10. Kim D. The Role of Vitamin D in Thyroid Diseases. *Int J Mol Sci* (2017) 18 (9):1949. doi: 10.3390/ijms18091949
11. Min J-H, Waters P, Vincent A, Cho H-J, Joo B-E, Woo S-Y, et al. Low Levels of Vitamin D in Neuromyelitis Optica Spectrum Disorder: Association With Disease Disability. *PloS One* (2014) 9(9):e107274. doi: 10.1371/journal.pone.0107274
12. Sintzel MB, Rametta M, Reder AT. Vitamin D and Multiple Sclerosis: A Comprehensive Review. *Neurol Ther* (2018) 7(1):59–85. doi: 10.1007/s40120-017-0086-4
13. Wu Y, Cai Y, Liu M, Zhu D, Guan Y. The Potential Immunoregulatory Roles of Vitamin D in Neuromyelitis Optica Spectrum Disorder. *Multiple Sclerosis Related Disord* (2020) 43:102156. doi: 10.1016/j.msard.2020.102156
14. BM Segal. Th17 Cells in Autoimmune Demyelinating Disease. *Seminars in Immunopathology*. (2010) 32(1):71–7. doi: 10.1007/s00281-009-0186-z
15. Wingerchuk DM, Banwell B, Bennett JL, Cabre P, Carroll W, Chitnis T, et al. International Consensus Diagnostic Criteria for Neuromyelitis Optica Spectrum Disorders. *Neurology* (2015) 85(2):177–89. doi: 10.1212/WNL.0000000000001729
16. Kimura A, Kishimoto T. IL-6: Regulator of Treg/Th17 Balance. *Eur J Immunol* (2010) 40(7):1830–5. doi: 10.1002/eji.201040391
17. Hsu H-C, Yang P, Wang J, Wu Q, Myers R, Chen J, et al. Interleukin 17–Producing T Helper Cells and Interleukin 17 Orchestrate Autoreactive Germinal Center Development in Autoimmune BXD2 Mice. *Nat Immunol* (2008) 9(2):166–75. doi: 10.1038/ni1552
18. Rodney C, Rodney S, Millis RM. Vitamin D and Demyelinating Diseases: Neuromyelitis Optica (NMO) and Multiple Sclerosis (Ms). *Autoimmune Dis* (2020) 2020:8718736. doi: 10.1155/2020/8718736
19. Shan Y, Tan S, Zhang L, Huang J, Sun X, Wang Y, et al. Serum 25-Hydroxyvitamin D3 Is Associated With Disease Status in Patients With Neuromyelitis Optica Spectrum Disorders in South China. *J Neuroimmunol* (2016) 299:118–23. doi: 10.1016/j.jneuroim.2016.09.006

20. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of Multiple Sclerosis: 2017 Revisions of the McDonald Criteria. *Lancet Neurol* (2018) 17(2):162–73. doi: 10.1016/S1474-4422(17)30470-2
21. Klein U, Rajewsky K, Küppers R. Human Immunoglobulin (Ig) M+ IgD+ Peripheral Blood B Cells Expressing the CD27 Cell Surface Antigen Carry Somatically Mutated Variable Region Genes: CD27 as a General Marker for Somatically Mutated (Memory) B Cells. *J Exp Med* (1998) 188(9):1679–89. doi: 10.1084/jem.188.9.1679
22. Hoepner R, Bagnoud M, Pistor M, Salmen A, Briner M, Synn H, et al. Vitamin D Increases Glucocorticoid Efficacy *via* Inhibition of Mtorc1 in Experimental Models of Multiple Sclerosis. *Acta Neuropathol* (2019) 138(3):443–56. doi: 10.1007/s00401-019-02018-8
23. Zhang Y, Leung DY, Goleva E. Anti-Inflammatory and Corticosteroid-Enhancing Actions of Vitamin D in Monocytes of Patients With Steroid-Resistant and Those With Steroid-Sensitive Asthma. *J Allergy Clin Immunol* (2014) 133(6):1744–52.e1. doi: 10.1016/j.jaci.2013.12.004
24. Gruver-Yates AL, Quinn MA, Cidlowski JA. Analysis of Glucocorticoid Receptors and Their Apoptotic Response to Dexamethasone in Male Murine B Cells During Development. *Endocrinology* (2014) 155(2):463–74. doi: 10.1210/en.2013-1473
25. Grayson J, Dooley N, Koski I, Blaese R. Immunoglobulin Production Induced *In Vitro* by Glucocorticoid Hormones: T Cell-Dependent Stimulation of Immunoglobulin Production Without B Cell Proliferation in Cultures of Human Peripheral Blood Lymphocytes. *J Clin Invest* (1981) 68(6):1539–47. doi: 10.1172/JCI110408
26. Quan C, ZhangBao J, Lu J, Zhao C, Cai T, Wang B, et al. The Immune Balance Between Memory and Regulatory B Cells in NMO and the Changes of the Balance After Methylprednisolone or Rituximab Therapy. *J Neuroimmunol* (2015) 282:45–53. doi: 10.1016/j.jneuroim.2015.03.016
27. Yamamoto EA, Nguyen JK, Liu J, Keller E, Campbell N, Zhang C-J, et al. Low Levels of Vitamin D Promote Memory B Cells in Lupus. *Nutrients* (2020) 12 (2):291. doi: 10.3390/nu12020291
28. Duddy M, Niino M, Adatia F, Hebert S, Freedman M, Atkins H, et al. Distinct Effector Cytokine Profiles of Memory and Naive Human B Cell Subsets and Implication in Multiple Sclerosis. *J Immunol* (2007) 178(10):6092–9. doi: 10.4049/jimmunol.178.10.6092
29. Kim S-H, Huh S-Y, Lee SJ, Joung A, Kim HJ. A 5-Year Follow-Up of Rituximab Treatment in Patients With Neuromyelitis Optica Spectrum Disorder. *JAMA Neurol* (2013) 70(9):1110–7. doi: 10.1001/jamaneurol.2013.3071
30. McLaughlin L, Clarke L, Khalilidehkordi E, Butzkueven H, Taylor B, Broadley SA. Vitamin D for the Treatment of Multiple Sclerosis: A Meta-Analysis. *J Neurol* (2018) 265(12):2893–905. doi: 10.1007/s00415-018-9074-6
31. Van Etten E, Stoffels K, Gysemans C, Mathieu C, Overbergh L. Regulation of Vitamin D Homeostasis: Implications for the Immune System. *Nutr Rev* (2008) 66(suppl_2):S125–34. doi: 10.1111/j.1753-4887.2008.00096.x
32. Heine G, Niesner U, Chang HD, Steinmeyer A, Zügel U, Zuberbier T, et al. 1, 25-Dihydroxyvitamin D3 Promotes IL-10 Production in Human B Cells. *Eur J Immunol* (2008) 38(8):2210–8. doi: 10.1002/eji.200838216
33. Matilainen JM, Räsänen A, Gynther P, Väisänen S. The Genes Encoding Cytokines IL-2, IL-10 and IL-12B are Primary 1α, 25 (OH) 2D3 Target Genes. *J Steroid Biochem Mol Biol* (2010) 121(1–2):142–5. doi: 10.1016/j.jsbmb.2010.03.020
34. Agnello L, Scazzone C, Sasso BL, Ragonese P, Milano S, Salemi G, et al. CYP27A1, CYP24A1, and RXR-α Polymorphisms, Vitamin D, and Multiple Sclerosis: A Pilot Study. *J Mol Neurosci* (2018) 66(1):77–84. doi: 10.1007/s12031-018-1152-9
35. Ramasamy A, Trabzuni D, Forabosco P, Smith C, Walker R, Dillman A, et al. Genetic Evidence for a Pathogenic Role for the Vitamin D3 Metabolizing Enzyme CYP24A1 in Multiple Sclerosis. *Multiple Sclerosis Related Disord* (2014) 3(2):211–9. doi: 10.1016/j.msard.2013.08.009

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REVIEW
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Emerging Biomarkers and Therapeutic Strategies for Refractory Bullous Pemphigoid

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Bullous pemphigoid (BP) is the most common autoimmune subepidermal blistering disorder in the elderly. Systemic and topical use of glucocorticoids and immunosuppressants has been shown to be effective in most patients. However, refractory BP patients are challenged to clinicians with severe clinical symptoms, resistance to treatment, and high relapse rate. How to predict and assess the refractory and severity of bullous pemphigoid is the key issue in clinical practice, and the urgent need for precision medicine in refractory patients is driving the search for biomarkers and biologics. Recently, some biomarkers, such as the level of specific autoantibodies and released cytokines, have been proposed as the potential parameters to reflect the disease severity and predict the treatment response and relapse of refractory BP. Moreover, new biologics targeting pathogenic antibodies, complement, Th2 axis, eosinophils, and Th17 axis have shown potent efficacy on refractory BP. Here, we review the literature and give an overview of emerging biomarkers and therapeutic strategies for refractory bullous pemphigoid to improve the prognosis of the patient.

Keywords: bullous pemphigoid, biomarkers, severity, refractory, relapse, prognosis, biologics

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INTRODUCTION

Bullous pemphigoid (BP) is an uncommon autoimmune subepidermal blistering disease, but accounts for about 70% of subepidermal bullous diseases, mainly affecting the elderly (1). It is estimated that the annual incidence of BP among different populations in the world is about 12–66 cases per million people (2). The typical clinical features of BP consist tension blisters on erythema or normal skin with intense itching. Histopathology shows subepidermal blisters and inflammatory cell infiltration dominated by eosinophils. Immunologically, IgG and/or C3 are deposited linearly along the basement membrane zone, characterized by the production of autoantibodies against the components of the hemidesmosomes BP180 and BP230 at the dermis-epidermis junction (3).

Genetic predisposing factors, such as HLA-DQβ1*0301 is associated to the occurrence of BP, UV exposure, thermal or electrical burns, trauma, drugs, virus infection, and other physical, chemical, and biological factors that are involved in the pathogenesis of susceptible individuals by mediating the loss of immune tolerance to the autoantigen (4). In terms of the pathological mechanism of BP, autoantibodies, immune cells, and inflammatory factors are all involved in the pathogenesis of BP. The interaction of autoantibodies with BP180 brings about the formation of blisters by the activating complement-dependent or independent signals to amplify the inflammatory pathway

(**Figure 1**) (5). Non-specific immunosuppressive therapy is effective in most patients. Unfortunately, refractory BP patients are resistant to glucocorticoids or immunosuppressants treatment or have a high relapse rate of 27.87%–53%, and most of the relapse occurs in the early stage of remission (within 6 months) (6). Besides, severe adverse reactions due to a long-term use of glucocorticoids or immunosuppressants, including infections, gastrointestinal intolerance, myelosuppression, hepatotoxicity, and even increased risk of cancer could not be ignored (7–9). Hereby, biomarkers for predicting the refractory BP and indicating optimal therapeutic strategies to control their symptoms are urgently required.

With a deep understanding of the pathogenesis of BP, several studies have reported that some parameters involved in the pathogenesis can be used as biomarkers for refractory BP. The purpose of this paper is to analyze the relationship between biomarkers and the disease activity, relapse, and response to treatment. Moreover, the clinical application of new biologics for refractory BP would be reviewed.

EMERGING BIOMARKERS IN BP

Anti-BP IgG and IgE

The reaction of specific autoantibodies produced by autoimmune abnormalities with BP180 is regarded as the central event in the pathogenesis of BP. Both anti-BP180 IgG and IgE could reduce the adhesion of keratinocytes by increasing the internalization of BP180 as well as mediating the recruitment and activation of immune cells through complement-dependent or complement-

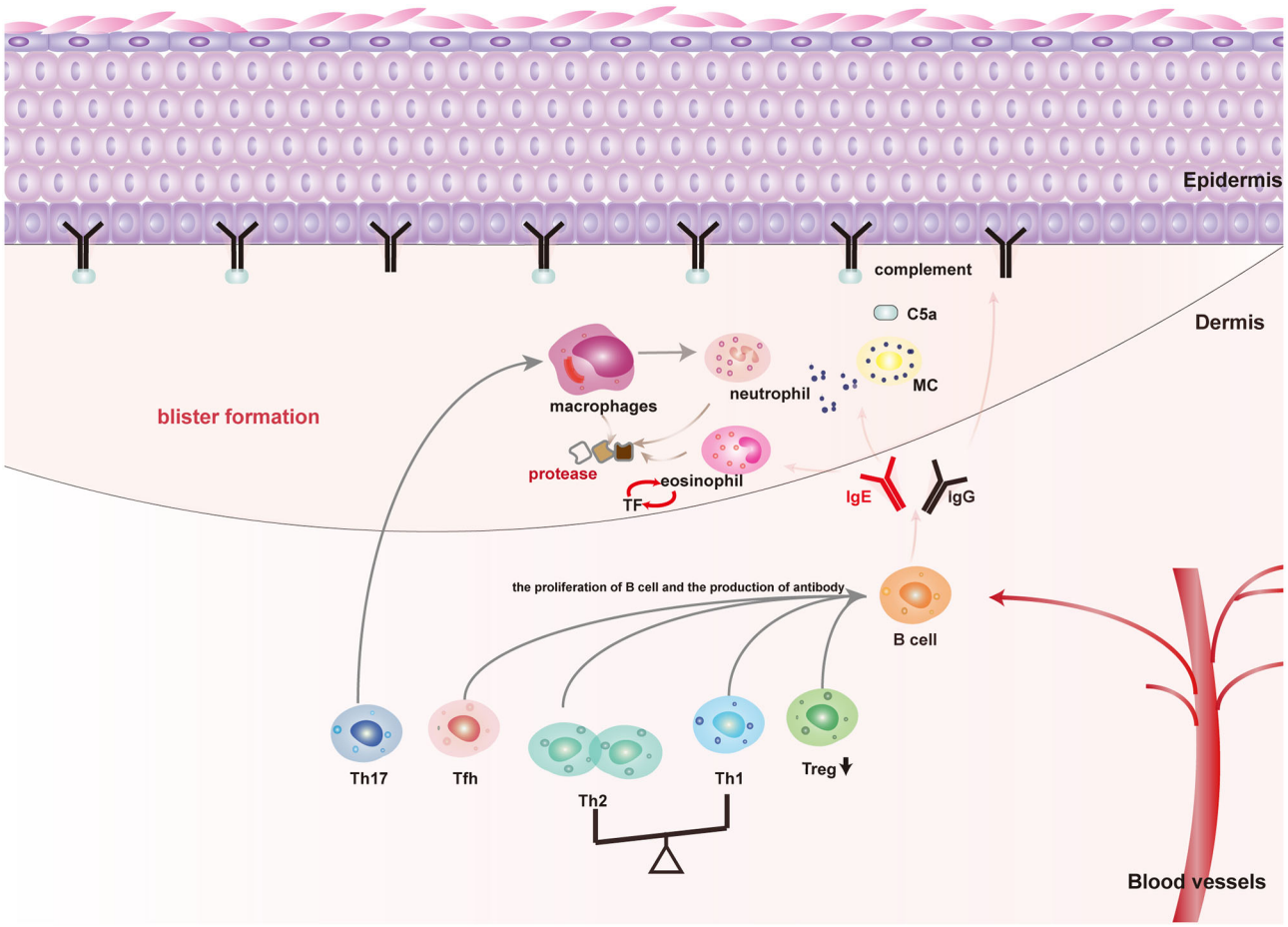


FIGURE 1 | The main pathogenic mechanism of blister formation in patients with BP: Autoimmune abnormalities such as dysfunction of Th cells and Treg cells lead to the production of anti-BP180 autoantibodies, among which BP180IgG and IgE are the main pathogenic antibodies for blister formation. (1) Mechanical damage: Both BP180IgG and IgE could bind to the NC16A domain of BP180, resulting in BP180 internalization, thus decreasing the adhesion; (2) Inflammatory injury: BP180IgG and IgE could also activate keratinocytes to release IL-8 and other cytokines by binding to BP180. Moreover, BP180IgG and IgE also activate mast cells in a complement-dependent or independent way to release cytokines and mMCP-4. These above cytokines and mMCP-4 recruit and activate inflammatory cells dominated by eosinophils and neutrophils to magnify the inflammatory response of skin lesions and cause tissue damage; (3) Specific pathways of mast cell activation: BP180IgG and IgE directly activate mast cells by binding to FcγR or FcεR of mast cells. BP180IgG binding BP180 also activate the complement cascade pathway to produce C5a, which binds to the C5aR of mast cells and indirectly causes mast cell activation.

independent pathways to release proteases and cytokines, thus participating in the formation of blisters (5).

BP180 IgG

BP180 ELISA values and direct immune fluorescence (DIF) are necessary to diagnose BP. The value of anti-BP180NC16A IgG ELISA is positive correlate with the disease severity in cross-sectional studies (10–15). The longitudinal follow-up of large samples during traditional treatment suggests that patients with a high level of BP180NC16A IgG indicate refractory BP who have an insufficient response to glucocorticoid and requiring a longer treatment time or a higher dose of glucocorticoid (12, 15–18).

Moreover, BP180IgG levels are of great clinical importance in predicting the risk of relapse. High titer of anti-BP180NC16AIgG (>27 IU/mL) is the only factor independently predicting the relapse of BP within one year after discontinuation of treatment, and the positive predictive value and the negative predictive value are 90.9% and 51.2%, respectively (19). However, in another study, positive DIF was of a better value than BP180 IgG ELISA values for predicting the relapse risk, with 59.0% sensitivity, 78.0% specificity, while 50.0% positive predictive values and 83.3% negative predictive value, respectively (20). When the ELISA-BP180 value was high or DIF shows positive, treatment to BP should not be ceased. Relapse is concerned with a slight deceleration of BP180 IgG levels between day 0 and day 60. A high level of BP180 at day 150 (23 U/mL) (21) and baseline (53.09 U/mL) could also predict relapse, the sensitivity was 84.2% and 81.3%, respectively. Meanwhile, the negative reaction to BP180 IgG and BP230 IgG ELISA may be a clue of non-relapse (22).

It should be noted that about 8% of BP cases have anti-BP230 but not anti-BP180 (BP230-type BP). Most studies showed that anti-BP230IgG levels was not related to disease severity in the presence of BP180 IgG (3). A large proportion of BP230-type cases showed a mild clinical phenotype, which may be related to the weak deposition of IgG1, IgG3, and complement in lesions, indicating the importance of IgG antibody subclass analysis (23). Consistently, as a blocking antibody, it is found that the IgG4 level increased with the improvement of the disease condition, so the change of the main antibody subclass from IgG1 to IgG4 may mean the improvement of the disease (24).

BP180 IgE

Recently, increasing evidence has shown that IgE is involved in the occurrence of BP, but contradictions are still in the relationship between IgE and disease severity, which may be due to the lack of a unified method for the quantitative detection of BP180IgE (3). Elevated IgE levels have been reported in 22%–100% of patients with BP (25). A few studies reported that the circulating level of BP180NC16A IgE in patients was not related to the severity of the disease (26–28). For example, Ma et al. believed that high titers of anti-BP180 IgG contributes mostly to the disease activity than the anti-BP180 IgE level in patients (28). However, since previous studies have verified the pathogenic effect of BP180IgE in cell culture and animal models, Omalizumab targeting IgE has been successfully used in patients with refractory BP (3). It agrees with the conclusion that high levels of circulating BP180NC16A IgE and total IgE in serum are correlated with higher BPDAI scores of BP patients (11, 13, 25, 29–32). It is an urgent need to explore and unify BP180 IgE detection methods with acceptable sensitivity and specificity to confirm this conclusion. On the other hand, as a low-affinity Fc receptor of IgE, the levels of CD23 in tissue or and sCD23 in serum are consistent with the severity of the disease (33–35), which also imply that IgE is another key participant in the pathogenesis of BP. In addition, based on the existence of high levels of anti-BP180IgG antibodies, the appearance of anti-BP180IgE supports that those patients need more active therapies for remission (31). The patients with a high level of anti-BP180 IgG need to jointly evaluate the level of anti-BP180 IgE in a subsequent treatment to more effectively identify refractory BP patients. Not only that, when the total serum IgE level remains high and the skin lesions could not be effectively controlled, Omalizumab can be considered (13, 36–38).

Chemokines

All infiltrating cells contribute to the pathogenesis of BP. Especially, the chemotaxis and activation of major effector cells such as eosinophils, neutrophils, and monocytes/macrophages in the lesions, directly leading to the blister formation through the release of proteases and cytokines (39). Chemotactic factors that attract inflammatory cells infiltrating should be attended to.

CXCL8 (IL-8), a neutrophil-attracting chemokine, in serum and blister fluid (BF) of patients could change with the fluctuation of the disease activity (40–42). CXCL9(MIG), CXCL10 (IP10), and Th2 chemokine, such as CCL2 (MCP-1), CCL17 (TARC), CCL18 (PARC), and CCL22 (MDC) cannot only attract T lymphocytes and monocytes to the lesion but also relate to the disease severity (43–48). In addition, elevated serum CXCL9 levels only occur in patients with a serious condition, and should be particularly vigilant against the disease deterioration (44, 47). CXCL10 induces monocytes and neutrophils to express MMP-9, which is involved in the relapse of BP. The serum CXCL10 levels can be upregulated by IL-17 and continue to rise or maintain high levels at 60 days or within the first year after treatment in relapse patients, which maintain and amplify the inflammatory response (49). What is more, CCL17 levels sharply reflect the disease activity with a higher sensitivity than BP180NC16A IgG, and could be viewed as a promising biomarker for predicting the early relapse of the disease (43).

Cytokines such as IL-5, CCL5 (RANTES), CCL11 (Eotaxin), and CCL26 (Eotaxin3) are involved in the proliferation and recruitment of eosinophils from the peripheral circulation to the lesions, and their levels always keep in line with the number of blisters (40, 44, 50–52). Particularly, IL-5 is the promising biomarker for disease severity, as IL-5 levels in serum and BF could reflect the disease severity in several different population studies. The count of peripheral blood eosinophils fluctuates in line with the disease severity in BP (30, 32, 53–58).

Cytokines

Inflammatory mediators, for instance, IL-1β, IL-6, and TNF-α, reflecting the disease severity, because serum levels of TNF-α,

IL-6 and blister contents of IL-1β and TNF-α are significantly higher in severe patients than in mild patients. But they are not specific enough for BP, because they rise in many other conditions (40–42, 59). In contrast, patients with severe clinical manifestations and treatment resistance are often accompanied by low levels of IL-10 secreted by Treg, and the increasing levels of IL-10 is related to clinical improvement in BP (60–62). These observations support the idea that a persistent immune activation exists in diseases that are relatively or absolutely deficient in IL-10, and the recovery of relatively defective Treg and IL-10 is helpful to alleviate the disease.

Moreover, some members of the TNF family, such as sCD40L, a proliferation-inducing ligand (APRIL), and B lymphocyte activating factor (BAFF), contribute to B cell proliferation and autoantibodies production, they are more sensitive than autoantibodies to reflect the treatment response and early relapse. They often occur before the appearance of skin lesions and the increase of BP180NC16A antibody levels, and decline after treatment, so that detection of their serum levels could distinguish the tendency of relapse in time (63–65). Likewise, IL-18, as well as IL-15 derived from macrophages and IL-21 secreted by Tfh cell increase in BF or serum of BP, which promote the proliferation of B cells and the production of antibodies, also correlate with the disease activity (42, 66–68).

In BP patients with a continuous remission and relapse in the first year of treatment, serum autoantibodies titers, as well as concentrations of cytokines belonging to the Th17 axis are different. IL-17 and IL-23 participate in a variety of pathophysiological mechanisms of relapse. To begin with, both IL-17 and IL-23 independently induce DNA extracellular traps (ETs) formation in patients with relapse; subsequently, they also stimulate monocytes and neutrophils to produce MMP-9, which contributes to the separation of the dermis and epidermis; eventually, they upregulate the expression of glucocorticoid receptor-β related to the resistance to glucocorticoid therapy (69, 70). During the one-year follow-up, Plée et al. found that the patients with a significantly decreased IL17 level within 0–60 days showed a continuous remission, while the patients whose IL-17 level remained high within 60 days after treatment had a higher risk of relapse. Similarly, patients with significantly elevated IL-23 and MMP-9 levels within 0–60 days were also prone to relapse (69).

However, we have doubts about the predictive value of serum inflammatory mediator levels not only because a variety of factors influence the inflammatory mediator levels in serum and make them unrepresentative of the levels in tissues, but because the dynamic monitoring of serum inflammatory mediator levels is costly.

Eosinophil and Eosinophil Cationic Protein

Eosinophils, as the main infiltration cell in the lesions of BP, play the central effect on blister formation and pruritus by secreting protease and cytokines like Eosinophil cationic protein (ECP) and IL-31 (3), it also provides several biomarkers to reflect disease severity and relapse.

ECP in BF released by eosinophils contributed to the blister formation, which keeps in line with BP severity (51, 58, 71, 72). Giusti et al. also found that the serum concentration of ECP decreased within 0–60 days after treatment, but it did not decrease in patients with subsequent relapse. The cutoff value for the decrease of ECP concentration from baseline to 60 days was 12.8ng/mL, the sensitivity, specificity, positive predictive value, and negative predictive value for judging the clinical remission of BP were 63.2%, 64.3%, 81.1%, and 41.8%, respectively (72).

Eosinophils are known to initiate the coagulation cascade at skin level *via* TF, which in BP promotes the recruitment of eosinophils in skin lesions and the expression of matrix metalloproteinases (MMP)-9, making the coagulation cascade become an auxiliary mechanism that is involved in blister formation. Moreover, serum and BF levels of F1+2 and D-dimer ascend in patients with a more severe disease and significantly descend with the remission of the disease (57, 58, 73). The detection of D-dimer has increased the diagnostic evidence of chronic urticaria in clinics (74). After excluding the influence of other diseases, the indicative effect of D-dimer on refractory BP is worth further exploring.

Others

Several experimental studies have proved the contribution of the classical pathway of a complement cascade to the formation of BP blisters, C5a produced by complement cascade could recruit and activate mast cells to initiate a downstream inflammatory cascade reaction (75). The deposition of autoantibodies and complement C3 along the dermis-epidermis junction is the diagnostic criteria of BP. Interestingly, a more abnormal complement activation is present in more active patients. The complement-fixation assay is a simple method to detect the complement activation by autoantibodies *in vitro*, which could reflect the BP activity (76). Due to the maturity and simplicity of this clinical assay, a larger sample of clinical trial will be necessary.

On the other hand, the activation of mast cells in the lesions is the earliest event in the formation of BP blister. It cannot only secrete inflammatory cytokines to promote subsequent infiltration of inflammatory cells such as neutrophils in skin lesions, but also release mast cell protease (MCP)-4 to activate major proteases such as MMP-9 that cause BP tissue damage (77). Trypsin in serum and BF, as reliable indicators of mast cell activation, seems to be consistent with the disease activity (78, 79), but more research is required to obtain a high-level evidence.

Modern techniques such as RNA sequencing help identify potential BP biomarkers. MiR-1291 is an endogenous non-coding RNA, which is also a possible biomarker of some autoimmune disorders. A recent study has found that MiR-1291 significantly increase in active patients, and the serum relative expression could reflect the BP activity, the sensitivity and specificity were 75.56% and 81.03%, respectively (80). The implementation of these techniques may not only help to find more potential biomarkers of diseases but also improve our understanding of the pathogenesis of BP. Some clinical characters are also linked to relevant aspects of the BP. At baseline, patients with generalized diseases and neurological diseases such as dementia have a higher risk of relapse (21).

It is necessary to assess whether the patients have neurological diseases or a wide range of disease areas as soon as possible to take more positive and reasonable measures.

In summary, both innate immunity and adaptive immunity play important roles in the pathogenesis of BP. Although a large number of laboratory parameters are related to refractory BP, we recommend that especially anti-BP180 NC16A IgG, anti-BP180 NC16A IgE, total IgE, the count of peripheral blood eosinophils, ECP, D-dimer, F1+2, IL-5,CXCL-8, CCL17, and IL-10 are the most promising indicators for severity, based on clinical relevance, repeatability (i.e., more than three studies from different centers showing the same correlation), and feasibility, and their value as biomarkers of disease severity deserves further investigation. Some potential markers such as IL-1β, IL-6, TNF-α, etc. are not specific enough, so they should be interpreted more carefully. However, due to the small number of large-scale studies on biomarkers for treatment resistance or relapse, the level of evidence is still very low, and more research is needed. Having said that, several large-scale studies have provided evidence of the clinical efficacy of biomarkers reflecting the risk of disease relapse or treatment resistance. Detecting BP180NC16AIgG antibody levels at baseline, serum levels of IL17, IL-23, CXCL10, CCL17, BAFF, APRIL, sCD40L, MMP-9, and ECP during treatment, and DIF or BP180NC16A IgG ELISA on the day ready to stop the treatment may help clinicians to identify the patients who are prone to relapse and take more active treatment and management measures to reduce the possibility of relapse., but it should be clear that complex autoantibody and cytokine disorders exist in BP, and it is often not accurate to use a biomarker alone. More large-scale clinical research is needed to explore new biomarkers or verify promising biomarkers that have been found, and the joint detection of these biomarkers is more meaningful to reflect the disease status.

EMERGING BIOLOGICS FOR BP

Currently, no biologics have been approved for BP, and the treatment mainly depends on traditional immunosuppressive therapies. However, high-dose of glucocorticoids and immunosuppressants cause obvious side effects, and are associated with a high relapse rate, precision medicine of refractory BP attracts much attention. With the emergence of biomarkers, more and more biologics targeting the pathogenic mechanism are entering the experimental stage or being used in the treatment of refractory BP in the clinic. We will focus on these biologics.

Biologics Targeting Pathogenic Antibodies

Pathogenic antibodies including BP180IgG and IgE are used as tools to identify refractory BP, and in some case reports, biologics specifically targeting them have shown great advantages in managing refractory BP.

Rituximab

Rituximab is a monoclonal antibody targeting the specific CD20 transmembrane glycoprotein of mature B cells. It causes B cell depletion and antibodies reduction through antibody-dependent cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and direct induction of apoptosis. Rituximab was originally used for non-Hodgkin's lymphoma and has been recommended by the international panel of experts for the first-line treatment of pemphigus (81, 82).

In recent times, Rituximab has been gradually applied to refractory BP. First-line combination therapy with rituximab and corticosteroids could significantly improve the complete remission rate of patients on the basis of reducing the dose of prednisone and not increasing the incidence of adverse reactions. Now, the dose of Rituximab for BP treatment has not been specified, almost all clinical applications use the recommended dose for non-Hodgkin's lymphoma or rheumatoid arthritis, that is, intravenous infusion of Rituximab 375 mg/m^2 per week for four weeks or 1,000 mg per week for two consecutive weeks (83).

It is worth noting the relapse and adverse reactions under the treatment of Rituximab. CD20 is not expressed on long-living antibody-producing plasma cells, even the peripheral blood B cells achieve complete consumption and the autoantibodies disappear, patients could only achieve temporary and partial remission, so it is very important to observe the tendency of relapse and to find a suitable maintenance treatment (84). Low peak BAFF levels and the increased proportion of memory B cells before B-cell recovery are helpful to identify BP patients who are prone to relapse or resistant to Rituximab (85). In addition, infusion-related adverse reactions are common and serious adverse events are rare, there are occasional neutropenia, skin toxicity (leukoclastic vasculitis and Stevens-Johnson syndrome, etc.), systemic infection, and even death (86–88). IVIg combined with Rituximab in the treatment of refractory BP could help patients to rebuild the immunomodulatory function to reduce the incidence of adverse events and achieve lasting remission (89). As a steroid protective agent, Rituximab has benefited some patients with refractory BP and brought a higher remission rate, but it is necessary to monitor the hemogram, blood pressure, and temperature, and take care of the infection and infusion-related adverse reactions.

Omalizumab

As the first targeted drug approved by the FDA for the treatment of chronic urticaria (74), Omalizumab was successfully cured a patient with refractory BP for the first time in 2009 (90), because it could prevent IgE from interacting with FcεR I on mast cells and other effector cells to reduce the release of inflammatory mediators by binding to the Fc region of free IgE, downregulating of FcεR I expression and dissociating the IgE- FcεR I complex.

A total of 16 studies have reported the efficacy of Omalizumab in the treatment of refractory BP. In these studies, most patients showed elevated total IgE and eosinophil levels and resistance to immunosuppressive therapy, that is, intractable pruritus or extensive skin lesions were difficult to control, or had complications such as osteoporosis and infections. After they were treated with Omalizumab, their itching, new blisters, and eosinophil count were significantly improved at the earliest within a week, the lesions basically disappeared, and the dose of glucocorticoid gradually decreased after an average of 3 months. Most patients could benefit from subcutaneous injection of 300 mg

Omalizumab every 4 weeks as a steroid protective agent or monotherapy, with varying maintenance time (up to 18 months), and no serious adverse reactions (36, 38, 90–102). In the absence of large-scale clinical trials, the recommended dose of Omalizumab is based on its use in asthma or chronic urticaria. During the period of treatment, peripheral blood eosinophil counts and FcεR I have been linked to response to Omalizumab, and followed the disease activity. Patients with a continuous downregulation of Fcε RI expression and eosinophil count are more likely to show a satisfactory response to Omalizumab (38, 99).

In the current case reports, the conclusions about Omalizumab and Rituximab for refractory BP are encouraging, but their efficacy and safety have not been tested in clinical trials, which is essential for eliminating bias and determining their options in the treatment of BP. Recently, an open-label, single group design, phase 3 clinical trial evaluating the efficacy and safety of Rituximab combined with Omalizumab in the treatment of BP is underway (NCT04128176).

Biologics Targeting Complements

Complement cascade reaction is involved in BP blister formation, biologics specifically blocking it may be beneficial to the treatment of BP.

BIVV009

BIVV009 (sutimlimab) is a humanized IgG4 monoclonal antibody, and specifically inhibits the first components subcomponent (C1s) of the complement system to interfere with the classical pathway of complement cascade. The safety and activity of BIVV009 for the treatment of BP have been tested in phase 1 clinical trial (NCT02502903) involving 10 active BP subjects. The results showed that 60 mg/kg infusion of BIVV009 four times a week was enough to inhibit the classical complement pathway as well as safe and tolerable in this elderly population. Only mild to moderate adverse events such as headache and fatigue were reported (103).

Given the success of phase I trials, FDA designated BIVV009 as an orphan drug of BP in August 2017, thus promoting future clinical development (75). A prospective, randomized, double-blind, placebo-controlled phase 1 clinical trial (NCT02502903) with a larger sample continues.

Avdoralimab

Avdoralimab is a specific antibody against C5aR1, which is safe for the treatment of solid tumors and rheumatoid arthritis. Karsten et al. have previously demonstrated that C5aR1 mediates the anti-BP180 IgG-induced pathogenicity, while C5aR2 has a protective effect (104). Therefore, more specific blocking of the C5a-C5aR1 axis by Avdoralimab is expected to be used in the treatment of BP. Therefore, 40 BP patients are expected to participate in an open-label, multicenter, randomized, parallel-group phase 2 clinical trial (NCT04563923).

Biologics Targeting Th2 Axis

Dupilumab, a monoclonal antibody against IL-4Rα, not only blocks the signal transduction mediated by IL-4 and IL-13, but also inhibits the secretion of IL-31 by eosinophils (101). It has been approved for the treatment of atopic dermatitis and chronic rhinosinusitis with moderate to severe asthma and nasal polyposis. In most patients with BP, the frequency of cells producing IL-4 and IL-13 in blood and lesions increases, and decreases as the disease improves. Dupilumab is also an effective inhibitor of Th2-related chemokines CCL17, CCL18, CCL22, and CCL26, which fluctuate synchronously with the disease severity. Therefore, Dupilumab could specifically block the pathogenic effects of these cytokines in BP (105). Recently, Dupilumab has been successfully used in refractory BP, especially for the relief of intractable itching (106).

In 2018, Dupilumab monotherapy successfully treated a patient with refractory BP for the first time (105). It also relieves that itching could not be controlled by Omalizumab in the treatment of BP (101). Abdat et al. reported the study with the largest sample. A total of 13 patients with refractory BP initially accepted the approved administration regimen for atopic dermatitis, that is, Dupilumab 600 mg was injected subcutaneously for the first time, and then 300 mg was injected subcutaneously every two weeks, but some patients could only achieve partial control of the disease, so the frequency of medication was mostly changed to once a week. Six of them received Dupilumab monotherapy, seven patients received a combination of glucocorticoid or methotrexate with Dupilumab for an average of 5 months. A total of 84.6% of the patients achieved remission of the disease, and there was no obvious adverse effect. Although 53.8% of the patients were cleared of the disease, 42.9% of them received Dupilumab more frequently than every two weeks. Wherein, the weekly frequency of treatment seems to be more conducive to remission (107).

Currently, a multicenter, randomized, double-blind, parallel-group, placebo-controlled clinical trial (NCT04206553) is in progress to evaluate the efficacy and safety of Dupilumab in adult patients with BP.

Biologics Targeting Eosinophils

As the main infiltrating cells of BP lesions and the main effector cells of blisters formation, eosinophils are involved in the pathogenesis of BP in many ways, and provide several biomarkers indicating the severity and refractory of BP. Targeting eosinophils is a candidate therapeutic strategy for BP.

Bertilimumab

Bertilimumab is a completely human monoclonal antibody targeting eotaxin-1 (CCL-11), while eotaxin-1 is mainly involved in the recruitment of eosinophils from the peripheral circulation to the skin lesion in BP. The use of Bertilimumab is designed to reduce eosinophil infiltration in BP lesions. An open, single-group, phase 2 clinical trial (NCT02226146) has been conducted to study the safety, efficacy, and pharmacodynamics of Bertilimumab in patients with newly diagnosed moderate to generalized bullous pemphigoid. The study included nine subjects with moderate to severe BP who were treated with Bertilimumab as a steroid protector for four weeks. Bertilimumab was well tolerated in all nine subjects. The BPDAI score decreased by 81%, and no serious drug-related adverse events were reported. Based on these results, FDA has granted an orphan drug designation to Bertilimumab in BP.

Benralizumab

Benralizumab is a humanized IgG1 κ monoclonal antibody against the IL-5R α subunit, which acts by blocking the downstream signaling of IL-5. It induces target cell killing mediated by NK cells through ADCC, which leads to the decrease of eosinophils and basophils in circulation. Considering the pathogenic role of IL-5 and eosinophils in BP, a multi-country, randomized, double-blind, parallel-group, placebo-controlled phase 3 clinical trial (NCT04612790) has just begun to investigate the effectiveness of Benralizumab for BP.

Biologics Targeting Th17 Axis

Th17 axis takes part in the blister formation and drug resistance in BP relapse. The patients who are prone to relapse may be the indications for biologics targeting it.

Ustekinumab

Ustekinumab is a humanized monoclonal antibody targeting the p40 subunit shared between IL-23 and IL-12. IL-23 often has a synergistic effect with IL-17 in BP relapse (108). To study the efficacy and safety of Ustekinumab combined with super-effective topical corticosteroids in the treatment of BP, an open-label, single-group design phase 2 clinical trial is in progress (NCT04117932).

Tildrakizumab

Tildrakizumab is an IL-23 inhibitor that specifically inhibits the p19 subunit of IL-23, thereby reducing its activity. It has been approved for the treatment of adult plaque psoriasis by the FDA (109). Recently, an open-label, single-group design phase 1 clinical trial (NCT04465292) has been conducted to evaluate the efficacy of Tildrakizumab for BP.

Obviously, biologics are promising therapeutic options for patients who are resistant to standard therapy. Rituximab, Omalizumab, and Dupilumab have been successfully used in clinic, and clinical trials of new biologics targeting different mechanisms have been gradually carried out. Furthermore, for the elderly with a high incidence of BP, it is prone to have contraindications of glucocorticoid and immunosuppressive, such as hypertension, arteriosclerosis, diabetes, osteoporosis, and cardiorenal insufficiency, and chronic recurrent course requires long-term and high-dose use of glucocorticoid, which increases the risk of adverse events. Although the local use of glucocorticoids is considered to be relatively safe, it is indeed difficult to implement for extensive lesions. For these patients, appropriate biologics should be available as a first-line therapy regimen by comprehensively evaluating the basic condition, so as to bring better prognosis for patients.

Markers for Prognosis

The mortality rate of BP patients one year after diagnosis is 9.3%–41% (110). Clinical features and biomarkers of patients that indicate the risk of death have been gradually discovered. Individual factors, such as advanced age, low Karnofsky score (≤40), comorbidity groups of the nervous system (including Parkinson's syndrome, dementia, and stroke), chronic basic diseases (such as heart failure and chronic kidney disease), and iatrogenic factors including long-term hospitalization and high-dose corticosteroids alone are associated with the increased risk of death. On the contrary, Statins and topical glucocorticoids or low-dose glucocorticoids combined with immunomodulators are protective factors associated with a reduced risk of death (9, 26, 110–116). Moreover, there are a few studies on biomarkers related to the risk of death. High levels of anti-BP180 autoantibodies (≥61 U/mL) at diagnosis have to do with an increased risk of death (26, 110). High erythrocyte sedimentation rate (ESR) and low serum albumin levels are also correlated with the risk of death in BP, but they could not be used as special biomarkers to predict the prognosis of BP, because they are easily influenced by other disorders (115). During treatment, more attention should be paid to the above signals indicating poor prognosis in order to better manage refractory BP.

CONCLUSION

Although there is increasing interest in finding reliable indicators for different aspects of BP, the level of evidence supporting existing biomarkers is still very low. We advocate researchers to conduct large-scale multicenter clinical trials as much as possible, and actively explore better detection methods to identify or confirm potential biomarkers. For refractory BP patients indicated by clinical manifestations or biomarkers and elderly patients with severe adverse reactions to high-dose glucocorticoids, biologics becomes the promising treatment, some of them have been of benefit to refractory BP patients. More randomized controlled trials are needed to determine their efficacy, safety, and availability for the treatment of patients with BP. All in all, monitoring and targeting these meaningful factors will play an important role in the management of refractory BP.

AUTHOR CONTRIBUTIONS

TZ, BP, and SG conceived this paper. TZ wrote this manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Joly P, Baricault S, Sparsa A, Bernard P, Bédane C, Duvert-Lehembre S, et al. Incidence and Mortality of Bullous Pemphigoid in France. *J Invest Dermatol* (2012) 132:1998–2004. doi: 10.1038/jid.2012.35
2. Zhao CY, Murrell DF. Outcome Measures for Autoimmune Blistering Diseases. *J Dermatol* (2015) 42:31–6. doi: 10.1111/1346-8138.12711
3. Messingham KN, Crowe TP, Fairley JA. The Intersection of IgE Autoantibodies and Eosinophilia in the Pathogenesis of Bullous Pemphigoid. *Front Immunol* (2019) 10:2331. doi: 10.3389/fimmu.2019.02331
4. Lo Schiavo A, Ruocco E, Brancaccio G, Caccavale S, Ruocco V, Wolf R. Bullous Pemphigoid: Etiology, Pathogenesis, and Inducing Factors: Facts and Controversies. *Clin Dermatol* (2013) 31:391–9. doi: 10.1016/j.clindermatol.2013.01.006
5. Liu Y, Li L, Xia Y. BP180 Is Critical in the Autoimmunity of Bullous Pemphigoid. *Front Immunol* (2017) 8:1752. doi: 10.3389/fimmu.2017.01752

6. Wang Y, Mao X, Wang Y, Zeng Y, Liu Y, Jin H, et al. Relapse of Bullous Pemphigoid: An Update on This Stubborn Clinical Problem. *Ann Med* (2018) 50:234–9. doi: 10.1080/07853890.2018.1443346
7. Binois R, Nadal M, Esteve E, De Muret A, Kerdraon R, Gheit T, et al. Cutaneous Kaposi Sarcoma During Treatment With Superpotent Topical Steroids and Methotrexate for Bullous Pemphigoid: Three Cases. *Eur J Dermatol* (2017) 27:369–74. doi: 10.1684/ejd.2017.3027
8. Gual A, Iranzo P, Mascaró JM Jr. Treatment of Bullous Pemphigoid With Low-Dose Oral Cyclophosphamide: A Case Series of 20 Patients. *J Eur Acad Dermatol Venereol* (2014) 28:814–8. doi: 10.1111/jdv.12155
9. Kalinska-Bienias A, Lukowska-Smorawska K, Jagielski P, Kowalewski C, Wozniak K. Mortality in Bullous Pemphigoid and Prognostic Factors in 1st and 3rd Year of Follow-Up in Specialized Centre in Poland. *Arch Dermatol Res* (2017) 309:709–19. doi: 10.1007/s00403-017-1772-x
10. Daneshpazhooh M, Ghiasi M, Lajevardi V, Nasiri N, Balighi K, Teimourpour A, et al. BPDAI and ABSIS Correlate With Serum Anti-BP180 NC16A IgG But Not With Anti-BP230 IgG in Patients With Bullous Pemphigoid. *Arch Dermatol Res* (2018) 310:255–9. doi: 10.1007/s00403-018-1817-9
11. Döpp R, Schmidt E, Chimanovitch I, Leverkus M, Bröcker EB, Zillikens D. IgG4 and IgE Are the Major Immunoglobulins Targeting the NC16A Domain of BP180 in Bullous Pemphigoid: Serum Levels of These Immunoglobulins Reflect Disease Activity. *J Am Acad Dermatol* (2000) 42:577–83. doi: 10.1067/mjd.2000.103986
12. Feng S, Wu Q, Jin P, Lin L, Zhou W, Sang H, et al. Serum Levels of Autoantibodies to BP180 Correlate With Disease Activity in Patients With Bullous Pemphigoid. *Int J Dermatol* (2008) 47:225–8. doi: 10.1111/j.1365-4632.2008.03473.x
13. Kalowska M, Ciepiela O, Kowalewski C, Demkow U, Schwartz RA, Wozniak K. Enzyme-Linked Immunoassay Index for Anti-NC16a IgG and IgE Auto-Antibodies Correlates With Severity and Activity of Bullous Pemphigoid. *Acta Derm Venereol* (2016) 96:191–6. doi: 10.2340/00015555-2101
14. Pomponi D, Di Zenzo G, Zennaro D, Calabresi V, Eming R, Zuzzi S, et al. Detection of IgG and IgE Reactivity to BP180 Using the ISAC® Microarray System. *Br J Dermatol* (2013) 168:1205–14. doi: 10.1111/bjd.12161
15. Zhou XP, Liu B, Xu Q, Yang Y, He CX, Zuo YG, et al. Serum Levels of Immunoglobulins G1 and G4 Targeting the Non-Collagenous 16A Domain of BP180 Reflect Bullous Pemphigoid Activity and Predict Bad Prognosis. *J Dermatol* (2016) 43:141–8. doi: 10.1111/1346-8138.13051
16. Amo Y, Ohkawa T, Tatsuta M, Hamada Y, Fujimura T, Katsuoka K, et al. Clinical Significance of Enzyme-Linked Immunosorbent Assay for the Detection of Circulating Anti-BP180 Autoantibodies in Patients With Bullous Pemphigoid. *J Dermatol Sci* (2001) 26:14–8. doi: 10.1016/s0923-1811(00)00149-3
17. Kobayashi M, Amagai M, Kuroda-Kinoshita K, Hashimoto T, Shirakata Y, Hashimoto K, et al. BP180 ELISA Using Bacterial Recombinant NC16a Protein as a Diagnostic and Monitoring Tool for Bullous Pemphigoid. *J Dermatol Sci* (2002) 30:224–32. doi: 10.1016/s0923-1811(02)00109-3
18. Schmidt E, Obe K, Bröcker EB, Zillikens D. Serum Levels of Autoantibodies to BP180 Correlate With Disease Activity in Patients With Bullous Pemphigoid. *Arch Dermatol* (2000) 136:174–8. doi: 10.1001/archderm.136.2.174
19. Bernard P, Reguiai Z, Tancrède-Bohin E, Cordel N, Plantin P, Pauwels C, et al. Risk Factors for Relapse in Patients With Bullous Pemphigoid in Clinical Remission: A Multicenter, Prospective, Cohort Study. *Arch Dermatol* (2009) 145:537–42. doi: 10.1001/archdermatol.2009.53
20. Ingen-Housz-Oro S, Plée J, Belmondo T, Maizières M, Pham BN, Hüe S, et al. Positive Direct Immunofluorescence Is of Better Value Than ELISA-BP180 and ELISA-BP230 Values for the Prediction of Relapse After Treatment Cessation in Bullous Pemphigoid: A Retrospective Study of 97 Patients. *Dermatology* (2015) 231:50–5. doi: 10.1159/000381143
21. Fichel F, Barbe C, Joly P, Bedane C, Vabres P, Truchetet F, et al. Clinical and Immunologic Factors Associated With Bullous Pemphigoid Relapse During the First Year of Treatment: A Multicenter, Prospective Study. *JAMA Dermatol* (2014) 150:25–33. doi: 10.1001/jamadermatol.2013.5757
22. Koga H, Teye K, Ishii N, Ohata C, Nakama T. High Index Values of Enzyme-Linked Immunosorbent Assay for BP180 at Baseline Predict Relapse in Patients With Bullous Pemphigoid. *Front Med (Lausanne)* (2018) 5:139. doi: 10.3389/fmed.2018.00139
23. Zheng M, Ujiie H, Iwata H, Muramatsu K, Yoshimoto N, Ito T, et al. Characteristics of IgG Subclasses and Complement Deposition in BP230-Type Bullous Pemphigoid. *J Eur Acad Dermatol Venereol* (2019) 33:595–600. doi: 10.1111/jdv.15325
24. Modre B, Allen J, Wojnarowska F. Does Class Switching Contribute to Remission in Bullous Pemphigoid? *Acta Derm Venereol* (1999) 79:127–31. doi: 10.1080/000155599750011345
25. Hashimoto T, Ohzono A, Teye K, Numata S, Hiroyasu S, Tsuruta D, et al. Detection of IgE Autoantibodies to BP180 and BP230 and Their Relationship to Clinical Features in Bullous Pemphigoid. *Br J Dermatol* (2017) 177:141–51. doi: 10.1111/bjd.15114
26. Holtsche MM, Goletz S, van Beek N, Zillikens D, Benoit S, Harman K, et al. Prospective Study in Bullous Pemphigoid: Association of High Serum Anti-BP180 IgG Levels With Increased Mortality and Reduced Karnofsky Score. *Br J Dermatol* (2018) 179:918–24. doi: 10.1111/bjd.16553
27. Kamata A, Kurihara Y, Funakoshi T, Takahashi H, Kuroda K, Hachiya T, et al. Basement Membrane Zone IgE Deposition Is Associated With Bullous Pemphigoid Disease Severity and Treatment Results. *Br J Dermatol* (2020) 182:1221–7. doi: 10.1111/bjd.18364
28. Ma L, Wang M, Wang X, Chen X, Zhu X. Circulating IgE Anti-BP180 Autoantibody and Its Correlation to Clinical and Laboratorial Aspects in Bullous Pemphigoid Patients. *J Dermatol Sci* (2015) 78:76–7. doi: 10.1016/j.jdermsci.2015.02.015
29. Cho YT, Liao SL, Wang LF, Chu CY. High Serum Anti-BP180 IgE Levels Correlate to Prominent Urticarial Lesions in Patients With Bullous Pemphigoid. *J Dermatol Sci* (2016) 83:78–80. doi: 10.1016/j.jdermsci.2016.03.009
30. Delli FS, Sotiriou E, Lazaridou E, Apalla Z, Lallas A, Vakirlis E, et al. Total IgE, Eosinophils, and Interleukins 16, 17A, and 23 Correlations in Severe Bullous Pemphigoid and Treatment Implications. *Dermatol Ther* (2020) 33:e13958. doi: 10.1111/dth.13958
31. Iwata Y, Komura K, Kodera M, Usuda T, Yokoyama Y, Hara T, et al. Correlation of IgE Autoantibody to BP180 With a Severe Form of Bullous Pemphigoid. *Arch Dermatol* (2008) 144:41–8. doi: 10.1001/archdermatol.2007.9
32. van Beek N, Lüttmann N, Huebner F, Recke A, Karl I, Schulze FS, et al. Correlation of Serum Levels of IgE Autoantibodies Against BP180 With Bullous Pemphigoid Disease Activity. *JAMA Dermatol* (2017) 153:30–8. doi: 10.1001/jamadermatol.2016.3357
33. Furukawa F, Kumagai S, Sakamoto Y, Takigawa M, Imamura S. Elevated Serum Levels of IgE-Binding Factor/Soluble CD23 in Bullous Pemphigoid. *J Dermatol Sci* (1994) 7:150–4. doi: 10.1016/0923-1811(94)90089-2
34. Maekawa N, Hosokawa H, Soh H, Kasahara M, Izumi H, Yodoi J, et al. Serum Levels of Soluble CD23 in Patients With Bullous Pemphigoid. *J Dermatol* (1995) 22:310–5. doi: 10.1111/j.1346-8138.1995.tb03394.x
35. Inaoki M, Sato S, Takehara K. Elevated Expression of CD23 on Peripheral Blood B Lymphocytes From Patients With Bullous Pemphigoid: Correlation With Increased Serum IgE. *J Dermatol Sci* (2004) 35:53–9. doi: 10.1016/j.jdermsci.2004.03.009
36. James T, Salman S, Stevenson B, Bundell C, Kelly G, Nolan D, et al. IgE Blockade in Autoimmunity: Omalizumab Induced Remission of Bullous Pemphigoid. *Clin Immunol* (2019) 198:54–6. doi: 10.1016/j.clim.2018.12.015
37. Kwon HJ, Ahn GR, Choi SY, Li K, Seo SJ. Explosive Bullous Pemphigoid With High Serum Total IgE: Serum IgE as a Biomarker That Reflects Disease Activity. *JAAD Case Rep* (2018) 4:352–4. doi: 10.1016/j.jdcr.2017.09.011
38. Yu KK, Crew AB, Messingham KA, Fairley JA, Woodley DT. Omalizumab Therapy for Bullous Pemphigoid. *J Am Acad Dermatol* (2014) 71:468–74. doi: 10.1016/j.jaad.2014.04.053
39. Genovese G, Di Zenzo G, Cozzani E, Berti E, Cugno M, Marzano AV. New Insights Into the Pathogenesis of Bullous Pemphigoid: 2019 Update. *Front Immunol* (2019) 10:1506. doi: 10.3389/fimmu.2019.01506
40. Ameglio F, D'Auria L, Bonifati C, Ferraro C, Mastroianni A, Giacalone B. Cytokine Pattern in Blister Fluid and Serum of Patients With Bullous

Pemphigoid: Relationships With Disease Intensity. *Br J Dermatol* (1998) 138:611–4. doi: 10.1046/j.1365-2133.1998.02169.x
41. Inaoki M, Takehara K. Increased Serum Levels of Interleukin (IL)-5, IL-6 and IL-8 in Bullous Pemphigoid. *J Dermatol Sci* (1998) 16:152–7. doi: 10.1016/s0923-1811(97)00044-3
42. Margaroli C, Bradley B, Thompson C, Brown MR, Giacalone VD, Bhatt L, et al. Distinct Compartmentalization of Immune Cells and Mediators Characterizes Bullous Pemphigoid Disease. *Exp Dermatol* (2020) 29:1191–8. doi: 10.1111/exd.14209
43. Suzuki M, Yamaguchi Y, Nakamura K, Kanaoka M, Matsukura S, Takahashi K, et al. Serum Thymus and Activation-Regulated Chemokine (TARC/CCL17) May Be Useful to Predict the Disease Activity in Patients With Bullous Pemphigoid. *J Eur Acad Dermatol Venereol* (2021) 35:e121–4. doi: 10.1111/jdv.16851
44. Nakashima H, Fujimoto M, Asashima N, Watanabe R, Kuwano Y, Yazawa N, et al. Serum Chemokine Profile in Patients With Bullous Pemphigoid. *Br J Dermatol* (2007) 156:454–9. doi: 10.1111/j.1365-2133.2006.07601.x
45. Kakinuma T, Wakugawa M, Nakamura K, Hino H, Matsushima K, Tamaki K. High Level of Thymus and Activation-Regulated Chemokine in Blister Fluid and Sera of Patients With Bullous Pemphigoid. *Br J Dermatol* (2003) 148:203–10. doi: 10.1046/j.1365-2133.2003.05066.x
46. Günther C, Carballido-Perrig N, Kopp T, Carballido JM, Pfeiffer C. CCL18 Is Expressed in Patients With Bullous Pemphigoid and Parallels Disease Course. *Br J Dermatol* (2009) 160:747–55. doi: 10.1111/j.1365-2133.2008.08979.x
47. Echigo T, Hasegawa M, Shimada Y, Inaoki M, Takehara K, Sato S. Both Th1 and Th2 Chemokines are Elevated in Sera of Patients With Autoimmune Blistering Diseases. *Arch Dermatol Res* (2006) 298:38–45. doi: 10.1007/s00403-006-0661-5
48. Nin-Asai R, Muro Y, Sekiya A, Sugiura K, Akiyama M. Serum Thymus and Activation-Regulated Chemokine (TARC/CCL17) Levels Reflect the Disease Activity in a Patient With Bullous Pemphigoid. *J Eur Acad Dermatol Venereol* (2016) 30:327–8. doi: 10.1111/jdv.12719
49. Riani M, Le Jan S, Plée J, Durlach A, Le Naour R, Haegeman G, et al. Bullous Pemphigoid Outcome Is Associated With CXCL10-Induced Matrix Metalloproteinase 9 Secretion From Monocytes and Neutrophils But Not Lymphocytes. *J Allergy Clin Immunol* (2017) 139:863–72.e3. doi: 10.1016/j.jaci.2016.08.012
50. Engineer L, Bhol K, Kumari S, Razzaque Ahmed A. Bullous Pemphigoid: Interaction of Interleukin 5, Anti-Basement Membrane Zone Antibodies and Eosinophils. A Preliminary Observation. *Cytokine* (2001) 13:32–8. doi: 10.1006/cyto.2000.0791
51. D'Auria L, Pietravalle M, Mastroianni A, Ferraro C, Mussi A, Bonifati C, et al. IL-5 Levels in the Serum and Blister Fluid of Patients With Bullous Pemphigoid: Correlations With Eosinophil Cationic Protein, RANTES, IgE and Disease Severity. *Arch Dermatol Res* (1998) 290:25–7. doi: 10.1007/s004030050272
52. Kagami S, Kai H, Kakinuma T, Miyagaki T, Kamata M, Sugaya M, et al. High Levels of CCL26 in Blister Fluid and Sera of Patients With Bullous Pemphigoid. *J Invest Dermatol* (2012) 132:249–51. doi: 10.1038/jid.2011.251
53. Bernard P, Venot J, Constant F, Bonnetblanc JM. Blood Eosinophilia as a Severity Marker for Bullous Pemphigoid. *J Am Acad Dermatol* (1987) 16:879–81. doi: 10.1016/s0190-9622(87)80227-x
54. Farnaghi F, Ehsani AH, Kamyab-Hesary K, Abbasian S, Seirafi H, Nasimi M. Correlation of Dermal and Blood Eosinophilia With Bullous Pemphigoid Disease Severity. *Int J Womens Dermatol* (2020) 6:171–5. doi: 10.1016/j.ijwd.2020.01.005
55. Kridin K. Peripheral Eosinophilia in Bullous Pemphigoid: Prevalence and Influence on the Clinical Manifestation. *Br J Dermatol* (2018) 179:1141–7. doi: 10.1111/bjd.16679
56. Messingham KN, Holahan HM, Frydman AS, Fullenkamp C, Srikantha R, Fairley JA. Human Eosinophils Express the High Affinity IgE Receptor, FcεRI, in Bullous Pemphigoid. *PloS One* (2014) 9:e107725. doi: 10.1371/journal.pone.0107725
57. Marzano AV, Tedeschi A, Fanoni D, Bonanni E, Venegoni L, Berti E, et al. Activation of Blood Coagulation in Bullous Pemphigoid: Role of Eosinophils, and Local and Systemic Implications. *Br J Dermatol* (2009) 160:266–72. doi: 10.1111/j.1365-2133.2008.08880.x
58. Tedeschi A, Marzano AV, Lorini M, Balice Y, Cugno M. Eosinophil Cationic Protein Levels Parallel Coagulation Activation in the Blister Fluid of Patients With Bullous Pemphigoid. *J Eur Acad Dermatol Venereol* (2015) 29:813–7. doi: 10.1111/jdv.12464
59. D'Auria L, Mussi A, Bonifati C, Mastroianni A, Giacalone B, Ameglio F. Increased Serum IL-6, TNF-Alpha and IL-10 Levels in Patients With Bullous Pemphigoid: Relationships With Disease Activity. *J Eur Acad Dermatol Venereol* (1999) 12:11–5. doi: 10.1111/j.1468-3083.1999.tb00801.x
60. Teraki Y, Hotta T, Shiohara T. Skin-Homing Interleukin-4 and -13-Producing Cells Contribute to Bullous Pemphigoid: Remission of Disease Is Associated With Increased Frequency of Interleukin-10-Producing Cells. *J Invest Dermatol* (2001) 117:1097–102. doi: 10.1046/j.0022-202x.2001.01505.x
61. Riani M, Muller C, Bour C, Bernard P, Antonicelli F, Le Jan S. Blister Fluid Induces MMP-9-Associated M2-Type Macrophages in Bullous Pemphigoid. *Front Immunol* (2019) 10:1858. doi: 10.3389/fimmu.2019.01858
62. Antiga E, Quaglino P, Volpi W, Pierini I, Del Bianco E, Bianchi B, et al. Regulatory T Cells in Skin Lesions and Blood of Patients With Bullous Pemphigoid. *J Eur Acad Dermatol Venereol* (2014) 28:222–30. doi: 10.1111/jdv.12091
63. Asashima N, Fujimoto M, Watanabe R, Nakashima H, Yazawa N, Okochi H, et al. Serum Levels of BAFF Are Increased in Bullous Pemphigoid But Not in Pemphigus Vulgaris. *Br J Dermatol* (2006) 155:330–6. doi: 10.1111/j.1365-2133.2006.07305.x
64. Watanabe R, Ishiura N, Nakashima H, Yazawa N, Kuwano Y, Tada Y, et al. Increased Serum Levels of Circulating CD40 Ligand in Patients With Bullous Pemphigoid: Preliminary Results. *Dermatology* (2007) 215:180–6. doi: 10.1159/000106573
65. Watanabe R, Fujimoto M, Yazawa N, Nakashima H, Asashima N, Kuwano Y, et al. Increased Serum Levels of a Proliferation-Inducing Ligand in Patients With Bullous Pemphigoid. *J Dermatol Sci* (2007) 46:53–60. doi: 10.1016/j.jdermsci.2006.12.008
66. Fang H, Shao S, Cao T, Lei J, Dang E, Zhang J, et al. Increased Expression of NLRP3 Inflammasome Components and Interleukin-18 in Patients With Bullous Pemphigoid. *J Dermatol Sci* (2016) 83:116–23. doi: 10.1016/j.jdermsci.2016.04.009
67. Li Q, Liu Z, Dang E, Jin L, He Z, Yang L, et al. Follicular Helper T Cells (Tfh) and IL-21 Involvement in the Pathogenesis of Bullous Pemphigoid. *PloS One* (2013) 8:e68145. doi: 10.1371/journal.pone.0068145
68. D'Auria L, Bonifati C, Cordiali-Fei P, Leone G, Picardo M, Pietravalle M, et al. Increased Serum Interleukin-15 Levels in Bullous Skin Diseases: Correlation With Disease Intensity. *Arch Dermatol Res* (1999) 291:354–6. doi: 10.1007/s004030050421
69. Plée J, Le Jan S, Giustiniani J, Barbe C, Joly P, Bedane C, et al. Integrating Longitudinal Serum IL-17 and IL-23 Follow-Up, Along With Autoantibodies Variation, Contributes to Predict Bullous Pemphigoid Outcome. *Sci Rep* (2015) 5:18001. doi: 10.1038/srep18001
70. Brulefert A, Le Jan S, Plée J, Durlach A, Bernard P, Antonicelli F, et al. Variation of the Epidermal Expression of Glucocorticoid Receptor-Beta as Potential Predictive Marker of Bullous Pemphigoid Outcome. *Exp Dermatol* (2017) 26:1261–6. doi: 10.1111/exd.13444
71. Czech W, Schaller J, Schöpf E, Kapp A. Granulocyte Activation in Bullous Diseases: Release of Granular Proteins in Bullous Pemphigoid and Pemphigus Vulgaris. *J Am Acad Dermatol* (1993) 29:210–5. doi: 10.1016/0190-9622(93)70170-x
72. Giusti D, Gatouillat G, Le Jan S, Plée J, Bernard P, Antonicelli F, et al. Eosinophil Cationic Protein (ECP), A Predictive Marker of Bullous Pemphigoid Severity and Outcome. *Sci Rep* (2017) 7:4833. doi: 10.1038/s41598-017-04687-5
73. Marzano AV, Tedeschi A, Berti E, Fanoni D, Crosti C, Cugno M. Activation of Coagulation in Bullous Pemphigoid and Other Eosinophil-Related Inflammatory Skin Diseases. *Clin Exp Immunol* (2011) 165:44–50. doi: 10.1111/j.1365-2249.2011.04391.x
74. Deza G, Ricketti PA, Giménez-Arnau AM, Casale TB. Emerging Biomarkers and Therapeutic Pipelines for Chronic Spontaneous Urticaria. *J Allergy Clin Immunol Pract* (2018) 6:1108–17. doi: 10.1016/j.jaip.2018.02.024

75. Kushner CJ, Payne AS. Increasing the Complement of Therapeutic Options in Bullous Pemphigoid. *J Invest Dermatol* (2018) 138:246–8. doi: 10.1016/j.jid.2017.09.026
76. Chiorean RM, Baican A, Mustafa MB, Lischka A, Leucuta DC, Feldrihan V, et al. Complement-Activating Capacity of Autoantibodies Correlates With Disease Activity in Bullous Pemphigoid Patients. *Front Immunol* (2018) 9:2687. doi: 10.3389/fimmu.2018.02687
77. Fang H, Zhang Y, Li N, Wang G, Liu Z. The Autoimmune Skin Disease Bullous Pemphigoid: The Role of Mast Cells in Autoantibody-Induced Tissue Injury. *Front Immunol* (2018) 9:407. doi: 10.3389/fimmu.2018.00407
78. Bieber K, Ernst AL, Tukaj S, Holtsche MM, Schmidt E, Zillikens D, et al. Analysis of Serum Markers of Cellular Immune Activation in Patients With Bullous Pemphigoid. *Exp Dermatol* (2017) 26:1248–52. doi: 10.1111/exd.13382
79. D'Auria L, Pietravalle M, Cordiali-Fei P, Ameglio F. Increased Tryptase and Myeloperoxidase Levels in Blister Fluids of Patients With Bullous Pemphigoid: Correlations With Cytokines, Adhesion Molecules and Anti-Basement Membrane Zone Antibodies. *Exp Dermatol* (2000) 9:131–7. doi: 10.1034/j.1600-0625.2000.009002131.x
80. Qiu L, Zhang L, Qi R, Gao X, Chen H, Xiao T. miR-1291 Functions as a Potential Serum Biomarker for Bullous Pemphigoid. *Dis Markers* (2020) 2020:9505312. doi: 10.1155/2020/9505312
81. Amber KT, Maglie R, Solimani F, Eming R, Hertl M. Targeted Therapies for Autoimmune Bullous Diseases: Current Status. *Drugs* (2018) 78:1527–48. doi: 10.1007/s40265-018-0976-5
82. Murrell DF, Peña S, Joly P, Marinovic B, Hashimoto T, Diaz LA, et al. Diagnosis and Management of Pemphigus: Recommendations of an International Panel of Experts. *J Am Acad Dermatol* (2020) 82:575–85.e1. doi: 10.1016/j.jaad.2018.02.021
83. Cho YT, Chu CY, Wang LF. First-Line Combination Therapy With Rituximab and Corticosteroids Provides a High Complete Remission Rate in Moderate-to-Severe Bullous Pemphigoid. *Br J Dermatol* (2015) 173:302–4. doi: 10.1111/bjd.13633
84. Chee R, Nagendran V, Bansal A, Casie Chetty SN, Harland C. B-Cell Targeted Therapy Alone May Not Be Effective in Bullous Pemphigoid. *Clin Exp Dermatol* (2007) 32:111–2. doi: 10.1111/j.1365-2230.2006.02267.x
85. Hall RP 3rd, Streilein RD, Hannah DL, McNair PD, Fairley JA, Ronaghy A, et al. Association of Serum B-Cell Activating Factor Level and Proportion of Memory and Transitional B Cells With Clinical Response After Rituximab Treatment of Bullous Pemphigoid Patients. *J Invest Dermatol* (2013) 133:2786–8. doi: 10.1038/jid.2013.236
86. Schmidt E, Seitz CS, Benoit S, Bröcker EB, Goebeler M. Rituximab in Autoimmune Bullous Diseases: Mixed Responses and Adverse Effects. *Br J Dermatol* (2007) 156:352–6. doi: 10.1111/j.1365-2133.2006.07646.x
87. Adler BL, Crew AB, Woodley DT. Early-Onset Neutropenia After Rituximab Therapy for Bullous Pemphigoid. *Clin Exp Dermatol* (2019) 44:334–6. doi: 10.1111/ced.13726
88. Schmidt E, Hunzelmann N, Zillikens D, Bröcker EB, Goebeler M. Rituximab in Refractory Autoimmune Bullous Diseases. *Clin Exp Dermatol* (2006) 31:503–8. doi: 10.1111/j.1365-2230.2006.02151.x
89. Ahmed AR, Shetty S, Kaveri S, Spigelman ZS. Treatment of Recalcitrant Bullous Pemphigoid (BP) With a Novel Protocol: A Retrospective Study With a 6-Year Follow-Up. *J Am Acad Dermatol* (2016) 74:700–8.e3. doi: 10.1016/j.jaad.2015.11.030
90. Fairley JA, Baum CL, Brandt DS, Messingham KA. Pathogenicity of IgE in Autoimmunity: Successful Treatment of Bullous Pemphigoid With Omalizumab. *J Allergy Clin Immunol* (2009) 123:704–5. doi: 10.1016/j.jaci.2008.11.035
91. Maglie R, Antiga E, Quintarelli L, Verdelli A, Caproni M. Dramatic Exacerbation of Bullous Pemphigoid Following Rituximab and Successful Treatment With Omalizumab. *Eur J Dermatol* (2019) 29:213–5. doi: 10.1684/ejd.2019.3499
92. Lonowski S, Sachsman S, Patel N, Truong A, Holland V. Increasing Evidence for Omalizumab in the Treatment of Bullous Pemphigoid. *JAAD Case Rep* (2020) 6:228–33. doi: 10.1016/j.jdcr.2020.01.002
93. Vico-Alonso C, Calleja-Algarra A, Aragón-Miguel R, Sánchez-Velázquez A, Velasco-Tamariz V, Ortiz-Romero PL, et al. Omalizumab as an Alternative Therapeutic Tool in the Treatment of Bullous Pemphigoid: A Case Report. *Dermatol Ther* (2019) 32:e12829. doi: 10.1111/dth.12829
94. Menzinger S, Kaya G, Schmidt E, Fontao L, Laffitte E. Biological and Clinical Response to Omalizumab in a Patient With Bullous Pemphigoid. *Acta Derm Venereol* (2018) 98:284–6. doi: 10.2340/00015555-2845
95. Balakirski G, Alkhateeb A, Merk HF, Leverkus M, Megahed M. Successful Treatment of Bullous Pemphigoid With Omalizumab as Corticosteroid-Sparing Agent: Report of Two Cases and Review of Literature. *J Eur Acad Dermatol Venereol* (2016) 30:1778–82. doi: 10.1111/jdv.13758
96. London VA, Kim GH, Fairley JA, Woodley DT. Successful Treatment of Bullous Pemphigoid With Omalizumab. *Arch Dermatol* (2012) 148:1241–3. doi: 10.1001/archdermatol.2012.1604
97. Navarro-Triviño FJ, Llamas-Molina JM, Ayen-Rodriguez A, Cancela-Díez B, Ruiz-Villaverde R. Dramatic Improvement of Bullous Pemphigoid With Omalizumab in an Elderly Patient. *Eur J Hosp Pharm* (2020) 0:1–3. doi: 10.1136/ejhpharm-2020-002418
98. Sinha S, Agrawal D, Sardana K, Kulhari A, Malhotra P. Complete Remission in a Patient With Treatment Refractory Bullous Pemphigoid After a Single Dose of Omalizumab. *Indian Dermatol Online J* (2020) 11:607–11. doi: 10.4103/idoj.IDOJ_438_19
99. Seyed Jafari SM, Gadaldi K, Feldmeyer L, Yawalkar N, Borradori L, Schlapbach C. Effects of Omalizumab on FcεRI and IgE Expression in Lesional Skin of Bullous Pemphigoid. *Front Immunol* (2019) 10:1919. doi: 10.3389/fimmu.2019.01919
100. Garrido PM, Alexandre MI, Travassos AR, Filipe P. Dipeptidyl-Peptidase IV Inhibitor-Associated Bullous Pemphigoid Efficiently Treated With Omalizumab. *Dermatol Ther* (2020) 33:e14160. doi: 10.1111/dth.14160
101. Seyed Jafari SM, Feldmeyer L, Bossart S, Simon D, Schlapbach C, Borradori L. Case Report: Combination of Omalizumab and Dupilumab for Recalcitrant Bullous Pemphigoid. *Front Immunol* (2020) 11:611549. doi: 10.3389/fimmu.2020.611549
102. Sarrazin M, Jouen F, Duvert-Lehembre S. Refractory Bullous Pemphigoid With IgE Anti-BP230 and IgG Anti-P200 Antibodies Successfully Treated With Omalizumab. *Ann Dermatol Venereol* (2021) 148:60–2. doi: 10.1016/j.annder.2020.08.053
103. Freire PC, Muñoz CH, Derhaschnig U, Schoergenhofer C, Firbas C, Parry GC, et al. Specific Inhibition of the Classical Complement Pathway Prevents C3 Deposition along the Dermal-Epidermal Junction in Bullous Pemphigoid. *J Invest Dermatol* (2019) 139:2417–24.e2. doi: 10.1016/j.jid.2019.04.025
104. Karsten CM, Beckmann T, Holtsche MM, Tillmann J, Tofern S, Schulze FS, et al. Tissue Destruction in Bullous Pemphigoid Can Be Complement Independent and May Be Mitigated by C5aR2. *Front Immunol* (2018) 9:488. doi: 10.3389/fimmu.2018.00488
105. Kaye A, Gordon SC, Deverapalli SC, Her MJ, Rosmarin D. Dupilumab for the Treatment of Recalcitrant Bullous Pemphigoid. *JAMA Dermatol* (2018) 154:1225–6. doi: 10.1001/jamadermatol.2018.2526
106. Seidman JS, Eichenfield DZ, Orme CM. Dupilumab for Bullous Pemphigoid With Intractable Pruritus. *Dermatol Online J* (2019) 25(11):130130/qt25q9w6r9. doi: 10.5070/D32511046147
107. Abdat R, Waldman RA, de Bedout V, Czernik A, McLeod M, King B, et al. Dupilumab as a Novel Therapy for Bullous Pemphigoid: A Multicenter Case Series. *J Am Acad Dermatol* (2020) 83:46–52. doi: 10.1016/j.jaad.2020.01.089
108. Erichsen CY, Jensen P, Kofoed K. Biologic Therapies Targeting the Interleukin (IL)-23/IL-17 Immune Axis for the Treatment of Moderate-to-Severe Plaque Psoriasis: A Systematic Review and Meta-Analysis. *J Eur Acad Dermatol Venereol* (2020) 34:30–8. doi: 10.1111/jdv.15879
109. Armstrong AW, Read C. Pathophysiology, Clinical Presentation, and Treatment of Psoriasis: A Review. *Jama* (2020) 323:1945–60. doi: 10.1001/jama.2020.4006
110. Monshi B, Gulz L, Piringer B, Wiala A, Kivaranovic D, Schmidt M, et al. Anti-BP180 Autoantibody Levels at Diagnosis Correlate With 1-Year Mortality Rates in Patients With Bullous Pemphigoid. *J Eur Acad Dermatol Venereol* (2020) 34:1583–9. doi: 10.1111/jdv.16363
111. Joly P, Roujeau JC, Benichou J, Picard C, Dreno B, Delaporte E, et al. A Comparison of Oral and Topical Corticosteroids in Patients With Bullous Pemphigoid. *N Engl J Med* (2002) 346:321–7. doi: 10.1056/NEJMoa011592

112. Roujeau JC, Lok C, Bastuji-Garin S, Mhalla S, Enginger V, Bernard P. High Risk of Death in Elderly Patients With Extensive Bullous Pemphigoid. *Arch Dermatol* (1998) 134:465–9. doi: 10.1001/archderm.134.4.465
113. Joly P, Benichou J, Lok C, Hellot MF, Saiag P, Tancrede-Bohin E, et al. Prediction of Survival for Patients With Bullous Pemphigoid: A Prospective Study. *Arch Dermatol* (2005) 141:691–8. doi: 10.1001/archderm.141.6.691
114. Rozenblat M, Halaj A, Rozenblat T, Fisher S, Sah M, Dodiuk-Gad RP, et al. Mortality and Risk Factors Among Israeli Bullous Pemphigoid Patients. *Arch Dermatol Res* (2019) 311:19–27. doi: 10.1007/s00403-018-1875-z
115. Liu YD, Wang YH, Ye YC, Zhao WL, Li L. Prognostic Factors for Mortality in Patients With Bullous Pemphigoid: A Meta-Analysis. *Arch Dermatol Res* (2017) 309:335–47. doi: 10.1007/s00403-017-1736-1
116. Cai SC, Allen JC, Lim YL, Chua SH, Tan SH, Tang MB. Mortality of Bullous Pemphigoid in Singapore: Risk Factors and Causes of Death in 359 Patients Seen at the National Skin Centre. *Br J Dermatol* (2014) 170:1319–26. doi: 10.1111/bjd.12806

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REVIEW
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Immunomodulatory Effect of MSCs and MSCs-Derived Extracellular Vesicles in Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is a common autoimmune connective tissue disease with unclear etiology and pathogenesis. Mesenchymal stem cell (MSC) and MSC derived extracellular vesicles (EVs) play important roles in regulating innate and adaptive immunity, which are involved in many physiological and pathological processes and contribute to the immune homeostasis in SLE. The effects of MSCs and EVs on SLE have been drawing more and more attention during the past few years. This article reviews the immunomodulatory effects and underlying mechanisms of MSC/MSC-EVs in SLE, which provides novel insight into understanding SLE pathogenesis and guiding the biological therapy.

Keywords: mesenchymal stem cells, extracellular vesicles, exosome, systemic lupus erythematosus, immunity

INTRODUCTION

SLE is a systemic autoimmune disease with multiple organs and multiple systems damages. It is characterized by abnormal activation of immune cells, abundant production of pathogenic autoantibodies and immune complexes deposition (1). The etiology and pathogenesis of SLE are complex and accompanied by immune disorders including abnormal proliferation, differentiation, activation and dysfunction of T cells, mononuclear-macrophage cells as well as B cells. Long term autoimmune disorders and sustained inflammation eventually cause tissue and organ damages (2, 3). Lupus nephritis is the most common and severe organ injury in SLE (4). At present, glucocorticoids and immunosuppressants are traditional treatments for SLE. However, there are still many refractory patients who are difficult to achieve clinical remission with high mortality. It brings a great economic burden and psychological pressure to SLE patients (5). As a result, investigating the optimal treatment strategy for SLE patients is still an urgent problem to be solved. Here, we provide an updated review of currently available information regarding dysregulated immune cells and mechanical molecules involved in SLE pathogenesis, which would be promising for investigating new biological approaches to SLE treatment.

Mesenchymal stem cells (MSCs) are pluripotent stem cells that are widely distributed in human body, such as bone marrow (BM) (6), umbilical cord (UC) (7), umbilical cord blood (UCB) (8), peripheral blood (9), placenta (PL) (10), adipose tissues (AT) (11), and dental pulp (DP) (12). It has been demonstrated that MSCs derived EVs possess strong biological activity similar to MSCs (13). MSCs-EVs exert immunomodulatory effects by inducing immune cells differentiation into cells with more anti-inflammatory or tolerant phenotype (14). MSC-EVs promote the chemotaxis of anti-inflammatory noncoding RNAs towards injured tissues and participate in regulating inflammatory and immune response to better enhance the healing process (15). Bone marrow derived mesenchymal stem cells (BM-MSCs) are found to suppress the proliferation of cancer cells and induced dormant states (16). MSCs have self-renewal, migration and immunosuppression functions under physiological conditions. However, some MSCs are dysregulated in immune microenvironment under pathological conditions. However, tumor-derived exosomes have been demonstrated to induce phenotypic and functional changes of MSCs, which can convert into cancer-associated cells (17). MSCs senescence can be found in SLE patients or lupus mice models. Changes of MSCs morphology and microstructure occur at the early stage in SLE patients with impaired immunosuppressive effects (18). Sun et al. have found that MSCs from SLE patients exhibit structural and functional defects, including slow growth, earlier aging and decreased vitality (18). In addition, a previous study has suggested abnormalities of multiple signaling pathways are involved in regulating actin cytoskeleton and cell cycling in BM-MSCs from SLE patients, such as MAPK and BMP/TGF-β signaling pathways (19). SLE patients exist defective immune regulation of BM-MSCs from SLE patients, characteristic of down-regulated microRNA let-7f (20). The study by Che N et al. has shown that BM-MSCs from lupus-like mice and SLE patients are deficient in suppressing B cell proliferation and differentiation (21). A previous study has shown that autologous MSCs from lupus patients are not effective in treating disease. However, whether it is possible to inhibit B cell proliferation seems to distinguish between effective MSCs and ineffective MSCs (22).

MSCs have immunomodulatory effects mainly through intercellular contact and paracrine pathways. MSC-EV is the main way for them to exert paracrine effects (23). MSC-EVs have stable membrane-like structure with a phospholipid bilayer. EVs contain substantial bioactive factors, such as nucleic acids, proteins and lipids. Some EVs delivering DNAs, mRNAs, circRNAs and lncRNAs have been demonstrated to play critical roles in regulating autoimmunity. MSC-EVs transfer bioactive molecules into the recipient cells mainly through three ways, namely, endocytosis, membrane fusion and specific receptor-ligand recognition. MSC-EVs can promote the transformation of inflammatory cell phenotypes (like M1 macrophages, DCs, Th1 and Th17 cells) into immunosuppressive phenotypes (like M2 macrophages, tolerance DCs and regulatory T cells) (24, 25). Accumulating studies have implicated that EVs derived non-coding RNAs play an important role in the pathogenesis of inflammatory and autoimmune diseases (24, 25). MSC-EVs have similar biological effects to MSCs, which are promising tools for tissue engineering and regenerative medicine (15, 26). MSC-EVs delivering molecules might not only be used as diagnostic biomarkers but therapeutic targets. Up to date, little has been known about the precise role of MSC-EVs derived bioactive molecules in SLE diseases.

MSC-EVs are important carriers for material transport and information exchange between MSC and other cells (27, 28). Exosomes are the most common type of EVs. They regulate T cells-, B cells- and other immune cells-mediated immune and inflammatory reactions (29, 30). MSC exosomes (MSC-Exo) encapsulating lipids, DNAs, RNAs and proteins are essential for intercellular communications (31). The role of MSC-Exo depends on its transferring bioactive molecules, such as proteins and RNAs (32). As a nano-scale natural carrier, exosomes can encapsulate and deliver bioactive substances produced by immune cells or tissue cells, such as nucleotides, peptides and lipids (33–35). MSC-Exo shows great potentials in intercellular information change between cells. Recently, non-coding RNAs have become a hot spot in oncology, rheumatology and other fields, such as circRNAs, lncRNAs, and miRNAs (36–40). Emerging evidence has elucidated that exosome-deriving miRNAs, circRNAs and other non-coding RNAs play important roles in SLE pathogenesis (41, 42). Therefore, non-coding RNA may serve as new diagnostic biomarkers or therapeutic targets for SLE. In this review, we have summarized currently available studies on the role of MSCs and MSCs-EVs in autoimmune diseases, primarily including SLE. These findings are useful for understanding SLE pathogenesis and exploring novel biomarkers for SLE diagnosis and treatment.

IMMUNOREGULATORY EFFECTS OF MSCs AND MSC-EVs ON T CELLS

Immune regulation dysfunction is the main cause for SLE (43). In particular, T cells play a key role in SLE pathogenesis. It has been illustrated that the interaction between MSCs and T cells is essential for maintaining immune balance. Various soluble immune regulatory factors, growth factors, non-coding RNA, proteins are involved in the interaction process through paracrine action (44). MSCs inhibit T cell proliferation and activation through EVs in a dose-dependent manner (45, 46). Accumulated studies have shown BM-MSCs inhibit T cells proliferation and differentiation by preventing them from entering S phase and G0/G1 phase of the cell cycle (47, 48). Fetal liver-derived MSCs (FL-MSCs) inhibit $CD4^+$ and $CD8^+$ T cells response and promote $CD4^+CD25^+Foxp3^+$Tregs response (**Table 1**) (49). MSCs can induce T cells differentiation from pro-inflammatory state to anti-inflammatory state mainly by inhibiting lymphocyte proliferation and pro-inflammatory cytokines production (57). Thus, the extensive wealth of in-vitro data has suggested MSCs exert immunomodulatory effect on T cells and might participate in maintaining the balance of

TABLE 1 | Mechanism of action with MSCs and EVs on T cells.

Cell type	Immune cell	Mechanism	Effect	Reference
FL-MSCs	CD4+CD8+T cell and Treg cell	–	inhibit CD4+T cells, CD8+T cells and promote Treg cells	(49)
MSC	Treg, Tfh	through an APC pathway	promotes Treg cells and inhibits the generation of Tfh	(49)
MSC-EVs	Th1 cell	regulating glycolysis and cytokine signaling pathways	inhibits T cell proliferation and Th1 differentiation	(39)
MSC-EVs	$CD4^+$T cell	EVs-encapsulating miR-23a-3p and post-transcriptionally regulated TGF-β receptor 2 in T cells	suppressive Th1 differentiation	(50)
MSC-Exo	T cell	P27kip1/Cdk2 pathway	suppressive activation T cell proliferation and cell cycle arrest	(51)
MSC-EVs	T cell	increasing IL-10 and TGF-β	promote T cells apoptosis and inhibit proliferation	(13)
AD-MSC miR-10a	Th17/Treg cell	regulating $Foxp3^+$ expression through TGF-β pathway	promote the differentiation of Th2 and Treg from naive $CD4^+$ T cells	(52)
UC-MSCs	T cell	through the COX2/PGE2/NF-κB signaling pathway	inhibiting T cell proliferation and DC differentiation	(53)
UC-MSCs	T cell	IFNGR1/JAK2/Stats signaling pathways, IFN-γ/IDO axis	suppressive T cell proliferation and promote Treg function	(54)
AD-MSC	T cell	through regulating TGF-β and PGE2	regulate the Th17/Treg balance	(55, 56)

Tfh, T follicular helper; MSC, Mesenchymal stem cells; MSC-EVs, MSC derived Extracellular Vesicles; MSC-exo, MSC derived Exsome; FL-MSC, Fetal liver-derived Mesenchymal stem cells; BM-MSC, bone marrow Mesenchymal stem cells; UC-MSC, umbilical cord Mesenchymal stem cells; AD-MSC, adipose MSC; APC, antigen presenting cells; IL-10, interleukin 10; IDO, Indoleamine 2,3-dioxygenase; PGE2, Prostaglandin E2; TLR4, Toll-like Receptor 4; Treg, Regulatory T; Th1, T-helper 1; Th17, T-helper 1.

immune microenvironment. It has been documented that MSCs reduce the activation of antigen presenting cells (APCs) by producing soluble factors, such as indoleamine-2,3-dioxygenase (IDO), prostaglandin E2 (PGE2) and IL-10. MSC promotes the proportion of regulatory T (Treg) cells and inhibits the generation of T follicular helper (Tfh) cells through an APC pathway (58). It has been well established that MSCs can induce T cells differentiation into Treg and Th2 cells but inhibit Th17 and Tfh cells differentiation and immune response. Accordingly, MSCs might serve as a useful treatment strategy for autoimmune diseases, including SLE.

MSC-EVs exert immunomodulatory effects similar to MSCs. MSC-EVs inhibit T cell proliferation and Th1 differentiation by regulating glycolysis and cytokine signaling pathways (39). A previous study has suggested MSC-EVs promote $CD4^+$ T lymphocytes differentiation toward a regulatory phenotype by EVs-encapsulating miR-23a-3p and post-transcriptionally regulated TGF-β receptor 2 in T cells (**Table 1**) (50). MSC-Exo suppresses the proliferation of T cells and induce cell cycle arrest through p27kip1/Cdk2 signaling pathway (**Table 1**) (51). MSC-Exo exerts immunosuppressive effects in autoimmune uveitis mice model by reducing T cell subsets and other inflammatory cells infiltration (59). Besides, MSCs-Exo enhances Treg generation *in vitro* and *vivo* (58). BM-MSC-EVs can prevent naive T cells differentiation into effector T cells and their activation (60). MSC-EVs promote the apoptosis of activated T cells, inhibit self-reactive lymphocytes proliferation, and produce more Tregs by increasing IL-10 and TGF-β (13). These findings have suggested the biological functions of MSC-EVs are similar to MSC that can regulate immune cells growth and function. Accordingly, MSC-EVs might be a promising cell-free therapy in autoimmune diseases.

MiRNAs are endogenous single-stranded non-coding RNAs that regulate approximately 30-70% of human genes. They play an important role in innate and adaptive immune responses by influencing cell proliferation, differentiation and apoptosis (61). Accumulated data has shown that miRNAs disruption and dysfunction can interfere with immune response, stimulate inflammatory cytokines release, initiate autoantibodies production and promote autoimmune diseases occurrence, such as SLE (62, 63). The expression of miR-146a in SLE patients serum exosomes is significantly decreased, which can promote MSCs senescence by targeting TRAF6 and inhibiting NF-κB signaling pathway activation (**Figure 1**) (64, 65). Besides, exosomal miR-146a plays a key role in regulating innate immunity by targeting toll-like receptors (TLRs), tumor necrosis factor (TNF)-associated family (TNAF), and interleukin-1 receptor-associated kinase 1 (IRAK1) (**Table 1**) (66). Accordingly, circulating exosomes encapsulating miR-146a may be a key biomarker for SLE. The study by Bolandi Z et al. has suggested adipose-derived mesenchymal stem cell exosomes (AD-MSC-Exo) miR-10a promote the differentiation of Th2 and Treg from naive $CD4^+$ T cells (52). AD-MSC-Exo miR-10a can regulate Th17 and Treg differentiation by regulating $Foxp3^+$ expression through TGF-β pathway (**Table 1**) (52). Taken together, MSCs carry molecular information of specific source cells and participate in intercellular communications.

Imbalance of Th1, Th2, Th17 and Treg results in inflammatory response and immune disorders in SLE (20, 67). Defective clearance of apoptotic cells (ACs) has been considered to be involved in the pathogenesis of SLE. It has been shown that human umbilical cord (UC) MSC possessed the ability to engulf ACs and then enhanced the immunosuppressive function by inhibiting T cell proliferation and DC differentiation through the COX2/PGE2/NF-κB signaling pathway (**Table 1**) (53). MSCs have significant improvements of hematological ingredient by upregulating Treg and downregulating Th17 (68). Interestingly, BM-MSCs and UC-MSCs from SLE patients have a defect in

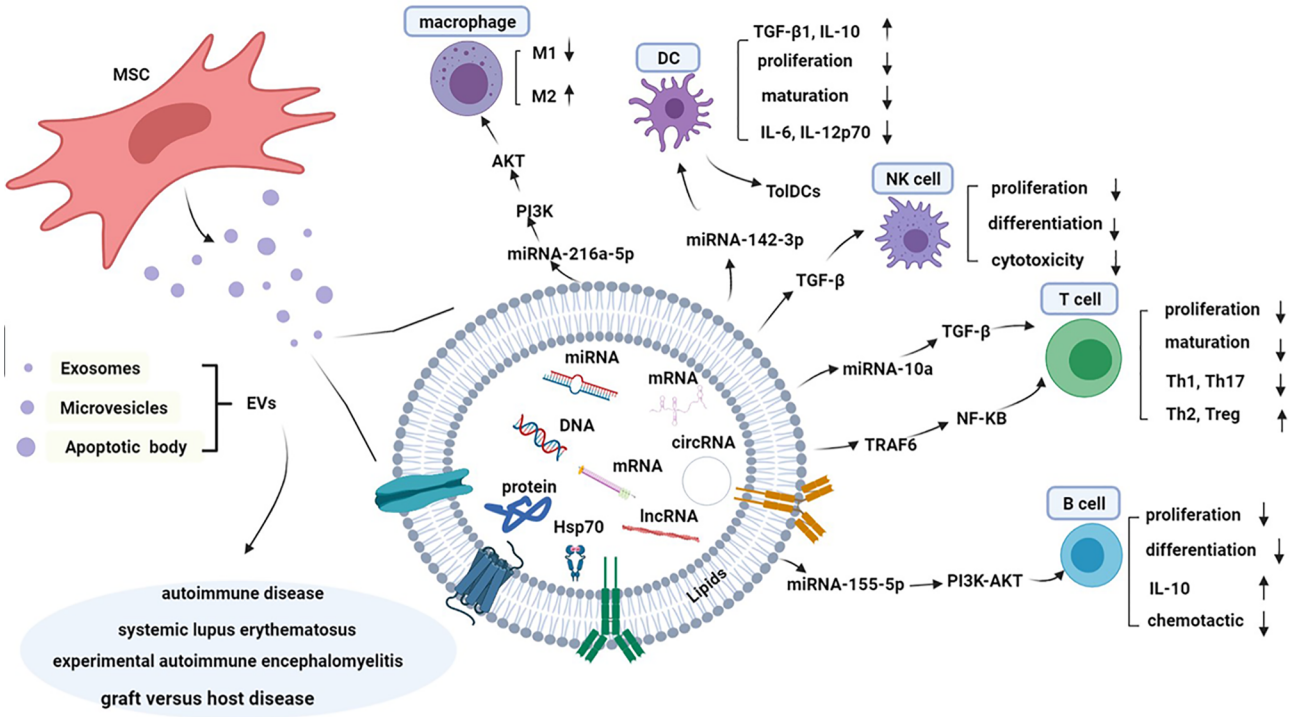


FIGURE 1 | Composition and mechanism of immunological tolerance of MSC-EVs in systemic lupus erythematosus. MSC-EVs are spheroidal shaped and two-layer lipid particles containing various types of protein, lipids, DNAs, non-coding RNAs, miRNAs, and mRNA, which cause genetic information exchange by various of signal pathway and reprogramming of the recipient cell. MSC-EVs can suppress the differentiation and proliferation of B cell by PI3K-AKT pathway, and reduce production of IL-10. Similarly, T cells play the suspensive role on the proliferation and maturation, while reduce production of Th17 and Th1, and improve function of Treg and Th2 through the TGF-β/NF-κB pathway. EVs can suppress the proliferation and maturation of DCs and induce tolerable DCs with low expression of costimulatory makers. Macrophages can transform to anti-inflammatory M2 phenotype after treating by MSC-EVs through the PI3K/AKT pathway. EVs can suppress the proliferation, differentiation and cytotoxicity of NK cells in a TGF-β dependent manner. MSC-EVs play an important role in the pathogenesis of autoimmune diseases, including SLE, graft *versus* host disease, experimental autoimmune encephalomyelitis, etc.

IDO activity and secretion, which is an enzyme that mediates tryptophan degradation into immunosuppression metabolites (54). However, allogeneic UC-MSC can produce extremely amount of IDO to inhibit T cell proliferation through IFNGR1/JAK2/Stats signaling pathway (**Table 1**) (54). Moreover, MSC restores the immune balance *via* regulating TGF-β and PGE2 and enhancing Treg/Th17 cells ratio in lupus mice (55, 56). In SLE patients, allogeneic UC-MSCs inhibit T cells response through IFN-γ/IDO axis (69), and induce more inducible Treg (iTreg) by generating anti-inflammatory cytokines, such as TGF-β1 (70, 71). Besides, BM-MSCs can alleviate lupus nephritis and improve mice survival rate by effectively inhibiting follicular helper T cell (Tfh) differentiation and IL-21 generation (72, 73).

REGULATION OF MSCs AND MSC-EVs ON B CELLS

Abundant activation and dysregulation of B cells is closely related to SLE pathogenesis. Pathogenic autoantibodies from B cells participate in SLE pathogenesis and mediate tissue damage, which are also involved in presenting antigens to self-reactive T cells (74). The activation of B cells leads to the production of a large number of autoreactivity autoantibodies involved in the disease process of SLE, including BAFF. A previous study has demonstrated that excessive expression of miR-152-3p is observed in SLE, resulting in increased BAFF Expression in SLE B-Cells by downregulating KLF5 (75). The study by Ma X et al. has shown that BM-MSC suppressed the excessive activation of B-cells *via* inhibiting BAFF production in MRL/lpr mice (**Table 2**) (76). Belimumab, an anti-BAFF antibody, has already been used as a treatment target for SLE, which is approved by FDA for Lupus in 2011. A published study has reported that MSCs inhibit the proliferation of B lymphocytes by keeping the cell cycle in the G1 and G0 phases and impair plasma cell formation and immunoglobulin secretion (81, 83). Besides, MSCs can indirectly inhibit B cell differentiation, maturation, and plasma cell differentiation as well as antibody production through directly contacting with T cells (**Table 2**) (77). Moreover, MSCs inhibit the proliferation, excessive activation and maturation of B cells by depleting tryptophan in the inflammatory microenvironment of human body **(Table 2)** (78). Most interestingly, MSCs enhance increased regulation B (Breg)

TABLE 2 | Mechanism of action with MSCs and EVs on B cell.

Cell type	Immune cell	Mechanism	Effect	Reference
BM-MSC	B cell	inhibition of BAFF production	suppress the excessive activation of B-cells	(76)
MSCs	B cell	directly contacting with T cells	inhibit B cell differentiation, maturation,	(77)
MSCs	B cell	by depleting tryptophan in the inflammatory microenvironment of human body	inhibits the proliferation, excessive activation and maturation of B cells	(78)
MSC	Breg cell	through SDF-1-CXCR7 axis	increased regulation B (Breg)	(77)
MSC-EVs miRNA-155-5p	B cell	targeting PI3K/AKT signaling pathway	inhibit activation of B cell	(79)
MSC	B cell	increased expression of CCL2 by CCL2-MST1-mTOR-STAT1 mediated metabolic signaling pathway	prevent inhibition differentiation, proliferation, and antibody secretion of B-cell	(80)
MSC	B cell	inhibit expression of maturation protein-1	inhibit B cells terminal differentiation	(81)
GMSCs	B cell	targeting of $CD39^{-}CD73$ signaling pathway	suppressing B cells and produce autoantibodies	(82)

MSC, Mesenchymal stem cells; BM-MSC, bone marrow Mesenchymal stem cells; GMSC, gingiva derived MSCs; SDF-1-CXCR7, Stromal-derived factor-1 and its receptor C-X-C chemokine receptor-7; Breg, regulation B cell; CCL2, chemokine CC motif ligand 2; CCL2-MST1-mTOR-STAT1, chemokine CC motif ligand 2-mammalian Sterile 20-like kinase 1- media Time of Repair-signal transducerand activator of transcription 1.

cell through SDF-1-CXCR7 axis, which plays a key role in maintaining immune tolerance and inhibiting immune and inflammatory responses (84). MSCs affect B cells chemotaxis by inhibiting the production of immunoglobulin IgG, IgM and IgA and downregulating related ligands expression. As a result, MSCs regulate B cell response and mediate immune suppression, whereas the potential molecular mechanism warrants to be investigated.

The immunomodulatory effects of MSC on B Cells and plasma cells are associated with EVs delivering soluble factors (85, 86). MSC-exo inhibits the proliferation of T cells and B cells (**Table 2**) (29). It has been well documented that MSC-EVs delivering miRNA-155-5p reduce the activation of B cell cycle progression by targeting PI3K/AKT signaling pathway(**Figure 1** and **Table 2**) (79). Nonetheless, more studies are warranted to further investigate the potential bioactive factors in MSC-EVs and their precise effects on immune cells function in SLE and other autoimmune diseases. Understanding the mechanisms of MSC-EVs in immune regulations may help for developing new therapeutic strategies for SLE.

It has been documented that toll-like receptor 7 (TLR7) is overexpressed in SLE, which drives autoreactive B cells activation and autoantibodies production through IFN-γ signal pathway in SLE (87–89). MSC enhances IL-10 production by activating the extracellular signal-related kinase (ERK) signal but suppresses the generation of TNF-α by downregulating TLR-7/NF-κB signal in murine macrophages (90). Intrinsic B cells with autoreactive B cell receptor (BCR) expression and long-lived plasma cells continuously producing autoantibodies are essential for the development of SLE (91). A previous study has implicated that MSCs derived from SLE patients have significantly reduced expression of CCL2 (21). Those MSCs prevent from the proliferation, differentiation, and antibody secretion of B-cell through CCL2-MST1-mTOR-STAT1 mediated metabolic signaling pathway (**Table 2**) (80). Elevated expression of IL-21 in serum of SLE patients promotes B cells differentiation, which thus leads to a large number of pathogenic autoantibodies aggravating SLE manifestations (92). MSCs exert inhibitory effects on the terminal differentiation of B cells through decreasing the expression of maturation protein-1 (**Table 2**) (81). In addition, human gingiva derived MSCs (GMSCs) directly suppress autoantibodies production and proteinuria and alleviate the histopathological progression of lupus nephritis through $CD39^{-}CD73$ signaling pathway (82). Thus, MSCs play a vital role in SLE pathogenesis by inhibiting B cells proliferation, differentiation and activation.

REGULATORY EFFECTS OF MSCs AND MSC-EVs ON DENDRITIC CELLS

DCs are the most important APCs, which are key cells in defending against infection and tumor. As APCs, DCs present exogenous antigens to secondary lymphoid tissues such as spleen and lymph nodes. In lupus nephritis, DC infiltrates the kidney to exacerbate inflammation. Shahir M et al. have found that MSC-Exo induces more tolerance DCs (tol-DCs) with low expression of costimulatory markers and IL-6, but increases the anti-inflammatory cytokines of IL-10 and TGF-β (**Figure 1** and **Table 3**) (93). Moreover, the induced tol-DCs further promote the differentiation of regulatory T cells, which play a protective role in SLE (93). MSC pretreated with IFN-γ inhibits DC maturation, activation, and antigen uptakes (**Table 3**) (94). Type I IFN is closely related to the severity of lupus nephritis, hematopoietic and central nervous system symptoms of SLE patients. Plasmacytoid DCs (pDCs) are considered to be the main source of type I IFN. Apart from pDCs, T cells, B cells and NK cells are also involved in IFN production in SLE (97). However, MSCs can inhibit the generation and function of pDCs (98). Accordingly, MSCs are ideal therapeutic way for SLE patients due to their inhibitory effects on pDCs and type I IFN. The regulatory effects of MSCs on DCs make them as promising treatment strategies for SLE patients.

Increasing studies have suggested the modulatory effects of MSC-EVs on immune cell functions. MSC-Exo transferring miRNA-155 and miRNA-146 have been found to regulate endotoxin-induced inflammatory response in DCs (99). Wang Y et al. have demonstrated that miR-142-3p was highly expressed in SLE, which induced pro-inflammatory moDCs involved in SLE pathogenesis (100). MSC-EVs are capable of attenuating DCs maturation and function. It has been found that some MSC-EVs derived miRNAs can decrease the expression of

TABLE 3 | Mechanism of action with MSCs and EVs on DC cell.

Cell type	Immune cell	Mechanism	Effect	Reference
MSC-Exo	tol-DCs	induces more tolerance DCs (tol-DCs) with low expression of costimulatory markers	increased anti-inflammatory cytokines IL-10 and TGF-β expression but decreased IL-6 expression, promotes the differentiation of regulatory T cells	(93)
MSC	DC	Combine with IFN-γ	inhibits DC maturation, activation, and antigen uptakes	(94)
MSC-Evs	DC	expression of anti-inflammatory factors (TGF-β1 and IL-10) and reduce the generation of proinflammatory cytokines (L-6 and IL-12p70)	attenuate DCs maturation and function	(95)
UC-MSCs	$CD1c^+$ DCs	upregulating serum FLT3L	Increased expression of $CD1c^+$ DCs	(96)

MSC, Mesenchymal stem cells; MSC-EVs, MSC derived Extracellular Vesicles; MSC-Exo, MSC derived Exsome; UC-MSC, umbilical cord Mesenchymal stem cells; IL-10, interleukin 10; TGF-β1, transforming growth factor-β1; DC, Dendritic cells; NK, Natural killer; IFN-γ, interferon; IL-6, interleukin-6.

mature markers CD83, CD38 and CD80 and proinflammatory cytokines (IL-6 and IL-12p70) but promote the expression of anti-inflammatory factors (TGF-β1 and IL-10) (**Figure 1** and **Table 3**) (95).

A number of studies have shown that allogeneic BM-MSCs inhibit SLE inflammatory response by upregulating tolerance DCs. After transplantation of allogenic UC-MSCs, peripheral blood $CD1c^+$ DCs and serum FLT3L can be significantly up-regulated in SLE patients, suggesting that UC-MSCs are useful for SLE treatment by inducing increased resistant $CD1c^+$ DCs (96). Nonetheless, more future studies are warranted to elucidate the underlying mechanism involved in MSCs and DCs interactions in SLE.

REGULATORY EFFECTS OF MSCs AND MSC-EVs ON NK CELLS

NK cells primarily mediate natural immune response and also play a crucial role in the pathogenesis of SLE (**Figure 1**). In SLE patients, the number of NK cells is significantly reduced, whose cytotoxic activity and cytokine profile are impaired. NK cells with higher expression of granzyme B are observed in active SLE patients, which can be further enhanced by IL-15 (101). Interactions between MSCs and NK cells are necessary for reducing NK cytotoxicity (102). A published study has confirmed that MSCs inhibit the activity of NK cells by regulating IDO and PGE2 (**Table 4**) (103, 104). BM-MSCs can inhibit NK cell proliferation induced by IL-12 and IL-21 but upregulate IFN-γ, IFN-α, perforin and granzyme in NK cells (**Table 4**) (105, 106). Besides, NK cells also exert regulatory effects on MSCs. NK cells promote MSCs recruitment and ROS production, which can downregulate MSCs activity (109). Interestingly, the sorted TLR4-positive MSC ($TLR4^+$MSC) has a strong inhibitory effect on NK cells by regulating the receptor NKG2D (107), suggesting the pivotal immunomodulatory effects of MSCs on NK cells.

Accumulating data has implicated that MSC-EVs inhibit NK cells proliferation by regulating G0 and G1 cell cycle phases (99). Exosomes from fetal liver MSCs (FL-MSCs) inhibit the proliferation, activation and cytotoxicity of NK cells by regulating TGF-β (**Table 4**) (108). Activin A is a member of the TGF-β superfamily. It has been demonstrated that UC-MSC produces a large amount of activin A, which inhibits IFN-γ production by downregulating T-bet in NK cells (110). However, whether MSC-EVs protect against SLE by inhibiting NK cells proliferation and function warrants to be further studied in the future. It is lacking substantial evidence to support the immunomodulatory effect of MSC-EVs on NK cells in lupus, either in animal models or in clinical studies. The precise effect and underlying mechanism of MSCs/MSC-EVs on NK cells in SLE warrant to be investigated in more future studies.

REGULATORY EFFECTS OF MSCs AND MSC-EVs ON MACROPHAGES

Imbalance of M1/M2 polarization and abnormal activation of macrophages are involved in the pathogenesis of SLE. Macrophages are responsible for clearing ACs. Increased ACs

TABLE 4 | Mechanism of action with MSCs and EVs on NK cell.

Cell type	Immune cell	Mechanism	Effect	Reference
MSC	NK cell	regulating indoleamine-2, 3-dioxygenase (IDO) and prostaglandin E2 (PGE2)	inhibit the activity of NK cells	(103, 104)
BM-MSCs	NK cell	inhibit IL-12 and IL-21	Suppression NK cell proliferation but increase IFN-γ and IFN-α production	(105, 106)
$TLR4^+$MSC	NK cell	–	inhibitory effect on NK cells and the receptor NKG2D	(107)
FL-MSC-EXO	NK cell	regulating TGF-β	inhibit the proliferation, activation and cytotoxicity of NK cells	(108)

MSC, Mesenchymal stem cells; FL-MSC, Fetal liver-derived Mesenchymal stem cells; BM-MSC, bone marrow Mesenchymal stem cells; UC-MSC, umbilical cord Mesenchymal stem cells; IDO, Indoleamine 2,3-dioxygenase; PGE2, Prostaglandin E2; TLR4, Toll-like Receptor 4; NK, Natural killer; $TLR4^+$MSC, TLR4-positive MSC; IL-12, interleukin 12; IL-21, interleukin 21; NKG2D, type II integral membrane protein; IFN-γ, interferon-γ; IFN-α, interferon-α.

due to dysregulated macrophages will activate autoreactive B cells, thereby leading to abundant autoantibodies and immune complexes deposition in multiple organs and tissues (111). The polarization and activation of macrophages are different depending on certain microenvironmental conditions. It has been well established that MSCs effectively weaken the phagocytosis and antigen presentation of macrophages. Besides, MSCs can induce macrophage polarization to M2 phenotype through TGF-β/Akt/FoxO1 pathway (**Table 5**) (112). Similarly, BM-MSCs can drive macrophages differentiation into anti-inflammatory phenotype M2, while they inhibit the differentiation of pro-inflammatory phenotype M1 (120). DNA methyltransferase 1 in SLE patients' peripheral blood mononuclear cells is increased. BM-MSC may down-regulate the expression of methyltransferase 1 through the MEK/ERK signaling pathway, thereby inhibiting self-activated peripheral blood mononuclear cells from SLE patients (121). UC-MSC can affect M1/M2 balance by regulating macrophage metabolic pathways (**Table 5**) (113). Accordingly, MSCs play a critical role in maintaining M1/M2 balance in SLE.

A number of studies have shown that MSC-Exo has lower immunogenicity and can inhibit the development and progression of experimental autoimmune encephalomyelitis, traumatic spinal cord injury and diabetes animal models by regulating M1/M2 balance and Th17/Treg ratio (122–124). A previous study has shown that MSC-EVs enhance the anti-inflammatory phenotype of regulatory macrophages by down-regulating IL-23 and IL-22 and promoting inflammation remission (**Table 5**) (114). At the same time, AD-MSCs derived EVs are capable of inducing macrophage polarization toward M2 under hypoxia conditions (**Table 5**) (115). MSC-Exo exerts anti-inflammatory properties by promoting macrophages differentiation toward M2 through miR-223/pKNOX1 pathway (**Table 5**) (116). MSC-EVs delivering miR-216a-5p can be transferred to macrophages and induce M2 macrophages polarization through TLR4/NF-κB/PI3K/Akt signaling cascade (**Figure 1** and **Table 5**) (117). Interestingly, MSC-Exo attenuates myocardial ischemia-reperfusion injury by enhancing M2 macrophages polarization through miRNA-182 and its downstream target, namely, toll-like receptor 4 (TLR4) (125). Therefore, MSCs and MSC-EVs can effectively regulate macrophages polarization toward M2 and inhibit macrophages-mediated inflammatory response. However, little is known about the effect of MSCs and MSC-EVs on macrophages in SLE. It has been shown that MSC mediated macrophage polarization depends on the IL-6 signaling pathway (126). UC-MSCs promote the proportion of $CD206^+$ M2 cells and the phagocytic activity of macrophages in an IL-6 dependent manner in SLE (127). Human UC-MSCs/exosomes attenuate the diffuse alveolar hemorrhage (DAH) induced inflammatory responses and alveolar hemorrhage through increasing M2 macrophage polarization in DAH patients or lupus mice (**Table 5**) (118, 119). All these findings have strongly suggested the pivotal role of MSC-EVs in macrophage polarization in SLE. MSC-Exo serves as a nanocarrier delivering bioactive molecules between immune cells and stem cells, which may be a promising treatment way for SLE.

During the past few years, immune metabolic disorders have been implicated in SLE pathogenesis, whereas the regulatory mechanism of MSC and MSC-EVs on the immune metabolic phenotype of macrophages and M1/M2 bias in SLE remains largely unclear. Blocking the reprogramming of macrophage metabolism and maintaining the balance of M1/M2 in the immune microenvironment would provide new insight into identifying valuable strategies for the biological treatment of SLE.

POTENTIAL USE OF MSCs AND MSC-EVs IN SLE TREATMENT

Current treatment strategies for SLE are mainly aimed at controlling and mitigating disease activity. Although Belimumab and Telitacicept have been approved for SLE treatment, the heterogeneity of SLE has led to the dilemma of current treatment status (128). Accordingly, identifying a more effective treatment strategy has become a top priority for SLE. Some studies have established that MSCs can attenuate the adverse effects of immunosuppressive drugs. A previous study

TABLE 5 | Mechanism of action with MSCs and EVs on macrophage.

Cell type	Immune cell	Mechanism	Effect	Reference
MSC	macrophage	Through TGF-β/Akt/FoxO1 pathway	toward M2 phenotype polarization	(112)
UC-MSC	macrophage	regulating macrophage metabolic pathways	affect M1/M2 balance	(113)
MSC-Exo	macrophage	down-regulating IL-23 and IL-22	enhances the anti-inflammatory phenotype of macrophages, promoting inflammation remission	(114)
AD-MSCs	macrophage	–	toward M2 phenotype polarization	(115)
MSC-Exo	macrophage	through miR-223/pKNOX1 pathway	promoting macrophages differentiation toward M2	(116)
MSC-EVs	macrophage	through TLR4/NF-κB/PI3K/Akt signaling cascade	toward M2 phenotype polarization	(117)
UC-MSC/ exosomes	macrophage	increased the proportion of M2 macrophage polarization	attenuate diffuse alveolar hemorrhage (DAH) induced inflammatory responses and alveolar hemorrhage	(118, 119)

MSC, Mesenchymal stem cells; MSC, Mesenchymal stem cells; MSC-EVs, MSC derived Extracellular Vesicles; MSC-exo, MSC derived Exsome; UC-MSC, umbilical cord Mesenchymal stem cells; AD-MSC, adipose MSC; TRAF6, IR7; IL-10, interleukin 10; IL-23, interleukin 23 ;IL-22 , interleukin 22; pKNOX1, PBX/knotted 1 homeobox 1; NF-KB, nuclear transcription factor-kappa B; PI3K, phosphoinositide 3-kinase; PGE2, Prostaglandin E2; TLR4, Toll-like Receptor 4; DAH, diffuse alveolar hemorrhage.

has implicated that MSCs combination with immunosuppressive drugs display distinct effects on T cell activation and bias (129). They exert a suppressive effect on proinflammatory T cell subsets and promote the activation and function of anti-inflammatory Treg cells (129). Besides, accumulating evidence has suggested that mycophenolate mofetil (MMF) can selectively suppress B cells proliferation (74). Lee HK et al. have found that prednisone (PD) or MMF in combination with MSCs showed better therapeutic effect than single therapy in lupus-prone MRL/lpr mice (130). Combination of MSCs with PD or MMF can prolong the survival, decrease autoantibody levels and inflammatory cytokines in serum, and reduce the inflammatory cell infiltration in kidney and spleen in lupus mice (130). Moreover, immunosuppressants can influence MSC transplantation (MSCT) mediated immune responses and prolong the efficacy of transplanted MSCs (131). Accordingly, the combination of MSCs and immunosuppressants may become a more effectively therapeutic strategy for SLE.

MSCs are characterized by high self-renewal ability, rapid expansion *in vivo* and *in vitro*, and low immunogenicity. They can participate in immune response through two ways: paracrine effects and cell-cell interaction directly. In SLE patients, autologous MSCs are defective in immunomodulatory and regenerative functions. Allogeneic mesenchymal stem cell transplantation (MSCT) has brought new hope to cure severe SLE patients (132, 133). Reconstruction of immune tolerance and tissue regeneration and repair are necessary for SLE treatment. MSCs are easy for isolation and purification, which have good therapeutic effects in MRL/lpr and (NZB/NZW) F1 mice (134). Currently available animal studies have provided strong evidence for the therapeutic potentials of MSCs in SLE. The use of MSCs in treating SLE patients has also been reported in some previously published studies. The clinical symptoms of patients are significantly improved and the disease activity index is significantly decreased, when UC-MSCs are applied to the treatment of refractory and severe SLE patients.

MSCT is effective and safe in treating SLE patients and lupus animal models, even though Deng D et al. have shown that allogeneic UC-MSCs did not exert effects on SLE patients (135). However, a previous study has revealed that metformin-treated AD-MSCs regulated the Th17/Treg balance in MRL/lpr mice, and metformin enhanced the immunomodulatory properties of AD-MSCs through AMPK and STAT1 signal (**Table 1**) (136). It has been demonstrated that transplantation of human MSC can significantly inhibit disease progression in MRL/lpr mice (137). The study by Tang X et al. has suggested that dental pulp MSC (DP-MSCs) could alleviate the disease symptoms of lupus-prone B6/lpr mice, especially reduce the kidney glomerular lesion and perivascular inflammation infiltration (138). MSCs are found to reduce proteinuria and serum anti-dsDNA levels, increase C3 and C4 levels and alter serum cytokine profiles by down-regulating the MyD88-NF-kB signaling pathway in NZBWF1 mice model (139). Most importantly, mounting clinical studies have shown intravenous infusion of UC-MSC is an available and safe practice in SLE treatment, which not only significantly declines SLE Disease Activity Index (SLEDAI) but also ameliorates renal function and systemic manifestations including hematopoietic and cutaneous systems in SLE patients (140, 141). It has been well documented that a repeated infusion of MSCs is feasible, and MSCT should be adopted again 6 months after the first time to avoid disease relapse in severe and refractory lupus patients (140, 142, 143). LN patients get to renal remission 12 months after allogeneic MSCT, including obvious renal function amelioration in parallel with significantly improved glomerular filtration rate (144). Long-term serial administration of human AD-MSCs can help to ameliorate SLE without any adverse effects, which decreases the level of anti-double-stranded DNA antibodies but significantly increases IL-10 and Treg cells (145). Taken together, MSCT is a promising treatment strategy for SLE as evidenced by both animal experiments and clinical tests. However, the safety and efficacy need to be investigated in more future studies with large sample size. Last but not the least, the effect of MSCs derived EVs including exosomes in SLE is not clear yet, although they have been demonstrated to possess similar biological effects as MSCs in SLE (146).

PERSPECTIVES

Besides tissue repair and regeneration capacities, MSCs and MS-EVs have strong anti-inflammatory and immunomodulatory effects. They can be ideal therapeutic strategy for SLE, particularly the refractory and severe SLE patients resistant to hormones and immunosuppressant drugs. Accumulating data has suggested MSC-EVs also have anti-inflammatory, anti-apoptosis, pro-angiogenesis and immunomodulatory effects in inflammatory and autoimmune diseases by transferring bioactive constitutes to specific cells. Most importantly, MSC-EVs have much less immunogenicity but similar biological function with MSCs themselves, which act as a representative cell-free treatment way. Moreover, MSCs are demonstrated to possess tumorigenic potentials due to gene mutations, genetic instability as well as excessive proliferation and differentiation. Therefore, MSC-EVs may be a better choice for SLE treatment in future. Nevertheless, genetic modification, metabolic recombination, and other priming of MSCs *in vitro* should be considered before MSC/MSC-EVs application for SLE treatment. The standardized methods for MSC/MSC-EVs isolation, quantification and quality control should also be seriously considered before using MSC/MSC-EVs in treating SLE and other immune diseases. Lastly, the time of infusion, the appropriate dosage, the interval of treatment, and the long-term safety of MSC/MSC-EVs treatment in SLE warrant further clinical evaluations in more studies with high quality.

AUTHOR CONTRIBUTIONS

CY, JZ, and YT carried out literature research and reviewed all articles. JW, HL, JW, JY, and LZ helped to extract information. CY and SY wrote the paper. JS, CY, and DX revised the manuscript. SY and DX edited and submitted the article. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Justiz Vaillant AA, Goyal A, Bansal P, Varacallo M. *Systemic Lupus Erythematosus*. Treasure Island FL: StatPearls (2021).
2. Yan S, Wang W, Gao G, Cheng M, Wang X, Wang Z, et al. Key Genes and Functional Coexpression Modules Involved in the Pathogenesis of Systemic Lupus Erythematosus. *J Cell Physiol* (2018) 233:8815–25. doi: 10.1002/jcp.26795
3. Tsokos GC, Lo MS, Costa Reis P, Sullivan KE. New Insights Into the Immunopathogenesis of Systemic Lupus Erythematosus. *Nat Rev Rheumatol* (2016) 12:716–30. doi: 10.1038/nrrheum.2016.186
4. Yu F, Haas M, Glassock R, Zhao MH. Redefining Lupus Nephritis: Clinical Implications of Pathophysiologic Subtypes. *Nat Rev Nephrol* (2017) 13:483–95. doi: 10.1038/nrneph.2017.85
5. Pons-Estel GJ, Alarcon GS, Scofield L, Reinlib L, Cooper GS. Understanding the Epidemiology and Progression of Systemic Lupus Erythematosus. *Semin Arthritis Rheumatol* (2010) 39:257–68. doi: 10.1016/j.semarthrit.2008.10.007
6. Gupta PK, Chullikana A, Rengasamy M, Shetty N, Pandey V, Agarwal V, et al. Efficacy and Safety of Adult Human Bone Marrow-Derived, Cultured, Pooled, Allogeneic Mesenchymal Stromal Cells (Stempeucel(R)): Preclinical and Clinical Trial in Osteoarthritis of the Knee Joint. *Arthritis Res Ther* (2016) 18:301. doi: 10.1186/s13075-016-1195-7
7. Van Pham P, Truong NC, Le PT, Tran TD, Vu NB, Bui KH, et al. Isolation and Proliferation of Umbilical Cord Tissue Derived Mesenchymal Stem Cells for Clinical Applications. *Cell Tissue Bank* (2016) 17:289–302. doi: 10.1007/s10561-015-9541-6
8. Romanov YA, Vtorushina VV, Dugina TN, Romanov AY, Petrova NV, Sukhikh GT. Human Umbilical Cord Tissue-Derived Multipotent Mesenchymal Stromal Cells Exhibit Maximum Secretory Activity in the Presence of Umbilical Cord Blood Serum. *Bull Exp Biol Med* (2020) 169:544–8. doi: 10.1007/s10517-020-04926-2
9. Calle A, Gutierrez-Reinoso MA, Re M, Blanco J, de la Fuente J, Monguio-Tortajada M, et al. Bovine Peripheral Blood MSCs Chemotax Towards Inflammation and Embryo Implantation Stimuli. *J Cell Physiol* (2021) 236:1054–67. doi: 10.1002/jcp.29915
10. Beeravolu N, McKee C, Alamri A, Mikhael S, Brown C, Perez-Cruet M, et al. Isolation and Characterization of Mesenchymal Stromal Cells From Human Umbilical Cord and Fetal Placenta. *J Vis Exp* (2017) 122:55224. doi: 10.3791/55224
11. Liu X, Xiang Q, Xu F, Huang J, Yu N, Zhang Q, et al. Single-Cell RNA-Seq of Cultured Human Adipose-Derived Mesenchymal Stem Cells. *Sci Data* (2019) 6:190031. doi: 10.1038/sdata.2019.31
12. Tomasello L, Mauceri R, Coppola A, Pitrone M, Pizzo G, Campisi G, et al. Mesenchymal Stem Cells Derived From Inflamed Dental Pulpal and Gingival Tissue: A Potential Application for Bone Formation. *Stem Cell Res Ther* (2017) 8:179. doi: 10.1186/s13287-017-0633-z
13. Kim H, Lee MJ, Bae EH, Ryu JS, Kaur G, Kim HJ, et al. Comprehensive Molecular Profiles of Functionally Effective MSC-Derived Extracellular Vesicles in Immunomodulation. *Mol Ther* (2020) 28:1628–44. doi: 10.1016/j.ymthe.2020.04.020
14. Aggarwal S, Pittenger MF. Human Mesenchymal Stem Cells Modulate Allogeneic Immune Cell Responses. *Blood* (2005) 105:1815–22. doi: 10.1182/blood-2004-04-1559
15. Fatima F, Ekstrom K, Nazarenko I, Maugeri M, Valadi H, Hill AF, et al. Non-Coding RNAs in Mesenchymal Stem Cell-Derived Extracellular Vesicles: Deciphering Regulatory Roles in Stem Cell Potency, Inflammatory Resolve, and Tissue Regeneration. *Front Genet* (2017) 8:161. doi: 10.3389/fgene.2017.00161
16. Ono M, Kosaka N, Tominaga N, Yoshioka Y, Takeshita F, Takahashi RU, et al. Exosomes From Bone Marrow Mesenchymal Stem Cells Contain a microRNA That Promotes Dormancy in Metastatic Breast Cancer Cells. *Sci Signal* (2014) 7:ra63. doi: 10.1126/scisignal.2005231
17. Lazennec G, Lam PY. Recent Discoveries Concerning the Tumor - Mesenchymal Stem Cell Interactions. *Biochim Biophys Acta* (2016) 1866:290–9. doi: 10.1016/j.bbcan.2016.10.004
18. Sun LY, Zhang HY, Feng XB, Hou YY, Lu LW, Fan LM. Abnormality of Bone Marrow-Derived Mesenchymal Stem Cells in Patients With Systemic Lupus Erythematosus. *Lupus* (2007) 16:121–8. doi: 10.1177/0961203306075793
19. Tang Y, Ma X, Zhang H, Gu Z, Hou Y, Gilkeson GS, et al. Gene Expression Profile Reveals Abnormalities of Multiple Signaling Pathways in Mesenchymal Stem Cell Derived From Patients With Systemic Lupus Erythematosus. *Clin Dev Immunol* (2012) 2012:826182. doi: 10.1155/2012/826182
20. Geng L, Tang X, Wang S, Sun Y, Wang D, Tsao BP, et al. Reduced Let-7f in Bone Marrow-Derived Mesenchymal Stem Cells Triggers Treg/Th17 Imbalance in Patients With Systemic Lupus Erythematosus. *Front Immunol* (2020) 11:233. doi: 10.3389/fimmu.2020.00233
21. Che N, Li X, Zhang L, Liu R, Chen H, Gao X, et al. Impaired B Cell Inhibition by Lupus Bone Marrow Mesenchymal Stem Cells Is Caused by Reduced CCL2 Expression. *J Immunol* (2014) 193:5306–14. doi: 10.4049/jimmunol.1400036
22. Collins E, Gu F, Qi M, Molano I, Ruiz P, Sun L, et al. Differential Efficacy of Human Mesenchymal Stem Cells Based on Source of Origin. *J Immunol* (2014) 193:4381–90. doi: 10.4049/jimmunol.1401636
23. Zhou J, Benito-Martin A, Mighty J, Chang L, Ghoroghi S, Wu H, et al. Retinal Progenitor Cells Release Extracellular Vesicles Containing Developmental Transcription Factors, microRNA and Membrane Proteins. *Sci Rep* (2018) 8:2823. doi: 10.1038/s41598-018-20421-1
24. Qiu G, Zheng G, Ge M, Wang J, Huang R, Shu Q, et al. Mesenchymal Stem Cell-Derived Extracellular Vesicles Affect Disease Outcomes *via* Transfer of microRNAs. *Stem Cell Res Ther* (2018) 9:320. doi: 10.1186/s13287-018-1069-9
25. Harrell CR, Jovicic N, Djonov V, Arsenijevic N, Volarevic V. Mesenchymal Stem Cell-Derived Exosomes and Other Extracellular Vesicles as New Remedies in the Therapy of Inflammatory Diseases. *Cells* (2019) 8:1605. doi: 10.3390/cells8121605
26. Tsiapalis D, O'Driscoll L. Mesenchymal Stem Cell Derived Extracellular Vesicles for Tissue Engineering and Regenerative Medicine Applications. *Cells* (2020) 9:991. doi: 10.3390/cells9040991
27. Yeo RW, Lai RC, Zhang B, Tan SS, Yin Y, Teh BJ, et al. Mesenchymal Stem Cell: An Efficient Mass Producer of Exosomes for Drug Delivery. *Adv Drug Delivery Rev* (2013) 65:336–41. doi: 10.1016/j.addr.2012.07.001
28. Raposo G, Stoorvogel W. Extracellular Vesicles: Exosomes, Microvesicles, and Friends. *J Cell Biol* (2013) 200:373–83. doi: 10.1083/jcb.201211138
29. Khare D, Or R, Resnick I, Barkatz C, Almogi-Hazan O, Avni B. Mesenchymal Stromal Cell-Derived Exosomes Affect mRNA Expression and Function of B-Lymphocytes. *Front Immunol* (2018) 9:3053. doi: 10.3389/fimmu.2018.03053
30. Fathollahi A, Hashemi SM, Haji Molla Hoseini M, Yeganeh F. *In Vitro* Analysis of Immunomodulatory Effects of Mesenchymal Stem Cell- and Tumor Cell-Derived Exosomes on Recall Antigen-Specific Responses. *Int Immunopharmacol* (2019) 67:302–10. doi: 10.1016/j.intimp.2018.12.009
31. Kalluri R, LeBleu VS. The Biology, Function, and Biomedical Applications of Exosomes. *Science* (2020) 367:eaau6977. doi: 10.1126/science.aau6977
32. Toh WS, Lai RC, Zhang B, Lim SK. MSC Exosome Works Through a Protein-Based Mechanism of Action. *Biochem Soc Trans* (2018) 46:843–53. doi: 10.1042/BST20180079
33. Xu D, Song M, Chai C, Wang J, Jin C, Wang X, et al. Exosome-Encapsulated miR-6089 Regulates Inflammatory Response *via* Targeting TLR4. *J Cell Physiol* (2019) 234:1502–11. doi: 10.1002/jcp.27014
34. Wang JH, Liu XL, Sun JM, Yang JH, Xu DH, Yan SS. Role of Mesenchymal Stem Cell Derived Extracellular Vesicles in Autoimmunity: A Systematic Review. *World J Stem Cells* (2020) 12:879–96. doi: 10.4252/wjsc.v12.i8.879

35. Yan S, Ren X, Yang J, Wang J, Zhang Q, Xu D. Exosomal miR-548c-5p Regulates Colorectal Cancer Cell Growth and Invasion Through HIF1A/CDC42 Axis. *Onco Targets Ther* (2020) 13:9875–85. doi: 10.2147/OTT.S273008
36. Xu Y, Deng W, Zhang W. Long Non-Coding RNA TUG1 Protects Renal Tubular Epithelial Cells Against Injury Induced by Lipopolysaccharide *via* Regulating microRNA-223. *BioMed Pharmacother* (2018) 104:509–19. doi: 10.1016/j.biopha.2018.05.069
37. Yan S, Liu G, Jin C, Wang Z, Duan Q, Xu J, et al. MicroRNA-6869-5p Acts as a Tumor Suppressor *via* Targeting TLR4/NF-kappaB Signaling Pathway in Colorectal Cancer. *J Cell Physiol* (2018) 233:6660–8. doi: 10.1002/jcp.26316
38. Liu W, Wang Z, Liu L, Yang Z, Liu S, Ma Z, et al. LncRNA Malat1 Inhibition of TDP43 Cleavage Suppresses IRF3-Initiated Antiviral Innate Immunity. *Proc Natl Acad Sci USA* (2020) 117:23695–706. doi: 10.1073/pnas.2003932117
39. Xu H, Chen W, Zheng F, Tang D, Liu D, Wang G, et al. Reconstruction and Analysis of the Aberrant lncRNA-miRNA-mRNA Network in Systemic Lupus Erythematosus. *Lupus* (2020) 29:398–406. doi: 10.1177/0961203320908927
40. Zhang J, Liu Y, Shi G. The circRNA-miRNA-mRNA Regulatory Network in Systemic Lupus Erythematosus. *Clin Rheumatol* (2021) 40:331–9. doi: 10.1007/s10067-020-05212-2
41. Guo G, Wang H, Ye L, Shi X, Yan K, Lin K, et al. Hsa_circ_0000479 as a Novel Diagnostic Biomarker of Systemic Lupus Erythematosus. *Front Immunol* (2019) 10:2281. doi: 10.3389/fimmu.2019.02281
42. Lodde V, Murgia G, Simula ER, Steri M, Floris M, Idda ML. Long Noncoding RNAs and Circular RNAs in Autoimmune Diseases. *Biomolecules* (2020) 10:1044. doi: 10.3390/biom10071044
43. Sharabi A, Tsokos GC. T Cell Metabolism: New Insights in Systemic Lupus Erythematosus Pathogenesis and Therapy. *Nat Rev Rheumatol* (2020) 16:100–12. doi: 10.1038/s41584-019-0356-x
44. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, et al. Mesenchymal Stem Cell-Mediated Immunosuppression Occurs *via* Concerted Action of Chemokines and Nitric Oxide. *Cell Stem Cell* (2008) 2:141–50. doi: 10.1016/j.stem.2007.11.014
45. Weiss ARR, Lee O, Eggenhofer E, Geissler E, Korevaar SS, Soeder Y, et al. Differential Effects of Heat-Inactivated, Secretome-Deficient MSC and Metabolically Active MSC in Sepsis and Allogenic Heart Transplantation. *Stem Cells* (2020) 38:797–807. doi: 10.1002/stem.3165
46. Cheng A, Choi D, Lora M, Shum-Tim D, Rak J, Colmegna I. Human Multipotent Mesenchymal Stromal Cells Cytokine Priming Promotes RAB27B-Regulated Secretion of Small Extracellular Vesicles With Immunomodulatory Cargo. *Stem Cell Res Ther* (2020) 11:539. doi: 10.1186/s13287-020-02050-6
47. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, et al. Bone Marrow Mesenchymal Stem Cells Inhibit the Response of Naive and Memory Antigen-Specific T Cells to Their Cognate Peptide. *Blood* (2003) 101:3722–9. doi: 10.1182/blood-2002-07-2104
48. Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone Marrow Mesenchymal Stem Cells Induce Division Arrest Anergy of Activated T Cells. *Blood* (2005) 105:2821–7. doi: 10.1182/blood-2004-09-3696
49. Yu Y, Valderrama AV, Han Z, Uzan G, Naserian S, Oberlin E. Human Fetal Liver MSCs Are More Effective Than Adult Bone Marrow MSCs for Their Immunosuppressive, Immunomodulatory, and Foxp3(+) T Reg Induction Capacity. *Stem Cell Res Ther* (2021) 12:138. doi: 10.1186/s13287-021-02176-1
50. Franco da Cunha F, Andrade-Oliveira V, Candido de Almeida D, Borges da Silva T, Naffah de Souza Breda C, Costa Cruz M, et al. Extracellular Vesicles Isolated From Mesenchymal Stromal Cells Modulate CD4(+) T Lymphocytes Toward a Regulatory Profile. *Cells* (2020) 9:1059. doi: 10.3390/cells9041059
51. Lee S, Kim S, Chung H, Moon JH, Kang SJ, Park CG. Mesenchymal Stem Cell-Derived Exosomes Suppress Proliferation of T Cells by Inducing Cell Cycle Arrest Through P27kip1/Cdk2 Signaling. *Immunol Lett* (2020) 225:16–22. doi: 10.1016/j.imlet.2020.06.006
52. Bolandi Z, Mokhberian N, Eftekhary M, Sharifi K, Soudi S, Ghanbarian H, et al. Adipose Derived Mesenchymal Stem Cell Exosomes Loaded With miR-10a Promote the Differentiation of Th17 and Treg From Naive CD4(+) T Cell. *Life Sci* (2020) 259:118218. doi: 10.1016/j.lfs.2020.118218
53. Zhang Z, Huang S, Wu S, Qi J, Li W, Liu S, et al. Clearance of Apoptotic Cells by Mesenchymal Stem Cells Contributes to Immunosuppression *via* PGE2. *EBioMedicine* (2019) 45:341–50. doi: 10.1016/j.ebiom.2019.06.016
54. Wang D, Feng X, Lu L, Konkel JE, Zhang H, Chen Z, et al. A CD8 T Cell/Indoleamine 2,3-Dioxygenase Axis Is Required for Mesenchymal Stem Cell Suppression of Human Systemic Lupus Erythematosus. *Arthritis Rheumatol* (2014) 66:2234–45. doi: 10.1002/art.38674
55. Sun L, Akiyama K, Zhang H, Yamaza T, Hou Y, Zhao S, et al. Mesenchymal Stem Cell Transplantation Reverses Multiorgan Dysfunction in Systemic Lupus Erythematosus Mice and Humans. *Stem Cells* (2009) 27:1421–32. doi: 10.1002/stem.68
56. Wang D, Huang S, Yuan X, Liang J, Xu R, Yao G, et al. The Regulation of the Treg/Th17 Balance by Mesenchymal Stem Cells in Human Systemic Lupus Erythematosus. *Cell Mol Immunol* (2017) 14:423–31. doi: 10.1038/cmi.2015.89
57. Chiossone L, Conte R, Spaggiari GM, Serra M, Romei C, Bellora F, et al. Mesenchymal Stromal Cells Induce Peculiar Alternatively Activated Macrophages Capable of Dampening Both Innate and Adaptive Immune Responses. *Stem Cells* (2016) 34:1909–21. doi: 10.1002/stem.2369
58. Zhang B, Yeo RWY, Lai RC, Sim EWK, Chin KC, Lim SK. Mesenchymal Stromal Cell Exosome-Enhanced Regulatory T-Cell Production Through an Antigen-Presenting Cell-Mediated Pathway. *Cytotherapy* (2018) 20:687–96. doi: 10.1016/j.jcyt.2018.02.372
59. Bai L, Shao H, Wang H, Zhang Z, Su C, Dong L, et al. Effects of Mesenchymal Stem Cell-Derived Exosomes on Experimental Autoimmune Uveitis. *Sci Rep* (2017) 7:4323. doi: 10.1038/s41598-017-04559-y
60. Del Fattore A, Luciano R, Pascucci L, Goffredo BM, Giorda E, Scapaticci M, et al. Immunoregulatory Effects of Mesenchymal Stem Cell-Derived Extracellular Vesicles on T Lymphocytes. *Cell Transpl* (2015) 24:2615–27. doi: 10.3727/096368915X687543
61. Xu HK, Chen LJ, Zhou SN, Li YF, Xiang C. Multifunctional Role of microRNAs in Mesenchymal Stem Cell-Derived Exosomes in Treatment of Diseases. *World J Stem Cells* (2020) 12:1276–94. doi: 10.4252/wjsc.v12.i11.1276
62. Long H, Wang X, Chen Y, Wang L, Zhao M, Lu Q. Dysregulation of microRNAs in Autoimmune Diseases: Pathogenesis, Biomarkers and Potential Therapeutic Targets. *Cancer Lett* (2018) 428:90–103. doi: 10.1016/j.canlet.2018.04.016
63. Jin F, Hu H, Xu M, Zhan S, Wang Y, Zhang H, et al. Serum microRNA Profiles Serve as Novel Biomarkers for Autoimmune Diseases. *Front Immunol* (2018) 9:2381. doi: 10.3389/fimmu.2018.02381
64. Li W, Liu S, Chen Y, Weng R, Zhang K, He X, et al. Circulating Exosomal microRNAs as Biomarkers of Systemic Lupus Erythematosus. *Clinics (Sao Paulo)* (2020) 75:e1528. doi: 10.6061/clinics/2020/e1528
65. Dong C, Zhou Q, Fu T, Zhao R, Yang J, Kong X, et al. Circulating Exosomes Derived-miR-146a From Systemic Lupus Erythematosus Patients Regulates Senescence of Mesenchymal Stem Cells. *BioMed Res Int* (2019) 2019:6071308. doi: 10.1155/2019/6071308
66. Labib DA, Shaker OG, El Refai RM, Ghoniem SA, Elmazny A. Association Between miRNA-146a and Polymorphisms of Its Target Gene, IRAK1, Regarding Susceptibility to and Clinical Features of Systemic Lupus Erythematous and Multiple Sclerosis. *Lab Med* (2019) 50:34–41. doi: 10.1093/labmed/lmy033
67. Talaat RM, Mohamed SF, Bassyouni IH, Raouf AA. Th1/Th2/Th17/Treg Cytokine Imbalance in Systemic Lupus Erythematosus (SLE) Patients: Correlation With Disease Activity. *Cytokine* (2015) 72:146–53. doi: 10.1016/j.cyto.2014.12.027
68. Li X, Wang D, Liang J, Zhang H, Sun L. Mesenchymal SCT Ameliorates Refractory Cytopenia in Patients With Systemic Lupus Erythematosus. *Bone Marrow Transpl* (2013) 48:544–50. doi: 10.1038/bmt.2012.184
69. Chen Y, Yu Q, Hu Y, Shi Y. Current Research and Use of Mesenchymal Stem Cells in the Therapy of Autoimmune Diseases. *Curr Stem Cell Res Ther* (2019) 14:579–82. doi: 10.2174/1574888X14666190429141421
70. Wang AYL, Loh CYY, Shen HH, Hsieh SY, Wang IK, Lee CM, et al. Human Wharton's Jelly Mesenchymal Stem Cell-Mediated Sciatic Nerve Recovery Is Associated With the Upregulation of Regulatory T Cells. *Int J Mol Sci* (2020) 21:6310. doi: 10.3390/ijms21176310

71. Darlan DM, Munir D, Putra A, Jusuf NK. MSCs-Released TGFbeta1 Generate CD4(+)CD25(+)Foxp3(+) in T-Reg Cells of Human SLE PBMC. *J Formos Med Assoc* (2021) 120:602–8. doi: 10.1016/j.jfma.2020.06.028
72. Yang X, Yang J, Li X, Ma W, Zou H. Bone Marrow-Derived Mesenchymal Stem Cells Inhibit T Follicular Helper Cell in Lupus-Prone Mice. *Lupus* (2018) 27:49–59. doi: 10.1177/0961203317711013
73. Jang E, Jeong M, Kim S, Jang K, Kang BK, Lee DY, et al. Infusion of Human Bone Marrow-Derived Mesenchymal Stem Cells Alleviates Autoimmune Nephritis in a Lupus Model by Suppressing Follicular Helper T-Cell Development. *Cell Transpl* (2016) 25:1–15. doi: 10.3727/096368915X688173
74. Yap DYH, Chan TM. B Cell Abnormalities in Systemic Lupus Erythematosus and Lupus Nephritis-Role in Pathogenesis and Effect of Immunosuppressive Treatments. *Int J Mol Sci* (2019) 20:6231. doi: 10.3390/ijms20246231
75. Luo S, Ding S, Liao J, Zhang P, Liu Y, Zhao M, et al. Excessive miR-152-3p Results in Increased BAFF Expression in SLE B-Cells by Inhibiting the KLF5 Expression. *Front Immunol* (2019) 10:1127. doi: 10.3389/fimmu.2019.01127
76. Ma X, Che N, Gu Z, Huang J, Wang D, Liang J, et al. Allogenic Mesenchymal Stem Cell Transplantation Ameliorates Nephritis in Lupus Mice *via* Inhibition of B-Cell Activation. *Cell Transpl* (2013) 22:2279–90. doi: 10.3727/096368912X658692
77. Rosado MM, Bernardo ME, Scarsella M, Conforti A, Giorda E, Biagini S, et al. Inhibition of B-Cell Proliferation and Antibody Production by Mesenchymal Stromal Cells Is Mediated by T Cells. *Stem Cells Dev* (2015) 24:93–103. doi: 10.1089/scd.2014.0155
78. Luk F, Carreras-Planella L, Korevaar SS, de Witte SFH, Borras FE, Betjes MGH, et al. Inflammatory Conditions Dictate the Effect of Mesenchymal Stem or Stromal Cells on B Cell Function. *Front Immunol* (2017) 8:1042. doi: 10.3389/fimmu.2017.01042
79. Adamo A, Brandi J, Caligola S, Delfino P, Bazzoni R, Carusone R, et al. Extracellular Vesicles Mediate Mesenchymal Stromal Cell-Dependent Regulation of B Cell PI3K-AKT Signaling Pathway and Actin Cytoskeleton. *Front Immunol* (2019) 10:446. doi: 10.3389/fimmu.2019.00446
80. Yang L, Li N, Yang D, Chen A, Tang J, Jing Y, et al. CCL2 Regulation of MST1-mTOR-STAT1 Signaling Axis Controls BCR Signaling and B-Cell Differentiation. *Cell Death Differ* (2021) 28:2616–33. doi: 10.1038/s41418-021-00775-2
81. Asari S, Itakura S, Ferreri K, Liu CP, Kuroda Y, Kandeel F, et al. Mesenchymal Stem Cells Suppress B-Cell Terminal Differentiation. *Exp Hematol* (2009) 37:604–15. doi: 10.1016/j.exphem.2009.01.005
82. Dang J, Xu Z, Xu A, Liu Y, Fu Q, Wang J, et al. Human Gingiva-Derived Mesenchymal Stem Cells Are Therapeutic in Lupus Nephritis Through Targeting of CD39(-)CD73 Signaling Pathway. *J Autoimmun* (2020) 113:102491. doi: 10.1016/j.jaut.2020.102491
83. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human Mesenchymal Stem Cells Modulate B-Cell Functions. *Blood* (2006) 107:367–72. doi: 10.1182/blood-2005-07-2657
84. Qin Y, Zhou Z, Zhang F, Wang Y, Shen B, Liu Y, et al. Induction of Regulatory B-Cells by Mesenchymal Stem Cells Is Affected by SDF-1alpha-CXCR7. *Cell Physiol Biochem* (2015) 37:117–30. doi: 10.1159/000430338
85. Fan L, Hu C, Chen J, Cen P, Wang J, Li L. Interaction Between Mesenchymal Stem Cells and B-Cells. *Int J Mol Sci* (2016) 17:650. doi: 10.3390/ijms17050650
86. Carreras-Planella L, Monguio-Tortajada M, Borras FE, Franquesa M. Immunomodulatory Effect of MSC on B Cells Is Independent of Secreted Extracellular Vesicles. *Front Immunol* (2019) 10:1288. doi: 10.3389/fimmu.2019.01288
87. Wang T, Marken J, Chen J, Tran VB, Li QZ, Li M, et al. High TLR7 Expression Drives the Expansion of CD19(+)CD24(hi)CD38(hi) Transitional B Cells and Autoantibody Production in SLE Patients. *Front Immunol* (2019) 10:1243. doi: 10.3389/fimmu.2019.01243
88. Jenks SA, Cashman KS, Zumaquero E, Marigorta UM, Patel AV, Wang X, et al. Distinct Effector B Cells Induced by Unregulated Toll-Like Receptor 7 Contribute to Pathogenic Responses in Systemic Lupus Erythematosus. *Immunity* (2018) 49:725–39 e6. doi: 10.1016/j.immuni.2018.08.015
89. Chodisetti SB, Fike AJ, Domeier PP, Singh H, Choi NM, Corradetti C, et al. Type II But Not Type I IFN Signaling Is Indispensable for TLR7-Promoted Development of Autoreactive B Cells and Systemic Autoimmunity. *J Immunol* (2020) 204:796–809. doi: 10.4049/jimmunol.1901175
90. Asami T, Ishii M, Fujii H, Namkoong H, Tasaka S, Matsushita K, et al. Modulation of Murine Macrophage TLR7/8-Mediated Cytokine Expression by Mesenchymal Stem Cell-Conditioned Medium. *Mediators Inflamm* (2013) 2013:264260. doi: 10.1155/2013/264260
91. Ma K, Du W, Wang X, Yuan S, Cai X, Liu D, et al. Multiple Functions of B Cells in the Pathogenesis of Systemic Lupus Erythematosus. *Int J Mol Sci* (2019) 20:6021. doi: 10.3390/ijms20236021
92. Feng Y, Yang M, Wu H, Lu Q. The Pathological Role of B Cells in Systemic Lupus Erythematosus: From Basic Research to Clinical. *Autoimmunity* (2020) 53:56–64. doi: 10.1080/08916934.2019.1700232
93. Shahir M, Mahmoud Hashemi S, Asadirad A, Varahram M, Kazempour-Dizaji M, Folkerts G, et al. Effect of Mesenchymal Stem Cell-Derived Exosomes on the Induction of Mouse Tolerogenic Dendritic Cells. *J Cell Physiol* (2020) 235:7043–55. doi: 10.1002/jcp.29601
94. Wheat WH, Chow L, Kurihara JN, Regan DP, Coy JW, Webb TL, et al. Suppression of Canine Dendritic Cell Activation/Maturation and Inflammatory Cytokine Release by Mesenchymal Stem Cells Occurs Through Multiple Distinct Biochemical Pathways. *Stem Cells Dev* (2017) 26:249–62. doi: 10.1089/scd.2016.0199
95. Reis M, Mavin E, Nicholson L, Green K, Dickinson AM, Wang XN. Mesenchymal Stromal Cell-Derived Extracellular Vesicles Attenuate Dendritic Cell Maturation and Function. *Front Immunol* (2018) 9:2538. doi: 10.3389/fimmu.2018.02538
96. Yuan X, Qin X, Wang D, Zhang Z, Tang X, Gao X, et al. Mesenchymal Stem Cell Therapy Induces FLT3L and CD1c(+) Dendritic Cells in Systemic Lupus Erythematosus Patients. *Nat Commun* (2019) 10:2498. doi: 10.1038/s41467-019-10491-8
97. Ethokie JM, Tomic SZ, Colic MJ. Cross-Talk Between Mesenchymal Stem/Stromal Cells and Dendritic Cells. *Curr Stem Cell Res Ther* (2016) 11:51–65. doi: 10.2174/1574888X10666150904114035
98. Hackstein H, Tschipakow I, Bein G, Nold P, Brendel C, Baal N. Contact-Dependent Abrogation of Bone Marrow-Derived Plasmacytoid Dendritic Cell Differentiation by Murine Mesenchymal Stem Cells. *Biochem Biophys Res Commun* (2016) 476:15–20. doi: 10.1016/j.bbrc.2016.05.108
99. Di Trapani M, Bassi G, Midolo M, Gatti A, Kamga PT, Cassaro A, et al. Differential and Transferable Modulatory Effects of Mesenchymal Stromal Cell-Derived Extracellular Vesicles on T, B and NK Cell Functions. *Sci Rep* (2016) 6:24120. doi: 10.1038/srep24120
100. Wang Y, Liang J, Qin H, Ge Y, Du J, Lin J, et al. Elevated Expression of miR-142-3p Is Related to the Pro-Inflammatory Function of Monocyte-Derived Dendritic Cells in SLE. *Arthritis Res Ther* (2016) 18:263. doi: 10.1186/s13075-016-1158-z
101. Lin SJ, Kuo ML, Hsiao HS, Lee PT, Lee WI, Chen JY, et al. Cytotoxic Function and Cytokine Production of Natural Killer Cells and Natural Killer T-Like Cells in Systemic Lupus Erythematosis Regulation With Interleukin-15. *Mediat Inflamm* (2019) 2019:4236562. doi: 10.1155/2019/4236562
102. Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. Interactions Between Human Mesenchymal Stem Cells and Natural Killer Cells. *Stem Cells* (2006) 24:74–85. doi: 10.1634/stemcells.2004-0359
103. Hu CD, Kosaka Y, Marcus P, Rashedi I, Keating A. Differential Immunomodulatory Effects of Human Bone Marrow-Derived Mesenchymal Stromal Cells on Natural Killer Cells. *Stem Cells Dev* (2019) 28:933–43. doi: 10.1089/scd.2019.0059
104. Chatterjee D, Marquardt N, Tufa DM, Beauclair G, Low HZ, Hatlapatka T, et al. Role of Gamma-Secretase in Human Umbilical-Cord Derived Mesenchymal Stem Cell Mediated Suppression of NK Cell Cytotoxicity. *Cell Commun Signal* (2014) 12:63. doi: 10.1186/s12964-014-0063-9
105. Najar M, Fayyad-Kazan M, Meuleman N, Bron D, Fayyad-Kazan H, Lagneaux L. Mesenchymal Stromal Cells of the Bone Marrow and Natural Killer Cells: Cell Interactions and Cross Modulation. *J Cell Commun Signal* (2018) 12:673–88. doi: 10.1007/s12079-018-0448-4
106. Gao L, OC M, Allen M, Liesveld J, McDavid A, Anolik JH, et al. Bone Marrow Mesenchymal Stem Cells From Patients With SLE Maintain an Interferon Signature During *In Vitro* Culture. *Cytokine* (2020) 132:154725. doi: 10.1016/j.cyto.2019.05.012

107. Lu Y, Liu J, Liu Y, Qin Y, Luo Q, Wang Q, et al. TLR4 Plays a Crucial Role in MSC-Induced Inhibition of NK Cell Function. *Biochem Biophys Res Commun* (2015) 464:541–7. doi: 10.1016/j.bbrc.2015.07.002
108. Fan Y, Herr F, Vernochet A, Mennesson B, Oberlin E, Durrbach A. Human Fetal Liver Mesenchymal Stem Cell-Derived Exosomes Impair Natural Killer Cell Function. *Stem Cells Dev* (2019) 28:44–55. doi: 10.1089/scd.2018.0015
109. Cui R, Rekasi H, Hepner-Schefczyk M, Fessmann K, Petri RM, Bruderek K, et al. Human Mesenchymal Stromal/Stem Cells Acquire Immunostimulatory Capacity Upon Cross-Talk With Natural Killer Cells and Might Improve the NK Cell Function of Immunocompromised Patients. *Stem Cell Res Ther* (2016) 7:88. doi: 10.1186/s13287-016-0353-9
110. Chatterjee D, Marquardt N, Tufa DM, Hatlapatka T, Hass R, Kasper C, et al. Human Umbilical Cord-Derived Mesenchymal Stem Cells Utilize Activin-A to Suppress Interferon-Gamma Production by Natural Killer Cells. *Front Immunol* (2014) 5:662. doi: 10.3389/fimmu.2014.00662
111. Ma C, Xia Y, Yang Q, Zhao Y. The Contribution of Macrophages to Systemic Lupus Erythematosus. *Clin Immunol* (2019) 207:1–9. doi: 10.1016/j.clim.2019.06.009
112. Liu F, Qiu H, Xue M, Zhang S, Zhang X, Xu J, et al. MSC-Secreted TGF-Beta Regulates Lipopolysaccharide-Stimulated Macrophage M2-Like Polarization *via* the Akt/FoxO1 Pathway. *Stem Cell Res Ther* (2019) 10:345. doi: 10.1186/s13287-019-1447-y
113. Selleri S, Bifsha P, Civini S, Pacelli C, Dieng MM, Lemieux W, et al. Human Mesenchymal Stromal Cell-Secreted Lactate Induces M2-Macrophage Differentiation by Metabolic Reprogramming. *Oncotarget* (2016) 7:30193–210. doi: 10.18632/oncotarget.8623
114. Hyvarinen K, Holopainen M, Skirdenko V, Ruhanen H, Lehenkari P, Korhonen M, et al. Mesenchymal Stromal Cells and Their Extracellular Vesicles Enhance the Anti-Inflammatory Phenotype of Regulatory Macrophages by Downregulating the Production of Interleukin (IL)-23 and IL-22. *Front Immunol* (2018) 9:771. doi: 10.3389/fimmu.2018.00771
115. Lo Sicco C, Reverberi D, Balbi C, Ulivi V, Principi E, Pascucci L, et al. Mesenchymal Stem Cell-Derived Extracellular Vesicles as Mediators of Anti-Inflammatory Effects: Endorsement of Macrophage Polarization. *Stem Cells Transl Med* (2017) 6:1018–28. doi: 10.1002/sctm.16-0363
116. He X, Dong Z, Cao Y, Wang H, Liu S, Liao L, et al. MSC-Derived Exosome Promotes M2 Polarization and Enhances Cutaneous Wound Healing. *Stem Cells Int* (2019) 2019:7132708. doi: 10.1155/2019/7132708
117. Xin D, Li T, Chu X, Ke H, Yu Z, Cao L, et al. Mesenchymal Stromal Cell-Derived Extracellular Vesicles Modulate Microglia/Macrophage Polarization and Protect the Brain Against Hypoxia-Ischemic Injury in Neonatal Mice by Targeting Delivery of miR-21a-5p. *Acta Biomater* (2020) 113:597–613. doi: 10.1016/j.actbio.2020.06.037
118. Chen X, Wei Q, Sun H, Zhang X, Yang C, Tao Y, et al. Exosomes Derived From Human Umbilical Cord Mesenchymal Stem Cells Regulate Macrophage Polarization to Attenuate Systemic Lupus Erythematosus-Associated Diffuse Alveolar Hemorrhage in Mice. *Int J Stem Cells* (2021) 14:331–40. doi: 10.15283/ijsc20156
119. Shi D, Wang D, Li X, Zhang H, Che N, Lu Z, et al. Allogeneic Transplantation of Umbilical Cord-Derived Mesenchymal Stem Cells for Diffuse Alveolar Hemorrhage in Systemic Lupus Erythematosus. *Clin Rheumatol* (2012) 31:841–6. doi: 10.1007/s10067-012-1943-2
120. Vasandan AB, Jahnavi S, Shashank C, Prasad P, Kumar A, Prasanna SJ. Human Mesenchymal Stem Cells Program Macrophage Plasticity by Altering Their Metabolic Status *via* a PGE2-Dependent Mechanism. *Sci Rep* (2016) 6:38308. doi: 10.1038/srep38308
121. Xiong H, Guo Z, Tang Z, Ai X, Qi Q, Liu X, et al. Mesenchymal Stem Cells Activate the MEK/ERK Signaling Pathway and Enhance DNA Methylation *via* DNMT1 in PBMC From Systemic Lupus Erythematosus. *BioMed Res Int* (2020) 2020:4174082. doi: 10.1155/2020/4174082
122. Liu W, Rong Y, Wang J, Zhou Z, Ge X, Ji C, et al. Exosome-Shuttled miR-216a-5p From Hypoxic Preconditioned Mesenchymal Stem Cells Repair Traumatic Spinal Cord Injury by Shifting Microglial M1/M2 Polarization. *J Neuroinflammation* (2020) 17:47. doi: 10.1186/s12974-020-1726-7
123. Fan B, Li C, Szalad A, Wang L, Pan W, Zhang R, et al. Mesenchymal Stromal Cell-Derived Exosomes Ameliorate Peripheral Neuropathy in a Mouse Model of Diabetes. *Diabetologia* (2020) 63:431–43. doi: 10.1007/s00125-019-05043-0
124. Riazifar M, Mohammadi MR, Pone EJ, Yeri A, Lasser C, Segaliny AI, et al. Stem Cell-Derived Exosomes as Nanotherapeutics for Autoimmune and Neurodegenerative Disorders. *ACS Nano* (2019) 13:6670–88. doi: 10.1021/acsnano.9b01004
125. Zhao J, Li X, Hu J, Chen F, Qiao S, Sun X, et al. Mesenchymal Stromal Cell-Derived Exosomes Attenuate Myocardial Ischaemia-Reperfusion Injury Through miR-182-Regulated Macrophage Polarization. *Cardiovasc Res* (2019) 115:1205–16. doi: 10.1093/cvr/cvz040
126. Philipp D, Suhr L, Wahlers T, Choi YH, Paunel-Gorgulu A. Preconditioning of Bone Marrow-Derived Mesenchymal Stem Cells Highly Strengthens Their Potential to Promote IL-6-Dependent M2b Polarization. *Stem Cell Res Ther* (2018) 9:286. doi: 10.1186/s13287-018-1039-2
127. Deng W, Chen W, Zhang Z, Huang S, Kong W, Sun Y, et al. Mesenchymal Stem Cells Promote CD206 Expression and Phagocytic Activity of Macrophages Through IL-6 in Systemic Lupus Erythematosus. *Clin Immunol* (2015) 161:209–16. doi: 10.1016/j.clim.2015.07.011
128. Wallace DJ, Ginzler EM, Merrill JT, Furie RA, Stohl W, Chatham WW, et al. Safety and Efficacy of Belimumab Plus Standard Therapy for Up to Thirteen Years in Patients With Systemic Lupus Erythematosus. *Arthritis Rheumatol* (2019) 71:1125–34. doi: 10.1002/art.40861
129. Hajkova M, Hermankova B, Javorkova E, Bohacova P, Zajicova A, Holan V, et al. Mesenchymal Stem Cells Attenuate the Adverse Effects of Immunosuppressive Drugs on Distinct T Cell Subopulations. *Stem Cell Rev Rep* (2017) 13:104–15. doi: 10.1007/s12015-016-9703-3
130. Lee HK, Kim KH, Kim HS, Kim JS, Lee JH, Ji A, et al. Effect of a Combination of Prednisone or Mycophenolate Mofetil and Mesenchymal Stem Cells on Lupus Symptoms in MRL.Fas(lpr) Mice. *Stem Cells Int* (2018) 2018:4273107. doi: 10.1155/2018/4273107
131. Hwang JW, Myeong SH, Lee NH, Kim H, Son HJ, Chang JW, et al. Immunosuppressant Drugs Mitigate Immune Responses Generated by Human Mesenchymal Stem Cells Transplanted Into the Mouse Parenchyma. *Cell Transpl* (2021) 30:9636897211019025. doi: 10.1177/09636897211019025
132. Zhu Y, Feng X. Genetic Contribution to Mesenchymal Stem Cell Dysfunction in Systemic Lupus Erythematosus. *Stem Cell Res Ther* (2018) 9:149. doi: 10.1186/s13287-018-0898-x
133. Wang D, Zhang H, Liang J, Wang H, Hua B, Feng X, et al. A Long-Term Follow-Up Study of Allogeneic Mesenchymal Stem/Stromal Cell Transplantation in Patients With Drug-Resistant Systemic Lupus Erythematosus. *Stem Cell Rep* (2018) 10:933–41. doi: 10.1016/j.stemcr.2018.01.029
134. Gu F, Molano I, Ruiz P, Sun L, Gilkeson GS. Differential Effect of Allogeneic *Versus* Syngeneic Mesenchymal Stem Cell Transplantation in MRL/lpr and (NZB/NZW)F1 Mice. *Clin Immunol* (2012) 145:142–52. doi: 10.1016/j.clim.2012.08.012
135. Deng D, Zhang P, Guo Y, Lim TO. A Randomised Double-Blind, Placebo-Controlled Trial of Allogeneic Umbilical Cord-Derived Mesenchymal Stem Cell for Lupus Nephritis. *Ann Rheum Dis* (2017) 76:1436–9. doi: 10.1136/annrheumdis-2017-211073
136. Jang SG, Lee J, Hong SM, Kwok SK, Cho ML, Park SH. Metformin Enhances the Immunomodulatory Potential of Adipose-Derived Mesenchymal Stem Cells Through STAT1 in an Animal Model of Lupus. *Rheumatol (Oxford)* (2020) 59:1426–38. doi: 10.1093/rheumatology/kez631
137. Zhou K, Zhang H, Jin O, Feng X, Yao G, Hou Y, et al. Transplantation of Human Bone Marrow Mesenchymal Stem Cell Ameliorates the Autoimmune Pathogenesis in MRL/lpr Mice. *Cell Mol Immunol* (2008) 5:417–24. doi: 10.1038/cmi.2008.52
138. Tang X, Li W, Wen X, Zhang Z, Chen W, Yao G, et al. Transplantation of Dental Tissue-Derived Mesenchymal Stem Cells Ameliorates Nephritis in Lupus Mice. *Ann Transl Med* (2019) 7:132. doi: 10.21037/atm.2019.02.41
139. Wu KH, Cheng CC, Li JP, Weng TF, Yang SF, Pan HH, et al. Toll-Like Receptor Signalling Associated With Immunomodulation of Umbilical Cord-Derived Mesenchymal Stem Cells in Mice With Systemic Lupus Erythematosus. *Lupus* (2020) 29:165–75. doi: 10.1177/0961203319898532
140. Wang D, Zhang H, Liang J, Li X, Feng X, Wang H, et al. Allogeneic Mesenchymal Stem Cell Transplantation in Severe and Refractory Systemic Lupus Erythematosus: 4 Years of Experience. *Cell Transpl* (2013) 22:2267–77. doi: 10.3727/096368911X582769c

141. Zhou T, Li HY, Liao C, Lin W, Lin S. Clinical Efficacy and Safety of Mesenchymal Stem Cells for Systemic Lupus Erythematosus. *Stem Cells Int* (2020) 2020:6518508. doi: 10.1155/2020/6518508
142. Wang D, Li J, Zhang Y, Zhang M, Chen J, Li X, et al. Umbilical Cord Mesenchymal Stem Cell Transplantation in Active and Refractory Systemic Lupus Erythematosus: A Multicenter Clinical Study. *Arthritis Res Ther* (2014) 16:R79. doi: 10.1186/ar4520
143. Sun L, Wang D, Liang J, Zhang H, Feng X, Wang H, et al. Umbilical Cord Mesenchymal Stem Cell Transplantation in Severe and Refractory Systemic Lupus Erythematosus. *Arthritis Rheumatol* (2010) 62:2467–75. doi: 10.1002/art.27548
144. Gu F, Wang D, Zhang H, Feng X, Gilkeson GS, Shi S, et al. Allogeneic Mesenchymal Stem Cell Transplantation for Lupus Nephritis Patients Refractory to Conventional Therapy. *Clin Rheumatol* (2014) 33:1611–9. doi: 10.1007/s10067-014-2754-4
145. Choi EW, Shin IS, Park SY, Park JH, Kim JS, Yoon EJ, et al. Reversal of Serologic, Immunologic, and Histologic Dysfunction in Mice With Systemic Lupus Erythematosus by Long-Term Serial Adipose Tissue-Derived Mesenchymal Stem Cell Transplantation. *Arthritis Rheumatol* (2012) 64:243–53. doi: 10.1002/art.33313
146. Sharma J, Hampton JM, Valiente GR, Wada T, Steigelman H, Young MC, et al. Therapeutic Development of Mesenchymal Stem Cells or Their Extracellular Vesicles to Inhibit Autoimmune-Mediated Inflammatory Processes in Systemic Lupus Erythematosus. *Front Immunol* (2017) 8:526. doi: 10.3389/fimmu.2017.00526

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What Basophil Testing Tells Us About CSU Patients – Results of the CORSA Study

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Basophil testing is the most effective single approach for diagnosing type-IIb autoimmune chronic spontaneous urticaria (TIIbaiCSU). A positive basophil test has been linked to long disease duration, higher disease activity, a poor response to antihistamines and omalizumab, and a better response to cyclosporine and fenebrutinib. As of now it is unclear what other features are connected to a positive basophil test in chronic spontaneous urticaria (CSU). We aimed to identify features of basophil test-positive CSU patients. We performed a cross-sectional study of 85 CSU patients. Basophil testing was done with the basophil activation test (BAT) and the basophil histamine release assay (BHRA). Data were analysed using SPSS: Student's t-test, Chi-square test, Odds Ratio, Spearman's correlation test. Of 85 CSU patients, 44% and 28% tested positive with the BAT and BHRA, respectively. These patients showed higher disease activity and impact, lower levels of disease control and total serum IgE, as well as higher rates of having a positive autologous serum skin test (ASST), angioedema, nocturnal symptoms, symptoms for >5 days/week, and thyroid autoantibodies. The ASST, by itself, was not a good predictor of basophil test results, but it predicted a positive basophil test in up to 100% of cases when combined with angioedema, thyroid autoantibodies or low IgE. In conclusion, a positive basophil test is linked to known features of TIIbaiCSU and novel characteristics including nocturnal symptoms. Further studies on basophil test-positive and -negative CSU patients can help to better understand CSU endotypes and to develop better management approaches.

Keywords: angioedema, autologous serum skin test (ASST), basophil activation test (BAT), basophil histamine release test, chronic spontaneous urticaria (CSU), anti-thyroperoxidase (anti-TPO), IgE (immunoglobulin E)

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INTRODUCTION

Chronic spontaneous urticaria (CSU) is a common and debilitating disease, affecting adults and children, with a marked impact on patients' quality of life (1–5). It is defined by the spontaneous appearance of transient itchy wheals (hives), angioedema, or both for more than 6 weeks (1–6). Recent advances have characterized CSU as an autoantibody-driven disease, where mast cells and basophils in the skin are activated through two distinct pathways (4, 5).

In type I autoimmune CSU (TIaiCSU, also called autoallergic CSU), IgE autoantibodies are cross-linked by self-antigens, for example thyroid peroxidase or interleukin 24 (2–5). In type IIb autoimmune CSU (TIIbaiCSU), IgG and IgM autoantibodies are directed against IgE receptors (or IgE itself) on the surface of mast cells and basophils (1–7).

In clinical practice, it is important to know if a patient has TIIbaiCSU, as TIaiCSU and TIIbaiCSU present distinct phenotypes. Patients with TIIbaiCSU have been shown to have an increased risk of developing other autoimmune diseases, of failing antihistamine and omalizumab treatment, and of having a better response to cyclosporine and fenebrutinib (8–11). They have also been linked to higher disease activity and impact, making effective treatment both critical and challenging. The identification of further characteristics that characterize both subgroups is still a matter of ongoing research (4).

TIIbaiCSU is diagnosed by the combination of three tests, i.e. the autologous serum skin test (ASST), the basophil activation test (BAT) or the basophil histamine release assay (BHRA), and an ELISA or Western Blot-based autoantibody assay (1). Testing of all CSU patients for TIIBaiCSU with these three test is difficult, for several reasons, including their availability and the high and increasing prevalence of CSU, of up to 1.4% (12).

Basophil testing, with the BAT or the BHRA, is the single best diagnostic test for TIIbaiCSU. The recent PURIST study found that the BAT and the BHRA were 69% and 88% predictive of TIIbaiCSU, respectively (4). In addition, several reports show that the BAT/BHRA alone can identify patients with higher disease activity, longer disease duration, and poorer response to omalizumab (1, 9, 13, 14). In contrast, the ASST, initially thought off as a good, inexpensive, globally available marker of TIIbaiCSU, is only 27% predictive of TIIbaiCSU (4). Moreover, the ASST is influenced by the intake of antihistamines (the first-line treatment and a treatment difficult to interrupt in patients with severe urticaria), it does not give a quantifiable result, and handling of biologic samples requires exceptional care, as reinjecting patients' sera has important safety requirements (15).

Basophil testing of patients with CSU is performed at many urticaria centers of reference and excellence [UCAREs (16)] and by a limited number of commercial vendors. Finding ways of selecting patients who benefit the most from performing TIIbaiCSU tests are needed; as diagnosing CSU patients with TIIbaiCSU has real implications for the patient and the physician.

The present CORSA (component-resolved screening for autoimmune chronic spontaneous urticaria) study has two main objectives: 1) to confirm known and identify as of yet unknown features of BAT/BHRA-positive CSU patients, 2) to identify combinations of clinical and laboratory markers of CSU patients that can help to guide patient selection for basophil testing.

Abbreviations: CSU, Chronic spontaneous urticaria; BAT, basophil activation test; BHRA, basophil histamine release assay; ASST, autologous serum skin test; UAS 7, urticaria activity score 7; DLQI, dermatology life quality index; UCT, urticaria control test; anti-TPO, anti-thyroperoxidase autoantibody; anti-Tg, anti-thyroglobulin autoantibody; TIaiCSU, type I autoimmune CSU; TIIbaiCSU, type IIb autoimmune CSU; ANA, antinuclear antibody; anti-ds-DNA, anti-double stranded DNA antibody; TsIgE, total serum IgE.

MATERIALS AND METHODS

Patient Selection and Study Conduct

We performed a cross-sectional, single-centre study of 85 patients with CSU with daily symptoms. Patients were evaluated during a 1-year follow-up to evaluate the therapy needed to achieve CSU control. Patients were considered eligible if they had active CSU, and if they were not, nor had ever been, on omalizumab or cyclosporine. Patients were excluded if they had not performed all 3 of the following: the ASST, the BAT and the BHRA.

