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Autoimmune pre-disease

Edited by
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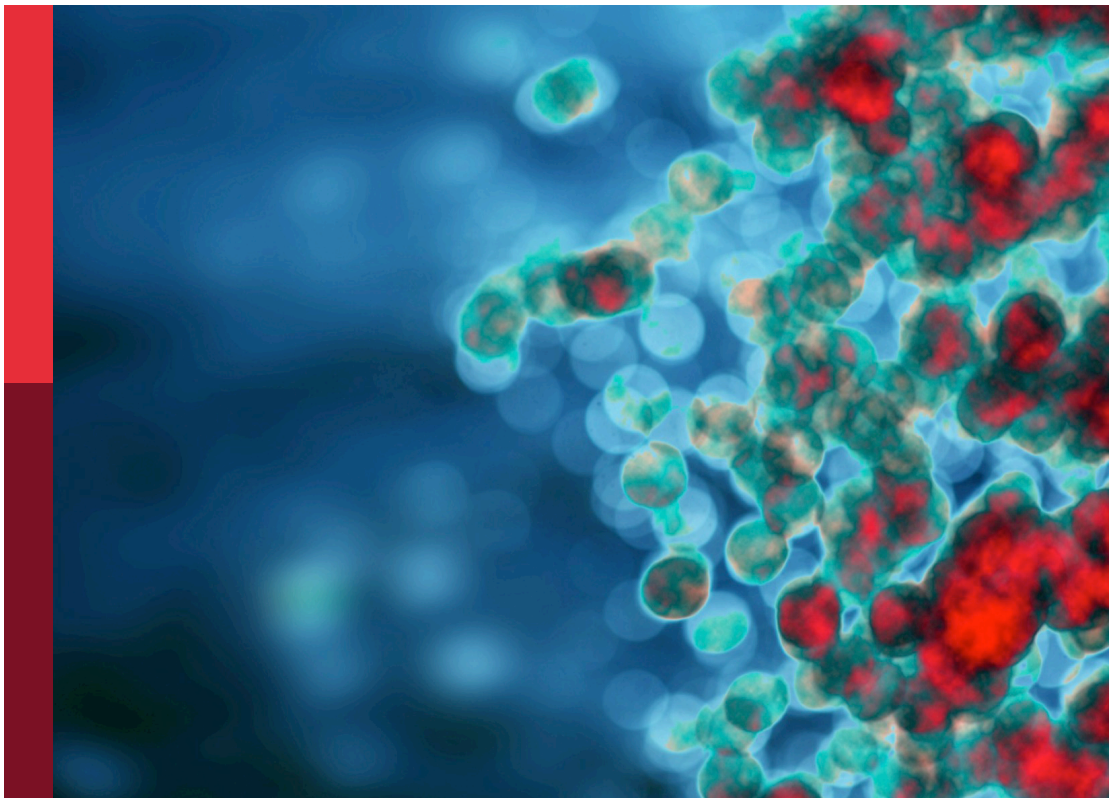

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Autoimmune pre-disease

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Editorial: Autoimmune pre-disease

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Editorial on the Research Topic
Autoimmune pre-disease

1 Introduction

According to the revised Witebsky postulates, diseases are of an autoimmune nature if, (i) the clinical phenotype can be reproduced through the transfer of autoantibodies and/or lymphocytes; (ii) the disease can be reproduced in experimental animal models; (iii) autoreactive T cells or autoantibodies are identified; and/or (iv) distinctive clinical observations, such as an HLA association, are found (1). These postulates mostly still hold true despite they were made 3 decades ago. However, in psoriasis, lichen sclerosus and lichen planus, which are considered chronic, non-communicable inflammatory diseases, autoreactive T cells and/or autoantibodies that potentially contribute to disease pathogenesis are also detected (2–5). Thus, rather than a clear distinction between autoimmune and chronic non-communicable inflammatory diseases, categorization of a given distinct disease may be better placed in the continuum between the two. Hence, within this research topic, we cover both "classic" autoimmune diseases, such as systemic lupus erythematosus (SLE), as well as non-communicable, inflammatory diseases with detectable autoimmunity, e.g., psoriasis.

Autoimmune diseases develop over a long time, which at least spans over several years. This is maybe best demonstrated by the presence of disease-specific autoantibodies years prior to diagnosis of the corresponding autoimmune disease (6–10). The presence of disease-specific autoantibodies, does not, however, reliably predict if clinical manifestation of the corresponding autoimmune disease will occur in the future. Therefore, definite biomarkers that define the transition (i) from health to autoimmunity, (ii) from autoimmunity to autoimmune disease, and (iii) to chronicity or resolution of inflammation (Figure 1) would allow to implement measures to slow or prevent disease progression. This would have a significant impact on affected individuals, as well as the healthcare system because the incidence of autoimmune and non-communicable inflammatory diseases is rising and there are still many unmet medical needs in the care of patients with these diagnoses (12, 13).

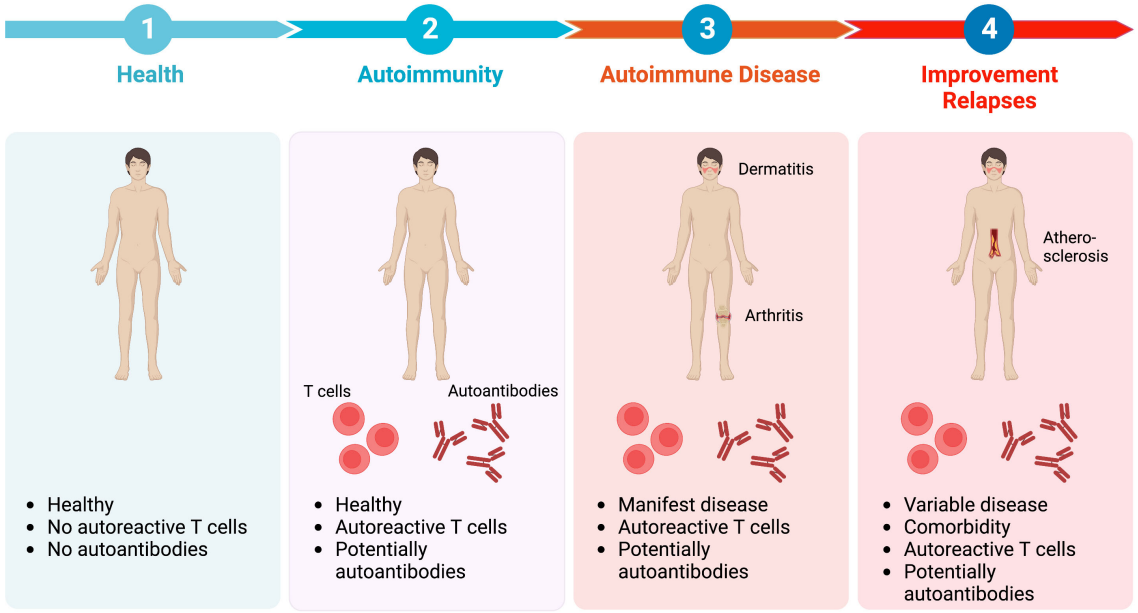

FIGURE 1
Transitional stages in the pathogenesis of autoimmune diseases. Regarding the transitional stages in the pathogenesis of autoimmune diseases, health (1) is defined by no inflammatory symptoms and absence of autoreactive T cells and autoantibodies. However, in approximately 10% of healthy individuals autoantibodies can be detected. At this stage of autoimmunity (2) no clinical symptoms are apparent. Progression towards overt autoimmune disease (3) occurs in some, but not all individuals with autoimmunity. Autoimmune disease is characterised by clinical symptoms, such as dermatitis or arthritis, as well as the detection of autoreactive T cells and/or autoantibodies. Current treatment options improve disease. However, adverse drug events as well as comorbidity are encountered frequently (4). Besides, clinical relapses of disease (flares) often occur, especially when treatment is tapered or withdrawn, because the underlying inflammatory sensitisation of tissue is sustained even during clinically silent phases (11). Image created with Biorender (www.biorender.com).

In total, 25 articles covering the topic of autoimmune pre-disease were submitted to the research topic "*Autoimmune Pre-Disease*". Ten of these articles were written by PhD- and MD-candidates from the DFG-funded Research Training Group "Autoimmune Pre Disease" (GRK 2633) that is dedicated to defining and modulating above-described transitional steps from heath to clinically manifest autoimmune disease. Some of these authors introduce themselves and their project with short clips on YouTube. For a structured description of all articles, we allocated each article to one of the following chapters: (i) From health to autoimmunity, (ii) From autoimmunity to autoimmune disease, (iii) Resolution of autoimmune disease, and (iv) New concepts in autoimmune disease.

2 From health to autoimmunity

In SLE, rheumatoid arthritis and type 1 diabetes (T1DM) the presence of disease-specific autoantibodies prior to clinical disease manifestation is well-established (6–10). This is less well characterized for autoantibodies targeting BP180 or BP230, which characterize and cause bullous pemphigoid (14). So far, large-scale studies demonstrated that these autoantibodies are also present in 0.9% to 2.2% of healthy volunteers (15, 16). Herein, Mai et al. review the significance of preclinical anti-BP180 autoantibodies. They conclude that in certain non-BP patient populations, the prevalence of BP180 autoantibodies is increased when compared to the appropriate controls. Some of these patient populations, e.g., neurological diseases or type 2 diabetes (T2DM), are associated with a higher risk to develop bullous pemphigoid. The increased prevalence of BP180 autoantibodies in T2DM may be confounded by the use of gliptins, oral antidiabetics that significantly increase the risk for subsequent manifestation of bullous pemphigoid (17, 18). This concept is challenged by the original data presented by Nätynki et al. demonstrating that the use of gliptins reduces levels of SDF-1/CXCL12 in bullous pemphigoid and T2DM, but does not increase autoantibodies against BP180 in diabetic patients. These findings are in line with a report from Japan (19). Hence, future studies are needed to clarify the impact of T2DM and gliptins on BP180 and BP230 autoantibodies. Schanzenbacher et al. outline that the complement system may contribute to the formation of autoantibodies. Complement can modulate autoimmune diseases at their initiation, as well as in the induction of tissue inflammation in the effector phase, as shown by Papara et al. Furthermore, shift work, circadian rhythm misalignment, and/or poor sleep may also lead to the formation of autoimmunity and autoimmune disease. These later aspects are covered in-depth in the review by Stenger et al..

Data from SLE demonstrated that cell death and dysregulated clearance of dead cells are key events in the induction of autoimmune diseases (20–22). In this Research Topic, Brieske et al. detail the impact of immunogenic cell death as driver of autoimmunity in granulomatosis with polyangiitis (GPA). GPA is an orphan anti-neutrophil cytoplasmic autoantibody (ANCA)-

associated vasculitis that, in addition to small-vessel vasculitis, is characterized by granulomatous inflammation (23). The dysregulated cell death in GPA leads to the release of damage-associated molecular patterns (DAMP) such as high mobility group box 1 (HMGB1). These, along with cytokines, contribute to the loss of tolerance towards the proteinase 3 autoantigen in GPA, that ultimately lead to autoantibody formation.

In an elaborate study Anaparti et al. documented alterations in the CD4 T cell compartment of autoantibody-positive, first-degree relative of patients with rheumatoid arthritis (RA). For this, they performed multicolor flow cytometry for immunophenotyping of CD4 cells from autoantibody-negative and autoantibody positive first-degree relatives of RA patients, as well as from RA patients themselves. Herein, they identified a higher frequency of a TIGIT+ CD4 T cell subset in autoantibody-positive, compared to autoantibody-negative first-degree relatives. This underscores the importance of immunophenotyping in patients and risk populations to unravel molecular pathways in autoimmune pre-diseases (24). Zotti et al. followed a similar approach. Contrasting flow cytometric characterization of the lymphocyte compartment they found significantly reduced frequencies of recent thymic emigrants and naïve T cells, and significantly increased frequencies of central memory T cells, TH2 and TH17 cells in antinuclear antibody (ANA)+ as opposed to ANA- healthy individuals. Furthermore, CD4+ T cells in ANA+ individuals were metabolically more active than those obtained from ANA- individuals.

3 From autoimmunity to autoimmune disease

The article by Sprow et al. has attracted the most reads within our Research Topic. Here the researchers investigated the risk of exacerbations of dermatomyositis and SLE patients following COVID-19 vaccination. The risk for disease exacerbation following COVID-19 vaccination was 22% for dermatomyositis and 8.6% for SLE patients. Independently of these findings, the authors conclude that, given the risks of COVID-19 infection, vaccination should nevertheless be performed in most patients with autoimmune skin diseases. This latter argument is of particular importance because patients with bullous pemphigoid experience an increased risk for COVID-19-associated mortality (25). Similar findings and conclusions were made in Australian and Italian patient cohorts (26, 27). Like infections, drugs are also known to trigger autoimmune diseases; recent examples are checkpoint- or dipeptidyl peptidase 4- inhibitors (17). Anti-TNF treatment-induced psoriasis, termed paradoxical psoriasis, is among the more peculiar drug-induced autoimmune diseases, because the same class of drugs is used to treat psoriasis (28). Herein, Xie et al. review the incidence and risk factors for paradoxical psoriasis in individuals with inflammatory bowel disease (IBD) treated with anti-TNF. In a cohort of 24,547 IBD patients, paradoxical psoriasis was observed in 4.6% of cases. Risk factors for paradoxical psoriasis manifestation were female sex, younger age at anti-TNF treatment initiation, smoking, ileocolonic Crohn's disease and use of adalimumab or certolizumab as compared to infliximab. IL-10 is another cytokine with (mostly) anti-inflammatory activity in autoimmune diseases (29). However, its' role in autoimmunity may be more complex as highlighted in the article by Biswas et al.. The authors point out that in SLE, IL-10 seems to be also a main driver of the extrafollicular pathogenic autoantibody response. Hence, its' pharmacological targeting needs to be well timed to achieve therapeutic effects.

Four articles within the research topic focused on an in-depth characterization of the transition from autoimmunity to autoimmune disease. Buhre et al. described the role of IgG subclass and Fc glycosylation shifts in the transition from pre- to inflammatory autoimmune diseases. They introduce the concept of a two-step model for the development of inflammatory autoimmune diseases, which isinitiated by a state of low- or non-inflammatory T/B cell responses that may shift towards more inflammatory T/B cell response. The shift to the inflammatory response is characterized by the IgG subclass distributions and IgG Fc glycosylation patterns. Hence, these might be used as biomarkers to detect this stepwise development towards autoimmune disease. Also in search of molecular markers for the development of clinical autoimmune disease important efforts were made by O'Neil et al., Niebuhr et al. and Wang et al.. In their study O`Neil et al. performed serum proteomics from first-degree relatives of patients with RA. Their analysis was stratified by the presence of absence of anti-citrullinated protein autoantibodies (ACPA). Overall, they identified 6 proteomic clusters. One of the clusters showed an enrichment of ACPA positive samples. Follow-up will determine if this dataset can be used to predict future disease onset. Wang et al. focused on Vogt-Koyanagi-Harada disease, an autoimmune inflammatory disease characterized by bilateral granulomatous uveitis, using publicly available expression datasets to identify hub genes that are potentially involved in pathogenesis. In total, six immune-related hub genes were identified. Dr. Niebuhr et al. contrasted T cell repertoires of CD45RO CD4 T cells obtained from bullous pemphigoid patients to those of age- and sex-matched controls. Interestingly, the diversity of TCR repertoires from peripheral CD4 T cells does not reflect the manifestation of bullous pemphigoid and is thus not suited to serve as a diagnostic marker.

4 Resolution of autoimmune disease

Once an autoimmune disease has manifested, current standard treatment options mostly aim to improve symptoms by immunosuppression, and (if needed) by hormone replacement; e.g., insulin. Current innovative treatments have significantly improved the outcomes. However, since causative treatment is not possible, these treatments must be long-term. Their adverse events and the underlying chronic inflammation may cause a significant comorbidity, especially metabolic and cardiovascular diseases. Early detection,individualization, and (in the future) potentially curative treatments will meet these medical needs in autoimmune diseases (12). Three articles of this Research Topic cover these aspects. In the paper by Song et al. the authors

demonstrate that a change in reimbursement practices led to a much shorter time to remission in pemphigus patients because of early initiation of rituximab treatment. This highlights that not only break-through discoveries in translational research but also political implementation of these findings into the health care system are essential for optimal patient care. By contrast to rituximab that is effective not only in pemphigus (30, 31), but also in several autoimmune diseases (32), Zeng et al., in their meta-analysis show that there is too little evidence to support the use of curcumin and curcuma longa extract in the treatment of RA. In contrast. as reviewed by Su et al. the hedgehog signaling pathway has recently emerged as a potential therapeutic target in rheumatic diseases.

5 New concepts in autoimmune disease

As mentioned above, the initial definition of autoimmune diseases still mostly holds true (1), but a clear separation between autoimmunity and chronic inflammation is challenged by new methodology and new insights. Using advanced flow cytometry Polakova et al. describe the detection of rare autoreactive T cell subsets in patients with pemphigus vulgaris. Detection of (auto) antigen-specific CD4+ T cells specific for defined antigens was performed according their CD154 expression following *in vitro* stimulation (33).

In addition to these methodological innovations, thorough clinical investigations can lead to new concepts. This is exemplified by the review on anti-desmoglein autoantibodies in oral lichen planus by Didona and Hertl. Oral lichen planus is considered a chronic inflammatory disease (2). Meanwhile, autoantibodies targeting desmoglein 1 and/or 3 are hallmarks of pemphigus (34). In their review, Didona and Hertl summarize the evidence regarding the presence and potential clinical relevance of desmoglein autoantibodies in oral lichen planus. The review by Opelka et al. also focuses on autoantibodies. Here, they raise the point that autoantibodies targeting different epitopes of the same autoantigen, specifically type XVII collagen (COL17), lead to distinct clinical phenotypes: bullous pemphigoid or mucous membrane pemphigoid.

Along this line, careful clinical investigations by Baker et al. identified three patients with cutaneous pemphigus vulgaris without history of mucosal lesions. These observations challenge the concept that mucosal disease manifestations are obligatory for a diagnosis of pemphigus vulgaris (34). In the same line Sielski et al. challenge the desmoglein compensation hypothesis that correlates clinical presentation of pemphigus with the profile of autoantibodies directed against desmoglein 1 and/or 3 (35). Of note, the authors find that approximately half of active pemphigus vulgaris and pemphigus foliaceus patients investigated presented with a combination of lesion morphology and anti-Dsg3/1 levels that align with the desmoglein compensation hypothesis, whilst in the other half of the patients this correlation was not observed. However, before this dogma can be definitively refuted, prospective investigations from different sites are required.

In the article by Saurabh et al. genome-wide association studies, polygenic scores and the UK Biobank were screened in order to develop polygenic scores for autoimmune diseases. They, however, find that only comparably highly prevalent autoimmune diseases are covered by the UK Biobank, and at the same time assessed by both genome-wide association studies- and polygenic scores catalogs.

Author contributions

All authors wrote, revised, and approve the submission of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SYSTEMATIC REVIEW
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Incidence of and Risk Factors for Paradoxical Psoriasis or Psoriasiform Lesions in Inflammatory Bowel Disease Patients Receiving Anti-TNF Therapy: Systematic Review With Meta-Analysis

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Background: Paradoxical psoriasis or psoriasiform lesions induced by anti-tumor necrosis factor (anti-TNF) therapies receive increasing attention worldwide. However, no comprehensive meta-analysis investigating the incidence estimates and risk factors for anti-TNF-induced psoriasis is currently available. We aimed to precisely quantify its incidence as well as risk factors in patients with inflammatory bowel disease (IBD).

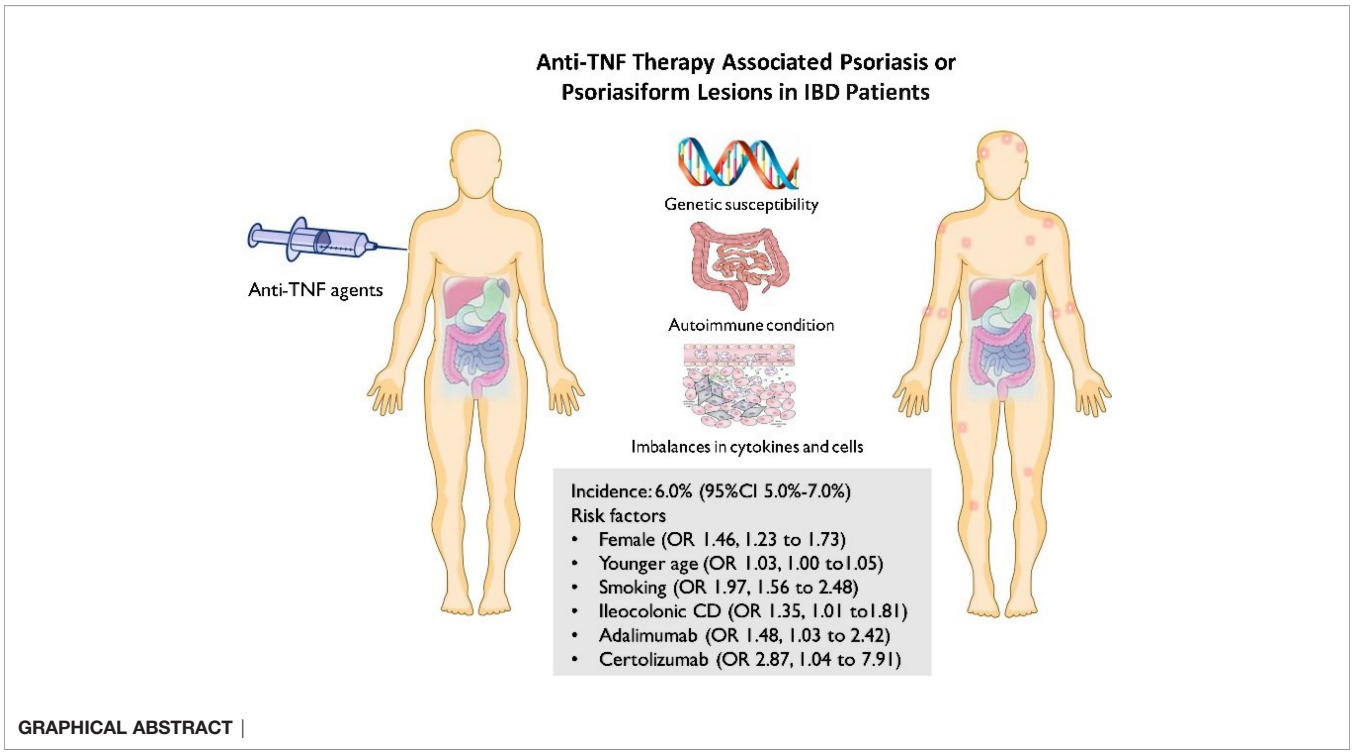
Methods: This study was registered on PROSPERO database under review registration number CRD42021233695. The electronic databases PubMed, EMBASE, and the Cochrane library were comprehensively searched for observational studies published as full-length papers in English and reporting the incidence and/or predictors for psoriasis or psoriasiform lesions in IBD patients. A random-effects meta-analysis was performed to calculate the pooled incidence. Pooled odds ratio (OR) and 95% confidence interval for potential predictors were combined using a fixed-effects or random-effects model.

Results: In total, 30 articles comprising 24,547 IBD patients treated by anti-TNF were finally included. The overall pooled incidence of psoriasis and/or psoriasiform lesions following anti-TNF therapy was 6.0% (5.0–7.0%; I^2 = 93.9%), with 6.9% (5.1–8.7%; I^2 = 92.4%) for psoriasiform lesions and 4.6% (3.6–5.6%; I^2 = 93.9%) for psoriasis. Multivariable meta-regression analysis indicated regions and populations that significantly contributed to the heterogeneity. A statistically higher risk for psoriasis or psoriasiform lesions during anti-TNF therapy was observed in female patients (OR 1.46, 1.23–1.73), those who are at a younger age at anti-TNF initiation (OR 1.03, 1.00–1.05), smokers (OR 1.97, 1.56–2.48), ileocolonic Crohn's disease patients (OR 1.48, 1.03–2.13), and those who are using adalimumab or certolizumab (*vs*. infliximab) (OR: 1.48 and 2.87 respectively).

Conclusions: The incidence of psoriasis or psoriasiform lesions was not uncommon in IBD patients following anti-TNF therapy. Female, younger age, smoker, ileocolonic Crohn's disease, and the types of anti-TNF were significantly associated with such risk. These findings may help gastroenterologists to make more individualized decisions and understand the mechanisms of this paradoxical phenomenon.

Systematic Review Registration: https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=233695, identifier CRD42021233695.

Keywords: inflammatory bowel disease, anti-tumor necrosis factor, psoriasis, psoriasiform lesions, risk factors, meta-analysis, incidence



GRAPHICAL ABSTRACT |

INTRODUCTION

Inflammatory bowel diseases (IBDs) are chronic inflammatory disorders of the gastrointestinal tract that affect approximately 10 million patients worldwide (1, 2). The introduction of biologics has dramatically transformed the therapeutic landscape of IBD. Among these, anti-tumor necrosis factor (anti-TNF), such as infliximab and adalimumab, are most extensively used in daily practice for a couple of decades (3). Accumulative evidence has demonstrated that anti-TNF can control the disease activity rapidly, exert a steroid-sparing effect, promote mucosal healing, improve the quality of life, and reduce the risk of surgery as well (3, 4).

With increasing use of these agents, however, some paradoxical inflammations, involving the skin, joints and lungs, have been described and received increasing attention in recent years (5). Of these, paradoxical psoriasis or psoriasiform lesion induced by anti-TNF therapies is one of the most extended concerned topics worldwide. Generally, anti-TNF treatments are commonly used for psoriasis therapy, but psoriasis and psoriasiform skin lesions are sometimes observed in IBD patients receiving anti-TNF therapies. Overall, IBD patients treated with anti-TNF therapy have a 2.4-fold increased risk of paradoxical psoriasis compared with nonusers of anti-TNF (6). Meanwhile, there is high inconsistency in the results of previous studies on the incidence of psoriasis or psoriasiform lesions in IBD patients with exposure to anti-TNF, varying from 1% (6) to more than 30% (7). The relatively small sample sizes and limited number of events lead to significant variation and imprecise incidence estimates and further preclude robust conclusions to be drawn from any of the individual studies. On the other hand, currently, the knowledge about the risk factor for psoriasis or psoriasiform lesions secondary to anti-TNF therapy in IBD patients is limited and contradictory—for example, some

studies have shown gender, smoking, and concomitant immunosuppressive agents to be associated with an increased risk of developing anti-TNF-induced psoriasis, while others have not (8–16).

To date, no comprehensive meta-analysis investigating the incidence estimates and risk factors for anti-TNF-induced psoriasis is currently available. To fill the gap, the present study is intended to precisely quantify the incidence of and risk factors for developing anti-TNF-induced psoriasis or psoriasiform lesions in IBD patients.

MATERIALS AND METHODS

This article was carried out and reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analysis (17). The methods were stipulated in a protocol that was registered with PROSPERO (CRD42021233695).

Literature Search and Inclusion Criteria

A literature search of English language publications was performed using the electronic databases PubMed, EMBASE, and the Cochrane Library from database inception to February 9, 2021. The search strategy was designed and conducted by an experienced medical librarian with input from the study investigators. The studies were identified by combining three search themes: the first theme, inflammatory bowel disease; the second theme, anti-TNF; and the third theme, a combination of the following terms: psoriasis, psoriasiform, dermatological, skin, and cutaneous. The detailed search strategies are available in **Supplementary Appendix S1**.

Studies were included if they were on IBD patients of all ages (including children) receiving anti-TNF treatment, were cohort studies or case–control studies, reported the incidence of and/or risk factors for psoriasis or psoriasiform lesions in IBD patients, and were full-text English articles. When duplicate publications were identified, only the article with the newest and most comprehensive information was included. We excluded studies with insufficient data of interest (such as those only presenting all dermatological events), meeting abstract, case report, editorial, review, or nonhuman investigations. Two investigators (WX and SX) independently evaluated the eligibility, and any discrepancy throughout was resolved by a third investigator (ZZ).

Data Extraction and Outcome Assessment

Data extraction of the eligible studies was conducted by two independent review authors (WX and SX) using piloted data extraction sheets: first author, publication year, country/countries, study design, data sources, setting, study period, the diagnosis of IBD, psoriasis, sample size, time period of observations, patients' demographics and clinical characteristics, number of patients developing psoriasis or psoriasiform lesions, risk factor of interests, and risk estimates. The methodological quality of each study was rated by the Newcastle–Ottawa Scale (NOS) which consists of three factors: patient selection (0–4 points), comparability of the study groups (0–2 points), and assessment of outcome (0–3 points) (18). All relevant studies were scored from 0 to 9 on the NOS to determine the study quality.

Data Synthesis and Analysis

All calculations and graphs were performed using Stata Statistical Software version 13.0. The incidence of psoriasis or psoriasiform lesions in IBD patients treated with anti-TNF therapy was pooled. The levels of heterogeneity were assessed by the I^2 statistic (I^2 >50% was considered as a statistically significant heterogeneity). If severe heterogeneity was present at I^2 >50%, the random-effects model (DerSimonian and Laird method) was chosen; otherwise, the fixed-effects model was adopted (Mantel–Haenszel method). The sources of heterogeneity were explored by using subgroup and meta-regression analyses. On the other hand, to explore the risk factors for developing psoriasis or psoriasiform lesions, the patients' demographics and clinical characteristics (sex, age, smoking, disease phenotype, type of anti-TNF therapy, *etc.*) were compared between IBD patients with and without psoriasis/psoriasiform lesions during anti-TNF therapy, if possible. Pooled odds ratios (ORs) with 95% CI were calculated as an effect measure. We extracted the risk estimates that were adjusted for most variables. When no raw data were available, relative risks and hazard ratios were taken as good estimates of OR, in line with previous reports (19–21). Sensitivity analyses were performed to assess the robustness of estimates. Graphical symmetry with funnel plot, as well as with Begg's and Egger's statistical tests, was produced to help detect publication bias. Trim-and-filled method was performed in the case of potential publication bias. A two-sided P-value <0.05 was considered statistically significant.

RESULTS

Study Selection and Characteristics

The study selection process is shown in **Supplementary Figure S1**. Initially, we retrieved 11,467 citations, of which 107 full-text articles were eligible for inclusion (6–16, 22–40). The characteristics of the included studies are presented in **Table 1**. In total, 30 citations were published between 2009 and 2020. All papers were based on retrospective or prospective cohorts, and majority of them originated from Europe and the USA. Psoriasis or psoriasiform lesions diagnosis was mostly judged by the treating gastroenterologist and/or dermatologist (biopsy if necessary) (8, 10, 13–16, 22, 26, 29–31, 33–37). The median NOS score in the included studies was 6, ranging from 5 to 8 (**Supplementary Table S1**).

The Incidence of Psoriasis or Psoriasiform Lesions

The incidence of psoriasis or psoriasiform lesions associated with anti-TNF therapy in IBD patients was reported by 29 articles, varying from 1.1 to 29.8%. In total, 913 cases were documented from 24,547 patients with IBD exposed to anti-TNF agents, corresponding to a crude incidence of 3.7%. The overall pooled incidence of psoriasis and/or psoriasiform lesions following anti-

TABLE 1 | Characteristics of the studies included in the meta-analysis.

Author, Reference	Year	Country	Data source	Study design	Enrollment period	Population	Number of IBD	Outcome	Diagnosis method	Number of events
Fidder et al. (22)	2009	Belgium	Monocenter	Retrospective	1994–2008	Adult	743	Psoriasiform lesions	Patient or treating physician	39
Rahier et al. (23)	2010	France	Multicenter	Retrospective	2004–2009	Adult	562	Psoriasiform lesions	NA	62
Baumgart et al. (24)	2011	Germany	Monocenter	Prospective	NA	Mixed	50	Psoriasiform lesions	NA	6
Hiremath et al. (25)	2011	USA	Monocenter	Retrospective	NA	Children	73	Psoriasis	NA	6
Guerra et al. (26)	2012	Spain	Multicenter	Retrospective	NA	Mixed	1,294	Psoriasis	Gastroenterologists and dermatologists; biopsy if necessary	21
Salgueiro et al. (27)	2013	Portugal	Monocenter	Retrospective	2002–2012	NA	132	Psoriasis	NA	11
Sherlock et al. (28)	2013	Canada	Monocenter	Retrospective	2000–2010	Children	172	Psoriasiform lesions	NA	18
Afzali et al. (29)	2014	USA	Monocenter	Retrospective	1998–2011	Mixed	1,004	Psoriasiform lesions	Gastroenterologists	27
Mälkönen et al. (7)	2014	Finland	Monocenter	Prospective	2011–2013	Children	84	Psoriasiform lesions	NA	25
Tillack et al. (30)	2014	Germany	Monocenter	Prospective	2010–2011	Adult	434	Psoriasiform lesions	Dermatologists	21
Włodarczyk et al. (31)	2014	Poland	Monocenter	Prospective	2012–2013	Adult	30	Psoriasiform lesions	Gastroenterologists and dermatologists	8
Pugliese et al. (32)	2015	Italy	Monocenter	Retrospective	2008–2013	Adult	402	Psoriasis		42
Fréling et al. (33)	2015	France	Monocenter	Retrospective	2000–2011	Adult	583	Psoriasiform lesions	Dermatologists	59
George et al. (34)	2015	USA	Monocenter	Retrospective	2004–2013	Mixed	72	Psoriasiform lesions	Dermatologists	18
Huang et al. (35)	2015	Canada	Monocenter	Retrospective	2013	Adult	71	Psoriasis	Gastroenterologists and dermatologist	2
Soh et al. (36)	2015	Republic of Korea	Monocenter	Retrospective	2002–2013	NA	500	Psoriasiform lesions	Dermatologist	13
Cleynen et al. (37)	2016	Belgium	Monocenter	Retrospective	1994–2009	Adult	917	Psoriasiform lesions, psoriasis	Dermatologist's clinical diagnosis (biopsy 42%)	81
Guerra et al. (38)	2016	Spain	Multicenter	Retrospective	Inception–2015	Mixed	7,415	Psoriasis	NA	125
Hellström et al. (39)	2016	Finland	Monocenter	Prospective	2013–2014	Adult	118	Psoriasiform lesions	NA	7
Protic et al. (40)	2016	Switzerland	Monocenter	Retrospective	2010–2013	NA	269	Psoriasis	NA	23
Vedak et al. (8)	2016	USA	Monocenter	Retrospective	2005–2014	Adult	765	Psoriasis	Dermatologists	35
Jeyarajah et al. (9)	2017	Ireland	Monocenter	Retrospective	2000–2015	NA	403	Psoriasis	NA	8
Peer et al. (10)	2017	Australia	Monocenter	Retrospective	2009–2013	Adult	270	Psoriasiform lesions	Dermatologist and biopsy	10
Andrade et al. (11)	2018	Portugal	Monocenter	Retrospective	2005–2015	Mixed	732	Psoriasis	NA	39
Bae et al. (6)	2018	Korea	Nationwide	Retrospective	2007–2016	Mixed	5,428	Psoriasis	NA	62
Sridhar et al. (12)	2018	USA	Monocenter	Retrospective	2010–2015	Children	409	Psoriasis	NA	33
Weizman et al. (13)	2018	Canada	Monocenter	Retrospective	2004–2016	Mixed	676	Psoriasis	Dermatologists and biopsy	72
Courbette et al. (14)	2019	France	Monocenter	Retrospective	2002–2014	Children	147	Psoriasis	Dermatologists	20
Cossio et al. (15)	2020	Canada	Monocenter	Retrospective	2013–2016	Children	343	Psoriasis	Dermatologists	20
Ya et al. (16)	2020	USA	Monocenter	Retrospective	2003–2015	Adult	97	Psoriasis	Dermatologist and biopsy	97

NA, not available; IBD, inflammatory bowel disease.

TNF therapy was 6.0% (95% CI, 5.0–7.0%; I^2 = 93.9%, n = 29 studies), with 6.9% (95% CI 5.1–8.7%; I^2 = 88.7%, n = 15 studies) for psoriasiform lesions and 4.6% (95% CI 3.6–5.6%; I^2 = 93.3%, n = 15 studies) for psoriasis, respectively (**Figures 1–3**). The results of the jackknife sensitivity analysis suggested that the pooled estimate was robust and not influenced excessively by omitting any single study, ranging from 5.7 to 6.5% (**Supplementary Table S2**). When tested for publication bias, P was 0.034 for Begg's test and 0.001 for Egger's test. Despite the existence of a publication bias, the sensitivity analyses of the trim-and-filled method showed that the result was roughly reliable. We further conducted a sensitivity analysis exclusively including the 23 studies that reported an incidence of *de novo* psoriasis/psoriasiform lesion, yielding a similar pooled incidence of 5.8% (4.8–6.9%) (6–15, 22, 24, 25, 27, 30–32, 34–38, 40) (**Supplementary Figure S2**).

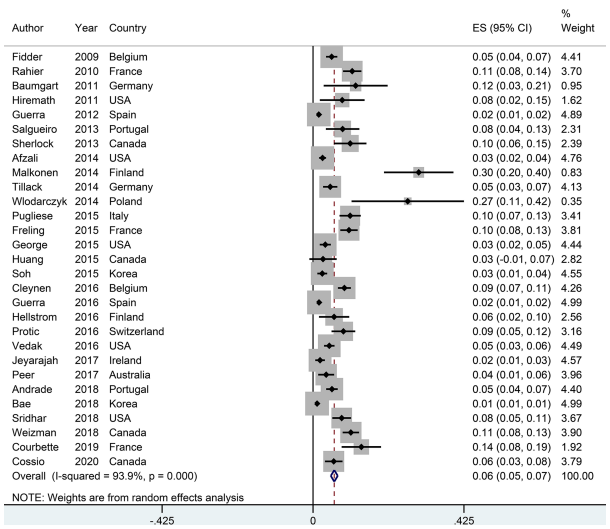


FIGURE 1 | Forest plots of incidence of psoriasiform lesions and/or psoriasis associated with anti-tumor necrosis factor therapy in inflammatory bowel disease patients.

In addition, the pooled incidence of psoriasis and/or psoriasiform reaction was separately analyzed according to gender (5.6%, 95% CI: 3.9–7.2% for female, n = 10 studies; 5.6%, 95% CI 3.9–7.2% for male, n = 10 studies), the types of IBD [4.8%, 95% CI: 3.7–5.9% for Crohn's disease (CD), n = 14 studies; 3.0%, 95% CI: 1.9–4.1% for ulcerative colitis (UC), n = 12 studies], and anti-TNF agents (5.4%, 95% CI: 4.2–6.5% for infliximab, n = 15 studies; 4.3%, 95% CI: 2.9–5.6% for adalimumab, n = 11 studies; 6.8%, 95% CI: 3.7–9.9% for certolizumab, n = 2 studies) (**Supplementary Figures S3**–**S9**).

Meta–Regression and Subgroup Analysis

Meta-regression analysis was conducted to investigate the source of heterogeneity. Nine covariates, including publication year, setting, study design, region, population, sample size, CD proportion, infliximab proportion, and study quality, were extracted from the 29 included studies. The univariable meta-regression identified three factors potentially related to the heterogeneity, including study design, region, population, and sample size (**Table 2**). As only 29 studies were included, we further included 3 factors in the multivariable meta-regression, showing region (coefficient -0.244, 95% CI: -0.046 to 0.003, P = 0.029) and population (coefficient 0.023, 95% CI -0.001 to 0.047, P = 0.062) to have significantly contributed to the heterogeneity (**Table 2**).

We further conducted several subgroup analyses according to region, setting and study design, sample size, diagnosis method, and study quality (**Table 3**). These results indicated that the heterogeneity can also be partially explained by the differences in region and population.

Risk Factors for Psoriasis or Psoriasiform Lesions

Data on clinical associations of psoriasis or psoriasiform lesions following anti-TNF therapy were available from 18 studies (6, 8, 9, 11–16, 25, 26, 30, 32–34, 36, 38, 40). With regard to demographic features, the risk of psoriasis or psoriasiform lesions during anti-TNF therapy was significantly higher in female patients (pooled OR: 1.46, 95% CI: 1.23–1.73, I^2 = 45.7%, n = 11 studies), with younger age at anti-TNF initiation (pooled OR: 1.03, 95% CI: 1.00–1.05, I^2 = 65.5%, n = 4 studies), and smoking (pooled OR: 1.97, 95% CI: 1.56–2.48, I^2 = 5.2%, n = 7 studies) (**Figure 4** and **Supplementary Figures S10**–**S12**). In the study of Fréling et al., IBD patients aged <28 years had a significantly higher risk of developing psoriasiform lesions compared with those >46 years (hazard ratio: 5.21, 95% CI:

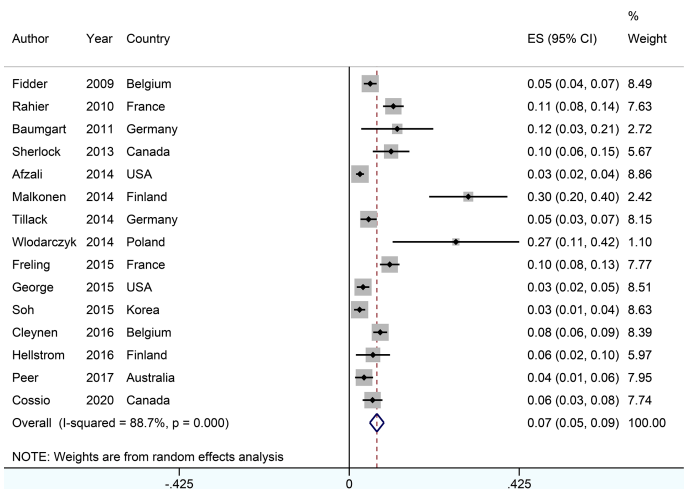


FIGURE 2 | Forest plots of incidence of psoriasiform lesions associated with anti-tumor necrosis factor therapy in inflammatory bowel disease patients.

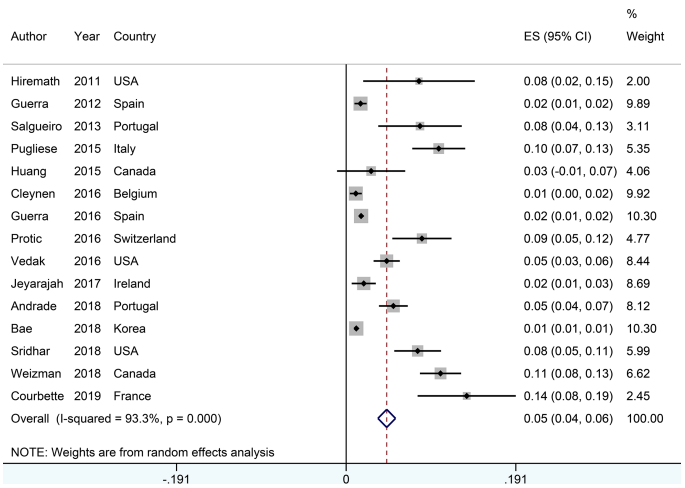


FIGURE 3 | Forest plots of incidence of psoriasis associated with anti-tumor necrosis factor therapy in inflammatory bowel disease patients.

2.43–11.16). No significant association was detected regarding white race, obesity/overweight, and family history of psoriasis (**Figure 4** and **Supplementary Figures S13**–**S16**).

In relation to combinable disease characteristics and treatment regimen, the presence of psoriasis or psoriasiform lesions following anti-TNF exposure was significantly higher in IBD patients receiving adalimumab (OR: 1.48, 95% CI: 1.03–2.13, I^2 = 52.8%, n = 6 studies) and certolizumab (OR: 2.87, 95% CI: 1.04–7.91, I^2 = 62.6%, n = 2 studies) than that of infliximab, but not associated with CD (*versus* UC), longer IBD duration, the presence of extraintestinal manifestations, and concomitant immunosuppressive therapy (**Figure 4** and **Supplementary Figures S17**–**S34**). Moreover, no significant differences were observed regarding disease behavior and location of CD and the extent of UC, except ileocolonic Crohn's disease with OR of 1.48 (1.03–2.13, I^2 = 30.3%, n = 5 studies) (**Figure 4** and **Supplementary Figures S17**–**S34**). The result of Egger's and Begg's tests showed no publication bias for the above-mentioned analyses (**Supplementary Table S3**).

In addition, other factors were included only in the systematic review rather than in the meta-analysis (**Supplementary Table S4**) because the assessment was performed only in 1 study. In this systematic review, no significant associations with psoriasis or psoriasiform lesions in IBD patients receiving anti-TNF therapy were observed, except acute psychological stressor (OR: 3.14, 95% CI: 1.10–8.93) (16) and body mass index (OR: 1.12, 95% CI: 1.01–1.24) (30).

DISCUSSION

To our knowledge, this is the first systematic review and meta-analysis to comprehensively investigate the incidence of and risk

TABLE 2 | Meta-regression for the source of heterogeneity of pooled incidence.

Variables	Number of studies	Coefficient	*P*-value	95% CI	Adjusted R^2
Univariate analysis					
Publication year	29	-0.002	0.575	-0.009, 0.005	-5.53%
Setting	29	-0.035	0.167	-0.085, 0.153	7.25%
Study design	29	0.055	0.066	-0.004, 0.111	-4.33%
Region	29	-0.023	0.073	-0.049, 0.023	10.60%
Population	27	0.0289	0.025	0.004, 0.054	26.52%
Sample size	29	-1.05e-05	0.044	-2.06e-05, -3.01 e-07	20.38%
CD proportion	23	0.012	0.294	-0.011, 0.351	-5.44%
IFX proportion	21	0.037	0.618	-0.116, 0.190	-7.63%
Psoriasis diagnosis	29	-0.016	0.407	-0.057, 0.024	-2.69%
Study outcome	29	-0.017	0.382	-0.058, 0.023	-4.99%
Study quality	29	0.115	0.275	-0.010, 0.033	0.85%
Multivariate analysis					
Region	27	-0.244	0.029	-0.046, 0.003	47.63%
Population		0.023	0.062	-0.001, 0.047	
Sample size		-5.66e-06	0.234	-1.52 e-05, 3.92e-06	

TABLE 3 | Subgroup analyses of pooled incidence of psoriasis or psoriasiform lesions associated with anti-tumor necrosis factor therapy in inflammatory bowel disease patients.