The CORSA study was approved by the corresponding ethics committee: *Comissão de Ética do Centro Hospitalar Universitário Lisboa Norte e Centro Académico Médico de Lisboa* (Ethics Committee authorization references 129/17 and 339/19). The study was conducted according to the Declaration of Helsinki, Good Clinical Practice and local regulations. All patients provided written informed consent.

Clinical Assessment of Patients

Data collected included: 1) date of CSU onset, 2) CSU duration, 3) presence of angioedema, 4) hives duration, 5) number of symptomatic days per week, 6) therapy necessary to achieve control (evaluated 12 months after patient enrolment), 7) presence of comorbid autoimmune diseases, 8) CSU activity as assessed with the Urticaria Activity Score 7 [UAS7 (9, 17)] CSU impact, evaluated with the Dermatology Life Quality Index [DLQI (18, 19)], and (8) CSU control per the Urticaria Control Test [UCT (20)]. The DLQI and UCT questionnaires were filled on the day of the ASST, and the UAS7 was calculated for the week prior to the ASST.

The ASST and BAT were performed according to clinical practice, and not for the purposes of the study. The BHRA was performed for this study from surplus serum collected from the patients.

Additional Measurements

Additional assessments included patient age, gender, total serum IgE, C3, C4, CH50, thyroid-stimulating hormone, free T4, thyroid auto-antibodies (anti-Tg and anti-TPO), ANA, anti-dsDNA, complete blood count, C-reactive protein, erythrocyte sedimentation rate, protein electrophoresis, immunoglobulins, liver and kidney function.

Patient history information was collected on a first visit to our urticaria outpatient clinic at our Immunoallergology Department. A second visit was scheduled within one month of the first visit. Patients were instructed to stop any antihistamine medication for 5 days and any systemic corticosteroids for at least 2 weeks prior to their second visit. On their second visit, venous blood was drawn to perform the ASST. The same blood was used to perform the BAT, the BHRA and the remaining blood tests. The UAS7, DLQI and UCT were collected on their second visit as well.

The Autologous Serum Skin Test (ASST)

The ASST was performed as previously reported (15, 21, 22). Venous blood was collected on the day of the test, centrifuged and the serum was used to perform the ASST (the same serum was also used to perform the BAT and BHRA). An intradermal test was performed, by injecting 50 µl of serum with an insulin needle and reading after 30 minutes. The test was considered positive when the diameter of the serum induced wheal was ≥1.5mm larger than that induced by saline and erythema was present.

The Basophil Activation Test (BAT)

The BAT was carried out by the Laboratory of Clinical Immunology, Faculdade de Medicina, Instituto de Medicina Molecular, Universidade de Lisboa, Lisbon, Portugal. The serum from the patients was exposed to heat complement-inactivation, for 30 minutes, before being tested at dilutions of 1:1, 1:5, 1:10. A positive control with N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP) and anti-FcεRI, and a negative control with saline solution (NaCl 0,9%), were used. IL-3 (stimulation buffer) and donor basophils were added to each tube, and double staining was performed with anti-CCR3-PE and anti-CD63-FITC monoclonal antibodies (Bühlmann, Switzerland) and incubated at 37°C for 15 minutes. Afterwards, erythrocytes were lysed for 10 minutes, and then the samples were washed (PBS buffer) and resuspended with the same solution. Data acquisition was performed by flow cytometry FACSCalibur (Becton-Dickinson Immunocytometry System, CA). The basophil population was identified as CCR3+ cells, and basophil activation was expressed as a proportion of CD63+ basophils corrected for the negative control and as a ratio of CD63 (%) of activated cells and negative control – stimulation index (SI). Data analysis was performed by Flow Jo (TreeStar, Ashland, or USA). Each serum was tested with basophils from three donors and the results were considered positive when there was a basophil activation percentage ≥ 5% activation, in response to the 1:1 serum dilution, and a stimulation index (SI) ≥2 in the basophils of at least one donor.

The Basophil Histamine Release Assay (BHRA)

The BHRA was carried out by RefLab ApS, Copenhagen, Denmark. Four buffy coats (obtained from the Danish National Hospital; Rigshospitalet, Copenhagen, Denmark), containing healthy donor basophils, were stored at 2-8°C overnight with IL-3 in a final concentration of 1 ng/ml. Buffy coats were washed in saline the following day and surface IgE was partially removed using a stripping buffer (pH 3.6, RefLab, Copenhagen, Denmark) before the cells were resuspended in Pipes buffer (RefLab, Copenhagen, Denmark). Stripped buffy coats were incubated at 37°C for 60 min with 20% patient serum and supernatants were then extracted for histamine quantification in the histamine release (HR) assay: Glass fiber-coated microtiter plates were loaded with 25 µl of each supernatant and incubated for 1 hour before histamine was measured using the ortho-phthaldialdehyde method and a highly sensitive fluorometer (Histareader 501, RefLab, Copenhagen, Denmark) according to RefLab instructions. The total histamine content was determined by lysing the basophils using 7% perchloric acid, and the histamine release was expressed as a percentage of the total. A response >16.5% of the spontaneous release was considered positive.

Statistical Analysis

Data were analysed using SPSS software version 22.0 (IBM Corporation, New York, USA). Laboratory and clinical data of patients were compared using students t-test for numerical data and chi-square test and odds ratio test for categorical data. Correlation between variables was calculated using the Spearman's correlation test.

The characteristics identified by the chi-square and student tests were used and combined to identify basophil test positive patients. For this analysis, we calculated the values of sensitivity, specificity, positive and negative predictive values for our sample.

Based on the data results, a decision tree was built using the CHAID algorithm (qui-squared automatic interaction detection) to enhance the ability to identify basophil test positive patients. The sensitivity, specificity, positive and negative predictive values were also calculated for these results.

In case of missing data, cases were excluded analysis by analysis, recalculating the N for the existing values. Results were reported as significant when the p was less than 0.05.

RESULTS

The BAT, BHRA, or Both Are Positive in a Significant Subset of CSU Patients

Of the 85 patients with CSU (81% female, average age of 46 ± 16 years), 37 (44%) and 24 (28%) were positive in the basophil activation test (BAT) and the basophil histamine release assay (BHRA), respectively. Both tests showed a significant correlation ($r=0.43$, $p<0.05$), with a 70% match of the results (with 21% of all patients showing double positivity and 49% of all patients showing double negativity).

CSU Patients With a Positive BAT or BHRA More Often Have Angioedema, Nocturnal Symptoms, and Wheals on Five or More Days per Week

Three clinical characteristics were statistically more frequent ($p<0.05$) in BAT-positive and in BHRA-positive compared to BAT/BHRA-negative patients (**Table 1**): 1) the occurrence of angioedema (BAT: 62% *vs* 35%; BHRA: 67% *vs* 39%); 2) the occurrence of nocturnal symptoms, sometimes causing

premature awakening (BAT: 70% *vs* 50%; BHRA: 79% *vs* 51%); and 3) the presence of symptoms on at least five days per week (BAT: 92% *vs* 65%; BHRA: 92% *vs* 70%).

CSU Patients With a Positive BAT or BHRA Have Higher Disease Activity and Impact, as Well as Higher Rates of Uncontrolled Disease

BAT-positive and BHRA-positive CSU patients had significantly higher disease activity as assessed by the UAS7 ($p<0.05$): 21.1 ± 9.7 *vs* 15.7 ± 10.6 for the BAT; and 22.2 ± 8.3 *vs* 16.4 ± 10.9 for the BHRA (**Table 2**). UAS7 values correlated ($p<0.05$), albeit weakly, with basophil activation in the BAT ($r=0.130$) and BHRA ($r=0.230$); the higher the disease activity, the greater the degree of basophil activation.

Disease impact, i.e. quality of life impairment assessed with the DLQI, was higher in CSU patients with a positive BAT or BHRA ($p<0.05$): 9.3 ± 6.8 *vs* 6.5 ± 5.6 for the BAT; and 10.3 ± 6.1 *vs* 6.7 ± 6.1 for the BHRA. CSU patients with a positive BAT or BHRA showed lower levels of disease control, as assessed by the UCT ($p<0.05$): 7.8 ± 4.1 *vs* 9.3 ± 3.9 for the BAT, and 7.7 ± 3.9 *vs* 9.0 ± 4.1 for the BHRA.

CSU Patients With a Positive BAT/BHRA Have Lower Levels of Total Serum IgE and Higher Rates of Thyroid Autoantibodies

BAT-positive and BHRA-positive CSU patients had significantly lower levels of total serum IgE as compared to negative patients ($p<0.05$): 91 ± 91 *vs* 395 ± 961 U/mL and 74 ± 69 *vs* 335 ± 858 U/ml, respectively (**Table 2**). In fact, the rate of CSU patients with a total serum IgE below 30 U/mL was significantly higher in BAT-positive and BHRA-positive patients ($p<0.05$): 41% *vs* 9% and 38% *vs* 17%, respectively (**Table 1**).

The rates of patients with autoantibodies to thyroid peroxidase (IgG-anti-TPO) or to thyroglobulin (IgG-anti-Tg) were higher in BAT-positive or BHRA-positive CSU patients, although this was not statistically significant: 35% *vs* 19% for the BAT, and 38% *vs* 22% for BHRA (**Table 1**). Also, the ratio of IgG-anti-TPO to total serum IgE was higher in BAT or BHRA positive patients: 5.7 ± 16.4 *vs* 0.2 ± 1.0 for the BAT ($p<0.05$), and 6.2 ± 17.1 *vs* 1.2 ± 7.1 for the BHRA ($p=0.192$; **Table 2**).

Autologous Serum Skin Testing Does Not Improve Patient Profiling by Basophil Testing

Basophil test-positive patients who also tested positive in the autologous serum skin test (ASST), when compared to those who were negative for both, showed a similar profile to the comparison between patients who were basophil test-positive and basophil test-negative, regardless of their ASST result, i.e. higher disease activity (UAS7 22.3 ± 9.5 *vs* 15.0 ± 10.3, $p<0.05$), higher disease impact (DLQI 9.7 ± 7.6 *vs* 6.2 ± 5.6, $p<0.05$), lower disease control (UCT 7.3 ± 4.2 *vs* 9.8 ± 3.4, $p<0.05$), lower total serum IgE (85.6 ± 97.8 *vs* 455.1 ± 1090.5, $p<0.05$), and higher ratio of IgG-anti-TPO to total serum IgE (3.0 ± 6.7 *vs* 0.3 ± 1.1, $p<0.05$). The combined use of basophil and autologous serum

TABLE 1 | Patients clinical and laboratorial data, according to the BAT and BHRA test result.

	BAT				BHRA			
	+ BAT (n=37)	- BAT (n=48)	Chi-square	Odds ratio	+ BHRA (n=24)	- BHRA (n=61)	Chi-square	Odds ratio
Positive ASST	23/37 (62%)	7/48 (15%)	**p<0.05**	9.622 (3.397-27.254)	13/24 (54%)	17/61 (28%)	**p<0.05**	3.059 (1.149-8.140)
Male Gender	7/37 (19%)	9/48 (19%)	p=0.984	1.011 (0.338-3.027)	4/24 (17%)	12/61 (20%)	p=0.750	0.817 (0.235-2.837)
Presence of angioedema	23/37 (62%)	17/48 (35%)	**p<0.05**	2.996 (1.231-7.292)	16/24 (67%)	24/61 (39%)	**p<0.05**	3.083 (1.143-8.315)
Presence of nocturnal CSU symptoms	26/37 (70%)	24/48 (50%)	**p<0.05**	2.364 (0.957-5.837)	19/24 (79%)	31/61 (51%)	**p<0.05**	3.677 (1.217-11.110)
Symptoms for ≥5 days/week	34/37 (92%)	31/48 (65%)	**p<0.05**	6.215 (1.660-23.274)	22/24 (92%)	43/61 (70%)	**p<0.05**	4.605 (0.979-21.664)
CSU for >6 months prior to the ASST	33/37 (89%)	44/48 (92%)	p=0.698	0.750 (0.175-3.222)	22/24 (92%)	55/61 (90%)	p=0.831	1.200 (0.225-6.406)
Hives duration >5 hours	18/37 (49%)	18/48 (38%)	p=0.302	1.579 (0.661-3.769)	13/24 (54%)	23/61 (37%)	p=0.167	1.953 (0.751-5.076)
Presence of anti-TPO/Tg autoantibodies	13/37 (35%)	9/47 (19%)	p=0.098	2.287 (0.848-6.165)	9/24 (38%)	13/60 (22%)	p=0.136	2.169 (0.775-6.074)
Presence of fT4/TSH abnormalities	7/37 (19%)	3/47 (6%)	p=0.078	3.422 (0.819-14.299)	3/24 (13%)	7/60 (12%)	p=0.915	1.082 (0.255-4.583)
Presence of ANA/anti-dsDNA autoantibodies	5/34 (15%)	4/47 (9%)	p=0.381	1.853 (0.459-7.490)	2/23 (9%)	7/58 (12%)	p=0.663	0.694 (0.133-3.619)
Presence of altered C3	2/33 (6%)	4/46 (9%)	p=0.663	0.677 (0.117-3.936)	3/23 (13%)	3/56 (5%)	p=0.208	2.842 (0.528-15.303)
Presence of altered C4	0/33 (0%)	2/46 (4%)	p=0.225	0.957 (0.899-1.017)	0/23 (0%)	2/56 (4%)	p=0.374	0.965 (0.918-1.014)
Presence of altered CH50	7/32 (22%)	9/45 (20%)	p=0.842	1.120 (0.368-3.404)	5/23 (22%)	11/54 (20%)	p=0.790	1.176 (0.356-3.891)
IgE<30 U/mL	15/37 (41%)	4/47 (9%)	**p<0.05**	7.330 (2.171-24.745)	9/24 (38%)	10/60 (17%)	**p<0.05**	3.000 (1.030-8.742)

BAT, basophil activation test; BHRA, basophil histamine release essay; ASST, autologous serum skin test; CSU, chronic spontaneous urticaria; anti-TPO, anti-thyroperoxidase; anti-Tg, anti-thyroglobulin; fT4, free thyroxine; TSH, thyroid stimulating hormone; ANA, antinuclear antibody; anti-dsDNA, anti-double stranded DNA.
Bold values highlight statistical significant values i.e. $p < 0.05$.

TABLE 2 | Patients clinical and laboratorial data, according to the BAT and BHRA test result.

	BAT			BHRA		
	+ BAT (n=37)	**- BAT (n=48)**	**Student test**	**+ BHRA (n=24)**	**- BHRA (n=61)**	**Student test**
Age (years)	44 ± 15	47 ± 17	p=0.332	45 ± 16	46 ± 16	p=0.977
UAS7 (mean ± SD)	21.1 ± 9.7	15.7 ± 10.6	**p<0.05**	22.2 ± 8.3	16.4 ± 10.9	**p<0.05**
DLQI (mean ± SD)	9.3 ± 6.8	6.5 ± 5.6	**p<0.05**	10.3 ± 6.1	6.7 ± 6.1	**p<0.05**
UCT (mean ± SD)	7.8 ± 4.1	9.3 ± 3.9	**p<0.05**	7.7 ± 3.9	9.0 ± 4.1	**p<0.05**
Total serum IgE (U/mL)	91 ± 91	395 ± 961	**p<0.05**	74 ± 69	335 ± 858	**p<0.05**
	+ BAT (n=37)	- BAT (n=47)	Student test	+ BHRA (n=24)	- BHRA (n=61)	Student test
Ratio IgG-anti-TPO/Total IgE	5.7 ± 16.4	0.2 ± 1.0	**p<0.05**	6.2 ± 17.1	1.2 ± 7.1	p=0.192

BAT, basophil activation test; BHRA, basophil histamine release essay; UAS7, urticaria activity score 7; DLQI, dermatological life quality index; UCT, urticaria control test. Bold values highlight statistical significant values i.e. p < 0.05.

skin testing identified the same features in double positive patients, as compared to those linked to positive basophil testing only (**Table 3**).

The ASST, by Itself, Is Not a Good Predictor of a Positive Basophil Test

Of the 85 CSU patients we investigated, 30 (35%) had a positive autologous skin test (ASST+). The ASST showed a weak correlation with the BAT (r=0.37, p<0.05) and with the BHRA (r=0.29, p<0.05). The probability (sensitivity) for a positive ASST to identify a patient with a positive BAT or BHRA was 62% and 56%, respectively.

The ASST, Combined With Other Clinical and Routine Laboratory Markers, Can Help to Select Patients for Basophil Testing

Next, we tested if the clinical features and laboratory markers linked to a positive BAT or BHRA predicted which patients would have a positive basophil test. By themselves, the sensitivity and specificity of angioedema, nocturnal wheals, wheals on five or more days per week, low IgE, elevated thyroid autoantibodies and high disease activity ranged from 29% to 89% and 35% to 90% for predicting a positive BAT or BHRA, respectively (**Table 4**).

TABLE 3 | Positive predictive value, negative predictive value, sensitivity, and specificity of a positive basophil test (BAT or BHRA) according to patients' characteristics.

Patients' characteristics	Positive predictive value	Negative predictive value	Sensitivity	Specificity
ASST(+)	80.0	61.8	53.3	85.0
UAS7≥16	59.6	55.3	62.2	52.5
Angioedema	62.5	55.6	55.6	62.5
Nocturnal symptoms	62.0	60.0	68.9	52.5
>5 days/week	60.6	73.7	88.9	35.0
Anti-TG/TPO	59.1	48.4	28.9	76.9
TSIgE<30	78.9	53.8	33.3	89.7
TSIgE<30 + Anti-TG/TPO	100.0	52.0	20.0	100
ASST(+) + Angioedema	86.4	58.7	42.2	92.5
ASST(+) + Anti-TG/TPO	90.0	50.7	20.0	97.4
ASST(+) + TSIgE<30	100.0	54.2	26.7	100.0

ASST, autologous serum skin test; anti-TPO, anti-thyroperoxidase; anti-Tg, anti-thyroglobulin; UAS7, urticaria activity score 7; TSIgE, Total serum IgE in (U/mL).

When the ASST was combined with angioedema, elevated thyroid autoantibodies or low IgE, it predicted a positive basophil test (either BAT or BHRA) with 92.5%, 97.4% and 100% specificity, respectively. In addition, patients with low IgE and elevated thyroid autoantibodies also had 100% specificity of identifying a positive basophil test. These combinations, however, showed low sensitivities, excluding many basophil test-positive patients.

To optimize the identification of patients with a positive basophil test (either BAT or BHRA), a decision tree was built using the CHAID algorithm (qui-squared automatic interaction detection). Applying the decision tree, the CSU patients were sequentially divided according to their clinical characteristics, until a final branch, after which patients were labelled as having either a positive basophil test or a negative basophil test (**Figure 1**).

Comparing the patients who were identified by this decision tree as being basophil test positive or negative with the actual BAT and BHRA results, we calculated that this decision tree showed a sensitivity of 76.7% and a specificity of 64.3% for correctly identifying the basophil test result of the patients.

DISCUSSION

The results of the CORSA study show that basophil test-positive patients (either BAT or BHRA) represent a distinct subset of CSU patients, with characteristics of TIIbaiCSU detailed by the recent PURIST study (4).

In our cohort, 44% and 28% of the patients were BAT-positive and BHRA-positive, respectively. This this is expected, as most studies report basophil test-positive results in 25% to 45% of the cases (8, 9, 23–25), with some studies going as high as 64% (26). However, even though our BAT and BHRA tests showed significant correlation and a 70% match in the results, this correlation was not perfect. The precise reason for this mismatch is currently unknown, however, not unexpected.

There are few studies directly comparing the two basophil tests in CSU. A previous study by our Department evaluated the correlation between ASST and BAT positivity in 48 CSU patients, and found a correlation coefficient of 0.79 (27). A study by Altrich et al. calculated a correlation coefficient between both tests of r=0.54, even though the concordance (positive versus negative) was high (75%); which is similar to the results presented in this study (28).

TABLE 4 | Patients clinical and laboratorial data, according to ASST plus basophil test double-positivity or double-negativity.

	ASST and basophil test double-positive patients (n=24)	ASST and basophil test double-negative patients (n=36)	Chi-square	Odds ratio	Student test
Male Gender	3 (13%)	7 (19%)	p=0.480	0.592 (0.137-2.560)	–
Presence of angioedema	19 (79%)	12 (33%)	**p<0.05**	7.600 (2.279-25.345)	–
Presence of nocturnal CSU symptoms	17 (71%)	16 (44%)	**p<0.05**	3.036 (1.012-9.107)	–
Symptoms for ≥5 days/week	23 (96%)	23 (64%)	**p<0.05**	13.000 (1.569-107.708)	–
Hives duration >5 hours	16 (67%)	13 (36%)	**p<0.05**	3.538 (1.193-10.499)	–
Presence of anti-TPO/Tg autoantibodies	9 (38%)	10 (28%)	p=0.471	1.500 (0.497-4.528)	–
Presence of fT4/TSH abnormalities	5 (21%)	3 (8%)	p=0.177	2.807 (0.602-13.091)	–
Presence of ANA/anti-dsDNA autoantibodies	2 (8%)	4 (11%)	p=0.780	0.775 (0.130-4.633)	–
IgE<30 U/mL	12 (50%)	4 (11%)	**p<0.05**	7.750 (2.084-28.815)	–
UAS7 (mean ± SD)	22.3 ± 9.5	15.0 ± 10.3	–	–	**p<0.05**
DLQI (mean ± SD)	9.7 ± 7.6	6.2 ± 5.6	–	–	**p<0.05**
UCT (mean ± SD)	7.3 ± 4.2	9.8 ± 3.4	–	–	**p<0.05**
Total serum IgE (U/mL)	85.6 ± 97.8	455.1 ± 1090.5	–	–	**p<0.05**
Ratio IgG-anti-TPO/Total IgE	3.0 ± 6.7	0.3 ± 1.1	–	–	**p<0.05**

ASST, autologous serum skin test; CSU, chronic spontaneous urticaria; anti-TPO, anti-thyroperoxidase; anti-Tg, anti-thyroglobulin; fT4, free thyroxine; TSH, thyroid stimulating hormone; ANA, antinuclear antibody; anti-dsDNA, anti-double stranded DNA; UAS7, urticaria activity score 7; DLQI, dermatological life quality index; UCT, urticaria control test.
Bold values highlight statistical significant values i.e. p < 0.05.

Another study, by Yasnowsky and co-workers, showed a correlation of r=0.6, although in this case the BAT test was evaluated using CD203c expression, which may have influenced these results (29). Szegedi and colleagues published results with a higher degree of correlation: r=0.91 when an atopic donor was used and r=0.7 when a non-atopic donor was used (30). However, these results highlight an important caveat; the correlation between basophil test results can change significantly with the characteristics of the donor of the basophils used. Additionally, in Szegedi's study, the same atopic and non-atopic donor were used for all the tests. In our study, different donors were used for the BAT and the BHRA, which can further explain the correlation discrepancies.

Not only do the BAT and BHRA use different techniques, which can impact the degree of basophil activation, it is hard to quantify the impact of using different basophil donors. This is also true for the tests individually. In the BAT and BHRA, the same serum tested at the same time with different donors, produces different degrees of basophil activation. The basophil test (BAT and BHRA) and ASST mismatch, on the other hand, was expected and has been described in the literature (24, 28–32).

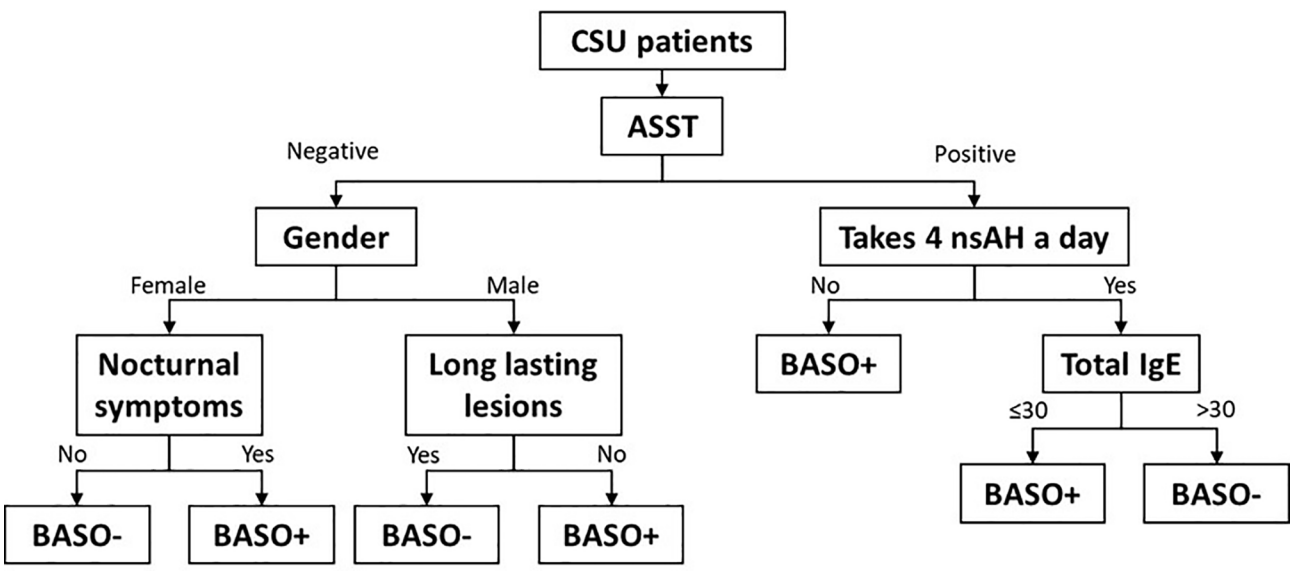


FIGURE 1 | Decision tree assessing patients with the highest probability of being basophil-positive (BASO+) and basophil-negative (BASO-) patients. BASO+ were accurately predicted by this model with a sensitivity of 76.7% (IC95% 61.4-88.2), a specificity of 64.3% (IC95% 48.0-78.5), a positive predictive value of 68.8% (IC95% 58.7-77.3), and a negative predictive value of 73.0% (IC95% 60.0-82.9).

The recent PURIST study, the first study to identify TIIbaiCSU patients using all three defining tests, provided important evidence of the distinctive characteristics of these patients. It showed that this patient subgroup presented higher disease activity (i.e. UAS7), lower total serum IgE, higher rates of thyroid autoantibodies and a higher IgG-anti-TPO to total serum IgE ratio (4). The results of the CORSA study show that our basophil test-positive patients exhibit a phenotype that is distinct from that of our basophil test-negative patients, and which is in agreement with the TIIbaiCSU profile of the patients of the PURIST study. Our basophil test-positive patients presented higher disease activity (UAS7), higher disease impact (DLQI), lower disease control (UCT), lower total serum IgE, higher rates of thyroid autoantibodies and a higher IgG-anti-TPO to total serum IgE ratio.

However, these results were based solely on basophil testing, one of the three tests required to identify TIIbaiCSU. Therefore, we re-analysed the phenotypes after adding the ASST (a second criterion for TIIbaiCSU). The results remained unchanged. This suggests that the basophil test is the more significant test for identifying patients with this more severe phenotype. In fact, most previous studies, which reported higher disease severity (24, 25), lower disease control (11), elevated thyroid autoantibodies (9, 14, 28), a non-response or slower response to omalizumab, and a better response to cyclosporin (8–11), based their findings on a single basophil test (BAT or BHRA) and not on all three tests needed to identify TIIbaiCSU. This makes the case for why basophil testing is so important for evaluating CSU patients for type IIb autoimmunity and how it can help to identify cases of severe CSU early on.

Using both the BAT and the BHRA, we were also able to identify novel clinical characteristics of basophil test-positive patients. The CORSA study basophil test-positive patients showed a higher frequency of angioedema, nocturnal symptoms, and wheals on five or more days per week; characteristics which may have influenced the poorer results on the UAS7, DLQI and UCT scores.

Despite its relevance, basophil testing meets real challenges as a solution for widespread implementation for all CSU patients, namely in terms of its availability, cost, and the high prevalence of CSU in the population. Identifying markers of basophil test-positive patients would be of great use in clinical practice, as they could select the best group of patients in whom to perform the test.

The ASST, the first attempt at such a marker, proved insufficient (1, 4). Our results confirm this and show a sensitivity of only 53% at identifying BAT+ and/or BHRA+ patients. In an attempt to improve these results, we used combinations of the ASST with the characteristics we identified in our basophil test-positive patients. ASST-positive patients with simultaneous presence of thyroid autoantibodies or a total serum IgE<30, showed a ≈100% probability (specificity) of having a positive basophil test. The combination of total serum IgE<30 and the presence of thyroid autoantibodies also showed 100% specificity. However, these combinations showed a very low sensitivity, and would leave out more basophil test-positive patients than the ASST.

A stepwise approach, employing a decision tree, using the characteristics associated with basophil test positivity, correctly identified most of our patients: it correctly identified 33 of 48 basophil test-positive patients and 27 of 37 basophil test-negative patients. This decision tree is easy to use and easily replicable and can be a promising tool to many physicians treating CSU patients. By this model, restricting the patients tested with a basophil test to those indicated, a physician could: 1) significantly reduce the number of patients in whom to ask for a basophil test to a cost-effective number, and 2) increase the number of positive basophil tests from the 25-45% average described in the literature for the whole CSU population, to about 70%.

This study has some limitations. It has a limited number of patients, from a single center, and these findings were not tested in a control group. There is a need for further studies to determine why TIIbaiCSU or a basophil-positive patient presents with more severe CSU, lower IgE, and higher thyroid autoantibodies. In addition, these findings need to be corroborated by future studies in larger and more diverse patient populations, and the proposed decision tree should be looked at as an initial attempt to identify basophil-positive patients, to be perfected by future studies. The identification of additional easily available clinical/laboratory parameters and their insertion in the model might be useful to increase the specificity and sensitivity of this algorithm.

In conclusion, basophil testing identifies a distinct subset of CSU patients, consistent with TIIbaiCSU. Using clinical and laboratory characteristics of TIIbaiCSU, it is possible to identify patients who are likely to have a positive basophil test, allowing for a more patient-specific approach to CSU patients in the future and a more cost-effective use of the basophil tests.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because it requires authorization by ethics committee. Requests to access the datasets should be directed to corresponding author JM.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comissão de Ética do Centro Hospitalar Universitário Lisboa Norte e Centro Académico Médico de Lisboa (Ethics Committee authorization references 129/17 and 339/19). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JM, KB, PS, and MM contributed to conception and design of the study. JM, PS, and KB organized the database, performed the statistical analysis. JM and MM wrote the first draft of the manuscript. KB, PS, MS, IW, JS, SA, AW, CC, and MP-B wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Zuberbier T, Aberer W, Asero R, Abdul Latiff AH, Baker D, Ballmer-Weber B, et al. The EAACI/GA²LEN/EDF/WAO Guideline for the Definition, Classification, Diagnosis and Management of Urticaria. *Allergy* (2018) 73:1393–414. doi: 10.1111/all.13397
2. Kolkhir P, Church MK, Weller K, Metz M, Schmetzer O, Maurer M. Autoimmune Chronic Spontaneous Urticaria: What We Know and What We do Not Know. *J Allergy Clin Immunol* (2017) 139:1772–81. doi: 10.1016/j.jaci.2016.08.050
3. Kolkhir P, Church MK, Weller K, Metz M, Schmetzer O, Maurer M. Reply. *J Allergy Clin Immunol* (2018) 141:1166–7. doi: 10.1016/j.jaci.2017.09.010
4. Schoepke N, Asero R, Ellrich A, Ferrer M, Gimenez-Arnau A, Grattan C EH, et al. Biomarkers and Clinical Characteristics of Autoimmune Chronic Spontaneous Urticaria: Results of the PURIST Study. *Allergy* (2019) 74:2427–36. doi: 10.1111/all.13949
5. Deza G, March-Rodríguez A, Sánchez S, Ribas-Llauradó C, Soto D, Pujol RM, et al. Relevance of the Basophil High-Affinity IgE Receptor in Chronic Urticaria: Clinical Experience From a Tertiary Care Institution. *J Allergy Clin Immunol Pract* (2019) 7:1619–26. doi: 10.1016/j.jaip.2019.01.026
6. Baumann K, Marcelino J, Skov PS, Santos M, Wyroslak I, Scheffel J, et al. Autologous Serum Skin Test Reactions in Chronic Spontaneous Urticaria Differ From Heterologous Cell Reactions. *J Eur Acad Dermatol Venereol* (2021) 35(6):1338–45. doi: 10.1111/jdv.17131
7. Konstantinou GN, Asero R, Ferrer M, Knol EF, Maurer M, Raap U, et al. EAACI Taskforce Position Paper: Evidence for Autoimmune Urticaria and Proposal for Defining Diagnostic Criteria. *Allergy* (2013) 68:27–36. doi: 10.1111/all.12056
8. Gericke J, Metz M, Ohanyan T, Weller K, Altrichter S, Skov PS, et al. Serum Autoreactivity Predicts Time to Response to Omalizumab Therapy in Chronic Spontaneous Urticaria. *J Allergy Clin Immunol* (2017) 139:1059–61. doi: 10.1016/j.jaci.2016.07.047
9. Iqbal K, Bhargava K, Skov PS, Falkencrone S, Grattan CE. A Positive Serum Basophil Histamine Release Assay Is a Marker for Ciclosporin-Responsiveness in Patients With Chronic Spontaneous Urticaria. *Clin Transl Allergy* (2012) 2:19. doi: 10.1186/2045-7022-2-19
10. Palacios T, Stillman L, Borish L, Lawrence M. Lack of Basophil CD203c-Upregulating Activity as an Immunological Marker to Predict Response to Treatment With Omalizumab in Patients With Symptomatic Chronic Urticaria. *J Allergy Clin Immunol Pract* (2016) 4:529–30. doi: 10.1016/j.jaip.2015.11.025
11. Endo T, Toyoshima S, Hayama K, Tagui M, Niwa Y, Ito M, et al. Relationship Between Changes in the 7-Day Urticaria Activity Score After Treatment With Omalizumab and the Responsiveness of Basophils to Fcεri Stimulation in Patients With Chronic Spontaneous Urticaria. *Asia Pac Allergy* (2020) 10:e12. doi: 10.5415/apallergy.2020.10.e12
12. Fricke J, Ávila G, Keller T, Weller K, Lau S, Maurer M, et al. Prevalence of Chronic Urticaria in Children and Adults Across the Globe: Systematic Review With Meta-Analysis. *Allergy* (2020) 75:423–32. doi: 10.1111/all.14037
13. Maurer M, Church MK, Marsland AM, Sussman G, Siebenhaar F, Vestergaard C, et al. Questions and Answers in Chronic Urticaria: Where do We Stand and Where do We Go? *J Eur Acad Dermatol Venereol* (2016) 30 Suppl 5:7–15. doi: 10.1111/jdv.13695
14. Grattan CE, O'Donnell BF, Francis DM, Niimi N, Barlow RJ, Seed PT, et al. Randomized Double-Blind Study of Cyclosporin in Chronic 'Idiopathic' Urticaria. *Br J Dermatol* (2000) 143:365–72. doi: 10.1046/j.1365-2133.2000.03664.x
15. Konstantinou GN, Asero R, Maurer M, Sabroe RA, Schmid-Grendelmeier P, Grattan CE. EAACI/GA(2)LEN Task Force Consensus Report: The Autologous Serum Skin Test in Urticaria. *Allergy* (2009) 64::1256–68. doi: 10.1111/j.1398-9995.2009.02132.x
16. Maurer M, Metz M, Bindslev-Jensen C, Bousquet J, Canonica GW, Church MK, et al. Definition, Aims, and Implementation of GA²LEN Urticaria Centers of Reference and Excellence. *Allergy* (2016) 71:1210–8. doi: 10.1111/all.12901
17. Mathias SD, Crosby RD, Zazzali JL, Maurer M, Saini SS. Evaluating the Minimally Important Difference of the Urticaria Activity Score and Other Measures of Disease Activity in Patients With Chronic Idiopathic Urticaria. *Ann Allergy Asthma Immunol* (2012) 108:20–4. doi: 10.1016/j.anai.2011.09.008
18. Moestrup K, Ghazanfar MN, Thomsen SF. Patient-Reported Outcomes (PROs) in Chronic Urticaria. *Int J Dermatol* (2017) 56:1342–8. doi: 10.1111/ijd.13668
19. Basra MK, Fenech R, Gatt RM, Salek MS, Finlay AY. The Dermatology Life Quality Index 1994-2007: A Comprehensive Review of Validation Data and Clinical Results. *Br J Dermatol* (2008) 159:997–1035. doi: 10.1111/j.1365-2133.2008.08832.x
20. Weller K, Groffik A, Church MK, Hawro T, Krause K, Metz M, et al. Development and Validation of the Urticaria Control Test: A Patient-Reported Outcome Instrument for Assessing Urticaria Control. *J Allergy Clin Immunol* (2014) 133:1365–72. doi: 10.1016/j.jaci.2013.12.1076
21. Yu L, Buttgereit T, Stahl Skov P, Schmetzer O, Scheffel J, Kocatürk E, et al. Immunological Effects and Potential Mechanisms of Action of Autologous Serum Therapy in Chronic Spontaneous Urticaria. *J Eur Acad Dermatol Venereol* (2019) 33:1747–54. doi: 10.1111/jdv.15640
22. Metz M, Gimenez-Arnau A, Borzova E, Grattan C, Magerl M, Maurer M. Frequency and Clinical Implications of Skin Autoreactivity to Serum Versus Plasma in Patients With Chronic Urticaria. *J Allergy Clin Immunol* (2009) 123:705–6. doi: 10.1016/j.jaci.2008.11.040
23. Kaplan AP. Basophil Histamine Release in Patients With Chronic Spontaneous Urticaria: Optimize or Minimize. *J Allergy Clin Immunol* (2019) 144:622–3. doi: 10.1016/j.jaci.2019.05.004
24. Curto-Barredo L, Yelamos J, Gimeno R, Mojal S, Pujol RM, Giménez-Arnau A. Basophil Activation Test Identifies the Patients With Chronic Spontaneous Urticaria Suffering the Most Active Disease. *Immun Inflamm Dis* (2016) 4:441–5. doi: 10.1002/iid3.125
25. Netchiporouk E, Moreau L, Rahme E, Maurer M, Lejtenyi D, Ben-Shoshan M. Positive CD63 Basophil Activation Tests Are Common in Children With Chronic Spontaneous Urticaria and Linked to High Disease Activity. *Int Arch Allergy Immunol* (2016) 171:81–8. doi: 10.1159/000451084
26. Hossein Zadeh Attar M, Merk HF, Kotliar K, Wurpts G, Röseler S, Moll-Slodowy S, et al. The CD63 Basophil Activation Test as a Diagnostic Tool for Assessing Autoimmunity in Patients With Chronic Spontaneous Urticaria. *Eur J Dermatol* (2019) 29:614–8. doi: 10.1684/ejd.2019.3680
27. Costa C. *Diagnóstico De Urticária Crónica Autoimune: Estudo De Activação Dos Basófilos Por Citometria De Fluxo*. Lisbon: Medicine, Medical Immunology Master, Lisbon Medical School, University of Lisbon (2008). Available at: http://hdl.handle.net/10451/1091.
28. Altrich ML, Halsey JF, Altman LC. Comparison of the In Vivo Autologous Skin Test With In Vitro Diagnostic Tests for Diagnosis of Chronic Autoimmune Urticaria. *Allergy Asthma Proc* (2009) 30:28–34. doi: 10.2500/aap.2009.30.3185
29. Yasnowsky KM, Dreskin SC, Efaw B, Schoen D, Vedanthan PK, Alam R, et al. Chronic Urticaria Sera Increase Basophil CD203c Expression. *J Allergy Clin Immunol* (2006) 117:1430–4. doi: 10.1016/j.jaci.2006.02.016
30. Szegedi A, Irinyi B, Gál M, Hunyadi J, Dankó K, Kiss E, et al. Significant Correlation Between the CD63 Assay and the Histamine Release Assay in Chronic Urticaria. *Br J Dermatol* (2006) 155:67–75. doi: 10.1111/j.1365-2133.2006.07205.x
31. Tedeschi A, Lorini M, Suli C, Asero R. Serum Interleukin-18 in Patients With Chronic Ordinary Urticaria: Association With Disease Activity. *Clin Exp Dermatol* (2007) 32:568–70. doi: 10.1111/j.1365-2230.2007.02450.x
32. Gentinetta T, Pecaric-Petkovic T, Wan D, Falcone FH, Dahinden CA, Pichler WJ, et al. Individual IL-3 Priming is Crucial for Consistent In Vitro Activation of Donor Basophils in Patients With Chronic Urticaria. *J Allergy Clin Immunol* (2011) 128:1227–34. doi: 10.1016/j.jaci.2011.07.021

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The COVID-19 Pandemic Affects Male Patients With Chronic Spontaneous Urticaria More Than Female Patients

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Introduction: The COVID-19 pandemic dramatically disrupts health care for patients with chronic diseases including chronic spontaneous urticaria (CSU). As of now, it is unknown if the effects of the pandemic in CSU are different than in other chronic diseases. We also do not know, if different groups of CSU patients, for example female and male patients, are affected differently.

Aim: To understand how CSU patients and subgroups are affected by the COVID-19 pandemic in their disease activity and control and treatment, using psoriasis as control.

Patients and Methods: We analyzed 399 patients (450 visits) with CSU or psoriasis assessed during August 2019, i.e. before the pandemic, or August 2020, i.e. during the pandemic, for changes in disease activity, disease control, and the treatment they used, and how these changes are linked to age, gender, and disease duration.

Results: Male but not female patients with CSU had markedly increased disease activity during the pandemic. CSU patients' age or disease duration were not linked to changes. Male and female patients with psoriasis showed similar increases in disease activity and decreases in disease control. The rate of omalizumab treatment, during the pandemic, was unchanged in male patients and increased in female patients with CSU. The efficacy of omalizumab treatment, during the pandemic, was reduced in male patients but not female patients with CSU.

Conclusion: Male but not female CSU patients, during the COVID-19 pandemic, show loss of disease control linked to loss of omalizumab efficacy. The reasons for this need to be investigated.

Keywords: chronic spontaneous urticaria, chronic skin diseases, COVID-19, psoriasis, pandemic (COVID19)

Abbreviations: ACARE, Angioedema Centers of Reference and Excellence; CU, Chronic Urticaria; CSDU, Chronic Skin Diseases Unit; CSU, Chronic Spontaneous Urticaria; DLQI, Dermatology Life Quality Index; IQR, interquartile range; PASI, Psoriasis Area and Severity Index; UAS, Urticaria Activity Score; UCARE, Urticaria Center of Reference and Excellence; UCT, Urticaria Control Test.

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INTRODUCTION

The COVID-19 pandemic caused by SARS-CoV-2 has caused ongoing challenges for health care systems across the globe[1]. One of them is the disruption of routine clinical care for patients with chronic diseases (1). Chronic diseases require continued monitoring, and patients are often in need of treatment adaptation. The COVID-19 pandemic, with lockdowns, travel restrictions, and a redistribution of health care resources towards testing and treating patients for COVID-19 has severely reduced the ability of patients with chronic diseases to obtain treatment and of physicians to provide it. This affects patients with various chronic diseases such as diabetes, chronic obstructive pulmonary disease, hypertension, asthma, cancer, depression, psoriasis and chronic urticaria (1–3).

Chronic urticaria (CU) is a common and disabling disease that manifests with pruritic wheals, angioedema, or both. The most common type of CU, chronic spontaneous urticaria (CSU), comes with highly fluctuating disease activity and is unpredictable in terms of when and where its signs and symptoms occur. Many environmental triggers such as stress and infections (and non-steroidal anti-inflammatory drugs that are used to treat them) can lead to increased disease activity, and they often do. Also, CSU is a disease that comes with impairment at work and in school, with sleep disturbance and sexual dysfunction (4), and can be hard to treat, as many patients do not achieve disease control with first and second line treatment, i.e. an antihistamine used at standard or higher than standard dose (5, 6). Psoriasis, like CSU, is a common, chronic, and disabling inflammatory skin disease. Unlike CSU, psoriasis manifests with stationary or progressing lesions, most commonly erythematous squamous plaques (7). Both CSU and psoriasis, in most patients, seriously reduce quality of life (6, 8).

Very recently, the COVID-CU study performed by the global network of Urticaria Centers of Reference and Excellence (UCAREs (9)) showed that the COVID-19 pandemic severely impairs patient care at urticaria specialist centers, markedly changes physician-patient interactions, and affects how patients are treated. Its results also indicate that CU is not linked to severe COVID-19, but often worsened by it (3, 10). The UCARE COVID-CU study investigated a limited number of CSU patients, few per country, across several countries affected differently by the pandemic, and it did not include controls (3). Because of the low number of patients included in the COVID-CU study, meaningful analyses of subpopulations of patients, e.g. male *vs* female or young *vs* old CU patients, were not possible. These limitations and the questions that emerged from the results of the COVID-CU study prompted the present study, which focused on the following questions: How is the care for patients with CSU and the management of their disease affected by the pandemic as compared to that of patients with other chronic inflammatory skin diseases, e.g. psoriasis? What are the effects of the pandemic on the levels of disease activity and on the treatment in patients with CSU as compared to patients with psoriasis? How has the pandemic affected the use of biologics in patients with CSU and psoriasis? Does the pandemic affect different CSU patients differently?

To address these questions, we chose a unique approach and made use of the fact that our UCARE is linked to the chronic skin diseases unit (CSDU) of our dermatology department, which provides outpatient services for patients with chronic inflammatory dermatoses including psoriasis (11). We selected the month of August of the past year, 2020, as our observation period, i.e. a time with intermediate numbers of SARS-CoV-2 infections and new COVID-19 cases in our country, after the first wave earlier that year. Our control observation period was the same month, August, of the previous year, 2019, i.e. before the pandemic. With the data from these two observation periods, from a sizeable number of CSU and psoriasis patients treated at our UCARE and CSDU, we analyzed the impact of the pandemic on both patient cohorts and subpopulations in terms of their outpatient care, disease activity and treatments.

PATIENTS AND METHODS

Study Design and Conduct

A total of 450 visits of 399 patients with CSU or psoriasis treated at our UCARE and CSDU during August of 2019 or August of 2020 were analyzed in this retrospective study. Of these 399 patients, 51 patients, 27 with CSU and 24 with psoriasis, were treated in both, August of 2019 and August of 2020. None of the patients analyzed had CSU and psoriasis.

Of the 450 patient visits, 184 (41%) were for CSU and 266 (59%) for psoriasis. In August of 2019, 113 patients with CSU visited our outpatient clinic, 83 of them female (73.5%). In August of 2020, 71 CSU patients, 50 of whom were female (70.4%), visited the outpatient clinic. The August 2019 and 2020 average age of CSU patients was 41.2 ± 14.2 years and 39.6 ± 11.7 years, respectively.

In August of 2019, a total of 215 patients with psoriasis visited the outpatient clinic, 121 of them female (56.3%). In August of 2020, 51 psoriasis patients, 24 of whom were female (47.1%), visited the outpatient clinic. The August 2019 and 2020 average age of psoriasis patients was 41.4 ± 16.0 years and 43.6 ± 16.8 years, respectively.

Informed consent was obtained from each patient who participated in the study. Approval for this study was obtained from the Ethics committee of Kayseri City Education and Research Hospital. We compared 2019 and 2020 numbers of outpatients seen during the month of August, in total and by disease, as well as patient demographic data. Patient consultations outside of these dates were not investigated in this study. No other criteria were used in the selection of patients.

Patient Assessment

The demographic information of patients was obtained from the hospital data management system. The parameters examined in

[1] WHO Director-General's opening remarks at the media briefing on COVID-19 - 5 March 2020 Available from: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19—5-march-2020 [Accessed October 10, 2020].

patients with CSU included age, gender, disease activity as assessed by use of the urticaria activity score (UAS), disease control as measured by the urticaria control test (UCT) and the treatment they used [antihistamine, omalizumab, other (cyclosporine, methotrexate, etc.)]. For 2019, UAS and UCT scores for 27 patients were missing, and also the treatment data for 23 of 27 CSU patients were not available in that period. In 2020, UAS and UCT scores were missing of only 1 patient.

The UAS consists of two questions, one on the intensity of itch and one on the number of wheals in the last 24 hours. It is scored from 0 to 6, with an itch score between 0 and 3 (0=none; 3=very severe) and a wheal score between 0 and 3 (0=none; 3=more than 50 wheals in last 24 hours) (5). The UCT measures disease control during the last 4 weeks; it consists of four questions, with a score between 0 and 4 assigned to every answer option. The scores for all 4 questions are summed up for the total UCT, which ranges from 0 (no control) to 16 (complete control) (12). A UCT score of 12 points or higher indicates well-controlled disease (5). In CSU patients, until August 2020, we had no patients who were positive for COVID-19 PCR. Since two of our patients had symptoms, they applied with the suspicion of COVID, but PCR was not positive.

The parameters examined in the psoriasis patients included in the study were their age, gender, disease activity as assessed with the psoriasis area and severity index (PASI), and the treatment they used (topical therapy, phototherapy, conventional treatments [acitretin, methotrexate, cyclosporine] and biological treatments [adalimumab, infliximab, certolizumab, ustekinumab, secukinumab, ixekizumab]). For 2019, treatment data were unavailable for 11 of 215 patients. In 2020, all psoriasis datasets were complete. The PASI is one of the most used tools for the assessment of psoriasis severity and combines the measurement of skin lesion severity and affected skin area in a total score that ranges from 0 (no disease) to 72 (maximal disease) (13). We categorized psoriasis patients with a total PASI score of 0 or 1, i.e. those with no or minimal disease, as having controlled disease and those with PASI >1 as having uncontrolled disease.

We did not have any vaccinated patients since there was no approved vaccine in the period up to the date we base our study on.

Statistical Analyses

The collected data were analyzed, and comparisons for statistically significant differences between groups were made using the IBM SPSS 25 package program. For continuous variables, the data were shown as mean ± standard deviation or median (interquartile range, IQR) and for categorical variables as frequencies (percentiles). Descriptive analyzes, chi-square test and Student t-test or Mann-Whitney U test were used. P values of less than 0.05 were considered to indicate significant differences.

RESULTS

During the Pandemic, Disease Activity in Male, but Not Female Patients With CSU Increases

Male patients with CSU had markedly increased disease activity during the pandemic (UAS: 3, 1-5) as compared to before the pandemic (UAS: 1, 0-2, p = 0.028, **Figure 1A** and **Table 1**). Male CSU patients, during the pandemic, also had lower levels of disease control (UCT: 11, 7-15) than before the pandemic (UCT: 15, 12-16; p = 0.029, **Figure 1B** and **Table 1**). Before the pandemic, 80% of male patients with CSU had well controlled disease, i.e. a UCT of 12 or more (**Table 1**). In 2020, the rate had dropped to 42.8% (p = 0.009; **Figure 1C** and **Table 1**). In contrast, disease activity in female patients with CSU was unchanged during the pandemic (UAS: 2, 1-4 in 2020 *vs* 2, 0-5 in 2019), as were levels of disease control (UCT: 12, 9-15 in 2020 *vs* 12, 6-16 in 2019) and rates of patients with well controlled disease (50.8% in 2019 *vs* 53.0% in 2020; **Figure 1** and **Table 1**). Unlike gender, CSU patients' age and disease duration were not linked to increased disease activity during the pandemic.

As for patients with psoriasis, male and female patients, during the pandemic, showed similar and increased levels of disease activity, i.e. higher PASI scores, than before the pandemic (**Table 1**). During the pandemic, the rates of patients with minimal disease, i.e. PASI 1 or 0, dropped from 60.6% in 2019 to 51.9% in males and from 72.7% in 2019 to 50.0% in females (**Figure 1** and **Table 1**).

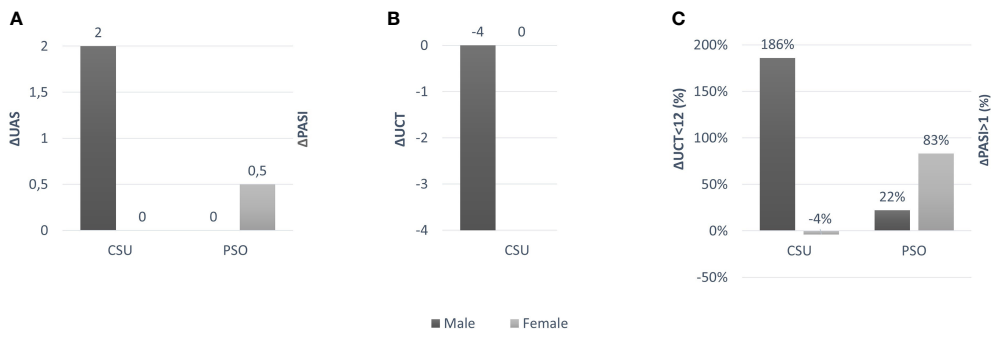


FIGURE 1 | **(A)** Difference in UAS and PASI values for female and male patients between 2019 and 2020; **(B)** Difference in UCT value for female and male patients between 2019 and 2020; **(C)** Difference in% between UCT <12 and PASI> 1 values in female and male patients between 2019 and 2020.

TABLE 1 | Number of outpatient visits, treatment and disease activity in female and male patients with psoriasis and CSU before and during the COVID-19 pandemic.

		Before the pandemic (2019)	During the pandemic (2020)	P value
CSU outpatient visits				**<0.0001**[¶]
Female patients		83	50 (-40%)	0.736[¶]
Male patients		30	21 (-30%)	
All patients		113	71 (-37%)	
Psoriasis outpatient visits				
Female patients		121	24 (-81%)	0.274[¶]
Male patients		94	27 (-62%)	
All patients		215	51 (-77%)	
Female patients with CSU	On omalizumab, n (%)	21 / 63 (33.3%)	33 / 50 (66.0%)	**0.001**[¶]
	UAS, median (IQR)	2 (0-5)	2 (1-4)	0.622[§]
	UCT, median (IQR)	12 (6-16)	12 (9-15)	0.858[§]
	UCT≥12, n (%)	31 / 61 (50.8%)	26 / 49 (53.0%)	0.815[¶]
Male patients with CSU	On omalizumab, n (%)	13 / 27 (48.1%)	12 / 21 (57.1%)	0.159[¶]
	UAS, median (IQR)	1 (0-2)	3 (1-5)	**0.028**[§]
	UCT, median (IQR)	15 (12-16)	11 (7-15)	**0.029**[§]
	UCT≥12, n (%)	20 / 25 (80.0%)	9 / 21 (42.8%)	**0.009**[¶]
All patients with CSU	On omalizumab, n (%)	34 / 90 (37.8%)	45 / 71 (63.4%)	**< 0.0001**[¶]
	UAS, median (IQR)	2 (0-4)	2 (1-4)	0.105[§]
	UCT, median (IQR)	12 (8-16)	11,5 (9-15)	0.302[§]
	UCT≥12, n (%)	51 / 86 (59.3%)	35 / 70 (50.0%)	0.245[¶]
Female patients with psoriasis	Biological therapy, n (%)	32 / 113 (28.3%)	11 / 24 (45.8%)	0.141[¶]
	PASI, median (IQR)	1 (0-2.0)	1.5 (0-4.35)	**0.030**[§]
	PASI≤1, n (%)	88 / 121 (72.7%)	12 / 24 (50.0%)	**0.028**[¶]
Male patients with psoriasis	Biological therapy, n (%)	25 / 91 (27.4%)	7 / 27 (25.9%)	0.276[¶]
	PASI, median (IQR)	1 (0-3.0)	1 (1.0-5.0)	**0.050**[§]
	PASI≤1, n (%)	57 / 94 (60.6%)	14 / 27 (51.9%)	0.414[¶]
All patients with psoriasis	Biological therapy, n (%)	57 / 204 (27.9%)	18 / 51 (35.3%)	0.060[¶]
	PASI, median (IQR)	1 (0-2.0)	1 (0.5-4.8)	**0.002**[§]
	PASI≤1, n (%)	145 / 215 (67.4%)	26 / 51 (51.0%)	**0.027**[¶]

CSU, Chronic Spontaneous Urticaria; IQR, interquartile range; PASI, Psoriasis Area and Severity Index; UAS, Urticaria Activity Score; UCT, Urticaria Control Test; ¶, Chi-squared test; §, Mann-Whitney U test.

Values in bold highlight statistically significant values.

The COVID-19 Pandemic Is Linked to Markedly Reduced Outpatient Visits of Both Male and Female Patients With CSU and Psoriasis

The number of monthly consultations of patients with CSU or psoriasis dropped from 328 before the pandemic to 122 (-63%) during the pandemic (**Table 1**). CSU outpatient visits went down to 71 per month during the pandemic as compared to 113 per month before the pandemic (-37%; -40% and -30% in female and male patients, respectively; **Table 1**). Outpatient visits of patients with psoriasis were reduced by 77% (-81% in female and -62% in male patients), from 215 per month before the pandemic to 51 per month during the pandemic, a significantly greater reduction than in CSU patients ($p < 0.0001$; **Table 1**).

The Rate of Omalizumab Treatment, During the Pandemic, Is Unchanged in Male Patients and Increased in Female Patients With CSU

With the decision taken by the Turkish Ministry of Health at the beginning of the pandemic period, all chronic patients would be able to take their medicines without applying to the hospital. Despite this decision, we identified patients who did not use their medication regularly. In male CSU patients using omalizumab, only 2 patients out of 13 could not use their medication regularly (15%) during the pandemic period. Similarly, in female CSU patients using omalizumab, 6 out of 33 patients could not take their medication regularly during the pandemic period (18%). The loss of disease control and increase in disease activity in male CSU patients during the pandemic was not due to a change in their rate of omalizumab treatment. Before the pandemic, 48.1% of male patients were treating with omalizumab, similar to during the pandemic, when the rate was 57.1% (**Table 1** and **Figure 2A**). Female patients with CSU showed increased rates of omalizumab treatment during the pandemic, 66.0% in 2020, up from 33.3% in 2019 ($p=0.001$; **Table 1** and **Figure 2A**). Overall, the use of omalizumab increased from 37.8%, in 2019, to 63.4%, in 2020.

In psoriasis, female patients also used biologics more frequently during the pandemic as compared to before the pandemic, and male patients also did not. Overall, the rate of psoriasis patients on biologic treatment increased to 35.3% from 27.9% during 2019 to 2020. Before the pandemic, 27.4% of male psoriasis patients were on biologic treatment, similar to during the pandemic, when the rate was 25.9%. Female patients with psoriasis showed increased and markedly higher rates of biologic treatment during the pandemic, 45.8% in 2020, up from 28.3% in 2019 (**Table 1** and **Figure 2A**).

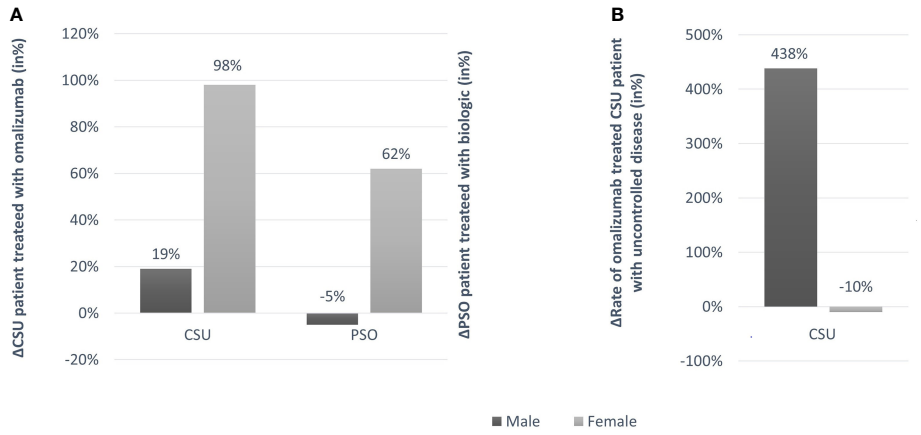


FIGURE 2 | **(A)** Change rates (in%) of CSU patients treated with omalizumab and psoriasis patients treated with biologics, in 2019 and 2020; **(B)** Change rate (in%) of CSU patients treated with omalizumab with uncontrolled disease in 2019 and 2020.

The Efficacy of Omalizumab Treatment, During the Pandemic, Is Reduced in Male Patients but Not Female Patients With CSU

Before the pandemic, 91.7% of male CSU patients treating with omalizumab had controlled disease, i.e. a UCT≥12. During the pandemic, CSU in male patients on omalizumab treatment was controlled in only 50% of patients (p=0.025). In contrast, female patients who treated with omalizumab had similar rates of controlled disease in 2019 and 2020, 63.2% and 66.7%, respectively (**Figure 2B**).

DISCUSSION

Our study shows, unexpectedly, that the current COVID-19 pandemic affects male and female patients differently, an effect not seen in psoriasis. Male patients experienced a drop in their rate of having CSU under control by more than 50% during the pandemic. This finding is explained, in part, by stagnant *vs* higher rates of omalizumab treatment and by markedly lower rates of response to omalizumab treatment in male *vs* female CSU patients during the pandemic. Why omalizumab is seemingly less effective in males during the pandemic is unknown. Several hypotheses are discussed below and should be tested in future studies.

That the COVID-19 pandemic comes with markedly reduced outpatient visits in patients with CSU confirms the results of the recent COVID-CU study (3). In our center, CSU patient visits were down by more than a third as compared to before the pandemic. This reduction in patient visits was not unique for CSU. In fact, outpatient visits of psoriasis patients were reduced even more, by more than two thirds. Importantly, male CSU patient visit numbers were as affected as those in females, slightly less actually, suggesting that their CSU deterioration during the pandemic did not occur primarily because they did not visit their physicians.

That male but not female CSU patients experienced loss of disease control during the pandemic is likely linked to differences in their omalizumab treatment rates during as compared to before the pandemic and differences in omalizumab efficacy. As for omalizumab treatment rates, one of two male CSU patients used omalizumab during the pandemic, and this was the same as before the pandemic. Female patients more often used omalizumab during the pandemic than before the pandemic, and their levels of disease activity and rates of controlled disease remained stable during the pandemic. This suggests that the pandemic, overall, aggravates CSU and increases CSU disease activity. In female patients, increased use of omalizumab comes with unchanged rates of controlled disease. In male patients, unchanged use of omalizumab comes with reduced rates of controlled disease. Interestingly, female patients with psoriasis, like female patients with CSU, also used biologics more frequently during the pandemic as compared to before the pandemic, and male patients with psoriasis, like male patients with CSU, also did not increase their use. But in psoriasis, both male and female patients showed similar rates of decreased disease control during the pandemic. It is tempting to speculate that this points to a higher impact of the pandemic on psoriasis in female patients, who experience similar rates of worsening as male patients, despite using biologics markedly more often. In any case, the situation for male CSU patients during the pandemic appears to be worse not only compared to female CSU patients but also compared to male patients with psoriasis. In male patients with psoriasis, rates of controlled disease during the pandemic dropped by 15% as compared to more than 50% in male CSU patients, with unchanged rates of biological treatment in both. The first likely reason for the loss of disease control in male but not female patients with CSU is that the pandemic translates to higher skin mast cell activation, driving increased symptom occurrence and disease activity across all patients with CSU, male and female. This is mitigated, in the female CSU patient population, by the higher number of patients treated with omalizumab. In the male

patient population, rates of omalizumab use are unchanged, and, consequently, loss of disease control is more frequent.

The second difference between male and female CSU patients linked to loss of disease control in the former but not the latter is the ability of omalizumab to control CSU. Only 50% of male omalizumab-treated patients reported controlled disease during the pandemic as compared to 92% before the pandemic, whereas two thirds of female omalizumab-treated patients experienced disease control during and before the pandemic. Why is the efficacy of omalizumab treatment, during the pandemic, reduced in male patients but not female patients with CSU? Our study does not and cannot answer this question. The least likely explanation is that the pharmacological effects of omalizumab, i.e. neutralization of IgE and downregulation of IgE receptors, are impaired by the pandemic, in male patients only. We cannot think of a mechanism that would explain this.

In addition, some of the effects of the pandemic that increase disease activity in CSU patients may be more prominent in males than in females. COVID-19 can exacerbate CSU, and it often does, in 3 of 10 affected patients and in 7 of 10 patients hospitalized because of COVID-19 (3). Did our male CSU patients have higher rates of COVID-19 than our female patients? Until August 2020, none of our CSU patients had been diagnosed with COVID-19 disease, however, we know that the incidence of COVID-19 at that time was 26.1 per 100,000 and the female:male ratio was 49:51[2]. This makes it unlikely that higher rates of CSU exacerbation in our male patients were due to higher rates of COVID-19. Pandemic-associated stress and anxiety, rather than COVID-19 itself, may have been more pronounced in males *vs* females. The pandemic comes with increased levels of stress in the general population, which is held to be linked to the fear of becoming infected, of financial hardship due to lockdowns or getting sick, of family members getting COVID-19, and of dying from COVID-19 (14). A recent study investigating the psychological burden of the pandemic on the CSU showed increased CSU activity during quarantine periods (15). On the date we base our study on (August 2020), our country had just come out of the first quarantine period. In our patient population, males are more often than females the breadwinners and providers of their families, and they also have higher numbers of social contacts and therefore risk of infection, so stress may have been more pronounced in males than females, and stress is an important driver of CSU disease activity. We acknowledge that this explanation is highly speculative and calls for further studies on pandemic-associated stress levels in males and females and their relevance for changes in CSU disease activity. Finally, male bias towards severe disease is a consistent feature of COVID-19 (16). It is, therefore, possible, that male COVID-19 patients with CSU experience disease exacerbation more often than female patients and that these exacerbations are more severe because of a more severe course of COVID-19. Interestingly, more severe COVID-19 in male patients as compared to female patients has been linked to differences in both the innate and adaptive immune system including T and B cell responses. Male patients produce less type 1 interferon (IFN), a potent anti-viral cytokine that is encoded, like many immune-related genes and regulatory elements involved in anti-viral immune responses, on the X chromosome (17, 18). Of note, type 1 IFN is known to suppress mast cell function, including histamine release, and poor IFN responses may, therefore, be linked to increased mast cell activation, which drives disease activity in patients with CSU (19).

Our study has several strengths and limitations. The strengths of this study include its sizeable patient numbers, the analysis of psoriasis as a control, and the fact that it was performed at an UCARE, the only in the region, making it the key referral center for patients with moderate or severe CSU. The limitations are that it was a single center and its retrospective design.

Taken together, our study demonstrates that the ongoing COVID-19 pandemic has a major impact on patients with CSU and psoriasis, and that this impact can differ across disease populations. Male but not female CSU patients, during the COVID-19 pandemic, show loss of disease control. The clinical learning from our findings is that the pandemic must be expected to increase the pathogenic drive and disease activity in CSU and other chronic inflammatory diseases. When this is met with increased use of effective treatment, rates of controlled disease are maintained. When the treatment remains unchanged, control is at risk of being lost.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

RE and HK performed statistical analysis and drafted the manuscript. AA, HK, and MRA were involved in patient recruitment and proof-reading of the manuscript. MM, MA, and KÖ were involved in study planning and proof-reading of the manuscript. RE, HK, and MM planned the study, coordinated the study, collected patient data, was involved in statistical analysis and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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[2] Republic of Turkey Ministry of Health Weekly Case Numbers https://covid19.saglik.gov.tr/TR-68640/haftalik-rapor–weekly-report.html [Accessed April 10, 2021].

REFERENCES

1. Chudasama YV, Gillies CL, Zaccardi F, Coles B, Davies MJ, Seidu S, et al. Impact of COVID-19 on Routine Care for Chronic Diseases A Global Survey of Views From Healthcare Professionals. *Diabetes Metab Syndrome: Clin Res Rev* (2020) 4(5):965–7. doi: 10.1016/j.dsx.2020.06.042
2. Elmas ÖF, Demirbaş A, Kutlu Ö, Bağcıer F, Metin MS, Özyurt K, et al. Psoriasis and COVID-19: A Narrative Review With Treatment Considerations. *Dermatol Ther* (2020) e13858. doi: 10.1111/dth.13858
3. Kocatürk E, Salman A, Cherrez-Ojeda I, Criado PR, Peter J, Comert-Ozer E, et al. The Global Impact of the COVID-19 Pandemic on the Management and Course of Chronic Urticaria. *Allergy* (2021) 76(3):816–30. doi: 10.1111/all.14687
4. Ertaş R, Erol K, Hawro T, Yılmaz H, Maurer M. Sexual Functioning Is Frequently and Markedly Impaired in Female Patients With Chronic Spontaneous Urticaria. *J Allergy Clin Immunol: In Pract* (2020) 8(3):1074–82. doi: 10.1016/j.jaip.2019.10.046
5. Zuberbier T, Aberer W, Asero R, Abdul Latiff AH, Baker D, Ballmer- eber B, et al. The EAACI/GA2373 LEN/EDF/WAO Guideline for the Definition, Classification, Diagnosis and Management of Urticaria. *Allergy* (2018) 73:1393–414. doi: 10.1111/all.13397
6. Maurer M, Ortonne JP, Zuberbier T. Chronic Urticaria: A Patient Survey on Quality-of-Life, Treatment Usage and Doctor-Patient Relation. *Allergy* (2009) 64:581–8. doi: 10.1111/j.1398-9995.2008.01853.x
7. Kamiya K, Kishimoto M, Sugai J, Komine M, Ohtsuki M. Risk Factors for the Development of Psoriasis. *Int J Mol Sci* (2019) 20(18):4347. doi: 10.3390/ijms20184347
8. Alper S, Akyol M, Atakan N, Başkan EB, Gürer MA, Koç E, et al. Türkiye Psoriasis Tedavi Kılavuzu- 2012. *Türkderm-Deri Hastalıkları Ve Frengi Arşivi* (2012) 46(Özel):1–36.
9. Maurer M, Metz M, Bindslev-Jensen C, Bousquet J, Canonica GW, Church MK, et al. Definition, Aims, and Implementation of GA 2 LEN Urticaria Centers of Reference and Excellence. *Allergy* (2016) 71(8):1210–8. doi: 10.1111/all.12901
10. Maurer M, Aberer W, Agondi R, Al-Ahmad M, Al-Nesf MA, Ansotegui I, et al. Definition, Aims, and Implementation of GA2LEN/HAEi Angioedema Centers of Reference and Excellence. *Allergy* (2020). doi: 10.1111/all.14293
11. Özyurt K, Ertaş R, Avcı A, Akkuş MR, Atasoy M. Chronic Skin Disease Unit and Internal Counseling in an Outpatient Dermatology Clinic: Collaboration of Dermatologists in Dermatology Practice. *İKSSTD* (2020) 12(2):156–8. doi: 10.5222/iksstd.2020.81994
12. Weller K, Groffik A, Church MK, Hawro T, Krause K, Metz M, et al. Development and Validation of the Urticaria Control Test: A Patient-Reported Outcome Instrument for Assessing Urticaria Control. *J Allergy Clin Immunol* (2014) 133(5):1365–72. doi: 10.1016/j.jaci.2013.12.1076
13. Başkan EB. Türkiye Psoriasis Tedavi Kılavuzu. *Türkderm-Deri Hast Ve Frengi Arşivi* (2016) 50:4–6.
14. Barzilay R, Moore TM, Greenberg DM, DiDomenico GE, Brown LA, White LK, et al. Resilience, COVID-19-Related Stress, Anxiety and Depression During the Pandemic in a Large Population Enriched for Healthcare Providers. *Transl Psychiatry* (2020) 10:291. doi: 10.1038/s41398-020-00982-4
15. Beyaz S, Demir S, Oztop N, Karadag P, Coskun R, Colakoglu B, et al. Psychological Burden of COVID-19 on Mild and Moderate Chronic Spontaneous Urticarial. *In Allergy Asthma Proc* (2021) 42(4):e107–15(9). doi: 10.2500/aap.2021.42.210026
16. Peckham H, de Gruijter NM, Raine C, Radziszewska A, Ciurtin C, Wedderburn LR, et al. Male Sex Identified by Global COVID-19 Meta-Analysis as a Risk Factor for Death and ITU Admission. *Nat Commun* (2020) 11:6317. doi: 10.1038/s41467-020-19741-6
17. Bastard P, Rosen LB, Zhang Q, Michailidis E, Hoffmann H-H, Zhang Y, et al. Autoantibodies Against Type I IFNs in Patients With Life-Threatening COVID-19. *Science* (2020) 370(6515):eabd4585. doi: 10.1126/science.abd4585
18. Zhang Q, Bastard P, Liu Z, Le Pen J, Moncada-Velez M, Chen J, et al. Inborn Errors of Type I IFN Immunity in Patients With Life-Threatening COVID-19. *Science* (2020) 370(6515):eabd4570. doi: 10.1126/science.abd4570
19. Kobayashi T, Shimabukuro-Demoto S, Tsutsui H, Toyama-Sorimachi N. Type I Interferon Limits Mast Cell–Mediated Anaphylaxis by Controlling Secretory Granule Homeostasis. *PLoS Biol* (2019) 17(11):e3000530. doi: 10.1371/%0Ajournal.pbio.3000530

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NLRP3 Inflammasome: Checkpoint Connecting Innate and Adaptive Immunity in Autoimmune Diseases

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Autoimmune diseases are a broad spectrum of human diseases that are characterized by the breakdown of immune tolerance and the production of autoantibodies. Recently, dysfunction of innate and adaptive immunity is considered to be a key step in the initiation and maintenance of autoimmune diseases. NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is a multimeric protein complex, which can detect exogenous pathogen irritants and endogenous danger signals. The main function of NLRP3 inflammasome is to promote secretion of interleukin (IL)-1β and IL-18, and pyroptosis mediated by caspase-1. Served as a checkpoint in innate and adaptive immunity, aberrant activation and regulation of NLRP3 inflammasome plays an important role in the pathogenesis of autoimmune diseases. This paper reviewed the roles of NLRP3 inflammasome in autoimmune diseases, which shows NLRP3 inflammasome may be a potential target for autoimmune diseases deserved further study.

Keywords: NLRP3, autoimmune diseases, inflammatory bowel disease, rheumatoid arthritis, type 1 diabetes, systemic lupus erythematosus

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INTRODUCTION

Autoimmune diseases are characterized by self-reactive cells and the overproduction of autoantibodies, which are led by the breakdown of immunological tolerance and aberrant autoreactive immune responses (1). Autoimmune diseases include organ-specific autoimmune diseases, such as type 1 diabetes, autoimmune thyroid diseases, and rheumatoid arthritis, and systemic autoimmune diseases, such as systemic lupus erythematosus and systemic sclerosis (2). Although the pathogenesis of autoimmune diseases is still unclear, numerous studies have shown that aberrant innate and adaptive immunity is involved in the pathogenesis of autoimmune diseases (3, 4). Recently, emerging appreciation showed that NLRP3 inflammasome plays an important role in recognizing innate immune signals and inducing autoreactive immune responses, which probably acts as a checkpoint in innate immunity to cause skewed adaptive immune responses (**Figure 1**).

Inflammasomes are protein complexes composed of three parts: a sensor, an adaptor, and an effector. Tschopp et al. firstly proposed the concept of inflammasomes in 2002 (5), following which several inflammasome subtypes were discovered, including NLRP1 inflammasome, NLRP3 inflammasome, absent in melanoma 2 (AIM2) inflammasome, etc. In most of the inflammasome subtypes, the component of adaptor is usually apoptosis-associated speck-like protein (ASC), which

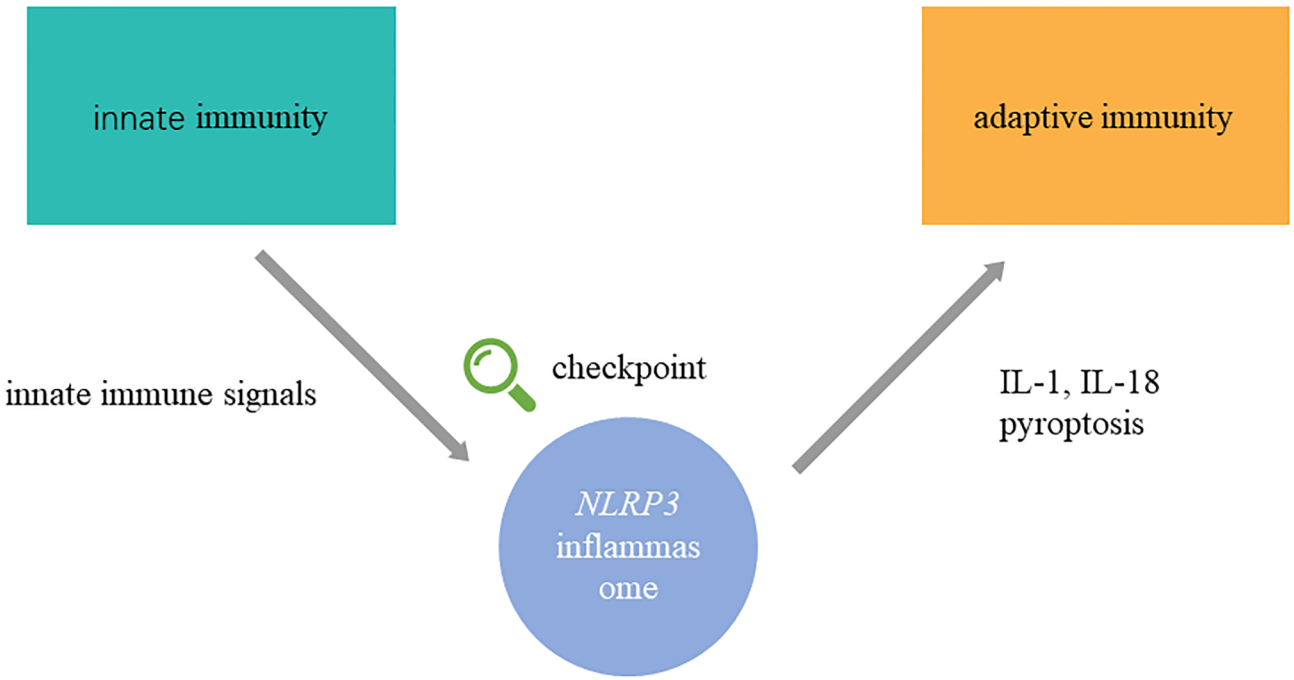


FIGURE 1 | NLRP3 inflammasome connects innate and adaptive immunity in autoimmune diseases. NLRP3 inflammasome is activated abnormally in autoimmune diseases. In the upstream process, exogenous pathogen irritants and endogenous danger signals initiate assembly of NLRP3 inflammasome. In the upstream process, IL-1β, IL-18, and pyroptosis, which are modulated by NLRP3 inflammasome, regulate adaptive immune response. Namely, NLRP3 inflammasome might participate in the transition from innate immunity to adaptive immunity in the pathogenesis of autoimmune diseases as a checkpoint. The detailed process of activation, assembly, regulation, and effects on adaptive immunity are shown in **Figure 2**.

contains a caspase activation and recruitment domain. The effector component is usually caspase-1. The differences among inflammasomes subtypes are the sensor component (**Figure 2**).

NOD-like receptor (NLR) is a typical type of pattern recognition receptors (PRRs), *via* which innate immune system recognizes pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). According to different subcellular localization, PRRs are classified into two categories: (a) PRRs located in the plasma membrane, which mainly play the role to recognize PAMPs and DAMPs, typically including Toll-like receptors (TLRs) and C-type lectin receptors (CLRs); (b) PRRs located in intracellular partitions, which mainly include NLRs, AIM2-like receptor (ALRs), and cytosolic sensor cyclic GMP-AMP (cGAMP) synthase (6, 7). NLRP3 inflammasome is one of the most widely studied inflammasomes. Effect of NLRP3 inflammasome to various physiological and pathogenic stimuli mainly includes caspase-1 activation, secretion of IL-1β and IL-18, and pyroptosis mediated by caspase-1. Under physiological conditions, inflammasomes play an important role in clearing pathogens and damaged cells, and serve as a critical composition of innate immune response. Whereas under pathological conditions, the overactivation of inflammasomes may trigger autoinflammatory and autoimmune responses and result in numerous diseases.

In this review, we summarized the references and presented that as the checkpoint, NLRP3 inflammasome connects innate and adaptive immunity in several autoimmune diseases, including inflammatory bowel disease, psoriasis, rheumatoid arthritis, systemic sclerosis, type 1 diabetes, systemic lupus erythematosus, and autoimmune thyroid diseases. Finally, we discussed the effect of new-onset inhibitors of NLRP3 inflammasome in autoimmune diseases, which implies their potential therapeutic value for clinical applications deserved further study.

STRUCTURE, ACTIVATION, AND REGULATION OF NLRP3 INFLAMMASOME

Structure

NLRP3 inflammasome consists of three components including NLRP3 scaffold, a pyrin domain (PYD), and a caspase recruitment domain (CARD), known as ASC, and caspase-1 (8). NLRP3 belongs to NLR protein family which includes 22 members widely expressed in human histiocytes, such as dendritic cells, macrophages, and monocytes. NLRP3 contains three segments (**Figure 2**): (a) PYD in amino-terminal; (b) a NACHT domain in central part: executes the function of NLRP3 self-association *via* ATPase activity; (c) a leucine-rich repeat domain (LRR domain) in carboxy-terminal: depresses NLRP3

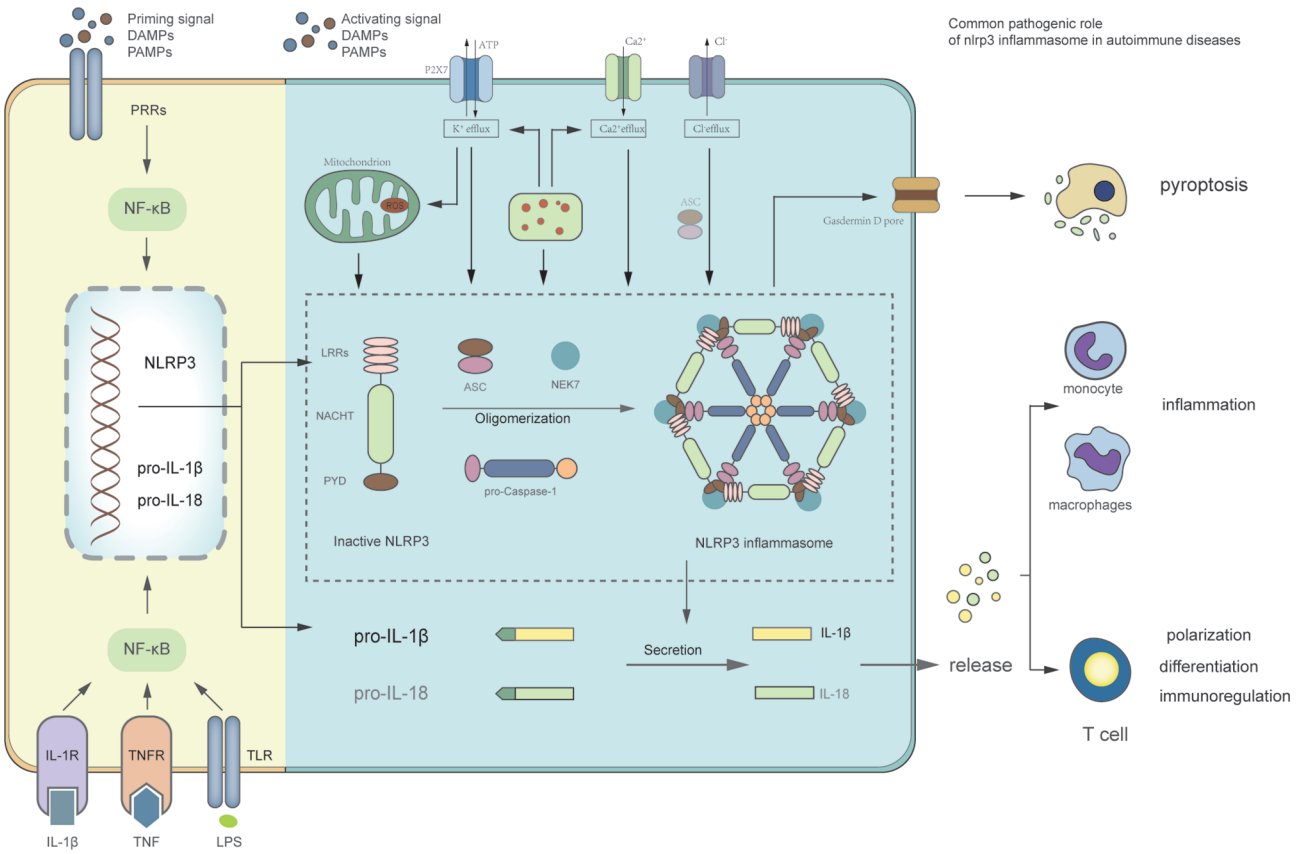


FIGURE 2 | Signaling pathway of NLRP3 inflammasome and the role of NLRP3 inflammasome in autoimmune diseases. The priming stage: the binding of cytokines or PAMPs to its receptors can activate NF-κB signaling pathway, which upregulates transcription of NLRP3, pro-IL-1β, and pro-IL-18. The activation stage: this stage is stimulated by PAMPs and DAMPs. The activating signals, including K^+ efflux, Ca^{2+}flux, Cl^- efflux, mitochondrial dysfunction, ROS production, lysosomal disruption, and NEK7 promote oligomerization of NLRP3, ASC, and pro-caspase-1 to format NLRP3 inflammasome complex. The active caspase-1 can cleave proinflammatory cytokines IL-1β and IL-18, and promote pyroptosis mediated by Gasdermin D. The role of NLRP3 inflammasome in autoimmune diseases: inflammation promoted by cytokines IL-1β and IL-18; adaptive immune dysfunction caused by cytokines, such as proliferation and differentiation of T cells; pyroptosis, which can cause histiocytic death such as insulin β-cells.

activation by inhibiting the ATPase activity of the NACHT domain. ASC includes PYD in amino-terminal and CARD in carboxy-terminal, which interact with each other to activate caspase-1. Caspase-1 consists of three parts from amino-terminal to carboxy-terminal: CARD, central large catalytic domain (p20), and small catalytic subunit domain (p10) (9, 10).

All of the substructures execute specific function in NLRP3 inflammasome assembly. Activated by upstream signals, the NACHT domains of NLRP3 interact with each other to induce NLRP3 oligomerization. And then the homotypic PYD-PYD interaction promotes ASC recruitments and formation of nucleates helical ASC filament. ASC recruits and activates caspase-1 *via* homotypic CARD-CARD interactions. At last, the clustered caspase-1 cleaves to a complex of p33 which comprises CARD and p20, a formation with proteolytical activation (11, **Figure 2**). NIMA-related kinase 7 (NEK7) is a new component in recent studies (**Figure 2**). Genetic research showed that NEK7 functioned as an indispensable component to NLRP3 inflammasome activation (12). NEK7 belongs to the family of mammalian NIMA-related kinases and is a serine or threonine kinase involved in mitosis. In the mediation of potassium efflux and mitochondrial reactive oxygen species (ROS), NEK7 binds to LRR domain of NLRP3, which is essential to NLRP3 inflammasome activation (13).

Activation

The activation of NLRP3 inflammasome includes two steps: firstly, it should be primed, sequentially activated. Priming is a preparation stage for subsequent responses. On the one hand, upregulated expression of NLRP3, caspase-1, and pro-IL-1β is induced by gene transcription and activation of nuclear factor-κB (NF-κB). This process is initiated through three ways (**Figure 2**): (a) PRRs, such as NOD2 or TLRs, recognize PAMPs and DAMPs; (b) cytokines directly activate NF-κB pathway, including tumor necrosis factor (TNF) and IL-1β; (c) lipopolysaccharide (LPS) upregulates IL-1β transcription by shifting specific metabolism status (14–16). On the other hand, NLRP3 post-translational modifications (PTMs) are induced in

the priming stage. NLRP3 is stabilized into a state in which the NLRP3 activity is auto-suppressed, but it still can respond to various signals (17). Importantly, NLRP3 PTMs occurs in the whole process of NLRP3 inflammasome activation, even in the unstimulated stage (18).

PAMPs and DAMPs stimuli varies in chemical properties and structures, and the direct binding of stimuli to NLRP3 are rarely detected. It is hypothesized that NLRP3 may sense common upstream signals which are induced by NLRP3 activators. This character of indirect activation is the core to understand molecular mechanism of NLRP3 activation. The comprehensive signal has not been determined, and relevant researches are contradictory. The common upstream signals identified include K^+ efflux, Ca^{2+} flux, Cl^- efflux, mitochondrial dysfunction, ROS production, lysosomal damage, trans-Golgi disassembly, metabolic changes, and so on. There are three typical consensus models of common upstream signals.

Model of Ion Fluxes

The ion fluxes are common triggers of NLRP3 inflammasome activation. They include K^+ efflux, Ca^{2+} flux, and Cl^- efflux, of which K^+ efflux is an indispensable upstream event. It was first proposed that ATP-mediated P2X purinoceptor 7 (P2X7) promoted production of mature IL-1β through K^+ efflux (19). And the high concentration of extracellular K^+ can depress NLRP3 inflammasome activation (20, 21). It suggests that intracellular low potassium status may trigger NLRP3 inflammasome activation. Meanwhile, the activated P2X7-ATP-dependent pore recruits a pannexin-1 hemi-channel, through which extracellular agonists could enter the cytoplasm and interact with NLRP3 inflammasome complex to promote mature IL-1β secretion (22, 23). In addition, the silica, particulate stimuli alum and calcium pyrophosphate crystals are demonstrated to induce K^+ efflux to trigger NLRP3 inflammasome activation (24). However, the mechanism of how intracellular decreased K^+ levels trigger NLRP3 activation has not been precisely illuminated. One of the presuppositions suggested that the drop in intracellular K^+ concentration may induce the conformational changes of NLRP3 to activate the following response (25, 26).

Model of Lysosomal Disruption

Particulate activation is a major factor to effect NLRP3 inflammasome. Self-derived particulate matter (such as uric acid, cholesterol crystals) and foreign-derived particulate matter (such as asbestos, alum, silica) could be endocytosed by lysosomal and cause subsequent lysosome membrane damages and release of the particulates and cathepsin B (27). NLRP3 inflammasome can be suppressed by broad-spectrum cathepsin inhibitors, such as CA-074-Me (a chemical cathepsin B inhibitor), which indicates that cathepsin is an important triggered signal of NLRP3 inflammasome (28, 29). Moreover, a variety of cathepsins exhibit function of promoting NLRP3 priming and activation, which is also blocked by CA-074-Me, and the NLRP3 activation process is rarely inhibited by the treatment of cathepsin B, X, L, or S gene deletion respectively (30). Hence, the redundancy among various cathepsin is vital for NLRP3 activation. In addition, lysosomal rupture can activate ion fluxes, including K^+ efflux and Ca^{2+} influx. It indicates that ion fluxes may be a common-converged point in different NLRP3 activation models (25, **Figure 2**).

Model of Mitochondrial Dysfunction and ROS

The activated NLRP3 inflammasome can eliminate dysfunctional mitochondria and reduce ROS, which are vital upstream events in NLRP3 inflammasome activation. NLRP3 inflammasome activation can be blocked by ROS scavenging agents or NAPDH oxidase inhibitors (31). NLRP3 inflammasome activation was also detected in mouse macrophages and human peripheral blood monocytes which are depleted of NAPDH oxidase activity (32). Further study is required to determine the comprehensive role of mitochondria in NLRP3 inflammasome activation. In addition, mitochondria can provide a docking site for assembly of NLRP3 inflammasome. Cardiolipin, mitochondrial antiviral signaling protein, and mitofusin 2 may serve as the connective point of NLRP3 to the mitochondria (33, 34).

The priming and activation stages result in a multimeric protein complex assembled by NLRP3, ASC and caspase-1. There are two major functions of activated caspase-1: (a) Promoting maturity and release of IL-1β and IL-18 by cleaving their precursors; (b) Initiating pyroptosis, a specific cell death between necrosis and apoptosis, by cleaving gasdermin D (11, 35). IL-1β and IL-18 are important members of the IL-1 family, which also include IL-1α, IL-33, IL 37, and so on. The IL-1 family play vital roles in inflammatory responses and immune regulation (36). IL-1β, as a typical pro-inflammatory cytokine, can induce autoinflammatory response and tissue destruction. It can improve macrophages' functions, recruit leukocytes *via* upregulating adhesion molecules and chemokines, and promote leukocytes to produce proinflammatory mediators (36–38). IL-1β, as a kind of T cell co-stimulatory factor, can provide pro-survival and proliferation signals for T cells (36), which also induce differentiation and polarization of T cell. IL-1β can promote naive $CD4^+$ T cells differentiation into Th17 cells and Th9 cells cooperating with other cytokines (39). IL-18, as a kind of IFN γ-inducing factor, can induce natural killer (NK) cells to product IFN-γ and IL-8 (40). IL-18 induces Th2 responses and promotes Th1 responses, synergizing with which induce IFN-γ production of T helper cells (36, 41). In epithelial cells, IL-18 regulates function of Th17 cell and Treg cell, which contributes to Th17/Treg imbalance (42).

Regulation

By recognizing pathological infections and endogenous danger signals, NLRP3 inflammasome can trigger immune responses and inflammatory responses. The regulation of NLRP3 inflammasome activation is as critical for immune regulation in immune homeostasis, as for inflammose function itself. The aberrant regulation can induce NLRP3 inflammasome overactivation and excessive cytokine production, resulting in skewed inflammatory responses, which might induce adaptive immune dysfunction. The statue can be switched from physiological defense to pathogenic damage by aberrant regulation.

The strict regulation of NLRP3 inflammasome activation is essential to maintain immune system homeostasis. More importantly, accurate understanding of the regulatory mechanisms is crucial to identify triggers of autoimmune diseases involved in NLRP3 inflammasome, and treatments targeted on it may show great therapeutic potential. Various potential regulation mechanisms have been reported, such as cell surface associated mucin 1, MicroRNA (miRNA), small heterodimer partner (**Table 1**). Clarifying traits of regulations from the following aspects might contribute to a more comprehensive understanding: positive or negative regulation; the specific stage on which the regulator targets, including priming stage and activation stage; and specific subcellular location, such as mitochondria and Golgi apparatus (**Table 1**).

PTMs of NLRP3 can participate in various phases of NLRP3 inflammasome activation and regulate innate immunity. Covalent additions including ubiquitylation, phosphorylation, and sumoylation have been reported in NLRP3 PTMs. For example, sumoylation by the protein E3 SUMO protein ligase MUL1 (also known as MAPL) depresses NLRP3 activation. However, both promoted and suppressed effect of phosphorylation on NLRP3 have been detected (54, 55). In addition, PTMs can interact with each other. Phosphorylation can promote deubiquitylation by interaction between BRCA1/BRCA2-containing complex subunit 3 and LRR domain of NLRP3 (54). It indicates that regulation of NLRP3 activation is a delicate and complex process.

Overreaction of innate immune responses may cause overactivity of cytokines and pyroptosis, which contribute to inflammatory and autoimmune diseases, the same as overreaction of skewed adaptive immune responses may cause hypersensitivity. Therefore, identifying critical triggers of the unbalanced immunoregulation would be an important direction in the future researches.

NLRP3 INFLAMMASOME IN AUTOIMMUNE DISEASES

Autoimmune diseases are characterized by loss of immunological tolerance and inappropriately autoreactive immune responses against histocytes and organs. However, the exact pathogenesis of autoimmune diseases has not been identified, and existing treatments are unsatisfactory (56). Current studies have proposed the role of NLRP3 inflammasome in autoimmune diseases and its clinical therapeutic potential (**Table 2**).

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is the most common intestinal tract disorders, characterized by dysfunction of innate immunity and aberrant inflammation in intestinal mucosa. It is comprised mainly of Crohn's disease (CD) and ulcerative colitis (UC) (133). Though the specific pathogenesis of IBD has not been elucidated until now, several researches have revealed that NLRP3 inflammasome activation is upregulated in IBD. Serum concentration of NLRP3 is elevated, positively correlated with serum IL-1β level and severity of IBD patients (134). Furtherly, NLRP3 inflammasome is activated in early stage of CD patients, whereas in late stage of UC patients, which implies that there are differences between the development of UC and DC (135).

TABLE 1 | Regulation of NLRP3 inflammasome.

Regulation	Effect on NLRP3 inflammasome activation		Stage		Subcellular location
	Promote	Inhibit	Priming stage	Activation stage	
Cell surface associated mucin 1 depresses phosphorylation of interleukin 1 receptor associated kinase 1 to downregulate activity of NF-kB (43).		√	√		Nucleus
A20 depresses degradation of NF-kB essential modulator by ubiquitination (44).		√	√		Nucleus
2,3,7,8-tetrachlorodibenzo-P-dioxin mediate aryl hydrocarbon receptor to enter the cell nucleus and combine to ARNT, which could bind to xenobiotic response element (45).		√	√		Nucleus
MicroRNA (miRNA) targets on specific region of NLRP3 mRNA to inhibit its expression (46).		√	√		Nucleus
Ubiquitylation of NLRP3, a type of post-translational modifications (PTMs) of NLRP3 (18).	√		√	√	
Proteins bind to PYCARD competitively, such as POPs (PYD-only proteins), COPs (CARD-only proteins), PYNOD (NLRPIO), and so on (47).		√		√	Cytoplasm
Leucine-rich repeat flightless-interacting protein recruits lightless I (the inhibitor protein of caspase substrate) (48).		√		√	Cytoplasm
Small heterodimer partner combines to NLRP3 competed with ASC (49).		√		√	Mitochondria
Cellular Fas-associated death domain-like IL-1β converting enzyme inhibitory protein interact with NLRP3 and pro-caspase-1 (50).	√			√	Cytoplasm
The functions of mitochondria, such as providing a docking site for NLRP3 inflammasome assembly (51, 52).	√			√	Mitochondria
Lysosomal disruption induced by phagocytosis of particulates (28).	√			√	Lysosome and cytoplasm
The trans-Golgi network is disassembled into vesicles, which recruit NLRP3 and promote NLRP3 aggregation (53).	√			√	Golgi

TABLE 2 | Studies of the roles of NLRP3 inflammasome in autoimmune diseases.

	Effect of NLRP3 inflammasome		Regulation of NLRP3 inflammasome activation and relevant pathway in autoimmune diseases.	
	Specific mechanism	**Common mechanism**	**Positive regulation**	**Negative regulation**
IBD	Disrupted inflammasome responses result in dysbiosis and increased colonization of pathobionts (57). .	Physiological condition: sense and respond to foreign milieu in the extracellular environment, *via* pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), mediate host immune responses to microbial infection and cellular damage.	Transporter 4 (58); Oxazolone (59); Protein tyrosine phosphatase non-receptor 22 (60).	Fasting-mimicking diet (61); Nutrient deprivation (62); CircRNA HECTD1 (63); Naringin (64); Carboxyamidotriazole (65); Growth differentiation factor 11 (66); Phloretin (67); Nuclear factor E2-related factor-2 (68); Cardamonin (69); sDR5-Fc fusion protein (70); Hydrogen sulfide (71); Cinnamaldehyde (72); BBG (a P2X7R blocker) and OLT1177 (73).
Psoriasis	IL-1 participates in pathogenesis partially (74).	Pathological conditions: 1) Histiocytic and organic inflammation promoted by cytokine IL-1 and IL-18; 2) Induce adaptive immune dysfunction *via* NLRP3 inflammasome activation, inducing the migration and differentiation of T cell by cytokines; 3) Pyroptosis modulated by activated caspase-1 of immunocyte and specific histocytes.	CD100/PlxnB2 (75); Tristetraprolin (TTP) downregulation (76); Tumor necrosis factor (TNF)-α (77); Acute-phase protein serum amyloid A (78).	Bay11-7082 (79); Datura Metel L (80).; Cycloastragenol (81); Cas9 RNP nanocomplexes (82).
RA	NLRP3 inflammasome activation contributes to Th1 differentiation in $CD4^+$ T cells (83); Induce Th2 differentiation and antibodies production (84).		TNF-α and calreticulin (85); Calcium-sensing receptor (86); Tofacitinib (87).	MCC950 (88); Protectin DX (89); Taraxasterol (90); Celastrol (91); Punicalagin (92); Hsa_circ_0044235 (93); hUCB-MSCs (94); A20 (44); tristetraprolin (95).
SSC	Downstream factors including IL-1, IL-18, and miR15 promote collagen synthesis and fibrosis (96, 97).		MiR-155 (97); Parvovirus B19 (98).	
T1DM	IL-1β induces the migration of proinflammatory cells into pancreatic islets (99, 100); IL-1β has direct cytotoxic effects on beta-cells (99, 100); Autoreactive T cells infiltrate pancreatic islets and cause beta-cell death (101).		LPS+ATP (102); Nitric oxide (103); Metabolic stress (104); Mitochondrial DNA (mDNA) (105).	Verapamil (106); Scutellarin (107); Ginsenoside Rg1 (108); Low-methoxyl pectin (109); fingolimod (110).
SLE	Autoantibodies induce NLRP3 inflammasome activation (82, 111); accumulation of NETs contributes to the pathogenesis (112, 113).		U1-small nuclear ribonucleoprotein (114); Glycogen synthase kinase 3β (115); Cyclic GMP-AMP synthase (116); Neutrophil extracellular traps (NETs) (117); Surface CXCR2 expression (118); Reactive oxygen species (119).	Xenon (120); Honokiol (121); Tris (dibenzylideneacetone) dipalladium (122); Cf-02 (123); Let-7f-5p (124); Magnolol Bay11-7082 (125); Curcumin (126); Melatonin (127); Lcariin (128); Piperine (129); Citral (130).
AITDs			Excessive iodine (131).	Yanghe decoction (132).

Downstream effects of NLRP3 inflammasome activation contribute to chronic inflammation, alterations in innate immune responses and disorders in mucosal immune response. These effects mainly include the consequent processes: pro-inflammatory cytokine release, macrophage hyperactivation with uncontrolled damage, and pyroptosis (136). *In vitro*, Monocarboxylate Transporter 4 contributed to intestinal enterocyte pyroptosis mediated by Caspase-1 through ERK1/2-NF-κB pathway, which can promote intestinal inflammation (58). Recent studies show that non-canonical endogenous irritants such as non-infectious stress conditions can also affect NLRP3 inflammasome activation. Fasting-mimicking diet reduced the expression of NLRP3 inflammasome and $CD4^+$ T cells percentage in peripheral blood and spleen to alleviate intestinal inflammation, which indicated that calorie restriction might modulate immune response *via* NLRP3 inflammasome (61). Yun et al. presented that NLRP3 inflammasome was removed by ubiquitin-mediated degradation, which was promoted by autophagy under nutrient deprivation (62). The downstream proinflammatory cytokines IL-1and IL-17E/25 could be suppressed to strengthen intestinal barrier function (62). CircRNA HECTD1 (circHECTD1) can alleviate UC by inhibiting NLRP3 inflammasome. And in Caco-2 cells, circHECTD1 can induce human antigen R (HuR) *via* miR-182-5p, which contributes to NLRP3 inflammasome activation by autophagy (63). NLRP3 inflammasome may have a double effect on IBD: some reports showed that IL-1β and IL-18 play a protective role in gastrointestinal inflammation. Oxazolone can stimulate maturation of pro-caspase-1 and pro-IL-1β, and colitis induced by oxazolone can be ameliorated by exogenous IL-1β or IL-18 (59). Higher sensitivity to oxazolone treatment and decreased IL-1β or IL-18 production were detected in

$NLRP3^{(-/-)}$ mice compared to wild-type mice, which indicates that IL-1β and IL-18 derived from NLRP3 inflammasome can protect against inflammation in gastrointestinal mucosa (59). In addition, IL-1β contributes to enhanced host defense against *Clostridium difficile* and *Citrobacter rodentium* during acute affection (137, 138). It induces ASC-dependent CXCL1 production to recruit neutrophils to the intestine, protects epithelial integrity, and reduces colonization (137, 138). There are several explanations for the protective effect. Marianne et al. presented that protein tyrosine phosphatase non-receptor 22 mediates dephosphorylation of NLRP3 inflammasome, resulting in its activation and release of mature IL-1β in mouse models (60). Under a physiological condition, this process contributes to effective host defense against harmful pathogens leading to gastrointestinal disorder and subsequent reconstitution (60). Meanwhile, another study had shown that NLRP3 inflammasome could promote neutrophil chemotaxis and antimicrobial secretions of the colon to sustain intestinal homeostasis (57). When epithelial barrier was destroyed, microbiota recruited immune cells to the lamina propria, and NLRP3 inflammasome activated in these immune cells played destructive effect (57). Therefore, NLRP3 inflammasome may have different effects in specific conditions, including physiological condition, infection by invading pathogenic bacteria and involving in innate immune disorder.

NLRP3 inflammasome inhibitors have been demonstrated to be effective in experimental models of IBD. Most inhibitors have a commonly downregulated effect on NLRP3, caspase-1, and pro-inflammatory cytokines. So far, therapeutic targets of NF-κB, Nuclear factor E2-related factor-2 (Nrf2), and ROS have been widely studied. Naringin, an activating ligand of peroxisome proliferator-activated receptor γ (PPARγ), can significantly decrease disease activity indexes (DAI), pathological damage of colon, and inflammation severity (64). Naringin alleviated UC *via* pressing NLRP3 inflammasome by activating PPARγ and degrading subsequent NF-κB activation (64). In a murine TNBS-induced colitis model, carboxyamidotriazolez downregulated NF-κB pathway by reduction of NF-κB p65 expression and phosphorylation of IκBα (65). Phloretin and Growth differentiation factor 11 play an anti-inflammatory effect targeting on the same pathway (66, 67). Nrf2 is a negative regulator for NLRP3 inflammasome by inhibiting priming of NLRP3 inflammasome (68). Cardamonin is a natural herbal extract of Alpinia katsumadai Hayata. Nrf2 and its target genes NQO1, Trx1, SOD2 and HO-1, especially NQO1, was elevated by cardamonin, which has the effect to depress NLRP3 inflammasome activation in a mouse model (69). And upregulation of Nrf2 has been detected after treatment of Resveratrol and Hydrogen sulfide (H_2S) (70, 71). ROS is considered as a second messenger in pro-inflammatory responses. H_2S can also reduce ROS production to prevent inflammation (71). Moreover, therapeutic strategies focused on miRNA has attracted more attention, Cinnamaldehyde can decrease MicroRNA-21 and miR-155 levels in colons and macrophages to ameliorate DSS-induced colitis (72). Recent studies showed that new mode of administration can also promote the efficacy of NLRP3 inflammasome inhibitors. The combined administration of BBG (a P2X7R blocker) and OLT1177 (a selective NLRP3 inhibitor) effectively alleviates UC by complementary effects (73). The effect of NLRP3 inflammasome inhibitors in IBD has not been comprehensively understood, which deserves further study.

Psoriasis

Psoriasis is a chronic inflammatory skin disease mediated by immune responses. In psoriasis biopsy, the expression of NLRP3, caspase-1, and IL-1β was significantly upregulated compared to non-lesional psoriatic skin (139). The single nucleotide polymorphisms (SNPs) studies show that the genetic mutations in NLRP3 are associated with psoriasis susceptibility in Chinese Han population (140). And the genetic mutations in *CARD8-C10X* (*rs2043211*) are associated with Psoriatic arthritis (PsA) in northern Swedish population (141). All these findings indicate that NLRP3 inflammasome may be involved in the occurrence and development of psoriasis.

PlxnB2 and its ligand (such as CD100) participate in neuronal development and immune responses (75). The bound of soluble CD100 to PlxnB2 can upregulate NF-κB pathway, which induces NLRP3 inflammasome activation in keratinocytes of psoriasis patients (75). In fibroblasts deriving from patients with psoriasis, abnormal inflammasome activity can be induced by downregulation of tristetraprolin TTP (76). TTP can directly target the degradation of NLRP3 mRNA; therefore, TTP downregulation may contribute to pathogenesis of psoriasis *via* NLRP3 inflammasome activation (76). Verma et al. found that TNF-α could activate NLRP3 inflammasome, which is independent of priming signals, and caspase-1 reactivity, plasma IL-1β and IL-18 are reduced in psoriasis patients treated with anti-TNF therapy (77). In mouse model of psoriasis, NLRP3 inflammasome keratinocytes can be activated by TNF-α to induce inflammation, which was induced by inhibiting autophagy *via* PI3K/AKT/mTOR signaling pathway (142). Overexpression of lncRNA MEG3 could alleviate inflammation by promoting autophagy (142). Serum amyloid A (SAA) serves as an important trigger to promote expression of IL-1β by activating NF-κB pathway in psoriatic keratinocytes (78). And IL-1β plays a key role in the skin diseases mediated by T helper type 17 cells (78). *In vitro*, miR-155 silencing can depress NLRP3/caspase-1 signal pathway to alleviate inflammatory responses (79). In summary, these findings indicate that CD100/PlxnB2, TTP, TNF, miR-155, and SAA might be potential therapeutic targets for psoriasis.

Herbal extracts have been demonstrated to be effective treatment for psoriasis. In psoriasis mouse models induced by imiquimod, the expression of TLR7, TLR8, p-NF-κB, and NLRP3 were obviously suppressed by administration of Datura metel L (80). The production of key inflammatory cytokines including IL-1β, IL-17, and IL-23 was also inhibited, which implied that Datura metel L. plays a protective role by depressing the pathway of TLR7/8-MyD88-NF-κb-NLRP3 (80). Paeonia lactiflora Pallas extract and BAY11-7082 have an inhibitory effect against immune responses in keratinocytes

probably by the similar pathway (79, 81). In addition, cycloastragenol specifically inhibits macrophages infiltration in dermis and pyroptosis mediated by NLRP3 inflammasome in mouse models (81). IL-1 inhibition might be another promising therapeutic strategy. Wan et al. reported a dissolvable microneedle, which consists of Cas9 RNP nanocomplexes and dexamethasone nanoparticles (82). The microneedle can be internalized by keratinocytes and immune cells, and disrupted NLRP3 inflammasome selectively to alleviate skin inflammations in a mouse model of psoriasis (82). Of note, new-onset plaque psoriasis has been shown to be a side effect in the RA patients treated with anti-IL-1 therapy (74). The role of NLRP3 inflammasome in psoriasis pathogenic mechanism is complicated, and the researches are comparatively limited, which deserves further studies.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common chronic autoimmune disease characterized by persistent synovial inflammation, pannus formation, destruction of cartilage and small diarthrodial joints, which is mainly caused by autoantibody secretion and aberrant immune responses (143). Various NLRP3-releated SNPs have been shown to be associated with susceptibility of RA. NLRP3 SNPs are associated with susceptibility of RA and anti-TNF responses in Caucasian population (144). In addition, genetic mutations of NLRP3 inflammasome may increase the risk of stroke and TIA in Swedish population, but not of myocardial infarction and angina pectoris (145).

Several studies have shown the hyperactivity of NLRP3 Inflammasome and downstream factors in RA. The intracellular levels of NLRP3, active caspase-1, pro-IL-1β, and active IL-1β in whole blood cells are increased in active RA patients (146). This increased level can also be detected with the treatment of TLR4 or TLR3 agonist (146). Guo et al. found that MCC950, a selective NLRP3 inhibitor, is able to depress NLRP3 inflammasome activation in monocyte and macrophages to infiltrate into the synovia, resulting in lesser joint inflammation and bone destruction (88). It indicates that NLRP3 Inflammasome is involved in the pathogenesis of RA. NLRP3 inflammasome activation has not been detected in fibroblast-like synoviocytes (FLS), which suggests that FLS may not produce IL-1β mediated by NLRP3 inflammation (147). TLR1-9 have been demonstrated to play a modulatory role in joint inflammation in the pathogenesis of RA (148). Downregulation of NOD2 gene expression can decrease NF-κB and pro-inflammatory cytokines in FLS of RA patients, which indicates that NOD2 may promote NLRP3 Inflammasome activation by effecting the priming stage (149). Both in FLS and human umbilical vein endothelial cells of RA patients, TNF-α/CRT dual signaling promotes NLRP3 Inflammasome activation *via* enhancing the effect of caspase-1 (85). TNF-α may serve as an initiator for HuR translocating to mediate NLRP3 Inflammasome activation (85). The typical activated models of Ca^{2+} and P2X7 are also detected to initiate activation of NLRP3 Inflammasome in RA patients (86).

Zhao et al. demonstrated that NLRP3 Inflammasome promotes Th17 cell differentiation to enhance the adaptive immune dysfunction of RA (150). Th17 has been proven to have a vital role in the downstream cascade reactions of NLRP3 inflammasome in RA. Jin et al. firstly found that protectin DX downregulates Th17 cells and pro-inflammatory cytokines and upregulates Tregs and anti-inflammatory cytokines by inhibiting NLRP3 inflammasome activation *via* miR-20a (89). Tofacitinib effectively ameliorates the severity of RA by restoring Treg/Th17 cell balance *via* reducing NLRP3 inflammasome activation in arthritic joints and draining lymph nodes (87). NLRP3 inflammasome can also mediate secretion of Fas-associated death domain, which is a pivotal adaptor molecule in innate immunity and inflammation (151). However, the expression of NLRP3 and pro-caspase-1 was decreased in the peripheral neutrophils (152). The active caspase-1 expression was increased, which was positively correlated with serum level of IL-18, which suggested that IL-18 mediated by active caspase-1 plays a pro-inflammatory role in neutrophils of RA, independently of NLRP3 inflammasome (152). Thus, different cell types should be noted in the pathogenesis of RA.

Taraxasterol exerts anti-inflammatory effects by depressing NLRP3 inflammasome pathway *via* downregulating the expression of NF-κB in RA patients (90). Celastrol, a quinone-methylated triterpenoid extracted from Tripterygium wilfordii, alleviates RA inflammation by suppressing the ROS/NF-κB/NLRP3 pathway (91). In collagen-induced arthritis mouse models, Punicalagin (an active substance extracted from pomegranate peel) inhibits phenotype polarization and pyroptosis of M1 macrophages by downregulating NF-κB signaling (92). Hsa_circ_0044235 has been found to inhibit NLRP3-mediated pyroptosis in FLSs (93). Other potential therapeutic strategies focus on negative regulation of NLRP3 inflammasome. Human umbilical cord blood-derived MSCs suppress the expression of NLRP3 Inflammasome *via* a paracrine loop of IL-1β signaling in CIA mouse models and mononuclear cells from RA patients (94). The deficiency of A20 in macrophages can enhance the effect of NLRP3 inflammasome by promoting the biological activity of caspase-1, IL-1β secretion, and pyroptosis in mouse models (44). Activation of NLRP3 Inflammasome and secretion of IL-1β can be inhibited by TTP expression and activation of TTP in both *in vivo* and *in vitro* models (95). TTP can be activated by dephosphorylation mediated by protein phosphatase 2A (PP2A), and Arctigenin (PP2A agonist) reduces monosodium urate (MSU) crystal-induced inflammation (95). It is indicated that TTP might be a potential target for inflammation induced by MSU crystals (95). These studies indicate that treatments aiming to inhibit NLRP3 inflammasome and IL-1β might be potential therapy for RA.

Systemic Sclerosis

Systemic sclerosis (SSc) is an idiopathic autoimmune disease targeting on connective tissue, characterized by autoimmune dysfunction, microvascular vessel alterations, and skin fibrosis. The fibrosis is triggered by inflammatory response, in which innate immunity serves as a key factor. However, the precise pathogenesis hasn't been clarified, and few effective treatments have been available until now.

In skin of patients with SSc, NLRP3 is upregulated and correlates positively with skin thickness (153). A resistant

feature to skin fibrosis can be detected in NLRP3$^{(-/-)}$ mice and ASC $^{(-/-)}$ mice, which implies that there is a correlativity between SNPs of NLRP3 and SSc (96). MiR-155 participates in innate immunity and adaptive immunity (154, 155). MiR-155 is involved in pulmonary fibrosis, hepatic fibrosis, as well as wound site fibrosis (156–158), and IL-1β can induce the expression of miR-155 (159). The expression of miR-155 was significantly increased in SSc lung fibroblasts, and fibrosis could be driven by inflammasome-dependent expression of miR-155 (97). Furthermore, there are positive feedbacks between NLRP3 inflammasome and miR-155, which explains sustaining fibrosis in SSc (97). MiR-155 might be a potential target for SSc, deserving future study.

Artlett et al. detected that caspase-1 inhibitor decreased secretion of IL-1β, IL-18, and collagens in dermal and lung fibroblasts of SSc, and reduced expression of α-smooth muscle actin in dermal fibroblasts (96). Shinohara presented that host receptors can identify pathogens to induce autoimmunity, which implies that infections and autoimmunity are closely connected (160). B19V infection induces caspase-1 mediated by NLRP3 inflammasome in monocytes of SSc patients (98). The expression of IL-1β and TNF-α can be induced by stimulus such as lipopolysaccharides (98). It indicates that viral components can enhance the sensitivity of NLRP3 inflammasome activation in monocytes (98). These findings may provide potential prevention strategy for SSc.

Type 1 Diabetes

Type 1 diabetes (T1D) is an autoimmune disease targeting insulin-producing pancreatic β-cells specifically mediated by T lymphocyte (161). Both innate immunity and adaptive immunity participate in the development of T1D, and NLRP3 inflammasome acts as an important component of innate immunity to induce insulitis and β-cell death (162, 163). Polymorphisms of NLRP3 inflammasome-related gene correlate with T1D. Pontillo et al. found that SNPs in NLRP3 had a correlation specifically with T1D in Brazilian population (164). In Norwegian population and Chinese Han population, NLRP1 was associated strongly with T1DM (165). Wu et al. found that SNPs in NLRP3 correlated specifically with T1D, especially in Latin American population (166). In Slovenian population, the association of *NLRP3* polymorphisms with T1D has not been observed (167). It is needed to confirm the specific gene polymorphisms of NLRP3 in different populations.

The important role of IL-1β in the pathogenesis of T1D has been determined in previous studies. IL-1β modulates pro-inflammatory cells to migrate into pancreatic islet, inducing direct cytotoxicity and β-cell apoptosis, which depends on the dose of IL-1β in T1D rat models (99, 100). Interestingly, NLRP3 inflammasome played a protective role in early stage of T1D in IRAK-M $^{(-/-)}$ NOD mice (168). In human islets, NLRP3 inflammasome can be activated, and secretion of IL-1β increases by the presence of LPS and ATP (102). As an upstream factor activating IL-1β, NLRP3 inflammasome may have a potential function in T1D. Resident peritoneal macrophages promote inflammation by inducing inflammatory cytokine/chemokine secretion such as IL-1β and upregulating the receptor expression of these proinflammatory cytokine/chemokine (103). Nitric oxide production is mediated by upregulated NLRP3/iNOS (nitric oxide synthase) pathway, which contributes to the proinflammatory state of resident PMs (103).

NLRP3 inflammasome has been shown to initiate and participate chronic complications of diabetes. Hyperglycemia can activate NLRP3 inflammasome, promote secretion of inflammatory cytokines, and trigger cascade of inflammatory response, finally inducing diabetic nephropathy, diabetic retinopathy, and so on (104, 169). Mitochondrial DNA activates NLRP3 inflammasome in endothelial cells *via* Ca^{2+} influx and mitochondrial ROS generation, which mediates endothelial dysfunction and vascular inflammation in diabetes complications (105). NLRP3 inflammasome activation can be detected in urothelial cells (170). It induces loss of in nerve density and Aδ-fibers, and leading to insensitivity of bladder fullness, which is a specific diabetic bladder dysfunction symptom (170). Verapamil, a calcium channel blocker, can diminish the release of IL-1β and TNF-α into the vitreous fluid and decrease retinal ganglion cell loss in diabetic retinopathy *via* inhibiting NLRP3 inflammasome mediated by TLR4 (106). Verapamil can also reduce pancreatic islets shrinkage and enhanced CD34 expression by interfering NLRP3 inflammasome assembly (106). In streptozotocin-induced mice with diabetic cardiomyopathy, the expression of NLRP3 inflammasome, IL-1β, and IL-18 in cardiac tissues was induced by nuclear NF-κB translocation, which can be inhibited by Scutellarin treatment (107). These researches imply that NLRP3 inhibitors could serve as a potential target for patients with diabetes.

MCC950, as an inhibitor of NLRP3 inflammasome, has been demonstrated to be a compelling treatment for diabetes in mouse models (171). Gao et al. found that Ginsenoside Rg1, a major active ingredient in ginseng, a traditional herb for diabetes in China, had a function to weaken NLRP3 activity in the liver and pancreas (108). In addition, low-methoxyl pectin can mediate decrease of NLRP3 inflammasome activation. And it can enhance cecal barrier function and shape intestinal homeostasis to ameliorate gut-pancreatic immune environment (109, 172). Low-methoxyl pectin may serve as a promising prevention drug of T1D. The TLR2/4, NF-κB, and NLRP3 inflammasome pathways are upregulated in intestinal tissues of NOD mice, which promotes the secretion of downstream signaling proteins such as IL-1β and IFN-γ (110). These downstream signaling proteins can induce activation and differentiation of T cells and the migration of these diabetogenic T cells to the pancreas (110). We propose that immunotherapy targeting on NLRP3 inflammasome is a promising approach to treat T1D.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by production of autoantibodies against nuclear components, deposition of immune complex, and multiorgan damage resulting from aberrations of immune response. Gene polymorphisms of NLRP3 were significantly

associated with susceptibility of SLE in Latin American individuals (173). However, in Chinese Han population, association between NLRP3 SNPs and SLE susceptibility has not been observed (174). It is warranted to study the relationship of gene polymorphisms of NLRP3 with SLE susceptibility in different populations. The expression of NLRP3 inflammasome, AIM2, and caspase-1 was increased in renal tissues, especially there was a positive relationship between the expression level of NLRP3 inflammasome and the activity index score in patients with SLE (175). Furthermore, the expression of NLRP3 mRNA was upregulated in macrophages, and the expression of AIM2 mRNA was decreased in female SLE patients (176). Meanwhile, in male SLE patients, the expression of AIM2 mRNA was increased, and SNP of CARD8 resulted in susceptibility of patients (176). It implied that there is a gender-dependent difference in mechanism of inflammasome activation in SLE patients.

Both innate and adaptive immunity are involved in the pathogenesis of SLE. Double-strand DNA and immune complex could activate NLRP3 inflammasome in histiocytes to promote inflammatory response mediated by IL-1β and IL-18 (49). Nucleic acid components including microbial nucleic acids, endogenous DNA, and endogenous RNA-containing U1-small nuclear ribonucleoprotein (U1-snRNP) can activate NLRP3 inflammasome in human monocytes *in vitro* (114). Shin MS et al. discovered that immune complexes could activate NLRP3 inflammasome by upregulating NF-kB pathway in animal models and patients with SLE (111). The caspase-1 activation and IL-1β production of bone marrow-derived macrophages was decreased after it was transfected with Glycogen synthase kinase 3β (GSK-3β) siRNA (115). The administration of GSK-3β inhibitor could alleviate severe proteinuria and nephritis, and anti-dsDNA antibody production, deposition of immune complex, and circulating cytokines were depressed (115). Zhao et al. suggested that GSK-3 promotes renal inflammation by activating NLRP3 inflammasome and mediating IL-1β release (115). Caspase-1 was activated especially in CD14-positive and CD16-positive monocytes from SLE patients, which were positively correlated with serum titers of anti-double-stranded DNA antibodies and negatively correlated with serum levels of complement component 3 and platelet count (116). NLRP3 inflammasome could be activated by cyclic GMP-AMP synthase stimulator of interferon genes pathway to promote caspase-1 activation and IL-1β secretion (116). Neutrophil extracellular traps (NETs) were also found to participate in the pathogenesis of SLE. NETs are a network consisting of chromatin fibers and granule-derived antimicrobial peptides (177). The impaired clearance of NETs and increased release of NETs are promoted by low-density granulocytes (LDGs), which contribute to accumulation of NETs in human monocytes *in vitro* (112, 113). NETs can activate caspase-1 with releasing of proinflammatory cytokines IL-1β and IL-18, and promote the formation of immune complex and type I interferon (117). Interestingly, IL-18 in turn induces perpetual NETosis in human neutrophils, which results in a feed-forward inflammatory loop (117). A recent study found that milk fat globule-EGF factor 8 (MFG-E8) could reduce neutrophil migration, accumulation, phagocytosis, and NETosis *via* reducing surface CXCR2 expression in pristane-induced lupus and patients with SLE (118).

NLRP3 inflammasome plays an important role in the development of SLE. Podocytes are highly differentiated epithelial cells and a critical component of glomerular basement membrane (GBM), which play a core role in maintaining the function of glomerular filtration (178). In the later stage of SLE, the expression of NLRP3 inflammasome and caspase-1 could be detected in podocytes in murine lupus models (115). The production of ROS promotes NLRP3 inflammasome activation in the podocytes line (119). Importantly, TLRs (such as TLR4) is expressed in glomerular podocytes of normal mice, which implies that podocytes also possess immune function in physiological condition (179). In addition, IL-18 release mediated by NLRP3 inflammasome is a potential pathogenic factor in cutaneous lupus lesions. Type I IFN is reported to be a main risk factor of cardiovascular disease (180). The feed-forward inflammatory loop mentioned above can also be detected in endothelial progenitor cells (181). Xenon can reduce NF-κB/NLRP3 inflammasome activation to ameliorating renal function in mouse models with spontaneous LN (120). Honokiol and dibenzylideneacetone (Tris) dipalladium both have potential therapeutic effect against accelerated and severe type of lupus nephritis by suppressing NLRP3 inflammasome activation (121, 122). The former interferes NLRP3 inflammasome *via* reducing NF-κB activation, suppressing reactive oxygen production and mitochondrial damage, and inducing sirtuin 1/autophagy axis activation (121). And the latter can reduce p38 MAPK signaling pathways and regulate the autophagy/NLRP3 inflammasome axis (122). Cf-02 can treat acute onset of severe lupus nephritis in mice by inhibiting the NF-κB/NLRP3 inflammasome axis and regulating T cell functions differentially (123).

Interestingly, NLRP3 inflammasome might have immunosuppressive effect on SLE (182–184). The expression of TGF-β target genes was depressed by deficiency of NLRP3 and ASC in mice models of spontaneous lupus-like autoimmunity (182). NLRP3 and ASC are demonstrated to downregulate TGF-β receptor signaling through SMAD2/3 phosphorylation, which contributes to the immunosuppressive effect (182). There are more and more new drugs targeting the NLRP3 inflammasome in the treatment for SLE, and some show to be effective (**Table 2**).

Autoimmune Thyroid Diseases

Autoimmune thyroid diseases (AITDs) are a series of thyroid diseases characterized by thyroid tissue damage and autoimmune disorders, including mainly Hashimoto's thyroiditis (HT) and Graves' disease (GD).

Guo et al. proposed firstly that multiple inflammasomes including NLRP3, NLRP1, NLRC4, and AIM2 participated in the development of AITDs (185). Excessive iodine promoted pyroptosis activity in thyroid follicular cells *via* the ROS-NF-κB-NLRP3 pathway which might be involved in the development of HT (186). However, Nagayama, Y., raised questions that Nthy-

ori 3-1 cells aren't capable of iodine uptake and the concentration of iodine (131). The role of NLRP3 inflammasome in Graves' disease (GD) has not been reported so far.

It is reported that Yanghe decoction (a traditional Chinese herbal formulation) can alleviate autoimmune thyroiditis in rat models *via* downregulating NLRP3 inflammasome and adjusting the imbalance Th17/Treg (132). There are very few studies concerning the effect of NLRP3 inflammasome in the pathogenesis of AITDs, deserving further study.

THE POSSIBLE PATHOGENESIS OF NLRP3 INFLAMMASOME IN AUTOIMMUNE DISEASES

NLRP3 inflammasome exists in various immune cells, including macrophages, granulocytes, antigen-presenting cells, B cells, and T cells. It has been found in tissues and cells such as platelet, podocyte, and keratinizing squamous epithelium of skin (187). As a vital component of innate immune system, NLRP3 inflammasome recognizes DAMPs and PAMPs, and initiates innate inflammatory responses *via* promoting proinflammatory cytokines secretion, which may also trigger adaptive immunity dysfunction (161). Besides its elementary physiological functions in immune responses, NLRP3 inflammasome also participates in pathogenesis process such as multiple autoimmune diseases. The common character of autoimmune diseases is histocyte and organ damage resulting from autoantibody overproduction, which leads to loss of immunological tolerance and aberrant autoreactive immune responses. The accurate role of NLRP3 inflammasome in autoimmune disease pathogenesis is complex and has not yet been completely illuminated.

Collectively, the effect of NLRP3 inflammasome in autoimmune diseases involves the following two aspects (**Table 2** and **Figure 2**): due to caspase-1 being the effector of inflammasome structure, IL-1β, IL-18, and pyroptosis modulated by activated caspase-1 play a major role in autoimmune diseases. Firstly, inflammation promoted by cytokines, especially IL-1, participates in the onset and development of most autoimmune diseases, such as RA and IBD (88, 188). Secondly, there is an emerging appreciation that NLRP3 inflammasome participates in adaptive immunity. Logically, NLRP3 inflammasome is involved in autoimmune diseases with adaptive immune dysfunction. For instance, IL-1 is a co-stimulatory factor and lymphocyte activating factor, which can provide signals of pro-survival and proliferation for T cells. It promotes autoreactive T cells to cause β-cell death (101). IL-1β induces migration of T cells into pancreatic islets by regulating chemotaxis (189). In RA, NLRP3 inflammasome of $CD4^+$ T cells promote Th1 differentiation, which is induced by IL-1β in a caspase-1-dependent manner (83). In other autoimmune diseases, NLRP3 inflammasome can also induce differentiation and polarization of Th2, Th17, and dendritic cells (84). Thirdly, pyroptosis mediated by activated caspase-1 promotes development of autoimmune diseases. In IBD, NLRP3 inflammasome triggers pyroptosis in a caspace-1-dependent manner to cause death of histocytes such as macrophages and dendritic cells (190). The release of cellular debris reacts with immune cells, resulting in an enhanced circle of inflammation (190). Generally, IL-1β, IL-18, and pyroptosis play a function by inflammation, activating adaptive immunity and immunological regulation. Interestingly, the pathogenesis of IBD and psoriasis is mainly inflammation, and the pathogenesis of T1DM, SLE, and AITDs mainly involves adaptive immune response. Meanwhile, the pathogenesis of RA and SSC involves both above factors. So distinguishing emphasis of different autoimmune disease pathogenesis, which might involve a spectrum from auto-inflammatory to adaptive immune response, may contribute to understand the specific and accurate role of NLRP3 inflammasome.

On the other hand, besides effect of NLRP3 inflammasome pathway, NLRP3 inflammasome can promote autoimmune diseases in some other manners. In SSC, NLRP3 inflammasome activation upregulates miR-155, which participates in collagen synthesis of keratinocytes (156, 157). In summary, NLRP3 inflammasome participates in the initiation of autoimmune diseases, which serves as a checkpoint in innate immunity and adaptive immune dysfunction (**Figure 1**). Compared with clinical application focusing on downstream adaptive immune response, chemical inhibitor targeting on NLRP3 inflammasome pathway might be a more potential therapeutic strategy.

NLRP3 inflammasome is significantly increased in autoimmune diseases such as T1D, IBD, SLE, RA, SSC, psoriasis, and AITDs. However, the protective role and decrease of NLRP3 inflammasome have also been detected in T1D, IBD, SLE, and psoriasis. The controversial results may be explained by hypothesis as follows: (a) In different stages of the disease, NLRP3 inflammasome may conduct completely inverse function. For example, an experiment conducted with IRAK-$M^{(-/-)}$ NOD mice, which is characterized by early onset and rapid progression of T1D, shows that NLRP3 inflammasome is a protective factor in the initial stage of T1D (168). (b) Despite the effect of NLRP3 inflammasome signaling pathway, NLRP3 inflammasome may also affect other bioactive substances and signaling pathways, by which it plays an opposite role. In SLE, NLRP3 inflammasome modulates TGF-β and IFN-I to conduct immunosuppressive effect (182, 191). (c) In some special pathways, NLRP3 inflammasome plays a protective role. Gastrointestinal tract is characterized by communication with external environment indirectly and existence of intestinal microflora. NLRP3 inflammasome strengthens anti-inflammatory effect to maintain intestine homeostasis but promotes modulation of T cell in a physiological condition. Therefore, deficiency and overt activation of NLRP3 inflammasome can both promote the development of IBD. In summary, future researches to identify specific mechanism of NLRP3 inflammasome in different histiocytes, disease stages and conditions are warranted.

There are several common pathways in different autoimmune diseases, such as autophagy, mitochondrial DNA, TLR4, Nrf2

pathway, Bay11-7082, P2X7 receptor, microRNA, and so on (**Table 2**). Moreover, there are specific and subtle differences between these similarly pathogenic mechanisms. For instance, the main function of miR-155 is to promote inflammation in keratinocyte of psoriasis and in macrophage of IBD; however, it drives fibrosis in fibroblasts of SSC. All these contrasts and connect in various mechanism indicate the complexity of NLRP3 inflammasome in the pathogenesis of autoimmune diseases. So, further studies are needed to focus on the comparison of mechanisms in different active sites, such as intestine and pancreatic β cell. In addition, previous studies have shown that NLRP3 inflammasome plays a protective role in physiological condition, and both the upregulation and downregulation of NLRP3 inflammasome pathway stimulated by specific factor will initiate pathogenic progress. As the mechanism of NLRP3 inflammasome assembly and downstream cytokines effect are more studied, the initiating mechanism of regulation dysfunction is of great importance in future researches. Just as avoidance of allergen to hypersensitivity, therapy focusing on initiating mechanism of regulation dysfunction will be a simple but effective therapeutic application. The inhibition of NLRP3 inflammasome pathway, such as Ginsenoside Rg1, low methoxyl pectin, and Bay11-7082, may be a potential therapeutic strategy for autoimmune diseases (**Table 2**). The inhibition involves three levels: structural proteins of NLRP3 inflammasome, cytokines, and the signaling pathway. However, the effect of some inhibitions is limited and scant. Some inhibitions cause serious side effects, and some inhibitions even show paradoxical pro-inflammatory effect. Newly plaque psoriasis could be detected in RA patients with anti-IL-1 treatment (74). It indicates that the role of NLRP3 inflammasome inhibition is complicated and associates with multiple mechanisms. Consequently, further researches are needed to focus on the following points: inhibitions that target on junction point of pathway may show precise efficacy; in addition, focusing on upstream mechanism and finding inhibition that blocks the switch of the whole pathogenic progress may bring breakthrough to therapy of autoimmune diseases.

CONCLUSION

In conclusion, as a platform sensing dangerous stimuli from endogenous or exogenous environment, NLRP3 inflammasome participates in both innate and adaptive immunity *via* modulating secretion of cytokines and pyroptosis. Increasing experiments show that NLRP3 inflammasome may play different pathogenic roles in autoimmune diseases, which provides a promising therapeutic option for autoimmune diseases. Therefore, delineating a comprehensive molecular mechanism of the complex role of NLRP3 inflammasome in autoimmune diseases deserves further study.

AUTHOR CONTRIBUTIONS

YWZ, the main author, contributed in literature review and summary, manuscript drafting. WY assisted in reviewing literature. WL revised the manuscript. YJZ, the corresponding author, contributed in designing, reviewing, and editing the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Davidson A, Diamond B. Autoimmune Diseases. *N Engl J Med* (2001) 345:340–50. doi: 10.1056/NEJM200108023450506
2. Papp G, Boros P, Nakken B, Szodoray P, Zeher M. Regulatory Immune Cells and Functions in Autoimmunity and Transplantation Immunology. *Autoimmune Rev* (2017) 16:435–44. doi: 10.1016/j.autrev.2017.03.011
3. Bentham J, Morris DL, Graham DSC, Pinder CL, Tombleson P, Behrens TW, et al. Genetic Association Analyses Implicate Aberrant Regulation of Innate and Adaptive Immunity Genes in the Pathogenesis of Systemic Lupus Erythematosus. *Nat Genet* (2015) 47:1457–64. doi: 10.1038/ng.3434
4. Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, et al. Innate Immunity and Intestinal Microbiota in the Development of Type 1 Diabetes. *Nature* (2008) 455:1109–13. doi: 10.1038/nature07336
5. Martinon F, Burns K, Tschopp J. The Inflammasome: A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of proIL-Beta. *Mol Cell* (2002) 10:417–26. doi: 10.1016/s1097-2765(02)00599-3
6. Medzhitov R, Preston-Hurlburt P, Janeway CA. A Human Homologue of the Drosophila Toll Protein Signals Activation of Adaptive Immunity. *Nature* (1997) 388:394–7. doi: 10.1038/41131
7. Seo GJ, Kim C, Shin WJ, Sklan EH, Eoh H, Jung JU. TRIM56-Mediated Monoubiquitination of cGAS for Cytosolic DNA Sensing. *Nat Commun* (2018) 9:613. doi: 10.1038/s41467-018-02936-3
8. Franchi L, Warner N, Viani K, Nuñez G. Function of Nod-Like Receptors in Microbial Recognition and Host Defense. *Immunol Rev* (2009) 227:106–28. doi: 10.1111/j.1600-065X.2008.00734.x
9. Schmidt FI, Lu A, Chen JW, Ruan J, Tang C, Wu H, et al. A Single Domain Antibody Fragment That Recognizes the Adaptor ASC Defines the Role of ASC Domains in Inflammasome Assembly. *J Exp Med* (2016) 213:771–90. doi: 10.1084/jem.20151790
10. Lu A, Magupalli VG, Ruan J, Yin Q, Atianand MK, Vos MR, et al. Unified Polymerization Mechanism for the Assembly of ASC-Dependent Inflammasomes. *Cell* (2014) 156:1193–206. doi: 10.1016/j.cell.2014.02.008
11. Boucher D, Monteleone M, Coll RC, Chen KW, Ross CM, Teo JL, et al. Caspase-1 Self-Cleavage Is an Intrinsic Mechanism to Terminate Inflammasome Activity. *J Exp Med* (2018) 215:827–40. doi: 10.1084/jem.20172222
12. Schmid-Burgk JL, Chauhan D, Schmidt T, Ebert TS, Reinhardt J, Endl E, et al. A Genome-Wide CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Screen Identifies NEK7 as an Essential Component

of NLRP3 Inflammasome Activation. *J Biol Chem* (2016) 291:103–9. doi: 10.1074/jbc.C115.700492
13. He Y, Zeng MY, Yang D, Motro B, Núñez G. NEK7 Is an Essential Mediator of NLRP3 Activation Downstream of Potassium Efflux. *Nature* (2016) 530:354–7. doi: 10.1038/nature16959
14. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, et al. Cutting Edge: NF-κb Activating Pattern Recognition and Cytokine Receptors License NLRP3 Inflammasome Activation by Regulating NLRP3 Expression. *J Immunol* (2009) 183:787–91. doi: 10.4049/jimmunol.0901363
15. Xing Y, Yao X, Li H, Xue G, Guo Q, Yang G, et al. Cutting Edge: TRAF6 Mediates TLR/IL-1r Signaling-Induced Nontranscriptional Priming of the NLRP3 Inflammasome. *J Immunol* (2017) 199:1561–6. doi: 10.4049/jimmunol.1700175
16. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate Is an Inflammatory Signal That Induces IL-1β Through HIF-1α. *Nature* (2013) 496:238–42. doi: 10.1038/nature11986
17. Liu J, Qian C, Cao X. Post-Translational Modification Control of Innate Immunity. *Immunity* (2016) 45:15–30. doi: 10.1016/j.immuni.2016.06.020
18. Juliana C, Fernandes-Alnemri T, Kang S, Farias A, Qin F, Alnemri ES. Non-Transcriptional Priming and Deubiquitination Regulate NLRP3 Inflammasome Activation. *J Biol Chem* (2012) 287:36617–22. doi: 10.1074/jbc.M112.407130
19. Walev I, Reske K, Palmer M, Valeva A, Bhakdi S. Potassium-Inhibited Processing of IL-1 Beta in Human Monocytes. *EMBO J* (1995) 14:1607–14. doi: 10.1002/j.1460-2075.1995.tb07149.x
20. Pétrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J. Activation of the NALP3 Inflammasome Is Triggered by Low Intracellular Potassium Concentration. *Cell Death Differ* (2007) 14:1583–9. doi: 10.1038/sj.cdd.4402195
21. Franchi L, Kanneganti TD, Dubyak GR, Núñez G. Differential Requirement of P2X7 Receptor and Intracellular K^+ for Caspase-1 Activation Induced by Intracellular and Extracellular Bacteria. *J Biol Chem* (2007) 282:18810–8. doi: 10.1074/jbc.M610762200
22. Schmid-Burgk JL, Gaidt MM, Schmidt T, Ebert TS, Bartok E, Hornung V. Caspase-4 Mediates Non-Canonical Activation of the NLRP3 Inflammasome in Human Myeloid Cells. *Eur J Immunol* (2015) 45:2911–7. doi: 10.1002/eji.201545523
23. Ketelut-Carneiro N, Silva GK, Rocha FA, Milanezi CM, Cavalcanti-Neto FF, Zamboni DS, et al. IL-18 Triggered by the NLRP3 Inflammasome Induces Host Innate Resistance in a Pulmonary Model of Fungal Infection. *J Immunol* (2015) 194:4507–17. doi: 10.4049/jimmunol.1402321
24. Liu H, You L, Wu J, Zhao M, Guo R, Zhang H, et al. Berberine Suppresses Influenza Virus-Triggered NLRP3 Inflammasome Activation in Macrophages by Inducing Mitophagy and Decreasing Mitochondrial ROS. *J Leukoc Biol* (2020) 108:253–66. doi: 10.1002/JLB.3MA0320-358RR
25. Muñoz-Planillo R, Kuffa P, Martínez-Colón G, Smith BL, Rajendiran TM, Núñez G. K^+ Efflux Is the Common Trigger of NLRP3 Inflammasome Activation by Bacterial Toxins and Particulate Matter. *Immunity* (2013) 38:1142–53. doi: 10.1016/j.immuni.2013.05.016
26. Meng G, Zhang F, Fuss I, Kitani A, Strober W. A Mutation in the Nlrp3 Gene Causing Inflammasome Hyperactivation Potentiates Th17 Cell-Dominant Immune Responses. *Immunity* (2009) 30:860–74. doi: 10.1016/j.immuni.2009.04.012
27. Chu J, Thomas LM, Watkins SC, Franchi L, Núñez G, Salter RD. Cholesterol-Dependent Cytolysins Induce Rapid Release of Mature IL-1beta From Murine Macrophages in a NLRP3 Inflammasome and Cathepsin B-Dependent Manner. *J Leukoc Biol* (2009) 86:1227–38. doi: 10.1189/jlb.0309164
28. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, et al. Silica Crystals and Aluminum Salts Activate the NALP3 Inflammasome Through Phagosomal Destabilization. *Nat Immunol* (2008) 9:847–56. doi: 10.1038/ni.1631
29. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, Reinheckel T, et al. The NALP3 Inflammasome Is Involved in the Innate Immune Response to Amyloid-Beta. *Nat Immunol* (2008) 9:857–65. doi: 10.1038/ni.1636
30. Orlowski GM, Colbert JD, Sharma S, Bogyo M, Robertson SA, Rock KL. Multiple Cathepsins Promote Pro-IL-1β Synthesis and NLRP3-Mediated IL-1β Activation. *J Immunol* (2015) 195:1685–97. doi: 10.4049/jimmunol.1500509
31. Dostert C, Pétrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate Immunee Activation Through Nalp3 Inflammasome Sensing of Asbestos and Silica. *Science* (2008) 320:674–7. doi: 10.1126/science.1156995
32. van Bruggen R, Köker MY, Jansen M, van Houdt M, Roos D, Kuijpers TW, et al. Human NLRP3 Inflammasome Activation Is Nox1-4 Independent. *Blood* (2010) 115:5398–400. doi: 10.1182/blood-2009-10-250803
33. Park S, Juliana C, Hong S, Datta P, Hwang I, Fernandes-Alnemri T, et al. The Mitochondrial Antiviral Protein MAVS Associates With NLRP3 and Regulates Its Inflammasome Activity. *J Immunol* (2013) 191:4358–66. doi: 10.4049/jimmunol.1301170
34. Ichinohe T, Yamazaki T, Koshiba T, Yanagi Y. Mitochondrial Protein Mitofusin 2 Is Required for NLRP3 Inflammasome Activation After RNA Virus Infection. *Proc Natl Acad Sci USA* (2013) 110:17963–8. doi: 10.1073/pnas.1312571110
35. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, et al. Cleavage of GSDMD by Inflammatory Caspases Determines Pyroptotic Cell Death. *Nature* (2015) 526:660–5. doi: 10.1038/nature15514
36. Dinarello CA. Immunological and Inflammatory Functions of the Interleukin-1 Family. *Annu Rev Immunol* (2009) 27:519–50. doi: 10.1146/annurev.immunol.021908.132612
37. Garlanda C, Dinarello CA, Mantovani A. The Interleukin-1 Family: Back to the Future. *Immunity* (2013) 39:1003–18. doi: 10.1016/j.immuni.2013.11.010
38. Chan AH, Schroder K. Inflammasome Signaling and Regulation of Interleukin-1 Family Cytokines. *J Exp Med* (2020) 217:e20190314. doi: 10.1084/jem.20190314
39. Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, et al. Development, Cytokine Profile and Function of Human Interleukin 17-Producing Helper T Cells. *Nat Immunol* (2007) 8:950–7. doi: 10.1038/ni1497
40. Poznanski SM, Lee AJ, Nham T, Lusty E, Larché MJ, Lee DA, et al. Combined Stimulation With Interleukin-18 and Interleukin-12 Potently Induces Interleukin-8 Production by Natural Killer Cells. *J Innate Immun* (2017) 9:511–25. doi: 10.1159/000477172
41. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 Is a Unique Cytokine That Stimulates Both Th1 and Th2 Responses Depending on Its Cytokine Milieu. *Cytokine Growth Factor Rev* (2001) 12:53–72. doi: 10.1016/s1359-6101(00)00015-0
42. Harrison OJ, Srinivasan N, Pott J, Schiering C, Krausgruber T, Ilott NE, et al. Epithelial-Derived IL-18 Regulates Th17 Cell Differentiation and Foxp3+ Treg Cell Function in the Intestine. *Mucosal Immunol* (2015) 8:1226–36. doi: 10.1038/mi.2015.13
43. Ng GZ, Menheniott TR, Every AL, Stent A, Judd LM, Chionh YT, et al. The MUC1 Mucin Protects Against Helicobacter Pylori Pathogenesis in Mice by Regulation of the NLRP3 Inflammasome. *Gut* (2016) 65:1087–99. doi: 10.1136/gutjnl-2014-307175
44. Vande Walle L, Van Opdenbosch N, Jacques P, Fossoul A, Verheugen E, Vogel P, et al. Negative Regulation of the NLRP3 Inflammasome by A20 Protects Against Arthritis. *Nature* (2014) 512:69–73. doi: 10.1038/nature13322
45. Huai W, Zhao R, Song H, Zhao J, Zhang L, Zhang L, et al. Aryl Hydrocarbon Receptor Negatively Regulates NLRP3 Inflammasome Activity by Inhibiting NLRP3 Transcription. *Nat Commun* (2014) 5:4738. doi: 10.1038/ncomms5738
46. Bauernfeind F, Rieger A, Schildberg FA, Knolle PA, Schmid-Burgk JL, Hornung V. NLRP3 Inflammasome Activity Is Negatively Controlled by miR-223. *J Immunol* (2012) 189:4175–81. doi: 10.4049/jimmunol.1201516
47. Le HT, Harton JA. Pyrin- and CARD-Only Proteins as Regulators of NLR Functions. *Front Immunol* (2013) 4:275. doi: 10.3389/fimmu.2013.00275
48. Jin J, Yu Q, Han C, Hu X, Xu S, Wang Q, et al. LRRFIP2 Negatively Regulates NLRP3 Inflammasome Activation in Macrophages by Promoting Flightless-I-Mediated Caspase-1 Inhibition. *Nat Commun* (2013) 4:2075. doi: 10.1038/ncomms3075
49. Yang CS, Kim JJ, Kim TS, Lee PY, Kim SY, Lee HM, et al. Small Heterodimer Partner Interacts With NLRP3 and Negatively Regulates Activation of the

NLRP3 Inflammasome. *Nat Commun* (2015) 6:6115. doi: 10.1038/ncomms7115
50. Wu Y, Kuo W, Wu Y, Yang K, Chen S, Jiang S, et al. Participation of C-FLIP in NLRP3 and AIM2 Inflammasome Activation. *Cell Death Differ* (2014) 21:451–61. doi: 10.1038/cdd.2013.165
51. Zhou R, Yazdi AS, Menu P, Tschopp J. A Role for Mitochondria in NLRP3 Inflammasome Activation. *Nature* (2011) 469(7329):221–5. doi: 10.1038/nature09663
52. Subramanian N, Natarajan K, Clatworthy MR, Wang Z, Germain RN. The Adaptor MAVS Promotes NLRP3 Mitochondrial Localization and Inflammasome Activation. *Cell* (2013) 153:348–61. doi: 10.1016/j.cell.2013.02.054
53. Chen J, Chen ZJ. PtdIns4P on Dispersed Trans-Golgi Network Mediates NLRP3 Inflammasome Activation. *Nature* (2018) 564:71–6. doi: 10.1038/s41586-018-0761-3
54. Song N, Liu Z, Xue W, Bai Z, Wang Q, Dai J, et al. NLRP3 Phosphorylation Is an Essential Priming Event for Inflammasome Activation. *Mol Cell* (2017) 68:185–97. e6. doi: 10.1016/j.molcel.2017.08.017
55. Mortimer L, Moreau F, MacDonald JA, Chadee K. NLRP3 Inflammasome Inhibition Is Disrupted in a Group of Auto-Inflammatory Disease CAPS Mutations. *Nat Immunol* (2016) 17:1176–86. doi: 10.1038/ni.3538
56. Chatenoud L. Immune Therapies of Autoimmune Diseases: Are We Approaching a Real Cure? *Curr Opin Immunol* (2006) 18:710–7. doi: 10.1016/j.coi.2006.09.004
57. Hirota SA, Ng J, Lueng A, Khajah M, Parhar K, Li Y, et al. NLRP3 Inflammasome Plays a Key Role in the Regulation of Intestinal Homeostasis. *Inflammation Bowel Dis* (2011) 17:1359–72. doi: 10.1002/ibd.21478
58. Wang Y, Zhou X, Zou K, Chen G, Huang L, Yang F, et al. Monocarboxylate Transporter 4 Triggered Cell Pyroptosis to Aggravate Intestinal Inflammation in Inflammatory Bowel Disease. *Front Immunol* (2021) 12:644862. doi: 10.3389/fimmu.2021.644862
59. Itani S, Watanabe T, Nadatani Y, Sugimura N, Shimada S, Takeda S, et al. NLRP3 Inflammasome has a Protective Effect Against Oxazolone-Induced Colitis: A Possible Role in Ulcerative Colitis. *Sci Rep* (2016) 6:39075. doi: 10.1038/srep39075
60. Spalinger MR, Kasper S, Gottier C, Lang S, Atrott K, Vavricka SR, et al. NLRP3 Tyrosine Phosphorylation Is Controlled by Protein Tyrosine Phosphatase PTPN22. *J Clin Invest* (2016) 126:1783–800. doi: 10.1172/JCI83669
61. Song S, Bai M, Ling Z, Lin Y, Wang S, Chen Y. Intermittent Administration of a Fasting-Mimicking Diet Reduces Intestinal Inflammation and Promotes Repair to Ameliorate Inflammatory Bowel Disease in Mice. *J Nutr Biochem* (2021) 96:108785. doi: 10.1016/j.jnutbio.2021.108785
62. Yun Y, Baek A, Kim DE. Autophagy Down-Regulates NLRP3-Dependent Inflammatory Response of Intestinal Epithelial Cells Under Nutrient Deprivation. *BMB Rep* (2021) 54(5):260–5. doi: 10.5483/BMBRep.2021.54.5.211
63. Xu Y, Tian Y, Li F, Wang Y, Yang J, Gong H, et al. Circular RNA HECTD1 Mitigates Ulcerative Colitis by Promoting Enterocyte Autophagy Via miR-182-5p/HuR Axis. *Inflamm Bowel Dis* (2021) izab188:1–16. doi: 10.1093/ibd/izab188
64. Cao H, Liu J, Shen P, Cai J, Han Y, Zhu K, et al. Protective Effect of Naringin on DSS-Induced Ulcerative Colitis in Mice. *J Agric Food Chem* (2018) 66:13133–40. doi: 10.1021/acs.jafc.8b03942
65. Du X, Chen W, Wang Y, Chen C, Guo L, Ju R, et al. Therapeutic Efficacy of Carboxyamidotriazole on 2,4,6-Trinitrobenzene Sulfonic Acid-Induced Colitis Model Is Associated With the Inhibition of NLRP3 Inflammasome and NF-κb Activation. *Int Immunopharmacol* (2017) 45:16–25. doi: 10.1016/j.intimp.2017.01.015
66. Wang L, Wang Y, Wang Z, Qi Y, Zong B, Liu M, et al. Growth Differentiation Factor 11 Ameliorates Experimental Colitis by Inhibiting NLRP3 Inflammasome Activation. *Am J Physiol Gastrointest Liver Physiol* (2018) 315:G909–20. doi: 10.1152/ajpgi.00159.2018
67. Zhang Z, Li S, Cao H, Shen P, Liu J, Fu Y, et al. The Protective Role of Phloretin Against Dextran Sulfate Sodium-Induced Ulcerative Colitis in Mice. *Food Funct* (2019) 10:422–31. doi: 10.1039/c8fo01699b
68. Liu X, Zhang X, Ding Y, Zhou W, Tao L, Lu P, et al. Nuclear Factor E2-Related Factor-2 Negatively Regulates NLRP3 Inflammasome Activity by Inhibiting Reactive Oxygen Species-Induced NLRP3 Priming. *Antioxid Redox Signal* (2017) 26:28–43. doi: 10.1089/ars.2015.6615
69. Wang K, Lv Q, Miao Y, Qiao S, Dai Y, Wei Z. Cardamonin, a Natural Flavone, Alleviates Inflammatory Bowel Disease by the Inhibition of NLRP3 Inflammasome Activation via an AhR/Nrf2/NQO1 Pathway. *Biochem Pharmacol* (2018) 155:494–509. doi: 10.1016/j.bcp.2018.07.039
70. Chen Y, Zheng Z, Li C, Pan Y, Tang X, Wang XJ. Synthetic Imine Resveratrol Analog 2-Methoxyl-3,6-Dihydroxyl-IRA Ameliorates Colitis by Activating Protective Nrf2 Pathway and Inhibiting NLRP3 Expression. *Oxid Med Cell Longev* (2019) 2019:7180284. doi: 10.1155/2019/7180284
71. Qin M, Long F, Wu W, Yang D, Huang M, Xiao C, et al. Hydrogen Sulfide Protects Against DSS-Induced Colitis by Inhibiting NLRP3 Inflammasome. *Free Radic Biol Med* (2019) 137:99–109. doi: 10.1016/j.freeradbiomed.2019.04.025
72. Qu S, Shen Y, Wang M, Wang X, Yang Y. Suppression of miR-21 and miR-155 of Macrophage by Cinnamaldehyde Ameliorates Ulcerative Colitis. *Int Immunopharmacol* (2019) 67:22–34. doi: 10.1016/j.intimp.2018.11.045
73. Sameh S, Mahmoud EY, Hossam S, Noha AA, Ruwyda ES, Farah HA, et al. BBG Enhances OLT1177-Induced NLRP3 Inflammasome Inactivation by Targeting P2X7R/NLRP3 and MyD88/NF-κb Signaling in DSS-Induced Colitis in Rats. *Life Sci* (2021) 270:119123. doi: 10.1016/j.lfs.2021.119123
74. González-López Ma, Martínez-Taboada Vm, González-Vela Mc, Fernández-Llaca H, Val-Bernal Jf. New-Onset Psoriasis Following Treatment With the Interleukin-1 Receptor Antagonist Anakinra. *Br J Dermatol* (2008) 158 (5):1146–8. doi: 10.1111/j.1365-2133.2008.08470.x
75. Zhang C, Xiao C, Dang E, Cao J, Zhu Z, Fu M, et al. CD100-Plexin-B2 Promotes the Inflammation in Psoriasis by Activating NF-κb and the Inflammasome in Keratinocytes. *J Invest Dermatol* (2018) 138(2):375–83. doi: 10.1016/j.jid.2017.09.005
76. Matteo B, Sebastian F, Claudia A, Roberta L, Andrea M, Sandra P, et al. Promoter Methylation Leads to Decreased ZFP36 Expression and Deregulated NLRP3 Inflammasome Activation in Psoriatic Fibroblasts. *Front Med* (2021) 7:579383. doi: 10.3389/fmed.2020.579383
77. Deepti V, Shora ZF, Gunnthorunn S, Cecilia BE, Charlotta S, Charlotta E. Enhanced Inflammasome Activity in Patients With Psoriasis Promotes Systemic Inflammation. *J Invest Dermatol* (2021) 141(3):586–595.e5. doi: 10.1016/j.jid.2020.07.012
78. Yu N, Liu S, Yi X, Zhang S, Ding Y. Serum Amyloid A Induces Interleukin-1β Secretion From Keratinocytes via the NACHT, LRR and PYD Domains-Containing Protein 3 Inflammasome. *Clin Exp Immunol* (2015) 179(2):344–53. doi: 10.1111/cei.12458
79. Irrera N, Vaccaro M, Bitto A, Pallio G, Pizzino G, Lentini M, et al. BAY 11-7082 Inhibits the NF-κb and NLRP3 Inflammasome Pathways and Protects Against IMQ-Induced Psoriasis. *Clin Sci* (2017) 131(6):CS20160645. doi: 10.1042/CS20160645
80. Yang B, Cheng Y, Liu Y, Liu Y, Tan J, Guan W, et al. Datura Metel L. Ameliorates Imiquimod-Induced Psoriasis-Like Dermatitis and Inhibits Inflammatory Cytokines Production Through TLR7/8-MyD88-NF-κb-NLRP3 Inflammasome Pathway. *Molecules* (2019) 24(11):2157. doi: 10.3390/molecules24112157
81. Deng G, Chen W, Wang P, Zhan T, Zheng W, Gu Z, et al. Inhibition of NLRP3 Inflammasome-Mediated Pyroptosis in Macrophage by Cycloastragenol Contributes to Amelioration of Imiquimod-Induced Psoriasis-Like Skin Inflammation in Mice. *Int Immunopharmacol* (2019) 74:105682. doi: 10.1016/j.intimp.2019.105682
82. Wan T, Pan Q, Ping Y. Microneedle-Assisted Genome Editing: A Transdermal Strategy of Targeting NLRP3 by CRISPR-Cas9 for Synergistic Therapy of Inflammatory Skin Disorders. *Sci Adv* (2021) 7 (11):eabe2888. doi: 10.1126/sciadv.abe2888
83. Arbore G, West EE, Spolski R, Robertson AAB, Klos A, Rheinheimer C, et al. T Helper 1 Immunity Requires Complement-Driven NLRP3 Inflammasome Activity in $CD4^+$ T Cells. *Science* (2016) 352:aad1210. doi: 10.1126/science.aad1210
84. Xue G, Jin G, Fang J, Lu Y. IL-4 Together With IL-1β Induces Antitumor Th9 Cell Differentiation in the Absence of TGF-β Signaling. *Nat Commun* (2019) 10(1):1376. doi: 10.1038/s41467-019-09401-9
85. Liu Y, Wei W, Wang Y, Wan C, Bai Y, Sun X, et al. TNF-α/Calreticulin Dual Signaling Induced NLRP3 Inflammasome Activation Associated With HuR

Nucleocytoplasmic Shuttling in Rheumatoid Arthritis. *Inflammation Res* (2019) 68:597–611. doi: 10.1007/s00011-019-01244-w
86. Jäger E, Murthy S, Schmidt C, Hahn M, Strobel S, Peters A, et al. Calcium-Sensing Receptor-Mediated NLRP3 Inflammasome Response to Calciprotein Particles Drives Inflammation in Rheumatoid Arthritis. *Nat Commun* (2020) 11:4243. doi: 10.1038/s41467-020-17749-6
87. Yang X, Zhan N, Jin Y, Ling H, Xiao C, Xie Z, et al. Tofacitinib Restores the Balance of γδtreg/γδt17 Cells in Rheumatoid Arthritis by Inhibiting the NLRP3 Inflammasome. *Theranostics* (2021) 11(3):1446–57. doi: 10.7150/thno.47860
88. Guo C, Fu R, Wang S, Huang Y, Li X, Zhou M, et al. NLRP3 Inflammasome Activation Contributes to the Pathogenesis of Rheumatoid Arthritis. *Clin Exp Immunol* (2018) 194:231–43. doi: 10.1111/cei.13167
89. Jin S, Sun S, Ling H, Ma J, Zhang X, Xie Z, et al. Protectin DX Restores Treg/T H 17 Cell Balance in Rheumatoid Arthritis by Inhibiting NLRP3 Inflammasome via miR-20a. *Cell Death Dis* (2021) 12(3):280. doi: 10.1038/s41419-021-03562-6
90. Chen J, Wu W, Zhang M, Chen C. Taraxasterol Suppresses Inflammation in IL-1β-Induced Rheumatoid Arthritis Fibroblast-Like Synoviocytes and Rheumatoid Arthritis Progression in Mice. *Int Immunopharmacolog* (2019) 70:274–83. doi: 10.1016/j.intimp.2019.02.029
91. Jing M, Yang J, Zhang L, Liu J, Xu S, Wang M, et al. Celastrol Inhibits Rheumatoid Arthritis Through the ROS-NF-κb-NLRP3 Inflammasome Axis. *Int Immunopharmacol* (2021) 98:107879. doi: 10.1016/j.intimp.2021.107879
92. Ge G, Bai J, Wang Q, Liang X, Tao H, Chen H, et al. Punicalagin Ameliorates Collagen-Induced Arthritis by Downregulating M1 Macrophage and Pyroptosis via NF-κb Signaling Pathway. *Sci China Life Sci* (2021) 64. doi: 10.1007/s11427-020-1939-1
93. Chen S, Luo Z, Chen X. Hsa_circ_0044235 Regulates the Pyroptosis of Rheumatoid Arthritis via MiR-135b-5p-SIRT1 Axis. *Cell Cycle (Georgetown Tex)* (2021) 20(12):1107–21. doi: 10.1080/15384101.2021.1916272
94. Shin TH, Kim HS, Kang TW, Lee BC, Lee HY, Kim YJ, et al. Human Umbilical Cord Blood-Stem Cells Direct Macrophage Polarization and Block Inflammasome Activation to Alleviate Rheumatoid Arthritis. *Cell Death Dis* (2016) 7:e2524. doi: 10.1038/cddis.2016.442
95. Lv L, Qin T, Huang Q, Jiang H, Chen F, Long F, et al. Targeting Tristetraprolin Expression or Functional Activity Regulates Inflammatory Response Induced by MSU Crystals. *Front Immunol* (2021) 12:675534. doi: 10.3389/fimmu.2021.675534
96. Artlett CM, Sassi-Gaha S, Rieger JL, Boesteanu AC, Feghali-Bostwick CA, Katsikis PD. The Inflammasome Activating Caspase 1 Mediates Fibrosis and Myofibroblast Differentiation in Systemic Sclerosis. *Arthritis Rheum* (2011) 63:3563–74. doi: 10.1002/art.30568
97. Artlett CM, Sassi-Gaha S, Hope JL, Feghali-Bostwick CA, Katsikis PD. Mir-155 Is Overexpressed in Systemic Sclerosis Fibroblasts and Is Required for NLRP3 Inflammasome-Mediated Collagen Synthesis During Fibrosis. *Arthritis Res Ther* (2017) 19:144. doi: 10.1186/s13075-017-1331-z
98. Zakrzewska K, Arvia R, Torcia MG, Clemente AM, Tanturli M, Castronovo G, et al. Effects of Parvovirus B19 *In Vitro* Infection on Monocytes From Patients With Systemic Sclerosis: Enhanced Inflammatory Pathways by Caspase-1 Activation and Cytokine Production. *J Invest Dermatol* (2019) 139:2125–33. doi: 10.1016/j.jid.2019.03.1144
99. Ohara-Imaizumi M, Cardozo AK, Kikuta T, Eizirik DL, Nagamatsu S. The Cytokine Interleukin-1beta Reduces the Docking and Fusion of Insulin Granules in Pancreatic Beta-Cells, Preferentially Decreasing the First Phase of Exocytosis. *J Biol Chem* (2004) 279:41271–4. doi: 10.1074/jbc.C400360200
100. Maedler K, Sergeev P, Ehses JA, Mathe Z, Bosco D, Berney T, et al. Leptin Modulates Beta Cell Expression of IL-1 Receptor Antagonist and Release of IL-1beta in Human Islets. *Proc Natl Acad Sci USA* (2004) 101:8138–43. doi: 10.1073/pnas.0305683101
101. Zheng Y, Wang Z, Zhou Z. miRNAs: Novel Regulators of Autoimmunity-Mediated Pancreatic β-Cell Destruction in Type 1 Diabetes. *Cell Mol Immunol* (2017) 14(6):488–96. doi: 10.1038/cmi.2017.7
102. Lebreton F, Berishvili E, Parnaud G, Rouget C, Bosco D, Berney T, et al. NLRP3 Inflammasome Is Expressed and Regulated in Human Islets. *Cell Death Dis* (2018) 9:726. doi: 10.1038/s41419-018-0764-x
103. Davanso MR, Crisma AR, Braga TT, Masi LN, Amaral CL, Leal VNC, et al. Macrophage Inflammatory State in Type 1 Diabetes: Triggered by NLRP3/iNOS Pathway and Attenuated by Docosahexaenoic Acid. *Clin Sci (London England: 1979)* (2021) 135(1):19–34. doi: 10.1042/CS20201348
104. Koenen TB, Stienstra R, van Tits LJ, de Graaf J, Stalenhoef AFH, Joosten LAB, et al. Hyperglycemia Activates Caspase-1 and TXNIP-Mediated IL-1beta Transcription in Human Adipose Tissue. *Diabetes* (2011) 60:517–24. doi: 10.2337/db10-0266
105. Pereira CA, Carlos D, Ferreira NS, Silva JF, Zanotto CZ, Zamboni DS, et al. Mitochondrial DNA Promotes NLRP3 Inflammasome Activation and Contributes to Endothelial Dysfunction and Inflammation in Type 1 Diabetes. *Front Physiol* (2019) 10:1557. doi: 10.3389/fphys.2019.01557
106. Eissa LD, Ghobashy WA, El-Azab MF. Inhibition of Thioredoxin-Interacting Protein and Inflammasome Assembly Using Verapamil Mitigates Diabetic Retinopathy and Pancreatic Injury. *Eur J Pharmacol* (2021) 901:174061. doi: 10.1016/j.ejphar.2021.174061
107. Xu L, Chen R, Zhang X, Zhu Y, Ma X, Sun G, et al. Scutellarin Protects Against Diabetic Cardiomyopathy via Inhibiting Oxidative Stress and Inflammatory Response in Mice. *Ann Palliative Med* (2021) 10(3):2481–93. doi: 10.21037/apm-19-516
108. Gao Y, Li J, Chu S, Zhang Z, Chen N, Li L, et al. Ginsenoside Rg1 Protects Mice Against Streptozotocin-Induced Type 1 Diabetic by Modulating the NLRP3 and Keap1/Nrf2/HO-1 Pathways. *Eur J Pharmacol* (2020) 866:172801. doi: 10.1016/j.ejphar.2019.172801
109. Wu C, Pan L, Niu W, Fang X, Liang W, Li J, et al. Modulation of Gut Microbiota by Low Methoxyl Pectin Attenuates Type 1 Diabetes in Non-Obese Diabetic Mice. *Front Immunol* (2019) 10:1733. doi: 10.3389/fimmu.2019.01733
110. Jia Li, Zhang Mi, Liu H, Sun J, Pan L. Early-Life Fingolimod Treatment Improves Intestinal Homeostasis and Pancreatic Immune Tolerance in Non-Obese Diabetic Mice. *Acta Pharmacologica Sin* (2021) 42(10):1620–9. doi: 10.1038/s41401-020-00590-4
111. Shin MS, Kang Y, Lee N, Wahl ER, Kim SH, Kang KS, et al. Self Double-Stranded (Ds)DNA Induces IL-1β Production From Human Monocytes by Activating NLRP3 Inflammasome in the Presence of anti-dsDNA Antibodies. *J Immunol* (2013) 190:1407–15. doi: 10.4049/jimmunol.1201195
112. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, et al. Netting Neutrophils Induce Endothelial Damage, Infiltrate Tissues, and Expose Immunostimulatory Molecules in Systemic Lupus Erythematosus. *J Immunol* (2011) 187:538–52. doi: 10.4049/jimmunol.1100450
113. Denny MF, Yalavarthi S, Zhao W, Thacker SG, Anderson M, Sandy AR, et al. A Distinct Subset of Proinflammatory Neutrophils Isolated From Patients With Systemic Lupus Erythematosus Induces Vascular Damage and Synthesizes Type I IFNs. *J Immunol* (2010) 184:3284–97. doi: 10.4049/jimmunol.0902199
114. Shin MS, Kang Y, Lee N, Kim SH, Kang KS, Lazova R, et al. U1-Small Nuclear Ribonucleoprotein Activates the NLRP3 Inflammasome in Human Monocytes. *J Immunol* (2012) 188:4769–75. doi: 10.4049/jimmunol.1103355
115. Zhao J, Wang H, Huang Y, Zhang H, Wang S, Gaskin F, et al. Lupus Nephritis: Glycogen Synthase Kinase 3β Promotion of Renal Damage Through Activation of the NLRP3 Inflammasome in Lupus-Prone Mice. *Arthritis Rheumatol* (2015) 67:1036–44. doi: 10.1002/art.38993
116. Inokuchi S, Mitoma H, Kawano S, Ayano M, Kimoto Y, Akahoshi M, et al. Activation of Caspase-1 Is Mediated by Stimulation of Interferon Genes and NLR Family Pyrin Domain Containing 3 in Monocytes of Active Systemic Lupus Erythematosus. *Clin Exp Rheumatol* (2021).
117. Kahlenberg JM, Carmona-Rivera C, Smith CK, Kaplan MJ. Neutrophil Extracellular Trap-Associated Protein Activation of the NLRP3 Inflammasome Is Enhanced in Lupus Macrophages. *J Immunol* (2013) 190:1217–26. doi: 10.4049/jimmunol.1202388
118. Huang W, Wu J, Yang H, Xiong Y, Jiang R, Cui T, et al. Milk Fat Globule-EGF Factor 8 Suppresses the Aberrant Immune Response of Systemic Lupus Erythematosus-Derived Neutrophils and Associated Tissue Damage. *Cell Death differentiation* (2017) 24(2):263–75. doi: 10.1038/cdd.2016.115
119. Fu R, Guo C, Wang S, Huang Y, Jin O, Hu H, et al. Podocyte Activation of NLRP3 Inflammasomes Contributes to the Development of Proteinuria in Lupus Nephritis. *Arthritis Rheumatol* (2017) 69:1636–46. doi: 10.1002/art.40155

120. Yang S, Hua K, Chu LJ, Hwu YK, Yang S, Wu C, et al. Xenon Blunts NF-κb/NLRP3 Inflammasome Activation and Improves Acute Onset of Accelerated and Severe Lupus Nephritis in Mice. *Kidney Int* (2020) 98(2):378–90. doi: 10.1016/j.kint.2020.02.033
121. Yang S, Hsu W, Wu C, Shang H, Liu F, Chen A. Accelerated, Severe Lupus Nephritis Benefits From Treatment With Honokiol by Immunoregulation and Differentially Regulating NF-κb/NLRP3 Inflammasome and Sirtuin 1/Autophagy Axis. *FASEB Journal: Off Publ Fed Am Societies Exp Biol* (2020) 34(10):13284–99. doi: 10.1096/fj.202001326R
122. Wu C, Hua K, Chu C, Yang S, Arbiser JL, Yang S, et al. Tris DBA Ameliorates Accelerated and Severe Lupus Nephritis in Mice by Activating Regulatory T Cells and Autophagy and Inhibiting the NLRP3 Inflammasome. *J Immunol (Baltimore Md: 1950)* (2020) 204(6):1448–61. doi: 10.4049/jimmunol.1801610
123. Yang S, Hua K, Yang C, Chen A, Weng J, Tsai Y, et al. Cf-02, a Novel Benzamide-Linked Small Molecule, Blunts NF-κb Activation and NLRP3 Inflammasome Assembly and Improves Acute Onset of Accelerated and Severe Lupus Nephritis in Mice. *FASEB J* (2021) 35(8):e21785. doi: 10.1096/fj.202100047R
124. Tan W, Gu Z, Leng J, Zou X, Chen H, Min F, et al. Let-7f-5p Ameliorates Inflammation by Targeting NLRP3 in Bone Marrow-Derived Mesenchymal Stem Cells in Patients With Systemic Lupus Erythematosus. *BioMed Pharmacother* (2019) 118:109313. doi: 10.1016/j.biopha.2019.109313
125. Zhao J, Zhang H, Huang Y, Wang H, Wang S, Zhao C, et al. Bay11-7082 Attenuates Murine Lupus Nephritis via Inhibiting NLRP3 Inflammasome and NF-κb Activation. *Int Immunopharmacol* (2013) 17:116–22. doi: 10.1016/j.intimp.2013.05.027
126. Zhao J, Wang J, Zhou M, Li M, Li M, Tan H. Curcumin Attenuates Murine Lupus via Inhibiting NLRP3 Inflammasome. *Int Immunopharmacol* (2019) 69:213–6. doi: 10.1016/j.intimp.2019.01.046
127. Bonomini F, Dos Santos M, Veronese FV, Rezzani R. NLRP3 Inflammasome Modulation by Melatonin Supplementation in Chronic Pristane-Induced Lupus Nephritis. *Int J Mol Sci* (2019) 20(14):3466. doi: 10.3390/ijms20143466
128. Su B, Ye H, You X, Ni H, Chen X, Li L. Icariin Alleviates Murine Lupus Nephritis via Inhibiting NF-κb Activation Pathway and NLRP3 Inflammasome. *Life Sci* (2018) 208:26–32. doi: 10.1016/j.lfs.2018.07.009
129. Peng X, Yang T, Liu G, Liu H, Peng Y, He L. Piperine Ameliorated Lupus Nephritis by Targeting AMPK-Mediated Activation of NLRP3 Inflammasome. *Int Immunopharmacol* (2018) 65:448–57. doi: 10.1016/j.intimp.2018.10.025
130. Ka S, Lin J, Lin T, Liu F, Chao L, Ho C, et al. Citral Alleviates an Accelerated and Severe Lupus Nephritis Model by Inhibiting the Activation Signal of NLRP3 Inflammasome and Enhancing Nrf2 Activation. *Arthritis Res Ther* (2015) 17:331. doi: 10.1186/s13075-015-0844-6
131. Nagayama Y. Commentary: Excessive Iodine Promotes Pyroptosis of Thyroid Follicular Epithelial Cells in Hashimoto's Thyroiditis Through the ROS-NF-κb-NLRP3 Pathway. *Front Endocrinol* (2020) 11:581. doi: 10.3389/fendo.2020.00581
132. Ma B, Chen D, Liu Y, Zhao Z, Wang J, Zhou G, et al. Yanghe Decoction Suppresses the Experimental Autoimmune Thyroiditis in Rats by Improving NLRP3 Inflammasome and Immune Dysregulation. *Front Pharmacol* (2021) 12:645354. doi: 10.3389/fphar.2021.645354
133. Goyette P, Labbé C, Trinh TT, Xavier RJ, Rioux JD. Molecular Pathogenesis of Inflammatory Bowel Disease: Genotypes, Phenotypes and Personalized Medicine. *Ann Med* (2007) 39:177–99. doi: 10.1080/07853890701197615
134. Chen Y, Wu D, Sun L. Clinical Significance of High-Mobility Group Box 1 Protein (HMGB1) and Nod-Like Receptor Protein 3 (NLRP3) in Patients With Ulcerative Colitis. *Med Sci Monit* (2020) 26:e919530. doi: 10.12659/MSM.919530
135. Lazaridis LD, Pistiki A, Giamarellos-Bourboulis EJ, Georgitsi M, Damoraki G, Polymeros D, et al. Activation of NLRP3 Inflammasome in Inflammatory Bowel Disease: Differences Between Crohn's Disease and Ulcerative Colitis. *Dig Dis Sci* (2017) 62:2348–56. doi: 10.1007/s10620-017-4609-8
136. Zhen Y, Zhang H. NLRP3 Inflammasome and Inflammatory Bowel Disease. *Front Immunol* (2019) 10:276. doi: 10.3389/fimmu.2019.00276
137. Hasegawa M, Kamada N, Jiao Y, Liu MZ, Núñez G, Inohara N. Protective Role of Commensals Against Clostridium Difficile Infection via an IL-1β-Mediated Positive-Feedback Loop. *J Immunol* (2012) 189:3085–91. doi: 10.4049/jimmunol.1200821
138. Alipour M, Lou Y, Zimmerman D, Bording-Jorgensen MW, Sergi C, Liu JJ, et al. A Balanced IL-1β Activity Is Required for Host Response to Citrobacter Rodentium Infection. *PloS One* (2013) 8:e80656. doi: 10.1371/journal.pone.0080656
139. Su F, Xia Y, Huang M, Zhang L, Chen L. Expression of NLPR3 in Psoriasis Is Associated With Enhancement of Interleukin-1β and Caspase-1. *Med Sci Monitor : Int Med J Exp Clin Res* (2018) 24:7909–13. doi: 10.12659/MSM.911347
140. Yu P, Hao S, Zheng H, Zhao X, Li Y. Association of NLRP1 and NLRP3 Polymorphisms With Psoriasis Vulgaris Risk in the Chinese Han Population. *BioMed Res Int* (2018) 2018:4714836. doi: 10.1155/2018/4714836
141. Juneblad K, Kastbom A, Johansson L, Rantapää-Dahlqvist S, Söderkvist P, Alenius G-M. Association Between Inflammasome-Related Polymorphisms and Psoriatic Arthritis. *Scandinavian J Rheumatol* (2021) 50(3):206–12. doi: 10.1080/03009742.2020.1834611
142. Tang Z, Zhang K, Lv S, Xu G, Zhang J, Jia H. LncRNA MEG3 Suppresses PI3K/AKT/mTOR Signalling Pathway to Enhance Autophagy and Inhibit Inflammation in TNF-α-Treated Keratinocytes and Psoriatic Mice. *Cytokine* (2021) 148:155657. doi: 10.1016/j.cyto.2021.155657
143. Chang X, He H, Zhu L, Gao J, Wei T, Ma Z, et al. Protective Effect of Apigenin on Freund's Complete Adjuvant-Induced Arthritis in Rats via Inhibiting P2X7/NF-κb Pathway. *Chem Biol Interact* (2015) 236:41–6. doi: 10.1016/j.cbi.2015.04.021
144. Mathews RJ, Robinson JI, Battellino M, Wong C, Taylor JC, Eyre S, et al. Evidence of NLRP3-Inflammasome Activation in Rheumatoid Arthritis (RA); Genetic Variants Within the NLRP3-Inflammasome Complex in Relation to Susceptibility to RA and Response to Anti-TNF Treatment. *Ann Rheum Dis* (2014) 73:1202–10. doi: 10.1136/annrheumdis-2013-203276
145. Kastbom A, Ärlestig L, Rantapää-Dahlqvist S. Genetic Variants of the NLRP3 Inflammasome Are Associated With Stroke in Patients With Rheumatoid Arthritis. *J Rheumatol* (2015) 42:1740–5. doi: 10.3899/jrheum.141529
146. Choulaki C, Papadaki G, Repa A, Kampouraki E, Kambas K, Ritis K, et al. Enhanced Activity of NLRP3 Inflammasome in Peripheral Blood Cells of Patients With Active Rheumatoid Arthritis. *Arthritis Res Ther* (2015) 17:257. doi: 10.1186/s13075-015-0775-2
147. Kolly L, Busso N, Palmer G, Talabot-Ayer D, Chobaz V, So A. Expression and Function of the NALP3 Inflammasome in Rheumatoid Synovium. *Immunology* (2010) 129:178–85. doi: 10.1111/j.1365-2567.2009.03174.x
148. Takakubo Y, Barreto G, Konttinen YT, Oki H, Takagi M. Role of Innate Immune Sensors, TLRs, and NALP3 in Rheumatoid Arthritis and Osteoarthritis. *J Long Term Eff Med Implants* (2014) 24:243–51. doi: 10.1615/jlongtermeffmedimplants.2014011295
149. Kim HW, Kwon YJ, Park BW, Song JJ, Park YB, Park MC. Differential Expressions of NOD-Like Receptors and Their Associations With Inflammatory Responses in Rheumatoid Arthritis. *Clin Exp Rheumatol* (2017) 35:630–7.
150. Zhao C, Gu Y, Zeng X, Wang J. NLRP3 Inflammasome Regulates Th17 Differentiation in Rheumatoid Arthritis. *Clin Immunol* (2018) 197:154–60. doi: 10.1016/j.clim.2018.09.007
151. Mouasni S, Gonzalez V, Schmitt A, Bennana E, Guillonneau F, Mistou S, et al. The Classical NLRP3 Inflammasome Controls FADD Unconventional Secretion Through Microvesicle Shedding. *Cell Death Dis* (2019) 10:190. doi: 10.1038/s41419-019-1412-9
152. Yang Z, Cao J, Yu C, Yang Q, Zhang Y, Han L. Caspase-1 Mediated Interleukin-18 Activation in Neutrophils Promotes the Activity of Rheumatoid Arthritis in a NLRP3 Inflammasome Independent Manner. *Joint Bone Spine* (2016) 83:282–9. doi: 10.1016/j.jbspin.2015.07.006
153. Martínez-Godínez MA, Cruz-Domínguez MP, Jara LJ, Domínguez-López A, Jarillo-Luna RA, Vera-Lastra O, et al. Expression of NLRP3 Inflammasome, Cytokines and Vascular Mediators in the Skin of Systemic Sclerosis Patients. *Israel Med Assoc Journal: IMAJ* (2015) 17(1):5–10.
154. O'Connell RM, Kahn D, Gibson WSJ, Round JL, Scholz RL, Chaudhuri AA, et al. MicroRNA-155 Promotes Autoimmune Inflammation by Enhancing

Inflammatory T Cell Development. *Immunity* (2010) 33:607–19. doi: 10.1016/j.immuni.2010.09.009
155. Gracias DT, Stelekati E, Hope JL, Boesteanu AC, Doering TA, Norton J, et al. The microRNA miR-155 Controls CD8(+) T Cell Responses by Regulating Interferon Signaling. *Nat Immunol* (2013) 14:593–602. doi: 10.1038/ni.2576
156. Bala S, Csak T, Saha B, Zatsiorsky J, Kodys K, Catalano D, et al. The Pro-Inflammatory Effects of miR-155 Promote Liver Fibrosis and Alcohol-Induced Steatohepatitis. *J Hepatol* (2016) 64(6):1378–87. doi: 10.1016/j.jhep.2016.01.035
157. Zheng L, Xu C, Chen W, Shen W, Ruan C, Zhu L, et al. MicroRNA-155 Regulates Angiotensin II Type 1 Receptor Expression and Phenotypic Differentiation in Vascular Adventitial Fibroblasts. *Biochem Biophys Res Commun* (2010) 400(4):483–8. doi: 10.1016/j.bbrc.2010.08.067
158. Yang L, Liu J, Bai X, Fan L, Han F, Jia W, et al. Acute Downregulation of miR-155 at Wound Sites Leads to a Reduced Fibrosis Through Attenuating Inflammatory Response. *Biochem Biophys Res Commun* (2014) 453:153–9. doi: 10.1016/j.bbrc.2014.09.077
159. Pottier N, Maurin T, Chevalier B, Puisségur MP, Lebrigand K, Robbe-Sermesant K, et al. Identification of Keratinocyte Growth Factor as a Target of microRNA-155 in Lung Fibroblasts: Implication in Epithelial-Mesenchymal Interactions. *PloS One* (2009) 4(8):e6718. doi: 10.1371/journal.pone.0006718
160. Shinohara ML. Yes, Research Matters. *PloS Pathog* (2017) 13:e1006420. doi: 10.1371/journal.ppat.1006420
161. Patin EC, Thompson A, Orr SJ. Pattern Recognition Receptors in Fungal Immunity. *Semin Cell Dev Biol* (2019) 89:24–33. doi: 10.1016/j.semcdb.2018.03.003
162. Esposito S, Toni G, Tascini G, Santi E, Berioli MG, Principi N. Environmental Factors Associated With Type 1 Diabetes. *Front Endocrinol (Lausanne)* (2019) 10:592. doi: 10.3389/fendo.2019.00592
163. Xia Y, Xie Z, Huang G, Zhou Z. Incidence and Trend of Type 1 Diabetes and the Underlying Environmental Determinants. *Diabetes Metab Res Rev* (2019) 35:e3075. doi: 10.1002/dmrr.3075
164. Pontillo A, Brandao L, Guimaraes R, Segat L, Araujo J, Crovella S. Two SNPs in NLRP3 Gene Are Involved in the Predisposition to Type-1 Diabetes and Celiac Disease in a Pediatric Population From Northeast Brazil. *Autoimmunity* (2010) 43:583–9. doi: 10.3109/08916930903540432
165. Sun X, Xia Y, Liu Y, Wang Y, Luo S, Lin J, et al. Polymorphisms in NLRP1 Gene Are Associated With Type 1 Diabetes. *J Diabetes Res* (2019) 2019:7405120. doi: 10.1155/2019/7405120
166. Wu Z, Wu S, Liang T. Association of NLRP3 Rs35829419 and Rs10754558 Polymorphisms With Risks of Autoimmune Diseases: A Systematic Review and Meta-Analysis. *Front Genet* (2021) 12:690860. doi: 10.3389/fgene.2021.690860
167. Smigoc Schweiger D, Goricar K, Hovnik T, Mendez A, Bratina N, Brecelj J, et al. Dual Role of PTPN22 But Not NLRP3 Inflammasome Polymorphisms in Type 1 Diabetes and Celiac Disease in Children. *Front Pediatr* (2019) 7:63. doi: 10.3389/fped.2019.00063
168. Liu H, Xu R, Kong Q, Liu J, Yu Z, Zhao C. Downregulated NLRP3 and NLRP1 Inflammasomes Signaling Pathways in the Development and Progression of Type 1 Diabetes Mellitus. *Biomedicine Pharmacotherapy* (2017) 94:619–26. doi: 10.1016/j.biopha.2017.07.102
169. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, et al. NLRP3 Inflammasomes Are Required for Atherogenesis and Activated by Cholesterol Crystals. *Nature* (2010) 464:1357–61. doi: 10.1038/nature08938
170. Hughes FM, Hirshman NA, Inouye BM, Jin H, Stanton EW, Yun CE, et al. NLRP3 Promotes Diabetic Bladder Dysfunction and Changes in Symptom-Specific Bladder Innervation. *Diabetes* (2019) 68:430–40. doi: 10.2337/db18-0845
171. Zhai Y, Meng X, Ye T, Xie W, Sun G, Sun X. Inhibiting the NLRP3 Inflammasome Activation With MCC950 Ameliorates Diabetic Encephalopathy in Db/Db Mice. *Molecules* (2018) 23(3):522. doi: 10.3390/molecules23030522
172. Wu C, Pan L, Luo Y, Niu W, Fang X, Liang W, et al. Low Methoxyl Pectin Protects Against Autoimmune Diabetes and Associated Caecal Dysfunction. *Mol Nutr Food Res* (2019) 63:e1900307. doi: 10.1002/mnfr.201900307
173. Lee YH, Bae SC. Association Between Functional NLRP3 Polymorphisms and Susceptibility to Autoimmune and Inflammatory Diseases: A Meta-Analysis. *Lupus* (2016) 25:1558–66. doi: 10.1177/0961203316644336
174. Su Z, Niu Q, Huang Z, Yang B, Zhang J. Association of Nucleotide-Binding Oligomerization Domain-Like Receptor Family Pyrin Domain-Containing Protein 3 Polymorphisms With Systemic Lupus Erythematosus Disease Activity and Biomarker Levels: A Case-Control Study in Chinese Population. *Med (Baltimore)* (2020) 99:e21888. doi: 10.1097/MD.0000000000021888
175. Huang T, Yin H, Ning W, Wang X, Chen C, Lin W, et al. Expression of Inflammasomes NLRP1, NLRP3 and AIM2 in Different Pathologic Classification of Lupus Nephritis. *Clin Exp Rheumatol* (2020) 38:680–90.
176. Yang C, Huang S, Chiang B. Sex-Dependent Differential Activation of NLRP3 and AIM2 Inflammasomes in SLE Macrophages. *Rheumatol (Oxford)* (2015) 54:324–31. doi: 10.1093/rheumatology/keu318
177. Brinkmann V, Zychlinsky A. Beneficial Suicide: Why Neutrophils Die to Make NETs. *Nat Rev Microbiol* (2007) 5:577–82. doi: 10.1038/nrmicro1710
178. Pavenstädt H, Kriz W, Kretzler M. Cell Biology of the Glomerular Podocyte. *Physiol Rev* (2003) 83:253–307. doi: 10.1152/physrev.00020.2002
179. Banas MC, Banas B, Hudkins KL, Wietecha TA, Iyoda M, Bock E, et al. TLR4 Links Podocytes With the Innate Immune System to Mediate Glomerular Injury. *J Am Soc Nephrol* (2008) 19:704–13. doi: 10.1681/ASN.2007040395
180. Denny MF, Thacker S, Mehta H, Somers EC, Dodick T, Barrat FJ, et al. Interferon-Alpha Promotes Abnormal Vasculogenesis in Lupus: A Potential Pathway for Premature Atherosclerosis. *Blood* (2007) 110:2907–15. doi: 10.1182/blood-2007-05-089086
181. Kahlenberg JM, Thacker SG, Berthier CC, Cohen CD, Kretzler M, Kaplan MJ. Inflammasome Activation of IL-18 Results in Endothelial Progenitor Cell Dysfunction in Systemic Lupus Erythematosus. *J Immunol* (2011) 187:6143–56. doi: 10.4049/jimmunol.1101284
182. Lech M, Lorenz G, Kulkarni OP, Grosser MOO, Stigrot N, Darisipudi MN, et al. NLRP3 and ASC Suppress Lupus-Like Autoimmunity by Driving the Immunosuppressive Effects of TGF-β Receptor Signalling. *Ann Rheum Dis* (2015) 74:2224–35. doi: 10.1136/annrheumdis-2014-205496
183. Thygesen SJ, Takizawa KE, Robertson AAB, Sester DP, Stacey KJ. Compromised NLRP3 and AIM2 Inflammasome Function in Autoimmune NZB/W F1 Mouse Macrophages. *Immunol Cell Biol* (2019) 97:17–28. doi: 10.1111/imcb.12193
184. Lu A, Li H, Niu J, Wu S, Xue G, Yao X, et al. Hyperactivation of the NLRP3 Inflammasome in Myeloid Cells Leads to Severe Organ Damage in Experimental Lupus. *J Immunol* (2017) 198:1119–29. doi: 10.4049/jimmunol.1600659
185. Guo Q, Wu Y, Hou Y, Liu Y, Liu T, Zhang H, et al. Cytokine Secretion and Pyroptosis of Thyroid Follicular Cells Mediated by Enhanced NLRP3, NLRP1, NLRC4, and AIM2 Inflammasomes Are Associated With Autoimmune Thyroiditis. *Front Immunol* (2018) 9:1197. doi: 10.3389/fimmu.2018.01197
186. Liu J, Mao C, Dong L, Kang P, Ding C, Zheng T, et al. Excessive Iodine Promotes Pyroptosis of Thyroid Follicular Epithelial Cells in Hashimoto's Thyroiditis Through the ROS-NF-κb-NLRP3 Pathway. *Front Endocrinol* (2019) 10:778. doi: 10.3389/fendo.2019.00778
187. Liu G, Mateer S, Hsu A, Goggins B, Tay H, Mathe A, et al. Platelet Activating Factor Receptor Regulates Colitis-Induced Pulmonary Inflammation Through the NLRP3 Inflammasome. *Mucosal Immunol* (2019) 12(4):862–73. doi: 10.1038/s41385-019-0163-3
188. Ranson N, Veldhuis M, Mitchell B, Fanning S, Cook AL, Kunde D, et al. NLRP3-Dependent and -Independent Processing of Interleukin (IL)-1β in Active Ulcerative Colitis. *Int J Mol Sci* (2018) 20(1):57. doi: 10.3390/ijms20010057
189. Hu C, Ding H, Li Y, Pearson JA, Zhang X, Flavell RA, et al. NLRP3 Deficiency Protects From Type 1 Diabetes Through the Regulation of Chemotaxis Into the Pancreatic Islets. *Proc Natl Acad Sci USA* (2015) 112:11318–23. doi: 10.1073/pnas.1513509112
190. Lewis KL, Reizis B. Dendritic Cells: Arbiters of Immunity and Immunological Tolerance. *Cold Spring Harb Perspect Biol* (2012) 4(8):a007401. doi: 10.1101/cshperspect.a007401

191. Yang Q, Yu C, Yang Z, Wei Q, Mu K, Zhang Y, et al. Deregulated NLRP3 and NLRP1 Inflammasomes and Their Correlations With Disease Activity in Systemic Lupus Erythematosus. *J Rheumatol* (2014) 41:444–52. doi: 10.3899/jrheum.130310

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Overexpressing Kallistatin Aggravates Experimental Autoimmune Uveitis Through Promoting Th17 Differentiation

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Kallistatin or kallikrein-binding protein (KBP) has been reported to regulate angiogenesis, inflammation and tumor progression. Autoimmune uveitis is a common, sight-threatening inflammatory intraocular disease. However, the roles of kallistatin in autoimmunity and autoreactive T cells are poorly investigated. Compared to non-uveitis controls, we found that plasma levels of kallistatin were significantly upregulated in patients with Vogt-Koyanagi-Harada (VKH) disease, one of the non-infectious uveitis. Using an experimental autoimmune uveitis (EAU) model induced by human interphotoreceptor retinoid-binding protein peptide 651-670 ($hIRBP_{651-670}$), we examined the effects of kallistatin on the pathogenesis of autoimmune diseases. Compared to wild type (WT) mice, kallistatin transgenic (KS) mice developed severe uveitis with dominant Th17 infiltrates in the eye. In addition, the proliferative antigen-specific T cells isolated from KS EAU mice produced increased levels of IL-17A, but not IFN-γ or IL-10 cytokines. Moreover, splenic $CD4^+$ T cells from naïve KS mice expressed higher levels of Il17a mRNA compared to WT naïve mice. Under Th17 polarization conditions, KS mice exhibited enhanced differentiation of naïve $CD4^+$ T cells into Th17 cells compared to WT controls. Together, our results indicate that kallistatin promotes Th17 differentiation and is a key regulator of aggravating autoinflammation in EAU. Targeting kallistatin might be a potential to treat autoimmune disease.