Subgroup	Number of studies	Pooled incidence (95% CI)	I^2
Region			
Europe	17	7.4% (5.7–9.1%)	94.6%
North America	9	6.0% (4.0–7.9%)	86.2%
Asia-Pacific	3	2.2% (5.0–7.0%)	77.0%
Setting			
Monocenter	25	6.8% (5.5–8.2%)	87.3%
Multicenter	4	2.5% (1.5–3.6%)	95.0%
Study Design			
Retrospective	24	5.6% (4.6–6.6%)	94.3%
Prospective	5	13.7% (6.2–21.2%)	87.5%
Population			
Children	6	11.01% (7.0–15.1%)	81.3%
Adult	12	6.6% (4.8–8.4%)	86.9%
Mixed	9	3.7% (2.6–4.7%)	93.3%
Sample size			
<500	16	8.2% (5.9–10.2%)	84.2%
≥500	13	4.9% (3.8–6.0%)	95.8%
Diagnosis method			
Gastroenterologist/dermatologist	15	5.6% (4.1–7.2%)	91.3%
Not reported	14	6.4% (5.0–7.7%)	94.1%
Newcastle–Ottawa Scale score			
<7	19	4.9% (3.9–5.9%)	91.8%
≥7	10	7.8% (5.5–10.1%)	91.0%

factors for psoriasis or psoriasiform lesions secondary to anti-TNF therapy in IBD patients. The overall estimated pooled incidence was 6.0% psoriasis and/or psoriasiform lesions in IBD patients following anti-TNF therapy. Regarding risk factors, female, younger age at anti-TNF therapy initiation, smoking, and adalimumab or certolizumab usage were significantly associated with an increased risk of developing psoriasis or psoriasiform lesions during anti-TNF therapy in IBD patients. These findings have the potential to inform clinical practice for more individualized decisions or precautions and may help us to understand the mechanism of this paradoxical phenomenon.

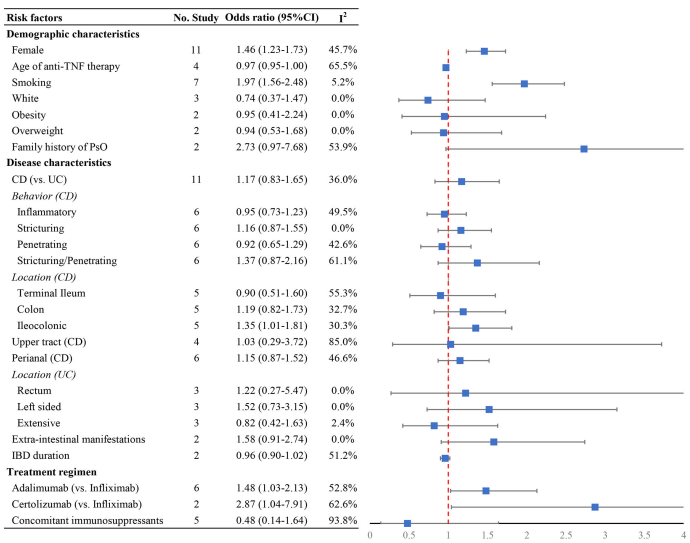


FIGURE 4 | Forest plots of the odds ratio for the risk of developing psoriasiform lesions and/or psoriasis in inflammatory bowel disease patients receiving anti-tumor necrosis factor therapy.

Anti-TNF agents have assumed the dominant position in the treatment of IBD over the past couple of decades. A large body of evidence confirms the overall good safety profile of the anti-TNF agents. However, with the increased use of these agents, paradoxical inflammation or autoimmune diseases induced by anti-TNF agents have been continuously reported, including cutaneous, articular, ocular, and neurological involvements (41, 42). Of these, paradoxical psoriasis or psoriasiform lesions, being the most prevalent and well-known paradoxical adverse events associated with anti-TNF agents, have been under intense investigation in recent years. At present, paradoxical psoriasis or psoriasiform lesions can no longer be considered rare in clinical routine, with incidence estimates of greater than 20% with the use of anti-TNF agents in some research. For the incidence of psoriasis/psoriasiform rash, our results, overall, were comparable to the results from the 2021 meta-analysis (43). The primary outcome of dermatological reactions in IBD patients receiving anti-TNF therapy from 26 studies was 19.4% (95% CI: 15.2–24.4%, I^2 = 95%) in this meta-analysis. In the secondary outcome of psoriasis/psoriasiform rash, there was a pooled incidence of 5.6% (95% CI: 4.2–7.4%, I^2 = 95%), with 6.1% (95% CI: 3.4–10.6%, I^2 = 96%) for infliximab therapy and 5.9% (95% CI: 2.5–13.5, I^2 = 93%) for adalimumab therapy. In addition to this, our work has reported more detailed and more specific information on anti-TNF associated psoriasis/psoriasiform rash. In the meta-regression and subgroup analyses, we also noticed that the regions and population contributed to the heterogeneity. Taken together, gastroenterologists should be aware of the paradoxical phenomenon, and the current findings could be instrumental in guiding therapeutic decision in clinical routine.

Currently, the molecular mechanisms and pathogenesis of paradoxical psoriasis/psoriasiform rash associated with anti-TNF agents are poorly understood, and multiple factors might be involved, including the genetic predisposition, preexisting autoimmune condition, and increased secretion and imbalance of cytokines and cells (interferon−α, Th1, Th2, Th17 cytokines, *etc.*). Clinically, the risk factors for developing psoriasis/psoriasiform rash after anti-TNF therapy are under exploration but are inconclusive. The present study, for the first time, has systematically reviewed the literature surrounding the risk factors. The meta-analyses revealed a statistically increased risk of developing psoriasis or psoriasiform lesions during anti-TNF therapy in IBD patients who are female, of a young age at anti-TNF therapy initiation, smoking, and using adalimumab or certolizumab. In the general population, psoriasis can manifest at any age, but with the highest peak between the ages of 20 and 40 years (44). The function of the immune system, and so does autoimmunity, is affected by various factors, including age (45). Overall, age is closely related to the strength of the immune system response, which is expected to decline in senescence (46, 47). From this aspect, the association between advanced age and low risk of psoriasis or psoriasiform lesions secondary to anti-TNF therapy can also be, in part, instinctively understood. In this study, we found that smoking, past and present, is the major risk factor for developing psoriasis during anti-TNF treatment in IBD patients. In fact, the adverse effects of smoking on psoriasis onset have been documented in the general population. The possible pathophysiological mechanisms of the associations included oxidative stress and free radical damage induced by smoking, which could trigger a cascade of systemic inflammation and the subsequent development of psoriasis (48, 49). However, it is still challenging to understand whether or how smoking work together with TNF blockade to orchestrate the psoriasis occurrence. *In vitro*, cigarette smoke chemical components could activate nuclear factor kappa-B activation and proinflammatory cytokine production, including IL-1β and IL-6 (50). The full blockade of TNF-α may impair the homeostasis of normal skin and cause an imbalance in cytokines and cells, which may be further exacerbated by the presence of smoking, and finally paradoxical adverse events occur (50). For paradoxical skin inflammation, the IFN-α pathway was considered to play a key role. However, cigarette smoking was found to decrease the production of IFN-α and increase the production of IFN-β *in vitro* (51). Unraveling the synergistic effect between smoking and TNF blockade on the incidence of paradoxical psoriasis can be extremely complex in people with IBD, yet smoking cessation before starting anti-TNF therapy merits consideration in IBD patients from the perspective of decreasing the risk of paradoxical adverse events. In addition, current evidence suggests that paradoxical inflammation during treatment with anti-TNF agents seems to be a drug class effect. In the present study, the significantly higher risk of adalimumab or certolizumab therapy than infliximab therapy was identified, although both of them were associated with an increased risk of paradoxical psoriasis or psoriasiform skin lesions. In fact, potential differences between adalimumab and infliximab in IBD have been reported. In a nationwide cohort study of biologic-naive adults with UC, the adalimumab-treated patients showed a substantially higher rate of all-cause hospitalization and serious infection requiring hospitalization and a trend toward a higher rate of UC-related hospitalization (52). Besides these, infliximab drug levels were found to be associated with the depth of remission in patients with CD, but no such relationship was detected for adalimumab (53). However, there is no plausible mechanism evidence to explicitly explain the difference among different types of anti-TNF-associated psoriasis or psoriasiform rash. Future clinical and basic science studies are needed to experimentally validate the presented findings.

However, there were several limitations to our study. First, due to the nature of observational design in the original studies, the present study is vulnerable to potential biases (information or selection bias), which cannot allow us to conclude definite causal relationships. Second, the heterogeneity for the pooled incidence among the studies was very high. For this, we performed a series of subgroup analyses, meta-regression, and risk factor exploration. To a large extent, they could explain the source of heterogeneity. Third, various diagnosis criteria of psoriasis or psoriasiform skin lesions were applied in the included studies, mostly by interview or read codes rather than by dermatologists. Establishing a close collaboration between gastroenterologists and dermatologists is necessary to overcome this limitation in the future. Fourth, not all

studies made enough adjustment for potential confounders, and we cannot fully unify the confounders, which can potentially lead to either an overestimation or an underestimation of the associations. Lastly, despite all the potential risk factors evaluated, for some of them, especially for disease activity, cumulative anti-TNF dosages were only included into the systematical review, and further investigations are required to explore their association.

CONCLUSION

In summary, the overall estimated pooled incidence of psoriasis/psoriasiform lesions secondary to anti-TNF therapy was 6% in IBD patients. Female, young age at anti-TNF therapy initiation, smoking, ileocolonic CD, and adalimumab or certolizumab use were associated with a substantially increased risk of developing psoriasis or psoriasiform lesions during anti-TNF therapy. These findings have the potential to inform clinical practice for more individualized decisions or precautions and may help us to understand the mechanism of this paradoxical phenomenon.

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.

AUTHOR CONTRIBUTIONS

ZZ conceptualized the study, participated in its design and coordination, and critically revised the manuscript. WX and SX contributed to data collection, analysis, and interpretation and drafted the manuscript. HH contributed to the process of data collection as a study investigator. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.847160/full#supplementary-material

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Autoimmune Skin Disease Exacerbations Following COVID-19 Vaccination

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Background: Vaccination against COVID-19 reduces the risk of severe COVID-19 disease and death. However, few studies have examined the safety of the COVID-19 vaccine in patients with autoimmune skin disease.

Objectives: We sought to determine the incidence of disease exacerbation in this population following COVID-19 vaccination as well as the associated factors.

Methods: We performed a chart review of all patients seen in the autoimmune skin disease clinic of the principal investigator during the study period. All patients included for analysis were systematically and prospectively asked about COVID-19 vaccination status, manufacturers, vaccine dates, autoimmune symptoms after the vaccine, and timing of symptom onset using a standardized template as part of their visit. Demographics and autoimmune disease diagnosis were also collected. Analysis used Chi-square and Fisher's exact tests.

Results: 402 subjects were included for analysis. 85.6% of patients were fully vaccinated, with 12.9% unvaccinated and 1.5% partially vaccinated. 14.8% of fully vaccinated patients reported worsening autoimmune signs and symptoms after the vaccine. Fully vaccinated dermatomyositis patients were more likely to report worsening autoimmune signs and symptoms after the vaccine (22.7%) than fully vaccinated lupus erythematosus patients (8.6%) (p=0.009). Patients fully vaccinated with the Moderna vaccine trended towards an increased likelihood of reporting worsening autoimmune signs and symptoms after the vaccine (19.1%) than those with the Pfizer-BioNTech vaccine (12.0%) (p=0.076). Of the patients who had autoimmune symptoms after vaccination, 20% had symptoms after the 1st dose, 82% after the 2nd dose, and 4% after the 3rd dose with median onset (95% confidence interval) of 7 (2,14), 14 (14,21), and 18 (7,28) days later, respectively.

Conclusions: More fully vaccinated dermatomyositis patients had exacerbation of autoimmune signs and symptoms after the vaccine than fully vaccinated lupus erythematosus patients. However, given the risks of COVID-19, clinicians should still promote vaccination in most patients with autoimmune skin disease.

Keywords: vaccination, COVID-19, autoimmune, connective tissue disease, skin

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INTRODUCTION

COVID-19, the disease resulting from infection with the SARS-CoV-2 virus, was officially declared a pandemic by the World Health Organization on March 11, 2020. Since then, COVID-19 has led to enormous amounts of death, altered how society approaches many aspects of daily life, and placed significant strain on healthcare systems worldwide (1). Development of vaccines targeting SARS-CoV-2 occurred at a historically quick pace and led to FDA emergency use authorization of two vaccines using mRNA technology administered in a two-dose series (Pfizer-BioNTech and Moderna) and one adenovirus-based vaccine administered in one dose (Johnson & Johnson). The Pfizer-BioNTech and Moderna vaccines eventually received full FDA approval, and booster doses became recommended 5 months after the initial two-dose vaccine series or 2 months after a Johnson & Johnson (J&J) vaccine.

All of the COVID-19 vaccines used in the United States have been shown to significantly reduce the risk of hospitalization and death (2). However, many patients with autoimmune diseases experience hesitancy with regards to receiving a COVID-19 vaccine (3). Patients with immune-mediated inflammatory diseases were not included in the COVID-19 vaccine clinical trials, leading to questions about vaccine efficacy and safety in this patient population (4, 5). A few studies have examined the incidence of flares in rheumatic disease patients following COVID-19 vaccination and found a range of 3%-13.4% (6–8). However, these studies used either online patient surveys or physician-reported registries to generate their data, which could be possible limitations in determining true flare incidence rates.

The exact mechanisms by which the COVID-19 vaccines could trigger autoimmune disease flares have not been fully elucidated, but some studies may give potential clues. New onset blistering diseases, including bullous pemphigoid (BP), have been reported following the COVID-19 vaccine (9, 10). One study showed that lesions in patients with new onset BP after COVID-19 vaccination had a high clonality of T cells reactive to SARS-CoV-2 derived epitopes while control tissues from prior to the widespread use of COVID-19 vaccines did not (10). This indicates that in these patients there was an ongoing adaptive immune response likely resulting from the vaccine and driving BP lesion formation (10). Thus, the COVID-19 vaccine may lead to an unintended T cell response that can result in new onset autoimmune disease or possibly drive flares in patients who already have an autoimmune disease diagnosis.

Given the lack of knowledge of the safety of the COVID-19 vaccine in patients with autoimmune disease, the aim of our study was to determine the incidence of disease exacerbation in patients with autoimmune skin disease following COVID-19 vaccination as well as to assess the factors associated with these flares.

METHODS

Study Design

All patients included for analysis were systematically and prospectively asked questions by the principal investigator (PI), author VPW, using a standardized template as part of their standard of care visit as shown here:

Did you get the vaccine? ***
When? ***
If yes, did you have a reaction to the vaccine? ***
When? ***
Which vaccine did you get? ***

We performed a chart review in Epic of all patients seen in the autoimmune skin disease clinic of the PI at the University of Pennsylvania during the study period to collect this prospectively collected data. Data was manually extracted and stored in a password-protected Excel file. Data collected included demographic data, autoimmune disease diagnosis, and data about the COVID-19 vaccine and reactions obtained from the template. Characteristics of the study population are summarized in **Table 1**. Patients were considered unvaccinated if they had never received a COVID-19 vaccine, partially vaccinated if they received only one dose of the Pfizer-BioNTech or Moderna vaccines, and fully vaccinated if they received at least one dose of the J&J vaccine or at least two doses of any vaccines. Patients were categorized into the following autoimmune disease diagnoses based on chart review: lupus erythematosus (LE), dermatomyositis (DM), morphea, pemphigus, bullous pemphigoid (BP), Behcet's disease, mucous membrane pemphigoid (MMP), and other. The pemphigus group included patients with pemphigus vulgaris and pemphigus foliaceus. The "other" group of autoimmune disease diagnoses included patients who had multiple overlapping autoimmune disease diagnoses as well as others not included here with only 1-2 patients per diagnosis. A full list of autoimmune disease diagnoses included in the "other" group can be found in **Table 2**. Responses recorded in the template were assessed to determine if the patient experienced an autoimmune disease exacerbation (e.g., increased erythema and itchiness of dermatomyositis skin lesions or increased swelling of joints in patients with lupus arthritis) with careful attention to distinguish from typical known vaccine-related adverse events (such as fever and sore arm). This assessment of whether or not patients experienced autoimmune disease exacerbations after receiving the COVID-19 vaccine was also collected. Patients' interim or initial history, physical exam findings, and assessment and plan were also collected and were assessed to determine if any patients who experienced autoimmune disease exacerbations after the vaccine required an escalation in treatment. Patients who experienced autoimmune disease exacerbations were called to ask if they had stopped any of their autoimmune disease treatments at the time of vaccination, as some patients stopped their immunosuppressive medications in an attempt to increase vaccine response. This study was approved by the institutional review board at the University of Pennsylvania and was carried out in accordance with the Declaration of Helsinki and the Health Insurance Portability and Accountability Act.

Statistical Analysis

Descriptive statistics were performed on patient demographic data, autoimmune disease diagnosis, vaccine manufacturer, and

TABLE 1 | Characteristics of the study population.

Characteristic	Patients (N=402) *No.* (%)
Age Group	
<18	1 (0.2)
18-30	28 (7.0)
31-40	35 (8.7)
41-50	63 (15.7)
51-60	102 (25.4)
61-70	103 (25.6)
71-80	52 (12.9)
80+	18 (4.5)
Sex	
Male	74 (18.4)
Female	328 (81.6)
Race	
White	262 (65.2)
Black	71 (17.7)
Asian/Pacific Islander	25 (6.2)
American Indian/Alaskan Native	1 (0.2)
Other	16 (4.0)
Unknown	27 (6.7)
Ethnicity	
Non-Hispanic	382 (95.0)
Hispanic	7 (1.7)
Unknown	13 (3.2)
Autoimmune Disease Diagnosis	
Lupus Erythematosus	105 (26.1)
Dermatomyositis	133 (33.1)
Morphea	13 (3.2)
Pemphigus	19 (4.7)
Bullous Pemphigoid	13 (3.2)
Behcet's Disease	2 (0.5)
Mucous Membrane Pemphigoid	10 (2.5)
Other	107 (26.6)
COVID-19 Vaccination Status	
Unvaccinated	52 (12.9)
Fully Vaccinated	344 (85.6)
Partially Vaccinated	6 (1.5)
COVID-19 Vaccine Manufacturer	First dose, second dose, third dose
Pfizer-BioNTech	187 (46.5), 184 (45.8), 25 (6.2)
Moderna	143 (35.6), 140 (34.8), 14 (3.5)
J&J	19 (4.7), 0 (0.0), 1 (0.2)
Unknown	1 (0.2), 1 (0.2), 0 (0.0)
n/a	52 (12.9), 77 (19.2), 362 (90.0)

timing of autoimmune disease symptom onset after vaccination. Further analyses used Chi-square and Fisher's exact tests. Analysis was conducted with IBM SPSS Statistics for Windows, Version 26.0 (Armonk, NY).

RESULTS

402 subjects were included for analysis. Ages ranged from 17-96 with a median age (95% confidence interval) of 58 (56,60). Most patients (81.6%) were female. Patients came from a variety of racial backgrounds, with 65.2% white and 17.7% Black. The study population was largely non-Hispanic (95.0%). Most patients (59.2%) had either LE or DM. Patients were largely fully vaccinated (85.6%). More patients received the Pfizer-BioNTech vaccine for all 3 doses than other vaccine manufacturers while J&J was given to the least number of patients.

TABLE 2 | Autoimmune diseases included as "other".

Other Autoimmune Diseases	
Alopecia areata	Mixed connective tissue disease
CREST syndrome	Multicentric reticulohistiocytosis
Cutaneous polyarteritis nodosa	Neutrophilic dermatosis
Cutaneous sarcoidosis	Neutrophilic urticarial dermatosis
Diabetic dermopathy	Overlap of multiple autoimmune diseases
Disseminated porokeratosis	PASH syndrome
Eczema	Primary Sjogren syndrome
Eczematous dermatitis	Psoriasis
Epidermolysis bullosa acquisita	Pyoderma gangrenosum
Erythema nodosum	Raynaud's phenomenon
Erythromelalgia	Rheumatoid nodules
Frontal fibrosing alopecia	Sneddon Wilkinson disease
Granuloma annulare	Solar urticaria
Hypocomplementemic urticarial vasculitis	Sweet's syndrome
IgA vasculitis	Systemic sclerosis
Lichen planopilaris	Thyroid acropachy
Lichen planus	Unclear blistering disease
Lichen sclerosis	Undifferentiated connective tissue disease
Lichenoid mucositis	Urticarial eruption
Livedo vasculopathy	Urticarial hypersensitivity reaction
Livedoid vasculitis	Vitiligo

Across all diagnoses, 14.8% of fully vaccinated patients had autoimmune disease exacerbations or flares after the COVID-19 vaccine. 45.1% of these patients required an escalation in treatment, representing 6.7% of all fully vaccinated patients. 19.6% of fully vaccinated patients who experienced flares had stopped their autoimmune disease treatment at the time of their COVID-19 vaccine. Most of these patients paused their autoimmune disease medications for 1-2 weeks starting at the time of receiving a vaccine dose. Fully vaccinated DM patients were more likely to report an autoimmune disease exacerbation after the COVID-19 vaccine (22.7%) than fully vaccinated LE patients (8.6%) (p=0.009) (**Figure 1**). The two fully vaccinated Behcet's disease patients both experienced significant flares after the COVID-19 vaccine. These patients were not included in the "other" category to draw attention to the severity of their flares. The proportion of fully vaccinated patients who had autoimmune disease exacerbations after COVID-19 vaccination varied by diagnosis (p=0.004) (**Figure 2**).

Figure 3 shows the proportion of fully vaccinated patients who reported autoimmune disease exacerbation by COVID-19 vaccine manufacturer. There was a trend towards more fully vaccinated patients who received the Moderna vaccine having an autoimmune disease exacerbation (19.1%) compared to Pfizer-BioNTech (12.0%) and J&J (10.5%) (p=0.198) (**Figure 3**).

The COVID-19 vaccination status of LE patients differed significantly from that of DM patients (p=0.005) (**Figure 4**). 89.5% of DM patients were fully vaccinated compared to 77.1% of LE patients (p=0.010). 42.9% of the LE patients in our sample were Black compared to 7.5% of DM patients. In both the LE and DM patient samples, a higher proportion of white patients were fully vaccinated than Black patients (**Figure 5**). There was a trend towards increased vaccination

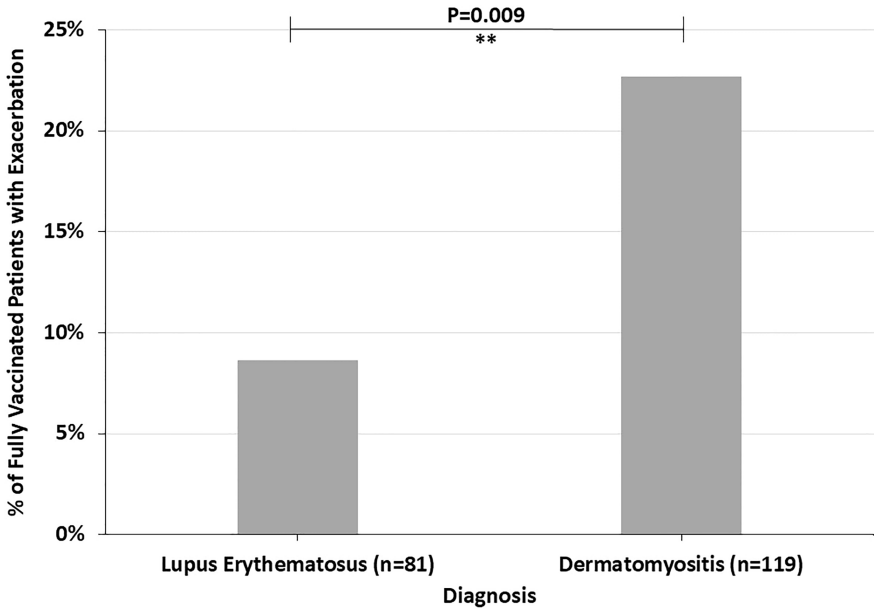

FIGURE 1 | The percent of fully vaccinated patients reporting symptoms of autoimmune disease exacerbation after the COVID-19 vaccine by diagnosis, highlighting the higher incidence in dermatomyositis patients compared to lupus erythematosus patients. **P ≤ 0.01.

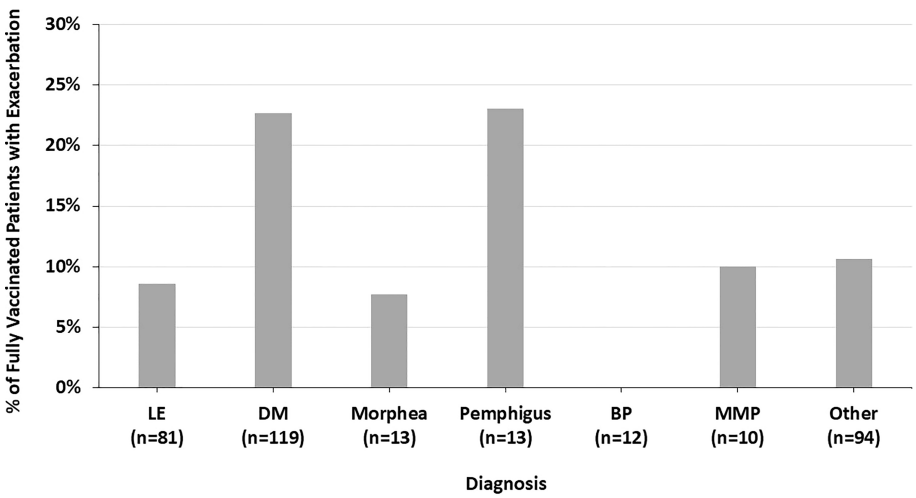

FIGURE 2 | The percent of fully vaccinated patients reporting symptoms of autoimmune disease exacerbation after the COVID-19 vaccine by diagnosis, highlighting the variation across all diagnoses seen.

rates in patients with DM, morphea, BP, Behcet's disease, and MMP compared to other diseases such as LE and pemphigus (p=0.061) (**Figure 6**).

Of the patients who had autoimmune exacerbations after vaccination, 20% had flares after the first dose, 82% after the second dose, and 4% after the third dose. Some patients experienced disease flares after multiple doses. The median time to autoimmune exacerbation onset (95% confidence interval) was 7 (2, 14), 14 (14, 21), and 18 (7, 28) days after the first, second, and third vaccine doses, respectively.

DISCUSSION

Several studies have shown that some autoimmune disease patients experience flares after the COVID-19 vaccine (6–8). However, these

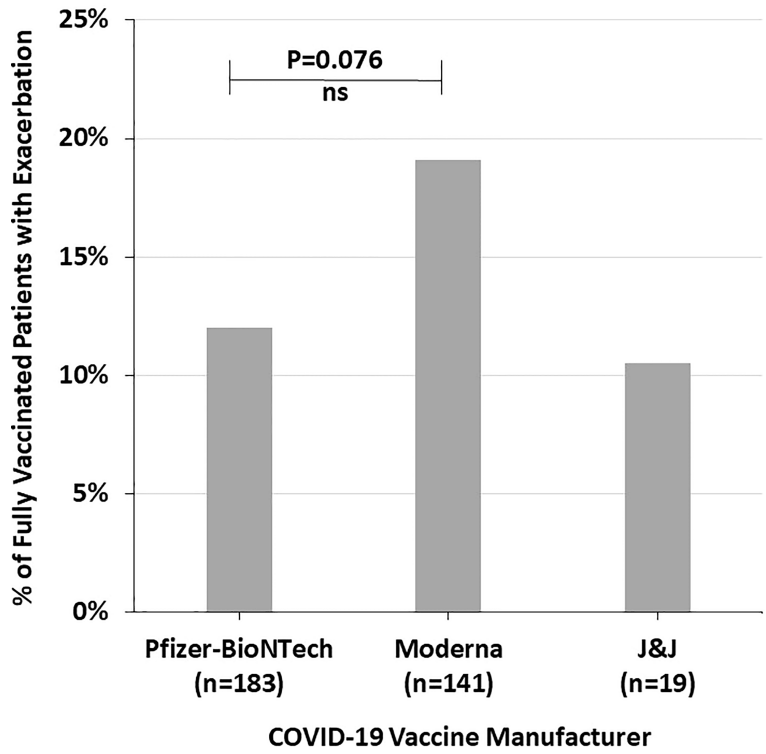

FIGURE 3 | The percent of fully vaccinated patients reporting symptoms of autoimmune disease exacerbation after the COVID-19 vaccine by vaccine manufacturer, highlighting the trend of increased incidence with the Moderna vaccine. NS, Not significant.

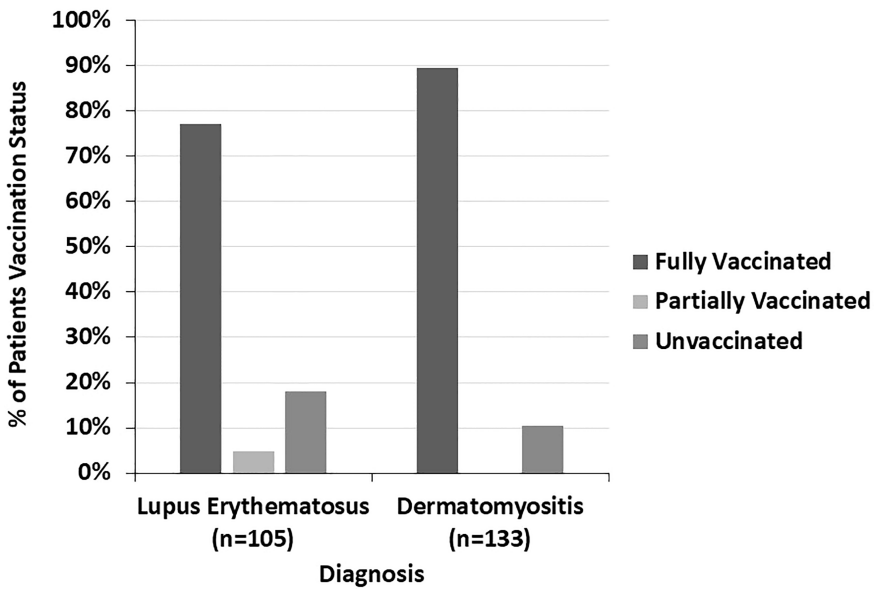

FIGURE 4 | The COVID-19 vaccination status of lupus erythematosus and dermatomyositis patients is shown here. A higher proportion of dermatomyositis patients were fully vaccinated compared to lupus erythematosus patients.

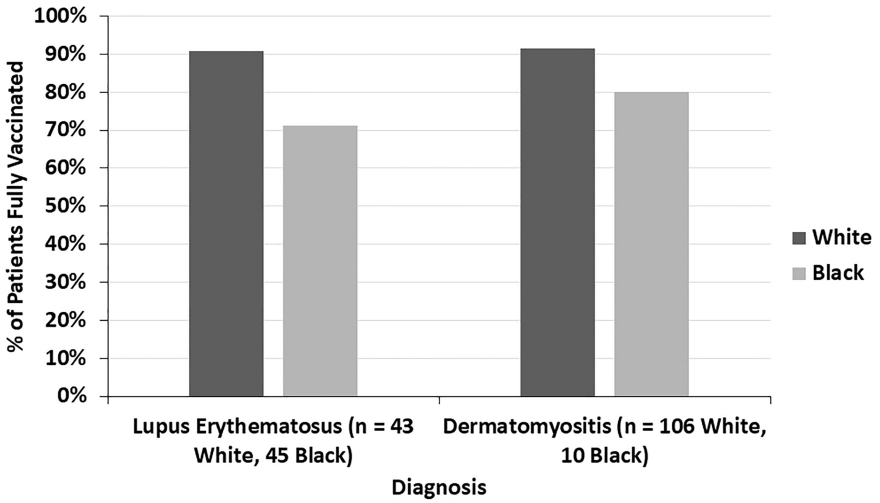

FIGURE 5 | The proportion of fully vaccinated lupus erythematosus and dermatomyositis patients is seen here by racial background (white and Black). In both diseases, a higher proportion of white patients were fully vaccinated compared to Black patients.

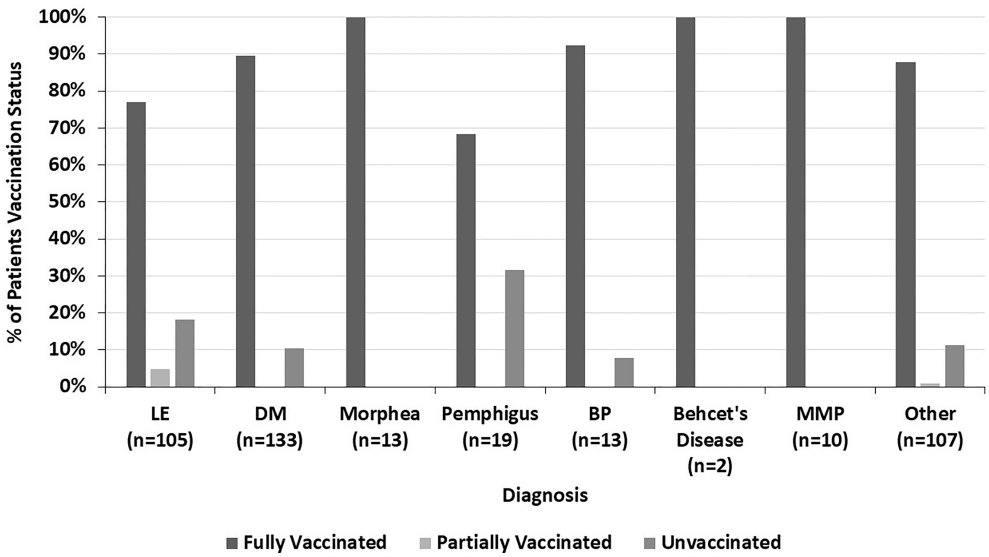

FIGURE 6 | The percent of patients who are fully vaccinated, partially vaccinated, and unvaccinated within each diagnosis, highlighting the variation across all diagnoses seen.

studies used either on-line patient surveys or physician-reported registries to generate their data, representing possible limitations in determining true flare incidence rates. In this study, we prospectively and systematically evaluated patients to determine if they experienced autoimmune disease exacerbations following the COVID-19 vaccine. The rate of fully vaccinated patients reporting autoimmune disease exacerbations in our study (14.8%) is higher than the previously reported incidences of rheumatic disease flares (3%-13.4%) following COVID-19 inoculation (6–8). Perhaps this difference results from differences in the populations sampled in our study compared to past studies. Past studies that used online patient surveys and physician-reported registries are more likely to include patients with mild autoimmune disease than patients from our study which were all recruited from a single tertiary care center that specializes in autoimmune skin disease with a high volume of moderate-to-severe autoimmune disease patients. Since these sicker patients have increased flare rates generally, it is possible that they also have increased flare rates with specific environmental triggers, such as the COVID-19 vaccine. As our study included patients from a dermatology clinic, it is also possible that the ubiquitous

prevalence of skin disease in our population played a role in the increased rate of disease exacerbations seen. As 19.6% of the fully vaccinated patients who experienced disease exacerbations in our study stopped their autoimmune disease treatment at the time of vaccination, it is possible that some of these flares were due to medication discontinuation. However, most patients who flared did not stop their medication for the vaccine and so it was not a factor contributing to their disease exacerbation.

The low rate of flares requiring an escalation in treatment in our study (6.7% of fully vaccinated patients or 45.1% of patients who flared) is reassuring and indicates that the COVID-19 vaccine is relatively safe and well tolerated by many patients with autoimmune skin disease. However, it does raise questions of risk-benefit analysis for patients who have had severe vaccine-related flares on the necessity of additional vaccine doses or booster shots. As new variant-specific vaccines are being developed, such as the Omicron-based vaccine, it is not clear either how safe these will be for autoimmune disease patients with past COVID-19 vaccine-related flares.

It is not totally obvious why the incidence of flares varied across diagnoses or specifically why DM patients were more likely to flare after the COVID-19 vaccine than LE patients. Only speculation is possible. Both DM and LE pathogenesis are believed to be in part driven by type 1 interferons which the COVID-19 vaccines have been shown to upregulate (11–13). However, the immune cells driving interferon expression in these diseases differ, with plasmacytoid dendritic cells playing a key role in LE interferon expression compared to myeloid dendritic cells in DM (14, 15). These differences in pathogenesis may be partly responsible for the differences in flares seen in these diseases after the COVID-19 vaccine. There is a lack of data comparing the flare rates of LE to DM patients, but studies looking at each disease individually report a lower rate of flares in LE than DM (16, 17). Thus, it is possible that more flares were seen in our study after the vaccine in DM patients than LE patients due to an increased tendency to flare at any given time.

Though the incidence in autoimmune disease exacerbation after vaccination did not differ significantly by COVID-19 vaccine manufacturer, there was a trend towards increased flares following the Moderna vaccine. Studies have shown the Moderna vaccine to be more immunogenic than the Pfizer-BioNTech or J&J vaccines, resulting in greater spike protein-specific antibody levels, neutralizing antibody levels, and spike protein-specific T cell responses (18). Data indicates that increased vaccine immunogenicity may correlate with increased vaccine-related side effects due to heightened immune system activation (18). This could explain why the more immunogenic Moderna vaccine had the highest rate of flares in our study as there was possibly an increased immune response with off-target autoimmune effects. There was also a trend towards the fewest autoimmune disease exacerbations occurring after the J&J vaccine in our study. This also seems to correlate with what is known about vaccine immunogenicity as J&J has been shown to result in lower levels of vaccine-induced antibodies and have a less robust T-cell response than the mRNA vaccines (18).

While the vaccination rate differed significantly between LE and DM patients with DM patients more likely to be fully vaccinated, this is likely explained in large part due to differences in the racial makeup of these two disease populations as well as variation in vaccine acceptance across racial backgrounds. COVID-19 vaccination rates among Black people in the US are lower than other racial groups (19). Black patients made up a larger share of LE patients in our study than DM patients and also had lower vaccination rates than white patients in both disease populations, which is consistent with previous data on differences in vaccination rates by race and helps to explain the differences observed across disease diagnoses.

Most patients who experienced autoimmune disease exacerbations after the COVID-19 vaccine in our study had increased symptoms after the second dose (82%). Studies have shown that higher levels of antibody response occur after the second dose of COVID-19 vaccines compared to the first dose (20). This increased humoral response may parallel the increased autoimmune response seen after the second COVID-19 vaccine dose in our study. Additionally, prior infection with COVID-19 has been shown to play a role in the level of immune response after each dose of the COVID-19 vaccine, with increased immunogenicity seen in previously infected individuals after the first vaccine dose (21). Thus, one possible mechanism for patients experiencing autoimmune disease exacerbations after the first vaccine dose is a prior COVID-19 infection which resulted in a more robust immune response after the first dose with increased autoimmune side effects.

There are several limitations to this study. Patients may have not accurately remembered details about their COVID-19 vaccine and any possible disease flares they experienced afterwards. Some patients were seen several months after they received the COVID-19 vaccine, increasing the possibility of recall bias. It is also possible that some patients who experienced flares temporally correlated with the COVID-19 vaccine were coincidental and that vaccine was not an autoimmune trigger. However, comparing the disease exacerbation rate by diagnosis and vaccine manufacturer helps to mitigate this potential limitation.

This study demonstrates that most patients with autoimmune skin disease tolerate the COVID-19 vaccine well, but a small number of patients do experience disease flares requiring an escalation in treatment (6.7%). Given the risks of hospitalization and death associated with COVID-19, clinicians should continue to promote vaccination in most 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 Institutional Review Board at the University of Pennsylvania. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GS, EK, MG, JC, and VW conceived and designed this study. GS collected data and drafted the manuscript and figures. MA, JD, and RF performed the statistical analysis. VW supervised the work. All authors revised the article and approved the submitted version.

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Efficacy and Safety of Curcumin and *Curcuma longa* Extract in the Treatment of Arthritis: A Systematic Review and Meta-Analysis of Randomized Controlled Trial

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Background: Modern pharmacological research found that the chemical components of *Curcuma longa L.* are mainly curcumin and turmeric volatile oil. Several recent randomized controlled trials (RCT) have shown that curcumin improves symptoms and inflammation in patients with arthritis.

Methods: Pubmed, Cochran Library, CNKI, and other databases were searched to collect the randomized controlled trials (RCTs). Then, the risk of bias of RCTs were assessed and data of RCTs were extracted. Finally, RevMan 5.3 was utilized for meta-analysis.

Results: Twenty-nine (29) RCTs involving 2396 participants and 5 types of arthritis were included. The arthritis included Ankylosing Spondylitis (AS), Rheumatoid Arthritis (RA), Osteoarthritis (OA), Juvenile idiopathic arthritis (JIA) and gout/hyperuricemia. Curcumin and Curcuma longa Extract were administered in doses ranging from 120 mg to 1500 mg for a duration of 4-36 weeks. In general, Curcumin and Curcuma longa Extract showed safety in all studies and improved the severity of inflammation and pain levels in these arthritis patients. However, more RCTs are needed in the future to elucidate the effect of Curcumin and Curcuma longa Extract supplementation in patients with arthritis, including RA, OA, AS and JIA.

Conclusion: Curcumin and Curcuma longa Extract may improve symptoms and inflammation levels in people with arthritis. However, due to the low quality and small quantity of RCTs, the conclusions need to be interpreted carefully.

Keywords: curcumin, *Curcuma longa* extract, rheumatoid arthritis, ankylosing spondylitis, osteoarthritis, juvenile idiopathic arthritis, systematic review, meta-analysis

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1 INTRODUCTION

Arthritis is a general term for various types of arthritic diseases, which are related to various factors such as degenerative diseases and autoimmunity. It is characterized by chronic inflammation of one or more joints, which usually causes pain and is often disabling. The main clinical symptoms are joint pain, swelling, stiffness, and limited mobility (1, 2). Epidemiology shows that arthritis is the most common in women, and the incidence of arthritis increases with age. Meanwhile, the prevalence of arthritis of different etiologies varies in the population (3, 4). Current research shows that there are more than 100 different forms of arthritis, with osteoarthritis (OA) and rheumatoid arthritis (RA) being the most common; other types mainly include arthritis associated with autoimmune diseases (5–7). Although these disorders have different etiologies, all of them are characterized by pain and limited mobility due to joint inflammation (7). At present, the drugs and non-drug methods for the treatment of arthritis are mainly related to the progression of joint pain and tissue joint inflammation, especially in the treatment of pain, the drugs are basically the same (8, 9). Among them, OA is a degenerative joint disease, the number of which is increasing with the aging of the population (10). According to the World Health Organization (WHO) survey, there are currently more than 400 million patients with osteoarthritis worldwide. In Asia, 1 in 6 people will develop OA at some point in their life. OA is more common in middle-aged and elderly people, and more women than men (11). Market research reports show that the OA therapeutics market was estimated at USD 6.8 billion in 2019 and is expected to reach USD 10.1 billion by 2024, growing at a CAGR of 8.1% from 2019 to 2024. The report shows that this increase is partly due to the rapid increase in the elderly and obese population and the consequent increase in the prevalence of OA (12–14). Rheumatoid arthritis (RA) is an autoimmune disease with erosive arthritis as the main symptom. The main symptoms are joint morning pain, swelling, pain and dysfunction. As a systemic inflammatory and destructive joint disease, the prevalence in the adult population worldwide is approximately 1-2% (15, 16). At present, RA is still difficult to cure, but standard diagnosis and treatment can achieve standard treatment. However, without regular treatment, it can lead to joint deformity and loss of function (17). Other types of arthritis are also associated with inflammation and pain, causing a huge burden on patients, but there is still no treatment for the underlying cause.

The main goal of current arthritis treatment is to reduce joint pain caused by joint inflammation, daily wear and tear of the joint, and muscle strain (18). Existing drugs for the treatment of arthritis are analgesics, steroids and non-steroidal anti-inflammatory drugs (NSAIDs), as well as biologically targeted drugs, which reduce symptoms such as severe pain and inflammation (19, 20). However, these drugs have a large number of side effects, which prevent them from providing sustained relief of disease symptoms and progression after long-term use. For example, the side effects of NSAIDs are severe gastrointestinal tract and insufficient pain relief after drug treatment, and biologically targeted drugs have immune disorders and adverse cardiovascular events (21–23). Therefore, the current treatment of arthritis has entered the stage of comprehensive management and treatment, and replacement therapy has gradually become an important part of the comprehensive management and treatment model (24, 25).

Curcuma longa L. is a potential alternative medicine for the treatment of arthritis, and they have been used as many ethnic medicines and gourmet condiments in several countries including China, Bangladesh, India and Pakistan (26). They have long been used as anti-inflammatory treatments in traditional Chinese medicine (TCM) and Ayurvedic medicines (27). The main components of turmeric are curcumin and demethoxycurcumin, bisdemethoxycurcumin and turmeric essential oil. Among them, curcumin is a natural compound, and current studies have shown that curcumin has good anti-inflammatory, immunosuppressive and anticancer properties (28–30). Evidence from multiple clinical trial studies suggests that curcumin can reduce the subjective experience of pain in patients with system-related disorders of muscle disease. Therefore, it is very important to systematically review the effects of *Curcuma longa L.* and curcumin in patients with arthritis.

2 MATERIALS AND METHODS

2.1 Protocol

This systematic review and meta-analysis were conducted strictly in accordance with the protocol (CRD42022286421) and PRISMA-guidelines (see **Supplementary Materials**).

2.2 Literature Search Strategy

Web of Science, Cochrane Library, PubMed, The ClinicalTrials.gov, China Biology Medicine (CBM), VIP Database, China National Knowledge Infrastructure (CNKI), MEDLINE Complete, Wanfang Database, Embase were searched for RCTs related to Curcumin and Curcuma longa Extract in the treatment of arthritis. The search period is from the establishment of the database to Fib. 2022. The search strategy of Pubmed and Embase is shown in **Table S1** as an example.

2.3 Selection Criteria

(1) Participants: Patients diagnosed with any type of arthritis by recognized standards. (2) Intervention: the intervention of experimental group is curcumin, with no restrictions on dosage, dosage form, and usage; the intervention of control group can be non-curcumin interventions such as placebo and conventional therapy. (3) Outcomes: Efficacy indicators, inflammatory indicators, adverse events. (4) Study design: RCTs

2.4 Literature Screening, Data Extraction and Quality Assessment

Two researchers independently reviewed the literature according to Selection criteria, and conducted literature screening and data extraction. If there are differences, they should be resolved through consultation and discussion. The literature quality evaluation uses the risk bias assessment tool of the systematic reviewer manual recommended by the Cochrane Collaboration to evaluate the methodological quality of the included studies (31). The content of the assessment includes random allocation

method, allocation concealment, whether blind method is used for participants, the completeness of the result data, whether there is selective reporting, and other biases.

2.5 Statistical Analysis

The RevMan 5.3 software recommended by the Cochrane Collaboration was used for meta-analysis. The risk ratio (RR) or mean difference (MD) and its 95% confidence interval (CI) are used as the efficacy and safety statistics. The $\chi 2$ test is used to evaluate the heterogeneity of the literature. $P \geq 0.1$ or $I\ 2 \leq 50\%$ means that the studies are homogeneous. The studies can be combined and analyzed using a fixed-effects model; otherwise, a random-effects model is used for analysis.

3 RESULTS

3.1 Results of the Search

According to the search strategy, 1981 related papers were initially retrieved. After deduplication, reading the title and abstract, and the full text, 30 RCTs were finally included, while 7 records were excluded (32–38) (**Figure 1**).