Keywords: kallistatin, uveitis, experimental autoimmune uveitis, autoimmune disease, immunology, interphotoreceptor retinoid-binding protein, Th17

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INTRODUCTION

Autoimmune uveitis or non-infectious uveitis (NIU) is a group of intraocular inflammatory disease, which can affect only the eyes, or it can be part of a systemic disease, and Vogt-Koyanagi-Harada (VKH) is one of NIU (1). Uveitis, if untreated, can cause a significant visual deficit and even blindness, which accounts for 10-15% of cases of severe visual impairment in the developed countries (2, 3). Due to easy recurrence, more frequent occurrence in younger adults (4), autoimmune uveitis can cause heavy social and economic burdens. However, the pathogenesis of autoimmune uveitis remains unknown. Traditional therapeutic drugs, such as corticosteroids and immunosuppressants, when used for long periods of time, can yield a spectrum of local or systemic side effects. Although the new biological agents are more targeted, there are still systemic side effects that are insurmountable (5, 6). Thus, there is an urgent need to study pathological mechanisms of the autoimmune uveitis and explore effective drugs with strong pertinence and safety to treat autoimmune uveitis.

Studies in experimental autoimmune uveitis (EAU), animal models for autoimmune panuveitis or posterior uveitis in the human have learned that autoreactive $CD4^+$ T cells play an important role in ocular inflammation (7–9). Antigen-responsive $CD4^+$ T cells from EAU mice transferred into naïve mice initiate autoimmune uveitis, and different effector T cells can induce experimental models with different pathological phenotypes (10–12). Interleukin 17 (IL-17) producing Th17 cells and IFN-γ-producing Th1 cells, have been thought to be important in the immunopathogenesis of uveitis and EAU (8, 13). The IFN-γ^+ IL-$17A^+$ phenotype, named Th1/Th17 subset, is associated with Th17-mediated diseases in human and animal models (14). And it has been recently shown that $CD4^+$ T cells or Th17 cells have substantial plasticity and display important functions in the tissue microenvironment during chronic inflammation (14–17). Nevertheless, the mechanisms by which these autoreactive T cells regulate the autoimmune response during EAU are not fully elucidated.

Kallistatin, also named SERPINA4, was originally identified as a tissue kallikrein-binding protein (KBP), a member of serine protease inhibitor (serpin) family (18–20). It has been previously reported that kallistatin exhibits various functions and protective effects in disease models of superficial angiogenesis or acute inflammation, such as arthritis, septic shock, renal injury, myocardial ischemia, hypertension and diabetic retinopathy (21–26). Increased circulating kallistatin levels have been observed in diabetic patients with microvascular complications (27, 28). And elevated kallistatin in diabetes has been suggested to promote the recruitment of macrophages and M1 polarization, aggravating the inflammatory responses and impairing the healing of diabetic wound, a chronic inflammation (29). All these findings suggest a potential role of kallistatin in modulating inflammation and immune system. However, how kallistatin affects autoimmune mediated inflammation remains poorly understood, and the roles of kallistatin in adaptive immune cells such as Th1 and Th17 cells have not yet been studied.

The primary aim of this study was to elucidate the roles of kallistatin in the pathogenesis of autoimmunity. We first detected the expression levels of kallistatin in the plasma of patients with VKH and controls. We additionally used kallistatin-transgenic (KS) mice and wild-type (WT) mice to induce EAU with human interphotoreceptor retinoid-binding protein (IRBP) peptide 651-670 ($hIRBP_{651-670}$), and compared the severity of EAU by various ocular imaging techniques and pathological verification. We also determined whether and how kallistatin overexpression regulates T-cell mediated autoimmune responses. Our results suggest that kallistatin overexpression enhances Th17 differentiation and exacerbates intra-ocular inflammation during EAU. Consequently, it provides interesting ways to think about therapeutic avenues for autoimmune diseases, such as uveitis.

MATERIALS AND METHODS

Human Subjects and Sample Collection

The studies involving human participants were reviewed and approved by the Ethics Committee of Tianjin Medical University Eye Hospital, Tianjin, China (No. 2016KY-14). All participants provided their written informed consent in this study. All VKH patients with initial onset, significant ocular inflammation and no treatments of systemic corticosteroids were diagnosed and recruited by ophthalmologist.

Animals

C57BL/6J (B6) female wild-type (WT) mice were purchased from Vital River Laboratory Animal Technology (Beijing, China). The kallistatin-transgenic mice with same background of B6 were provided as a gift from Dr. Jianxing Ma (University of Oklahoma Health Sciences Center), and the transgene expression was confirmed by real-time PCR (RT-PCR) of spleen and draining lymph nodes (dLNs) RNA using the genotyping primers. Mice were bred and maintained under specific-pathogen-free conditions. Animal care and involving experiments were performed according to the guidelines approved by the Animal Care and Use Committee of the Tianjin Medical University, and mice were used at 6 to 10 weeks of age.

LC-MS/MS Analysis for Proteomics

Samples were collected and prepared for LC-MS/MS analysis. Briefly, the plasma samples were lysed by 8M urea lysate. After reduction and alkylation, samples were trypsinized with sequencing grade modified trypsin (Promega, USA) for 16 h at 37°C to get the peptide segments. Plasma-derived tryptic peptide fragments were eluted with 0.1% FA and 40% CAN buffer and dried *via* Integrated SpeedVac (Thermo Fisher, USA). Peptide mixtures were loaded onto a self-packed trap column (100μm×2cm, Durashell C18 3 μm, 120A°), separated on a homemade analytical C18 column (150μm×15cm, 1.9 μm),

and monitored on an electrospray-ionization Triple TOF 6600 mass spectrometers (AB SCIEX, USA). The elution was performed over a gradient 120 min with buffer B ranging from 5% to 80%.

EAU Induction

Mice were injected subcutaneously with 250 μg of human interphotoreceptor retinoid binding protein peptide [LAQGAYRTAVDLESLASQLT ($hIRBP_{651-670}$), Shanghai Hanhong Chemical Co., Ltd., Shanghai, China] emulsified 1:1 v/v in complete Freund's adjuvant (CFA, Sigma Aldrich) containing 3.5 mg/mL mycobacterium tuberculosis H37RA (BD Biosciences). A total of 200 μL emulsion was distributed along the base of the tail (100 μL) and both thighs (50 μL each). In all immunizations, 500 ng pertussis toxin (List Biological Laboratories Inc.), per mouse was injected intraperitoneally (i.p.) immediately on the day of immunization and the following day.

Clinical and Histopathological Scoring

Clinical disease was scored at regular intervals (3 times a week) by indirect fundoscopy (Hein, Germany). The clinical signs of EAU were assessed with a 0–4 scoring system, according to the criteria previously published by Caspi's group (30). In brief, the clinical scale is as follows: 0 = No inflammation; 0.5 = trace disease, 1 to 2 very small, peripheral focal lesions; 1 = minimally active, localized, <5 focal lesions; ≤1 linear lesion; 2 = moderately active, multiple (>5) lesions; severe vasculitis; <5 linear lesions; 3 = Active, multiple diffuse lesions; 4 = very active, large retinal detachment or hemorrhage. For histology, animals were sacrificed 21 days after immunization, eyes were surgically resected, fixed in formalin for 24 h, and embedded in paraffin. Then 4-μm-thick sections of the pupil and optic nerve axis were made and stained with hematoxylin-eosin (H&E) staining. The retinal histopathological changes were also graded on a scale of 0–4, according to previously published criteria (30).

Retinal Imaging Evaluations

Retinal imaging evaluations were conducted with spectral-domain optical coherence tomography (SD-OCT, Heidelberg Engineering, Heidelberg, Germany) and retinal imaging microscope (Micron IV; Phoenix Research Labs, Pleasanton, CA, USA). ImageJ software (National Institutes of Health, USA) was used to quantify the numbers of hyperreflective foci in the vitreous cavity of the SD-OCT image. The fundus images under visible light were collected by retinal imaging microscope.

Isolation of Infiltrating Cells From Eyes

The eyes of mice were collected on the indicated days after immunization. After removal of the cornea, lens, optic nerve and excess connective tissue, the rest of the eyes was ground up on the steel screen, followed by digestion with RPMI1640 containing 1 mg/ml collagenase D (Roche) for 1 h at 37°C, a shaking speed of 220 rpm. After passing through the 70μm strainer, the single cell suspension from inflamed eyes was collected.

Flow Cytometry Analysis

Aliquots of single cells (1×10^6) were stained with APC-labeled anti-mouse CD4 (BioLegend, Cat# 100412) antibodies or CD25 (BioLegend, Cat# 101908), CD62L (BioLegend, Cat# 104407), CD44 (BioLegend, Cat# 103035) or other appropriately labeled antibodies. Samples were then washed and resuspended with staining buffer [PBS containing 5% fetal bovine serum (FBS)], followed by immediately flow analysis. For intracellular staining, cells were further fixed and permeabilized with fixation/permeabilization buffer (BioLegend). Intracellular staining was performed with FITC-conjugated anti-mouse IFN-γ (BioLegend, Cat# 505806) antibodies or PE-labeled anti-mouse IL-17A antibodies (BioLegend, Cat# 506908) in permeabilization wash buffer. Flow cytometric analysis was performed on flow cytometry ($FACS_{calibur}$, BD Biosciences).

Assays for *Ex Vivo* T Cell Recall and IRBP-Specific T Cell Proliferation

T cells from spleens of WT or KS mice were isolated on day 14-16 after immunization using nylon wool column and stimulated at 4×10^5 cells/well with different concentrations of $IRBP_{651-670}$ in the presence of 1×10^5 irradiated syngeneic APCs in 96-well plates. The cells were cultured in the incubator for 60 hours, and BrdU was incorporated into the culture medium at the last 16 hours to evaluate cell proliferation, following the manufacturer's protocol of BrdU assay kit (Millipore, 11647229001). Secreted IL-17A, IFN-γ and IL-10 cytokines in the supernatants were determined using ELISA kits according to the manufacturer's instructions (R&D Systems).

RNA Isolation, Reverse Transcription of cDNA, and Real-Time Quantitative PCR

Cells or tissue samples from the spleen, dLNs, and eye were homogenized and lysed with 1ml TRIzol (Ambion, 15596018). Total RNA was reverse transcribed to cDNA using the superscript First-Strand Synthesis System and random hexamer primers (Thermo Fisher Scientific, K1622), according to the manufacturer's instructions. The cDNA was then used as a template for real-time PCR using SYBER Green Master Mix (Roche, 41472600) and gene specific primers. For each sample, the transcript copy numbers were normalized to housekeeping gene glyceraldehyde phosphate dehydrogenase (*Gapdh*), and the fold induction compared to the control was calculated. The following gene-specific primers were used for analysis:

SERPINA4: F-AGGGAAGATTGTGGATTTGG, R-ATGAAG ATACCAGTGATGCTC;

Gapdh: F-CCTGTTGCTGTAGCCGTATTCA, R-CCAGGT TGTCTCCTGCGACTT;

Il17a: F-CTGGAGGATAACACTGTGAGAGT, R-TGCTGAA TGGCGACGGAGTTC;

Il17f: F-GAGGATAACACTGTGAGAGTTGAC, R-GAGTTCA TGGTGCTGTCTTCC;

Il21: F-TCATCATTGACCTCGTGGCCC, R-ATCGTACTT CTCCACTTGCAATCCC;

Il22: F-CATGCAGGAGGTGGTACCTT, R-CAGACGC AAGCATTTCTC AG;

Il23: F-AATAATGTGCCCCGTATCCA, R-AGGCTCCC CTTTGAAGATGT;

Ifng: F-GATGCATTCATGAGTATTGCCAAGT, R-GTGGACC ACTCGGATGAGCTC;

Tnfα: F-ATCATCTTCTCAAAATTCGAGTGA, R-TTGAGA TCCATGCCGTTGG;

Gmcsf: F-TTTACTTTTCCTGGGCATTG, R-TAGCTG GCTGTCATGTTCAA;

Foxp3: F-GGCCCTTCTCCAGGACAGA, R-GCTGATC ATGGCTGGGTTGT;

In Vitro T Cell Activation

$CD4^+$ T cells were isolated from single-cell suspensions of spleen and dLNs using a mouse $CD4^+$ T cell isolation kit (Invitrogen, 11415D), according to the manufacturer's instructions. 4×10^5 purified T cells were seeded in 96-well plates pre-coated with 5 μg/ml of anti-CD3 mAb (Bio X Cell, BP0001-1) overnight in the presence of 1 μg/ml of anti-CD28 mAb (Bio X Cell, BP0015-1) and 1,000 U/ml of recombinant mouse IL-2 (R&D, 402-ML). T cells were collected and stained with PE-labeled CD69 (BioLegend, Cat# 104507), followed by flow cytometry analysis.

Th17 Differentiation Assay

Naïve $CD4^+CD25^-62L^{hi}CD44^{lo}$ T cells were isolated from pooled single-cell suspensions of spleen and dLNs by a BD FACS Aria III flow cytometer (BD Biosciences). Purified naïve T cells were stimulated with plate-bound anti-CD3 mAb (R&D) for 5 days in the presence of polarizing cytokines and blocking antibodies, according to the manufacturer's protocol of the mouse Th17 cell differentiation kit (R&D, Cat# CDK017). We cultured 1.25×10^5 cells/well in 96-well plates with a total volume of 0.2 ml/well of culture medium. The mouse Th17 differentiation media were refreshed on day 3 by adding an equal volume of Th17 differentiation fresh media. The IL17A productions in the supernatants were measured by ELISA following the manufacturer's instructions.

Statistical Analysis

We performed the statistical analyses with Prism 8 (GraphPad software). The two-tailed student's t test was applied for the statistical comparison between two groups, and one-way ANOVA was used for three or more sets of data. The clinical score and T cell proliferation was analyzed by repeated measurement ANOVA (two-way ANOVA), using mixed models. Post-ANOVA comparisons were made using the Sidak correction. The data were expressed as means ± SEM. The p value less than 0.05 was considered statistically significant.

RESULTS

Elevated Expression of Kallistatin Protein in VKH Patients

To evaluate the levels of kallistatin in uveitis patients, we collected the plasma samples from 16 VKH patients and 28 non-uveitis controls, and determined the expression of kallistatin protein by proteomics analysis. The demographics of VKH patients and control subjects were summarized, as shown in **Figure 1B**. In the proteomics data, the proteins with a difference >1.5-fold and p value < 0.05 were considered differentially expressed proteins (DEPs), and kallistatin protein was identified as one of them. Compared to the non-uveitis controls, kallistatin proteins were highly expressed with a log2 fold change 2.07 in the VKH samples (**Figure 1A**, p = 0.0002). The proteomics analysis indicated that kallistatin was overexpressed in the plasma of VKH patients.

Kallistatin-Transgenic Mice Develop Severe EAU

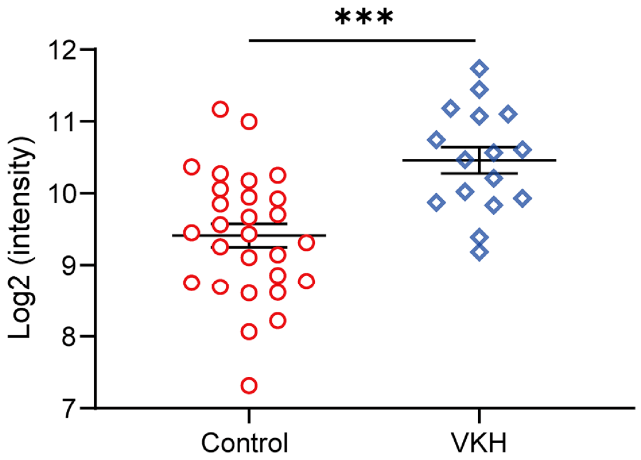
To investigate whether overexpressing kallistatin affects autoimmunity such as uveitis, we induced EAU with $hIRBP_{651-670}$ in WT and kallistatin-overexpression mice (13 mice each group). The efficiency of human kallistatin gene transfer was detected by real-time qPCR analysis. Both in the spleen (**Figure S1A**) and dLNs (**Figure S1B**) of KS mice, mRNA levels of kallistatin were significantly increased compared to WT controls. The schematic diagram (**Figure 2A**) outlines the general procedure of disease induction and examination. We first tracked and recorded the development of EAU by indirect ophthalmoscope from day 10 to day 25 after immunization. As seen in **Figure 2B**, the clinical score of EAU in KS mice was significantly higher than WT mice. To observe EAU more objectively we then used retinal imaging and SD-OCT 14-17 days after disease induction. The representative fundus images obtained by retinal imaging showed that more severe vasculitis, linear or diffuse lesions were observed in the fundus of KS mice than WT mice (**Figure 2C**). The SD-OCT B-scan imaging correlated morphological features with inflammation; that is, more hyper-reflective foci of inflammatory cells in the vitreous cavity of KS mice. Accordingly, more retinal foldings and disruptions of the inner segment/outer segment (IS/OS) junction were seen in the KS mice (**Figure 2D**). Next, we quantitated the foci in the vitreous cavity using Image J and found that KS mice had significantly higher numbers of the foci than WT control mice (p = 0.0043, **Figure 2E**). These imaging results indicated that KS mice had increased choroidal focal or lineal chorioretinal lesions.

Pathological examination on day 21 after immunization was consistent with the *in vivo* ocular imaging results, showing a significant increased leukocyte infiltration and retinal folds in KS mice (**Figure 2F**). KS mice exhibited significantly higher scores compared to control mice (2.467 *vs* 1.429, **Figure 2G**). Collectively, all ocular imaging data and histopathological analysis demonstrated that kallistatin overexpression results in increased EAU severity.

Overexpression of Kallistatin Results in Increased Th17 Infiltrates in the Eye and Lymphoid Organs During EAU

It's been known that effector $CD4^+$ T cells, especially Th1 and Th17 cells, play a pathogenic role in EAU development (1, 7, 31, 32). To

A



B

	Age (mean ± SD)	Gender (M/F)
Control	37.6 ± 8.2	15/13
VKH	47.4 ± 12.7	8/8

FIGURE 1 | Proteomics analysis of VKH patients identifies upregulation of kallistatin protein. The 48 participants with primary diagnosis (Control: n = 28; VKH: n = 16) were enrolled in the proteomics analysis. **(A)** The plasma levels of kallistatin protein in controls and VKH patients were analyzed by mass spectrometry. **(B)** Demographics of VKH patients and control participants. The mass spectrometry data were log2 transformed and quantile normalized using the R programming language package. Differential analysis of protein expression was performed using t test. Data are shown as mean ± SEM. ***P < 0.001.

determine whether kallistatin overexpression affects effector $CD4^+$ T cells after EAU induction. We collected the cells from eyes, spleens and dLNs of WT and KS mice on day 14-16 post-immunization (p.i.) and stimulated with PMA/ion plus brefeldin for 5 h and analyzed the cells using flow cytometry. As shown in **Figures 3A–F**, the percentages of $CD4^+$ $IL\text{-}17^+$ (Th17) cells in the eyes and peripheral immune organs (spleen and dLNs) of KS mice were significantly higher than WT controls. The frequency of $CD4^+$ IFN-γ^+ (Th1) cells in the local eyes was comparable between KS mice and WT control mice (**Figures 3A, B**) and, the Th1 subset in the spleen and dLNs was significantly decreased in KS mice compared to WT mice (**Figures 3C–F**). Although $CD4^+$ $IL\text{-}17^+$ IFN-γ^+ (Th17/Th1) subsets were observed in local eyes, there was no significant difference between WT and KS mice, and this subpopulation was detected at low levels (<1%) in both spleen and dLNs, as demonstrated in **Figure 3**. These results indicated that the overexpression of kallistatin affected pathogenic $CD4^+$ T cells toward Th17 profile during EAU.

Kallistatin Overexpression Promotes IRBP-Specific T Cell Proliferation

To further determine that kallistatin overexpression affects IRBP-specific T cells, we measured the antigen specific responses of T cells from immunized KS and WT control mice. T cells and APCs from spleen of either WT or KS EAU mice were collected on day 14-16 p.i. and stimulated with increasing doses of the immunizing antigen. As shown in **Figure 4A**, T cells from immunized KS mice had an enhanced proliferative response, especially at 3 μg/ml of $hIRBP_{651\text{-}670}$. Since both uveitogenic Th1 and Th17 cells are pathogenic for EAU induction (33, 34), we determined whether KS overexpression preferentially affected IFN-γ^+ and/or $IL\text{-}17^+$ autoreactive T cells. Supernatants from T cells stimulated with the highest concentration of $IRBP_{651\text{-}670}$ (as in **Figure 4A**) were collected and cytokines of IFN-γ, IL-17A and IL-10 were determined by ELISA. As shown in **Figure 4B**, the amounts of IL-17 released into the culture supernatants by T cells from KS EAU mice were markedly higher than those produced by T cells from WT control mice. Although the levels of IFN-γ were significantly higher in KS cocultured system than those in WT system, the differences were small. Whereas IL-10 cytokines were similar between KS and WT EAU mice.

Kallistatin Overexpression Directly Affects IRBP-Specific T Cells and Favors The Release Of IL-17A Production

To distinguish whether the high responsiveness of autoreactive T cells in KS animals resulted from dysfunction of autoreactive T

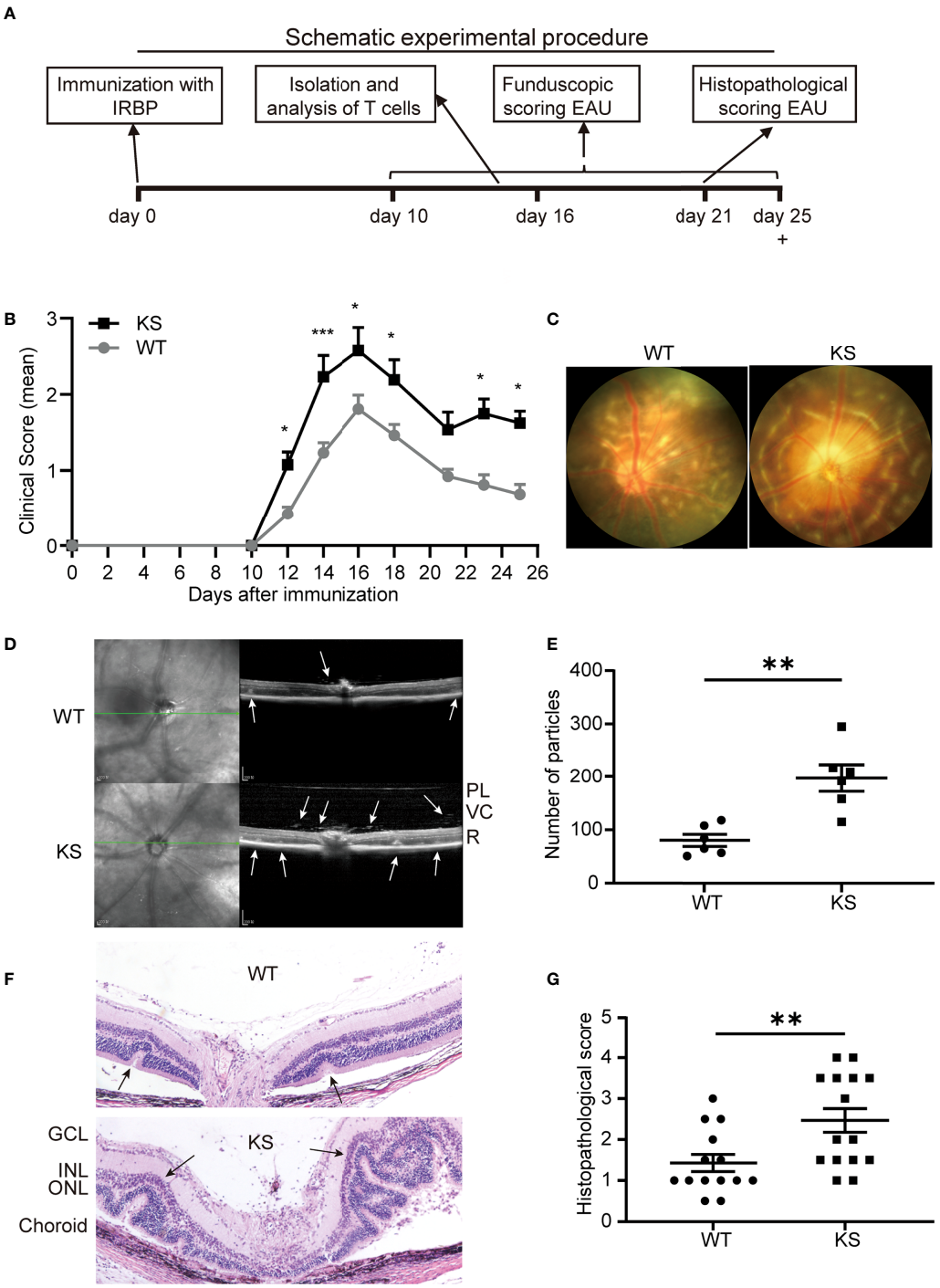


FIGURE 2 | Kallistatin-transgenic (KS) mice develop exacerbated EAU. **(A)** A schematic experimental procedure of EAU induction and examination. **(B)** EAU clinical score of WT and KS mice (n = 13 in each group). Age- and sex-matched WT and KS mice were immunized with $hIRBP_{651-670}$, and their eyes were examined by indirect ophthalmoscope and the clinical score was recorded from day 10 to day 25. Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***P < 0.001, two-way ANOVA. **(C)** Representative images of the eyes in WT and KS mice on day 16 after EAU induction by retinal imaging microscope. **(D)** Representative images showing fundus condition by SD-OCT. Hyper-reflective foci in the vitreous cavity and retinal folds (white arrows) were observed around the optical nerve. PL, posterior lens margin; VC, vitreous cavity; R, retina. **(E)** Particles in the SD-OCT images were assessed and calculated using ImageJ. **(F)** Pathological representatives of hematoxylin and eosin (H&E) staining of the eye sections from WT and KS mice on day 21 after immunization. The retinal folds and protrusions near the optic disk were indicated by the black arrows. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Original magnification, 20X. **(G)** Histopathological scores for EAU in WT and KS mice at day 21 post immunization. Data are presented as the mean ± SEM. N = 6 **(E)** or > 6 **(G)**; **p < 0.01, Mann-Whitney test.

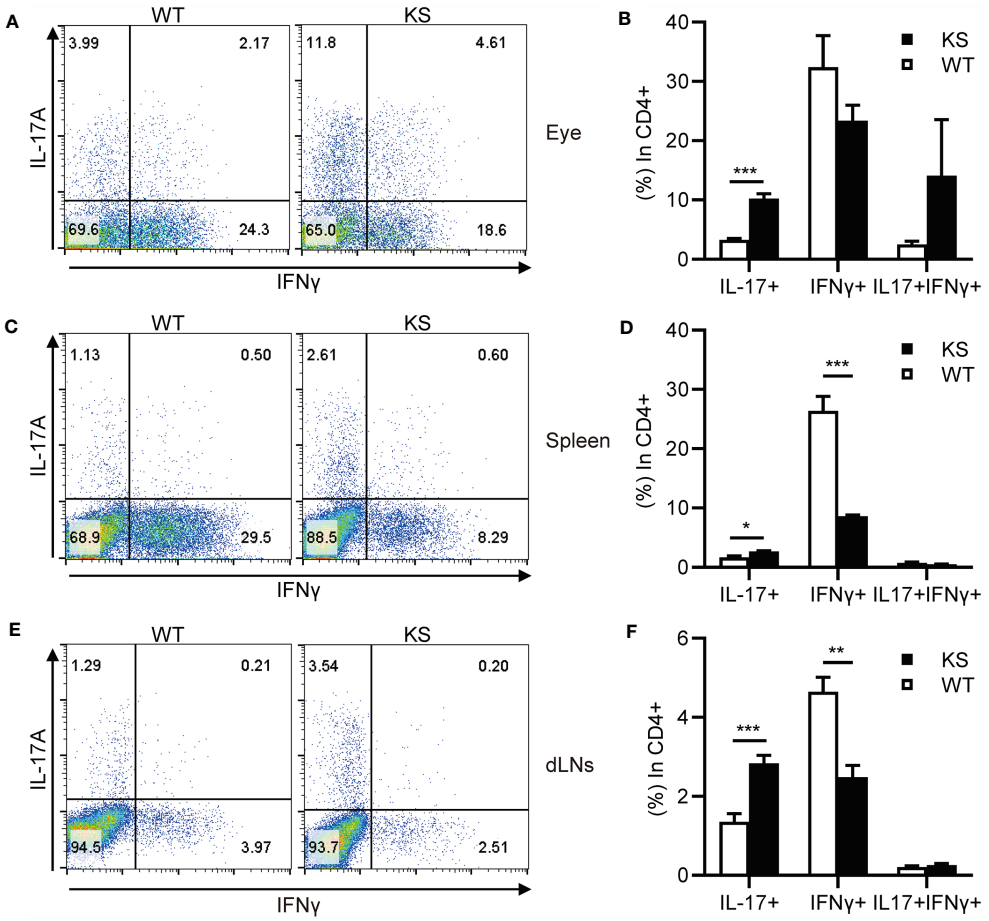


FIGURE 3 | KS mice show an increased accumulation of autoreactive effector T cells and a skewing toward Th17 infiltration after EAU immunization. The cells harvested from eye, spleen or dLNs of WT and KS mice 14-16 days after immunization were stimulated with PMA, ionomycin plus brefeldin for 5 h, then stained with anti-CD4 mAb and intracellular anti-IL-17A and anti-IFN-γ mAbs, followed by FACS analysis. **(A, C, E)** FACS plot are representative of three similar independent experiments. **(B, D, F)** Bar diagram are shown for frequencies of IL-17A$^+$, IFN-γ$^+$ or IL-17A$^+$IFN-γ$^+$ cells in total CD4$^+$ T cells from immunized WT and KS mice. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***P < 0.001. (Unpaired Student's t test).

cells or APCs, we performed cross-tests in which T cell proliferation was measured using several combinations of responder T cells and APCs from $IRBP_{651-670}$-immunized KS or WT control mice. T cells from KS mice showed higher responses to increasing doses of immunizing antigen in the presence of APCs from either KS or WT control mice, whereas T cells from control mice reacted well but lower than KS mice in the presence of APCs from control mice and KS mice (**Figures 5A, C**), indicating that overexpressing KS directly affects T cell hyperresponsiveness in KS mice. In addition, the levels of IL-17A secreted by KS-derived T cells were higher than those WT-derived T cells, whereas the levels of IFN-γ and IL-10 released were not significantly different (**Figures 5B, D**). However, when the autoreactive T cells were from KS mice, and APCs were derived either from WT or KS, there was no significant difference in T cell proliferation and three cytokine productions between the two coculture systems (**Figures 5E, F**). These results suggest that kallistatin overexpression is associated with the enhanced reactivation of IRBP-specific T cells, especially favoring Th17 cells.

Overexpressing Kallistatin Favors Th17 Cell Differentiation

To further investigate the effect of kallistatin on Th17 cells, we first isolated CD4$^+$ T cells from spleen of WT and KS mice, and identified the genes differently expressed in T cell subsets. As shown in **Figure 6**, only Il17a mRNA in CD4$^+$ T cells derived from KS mice was significantly higher than WT mice, whereas the expressions of other mRNAs, including *Il17f, Il21, Il22, Il23, Ifng, Tnfα, Gmcsf* and *Foxp3* were not significant different between KS and control mice, indicating that overexpressing kallistatin might only regulate IL-17A expression.

We then assessed the potential contribution of kallistatin to Th17 differentiation. Naïve CD4$^+$CD25$^-$62L^{hi}CD44^{lo} T cells sorted from the spleen and dLNs of KS or WT mice were

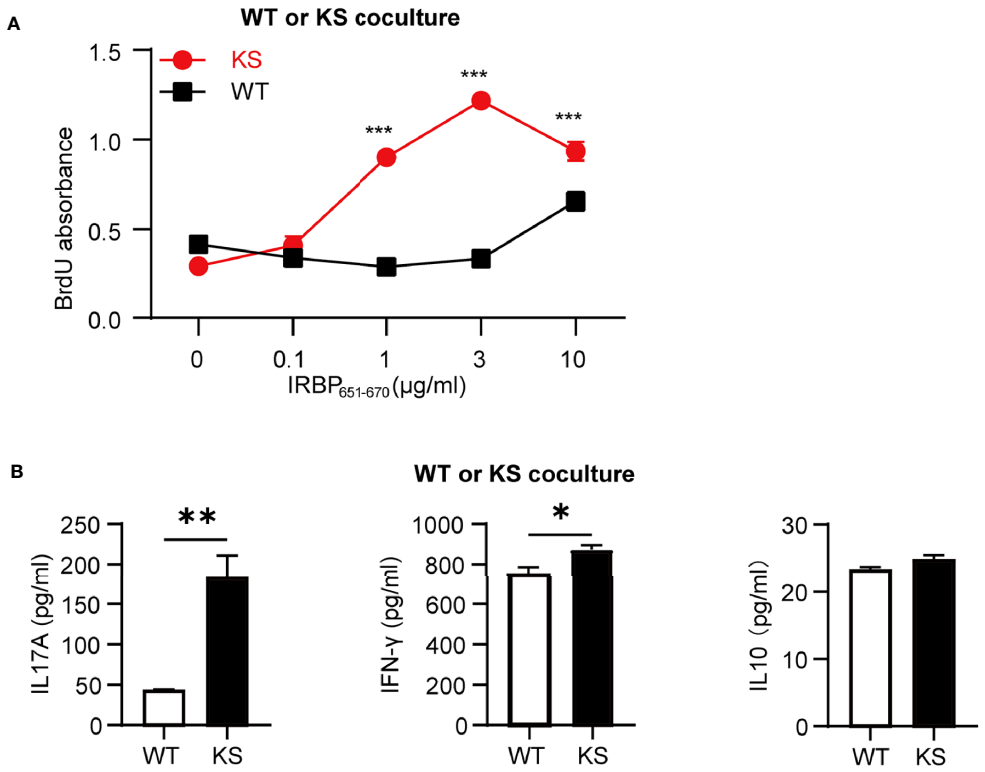


FIGURE 4 | Kallistatin overexpression favors the proliferation of antigen-specific T cells. Responder T cells (TCs) and irradiated antigen presenting cells (APCs) isolated from spleen of WT or KS mice 14-16 days after immunization were stimulated with increasing concentrations of hIRBP$_{651-670}$ antigen. Both the responder T cells and the irradiated APCs were from the same mice, either WT mice or KS mice. **(A)** The cell proliferation was measured after coculture for 60 h by incorporation of BrdU during the last 16-18 h of culture. **(B)** The supernatants of 10 μg/ml hIRBP$_{651-670}$ peptide-stimulated coculture were collected, and the cytokine levels of IL-17A, IFN-γ and IL-10 were then determined by ELISA. Graphs are representative of two similar experiments (n = 3 mice per group). Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***P < 0.001; **(A)** two-way ANOVA followed by a Sidak *post hoc* test; **(B)** Student's t test.

cultured under Th17 polarization condition for 5 days. As shown in **Figures 7A, B**, compared with WT mice, differentiated T cells from KS mice had significantly elevated percentages of IL-17A-expressing cells. We also determined the levels of IL-17A cytokine in the supernatants, which were significantly higher than controls (**Figure 7C**). In addition, we examined the expression of inflammatory cytokines under Th17 polarized conditions. A remarkable increase in mRNA levels of *Il17a*, *Il17f* and *Il22*, *Il23*, but not *Ifng* and *Foxp3*, was observed in naïve T cells from KS mice (**Figure 7D**). These data together indicate that overexpressing kallistatin boosts Th17 polarization.

Kallistatin Overexpression Has No Effects on Activation and Proliferation of T Cells From Naïve Mice

To assess the effects of kallistatin on CD4$^+$ T cells from naïve mice, we purified CD4$^+$ T cells from spleens and dLNs of WT or KS mice, and incubated them in the presence of anti-CD3 and anti-CD28 antibodies (mAbs). After 5 h, the activation status of CD4$^+$ T cells was evaluated by flow cytometry with the mAb against CD69, a marker of early T cell activation. We found that WT and KS mice had similar CD69 levels in the CD4$^+$ T cells, both in the resting and activated status (**Figures 8A, B**). In addition, proliferative responses of CD4$^+$ T cells to CD3/CD28 stimulation were not different between WT and KS groups determined by BrdU incorporation assay (**Figure 8C**). These data suggest that overexpressing kallistatin doesn't affect the activation and proliferation of CD4$^+$ T cells from naïve mice *in vitro*.

DISCUSSION

It has been reported that kallistatin exhibits various functions in angiogenesis, oxidative stress and inflammatory activities (21, 23, 24). However, much less attention has been paid to the role of kallistatin in the pathogenesis of autoimmune diseases. In this study, we identify the highly expressed kallistatin protein in VKH patients for the first time. Furthermore, we establish an association between overexpressing kallistatin and a mouse model of autoimmune uveitis, that is, KS mice are more susceptible to EAU induction. Overexpressing kallistatin

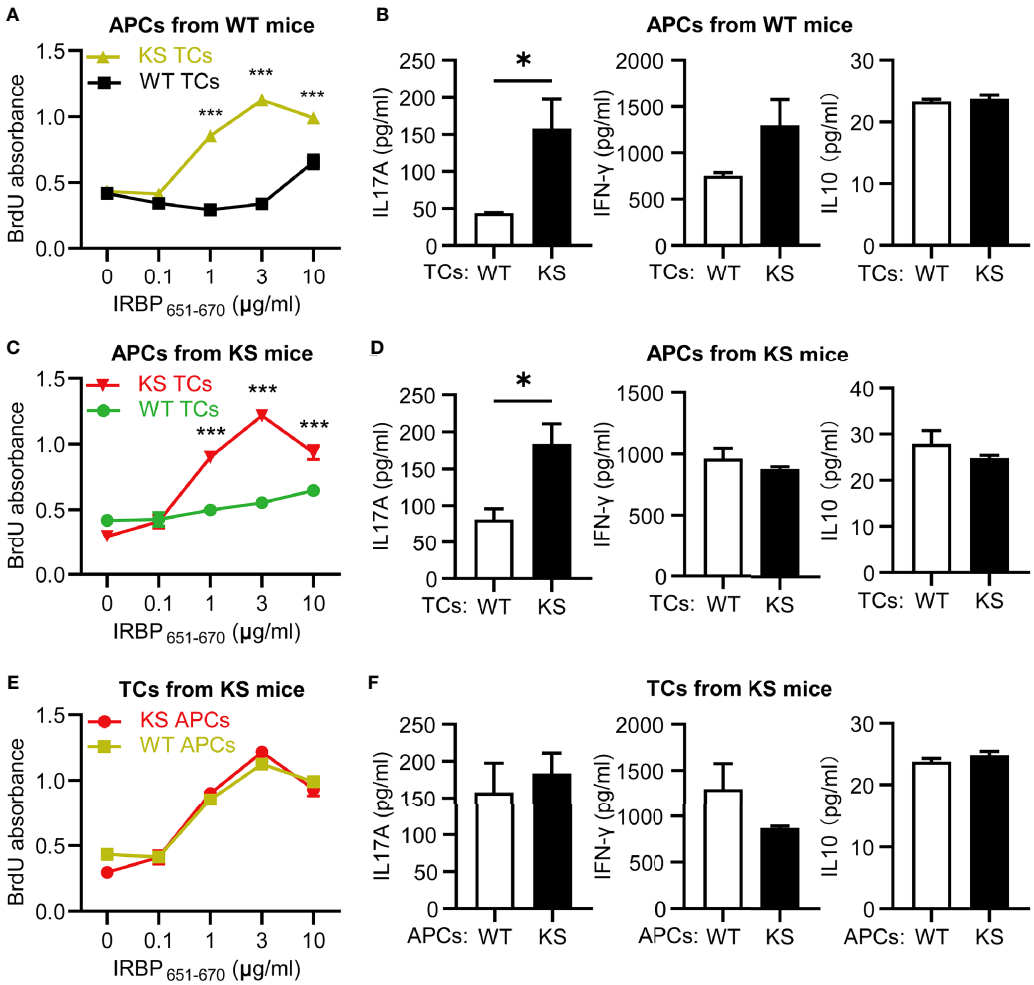


FIGURE 5 | Kallistatin overexpression directly affects IRBP-specific T cells and favors the release of IL-17A production. T cells, either WT or KS, after 60 h in coculture with splenic APCs isolated from WT or KS mice. **(A, C, E)** Proliferation of responder T cells was determined by BrdU incorporation. **(B, D, F)** The supernatants in the presence of 10 μg/ml hIRBP$_{651-670}$ peptide were collected, and levels of IL-17A, IFN-γ and IL-10 coproduction were measured by ELISA. **(A, B)** The responder T cells were from either WT or KS mice, while the irradiated APCs were from WT mice. **(C, D)** T cells from WT or KS mice were cocultured with APCs from KS mice. **(E, F)** Irradiated APCs from WT or KS mice were cocultured with T cells from KS mice. Graphs are representative of two similar experiments (n=3 animals per group). Data are shown as mean ± SEM. *p < 0.05, ***P < 0.001; **(A, C, E)** two-way ANOVA followed by a Sidak *post hoc* test; **(B, D, F)** Unpaired Student's t test.

promotes Th17 polarization *in vivo* and *in vitro*. Accordingly, there is a dominant Th17 subset in diseased eyes, and upregulated antigen-specific responses of Th17 cells in immunized KS mice. These observations support a concept that a high level of kallistatin promotes adaptive responses to uveitis by enhancing differentiation of CD4$^+$ T cells towards Th17 effector cell lineage. A critical finding of our study is that overexpressing kallistatin is directly responsible for the proliferation and IL-17A production of hIRBP$_{651-670}$–specific CD4$^+$ T cells, leading to an aggravated uveitogenic phenotype.

Our data show that kallistatin levels are elevated in VKH patients compared to those non-uveitis subjects, which raises the intriguing possibility that kallistatin might be involved in the regulation of inflammation of uveitis. We further reveal that transgenic elevation of human kallistatin in mice contributes to developing EAU. In contrast to our findings of EAU in KS mice, EAU in KS mice immunized with IRBP$_{1-20}$ peptide was reported to be milder than WT mice (35). The contradictory results might be attributable to the different animal models and cell types. One of major differences is that the IRBP peptides used for generation of EAU are different between two labs. We used hIRBP$_{651-670}$, whereas the other lab used IRBP$_{1-20}$. It has been reported that different IRBP peptides and model-induced methods elicit different uveitogenic and immunological responses (1, 8). IRBP$_{651-670}$ elicits severer EAU disease and specific T cell responses than IRBP$_{1-20}$ in B6 mice with the H-2b haplotype

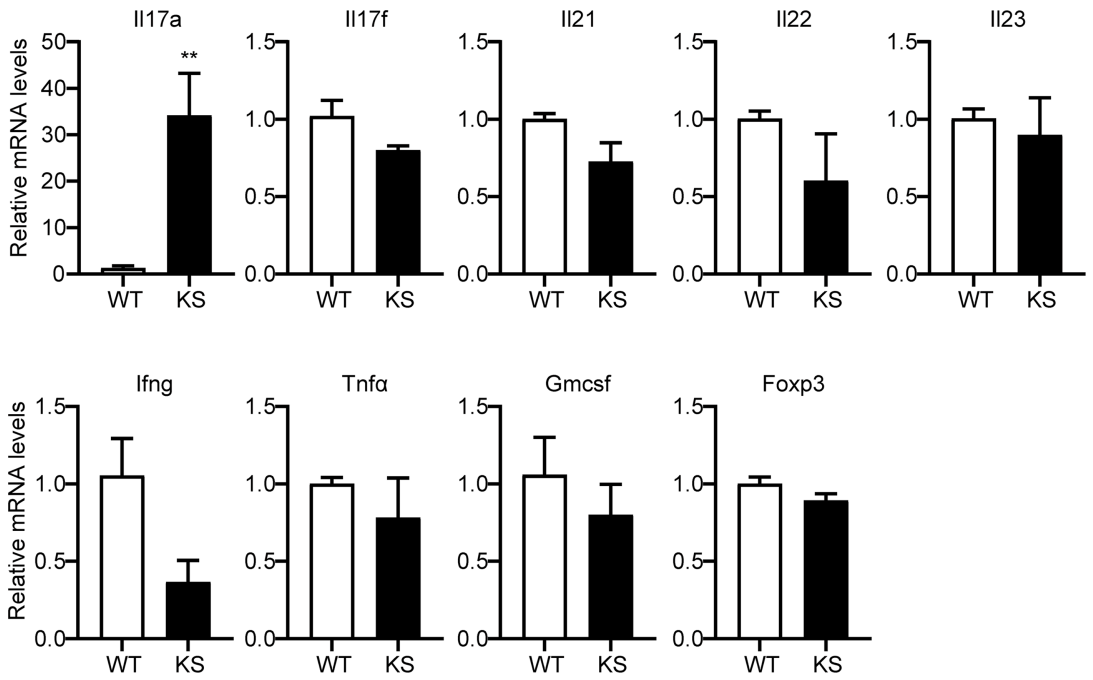


FIGURE 6 | Kallistatin overexpression favors mRNA expression of Il17a in $CD4^+$ T cells. The total $CD4^+$ T cells were sorted from spleens of unimmunized (naïve) WT or KS mice using a mouse $CD4^+$ T cell enrichment kit. Quantitative real-time PCR analysis was performed to determine the relative mRNA expression levels of *Il17a, Il17f, Il21, Il22, Il23, Ifng, Tnfα, Gmcsf* and *Foxp3*. The gene levels were normalized using *Gapdh*, and the level of each gene was expressed as a ratio of WT group. Data are mean ± SEM (n = 3-5 mice). **p < 0.01. (Unpaired Student's t test).

(36). We further evidenced that severe EAU was induced by $IRBP_{651\text{-}670}$ in KS mice, supported by their high responses of IRBP-specific T cell proliferation and elevated Th17 cell generation. Additionally, Fauziyya et al. was more interested in Treg cells in the resolution and post-EAU phases (35), while we mainly focused on Th1 and Th17 inflammatory cells at onset and peak phases of the disease. In their study (35), neither the effects of overexpressing kallistatin on Th1 or Th17 cells in EAU nor the mechanisms of kallitatin inhibiting EAU have been studied. These results may reflect diverse characteristics of kallistatin in different models and possible different activities depending on the stage of the disease. In any cases, additional studies are needed to validate these hypotheses.

Accumulating evidence suggests that $CD4^+$ T cells differentiate into functionally distinct subpopulations, protective or pathogenic, and mediate the balance of the immune responses (14, 37). It's been strongly supported that either a Th17 or a Th1 effector response can drive EAU depending on the animal model (8, 34, 38, 39). We demonstrated here that compared to WT mice, Th17 infiltrates at peak stage of a monophasic EAU model were increased in the eye and peripheral lymphoid organs of KS mice. In contrast, Th1 subset was significantly decreased in the spleen and dLNs of KS mice but not in the eye. This difference could be as a result of the different milieu of secondary lymphoid organs and local eye. Additionally, we did not observe that overexpressing kallistatin impacted the IFN-γ^+ IL-$17A^+$ subset or $Foxp3^+$ Treg subset (data not shown) during EAU. These data may explain why KS mice developed severe EAU, as the overexpression of kallistatin promotes autoimmune inflammation by providing a better setting for effector Th17 instead of Th1 cells in the local tissues. Previous study has also demonstrated that Th1 cytokine such as IFN-γ inhibits the development of Th17 (15).

The proliferative and pathogenic analyses of EAU splenocytes suggest that kallistatin overexpression contributes to the reactivation of autoreactive T cells in response to $IRBP_{651\text{-}670}$ antigen. Meanwhile, we have demonstrated that $IRBP_{651\text{-}670}$-specific T cells, but not APCs, from KS mice exhibit a more activated and aggressive phenotype. In line with this, kallistatin favors $IRBP_{651\text{-}670}$-reactive T cells releasing IL17A, but not IFN-γ or IL10. These suggest that autoreactive T cells, especially Th17 cells play a central role in controlling accelerated inflammation of KS mice during EAU. This affirmation is supported by the work of Dody et al, who states that IFN-γ is not necessary for the pathogenesis of EAU (40).

Our mRNA data of purified T cells show a higher level of IL17A in unimmunized KS mice than that in naïve WT mice, confirming the critical role of kallistatin in a pro-Th17 cytokine milieu, which may be mainly related to the expression of IL17A. Although IL-17A and IL-17F are highly homologous, they perform distinct functions: IL-17A plays important roles in inflammation, autoimmunity, and host defenses against

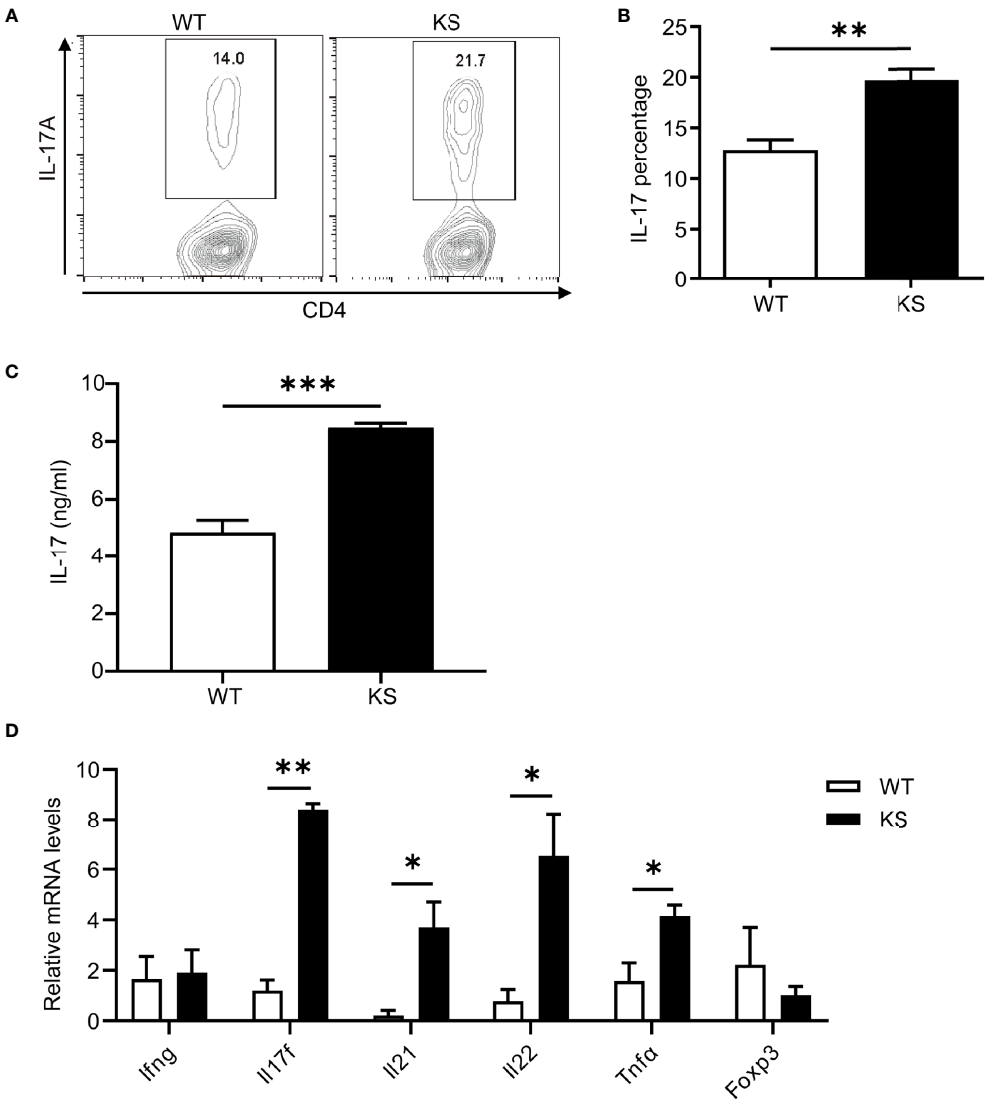


FIGURE 7 | Kallistatin favors differentiation of CD4$^+$ naïve T cells to effector Th17 cells. Naïve CD4$^+$ T cells purified from spleen and dLNs in WT and KS mice and cultured under Th17 polarizing conditions. **(A)** On day 5 of differentiation, the cells were then stimulated with PMA, ionomycin and brefeldin for 5 h, followed by flow cytometry analysis. **(B)** Bar diagram is shown for IL-17A$^+$ frequencies in CD4$^+$ T cells of WT and KS mice. **(C)** The supernatants harvested at 48 h of culture were assessed for IL-17A by ELISA. **(D)** Cells collected after 48h culture were determined for the mRNA expression of indicated cytokines by real-time qPCR analysis. Data are representative of three individual experiments and graphs are shown as mean values ± SEM. n = 3, *p < 0.05, **p < 0.01, ***P < 0.001. (Unpaired Student's t test).

bacterial and fungal infections; whereas IL-17F is mainly involved in mucosal host defense mechanisms (41). Furthermore, under Th17-polarizing conditions, KS naïve CD4$^+$ T cells showed a higher percentage of IL17A$^+$ populations, and increased mRNA levels of Th17 signature cytokines, such as IL17A, IL17F, IL21 and IL22. Despite the addition of various stimuli factors under *in vitro* polarized conditions, the *in vivo* and *in vitro* milieus are different, the *in vivo* regulatory mechanisms are relatively complex and more unclear. This may explain why only IL-17 was elevated and neither IL-21 nor IL-22 was elevated in our vivo mRNA data. These data together suggest that kallistatin gives rise to a high differentiation of naïve T cells into Th17 cells. In addition, our data showed that kallistatin did not activate the T cells of naïve mice by CD3/CD28 mAbs *in vitro*, suggesting that increased kallistatin helps to promote antigen-specific T cells during the development of autoimmunity. However, the detailed mechanism by which kallistatin regulates Th17 cells remains to be further clarified.

In conclusion, the results of our study demonstrate that overexpressing kallistatin favors Th17 generation which promotes autoimmune responses. We first demonstrated that

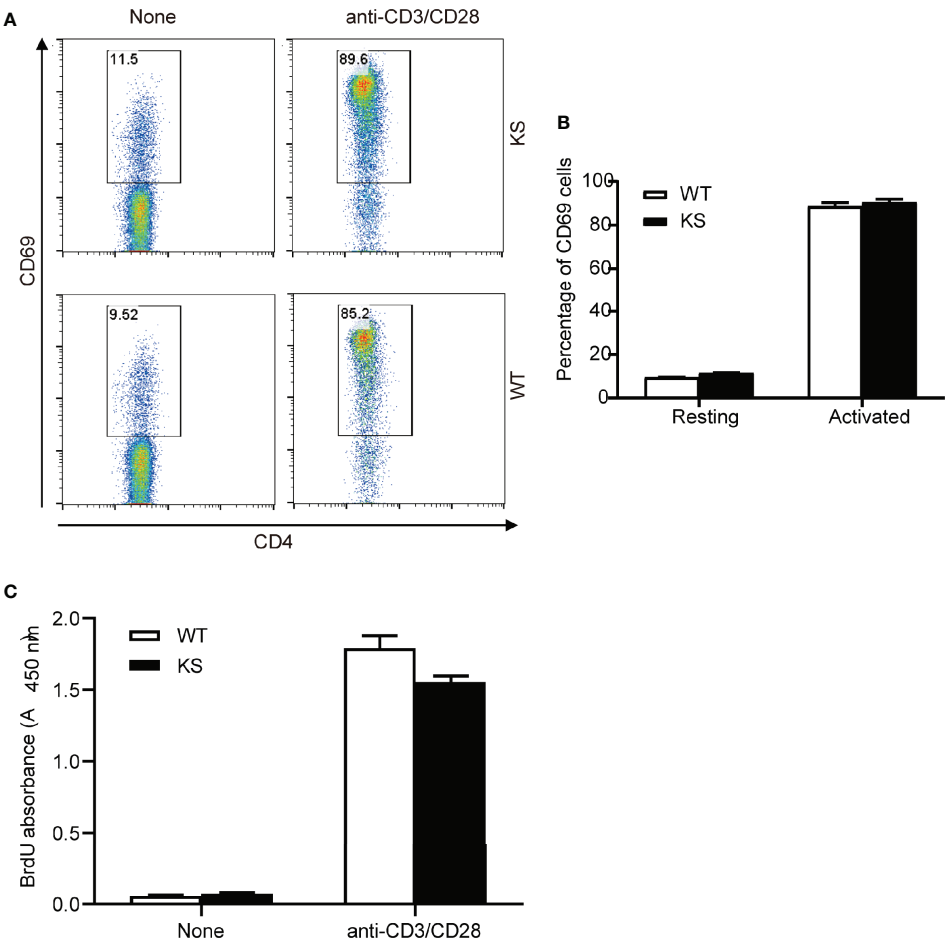


FIGURE 8 | Effects of Kallistatin overexpression on T cell activation and proliferation of naïve mice. $CD4^+$ T cells purified from naïve WT and KS mice were stimulated without (None) or with anti-CD3 and anti-CD28 mAbs. **(A, B)** After stimulation for 5 h, cells were collected and washed, followed by staining for CD69 and flow cytometry analysis. Representative FACS plot **(A)** and bar diagram **(B)** for frequencies of $CD69^+$ T cells are shown. **(C)** After stimulation for 24 h, the proliferation of T cells was detected by BrdU incorporation. Data are representative from three independent experiments and shown as mean ± SEM. (Unpaired Student's t test).

kallistatin was highly expressed in VKH patients. In EAU, high levels of kallistatin promoted Th17 polarization, and increased proportions of $CD4^+IL17A^+$ cells in local eye and peripheral immune organs, thereby aggravating the intraocular inflammation. These findings provide an insight into the roles of kallistatin in Th17 polarization and excessive inflammatory reactions in EAU. Hence, modulating kallistatin levels and reducing Th17 cells might be a potential therapeutic strategy for Th17 mediated autoimmune diseases.

DATA AVAILABILITY STATEMENT

The raw data that supports the conclusions of this article will be made available by request to the corresponding author, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Tianjin Medical University Eye Hospital (Tianjin, China). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Care and Use Committee, Tianjin Medical University.

AUTHOR CONTRIBUTIONS

XZ, HS, and NC designed the research and interpreted data. J-XM Lab generated and provided the transgenic mice. NC, SC, XC, ZZ, and LW performed experiments and analyzed data. KLG mated and bred the mice. NC wrote the manuscript. HS and XZ reviewed

the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.756423/full#supplementary-material

REFERENCES

1. Caspi RR. A Look at Autoimmunity and Inflammation in the Eye. *J Clin Invest* (2010) 120(9):3073–83. doi: 10.1172/JCI42440
2. Rothova A, Suttorp-van Schulten MS, Frits Treffers W, Kijlstra A. Causes and Frequency of Blindness in Patients With Intraocular Inflammatory Disease. *Br J Ophthalmol* (1996) 80(4):332–6. doi: 10.1136/bjo.80.4.332
3. Gritz DC, Wong IG. Incidence and Prevalence of Uveitis in Northern California; the Northern California Epidemiology of Uveitis Study. *Ophthalmology* (2004) 111(3):491–500. doi: 10.1016/j.ophtha.2003.06.014
4. Prete M, Guerriero S, Dammacco R, Fatone MC, Vacca A, Dammacco F, et al. Autoimmune Uveitis: A Retrospective Analysis of 104 Patients From a Tertiary Reference Center. *J Ophthalmic Inflamm Infect* (2014) 4:17. doi: 10.1186/s12348-014-0017-9
5. Liu D, Ahmet A, Ward L, Krishnamoorthy P, Mandelcorn ED, Leigh R, et al. A Practical Guide to the Monitoring and Management of the Complications of Systemic Corticosteroid Therapy. *Allergy Asthma Clin Immunol* (2013) 9 (1):30. doi: 10.1186/1710-1492-9-30
6. Rosenbaum JT. Future for Biological Therapy for Uveitis. *Curr Opin Ophthalmol* (2010) 21(6):473–7. doi: 10.1097/ICU.0b013e32833f00b3
7. Amadi-Obi A, Yu CR, Liu X, Mahdi RM, Clarke GL, Nussenblatt RB, et al. TH17 Cells Contribute to Uveitis and Scleritis and are Expanded by IL-2 and Inhibited by IL-27/Stat1. *Nat Med* (2007) 13(6):711–8. doi: 10.1038/nm1585
8. Luger D, Silver PB, Tang J, Cua D, Chen Z, Iwakura Y, et al. Either a Th17 or a Th1 Effector Response can Drive Autoimmunity: Conditions of Disease Induction Affect Dominant Effector Category. *J Exp Med* (2008) 205 (4):799–810. doi: 10.1084/jem.20071258
9. Lee RW, Nicholson LB, Sen HN, Chan CC, Wei L, Nussenblatt RB, et al. Autoimmune and Autoinflammatory Mechanisms in Uveitis. *Semin Immunopathol* (2014) 36(5):581–94. doi: 10.1007/s00281-014-0433-9
10. Foxman EF, Zhang M, Hurst SD, Muchamuel T, Shen D, Wawrousek EF, et al. Inflammatory Mediators in Uveitis: Differential Induction of Cytokines and Chemokines in Th1- Versus Th2-Mediated Ocular Inflammation. *J Immunol* (2002) 168(5):2483–92. doi: 10.4049/jimmunol.168.5.2483
11. Jager A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. Th1, Th17, and Th9 Effector Cells Induce Experimental Autoimmune Encephalomyelitis With Different Pathological Phenotypes. *J Immunol* (2009) 183(11):7169–77. doi: 10.4049/jimmunol.0901906
12. Rizzo LV, Silver P, Wiggert B, Hakim F, Gazzinelli RT, Chan CC, et al. Establishment and Characterization of a Murine CD4+ T Cell Line and Clone That Induce Experimental Autoimmune Uveoretinitis in B10. *A Mice J Immunol* (1996) 156(4):1654–60.
13. Wakefield D, Li Q, McCluskey P, Nussenblatt RB, Chan CC. Immunohistochemical Localization of T Lymphocytes and Macrophages and Expression of Interferon Gamma and Defensin in Uveitis. *Ocul Immunol Inflamm* (1994) 2(3):153–9. doi: 10.3109/09273949409057071
14. Chen YH, Eskandarpour M, Gondrand A, Zhang X, Gu R, Galatowicz G, et al. Functionally Distinct IFN-Gamma(+) IL-17a(+) Th Cells in Experimental Autoimmune Uveitis: T-Cell Heterogeneity, Migration, and Steroid Response. *Eur J Immunol* (2020) 50(12):1941–51. doi: 10.1002/eji.202048616
15. Lee YK, Turner H, Maynard CL, Oliver JR, Chen D, Elson CO, et al. Late Developmental Plasticity in the T Helper 17 Lineage. *Immunity* (2009) 30 (1):92–107. doi: 10.1016/j.immuni.2008.11.005
16. Maggi L, Santarlasci V, Capone M, Rossi MC, Querci V, Mazzoni A, et al. Distinctive Features of Classic and Nonclassic (Th17 Derived) Human Th1 Cells. *Eur J Immunol* (2012) 42(12):3180–8. doi: 10.1002/eji.201242648
17. Sallusto F, Cassotta A, Hoces D, Foglierini M, Lanzavecchia A. Do Memory CD4 T Cells Keep Their Cell-Type Programming: Plasticity Versus Fate Commitment? T-Cell Heterogeneity, Plasticity, and Selection in Humans. *Cold Spring Harb Perspect Biol* (2018) 10(3):a029421. doi: 10.1101/cshperspect.a029421
18. Chen VC, Chao L, Chao J. Roles of the P1, P2, and P3 Residues in Determining Inhibitory Specificity of Kallistatin Toward Human Tissue Kallikrein. *J Biol Chem* (2000) 275(49):38457–66. doi: 10.1074/jbc.M005605200
19. Zhou GX, Chao L, Chao J. Kallistatin: A Novel Human Tissue Kallikrein Inhibitor. Purification, Characterization, and Reactive Center Sequence. *J Biol Chem* (1992) 267(36):25873–80. doi: 10.1016/S0021-9258(18)35690-4
20. Chao J, Tillman DM, Wang MY, Margolius HS, Chao L. Identification of a New Tissue-Kallikrein-Binding Protein. *Biochem J* (1986) 239(2):325–31. doi: 10.1042/bj2390325
21. Chao J, Guo Y, Chao L. Protective Role of Endogenous Kallistatin in Vascular Injury and Senescence by Inhibiting Oxidative Stress and Inflammation. *Oxid Med Cell Longev* (2018) 2018:4138560. doi: 10.1155/2018/4138560
22. Chao J, Yin H, Yao YY, Shen B, Smith RSJr., Chao L. Novel Role of Kallistatin in Protection Against Myocardial Ischemia-Reperfusion Injury by Preventing Apoptosis and Inflammation. *Hum Gene Ther* (2006) 17(12):1201–13. doi: 10.1089/hum.2006.17.1201
23. Li P, Bledsoe G, Yang ZR, Fan H, Chao L, Chao J. Human Kallistatin Administration Reduces Organ Injury and Improves Survival in a Mouse Model of Polymicrobial Sepsis. *Immunology* (2014) 142(2):216–26. doi: 10.1111/imm.12242
24. Shen B, Gao L, Hsu YT, Bledsoe G, Hagiwara M, Chao L, et al. Kallistatin Attenuates Endothelial Apoptosis Through Inhibition of Oxidative Stress and Activation of Akt-eNOS Signaling. *Am J Physiol Heart Circ Physiol* (2010) 299 (5):H1419–27. doi: 10.1152/ajpheart.00591.2010
25. Shen B, Hagiwara M, Yao YY, Chao L, Chao J. Salutary Effect of Kallistatin in Salt-Induced Renal Injury, Inflammation, and Fibrosis *via* Antioxidative Stress. *Hypertension* (2008) 51(5):1358–65. doi: 10.1161/HYPERTENSIONAHA.107.108514
26. Yin H, Gao L, Shen B, Chao L, Chao J. Kallistatin Inhibits Vascular Inflammation by Antagonizing Tumor Necrosis Factor-Alpha-Induced Nuclear Factor kappaB Activation. *Hypertension* (2010) 56(2):260–7. doi: 10.1161/HYPERTENSIONAHA.110.152330
27. McBride JD, Jenkins AJ, Liu X, Zhang B, Lee K, Berry WL, et al. Elevated Circulation Levels of an Antiangiogenic SERPIN in Patients With Diabetic Microvascular Complications Impair Wound Healing Through Suppression of Wnt Signaling. *J Invest Dermatol* (2014) 134(6):1725–34. doi: 10.1038/jid.2014.40
28. Jenkins AJ, McBride JD, Januszewski AS, Karschimkus CS, Zhang B, O'Neal DN, et al. Increased Serum Kallistatin Levels in Type 1 Diabetes Patients With Vascular Complications. *J Angiogenes Res* (2010) 2:19. doi: 10.1186/2040-2384-2-19
29. Feng J, Dong C, Long Y, Mai L, Ren M, Li L, et al. Elevated Kallikrein-Binding Protein in Diabetes Impairs Wound Healing Through Inducing Macrophage M1 Polarization. *Cell Commun Signal* (2019) 17(1):60. doi: 10.1186/s12964-019-0376-9

30. Caspi RR. Experimental Autoimmune Uveoretinitis in the Rat and Mouse. *Curr Protoc Immunol* (2003) Chapter 15:Unit 15 6. doi: 10.1002/0471142735.im1506s53
31. Nussenblatt RB, Rodrigues MM, Wacker WB, Cevario SJ, Salinas-Carmona MC, Gery I. Cyclosporin a. Inhibition of Experimental Autoimmune Uveitis in Lewis Rats. *J Clin Invest* (1981) 67(4):1228–31. doi: 10.1172/jci110138
32. Salinas-Carmona MC, Nussenblatt RB, Gery I. Experimental Autoimmune Uveitis in the Athymic Nude Rat. *Eur J Immunol* (1982) 12(6):480–4. doi: 10.1002/eji.1830120606
33. Caspi R. Autoimmunity in the Immune Privileged Eye: Pathogenic and Regulatory T Cells. *Immunol Res* (2008) 42(1-3):41–50. doi: 10.1007/s12026-008-8031-3
34. Cox CA, Shi G, Yin H, Vistica BP, Wawrousek EF, Chan CC, et al. Both Th1 and Th17 are Immunopathogenic But Differ in Other Key Biological Activities. *J Immunol* (2008) 180(11):7414–22. doi: 10.4049/jimmunol.180.11.7414
35. Muhammad F, Avalos PN, Mursalin MH, Ma JX, Callegan MC, Lee DJ. Kallistatin Attenuates Experimental Autoimmune Uveitis by Inhibiting Activation of T Cells. *Front Immunol* (2020) 11:975. doi: 10.3389/fimmu.2020.00975
36. Mattapallil MJ, Silver PB, Cortes LM, St Leger AJ, Jittayasothorn Y, Kielczewski JL, et al. Characterization of a New Epitope of IRBP That Induces Moderate to Severe Uveoretinitis in Mice With H-2b Haplotype. *Invest Ophthalmol Vis Sci* (2015) 56(9):5439–49. doi: 10.1167/iovs.15-17280
37. Yan J, Wang R, Horng T. mTOR Is Key to T Cell Transdifferentiation. *Cell Metab* (2019) 29(2):241–2. doi: 10.1016/j.cmet.2019.01.008
38. Zhang R, Qian J, Guo J, Yuan YF, Xue K. Suppression of Experimental Autoimmune Uveoretinitis by Anti-IL-17 Antibody. *Curr Eye Res* (2009) 34(4):297–303. doi: 10.1080/02713680902741696
39. Ke Y, Liu K, Huang GQ, Cui Y, Kaplan HJ, Shao H, et al. Anti-Inflammatory Role of IL-17 in Experimental Autoimmune Uveitis. *J Immunol* (2009) 182(5):3183–90. doi: 10.4049/jimmunol.0802487
40. Avichezer D, Chan CC, Silver PB, Wiggert B, Caspi RR. Residues 1-20 of IRBP and Whole IRBP Elicit Different Uveitogenic and Immunological Responses in Interferon Gamma Deficient Mice. *Exp Eye Res* (2000) 71(2):111–8. doi: 10.1006/exer.2000.0860
41. Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional Specialization of Interleukin-17 Family Members. *Immunity* (2011) 34(2):149–62. doi: 10.1016/j.immuni.2011.02.012

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CASE REPORT
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Vedolizumab for the Management of Refractory Behçet's Disease: From a Case Report to New Pieces of Mosaic in a Complex Disease

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Objectives: When treating Behçet's disease (BD), anti-tumor necrosis factor (TNF)-α agents have become a second-line therapy when conventional immunosuppressive drugs have failed. However, in the case of failure of treatment with anti-TNFα drugs, further options are limited. Based on previous reports of the efficacy of vedolizumab (VDZ) in inflammatory bowel diseases, we decided to administer VDZ to treat a patient with intestinal BD.

Methods: We present the case of a 49-year-old female patient with BD. Her clinical manifestations included erythema nodosum, oro-genital ulcers, positive Pathergy test, positive HLA-B51, and biopsy-proven intestinal BD. The patient was unsuccessfully treated with conventional immunosuppressive and several biological agents.

Results: Treatment with VDZ was started intravenously at a dose of 300 mg at 0, 2, and 6 weeks and then every 4 weeks. After the second dose of VDZ, the patient reported a marked improvement of intestinal BD and a concomitant amelioration of arthralgia, erythema nodosum lesions and aphthosis. Clinical remission was achieved at 6 months after starting VDZ.

Conclusion: VDZ might represent a valid option to treat patients with BD who are non-responsive to standard treatments or anti-TNFα agents, particularly, those cases with intestinal involvement.

Keywords: vedolizumab, Behçet disease, biological drug, intestinal Behçet, biological therapy

INTRODUCTION

First identified in 1937, Behçet's disease (BD) is a multisystemic inflammatory condition often described as a part of the vasculitic spectrum, characterized by recurrent oral and genital aphthosis, skin lesions, uveitis, and, less frequently, neurologic, articular, and gastrointestinal involvement (1). BD is mostly prevalent in countries along the ancient "Silk Road", from the Mediterranean area to the far East, where it is associated with a significant prevalence of the major histocompatibility

complex antigen HLA-B51 (2, 3). Before the availability of biological agents, options for the treatment of BD were limited to corticosteroids and conventional immunosuppressive drugs (1). The recent off-label use of biologic drugs such as infliximab and adalimumab, two anti-TNFα monoclonal antibodies, has improved the therapeutic armamentarium for refractory cases (4, 5). Nevertheless, not all patients fully respond to anti-TNFα agents, and it is quite common to experience a loss of efficacy over time in patients who initially had a beneficial effect (6).

Vedolizumab is a new biologic agent with a specific intestinal tropism, recently approved for the treatment of inflammatory bowel diseases (IBDs) (7, 8). VDZ binds to the α4β7 integrin, a glycoprotein expressed on the cell surface of circulating B and T lymphocytes, and blocks the interaction between the integrin and the mucosal addressin cell adhesion molecule 1 on the endothelium of intestinal blood vessels (9). The rationale for the use of VDZ in BD patients relies on the similar gastrointestinal involvement of the two conditions. In fact, a growing body of evidence is suggesting that IBDs and BD may be closely related and part of a common disease spectrum rather than distinct disease entities (10).

Herein we describe the case of a patient with BD with gastrointestinal involvement refractory and/or intolerant to several previous therapeutic approaches (including both conventional and biological disease modifying anti-rheumatic drugs, DMARDs) but who was successfully treated with vedolizumab (VDZ) and represents, to our knowledge, the first report about the use of VDZ in BD.

CASE REPORT

We present the case of a 49-year-old female patient. Her clinical history was unremarkable, aside from cutaneous psoriasis until the age of 35 when, during the post-partum period of her second pregnancy, she presented with a new onset of fever, diarrhea, and ankle arthritis. The concomitant presence of erythema nodosum and oro-genital ulcers along with the negativity for antinuclear antibodies, anti-double-stranded DNA antibodies, and normal levels of C3 and C4 supported a BD diagnosis. Colchicine and low doses of corticosteroids were started, with improvement on the aphthosis and the erythema nodosum, but with no changes in the persistence of abdominalgia and diarrhea. After a careful investigation to rule out any concomitant infectious disease, treatment with cyclosporine was started, which initially only elicited limited benefit and was subsequently suspended due to the onset of dizziness and worsening of intestinal symptoms. The ileo-colonoscopy was consistent with ileocolitis with two sigmoid colon irregularities of aphtoid aspect adjacent to macroscopically normal-appearing mucosa. The colon biopsy results reported nonspecific chronic inflammation with follicular hyperplasia of the lymphoreticular tissue with no presence of granulomas, confirming the histological diagnosis of BD.

The list of main investigations undergone by the patient is presented in **Table 1**. The patient attended other centers, from 2011 to 2017, where she was treated with different cDMARDs and bDMARDs as follows: sulfasalazine, which was suspended for increased liver enzymes, while azathioprine, adalimumab, infliximab, golimumab, and certolizumab were all discontinued due to lack of response. Secukinumab, introduced in 2018, was stopped regardless of the beneficial effects on BD symptoms due to the development of an anxious–depressive syndrome with suicidal thoughts, which spontaneously subsided when secukinumab was suspended. In 2019, a further attempt with golimumab was performed, with no clinical benefit and no control on the persistence of erythema nodosum, aphthosis, and diarrhea.

In November 2020, the woman was referred to our center (CMID, S. Giovanni Bosco Hub, Turin, Italy) for further evaluation. She presented with arthralgia (mainly affecting the wrists, metacarpophalangeal joints, and shoulders) and recurrent ulcerations and folliculitis on her back, forehead, and malar area. During the first evaluation, her main complaints were abdominal pain and persistent diarrhea which constantly impact her quality of life and were only partially responsive to symptomatic treatment. The physical examination showed two areas of erythema nodosum on the lower limbs (**Figure 1**). The Pathergy test and HLA-B51 were positive. Her laboratory profile was consistent with active BD, characterized by markedly increased erythrocyte sedimentation rate and C-reactive protein (26 mg/dl). After a multidisciplinary evaluation, taking into account the scarce efficacy of both cDMARDs and anti-TNFα strategies and the persistence of gastrointestinal symptoms, we decided to start treatment with

TABLE 1 | Previous investigations undergone by the patient.

Blood count	WBC 10,000 (cells/μl); neutrophils 7,210; lymphocytes 2,470; RBC 5,700; Hb 11.3 g/dl; MCV 62 fL; PLTs 248,000
Blood tests	Crea 0.74, AST 21, ALT 29, GGT 19, total protein 73, C3 1.65 g/l, C4 0.26 g/l, ESR 25, CRP 26, ANA pos 1:640, ENA neg, anti-dsDNA neg, antiphospholipid antibodies neg, HBV-DNA: neg; HCV-RNA: neg; Normal complete urine test, Uroculture: neg.
Shoulder MRI	Signs of acromioclavicular fibroarthrosis, compression of the myo-tendon tract of the supraspinatus in which the tendon shows a inhomogeneous character in the pre-insertion anterior area due to tendinosis; signal alteration of the medullary bone component of the humeral shaft
Cerebral MRI	Unremarkable
Pelvis MRI	Initial sacrum—ileitis of the right sacro-iliac joint
Abdominal echography	Steatotic liver disease, free of focal lesions. Outcomes of cholecystectomy. Mild hypersplenomegaly (diameter 12.5 cm). Anechoic oval formation at uterine level with a diameter of 18 mm, compatible with a follicle
Abdominal TC	Thickening of the wall of the small intestine. At the level of an intestinal loop in the left iliac area between the bladder and the acetabular region, the wall appears markedly thickened, inhomogeneous, and with discrete effusion.
Colonoscopy/ Colon biopsy	Mucosal irregularities of aphtoid aspect at the sigmoid colon level. Mucosal flaps of the large intestine with mild atrophy, chronic interstitial inflammation, edema, and hyperplasia of the muscularis mucosae.

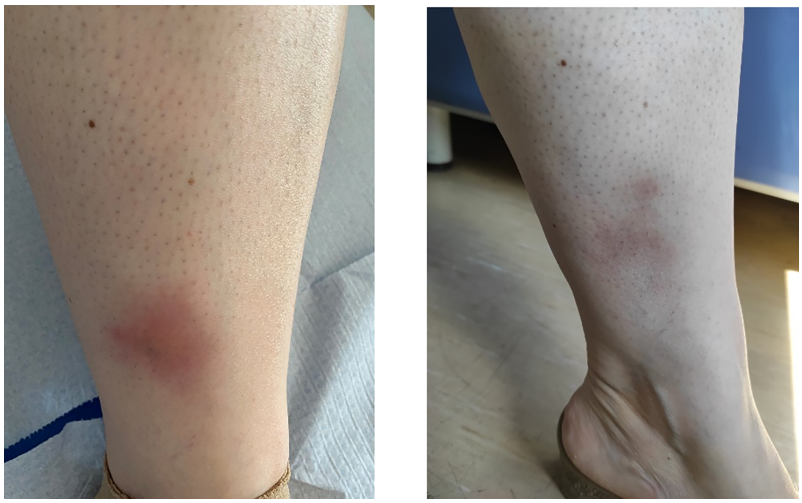


FIGURE 1 | Active tibial erythema nodosum at the first presentation of the patient, before starting with vedolizumab (left panel) and after 3 months (right panel).

VDZ, which was administered intravenously in monotherapy at a dose of 300 mg at 0, 2, and 6 weeks and then every 4 weeks.

After the second dose of VDZ, the patient reported a marked improvement when considering gastrointestinal symptoms and arthralgia, with a decrease in the Behçet's Disease Current Activity Form 2006 (11) score from 6 (at the VDZ starting time) to 0 (after 6 months of follow-up). A significant reduction of the multiple erythema nodosum lesions affecting both legs was progressively observed, with a complete resolution after the third dose and parallel amelioration of the associated pain.

At 6 months of follow-up, no side effects were observed, and the patient did not experience further episodes of aphthosis.

DISCUSSION

In this study, we reported the case of a woman with BD complicated by biopsy-proven gastrointestinal involvement, who was unresponsive to conventional treatment and various anti-TNFα agents, and who experienced a marked resolution of the gastrointestinal symptoms when treated with VDZ.

The patient was diagnosed with BD in 2007 because of recurrent oral and genital aphthosis, erythema nodosum, arthritis, and intestinal involvement. Since BD diagnosis is not characterized by specific laboratory, radiologic, or histologic findings, the diagnosis of this condition relies mainly on clinical evaluation (1). The most recent classification criteria are the International Criteria for Behçet Disease, published in 2006 and revised in 2014, which require a score of 4 from the following list: oral aphthosis, genital aphthosis, ocular manifestations, skin involvement, vascular manifestations, neurologic manifestation, and positive Pathergy test (12). Gastrointestinal involvement in BD remains infrequent. Interestingly, geographical differences among BD patients have been reported, with colon involvement being more frequent in patients from Europe and North America, while ileocecal involvement appears to be more common in Japan (13).

The aim of the treatment in BD is maintaining remission and improving the quality of life of the patient, but new therapeutic approaches are still needed. **Figure 2** illustrates the current therapeutic options for BD treatment and their main application related to clinical symptoms.

For many years, the only bDMARDs available for the management of BD were anti-TNFα agents. More recently, anti-cytokine-related (*e.g.*, IL-6) strategies have been suggested as therapeutic options for BD, with a good safety and efficacy profile (14, 15). Recently approved for IBDs, VDZ, targeting a completely novel mechanism of action, could represent a new tool in the therapeutic armamentarium of BD specialists. VDZ can be considered as an "integrin antagonist" due to its ability of binding to the α4β7 integrin (a molecule identifiable on T-lymphocytes and mononuclear cells). This gastrointestinal-specific interaction reduces the side effects associated with systemic immunosuppression. The severe intestinal involvement of the patient discussed in our study was the main reason for switching to VDZ from anti-TNFα agents. Despite the specific gastrointestinal effect, in several studies VDZ has proved to be effective also when managing the extraintestinal manifestations of IBDs (*i.e.*, skin involvement, uveitis, and arthritis), even if the mechanism remains unknown to this day (16, 17). These observations are in line with our case, as during follow-up, after 6 months of treatment with VDZ, our patient remained asymptomatic and without any sign of active inflammation. Of note is that no side effects were reported.

Meant to prevent the migration of inflammatory mononuclear cells into the inflamed mucosa in patients with IBD, VDZ could also mitigate the inflammatory process in BD (3), limiting the interaction of lymphocyte integrins (as expressed by CD4+ and CD8+ naive T cells and CD4+ and CD8+ memory T cells) and their endothelial ligands (18).

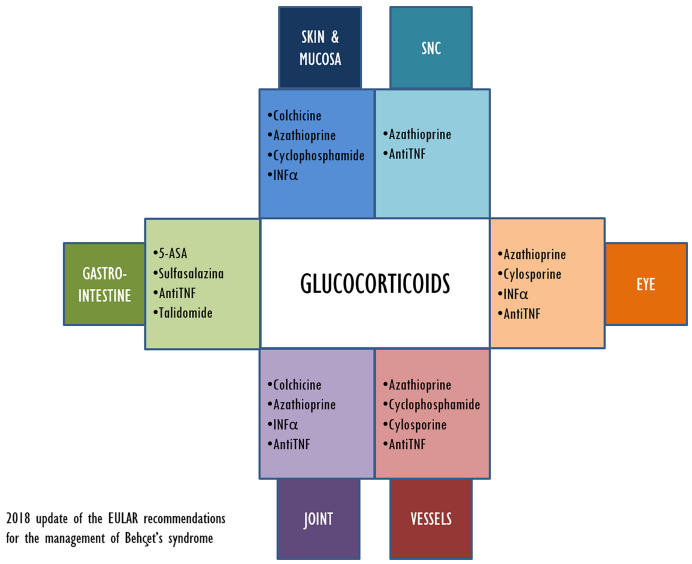


FIGURE 2 | 2018 update of the EULAR recommendations for the management of Beçhet's syndrome.

CONCLUSION

In conclusion, this case shows the efficacy of VDZ in inducing remission at 6 months of follow-up in a patient not responsive to anti-TNFα agents. When treating BD, the anti-TNFα agents have become a standard when conventional immunosuppressive drugs have failed. However, in case of anti-TNFα agents failure, the options are limited. A switch to another anti-TNFα drug is a current possibility, but in our case it was not effective. Based on previous reports of its efficacy in IBDs, we decided to administer VDZ to treat the intestinal involvement of our patient. This led not only to a satisfactory gastrointestinal response but also to the concomitant disappearing of ulcerations, arthralgia, and a reversion of the skin lesions.

Some limitations should be acknowledged, mainly the short follow-up which is currently up to 6 months. Further larger cohort studies with a longer follow-up are needed to provide more evidence on these preliminary findings, especially in regard to the dosage of VDZ, the duration of treatment, the compatibility with other immunosuppressive agents, and the efficacy on patients without intestinal involvement. However, VDZ might represent a valid option for the treatment of patients with BD who are not responsive to standard treatment or anti-TNF agents.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical approval was not provided for this study on human participants because the study is a case report. Written consensus was retrieved by the patient and the research was run in accordance with the declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MA participated in data collection, interpretation of results, and drafting of the manuscript. MR, DRos, EM, SB, SS, and DRoc participated in data collection, interpretation of results, manuscript preparation, and final review. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Akdeniz N, Elmas ÖF, Karadağ AS. Behçet Syndrome: A Great Imitator. *Clin Dermatol* (2019) 37(3):227–39. doi: 10.1016/j.clindermatol.2019.01.001
2. De Menthon M, LaValley MP, Maldini C, Guillevin L, Mahr A. HLA-B51/B5 and the Risk of Behçet's Disease: A Systematic Review and Meta-Analysis of Case-Control Genetic Association Studies. *Arthritis Care Res* (2009) 61 (10):1287–96. doi: 10.1002/art.24642
3. Leccese P, Alpsoy E. Behçet's Disease: An Overview of Etiopathogenesis. *Front Immunol* (2019) 10:1067(MAY). doi: 10.3389/fimmu.2019.01067
4. Esatoglu SN, Hatemi G. Update on the Treatment of Behçet's Syndrome. *Intern Emerg Med* (2019) 14(5):661–75. doi: 10.1007/s11739-019-02035-1

5. Alpsoy E, Leccese P, Emmi G, Ohno S. Treatment of Behçet's Disease: An Algorithmic Multidisciplinary Approach. *Front Med* (2021) 8:624795. doi: 10.3389/fmed.2021.624795
6. Caso F, Costa L, Rigante D, Lucherini OM, Caso P, Bascherini V, et al. Biological Treatments in Behçet's Disease: Beyond Anti-TNF Therapy. *Mediators Inflamm* (2014) 2014:107421. doi: 10.1155/2014/107421
7. Balzola F, Cullen G, Ho GT, Russell R. Vedolizumab as Induction and Maintenance Therapy for Crohn's Disease: Commentary. *Inflammation Bowel Dis Monit* (2014) 14(2):55–6. doi: 10.1056/NEJMoa1215739
8. Feagan BG, Rutgeerts P, Sands BE, Hanauer S, Colombel JF, Sandborn WJ, et al. Vedolizumab as Induction and Maintenance Therapy for Ulcerative Colitis. *N Engl J Med* (2013) 369(8):699–710. doi: 10.1056/nejmoa1215734
9. Wyant T, Fedyk E, Abhyankar B. An Overview of the Mechanism of Action of the Monoclonal Antibody Vedolizumab. *J Crohn's Colitis* (2016) 10 (12):1437–44. doi: 10.1093/ecco-jcc/jjw092
10. Kim ES, Chung WC, Lee KM, Lee BI, Choi H, Han SW, et al. A Case of Intestinal Behcet's Disease Similar to Crohn's Colitis. *J Korean Med Sci* (2007) 22(5):918–22. doi: 10.3346/jkms.2007.22.5.918
11. C HJ, S MR, R HJ, B HJ. Cross-Cultural Adaptation and Validation of the Behcet's Disease Current Activity Form in Korea. *Korean J Intern Med* (2015) 30(5):714–8. doi: 10.3904/KJIM.2015.30.5.714
12. Davatchi F, Assaad-Khalil S, Calamia KT, Crook JE, Sadeghi-Abdollahi B, Schirmer M, et al. The International Criteria for Behçet's Disease (ICBD): A Collaborative Study of 27 Countries on the Sensitivity and Specificity of the New Criteria. *J Eur Acad Dermatol Venereol* (2014) 28(3):338–47. doi: 10.1111/jdv.12107
13. Bayraktar Y, Özaslan E, Van Thiel DH. Gastrointestinal Manifestations of Behcet's Disease. *J Clin Gastroenterol* (2000) 30(2):144–54. doi: 10.1097/00004836-200003000-00006
14. Akiyama M, Kaneko Y, Takeuchi T. Effectiveness of Tocilizumab in Behcet's Disease: A Systematic Literature Review. *Semin Arthritis Rheumatol* (2020) 50 (4):797–804. doi: 10.1016/j.semarthrit.2020.05.017
15. Atienza-Mateo B, Beltrán E, Hernández-Garfella M, Valls Pascual E, Martínez-Costa L, Atanes A, et al. Tocilizumab in Behçet's Disease With Refractory Ocular and/or Neurological Involvement: Response According to Different Clinical Phenotypes. *Clin Exp Rheumatol* (2021) 132(5):37–42.
16. Fleisher M, Marsal J, Lee SD, Frado LE, Parian A, Korelitz BI, et al. Effects of Vedolizumab Therapy on Extraintestinal Manifestations in Inflammatory Bowel Disease. *Dig Dis Sci* (2018) 63(4):825–33. doi: 10.1007/s10620-018-4971-1
17. Hanzel J, Ma C, Casteele NV, Khanna R, Jairath V, Feagan BG. Vedolizumab and Extraintestinal Manifestations in Inflammatory Bowel Disease. *Drugs* (2021) 81(3):333–47. doi: 10.1007/s40265-020-01460-3
18. Mitroulis I, Alexaki VI, Kourtzelis I, Ziogas A, Hajishengallis G, Chavakis T. Leukocyte Integrins: Role in Leukocyte Recruitment and as Therapeutic Targets in Inflammatory Disease. *Pharmacol Ther* (2015) 147:123–35. doi: 10.1016/j.pharmthera.2014.11.008

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Tetracyclines Diminish *In Vitro* IFN-γ and IL-17-Producing Adaptive and Innate Immune Cells in Multiple Sclerosis

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Introduction: Limited data from clinical trials in multiple sclerosis (MS) reported that minocycline, a widely used antibiotic belonging to the family of tetracyclines (TCs), exerts a beneficial short-lived clinical effect A similar anti-inflammatory effect of minocycline attributed to a deviation from Th1 to Th2 immune response has been reported in experimental models of MS. Whether such an immunomodulatory mechanism is operated in the human disease remains largely unknown.

Aim: To assess the *in vitro* immunomodulatory effect of tetracyclines, and in particular minocycline and doxycycline, in naïve and treated patients with MS.

Material and Methods: Peripheral blood mononuclear cells from 45 individuals (35 MS patients, amongst which 15 naïve patients and 10 healthy controls, HCs) were cultured with minocycline or doxycycline and conventional stimulants (PMA/Ionomycin or IL-12/IL-18). IFN-γ and IL-17 producing T-, NK- and NKT cells were assessed by flow cytometry. The effect of TCs on cell viability and apoptosis was further assessed by flow cytometry with Annexin V staining.

Results: Both tetracyclines significantly decreased, in a dose dependent manner, IFN-γ production in NKT and $CD4^+$ T lymphocytes from MS patients (naïve or treated) stimulated with IL-12/IL-18 but did not decrease IFN-γ producing $CD8^+$ T cells from naive MS or treated RRMS patients. They also decreased IL-17^+ T and NKT cells following PMA and Ionomycin-stimulation. Tetracyclines did not affect the viability of cell subsets.

Conclusion: Tetracyclines can *in vitro* suppress IFN-γ and IL-17- producing cells from MS patients, and this may explain their potential therapeutic effect *in vivo*.

Keywords: multiple sclerosis, doxycycline, minocycline, proinflammatory, interferon-γ, interleukin-17, NKT cells

INTRODUCTION

Multiple Sclerosis (MS) is a chronic autoimmune inflammatory demyelinating disease of the central nervous system (CNS) (1). Based on disease course, patients can be classified into three main categories, relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS) (2). The autoimmune pathogenesis of MS is well established by translational research in patients with MS and studies based on experimental autoimmune encephalomyelitis (EAE), a model of MS (3, 4).

The activation of myelin-specific T cells followed by dysregulation of Th1, Th2 and Th17 cytokines is believed to be a major event during the initiation phase of MS (5). $CD4^+$ Th1 cells produce inflammatory mediators, such as interferon-γ (IFN-γ) which lead to autoantigen-specific inflammatory attack resulting in myelin degeneration (6, 7). IL-12 and IL-18, which can synergistically induce high levels of IFN-γ expression (8), are found elevated in MS and correlate with disease activity (9, 10). In addition to Th1 cells, it is now believed that pro-inflammatory Th17 and other IFN-γ and IL-17 producing cells, such as Th17, NK and NKT cells are also significantly involved in the initiation and perpetuation of the disease (11–13). Sharp reduction of CNS-penetrating IFN-γ and IL-17 producing cells is directly linked with disease prevention or disease remission in patients with MS (14, 15).

Current therapeutic agents in MS mainly affect pro-inflammatory cytokine production (15, 16), but they lack desirable efficacy (17–19).

In addition to their well-defined antimicrobial-bacteriostatic activity, tetracyclines (TCs) (20, 21), such as minocycline and doxycycline, demonstrate neuroprotective, anti-apoptotic, anti-inflammatory and immunomodulatory properties (22–25), and have been clinically tested with encouraging results in autoimmune diseases, such as rheumatoid arthritis and MS (26–29). For example, in a recent randomized, controlled clinical trial, conversion from a clinically isolated syndrome to MS at six months was significantly lower in the minocycline compared to placebo group, but this effect was short-lived (27). Other trials failed to report an efficacy of TCs in MS (30), contradicting data from animals whereby minocycline dramatically suppresses ongoing disease activity and remarkably limits EAE disease progression (31–33). Amongst the mechanisms involved in their immunomodulatory action are inhibition on IFN-γ and TNF-α production by T-lymphocytes or monocytes (34–37). An anti-inflammatory effect of minocycline has been reported in EAE when combined with other treatments such as IFN-β, steroids, glatiramer acetate, and atorvastatin (32, 38–40). Also, Popovic et al. reported a dramatic suppression of disease activity in EAE by minocycline and a deviation of MOG-specific T cell response towards a Th2-like response (31).

The precise cellular source and mechanism of cytokine modulation by TCs in MS patients, remains largely unknown and studies conducted so far limited their assessment in particular cell-subsets, selecting either TC but not both. The aim of our study was to comparatively investigate for the first time the *in vitro* effect of both minocycline and doxycycline on IFN-γ and IL-17 production by peripheral cell populations in patients with MS (newly-diagnosed naive and treated RRMS).

Abbreviations: CNS, Central Nervous System; EAE, Experimental Autoimmune encephalomyelitis; FCS, Fetal Calf Serum; MoAb, Monoclonal Antibody; MS, Multiple Sclerosis; PMA, Phorbol Myristate Acetate; TC, Tetracycline.

PATIENTS AND METHODS

Patients and Healthy Controls

A total of 35 patients with MS diagnosed according to the McDonald criteria (41) were included in the study, including 15 naïve (12 females, mean age of 41.4 ± 13.4 years, range 19-59) and 20 RRMS (13 females, mean age of 40.9 ± 11.4 years, range 20-61) with a mean disease duration of (11.2 ± 8.8 years, range 1-33). All patients attended the Outpatient Clinics of the Neurology Department, of the University General Hospital of Larissa, Greece. RRMS patients (9 at relapse) were on standard treatment (glatiramer acetate, n=3; natalizumab, n=6; fingolimod, n=5; fampridine, n=1; and IFN-β n=5). Blood collection of all naive MS patients was performed before initiation of corticosteroid or other drug treatment. Ten age and sex-matched healthy individuals (7 females, mean age of 37.3 ± 10.4 years) were included as healthy controls (HCs). Patients and healthy HCs had not received TCs for at least three months before blood collection.

All experimental protocols were approved by the Local Institution's Ethical Committee of the University General Hospital of Larissa, University of Thessaly while written informed consent has been obtained from all study participants according to the declaration of Helsinki.

PBMC Isolation and Cryopreservation

Peripheral blood samples (20-30mL) from MS patients and HCs were collected by venipuncture in preservative-free heparin tubes (50 U/mL) and aliquots were layered onto an equal volume of Ficoll-Hypaque (10ml Lymphoprep™) density gradient solution (Axis-Shield, Oslo, Norway). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation at 300g, washed twice with RPMI-1640 (GIBCO™ -Thermo Fisher Scientific, Waltham, MA, USA), counted, and their viability, determined by trypan blue exclusion, routinely exceeded 95%. Cells were re-suspended in freezing medium containing 10% DMSO and 70% fetal calf serum (FCS), aliquoted into cryogenic vials (Corning™, Thermo Fisher Scientific), kept at −80°C for one day, and then stored in liquid nitrogen tanks until used.

Reagents

TCs (minocycline hydrochloride and doxycycline hyclate, ≥ 98% pure) were purchased from Cayman Chemical Company, Michigan USA. Both antibiotics were reconstituted in DMSO, further aliquoted at small volumes and stored at a final concentration of 5 mg/ml. TCs were supplemented simultaneously with cell stimuli (see below) at a final concentration of 50μg/ml unless otherwise stated and remained in culture for 5 hours (see *Results* section).

DMSO concentration in TC-supplemented cultures was less than 0.1%. The same DMSO concentration (0.1%) was also added to control cultures in the absence of TCs, to exclude a direct cytotoxic effect of DMSO. Recombinant IL-12 and IL-18 were purchased from R and D Systems Inc (Abington, UK) and used at a final concentration of 20 ng/ml and 25 ng/ml, respectively (8). Phorbol 12-myristate 13-acetate (PMA) and Ionomycin were obtained from Sigma-Aldrich-Merck (Gillingham, UK) and were used at 20-50 ng/ml, and 1μg/ml, respectively in order to conventionally stimulate PBMCs in a non-specific manner (42).

Phenotypic Analysis of Peripheral Blood T and Innate Cells by Flow Cytometry

Phenotypic assessment and enumeration of peripheral blood subpopulations was performed using standard monoclonal antibodies (MoAbs) panels and protocols as described previously (43, 44). Briefly, upon thawing PBMCs were washed with serum-free RPMI-1640, counted to confirm more than 95% cell viability, pelleted, and re-suspended at 10^6 cells/mL in RPMI culture medium supplemented with L-glutamine and 10% heat-inactivated FCS (Biosera Europe, Nuaille, France). PBMCs were seeded in 24-well plates and allowed to rest at 37°C in a CO^2 incubator for at least one hour before stimulation. In this study we used the following mouse anti-human MoAbs: (FITC)-conjugated anti-CD3 (clone UCHT-1), (PE)- and (PE-Cy7)-conjugated anti-CD56 (clones C5.9 and B159), (PE)- and (PE-Cy7)-conjugated anti-CD4 (clone RPA-T4) and PE conjugated anti-CD8 (clone SK1). All MoAbs were obtained from BD Biosciences (Mountain View, CA, USA) and Merck-Millipore (Burlington, MA, USA). Isolated PBMCs (0.5-1 x 10^6cells) were washed in phosphate-buffered saline (PBS) and re-suspended in staining buffer (PBS plus 1% FCS plus 0.09% sodium azide) and then incubated with labeled MoAbs specific for cell surface antigens for 30 minutes on ice and fixed with paraformaldehyde (2%). Background auto-fluorescence was monitored by equivalent 4-colour matched isotype control mouse anti-human MoAbs and formed the basis to set the cut-off for surface-positive cell discrimination. Flow cytometric analysis was performed in Guava® EasyCyte8 (Merck-Millipore, Burlington, MA, USA) benchtop flow cytometer using logarithmic amplification and a forward and side scatter-based gate for total lymphocyte populations. At least $3x10^5$ events within the lymphocyte gate were collected for accurate measurement of infrequent cell subtypes.

Flow Cytometric Analysis of Apoptotic Cells by Annexin V

IL-12 and IL-18 activated PBMCs supplemented with 50μg/ml minocycline or doxycycline were collected and stained with FITC-labeled Annexin V (BioLegend, San Diego, CA, USA), which is used to specifically target and identify apoptotic cells (45), in the presence of Annexin V binding buffer according to manufacturer's instructions. Experiments in the absence of IL-12 and IL-18 were also performed as controls. Lymphocyte subsets were identified using fluorescent labeled mAbs directed against lymphocyte surface markers (section 2.4) and subjected to conventional FACS analysis.

Intracellular IFN-γ Production by Peripheral Blood Cell Subsets

In order to measure intracellular IFN-γ protein production by peripheral T cells and innate NK and NKT cells, PBMCs were left untreated, or cultured in 10% RPMI supplemented with 20 ng/ml PMA plus 1μg/ml ionomycin for 5 hours in the presence of Brefeldin A (GolgiPlug™, BD Biosciences). Cells were surface stained, fixed and subsequently permeabilized using commercially available Perm/Wash buffers (BD Biosciences). Intracellular IFN-γ protein was detected using APC-conjugated MoAbs (clone 4S.B3) obtained from BD Biosciences.

Intracellular IL-17 Production by Th17 Cells

In order to measure intracellular IL-17 protein production by peripheral Th17 cells, PBMCs were left untreated or cultured in 10% RPMI supplemented with 50 ng/ml PMA plus 1μg/ml ionomycin for 5 hours in the presence of Brefeldin A. Cells were surface stained, fixed and subsequently permeabilized using commercially available Perm/Wash buffers (BD Biosciences). Intracellular IL-17 was detected using FITC- and PE-conjugated MoAbs clones (BL-168) all obtained from BD Biosciences and Merck-Millipore.

Statistical Analysis

Percentages of cells expressing cell surface markers and mean fluorescence intensities (MFI) were described as median of the individuals in each group. Variation in each patient group was defined by standard deviation (SD). Differences between healthy controls and patients and between patient groups one-way analysis of variance (ANOVA) and the nonparametric Mann-Whitney test. P-values smaller than 0.05 were considered significant. All graphs and statistical calculations were performed with Graph Pad Prism 9 software.

RESULTS

Tetracyclines Decreased IFN-γ Producing Cells in a Dose-Dependent Manner

To assess the *in vitro* effect of either minocycline or doxycycline on IFN-γ production, antibiotics in isolation were supplemented in PBMC cell cultures at different concentrations ranging from of 0.1μg - 50μg/ml, similarly to previous reports in whole blood cultures and THP-1 human monocytes (46, 47). These concentrations are within the *in vivo* pharmacological plasma concentration levels noted following oral tetracycline administration; in human subjects who have taken oral doxycycline (200mg), doxycycline plasma concentrations (Cmax) of 1.5 to 7.0 μg/ml were usually reached within 3 h, and the drug had a half-life of 14 to 24 h (48, 49). Also, such TCs concentrations were administered in accordance with *in vivo* studies of experimental endotoxemia where doxycycline and other TCs are efficacious in downregulating inflammatory cytokines and preventing shock when the drug was injected immediately following the LPS challenge (50).

IFN-γ production was measured following stimulation with IL-12 plus IL-18 or PMA plus ionomycin for 5 hours on the basis of TCs optimal Cmax and limited half-life *in vivo*, as well as our previous published data, where both stimuli induce kinase (p38 MAPK) activation and robust IFN-γ production within 2-6hrs post stimulation in innate and adaptive cells (8).

Supplementary Figures 1, **2** show cumulative data from TC dose-response experiments in MS patients (n=3) and HCs (n=3). Minocycline or doxycycline decreased IFN-γ production by innate and adaptive T cells in a dose-dependent manner when IL-12 plus IL-18 was used for cell stimulation. Both drugs clearly inhibited cytokine expression in $CD4^+$ T cells and NKT cells at pharmacological concentrations (1μg/ml-10μg/ml). However, the maximal inhibitory effect was noted when IL-12 plus IL-18 and 50μg/ml minocycline or doxycycline were used (see below). In all tested concentrations there was no detrimental effect of TCs on cell viability, as assessed by microscopic evaluation and trypan blue exclusion in line with previously published observations (47). The effect of TCs on cell viability was further assessed using Annexin V staining. As shown in **Supplementary Figures 3**, **4** neither antibiotic exerted a toxicity effect influencing the viability, apoptosis and percentages of any cell subset at the maximal concentration (50μg/ml). Neither minocycline nor doxycycline exerted an inhibitory effect on IFN-γ producing $CD3^+$ and non $CD3^+$ cell subsets from naive MS and RRMS patients, after stimulation with PMA and Ionomycin (**Supplementary Figures 2**, **6**). There was a marginal statistically significant decrease in IFN-γ^+ NKT ($CD3^+CD56^+$) cells following treatment with doxycycline ($p = 0.04$, $n = 9$) (**Supplementary Figure 7**). Thus, all subsequent experiments were performed using IL-12 plus IL-18 and minocycline or doxycycline at 50μg/ml.

Tetracyclines Decreased IFN-γ Producing $CD4^+$ T Cells in MS

The flow cytometric gating strategy followed for phenotypic analysis of different cell subsets, including $CD4^+$ T cells, is shown at **Supplementary Figures 3**, **5**. In general, we observed a statistically significant reduction in IFN-γ producing $CD4^+$ T cells from both MS patients and HCs in the presence of minocycline or doxycycline. **Figure 1** illustrates representative cases and cumulative data. The percentage of IFN-γ^+ $CD4^+$ T lymphocytes following IL-12 plus IL-18 stimulation in naïve MS patients (n=15) decreased from 1.1 ± 0.41% to 0.53 ± 0.21% in the presence of minocycline ($p = 0.004$) and to 0.42 ± 0.20% in the presence of doxycycline ($p = 0.001$). The percentage of IFN-γ^+ $CD4^+$ T cells in RRMS patients (n=20) decreased from 0.89 ± 0.43% to 0.53 ± 0.27% in the presence of minocycline ($p = 0.009$) and to 0.44 ± 0.22% in the presence of doxycycline ($p = 0.002$). In HCs (n=10), the percentage of IFN-γ^+ $CD4^+$ T lymphocytes was

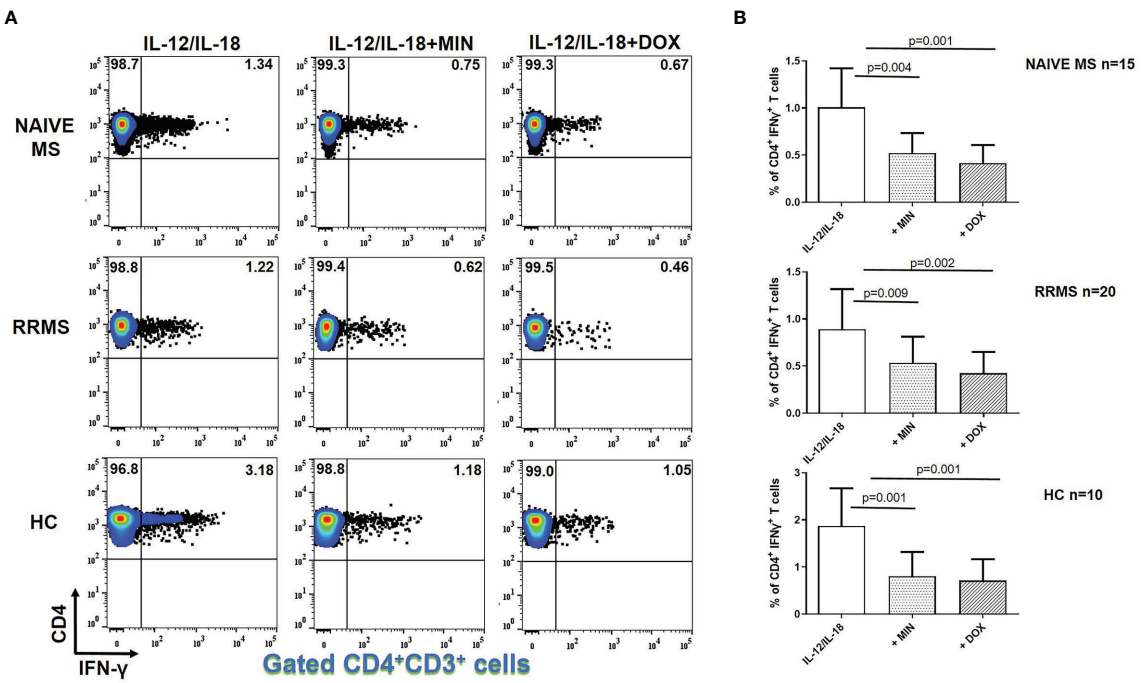


FIGURE 1 | Tetracycline-mediated inhibition of $CD4^+$ IFNγ$^+$ T cells. PBMCs from naïve MS patients (n=15), RRMS patients (n=20) and HCs (n=10) were seeded in cell culture plates (1 x 10^6 per well) and stimulated with IL-12 plus IL-18 (IL-12/IL-18), IL-12/IL-18 plus minocycline (MIN) and IL-12/IL-18 plus doxycycline (DOX) for 5h. Cells were collected, washed, surface stained with appropriate monoclonal antibodies an analyzed for intracellular IFN-γ production by flow cytometry (see also methods section). Individual cell subsets were sub-gated according to expression of CD3 and CD4 surface epitopes. **(A)** Flow cytometry dot-plots showing the frequency of $CD4^+$IFNγ$^+$ T cells in IL-12/IL-18, IL-12/IL-18/MIN and IL-12/IL-18/DOX treated cells from representative MS cases and HCs. **(B)** Box and whiskers graphical representation showing significant reduction in the percentages of IFN-γ-producing $CD4^+$ T cells in the presence of tetracyclines in naïve MS, RRMS and HCs.

reduced from 1.87 ± 0.79% to 0.80 ± 0.51% in the presence of minocycline (p = 0.0015) and to 0.71 ± 0.45% in the presence of doxycycline (p = 0.001) (**Figure 1**).

Minocycline and doxycycline did not decrease IFN-γ producing $CD8^+$ T cells from naive MS (n=15) or RRMS patients (n=20) (**Figure 2**). However, in HCs (n=10), the percentage of IFN-γ^+CD8^+ T lymphocytes was reduced from 3.43 ± 0.45% to 2.18 ± 0.78% in the presence of minocycline (p = 0.001) and to 2.10 ± 0.75% following doxycycline supplementation (p = 0.001) (**Figure 2**).

Tetracyclines Decreased IFN-γ Producing NKT Cells in MS

IFN-γ^+ NKT lymphocytes following IL-12 plus IL-18 stimulation in naïve MS (n=15) patients decreased from 6.49 ± 4.10% to 3.61 ± 2.39% in the presence of minocycline (p = 0.02) and to 2.52 ± 1.87% in the presence of doxycycline (p = 0.003) (**Figure 3**). The percentage of IFN-γ^+ NKT cells in RRMS patients (n=20) decreased from 7.34 ± 4.40% to 3.92 ± 2.51% in the presence of minocycline (p = 0.01) and to 2.40 ± 1.89% in the presence of doxycycline (p = 0.001). In HCs (n=10), the percentage of IFN-γ^+ NKT lymphocytes was reduced from 14.73 ± 7.53% to 7.35 ± 4.27% following minocycline supplementation (p = 0.03) and to 5.41 ± 3.07% following doxycycline supplementation (p = 0.005).

Tetracyclines Did Not Affect IFN-γ Producing NK Cells in MS

In IL-12 and IL-18- stimulated NK cells, there was no difference in IFN-γ^+ cells in the presence of minocycline or doxycycline in naïve MS (n=15), RRMS (n=20) or HCs (n=10) (**Figure 4**).

Tetracyclines Decreased IL-17A Producing $CD4^+$ T Cell and NKT Cells in MS

We also investigated the effect of TCs on IL-17A production in PMA plus ionomycin activated PBMCs from patients with naïve MS (n=9), RRMS patients (n=9) and HCs (n=9). As shown in **Figure 5**, TCs decreased IL-17 production from CD4-expressing cells. These consisted of $CD3^+CD4^+$ T cells and $CD56^+CD4^+$ NKT cells. In patients with naïve MS the percentage of $CD4^+$IL-$17A^+$ T cells decreased from 1.77 ± 1.33% to 0.74 ± 0.43% in the presence of minocycline (p = 0.02) and to 0.55 ± 0.44% in the presence of doxycycline (p = 0.01) (**Figure 5**). In RRMS patients the percentage of $CD4^+$IL-$17A^+$ T cells decreased from 2.14 ± 1.68% to 1.2 ± 0.9% in the presence of minocycline (p = 0.04) and to 0.76 ± 0.71% in the presence of doxycycline (p = 0.02). In HCs the percentage of CD4+IL-17A+ T cells decreased from 1.35 ± 0.51% to 0.64 ± 0.35% in the presence of minocycline (p = 0.01) and to 0.43 ± 0.29% in the presence of doxycycline (p = 0.02).

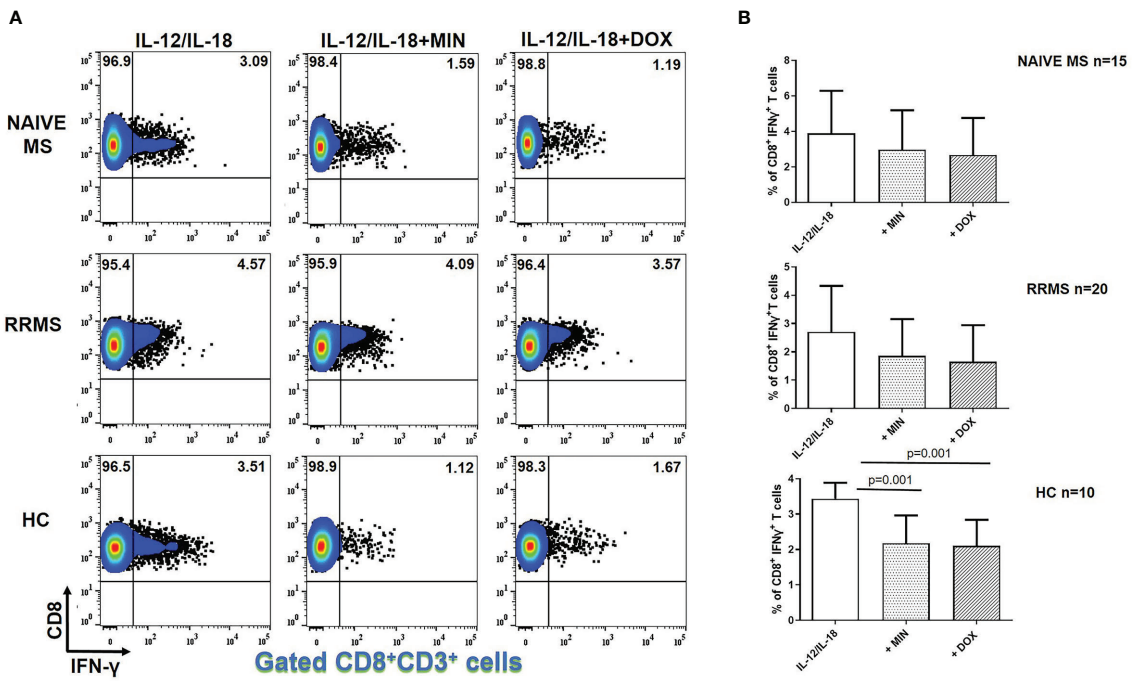


FIGURE 2 | Tetracycline-mediated inhibition of $CD8^+$ IFNγ^+ T cells. PBMCs from naïve MS patients (n=15), RRMS patients (n=20) and HCs (n=10) were seeded in cell culture plates (1 x 10^6 per well) and stimulated with IL-12 plus IL-18 (IL-12/IL-18), IL-12/IL-18 plus minocycline (MIN) and IL-12/IL-18 plus doxycycline (DOX) for 5h. Cells were collected, washed, surface stained with appropriate monoclonal antibodies an analyzed for intracellular IFN-γ production by flow cytometry (see also methods section). Individual cell subsets were sub-gated according to expression of CD3 and CD8 surface epitopes. **(A)** Flow cytometry dot-plots showing the frequency of $CD8^+$IFNγ^+ T cells in IL-12/IL-18, IL-12/IL-18/MIN and IL-12/IL-18/DOX treated cells from representative MS cases and HCs. **(B)** Box and whiskers graphical representation showing significant reduction in the percentages of IFNγ-producing $CD8^+$ T cells in the presence of tetracyclines in HCs but not naïve MS and RRMS patients.

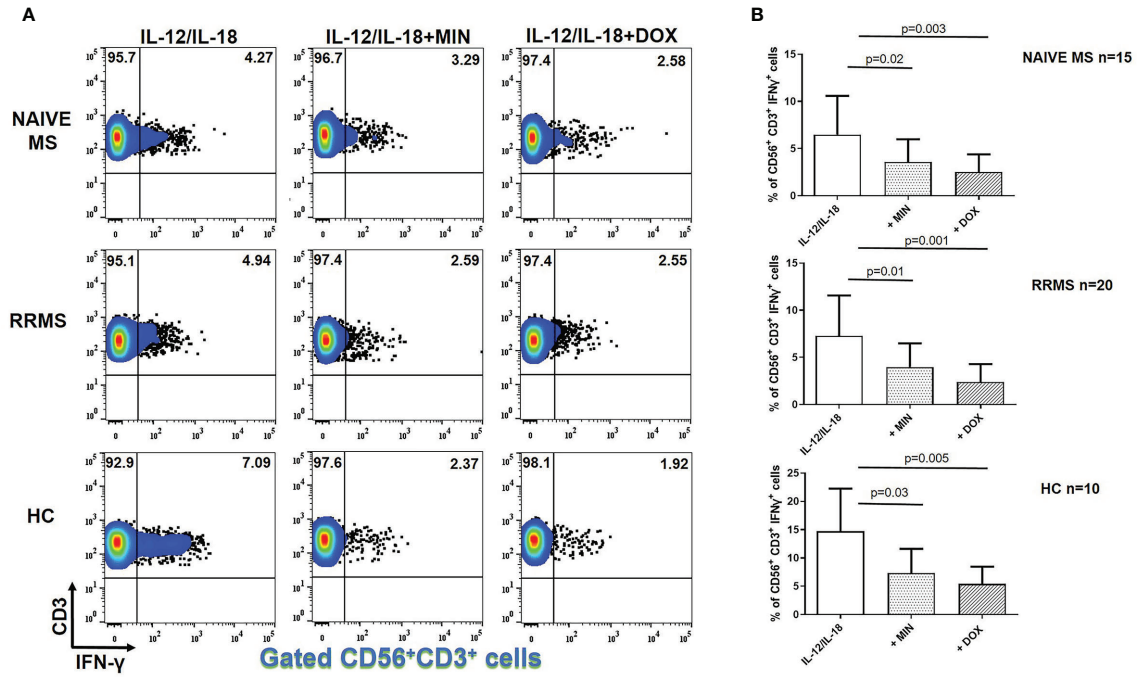


FIGURE 3 | Tetracycline-mediated inhibition of $CD56^+CD3^+IFN\text{-}\gamma^+$ T cells. PBMCs from naïve MS patients (n=15), RRMS patients (n=20) and HCs (n=10) were seeded in cell culture plates (1 x 10^6 per well) and stimulated with IL-12 plus IL-18 (IL-12/IL-18), IL-12/IL-18 plus minocycline (MIN) and IL-12/IL-18 plus doxycycline (DOX) for 5h. Cells were collected, washed, surface stained with appropriate monoclonal antibodies an analyzed for intracellular IFN-γ production by flow cytometry (see also methods section). Individual cell subsets were sub-gated according to expression of CD3 and CD56 surface epitopes. **(A)** Flow cytometry dot-plots showing the frequency of $IFN\text{-}\gamma^+$ NKT cells (gated $CD56^+CD3^+$ cells two-dimensionally depicted in CD3 *vs* IFN-γ plots) in IL-12/IL-18, IL-12/IL-18/MIN and IL-12/IL-18/DOX treated cells from representative MS cases. **(B)** Box and whiskers graphical representation showing significant reduction in the percentages of IFN-γ-producing NKT cells in the presence of tetracyclines in naïve MS, RRMS and HCs.

The percentage of $CD4^+IL\text{-}17A^+$ NKT lymphocytes in naïve MS patients (n=9) decreased from 1.49 ± 0.80% to 0.69 ± 0.33% in the presence of minocycline (p = 0.008) and to 0.48 ± 0.17% in the presence of doxycycline (p = 0.001). The percentage of $CD4^+IL\text{-}17A^+$ NKT lymphocytes in RRMS patients (n=9) decreased from 2.59 ± 0.90% to 1.09 ± 0.43% in the presence of minocycline (p = 0.009) and to 0.8 ± 0.55% in the presence of doxycycline (p = 0.006). The percentage of $CD4^+IL\text{-}17A^+$ NKT lymphocytes in HCs (n=9) decreased from 1.63 ± 0.75% to 0.87 ± 0.54% in the presence of minocycline (p = 0.04) and to 0.84 ± 0.64% in the presence of doxycycline (p = 0.04).

DISCUSSION

The major finding of our study is that of an inhibitory effect of minocycline and doxycycline on IFN-γ and IL-17 producing cells in a cell subset-related, dose-dependent manner in patients with MS, with the most prominent effect being noted in $CD4^+$ T and NKT lymphocytes.

Our results are of importance for studies conducted in humans, as previous studies in MS have only been limited to EAE, with their findings emphasizing an anti-inflammatory role of antibiotics and in particular minocycline, in synergy with prednisone (51), IFN-β1 (52) glatiramer acetate (38) or atorvastatin (39). While studies in EAE showed that minocycline attenuates the disease by reducing T cell infiltration into the spinal cord without affecting the cytokine production profile (33), our human data clearly demonstrate significant reduction of IFN-γ (and/or IL-17) by both T cells and NK/NKT cells, the inhibition varying amongst different cell types. No clear data on the effect of doxycycline on pro-inflammatory cytokine production in EAE currently exist, though our human data support the notion that this antibiotic also diminishes the ability of adaptive and innate immunity cells to produce IFN-γ and IL-17. In support, doxycycline decreased inflammatory infiltration of T-cell, B-cell and macrophage infiltration, sharply diminished IL-17 production and attenuated demyelination in sciatic nerves of rats with experimental autoimmune neuritis, a model of human inflammatory demyelinating polyneuropathies (53).

In our cohort of patients with MS, a comparable TC-mediated reduction of cytokine production was noted between naïve and treated RRMS patients. Subsequent stratified analysis amongst patients receiving different therapies revealed no statistically significant differences regarding the levels of TC-mediated cytokine inhibition within $CD4^+$ T and NKT cells. It remains

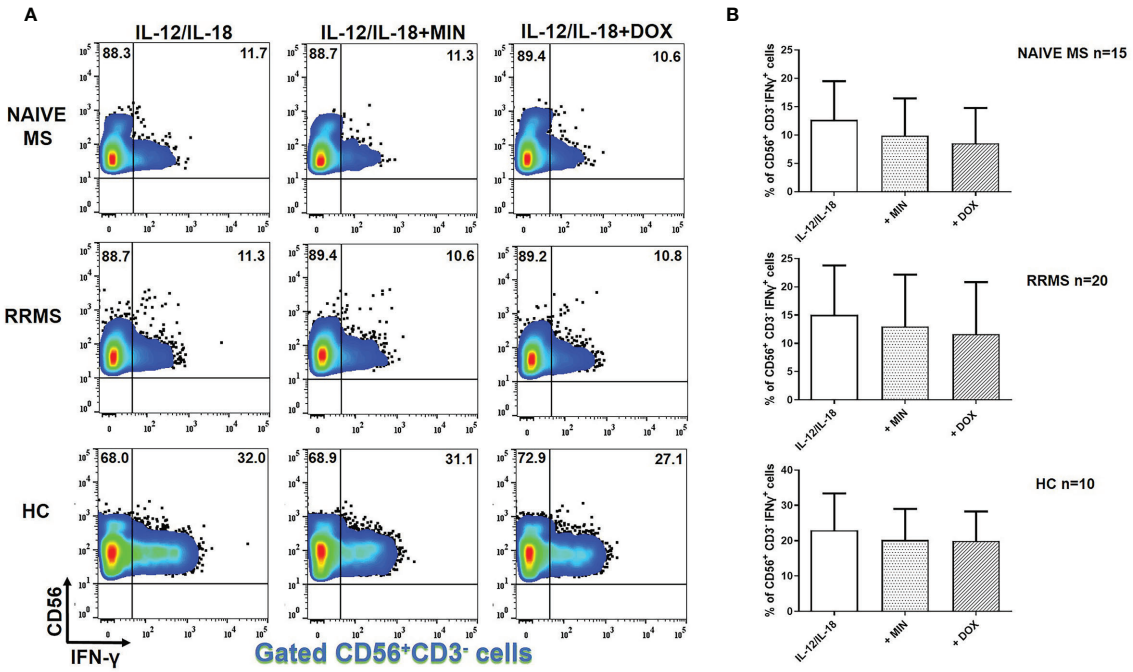


FIGURE 4 | Tetracyclines have no significant effect on IFN-γ$^+$ NK cells. PBMCs from naïve MS patients (n=15), RRMS patients (n=20) and HCs (n=10) were seeded in cell culture plates (1 x 10^6 per well) and stimulated with IL-12 plus IL-18 (IL-12/IL-18), IL-12/IL-18 plus minocycline (MIN) and IL-12/IL-18 plus doxycycline (DOX) for 5h. Cells were collected, washed, surface stained with appropriate monoclonal antibodies an analyzed for intracellular IFN-γ production by flow cytometry (see also methods section). Individual cell subsets were sub-gated according to expression of CD3 and CD56 surface epitopes. **(A)** Flow cytometry dot-plots showing the frequency of CD56$^+$CD3$^-$IFNγ$^+$ NK cells (gated CD56$^+$CD3$^-$ cells two-dimensionally depicted in CD56 *vs* IFN-γ plots) in IL-12/IL-18, IL-12/IL-18/MIN and IL-12/IL-18/DOX treated cells from representative MS cases. **(B)** Box and whiskers graphical representation show no significant reduction in the percentages of IFN-γ-producing NK cells in the presence of tetracyclines in either HCs or MS patients.

to be seen whether the *in vitro* effect of TC could be augmented following supplementation of MS-related immunomodulatory agents and whether this effect could be seen *in vivo*.

Clinical trials using minocycline, alone or in combination with conventional therapeutic agents, have produced inconclusive results, some demonstrating beneficial effect on the risk of conversion from a clinically isolated syndrome to MS (27), while others failed to show a superiority of minocycline when added to IFN β-1a (30).

Those studies by no means can de-emphasize the importance of our *in vitro* study. In rheumatoid arthritis, a typical autoimmune disease, both experimental and human data reveal a beneficial effect of TCs in combination with methotrexate over methotrexate alone, especially in early disease state, suggesting that timing of initiation of TCs may be of importance (54, 55). This effect is clearly attributed to the dose-dependent inhibition of T cell proliferation and reduction of IFN-γ and other pro-inflammatory cytokines (56), a finding that perfectly fits with those obtained in our study.

The inhibitory effect of antibiotics on IFN-γ was noted after the physiological stimulation of PBMC with IL-12 and IL-18 but not with PMA and ionomycin, a phenomenon, which requires further discussion. Previous work has shown that the effect of minocycline on cytokine production by T-cells and monocytes is stimulus specific, as T cells stimulated by a Ca^2+-independent manner exhibited a decrease in TNF-alpha mRNA in the presence of minocycline, whereas the TNF-alpha mRNA level remained unaffected by minocycline when cells were stimulated in a Ca^2+-dependent manner, like in the case of PMA/ionomycin stimulation (36, 56). The limitation of the *in vitro* studies, even if those are conducted in patients with MS and not in animal models of MS, cannot be ignored. While no *in vivo* studies have looked at the effect of TCs in IFN-γ or IL-17 producing T and NK cell-subsets in patients with MS, a study in EAE has provided data of interest. An early report by Popovic et al. (31) found that minocycline administration suppressed migration of inflammatory cells into CNS and further activation by a direct effect on the cytokine milieu in EAE. Treatment with minocycline shifted the balance from Th-1 to Th-2 and resulted in enhanced IL-10, reduced TNF-α and a minimal effect on IFN-γ production, as measured by ELISA in cell-culture supernatants (31). The investigation of cell-subset T cell specific cytokine production was not included in the aims of that study. Those latter data further emphasize the need for a vigorous attempt to assess the *in vivo* the effect of TCs on T-cell activation and cell-subset specific pro- and anti-inflammatory cytokine production in patients with MS in well-designed clinical trials.

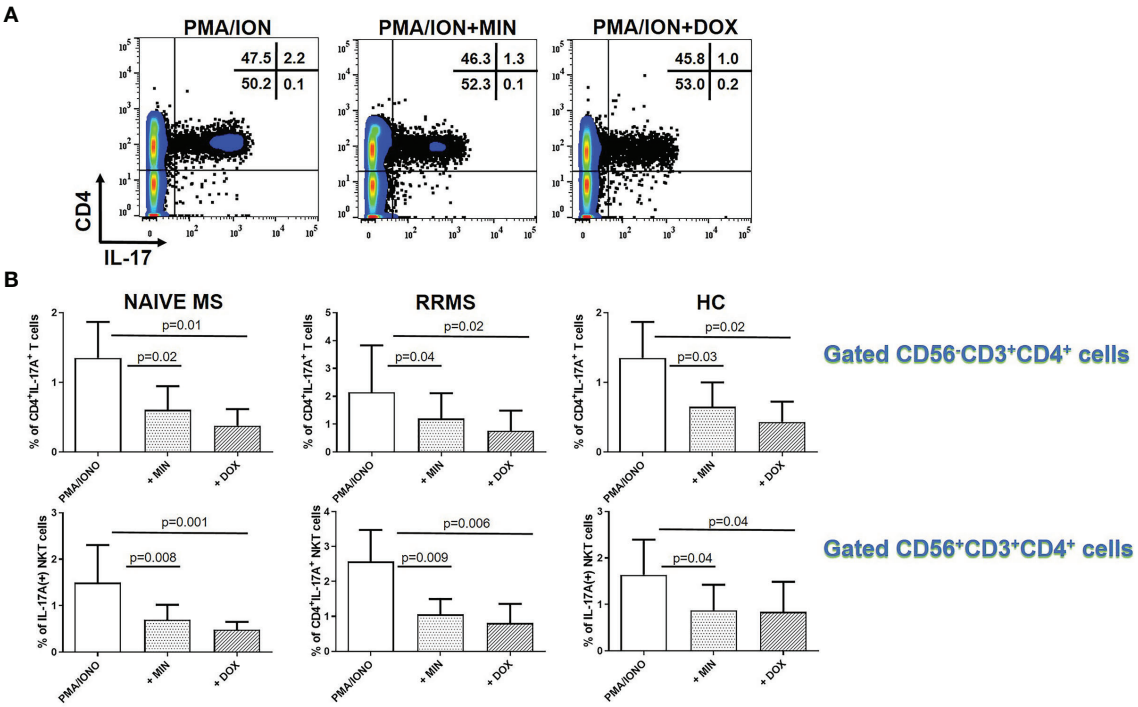


FIGURE 5 | Tetracycline-mediated inhibition of $CD4^+CD3^+IL\text{-}17A^+$ T cells and $CD56^+CD4^+IL\text{-}17A^+$ T (NKT) cells. PBMCs from naïve MS patients (n=9), RRMS patients (n=9) and HCs (n=9) were seeded in cell culture plates (1 x 10^6 per well) and stimulated with PMA plus ionomycin (PMA/ION), PMA/ION plus minocycline (MIN) and PMA/ION plus doxycycline (DOX) for 5h. Cells were collected, washed, surface stained with appropriate monoclonal antibodies an analyzed for intracellular IL-17A production by flow cytometry (see also methods section). **(A)** Flow cytometry dot-plots showing the frequency of total $CD4^+IL\text{-}17A^+$ cells in PMA/IONO, PMA/IONO/MIN and PMA/IONO/DOX treated cells from a representative naïve MS case. **(B)** $CD4^+IL\text{-}17A^+$ cells were sub-divided according to expression of CD3 or CD56 surface epitopes. Box and whiskers graphical representation showing significant reduction in the percentages of IL-17A-producing T and NKT cells in the presence of tetracyclines in naïve MS patients, RRMS patients and HCs.

CONCLUSION

In conclusion, this is the first *in vitro* study to show that minocycline and doxycycline have a significant inhibitory effect in $CD4^+$ T and NKT cell producing IFN-γ and/or IL-17 in patients with MS (naïve or under treatment). If this is proved *in vivo* in prospectively collected biological material from MS patients under treatment with these tetracyclines, it may have potential clinical relevance. *In vivo* inhibition of IFN-γ (and possibly IL-17)-producing NKT cells, for example, is a favorable pre-requisite for successful remission in patients with MS (57–59), and the effect of tetracyclines towards achieving this goal may provide an additional therapeutic tool, most likely in combination with standard treatment regimen in stratified cohorts of patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics and Scientific Committee, University General Hospital of Larissa, University of Thessaly, Faculty of Medicine, School of Health Sciences. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DF, AM, and ED contributed equally. LS, GH, and DB had the original idea. AM, ED, LS, GH and DB scripted considerable part of original draft. DF, AM, ED, CK, LS, and DB had performed extensive literature search and scripted relevant parts of the manuscript. DF, ED, VT, AA, VS, CT, and GH collected clinical and biological material and clinical data. DF, AM and CL analyzed the data. DF and DB prepared the artwork. DB, DF, and AM had edited the final version of the manuscript. DB and ED provided overall supervision. All authors performed extensive literature search and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.739186/full#supplementary-material

Supplementary Figure 1 | IFN-γ producing PBMC subsets following stimulation with IL-12 plus IL-18 in the presence of different doses of tetracyclines. The *in vitro* effect of either minocycline or doxycycline supplemented at different concentrations ranging from of 0.1μg - 50μg/ml on IFN-γ production was assessed in PBMC cell cultures of MS patients (n=3) and HCs (n=3) following stimulation with IL-12 plus IL-18 for 5 hours (Box and whiskers graphical representation of cumulative data with standard deviation errors bars are shown). *P < 0.05 **P < 0.005 ***P < 0.0005.

Supplementary Figure 2 | IFN-γ producing PBMC subsets following stimulation with PMA plus ionomycin in the presence of different doses of tetracyclines. The *in vitro* effect of either minocycline or doxycycline supplemented at different concentrations ranging from of 0.1μg - 50μg/ml on IFN-γ production was assessed in PBMC cell cultures of MS patients (n=3) and HCs (n=3) following stimulation with PMA plus ionomycin for 5 hours. (Box and whiskers graphical representation of cumulative data with standard deviation errors bars are shown). Data show a dose-depending inhibition only in IFN-γ producing NKT cells *P < 0.05 (see also **Supplementary Figure 7**).

Supplementary Figure 3 | Tetracyclines do not affect PBMC flow cytometric forward/side scatter characteristics and proportions of sub-gated cell subsets. Representative flow cytometric assessment (MS case) of the *in vitro* effect of either minocycline or doxycycline supplemented at the highest concentration (50μg/ml) on PBMC cell viability and on cytometric analysis characteristics based on size/granularity (FSC/SSC) and phenotypic discrimination of the proportions of individual cell subset.

Supplementary Figure 4 | Tetracyclines do not induce apoptosis of cell subsets. Representative flow cytometric illustration (MS case) of the *in vitro* effect of either minocycline or doxycycline supplemented at the highest concentration (50μg/ml) on apoptosis of sub-gated cell subpopulations of CD4 and CD8 T cells assessed by annexin V staining. Data are suggestive of lack of an *in vitro* effect of tetracyclines in inducting apoptosis and affecting viability.

Supplementary Figure 5 | Representative flow cytometric gating strategy of tetracycline treated IL-12 plus IL-18 stimulated PBMC subsets. Individual cell subsets from a representative MS case were sub-gated according to expression of CD3, CD4, CD8 and CD56 surface markers. All surface epitopes were sufficiently maintained and detected following cell activation with IL-12 plus IL-18 in the presence of minocycline and doxycycline.

Supplementary Figure 6 | Flow cytometric illustration of tetracycline-mediated effects on IFNγ production by PMA/ionomycin and IL-12 plus IL-18 stimulated $CD3^+$ and non-$CD3^+$ cell subsets from MS patients. Data of the effect of tetracyclines using different stimuli in a representative naïve MS case are shown in A and B and in a representative RRMS are shown in C and D. PBMCs were analyzed for IFN-γ production by flow cytometry following minocycline (MIN) or doxycycline (DOX) supplementation (50μg/ml) and simultaneous treatment with PMA plus ionomycin **(A, C)** or IL-12 plus IL-18 stimulation **(B, D)**. $CD3^+$ and non-$CD3^+$ cell subsets were sub-gated according to expression of CD3 surface epitope.

Supplementary Figure 7 | Doxycycline-mediated inhibition of $CD56^+CD3^+IFN\gamma^+$ T cells subsets following stimulation with PMA plus ionomycin. PBMCs from naïve MS patients (n=9) were analyzed for IFN-γ production by flow cytometry following minocycline (MIN) or doxycycline (DOX) supplementation and simultaneous PMA plus ionomycin stimulation (see also methods section). NKT cells were sub-gated according to expression of CD3 and CD56 surface markers. **(A)** CD3 versus IFN-γ Flow cytometry dot-plots in sub-gated NKT cells showing the frequency of IFN-γ^+ NKTs following PMA/IONO, PMA/IONO/MIN and PMA/IONO/DOX treatment. **(B)** Box and whiskers graphical representation showing significant reduction in the percentages of IFN-γ-producing NKT cells in the presence of doxycycline in naïve MS.

Supplementary Figure 8 | $CD4^+$IFN-γ^+ cells represent T helper 1 (TH1) cells and not other $CD4^+$ subsets like macrophage or DC subsets. PBMCs from MS patients were analyzed for IFN-γ production by flow cytometry following minocycline (MIN) or doxycycline (DOX) supplementation and IL-12 plus IL-18 stimulation (see also methods section).Th1 cells were assessed by staining against CD3, CD4 and intracellular IFN-γ simultaneously. A representative case of n=5 is illustrated where the effect of minocycline and doxycycline on IFN-γ production is clearly seen on $CD3^+$ and $CD4^+$ ie Th1 cells.

REFERENCES

1. Ghasemi N, Razavi S, Nikzad E. Multiple Sclerosis: Pathogenesis, Symptoms, Diagnoses and Cell-Based Therapy. *Cell J* (2017) 19(1):1–10. doi: 10.22074/cellj.2016.4867
2. Lublin FD, Reingold SC, Cohen JA, Cutter GR, Sorensen PS, Thompson AJ, et al. Defining the Clinical Course of Multiple Sclerosis: The 2013 Revisions. *Neurology* (2014) 83(3):278–86. doi: 10.1212/WNL.0000000000000560
3. Dargahi N, Katsara M, Tselios T, Androutsou ME, de Courten M, Matsoukas J, et al. Multiple Sclerosis: Immunopathology and Treatment Update. *Brain Sci* (2017) 7(7):78. doi: 10.3390/brainsci7070078
4. Mastorodemos V, Ioannou M, Verginis P. Cell-Based Modulation of Autoimmune Responses in Multiple Sclerosis and Experimental Autoimmmune Encephalomyelitis: Therapeutic Implications. *Neuroimmunomodulation* (2015) 22(3):181–95. doi: 10.1159/000362370
5. Rostami A, Ciric B. Role of Th17 Cells in the Pathogenesis of CNS Inflammatory Demyelination. *J Neurol Sci* (2013) 333(1-2):76–87. doi: 10.1016/j.jns.2013.03.002
6. Dettke M, Scheidt P, Prange H, Kirchner H. Correlation Between Interferon Production and Clinical Disease Activity in Patients With Multiple Sclerosis. *J Clin Immunol* (1997) 17(4):293–300. doi: 10.1023/A:1027374615106
7. Ottum PA, Arellano G, Reyes LI, Iruretagoyena M, Naves R. Opposing Roles of Interferon-Gamma on Cells of the Central Nervous System in Autoimmune Neuroinflammation. *Front Immunol* (2015) 6:539. doi: 10.3389/fimmu.2015.00539
8. Mavropoulos A, Sully G, Cope AP, Clark AR. Stabilization of IFN-Gamma mRNA by MAPK P38 in IL-12- and IL-18-Stimulated Human NK Cells. *Blood* (2005) 105(1):282–8. doi: 10.1182/blood-2004-07-2782
9. Comabella M, Balashov K, Issazadeh S, Smith D, Weiner HL, Khoury SJ. Elevated Interleukin-12 in Progressive Multiple Sclerosis Correlates With Disease Activity and Is Normalized by Pulse Cyclophosphamide Therapy. *J Clin Invest* (1998) 102(4):671–8. doi: 10.1172/JCI3125
10. Fassbender K, Mielke O, Bertsch T, Muehlhauser F, Hennerici M, Kurimoto M, et al. Interferon-Gamma-Inducing Factor (IL-18) and Interferon-Gamma in Inflammatory CNS Diseases. *Neurology* (1999) 53(5):1104–6. doi: 10.1212/WNL.53.5.1104

11. McGinley AM, Edwards SC, Raverdeau M, Mills KHG. Th17cells, Gammadelta T Cells and Their Interplay in EAE and Multiple Sclerosis. *J Autoimmun* (2018) S0896-8411(18)30007-6. doi: 10.1016/j.jaut.2018.01.001
12. El-Behi M, Ciric B, Dai H, Yan Y, Cullimore M, Safavi F, et al. The Encephalitogenicity of T(H)17 Cells Is Dependent on IL-1- and IL-23-Induced Production of the Cytokine GM-CSF. *Nat Immunol* (2011) 12 (6):568–75. doi: 10.1038/ni.2031
13. Van Kaer L, Postoak JL, Wang C, Yang G, Wu L. Innate, Innate-Like and Adaptive Lymphocytes in the Pathogenesis of MS and EAE. *Cell Mol Immunol* (2019) 16(6):531–9. doi: 10.1038/s41423-019-0221-5
14. da Costa DS, Hygino J, Ferreira TB, Kasahara TM, Barros PO, Monteiro C, et al. Vitamin D Modulates Different IL-17-Secreting T Cell Subsets in Multiple Sclerosis Patients. *J Neuroimmunol* (2016) 299:8–18. doi: 10.1016/j.jneuroim.2016.08.005
15. Wing AC, Hygino J, Ferreira TB, Kasahara TM, Barros PO, Sacramento PM, et al. Interleukin-17- and Interleukin-22-Secreting Myelin-Specific CD4(+) T Cells Resistant to Corticoids Are Related With Active Brain Lesions in Multiple Sclerosis Patients. *Immunology* (2016) 147(2):212–20. doi: 10.1111/imm.12552
16. Petek-Balci B, Coban A, Shugaiv E, Turkoglu R, Ulusoy C, Icoz S, et al. Predictive Value of Early Serum Cytokine Changes on Long-Term Interferon Beta-1A Efficacy in Multiple Sclerosis. *Int J Neurosci* (2015) 125(5):352–6. doi: 10.3109/00207454.2014.939747
17. Longbrake EE, Racke MK. Why did IL-12/IL-23 Antibody Therapy Fail in Multiple Sclerosis? *Expert Rev Neurother* (2009) 9(3):319–21. doi: 10.1586/14737175.9.3.319
18. Dumitrescu L, Constantinescu CS, Tanasescu R. Recent Developments in Interferon-Based Therapies for Multiple Sclerosis. *Expert Opin Biol Ther* (2018) 18(6):665–80. doi: 10.1080/14712598.2018.1462793
19. Avasarala J. It's Time for Combination Therapies: In Multiple Sclerosis. *Innov Clin Neurosci* (2017) 14(5-6):28–30.
20. Chopra I, Hawkey PM, Hinton M. Tetracyclines, Molecular and Clinical Aspects. *J Antimicrob Chemother* (1992) 29(3):245–77. doi: 10.1093/jac/29.3.245
21. Saivin S, Houin G. Clinical Pharmacokinetics of Doxycycline and Minocycline. *Clin Pharmacokinet* (1988) 15(6):355–66. doi: 10.2165/00003088-198815060-00001
22. Sapadin AN, Fleischmajer R. Tetracyclines: Nonantibiotic Properties and Their Clinical Implications. *J Am Acad Dermatol* (2006) 54(2):258–65. doi: 10.1016/j.jaad.2005.10.004
23. Garrido-Mesa J, Rodriguez-Nogales A, Algieri F, Vezza T, Hidalgo-Garcia L, Garrido-Barros M, et al. Immunomodulatory Tetracyclines Shape the Intestinal Inflammatory Response Inducing Mucosal Healing and Resolution. *Br J Pharmacol* (2018) 175(23):4353–70. doi: 10.1111/bph.14494
24. Chen X, Ma X, Jiang Y, Pi R, Liu Y, Ma L. The Prospects of Minocycline in Multiple Sclerosis. *J Neuroimmunol* (2011) 235(1-2):1–8. doi: 10.1016/j.jneuroim.2011.04.006
25. Maier K, Merkler D, Gerber J, Taheri N, Kuhnert AV, Williams SK, et al. Multiple Neuroprotective Mechanisms of Minocycline in Autoimmune CNS Inflammation. *Neurobiol Dis* (2007) 25(3):514–25. doi: 10.1016/j.nbd.2006.10.022
26. Kloppenburg M, Breedveld FC, Terwiel JP, Mallee C, Dijkmans BA. Minocycline in Active Rheumatoid Arthritis. A Double-Blind, Placebo-Controlled Trial. *Arthritis Rheum* (1994) 37(5):629–36. doi: 10.1002/art.1780370505
27. Metz LM, Li DKB, Traboulsee AL, Duquette P, Eliasziw M, Cerchiaro G, et al. Trial of Minocycline in a Clinically Isolated Syndrome of Multiple Sclerosis. *N Engl J Med* (2017) 376(22):2122–33. doi: 10.1056/NEJMoa1608889
28. Minagar A, Alexander JS, Schwendimann RN, Kelley RE, Gonzalez-Toledo E, Jimenez JJ, et al. Combination Therapy With Interferon Beta-1a and Doxycycline in Multiple Sclerosis: An Open-Label Trial. *Arch Neurol* (2008) 65(2):199–204. doi: 10.1001/archneurol.2007.41
29. Zabad RK, Metz LM, Todoruk TR, Zhang Y, Mitchell JR, Yeung M, et al. The Clinical Response to Minocycline in Multiple Sclerosis Is Accompanied by Beneficial Immune Changes: A Pilot Study. *Mult Scler* (2007) 13(4):517–26. doi: 10.1177/1352458506070319
30. Sorensen PS, Sellebjerg F, Lycke J, Farkkila M, Creange A, Lund CG, et al. Minocycline Added to Subcutaneous Interferon Beta-1a in Multiple Sclerosis: Randomized RECYCLINE Study. *Eur J Neurol* (2016) 23(5):861–70. doi: 10.1111/ene.12953
31. Popovic N, Schubart A, Goetz BD, Zhang SC, Linington C, Duncan ID. Inhibition of Autoimmune Encephalomyelitis by a Tetracycline. *Ann Neurol* (2002) 51(2):215–23. doi: 10.1002/ana.10092
32. Brundula V, Rewcastle NB, Metz LM, Bernard CC, Yong VW. Targeting Leukocyte Mmps and Transmigration: Minocycline as a Potential Therapy for Multiple Sclerosis. *Brain* (2002) 125(Pt 6):1297–308. doi: 10.1093/brain/awf133
33. Nikodemova M, Lee J, Fabry Z, Duncan ID. Minocycline Attenuates Experimental Autoimmune Encephalomyelitis in Rats by Reducing T Cell Infiltration Into the Spinal Cord. *J Neuroimmunol* (2010) 219(1-2):33–7. doi: 10.1016/j.jneuroim.2009.11.009
34. Garrido-Mesa N, Zarzuelo A, Galvez J. Minocycline: Far Beyond an Antibiotic. *Br J Pharmacol* (2013) 169(2):337–52. doi: 10.1111/bph.12139
35. Giuliani F, Hader W, Yong VW. Minocycline Attenuates T Cell and Microglia Activity to Impair Cytokine Production in T Cell-Microglia Interaction. *J Leukoc Biol* (2005) 78(1):135–43. doi: 10.1189/jlb.0804477
36. Kloppenburg M, Verweij CL, Miltenburg AM, Verhoeven AJ, Daha MR, Dijkmans BA, et al. The Influence of Tetracyclines on T Cell Activation. *Clin Exp Immunol* (1995) 102(3):635–41. doi: 10.1111/j.1365-2249.1995.tb03864.x
37. Krakauer T, Buckley M. Doxycycline Is Anti-Inflammatory and Inhibits Staphylococcal Exotoxin-Induced Cytokines and Chemokines. *Antimicrob Agents Chemother* (2003) 47(11):3630–3. doi: 10.1128/AAC.47.11.3630-3633.2003
38. Giuliani F, Metz LM, Wilson T, Fan Y, Bar-Or A, Yong VW. Additive Effect of the Combination of Glatiramer Acetate and Minocycline in a Model of MS. *J Neuroimmunol* (2005) 158(1-2):213–21. doi: 10.1016/j.jneuroim.2004.09.006
39. Luccarini I, Ballerini C, Biagioli T, Biamonte F, Bellucci A, Rosi MC, et al. Combined Treatment With Atorvastatin and Minocycline Suppresses Severity of EAE. *Exp Neurol* (2008) 211(1):214–26. doi: 10.1016/j.expneurol.2008.01.022
40. Nikodemova M, Watters JJ, Jackson SJ, Yang SK, Duncan ID. Minocycline Down-Regulates MHC II Expression in Microglia and Macrophages Through Inhibition of IRF-1 and Protein Kinase C (PKC)Alpha/Betaii. *J Biol Chem* (2007) 282(20):15208–16. doi: 10.1074/jbc.M611907200
41. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of Multiple Sclerosis: 2017 Revisions of the Mcdonald Criteria. *Lancet Neurol* (2018) 17(2):162–73. doi: 10.1016/S1474-4422(17)30470-2
42. Skyvalidas D, Mavropoulos A, Tsiogkas S, Dardiotis E, Liaskos C, Mamuris Z, et al. Curcumin Mediates Attenuation of Pro-Inflammatory Interferon Gamma and Interleukin 17 Cytokine Responses in Psoriatic Disease, Strengthening Its Role as a Dietary Immunosuppressant. *Nutr Res* (2020) 75:95–108. doi: 10.1016/j.nutres.2020.01.005
43. Mavropoulos A, Simopoulou T, Varna A, Liaskos C, Katsiari CG, Bogdanos DP, et al. Breg Cells Are Numerically Decreased and Functionally Impaired in Patients With Systemic Sclerosis. *Arthritis Rheumatol* (2016) 68(2):494–504. doi: 10.1002/art.39437
44. Mavropoulos A, Zafiriou E, Simopoulou T, Brotis AG, Liaskos C, Roussaki-Schulze A, et al. Apremilast Increases IL-10-Producing Regulatory B Cells and Decreases Proinflammatory T Cells and Innate Cells in Psoriatic Arthritis and Psoriasis. *Rheumatol (Oxford)* (2019) 58(12):2240–50. doi: 10.1093/rheumatology/kez204
45. Domae E, Hirai Y, Ikeo T, Goda S, Shimizu Y. Cytokine-Mediated Activation of Human *Ex Vivo*-Expanded Vgamma9Vdelta2 T Cells. *Oncotarget* (2017) 8 (28):45928–42. doi: 10.18632/oncotarget.17498
46. Cazalis J, Tanabe S, Gagnon G, Sorsa T, Grenier D. Tetracyclines and Chemically Modified Tetracycline-3 (CMT-3) Modulate Cytokine Secretion by Lipopolysaccharide-Stimulated Whole Blood. *Inflammation* (2009) 32 (2):130–7. doi: 10.1007/s10753-009-9111-9
47. Tai K, Iwasaki H, Ikegaya S, Ueda T. Minocycline Modulates Cytokine and Chemokine Production in Lipopolysaccharide-Stimulated THP-1 Monocytic Cells by Inhibiting Ikappab Kinase Alpha/Beta Phosphorylation. *Transl Res* (2013) 161(2):99–109. doi: 10.1016/j.trsl.2012.10.001
48. Newton PN, Chaulet JF, Brockman A, Chierakul W, Dondorp A, Ruangveerayuth R, et al. Pharmacokinetics of Oral Doxycycline During Combination Treatment of Severe Falciparum Malaria. *Antimicrob Agents Chemother* (2005) 49(4):1622–5. doi: 10.1128/AAC.49.4.1622-1625.2005

49. Welling PG, Koch PA, Lau CC, Craig WA. Bioavailability of Tetracycline and Doxycycline in Fasted and Nonfasted Subjects. *Antimicrob Agents Chemother* (1977) 11(3):462–9. doi: 10.1128/AAC.11.3.462
50. Milano S, Arcoleo F, D'Agostino P, Cillari E. Intraperitoneal Injection of Tetracyclines Protects Mice From Lethal Endotoxemia Downregulating Inducible Nitric Oxide Synthase in Various Organs and Cytokine and Nitrate Secretion in Blood. *Antimicrob Agents Chemother* (1997) 41(1):117–21. doi: 10.1128/AAC.41.1.117
51. Chen X, Pi R, Liu M, Ma X, Jiang Y, Liu Y, et al. Combination of Methylprednisolone and Minocycline Synergistically Improves Experimental Autoimmune Encephalomyelitis in C57 BL/6 Mice. *J Neuroimmunol* (2010) 226(1-2):104–9. doi: 10.1016/j.jneuroim.2010.05.039
52. Giuliani F, Fu SA, Metz LM, Yong VW. Effective Combination of Minocycline and Interferon-Beta in a Model of Multiple Sclerosis. *J Neuroimmunol* (2005) 165(1-2):83–91. doi: 10.1016/j.jneuroim.2005.04.020
53. Yi C, Zhang Z, Wang W, Zug C, Schluesener HJ, Zhang Z. Doxycycline Attenuates Peripheral Inflammation in Rat Experimental Autoimmune Neuritis. *Neurochem Res* (2011) 36(11):1984–90. doi: 10.1007/s11064-011-0522-2
54. Kloppenburg M, Mattie H, Douwes N, Dijkmans BA, Breedveld FC. Minocycline in the Treatment of Rheumatoid Arthritis: Relationship of Serum Concentrations to Efficacy. *J Rheumatol* (1995) 22(4):611–6.
55. Kloppenburg M, Terwiel JP, Mallee C, Breedveld FC, Dijkmans BA. Minocycline in Active Rheumatoid Arthritis. A Placebo-Controlled Trial. *Ann N Y Acad Sci* (1994) 732:422–3. doi: 10.1111/j.1749-6632.1994.tb24773.x
56. Kloppenburg M, Brinkman BM, de Rooij-Dijk HH, Miltenburg AM, Daha MR, Breedveld FC, et al. The Tetracycline Derivative Minocycline Differentially Affects Cytokine Production by Monocytes and T Lymphocytes. *Antimicrob Agents Chemother* (1996) 40(4):934–40. doi: 10.1128/AAC.40.4.934
57. Zaguia F, Saikali P, Ludwin S, Newcombe J, Beauseigle D, McCrea E, et al. Cytotoxic NKG2C+ CD4 T Cells Target Oligodendrocytes in Multiple Sclerosis. *J Immunol* (2013) 190(6):2510–8. doi: 10.4049/jimmunol.1202725
58. Fujii C, Kondo T, Ochi H, Okada Y, Hashi Y, Adachi T, et al. Altered T Cell Phenotypes Associated With Clinical Relapse of Multiple Sclerosis Patients Receiving Fingolimod Therapy. *Sci Rep* (2016) 6:35314. doi: 10.1038/srep35314
59. Araki M, Kondo T, Gumperz JE, Brenner MB, Miyake S, Yamamura T. Th2 Bias of CD4+ NKT Cells Derived From Multiple Sclerosis in Remission. *Int Immunol* (2003) 15(2):279–88. doi: 10.1093/intimm/dxg029

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Tocilizumab (TCZ) Decreases Angiogenesis in Rheumatoid Arthritis Through Its Regulatory Effect on miR-146a-5p and EMMPRIN/CD147

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Background: Angiogenesis is a major contributor to the development of inflammation during Rheumatoid arthritis (RA), as the vascularization of the pannus provides nutrients and oxygen for the infiltrating immune cells and proliferating synoviocytes. Tocilizumab (TCZ) is an anti-IL-6 receptor antibody that is used in the treatment of RA patients, and has been shown to exert anti-inflammatory effects. However, its effects on angiogenesis are not fully elucidated, and the molecular mechanisms regulating this effect are unknown.