3.2 Description of Included Trials

The included RCTs involved 5 types of arthritis: RA, AS, OA, Juvenile idiopathic arthritis (JIA) and gout/hyperuricemia; they also involved 11 countries: Iran, India, China, Australia, Belgium, Armenia, Indonesia, Thailand, Japan, Italy, Romania. The study size is between 20-200 participants. The drugs in the experimental group involved curcumin, curcuminoids and Curcuma longa Extract, and their types of preparation were different. 39, 40 and 41 used two different doses of curcumin intervention (high-dose group and low-dose group), so they are divided into 39 a (low-dose group), 39 b (high-dose group), 40 (low-dose group), 40 (high-dose group), 41 (low-dose group) and 41 (high-dose group). The interventions of Chandran et al. (42) were divided into Curcumin 500 mg and Curcumin 500 mg+diclofenac sodium 50 mg, so they were also divided into Chandran et al. (42) (Curcumin only) and Chandran et al. (42) (Curcumin +Diclofenac sodium). At the same time, their control group was divided into two groups, matching the two experimental groups. 43 include 2 experimental groups and 2 control groups; it was divided into 43 (Curcuma longa Extract v.s. placebo) and 43 (Curcuma longa Extract+Glucosamine v.s. Glucosamine). The details of study characteristics are presented in **Table 1**.

3.3 Risk of Bias Assessments

3.3.1 Selection Bias

Nine RCTs did not described the random sequence generation methods and were rated as unclear risk of bias (39, 40, 44, 49, 51, 54, 60, 63–65). Other studies have explained the method of generating random sequences, so they are assessed as low risk of bias.

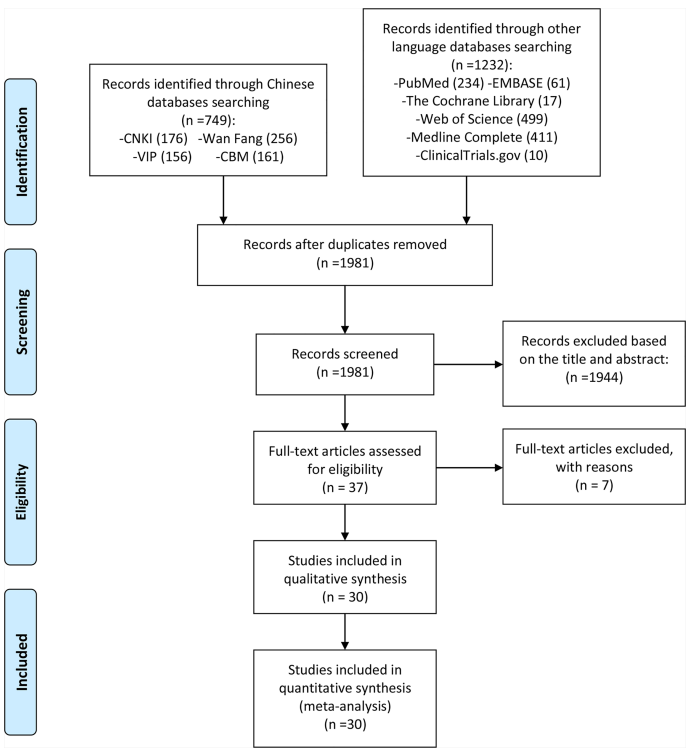

FIGURE 1 | Flow diagram of clinical trials.

TABLE 1 | The characteristics of the included studies.

Disease	RCTs	Country	Number of Participants (Female/male)		Intervention		Outcomes	Age (years)		Course of disease (years)		Duration
			Trial	Control	Trial	Control		Trial	Control	Trial	Control	
RA	Javadi et al. (44)	Iran	21/3	23/2	Curcumin nanomicelles 40mg Tid	wheat flour (placebo) 500mg Tid	DAS28, Tender joint count, Swollen joint count, ESR, adverse events	53.71 ± 2.75	56.28 ± 2.5	8.5 ± 9.08	7.36 ± 5.29	12 weeks
	Amalraj et al. (39)	India	8/16	7/5	Curcumin 250mg or 500mg	placebo	DAS28, ESR, CRP, Tender joint count, Swollen joint count, RF, adverse events	36.7 ± 10.7 (250mg); 38.3 ± 5.8 (500mg)	39.6 ± 8.8	–	–	12 weeks
	Jacob et al. (40)	India	7/9	5/3	Curcumin 250mg or 500mg	placebo	DAS28, ESR, CRP, RF	18-65	18-65	–	–	12 weeks
	Chandran et al. (42)	India	24/6	14/1	Curcumin 500 mg or Curcumin 500 mg+diclofenac sodium 50 mg	Diclofenac sodium 50 mg	DAS28, ESR, CRP, adverse events	47.8 ± 8.60 (Curcumin only); 47 ± 16.22 (combination)	48.87 ± 10.78	–	–	8 weeks
	Lin et al. (45)	China	32/32	33/31	Curcumin 100 mg Tid +Methotrexate 12.5mg once a week	Methotrexate 12.5mg once a week	DAS28, adverse events	48.39 ± 9.70	45 ± 10.31	9.43 ± 7.93	11.94 ± 8.02	12 weeks
	Pourhabibi-Zarandi et al. (46)	Iran	22/0	22/0	Curcumin 500 mg	placebo	ESR, CRP	50.68 ± 9.93	50.36 ± 9.70	9.77 ± 3.49	8.09 ± 3.13	8 weeks
OA	Hashemzadeh et al. 2007 (47)	Iran	29/7	31/4	Curcuminoids (SinaCurcumin™) 40mg	Placebo	WOMAC score, adverse events	54.11 ± 5.80	56.54 ± 5.77	4.51 ± 0.48	4.71 ± 0.48	6 weeks
		Italy	14/9	19/8	CartiJoint Forte (containing glucosamine hydrochloride (GH), chondroitin sulfate (CS) and Bio-Curcumin) + physical therapy	Placebo + physical therapy	VAS, WOMAC score, adverse events	71.3 ± 8.8	71.0 ± 8.0	6.8 ± 7.6	7.2 ± 6.0	8 weeks
	Haroyan et al. (48)	Armenia	62/5	65/3	Curcuminoids 999mg (CuraMed® 1500mg)	Placebo	WOMAC score, ESR, CRP, adverse events	54.65 ± 8.84	56.04 ± 8.55	–	–	12 weeks
	Nakagawa et al. (49)	Japan	14/4	18/5	Curcumin 180 mg	Placebo	VAS, adverse events	71.9 ± 5.3	66.1 ± 7.2	–	–	8 weeks
	Madhu et al. (43)	India	41/19	42/18	Curcuma longa Extract 1000 mg or Curcuma longa Extract 1000 mg+ Glucosamine 1500 mg	Glucosamine 1500 mg or Placebo (Microcrystalline cellulose) 800mg	VAS, adverse events	56.63 ± 10.58; 58.17 ± 9.30	56.80 ± 7.99; 56.77 ± 9.98	–	–	6 weeks
	Srivastava et al. (50)	India	53/25	50/32	Curcuma longa Extract 500 mg +Diclofenac 50 mg	Placebo 500mg +Diclofenac 50 mg	VAS, WOMAC score, adverse events	50.23 ± 8.08	50.27 ± 8.63	–	–	16 weeks
	Khanna et al. (51)	India	42/38		Curcumagalactomannosides 400mg + glucosamine hydrochloride 500mg	Chondroitin sulphate 415 mg+ glucosamine hydrochloride 500 mg	VAS, WOMAC score	53.4 ± 6.64	51.5 ± 5.95	–	–	12 weeks
	Shep et al. (52)	India	48/21	45/25	Curcumin (BCM-95®) 1500 mg	Diclofenac sodium 100mg	VAS, KOOS, adverse events	53.09 ± 4.17	52.14 ± 3.76	0.62 ± 0.29	0.62 ± 0.26	4 weeks
	Thomas et al. (53)	India	16/19	21/16	Curcuminoids 500mg	Chondroitin sulphate 830 mg+	VAS, WOMAC score, adverse events	51.7 ± 5.52	52.3 ± 4.59	3.51 ± 0.56	3.38 ± 0.72	6 weeks

(Continued)

TABLE 1 | Continued

Disease	RCTs	Country	Number of Participants (Female/male)		Intervention		Outcomes	Age (years)		Course of disease (years)		Duration
			Trial	Control	Trial	Control		Trial	Control	Trial	Control	
						glucosamine hydrochloride 1000 mg						
	Wang et al. (24)	Australia	18/18	21/13	Curcuma longa Extract 1000 mg	Placebo	VAS, WOMAC score, adverse events	61.3 ± 8.5	62.4 ± 8.8	–	–	12 weeks
	Pinsornsak et al. (54)	Thailand	62/13		Curcumin 1000 mg+diclofenac 75 mg	Diclofenac 75 mg	VAS	–	–	–	–	12 weeks
	Jamali et al. (55)	Iran	22/14	23/13	Curcumin ointment	Placebo (Vaseline ointment)	VAS, adverse events	68.86 ± 6.27	67.94 ± 6.72	7.22 ± 4.46	6.91 ± 4.68	6 weeks
	Kuptniratsaikul et al. (56)	Thailand	41/11	45/10	Curcuma longa Extract 2000 mg	Ibuprofen 800 mg	VAS, adverse events	61.4 ± 8.7	60.0 ± 8.4	1.59 ± 1.63	1.86 ± 2.2	6 weeks
	Kuptniratsaikul et al. (57)	Thailand	157/14	139/21	Curcuma longa Extract 1500 mg	Ibuprofen 1200 mg	WOMAC score, adverse events	60.3 ± 6.8	60.9 ± 6.9	4.28 ± 4.45	4.33 ± 4.31	4 weeks
	Panahi et al. (58)	Iran	14/5	17/4	Curcuminoid 1500mg	Placebo (inert starch)	VAS, WOMAC score, adverse events	57.32 ± 8.78	57.57 ± 9.05	–	–	6 weeks
	Lopresti et al. (59)	Australia	24/27	26/24	Curcuma longa Extract (Curcugen) 1000mg	Placebo	KOOS, adverse events	59.59 ± 6.57	57.92 ± 6.22	–	–	8 weeks
	Henrotin et al. (41)	Belgium	79/17	34/11	Curcuma longa Extract 280mg or 197mg	Placebo	VAS, KOOS, adverse events	60.9 ± 9.78; 61.4 ± 7.49	63.3 ± 7.69	7.41 ± 7.29; 6.6 ± 4.67	7.6 ± 9.3	12 weeks
	Kertia et al. (60)	Indonesia	24/15	29/12	Curcuminoid 90mg	Diclofenac sodium 90mg	COX-2	64.05 ± 8.83	64.56 ± 8.86	3.44 ± 2.72	3.36 ± 2.57	4 weeks
	Panahi et al. (66)	Iran	14/5	17/4	Curcuminoids (C3 complex®) 1500 mg	Placebo (inert starch)	SOD, GSH, MDA	57.32 ± 8.78	57.57 ± 9.05	–	–	6 weeks
	Gupte et al. (61)	India	11/6	23/2	Curcuma longa Extract 800mg	Ibuprofen + placebo	VAS, WOMAC score, adverse events	57 ± 7.5	54 ± 8	almost 1-11		6 weeks
AS	Ahmadi et al. (Ahmadi et al., 2020 (62)	Iran	12/0	12/0	Nanocurcumin	Placebo	Inflammatory factor	23-32	27-46	3-22	6-17	16 weeks
Juvenile oligoarthritis	Ailioaie and Ailioaie (63)	Romania	16 (not known)	16 (not known)	Protein Curcumin Complex 1800mg	Placebo	VAS, ACR Pedi30, ACR Pedi50, ACR Pedi70, ACR Pedi90, adverse events	8-16		–	–	36 weeks
	Ailioaie and Ailioaie (64)	Romania	28 (not known)	20 (not known)	Ultra Bioavailable Curcumin 1200mg	Placebo	JADAS-71	Mean: 13.8		–	–	24 weeks
Gout/ hyperuricemia	Bupparenoo et al. (65)	Thailand	20 (8/12)	19 (7/12)	Curcumin 1000mg	Placebo	Serum uric acid, urine uric acid clearance, fasting plasma glucose, blood lipids, adverse events	55.5 ± 8.7	55.2 ± 13.0	–	–	8 weeks

63 and 39, 40, 45, 51, 60, 64, 66 did not specify whether to perform allocation concealment and were therefore assessed as unclear risk of bias. Other studies have described the method of allocation concealment, so they are assessed as low risk of bias.

3.3.2 Performance Bias and Detection Bias

39, 40, 42, 49–51, 63 and 54, 58, 64, 65 stated that they used blinding, but did not describe how the blinding was performed, and was rated as unclear risk of bias. 45, 52, 53, 61 did not describe whether blinding was used, and its primary outcome is subjective evaluation index, which is easily affected by non-blinding, so it is assessed as a high risk of bias. 56 described only the blinding for outcome assessment and not the blinding of patients, it was rated as low risk for blinding of outcome assessment and high risk for bling of participants and personnel. 43 used blinding only on the participants, not the measurers, and thus it was rated as having low risk of bias in performance bias and having high risk of bias in detection bias. The other RCTs described the method blind implementation to patients and researchers or their outcomes are objective indicators, and are therefore considered to be a low risk of bias.

3.3.3 Attrition Bias and Reporting Bias

The remaining RCTs did not have incomplete outcomes or the reasons for the missing and the number are balanced, hence they are therefore assessed as low risk of bias. Allioaie and Ailioaie 63 and Allioaie and Ailioaie 64 only have abstracts and no proposals for outcomes, so we do not have enough information to rate whether there is selective reporting, so it is assessed as unclear risk of bias. The other RCTs do not have selective reporting and are therefore considered to be a low risk of bias.

3.3.4 Other Potential Bias

Ailioaie and Ailioaie 2018 only have abstracts, so we do not have enough information to rate whether there is selective reporting, so it is assessed as unclear risk of bias. 39–41, 49, 51, 53, 54, 59, 67, 68 claimed that authors have received funding from company that produces curcumin or Curcuma longa Extract; or that claimed that authors are the employees of company that produces curcumin or Curcuma longa Extract, hence they were rated as high risk of bias. Other sources of bias were not observed in 5 RCTs; therefore, the risks of other bias of the RCTs were low. (**Figure 2**)

3.4 The Outcomes for RA

3.4.1 Efficacy Indicators

(1) DAS28: Five (5) RCTs reported the DAS28. The result of heterogeneity analysis was I2 = 85% and P<0.00001, which showed that there was statistical heterogeneity among the 5 studies, so the random effects model was used. The results of Meta analysis showed that there was a statistical difference between the experimental group and the control group (P<0.0001), which indicates that curcumin may decrease DAS28 [WMD -1.06 (-1.53, -0.59)] (**Figure 3**).

(2) tender joint count: Two (2) RCTs reported the tender joint count. The result of heterogeneity analysis was I2 = 95% and P<0.00001, which showed that there was statistical heterogeneity among the 2 studies, so the random effects model was used. The results of Meta analysis showed that the difference between the experimental group and control group is of no statistical significance [SMD -3.91 (-8.60, 0.78), P=0.10] (**Figure 4A**).

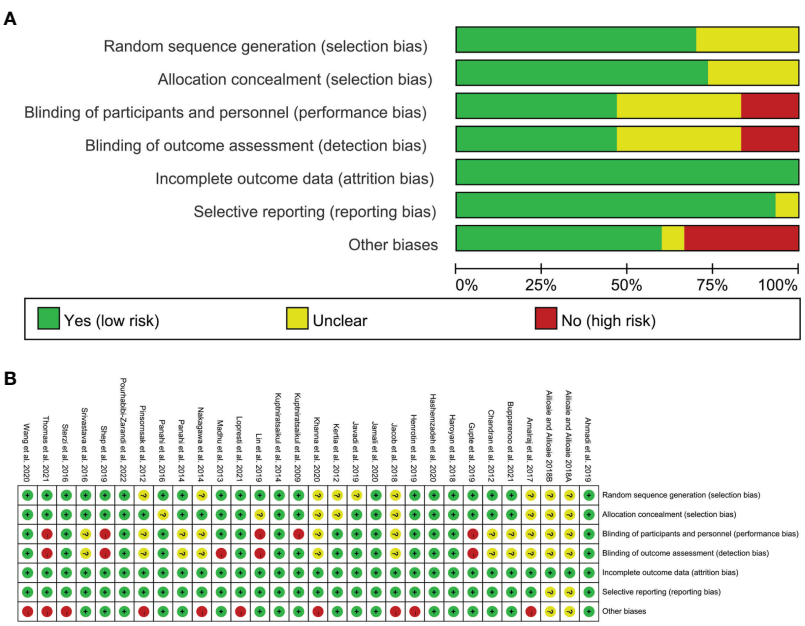

FIGURE 2 | Risk of bias [**(A)** graph; **(B)** summary].

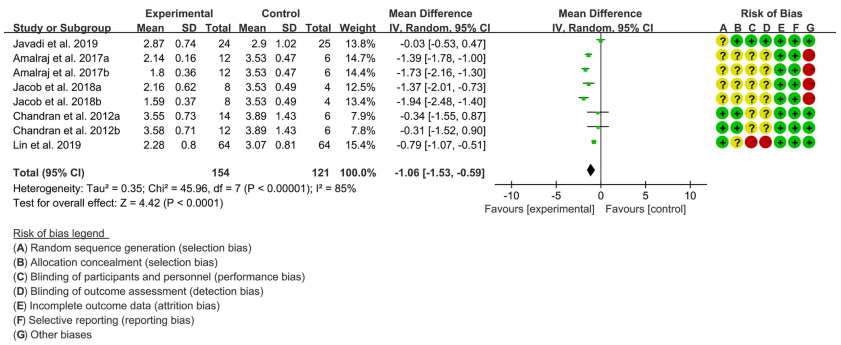

FIGURE 3 | The results of DAS28.

(3) swollen joint count: Two (2) RCTs reported the swollen joint count. The result of heterogeneity analysis was I2 = 95% and P<0.00001, which showed that there was statistical heterogeneity among the 2 studies, so the random effects model was used. The results of Meta analysis showed that the difference between the experimental group and control group is of no statistical significance [SMD -3.75 (-8.32, 0.81), P=0.11] (**Figure 4B**).

3.4.2 Inflammatory Indicator

(1) ESR: Five (5) RCTs reported the ESR. The result of heterogeneity analysis was I2 = 91% and P<0.00001, which showed that there was statistical heterogeneity among the 5 studies, so the random effects model was used. The results of Meta analysis showed that there was a statistical difference between the experimental group and the control group (P<0.0001), which indicates that curcumin may decrease ESR [SMD -3.09 (-4.60, -1.58)] (**Figure 5A**).

(2) CRP: Four (4) RCTs reported the CRP. Since Chandran and Goel (42) differed in baseline CRP, the endpoint results were not comparable and the data were excluded. The result of heterogeneity analysis was I2 = 93% and P<0.00001, which showed that there was statistical heterogeneity among the 4 studies, so the random effects model was used. The results of Meta analysis showed that there was a statistical difference between the experimental group and the control group (P=0.0005), which indicates that curcumin may decrease CRP [WMD -0.35 (-0.55, -0.15)] (**Figure 5B**).

(3) RF: Two (2) RCTs reported RF. The result of heterogeneity analysis was I2 = 0% and P=0.97, which showed that there was no statistical heterogeneity among the 2 studies, so the fixed effects model was used. The results of Meta analysis

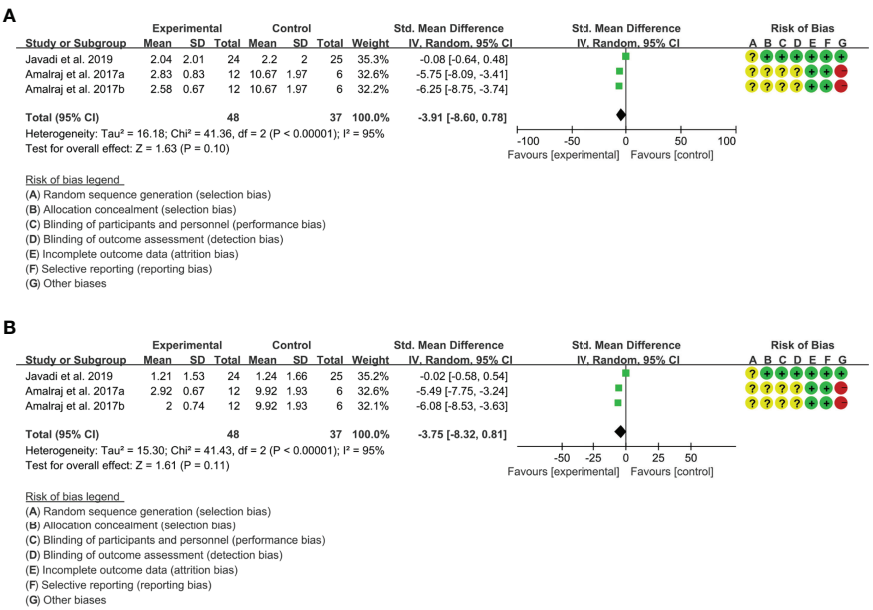

FIGURE 4 | **(A)** Tender joint count; **(B)** Swollen joint count.

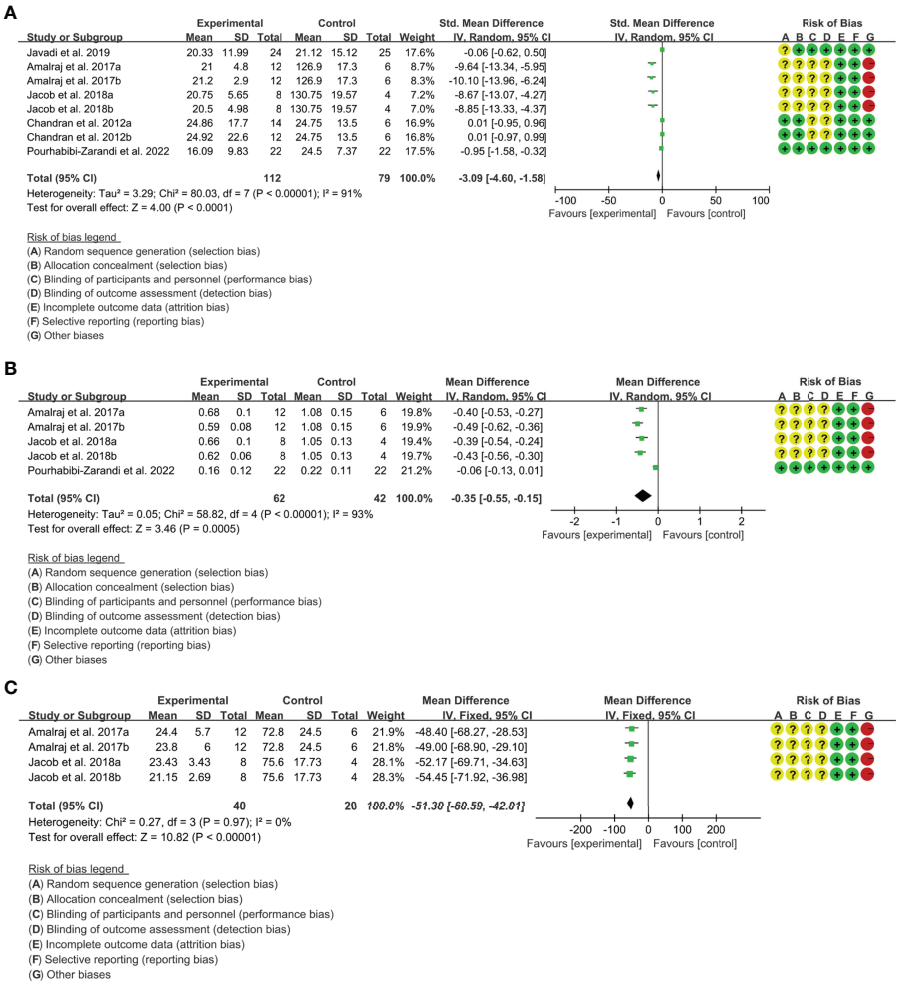

FIGURE 5 | The results of ESR **(A)**, CRP **(B)** and RF **(C)**.

showed that there was a statistical difference between the experimental group and the control group (P<0.00001), which indicates that curcumin may decrease RF [WMD -51.30 (-60.59, -42.01)] (**Figure 5C**).

3.4.3 Adverse Events

Four (4) RCTs reported the adverse events. The result of heterogeneity analysis was I2 = 0% and P=0.93, which showed that there was no statistical heterogeneity among the 4 studies, so the fixed effects model was used. The results of Meta analysis showed that the difference between the experimental group and control group is of no statistical significance [RR 0.36 (0.11, 1.15), P=0.08] (**Figure 6**).

3.5 The Outcomes for OA

3.5.1 Efficacy Indicators

The efficacy indicators include pain (include VAS and WOMAC-pain), physical function and stiffness. The efficacy indicators were divided into subgroups according to the intervention methods (**Table 2**).

(1) Pain: The results of Meta analysis showed that there was a statistical difference between the experimental group and the control group (VAS: P<0.0001; WOMAC-pain: P<0.00001), which indicates that curcumin may decrease VAS and WOMAC-pain [VAS: SMD -2.03 (-3.03, -1.03); WOMAC-pain: SMD -0.69 (-0.83, -0.55)] (**Figures 7**, **8**).

(2) Physical function: The results of Meta analysis showed that there was a statistical difference between the experimental group and the control group (P=0.001), which indicates that curcumin may decrease WOMAC-physical function [SMD -1.65 (-2.65, -0.64)] (**Figure 9**).

(3) Stiffness: The results of Meta analysis showed that there was a statistical difference between the experimental group and the control group (P=0.0007), which indicates that curcumin may decrease WOMAC-stiffness [SMD -0.22 (-0.35, -0.09)] (**Figure 10**).

3.5.2 Inflammatory Indicators

The inflammatory indicators include ESR, CRP and COX-2.

(1) ESR: Two (2) RCTs reported the ESR. The result of heterogeneity analysis was I2 = 0% and P=0.41, which showed that there was no statistical heterogeneity among the 2 studies, so

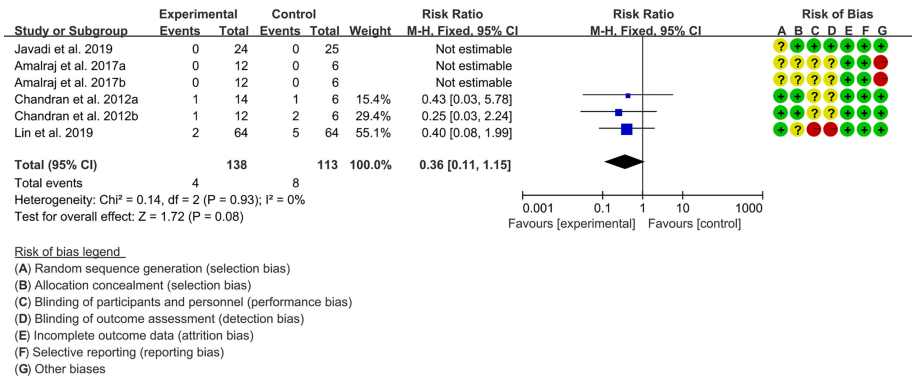

FIGURE 6 | Adverse events.

TABLE 2 | The subgroup analysis of OA efficacy indicators and adverse events.

Outcomes	Subgroup	Overall effect					Heterogeneity test			Figure
		MD	95%CI	P	I^2 (%)	P	Statistical method	Studies (N)	Sample size (N)	
VAS	Curcuma longa Extract and curcumin (C.) v.s. placebo	-1.33	[-2.23, -0.43]	P=0.004	94	P<0.0001	Random	6	431	**Figure 7**
	C. v.s. NSAIDs	-0.07	[-0.32, 0.19]	P=0.62	0	P=0.54	Random	2	230	
	C.+ NSAIDs v.s. NSAIDs	-9.37	[-10.45, -8.28]	P<0.0001	–	–	Random	1	160	
	C. v.s. Articular cartilage nutrition drug	-2.71	[-5.91, 0.39]	P=0.09	98	P<0.0001	Random	2	204	
WOMAC-pain	C. v.s. placebo	-0.66	[-0.88, -0.43]	P<0.0001	34	P=0.21	Fixed	4	315	**Figure 8**
	C. v.s. NSAIDs	0.04	[-0.18, 0.25]	P=0.72	–	–	Fixed	1	331	
	C.+ NSAIDs v.s. NSAIDs	-4.1	[-4.65, -3.55]	P<0.0001	–	–	Fixed	1	160	
	C. v.s. Articular cartilage nutrition drug	-1.33	[-1.69, -0.98]	P<0.0001	1	P=0.32	Fixed	2	152	
WOMAC-physical function	C. v.s. placebo	-0.79	[-1.27, -0.31]	P=0.001	75	P=0.008	Random	4	315	**Figure 9**
	C. v.s. NSAIDs	0.07	[-0.14, 0.29]	p=0.51	–	–	Random	1	331	
	C.+ NSAIDs v.s. NSAIDs	-3.81	[-4.34, -3.29]	P<0.00001	–	–	Random	1	160	
	C. v.s. Articular cartilage nutrition drug	-3.1	[-4.34, -1.86]	P<0.00001	84	P=0.01	Random	2	152	
WOMAC-stiffness	C. v.s. placebo	-0.35	[-0.57, -0.12]	P=0.002	26	P=0.25	Fixed	4	315	**Figure 10**
	C. v.s. NSAIDs	0.05	[-0.17, 0.27]	P=0.65	–	–	Fixed	1	331	
	C.+ NSAIDs v.s. NSAIDs	-0.45	[-0.77, -0.14]	P=0.005	–	–	Fixed	1	160	
	C. v.s. Articular cartilage nutrition drug	-0.32	[-0.64, -0.00]	P=0.05	0	P=0.49	Fixed	2	152	
Adverse events	C. v.s. placebo	1.18	[0.71, 1.94]	P=0.52	25	P=0.25	Random	8	629	**Figure 13**
	C. v.s. NSAIDs	0.55	[0.34, 0.88]	p=0.01	70	P=0.03	Random	3	561	
	C.+ NSAIDs v.s. NSAIDs	0.53	[0.10, 2.79]	P=0.45	–	–	Random	1	160	
	C. v.s. Articular cartilage nutrition drug	0.58	[0.27, 1.24]	P=0.16	0	P=0.49	Random	2	158	

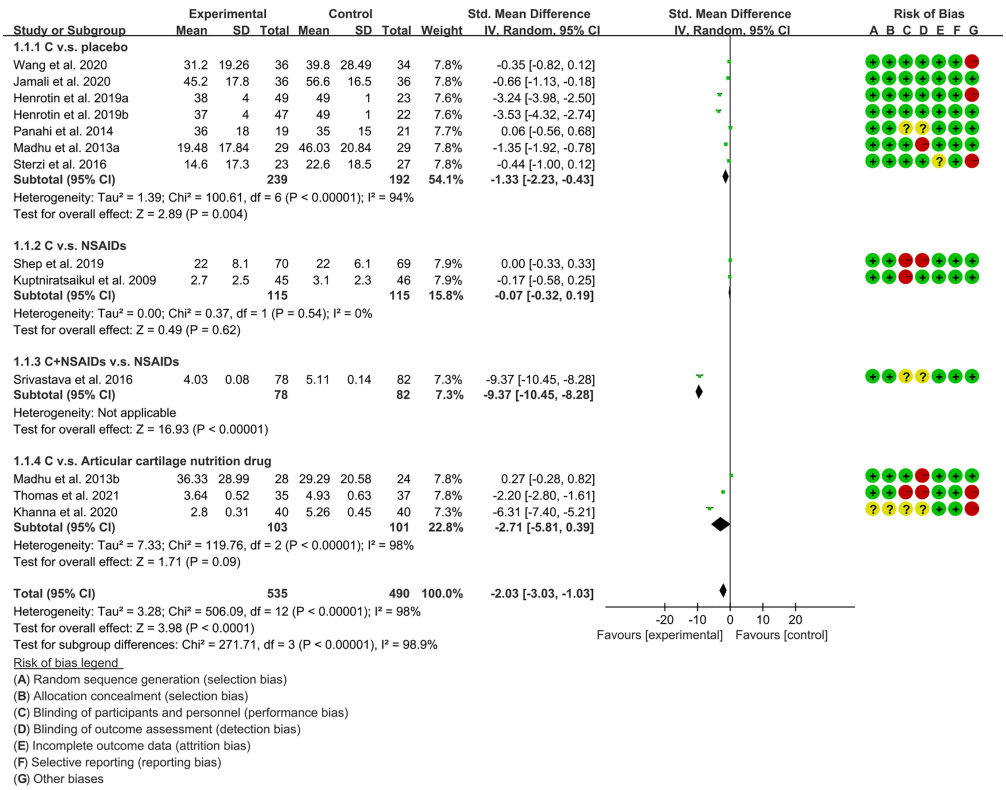

FIGURE 7 | The results of VAS.

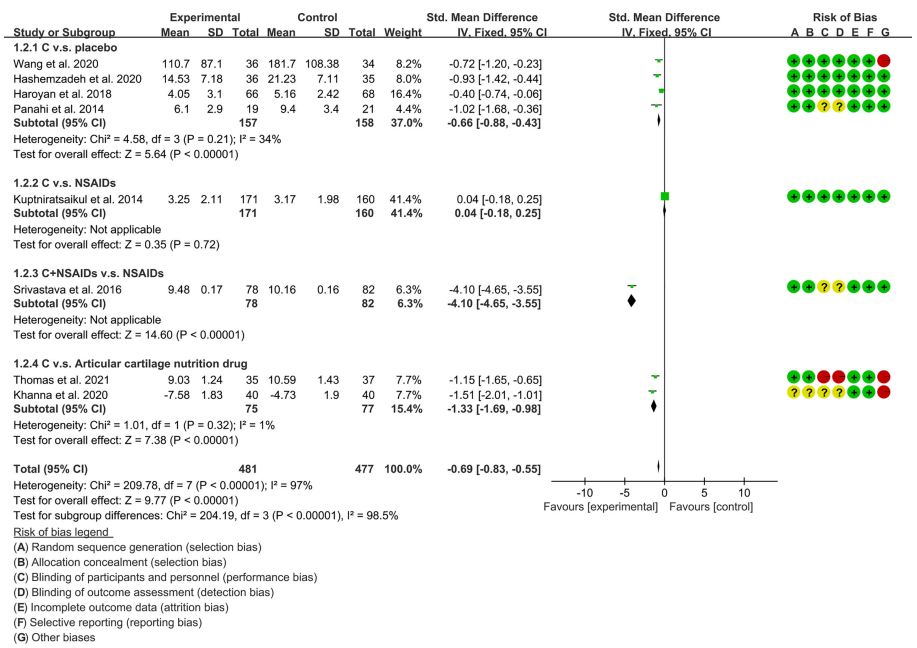

FIGURE 8 | WOMAC-pain.

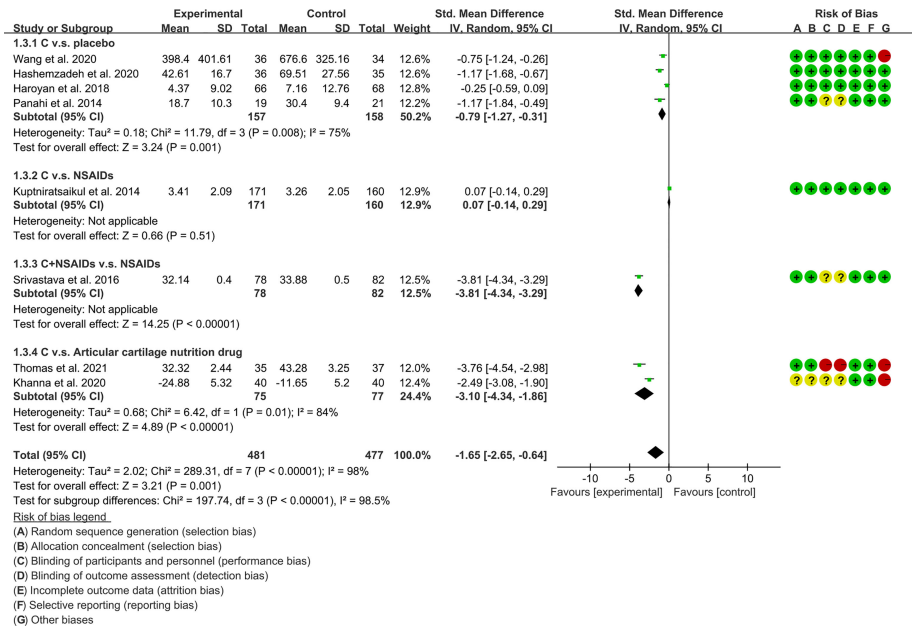

FIGURE 9 | WOMAC-physical function.

the fixed effects model was used. The results of Meta analysis showed that there was a statistical difference between the experimental group and the control group (P<0.0001), which indicates that curcumin may decrease ESR [WMD -1.00 (-1.26, -0.74)] (**Figure 11A**).

(2) CRP: Three (3) RCTs reported the CRP. The result of heterogeneity analysis was I2 = 94% and P<0.00001, which showed that there was statistical heterogeneity among the 3 studies, so the random effects model was used. The results of Meta analysis showed that there was no a statistical difference

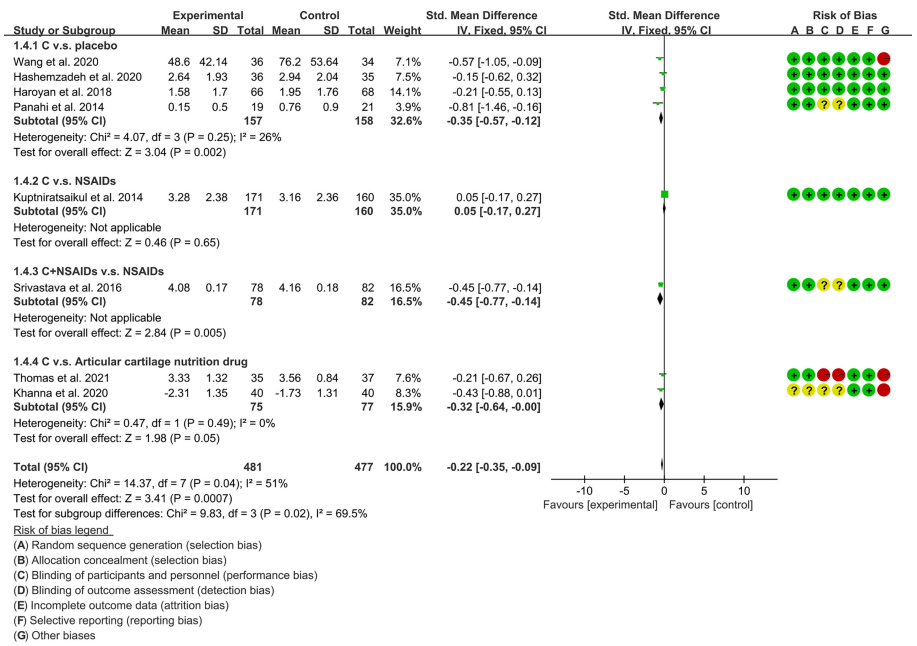

FIGURE 10 | WOMAC -stiffness.

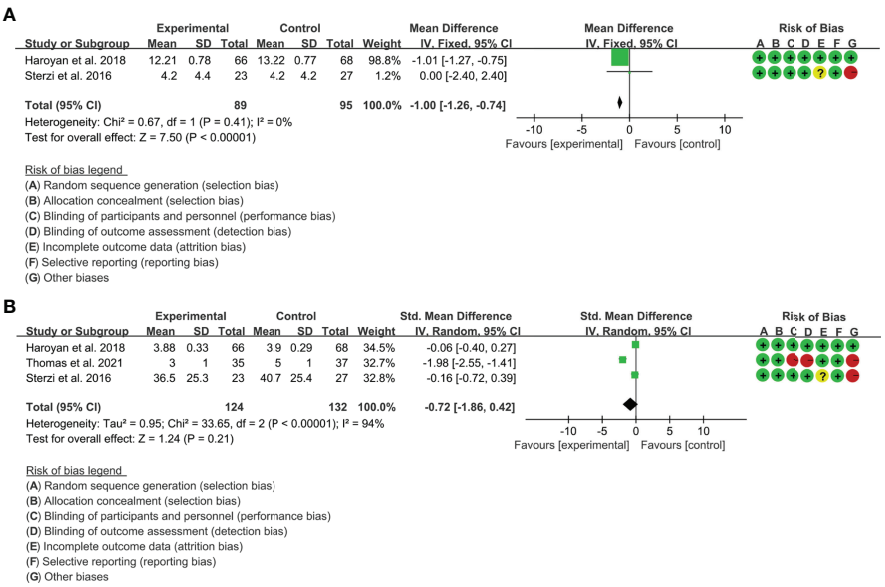

FIGURE 11 | The results of ESR **(A)** and CRP **(B)**.

between the experimental group and the control group [SMD -1.00 (-1.86, 0.42), P=0.21] (**Figure 11B**).

(3) COX-2 was only reported by 60, who found no significant difference in COX-2 between the diclofenac sodium group and the curcumin group (P=0.89).

3.5.3 Oxidative Stress Related Outcomes

The indicators related to oxidative stress include SOD, GSH, and MDA.

Two (2) RCTs reported MDA. The result of heterogeneity analysis was I2 = 94%, P<0.0001, which showed that there was statistical heterogeneity among the 2 studies, so the random effects model was used. The results of Meta analysis showed that there was a statistical difference between the experimental group and the control group (P=0.02), which indicates that curcumin may decrease MDA [WMD -2.06, (-3.80 to -0.32)]. (**Figure 12**).

Only Panahi et al., 2016 reported improvements in SOD and GSH and found higher serum SOD activity in the curcumin group compared to placebo (P<0.001). However, there was no statistically significant difference in GSH levels between the curcumin group and the placebo group (P=0.064).

3.5.4 Adverse Events

The subgroup analysis results were shown in **Table 2**. 49, 53, 61 and 67 claimed that no serious adverse events were observed in either the experimental group or the control group. The summary result showed a borderline difference [RR 0.74, (0.54, 1.00), P=0.05; random effect model]. (**Figure 13**). If the study increases, it may be possible to find fewer adverse events with the addition of curcumin.

3.6 The Outcomes for AS

Only Ahmadi et al., 2020 (62) reported on AS. They reported that 12 patients received Nanocurcumin and 12 received Placebo. They found that the AS patients in the Nanocurcumin group had significantly increased Treg cells, increased IL-10 and TGF-β levels, and decreased IL-6 levels compared to control group. They also found that Nanocurcumin decreased the expressions

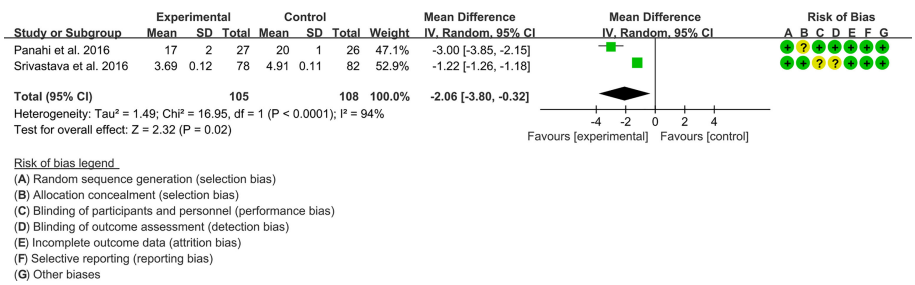

FIGURE 12 | The results of MDA.

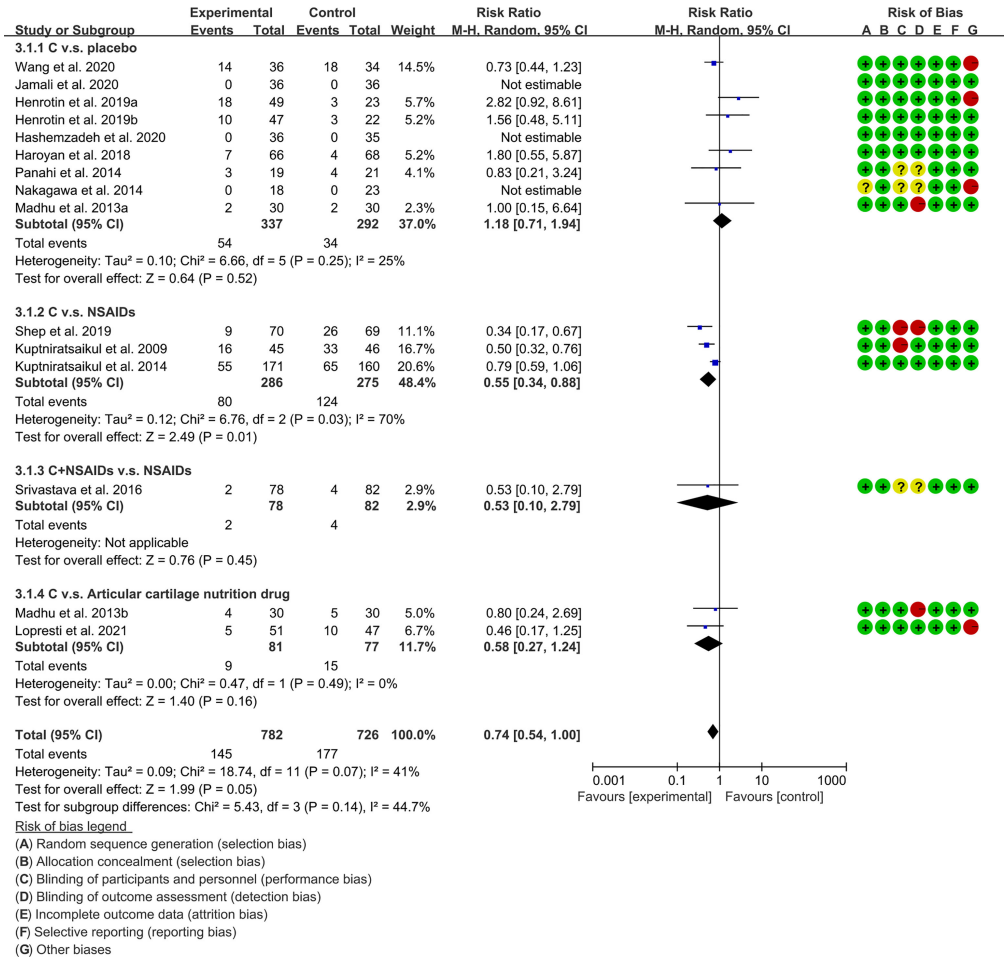

FIGURE 13 | Adverse events.

of miR-17 and miR-27 and increased the expressions of miR-146a and FoxP3 (P<0.05).