Methods: We evaluated the concentrations of several pro- and anti-angiogenic factors and the expression levels of several microRNA molecules that are associated with RA and angiogenesis in serum samples obtained from 40 RA patients, before and 4 months after the initiation of TCZ treatment. Additionally, we used an *in vitro* co-culture system of fibroblasts (the HT1080 cell line) and monocytes (the U937 cell line) to explore the mechanisms of TCZ action.

Results: Serum samples from RA patients treated with TCZ exhibited reduced circulating levels of EMMPRIN/CD147, enhanced expression of circulating miR-146a-5p and miR-150-5p, and reduced the angiogenic potential as was manifested by the lower number of tube-like structures that were formed by EaHy926 endothelial cell line. *In vitro*, the accumulation in the supernatants of the pro-angiogenic factors EMMPRIN, VEGF and MMP-9 was increased by co-culturing the HT1080 fibroblasts and the U937 monocytes, while the accumulation of the anti-angiogenic factor thrombospondin-1 (Tsp-1) and the expression levels of miR-146a-5p were reduced. Transfection of HT1080 cells with the miR-146a-5p mimic, decreased the accumulation of EMMPRIN, VEGF and MMP-9. When we neutralized EMMPRIN with a blocking antibody, the supernatants derived from

these co-cultures displayed reduced migration, proliferation and tube formation in the functional assays.

Conclusions: Our findings implicate miR-146a-5p in the regulation of EMMPRIN and propose that TCZ affects angiogenesis through its effects on EMMPRIN and miR-146a-5p.

Keywords: rheumatoid arthritis (RA), tocilizumab (TCZ), angiogenesis, EMMPRIN/CD147, miR-146a-5p, thrombospondin-1 (Tsp-1)

INTRODUCTION

Tocilizumab (TCZ) in RA Treatment

Rheumatoid arthritis (RA) is a chronic autoimmune disease that causes joint inflammation, damage and bone erosion, as well as many systemic manifestations. The pathophysiology of RA is based on a complex network of pro-inflammatory cytokines, especially interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) (1, 2). The binding of IL-6 to its membranal receptor or to its soluble receptor and the subsequent binding of the complex to the gp130 receptor chains evokes signals, primarily through JAK/STAT3 activation, but also through the activation of the MAPKs and PI3K/Akt pathways (3) that give rise to the pleotropic effects of this cytokine. These pathways, together with TNFα and IL-1β, synergistically activate pro-inflammatory and pro-angiogenic molecules (4, 5). This accounts for the pro-inflammatory effects of IL-6 including neutrophil and monocyte recruitment, endothelial cell activation, B cell stimulation leading to autoantibody production, and induction of acute phase reactants such as C-reactive protein (CRP) and serum amyloid A (SAA) (3). Furthermore, IL-6 together with TNFα drive the proliferation of fibroblast-like synoviocytes (FLS) and promote their local secretion of a myriad of cytokines (6), as well as their stimulation of angiogenesis through phosphorylation of STAT3 and induction of vascular endothelial growth factor (VEGF) production (7, 8).

The humanized anti-IL-6 receptor monoclonal antibody tocilizumab (TCZ) blocks both the *cis* and *trans* signaling pathways of IL-6. Although it is known to lead to the accumulation of IL-6 in the serum of treated patients (2), TCZ reduces the serum levels of pro-inflammatory cytokines [e.g., RANKL, MIF, chemerin, IL-21 (9, 10)], reduces CRP and SAA levels, and ameliorates the systemic manifestation of RA, such as pain, fatigue, and anemia (1, 2, 10). While the effects of TCZ on angiogenesis have yet to be investigated in depth, TCZ has been shown to reduce VEGF serum levels in complete Freund's adjuvant (CFA)-induced arthritic rats (11), and to reduce the mean vessel density (MVD) in the synovium of RA patients as evaluated by immunohistochemical staining for the endothelial marker CD31 (12). However, the detailed mechanisms of action, which enable TCZ to inhibit angiogenesis, are not yet understood.

To allow immune cells to infiltrate, and to supply the growing metabolic needs of these cells and of the proliferating FLS for oxygen and nutrients, new blood vessels must be formed in the expanding pannus. Therefore, angiogenesis is considered critical to pannus formation. The increasing number of cells results in local hypoxia, which stimulates the production of pro-angiogenic factors (e.g., VEGF). Thus, an excess of pro-angiogenic relative to anti-angiogenic mediators is generated, and this imbalance switches on angiogenesis and increases blood vessel density to help sustain pannus progression (7).

Here we investigate the role of the several pro-angiogenic factors: VEGF, which promotes endothelial cell migration, proliferation, and tube formation (13), and is the most potent pro-angiogenic factor known; matrix metalloproteinases (MMPs) which degrade the basement membrane and allow endothelial cell migration, particularly MMP-9; the neutrophil gelatinase associated lipocalin (NGAL), which can bind to and protect MMP-9 from degradation (14); and EMMPRIN/CD147 which can induce both VEGF and MMPs secretion and therefore is considered a pro-angiogenic factor (15). On the other hand, we also study thrombospondin-1 (Tsp-1), which is an example of a potent endogenous inhibitor of angiogenesis (16, 17).

MicroRNAs (miRNAs) are small (20-23 nucleotides long) non-coding RNA molecules, which function in post-transcriptional regulation of gene expression *via* by base-pairing with complementary mRNA sequences leading to their silencing by cleavage or inhibited translation. Thus, miRNAs have been implicated in the regulation of many cellular processes, including angiogenesis, particularly in the context of malignancy (18–22). Several miRNAs have previously been implicated in the pathogenesis of RA, some of which are also known to be involved in the regulation of angiogenesis (18, 20, 23–25). Of these, we selected 9 miRNAs to examine the effect of TCZ on their circulating levels. For instance, we have previously linked the regulation of EMMPRIN expression to miR-146a-5p in cancer cells (26, 27), and miR-146a-5p has been linked to angiogenesis in the context of RA (28). However, little is known about the involvement of miRNAs in angiogenesis in the context of RA.

The goal of the research presented here was to evaluate the effects of TCZ on levels of pro- and anti-angiogenic factors found in sera of RA patients, as well as on the accumulation of several

Abbreviations: bDMARDs, biologic disease modifying anti-rheumatic drugs; cDMARDs, conventional disease modifying anti-rheumatic drugs; CRP, C-reactive protein; EMMPRIN/CD147, extracellular matrix metalloproteinase inducer; FLS, fibroblast-like synoviocytes; IL, interleukin; JAK, Janus Kinase; MAPK, mitogen activated protein kinase; miRNA, microRNA; MMPs, matrix metalloproteinases; MVD, mean vessel density; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NGAL, neutrophil gelatinase associated lipocalin (lipocalin-2); RA, Rheumatoid arthritis; SAA, serum amyloid A; STAT, signal transducers and activators of transcription; VEGF, vascular endothelial growth factor; TCZ, tocilizumab; TNFα, tumor necrosis factor alpha; Tsp-1, thrombospondin-1.

circulating miRNAs known to be associated with angiogenesis. Our findings, including the regulatory role of miR-146a-5p on angiogenesis, were then corroborated in an *in vitro* co-culture system of human monocyte and fibroblast cell lines.

METHODS

Patients

The study cohort included 40 patients diagnosed with RA, who fulfilled the 2010 EULAR and ACR classification criteria for RA (29) with active disease according to the disease activity score in 28 joints (DAS28-CRP) score (DAS≥3.2) (30) and initiating treatment with tocilizumab infusion (8mg/kg every 4 weeks). The patients were recruited consecutively from the Rheumatology Clinic in Carmel Medical Center and Bnei Zion Medical Center, Haifa, Israel, after failure of conventional disease modifying anti-rheumatic drugs (cDMARDs). All other medical decisions were at the physicians' discretion. All patients were examined and blood samples were obtained before TCZ infusion at enrollment (referred to as "Before") and 4 months after the beginning of treatment (referred to as "After 4 m"). Blood was immediately centrifuged, and serum samples were stored at -80°C until further analysis. Response to treatment was assessed according to the EULAR response criteria (30). We further stratified our patients to "responders"- those patients who experienced an improvement in their DAS-28-CRP score ≧ 1.2- and their total DAS28-CRP score was <5.1, and to "non-responders" - patients who demonstrated no change or a change < 1.2 in DAS-28-CRP score (30).

Patients diagnosed with additional inflammatory disease or active neoplastic diseases were excluded from the study. The study was approved by Carmel Hospital Institutional Review Board (Helsinki committee CMC-0018-11) and all patients signed an informed consent form.

Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrations of EMMPRIN, VEGF, MMP-9, IL-6, NGAL, and Tsp-1 were measured using commercial DuoSet ELISA kits (R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Duplicate serum and supernatant samples were diluted according to preliminary calibration experiments. To deterimine cytokine concentrations in serum samples, samples tested for EMMPRIN, MMP-9 and NGAL were diluted 1:100; samples tested for VEGF and IL-6 were diluted 1:4; and samples tested for Tsp-1 were diluted 1:1000. To determine the cytokine concentrations in supernatants derived from cultured cells, samples were diluted 1:100, except for Tsp-1 (1:1000). The human high sensitivity CRP (hsCRP) was measured by ELISA kit (AssayPro, St. Charles, MO), and samples were diluted 1:4,000.

Quantitative Real-Time PCR (qPCR) Analyses

Total RNA was extracted from 200 µl of serum derived from the RA patients at the different time points or from $4x10^5$ HT1080 cells, using the total RNA purification kit (Norgen Biotek, Ontario, Canada) according to the manufacturer's instructions. To assess the expression of specific miRNAs, 350 ng of total RNA were reverse transcribed at 37°C for 1 hour using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific/ Applied Biosystems, CA) and a mixture of the 5xRT primers for each of the miRNA examined (Thermo Fisher Scientific/Applied Biosystems). The miRNAs measured (miR-16-5p, miR-21-5p, miR-132-3p, miR-146a-5p, miR-150-5p, miR-155-5p, miR-203a-3p, miR-221-3p, and miR-323a-3p) and the RNU6B (U6) small RNA endogenous control were amplified in triplicates using the TaqMan microRNA assay kit (Thermo Fisher Scientific/Applied Biosystems) according to the manufacturer's instructions. The reaction was carried out for 40 cycles, each of 15 sec at 95°C and 60 sec at 60°C, using the StepOne real-time PCR (Thermo Fisher Scientific/Applied Biosystems). The comparative method ($2^{-\Delta\Delta CT}$) was used for relative quantification, and serum samples from the first visit (before the initiation of TCZ treatment) served as a calibrator in each experiment.

To date, there is still a debate as to which miRNA or small RNA molecule is best to use for normalization strategy for circulating miRNAs (31). Since miR-16 and U6 are two of the most frequently used reference genes, we compared their stability in the serum samples of patients before and 4 months after initiation of TCZ treatment, and chose U6 which demonstrated better stability for the normalization (median U6 C_T values of 30.4, IQR 31.4, 28.9; miR-16 median CT value 25.95. IQR 27.91, 23.95) (**Figure S1**).

Cultured Cells

To study the interactions between fibroblasts and monocytes, we co-cultured the human fibrosarcoma cell line HT1080 (ATCC CCL-12012) and the monocyte-like U937 cells (ATCC 1593). HT1080 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Biological Industries, IL), 10% fetal calf serum (FCS), 1% amphotericin B, 1% L-glutamine, 1% non-essential amino acids (NEAA), and 1% antibiotics, with the addition of 25% conditioned medium supplement derived from the human promyelocytic leukemic cell line HL60 (ATCC CRL-240) which secretes fibroblast growth factor-2 (FGF-2). U937 cells were cultured in RPMI-1640 medium, 10% FCS, amphotericin B (27 µM) and 1% antibiotics (Pen-strep-neomycin). The human endothelial cell line EaHy926 (ATCC CRL-2922) was cultured in DMEM with 10% FCS, 1% glutamine, 2% HAT (mixture of hypoxanthine, aminopterin, and thymidine), and 1% antibiotics. All tissue culture reagents were purchased from Biological Industries, Beit Ha'emek, Israel.

All cell lines were split twice a week at a ratio of 1:4. To avoid the masking of signals, after cells were seeded in plates and allowed to adhere, medium was replaced with serum-starved medium with 0.1% BSA for the duration of the experiment. All cell lines were regularly tested for morphological changes and presence of mycoplasma.

HT1080 ($4x10^5$ cells) or U937 ($4x10^5$ cells) were cultured separately or in co-culture, with or without the strong MMP-9 inducer TNFα (1ng/mL) or alternatively with recombinant IL-6 (20 ng/ml, PeproTech Asia, Rehovot, Israel), and after 48 hours,

supernatants were collected for further analysis. In some experiments, increasing amounts of the recombinant EMMPRIN protein (R&D systems, Minneapolis, MN) was added as indicated, or alternatively, the anti-EMMPRIN blocking antibody (2 ng/ml, Biolegend, San Diego, CA) was added to some of the wells. In other experiments the NF-κB inhibitor Bay 11-7082 (Merck/Sigma-Aldrich, Darmstadt, Germany) or the JAK/STAT inhibitor tofacitinib (Merck/Calbiochem, Darmstadt, Germany) were added to the cultured cells. In experiments where RNA was extracted, HT1080 and U937 cells were separated by an insert (0.4 μm pore size) allowing exchange of soluble nutrients and proteins but precluding cell-cell contact.

Isolation of Monocyte-Enriched PBMC

Blood (18 ml) was taken from healthy volunteers in the presence of 0.2% EDTA. Peripheral blood mononuclear cells (PBMC) were separated on Histopaque gradient (Sigma-Aldrich, St. Louis, MO) and then washed twice with phosphate-buffered saline (PBS). PBMC were counted and plated in 60-mm dishes at a concentration of $80x10^6$ cells/plate in 3 ml of DMEM medium supplemented with 20% FCS for 3 hours. To enrich for monocytes, non-adherent cells were washed twice with PBS, and monocyte-enriched PBMCs were detached using a scraper and counted again. $8x10^4$ PBMCs were co-cultured with $8x10^4$ HT1080 cells for 48 hours in serum starvation medium with 0.1% BSA, separated by an insert (0.4 μm pore size), with or without the addition of TNFα (1 ng/ml) or IL-6 (20 ng/ml), and in some of the experiments with or without TCZ (500 μg/ml) as described before. This part of the study was approved by Carmel Hospital Institutional Review Board (Helsinki committee CMC-0018-11), and all healthy volunteers signed an informed consent form.

Wound Assay (*In Vitro*)

EaHy926 cells were seeded (10^5 cells/well) in 96-well plates and cultured to confluency. The monolayer was then scratched using a toothpick, and the non-adherent cells were removed by washing with PBS. At this point, supernatants from the HT1080 and U937 co-cultures (diluted 1:2) with or without the addition of the anti-EMMPRIN antibody (2 ng/ml), were added to the endothelial layer. Images of the scratch site were acquired immediately after scratching the cell monolayer (T0) and 24 h later (T24) (Moticam 2MP, magnification x4), and the wound area was measured at both times using the ImagePro plus 4.5 software (Media Cybernetics, Inc., Rockville, MD). The subtraction of the area at T0 from the area measured at T24 reflected the area to which the endotheial cells migrated in wound closure.

Tube Formation Assay (*In Vitro*)

EaHy926 cells ($8x10^4$ cells/well) were plated in triplicates in DMEM with 2% FCS on 96-well plates which were previously coated at 4°C with Coultrex® reduced growth factor basement membrane extract (Travigen, Gaithersburg, MD) and polymerized at 37°C for 2 hours. Serum samples (diluted 1:4) or supernatnats from the HT1080 and U937 co-cultures (diluted 1:2) were added. Images were obtained after 6 hours of incubation (Moticam 2MP, magnification x4), and the number of closed lumens were counted in two separate fields.

Transfection of HT1080 Cells With miR-146-5p Mimic or Anti-miR-146a-5p

HT1080 cells (10^4 cells) were seeded in a 96-well plate in 100μl of full medium and incubated overnight. The Lipofectamine RNAi MAX (Ambion, Austin, TX) was diluted 1:25 in Opti-MEM medium and combined with an Opti-MEM medium containing 30 nM of miRNA-146a-5p mimic, anti-miR-146a-5p, or their respective negative controls (all from Thermo Fisher Scientific/Ambion) to create miRNA-lipid complex that was added to each well and incubated overnight. Cells were then washed with PBS, and were incubated with serum-starved medium with 0.1% BSA, with or without addition of U937 cells for additional 48 hours, before collecting the supernatants for further analysis.

Statistics

All values are presented as means ± standard error of measurement (SEM). The nonparametric one-way analysis of variance (ANOVA) test was used to compare multiple groups, followed by the Bonferroni's multiple post-hoc comparison test. In the patient's data, two groups were compared with the two-tailed Mann-Whitney U test, or if paired, with the Wilcoxon matched-paired signed rank test. In the *in vitro* experiments, two groups were compared using he unpaired student t test. Detais of each analysis are provided in the figure legends. P values exceeding 0.05 were not considered significant.

RESULTS

Study Population

The age of the study population was 57.5 ± 11.1 years, with disease duration of 7.7 ± 5.6 years, 33 (82.5%) were female and 53.9% were positive for rheumatoid factor. The demographic and clinical data of the patients participating in the study are summarized in **Table 1**. All patients continued treatment with TCZ during the study period of 4 months, 25/40 (62.5%) patients were classified as "responders" according to EULAR criteria. Notably, after 4 months of treatment, the mean DAS-28 CRP dropped from 5.47 (IQR 6.1, 4.75) to 3.52 (IQR 4.81, 2.8), tender joint count decreased from 12.5 ± 1.02 to 5.9 ± 0.82, and the swollen joint count decreased from 9.25 ± 0.85 to 4.07 ± 0.66.

As expected, serum IL-6 levels were increased after 4 months of treatment (**Figure S2A**) with higher levels in responders than in non-responders (**Figure S2C**). The hsCRP levels were higher in the RA patients before treatment, and were significantly reduced after 4 months of treatment. However, no difference in hsCRP levels was found between responders and non-responders (**Figures S2B, D**).

TCZ Affects the Concentrations of Pro-Angiogenic Factors

Comparing the levels of pro-angiogenic factors, we noted a drop in EMMPRIN after 4 months of TCZ (**Figure 1A**), an increase in

TABLE 1 | Demographic, clinical characteristics, underlying diseases, and treatment of the study groups.

	Non-responding Patiens	Responding patients	Total RA patients	P values: Responding vs. non-responding
No. participants	15	25	40	
Sex: Female (%)	14 (93.3%)	19 (76%)	33 (82.5%)	ns
Age (years) ± SD	56.9 ± 3.3	57.9 ± 2.1	57.53 ± 11.1	ns
Disease Duration	8 ± 1	7.52 ± 1.3	7.7 ± 5.6	ns
Tobacco use (%)	3 (20%)	7 (28%)	10 (25%)	ns
RF positive (%)	8 (53.3%)	13 (52%)	21 (53.9%)	ns
Anti-CCP positive (%)	3 (20%)	9 (36%)	12 (30%)	ns
Comorbidities:				
Hypertension	3 (20%)	9 (36%)	12 (30%)	ns
Hyperlipidemia	8 (53.3%)	11 (44%)	19 (47.5%)	ns
Diabetes mellitus (DM)	5 (33.3%)	3 (12%)	8 (20%)	ns
Chronic obstructive pulmonary disease (COPD)	0 (0%)	1 (4%)	1 (2.5%)	ns
Ischemic heart disease (IHD)	0 (0%)	1 (4%)	1 (2.5%)	ns
Prior malignancy	1 (6.6%)	0 (0%)	1 (2.5%)	ns
Medications (at baseline):				
Methotrexate (MTX)	12 (80%)	10 (40%)	27 (67.5%)	0.0217
Sulfasalazine (SSZ)	1 (6.6%)	4 (16%)	5 (12.5%)	ns
Hydroxycholoroquine (HCQ)	1 (6.6%)	3 (12%)	4 (10%)	ns
Leflunomide (LEF)	0 (0%)	3 (12%)	3 (7.5%)	ns
Corticosteroid dose in milligrams (mean + SD)	3 ± 5.9	7.2 ± 10.5	5.62 ± 9.2	ns

Anti-CCP, anti-cyclic citrullinated peptide; ns, not significant; RF, rheumatoid factor; SD, standard deviation.

NGAL (**Figure 1C**), and no significant change in MMP-9 or VEGF levels (**Figures 1B, D**). Likewise, no change occurred in the serum levels of MMP-3 and MMP-7 (data not shown). The serum levels of the anti-angiogenetic factors Tsp-1 (**Figure 1E**) and endostatin (data not shown) were also unchanged. Because the angiogenic switch is turned on when the concentrations of pro-angiogenic factors exceed those of anti-angiogenic factors, we calculated the ratio between EMMPRIN (as a pro-angiogenic factor) and Tsp-1 (as an anti-angiogenic factor) for each patient before and 4 months after initiating TCZ, and found a significant decrease following 4 months of treatment (**Figure 1F**).

To show that TCZ affected the balance between serum levels of pro- and anti-angiogenic factors, we assessed their angiogenic potential directly on endothelial cells using the tube formation assay. Serum samples before and 4 months after initiation of TCZ treatment were incubated with the endothelial cell line EaHy926, and the number of closed lumens generated, reflecting the angiogenic potential, was quantified. We show that in accordance with the EMMPRIN levels and the EMMPRIN/Tsp-1 ratio, the endothelial cells generated a reduced number of closed lumens after 4 months of TCZ treatment with thicker layers of cells between the lumens, demonstrating reduced angiogenesis (**Figures 1G, H**).

Patients Responding to TCZ Treatment Demonstrate Reduced EMMPRIN/Tsp-1 Ratio

To investigate the correlation between the effects of TCZ on angiogenic factors and the clinical response which was observed in treated patients, we stratified the patients into responders and non-responders, resulting in 15 RA patients who did not respond to TCZ treatment and 25 RA patients who responded well to TCZ treatment according to EULAR criteria (30). Only NGAL levels were increased in the responders relative to the non-responders (**Figure 2C**). Levels of VEGF, MMP-9 and Tsp-1, and surprisingly even EMMPRIN levels, were not different between responders and non-responders (**Figures 2A, B, D, E**). However, although each one of these factors separately did not reveal a difference between responders and non-responders, the ratio between EMMPRIN and Tsp-1 was reduced in the responding patients (**Figure 2F**), indicating the usefulness of this ratio in evaluating the angiogenic potential of treated patients.

TCZ Affects the Serum Expression of miR-146a-5p and miR-150-5p

We next asked whether miRNAs are involved in the regulation of angiogenesis in RA and whether TCZ affects their expression. We selected 9 miRNAs whose expression has been linked to angiogenesis in previous studies and which were also shown to have dysregulated expression in RA (25), and followed their expression in RA patients before and after initiation of TCZ treatment. We chose to examine the level of circulating miRNAs, as those are known to be stable and protected from RNase activity within exosomes or when complexed with serum proteins (20). We show that no change occurred in the levels of the miRNAs tested except for the levels of miR-146a-5p and miR-150-5p which were significantly increased after 4 months of TCZ treatment relative to treatment initiation (**Figure 3**). However, no difference was found in the levels of all the serum miRNAs, including miR-146a-5p and miR-150-5p, between patients considered responders and non-responders to TCZ (data not shown).

EMMPRIN, MMP-9 and VEGF Are Increased, Whereas Tsp-1 Is Reduced in Co-Cultured HT1080 and U937 Cells

To explore the mechanisms responsible for the changes observed in the patient serum samples after 4 months of TCZ treatment, we

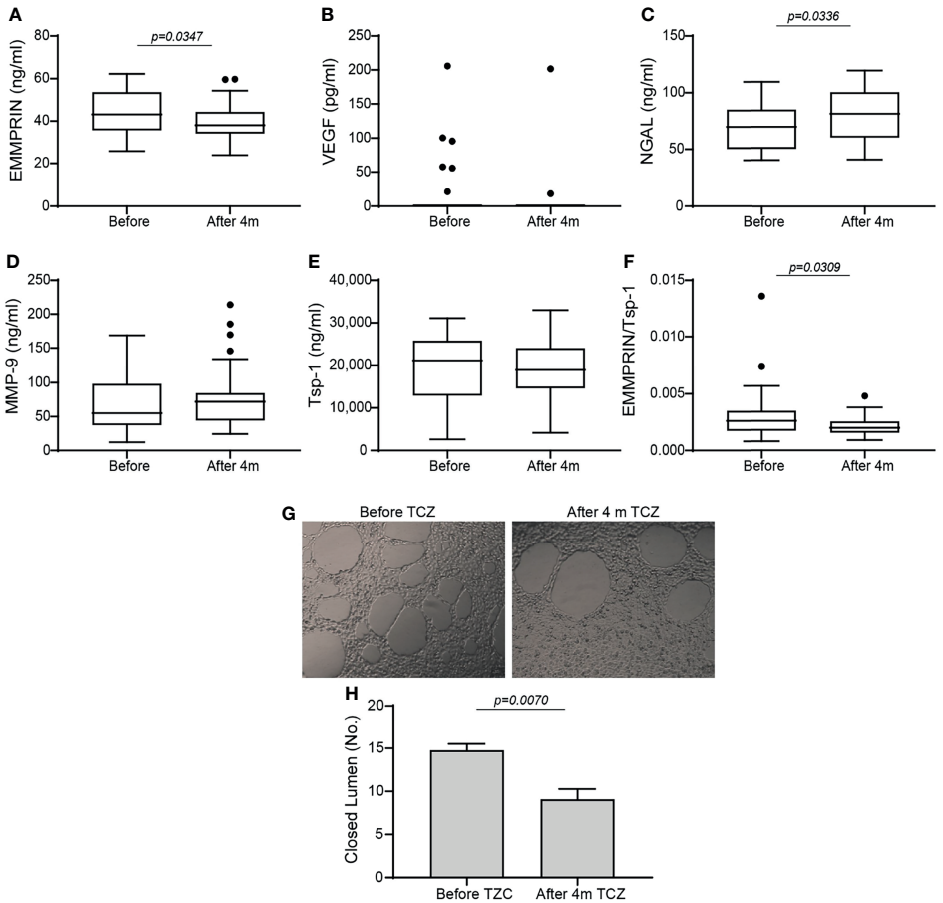


FIGURE 1 | Angiogenic factors in serum samples of RA patients before and 4 months after initiation of TCZ treatment. Box plot representing the concentrations of angiogenic mediators in the serum of RA patients before and 4 months after initiation of TCZ treatment, as determined in duplicates for each sample. **(A)** EMMPRIN, **(B)** VEGF, **(C)** NGAL, **(D)** MMP-9, **(E)** thrombospondin-1 (Tsp-1), and **(F)** the ratio between EMMPRIN and Tsp-1 as a suggested measure of enhanced angiogenesis. The non-parametric Mann-Whitney test was used to compare between the concentrations of each of the cytokines before and 4 months after TCZ treatment (n=40). **(G)** Tube formation assay with **(H)** closed lumen quantitation, to assess the direct angiogenic potential of serum samples on EaHy926 endothelial cell. The tube formation assay was carried out in triplicates with serum samples from selected patients, and the unpaired student *t* test was used to determine the effects of TCZ in the tube formation assay (n=4).

turned to an *in vitro* co-culture system of HT1080 fibroblasts and U937 monocytes, similar to our previous study with a different monocytic cell line (32). Levels of secreted EMMPRIN, VEGF and MMP-9 in the U937 single cultures were minimal (**Figures 4A–C**). In co-cultures, EMMPRIN and VEGF levels were synergistically elevated after 48 hours of incubation without TNFα relative to the levels in the single culture of HT1080 (both by about 1.7 fold, $p<0.05$), whereas MMP-9 levels showed no significant change. The addition of TNFα increased MMP-9 levels in the co-culture (by 2.2 fold, $p<0.001$), but not those of EMMPRIN or VEGF (**Figures 4A–C**), consistent with the known inducing activity of TNFα on MMP-9 (33). Levels of the anti-angiogenic factor Tsp-1 were reduced in the co-culture relative to the single culture of HT1080 (by 2 fold, $p<0.001$), and the presence of TNFα further reduced them (by 1.9 fold, $p<0.05$, **Figure 4D**). The ratio between EMMPRIN and Tsp-1 was increased by the co-culture relative to the single culture of HT1080 cells (by 2 fold, <0.05), and the addition of TNFα further increased it (by 2.3 fold, $p<0.05$, **Figure 4E**).

EMMPRIN Expression Promotes VGEF and MMP-9 Expression *In Vitro*, and Neutralization of EMMPRIN Activity Reduces Angiogenesis

We next asked whether EMMPRIN is directly involved in the pro-angiogenic effects of the co-culture. To this end, we incubated each cell type alone with increasing amounts of human recombinant EMMPRIN protein. In both cell lines, the addition of TNFα was necessary to elevate MMP-9 levels, and a significant increase in MMP-9 level (by about 2 fold, $p<0.05$, **Figures 5A, C**) was observed upon adding a concentration of 500 ng/ml of recombinant EMMPRIN relative to the addition of

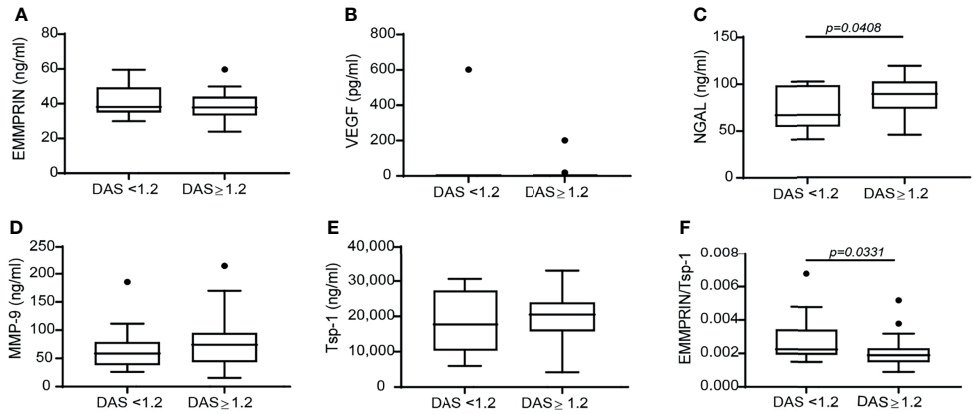


FIGURE 2 | The change in the disease activity score DAS28 is not associated with a difference in the concentration of angiogenic factors, except for the ratio EMMPRIN/Tsp-1. Patients were stratified into responders and non-responders according to the change in their DAS28 score to assess the effects of TCZ on the concentrations of the angiogenic factors. **(A)** EMMPRIN, **(B)** VEGF, **(C)** NGAL, **(D)** MMP-9, **(E)** Tsp-1, and **(F)** the ratio between EMMPRIN and Tsp-1 as a suggested measure of enhanced angiogenesis. The non-parametric Mann-Whitney test was used to compare between the concentrations of each of the cytokines before and 4 months after TCZ treatment (n=40).

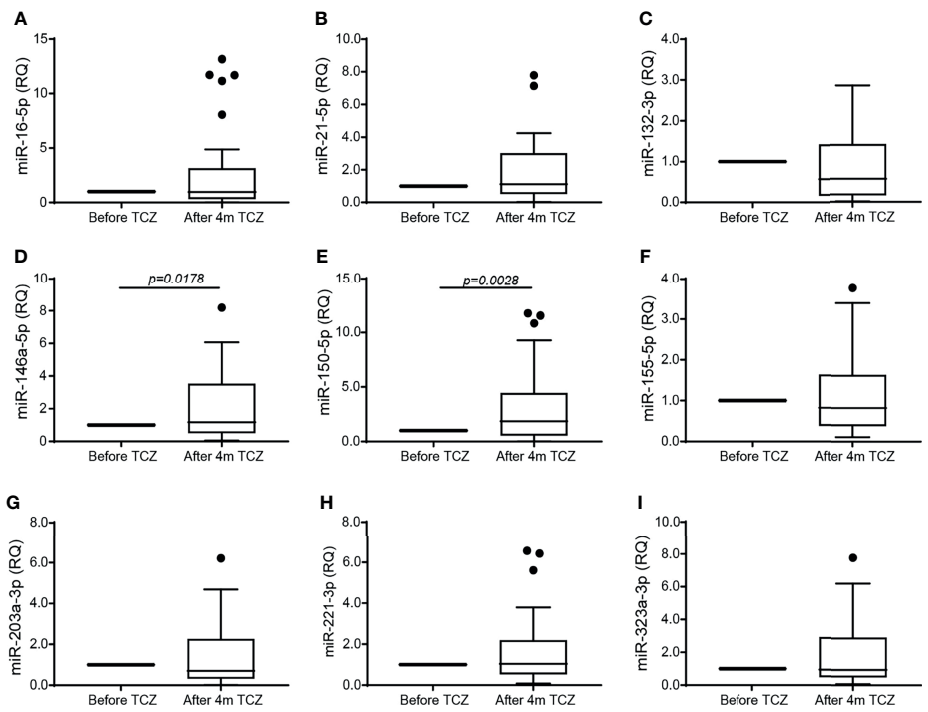


FIGURE 3 | TCZ treatment increases the serum expression of miR-146a-5p and miR-150. Total RNA was extracted from serum samples before and 4 months after initiation of TCZ treatment, and the expression levels of 9 different microRNA known to be involved in angiogenesis and in RA were determined using comparative CT method for relative quantification, where each patient served as its own control. **(A)** miR-16-5p, **(B)** miR-21-5p, **(C)** miR-132-3p, **(D)** miR-146a-5p, **(E)** miR-150-5p, **(F)** miR-155-5p, **(G)** miR-203a-3p, **(H)** miR-223-3p, **(I)** miR-323a-3p. The Wilcoxon matched-paired signed rank test was used to compare between the concentrations of each of the cytokines before and 4 months after TCZ treatment (n=37).

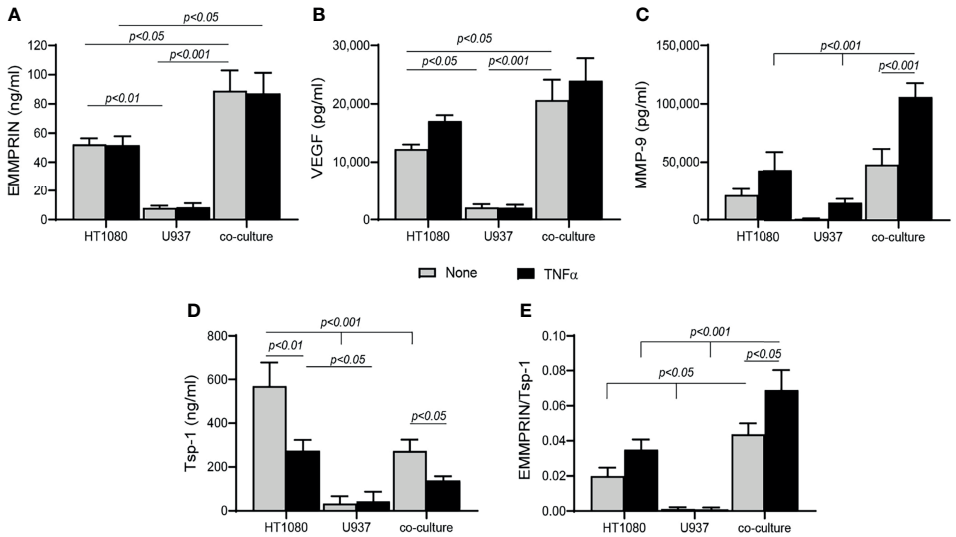


FIGURE 4 | Co-culture enhances angiogenesis. HT1080 cells ($4x10^5$ cells/well in 800 µL) were cultured alone or in co-culture with U937 monocytes at a ratio of 1:1, in the absence or presence of TNFα (1ng/mL). Supernatants were collected after 48h of incubation and the concentrations of **(A)** EMMPRIN, **(B)** VEGF, **(C)** MMP-9, and **(D)** Tsp-1 were determined by ELISA. **(E)** The EMMPRIN/Tsp-1 ratio was calculated for each repetition. The two-way analysis of variance (ANOVA) test was used to compare multiple groups, followed by the *post-hoc* Bonferroni's multiple comparison test (n=6-7 in all groups).

TNFα alone to each cell line. On the other hand, TNFα had no influence on the expression of VEGF, and the addition of 500 ng/ml of recombinant EMMPRIN increased VEGF in HT1080 cells (about 2 fold, p<0.01), but not in U937 cells (**Figures 5B, D**).

The neutralizing anti-EMMPRIN antibody was added to the two cell types co-cultured in the presence of TNFα, and after 48 hours of incubation, the accumulation of VEGF and MMP-9 in the supernatants was significantly reduced (by 1.8 and 1.4 respectively, p<0.05, **Figures 5E, F**).

Next, the overall contribution of EMMPRIN to the angiogenic potential of the supernatants was examined in functional *in vitro* assays. Conditioned media (CM) were collected from the TNFα-induced fibroblast-monocyte co-cultures, and EMMPRIN's activity was neutralized by the addition of the anti-EMMPRIN antibody. These treated CM were then incubated with EaHy926 endothelial cells, and the effect was compared to the non-neutralized CM. Neutralization of EMMPRIN activity reduced the ability of endothelial cells to form tube-like structures (by 1.6 fold, p=0.071, **Figures 6A, C**), or to migrate and close the gaps formed by a scratch (by 1.4, p=0.008, **Figures 6B, C**).

TCZ Regulates miR-146a-5p, Which in Turn Regulates EMMPRIN Expression in HT1080 Cells

Both miR-146a-5p and miR-150-5p were elevated in the serum of RA patients following 4 months of TCZ treatment. Since we have previously demonstrated that miR-146a-5p regulates EMMPRIN expression in tumor cells, we chose to focus on this miRNA and to explore whether it regulates EMMPRIN expression in fibroblasts and monocytes, and whether TCZ treatment affects EMMPRIN and angiogenesis through this regulatory pathway. We evaluated the expression of miR-146a-5p in each cell type in single cultures and in co-cultures in the presence of TNFα, compared to the single cultures without TNFα, which served as calibrators (indicated by the dashed line). For HT1080 cells, the co-culture reduced the levels of miR-146a-5p expression (by 3.5 fold, p=0.0024), while U937 showed no significant change (**Figure 7F**).

Next, we asked whether overexpression of miR-146a-5p would affect the expression levels of EMMPRIN, VEGF and MMP-9. HT1080 cells were transfected either with the miR-146a-5p mimic or with the anti-miR-146a-5p, as well as with their respective negative controls, and after 24 hours the transfected cells were incubated in co-culture with TNFα. Transfection of the anti-miR-146a-5p increased EMMPRIN levels (by 1.53 fold, p<0.05), whereas the miR-146-5p mimic reduced EMMPRIN, VEGF and MMP-9 levels (by 1.3, 2.3 and 2.2 fold, respectively, p<0.05, **Figures 7A–D**), and the negative controls did not differ from the co-culture with the non-transfected cells.

To assess the effects of TCZ on angiogenesis potential, we added increasing amounts of the drug to the co-cultured cells with TNFα, and observed that EMMPRIN, VEGF and MMP-9 were all increased (by 1.5 fold, p<0.05) at a concentration of 500 µg/ml relative to co-cultured cell without the drug (**Figure 7E**). Lastly, we show that TCZ at 500 µg/ml reduced miR-146a-5p expression levels in the HT1080 cells (by 1.6 fold, p<0.001), but not in the U937 cells.

To ask whether the NF-κB or the STAT pathways are involved in the regulation of EMMPRIN and miR-146a-5p expression, we co-cultured the HT1080 and U937 cells in the presence of TNFα and added either the NF-κB inhibitor

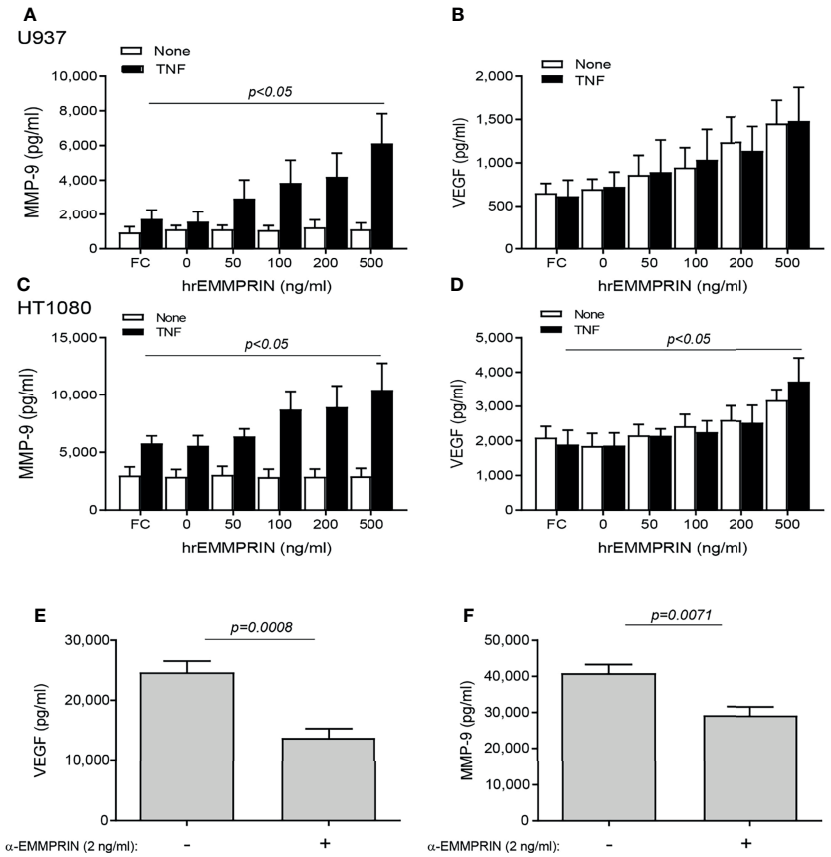


FIGURE 5 | EMMPRIN regulates the increase in VEGF and MMP-9. Cells were seeded in single cultures ($2x10^4$ cells/well in 200 μL for HT1080 or U937), with or without TNFα (1ng/mL), and increasing concentrations of recombinant human EMMPRIN or the IgG Fc fragment (Fc, at 200 ng/ml) were added as indicated. After 48h of incubation, supernatants were collected and concentrations of **(A, C)** MMP-9 and **(B, D)** VEGF were determined by ELISA (n=5-6 in all groups). The one-way ANOVA test, followed by the Dunn's multiple *post-hoc* comparison test were used. The involvement of EMMPRIN was demonstrated by adding the blocking anti-EMMPRIN antibody (2 ng/ml) to a co-culture of HT1080 and U937 cells ($2x10^4$ cell/well each) in the presence of TNFα (1 ng/mL). After 48h, the concentration of **(E)** VEGF and **(F)** MMP-9 were estimated by ELISA. The unpaired *t* test was used (n=7 in each group).

Bay 11-7082 or the JAK/STAT inhibitor tofacitinib. We show that each of these inhibitors increased the expression of EMMPRIN in the supernatants of the co-cultured cells, relative to the control without the inhibitors (by about 1.3 fold, p<0.05), whereas the expression of miR-146a-5p was reduced by the inhibitors only in the HT1080 cells and not in the U937 cells (by 1.5-1.8 fold, p<0.05, **Figures 7H, I**).

TCZ Inhibits miR-146a-5p and Increases EMMPRIN Expression in Co-Cultured HT1080 and U937, Regardless of the Stimulation of the Cells With Either TNFα or IL-6

Essentially, TCZ is designed to inhibit the IL-6 pathway by blocking IL-6R signaling. We, however, used TNFα as the primary stimulus in this study, as it a strong inducer of both MMP-9 and IL-6 (**Figure S3**). However, to confirm that IL-6 in the absence of TNFα could also affect miR-146a-5p and EMMPRIN expression, we stimulated the co-cultured HT1080 and U937 cells with IL-6 and observed that, similar to the presence of TNFα but to a lesser degree, EMMPRIN secretion was increased (by 1.25 fold, p<0.01, **Figure 8A**), whereas the expression of miR-146a-5p was reduced only in the HT1080 cells and not in the U937 cells, relative to non-stimulated cells (by 1.9 fold, p<0.05, **Figure 8B**). TCZ further increased EMMPRIN secretion (by 1.4 fold, p<0.001, **Figure 8C**) and reduced miR-146a-5p expression only in the HT1080 fibroblast cells (by 1.5 fold, p<0.05, **Figure 8D**). The effect on EMMPRIN was mediated by miR-146a-5p, as inhibiting it with its antagomir anti-miR-146a or overexpressing it by transfecting HT1080 cells with the miR-146a-5p mimic resulted in significant increased or decreased amounts of secreted EMMPRIN, respectively (**Figures 8E, F**). Lastly, by using the NF-κB pathway inhibitor Bay 11-7082 or the JAK/STAT pathway inhibitor tofacitinib in the presence of IL-6, we show that EMMPRIN is increased (by about 1.6 fold, p<0.05, **Figure 8F**) and miR-146a-5p is reduced only in the HT1080 cells (by about 1.5 fold, p<0.05, **Figure 8G**), in a similar way to what we demonstrated in the presence of TNFα.

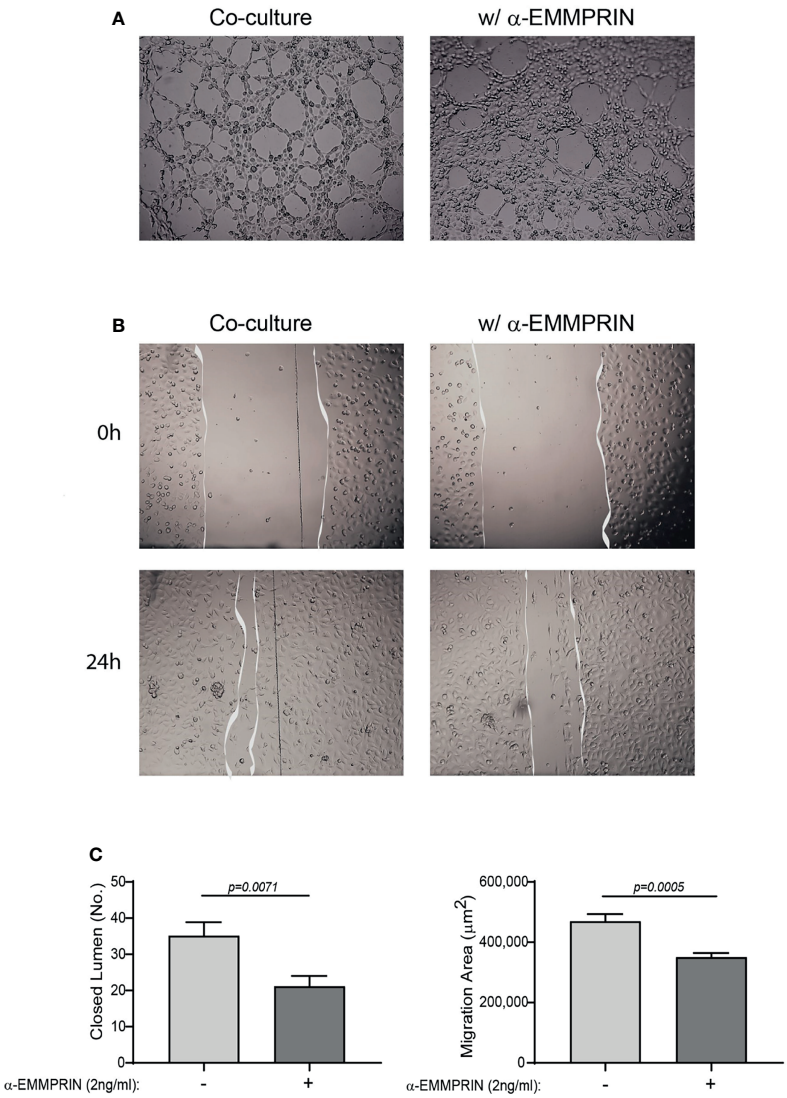


FIGURE 6 | EMMPRIN mediates the angiogenic function of the co-culture. Supernatants derived from the HT1080 and U937 co-culture experiments were collected, and diluted 1:2 in full medium with or without the addition of blocking anti-EMMPRIN antibody (2 ng/mL). This mixture was added to EaHy926 endothelial cells ($8x10^4$ cells/well in 200 μL) seeded on a Coulterx® layer, and incubated for 6 hours. **(A)** Representative images (magnification ×4), and (**C**, left panel) quantification of the closed lumen tube-like structures. The unpaired t test was used (n=10). Alternatively, confluent monolayer of EaHy926 cells was scratched with a toothpick, detached cells were washed away, and cells were allowed to migrate for 24 hours to close the wound. **(B)** Representative images obtained at the beginning of the experiment (0 h) and after 24 hours (magnification ×4). (**C**, right panel) The area of the scratch at T24 was subtracted from the area at T0, to calculate the area endothelial cells migrated to. The unpaired *t* test was used (n=9).

Co-Culture of the Primary Monocyte-Enriched PBMC With the HT1080 Fibroblasts Increase EMMPRIN, VEGF and MMP-9, and Reduce Tsp-1, and Addition of TCZ Reduce miR-146a-5p and Increase EMMPRIN

As we have used two cell lines in our study that do not necessarily represent physiological conditions, we next wanted to confirm our main results with primary monocytes. We have isolated PBMC from healthy donors, and co-cultured them with the HT1080 cell line. Similar to the previous results of the co-cultured HT1080 and U937 cells, we show here that the levels of secreted EMMPRIN, VEGF and MMP-9 in HT1080 cells co-cultured with primary monocytes-enriched PBMC were increased after 48 hours of incubation relative to single HT1080 culture (**Figures 9A-F**). While IL-6 did not have an additional effect on the co-cultured cells, TNFα enhanced the secretion of MMP-9 and VEGF relative to unstimulated cells. In contrast, relative to the HT1080 single culture, the secreted levels of Tsp1 were decreased either by the co-culture or by the addition of the cytokines (**Figures 9G, H**). Hence, the ratio EMMPRIN/Tsp-1 was increased by the co-culture relative to the

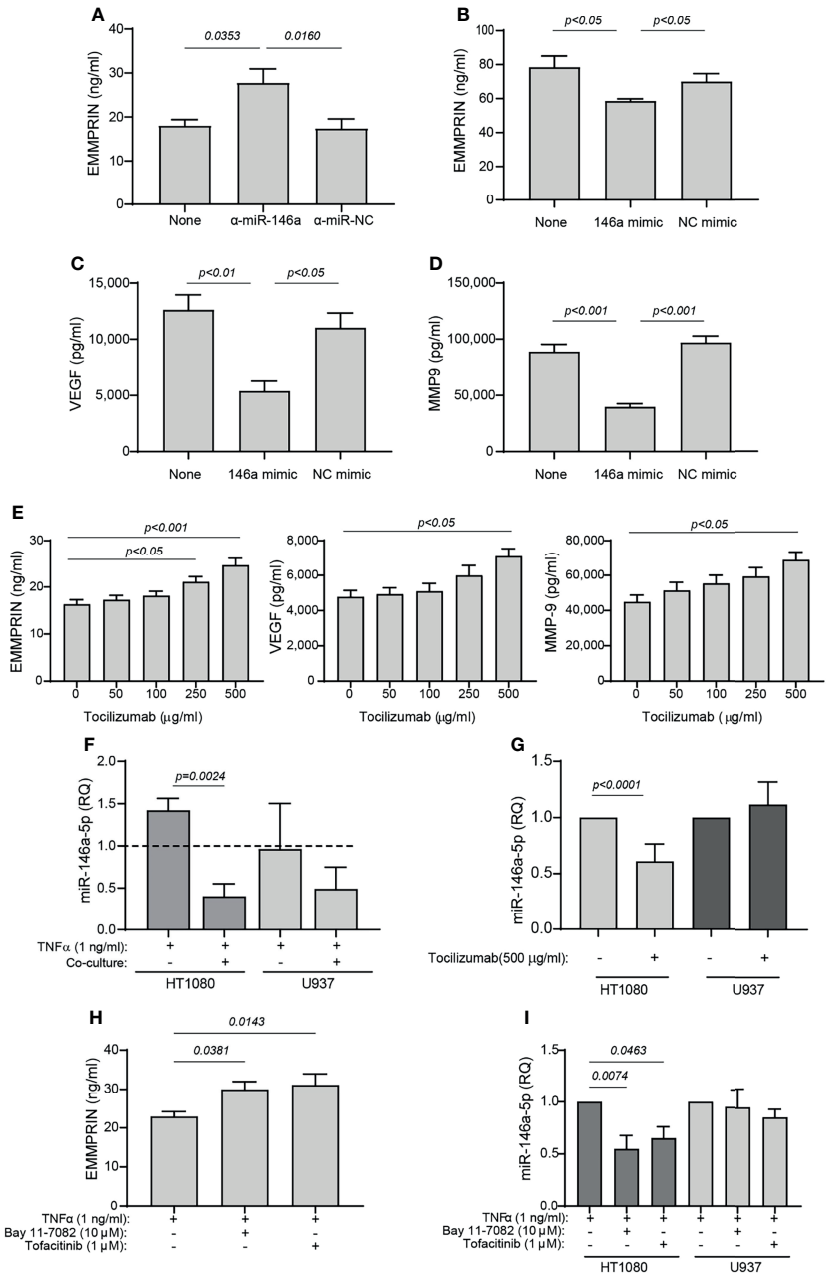


FIGURE 7 | TCZ affects the expression levels of miR-146a and EMMPRIN. **(A–D)** HT1080 cells (10^4 cells/well in 100 μL) were transfected with **(A)** anti-miR-146a-5p or its negative control, or with **(B–D)** miR-146a-5p mimic or its scrambled negative control (NC mimic). After transfection, cells were co-cultured with U937 cells with the addition of TNFα (1 ng/ml), and 48 hours later the concentrations of **(A, B)** EMMPRIN, **(C)** VEGF and **(D)** MMP-9 were determined by ELISA. The one-way ANOVA test, followed by the Bonferroni's multiple *post-hoc* comparison test were used to assess the effects of the mimic and anti-miR-146a (n=5-6 for each group). **(E)** Increasing amounts of TCZ were added to HT1080 and U937 co-cultures with TNFα (1 ng/ml), and the concentrations of EMMPRIN, VEGF and MMP-9 were determined by ELISA. The one-way ANOVA test, followed by the *post-hoc* Dunn's multiple comparison test were used (n=13 in each group). **(F)** Total RNA was extracted from HT1080 or U937 cells that were co-cultured with or without TNFα (1 ng/ml), and the expression levels of miR-146a-5p were determined in each of the cell types compared to single cultures without TNFα (dashed line). The unpaired student *t* test was used to compare between groups within each cell line. (n=5 in each group). **(G)** Expression levels of miR-146a-5p in co-cultures of HT1080 and U937 cells with or without the addition of TCZ (500 μg/ml) were determined in each cell type. The student *t* test was used to compare between the groups in each cell line (n=9-10 in each group). Lastly, HT1080 and U937 cells were incubated in co-culture for 48 hours in the presence of TNFα (1 ng/ml), with or without the NF-κB inhibitor Bay 11-7082 (10 μM) or the JAK/STAT inhibitor Tofacitinib (1 μM). **(H)** EMMPRIN concentrations and **(I)** miR-146a-5p expression levels were determined. The one-way ANOVA test, followed by the Bonferroni's multiple *post-hoc* comparison test were used to assess the effects of the inhibitors on EMMPRIN and miR-146a expression (n=5).

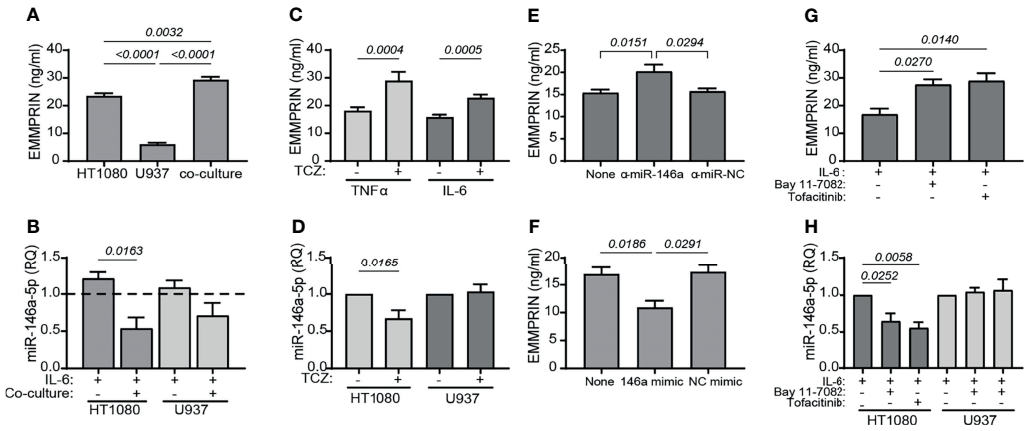


FIGURE 8 | IL-6 affects EMMPRIN and miR-146-5p expression similarly to TNFα. **(A, B)** HT1080 and U937 cells ($3x10^5$ cells in 800 μL) were co-cultured for 48h in the presence of recombinant IL-6 (20 ng/ml), and **(A)** EMMPRIN concentrations or **(B)** miR-146a-5p expression levels in each cell types were determined. The one-way ANOVA test, followed by the Bonferroni's multiple *post-hoc* comparison test were used (n=4). **(C, D)** HT1080 and U937 cells were co-cultured with **(C)** TNFα (1 ng/ml) or IL-6 (20 ng/ml), or **(D)** with IL-6 (20 ng/ml) only, and with or without the addition of TCZ (500 μg/ml), and the effects on **(C)** EMMPRIN concentrations or **(D)** miR-146a-5p expression levels in each cell type were measured. The one-way ANOVA test, followed by the Bonferroni's multiple *post-hoc* comparison test were used (n=4). **(E, F)** HT1080 cells (10^4 cells/well in 100 μL) were transfected with **(E)** anti-miR-146a-5p or its negative control (α-miR-NC), or with **(F)** miR-146a-5p mimic or its negative control (NC mimic). After transfection, cells were co-cultured with U937 cells with the addition of IL-6 (20 ng/ml), and 48h later the concentrations of EMMPRIN were determined. The one-way ANOVA test, followed by the Bonferroni's multiple *post-hoc* comparison test were used to assess the effects of the mimic and anti-miR-146a (n=4-5 for each group). Lastly, **(G, H)** HT1080 and U937 cells ($3x10^5$ cells in 800 μL) were incubated in co-culture for 48h in the presence of IL-6 (20 ng/ml) and with or without the NF-κB inhibitor Bay 11-7082 (10 μM) or the JAK/STAT inhibitor Tofacitinib (1 μM). The concentrations of **(G)** EMMPRIN and **(H)** miR-146a-5p expression levels were measured. The one-way ANOVA test, followed by the Bonferroni's multiple *post-hoc* comparison test were used to assess the effects of the inhibitors on EMMPRIN and miR-146a-5p expression (n=5).

single culture of HT1080 cells, and the addition of TNFα, but not IL-6, further increased it (**Figure 9I**). The co-culture in the presence of TNFα or IL-6 also reduced the expression of miR-146a-5p in the HT1080 cells, but not in the monocyte-enriched PBMC, relative to the HT1080 single culture (**Figures 9L, M**).

Similar to the previous results, the addition of TCZ enhanced EMMPRIN secretion and reduced miR-146a-5p expression in the HT1080, but not in the monocyte-enriched PBMC (**Figures 9K, N, O**). Thus, the monocyte-enriched PBMC confirmed that primary monocytes behave in a similar way to the U937 monocytic-like cell line, and promote pro-angiogenic changes in the HT1080 fibroblast cell line.

DISCUSSION

Angiogenesis is an important process in the pathophysiology of RA (34), but the mechanisms regulating it are yet unclear. TCZ is a biologic agent indicated for the treatment of RA (2), but its effects on pathological angiogenesis have not been sufficiently studied. Here we demonstrate that EMMPRIN (known to be a pro-angiogenic factor in the tumor microenvironment (35)) is involved in angiogenesis in RA patients and in a co-culture of fibroblasts and monocytes *in vitro*. Furthermore, we demonstrate that TCZ affects the angiogenic process, at least partially, through its effects on pro-angiogenic factors, particularly EMMPRIN and its regulator miR-146a-5p. We also demonstrate that the ratio between EMMPRIN and Tsp-1 levels is a useful measure of the angiogenic state in RA patients.

The effects of TCZ on our RA patient cohort concurred with the known effects of TCZ, showing clinical improvement in arthritis, and causing reductions in DAS28 scores and high sensitivity CRP levels. Also, in accordance with previous observations, IL-6 serum levels increased in RA patients following initiation of TCZ treatment (**Figure S2**) (2, 36).

We show here that after 4 months of treatment, TCZ reduced EMMPRIN serum levels as well as the EMMPRIN/Tsp-1 ratio (**Figure 1**). With the help of the functional tube-formation assay, we were able to demonstrate a direct effect of EMMPRIN on endothelial cells (**Figure 6**). However, despite the reduction in EMMPRIN, with its known ability to induce VEGF and MMPs (15, 35), no parallel reduction in the serum levels of VEGF or MMP-9 occurred in the sera of RA patients (**Figure 1**). This finding may be explained by the presence of alternative signaling pathways to that of EMMPRIN which may induce VEGF and MMP-9 secretion, such as TNFα, a known inducer of MMP-9 (33) or tissue hypoxia, a known inducer of VEGF (37, 38). Thus, the inhibition of the IL-6 signaling pathway by TCZ may not be sufficient to reduce the serum concentrations of these mediators in the synovial microenvironment, which is rich in pro-inflammatory cytokines.

When setting up the *in vitro* system, we chose to stimulate the cells with TNFα, a known inducer of both MMP-9 and IL-6. We show that TNFα induced IL-6 levels in the HT1080 cells, and reduced the shedding of IL-6R expression in U937 cells (**Figure S3**). Thus, the ability of TCZ to inhibit IL-6 signaling could be mimicked in our *in vitro* system. Since TCZ is an inhibitor of IL-6 signaling and TNFα might have different effects, we further

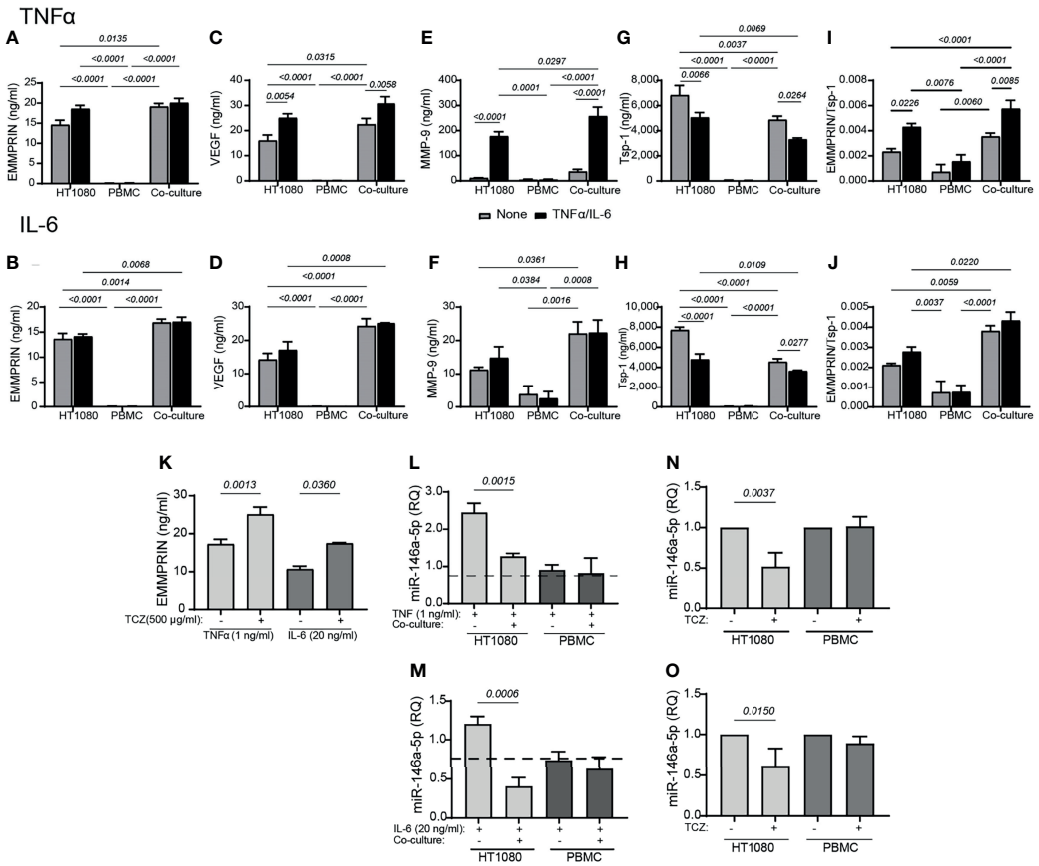


FIGURE 9 | Primary monocyte-enriched PBMC co-cultured with HT1080 fibroblasts enhance the secretion of EMMPRIN, VEGF and MMP-9, and TCZ enhances EMMPRIN and reduces miR-146a-5p expression. HT1080 cells ($8x10^4$ cells/well in 650 µL) were cultured alone or in co-culture with monocyte-enriched PBMCs at a ratio of 1:1, in the absence or presence of TNFα (1ng/mL, top panel) or IL-6 (20 ng/ml, lower panel). Supernatants were collected after 48h of incubation and the concentrations of **(A, B)** EMMPRIN, **(C, D)** VEGF, **(E, F)** MMP-9, and **(G, H)** Tsp-1 were determined by ELISA. **(I, J)** The EMMPRIN/Tsp-1 ratio was calculated for each repetition. The two-way analysis of variance (ANOVA) test was used to compare multiple groups, followed by the *post-hoc* Bonferroni's multiple comparison test (n=4-5 in all groups). Co-cultured HT1080 cells and monocyte-enriched PMBC were incubated in the presence or absence of TCZ (500 µg/ml), and **(K)** their levels of secreted of EMMPRIN were determined. The unpaired student *t* test was used to compare between groups within each cell line (n=4-5 in each group). Total RNA was extracted from HT1080 or monocyte-enriched PBMC that were co-cultured with or without **(L)** TNFα (1 ng/ml) or **(M)** IL-6 (20 ng/ml) for 48 hours, and the expression levels of miR-146a-5p were determined in each of the cell types compared to single cultures without the cytokines (dashed line). The unpaired student *t* test was used to compare between groups within each cell line. (n=4-5 in each group). Lastly, the effects of TCZ on the expression levels of miR-146a-5p in each cell type in the presence of **(N)** TNF (1 ng/ml) or **(O)** IL-6 (20 ng/ml) were determined. The unpaired student *t* test was used to compare between groups within each cell line. (n=4-5 in each group).

verified these results by repeating the experiment in the presence of IL-6 alone (**Figure 8**), and demonstrated a similar ability of TCZ to inhibit miR-146a-5p expression levels and enhance EMMPRIN secretion. However, it is still unclear whether TNFα acts directly on miR-146a-5p and EMMPRIN, or whether it works through its ability to induce IL-6. The findings in our study should be further verified by using primary human synoviocytes, however, collection of synovial fluid was not part of our approved protocol, and we could not isolate such cells.

Next, we demonstrate that EMMPRIN is directly involved in the regulation of angiogenesis using an *in vitro* co-culture system, as its levels were increased in the media of co-cultured fibroblasts and monocytes together with those of VEGF and MMP-9 (**Figure 4**), and the anti-EMMPRIN antibody reduced these levels while recombinant EMMPRIN increased them (**Figure 5**). This involvement was further established by its direct effects on migration, proliferation, and tube-formation of the endothelial cells in the scratch and tube formation assays, and the ability of an anti-EMMPRIN antibody to reduce them (**Figure 6**). Of note, the obvious inconsistency between the unchanged serum levels of VEGF and MMP-9 in the RA patients (**Figure 1**) and their elevated levels in the *in vitro* system may suggest the involvement of additional factors in their regulation, including other cell types and multiple cytokines, which may not have been present in the isolated *in vitro* system. This premise is also supported by the fact that monocyte-enriched PBMC produced similar results to those of the U937 cells when co-cultured with the HT1080 cells (**Figure 9**). Such differences between the *in vivo* and *in vitro*

systems are exemplified by reduction in the pro-angiogenic factor TGFβ in the patients after TCZ treatment, whereas in the *in vitro* setting TGFβ levels were unchanged by TCZ treatment (**Figure S4**).

Although no difference was detected in EMMPRIN, VEGF, MMP-9 or Tsp-1 serum levels between patients who responded well to TCZ treatment and those who were unresponsive to therapy, the ratio between EMMPRIN and Tsp-1 was reduced in the responding patients compared to the non-responders (**Figure 2**). This suggest that use of the EMMPRIN/Tsp-1 ratio, which takes into account small changes in the balance between pro- and anti-angiogenic factors, might be a reliable way to assess the angiogenic status in patients. However, additional studies are necessary to establish the validity of this proposed parameter in assessing the effects of conventional or biologic DMARDs on angiogenesis in RA patients.

Among the pro-angiogenic factors we tested, NGAL was significantly elevated in TCZ-treated patients, as well as in responding patients relative to non-responders (**Figures 1**, **2**). NGAL is known to form heterodimers with MMP-9, thus protecting the latter from degradation. In addition, NGAL has been shown to regulate VEGF expression and to promote angiogenesis (39). Previous reports demonstrated higher levels of NGAL in the serum of RA patients compared to healthy controls, suggesting that it promotes angiogenesis (40). In this regard, the observation of elevated levels of NGAL in RA patients after TCZ administration is contrary to the general anti-angiogenic effects mediated by TCZ. However, notably, NGAL plays many other pleiotropic roles unrelated to its role in angiogenesis, such as in protecting against bacterial infection by acting as an iron-carrying protein, in modulating oxidative stress, in promoting neutrophil chemotaxis, and in regulating thermogenesis and lipid metabolism by acting as an adipokine (10, 30). Moreover, although NGAL normally protects MMP-9 from degradation, we did not observe any elevation in MMP-9 serum levels in RA patients. This finding further supports the notion that the primary role played by NGAL in RA is unrelated to angiogenesis, and that its elevation after initiation of TCZ treatment may be related to its role as an adipokine. This possibility must be carefully explored in a follow-up study.

From the nine miRNAs that we examined, we observed that only miR-146a-5p and miR-150-5p were elevated after 4 months of treatment (**Figure 3**). Previous studies have implicated these two miRNAs in the inflammation that is driving RA. The long non-coding RNA LINC01197 which exhibits low levels in RA patients, normally acts as a sponge that binds miR-150-5p, thereby leading to enhanced thrombospondin-2 levels and reduced inflammation (41). In agreement with our findings, previous studies have shown increased levels of miR-146a-5p in peripheral blood mononuclear cells (PBMC) derived from RA patients (42), and the increased miR-146a-5p levels found in synovial fluid and in PBMC derived from RA patients were linked to decreased apoptosis in $CD4^+$ T cells derived from RA patients (43). Outside the context of RA, TCZ was shown to increase the serum levels of miR-146a-5p in COVID-19 patients, and its levels could predict response to the drug (44). However, we are unaware of any other previously published studies examining the effects of TCZ on the expression of the particular miRNAs we chose to examine in our study, and only one study demonstrated an increase in the level of a different miRNA - miR-148a - by TCZ in neutrophils isolated from RA patients *in vitro* (45).

Since we have already previously shown that miR-146a-5p participates in the regulation of EMMPRIN expression in tumor cells (26, 27), we suspected that this miRNA was also involved in EMMPRIN regulation in fibroblasts, and therefore focused on this miRNA in our *in vitro* experiments. We demonstrate that transfection of the fibroblast cell line HT1080 with the miR-146a-5p mimic resulting in overexpression of miR-146a-5p in the HT1080 cells, reduced the secretion of EMMPRIN, and subsequently of VEGF and MMP-9, implicating this miRNA in the regulation of EMMPRIN in fibroblasts (**Figures 7B–D**). In contrast, inhibiting miR-146a-5p activity by transfecting the HT1080 cells with its antagomir increased EMMPRIN secretion (**Figure 7A**), whether the cells were stimulated with TNFα or with IL-6. Thus, miR-146a-5p is involved in the regulation of EMMPRIN expression in the fibroblast cell line, directly or indirectly by affecting other regulators of EMMPRIN.

We note that TCZ decreased miR-146a-5p levels and increased EMMPRIN levels in fibroblasts *in vitro* (**Figures 7E, G**, **8C, D**, **9N, O**), whereas it increased miR-146a-5p levels and decreased EMMPRIN levels in the serum samples from treated RA patients (**Figures 1**, **3**). These inconsistencies may be explained by the difference between the *in vitro* and *in vivo* systems or by differing effects exerted by TCZ on different cell types. Indeed we could observe that the effects of TCZ on miR-146a-5p were specific to the HT1080 fibroblasts, and TCZ had no significant effect on the miR-146a-5p levels in the monocytic U937 cells or in the monocyte-enriched PBMCs (**Figures 7G**, **8D**, **9N, O**). Alternatively, the serum may reflect the state in the synovium, where interactions between fibroblasts and many other cell types may generate a balance different from that observed in the *in vitro* co-culture system involving only two cell-lines. However, despite these inconsistencies, both the *in vivo* and the *in vitro* systems reflect a strong link between miR-146a-5p, EMMPRIN and the angiogenic process, and demonstrate the ability of TCZ to intervene in this process.

The induction of miR-146a-5p is mostly attributed to stimulators activating the NF-κB pathway, such as TNFα and IL-6 (46, 47), and accordingly its levels have been shown to increase in RA patients (47). Therefore, the presence of TNFα or IL-6 in our *in vitro* system can explain the upregulation of miR-146a-5p compared to non-stimulated cells (**Figure 7F**), and the inhibitory effect of TCZ on the expression of miR-146a-5p (**Figures 7G**, **8D**, **9N, O**) might suggest a disruption of this pathway. This assumption is supported by the cooperation between TNFα-induced NF-κB and the JAK/STAT3 pathway which has recently been demonstrated in brain pericytes (48), and by the inhibitory effects of TCZ on NF-κB in a rat model of sepsis (49). Furthermore, by inducing IL-6, TNFα could indirectly activate the JAK/STAT pathway. To explore whether the NF-κB or the JAK/STAT pathways are involved in the regulation on

miR-146a-5p expression in our system, we used the NF-κB inhibitor Bay 11-7082 and the JAK/STAT inhibitor tofacitinib, and observed an increase in EMMPRIN secretion and a decrease in miR-146a-5p expression, regardless of whether TNFα or IL-6 were used to stimulate the cells (**Figures 7H, I**, **8G, H**). Thus, we suggest that TCZ helps regulate the TNFα/IL-6-induced expression of miR-146a-5p through interference with both the NF-κB and JAK/STAT pathways, and consequently controls the expression of EMMPRIN and thereby of angiogenesis. Further investigation is required to map the exact nature of this interference.

CONCLUSION

In summary, we establish an important role for EMMPRIN in mediating pro-angiogenic signals in RA patients and demonstrate a strong link between miR-146a-5p expression and the regulation of EMMPRIN secretion. Importantly, we show that TCZ reduces the angiogenic potential in RA patients, and we suggest that this is partially due to the ability of TCZ to interfere with the expression of miR-146a-5p, leading to changes in EMMPRIN levels. We also suggest that the ratio between EMMPRIN and Tsp-1 may reflect the angiogenic status in RA patients more accurately than any one factor alone.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board at Carmel Medical Center (CMC-0018-11). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS, ES, AK, and LZ performed the experiments. JF, TG, AH, ME, IR, LK, and DZ recruited the patients. JF secured the funding. TG, JF, AH, ME, IR, and LK reviewed and edited the paper. MR and DZ designed the work, analyzed and interpreted the data, and drafted the paper. All authors contributed to the article and approved the submitted version.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.739592/full#supplementary-material

Supplementary Figure S1 | TCZ treatment does not change U6 expression in RA patients. The expression levels U6 and miR-16 manifested by the C_T values of patients before and 4 months following initiation of TCZ treatment were determined by qPCR as described (n=37). The change in the expression of U6 was lower relative to the change in miR-16, and therefore, it was chosen for the normalization of the rest of the studied miRNAs.

Supplementary Figure S2 | TCZ increases serum levels of IL-6 and decreases those of hsCRP. Box plot representing the concentrations of **(A)** IL-6 and **(B)** hsCRP in the serum of RA patients before and after 4 months of TCZ treatment in comparison to healthy volunteers (controls). Patients were stratified into "responders" and "non-responders" according to the change in their DAS-28 score (≧1.2 vs <1.2, respectively), and the effects of TCZ on the concentrations of **(C)** IL-6 and **(D)** hsCRP are indicated. The non-parametric Mann-Whitney test was used to compare between the concentrations of each of the cytokines before and 4 months after TCZ treatment (n=40).

Supplementary Figure S3 | TNFα induces IL-6 in HT1080 cells and decreases IL-6R in U937 cells. HT1080 cells ($4x10^5$ cells) were cultured alone or in co-culture with U937 monocytes at a ratio of 1:1, in the absence or presence of TNFα (1ng/mL) or TCZ (500 μg/ml). Supernatants were collected after 48h of incubation and the concentrations of **(A)** IL-6, and **(B)** IL-6R were determined by ELISA. The one-way ANOVA test followed by the Bonferroni's multiple *post-hoc* comparison test was used (n=6 in all groups).

Supplementary Figure S4 | TGFβ is reduced after TCZ treatment in RA patients, but not in the *in vitro* co-culture. **(A)** HT1080 cells ($4x10^5$ cells/well in 800 μL) were cultured alone or in co-culture with U937 monocytes at a ratio of 1:1, in the absence or presence of TNFα (1ng/mL). Supernatants were collected after 48h of incubation and the concentrations of TGFβ were unchanged. **(B)** Box plot representing the concentrations of angiogenic mediators in the serum of RA patients before and 4 months after initiation of TCZ treatment, as determined in duplicates for each sample.

REFERENCES

1. Biggioggero M, Crotti C, Becciolini A, Favalli EG. Tocilizumab in the Treatment of Rheumatoid Arthritis: An Evidence-Based Review and Patient Selection. *Drug Des Devel Ther* (2019) 13:57–70. doi: 10.2147/DDDT.S150580
2. Ogata A, Kato Y, Higa S, Yoshizaki K. IL-6 Inhibitor for the Treatment of Rheumatoid Arthritis: A Comprehensive Review. *Mod Rheumatol* (2019) 29:258–67. doi: 10.1080/14397595.2018.1546357
3. Rose-John S. Interleukin-6 Signalling in Health and Disease [Version 1 ; Peer Review : 3 Approved]. *F1000Research* (2020) 9:1013 (11 pages). doi: 10.12688/f1000research.26058.1
4. Kaur S, Bansal Y, Kumar R, Bansal G. A Panoramic Review of IL-6: Structure, Pathophysiological Roles and Inhibitors. *Bioorganic Med Chem* (2020) 28:115327. doi: 10.1016/j.bmc.2020.115327
5. Nakahara H, Song J, Sugimoto M, Hagihara K, Kishimoto T, Yoshizaki K, et al. Anti-Interleukin-6 Receptor Antibody Therapy Reduces Vascular Endothelial Growth Factor Production in Rheumatoid Arthritis. *Arthritis Rheum* (2003) 48:1521–9. doi: 10.1002/art.11143
6. Srirangan S, Choy EH. The Role of Interleukin 6 in the Pathophysiology of Rheumatoid Arthritis. *Ther Adv Musculoskelet Dis* (2010) 2:247–56. doi: 10.1177/1759720X10378372
7. Marrelli A, Cipriani P, Liakouli V, Carubbi F, Perricone C, Perricone R, et al. Angiogenesis in Rheumatoid Arthritis: A Disease Specific Process or a

Common Response to Chronic Inflammation? *Autoimmun Rev* (2011) 10:595–8. doi: 10.1016/j.autrev.2011.04.020
8. Tartour E, Pere H, Maillere B, Terme M, Merillon N, Taieb J, et al. Angiogenesis and Immunity: A Bidirectional Link Potentially Relevant for the Monitoring of Antiangiogenic Therapy and the Development of Novel Therapeutic Combination With Immunotherapy. *Cancer Metastasis Rev* (2011) 30:83–95. doi: 10.1007/s10555-011-9281-4
9. Kasama T, Isozaki T, Takahashi R, Miwa Y. Clinical Effects of Tocilizumab on Cytokines and Immunological Factors in Patients With Rheumatoid Arthritis. *Int Immunopharmacol* (2016) 35:301–6. doi: 10.1016/j.intimp.2016.03.016
10. Favalli EG. Understanding the Role of Interleukin-6 (IL-6) in the Joint and Beyond: A Comprehensive Review of IL-6 Inhibition for the Management of Rheumatoid Arthritis. *Rheumatol Ther* (2020) 7:473–516. doi: 10.1007/s40744-020-00219-2
11. Abdel-Maged AES, Gad AM, Abdel-Aziz AK, Aboulwafa MM, Azab SS. Comparative Study of Anti-VEGF Ranibizumab and Interleukin-6 Receptor Antagonist Tocilizumab in Adjuvant-Induced Arthritis. *Toxicol Appl Pharmacol* (2018) 356:65–75. doi: 10.1016/j.taap.2018.07.014
12. Hirohata S, Abe A, Murasawa A, Kanamono T, Tomita T, Yoshikawa H. Differential Effects of IL-6 Blockade Tocilizumab and TNF Inhibitors on Angiogenesis in Synovial Tissues From Patients With Rheumatoid Arthritis. *Mod Rheumatol* (2017) 27:766–72. doi: 10.1080/14397595.2016.1259717
13. Elshabrawy HE, Chen Z, Volin MV, Ravella S, Virupannavar S, Shahrara S. The Pathogenic Role of Angiogenesis in Rheumatoid Arthritis. *Angiogenesis* (2015) 18:433–48. doi: 10.1007/s10456-015-9477-2
14. Santiago-Sánchez GS, Pita-Grisanti V, Quiñones-Díaz B, Gumpper K, Cruz-Monserrate Z, Vivas-Mejía PE. Biological Functions and Therapeutic Potential of Lipocalin 2 in Cancer. *Int J Mol Sci* (2020) 21:4365–80 doi: 10.3390/ijms21124365
15. Muramatsu T. Basigin (CD147), a Multifunctional Transmembrane Glycoprotein With Various Binding Partners. *J Biochem* (2016) 159:481–90. doi: 10.1093/jb/mvv127
16. Sherbet GV. Endogenous Inhibitors of Angiogenesis. In: GV Sherbet, editor. *Mol Approach to Cancer Manag*, vol. p . London: Academic Press, Elsevier Inc (2017). p. 113–30.
17. Kaur S, Martin-Manso G, Pendrak ML, Garfield SH, Isenberg JS, Roberts DD. Thrombospondin-1 Inhibits VEGF Receptor-2 Signaling by Disrupting its Association With CD47. *J Biol Chem* (2010) 285:38923–32. doi: 10.1074/jbc.M110.172304
18. Salinas-Vera YM, Marchat LA, Gallardo-Rincón D, Ruiz-García E, Astudillo-De la Vega H, Echavarría-Zepeda R, et al. AngiomiRs: MicroRNAs Driving Angiogenesis in Cancer (Review). *Int J Mol Med* (2019) 43:657–70. doi: 10.3892/ijmm.2018.4003
19. Sun LL, Li WD, Lei FR, Li XQ. The Regulatory Role of microRNAs in Angiogenesis-Related Diseases. *J Cell Mol Med* (2018) 22:4568–87. doi: 10.1111/jcmm.13700
20. Wang Y, Wang L, Chen C, Chu X. New Insights Into the Regulatory Role of microRNA in Tumor Angiogenesis and Clinical Implications. *Mol Cancer. Mol Cancer;* (2018) 17:1–10. doi: 10.1186/s12943-018-0766-4
21. Kumar V. Toll-Like receptors in Sepsis-Associated Cytokine Storm and Their Endogenous Negative Regulators as Future Immunomodulatory Targets. *Int Immunopharmacol* (2020) 89:107087–111. doi: 10.1016/j.intimp.2020.107087
22. Singh A, Patro PS, Aggarwal A. MicroRNA-132, miR-146a, and miR-155 as Potential Biomarkers of Methotrexate Response in Patients With Rheumatoid Arthritis. *Clin Rheumatol* (2019) 38:877–84. doi: 10.1007/s10067-018-4380-z
23. Fu H, Hu D, Zhang L, Tang P. Role of Extracellular Vesicles in Rheumatoid Arthritis. *Mol Immunol* (2018) 93:125–32. doi: 10.1016/j.molimm.2017.11.016
24. Madanecki P, Kapoor N, Bebok Z, Ochocka R, Collawn JF, Bartoszewski R. Regulation of Angiogenesis by Hypoxia: The Role of microRNA. *Cell Mol Biol Lett* (2013) 18:47–57. doi: 10.2478/s11658-012-0037-0
25. Churov AV, Oleinik EK, Knip M. MicroRNAs in Rheumatoid Arthritis: Altered Expression and Diagnostic Potential. *Autoimmun Rev* (2015) 14:1029–37. doi: 10.1016/j.autrev.2015.07.005
26. Simanovich E, Brod V, Rahat MM, Rahat MA. Function of miR-146a-5p in Tumor Cells As a Regulatory Switch Between Cell Death and Angiogenesis: Macrophage Therapy Revisited. *Front Immunol* (2018) 8:1931/full. doi: 10.3389/fimmu.2017.01931/full
27. Perske C, Lahat N, Sheffy Levin S, Bitterman H, Hemmerlein B, Rahat MA. Loss of Inducible Nitric Oxide Synthase Expression in the Mouse Renal Cell Carcinoma Cell Line RENCA is Mediated by microRNA miR-146a. *Am J Pathol* (2010) 177:2046–54. doi: 10.2353/ajpath.2010.091111
28. Sun W, Ma J, Zhao H, Xiao C, Zhong H, Ling H, et al. Resolvin D1 Suppresses Pannus Formation *via* Decreasing Connective Tissue Growth Factor Caused by Upregulation of miRNA-146a-5p in Rheumatoid Arthritis. *Arthritis Res Ther* (2020) 22:1–14. doi: 10.1186/s13075-020-2133-2
29. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, et al. 2010 Rheumatoid Arthritis Classification Criteria: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum* (2010) 62:2569–81. doi: 10.1002/art.27584
30. Fransen J, van Riel PLCM. The Disease Activity Score and the EULAR Response Criteria. *Rheum Dis Clin North Am* (2009) 35:745–57. doi: 10.1016/j.rdc.2009.10.001
31. Donati S, Ciuffi S, Brandi ML. Human Circulating miRNAs Real-Time qRT-PCR-Based Analysis: An Overview of Endogenous Reference Genes Used for Data Normalization. *Int J Mol Sci* (2019) 20:1–19. doi: 10.3390/ijms20184353
32. Rahat MA, Safieh M, Simanovich E, Pasand E, Gazitt T, Haddad A, et al. The Role of EMMPRIN/CD147 in Regulating Angiogenesis in Patients With Psoriatic Arthritis. *Arthritis Res Ther* (2020) 22:240–52. doi: 10.1186/s13075-020-02333-6
33. St-Pierre Y, Couillard J, Van Themsche C. Regulation of MMP-9 Gene Expression for the Development of Novel Molecular Targets Against Cancer and Inflammatory Diseases. *Expert Opin Ther Targets* (2004) 8:473–89. doi: 10.1517/14728222.8.5.473
34. Cantatore FP, Maruotti N, Corrado A, Ribatti D. Anti-Angiogenic Effects of Biotechnological Therapies in Rheumatic Diseases. *Biol Targets Ther* (2017) 11:123–8. doi: 10.2147/BTT.S143674
35. Grass GD, Toole BP. How, With Whom and When: An Overview of CD147-Mediated Regulatory Networks Influencing Matrix Metalloproteinase Activity. *Biosci Rep* (2016) 36:e00283 (16 pages). doi: 10.1042/BSR20150256
36. Nishimoto N, Terao K, Mima T, Nakahara H, Takagi N, Kakehi T. Mechanisms and Pathologic Significances in Increase in Serum Interleukin-6 (IL-6) and Soluble IL-6 Receptor After Administration of an Anti-IL-6 Receptor Antibody, Tocilizumab, in Patients With Rheumatoid Arthritis and Castleman Disease. *Blood* (2008) 112:3959–64. doi: 10.1182/blood-2008-05-155846
37. Metzger CS, Koutsimpelas D, Brieger J. Transcriptional Regulation of the VEGF Gene in Dependence of Individual Genomic Variations. *Cytokine* (2015) 76:519–26. doi: 10.1016/j.cyto.2015.07.015
38. Berra E, Milanini J, Richard DE, Le Gall M, Viñals F, Gothié E, et al. Signaling Angiogenesis *via* P42/P44 MAP Kinase and Hypoxia. *Biochem Pharmacol* (2000) 60:1171–8. doi: 10.1016/S0006-2952(00)00423-8
39. Yang J, McNeish B, Butterfield C, Moses MA. Lipocalin 2 is a Novel Regulator of Angiogenesis in Human Breast Cancer. *FASEB J* (2013) 27:45–50. doi: 10.1096/fj.12-211730
40. Gulkesen A, Akgol G, Poyraz AK, Aydin S, Denk A, Yildirim T, et al. Lipocalin 2 as a Clinical Significance in Rheumatoid Arthritis. *Cent Eur J Immunol* (2017) 42:269–73. doi: 10.5114/ceji.2017.70969
41. Zhao F, Dong J, Guo J, Bi L. Inhibiting Role of Long non-Coding RNA LINC01197 in Inflammation in Rheumatoid Arthritis Through the microRNA-150/THBS2 Axis. *Exp Cell Res* (2020) 394:112136. doi: 10.1016/j.yexcr.2020.112136
42. Pauley KM, Satoh M, Chan AL, Bubb MR, Reeves WH, Chan EKL. Upregulated miR-146a Expression in Peripheral Blood Mononuclear Cells From Rheumatoid Arthritis Patients. *Arthritis Res Ther* (2008) 10:1–10. doi: 10.1186/ar2493
43. Li J, Wan Y, Guo Q, Zou L, Zhang J, Fang Y, et al. Altered microRNA Expression Profile With miR-146a Upregulation in CD4+T Cells From Patients With Rheumatoid Arthritis. *Arthritis Res Ther* (2010) 12:1–12. doi: 10.1186/ar3006
44. Sabbatinelli J, Giuliani A, Matacchione G, Latini S, Laprovitera N, Pomponio G, et al. Decreased Serum Levels of the Inflammaging Marker miR-146a are Associated With Clinical non-Response to Tocilizumab in COVID-19 Patients. *Mech Ageing Dev* (2021) 193:111413. doi: 10.1016/j.mad.2020.111413
45. de la Rosa IA, Perez-Sanchez C, Ruiz-Limon P, Patiño-Trives A, Torres-Granados C, Jimenez-Gomez Y, et al. Impaired microRNA Processing in Neutrophils From Rheumatoid Arthritis Patients Confers Their Pathogenic

Profile. Modulation by Biol Therapies. *Haematologica* (2020) 105:2250–61. doi: 10.3324/haematol.2018.205047
46. Paterson MR, Kriegel AJ. MiR-146a/B: A Family With Shared Seeds and Different Roots. *Physiol Genomics* (2017) 49:243–52. doi: 10.1152/physiolgenomics.00133.2016
47. Bae SC, Lee YH. MiR-146a Levels in Rheumatoid Arthritis and Their Correlation With Disease Activity: A Meta-Analysis. *Int J Rheum Dis* (2018) 21:1335–42. doi: 10.1111/1756-185X.13338
48. Matsumoto J, Dohgu S, Takata F, Machida T, Bölükbaşi Hatip FF, Hatip-Al-Khatib I, et al. TNF-α-Sensitive Brain Pericytes Activate Microglia by Releasing IL-6 Through Cooperation Between Iκb-Nfκb and JAK-STAT3 Pathways. *Brain Res* (2018) 1692:34–44. doi: 10.1016/j.brainres.2018.04.023
49. Ibrahim YF, Moussa RA, Bayoumi AMA, Ahmed ASF. Tocilizumab Attenuates Acute Lung and Kidney Injuries and Improves Survival in a Rat Model of Sepsis *via* Down-Regulation of NF-κb/JNK: A Possible Role of P-Glycoprotein. *Inflammopharmacology* (2020) 28:215–30. doi: 10.1007/s10787-019-00628-y

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REVIEW
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Toward Overcoming Treatment Failure in Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is an autoimmune disorder characterized by inflammation and bone erosion. The exact mechanism of RA is still unknown, but various immune cytokines, signaling pathways and effector cells are involved. Disease-modifying antirheumatic drugs (DMARDs) are commonly used in RA treatment and classified into different categories. Nevertheless, RA treatment is based on a "trial-and-error" approach, and a substantial proportion of patients show failed therapy for each DMARD. Over the past decades, great efforts have been made to overcome treatment failure, including identification of biomarkers, exploration of the reasons for loss of efficacy, development of sequential or combinational DMARDs strategies and approval of new DMARDs. Here, we summarize these efforts, which would provide valuable insights for accurate RA clinical medication. While gratifying, researchers realize that these efforts are still far from enough to recommend specific DMARDs for individual patients. Precision medicine is an emerging medical model that proposes a highly individualized and tailored approach for disease management. In this review, we also discuss the potential of precision medicine for overcoming RA treatment failure, with the introduction of various cutting-edge technologies and big data.

Keywords: rheumatoid arthritis, DMARDs, biomarker, treatment failure, precision medicine

INTRODUCTION

Rheumatoid arthritis (RA), an autoimmune disorder that preferentially attacks the joints, affects approximately 1% of people worldwide (1). RA patients experience morning stiffness in the early stage, which manifests as facet joint pain, swelling, and synovitis. In the late stage, small focal necrosis and granulation tissue pannus formation appear, spreading to the cartilage surface, accompanied by symmetrical polyarticular swelling, bone erosion and pain mainly in the interphalangeal and metacarpophalangeal joints and limited mobility (2). Finally, granulation

tissue and fibrous tissue adhesion appear on the articular surface, forming deformity symptoms such as ankylosis and joint subluxation. Most patients also present with extra-articular multisystem involvement in skin, blood, kidneys and lungs, further aggravating the condition (2).

The exact mechanism of RA development is unknown, but both genetic and environmental factors are contributory. Various proinflammatory cytokines and immune cells are involved in RA pathophysiology (3). In the progress of RA, the synovium is infiltrated by leukocytes and synovial fluid is inundated with pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-6, IL-17 and IL-1β (4). These cytokines induce an inflammatory cascade characterized by interactions of fibroblast-like synoviocytes (FLS) with innate immune cells, including macrophages, monocytes, dendritic cells and mast cells, as well as adaptive immune cells such as B and T cells. TNF also promotes bone resorption and bone erosion. IL-1 indirectly stimulates osteoclast formation. IL-6 aggravates pathogenic effects in RA by enhancing the inflammatory effects of IL-1 and TNF (4). An imbalance between osteoblasts/osteoclasts and regulatory T (Treg)/T helper (Th)17 cells are typical characteristics of RA (5).

RA requires the combined effects of different signaling pathways, such as receptor activator of nuclear factor kappa B ligand (RANKL)/receptor activator of nuclear factor kappa B (RANK)/osteoprotegerin (OPG) and IL-6/glycoprotein 130 (gp130)/janus kinase (JAK)/signal transducer and activator of transcription (STAT). For RANKL/RANK/OPG signaling, binding of RANKL to RANK induces nuclear factor kappa B (NF-κB) activation, which upregulates levels of pro-inflammatory cytokines (IL-1, IL-6 and TNF) and mediates proliferation of T and B cells (6). OPG binds explicitly to RANKL and inhibits RANKL activity by preventing its binding to RANK. RANKL promotes the differentiation and production of osteoclasts. For IL-6/gp130/JAK/STAT signaling, IL-6 binds to IL-6 receptor (IL-6R). The IL-6/IL-6R complex interacts with gp130 to induce its dimerization and initiate intracellular signaling *via* JAK/STAT pathway, thus increasing T cell activity, inhibiting FLS apoptosis, allowing B cell maturation and stimulating differentiation of naive T cells into Th17 cells (7).

To assess disease activity of RA, the American College of Rheumatology (ACR), the European League Against Rheumatism (EULAR) and the World Health Organization/International League Against Rheumatism (WHO/ILAR) have established a core set of variables, which include swollen joint count (SJC), tender joint count (TJC), physician's global assessment of disease activity (PhGA), patient's global assessment of disease activity (PtGA), patient's assessment of pain, patient's assessment of physical function, and level of an acute phase reactant (APR, either C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR)) (8). Based on these variables, composite measurement tools with strong clinimetric properties have been developed. Among these tools are dichotomous indices like the ACR response criteria (ACR20, 50 and 70) (9), and continuous scores like the Disease Activity Score for 28 joints (DAS28), the Clinical Disease Activity Index (CDAI) and the Simplified Disease Activity Index (SDAI) (10). ACR20, 50 and 70 are based on improvement of at least 20%, 50% and 70% in both TJC and SJC, and three of the five additional core set of variables listed above, respectively. DAS28 considers TJC and SJC of 28 joints, PtGA, plus level of an APR (either ESR or CRP) (11). CDAI is based on the simple summation of TJC and SJC of 28 joints, along with PhGA and PtGA (12). SDAI is the arithmetic sum of TJC and SJC of 28 joints, PhGA, PtGA and level of an APR (CRP) (12). These tools allow better standardization and interpretation of disease activity of RA and patient response to therapy.

The EULAR has updated its recommendations for the management of RA in 2019, which are regarded as the main guidelines worldwide. In this update, most of the recommendations remain unchanged when reviewing its first version one decade ago and the updates in 2013 and 2016. The target of treatment remains as sustained remission (according to the ACR-EULAR definition) or low disease activity, and the major focus continues to be pharmacological therapy with disease-modifying antirheumatic drugs (DMARDs) (13). The DMARDs are divided into conventional synthetic (cs) DMARDs (such as methotrexate, leflunomide and sulfasalazine), biological (b) DMARDs [TNF inhibitors (infliximab, etanercept, adalimumab, certolizumab pegol and golimumab), a T cell co-stimulation inhibitor (abatacept), a cluster of differentiation 20 (CD20) inhibitor (rituximab), IL-6R inhibitors (tocilizumab and sarilumab) and biosimilar (bs) DMARDs)] and targeted synthetic (ts) DMARDs [JAK inhibitors (such as tofacitinib, baricitinib and upadacitinib)] (13).

Over the past years, the management of RA has progressed remarkably, encompassing the development of the above measurement tools and approvals of various DMARDs. However, the response is not universal for any treatment option. A large number of clinical trials have demonstrated that substantial proportions of RA patients experience treatment failure after receiving csDMARDs and even bDMARDs and tsDMARDs (14–19). Treatment failure is defined as nonresponse or limited efficacy (16, 17, 20), including initial lack of response, responsiveness over time, and inadequate response (partial response). To date, continuous efforts have been made toward overcoming treatment failure in RA patients, such as identification of biomarkers for response or nonresponse to DMARDs, exploration of the reasons for loss of efficacy, development of sequential or combinational DMARDs strategies either within the same or different mechanistic class, and approval of new DMARDs (13). Some of them provide valuable insights that can help to improve the design of future clinical trials and enable accurate clinical medication (21).

Due to the striking heterogeneity of RA, people realize that the current efforts are far from enough to recommend specific DMARDs for individual patients, which is also highlighted by EULAR as an important issue to be addressed in the future (13). Precision medicine, also called personalized medicine, is an emerging medical model that proposes a highly individualized and tailored approach for patient management by accounting for individual variability in genes, environment, and lifestyle, instead of a one-drug-fits-all model (22). It involves the ability to classify individuals into subpopulations that are susceptible or responsive to a specific treatment (23). Precision medicine is in

its infancy and has not become a routine practice in RA. But it is anticipated that precision medicine would have tremendous potential to address the treatment failure for RA (24).

In this review, we summarize the current efforts in identifying biomarkers for DMARDs, exploring the reasons for loss of efficacy, developing sequential or combinational DMARDs strategies and approving of new DMARDs, toward overcoming treatment failure in RA. We also discuss the opportunities and advantages of precision medicine approaches to make a breakthrough in diagnosis, prognosis, and treatment selection for RA.

csDMARDs

Methotrexate: Mechanism, Biomarkers and Alternative Therapy

Despite the wealth of new agents, methotrexate approved by FDA in 1988 remains the primary starting therapy and anchor drug for the treatment of RA, owing to its inexpensive cost, extended safety record, and weekly treatment regimen (25). Mechanism of action in methotrexate is not fully understood. DMARD activity of methotrexate is thought to be due to its polyglutamated form and several mechanisms have been proposed to explain the clinical efficacy in RA, including generation of reactive oxygen species, antagonism of folate-dependent processes, inhibition of methyl-donor production, downregulation of adhesion-molecule, eicosanoids and matrix metalloproteinases (MMPs) expression, modification of cytokine profiles, stimulation of adenosine signaling, inhibition of RANKL/RANK/OPG and JAK/STAT pathways (26–28). Clinical trials with methotrexate monotherapy demonstrate that only 40% of patients with early RA obtain a good response based on ACR50 criteria (29).

Currently, adenosine signaling carries the most robust data for the action of methotrexate in RA (30). Both adenosine A2A receptor (ADORA2A) and A3 receptor (ADORA3) are required for the anti-inflammatory effects of methotrexate (31). The expression of ADORA2A and ADORA3 is increased on immune cells and inversely correlated with disease activity in RA patients (32). It is possible that RA patients with low expression of adenosine receptors will be less responsive to methotrexate. In a study with methotrexate monotherapy, RA patients were categorized into three groups, i.e. good, moderate and nonresponders. A low level of baseline *ADORA3* mRNA expression in blood is associated with nonresponse to methotrexate and could serve as a potential biomarker for distinguishing response to methotrexate therapy in RA (33). Adenosine signaling through ADORA2A leads to the development of Tregs expressing both CD39 and CD73 that may decrease T cell activation (34, 35). A prospective study found that RA patients who did not respond to methotrexate had lower pretreatment CD39 expression on Tregs than methotrexate-responsive patients or healthy controls, suggesting that low expression of CD39 on Tregs could be a biomarker for identifying methotrexate-resistant RA patients (34). Clinical observations suggested that RA patients who had a high intake of adenosine receptor antagonists (such as caffeine) had impaired methotrexate responsiveness, which was consistent with data from animal models (36, 37). However, conflicting evidence manifested that methotrexate efficacy was not affected by adenosine receptor antagonists (38, 39).

In addition to the adenosine signaling, exploration of pharmacometabolic markers, expression and polymorphisms of genes linked to the action of methotrexate is underway to identify methotrexate-responsive or nonresponsive signatures. In a longitudinal study, an increase in methotrexate polyglutamates in erythrocytes was associated with lower disease activity of RA and thought to be a tool for monitoring methotrexate response (40). Changing from oral to subcutaneous methotrexate resulted in increased methotrexate polyglutamates and achieved a better improvement of disease activity of RA (41). A low baseline folate level was associated with a poor response to methotrexate and folate polyglutamate partially antagonized methotrexate efficacy (40, 42). Breast cancer resistance protein [BCRP, gene symbol *ATP Binding Cassette Subfamily G Member 2* (*ABCG2*)] is an ATP-binding cassette efflux transporter that plays an important role in multidrug resistance (43). BCRP transported both methotrexate and polyglutamylated methotrexate and participated in methotrexate resistance (44). Good response to methotrexate was associated with a decrease in expression of BCRP in RA patients (45), while the association of BCRP polymorphisms with the effectiveness of methotrexate was not observed (46), suggesting that BCRP expression was not genetically determined, but might be associated with environmental factors (45). BCRP inhibition could be a strategy for overcoming nonresponse to methotrexate. Consistently, a six-month randomized, double-blind trial enrolling 148 RA patients showed that individuals with partial responses to methotrexate had clinical improvement after combination therapy with an FDA-approved BCRP inhibitor cyclosporine and methotrexate (47). Other studies found that baseline FcγRIIIa expression on CD14+ monocytes was negatively associated to methotrexate response in patients with early RA (48). Circulating miR-10a was upregulated in RA patients with good methotrexate response (49). *Human leukocyte antigen (HLA)-DRB1* shared epitope alleles were linked to a lack of response to methotrexate at the genomic level (50). Stratification based on *HLA-DRB4* allele expression revealed distinct innate and adaptive immune transcriptional patterns in early RA and response to methotrexate therapy could be suggested by a preponderance of innate but not adaptive immune activation (51). A number of single-nucleotide polymorphisms have been investigated for the prediction of methotrexate treatment response. Patients with *solute carrier family 19 A (SLC19A)* rs1051266, *dihydrofolate reductase (DHFR)* rs836788 and *thymidylate synthetase (TYMS)* rs2244500 showed response to methotrexate, while patients with *5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (ATIC)* rs7563206, *TYMS* rs3786362 and rs2847153 showed reduced effectiveness of methotrexate (52). Patients with *folylpolyglutamate synthetase (FPGS)* rs1544105-AA or -AG and *TYMS* rs2853539-AA genotype were associated

with poor response to methotrexate (53). An analysis of the -174 (rs1800795) -GC *IL-6* gene promoter polymorphism in RA patients revealed that genotype -GG may be associated with a poorer response to methotrexate when compared to genotypes -GC and -CC (54), which was in disagreement with another study identifying no association between -GG genotype or G allele and risk of therapeutic failure using different measures for defining response to therapy (55) (**Table 1**).

In RA patients with treatment failure of methotrexate, combination therapy is an attractive alternative strategy. A series of clinical trials (such as an observational and descriptive CONAART study enrolling 106 RA patients, a 24-week, randomized, double-blind, controlled SLCTR study enrolling 40 patients and a 48-week, randomized, double-blind, placebo-controlled study enrolling 263 patients) showed that leflunomide in combination with methotrexate was effective for RA patients who did not respond to methotrexate (65, 66). In a 12-month, multicenter, randomized, double-blind, placebo-controlled, parallel-design, dose-finding phase II trial, 115, 105, and 119 RA patients with inadequate response to methotrexate were grouped to receive 2 mg/kg abatacept, 10 mg/kg abatacept, and placebo, in addition to continued methotrexate treatment, respectively. Results showed that 10 mg/kg abatacept presented better anti-inflammatory effects than either 2 mg/kg abatacept and placebo (67). In a double-blind, randomized, parallel-arm MUSICA trial enrolling 309 methotrexate nonresponders, patients were randomly assigned to receive either a high dose (20 mg/week) or a low dose (7.5 mg/week) of methotrexate and received open-label adalimumab for 24 weeks. Adalimumab treatment resulted in a rapid improvement in clinical indices in both groups, which is consistent with results from an OPTIMA study (a 78-week, randomized, double-blind, double-period phase 4 trial enrolling 348 methotrexate inadequate responders) (68) and a PREMIER study (a 2-year, randomized, double-blind, placebo-controlled phase 3 trial enrolling 177 methotrexate inadequate responders) (68). In a prospective, randomized, controlled SURPRISE study enrolling 223 RA patients, tocilizumab in combination with methotrexate more rapidly deceased inflammation than tocilizumab switched from methotrexate, resulting in greater clinical effectiveness and avoidance of joint damage (69).

Leflunomide: Mechanism, Biomarkers and Alternative Therapy

Leflunomide, approved by the FDA in 1998, is the first choice if methotrexate is contraindicated according to the latest EULAR recommendations (13). Leflunomide acts *via* its active metabolite A77 1726 after the metabolic opening of the isoxazole ring. Its primary target is thought to be dihydroorotate dehydrogenase (DHODH), an enzyme involved in *de novo* pyrimidine production (70). Leflunomide inhibits DHODH activity, resulting in nucleotide depletion, leading to cell cycle arrest and reproduction of rapidly dividing cells, particularly lymphocytes (71). Tyrosine kinases such as Lck and JAK3 in activated T and B cells are also targets of leflunomide (72). Clinical trials have shown that only 40-50% of RA patients taking

TABLE 1 | Potential biomarkers for response or partial response/nonresponse to csDMARDs.

csDMARDs	Biomarkers for response	Sample size	Reference	Biomarkers for partial response/nonresponse	Sample size	Reference
Methotrexate	Increased methotrexate polyglutamates in erythrocytes	285	(40)	Low level of baseline ADORA3 mRNA expression in blood	100	(33)
	A decrease in expression of BCRP	24	(45)	Lower pretreatment expression of CD39 on Tregs	122	(34)
	Upregulated circulating miR-10a	30	(49)	High intake of adenosine receptor antagonists	39	(36)
	Preponderance of innate immune activation	68	(51)	Low baseline folate level	226	(42)
	SLC19A rs1051266, DHFR rs836788, and TYMS rs2244500	35	(52)	Higher baseline FcγRIIIa expression on CD14+ monocytes	38	(48)
				HLA-DRB1 shared epitope alleles	102	(50)
				ATIC rs7563206, TYMS rs3786362 and rs2847153	35	(52)
				FPGS rs1544105-AA or -AG and TYMS rs2853539-AA	281	(53)
				IL-6 rs1800795 -GC	70	(54)
Leflunomide	Higher A77 1726 steady-state plasma concentration	67	(56)	DHODH rs3213422 A allele	147	(57)
	Estrogen receptor 1 rs9340799-rs2234693 A/T haplotype	115	(58)	Estrogen receptor 1 rs9340799-rs2234693 G/C	115	(58)
				IL-6 rs1800795 -GG	96	(55)
				Higher serum baseline CRP level	250	(59)
Sulfasalazine	Higher exosomal miR-328 in plasma	33	(60)	Higher serum P-gp level	151	(61)
	BCRP rs2231142 -AC or -AA genotype	229	(62)	Increased level of BCRP	229	(62)
	Low interferon IFN/IL-4 ratio	11	(63)			
	HLA-B27-positive	132	(64)			
	Low level of soluble IL-2 receptor	195	(63)			

ADORA3, adenosine A3 receptor; ATIC, ribonucleotide formyltransferase; BCRP, breast cancer resistance protein; CRP, C-reactive protein; DHFR, dihydrofolate reductase; DHODH, dihydroorotate dehydrogenase; FPGS, folylpolyglutamate synthetase; HLA, human leukocyte antigen; IFN, interferon; P-gp, P-glycoprotein; SLC19A, solute carrier family 19 A; TYMS, thymidylate synthetase.

leflunomide fulfilled the ACR response criteria for a 20% reduction in disease activity (59).

DHODH is located on the inner membrane of mitochondria (73). The human DHODH gene is relatively conserved, with only one common missense polymorphism (rs3213422) in the first exon (19C>A). This polymorphism led to Gln7Lys amino acid substitution in the cationic N-terminal region of the DHODH polypeptide, which was essential for transport and correct insertion into the mitochondrial inner membrane (57). A study reported that RA patients with A allele had a worse response to leflunomide than patients with the C allele (57). A proposed mechanism was that the amino acid substitution generated by the missense polymorphism in *DHODH* might block its import into mitochondria and subsequently affect the action of leflunomide (57). However, another study did not replicate the association between leflunomide response and rs3213422 in a smaller cohort of indviduals (74). Cytochromes P450 (CYP) enzymes, including CYP1A2 and CYP2C19, may be implicated in the conversion of leflunomide to A77 1726. Better response to leflunomide was accompanied by higher A77 1726 steady-state plasma concentration, which was influenced by *CYP2C19**2 allele rather than *CYP1A2* polymorphism (56). Evidence demonstrated that the efficacy of DMARDs is more effective in men than in women and estrogens play important roles in the immune response. A study found that the A/T haplotype of the *estrogen receptor 1 (ESR1)* rs9340799-rs2234693 was related with a better sensitivity to leflunomide, while the G/C haplotype was associated with a worse response (58). Researchers also evaluated the influence of the rs1800795-GC *IL-6* gene promoter polymorphism on the therapeutic failure of leflunomide (54, 55, 75). RA patients with *IL-6* rs1800795-GG genotype had a higher risk of failure in therapeutic response to leflunomide when compared to patients with -GC (55), which was contrary to other observations of noninfluence of the rs1800795-GC *IL-6* gene polymorphism on response to leflunomide that used different measures for defining response to therapy (54, 75). Similar to that of methotrexate, drug efflux transporter BCRP was reported to interact with leflunomide and A771726, and an increased level of BCRP might contribute to inadequate response to leflunomide (76). In a 12-month open, prospective trial enrolling 106 RA patients, the combination of a BCRP inhibitor cyclosporine and leflunomide provided statistically significant benefit (77), suggesting that BCRP inhibition could be a potential approach for improving the nonresponse to leflunomide.

Recently, our group found that RA individuals with limited efficacy of leflunomide could be distinguished by higher serum baseline CRP level. Besides the immunomodulation *via* A77 1726, we revealed that leflunomide itself induced aryl hydrocarbon receptor (AHR)-AHR nuclear translocator (ARNT) interaction to inhibit hepatic CRP production and attenuate bone erosion in arthritic rat models. Nevertheless, enforced CRP expression upregulated hypoxia-inducible factor 1α (HIF1α), which competed with AHR for ARNT association and interfered leflunomide-AHR-CRP signaling, leading to nonresponse to leflunomide in arthritic rat models. Hepatocyte-specific HIF1α deletion or an FDA-approved HIF1α inhibitor Acriflavine re-activated leflunomide-AHR-CRP signaling to inhibit bone erosion in leflunomide-nonresponsive animals. This study presented a precision medicine-based therapeutic strategy for overcoming nonresponse to leflunomide in RA (59). In addition, we also performed a 48-week, randomized, controlled clinical trial enrolling 123 RA patients, and showed that leflunomide combined with ligustrazine extracted the Chinese herb Chuanxiong, which was an approved drug in China and had the capacity to inhibit HIF1α expression (78), could significantly reduce disease activity (79). Regarding other alternative treatment options, leflunomide plus infliximab present a general improvement in disease control compared with leflunomide alone in an open, multicenter, retrospective study (80). In a 24 week, double-blind phase of the multicenter, international RELIEF study enrolling 106 inadequate responders to leflunomide, the trend of benefit was indicated for combining leflunomide with sulfasalazine compared with switching to sulfasalazine alone (81) (**Table 1**).

Sulfasalazine: Mechanism, Biomarkers and Alternative Therapy

Based on the latest EULAR recommendation, sulfasalazine approved by FDA in 1996 is also considered as part of the (first) treatment strategy in RA patients with a contraindication to methotrexate, which is in parallel with leflunomide (13). Among the above agents, sulfasalazine has an acceptable safety profile during pregnancy (82, 83). The mechanism of sulfasalazine is not entirely understood. It is unknown if sulfasalazine or its metabolites such as sulfapyridine and 5-aminosalicylic acid have a role in its anti-inflammatory actions. It is suggested that sulfasalazine inhibits TNF expression by suppressing NF-κB and by inducing caspase 8-induced apoptosis in macrophages (84). Sulfasalazine inhibits osteoclast formation by suppression of RANKL and stimulation of osteoprotegerin (85). Sulfasalazine induces the conversion of adenine nucleotides to adenosine (86). Sulfapyridine and 5-aminosalicylic acid inhibit B cell function and suppress the production of IgM and IgG (87). Sulfapyridine inhibits chemokines IL-8, growth-related gene product-alpha (gro alpha), and monocyte chemotactic protein-1 (MCP-1/CCL2) (88). 5-aminosalicylic acid (5-ASA) inhibits NF-κB signaling by inducing phosphorylation and activation of adenosine monophosphate-activated protein kinase (89). Studies have confirmed that the ACR20 response in RA patients does not exceed 50% after 6 months of sulfasalazine treatment (90, 91).

Research showed that sulfasalazine also interacted primarily with the above-mentioned drug efflux transporter BCRP. BCRP knockout mice had a more than 100-folds increase in plasma concentration of sulfasalazine compared with wild-type (WT) mice (62). Sulfasalazine bioavailability in BCRP knockout mice was 97% compared to 3% in WT mice (92). Of note, treatment of WT mice with a BCRP inhibitor (gefitinib) resulted in a significant increase in plasma concentration and bioavailability of sulfasalazine (92). This suggests that BCRP could be a therapeutic target for eliminating nonresponse to sulfasalazine. Another study found a circulating intestine-derived exosomal

miR-328 in plasma, which negatively regulated BCRP expression and resulted in a high plasma concentration of sulfasalazine (60), could be a biomarker of sulfasalazine responsiveness. P-glycoprotein (P-gp), like BCRP, is another drug efflux transporter. It was reported that serum P-gp level was higher in patients with active RA compared to inactive RA patients (93). Serum P-gp level was negatively correlated with sulfasalazine efficacy (61). Sulfasalazine oral bioavailability was markedly increased 2-3 folds in P-gp knockout rats (94). P-gp on Th1 cells participated in the drug resistance to sulfasalazine in RA (95). These results inspire a hypothesis that blockage of P-gp may mimic the effectiveness of BCRP inhibition in overcoming the nonresponse to sulfasalazine. However, it was discouraging to observe that a P-gp inhibitor verapamil could not reverse sulfasalazine nonresponse (96). We assumed that BCRP was mainly responsible for efflux transport of sulfasalazine because plasma concentration of sulfasalazine was more significantly increased in BCRP knockout animals (more than 100 folds) when compared to that in P-gp knockout animals (2-3 folds). It is possible that pharmacological inhibition of P-gp by verapamil could be compensated by the powerful BCRP function in the efflux of sulfasalazine, which should be verified in future studies. Regarding the gene polymorphism, an association between *ABCG2* genotype and remission was found, and carriers of the loss of function alleles (that is, *ABCG2* rs2231142 -AC or -AA genotype) had higher plasma sulfasalazine concentrations (62). Other reports indicated that a low interferon (IFN)/IL-4 ratio is associated with a better response to sulfasalazine (63). HLA-B27-positive patients presented a better response to sulfasalazine (64). A low level of soluble IL-2 receptor predicted remission in early RA patients treated with sulfasalazine (63). Sulfasalazine responders had lower serum MMP-3 values compared to partial responders or nonresponders (97) (**Table 1**).

In an 18-month, randomized, double-blind, placebo-controlled MASCOT study, 165 RA patients who were nonresponders to 6-month sulfasalazine therapy were grouped to receive methotrexate, sulfasalazine and a combination of sulfasalazine and methotrexate for additional 12 months, respectively. The combination significantly decreased DAS and improved the ACR scores when compared to either drug alone (98). This study, together with other randomized controlled trials, were included in a meta-analysis, which suggested that the addition of methotrexate to sulfasalazine is a therapeutic option in SSZ sulfasalazine failure (99). In a 2-year, double-blind, randomized study, 260 sulfasalazine nonresponders were randomly assigned to etanercept, sulfasalazine and etanercept plus sulfasalazine, respectively. A significant improvement was seen in the group treated with etanercept plus sulfasalazine when compared to the other two groups (100). In a 52-week, multicenter, double-blind, parallel-group trial, a total of 123 DMARDs (including sulfasalazine) nonresponders were randomized to receive tacrolimus (an inhibitor targeting calcineurin, which is involved in the production of IL-2) or placebo. Data showed that tacrolimus was helpful for achieving a better clinical response according to ACR20 and EULAR response criteria (101).

bDMARDs TARGETING TNF

Infliximab: Mechanism, Biomarkers and Alternative Therapy

TNF has been identified its central role in RA at the end of the last century. At the time, little was known about the mechanisms of csDMARDs and people had no better choice for treating RA. This led to a question about whether blockade of TNF could serve as a treatment method. With the development of monoclonal antibodies, this question was firstly answered. In 1992, cA2, now known as infliximab, was produced to confirm that the inflammation driving RA could be suppressed by TNF blockade (102). Infliximab was approved by the FDA in 1999 for RA treatment and attracted an inordinate amount of attention over the past several decades (103). According to the most recent EULAR recommendation, if the treatment goal is not met with the initial csDMARD strategy and there are poor prognostic factors, a bDMARD should be added (13). TNF inhibitors are now the most frequently used bDMARDs (104) and infliximab serves as a first-in-class TNF inhibitor (103). Infliximab is an intravenous administrated, chimeric monoclonal IgG1κ antibody composed of human constant (75%) and murine variable (25%) regions (105). Infliximab binds to both soluble and transmembrane forms of TNF with high affinity, inducing the downregulation of local and systemic pro-inflammatory cytokines (e.g., IL-6), the reduction of lymphocyte and leukocyte migration to sites of inflammation, the induction of apoptosis in TNF-producing cells and the reduction of levels of endothelial adhesion molecules and APR (105). Only approximately 50% of RA patients showed ACR20 response after receiving infliximab treatment (106, 107).

Anti-drug antibodies (ADAs), generated by a T-cell dependent or independent B cell activation pathway, primarily contribute to a poor clinical outcome of biological treatment (108–110). In fact, there are two types of ADAs that can be produced: non-neutralizing antibodies that bind to the medication alongside TNF and neutralizing antibodies that compete with TNF for the antigen-binding site (paratope). Neutralizing antibodies can therefore immediately inhibit the working mechanism of the anti-TNF agents (111). Several clinical trials demonstrated that ADAs might be associated with treatment failure of infliximab (112, 113). Over 40% of patients treated with infliximab developed ADAs (114). Of interest, it was reported that concomitant administration of csDMARDs such as methotrexate might decrease ADAs and prolong therapeutic efficacy (115). Furthermore, the emergence of ADAs may be related with lower serum concentrations of (free) infliximab and a lower clinical response (116). Low infliximab serum concentrations, even 2 months after treatment commencement, were associated with the production of ADAs and predicted later treatment failure (117). However, another study found that lack of response could be due to a lack of infliximab, rather than the presence of ADAs (118), which in line with a hypothesis that drug tolerance was not directly related to the quantity of anti-drug antibodies, but rather depended on the size of the response in relation to the amount of drug that could be neutralized (115).

S100 calcium-binding protein A4 (S100A4) is a metastasis-inducing protein, which promotes the inflammatory response of mononuclear cells *via* the Toll-like receptors (TLR4) signaling in RA (119). It was reported that high S100A4 level was associated with inadequate response to infliximab, ADAs production and high levels of survivin and FMS-like tyrosine kinase 3 (Flt3) ligand (120). Flt3 ligand is a differentiation factor that has predictive value in the preclinical diagnosis of RA (121). Survivin is a downstream molecule of Flt3 signaling and high survivin level predicted poor clinical response to infliximab in RA patients (121, 122). It was proposed that S100A4, survivin and Flt3 ligand could form a new cluster of predictive biomarkers for infliximab nonresponders (120). Another study developed a customized low-density microarray for monitoring mRNA expression in peripheral blood cells, which was helpful for identifying a unique set of genes with differential expressions in infliximab responders and nonresponders. It was important to note that TNF-α itself did not differ significantly between responders and nonresponders, while a clear difference was observed in the kinetics of IFN-related genes during infliximab treatment between the two groups. Specifically, there was sustained inhibition of the IFN signature in responders and reappearance of the signature in nonresponders during infliximab treatment. The underlying mechanism remains to be clarified, and such knowledge will likely identify new therapeutic targets for RA (123). In addition, other data showed that AP-1-associated adaptor complex subunit responsible for protein transport between membrane compartments in receptor-mediated endocytosis was significantly downregulated in peripheral mononuclear cells of infliximab nonresponders (123). TNF receptor recycling was inhibited in nonresponders to infliximab (123). Human immunoglobulin allotypes in the IgG_1 heavy chain (G_1m1 and G_1m17 allotypes) were associated with response to infliximab (124). A significantly decreased CRP level was a predictor of good response with infliximab treatment (125). Patients with low-affinity homozygotes, *fragment crystallizable (Fc) fragment of IgG receptor (FCGR)2A* and *FCGR3A* alleles showed better response to infliximab (126). The baseline level of IgG antibodies against centromere protein F was significantly increased in infliximab-responders (127). In patients with early RA, infliximab < 0.2 μg/mL, and ADAs development were associated with treatment failure and were more common in females (112). TNF level in the intimal lining layer and synovial sublining and number of macrophages, macrophage subsets and T cells were significantly higher in responders than in nonresponders (128). Infliximab responders had a higher number of CD4+CD25+ T cells than nonresponders at baseline (129).

For the gene polymorphism, a series of studies showed that RA patients carrying *TNF-α* rs1800629 -GG genotype were better responders to infliximab, while the presence of A allele significantly decreases the response to infliximab (130–133). *TNF receptor superfamily member 1B* (*TNFRSF1B*, codes TNF receptors 2 (TNFR2)) rs1061622-GG or -TG was related to a lower responsiveness to infliximab (134, 135), while -TT genotype of the *TNFRSF1B* rs1061622 was a predictor of good response to infliximab (136, 137). A possible explanation was that the rs1061622 T>G induced an amino acid substitution at codon 196 (M196R), which located in the fourth cysteine-rich domain of the extracellular region of TNFR2. The R allele elicited a high inflammatory response *via* the TNF- pathway, which could explain the poor response to anti-TNF medication (134). Studies also confirmed that patients with *TNFRSF1B* rs3397-CC and *TNFRSF1B* rs1061631-AA genotypes had an increased risk for nonresponse to infliximab (135). *TNF receptor superfamily member 1A (TNFRSF1A*, codes TNF receptors 1 (TNFR1)) rs767455-AA genotype was associated with a worse EULAR response than -AG or -GG genotype (138). RA patients with homozygous rs396991 polymorphism (V158F) in *FCGR3A* had good response to infliximab (139) (**Table 2**).

In a cohort study, in the context of methotrexate, 95 consecutive patients with RA who were first treated with infliximab were switched to etanercept due to a lack of response (either primary, secondary, or with toxicity). Significant DAS28 reductions and ACR response were reported in the overall cohort and nonresponse subtype groups after 12 weeks of medication. Sixty-one percent of the group received a moderate or good EULAR score, confirming that etanercept was successful in patients who did not respond to infliximab (180). In a 12-week, multicenter, open-label clinical study involving 6610 difficult-to-treat patients using DMARDs (including 11% infliximab), results showed that adalimumab alone or in combination with standard DMARDs was effective to improving ACR20 response and EULAR response (181). In a 12-week, double-blind period of the phase IIIb trial, 1063 DMARDs nonresponders (37.6% had previous TNF inhibitor use including infliximab) were randomized to certolizumab pegol or placebo. Certolizumab pegol was linked to faster and more consistent clinical responses as well as increased physical function (182). In a prospective, 12-week, open label, single-arm, observational trial, 25 patients were enrolled, 18 of whom had stopped taking infliximab due to inefficacy, and 22 who had completed 12 weeks of switching etanercept medication. After 12 weeks, 64% of patients had an ACR20 response (183).

Etanercept: Mechanism, Biomarkers and Alternative Therapy

Etanercept was originally developed for treating sepsis but failed in clinical trials. It was then tested for treating RA (184). Etanercept was approved by FDA in 1998 (185), one year ahead of infliximab. Etanercept is a soluble fusion protein consisting of two human 75 kD TNFR 2, each linked to an Fc portion of human IgG_1 (186). Functioning as a decoy receptor, etanercept binds to both TNF-α and TNF-β with much greater affinity than endogenous soluble TNFRs, which is unique from other TNF inhibitors that are variants of anti-TNF antibodies (187). TNF inhibition with etanercept modifies various physiologic responses caused or regulated by TNF, including the expression of adhesion molecules involved in leukocyte migration, serum levels of cytokines (e.g., IL-6), and serum levels of MMP-3 (188–190). Only approximately 40% patients achieved an ACR50 response when treated by etanercept monotherapy (191, 192).

TABLE 2 | Potential biomarkers for response or partial response/nonresponse to bDMARDs targeting TNF.

bDMARDs	Biomarkers for response	Sample size	Reference	Biomarkers for partial response/nonresponse	Sample size	Reference
Infliximab	Sustained inhibition of the IFN signature	18	(123)	ADAs production	128;26;69	(112, 113, 117)
	G_1m1 and G_1m17 allotypes	1037	(124)	Lack of infliximab	94	(118)
	Decreased CRP level	207	(125)	High S100A4 level	87	(120)
	Patients with low-affinity homozygotes, Fc fragment of FCGR2A and FCGR3A alleles	91	(126)	High survivin level	87	(122)
	Increased baseline level of IgG antibodies against centromere protein F	185	(127)	Downregulated AP-1-associated adaptor complex subunit	18	(123)
	Higher TNF level and number of macrophages, macrophage subsets and T cells	143	(128)	TNF receptor recycling was inhibited	18	(123)
	A higher number of CD4+CD25+ T cells at baseline	44	(129)	Infliximab < 0.2 μg/mL, and ADAs development	128	(112)
	TNF-α rs1800629 -GG	59;54	(130, 131)	TNF-α rs1800629 A allele	54;692	(131, 133)
	TNF-α rs1800629 G alleles	2127	(132)	TNFRSF1B rs1061622-GG or -TG	148;2637	(134, 135)
	TNFRSF1B rs1061622-TT	175;105	(136, 137)	TNFRSF1B rs3397-CC and TNFRSF1B rs1061631-AA	2637	(135)
	FCGR3A homozygous rs396991	41	(139)	TNFRSF1A rs767455-AA	280	(138)
Etanercept	TNF-α rs1800629-GG	86;86	(140, 141)	NUBPL rs2378945 A allele	755	(142)
	TNF-α rs1799724-TT or -CT	280	(138)	IL-10 promoter microsatellite allele IL10.G13	50	(143)
	TNFRSF1A rs767455-AA	280	(138)	Combination of TGF-β1 codon 25 C and IL-1RN intron 2 A2 allele	123	(144)
	TNFRSF1B rs1061622-TT	175	(136)	More methylated 4 CpG within exon 7 of LRPAP1	72	(145)
	TNFRSF1B rs1061622-TT	105	(137)			
	The combination of TNF-α rs1800629-GG and IL-10 rs1800896-GG	123	(144)			
	IL-6 rs1800795-GG	73;77;199	(146–148)			
	low TNF-α and IL-6 production	73	(146)			
	IL-10 promoter microsatellite allele IL10.R3 and the haplotype R3-G9	50	(143)			
	Higher expression of CD84	2706	(149)			
	Increased isoleucine, leucine, valine, alanine, glutamine	27	(150)			
	Increased tyrosine, glucose and 3-hydroxybutyrate	27	(150)			
	Higher baseline serum CRP, IL-1β and IL-17A	128	(151)			
Adalimumab	Finer ACPA specificities in ACPA-negative	286	(152)	Carrying the same IgG allotype as present on the adalimumab IgG	250	(153, 154)
	ACPA positive	646	(155)	< 9.4% of SIRPα/β-expressing memory B cells	57	(156)
	Decreased CD68 and MMP-3 expression in the synovium	5	(157)	The presence of ACPA	642	(158)
	Lower chemokine receptor 6 expression	48	(159)	Elevated baseline NLR and PLR	410	(160)
	Increased Th17 and Th1	48	(159)	TNF-α rs1800629 G haplotype in a homozygous form	388	(161)
	Elevated baseline CXCL10 and CXCL13	29	(162)			
	Increased expression of CD11c	27	(163)			
	Higher MRP 8/14	170	(164)			
	High sICAM1 and low CXCL13	69	(165)			
	TNF-α rs1800629-GG	81	(166)			
	TNF-α rs1799724-TT	280	(138)			
	TNFR1 rs767455-AA	280	(138)			
	TNFR2 676 rs1061622-TT	105	(137)			
	Low-affinity FCGR2A-R(A)* allele	302	(167)			
	IL-6 rs1800795-GG	199	(168)			
Certolizumab pegol	No Data	N/A	N/A	Low drug concentration	40	(169)
				Early response to certolizumab pegol	198	(170)
				Failure to achieve improvement in DAS28 within the first 3 months of therapy	783	(171)
				Failure to achieve improvements in DAS28(ESR) within the first 3 months	955	(172)
				Failure to achieve improvements in SJC or CDAI within the first 3 months	955	(172)
				CDAI nonresponse at 3 months	574	(173)

(Continued)

TABLE 2 | Continued

bDMARDs	Biomarkers for response	Sample size	Reference	Biomarkers for partial response/nonresponse	Sample size	Reference
				High serum pretreatment ratio of type I IFNβ/α (> 1.3) or undetectable type I IFN	124;15	(174, 175)
Golimumab	Golimumab concentration ≥ 1.0 mg/L	91	(176)	Sustained increase of IL-6, CRP, IL-2 receptor alpha chain, and MMP-1	138	(177)
	Decreased in serum amyloid A, E-selectin and MMP-9	137	(178)			
	Lower HSQ, ESR (or CRP) and TJC (or SJC) scores	3280	(179)			
	Being male, younger age, and absence of comorbidities	3280	(179)			

ACPA, Anti-citrullinated protein antibodie; ADAs, anti-drug antibodies; CD, Cluster of Differentiation; CDAI, Clinical Disease Activity Index; CRP, C-reactive protein; CXCL, C-X-C motif chemokine ligand 10; DAS, disease activity score; ESR, erythrocyte sedimentation rate; FCGR, Fc fragment of IgG receptor; HSQ, Health Status Questionnaire; IFN, interferon; IL-1RN, Interleukin 1 receptor antagonist; IL-2R, interleukin-2 receptor subunit; LRPAP1, LDL Receptor Related Protein Associated Protein 1; MMP, matrix metallopeptidase; MRP8/14, Myeloid-related protein 8/14; NLR, neutrophil-to-lymphocyte ratio; NUBPL, Nucleotide Binding Protein Like; PLR, platelet-to-lymphocyte ratio; RF, rheumatoid factor; SIRPα/β, Signal regulatory protein α/β; SJC, swollen joint count; Th, T helper; TJC, tender joint count; TNF, Tumor necrosis factor; TNFRSF, tumor necrosis factor receptor superfamily.

ADAs against etanercept were not consistently detected (193, 194) and had no relationship with reduced clinical response (195, 196), while low etanercept level was associated with nonresponse (197).This could be explained by the following two reasons. Firstly, etanercept formed smaller immune complexes compared to infliximab when bound to TNF, which might reduce uptake by antigen-presenting cells (198). Secondly, only the fusion part of the etanercept protein contained foreign epitopes while the TNF binding area did not, which led to low immunogenicity (197). Same as infliximab, *TNF-α* rs1800629-GG was associated with better response to etanercept than -AA or -AG (140, 141). *TNF-α* rs1799724-TT or -CT were associated with better response than -CC (199). *TNF-α* rs1799724-TT showed better response than C allele carriers (138). *TNFRSF1A* rs767455-AA was associated with better response than -GG (138); *TNFRSF1B* rs1061622-TT was associated with better response to etanercept (136, 137). A combination of alleles (*TNF-α* rs1800629-GG and *IL-10* rs1800896-GG) was associated with good response to etanercept (144). Other polymorphisms were also contributory. Several studies have confirmed that *IL-6* rs1800795-GG was associated with better response than -GC or -CC (146–148). Patients with the combination of *IL-6* rs1800795-GG and *TNF-α* rs1800629-GG genotype were more frequent among the responders compared to those with other combined genotypes. Patients with low TNF- and IL-6 production were the best responders to etanercept therapy (146). *Nucleotide-binding protein-like (NUBPL)* rs2378945 minor allele (A) had a significant association with poor response to etanercept (142). The *IL-10* promoter microsatellite allele IL10.R3 and the haplotype R3-G9 were considerably more prevalent in patients who responded well to etanercept, whereas IL10.G13 was more common in patients who responded moderately or no response (143).

A combination of C allele in codon 25 of the *transforming growth factor beta 1 (TGF-β1)* gene and the A2 allele in intron 2 of the *interleukin 1 receptor antagonist* (*IL-1RN*, codes IL-1Ra) gene, were associated with nonresponse to etanercept (144). On one hand, TGF-1 has been shown to suppress T-cell functions such as proliferation and differentiation of cytotoxic T-cells and T-helper cells (200). The homozygous genotype CC, whether at codon 10 or codon 25, was highly related with reduced TGF-1 production (201). On the other hand, patients carrying the *IL-1RN* A2 allele had increased production of IL-1 and possibly decreased IL-1Ra (202–204). It was the above two reasons that contributed to nonresponse to etanercept. In addition, 4 CpG within exon 7 of *LDL receptor related protein associated protein 1 (LRPAP1)* were observed to be more methylated in nonresponders (145). LRPAP1 is a receptor for TGF-β1 (205). We speculated that *LRPAP1* methylation blocked the function of TGF-β1 and then induced etanercept nonresponse. Thus, DNA methylation inhibitor might be helpful for such patients (206). Moreover, higher expression of CD84 was associated with better response to etanercept (149). Increased isoleucine, leucine, valine, alanine, glutamine, tyrosine, glucose and 3-hydroxybutyrate levels were associated with good response to etanercept (150). Higher baseline serum CRP, IL-1β and IL-17A were associated with better response to etanercept (151) (**Table 2**).

In an open-label, single-blind clinical trial, 28 patients with an inadequate response to etanercept were randomized 1:1 to receive infliximab, or to continue etanercept, with background methotrexate treatment. At week 16, 62% of infliximab-treated patients had ACR20 responses, compared to 29% of etanercept-treated patients (207). A multicenter, randomized, double-blind, placebo-controlled, phase III trial enrolled 461 patients with nonresponse to TNF inhibitors (including etanercept). Patients with continued background csDMARDs treatment were assigned in a 1:1:1 ratio to receive subcutaneous injections of placebo, 50 mg golimumab, or 100 mg golimumab. 140 patients achieved ACR20 at week 14, including 18% patients on placebo, 35% patients on 50 mg golimumab, and 38% patients on 100 mg golimumab, suggesting golimumab was a good choice for patients who had previously received one or more TNF-α inhibitors (208). These results were confirmed in a long-term extension, multicenter, randomized, double-blind, placebo-controlled, phase 3 GO-AFTER study with up to five years of

therapy (209). Another trial included 18 RA patients who were first treated with etanercept and subsequently switched to infliximab due to inefficacy. The mean best DAS28 after switching to infliximab was considerably better than the previous result, indicating that a trial of infliximab was reasonable for such patients (210).

Adalimumab: Mechanism, Biomarkers and Alternative Therapy

Adalimumab, approved by FDA in 2002, is the first fully human, high-affinity, recombinant IgG_1 anti-TNF monoclonal antibody (211). It has high selectivity and affinity for TNF, a low degree of immunogenicity, and a half-life comparable to that of IgG_1, allowing every-other-week dosing for patients (211). Adalimumab exerts its therapeutic effects by blocking the interaction of TNF with the p55 and p75 cell surface TNFR (211). By blocking TNF signaling, MMP-1 and MMP-3 are downregulated and osteoclast maturation and activation are inhibited (190, 212). Only approximately 40% of RA patients showed ACR50 response after receiving adalimumab monotherapy (213).

ADAs rate of adalimumab was 28% and RA patients carrying the same IgG allotype as present on the adalimumab IgG had a high frequency of ADAs (153, 154), suggesting that these patients might not gain substantial clinical benefit from adalimumab treatment. In addition, a frequency of < 9.4% of signal regulatory protein (SIRP)α/β-expressing memory B cells predicted RA patients that would develop ADAs, and consequentially failed to respond to treatment (156). It was postulated that evaluating the percentage of SIRP/-expressing memory B cells in patients prior to adalimumab treatment could be a valuable biomarker for identifying a subset of active RA patients who will develop ADAs and develop nonresponse to adalimumab (156). Interestingly, there was no functional data showing the role of SIRP in B cells, while SIRPα was reported to be a critical regulator of myeloid cell activation *via* binding to CD47 and SIRPα/CD47 axis limited the efficacy of tumor-opsonising antibodies (214). Thus, it is necessary to explore the underlying mechanism involving SIRPα/β-expressing memory B cells in ADAs response of adalimumab. In addition to SIRPα/β-expressing memory B cells, it was suggested that the existence of other specific risk factors, genetic or environmental, predisposed some individuals to develop adalimumab ADAs (156). For example, smoking could predict ADAs development (215) and RA patients with over 1 year disease duration or with an initial DAS28 over 3.2 have a higher risk of ADAs positivity (215). In addition to ADAs, the status of anti-citrullinated protein/peptide antibody (ACPA) could affect the efficacy of adalimumab. Finer ACPA specificities in ACPA-negative might be predictive of response to treatment (adalimumab or methotrexate) (152) and adalimumab was more effective in patients who were ACPA positive than in those who were ACPA negative at baseline (155). However, another study reported a contradictory result that the presence of ACPA was associated with a reduced response to TNF inhibitors including adalimumab (158). Decreased CD68 and MMP-3 expression in the synovium was associated with a good response to adalimumab (157). RA patients with response to adalimumab had significantly lower chemokine receptor 6 (CCR6) expression and increased Th17 and Th1 (159). Elevated baseline levels of chemokine (C-X-C motif) ligand (CXCL)10 and CXCL13 were associated with favorable response to adalimumab (162). Increased expression of CD11c was correlated with a good response to adalimumab (163). Higher myeloid-related protein (MRP)8/14 levels predicted good adalimumab response (164). Elevated baseline neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) were associated with a higher risk of nonresponse to adalimumab (160). RA patients with high soluble intercellular adhesion molecule 1 (sICAM1) and low CXCL13 had a good clinical response to adalimumab (165) (**Table 2**).

Polymorphisms of TNF and TNFR also influenced response to adalimumab. A study showed that *TNF-α* rs1800629-GG was associated with a better response to adalimumab than -GA or -AA (166). In contrast, another study reported that *TNF-α* rs1800629 G haplotype in a homozygous form was associated with a lower response (161). Two meta-analyses failed to demonstrate that the rs1800629 G/A genotype, whether heterozygous or homozygous, is linked to a poor response to anti-TNF medication treatment (216, 217). In *TNF-α* rs1800629-GG patients, ACPA status did not affect the clinical response to adalimumab (218). *TNF-α* rs1799724-TT showed a better response than C allele carriers with adalimumab treatment (138). *TNFR1* 36 (rs767455) -AA was associated with a better response to adalimumab than -GG (138); RA patients with *TNFR2* 676 (rs1061622) -TT demonstrated a better response compared to those with -TG (137). Other polymorphisms were also studied. When RA patients treated with adalimumab, low-affinity *Fc gamma receptors 2A (FCGR2A)*-R(A)* allele shows a better EULAR good response (167), *IL-6* rs1800795-GG was associated with a better response than -GC or -CC (168).

In a 52-week, double-blind, placebo-controlled, active-controlled phase III study, 1305 patients were randomized 3:3:2 to placebo, baricitinib (a JAK inhibitor) or adalimumab. At week 16, adalimumab nonresponders received rescue treatment with baricitinib. Results showed that switching from adalimumab to baricitinib was associated with improvements in disease management, physical function, and pain (219). In a 24-weeks Single-Arm study, 90 patients discontinued prior adalimumab treatment and continued methotrexate combined with etanercept for 24 weeks. ACR response data demonstrated that switching to etanercept was a therapeutic option in patients with RA who failed adalimumab treatment. ADAs response was examined to explain the treatment failure in this study. It was shown that patients with nonresponse to adalimumab produced higher anti-adalimumab antibodies, which did not cross-react with etanercept and provided additional support for switching to etanercept (220). In a 48-week, randomized, double-blind, SELECT-COMPARE study, 1629 patients were grouped 2:2:1 to upadacitinib (a JAK inhibitor), placebo or adalimumab, with stable background methotrexate. Upadacitinib in combination with methotrexate demonstrated superior clinical and functional

responses versus adalimumab combined with methotrexate (221). Patients who did not respond well to adalimumab saw clinically significant improvements after switching to upadacitinib (221).

Certolizumab Pegol: Mechanism, Biomarkers and Alternative Therapy

Certolizumab pegol, approved by FDA in 2009, is an antigen-binding fragment (Fab) of a recombinant humanized monoclonal antibody conjugated to PEG. PEGylation enables the increase of the plasma half-life and solubility and reduces the immunogenicity and protease sensitivity (222). Certolizumab pegol binds to TNF with greater affinity and is more effective than adalimumab and infliximab at neutralizing soluble TNF-mediated signaling, but has equal or lesser potency than etanercept (223). Certolizumab pegol may be more effective in penetrating inflamed arthritic tissue than other anti-TNF medications and it cannot be actively transported through the placenta during pregnancy (222). ACR20 and ACR50 response in RA patients was only about 45% and 23% after 6-month treatment of certolizumab pegol, respectively (224).

Like other biologic agents, certolizumab pegol elicited immunogenic response, resulting in the formation of ADAs with a high incidence of about 65% (169). Further research showed that >97% of ADAs to certolizumab pegol was directed to the paratope of the drug and were thus neutralizing, indicating these patients with neutralizing ADAs had especially higher risk for drug nonresponse (225). However, a recent study advocated not to overvalue ADAs in a clinical setting, unless certolizumab pegol concentration was low, as they found that the drug concentration but not the presence of ADAs was highly correlated with the capacity to neutralize TNF (169). Clinimetric measurements were found to be associated with nonresponse to certolizumab pegol during course of treatment. Early response to certolizumab pegol predicted long-term outcomes (170). Failure to achieve improvement in DAS28 within the first 3 months of therapy was predictive of a low probability of achieving low disease activity at 12 months using certolizumab pegol (171). Failure to achieve improvements in DAS28(ESR), SJC or CDAI within the first 3 months of certolizumab pegol therapy was associated with a low chance of achieving low disease activity at 7 months (172). CDAI nonresponse at 3 months was a predictor of failure to achieve the therapeutic target of low disease activity at 12 months in patients with RA initiating treatment with certolizumab pegol (173). Studies observed that RA patients who had high serum pretreatment ratio of type I IFNβ/α (> 1.3) or undetectable type I IFN were likely to have EULAR nonresponse to TNF inhibitors (including certolizumab pegol) (174, 175). Mechanically, the pattern of gene expression that differed between the response and nonresponse groups suggested that canonical type I IFN pathway signaling *via* JAK/STAT was increased in peripheral blood classical monocytes of RA patients who were likely to respond to TNF inhibition, whereas JAK/STAT-independent non-canonical IFNβ-IFNAR1 signaling was increased in nonclassical monocytes of those who were not likely to respond to TNF inhibition (175, 226). Notably, JAK1 expression was absent in both classical and nonclassical monocytes from the patients with undetectable IFN or IFNβ/α > 1.3, suggesting JAK1 could be a predictive factor for nonresponders to TNF inhibitors (175) (**Table 2**).

In a 104-week, randomized, single-blind (double-blind until week 12 and investigator blind thereafter), parallel-group, head-to-head superiority study, 457 RA patients were treated by certolizumab pegol plus methotrexate or adalimumab plus methotrexate. At week 12, 65 nonresponders to certolizumab pegol were switched to adalimumab and 57 non-responders to adalimumab were switched to certolizumab pegol. Certolizumab pegol plus methotrexate was not found to be superior to adalimumab plus methotrexate. For patients with primary therapy failure, clinical benefit could be observed after drug switching in both groups (227). In a 2-year, phase 2a, double-blind, proof-of-concept study, 52 RA patients with inadequate response to certolizumab pegol received certolizumab pegol plus bimekizumab (a monoclonal IgG1 antibody that selectively inhibits IL-17A and IL-17F). Data showed that reduction of DAS28 was greater in the group treated with bimekizumab in combination with certolizumab pegol compared with the group treated with certolizumab pegol plus placebo after 20-week treatment (228). This suggested that the add-on therapy of bimekizumab was of great clinical significance for nonresponsive patients to certolizumab pegol.

Golimumab: Mechanism, Biomarkers and Alternative Therapy

Golimumab, a fully human IgG1κ monoclonal antibody with directed against the soluble and membrane bound forms of TNF-α, was the latest TNF inhibitor approved by the FDA in 2009 (229, 230). As a newer, second-generation TNF inhibitor, the clinical experience of golimumab was less in comparison with the older ones such as infliximab, etanercept and adalimumab (230). Different with other TNF inhibitors, golimumab has a specific mode of action: it binds to a distinct epitope on TNF-α that does not overlap with the binding residues of TNFR2, but the complex sterically hinders TNFR2 as well as TNFR1 from associating with TNF-α (231). In combination with methotrexate, golimumab has a UK marketing authorization for RA therapy (232), which may in part be attributable to the concomitant use of methotrexate reduces the clearance of golimumab by approximately 35% (230). A GO-BEFORE trial enrolling 637 RA patients and a GO-FORWARD trial enrolling 444 RA patients demonstrated that golimumab achieved ACR50 response in approximately 40-50% of patients (233, 234).

Regarding immunogenicity of golimumab, complementarity determining region loop grafting was developed to reduce some of the immunogenic issues associated with chimeric antibodies (235). A study showed that only 6.5% of golimumab-treated patients developed ADAs (236). However, other studies detected 31.7% of ADAs for golimumab using a more sensitive method (237). Some patients showed good response to golimumab even with a presence of ADAs and the numbers of ADAs-positive patients were insufficient to determine whether these ADAs are associated with an impaired clinical response (196). Golimumab

concentration ≥ 1.0 mg/L was associated with improved treatment response (176). Larger magnitudes of the decrease in serum amyloid A (SAA), E-selectin and MMP-9 were observed in responders to golimumab plus methotrexate relative to nonresponders (178). Greater likelihood of low disease activity and remission were associated with being male, younger age, lower health assessment questionnaire, ESR (or CRP) and TJC (or SJC) scores and absence of comorbidities in golimumab-treated RA patients (179). Sustained increase of markers including IL-6, CRP, IL-2 receptor alpha chain, and MMP-1, was presented in golimumab-inadequate responders (177) (**Table 2**).

Currently, alternative therapies for golimumab are poorly studied. In a latest study, according to real-life data extracted from 11 Austrian social health insurance funds covering 86% of the Austrian population, 7637 RA patients on bDMARD therapy were retrieved in total. Golimumab was prescribed in 15% RA patients. After golimumab failure, patients were often switched to an IL-6R antagonist tocilizumab and efficacy was waiting to be determined (238).

bDMARDs BLOCKING T CELLS, CD20 AND IL-6R

Abatacept: Mechanism, Biomarkers and Alternative Therapy

Beyond TNF, CD28 signaling play a key role in T cell process and RA development (239). According to ACR guideline, RA patients with their first TNF Inhibitor failure could switch to abatacept (240). Abatacept, which was approved by the FDA in 2005, is a soluble, recombinant, fully humanized fusion protein that consists of the extracellular domain of cytotoxic T-lymphocyte antigen 4 (CTLA-4) and the Fc portion of IgG1 that has been modified to reduce the Fc region's capacity to induce antibody-dependent and complement-dependent cytotoxicity (241). Abatacept is the first biological drug to primarily target T-cell activation in RA. Abatacept works therapeutically by binding to the costimulatory molecules CD80 and CD86 on antigen-presenting cells (APCs), preventing them from interacting with CD28 on T cells (241). Abatacept also functions through regulating macrophages (242, 243), monocytes (244) and B cells (245–248). Abatacept significantly decreases expression of IFN-γ, IL-1β, MMP-1 and MMP-3 (247). There are two approved formulations for abatacept, intravenous and subcutaneous, which have similar efficacy and safety profile (249). Immunogenicity for abatacept is low and transient, and do not interfere with clinical response (250). Abatacept can be used in conjunction with csDMARDs or as a monotherapy. However, because of an increased risk of infections and malignancies without a significant improvement in efficacy, concurrent treatment with abatacept and other bDMARDs is not indicated (251). In two phase III trials, patients treated with abatacept achieve ACR20 at 66% (252) and 50% (253), respectively.

Rheumatoid factor (RF) was the first autoantibody to be discovered in RA patients (254). A pooled study of data from 9 observational RA registries in Europe found that RF positivity was related with improved abatacept medication efficacy (255). However, A meta-analysis of clinical trials found that no association was found between abatacept response and RF (256). Recently, RF seropositivity could predict increased abatacept retention and abatacept showed preferential efficacy in patients with high-titer RF (257, 258). ACPA/anti-cyclic citrullinated peptides (anti-CCP, a surrogate for ACPA) (259), added to the 2010 ACR/EULAR diagnostic criteria (260), is a hallmark of RA and plays a role in disease pathogenesis (261). A retrospective observational cohort study found there was significantly higher clinical response and drug retention rate in ACPA-positive patients treated with abatacept (262). In an AMPLE trial, abatacept was more effective in patients who were positive for anti-CCP than those who were negative for anti-CCP at baseline (155). Data from the AVERT trial showed that abatacept patients who were anti-CCP IgM positive at baseline had stronger clinical effectiveness than those who were anti-CCP IgM negative at baseline (263). Patients in clinical trials are often a selected population that may not reflect the diverse population observed in ordinary practice settings. As a result, more real-world data are needed to investigate the relationship between anti-CCP status and abatacept therapy effects. According to real-world data from US clinical practices, better clinical response was observed in anti-CCP positive patients (264), which was consistent with a real-world ACTION study reporting that anti-CCP positive status was associated with greater efficacy of abatacept than seronegative status (257). The real-world ACTION study also discovered that double ACPA/RF positive led in increased abatacept retention rates (265). A major limitation still exists in these studies, i.e., they categorized patients according to ACPA/anti-CCP status (e.g., positive vs. negative) rather than titers. Most recently, RA patients treated with abatacept were classified based on ACPA/anti-CCP titers. Results showed that clinical effect of abatacept was most pronounced in patients with high-titer ACPA (258). However, this seems to be contradicting with other two studies, which found that sustained response to abatacept was associated with an early reduction in ACPA titers (266), and abatacept was more effective in patients who showed decreasing anti-CCP antibody titers during treatment (267). Taken together, although ACPA/anti-CCP has been used as a biomarker of disease progression in RA patients for decades, its exact relationship with abatacept response still needs to be explored.

Furthermore, in RA, a large baseline number of circulating CD28 negative T cells may indicate nonresponse to abatacept (268). RNA elongation, apoptosis-related, and NK cell-specifically expressed genes were upregulated in abatacept nonresponders, while inflammasome genes were upregulated in infliximab nonresponders and B cell-specifically expressed genes were downregulated in tocilizumab nonresponders (269). When RA patients with *CTLA-4* rs5742909 T or *CTLA-4* rs231775 G polymorphisms received abatacept, they had a greater EULAR response and lower disease activity (270). By metabolomic analysis, low level of 3-aminobutyric acid and high levels of quinic acid and citrate were observed in responders to abatacept

treatment (271). Serum CXCL10 level was associated with better response to abatacept (272). A higher level of CD24-high and CD27 positive regulatory B cells at baseline was associated with DAS28 remission and a good EULAR response in abatacept-treated patients (273). Reduced type I IFN score, and higher expression of dendritic cells-related genes (*Basic Leucine Zipper ATF-Like Transcription Factor 2 (BATF)*, *Lysosomal Associated Membrane Protein 3 (LAMP-3)*, *CD83*, *C-type Lectin Domain Family 4 Member A (CLEC4A)*, *Indoleamine 2, 3-dioxygenase 1 (IDO)*, *interferon regulatory factor (IRF)7*, *STAT1*, *STAT2* and *TNF Superfamily Member 10 (TNFSF10)*) could be used as biomarkers to predict good response to abatacept (274). Increased dickkopf (Dkk)-1 serum level and sclerostin might indicate a poor prognosis and resistance to abatacept treatment in RA patients (275). Increased cartilage oligomeric matrix protein level served as a strong predictive biomarker for inadequate response to abatacept treatment for RA patients with a first TNF inhibitor failure (276) (**Table 3**).

In a multicenter, retrospective study, RA patients initially treated with abatacept (n = 76, most of them discontinued abatacept due to lack of effectiveness) were switched to either TNF inhibitors (adalimumab, certolizumab pegol, etanercept, golimumab, infliximab) or tocilizumab. Drug retention was estimated after 24 months. Switching to tocilizumab resulted in higher retention due to efficacy, although total retention was comparable when compared to TNF inhibitors (315). In a retrospective cohort study involving 550 RA patients treated with abatacept, 25 inadequate responders underwent an add-on macrolide calcineurin inhibitor tacrolimus therapy. At week 24, 40.0% of patients achieved low disease activity or remission, and the EULAR moderate or good response was 72.0% (316).

Rituximab: Mechanism, Biomarkers and Alternative Therapy

B cells play a critical role of in pathogenesis of RA (317). In 2001, a pilot trial evaluating B cell depletive therapy in RA patients was successfully performed (318). Rituximab, which was approved by the FDA in 2006, is a chimeric mouse/human monoclonal antibody that targets the transmembrane protein CD20 molecule on the surfaces of B cells, causing apoptosis through antibody- and complement-dependent cytotoxicity (319). Rituximab monotherapy and/or in combination with methotrexate is recommended as a treatment option for RA patients who have inadequate response to TNF inhibitors and thus serves as a second-line bDMARD (320, 321). Although no fetus damage has been reported in pregnancies exposed to rituximab within 6 months, it should be considered only when no other therapeutic option is available (322). Only approximately 50% of patients achieved ACR20 response after rituximab treatment (323).

Normal cells are resistant to the complement-mediated lysis through complement regulatory proteins (CRPs), including CD55, CD59, CD46 and CD35 (324). A study showed that increased CD46 expression in peripheral B cells, but not CD35, seemed to be able to predict nonresponders (278). CD46 reduced complement-mediated lysis, one of the mechanisms of action of rituximab, thus decreasing the effectiveness of rituximab (278). It is possible that CD46 inhibitor monotherapy or combined with rituximab could be an alternative strategy for nonresponders to rituximab. Another study demonstrated that depleting CD46 from the cell surface by Ad35K++ sensitized complement-dependent cytotoxicity triggered by rituximab in CD20-positive B-cell malignancies (325, 326). However, effects of CD46 inhibition have not been validated in RA patients who do not respond to rituximab. Roles of CD55 and CD59 were also investigated in RA patients. There was no correlation between the expression levels of CD55 or CD59 at baseline or after treatment and the frequencies of B cell subsets after rituximab treatment or the extent of B cell depletion (280). Apart from these studies, persistence of switched memory B cells in lymphoid tissues was related to rituximab nonresponse (280). Structurally like infliximab, about 11% of rituximab-treated patients developed ADAs, which might influence treatment efficacy and tolerability of rituximab (282). Low or absence of baseline IFN type I response genes expression was associated with good response to rituximab (277). Decreased in expression of mammalian target of rapamycin (mTOR), p21, caspase 3, unc-51 like autophagy activating kinase 1 (ULK1), TNFα, IL-1β, and cathepsin K was predictive of better rituximab response (279). A significant reduction in circulating CD4+ T cell number was observed in RA patients with good response to rituximab (281). Depletion of CD19+/-CD27++CD38++ preplasma cells could be a predictor of good response (283).

Polymorphisms related to rituximab therapy have been well studied. The *IL-6* rs1800795-CC served as a predictor of nonresponse to rituximab in RA patients, while patients with -GC or -GG was more susceptible to rituximab (284). There was a significant correlation between this homozygosis polymorphism in the promoter region with a higher IL-6 expression level (327). It was rational that IL-6R inhibitor tocilizumab could be used as a companion to rituximab treatment in nonresponders (293, 328). *FCGR2A* polymorphism rs1801274-TT was associated with better response to rituximab (286). Several studies suggested that *FCGR3A* rs396991 genotypes, either in heterozygous or homozygous conditions, were associated with different response rates to rituximab. *FCGR3A* rs396991 G allele were associated with better response to rituximab (286, 288, 289). Paradoxically, patients with rs396991 -GT showed the highest response rate, when compared to patients with rs396991-TT or rs396991-GG (291). Homozygous carriers of the *B-cell activating factor belonging to the TNF family (BAFF)* rs9514828 C served as a better response marker to rituximab as well as the homozygotes *BAFF* rs9514828 T (292). *IRF5* rs2004640-TG (294), *TGFβ1* rs1800471-GC or -CT (295) was related to good response to rituximab. The TTTT haplotype in promoter region of B cell stimulator gene was associated with good response to rituximab therapy in RF and/or ACPA seropositive RA patients (296).

A series of studies focused on the relationships between rituximab efficacy and B cells. Incomplete depletion of baseline peripheral blood B cell receptor repertoire might predict clinical nonresponse (285). The fast rebuilding of functional B cells might be present in rituximab nonresponders (287). Total lymphocyte counts >2910/μL combined with plasmablast

TABLE 3 | Potential biomarkers for response or partial response/nonresponse to bDMARDs blocking T cells, CD20 and IL-6R.

bDMARDs	Biomarkers for response	Sample size	Reference	Biomarkers for partial response/nonresponse	Sample size	Reference
Abatacept	RF positivity	2942	(255)	High circulating CD28 negative T cells at baseline	32	(268)
	RF seropositivity and high-titer RF	2350;40	(257, 258)	Upregulated RNA elongation, apoptosis-related expressed genes	209	(269)
	ACPA-positive	553	(262)	NK cell-specifically expressed genes	209	(269)
	Anti-CCP positive at baseline	646;2281;2350	(155, 257, 264)	Increased dickkopf-1 serum level and sclerostin	59	(275)
	Anti-CCP IgM positive at baseline	511	(263)	Increased cartilage oligomeric matrix protein level	30	(276)
	High-titer ACPA	40	(258)			
	An early reduction in ACPA titers	149	(266)			
	Decreasing anti-CCP antibody titers	109	(267)			
	CTLA-4 rs5742909 T or CTLA-4 rs231775 G	109	(270)			
	Low level 3-aminobutyric acid; high level quinic acid and citrate	43	(271)			
	Decreased serum CXCL10 level	25	(272)			
	Baseline higher level of CD24-high and CD27 positive regulatory B cells	38	(273)			
	Reduced type I IFN score; higher expression of dendritic cells-related genes	168	(274)			
Rituximab	Low or absence of baseline IFN type I response genes expression	226	(277)	Increased CD46 expression in peripheral B cells	10	(278)
	Decreased mTOR, p21, caspase 3, ULK1, TNFα, IL-1β, and cathepsin K	42	(279)	Persistence of switched memory B cells in lymphoid tissues	14	(280)
	Reduction in circulating CD4+ T cell number	33	(281)	ADAs formation	96	(282)
	Depletion of CD19+/-CD27++CD38++ preplasma cells	31	(283)	IL-6 rs1800795-CC	142	(284)
	IL-6 rs1800795-GC or -GG	142	(284)	Incomplete depletion of baseline peripheral blood B cell receptor repertoire	24	(285)
	FCGR2A rs1801274-TT	142	(286)	Fast rebuilding of functional B cells	26	(287)
	FCGR3A rs396991 G allele	142;224;212	(286, 288, 289)	Total lymphocyte >2910/μL combined with plasmablast >2.85% at baseline	44	(290)
	FCGR3A rs396991-GT	177	(291)	Higher circulating preplasma at baseline and incomplete B cell depletion	158	(20)
	Homozygotes BAFF rs9514828 C and rs9514828 T	224	(292)	Persistently high serum IL-6 level	51	(293)
	IRF5 rs2004640-TG	115	(294)			
	TGFβ1 rs1800471-GC or -CT	63	(295)			
	TTTT haplotype in promoter region of B cell stimulator gene	269	(296)			
	Higher initial depth of B cell depletion	180	(297)			
Tocilizumab	IL-6R 12083537-AA	171	(298)	IL-6R rs12083537 A allele and the rs4329505 C allele	79	(299)
	CD69 rs11052877 A alleles	79	(300)	High sICAM1 and low CXCL13 at the synovial level	69	(165)
	FCGR3A rs396991-TT	142	(286)	Higher enrichment of TNF-induced gene transcripts	65	(301)
	RF positivity at baseline	23	(256)			
	High baseline CRP level	204	(302)			
	Soluble IL-6R low at baseline	43	(303)			
	Upregulated gene IFI6, MX2, OASL and one encoding metallothionein-1G	60	(304)			
	Low serum D-dimer and IL-1β levels	65	(305)			
	Pre-treatment serum 14-3-3η levels	149	(306)			
	Increased TRAV8-3, EPHA4, CCDC32, and a decrease of DHFR	13	(307)			
	High soluble gp130Fc	138	(308)			
	Low IL-17A level	88	(309)			
	A higher baseline NK cell count	92	(310)			
	Low sICAM1 and high CXCL13	69	(165)			
Sarilumab	ACPA positive	2108	(311)	No data	N/A	N/A
	RF positive and/or CCP positive	1743	(312)			

(Continued)

TABLE 3 | Continued

bDMARDs	Biomarkers for response	Sample size	Reference	Biomarkers for partial response/nonresponse	Sample size	Reference
	Patients received 200 mg sarilumab every 2 weeks	1743	(312)			
	Elevated baseline level of IL-6	1193	(313)			
	Lower level of sICAM1 at baseline	291	(314)			

ACPA, Anti-citrullinated protein antibodie; ADAs, anti-drug antibodies; CASP3, caspase 3; CCDC32, Coiled-Coil Domain Containing 32; CCP, cyclic citrullinated peptide; CD, Cluster of Differentiation; CDAI, Clinical Disease Activity Index; CRP, C-reactive protein; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CTSK, cathepsin K; CXCL, The chemokine (C-X-C motif) ligand; DHFR, dihydrofolate reductase; EPHA4, ephrin type-A receptor 4; FCGR, Fc fragment of IgG receptor; HAQ, Health Assessment Questionnaire; IFI6, Interferon Alpha Inducible Protein 6; IFN, interferons; MX2, MX Dynamin Like GTPase 2; NK, Natural killer; OASL, 2'-5'-Oligoadenylate Synthetase Like; RF, Rheumatoid Factor; sICAM1, soluble intercellular adhesion molecule-1; TGFβ1, transforming growth factor beta 1; TNF, Tumor necrosis factor; TRAV, T Cell Receptor Alpha Variable.

frequency >2.85% at baseline predicted rituximab nonresponse (290). Higher initial depth of B cell depletion was associated with good response to rituximab (297). Patients with RA who did not respond to an initial cycle of rituximab had larger circulating preplasma cell counts and incomplete B cell depletion, whereas an extra cycle of rituximab delivered prior to total B cell repopulation improved B cell depletion and clinical response (20). However, another study found that despite adequate B cell depletion, failed rituximab therapy still existed in some RA patients, and nonresponse to rituximab was associated with persistently high serum IL-6 level (293). Further, in a single-center, prospective, observational database, 51 RA patients who had discontinued rituximab therapy owing to inefficacy received either a T cell costimulation inhibitor abatacept or IL-6R inhibitor tocilizumab. After 6-month treatment, reduction of disease activity score (DAS28-ESR) and swollen joint count was more significant in tocilizumab-treated patients than in abatacept-treated patients, suggesting that IL-6-directed therapy might be a more logical and effective treatment choice than T cell costimulation blockade in RA patients with failed rituximab therapy (293). Consistently, in an investigator-led, industry-supported, prospective, longitudinal, multinational CERERRA database, 265 RA patients were analyzed and majority of them (78%) had stopped rituximab owing to ineffectiveness (328). 90 patients were prescribed abatacept, 86 were started on tocilizumab and the remaining 89 patients received TNF inhibitors (including etanercept, adalimumab, infliximab, certolizumab pegol and golimumab). After 6-month treatment, patients treated with tocilizumab had a greater decline of DAS28-ESR and better EULAR response than patients treated with TNF inhibitors or abatacept (328) (**Table 3**).