3.7 The Outcomes for JIA

Two RCTs reported on JIA. Ailioaie et al., 63 enrolled 32 patients, ages 8-16, and randomly assigned them to curcumin group (receiving 600 mg three times a day) or placebo group for 9 months, while all patients received standard treat. Their study showed that compared with the control group, ACR Pedi30, ACR Pedi50, ACR Pedi70, and ACR Pedi90 were significantly improved in the curcumin group (P<0.05), and the addition of curcumin (1800mg/day) did not increase the incidence of adverse events. In another study, 48 children (mean age, 13.8 years) with extensive oligoarticular and polyarticular JIA were randomly assigned to experiment group (receiving curcumin 1,200 mg+blue laser) or control group (receiving placebo) for 6 months. They found that curcumin+blue laser reduced disease activity according to the Disease Activity Score (JADAS-71) and pain levels (0-10 cm VAS), it also increased their functional activities of daily living (CHAQ scores) compared to placebo (64).

3.8 The Outcomes for Gout/Hyperuricemia

Only 65 reported on hyperuricemia. They found that curcumin intervention tended to reduce serum uric acid compared with placebo, but the difference was not statistically significant (P=0.532). There were no significant differences in urine uric acid clearance, fasting plasma glucose, and blood lipids between the two groups (P>0.05). Compared with the placebo group, curcumin did not increase the incidence of adverse events (P>0.05), and the most common adverse event was diarrhea.

4 DISCUSSION

The mechanism of Curcumin and Curcuma longa Extract in the treatment of arthritis is shown in **Figure 14**.

4.1 Curcumin and *Curcuma longa* Extract for RA

This study systematically evaluated the clinical efficacy and safety of curcumin in the treatment of RA. The clinical efficacy

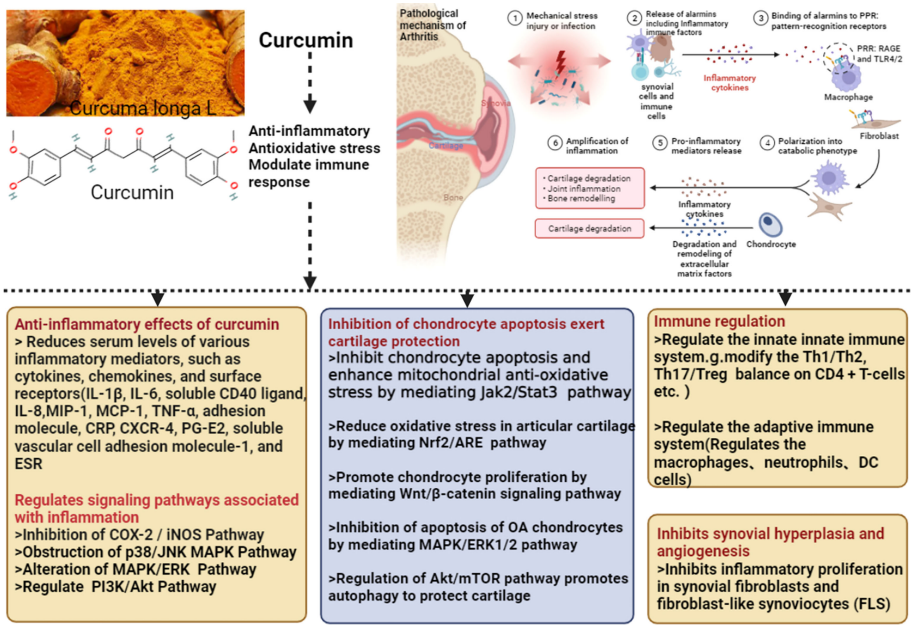

FIGURE 14 | The mechanism of Curcumin and Curcuma longa Extract in the treatment of arthritis.

indicators DAS28, ESR, CRP and RF were lower than those in the control group, indicating that curcumin may improve the symptoms of RA and inhibit the inflammatory response. For safety, the addition of curcumin may not increase the probability of adverse events.

Sun et al. constructed a model of destabilization of the medial meniscus (DMM) in mice. They found that curcumin can reduce the expression of IL-1β, interferon-γ (IFN-γ), IL17α, IL-18, TNF-α, (vascular cell adhesion molecule 1 (VCAM1), and inhibit the inflammatory response in mice (69). This is similar to the findings of this study. This meta-analysis shows that curcumin can reduce inflammation indicators (RF, ESR, CRP). Recent studies have shown that curcumin may inhibit osteoclast (OC) differentiation in RA patients by down-regulating the expression of RANK gene and related proteins (70). Curcumin combined with methotrexate can effectively improve the joint and systemic symptoms of RA patients, and can improve bone destruction, and has a significant intervention effect on the RANK/RANKL/OPG system (45). Curcumin can also inhibit the proliferation of fibroblast-like synovial cells (RA-FLS) and reduce the secretion of TNF-α and IL-6 in RA patients (71). Curcumin has a therapeutic effect on rats with type II collagen-induced arthritis (CIA). It can effectively inhibit macrophage-related inflammation and reduce the synovial homogenate of joints. It can also reduce the degradation of IκBα and the expression of COX-2 in RAW 264.7 cells (72). Xu et al. found that curcumin can reduce osteoclast production by inhibiting NF-κB signaling activation in RA (73). Curcumin can inhibit synovial angiogenesis in adjuvant arthritis (AA) rats. The mechanism may be related to reducing the expression of HIF-1α and down-regulating the expression of target genes VEGF and VEGFR, which may be one of the mechanisms of its treatment of RA (74).

4.2 Curcumin and *Curcuma longa* Extract for OA

The meta-analysis of OA found that: (1) Compared with placebo, Curcumin and Curcuma longa Extract may reduce pain, improve joint function, and improve joint stiffness; and the addition of Curcumin and Curcuma longa Extract did not increase adverse events. (2) Curcumin and Curcuma longa Extract and NSAIDs have similar effects in improving joint pain, function, and stiffness, but with a lower incidence of adverse events. However, when curcumin was used in combination with NSAIDs, it improved joint pain, function, and stiffness more than NSAIDs alone, without increasing the rate of adverse events. But, due to the small number of RCTs, definitive conclusions are difficult to draw. (3) Compared with articular cartilage nutritional drugs, Curcumin and Curcuma longa Extract may improve joint pain, joint function, and joint stiffness without increasing the incidence of adverse events. (4) Compared with the control group, Curcumin and Curcuma longa Extract could reduce ESR and MDA levels. For SOD, GSH and COX-2, no clear conclusions could be drawn due to the small number of RCTs.

OA is a common chronic disease that mainly affects the knee joint, resulting in joint pain and loss of function (10). Knee OA carries a high societal cost, but management options are few and not ideal (75). Current medical treatment options are limited to analgesics, intra-articular corticosteroids, and NSAIDs (76). Although they have some efficacy in relieving pain, they are

associated with gastrointestinal, renal, and cardiovascular complications and are generally contraindicated in patients with comorbidities (77). *Curcuma longa L.* has a long history of medicinal use (78–80). Curcumin, the main and most pharmacologically active ingredient in *Curcuma longa L.*, is "generally recognized as safe" by the US FDA (81–83). Current *in vitro* and preclinical studies have demonstrated the potential of curcumin, Curcuma longa Extract, and other Curcuma longa Extract multi-herbal preparations to delay OA progression and relieve OA-related pain (84–86).

Compared to previous systematic reviews (86, 87), this study pooled the largest number of RCTs, including more evidence and relevant content. This improves the quality and evidence of this study to provide more realistic and precise effect sizes. In addition, we found that OA patients in the curcumin group may be less likely to initiate pain medication and more likely to discontinue their existing pain medication because of its efficacy and better safety profile compared to the NSAIDs group. A previous systematic review report on the effects of Curcuma longa Extract on chronic inflammatory diseases, including rheumatic diseases, showed no significant between-group differences in inflammatory markers between Curcuma longa Extract and placebo (88), however, our results found that Curcuma longa Extract could improve ESR in OA patients. Although Curcumin and Curcuma longa Extract are effective and safe for OA, these results are only from short-term studies (maximum follow-up of 16 weeks), but are expected to be effective and safe drugs, as most current OA drug treatments have poor safety profiles (89). Furthermore, the meta-analyses showed significant heterogeneity, which could be explained by study-level covariates such as BMI and age.

4.3 Curcumin and *Curcuma longa* Extract for AS

AS mainly affects the axial skeleton, sacroiliac joints, and peripheral joints, causing structural changes and dysfunction, and is a chronic autoimmune inflammatory disease (90–92). Chronic inflammation of the spine resulting from the progression of AS leads to the formation of new bone on the spine, ultimately resulting in spinal immobility and stiffness (93). Previous studies have suggested that the pathogenesis of AS may be related to bacterial infection and human leukocyte antigen B27 (HLA-B27), and recent studies also suggest that T lymphocytes may mediate AS (94, 95). This notion is supported by changes in peripheral blood (PB) CD4+ T cell frequency in patients with AS, including an increase in Th17 frequency with Th2 and a decrease in CD4+CD25+ regulatory T (Treg) cells (93, 95–97). Numerous studies have shown that patients with AS have a decreased Treg/Th17 ratio, suggesting that immune phenotype changes may be one of the pathogenesis of AS, and that regulating the balance of Treg/Th17 may reduce disease activity (96, 98, 99). Treg/Th17 functional balance is critical for the prevention of autoimmune and inflammatory diseases by preventing deleterious damage to the host and generating an effective immune response.

The current flow cytometry analysis of PB from AS patients showed that daily treatment with nanocurcumin for 4 months significantly increased the percentage of PB Treg cells compared to patients receiving placebo. Recent studies have found that curcumin can enhance Treg differentiation by increasing the expression of FoxP3 (94, 96). The results showed that FoxP3 gene expression was significantly increased in AS patients after nano-curcumin treatment, which confirmed the effect of nano-curcumin in enhancing Treg cells in these patients. In conclusion, this RCT study showed that administration of nanocurcumin (80 mg/kg bw/day) for 4 months increased the Treg population and the expression levels of FoxP3, TGF-β and IL-10, as well as inhibited the IL-6 cytokine level. Furthermore, nanocurcumin could effectively alter the expression of Treg-related miRNAs (decreased miR-17, miR-27 and increased miR-146a) during the follow-up of AS patients. More research is still needed in the future to further explore the exact biological process that curcumin modulates in AS patients. In addition, higher-quality multiple RCTs provide higher-quality evidence, thereby providing clinical value.

4.4 Curcumin and *Curcuma longa* Extract for JIA

The pathogenesis of JIA is related to a variety of factors, including genetic factors, immune responses, and environmental exposures. The pathogenesis of JIA is associated with aberrant activation of phagocytes (monocytes, macrophages, and neutrophils), suppression of Treg cells, hyperactivation of Th1 and Th17 cells, activation of NF-κB, and proinflammatory cytokines (IL-1, IL-6, IL-17, IL-18, IL-21, IL-22, IL-23, Interferon-γ [IFNγ] and TNF-α) (100–103). Currently, immunomodulatory drugs are the cornerstone of the treatment of JIA for these pathological processes of immune inflammation. Ailioaie et al., 63 included 32 children (ages 8-16 years) with JIA. They found that compared with the control group, ACR Pedi30, ACR Pedi50, ACR Pedi70, and ACR Pedi90 were significantly improved in the curcumin group ($P<0.05$), and the addition of curcumin (1800mg/day) did not increase the incidence of adverse events. In another study, 48 children (mean age, 13.8 years) with extensive oligoarticular and polyarticular JIA were randomly assigned to experiment group (receiving curcumin 1,200 mg+blue laser) or control group (receiving placebo) for 6 months. They found that curcumin+blue laser reduced disease activity according to the Disease Activity Score (JADAS-71) and pain levels (0-10 cm VAS), it also increased their functional activities of daily living (CHAQ scores) compared to placebo (64)

In the Miserocchi et al. (104) cohort study, 27 patients (age 17.4 ± 8.9 years) with oligoarticular JIA-associated uveitis were recruited and received curcumin (500 mg per day) and JIA standard of care for 12 months. Uveitis is a serious extra-articular

complication of JIA. The severity of uveitis was assessed by slit-lamp examination and FC500 laser flare at baseline and 1, 3, 6, 9, and 12 months after curcumin initiation. A total of 22 patients (81%) had inactive uveitis at the end of the study. Five patients remained stable, and three developed uveitis flares. During the study period, curcumin supplements were well tolerated and no one experienced ocular side effects or allergic reactions [7]. Although the above studies have shown curcumin in JIA with considerable evidence of its clinical adjunctive value, more large-scale, randomized, placebo-controlled RCTs are needed in the future to confirm or revised the findings of these RCTs.

4.5 Curcumin and *Curcuma longa* Extract for Gout/Hyperuricemia

Hyperuricemia and gout is a metabolic abnormal syndrome caused by disturbance of purine metabolism. Because blood uric acid exceeds its saturation in blood or tissue fluid, sodium urate crystals are formed and deposited in the local joints, which induces local inflammation and tissue destruction symptoms (105, 106). The deposition of sodium urate crystals in the kidneys can cause acute kidney disease, chronic interstitial nephritis or kidney stones, which is called uric acid nephropathy. The common symptoms of hyperuricemia and gout are joint swelling and pain, starting from the feet, and then to the finger joints or wrists. Epidemiology shows that the number of patients with hyperuricemia and gout is increasing year by year worldwide, and it is expected to reach 1.42 billion in 2030 (107, 108). Patients with hyperuricemia and gout have poor compliance, with more than 50% of patients failing to follow doctor's orders for examination or re-examination, and the number of hyperuricemia and gout patients is still increasing (109; 110). Curcumin has shown potential effects in hyperuricemia and gout (65). Basic studies have shown that curcumin can inhibit the degradation of IκBα, the activation of NF-κB signaling pathway, and the inflammatory genes downstream of NF-κB in monosodium urate-stimulated THP-1-derived macrophages (111). Curcumin protected THP-1 and RAW264.7 cells from monosodium urate-induced mitochondrial damage by preventing the reduction of mitochondrial membrane potential, reducing mitochondrial reactive oxygen species, and then inhibiting the activity of the NLRP3 inflammasome. Animal studies have also shown that intraperitoneal injection of curcumin attenuates monosodium urate crystal-induced paw and ankle swelling, inflammatory cell infiltration, and MPO activity in a mouse model of acute gout. These results were related to inhibition of IκBα degradation, phosphorylation levels of NF-κB subunits (p65 and p50) (111). *In vitro* and *in vivo* studies on hyperuricemia have shown that curcumin and its degradation products have xanthine oxidase inhibition (112–114) and uric acid production by inhibiting URAT1 (115). Therefore, the drug may be effective in reducing serum uric acid. In addition, curcumin has been reported to reduce serum uric acid levels in other diseases (non-alcoholic fatty liver disease (NAFLD) and diabetes mellitus) (116; 117).

4.6 The Safety of Curcumin and *Curcuma longa* Extract

According to RCTs reporting adverse events, Curcumin and Curcuma longa Extract did not increase the occurrence of adverse events. According to the report of Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) and European Food Safety Authority (EFSA), the acceptable daily intake (ADI) value of curcumin is 0-3 mg/kg; it is also approved by the US Food and Drug Administration as a botanical (79). According to the relevant safety and toxicity clinical trials, the acceptable dose of curcumin to obtain the maximum efficacy is 4-8 g/d. The dose of 8 g/d curcumin was shown to be safe in both phase I and II clinical trials (118), and it has been reported that curcumin up to 12 g/d is still tolerated by humans (119). Studies have shown that curcumin has no obvious sub-chronic toxicity damage after animal toxicity test, and has no potential mutagenic or teratogenic effects (120). For example, Krishnaraju et al. conducted toxicological evaluations on the safety of demethyl curcumin (DC) through acute oral administration, acute skin, primary skin and eye irritation, and dose-dependent 90-day subchronic toxicity studies. They found that the acute oral median lethal dose (LDso) of DC in female SD rats was >5 000 mg/kg, and the acute dermal LD50 was >2000 mg/kg, and no weight change or adverse effects were observed after autopsy, which proved the broad-spectrum safety of DC (121). Dandekar P et al. evaluated the toxicology of curcumin-loaded nanoparticles. The results of acute toxicity studies showed that the dose of 2000 mg/kg was non-toxic, and the subacute toxicity studies demonstrated the safety of long-term administration at the usual therapeutic dose of 100 mg/kg curcumin and twice the therapeutic dose (122).

4.7 The Strengths and Limitations

The strengths of this study is that to our knowledge, this is the first systematic review and meta-analysis of RCTs on the efficacy of turmeric and its curcumin on arthritis. The limitations of this study are: (1) Because curcumin has not been widely used in clinical practice, the sample size of the included studies is limited. (2) There may be omissions in the collection of documents and data extraction or the researcher's subjective judgment is not strict. (3) The RCTs included in this study were at high risk of bias. The authors of some RCTs were funded by drug manufacturers or were employees, which may introduce bias. (4) Languages are limited to English and Chinese, and related studies in other languages are not included.

5 CONCLUSION

In summary, Curcumin and Curcuma longa Extract may improve symptoms and inflammation levels in people with arthritis. However, due to the low quality and small quantity of RCTs, the conclusions need to be interpreted carefully. Limited by the sample size of the included studies, large-sample, multi-center clinical trials are still needed for correction or verification.

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.

AUTHOR CONTRIBUTIONS

TY and LZ contributed equally to this work. All authors contributed to the article and approved the submitted version.

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Figure 14 was created with BioRender.com.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.891822/full#supplementary-material

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Identification of immune hub genes participating in the pathogenesis and progression of Vogt-Koyanagi-Harada disease

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Background: Vogt-Koyanagi-Harada (VKH) disease is an autoimmune inflammatory disorder characterized by bilateral granulomatous uveitis. The objective of this study was to identify immune hub genes involved in the pathogenesis and progression of VKH disease.

Methods: High throughput sequencing data were downloaded from the Gene Expression Omnibus (GEO) and an immune dataset was downloaded from ImmPort. Immune differentially expressed genes (DEGs) were obtained from their intersection in the GEO and ImmPort datasets. Immune hub genes for VKH disease were selected through differential expression analyses, including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Disease Ontology (DO), protein-protein interaction (PPI) network, and clustering analyses. Confidence in the immune hub genes was subsequently validated using box plots and receiver operating characteristic (ROC) curves.

Results: A total of 254 DEGs were screened and after the intersection with ImmPort, 20 genes were obtained as immune DEGs. Functional enrichment analysis indicated that the key genes were mainly involved in several types of immune pathways (such as the lymphocyte mediated and leukocyte mediated immune responses, natural killer cell mediated cytotoxicity, and antigen binding) and immunodeficiency diseases. Following PPI network analysis, the top seven genes in cluster 1 were selected as potential immune hub genes in VKH. After evaluating the accuracy of the hub genes, one gene (GNLY) was excluded because its expression level was statistically similar in VKH patients and healthy controls. Finally, six immune hub genes, namely KLRC2, KLRC3 SH2D1B, GZMB, KIR2DL3, and KIR3DL2 were identified as playing important roles in the occurrence and development of VKH disease.

Conclusion: Six immune hub genes (KLRC2, KLRC3 SH2D1B, GZMB, KIR2DL3, and KIR3DL2) identified by our bioinformatics analyses may provide new diagnostic and therapeutic targets for VKH disease.

KEYWORDS

Vogt-Koyanagi-Harada disease, functional enrichment analyses, protein-protein interaction network analysis, immune hub genes, receiver operating characteristic curves

Introduction

Vogt-Koyanagi-Harada (VKH) disease is an autoimmune inflammatory disorder with both intraocular and extraocular manifestations. Heavily pigmented races, such as Asians (1–3), Latin Americans (4, 5), and Middle Easterners (6–8), have high incidence rates of VKH disease, women are more susceptible than men (9, 10) and 20-to-50-year-olds are primarily affected (11). The disease progresses through prodromal, acute uveitic, chronic convalescent and chronic recurrent phases (12) and patients characteristically present with bilateral granulomatous uveitis. Retinal detachments, disk edema, vitritis and eventually sunset glow fundus are typical intraocular findings in VKH disease, accompanied by a series of systemic symptoms, such as headache, tinnitus, poliosis, vitiligo, and meningismus (13). The etiology and pathogenesis of the disease have not been fully clarified. Although melanocytes have been widely acknowledged as targets of autoimmune responses in VKH disease (14, 15), microbial infection (16), gene susceptibility (17), immune-related cells and pathways also have vital roles in its pathogenesis. For example, CD4+ T cells, Th1 cells, Th17 cells, and a series of cytokines have been found to be associated with the disease (14, 18–22). However, the underlying molecular mechanisms of the pathogenesis of VKH disease remain to be elucidated. Therefore, further research is essential to better understand its occurrence and progression.

Since the ocular tissue samples of patients with VKH disease (such as aqueous humor and choroid) are difficult to obtain, basic research on the pathogenesis of this disease is challenging, limiting the scope to further understand the mechanisms of immune-related cells and pathways involved in its progression. The rapid development of high throughput sequencing technology has facilitated the availability of key genes for the identification of diagnostic and therapeutic biomarkers of VKH disease. Since blood samples are easier to obtain than ocular tissues, evaluating gene expression in the peripheral blood of VKH patients may help enhance comprehension of the pathogenesis of VKH disease. To date, no bioinformatics analysis has focused on the mechanism of immune genes in VKH disease. Thus, we analyzed VKH disease-related datasets to screen differentially expressed genes (DEGs) in peripheral blood from VKH patients and healthy controls and then intersected these DEGs with an immune dataset to obtain immune DEGs. Subsequently, functional enrichment analyses of immune DEGs were conducted. Protein-protein interaction (PPI) network and clustering analyses were conducted to identify potential immune hub genes, and their value in clinical diagnosis of VKH disease was predicted using receiver operating characteristic (ROC) curves.

Materials and methods

High throughput sequencing data

Clinical data from patients with VKH disease and healthy controls were obtained from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). The dataset GSE166663 was downloaded, including a total of 14 samples, among which seven were from VKH patients and seven from healthy controls. The consent of patients and the approval of ethics committee were unnecessary because the data were downloaded from public databases.

Differential expression analysis

The "limma" and "pheatmap" packages of R software (version 4.1.3) (https://www.r-project.org/) were used to perform differential expression analysis and to draw a heatmap. The expression profiles of VKH patients and healthy controls were compared to identify the DEGs. A t-test was used to determine P-values in DEG analysis.

Genes retained were selected using the criteria of P-value <0.05 and |log2(Fold Change)|>1. A dataset including 1793 genes from the immune database (ImmPort, https://www.immport.org) was also obtained and intersected with GSE166663 to identify immune DEGs. The "VennDiagram" package of R software (version 4.1.3) was employed to generate a Venn diagram.

Functional enrichment analyses

The "clusterProfiler", "org.Hs.eg.db", "enrichplot", "ggplot2", "GSEABase", and "DOSE" packages of R software (version 4.1.3) were used to obtain the Gene Ontology (GO) functions, Kyoto Encyclopedia of Genes and Genomes (KEGG)

enrichment, and to conduct Disease Ontology (DO) analyses of immune DEGs and visualize the obtained data. P values <0.05 were considered statistically significant.

Protein-protein interaction network

The STRING database (https://cn.string-db.org/) was utilized to predict PPIs with the protein species set to "HomoSapiens" and the lowest interaction threshold set to "low confidence" (0.15). The PPI network was then visualized using Cytoscape software (https://cytoscape.org/). Further clustering analysis was conducted using molecular complex detection (MCODE), an application of Cytoscape. Genes in the key sub-network were selected as potential immune hub genes.

Validation of hub genes

Box plots were drawn using the "ggpubr" package of R software (version 4.1.3) and showed the potential immune hub gene expression levels in VKH patients and healthy controls. ROC curves were drawn using the "pROC" package of R software (version 4.1.3) to assess the levels of potential immune hub genes in VKH disease. Immune hub genes were selected using the criteria of P-value <0.05 and area under curve (AUC) value >0.8.

Results

Identification of immune DEGs

A high throughput sequencing dataset, GSE166663, was downloaded from GEO and we compared VKH patients with healthy controls to obtain DEGs. The top 50 DEGs identified from the high throughput sequencing dataset are shown in the heatmap (Figure 1A). Intersection analysis identified 13 downregulated genes and seven upregulated genes (Table 1). The Venn diagram of immune DEGs is shown in Figure 1B.

Functional enrichment analysis of immune DEGs

Functional enrichment analysis of immune DEGs was performed using R software (version 4.1.3). The results of GO analysis revealed that the significantly enriched genes were mainly involved in humoral immune response, lymphocyte mediated and leukocyte mediated immunity, and antigen binding (Figure 2). KEGG analysis indicated that antigen processing and presentation, natural killer cell mediated cytotoxicity, and graft-versus-host disease were significantly enriched in the gene sets (Figure 3A). Finally, DO analysis showed that the immune DEGs primarily participated in the progression of human immunodeficiency virus infectious disease (Figure 3B).

Protein-protein interaction network analysis of immune DEGs

PPI network analysis was performed using the STRING online database and Cytoscape software. After removing eight genes which were not related to other molecules, the PPI network contained a total of 12 nodes and 31 edges. In Figure 4A, nodes represent genes and edges represent interactions between genes, with the upregulated genes marked in red and downregulated genes marked in blue. Clustering analysis established one key module (Figure 4B) and seven key genes (KIR2DL3, KIR3DL2, SH2D1B, KLRC3, KLRC2, GNLY, and GZMB) identified using MCODE were selected as potential hub genes for VKH disease.

Identification of immune hub gene expression levels and diagnostic values

To validate these potential immune hub genes, both the box plots and the ROC curves with AUC calculated were made with R software (version 4.1.3). Box plots were used to validate the expression levels of the seven immune potential hub genes (Figure 5). Significantly lower expression levels of KLRC2 (P=0.035), KLRC3 (P=0.017), SH2D1B (P=0.0019), GZMB (P=0.023), KIR2DL3 (P=0.0021), and KIR3DL2 (P=0.0048) were found in VKH patients than in healthy controls. However, the expression level of GNLY (P>0.05) showed no statistically significant difference between the two groups. Thus, GNLY could not be considered as an immune hub gene in VKH disease. ROC curves were used to assess the sensitivity and specificity of the key genes in diagnosing VKH disease (Figure 6). The AUC values >0.8 in all seven potential hub genes indicated their diagnostic value for VKH diseases. After excluding GNLY as explained above, KLRC2, KLRC3 SH2D1B, GZMB, KIR2DL3, and KIR3DL2 were validated as six immune hub genes in both expression levels and diagnostic values.

Discussion

In the present study, immune-related key genes involved in VKH disease were identified and the role of immune mechanisms in VKH disease was furtherly explored. Moreover, six immune hub genes were identified and verified as having diagnostic value in VKH disease. The results of both

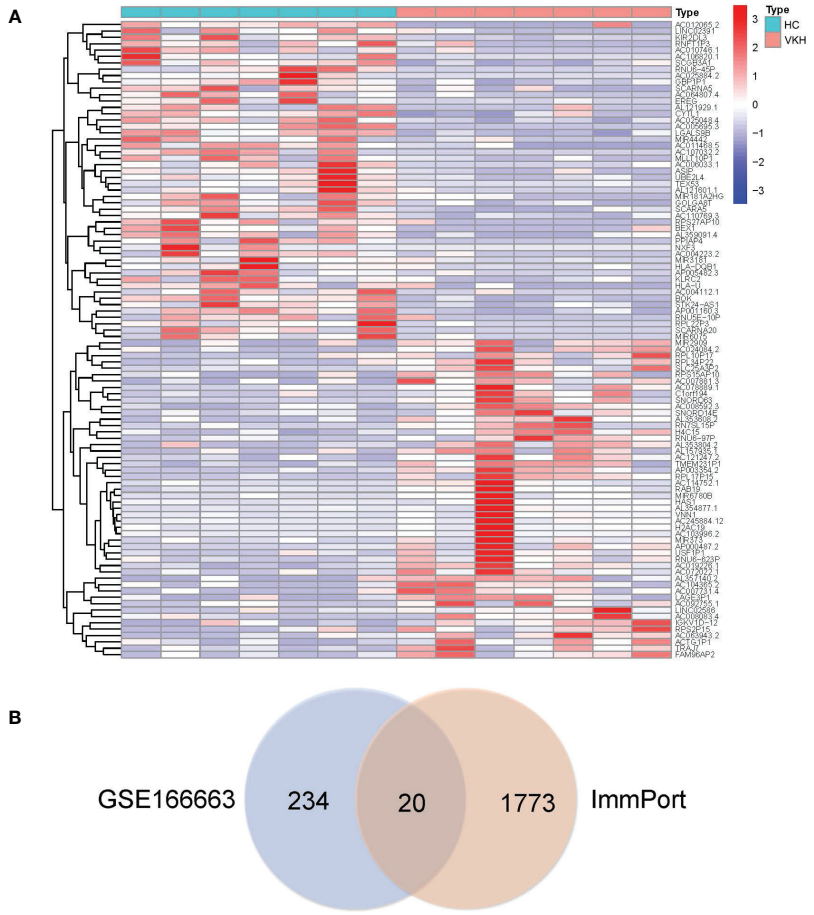

FIGURE 1
In total, 254 differentially expressed genes (DEGs) were identified in Vogt-Koyanagi-Harada (VKH) patients and healthy controls (HC) and the top 50 DEGs are shown in **(A)** the heatmap, with significantly up-regulated genes marked in red and significantly down-regulated genes marked in blue. **(B)** Venn diagram of immune DEGs. Clinical dataset of VKH disease (GSE166663) and immune dataset (ImmPort) were intersected to identify immune DEGs.

GO and KEGG enrichment analyses indicated that the gene sets were primarily enriched in several types of immune pathways, including lymphocyte mediated and leukocyte mediated immune responses, natural killer cell mediated cytotoxicity, and antigen binding, which are closely related to the pathogenesis of VKH disease. In addition, DO analysis suggested that the immune DEGs were mainly involved in human immunodeficiency virus infectious disease. As VKH disease is an autoimmune inflammatory disorder with virus infection playing an important role in its pathogenesis (12, 23), the results of the present DO analysis were consistent with those of previous studies. Since monocytes were thought to be significantly involved in the development of autoimmune diseases, peripheral blood mononuclear cells (PBMCs) have gradually become a breakthrough in studying the pathogenesis of VKH disease. Single-cell RNA sequencing (scRNA-seq), the latest advanced technology, has been used in a recent study, identifying six subpopulations of human blood monocytes, among which the proinflammatory monocyte subpopulation is a promising therapeutic target for treating VKH disease (24). Another research purified the CD4+ T cells from PBMCs and extracted total RNA of CD4+ T cells. RNA-seq was conducted, revealing that circular RNAs (cicRNAs) may have an important immunomodulatory function in the development of VKH disease (25). As the existing researches have revealed the crucial role of immune cells and molecules in the development of VKH disease, our research group innovatively intersected the screened DEGs between VKH patients and healthy controls with the immune dataset and finally found out six immune hub genes in VKH disease, furtherly providing a new insight into the pathogenesis and treatment of this disease.

It has been revealed in previous studies that autoimmune response against melanin in multiple organs may cause the clinical manifestations of VKH disease and that cell mediated

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TABLE 1 Immune differentially expressed genes of Vogt-Koyanagi-Harada disease.

Gene symbol	log_2FC	P-value	Entrez ID
HLA-DQB1	-2.10	0.0262	3119
KIR2DL3	-1.87	0.0006	3804
KIR3DL2	-1.19	0.0070	3812
KLRC2	-2.04	0.0400	3822
KLRC3	-1.04	0.0379	3823
SFTPD	-1.57	0.0111	6441
GNLY	-1.11	0.0041	10578
PLXNA4	-1.02	0.0262	91584
EREG	-3.09	0.0151	2069
SCGB3A1	-2.21	0.0048	92304
SH2D1B	-1.78	0.0006	117157
GZMB	-1.56	0.0006	3002
TRDJ1	-1.55	0.0070	28522
HSPA1A	1.14	0.0379	3303
SLPI	1.54	0.0262	6590
IGHV2-26	1.75	0.0252	28455
IGHV3-15	1.57	0.0007	28448
IGKV1D-12	1.93	0.0200	28903
IGLV8-61	1.37	0.0262	28774
TRAJ7	2.29	0.0007	28522

immune responses play an important role in its occurrence and progression (26–28). Among all the immune-related cells, T cells have proven crucial in the development of VKH diseases. Since the experimental autoimmune uveitis model of VKH became widely accepted in the 1990s, several animal experiments have indicated that with leukocyte infiltration of the retina, CD4+ T cells, Th1 cells and Th17 cells may be activated to trigger autoimmune responses and ocular inflammation (29, 30). Since it is difficult to obtain aqueous humor or choroid samples from VKH patients, studies of peripheral blood lymphocytes have been widely conducted to identify the immune responses involved in human VKH disease. Those studies have shown that CD4+CD25high Treg cells, which may suppress the proliferation of CD4+CD25- T cells, are deficient in VKH patients (31), clarifying how CD4+ T cells cause autoimmune responses in affected human eyes. In addition, Th1 cells (14, 19, 21), Th 17 cells (18, 32), and their related cytokines (such as IL-6, IL-12, IL-17, IL-23, and IFN-γ) were found to be involved in the pathogenesis of human VKH disease. The finding that uveal pigment is an antigen in the development of VKH disease (33) suggested that antigen binding plays an important role in its pathogenesis. It has been widely accepted that the combination of CD4+ T cells and melanocyte-related proteins plays an important role in the pathogenesis of VKH disease (34). In addition, Sugita et al. found that MART-1 is a vital antigen in HLA-A2+ VKH patients (15). Tyrosinase family proteins, especially TRP1 and TPR2, have also been found to play a part in the progression of VKH disease (35). These previous studies provide context for the results of our functional enrichment analyses and indicate that the immune DEGs are pivotal in the pathogenesis and development of VKH disease.

Among the six immune hub genes identified in the present analysis, KIR2DL3 and KIR3DL2 have been found to be related to VKH disease. KIR2DL3 and KIR3DL2, members of inhibitory killer immunoglobulin-like receptors (KIR), may prevent the activation of natural killer cells and T cells (36). A number of KIR genes and human leukocyte antigen (HLA) genes (ligands for KIRs) have previously been used to analyze the gene susceptibility of autoimmune diseases (37). Studies have shown that some inhibitory KIR-HLA combinations are lower in VKH patients than in healthy controls, consistent with our bioinformatics analysis results. In addition to inhibitory KIRs, activating KIRs also play an important part in the pathogenesis in VKH disease, with higher levels of activating KIR-HLA bindings in VKH patients than in healthy controls (38–40).

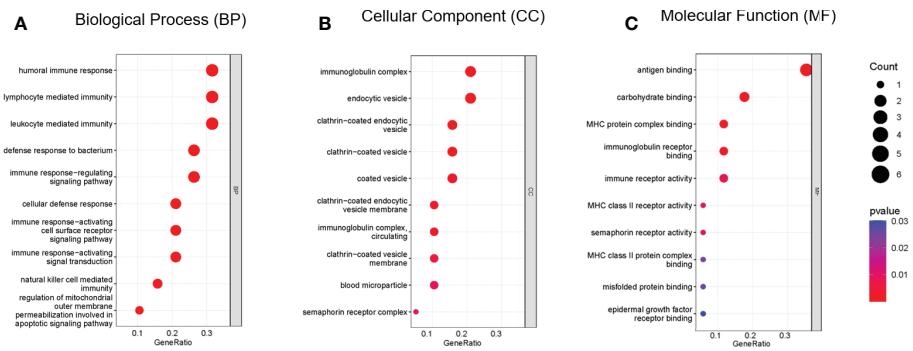

FIGURE 2
Bubble plot of gene ontology (GO) functional analysis included the respective top 10 terms in **(A)** biological process, **(B)** cellular component, and **(C)** molecular function, indicating that the immune differentially expressed genes (DEGs) were mainly involved in humoral immune response, lymphocyte mediated immunity, leukocyte mediated immunity, and antigen binding ($P<0.05$).

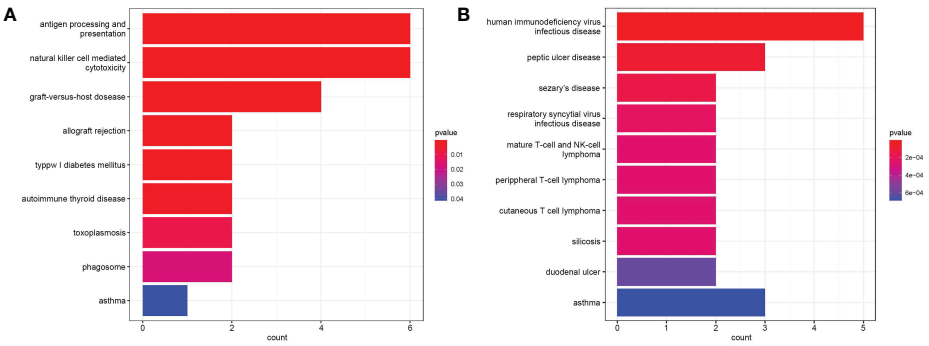

FIGURE 3
(A) Bar plot of results from Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analysis indicating that antigen processing and presentation, natural killer cell mediated cytotoxicity, and graft-versus-host disease were most significantly activated in the gene sets ($P<0.05$). **(B)** Bar plot of disease ontology (DO) functional analysis showed the top 10 diseases, with the immune differentially expressed genes (DEGs) primarily participating in the pathogenesis of human immunodeficiency virus infectious disease ($P<0.05$).

Other immune hub genes are also key to the human immune system. KLRC2 and KLRC3 are both members of killer cell lectin like receptor C (KLRC), a gene family located within the natural killer complex, which can regulate specific humoral and cell-mediated immunity. Although there has been no research to date on the role of KLRCs in the pathogenesis of VKH disease, they have been found to be involved in the development of other autoimmune diseases and tumors. For example, Fatma et al. found that KLRC2+ CD4+ T cells target oligodendrocytes in multiple sclerosis (41). Moreover, the study conducted by Mathilde et al. found that KLRC3 overexpressed in glioblastoma undifferentiated cells and furtherly revealed that the gene expression of KLRC3 was related to glioblastoma aggressiveness (42). SH2 domain containing 1 B (SH2D1B) regulates signal transduction through receptors expressed on the surface of antigen-presenting cells. It is mainly expressed in innate immune cells and the expression of SH2D1B is associated with antigen presentation in human cells. Yasser et al. exposed human cells to SH2D1B-overexpression vaccines, and found that SH2D1B could improve antigen presentation in innate immune cells (43). Granzyme B (GZMB) can encode proteins, mainly including the granzyme subfamily of proteins and peptidase S1 family of serine proteases, process cytokines, and degrade extracellular matrix proteins, thus playing an important

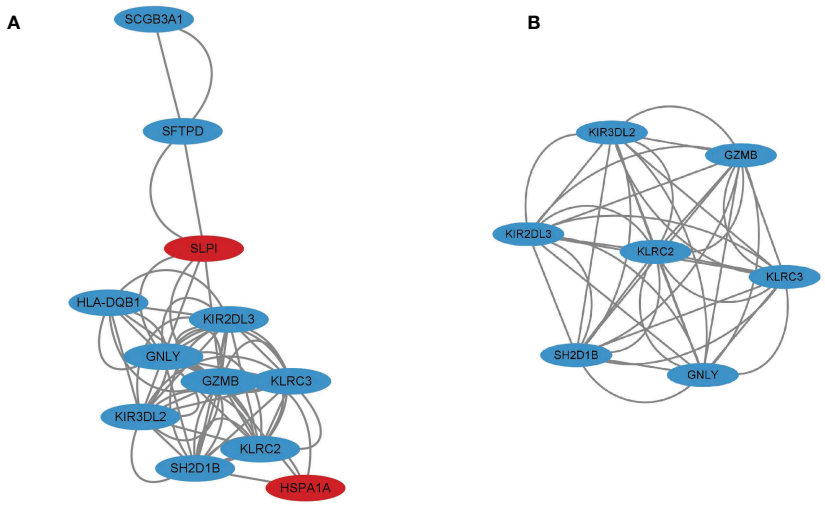

FIGURE 4
(A) Protein-protein interaction (PPI) network for 12 immune differentially expressed genes (DEGs) with the upregulated genes marked in red and the downregulated genes marked in blue. **(B)** Cluster 1 consisting of seven genes was constructed using MCODE.

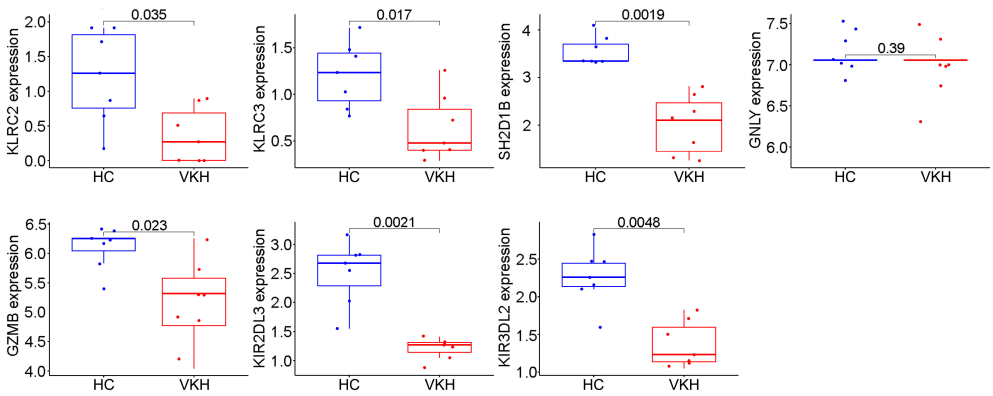

FIGURE 5
Validation of immune potential hub genes in the gene expression level. The expressions of KLRC2, KLRC3, SH2D1B, GZMB, KIR2DL3, and KIR3DL2 were significantly lower in Vogt-Koyanagi-Harada patients (VKH) than in healthy controls (HC). The expression level of GNLY was not statistically significantly different between the two groups.

role in chronic inflammation. Similar to KLRCs, there have been studies showing its role in autoimmune diseases. For example, overexpression of CD4+ GZMB+ CTL cells were found in Sjögren's syndrome (44). GZMB was also proved important in the pathogenesis of inflammatory skin diseases due to GZMB-mediated proteolysis involved in processes such as tissue remodeling and autoantigen generations (45). All the six immune hub genes identified in the present study are relevant to autoimmune responses, and further research is needed to better understand their involvement in VKH disease.

Conclusion

All in all, we obtained 20 immune DEGs (13 downregulated genes and seven upregulated genes) in VKH disease and finally screened out six immune hub genes (KLRC2, KLRC3 SH2D1B, GZMB, KIR2DL3, and KIR3DL2) associated with VKH disease, some of which have not been mentioned in the present researches of VKH disease until now. As far as we know, this is the first research to find out immune hub genes in the pathogenesis of VKH disease. Further analyses validating the

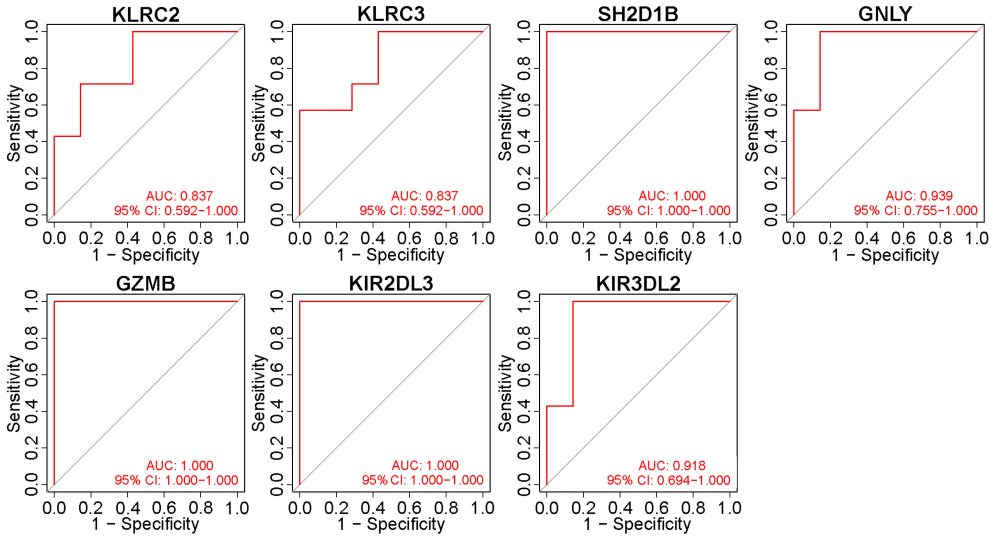

FIGURE 6
Validation of the diagnostic value of immune potential hub genes. Receiver operating characteristic values and area under the curve (AUC) statistics indicated that all seven potential hub genes had diagnostic value for Vogt-Koyanagi-Harada disease.

expression levels and diagnostic levels of these hub genes are of great significance to provide new diagnostic and therapeutic targets of VKH disease in future works. However, it should be noted that this study only includes bioinformatic analyses, thus, further experimental validations are necessary.

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

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

JJ, PG, and YW designed the experiments. YW performed the experiments. YW, YJ, JW, NS, ZT, and HG wrote the manuscript and analyzed the data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Anaphylatoxins spark the flame in early autoimmunity

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The complement system (CS) is an ancient and highly conserved part of the innate immune system with important functions in immune defense. The multiple fragments bind to specific receptors on innate and adaptive immune cells, the activation of which translates the initial humoral innate immune response (IR) into cellular innate and adaptive immunity. Dysregulation of the CS has been associated with the development of several autoimmune disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), ANCA-associated vasculitis, and autoimmune bullous dermatoses (AIBDs), where complement drives the inflammatory response in the effector phase. The role of the CS in autoimmunity is complex. On the one hand, complement deficiencies were identified as risk factors to develop autoimmune disorders. On the other hand, activation of complement can drive autoimmune responses. The anaphylatoxins C3a and C5a are potent mediators and regulators of inflammation during the effector phase of autoimmunity through engagement of specific anaphylatoxin receptors, i.e., C3aR, C5aR1, and C5aR2 either on or in immune cells. In addition to their role in innate IRs, anaphylatoxins regulate humoral and cellular adaptive IRs including B-cell and T-cell activation, differentiation, and survival. They regulate B- and T-lymphocyte responses either directly or indirectly through the activation of anaphylatoxin receptors *via* dendritic cells that modulate lymphocyte function. Here, we will briefly review our current understanding of the complex roles of anaphylatoxins in the regulation of immunologic tolerance and the early events driving autoimmunity and the implications of such regulation for therapeutic approaches that target the CS.