Tocilizumab: Mechanism, Biomarkers and Alternative Therapy

IL-6 is one of the most abundant pro-inflammatory cytokines in RA. It can signal through two distinct mechanisms. In the cis-signaling, IL-6 binds to its membrane IL-6R which is mainly expressed in hepatocytes and hematopoietic cells (T cells, monocytes/macrophages, B cells and neutrophils). In the trans-signaling, IL-6 binds to its soluble IL-6R. The complex consisting of IL-6 and membrane and soluble IL-6R associates with gp130, resulting in the activation of downstream signaling events *via* JAK/STAT (329). The option to target IL-6R rather than IL-6 itself was chosen after considering that receptor concentrations have less interpatient variability than IL-6 concentrations, potentially simplifying dose and regimen selection (330). Approved by FDA in 2010, tocilizumab is the first anti-IL-6Rα humanized IgG_1/kappa monoclonal antibody, used for the treatment of moderate to severe RA (331). Tocilizumab targets and neutralizes both soluble and membrane-bound IL-6R, resulting in inhibition of IL-6-mediated inflammatory activities (332). Tocilizumab can be either applied in combination with methotrexate or used as a monotherapy (330, 333, 334). Compared with TNF inhibitors, tocilizumab monotherapy improves the healing of focal bone erosions in RA patients and outperforms methotrexate or other csDMARDs in terms of lowering RA symptoms (330). Tocilizumab treated patients achieved an approximately 50% ACR20 response rate (335, 336).

Tocilizumab-subcutaneous and -intravenous treatment had a low immunogenicity risk, whether used alone or in combination with csDMARDs (337). The development of ADAs in a small fraction of patients had no noticeable impact on the efficacy of tocilizumab (337). Several studies investigated whether polymorphisms of genes were associated with response to tocilizumab therapy. A study with 79 RA patients enrolled reported that the major allele (A) of rs12083537 and the minor allele (C) of rs4329505 with *IL-6R* were associated with poor tocilizumab response (299). But another study with 171 RA patients enrolled found that 12083537-AA could predict better EULAR response to tocilizumab (298). Further, a larger cohort of 927 patients demonstrated no association between them (338). A genome-wide association analysis implicated the involvement of 8 loci (*CD69* rs11052877; *GalNAc-T-Like Protein 4 (GALNTL4)* rs4910008; *Ecto-NOX Disulfide-Thiol Exchanger 1 (ENOX1)* rs9594987, rs10108210 and rs703297; *Potassium Voltage-Gated Channel Interacting Protein 1 (KCNIP1)* rs703505; *C-Type Lectin Domain Family 2 Member D (CLEC2D)* rs1560011; *Solute Carrier Family 9 Member A7 (SLC9A7)* rs7055107) with response to tocilizumab (339). Relationship between *CD69* rs11052877 A alleles and good tocilizumab response was further validated in a study with 79 RA patients enrolled (300). In contrast, another study concluded that *CD69* rs11052877 A/G genetic polymorphism was not useful as a predictor of tocilizumab response in RA patients (340). Data from 87 patients suggested that *FCGR3A* rs396991-TT could be used to predict better EULAR response (286). However, no relationship

between rs396991 and EULAR response was shown in a research enrolling 171 RA patients (298). Due to the small sample sizes and/or conflicting findings, larger studies are necessary to resolve whether the above genetic variations had real impact on therapeutic response to tocilizumab.

Features of pre-treatment disease activity had been demonstrated to be associated with response to tocilizumab in RA patients. A meta-analysis found that RF positivity at baseline predicted better response to tocilizumab (256). Several studies, however, found no link between RF positive and response (341, 342). A high baseline CRP level could serve as a predictor of better response to tocilizumab (302). Patients with a strong acute phase response, extra-articular symptoms, and a history of DMARDs and biological treatments may be more likely to respond quickly to tocilizumab. However, no parameter was likely to predict reaction if examined separately (343). A significantly higher proportion of patients in soluble IL-6R-low group achieved SDAI remission compared with those in soluble IL-6R-high group (303). Upregulation of gene *Interferon Alpha Inducible Protein 6 (IFI6)*, *MX Dynamin Like GTPase 2 (MX2)*, *2'-5'-Oligoadenylate Synthetase Like (OASL)* and one encoding metallothionein-1G in peripheral blood mononuclear cells was observed in tocilizumab good/moderate responders (304). Low blood D-dimer and IL-1 levels at 4 weeks were found to predict favorable treatment response to tocilizumab at 52 weeks in a population of 65 patients (305). In patients treated with tocilizumab, pre-treatment blood 14-3-3 levels predicted 1-year DAS28 remission (306). Patients with increased T Cell Receptor Alpha Variable 8-3 (TRAV8-3), EPH Receptor A4 (EPHA4), Coiled-Coil Domain Containing 32 (CCDC32), and a decrease of DHFR presented good response to tocilizumab (307). High soluble gp130Fc strongly predicted good response to tocilizumab (308). Low IL-17A level was linked to higher response rate of tocilizumab (309). A higher baseline NK cell count was associated with better clinical remission after treatment with tocilizumab (310). A serological cytokine signature showed that patients with high sICAM1 and low CXCL13 at the synovial level was negatively associated with the ACR50 response to tocilizumab, whereas patients with low sICAM1 and high CXCL13 showed good tocilizumab response (165). The presence of more TNF-induced gene transcripts in synovial samples was linked to a poor response to tocilizumab (301) (**Table 3**).

In a multicenter, retrospective study, 145 RA patients initially treated with tocilizumab (most of them discontinued tocilizumab due to lack of effectiveness) were switched to TNF inhibitors (adalimumab, certolizumab pegol, etanercept, golimumab, infliximab), abatacept or JAK inhibitors. After 24 months, drug retention was estimated. Switching to abatacept in tocilizumab-treated patients led to higher retention (315). In an open-label, non-randomized phase 3 study, 519 RA patients with inadequate response to conventional synthetic DMARDs received tocilizumab. 213 partial responders continued tocilizumab treatment and 27 nonresponders were switched to an CD20 inhibitor rituximab. At week 32, half of early partial responders benefitted from continuing tocilizumab and switching non-responders to rituximab seemed feasible (344). In a retrospective, observational clinical study, 63 nonresponders from 527 RA patients treated with tocilizumab were switched to TNF inhibitors (infliximab, adalimumab, etanercept, golimumab, and certolizumab pegol) or abatacept. The proportion of patients achieving CDAI ≤ 10 at week 24 was significantly higher in patient treated with TNF inhibitors than those treated with abatacept, and the values of the CDAI at week 24 showed the same tendency (345).

Sarilumab: Mechanism, Biomarkers and Alternative Therapy

Sarilumab, a completely human IgG1 monoclonal antibody authorized by the FDA in 2017, specifically binds to soluble and membrane-bound IL-6R and blocks IL-6-mediated cis- and trans-signalling (346). Sarilumab presented good therapeutic efficacy when administered in combination with csDMARD in patients with inadequate response to methotrexate or at least one TNF inhibitor (346). It should be noted that sarilumab was developed in mice engineered to produce human antibodies with an affinity for human IL-6R 20-fold greater than tocilizumab (347). Preclinical findings showed that sarilumab inhibited IL-6 signaling in a dose-dependent manner at a lower concentration than tocilizumab, with no evidence of complement-dependent or antibody-dependent cytotoxicity (348). Sarilumab on the background of methotrexate significantly suppresses CRP level (349), biomarkers of bone resorption (RANKL and RANKL/OPG), bone and cartilage destruction and synovial inflammation (350). Approximately 60% of sarilumab-treated patients achieved an ACR20 response (351, 352).

Currently, only a few studies have been undertaken to identify potential biomarkers that can predict sarilumab response or nonresponse in RA patients, and alternative therapy in sarilumab nonresponders is not reported as sarilumab is approved very recently. In biomarker analysis of two phase III trials (MOBILITY involving RA patients with inadequate response to prior methotrexate and TARGET involving RA patients with inadequate response to prior TNF inhibitors), ADAs response rates were 5.6% (150 sarilumab) and 4.0% (200 mg sarilumab) and neutralizing antibodies were detected at 1.6% and 1.0% (346). Likewise, 7% of RA patients received sarilumab monotherapy in a MONARCH study exhibited an ADAs presentation but no detectable neutralizing antibodies (353). The development of ADAs was not connected with adverse effects or loss of efficacy, although it may have an impact on sarilumab pharmacokinetics (346). A phase III multicenter, randomized, controlled studies indicated that sarilumab might be more effective in RA patients who were ACPA positive (311). Also, better clinical response to sarilumab was consistently observed among patients who were RF positive and/or CCP positive in MOBILITY and TARGET studies (312). Regardless of autoantibody status, there was a more robust response in patients received 200 mg sarilumab every 2 weeks (312). Patients with elevated baseline IL-6 levels were found to have a better response to sarilumab compared to methotrexate or adalimumab than patients with normal IL-6 levels (313). Lower level of sICAM-1 at

baseline was predictive of improved response to sarilumab (314) (**Table 3**).

tsDMARDs TARGETING JAKS

Tofacitinib: Mechanism, Biomarkers and Alternative Therapy

Even through development of bDMARDs revolutionizes treatment of RA (103), these bDMARDs bring up new issues, e.g., formation of neutralizing antibodies, biologics-related toxicity and infusion-related adverse effects (354). With discovery more than 20 years ago, JAKs attract much attention since they are the most important signaling transducers (355). When triggered by cytokines such as TNF-α and IL-6, JAKs phosphorylate STATs, causing dimerization and translocation of STATs to the nucleus, where they control inflammation-related genes (7, 356). In other words, JAK inhibition blocks the action of all dependent cytokines ("many birds with one shot") (357). The ACR and EULAR affirm that JAK inhibitors could be a viable option for RA patients who are refractory to methotrexate monotherapy and viewed on equal footing with TNF inhibitors and non-TNF biologics such as abatacept, tocilizumab and rituximab (13, 240). As the first JAK inhibitor approved by FDA in 2012, tofacitinib preferentially inhibits JAK1 and/or JAK3, and to a lesser extent of JAK2 (358, 359). Tofacitinib reduced JAK1/JAK3-mediated signaling of IL-2, IL-4, IL-6, IL-7, IL-15 and IL-21, as well as IFNα and IFNγ, resulting in the regulation of inflammatory response (360). Tofacitinib also reduced levels of CRP, C-C motif chemokine ligand (CCL)2, CXCL10, CXCL13, MMP-1 and MMP-3 (361). In the background methotrexate, either 5 mg or 10 mg tofacitinib achieved more than 50% ACR20 response in patients with methotrexate inadequate response (359).

High baseline musculoskeletal ultrasound (MSUS) and the multi-biomarker disease activity (MBDA) score could predict tofacitinib nonresponse at week 12 (362). miR-432-5p was significantly downregulated in RA patients who were responsive to tofacitinib therapy (363). Lower levels of IFN-γ, IL-6, IL-17 and higher levels of IL-35 were found in tofacitinib responders than in nonresponders (364). Lower age, CRP, ACPA, and DKK-1 indicated the good treatment effects of tofacitinib therapy on bone mineral density changes (365). MMP-3 had higher pre-treatment values correlating with improved tofacitinib response (361). Clinical improvement with tofacitinib therapy correlated with reductions in STAT1 and STAT3 phosphorylation (361). The available evidence is insufficient to support alternative therapy for tofacitinib because limited clinical trials have been conducted (13) (**Table 4**).

Baricitinib: Mechanism, Biomarkers and Alternative Therapy

Approved by FDA in 2018, baricitinib is the second JAK inhibitor for RA treatment that selectively and reversibly inhibits JAK1 and JAK2 and then modulates JAK-STATs intracellular signaling pathways (369, 370). Baricitinib also inhibits the effects of JAK3, TyK2, tyrosine-protein kinase Met (c-MET) and Checkpoint kinase 2 (Chk2) (371). Baricitinib could reduce pannus formation and bone damage in multiple murine models of arthritis (371), and also present an osteoprotective effect, increasing mineralization in bone-forming cells in phase III studies (372). After treatment, mean serum values of IgG, IgM and IgA were decreased and remained stable below baseline (373). Treatment with baricitinib did not result in increased autoantibody (RF and ACPA) titers (374). Caution is recommended when leflunomide or teriflunomide was co-administered with baricitinib (373). Baricitinib demonstrated a consistent, beneficial treatment effects in bDMARD-refractory patients (375). Baricitinib also ameliorated disease progression in RA patients who were naïve to DMARDs or respond inadequately to csDMARDs, and the beneficial effects were similar to those observed with adalimumab (376). In two clinical trials, RA patients treated with baricitinib achieved ACR20 for 77% and 55%, respectively (377, 378).

High titers of anti-carbamylated vimentin (CarbV) IgA and IgG antibodies were associated with a greater clinical response to

TABLE 4 | Potential biomarkers for response or partial response/nonresponse to tsDMARDs targeting JAKs.

tsDMARDs	Biomarkers for response	Sample size	Reference	Biomarkers for partial response/nonresponse	Sample size	Reference
Tofacitinib	Downregulated miR-432-5p	16	(363)	High baseline MSUS and MBDA score	25	(362)
	Lower levels of IFN-γ, IL-6, IL-17 and higher levels of IL-35	32	(364)			
	Lower age, CRP, ACPA, and DKK-1	26	(365)			
	Higher pre-treatment MMP-3 values	14	(361)			
	Reductions in STAT1 and STAT3 phosphorylation	14	(361)			
Baricitinib	High titers of CarbV IgA and IgG antibodies	584	(366)	Previous use of bDMARDs (non-TNF inhibitors) or JAK inhibitors	113	(367)
	No previous targeted DMARD (b or tsDMARDs) use	113	(367)			
	Lower DAS28-CRP score at baseline	113	(367)			
Upadacitinib	Higher levels of IL-17A, IL-17C, CCL11, CCL20, and TIMP4	300	(368)	No data	N/A	N/A

ACPA, Anti–citrullinated protein antibody; CCL, C-C Motif Chemokine Ligand; CRP, C-reactive protein; DKK, Dickkopf; IFN, interferon; JAK, Janus kinase; MBDA, Multi-Biomarker Disease Activity; MMP, matrix metalloproteinase; MSUS, Musculoskeletal Ultrasound; STAT1, signal transducer and activator of transcription 1; TNF, tumour necrosis factor.

baricitinib as measured by SDAI and DAS28-high-sensitivity CRP (366). Patients who had previously used bDMARDs (non-TNF inhibitors) or JAK inhibitors had decreased rates of DAS28-CRP improvement when treated with baricitinib (367). No previous targeted DMARD (b or tsDMARDs) use was associated with the achievement of low disease activity (367). However, this result was contradicted to another study in which baricitinib nonresponse was not related to prior use of one or more of bDMARDs (375). Besides, baseline characteristics (excluding DAS28-CRP score) did not substantially affect the clinical response to baricitinib in RA patients (379), but a lower DAS28-CRP score at baseline was associated with the achievement of low disease activity (367). Taken together, predicting response to baricitinib by previous treatment with DMARDs and baseline characteristics still needs further investigation (**Table 4**).

Upadacitinib: Mechanism, Biomarkers and Alternative Therapy

Unlike tofacitinib (359) and baricitinib (380), upadacitinib is engineered to be selective for JAK1 and serves as the second-generation JAK inhibitor approved by FDA in 2019 for RA (381). The rationale for selectively targeting JAK1 is that the anti-inflammatory effect should be maintained *via* inhibiting JAK1, but effects on undesired JAK2- and JAK3-dependent processes should be minimized (382, 383). Upadacitinib was shown to be >40-fold, 130-fold and 190-fold more selective for JAK1 versus JAK2, JAK3 and TYK2, respectively (383). Upadacitinib may be used as monotherapy or in combination with methotrexate in active RA patients with inadequate response to cs or bDMARDs (384). It has been demonstrated that upadacitinib was more effective than adalimumab in RA patients who were concomitantly receiving methotrexate (221). Upadacitinib could decrease circulating numbers of lymphocytes, neutrophils and NK cells with no significant changes in RF and ACPA levels (383, 385–387). In three studies (SELECT-NEXT, SELECT-BEYOND and SELECT-MONOTHERAPY), rates of ACR20 were 64%, 65% and 68% in RA patients treated with 15 mg upadacitinib, and 66%, 56% and 71% in patients treated with 30 mg upadacitinib, respectively (388–390).

Clinical response with upadacitinib treatment was mainly associated with slightly higher levels of the IL-17A, IL-17C, CCL11, CCL20, and Tissue inhibitor of metalloproteinases-4 (TIMP4) (368). A study showed that adalimumab appeared to affect M1 macrophages, while upadacitinib appeared to affect T cells preferentially (368), which was in line with another study (391). This modulatory pattern by upadacitinib was consistent with its wide cytokine receptor inhibition profile as compared to specific TNF inhibition and could account, at least in part, for superior efficacy of upadacitinib over adalimumab (368). In a randomized, double-blinded SELECT-COMPARE study enrolled 651 upadacitinib-treated patients, a total of 39% (252/651, including non- and incomplete-responders) patients were randomized to adalimumab. Low disease activity was achieved in 36% nonresponders and 45% incomplete-responders after switching for 6 months (392) (**Table 4**).

PRECISION MEDICINE AND RA

Over the past decades, treatment of RA always depends on "trial-and-error" methods of finding a DMARD that works (393). Regarding the above-mentioned clinical trials enrolling RA patients who have failed initial DMARDs therapy, we can see that successive conventional or biologic switching, either within the same or different mechanistic class, is advocated as an alternative therapy by an experience-oriented principle. This leads to the apparent disadvantage of continuously exposing patients to multiple drugs that they do not respond to, with unnecessary side-effects, delaying the use of effective agents, and causing a serious economic burden to society (19). According to the current ACR and EULAR guidelines for managing RA, it is still a great challenge to choose the right treatment for the right patients.

The most recent EULAR recommendations provide an updated research agenda that highlights important issues to be addressed in the future, such as the safety and efficacy of various drug sequences and combinations, the discovery of biomarkers to stratify patients and predict therapeutic response, and the reasons for efficacy loss (13). It is worth noting that these issues fall into the category of the emerging precision medicine approach for disease treatment, which is a medical model that proposes the customization of healthcare, with diagnosis, prognosis and treatment being tailored to different subgroups of patients (394). Precision medicine in RA has been recently discussed regarding its great potential in allowing a better diagnosis (RA vs. non-RA), finding biomarkers for preferential treatment selection in patients (responders vs. nonresponders), as well as understanding the prognosis of the disease (progressor vs. non-progressor) (24). Precision medicine is believed to lead to the next revolution for overcoming treatment failure in RA, with the introduction of cutting-edge technologies and big data, especially the multi-omics, single-cell analysis, bioinformatics and biostatistics (**Figure 1**).

MULTI-OMICS IN PRECISION MEDICINE OF RA

Multi-omics is the integration of datasets generated from genomics, transcriptomics, epigenomics, proteomics and metabolomics (395). There has been a growing trend of studies, which utilize high-throughput multi-omics analyses to achieve personalized health care, especially through prediction of disease risk and early intervention for a potentially better outcome (396). Despite much hoopla based primarily on oncology data, the progress of multi-omics in autoimmunity is currently restricted (397). Previously, genomics, transcriptomics and epigenetics have been used separately to characterize the molecular basis of treatment efficacy in RA patients (145, 274, 398–400). However, the molecular effects of DMARDs from multi-omics perspectives are unknown. Recently, researchers reported longitudinal monitoring of the drug response at multi-omics levels in RA patients (401). They revealed that

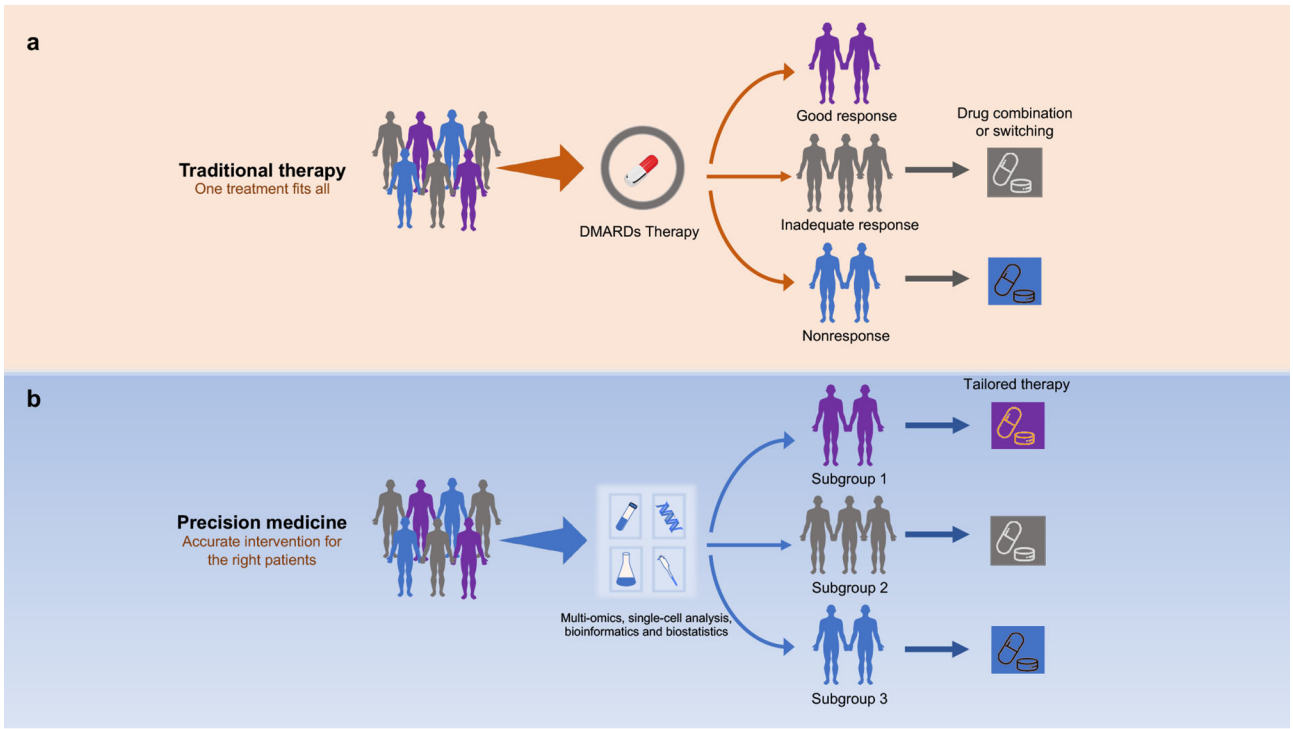


FIGURE 1 | Comparison between traditional therapy and precision medicine.

DMARDs treatments altered the molecular profile of RA patients closer to that of healthy individuals at the transcriptome, serum proteome, and immunophenotype levels. Effects of infliximab and tocilizumab on this molecular profile which defined stable clinical remission were greater than that of methotrexate. Tocilizumab normalized some specific molecular markers that methotrexate and infliximab did not modify, implying that tocilizumab was a more potent treatment for RA at the molecular level (401). Moreover, researchers also identified molecular signatures in transcripts and serum proteins that were resistant to DMARDs. These signals were linked to RA independently of known disease severity indices and were mostly explained by a neutrophil, monocyte, and lymphocyte imbalance. This knowledge will facilitate the identification of biomarkers and drug discovery and contribute to the development of precision therapy for RA (401). Besides, another study performed a sequential multi-omics analysis integrating transcriptomics and genomics to identify gene signatures associated with the response to anti-TNF therapy in RA patients (402). Using transcriptomic data from the RA synovium, thirteen gene co-expressed modules were found to be associated with anti-TNF response. At the genetic level, two of these modules were confirmed to be associated with the anti-TNF response using two independent GWAS cohorts of RA patients. Functional analysis suggested that nucleotide metabolism and Tregs could mediate the response to anti-TNF therapy. These findings demonstrated the existence of a drug-specific genetic foundation for an anti-TNF response, allowing for therapy stratification in the quest for response biomarkers in RA (402). In addition, the latest study conducted multi-omics and machine learning to predict response to anti-TNF therapies in RA patients. Transcription and/or DNA methylome signatures were found to be associated with response to different anti-TNF therapy in peripheral blood mononuclear cells (PBMCs), monocytes, and CD4+ T cells from RA patients (403). Further, transcription signatures in responders to adalimumab and etanercept were divergent in PBMCs, and this phenomenon was reproduced in monocytes and CD4+ T cells. Differentially methylated sites in etanercept responders but not in adalimumab responders were substantially hypermethylated (403). Finally, machine learning models based on these molecular signatures were built to reliably predict response prior to anti-TNF treatment, paving the way for tailored anti-TNF therapeutic treatment regimens (403).

SINGLE-CELL ANALYSIS IN PRECISION MEDICINE OF RA

Unlike traditional omics research, researchers have discovered that cells differ dramatically at the transcriptome, proteomic, and epigenomic levels among tissues, organs, organ systems, and organisms. Individual immune cell coordination is crucial in RA for the production of efficient immune responses to infections

while immunological tolerance is maintained to protect the host. Furthermore, when immune responses are dysregulated, pathologically essential cells may constitute only a minor proportion of the immune system. Examining the roles of particular immune cells in etiology, disease progression, and medication failure should yield valuable insights into RA (404). Single-cell analysis investigates genomes, transcriptomics, proteomics, metabolomics, and cell-cell interactions in individual cells, leading to a higher resolution of cellular distinctions and a better understanding of an individual cell activity in the context of its microenvironment (405–407). It enables the high-dimensional dissection of single cells at multi-omics levels, which could facilitate the discovery of new biomarkers and stratified RA patients into more precise subgroups (408). As mentioned in a subsection of certolizumab pegol, the circulating T1IFN ratio linked to remarkably diverse gene expression patterns in monocytes of RA patients, and certain transcripts such as JAK1 were very informative and could indicate alternative treatment paths in individuals anticipated to be non-responders to anti-TNF therapy. This work was done mainly using a novel single-cell PCR approach, which was similar to single-cell sequencing (175). Another study described a robust statistical method to test for disease associations with single-cell data called MASC (Mixed-effects modeling of Associations of Single Cells). This approach enabled modeling of technical and inter-individual variance as random effects, allowing robust detection of disease-associated cellular populations. Using MASC to analyze CD4+ T cells from blood of RA patients, researchers discovered a population of memory CD4+ T cells known as CD27-HLA-DR+ that was enlarged in the circulation of RA patients. Further, CD27-HLA-DR+ T cells were enriched within inflamed RA joints, rapidly produced IFN-γ and cytolytic factors, and contracted with successful treatment of RA (409). Furthermore, the repertoires of B cell receptors, which may be collected using single cell-resolution sequencing technology, carry a personal history of antigen exposure for a donor (410). Single-cell sequencing of plasmablasts derived from RA patients revealed the presence of B cell receptors specific for CCP and other RA-associated autoantigens (411). Plasmablasts from ACPA-positive patients were sequenced, and both IgA-secreting and IgG-secreting clones were found to be responsive to common RA autoantigens (412). A longitudinal analysis of plasmablasts from individuals with clinically apparent RA revealed the presence of persistent IgA-producing cells that underwent ongoing affinity maturation and produced ACPAs (413). As the complex relationship between the response of DMARDs and the ACPA/anti-CCP has been discovered, single-cell functional B cell receptors sequencing is likely to provide new insights into precision medicine of RA.

BIOINFORMATICS AND BIOSTATISTICS IN PRECISION MEDICINE OF RA

Nowadays, there is an exponentially increasing number of databases integrating personal omics and volume of healthcare data (414, 415). However, due to the nature and complexity of such data, immediate interpretation or usage by healthcare practitioners is frequently out of the question. Biostatistics and bioinformatics pertain to the acquisition and interpretation of the quantitative data. No sharp delineation exists between them. Bioinformatics tends to deal with data in many dimensions, so-called "big data" (416), while biostatistics is a building block for the complex data analytics methods in bioinformatics (417). Integration of bioinformatics and biostatistics facilitate the establishment of sophisticated methods based on omics and advanced mathematics, such as artificial intelligence, machine learning and deep learning (415, 418–420). In a nutshell, artificial intelligence seeks to increase cognitive abilities and performance of computers in order to tackle complicated and massive data-oriented challenges by identifying interaction patterns among variables, learning from experiences, planning, and anticipating better directions. Machine learning is a subfield of artificial intelligence that employs and proposes various algorithms for learning from large amounts of data and revealing multifaceted relationships between data features in order to predict accuracy in various contexts and support decision-making processes, whereas deep learning is a dominant approach based on artificial neural networks (421). These methods have been widely utilized in oncology field to identify individuals at risk, to predict which prevention strategies work best on patients, to automatically screen different subtypes of diseases, or to perform drug repurposing (422). In recent years, application of these methods in RA is burgeoning. A study, for example, offered an automatic method for detecting RA disease activity in an electronic medical record. Different machine learning models were developed and tested using a training set of clinical notes and laboratory data. The models extracted terms such as synovitis, pain, or stiffness as input features by using a text analysis tool on clinical notes, and also used laboratory values of CRP or ESR. Disease activity of each RA patient was predicted by different DAS28 score. This study demonstrated that automatically discovering RA disease activity from electronic medical record data was, in principle, a learnable task, with results approximating human performance (423). Deep learning was also applied to forecast RA disease activity. Researchers classified disease activity into two categories: remission/low and moderate/high. Demographics, past CDAI score, ESR and CRP level, DMARDs, oral and injectable glucocorticoids, and autoantibodies (RF and/or ACPAs) were all taken into account. Results showed that CDAI was the most important feature for prediction of disease, followed by cortisone injections and CRP (424). Deep learning algorithms have been utilized in image processing to find patterns in images (so-called convolutional neural networks). This sort of neural network has been utilized to detect bone erosions (425) and differentiate RA patients from healthy participants from conventional hand radiographs (426). As discussed in the above subsection of multi-omics in precision medicine of RA, the combination of multi-omics and/or clinical data with machine learning could be used to predict response to DMARDs in RA patients (403). Transcription and/or DNA

methylome signatures were found to be associated with response to different anti-TNF therapy in PBMCs, monocytes, and CD4+ T cells from RA patients (403). Machine learning models based on these molecular signatures were developed to accurately predict response before anti-TNF treatment. In another study, researchers used a regression model to predict the response to anti-TNF therapy after methotrexate failure, considering of demographic and clinical data in addition to genetic data (single nucleotide polymorphisms). The model classified the response to anti-TNF treatment with 78% accuracy (427).

CONCLUSION AND PERSPECTIVES

Over the past few decades, researchers have delivered continuous efforts as above mentioned toward overcoming treatment failure in RA. In fact, these efforts have driven the diagnosis, treatment and prognosis of RA entering an early stage of precision medicine, which was commonly described as personalized medicine prior to the proposal of precision medicine. These efforts lead to a massive step when compared to the earlier era in which RA is seen as a devastating and stubborn disease. While gratifying, researchers have realized that even through sequential development of csDMARDs, bDMARDs and tsDMARDs has gradually improved treatment outcome of RA, response rates seem to reach ceiling (approximately 40-60%) in different clinical trials with DMARDs monotherapy or combination therapy in RA patients. Notably, this response ceiling is observed irrespective of the mode of action of the different types of DMARDs or their diverse specific cellular, molecular and signaling targets, such as CD20, TNF, IL-6, CD80-CD86, and the JAK-STAT pathways (18, 428). Although emerging data suggest that a higher response threshold could be reached, breaking through the response ceiling has been proven particularly difficult. This may be because that, on one hand, RA is a highly heterogeneous and complex disease with unclear understanding of mechanisms. On the other hand, small sample sizes and insufficient technologies lead to conflicting or uncertain conclusions as well as slow renewal of knowledge in basic research and translational studies of RA. Precision medicine refers more appropriately to the generation of criteria for advanced taxonomy of patients, producing models to identify and classify clinical decisions for each disease phenotype. This new perspective on patient evaluation can make use of both fundamental laboratory and clinical analyses as well as large data provided by cutting-edge technologies, as previously discussed. Precision medicine is exhibiting great potentials to be a more efficient way to overcome the treatment failure and has begun to emerge in RA studies. Undoubtedly, there are also some limitations of precision medicine, such as high cost of sequencing, existence of ethical issues, difficulty in collection and storage of large amount of data, and lack of easy and standardized approaches for data interpretation for doctors and other healthcare providers. It is believed that most of them will be solved with the rise of new technologies and algorithms. In the foreseeable future, RA patients will ultimately be precisely classified, receive their tailored therapy, and avoid wasting time during months or years of ineffective treatment. Precision medicine will also generate sufficient data to elucidate the molecular foundation underlying drug failure and push the development of next-generation DMARDs.

AUTHOR CONTRIBUTIONS

CL, AL, and DH supervise and revise the manuscript. ZW writes and edits the manuscript. JH and DX provides their professional expertise. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Gibofsky A. Epidemiology, Pathophysiology, and Diagnosis of Rheumatoid Arthritis: A Synopsis. *Am J Manag Care* (2014) 20(7 Suppl):S128–35.
2. Jeffery RC. Clinical Features of Rheumatoid Arthritis. *Medicine* (2014) 42 (5):231–6. doi: 10.1016/j.mpmed.2014.02.011
3. Choy E. Understanding the Dynamics: Pathways Involved in the Pathogenesis of Rheumatoid Arthritis. *Rheumatol (Oxford)* (2012) 51 (Suppl 5):v3–11. doi: 10.1093/rheumatology/kes113
4. Feldmann M, Maini SR. Role of Cytokines in Rheumatoid Arthritis: An Education in Pathophysiology and Therapeutics. *Immunol Rev* (2008) 223:7–19. doi: 10.1111/j.1600-065X.2008.00626.x
5. Smolen JS, Aletaha D, McInnes IB. Rheumatoid Arthritis. *Lancet* (2016) 388 (10055):2023–38. doi: 10.1016/s0140-6736(16)30173-8
6. Walsh MC, Choi Y. Biology of the RANKL-RANK-OPG System in Immunity, Bone, and Beyond. *Front Immunol* (2014) 5:511. doi: 10.3389/fimmu.2014.00511
7. Malemud CJ. The Role of the JAK/STAT Signal Pathway in Rheumatoid Arthritis. *Ther Adv Musculoskelet Dis* (2018) 10(5-6):117–27. doi: 10.1177/1759720X18776224
8. Salaffi F, Ciapetti A. Clinical Disease Activity Assessments in Rheumatoid Arthritis Part of. in. *Int J Clin Rheumatol* (2013) 8(3):347–60. doi: 10.2217/ijr.13.24
9. Schoels M, Alasti F, Smolen JS, Aletaha D. Evaluation of Newly Proposed Remission Cut-Points for Disease Activity Score in 28 Joints (DAS28) in Rheumatoid Arthritis Patients Upon IL-6 Pathway Inhibition. *Arthritis Res Ther* (2017) 19(1):155. doi: 10.1186/s13075-017-1346-5
10. Heegaard C, Dreyer L, Egsmose C, Madsen OR. Test-Retest Reliability of the Disease Activity Score 28 CRP (DAS28-CRP), the Simplified Disease

Activity Index (SDAI) and the Clinical Disease Activity Index (CDAI) in Rheumatoid Arthritis When Based on Patient Self-Assessment of Tender and Swollen Joints. *Clin Rheumatol* (2013) 32(10):1493–500. doi: 10.1007/s10067-013-2300-9
11. Carpenter L, Norton S, Nikiphorou E, Kiely P, Walsh DA, Dixey J, et al. Validation of Methods for Converting the Original Disease Activity Score (DAS) to the DAS28. *Rheumatol Int* (2018) 38(12):2297–305. doi: 10.1007/s00296-018-4184-0
12. Smolen JS, Aletaha D. Scores for All Seasons: SDAI and CDAI. *Clin Exp Rheumatol* (2014) 32(5 Suppl 85):S–75-9.
13. Smolen JS, Landewe RBM, Bijlsma JWJ, Burmester GR, Dougados M, Kerschbaumer A, et al. EULAR Recommendations for the Management of Rheumatoid Arthritis With Synthetic and Biological Disease-Modifying Antirheumatic Drugs: 2019 Update. *Ann Rheum Dis* (2020) 79(6):685–99. doi: 10.1136/annrheumdis-2019-216655
14. Hyrich KL, Lunt M, Dixon WG, Watson KD, Symmons DP, Register BSRB. Effects of Switching Between Anti-TNF Therapies on HAQ Response in Patients Who do Not Respond to Their First Anti-TNF Drug. *Rheumatol (Oxford)* (2008) 47(7):1000–5. doi: 10.1093/rheumatology/ken127
15. Rubbert-Roth A, Finckh A. Treatment Options in Patients With Rheumatoid Arthritis Failing Initial TNF Inhibitor Therapy: A Critical Review. *Arthritis Res Ther* (2009) 11 Suppl:1, S1. doi: 10.1186/ar2666
16. Mittal N, Mittal R, Sharma A, Jose V, Wanchu A, Singh S. Treatment Failure With Disease-Modifying Antirheumatic Drugs in Rheumatoid Arthritis Patients. *Singapore Med J* (2012) 53(8):532–6.
17. Gomes JL, Sepriano A, Eusebio M, Serra S, Fonseca JE, Saavedra MJ, et al. Predictors and Causes of First-Line Biologic Agent Discontinuation in Rheumatoid Arthritis: Data From Reuma.Pt. *Acta Reumatol Port* (2019) 44(1):57–64.
18. Favalli EG, Raimondo MG, Becciolini A, Crotti C, Biggioggero M, Caporali R. The Management of First-Line Biologic Therapy Failures in Rheumatoid Arthritis: Current Practice and Future Perspectives. *Autoimmun Rev* (2017) 16(12):1185–95. doi: 10.1016/j.autrev.2017.10.002
19. Astorri E, Nerviani A, Bombardieri M, Pitzalis C. Towards a Stratified Targeted Approach With Biologic Treatments in Rheumatoid Arthritis: Role of Synovial Pathobiology. *Curr Pharm Des* (2015) 21(17):2216–24. doi: 10.2174/1381612821666150310145758
20. Vital EM, Dass S, Rawstron AC, Buch MH, Goeb V, Henshaw K, et al. Management of Nonresponse to Rituximab in Rheumatoid Arthritis: Predictors and Outcome of Re-Treatment. *Arthritis Rheum* (2010) 62 (5):1273–9. doi: 10.1002/art.27359
21. Mahler M, Martinez-Prat L, Sparks JA, Deane KD. Precision Medicine in the Care of Rheumatoid Arthritis: Focus on Prediction and Prevention of Future Clinically-Apparent Disease. *Autoimmun Rev* (2020) 19(5):102506. doi: 10.1016/j.autrev.2020.102506
22. Denny JC, Collins FS. Precision Medicine in 2030—Seven Ways to Transform Healthcare. *Cell* (2021) 184(6):1415–9. doi: 10.1016/j.cell.2021.01.015
23. Redekop WK, Mladsi D. The Faces of Personalized Medicine: A Framework for Understanding Its Meaning and Scope. *Value Health* (2013) 6(6, Supplement):S4–9. doi: 10.1016/j.jval.2013.06.005
24. Aletaha D. Precision Medicine and Management of Rheumatoid Arthritis. *J Autoimmun* (2020) 110:102405. doi: 10.1016/j.jaut.2020.102405
25. Salliot C, van der Heijde D. Long-Term Safety of Methotrexate Monotherapy in Patients With Rheumatoid Arthritis: A Systematic Literature Research. *Ann Rheum Dis* (2009) 68(7):1100–4. doi: 10.1136/ard.2008.093690
26. Thomas S, Fisher KH, Snowden JA, Danson SJ, Brown S, Zeidler MP. Methotrexate Is a JAK/STAT Pathway Inhibitor. *PLoS One* (2015) 10(7): e0130078. doi: 10.1371/journal.pone.0130078
27. Revu S, Neregard P, af Klint E, Korotkova M, Catrina AI. Synovial Membrane Immunohistology in Early-Untreated Rheumatoid Arthritis Reveals High Expression of Catabolic Bone Markers That is Modulated by Methotrexate. *Arthritis Res Ther* (2013) 15(6):R205. doi: 10.1186/ar4398
28. Brown PM, Pratt AG, Isaacs JD. Mechanism of Action of Methotrexate in Rheumatoid Arthritis, and the Search for Biomarkers. *Nat Rev Rheumatol* (2016) 12(12):731–42. doi: 10.1038/nrrheum.2016.175
29. Hazlewood GS, Barnabe C, Tomlinson G, Marshall D, Devoe D, Bombardier C. Methotrexate Monotherapy and Methotrexate Combination Therapy With Traditional and Biologic Disease Modifying Antirheumatic Drugs for Rheumatoid Arthritis: Abridged Cochrane Systematic Review and Network Meta-Analysis. *BMJ* (2016) 353:i1777. doi: 10.1136/bmj.i1777
30. Cronstein BN, Sitkovsky M. Adenosine and Adenosine Receptors in the Pathogenesis and Treatment of Rheumatic Diseases. *Nat Rev Rheumatol* (2017) 13(1):41–51. doi: 10.1038/nrrheum.2016.178
31. Montesinos MC, Desai A, Delano D, Chen JF, Fink JS, Jacobson MA, et al. Adenosine A2A or A3 Receptors are Required for Inhibition of Inflammation by Methotrexate and its Analog MX-68. *Arthritis Rheum* (2003) 48(1):240–7. doi: 10.1002/art.10712
32. Varani K, Padovan M, Vincenzi F, Targa M, Trotta F, Govoni M, et al. A2A and A3 Adenosine Receptor Expression in Rheumatoid Arthritis: Upregulation, Inverse Correlation With Disease Activity Score and Suppression of Inflammatory Cytokine and Metalloproteinase Release. *Arthritis Res Ther* (2011) 13(6):R197. doi: 10.1186/ar3527
33. Singh A, Misra R, Aggarwal A. Baseline Adenosine Receptor mRNA Expression in Blood as Predictor of Response to Methotrexate Therapy in Patients With Rheumatoid Arthritis. *Rheumatol Int* (2019) 39(8):1431–8. doi: 10.1007/s00296-019-04344-2
34. Peres RS, Liew FY, Talbot J, Carregaro V, Oliveira RD, Almeida SL, et al. Low Expression of CD39 on Regulatory T Cells as a Biomarker for Resistance to Methotrexate Therapy in Rheumatoid Arthritis. *Proc Natl Acad Sci USA* (2015) 112(8):2509–14. doi: 10.1073/pnas.1424792112
35. Vincenzi F, Padovan M, Targa M, Corciulo C, Giacuzzo S, Merighi S, et al. A (2A) Adenosine Receptors are Differentially Modulated by Pharmacological Treatments in Rheumatoid Arthritis Patients and Their Stimulation Ameliorates Adjuvant-Induced Arthritis in Rats. *PloS One* (2013) 8(1): e54195. doi: 10.1371/journal.pone.0054195
36. Nesher G, Mates M, Zevin S. Effect of Caffeine Consumption on Efficacy of Methotrexate in Rheumatoid Arthritis. *Arthritis Rheum* (2003) 48(2):571–2. doi: 10.1002/art.10766
37. Montesinos MC, Yap JS, Desai A, Posadas I, McCrary CT, Cronstein BN. Reversal of the Antiinflammatory Effects of Methotrexate by the Nonselective Adenosine Receptor Antagonists Theophylline and Caffeine: Evidence That the Antiinflammatory Effects of Methotrexate are Mediated *via* Multiple Adenosine Receptors in Rat Adjuvant Arthritis. *Arthritis Rheum* (2000) 43 (3):656–63. doi: 10.1002/1529-0131(200003)43:3<656::Aid-anr23>3.0.Co;2-h
38. Andersson SE, Johansson LH, Lexmüller K, Ekström GM. Anti-Arthritic Effect of Methotrexate: Is it Really Mediated by Adenosine? *Eur J Pharm Sci* (2000) 9(4):333–43. doi: 10.1016/s0928-0987(99)00073-1
39. Benito-Garcia E, Heller JE, Chibnik LB, Maher NE, Matthews HM, Bilics JA, et al. Dietary Caffeine Intake Does Not Affect Methotrexate Efficacy in Patients With Rheumatoid Arthritis. *J Rheumatol* (2006) 33(7):1275–81.
40. de Rotte MCFJ, den Boer E, de Jong PHP, Pluijm SMF, Bulatović Ćalasan M, Weel AE, et al. Methotrexate Polyglutamates in Erythrocytes are Associated With Lower Disease Activity in Patients With Rheumatoid Arthritis. *Ann Rheum Dis* (2015) 74(2):408. doi: 10.1136/annrheumdis-2013-203725
41. Stamp LK, Barclay ML, O'Donnell JL, Zhang M, Drake J, Frampton C, et al. Effects of Changing From Oral to Subcutaneous Methotrexate on Red Blood Cell Methotrexate Polyglutamate Concentrations and Disease Activity in Patients With Rheumatoid Arthritis. *J Rheumatol* (2011) 38(12):2540–7. doi: 10.3899/jrheum.110481
42. Dervieux T, Furst D, Lein DO, Capps R, Smith K, Caldwell J, et al. Pharmacogenetic and Metabolite Measurements are Associated With Clinical Status in Patients With Rheumatoid Arthritis Treated With Methotrexate: Results of a Multicentred Cross Sectional Observational Study. *Ann Rheum Dis* (2005) 64(8):1180. doi: 10.1136/ard.2004.033399
43. Litman T, Druley TE, Stein WD, Bates SE. From MDR to MXR: New Understanding of Multidrug Resistance Systems, Their Properties and Clinical Significance. *Cell Mol Life Sci* (2001) 58(7):931–59. doi: 10.1007/pl00000912
44. Volk EL, Schneider E. Wild-Type Breast Cancer Resistance Protein (BCRP/ABCG2) is a Methotrexate Polyglutamate Transporter. *Cancer Res* (2003) 63 (17):5538–43.
45. Muto S, Minamitani N, Ogura T, Nakajima A, Nakagawa K, Masaka T, et al. Good Response to Methotrexate Is Associated With a Decrease in the Gene Expression of ABCG2, a Drug Transporter, in Patients With Rheumatoid Arthritis. *Mod Rheumatol* (2021) 31(6):1079–86. doi: 10.1080/14397595.2021.1879429

46. Hakamata J, Kaneko Y, Shimizu M, Yamaoka K, Maruyama J, Takeuchi T, et al. Factors Predicting the Therapeutic Response to Methotrexate in Japanese Patients With Rheumatoid Arthritis: A Hospital-Based Cohort Study. *Biol Pharm Bull* (2018) 41(9):1414–22. doi: 10.1248/bpb.b18-00247
47. Tugwell P, Pincus T, Yocum D, Stein M, Gluck O, Kraag G, et al. Combination Therapy With Cyclosporine and Methotrexate in Severe Rheumatoid Arthritis. The Methotrexate-Cyclosporine Combination Study Group. *N Engl J Med* (1995) 333(3):137–41. doi: 10.1056/nejm199507203330301
48. Cooper DL, Martin SG, Robinson JI, Mackie SL, Charles CJ, Nam J, et al. Fcγriiia Expression on Monocytes in Rheumatoid Arthritis: Role in Immune-Complex Stimulated TNF Production and non-Response to Methotrexate Therapy. *PloS One* (2012) 7(1):e28918. doi: 10.1371/journal.pone.0028918
49. Hong H, Yang H, Xia Y. Circulating miR-10a as Predictor of Therapy Response in Rheumatoid Arthritis Patients Treated With Methotrexate. *Curr Pharm Biotechnol* (2018) 19(1):79–86. doi: 10.2174/1389201019666180417155140
50. O'Dell JR, Nepom BS, Haire C, Gersuk VH, Gaur L, Moore GF, et al. HLA-DRB1 Typing in Rheumatoid Arthritis: Predicting Response to Specific Treatments. *Ann Rheum Dis* (1998) 57(4):209–13. doi: 10.1136/ard.57.4.209
51. Stuhlmüller B, Mans K, Tandon N, Bonin MO, Smiljanovic B, Sörensen TA, et al. Genomic Stratification by Expression of HLA-DRB4 Alleles Identifies Differential Innate and Adaptive Immune Transcriptional Patterns - A Strategy to Detect Predictors of Methotrexate Response in Early Rheumatoid Arthritis. *Clin Immunol* (2016) 171:50–61. doi: 10.1016/j.clim.2016.08.013
52. Eektimmerman F, Swen JJ, Madhar MB, Allaart CF, Guchelaar HJ. Predictive Genetic Biomarkers for the Efficacy of Methotrexate in Rheumatoid Arthritis: A Systematic Review. *Pharmacogenomics J* (2020) 20(2):159–68. doi: 10.1038/s41397-019-0098-9
53. Sharma S, Das M, Kumar A, Marwaha V, Shankar S, Singh P, et al. Purine Biosynthetic Pathway Genes and Methotrexate Response in Rheumatoid Arthritis Patients Among North Indians. *Pharmacogenet Genomics* (2009) 19(10):823–8. doi: 10.1097/fpc.0b013e328331b53e
54. Pawlik A, Czerny B, Dabrowska-Zamojcin E, Górnik W, Poziomkowska I, Gawrońska-Szklarz B, et al. The Influence of IL-6 Polymorphism on Efficacy of Treatment of Rheumatoid Arthritis Patients With Methotrexate and Prednisone. *Pol Arch Med Wewn* (2005) 114(3):843–7.
55. Ruiz-Padilla AJ, Gamez-Nava JI, Saldaña-Cruz AM, Murillo-Vazquez JD, Vazquez-Villegas ML, Zavaleta-Muñiz SA, et al. The -174g/C Interleukin-6 Gene Promoter Polymorphism as a Genetic Marker of Differences in Therapeutic Response to Methotrexate and Leflunomide in Rheumatoid Arthritis. *BioMed Res Int* (2016) 2016:4193538. doi: 10.1155/2016/4193538
56. Bohanec Grabar P, Grabnar I, Rozman B, Logar D, Tomsic M, Suput D, et al. Investigation of the Influence of CYP1A2 and CYP2C19 Genetic Polymorphism on 2-Cyano-3-Hydroxy-N-[4-(Trifluoromethyl)Phenyl]-2-Butenamide (A77 1726) Pharmacokinetics in Leflunomide-Treated Patients With Rheumatoid Arthritis. *Drug Metab Dispos* (2009) 37(10):2061–8. doi: 10.1124/dmd.109.027482
57. Pawlik A, Herczynska M, Kurzawski M, Safranow K, Dziedziejko V, Drozdzik M. The Effect of Exon (19C>A) Dihydroorotate Dehydrogenase Gene Polymorphism on Rheumatoid Arthritis Treatment With Leflunomide. *Pharmacogenomics* (2009) 10(2):303–9. doi: 10.2217/14622416.10.2.303
58. Dziedziejko V, Kurzawski M, Safranow K, Chlubek D, Pawlik A. The Effect of ESR1 and ESR2 Gene Polymorphisms on the Outcome of Rheumatoid Arthritis Treatment With Leflunomide. *Pharmacogenomics* (2011) 12(1):41–7. doi: 10.2217/pgs.10.164
59. Liang C, Li J, Lu C, Xie D, Liu J, Zhong C, et al. Hif1α Inhibition Facilitates Leflunomide-AHR-CRP Signaling to Attenuate Bone Erosion in CRP-Aberrant Rheumatoid Arthritis. *Nat Commun* (2019) 10(1):4579. doi: 10.1038/s41467-019-12163-z
60. Gotanda K, Hirota T, Saito J, Fukae M, Egashira Y, Izumi N, et al. Circulating Intestine-Derived Exosomal miR-328 in Plasma, a Possible Biomarker for Estimating BCRP Function in the Human Intestines. *Sci Rep* (2016) 6:32299. doi: 10.1038/srep32299
61. Perez-Guerrero EE, Gonzalez-Lopez L, Munoz-Valle JF, Vasquez-Jimenez JC, Ramirez-Villafana M, Sanchez-Rodriguez EN, et al. Serum P-Glycoprotein Level: A Potential Biomarker of DMARD Failure in Patients With Rheumatoid Arthritis. *Inflammopharmacology* (2018) 26:1375–81. doi: 10.1007/s10787-018-0529-2
62. Wiese MD, Alotaibi N, O'Doherty C, Sorich MJ, Suppiah V, Cleland LG, et al. Pharmacogenomics of NAT2 and ABCG2 Influence the Toxicity and Efficacy of Sulphasalazine Containing DMARD Regimens in Early Rheumatoid Arthritis. *Pharmacogenomics J* (2014) 14(4):350–5. doi: 10.1038/tpj.2013.45
63. Romão VC, Canhão H, Fonseca JE. Old Drugs, Old Problems: Where do We Stand in Prediction of Rheumatoid Arthritis Responsiveness to Methotrexate and Other Synthetic DMARDs? *BMC Med* (2013) 11(1):17. doi: 10.1186/1741-7015-11-17
64. Varbanov B, Mircheva I. Efectiveness of Treatment With Sulfasalasine in Juvenile Idiopathic Arthritis. *Pediatriya* (2013) 53:59–61.
65. Wijesinghe H, Galappatthy P, de Silva R, Seneviratne SL, Saravanamuttu U, Udagama P, et al. Leflunomide is Equally Efficacious and Safe Compared to Low Dose Rituximab in Refractory Rheumatoid Arthritis Given in Combination With Methotrexate: Results From a Randomized Double Blind Controlled Clinical Trial. *BMC Musculoskeletal Disord* (2017) 18(1):310–0. doi: 10.1186/s12891-017-1673-3
66. Kremer J, Genovese M, Cannon GW, Caldwell J, Cush J, Furst DE, et al. Combination Leflunomide and Methotrexate (MTX) Therapy for Patients With Active Rheumatoid Arthritis Failing MTX Monotherapy: Open-Label Extension of a Randomized, Double-Blind, Placebo Controlled Trial. *J Rheumatol* (2004) 31(8):1521–31.
67. Weisman MH, Durez P, Hallegua D, Aranda R, Becker JC, Nuamah I, et al. Reduction of Inflammatory Biomarker Response by Abatacept in Treatment of Rheumatoid Arthritis. *J Rheumatol* (2006) 33(11):2162–6.
68. Smolen JS, van Vollenhoven RF, Florentinus S, Chen S, Suboticki JL, Kavanaugh A. Predictors of Disease Activity and Structural Progression After Treatment With Adalimumab Plus Methotrexate or Continued Methotrexate Monotherapy in Patients With Early Rheumatoid Arthritis and Suboptimal Response to Methotrexate. *Ann Rheum Dis* (2018) 77(11):1566–72. doi: 10.1136/annrheumdis-2018-213502
69. Kaneko Y, Atsumi T, Tanaka Y, Inoo M, Kobayashi-Haraoka H, Amano K, et al. Comparison of Adding Tocilizumab to Methotrexate With Switching to Tocilizumab in Patients With Rheumatoid Arthritis With Inadequate Response to Methotrexate: 52-Week Results From a Prospective, Randomised, Controlled Study (SURPRISE Study). *Ann Rheum Dis* (2016) 75(11):1917–23. doi: 10.1136/annrheumdis-2015-208426
70. Davis JP, Cain GA, Pitts WJ, Magolda RL, Copeland RA. The Immunosuppressive Metabolite of Leflunomide Is a Potent Inhibitor of Human Dihydroorotate Dehydrogenase. *Biochemistry* (1996) 35(4):1270–3. doi: 10.1021/bi952168g
71. Breedveld FC, Dayer J-M. Leflunomide: Mode of Action in the Treatment of Rheumatoid Arthritis. *Ann Rheum Dis* (2000) 59(11):841–9. doi: 10.1136/ard.59.11.841
72. Kirsch BM, Zeyda M, Stuhlmeier K, Grisar J, Smolen JS, Watschinger B, et al. The Active Metabolite of Leflunomide, A77 1726, Interferes With Dendritic Cell Function. *Arthritis Res Ther* (2005) 7(3):R694. doi: 10.1186/ar1727
73. Fang J, Uchiumi T, Yagi M, Matsumoto S, Amamoto R, Takazaki S, et al. Dihydro-Orotate Dehydrogenase is Physically Associated With the Respiratory Complex and its Loss Leads to Mitochondrial Dysfunction. *Biosci Rep* (2013) 33(2):e00021. doi: 10.1042/BSR20120097
74. O'Doherty C, Schnabl M, Spargo L, Cleland LG, James M, Proudman SM, et al. Association of DHODH Haplotype Variants and Response to Leflunomide Treatment in Rheumatoid Arthritis. *Pharmacogenomics* (2012) 13(12):1427–34. doi: 10.2217/pgs.12.118
75. Pawlik A, Herczyńska M, Kurzawski M, Safranow K, Dziedziejko V, Juzyszyn Z, et al. IL-1beta, IL-6, and TNF Gene Polymorphisms do Not Affect the Treatment Outcome of Rheumatoid Arthritis Patients With Leflunomide. *Pharmacol Rep* (2009) 61(2):281–7. doi: 10.1016/s1734-1140(09)70033-7
76. Kis E, Nagy T, Jani M, Molnar E, Janossy J, Ujhellyi O, et al. Leflunomide and its Metabolite A771726 are High Affinity Substrates of BCRP: Implications for Drug Resistance. *Ann Rheum Dis* (2009) 68(7):1201–7. doi: 10.1136/ard.2007.086264
77. Karanikolas G, Charalambopoulos D, Andrianakos A, Antoniades C, Katsilambros N. Combination of Cyclosporine and Leflunomide Versus Single Therapy in Severe Rheumatoid Arthritis. *J Rheumatol* (2006) 33(3):486–9. doi: 10.1136/ard.2007.086264

78. Lu C, Xu W, Shao J, Zhang F, Chen A, Zheng S. Nrf2 Activation Is Required for Ligustrazine to Inhibit Hepatic Steatosis in Alcohol-Preferring Mice and Hepatocytes. *Toxicol Sci* (2016) 155(2):432–43. doi: 10.1093/toxsci/kfw228
79. Zhang C, Guan D, Jiang M, Liang C, Li L, Zhao N, et al. Efficacy of Leflunomide Combined With Ligustrazine in the Treatment of Rheumatoid Arthritis: Prediction With Network Pharmacology and Validation in a Clinical Trial. *Chin Med* (2019) 14(1):26. doi: 10.1186/s13020-019-0247-8
80. Hansen KE, Cush J, Singhal A, Cooley DA, Cohen S, Patel SR, et al. The Safety and Efficacy of Leflunomide in Combination With Infliximab in Rheumatoid Arthritis. *Arthritis Rheum* (2004) 51(2):228–32. doi: 10.1002/art.20228
81. Dougados M, Emery P, Lemmel EM, Zerbini CA, Brin S, van Riel P. When a DMARD Fails, Should Patients Switch to Sulfasalazine or Add Sulfasalazine to Continuing Leflunomide? *Ann Rheum Dis* (2005) 64(1):44–51. doi: 10.1136/ard.2003.016709
82. Götestam Skorpen C, Hoeltzenbein M, Tincani A, Fischer-Betz R, Elefant E, Chambers C, et al. The EULAR Points to Consider for Use of Antirheumatic Drugs Before Pregnancy, and During Pregnancy and Lactation. *Ann Rheum Dis* (2016) 75(5):795–810. doi: 10.1136/annrheumdis-2015-208840
83. Kesharwani D, Paliwal R, Satapathy T, Das Paul S. Rheumatiod Arthritis: An Updated Overview of Latest Therapy and Drug Delivery. *J Pharmacopuncture* (2019) 22(4):210–24. doi: 10.3831/KPI.2019.22.029
84. Rodenburg R, Ganga A, Lent P, Putte L, Venrooij W. The Antiinflammatory Drug Sulfasalazine Inhibits Tumor Necrosis Factor α Expression in Macrophages by Inducing Apoptosis. *Arthritis Rheum* (2000) 43:1941–50. doi: 10.1002/1529-0131(200009)43:9<1941::AID-ANR4>3.0.CO;2-O
85. Plosker GL, Croom KF. Sulfasalazine: A Review of its Use in the Management of Rheumatoid Arthritis. *Drugs* (2005) 65(13):1825–49. doi: 10.2165/00003495-200565130-00008
86. Morabito L, Montesinos MC, Schreibman DM, Balter L, Thompson LF, Resta R, et al. Methotrexate and Sulfasalazine Promote Adenosine Release by a Mechanism That Requires Ecto-5'-Nucleotidase-Mediated Conversion of Adenine Nucleotides. *J Clin Invest* (1998) 101(2):295–300. doi: 10.1172/jci1554
87. MacDermott RP, Schloemann SR, Bertovich MJ, Nash GS, Peters M, Stenson WF. Inhibition of Antibody Secretion by 5-Aminosalicylic Acid. *GastroenterolOGY* (1989) 96(2 Pt 1):442–8. doi: 10.1016/0016-5085(89)91569-2
88. Volin M, Campbell P, Connors M, Woodruff D, Koch A. The Effect of Sulfasalazine on Rheumatoid Arthritic Synovial Tissue Chemokine Production. *Exp Mol Pathol* (2002) 73:84–92. doi: 10.1006/exmp.2002.2460
89. Choi J, Fenando A. Sulfasalazine. In: *StatPearls*. Treasure Island (FL: StatPearls Publishing Copyright © 2021, StatPearls Publishing LLC. (2021).
90. Huang R, Tang Y, Zeng J. Comparison of the Effects of Tripterygii Totorum and Sulfasalazine on Rheumatoid Arthritis: A Retrospective Cohort Study. *Trop J Pharm Res* (2020) 19:421–5. doi: 10.4314/tjpr.v19i2.26
91. Scott DL, Smolen JS, Kalden JR, van de Putte LB, Larsen A, Kvien TK, et al. Treatment of Active Rheumatoid Arthritis With Leflunomide: Two Year Follow Up of a Double Blind, Placebo Controlled Trial Versus Sulfasalazine. *Ann Rheum Dis* (2001) 60(10):913–23. doi: 10.1136/ard.60.10.913
92. Zaher H, Khan AA, Palandra J, Brayman TG, Yu L, Ware JA. Breast Cancer Resistance Protein (BCRP/ABCG2) is a Major Determinant of Sulfasalazine Absorption and Elimination in the Mouse. *FASEB J* (2006) 20(5):A1143–3. doi: 10.1096/fasebj.20.5.A1143-c
93. Pascual-Ramos V, Atisha-Fregoso Y, Lima G, Fragoso-Loyo H, Jákez-Ocampo J, Contreras-Yáñez I, et al. Rheumatoid Arthritis (RA) Disease Activity Predicts Function of ABCB1 (P-Gp) and ABCG2 (BCRP1) Drug-Efflux Transporters. *Gac Med Mex* (2016) 152(6):582–6.
94. Zamek-Gliszczynski MJ, Bedwell DW, Bao JQ, Higgins JW. Characterization of SAGE Mdr1a (P-Gp), Bcrp, and Mrp2 Knockout Rats Using Loperamide, Paclitaxel, Sulfasalazine, and Carboxydichlorofluorescein Pharmacokinetics. *Drug Metab Dispos* (2012) 40(9):1825–33. doi: 10.1124/dmd.112.046508
95. Yudoh K, Matsuno H, Nakazawa F, Yonezawa T, Kimura T. Increased Expression of Multidrug Resistance of P-Glycoprotein on Th1 Cells Correlates With Drug Resistance in Rheumatoid Arthritis. *Arthritis Rheum* (1999) 42(9):2014–5. doi: 10.1002/1529-0131(199909)42:9<2014::Aid-anr32>3.0.Co;2-r
96. van der Heijden J, de Jong MC, Dijkmans BAC, Lems WF, Oerlemans R, Kathmann I, et al. Development of Sulfasalazine Resistance in Human T Cells Induces Expression of the Multidrug Resistance Transporter ABCG2 (BCRP) and Augmented Production of TNFα. *Ann Rheum Dis* (2004) 63(2):138–43. doi: 10.1136/ard.2002.005249
97. Lee A, Park K, Choi SJ, Seo DH, Kim K, Kim HS, et al. Prediction of Antiarthritic Drug Efficacies by Monitoring Active Matrix Metalloproteinase-3 (MMP-3) Levels in Collagen-Induced Arthritic Mice Using the MMP-3 Probe. *Mol Pharm* (2014) 11(5):1450–8. doi: 10.1021/mp400622q
98. Capell HA, Madhok R, Porter DR, Munro RA, McInnes IB, Hunter JA, et al. Combination Therapy With Sulfasalazine and Methotrexate is More Effective Than Either Drug Alone in Patients With Rheumatoid Arthritis With a Suboptimal Response to Sulfasalazine: Results From the Double-Blind Placebo-Controlled MASCOT Study. *Ann Rheum Dis* (2007) 66(2):235–41. doi: 10.1136/ard.2006.057133
99. Schipper LG, Fransen J, Barrera P, Van Riel PLCM. Methotrexate in Combination With Sulfasalazine is More Effective in Rheumatoid Arthritis Patients Who Failed Sulfasalazine Than in Patients Naïve to Both Drugs. *Rheumatology* (2009) 48(7):828–33. doi: 10.1093/rheumatology/kep090
100. Combe B, Codreanu C, Fiocco U, Gaubitz M, Geusens PP, Kvien TK, et al. Efficacy, Safety and Patient-Reported Outcomes of Combination Etanercept and Sulfasalazine Versus Etanercept Alone in Patients With Rheumatoid Arthritis: A Double-Blind Randomised 2-Year Study. *Ann Rheum Dis* (2009) 68(7):1146–52. doi: 10.1136/ard.2007.087106
101. Kawai S, Takeuchi T, Yamamoto K, Tanaka Y, Miyasaka N. Efficacy and Safety of Additional Use of Tacrolimus in Patients With Early Rheumatoid Arthritis With Inadequate Response to DMARDs–a Multicenter, Double-Blind, Parallel-Group Trial. *Mod Rheumatol* (2011) 21(5):458–68. doi: 10.1007/s10165-011-0425-8
102. Elliott MJ, Maini RN, Feldmann M, Long-Fox A, Charles P, Katsikis P, et al. Treatment of Rheumatoid Arthritis With Chimeric Monoclonal Antibodies to Tumor Necrosis Factor Alpha. *Arthritis Rheum* (1993) 36(12):1681–90. doi: 10.1002/art.1780361206
103. Melsheimer R, Geldhof A, Apaolaza I, Schaible T. Remicade(®) (Infliximab): 20 Years of Contributions to Science and Medicine. *Biologics: Targets Ther* (2019) 13:139–78. doi: 10.2147/BTT.S207246
104. Chaabo K, Kirkham B. Rheumatoid Arthritis - Anti-TNF. *Int Immunopharmacol* (2015) 27(2):180–4. doi: 10.1016/j.intimp.2015.04.051
105. Klotz U, Teml A, Schwab M. Clinical Pharmacokinetics and Use of Infliximab. *Clin Pharmacokinet* (2007) 46(8):645–60. doi: 10.2165/00003088-200746080-00002
106. Harriman G, Harper LK, Schaible TF. Summary of Clinical Trials in Rheumatoid Arthritis Using Infliximab, an Anti-TNFα Treatment. *Ann Rheum Dis* (1999) 58(suppl 1):I61–4. doi: 10.1136/ard.58.2008.i61
107. Kim J, Ryu H, Yoo DH, Park SH, Song GG, Park W, et al. A Clinical Trial and Extension Study of Infliximab in Korean Patients With Active Rheumatoid Arthritis Despite Methotrexate Treatment. *J Korean Med Sci* (2013) 28(12):1716–22. doi: 10.3346/jkms.2013.28.12.1716
108. Talotta R, Rucci F, Canti G, Scaglione F. Pros and Cons of the Immunogenicity of Monoclonal Antibodies in Cancer Treatment: A Lesson From Autoimmune Diseases. *Immunotherapy* (2019) 11(3):241–54. doi: 10.2217/imt-2018-0081
109. Vos Q, Lees A, Wu ZQ, Snapper CM, Mond JJ. B-Cell Activation by T-Cell-Independent Type 2 Antigens as an Integral Part of the Humoral Immune Response to Pathogenic Microorganisms. *Immunol Rev* (2000) 176:154–70. doi: 10.1034/j.1600-065x.2000.00607.x
110. Atzeni F, Talotta R, Salaffi F, Cassinotti A, Varisco V, Battellino M, et al. Immunogenicity and Autoimmunity During Anti-TNF Therapy. *Autoimmun Rev* (2013) 12(7):703–8. doi: 10.1016/j.autrev.2012.10.021
111. van Schie KA, Hart MH, de Groot ER, Kruithof S, Aarden LA, Wolbink GJ, et al. The Antibody Response Against Human and Chimeric Anti-TNF Therapeutic Antibodies Primarily Targets the TNF Binding Region. *Ann Rheum Dis* (2015) 74(1):311–4. doi: 10.1136/annrheumdis-2014-206237
112. Hambardzumyan K, Hermanrud C, Marits P, Vivar N, Ernestam S, Wallman JK, et al. Association of Female Sex and Positive Rheumatoid Factor With Low Serum Infliximab and Anti-Drug Antibodies, Related to Treatment Failure in Early Rheumatoid Arthritis: Results From the SWEFOT Trial Population. *Scand J Rheumatol* (2019) 48(5):362–6. doi: 10.1080/03009742.2019.1602670

113. Eng GP, Bouchelouche P, Bartels EM, Bliddal H, Bendtzen K, Stoltenberg M. Anti-Drug Antibodies, Drug Levels, Interleukin-6 and Soluble TNF Receptors in Rheumatoid Arthritis Patients During the First 6 Months of Treatment With Adalimumab or Infliximab: A Descriptive Cohort Study. *PloS One* (2016) 11(9):e0162316. doi: 10.1371/journal.pone.0162316
114. Wolbink GJ, Vis M, Lems W, Voskuyl AE, de Groot E, Nurmohamed MT, et al. Development of Antiinfliximab Antibodies and Relationship to Clinical Response in Patients With Rheumatoid Arthritis. *Arthritis Rheum* (2006) 54 (3):711–5. doi: 10.1002/art.21671
115. Murdaca G, Negrini S, Greco M, Schiavi C, Giusti F, Borro M, et al. Immunogenicity of Infliximab and Adalimumab. *Expert Opin Drug Saf* (2019) 18(5):343–5. doi: 10.1080/14740338.2019.1602117
116. Radstake TR, Svenson M, Eijsbouts AM, van den Hoogen FH, Enevold C, van Riel PL, et al. Formation of Antibodies Against Infliximab and Adalimumab Strongly Correlates With Functional Drug Levels and Clinical Responses in Rheumatoid Arthritis. *Ann Rheum Dis* (2009) 68 (11):1739–45. doi: 10.1136/ard.2008.092833
117. Kalden JR, Schulze-Koops H. Immunogenicity and Loss of Response to TNF Inhibitors: Implications for Rheumatoid Arthritis Treatment. *Nat Rev Rheumatol* (2017) 13(12):707–18. doi: 10.1038/nrrheum.2017.187
118. Siljehult F, Ärlestig L, Eriksson C, Rantapää-Dahlqvist S. Concentrations of Infliximab and Anti-Drug Antibodies in Relation to Clinical Response in Patients With Rheumatoid Arthritis. *Scand J Rheumatol* (2018) 47(5):345–50. doi: 10.1080/03009742.2018.1433232
119. Cerezo LA, Remáková M, Tomčik M, Gay S, Neidhart M, Lukanidin E, et al. The Metastasis-Associated Protein S100A4 Promotes the Inflammatory Response of Mononuclear Cells *via* the TLR4 Signalling Pathway in Rheumatoid Arthritis. *Rheumatology* (2014) 53(8):1520–6. doi: 10.1093/rheumatology/keu031
120. Erlandsson MC, Forslind K, Andersson SEM, Lund A, Bokarewa MI. Metastasin S100A4 is Increased in Proportion to Radiographic Damage in Patients With RA. *Rheumatology* (2012) 51(5):932–40. doi: 10.1093/rheumatology/ker362
121. Andersson SEM, Svensson MND, Erlandsson MC, Dehlin M, Andersson KME, Bokarewa MI. Activation of Fms-Like Tyrosine Kinase 3 Signaling Enhances Survivin Expression in a Mouse Model of Rheumatoid Arthritis. *PloS One* (2012) 7(10):e47668–8. doi: 10.1371/journal.pone.0047668
122. Isgren A, Forslind K, Erlandsson M, Axelsson C, Andersson S, Lund A, et al. High Survivin Levels Predict Poor Clinical Response to Infliximab Treatment in Patients With Rheumatoid Arthritis. *Semin Arthritis Rheum* (2012) 41 (5):652–7. doi: 10.1016/j.semarthrit.2011.08.005
123. Sekiguchi N, Kawauchi S, Furuya T, Inaba N, Matsuda K, Ando S, et al. Messenger Ribonucleic Acid Expression Profile in Peripheral Blood Cells From RA Patients Following Treatment With an Anti-TNF-Alpha Monoclonal Antibody, Infliximab. *Rheumatol (Oxford)* (2008) 47(6):780–8. doi: 10.1093/rheumatology/ken083
124. Montes A, Perez-Pampin E, Navarro-Sarabia F, Moreira V, de la Serna AR, Magallares B, et al. Genomics Study: Rheumatoid Arthritis Response to Treatment Across IgG1 Allotype – Anti-TNF Incompatibility: A Case-Only Study. *Arthritis Res Ther* (2015) 17(1):63. doi: 10.1186/s13075-015-0571-z
125. Buch MH, Seto Y, Bingham SJ, Bejarano V, Bryer D, White J, et al. C-Reactive Protein as a Predictor of Infliximab Treatment Outcome in Patients With Rheumatoid Arthritis: Defining Subtypes of Nonresponse and Subsequent Response to Etanercept. *Arthritis Rheum* (2005) 52(1):42–8. doi: 10.1002/art.20711
126. Canete JD, Suarez B, Hernandez MV, Sanmarti R, Rego I, Celis R, et al. Influence of Variants of Fc Gamma Receptors IIA and IIIA on the American College of Rheumatology and European League Against Rheumatism Responses to Anti-Tumour Necrosis Factor Alpha Therapy in Rheumatoid Arthritis. *Ann Rheum Dis* (2009) 68(10):1547–52. doi: 10.1136/ard.2008.096982
127. Lourido L, Ruiz-Romero C, Picchi F, Diz-Rosales N, Vilaboa-Galan S, Fernandez-Lopez C, et al. Association of Serum Anti-Centromere Protein F Antibodies With Clinical Response to Infliximab in Patients With Rheumatoid Arthritis: A Prospective Study. *Semin Arthritis Rheum* (2020) 50(5):1101–8. doi: 10.1016/j.semarthrit.2020.06.010
128. Wijbrandts CA, Dijkgraaf MG, Kraan MC, Vinkenoog M, Smeets TJ, Dinant H, et al. The Clinical Response to Infliximab in Rheumatoid Arthritis is in Part Dependent on Pretreatment Tumour Necrosis Factor Alpha Expression in the Synovium. *Ann Rheum Dis* (2008) 67(8):1139–44. doi: 10.1136/ard.2007.080440
129. Julia A, Erra A, Palacio C, Tomas C, Sans X, Barcelo P, et al. An Eight-Gene Blood Expression Profile Predicts the Response to Infliximab in Rheumatoid Arthritis. *PloS One* (2009) 4(10):e7556. doi: 10.1371/journal.pone.0007556
130. Mugnier B, Balandraud N, Darque A, Roudier C, Roudier J, Reviron D. Polymorphism at Position –308 of the Tumor Necrosis Factor α Gene Influences Outcome of Infliximab Therapy in Rheumatoid Arthritis. *Arthritis Rheumatism* (2003) 48(7):1849–52. doi: 10.1002/art.11168
131. Seitz M, Wirthmüller U, Möller B, Villiger PM. The -308 Tumour Necrosis Factor-Alpha Gene Polymorphism Predicts Therapeutic Response to TNFalpha-Blockers in Rheumatoid Arthritis and Spondyloarthritis Patients. *Rheumatol (Oxford)* (2007) 46(1):93–6. doi: 10.1093/rheumatology/kel175
132. Zeng Z, Duan Z, Zhang T, Wang S, Li G, Gao J, et al. Association Between Tumor Necrosis Factor-α (TNF-α) Promoter -308 G/A and Response to TNF-α Blockers in Rheumatoid Arthritis: A Meta-Analysis. *Mod Rheumatol* (2013) 23(3):489–95. doi: 10.1007/s10165-012-0699-5
133. O'Rielly DD, Roslin NM, Beyene J, Pope A, Rahman P. TNF-α –308 G/A Polymorphism and Responsiveness to TNF-α Blockade Therapy in Moderate to Severe Rheumatoid Arthritis: A Systematic Review and Meta-Analysis. *Pharmacogenomics J* (2009) 9(3):161–7. doi: 10.1038/tpj.2009.7
134. Rooryck C, Barnetche T, Richez C, Laleye A, Arveiler B, Schaeverbeke T. Influence of FCGR3A-V212F and TNFRSF1B-M196R Genotypes in Patients With Rheumatoid Arthritis Treated With Infliximab Therapy. *Clin Exp Rheumatol* (2008) 26(2):340–2.
135. Canet LM, Filipescu I, Cáliz R, Lupiañez CB, Canhão H, Escudero A, et al. Genetic Variants Within the TNFRSF1B Gene and Susceptibility to Rheumatoid Arthritis and Response to Anti-TNF Drugs: A Multicenter Study. *Pharmacogenet Genomics* (2015) 25(7):323–33. doi: 10.1097/fpc.0000000000000140
136. Fabris M, Tolusso B, Di Poi E, Assaloni R, Sinigaglia L, Ferraccioli G. Tumor Necrosis Factor-Alpha Receptor II Polymorphism in Patients From Southern Europe With Mild-Moderate and Severe Rheumatoid Arthritis. *J Rheumatol* (2002) 29(9):1847–50.
137. Ongaro A, De Mattei M, Pellati A, Caruso A, Ferretti S, Masieri FF, et al. Can Tumor Necrosis Factor Receptor II Gene 676T>G Polymorphism Predict the Response Grading to Anti-TNFalpha Therapy in Rheumatoid Arthritis? *Rheumatol Int* (2008) 28(9):901–8. doi: 10.1007/s00296-008-0552-5
138. Swierkot J, Bogunia-Kubik K, Nowak B, Bialowas K, Korman L, Gebura K, et al. Analysis of Associations Between Polymorphisms Within Genes Coding for Tumour Necrosis Factor (TNF)-Alpha and TNF Receptors and Responsiveness to TNF-Alpha Blockers in Patients With Rheumatoid Arthritis. *Joint Bone Spine* (2015) 82(2):94–9. doi: 10.1016/j.jbspin.2014.08.006
139. Morales-Lara MJ, Conesa-Zamora P, Garcia-Simon MS, Pedrero F, Santaclara V, Perez-Guillermo M, et al. Association Between the FCGR3A V158F Polymorphism and the Clinical Response to Infliximab in Rheumatoid Arthritis and Spondyloarthritis Patients. *Scand J Rheumatol* (2010) 39(6):518–20. doi: 10.3109/03009741003781969
140. Seitz M, Wirthmüller U, Möller B, Villiger PM. The –308 Tumour Necrosis Factor-α Gene Polymorphism Predicts Therapeutic Response to TNFα-Blockers in Rheumatoid Arthritis and Spondyloarthritis Patients. *Rheumatology* (2007) 46(1):93–6. doi: 10.1093/rheumatology/kel175
141. Guis S, Balandraud N, Bouvenot J, Auger I, Toussirot E, Wendling D, et al. Influence of –308 a/G Polymorphism in the Tumor Necrosis Factor α Gene on Etanercept Treatment in Rheumatoid Arthritis. *Arthritis Care Res* (2007) 57(8):1426–30. doi: 10.1002/art.23092
142. Ferreiro-Iglesias A, Montes A, Perez-Pampin E, Canete JD, Raya E, Magro-Checa C, et al. Evaluation of 12 GWAS-Drawn SNPs as Biomarkers of Rheumatoid Arthritis Response to TNF Inhibitors. A Potential SNP Association With Response to Etanercept. *PloS One* (2019) 14(2): e0213073. doi: 10.1371/journal.pone.0213073
143. Schotte H, Schluter B, Drynda S, Willeke P, Tidow N, Assmann G, et al. Interleukin 10 Promoter Microsatellite Polymorphisms are Associated With Response to Long Term Treatment With Etanercept in Patients With Rheumatoid Arthritis. *Ann Rheum Dis* (2005) 64(4):575–81. doi: 10.1136/ard.2004.027672

144. Padyukov L, Lampa J, Heimburger M, Ernestam S, Cederholm T, Lundkvist I, et al. Genetic Markers for the Efficacy of Tumour Necrosis Factor Blocking Therapy in Rheumatoid Arthritis. *Ann Rheum Dis* (2003) 62(6):526–9. doi: 10.1136/ard.62.6.526
145. Plant D, Webster A, Nair N, Oliver J, Smith SL, Eyre S, et al. Differential Methylation as a Biomarker of Response to Etanercept in Patients With Rheumatoid Arthritis. *Arthritis Rheumatol* (2016) 68(6):1353–60. doi: 10.1002/art.39590
146. Jančić I, Šefik-Bukilica M, Živojinović S, Damjanov N, Spasovski V, Kotur N, et al. Influence of Promoter Polymorphisms of the TNF-α (-308g/A) and IL-6 (-174g/C) Genes on Therapeutic Response to Etanercept in Rheumatoid Arthritis. *J Med Biochem* (2015) 34(4):414–21. doi: 10.2478/jomb-2014-0060
147. Jančić I, Arsenović-Ranin N, Šefik-Bukilica M, Živojinović S, Damjanov N, Spasovski V, et al. -174g/C Interleukin-6 Gene Promoter Polymorphism Predicts Therapeutic Response to Etanercept in Rheumatoid Arthritis. *Rheumatol Int* (2013) 33(6):1481–6. doi: 10.1007/s00296-012-2586-y
148. Dávila-Fajardo CL, Márquez A, Pascual-Salcedo D, Moreno Ramos MJ, García-Portales R, Magro C, et al. Confirmation of –174G/C Interleukin-6 Gene Promoter Polymorphism as a Genetic Marker Predicting Antitumor Necrosis Factor Treatment Outcome. *Pharmacogenet Genomics* (2014) 24 (1):1–5. doi: 10.1097/FPC.0000000000000013
149. Cui J, Stahl EA, Saevarsdottir S, Miceli C, Diogo D, Trynka G, et al. Genome-Wide Association Study and Gene Expression Analysis Identifies CD84 as a Predictor of Response to Etanercept Therapy in Rheumatoid Arthritis. *PloS Genet* (2013) 9(3):e1003394. doi: 10.1371/journal.pgen.1003394
150. Priori R, Casadei L, Valerio M, Scrivo R, Valesini G, Manetti C. (1)H-NMR-Based Metabolomic Study for Identifying Serum Profiles Associated With the Response to Etanercept in Patients With Rheumatoid Arthritis. *PloS One* (2015) 10(11):e0138537. doi: 10.1371/journal.pone.0138537
151. Zhang B, Jiang W. IL-1β, IL-17a, CRP and Biologics History Might Serve as Potential Markers for Clinical Response to Etanercept in Rheumatoid Arthritis Patients. *Inflammopharmacology* (2019) 27(6):1123–30. doi: 10.1007/s10787-019-00624-2
152. Ling SF, Nair N, Verstappen SMM, Barton A, Zucht H-D, Budde P, et al. Proteomic Analysis to Define Predictors of Treatment Response to Adalimumab or Methotrexate in Rheumatoid Arthritis Patients. *Pharmacogenomics J* (2020) 20(3):516–23. doi: 10.1038/s41397-019-0139-4
153. Vaisman-Mentesh A, Gutierrez-Gonzalez M, DeKosky BJ, Wine Y. The Molecular Mechanisms That Underlie the Immune Biology of Anti-Drug Antibody Formation Following Treatment With Monoclonal Antibodies. *Front Immunol* (2020) 11:1951(1951). doi: 10.3389/fimmu.2020.01951
154. Bartelds GM, de Groot E, Nurmohamed MT, Hart MH, van Eede PH, Wijbrandts CA, et al. Surprising Negative Association Between IgG1 Allotype Disparity and Anti-Adalimumab Formation: A Cohort Study. *Arthritis Res Ther* (2010) 12(6):R221. doi: 10.1186/ar3208
155. Sokolove J, Schiff M, Fleischmann R, Weinblatt ME, Connolly SE, Johnsen A, et al. Impact of Baseline Anti-Cyclic Citrullinated Peptide-2 Antibody Concentration on Efficacy Outcomes Following Treatment With Subcutaneous Abatacept or Adalimumab: 2-Year Results From the AMPLE Trial. *Ann Rheum Dis* (2016) 75(4):709–14. doi: 10.1136/annrheumdis-2015-207942
156. Magill L, Adriani M, Berthou V, Chen K, Gleizes A, Hacein-Bey-Abina S, et al. Low Percentage of Signal Regulatory Protein Alpha/Beta(+) Memory B Cells in Blood Predicts Development of Anti-Drug Antibodies (ADA) in Adalimumab-Treated Rheumatoid Arthritis Patients. *Front Immunol* (2018) 9:2865. doi: 10.3389/fimmu.2018.02865
157. Kanbe K, Chiba J, Nakamura A. Decrease of CD68 and MMP-3 Expression in Synovium by Treatment of Adalimumab for Rheumatoid Arthritis. *Int J Rheum Dis* (2011) 14(3):261–6. doi: 10.1111/j.1756-185X.2011.01643.x
158. Potter C, Hyrich KL, Tracey A, Lunt M, Plant D, Symmons DP, et al. Association of Rheumatoid Factor and Anti-Cyclic Citrullinated Peptide Positivity, But Not Carriage of Shared Epitope or PTPN22 Susceptibility Variants, With Anti-Tumour Necrosis Factor Response in Rheumatoid Arthritis. *Ann Rheum Dis* (2009) 68(1):69–74. doi: 10.1136/ard.2007.084715
159. Aerts NE, De Knop KJ, Leysen J, Ebo DG, Bridts CH, Weyler JJ, et al. Increased IL-17 Production by Peripheral T Helper Cells After Tumour Necrosis Factor Blockade in Rheumatoid Arthritis is Accompanied by Inhibition of Migration-Associated Chemokine Receptor Expression. *Rheumatol (Oxford)* (2010) 49 (12):2264–72. doi: 10.1093/rheumatology/keq224
160. Lee HN, Kim YK, Kim GT, Ahn E, So MW, Sohn DH, et al. Neutrophil-To-Lymphocyte and Platelet-to-Lymphocyte Ratio as Predictors of 12-Week Treatment Response and Drug Persistence of Anti-Tumor Necrosis Factor-Alpha Agents in Patients With Rheumatoid Arthritis: A Retrospective Chart Review Analysis. *Rheumatol Int* (2019) 39(5):859–68. doi: 10.1007/s00296-019-04276-x
161. Miceli-Richard C, Comets E, Verstuyft C, Tamouza R, Loiseau P, Ravaud P, et al. A Single Tumour Necrosis Factor Haplotype Influences the Response to Adalimumab in Rheumatoid Arthritis. *Ann Rheum Dis* (2008) 67(4):478–84. doi: 10.1136/ard.2007.074104
162. Han BK, Kuzin I, Gaughan JP, Olsen NJ, Bottaro A. Baseline CXCL10 and CXCL13 Levels are Predictive Biomarkers for Tumor Necrosis Factor Inhibitor Therapy in Patients With Moderate to Severe Rheumatoid Arthritis: A Pilot, Prospective Study. *Arthritis Res Ther* (2016) 18:93. doi: 10.1186/s13075-016-0995-0
163. Stuhlmuller B, Haupl T, Hernandez MM, Grutzkau A, Kuban RJ, Tandon N, et al. CD11c as a Transcriptional Biomarker to Predict Response to Anti-TNF Monotherapy With Adalimumab in Patients With Rheumatoid Arthritis. *Clin Pharmacol Ther* (2010) 87(3):311–21. doi: 10.1038/clpt.2009.244
164. Choi IY, Gerlag DM, Herenius MJ, Thurlings RM, Wijbrandts CA, Foell D, et al. MRP8/14 Serum Levels as a Strong Predictor of Response to Biological Treatments in Patients With Rheumatoid Arthritis. *Ann Rheum Dis* (2015) 74(3):499–505. doi: 10.1136/annrheumdis-2013-203923
165. Dennis GJr., Holweg CT, Kummerfeld SK, Choy DF, Setiadi AF, Hackney JA, et al. Synovial Phenotypes in Rheumatoid Arthritis Correlate With Response to Biologic Therapeutics. *Arthritis Res Ther* (2014) 16(2):R90. doi: 10.1186/ar4555
166. Cuchacovich M, Soto L, Edwardes M, Gutierrez M, Llanos C, Pacheco D, et al. Tumour Necrosis Factor (TNF)alpha -308 G/G Promoter Polymorphism and TNFalpha Levels Correlate With a Better Response to Adalimumab in Patients With Rheumatoid Arthritis. *Scand J Rheumatol* (2006) 35(6):435–40. doi: 10.1080/03009740600904284
167. Dávila-Fajardo CL, van der Straaten T, Baak-Pablo R, Medarde Caballero C, Cabeza Barrera J, Huizinga TW, et al. FcGR Genetic Polymorphisms and the Response to Adalimumab in Patients With Rheumatoid Arthritis. *Pharmacogenomics* (2015) 16(4):373–81. doi: 10.2217/pgs.14.178
168. Dávila-Fajardo CL, Márquez A, Pascual-Salcedo D, Moreno Ramos MJ, García-Portales R, Magro C, et al. Confirmation of -174G/C Interleukin-6 Gene Promoter Polymorphism as a Genetic Marker Predicting Antitumor Necrosis Factor Treatment Outcome. *Pharmacogenet Genomics* (2014) 24 (1):1–5. doi: 10.1097/fpc.0000000000000013
169. Berkhout LC, Vogelzang EH, Hart MM, Loeff FC, Dijk L, Derksen NIL, et al. The Effect of Certolizumab Drug Concentration and Anti-Drug Antibodies on TNF Neutralisation. *Clin Exp Rheumatol* (2020) 38(2):306–13. doi: 10.1136/annrheumdis-2018-eular.4399
170. Takeuchi T, Yamamoto K, Yamanaka H, Ishiguro N, Tanaka Y, Eguchi K, et al. Early Response to Certolizumab Pegol Predicts Long-Term Outcomes in Patients With Active Rheumatoid Arthritis: Results From the Japanese Studies. *Mod Rheumatol* (2015) 25(1):11–20. doi: 10.3109/14397595.2014.904475
171. van der Heijde D, Keystone EC, Curtis JR, Landewé RB, Schiff MH, Khanna D, et al. Timing and Magnitude of Initial Change in Disease Activity Score 28 Predicts the Likelihood of Achieving Low Disease Activity at 1 Year in Rheumatoid Arthritis Patients Treated With Certolizumab Pegol: A *Post-Hoc* Analysis of the RAPID 1 Trial. *J Rheumatol* (2012) 39(7):1326–33. doi: 10.3899/jrheum.111171
172. Weinblatt ME, Fleischmann R, van Vollenhoven RF, Emery P, Huizinga TW, Cutolo M, et al. Twenty-Eight-Week Results From the REALISTIC Phase IIIb Randomized Trial: Efficacy, Safety and Predictability of Response to Certolizumab Pegol in a Diverse Rheumatoid Arthritis Population. *Arthritis Res Ther* (2015) 17:325. doi: 10.1186/s13075-015-0841-9
173. Saraux A, Flipo R-M, Fagnani F, Massol J, Cukierman G, Joubert J-M, et al. Early non-Response to Certolizumab Pegol in Rheumatoid Arthritis Predicts Failure to Achieve Low Disease Activity at 1 Year: Data From a Prospective Observational Study. *RMD Open* (2020) 6(1):e000991. doi: 10.1136/rmdopen-2019-000991
174. Wampler Muskardin T, Vashisht P, Dorschner JM, Jensen MA, Chrabot BS, Kern M, et al. Increased Pretreatment Serum IFN-β/α Ratio Predicts non-

Response to Tumour Necrosis Factor α Inhibition in Rheumatoid Arthritis. *Ann Rheum Dis* (2016) 75(10):1757–62. doi: 10.1136/annrheumdis-2015-208001
175. Wampler Muskardin TL, Fan W, Jin Z, Jensen MA, Dorschner JM, Ghodke-Puranik Y, et al. Distinct Single Cell Gene Expression in Peripheral Blood Monocytes Correlates With Tumor Necrosis Factor Inhibitor Treatment Response Groups Defined by Type I Interferon in Rheumatoid Arthritis. *Front Immunol* (2020) 11:1384(1384). doi: 10.3389/fimmu.2020.01384
176. Gehin JE, Warren DJ, Syversen SW, Lie E, Sexton J, Loli L, et al. Serum Golimumab Concentration and Anti-Drug Antibodies are Associated With Treatment Response and Drug Survival in Patients With Inflammatory Joint Diseases: Data From the NOR-DMARD Study. *Scand J Rheumatol* (2021) 50 (6):445–54. doi: 10.1080/03009742.2021.1875040
177. Guo X, Wang S, Godwood A, Close D, Ryan PC, Roskos LK, et al. Pharmacodynamic Biomarkers and Differential Effects of TNF- and GM-CSF-Targeting Biologics in Rheumatoid Arthritis. *Int J Rheum Dis* (2019) 22 (4):646–53. doi: 10.1111/1756-185X.13395
178. Visvanathan S, Wagner C, Rojas J, Kay J, Dasgupta B, Matteson EL, et al. E-Selectin, Interleukin 18, Serum Amyloid a, and Matrix Metalloproteinase 9 are Associated With Clinical Response to Golimumab Plus Methotrexate in Patients With Active Rheumatoid Arthritis Despite Methotrexate Therapy. *J Rheumatol* (2009) 36(7):1371–9. doi: 10.3899/jrheum.080755
179. Vastesaeger N, Kutzbach AG, Amital H, Pavelka K, Lazaro MA, Moots RJ, et al. Prediction of Remission and Low Disease Activity in Disease-Modifying Anti-Rheumatic Drug-Refractory Patients With Rheumatoid Arthritis Treated With Golimumab. *Rheumatol (Oxford)* (2016) 55 (8):1466–76. doi: 10.1093/rheumatology/kew179
180. Buch MH, Bingham SJ, Bejarano V, Bryer D, White J, Emery P, et al. Therapy of Patients With Rheumatoid Arthritis: Outcome of Infliximab Failures Switched to Etanercept. *Arthritis Care Res* (2007) 57(3):448–53. doi: 10.1002/art.22617
181. Burmester GR, Mariette X, Montecucco C, Monteagudo-Sáez I, Malaise M, Tzioufas AG, et al. Adalimumab Alone and in Combination With Disease-Modifying Antirheumatic Drugs for the Treatment of Rheumatoid Arthritis in Clinical Practice: The Research in Active Rheumatoid Arthritis (ReAct) Trial. *Ann Rheum Dis* (2007) 66(6):732–9. doi: 10.1136/ard.2006.066761
182. Weinblatt ME, Fleischmann R, Huizinga TW, Emery P, Pope J, Massarotti EM, et al. Efficacy and Safety of Certolizumab Pegol in a Broad Population of Patients With Active Rheumatoid Arthritis: Results From the REALISTIC Phase IIIb Study. *Rheumatol (Oxford)* (2012) 51(12):2204–14. doi: 10.1093/rheumatology/kes150
183. Haraoui B, Keystone EC, Thorne JC, Pope JE, Chen I, Asare CG, et al. Clinical Outcomes of Patients With Rheumatoid Arthritis After Switching From Infliximab to Etanercept. *J Rheumatol* (2004) 31(12):2356–9. doi: 10.1093/rheumatology/kes115
184. Marshall JC. Such Stuff as Dreams are Made on: Mediator-Directed Therapy in Sepsis. *Nat Rev Drug Discovery* (2003) 2(5):391–405. doi: 10.1038/nrd1084
185. Azevedo VF, Galli N, Kleinfelder A, D'Ippolito J, Urbano PCM. Etanercept Biosimilars. *Rheumatol Int* (2015) 35(2):197–209. doi: 10.1007/s00296-014-3080-5
186. Moreland LW, Baumgartner SW, Schiff MH, Tindall EA, Fleischmann RM, Weaver AL, et al. Treatment of Rheumatoid Arthritis With a Recombinant Human Tumor Necrosis Factor Receptor (P75)–Fc Fusion Protein. *New Engl J Med* (1997) 337(3):141–7. doi: 10.1056/NEJM199707173370301
187. Haraoui B, Bykerk V. Etanercept in the Treatment of Rheumatoid Arthritis. *Ther Clin Risk Manage* (2007) 3(1):99–105. doi: 10.2147/tcrm.2007.3.1.99
188. Cao Z, Ding X, Huang Y, Liu S, Lin J, Lyu H, et al. Etanercept Inhibits Synovial Inflammation and Reduces the Expression of Adhesion Related Molecules in Synovial Tissues of Patients With Rheumatoid Arthritis. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* (2015) 31(4):511–5, 519.
189. Degboé Y, Rauwel B, Baron M, Boyer J-F, Ruyssen-Witrand A, Constantin A, et al. Polarization of Rheumatoid Macrophages by TNF Targeting Through an IL-10/STAT3 Mechanism. *Front Immunol* (2019) 10:3(3). doi: 10.3389/fimmu.2019.00003
190. Catrina AI, Lampa J, Ernestam S, af Klint E, Bratt J, Klareskog L, et al. Anti-Tumour Necrosis Factor (TNF)-Alpha Therapy (Etanercept) Down-Regulates Serum Matrix Metalloproteinase (MMP)-3 and MMP-1 in Rheumatoid Arthritis. *Rheumatol (Oxford)* (2002) 41(5):484–9. doi: 10.1093/rheumatology/41.5.484
191. Moreland LW, Schiff MH, Baumgartner SW, Tindall EA, Fleischmann RM, Bulpitt KJ, et al. Etanercept Therapy in Rheumatoid Arthritis. A Randomized, Controlled Trial. *Ann Intern Med* (1999) 130(6):478–86. doi: 10.7326/0003-4819-130-6-199903160-00004
192. Bathon JM, Martin RW, Fleischmann RM, Tesser JR, Schiff MH, Keystone EC, et al. A Comparison of Etanercept and Methotrexate in Patients With Early Rheumatoid Arthritis. *N Engl J Med* (2000) 343(22):1586–93. doi: 10.1056/nejm200011303432201
193. Moots RJ, Xavier RM, Mok CC, Rahman MU, Tsai WC, Al-Maini MH, et al. The Impact of Anti-Drug Antibodies on Drug Concentrations and Clinical Outcomes in Rheumatoid Arthritis Patients Treated With Adalimumab, Etanercept, or Infliximab: Results From a Multinational, Real-World Clinical Practice, non-Interventional Study. *PloS One* (2017) 12(4):e0175207. doi: 10.1371/journal.pone.0175207
194. Anderson PJ. Tumor Necrosis Factor Inhibitors: Clinical Implications of Their Different Immunogenicity Profiles. *Semin Arthritis Rheum* (2005) 34(5 Suppl1):19–22. doi: 10.1016/j.semarthrit.2005.01.005
195. Herszényi K, Jókai H, Rencz F, Brodszky V, Nagy E, Holló P. Antidrug Antibody Formation During Tumor Necrosis Factor α Inhibitor Treatment of Severe Psoriatic Patients in the Real-Life Practice. *Postepy dermatologii i alergologii* (2019) 36(5):589–94. doi: 10.5114/ada.2019.89507
196. van Schouwenburg PA, Rispens T, Wolbink GJ. Immunogenicity of Anti-TNF Biologic Therapies for Rheumatoid Arthritis. *Nat Rev Rheumatol* (2013) 9(3):164–72. doi: 10.1038/nrrheum.2013.4
197. Jamnitski A, Krieckaert CL, Nurmohamed MT, Hart MH, Dijkmans BA, Aarden L, et al. Patients Non-Responding to Etanercept Obtain Lower Etanercept Concentrations Compared With Responding Patients. *Ann Rheum Dis* (2012) 71(1):88–91. doi: 10.1136/annrheumdis-2011-200184
198. Kim MS, Lee SH, Song MY, Yoo TH, Lee BK, Kim YS. Comparative Analyses of Complex Formation and Binding Sites Between Human Tumor Necrosis Factor-Alpha and its Three Antagonists Elucidate Their Different Neutralizing Mechanisms. *J Mol Biol* (2007) 374(5):1374–88. doi: 10.1016/j.jmb.2007.10.034
199. Kang CP, Lee KW, Yoo DH, Kang C, Bae SC. The Influence of a Polymorphism at Position –857 of the Tumour Necrosis Factor α Gene on Clinical Response to Etanercept Therapy in Rheumatoid Arthritis. *Rheumatology* (2005) 44(4):547–52. doi: 10.1093/rheumatology/keh550
200. Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, et al. Production of Transforming Growth Factor Beta by Human T Lymphocytes and its Potential Role in the Regulation of T Cell Growth. *J Exp Med* (1986) 163(5):1037–50. doi: 10.1084/jem.163.5.1037
201. Rafiei A, Hajilooi M, Shakib RJ, Alavi SA. Transforming Growth Factor-Beta1 Polymorphisms in Patients With Brucellosis: An Association Between Codon 10 and 25 Polymorphisms and Brucellosis. *Clin Microbiol Infect* (2007) 13(1):97–100. doi: 10.1111/j.1469-0691.2006.01575.x
202. Tountas NA, Casini-Raggi V, Yang H, Di Giovine FS, Vecchi M, Kam L, et al. Functional and Ethnic Association of Allele 2 of the Interleukin-1 Receptor Antagonist Gene in Ulcerative Colitis. *Gastroenterology* (1999) 117(4):806–13. doi: 10.1016/s0016-5085(99)70338-0
203. Schrijver HM, van As J, Crusius JB, Dijkstra CD, Uitdehaag BM. Interleukin (IL)-1 Gene Polymorphisms: Relevance of Disease Severity Associated Alleles With IL-1beta and IL-1ra Production in Multiple Sclerosis. *Mediators Inflammation* (2003) 12(2):89–94. doi: 10.1080/0962933031000097691
204. Huang CM, Wu MC, Wu JY, Tsai FJ. Interleukin-1 Receptor Antagonist Gene Polymorphism in Chinese Patients With Systemic Lupus Erythematosus. *Clin Rheumatol* (2002) 21(3):255–7. doi: 10.1007/s10067-002-8292-5
205. Boucher P, Li WP, Matz RL, Takayama Y, Auwerx J, Anderson RG, et al. LRP1 Functions as an Atheroprotective Integrator of TGFbeta and PDFG Signals in the Vascular Wall: Implications for Marfan Syndrome. *PloS One* (2007) 2(5):e448. doi: 10.1371/journal.pone.0000448
206. Ciechomska M, Roszkowski L, Maslinski W. DNA Methylation as a Future Therapeutic and Diagnostic Target in Rheumatoid Arthritis. *Cells* (2019) 8 (9):953. doi: 10.3390/cells8090953
207. Furst DE, Gaylis N, Bray V, Olech E, Yocum D, Ritter J, et al. Open-Label, Pilot Protocol of Patients With Rheumatoid Arthritis Who Switch to Infliximab After an Incomplete Response to Etanercept: The Opposite Study. *Ann Rheum Dis* (2007) 66(7):893–9. doi: 10.1136/ard.2006.068304

208. Smolen JS, Kay J, Doyle MK, Landewé R, Matteson EL, Wollenhaupt J, et al. Golimumab in Patients With Active Rheumatoid Arthritis After Treatment With Tumour Necrosis Factor α Inhibitors (GO-AFTER Study): A Multicentre, Randomised, Double-Blind, Placebo-Controlled, Phase III Trial. *Lancet* (2009) 374(9685):210–21. doi: 10.1016/S0140-6736(09)60506-7
209. Smolen JS, Kay J, Doyle M, Landewé R, Matteson EL, Gaylis N, et al. Golimumab in Patients With Active Rheumatoid Arthritis After Treatment With Tumor Necrosis Factor α Inhibitors: Findings With Up to Five Years of Treatment in the Multicenter, Randomized, Double-Blind, Placebo-Controlled, Phase 3 GO-AFTER Study. *Arthritis Res Ther* (2015) 17(1):14–4. doi: 10.1186/s13075-015-0516-6
210. van Vollenhoven R, Harju A, Brannemark S, Klareskog L. Treatment With Infliximab (Remicade) When Etanercept (Enbrel) has Failed or Vice Versa: Data From the STURE Registry Showing That Switching Tumour Necrosis Factor Alpha Blockers can Make Sense. *Ann Rheum Dis* (2003) 62(12):1195–8. doi: 10.1136/ard.2003.009589
211. Mease PJ. Adalimumab in the Treatment of Arthritis. *Ther Clin Risk Manage* (2007) 3(1):133–48. doi: 10.2147/tcrm.2007.3.1.133
212. Catrina AI, af Klint E, Ernestam S, Catrina SB, Makrygiannakis D, Botusan IR, et al. Ulfgren: Anti-Tumor Necrosis Factor Therapy Increases Synovial Osteoprotegerin Expression in Rheumatoid Arthritis. *Arthritis Rheum* (2006) 54(1):76–81. doi: 10.1002/art.21528
213. Breedveld FC, Weisman MH, Kavanaugh AF, Cohen SB, Pavelka K, van Vollenhoven R, et al. The PREMIER Study: A Multicenter, Randomized, Double-Blind Clinical Trial of Combination Therapy With Adalimumab Plus Methotrexate Versus Methotrexate Alone or Adalimumab Alone in Patients With Early, Aggressive Rheumatoid Arthritis Who had Not had Previous Methotrexate Treatment. *Arthritis Rheum* (2006) 54(1):26–37. doi: 10.1002/art.21519
214. Zhang J, Jin S, Guo X, Qian W. Targeting the CD47-Sirpα Signaling Axis: Current Studies on B-Cell Lymphoma Immunotherapy. *J Int Med Res* (2018) 46(11):4418–26. doi: 10.1177/0300060518799612
215. Quistrebert J, Hässler S, Bachelet D, Mbogning C, Musters A, Tak PP, et al. Incidence and Risk Factors for Adalimumab and Infliximab Anti-Drug Antibodies in Rheumatoid Arthritis: A European Retrospective Multicohort Analysis. *Semin Arthritis Rheum* (2019) 48(6):967–75. doi: 10.1016/j.semarthrit.2018.10.006
216. Lee YH, Ji JD, Bae SC, Song GG. Associations Between Tumor Necrosis Factor-Alpha (TNF-Alpha) -308 and -238 G/A Polymorphisms and Shared Epitope Status and Responsiveness to TNF-Alpha Blockers in Rheumatoid Arthritis: A Metaanalysis Update. *J Rheumatol* (2010) 37(4):740–6. doi: 10.3899/jrheum.090707
217. Pavy S, Toonen EJ, Miceli-Richard C, Barrera P, van Riel PL, Criswell LA, et al. Tumour Necrosis Factor Alpha -308G->A Polymorphism is Not Associated With Response to TNFalpha Blockers in Caucasian Patients With Rheumatoid Arthritis: Systematic Review and Meta-Analysis. *Ann Rheum Dis* (2010) 69(6):1022–8. doi: 10.1136/ard.2009.117622
218. Soto L, Sabugo F, Catalan D, Wurmann P, Cermenatti T, Gatica H, et al. The Presence of Anti-Citrullinated Protein Antibodies (ACPA) Does Not Affect the Clinical Response to Adalimumab in a Group of RA Patients With the Tumor Necrosis Factor (TNF) α-308 G/G Promoter Polymorphism. *Clin Rheumatol* (2011) 30(3):391–5. doi: 10.1007/s10067-011-1679-4
219. Tanaka Y, Fautrel B, Keystone EC, Ortmann RA, Xie L, Zhu B, et al. Clinical Outcomes in Patients Switched From Adalimumab to Baricitinib Due to non-Response and/or Study Design: Phase III Data in Patients With Rheumatoid Arthritis. *Ann Rheum Dis* (2019) 78(7):890–8. doi: 10.1136/annrheumdis-2018-214529
220. Bessette L, Khraishi M, Kivitz AJ, Kaliyaperumal A, Grantab R, Poulin-Costello M, et al. Single-Arm Study of Etanercept in Adult Patients With Moderate to Severe Rheumatoid Arthritis Who Failed Adalimumab Treatment. *Rheumatol Ther* (2017) 4(2):391–404. doi: 10.1007/s40744-017-0079-x
221. Fleischmann R, Pangan AL, Song IH, Mysler E, Bessette L, Peterfy C, et al. Upadacitinib Versus Placebo or Adalimumab in Patients With Rheumatoid Arthritis and an Inadequate Response to Methotrexate: Results of a Phase III, Double-Blind, Randomized Controlled Trial. *Arthritis Rheumatol* (2019) 71(11):1788–800. doi: 10.1002/art.41032
222. Pasut G. Pegylation of Biological Molecules and Potential Benefits: Pharmacological Properties of Certolizumab Pegol. *BioDrugs* (2014) 28 Suppl:1, S15–23. doi: 10.1007/s40259-013-0064-z
223. Lim H, Lee SH, Lee HT, Lee JU, Son JY, Shin W, et al. Structural Biology of the TNFα Antagonists Used in the Treatment of Rheumatoid Arthritis. *Int J Mol Sci* (2018) 19(3):768. doi: 10.3390/ijms19030768
224. Fleischmann R, Vencovsky J, van Vollenhoven RF, Borenstein D, Box J, Coteur G, et al. Efficacy and Safety of Certolizumab Pegol Monotherapy Every 4 Weeks in Patients With Rheumatoid Arthritis Failing Previous Disease-Modifying Antirheumatic Therapy: The FAST4WARD Study. *Ann Rheum Dis* (2009) 68(6):805–11. doi: 10.1136/ard.2008.099291
225. van Schie K, Hart M, Groot E, Kruithof S, Aarden LA, Wolbink GJ, et al. Response to: 'The Antibody Response Against Human and Chimeric Anti-TNF Therapeutic Antibodies Primarily Targets the TNF Binding Region' by Rinaudo-Gaujous Et al. *Ann Rheum Dis* (2014) 74(8):e41. doi: 10.1136/annrheumdis-2014-206237
226. de Weerd NA, Vivian JP, Nguyen TK, Mangan NE, Gould JA, Braniff SJ, et al. Structural Basis of a Unique Interferon-β Signaling Axis Mediated *via* the Receptor IFNAR1. *Nat Immunol* (2013) 14(9):901–7. doi: 10.1038/ni.2667
227. Smolen JS, Burmester GR, Combe B, Curtis JR, Hall S, Haraoui B, et al. Head-To-Head Comparison of Certolizumab Pegol Versus Adalimumab in Rheumatoid Arthritis: 2-Year Efficacy and Safety Results From the Randomised EXXELERATE Study. *Lancet* (2016) 388(10061):2763–74. doi: 10.1016/S0140-6736(16)31651-8
228. Glatt S, Taylor PC, McInnes IB, Schett G, Landewe R, Baeten D, et al. Efficacy and Safety of Bimekizumab as Add-on Therapy for Rheumatoid Arthritis in Patients With Inadequate Response to Certolizumab Pegol: A Proof-of-Concept Study. *Ann Rheum Dis* (2019) 78(8):1033–40. doi: 10.1136/annrheumdis-2018-214943
229. Campbell J, Lowe D, Sleeman MA. Developing the Next Generation of Monoclonal Antibodies for the Treatment of Rheumatoid Arthritis. *Br J Pharmacol* (2011) 162(7):1470–84. doi: 10.1111/j.1476-5381.2010.01183.x
230. Pelechas E, Voulgari PV, Drosos AA. Golimumab for Rheumatoid Arthritis. *J Clin Med* (2019) 8(3):387. doi: 10.3390/jcm8030387
231. Ono M, Horita S, Sato Y, Nomura Y, Iwata S, Nomura N. Structural Basis for Tumor Necrosis Factor Blockade With the Therapeutic Antibody Golimumab. *Protein Sci* (2018) 27(6):1038–46. doi: 10.1002/pro.3407
232. Stevenson M, Archer R, Tosh J, Simpson E, Everson-Hock E, Stevens J, et al. Adalimumab, Etanercept, Infliximab, Certolizumab Pegol, Golimumab, Tocilizumab and Abatacept for the Treatment of Rheumatoid Arthritis Not Previously Treated With Disease-Modifying Antirheumatic Drugs and After the Failure of Conventional Disease-Modifying Antirheumatic Drugs Only: Systematic Review and Economic Evaluation. *Health Technol Assess* (2016) 20(35):1–610. doi: 10.3310/hta20350
233. Emery P, Fleischmann RM, Strusberg I, Durez P, Nash P, Amante EJ, et al. Efficacy and Safety of Subcutaneous Golimumab in Methotrexate-Naive Patients With Rheumatoid Arthritis: Five-Year Results of a Randomized Clinical Trial. *Arthritis Care Res (Hoboken)* (2016) 68(6):744–52. doi: 10.1002/acr.22759
234. Keystone EC, Genovese MC, Hall S, Bae SC, Han C, Gathany TA, et al. Safety and Efficacy of Subcutaneous Golimumab in Patients With Active Rheumatoid Arthritis Despite Methotrexate Therapy: Final 5-Year Results of the GO-FORWARD Trial. *J Rheumatol* (2016) 43(2):298–306. doi: 10.3899/jrheum.150712
235. Jones PT, Dear PH, Foote J, Neuberger MS, Winter G. Replacing the Complementarity-Determining Regions in a Human Antibody With Those From a Mouse. *Nature* (1986) 321(6069):522–5. doi: 10.1038/321522a0
236. Zidi I, Bouaziz A, Mnif W, Bartegi A, Al-Hizab FA, Amor NB. Golimumab Therapy of Rheumatoid Arthritis: An Overview. *Scand J Immunol* (2010) 72(2):75–85. doi: 10.1111/j.1365-3083.2010.02423.x
237. Leu JH, Adedokun OJ, Gargano C, Hsia EC, Xu Z, Shankar G. Immunogenicity of Golimumab and its Clinical Relevance in Patients With Rheumatoid Arthritis, Psoriatic Arthritis and Ankylosing Spondylitis. *Rheumatol (Oxford)* (2019) 58(3):441–6. doi: 10.1093/rheumatology/key309
238. Nell-Duxneuner V, Reichardt B, Stamm T. AB0312 Real-Life Data on the Use of Biological DMARDS in Rheumatoid Arthritis in Austria With Special

Attention to Switching After First BDMARD Failure. *Ann Rheum Dis* (2020) 79(Suppl 1):1455–5. doi: 10.1136/annrheumdis-2020-eular.5723
239. Esensten JH, Helou YA, Chopra G, Weiss A, Bluestone JA. CD28 Costimulation: From Mechanism to Therapy. *Immunity* (2016) 44(5):973–88. doi: 10.1016/j.immuni.2016.04.020
240. Fantus S, Ruderman E. Updates in the Treatment of Rheumatoid Arthritis. *Curr Treat Options Rheumatol* (2021) 7. doi: 10.1007/s40674-021-00173-2
241. Abatacept (Orencia) for Rheumatoid Arthritis. *Med Lett Drugs Ther* (2006) 48(1229):17–8.
242. Bozec A, Zaiss MM, Kagwiria R, Voll R, Rauh M, Chen Z, et al. T Cell Costimulation Molecules CD80/86 Inhibit Osteoclast Differentiation by Inducing the IDO/tryptophan Pathway. *Sci Transl Med* (2014) 6 (235):235ra60. doi: 10.1126/scitranslmed.3007764
243. Axmann R, Herman S, Zaiss M, Franz S, Polzer K, Zwerina J, et al. CTLA-4 Directly Inhibits Osteoclast Formation. *Ann Rheum Dis* (2008) 67(11):1603–9. doi: 10.1136/ard.2007.080713
244. Bonelli M, Ferner E, Göschl L, Blüml S, Hladik A, Karonitsch T, et al. Abatacept (CTLA-4IG) Treatment Reduces the Migratory Capacity of Monocytes in Patients With Rheumatoid Arthritis. *Arthritis Rheum* (2013) 65(3):599–607. doi: 10.1002/art.37787
245. O'Neill SK, Cao Y, Hamel KM, Doodes PD, Hutas G, Finnegan A. Expression of CD80/86 on B Cells is Essential for Autoreactive T Cell Activation and the Development of Arthritis. *J Immunol* (2007) 179 (8):5109–16. doi: 10.4049/jimmunol.179.8.5109
246. Gazeau P, Alegria GC, Devauchelle-Pensec V, Jamin C, Lemerle J, Bendaoud B, et al. Memory B Cells and Response to Abatacept in Rheumatoid Arthritis. *Clin Rev Allergy Immunol* (2017) 53(2):166–76. doi: 10.1007/s12016-017-8603-x
247. Buch MH, Boyle DL, Rosengren S, Saleem B, Reece RJ, Rhodes LA, et al. Mode of Action of Abatacept in Rheumatoid Arthritis Patients Having Failed Tumour Necrosis Factor Blockade: A Histological, Gene Expression and Dynamic Magnetic Resonance Imaging Pilot Study. *Ann Rheum Dis* (2009) 68(7):1220–7. doi: 10.1136/ard.2008.091876
248. Scarsi M, Paolini L, Ricotta D, Pedrini A, Piantoni S, Caimi L, et al. Abatacept Reduces Levels of Switched Memory B Cells, Autoantibodies, and Immunoglobulins in Patients With Rheumatoid Arthritis. *J Rheumatol* (2014) 41(4):666–72. doi: 10.3899/jrheum.130905
249. Genovese MC, Covarrubias A, Leon G, Mysler E, Keiserman M, Valente R, et al. Subcutaneous Abatacept Versus Intravenous Abatacept: A Phase IIIb Noninferiority Study in Patients With an Inadequate Response to Methotrexate. *Arthritis Rheum* (2011) 63(10):2854–64. doi: 10.1002/art.30463
250. Weinblatt ME, Genovese MC, Schiff MH, Westhovens R, Alten R, Delaet I, et al. FRI0191 Immunogenicity is Low and Transient With Intravenous Abatacept Therapy. *Ann Rheum Dis* (2013) 71(Suppl 3):377–7. doi: 10.1136/annrheumdis-2012-eular.2648
251. Köller MD. Abatacept: The Evidence for its Place in the Treatment of Rheumatoid Arthritis. *Core Evid* (2008) 2(3):163–72.
252. Schiff M, Keiserman M, Codding C, Songcharoen S, Berman A, Nayiager S, et al. Efficacy and Safety of Abatacept or Infliximab vs Placebo in ATTEST: A Phase III, Multi-Centre, Randomised, Double-Blind, Placebo-Controlled Study in Patients With Rheumatoid Arthritis and an Inadequate Response to Methotrexate. *Ann Rheum Dis* (2008) 67(8):1096–103. doi: 10.1136/ard.2007.080002
253. Genovese MC, Schiff M, Luggen M, Becker JC, Aranda R, Teng J, et al. Efficacy and Safety of the Selective Co-Stimulation Modulator Abatacept Following 2 Years of Treatment in Patients With Rheumatoid Arthritis and an Inadequate Response to Anti-Tumour Necrosis Factor Therapy. *Ann Rheum Dis* (2008) 67(4):547–54. doi: 10.1136/ard.2007.074773
254. Ingegnoli F, Castelli R, Gualtierotti R. Rheumatoid Factors: Clinical Applications. *Dis Markers* (2013) 35:726598. doi: 10.1155/2013/726598
255. Gottenberg JE, Courvoisier DS, Hernandez MV, Iannone F, Lie E, Canhão H, et al. Brief Report: Association of Rheumatoid Factor and Anti-Citrullinated Protein Antibody Positivity With Better Effectiveness of Abatacept: Results From the Pan-European Registry Analysis. *Arthritis Rheumatol* (2016) 68 (6):1346–52. doi: 10.1002/art.39595
256. Maneiro RJ, Salgado E, Carmona L, Gomez-Reino JJ. Rheumatoid Factor as Predictor of Response to Abatacept, Rituximab and Tocilizumab in Rheumatoid Arthritis: Systematic Review and Meta-Analysis. *Semin Arthritis Rheum* (2013) 43(1):9–17. doi: 10.1016/j.semarthrit.2012.11.007
257. Alten R, Mariette X, Lorenz HM, Nüßlein H, Galeazzi M, Navarro F, et al. Predictors of Abatacept Retention Over 2 Years in Patients With Rheumatoid Arthritis: Results From the Real-World ACTION Study. *Clin Rheumatol* (2019) 38(5):1413–24. doi: 10.1007/s10067-019-04449-w
258. Bozec A, Luo Y, Engdahl C, Figueiredo C, Bang H, Schett G. Abatacept Blocks Anti-Citrullinated Protein Antibody and Rheumatoid Factor Mediated Cytokine Production in Human Macrophages in IDO-Dependent Manner. *Arthritis Res Ther* (2018) 20(1):24. doi: 10.1186/s13075-018-1527-x
259. Ioan-Facsinay A, el-Bannoudi H, Scherer HU, van der Woude D, Ménard HA, Lora M, et al. Anti-CCP Antibodies are a Collection of ACPA That are Cross-Reactive to Multiple Citrullinated Antigens. *Ann Rheum Dis* (2010) 69 (Suppl 2):A8–8. doi: 10.1136/ard.2010.129577s
260. Kay J, Upchurch KS. ACR/EULAR 2010 Rheumatoid Arthritis Classification Criteria. *Rheumatology* (2012) 51(suppl_6):vi5–9. doi: 10.1093/rheumatology/kes279
261. Puszczewicz M, Iwaszkiewicz C. Role of Anti-Citrullinated Protein Antibodies in Diagnosis and Prognosis of Rheumatoid Arthritis. *Arch Med Sci: AMS* (2011) 7(2):189–94. doi: 10.5114/aoms.2011.22067
262. Kida D, Takahashi N, Kaneko A, Hirano Y, Fujibayashi T, Kanayama Y, et al. A Retrospective Analysis of the Relationship Between Anti-Cyclic Citrullinated Peptide Antibody and the Effectiveness of Abatacept in Rheumatoid Arthritis Patients. *Sci Rep* (2020) 10(1):19717. doi: 10.1038/s41598-020-76842-4
263. Huizinga TWJ, Connolly SE, Johnsen A, Zhu J, Furst DE, Bykerk VP, et al. THU0114 Effect of Anti-Cyclic Citrullinated Peptide 2 Immunoglobulin M Serostatus on Efficacy Outcomes Following Treatment With Abatacept Plus Methotrexate in the Avert Trial. *Ann Rheum Dis* (2015) 74(Suppl 2):234–5. doi: 10.1136/annrheumdis-2015-eular.1983
264. Harrold LR, Litman HJ, Connolly SE, Kelly S, Hua W, Alemao E, et al. Effect of Anticitrullinated Protein Antibody Status on Response to Abatacept or Antitumor Necrosis Factor-α Therapy in Patients With Rheumatoid Arthritis: A US National Observational Study. *J Rheumatol* (2018) 45 (1):32–9. doi: 10.3899/jrheum.170007
265. Alten R, Mariette X, Lorenz H-M, Galeazzi M, Cantagrel A, Nüßlein HG, et al. Real-World Predictors of 12–Month Intravenous Abatacept Retention in Patients With Rheumatoid Arthritis in the ACTION Observational Study. *RMD Open* (2017) 3(2):e000538. doi: 10.1136/rmdopen-2017-000538
266. Endo Y, Koga T, Kawashiri SY, Morimoto S, Nishino A, Okamoto M, et al. Anti-Citrullinated Protein Antibody Titre as a Predictor of Abatacept Treatment Persistence in Patients With Rheumatoid Arthritis: A Prospective Cohort Study in Japan. *Scand J Rheumatol* (2020) 49(1):13–7. doi: 10.1080/03009742.2019.1627411
267. Ogawa N, Ohashi H, Ota Y, Kobori K, Suzuki M, Tsuboi S, et al. Multicenter, Observational Clinical Study of Abatacept in Japanese Patients With Rheumatoid Arthritis. *Immunol Med* (2019) 42(1):29–38. doi: 10.1080/25785826.2019.1605036
268. Scarsi M, Ziglioli T, Airo P. Baseline Numbers of Circulating CD28-Negative T Cells may Predict Clinical Response to Abatacept in Patients With Rheumatoid Arthritis. *J Rheumatol* (2011) 38(10):2105–11. doi: 10.3899/jrheum.110386
269. Nakamura S, Suzuki K, Iijima H, Hata Y, Lim CR, Ishizawa Y, et al. Identification of Baseline Gene Expression Signatures Predicting Therapeutic Responses to Three Biologic Agents in Rheumatoid Arthritis: A Retrospective Observational Study. *Arthritis Res Ther* (2016) 18:159. doi: 10.1186/s13075-016-1052-8
270. Marquez Pete N, Maldonado Montoro MDM, Pérez Ramírez C, Sánchez Martín A, Martínez de la Plata JE, Martínez Martínez F, et al. Impact of Single-Nucleotide Polymorphisms of CTLA-4, CD80 and CD86 on the Effectiveness of Abatacept in Patients With Rheumatoid Arthritis. *J Personalized Med* (2020) 10(4):220. doi: 10.3390/jpm10040220
271. Takahashi S, Saegusa J, Onishi A, Morinobu A. Biomarkers Identified by Serum Metabolomic Analysis to Predict Biologic Treatment Response in Rheumatoid Arthritis Patients. *Rheumatol (Oxford)* (2019) 58(12):2153–61. doi: 10.1093/rheumatology/kez199
272. Yukawa K, Mokuda S, Kohno H, Oi K, Kuranobu T, Tokunaga T, et al. Serum CXCL10 Levels are Associated With Better Responses to Abatacept Treatment of Rheumatoid Arthritis. *Clin Exp Rheumatol* (2020) 38(5):956–63.

273. Salomon S, Guignant C, Morel P, Flahaut G, Brault C, Gourguechon C, et al. Th17 and CD24(hi)CD27(+) Regulatory B Lymphocytes are Biomarkers of Response to Biologics in Rheumatoid Arthritis. *Arthritis Res Ther* (2017) 19 (1):33. doi: 10.1186/s13075-017-1244-x
274. Yokoyama-Kokuryo W, Yamazaki H, Takeuchi T, Amano K, Kikuchi J, Kondo T, et al. Identification of Molecules Associated With Response to Abatacept in Patients With Rheumatoid Arthritis. *Arthritis Res Ther* (2020) 22(1):46. doi: 10.1186/s13075-020-2137-y
275. Shin-Ya K, Yushiro E, Ayako N, Momoko O, Sosuke T, Ayuko T, et al. Association Between Serum Bone Biomarker Levels and Ultrasonographic Response in Abatacepttreated Rheumatoid Arthritis Patients. *Res Square* (2021) 22(1):506. doi: 10.21203/rs.3.rs-25419/v1
276. Nguyen MVC, Adrait A, Baillet A, Trocmé C, Gottenberg JE, Gaudin P. Identification of Cartilage Oligomeric Matrix Protein as Biomarker Predicting Abatacept Response in Rheumatoid Arthritis Patients With Insufficient Response to a First Anti-TNFα Treatment. *Joint Bone Spine* (2019) 86(3):401–3. doi: 10.1016/j.jbspin.2018.09.005
277. de Jong TD, Sellam J, Agca R, Vosslamber S, Witte BI, Tsang ASM, et al. A Multi-Parameter Response Prediction Model for Rituximab in Rheumatoid Arthritis. *Joint Bone Spine* (2018) 85(2):219–26. doi: 10.1016/j.jbspin.2017.02.015
278. Viecceli D, Garcia MP, Schneider L, Alegretti AP, Silva CK, Ribeiro AL, et al. Correlation Between Cellular Expression of Complement Regulatory Proteins With Depletion and Repopulation of B-Lymphocytes in Peripheral Blood of Patients With Rheumatoid Arthritis Treated With Rituximab. *Rev Bras Reumatol Engl Ed* (2017) 57(5):385–91. doi: 10.1016/j.rbre.2016.09.007
279. Tchetina EV, Pivanova AN, Markova GA, Lukina GV, Aleksandrova EN, Aleksankin AP, et al. Rituximab Downregulates Gene Expression Associated With Cell Proliferation, Survival, and Proteolysis in the Peripheral Blood From Rheumatoid Arthritis Patients: A Link Between High Baseline Autophagy-Related ULK1 Expression and Improved Pain Control. *Arthritis* (2016) 2016:4963950. doi: 10.1155/2016/4963950
280. Ramwadhdoebe TH, van Baarsen LGM, Boumans MJH, Bruijnen STG, Safy M, Berger FH, et al. Effect of Rituximab Treatment on T and B Cell Subsets in Lymph Node Biopsies of Patients With Rheumatoid Arthritis. *Rheumatol (Oxford England)* (2019) 58(6):1075–85. doi: 10.1093/rheumatology/key428
281. Piantoni S, Scarsi M, Tincani A, Airo P. Circulating CD4+ T-Cell Number Decreases in Rheumatoid Patients With Clinical Response to Rituximab. *Rheumatol Int* (2015) 35(9):1571–3. doi: 10.1007/s00296-015-3295-0
282. Einarsson JT, Evert M, Geborek P, Saxne T, Lundgren M, Kapetanovic MC. Rituximab in Clinical Practice: Dosage, Drug Adherence, Ig Levels, Infections, and Drug Antibodies. *Clin Rheumatol* (2017) 36(12):2743–50. doi: 10.1007/s10067-017-3848-6
283. Bergantini L, d'Alessandro M, Cameli P, Vietri L, Vagaggini C, Perrone A, et al. Effects of Rituximab Therapy on B Cell Differentiation and Depletion. *Clin Rheumatol* (2020) 39(5):1415–21. doi: 10.1007/s10067-020-04996-7
284. Fabris M, Quartuccio L, Lombardi S, Benucci M, Manfredi M, Saracco M, et al. Study on the Possible Role of the -174G>C IL-6 Promoter Polymorphism in Predicting Response to Rituximab in Rheumatoid Arthritis. *Reumatismo* (2010) 62(4):253–8. doi: 10.4081/reumatismo.2010.253
285. Pollastro S, Klarenbeek PL, Doorenspleet ME, van Schaik BDC, Esveldt REE, Thurlings RM, et al. Non-Response to Rituximab Therapy in Rheumatoid Arthritis is Associated With Incomplete Disruption of the B Cell Receptor Repertoire. *Ann Rheum Dis* (2019) 78(10):1339–45. doi: 10.1136/annrheumdis-2018-214898
286. Jimenez Morales A, Maldonado-Montoro M, Martinez de la Plata JE, Perez Ramirez C, Daddaoua A, Alarcon Payer C, et al. FCGR2A/FCGR3A Gene Polymorphisms and Clinical Variables as Predictors of Response to Tocilizumab and Rituximab in Patients With Rheumatoid Arthritis. *J Clin Pharmacol* (2019) 59(4):517–31. doi: 10.1002/jcph.1341
287. Konthur Z, Wiemkes MM, Haeupl T, Burmester GR, Skriner K. FRI0201 Rituximab Treated Non Responder Rheumatoid Arthritis Patients Are Generating A New Autoantibody Repertoire. *In: BMJ Publishing Group Ltd* (2016) 75:503. doi: 10.1136/annrheumdis-2017-eular.6978
288. Ruyssen-Witrand A, Rouanet S, Combe B, Dougados M, Le Loët X, Sibilia J, et al. Fcγ Receptor Type IIIA Polymorphism Influences Treatment Outcomes in Patients With Rheumatoid Arthritis Treated With Rituximab. *Ann Rheum Dis* (2012) 71(6):875–7. doi: 10.1136/annrheumdis-2011-200337
289. Quartuccio L, Fabris M, Pontarini E, Salvin S, Zabotti A, Benucci M, et al. The 158vv Fcgamma Receptor 3A Genotype is Associated With Response to Rituximab in Rheumatoid Arthritis: Results of an Italian Multicentre Study. *Ann Rheum Dis* (2014) 73(4):716–21. doi: 10.1136/annrheumdis-2012-202435
290. Stradner MH, Dejaco C, Brickmann K, Graninger WB, Brezinschek HP. A Combination of Cellular Biomarkers Predicts Failure to Respond to Rituximab in Rheumatoid Arthritis: A 24-Week Observational Study. *Arthritis Res Ther* (2016) 18:190. doi: 10.1186/s13075-016-1091-1
291. Kastbom A, Cöster L, Arlestig L, Chatzidionysiou A, van Vollenhoven RF, Padyukov L, et al. Influence of FCGR3A Genotype on the Therapeutic Response to Rituximab in Rheumatoid Arthritis: An Observational Cohort Study. *BMJ Open* (2012) 2(5):e001524. doi: 10.1136/bmjopen-2012-001524
292. Ruyssen-Witrand A, Rouanet S, Combe B, Dougados M, Le Loet X, Sibilia J, et al. Association Between -871C>T Promoter Polymorphism in the B-Cell Activating Factor Gene and the Response to Rituximab in Rheumatoid Arthritis Patients. *Rheumatol (Oxford)* (2013) 52(4):636–41. doi: 10.1093/rheumatology/kes344
293. Das S, Vital EM, Horton S, Bryer D, El-Sherbiny Y, Rawstron AC, et al. Abatacept or Tocilizumab After Rituximab in Rheumatoid Arthritis? An Exploratory Study Suggests non-Response to Rituximab is Associated With Persistently High IL-6 and Better Clinical Response to IL-6 Blocking Therapy. *Ann Rheum Dis* (2014) 73(5):909–12. doi: 10.1136/annrheumdis-2013-204417
294. Juge PA, Gazal S, Constantin A, Mariette X, Combe B, Tebib J, et al. Variants of Genes Implicated in Type 1 Interferon Pathway and B-Cell Activation Modulate the EULAR Response to Rituximab at 24 Weeks in Rheumatoid Arthritis. *RMD Open* (2017) 3(2):e000448. doi: 10.1136/rmdopen-2017-000448
295. Daien CI, Fabre S, Rittore C, Soler S, Daien V, Tejedor G, et al. TGF Beta1 Polymorphisms are Candidate Predictors of the Clinical Response to Rituximab in Rheumatoid Arthritis. *Joint Bone Spine* (2012) 79(5):471–5. doi: 10.1016/j.jbspin.2011.10.007
296. Fabris M, Quartuccio L, Vital E, Pontarini E, Salvin S, Fabro C, et al. The TTTT B Lymphocyte Stimulator Promoter Haplotype is Associated With Good Response to Rituximab Therapy in Seropositive Rheumatoid Arthritis Resistant to Tumor Necrosis Factor Blockers. *Arthritis Rheum* (2013) 65 (1):88–97. doi: 10.1002/art.37707
297. Md Yusof MY, Candelier JB, Dass S, Rawstron A, Vital EM, Emery P. SAT0175 Validation of B Cell Depletion as A Predictor of Clinical Response and Efficacy of Retreatment of Non-Response in Rheumatoid Arthritis. *Ann Rheum Dis* (2016) 75(Suppl 2):731–1. doi: 10.1136/annrheumdis-2016-eular.6122
298. Luxembourger C, Ruyssen-Witrand A, Ladhari C, Rittore C, Degboe Y, Maillefert JF, et al. A Single Nucleotide Polymorphism of IL6-Receptor is Associated With Response to Tocilizumab in Rheumatoid Arthritis Patients. *Pharmacogenomics J* (2019) 19(4):368–74. doi: 10.1038/s41397-019-0072-6
299. Enevold C, Baslund B, Linde L, Josephsen NL, Tarp U, Lindegaard H, et al. Interleukin-6-Receptor Polymorphisms Rs12083537, Rs2228145, and Rs4329505 as Predictors of Response to Tocilizumab in Rheumatoid Arthritis. *Pharmacogenet Genomics* (2014) 24(8):401–5. doi: 10.1097/fpc.0000000000000071
300. Maldonado-Montoro M, Canadas-Garre M, Gonzalez-Utrilla A, Plaza-Plaza JC, Calleja-Hernandez MY. Genetic and Clinical Biomarkers of Tocilizumab Response in Patients With Rheumatoid Arthritis. *Pharmacol Res* (2016) 111:264–71. doi: 10.1016/j.phrs.2016.06.016
301. De Groof A, Ducreux J, Humby F, Nzeusseu Toukap A, Badot V, Pitzalis C, et al. Higher Expression of TNFalpha-Induced Genes in the Synovium of Patients With Early Rheumatoid Arthritis Correlates With Disease Activity, and Predicts Absence of Response to First Line Therapy. *Arthritis Res Ther* (2016) 18:19. doi: 10.1186/s13075-016-0919-z
302. Pers YM, Fortunet C, Constant E, Lambert J, Godfrin-Valnet M, De Jong A, et al. Predictors of Response and Remission in a Large Cohort of Rheumatoid Arthritis Patients Treated With Tocilizumab in Clinical Practice. *Rheumatol (Oxford)* (2014) 53(1):76–84. doi: 10.1093/rheumatology/ket301
303. Nishina N, Kikuchi J, Hashizume M, Yoshimoto K, Kameda H, Takeuchi T. Baseline Levels of Soluble Interleukin-6 Receptor Predict Clinical Remission in Patients With Rheumatoid Arthritis Treated With Tocilizumab: Implications for Molecular Targeted Therapy. *Ann Rheum Dis* (2014) 73 (5):945–7. doi: 10.1136/annrheumdis-2013-204137

304. Sanayama Y, Ikeda K, Saito Y, Kagami S, Yamagata M, Furuta S, et al. Prediction of Therapeutic Responses to Tocilizumab in Patients With Rheumatoid Arthritis: Biomarkers Identified by Analysis of Gene Expression in Peripheral Blood Mononuclear Cells Using Genome-Wide DNA Microarray. *Arthritis Rheumatol* (2014) 66(6):1421–31. doi: 10.1002/art.38400
305. Okano T, Inui K, Tada M, Sugioka Y, Mamoto K, Wakitani S, et al. Levels of Interleukin-1 Beta can Predict Response to Tocilizumab Therapy in Rheumatoid Arthritis: The PETITE (Predictors of Effectiveness of Tocilizumab Therapy) Study. *Rheumatol Int* (2016) 36(3):349–57. doi: 10.1007/s00296-015-3379-x
306. Hirata S, Marotta A, Gui Y, Hanami K, Tanaka Y. Serum 14-3-3η Level is Associated With Severity and Clinical Outcomes of Rheumatoid Arthritis, and its Pretreatment Level is Predictive of DAS28 Remission With Tocilizumab. *Arthritis Res Ther* (2015) 17(1):280. doi: 10.1186/s13075-015-0799-7
307. Mesko B, Poliska S, Szamosi S, Szekanecz Z, Podani J, Varadi C, et al. Peripheral Blood Gene Expression and IgG Glycosylation Profiles as Markers of Tocilizumab Treatment in Rheumatoid Arthritis. *J Rheumatol* (2012) 39(5):916–28. doi: 10.3899/jrheum.110961
308. Uno K, Yoshizaki K, Iwahashi M, Yamana J, Yamana S, Tanigawa M, et al. Pretreatment Prediction of Individual Rheumatoid Arthritis Patients' Response to Anti-Cytokine Therapy Using Serum Cytokine/Chemokine/Soluble Receptor Biomarkers. *PloS One* (2015) 10(7):e0132055. doi: 10.1371/journal.pone.0132055
309. Lee SJ, Park W, Park SH, Shim SC, Baek HJ, Yoo DH, et al. Low Baseline Interleukin-17A Levels are Associated With Better Treatment Response at 12 Weeks to Tocilizumab Therapy in Rheumatoid Arthritis Patients. *J Immunol Res* (2015) 2015:487230. doi: 10.1155/2015/487230
310. Daïen CI, Gailhac S, Audo R, Mura T, Hahne M, Combe B, et al. High Levels of Natural Killer Cells Are Associated With Response to Tocilizumab in Patients With Severe Rheumatoid Arthritis. *Rheumatol (Oxford)* (2015) 54(4):601–8. doi: 10.1093/rheumatology/keu363
311. Genovese MC, Fleischmann R, Kivitz A, Lee E-B, van Hoogstraten H, Kimura T, et al. Efficacy and Safety of Sarilumab in Combination With csDMARDs or as Monotherapy in Subpopulations of Patients With Moderately to Severely Active Rheumatoid Arthritis in Three Phase III Randomized, Controlled Studies. *Arthritis Res Ther* (2020) 22(1):139. doi: 10.1186/s13075-020-02194-z
312. Emery P, van Hoogstraten H, Mangan EK, van Adelsberg J, Fan C, Kivitz A, et al. SAT0178 Clinical Responses of Sarilumab Based on Rheumatoid Factor and Anti–Cyclic Citrullinated Peptide Antibody Status in Patients With Rheumatoid Arthritis. *Ann Rheum Dis* (2016) 75(Suppl 2):732–2. doi: 10.1136/annrheumdis-2016-eular.4415
313. Boyapati A, Msihid J, Schwartzman S, Choy E, Burmester G, Lam G, et al. 069 High Baseline Serum IL-6 Identifies a Subgroup of Rheumatoid Arthritis Patients With Rapid Joint Damage and Clinical Progression and Predicts Increased Sarilumab Treatment Response. *Rheumatology* (2018) 58(2019):L08. doi: 10.1093/rheumatology/kez106.068
314. Gabay C, Msihid J, Zilberstein M, Paccard C, Lin Y, Graham NMH, et al. Identification of Sarilumab Pharmacodynamic and Predictive Markers in Patients With Inadequate Response to TNF Inhibition: A Biomarker Substudy of the Phase 3 TARGET Study. *RMD Open* (2018) 4(1):e000607. doi: 10.1136/rmdopen-2017-000607
315. Ebina K, Hirano T, Maeda Y, Yamamoto W, Hashimoto M, Murata K, et al. Drug Retention of Secondary Biologics or JAK Inhibitors After Tocilizumab or Abatacept Failure as First Biologics in Patients With Rheumatoid Arthritis -the ANSWER Cohort Study. *Clin Rheumatol* (2020) 39(9):2563–72. doi: 10.1007/s10067-020-05015-5
316. Suzuki M, Takahashi N, Kida D, Hirano Y, Kato T, Yabe Y, et al. Clinical Effectiveness and Safety of Additional Administration of Tacrolimus in Rheumatoid Arthritis Patients With an Inadequate Response to Abatacept: A Retrospective Cohort Study. *Int J Rheum Dis* (2019) 22(12):2199–205. doi: 10.1111/1756-185X.13731
317. Silverman GJ, Carson DA. Roles of B Cells in Rheumatoid Arthritis. *Arthritis Res Ther* (2003) 5(4):S1. doi: 10.1186/ar1010
318. Boumans MJH, Tak PP. Rituximab Treatment in Rheumatoid Arthritis: How Does it Work? *Arthritis Res Ther* (2009) 11(6):134. doi: 10.1186/ar2852
319. Schioppo T, Ingegnoli F. Current Perspective on Rituximab in Rheumatic Diseases. *Drug Des Devel Ther* (2017) 11:2891–904. doi: 10.2147/dddt.S139248
320. Mian A, Ibrahim F, Scott DL. A Systematic Review of Guidelines for Managing Rheumatoid Arthritis. *BMC Rheumatol* (2019) 3(1):42. doi: 10.1186/s41927-019-0090-7
321. BMJ Group. Rituximab and ▼Abatacept for Rheumatoid Arthritis. *Drug Ther Bull* (2008) 46(8):57–61. doi: 10.1136/dtb.2008.07.0017
322. Das G, Damotte V, Gelfand JM, Bevan C, Cree BAC, Do L, et al. Rituximab Before and During Pregnancy: A Systematic Review, and a Case Series in MS and NMOSD. *Neurology(R) Neuroimmunol Neuroinflamm* (2018) 5(3):e453–3. doi: 10.1212/NXI.0000000000000453
323. Cohen SB, Emery P, Greenwald MW, Dougados M, Furie RA, Genovese MC, et al. Rituximab for Rheumatoid Arthritis Refractory to Anti–Tumor Necrosis Factor Therapy: Results of a Multicenter, Randomized, Double-Blind, Placebo-Controlled, Phase III Trial Evaluating Primary Efficacy and Safety at Twenty-Four Weeks. *Arthritis Rheumatism* (2006) 54(9):2793–806. doi: 10.1002/art.22025
324. Kim DD, Song W-C. Membrane Complement Regulatory Proteins. *Clin Immunol* (2006) 118(2):127–36. doi: 10.1016/j.clim.2005.10.014
325. Beyer I, Cao H, Persson J, Wang H, Liu Y, Yumul R, et al. Transient Removal of CD46 is Safe and Increases B-Cell Depletion by Rituximab in CD46 Transgenic Mice and Macaques. *Mol Ther* (2013) 21(2):291–9. doi: 10.1038/mt.2012.212
326. Wang H, Liu Y, Li Z, Hemminki A, Lieber A. 657. A CD46-Targeting Recombinant Protein That Increases the Therapeutic Efficacy of Monoclonal Antibodies for Cancer. *Mol Ther* (2010) 18:S256. doi: 10.1016/S1525-0016(16)38098-4
327. El-Azkalany G, Gheita P. A. B and R. B: Clinical Significance of Serum Interleukin-6 and ◆174 G/C Promoter Polymorphism in Rheumatoid Arthritis Patients. *Egypt Rheumatol* (2014) 35(2013):107–13. doi: 10.1016/j.ejr.2012.11.002
328. Walker UA, Jaeger VK, Chatzidionysiou K, Hetland ML, Hauge EM, Pavelka K, et al. Rituximab Done: What's Next in Rheumatoid Arthritis? A European Observational Longitudinal Study Assessing the Effectiveness of Biologics After Rituximab Treatment in Rheumatoid Arthritis. *Rheumatol (Oxford)* (2016) 55(2):230–6. doi: 10.1093/rheumatology/kev297
329. Heinrich PC, Behrmann I, Müller-Newen G, Schaper F, Graeve L. Interleukin-6-Type Cytokine Signalling Through the Gp130/Jak/STAT Pathway. *Biochem J* (1998) 334(Pt 2)(Pt 2):297–314. doi: 10.1042/bj3340297
330. Choy EH, De Benedetti F, Takeuchi T, Hashizume M, John MR, Kishimoto T. Translating IL-6 Biology Into Effective Treatments. *Nat Rev Rheumatol* (2020) 16(6):335–45. doi: 10.1038/s41584-020-0419-z
331. Dayer JM, Choy E. Therapeutic Targets in Rheumatoid Arthritis: The Interleukin-6 Receptor. *Rheumatol (Oxford)* (2010) 49(1):15–24. doi: 10.1093/rheumatology/kep329
332. Rose-John S, Winthrop K, Calabrese L. The Role of IL-6 in Host Defence Against Infections: Immunobiology and Clinical Implications. *Nat Rev Rheumatol* (2017) 13(7):399–409. doi: 10.1038/nrrheum.2017.83
333. Cortellini G, Mascella F, Simoncelli M, Lippolis D, Focherini MC, Cortellini F, et al. Effective Desensitization to Tocilizumab in Delayed Hypersensitivity Reaction. *Pharmacology* (2018) 102(1–2):114–6. doi: 10.1159/000490419
334. Ogata A, Hirano T, Hishitani Y, Tanaka T. Safety and Efficacy of Tocilizumab for the Treatment of Rheumatoid Arthritis. *Clin Med Insights Arthritis Musculoskeletal Disord* (2012) 5:27–42. doi: 10.4137/CMAMD.S7371
335. Kremer JM, Blanco R, Brzosko M, Burgos-Vargas R, Halland AM, Vernon E, et al. Tocilizumab Inhibits Structural Joint Damage in Rheumatoid Arthritis Patients With Inadequate Responses to Methotrexate: Results From the Double-Blind Treatment Phase of a Randomized Placebo-Controlled Trial of Tocilizumab Safety and Prevention of Structural Joint Damage at One Year. *Arthritis Rheum* (2011) 63(3):609–21. doi: 10.1002/art.30158
336. Emery P, Keystone E, Tony HP, Cantagrel A, van Vollenhoven R, Sanchez A, et al. IL-6 Receptor Inhibition With Tocilizumab Improves Treatment Outcomes in Patients With Rheumatoid Arthritis Refractory to Anti-Tumour Necrosis Factor Biologicals: Results From a 24-Week Multicentre Randomised Placebo-Controlled Trial. *Ann Rheum Dis* (2008) 67(11):1516–23. doi: 10.1136/ard.2008.092932

337. Burmester GR, Choy E, Kivitz A, Ogata A, Bao M, Nomura A, et al. Low Immunogenicity of Tocilizumab in Patients With Rheumatoid Arthritis. *Ann Rheum Dis* (2017) 76(6):1078–85. doi: 10.1136/annrheumdis-2016-210297
338. Wang J, Platt A, Upmanyu R, Germer S, Lei G, Rabe C, et al. IL-6 Pathway-Driven Investigation of Response to IL-6 Receptor Inhibition in Rheumatoid Arthritis. *BMJ Open* (2013) 3(8):e003199. doi: 10.1136/bmjopen-2013-003199
339. Wang J, Bansal A, Martin M, Germer S, Benayed R, Essioux L, et al. Genome-Wide Association Analysis Implicates the Involvement of Eight Loci With Response to Tocilizumab for the Treatment of Rheumatoid Arthritis. *Pharmacogenomics J* (2012) 13:235–41. doi: 10.1038/tpj.2012.8
340. Díaz-Villamarín X, Dávila-Fajardo C, González-Medina M, Blánquez-Martínez D, Moreno-Raya P, Cabeza-Barrera J. CP-086 CD69 a<G (RS11052877) Genetic Polymorphism on the Response to Tocilizumab in Rheumatoid Arthritis Patients. *Eur J Hosp Pharm* (2017) 24(Suppl 1):A38–8. doi: 10.1136/ejhpharm-2017-000640.85
341. Burmester GR, Feist E, Kellner H, Braun J, Iking-Konert C, Rubbert-Roth A. Effectiveness and Safety of the Interleukin 6-Receptor Antagonist Tocilizumab After 4 and 24 Weeks in Patients With Active Rheumatoid Arthritis: The First Phase IIIb Real-Life Study (TAMARA). *Ann Rheum Dis* (2011) 70(5):755–9. doi: 10.1136/ard.2010.139725
342. Jones G, Ding C. Tocilizumab: A Review of its Safety and Efficacy in Rheumatoid Arthritis. *Clin Med Insights Arthritis Musculoskelet Disord* (2010) 3:81–9. doi: 10.4137/cmamd.S4864
343. Narváez J, Magallares B, Díaz Torné C, Hernández MV, Reina D, Corominas H, et al. Predictive Factors for Induction of Remission in Patients With Active Rheumatoid Arthritis Treated With Tocilizumab in Clinical Practice. *Semin Arthritis Rheum* (2016) 45(4):386–90. doi: 10.1016/j.semarthrit.2015.07.001
344. Dorner T, Schulze-Koops H, Burmester GR, Iking-Konert C, Schmalzing M, Engel A, et al. Early and Late Responses in Patients With Rheumatoid Arthritis Who Were Conventional Synthetic Disease-Modifying Anti-Rheumatic Drug Inadequate Responders and Were Treated With Tocilizumab or Switched to Rituximab: An Open-Label Phase 3 Trial (MIRAI). *Clin Exp Rheumatol* (2019) 37(6):937–45.
345. Akiyama M, Kaneko Y, Kondo H, Takeuchi T. Comparison of the Clinical Effectiveness of Tumour Necrosis Factor Inhibitors and Abatacept After Insufficient Response to Tocilizumab in Patients With Rheumatoid Arthritis. *Clin Rheumatol* (2016) 35(11):2829–34. doi: 10.1007/s10067-016-3227-8
346. Lamb YN, Deeks ED. Sarilumab: A Review in Moderate to Severe Rheumatoid Arthritis. *Drugs* (2018) 78(9):929–40. doi: 10.1007/s40265-018-0929-z
347. Rafique A, Martin J, Blome M, Huang T, Ouyang A, Papadopoulos N. Evaluation of the Binding Kinetics and Functional Bioassay Activity of Sarilumab and Tocilizumab to the Human IL-6 Receptor (IL-6r) α. *Ann Rheum Dis* (2014) 72:A797–7. doi: 10.1136/annrheumdis-2013-eular.2360
348. Wang L-H, Xue Y, Liu X, Luo F, Kelly L, Huang T, et al. FRI0020 Preclinical Development of Sarilumab, the First Fully Human Monoclonal Antibody (MAB) Against IL-6r Alpha: Utilization and Value of Double Humanized Animal Model. *Ann Rheum Dis* (2013) 72(Suppl 3):A375–5. doi: 10.1136/annrheumdis-2013-eular.1148
349. Huizinga TW, Fleischmann RM, Jasson M, Radin AR, van Adelsberg J, Fiore S, et al. Sarilumab, a Fully Human Monoclonal Antibody Against IL-6rα in Patients With Rheumatoid Arthritis and an Inadequate Response to Methotrexate: Efficacy and Safety Results From the Randomised SARIL-RA-MOBILITY Part A Trial. *Ann Rheum Dis* (2014) 73(9):1626–34. doi: 10.1136/annrheumdis-2013-204405
350. Boyapati A, Msihid J, Fiore S, van Adelsberg J, Graham NM, Hamilton JD. Sarilumab Plus Methotrexate Suppresses Circulating Biomarkers of Bone Resorption and Synovial Damage in Patients With Rheumatoid Arthritis and Inadequate Response to Methotrexate: A Biomarker Study of MOBILITY. *Arthritis Res Ther* (2016) 18(1):225. doi: 10.1186/s13075-016-1132-9
351. Genovese MC, Fleischmann R, Kivitz AJ, Rell-Bakalarska M, Martincova R, Fiore S, et al. Sarilumab Plus Methotrexate in Patients With Active Rheumatoid Arthritis and Inadequate Response to Methotrexate: Results of a Phase III Study. *Arthritis Rheumatol* (2015) 67(6):1424–37. doi: 10.1002/art.39093
352. Fleischmann R, van Adelsberg J, Lin Y, Castelar-Pinheiro GD, Brzezicki J, Hrycaj P, et al. Sarilumab and Nonbiologic Disease-Modifying Antirheumatic Drugs in Patients With Active Rheumatoid Arthritis and Inadequate Response or Intolerance to Tumor Necrosis Factor Inhibitors. *Arthritis Rheumatol* (2017) 69(2):277–90. doi: 10.1002/art.39944
353. Burmester GR, Lin Y, Patel R, van Adelsberg J, Mangan EK, Graham NM, et al. Efficacy and Safety of Sarilumab Monotherapy Versus Adalimumab Monotherapy for the Treatment of Patients With Active Rheumatoid Arthritis (MONARCH): A Randomised, Double-Blind, Parallel-Group Phase III Trial. *Ann Rheum Dis* (2017) 76(5):840–7. doi: 10.1136/annrheumdis-2016-210310
354. Ebina K, Hashimoto M, Yamamoto W, Hirano T, Hara R, Katayama M, et al. Drug Tolerability and Reasons for Discontinuation of Seven Biologics in 4466 Treatment Courses of Rheumatoid Arthritis—the ANSWER Cohort Study. *Arthritis Res Ther* (2019) 21(1):91. doi: 10.1186/s13075-019-1880-4
355. Schwartz DM, Kanno Y, Villarino A, Ward M, Gadina M, O'Shea JJ. JAK Inhibition as a Therapeutic Strategy for Immune and Inflammatory Diseases. *Nat Rev Drug Discov* (2017) 17(1):78. doi: 10.1038/nrd.2017.267
356. Rosengren S, Corr M, Firestein GS, Boyle DL. The JAK Inhibitor CP-690,550 (Tofacitinib) Inhibits TNF-Induced Chemokine Expression in Fibroblast-Like Synoviocytes: Autocrine Role of Type I Interferon. *Ann Rheum Dis* (2012) 71(3):440–7. doi: 10.1136/ard.2011.150284
357. Kotyla PJ. Are Janus Kinase Inhibitors Superior Over Classic Biologic Agents in RA Patients? *BioMed Res Int* (2018) 2018:7492904–7492904. doi: 10.1155/2018/7492904
358. Ghoreschi K, Jesson MI, Li X, Lee JL, Ghosh S, Alsup JW, et al. Modulation of Innate and Adaptive Immune Responses by Tofacitinib (CP-690,550). *J Immunol* (2011) 186(7):4234–43. doi: 10.4049/jimmunol.1003668
359. Dhillon S. Tofacitinib: A Review in Rheumatoid Arthritis. *Drugs* (2017) 77(18):1987–2001. doi: 10.1007/s40265-017-0835-9
360. Meyer DM, Jesson MI, Li X, Elrick MM, Funckes-Shippy CL, Warner JD, et al. Anti-Inflammatory Activity and Neutrophil Reductions Mediated by the JAK1/JAK3 Inhibitor, CP-690,550, in Rat Adjuvant-Induced Arthritis. *J Inflamm (Lond)* (2010) 7:41. doi: 10.1186/1476-9255-7-41
361. Boyle DL, Soma K, Hodge J, Kavanaugh A, Mandel D, Mease P, et al. The JAK Inhibitor Tofacitinib Suppresses Synovial JAK1-STAT Signalling in Rheumatoid Arthritis. *Ann Rheum Dis* (2015) 74(6):1311–6. doi: 10.1136/annrheumdis-2014-206028
362. Razmjou AA, Brook J, Elashoff D, Kaeley G, Choi S, Kermani T, et al. Ultrasound and Multi-Biomarker Disease Activity Score for Assessing and Predicting Clinical Response to Tofacitinib Treatment in Patients With Rheumatoid Arthritis. *BMC Rheumatol* (2020) 4:55. doi: 10.1186/s41927-020-00153-4
363. Fernández-Ruiz JC, Ramos-Remus C, Sánchez-Corona J, Castillo-Ortiz JD, Castañeda-Sánchez JJ, Bastian Y, et al. Analysis of miRNA Expression in Patients With Rheumatoid Arthritis During Remission and Relapse After a 5-Year Trial of Tofacitinib Treatment. *Int Immunopharmacol* (2018) 63:35–42. doi: 10.1016/j.intimp.2018.07.028
364. Li Y, Yuan L, Yang J, Lei Y, Zhang H, Xia L, et al. Changes in Serum Cytokines May Predict Therapeutic Efficacy of Tofacitinib in Rheumatoid Arthritis. *Mediators Inflamm* (2019) 2019:5617431. doi: 10.1155/2019/5617431
365. Hamar A, Szekanecz Z, Pusztai A, Czókolyová M, Végh E, Pethő Z, et al. Effects of One-Year Tofacitinib Therapy on Bone Metabolism in Rheumatoid Arthritis. *Osteoporosis Int* (2021) 32(8):1621–9. doi: 10.1007/s00198-021-05871-0
366. López-Romero P, Martinez-Gamboa L, Bang H, de la Torre I, Holzkämper T, Feist E. Assessment of the Association of Baseline Anti-CarbV and Anti-MCV Antibodies With Response to Treatment and Radiographic Progression in an RA Population Treated With Either Methotrexate or Baricitinib: *Post-Hoc* Analyses From RA-BEGIN. *Arthritis Res Ther* (2020) 22(1):193. doi: 10.1186/s13075-020-02284-y
367. Takahashi N, Asai S, Kobayakawa T, Kaneko A, Watanabe T, Kato T, et al. Predictors for Clinical Effectiveness of Baricitinib in Rheumatoid Arthritis Patients in Routine Clinical Practice: Data From a Japanese Multicenter Registry. *Sci Rep* (2020) 10(1):21907. doi: 10.1038/s41598-020-78925-8
368. Sornasse T, Song IH, Radstake T, Mcinnes I. FRI0026 Proteomics Analysis Comparing the Mode OF Action of Upadacitinib and Adalimumab Head to

Head in RA Identifies Novel, Discrete Early Immune Pathway Modulation in the Select-Compare Phase 3 Study. *Ann Rheum Dis* (2020) 79(Suppl 1):585–6. doi: 10.1136/annrheumdis-2020-eular.1908

369. Shi JG, Chen X, Lee F, Emm T, Scherle PA, Lo Y, et al. The Pharmacokinetics, Pharmacodynamics, and Safety of Baricitinib, an Oral JAK 1/2 Inhibitor, in Healthy Volunteers. *J Clin Pharmacol* (2014) 54 (12):1354–61. doi: 10.1002/jcph.354
370. Two New Drugs for Rheumatoid Arthritis. *Drug Ther Bull* (2017) 55(9):102–5. doi: 10.1136/dtb.2017.8.0526
371. Fridman JS, Scherle PA, Collins R, Burn TC, Li Y, Li J, et al. Selective Inhibition of JAK1 and JAK2 is Efficacious in Rodent Models of Arthritis: Preclinical Characterization of INCB028050. *J Immunol* (2010) 184(9):5298–307. doi: 10.4049/jimmunol.0902819
372. Emery P, Durez P, Hueber AJ, de la Torre I, Larsson E, Holzkämper T, et al. Baricitinib Inhibits Structural Joint Damage Progression in Patients With Rheumatoid Arthritis-a Comprehensive Review. *Arthritis Res Ther* (2021) 23 (1):3–3. doi: 10.1186/s13075-020-02379-6
373. Agency EM. Olumiant (Baricitinib) Film-Coated Tablets: EU Summary of Product Characteristics. In: *European Medicines Agency*. Amsterdam, The Netherlands: European Medicines Agency (2018).
374. Tanaka Y, McInnes IB, Taylor PC, Byers NL, Chen L, de Bono S, et al. Characterization and Changes of Lymphocyte Subsets in Baricitinib-Treated Patients With Rheumatoid Arthritis: An Integrated Analysis. *Arthritis Rheumatol* (2018) 70(12):1923–32. doi: 10.1002/art.40680
375. Genovese MC, Kremer JM, Kartman CE, Schlichting DE, Xie L, Carmack T, et al. Response to Baricitinib Based on Prior Biologic Use in Patients With Refractory Rheumatoid Arthritis. *Rheumatol (Oxford)* (2018) 57(5):900–8. doi: 10.1093/rheumatology/kex489
376. Emery P, Durez P, Hueber A, Torre I, Larsson E, Holzkaemper T, et al. Baricitinib Inhibits Structural Joint Damage Progression in Patients With Rheumatoid Arthritis – a Comprehensive Review. *Arthritis Res Ther* (2021) 23(1):3. doi: 10.1186/s13075-020-02379-6
377. Fleischmann R, Schiff M, van der Heijde D, Ramos-Remus C, Spindler A, Stanislav M, et al. Baricitinib, Methotrexate, or Combination in Patients With Rheumatoid Arthritis and No or Limited Prior Disease-Modifying Antirheumatic Drug Treatment. *Arthritis Rheumatol* (2017) 69(3):506–17. doi: 10.1002/art.39953
378. Taylor PC, Keystone EC, van der Heijde D, Weinblatt ME, Del Carmen Morales L, Reyes Gonzaga J, et al. Baricitinib Versus Placebo or Adalimumab in Rheumatoid Arthritis. *N Engl J Med* (2017) 376(7):652–62. doi: 10.1056/NEJMoa1608345
379. Kremer JM, Schiff M, Muram D, Zhong J, Alam J, Genovese MC. Response to Baricitinib Therapy in Patients With Rheumatoid Arthritis With Inadequate Response to csDMARDs as a Function of Baseline Characteristics. *RMD Open* (2018) 4(1):e000581. doi: 10.1136/rmdopen-2017-000581
380. Al-Salama ZT, Scott LJ. Baricitinib: A Review in Rheumatoid Arthritis. *Drugs* (2018) 78(7):761–72. doi: 10.1007/s40265-018-0908-4
381. Mohamed MF, Klunder B, Othman AA. Clinical Pharmacokinetics of Upadacitinib: Review of Data Relevant to the Rheumatoid Arthritis Indication. *Clin Pharmacokinet* (2020) 59(5):531–44. doi: 10.1007/s40262-019-00855-0
382. Biggioggero M, Becciolini A, Crotti C, Agape E, Favalli EG. Upadacitinib and Filgotinib: The Role of JAK1 Selective Inhibition in the Treatment of Rheumatoid Arthritis. *Drugs Context* (2019) 8:212595. doi: 10.7573/dic.212595
383. Parmentier JM, Voss J, Graff C, Schwartz A, Argiriadi M, Friedman M, et al. *In Vitro* and *In Vivo* Characterization of the JAK1 Selectivity of Upadacitinib (ABT-494). *BMC Rheumatol* (2018) 2(1):23. doi: 10.1186/s41927-018-0031-x
384. Conaghan PG, Mysler E, Tanaka Y, Da Silva-Tillmann B, Shaw T, Liu J, et al. Upadacitinib in Rheumatoid Arthritis: A Benefit–Risk Assessment Across a Phase III Program. *Drug Saf* (2021) 44(5):515–30. doi: 10.1007/s40264-020-01036-w
385. Kremer JM, Emery P, Camp HS, Friedman A, Wang L, Othman AA, et al. A Phase IIb Study of ABT-494, a Selective JAK-1 Inhibitor, in Patients With Rheumatoid Arthritis and an Inadequate Response to Anti–Tumor Necrosis Factor Therapy. *Arthritis Rheumatol* (2016) 68(12):2867–77. doi: 10.1002/art.39801
386. Genovese MC, Smolen JS, Weinblatt ME, Burmester GR, Meerwein S, Camp HS, et al. Efficacy and Safety of ABT-494, a Selective JAK-1 Inhibitor, in a Phase IIb Study in Patients With Rheumatoid Arthritis and an Inadequate Response to Methotrexate. *Arthritis Rheumatol* (2016) 68(12):2857–66. doi: 10.1002/art.39808
387. Strand V, Pope J, Tundia N, Friedman A, Camp HS, Pangan A, et al. Upadacitinib Improves Patient-Reported Outcomes in Patients With Rheumatoid Arthritis and Inadequate Response to Conventional Synthetic Disease-Modifying Antirheumatic Drugs: Results From SELECT-NEXT. *Arthritis Res Ther* (2019) 21(1):272. doi: 10.1186/s13075-019-2037-1
388. Burmester GR, Kremer JM, Van den Bosch F, Kivitz A, Bessette L, Li Y, et al. Safety and Efficacy of Upadacitinib in Patients With Rheumatoid Arthritis and Inadequate Response to Conventional Synthetic Disease-Modifying Anti-Rheumatic Drugs (SELECT-NEXT): A Randomised, Double-Blind, Placebo-Controlled Phase 3 Trial. *Lancet* (2018) 391(10139):2503–12. doi: 10.1016/s0140-6736(18)31115-2
389. Genovese MC, Fleischmann R, Combe B, Hall S, Rubbert-Roth A, Zhang Y, et al. Safety and Efficacy of Upadacitinib in Patients With Active Rheumatoid Arthritis Refractory to Biologic Disease-Modifying Anti-Rheumatic Drugs (SELECT-BEYOND): A Double-Blind, Randomised Controlled Phase 3 Trial. *Lancet* (2018) 391(10139):2513–24. doi: 10.1016/s0140-6736(18)31116-4
390. Smolen JS, Pangan AL, Emery P, Rigby W, Tanaka Y, Vargas JI, et al. Upadacitinib as Monotherapy in Patients With Active Rheumatoid Arthritis and Inadequate Response to Methotrexate (SELECT-MONOTHERAPY): A Randomised, Placebo-Controlled, Double-Blind Phase 3 Study. *Lancet* (2019) 393(10188):2303–11. doi: 10.1016/S0140-6736(19)30419-2
391. Sornasse T, Sokolove J, McInnes I. THU0181 Treatment With Upadacitinib Results in the Normalization of Key Pathobiologic Pathways in Patients With Rheumatoid Arthritis: Biomarker Results From the Phase 3 Select-Next and Select-Beyond Studies. *Ann Rheum Dis* (2019) 78(Suppl 2):365–6. doi: 10.1136/annrheumdis-2019-eular.2994
392. Fleischmann RM, Blanco R, Hall S, Thomson GTD, Van den Bosch FE, Zerbini C, et al. Switching Between Janus Kinase Inhibitor Upadacitinib and Adalimumab Following Insufficient Response: Efficacy and Safety in Patients With Rheumatoid Arthritis. *Ann Rheum Dis* (2021) 80(4):432–9. doi: 10.1136/annrheumdis-2020-218412
393. Bluett J, Barton A. Precision Medicine in Rheumatoid Arthritis. *Rheum Dis Clin North Am* (2017) 43(3):377–87. doi: 10.1016/j.rdc.2017.04.008
394. Yau TO. Precision Treatment in Colorectal Cancer: Now and the Future. *JGH Open* (2019) 3(5):361–9. doi: 10.1002/jgh3.12153
395. Olivier M, Asmis R, Hawkins GA, Howard TD, Cox LA. The Need for Multi-Omics Biomarker Signatures in Precision Medicine. *Int J Mol Sci* (2019) 20 (19):4781. doi: 10.3390/ijms20194781
396. Subramanian I, Verma S, Kumar S, Jere A, Anamika K. Multi-Omics Data Integration, Interpretation, and Its Application. *Bioinf Biol Insights* (2020) 14:1177932219899051–1177932219899051. doi: 10.1177/1177932219899051
397. Putignani L, Gasbarrini A, Dallapiccola B. Potential of Multiomics Technology in Precision Medicine. *Curr Opin Gastroenterol* (2019) 35 (6):491–8. doi: 10.1097/mog.0000000000000589
398. Oswald M, Curran ME, Lamberth SL, Townsend RM, Hamilton JD, Chernoff DN, et al. Modular Analysis of Peripheral Blood Gene Expression in Rheumatoid Arthritis Captures Reproducible Gene Expression Changes in Tumor Necrosis Factor Responders. *Arthritis Rheumatol* (2015) 67(2):344–51. doi: 10.1002/art.38947
399. Ducreux J, Durez P, Galant C, Nzeusseu Toukap A, Van den Eynde B, Houssiau FA, et al. Global Molecular Effects of Tocilizumab Therapy in Rheumatoid Arthritis Synovium. *Arthritis Rheumatol* (2014) 66(1):15–23. doi: 10.1002/art.38202
400. Cuppen BVJ, Rossato M, Fritsch-Stork RDE, Concepcion AN, Linn-Rasker SP, Bijlsma JWJ, et al. RNA Sequencing to Predict Response to TNF-α Inhibitors Reveals Possible Mechanism for Nonresponse in Smokers. *Expert Rev Clin Immunol* (2018) 14(7):623–33. doi: 10.1080/1744666x.2018.1480937
401. Tasaki S, Suzuki K, Kassai Y, Takeshita M, Murota A, Kondo Y, et al. Multi-Omics Monitoring of Drug Response in Rheumatoid Arthritis in Pursuit of Molecular Remission. *Nat Commun* (2018) 9(1):2755. doi: 10.1038/s41467-018-05044-4

402. Aterido A, Cañete JD, Tornero J, Blanco F, Fernández-Gutierrez B, Pérez C, et al. A Combined Transcriptomic and Genomic Analysis Identifies a Gene Signature Associated With the Response to Anti-TNF Therapy in Rheumatoid Arthritis. *Front Immunol* (2019) 10:1459(1459). doi: 10.3389/fimmu.2019.01459
403. Tao W, Concepcion AN, Vianen M, Marijnissen ACA, Lafeber FPGJ, Radstake TRDJ, et al. Multiomics and Machine Learning Accurately Predict Clinical Response to Adalimumab and Etanercept Therapy in Patients With Rheumatoid Arthritis. *Arthritis Rheumatol (Hoboken NJ)* (2021) 73(2):212–22. doi: 10.1002/art.41516
404. Cheung P, Khatri P, Utz PJ, Kuo AJ. Single-Cell Technologies — Studying Rheumatic Diseases One Cell at a Time. *Nat Rev Rheumatol* (2019) 15(6):340–54. doi: 10.1038/s41584-019-0220-z
405. Reyfman PA, Walter JM, Joshi N, Anekalla KR, McQuattie-Pimentel AC, Chiu S, et al. Single-Cell Transcriptomic Analysis of Human Lung Provides Insights Into the Pathobiology of Pulmonary Fibrosis. *Am J Respir Crit Care Med* (2019) 199(12):1517–36. doi: 10.1164/rccm.201712-2410OC
406. Hwang B, Lee JH, Bang D. Single-Cell RNA Sequencing Technologies and Bioinformaticspipelines. *Exp Mol Med* (2018) 50(8):1–14. doi: 10.1038/s12276-018-0071-8
407. Luecken MD, Theis FJ. Current Best Practices in Single-Cell RNA-Seq Analysis: A Tutorial. *Mol Syst Biol* (2019) 15(6):e8746. doi: 10.15252/msb.20188746
408. Lee J, Hyeon DY, Hwang D. Single-Cell Multiomics: Technologies and Data Analysis Methods. *Exp Mol Med* (2020) 52(9):1428–42. doi: 10.1038/s12276-020-0420-2
409. Fonseka CY, Rao DA, Teslovich NC, Korsunsky I, Hannes SK, Slowikowski K, et al. Mixed-Effects Association of Single Cells Identifies an Expanded Effector CD4(+) T Cell Subset in Rheumatoid Arthritis. *Sci Transl Med* (2018) 10(463):eaaq0305. doi: 10.1126/scitranslmed.aaq0305
410. Xu ZC, Li SL, Rozewicki J, Yamashita K, Teraguchi S, Inoue T, et al. Functional Clustering of B Cell Receptors Using Sequence and Structural Features. *Mol Syst Design Eng* (2019) 4(4):769–78. doi: 10.1039/c9me00021f
411. Tan YC, Kongpachith S, Blum LK, Ju CH, Lahey LJ, Lu DR, et al. Barcode-Enabled Sequencing of Plasmablast Antibody Repertoires in Rheumatoid Arthritis. *Arthritis Rheumatol* (2014) 66(10):2706–15. doi: 10.1002/art.38754
412. Kinslow JD, Blum LK, Deane KD, Demoruelle MK, Okamoto Y, Parish MC, et al. Elevated IgA Plasmablast Levels in Subjects at Risk of Developing Rheumatoid Arthritis. *Arthritis Rheumatol* (2016) 68(10):2372–83. doi: 10.1002/art.39771
413. Elliott SE, Kongpachith S, Lingampalli N, Adamska JZ, Cannon BJ, Mao R, et al. Affinity Maturation Drives Epitope Spreading and Generation of Proinflammatory Anti-Citrullinated Protein Antibodies in Rheumatoid Arthritis. *Arthritis Rheumatol* (2018) 70(12):1946–58. doi: 10.1002/art.40587
414. Chen R, Mias GI, Li-Pook-Than J, Jiang L, Lam HY, Chen R, et al. Personal Omics Profiling Reveals Dynamic Molecular and Medical Phenotypes. *Cell* (2012) 148(6):1293–307. doi: 10.1016/j.cell.2012.02.009
415. Gligorijević V, Malod-Dognin N, Pržulj N. Integrative Methods for Analyzing Big Data in Precision Medicine. *Proteomics* (2016) 16(5):741–58. doi: 10.1002/pmic.201500396
416. Hobbs BP, Berry DA, Coombes KR. 17 - Biostatistics and Bioinformatics in Clinical Trials. In: JE Niederhuber, JO Armitage, MB Kastan, JH Doroshow and JE Tepper, editors. *Abeloff's Clinical Oncology*, *Sixth Edition*. Philadelphia: Elsevier (2020). doi: 10.1016/B978-0-323-47674-4.00017-7
417. Kashyap H, Ahmed HA, Hoque N, Roy S, Bhattacharyya DK. Big Data Analytics in Bioinformatics: Architectures, Techniques, Tools and Issues. *Netw Model Anal Health Inf Bioinf* (2016) 5(1):28. doi: 10.1007/s13721-016-0135-4
418. Bersanelli M, Mosca E, Remondini D, Giampieri E, Sala C, Castellani G, et al. Methods for the Integration of Multi-Omics Data: Mathematical Aspects. *BMC Bioinf* (2016) 17(2):S15. doi: 10.1186/s12859-015-0857-9
419. Ritchie MD, Holzinger ER, Li R, Pendergrass SA, Kim D. Methods of Integrating Data to Uncover Genotype–Phenotype Interactions. *Nat Rev Genet* (2015) 16(2):85–97. doi: 10.1038/nrg3868
420. Kristensen VN, Lingjærde OC, Russnes HG, Vollan HKM, Frigessi A, Børresen-Dale A-L. Principles and Methods of Integrative Genomic Analyses in Cancer. *Nat Rev Cancer* (2014) 14(5):299–313. doi: 10.1038/nrc3721
421. Ahmed Z, Mohamed K, Zeeshan S, Dong X. Artificial Intelligence With Multi-Functional Machine Learning Platform Development for Better Healthcare and Precision Medicine. *Database: J Biol Database Curation* (2020) 2020:baaa010. doi: 10.1093/database/baaa010
422. Nagy M, Radakovich N, Nazha A. Machine Learning in Oncology: What Should Clinicians Know? *JCO Clin Cancer Inf* (2020) 4):799–810. doi: 10.1200/CCI.20.00049
423. Lin C, Karlson EW, Canhao H, Miller TA, Dligach D, Chen PJ, et al. Automatic Prediction of Rheumatoid Arthritis Disease Activity From the Electronic Medical Records. *PloS One* (2013) 8(8):e69932–2. doi: 10.1371/journal.pone.0069932
424. Norgeot B, Glicksberg BS, Trupin L, Lituiev D, Gianfrancesco M, Oskotsky B, et al. Assessment of a Deep Learning Model Based on Electronic Health Record Data to Forecast Clinical Outcomes in Patients With Rheumatoid Arthritis. *JAMA Netw Open* (2019) 2(3):e190606. doi: 10.1001/jamanetworkopen.2019.0606
425. Murakami S, Hatano K, Tan J, Kim H, Aoki T. Automatic Identification of Bone Erosions in Rheumatoid Arthritis From Hand Radiographs Based on Deep Convolutional Neural Network. *Multimedia Tools Appl* (2018) 77(9):10921–37. doi: 10.1007/s11042-017-5449-4
426. Üreten K, Erbay H, Maraş HH. Detection of Rheumatoid Arthritis From Hand Radiographs Using a Convolutional Neural Network. *Clin Rheumatol* (2020) 39(4):969–74. doi: 10.1007/s10067-019-04487-4
427. Guan Y, Zhang H, Quang D, Wang Z, Parker SCJ, Pappas DA, et al. Machine Learning to Predict Anti-Tumor Necrosis Factor Drug Responses of Rheumatoid Arthritis Patients by Integrating Clinical and Genetic Markers. *Arthritis Rheumatol* (2019) 71(12):1987–96. doi: 10.1002/art.41056
428. Feldmann M, Maini R. Can We Get Closer to a Cure for Rheumatoid Arthritis? *Arthritis Rheumatol (Hoboken NJ)* (2015) 67(9):2283–91. doi: 10.1002/art.39269

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Decreased Absolute Number of Circulating Regulatory T Cells in Patients With Takayasu's Arteritis

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Background: Takayasu's arteritis (TA) is a type of primary large vessel vasculitis. Th1, Th17, and Tfh cells have been reported to be associated with TA relapse. However, the relationship between regulatory T cells (Tregs) and TA remains unclear.