KEYWORDS

complement, anaphylatoxins, C3a, C5a, early autoimmunity, break of tolerance

Introduction

The complement system (CS) is an ancient and highly conserved part of the innate immune response (IR) comprising soluble proteins and membrane-bound receptors bridging innate immunity and adaptive immunity (1). Aside from its well-appreciated canonical activation pathways, non-canonical mechanisms have been recently described, which orchestrate the cleavage and activation of complement factors both in the circulation and intracellularly in immune cells (2). The broad implications of complement activation for health and disease have been reviewed elsewhere (3). Canonical complement activation occurs *via* three different pathways, i.e., the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP), all of which converge at the level of C3, eventually resulting in terminal pathway (TP) activation and subsequent membrane attack complex (MAC) formation (4). While the CP and the LP have critical roles in the initiation of the complement cascade and/or pathogen recognition, the AP accounts for amplification and the majority of terminal complement activation (5). In autoantibody-mediated autoimmune diseases, the deposition of immunoglobulin G (IgG) immune complexes can activate the CP (6). During this process, several cleavage products of C3 and C5 are formed that can activate multiple cells of the immune system *via* their corresponding complement receptors (7–9). The two cleavage fragments C3a and C5a, the so-called "anaphylatoxins", significantly contribute to inflammation and the activation of cells through ligation of their cognate anaphylatoxin receptors C3aR, C5aR1, and C5aR2. Anaphylatoxins are potent chemoattractants that recruit several types of phagocytes to the site of inflammation and mobilize reactive oxygen species in macrophages (10), eosinophils (11), and neutrophils (12). Due to their strong pro-inflammatory properties, they significantly contribute to the pathogenesis of many acute and chronic inflammatory diseases (13).

Autoimmune diseases are a group of chronic inflammatory diseases in which a combination of genetic and environmental factors leads to activation of self-reactive lymphocytes that escaped the multiple layers of central and peripheral tolerance (14). The underlying mechanisms leading to the loss of self-tolerance are multifaceted (15). Most autoreactive lymphocytes are removed at two main checkpoints, i.e., the thymus and the bone marrow by central tolerance mechanisms including deletion and editing. After lymphocytes exit the primary lymphoid organs, several mechanisms of peripheral tolerance ensure that many self-reactive lymphocytes, which escaped central tolerance, are removed from the system (16–18). For this complex process to be successful, a tightly regulated interplay of dendritic cells (DCs), $CD4^+$ T cells, and B cells is required. Dysregulation can lead to a break of tolerance that initiates and drives the early phase of autoimmunity, followed by the effector phase where innate and adaptive effector cells promote multiple inflammatory responses. The CS is critically involved in the immunopathology of several autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and autoimmune bullous dermatoses (AIBDs) such as bullous pemphigoid (BP) and epidermolysis bullosa acquisita (EBA) where it significantly shapes the effector phase of such diseases by recruiting effector cells to the sites of inflammation (19–24).

During the past decade, our understanding of the mechanisms underlying complement-mediated inflammation during the effector phase of several autoimmune diseases has markedly improved. In contrast, we are still at the beginning to delineate the multiple (path)ways by which the CS contributes to the initiation of autoimmunity. Here, we provide an overview of our current understanding and potential future developments in the field.

The dual role of complement in autoimmunity

The role of complement in autoimmunity is complex. On the one hand, complement activation is associated with the progression of several autoimmune disorders (19, 20, 23, 24). On the other hand, complement can also protect from autoimmunity.

The deficiency of complement factors that drive the activation of the CP such as C1, C2, and C4 is strongly associated with the development of SLE (25). As part of the C1 complex, binding of C1q to IgG or IgM immune complexes results in activation of the CP (26). Roughly 90% of patients with deficiency in C1q develop lupus-like manifestations (27). Recently, an elegant study shed new light on the role of C1q in the development of SLE (28). The authors demonstrated that C1q limits tissue damage by acting as a "metabolic rheostat" for effector $CD8^+$ T cells that drive autoimmune inflammation through the generation of autoantigen fragments *via* granzyme B. In contrast to patients with C1q deficiency, only 10%–20% of patients with a C2 deficiency develop lupus (29). The milder disease manifestation in C2-deficient patients might be explained by a C2 bypass mechanism that leads to activation of terminal complement by C1q and mannose-binding lectin (MBL) (30).

While the incidence of SLE among C3 deficiency is very low (19), reports for C4 deficiency differ depending on the ancestral and ethnic background of the patients (31–35). In a study with over 6,000 lupus patients and healthy controls of European ancestry, both C4 isoforms appeared to be protective relative to complete C4 deficiency. However, patients deficient in C4A were at a higher relative risk than patients deficient in C4B (36). When either human C4A or C4B was expressed in a lupus-susceptible strain (37), mice expressing C4A developed less

humoral autoimmunity than C4B-expressing mice. This included a decrease in the number of germinal centers (GCs), autoreactive B-2 cells, autoantibodies, and memory B cells, where the higher efficiency of C4A in inducing self-antigen clearance was associated with the follicular exhaustion of autoreactive B-2 cells. In summary, recent findings provided detailed insights into the mechanisms underlying the protective effects of C1q in the context of autoimmunity; however, the picture regarding the protective effects of C2 and C4 is still sketchy and demands further studies.

In contrast to the protective effect of C1q, C2, and C4, C3 cleavage fragments serve as important cofactors to mount a strong humoral IR. An elegant series of experiments from the Carroll lab demonstrated that binding of C3d-opsonized antigens to complement receptor 2 (CR2; CD21) serves as an important mechanism to foster the uptake of immune complexes by naive B-2 cells within the lymphatics and deliver them to follicular DCs (FDCs) in the B-cell compartment. Furthermore, they identified CR2/CD21 as an important coreceptor for the CD19/CD81 complex that augmented B-cell receptor (BCR)-mediated activation through antigen-tagged C3d that links the CD21/CD19/CD81 complex with the BCR. Finally, CR2 is critical to retain antigens on FDCs, which is crucial for the GC reaction and formation of memory B cells (38).

C3 activation initiates the formation of C5 convertases that activate the terminal pathway. This pathway is characterized by the cleavage of C5 into C5a and C5b, the latter of which initiates the formation of the C5b-9 complex that can form pores as the MAC and destroy pathogens. Aside from its beneficial cytolytic effector functions, the MAC also contributes to inflammation and tissue damage and is closely linked to several autoimmune diseases, such as SLE, where its deposition is associated with disease intensity and used as a marker for treatment response (39). The smaller C5 cleavage, C5a, binds to two distinct receptors, i.e., C5aR1 (CD88) that mediates many of the effector functions of C5a and C5aR2 (C5L2; GPR77), which has initially been considered a mere decoy receptor due to its missing coupling to G-proteins. However, more recent findings identified several C5aR2-mediated functions in inflammation and immunity either in concert with C5aR1 or even independent of C5aR1 (40–43). C5a is a crucial player in the effector phase of various autoimmune disorders, where it drives disease progression through the recruitment and activation of neutrophils and macrophages, depending on the disease (44–49).

By binding to its cognate C3aR, the second anaphylatoxin, C3a, adds to the inflammatory response by activation of innate and adaptive immune cells. In addition to its function as a chemoattractant and activator of eosinophils and mast cells, C3a regulates B-cell and T-cell responses (50, 51). Similar to C5a, C3a plays important roles in the effector phase of different autoimmune disorders including SLE and autoimmune encephalitis (52, 53), where C3a/C3aR signaling promotes the infiltration of neutrophils and macrophages/monocytes. Also, elevated levels of C3a have been associated with disease progression in RA and SLE (54, 55). Of note, C3a can also exhibit anti-inflammatory properties such as preventing the mobilization and degranulation of neutrophils in acute inflammation (56). In summary, C3 cleavage fragments promote humoral autoimmune responses, the MAC can facilitate tissue damage in the context of autoimmunity, and the chemoattractant properties of anaphylatoxins orchestrate the effector phase of many autoimmune disorders.

From the break of tolerance to early autoimmunity

The maintenance of tolerance underlies a complex interplay between DCs, T follicular helper (TFH) cells, and B cells, where dysregulation can lead to a break of tolerance and the development of autoimmunity. Here, we discuss our current understanding of anaphylatoxin receptor signaling as a regulator of early autoimmunity.

DCs are specialized in priming different types of effector T cells and thus possess the unique ability to control both immunity and tolerance. DCs capture antigens at several mucosal surfaces and then migrate to the lymph nodes, where major histocompatibility complex (MHC)-I- or II-loaded peptides are recognized by T cells *via* the T-cell receptor (TCR) (57). Immature DCs can keep tolerance by presenting self-antigens to T cells in the absence of appropriate costimulation. After receiving appropriate stimuli from pattern recognition receptors, they can differentiate into mature DCs (58) that show a reduced endocytic activity associated with a strong upregulation of MHC-II and costimulatory molecule expression (59). These changes enable them to efficiently drive activation of naive T cells and their differentiation into distinct effector T cells through immunomodulatory signals mediated *via* cell-to-cell contacts and the release of a defined set of cytokines such as IL-12, IL-23, and IL-6 (57, 60).

Activation of C5aR1 on DCs has a strong impact on proliferation and differentiation of naive T cells (48). In anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), ANCAs produced by autoreactive B-2 cells activate neutrophils, resulting in direct endothelial injury and extensive glomerular deposition of myeloperoxidase (MPO) (61, 62). Here, the response of MPO-specific T cells to glomerular MPO, mediated by C5aR1 on DCs, contributes significantly to necrotizing glomerulonephritis (63, 64). In experimental anti-MPO glomerulonephritis, genetic or pharmacologic C5aR1 targeting resulted in attenuated T_H1 immunity and increased frequency of regulatory T cells (Tregs) eventually mitigating autoimmunity to MPO (48) (Figure 1A).

Furthermore, a strong link between complement receptor signaling and the regulation of DCs and TFH cells in the context of immunological tolerance has been described. Activation of the C5/C5a/C5aR1 axis controlled the development of maladaptive T_H2/T_H17 development by shifting the balance between immunogenic pulmonary $CD11b^+$ conventional DCs (cDCs) and tolerogenic plasmacytoid DCs (pDCs), thereby regulating Th2 cytokine production (65–67). Recently, pulmonary $C5aR1^+$ and $C5aR1^-$ cDC2 subsets have been described (68), which showed a distinct impact on cDC function after one-time allergen exposure. *Ex vivo* allergen pulsing resulted in low expression of CD40 and MHC-II in the $C5aR1^+$ cDC2 subset, leading to minor antigen-specific proliferation of $CD4^+$ T cells. In sharp contrast, missing C5aR1 activation either in $C5aR1^-$ cDC2s or by C5aR1 targeting induced strong $CD4^+$ T-cell proliferation, suggesting that C5aR1 activation on pulmonary cDC2s controls pulmonary tolerance toward aeroallergens by downregulation of CD40 (Figure 1B). Furthermore, several studies found C5aR1 activation on T cells as a key mechanism to control T_H1 differentiation both in mice and man (69–73). For example, in a model of lupus-like chronic graft-versus-host disease (GvHD), genetic or pharmacological ablation of C5aR1 in $CD4^+$ T cells protected from the generation and expansion of TFH cells, GC B cells, and autoantibodies (74). Furthermore, C5aR1 antagonism initiated in mice with established bronchiolitis obliterans syndrome ameliorated disease manifestation and reduced the associated differentiation of TFH and GC B cells. These findings emphasize the critical role of C5aR1 in supporting TFH cell differentiation and its subsequent impact on the GC reaction and (auto)antibody production (Figure 2A).

In addition to its impact on DC and TFH functions, anaphylatoxin receptor activation also regulates the function of Tregs. Natural $CD4^+$ $FoxP3^+$ Tregs (nTregs) are crucial for immune homeostasis, the persistence of self-tolerance, and hence underlie strict control mechanisms to ensure protective immunity (75–77). Based on findings showing that the activation, differentiation, and expansion of conventional $CD4^+$ CD25 T cells are linked to C3aR and C5aR1 signaling (69, 78–80), Kwan et al. (81) investigated the modulation of

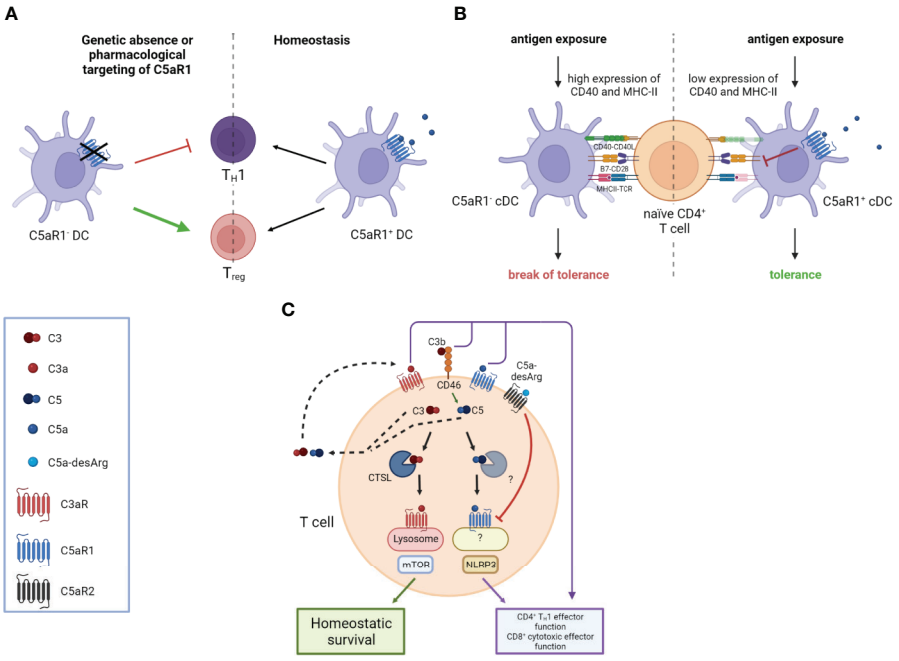

FIGURE 1
Impact of the anaphylatoxins on DC-mediated and intrinsic T cell activation. **(A)** The genetic absence or pharmacological targeting of C5aR1 on DCs leads to attenuated T helper type 1 (T_H1) immunity and an increased frequency of Regulatory T cells (Tregs). **(B)** C5aR1 signaling on naïve mucosal conventional DC2 (cDC2) controls the expression of CD40 and MHC-II which determines the threshold of naïve $CD4^+$ T cell activation. Mucosal antigen exposure is associated with decreased C5aR1 expression; the lack of C5aR1 expression in cDC2s releases the break on CD40 and MHC-II expression resulting in strong $CD4^+$ T cell proliferation and the break of mucosal tolerance. **(C)** T cell activation triggers the secretion of preformed C3 and C5 into the extracellular space, which can be cleaved into C3a, C3b, C5a, and C5b by canonical and non-canonical mechanisms. Binding of these complement fragments to their respective receptors on the T cell induces $CD4^+$ T_H1 and $CD8^+$ effector T cell functions. C3 and C5 are also processed intracellularly by proteases such as cathepsin L (CTSL) in the case of C3 and an unknown protease in the case of C5, respectively. Intracellular C3a is critical to maintain low-level mechanistic target of rapamycin (mTOR) activity by binding to C3aR on lysosomes, thereby contributing to the homeostatic survival of $CD4^+$ T cells. The cleavage of intracellular C5 into C5a and C5b is enhanced by CD46-mediated signaling. C5a engages C5aR1 triggering NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome assembly, eventually driving T_H1 differentiation of $CD4^+$ T cells and $CD8^+$ effector T cell functions. Importantly, autocrine engagement of surface-expressed C5aR2 by C5a-desArg can control intracellular C5aR1 activity. Created in BioRender.com.

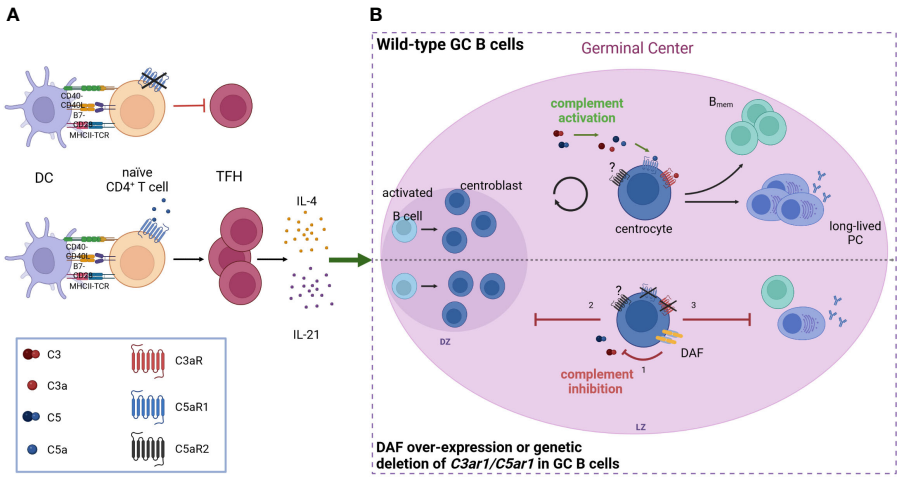

FIGURE 2
Anaphylatoxin receptor activation on TFH cells and GC B cells controls the production of IgG (auto)antibodies **(A)** C5aR1 signaling on CD4+ T cells facilitates the expansion of TFH cells and their subsequent production of IL-4 and IL-21, which are crucial for the germinal center reaction. The absence of C5aR1 signaling leads to an attenuated TFH cell expansion and reduced GC reaction. **(B)** During the GC reaction, C3a and C5a engage their receptors, C3aR and C5aR1 respectively, on GC B cells, driving B cell proliferation and differentiation into memory B cells (B_{mem}) as well as long-lived plasma cells (PC). When complement activation is inhibited (bottom) either by decay-accelerating factor (DAF, CD55) over-expression or deletion of C3aR/C5aR1 on GC B cells, (1) GCs collapse prematurely due to impaired dark zone re-entry and affinity maturation (2), resulting in decreased generation of Bmem and long-lived PCs (3). Created in BioRender.com.

nTreg functions by C3a and C5a. They found that C3aR and C5aR1 activation on nTregs inhibited their function by inducing phosphorylation of the transcription factor Foxo1, resulting in reduced FoxP3 expression on nTregs.

More recent work by Liszewski et al. (82) unraveled a novel and unexpected role for an intracellular CS, which they termed the "complosome," that regulates key metabolic pathways critical for the survival of peripheral human T cells and their effector functions. They showed that circulating human $CD4^+$ and $CD8^+$ T cells continuously generate low levels of C3a and C3b by cathepsin L-mediated cleavage of intracellular C3, resulting in mammalian target of rapamycin (mTOR) activation *via* lysosome-bound C3aR engagement and interaction of complosome C3b with surface-bound CD46. These mechanisms were shown to be crucial for T-cell homeostasis, $CD4^+$ T_H1 effector function, and $CD8^+$ cytotoxic T-cell effector activity (83). This interesting finding implicates that human T_H1 and T_H17 responses are regulated by autocrine and intracellular complement activation, shedding new light on the role of complement in controlling immunological tolerance (Figure 1C).

In addition to its impact on cellular immunity, anaphylatoxins also regulate humoral IR. Effective humoral IRs rely on high-affinity antibodies generated by affinity maturation in the GCs within the secondary lymphoid organs (84, 85). Here, B-2 cells go through repeated cycles of somatic hypermutation, clonal expansion, and affinity-governed positive selection. Positive selection is orchestrated by costimulatory signals from TFH cells that have been recruited to the GCs after antigen capture. Depending on these signals, non-self-reactive GC B cells survive and proliferate, whereas self-reactive GC B cells undergo either further differentiation or cell death (85–88). In this stringently regulated process, mTOR signaling and expression of the proto-oncogene c-MYC exert crucial functions (89, 90). In a recent study, Cumpelik et al. (91) found downregulation of the complement inhibitor decay-accelerating factor (DAF, CD55) in GC B cells *via* B-cell lymphoma 6 (Bcl-6) associated with simultaneously increased expression of MAC inhibitor CD59. The reduced complement regulation resulted in increased C3 and C5 cleavage on GC B cells leading to increased generation of C3a and C5a and consecutive enhanced C3aR and C5aR1 signaling. Importantly, this process was indispensable for positive selection and GC function, as disruption of this pathway decreased mTOR activity in response to BCR-CD40 signaling, eventually leading to a premature GC collapse and defective affinity maturation (Figure 2B).

Furthermore, combined C3aR and C5aR1 signaling was shown to modulate antibody production and class switch recombination of B-2 cells (92). Using $C3aR^{-/-}C5aR1^{-/-}$ mice, Paiano et al. found that C3aR/C5aR1 signal transduction was indispensable for CD40 upregulation, IL-6 production, proliferation, and IL-21 production by follicular $CD4^+$ T cells. Furthermore, using immunized mice deficient in systemic C3 and C5 and transfecting them with wild-type bone marrow (BM), the study showed that locally produced complement was necessary for this signaling pathway and sufficient for the initial B-2 antibody response.

In addition to B-2 cells that generate high-affinity antibodies against foreign antigens, B-1 cells mediate the first line of

immune defense through low-affinity natural IgM antibodies. Interestingly, B-1 cells have also been shown to drive the establishment of autoimmune-mediated diseases, such as type 1 diabetes (93) and SLE (94). Their regulation is also highly dependent on C5a (95), as the C5a/C5aR1 axis controls the trafficking of B-1 cells into the BM, the peritoneal cavity, and from the BM to the spleen, emphasizing the importance of the C5a/C5aR1 axis in early autoimmunity.

Based on a growing body of evidence showing that complement not only controls the effector phase of many autoimmune disorders but the early events of humoral and cellular adaptive immune responses, complement pathways and mediators have sparked the interest as therapeutic targets to treat autoimmune disorders (96). At this point, only a few complement inhibitors have been approved for therapeutic use, including the C5 inhibitor eculizumab, the plasma C1 protease inhibitor (C1INH), the C3 inhibitor pegcetacoplan, and the C5aR1 antagonist avacopan. While eculizumab treatment has been approved for the treatment of paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), and neuromyelitis optica spectrum disorders (NMOSDs), C1INH is used for the treatment of hereditary angioedema (96). Pegcetacoplan is currently approved for the treatment of PNH (97) and the first C5aR1 inhibitor, avacopan, for the treatment of AAV (98).

Conclusion

Apart from the well-known functions of recruiting and activating innate effector cells that drive the pro-inflammatory environment of many autoimmune diseases, anaphylatoxin receptor signaling appears to also ignite the early events of humoral and adaptive immunity, leading to the loss of tolerance as a first step to induce autoimmunity. Exemplarily, local generation of C3a and C5a in tissues or inside DCs, T cells, and B cells and activation of their cognate anaphylatoxin receptors in an autocrine or paracrine fashion function as one important rheostat to keep tissue homeostasis and immunologic tolerance. The growing understanding of the multiple facets of anaphylatoxin functions opens new perspectives for spatially and temporarily tailored targeting strategies that consider the interindividual differences in immune responses. A few drugs are already Food and Drug Administration (FDA)-approved that target the complement system at the level of C3, C5 or more specifically inhibit C5a-mediated C5aR1 activation. The multiple complement inhibitors that are currently tested in clinical trials open up a wide range of new treatment options for clinicians to inhibit the distinct activation pathways or complement fragment-mediated activation of specific complement receptors (99, 100). However, the already approved drugs and the compounds tested in ongoing clinical trials are designed for the treatment of acute inflammation. In light of the impact on humoral and cellular adaptive immune responses of C3 and C5 cleavage fragments, it will be of major importance in future studies to define endpoints in clinical studies of autoimmune diseases that also take these crucial functions of complement mediator molecules into account. Also, it will be crucial to discriminate between intracellular and extracellular complement and complement receptor targeting given the major impact of complosome activation for T-cell activation in particular (101).

Open questions

- How can we translate our findings that anaphylatoxins shape early autoimmunity into appropriate therapeutic approaches?
- When do we target the CS?
- Where do we target the CS, i.e., extracellular complement vs. intracellular complement?
- What is the contribution of C5aR2 to early autoimmunity?

Author contributions

JS wrote the first draft of the manuscript. JK, CK, and JS wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Use of gliptins reduces levels of SDF-1/CXCL12 in bullous pemphigoid and type 2 diabetes, but does not increase autoantibodies against BP180 in diabetic patients

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The use of dipeptidyl peptidase 4 (DPP4) inhibitors, (also known as gliptins), is associated with an increased risk of bullous pemphigoid (BP), an autoimmune blistering skin disease. To explore the mechanism behind gliptin-associated BP we investigated circulating autoantibodies against the major BP autoantigen BP180 in serum samples from patients with type 2 diabetes (T2D) with preceding gliptin medication (n = 136) or without (n = 136). Sitagliptin was the most frequently prescribed gliptin (125/136 patients). Using an ELISA assay, we showed that IgG autoantibodies against the immunodominant NC16A domain of BP180 were found in 5.9% of gliptin treated and in 6.6% of non-gliptin treated T2D patients. We found that 28% of gliptin treated patients had IgG autoantibodies recognizing the native full-length BP180 in ELISA, but among non-gliptin treated the seropositivity was even higher, at 32%. Further ELISA analysis of additional serum samples (n = 57) found no major changes in the seropositivity against BP180 during a follow-up period of about nine years. In immunoblotting, full-length BP180 was recognized by 71% of gliptin treated and 89% of non-gliptin treated T2D patients, but only by 46% of the age-and sex-matched controls. The chemokine stromal derived factor-1(SDF-1/CXCL12) is one of the major substrates of DPP4. Immunostainings showed

that the expression of SDF-1 was markedly increased in the skin of BP patients, but not affected by prior gliptin treatment. We found that the use of gliptins decreased the serum level of SDF-1α in both BP and T2D patients. Our results indicate that the autoantibodies against the linear full-length BP180 are common in patients with T2D, but seropositivity is unaffected by the use of sitagliptin.

KEYWORDS

bullous pemphigoid, autoimmunity, BP180, gliptins, DPP4, SDF-1 (CXCL12)

Introduction

Bullous pemphigoid (BP) is the most common autoimmune subepidermal blistering skin disease primarily affecting elderly people. It is characterized by intense pruritus and tense bullae (1). The major target of BP IgG autoantibodies is BP180, a transmembrane hemidesmosomal protein of basal keratinocytes (2). Around 85% of BP autoantibodies target the juxtamembranous extracellular 16th non-collagenous (NC16A) domain of BP180, and levels of anti-NC16A IgG antibodies correlate with the severity of BP (1, 3).

Epidemiological data have convincingly demonstrated that the most evident risk factors for BP are certain neurological diseases (4–7) and the use of dipeptidyl peptidase 4 (DPP4) inhibitors (gliptins) (8–14). Gliptins are oral anti-diabetic drugs that are widely used to treat type 2 diabetes (T2D) (15), but the exact mechanism by which the use of gliptins increases the risk for BP is unknown (13). Furthermore, data about the clinical and immunological similarities and differences of regular BP (rBP) and gliptin-associated BP (BP+g) are scarce and partially conflicting (13). The target of gliptins, the DPP4 protein (also known as CD26), is ubiquitously expressed and has multiple functions in various cell types (16). The various effects of the inhibition of DPP4/CD26 activity include, for example, regulation of inflammatory cells such as T lymphocytes (17). In addition to BP, DPP4 and its inhibition have been linked to several other skin diseases including psoriasis, atopic dermatitis, T-cell lymphoma, hypertrophic scars, and sclerotic disorders (17).

One of the best characterized substrates of DPP4 is stromal cell-derived factor-1 (SDF-1 or CXCL12), a lymphocyte and monocyte attracting chemokine (18). Alternative splicing generates several SDF-1 isoforms of which SDF-1α is the most common (19). DPP4 cleaves the first two amino (N)-terminal amino acids of SDF-1 and inhibits its binding to CXC chemokine receptor 4 (CXCR4) or atypical chemokine receptor 3 (ACKR3, previously named CXCR7) (18, 20–22). CXCR4 is ubiquitously expressed in most cells and the SDF-1/CXCR4 axis participates in embryonic neural and vascular development and later maintains homeostasis in tissues e.g., during leukocyte trafficking and skin inflammation (18). In the skin the expression of SDF-1 is increased during wound healing in fibroblasts and injured keratinocytes (23, 24). Moreover, the cutaneous expression of CXCR4 and its ligand SDF-1 are upregulated in patients with atopic dermatitis (25), psoriasis and keratinocyte-originating basal and squamous cell carcinomas (26).

We and others have previously found that patients with an elevated risk for developing BP i.e., those with neurological disorder such as Alzheimer's disease, multiple sclerosis, and Parkinson's disease, have an increased prevalence of BP180 autoantibodies (27–30). In the present study we investigate how the use of gliptins influences the rates of seropositivity for anti-BP180 IgG autoantibodies in patients with T2D. Given the major role of SDF-1 as a DPP4 substrate and lymphocyte attractant, and its involvement in inflammatory skin diseases, we have also analyzed the amount of SDF-1 in serum and skin samples from patients with BP.

Material and methods

Patients

Patients with T2D treated with gliptin medication (T2D+g) and age- and sex-matched T2D patients who were not using gliptins were recruited from the ARTEMIS (31) and OPERA (32) study cohorts. Information concerning medication, skin diseases and cutaneous symptoms was collected from hospital records and patient questionnaires. BP serum and skin samples were collected in the Department of Dermatology, Oulu University Hospital at the time of diagnosis as described earlier (33). Patients using gliptins at the time of BP diagnosis were designated as BP+g. Age-matched healthy control sera were collected in Kuopio University Hospital from patients attending the hospital for knee replacement operations (30). Additional age-matched healthy control sera were obtained from

the OPERA cohort and the Northern Finland Biobank Borealis, Oulu, Finland (https://www.oulu.fi/university/node/38474). All subjects gave written informed consent before the sample collection. The study was performed according to the principles of the Declaration of Helsinki. Ethics Committees of the Northern Ostrobothnia Hospital District and Kuopio University Hospital approved the collection of patient samples. All available patients were invited to attend a follow-up visit approximately 9 years after the date of baseline sample collection. ELISA assays were performed on samples obtained at study baseline, and again at the follow-up visits, but only baseline samples were analyzed by immunoblotting.

ELISA assays

A commercial MESACUP BP180 ELISA kit (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) was used to measure serum BP180-NC16A IgG autoantibodies according to the manufacturer's instructions. The cut-off value for positivity was set at ≥ 9 U/ml.

The methodology for the detection of full-length BP180 (FL-BP180) autoantibodies has been previously described (34). Briefly, 96 well plates (Nunc Maxisorp, Thermo Fisher Scientific, Inc., Waltham, MA, USA) were coated with 125 ng/well of mammalian cell expressed DDDDK-tagged BP180 protein. Plates were incubated with 1:100 serum dilutions. Absorbances were measured at 450 nm using a Victor2 1420 multilabel plate reader (Wallac, Turku, Finland). The cut-off value for positivity was determined by obtaining the maximal Youden index value from a ROC curve analysis using 16 known seropositive BP samples and 27 samples from age-matched healthy controls. SPSS software was used to determine a cut-off value of 1.950, with sensitivity of 0.938 and specificity of 0.889 (Supplementary Table 1 and Supplementary Figure 1).

Serum SDF-1α levels were measured using a human CXCL12/SDF-1α Immunoassay (Quantikine® ELISA DSA00, R&D Systems Inc., MN, USA) according to the manufacturer's instructions.

Immunoblotting and epitope mapping

FL-BP180 was expressed in COS7 cells transfected with human BP180 cDNA (35) and prepared as described previously (36). Fifty ng of each recombinant human glutathione-S-transferase (GST)-BP180-fusion protein expressed in *E. coli* spanning most of the BP180 polypeptide were used as an antigen. Immunoblotting and preparation of GST-BP180 fusion proteins were performed as described previously (30). Serum samples were diluted to 1:100 in 5% non-fat milk-TBS-0.1% Tween-20 and 1:50 000 peroxidase-conjugated anti-human IgG (Sigma-Aldrich, St. Louis, MO, USA) was used as a secondary antibody. Anti-GST (1:3000, Thermo Fisher Scientific, Rockford, IL, USA) with peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich) were used to detect fusion proteins. Protein bands were visualized with ECL Prime substrate (GE Healthcare, Buckinghamshire, UK) on a LAS Imager 3000 (Fujifilm, Tokyo, Japan). Epitope mapping data of 14 age and sex-matched healthy controls from our previous work (30) were used as a control.

Immunohistochemistry

Three-µm thick sections of formalin-fixed and paraffin-embedded lesional skin samples of BP patients (n = 4 gliptin-treated, n = 7 without gliptin usage) and healthy control skin (n = 5) were deparaffinized and rehydrated. After heat-induced antigen retrieval in 10 mM Tris-1 mM EDTA (pH 9.0), the sections were immunostained with anti-human SDF-1α antibody (aa 1–93, 1:20000, PA5-114344, Invitrogen/Thermo Fisher Scientific, Inc.) and a Rabbit-specific HRP/DAB (ABC) detection IHC kit (Abcam, Cambridge, UK). The average number of strongly stained infiltrated SDF-1 positive cells was calculated from the three areas with the most abundant positive cells in the epidermis and dermis using a x40 high power field objective. Stained cells in the blister fluid and inside capillaries were excluded from the analysis.

Data analysis

Statistical analyses were conducted using the IBM SPSS software (v. 27) (IBM, Armonk, NY, USA). Differences between groups in FL-BP180 and BP180-NC16A levels (non-normally distributed, homoscedastic, symmetric distributions within groups, unequal group sizes) were analyzed using the Kruskal-Wallis test with Dunn's corrected P values for multiple comparisons. Differences within the BP and T2D groups in SDF-1α measurements (non-normally distributed, heteroscedastic, unequal group sizes) were analyzed separately (2 families, 3 or 4 comparisons per family) using Welch's ANOVA and Dunnett's T3 adjustment for multiple comparisons. Mean and median values, 95% confidence intervals and percentiles were reported, as appropriate. The numbers of immunohistochemically stained SDF-1 positive cells in the skin samples were compared using the Kruskal-Wallis test with Dunn's corrected P values for multiple comparisons. In epitope mapping, antibody-detected bands were densitometrically analyzed using the ImageJ software package (NIH, Bethesda, MD, USA) and classified in an ordinal scale: "0" = no band, "1" = weak, "2" = strong, "3" = very strong, as described previously (36). Differences between groups in epitope mapping were compared pairwise using the Fisher-Freeman-Halton exact test. A two-tailed P value of 0.05 or less was considered statistically significant.

Results

Study population

Our study included 136 T2D patients treated with gliptin medication (T2D+g) and 136 age- and sex-matched T2D patients who were not using gliptins from the ARTEMIS (31) and OPERA (32) study cohorts (Table 1). After an average interval of nine years after the baseline sampling (range 7–13 years), 179 patients were invited for the follow-up visit, 57 accepted the invitation and were subjected to serum sampling (Table 1). Total skin examination was performed to 28 patients who either had increased anti-BP180-NC16A values or reported skin symptoms in patient questionnaire.

Gliptin medication does not affect the prevalence of autoantibodies against folded BP180 in patients with type 2 diabetes

Although the exact details of the timing and duration of gliptin medication were not obtained in all cases, the information of type of gliptin used and other medications was available for all patients. At baseline 125 gliptin users had sitagliptin as the prescribed agent, eight had vildagliptin, two had linagliptin and one had saxagliptin. When serum samples were analyzed by ELISA, in the T2D+g group, 8 out of 136 sera (5.9%) were classified as positive for anti-BP180-NC16A IgG antibodies, as were 9 out of 136 (6.6%) in the non-gliptin T2D group (Figure 1A and Table 1).

The data collected for the 57 cases who participated to follow-up visit revealed that 21 of them had continued the use of gliptins from baseline to follow-up visit and four of them had not been using gliptin during the whole study period. Before the follow-up sampling 16 patients had stopped using gliptins and 16 patients who had not been receiving gliptins at baseline had started their use before the follow-up visit. At the follow-up sitagliptin was the prescribed agent for 36 patients and vildagliptin for one patient. Three patients who had BP180-NC16A ELISA values just above positive cut-off at baseline, remained weakly positive (9.9–11.6 U/ml) at follow-up. One gliptin user with a negative baseline value (8.8 U/ml) turned weakly positive (10.9 U/ml) although he/she had discontinued gliptin use during the follow-up period. Taken together, there were no major changes in anti-BP180-NC16A IgG positivity in either the T2D or T2D+g groups during the follow-up period (Figures 1A, B and Table 1).

All 272 baseline samples and 57 follow-up samples were further analyzed by ELISA for FL-BP180 seropositivity (Figure 1C). We found that 28% (38/136) of T2D+g sera recognized FL-BP180, but among the T2D cases the

TABLE 1 Characteristics and presence of BP180 IgG autoantibodies of T2D patients with (+g) or without use of gliptins at baseline and after a 9-year follow-up.

	T2D[†]	T2D+g[‡]	P value
n	136	136	
Females n (%)	47 (35)	48 (35)	ns.[§]
Age (mean ± SD[¶] years)	66.7 ± 8.0	67.2 ± 7.8	ns.
BP180-NC16A-ab U/ml median (range)	2.0 (0.0–26.4)	2.3 (0.0–40.8)	ns.
BP180-NC16A-ab positives n (%)	9 (7)	8 (6)	ns.
FL-BP180-ab U/ml median (range)	0.9 (0.0–21.9)	0.1 (0.0–29.5)	ns.
FL-BP180-ab positives n (%)	44 (32)	38 (28)	ns.
	Follow-up	**Follow-up**	
n	20	37	
Females n (%)	4 (20)	11 (30)	ns.
Age (mean ± SD years)	73.3 ± 6.7	73.0 ± 6.8	ns.
BP180-NC16A-ab U/ml median (range)	2.8 (1.5–12.2)	2.4 (1.0–18.8)	ns.
BP180-NC16A-ab positives n (%)	2 (10)	2 (5)	ns.
FL-BP180-ab U/ml median (range)	0.0 (0.0–2.0)	0.0 (0.0–90.3)	ns.
FL-BP180-ab positives n (%)	1 (5)	7 (19)	ns.

Follow-up cases were classified to T2D and T2D+g groups according to their use of gliptins at the time of follow-up, regardless of their use of gliptins at baseline. During the follow-up, 16 T2D cases started to use gliptins and were switched to the T2D+g group and 16 T2D+g cases stopped using gliptins and were switched to T2D group. Four T2D and 21 T2D+g cases remained in their respective groups from baseline to follow-up. IgG autoantibody levels in serum samples were measured using BP180-NC16A ELISA and full-length (FL) BP180 ELISA.
[†]Type 2 diabetes.
[‡]Type 2 diabetes with gliptins.
[§]Nonsignificant.
[¶]Standard deviation.

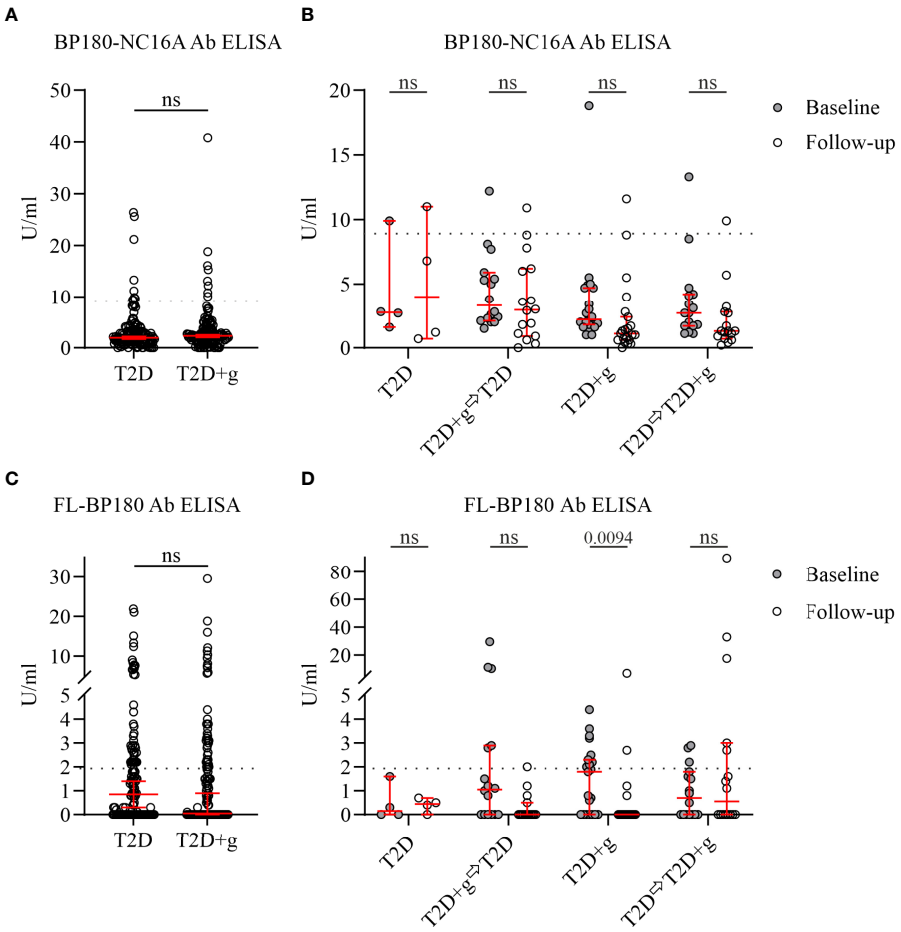

FIGURE 1
IgG autoantibodies against the NC16A domain of BP180 and full-length BP180 in serum samples of T2D patients with (+g) or without use of gliptins. Commercially available BP180-NC16A ELISA and full-length (FL) BP180 ELISA (in-house) with a cut-off value of 9.0 and 1.950 U/ml (dashed line), respectively, were used. n = 136 for both T2D and T2D+g groups at baseline **(A, C)**. In total, 57 follow-up serum samples were analyzed (B and D). **(A)** There was no significant difference in serum anti-BP180-NC16A IgG antibody levels between T2D and T2D+g groups at baseline. **(B)** Similar amounts of anti-BP180-NC16A IgG antibodies and numbers of positive cases were seen when the same individual cases were compared between baseline and follow-up. **(C)** Serum anti-FL-BP180 IgG antibody levels were similar in T2D and T2D+g groups at baseline. **(D)** Significant changes were seen between baseline and follow-up in anti-FL-BP180 IgG antibody levels in the T2D+g group: the number of positive cases decreased from 9 to 2 (one case was positive both in baseline and follow-up). Whiskers indicate 5th and 95th percentiles. Statistically significant differences between groups with associated two-sided P values are shown. ⇨, switch to; +g, gliptins; Ab, antibodies; BP, bullous pemphigoid; Ctrl, control; ns; nonsignificant; T2D, type 2 diabetes; U/ml, units per milliliter.

proportion of seropositive samples was higher, at 32% (44/136) (Table 1). Only 14% (8/57) of follow-up samples were positive (Table 1). In T2D patients who were gliptin users both at the baseline and follow-up, average level of IgG antibodies against FL-BP180 decreased from baseline to follow-up (Figure 1D). Also, the group that had discontinued gliptin usage before follow-up sampling had lower average anti-FL-BP180 antibody levels and fewer positive cases at follow-up than baseline, but the difference was not statistically significant (Figure 1D). In other groups, there were no clear changes in FL-BP180 antibody levels or positivity at the group level.

A diagnosis of BP was present in one T2D+g female patient at the age of 66 years (four years after the baseline, in 2012) who had initially been treated with sitagliptin but was changed to vildagliptin medication before BP onset. The diagnosis of BP was based on positive direct immunofluorescence findings, but the BP180-NC16A ELISA was negative. The patient's BP180-NC16A ELISA value was 2.6 U/ml at baseline in 2008 and 7.8 U/ml at follow-up in 2020. Her FL-BP180 ELISA was negative at both baseline and follow-up. No other patients who was positive for anti-BP180-NC16A or FL-BP180 autoantibodies had skin symptoms suggestive of BP.

Use of gliptins does not induce major changes in anti-BP180 IgG autoantibody profiles in patients with type 2 diabetes

A subset of patient sera containing all BP180-NC16A ELISA positive (T2D+g: n = 8; T2D: n = 9), and ELISA negative baseline samples (T2D+g: n = 9, including the only BP T2D+g case; T2D: n = 9), and healthy controls (n = 13) were analyzed by immunoblotting against FL-BP180 (Figures 2A, B). We found that 89% T2D sera and 71% of T2D+g sera detected linear FL-BP180 in immunoblotting, while 46% of control sera contained anti-BP180 autoantibodies (Table 2). The recognition of FL-BP180 in immunoblotting showed a weak tendency towards correlation with the FL-BP180 ELISA positivity in T2D group (Table 2).

The same T2D+g, T2D and control serum samples whose anti-FL-BP180 autoantibodies were analyzed in immunoblotting were subjected to detailed epitope mapping using GST-fusion proteins (FP) covering the whole BP180 polypeptide (Figure 2A). Each serum detected a unique combination of several fusion proteins. When compared to previously published control serum results (30), T2D sera recognized intracellular and mid-extracellular epitopes slightly more frequently (Figures 2C, D). In addition, FP7 (aa 661–825) and FP13 (aa 1278–1497) were significantly more frequently recognized by T2D than T2D+g sera (Figures 2C, D, Supplementary Tables 2–4). Only one T2D +g patient had serum antibodies targeting the NC16A domain (FP5) in immunoblotting. Accordingly, the baseline and follow-up samples of this patient were positive in the BP180-NC16A ELISA, but no BP-related skin symptoms were found by the clinical examination.

Gliptin treatment reduces the serum level of SDF-1α in patients with type 2 diabetes and bullous pemphigoid

Another approach to understand the mechanism behind gliptin-associated BP was to investigate how the use of gliptins in patients with BP and T2D modifies the amount of the

TABLE 2 In immunoblotting full-length BP180 was weakly recognized by most T2D patient sera and at a lower frequency among T2D+g group.

	FL-IB‡ pos.	*FL-IB neg.*	
	T2D† (n = 18)		
NC16A-ELISA pos. (n = 9)	***FL-IB pos.***	***FL-IB neg.***	Sum
FL-ELISA pos.	3 (33%)	0 (0%)	3 (33%)
FL-ELISA neg.	5 (56%)	1 (11%)	6 (67%)
Sum	8 (89%)	1 (11%)	9 (100%)
NC16A-ELISA neg. (n = 9)			Sum
FL-ELISA pos.	2 (22%)	0 (0%)	2 (22%)
FL-ELISA neg.	6 (67%)	1 (11%)	7 (78%)
Sum	8 (89%)	1 (11%)	9 (100%)
Total (n = 18)	**16 (89%)**	**2 (11%)**	**18 (100%)**
	T2D+g§ (n = 17)		
	FL-IB pos.	***FL-IB neg.***	
NC16A-ELISA pos. (n = 8)			Sum
FL-ELISA pos.	2 (25%)	1 (13%)	3 (38%)
FL-ELISA neg.	4 (50%)	1 (13%)	5 (63%)
Sum	6 (75%)	2 (25%)	8 (100%)
NC16A-ELISA neg. (n = 9)			Sum
FL-ELISA pos.	0 (0%)	1 (11%)	1 (11%)
FL-ELISA neg.	6 (67%)	2 (22%)	8 (89%)
Sum	6 (67%)	3 (33%)	9 (100%)
Total (n = 17)	**12 (71%)**	**5 (29%)**	**17 (100%)**
	Controls (n = 13)		
	FL-IB pos.	***FL-IB neg.***	Sum
Total (n = 13)	**6 (46%)**	**7 (54%)**	**13 (100%)**

IgG autoantibodies targeting full-length BP180 in NC16A-ELISA positive and negative T2D and T2D+g sera and healthy control sera were analyzed by immunoblotting. IgG autoantibodies against native full-length BP180 and BP180-NC16A were measured by ELISA. The distribution of the (positive/negative) status in immunoblotting (FL-IB) and full-length BP180 ELISA (FL-ELISA) is shown.
†Type 2 diabetes.
‡Full-length BP180 immunoblotting.
§Type 2 diabetes + gliptins.