Objective: To analyze the levels of circulating lymphocytes, especially Treg cells ($CD4^+CD25^+FOXP3^+$ T cells) and serum cytokines in TA patients and explore their relationship with their changes and TA disease activity.

Methods: A total of 57 TA patients and 43 sex- and age-matched healthy controls (HCs) were enrolled. According to NIH standards, 36 patients had active disease status. Flow cytometry combined with counting was used to detect the absolute numbers and ratios of Th1, Th2, Th17, and Treg cells in the peripheral blood of all the subjects. Magnetic bead-based multiplex immunoassay was used to detect cytokines.

Results: Compared to HCs, the absolute number and proportion of peripheral Treg cells in TA patients was significantly decreased, while Th17 cells were significantly increased. Furthermore, compared to the inactive group, the TA active group had significantly increased levels of interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-α, but lower IL-10 levels. The absolute number of Th2 cells was negatively associated with platelet (PLT) and NIS scores in TA patients. The proportion of Th2 cells was negatively associated with the erythrocyte sedimentation rate in TA patients. After treatment, Treg cells were markedly increased.

Conclusion: There was a Th17-Treg cell imbalance with a significant reduction in peripheral Treg cells and an increase in Th17 cells in TA patients compared to the HCs. The levels of IL-6, IL-10, IL-17, and TNF-α appeared to be related to disease activity.

Keywords: Takayasu's arteritis, Treg cell, Th17 cell, IL-6, TNF-α

INTRODUCTION

Takayasu's arteritis (TA), a primary large vessel vasculitis, causes chronic, progressive, and non-specific inflammation of the aorta and its main branches, and stenosis and occlusion of various arteries, which leads to ischemic manifestations. A variety of immune dysfunctions are involved in the occurrence and development of TA (1).

Infiltration of a variety of inflammatory cells in the blood-vessel walls is the main pathological manifestation of TA. During pathological bodily states, such as infections, dendritic cells (DCs) are activated and naïve T cells differentiate into helper T (Th) cells (namely, Th1, Th2, and Th17), which may lead to TA (2).

Recent studies have demonstrated that Th17 cells are upregulated in TA and play an important role in its pathogenesis (3, 4). Regulatory T cells (Tregs) are the key anti-inflammatory cells of the immune response. Zhang et al. (5) found that overactivation of mTORC1 in TA patients can upregulate the expression of Th1 and Th17 cells. Meanwhile, the expression of $CD8^+$ Treg remained normal. However, $CD4^+CD25^+FOX3^+$ Tregs were not studied.

This study aimed to analyze the levels of circulating lymphocyte subsets, especially Treg cells ($CD4^+CD25^+FOXP3^+$ T cells),and cytokines in TA patients, and to explore their relationship with TA disease activity. These findings lead to novel methods of TA diagnosis and treatment.

MATERIALS AND METHODS

Patients

A total of 57 newly diagnosed and untreated TA patients, who visited the Department of Rheumatology and Immunology of the Second Hospital of Shanxi Medical University between March 2016 and May 2019 and met the 1990 American College of Rheumatology TA classification criteria (6) were enrolled. TA patients were 18–51 years (39.05 ± 14.86 years), the male-to-female ratio was 1:5.3. We also enrolled 43 age- and sex-matched healthy adults as healthy controls (HCs) by frequency matching. Informed consent was obtained from all the participants and the study was approved by the Institutional Review Board of the Second Hospital of Shanxi Medical University.

Clinical Indicators

ESR was measured using the Westergren method, C-reactive protein (CRP) was analyzed using immune turbidimetry. Platelets were measured using light transmission aggregometry. Magnetic bead-based multiplex immunoassays were used to detect serum interleukin (IL)-6, IL-10, IL-17, and tumor necrosis factor (TNF)-α levels. In accordance with the National Institutes of Health (NIH) standard (7), TA disease activity was evaluated on the basis of the NIS score (arteritis inactive group ≤1; TA active group: ≥2). Based on the NIS score, the disease active and inactive groups comprised 36 and 21 patients, respectively.

CD4+ T Lymphocyte Subset Detection

(1) Th1, Th2, and Th17 Cell cultures and Labeling: An 80 μl blood sample together with 10 μl phorbol myristate acetate working solution (final concentration, 30 ng/ml), 10 μl ionomycin working solution (final concentration, 750 ng/ml), and 1 μl GolgiStop was incubated at 37°C and 5% CO_2 for 5 h. The samples were then divided into two tubes, followed by staining with anti-CD4-FITC antibodies at room temperature in the dark for 30 min. To the tubes was added 1 ml freshly prepared fixation/permeabilization solution; the tubes were then placed in an incubator at 4°C in the dark for 30 min. Anti-IL-4-PE and anti-interferon gamma (IFN-γ)-APC were added to tube A; Anti-FITC-CD4 and anti-IFN-γ-APC (intracellular staining) were used to detect Th1 cells, while anti-FITC-CD4, and anti-IL-4-PE (intracellular staining) were used to detect Th2 cells. Anti-human IL-17-PE (intracellular staining) was added to tube B for Th17-cell analysis. The two tubes of cells were stored at room temperature for 30 min in the dark and then washed with phosphate-buffered saline (PBS). The absolute numbers of $CD4^+$ T lymphocyte subsets were automatically detected using BD Multitest software (BD Biosciences, Franklin Lakes, NJ, USA). All immunofluorescence antibodies were purchased from BD Biosciences.

(2) Detection of Treg Cells: Anti-CD4-FITC and anti-CD25-APC were added to an 80 μl blood sample and incubated at room temperature in the dark for 30 min. Then, 1 ml freshly prepared fixation/permeabilization solution was added to each tube, mixed, and incubated at 4°C for 30 min. AntiFOXP3-PE (intracellular staining) was added and incubated at room temperature for 30 min in the dark, followed by washing with PBS and detection of Treg cells using flow cytometry. All immunofluorescence antibodies were purchased from BD Biosciences.

(3) Flow cytometry: The stained cells were measured using flow cytometry (Calibur; BD Biosciences) within 24 h. Based on the scatter plot of the forward angular scattered light relative to the lateral angular dispersive light (side scatter (SSC)), the lymphocytes were gated to distinguish them. CD4 was used to distinguish $CD4^+$ T cells from the SSC gate; 10,000 cells from the gate were taken. The relative percentages were obtained and analyzed using CellQuest software. The absolute number of cells in each subgroup was calculated using the following equation: absolute cell number = percentage of positive cells in each subset × absolute number of $CD4^+$ T cells (cells/μl) cells/μl whole blood (**Figure 1**).

Detection of Cytokine Levels by Cytometric Bead Array

The serum was separated from 4 ml of venous blood after 1–2 h and stored at −20°C. IL-6, IL-10, IL-17, and TNF-α were measured using flow cytometry. A cytometric bead array (CBA) kit was purchased from Jiangsu Sage Biotechnology Co. Ltd. (Jiangsu, China) and used according to the manufacturer's instructions. The results were expressed as pg/ml.

Statistical Analysis

PASS 11.0 software was used for statistical treatment for sample size. Previous studies have obtained the mean and standard deviation of each group. The sample size is 1:1, the two-sided test is 0.05, and the minimum test power is 0.8. It is calculated that the healthy control group and TA group requires 38 cases, and the active group and disease-active group requires16 cases. SPSS Statistics l24.0 software (IBM Corp., Armonk, NY, USA) was

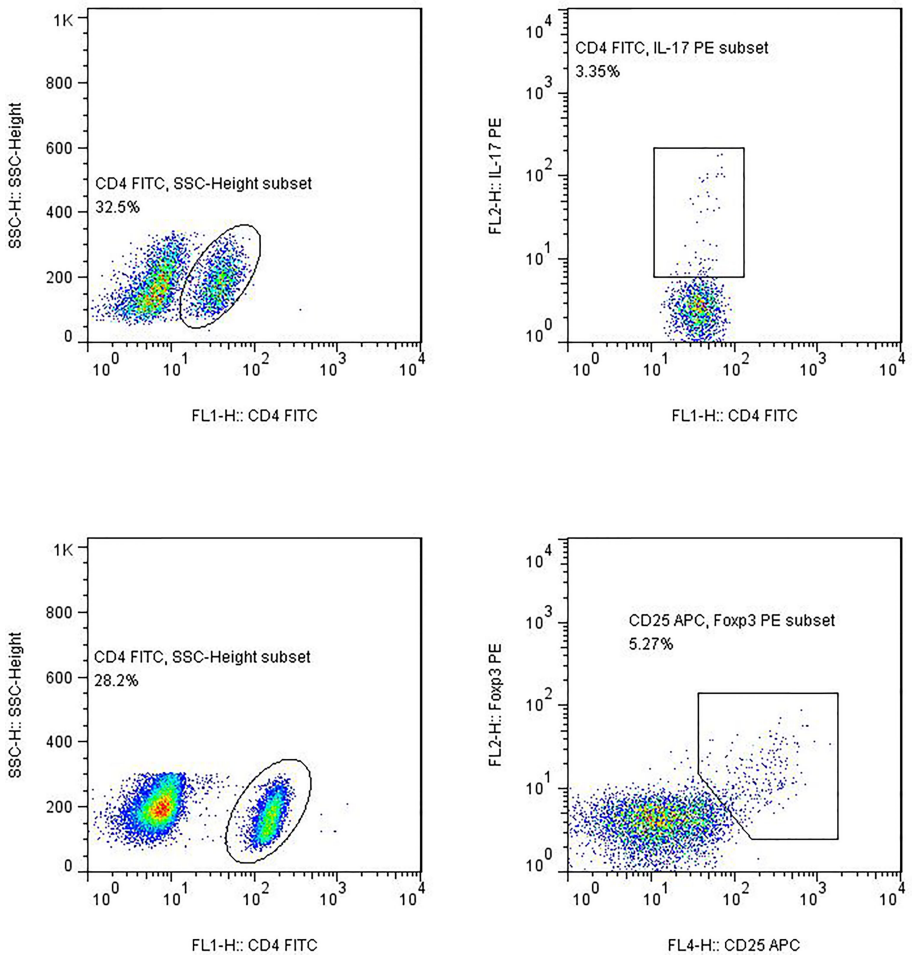


FIGURE 1 | Gating for Th17 cells and Treg cells.

used for the statistical analysis. Data are expressed as mean ± standard deviation (mean ± SD). Normal measurement data were compared between groups using the independent sample t-test. The data distribution was tested using a non-parametric test. Normally distributed variables were analyzed using Pearson correlation analysis, while non-normally distributed variables were analyzed using Spearman correlation analysis. GraphPad (GraphPad Software Inc., San Diego, CA, USA) was used to perform receiver operating characteristic (ROC) curve analysis, to determine the accuracy of cytokine level for predicting TA disease activity. The paired t-test was used to assess the differences in variables before and after treatment. P-values <0.05 were considered significant.

RESULTS

Absolute Number of Th1, Th2, Th17, and Tregs, and the Th17/Treg Ratio

The absolute numbers of Th1 cells were increased in TA (185.5 ± 145.0 *vs* 115.3 ± 66.9 cells/μl, p = 0.019) (**Figure 1**). The absolute numbers and proportion of Th17 cells were also markedly increased in TA (11.9 ± 9.3 *vs* 4.6 ± 1.6 cells/μl, p <0.001; 1.3 ± 0.9% *vs* 0.7 ± 0.3%, p <0.001). We also observed a significant decline in the absolute numbers and proportion of peripheral CD4+ Treg cells in the TA patients compared to the HCs (30.2 ± 14.2 *vs* 37.1 ± 9.2 cells/μl, p = 0.001; 3.4 ± 1.6% *vs* 5.5 ± 1.1%, p <0.001). There were no differences in the absolute numbers or proportion of Th2 cells, between the TA patients and the HCs (9.9 ± 5.9 *vs* 8.3 ± 5.0 cells/μl, p = 0.179; 1.1 ± 0.7% *vs* 1.2 ± 0.7%, p = 0.277).

Compared to the inactive TA group, we observed increased numbers of peripheral Th2 cells in the active TA group (8.4 ± 5.0 *vs* 12.3 ± 6.5 cells/μl, p = 0.026). There were no differences in the absolute numbers or proportion of Th1 cells between the groups (158.2 ± 121.8 *vs* 232.4 ± 171.0 cells/μl, p = 0.132; 19.1 ± 14.5% *vs* 22.2 ± 12.9%, p = 0.254). Neither the absolute number nor the proportion of Th17 cells was increased (12.0 ± 10.0 *vs* 11.7 ± 8.0 cells/μl, p = 0.679; 1.3 ± 0.9% *vs* 1.2 ± 0.7%, p = 0.882). Similarly, there were no significant differences in Treg cells between the active and inactive groups (30.0 ± 15.3 *vs* 30.7 ± 12.4 cells/μl, p = 0.697; 3.4 ± 1.3% *vs* 3.5 ± 2.0%, p = 0.597) (**Figure 2**).

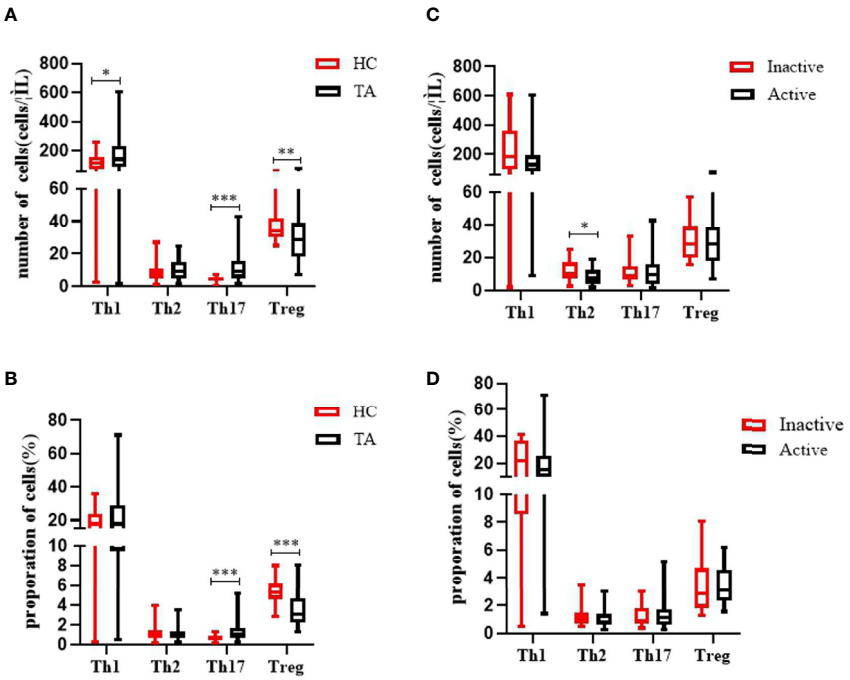


FIGURE 2 | Characteristics of the absolute numbers and proportions of Th17 cells and CD4Treg cells in the PB of patients with TA. **(A, B)** The levels of Th1 cells and Th17 cells in PB were significantly increased in patients with TA (n = 57). The absolute number and the proportion of CD4Treg cells were significantly decreased in TA (n = 57). There were no significant changes of the absolute numbers and proportion of Th2 in PB between healthy controls (n = 43). **(C, D)** The absolute number of Th2 cells in PB were significantly decreased in active patients with TA (n = 36). Neither the absolute number nor proportion of Th1, Th17, and Treg cells were altered significantly between active TA patients (n = 36) and inactive TA patients (n = 21). Data were presented as mean ± SD. Shown are the significant differences assessed by the Mann–Whitney U test. *P < 0.05; **P < 0.001, ***P < 0.001. P < 0.05 was considered statistically significant. TA, Takayasu's arteritis; PB, peripheral blood; Tregs, regulatory T cells.

Il-6, TNF-α, and IL-17 Increased in Disease-Active Group

The active disease group had significantly higher IL-6 levels (23.7 ± 16.0 *vs* 9.4 ± 5.7 pg/ml, p <0.001), and lower IL-10 levels (6.8 ± 3.6 *vs* 15.2 ± 12.4 pg/ml, p = 0.024) compared to the inactive group. The concentrations of IL-17 (14.6 ± 10.2 *vs* 7.9 ± 7.0 pg/ml, p = 0.016) and TNF-α (8.6 ± 7.6 *vs* 3.3 ± 2.0 pg/ml, p = 0.001) (**Figure 3**).

ROC Curve for Analysis for the Prediction of TA Disease Activity

Based on the NIS scores, the areas under the ROC curve (AUCs) for IL-6, IL-10, IL-17, and TNF-α were 0.827 (sensitivity and specificity, 87.9 and 61.9%, respectively); 0.683 (47.6 and 90.9%), 0.696 (66.7 and 76.2%), and 0.762 (57.6 and 85.7%), respectively (**Figure 4**).

The Absolute Number of Th2 cells Was Negatively Correlated With ESR and NIS

Vascular stenosis was classified as mild (score 1, <30%), moderate (score 2, 30–69%), severe (score 3, 70–99%), or occlusive (score 4, >99%). The correlations between the scores and the absolute and relative numbers of $CD4^+$ T cell subsets were analyzed.

The absolute number of Th2 cells was negatively associated with platelets (PLT), C3 and C4, and NIS scores in the TA patients (r = −0.366, p = 0.016; r = −0.390, p = 0.007; r = −0.435, p = 0.002; and r = −0.295, p = 0.047, respectively). The proportion of Th2 cells was negatively associated with the ESR in the TA patients (r = −0.342, p = 0.048). The absolute number of Th17 cells correlated negatively with C3 in TA patients (r = −0.625, p = 0.002).There was no significant correlation with PLT, IgG, IgA, IgM, C4, ESR, CRP, NIS, vascular stenosis, IL-6, IL-10, IL-17, or TNF-α (p >0.05). The proportion of Th17 cells was not significantly correlated with the levels of PLT, IgG, IgA, IgM, C3, C4, ESR, CRP, NIS, vascular stenosis, IL-6, IL-10, IL-17 or TNF-α (p >0.05). The Th17/Treg ratio and Treg cells were not significantly correlated with the levels of PLT, IgG, IgA, IgM, C3, C4, ESR, CRP, NIS, vascular stenosis, IL-6, IL-10, IL-17 or TNF-α (p >0.05) (**Figure 5** and **Table 1**).

Treg Cell Counts Increased After Treatment

In total, 57 patients were recruited to this study, and we observed changes in various indicators of TA before and after treatment. In the patients treated with glucocorticoids and immunosuppressants, the numbers of Th17 cells showed a downward trend after treatment, but the difference was not significant. The absolute

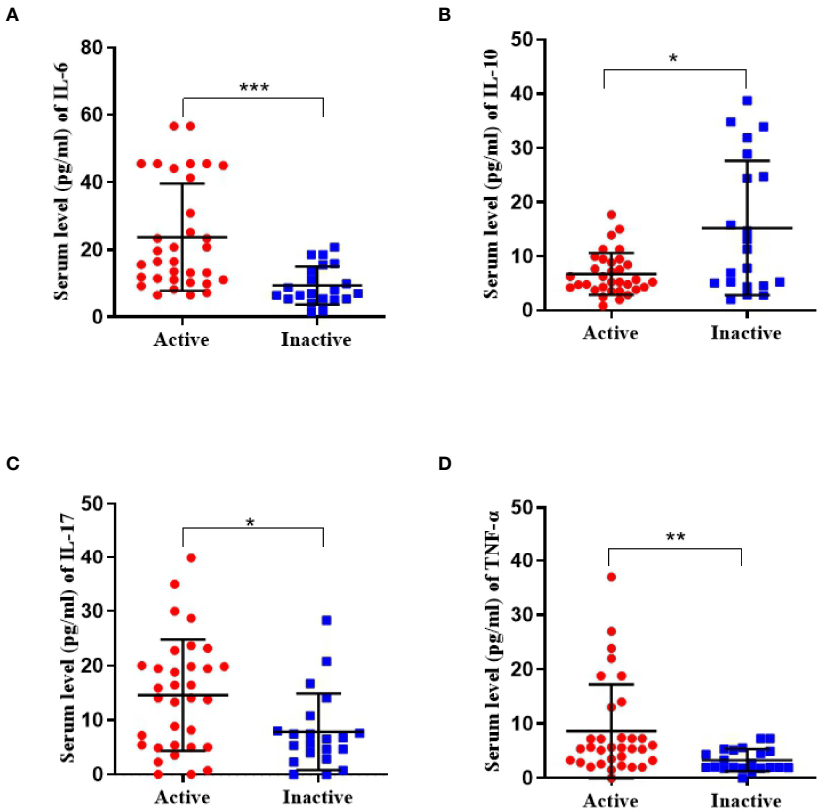


FIGURE 3 | Serum concentrations of cytokine (including IL-6, IL-10, IL-17, and TNF-α) in active TA patients (n = 36) and inactive TA patients (n = 21). **(A, C, D)** The concentration of IL-6, IL-17, and TNF-α was significantly upregulated. **(B)** That of IL-10 was found reduced. Data were presented as mean ± SD. Shown significant differences are assessed by the Mann–Whitney U test. *P < 0.05; **P < 0.001, ***P < 0.001. P < 0.05 was considered statistically significant.

number of Treg cells after conventional treatment was significantly higher before treatment (p = 0.004) (**Table 2**).

DISCUSSION

TA is a chronic and non-specific full-thickness arteritis of unknown etiology. Its pathogenesis is related to genetic factors, endocrine abnormalities, immune dysfunction, and the inflammatory response of cytokines. Activated T lymphocytes promote arterial inflammation, and a variety of T lymphocyte subsets and cytokines are involved in the pathogenesis of TA (8).$CD4^+$ T lymphocytes are also known as key cell participants in vasculitis (9). It is known that immune cells such as Th1 and Th17 cells and their secreted cytokines are mainly involved in the pathogenesis of TA; the role of $CD4^+$ T cells of TA has always been controversial. Previous studies have suggested that Th1 and Th2 cells involved in cellular and humoral immunity play a major role in the immune system. In recent years, it has been discovered that Th17 and Treg cells from the same source but with different immune functions play an important role in autoimmune diseases. The basis of the body's autoimmune balance is the Th17/Treg. Once the balance is broken, it will lead to the occurrence of autoimmune diseases.

Th17 cells are an important subgroup of $CD4^+$ T cells, which play a major role in causing inflammation in autoimmune diseases. Excessive expression of Th17 cells and related cytokines can tend inflammatory cells to accumulate on the blood vessel wall, stimulate fibroblasts and macrophages at the same time, induce the production of a variety of inflammatory cytokines, cause repeated inflammation and endothelial damage, leading to large arteries Arterial wall thickening and thrombosis in patients with inflammation, stenosis, and occlusive disease can occur in severe cases (10). Regulatory T cells (Regulatory T cells, Treg cells) are an important subgroup of T cells, which enable the body to maintain immune tolerance and inhibit the occurrence of autoimmune diseases. After the inflammation occurs, antigen-presenting cells such as dendritic cells and macrophages are activated to induce Treg cells to reach the site of inflammation, and downregulate the expression of autoreactive T cells and inflammatory cytokines by secreting inhibitory cytokine IL-10, exerting immune tolerance to maintain the body's own immune balance. Th17 cells and Treg cells are closely related in the differentiation process and can be transformed into each other in a specific cytokine microenvironment. When the immune system is stable, IL-10 produced by the immune system can inhibit the production of effector T cells and promote $Foxp3^+$

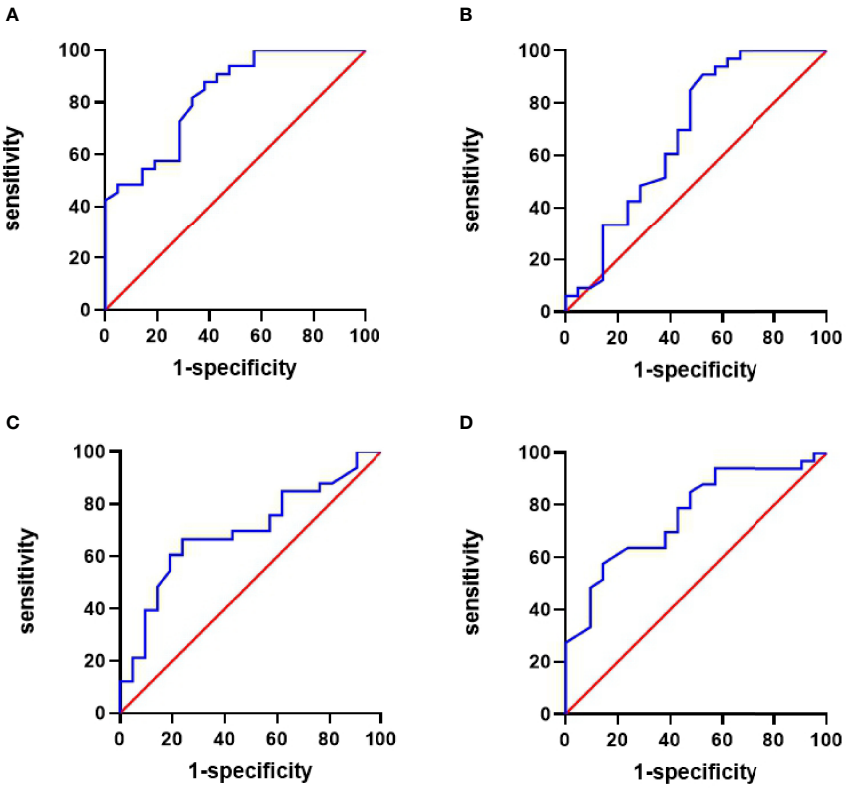


FIGURE 4 | Receiver operating characteristic (ROC) curve of cytokines for predicting the activity of TA patients. **(A)** The area under the ROC curve (AUC) of IL-6 was 0.827, sensitivity 87.9%, and specificity 61.9%. **(B)** AUC of IL-10 was 0.683, sensitivity 47.6%, and specificity 90.9%. **(C)** The AUC of IL-17 was 0.696, sensitivity 66.7%, and specificity 76.2%. **(D)** AUC of TNF-α was 0.762, sensitivity 57.6%, and specificity 85.7%.

Treg to differentiate and maintain autoimmune tolerance; when the body has an inflammatory infiltration, the activated immune system will produce IL-6 and TNF-α to promote Th17 cells to induce inflammation, while inhibiting IL-10 mediated Treg cell production. Th17/Treg balance is a key factor in the body's own immune balance, and it plays an important role in immune defense and immune stability. The imbalance of the ratio of pro-inflammatory Th17 cells and inhibitory Treg cells mediates the occurrence and development of a variety of vascular inflammatory autoimmune diseases (11–13).

Although the pathogenesis of aortitis remains unclear, there is considerable evidence that TA occurs due to the disruption of the original immune balance. Overactivation of T cells and decreased fever of Treg cells may lead to immune imbalance. Our results do not represent the truth of the pathogenesis. But it can reflect certain trends to some extent. We demonstrated that the absolute number of circulating Treg cells was significantly lower in the TA patients, even in the inactive group, than in the HCs, which suggests that the Treg cell reduction may be involved in disease onset. Treg cells are an important T cell subgroup that maintains immune tolerance and suppresses autoimmune diseases. Downregulation of Treg cell expression is associated with a variety of autoimmune diseases (14–16). A decrease in the absolute numbers and function of Treg cells can lead to disease. However, no difference in Treg expression was found between our active and inactive groups, which suggests that disease activity may not be related to Treg cell expression.

We also observed an increase in the absolute numbers of pro-inflammatory T-cell subsets (Th1, Th2, and Th17 cells) in TA patients. The absolute numbers and proportion of Th17 cells were significantly higher in the TA patients than the HCs, consistent with the findings of Misra (3) and Saadoun (17). Although Th17 cells did not correlated with disease activity, the IL-17 levels were significantly higher in the active compared to inactive group, which suggests that IL-17 producing cells not only increased in number, but also in terms of function. However, large-sample cohort studies are required to further explore the role of Th17 and Treg cells in TA pathogenesis.

Th1 cells are one of the Th cell subgroups with pro-inflammatory effects, which can exert their inflammatory effects by secreting IFN-γ and promote cellular immunity. Previous studies have believed that Th17 cells are the main immune cells in the acute phase, while Th1 cells are the cells for chronic inflammation of blood vessels. This study did not find that Th1 cells are abnormally expressed in TA patients, which may be proportional (36/57) to the patients in the acute phase in this study. Largely related Th2 cells can secrete IL-4 and other cytokines, which play an important role in fighting parasitic

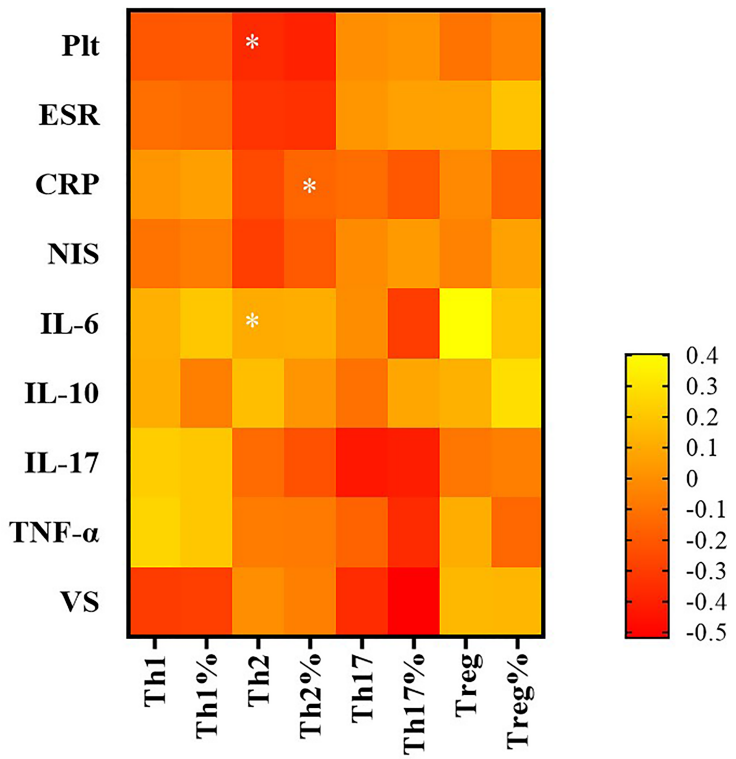


FIGURE 5 | The correlation between $CD4^+$ T subsets and clinical indicators by Spearman correlation test. In TA patients, that of Th2 cells was negatively associated with platelet (PLT) and NIS score; the proportion of Th2 cells was negatively associated with erythrocyte sedimentation rate (ESR). *P < 0.05; P < 0.05 was considered statistically significant.

TABLE 1 | Correlation analysis.

	Th17 (cells/μl)		Th17%		Tregs (cells/μl)		Treg%	
	r	P	r	P	r	P	R	P
Plt (*109/L)	−0.007	0.966	0.011	0.942	−0.106	0.481	−0.047	0.756
ESR (mm/h)	0.020	0.900	0.061	0.696	0.066	0.661	0.184	0.222
CRP (mg/L)	−0.126	0.476	−0.201	0.255	−0.026	0.866	−0.166	0.270
NIS (score)	−0.019	0.902	0.038	0.800	−0.050	0.742	0.063	0.676
VS (score)	−0.316	0.248	−0.519	0.084	0.143	0.657	0.136	0.613
IL-6 (pg/ml)	−0.011	0.958	−0.300	0.136	0.403	0.141	0.186	0.363
IL-10 (pg/ml)	−0.113	0.583	0.079	0.701	0.118	0.565	0.282	0.162
IL-17 (pg/ml)	−0.438	0.069	−0.415	0.087	−0.094	0.711	−0.063	0.803
TNF-α (pg/ml)	−0.164	0.423	−0.364	0.068	0.110	0.594	−0.143	0.484
	Th1 (cells/μl)		**Th1%**		**Th2 (cells/μl)**		**Th2%**	
	r	P	r	P	r	P	r	P
Plt (*109/L)	−0.211	0.174	−0.201	0.197	−0.366	0.016*	−0.400	0.008
ESR (mm/h)	−0.118	0.505	−0.138	0.436	−0.333	0.054	−0.342	0.048*
CRP (mg/L)	0.023	0.884	0.059	0.709	−0.252	0.103	−0.152	0.332
NIS (score)	−0.104	0.491	−0.071	0.638	−0.295	0.047*	−0.196	0.191
VS (score)	0.100	0.757	−0.004	0.991	−0.118	0.715	−0.410	0.185
IL-6 (pg/ml)	0.116	0.548	0.198	0.304	0.096	0.620	0.107	0.579
IL-10 (pg/ml)	0.000	1.00	−0.062	0.749	0.168	0.384	0.017	0.929
IL-17 (pg/ml)	0.220	0.365	0.199	0.414	−0.135	0.583	−0.228	0.348
TNF-α (pg/ml)	0.248	0.195	0.200	0.299	−0.071	0.715	−0.078	0.686

*By Spearman correlation test, *P <0.05. P <0.05 was considered statistically significant. VS, Vascular Stenosis.*

TABLE 2 | Treg cells were increased after treatment.

	Before	After	P
Th17	13.49 (8.76, 25.52)	12.44 (4.57, 28.06)	0.454
Th17%	1.69 ±0.93	1.45 ±0.68	0.384
Treg	23.96 (16.28, 39.5)	44.46 (33.67, 56.15)	0.004**
Treg%	2.3 (1.74, 4.48)	5.03 (2.31, 7.96)	0.118

*Absolute numbers of $CD4^+$ T subsets, especially Tregs, were significantly increased in patients after treatment. The normal distribution data were expressed as mean± standard deviation (SD), nonnormal distribution data were expressed as median four quantile method [M (P25, P75)]. *P < 0.05; **P < 0.01. P < 0.05 was considered statistically significant.*

infections and allergic diseases. In autoimmune diseases, Th2 cells have an inhibitory effect on inflammation, and can inhibit the differentiation of Th1 cells by secreting IL-4, thereby reducing a variety of inflammatory factors secreted by Th1 cells and inhibiting the corresponding immune response. Similar to our study, in the study of Kong et al. (10), there was no statistical difference in the proportion of Th2 cells in TA patients compared with healthy controls. Therefore, Th2 cells have no decisive role in the pathogenesis of TA. However, Th2 cells in patients with active TA were significantly reduced compared with the inactive group. Further research found that Th2 cells can predict TA disease activity: Th2 cell expression is negatively correlated with inflammatory indicators such as ESR, NIS score, and PLT, suggesting that Th2 cells have weakened anti-inflammatory effects. Lead to disease activity; still need to increase the sample size for further research.

Interestingly, we also observed higher serum levels of IL-6, IL-17, and TNF-α in active TA patients compared to inactive patients, while the levels of IL-10 were decreased. The AUC values for IL-6, IL-10, IL-17, and TNF-α, for predicting TA disease activity were 0.827, 0.683, 0.696, and 0.762, respectively, reflecting some degree of predictive value, IL-6 and IL-17 are pro-inflammatory factors secreted by Th17 cells. IL-6 can promote T cell activation and exacerbate the inflammatory response and is an important initiator of Th17 differentiation (18). Savioli et al. found that IL-6 expression in active TA patients was higher compared to those in remission (19), in agreement with our results. Refractory arteritis has been treated successfully with IL-6 antagonists (20). IL-17 is a strong inflammatory factor, that can activate T cells and stimulate endothelial cells, epithelial cells, and fibroblasts to induce inflammation in TA, and IL-17 also has a synergistic effect with TNF-α, and upregulates IL-6 expression to jointly regulate the inflammatory response (21).The increased IL-6 and IL-17 expression seen in our active TA patients suggested that the inflammatory cytokines secreted by Th17 were involved in the development of TA.

IL-10 is a typical anti-inflammatory cytokine, mainly produced by macrophages and Treg cells. It has a variety of immune regulation and inflammatory effects, and can transmit negative feedback signals, suppress immune system activation, inhibit the activation of macrophages, and reduce cytokine production by T cells. The results of this study showed that IL-10 expression, decreased with disease activity, consistent with the change in Treg-cell expression. This suggests that TA activity could be alleviated by regulating the changes in IL-10 levels and inhibiting the secretion of inflammatory factors.

Another advantage of our study was that Treg cells were labeled by anti-CD4/CD25/FoxP3 antibodies. FoxP3, regarded as the most specific marker of Treg cells, was truly important for the suppressive function of Treg cells. Our results showed that the number of Treg cells defined as $CD4^+CD25^+FoxP3^+$ was obviously lower than those labeled by $CD4^+CD25^+$, suggesting that $CD4^+CD25^+$ T cells could not exhibit the true level of Treg cells. Moreover, we observed, in our study, the changes of the proportion and absolute number of cells were sometimes not very consistent, and the change of the percentage of one subset is not only absolutely due to changes in its cell number, but the changes in the number of other cells. Thus, proportion of cells should not completely replace the absolute number of cells to represent the cellular level.

Despite the remarkable and clinically relevant findings in this study, there are some drawbacks to our study. The number of TA patients was not large. We further need to expand the sample size to study the pathogenesis of TA.

CONCLUSION

Our study suggests that the vascular inflammation seen in the TA patients is closely related to decreased absolute numbers of Treg cells, and increased Th17 cell numbers. High levels of IL-6, IL-17, and TNF-α may also contribute to disease activity. Further investigation of the causes of decreased Treg cells is required, as this may be a useful indicator for disease activity and a potential target for TA treatment. Through *in vitro* amplification or modification of Treg cells, anti-inflammatory effects may be promoted in tissues and inflammatory microenvironments to achieve control over diseases. This phenomenon may inform new strategies for immune cell therapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Department of Rheumatology, The Second

Hospital of Shanxi Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-FX and X-FL participated in the study design. JL, Y-L and G-YL were in charge of providing data. WJ and Z-LF participated in the data collection and interpreted the data. WJ, J-PC, and C-LY performed the statistical analysis under the supervision of J-FX and X-FL. WJ wrote the draft manuscript, and J-FX and CG helped to revise the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Russo RAG, Katsicas MM. Takayasu Arteritis. *Front Pediatr* (2018) 6:265. doi: 10.3389/fped.2018.00265
2. Matsumoto K, Suzuki K, Yoshimoto K, Seki N, Tsujimoto H, Chiba K, et al. Significant Association Between Clinical Characteristics and Changes in Peripheral Immuno-Phenotype in Large Vessel Vasculitis. *Arthritis Res Ther* (2019) 21(1):304. doi: 10.1186/s13075-019-2068-7
3. Misra DP, Chaurasia S, Misra R. Increased Circulating Th17 Cells, Serum IL-17A, and IL-23 in Takayasu Arteritis. *Autoimmune Dis* (2016) 2016:7841718. doi: 10.1155/2016/7841718
4. Bereswill S, Muñoz M, Fischer A, Plickert R, Haag LM, Otto B, et al. Anti-Inflammatory Effects of Resveratrol, Curcumin and Simvastatin in Acute Small Intestinal Inflammation. *PloS One* (2010) 5(12):e15099. doi: 10.1371/journal.pone.0015099
5. Zhang J, Zhao L, Wang J, Cheng Z, Sun M, Zhao J, et al. Targeting Mechanistic Target of Rapamycin Complex 1 Restricts Proinflammatory T Cell Differentiation and Ameliorates Takayasu Arteritis. *Arthritis Rheumatol* (2020) 72(2):303–5. doi: 10.1002/art.41084
6. Arend WP, Michel BA, Bloch DA, Hunder GG, Calabrese LH, Edworthy SM, et al. The American College of Rheumatology 1990 Criteria for the Classification of Takayasu Arteritis. *Arthritis Rheum* (1990) 33:1129–34. doi: 10.1002/art.1780330811
7. Kerr GS, Hallahan CW, Giordano J, Leavitt RY, Fauci AS, Rottem M, et al. Takayasu Arteritis. *Ann Intern Med* (1994) 120(11):919–29. doi: 10.7326/0003-4819-120-11-199406010-00004
8. Samson M, Bonnotte B. Pathogenesis of Large Vessel Vasculitis. *Rev Med Interne* (2016) 37(4):264–73. doi: 10.1016/j.revmed.2015.10.350
9. O'Connor MB, O'Donovan N, Bond U, Phelan MJ. Successful Treatment of Takayasu Arteritis With Rituximab as a First-Line Immunosuppressant. *BMJ Case Rep* (2017) 2017. doi: 10.1136/bcr-2016-217313
10. Kong X, Sun Y, Ma L, Chen H, Wei L, Wu W, et al. The Critical Role of IL-6 in the Pathogenesis of Takayasu Arteritis. *Clin Exp Rheumatol* (2016) 34(suppl 9):S21–7.
11. Mekinian A, Resche-Rigon M, Comarmond C, Soriano A, Constans J, Alric L, et al. Efficacy of Tocilizumab in Takayasu Arteritis:Multicenter Retrospective Study of 46 Patients. *J Autoimmun* (2018) 91:55–60. doi: 10.1016/j.jaut.2018.04.002
12. Ferri L, Rousseau S, Olivenstein R, Bérubé J, Roussel L, Yao Y, et al. IL-17 Promotes P38 Mapkdependent Endothelial Activation Enhancing Neutrophil Recruitment to Sites of Inflammation. *J Immunol* (2010) 184(8):4531–7. doi: 10.4049/jimmunol.0903162
13. Lima VV, Zemse SM, Chiao CW, Bomfim GF, Tostes RC, Clinton WR, et al. Interleukin-10 Limits Increased Blood Pressure and Vascular Rhoa/Rho-Kinase Signaling in Angiotensin II-Infused Mice. *Life Sci* (2016) 145:137–43. doi: 10.1016/j.lfs.2015.12.009
14. Talaat RM, Mohamed SF, Bassyouni IH, Raouf AA. Thl/Th2/Thlt/Treg Cytokine Imbalance in Systemic Lupus Erythematosus(SLE)Patients: Correlation With Disease Activity. *Cytokine* (2015) 72:146–53. doi: 10.1016/j.cyto.2014.12.027
15. Coca A, Sanz I. Updates on B—Cell Immunotherapies for Systemic Lupus Erythematosus and Sjogren'syndrome. *Curr Opin Rheumatol* (2012) 24:451–6. doi: 10.1097/BOR.0b013e32835707e4
16. Cooles FA, Isaacs JD, Anderson AE. Treg Cells in Rheumatoid Arthritis: An Update. *Curr Opin Rheumatol Rep* (2013) 15:352. doi: 10.1007/s11926-013-0352-0
17. Saadoun D, Garrido M, Comarmond C, Desbois AC, Domont F, Savey L, et al. Th1 and Th17 Cytokines Drive Inflammation in Takayasu Arteritis. *Arthritis Rheumatol* (2015) 67(5):1353–60. doi: 10.1002/art.39037
18. Nishino Y, Tamai M, Kawakami A, Koga T, Makiyama J, Maeda Y, et al. Serum Levels of BAFF for Assessing the Disease Activity of Takayasu Arteritis. *Clin Exp Rheumatol* (2010) 28(1 Suppl 57):14–7 doi: 10.1186/ar2917.
19. Savioli B, Abdulahad WH, Brouwer E, Kallenberg CGM, de Souza AWS. Are Cytokines and Chemokines Suitable Biomarkers for Takayasu Arteritis? *Autoimmun Rev* (2017) 16(10):1071–8. doi: 10.1016/j.autrev.2017.07.023
20. Nakaoka Y, Isobe M, Takei S, Tanaka Y, Ishii T, Yokota S, et al. Efficacy and Safety of Tocilizumab in Patients With Refractory Takayasu Arteritis: Results From a Randomised, Double-Blind, Placebo-Controlled, Phase 3 Trial in Japan (the TAKT Study). *Ann Rheumatol Dis* (2018) 77(3):348–54. doi: 10.1136/annrheumdis-2017-211878
21. Ruddy MJ, Wong GC, Liu XK, Yamamoto H, Kasayama S, Kirkwood KL, et al. Functional Cooperation Between Interleukin-17 and Tumor Necrosis Factor-Alpha is Mediated by CCAAT/Enhancer-Binding Protein Family Members. *J Biol Chem* (2004) 279(4):2559–67. doi: 10.1074/jbc.M308809200

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Inhibiting TLR7 Expression in the Retinal Pigment Epithelium Suppresses Experimental Autoimmune Uveitis

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Experimental autoimmune uveitis (EAU), a model of human uveitis, is an organ-specific, T cell-mediated autoimmune disease. Autoreactive T cells can penetrate the blood-retinal barrier, which is a physical defense composed of tight junction-linked retinal pigment epithelial (RPE) cells. RPE cells serve as antigen-presenting cells (APCs) in the eye since they express MHC class I and II and Toll-like receptors (TLRs). Although previous studies have shown that supplementation with TLR agonists exacerbates uveitis, little is known about how TLR signaling in the RPE contributes to the development of uveitis. In this study, we isolated the RPE from EAU mice, which were induced by active immunization (aEAU) or adoptive transfer of antigen-specific T cells (tEAU). The expression of TLRs on RPE was determined, and both aEAU and tEAU mice exhibited induced *tlr7* expression. The TLR7 agonist R848 was shown to induce aggressive disease progression, along with significantly elevated levels of the uveopathogenic cytokine IL-17. Furthermore, not only IL-17 but also R848 appeared to enhance the inflammatory response and to impair the barrier function of the RPE, indicating that TLR7 signaling is involved in the pathogenesis of EAU by affecting the behaviors of the RPE and consequently allowing the infiltration of autoreactive T cells intraocularly. Finally, local application of shRNA against TLR7 delivered by recombinant AAV effectively inhibited disease severity and reduced IFN-γ and IL-17. Our findings highlight an immunomodulatory role of RPE TLR7 in EAU development and provide a potential therapeutic strategy for autoimmune uveitis.

Keywords: autoimmune uveitis, barrier function, EAU, IL-17, RPE, TLR7

INTRODUCTION

The blood-retinal barrier (BRB) is a physical barrier composed of a layer of tight junction-linked retinal pigment epithelial (RPE) cells that provides an impermeable shield to prevent cells and molecules from entering the tissue of the retina (1, 2). The BRB separates the inner side of the eyes from the blood circulation and contributes to maintaining immune privilege in the ocular environment. Immune privilege involves tolerance to the introduction of antigens and does not to elicit an unexpected immune response (3). Impairment of the BRB might cause the influx of immune cells; thus, these cells might recognize rare self-antigens and trigger autoreactivity in the eye (4, 5). Uveitis is one of the most common autoimmune diseases associated with ocular blindness and is responsible for 5-25% of legal blindness in the population (6). Patients with autoimmune uveitis tend to have strong major histocompatibility complex (MHC) associations and exhibit ocular infiltration of autoreactive T cells in response to retinal antigens such as interphotoreceptor retinoid-binding protein (IRBP), retinal arrestin and recoverin (7). Evidence suggests that the activation of autoreactive T cells specific to IRBP is cross-reactive with the gut microbiota (8). Corticosteroids are still the major therapeutic agent used to treat uveitis (9), although immunosuppressants (such as cyclosporin A, azathioprine and rapamycin) (10) and biologics (such as anti-TNFα therapy) (11) are also used as adjunctive therapies.

Experimental autoimmune uveitis (EAU) is an animal model of human uveitis. The actively induced EAU (aEAU) model is the most common animal model, in which C57BL/6 mice are actively immunized with the uveitogenic antigen peptide $IRBP_{1\text{-}20}$ emulsified with complete Freud's adjuvant (CFA) by subcutaneous injection in combination with intraperitoneal injection of pertussis toxin (PTX) to break the BRB (12). Autoimmune uveitis can also be induced by the adoptive transfer of autoreactive $CD4^+$ T cells (13). In an adoptively transferred EAU (tEAU) model (14), the use of T cell inhibitors, including cyclosporine A and FK506 (15, 16), verified the essential role of autoreactive T cells in the development of uveitis. Two major uveopathogenic T cell subgroups, Th1 and Th17, are involved and exert different impacts on disease progression (17). In the early stage, the Th1-derived cytokine IFN-γ was thought to be the major uveopathogenic cytokine in naïve recipient animal models (18). In contrast, the Th17 response was shown to participate in the late phase of EAU (19). IL-17-secreting T cells exhibit a strong antigen-specific response (20), and anti-IL-17 antibody treatment attenuates EAU development (21).

In addition to uveopathogenic cytokines, Toll-like receptors (TLRs) might also be involved in uveitis development. Subretinal delivery of TLR agonists induced the ocular infiltration of leukocytes and triggered uveitis in mice (22), and subcutaneous injection of several TLR agonists exacerbated aEAU disease severity (23). Moreover, mice deficient in myeloid differentiation primary response gene 88 (Myd88), which is a downstream signal of TLRs, were completely resistant to the induction of aEAU (24). In other autoimmune diseases, Myd88 overexpression in dendritic cells (DCs) could result in systemic lupus erythematosus (SLE)-like disease (25). Stimulation of TLR2 and TLR4 could enhance the inflammatory response in rheumatoid arthritis (RA) (26), and the expression of TLRs was found to be associated with the incidence of inflammatory bowel disease (IBD) and the pathogenesis of Behcet's disease (27, 28). TLRs are mainly expressed by antigen-presenting cells (APCs), which bridge innate and adaptive immunity (29). In the ocular environment, RPE cells serve as APCs, express MHC class I and II (30), and induce inflammatory cytokines such as IL-6, IL-8 and MCP-1 (31) through TLR signaling. In the current study, we sought to test the hypothesis that specific TLR signaling in RPE cells is able to regulate uveopathogenic inflammation in uveitis. The induced expression of TLR7 on RPE cells from EAU mice was observed, and its role in disease development was investigated by using agonists or shRNA-mediated inhibition. Additionally, we elucidated the effects of the TLR7 agonist R848 and uveopathogenic cytokine IL-17 on the production of the inflammatory cytokine IL-6 and the impairment of barrier function in RPE cells.

MATERIALS AND METHODS

Animals

Wild-type C57BL/6 (B6) mice (6-7 weeks old) were obtained from the National Laboratory Animal Center, Taiwan, and kept in the animal facilities at Chang Gung University. Animal care and experiments were approved by and performed in accordance with the Institutional Animal Care and Use Committee of Chang Gung University, Taiwan.

Actively Induced Experimental Autoimmune Uveitis in Mice

According to a previous study (12), the aEAU animal model was established on the day 0 by subcutaneous immunization of an emulsion containing 100 μl of human $IRBP_{1\text{-}20}$ peptide (2 μg/μl) in 100 μl of CFA (Sigma-Aldrich), distributed over 4 spots on the tail base to flank. A single dose (100 ng) of intraperitoneal injection of PTX was used as a costimulatory adjuvant. Age- and sex-matched B6 mice that did not undergo immunization were used as disease-free controls. aEAU mice were sacrificed on the 12th or 28th day after disease induction. The eyes and spleen were harvested, and TLR expression and immune responses were analyzed.

Adoptively Transferred Experimental Autoimmune Uveitis

Nylon wool-purified splenic T cells were isolated from day-12 aEAU mice and cultured in the presence of $IRBP_{1\text{-}20}$ (10 μg/mL) for 2 days (14). The tEAU animal model was then established by intraperitoneal injection of IRBP-specific T cells (5×10^6/mouse) into naïve 6-week-old B6 mice.

Primary RPE Cell Isolation

The method was performed according to previous studies with modifications (32–34). After enucleation of infant mice (10-14 days old) or adult EAU mice, the eyeballs were cut into halves

along the circumferential line to create a ciliary body-free posterior eyecup. The retinal layer was removed from the eyecup, which was pretreated with trypsin, and RPE tissue was scraped (**Supplementary Figure 1**) and minced into a single-cell suspension in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific) containing 10% (v/v) fetal bovine serum (FBS) (Gibco).

Cell Culture and Culture Reagents

Primary RPE cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). Splenocytes and splenic T cells were cultured in minimum essential medium α (α-MEM) (Gibco) containing 10% FBS, 55 nM β-mercaptoethanol (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin. ID8 cells were maintained in DMEM with 3% (v/v) FBS and 1× ITS (Sigma-Aldrich, Merck) containing 5 μg/mL insulin and transferrin and 5 ng/mL sodium selenite. The $IRBP_{1-20}$ (GPTHLFQPSLVLDMAKVLLD) peptide was synthesized by the Peptide Synthesis Core Facility of Academia Sinica, Taiwan.

Gene and Protein Expression Analysis in RPE Cells

Total RNA was extracted from cultured primary RPE cells or RPE cells that were freshly isolated from EAU mice by the TRIzol (Invitrogen, Thermo Fisher Scientific) method. Reverse transcription and real-time quantitative PCR were performed by an iScript cDNA Synthesis Kit and iQ SYBR Green Supermix with a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories). A total of 10 targets were analyzed, and the primer sets used were published elsewhere (35–37). The production of the proinflammatory cytokine IL-6 by RPE cells was analyzed by a Ready-Set-Go! ELISA kit (eBioscience, Thermo Fisher Scientific). Specific signaling protein levels were identified by MILLIPLEX® multiplex assays for Luminex (Millipore, Merck) according to the manufacturer's instructions.

Electric Cell-Substrate Impedance Sensing (ECIS) Analysis

Primary RPE cells were seeded at a density of 7×10^4 cells/well in ECIS 8W10E culture dishes. When the cell confluence reached 100% (≃16 hours), recombinant mouse IL-17 (PeproTech) or R848 was added to the RPE cultures. The level of impedance at low frequency (400 Hz) was monitored by an ECIS system (Applied BioPhysics), and the effects on barrier function during the entire culture period were determined according to the manufacturer's instructions.

Generation of Recombinant Adeno-Associated Viral (AAV) Vectors and rAAV-TLR7-shRNA

TLR7-specific shRNA (5'-GCCCTTTACCTGGATGGAAAC-3') and scramble shRNA were subcloned into the pAAV-IRES-GFP plasmid (Stratagene) AAV vector and were named pAAV-shTLR7 and pAAV-scramble, respectively (**Supplementary Figure 2**). Recombinant AAV-8 virus production was performed with the AAV helper system by the Institute of Biomedical Sciences of Academia Sinica, Taiwan. Titers of rAAV-TLR7-shRNA (rAAV.shTLR7) and rAAV-scramble-shRNA (rAAV.sc) were determined by RT-qPCR analysis for green florescence protein (GFP) by calculating the viral genome copy number.

Subretinal Treatment With R848 and rAAV-TLR7-shRNA

The TLR7 agonist R848 (4 μg/mouse) (Resiquimod; InvivoGen) and rAAV-TLR7-shRNA (4×10^{10} vg/mouse) were subretinally injected into both eyes of tEAU mice on day 0. tEAU mice treated with balanced salt solution (BSS) or rAAV-scramble shRNA served as controls. After 12 days of treatment, clinical disease severity was examined by fundoscopy as described previously (38, 39). Treated tEAU mice were sacrificed on the 28th day after disease induction. The eyes and spleen were harvested, and gene expression, histopathology and the immune response were analyzed *in vitro*.

Disease Severity Scoring by Fundoscopy

From the 5th day to 28th day after disease induction, disease severity scoring was performed by fundoscopy every 3 weekdays; the final evaluation was conducted on the 28th day. After anesthetizing and dilating the pupil with proparacaine 0.5% and tropicamide 1% (ALCON), the clinical score of uveitis in each eye was graded based on the degree of cell infiltration as published elsewhere (38, 39). The severity score was graded from 0 to 4 as follows: 0, no change; 0.5 to 3, few focal lesions to a pattern of linear lesions, increasing vasculitis, neovascularization, retinal hemorrhage, and papilledema; and 4, large retinal detachment and retinal atrophy.

Histopathology

The eyeballs were collected and fixed in 3.7% formaldehyde on the 28th day after tEAU induction. The fixed tissues were embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich).

Immune Cell Phenotyping

Splenocytes were obtained from untreated tEAU mice, and samples containing 10^5 cells were labeled with anti-CD4-PerCP (BD Biosciences) and anti-CD25-APC (eBioscience) to examine surface marker expression. Intracellular Foxp3 analysis was performed using anti-Foxp3-PE (eBioscience) and the Foxp3/transcription factor staining buffer set (eBioscience) according to the manufacturer's instructions. Flow cytometric analysis was performed by an Attune NxT flow cytometer (Thermo Fisher Scientific), and the data were analyzed by FlowJo software (Clever.ly, BD Biosciences).

IRBP-Specific Immune Responses

Splenocytes were isolated from the treated and untreated tEAU mice and cultured in the presence of $IRBP_{1-20}$ (10 μg/mL) at a density of 4×10^6 cells/well in 24-well plates for 4 days. The levels of

the cytokines IFN-γ, IL-17, IL-10 and IL-6 were analyzed using Ready-Set-Go! ELISA kits (eBioscience, Thermo Fisher Scientific).

Statistical Analysis

Nonparametric analyses, including the Mann-Whitney U test or Wilcoxon signed rank test, were performed by statistical tools in GraphPad Prism software. A statistically significant difference was defined as one with a p-value less than 0.05.

RESULTS

TLR7 Expression Was Significantly Upregulated in Uveitis

TLRs seem to be expressed on RPE cells, so we hypothesized that TLR signaling is associated with the development of uveitis. First, primary RPE cells were freshly isolated from EAU mice, and TLR expression was analyzed. To ensure the purity of RPE cells, the expression of RPE65 was examined in freshly obtained RPE cells. The real-time RT-PCR results show that *rpe65* expression was significantly increased in freshly isolated RPE cells compared with the retina, which is the tissue adjacent to the retinal pigment epithelium (**Figure 1A**). The presence of RPE65 in these RPE cells was also confirmed by immunofluorescence analysis (**Figure 1B**).

Next, we examined which TLRs were involved in the development of EAU, and the expression levels of different TLRs were analyzed in RPE cells from EAU mice. Primary RPE cells were obtained from aEAU and tEAU animals and analyzed. **Figure 1C** shows significantly increased levels of *tlr7* expression in both aEAU and tEAU mice. However, RPE cells from only aEAU animals exhibited upregulated *tlr4* expression levels, indicating that the adjuvant in the aEAU induction protocol induced *tlr4* expression. This result suggests that the induced expression of *tlr7* but not *tlr4* contributes directly to the induction or development of EAU.

The TLR7 Agonist R848 Exacerbated Disease Severity in EAU Mice

To clarify whether TLR7 contributes to the development of autoimmune uveitis, the TLR7 agonist R848 was used to

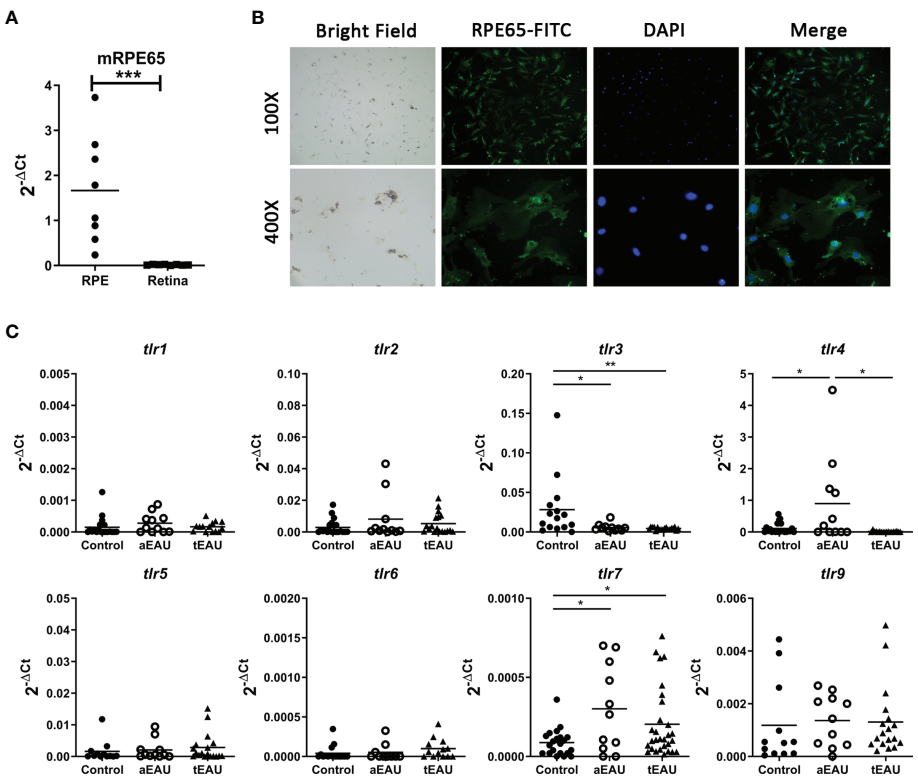


FIGURE 1 | Significantly increased levels of tlr7 expression were found in freshly isolated RPE cells from aEAU and tEAU mice. **(A)** Primary RPE cells were isolated from adult C57BL/6 mice, RNA was extracted, and the expression of the RPE-specific molecule RPE65 was analyzed by RT-qPCR. Retinal layers, which are adjacent tissues in the retinal pigment epithelium, served as negative controls. **(B)** Primary RPE cells were isolated from young C57BL/6 mice (10-14 days old) and cultured to amplify their numbers until further use. The protein level of RPE65 on the cell surface was assessed by immunofluorescence with FITC-conjugated anti-RPE65, and the nuclei of cells were stained with DAPI. The results are displayed at magnifications of 100× and 400×. **(C)** C57BL/6 mice were induced to develop aEAU and tEAU by active immunization or adoptive transfer of IRBP-specific T cells, respectively. On the day of sacrifice, primary RPE cells were obtained from aEAU mice and tEAU mice, and RPE cells from healthy mice served as controls. RNA was purified and analyzed by RT-qPCR to quantify the expression levels of different TLRs (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

identify the effects of TLR7 on a mouse model of tEAU. On the day of tEAU induction, 6-week-old B6 mice received an intraperitoneal injection of IRBP-specific T cells (5×10^6/mouse) and subretinal injection of R848 (4 μg/mouse). Healthy C57BL/6 mice without EAU induction were administered a subretinal injection of R848 and served as disease-free controls (data not shown). As shown in **Figure 2**, tEAU mice treated subretinally with R848, exhibited significantly higher *tlr7* gene expression in RPE cells than tEAU mice that received BSS treatment (**Figure 2A**). **Figure 2B** shows the disease severity curves of the mice receiving R848 or BSS as controls, and the severity score was determined by fundoscopy during the period of disease induction and development (38, 39). In tEAU mice that received R848 treatment, disease peaked at days 8-13 after induction, and severe disease lasted until the day of sacrifice. In contrast, in the absence of R848 treatment, disease peaked on day 19 in mice that received BSS treatment. The peak score in R848-treated mice was approximately 30% higher than that in BSS-treated control mice. **Figure 2C** summarizes the disease severity distribution in tEAU animals. Overall, 37.5% of BSS-treated tEAU mice developed mild disease (score ≤ 1), while the percentage was 5.8% in the R848-treated group. Moreover, 17.6% of R848-treated mice exhibited very severe disease (score=4). Fundoscopy (**Figure 2D**) and histological analysis (**Figure 2E**) showed many more infiltrated cells in the ocular fundus, along with neovascularization, disordered structures and significant morphological changes, in the R848-treated group, indicating that R848 exacerbated ocular inflammatory cell infiltration and impaired retinal tissue structure.

Treatment with R848 exacerbated ocular inflammation. To examine whether R848 affected antigen-specific immunity, the production of pathogenic cytokines associated with uveitis, IFN-γ and IL-17, and IL-6 by splenocytes in response to IRBP in R848-treated tEAU mice were compared with those from BSS-treated tEAU mice. The levels of the cytokines IFN-γ, IL-17 and IL-6 were relatively increased in R848-treated tEAU mice (**Figure 3A**). The IRBP-specific IL-17 level was significantly elevated. In addition, the gene expression of *tgf-β* was upregulated in RPE cells from the tEAU+R848 group compared to the BSS-treated group (**Figure 3B**). To examine the direct effects of R848 on RPE cells, we treated primary RPE cells with different doses of R848 and analyzed the production of IL-6, which is a proinflammatory cytokine that is produced in response to specific pathogen-associated molecular patterns (PAMPs). R848 (30 and 300 ng/mL) could significantly induce RPE cell production of IL-6. Moreover, IL-6 production was significantly increased when RPE cells were stimulated with

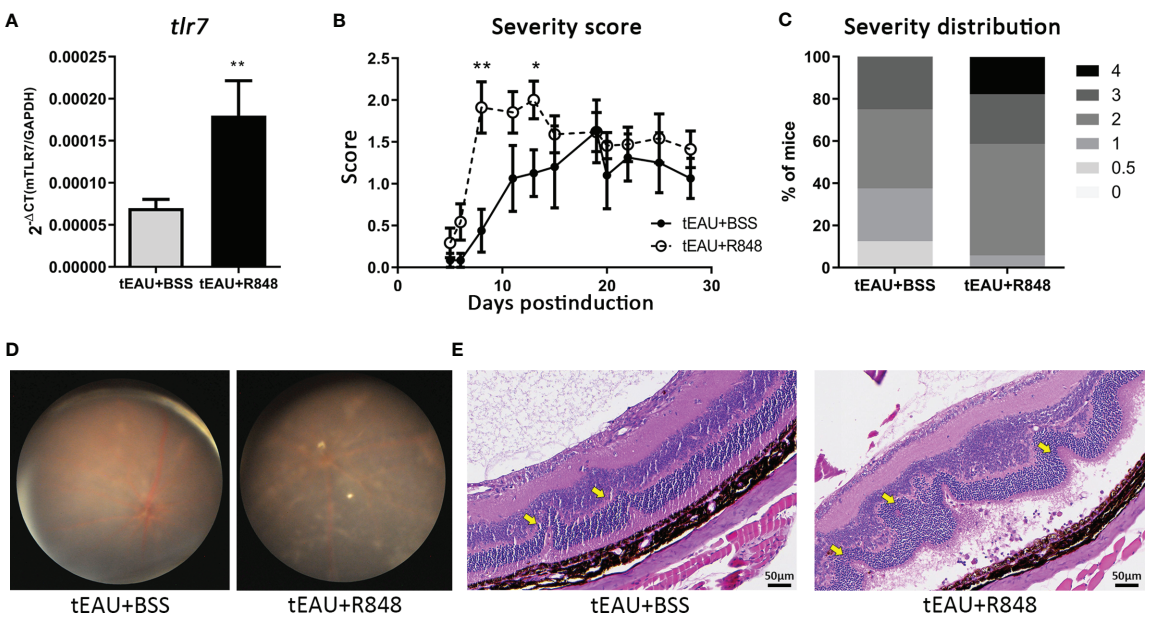


FIGURE 2 | Subretinal delivery of the TLR7 agonist R848 induced TLR7 expression and exacerbated disease severity in the tEAU model. C57BL/6 mice were adoptively transferred with IRBP-specific T cells obtained from aEAU mice to induce tEAU. On the same day, tEAU mice were subretinally injected with the TLR7 agonist R848 (2 μg/eye) (tEAU+R848, n=17). The mice that received injections of BSS served as controls (tEAU+BSS, n=8). The eyes of these mice were monitored by fundoscopy and scored regularly. On the day of sacrifice, splenocytes and one eye from each mouse were harvested for autoimmunity and histological analysis. **(A)** Primary RPE cells were obtained from the other eye from ach tEAU mouse, and tlr7 gene expression in these RPE cells was determined by RT-qPCR. **(B)** Disease progression was monitored as scheduled, and the recorded disease score is shown as the mean ± SD. (*$p < 0.05$; and **$p < 0.01$). **(C)** The severity distribution of disease on day 28 before sacrifice was summarized among tEAU mice treated with or without R848. **(D)** Representative images showing fundoscopic analysis of tEAU mice treated with or without R848 on day 28 before sacrifice. **(E)** The eyes from the two groups of tEAU mice were fixed in 3.7% formaldehyde, sectioned and stained with H&E for ocular histopathological analysis (magnification: 200×).

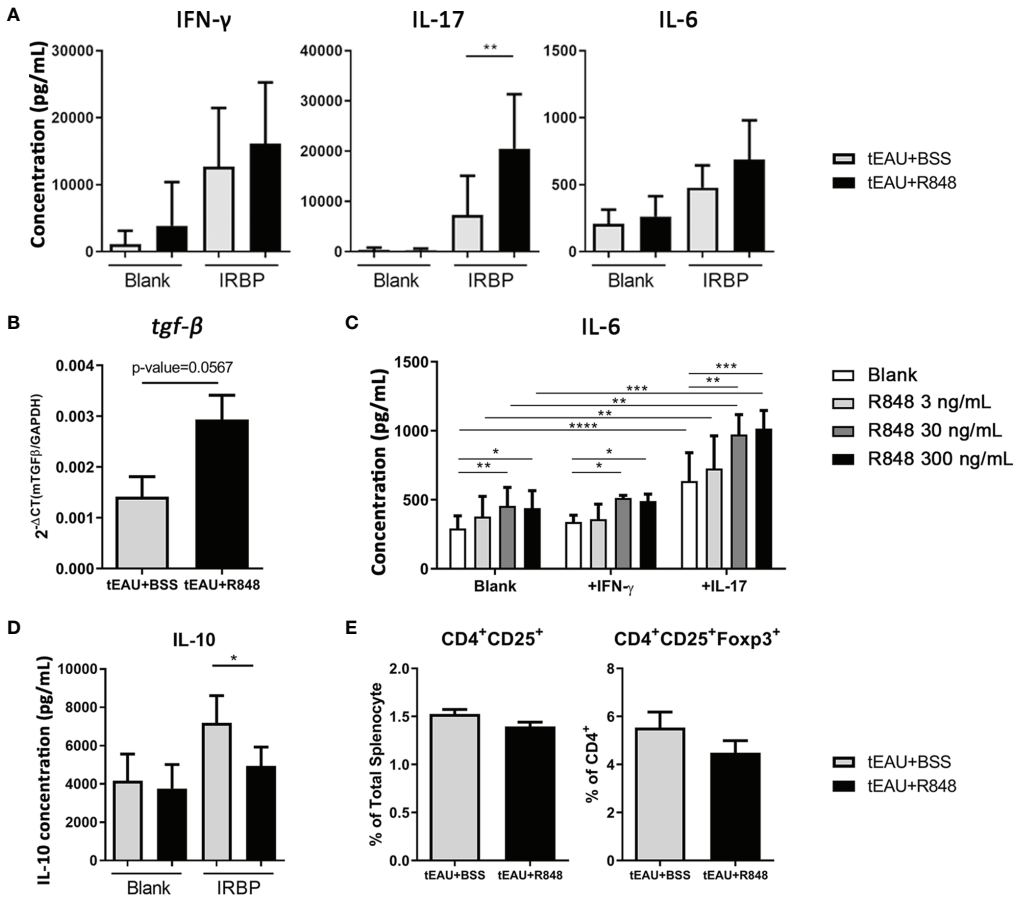


FIGURE 3 | R848 enhanced the production of pathogenic and inflammatory cytokines. Splenocytes and RPE cells were freshly obtained from R848-treated tEAU mice (tEAU+R848, n=17) and control animals (tEAU+BSS, n=8) on the day of sacrifice. **(A)** Splenocytes were cultured with 10 μg/mL IRBP for 96 hours, and the levels of IFN-γ, IL-17 and IL-6 in the supernatant were measured by ELISA. **(B)** RNA was purified from primary RPE cells, and tgf-β gene expression was quantified by RT-qPCR. **(C)** Primary RPE cells were cultured and stimulated with a TLR7 agonist (R848) in the presence or absence of the uveitis-associated cytokines IFN-γ and IL-17. The culture supernatants were collected at 24 hours, and the levels of the proinflammatory cytokine IL-6 were measured by ELISA. **(D)** Splenocytes were cultured in to the presence of 10 μg/mL IRBP for 96 hours, and the levels of IL-10 in the culture supernatants were determined by ELISA. **(E)** The presence of Tregs was assessed by flow cytometric analysis of $CD4^+CD25^+$ or $CD4^+CD25^+FoxP3^+$ T cells. These data are representative of at least three independent experiments and presented as the mean ± SD (*p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001).

both R848 and recombinant IL-17, revealing a synergistic effect (**Figure 3C**). In contrast, treatment with the TLR7 antagonist hydroxychloroquine sulfate (HCQ) (4000 ng/mL) significantly reduced IL-6 production. Although the addition of IL-17 enhanced IL-6 levels, it failed to restore the suppressive effect of HCQ on IL-6 production (**Supplementary Figure 3**).

R848 Impaired Barrier Function in Primary RPE Cells

Because IL-17 can impair the expression of tight junction proteins in ARPE-19 cells (40), we next determined whether IL-17 combined with TLR7 signaling to affect the barrier function of RPE cells. Impedance was measured to quantify the integrity of the epithelial barrier by ECIS analysis. **Figure 4A** shows an instant decrease in impedance in primary RPE cells after treatment with recombinant IL-17, indicating an impairment of barrier function. Statistical analysis showed a significant difference between the IL-17-treated and untreated groups, with an approximately 10% decrease from 6 to 24 hours after treatment (**Figure 4B**). Likewise, R848 treatment also resulted in dysfunction of the epithelial barrier in primary RPE cells, and **Figure 4C** shows that R848 induced a dose-dependent reduction from 3 ng/mL to 30 ng/mL. Treatment with 30 ng/mL R848 reduced the impedance to 90% (**Figure 4D**). Moreover, under the stimulation of IL-17 or R848, the expression of tight junction-associated genes was down-regulated. In particular, treatment of IL-17 significantly reduced the expression of *claudin1* (**Figure 4E**). These results indicated that the TLR7

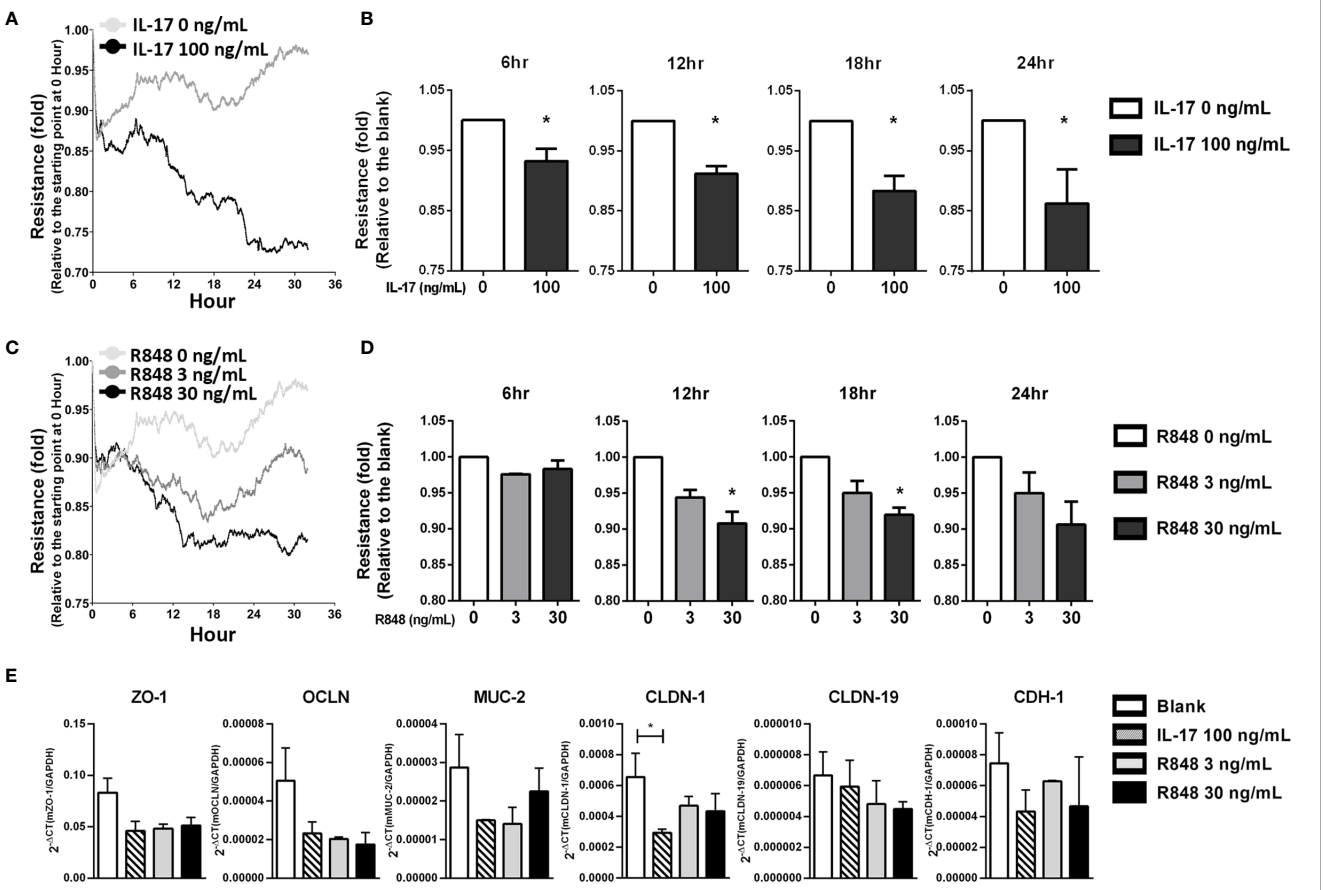


FIGURE 4 | R848 plus IL-17 significantly impaired the barrier function of primary RPE cells *in vitro*. Primary RPE cells were isolated from young C57BL/6 mice (10-14 days old), expanded and cultured in ECIS 8W10E culture plates for functional analyses. **(A)** To determine the effects of IL-17 on barrier function in RPE cells, 0 or 100 ng/mL IL-17 was added to cultures containing 100% confluent RPE cells, and barrier function was examined by ECIS. The line graph shows the representative results. The X-axis indicates the time after stimulation with IL-17. The Y-axis indicates the fold change in the resistance level at each time point relative to 0 hours. **(C)** The ECIS assays were performed to identify the effects of R848, 0, 3 or 30 ng/mL R848 was added to the cultures, and ECIS analysis was performed according to the schedule. **(B, D)** The data in the bar chart were normalized to each corresponding blank (untreated) group. The data shown are representative of at least three experiments and presented as the mean ± SD (*$p < 0.05$). **(E)** Primary RPE cells were cultured and stimulated with IL-17 (100 ng/mL) or R848 (3 or 30 ng/mL) for 6 hours, RNA was isolated and the tight junction-associated gene expressions were examined by RT-qPCR. These data are representative of at least two independent experiments and presented as the mean ± SD. (*$p < 0.05$).

agonist R848 exacerbated disease by increasing inflammatory cytokine production and impairing barrier function. This result suggested that the increase in *tlr7* gene expression was associated with the exacerbation of uveitis.

Subretinal Delivery of rAAV.shTLR7 Ameliorated Uveitis Severity

RNA interference is widely used to manipulate gene expression and is considered a powerful therapeutic tool. To investigate whether *tlr7* gene expression is directly associates with the induction of uveitis and serves as a potential therapeutic target, a recombinant AAV (rAAV) delivering an shRNA against TLR7 was used. Before performing the treatment, we first identified the appropriate serotype of rAAV with robust efficiency in primary RPE cells. rAAV-8 had the highest infection rate according to GFP expression, followed by rAAV-9 (**Figure 5A**). Next, rAAV.shTLR7 was constructed, and its inhibitory effect on TLR7 expression was examined. **Figure 5B** shows that infection with rAAV.shTLR7 dose- and time-dependently inhibited *tlr7* expression in primary RPE cells at a viral load of 2 or 5×10^{10} vg/mL at 24 or 48 hours *in vitro*. To assess the ability of rAAV.shTLR7 to downregulate TLR7 levels, RPE cells were isolated from tEAU mice that received rAAV.shTLR7, and TLR7 expression was compared with that in rAAV.sc-treated tEAU animals. The mice treated with rAAV.shTLR7 showed lower *tlr7* gene expression in the RPE layers than rAAV.sc-treated mice (**Figure 5C**).

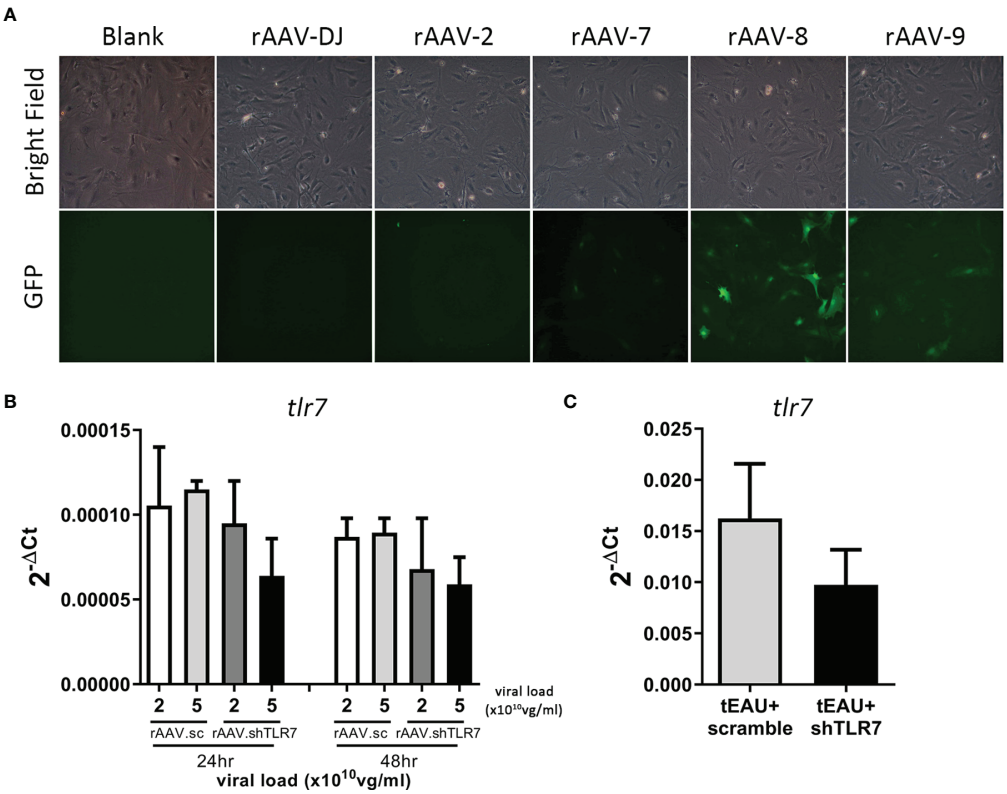


FIGURE 5 | rAAV.shTLR7 inhibited tlr7 gene expression in primary RPE cells *in vivo* and *in vitro*. **(A)** Primary RPE cells were infected with different serotypes of rAAV-GFP (1×10^{11} vg/mL) (rAAV-DJ, rAAV-2, rAAV-7, rAAV-8, or rAAV-9), and GFP expression levels were determined to assess the infection efficiency of rAAVs by fluorescence microscopy. **(B)** Primary RPE cells were infected with 2 or 5 $\times10^{10}$ vg/mL rAAV.sc or rAAV.shTLR7 for 24-48 hours, RNA was isolated, and tlr7 gene expression was determined by RT-qPCR. The data from two representative experiments are shown and presented as the mean ± SD. **(C)** C57BL/6 mice were adoptively transferred with IRBP-specific T cells, which were obtained from aEAU, mice to induce tEAU disease. On the same day, tEAU mice were subretinally injected with rAAV.sc or rAAV.shTLR7. On the day of sacrifice, the primary RPE layer was freshly obtained from the animals, and tlr7 gene expression was examined by RT-qPCR.

To evaluate whether rAAV.shTLR7 could treat autoimmune uveitis, on the day of tEAU induction, the mice were treated with rAAV (4×10^{10} vg/mouse) by subretinal injection, and the severity score was monitored during disease development (**Figure 6A**). After the incidence of the highest score between these two groups was analyzed, an almost 25% reduction in the disease score in mice treated with rAAV.shTLR7 compared with mice treated with rAAV.sc was observed (**Figure 6B**). No mice with severe disease (score≥3) were observed in the rAAV.shTLR7-treated group, while 40% were present in the rAAV.sc-treated group (**Figure 6B**). Histological analysis and fundoscopy examination also revealed disease lesions with improved pathological morphology and lower cell infiltration in ocular after being treated with rAAV.shTLR7 (**Figures 6C, D**). In addition, compared with mice in the rAAV.sc group, mice treated with rAAV.shTLR7 exhibited reduced production of the pathogenic uveitis cytokines IFN-γ and IL-17 by splenocytes in response to IRBP (**Figure 6E**), although no significant difference was found in the production of IL-10 (**Figure 6F**) or Treg frequency (**Figure 6G**). Similar results were found in aEAU animal model. The aEAU mice treated with rAAV.shTLR7 showed lower *tlr7* gene expression in the RPE layers and disease severity (**Supplementary Figures 4A, B**). Histological analysis and fundoscopy examination indicated improved pathology after being treated with rAAV.shTLR7 (**Supplementary Figures 4C, D**). Reduction of pathogenic cytokines IFN-γ and IL-17 were observed in splenocytes in response to IRBP in rAAV.shTLR7 group (**Supplementary Figure 4E**), No significant difference in Treg frequency and/or IRBP-specific Treg response was found in these groups of mice (data not shown). Collectively, the above results in aEAU and tEAU models suggested that TLR7-mediated responses were involved in the underlying mechanism contributing to inflammation and disease development.

DISCUSSION

In this study, we revealed the significance of TLR7 signaling in the uveopathogenic inflammatory response and maintaining the barrier function of RPE cells. First, we demonstrated different

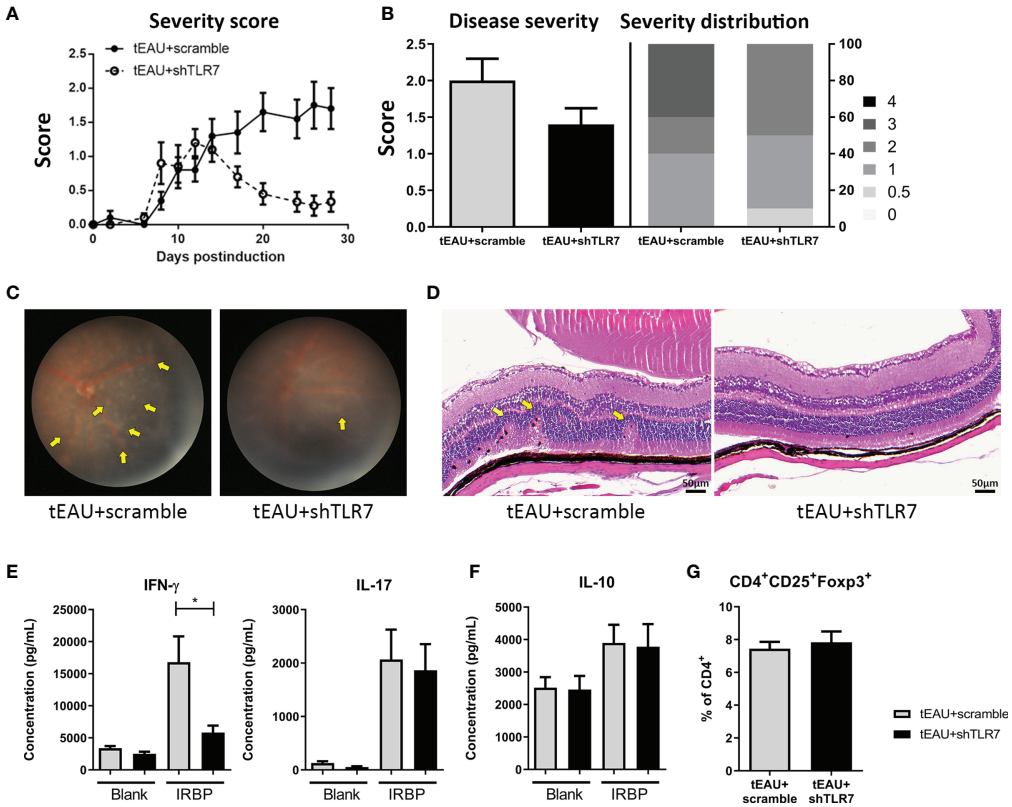


FIGURE 6 | Subretinal delivery of rAAV.shTLR7 reduced disease severity in the tEAU model. C57BL/6 mice were adoptively transferred with IRBP-specific T cells obtained from aEAU mice to induce tEAU. On the same day, tEAU mice were subretinally injected with rAAV.shTLR7 (tEAU+shTLR7, n=10). The mice that received injections of rAAV.sc served as controls (tEAU+scramble, n=10). The eyes of these mice were monitored and scored regularly by fundoscopy. On the day of sacrifice, splenocytes and one eye from each mouse were harvested for autoimmunity and histological analyses. **(A)** Eyes of EAU mice were monitored by fundoscopy and scored regularly. Disease severity was monitored and the recorded disease score is shown as the mean ± SEM. **(B)** The left panel shows the disease severity, which indicates the most severe scores of individual mice during disease progression, and the right panel shows the disease distribution on day 28 among the tEAU mice treated with rAAV.shTLR7 or rAAV.sc. **(C)** The representative images showing fundoscopic analysis of tEAU mice treated with rAAV.sc (left) or rAAV.shTLR7 (right) on day 28 before sacrifice. **(D)** The eyes of tEAU mice in the two groups were fixed in 3.7% formaldehyde, sectioned and stained with H&E for ocular histopathological analysis (magnification: 200×). **(E)** Splenocytes were cultured in to the presence of 10 μg/mL IRBP for 96 hours, and the levels of the pathogenic cytokines IFN-γ and IL-17 and IL-6 in the supernatant were measured by ELISA. **(F)** The levels of IL-10 in the splenocyte culture supernatant with or without IRBP (10 μg/mL) were measured by ELISA. **(G)** Splenocytes were freshly isolated, and the phenotypes of Tregs ($CD4^+CD25^+Foxp3^+$) were analyzed by flow cytometry. The data are presented as the mean ± SD. (*$p < 0.05$).

patterns of *tlr* expression in aEAU and tEAU. To avoid the effects of adjuvants that serve as TLR ligands, the tEAU model was used throughout the study. Compared with that in healthy controls, *tlr4* expression was elevated only in aEAU mice but not in tEAU mice (**Figure 1C**), indicating that the induction of *tlr4* expression might result from the presence of adjuvants during the induction of aEAU. It was indeed reported that MTB, a major component of CFA, could trigger an immune response through TLR2, TLR4 and TLR9 (24, 41); moreover, PTX appeared to not only enhance the Th1 response but also stimulate TLR4 in DCs (42, 43).

Significantly increased levels of *tlr7* and decreased *tlr3* expression were found in the aEAU and tEAU groups (**Figure 1C**). It is well known that APCs stimulated with immunogenic DAMPs might cause immune-mediated inflammatory diseases (IMIDs) (44), including autoimmune diseases (45, 46). The epicutaneous application of imiquimod, a TLR7 agonist, was able to induce acute cutaneous inflammation, and plasmacytoid dendritic cells (pDCs) acted as primary sensors. The inflammatory reaction was initiated by pDCs and showed a deviation toward the IL-23/TH17 axis, the typical molecular signature of human psoriasis (47). Moreover, endogenous nucleic acids have been shown to activate DCs and autoreactive B cells *via* TLR7 signaling, resulting in the development of SLE (48, 49). For TLR7-mediated T cell responses, activation of TLR7 signaling appeared to inhibit the differentiation of Th1 and Th17 cells and thus ameliorated the development and disease severity in the experimental autoimmune encephalomyelitis (EAE) animal model (50).

In addition, single-stranded RNA (ssRNA) is the natural ligand of TLR7, and worldwide pandemic diseases such as flu and COVID-19 are all caused by RNA viruses (51, 52). S Diebold et al. showed that innate responses to ssRNA, such as that of influenza virus, were critically dependent on TLR7, and this response required endosomal recognition of influenza genomic RNA, which then induced TLR7-dependent production of inflammatory cytokines (51). In a recent clinical trial, the combination of adjuvants containing TLR7/8 agonists and inactive SARS-CoV-2 virus enhanced the IgG2a/IgG1 ratio and Th1-biased immunity mediated by antigen-specific IFN-γ-expressing helper T cells, which provided longer protective immunity (53, 54).

Regarding the role of TLR3 signaling, although Fang et al. demonstrated that subcutaneous injection of TLR2, 3, 4, and 9 agonists could exacerbate uveitis, knockout of these genes in mice except MyD88 still resulted in the development of uveitis with similar severity (23), indicating that MyD88 but not TLR2, 3, 4 or 9 is essential for the induction of EAU. In our study, although there appeared to be no elevated levels of *tlr3* in both EAU animal models *in vivo*, the basal level of *tlr3* was much higher than *tlr7* **Figure 1C**). In fact, S Chen et al. targeted *tlr3* and also demonstrated a successful therapeutic strategy in treating EAU by chitosan-loaded TLR3-siRNA, according to the result of the induced *tlr3* level in primary RPE cells by TLRs' agonist *in vitro* (55), suggesting the involvement of TLR3 in the development of EAU.

Accordingly, TLR7 expression appears to serve as a potential target for manipulating the inflammatory responses in EAU. In this study, local delivery of the TLR7 agonist to tEAU mice significantly enhanced disease severity, which was followed by elevated *tlr7* expression in RPE cells (**Figures 2A–C**). Additionally, in R848-treated tEAU mice, there appeared to be fewer $CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$ Tregs (**Figure 3E**) as well as reduced levels of the regulatory cytokine IL-10 produced by splenocytes in response to the autoantigen IRBP (**Figure 3D**), indicating the induction of regulatory responses in an antigen-specific manner. Although the expression of *tgf-β* was also increased in the R848-treated group (**Figure 3B**), this factor was considered to collaborate with IL-6, which was induced by R848 (**Figures 3A, C**), and consequently enhanced the production of IL-17 (56, 57). This result suggested that R848 could exacerbate Th17-mediated inflammation in EAU mice.

Previous studies have shown that IL-17 and other Th17 cytokines significantly impair the function of the epithelial barrier in the airway (58). In Sjogren's syndrome, IL-17 secreted by infiltrating lymphocytes disrupts the integrity of the tight junction barrier by downregulating claudin-4 and ZO-1 expression in submandibular glands (59). Moreover, IL-17 treatment resulted in the impairment of barrier function (**Figures 4A, B**), which was consistent with a previous study that showed that IL-17 profoundly disturbed the distribution of tight function proteins (TJPs) and consequently affected the barrier function of ARPE-19 cells (40). Similarly, R848 treatment affected barrier function in primary mouse RPE cells (**Figures 4C, D**), which may be a result of reduced TJP expression through an R848-induced IL-17 response (**Figure 4E**). Moreover, there appeared to be upregulation of Akt and NF-κB (**Supplementary Figure 5**), which are known to be positively associated with the loss of barrier function in epithelial cells (60). Additionally, R848 may have more effects than the production of immune-associated molecules and the impairment of barrier function of RPE cells. Indeed, the R848-induced responses in RPE cells also include an increase in ERK/CREB and the upregulation of STAT5 (**Supplementary Figure 5**). The former is known to positively correlate with reactive oxygen species (ROS)-mediated damage to RPE cells in age-related macular degeneration (AMD) (61), and the latter has been shown to contribute to the proliferation, differentiation and apoptosis of hematopoietic cells (62). These results suggested that several transcription factors involved in signaling pathways might be important in R848-induced responses in RPE cells and contribute to inflammatory responses in autoimmune uveitis.

Chloroquine, an antagonist of TLR7, has been used clinically to treat malaria infection since World War II (63). In fact, chloroquine is also commonly used to treat several autoimmune diseases, such as SLE, RA and Sjogren's syndrome (64–66). In our study, TLR7 antagonist HCQ appeared to reduce disease severity slightly in tEAU mice (data not shown). However, retinal toxicity and macular retinopathy are well-known side effects of the TLR7 antagonist HCQ (67), a derivative of chloroquine. This finding led us to adapt an RNAi strategy to reduce/interfere with TLR7 signaling *via* gene therapy combined with an rAAV delivery system (**Figure 5**). The results might not be very promising, but they indeed showed the possibility of ameliorating disease severity and suppressing autoreactive immune responses in both tEAU and aEAU (**Figure 6** and **Supplementary Figure 4**). It was also noted that TLR7 expression was extremely low not only in the retinal pigment epithelium but also in primary RPE cells (**Supplementary Figure 6**) when we attempted to validate the efficacy of pAAV-shTLR7 *in vitro*. In fact, to confirm the suppressive effect of pAAV-shTLR7, we further used the ID8 cell line, which was accidentally found to have increased *tlr7* expression, to assess the efficiency of *tlr7* inhibition.

In summary, our results elucidate the role of TLR7 signaling in EAU pathogenesis by affecting the characteristics of RPE and pathogenic T cells in uveitis. Pathogenic T cells might shape RPE cells and induce higher TLR7 expression through direct or indirect pathways by the stimulation of DAMPs that might have been produced by T cell-mediated tissue damage in uveitis. TLR7 signaling appears to induce increased IL-6 production by RPE cells and skew T cells toward more aggressive uveopathogenic phenotypes with more Th1 and Th17 cells and fewer Tregs. Moreover, TLR7 signaling also affects the barrier function of RPE cells, and such impaired BRB function in uveitis results in a positive feedback loop with increased pathogenic T cell infiltration in the ocular environment, leading to the exacerbation of uveitis severity (**Figure 7**). Therefore, TLR7 signaling changes RPE cells from

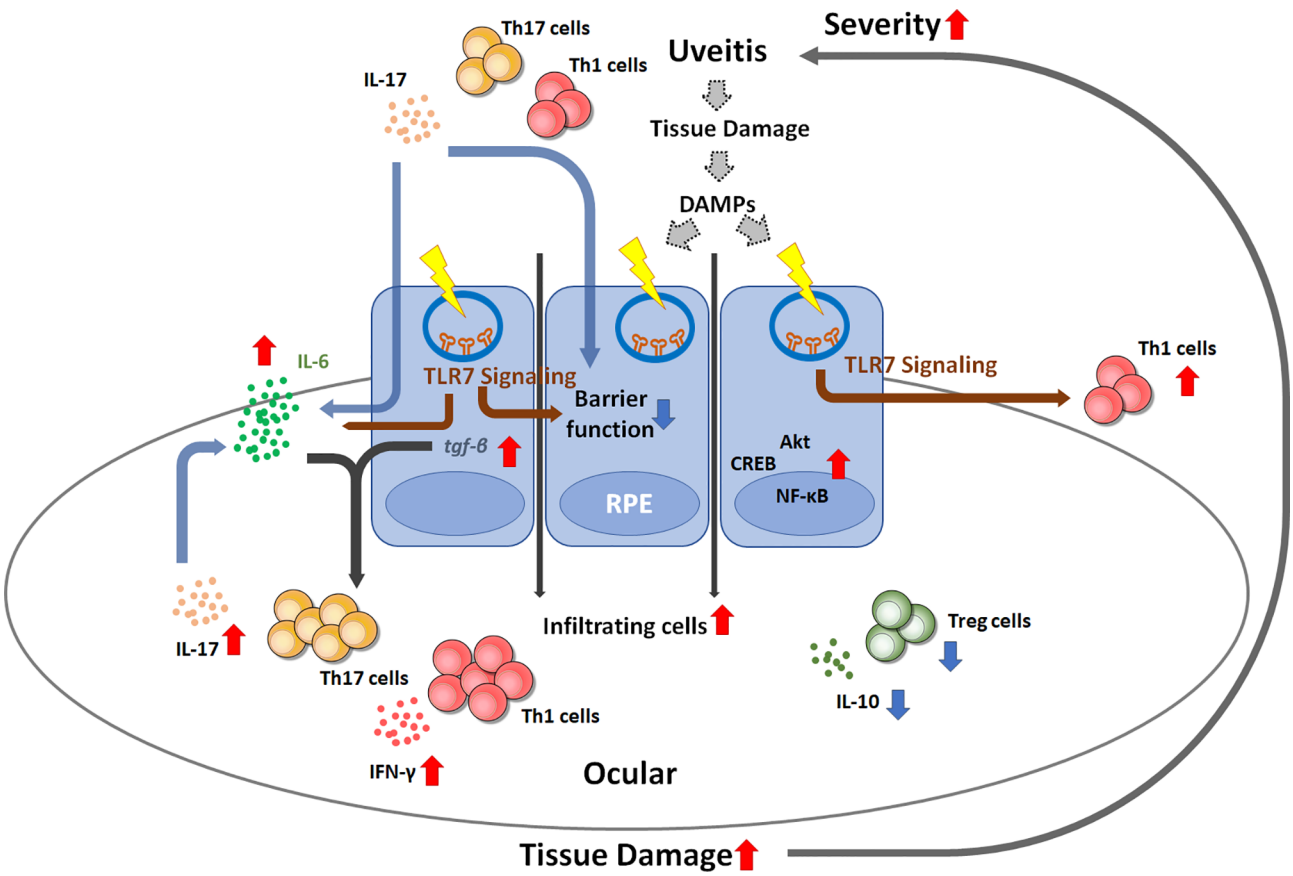


FIGURE 7 | Schematic diagram showing the possible mechanism by which TLR7 signaling contributes to uveitis disease progression. Once uveitis develops and progresses, autoreactive T cells can trigger TLR7 signaling in RPE cells by direct stimulation or DAMP-induced signaling. TLR7 signaling activation results in impaired barrier function, enhanced IL-6 production and inflammatory signaling activation, which consequently enhances Th1 and Th17 differentiation and reduces regulatory T cells. The resultant increase in IL-17 may impair the barrier function of RPE cells. These events can create a positive feedback loop and lead to the exacerbation of uveitis inflammation.

a protective cell type toward an inflammatory enhancer and impairs barrier function. These findings provide a possible mechanism that results in autoimmune uveitis and a potential therapeutic strategy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Chang Gung University, Taiwan.

AUTHOR CONTRIBUTIONS

S-ML and C-RS conceived and designed the experiments. S-ML, Y-SH, W-HH, W-CY, and M-HL performed the experiments. S-ML and C-RS analyzed the data. Y-SH, C-LL, C-NS, and C-RS contributed to the protocol, reagents, materials and analysis tools. S-ML and C-RS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.736261/full#supplementary-material

REFERENCES

1. Sugita S. Role of Ocular Pigment Epithelial Cells in Immune Privilege. *Arch Immunol Ther Exp (Warsz)* (2009) 57(4):263–8. doi: 10.1007/s00005-009-0030-0
2. Benhar I, London A, Schwartz M. The Privileged Immunity of Immune Privileged Organs: The Case of the Eye. *Front Immunol* (2012) 3:296. doi: 10.3389/fimmu.2012.00296
3. Medawar PB. Immunity to Homologous Grafted Skin; the Fate of Skin Homografts Transplanted to the Brain, to Subcutaneous Tissue, and to the Anterior Chamber of the Eye. *Br J Exp Pathol* (1948) 29(1):58–69.
4. Luna JD, Chan CC, Derevjanik NL, Mahlow J, Chiu C, Peng B, et al. Blood-Retinal Barrier (BRB) Breakdown in Experimental Autoimmune Uveoretinitis: Comparison With Vascular Endothelial Growth Factor, Tumor Necrosis Factor Alpha, and Interleukin-1beta-Mediated Breakdown. *J Neurosci Res* (1997) 49(3):268–80. doi: 10.1002/(sici)1097-4547(19970801)49:3<268::aid-jnr2>3.0.co;2-a
5. Takeda A, Yanai R, Murakami Y, Arima M, Sonoda KH. New Insights Into Immunological Therapy for Retinal Disorders. *Front Immunol* (2020) 11:1431. doi: 10.3389/fimmu.2020.01431
6. Miserocchi E, Fogliato G, Modorati G, Bandello F. Review on the Worldwide Epidemiology of Uveitis. *Eur J Ophthalmol* (2013) 23(5):705–17. doi: 10.5301/ejo.5000278
7. Rizzo LV, Silver P, Wiggert B, Hakim F, Gazzinelli RT, Chan CC, et al. Establishment and Characterization of a Murine CD4+ T Cell Line and Clone That Induce Experimental Autoimmune Uveoretinitis in B10.A Mice. *J Immunol* (1996) 156(4):1654–60.
8. Zarate-Blades CR, Horai R, Mattapallil MJ, Ajami NJ, Wong M, Petrosino JF, et al. Gut Microbiota as a Source of a Surrogate Antigen That Triggers Autoimmunity in an Immune Privileged Site. *Gut Microbes* (2017) 8(1):59–66. doi: 10.1080/19490976.2016.1273996
9. McGhee CN. Pharmacokinetics of Ophthalmic Corticosteroids. *Br J Ophthalmol* (1992) 76(11):681–4. doi: 10.1136/bjo.76.11.681
10. Babu K, Mahendradas P. Medical Management of Uveitis - Current Trends. *Indian J Ophthalmol* (2013) 61(6):277–83. doi: 10.4103/0301-4738.114099
11. Cordero-Coma M, Sobrin L. Anti-Tumor Necrosis Factor-Alpha Therapy in Uveitis. *Surv Ophthalmol* (2015) 60(6):575–89. doi: 10.1016/j.survophthal.2015.06.004
12. Caspi RR, Roberge FG, Chan CC, Wiggert B, Chader GJ, Rozenszajn LA, et al. A New Model of Autoimmune Disease. Experimental Autoimmune Uveoretinitis Induced in Mice With Two Different Retinal Antigens. *J Immunol* (1988) 140(5):1490–5.
13. Caspi RR, Roberge FG, McAllister CG, el-Saied M, Kuwabara T, Gery I, et al. T Cell Lines Mediating Experimental Autoimmune Uveoretinitis (EAU) in the Rat. *J Immunol* (1986) 136(3):928–33.
14. Shao H, Liao T, Ke Y, Shi H, Kaplan HJ, Sun D. Severe Chronic Experimental Autoimmune Uveitis (EAU) of the C57BL/6 Mouse Induced by Adoptive Transfer of IRBP1-20-Specific T Cells. *Exp Eye Res* (2006) 82(2):323–31. doi: 10.1016/j.exer.2005.07.008
15. Fujino Y, Okumura A, Nussenblatt RB, Gery I, Mochizuki M. Cyclosporine-Induced Specific Unresponsiveness to Retinal Soluble Antigen in Experimental Autoimmune Uveoretinitis. *Clin Immunol Immunopathol* (1988) 46(2):234–48. doi: 10.1016/0090-1229(88)90186-9
16. Kawashima H, Mochizuki M. Effects of a New Immunosuppressive Agent, FK 506, on the Efferent Limb of the Immune Responses. *Exp Eye Res* (1990) 51(5):565–72. doi: 10.1016/0014-4835(90)90087-B
17. Luger D, Silver PB, Tang J, Cua D, Chen Z, Iwakura Y, et al. Either a Th17 or a Th1 Effector Response can Drive Autoimmunity: Conditions of Disease Induction Affect Dominant Effector Category. *J Exp Med* (2008) 205(4):799–810. doi: 10.1084/jem.20071258
18. Xu H, Rizzo LV, Silver PB, Caspi RR. Uveitogenicity Is Associated With a Th1-Like Lymphokine Profile: Cytokine-Dependent Modulation of Early and Committed Effector T Cells in Experimental Autoimmune Uveitis. *Cell Immunol* (1997) 178(1):69–78. doi: 10.1006/cimm.1997.1121
19. Yoshimura T, Sonoda KH, Miyazaki Y, Iwakura Y, Ishibashi T, Yoshimura A, et al. Differential Roles for IFN-Gamma and IL-17 in Experimental Autoimmune Uveoretinitis. *Int Immunol* (2008) 20(2):209–14. doi: 10.1093/intimm/dxm135
20. Peng Y, Han G, Shao H, Wang Y, Kaplan HJ, Sun D. Characterization of IL-17+ Interphotoreceptor Retinoid-Binding Protein-Specific T Cells in Experimental Autoimmune Uveitis. *Invest Ophthalmol Vis Sci* (2007) 48(9):4153–61. doi: 10.1167/iovs.07-0251
21. Zhang R, Qian J, Guo J, Yuan YF, Xue K. Suppression of Experimental Autoimmune Uveoretinitis by Anti-IL-17 Antibody. *Curr Eye Res* (2009) 34(4):297–303. doi: 10.1080/02713680902741696
22. Allensworth JJ, Planck SR, Rosenbaum JT, Rosenzweig HL. Investigation of the Differential Potentials of TLR Agonists to Elicit Uveitis in Mice. *J Leukoc Biol* (2011) 90(6):1159–66. doi: 10.1189/jlb.0511249
23. Fang J, Fang D, Silver PB, Wen F, Li B, Ren X, et al. The Role of TLR2, TRL3, TRL4, and TRL9 Signaling in the Pathogenesis of Autoimmune Disease in a Retinal Autoimmunity Model. *Invest Ophthalmol Vis Sci* (2010) 51(6):3092–9. doi: 10.1167/iovs.09-4754
24. Su SB, Silver PB, Grajewski RS, Agarwal RK, Tang J, Chan CC, et al. Essential Role of the MyD88 Pathway, But Nonessential Roles of TLRs 2, 4, and 9, in the Adjuvant Effect Promoting Th1-Mediated Autoimmunity. *J Immunol* (2005) 175(10):6303–10. doi: 10.4049/jimmunol.175.10.6303
25. Lamagna C, Scapini P, van Ziffle JA, DeFranco AL, Lowell CA. Hyperactivated MyD88 Signaling in Dendritic Cells, Through Specific Deletion of Lyn Kinase, Causes Severe Autoimmunity and Inflammation. *Proc Natl Acad Sci U.S.A.* (2013) 110(35):E3311–20. doi: 10.1073/pnas.1300617110
26. Sacre SM, Andreakos E, Kiriakidis S, Amjadi P, Lundberg A, Giddins G, et al. The Toll-Like Receptor Adaptor Proteins MyD88 and Mal/TIRAP Contribute to the Inflammatory and Destructive Processes in a Human Model of Rheumatoid Arthritis. *Am J Pathol* (2007) 170(2):518–25. doi: 10.2353/ajpath.2007.060657
27. Cario E. Toll-Like Receptors in Inflammatory Bowel Diseases: A Decade Later. *Inflammation Bowel Dis* (2010) 16(9):1583–97. doi: 10.1002/ibd.21282
28. Seoudi N, Bergmeier LA, Hagi-Pavli E, Bibby D, Curtis MA, Fortune F. The Role of TLR2 and 4 in Behcet's Disease Pathogenesis. *Innate Immun* (2014) 20(4):412–22. doi: 10.1177/1753425913498042
29. West MA, Wallin RP, Matthews SP, Svensson HG, Zaru R, Ljunggren HG, et al. Enhanced Dendritic Cell Antigen Capture *via* Toll-Like Receptor-Induced Actin Remodeling. *Science* (2004) 305(5687):1153–7. doi: 10.1126/science.1099153
30. Detrick B, Rodrigues M, Chan CC, Tso MO, Hooks JJ. Expression of HLA-DR Antigen on Retinal Pigment Epithelial Cells in Retinitis Pigmentosa. *Am J Ophthalmol* (1986) 101(5):584–90. doi: 10.1016/0002-9394(86)90949-9
31. Kumar MV, Nagineni CN, Chin MS, Hooks JJ, Detrick B. Innate Immunity in the Retina: Toll-Like Receptor (TLR) Signaling in Human Retinal Pigment Epithelial Cells. *J Neuroimmunol* (2004) 153(1-2):7–15. doi: 10.1016/j.jneuroim.2004.04.018
32. Chang CW, Roque RS, Defoe DM, Caldwell RB. An Improved Method for Isolation and Culture of Pigment Epithelial Cells From Rat Retina. *Curr Eye Res* (1991) 10(11):1081–6. doi: 10.3109/02713689109020348
33. Gibbs D, Williams DS. Isolation and Culture of Primary Mouse Retinal Pigmented Epithelial Cells. *Adv Exp Med Biol* (2003) 533:347–52. doi: 10.1007/978-1-4615-0067-4_44
34. Sugita S, Kawazoe Y, Imai A, Usui Y, Takahashi M, Mochizuki M. Suppression of IL-22-Producing T Helper 22 Cells by RPE Cells *via* PD-L1/PD-1 Interactions. *Invest Ophthalmol Vis Sci* (2013) 54(10):6926–33. doi: 10.1167/iovs.13-12703
35. Xin-Zhao Wang C, Zhang K, Aredo B, Lu H, Ufret-Vincenty RL. Novel Method for the Rapid Isolation of RPE Cells Specifically for RNA Extraction and Analysis. *Exp Eye Res* (2012) 102:1–9. doi: 10.1016/j.exer.2012.06.003
36. Neal MD, Sodhi CP, Jia H, Dyer M, Egan CE, Yazji I, et al. Toll-Like Receptor 4 Is Expressed on Intestinal Stem Cells and Regulates Their Proliferation and Apoptosis *via* the P53 Up-Regulated Modulator of Apoptosis. *J Biol Chem* (2012) 287(44):37296–308. doi: 10.1074/jbc.M112.375881
37. Quigley M, Martinez J, Huang X, Yang Y. A Critical Role for Direct TLR2-MyD88 Signaling in CD8 T-Cell Clonal Expansion and Memory Formation Following Vaccinia Viral Infection. *Blood* (2009) 113(10):2256–64. doi: 10.1182/blood-2008-03-148809
38. Thurau SR, Chan CC, Nussenblatt RB, Caspi RR. Oral Tolerance in a Murine Model of Relapsing Experimental Autoimmune Uveoretinitis (EAU): Induction of Protective Tolerance in Primed Animals. *Clin Exp Immunol* (1997) 109(2):370–6. doi: 10.1046/j.1365-2249.1997.4571356.x

39. Copland DA, Wertheim MS, Armitage WJ, Nicholson LB, Raveney BJ, Dick AD. The Clinical Time-Course of Experimental Autoimmune Uveoretinitis Using Topical Endoscopic Fundal Imaging With Histologic and Cellular Infiltrate Correlation. *Invest Ophthalmol Vis Sci* (2008) 49(12):5458–65. doi: 10.1167/iovs.08-2348
40. Chen Y, Yang P, Li F, Kijlstra A. The Effects of Th17 Cytokines on the Inflammatory Mediator Production and Barrier Function of ARPE-19 Cells. *PloS One* (2011) 6(3):e18139. doi: 10.1371/journal.pone.0018139
41. Reiling N, Holscher C, Fehrenbach A, Kroger S, Kirschning CJ, Goyert S, et al. Cutting Edge: Toll-Like Receptor (TLR)2- and TLR4-Mediated Pathogen Recognition in Resistance to Airborne Infection With Mycobacterium Tuberculosis. *J Immunol* (2002) 169(7):3480–4. doi: 10.4049/jimmunol.169.7.3480
42. Agarwal RK, Sun SH, Su SB, Chan CC, Caspi RR. Pertussis Toxin Alters the Innate and the Adaptive Immune Responses in a Pertussis-Dependent Model of Autoimmunity. *J Neuroimmunol* (2002) 129(1-2):133–40. doi: 10.1016/s0165-5728(02)00203-5
43. Wang ZY, Yang D, Chen Q, Leifer CA, Segal DM, Su SB, et al. Induction of Dendritic Cell Maturation by Pertussis Toxin and Its B Subunit Differentially Initiate Toll-Like Receptor 4-Dependent Signal Transduction Pathways. *Exp Hematol* (2006) 34(8):1115–24. doi: 10.1016/j.exphem.2006.04.025
44. Kuek A, Hazleman BL, Ostor AJ. Immune-Mediated Inflammatory Diseases (IMIDs) and Biologic Therapy: A Medical Revolution. *Postgrad Med J* (2007) 83(978):251–60. doi: 10.1136/pgmj.2006.052688
45. Yun J, Jiang G, Wang Y, Xiao T, Zhao Y, Sun D, et al. The HMGB1-CXCL12 Complex Promotes Inflammatory Cell Infiltration in Uveitogenic T Cell-Induced Chronic Experimental Autoimmune Uveitis. *Front Immunol* (2017) 8:142. doi: 10.3389/fimmu.2017.00142
46. Zhai X, Zhang Y, Xin S, Cao P, Lu J. Insights Into the Involvement of Circular RNAs in Autoimmune Diseases. *Front Immunol* (2021) 12:622316. doi: 10.3389/fimmu.2021.622316
47. Garzorz-Stark N, Lauffer F, Krause L, Thomas J, Atenhan A, Franz R, et al. Toll-Like Receptor 7/8 Agonists Stimulate Plasmacytoid Dendritic Cells to Initiate TH17-Deviated Acute Contact Dermatitis in Human Subjects. *J Allergy Clin Immunol* (2018) 141(4):1320–33 e11. doi: 10.1016/j.jaci.2017.07.045
48. Santiago-Raber ML, Baudino L, Izui S. Emerging Roles of TLR7 and TLR9 in Murine SLE. *J Autoimmun* (2009) 33(3-4):231–8. doi: 10.1016/j.jaut.2009.10.001
49. Hoffmann MH, Skriner K, Herman S, Baumann C, Steiner CW, Ospelt C, et al. Nucleic Acid-Stimulated Antigen-Presenting Cells Trigger T Cells to Induce Disease in a Rat Transfer Model of Inflammatory Arthritis. *J Autoimmun* (2011) 36(3-4):288–300. doi: 10.1016/j.jaut.2011.02.007
50. Ye J, Wang Y, Liu X, Li L, Opejin A, Hsueh EC, et al. TLR7 Signaling Regulates Th17 Cells and Autoimmunity: Novel Potential for Autoimmune Therapy. *J Immunol* (2017) 199(3):941–54. doi: 10.4049/jimmunol.1601890
51. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate Antiviral Responses by Means of TLR7-Mediated Recognition of Single-Stranded RNA. *Science* (2004) 303(5663):1529–31. doi: 10.1126/science.1093616
52. van der Made CI, Simons A, Schuurs-Hoeijmakers J, van den Heuvel G, Mantere T, Kersten S, et al. Presence of Genetic Variants Among Young Men With Severe COVID-19. *Jama* (2020) 324(7):663–73. doi: 10.1001/jama.2020.13719
53. Ganneru B, Jogdand H, Daram VK, Das D, Molugu NR, Prasad SD, et al. Th1 Skewed Immune Response of Whole Virion Inactivated SARS CoV 2 Vaccine and Its Safety Evaluation. *iScience* (2021) 24(4):102298. doi: 10.1016/j.isci.2021.102298
54. Ella R, Reddy S, Jogdand H, Sarangi V, Ganneru B, Prasad S, et al. Safety and Immunogenicity of an Inactivated SARS-CoV-2 Vaccine, BBV152: Interim Results From a Double-Blind, Randomised, Multicentre, Phase 2 Trial, and 3-Month Follow-Up of a Double-Blind, Randomised Phase 1 Trial. *Lancet Infect Dis* (2021) 21(7):950–61. doi: 10.1016/S1473-3099(21)00070-0
55. Chen S, Yan H, Sun B, Zuo A, Liang D. Subretinal Transfection of Chitosan-Loaded TLR3-siRNA for the Treatment of Experimental Autoimmune Uveitis. *Eur J Pharm Biopharm* (2013) 85(3 Pt A):726–35. doi: 10.1016/j.ejpb.2013.09.005
56. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming Growth Factor-Beta Induces Development of the T(H)17 Lineage. *Nature* (2006) 441(7090):231–4. doi: 10.1038/nature04754
57. Qin H, Wang L, Feng T, Elson CO, Niyongere SA, Lee SJ, et al. TGF-Beta Promotes Th17 Cell Development Through Inhibition of SOCS3. *J Immunol* (2009) 183(1):97–105. doi: 10.4049/jimmunol.0801986
58. Ramezanpour M, Moraitis S, Smith JL, Wormald PJ, Vreugde S. Th17 Cytokines Disrupt the Airway Mucosal Barrier in Chronic Rhinosinusitis. *Mediators Inflammation* (2016) 2016:9798206. doi: 10.1155/2016/9798206
59. Zhang LW, Cong X, Zhang Y, Wei T, Su YC, Serrao AC, et al. Interleukin-17 Impairs Salivary Tight Junction Integrity in Sjogren's Syndrome. *J Dent Res* (2016) 95(7):784–92. doi: 10.1177/0022034516634647
60. Amasheh M, Fromm A, Krug SM, Amasheh S, Andres S, Zeitz M, et al. TNFalpha-Induced and Berberine-Antagonized Tight Junction Barrier Impairment via Tyrosine Kinase, Akt and NFkappaB Signaling. *J Cell Sci* (2010) 123(Pt 23):4145–55. doi: 10.1242/jcs.070896
61. Chong CM, Zheng W. Artemisinin Protects Human Retinal Pigment Epithelial Cells From Hydrogen Peroxide-Induced Oxidative Damage Through Activation of ERK/CREB Signaling. *Redox Biol* (2016) 9:50–6. doi: 10.1016/j.redox.2016.06.002
62. Nosaka T, Kawashima T, Misawa K, Ikuta K, Mui AL, Kitamura T. STAT5 as a Molecular Regulator of Proliferation, Differentiation and Apoptosis in Hematopoietic Cells. *EMBO J* (1999) 18(17):4754–65. doi: 10.1093/emboj/18.17.4754
63. Fox R. Anti-Malarial Drugs: Possible Mechanisms of Action in Autoimmune Disease and Prospects for Drug Development. *Lupus* (1996) 5(Suppl 1):S4–10. doi: 10.1177/096120339600500103l
64. O'Neill PM, Bray PG, Hawley SR, Ward SA, Park BK. 4-Aminoquinolines–past, Present, and Future: A Chemical Perspective. *Pharmacol Ther* (1998) 77(1):29–58. doi: 10.1016/s0163-7258(97)00084-3
65. Sun S, Rao NL, Venable J, Thurmond R, Karlsson L. TLR7/9 Antagonists as Therapeutics for Immune-Mediated Inflammatory Disorders. *Inflammation Allergy Drug Targets* (2007) 6(4):223–35. doi: 10.2174/187152807783334300
66. Gottschalk TA, Tsantikos E, Hibbs ML. Pathogenic Inflammation and Its Therapeutic Targeting in Systemic Lupus Erythematosus. *Front Immunol* (2015) 6:550. doi: 10.3389/fimmu.2015.00550
67. Stern EM, Johnson JS, Mazzulla DA. Highly Accelerated Onset of Hydroxychloroquine Macular Retinopathy. *Ochsner J* (2017) 17(3):280–83.

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High Dimensional Analyses of Circulating Immune Cells in Psoriatic Arthritis Detects Elevated Phosphorylated STAT3

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Psoriatic arthritis (PsA) is a chronic inflammatory arthritis, affecting up to 40% of patients with psoriasis. Constitutive expression by CD4+ T cells of an active form of STAT3, a signal transducer and transcription factor, has been shown to induce many of the major features of PsA in an animal model. We used high dimensional mass cytometry (CyTOF) to probe *ex-vivo* levels of phosphorylated STAT3 (pSTAT3) in circulating immune cell subpopulations from PsA patients during active and inactive states. We evaluated the frequency of 16 immune cell populations and the levels of the activated forms of STAT3 (pSTAT3) and, for comparison, STAT1 (pSTAT1) and Src (pSrc) in whole blood fixed shortly after collection. In addition to PsA patients, we studied active rheumatoid arthritis (RA) patients. Increased levels of pSTAT3 were found in all the CD4+ T cell subsets analyzed, specifically, Th1, Th2, Th17, T follicular helper (Tfh) and T regulatory (Treg) as well as in CD14+CD16- (classical) monocytes from active PsA patients compared to inactive patients. After correcting for body mass index (BMI), smoking and conventional disease modifying antirheumatic drugs (c-DMARDs), levels of pSTAT3 levels remained increased in Th1 and Tfh CD4+ T cells, and in CD14+CD16- monocytes from active patients compared to inactive patients. No differences between the patient groups were observed for pSTAT1 or pSrc. No differences were found between the active PsA and active RA groups after correction for multiple testing. During active PsA, circulating Th1 and Tfh CD4+ T cells, and CD14+CD16- monocytes expressing high levels of pSTAT3 may play a role in PsA pathophysiology, perhaps by migration to inflamed sites.

Keywords: psoriatic arthritis, STAT3, CD4+ T cells, monocytes, rheumatoid arthritis

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INTRODUCTION

Psoriatic arthritis (PsA), a chronic inflammatory arthritis belonging to the group of spondyloarthropathies, affects 6-41% of patients with psoriasis (1). A recent meta-analysis found a pooled prevalence of 133 for every 100,000 persons and an incidence of 83 for every 100,000 individuals (2). In most adult cases, arthritis develops around 10 years after the appearance of psoriasis, with the disease affecting males and females equally (3). Psoriatic arthritis also occurs in children, with distinguishing features, such as a bimodal age of onset distribution (4). PsA is a systemic disease, involving the musculoskeletal system, the skin and nails (3, 5). The term psoriatic disease has been used to encompass psoriasis, PsA and associated comorbidities (6). Psoriatic disease is associated with uveitis and inflammatory bowel disease, and an increased risk of comorbidities, especially metabolic syndrome and cardiovascular disease (7, 8). There are no specific biomarkers for PsA, and the diagnosis is based on clinical and imaging findings.

Genetic, environmental and immune-related mechanisms have been implicated in PsA pathogenesis. It is not fully understood how interactions among these different factors lead to manifestations of PsA, and initiation of the disease either at the skin site or at the entheses has been proposed (3). Many immune components, both innate and adaptive, have been associated with the inflammatory response in PsA (9). Among these, T cells and other cell types producing IL-17 have been shown to be of fundamental importance, based on evidence from genetic and tissue studies, animal models and, more recently, response to therapy (10–12). The transcription factor STAT3 has been shown to be essential for the development of Th17 cells (13). Recently, work by Yang et al. described an animal model in which overexpression of STAT3C, a constitutively active form of STAT3, in CD4+ T cells led to expression of many of the major features of PsA, including psoriasis-like skin lesions, tendinitis/enthesitis and arthritis (14, 15). This work points to a crucial role of STAT3 in PsA pathogenesis, building upon an extensive literature showing the importance of STAT3 in psoriasis (16) and PsA, including a STAT3 polymorphism (STAT3 rs744166∗G allele) that has been found to be associated with PsA (17). In a commentary to Yang et al. (14), Mountz (15) proposes a model in which dysregulation of STAT3 expression in CD4+ T cells is the 'initiating event,' driving both skin disease and musculoskeletal disease through the induction of Th17 and Th22 cells. In this model, Th17 cells would be mostly involved in the development of psoriatic arthritis, and Th22 cells, which express the chemokine receptor CCR10, would drive the development of psoriasis. The higher expression of STAT3 would underlie both these outcomes.

Given the complexity of the immune landscape in PsA, it is likely that, at different stages of the disease, distinct immune phenotypes can be detected and shed light on the disease immunopathology. In this study, we used the high dimensionality of mass cytometry to measure expression of phosphorylated STAT3 (pSTAT3) in non-manipulated, *ex vivo*, circulating immune populations during active and inactive PsA. We also analyzed samples from active rheumatoid arthritis (RA) for comparison. Mass cytometry, also known as cytometry by time-of-flight (CyTOF), is a novel platform combining flow cytometry and mass spectrometry that allows assessment of overall heterogeneity and degree of similarity between subsets of immune cells, based on a large number of parameters. Studies using CyTOF in rheumatic diseases have been reported (18, 19), and use of this approach in these diseases has been reviewed recently (20).

PATIENTS, MATERIALS AND METHODS

Patients

Demographic, clinical and laboratory parameters and treatment data were collected from 27 patients with PsA fulfilling CASPAR criteria (21) and 14 patients with RA fulfilling 2010 EULAR/ACR classification criteria (**Table 1**) (22). All patients provided informed consent, and the study was approved by the IRB at Carmel Medical Center (CMC 0044-11). The PsA study population consisted of 12 patients with inactive disease, as defined by minimal disease activity (MDA) ≥ 5, and 15 patients with active disease (MDA<5) (23). All RA patients were classified as having moderate to high disease activity, according to the clinical disease activity index (CDAI) score (24).

Samples

Four ml of blood was collected from each subject in EDTA tubes and promptly treated with Smart Tube fixative in a proportion of 1.4 parts Proteomic Stabilizer PROT1 (Smart Tube, Inc. San Carlos, CA) to 1 part whole blood, and frozen at -80°C. The fixed whole blood was thawed in a 4°C cold room. Following complete thawing, samples were diluted 1:5 with 1X Smart Tube Thaw-Lyse Buffer (Smart Tube, Inc.), mixed 5 times and incubated for 10 min at room temperature (RT). Samples were then centrifuged at 600 x g for 8 min at RT. Supernatant was decanted, and the pellet was re-suspended in 25-50 ml of Thaw-Lyse Buffer. This procedure was performed in total 3 times, followed by 2 washes with 25 ml of thaw Lyse Buffer 2 (Smart Tube, Inc). The resulting pellet was re-suspended in 1.6 ml of 0.22 μm filtered CyFacs buffer [1 x PBS (Rockland, Limerick, PA), 1% bovine serum albumin (Sigma) and 0.05% sodium azide (Sigma) in Milli-Q water] and stored at 4°C overnight in a 96 well deep well polypropylene plate (VWR, Radnor, PA).

Antibodies

The antibody panel is shown on **Supplementary Table 1**. Antibodies were titrated using fixed blood from a healthy adult control. Antibodies were freshly combined for each experiment in a volume of 50 μl per sample and filtered using a 0.1μm Durapore PVDF filter (Millipore Sigma, St. Louis, MO) at 14,000 x g for 5 min at RT.

Staining

The staining was performed in a 96 well deep well plate at RT. The cells, initially resuspended in 1.6 ml of CyFacs buffer, were

TABLE 1 | Characteristics of the study patient population.

	Active PsA	Inactive PsA	Active RA	p-valueActive PsA - Inactive PsA[1]	p-valueActive PsA – Active RA[1]
Number	15	12	14		
Age (years)[2]	54.60±15.52	58.67±11.32	58.71±13.76	0.68	0.6
Disease Duration Arthritis (years)[2]	7.87±4.96	13.75±12.11	9.36±11.83	0.21	0.48
Disease Duration: Psoriasis (years)[2]	17.93±12.60	25.75±12.62			
Body Mass Index[2]	30.57±4.61	24.62± 3.36	30.29±7.18	**0.001**	0.88
Gender(female)	11 (73.3%)	8 (66.7%)	11 (78.6%)	1.0	1.0
Smoking	9 (64.3%)	2 (16.7%)	4 (28.6%)	**0.02**	0.12
Ethnicity: Jewish	12 (80 %)	12 (100%)	11 (78.6%)	0.23	1.0
Arab	3 (20.0%)	0	3 (21.4%)	0.23	1.0
Comorbidities					
Hypertension	4 (26.7%)	2 (16.7%)	5 (35.7%)	0.67	0.70
Hyperlipidemia	6 (40%)	4 (33.3%)	7 (50.0%)	1.00	0.59
Diabetes Mellitus	3 (20%)	0	0	0.23	0.22
Asthma	1 (6.7%)	0	1 (7.1 %)	1.0	1.0
Ischemic Heart Disease	2 (13.3%)	0	0		1.0
Thyroid Diseases	1 (6.7%)	1 (8.3%)	4 (28.6%)	1.0	0.17
Medication					
c-DMARDS[3]	5 (33.3%)	9 (75.0%)	4 (28.6%)	**0.03**	0.68
Methotrexate	5 (33.3%)	8 (66.7%)	4 (28.6%)	1.0	0.68
Leflunomide	1 (6.7%)	0	0	1.0	1.0
Salazopyrine	2 (13.3%)	3 (25.0%)	0	0.63	0.48
Cyclosporine	1 (6.7%)	0	0	1.0	1.0
Apremilast	1 (6.7%)	0	0	1.0	1.0
b-DMARDS[4]	5 (33.3%)	5 (41.7%)	0	0.71	**0.04**
Anti TNFα	3 (20%)	|5 (41.7%)	0	0.40	0.22
Etanercept	3 (20%)	3 (25.0%)	0	1.0	0.22
Golimumab	0	1 (8.3%)	0	0.44	1.0
Adalimumab	1 (6.7%)	0	0	1.0	1.0
Infliximab	1 (6.7%)	1(8.3%)	0	1.0	1.0
Secukinumab	2 (13.3%)	0	0	0.49	0.48
Ustekinumab	1 (6.7%)	0	0	1.0	1.0

[1]: t test or Mann Whitney test were used to compare continuous variables between two independent groups. Chi-squared test or exact test for small sample were used to compare categorical variables between groups; the bold values denote statistical significance. [2]: Continuous variables are presented as mean ± SD. [3]: c-DMARDS: conventional disease-modifying antirheumatic drugs; [4] : b-DMARDS: biological disease-modifying antirheumatic drugs. For medication, the number of patients refers to the total number of patients on the particular medication in each group.

pelleted by centrifugation at 974 x g at 4°C. Supernatant was aspirated using a manifold to control the amount of supernatant left. Following aspiration, the plate was vortexed to ensure resuspension of the cell pellet. Fc receptors were blocked using 5 µl of Human TruStain FcX™ (BioLegend, San Diego, CA) for 10 min. At the end of the incubation, 50 µl of filtered antibodies for surface antigens (**Supplementary Table 1**) was added to each well and mixed gently with the cells. The plate was incubated for 30 min at RT with gentle vortexing after 15 min. Cells were then washed twice with 1.6 ml of CyFacs Buffer and fixed with 1.6% of formaldehyde (Thermo Scientific, Rockford, IL) for 10 min at RT, followed by 2 washes with CyPBS. Cells were then permeabilized with 90% methanol (Sigma), on ice for 30 min, followed by 2 washes with CyPBS. Antibodies to intracellular antigens (**Supplementary Table 1**) were added to the cells and incubated for 30 min at RT with a gentle vortexing after 15 min, and cells were washed once with 1.6 ml of CyPBS. To enable cell identification based on DNA content, cells were labeled with 0.125 nM iridium (191Ir and 193Ir) (Cell-ID™ Intercalator-Ir, Fluidigm, South San Francisco, CA), for 20 min at RT, in a volume of 300 µl. Cells were then washed 5 times before CyTOF acquisition: 2 times with CyFacs buffer and 3 times with 0.22 µm filtered MilliQ water; the last 2 washes were performed just before acquisition.

Mass Cytometry

A Helios mass cytometer (Fluidigm) was used for sample acquisition. Before acquisition, the machine was tuned with Tuning Solution (Fluidigm) and a bead sensitivity test was performed using EQ™ Four Element Calibration Beads (Fluidigm). The samples were resuspended in a solution of 1:10 Calibration Beads in CyWater to obtain a concentration of approximately 1 x 10^6 cell/ml. The fluidics system SuperSampler (VictorianAirships, Alamo, CA) was used for injection of cells into the instrument. Sample files were normalized using calibration beads.

Mass Cytometry Data Analysis

Cell subpopulations were manually determined using FlowJo version 10.5.2 (FlowJo, LLC). **Supplementary Figure 1** shows the gating strategy. Briefly, cell events (intact cells) were identified as Ir191/193 double positive events, and doublets were excluded on the basis of higher DNA content (Ir191) and longer event length. Immune cell frequencies are expressed as frequency of mononuclear cells, defined as CD45+, CD66a- cells, except for granulocytes that are expressed as frequency of total leukocytes. CD4+ T cell subsets were defined based on Kunicki et al. (25): from gated CD3+CD4+ cells, the Tfh subset was defined as CXCR5+ cells; the CXCR5- cells were then defined as

follows: the Th1 subset was defined as CXCR3+ CCR4- cells, the Th2 subset as CCR4+CXCR3-, Th17 as CCR6+CD161+, and the Treg subset as $CD127^{lo/-}CD25^{hi}$ $FOXP3^{+}$ (**Supplementary Figure 1**). For levels of phosphorylated signaling proteins, the signal intensity from a whole given population was measured, as the distribution was unimodal; the geometric mean (gmean, as defined by FlowJo software) of the signal intensity for each value was expressed as the hyperbolic arcsine of the gmean divided by a cofactor parameter (value=5) (arcsinh transformed).

Statistical Analysis

We analyzed the differences between PsA patients at active and inactive states and the differences between the active PsA and active RA patients. Sixty-four conditions were analyzed: 16 cell subpopulations and 3 phosphorylated signaling proteins. Group to group analyses were performed using unpaired t test with Welch's correction, using GraphPad Prism version 9.1.0.

Data were analyzed using significance analysis of microarray (SAM) analysis (26), a non-parametric method that performs correction for multiple testing, using the "samr" package in R through GitHub (https://github.com/MikeJSeo/SAM). Two class unpaired SAM analyses were used to analyze the groups, with a false discovery rate (FDR) set at <1% (q<0.01).

To analyze the contribution of variables that differed between active and inactive PsA patients, we performed linear regression model with bootstrapping for deriving robust estimates of standard errors and confidence intervals for estimates regression coefficients. This allowed estimation of the contribution of BMI, smoking and conventional disease-modifying anti-rheumatic drugs (c-DMARDS). All tests were two-sided with a p<=0.05 considered statistically significant. Statistical analyses were performed using SPSS version 24.0 (IBM, Armonk, New York, USA).

Correlations between phosphoproteins levels and disease parameters were performed using Spearman's rank correlation in the GraphPad Prism version 9.1.0.

Heatmaps

Heat maps were created using the Morpheus visualization and analysis software: https://software.broadinstitute.org/morpheus/. Values were transformed by subtracting row median and dividing by row median absolute deviation. Clustering was performed by hierarchical clustering using Euclidean distance with average linkage.

RESULTS

Study Population

The PsA population had an average age of 58.25 ± 12.22 years, and 70% (n=19) were female. The patients with active PsA were more obese (BMI=30.57 ± 4.61) and more often smokers (64%), compared to patients with inactive PsA. Fewer active compared to inactive PsA patients were using conventional (c-)DMARDS (33% to 75%). There were no other statistically significant differences in demographic parameters, comorbidities or medications (**Table 1**) between patients with active versus inactive disease. In the active PsA group, the mean tender joint count was 14.13 ± 9.32, swollen joints 11.27 ± 6.81, Psoriasis Area and Severity Index (PASI) score 2.77 ± 2.82 and enthesitis score of 5.14 ± 7.54. The calculated minimal disease activity (MDA) score in active PsA patients was 0 in 4 patients, 1 in 8 patients and 2 in 3 patients. In the inactive PsA group, 5 patients had an MDA score of 5, 4 patients a score of 6 and 3 patients scored 7. In the RA group, 11 (79%) patients were female, and the mean age was 58.71 ± 13.76 years. The calculated mean CDAI score was 33.19 ± 14.61. No RA patient was on biologic (b)-DMARDS, compared to 33% in the active PsA group. There were no statistically significant differences in demographic and comorbidities between active PsA patients and active RA patients (**Table 1**).

Comparisons Between Active and Inactive Psoriatic Arthritis Patients

We compared the frequency of 16 immune cell subpopulations between active and inactive PsA. No significant differences in the frequency of these subpopulations were found between the two PsA groups, using group to group comparisons (**Figures 1A, B**). Next, we compared the level of phosphorylated proteins (pSTAT1, pSTAT3 and pSrc) in these 16 subpopulations between the two groups of PsA patients. Higher levels of pSTAT3 were found in all CD4+ T cell subsets analyzed, defined as detailed in Kunicki et al. (25), as well as in CD14+CD16- (classical) and CD14+CD16+ (intermediate) monocytes from active compared to inactive PsA patients, using group to group comparisons (**Figures 2A, B** and **Supplementary Tables 2**, **3**).

We next analyzed all data using SAM (significance analysis of microarrays): frequency of 16 immune cell subpopulations, and the baseline levels of the 3 phosphoproteins in all subpopulations, for a total of 64 comparisons. At FDR<1% (q<0.01), levels of pSTAT3 in all CD4+ T cell subsets analyzed (Th1, Th2, Th17, Treg, Tfh), as well as in CD14+CD16- monocytes (classical) were significantly different between active and inactive PsA, whereas levels in CD14+CD16+ monocytes were similar between the 2 groups (**Figures 2A, B**). Next, using the SAM results, we generated a heat map to visualize the baseline status of the 3 phosphorylated signaling proteins (pSTAT1, pSTAT3 and pSrc) in all subpopulations analyzed. As can be seen in the heat map in **Figure 2C**, the level of signaling from pSTAT3 in the CD4+ T cell subsets as well as in CD14+ monocytes are noticeably higher, albeit heterogeneous, in the active PsA group and decreased in the inactive group. In contrast, levels of pSTAT1 or pSrc did not differ between the PsA groups in the immune subpopulations assessed (**Supplementary Figure 2**).

As active PsA patients have higher level of BMI and smoking and lower level of c-DMARD usage than inactive patients (**Table 1**), we performed a bootstrap analysis that showed that levels of pSTAT3 in Th1 and Tfh CD4+ T cells, as well as in CD14+CD16- monocytes, stay significantly higher in active PsA patients, after correction for these confounding variables (**Tables 2**–**4**). The difference between the patient groups is no longer significant for Th17, Treg and Th2 CD4+ subsets after correction (**Supplementary Tables 4**–**6**).

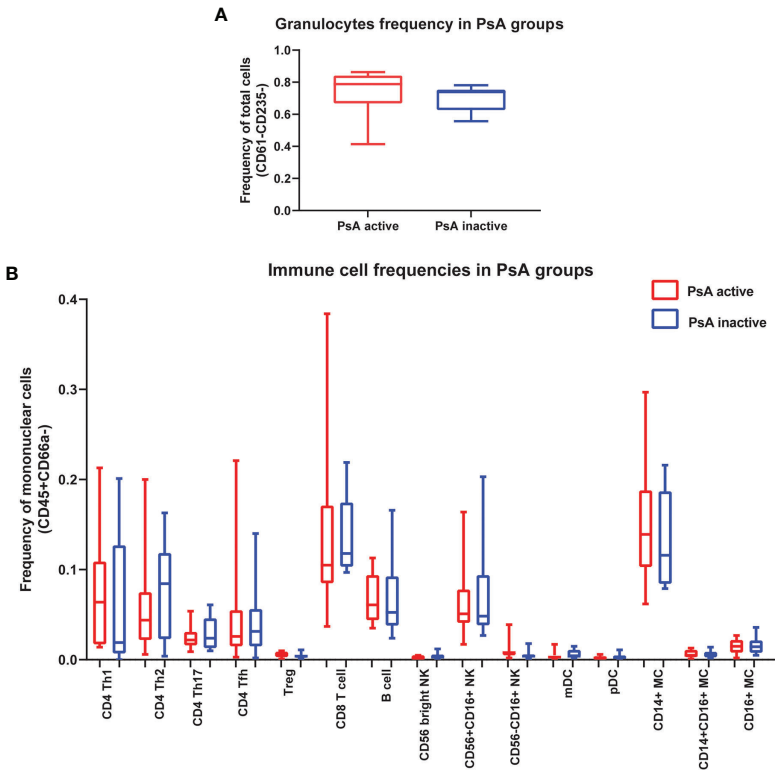


FIGURE 1 | No differences in immune cell frequencies in whole blood between patients with active or inactive PsA. **(A)** Frequency of granulocytes is expressed as frequency of leukocytes. **(B)** Frequencies of 15 immune cell subpopulations expressed as frequency of total mononuclear cells (CD45+CD66a-). Data are shown as box plots extending from the 25th to 75th percentiles, and the whiskers from the minimum to the maximum point; middle line represents the median. Samples from 15 active PsA patients and 12 inactive PsA patients were tested. Group to group comparison using unpaired t test with Welch's correction, p>0.05 in all comparisons. Tfh, T follicular helper; mDC, myeloid Dendritic cell; pDC, plasmacytoid Dendritic cell; MC, Monocyte.

PsA samples were clustered using the level of pSTAT3 in Th1 and Tfh CD4+ T cells and CD14+ monocytes. The majority of the active samples clusters in 2 adjacent clusters, while half of the inactive samples cluster away from the active samples. Between these 2 groups, there is a mixed group of active and inactive samples (**Figure 3**).

We next examined whether the levels of pSTAT3 in Th1 or Tfh cells CD4+ T cells or in CD14+CD16- monocytes were correlated with tender joints (TJ), swollen joints (SJ), enthesitis or C reactive protein (CRP). There was no correlation between enthesitis or CRP and levels of pSTAT3 in Th1, Tfh or CD14 monocytes (**Supplementary Tables 7**, **8**). Modest negative correlations (r between -0.43 and -0.47) were observed between tender joints or swollen joints and levels of pSTAT3 in Th1 and Tfh cells, although without reaching significance (**Supplementary Figures 3A, B**), while weak negative (r ~ -0.2), non-significant correlations with pSTAT3 in CD14+ monocytes and tender joints or swollen joints were observed (**Supplementary Figure 3C**).

Comparisons Between Active Psoriatic Arthritis Patients and Active Rheumatoid Arthritis

We also performed a comparison between active PsA and active RA. No significant difference in cell frequency for 16 immune subpopulations was observed between these patient groups (**Supplementary Figure 4**). Levels of phosphorylated proteins were also similar between the two groups (**Supplementary Figure 5**), except for the higher level of pSrc in granulocytes in active PsA (**Supplementary Figure 6**), which was not significant after performing SAM analysis. No correlations were observed between CRP (N=9), TJ or SJ (N=13 for both) in active RA and any phosphoproteins using Spearman's correlation.

DISCUSSION

In this study, we describe findings of elevated levels of phosphorylated STAT3 (pSTAT3) in CD4+ T cell subsets and CD14+CD16- (classical) monocytes in active PsA patients, in comparison to patients with inactive disease (inactive PsA). To the best of our knowledge, this is the first report of higher level of pSTAT3 in circulating CD4+ T cells subsets and CD14+CD16- monocytes, measured directly in *ex vivo* cells from PsA patients with active disease. Levels of other signaling proteins analyzed - pSTAT1 and pSrc - were similar between active and inactive PsA patients in all cell types assessed. Elevated pSTAT3 in PsA has been described previously in other tissues or in total T cells. Fiocco

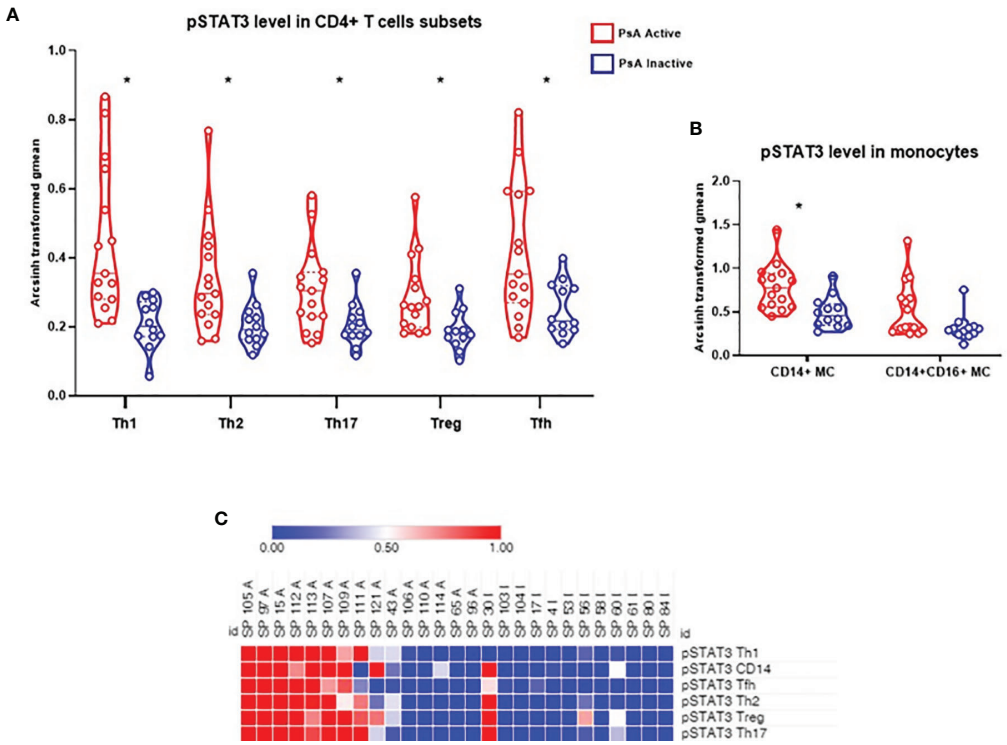


FIGURE 2 | Levels of pSTAT3 are increased in CD4+ T cell subsets and in classical monocytes during active PsA. **(A)** Signal intensity of pSTAT3 in CD4+ T cell subsets from PsA patients. **(B)** Signal intensity in monocytes: CD14+CD16- (classical) subset and CD14+CD16+ (Intermediate) subset. Data expressed as arcsinh transformed geometric mean (gmean), displayed as violin plots, with the straight line representing the median and dashed lines the quartiles. Samples from 15 active PsA patients and 12 inactive PsA patients were tested. Group to group comparison using unpaired t test with Welch's correction, all values p<0.05, see **Supplementary Tables 2**, **3** for details. *: significant difference between the 2 groups by two class unpaired SAM analysis, FDR <1% (q<0.01). **(C)** Heat map showing STAT3 phosphorylation signal in CD4+ T cell subsets and CD14+ (classical) monocytes). A, Active PsA; I, Inactive PsA; Tfh, T follicular helper; MC, Monocytes.

et al. (27, 28), found higher levels of pSTAT3, but also of other signaling proteins, on total T cell lysates from synovial fluid of PsA patients compared to T cells from peripheral blood mononuclear cells (PBMC) from healthy donors. These synovial fluid T cells were isolated through a multistep process, which may have affected the signaling profile, and the comparison to circulating cells may be another confounder. In a brief report, Raychaudhuri et al. (29) showed that sorted and activated CD3+ T cells from peripheral blood, upon activation with IL-23, showed increased pSTAT3, but the effect was similar in cells from both PsA patients and healthy controls. Elevated pSTAT3 and pSTAT1 was observed in PsA synovial tissue from PsA in comparison to tissue from osteoarthritis patients (30), indicating that there is activation of the JAK-STAT pathway at inflammatory sites.

TABLE 2 | Bootstrapping analysis for pSTAT3 in CD4+ Th1 cells.

Bootstrap for Coefficients

Model		B	Bootstrap (based on 999 bootstrap samples)				
			Bias	Standard Error	Significance (2-tailed)	95% Confidence Interval	
						Lower	Upper
1	(Constant)	3.272	.060	.594	.003	2.229	4.521
	Group	-0.940	-.026	.306	.011	-1.585	-.331
	Smoke	0.160	-.012	.413	.671	-.634	1.024
	cDMARDS	-0.713	.018	.347	.070	-1.427	-.026
	BMI	0.211	-.018	.292	.425	-.439	.780

cDMARDs, conventional disease-modifying antirheumatic drugs. For smoke and cDMARDS, the variables are yes/no for each condition. BMI, Body Mass Index; for this variable grouping was based on normal weight (below 25) versus obese (above 25).

TABLE 3 | Bootstrapping analysis for pSTAT3 in CD4+ Tfh cells.

Bootstrap for Coefficients							
Model		**B**	**Bootstrap (based on 998 bootstrap samples)**				
			Bias	**Std. Error**	**Sig. (2-tailed)**	**95% Confidence Interval**	
						Lower	**Upper**
1	(Constant)	2.982	.042	.596	.001	1.862	4.338
	Group	-0.741	-.013	.346	.044	-1.464	-.041
	Smoke	-0.210	-.031	.364	.538	-.963	.503
	cDMARDS	-0.499	-.009	.362	.180	-1.306	.160
	BMI	0.299	-.002	.300	.291	-.331	.868

cDMARDs, conventional disease-modifying antirheumatic drugs. For smoke and cDMARDS, the variables are yes/no for each condition. BMI, Body Mass Index; for this variable grouping was based on normal weight (below 25) versus obese (above 25).

TABLE 4 | Bootstrapping analysis for pSTAT3 in CD14+CD16- monocytes.

Bootstrap for Coefficients							
Model		**B**	**Bootstrap (based on 1000 bootstrap samples)**				
			Bias	**Std. Error**	**Sig. (2-tailed)**	**95% Confidence Interval**	
						Lower	**Upper**
1	(Constant)	5.315	-0.082	1.077	0.002	2.890	7.248
	Group	-1.464	0.054	0.594	0.024	-2.471	-0.060
	Smoke	-0.090	0.024	0.700	0.888	-1.238	1.501
	cDMARDS	-0.313	0.003	0.612	0.616	-1.617	0.820
	BMI_NW	0.930	0.044	0.560	0.099	-0.115	2.052

cDMARDs, conventional disease-modifying antirheumatic drugs. For smoke and cDMARDS, the variables are yes/no for each condition. BMI, Body Mass Index; for this variable grouping was based on normal weight (below 25) versus obese (above 25).

In our study, elevated levels of pSTAT3 in active PsA patients, compared to patients with inactive disease, were found in all circulating CD4+ T cells analyzed. These findings agree with results from a murine model showing that expression of STAT3C in CD4+ T cells led to appearance of PsA-like symptoms (14). However, in our study, levels of pSTAT3 in circulating Th17, Treg and Th2 CD4+ T cells were not different in active PsA patients after correction for BMI, smoking (both higher in active patients) and use of c-DMARDs (lower in active PsA). These factors have been shown to affect activation of STAT3 in different directions and may affect subsets of circulating CD4+ cells in distinct ways. Smoke has been shown to activate STAT3 in the lung (31) and cigar smoke extract has been shown to activate STAT3 phosphorylation in a murine macrophage cell line (32). In contrast, c-DMARDS, such as methotrexate (MTX), have been shown to reduce levels of pSTAT3 (33).

Higher BMI in the active PsA group is consistent with several lines of evidence that indicate that obesity plays an important role in PsA. STAT3 is the main signaling factor of IL-6, and obesity has been shown to chronically activate intracellular JAK-STAT3 signaling through increased levels of IL-6 and leptin (34). Of particular importance to PsA, obesity increases the chance of developing the disease (8, 35). In addition, juvenile PsA patients have been found to have higher rate of obesity compared to polyarticular juvenile idiopathic arthritis (JIA) patients or to the US pediatric population (36). Furthermore, obesity is associated with reduced treatment response in PsA (37). Obesity has been shown to be associated with induction of Th17 cells, but not with Th1 or Treg cells (38, 39). Thus, elevated pSTAT3 in the circulating CD4+ Th17 (and perhaps other helper subsets) in active PsA patients in our study could be partially driven by factors associated with obesity.

For circulating Th1 and Tfh CD4+ T cells, higher expression of pSTAT3 in active PsA compared to inactive PsA was independent of group variables, suggesting an intrinsic role for these cells in PsA. However, in contrast to Th17 cells, Th1 differentiation appears largely independent of STAT3. Indeed, higher expression of IFNγ has been described in STAT3-deficient CD4+ T cells (40), suggesting that STAT3 may inhibit Th1 development. Knocking out STAT3 and mTOR decreased IL-10 production in Th1 cells, but did not affect IFNγ production (41). Although STAT3 does not seem to play a role in Th1 development, increased levels of pSTAT3 in Th1 cells have been found to contribute to organ damage in a model of acute liver injury, suggesting a potential role of these cells in promoting disease pathogenesis (42).

The presence of Th1 cells in psoriatic lesions has been noted for quite some time. However, the role of Th1 cells and IFNγ in psoriasis or PsA is still unclear. In psoriatic plaques, IFNγ producing Th1 cells are abundant, following an initial phase where Th17 predominates (43); expression of IFNγ and IFN-inducible genes is also upregulated. However, a small trial with an anti-IFNγ antibody showed only minimal efficacy in the treatment of psoriasis, in contrast to inhibition of IL-17

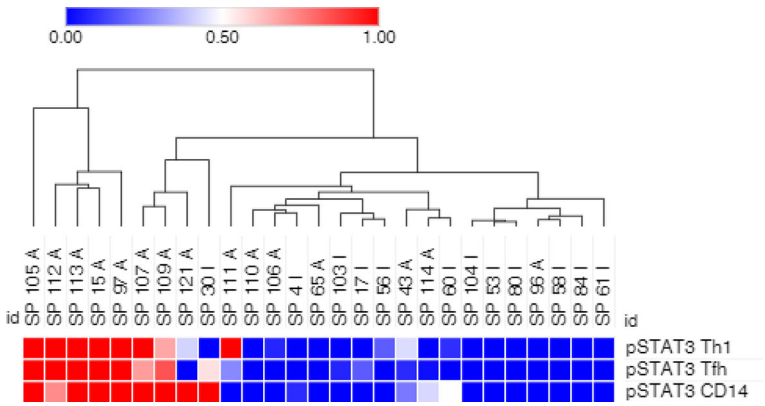


FIGURE 3 | pSTAT3 levels in Th1 and Tfh CD4+ T cells, and in CD14+ monocytes partially cluster PsA samples. Arcsinh transformed geometric mean of pSTAT3 phosphorylated target in Th1 and Tfh CD4+ T cells and CD14+ monocytes was used. Clustering by hierarchical clustering using Euclidean distance with average linkage. A, active PsA; I, inactive PsA.

activity (44). More recently, Diani et al. described that Th1 (and Tc1) cells expressing the chemokine receptor CXCR3 are enriched in synovial fluid (SF), in contrast to the blood, of PsA patients (45), paralleling a marked increase in CXCL10, the ligand of CXCR3, in the SF of PsA patients. Similar observations were also described in the skin of psoriasis patients (46). These findings suggest a potential role for Th1 (and Tc1) cells that are recruited from the blood in response to CXCL10 chemokine, both in psoriasis and in PsA (47). Expansion of synovial CXCR3+ CD8+ T cells has been also described by Penkava et al. using single-cell sequencing (48). Interestingly, we found that levels of pSTAT3 in circulating Th1 and Tfh cells were negatively correlated with the number of tender or swollen joints, although without reaching statistical significance. Th1 (and perhaps Tfh) cells expressing high levels of pSTAT3 might be recruited to sites of inflammation and contribute to tissue damage in PsA, although how these cells would induce damage, and the exact role of IFNγ in these processes, is currently unclear.

Tfh are a subset of CD4+ T cells in the germinal center that express CXCR5 and provide help to B cells for antibody production; circulating Tfh (cTfh) are considered a counterpart of Tfh, and alterations in the frequency of cTfh have been described in several autoimmune conditions (49). cTfh have been found to be increased in the circulation of patients with psoriasis (49), although pediatric PsA patients had a similar percentage of cTfh in comparison to healthy controls (50). Different subsets of T follicular (Tf) cells have been described, both in secondary lymphoid organs as well as in the circulation (51), including regulatory Tf (Tfr). We did not assess these subsets in our study, and further examination of Tfh associated markers is needed to validate our results (52). Nevertheless, an increase in IL-6/pSTAT3 signaling was found to favor development of Tfh over Tfr (53), suggesting that cTfh with high pSTAT3 might represent preferentially an effector rather than a regulatory subset and thus potentially contribute to disease immunopathology.

We also find that CD14+CD16- monocytes express higher pSTAT3 in active PsA patients. Although the percentage of CD14+ monocytes was not increased in the active compared to the inactive group, enhanced pSTAT3 expression could be related to osteoclast development in active PsA patients (54).

PsA has some similarities with RA, which is also a chronic inflammatory arthritic disease (55), and we did not find differences between active PsA and active RA in the parameters we tested, including pSTAT3 levels, after correction for multiple testing. Constitutive pSTAT3 has been described in CD4+ and CD8+ T cells, as well as in $CD14^{+}$ monocytes from RA patients (56). Jak inhibitors have shown efficacy for treating RA and PsA (57–61), consistent with a potentially important role of STAT3 in both diseases.

STAT3 is able to interact with several different transcription factors in different cell types, and activate diverse sets of genes, leading to distinct phenotypes in different conditions (62). Consistent with this, STAT3 is involved in various cellular processes including inflammation, proliferation, cell growth and differentiation and apoptosis. STAT3 is activated by several pro-inflammatory cytokines, such as IL-6, that may play a role in promoting STAT3 activation in PsA. In preliminary experiments, we found that serum from active PsA patients induces increased phosphorylation of STAT3 in CD4+ T cells from a healthy donor compared to serum from an inactive PsA patient (**Supplementary Figure 7**), suggesting the presence of circulating factors that promote STAT3 activation in active PsA patients.

This study has several limitations. We analyzed a small, heterogeneous sample, and further studies with larger number of patients are needed to confirm our findings. The absence of a healthy control precludes evaluating if PsA inactive patients have returned to healthy levels or are still in a state of compensated inflammation, as we described previously in systemic juvenile idiopathic arthritis (sJIA) (63). Furthermore, diversity within the major CD4+ T cell subsets, as well as within monocytes, was not analyzed and may reveal additional relationships between these

cells and PsA. For example, subsets of Th17, such as Th1-like Th17 cells, have been described in autoimmune diseases such as RA (64, 65). These ex-Th17 cells lose expression of IL-17 and produce IFNγ; interestingly, they gain the expression of CXCR3 (66) and could respond to the CXCL10 chemokine. Further studies on the functional capability of these cells will also be necessary to investigate the relevance of the higher pSTAT3 expression in circulating cells in PsA.

In summary, our results show that increased STAT3 signaling in circulating CD4+ T cells, especially Th1 and Tfh, as well as CD14+CD16- monocytes, is associated with active PsA. Evidence suggests that the elevated expression of STAT3 is associated with an effector rather than regulatory function for these cells. These cells may be recruited from the circulation into sites of inflammation and make an important contribution to PsA immunopathology.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the IRB at Carmel Medical Center (CMC 0044-11). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CM, DZ, and EM designed the research, interpreted the results and wrote the manuscript. CM and SR performed experiments and analyzed data. IL performed statistical analysis. AH and ME performed patient sample collection and processing. DS performed experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.758418/full#supplementary-material

REFERENCES

1. Ogdie A, Weiss P. The Epidemiology of Psoriatic Arthritis. *Rheum Dis Clin North Am* (2015) 41(4):545–68. doi: 10.1016/j.rdc.2015.07.001
2. Scotti L, Franchi M, Marchesoni A, Corrao G. Prevalence and Incidence of Psoriatic Arthritis: A Systematic Review and Meta-Analysis. *Semin Arthritis Rheum* (2018) 48(1):28–34. doi: 10.1016/j.semarthrit.2018.01.003
3. Ritchlin CT, Colbert RA, Gladman DD. Psoriatic Arthritis. *N Engl J Med* (2017) 376(10):957–70. doi: 10.1056/NEJMra1505557
4. Zisman D, Stoll ML, Butbul Aviel Y, Mellins ED. Juvenile Psoriatic Arthritis: A Report From the GRAPPA 2017 Annual Meeting. *J Rheumatol Suppl* (2018) 94:11–6. doi: 10.3899/jrheum.180131
5. McGonagle D, Lories RJ, Tan AL, Benjamin M. The Concept of a "Synovio-Entheseal Complex" and its Implications for Understanding Joint Inflammation and Damage in Psoriatic Arthritis and Beyond. *Arthritis Rheum* (2007) 56(8):2482–91. doi: 10.1002/art.22758
6. Scarpa R, Caso F, Costa L, Peluso R, Del Puente A, Olivieri I. Psoriatic Disease 10 Years Later. *J Rheumatol* (2017) 44(9):1298–301. doi: 10.3899/jrheum.161402
7. Feld J, Nissan S, Eder L, Rahat MA, Elias M, Rimar D, et al. Increased Prevalence of Metabolic Syndrome and Adipocytokine Levels in a Psoriatic Arthritis Cohort. *J Clin Rheumatol* (2018) 24(6):302–7. doi: 10.1097/RHU.0000000000000721
8. Ferguson LD, Siebert S, McInnes IB, Sattar N. Cardiometabolic Comorbidities in RA and PsA: Lessons Learned and Future Directions. *Nat Rev Rheumatol* (2019) 15(8):461–74. doi: 10.1038/s41584-019-0256-0
9. Veale JD, Fearon U. The Pathogenesis of Psoriatic Arthritis. *Lancet* (2018) 391(10136):2273–84. doi: 10.1016/S0140-6736(18)30830-4
10. Bravo A, Kavanaugh A. Bedside to Bench: Defining the Immunopathogenesis of Psoriatic Arthritis. *Nat Rev Rheumatol* (2019) 15(11):645–56. doi: 10.1038/s41584-019-0285-8
11. McGonagle DG, McInnes IB, Kirkham BW, Sherlock J, Moots R. The Role of IL-17A in Axial Spondyloarthritis and Psoriatic Arthritis: Recent Advances and Controversies. *Ann Rheum Dis* (2019) 78(9):1167–78. doi: 10.1136/annrheumdis-2019-215356
12. Durham LE, Kirkham BW, Taams LS. Contribution of the IL-17 Pathway to Psoriasis and Psoriatic Arthritis. *Curr Rheumatol Rep* (2015) 17(8):55. doi: 10.1007/s11926-015-0529-9
13. Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, et al. STAT3 Regulates Cytokine-Mediated Generation of Inflammatory Helper T Cells. *J Biol Chem* (2007) 282(13):9358–63. doi: 10.1074/jbc.C600321200
14. Yang L, Fanok MH, Mediero-Munoz A, Fogli LK, Corciulo C, Abdollahi S, et al. Augmented Th17 Differentiation Leads to Cutaneous and Synovio-Entheseal Inflammation in a Novel Model of Psoriatic Arthritis. *Arthritis Rheumatol* (2018) 70(6):855–67. doi: 10.1002/art.40447
15. Mountz JD. Editorial: STATus of STAT3 in Psoriatic Arthritis. *Arthritis Rheumatol* (2018) 70(6):801–4. doi: 10.1002/art.40445
16. Calautti E, Avalle L, Poli V. Psoriasis: A STAT3-Centric View. *Int J Mol Sci* (2018) 19(1):171. doi: 10.3390/ijms19010171
17. Cenit MC, Ortego-Centeno N, Raya E, Callejas JL, Garcia-Hernandez FJ, Castillo-Palma MJ, et al. Influence of the STAT3 Genetic Variants in the Susceptibility to Psoriatic Arthritis and Behcet's Disease. *Hum Immunol* (2013) 74(2):230–3. doi: 10.1016/j.humimm.2012.10.019
18. Bader L, Gullaksen S-E, Blaser N, Brun M, Bringeland GH, Sulen A, et al. Candidate Markers for Stratification and Classification in Rheumatoid Arthritis. *Front Immunol* (2019) 10:1488–. doi: 10.3389/fimmu.2019.01488
19. Leong JY, Chen P, Yeo JG, Ally F, Chua C, Nur Hazirah S, et al. Immunome Perturbation is Present in Patients With Juvenile Idiopathic Arthritis Who are in Remission and Will Relapse Upon Anti-Tnfα Withdrawal. *Ann Rheum Dis* (2019) 78(12):1712–21. doi: 10.1136/annrheumdis-2019-216059
20. Cheung P, Khatri P, Utz PJ, Kuo AJ. Single-Cell Technologies - Studying Rheumatic Diseases One Cell at a Time. *Nat Rev Rheumatol* (2019) 15(6):340–54. doi: 10.1038/s41584-019-0220-z

21. Taylor W, Gladman D, Helliwell P, Marchesoni A, Mease P, Mielants H. Classification Criteria for Psoriatic Arthritis: Development of New Criteria From a Large International Study. *Arthritis Rheum* (2006) 54(8):2665–73. doi: 10.1002/art.21972
22. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO3rd, et al. Rheumatoid Arthritis Classification Criteria: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum* (2010) 62(9):2569–81. doi: 10.1002/art.27584
23. Coates LC, Fransen J, Helliwell PS. Defining Minimal Disease Activity in Psoriatic Arthritis: A Proposed Objective Target for Treatment. *Ann Rheum Dis* (2010) 69(1):48–53. doi: 10.1136/ard.2008.102053
24. Aletaha D, Martinez-Avila J, Kvien TK, Smolen JS. Definition of Treatment Response in Rheumatoid Arthritis Based on the Simplified and the Clinical Disease Activity Index. *Ann Rheum Dis* (2012) 71(7):1190–6. doi: 10.1136/annrheumdis-2012-201491
25. Kunicki MA, Amaya Hernandez LC, Davis KL, Bacchetta R, Roncarolo MG. Identity and Diversity of Human Peripheral Th and T Regulatory Cells Defined by Single-Cell Mass Cytometry. *J Immunol* (2018) 200(1):336–46. doi: 10.4049/jimmunol.1701025
26. Tusher VG, Tibshirani R, Chu G. Significance Analysis of Microarrays Applied to the Ionizing Radiation Response. *Proc Natl Acad Sci USA* (2001) 98(9):5116–21. doi: 10.1073/pnas.091062498
27. Fiocco U, Martini V, Accordi B, Caso F, Costa L, Oliviero F, et al. Transcriptional Network Profile on Synovial Fluid T Cells in Psoriatic Arthritis. *Clin Rheumatol* (2015) 34(9):1571–80. doi: 10.1007/s10067-015-3002-2
28. Fiocco U, Accordi B, Martini V, Oliviero F, Facco M, Cabrelle A, et al. JAK/STAT/Pkcδ Molecular Pathways in Synovial Fluid T Lymphocytes Reflect the *In Vivo* T Helper-17 Expansion in Psoriatic Arthritis. *Immunol Res* (2014) 58(1):61–9. doi: 10.1007/s12026-013-8481-0
29. Raychaudhuri SK, Abria C, Raychaudhuri SP. Regulatory Role of the JAK STAT Kinase Signalling System on the IL-23/IL-17 Cytokine Axis in Psoriatic Arthritis. *Ann Rheum Dis* (2017) 76(10):e36. doi: 10.1136/annrheumdis-2016-211046
30. Gao W, McGarry T, Orr C, McCormick J, Veale DJ, Fearon U. Tofacitinib Regulates Synovial Inflammation in Psoriatic Arthritis, Inhibiting STAT Activation and Induction of Negative Feedback Inhibitors. *Ann Rheum Dis* (2016) 75(1):311–5. doi: 10.1136/annrheumdis-2014-207201
31. Geraghty P, Wyman AE, Garcia-Arcos I, Dabo AJ, Gadhvi S, Foronjy R. STAT3 Modulates Cigarette Smoke-Induced Inflammation and Protease Expression. *Front Physiol* (2013) 4:267. doi: 10.3389/fphys.2013.00267
32. Yuan F, Fu X, Shi H, Chen G, Dong P, Zhang W. Induction of Murine Macrophage M2 Polarization by Cigarette Smoke Extract *via* the JAK2/STAT3 Pathway. *PloS One* (2014) 9(9):e107063. doi: 10.1371/journal.pone.0107063
33. Thomas S, Fisher KH, Snowden JA, Danson SJ, Brown S, Zeidler MP. Methotrexate Is a JAK/STAT Pathway Inhibitor. *PloS One* (2015) 10(7):e0130078. doi: 10.1371/journal.pone.0130078
34. Wunderlich CM, Hovelmeyer N, Wunderlich FT. Mechanisms of Chronic JAK-STAT3-SOCS3 Signaling in Obesity. *Jakstat* (2013) 2(2):e23878. doi: 10.4161/jkst.23878
35. Soltani-Arabshahi R, Wong B, Feng BJ, Goldgar DE, Duffin KC, Krueger GG. Obesity in Early Adulthood as a Risk Factor for Psoriatic Arthritis. *Arch Dermatol* (2010) 146(7):721–6. doi: 10.1001/archdermatol.2010.141
36. Samad A, Stoll ML, Lavi I, Hsu JJ, Strand V, Robinson TN, et al. Adiposity in Juvenile Psoriatic Arthritis. *J Rheumatol* (2018) 45(3):411–8. doi: 10.3899/jrheum.170598
37. Galindez E, Carmona L. Is Obesity in Psoriatic Arthritis Associated With a Poorer Therapeutic Response and More Adverse Effects of Treatment With an Anchor Drug? *Reumatol Clin* (2016) 12(6):307–12. doi: 10.1016/j.reumae.2015.12.002
38. Winer S, Paltser G, Chan Y, Tsui H, Engleman E, Winer D, et al. Obesity Predisposes to Th17 Bias. *Eur J Immunol* (2009) 39(9):2629–35. doi: 10.1002/eji.200838893
39. Endo Y, Asou HK, Matsugae N, Hirahara K, Shinoda K, Tumes DJ, et al. Obesity Drives Th17 Cell Differentiation by Inducing the Lipid Metabolic Kinase, Acc1. *Cell Rep* (2015) 12(6):1042–55. doi: 10.1016/j.celrep.2015.07.014
40. Ma CS, Wong N, Rao G, Nguyen A, Avery DT, Payne K, et al. Unique and Shared Signaling Pathways Cooperate to Regulate the Differentiation of Human CD4+ T Cells Into Distinct Effector Subsets. *J Exp Med* (2016) 213(8):1589–608. doi: 10.1084/jem.20151467
41. Sun M, Wu W, Chen L, Yang W, Huang X, Ma C, et al. Microbiota-Derived Short-Chain Fatty Acids Promote Th1 Cell IL-10 Production to Maintain Intestinal Homeostasis. *Nat Commun* (2018) 9(1):3555. doi: 10.1038/s41467-018-05901-2
42. Zhang S, Dai X, Shi Y, Zhu X, Dai Y, Qian X, et al. CHI3L1 Alleviate Acute Liver Injury by Inhibiting Th1 Cells Differentiation Through STAT3 Signaling Pathway. *Ann Transl Med* (2021) 9(7):529. doi: 10.21037/atm-20-6127
43. Christophers E, Metzler G, Röcken M. Bimodal Immune Activation in Psoriasis. *Br J Dermatol* (2014) 170(1):59–65. doi: 10.1111/bjd.12631
44. Harden JL, Johnson-Huang LM, Chamian MF, Lee E, Pearce T, Leonardi CL, et al. Humanized Anti-IFN-γ (HuZAF) in the Treatment of Psoriasis. *J Allergy Clin Immunol* (2015) 135(2):553–6. doi: 10.1016/j.jaci.2014.05.046
45. Diani M, Casciano F, Marongiu L, Longhi M, Altomare A, Pigatto PD, et al. Increased Frequency of Activated CD8(+) T Cell Effectors in Patients With Psoriatic Arthritis. *Sci Rep* (2019) 9(1):10870. doi: 10.1038/s41598-019-47310-5
46. Diani M, Galasso M, Cozzi C, Sgambelluri F, Altomare A, Cigni C, et al. Blood to Skin Recirculation of CD4(+) Memory T Cells Associates With Cutaneous and Systemic Manifestations of Psoriatic Disease. *Clin Immunol* (2017) 180:84–94. doi: 10.1016/j.clim.2017.04.001
47. Casciano F, Pigatto PD, Secchiero P, Gambari R, Reali E. T Cell Hierarchy in the Pathogenesis of Psoriasis and Associated Cardiovascular Comorbidities. *Front Immunol* (2018) 9:1390. doi: 10.3389/fimmu.2018.01390
48. Penkava F, Velasco-Herrera MDC, Young MD, Yager N, Nwosu LN, Pratt AG, et al. Single-Cell Sequencing Reveals Clonal Expansions of Pro-Inflammatory Synovial CD8 T Cells Expressing Tissue-Homing Receptors in Psoriatic Arthritis. *Nat Commun* (2020) 11(1):4767. doi: 10.1038/s41467-020-18513-6
49. Gensous N, Charrier M, Duluc D, Contin-Bordes C, Truchetet ME, Lazaro E, et al. T Follicular Helper Cells in Autoimmune Disorders. *Front Immunol* (2018) 9:1637. doi: 10.3389/fimmu.2018.01637
50. Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human Blood CXCR5(+)CD4(+) T Cells are Counterparts of T Follicular Cells and Contain Specific Subsets That Differentially Support Antibody Secretion. *Immunity* (2011) 34(1):108–21. doi: 10.1016/j.immuni.2010.12.012
51. Deng J, Wei Y, Fonseca VR, Graca L, Yu D. T Follicular Helper Cells and T Follicular Regulatory Cells in Rheumatic Diseases. *Nat Rev Rheumatol* (2019) 15(8):475–90. doi: 10.1038/s41584-019-0254-2
52. Vella LA, Buggert M, Manne S, Herati RS, Sayin I, Kuri-Cervantes L, et al. T Follicular Helper Cells in Human Efferent Lymph Retain Lymphoid Characteristics. *J Clin Invest* (2019) 129(8):3185–200. doi: 10.1172/JCI125628
53. Niu Q, Huang ZC, Wu XJ, Jin YX, An YF, Li YM, et al. Enhanced IL-6/Phosphorylated STAT3 Signaling is Related to the Imbalance of Circulating T Follicular Helper/T Follicular Regulatory Cells in Patients With Rheumatoid Arthritis. *Arthritis Res Ther* (2018) 20(1):200. doi: 10.1186/s13075-018-1690-0
54. Yang Y, Chung MR, Zhou S, Gong X, Xu H, Hong Y, et al. STAT3 Controls Osteoclast Differentiation and Bone Homeostasis by Regulating NFATc1 Transcription. *J Biol Chem* (2019) 294(42):15395–407. doi: 10.1074/jbc.RA119.010139
55. Merola JF, Espinoza LR, Fleischmann R. Distinguishing Rheumatoid Arthritis From Psoriatic Arthritis. *RMD Open* (2018) 4(2):e000656. doi: 10.1136/rmdopen-2018-000656
56. Kuuliala K, Kuuliala A, Koivuniemi R, Oksanen S, Hamalainen M, Moilanen E, et al. Constitutive STAT3 Phosphorylation in Circulating CD4+ T Lymphocytes Associates With Disease Activity and Treatment Response in Recent-Onset Rheumatoid Arthritis. *PloS One* (2015) 10(9):e0137385. doi: 10.1371/journal.pone.0137385
57. Gladman D, Rigby W, Azevedo VF, Behrens F, Blanco R, Kaszuba A, et al. Tofacitinib for Psoriatic Arthritis in Patients With an Inadequate Response to TNF Inhibitors. *N Engl J Med* (2017) 377(16):1525–36. doi: 10.1056/NEJMoa1615977
58. Mease P, Hall S, FitzGerald O, van der Heijde D, Merola JF, Avila-Zapata F, et al. Tofacitinib or Adalimumab Versus Placebo for Psoriatic Arthritis. *N Engl J Med* (2017) 377(16):1537–50. doi: 10.1056/NEJMoa1615975

59. Mease P, Coates LC, Helliwell PS, Stanislavchuk M, Rychlewska-Hanczewska A, Dudek A, et al. Efficacy and Safety of Filgotinib, a Selective Janus Kinase 1 Inhibitor, in Patients With Active Psoriatic Arthritis (EQUATOR): Results From a Randomised, Placebo-Controlled, Phase 2 Trial. *Lancet* (2018) 392 (10162):2367–77. doi: 10.1016/S0140-6736(18)32483-8
60. Taylor PC. Clinical Efficacy of Launched JAK Inhibitors in Rheumatoid Arthritis. *Rheumatology (Oxford England)* (2019) 58(Supplement_1):i17–26. doi: 10.1093/rheumatology/key225
61. Harrington R, Al Nokhatha SA, Conway R. JAK Inhibitors in Rheumatoid Arthritis: An Evidence-Based Review on the Emerging Clinical Data. *J Inflamm Res* (2020) 13:519–31. doi: 10.2147/JIR.S219586
62. Vallania F, Schiavone D, Dewilde S, Pupo E, Garbay S, Calogero R, et al. Genome-Wide Discovery of Functional Transcription Factor Binding Sites by Comparative Genomics: The Case of Stat3. *Proc Natl Acad Sci* (2009) 106 (13):5117–22. doi: 10.1073/pnas.0900473106
63. Macaubas C, Nguyen KD, Peck A, Buckingham J, Deshpande C, Wong E, et al. Alternative Activation in Systemic Juvenile Idiopathic Arthritis Monocytes. *Clin Immunol* (2012) 142(3):362–72. doi: 10.1016/j.clim.2011.12.008
64. Basdeo SA, Cluxton D, Sulaimani J, Moran B, Canavan M, Orr C, et al. Ex-Th17 (Nonclassical Th1) Cells Are Functionally Distinct From Classical Th1 and Th17 Cells and Are Not Constrained by Regulatory T Cells. *J Immunol* (2017) 198(6):2249–59. doi: 10.4049/jimmunol.1600737
65. Yang P, Qian FY, Zhang MF, Xu AL, Wang X, Jiang BP, et al. Th17 Cell Pathogenicity and Plasticity in Rheumatoid Arthritis. *J Leukoc Biol* (2019) 106 (6):1233–40. doi: 10.1002/JLB.4RU0619-197R
66. Sallusto F, Zielinski CE, Lanzavecchia A. Human Th17 Subsets. *Eur J Immunol* (2012) 42(9):2215–20. doi: 10.1002/eji.201242741

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REVIEW
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Inflammasome and Its Therapeutic Targeting in Rheumatoid Arthritis

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Inflammasome is a cytoplasmic multiprotein complex that facilitates the clearance of exogenous microorganisms or the recognition of endogenous danger signals, which is critically involved in innate inflammatory response. Excessive or abnormal activation of inflammasomes has been shown to contribute to the development of various diseases including autoimmune diseases, neurodegenerative changes, and cancers. Rheumatoid arthritis (RA) is a chronic and complex autoimmune disease, in which inflammasome activation plays a pivotal role in immune dysregulation and joint inflammation. This review summarizes recent findings on inflammasome activation and its effector mechanisms in the pathogenesis of RA and potential development of therapeutic targeting of inflammasome for the immunotherapy of RA.

Keywords: inflammasome, autoimmunity, immunotherapy, rheumatoid arthritis, inflammation

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease that manifests as persistent inflammation of the synovial joints, leading to synovial tissue proliferation, cartilage erosion and consequent joint deformation with functional limitations (1–3). RA occurs at any age, there are more than 20 million prevalent cases of RA, given the general increase in life expectancy worldwide, and the number of elderly patients with RA is increasing annually. Globally, the age-standardized point annual incidence of RA has increased by 8.2% compared to 1990 (4, 5). The ratio of male patients to female patients with RA is approximately 1:3, which is possibly associated with the stimulation of the immune system by estrogen (6, 7). The onset of RA is also associated with pregnancy and menopause (8, 9). Although the exact pathogenesis of RA remains unclear, genetics, smoking, obesity, infections, periodontal disease, and even gut microbiota are currently thought to be associated with the development of RA (10, 11). For example, the HLA-DRB1 gene within the human leukocyte antigen (HLA) locus is associated with increased susceptibility and severity of RA, although genetic susceptibility factors for RA are significantly different between Asian and European populations, the HLA-DRB1 gene is a common susceptibility gene in all populations (12, 13). The DRB1 shared epitope allele also synergizes with smoking and increases the risk of anti-citrullinated protein antibody (ACPA)-positive RA (14). The levels of anti-cyclic citrullinated peptide (CCP) IgG antibodies, rheumatoid factor (RF), erythrocyte sedimentation rate (ESR) and

C-reactive protein (CRP) in blood reflect the extent of inflammation and tissue damage in RA patients (15). Interleukin (IL)-1β, IL-6, IL-18 and tumor necrosis factor (TNF) are the major proinflammatory cytokines in RA. IL-1β enhances the secretion of chemokines and cytokines, promotes Th17 cell differentiation and reduces the synthesis of cartilage components (15). Moreover, the severity of RA is positively correlated with serum IL-18 levels (16–18). These findings indicate an important role of inflammation in the pathogenesis of RA.

The human immune system is composed of the innate immune system and the adaptive immune system. The innate immune system consists of anatomical barriers (skin, mucous membranes), hematopoietic cells (such as macrophages, dendritic cells, monocytes), nonhematopoietic cells (such as epithelial cells), and the complement system (19). Unlike the adaptive immune cells, which are antigen-specific and capable of generating immunological memory, innate immune cells are pre-programmed to recognize molecules shared by broad categories of pathogens or pathological situations, such as pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), homeostasis-altering molecular processes (HAMPs), and pattern recognition receptors (PRRs) (20, 21). Based on their locations, PRRs are categorized into membrane PRRs, cytoplasmic PRRs, and secretory PRRs. Based on their structures, PRRs are categorized into Toll-like receptors (TLRs), NOD-like receptors (NLRs), c-type lectin receptors (CLRs), and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (22, 23). NLRs are cytoplasmic PRRs that play a bridging role between innate and adaptive immunity by activating a variety of inflammatory factor precursors and inducing the release of inflammatory factors (22, 23). Inflammation itself is a protective mechanism of the organism in response to internal and external stimuli; a moderate inflammatory response contributes to the stability of the body's internal environment, whereas excessive or persistent inflammation will lead to cancer or other diseases (22, 23). Since the early response to inflammatory reactions is achieved mainly by stimulating inflammasomes, it is particularly important to understand the activation process of inflammasomes (24–26). However, current knowledge of about the role of inflammasomes in the pathogenesis of RA remains incomplete. This review will systematically describe the classification, structure, and activation mechanisms of inflammasomes and discuss about the role of inflammasomes and their therapeutic targeting in RA treatment.

OVERVIEW OF INFLAMMASOMES

In 2002, Martinon et al. firstly described inflammasomes as multiprotein platforms formed by organisms in response to various pathogenic or physiological factors (27). These oligomeric protein complexes can respond to a variety of ligands and have unique activation and regulatory mechanisms. There are two types of inflammasomes: the canonical inflammasomes that activate caspase-1, including NLRP1, NLRP3, NOD-like receptor family apoptosis inhibitory protein (NAIP)-NLRC4, NLRP6, NLRP7, NLRP9, NLRP12, absent in melanoma (AIM) 2, and pyrin inflammasomes; The noncanonical inflammasomes can activate caspase-4/5 (human) or caspase-11 (murine) (27–30) **(Figure 1)**. Canonical inflammasomes are composed of three components: a sensor molecule (responsible for DAMP/PAMP recognition), an adapter protein [apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC)], and an effector molecule (pro-caspase-1) (31). The sensors include members of the NLR family, the AIM2-like receptor (ALR) family and pyrin, recognizing specific ligands to promote the assembly of inflammasomes. NLRs are composed of the N-terminal effector domains, pyrin domain (PYD) or caspase recruitment domain (CARD) or baculovirus inhibitor of apoptosis protein repeat (BIR), the central nucleotide-binding domain (NBD) or NACHT domain and the C-terminal leucine-rich repeat (LRR). According to the N-terminal domains, NLRs can be divided into five subfamilies: NLRA, NLRB, NLRC, NLRP and NLRX1. The human genome contains 23 NLR genes, and the mouse genome contains more than 30 NLR genes. These genes are expressed in a variety of tissues and cells; however, only a few NLR proteins form inflammasomes, and most known inflammasomes contain NLR structures (32–34). Sensors of the ALR family include AIM2 and human interferon (IFN)-g-inducible protein (IFI)16 (29, 35). ASC consists of a PYD and a CARD. ASCs mediate the oligomerization of components of inflammasomes and the signaling for caspase activation through homotypic PYD-PYD or CARD-CARD interactions coupled to upstream PRRs. These inflammasomes that require the adapter protein ASC for activation and assembly are called ASC-dependent inflammasomes, including NLRP3 and AIM2. ASC-independent inflammasomes such as NLRP1 and NAIP-NLRC4 directly activate caspase-1 *via* the CARD domain (36, 37). Caspases that are involved in inflammatory responses include human caspases-1, caspases-4 and caspases-5, as well as mouse caspases-1 and caspases-11 (38, 39). Active inflammatory caspases exert proinflammatory effects by cleaving pro-IL-1β and pro-IL-18 into active IL-1β and IL-18 *via* protein hydrolysis and promote pore-forming protein gasdermin D (GSDMD) cleavage to induce pyroptosis (38) **(Figure 2)**. This review focuses on the accumulation of PRR on the caspase-1 activation platform and inflammasomes in RA. This platform regulates the synthesis and activation of IL-1β and IL-18, which are the main inflammatory cytokines involved in RA.

NLRP3

NLRP3 is an important inflammasomethathas beenextensively investigated and can be activated by multiple factors. Endogenous molecules include adenosine triphosphate (ATP), the heat shock protein HSP70, and uric acid crystals monosodium urate (MSU). Exogenous activators include lipopolysaccharides (LPS), components of the cell wall, microbial-specific nucleic acid structures, *Candida albicans*, and influenza viruses (40–44). Generally, NLRP3 has three distinct activation mechanisms, including altered ion flow

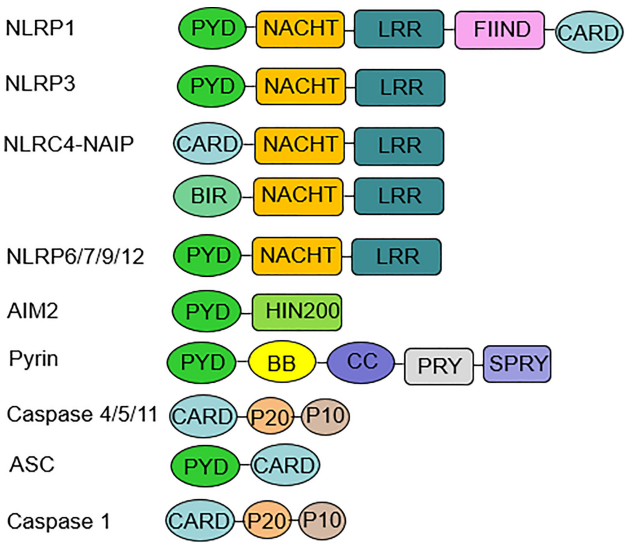


FIGURE 1 | Inflammasome components and domain structure. Inflammasome complexes are formed by the oligomerization of several protein domains. A typical inflammasome consists of three parts: a sensor molecule, the adaptor protein ASC and the effector molecule pro-caspase-1. The sensors include NLRs, AIM2, and pyrin. NLRP3, 6, 7, 9, and 12 sensors all have a PYD at the N-terminus, an NBD or NACHT in the middle, and an LRR domain at the C-terminus. AIM2 consists of a PYD at the N-terminus and a HIN-200 domain at the C-terminus. ASC is required for the formation of NLRP3, 6, 7, 9, 12, and AIM2 inflammasomes. ASC mediates signaling to promote pro-caspase-1 activation through homotypic PYD-PYD or CARD-CARD interactions. The NLRP1 sensor also has a PYD at the N-terminus and a CARD at the C-terminus, which can bind directly to caspase-1 independent of ASC, and NLRP1 has a unique FIIND domain that is involved in inflammasome activation through its own protein hydrolysis. NLRC4 has an N-terminal CARD, an intermediate NBD or NACHT, and a C-terminal LRR domain. NAIP is required for NLRC4 to recognize PAMP, and then NLRC4 self-activates and oligomerizes to form the NAIP-NLRC4 inflammasome. Noncanonical inflammasomes include human caspase-4, caspase-5, and murine caspase-11. *ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; AIM2, absent in melanoma AIM2; PYD, pyrin domain; NBD, nucleotide-binding domain; CARD, caspase recruitment domain; PAMP, pathogen-associated molecular patterns.*

(K^+efflux, Cl^- flow and Ca^+ flow), mitochondria-derived ROS production, and lysosomal rupture (45–49). Activation of typical NLRP3 requires two steps: microbial or endogenous cytokines bind to cell membrane receptors to activate nuclear factor-κB (NF-κB), increasing the expression levels of NLRP3 and pro-IL-1β, and then PAMPs/DAMPs/HAMPs trigger activation signals that recruit ASC and caspase-1 to form an inflammasome complex, which in turn drives caspase-1 self-cleavage and activation. Activated caspase-1 induces pro-IL-1β and pro-IL-18 cleavage to produce IL-1β and IL-18, which promote inflammatory responses, as well as shear GSDMD proteins, which induce pyrolysis (50, 51). Inflammasomes can also be activated *via* a noncanonical pathway. LPS release from gram-negative bacteria can lead to noncanonical inflammasome activation by initiating caspase-4/5 or murine caspase-11 signaling. Activation of these caspases promotes pore generation and K^+ efflux from the cytoplasm to activate NLRP3 inflammasomes, which subsequently induce IL-1β and IL-18 maturation in a manner similar to the canonical inflammasome pathway (52–54). Recent studies have shown that Msn family kinase MINK1 is directly involved in regulating NLRP3 inflammasome (55).

AIM2

AIM2 is well known for its ability to recognize intracellular double-stranded DNA (dsDNA), notably, host or pathogen-derived DNA in the cytoplasm (35). In normal cells, DNA is in the nucleus while the presence of DNA in the cytoplasm indicates compromised nuclear membrane integrity or infection (56). AIM2 is composed of two domains: the amino-terminal PYD and the carboxy-terminal HIN-200 domain (27). AIM2 binds to dsDNA in a sequence-independent manner, requiring a dsDNA length of 70 bp for activation in human and mouse cells, so the assembly of AIM2 is influenced by the length of the dsDNA (57, 58). Although AIM2 is considered as a cytoplasmic receptor, it is found that murine AIM2 is transported to the nucleus in response to ionizing radiation-induced DNA damage (59). However, in human monocytes, the cGAS-STING axis replaces the DNA inflammasome sensor function of AIM2 and triggers cell death in direct response to cytoplasmic DNA *via* the cGAS-STING-lysosome-NLRP3 pathway (60). In the presence of IFN-α, AIM2 can participate in the *Toxoplasma* response (61). All of these results suggest that AIM2 function in human cells may differ depending on the environment, and the exact mechanisms need to be further investigated. A recent study showed that AIM2 exacerbates atherosclerosis during clonal hematopoiesis and that treatment targeting inflammasomes may also reduce the risk of cardiovascular disease (62).

NLRP1

Human NLRP1 was the first inflammasome to be identified and has two extra domains compared with NLRP3 (**Figure 1**). The

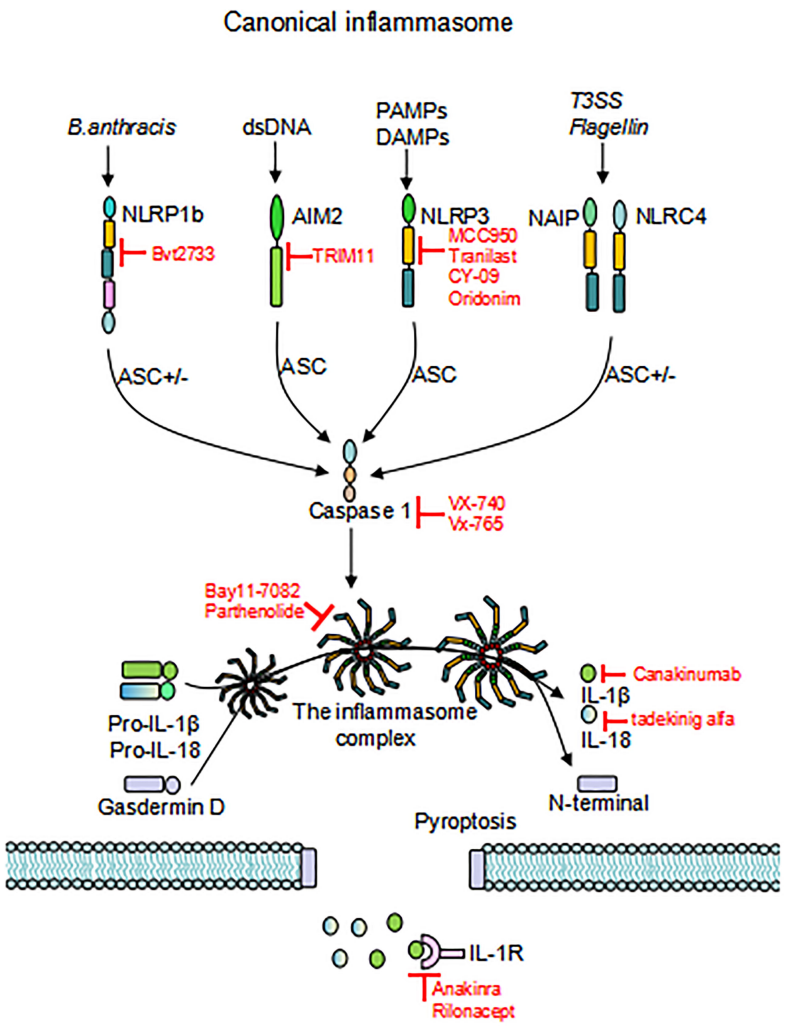


FIGURE 2 | Activation of inflammasomes. Different inflammasome sensors sense different ligands, canonical inflammasome activation occurs through the response to PAMP/DAMP/HAMP. Upon initiation and activation, the inflammasome complex assembles, inducing caspase-1 self-cleavage and activation, and cleaves GSDMD to release the N-terminal domain and induce pyroptosis. Activated caspase-1 promotes cleavage of pro-IL-1β and pro-IL-18 to activate IL-1β and IL-18, which are released through the GSDMD pore. Inhibitors that target inflammasomes are also depicted in the Figure. *"ASC+/-"means this activation pathway is independent of ASC; PAMP, pathogen-associated molecular patterns; DAMP, damage-associated molecular patterns; HAMP, homeostasis-altering molecular processes; GSDMD, gasdermin D.*

function-to-find (FIIND) domain is currently thought to be present in only two inflammasomes, NLRP1 and CARD8. The exact mechanism of the regulation of NLRP1 is unclear (63). Unlike human NLRP1, mouse NLRP1 carries three paralogs (a-c), of which NLRP1b has been most well characterized and can be activated by the anthrax lethal toxin produced by *Bacillus anthracis* (64, 65). The eukaryote *Toxoplasma gondii* and the bacterium *Shigella flexneri* can activate human NLRP1 and murine NLRP1b (66, 67). Recent studies have shown that multiple viral proteases can also activate NLRP1 (68). Cytosolic dipeptidyl peptidase 9 (DPP9) can inhibit NLRP1 activation by closing the C-terminus of NLRP1, and the ZU5 domain is required for autoinhibition of human NLRP1 (69, 70). NLRP1 is the most prominent inflammasome manifested in human skin diseases. NLRP1 mutants lacking PYD are more likely to form ASC spots, whereas in AIM2 and NLRP3, PYD enhances ASC assembly, so the role of PYD varies in different inflammasomes (71).

NAIP-NLRC4

The intermediate structure of NAIP-NLRC4 is NACHT (also known as NOD, a characteristic domain shared by the NLR family that mediates its oligomerization) with LRRs at the C-terminus that recognize and bind ligands (72). Human and mouse NAIP-NLRC4 can be activated by type three secretion system (T3SS) proteins and flagellin (73, 74). Only one NAIP

protein isoform exists in humans, whereas mice have seven NAIP paralogs that can each bind different ligands (74). NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, NAIP5 and NAIP6 recognize bacterial flagellin, and the ligands for NAIP3, 4, and 7 are currently unknown (75, 76). NAIP recognizes PAMP to activate NLRC4, and then NLRC4 self-activates, oligomerizes and forms NAIP-NLRC4 inflammasomes, causing a subsequent series of cascade reactions (77). Unlike NLRP3, NLRC4 activation is not ASC-dependent, and the secretion of IL-1β and IL-18 is reduced in the absence of ASC, while cell scorching is unaffected (78). These findings indicate that ASCs play a critical role in the secretion of proinflammatory cytokines.

Other Inflammasomes

NLRP6 recruits ASC to form inflammasomes with caspase-1/11 and plays a role in intestinal diseases (79). The mRNA and protein levels of NLRP6 are reduced in fibroblast-like synovial cells (FLSs) and synovial tissue of RA patients. Overexpression of NLRP6 in RA-FLSs was associated with suppressed activation of NF-κB and reduced proinflammatory cytokines (80). NLRP6 is a negative regulator of inflammation in RA. There are no detailed reports on NLRP7, NLRP9, and pyrin inflammatory vesicles in RA. NLRP12 exhibits inflammasome properties in some specific infections but acts as a negative regulator in intestinal diseases (81). Recent studies have shown that NLRP12 knockout ($NLRP12^{-/-}$) in a mouse model of antigen-induced arthritis (AIA) with an increased Th17-associated inflammatory response develops more severe arthritis, and NLRP12 negatively regulates STAT3 phosphorylation of the IL-6 pathway (82). Overexpression of NLRP12 inhibited the proliferation of RA-FLSs and downregulated inflammatory cytokines, including IL-6, IL-1β, and TNF-α. NLRP12 knockdown promoted the phosphorylation of NF-κB, ERK, JNK, and p38, indicating NLRP12 is also a negative regulator of inflammation in RA (83).

INFLAMMASOME IN RA

NLRP3

Expression of NLRP3 in Animals

Inflammasomes have been studied in animal models of RA and in humans to help understand their role in pathogenesis. Synovial NLRP3 expression is increased in the collagen-induced arthritis (CIA) model, and positively correlates with radiological destruction and arthritis severity (84, 85). ASC knockout ($ASC^{-/-}$) mice are protected from arthritis, while caspase-1 knockout ($caspase\text{-}1^{-/-}$) and NLRP3 knockout ($NLRP3^{-/-}$) mice are susceptible to CIA (86). The expression of NLRP3 inflammasomes is also increased in the adjuvant arthritis (AA) model. Silence of the NLRP3 gene downregulated matrix metalloproteinase (MMP)-1 and IL-1β (87).

Expression of NLRP3 in Humans

Several studies have demonstrated that NLRP3 is activated in RA patients. NLRP3 and IL-1β secretion are elevated in peripheral blood mononuclear cells (PBMCs) from RA patients (88, 89). It was also found that miR-33 level was significantly increased in PBMCs from RA patients, which enhanced NLRP3 inflammatory vesicle expression (90). IL-18 and IL-1β levels in bronchoalveolar lavage fluid (BALF) were also elevated in RA-usual interstitial pneumonia (RA-UIP) patients (91). Unlike macrophages or monocytes, NLRP3 mRNA levels, ASC and pro-caspase-1 levels were reduced in neutrophils from RA patients, while the level of active caspase-1 was elevated and positively correlated with the CRP-based 28 joint disease activity score (DAS28-CRP). Caspase-1 activation was not correlated with IL-1β levels but positively correlated with serum IL-18 levels (18). Notably, NLRP3 was activated in $CD4^{+}$T cells of RA patients, and its activation correlated with serum IL-17A concentrations and disease activity. Th17 cell differentiation was inhibited after NLRP3 knockdown, suggesting that NLRP3 not only increased inflammatory cytokines in RA patients but also exerted pathogenic effects by promoting Th17 cell differentiation (92). NLRP3 and ASC expression in synovial tissue of RA patients were higher than those in osteoarthritis patients (93). Interestingly, IL-1β levels were higher in ACPA-positive RA patients, and ACPA may activate the Akt/NF-κB signaling pathway through enhanced interaction with CD147, stimulating IL-1β production by macrophages (94). Two recent studies have shown that calcium-sensitive receptors (CaSR) in RA patients can mediate NLRP3 inflammasome activation, increase IL-1β levels and exacerbate joint and systemic inflammation (95). Complement C1q can act synergistically with PTX3 to promote NLRP3 inflammasome scorching and hyperactivation in RA patients (96).

Genetic Polymorphisms of NLRP3 in RA

Genetic polymorphisms of inflammasomes are associated with the inheritance of RA (**Table 1**). It has been shown that single nucleotide polymorphisms (SNPs) in NLRP3 and CARD8 are related to increased susceptibility to RA and response to anti-TNF therapy (97, 98). Carriers of the NLRP3 (rs10754558) gene variant were more likely to have a negative response to anti-TNF treatment (99). A study from Brazil also confirmed that polymorphisms in CARD8 and NLRP3 wererelated to RA susceptibility and disease severity (100). A study from northern Sweden showed that CARD8-X was related to disease severity in early RA (101). Another Swedish study showed that genetic variants in NLRP3 were associated with the risk of transient ischemic attack (TIA) or stroke in RA patients (102). Genetic polymorphisms in cryopyrin (CIAS1) and TUCAN (CARD8) were related to both RA disease severity and susceptibility (104). However, contradictory results were also reported, with polymorphisms in NLRP3 (p.Q705K) and CARD8 (p.C10X) not related to RA susceptibility in French or Tunisian populations (103). While a retrospective study of 1530 patients with RA in Spain concluded that CARD8 rs2043211 gene variants were not associated with the severity of cardiovascular disease development and disease susceptibility in RA patients, it is controversial whether inflammasomes are related to RA-complicated cardiovascular disease (105).

TABLE 1 | The relationship between inflammasome SNPs and RA.

	SNP	Study population	Association	Ref.
NLRP3	rs10159239	Caucasian	associated with RA susceptibility and anti-TNF response	(97)
	rs4612666	Denmark	associated with anti-TNF response	(98)
	rs10754558	Denmark	associated with anti-TNF response	(99)
	rs10754558	Brazil	associated with RA susceptibility and severity	(100)
	rs35829419	Sweden	not associated with an increased susceptibility	(101)
	rs35829419	Sweden	associated with an increased risk of stroke/transient ischemic attack	(102)
	rs35829419	France, Tunisia	not associated with an increased susceptibility	(103)
CARD8	rs16981845	Caucasian	associated with RA susceptibility and anti-TNF response	(97)
	rs2043211	Brazil	associated with RA susceptibility and severity	(100)
	rs2043211	Sweden	associated with a worse disease course in early RA	(101)
	rs2043211	Sweden	not associated with any type of CV event	(102)
	rs2043211	Sweden	associated with RA susceptibility and severity	(104)
	rs2043211	France, Tunisia	not associated with an increased susceptibility	(103)
	rs2043211	Spain	not associated with RA susceptibility and the development of CV disease	(105)
NLRP1	rs878329G	Han Chinese	Increase risk of RA	(106)
	rs6502867 T/C	Chinese Singaporean	not associated with risk of RA	(107)
	rs6502867 C/T	Chinese	not associated with risk of RA	(108)
	rs878329 C/G			

SNP, single nucleotide polymorphisms; CV, cardiovascular.

Inhibition of NLRP3-Associated Signaling Pathway

Inhibition of NLRP3-associated signaling pathways may become an effective way to treat NLRP3-mediated diseases. Some studies have shown that overexpression of miRNA-20a resulted in reduced NLRP3 expression and decreased secretion of inflammatory cytokines, including MMP-1 and IL-1β. MicroRNA-20a may downregulate Thioredoxin-interacting protein (TXNIP) expression, thereby inhibiting the NLRP3 inflammasome (87). Protectin DX (PDX) was also shown to inhibit NLRP3 expression *via* the miRNA-20a pathway, regulate Treg/Th17 cell homeostasis and significantly delay disease progression in CIA models (109). A recent study showed that IL-6 could induce activation of the NLRP3 inflammasome *via* the cathepsin B (CTSB)/S100A9-mediated pathway and promote joint inflammation in CIA mice, suggesting that the IL-6/NLRP3 pathway may also be a novel target for RA therapy (110). In addition, tofacitinib restores the cellular balance of γδ-Treg/γδ-T17 cells in the CIA model, and a balanced γδ-Treg/γδ-T17 cell ratio inhibits NLRP3 expression and reduces IL-1β secretion (111). Recent studies have shown increased expression of long noncoding RNA myocardial infarction-associated transcript (lncRNA MIAT) in the myocardial tissue and synovium of CIA mice. LncRNA MIAT suppresses TNF-α and IL-1β expression but is inhibited by the ATP-activated NLRP3 inflammasome. Macrophage infiltration is increased in CIA tissues, and LPS-induced macrophage inflammation can in turn upregulate lncRNA MIAT expression; thus, lncRNA MIAT in macrophages may become a new target for RA therapy (112).

AIM2

With the in-depth study of NLRP3, NLRP3-associated AIM2 inflammasomes as cytoplasmic receptor are becoming the focus of recent research in RA pathogenesis. Pannus is formed in the RA joint due to vascular hyperplasia, and there is a hypoxic microenvironment inside the joint. Hypoxia causes mitochondrial or nuclear DNA damage, and since mtDNA is closer to the respiratory chain, it is more likely to be damaged in oxidative stress. RA patients have higher mtDNA levels in the plasma and synovial tissue than those in healthy controls, and are more likely to activate AIM2 inflammasomes (113–115). A meta-analysis showed that AIM2 gene expression levels were significantly upregulated in PBMCs from RA patients (116). Arthritis-prone mice with AIM2 deficiency exhibited significantly attenuated joint inflammation and histopathological changes (117, 118). Two new studies showed different results: monocytes in RA patients were more likely to release IL-1β in the absence of AIM2 inflammasome signaling (119). The serum AIM2 levels were lower in RA patients than that in healthy controls, while the levels of caspase-1, ASC, IL-1β, and molecules associated with AIM2 inflammasomes, were higher than those in healthy controls, plus positively correlated with the levels of CRP and ESR. AIM2 levels were higher in FLSs of RA patients than those in osteoarthritis (OA), and FLS proliferation was inhibited by silencing AIM2 in FLSs. Therefore, despite the inconsistent results, AIM2 remains a target for RA therapy (120). TRIM11 is a negative regulator of AIM2, which can degrade AIM2 in a p62-dependent manner (121).

NLRP1

In the adjuvant arthritis (AA) rat model, the NALP1 inflammasome was activated, and carboxyamidotriazole (CAI) reduced proinflammatory cytokine secretion by inhibiting the NF-κB signaling pathway and suppressing NALP1 activation, which may be beneficial for RA treatment (122). An inhibitor of 11 β-hydroxysteroid dehydrogenase 1 (11β-HSD1), bvt2733, has been shown to reduce joint symptoms and decrease serum IL-1β, IL-17, TNF-α and IL-6 levels in CIA mice by inhibiting NLRP1 inflammasomes and NF-κB signaling pathways (123). The purinergic receptor P2X4 antisense oligonucleotide (AS-ODN) plays a therapeutic role in reducing the clinical scores of CIA by inhibiting the NLRP1 signaling pathway (124). A study with a

large sample size showed that the NLRP1 and NLRP3 genes were associated with RA, *via* analyzing PBMCs expression profiles in RA patients (125). A study from France also suggested that mutations in the NLRP1 gene may be related to the development of RA (126). A study by Sui et al. showed that the rs878329 G allele in NLRP1 correlated with the risk of RA, and the polymorphism of the NLRP1 gene was associated with the incidence of RA in the Han population (106). However, there were also different results: polymorphisms in NLRP1 rs6502867 T/C were shown not related to the risk of developing RA in Chinese Singaporeans (107), and another study also showed that genetic polymorphisms in NLRP1 rs6502867 C/T and rs878329 C/G were not associated with RA (108). Ethnicities, geographic locations, lifestyles, and the sample sizes may affect the conclusions. Whether NLRP1 diversity is associated with RA susceptibility or severity remains to be investigated in more depth.

NAIP-NLRC4

Although much less research has been done on NLRC4 in RA, a recent study showed significantly elevated NLRC4 and NLRP3 expression in monocytes from RA patients, supporting a role of inflammasomes in RA (100).

INFLAMMASOMES AND RA THERAPY

The role of various inflammasomes is increasingly recognized in autoimmune diseases. Thus, targeting inflammasomesor their associated cytokines may become new strategies for therapeutic intervention. Several inhibitors of inflammasomes have been identified, including those that directly inhibit NLRP3 inflammasomes and indirectly inhibit caspase-1 or IL-1 signaling pathways (**Figure 2**).

NLRP3 Inhibitors

The drug glyburide for the treatment of type 2 diabetes (T2D) selectively inhibits NLRP3 inflammasomes, and the inhibition of NLRP3 by glyburide demonstrates for the first time that selective pharmacological inhibition is feasible (127). Another NLRP3 inhibitor, MCC950, has the same high specificity as glyburide but has no inhibitory effect on AIM2, Pyrin, NLRP1, and NAIP-NLRC4 inflammasomes. It prevents ASC oligomerization by inhibiting NLRP3 activation, downregulating IL-1β secretion (128). A recent study showed that MCC950 could directly target the NACHT domain of NLRP3 and block ATP hydrolysis to inhibit NLRP3 activation (129). Although MCC950 is a potent and specific small molecule inhibitor of NLRP3 and has shown beneficial effects in models of myocardial infarction, atherosclerosis, colitis, airway and skin inflammation, phase II clinical trials of MCC950 in RA were discontinued due to its hepatoxicity (130, 131).

The anti-allergy drug tranilast (TR) is also a direct NLRP3 inhibitor, which binds to the NACHT domain of NLRP3, inhibiting NLRP3 assembly by blocking its oligomerization (132). TR has shown beneficial effects in mouse models of T2D, cryopyrin-associated periodic syndrome (CAPS) and gouty arthritis (132). TR was evaluated for safety and efficacy in CAPS patients in a phase 2 open-label clinical trial (NCT03923140) (131, 133). Bay 11–7082 and parthenolide directly inhibit NLRP3 and also inhibit caspase-1 activity, but are not suitable for clinical development due to the potential for widespread immunosuppression (134). CY-09, oridonin, and derivatives of acrylamide (e.g., INF58) all directly inhibit NLRP3 (135–137). Studies have also shown that human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) ameliorated CIA in the mouse model to a similar extent as etanercept. hUCB-MSCs can modulate multiple cytokine pathways and may be a favorable candidate for the treatment of patients with refractory RA (138). Taraxerol significantly inhibited IL-1β-induced proinflammatory cytokines, including IL-6, IL-8 and TNF-α *in vitro* and inhibited NLRP3 inflammasome expression in a model (139). Cinnamaldehyde (CA) is also a promising drug for RA therapy. It reduces the joint inflammatory response in RA rat models, especially cytokines associated with IL-1β. CA may inhibit the activation of the NLRP3 inflammasome and suppress disease progression by regulating the succinate/HIF-1α axis (140). Recently, RRx-001, a well-tolerated anticancer agent, has been identified as a potent covalent NLRP3 inhibitor and may serve as a new potential therapeutic agent for NLRP3-driven diseases (141). All of the above are in the experimental stage and have not yet been applied for the treatment of autoimmune diseases in humans. Research on NLRP3 inhibitors is rapidly advancing, and the promising compounds with good safety profiles, high specificity and low cost will provide benefits for the treatment of patients.

Caspase-1 Inhibitors

Caspase-1 is common to all canonical inflammasomes, and the development of selective inhibitors of caspase-1 protease is a hotspot in the pharmaceutical industry in recent years (142). VX-740 (pralnacasan) and its analog VX-765 (belnacasan) can be metabolized to VRT-18858 and VRT-043198, respectively. VX-740 attenuates both RA and OA knee osteoarthritis injury, and VX-765 inhibits cytokine secretion and reduces disease severity in models of skin inflammation as well as RA (143, 144). Although VX-740 showed good anti-inflammatory performance in phase I and II clinical trials in RA patients, the trials were discontinued due to its hepatotoxicity (145). VX-765 failed to meet the stated endpoints in phase II clinical trials, although it reduced the release of IL-1β and IL-18 in mice and reduced seizures in a mouse model of chronic epilepsy (NCT01501383) (143, 146, 147).

IL-1/IL-18 Blockades

IL-1β and IL-18 are the major inflammatory cytokines activated by various types of inflammasomes and are involved in the pathogenesis of several autoimmune diseases. Therefore, blocking IL-1 and IL-18 would be a more desirable therapeutic strategy. Three biological anti-IL-1 agents have been approved for clinical use: anakinra, a human recombinant interleukin-1 receptor antagonist (IL-1Ra) that competitively inhibits IL-1α and IL-1β; canakinumab, a fully human anti-IL-1β monoclonal antibody; and rilonacept, an IL-1 inhibitor (IL-1 Trap). Anakinra

was firstly developed for use in patients who had no response to conventional therapy for RA. Anakinra inhibited disease activity in RA patients but was later found to be less effective than TNF-α blockers (148, 149). Therefore, anakinra is currently used in the treatment of adult Still's disease (AOSD), Schnitzler syndrome (SchS), and systemic juvenile idiopathic arthritis (SJIA) and has also shown better results and safety in patients with gout (148). Anakinra was found in a recent small multicenter and randomized clinical trial to improve inflammatory and glycemic parameters in patients with RA and T2D (NCT02236481) (150). Since anakinra has a half-life of only 4~6 hours, it requires frequent injections, with the resulting potential risk of infection, whereas canakinumab has a half-life of 26 days and showed a better treatment response and a higher safety profile in patients with active RA in a phase II multicenter randomized and double-blind trial (NCT00784628) (151). Additionally, canakinumab has shown beneficial effects in active SJIA, autoinflammatory recurrent fever syndromes, atherosclerosis, and lung cancer (152–156). The third agent, Rilonacept, is mainly used to treat gout in children and adults with CAPS as well as SchS (157–159).

Currently, two IL-18 blockers are being explored in clinical trials. Tadekinigalfa, a recombinant human IL-18 binding protein, is both effective and safe in phase II clinical trials in AOSD (NCT02398435) (160). GSK1070806 is a recombinant human IL-18 neutralizing antibody currently under phase II clinical trials for the treatment of moderate to severe Crohn's disease (NCT03681067) (131). If these clinical trials are proved effective, it will be possible to extend inhibitors of IL-18 to the treatment of other autoimmune diseases with abnormally high level of IL-18.

CONCLUSIONS

RA is a complex autoimmune disease caused by multiple environmental and genetic factors. Over the past decades, there have been significant advances in understanding the pathogenesis of RA. Compelling evidence indicates that inflammasomes play a critical role in the RA disease process. In recent years, reports on elucidation of different mechanisms of inflammasome activation and regulation have also made it possible to design effective inflammasome inhibitors. Advanced technologies such as solution-state NMR, X-ray crystallography, and cryo-EM have all contributed to the characterization of the high-resolution structure of receptor/ligand-driven induced conformational changes. Thus, further understanding of the effector mechanisms of inflammasome activation and immune regulation will not only provide new insight in RA pathogenesis but also facilitate the development of novel therapeutic strategies for the treatment of RA and other autoimmune diseases.

AUTHOR CONTRIBUTIONS

QJ and DC drafted the manuscript and designed the figures and tables. XW, EH, QW, CW, GY, LL, and DC revised the manuscript. DC, LL, and GY conceived the topic. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Smolen JS, Aletaha D, McInnes IB. Rheumatoid Arthritis. *Lancet* (2016) 388 (10055):2023–38. doi: 10.1016/s0140-6736(16)30173-8
2. Aletaha D, Smolen JS. Diagnosis and Management of Rheumatoid Arthritis: A Review. *JAMA* (2018) 320(13):1360–72. doi: 10.1001/jama.2018.13103
3. Xiao F, Han M, Rui K, Ai X, Tian J, Zhang W, et al. New Insights Into Follicular Helper T Cell Response and Regulation in Autoimmune Pathogenesis. *Cell Mol Immunol* (2021) 18(6):1610–2. doi: 10.1038/s41423-021-00688-7
4. Crowson CS, Matteson EL, Myasoedova E, Michet CJ, Ernste FC, Warrington KJ, et al. The Lifetime Risk of Adult-Onset Rheumatoid Arthritis and Other Inflammatory Autoimmune Rheumatic Diseases. *Arthritis Rheumatol* (2011) 63(3):633–9. doi: 10.1002/art.30155
5. Safiri S, Kolahi AA, Hoy D, Smith E, Bettampadi D, Mansournia MA, et al. Global, Regional and National Burden of Rheumatoid Arthritis 1990-2017: A Systematic Analysis of the Global Burden of Disease Study 2017. *Ann Rheum Dis* (2019) 78(11):1463–71. doi: 10.1136/annrheumdis-2019-215920
6. Tan IJ, Peeva E, Zandman-Goddard G. Hormonal Modulation of the Immune System - A Spotlight on the Role of Progestogens. *Autoimmun Rev* (2015) 14(6):536–42. doi: 10.1016/j.autrev.2015.02.004
7. Pierdominici M, Maselli A, Colasanti T, Giammarioli AM, Delunardo F, Vacirca D, et al. Estrogen Receptor Profiles in Human Peripheral Blood Lymphocytes. *Immunol Lett* (2010) 132(1-2):79–85. doi: 10.1016/j.imlet.2010.06.003
8. Cooper GS, Stroehla BC. The Epidemiology of Autoimmune Diseases. *Autoimmun Rev* (2003) 2(3):119–25. doi: 10.1016/s1568-9972(03)00006-5
9. Østensen M, Villiger PM, Förger F. Interaction of Pregnancy and Autoimmune Rheumatic Disease. *Autoimmun Rev* (2012) 11(6-7):A437–46. doi: 10.1016/j.autrev.2011.11.013
10. Jiang Q, Yang G, Xiao F, Xie J, Wang S, Lu L, et al. Role of Th22 Cells in the Pathogenesis of Autoimmune Diseases. *Front Immunol* (2021) 12:688066. doi: 10.3389/fimmu.2021.688066
11. Jiang Q, Yang G, Liu Q, Wang S, Cui D. Function and Role of Regulatory T Cells in Rheumatoid Arthritis. *Front Immunol* (2021) 12:626193. doi: 10.3389/fimmu.2021.626193

12. Wordsworth P, Bell J. Polygenic Susceptibility in Rheumatoid Arthritis. *Ann Rheum Dis* (1991) 50(6):343–6. doi: 10.1136/ard.50.6.343
13. Lee HS, Korman BD, Le JM, Kastner DL, Remmers EF, Gregersen PK, et al. Genetic Risk Factors for Rheumatoid Arthritis Differ in Caucasian and Korean Populations. *Arthritis Rheumatol* (2009) 60(2):364–71. doi: 10.1002/art.24245
14. Baka Z, Buzás E, Nagy G. Rheumatoid Arthritis and Smoking: Putting the Pieces Together. *Arthrit Res Ther* (2009) 11(4):238. doi: 10.1186/ar2751
15. Alippe Y, Mbalaviele G. Omnipresence of Inflammasome Activities in Inflammatory Bone Diseases. *Semin Immunopathol* (2019) 41(5):607–18. doi: 10.1007/s00281-019-00753-4
16. Kuwabara T, Ishikawa F, Kondo M, Kakiuchi T. The Role of IL-17 and Related Cytokines in Inflammatory Autoimmune Diseases. *Mediators Inflamm* (2017) 2017:3908061. doi: 10.1155/2017/3908061
17. Wähämaa H, Schierbeck H, Hreggvidsdottir HS, Palmblad K, Aveberger AC, Andersson U, et al. High Mobility Group Box Protein 1 in Complex With Lipopolysaccharide or IL-1 Promotes an Increased Inflammatory Phenotype in Synovial Fibroblasts. *Arthrit Res Ther* (2011) 13(4):R136. doi: 10.1186/ar3450
18. Yang Z, Cao J, Yu C, Yang Q, Zhang Y, Han L. Caspase-1 Mediated Interleukin-18 Activation in Neutrophils Promotes the Activity of Rheumatoid Arthritis in a NLRP3 Inflammasome Independent Manner. *Joint Bone Spine* (2016) 83(3):282–9. doi: 10.1016/j.jbspin.2015.07.006
19. Lamkanfi M, Dixit VM. Inflammasomes and Their Roles in Health and Disease. *Annu Rev Cell Dev Biol* (2012) 28:137–61. doi: 10.1146/annurev-cellbio-101011-155745
20. Kanneganti TD, Lamkanfi M, Núñez G. Intracellular NOD-Like Receptors in Host Defense and Disease. *Immunity* (2007) 27(4):549–59. doi: 10.1016/j.immuni.2007.10.002
21. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in Health and Disease. *Nature* (2012) 481(7381):278–86. doi: 10.1038/nature10759
22. Takeuchi O, Akira S. Pattern Recognition Receptors and Inflammation. *Cell* (2010) 140(6):805–20. doi: 10.1016/j.cell.2010.01.022
23. Macleod C, Bryant CE. Visualising Pattern Recognition Receptor Signalling. *Biochem Soc Trans* (2017) 45(5):1077–85. doi: 10.1042/bst20160459
24. Makkar R, Behl T, Bungau S, Kumar A, Arora S. Understanding the Role of Inflammasomes in Rheumatoid Arthritis. *Inflammation* (2020) 43(6):2033–47. doi: 10.1007/s10753-020-01301-1
25. Schroder K, Tschopp J. The Inflammasomes. *Cell* (2010) 140(6):821–32. doi: 10.1016/j.cell.2010.01.040
26. Huang Y, Xu W, Zhou R. NLRP3 Inflammasome Activation and Cell Death. *Cell Mol Immunol* (2021) 18(9):2114–27. doi: 10.1038/s41423-021-00740-6
27. Martinon F, Burns K, Tschopp J. The Inflammasome: A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of proIL-Beta. *Mol Cell* (2002) 10(2):417–26. doi: 10.1016/s1097-2765(02)00599-3
28. Radian AD, de Almeida L, Dorfleutner A, Stehlik C. NLRP7 and Related Inflammasome Activating Pattern Recognition Receptors and Their Function in Host Defense and Disease. *Microbes Infect* (2013) 15(8-9):630–9. doi: 10.1016/j.micinf.2013.04.001
29. Kerur N, Veettil MV, Sharma-Walia N, Bottero V, Sadagopan S, Otageri P, et al. IFI16 Acts as a Nuclear Pathogen Sensor to Induce the Inflammasome in Response to Kaposi Sarcoma-Associated Herpesvirus Infection. *Cell Host Microbe* (2011) 9(5):363–75. doi: 10.1016/j.chom.2011.04.008
30. Xue Y, Enosi Tuipulotu D, Tan WH, Kay C, Man SM. Emerging Activators and Regulators of Inflammasomes and Pyroptosis. *Trends Immunol* (2019) 40(11):1035–52. doi: 10.1016/j.it.2019.09.005
31. Latz E, Xiao TS, Stutz A. Activation and Regulation of the Inflammasomes. *Nat Rev Immunol* (2013) 13(6):397–411. doi: 10.1038/nri3452
32. Chen L, Cao SQ, Lin ZM, He SJ, Zuo JP. NOD-Like Receptors in Autoimmune Diseases. *Acta Pharmacol Sin* (2021) 42(11):1742–56. doi: 10.1038/s41401-020-00603-2
33. Di Virgilio F. The Therapeutic Potential of Modifying Inflammasomes and NOD-Like Receptors. *Pharmacol Rev* (2013) 65(3):872–905. doi: 10.1124/pr.112.006171
34. de Zoete MR, Palm NW, Zhu S, Flavell RA. Inflammasomes. *Cold Spring Harb Perspect Biol* (2014) 6(12):a016287. doi: 10.1101/cshperspect.a016287
35. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, et al. AIM2 Recognizes Cytosolic dsDNA and Forms a Caspase-1-Activating Inflammasome With ASC. *Nature* (2009) 458(7237):514–8. doi: 10.1038/nature07725
36. Jones JW, Kayagaki N, Broz P, Henry T, Newton K, O'Rourke K, et al. Absent in Melanoma 2 Is Required for Innate Immune Recognition of Francisella Tularensis. *Proc Natl Acad Sci USA* (2010) 107(21):9771–6. doi: 10.1073/pnas.1003738107
37. Broz P, Newton K, Lamkanfi M, Mariathasan S, Dixit VM, Monack DM. Redundant Roles for Inflammasome Receptors NLRP3 and NLRC4 in Host Defense Against Salmonella. *J Exp Med* (2010) 207(8):1745–55. doi: 10.1084/jem.20100257
38. Man SM, Kanneganti TD. Converging Roles of Caspases in Inflammasome Activation, Cell Death and Innate Immunity. *Nat Rev Immunol* (2016) 16(1):7–21. doi: 10.1038/nri.2015.7
39. Martinon F, Tschopp J. Inflammatory Caspases: Linking an Intracellular Innate Immune System to Autoinflammatory Diseases. *Cell* (2004) 117(5):561–74. doi: 10.1016/j.cell.2004.05.004
40. Kufer TA, Banks DJ, Philpott DJ. Innate Immune Sensing of Microbes by Nod Proteins. *Ann NY Acad Sci* (2006) 1072:19–27. doi: 10.1196/annals.1326.020
41. Craven RR, Gao X, Allen IC, Gris D, Bubeck Wardenburg J, McElvania-Tekippe E, et al. Staphylococcus Aureus Alpha-Hemolysin Activates the NLRP3-Inflammasome in Human and Mouse Monocytic Cells. *PLoS One* (2009) 4(10):e7446. doi: 10.1371/journal.pone.0007446
42. Muruve DA, Pétrilli V, Zaiss AK, White LR, Clark SA, Ross PJ, et al. The Inflammasome Recognizes Cytosolic Microbial and Host DNA and Triggers an Innate Immune Response. *Nature* (2008) 452(7183):103–7. doi: 10.1038/nature06664
43. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, et al. Cryopyrin Activates the Inflammasome in Response to Toxins and ATP. *Nature* (2006) 440(7081):228–32. doi: 10.1038/nature04515
44. Martinon F, Pétrilli V, Mayor A, Tardivel A, Tschopp J. Gout-Associated Uric Acid Crystals Activate the NALP3 Inflammasome. *Nature* (2006) 440(7081):237–41. doi: 10.1038/nature04516
45. Di A, Xiong S, Ye Z, Malireddi RKS, Kometani S, Zhong M, et al. The TWIK2 Potassium Efflux Channel in Macrophages Mediates NLRP3 Inflammasome-Induced Inflammation. *Immunity* (2018) 49(1):56–65.e4. doi: 10.1016/j.immuni.2018.04.032
46. Tang T, Lang X, Xu C, Wang X, Gong T, Yang Y, et al. CLICs-Dependent Chloride Efflux is an Essential and Proximal Upstream Event for NLRP3 Inflammasome Activation. *Nat Commun* (2017) 8(1):202. doi: 10.1038/s41467-017-00227-x
47. Lee GS, Subramanian N, Kim AI, Aksentijevich I, Goldbach-Mansky R, Sacks DB, et al. The Calcium-Sensing Receptor Regulates the NLRP3 Inflammasome Through Ca2+ and cAMP. *Nature* (2012) 492(7427):123–7. doi: 10.1038/nature11588
48. Zhong Z, Liang S, Sanchez-Lopez E, He F, Shalapour S, Lin XJ, et al. New Mitochondrial DNA Synthesis Enables NLRP3 Inflammasome Activation. *Nature* (2018) 560(7717):198–203. doi: 10.1038/s41586-018-0372-z
49. Lučiūnaitė A, McManus RM, Jankunec M, Rácz I, Dansokho C, Dalgėdienė I, et al. Soluble Aβ Oligomers and Protofibrils Induce NLRP3 Inflammasome Activation in Microglia. *J Neurochem* (2020) 155(6):650–61. doi: 10.1111/jnc.14945
50. Elliott EI, Sutterwala FS. Initiation and Perpetuation of NLRP3 Inflammasome Activation and Assembly. *Immunol Rev* (2015) 265(1):35–52. doi: 10.1111/imr.12286
51. Jo EK, Kim JK, Shin DM, Sasakawa C. Molecular Mechanisms Regulating NLRP3 Inflammasome Activation. *Cell Mol Immunol* (2016) 13(2):148–59. doi: 10.1038/cmi.2015.95
52. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, et al. Non-Canonical Inflammasome Activation Targets Caspase-11. *Nature* (2011) 479(7371):117–21. doi: 10.1038/nature10558
53. Shi J, Zhao Y, Wang Y, Gao W, Ding J, Li P, et al. Inflammatory Caspases Are Innate Immune Receptors for Intracellular LPS. *Nature* (2014) 514(7521):187–92. doi: 10.1038/nature13683
54. Baker PJ, Boucher D, Bierschenk D, Tebartz C, Whitney PG, D'Silva DB, et al. NLRP3 Inflammasome Activation Downstream of Cytoplasmic LPS Recognition by Both Caspase-4 and Caspase-5. *Eur J Immunol* (2015) 45(10):2918–26. doi: 10.1002/eji.201545655

55. Zhu K, Jin X, Chi Z, Chen S, Wu S, Sloan RD, et al. Priming of NLRP3 Inflammasome Activation by Msn Kinase MINK1 in Macrophages. *Cell Mol Immunol* (2021) 18(10):2372–82. doi: 10.1038/s41423-021-00761-1
56. Di Micco A, Frera G, Lugrin J, Jamilloux Y, Hsu ET, Tardivel A, et al. AIM2 Inflammasome Is Activated by Pharmacological Disruption of Nuclear Envelope Integrity. *Proc Natl Acad Sci USA* (2016) 113(32):E4671–80. doi: 10.1073/pnas.1602419113
57. Jin T, Perry A, Jiang J, Smith P, Curry JA, Unterholzner L, et al. Structures of the HIN Domain:DNA Complexes Reveal Ligand Binding and Activation Mechanisms of the AIM2 Inflammasome and IFI16 Receptor. *Immunity* (2012) 36(4):561–71. doi: 10.1016/j.immuni.2012.02.014
58. Matyszewski M, Morrone SR, Sohn J. Digital Signaling Network Drives the Assembly of the AIM2-ASC Inflammasome. *Proc Natl Acad Sci USA* (2018) 115(9):E1963–72. doi: 10.1073/pnas.1712860115
59. Hu B, Jin C, Li HB, Tong J, Ouyang X, Cetinbas NM, et al. The DNA-Sensing AIM2 Inflammasome Controls Radiation-Induced Cell Death and Tissue Injury. *Science* (2016) 354(6313):765–8. doi: 10.1126/science.aaf7532
60. Gaidt MM, Ebert TS, Chauhan D, Ramshorn K, Pinci F, Zuber S, et al. The DNA Inflammasome in Human Myeloid Cells Is Initiated by a STING-Cell Death Program Upstream of NLRP3. *Cell* (2017) 171(5):1110–24.e18. doi: 10.1016/j.cell.2017.09.039
61. Fisch D, Bando H, Clough B, Hornung V, Yamamoto M, Shenoy AR, et al. Human GBP1 Is a Microbe-Specific Gatekeeper of Macrophage Apoptosis and Pyroptosis. *EMBO J* (2019) 38(13):e100926. doi: 10.15252/embj.2018100926
62. Fidler TP, Xue C, Yalcinkaya M, Hardaway B, Abramowicz S, Xiao T, et al. The AIM2 Inflammasome Exacerbates Atherosclerosis in Clonal Haematopoiesis. *Nature* (2021) 592(7853):296–301. doi: 10.1038/s41586-021-03341-5
63. D'Osualdo A, Weichenberger CX, Wagner RN, Godzik A, Wooley J, Reed JC. CARD8 and NLRP1 Undergo Autoproteolytic Processing Through a ZU5-Like Domain. *PLoS One* (2011) 6(11):e27396. doi: 10.1371/journal.pone.0027396
64. Levinsohn JL, Newman ZL, Hellmich KA, Fattah R, Getz MA, Liu S, et al. Anthrax Lethal Factor Cleavage of Nlrp1 Is Required for Activation of the Inflammasome. *PLoS Path* (2012) 8(3):e1002638. doi: 10.1371/journal.ppat.1002638
65. Chavarría-Smith J, Vance RE. Direct Proteolytic Cleavage of NLRP1B Is Necessary and Sufficient for Inflammasome Activation by Anthrax Lethal Factor. *PLoS Path* (2013) 9(6):e1003452. doi: 10.1371/journal.ppat.1003452
66. Ewald SE, Chavarria-Smith J, Boothroyd JC. NLRP1 Is an Inflammasome Sensor for Toxoplasma Gondii. *Infect Immun* (2014) 82(1):460–8. doi: 10.1128/iai.01170-13
67. Sandstrom A, Mitchell PS, Goers L, Mu EW, Lesser CF, Vance RE. Functional Degradation: A Mechanism of NLRP1 Inflammasome Activation by Diverse Pathogen Enzymes. *Science* (2019) 364(6435):eaau1330. doi: 10.1126/science.aau1330
68. Tsu BV, Beierschmitt C, Ryan AP, Agarwal R, Mitchell PS, Daugherty MD. Diverse Viral Proteases Activate the NLRP1 Inflammasome. *Elife* (2021) 10:e60609. doi: 10.7554/eLife.60609
69. Hollingsworth LR, Sharif H, Griswold AR, Fontana P, Mintseris J, Dagbay KB, et al. DPP9 Sequesters the C Terminus of NLRP1 to Repress Inflammasome Activation. *Nature* (2021) 592(7856):778–83. doi: 10.1038/s41586-021-03350-4
70. Huang M, Zhang X, Toh GA, Gong Q, Wang J, Han Z, et al. Structural and Biochemical Mechanisms of NLRP1 Inhibition by DPP9. *Nature* (2021) 592(7856):773–7. doi: 10.1038/s41586-021-03320-w
71. Zhong FL, Mamaï O, Sborgi L, Boussofara L, Hopkins R, Robinson K, et al. Germline NLRP1 Mutations Cause Skin Inflammatory and Cancer Susceptibility Syndromes *via* Inflammasome Activation. *Cell* (2016) 167(1):187–202.e17. doi: 10.1016/j.cell.2016.09.001
72. Fusco WG, Duncan JA. Novel Aspects of the Assembly and Activation of Inflammasomes With Focus on the NLRC4 Inflammasome. *Int Immunol* (2018) 30(5):183–93. doi: 10.1093/intimm/dxy009
73. Franchi L, Amer A, Body-Malapel M, Kanneganti TD, Ozören N, Jagirdar R, et al. Cytosolic Flagellin Requires Ipaf for Activation of Caspase-1 and Interleukin 1beta in Salmonella-Infected Macrophages. *Nat Immunol* (2006) 7(6):576–82. doi: 10.1038/ni1346
74. Zhao Y, Yang J, Shi J, Gong YN, Lu Q, Xu H, et al. The NLRC4 Inflammasome Receptors for Bacterial Flagellin and Type III Secretion Apparatus. *Nature* (2011) 477(7366):596–600. doi: 10.1038/nature10510
75. Kofoed EM, Vance RE. Innate Immune Recognition of Bacterial Ligands by NAIPs Determines Inflammasome Specificity. *Nature* (2011) 477(7366):592–5. doi: 10.1038/nature10394
76. Zhao Y, Shi J, Shi X, Wang Y, Wang F, Shao F. Genetic Functions of the NAIP Family of Inflammasome Receptors for Bacterial Ligands in Mice. *J Exp Med* (2016) 213(5):647–56. doi: 10.1084/jem.20160006
77. Indramohan M, Stehlik C, Dorfleutner A. COPs and POPs Patrol Inflammasome Activation. *J Mol Biol* (2018) 430(2):153–73. doi: 10.1016/j.jmb.2017.10.004
78. Duncan JA, Canna SW. The NLRC4 Inflammasome. *Immunol Rev* (2018) 281(1):115–23. doi: 10.1111/imr.12607
79. Zheng D, Kern L, Elinav E. The NLRP6 Inflammasome. *Immunology* (2021) 162(3):281–9. doi: 10.1111/imm.13293
80. Lin Y, Luo Z. NLRP6 Facilitates the Interaction Between TAB2/3 and TRIM38 in Rheumatoid Arthritis Fibroblast-Like Synoviocytes. *FEBS Lett* (2017) 591(8):1141–9. doi: 10.1002/1873-3468.12622
81. Tuladhar S, Kanneganti TD. NLRP12 in Innate Immunity and Inflammation. *Mol Aspects Med* (2020) 76:100887. doi: 10.1016/j.mam.2020.100887
82. Prado DS, Veras FP, Ferreira RG, Damasceno LEA, Melo PH, Zamboni DS, et al. NLRP12 Controls Arthritis Severity by Acting as a Checkpoint Inhibitor of Th17 Cell Differentiation. *FASEB J* (2020) 34(8):10907–19. doi: 10.1096/fj.202000795R
83. Zhang X, Nan H, Guo J, Liu J. NLRP12 Reduces Proliferation and Inflammation of Rheumatoid Arthritis Fibroblast-Like Synoviocytes by Regulating the NF-κb and MAPK Pathways. *Eur Cytokine Netw* (2021) 32(2):15–22. doi: 10.1684/ecn.2021.0465
84. Zhang Y, Zheng Y, Li H. NLRP3 Inflammasome Plays an Important Role in the Pathogenesis of Collagen-Induced Arthritis. *Mediators Inflamm* (2016) 2016:9656270. doi: 10.1155/2016/9656270
85. Guo C, Fu R, Wang S, Huang Y, Li X, Zhou M, et al. NLRP3 Inflammasome Activation Contributes to the Pathogenesis of Rheumatoid Arthritis. *Clin Exp Immunol* (2018) 194(2):231–43. doi: 10.1111/cei.13167
86. Ippagunta SK, Brand DD, Luo J, Boyd KL, Calabrese C, Stienstra R, et al. Inflammasome-Independent Role of Apoptosis-Associated Speck-Like Protein Containing a CARD (ASC) in T Cell Priming Is Critical for Collagen-Induced Arthritis. *J Biol Chem* (2010) 285(16):12454–62. doi: 10.1074/jbc.M109.093252
87. Li XF, Shen WW, Sun YY, Li WX, Sun ZH, Liu YH, et al. MicroRNA-20a Negatively Regulates Expression of NLRP3-Inflammasome by Targeting TXNIP in Adjuvant-Induced Arthritis Fibroblast-Like Synoviocytes. *Joint Bone Spine* (2016) 83(6):695–700. doi: 10.1016/j.jbspin.2015.10.007
88. Ruscitti P, Cipriani P, Di Benedetto P, Liakouli V, Berardicurti O, Carubbi F, et al. Monocytes From Patients With Rheumatoid Arthritis and Type 2 Diabetes Mellitus Display an Increased Production of Interleukin (IL)-1β *via* the Nucleotide-Binding Domain and Leucine-Rich Repeat Containing Family Pyrin 3(NLRP3)-Inflammasome Activation: A Possible Implication for Therapeutic Decision in These Patients. *Clin Exp Immunol* (2015) 182(1):35–44. doi: 10.1111/cei.12667
89. Choulaki C, Papadaki G, Repa A, Kampouraki E, Kambas K, Ritis K, et al. Enhanced Activity of NLRP3 Inflammasome in Peripheral Blood Cells of Patients With Active Rheumatoid Arthritis. *Arthrit Res Ther* (2015) 17(1):257. doi: 10.1186/s13075-015-0775-2
90. Xie Q, Wei M, Zhang B, Kang X, Liu D, Zheng W, et al. MicroRNA−33 Regulates the NLRP3 Inflammasome Signaling Pathway in Macrophages. *Mol Med Rep* (2018) 17(2):3318–27. doi: 10.3892/mmr.2017.8224
91. Lasithiotaki I, Giannarakis I, Tsitoura E, Samara KD, Margaritopoulos GA, Choulaki C, et al. NLRP3 Inflammasome Expression in Idiopathic Pulmonary Fibrosis and Rheumatoid Lung. *Eur Respir J* (2016) 47(3):910–8. doi: 10.1183/13993003.00564-2015
92. Zhao C, Gu Y, Zeng X, Wang J. NLRP3 Inflammasome Regulates Th17 Differentiation in Rheumatoid Arthritis. *Clin Immunol* (2018) 197:154–60. doi: 10.1016/j.clim.2018.09.007
93. Liu Y, Wei W, Wang Y, Wan C, Bai Y, Sun X, et al. TNF-α/Calreticulin Dual Signaling Induced NLRP3 Inflammasome Activation Associated With HuR

Nucleocytoplasmic Shuttling in Rheumatoid Arthritis. *Inflamm Res* (2019) 68(7):597–611. doi: 10.1007/s00011-019-01244-w
94. Dong X, Zheng Z, Lin P, Fu X, Li F, Jiang J, et al. ACPAs Promote IL-1β Production in Rheumatoid Arthritis by Activating the NLRP3 Inflammasome. *Cell Mol Immunol* (2020) 17(3):261–71. doi: 10.1038/s41423-019-0201-9
95. Jäger E, Murthy S, Schmidt C, Hahn M, Strobel S, Peters A, et al. Calcium-Sensing Receptor-Mediated NLRP3 Inflammasome Response to Calciprotein Particles Drives Inflammation in Rheumatoid Arthritis. *Nat Commun* (2020) 11(1):4243. doi: 10.1038/s41467-020-17749-6
96. Wu XY, Li KT, Yang HX, Yang B, Lu X, Zhao LD, et al. Complement C1q Synergizes With PTX3 in Promoting NLRP3 Inflammasome Over-Activation and Pyroptosis in Rheumatoid Arthritis. *J Autoimmun* (2020) 106:102336. doi: 10.1016/j.jaut.2019.102336
97. Mathews RJ, Robinson JI, Battellino M, Wong C, Taylor JC, Eyre S, et al. Evidence of NLRP3-Inflammasome Activation in Rheumatoid Arthritis (RA); Genetic Variants Within the NLRP3-Inflammasome Complex in Relation to Susceptibility to RA and Response to Anti-TNF Treatment. *Ann Rheum Dis* (2014) 73(6):1202–10. doi: 10.1136/annrheumdis-2013-203276
98. Sode J, Vogel U, Bank S, Andersen PS, Thomsen MK, Hetland ML, et al. Anti-TNF Treatment Response in Rheumatoid Arthritis Patients Is Associated With Genetic Variation in the NLRP3-Inflammasome. *PLoS One* (2014) 9(6):e100361. doi: 10.1371/journal.pone.0100361
99. Sode J, Vogel U, Bank S, Andersen PS, Hetland ML, Locht H, et al. Genetic Variations in Pattern Recognition Receptor Loci Are Associated With Anti-TNF Response in Patients With Rheumatoid Arthritis. *PLoS One* (2015) 10 (10):e0139781. doi: 10.1371/journal.pone.0139781
100. Addobbati C, da Cruz HLA, Adelino JE, Melo Tavares Ramos AL, Fragoso TS, Domingues A, et al. Polymorphisms and Expression of Inflammasome Genes Are Associated With the Development and Severity of Rheumatoid Arthritis in Brazilian Patients. *Inflamm Res* (2018) 67(3):255–64. doi: 10.1007/s00011-017-1119-2
101. Kastbom A, Johansson M, Verma D, Söderkvist P, Rantapää-Dahlqvist S. CARD8 P.C10X Polymorphism Is Associated With Inflammatory Activity in Early Rheumatoid Arthritis. *Ann Rheum Dis* (2010) 69(4):723–6. doi: 10.1136/ard.2008.106989
102. Kastbom A, Ärlestig L, Rantapää-Dahlqvist S. Genetic Variants of the NLRP3 Inflammasome Are Associated With Stroke in Patients With Rheumatoid Arthritis. *J Rheumatol* (2015) 42(10):1740–5. doi: 10.3899/jrheum.141529
103. Ben Hamad M, Cornelis F, Marzouk S, Chabchoub G, Bahloul Z, Rebai A, et al. Association Study of CARD8 (P.C10X) and NLRP3 (P.Q705K) Variants With Rheumatoid Arthritis in French and Tunisian Populations. *Int J Immunogenet* (2012) 39(2):131–6. doi: 10.1111/j.1744-313X.2011.01070.x
104. Kastbom A, Verma D, Eriksson P, Skogh T, Wingren G, Söderkvist P. Genetic Variation in Proteins of the Cryopyrin Inflammasome Influences Susceptibility and Severity of Rheumatoid Arthritis (the Swedish TIRA Project). *Rheumatol (Oxford)* (2008) 47(4):415–7. doi: 10.1093/rheumatology/kem372
105. García-Bermúdez M, López-Mejías R, González-Juanatey C, Corrales A, Castañeda S, Ortiz AM, et al. CARD8 Rs2043211 (P.C10X) Polymorphism Is Not Associated With Disease Susceptibility or Cardiovascular Events in Spanish Rheumatoid Arthritis Patients. *DNA Cell Biol* (2013) 32(1):28–33. doi: 10.1089/dna.2012.1836
106. Sui J, Li H, Fang Y, Liu Y, Li M, Zhong B, et al. NLRP1 Gene Polymorphism Influences Gene Transcription and Is a Risk Factor for Rheumatoid Arthritis in Han Chinese. *Arthritis Rheumatol* (2012) 64(3):647–54. doi: 10.1002/art.33370
107. Goh LL, Yong MY, See WQ, Chee EYW, Lim PQ, Koh ET, et al. NLRP1, PTPN22 and PADI4 Gene Polymorphisms and Rheumatoid Arthritis in ACPA-Positive Singaporean Chinese. *Rheumatol Int* (2017) 37(8):1295–302. doi: 10.1007/s00296-017-3762-x
108. Sun R, Huang Y, Zhang H, Liu R. MMP-2, TNF-α and NLRP1 Polymorphisms in Chinese Patients With Ankylosing Spondylitis and Rheumatoid Arthritis. *Mol Biol Rep* (2013) 40(11):6303–8. doi: 10.1007/s11033-013-2743-8
109. Jin S, Sun S, Ling H, Ma J, Zhang X, Xie Z, et al. Protectin DX Restores Treg/T(h)17 Cell Balance in Rheumatoid Arthritis by Inhibiting NLRP3 Inflammasome *via* miR-20a. *Cell Death Dis* (2021) 12(3):280. doi: 10.1038/s41419-021-03562-6
110. Wang H, Wang Z, Wang L, Sun L, Liu W, Li Q, et al. IL-6 Promotes Collagen-Induced Arthritis by Activating the NLRP3 Inflammasome Through the Cathepsin B/S100A9-Mediated Pathway. *Int Immunopharmacol* (2020) 88:106985. doi: 10.1016/j.intimp.2020.106985
111. Yang X, Zhan N, Jin Y, Ling H, Xiao C, Xie Z, et al. Tofacitinib Restores the Balance of γδtreg/γδt17 Cells in Rheumatoid Arthritis by Inhibiting the NLRP3 Inflammasome. *Theranostics* (2021) 11(3):1446–57. doi: 10.7150/thno.47860
112. Wang Z, Kun Y, Lei Z, Dawei W, Lin P, Jibo W. LncRNA MIAT Downregulates IL-1β, TNF-α to Suppress Macrophage Inflammation But Is Suppressed by ATP-Induced NLRP3 Inflammasome Activation. *Cell Cycle* (2021) 20(2):194–203. doi: 10.1080/15384101.2020.1867788
113. Lee SH, Chang DK, Goel A, Boland CR, Bugbee W, Boyle DL, et al. Microsatellite Instability and Suppressed DNA Repair Enzyme Expression in Rheumatoid Arthritis. *J Immunol* (2003) 170(4):2214–20. doi: 10.4049/jimmunol.170.4.2214
114. Moodley D, Mody G, Patel N, Chuturgoon AA. Mitochondrial Depolarisation and Oxidative Stress in Rheumatoid Arthritis Patients. *Clin Biochem* (2008) 41 (16-17):1396–401. doi: 10.1016/j.clinbiochem.2008.08.072
115. Hajizadeh S, DeGroot J, TeKoppele JM, Tarkowski A, Collins LV. Extracellular Mitochondrial DNA and Oxidatively Damaged DNA in Synovial Fluid of Patients With Rheumatoid Arthritis. *Arthrit Res Ther* (2003) 5(5):R234–40. doi: 10.1186/ar787
116. Afroz S, Giddaluru J, Vishwakarma S, Naz S, Khan AA, Khan N. A Comprehensive Gene Expression Meta-Analysis Identifies Novel Immune Signatures in Rheumatoid Arthritis Patients. *Front Immunol* (2017) 8:74. doi: 10.3389/fimmu.2017.00074
117. Baum R, Sharma S, Carpenter S, Li QZ, Busto P, Fitzgerald KA, et al. Cutting Edge: AIM2 and Endosomal TLRs Differentially Regulate Arthritis and Autoantibody Production in DNase II-Deficient Mice. *J Immunol* (2015) 194(3):873–7. doi: 10.4049/jimmunol.1402573
118. Jakobs C, Perner S, Hornung V. AIM2 Drives Joint Inflammation in a Self-DNA Triggered Model of Chronic Polyarthritis. *PLoS One* (2015) 10(6): e0131702. doi: 10.1371/journal.pone.0131702
119. Méndez-Frausto G, Medina-Rosales MN, Uresti-Rivera EE, Baranda-Cándido L, Zapata-Zúñiga M, Bastián Y, et al. Expression and Activity of AIM2-Inflammasome in Rheumatoid Arthritis Patients. *Immunobiology* (2020) 225(2):151880. doi: 10.1016/j.imbio.2019.11.015
120. Chen Y, Fujuan Q, Chen E, Yu B, Zuo F, Yuan Y, et al. Expression of AIM2 in Rheumatoid Arthritis and Its Role on Fibroblast-Like Synoviocytes. *Mediators Inflamm* (2020) 2020:1693730. doi: 10.1155/2020/1693730
121. Liu T, Tang Q, Liu K, Xie W, Liu X, Wang H, et al. TRIM11 Suppresses AIM2 Inflammasome by Degrading AIM2 *via* P62-Dependent Selective Autophagy. *Cell Rep* (2016) 16(7):1988–2002. doi: 10.1016/j.celrep.2016.07.019
122. Zhu L, Li J, Guo L, Yu X, Wu D, Luo L, et al. Activation of NALP1 Inflammasomes in Rats With Adjuvant Arthritis; a Novel Therapeutic Target of Carboxyamidotriazole in a Model of Rheumatoid Arthritis. *Br J Pharmacol* (2015) 172(13):3446–59. doi: 10.1111/bph.13138
123. Zhang L, Dong Y, Zou F, Wu M, Fan C, Ding Y. 11β-Hydroxysteroid Dehydrogenase 1 Inhibition Attenuates Collagen-Induced Arthritis. *Int Immunopharmacol* (2013) 17(3):489–94. doi: 10.1016/j.intimp.2013.07.015
124. Li F, Guo N, Ma Y, Ning B, Wang Y, Kou L. Inhibition of P2X4 Suppresses Joint Inflammation and Damage in Collagen-Induced Arthritis. *Inflammation* (2014) 37(1):146–53. doi: 10.1007/s10753-013-9723-y
125. Glinsky GV. SNP-Guided microRNA Maps (MirMaps) of 16 Common Human Disorders Identify a Clinically Accessible Therapy Reversing Transcriptional Aberrations of Nuclear Import and Inflammasome Pathways. *Cell Cycle* (2008) 7(22):3564–76. doi: 10.4161/cc.7.22.7073
126. Grandemange S, Sanchez E, Louis-Plence P, Tran Mau-Them F, Bessis D, Coubes C, et al. A New Autoinflammatory and Autoimmune Syndrome Associated With NLRP1 Mutations: NAIAD (NLRP1-Associated Autoinflammation With Arthritis and Dyskeratosis). *Ann Rheum Dis* (2017) 76(7):1191–8. doi: 10.1136/annrheumdis-2016-210021
127. Lamkanfi M, Mueller JL, Vitari AC, Misaghi S, Fedorova A, Deshayes K, et al. Glyburide Inhibits the Cryopyrin/Nalp3 Inflammasome. *J Cell Biol* (2009) 187(1):61–70. doi: 10.1083/jcb.200903124

128. Coll RC, Robertson AA, Chae JJ, Higgins SC, Muñoz-Planillo R, Inserra MC, et al. A Small-Molecule Inhibitor of the NLRP3 Inflammasome for the Treatment of Inflammatory Diseases. *Nat Med* (2015) 21(3):248–55. doi: 10.1038/nm.3806
129. Coll RC, Hill JR, Day CJ, Zamoshnikova A, Boucher D, Massey NL, et al. MCC950 Directly Targets the NLRP3 ATP-Hydrolysis Motif for Inflammasome Inhibition. *Nat Chem Biol* (2019) 15(6):556–9. doi: 10.1038/s41589-019-0277-7
130. Mangan MSJ, Olhava EJ, Roush WR, Seidel HM, Glick GD, Latz E. Targeting the NLRP3 Inflammasome in Inflammatory Diseases. *Nat Rev Drug Discovery* (2018) 17(8):588–606. doi: 10.1038/nrd.2018.97
131. Chauhan D, Vande Walle L, Lamkanfi M. Therapeutic Modulation of Inflammasome Pathways. *Immunol Rev* (2020) 297(1):123–38. doi: 10.1111/imr.12908
132. Huang Y, Jiang H, Chen Y, Wang X, Yang Y, Tao J, et al. Tranilast Directly Targets NLRP3 to Treat Inflammasome-Driven Diseases. *EMBO Mol Med* (2018) 10(4):e8689. doi: 10.15252/emmm.201708689
133. Darakhshan S, Pour AB. Tranilast: A Review of Its Therapeutic Applications. *Pharmacol Res* (2015) 91:15–28. doi: 10.1016/j.phrs.2014.10.009
134. Juliana C, Fernandes-Alnemri T, Wu J, Datta P, Solorzano L, Yu JW, et al. Anti-Inflammatory Compounds Parthenolide and Bay 11-7082 Are Direct Inhibitors of the Inflammasome. *J Biol Chem* (2010) 285(13):9792–802. doi: 10.1074/jbc.M109.082305
135. Jiang H, He H, Chen Y, Huang W, Cheng J, Ye J, et al. Identification of a Selective and Direct NLRP3 Inhibitor to Treat Inflammatory Disorders. *J Exp Med* (2017) 214(11):3219–38. doi: 10.1084/jem.20171419
136. He H, Jiang H, Chen Y, Ye J, Wang A, Wang C, et al. Oridonin Is a Covalent NLRP3 Inhibitor With Strong Anti-Inflammasome Activity. *Nat Commun* (2018) 9(1):2550. doi: 10.1038/s41467-018-04947-6
137. Cocco M, Miglio G, Giorgis M, Garella D, Marini E, Costale A, et al. Design, Synthesis, and Evaluation of Acrylamide Derivatives as Direct NLRP3 Inflammasome Inhibitors. *ChemMedChem* (2016) 11(16):1790–803. doi: 10.1002/cmdc.201600055
138. Shin TH, Kim HS, Kang TW, Lee BC, Lee HY, Kim YJ, et al. Human Umbilical Cord Blood-Stem Cells Direct Macrophage Polarization and Block Inflammasome Activation to Alleviate Rheumatoid Arthritis. *Cell Death Dis* (2016) 7(12):e2524. doi: 10.1038/cddis.2016.442
139. Chen J, Wu W, Zhang M, Chen C. Taraxasterol Suppresses Inflammation in IL-1β-Induced Rheumatoid Arthritis Fibroblast-Like Synoviocytes and Rheumatoid Arthritis Progression in Mice. *Int Immunopharmacol* (2019) 70:274–83. doi: 10.1016/j.intimp.2019.02.029
140. Liu P, Wang J, Wen W, Pan T, Chen H, Fu Y, et al. Cinnamaldehyde Suppresses NLRP3 Derived IL-1β *via* Activating Succinate/HIF-1 in Rheumatoid Arthritis Rats. *Int Immunopharmacol* (2020) 84:106570. doi: 10.1016/j.intimp.2020.106570
141. Chen Y, He H, Lin B, Chen Y, Deng X, Jiang W, et al. RRx-001 Ameliorates Inflammatory Diseases by Acting as a Potent Covalent NLRP3 Inhibitor. *Cell Mol Immunol* (2021) 18(6):1425–36. doi: 10.1038/s41423-021-00683-y
142. Cornelis S, Kersse K, Festjens N, Lamkanfi M, Vandenabeele P. Inflammatory Caspases: Targets for Novel Therapies. *Curr Pharm Des* (2007) 13(4):367–85. doi: 10.2174/138161207780163006
143. Wannamaker W, Davies R, Namchuk M, Pollard J, Ford P, Ku G, et al. (S)-1-((S)-2-{[1-(4-Amino-3-Chloro-Phenyl)-Methanoyl]-Amino}-3,3-Dimethyl-Butanoyl)-Pyrrolidine-2-Carboxylic Acid ((2R,3S)-2-Ethoxy-5-Oxo-Tetrahydro-Furan-3-Yl)-Amide (VX-765), an Orally Available Selective Interleukin (IL)-Converting Enzyme/Caspase-1 Inhibitor, Exhibits Potent Anti-Inflammatory Activities by Inhibiting the Release of IL-1beta and IL-18. *J Pharmacol Exp Ther* (2007) 321(2):509–16. doi: 10.1124/jpet.106.111344
144. Rudolphi K, Gerwin N, Verzijl N, van der Kraan P, van den Berg W. Pralnacasan, an Inhibitor of Interleukin-1beta Converting Enzyme, Reduces Joint Damage in Two Murine Models of Osteoarthritis. *Osteoarthritis Cartilage* (2003) 11(10):738–46. doi: 10.1016/s1063-4584(03)00153-5
145. Strand V, Sokolove J. Randomized Controlled Trial Design in Rheumatoid Arthritis: The Past Decade. *Arthrit Res Ther* (2009) 11(1):205. doi: 10.1186/ar2555
146. Maroso M, Balosso S, Ravizza T, Iori V, Wright CI, French J, et al. Interleukin-1β Biosynthesis Inhibition Reduces Acute Seizures and Drug Resistant Chronic Epileptic Activity in Mice. *Neurotherapeutics* (2011) 8 (2):304–15. doi: 10.1007/s13311-011-0039-z
147. Bialer M, Johannessen SI, Levy RH, Perucca E, Tomson T, White HS. Progress Report on New Antiepileptic Drugs: A Summary of the Eleventh Eilat Conference (EILAT Xi). *Epilepsy Res* (2013) 103(1):2–30. doi: 10.1016/j.eplepsyres.2012.10.001
148. Calabrese LH. Anakinra Treatment of Patients With Rheumatoid Arthritis. *Ann Pharmacother* (2002) 36(7-8):1204–9. doi: 10.1345/aph.1A396
149. Kalliolias GD, Liossis SN. The Future of the IL-1 Receptor Antagonist Anakinra: From Rheumatoid Arthritis to Adult-Onset Still's Disease and Systemic-Onset Juvenile Idiopathic Arthritis. *Expert Opin Investig Drugs* (2008) 17(3):349–59. doi: 10.1517/13543784.17.3.349
150. Ruscitti P, Masedu F, Alvaro S, Airò P, Battafarano N, Cantarini L, et al. Anti-Interleukin-1 Treatment in Patients With Rheumatoid Arthritis and Type 2 Diabetes (TRACK): A Multicentre, Open-Label, Randomised Controlled Trial. *PLoS Med* (2019) 16(9):e1002901. doi: 10.1371/journal.pmed.1002901
151. Alten R, Gomez-Reino J, Durez P, Beaulieu A, Sebba A, Krammer G, et al. Efficacy and Safety of the Human Anti-IL-1β Monoclonal Antibody Canakinumab in Rheumatoid Arthritis: Results of a 12-Week, Phase II, Dose-Finding Study. *BMC Musculoskel Disord* (2011) 12:153. doi: 10.1186/1471-2474-12-153
152. Orrock JE, Ilowite NT. Canakinumab for the Treatment of Active Systemic Juvenile Idiopathic Arthritis. *Expert Rev Clin Pharmacol* (2016) 9(8):1015–24. doi: 10.1080/17512433.2016.1204910
153. De Benedetti F, Gattorno M, Anton J, Ben-Chetrit E, Frenkel J, Hoffman HM, et al. Canakinumab for the Treatment of Autoinflammatory Recurrent Fever Syndromes. *N Engl J Med* (2018) 378(20):1908–19. doi: 10.1056/NEJMoa1706314
154. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, et al. Antiinflammatory Therapy With Canakinumab for Atherosclerotic Disease. *N Engl J Med* (2017) 377(12):1119–31. doi: 10.1056/NEJMoa1707914
155. Ridker PM, Howard CP, Walter V, Everett B, Libby P, Hensen J, et al. Effects of Interleukin-1β Inhibition With Canakinumab on Hemoglobin A1c, Lipids, C-Reactive Protein, Interleukin-6, and Fibrinogen: A Phase IIb Randomized, Placebo-Controlled Trial. *Circulation* (2012) 126(23):2739–48. doi: 10.1161/circulationaha.112.122556
156. Ridker PM, MacFadyen JG, Thuren T, Everett BM, Libby P, Glynn RJ. Effect of Interleukin-1β Inhibition With Canakinumab on Incident Lung Cancer in Patients With Atherosclerosis: Exploratory Results From a Randomised, Double-Blind, Placebo-Controlled Trial. *Lancet* (2017) 390(10105):1833–42. doi: 10.1016/s0140-6736(17)32247-x
157. Schumacher HR Jr, Sundy JS, Terkeltaub R, Knapp HR, Mellis SJ, Stahl N, et al. Rilonacept (Interleukin-1 Trap) in the Prevention of Acute Gout Flares During Initiation of Urate-Lowering Therapy: Results of a Phase II Randomized, Double-Blind, Placebo-Controlled Trial. *Arthritis Rheumatol* (2012) 64(3):876–84. doi: 10.1002/art.33412
158. Hoffman HM, Throne ML, Amar NJ, Cartwright RC, Kivitz AJ, Soo Y, et al. Long-Term Efficacy and Safety Profile of Rilonacept in the Treatment of Cryopryin-Associated Periodic Syndromes: Results of a 72-Week Open-Label Extension Study. *Clin Ther* (2012) 34(10):2091–103. doi: 10.1016/j.clinthera.2012.09.009
159. Krause K, Weller K, Stefaniak R, Wittkowski H, Altrichter S, Siebenhaar F, et al. Efficacy and Safety of the Interleukin-1 Antagonist Rilonacept in Schnitzler Syndrome: An Open-Label Study. *Allergy* (2012) 67(7):943–50. doi: 10.1111/j.1398-9995.2012.02843.x
160. Gabay C, Fautrel B, Rech J, Spertini F, Feist E, Kötter I, et al. Open-Label, Multicentre, Dose-Escalating Phase II Clinical Trial on the Safety and Efficacy of Tadekinig Alfa (IL-18BP) in Adult-Onset Still's Disease. *Ann Rheum Dis* (2018) 77(6):840–7. doi: 10.1136/annrheumdis-2017-212608

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GLOSSARY

HLA	human leukocyte antigen
ACPA	anti-citrullinated protein antibody
CCP	cyclic citrullinated peptide
RF	rheumatoid factor
ESR	erythrocyte sedimentation rate
CRP	C-reactive protein
TNF	tumor necrosis factor
PAMPs	pathogen-associated molecular patterns
DAMPs	damage-associated molecular patterns
HAMPs	homeostasis-altering molecular processes
PRRs	pattern recognition receptors
TLRs	Toll-like receptors
NLRs	NOD-like receptors
CLRs	c-type lectin receptors
RLRs	retinoic acid-inducible gene (RIG)-I-like receptors
AIM2	absent in melanoma 2
CARD	caspase recruitment domain
ASC	apoptosis-associated speck-like protein containing a CARD
PYD	pyrin domain
BIR	baculovirus inhibitor of apoptosis protein repeat
NBD	the central nucleotide-binding domain
LRR	leucine-rich repeat
IFI16	human interferon (IFN)-g-inducible protein 16
ATP	adenosine triphosphate
MSU	uric acid crystals monosodium urate
LPS	lipopolysaccharides
NF-κB	nuclear factor-κB
dsDNA	double-stranded DNA
T3SS	type three secretion system
FLS	fibroblast-like synovial
AIA	antigen-induced arthritis
CIA	collagen-induced arthritis
AA	adjuvant arthritis
PBMC	peripheral blood mononuclear cell
SNP	single nucleotide polymorphism
T2D	type 2 diabetes
CAPS	cryopyrin-associated periodic syndrome

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Causal Relationship Between Gut Microbiota and Autoimmune Diseases: A Two-Sample Mendelian Randomization Study

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Background: Growing evidence has shown that alterations in gut microbiota composition are associated with multiple autoimmune diseases (ADs). However, it is unclear whether these associations reflect a causal relationship.

Objective: To reveal the causal association between gut microbiota and AD, we conducted a two-sample Mendelian randomization (MR) analysis.

Materials and Methods: We assessed genome-wide association study (GWAS) summary statistics for gut microbiota and six common ADs, namely, systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, type 1 diabetes (T1D), and celiac disease (CeD), from published GWASs. Two-sample MR analyses were first performed to identify causal bacterial taxa for ADs in discovery samples. Significant bacterial taxa were further replicated in independent replication outcome samples. A series of sensitivity analyses was performed to validate the robustness of the results. Finally, a reverse MR analysis was performed to evaluate the possibility of reverse causation.

Results: Combining the results from the discovery and replication stages, we identified one causal bacterial genus, *Bifidobacterium*. A higher relative abundance of the *Bifidobacterium* genus was associated with a higher risk of T1D [odds ratio (OR): 1.605; 95% CI, 1.339–1.922; $P_{FDR} = 4.19 \times 10^{-7}$] and CeD (OR: 1.401; 95% CI, 1.139–1.722; $P_{FDR} = 2.03 \times 10^{-3}$), respectively. Further sensitivity analyses validated

the robustness of the above associations. The results of reverse MR analysis showed no evidence of reverse causality from T1D and CeD to the *Bifidobacterium* genus.

Conclusion: This study implied a causal relationship between the *Bifidobacterium* genus and T1D and CeD, thus providing novel insights into the gut microbiota-mediated development mechanism of ADs.

Keywords: Mendelian randomization, gut microbiota, autoimmune disease (AD), type 1 diabetes, celiac disease

INTRODUCTION

Autoimmune diseases (ADs) are conditions in which an individual's immune system mistakenly attacks its host's tissues. Patients with ADs often endure lifelong debilitating symptoms, loss of organ function, reduced productivity at work, and high medical expenses. ADs are considered a significant cause of morbidity and mortality worldwide. Accumulating evidence demonstrates a steady rise in the incidence of ADs over the last few decades (1).

Although the etiology and pathogenesis of ADs are not fully understood, genetic components, environmental factors, and their interactions have great significance in their development. In addition, growing evidence suggests that alterations in gut microbiota composition are closely related to autoimmunity (2, 3). The gut microbiota is defined as the community of microorganisms that live in the human gastrointestinal tract. Gut microbial dysbiosis has been observed in many AD studies. For example, multiple studies reported a decrease of *Firmicutes*/*Bacteroidetes* ratio in systemic lupus erythematosus (SLE) patients and type 1 diabetes (T1D) patients (4, 5). A case-control study reported an increased abundance of *Methanobrevibacter* and *Akkermansia* and decreased abundance of *Butyricimonas* in patients with multiple sclerosis (MS) (6). Chen et al. (7) found that rheumatoid arthritis (RA) patients had a decrease in *Faecalibacterium* and expansion of *Eggerthella* and *Collinsella*.

All the above gut microbiota–AD associations were derived from cross-sectional studies, leaving the causal nature of these associations elusive. However, establishing causal relationships not only deepens the understanding of gut microbiota-derived AD pathogenesis but also has the capacity to guide microbiota-orientated interventions against AD in the clinic. Therefore, there is an urgent need to elucidate the causal relationship between the gut microbiota and various types of AD.

Mendelian randomization (MR) is a statistical approach that implies causal association from an exposure to an outcome. It uses genetic variants associated with exposure as a surrogate for exposure to assess the association between the surrogate and the outcome (8). Thanks to fruitful findings from large-scale genome-wide association studies (GWASs) conducted to date at both gut microbiota and disease levels (9–11), MR analysis has been widely applied to various scenarios, including the causal associations between gut microbiota and AD. In previous studies, García-Santisteban et al. (12) performed an MR analysis and identified a causal association between gut microbiota composition and celiac disease (CeD). Another study by Inamo (13) identified no causal association between gut microbiota composition and RA. The above two studies fall short in that they treat gut microbiota composition as a whole without distinguishing specific taxa, while different microbial taxa may have distinct effects on human health. During the preparation of this article, Zhang et al. (14) and Xiang et al. (15) investigated the causal effects of specific microbial taxa on two ADs, inflammatory bowel disease (IBD) and SLE. However, studies on other ADs are still sparse.

In the present study, aiming to investigate the causal relationship between gut microbiota and a broad range of ADs, we conducted a comprehensive two-sample MR analysis of six ADs, including SLE, RA, IBD, MS, T1D, and CeD.

MATERIALS AND METHODS

Ethics Statement

Our analysis used publicly available GWAS summary statistics. No new data were collected, and no new ethical approval was required. The flowchart of the study is shown in **Figure 1**. Briefly, gut microbiota served as the exposure, while ADs served as the outcome. Single-nucleotide polymorphisms (SNPs) significantly associated with specific gut microbiota taxa were selected as instrumental variables (IVs) based on strict inclusion and exclusion criteria. Outcome samples included both discovery and replication samples. A series of sensitivity analyses was performed for significant associations. Finally, reverse MR analysis was performed to mitigate the potential impact of ADs on the causal gut microbiota.

Gut Microbiota Sample

Summary statistics for gut microbial taxa were obtained from a large-scale multi-ethnic GWAS meta-analysis that included 18,340 individuals from 24 cohorts (16). The microbial composition was profiled by targeting three distinct variable regions of the 16S rRNA gene. To account for differences in sequencing depth, all datasets were rarefied to 10,000 reads per sample. Taxonomic classification was performed using direct taxonomic binning. In each cohort, only the taxa present in more than 10% of the samples were included to explore the effect of host genetics on the abundance of gut bacterial taxa. The study-wide cutoffs included an effective sample size of at least 3,000 individuals and presence in at least three cohorts. A

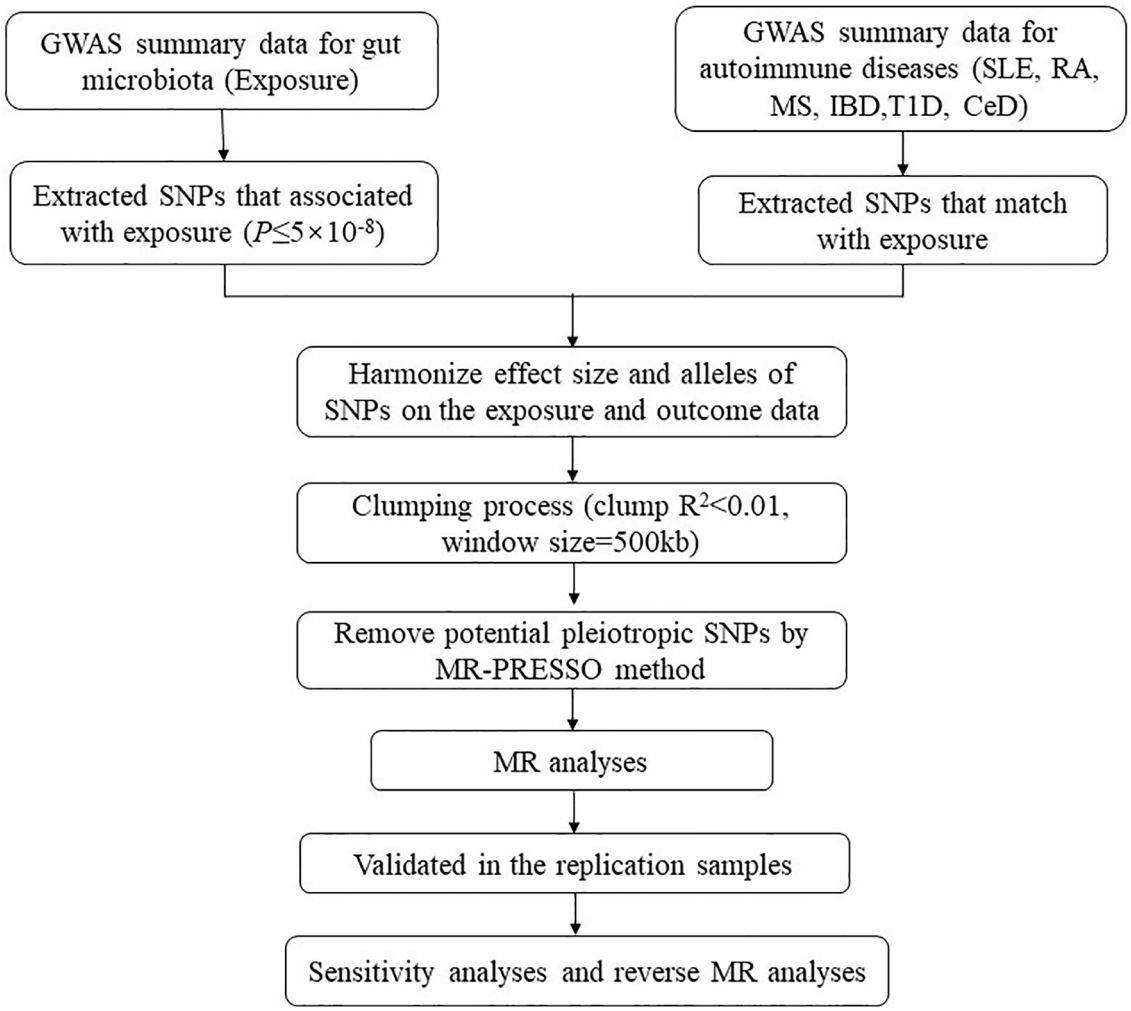


FIGURE 1 | The flowchart of the study. The whole workflow of MR analysis. MR, Mendelian randomization; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; MS, multiple sclerosis; IBD, inflammatory bowel disease; T1D, type 1 diabetes; CeD, celiac disease.

total of 211 taxa (131 genera, 35 families, 20 orders, 16 classes, and 9 phyla) were included. After adjustment for age, sex, technical covariates, and genetic principal components, Spearman's correlation analysis was performed to identify genetic loci that affected the covariate-adjusted abundance of bacterial taxa. More details on the microbiota data were described elsewhere (16).

Autoimmune Disease Discovery Samples

In the discovery stage, GWAS summary statistics for each of the six ADs were extracted from publicly available GWAS analyses. Summary statistics for SLE were obtained from a publicly available GWAS meta-analysis, including 7,219 cases and 15,991 controls of European ancestry (17). Summary statistics for RA were extracted from a GWAS meta-analysis, including 14,361 RA cases and 43,923 controls of European ancestry from 18 studies (18). Summary statistics for IBD were obtained from a GWAS meta-analysis of 25,042 IBD cases and 34,915 controls of European ancestry (19). Summary statistics of MS were derived from the discovery stage of the latest GWAS meta-analysis of the International MS Genetics Consortium (IMSGC), including 14,802 MS cases and 26,703 controls of European ancestry (20). Summary statistics of T1D were derived from a GWAS with 6,683 T1D cases and 12,173 controls of European ancestry (21). Finally, summary statistics of CeD were obtained from a GWAS meta-analysis, including 12,041 CeD cases and 12,228 controls (22). Detailed information on the datasets is provided in **Table 1**.

Autoimmune Disease Replication Samples

Significant bacterial taxa identified in the discovery stage were replicated during the replication stage. The replication outcome samples for RA, IBD, MS, and T1D were obtained from the UK Biobank study, which is a large prospective cohort study with

TABLE 1 | Autoimmune diseases GWAS samples used in this study.

Stage	Trait	N. cases	N. controls	Populations	Reference
Discovery	SLE	7,219	15,991	European	Bentham et al. (17)
	RA	14,361	43,923	European	Okada et al. (18)
	IBD	25,042	34,195	European	de Lange et al. (19)
	MS	14,802	26,703	European	Patsopoulos et al. (20)
	T1D	6,683	12,173	European	Onengut-Gumuscu et al. (21)
	CeD	12,041	12,228	97% European	Trynka et al. (22)
Replication	SLE	907	1,524	Spain	Julià et al. (23)
	RA	5,082	447,182	British	UKBB (data filed: 20002)
	IBD	3,878	448,386	British	UKBB (data filed: 20002)
	MS	1,406	450,858	British	UKBB (data filed: 41202 41204)
	T1D	3,041	449,223	British	UKBB (data filed: 41202 41204)
	CeD	4,533	10,750	European	Dubois et al. (24)

SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; MS, multiple sclerosis; IBD, inflammatory bowel disease; T1D, type 1 diabetes; CeD, celiac disease; GWAS, genome-wide association study; UKBB, UK Biobank.

approximately 500,000 participants aged 40–69 years from 22 centers across the United Kingdom. The replication sample of SLE was a single GWAS from Spain, including 907 patients with SLE and 1,524 healthy controls (23). The replication sample for CeD is a GWAS meta-analysis of five samples, including 4,533 CeD cases and 10,750 controls of European ancestry (24). Detailed information on the replication samples is presented in **Table 1**.

Selection of Instrumental Variables

The 211 bacterial taxa were categorized at six taxonomic levels. Of these, the genus is the smallest and most specific taxonomic level. To identify each causal bacteria group as specifically as possible, we analyzed 131 bacterial taxa at the genus level only. Fourteen taxa with unknown groups were excluded, meaning 117 bacterial taxa were included in the subsequent MR analysis.

SNPs associated with gut bacterial taxa at the genome-wide significance threshold $P < 5.0 \times 10^{-8}$ were selected as potential IVs. A series of quality control steps was implemented to select eligible IVs. First, SNPs with inconsistent alleles between the exposure and outcome samples (i.e., A/G vs. A/C) were excluded. Second, palindromic A/T or G/C alleles were excluded. Third, SNPs within each bacterial taxon were clumped to retain only independent SNPs. The linkage disequilibrium (LD) threshold for clumping was set to $r^2 < 0.01$, and the clumping window size was set to 500 kb. LD was estimated based on the European-based 1,000 Genome Projects reference panel. Fourth, the MR pleiotropy residual sum and outlier (MR-PRESSO) test was applied to detect potential horizontal pleiotropy and to eliminate the effects of pleiotropy by removing outliers (25). Finally, to assess the strength of the selected SNPs, the following equation was used to calculate the *F* statistics for each bacterial taxon:

$$F = \frac{R^2(n-1-k)}{(1-R^2)k}$$

where R^2 is the portion of exposure variance explained by the IVs, n is the sample size, and k is the number of IVs. An *F*-statistic ≥10 indicates no strong evidence of weak instrument bias (26). IVs with *F*-statistics less than <10 were considered weak IVs and were excluded.

Statistical Analysis

We performed MR analysis to estimate the causal effect of the gut microbiota on the six ADs. For bacterial genera containing only one SNP, the Wald ratio method was used for the MR analysis. The causal effect was calculated by dividing the SNP-outcome effect estimated by the SNP-exposure effect estimate. For bacterial genera containing multiple SNPs, multiple tests, including fixed-/random-effects inverse variance weighted (IVW) test (27), weighted median method, and MR-Egger regression test were performed. Cochrane's *Q* test was performed to assess the heterogeneity among SNPs associated with each bacterial genus. In the presence of heterogeneity ($P < 0.05$), the random-effects IVW test was used instead to provide a more conservative but robust estimate. The weighted median test can generate consistent estimates when ≥50% of the weights come from valid IVs (28). The MR-Egger regression test allows pleiotropy present in more than 50% of IVs (29).

Significant genera identified in the discovery samples were replicated in replication samples. The replication MR analysis procedure was the same as that used in the discovery stage. To evaluate the robustness of the identified causal associations, we performed two sensitivity analyses, including the MR-Egger intercept test and leave-one-out analysis. The intercept of the MR-Egger regression test can provide an estimate of the degree of directional pleiotropy (29). The leave-one-out analysis was performed to evaluate whether the significant results were driven by a single SNP.

Reverse Mendelian Randomization Analysis

To explore whether ADs have any causal impact on the identified significant bacterial genus, we also performed a reverse MR analysis (i.e., ADs as exposure and the identified causal bacterial genus as outcome) using SNPs that are associated with ADs as IVs.

All statistical analyses were conducted using R (version 4.0.3). The IVW, weighted median, and MR-Egger regression methods were performed using the "*TwoSampleMR*" package (version

0.5.4). The MR-PRESSO test was performed using the "*MRPRESSO*" package. The statistical significance of the MR effect estimates was defined as a false discovery rate (FDR) of <5% to adjust for multiple testing.

RESULTS

Selection of Instrumental Variables

After a series of quality control steps, 32 SNPs associated with 13 genera were selected as IVs. Specifically, 19 independent SNPs ($P < 5.0 \times 10^{-8}$, $r^2 < 0.01$) were associated with 13 genera for SLE, 17 independent SNPs were associated with 12 genera for RA, 19 SNPs were associated with 13 genera for MS, 18 SNPs were associated with 12 genera for IBD, 7 SNPs were associated with 3 genera for T1D, and 6 SNPs were associated with 3 genera for CeD (**Supplementary Table S1**). No evidence of pleiotropic effects was detected by the MR-PRESSO global test ($P > 0.05$). The *F*-statistics of IVs ranged between 29.78 and 2,074.13, all largely >10, indicating no evidence of weak instrument bias (**Supplementary Table S2**).

Causal Effects of Gut Microbiota on Autoimmune Diseases

In the discovery stage, the genetically predicted relative abundance of two genera, *Bifidobacterium* and *Ruminococcus*, was associated with the risk of SLE, MS, T1D, and CeD. *Ruminococcus* was also associated with the risk of IBD (**Table 2**). Specifically, a higher genetically predicted *Bifidobacterium* level was associated with a lower risk of SLE [odds ratio (OR): 0.565, 95% confidence interval (CI): 0.426–0.748, $P_{FDR} = 8.53 \times 10^{-4}$]. In contrast, a higher genetically predicted *Bifidobacterium* was associated with a higher risk of MS (OR: 1.384, 95% CI: 1.128–1.700, $P_{FDR} = 0.012$), T1D (OR: 1.605, 95% CI: 1.339–1.922, $P_{FDR} = 4.19 \times 10^{-7}$), and CeD (OR: 1.401, 95% CI: 1.139–1.722, $P_{FDR} = 2.03 \times 10^{-3}$). These associations were also supported by the weighted median method, as shown in **Table 2**. The genetically predicted *Ruminococcus* level was associated with a higher risk of SLE (OR: 5.593, 95% CI: 2.079–15.045, $P_{FDR} = 4.22 \times 10^{-3}$), IBD (OR: 2.141, 95% CI: 1.425–3.216, $P_{FDR} = 2.92 \times 10^{-3}$), and MS (OR: 2.890, 95% CI: 1.669–5.003, $P_{FDR} = 1.96 \times 10^{-3}$). But its associations with T1D and CeD were negative (OR: 0.122, 95% CI: 0.0661–0.224, $P_{FDR} = 3.38 \times 10^{-11}$) and CeD (OR: 0.352, 95% CI: 0.195–0.635, $P_{FDR} = 1.57 \times 10^{-3}$). As shown in **Supplementary Table S2**, there was no evidence of a causal association between any microbial taxa and RA.

These two genera *Bifidobacterium* and *Ruminococcus* were replicated in the replication samples. The causal effects of the *Bifidobacterium* genus on T1D and CeD were successfully replicated, as shown in **Table 3**. The effect direction was consistent with that in the discovery sample, which strengthened the confidence of the true causal associations.

Sensitivity Analyses

No evidence of heterogeneity was observed between the genetic IVs for *Bifidobacterium* (**Supplementary Table S3**). None of the MR-Egger regression intercepts deviated from null, indicating no evidence of horizontal pleiotropy (all intercept $P > 0.05$) (**Supplementary Table S4**). Additionally, the leave-one-out analysis showed no marked difference in causal estimations of *Bifidobacterium* on T1D and CeD, suggesting

TABLE 2 | Significant MR analysis results in the discovery samples.

Traits (outcome)	Bacterial taxa (exposure)	MR method	No. SNP	*F*-statistics	OR	95% CI	*P*	P_{FDR}
SLE	*Bifidobacterium*	IVW (fixed)	6	2074.13	0.565	0.426–0.748	6.56×10^{-5}	**8.53×10^{-4}**
		Weighted median			0.508	0.353–0.730	2.50×10^{-4}	**3.35×10^{-3}**
		MR-Egger			0.776	0.132–4.538	0.792	0.819
	Ruminococcus	Wald ratio	1	31.33	5.593	2.079–15.045	6.50×10^{-4}	**4.22×10^{-3}**
IBD	*Bifidobacterium*	IVW (fixed)	6	1905.96	1.182	1.039–1.345	0.011	0.064
		Weighted median			1.182	1.009–1.384	0.037	0.188
		MR-Egger			1.226	0.641–2.344	0.561	0.767
	Ruminococcus	Wald ratio	1	31.33	2.141	1.425–3.216	2.43×10^{-4}	**2.92×10^{-3}**
MS	*Bifidobacterium*	IVW (fixed)	6	2074.13	1.384	1.128–1.698	1.84×10^{-3}	**0.012**
		Weighted median			1.439	1.104–1.877	7.19×10^{-3}	**0.047**
		MR-Egger			1.024	0.348–3.011	0.967	0.970
	Ruminococcus	Wald ratio	1	31.33	2.890	1.669–5.003	1.51×10^{-4}	**1.96×10^{-3}**
T1D	*Bifidobacterium*	IVW (fixed)	5	1804.95	1.605	1.339–1.922	2.79×10^{-7}	**4.19×10^{-7}**
		Weighted median			1.745	1.405–2.167	4.66×10^{-7}	**6.99×10^{-7}**
		MR-Egger			3.046	0.580–15.992	0.279	0.419
	Ruminococcus	Wald ratio	1	27.47	0.122	0.0661–0.224	1.13×10^{-11}	**3.38×10^{-11}**
CeD	*Bifidobacterium*	IVW (fixed)	4	981.22	1.401	1.139–1.722	1.35×10^{-3}	**2.03×10^{-3}**
		Weighted median			1.463	1.149–1.863	1.96×10^{-3}	**2.95×10^{-3}**
		MR-Egger			2.079	0.646–6.680	0.344	0.516
	Ruminococcus	Wald ratio	1	31.32	0.352	0.195–0.635	5.25×10^{-4}	**1.57×10^{-3}**

No. SNP is the number of SNPs being used as IVs.

Significant P_{FDR} was marked in bold.

MR, Mendelian randomization; SNP, single-nucleotide polymorphism; IVW, inverse-variance weighted; OR, odds ratio; CI, confidence interval; P_{FDR}, P value corrected by false discovery rate (FDR); SLE, systemic lupus erythematosus; IBD, inflammatory bowel disease; MS, multiple sclerosis; T1D, type 1 diabetes; CeD, celiac disease.

TABLE 3 | Results of the identified bacterial taxa in the replication samples.

Traits (outcome)	Bacterial taxa (exposure)	MR methods	No. SNP	*F*-statistics	OR	95% CI	*P*	P_{FDR}
SLE	*Bifidobacterium*	IVW (fixed)	6	2,074.13	1.269	0.929–1.734	0.155	0.155
		Weighted median			1.175	0.801–1.723	0.410	0.410
		MR-Egger			0.849	0.124–5.808	0.876	0.876
	Ruminococcus	Wald ratio test	1	31.33	4.314	0.576–32.286	0.155	0.309
IBD	*Bifidobacterium*	IVW (fixed)	5	1,557.97	0.998	0.998–1.002	0.761	0.111
		Weighted median			0.998	0.995–1.001	0.142	0.142
		MR-Egger			1.002	0.987–1.016	0.845	0.850
	Ruminococcus	Wald ratio test	1	31.33	0.995	0.989–1.001	0.111	0.142
MS	*Bifidobacterium*	IVW (fixed)	5	1,557.97	1.001	0.999–1.003	0.070	0.140
		Weighted median			1.001	1.000–1.003	0.038	0.076
		MR-Egger			1.000	0.993–1.008	0.938	0.938
	Ruminococcus	Wald ratio test	1	31.33	1.002	0.998–1.006	0.312	0.312
T1D	*Bifidobacterium*	IVW (fixed)	5	1,557.97	1.002	1.001–1.004	8.58×10^{-4}	$\mathbf{1.72 \times 10^{-3}}$
		Weighted median			1.002	1.000–1.004	6.42×10^{-3}	**0.013**
		MR-Egger			1.000	0.991–1.009	0.965	0.965
	Ruminococcus	Wald ratio test	1	31.33	0.997	0.991–1.002	0.247	0.247
CeD	*Bifidobacterium*	IVW (fixed)	6	2,207.82	1.643	1.300–2.076	3.16×10^{-5}	$\mathbf{3.16 \times 10^{-5}}$
		Weighted median			1.755	1.314–2.343	1.38×10^{-4}	$\mathbf{1.38 \times 10^{-4}}$
		MR-Egger			1.202	0.219–6.589	0.842	0.842

No. SNP is the number of SNPs being used as IVs.

Significant P_{FDR} was marked in bold.

MR, Mendelian randomization; SNP, single-nucleotide polymorphism; IV, instrumental variable; IVW, inverse-variance weighted; OR, odds ratio; CI, confidence interval; P_{FDR}, P value corrected by false discovery rate (FDR); SLE, systemic lupus erythematosus; IBD, inflammatory bowel disease; MS, multiple sclerosis; T1D, type 1 diabetes; CeD, celiac disease.

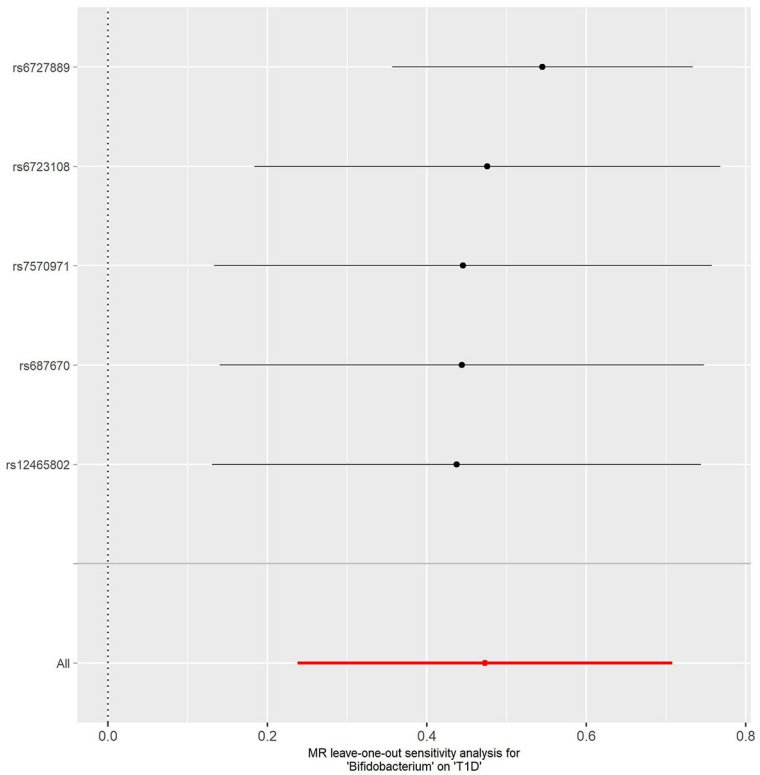


FIGURE 2 | Leave-one-out analysis of the causal effect of *Bifidobacterium* on T1D. Red lines represent estimations from the IVW test. T1D, type 1 diabetes; IVW, inverse variance weighted.

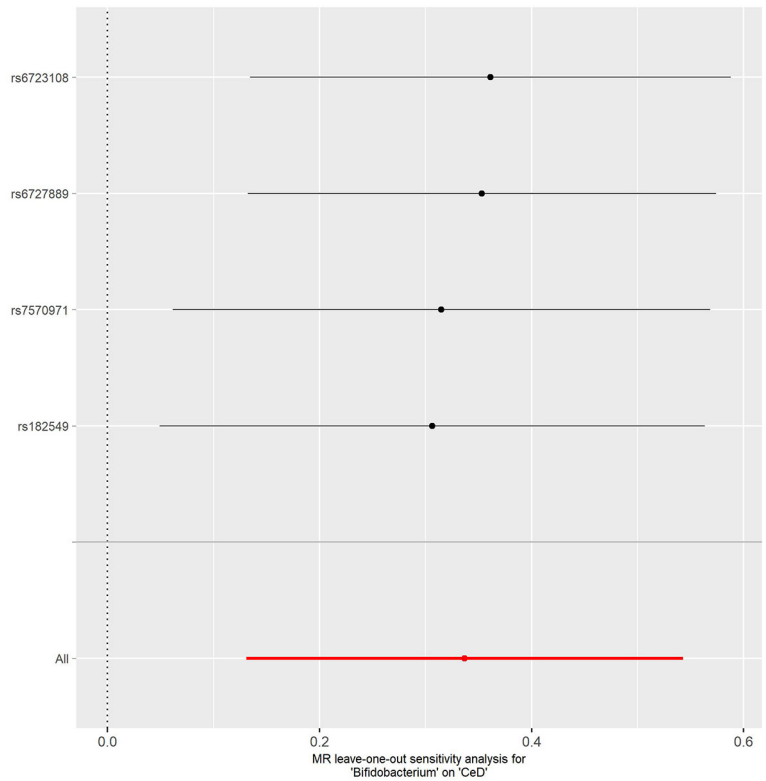


FIGURE 3 | Leave-one-out analysis of the causal effect of *Bifidobacterium* on CeD. Red lines represent estimations from the IVW test. CeD, celiac disease; IVW, inverse variance weighted.

that none of the identified causal associations were driven by any single IV (**Figures 2**, **3**). In reverse MR analysis, there was no evidence of a causal effect of T1D and CeD on *Bifidobacterium* (**Table 4**). Detailed information on the IVs used in the reverse MR analyses is shown in **Supplementary Table S5**.

DISCUSSION

In this study, we performed two-sample MR analyses to investigate the causal association between gut microbiota and six common ADs (SLE, RA, MS, IBD, T1D, and CeD). Combining evidence from both discovery and replication samples, we identified that the bacterial genus *Bifidobacterium* was causally associated with the risk of T1D and CeD.

Bifidobacterium is the primary microbe that colonizes the human gut. Previous observational studies have demonstrated that *Bifidobacterium* plays an important role in the pathogenesis of multiple ADs. However, observational studies have yielded conflicting results regarding the effect pattern. Two case-control studies showed that the relative abundance of *Bifidobacterium* was higher in T1D patients than that in controls (30, 31). Similarly, a higher relative abundance of *Bifidobacterium* was observed in patients with CeD (32). In line with these studies, our study suggested that the increased relative abundance of *Bifidobacterium* was causally associated with a higher risk of T1D and CeD, indicating its harmful effect on both diseases. In contrast, several other studies observed a lower relative abundance of *Bifidobacterium* in T1D and CeD patients, suggesting its protective effect (33–35).

Recent studies have shown that probiotic intervention, mainly of the *Lactobacillus* and *Bifidobacterium* genera, can effectively attenuate the progression of multiple ADs, including T1D and CeD. In a double-blinded, placebo-controlled trial, probiotic intervention with *Bifidobacterium breve* BR03 and *B. breve* B632 has shown a positive effect on decreasing the production of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) in children with CeD on a gluten-free diet (36). In contrast, Smecuol et al. (37) did not detect significant changes in TNF-α in CeD patients treated with *Bifidobacterium infantis*. Similarly, Groele et al. (38) reported that administration of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 had no significant effect on maintaining the residual pancreatic beta-cell function in children with newly diagnosed T1D. There was no significant difference in cytokine levels and intestinal permeability (zonulin levels) between the probiotics and placebo groups (38).

Some functional studies have shown evidence of the anti-inflammatory effects of *Bifidobacterium*, while others have

TABLE 4 | Reverse causal association between ADs and gut microbiota.

Exposure	Outcome	MR method	No. SNP	OR	95% CI	*P*
T1D	*Bifidobacterium*	IVW	39	0.991	0.973–1.009	0.31
		Weighted median		0.993	0.964–1.023	0.66
		MR-Egger		0.990	0.995–1.026	0.58
CeD	*Bifidobacterium*	IVW	105	0.998	0.993–1.003	0.40
		Weighted median		0.998	0.990–1.005	0.56
		MR-Egger		0.993	0.986–1.000	0.08

No. SNP is the number of SNPs being used as IVs.
MR, Mendelian randomization; AD, autoimmune disease; SNP, single-nucleotide polymorphism; IV, instrumental variable; IVW, inverse-variance weighted; OR, odds ratio; CI, confidence interval; T1D, type 1 diabetes; CeD, celiac disease.

reported its pro-inflammatory effects. A previous study showed that *Bifidobacterium adolescentis* significantly increased Th17 cell levels in several other gut-associated organs, while elevated Th17 cell responses have been associated with autoimmune/inflammatory disease in both mice and humans (38). In addition, López et al. (39) reported that some *Bifidobacterium bifidum* strains could induce the secretion of large amounts of interleukin IL-17 and promote Th17 cell polarization. Combining evidence from observational studies, MR analysis, clinical trials, and functional studies, we speculated that the positive and negative effects of *Bifidobacterium* on ADs may be species- and strain-specific. The causal relationship between *Bifidobacterium* and ADs needs to be further explored at more specialized levels (i.e., species level and strain level).

In previous studies, Zhang et al. (14) and Xiang et al. (15) performed MR analyses to investigate the effects of gut microbiota on IBD and SLE, respectively. Our study differs from their studies in the following three aspects: First, our study is more comprehensive in its investigation of ADs. Unlike the above two studies that analyzed two separate diseases, we comprehensively analyzed six common AD diseases. This will give us an opportunity to evaluate common gut microbiota that are causally related to multiple ADs. Second, the quality control procedure for selecting IVs was stricter in our study. We selected independent GWAS SNPs as IVs and conducted a series of sensitivity analyses, including horizontal pleiotropy assessment and reverse MR analysis, to maximally fulfill basic MR assumptions. In contrast, the above two studies used a fairly loose *P*-value threshold ($P < 1 \times 10^{-5}$) to select eligible IVs. Third, Zhang et al. (14) used summary-level data of gut microbiota in a relatively small sample size (N = 1,126 twin pairs). Instead, the sample size in the present study was much larger (N = 18,340). Meanwhile, the causal associations identified in the discovery stage were further replicated in independent replication outcome samples, which enhanced the confidence of the true causal relationship.

Nevertheless, our study had several limitations. First, while the majority of participants in the GWAS summary data used in our study were of European ancestry, a small number of the gut microbiota data were taken from sets consisting of other races, which may partially bias our estimates. Second, bacterial taxa were only analyzed at the genus level but not at a more specialized level such as species or strain levels. When microbiota GWASs use more advanced shotgun metagenomic sequencing analysis, the results can be more specific and accurate. Third, our study used gut microbiota data from a meta-analysis of mostly adult individuals, whereas the CeD study was conducted in children. Finally, most ADs are more prevalent in women than in men (e.g., SLE, RA, and MS). However, our study did not analyze the two genders separately, which may have influenced our results. It would be helpful to perform a gender-specific MR analysis in future endeavors.

In conclusion, our findings support the potentially causal effects of the *Bifidobacterium* genus on T1D and CeD. Although *Bifidobacterium* is generally considered beneficial bacteria, specific species and strains of *Bifidobacterium* may have varying effects on human health. Therefore, the potential mechanisms of specific species and strains of *Bifidobacterium* in the development of AD need to be further investigated.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

LZ and Y-FP designed the research. QX and J-JN collected the data. QX and BL analyzed the data. J-JN, B-XH, S-SY, X-TW, G-JF, and HZ performed the literature search. QX and BL drafted the article. Y-FP and LZ jointly supervised the study. All authors were involved in writing the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.746998/full#supplementary-material

REFERENCES

1. Lerner A, Jeremias P, Matthias T. The World Incidence and Prevalence of Autoimmune Diseases is Increasing. *Int J Celiac Dis* (2015) 3:151–5.
2. De Luca F, Shoenfeld Y. The Microbiome in Autoimmune Diseases. *Clin Exp Immunol* (2019) 195:74–85. doi: 10.1111/cei.13158
3. Chen B, Sun L, Zhang X. Integration of Microbiome and Epigenome to Decipher the Pathogenesis of Autoimmune Diseases. *J Autoimmun* (2017) 83:31–42. doi: 10.1016/j.jaut.2017.03.009
4. López P, de Paz B, Rodríguez-Carrio J, Hevia A, Sánchez B, Margolles A, et al. Th17 Responses and Natural IgM Antibodies are Related to Gut Microbiota Composition in Systemic Lupus Erythematosus Patients. *Sci Rep* (2016) 6:24072. doi: 10.1038/srep24072
5. Murri M, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, Soriguer F, et al. Gut Microbiota in Children With Type 1 Diabetes Differs From That in Healthy Children: A Case-Control Study. *BMC Med* (2013) 11:46. doi: 10.1186/1741-7015-11-46
6. Jangi S, Gandhi R, Cox LM, Li N, von Glehn F, Yan R, et al. Alterations of the Human Gut Microbiome in Multiple Sclerosis. *Nat Commun* (2016) 7:12015. doi: 10.1038/ncomms12015
7. Chen J, Wright K, Davis JM, Jeraldo P, Marietta EV, Murray J, et al. An Expansion of Rare Lineage Intestinal Microbes Characterizes Rheumatoid Arthritis. *Genome Med* (2016) 8(1):43. doi: 10.1186/s13073-016-0299-7
8. Emdin CA, Khera AV, Kathiresan S. Mendelian Randomization. *JAMA* (2017) 318(19):1925–6. doi: 10.1001/jama.2017.17219
9. Goodrich JK, Davenport ER, Beaumont M, Jackson MA, Knight R, Ober C, et al. Genetic Determinants of the Gut Microbiome in UK Twins. *Cell Host Microbe* (2016) 19(5):731–43. doi: 10.1016/j.chom.2016.04.017
10. Wang J, Thingholm LB, Skiecevičienė J, Rausch P, Kummen M, Hov JR, et al. Genome-Wide Association Analysis Identifies Variation in Vitamin D Receptor and Other Host Factors Influencing the Gut Microbiota. *Nat Genet* (2016) 48(11):1396–406. doi: 10.1038/ng.3695
11. Bonder MJ, Kurilshikov A, Tigchelaar EF, Mujagic Z, Imhann F, Vila AV, et al. The Effect of Host Genetics on the Gut Microbiome. *Nat Genet* (2016) 48 (11):1407–12. doi: 10.1038/ng.3663
12. García-Santisteban I, Cilleros-Portet A, Moyua-Ormazabal E, Kurilshikov A, Zhernakova A, Garcia-Etxebarria K, et al. A Two-Sample Mendelian Randomization Analysis Investigates Associations Between Gut Microbiota and Celiac Disease. *Nutrients* (2020) 12(5):1420. doi: 10.3390/nu12051420
13. Inamo J. Non-Causal Association of Gut Microbiome on the Risk of Rheumatoid Arthritis: A Mendelian Randomisation Study. *Ann Rheum Dis* (2021) 81(1):e3. doi: 10.1136/annrheumdis-2019-216565
14. Zhang ZJ, Qu HL, Zhao N, Wang J, Wang XY, Hai R, et al. Assessment of Causal Direction Between Gut Microbiota and Inflammatory Bowel Disease: A Mendelian Randomization Analysis. *Front Genet* (2021) 12:631061. doi: 10.3389/fgene.2021.631061
15. Xiang K, Wang P, Xu Z, Hu YQ, He YS, Chen Y, et al. Causal Effects of Gut Microbiome on Systemic Lupus Erythematosus: A Two-Sample Mendelian Randomization Study. *Front Immunol* (2021) 12:667097. doi: 10.3389/fimmu.2021.667097
16. Kurilshikov A, Medina-Gomez C, Bacigalupe R, Radjabzadeh D, Wang J, Demirkan A, et al. Large-Scale Association Analyses Identify Host Factors Influencing Human Gut Microbiome Composition. *Nat Genet* (2021) 53 (2):156–65. doi: 10.1038/s41588-020-00763-1
17. Bentham J, Morris DL, Graham DSC, Pinder CL, Tombleson P, Behrens TW, et al. Genetic Association Analyses Implicate Aberrant Regulation of Innate and Adaptive Immunity Genes in the Pathogenesis of Systemic Lupus Erythematosus. *Nat Genet* (2015) 47(12):1457–64. doi: 10.1038/ng.3434
18. Okada Y, Wu D, Trynka G, Raj T, Terao C, Ikari K, et al. Genetics of Rheumatoid Arthritis Contributes to Biology and Drug Discovery. *Nature* (2014) 506(7488):376–81. doi: 10.1038/nature12873
19. de Lange KM, Moutsianas L, Lee JC, Lamb CA, Luo Y, Kennedy NA, et al. Genome-Wide Association Study Implicates Immune Activation of Multiple Integrin Genes in Inflammatory Bowel Disease. *Nat Genet* (2017) 49(2):256–61. doi: 10.1038/ng.3760
20. International Multiple Sclerosis Genetics Consortium. Multiple Sclerosis Genomic Map Implicates Peripheral Immune Cells and Microglia in Susceptibility. *Sci (New York NY)* (2019) 365(6460):eaav7188. doi: 10.1126/science.aav7188
21. Onengut-Gumuscu S, Chen WM, Burren O, Cooper NJ, Quinlan AR, Mychaleckyj JC, et al. Fine Mapping of Type 1 Diabetes Susceptibility Loci and Evidence for Colocalization of Causal Variants With Lymphoid Gene Enhancers. *Nat Genet* (2015) 47(4):381–6. doi: 10.1038/ng.3245
22. Trynka G, Hunt KA, Bockett NA, Romanos J, Mistry V, Szperl A, et al. Dense Genotyping Identifies and Localizes Multiple Common and Rare Variant Association Signals in Celiac Disease. *Nat Genet* (2011) 43(12):1193–201. doi: 10.1038/ng.998
23. Julià A, López-Longo FJ, Pérez Venegas JJ, Bonàs-Guarch S, Olivé À, Andreu JL, et al. Genome-Wide Association Study Meta-Analysis Identifies Five New Loci for Systemic Lupus Erythematosus. *Arthritis Res Ther* (2018) 20(1):100. doi: 10.1186/s13075-018-1604-1
24. Dubois PC, Trynka G, Franke L, Hunt KA, Romanos J, Curtotti A, et al. Multiple Common Variants for Celiac Disease Influencing Immune Gene Expression. *Nat Genet* (2010) 42:295–302. doi: 10.1038/ng.543
25. Verbanck M, Chen CY, Neale B, Do R. Detection of Widespread Horizontal Pleiotropy in Causal Relationships Inferred From Mendelian Randomization Between Complex Traits and Diseases. *Nat Genet* (2018) 50(5):693–8. doi: 10.1038/s41588-018-0099-7
26. Pierce BL, Ahsan H, Vanderweele TJ. Power and Instrument Strength Requirements for Mendelian Randomization Studies Using Multiple Genetic Variants. *Int J Epidemiol* (2011) 40(3):740–52. doi: 10.1093/ije/dyq151
27. Burgess S, Butterworth A, Thompson SG. Mendelian Randomization Analysis With Multiple Genetic Variants Using Summarized Data. *Genet Epidemiol* (2013) 37(7):658–65. doi: 10.1002/gepi.21758
28. Bowden J, Davey Smith G, Haycock PC, Burgess S. Consistent Estimation in Mendelian Randomization With Some Invalid Instruments Using a Weighted Median Estimator. *Genet Epidemiol* (2016) 40(4):304–14. doi: 10.1002/gepi.21965
29. Bowden J, Davey Smith G, Burgess S. Mendelian Randomization With Invalid Instruments: Effect Estimation and Bias Detection Through Egger Regression. *Int J Epidemiol* (2015) 44(2):512–25. doi: 10.1093/ije/dyv080
30. Maffeis C, Martina A, Corradi M, Quarella S, Nori N, Torriani S, et al. Association Between Intestinal Permeability and Faecal Microbiota Composition in Italian Children With Beta Cell Autoimmunity at Risk for Type 1 Diabetes. *Diabetes/Metabol Res Rev* (2016) 32(7):700–9. doi: 10.1002/dmrr.2790
31. Brown CT, Davis-Richardson AG, Giongo A, Gano KA, Crabb DB, Mukherjee N, et al. Gut Microbiome Metagenomics Analysis Suggests a Functional Model for the Development of Autoimmunity for Type 1 Diabetes. *PloS One* (2011) 6():e25792. doi: 10.1371/journal.pone.0025792

32. Nistal E, Caminero A, Vivas S, Ruiz de Morales JM, Sáenz de Miera LE, Rodríguez-Aparicio LB, et al. Differences in Faecal Bacteria Populations and Faecal Bacteria Metabolism in Healthy Adults and Celiac Disease Patients. *Biochimie* (2012) 94(8):1724–9. doi: 10.1016/j.biochi.2012.03.025
33. Pinto E, Anselmo M, Calha M, Bottrill A, Duarte I, Andrew PW, et al. The Intestinal Proteome of Diabetic and Control Children Is Enriched With Different Microbial and Host Proteins. *Microbiol (Read Engl)* (2017) 163 (2):161–74. doi: 10.1099/mic.0.000412
34. Salamon D, Sroka-Oleksiak A, Kapusta P, Szopa M, Mrozińska S, Ludwig-Słomczyńska AH, et al. Characteristics of Gut Microbiota in Adult Patients With Type 1 and Type 2 Diabetes Based on Next–Generation Sequencing of the 16S rRNA Gene Fragment. *Polish Arch Intern Med* (2018) 128(6):336–43. doi: 10.20452/pamw.4246
35. Leiva-Gea I, Sánchez-Alcoholado L, Martín-Tejedor B, Castellano-Castillo D, Moreno-Indias I, Urda-Cardona A, et al. Gut Microbiota Differs in Composition and Functionality Between Children With Type 1 Diabetes and MODY2 and Healthy Control Subjects: A Case-Control Study. *Diabetes Care* (2018) 41(11):2385–95. doi: 10.2337/dc18-0253
36. Klemenak M, Dolinšek J, Langerholc T, Di Gioia D, Mičetić-Turk D. Administration of Bifidobacterium Breve Decreases the Production of TNF-α in Children With Celiac Disease. *Digest Dis Sci* (2015) 60(11):3386–92. doi: 10.1007/s10620-015-3769-7
37. Smecuol E, Hwang HJ, Sugai E, Corso L, Cherñavsky AC, Bellavite FP, et al. Exploratory, Randomized, Double-Blind, Placebo-Controlled Study on the Effects of Bifidobacterium Infantis Natren Life Start Strain Super Strain in Active Celiac Disease. *J Clin Gastroenterol* (2013) 47(2):139–47. doi: 10.1097/MCG.0b013e31827759ac
38. Groele L, Szajewska H, Szalecki M, Świderska J, Wysocka-Mincewicz M, Ochocińska A, et al. Lack of Effect of Lactobacillus Rhamnosus GG and Bifidobacterium Lactis Bb12 on Beta-Cell Function in Children With Newly Diagnosed Type 1 Diabetes: A Randomised Controlled Trial. *BMJ Open Diabetes Res Care* (2021) 9(1):e001523. doi: 10.1136/bmjdrc-2020-001523
39. López P, Gueimonde M, Margolles A, Suárez A. Distinct Bifidobacterium Strains Drive Different Immune Responses *In Vitro*. *Int J Food Microbiol* (2010) 138(1-2):157–65. doi: 10.1016/j.ijfoodmicro.2009.12.023

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Humoral and Cellular Immune Responses to SARS-CoV-2 mRNA Vaccination in Patients with Multiple Sclerosis: An Israeli Multi-Center Experience Following 3 Vaccine Doses

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Background: Immunomodulatory/immunosuppressive activity of multiple sclerosis (MS) disease modifying therapies (DMTs) might affect immune responses to SARS-CoV-2 exposure or vaccination in patients with MS (PwMS). We evaluated the effect of DMTs on humoral and cell-mediated immune responses to 2 and 3 vaccinations and the longevity of SARS-Cov-2 IgG levels in PwMS.

Methods: 522 PwMS and 68 healthy controls vaccinated with BNT162b2-Pfizer mRNA vaccine against SARS-CoV-2, or recovering from COVID-19, were recruited in a nation-wide multi-center study. Blood was collected at 3 time-points: 2-16 weeks and ~6 months post 2nd vaccination and 1-16 weeks following 3rd vaccination. Serological responses were measured by quantifying IgG levels against the spike-receptor-binding-domain of SARS-CoV-2, and cellular responses (in a subgroup analysis) by quantifying IFNγ secretion in blood incubated with COVID-19 spike-antigen.

Results: 75% PwMS were seropositive post 2nd or 3rd vaccination. IgG levels decreased by 82% within 6 months from vaccination ($p<0.0001$), but were boosted 10.3 fold by the 3rd vaccination ($p<0.0001$), and 1.8 fold compared to ≤3m post 2nd vaccination ($p=0.025$). Patients treated with most DMTs were seropositive post 2nd and 3rd vaccinations, however only 38% and 44% of ocrelizumab-treated patients and 54% and 46% of fingolimod-treated patients, respectively, were seropositive. Similarly, in COVID-19-recovered patients only 54% of ocrelizumab-treated, 75% of fingolimod-

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treated and 67% of cladribine-treated patients were seropositive. A time interval of ≥5 months between ocrelizumab infusion and vaccination was associated with higher IgG levels (p=0.039 post-2nd vaccination; p=0.036 post-3rd vaccination), and with higher proportions of seropositive patients. Most fingolimod- and ocrelizumab-treated patients responded similarly to 2nd and 3rd vaccination. IFNγ-T-cell responses were detected in 89% and 63% of PwMS post 2nd and 3rd vaccination, however in only 25% and 0% of fingolimod-treated patients, while in 100% and 86% of ocrelizumab-treated patients, respectively.

Conclusion: PwMS treated with most DMTs developed humoral and T-cell responses following 2 and 3 mRNA SARS-CoV-2 vaccinations. Fingolimod- or ocrelizumab-treated patients had diminished humoral responses, and fingolimod compromised the cellular responses, with no improvement after a 3rd booster. Vaccination following >5 months since ocrelizumab infusion was associated with better sero-positivity. These findings may contribute to the development of treatment-stratified vaccination guidelines for PwMS.

Keywords: autoimmunity, COVID-19, humoral response, IgG, multiple sclerosis, SARS-CoV-2, T-cell immune response, disease modifying therapies (DMTs)

INTRODUCTION

With the outbreak of the COVID-19 pandemic and the rapid development of SARS-CoV-2 vaccines worldwide, concern was raised that the immunomodulatory/immunosuppressive effects of multiple sclerosis (MS) disease modifying therapies (DMTs) might affect COVID-19 disease severity as well as the development of humoral and cellular immunity after SARS-CoV-2 exposure or vaccination (1). In Israel, a nationwide COVID-19 vaccination program was rapidly launched starting December 2020, based solely upon the BNT162b2 (Pfizer-BioNTech) mRNA vaccine, offering two doses administered 3 weeks apart, and a third vaccine booster ≥ 5 months after the 2nd dose (2), a vaccination regime associated with a good safety profile (3). All PwMS are recommended by the National Multiple Sclerosis Society of the United States and by the MS International Federation to be vaccinated against SARS-CoV-2 (4, 5). With the variety of available DMTs, that exert their action through diverse immunological mechanisms, it is of high importance to investigate the immune response to SARS-CoV-2 vaccination in PwMS treated with various immunotherapies, in order to develop optimal and treatment-stratified guidelines. The immune response to SARS-CoV-2 consists of both a cellular and a humoral response, but measurement of SARS-CoV-2 IgG is widely used to identify persons who have recovered from COVID-19 infection or as a confirmation of a sufficient vaccine response, based on the fact that the levels of neutralizing antibodies have been shown to be highly predictive of SARS-CoV-2 immune protection (6). Initial reports have demonstrated that while most MS patients show a positive humoral immune response shortly after the 2nd SARS-CoV-2 mRNA vaccination, a considerable proportion of patients treated with fingolimod or ocrelizumab do not develop antibodies (7–12). In light of the immunological effects of the DMTs, it is important to evaluate the effect of the patients' immune response to the vaccine and to COVID-19 over time and the effects of the third dose of vaccine in patients treated with ocrelizumab and fingolimod. In this nation-wide multi-center prospective study we examined the levels of anti-SARS-CoV-2 antibodies in PwMS treated with most available DMTs and in healthy controls (HC), during a period of at least 6 months following the 2nd SARS-CoV-2 vaccination and at 1-16 weeks following a 3rd vaccine booster. In addition, we evaluated the cell-mediated immune responses against the COVID-19 spike protein in a subgroup of the participants.

MATERIALS AND METHODS

Participants and Sampling

This observational multi-center prospective study was conducted in the MS centers at 5 major Israeli hospitals: Hadassah Medical Center, Tel Aviv Sourasky Medical Center, Barzilai Medical Center, Rabin Medical Center and Carmel Medical Center, in compliance with the principles of the Declaration of Helsinki, as approved by the Ethics Committees of each hospital. The study included 522 PwMS and 68 HC, that were recently vaccinated or about to be vaccinated against SARS-CoV-2 with the mRNA vaccine BNT162b2 (Pfizer-BioNTech), or who recovered from COVID-19. All participants signed a written informed consent prior to the study procedures. Demographic and clinical data were recorded at recruitment. Serum was collected 2-16 weeks (mean=7.5 weeks, median=6.9 weeks) after the 2nd vaccine dose (time-point 1), ~6 months (17-39 weeks, mean and median=25 weeks.) after the 2nd vaccine dose (time-point 2) and 1-16 weeks (mean=7.2 weeks, median=6.7 weeks) after the 3rd vaccine booster (time-point 3) and kept at -80^{0}C until assessed. In a subgroup of 39 PwMS and 8 HC, whole blood was collected at the same time-points and used for assessment of the anti-spike protein T-cell response.

SARS-CoV-2 IgG

The humoral response was measured at a centralized laboratory (the Clinical Virology Laboratory at Hadassah Medical Center, Jerusalem) using the spike receptor-binding domain (RBD) Architect SARS-CoV-2 IgG II Quant Assay (Abbott). Serum positivity was determined in accordance with the definition of The Israeli Health Ministry at: ≥50 arbitrary units per ml in vaccinated and/or COVID-19-recovered persons.

T Cell Reactivity

For evaluation of SARS-CoV-2 spike-specific T-cell responses, a quantitative assay SARS-CoV-2 IGRA stimulation tube set (EuroImmun, Germany) was used according to manufacturer's protocol. The IFNγ-T cell response assay was carried out at the Israel Institute for Biological Research (Ness Ziona, Israel). Heparinized whole-blood samples were stimulated *ex-vivo* with the COVID-19 spike protein for 24 hours, then plasma was collected and secreted IFNγ was quantified by ELISA (ELISA DuoSet, R&D Systems, Minneapolis, Minnesota, USA). A similar sample without antigen stimulation was used as control. Values above 25 pg/ml of IFNγ spike-specific response were considered positive.

Statistical Analysis

Data was analyzed using SPSS v24. The antibody levels were transformed on a Log10 scale, to normalize their distribution. A general univariate linear model with Bonferonni adjustment for multiple comparisons was used to compare IgG levels between groups of demographic or clinical variables, adjusted for time from vaccination/infection to blood collection (as samples were collected within a relatively large interval of 2-16 weeks post-vaccine or longer post-COVID-19), age and gender. Since the number of samples from each participant varied, we used a generalized linear model (generalized estimated equation model) to compare IgG levels between time-points, where comparison of 1st and 3rd time-point was adjusted for time from vaccination to blood collection. A multiple linear regression model was used to assess correlation between IgG levels and variables, adjusted for time between vaccination and blood collection, age or gender. P value at <0.05 was considered significant.

RESULTS

Demographic and Clinical Characteristics of the Participants

We recruited for this prospective study 522 PwMS and 68 HC, who were vaccinated, about to be vaccinated, or who had been infected with SARS-CoV-2. **Table 1** summarizes their demographic and clinical characteristics. There was no significant difference between PwMS and HC regarding gender, BMI and smoking status, with the exception of a small age difference between the healthy participants and the MS patients (50 vs. 46 years, p=0.013). 93% of PwMS and 96% of HC received the COVID-19 vaccine, and 61 PwMS (12%) and 5 HC (7.4%) had a confirmed COVID-19 infection before (44 and 4, respectively) or during the study (17 and 1, respectively). COVID-19 infection was reported in 10 PwMS following the 2nd vaccination and in 3 patients following the 3rd vaccine booster, while in none of the HC after these vaccinations.

Serological Response in Vaccinated and Post-COVID-19 Participants

The serological response to SARS-CoV-2 vaccine was measured by detecting the anti-SARS-CoV-2 IgG level at 3 time-points: 2-16 weeks (≤3 months) and at 17-39 weeks (~6 months) after the 2nd vaccination, and at 1-16 weeks (≤3 months) after the 3rd vaccine booster. **Table 2** summarizes the IgG levels at each time-point in vaccinated PwMS and HC, as well as the IgG levels post-COVID-19 infection in unvaccinated PwMS. Supplementary data on additional DMTs can be found in **Supplementary Table 1**. 322 samples were available at ≤3 months from 2nd vaccination (mean=7.5 weeks, median=6.9 weeks), 159 samples at ~6 months from 2nd vaccination (mean=25.1 weeks, median=24.6 weeks) and 172 samples at ≤3 months following the 3rd vaccine booster in PwMS (mean=7.2 weeks, median=6.7 weeks). In total, 75.5% of PwMS and 98% of HC were seropositive after the 2nd vaccination, and 78.5% PwMS and 100% HC were seropositive after the 3rd vaccine booster. After 2 vaccinations, the mean IgG level was slightly lower in PwMS compared to HC (p=0.001) (**Figure 1A**). 72% of COVID-19-recovered and non-vaccinated patients had positive IgG levels, obtained after an average time of 30 weeks (median=27 weeks) post- infection, not different from the mean IgG level in vaccinated PwMS (p=0.6), when adjusted for time since infection/vaccination (**Table 1** and **Figure 1A**). IgG levels dropped by 82% in MS patients [OR=0.53, 95%CI: (0.47, 0.59), p<0.0001] and by 76% in healthy individuals [OR=0.50, 95%CI: (0.40, 0.63), p<0.0001] at time-point 2, 6 months after the 2nd vaccination (**Figures 1B, C**). In PwMS the 3rd vaccine booster increased the IgG levels at time-point 3 10 fold compared with the levels at time-point 2 (~6 months post 2nd vaccine) [OR=2.19, 95%CI: (1.75, 2.73), p<0.0001], and 1.8 fold compared with the levels at time-point 1 (≤3 months post 2nd vaccine) [OR=1.21, 95%CI: (1.02, 1.43), p=0.025] (**Figure 1B**). In the healthy participants the 3rd vaccine booster similarly increased the IgG levels 12.6 fold compared with the levels at time-point 2 [OR=3.58, 95%CI: (2.76, 4.64), p<0.0001], and 3 fold compared with the levels at time-point 1 [OR=1.40, 95%CI: (1.05, 1.87), p=0.023] (**Figure 1C**). While IgG levels correlated negatively with time since the 2nd vaccination in vaccinated MS patients [B=-0.038 ± 0.019, 95%CI: (-0.075, -0.001), p=0.043] (**Figure 1D**) and in vaccinated healthy individuals [B=-0.08 ± 0.019, 95%CI: (-0.12, -0.04), p=0.00013] (**Figure 1E**), no correlation was found between IgG levels and time since COVID-19 infection (COVID-19- recovered, unvaccinated patients), measured for a time interval of 4-72 weeks (mean=30 weeks) [B=0.005 ± 0.012, 95%CI: (-0.02, 0.03),p=0.7] (**Figure 1F**).

Effect of Demographic Factors on the Serological Response

We assessed whether the serological response after the 2nd vaccination was affected by demographic parameters (**Figures 1G–L**). Mean IgG levels were higher in female than

TABLE 1 | Demographic and clinical characteristics of study participants.

Study Population	MS	Healthy	p-value
N	522	68	
Age (years)	46 ± 0.6	50 ± 1.7	**0.013**
Gender Female N (%)	336 (65%)	36 (53%)	0.4
Smoking (Yes)	16.6%	12.3%	0.5
BMI mean ± SE (N)	24 ± 0.3 (219)	25 ± 0.6 (32)	0.3
EDSS (N)	3.2 ± 0.1 (480)		
Vaccinated N (%):	484 (93%)	65 (95.5%)	
No vaccines and no COVID-19	11	0	
1 vaccine only + COVID-19	10	0	
≥2 vaccines	474	65	
3 vaccines	186	23	
Confirmed COVID-19 N (%):	61 (12.0)	5 (7.4)	
only COVID-19, no vaccine	26	3	
Pre- vaccination	18	1	
Post 1st vaccine	4	1	
Post 2nd vaccine	10	0	
Post 3rd vaccine	3	0	
MS Therapy (N):			
Untreated	47		
Interferon beta	33		
Glatiramer Acetate	33		
Natalizumab	38		
S1PR modulator	63		
Teriflunomide	21		
Alemtuzumab	6		
Cladribine	35		
Dimethyl/Diroximel Fumarate	81		
Anti CD-20 monoclonal antibody	155		
Azathioprine	3		
other	6		

F, Female; M, Male; DMT, Disease modifying therapy; EDSS, Expanded Disability Status Scale; S1PR, Sphingosine 1-phosphate receptor.
Bold, significant p-value.

in male PwMS (F=6.49, p=0.011) (**Figure 1G**). No gender-related difference was found in the small cohort of HC (data not shown). No difference was found in PwMS between smokers and non-smokers (F=0.1, P=0.7) (**Figure 1H**), and IgG levels did not correlate with body mass index (BMI) [B=-0.009 ± 0.024, 95%CI: (-0.056, -0.038), p=0.7] (**Figure 1I**). Age negatively correlated with IgG levels, both in vaccinated MS patients [B= -0.026 ± 0.006, 95%CI: (-0.038,-0.015), p<0.0001] (**Figure 1J**) and in vaccinated healthy individuals [B=-0.015 ± 0.005, 95%CI: (-0.025, -0.004), p=0.006] (**Figure 1K**), but not in MS patients post-COVID-19 infection [B=-0.018± 0.016, 95%CI: (-0.05, 0.014), p=0.3] (**Figure 1L**).

Effect of DMTs on the Serological Response

Table 2 and **Supplementary Table 1** present the IgG levels according to the DMT that patients were receiving. Patients treated with most DMTs had a positive IgG response both after the 2nd and the 3rd vaccination, with IgG levels similar to untreated MS patients. However patients treated with the S1PR1 modulator fingolimod and the anti-CD20 therapy ocrelizumab had lower IgG levels after both the 2nd and 3rd vaccination (both p<0.0001), with only 38% of ocrelizumab-treated patients and 54% of fingolimod-treated patients being seropositive after the 2nd vaccine dose, and 44% and 46% respectively being seropositive after the 3rd vaccine booster (**Table 2**). Patients receiving other S1PR-modulators ponesimod or siponimod were seropositive both after the 2nd and the 3rd vaccination, but with relatively low IgG levels compared to untreated patients. Interestingly, patients receiving the anti-CD-20 therapy ofatumumab were all seropositive both after the 2nd and the 3rd vaccination, with relatively normal IgG levels (**Table 2** and **Supplementary Table 1**). In contrast, two patients receiving rituximab (a first generation anti-CD20 therapy) had very low IgG levels, only one being borderline seropositive (**Supplementary Table 1**). 100% of cladribine-treated patients were seropositive after the 2nd and the 3rd vaccination, and their IgG levels were comparable to untreated patients (**Table 2**). Although the number of unvaccinated patients who recovered from COVID-19 infection was small (N=32), a similar trend was observed with a positive IgG response in patients under treatment with most DMTs, with the exception of ocrelizumab [7/13 (54%) positivity], fingolimod [3/4 (75%) positivity], siponimod (0/1 positivity) and cladribine [2/3 (67%) positivity] at time of sample collection (**Table 2**).

Relation Between Time Since Last Ocrelizumab Infusion and IgG Response

Since anti-CD20 therapies are expected to have a lowering effect on antibody production after vaccination, and since ocrelizumab is administered periodically every 6 months, it is important to

TABLE 2 | IgG index following SARS-CoV-2 vaccination or COVID-19.