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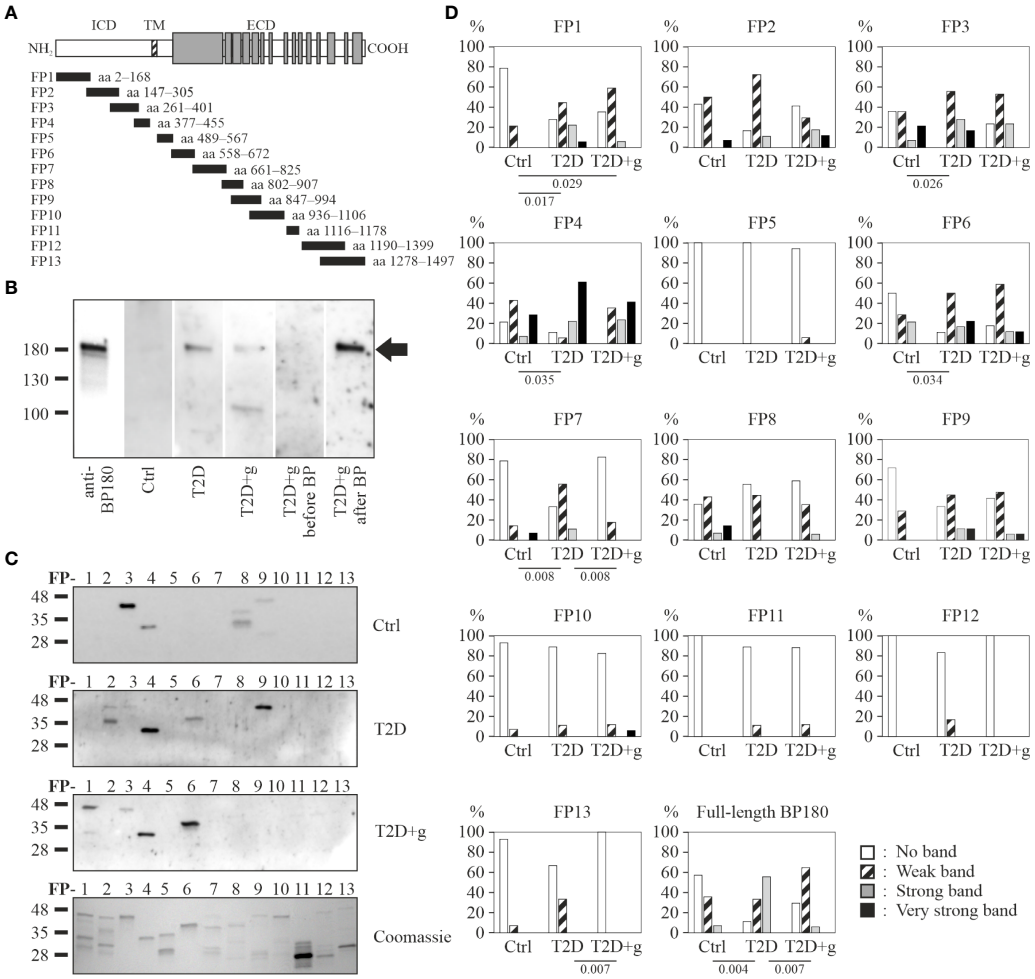

FIGURE 2
Detection of BP180 epitopes by IgG autoantibodies of T2D patients with (+g) or without use of gliptins and controls in immunoblotting. **(A)** Schematic representation of the full-length BP180 polypeptide and glutathione-S-transferase-BP180 fusion proteins. **(B)** Examples of recombinant FL-BP180 recognition by a specific BP180-antibody and serum of a healthy control, a T2D patient, a T2D+g patient and a T2D+g patient before and after BP diagnosis. The arrow indicates the BP180 band. **(C)** Fusion proteins were immunoblotted with 18 T2D, 17 T2D+g and 14 control serum samples. A representative Coomassie blue-stained gel of size-separated fusion proteins is also shown. **(D)** Relative frequencies of densitometrically classified immunoblotting signal intensities for indicated fusion proteins and statistically significant differences between groups with corresponding two-tailed P values are shown. Ctrl, control; ECD, extracellular domain; FP, fusion protein; ICD, intracellular domain; TM, transmembrane domain.

chemokine SDF-1, one of the best characterized substrates of DPP4. We measured SDF-1α levels by ELISA in serum samples from patients with regular BP (rBP, n = 27, mean age ± SD = 81.6 ± 8.4 years), BP patients using gliptins (BP+g, n = 20, 77.7 ± 8.8 years) and age-matched healthy controls (n = 20, 79.4 ± 2.1 years). The level of SDF-1α of patients with rBP was similar to that found in controls (P = 0.4753) (Figure 3A). The serum level of SDF-1α in BP+g patients was significantly lower than that in rBP patients (P <0.0001) and healthy controls (P <0.0001).

We also measured the amount of serum SDF-1α in the T2D+g (n = 22, 67.5 ± 9.6 years), T2D (n = 21, 64.5 ± 7.1 years), and age-matched healthy control groups (n=18, 62.7 ± 13.1 years). As was the case in BP patients, SDF-1α serum levels were significantly lower in T2D+g patients than in T2D patients (P = 0.0035). Compared to the healthy controls the level of SDF-1α was higher in patients with T2D (P = 0.0011) and lower in T2D+g patients

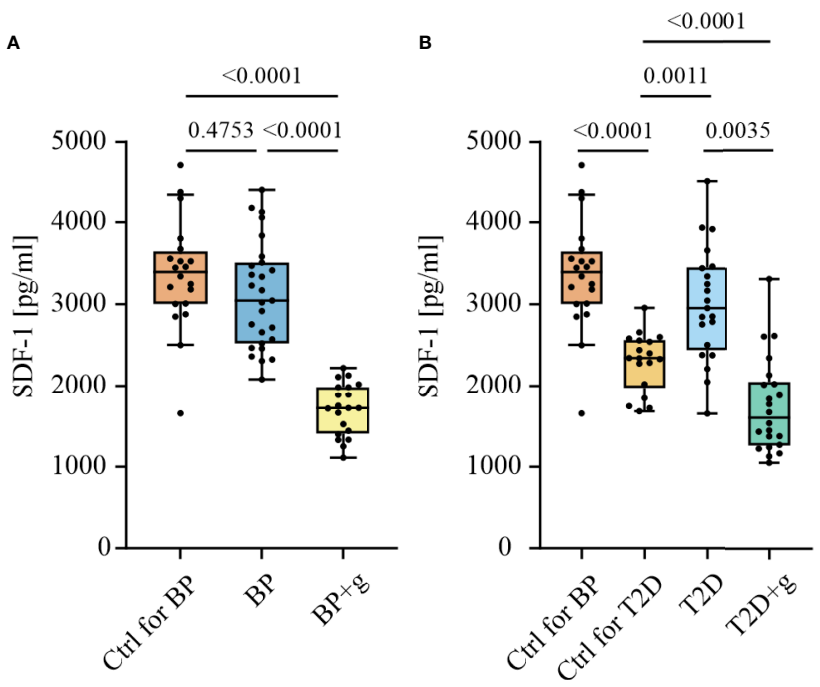

FIGURE 3
Concentrations of SDF-1α in serum samples of BP and T2D patients with (+g) or without use of gliptins. Serum SDF-1α levels were measured using human CXCL12/SDF-1α ELISA. **(A)** The use of gliptins was associated with decreased SDF-1α levels in BP patients (n = 20 for BP+g, 27 for BP and 20 for healthy age-matched control group [Ctrl for BP, 79.4 ± 2.1 years]). **(B)** The use of gliptins was associated with decreased SDF-1α levels in T2D patients (n = 22 for T2D+g, 21 for T2D and 18 for healthy age-matched control group [Ctrl for T2D, 62.7 ± 13.1 years]). Boxes depict the 25th and 75th percentiles (interquartile range) and line in the box is the median. Whiskers indicate 1.5 times the interquartile range. Statistically significant differences between groups with corresponding two-tailed P values are shown. +g, gliptin; BP, bullous pemphigoid; pg/ml, picograms per milliliter; T2D, type 2 diabetes; Ctrl, control.

(P<0.0001). SDF-1α levels of elderly BP controls were higher than in the younger T2D controls (P <0.0001) (Figure 3B). Serum SDF-1α levels did not correlate with BP180-NC16A-levels in either the BP or T2D patient groups (data not shown).

The expression of SDF-1 is increased in the skin of patients with bullous pemphigoid

Finally, we analyzed the expression of SDF-1 in the lesional rBP and BP+g skin, and healthy control skin. Immunostaining of the healthy skin was negative for SDF-1 in the epidermis and only a few immune cells showing strong staining were observed on the whole skin section (Figure 4A). Faint SDF-1 immunostaining was detected in capillary endothelial cells in both healthy control and BP skin. Independently of the gliptin status, strongly stained infiltrated cells were detected in the epidermis, dermis, and blister fluid in BP lesional skin (Figure 4B). SDF-1 was detected in epidermal keratinocytes, with the staining strongest at the blister roof and blister margins in the upper layers of the epidermis (Figures 4B, C). No statistical differences were found in the numbers of strongly stained immune cells between skin samples taken from patients with rBP and BP+g (data not shown).

Discussion

In many autoimmune diseases, including type 1 diabetes, multiple sclerosis and rheumatoid arthritis, autoantibodies have been detected before clinical onset of symptoms (37). Accordingly, in a recent retrospective study of 18 BP patients, 22% were found to be seropositive for antibodies against BP180 and/or BP230, another BP-associated autoantigen, 1-5 months before the BP diagnosis. Interestingly, some cases had detectable autoantibodies over 10 years before the diagnosis (38). We and others have previously shown individuals who carry an elevated risk for developing BP due to Alzheimer's disease or multiple sclerosis have IgG autoantibodies targeting the immunodominant NC16A domain and FL-BP180, despite the absence of clinical symptoms of BP (27–30). Although the risk of BP for patients being treated with gliptins for diabetes is at the same level or even higher than that of patients with neurological diseases (4, 14),

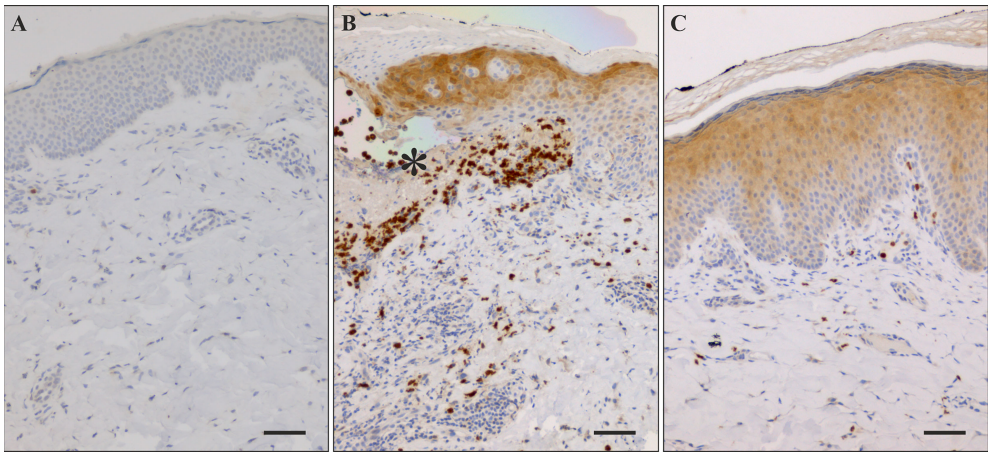

FIGURE 4
Immunostaining of SDF-1 in healthy control skin and lesional BP skin. **(A)** Immunohistochemical staining of SDF-1 was negative in healthy control skin. **(B)** Numerous SDF-1 positive cells were detected near blisters and in blister fluid (asterisk) in lesional BP skin along with strong staining of epidermis on the blister roof and on the margins of the blister. **(C)** In BP perilesional skin sections without blister there is strong staining of the upper layers of the epidermis and relatively few positive cells in the dermis. Scale bar = 50 μm.

studies concerning the preceding autoantibody levels before the diagnosis of gliptin-associated BP are scarce. Here, we investigated putative differences in the serological immunity against BP180 of patients with T2D. By using ELISA, we found that the prevalence of serum IgG-autoantibodies against folded BP180-NC16A or FL-BP180 was similar among gliptin using and non-using T2D patients, but higher than we have previously found for healthy controls (7.5%) (30). Our results echo the findings of a Japanese study, which revealed no statistically significant difference in the prevalence of BP-autoantibodies based on gliptin treatment for T2D, although a tendency towards higher levels of anti-FL-BP180 autoantibodies was found in gliptin users (39).

In immunoblotting 71% of our patients with T2D+g had IgG autoantibodies against denatured FL-BP180, but the proportion was even higher among T2D patients without gliptin medication, at 89%. Almost equal shares of BP180-NC16A ELISA positive and negative T2D+g and T2D samples recognized FL-BP180 in immunoblotting. Also 46% of the healthy control sera detected FL-BP180, which is in accordance with previous findings of our group and others (36, 40). Our previous immunoblotting works have shown that FL-BP180 is frequently recognized by serum samples from patients with Alzheimer's disease, multiple sclerosis, dermatitis herpetiformis and coeliac disease. Of note is that those samples displayed various proportions of BP180-NC16A ELISA positive and negative sera (28, 30, 36). The high proportion of FL-BP180 recognition in immunoblotting could be explained by the fact that the Alzheimer's disease and T2D populations tend to be older than those with the other conditions. Indeed, elderly people have been shown to carry autoantibodies against BP180 but with low titer and low affinities (28, 40). This suggests the presence of "naturally occurring autoantibodies" against cryptic linear epitopes present in the FL-BP180 (30). Resembling our previous results in patients with Alzheimer's disease (30), the present study found a modest increase in the recognition of intracellular epitopes by T2D/T2D+g sera compared to controls. Although the T2D groups recognized the FP4 containing BP180 aa 377–455, the sera of T2D patients without gliptin usage had higher intensity bands than the T2D+g sera. Similarly, in our previous study of gliptin-associated BP, FP4 was more strongly detected by the non-gliptin-BP than gliptin-BP sera (33). Levels of autoantibodies against this linear epitope were also elevated in patients with multiple sclerosis (30). Together with the previously described gliptin-dependent recognition of FP4 by BP autoantibodies, the subtle differences between the epitope spectra of non-gliptin-T2D and gliptin-T2D samples suggest that gliptins might modify the degradation and/or antigen presentation of BP180.

The follow-up period of nine years should have revealed any incidence of BP or its autoantibodies amongst gliptin users, since the mean latency between the initiation of gliptin usage and the development of BP in different reports ranges from 3 to 27 months (8, 9, 12, 14, 33, 41). Although we were able to review the medical records of most participants, only 57 of the initial 179 attended the follow-up sampling, probably due to the Covid-19 pandemic. During the study period sitagliptin has been the most prescribed gliptin in Finland (www.kela.fi/web/en/697), and consequently also in our T2D patients, sitagliptin was the most commonly prescribed gliptin. A study utilizing the EudraVigilance pharmacovigilance database revealed that vildagliptin was the gliptin most commonly reported to induce

BP, having about 20-fold higher proportional reporting ratio than sitagliptin (41). Several other studies, including Finnish population studies have similarly reported that the risk of BP is greater with vildagliptin than with sitagliptin (8, 14, 42). Because the majority of T2D patients in our study were using sitagliptin, we were not able to make any comparison between gliptins. However, an interesting detail is that the only T2D patient in our study population who developed BP during the follow-up period had initially used sitagliptin and developed BP after their medication was switched to vildagliptin.

To the best of our knowledge, the amount of SDF-1 in patients with BP has not previously been addressed. This is rather surprising considering the significant role of gliptins as a risk factor for BP, since SDF-1 is a well characterized substrate of DPP4 *in vivo* and is involved in inflammatory processes and lymphocyte chemoattraction (43). Because DPP4 inactivates many proinflammatory cytokines, inhibiting DPP4 with gliptins could be expected to lead to increased proinflammatory cytokine expression. Also, central B cell tolerance is dependent on the interaction of CXCR4 and SDF-1 as autoreactive B cells have a high expression of CXCR4 receptor, and SDF-1 signaling is required for the retainment of autoantibody-producing B cells in bone marrow (44). The blockade of CXCR4 in a mouse model elevated the number of autoreactive B cells in the spleen and blood (44). Gliptin-associated inactivation of SDF-1 could result in an increase of autoantibody-producing B cells in periphery. Finally, being a critical organizer of germinal centers during somatic hypermutation, changes in SDF-1 levels may also modify autoantibody production (45–47). In comparison to control samples, we detected stronger SDF-1 staining in epidermal keratinocytes in BP lesional skin and in infiltrated inflammatory cells in the blister area but did not find any significant differences in SDF-1 immunostaining between rBP or BP+g skin. This is not parallel to decreased SDF-1α levels in BP+g serum but could be explained by the fact that ELISA assay detects specifically SDF-1α while the antibody used in immunohistochemistry recognizes all SDF-1 forms. In addition, it has been shown that the local paracrine expression of SDF-1 in tissues is not tightly bound to systemic levels in serum (48). Faint SDF-1 positivity was found in vascular endothelial cells of both healthy control skin and BP lesional skin. It has previously been shown that in the healthy skin SDF-1 is mainly expressed in dermal fibroblasts, vascular endothelial cells, and epidermal dendritic cells (Langerhans cells) (26). The cutaneous expression of SDF-1 is greater in the margins of healing wounds (23, 24, 49, 50) and in skin diseases such as atopic dermatitis, psoriasis and keratinocyte cancers (25, 26). DPP4/CD26 is highly expressed on the surface of T cells, and it may have a role in T cell differentiation, maturation, and proliferation (51). Gliptins could affect T cell migration by activating/inhibiting chemokines that are DPP4 substrates. For instance, the N-terminal amino acids of SDF-1 are required for binding to CXCR4 and therefore cleavage of the N-terminus by DPP4 blocks the chemotactic effect on lymphocytes (reviewed in 18). Sitagliptin treatment has been shown to change the T cell subpopulations of T2D patients, particularly lowering numbers of Th17 and regulatory T cells (Tregs) (52, 53). Tregs maintain immune tolerance by suppressing T cell activation (54). Dysfunction of Tregs was shown to induce BP-autoantibody production (55, 56) and depletion of Tregs induced inflammation and blistering in a mouse model of BP (57). However, a recent Japanese study found no significant difference in the counts and subpopulations of circulating Tregs between BP+g patients and controls while numbers of Tregs were increased in rBP (58). It remains to be clarified whether gliptins may enhance BP development in T2D patients' skin *via* an imbalance in lymphocyte subpopulations and BP180-specific Tregs.

Our results confirmed the previous findings that serum SDF-1α levels are high in the elderly (59, 60) and in patients with T2D (61). Despite the increased expression in the BP skin, the level of SDF-1α in our patients with rBP was similar to those of the age-matched controls. However, we found that the levels of circulating SDF-1α were lower in BP and T2D patients using gliptins than in those without gliptin treatment. Of note is that the ELISA assay we used does not discriminate between the full-length (aa 1–68) and DPP4 trimmed (aa 3–68) SDF-1α. In line with our results, the use of gliptins for three months has been shown to decrease circulating total SDF-1α levels in T2D patients (62, 63). In a more specific analysis circulating levels of intact SDF-1α increased while levels of truncated (DPP4-cleaved) SDF-1α decreased both in T2D patients using sitagliptin (64, 65) and in animals with gliptin administration (66). Platelets are a major source and site of storage of SDF-1 (67) and recently many proteins associated with platelet degranulation have been detected in BP blister fluid (68). Also, elevated serum levels of sP-selectin in BP patients reflect platelet activation (69). T2D patients have enhanced platelet activation and increased risk for atherosclerosis (70). Gliptins may regulate platelet functions; sitagliptin has been shown to reduce platelet aggregation (71, 72). Taken together, high serum levels of SDF-1α could be a sign of platelet activation in both BP and T2D patients and in elderly controls (73), but it seems that the decline of circulating SDF-1α is a BP-independent gliptin-induced phenomenon. One possible explanation for gliptin-induced decline of SDF-1α is the ACKR3-mediated scavenging of SDF-1 during prolonged use of gliptins (48, 74).

Currently we have limited knowledge concerning the mechanism and timing of progression from a preclinical state with BP autoantibodies to clinical disease and whether these vary in BP patients with different risk factors. Here we found that the use of a gliptins, more specifically sitagliptin, does not induce significant appearance of anti-BP180 autoantibodies but modulates levels of SDF-1α decreasing its total serum concentration in both T2D and BP patients. Considering the limitations caused by small study populations, it seems that seropositivity against BP180 is more common among patients with neurological risk factors than gliptin users. Although rather challenging to implement, we need further research using serum samples obtained at various time points well-

ahead of the onset of BP symptoms from a large number of patients with various risk factors.

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 Ethics Committee of the Northern Ostrobothnia Hospital District and Ethics Committee of the Kuopio University Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: JT, NK, KT. Methodology: AN, PL, JT, OV, LH, KI, S-KH, OU, JJ, NK, KT. Analysis: AN, JT, PL, KT. Supervision: JT, NK, KT. Writing: – original draft preparation: AN, JT, NK, KT. Writing – Review and editing: AN, PL, JT, OV, LH, KI, S-KH, OU, JJ, NK, KT. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2022.942131/full#supplementary-material

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Increased frequency of TIGIT+ CD4 T Cell subset in autoantibody-positive first-degree relatives of patients with rheumatoid arthritis

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Background: Despite immune cell dysregulation being an important event preceding the onset of rheumatoid arthritis (RA), the phenotype of T and B cells in preclinical RA is less understood. The aim of this study was to characterize T and B cell populations in RA patients and their autoantibody (aAb) negative and positive first-degree relatives (FDR).

Methods: Cryopreserved peripheral blood mononuclear cells (PBMCs) collected at scheduled visits from aAb-(n=25), and aAb+ FDR (n=10) and RA patients (n=13) were thawed and stained using optimized antibody cocktails as per a specific 13-color T or B cell panel. Immunophenotyping was performed using a Cytoflex LX (Beckman-Coulter) flow cytometer and FlowJo software was used for analyzing the frequency of immune cell populations.

Results: Multicolor flow cytometry experiments identified an increased TIGIT expression in circulating lymphocytes of aAb+ FDR and RA patients, relative to aAb- FDR ($P<0.01$). These TIGIT$^+$ T cells exhibited a memory phenotype and expressed high levels of PD-1, ICOS, HLA-DR, CXCR3 and CXCR5. Moreover, increased TIGIT$^+$ CD4 T cell frequency correlated with the frequency of PD-1$^+$ CD4 T cells ($r = 0.4705$: $P = 0.0043$) and circulating levels of ACPA and RF. We also identified a decreased frequency of CD27+IgD- switched memory B cells in RA patients ($P < 0.01$), while increased frequency of TIGIT+ CD4 T cells in FDR correlated with the frequency of PD1$^+$PTEN$^+$ B cells ($r = 0.6838$, $P = 0.0004$) and autoantibody positivity ($P = 0.01$).

Conclusion: We demonstrate TIGIT as a distinct CD4 T cell marker for differentiating aAb- FDR from aAb+FDR and might play a critical role in regulating T and B cell crosstalk in preclinical RA.

KEYWORDS

TIGIT, PD-1, rheumatoid arthritis, first-degree relatives (FDRs), multicolor flow cytometry (MFC), immunophenotyping analysis

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic, immune mediated synovial inflammation leading to cartilage and bone destruction, joint deformity, and functional loss (1). Considerable evidence has been accumulated to suggest that seropositive RA evolves as a continuum involving distinct preclinical phases of systemic autoimmunity and immune dysregulation occurring prior to onset of clinically classifiable inflammatory arthritis (IA) (1). Currently, this preclinical phase of RA is primarily characterized by the detection of circulating RA-associated autoantibodies such as anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF), in some cases these being detected years before the onset of clinically detectable joint inflammation (1, 2).

The immunological basis for the development of RA autoantibodies during the preclinical phase, and the maturation of the autoimmunity to ultimately become pathogenic, remains an area of considerable interest and research activity. Based on our current understanding, these immunological processes require complex interactions between T and B lymphocyte populations that involve specific cognate recognition of autoantigens, as well as antigen-independent regulatory mechanisms (3). As such, it has been proposed that an expanded pathogenic $CD4^+$ T-cell population mediates activation of autoreactive B cell recognizing post-translationally modified self-antigens and resulting in the production of matured autoantibodies directed towards these autoantigens (4, 5). These processes typically occur in germinal centers of lymphoid tissues and involve a well characterized population of T follicular helper cells (Tfh) expressing CXCR5 and PD-1 (6). Of considerable interest has been the demonstration of a distinct population of activated CD4+ T cells, both in the blood and the affected synovial tissues, characterized by $PD\text{-}1^{hi}CXCR5^-$ expression, and labelled as peripheral-helper T cells (Tph cells) (7). This unique population demonstrates an increased expression of IL-21 (interleukin 21), CXCL13, and ICOS, these being involved in B-cell differentiation, migration to the inflamed tissues, and autoantibody production within the synovium (7). Tph cells also express TIGIT, an immune checkpoint receptor expressed primarily on regulatory T cells, activated T cells, B cells and natural killer cells and inhibits effector T cell responses (8). Importantly, it has been demonstrated that there is an increased frequency of these $PD\text{-}1^{hi}CXCR5^-$ Tph cells in patients with early-RA, active-RA, and even those in clinical remission (7, 9). Moreover, recent studies showed that during the preclinical phase there was a decreased frequency of naïve and regulatory T cells, along with an increased frequency of inflammation-related cells (10, 11). Indeed, it has been proposed that the CD4+ T cell dysregulation could be utilized as a predictor of progression towards disease development (10, 11).

Our aim of this study was to identify the differences in the phenotype of T and B cell populations in autoantibody-positive (aAb+) first-degree relatives (FDR) of RA patients, compared to both unaffected FDR without detectable RA autoantibodies (aAb- FDR), as well as to RA patients with established disease. We hypothesized that individuals with detectable RA autoantibodies, but no clinically evident disease represent an intermediate phenotype exhibiting some of the immunological features seen in the circulating lymphocytes of patients with established RA.

Methods

Study design

To better understand the preclinical stages of RA, we have assembled a cohort of FDR of Indigenous North American (First Nations, FN) RA patients, this population being known to have a high prevalence of RA autoantibodies, and to have an increased risk of future RA development (12, 13). Study participants were recruited from urban and rural First Nations (FN) communities in Central Canada. The Research Ethics Board of the University of Manitoba approved the overall design of the study and consent forms (HS2005:093, HS14453; Early Identification of Rheumatoid Arthritis in First Nations). The conduct of the study was guided by the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans – TCPS 2 (2018) Chapter 9: Research Involving the First Nations, Inuit and Métis Peoples of Canada and the principles of Community Based Participatory Research, a cornerstone of the Canadian Institutes of Health Research guidelines for Aboriginal health research (http://www.cihr-irsc.gc.ca/e/29134.html). This First Nations prospective longitudinal study includes oversight from Indigenous community members and Elders and includes signed research agreements, developed through mutual collaborations with First Nations communities. Additionally, an Arthritis Advisory Committee, with Indigenous community representatives collaborates on proposed research work. Study participants provided free, informed consent. Within this cohort

of FDR, we identified individuals in whom ACPA and/or RF were detectable, and in a subset of these individuals for whom peripheral blood mononuclear cells were available for study.

This sub-study was undertaken at a single timepoint in three distinct groups: (1) aAb+ RA patients (ACPA and/or RF), all of whom met the 2010 ACR/EULAR criteria (n=13), (2) aAb+ FDR with detectable ACPA or RF titers (>20 U/mL), but without any clinical evidence of joint or systemic inflammation (n=10), and (3) unaffected aAb- FDR (n=25) without any arthralgia, systemic inflammation and detectable ACPA or RF titers. RA patients and FDR studied here were, in most cases, not related. As such, these groups represent a hypothesized continuum, where the aAb+ unaffected group represents an intermediate phenotype that, in some cases, leads to the future development of clinically classifiable RA. Characteristics of the study participants are shown in Table 1.

Sample collection, storage, serology and PBMC isolation

Venous blood was collected into BD Vacutainer SST™ serum separation tubes (BD Biosciences, US) by a trained phlebotomist or study nurse, allowed to clot for 35 minutes and then centrifuged to separate the serum. Aliquots of sera were stored at −20°C until required for serology assays. C-reactive protein (CRP) levels were measured using a human high-sensitivity CRP (hs-CRP) ELISA kit (Biomatik, Canada) as per the manufacturer's instructions. ACPA titer was determined using either the anti-CCP2 on a BioPlex® 2200 System (Bio-Rad, US) or anti-CCP3 kits (Inova Diagnostics Inc, San Diego, CA). ACPA seropositivity status was considered negative if the titer was below manufacturer's standardized assay cutoff (< 20U/mL, also known as upper limit normal or ULN) (13). For peripheral blood mononuclear cells (PBMC), venous blood was collected into heparinized vacutainers (BD) and isolation was performed using SepMate™ tubes (StemCell Technologies) as per the manufacturer's protocol. Isolated PBMCs were cryopreserved in a freezing medium (90% fetal bovine serum and 10% DMSO) and stored in liquid nitrogen.

Multi-color flow cytometry

T and B cell immunophenotyping was performed by multicolor flow cytometry on frozen PBMCs. Cells were thawed, washed with PBS + 1%BSA solution and surface-stained for either T or B cell markers at 4°C for 30 minutes. Stained cells were run on Cytoflex LX (Beckman-Coulter). OneComp eBeads™ (Thermofisher Scientific) were used as compensation controls, while FMO (fluorescence minus one) controls were used as negative controls. $CD4^+$ T cells were represented as percent of $CD3^+dump^-$ ($CD19^+$/$CD14^+$/$CD56^+$/viability) population, while B cells were represented as a percent of $CD19^+dump^-$ ($CD14^+$/$CD56^+$/$CD3^+$/viability) population. Antibody details are listed in the Supplementary Table S1. $CD4^+$ T cell and $CD19^+$ B cell subset gating strategy is presented in Supplementary Figure S1.

Data analysis & statistics

Flow cytometry data was analyzed by FlowJo (v10.8). Graphs and statistics were generated using GraphPad Prism (v9.1). Compensated fcs (flow cytometry standard) data files were

TABLE 1 Baseline characteristics of the study population: All values are reported as either mean (SD) or n (%).

	FDR		RA(n=13)
	aAb-(n=25)	aAb+(n=10)	
Age, years, mean (SD)	43.6 (10.1)	47.7 (11.2)	44.1 (12.5)
Female, n (%)	11 (44)	4 (40)	9 (69.23)
CRP, mg/L, mean (SD)	2.7 (1.9)	3.7 (3.7)	15.5 (13.4)$^{\$}$
BMI, kg/m2, mean (SD)	28.9 (6.5)	29.3 (5.5)	28.3 (5.4)
DAS28, mean (SD)	–	–	3.9 (1.5)
Ever Smokers (%)	22 (88)	9 (90)	10 (77)
Autoantibody(aAb) status*			
RF+ only (%)	0	3 (30)	1(7.7)
ACPA+ only (%)	0	3 (30)	1 (7.7)
ACPA+ and RF+ (%)	0	4 (40)	7 (53.8)
ACPA-/RF- (%)	25 (100)	0	2 (15.4)

*Autoantibody status is available for two RA patients.
$^{\$}$ = $P<0.05$ compared to aAb- or aAb+ FDR; analyzed by Mann-Whitney test.
RA, Rheumatoid Arthritis; FDR, first-degree relative; aAb, autoantibody; CRP, C-reactive protein; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibody; BMI, body mass index; DAS28, disease activity score 28.

pre-processed using CytoNorm plugin for batch normalization and FlowClean plugin to remove unwanted events (14, 15). Gates were set to define a population subset using FMOs as negative control. Mann-Whitney U test, Kruskal-Wallis test with Dunn's *post-hoc* correction, Wilcoxon matched-pairs signed test, Spearman rank correlation analyses or regression analyses were used for statistical comparison as per the requirement. *P*-values < 0.05 were considered as statistically significant.

Results

Study population

Table 1 outlines the characteristics of these three groups. There were no significant differences in age, sex, BMI, or smoking frequency between the groups. All the RA patients met 2010 ACR criteria, had established disease with a mean DAS28 score of 3.9 ± 1.5, and were receiving stable doses of disease-modifying anti-rheumatic drugs. Of these RA patients, 53.8% (7/13) were seropositive for both ACPA and RF, 7.7% (1/13) were either positive for ACPA or RF and 15.4% (2/13) were negative for both ACPA and/or RF. Autoantibody data was unavailable for 2 RA patients. Neither of the two FDR groups had clinically detectable RA, although as we previously reported, joint symptoms were prevalent in this population irrespective of their seropositivity status (16). Of note, the aAb+ FDR group was comprised of 3 individuals who were positive for ACPA only (222.5 ± 83.2 U/mL; mean ± SD), 3 individuals who were positive for RF only (27.9 ± 8.9 IU/mL; mean ± SD), and 4 individuals who were positive for both ACPA (59.6 ± 21.9 U/mL) and RF (231.5 ± 331 IU/mL).

RA patients and aAb+ FDR have increased frequency of TIGIT-expressing CD4$^+$ T cells

Relative to aAb-FDR, RA patients and aAb+ FDR had a significantly higher frequency of TIGIT$^+$ CD4 T cells and TIGIT: PD-1 ratio (P < 0.01; Figures 1A, B; Figure S1 shows gating strategy of TIGIT$^+$ CD4 T cells). Frequency of TIGIT$^+$ and PD-

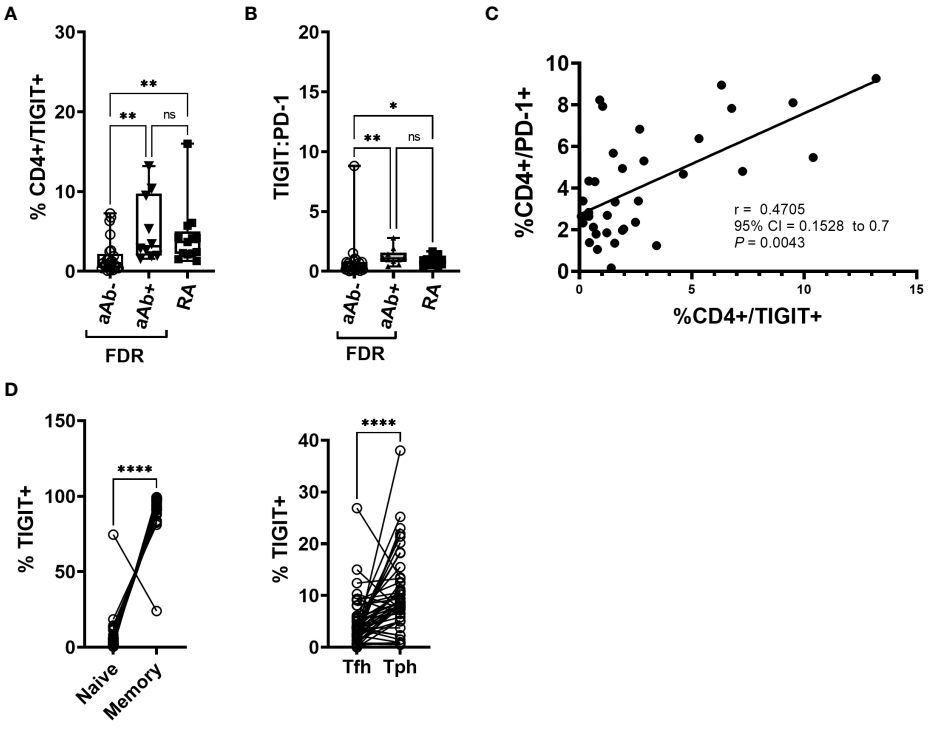

FIGURE 1
(A) Box-whiskers plot showing the frequency of CD4+ cells positive for TIGIT between aAb-FDR (n=25), aAb+FDR (n=10) and RA patients (n=13). *P<0.05, **P<0.01; Data was analyzed using Kruskal-Wallis method with Dunn's *post-hoc* test. **(B)** Box-whiskers plot showing TIGIT: PD-1 ratio between aAb-FDR (n=25), aAb+FDR (n=10) and RA patients (n=13). *P<0.05, **P<0.01; Data was analyzed using Kruskal-Wallis method with Dunn's *post-hoc* test. **(C)** Figure showing Spearman rank correlation plot between the frequency of TIGIT+ vs PD-1+ CD4 T cells. **(D)** Plot showing the frequency of naïve, memory, Tph and Tfh cells in the TIGIT+ fraction in all the subjects (n=48). Data analyzed by Wilcoxon matched - pairs signed rank test. ****P<0.0001. ns, non-significant.

1$^+$ CD4 T cells was used to calculate the ratio. We also observed a strong correlation between TIGIT$^+$ and PD-1$^+$ CD4 T cells in FDR (P = 0.0043; Figure 1C). We observed that ~93% of memory (CD4$^+$ CD45RA$^-$) T cells were TIGIT$^+$ (Figure 1D) and were found at a higher frequency in the Tph fraction (CXCR5$^-$PD-1^{hi}) than in the Tfh fraction (CXCR5$^+$ PD-1^{hi}; mean 10.18 vs 4.723; P < 0.001; Figure 1D). Expression of other phenotypic markers on the surface of peripheral blood CD4$^+$ T cells was similar between aAb-FDR and aAb+FDR and RA patients (Figure S2). We also did not observe any significant differences in the proportion of major memory CD4$^+$ T cell subsets between the two groups based on CCR7 or CD45RA expression (Figure S2).

Characterization of TIGIT$^+$ CD4 T cells across the entire study population showed that a higher proportion of these cells were positive for HLA-DR, Ki-67, PD-1, ICOS, CXCR3 and CXCR5, compared to TIGIT- CD4 T cells (Figure 2). CCR2 shows the opposite trend, with TIGIT$^+$ cells showing significantly lower expression of this regulatory T cell-associated marker (Figure 2A). Interestingly, the TIGIT$^+$ population shows selective phenotypic differences between aAb+ and aAb- FDR groups, with lower frequencies of Ki-67 and HLA-DR expression (Figure S3). While we observed a decrease in the frequency of TIGIT- CD4 T cells in aAb+FDR and RA patients relative to aAb-FDR (Figure 2B), these cell subsets did show any difference in the expression of phenotypic markers such as Ki-67 and HLA-DR (Figure S4). Taken together, these findings suggest that TIGIT$^+$ generally cells have a higher activation status, exhibit a stronger proliferation index and better migratory capacity compared to TIGIT- cells. Expression of some activation and proliferation markers on TIGIT$^+$ CD4 T cells is lowered in autoantibody-positive FDR, potentially indicating that chronic stimulation leads to a decreased activation status and a reduced proliferative capacity.

Frequency of TIGIT$^+$ CD4 T cells in FDR correlate with the frequency of PD-1$^+$ and PTEN$^+$ B cells

TIGIT expression on CD4 T cells facilitates T-B cell interactions and promotes B-cell differentiation into antibody-

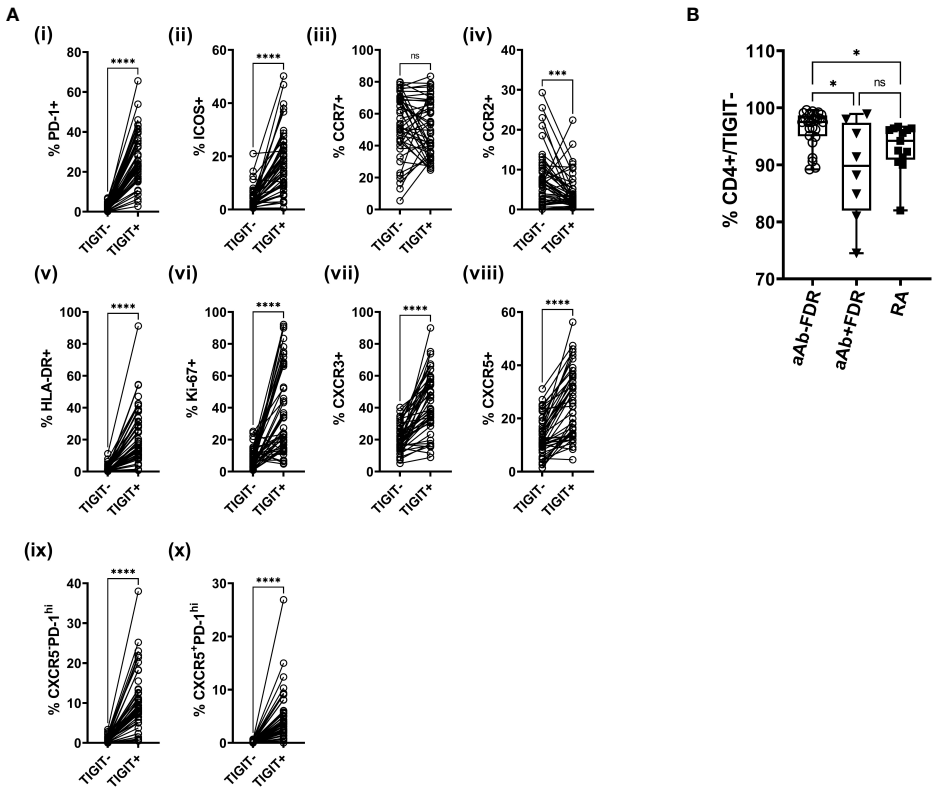

FIGURE 2
(A) Plots showing the frequency of TIGIT$^+$ and TIGIT$^-$ CD4 T cells expressing various phenotypic markers in the entire study population (i -x; n = 48). Data was analyzed using Wilcoxon matched pairs signed rank test. ***P<0.001, ****P<0.0001 ns, non-significant. **(B)** Box-whiskers plot showing the frequency of TIGIT$^-$ CD4+ cells between aAb-FDR (n=25), aAb+FDR (n=10) and RA patients (n=13). *P<0.05; Data was analyzed using Kruskal-Wallis method with Dunn's *post-hoc* test. ns, non-significant.

secreting plasmablasts (8). Therefore, we next analyzed the phenotype of $CD19^+$ B cells in the peripheral blood of a subset of FDR (both aAb- and aAb+) and RA patients on whom CD4 T cell phenotyping was done (Figure 3 and S5). Relative to aAb- FDR, RA patients demonstrated a high frequency of $CD27^-$ naïve B cells. The frequency of $CD27^+$ memory B cell population, including switched memory B cell subsets (B_{SM}; $CD27^+IgD^-$) was lower in RA patients compared to aAb- FDR (Figures 3A, B). Further analysis of individual markers showed no significant differences between the three groups (Figure S5). However, we observed a strong correlation between the frequency of $TIGIT^+$ CD4 T cells and $PD\text{-}1^+$ or $PTEN^+$ B cells (P = 0.0011 and P = 0.0056 respectively, Figure 3C). The frequency of $TIGIT^+$ CD4 T cells also correlated with $PD1^+PTEN^+$ B cells (r = 0.7322, 95% CI = 0.4045 to 0.8933, P=0.004) indicating an association between the expression of co-inhibitory receptors on T and B cells supposedly mediated through PI-3K signaling. Of note, no such correlation was observed in the RA patients.

Discussion

Currently, the most widely accepted biomarker for determining the risk of future RA development is the detection of circulating RA autoantibodies, particularly ACPA and RF, in otherwise clinically unaffected individuals. We and others have shown that a proportion of these RA autoantibody positive individuals will ultimately develop clinically definable RA, albeit after a variable period of follow-up (2, 13). The immunological mechanisms that underlie the development of the RA autoantibodies, and the progression towards pathogenic autoimmunity in specific individuals, remain unclear. Although alterations in circulating cytokines and chemokines detected in readily accessible serum/plasma samples have provided important clues in this regard (3, 10, 17, 18), disturbances in immune cell populations have not been defined, primarily because of the difficulty in obtaining suitable samples from at-risk individuals. In the current study, we used multiparametric flow cytometry to profile peripheral blood T and B lymphocytes in a cohort of RA autoantibody positive at-risk individuals and compared them to autoantibody negative individuals from the same population, and to RA patients with established disease. Our results support the hypothesis that individuals with detectable RA autoantibodies, but no clinically evident disease represent an intermediate phenotype exhibiting some of the immunological features seen in the circulating $CD4^+$ T lymphocytes of patients with established RA, particularly an increased expression of the immune checkpoint receptor TIGIT, primarily among $PD\text{-}1^+$ cell subsets.

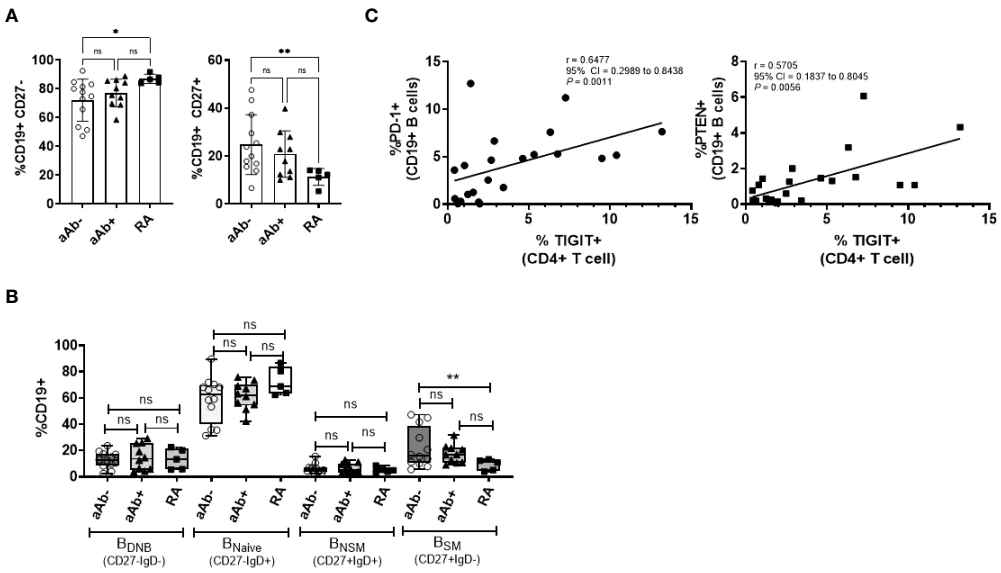

FIGURE 3
(A) Box-whiskers plot showing the frequency of $CD19^+CD27^-$ (naïve) and $CD19^+CD27^+$ (memory) B cells between aAb-FDR (n=12), aAb+FDR (n=10) and RA patients (n=5). **(B)** Box-whiskers plot showing the frequency of B-cell subsets between aAb-FDR, aAb+FDR and RA patients. **(C)** Spearman correlation plots showing the relationship between the frequency of TIGIT+ CD T cells (x-axis) and frequency of $PD\text{-}1^+$ or $PTEN^+$ (y-axis) in FDR (n=34). Data was analyzed by Kruskal-Wallis test with Dunn's *post-hoc* analysis. *P<0.05, **P<0.01, ns, non-significant.