IgG AU/mL Mean ± SE [range] (% IgG positive)	Post COVID-19 no vaccine	Time-point 1: ≤3m Post 2nd vaccine	Time-point 2: ~6m Post 2nd vaccine	Time-point 3: ≤3m Post 3rd vaccine
MS patients				
Untreated	N=2 661 ± 480 [180 – 1141] (100%)	N=28 7778 ± 1502 [770 – 32497] (100%)	N=17 1269 ± 681 [120 – 12046] (100%)	N=14 18640 ± 4167 [454 – 40000] (100%)
Alemtuzumab	N=2 73 ± 0 [73–73] (100%)	N=3 8414 ± 3888 [761 – 13329] (100%)	N=1 1366 (100%)	
Anti CD-20 mAb (Ocrelizumab)	N=13 709 ± 586 [0 – 7722] (54%)	N=89 290 ± 113 [0 – 7722] (38%)	N=38 137 ± 37 [0 – 992] (42%)	N=43 2072 ± 938 [0 – 36108] (44%)
Anti CD-20 mAb (Ofatumumab)		N=4 2120 ± 1344 [120 – 5826] (100%)		N=2 5210 ± 5021 [189–10230] (100%)
Cladribine	N=3 13661 ± 13172 [44 – 40000] (67%)	N=23 5923 ± 1096 [162 – 22148] (100%)	N=11 806 ± 181 [42 – 1991] (91%)	N=9 9826 ± 2691 [643 – 23144] (100%)
Dimethyl Fumarate	N=2 181 ± 75 [106 – 256] (100%)	N=52 8401 ± 1217 [610 – 32938] (100%)	N=28 708 ± 139 [6 – 3813] (96%)	N=32 13813 ± 2226 [420 – 40000] (100%)
Glatiramer Acetate		N=22 6984 ± 1577 [153 – 33131] (100%)	N=9 1283 ± 633 [152 – 6146] (100%)	N=8 10748 ± 2567 [2902 – 21115] (100%)
Interferon β (Avonex, Plegridy, Betaferon, Rebif)	N=1 22699 (100%)	N=19 7832 ± 2182 [248 – 40000] (100%)	N=15 1649 ± 971 [35 – 14783] (93%)	N=13 16509 ± 3639 [1649 – 40000] (100%)
Natalizumab	N=3 288 ± 164 [90 – 614] (100%)	N=19 9698 ± 2458 [161 – 40000] (100%)	N=12 1372 ± 573 [259 – 7258] (100%)	N=14 7149 ± 2592 [792 – 33990] (100%)
S1PR modulator (Fingolimod)	N=4 298 ± 164 [29 – 777] (75%)	N=37 1494 ± 670 [0 – 19543] (54%)	N=13 200 ± 143 [0 – 1856] (31%)	N=24 325 ± 191 [0 – 4586] (46%)
S1PR modulator (Siponimod)	N=1 25 (0%)	N=3 1278 ± 1095 [69–3464] (100%)	N=1 52 (100%)	
Teriflunomide		N=15 6054 ± 1356 [439 – 16216] (100%)	N=7 2063 ± 1240 [244 – 9366] (100%)	N=10 13886 ± 4039 [568 – 40000] (100%)
Healthy		N=53 3537 ± 725 [42 – 27876] (98%)	N=16 855 ± 371 [108 – 5790] (100%)	N=21 10765 ± 2096 [787 – 33761] (100%)

AU, arbitrary units; IgG, immunoglobulin G; m, months; mAb, monoclonal antibody; SE, standard error; S1P, Sphingosine 1-phosphate receptor.

appreciate the preferred timing for immunization between treatment doses. We evaluated the anti SARS-CoV-2 spike protein IgG levels according to the time interval between last ocrelizumab infusion and vaccine administration (**Figure 2**, **Supplementary Figure 1AB** and **Supplementary Table 2**). Only a very weak, although significant, correlation was found between the time since previous ocrelizumab infusion and IgG levels after the 2nd vaccination [B=0.02 ± 0.01, 95%CI: (0.0, 0.04), p=0.048] (**Supplementary Figure 1A**) or after the 3rd vaccine booster [B=0.048 ± 0.022, 95% CI (0.00, 0.09), p=0.041] (**Supplementary Figure 1B**). Mean IgG levels were significantly higher in patients with a ≥ 5 months interval between last infusion and the 1st vaccine

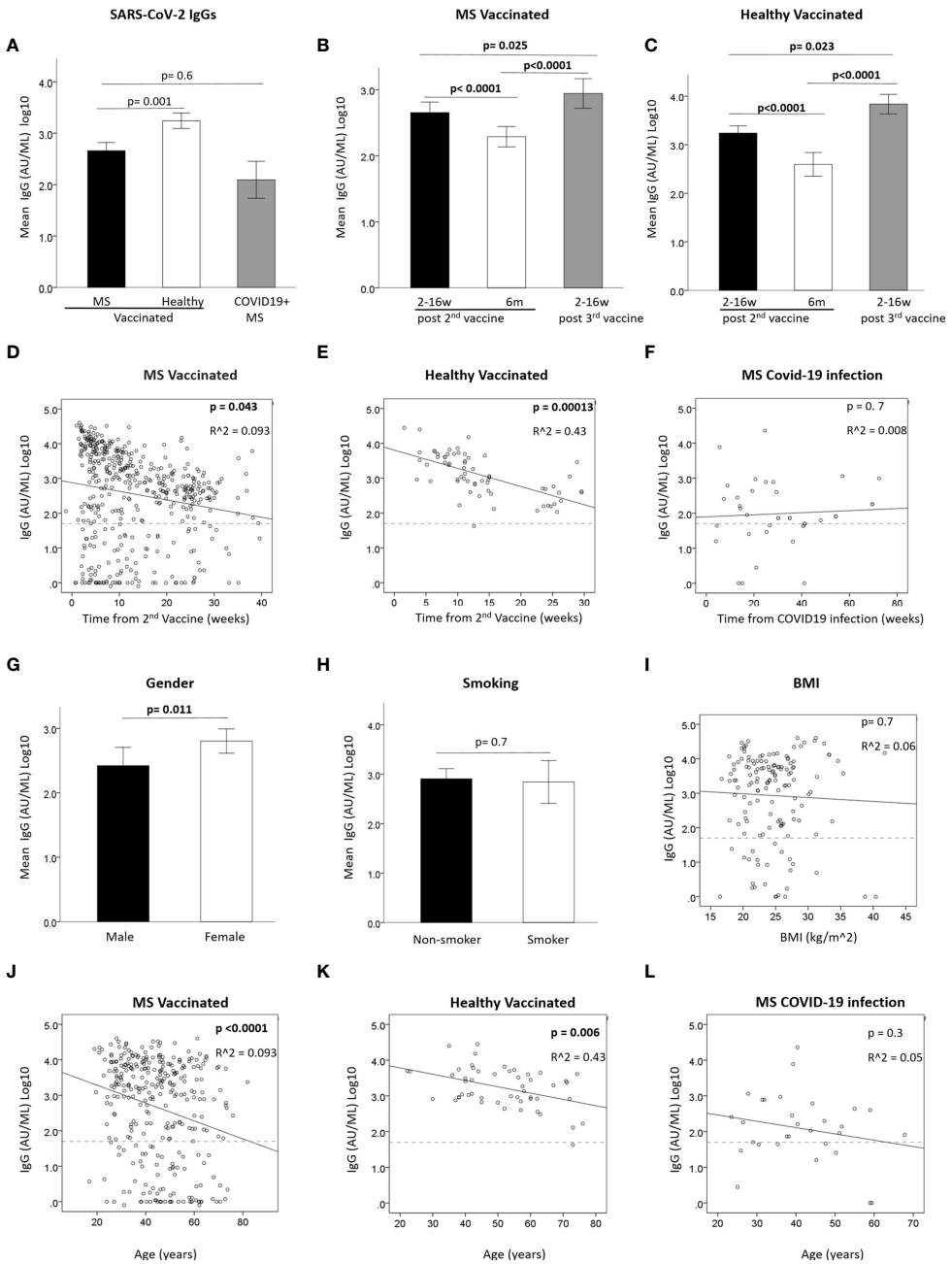


FIGURE 1 | **(A)** Mean IgG levels after 2nd vaccine in vaccinated PwMS (N=309) and healthy (N=48) and in unvaccinated COVID-19-recovered PwMS (N=29). **(B, C)** Mean IgG level in vaccinated PwMS (N=479) **(B)** or healthy (N=65) **(C)** at 3 time-points: <3months and 6 months from 2nd vaccine and <3 months from 3rd vaccine. **(D, E)** Correlation between IgG and time since 2nd vaccine in vaccinated PwMS (N=311) **(D)** or healthy) (N=46) **(E)**. **(F)** Correlation between IgG and time since COVID-19 infection in unvaccinated PwMS. **(G)** mean IgG difference between male (N=107) and female (N=204) PwMS after 2nd vaccine. **(H)** mean IgG difference between non-smokers (N=166) and smokers (N=37) PwMS after 2nd vaccine. **(I)** Correlation between IgG post 2nd vaccine and BMI in PwMS (N=144). **(J, K)** Correlation between IgG after 2nd vaccine and age in vaccinated PwMS (N=311) **(J)** or healthy (N=46) **(K)**. **(L)** Correlation between IgG and age in unvaccinated PwMS recovered from COVID-19 (N=38). Dashed horizontal grey line represents minimum seropositive border (log10(50AU/ml).

dose (**Figure 2A** and **Supplementary Table 2**) or the 3rd vaccine booster (**Figure 2B** and **Supplementary Table 2**), compared to patients with an interval of < 5 months (F=4.43, p=0.039 and F=4.76, p=0.036, respectively). Similarly, patients with a ≥6 months interval had significantly higher IgG levels than patients with < 6 months between last infusion and 1st (**Figure 2A**) or 3rd vaccine (**Figure 2B**) (F= 5.19, p=0.026 and F=4.43, p=0.042, respectively) (**Supplementary Table 2**). Furthermore, the percentage of

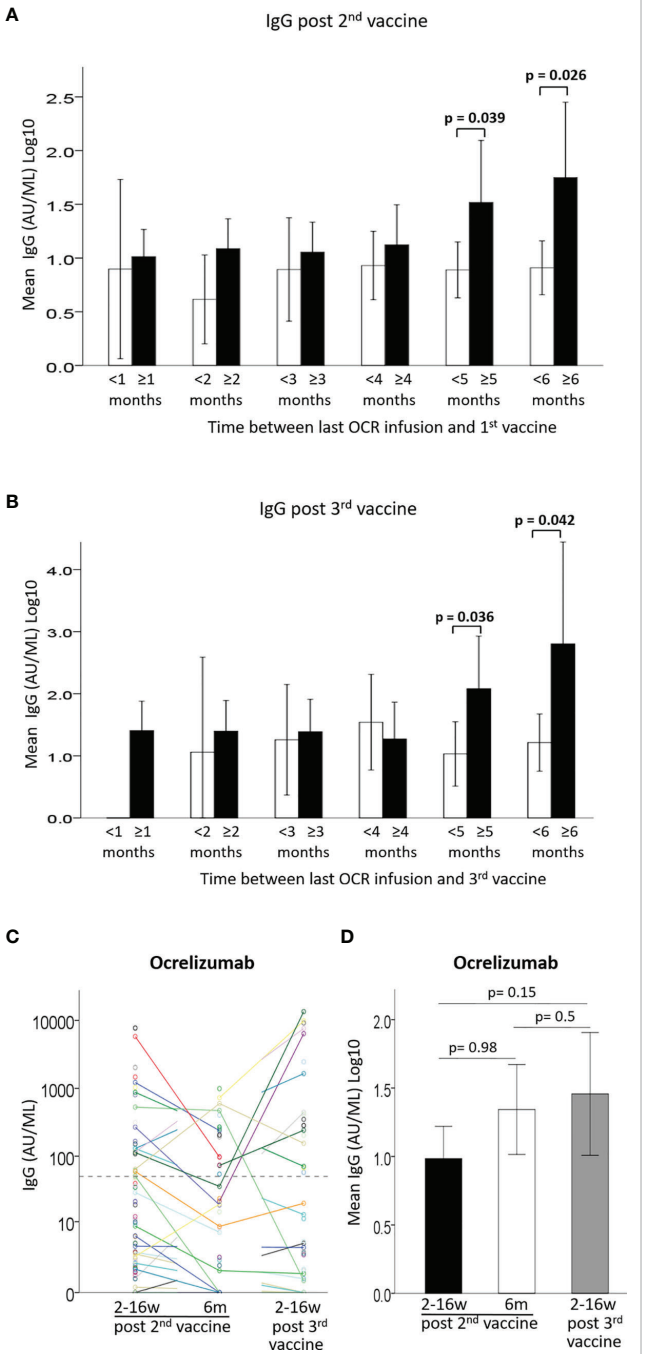


FIGURE 2 | **(A)** Mean IgG levels after 2nd vaccine in patients with < (white) or ≥ (black) 1-6 months between last Ocrelizumab infusion and 1st vaccine. **(B)** Mean IgG levels after 3rd vaccine in patients with < (white) or ≥ (black) 1-6 months between last Ocrelizumab infusion and 3rd vaccine. **(C)** IgG levels of Ocrelizumab-treated patients at 3 time-points: ≤3months and 6 months from 2nd vaccine and ≤3 months from 3rd vaccine. **(D)** Comparison of IgG levels in Ocrelizumab-treated patients at 3 time-points: ≤3months (N=89) and 6 months (N=38) from 2nd vaccine and ≤3 months from 3rd vaccine (N=43). Dashed horizontal grey line represents minimum seropositive border (log10(50AU/ml).

seropositive patients was higher in patients with ≥5 months interval than in those with <5 months interval between last infusion and 1st or 3rd vaccine (44% vs. 26% and 62% vs. 30%, respectively), and higher in patients with ≥6 months interval than in those with <6 months interval (60% vs. 25% and 75% vs. 36%, respectively) (**Supplementary Table 2**). Comparing the IgG levels in ocrelizumab-treated patients after the 2nd and 3rd vaccinations revealed a similar pattern for most patients, e.g. those who had a positive response after the first 2 vaccine doses were most likely to be also seropositive after the 3rd vaccination, while most patients with an insufficient response after the 2nd vaccination remained mostly seronegative after the 3rd vaccination, although some patients did benefit from the booster (**Figure 2C**). Using a generalized linear model to compare IgG levels between the 3 time-points we found no differences between time-point 1 and 2 post 2nd vaccination [OR1.00, 95%CI: (0.73, 1.39), p=0.98], between time-point 2 post 2nd vaccine and time-point 3 post 3rd vaccination [OR1.25, 95%CI: (0.70, 2.2), p=0.5], or between time-point 1 post-2nd vaccination and time-point 3 post-3rd vaccination [OR1.38, 95%CI: (0.89, 2.1), p=0.2] (**Figure 2D**).

IgG Levels in Fingolimod-Treated Patients

IgG levels did not correlate with absolute lymphocyte counts at time of vaccination in fingolimod-treated patients [B=0.537 ± 0.64, 95% CI: (-0.77, 1.85), p=0.4] (**Figure 3A**). Comparison of the IgG response after the 2nd and 3rd vaccination showed a similar pattern for most fingolimod-treated patients, with seronegative patients remaining negative also after the 3rd vaccination (**Figure 3B**). A generalized linear model comparing IgG levels between the 3 time-points revealed that the IgG levels were reduced between time-point 1 and 2 after the 2nd vaccination [OR=0.53, 95%CI: (0.36, 0.78), p=0.001], and increased between 2nd and 3rd time-point following the vaccine booster [OR=1.88, 95% CI: (1.42, 2.49), p<0.0001]; however, the 3rd vaccine booster did not increase the IgG levels further than at time-point 1 (post 2nd vaccination) [OR=0.76, 95%CI: (0.48, 1.21), p=0.3] (**Figure 3C**).

INFγ Immune Cell Response

T-cell immune response to the vaccine was assessed by measuring IFNγ secretion in response to incubation of whole blood with the SARS-CoV-2 spike protein. Data were available from 26 patients and 6 healthy participants after 2nd vaccine, along with their serological assay, and from 16 patients and 2 healthy participants after the 3rd vaccine booster (**Table 3** and **Supplementary Table 3**). There was no significant difference in IFNγ-T-cell response levels between PwMS and healthy participants after the 2nd vaccination (p=0.8) or after the 3rd vaccine booster (p=0.2). 100% of healthy participants and 88.5% of MS patients had a positive IFNγ-T cell response after the 2nd vaccination, with 100% and 63% positivity after the 3rd vaccine booster, respectively. Only 25% (1/4) of fingolimod-treated patients had a positive T cell response after the 2nd vaccination and 0% (0/4) after the 3rd vaccination, including patients who were seropositive. In contrast, all ocrelizumab-treated patients (5/5) had a positive T cell response after the 2nd vaccine, despite

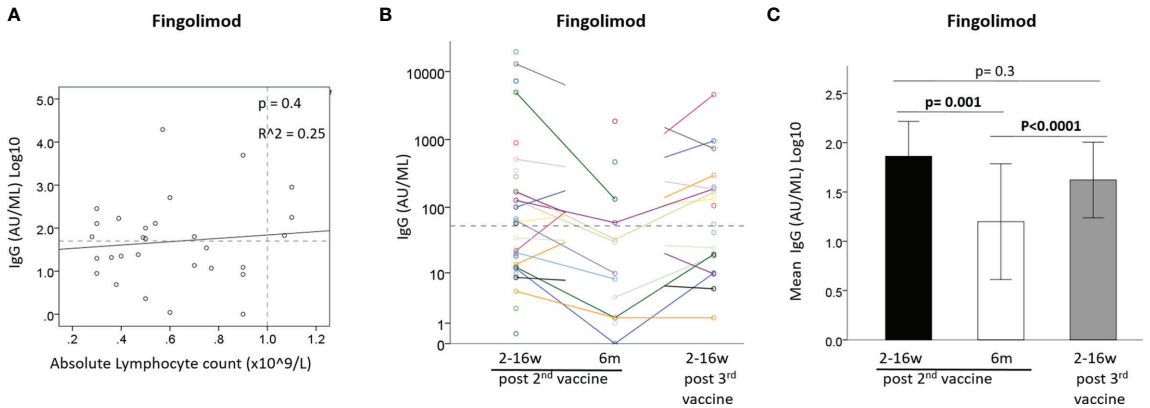


FIGURE 3 | **(A)** Correlation between IgG after 2nd vaccine and absolute lymphocyte count in Fingolimod-treated patients (N=29). **(B)** IgG levels of Fingolimod-treated patients at 3 time-points: ≤3months and 6 months from 2nd vaccine and ≤3 months from 3rd vaccine. **(C)** Mean IgG levels in Fingolimod-treated patients at 3 time-points: ≤3months and 6 months from 2nd vaccine and ≤3 months from 3rd vaccine. Dashed horizontal grey line represents minimum seropositive border (log10 (50AU/ml), dashed vertical line represent normal lymphocyte count border.

58% being seronegative, and 86% (6/7) showed a T cell response after the 3rd vaccine (**Table 3**). One teriflunomide-treated patient did not develop an IFNγ-T cell response post 3rd vaccination, while all of the remaining patients treated with various other DMTs had a positive T cell response.

DISCUSSION

To the best of our knowledge, this is the first comprehensive prospective study on the magnitude and durability of SARS-CoV-2 IgG levels combined with T cell responses in PwMS, treated with various DMTs, for over 6 months from vaccination and following a 3rd vaccine booster. Vaccination is the major available tool to control and fight the pandemic of COVID-19. Several vaccines have been developed worldwide, using different strategies and platforms, including the novel strategy of vaccination with the SARS-CoV-2 spike protein mRNA as in the case of the BNT162b2 (Pfizer/BioNTech,Inc) and the mRNA-1273 (Moderna Tx,Inc) vaccines. These mRNA vaccines appear to be safe and have not been associated with increased incidence of MS disease activation (13–16). In general, the use of DMTs in PwMS has not been found to significantly affect COVID-19 disease course (17), although anti-CD20 therapies have been associated with higher incidence of COVID-19 infection (18) and with a possibly increased risk of severe disease course (19).

An effective, long-term, memory immune response is driven by the adaptive immune system, consisting of both a humoral response mediated by B cells, producing antigen-specific antibodies, and a T cell-mediated response, causing destruction of virus-infected cells and necessary for the development of plasma cells and memory B cells. Thus, measurement of both the humoral and the cell-mediated responses is required to precisely estimate the immune response to SARS-CoV-2 vaccine. However, the relatively easily accessible measurement of anti SARS-CoV-2 antibody levels in the serum is the common and mostly used method to identify individuals who recovered from COVID-19 infection or to confirm a sufficient vaccine response, especially since SARS-CoV-2 neutralizing antibodies were shown to be highly predictive of SARS-CoV-2 immune protection (6). Evidence for positive humoral immune responses to the SARS-CoV-2 vaccine in MS patients is accumulating (9, 10, 12, 20, 21). Our current study provides longitudinal accumulating data regarding the IgG serum levels following >6 months follow-up and also after a 3rd vaccine booster. Our findings show that IgG levels to the SARS-CoV-2 spike protein in MS patients were slightly lower than in healthy individuals, declined by >80% within 6 months from the 2nd vaccination, and were significantly increased following a 3rd vaccine booster. A similar decline in IgG levels at 6 months from vaccination and a beneficial effect of the 3rd vaccine booster was also demonstrated in the healthy cohort. Interestingly, we did not detect a negative correlation between IgG levels after COVID-19 infection and time, suggesting a more robust and long-lasting immune response to the SARS-CoV-2 virus following natural infection vs vaccination. However, the median time between COVID-19-infection and blood collection in our cohort was 27 weeks, thus may not being able to capture the major, initial decline in IgG levels. Antibody levels following COVID-19 infection were shown to be associated with COVID-19 disease severity (a parameter that was not registered in our study), and were reported to start declining after 60 days, but still to be detectable for at least 120 days (22). For the COVID-19-recovered patients in our study, the follow up data were limited, as the majority of these patients received the recommended post–infection vaccination. Interestingly, we found higher IgG levels in vaccinated female than in male patients, not reported in previous reports (20, 23). While we did not detect a similar difference in IgG levels between genders in HC, probably due to the relatively small cohort, both levels of IgGs and of neutralizing antibodies have been shown to be higher in females than in males receiving the BNT162b2 Covid-19 Vaccine, especially in association with age (24), a difference that at least in part is thought to be hormone-mediated (25). The negative correlation between IgG levels and age in vaccinated PwMS or healthy

TABLE 3 | Serological and IFNγ-T cell immune response.

Participant	N	Time-point 1: 2-16w post 2nd vaccine		Time-point 2: ~6m post 2nd vaccine		Time-point 3: 1-16w post 3rd vaccine	
		IgG response	T cell response	IgG response	T cell response	IgG response	T cell response
Untreated	1	+	+				
	2					+	+
	3					+	+
	4			+	+		
	5	+	+				
Cladribine	1			+	+		
Dimethyl	1	+	+				
Fumarate	2	+	+				
	3					+	+
Fingolimod	1	+	−			+	−
	2	+	−				
	3	−	+				
	4	−	−				
	5					−	−
	6					−	−
	7					−	−
Ponesimod	1	+	+				
Siponimod	1	+	+				
Glat. Acetate	1	+	+				
IFNβ (Rebif)	1	+	+				
Methotrexte	1	+	+				
Natalizumab	1	+	+				
	2	+	+				
	3	+	+				
	4	+	+				
Ocrelizumab	1	−	+				
	2	+	+				
	3	−	+			−	+
	4	−	+				
	5					−	+
	6					−	+
	7					+	+
	8			−	+		
	9					+	−
	10					+	+
	11					+	+
Ofatumumab	1	+	+				
	2	+	+			+	+
Teriflunomide	1					+	−
Healthy	1	+	+				
	2	+	+				
	3	+	+				
	4	+	+				
	5	+	+				
	6	+	+				
	7					+	+
	8					+	+

+: IgG≥ 50 AU/ml serology, or ≥ 25 pg/ml of Spike-specific IFNγ response. -: IgG<50AU/ml serology, or <25 pg/ml of Spike-specific IFNγ response.

participants presented in our study has also been suggested elsewhere (11, 26), and confirms the potential elevated risk of COVID-19 infection in elderly people.

Our data show, similarly to previous reports, that MS patients treated with most DMTs develop a positive humoral and cell-mediated immune response to the mRNA vaccine, which in general does not differ significantly from that observed in untreated patients or healthy individuals. The reduced humoral response and low frequency of seropositive patients that was observed in PwMS treated with fingolimod and ocrelizumab are in line with several recent studies (7, 8, 10–12, 20, 21, 27–30). In addition, we could show that a similar humoral response and low frequency of seropositive patients is also observed after the 3rd vaccine booster in the patients treated with fingolimod or ocrelizumab, and that in those patients (in contrast to healthy individuals and PwMS in general), the 3rd vaccination did not boost the IgG levels further than the first 2 vaccine doses. One recent study reported similarly that only 1 out of 16 ocrelizumab-treated patients was seropositive after the 3rd BNT162b2 vaccine booster (29). It seems thus, that while some PwMS treated with S1PR-modulators or anti-CD20 therapies

do benefit from the additional vaccine dose, an optional vaccine strategy for these patients should be considered. On the other hand, we found that the vast majority of ocrelizumab-treated patients developed a positive anti-spike protein IFNγ-T-cell immune response (both after the 2nd and the 3rd vaccinations) and thus, despite the lack of a sufficient humoral response, they may carry a relative protection against COVID-19 infection or severe disease. Similar results were recently reported by other investigators: Gadani et al. found that 96.9% of ocrelizumab patients developed a T-cell response (30), Tortorella et al. reported a 92% positive T-cell response rate (21) and Brill et al. found a response rate of 89.7% (11). Aposolidis et al. reported positive CD-4 and CD-8 T-cell responses to vaccination in all anti CD-20-treated patients, but these responses were somewhat skewed, with reduced follicular helper T (T_{FH}) cell development and elevated CD8 T-cell responses, while Th1 responses were preserved, especially in patients who were seronegative (31). Although these findings of relatively preserved T-cell-mediated anti-COVID-19 responses are encouraging, studies with larger patient cohorts, including follow-up on the risk and outcome of COVID-19 infection are needed to accurately estimate the vaccine-induced protection in anti-CD20- treated patients. Interestingly, we observed both positive humoral and cellular immune responses to the vaccine in patients treated with another anti-CD20 mAB - ofatumumab. We are not aware of published data on the response to vaccination in these patients, however, one study found that COVID-19- recovered ofatumumab-treated, B-cell depleted patients were seronegative for anti SARS-Cov-2 antibodies, but developed a positive cellular response (32), while another study reported a positive anti-SARS-CoV-2 antibody response after COVID-19 in a B-cell depleted ofatumumab-treated patient (33). Confirmation of this finding in a larger group of ofatumumab-treated patients will be of high interest.

Anti-CD20 therapies such as ocrelizumab reduce the number of circulating B cells, thus preventing a sufficient B cell response to antigens and the development of antibodies, an effect which is likely to persist until sufficient B cell reconstitution occurs. It would therefore be of interest to determine the optimal time interval between the infusion and vaccination that would allow for sufficient reconstitution of B cells to enable an effective humoral immune response. This issue has been studied by a few groups but remains debatable; while in some reports there was a significant correlation between time from last treatment to vaccination and the IgG levels (11, 20, 30), others could not confirm this finding (21). In our study, IgG levels correlated only very weakly with the time between infusion and vaccination, both after the 2nd and the 3rd vaccine administration. One study found that 143 days following ocrelizumab infusion is the time-point when IgG starts to increase (20). We found that a time interval of more than 5 months between ocrelizumab infusion and vaccination allows for higher IgG levels than a time interval of <5 months, and a similar difference was also observed in patients vaccinated ≥6 months vs. those vaccinated <6 months after ocrelizumab infusion. Our data also showed that a higher proportion of ocrelizumab-treated patients were seropositive if they were at least 5 months from last infusion at time of vaccine, and in patients with ≥ 6 months time interval, sero-positivity increased to 75% post the 3rd vaccination. Based on our data, and since the humoral response to the vaccine is effective about 14 days after vaccination (34), we recommend that immunization should be timed to the window of 5 months after the last ocrelizumab dose and two weeks before the next dose of ocrelizumab. It was recently suggested that measuring the association between IgG response and B cell reconstitution, rather than the time interval since infusion, is more useful for determining the optimal timing of vaccination administration in ocrelizumab-treated patients (31). A recent study found that the mRNA-1273 vaccine, containing 100 micrograms of the spike protein mRNA, induced higher IgG levels in MS patients compared to the BNT162b2 mRNA vaccine which contains only 30 micrograms of mRNA (20). Based on this finding, the investigators suggested that the mRNA-1273 vaccine would be preferable as vaccination booster in PwMS on anti-CD20 therapy or fingolimod, who did not develop efficient humoral responses following BNT162b2 vaccination.

Our findings indicate that the vast majority of patients treated with fingolimod fail to mount an IFNγ-T-cell immune response. Tortorella et al. similarly found a T-cell response in only 14.3% of fingolimod-treated patients (21). With both reduced or insufficient humoral and cell-mediated immune responses, these patients may be at increased risk of COVID-19 infection and severe disease. Interestingly, it has been suggested that the immunosuppressive effects of fingolimod could be beneficial for prevention of acute respiratory distress syndrome in patients with severe COVID-19, by reducing the cytokine storm (35), and in a recent study fingolimod-treated PwMS were not found to be at increased risk of severe COVID-19 (19). Our data did not confirm a previous report of correlation between IgG response and lymphocyte count in fingolimod-treated patients (20).

Our study has certain limitations. Due to the rapid vaccination program in Israel, the serum samples after both the 2nd and the 3rd vaccines were collected during a variable and rather long time period (2-16 weeks, median ~7 weeks) when patients visited the outpatient clinics, and thus the antibody measurement does not represent the peak of post-vaccination humoral response in every patient. Since there is a natural reduction in IgG levels with time, adjustment for time between vaccination and blood collection was integrated in the statistical analysis. The number of patients treated with other than fingolimod or ocrelizumab S1PR-modulators or anti-CD20 therapies was small, thus limiting any conclusion-making on their effects on the vaccine response. The number of patients assessed for T-cell mediated responses (assessed only in a sub-group of patients) was also low and not sufficiently representative for each DMT, but still our findings add to information emerging from other sites. Data on COVID-19 infection before and during the follow-up period were based only on reports by the participants attending the clinics, and was not intended for interpretation regarding the efficacy of vaccination and the risk of infection in our study population. Since our samples were collected before the outbreak of the latest Omicron variant in Israel, which seems to have the capability to escape immune-surveillance, the impact of the vaccination program on Omicron and future variants is yet to be determined. However, the information on the immune responses of PwMS after the first 3 mRNA vaccines is likely to be relevant for future vaccine development.

The merit of this study is the longitudinal follow-up of IgG levels and cell-mediated responses after 2 vaccinations and the response to a 3rd vaccine booster in PwMS treated with a wide variety of DMTs. While for most DMTs, the humoral and cell-mediated responses appear to be similar to those of untreated PwMS, the finding of sero-negativity in >50% of patients treated with the S1PR-modulator fingolimod and the anti-CD20 therapy ocrelizumab even after a 3 vaccine booster, along with the lack of cell-mediated immune response in the vast majority of fingolimod-treated patients, may suggest that the strategy of boosting the immune system with additional vaccine doses is not effective enough for these patients and that other vaccination strategies should be considered. Such considerations could include optimization of the timing between vaccine administrations, specifically in the context of MS immunotherapies, use of combinational vaccines based on different development platforms and targets, optimization of the vaccine dose and choice of appropriate vaccine in relation to its ability to induce higher antibody levels. In general, efforts should be focused on development of optimal vaccine strategies aiming at improving immunogenicity and long-lasting immunity, tailored for each PwMS under treatment with a specific immunotherapy. Based on the cumulative data until today, updated recommendations about the type and timing of SARS-CoV-2 vaccinations of MS patients are needed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Helsinki Committee, Barzilai Medical Center; Helsinki Committee, Carmel Medical Center; Helsinki Committee, Hadassah Medical Center; Helsinki Committee, Tel Aviv Sourasky Medical Center; Helsinki Committee, Rabin Medical Center;. The patients/participants provided their written informed consent to participate in this study.

THE ISRAELI NEUROIMMUNOLOGY STUDY GROUP ON COVID-19 VACCINATION IN MULTIPLE SCLEROSIS

The Israeli Neuroimmunology Study Group on COVID-19 vaccination in Multiple Sclerosis:

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AUTHOR CONTRIBUTIONS

AM conceptualized and designed the study, contributed to acquisition and interpretation of the data and revised the manuscript for intellectual content. ES-R designed the study, analyzed and interpreted the data, drafted the manuscript. RM, DK and AK contributed to the design of the study, acquisition and interpretation of the data, and revised the manuscript for intellectual content. MH contributed to acquisition and interpretation of the data and revised the manuscript for intellectual content. EB-H contributed to the acquisition and analysis of the data. The Israeli Neuroimmunology Study Group on COVID-19 vaccination in Multiple Sclerosis contributed to acquisition of the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.868915/full#supplementary-material

Supplementary Figure 1 | (A) Correlation between IgG after 2nd vaccine and time between Ocrelizumab infusion and 1st vaccine (N=81). **(B)** Correlation between IgG post 3rd vaccine and time between Ocrelizumab infusion and 3rd vaccine (N=40). OCR- ocrelizumab. Dashed horizontal grey line represents minimum seropositive border (log10(50AU/ml).

REFERENCES

1. Sellner J, Rommer PS. Multiple Sclerosis and SARS-CoV-2 Vaccination: Considerations for Immune-Depleting Therapies. *Vaccines* (2021) 9:1–12. doi: 10.3390/vaccines9020099
2. Bar-On YM, Goldberg Y, Mandel M, Bodenheimer O, Freedman L, Kalkstein N, et al. Protection of BNT162b2 Vaccine Booster Against Covid-19 in Israel. *N Engl J Med* (2021) 385:1393–400. doi: 10.1056/nejmoa2114255
3. Barda N, Dagan N, Ben-Shlomo Y, Kepten E, Waxman J, Ohana R, et al. Safety of the BNT162b2 mRNA Covid-19 Vaccine in a Nationwide Setting. *N Engl J Med* (2021) 385:1078–90. doi: 10.1056/nejmoa2110475
4. *The National Multiple Sclerosis Society*. Available at: https://www.nationalmssociety.org/coronavirus-covid-19-information/multiple-sclerosis-and-coronavirus/covid-19-vaccine-guidance. (Accessed January 18.2022).
5. https://www.msif.org/news/2020/02/10/the-coronavirus-and-ms-what-you-need-to-know/ (Accessed January 28, 2022).
6. Khoury DS, Cromer D, Reynaldi A, Schlub TE, Wheatley AK, Juno JA, et al. Neutralizing Antibody Levels are Highly Predictive of Immune Protection From Symptomatic SARS-CoV-2 Infection. *Nat Med* (2021) 27:1205–11. doi: 10.1038/s41591-021-01377-8
7. Guerrieri S, Lazzarin S, Zanetta C, Nozzolillo A, Filippi M, Moiola L. Serological Response to SARS-CoV-2 Vaccination in Multiple Sclerosis Patients Treated With Fingolimod or Ocrelizumab: An Initial Real-Life Experience. *J Neurol* (2021) 269(1):39–43. doi: 10.1007/s00415-021-10663-x
8. Gadani SP, Reyes-mantilla M, Jank L, Harris S, Douglas M, Smith MD, et al. Discordant Humoral and T Cell Immune Responses to SARS-CoV-2 Vaccination in People With Multiple Sclerosis on Anti-CD20 Therapy Sachin. *EBioMedicine* (2021) 73:103636. doi: 10.1016/j.ebiom.2021.103636
9. Boekel L, Steenhuis M, Hooijberg F, Besten YR, van Kempen ZLE, Kummer LY, et al. Antibody Development After COVID-19 Vaccination in Patients With Autoimmune Diseases in the Netherlands: A Substudy of Data From Two Prospective Cohort Studies. *Lancet Rheumatol* (2021) 3:e778–88. doi: 10.1016/s2665-9913(21)00222-8
10. Bigaut K, Kremer L, Fleury M, Lanotte L, Collongues N, de Seze J. Impact of Disease-Modifying Treatments on Humoral Response After COVID-19 Vaccination: A Mirror of the Response After SARS-CoV-2 Infection. *Rev Neurol (Paris)* (2021). 177(10):1237–40. doi: 10.1016/j.neurol.2021.05.001
11. Brill L, Ariel R, Omri Z, Nitzan H, Esther O-D, WD G, et al. Humural and T-Cell Response to SARS-CoV-2 Vaccination in Patients With MS Treated With Ocrelizumab. *JAMA Neurol* (2021) 23:e213599. doi: 10.1001/jamaneurol.2021.3599
12. Achiron A, Mandel M, Dreyer-Alster S, Harari G, Magalashvili D, Sonis P, et al. Humoral Immune Response to COVID-19 mRNA Vaccine in Patients With Multiple Sclerosis Treated With High-Efficacy Disease-Modifying Therapies. *Ther Adv Neurol Disord* (2021) 14:1–8. doi: 10.1177/17562864211012835
13. Bellucci G, Rinaldi V, Buscarinu MC, Reniè R, Bigi R, Pellicciari G, et al. Multiple Sclerosis and SARS-CoV-2: Has the Interplay Started? *Front Immunol* (2021) 12:755333. doi: 10.3389/fimmu.2021.755333
14. Lotan I, Wilf-Yarkoni A, Friedman Y, Stiebel-Kalish H, Steiner I, Hellmann MA. Safety of the BNT162b2 COVID-19 Vaccine in Multiple Sclerosis (MS): Early Experience From a Tertiary MS Center in Israel. *Eur J Neurol* (2021) 28:3742–8. doi: 10.1111/ene.15028
15. Achiron A, Dolev M, Menascu S, Zohar DN, Dreyer-Alster S, Miron S, et al. COVID-19 Vaccination in Patients With Multiple Sclerosis: What We Have Learnt by February 2021. *Mult Scler J* (2021) 27:864–70. doi: 10.1177/13524585211003476
16. Briggs FBS, Mateen FJ, Schmidt H, Currie KM, Siefers HM, Crouthamel S, et al. COVID-19 Vaccination Reactogenicity in Persons With Multiple Sclerosis. *Neurol Neuroimmunol Neuroinflamm* (2022) 9:e1104. doi: 10.1212/nxi.0000000000001104
17. Chaudhury F, Bulka H, Rathnam AS, Said OM, Lin J, Lorigan H, et al. COVID-19 in Multiple Sclerosis Patients and Risk Factors for Severe Infection. *J Neurol Sci* (2020) 418:117147. doi: 10.1016/j.jns.2020.117147
18. Reder AT, Centonze D, Naylor ML, Nagpal A, Rajbhandari R, Altincatal A, et al. COVID-19 in Patients With Multiple Sclerosis: Associations With Disease-Modifying Therapies. *CNS Drugs* (2021) 35:317–30. doi: 10.1007/s40263-021-00804-1
19. Sormani MP, De Rossi N, Schiavetti I, Carmisciano L, Cordioli C, Moiola L, et al. Disease-Modifying Therapies and Coronavirus Disease 2019 Severity in Multiple Sclerosis. *Ann Neurol* (2021) 89:780–9. doi: 10.1002/ana.26028
20. Sormani MP, Inglese M, Schiavetti I, Carmisciano L, Laroni A, Lapucci C, et al. Effect of SARS-CoV-2 mRNA Vaccination in MS Patients Treated With Disease Modifying Therapies. *EBioMedicine* (2021) 72:103581. doi: 10.1016/j.ebiom.2021.103581
21. Tortorella C, Aiello A, Gasperini C, Agrati C, Castilletti C, Ruggieri S, et al. Humoral- and T-Cell–Specific Immune Responses to SARS-CoV-2 mRNA Vaccination in Patients With MS Using Different Disease-Modifying Therapies. Neurology (2022) 98(5):e541–54 doi: 10.1212/wnl.0000000000013108.
22. Arkhipova-Jenkins I, Helfand M, Armstrong C, Gean E, Anderson J, Paynter RA, et al. Antibody Response After SARS-CoV-2 Infection and Implications for Immunity: A Rapid Living Review. *Ann Intern Med* (2021) 174:811–21. doi: 10.7326/M20-7547
23. Wall EC, Wu M, Harvey R, Kelly G, Warchal S, Sawyer C, et al. Neutralising Antibody Activity Against SARS-CoV-2 VOCs B.1.617.2 and B.1.351 by BNT162b2 Vaccination. *Lancet* (2021) 397:2331–3. doi: 10.1016/S0140-6736(21)01290-3
24. Levin EG, Lustig Y, Cohen C, Fluss R, Indenbaum V, Amit S, et al. Waning Immune Humoral Response to BNT162b2 Covid-19 Vaccine Over 6 Months. *N Engl J Med* (2021) 385:e84. doi: 10.1056/nejmoa2114583
25. Ciarambino T, Para O, Giordano M. Immune System and COVID-19 by Sex Differences and Age. *Women's Heal* (2021) 17:17455065211022262. doi: 10.1177/17455065211022262
26. Chvatal-Medina M, Mendez-Cortina Y, Patiño PJ, Velilla PA, Rugeles MT. Antibody Responses in COVID-19: A Review. *Front Immunol* (2021) 12:633184. doi: 10.3389/fimmu.2021.633184
27. Novak F, Christine A, Nielsen C, Holm DK, Kamilla Ø, Bystrup A, et al. Humoral Immune Response Following SARS-CoV-2 mRNA Vaccination Concomitant to Anti-CD20 Therapy in Multiple Sclerosis ☆. *Mult Scler Relat Disord* (2021) 56:103251. doi: 10.1016/j.msard.2021.103251
28. Gallo A, Capuano R, Donnarumma G, Bisecco A, Grimaldi E, Conte M, et al. Preliminary Evidence of Blunted Humoral Response to SARS-CoV-2 mRNA Vaccine in Multiple Sclerosis Patients Treated With Ocrelizumab. *Neurol Sci* (2021) 42:3523–6. doi: 10.1007/s10072-021-05397-7
29. Achtnichts L, Jakopp B, Oberle M, Nedeltchev K, Fux CA, Sellner J, et al. Humoral Immune Response After the Third SARS-CoV-2 mRNA Vaccination in CD20 Depleted People With Multiple Sclerosis. *Vaccines* (2021) 9:1470. doi: 10.3390/vaccines9121470
30. Gadani SP, Reyes-Mantilla M, Jank L, Harris S, Douglas M, Smith MD, et al. Discordant Humoral and T Cell Immune Responses to SARS-CoV-2 Vaccination in People With Multiple Sclerosis on Anti-CD20 Therapy. *EBioMedicine* (2021) 73:34462762. doi: 10.1016/j.ebiom.2021.103636
31. Apostolidis SA, Kakara M, Painter MM, Goel RR, Mathew D, Lenzi K, et al. Cellular and Humoral Immune Responses Following SARS-CoV-2 mRNA Vaccination in Patients With Multiple Sclerosis on Anti-CD20 Therapy. *Nat Med* (2021) 27:1990–2001. doi: 10.1038/s41591-021-01507-2
32. Adamec I, Rogi D. Humoral and Cellular Immunity in Convalescent COVID-19 People With Multiple Sclerosis Treated With Ofatumumab. *J Neuroimmunol* (2022) 362:577788. doi: 10.1016/j.jneuroim.2021.577788
33. Flores-Gonzalez RE, Hernandez J, Tornes L, Rammohan K, Delgado S. Development of SARS-CoV-2 IgM and IgG Antibodies in a Relapsing Multiple Sclerosis Patient on Ofatumumab. *Mult Scler Relat Disord* (2021) 49:2020–2. doi: 10.1016/j.msard.2021.102777
34. Doria-Rose N, Suthar MS, Makowski M, O'Connell S, McDermott AB, Flach B, et al. Antibody Persistence Through 6 Months After the Second Dose of mRNA-1273 Vaccine for Covid-19. *N Engl J Med* (2021) 384:2259–61. doi: 10.1056/nejmc2103916
35. Tasat DR, Yakisich JS. Rationale for the Use of Sphingosine Analogues in COVID-19 Patients. *Clin Med J R Coll Phys London* (2021) 21:E84–7. doi: 10.7861/CLINMED.2020-0309

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PD-L1 Inhibits T Cell-Induced Cytokines and Hyaluronan Expression *via* the CD40-CD40L Pathway in Orbital Fibroblasts From Patients With Thyroid Associated Ophthalmopathy

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Thyroid associated ophthalmopathy (TAO), characterized by T cell infiltration and orbital fibroblast activation, is an organ-specific autoimmune disease which is still short of effective and safety therapeutic drugs. The PD-1/PD-L1 pathway has been reported hindering the progression of Graves' disease to some extent by inhibiting T cell activity, and tumor therapy with a PD-1 inhibitor caused some adverse effects similar to the symptoms of TAO. These findings suggest that the PD-1/PD-L1 pathway may be associated with the pathogenesis of TAO. However, it remains unknown whether the PD-1/PD-L1 pathway is involved in orbital fibroblast activation. Here, we show that orbital fibroblasts from patients with TAO do not express PD-L1. Based on *in vitro* OF-T cell co-culture system, exogenous PD-L1 weakens T cell-induced orbital fibroblast activation by inhibiting T cell activity, resulting in reduced production of sICAM-1, IL-6, IL-8, and hyaluronan. Additionally, exogenous PD-L1 treatment also inhibits the expression of CD40 and the phosphorylation levels of MAPK and NF-κB pathways in orbital fibroblasts of the OF-T cell co-culture system. Knocking down CD40 with CD40 siRNA or down-regulating the phosphorylation levels of MAPK and NF-κB pathways with SB203580, PD98059, SP600125, and PDTC can both reduce the expression of these cytokines and hyaluronan. Our study demonstrates that the orbital immune tolerance deficiency caused by the lack of PD-L1 in orbital fibroblasts may be one of the causes for the active orbital inflammation in TAO patients, and the utilization of exogenous PD-L1 to reconstruct the orbital immune tolerance microenvironment may be a potential treatment strategy for TAO.

Keywords: PD-L1, T cell, orbital fibroblast, CD40, thyroid associated ophthalmopathy

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INTRODUCTION

Thyroid associated ophthalmopathy (TAO), also designated as Graves' ophthalmopathy (GO), is a prevalent organ-specific autoimmune disease that has a deteriorating effect on patients' appearance and vision. TAO most often occurs in association with thyroid disease, such as hyperthyroid and hypothyroid. It is most frequently found complicating Graves' disease (GD), with a prevalence of more than ninety percent (1–3). Currently, it is difficult to achieve desired results for the treatment of TAO. Corticosteroids, orbital irradiation and surgical decompression have been the mainstay of therapeutic strategies (4–6). However, current medical therapies have limited efficacy, and they are often associated with side effects and other safety concerns (4, 5, 7). In recent years, breakthroughs have been made in the research of immunosuppressants for TAO. Rituximab, Teprotumumab, Tocilizumab, Thalidomide and other drugs, which have been used in the treatment of TAO, partially improve proptosis, diplopia and orbital inflammation of TAO patients (4, 5, 8–10). Due to the insufficient experiences in clinical application, however, the efficacy and safety profile of these drugs remains to be further studied and fully elucidated (4, 5, 8–10). The pathogenesis of TAO is still incompletely understood, which is the result of cellular immunity and humoral immunity under the influence of genetic, environmental and other factors (1–3). A large number of inflammatory cells, mainly T cells, and a few B cells, plasma cells, neutrophils, macrophages, etc., exist in the orbital adipose tissue and the intermuscular space of extraocular muscles from TAO patients (11). T cell, which plays important roles in antigen recognition, orbital fibroblast (OF) activation, B cell differentiation, cytokines release, and hyaluronan (HA) aggregation, is the crucial immune cell mediating orbital inflammation, adipose hyperplasia and extraocular muscle fibrosis in TAO patients (3, 11, 12). Therefore, it is essential to inhibit T cell activity for the treatment of TAO. However, there is still a lack of effective T cell-targeted drugs for the treatment of TAO, which leaves a big gap for the research of TAO therapy.

Recent researches have unveiled a close association between GD and the PD-1/PD-L1 pathway. Programmed cell death-1 (PD-1, or CD279), is a cell surface receptor that functions as an immune checkpoint and plays a crucial role in modulating T cell exhaustion. And its ligand, programmed death-ligand 1 (PD-L1, or CD274), is mainly expressed on various tumor cells (13, 14). PD-1/PD-L1 is the most crucial negative costimulatory pathway in the immune tolerance of tumor cells (14). The immunosuppression of the PD-1/PD-L1 pathway can attenuate T cell activity, accordingly helping tumor cells escape the immune attack, which plays an important role in promoting the progression of tumor (15, 16). In recent years, PD-1/PD-L1 inhibitors are studied extensively and a few of them have been approved for the treatment of certain types of tumors (17, 18). However, it has also been reported that tumor treatment by inhibiting the PD-1/PD-L1 signaling pathway was likely to cause immune imbalance, resulting in autoimmune diseases, such as multiple sclerosis, inflammatory bowel disease, Hashimoto's thyroiditis, and rheumatoid arthritis (19, 20). More importantly, the application of a PD-1 inhibitor presented clinical symptoms of TAO, including exophthalmos and enlargement of extraocular muscles (21). Interestingly, the PD-1/PD-L1 pathway is also operative in the pathogenesis of GD and may be a compensatory mechanism to restrain the autoimmune system but probably not to the extent of hindering the progression of GD (22–25). Due to the absence of research about the PD-1/PD-L1 pathway in TAO, however, the role of the PD-1/PD-L1 pathway in the pathogenesis of TAO remains unclear.

In this study, we initially performed an investigation and it was found that OFs did not express PD-L1. This suggested that the orbital immune tolerance deficiency caused by the lack of PD-L1 in OFs may be involved in the pathogenesis of TAO. Then, we constructed a co-culture system of OFs and T cells, utilizing exogenous PD-L1 in an attempt to simulate the orbital immune tolerance microenvironment and discover a potential TAO therapeutic strategy targeting T cell. Additionally, we have also studied the specific mechanism of T cell-induced OF activation *via* the CD40-CD40L pathway.

MATERIALS AND METHODS

Specimen Procurement

From July 2020 to October 2021, 13 subjects, including 8 patients with active TAO (CAS ≥ 3; CAS: clinical activity score) and 5 negative controls (healthy subjects without any known ophthalmopathy or other disease), as **Tables S1**, **S2** showed were recruited from Daping Hospital, Army Medical University (Chongqing, China). Informed consent was obtained from each subject. The study protocol was approved by the Ethics Committee of Daping Hospital, Army Medical University (Chongqing, China), and adhered to the tenets of the Declaration of Helsinki. Orbital connective tissue samples were obtained as surgical waste during orbital decompression for acute TAO patients or blepharoplasty for healthy subjects. Peripheral venous blood samples were collected at the beginning of both TAO patients' and healthy subjects' admission.

PD-L1 Treatment

Activated T cells were treated with either PBS, recombinant human PD-L1 protein (10 ug/mL; R&D Systems, Minneapolis, MN, USA), PD-L1 combined with goat anti-human IgG (10 ug/mL; R&D Systems, Minneapolis, MN, USA), or PD-L1 combined with PD-L1 neutralizing antibody (10 ug/mL; Abcam, Cambridge, UK), and the supernatants were subjected to ELISA after a 72-hour culture to detect the secretion of IFN-γ, IL-1β, TNF-α, and IL-2. Lymphocytes isolated from peripheral venous blood of TAO patients were treated with either PBS, recombinant human PD-L1 protein (10 ug/mL), PD-L1 combined with goat anti-human IgG (10 ug/mL), or PD-L1 combined with PD-L1 neutralizing antibody (10 ug/mL) for 48 hours, and the proportion of CD3+CD40L+ cells were determined by FCM. OFs from patients with TAO (TAO-OFs) were treated with either PBS, PD-L1 (10 ug/mL), autologous

activated T cells (OFs: T cells = 1: 10), or PD-L1 combined with T cells for 24 hours. TAO-OFs were subjected to flow cytometry (FCM) and immunofluorescence staining (IF) for CD40 expression, and the total protein extracted from TAO-OF layers were subjected to WB to detect the expression levels of p38, ERK1/2, JNK, and NF-κB in TAO-OFs. After a 48-hour co-culturing, the supernatants were subjected to analysis of sICAM-1, IL-6, IL-8, and HA production by ELISA.

Assessment of Cytokines and Hyaluronan Levels

Cytokines and Hyaluronan (HA) were assessed in triplicate by ELISA. TAO-OFs were treated with either PBS, IFN-γ (100 U/mL; Sino Biological Inc., Beijing, China), sCD40L (100 ng/mL; Sino Biological Inc., Beijing, China), or IFN-γ combined with sCD40L for 48 hours. TAO-OFs and CD40-knockdown TAO-OFs were co-cultured with autologous T cells (OFs: T cells = 1: 10) for 48 hours or not. TAO-OFs were stimulated with either PBS, IFN-γ neutralizing antibody (1 ug/mL; Proteintech, ORD, USA), autologous T cells, and IFN-γ Ab combined with and T cells for 48 hours. TAO-OFs were treated with either SB203580 (30 uM, p38 inhibitor), PD98059 (30 uM, ERK1/2 inhibitor), SP600125 (30 uM, JNK inhibitor), or PDTC (100 uM, NF-κB inhibitor) (all from MedChemExpress, NJ, USA) for 30 minutes, and then co-cultured with autologous T cells for 24 hours or not. Supernatant of cell culture from each experiment was collected and centrifuged at 1000 g for 10 minutes to remove debris. HA, IL-6, IL-8 (all from R&D Systems, Minneapolis, MN, USA), and sICAM-1 (4A Biotech, Beijing, China) were quantified in triplicate by ELISA according to the manufacturer's protocols.

Small Interfering RNA Transfection

TAO-OFs were plated and transiently transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) with either 50 nM of CD40 siRNA or negative control siRNA (GeneBio Co., Shanghai, China) according to the manufacturer's protocol. To detect the transfection efficiency, cells were incubated for 48 hours before being analyzed by RT-qPCR and incubated for 72 hours before being analyzed by FCM. TAO-OFs and CD40-knockdown TAO-OFs were co-cultured with T cells (OFs: T cells = 1: 10) for 24 hours or not, the total protein extracted from TAO-OF layers were subjected to western blot (WB) to detect the expression levels of p38, ERK1/2, JNK, and NF-κB in TAO-OFs.

Analysis of CD40 Expression

The CD40 expression in TAO-OFs was detected by FCM and IF. TAO-OFs were treated with either PBS, IFN-γ (100 U/mL), IFN-γ (100 U/mL) combined with IFN-γ Ab (1 ug/mL), autologous T cells (OFs: T cells = 1: 10), IFN-γ Ab combined with T cells, or PD-L1 combined with T cells for 24 hours. Then, TAO-OFs were subjected to FCM and IF for CD40 expression.

Statistics

Each experiment was repeated at least three times and data were graphed using GraphPad Prism Version 8.0 software (GraphPad Software, La Jolla, CA, USA). Statistical analyses were performed using SPSS version 23.0 software (IBM Corp., Armonk, NY, USA). Paired student's *t*-test was used as appropriate. All values were expressed as mean ± SD and statistical significance was defined as values of $P < 0.05$ (*); $P < 0.01$ (**); and $P < 0.001$ (***).

RESULTS

PD-L1 Inhibited the Activity of T cells

As activated T cells have been proposed to express abundant PD-1 (13, 14), we initially determined the proportion of PD-1+ cells in CD4+ T cells and CD8+ T cells by FCM, respectively. Our results showed that the expression of PD-1 by CD4+ T cells and CD8+ T cells from peripheral venous blood of TAO patients was significantly higher than that of healthy subjects, and the proportion of PD-1+ cells in CD8+ T cells was higher than that in CD4+ T cells (**Figure 1A**).

The general model for the immunosuppression mediated by PD-1/PD-L1 is based on the interaction between PD-L1 on the tumor cells and PD-1 on T cells. It has been reported that PD-L1 treatment resulted in inhibiting T cell proliferation and decreased IL-2, IL-4, IL-10, IFN-γ, and TNF-α secretion (26). Therefore, we next sought to detect the inhibition of PD-L1 exerting on T cells. Recombinant human PD-L1 protein was used to treat T cells, and the supernatants were subjected to ELISA after a 48-hour culture. T cells included in lymphocytes, which were isolated from peripheral venous blood of TAO patients, were enriched and activated by anti-human CD3/CD28 monoclonal antibody beads (**Figures S1A, S1B**). It was found that the production of IFN-γ, IL-1β, TNF-α, and IL-2 in T cells significantly decreased after PD-L1 treatment, and the neutralizing antibody to PD-L1 inhibited this effect (**Figures 1B**, **S1C**). We then detected the expression of CD40L in T cells by FCM. The results showed that PD-L1 significantly inhibited the expression of CD40L in T cells, and the neutralizing antibody to PD-L1 blocked the inhibition too (**Figure 1C**).

The Expression of PD-L1 in OFs and the Effect Exogenous PD-L1 Exerted on the OF-T Cell Co-Culture System

To investigate whether OFs expressed PD-L1, eight OF samples from TAO patients (TAO-OFs) and five OF samples from negative controls (NC-OFs) were collected and FCM was performed. RB1, a tumor cell line of retinoblastoma, was selected as a positive control in this experiment. It was found that PD-L1 was low expressed by OFs (**Figure 2A**). In addition, we also detected the expression of PD-1 in OFs with a positive control of activated T cells, and our data showed that PD-1 was low expressed in OFs too (**Figure 2B**).

Furthermore, the OF-T cell (activated T cells) co-culture system was treated with recombinant human PD-L1 protein for 48 hours, and the supernatants were subjected to ELISA. As shown in **Figures 2C, D**, **S2C**, the expressions of sICAM-1, IL-6, IL-8, CCL2, and HA in the OF-T cell co-culture system treated with PD-L1 were significantly decreased.

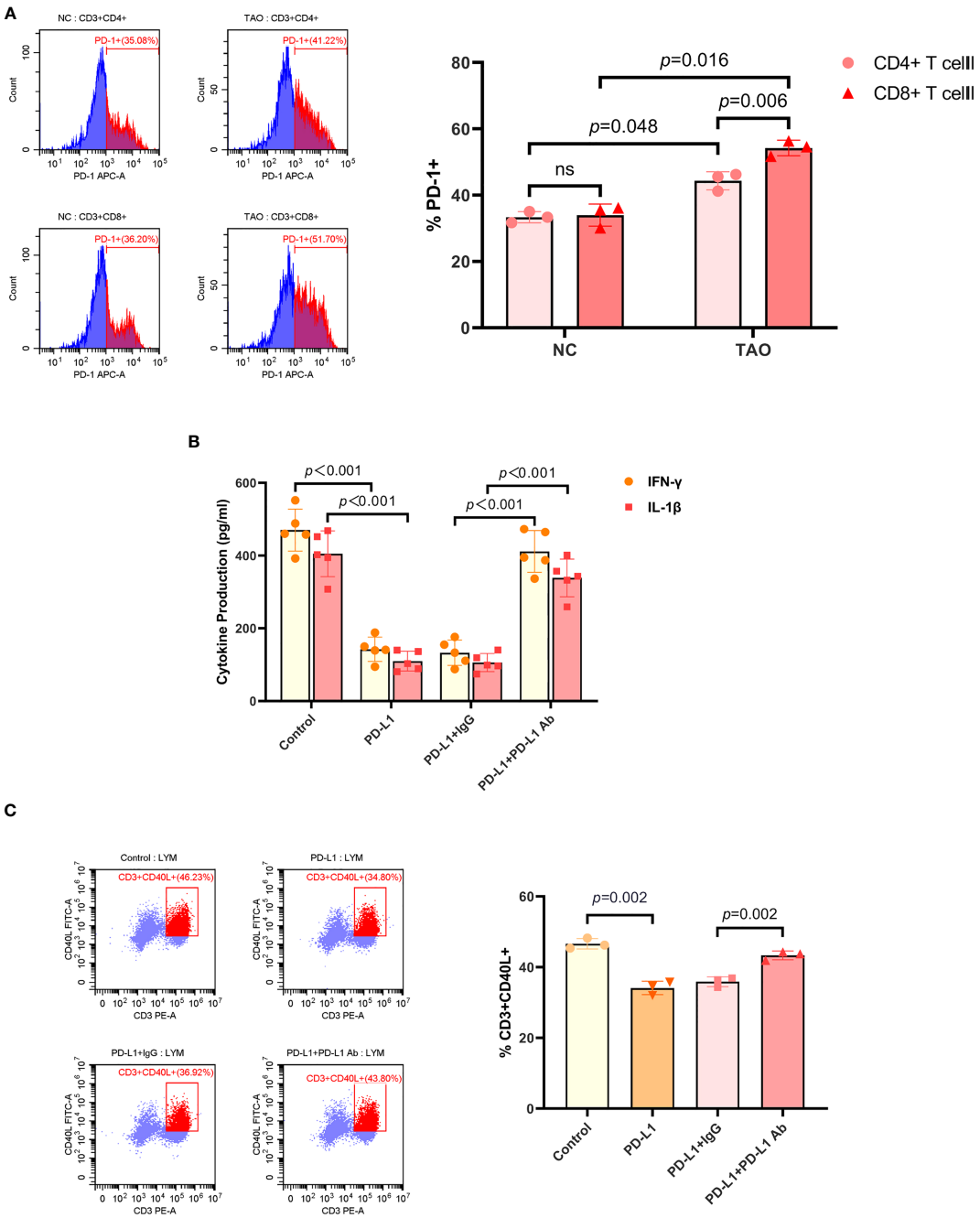


FIGURE 1 | PD-L1 inhibited the activity of T cell. **(A)** Lymphocytes isolated from peripheral venous blood of TAO patients compared to negative controls were subjected to FCM to detect the proportion of PD-1+ cells in CD4+ T cells and CD8+ T cells. **(B)** T cells were treated with PBS, recombinant human PD-L1 protein (10 ug/mL), PD-L1 combined with goat anti-human IgG (10 ug/mL), and PD-L1 combined with PD-L1 neutralizing antibody (10 ug/mL), respectively. And the supernatants were subjected to ELISA after a 72-hour culture to detect the secretion of IFN-γ and IL-1β. **(C)** Lymphocytes isolated from peripheral venous blood of TAO patients were treated with PBS, recombinant human PD-L1 protein (10 ug/mL), PD-L1 combined with goat anti-human IgG (10 ug/mL), and PD-L1 combined with PD-L1 neutralizing antibody (10 ug/mL) for 48 hours, respectively. And the proportion of CD3+CD40L+ cells were determined by FCM. Data are expressed as mean ± SD of three or more repetitions. ns, no significance. Representative of three or more independent experiments using cells from a different donor.

To figure out which cell (OF or T cell) secreted these cytokines and hyaluronan, we further examined these molecules in cytoplasm and the relevant mRNA. The results showed that T cells expressed a small amount of sICAM-1 which was much less than that of OFs, and hardly expressed IL-6, IL-8, and HA. The expressions of these molecules by OFs in OF-T cell co-culture system were significantly reduced upon PD-L1 treatment (**Figures 2E, F**).

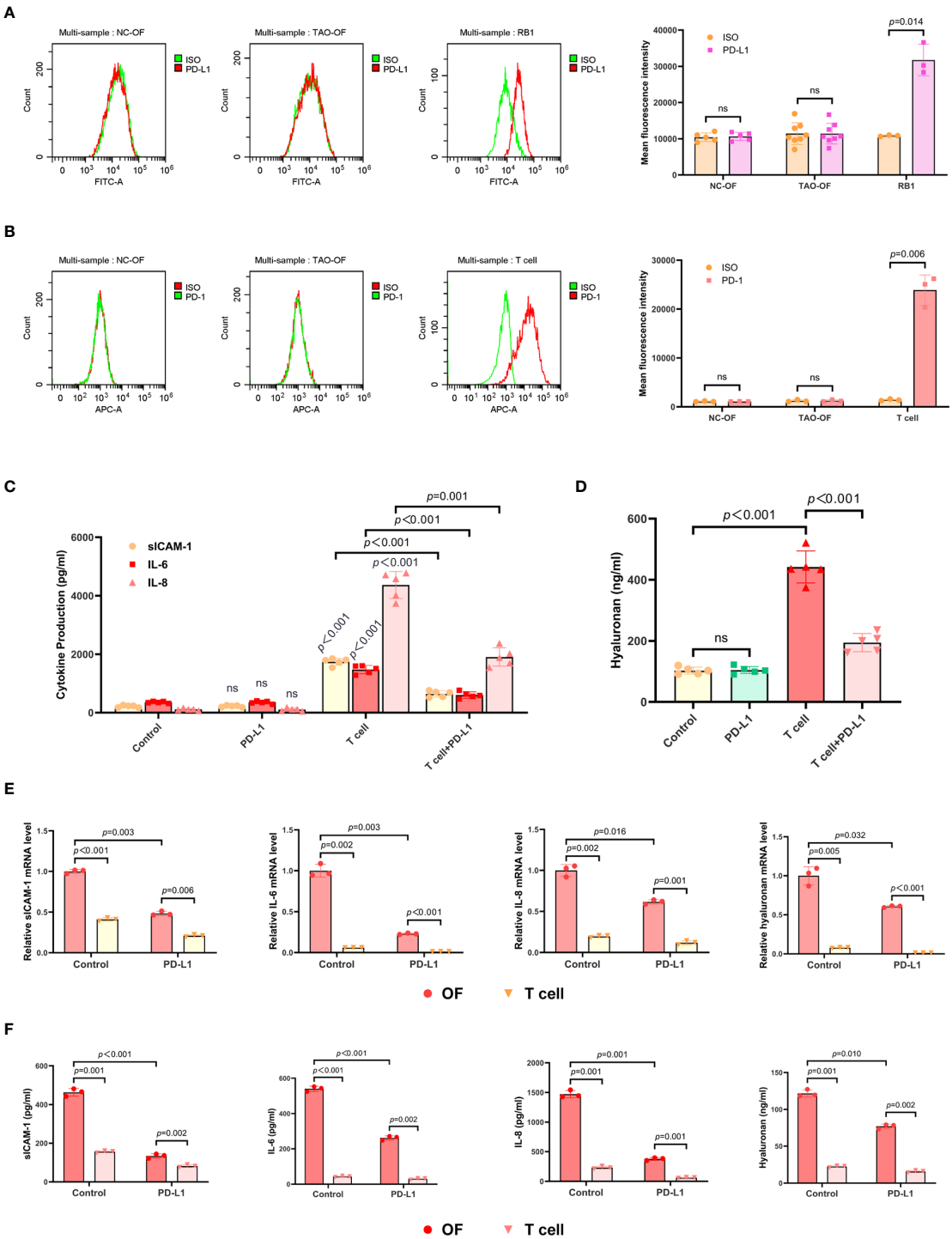


FIGURE 2 | The expression of PD-L1 in OFs and the effect exogenous PD-L1 exerted on the OF-T cell co-culture system. **(A)** OFs from TAO patients and negative controls were subjected to FCM to detect the expression of PD-L1. **(B)** OFs from TAO patients and negative controls were subjected to FCM to detect the expression of PD-1. **(C, D)** TAO-OFs were stimulated with PBS, PD-L1 (10 ug/mL), autologous T cells (OFs: T cells = 1: 10), and PD-L1 combined with T cells for 48 hours, respectively. And the supernatants were subjected to analysis of sICAM-1, IL-6, IL-8, **(C)** and HA **(D)** production by ELISA. **(E, F)** TAO-OFs were co-cultured with autologous T cells (OFs: T cells = 1: 10) for 48 hours, and the two cells were subjected to qPCR to assay mRNA expressions of sICAM-1, IL-6, IL-8, and HA **(E)**, or to ELISA to detect the production of sICAM-1, IL-6, IL-8, and HA in cytoplasm **(F)**. Data are expressed as mean ± SD of three or more repetitions. ns, no significance. Representative of three or more independent experiments using cells from a different donor.

T Cells Induced the Production of sICAM-1, IL-6, IL-8, and HA in TAO-OFs *via* the CD40-CD40L Pathway

T cells induced the activation of TAO-OFs *via* the CD40-CD40L pathway, leading to considerably high expression of HA and cytokines (27–30). Therefore, we first examined the expression of CD40 in OFs. Five TAO-OF samples and five NC-OF samples were collected respectively and subjected to FCM. The results showed that NC-OFs expressed a small amount of CD40, and the expression of CD40 in TAO-OFs was significantly higher (**Figure S3A**).

Peripheral venous blood samples from TAO patients and negative controls were collected, then lymphocytes were isolated and subjected to FCM. The results showed that CD4+ T cells from negative controls expressed a small amount of CD40L, whereas CD8+ T cells almost did not, and the proportion of CD40L+ cells in either CD4+ T cells or CD8+ T cells of TAO samples was increased apparently (**Figure 3A**).

As depicted above, CD40 was involved in stimulating HA and cytokine synthesis (11, 27–30). To verify this conclusion, we first treated TAO-OFs with either sCD40L, IFN-γ, or the combination. The supernatants were subjected to analysis of HA and cytokines contents by ELISA. It was found that, sCD40L, but not IFN-γ, slightly increased the secretion of sICAM-1, IL-6, IL-8, and HA. While significant increases of these cytokines and HA could be detected in TAO-OFs treated with sCD40L combined with IFN-γ (**Figures 3B, C**).

Then, CD40-knockdown TAO-OFs were used in the experiments. CD40 siRNA was transiently transfected into TAO-OFs. Cell monolayers were collected for total RNA extraction after a 48-hour culture, and RT- qPCR was performed. As **Figure S3B** showed, TAO-OFs transfected with CD40 siRNA displayed decreased levels of CD40 mRNA under autologous T cell stimulation compared with TAO-OFs transfected with NC siRNA. In addition, cells cultured for 72 hours were subjected to FCM and the results revealed that the expression of CD40 protein in TAO-OFs transfected with CD40 siRNA was also significantly reduced (**Figure S3C**). We co-cultured autologous T cells with CD40 knock-down TAO-OFs, and the production of cytokines and HA were assessed by ELISA. As shown in **Figures 3D, E**, no significant difference was detected in the production of cytokines or HA between the CD40 siRNA transfected group and the negative control group. While they were significantly increased when stimulated by T cells. However, they showed relatively lower increases in the group transfected with CD40 siRNA followed by T cell stimulation.

T Cells Up-Regulated the Expression of CD40 in TAO-OFs by Secreting IFN-γ

Activated T cells were known to secrete IFN-γ (31, 32). Additionally, it has been reported that IFN-γ stimulated the overexpression of CD40 in TAO-OFs (27–29). To verify this view, we first stimulated TAO-OFs with autologous T cells (OFs: T cells = 1: 10), T cells combined with IFN-γ neutralizing antibody, or T cells combined with recombinant human PD-L1 protein, and then detected the expression of CD40 in TAO-OFs by IF and FCM. IFN-γ-treated TAO-OFs and untreated TAO-OFs were used as the positive control and the negative control, respectively. It was shown that the expression of CD40 in TAO-OFs increased significantly when stimulated by autologous T cells, while both IFN-γ Ab and PD-L1 inhibited the stimulation (**Figures 4A, B**).

Then, we detected serum IFN-γ levels from TAO patients and negative controls by ELISA. It was found that the content of IFN-γ in serum from TAO patients was significantly high than that of negative controls. The IFN-γ content of PBMCs culture supernatant was also measured and the analogous result was obtained (**Figure 4C**).

As showed above, T cells induced the production of cytokines and HA in TAO-OFs *via* the CD40-CD40L pathway. Hence, we utilized IFN-γ neutralizing antibody to treat the OF-T cell co-culture system, and the results showed the production of sICAM-1, IL-6, IL-8, and HA in TAO-OFs stimulated by T cells after blocking IFN-γ were also reduced (**Figures 4D, E**).

PD-L1 Down-Regulated the Phosphorylation Levels of MAPK and NF-κB Pathway Proteins in TAO-OFs From the OF-T Cell Co-Culture System *via* the CD40-CD40L Pathway

To investigate how PD-L1 affected the pathways in TAO-OFs from the OF-T cell co-culture system, we first analyzed the effect of CD40 on the expression of the phosphorylation of MAPK and NF-κB signaling proteins in TAO-OFs. After transfection of CD40 siRNA followed by a 24-hour co-culture with autologous T cells, the TAO-OF layers were harvested for total protein extraction and the expression levels of MAPKs (p38, ERK1/2, JNK) and NF-κB p65 were detected by western blot. It was found that there was no significant difference in the expressions of these signaling proteins in the group transfected with CD40 siRNA compared with the negative control group. However, the phosphorylation levels of all the signaling proteins were significantly increased when stimulated by T cells. In contrast, TAO-OFs transfected with CD40 siRNA under the stimulation of T cells showed relatively lower phosphorylation levels of the four signaling proteins (**Figure 5A**).

In the second place, the OF-T cell co-culture system was treated with PD-L1 for 24 hours, and the total protein extracted from TAO-OF layers was subjected to western blot. Our data showed that PD-L1 had no direct effect on the expression of total p38, ERK1/2, JNK and NF-κB p65 proteins. However, compared with the untreated group, the phosphorylation levels of these signaling proteins in TAO-OFs from the OF-T cell co-culture system treated with PD-L1 were significantly down-regulated (**Figure 5B**).

The Production of sICAM-1, IL-6, IL-8, and HA in TAO-OFs From the OF-T Cell Co-Culture System Were Regulated by the MAPK and NF-κB Signaling Pathways

Based on several previous reports demonstrating the involvements of the MAPK and NF-κB signaling pathways in the production of sICAM-1, IL-6 and IL-8 by TAO-OFs (27, 33–36), we further investigated the role of MAPK and NF-κB signaling pathways in T cell-induced cytokines secretion in TAO-OFs. TAO-OFs were

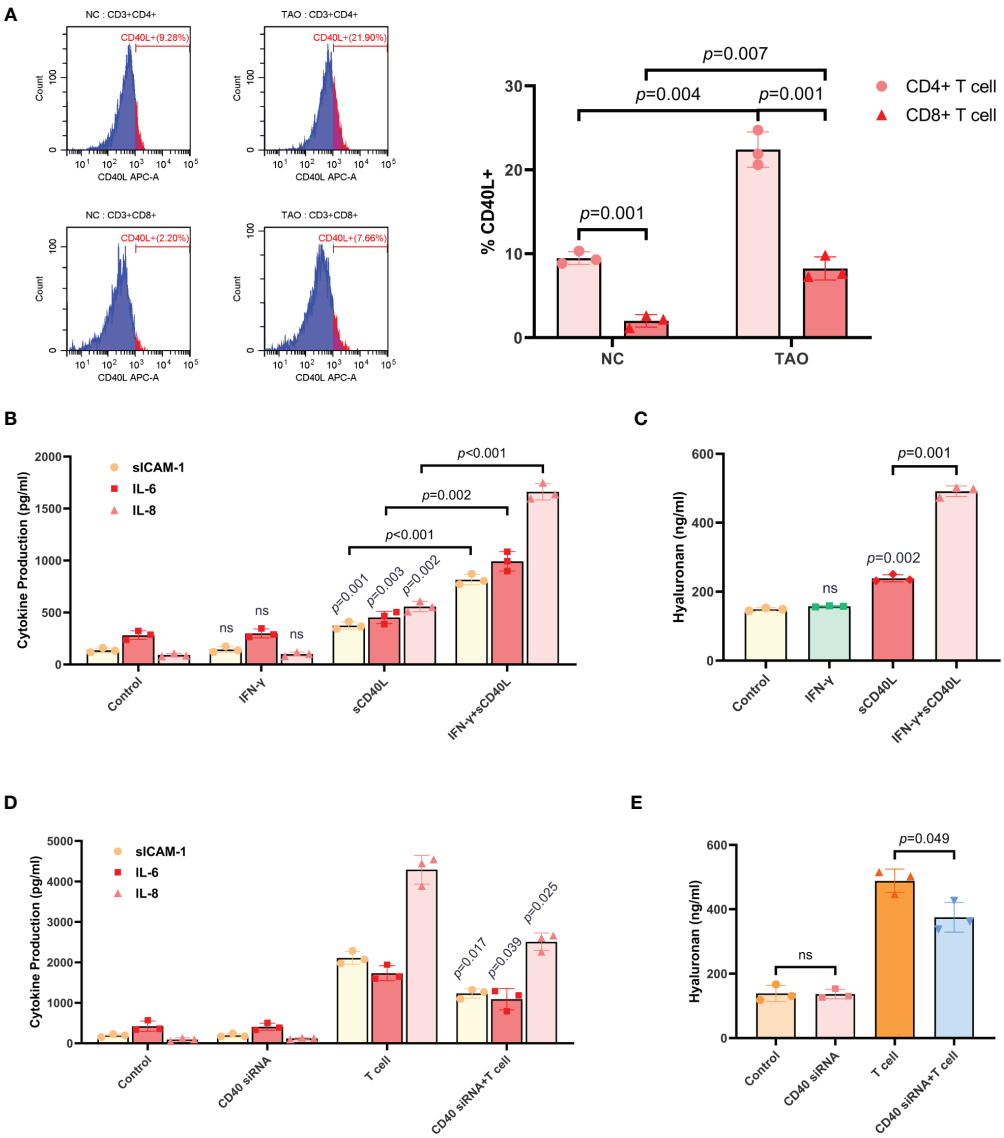


FIGURE 3 | T cells induced the production of sICAM-1, IL-6, IL-8, and HA in TAO-OFs *via* the CD40-CD40L pathway. **(A)** FCM of lymphocytes isolated from peripheral venous blood of TAO patients compared to negative controls demonstrating differential proportion of CD40L+ cells in CD4+ T cells and CD8+ T cells. **(B, C)** TAO-OFs were treated with PBS, IFN-γ (100 U/mL), sCD40L (100 ng/mL), and IFN-γ combined with sCD40L for 48 hours, respectively. And the supernatants were subjected to analysis of sICAM-1, IL-6, IL-8, **(B)** and HA **(C)** contents by ELISA. **(D, E)** TAO-OFs and CD40-knockdown TAO-OFs were co-cultured with autologous T cells (OFs: T cells = 1: 10) for 48 hours or not, and the supernatants were subjected to ELISA to detect the production of sICAM-1, IL-6, IL-8, **(D)** and HA **(E)**. Data are expressed as mean ± SD of triplicates. ns: no significance. Representative of three independent experiments using cells from a different donor.

treated with either SB203580, PD98059, SP600125, or PDTC for 30 minutes, and then co-cultured with autologous T cells for 24 hours or not. The supernatants were subjected to ELISA subsequently. The data demonstrated that the production of sICAM-1, IL-6, IL-8, and CCL2 did not change in the supernatants from TAO-OFs treated with inhibitors only, whereas, they were significantly reduced in the supernatants from TAO-OFs treated with inhibitors followed by co-culturing with T cells (**Figures 6A, B**, **S2D**). Furthermore, the production of HA in co-culture groups treated with inhibitors was significantly decreased (**Figures 6C, D**). In addition, no correlation could be detected between HA secretion and MAPK inhibitors without T cell stimulation (**Figure 6C**), however, the secretion of HA was inhibited by PDTC alone (**Figure 6D**).

DISCUSSION

PD-1/PD-L1, a crucial inhibitory signaling pathway in regulating T cell activity, functions as a suppressor in the progression of Graves' disease. However, the role of the PD-1/PD-L1 pathway in the

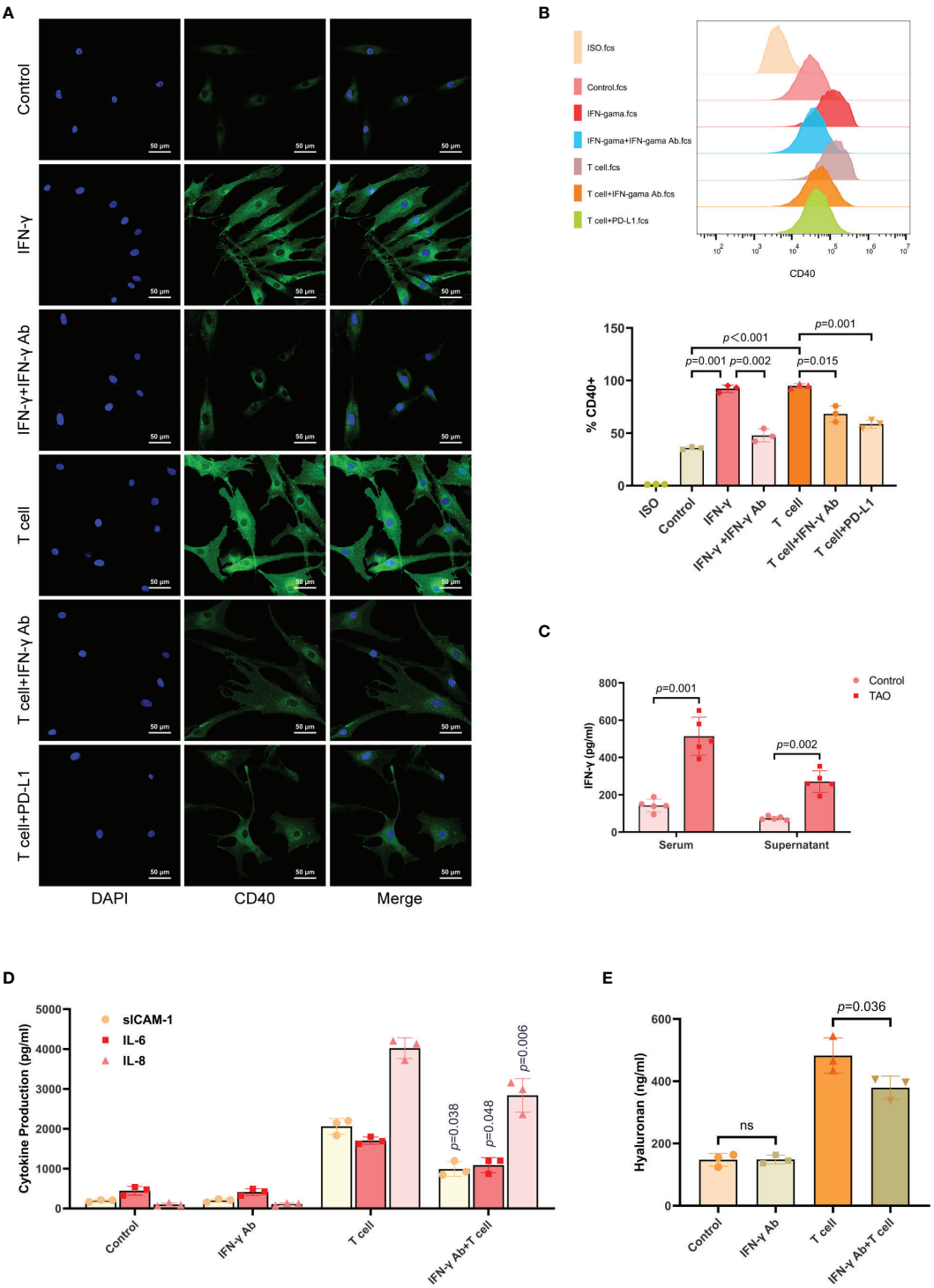


FIGURE 4 | T cells up-regulated the expression of CD40 in TAO-OFs by secreting IFN-γ. **(A, B)** TAO-OFs were stimulated with PBS, IFN-γ (100 U/mL), IFN-γ combined with IFN-γ neutralizing antibody (1 ug/mL), autologous T cells (OFs: T cells = 1: 10), T cells combined with IFN-γ Ab, and T cells combined with PD-L1 for 24 hours, respectively. And TAO-OFs were subjected to IF **(A)** and FCM **(B)** for CD40 expression. **(C)** Comparison of IFN-γ concentration in serum and lymphocyte culture supernatants between TAO patients and negative controls by ELISA. Lymphocytes were cultured in RPMI 1640 containing 10% FBS for 72 hours. **(D, E)** TAO-OFs were stimulated with PBS, IFN-γ Ab (1 ug/mL), autologous T cells, and T cells combined with IFN-γ Ab for 48 hours, respectively. And the supernatants were subjected to ELISA for sICAM-1, IL-6, IL-8, **(E)** and HA **(D)** production. Data are expressed as mean ± SD of three or more repetitions. ns, no significance. Representative of three or more independent experiments using cells from a different donor.

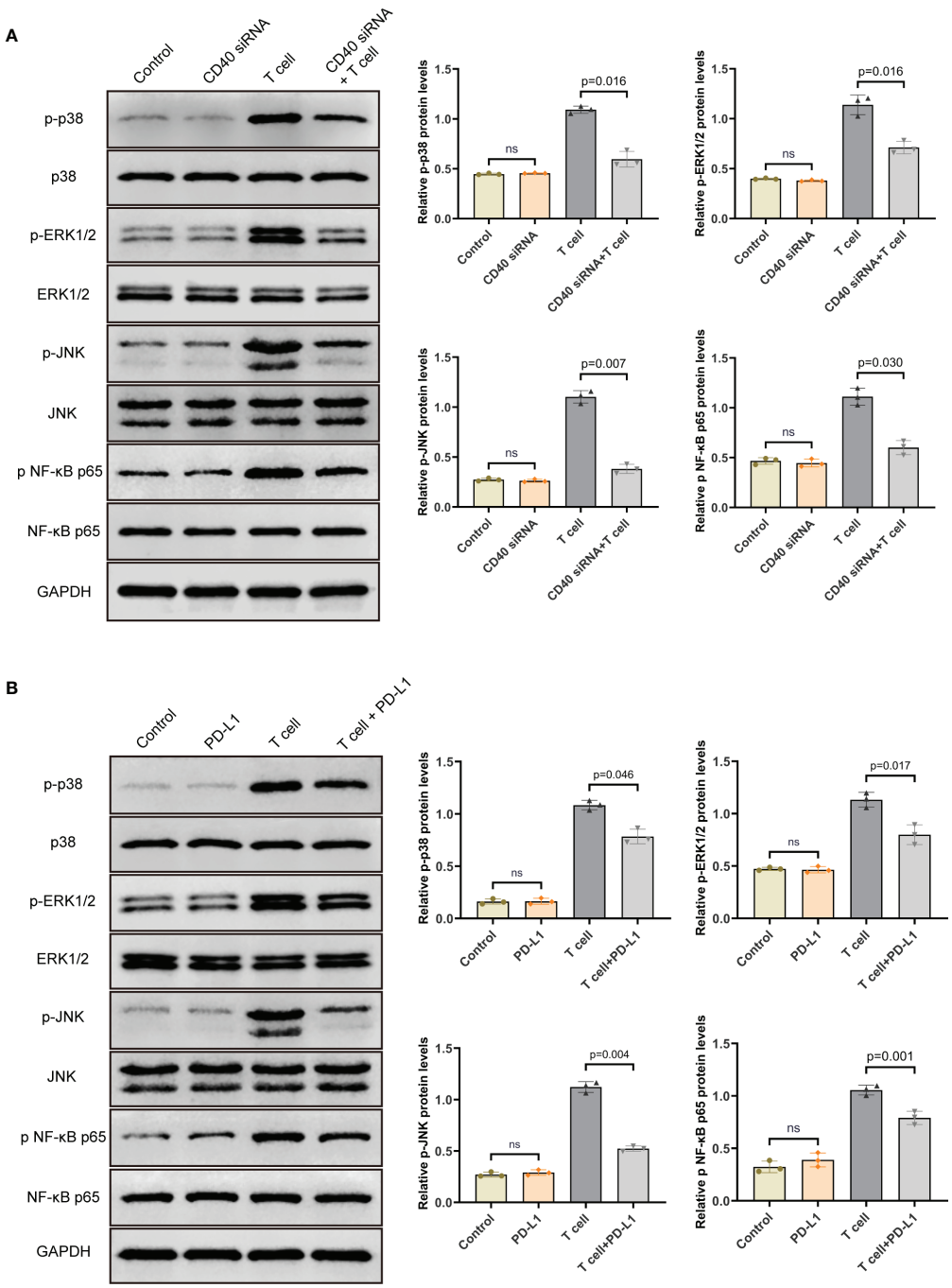


FIGURE 5 | PD-L1 down-regulated the phosphorylation levels of MAPK and NF-κB pathway proteins in TAO-OFs from the OF-T cell co-culture system *via* the CD40-CD40L pathway. **(A)** TAO-OFs and CD40-knockdown TAO-OFs were co-cultured with autologous T cells (OFs: T cells = 1: 10) for 24 hours or not, the total protein extracted from TAO-OF layers was subjected to western blot to detect the expression levels of p38, ERK1/2, JNK and NF-κB p65 in TAO-OFs. **(B)** TAO-OFs were treated with PBS, PD-L1 (10 ug/mL), autologous T cells (OFs: T cells = 1: 10), and T cells combined with PD-L1 for 24 hours, respectively. And the total protein extracted from TAO-OF layers was subjected to western blot to detect the expression levels of p38, ERK1/2, JNK and NF-κB p65 in TAO-OFs. Data are expressed as mean ± SD of triplicates. ns, no significance. Representative of three independent experiments using cells from a different donor.

pathogenesis of TAO remains unclear. In the present study, we determine that TAO-OFs do not express PD-L1, and exogenous PD-L1 attenuates CD40-CD40L-mediated T cells activating TAO-OFs by inhibiting T cell function, thus inhibiting immune inflammation and HA aggregation in the orbit of TAO patients (**Figure 7**).

T cells are the key immune cells that induce the activation of OFs and promote the development of TAO (11). In the clinic, the

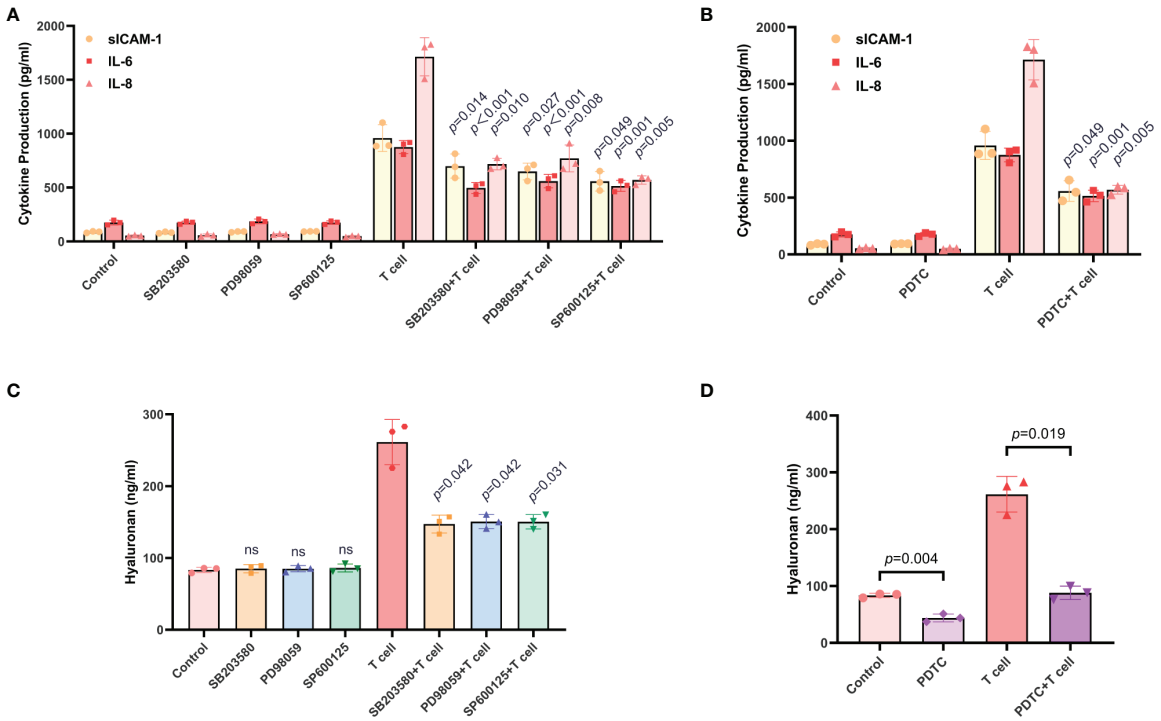


FIGURE 6 | The production of sICAM-1, IL-6, IL-8, and HA in TAO-OFs from the OF-T cell co-culture system were regulated by the MAPK and NF-κB signaling pathways. **(A–D)** TAO-OFs were treated with SB203580 (30 uM), PD98059 (30 uM), SP600125 (30 uM), and PDTC (100 uM) for 30 minutes, respectively, and then co-cultured with autologous T cells (OFs: T cells = 1: 10) for 24 hours or not. The supernatants were subjected to analysis of sICAM-1, IL-6, IL-8, **(A, B)** and HA **(C, D)** production by ELISA. Data are expressed as mean ± SD of triplicates. ns, no significance. Representative of three independent experiments using cells from a different donor.

use of some broad-spectrum immunosuppressants, such as corticosteroids, in the treatment of TAO can significantly inhibit T cell activity (4, 5). But if the activity of T cells can be inhibited in a targeted and safe way, we might achieve a better therapeutic result. Turning attention to tumor immunity, we focus on PD-1/PD-L1, a negative immune costimulatory pathway, which plays a vital role in protecting tumor cells from immune attack (13, 14). PD-L1 inhibits the activation of T cells and reduces the cytokines secretion of T cells (13, 14). Our data shows that PD-L1 also inhibits the expression of CD40L in T cells, which is in line with the finding in an inflammatory model (37). Recent studies suggested that PD-1/PD-L1 could partially inhibit the pathogenesis of GD (22–25). Moreover, the application of a PD-1 inhibitor resulted in some symptoms similar to TAO (21). We, therefore, wonder whether PD-1/PD-L1 also plays a certain inhibitory role in the pathogenesis of TAO. In the present study, it is found that PD-1 and PD-L1 were low expressed on the surface of OFs from both TAO patients and negative controls. This result suggests that the immune tolerance mechanism of the PD-1/PD-L1 pathway is absent in OFs, which may be one of the causes for the active orbital inflammatory response in TAO patients. Previous studies have demonstrated that CD34+ orbital fibroblasts (OFs), migrating from the circulation into the orbit of TAO patients but not healthy subjects, were the crucial cells that initiate the onset of TAO by expressing abundant cytokines and differentiating further into myofibroblasts and adipocytes (1, 3, 38). We then attempt to construct a co-culture system of TAO-OFs and activated T cells upon PD-L1 treatment. Our data indicate that exogenous PD-L1 significantly reduces the production of sICAM-1, IL-6, IL-8, CCL2, and HA in the OF-T cell co-culture system. We have conducted experiments to examine the levels of sICAM-1, IL-6, IL-8, and hyaluronan from NC-OFs co-cultured with activated T cells upon PD-L1 treatment. The results showed significant differences of these molecule expressions between NC-OFs versus TAO-OFs co-cultured with activated T cells either upon PD-L1 treatment or not. Additionally, the relative reduced expression of CD40 and phosphorylation levels of MAPKs and NF-κB p65 are also observed in TAO-OFs.

Previous reports suggested that activated T cells induce the proliferation of TAO-OFs (39), and significant increases of sICAM-1, IL-6 and IL-8 could be induced by sCD40L combined with IFN-γ in TAO-OFs (11, 28–30). In this study, the production of HA is also up-regulated. Our results also indicate that the secretions of these cytokines and HA in TAO-OFs are significantly up-regulated by activated T cells (40–42). It has been reported that CD40 was highly expressed in TAO-OFs compared with NC-OFs, and IFN-γ stimulated the overexpression of CD40 in TAO-OFs (27–29). T cells also induce the increased CD40 expression of TAO-OFs in our

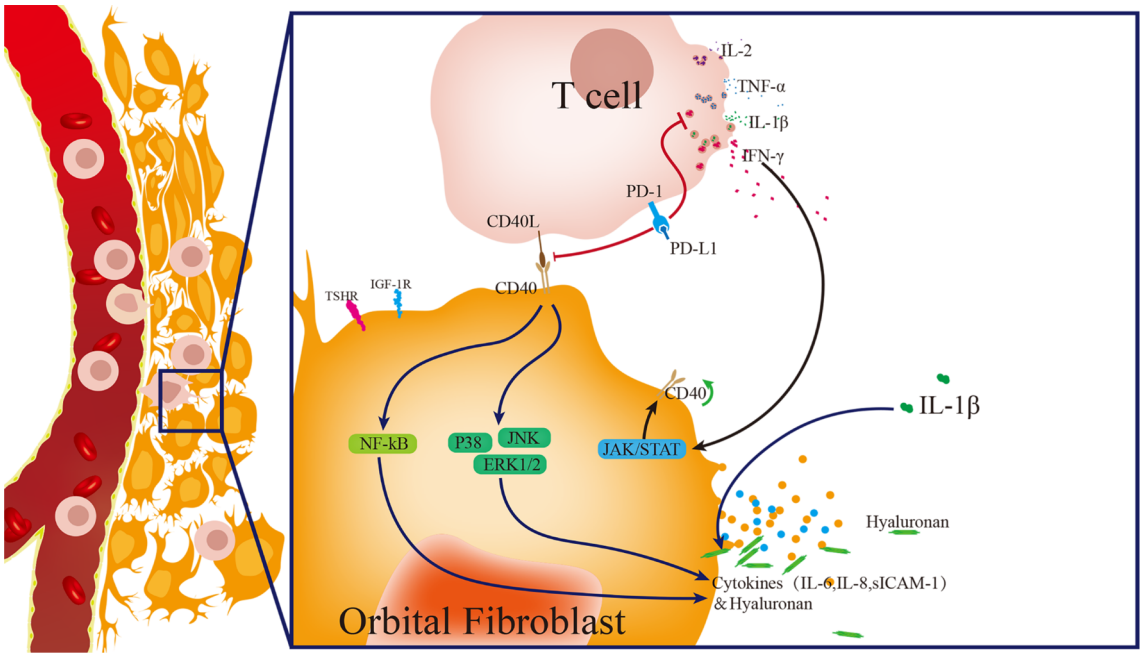


FIGURE 7 | Model of pathogenesis of TAO induced by T cells activating OFs, and PD-L1 blocking the progression of TAO. Activated T cells express abundant CD40L and release a large amount of cytokines, such as IL-1β, IL-2, IFN-γ and TNF-α. IFN-γ up-regulates the expression of CD40 in OFs. IL-1β induces the high expression of cytokines in OFs. T cells activate OFs *via* CD40-CD40L costimulatory molecules, thus up-regulating the phosphorylation of the downstream pathway proteins, including p38, ERK 1/2, JNK and NF-κB p65, and inducing the high production of sICAM-1, IL-6, IL-8, and hyaluronan. PD-L1 inhibits T cell activity, and thus weakens T cell-induced OFs activation, thereby significantly reducing the production of cytokines and hyaluronan in OFs.

study. Therefore, we speculate that the high expression of CD40 in TAO-OFs may be due to the active immune system, where activated T cells infiltrate into the orbit and secrete large amounts of IFN-γ to up-regulate the expression of CD40 in TAO-OFs. In addition, the proportion of CD40L+ cells was increased in lymphocytes from TAO patients. It's clear that CD40L, highly expressed on the surface of T cells, binding to CD40 expressed on the surface of TAO-OFs, stimulates the activation of TAO-OFs and induces the production of cytokines and HA.

CD40-CD40L, also known as a pair of costimulatory molecules, is essential for cellular immune responses (43). It has also been shown to act as an upstream master switch for the MAPK and NF-κB signaling pathways (43–45). Previous studies showed that sCD40L stimulated the secretion of cytokines and HA by up-regulating the phosphorylation levels of MAPK and NF-κB signaling pathways in TAO-OFs (27, 29, 33–36, 46). Our results indicate that activated T cells show analogous effects. When the CD40 gene of TAO-OFs was knocked down, the phosphorylation levels of p38, ERK1/2, JNK, and NF-κB p65 induced by T cells were significantly decreased, as well as the production of cytokines and HA. In addition, T cell-induced production of cytokines and HA in TAO-OFs were also reduced by inhibitors of MAPK and NF-κB p65 pathway proteins. These results suggest that T cells up-regulate the phosphorylation levels of MAPK and NF-κB signaling pathways in TAO-OFs *via* the CD40-CD40L costimulatory pathway, thus inducing the production of cytokines and HA.

Previous studies reported that MAPKs activation led to a substantial increase of HA synthesis in TAO-OFs (29, 41, 47). However, a contrary conclusion was presented in another research (48), which suggested PD98059, an ERK1/2 inhibitor, up-regulating IGF-1-induced HA secretion in TAO-OFs. Due to the shortage of researches in this aspect, the causes for this divergence are unclear, and the distinction of stimuli may be taken into account. Furthermore, our results show that the secretion of HA in TAO-OFs is also closely associated with the NF-κB pathway, which has never been reported in TAO before. The conclusions drawn by several studies in other models (49–51), including aortic smooth muscle cells, endothelial cells and type-B synoviocytes, were in agreement with it. Interestingly, our data suggest that PDTC not only inhibits T cell-induced HA secretion in TAO-OFs but also decreases the basal HA secretion of TAO-OFs. This is not consistent with a previous study with endothelial cells (51). One reason for this may be that TAO-OFs can secrete a small number of cytokines, including IL-1β, without external stimulus, which in turn induce the production of HA in TAO-OFs *via* activating the NF-κB pathway, while the basal secretion of HA in endothelial cells is relatively lower.

These results suggest that PD-L1 can block T cell-induced activation of TAO-OFs in two ways. PD-L1 not only down-regulates CD40L expression in T cells but also reduces IFN-γ secretion, thus attenuating T cells stimulated CD40 expression in TAO-OFs. In other words, PD-L1 inhibits the activity of T cells, and thus weakens T cell-induced TAO-OF activation *via* the

CD40-CD40L costimulatory pathway, thereby down-regulating the phosphorylation levels of p38, ERK1/2, JNK, and NF-κB p65 pathway proteins, and significantly reducing the production of sICAM-1, IL-6, IL-8, and HA in TAO-OFs (**Figure 7**). Therefore, the reconstruction of immune tolerance utilizing the PD-1/PD-L1 pathway may appear to be a novel approach for the treatment of TAO and other autoimmune diseases in the future.

In this study, the pathological mechanism of T cell-activated OFs initiating the pathogenesis of TAO has been revealed preliminarily. However, the role of TSHR and IGF-1R, the common antigens of TAO, in the pathogenesis of TAO have not been discussed. Additionally, we have shown that PD-L1 can be applied to efficiently inhibit T cell-induced production of cytokines and HA in TAO-OFs *in vitro*. However, considering the current TAO animal modeling methods are inefficient and difficult to replicate (52–54), further verification has not been performed *in vivo* for a firm conclusion.

In summary, the current study presents persuasive evidence that the PD-1/PD-L1 pathway is deficient in the orbit of both TAO patients and healthy subjects, and explores the possibility of utilizing exogenous PD-L1 to reconstruct the orbital immune tolerance microenvironment in patients with TAO. More importantly, the application of PD-L1 *in vitro* weakens T cell-induced OF activation by inhibiting T cell activity, thus inhibiting immune-inflammatory reaction and HA aggregation in the orbital region of TAO patients. These findings may provide new intervention strategies for the pathogenesis of TAO, and may also be expected to shed light on the treatment of other autoimmune diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Daping Hospital, Army Medical University, Chongqing, China. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZBL and YL contributed equally to this paper. ZBL, XZ, and HFY formulated the concept and designed the study. XB, XYG, and HFY collected the specimen. ZBL, YL, QJG, and BH performed the experiments. MML, AJS, and XHL analyzed the data. ZBL and YL drafted the paper. ZBL, YL, FLL, XZ and HFY participated in discussions associated with the paper. XZ and HFY revised the paper. All authors read and approved the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.849480/full#supplementary-material

REFERENCES

1. Taylor PN, Zhang L, Lee RWJ, Muller I, Ezra DG, Dayan CM, et al. New Insights Into the Pathogenesis and Nonsurgical Management of Graves Orbitopathy. *Nat Rev Endocrinol* (2020) 16(2):104–16. doi: 10.1038/s41574-019-0305-4
2. Khong JJ, McNab AA, Ebeling PR, Craig JE, Selva D. Pathogenesis of Thyroid Eye Disease: Review and Update on Molecular Mechanisms. *Br J Ophthalmol* (2016) 100(1):142–50. doi: 10.1136/bjophthalmol-2015-307399
3. Smith TJ, Janssen J. Insulin-Like Growth Factor-I Receptor and Thyroid-Associated Ophthalmopathy. *Endocr Rev* (2019) 40(1):236–67. doi: 10.1210/er.2018-00066
4. Wiersinga WM. Advances in Treatment of Active, Moderate-to-Severe Graves' Ophthalmopathy. *Lancet Diabetes Endocrinol* (2017) 5(2):134–42. doi: 10.1016/s2213-8587(16)30046-8
5. Ruchala M, Sawicka-Gutaj N. Advances in the Pharmacological Treatment of Graves' Orbitopathy. *Expert Rev Clin Pharmacol* (2016) 9(7):981–9. doi: 10.1586/17512433.2016.1165606
6. Boboridis KG, Bunce C. Surgical Orbital Decompression for Thyroid Eye Disease. *Cochrane Database Syst Rev* (2011) 12):CD007630. doi: 10.1002/14651858.CD007630.pub2
7. Han R, Smith TJ. T Helper Type 1 and Type 2 Cytokines Exert Divergent Influence on the Induction of Prostaglandin E2 and Hyaluronan Synthesis by Interleukin-1beta in Orbital Fibroblasts: Implications for the Pathogenesis of Thyroid-Associated Ophthalmopathy. *Endocrinology* (2006) 147(1):13–9. doi: 10.1210/en.2005-1018
8. Smith TJ. Challenges in Orphan Drug Development: Identification of Effective Therapy for Thyroid-Associated Ophthalmopathy. *Annu Rev Pharmacol Toxicol* (2019) 59:129–48. doi: 10.1146/annurev-pharmtox-010617-052509
9. Minakaran N, Ezra DG. Rituximab for Thyroid-Associated Ophthalmopathy. *Cochrane Database Syst Rev* (2013) 5):CD009226. doi: 10.1002/14651858.CD009226.pub2
10. Zhang C, Zhang X, Ma L, Peng F, Huang J, Han H. Thalidomide Inhibits Adipogenesis of Orbital Fibroblasts in Graves' Ophthalmopathy. *Endocrine* (2012) 41(2):248–55. doi: 10.1007/s12020-012-9600-8

11. Del Prete G, Romagnani S. The Role of TH1 and TH2 Subsets in Human Infectious Diseases. *Trends Microbiol* (1994) 2(1):4–6. doi: 10.1016/0966-842x(94)90336-0
12. Chen B, Tsui S, Smith TJ. IL-1 Beta Induces IL-6 Expression in Human Orbital Fibroblasts: Identification of an Anatomic-Site Specific Phenotypic Attribute Relevant to Thyroid-Associated Ophthalmopathy. *J Immunol* (2005) 175(2):1310–9. doi: 10.4049/jimmunol.175.2.1310
13. Sun C, Mezzadra R, Schumacher TN. Regulation and Function of the PD-L1 Checkpoint. *Immunity* (2018) 48(3):434–52. doi: 10.1016/j.immuni.2018.03.014
14. Konstantinidou M, Zarganes-Tzitzikas T, Magiera-Mularz K, Holak TA, Domling A. Immune Checkpoint PD-1/PD-L1: Is There Life Beyond Antibodies? *Angew Chem Int Ed Engl* (2018) 57(18):4840–8. doi: 10.1002/anie.201710407
15. Lu S, Stein JE, Rimm DL, Wang DW, Bell JM, Johnson DB, et al. Comparison of Biomarker Modalities for Predicting Response to PD-1/PD-L1 Checkpoint Blockade: A Systematic Review and Meta-Analysis. *JAMA Oncol* (2019) 5(8):1195–204. doi: 10.1001/jamaoncol.2019.1549
16. Li Y, Kilani RT, Pakyari M, Leung G, Nabai L, Ghahary A. Increased Expression of PD-L1 and PD-L2 in Dermal Fibroblasts From Alopecia Areata Mice. *J Cell Physiol* (2018) 233(3):2590–601. doi: 10.1002/jcp.26134
17. Postow MA, Callahan MK, Wolchok JD. Immune Checkpoint Blockade in Cancer Therapy. *J Clin Oncol* (2015) 33(17):1974–82. doi: 10.1200/JCO.2014.59.4358
18. Wu X, Gu Z, Chen Y, Chen B, Chen W, Weng L, et al. Application of PD-1 Blockade in Cancer Immunotherapy. *Comput Struct Biotechnol J* (2019) 17:661–74. doi: 10.1016/j.csbj.2019.03.006
19. Kuol N, Stojanovska L, Nurgali K, Apostolopoulos V. PD-1/PD-L1 in Disease. *Immunotherapy* (2018) 10(2):149–60. doi: 10.2217/imt-2017-0120
20. Ferrara R, Mezquita L, Texier M, Lahmar J, Audigier-Valette C, Tessonnier L, et al. Hyperprogressive Disease in Patients With Advanced Non-Small Cell Lung Cancer Treated With PD-1/PD-L1 Inhibitors or With Single-Agent Chemotherapy. *JAMA Oncol* (2018) 4(11):1543–52. doi: 10.1001/jamaoncol.2018.3676
21. Sagiv O, Kandl TJ, Thakar SD, Thuro BA, Busaidy NL, Cabanillas M, et al. Extraocular Muscle Enlargement and Thyroid Eye Disease-Like Orbital Inflammation Associated With Immune Checkpoint Inhibitor Therapy in Cancer Patients. *Ophthalmic Plast Reconstr Surg* (2019) 35(1):50–2. doi: 10.1097/IOP.0000000000001161
22. Wang X, Wu M, Cao Y, Zhang Z, Guo F, Li X, et al. Exploring the Role of Programmed Cell Death Protein 1 and its Ligand 1 in Eye Diseases. *Crit Rev Clin Lab Sci* (2019) 56(1):18–32. doi: 10.1080/10408363.2018.1522292
23. Álvarez-Sierra D, Marín-Sánchez A, Ruiz-Blázquez P, de Jesús Gil C, Iglesias-Felip C, González Ó, et al. Analysis of the PD-1/PD-L1 Axis in Human Autoimmune Thyroid Disease: Insights Into Pathogenesis and Clues to Immunotherapy Associated Thyroid Autoimmunity. *J Autoimmun* (2019) 103:102285. doi: 10.1016/j.jaut.2019.05.013
24. Pyzik A, Grywalska E, Matyjaszek-Matuszek B, Smolen A, Pyzik D, Rolinski J. Frequencies of PD-1- Positive T CD3+CD4+, T CD3+CD8+ and B CD19+ Lymphocytes in Female Patients With Graves' Disease and Healthy Controls-Preliminary Study. *Mol Cell Endocrinol* (2017) 448:28–33. doi: 10.1016/j.mce.2017.03.006
25. Han H, Fu X, Huang J, Zhang X, Yu J. PD-1/PD-L1 Affects Graves Progression Through Lymphocytes on the Proliferation, Apoptosis and Inflammatory Cytokine Secretion of Thyroid Follicular Epithelial Cells. *J Toxicol Sci* (2020) 45(11):701–11. doi: 10.2131/jts.45.701
26. Fu W, Lei C, Liu S, Cui Y, Wang C, Qian K, et al. CAR Exosomes Derived From Effector CAR-T Cells Have Potent Antitumour Effects and Low Toxicity. *Nat Commun* (2019) 10(1):4355. doi: 10.1038/s41467-019-12321-3
27. Hwang CJ, Afifiyan N, Sand D, Naik V, Said J, Pollock SJ, et al. Orbital Fibroblasts From Patients With Thyroid-Associated Ophthalmopathy Overexpress CD40: CD154 Hyperinduces IL-6, IL-8, and MCP-1. *Invest Ophthalmol Vis Sci* (2009) 50(5):2262–8. doi: 10.1167/iovs.08-2328
28. Vogel JD, West GA, Danese S, de la Motte C, Phillips MH, Strong SA, et al. CD40-Mediated Immune-Nonimmune Cell Interactions Induce Mucosal Fibroblast Chemokines Leading to T-Cell Transmigration. *Gastroenterology* (2004) 126(1):63–80. doi: 10.1053/j.gastro.2003.10.046
29. Cao HJ, Wang HS, Zhang Y, Lin HY, Phipps RP, Smith TJ. Activation of Human Orbital Fibroblasts Through CD40 Engagement Results in a Dramatic Induction of Hyaluronan Synthesis and Prostaglandin Endoperoxide H Synthase-2 Expression. Insights Into Potential Pathogenic Mechanisms of Thyroid-Associated Ophthalmopathy. *J Biol Chem* (1998) 273(45):29615–25. doi: 10.1074/jbc.273.45.29615
30. Sempowski GD, Rozenblit J, Smith TJ, Phipps RP. Human Orbital Fibroblasts are Activated Through CD40 to Induce Proinflammatory Cytokine Production. *Am J Physiol* (1998) 274(3):C707–14. doi: 10.1152/ajpcell.1998.274.3.C707
31. Zhou L, Chong MMW, Littman DR. Plasticity of CD4+ T Cell Lineage Differentiation. *Immunity* (2009) 30(5):646–55. doi: 10.1016/j.immuni.2009.05.001
32. St Paul M, Ohashi PS. The Roles of CD8(+) T Cell Subsets in Antitumor Immunity. *Trends Cell Biol* (2020) 30(9):695–704. doi: 10.1016/j.tcb.2020.06.003
33. Li H, Yuan Y, Zhang Y, He Q, Xu R, Ge F, et al. Celastrol Inhibits IL-1β-Induced Inflammation in Orbital Fibroblasts Through the Suppression of NF-κb Activity. *Mol Med Rep* (2016) 14(3):2799–806. doi: 10.3892/mmr.2016.5570
34. Zhao LQ, Wei RL, Cheng JW, Cai JP, Li Y. The Expression of Intercellular Adhesion Molecule-1 Induced by CD40-CD40L Ligand Signaling in Orbital Fibroblasts in Patients With Graves' Ophthalmopathy. *Invest Ophthalmol Vis Sci* (2010) 51(9):4652–60. doi: 10.1167/iovs.09-3789
35. Virakul S, Phetsuksiri T, van Holten-Neelen C, Schrijver B, van Steensel L, Dalm V, et al. Histamine Induces NF-kappaB Controlled Cytokine Secretion by Orbital Fibroblasts *via* Histamine Receptor Type-1. *Exp Eye Res* (2016) 147:85–93. doi: 10.1016/j.exer.2016.05.005
36. van Steensel L, Paridaens D, Dingjan GM, van Daele PL, van Hagen PM, Kuijpers RW, et al. Platelet-Derived Growth Factor-BB: A Stimulus for Cytokine Production by Orbital Fibroblasts in Graves' Ophthalmopathy. *Invest Ophthalmol Vis Sci* (2010) 51(2):1002–7. doi: 10.1167/iovs.09-4338
37. Cui X, Ma C, Vasudevaraja V, Serrano J, Tong J, Peng Y, et al. Dissecting the Immunosuppressive Tumor Microenvironments in Glioblastoma-On-a-Chip for Optimized PD-1 Immunotherapy. *eLife* (2020) 9:e52253. doi: 10.7554/eLife.52253
38. Douglas RS, Afifiyan NF, Hwang CJ, Chong K, Haider U, Richards P, et al. Increased Generation of Fibrocytes in Thyroid-Associated Ophthalmopathy. *J Clin Endocrinol Metab* (2010) 95(1):430–8. doi: 10.1210/jc.2009-1614
39. Feldon SE, Park DJ, O'Loughlin CW, Nguyen VT, Landskroner-Eiger S, Chang D, et al. Autologous T-Lymphocytes Stimulate Proliferation of Orbital Fibroblasts Derived From Patients With Graves' Ophthalmopathy. *Invest Ophthalmol Vis Sci* (2005) 46(11):3913–21. doi: 10.1167/iovs.05-0605
40. Xia N, Zhou S, Liang Y, Xiao C, Shen H, Pan H, et al. CD4+ T Cells and the Th1/Th2 Imbalance are Implicated in the Pathogenesis of Graves' Ophthalmopathy. *Int J Mol Med* (2006) 17(5):911–6. doi: 10.3892/ijmm.17.5.911
41. Watanabe M, Yamamoto N, Matsuzuka F, Miyauchi A, Iwatani Y. Decrease of CD154 Intensity on Peripheral CD4+ T Cells in Autoimmune Thyroid Disease. *Clin Exp Immunol* (2004) 136(3):555–8. doi: 10.1111/j.1365-2249.2004.02482.x
42. Kahaly GJ, Shimony O, Gellman YN, Lytton SD, Eshkar-Sebban L, Rosenblum N, et al. Regulatory T-Cells in Graves' Orbitopathy: Baseline Findings and Immunomodulation by Anti-T Lymphocyte Globulin. *J Clin Endocrinol Metab* (2011) 96(2):422–9. doi: 10.1210/jc.2010-1424
43. Pluvinet R, Olivar R, Krupinski J, Herrero-Fresneda I, Luque A, Torras J, et al. CD40: An Upstream Master Switch for Endothelial Cell Activation Uncovered by RNAi-Coupled Transcriptional Profiling. *Blood* (2008) 112(9):3624–37. doi: 10.1182/blood-2008-03-143305
44. Harigai M, Hara M, Kawamoto M, Kawaguchi Y, Sugiura T, Tanaka M, et al. Amplification of the Synovial Inflammatory Response Through Activation of Mitogen-Activated Protein Kinases and Nuclear Factor kappaB Using Ligation of CD40 on CD14+ Synovial Cells From Patients With Rheumatoid Arthritis. *Arthritis Rheum* (2004) 50(7):2167–77. doi: 10.1002/art.20340
45. Yang M, Xu Z, Zhuang Z. Macrophages Affect Immune Inflammation and Proliferation in Benign Prostatic Hyperplasia *via* Androgen Receptor and

CD40/CD40L Signaling Pathway. *Tissue Cell* (2020) 64:101343. doi: 10.1016/j.tice.2020.101343
46. Gillespie EF, Raychaudhuri N, Papageorgiou KI, Atkins SJ, Lu Y, Charara LK, et al. Interleukin-6 Production in CD40-Engaged Fibrocytes in Thyroid-Associated Ophthalmopathy: Involvement of Akt and NF-Kappab. *Invest Ophthalmol Vis Sci* (2012) 53(12):7746–53. doi: 10.1167/iovs.12-9861
47. Terazawa S, Nakano M, Yamamoto A, Imokawa G. Mycosporine-Like Amino Acids Stimulate Hyaluronan Secretion by Up-Regulating Hyaluronan Synthase 2 *via* Activation of the P38/MSK1/CREB/c-Fos/AP-1 Axis. *J Biol Chem* (2020) 295(21):7274–88. doi: 10.1074/jbc.RA119.011139
48. Zhang L, Grennan-Jones F, Lane C, Rees DA, Dayan CM, Ludgate M. Adipose Tissue Depot-Specific Differences in the Regulation of Hyaluronan Production of Relevance to Graves' Orbitopathy. *J Clin Endocrinol Metab* (2012) 97(2):653–62. doi: 10.1210/jc.2011-1299
49. Caon I, Bartolini B, Moretto P, Parnigoni A, Carava E, Vitale DL, et al. Sirtuin 1 Reduces Hyaluronan Synthase 2 Expression by Inhibiting Nuclear Translocation of NF-KappaB and Expression of the Long-Noncoding RNA HAS2-As1. *J Biol Chem* (2020) 295(11):3485–96. doi: 10.1074/jbc.RA119.011982
50. Kao JJ. The NF-kappaB Inhibitor Pyrrolidine Dithiocarbamate Blocks IL-1beta Induced Hyaluronan Synthase 1 (HAS1) mRNA Transcription, Pointing at NF-kappaB Dependence of the Gene HAS1. *Exp Gerontol* (2006) 41(6):641–7. doi: 10.1016/j.exger.2006.04.003
51. Vigetti D, Genasetti A, Karousou E, Viola M, Moretto P, Clerici M, et al. Proinflammatory Cytokines Induce Hyaluronan Synthesis and Monocyte Adhesion in Human Endothelial Cells Through Hyaluronan Synthase 2 (HAS2) and the Nuclear Factor-KappaB (NF-Kappab) Pathway. *J Biol Chem* (2010) 285(32):24639–45. doi: 10.1074/jbc.M110.134536
52. Ungerer M, Fassbender J, Li Z, Munch G, Holthoff HP. Review of Mouse Models of Graves' Disease and Orbitopathy-Novel Treatment by Induction of Tolerance. *Clin Rev Allergy Immunol* (2017) 52(2):182–93. doi: 10.1007/s12016-016-8562-7
53. Banga JP, Moshkelgosha S, Berchner-Pfannschmidt U, Eckstein A. Modeling Graves' Orbitopathy in Experimental Graves' Disease. *Horm Metab Res* (2015) 47(10):797–803. doi: 10.1055/s-0035-1555956
54. Nagayama Y, Nakahara M, Abiru N. Animal Models of Graves' Disease and Graves' Orbitopathy. *Curr Opin Endocrinol Diabetes Obes* (2015) 22(5):381–6. doi: 10.1097/MED.0000000000000186

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Selective Expansion of Tregs Using the IL-2 Cytokine Antibody Complex Does Not Reverse Established Alopecia Areata in C3H/HeJ Mice

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Alopecia areata (AA) is an autoimmune disease mediated by NKG2D-expressing cytotoxic T lymphocytes destroying hair follicles in the skin. It is one of the most common autoimmune diseases, but there is no effective treatment modality approved by the FDA. Regulatory T cells (Tregs) are crucial for suppressing autoreactive T cells, and in the skin, they promote hair growth by inducing anagen. Based on this, we tested the therapeutic potential of expanded Tregs in AA using the C3H/HeJ mouse model. In mice with AA, NKG2D-expressing CD8 T cells widely infiltrate both haired and hairless skin areas, which have tissue-resident memory T-cell phenotypes. Tregs in the skin express CD25, CTLA-4, GATA-3, and Jagged1 and efficiently proliferate with IL-2 cytokine antibody complex. However, expanding Tregs in the skin did not induce anagen in normal mice, indicating that they are necessary but not sufficient for anagen induction. Also, they fail to suppress autoreactive CD8 T cells in the skin to reverse established AA in C3H/HeJ mice. These results suggest that Treg expansion alone is not sufficient for AA treatment, and combined immunotherapy is required.

Keywords: alopecia areata (AA), Treg, IL-2, anagen, hair

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INTRODUCTION

Alopecia areata (AA) is the most frequent cause of inflammation-induced hair loss affecting 0.1%–0.2% of the general population at any given point in time, with an estimated lifetime risk of 2.1% (1, 2). It is mediated by NKG2D-expressing autoreactive cytotoxic T lymphocytes (CTL), which destroy hair follicles (HFs), leading to transient or permanent hair loss. Genetic and environmental factors provoke the collapse of immune privilege in HFs, and other stressors induce the activation of NKG2D+ CTL in the skin and its draining lymph nodes (LNs) (3). Activated CTLs express high levels of IFN-γ, which upregulates MHC I and II expressions on HF stem cells and induce the presentation of follicle-associated auto-antigens (4). RNA profiling revealed that AA lesions in mice and humans contain increased levels of granzymes A and B and cytokine IL-15, and IL-15 can effectively increase granzyme B expression in auto-reactive T cells (5). However, blocking these cytokines after disease onset has no

therapeutic effects and there is no effective treatment modality that can permanently reverse this process (5).

Janus kinase (JAK)/signal transducer and activator of transcription protein (STAT) signaling mediates various cellular events, including cytokine signaling cascade of lymphocytes and quiescent status of HF stem cells (6). Strikingly, Jak inhibitors (JAKis) could reverse established AA in C3H/HeJ mice and human patients (5, 7), and its derivatives are under clinical trials. However, JAKis increase sensitivity to infection and cancer by nonspecific suppression of immune cell activation. Also, human patients invariably relapse upon treatment cessation, indicating that its therapeutic effect is transient (8–10).

Regulatory T cells (Tregs) suppress the activation of autoreactive T cells, and their therapeutic potential to treat autoimmune disease has been successfully tested in several autoimmune diseases, including inflammatory bowel diseases (IBDs) and type I diabetes (T1D) (11–14). Interestingly, Tregs in the skin promote hair growth by stimulating the proliferation of HF stem cells through the expression of Notch ligand family member, Jagged1 (15). Based on these findings, we hypothesized that Treg expansion in the skin could be a treatment modality of AA by inhibiting HF-reactive CTLs and facilitating hair growth by anagen induction. To address this issue, we set up the C3H/HeJ mouse model of AA as previously described (16). In this mouse, we found that NKG2D+ CTLs in the AA lesion have the tissue-resident memory T-cell phenotype. To expand Tregs in the skin, we used intradermal (ID) injection of IL-2/anti-IL-2 antibody complex (IL-2c), which induces selective expansion of Tregs expressing Jagged1, CTLA-4, and GITR. However, although Tregs expanded about 8- to 10-fold in the skin, it was not sufficient to induce anagen in normal mice. In mice with AA, expanded Tregs also failed to decrease the number of pathogenic CD8 T cells and resume hair growth. These results indicate that expanded Tregs have limited therapeutic potential in AA, unlike other autoimmune diseases.

MATERIALS AND METHODS

Mice

C57BL/6 and C3H/HeJ mice were purchased from the Jackson Laboratory and maintained under specific pathogen-free conditions at POSTECH. Experiments were performed in compliance with institutional guidelines as approved by the Institutional Animal Care and Use Committee of POSTECH (2013–01–0012).

Animal Model of Alopecia Areata

AA mice were generated as previously described (16). Briefly, SDLNs were removed from mice that spontaneously developed AA, and cells were cultured with AR10 (advanced RPMI 1640, Gibco) containing 10% FBS (Atlas Biologicals), 2 mM GlutaMAX (Welgene), and 100 U/ml penicillin–streptomycin (Welgene) supplemented with IL-2, IL-7, and IL-15. Cells were stimulated with anti-CD3 and CD28-coated microbeads (Dynabeads, Thermofisher) and intradermally transferred to at least 10-week-old normal-haired C3H/HeJ mice during the second telogen phase.

Single-Cell Isolation

To make a single-cell suspension of mouse skin, minced skin tissue was incubated in RPMI media containing 10% FBS, 1% HEPES, and 100 U/ml penicillin–streptomycin (Welgene) supplemented with 2 mg/ml collagenase D (Roche), 0.1 mg/ml DNase I (Biosesang), and 0.5 mg/ml hyaluronidase (Biosesang) at 37°C for 50 min. Then, an additional 20 ml of media was added and shaken by hand for 30–45 s, and the cell suspension was filtered through a sterile 40-μl cell strainer into a new 50-ml conical tube. The cell suspension was then pelleted and re-suspended in PBS for cell counting and staining. Perfused lung tissues were harvested, minced, and digested in 5 ml of RPMI media containing 2 mg/ml collagenase D and 0.1 mg/ml DNase I on a shaker at 37°C for 45 min followed by filtration through a 70-μm strainer. Mononuclear cells were obtained after 40% and 70% Percoll (Merck) gradient centrifugation at 2,000 rpm for 20 min at RT. Liver tissue was minced and filtered through a 70-μm strainer, and mononuclear cells were isolated after 40% and 70% Percoll gradient centrifugation.

Immunofluorescence

Skin tissue was fixed in 4% paraformaldehyde (PFA, Electron Microscopic Science) for 1 h, washed with PBS, and left in 30% sucrose overnight before embedding in OCT. Tissue sections were stained primarily with anti-CD3-BV480 (1:1,000, 17A2), anti-CD4-AF488 (1:250, RM4-5), anti-CD8a-PE (1:250, 53-6.7), anti-FoxP3-APC (1:250, FJK-16s), and anti-GL3-biotin (1:250, GL3), followed by secondary SA-AF750 (1:10,000) at RT. Slides were then washed in PBS and mounted with DAPI-containing medium. Images were obtained using Leica DM6B with the THUNDER system.

IL-2 Treatment

Human IL-2 (hIL-2, GenScript), anti-hIL-2 antibody (BD, clone 5344.111), and mouse IL-2 Fc (absolute antibody) were used for Treg expansion. The IL-2 cytokine antibody complex (IL-2c) was made by mixing 1 μg of hIL-2 and 5 μg of 5344.111 per 18 g of mice weight.

Local Treatment of Ruxolitinib

The affected dorsal skin of C3H/HeJ mice with AA was treated daily for 12 weeks with ruxolitinib dissolved in 10% DMSO and mixed with Aquaphor as 0.5% ointment as described previously (17).

Anagen Induction

The dorsal hair of 60-day-old C57BL/6 mice was shaved with a clipper before the treatment. Control mice were treated with topical 10% DMSO (vehicle control) or 2% ruxolitinib (APExBIO), as described previously (7). IL-2c was IP injected for three consecutive days, and hair growth was monitored until day 80.

Ex Vivo Expansion of Treg

Ex vivo expansion of Treg was performed as previously described (18). Briefly, spleens of Thy1.1$^+$ C57BL/6 mice were single-cell isolated and enriched for CD4 T cells by using the CD4 T cell isolation kit (Miltenyi Biotec). CD25$^+$ cells were isolated using the FACSAria II cell sorter (BD Biosciences). After checking the purity (≥95%), Tregs were cultured in RPMI media containing 10% FBS, 100 U/ml penicillin–streptomycin (Welgene), 1× GlutaMAX-1, 1 mM sodium pyruvate, 10 mM HEPES, 1× nonessential amino acids, 10 μM 2-mercaptoethanol, and 2,000 IU/ml of recombinant human IL-2 (hIL-2, GenScript). Tregs were seeded into 12-well plates with anti-CD3 and CD28-coated microbeads (Dynabeads, Thermofisher) at a 2:1 cell-to-bead ratio. On days 2, 4, 6, 9, and 11, culture volume was doubled by adding hIL-2 supplemented fresh medium.

Flow Cytometry

Isolated mononuclear cells were stained with surface markers for 30 min at 4°C, and dead cells were excluded by staining Zombie Aqua Fixable viability dye (BioLegend). For intracellular staining, surface-stained cells were fixed and permeabilized with the Foxp3/transcription factor staining buffer set (Thermo Fisher Scientific). The following fluorescent dye-labeled antibodies were purchased from BD Biosciences, BioLegend, and eBioscience: anti-CD4-BUV395 (1:400, GK1.5), anti-CD25-APC (1:400, PC61), anti-CD314 (NKG2D)-PE (1:200, CX5), anti-CD62L-PerCP-Cy5.5 (1:400, MEL-14), anti-CD103-APC (1:400), anti-CD44-AF700 (1:400, IM7), anti-TCRβ-APC-Cy7 (1:400, H57-597), anti-CD45.2-BV605 (1:200, 104), anti-CD8-BV650 (1:400, 53-6.7), anti-CD11b-BV711 (1:1,000, M1/70), anti-B220-BV711 (1:400, RA3-6B2), anti-CD357 (GITR)-PE-Cy7 (1:400, DTA-1), anti-CD339 (Jagged1)-PE (1:100, HMJ1-29), anti-Eomes-AF488 (1:200, Dan11mag), anti-T-bet-PE-Cy7 (1:400, 4B10), anti-FoxP3-PE-CF594 (1:400, MF23), anti-GATA3-PE (1:400, TWAJ), and anti-CD152 (CTLA-4)-PE-Texas Red (1:400, UC10-4F10-11). Stained cells were analyzed using BD LSR Fortessa, and data were analyzed using Flow Jo software (Tree Star).

Statistical Analysis

Statistical analyses were performed with Prism software (GraphPad). *p*-values were calculated using a two-tailed unpaired Student's *t*-test.

RESULTS

Tissue-Resident Memory CD8 T Cells Infiltrate in Skin Lesion of C3H/HeJ Mice With AA

We generated a mouse model of AA by transferring cultured skin-draining LN cells obtained from C3H/HeJ mice that spontaneously developed AA to normal-haired C3H/HeJ mice as previously described (16) (**Figure S1**). Immunofluorescence staining of hairless skin lesions shows a dense infiltration of CD8 T cells, destroying a normal architecture of HFs (**Figure 1A**, right). Interestingly, the haired skin of C3H/HeJ mice with AA also showed linear infiltration of NKG2D-expressing CD8 T cells alongside the border of intact HFs (**Figure 1A**, middle). These features suggest that pathogenic CD8 T cells infiltrate before the onset of hair loss. In flow cytometric analysis, CD8 T cells from both haired and hairless areas express NKGD2 (**Figures 1B, C**). Besides NKG2D, they also expressed TBET and CD103 but not Eomes (**Figure 1D**). These phenotypes are similar to tissue-resident memory (Trm) T cells defined after viral infection in the skin and lung (19–21). In Trm T cells, TBET upregulates the IL-15 receptor, and downregulation of Eomes promotes CD103 expression, enabling their long-term survival and tissue retention, respectively. Therefore, it seems that pathogenic CTLs in AA are tissue-resident memory cells chronically destroying HFs. We also found that NKG2D-expressing CD8 T cells accumulate in the skin draining lymph node (SDLN) but not in the spleen and mLN, suggesting that they have a limited capacity for systemic circulation (**Figure S2**). Interestingly, NKG2D-expressing CD8 T cells were also found in the lung and liver of C3H/HeJ mice with AA. Although its pathological role and clinical significance are unclear, this result suggests that NKG2D-expressing CTLs also distribute outside the skin. Overall, these features show that tissue-resident NKG2D-expressing CTLs accumulate in the lesional and non-lesional skin, lung, and liver in C3H/HeJ mice involved with AA.

IL-2c Effectively Expands Tregs in the Skin

JAKi could reverse established AA in C3H/HeJ mice and humans (5). However, the patients invariably relapsed upon the cessation of JAKi administration (22). We tested this in C3H/HeJ mice with AA by applying ruxolitinib for 12 weeks and checking them after 10 weeks of treatment endpoint (**Figure S3**). We found that the mice relapsed after treatment cessation, indicating that the treatment effect of JAKi is transient. To test whether expanded Tregs could reverse established AA, we used IL-2, a potent stimulator of Tregs. A previous study showed that three consecutive injections (D0, D1, and D2) of IL-2c (1 μg of mIL-2 plus 5 μg of JES6-1A12) expanded Tregs most efficiently at D4 or D5 in the spleen (23). We found that three consecutive injections are more efficient than one or two injections in the skin, SDLN, and spleen (**Figures S4A, B**). However, prolonged daily injections of IL-2c up to 9 days did not overtly increase the Treg/CD8 T cell ratio in blood after day 5 (**Figure S4**). Next, we compared the effects of equal amounts of IL-2 (1 μg) between pure IL-2, IL-2 Fc, and IL-2c for the expansion of Tregs (**Figures 2A, B**). With three consecutive injections of each reagent containing 1 μg of IL-2, only IL-2c can effectively augment Tregs in the skin, spleen, and SDLN (**Figures 2A, B**). IL-2c-injected mice had more than nine times the number of Tregs compared to control mice, while CD8 T cells were not significantly affected (**Figures 2C, D**). We stained Foxp3 and other T-cell markers in the tissue section and observed that expanded Tregs are located between HFs in the skin (**Figure 2E**). After IL-2c injection, the increased Tregs gradually decreased with a half-life of about a week, and their numbers were normalized after 3 weeks (**Figures 2F, G**).

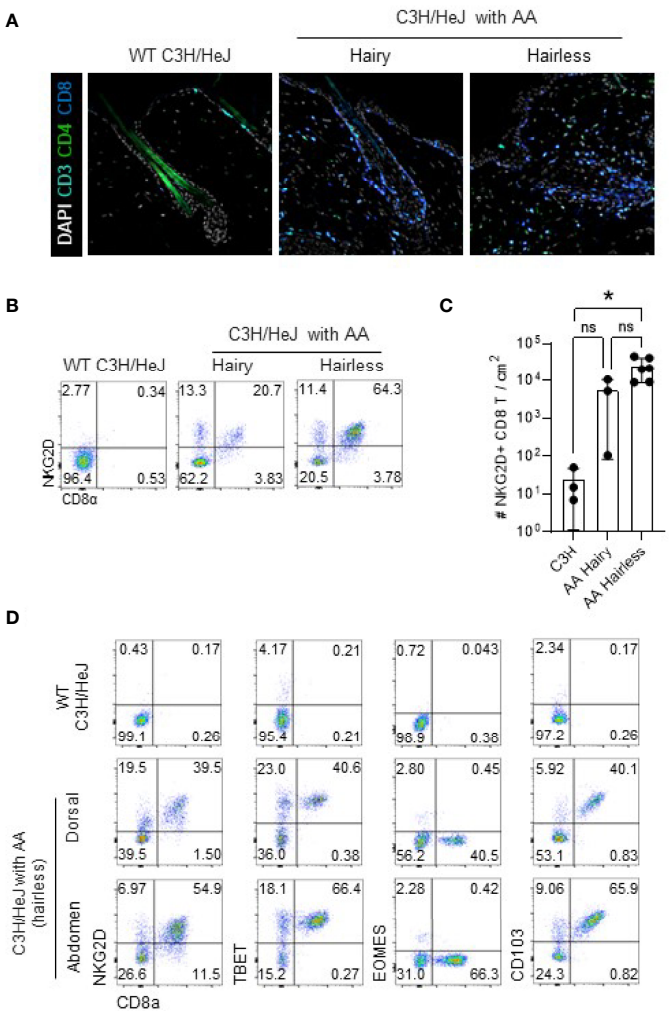


FIGURE 1 | NKG2D$^+$ TBET$^+$ Eomes$^-$ CD103$^+$ CTL cells infiltrate lesional and non-lesional skin of AA. **(A)** Representative immunofluorescence images show CD8 and CD4 T cells around the HF in the dorsal skin of indicated mice. **(B, C)** Representative dot plots show NKG2D$^+$ CD8 T cells in the dorsal skin of indicated mice after gating on live CD45$^+$CD19$^-$CD11b$^-$ cells **(B)**. Graph shows statistical analysis in normal C3H/HeJ mice (n = 3) and hairy (n = 3) and hairless (n = 6) skin of C3H/HeJ mice with AA. **(D)** Representative dot plots show expression of NKG2D, TBET, Eomes, and CD103 after gating on live CD45$^+$CD19$^-$CD11b$^-$ cells in indicated mice. Representative results from more than three independent experiments are shown. Horizontal bars indicate mean values, error bars show SD, and each dot represents an individual mouse **(C)**. An unpaired two-tailed t-test was used. *$p < 0.05$. CTL, cytotoxic T lymphocytes; HF, hair follicle. ns, non-significant.

We additionally tested whether ID injection of IL-2c is more effective than IP injection for the expansion of Tregs in the skin (**Figure 3**). Although there was no statistical significance, we found that, compared to IP injection, ID injection induced the expansion of the Tregs more stably, in both ipsilateral and contralateral injection sides. Furthermore, expanded Tregs in the skin maintained the expression of CD25, CTLA-4, GITR, and GATA-3 in C3H mice (**Figures 3A, B**). CD25, CTLA-4, and GITR were also expressed in B6 mice (**Figure S5**), and GATA-3 is a known marker of skin-resident Tregs in B6 mice (24), showing no strain-specific differences. We also confirmed the selective expansion of Tregs in C3H mice by an abrupt increase of CD8 T/Treg ratio upon IL-2c injection (**Figure 3C**). Overall, these results show that ID injection of IL-2c is a favorable method for Treg expansion in the skin of B6 and C3H/HeJ mice.

Expanded Skin Tregs by IL-2c Express Jagged1, But Does Not Induce Anagen

Tregs in the skin, but not in SDLN, express Jagged 1, and maintained their expressions after IL-2c treatment (**Figures 4A, B**). A previous study reported that Tregs expressing Jagged1 are required for the telogen-to-anagen transition of HF stem cells (15). To further address the issues of whether the expanded Tregs would facilitate anagen induction, we compared hair growth in

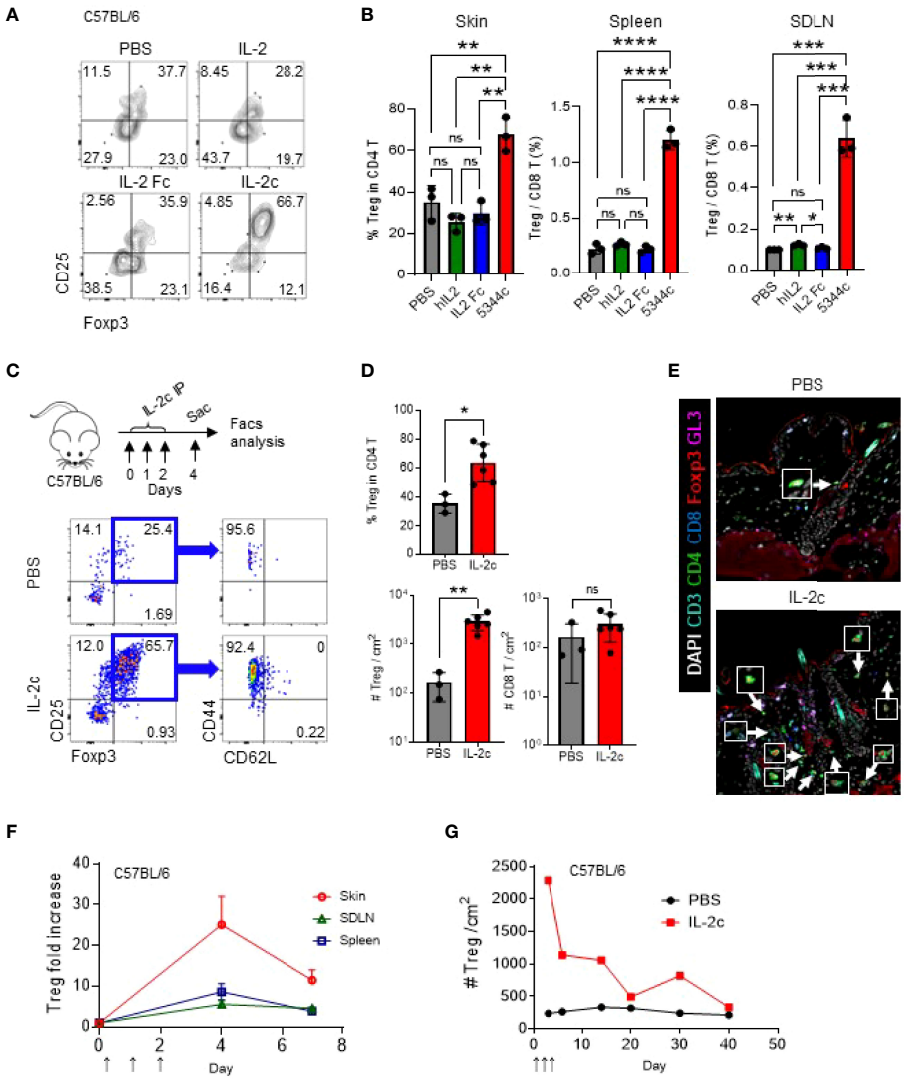


FIGURE 2 | IL-2c effectively expands Tregs in the skin. **(A, B)** Six- to 12-week-old female C57BL/6 mice were intraperitoneally injected with IL-2 (1 μg per 18 g), IL-2 Fc (2.67 μg per 18 g), IL-2c (1 μg of IL-2 and 5 μg of 5344.111 per 18 g) on days 0, 1, and 2, and analyzed on day 4. Representative FACS plots show Tregs in the skin **(A)**, and graphs show quantification of Tregs in indicated organs **(B)**. **(C, D)** Six- to twelve-week-old female C57BL/6 mice were IL-2c injected on days 0, 1, and 2 and analyzed on day 4. Experimental schemes are shown (**C**, top), and representative dot plots show skin Tregs in PBS- or IL-2c-injected mice (**C**, bottom). Graphs show quantification of Tregs in the skin and CD8 T cells in PBS- or IL-2c-injected mice (n = 3–6) **(D)**. **(E)** Representative images of skin Tregs of PBS- or IL-2c-injected mice harvested on day 4. Arrows depict Tregs. **(F, G)** Graphs show Treg ratios between IL-2c-injected and non-injected mice in indicated organs **(F)** and kinetics of skin Tregs of PBS- or IL-2c-injected mice **(G)**. Results are from two independent experiments. Horizontal bars indicate mean values, error bars indicate SD, and each dot represents an individual mouse **(B, D)**. An unpaired two-tailed t-test was used. *$p < 0.05$, **$p < 0.01$, ***p < 0.001, ****$p < 0.0001$, ns, non-significance ($p > 0.05$).

mice treated with DMSO, IL-2c, and ruxolitinib at the early telogen phase after shaving (**Figures 4C, D**). As shown previously (7), ruxolitinib induced anagen by day 82, but IL-2c had no effect until day 90 when normal anagen transition occurs. Ruxolitinib is a known negative regulator of Tregs (25), and consistent with this, we found that the frequency of Tregs among CD4 T cells decreased after its treatment (**Figure 4E**). Therefore, it seems like the anagen induction of ruxolitinib is independent of Tregs, and Tregs expressing Jagged 1 are required but not sufficient for the anagen induction in WT mice.

Expanded Tregs by IL-2c Do Not Reverse Established AA

Finally, we tested whether expanded Tregs inhibit autoreactive T cells in AA upon ID injection of IL-2c. Based on the expansion kinetics of Tregs in the skin after IL-2c injection (**Figures 2F**, **S4**), we ID injected IL-2c three times a week for 6 weeks in C3H/HeJ mice with AA as indicated in **Figure 5A**. However, upon completing treatment for 6 weeks, we found no difference in the hairless skin area before and after treatment (**Figure 5B**). In IL-2c-treated mice, imaging analysis revealed

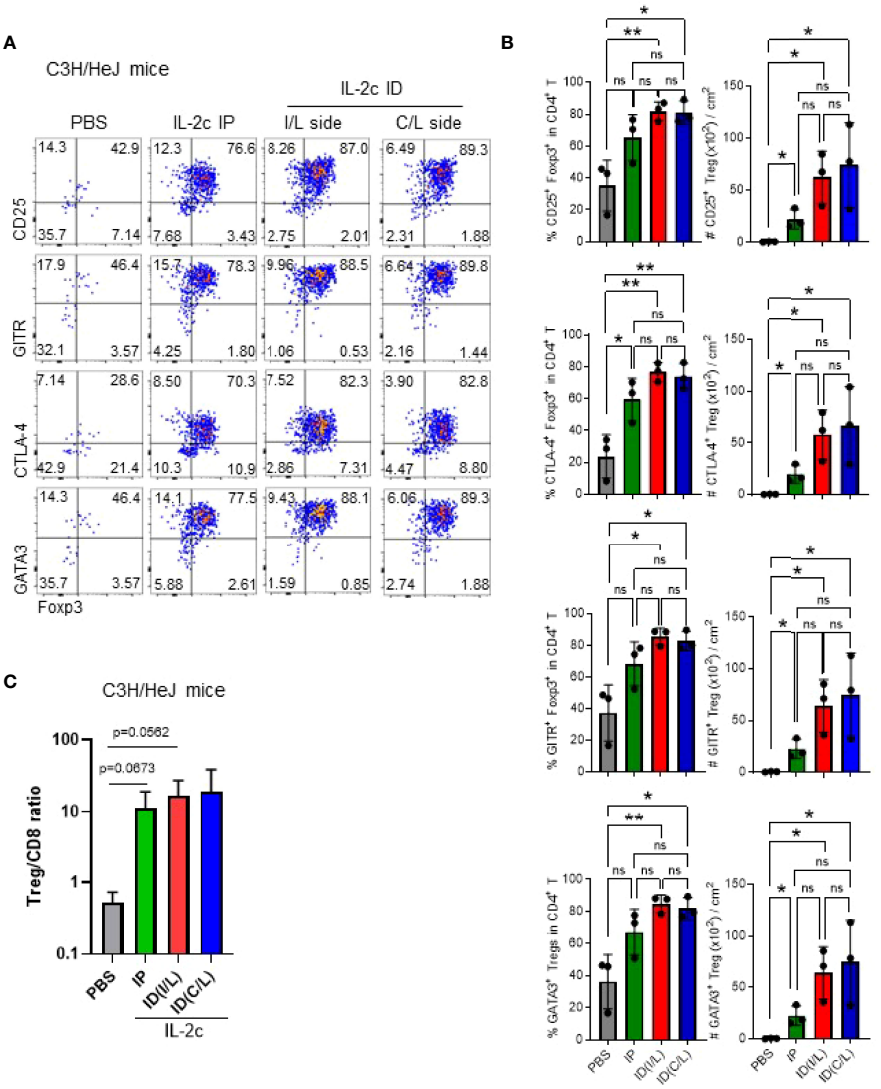


FIGURE 3 | Intradermal injection of IL-2c efficiently expands Tregs in C3H/HeJ mice. Female C3H/HeJ mice were intraperitoneally or intradermally injected with IL-2c on days 0, 1, and 2 and analyzed on day 4. **(A)** Representative dot plots show skin Tregs among total CD4 T cells pre-gated on live CD45.2$^+$CD11b$^-$B22 0$^-$TCRβ$^+$CD8$^-$ cells. **(B)** Graphs show the frequencies and absolute numbers of Tregs expressing indicated markers in each group (n = 3). Horizontal bars indicate mean values, error bars indicate SD, and each dot represents an individual mouse. An unpaired two-tailed t-test was used. *$p < 0.05$, **$p < 0.01$. ns, non-significance ($p > 0.05$).

expanded Tregs around the HFs (**Figure 5C**). Flow cytometric analysis showed a more than tenfold increase of Tregs among total CD4 T cells in the skin (**Figure 5D**). The fold ratio of CD8 T cells over the Tregs was 320 without IL-2c injections, which decreased to 15 with IL-2c treatment. Therefore, IL-2c selectively induced Tregs in the skin. However, CD8 T cells were still present around the HFs (**Figure 5C**). In addition, flow cytometric analysis showed that NKG2D- and TBET-expressing CD8 T cells are persistent in the skin and SDLN of IL-2c-treated mice (**Figures 5D, E**). Overall, these results indicate that Treg expansion in the skin with IL-2c is not sufficient for the reversal of established AA.

DISCUSSION

In this report, we used the C3H/HeJ mouse model of AA and showed that infiltrated T cells in the skin have the phenotype of Trm T cells. Using this mouse, we further tested whether expanded Tregs using IL-2c can promote hair growth by inducing anagen and suppressing autoreactive T cells in the skin. Among various modalities to expand Tregs, ID injections of IL-2c could selectively expand skin Tregs by about 8- to 10-fold (**Figure 2**). In addition, expanded Tregs expressed Jagged1 (**Figure 4A**), required for anagen transition of HF stem cells (5). Based on these findings, we expect that IL-2c administration

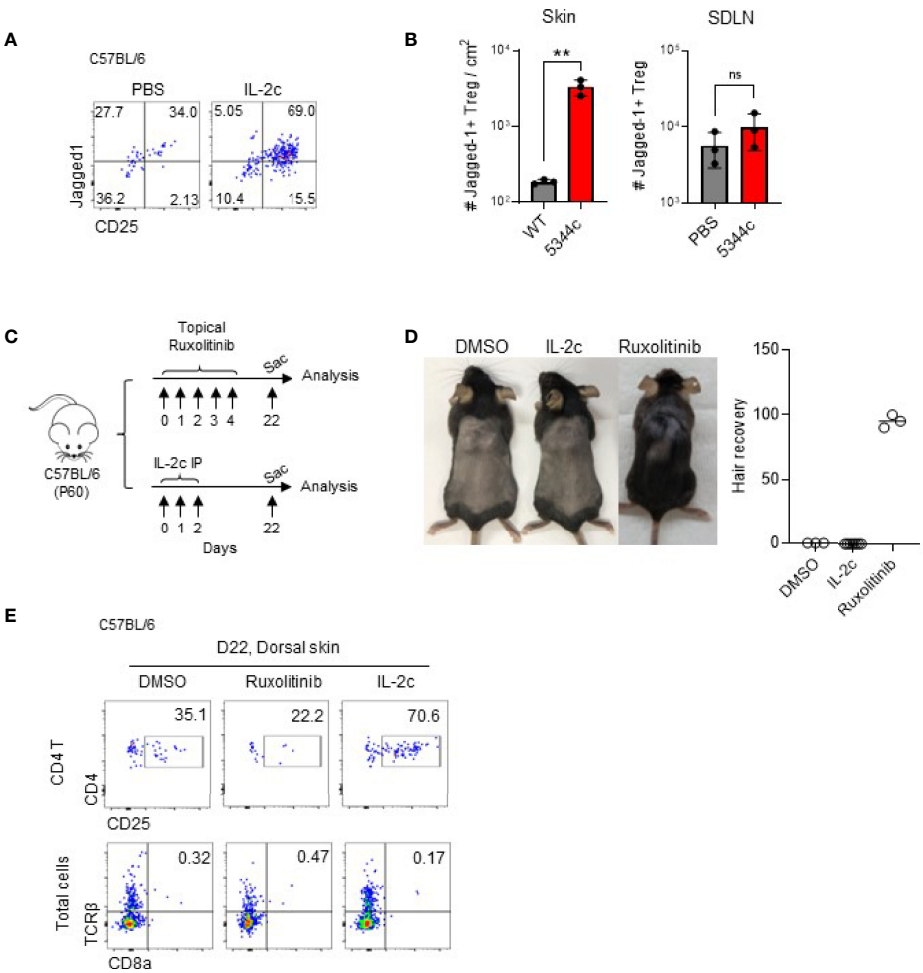


FIGURE 4 | Jagged1$^+$ expanded Tregs do not induce anagen. **(A, B)** Six- to 12-week-old female C57BL/6 mice were intraperitoneally injected with IL-2c on days 0, 1, and 2 and analyzed on day 4. Representative dot plots show Jagged1 expression in Foxp3$^+$ CD4 T cells **(A)**. Graphs show quantifications of Jagged1$^+$ skin Tregs in PBS- and IL-2c-injected mice (n = 3) **(B)**. **(C, D)** The experimental scheme shows that 60-day-old female C57BL/6 mice were shaved and topically treated with DMSO or ruxolitinib, or intraperitoneally injected with IL-2c. Mice were analyzed 22 days after treatment **(C)**. Representative photos show hair growth in each group **(D)**. The graph shows the percentages of hair recovery in shaved skin (n = 3–6). **(E)** Representative dot plots show frequencies of Tregs and CD8 T cells in the indicated group of mice. Results are from two independent experiments. Horizontal bars indicate mean values, error bars indicate SD, and each dot represents an individual mouse. An unpaired two-tailed t-test was used. $^{**}p < 0.01$; ns, non significance ($p > 0.05$).

can reverse AA by re-establishing the immune privilege around the HFs and inducing telogen-to-anagen transition. However, contrary to our expectations, expanded Jagged1$^+$ skin Tregs neither induced anagen (**Figure 4**) nor reversed established AA lesion by suppressing pathogenic CTLs (**Figure 5**).

In human patients and the C3H/HeJ mouse model of AA, the number of Tregs in the lesion and blood is significantly reduced (26, 27). In addition, compared to healthy control, the IL-10 or TGF-β secretion ability of Tregs in AA showed no significant difference, but the ability to suppress the proliferation of pathogenic T cells is impaired (28). Co-transfer of pathogenic CD8 T or CD25$^-$ CD4 T cells with Tregs attenuated the development of AA (29), and depletion of Tregs using anti-FR4 antibody accelerated hair loss in the mouse model of AA (30). Overall, these results indicate that Tregs have a critical role in regulating AA. Based on this, we further tested whether the ID transfer of *ex vivo* expanded Tregs followed by IL-2c administration can boost overall Treg numbers (**Figure S6**). This strategy, however, does not increase the efficiency of IL-2c that effectively expanded endogenous Tregs in the skin.

IL-2 receptor is composed of either IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132) on Tregs or IL-2Rβ and IL-2Rγ on effector T cells. Due to IL-2Rα (CD25) on Tregs, they have a 100-fold higher affinity to IL-2 than effector T cells (31). Because of this, low-dose IL-2 can selectively stimulate Tregs, which are attractive therapeutic modalities in several autoimmune or inflammatory diseases (14, 32). In human patients and mouse models with AA, defective Treg numbers and functions have been proposed, and a small-scale clinical trial suggested that low-dose IL-2 would have therapeutic effects in human patients (33).

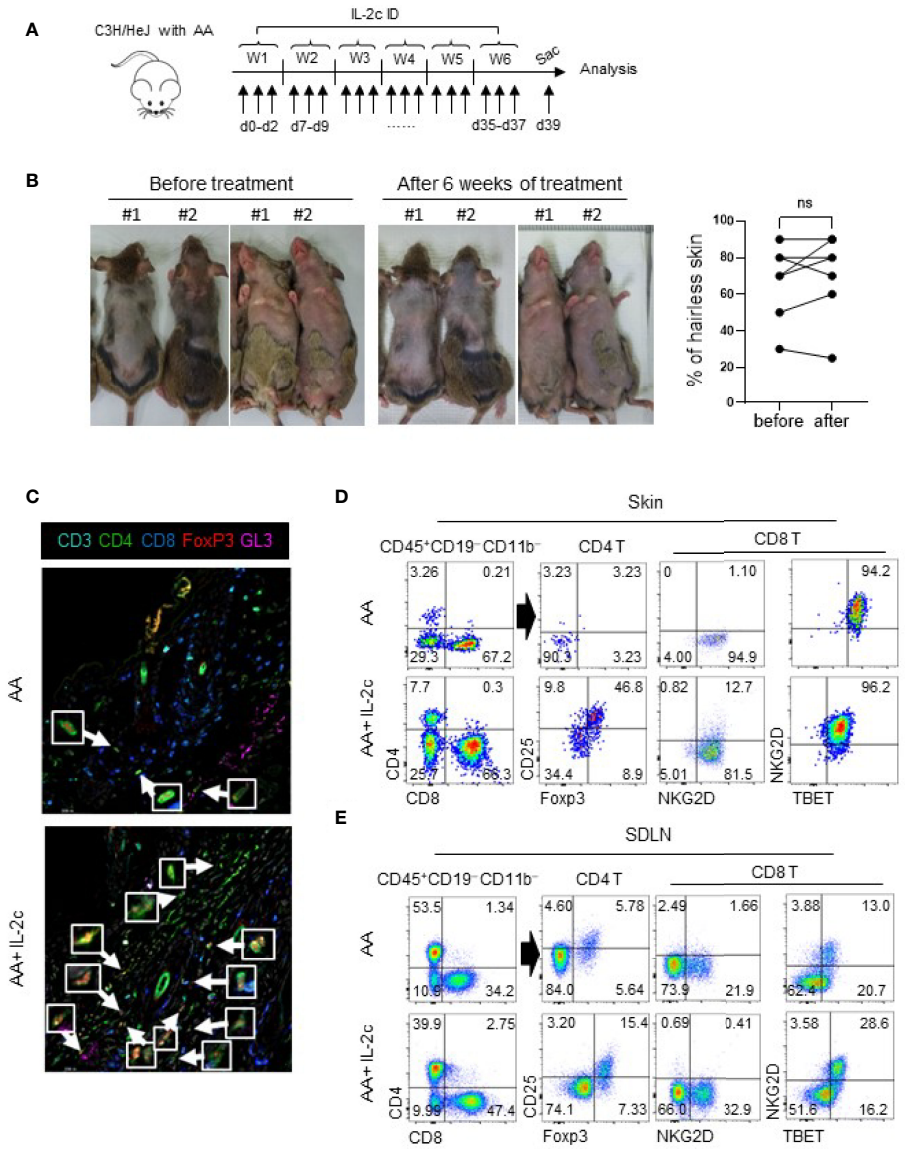


FIGURE 5 | Expanded Tregs by IL-2c do not reverse established AA. **(A)** The experimental scheme shows that C3H/HeJ mice with AA were intradermally injected with IL-2c three times a week for 6 weeks and sacrificed on day 39. **(B)** Representative photos show before and after treatment. The graph shows portions of the hairless area before and after treatment (n = 6). **(C)** Representative images show skin Tregs of AA involved C3H/HeJ mice with or without IL-2c injections. Arrows depict Tregs. **(D, E)** Representative dot plots show Tregs and CD8 T cells in the skin and SDLN of AA mice with or without IL-2c injections. Results are pooled from three independent experiments. ns, non significance ($p > 0.05$).

However, a multicenter prospective placebo-controlled study failed to show the significant therapeutic advantages of low-dose IL-2 in AA (34). This study showed that low-dose IL-2 could expand naïve Tregs for up to 12 months after IL-2 administration. However, Tregs with memory phenotype remained unaffected, suggesting that specific Treg populations could be critical for re-establishing tolerance. One caveat of using low-dose IL-2 treatment is determining its optimal dose for each individual because its slight change can activate STAT5 signaling on conventional memory T cells and NK cells (35). Also, due to the short half-life of IL-2, patients need to be administrated frequently at close intervals (36).

To overcome the issues of using pure IL-2, we used IL-2c, a complex of IL-2 and anti-IL-2 antibodies. IL-2c has dramatically extended the *in vivo* half-life of IL-2 and can preferentially stimulate effector T cells or Tregs dependent on the tertiary structure of the complex (37–41). These properties are applicable for tumor immunotherapy and inflammatory diseases, respectively. For example, selectively expanded Tregs by IL-2c showed promising results in allograft survival, prevention of

arthritis, experimental autoimmune encephalomyelitis (EAE), graft-versus-host disease (GvHD), and T1D (42, 43) (23, 44). The previous report used anti-human IL-2 (F5111) or anti-mouse IL-2 (JES6-1A12) antibodies and showed their therapeutic effect in diabetic NOD mice (44). In this report, we used an anti-human IL-2 antibody (BD Bioscience, clone 5344.111), which has a similar effect of Treg expansion compared to JES6-1A12. However, we found that expanded Tregs in the skin neither induce anagen nor reverse established AA.

Another form of engineered IL-2 is IL-2 mutein (Fc.IL-2), which fused murine IgG2a Fcv with IL-2 mutated on the CD122 binding site (45). It has increased *in vivo* half-life (79.7 h) and requires CD25 for efficient receptor binding, which induces long-term expansion of Tregs, preventing T1D onset in NOD mice. They showed that IL-2 mutein has a better *in vivo* efficacy to expand Tregs than IL-2c in a single injection scheme. However, we found that three consecutive injections are better than a single injection (**Figures S4A, B**), and mutant proteins have the issue of immunological rejection with repetitive usage. Therefore, comparing the *in vivo* efficiencies of IL-2c and IL-2 muteins requires further investigation.

In this study, we used female C3H/HeJ mice whose 30%–90% of the entire skin is affected by AA (**Figure 5**) to mimic the condition of human patients with alopecia totalis (AT) or alopecia universalis (AU) . It is possible that early treatment of IL-2c before the full establishment of AA in C3H/HeJ mice might inhibit disease progression. Combination therapy with depletion of CD8 T cells or other chemicals that can deplete Trm T cells in inflamed tissues (e.g., JAKi) would be worthy of testing for future therapeutic potential. Overall, our study suggests the need for combination treatment other than IL-2c for AA.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of POSTECH (2013–01–0012).

AUTHOR CONTRIBUTIONS

EL designed and performed experiments, analyzed data, and wrote the draft. MK performed immunofluorescence. YL supervised the study, analyzed data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.874778/full#supplementary-material

REFERENCES

1. Mirzoyev SA, Schrum AG, Davis MDP, Torgerson RR. Lifetime Incidence Risk of Alopecia Areata Estimated at 2.1% by Rochester Epidemiology Project, 1990-2009. *J Invest Dermatol* (2014) 134:1141–2. doi: 10.1038/jid.2013.464
2. Gilhar A, Etzioni A, Paus R. Alopecia Areata. *N Engl J Med* (2012) 366:1515–25. doi: 10.1056/NEJMra1103442
3. Rajabi F, Drake LA, Senna MM, Rezaei N. Alopecia Areata: A Review of Disease Pathogenesis. *Br J Dermatol* (2018) 179:1033–48. doi: 10.1111/bjd.16808
4. Gilhar A, Kam Y, Assy B, Kalish RS. Alopecia Areata Induced in C3H/HeJ Mice by Interferon-Gamma: Evidence for Loss of Immune Privilege. *J Invest Dermatol* (2005) 124:288–9. doi: 10.1111/j.0022-202X.2004.23580.x
5. Xing LZ, Dai ZP, Jabbari A, Cerise JE, Higgins CA, Gong WJ, et al. Alopecia Areata is Driven by Cytotoxic T Lymphocytes and is Reversed by JAK Inhibition. *Nat Med* (2014) 20:1043–9. doi: 10.1038/nm.3645
6. Schwartz DM, Kanno Y, Villarino A, Ward M, Gadina M, O'Shea JJ. JAK Inhibition as a Therapeutic Strategy for Immune and Inflammatory Diseases. *Nat Rev Drug Discov* (2017) 16:843–62. doi: 10.1038/nrd.2017.201
7. Harel S, Higgins CA, Cerise JE, Dai Z, Chen JC, Clynes R, et al. Pharmacologic Inhibition of JAK-STAT Signaling Promotes Hair Growth. *Sci Adv* (2015) 1: e1500973. doi: 10.1126/sciadv.1500973
8. Dillon KL. A Comprehensive Literature Review of JAK Inhibitors in Treatment of Alopecia Areata. *Clin Cosmet Investig Dermatol* (2021) 14:691–714. doi: 10.2147/CCID.S309215
9. Gilhar A, Keren A, Paus R. JAK Inhibitors and Alopecia Areata. *Lancet* (2019) 393:318–9. doi: 10.1016/S0140-6736(18)32987-8
10. Wang EHC, Sallee BN, Tejeda CI, Christiano AM. JAK Inhibitors for Treatment of Alopecia Areata. *J Invest Dermatol* (2018) 138:1911–6. doi: 10.1016/j.jid.2018.05.027
11. Raffin C, Vo LT, Bluestone JA. Treg Cell-Based Therapies: Challenges and Perspectives. *Nat Rev Immunol* (2020) 20:158–72. doi: 10.1038/s41577-019-0232-6
12. Ujiie H. Regulatory T Cells in Autoimmune Skin Diseases. *Exp Dermatol* (2019) 28:642–6. doi: 10.1111/exd.13535
13. Dominguez-Villar M, Hafler DA. Regulatory T Cells in Autoimmune Disease. *Nat Immunol* (2018) 19:665–73. doi: 10.1038/s41590-018-0120-4
14. Klatzmann D, Abbas AK. The Promise of Low-Dose Interleukin-2 Therapy for Autoimmune and Inflammatory Diseases. *Nat Rev Immunol* (2015) 15:283–94. doi: 10.1038/nri3823
15. Ali N, Zirak B, Rodriguez RS, Pauli ML, Truong HA, Lai K, et al. Regulatory T Cells in Skin Facilitate Epithelial Stem Cell Differentiation. *Cell* (2017) 169:1119–29.e11. doi: 10.1016/j.cell.2017.05.002
16. Wang EHC, Khosravi-Maharlooei M, Jalili RB, Yu R, Ghahary A, Shapiro J, et al. Transfer of Alopecia Areata to C3H/HeJ Mice Using Cultured Lymph Node-Derived Cells. *J Invest Dermatol* (2015) 135:2530–2. doi: 10.1038/jid.2015.176
17. Divito SJ, Kupper TS. Inhibiting Janus Kinases to Treat Alopecia Areata. *Nat Med* (2014) 20:989–90. doi: 10.1038/nm.3685
18. Sarkar D, Biswas M, Liao G, Seay HR, Perrin GQ, Markusic DM, et al. Ex Vivo Expanded Autologous Polyclonal Regulatory T Cells Suppress Inhibitor Formation in Hemophilia. *Mol Ther Methods Clin Dev* (2014) 1:14030. doi: 10.1038/mtm.2014.30
19. Snyder CM. Front-Line Memory T Cells Think Outside the T-Box. *Mostly Immun* (2015) 43:1030–2. doi: 10.1016/j.immuni.2015.11.020

20. Mackay LK, Wynne-Jones E, Freestone D, Pellicci DG, Mielke LA, Newman DM, et al. T-Box Transcription Factors Combine With the Cytokines TGF-Beta and IL-15 to Control Tissue-Resident Memory T Cell Fate. *Immunity* (2015) 43:1101–11. doi: 10.1016/j.immuni.2015.11.008
21. Laidlaw BJ, Zhang N, Marshall HD, Staron MM, Guan T, Hu Y, et al. CD4+ T Cell Help Guides Formation of CD103+ Lung-Resident Memory CD8+ T Cells During Influenza Viral Infection. *Immunity* (2014) 41:633–45. doi: 10.1016/j.immuni.2014.09.007
22. Gilhar A, Laufer-Britva R, Keren A, Paus R. Frontiers in Alopecia Areata Pathobiology Research. *J Allergy Clin Immunol* (2019) 144:1478–89. doi: 10.1016/j.jaci.2019.08.035
23. Webster KE, Walters S, Kohler RE, Mrkvan T, Boyman O, Surh CD, et al. *In Vivo* Expansion of T Reg Cells With IL-2-mAb Complexes: Induction of Resistance to EAE and Long-Term Acceptance of Islet Allografts Without Immunosuppression. *J Exp Med* (2009) 206:751–60. doi: 10.1084/jem.20082824
24. Whibley N, Tucci A, Powrie F. Regulatory T Cell Adaptation in the Intestine and Skin. *Nat Immunol* (2019) 20:386–96. doi: 10.1038/s41590-019-0351-z
25. Elli EM, Barate C, Mendicino F, Palandri F, Palumbo GA. Mechanisms Underlying the Anti-Inflammatory and Immunosuppressive Activity of Ruxolitinib. *Front Oncol* (2019) 9:1186. doi: 10.3389/fonc.2019.01186
26. Zoller M, McElwee KJ, Engel P, Hoffmann R. Transient CD44 Variant Isoform Expression and Reduction in CD4(+)/CD25(+) Regulatory T Cells in C3H/HeJ Mice With Alopecia Areata. *J Invest Dermatol* (2002) 118:983–92. doi: 10.1046/j.1523-1747.2002.01745.x
27. Hamed FN, Astrand A, Bertolini M, Rossi A, Maleki-Dizaji A, Messenger AG, et al. Alopecia Areata Patients Show Deficiency of FOXP3+CD39+ T Regulatory Cells and Clonotypic Restriction of Treg TCRbeta-Chain, Which Highlights the Immunopathological Aspect of the Disease. *PLos One* (2019) 14:e0210308. doi: 10.1371/journal.pone.0210308
28. Shin BS, Furuhashi T, Nakamura M, Torii K, Morita A. Impaired Inhibitory Function of Circulating CD4+CD25+ Regulatory T Cells in Alopecia Areata. *J Dermatol Sci* (2013) 70:141–3. doi: 10.1016/j.jdermsci.2013.01.006
29. McElwee KJ, Freyschmidt-Paul P, Hoffmann R, Kissling S, Hummel S, Vitacolonna M, et al. Transfer of CD8(+) Cells Induces Localized Hair Loss Whereas CD4(+)/CD25(-) Cells Promote Systemic Alopecia Areata and CD4(+)/CD25(+) Cells Blockade Disease Onset in the C3H/HeJ Mouse Model. *J Invest Dermatol* (2005) 124:947–57. doi: 10.1111/j.0022-202X.2005.23692.x
30. Dai Z, Wang EHC, Petukhova L, Chang Y, Lee EY, Christiano AM. Blockade of IL-7 Signaling Suppresses Inflammatory Responses and Reverses Alopecia Areata in C3H/HeJ Mice. *Sci Adv* (2021) 7:eabd1866. doi: 10.1126/sciadv.abd1866
31. Rickert M, Wang X, Boulanger MJ, Goriatcheva N, Garcia KC. The Structure of Interleukin-2 Complexed With Its Alpha Receptor. *Science* (2005) 308:1477–80. doi: 10.1126/science.1109745
32. Grasshoff H, Comduhr S, Monne LR, Muller A, Lamprecht P, Riemekasten G, et al. Low-Dose IL-2 Therapy in Autoimmune and Rheumatic Diseases. *Front Immunol* (2021) 12:648408. doi: 10.3389/fimmu.2021.648408
33. Castela E, Le Duff F, Butori C, Ticchioni M, Hofman P, Bahadoran P, et al. Effects of Low-Dose Recombinant Interleukin 2 to Promote T-Regulatory Cells in Alopecia Areata. *JAMA Dermatol* (2014) 150:748–51. doi: 10.1001/jamadermatol.2014.504
34. Le Duff F, Bouaziz JD, Fontas E, Ticchioni M, Viguier M, Dereure O, et al. Low-Dose IL-2 for Treating Moderate to Severe Alopecia Areata: A 52-Week Multicenter Prospective Placebo-Controlled Study Assessing Its Impact on T Regulatory Cell and NK Cell Populations. *J Invest Dermatol* (2021) 141:933–6.e6. doi: 10.1016/j.jid.2020.08.015
35. Yu A, Snowhite I, Vendrame F, Rosenzwajg M, Klatzmann D, Pugliese A, et al. Selective IL-2 Responsiveness of Regulatory T Cells Through Multiple Intrinsic Mechanisms Supports the Use of Low-Dose IL-2 Therapy in Type 1 Diabetes. *Diabetes* (2015) 64:2172–83. doi: 10.2337/db14-1322
36. Donohue JH, Rosenberg SA. The Fate of Interleukin-2 After *In Vivo* Administration. *J Immunol* (1983) 130:2203–8. Available at: https://www.jimmunol.org/content/130/5/2203
37. Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. Selective Stimulation of T Cell Subsets With Antibody-Cytokine Immune Complexes. *Science* (2006) 311:1924–7. doi: 10.1126/science.1122927
38. Spangler JB, Tomala J, Luca VC, Jude KM, Dong S, Ring AM, et al. Antibodies to Interleukin-2 Elicit Selective T Cell Subset Potentiation Through Distinct Conformational Mechanisms. *Immunity* (2015) 42:815–25. doi: 10.1016/j.immuni.2015.04.015
39. Boyman O, Sprent J. The Role of Interleukin-2 During Homeostasis and Activation of the Immune System. *Nat Rev Immunol* (2012) 12:180–90. doi: 10.1038/nri3156
40. Kim J, Lee JY, Park SY, Lee YJ, Kim MS. Crystal Structure of Human Interleukin-2 in Complex With TCB2, A New Antibody-Drug Candidate With Antitumor Activity. *Oncoimmunology* (2021) 10:1899671. doi: 10.1080/2162402X.2021.1899671
41. Lee JY, Lee E, Hong SW, Kim D, Eunju O, Sprent J, et al. TCB2, a New Anti-Human Interleukin-2 Antibody, Facilitates Heterodimeric IL-2 Receptor Signaling and Improves Anti-Tumor Immunity. *Oncoimmunology* (2020) 9:1681869. doi: 10.1080/2162402X.2019.1681869
42. Boyman O, Krieg C, Letourneau S, Webster K, Surh CD, Sprent J. Selectively Expanding Subsets of T Cells in Mice by Injection of Interleukin-2/Antibody Complexes: Implications for Transplantation Tolerance. *Transplant Proc* (2012) 44:1032–4. doi: 10.1016/j.transproceed.2012.01.093
43. Lee SY, Cho ML, Oh HJ, Ryu JG, Park MJ, Jhun JY, et al. Interleukin-2/Anti-Interleukin-2 Monoclonal Antibody Immune Complex Suppresses Collagen-Induced Arthritis in Mice by Fortifying Interleukin-2/STAT5 Signalling Pathways. *Immunology* (2012) 137:305–16. doi: 10.1111/imm.12008
44. Trotta E, Bessette PH, Silveria SL, Ely LK, Jude KM, Le DT, et al. A Human Anti-IL-2 Antibody That Potentiates Regulatory T Cells by a Structure-Based Mechanism. *Nat Med* (2018) 24:1005–14. doi: 10.1038/s41591-018-0070-2
45. Khoryati L, Pham MN, Sherve M, Kumari S, Cook K, Pearson J, et al. An IL-2 Mutein Engineered to Promote Expansion of Regulatory T Cells Arrests Ongoing Autoimmunity in Mice. *Sci Immunol* (2020) 5:eaba5264. doi: 10.1126/sciimmunol.aba5264

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