Our findings are consistent with those made by Ponchel et al. and others, suggesting that T and B cell dysregulation is observed in the preclinical stages of RA and supports the idea of using multiparametric T cell quantification as a clinical tool for predicting stages of preclinical RA (10, 11). ACPA are RF are insufficient in identifying individuals at a high-risk of disease progression as we know that there is a high likelihood of seroconversion to an autoantibody-negative state in those FDR who are non-progressors (13). As a result, there is an increased need of reliable biomarkers that can predict individuals at different stages along the RA continuum and help us identify individuals 'at-risk' of disease progression. Multi-parametric immune cell profiling allows us to stratify individuals along the RA continuum and allows us to develop hypotheses and explore mechanisms underlying these different stages of disease development, which can eventually lead to a targeted interventions (10).

Elevated TIGIT expression in aAb+ RA patients concurs with the observations made by Luo et al. and others, who found an increased TIGIT expression both in the peripheral and synovial CD4 T lymphocytes of RA patients (19–21). In RA patients, increased TIGIT expression correlated with disease activity, was an independent predictor of RA treatment response, and inhibited CD4 T cell effector responses such as cell proliferation and secretion of proinflammatory cytokines IFN-γ and IL-17 (19, 20). For the first time, we demonstrate an expanded $TIGIT^+$ CD4 T cell subset in aAb+FDR, some of whom might progress towards developing inflammatory arthritis. Our study also indicates the presence of a crosstalk between the presence of circulating RA autoantibodies and TIGIT expression on CD4 T cells. In FDR, increased TIGIT expression on CD4 T cells, along with reduced CCR2, CXCR3 and Ki-67 expression on $TIGIT^+$ CD4 T cells and correlation with ACPA and RF indicates an autoantibody-mediated polarization of CD4 T cells to either an exhaustive or a regulatory phenotype (11, 22). This hypothesis is supported by the observations made by Hunt et al. who demonstrated an expansion of regulatory T cells in at-risk seropositive individuals prior to onset of inflammatory arthritis (11). Most importantly, expression of TIGIT defines a distinct regulatory T cell population that exhibit an activated phenotype and suppress Th1 and Th17 cell differentiation and effector responses (23). Analysis of T cells in individuals who are prospectively followed till they develop inflammatory arthritis will provide us further insights into the functional role of $TIGIT^+$ T cells in RA.

We demonstrated a strong correlation between $TIGIT^+$ CD4 T cells and $PD1^+PTEN^+$ B cells (r = 0.7322, 95% PI = 0.4045 to 0.8933, $P < 0.0004$). PTEN and PD-1 are essential for maintaining peripheral B cell tolerance against autoantigens and mediate B cell functions such as antibody class switching, somatic hypermutation, migration into germinal centers in the synovium and memory B cell differentiation on plasmablasts (7, 21). In our study, we also observed an increased in TIGIT: PD-1 ratio in both RA patients and aAb+FDR along with a positive correlation between TIGIT positivity of T cells and the frequency of $PD1^+PTEN^+$ B cells. These findings point towards a novel regulatory function of $TIGIT^+$ T cells in determining the fate of memory B cells in an environment that contains increased amounts of cytokines such as IL-2, IL-15, IL-12 and IFNα (8, 24). While functional studies are needed to demonstrate these events, we are also interested in exploring the antibody repertoire of increased $CD27^+$ memory B cells (switched and non-switched), the relative percentage of those being a citrulline-reactive and the role of TIGIT in this process.

The strength of our study is the characterization of T and B cell immunophenotype in the same sample, which allows us to correlate and study the relationship between different immune compartments within the individual with acceptable confidence. A major limitation of our study is the lack of data that can demonstrate the function of TIGIT on T cells in the absence or presence of autoantibodies COVID-related logistical complications made it unfeasible for us to obtain PBMCs required for performing functional assays to assess the function of above-mentioned cell populations. These issues were also central to the low sample size observed in our study. We also acknowledge the fact that our study was performed on samples only from First Nations communities, which has been the primary focus of our research program owing to their increased genetic susceptibility to RA (13). Interestingly, irregular immune cell phenotype and an altered serum cytokine pattern observed in seropositive FDR concurs with the findings observed in other populations, thus advocating the need of undertaking many such studies on a larger sample size to better define immune cell perturbations during the preclinical period (5, 10, 11). Although long-term outcomes for these individuals would be of considerable interest, there was insufficient follow-up at the time of this manuscript to provide meaningful data.

In conclusion, we highlighted the role of co-inhibitory receptors on T and B cells, and their crosstalk with inflammatory cytokines in modulating adaptive immune responses to autoantigens during the preclinical stages of RA. Our findings provide compelling evidence showing a distinct preclinical immune activation in seropositive FDR, which is dependent on the appearance of RA-associated autoantibodies. Our study also found value in using T and B cell immune profiling as a clinical predictor of RA onset. Our future plan is to develop a model by combining immunophenotyping data, with serum cytokines and other known parameters of preclinical RA and evaluate its clinical applicability in a larger cohort of 'at-risk' people for RA prediction. We foresee a long-term use of such models as an outcome measure in intervention studies aimed at RA prevention.

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 Research Ethics Board, University of Manitoba. The patients/participants provided their written informed consent to participate in this study.

Author contributions

VA, ST, and HE-G conceived research concept; VA, ST and CZ performed experimental work, VA analyzed data, VA and LO'N prepared figures, CZ, LO'N, AM, and HE-G assisted in data analysis, IS assisted in patient recruitment, IS and XM assisted in sample acquisition and storage, and VA and HE-G. drafted, and revised the manuscript, and all the co-authors participated in editing 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.2022.932627/full#supplementary-material

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IL-10 revisited in systemic lupus erythematosus

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IL-10 is a cytokine with pleiotropic functions, particularly known for its suppressive effects on various immune cells. Consequently, it can limit the pathogenesis of inflammatory diseases, such as multiple sclerosis (MS), inflammatory bowel disease, Crohn's disease, and Epidermolysis bullosa acquisita, among others. Recent evidence however indicates that it plays dual roles in Systemic lupus Erythematosus (SLE) where it may inhibit pro-inflammatory effector functions but seems to be also a main driver of the extrafollicular antibody response, outside of germinal centers (GC). In line, IL-10 promotes direct differentiation of activated B cells into plasma cells rather than stimulating a GC response. IL-10 is produced by B cells, myeloid cells, and certain T cell subsets, including extrafollicular T helper cells, which are phenotypically distinct from follicular helper T cells that are relevant for GC formation. In SLE patients and murine lupus models extrafollicular T helper cells have been reported to support ongoing extrafollicular formation of autoreactive plasma cells, despite the presence of GCs. Here, we discuss the role of IL-10 as driver of B cell responses, its impact on B cell proliferation, class switch, and plasma cells.

KEYWORDS

IL-10, B cells, systemic lupus erythematosus, extrafollicular T cells, autoimmunity

Introduction

In the context of T-dependent (TD) immune responses (i.e. response to protein antigens), B cells can form short-lived plasma cells, memory B cells and long-lived plasma cells, eventually yielding a complex humoral response consisting of antibodies of various immunoglobulin (Ig) subtypes. The antigen-binding affinities of secreted antibodies range from relatively low dissociation constants (Kd) of below 10^{-6} moles/liter to very high Kd values of approximately 10^{-10} moles/liter (1–4). Antibodies against proteins of infectious agents tend to be of highest affinities, whereas autoantibodies can reach affinities of reported Kd of 10^{-7} moles/liter (5).

Low affinity antibodies are formed early in the GC reaction and during the extrafollicular pathway. In the latter, activated B cells directly differentiate into plasmablasts within extrafollicular foci of secondary lymphoid organs, avoiding GC formation (6–8). High affinity antibodies are typically formed in the context of the follicular pathway. There activated B cells initiate a GC reaction transforming a primary follicle into a secondary follicle (GC), eventually yielding hypermutated and affinity selected memory B cells and plasma cells producing antibodies with high affinity. Antibodies formed within both the follicular and the extrafollicular pathway play critical roles in the immediate immune defense against pathogens, as well as the development of autoimmune and allergic diseases (9–14). The extrafollicular antibody response is fast, peaking within a few days after immunization/infection to provide the first line adaptive humoral response. This early response consists mainly of IgM, but can also include a considerable proportion of switched isotypes, such as IgG1 (15). The follicular response generates memory B cells and long-lived plasma cells which can produce antibodies of all subclasses, which could persists for decades (9–14). If the same antigen-activated B cell uses asymmetric division to initiate a follicular and extrafollicular response simultaneously, or if different B cells are attracted into the two pathways is unknown so far (12).

Sources and functions of IL-10

Cytokines are important co-stimulatory factors that can modulate the nature of TD follicular and extrafollicular responses. They serve as B cell differentiation factors, support the survival of antibody-secreting plasmablasts and plasma cells and are key factors controlling antibody class switch. IL-10 is a major B cell stimulating cytokine, recently reported to be a crucial driver of the extrafollicular B cell response. It is a pleiotropic cytokine with potent immune tolerogenic effects (16). The receptor for IL-10 is a transmembrane spanning molecule comprising of the two subunits IL-10R1 and IL-10R2 (17). After activation and receptor dimerization, the JAK-STAT pathway gets activated resulting in the upregulation of STAT3.

When it was first discovered, IL-10 was described as Cytokine Synthesis Inhibitory Factor (CSIF) because it suppressed the activation and subsequent cytokine production by T helper (Th)1 cells. Originally described as part of the Th2 cytokine pattern, it was later realized that IL-10 is also produced by late Th1 cells (18–20). As of today, the cellular source of IL-10 has been broadened to include many hematopoietic and non-hematopoietic cell types, including B cells, malignant and non-malignant plasmablasts and plasma cells, cytotoxic T cells, natural killer cells, regulatory T cells, dendritic cells, among others (21–26).

One function of IL-10 is to suppress excessive pro-inflammatory action that might result in tissue damage. Accordingly, IL-10 deficient mice develop severe colitis (27). The cytokine inhibits the upregulation of MHC II and co-stimulatory molecules on antigen presenting cells (APC), thereby inhibiting the production of inflammatory cytokines (28, 29). Dendritic cell specific deletion of the IL-10 receptor limits chemokine production in tissues (30). It further prevents unwarranted T cell activation and subsequent proliferation that might result in inflammatory effector functions (31, 32). Plasma cell derived IL-10 has a local impact on adjacent neutrophils and myeloid cells (33, 34). IL-10 production from both B and T cells has important immunosuppressive functions, but while CD4 specific IL-10 KO mice show indications of severe inflammatory bowel disease with a high penetrance (35), there is no such indication in B specific IL-10 KO mice. Hence, indicating that IL-10 production of the two distinct lymphocyte types exhibit non-redundant functions.

IL-10 plays a protective role in MS and in the murine model of this autoimmune disorder of the central nervous system, experimental autoimmune encephalomyelitis (EAE) (36, 37). The cytokine is expressed at increased levels in remission phases of and its murine model EAE (38). B lineage derived IL-10 was shown to be critical for the disease course of EAE, with plasmablasts the critical source of the inhibitory cytokine (31).

In a mouse model for the autoimmune skin blistering disease Epidermolysis bullosa acquisita (EBA), IL-10 blockade has been shown to largely downregulated disease pathogenicity by inhibiting innate effector functions, while induction of IL-10+ plasma cells could inhibit the disease (33). In conclusion, IL-10 is produced by various cell types, has multiple functions, could down-regulate inflammatory effector cells but can also drive inflammation *via* promotion of the production of autoantibodies.

IL-10 influence of B cell proliferation, differentiation, and Ig class switch

Several cytokines are important co-stimulatory factors for the B cell response (39). Among them is IL-10, which has been known for long to promote B cell activation, differentiation and antibody class switch *in vitro* (40). Recent studies now imply that IL-10 could be of crucial relevance for B cell differentiation in SLE and murine lupus, particularly for the extrafollicular response (41–44).

In an *in vitro* setup using CD40 stimulated human B cells, IL-10 induced B cell proliferation, comparable with the effect of IL-4, which is a cytokine that plays a prominent role for the

survival and proliferation of cultures GC B cells and *in vitro* generated GC-like cells (45–47). IL-10 but not IL-4 could stimulate the secretion of antibodies into the culture (45).

A separate study focused on the role of IL-10 on GC B cells, also in an *in vitro* setup. Tonsillar B cells were cultured in the presence of CD40L and a follicular dendritic cell (FDC) like cell line, along with different cytokines. In the presence of IL-10, GC B cells serially differentiated into CD20+ CD38- memory B cells and subsequently into CD20-CD38+ plasma cells. However, in the absence of IL-10, plasma cell differentiation was impaired (48). In another study, cultured tonsillar human B cells, either activated through their Ig receptor or CD40, were seen to undergo proliferation and subsequent differentiation to antibody secreting cells in an IL-10 dependent manner (45). IL-10 can also promote the secretion of IgM, IgG1 and IgG3 in cultures of stimulated naïve tonsillar surface IgD+ B cells (49). A follow-up study than showed that IL-10 initiated the formation of switch circles, i.e. fragments of IgH DNA excised during class-switch recombination, indicating that IL-10 is actually supporting class switch recombination (50). Other studies using cultures of human B cells from peripheral blood or isolated tonsils provided further evidence that IL-10 can support the proliferation, Ig class switch and antibody secretion (51, 52). There is some evidence that B cell derived IL-10 could promote plasma cell differentiation and the secretion of antibodies of the IgM and IgG classes in an autocrine manner (53).

IL-10+ extrafollicular T helper cells as drivers of a persistent extrafollicular response in SLE

In the GC, B cells interact with a specialized subset of CD4+ T cells called follicular helper T (Tfh) cells which can phenotypically be characterized as CD4+ CXCR5+ ICOS+ PD1+ cells (54). Downregulation of chemokine receptor CCR7 and upregulation of CXCR5 allow Tfh cells to migrate towards B cells of secondary lymphoid tissues in the B cell follicles (55). Here, they interact with the antigen-activated B cells. Tfh cells provide IL-21, a cytokine required for a normal GC function (56). As shown in SLE, IL-10 is of crucial importance to drive the extrafollicular plasma cell formation outside the GC, independent of IL-21 (42). Multiple lines of evidence indicate that the extrafollicular plasma cell response is supported by a T helper cell population distinct from Tfh cells. These extrafollicular T helper cells express PD-1, but lack the expression of the chemokine receptor CXCR5, indicating that they are not able to migrate to the B cell follicles. Instead they express CCR6, with or without expression of CXCR3. Extrafollicular helper T cells do not produce IL-21, but seem to support extrafollicular plasma cell responses and antibody production mainly through IL-10 (42). In SLE patients, extrafollicular helper T cells have been identified in the peripheral blood, in the lymph nodes and tubular interstitial spaces of the kidneys (42, 44). Extrafollicular helper T cells were seen to stimulate plasmablast formation and IgG-autoantibody production of Toll-like receptor stimulated naïve and memory B cells from SLE patients in an IL-10 dependent manner. Moreover, murine IL-10+ extrafollicular helper T cells were shown to provide B cell help and support the production of IgG antibodies in pristane-induced lupus and after immunization with the model antigen ovalbumin, *in vivo*. In these systems, autoantibodies and anti-ovalbumin-antibodies were approximately 30% and 50% reduced in B cell specific IL-10 receptor knock out mice, respectively. Hence, indicating that B cell help from extrafollicular helper T cells is indeed partly dependent on IL-10 (42). Similarly, T cell derived IL-10 seems to support the extrafollicular antibody response to infection with plasmodium (57).

The opposing roles of IL-10 in SLE

There have been numerous studies indicating that IL-10 may play multiple and opposing roles in murine lupus. As discussed above, there is good evidence that IL-10 supports the proliferation and differentiation of autoreactive B cells into plasma cells in SLE, thereby contributing to disease progression (42, 44).

However, studies in murine models indicate that IL-10 can also play a protective role in lupus. Research done on lupus-prone B6.Sle1.Sle2.Sle3 mice depicted elevated levels of IL-10 from B lineage cells and CD4+ T cells. Transduced continuous overexpression of low levels of IL-10 in skeletal muscle cells resulted in a delay of autoantibodies. It further resulted in reduced renal pathology as depicted by reduced amounts of IgG and C3 deposits in the glomeruli (58). Because increased IL-10 impaired the activation of T cells in this model, it is difficult to draw conclusions on the direct role of IL-10 on B cell function. However, it indicates that the anti-inflammatory role of IL-10 plays a beneficial role in the disease. Accordingly, B cell specific deletion of IL-10 in Lyn deficient mice, another lupus model, lead to increased disease severity, but did not alter plasma cell counts (59). Hence indicating that B cell derived IL-10 can inhibit lupus pathology but has no impact on the overall B cell activation. In B6.NZMc1c4 mice, yet another lupus model, genetic deletion of IL-10 lead to increased production of autoantibodies (60). If this was due to a direct effect IL-10 has on B cells, or the consequence of the well-known inhibition of T helper cell differentiation (56), remains to be established.

The dual and opposing roles IL-10 has in lupus was recently depicted in New Zealand Black x New Zeeland White F1 mice were *in vitro* experiments revealed pro- and anti-inflammatory IL-10 effects (61). *In vivo* blockade of IL-10 starting after start of

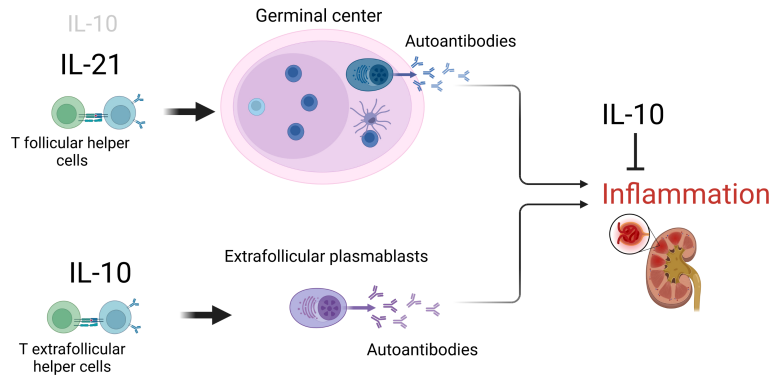

FIGURE 1
The opposing role of IL-10 in SLE. The cytokine drives the continuous formation of pro-inflammatory autoantibodies, particularly outside GCs, but can also limit inflammatory effector functions. Figure created with BioRender.com.

the disease at the age of 5 months increase production of autoantibodies and lupus pathogenesis (61). In another study however, continuous administration of blocking anti-IL-10 antibodies at birth, delayed the onset of autoantibody production and also disease symptoms such as proteinuria, glomerulonephritis (62). Together, these studies indicate that IL-10 blockade had opposing effects on the development of autoantibodies when applied before disease onset or afterwards. Since the extrafollicular response is expected to precede the follicular response, it is possible that early blockade of IL-10 blocks the initiation of the pathogenic extrafollicular B cell response hence delaying the onset of the disease. Blockade of IL10 later on may still inhibit the extrafollicular response, but the IL-10 independent follicular response could take over. IL-10 inhibition may even boost the follicular response because of the inhibitory effect the cytokine has on the formation of CD4 T helper cells. Hence, it is possible that after disease onset the IL-10 blockade shifts the balance between the extrafollicular and the follicular response, without changing the overall production of autoantibodies too much.

Conclusion

IL-10 is a potent suppressor of inflammatory effector functions. However, like several other cytokines, it can also promote B cell proliferation, differentiation and class switch. It had been recognized for long that IL-10 plays important, but redundant functions in the follicular B response in GCs. Recent evidence indicates that the cytokine is of even greater importance as a promoter of the extrafollicular B cell response outside the GC (Figure 1). In SLE, a specialized subset of extrafollicular helper T cells seems to be crucial to drive the extrafollicular autoantibody B cell response and disease pathogenesis, *via* IL-10. If that holds true also for other autoimmune diseases, remains to be elucidated.

Author contributions

RM and SB performed the literature search and drafted the original manuscript. SB drew the figure. KB revised the manuscript. All the authors approved the final version of the manuscript.

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Conflict of interest

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Clinical impact and a prognostic marker of early rituximab treatment after rituximab reimbursement in Korean pemphigus patients

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Pemphigus is an autoimmune mucocutaneous blistering disease caused by autoantibodies against desmogleins. Rituximab effectively treats pemphigus by inducing remission and rapidly reducing corticosteroid dosage. In Korea, the high cost of rituximab had been a burden until the National Health Insurance began to cover 90% of rituximab costs *via* reimbursement for severe pemphigus patients. We analyzed 214 patients with pemphigus who were treated with their first round of rituximab. The time to initiate rituximab and the time to partial remission under minimal therapy (PRMT) were both significantly shorter after the rituximab reimbursement policy. The total steroid intake for PRMT and complete remission (CR) was less in patients who were diagnosed after the reimbursement. The interrupted time series (ITS) model, a novel analysis method to evaluate the effects of an intervention, showed a decrease in total systemic corticosteroid intake until PRMT after reimbursement began. In peripheral blood mononuclear cells from patients with pemphigus vulgaris, the relative frequencies of desmoglein 3-specific $CD11c^{+}CD27^{-}IgD^{-}$ atypical memory B cells positively correlated with the periods from disease onset to rituximab treatment and to PRMT and the total systemic corticosteroid intake until PRMT. We found that early rituximab therapy, induced by the reimbursement policy, shortened the disease course and reduced the total corticosteroid use by pemphigus patients. The decreased frequency of circulating desmoglein-specific atypical memory B cells can be used as a surrogate marker for a good prognosis after rituximab.

KEYWORDS

pemphigus, rituximab, reimbursement, biomarker, autoimmune bullous diseases

Introduction

Pemphigus is an autoimmune blistering disease that affects the skin, the oral cavity, and other mucosal surfaces. Autoantibodies against the cell-surface proteins desmoglein (Dsg) 1 and Dsg3 are pathogenic (1). Intraepidermal blisters with acantholysis and erosion take place, and the failure to manage them can cause life-threatening infections, along with severe pain and poor quality of life (2). Anti-Dsg IgG autoantibodies are necessary and sufficient to induce acantholytic blisters (3, 4). Pathogenic Dsg3-specific B cells have memory phenotypes and undergo somatic hypermutation in pemphigus vulgaris (5), although some germline-reverted antibodies can bind to Dsg3 (6).

The initial treatment of pemphigus involves a high dosage of systemic corticosteroids, which markedly reduces the mortality of patients (7). The combination of high-dose systemic steroids and conventional immunosuppressants, such as mycophenolate mofetil and azathioprine, has been a standard treatment for severe pemphigus for several decades (8). Rituximab is an anti-CD20 monoclonal antibody that depletes B cells and is greatly effective in treating various types of severe pemphigus (9–12). Although rituximab is now used as the first-line treatment for pemphigus in Europe and the U.S (12)., it is still unclear whether early rituximab treatment has a beneficial effect on pemphigus.

Like other biologics, rituximab is an expensive medicine, putting a financial burden on clinicians and patients. Many patients cannot afford rituximab, which causes them to stay on high-dose corticosteroids. In February 2018, South Korea's National Health Insurance (NHI) began to cover the high cost of rituximab for severe pemphigus patients, in which the government reimburses 90% of the original cost of rituximab. This intervention is referred to as the rituximab reimbursement policy. The study aims to address the clinical effect of early rituximab treatment in pemphigus. We also analyzed the effect of the implementation of the rituximab reimbursement policy on the clinical outcomes of Korean pemphigus patients. Throughout the statistical analysis, a novel model of ITS was used to evaluate the impact of new government reimbursement policies more practically. Furthermore, we examined the peripheral blood mononuclear cell (PBMC) analyses of 17 pemphigus vulgaris patients to identify subtypes of Dsg3-specific B cells related to the prognosis of rituximab treatment.

Materials and methods

Study population

For the first part of the study, we collected data on patients who visited the Gangnam Severance Hospital with pemphigus from 1 January 2014 to 31 December 2020 (IRB No. 3-2021-0138). Patients were enlisted if they met the detailed inclusion and exclusion criteria. The inclusion criteria were (i) patients diagnosed as pemphigus vulgaris and pemphigus foliaceus by skin biopsy, and direct and indirect immunofluorescent tests; and (ii) patients who were treated with rituximab under a rheumatoid arthritis protocol (two 1,000-mg biweekly infusions of rituximab). The exclusion criteria included pemphigus patients who did not follow the appointment schedule more than three times after rituximab treatment. In this study, 214 patients were analyzed for time to the first rituximab treatment, total steroid intake over the first year of treatment, total steroid intake over the six months following rituximab treatment, and time from onset to rituximab treatment. To investigate the time to PRMT, total steroid intake to PRMT, and total steroid intake after rituximab treatment until PRMT, we excluded 39 patients who did not reach PRMT after the diagnosis of pemphigus. To measure the total steroid intake to achieve CR, 50 patients were excluded for not reaching CR after the diagnosis. Peripheral blood samples from the patients with 17 pemphigus vulgaris were collected at the start of their first rituximab treatment (IRB No. 3-2019-0191). All the necessary patient information was collected.

Variables

In February 2018, the NHI of South Korea began to reimburse rituximab for severe pemphigus patients (Figure 1A). The dependent variables of this study were time to the first rituximab treatment, total steroid intake over the first year of treatment, total steroid intake over six months following rituximab treatment, time to PRMT, total steroid intake after rituximab treatment until PRMT, time from onset to rituximab treatment, time from onset to PRMT, and total steroid intake to PRMT and CR. The definition of each variable is explained more in detail in Supplementary Table S1. PRMT means the moment when the symptoms and general condition of the patient stabilize, so that new lesions are rarely developed and healed within one week under minimal therapy (prednisone ≤10 mg/day). CR indicated the point when the patient maintained a disease-free condition without new lesions under the least amount of prednisone (≤2.5 mg/day).

To evaluate the implementation of rituximab reimbursement for interrupted time series (ITS) analyses, this study included "policy" and "trend after policy" as variables. The "policy" variable was defined as 0 before the implementation of rituximab reimbursement and 1 after the implementation of rituximab reimbursement. This variable refers to a prompt change in rituximab treatment just after the rituximab reimbursement began in February 2018. "Trend after policy" covers the continuous time (in months) starting in February 2018. This refers to the trend in outcomes after the

Abbreviations: DN, double negative; Dsg, desmoglein; ITS, interrupted time series; NHI, National Health Insurance; PRMT, partial remission under minimal therapy; RTX, Rituximab.

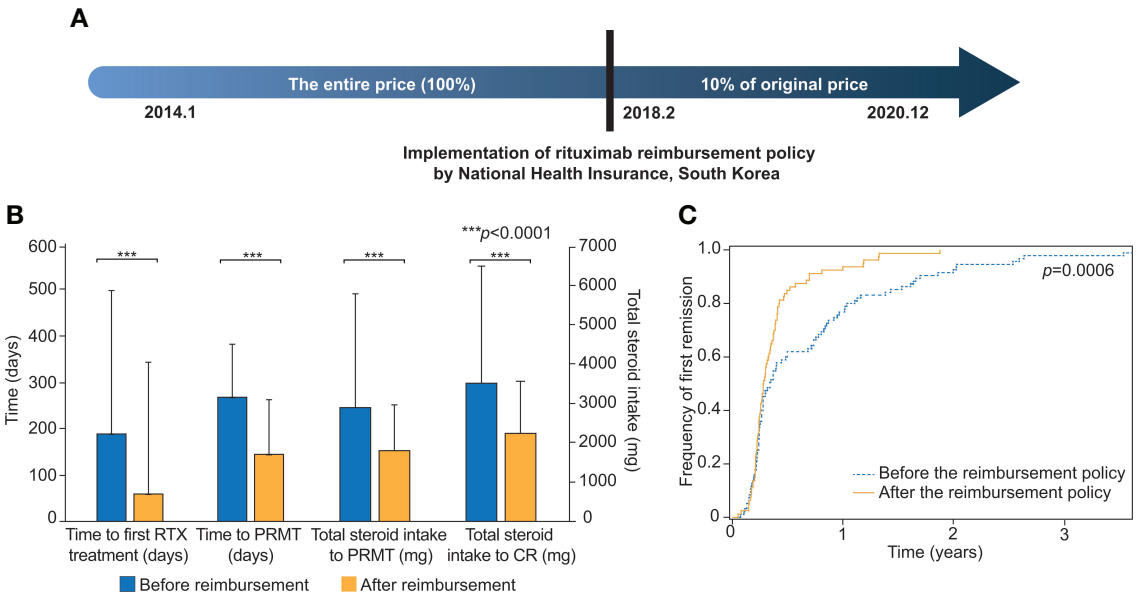

FIGURE 1
Comparisons of clinical outcomes before and after the implementation of the rituximab reimbursement policy. **(A)** Implementation of the rituximab reimbursement policy by the National Health Insurance of South Korea. Pemphigus patients have paid only 10% of what they used to pay for rituximab treatment since Feb. 2018. **(B)** The comparisons of time to the first rituximab treatment, time to the first PRMT, total steroid intake to PRMT, and total steroid intake to CR are based on the two groups of patients who were diagnosed before and after the implementation of the rituximab reimbursement policy. **(C)** Cumulative incidence curves for the first remission in patients diagnosed before and after the rituximab reimbursement policy. The *p*-value was obtained from Log-rank test. PRMT, Partial remission under minimal therapy; CR, Complete remission.

implementation of the policy. For covariates, we adjusted for age (≤29 years, 30 to 39 years, 40 to 49 years, 50 to 59 years, and ≥60 years), sex (male, female), and type of pemphigus (pemphigus vulgaris, pemphigus foliaceus).

Flow-cytometric analysis

PBMCs were obtained from blood samples of patients using Ficoll–Paque (GE Healthcare, Chicago, Ill). Dead cells were excluded with the LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen, Carlsbad, CA). After blocking with Fc receptor blocking solution (Biolegend, San Diego, CA), we incubated cells with 0.5 μg biotinylated human Dsg3 protein (CUSABIO, Wuhan, China) at 4°C for 1 h and made Dsg3 protein tetramers using streptavidin-BV421 and PE (Invitrogen). Then, the cells were stained with fluorescent-conjugated monoclonal antibody against CD3-BV510 (UCTH1), CD10-AF700 (HI10a), CD11c-BV605 (B-ly6), CD14-BV510 (MφP9), CD19-APC-Cy7 (SJ25C1), CD20-PerCP-Cy5.5 (2H7), CD27-BV711 (M-T271), CD38-PE-Cy7 (HIT2), IgD-BV786 (IA6-2) (all from BD Biosciences, Franklin Lakes, NJ), CD24-PE-CF594 (ML5) (Biolegend), and FcRL5-APC (509F6) (Invitrogen). Cells were detected with a BD LSRFortessa™ X-20 Cell Analyzer (BD Biosciences). For flow cytometric analysis, we have employed an established flow cytometry gating strategy as previously described (13) to detect antigen-specific B cells and atypical memory B cells. The data were analyzed by the FlowJo software package (BD Biosciences), and plots were generated using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA).

Statistical analysis

The chi-square test was used to compare demographic characteristics between the two groups. Then, the means and standard deviations of the dependent variables were compared using the t-test. An ITS method using a generalized linear model was applied to investigate the effect of the rituximab reimbursement policy on dependent variables. As previously described in various studies using ITS (14), our regression analysis equation is

$$Y_{it} = \beta 0 + \beta 1 \times Time_t + \beta 2 \times Policy_t + \beta 3 \times Time\ after\ policy_t + \beta 4 \times X_{it} + e_{it}$$

where Y = dependent variables; i = each patient; t = time period; *time* = a continuous variable starting in January 2014; *Policy*: a binary variable (0 before the implementation of rituximab reimbursement; 1 after the implementation of rituximab

reimbursement); *time after policy*: a continuous variable starting in February 2018; X = independent variables; and e = the error term. The statistical analysis was performed in SAS software, version 9.4 (SAS Institute, Cary, NC, USA). A p-value of <0.05 was considered to indicate a statistically significant result. In flow cytometry analysis, the Student's t-test was used to determine the statistical significance between comparable parameters. Pearson's correlation analysis was used to measure the strength of relationships between variables. A p-value of <0.05 was considered statistically significant. The statistical analysis of the data was performed using GraphPad Prism 9 (GraphPad Software Inc.).

Results

Patient characteristics

A total of 214 patients were included in this study. There were 153 patients with pemphigus vulgaris (71.5%) and 61 with pemphigus foliaceus (28.5%). The male-to-female ratio was nearly 1:1, and the patients who were 50 or older comprised approximately 60% of the whole population. There were 105 pemphigus patients who were diagnosed before the implementation of the rituximab reimbursement policy (49.1%) and 109 patients diagnosed after the implementation (50.9%). There was no statistically significant difference between the two groups in terms of sex, age, year of diagnosis, or type of pemphigus. Patient characteristics are shown in Table 1.

Clinical outcomes are improved after the rituximab reimbursement policy

We evaluated the differences in two patient groups: those who were diagnosed before the policy and those who were diagnosed after the policy (Table 2; Figure 1B). The mean time between the first visit to the clinic and the initiation of RTX treatment was 189 days (SD = 310) before the policy and 59 days (SD = 115) after the policy (p <0.0001). The mean value of the total amount of steroids that the patient had taken for the first year of treatment was 2,789 mg (SD = 1,305) before the policy. This was substantially reduced to 2,397 mg (SD = 1,333) after the policy (p = 0.031). The total steroid intake over the six months following RTX treatment (1,673 mg vs. 1,467 mg) and the duration from the onset of the disease to the start of RTX treatment (635 days vs. 456 days) also showed lower values in the group after the policy, but neither was statistically significant (p = 0.085, p = 0.090, respectively).

Then, the outcomes from both groups were examined, incorporating PRMT. The mean time from the first visit to our clinic to PRMT was 268 days (SD = 285) before the policy,

TABLE 1 General characteristics of the study population according to the timepoint of diagnosis of pemphigus[1].

Variables	Total		Before policy (2014.1.–2018. 1.)		After policy (2018.2.–2020. 12.)		*P*-value[2]
Number of patients	**214**	**(100.0)**	**105**	**(49.1 of total)**	**109**	**(50.9 of total)**	
Sex							0.689
Male	103	(48.1)	52	(49.5)	51	(46.8)	
Female	111	(51.9)	53	(50.5)	58	(53.2)	
Age							0.944
≤29	14	(6.5)	8	(7.6)	6	(5.5)	
30–39	22	(10.3)	11	(10.5)	11	(10.1)	
40–49	52	(24.3)	26	(24.8)	26	(23.9)	
50–59	60	(28.0)	30	(28.6)	30	(27.5)	
60+	66	(30.8)	30	(28.6)	36	(33.0)	
Year of diagnosis							
2014	6	(2.8)	6	(5.7)	0	(0.0)	
2015	33	(15.4)	33	(31.4)	0	(0.0)	
2016	36	(16.8)	36	(34.3)	0	(0.0)	
2017	25	(11.7)	25	(23.8)	0	(0.0)	
2018	39	(18.2)	5	(4.8)	34	(31.2)	
2019	44	(20.6)	0	(0.0)	44	(40.4)	
2020	31	(14.5)	0	(0.0)	31	(28.4)	
Types							0.778
Pemphigus vulgaris	153	(71.5)	76	(72.4)	77	(70.6)	
Pemphigus foliaceus	61	(28.5)	29	(27.6)	32	(29.4)	

[1]Data shown are number (percentage).
[2]P values are calculated by the chi-squared test.

TABLE 2 Changes in clinical outcomes before and after the implementation of the rituximab reimbursement policy.

Variables	Total	Before policy (2014.1.–2018. 1.)				After policy (2018.2.–2020. 12.)				*P*-value[1]
	N	N	Mean	±	SD	N	Mean	±	SD	
Time to first RTX treatment (days)	214	105	189	±	310	109	59	±	115	<.0001
Total steroid intake for first year of treatment (mg)	214	105	2,789	±	1,305	109	2,397	±	1,333	0.0309
Total steroid intake for six months following RTX treatment (mg)	214	105	1,673	±	914	109	1,467	±	822	0.0846
Time from onset to RTX treatment (days)	214	105	635	±	822	109	456	±	708	0.0896
Time to PRMT (days)[2]	175	95	268	±	285	80	145	±	118	0.0002
Total steroid intake after RTX treatment until PRMT (mg)[2]	175	95	1,309	±	1,272	80	1,152	±	857	0.3330
Total steroid intake to PRMT (mg)[2]	175	95	2,902	±	2,827	80	1,805	±	1,166	0.0008
Total steroid intake to CR (mg)	164	76	3,573		3,058	88	2,239		1,023	0.0002

[1]P values are calculated by t-test.
[2]Excluding those who did not reach PRMT after diagnosis of pemphigus.
RTX, Rituximab; PRMT, Partial remission under minimal therapy; CR, Complete remission.

and this was decreased to 145 days (SD = 118) after the reimbursement began (p = 0.0002). The mean total corticosteroid intake until the patient reached PRMT was 2,902 mg (SD = 2,827) before the policy and 1,805 mg (SD = 1,166) after the policy (p = 0.0008). The mean total corticosteroid intake until the patient achieved CR was 3,573 mg (SD = 3,058) before the reimbursement and 2,239 mg (SD = 1,023) after the reimbursement (p = 0.0002) (Table 2). The cumulative incidences of the first partial remission for the patients diagnosed before the reimbursement policy and after the reimbursement policy are shown in Figure 1C. The time to achieving partial remission was significantly faster among patients diagnosed after the implementation of rituximab reimbursement (p = 0.0006 from the log-rank test). There was no significant difference in the percentage of patients who achieved CR, relapsed, and experienced complications between the two groups (Supplementary Table S2).

The rituximab reimbursement policy reduces total steroid intake following rituximab

The implementation of the rituximab reimbursement policy by the NHI triggered a decrease in the total amount of corticosteroid intake throughout the treatment period (Figure 2; Table 3). The trend in total steroid intake for the first year of treatment was not statistically significant before the policy (β = 2.31, p = 0.792). The total steroid intake showed decreasing trends after the new reimbursement policy was implemented (β = −79.58, p <0.0001). There was no significant change in either the level or the trend of total steroid intake until PRMT was reached after the reimbursement policy. Before the policy, the amount of total steroid intake after the administration of rituximab until reaching PRMT and for six months afterward showed a slightly increasing trend, but not a significant one (total

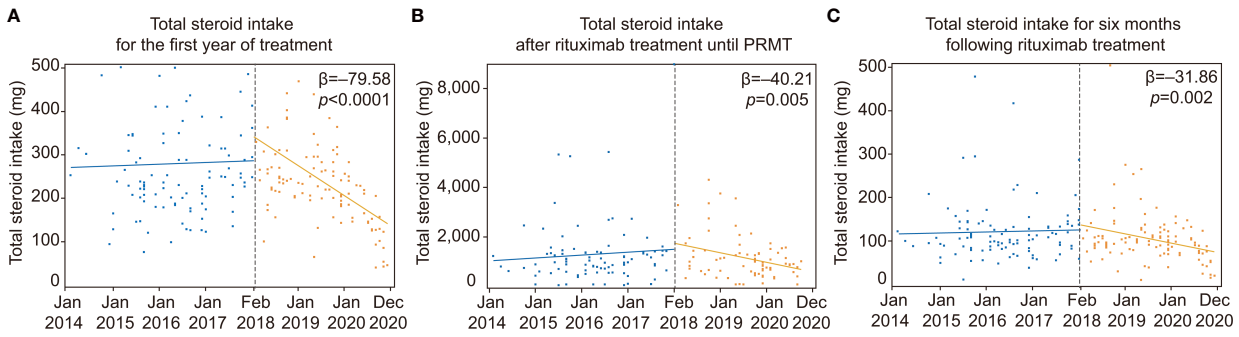

FIGURE 2
ITS analysis of changes in total steroid intake. ITS analysis of the implementation of rituximab reimbursement. **(A)** Total steroid intake for the first year of treatment before and after the rituximab reimbursement policy. **(B)** Changes in total steroid intake after rituximab treatment until achieving PRMT before and after the rituximab reimbursement policy. **(C)** Total steroid intake over the six months following rituximab treatment before and after the rituximab reimbursement policy. PRMT, Partial remission under minimal therapy; ITS, Interrupted time series.

TABLE 3 Results of the interrupted time series analysis for clinical outcomes[1].

	Time before implementation of RTX reimbursement (by month)			Implementation of RTX reimbursement				Time after implementation of RTX reimbursement (by month)		
				Before (~2018.01.)	After (2018.02. ~)					
	β	S.E.	*P*-value	β	β	S.E.	*P*-value	β	S.E.	*P*-value
Total steroid intake over first year of treatment (mg)	2.31	8.76	0.7924	Ref.	1,027.99	330.77	0.0019	−79.58	13.91	<.0001
Total steroid intake to PRMT (mg)[2]	−15.43	13.67	0.2590	Ref.	382.22	507.41	0.4513	−36.60	22.13	0.0982
Total steroid intake after RTX treatment until PRMT (mg)[2]	7.11	8.09	0.3797	Ref.	399.70	333.52	0.2307	−40.21	14.33	0.0050
Total steroid intake over six months following RTX treatment (mg)	3.38	6.33	0.5937	Ref.	281.85	224.61	0.2095	−31.86	10.01	0.0015
Time from onset to RTX treatment (days)	0.92	4.76	0.8466	Ref.	−318.27	128.95	0.0136	3.61	6.49	0.5779

[1]Adjusted factors included sex, age, and type of pemphigus.
[2]Excluding those who did not reach PRMT after diagnosis of pemphigus.
RTX, Rituximab; PRMT, Partial remission under minimal therapy.

steroid intake until PRMT: $\beta = 7.11$, $p = 0.380$; total steroid intake over six months $\beta = 3.38$, $p = 0.594$). There was a considerable decreasing trend in the total amount of steroids taken until the PRMT after the introduction of the new policy ($\beta = -40.21$, $p = 0.005$). The total amount of steroids over the six months after rituximab markedly decreased after the policy ($\beta = -31.86$, $p = 0.002$). The introduction of the new policy significantly decreased the time from the onset of the disease to the administration of rituximab ($\beta = -318.27$, $p = 0.014$). However, the time trend after the policy was not significant ($\beta = 3.61$, $p = 0.578$).

Circulating Dsg3-specific atypical memory B cells are associated with early rituximab treatment and disease prognosis after rituximab treatment

To find a prognostic marker for pemphigus after rituximab treatment, we analyzed B cells in PBMCs from 17 pemphigus vulgaris patients by flow cytometry using a gating strategy (Supplementary Figure S1). By using the recombinant human Dsg3 tetramer, we detected circulating Dsg3-specific B cells. There was no correlation between the time from the onset of pemphigus to rituximab infusion and the proportion of Dsg3-specific B cells among $CD19^+$ B cells in patients ($p = 0.314$) (Figure 3A). We detected a few $CD27^+CD38^+$ antibody-secreting cells (ASCs), including $CD24^-CD38^+$ plasmablasts, among the circulating Dsg3-specific B cells. We subdivided non-naïve B cells into $CD27^+IgD^-$ switched memory B cells, $CD27^+IgD^+$ unswitched memory B cells, and $CD27^-IgD^-$ double-negative (DN) B cells. The relative frequencies of all three subtypes of non-naïve B cells in $CD19^+$ B cells and Dsg3-specific B cells did not correlate with time to initiating rituximab (Supplementary Figure S2). However, among the three subtypes of memory B cells, we found that DN B cells were significantly enriched in Dsg3-specific B cells when compared to the total $CD19^+$ B cells (Figure 3B). $CD11c^+$ cells are enriched in Dsg3-specific DN B cells, and more than 90% of these cells are $CD11c^+FcRL5^+$ atypical memory B cells (Figures 3C, D). We thus investigated atypical memory B cells in Dsg3-specific DN B cells by using the CD11c marker. We found that the relative frequencies of $CD11c^+$ atypical memory B cells in Dsg3-specific B cells were positively correlated with the time to initiating rituximab ($R^2 = 0.492$, $p = 0.002$) (Figure 3E). Recognizing that the frequency of Dsg3-specific $CD11c^+$ atypical memory B cells may have prognostic value, we conducted further analyses using other clinical outcomes. The frequency of this subset was positively correlated with the time from disease onset to PRMT ($R^2 = 0.485$, $p = 0.002$) and the total amount of corticosteroid intake until the patient reached PRMT ($R^2 = 0.416$, $p = 0.005$) (Figures 3F, G).

Discussion

When evaluating the impact of the rituximab reimbursement policy of NHI, we found that the time to the first rituximab dose was shortened in the patients who were diagnosed after the reimbursement started. Thus, comparisons between groups before and after the reimbursement provide important evidence for understanding the effect of early rituximab treatment for pemphigus patients. In a previous study with 107 pemphigus patients treated with rituximab, the early treatment group (≤6 months after diagnosis) achieved a higher rate of remission than

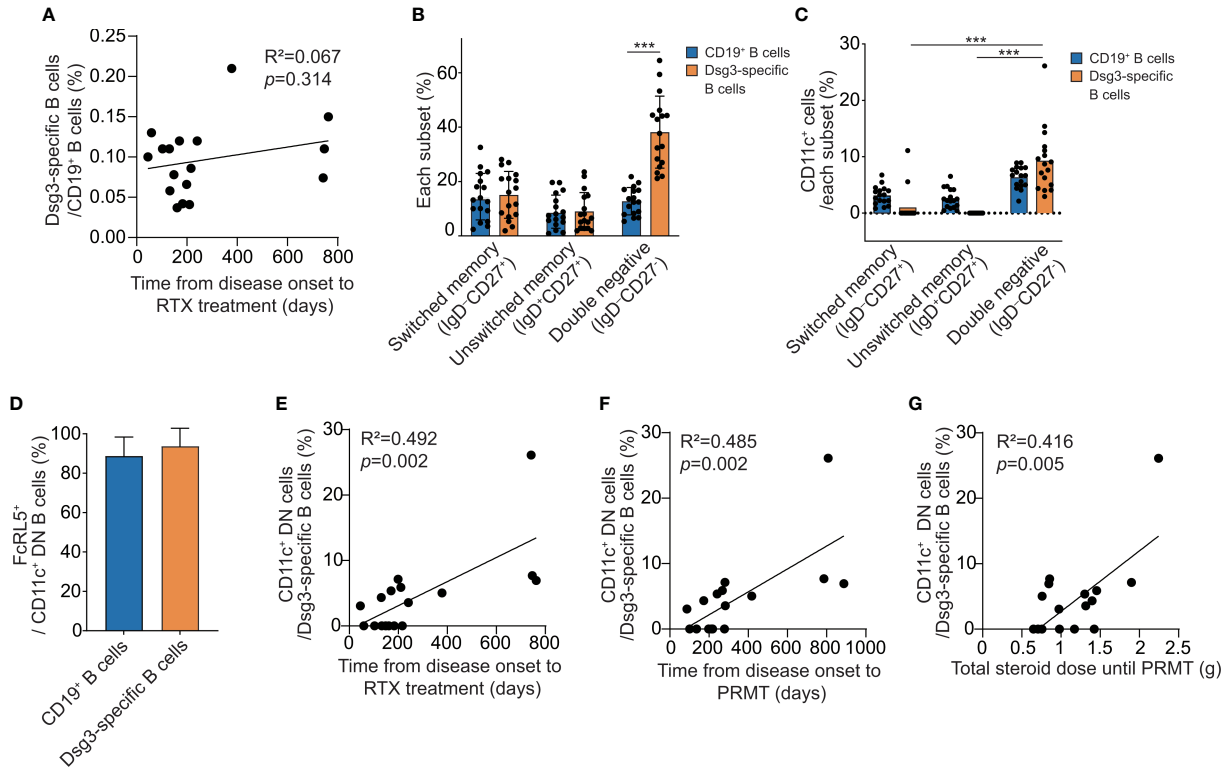

FIGURE 3
The analysis of Dsg3-specific B cell subsets associated with clinical outcome after rituximab treatment. **(A)** Correlation between the time from disease onset to rituximab treatment and the proportion of Dsg3-specific B cells within $CD19^+$ B cells. **(B)** The proportions of switched memory (IgD^-CD27^+) B cells, unswitched memory (IgD^+CD27^+) B cells and DN (IgD^-CD27^-) B cells among $CD19^+$ and Dsg3-specific B cells were compared. **(C)** The proportions of $CD11c^+$ cells were compared among switched memory, unswitched memory, and DN cells from $CD19^+$ and Dsg3-specific B cells. **(D)** The proportions of $FcRL5^+$ cells in Dsg3-specific $CD11c^+$ DN B cells and $CD19^+CD11c^+$ DN B cells are shown. **(E–G)** Correlation analyses of the proportion of $CD11c^+$ cells among Dsg3-specific DN B cells were performed for **(E)** the period from the onset to the administration of rituximab, **(F)** the time from the onset of the disease to reaching PRMT, and **(G)** the total corticosteroid intake until reaching PRMT. ***p <0.0001. RTX, Rituximab; DN, Double-negative; PRMT, Partial remission under minimal therapy.

the late treatment group (>6 months after diagnosis) (15). In another study with 95 pemphigus patients receiving rituximab, the early treatment group achieved remission sooner and had a longer duration of remission (16). In addition to time to remission, we found a decrease in steroid intake before remission in the group diagnosed after the reimbursement, indicating that early-treated patients may experience a more rapid decline in the disease state after rituximab than late-treated patients.

ITS design is a powerful analysis for evaluating the longitudinal effects of a sudden change or intervention (17). Often combined with segmented regression, it visualizes the consequence of an intervention and clarifies its significance using a statistical approach (18). In the ITS analyses, we observed that the implementation of the reimbursement policy significantly reduced the trend of total steroid intake for the first year of treatment. Additionally, the amounts of steroids taken in the six months after rituximab infusion and until PRMT were both decreased in the patient group after the reimbursement. These results strongly suggest that early rituximab treatment is beneficial for patient outcomes. Contrary to the results from the ITS analysis, there was no difference in the total amount of steroids taken for six months after rituximab treatment when the two groups were directly compared directly. Given that ITS analysis reflects complex factors during treatment and incorporates underlying trends (19), we think that the findings from ITS analysis are more credible when comparing the trends before and after the intervention. Also, it is interesting to note that total steroid intake levels spiked at the time the rituximab reimbursement policy was initiated. We infer that many patients with severe pemphigus who could not afford rituximab were enrolled at that time. This real-world evidence highlights the value of ITS analysis.

Atypical memory B cells were first considered to be exhausted or activated B cells, but recent studies suggest that these cells are precursors of plasma cells mediated by the extrafollicular pathway (20, 21). They are observed in $CD27^-IgD^-$ DN B cells and are characterized by the overexpression of t-bet, FcRL5, and

CD11c and downregulation of FcRL4, CXCR5, and CD21 (20, 22, 23). We also observed that most CD11c$^+$ DN B cells were FcRL5$^+$ cells in Dsg3-specific B cells from pemphigus vulgaris patients. An increase in atypical memory B cells is observed in various autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome, and multiple sclerosis (24–27), and is associated with female dominance in autoimmunity (28). These cells have similar features as age-associated B cells, which are enriched in the old-age population (25, 29), and SARS-CoV-2-specific atypical memory B cells were increased in the peripheral blood of COVID-19 patients and vaccinees (13, 30).

Among Dsg-specific B cells, we suggest that Dsg-specific atypical memory B cells may forecast a favorable prognosis after rituximab treatment in pemphigus patients. Consistent with our data, a recent study showed an increase in CD11c$^+$ B-cell frequency in pemphigus patients during active disease and a decrease after treatment (31). However, given that most autoreactive B cells in pemphigus undergo somatic hypermutation (5, 32), it is still unclear whether pemphigus is directly induced by Dsg-specific atypical memory B cells of the extrafollicular origin. Both pathogenic autoreactive ASCs in secondary lymphoid organs and autoreactive atypical memory B cells in peripheral blood can grow in number following disease progression. Since it is impractical to measure the frequency of pathogenic plasma cells in secondary lymphoid organs, we expect autoreactive atypical memory B cells to be a surrogate marker for predicting the prognosis of the disease and the response to rituximab.

After reimbursement policy, the period from the diagnosis of pemphigus to the initiation of rituximab has been significantly shortened, with substantial reductions in steroid doses throughout the course from the time of diagnosis. We also observed a positive correlation between the duration from the onset of the disease to rituximab infusion and the proportion of atypical CD11c$^+$ B cells among Dsg3-specific B cells. Based on these findings, we expect that Dsg-specific ASCs will expand over time. The shorter the time to rituximab, the lower the number of Dsg-specific ASCs, so the disease can subside more quickly with lower doses of steroids. Although the time between the actual onset of the disease and rituximab infusion was not significantly different between the two groups, we believe that a further study with a larger cohort and prospective design may find a close relationship between the disease duration and the effectiveness of rituximab.

In summary, our study revealed that Korea's reimbursement policy allowed early infusion of rituximab, which led to faster achievement of remission. A novel approach with ITS analyses provided evidence of a significant reduction in the trend of total steroid intake throughout the treatment period, which may relate to decreased morbidity and mortality. Furthermore, through PBMC analysis, we have highlighted the value of Dsg-specific CD11c$^+$ atypical memory B cells as a prognostic marker for pemphigus patients.

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 Gangnam Severance Hospital IRB No. 3-2021-0138 and No. 3-2019-0191. The patients/participants provided their written informed consent to participate in this study.

Author contributions

The authors confirm contribution to the paper as follows: Study conception and design: AS, S-CK, JS, and JK. Data collection: AS and SL. Analysis and interpretation of results: JJ, AS, AL, SM, JK, and JS. Draft manuscript preparation: JJ, AS, and JK. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.932909/full#supplementary-material

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A survey of genome-wide association studies, polygenic scores and UK Biobank highlights resources for autoimmune disease genetics

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Autoimmune diseases share a general mechanism of auto-antigens harming tissues. Still. they are phenotypically diverse, with genetic as well as environmental factors contributing to their etiology at varying degrees. Associated genomic loci and variants have been identified in numerous genome-wide association studies (GWAS), whose results are increasingly used for polygenic scores (PGS) that are used to predict disease risk. At the same time, a technological shift from genotyping arrays to next generation sequencing (NGS) is ongoing. NGS allows the identification of virtually all - including rare - genetic variants, which in combination with methodological developments promises to improve the prediction of disease risk and elucidate molecular mechanisms underlying disease. Here we review current, publicly available autoimmune disease GWAS and PGS data based on information from the GWAS and PGS catalog, respectively. We summarize autoimmune diseases investigated, respective studies conducted and their results. Further, we review genetic data and autoimmune disease patients in the UK Biobank (UKB), the largest resource for genetic and phenotypic data available for academic research. We find that only comparably prevalent autoimmune diseases are covered by the UKB and at the same time assessed by both GWAS and PGS catalogs. These are systemic (systemic lupus erythematosus) as well as organ-specific, affecting the gastrointestinal tract (inflammatory bowel disease as well as specifically Crohn's disease and ulcerative colitis), joints (juvenile ideopathic arthritis, psoriatic arthritis, rheumatoid arthritis, ankylosing spondylitis), glands (Sjögren syndrome), the nervous system (multiple sclerosis), and the skin (vitiligo).

KEYWORDS

autoimmune disease, genetics, genome-wide association study, GWAS, polygenic scores, genetic risk, UK Biobank, experimental factor ontology

Introduction

Autoimmune diseases are a range of diseases in which the immune response to self-antigens results in damage or dysfunction of tissues. It can be systemic or can affect specific organs or body systems. Autoimmune diseases are characterized by a multifactorial etiology, in which genetic factors interplay with environmental factors. Estimates of heritability, that is, variability in occurrence of autoimmune disease explained by genetic factors, vary considerably and have been reported to be between 42 and 91% for pediatric age-of-onset and lower for adult onset cases (1). Variation in the human major histocompatibility complex (MHC) regions harboring the human leukocyte antigen (HLA) genes is most strongly linked to autoimmune disease (2). Beside HLA, other genetic loci are shared between autoimmune diseases, with first investigations finding 47/107 (44%) immune-mediated disease risk variants associated with multiple, but not all such diseases (3), and later work identifying 244 shared disease loci (4). Accordingly, efforts are ongoing to unravel shared disease mechanisms based on shared genetic profiles (5, 6). Such genetics-driven systems approaches to autoimmune disease can largely benefit from public resources of genome and phenotype data as well as derived information. Here we perform a survey of autoimmune disease-related content in three such resources: (i) The NHGRI-EBI GWAS catalog (7) reporting on genome-wide association studies (GWAS), (ii) the newly established PGS catalog (8) having information on polygenic scores (PGS) and (iii) the UK Biobank (UKB) holding genetic and phenotypic data of ~500,000 people from the UK (9) (for details on data processing, see https://github.com/iwohlers/2022_autoimmune_review). While GWAS aim to identify associations of a large number of genetic variants with phenotypes or traits (10), the main goal of PGS studies is to estimate the risk of developing a disease or of the presence of a specific trait depending on the genetic profiles (11).

Autoimmune diseases and their relationships within biomedical ontologies

An ontology is a controlled vocabulary, formalizing domain knowledge into terms and their relationships. The Experimental Factor Ontology (EFO) is a biomedical ontology curated by the European Bioinformatics Institute (EBI) and used by the GWAS and PGS catalog for the purpose of disease classification (12). The part of EFO relating to autoimmune disease is shown in Suppl. Table S1 (EFO version 3.42.0; https://bioportal.bioontology.org/ontologies/EFO). It contains "is a" relationships between "parent" and "child" terms, e.g., rheumatoid arthritis (child term) is an autoimmune disease (parent term). The ontology branch of child terms for autoimmune disease contains 120 terms organized in up to five levels (Suppl. Table S1). For the disease-related part of EFO, some terms have been taken from other ontologies (denoted by IDs not starting with "EFO"). Within the autoimmune disease sub-branch, 13 terms are taken from the Mondo disease ontology that is also curated by EBI [https://bioportal.bioontology.org/ontologies/MONDO; (13)]. Further, three terms are taken from Orphanet, an online database of rare diseases and orphan drugs (Copyright, INSERM 1997. available at http://www.orpha.net) and one term from the disease ontology [www.disease-ontology.org; (14)], cf. Suppl. Table S2.

Genomics data, genetic variation and notable reference resources

Differences between genomic sequences are called genetic variations. They are classified into single nucleotide variants (SNVs), for which the base at a single position differs, indels, which are insertions or deletions of size up to 50 bases, and structural variants (SVs), which are genomic alterations of a size larger than 50 bases. SNVs commonly found in a population are also called single nucleotide polymorphisms (SNPs). Genotyping arrays assess predefined, common variants, i.e., SNPs. Genetic variations are specified with respect to a specific reference genome. For humans, this is GRCh38, the genome of the Genome Reference Consortium, with its latest version 38.

Human SNVs have been well characterized. The first milestone was the whole genome sequencing performed as part of the 1000 Genomes Project, which resulted in "a global reference of human genetic variation" based on the genomic data of 2,504 individuals from 26 populations (15). The 1000G-based genetic variation with respect to the reference genome was overall 84.7 million SNVs, 3.6 million indels and ~60,000 structural variants; each individual carried 4.1 million to 5.0 million sites that differed from the reference genome. This first comprehensive catalog of genetic variation was later extended by whole genome sequencing of the Human Genome Diversity Project (n=929) (16). Of the many, often national, genome sequencing initiatives, gnomAD [n=71,702 whole genome sequenced; (17)], Topmed [n=53,831 whole genome sequenced; (18)] and the UK Biobank [UKB, n=150,119 whole genomes sequenced; (19)] stand out in terms of sample size. Within the UKB cohort, 585.0 million SNVs, representing 7.0% of all possible human SNVs, 58.7 million indels and ~900,000 SVs have been identified (19). Many of the SNVs, 46%, are carried by

only one sequenced individual (called "singletons") and only 3.4% (~20 million) have a frequency of more than 0.1% (19).

Autoimmune disease genome-wide association studies (GWAS)

The main goal of GWAS is to identify associations of genetic variants with a phenotype or trait without prior knowledge about their genomic location. Although GWAS could in principle use different kinds of genetic variants, to date almost always SNPs are utilized (11). GWAS then consist of testing for associations of SNPs with the phenotype or trait of interest. Since the first GWAS about twenty years ago (20), more than 5,700 analyses have been conducted, yielding more than 3,300 traits established to be statistically associated with genetic variants (10).

Testing for associations between a phenotype or a trait and genetic variants is based on a statistical model, and the type of the model used depends on whether the phenotype or trait is continuous (e.g. Body Mass Index (BMI)) or dichotomous (e.g. presence or absence of an autoimmune disease). In the case of a continuous phenotype or trait, a linear regression model is most commonly used, whereas logistic regression is mostly applied for dichotomous ones. Typically, the models are estimated for each single variant separately. The typical GWAS output comprises, for each variant, a report giving the ID of the variant according to dbSNP (21), the effect allele, the statistical effect and the corresponding p-value. Since GWAS test a large number of genetic variants at the same time, the statistical significance threshold has to be corrected to avoid false positive results. The widely used approach for this aim is the so-called Bonferroni correction (10, 22), consisting of dividing the overall statistical significance threshold by the total number of independent tests, in this case, the tested independent variants. As a consequence, a threshold of $5*10^{-8}$ is commonly used in practice, since the human genome contains approximately one million common, independent variants (10).

The GWAS catalog is a publicly available, manually curated resource, which contains published GWAS and association results and is developed by the NHGRI and EMBL-EBI (7). Catalog data is provided for the latest reference genome version (GRCh38.p13) and variant database version (dbSNPBuild 154). GWAS catalog source files of studies and associations have been used in our survey (files gwas-catalog-studies_ontology-annotated.tsv and gwas-catalog-associations_ontology-annotated.tsv from http://ftp.ebi.ac.uk/pub/databases/gwas/releases/2022/05/23/), and entries with "MAPPED_TRAIT_URI" an autoimmune EFO IDs (Suppl. Table S2) were extracted. Overall, the GWAS catalog studies contain 442 autoimmune disease GWAS ("STUDY ACCESSION") published between 2006 and 2022 in 58 different journals with 221 unique PubMed IDs (Suppl. Table S3); these studies have been conducted on 377 different datasets (according to column "INITIAL SAMPLE SIZE" in Supplementary Table S3). A subset of studies (n=179 (47%)) reported no genome-wide significant variants.

The GWAS catalog contains 5,023 associations that cover 41 autoimmune diseases (according to EFO ID) based on 253 datasets (according to column "INITIAL SAMPLE SIZE") relating to 200 unique PubMed IDs (Suppl. Table S4). These associations correspond to 3,212 unique SNPs (according to column "SNPS") and 1,760 unique genes or gene combinations reported in the literature (column "REPORTED GENE(S)"; Supplementary Table S4).

Polygenic scores (PGS) developed for autoimmune diseases

GWAS are typically used for traits with an underlying polygenic architecture, that is, many genetic variants just show small effect sizes on the phenotype or trait of interest. As a result, prediction performances of single associated variants are generally poor. Therefore, polygenic scores, also termed "risk scores" if applied to a disease, are used to overcome these limitations. The main idea of predictive models based on polygenic scores is to combine effects of single genetic variants to expect a stronger association with the response phenotype or trait. The standard approach used for quantifying genetic liability in the prediction of disease risks are weighted polygenic scores (11). Based on this, PGSs are generally obtained as weighted sum scores of risk alleles using effect sizes from GWAS. More recently, new statistical machine learning approaches have emerged as a powerful approach for the computation of PGS (23).

The PGS catalog is a database and website established with the aim of making published PGS easily available and allowing their systematic evaluation (8). We obtained PGS catalog source files from https://www.pgscatalog.org/downloads and extracted from the respective files *via* EFO IDs all information related to autoimmune diseases: polygenic scores (Suppl. Table S5), score development samples (Suppl. Table S6), performance evaluation metrics (Suppl. Table S7) and evaluation samples (Suppl. Table S8). The database contains 18 autoimmune diseases for which 47 polygenic scores are published in 14 papers between 2018 and 2022 (cf. Suppl. Table S5). These have been developed with 15 different computational methods, mostly with the tools snpnet [n=18 scores; (24)], using genome-wide significant GWAS variants (n=7 scores), LDPred [n=6; version 1 and 2; (25, 26)] or by applying pruning and threshold (n=4). Corresponding to the method or tool applied for PGS constructions, the number of variants considered in the scores ranges from 3 to 6,907,112. Many cohorts of mainly European and/or East Asian (largely Chinese) ancestry have

been used for score development, mainly as source GWAS underlying the respective score, but also for parameter training (Suppl. Table S6). Further, a large number of PGS have been developed using the UK Biobank. Autoimmune PGS have been evaluated in 124 data sets (Suppl. Table S7) yielding 225 performance assessments in 16 publications (Suppl. Table S8). The most common performance measure is the Area Under the Receiver-Operating Characteristic Curve (AUROC), which shows the fraction of individuals incorrectly classified as having the disease (false positive rate) versus the fraction of individuals correctly classified as having the disease (true positive rate) at different PGS score thresholds. An AUROC value of 0.5 corresponds to a random and a value of 1.0 to a perfect classification. Typically, AUROC classification performance of autoimmune PGS are in the range from 0.56 to 0.99 (provided for n=124; Suppl. Table S8). Overall, according to the PGS catalog, 16 different publications either constructed and/or evaluated an autoimmune disease PGS (columns "PGS Publication (PGP) ID" of Suppl. Table S5 and Suppl. Table S8).

Genetic data and autoimmune diseases covered by the UK Biobank

The UK Biobank (UKB) is the largest resource for human genomic and phenotypic data available for global academic health research, containing data from approximately 500,000 individuals from the United Kingdom (9). Its first release in 2018 contained UK Biobank Axiom Array-based genotypes (m=825,927) imputed to m~96 million variants of these individuals (9). In 2021, whole exome sequencing data of 454,787 of its individuals was released (with m~2 million exonic SNVs) (27). In 2022 whole genome sequencing-based variants for n=150,119 individuals resulted in overall m~585 million SNVs, ~59 million indels, 2.5 million microsatellites and 900k structural variants (19), representing a nearly complete variant profile for these individuals.

UK Biobank provides medical diagnosis according to the International Statistical Classification of Diseases and Related Health Problems (ICD) of the World Health Organization (WHO), whose current version is ICD-10. Besides ICD-10 codes gathered from medical records, UK Biobank provides diagnoses that are self-reported by participants, referred to by dedicated UKB-internal IDs (starting with "20002_"). To extract autoimmune disease information from UK Biobank, we used the mapping file internal to the ontology mapping tool Zooma of the EMBL-EBI ontology lookup service (28) which was generated as part of a large-scale, comprehensive mapping of UK Biobank ICD-10 codes and self-reported diseases to EFO terms (https://github.com/EBISPOT/EFO-UKB-mappings). We find that 20 of 120 autoimmune EFO IDs (cf. Suppl. Table S2) correspond to patients and patient genotypes within UK Biobank (Suppl. Table S9), of which 9 have both self-reported and ICD-10 diagnosis, 6 are only self-reported and 5 only have ICD-10-based information. The number of respective patients ranges from 13 (reactive arthritis with ICD-10 code M03) to 12,556 (rheumatoid arthritis with ICD-10 code M06) (Suppl. Table S9).

Overlap between autoimmune diseases assessed by GWAS, PGS and UKB

We investigated for which autoimmune diseases the GWAS catalog, the PGS catalog and UK Biobank contain information by comparing the respective autoimmune EFO IDs covered by each resource. Overall, there are 120 autoimmune disease EFO IDs (Suppl. Table S2) representing different levels of diagnosis (Suppl. Table S1). Of those, the GWAS catalog covers 41 EFO IDs (Suppl. Table S4) and the PGS catalog 18 EFO IDs (Suppl. Table S5). As the UK Biobank does not use EFO IDs, we used a published mapping of EFO IDs to UKB data fields instead, as described in the last section and provided in Suppl. Table S9. This assigned 20 EFO IDs to traits in UKB (Suppl. Table S9). The overlap of autoimmune diseases covered by the three resources is shown in Figure 1. Out of 120 EFO IDs, only 48 autoimmune diseases are present in any of the three databases, most in the GWAS catalog. The GWAS catalog is sharing 15 autoimmune disease EFO IDs with the PGS catalog and 15 can be mapped to UK Biobank. Further, 12 disease EFO IDs are shared between PGS catalog and UK Biobank. There are 11 autoimmune disease EFO IDs common to all three databases. They relate to: ankylosing spondylitis, appendicitis, Crohn's disease, inflammatory bowel disease, juvenile idiopathic arthritis, psoriatic arthritis, rheumatoid arthritis, Sjögren syndrome, systemic lupus erythematosus, ulcerative colitis and vitiligo. Several of the autoimmune diseases related to EFO IDs that are not shared by all three resources are cases that highlight limitations with respect to the definition of terms and relationships within the EFO and in the mapping of EFO terms to external codes and identifiers, which may not be one-on-one and needs disease-specific knowledge (for details see caption of Figure 1).

We have investigated more closely the 10 autoimmune diseases with most GWAS studies according to GWAS catalog (Table 1). They are systemic lupus erythematosus (29), rheumatoid arthritis (30), multiple sclerosis (31), inflammatory bowel disease (32) with its two subtypes Crohn's disease and ulcerative colitis, vitiligo (33), Sjögren syndrome (34), Grave's disease (35), and Behcet's syndrome

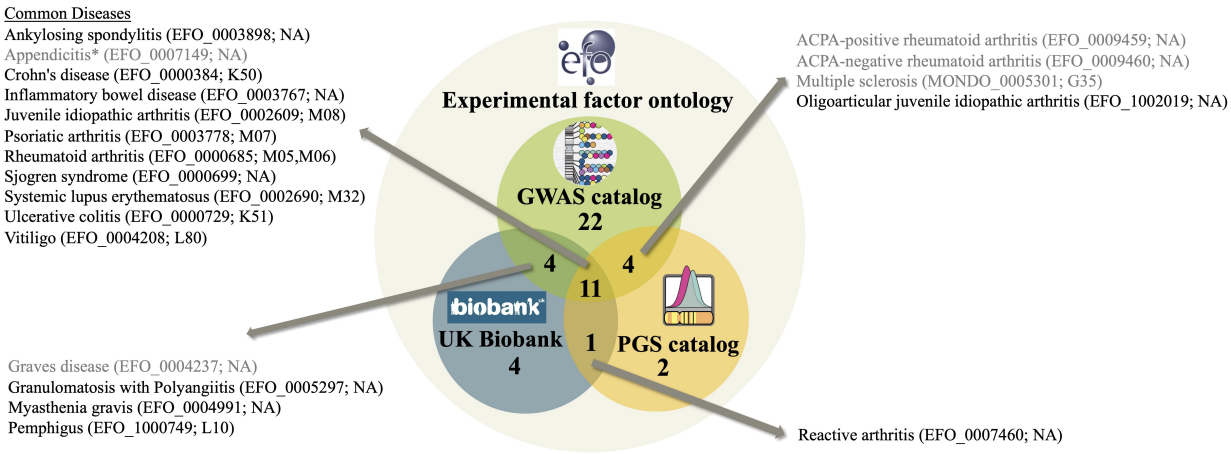

FIGURE 1
A Venn diagram representing the number of autoimmune diseases in the experimental factor ontology (EFO) overlapping with GWAS catalog and PGS catalog covered EFO IDs as well as UK Biobank data field matched according to Suppl. Table S9. EFO IDs of diseases in more than two resources are listed together with their name according to EFO and their UKB ICD-10 code according to Suppl. Table S9. Diseases shaded gray are affected by issues with disease definition and classification compromising the mapping. These issues are: (i) Appendicitis is classified as an autoimmune disease in EFO because it is a child term of inflammatory bowel disease, however, it is not considered an autoimmune disease. (ii) Grave's disease EFO child terms are in PGS, not the EFO ID of Grave's disease itself though. (iii) ACPA-positive and ACPA-negative rheumatoid arthritis is not mappable to UKB. UKB, however, contains seropositive and other rheumatoid arthritis, a distinction not covered by EFO. (iv) UKB has information on multiple sclerosis, yet since the recent EFO version was updated to using the MONDO ID for multiple sclerosis, the mapping to UKB data fields failed. "NA" denotes that mapping to UKB is not available for the respective EFO ID.

(36). The GWAS catalog association data of the top 10 autoimmune diseases (underlying Table 1) are provided in Supplementary Tables S10-S19. The most GWAS-studied autoimmune disease is systemic lupus erythematosus, for which 37 different GWAS have been performed, the largest one using 13,377 cases and 194,993 controls. These studies have reported 788 unique SNPs and 439 unique genes or gene combinations. In the PGS catalog, six studies are noted on systemic lupus erythematosus, which report six different risk scores. These PGS have been evaluated in 32 settings. The largest number of cases has been analyzed for inflammatory bowel disease (n=25,042), the lowest for Sjögren syndrome (n=1,599). Overall, the number of independent genomic loci associated with disease increases with the number of studies and cases (Table 1).

Discussion

We systematically reviewed the autoimmune disease-related content of the GWAS catalog of variant associations, the PGS catalog of polygenic scores and the UK Biobank of genomic and phenotypic data. These curated data sources and the ease of obtaining and querying them have already and will continue to unravel genetic and molecular underpinnings of autoimmune disease (37). An example are the currently ongoing 61 UKB-approved projects that are related to autoimmune disease (keyword search "autoimmune disease", June 13th, 2022). Our survey shows that the catalog of autoimmune GWAS studies and associations is already very comprehensive and generated in more than a decade. PGS for autoimmune diseases are rather few, very novel and largely developed within the last three years. Accordingly, in the polygenic disease genetics field, research efforts go into two interrelated directions: (i) unraveling specific functional effects of variants and (ii) combining effect estimates for a better personalized risk prediction. Computational approaches toward the first aim are the association of risk alleles with molecular traits (38) and the identification of functional variants *via* so-called fine-mapping (39). Although there is progress in the field, it is still a long way from variant associations to molecular disease mechanisms as well as treatments (37). Toward the second aim, polygenic scores are still being improved, for example by considering rare variants (40) or inclusion of functional information (41). Optimizing prediction performance is non-trivial, since machine learning models need to be calibrated to generalize to unseen data, i.e. overfitting of training data prevented (42). The AUROC of current autoimmune disease PGS varies widely (range 0.56-0.99; typically 0.6-0.8). Further

TABLE 1 The ten autoimmune diseases (defined by EFO term) which have the highest number of GWAS studies registered at the GWAS catalog. Displayed is the summary of information obtained from GWAS catalog, PGS catalog and UK Biobank. With respect to GWAS catalog, this is the number of unique studies (according to column "STUDY ACCESSION" of Suppl. Table S3), the highest number of cases with corresponding number of controls, the number of unique variants reported (according to column "SNP_ID_CURRENT" of Suppl. Table S4), the number of independent, associated genomic loci reported in the literature, the number of unique genes or gene combinations reported in the respective publications (according to column "REPORTED GENE(S)" of Suppl. Table S4). With respect to PGS catalog, reported are the number of unique studies (according to column "PGS Publication (PGP) ID" of Suppl. Table S5 and Suppl. Table S8), unique scores developed (according to column "Polygenic Score (PGS) ID" of Suppl. Table S5), the range of variants utilized in the scores for the respective disease and the number of performance evaluations in independent samples (according to column "PGS Performance Metric (PPM) ID" of Suppl. Table S8). Finally, for the UK Biobank, the UKB data field, ICD-10 code (if available in UKB) and patient number according to Suppl. Table S9 is provided.

Trait	EFO IDs	GWAS catalog						PGS catalog				UK Biobank		
		# Studies	# Cases	# Controls	# SNPs	# Loci	# Gene	# Studies	# PGS	# Variants	# PGS Eval.	Data Field	ICD10 Code	# Indiv.
Systemic lupus erythematosus	EFO_0002690	37	13,377	194,993	788	132[1]	439	6	6	41- 293,684	32	131894	M32	1,053
Rheumatoid arthritis	EFO_0000685	37	22,628	288,664	421	>150[2]	249	3	6	3- 95,083	33	131850	*M06	12,556
Multiple sclerosis	MONDO_0005301	27	14,802	26,703	603	233[3]	479	3	5	36- 129,077	25	131042	G35	2,518
Crohn's disease	EFO_0000384	27	12,924	21,442	411	>200[4]	265	1	2	220-257	9	131626	K50	3,355
Ulcerative colitis	EFO_0000729	25	12,366	33,609	295	>200[4]	184	2	4	179-566,637	26	131628	K51	6,451
Inflammatory bowel disease	EFO_0003767	12	25,042	34,915	387	>200[4]	238	3	2	195-690,7112	7	_**	_**	_**
Vitiligo	EFO_0004208	10	2,853	37,405	91	49[5]	80	3	3	42-77	10	131802	L80	1,201
Sjogren syndrome	EFO_0000699	10	1,599	658,316	48	25[6]	42	1	1	7	5	20002_1382	_	572***
Grave's disease	EFO_0004237	8	4,487	629,598	74	12[7]	27	_	_	_	_	20002_1522	_	183***
Behcet's syndrome	EFO_0003780	8	3,197	5,785	40	21[8]	35	_	_	_	_	41202	_	18****

[1] (29); [2] (30); [3] (31); [4] (32); [5] (33); [6] (34); [7](35); [8] (36); *Excludes seropositive rheumatoid arthritis (M05) with 1,401 patients ** K50+K51 *** Self-reported **** Based on medical history of hospital patients.

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evaluation of polygenic scores in more cohorts and systematic comparisons, facilitated by the PGS catalog, will help gaining further insights into PGS predictive performance for individual autoimmune diseases. Perspectively, PGS can be amended with other biomedical, clinical and behavioral data. Such rich, combined data sources together with recent developments in artificial intelligence promise to improve prediction of disease and personalized treatment options (43). Finally, polygenic scores can be used to investigate interactions of genetic and environmental factors, which is particularly relevant for autoimmune diseases, in which environmental factors play a key role (44).

Author contributions

RS and IW summarized data and generated tables and figures. RS, CF, and IW wrote the first draft of the manuscript. All authors contributed to writing and editing the manuscript. All authors approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.972107/full#supplementary-material

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The significance of preclinical anti-BP180 autoantibodies

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Bullous pemphigoid (BP) is the most common autoimmune subepidermal blistering disease. Although the pathomechanism of BP onset has yet to be elucidated in detail, BP autoantibodies targeting two hemidesmosomal components, BP180 and BP230, are known to play a pivotal role in BP pathogenesis. Thus, the detection and measurement of BP autoantibodies are necessary for diagnosing BP and monitoring the disease activity. Immune assays such as immunofluorescence microscopy, immunoblotting, and ELISAs using BP180 and BP230 detect BP autoantibodies in most BP cases with high specificity; however, BP autoantibodies are sometimes detected in BP patients before the onset of this disease. BP autoantibodies that are detected in patients without typical tense blisters are defined as "preclinical BP autoantibodies". These preclinical BP autoantibodies are detected even in a low percentage of normal healthy individuals. Although the importance of preclinical BP autoantibodies remains elusive, these autoantibodies might be a potential risk factor for subsequent BP development. Therefore, previous comparative epidemiological studies have focused on the prevalence of preclinical BP autoantibodies in populations susceptible to BP (e.g., the elderly) or in diseases with a higher risk of comorbid BP. This mini-review summarizes the literature on the prevalence of preclinical BP autoantibodies in patients with various conditions and diseases, and we discuss the significance of preclinical BP autoantibody detection.

KEYWORDS

bullous pemphigoid, autoantibodies, BP180, anti-BP180 autoantibodies, autoimmune disease, aging, diabetes mellitus, neurological disease

Abbreviations: BP, bullous pemphigoid; BMZ, basement membrane zone; NC16A, the 16th non-collagenous A domain; ELISA, enzyme-linked immunosorbent assay; DIF, direct immunofluorescence analysis; IIF, indirect immunofluorescence analysis; ssIIF, 1M NaCl-split skin indirect immunofluorescence analysis; SD, standard deviation; MS, multiple sclerosis; DM, diabetes mellitus; DPP4i, dipeptidyl peptidase-IV inhibitor; DPP4i-BP, dipeptidyl peptidase-IV inhibitor-associated bullous pemphigoid; PUPPP, polymorphic urticarial papules and plaques of pregnancy.

Introduction

Bullous pemphigoid (BP), a major autoimmune blistering disease, is characterized clinically by tense blisters and/or urticarial erythema and histologically by subepidermal blisters with abundant eosinophilic infiltration (1, 2). Autoantibodies directed to the basement membrane zone (BMZ) play an essential role in BP pathogenesis. BP autoantibodies mainly target two hemidesmosomal components: BP180 and BP230, and anti-BP180 autoantibodies have been recognized as the major pathogenic autoantibodies in BP (3–5). Furthermore, the 16th non-collagenous (NC16A) domain has been identified as a major pathogenic epitope of BP autoantibodies because 80–90% of BP sera show positive reactivity to the NC16A domain within BP180 (6, 7).

Regarding BP diagnosis, the detection of BP autoantibodies is necessary in addition to clinical and histological findings (1, 8). However, the presence of BP autoantibodies is not a sufficient condition for diagnosis. In fact, the production of BP autoantibodies precedes clinical tense blister formation in certain BP cases (9). Therefore, previous studies have investigated the prevalence of preclinical BP autoantibodies as a potential risk for subsequent BP development in various conditions, including aging, pruritus, collagen diseases, and neurological disorders.

Detection methods for BP autoantibodies

BP autoantibodies are present in BP patients in two forms: tissue-bound autoantibodies in lesional skin and circulating autoantibodies in peripheral blood. The former is detected with direct immunofluorescence (DIF) microscopy; the latter is detected with indirect immunofluorescence (IIF) microscopy, ELISA, and immunoblotting. With DIF, which is the most sensitive method of finding BP autoantibodies, those antibodies show up as the linear deposition of IgG and/or C3 at the dermal-epidermal junction of lesional skin (10). Several studies suggested a new subset of IgG4-dominant BP, which predominantly has IgG4 autoantibodies without complement activation at the BMZ (11–13). However, the linear deposition of C3 at the BMZ is a promising diagnostic sign for BP (14). The limitation of DIF is that it is difficult to differentiate autoantibodies of BP from those of other subepidermal autoimmune blistering diseases.

IIF semi-quantitates the titration of circulating BP autoantibodies using skin or esophagus cryosections from a healthy individual. Using 1 M NaCl-split skin sections, IIF allows differentiation between BP and other autoimmune subepidermal blistering skin diseases. In 1 M NaCl-split skin IIF (ssIIF), reactivity against the epidermal side corresponds to BP, while reactivity against the dermal side corresponds to anti-laminin 332 mucous membrane pemphigoid, epidermolysis bullosa acquisita, and anti-p200 pemphigoid (also called anti-laminin gamma 1 pemphigoid). Immunoblotting using epidermal extracts or recombinant proteins of BP autoantigens is also available to detect circulating BP autoantibodies in limited facilities.

ELISA using the BP180 NC16A recombinant protein (BP180 NC16A ELISA) is a commercially available method of detecting and quantifying BP autoantibodies. BP180 NC16A ELISA has a sensitivity of 84.4% and a specificity of 98.9% in diagnosing BP (6). Furthermore, the titration of BP autoantibodies quantified by BP180 NC16A ELISA closely correlates with BP activity (15); thus, BP180 NC16A ELISA is also useful for monitoring BP. Also, an ELISA using tetrameric NC16A recombinant proteins is commercially available (16). A few laboratories have developed ELISAs that use non-NC16A regions of BP180 and full-length BP180 to diagnose BP. Hofmann et al. showed that 54/116 (46.6%) of BP sera reacted to BP180 COOH-terminal regions, i.e., aa1351–1497 (17). Using a random BP180 epitope library displayed on a bacteriophage, 31/57 (54.4%) of BP sera reacted to at least one additional region other than BP180 NC16A, aa490–562, within the extracellular (36.8%) or intracellular (28.1%) domains of BP180 (18). An ELISA using BP180 aa1080–1107 and aa1331–1404 detected anti-BP180 autoantibodies in 32/78 (41.0%) of BP sera (19). A multicenter prospective study showed that distinct epitopes of the BP180 ectodomain other than BP180 NC16A are recognized by 95.9% of BP sera (20). An ELISA using human full-length BP180 recombinant proteins (a full-length BP180 ELISA) was able to detect anti-BP180 autoantibodies targeting the NC16A domain as well as other regions of BP180 for 83.5% of BP sera (21).

BIOCHIP is a unique IIF-based serologic diagnostic assay for detecting autoantibodies of autoimmune blistering diseases (22). The BIOCHIP technique provides autoimmune blistering-related antibody profiles of patient sera in a single incubation on the mosaic slide. BIOCHIP contains a recombinant BP180 NC16A protein, and the sensitivity of anti-BP180 NC16A autoantibodies in BP detected by BIOCHIP is 55.3–100.0% (22–31).

Although IIF, WB, ELISA, and BIOCHIP can detect anti-BP180 antibodies, the results can differ. Among BP180 ELISA-positive BP patients, positive rates for IIF, immunoblot, and BIOCHIP were 77.3–83.3%, 69.0–95.5%, and 83.3–100.0%, respectively (25, 26, 29, 32, 33). Low-titer anti-BP180 autoantibodies detected by ELISA may be confirmed by IIF or immunoblotting.

Prevalence of preclinical anti-BP180 autoantibodies in normal healthy individuals

We reviewed the studies evaluating preclinical anti-BP180 autoantibodies in normal healthy individuals and listed representative studies whose populations exceeded 50 (Table 1). BP180 NC16A ELISA detected anti-BP180 autoantibodies in 0.0–4.2% of normal healthy individuals (6, 16, 19, 35–40, 42, 43). Wieland et al. showed that 14 (4.15%) of 337 unaffected subject sera were positive by BP180 NC16A ELISA, and the mean titer of ELISA-positive sera was 16.2 ± 6.3 (mean ± standard deviation, SD, cutoff value <9.0) (39). According to other studies, the mean titers of normal subjects by BP180 NC16A ELISA were 2.1–2.5 (35, 37, 43). The mean titer of ELISA-positive sera was higher in the Wieland et al. study than in the other studies. However, these ELISA-positive sera had no positive reactivities toward a monkey esophagus by IIF (39). ELISAs using the mid-portion (aa490–811) or the C-terminal domain (aa1351–1497) of BP180 detected anti-BP180 autoantibodies in 1.4% and 4.2% of sera from normal healthy individuals, respectively (17).

Regarding immunoblotting, none of the normal control sera showed positive reactivity against GST-fusion proteins containing 42 amino acids of the NC16A region nor 49 amino acids of the C-terminal region of BP180 (34). Immunoblotting using epidermal extracts detected BP autoantibodies in 31% of healthy individuals, while BP180 NC16A ELISA did not detect BP autoantibodies in a population of those who were seropositive to epidermal extracts (38). For BIOCHIP, 0.5–1.0% of healthy blood donors showed reactivity to the NC16A domain of BP180 (22, 41).

An observational study to evaluate the prevalence of anti-BP180 antibodies in healthy volunteers by using a full-length BP180 ELISA was conducted in Japan (44). 2.2% of serum samples from the healthy volunteers possessed anti-BP180 autoantibodies without the volunteers having any skin lesions. The mean titer ± SD of ELISA-positive sera was 13.9 ± 16.4 (cutoff value <4.64), whereas the mean titer of the parent population was 0.8 ± 3.2. In addition, 12/23 (52.1%) of ELISA-positive sera were positive by IIF using monkey esophagus. Intriguingly, the existence of anti-BP180 autoantibodies is associated with a history of bone fracture and the administration of anti-osteoporosis drugs (44).

TABLE 1 The prevalences of anti-BP180 autoantibodies in normal healthy individuals.

Paper	Year	Subjects	Positive rate of anti-BP180 auto-antibodies	Subject attributes	Assay
Nakatani et al. (34)	1998	50	0.0%	Normal control sera	Immunoblotting using BP180 NC16A
		50	0.0%	Normal control sera	Immunoblotting using the BP180 C-terminus
Kobayashi et al. (6)	2002	336	1.5%	Normal individuals	BP180 NC16A ELISA
Hofmann et al. (17)	2002	72	1.4%	Normal human sera	ELISA using recombinant BP180 aa490–811
		72	4.2%	Normal human sera	ELISA using recombinant BP180 aa1351–1497
Mariotti et al. (19)	2004	85	1.2%	Healthy volunteers	ELISA using recombinant BP180 aa1080–1107 and 1311–1404
		70	0.0%	Healthy volunteers	BP180 NC16A ELISA
Sakuma-Oyama et al. (35)	2004	60	3.3%	Normal volunteers	BP180 NC16A ELISA
Yoshida et al. (36)	2005	336	1.5%	Normal control sera	BP180 NC16A ELISA
Powell et al. (37)	2005	166	0.0%	Control subjects	BP180 NC16A ELISA
Sitaru et al. (16)	2007	494	2.0%	Healthy donors	BP180 tetrameric NC16A ELISA
Desai et al. (38)	2008	61	31.1%	Healthy subjects and volunteers	Immunoblotting using epidermal extracts
		20	0.0%	Immunoblot-positive subjects	BP180 NC16A ELISA
Wieland et al. (39)	2010	337	4.2%	Unaffected subjects	BP180 NC16A ELISA
van Beek et al. (22)	2012	100	1.0%	Healthy blood donors	BIOCHIP
van Beek et al. (40)	2014	50	0.0%	Blood donors	BP180 NC16A ELISA
		50	0.0%	Blood donors	BP180 tetrameric NC16A ELISA
Prüßmann et al. (41)	2015	7063	0.5%	Normal blood donors	BIOCHIP
Recke et al. (42)	2016	75	2.7%	Healthy controls	BIOCHIP and BP180 NC16A ELISA
Tuusa et al. (43)	2019	140	1.4%	Healthy control subjects	BP180 NC16A ELISA
Mai et al. (44)	2022	1035	2.2%	Normal blood donors	Full-length BP180 ELISA

We also compared the prevalence of preclinical anti-BP180 autoantibodies among the detection systems. The mean ± SD (range) prevalence for immunoblot, ELISA, and BIOCHIP was 10.4 ± 18.0 (0–31.1), 1.6 ± 1.4 (0.0–4.2), 1.4 ± 1.2 (0.5–2.7), respectively (6, 16, 17, 19, 22, 34–44). Although the high prevalence of anti-BP180 autoantibodies detected by immunoblotting in the Desai et al. study was not confirmed by ELISA (38), immunoblotting might detect preclinical anti-BP180 autoantibodies more efficiently than the other detection methods do.

Prevalence of preclinical anti-BP180 autoantibodies in BP-susceptible populations or in patients with diseases associated with comorbid BP

We also reviewed the studies evaluating preclinical anti-BP180 autoantibodies in various medical conditions and listed the representative studies whose populations exceeded 20 (Table 2).

Aging and pruritus

BP predominantly occurs in individuals over age 60 (56). In a retrospective observational study of 869 patients with BP in the United Kingdom, the median age at presentation for BP was 80 years (57). Two epidemiological studies found the incidence of BP to be significantly higher for people in their ninth or tenth decade than for people of other ages (58, 59). Thus, aging has been recognized as a significant risk factor for BP onset. Several studies investigated the prevalence of anti-BP180 autoantibodies in the elderly (40, 45–49, 60). Interestingly, Hachisuka et al. showed no preclinical anti-BP180 autoantibodies in 32 normal elderly individuals (46). In addition, large retrospective studies using unaffected subjects or a general population in Japan did not find a significant difference between age and the prevalence of anti-BP180 autoantibodies (39, 44).

Pruritus is a common problem among elderly people (61), and pruritus may develop in the early stage of BP (2, 62–64). Several studies reported the prevalence of preclinical anti-BP180 autoantibodies in elderly patients with pruritic disorders (40, 47–49). Hofmann et al. showed that three (12.0%) of 25 elderly patients with pruritic disorders had anti-BP180 autoantibodies, and the mean titer ± SD of these positive sera was 18.1 ± 4.6 (47). However, DIF, ssIIF, and immunoblotting using cultured keratinocyte extracts were negative among all elderly patients with pruritic disorders. Feliciani et al. investigated preclinical anti-BP180 autoantibodies using ELISAs with the mid-portion (aa490–812) or C-terminus (aa1048–1465) of BP180 in elderly patients with pruritic disorders (48). Both BP180 mid-portion and C-terminus ELISAs detected preclinical BP autoantibodies in two (13.3%) of 15 elderly patients with pruritic disorders, whereas one (4.0%) of 25 elderly patients with immediate-type allergy had preclinical anti-BP180 autoantibodies. Fania et al. showed a higher prevalence of preclinical anti-BP180 autoantibodies (23.8%) in elderly patients with pruritic disorders than that in elderly patients with allergic diseases (4.2%) by BP180 N-terminus and C-terminus ELISAs (49). In contrast, van Beek et al. found no significant difference in the prevalence of anti-BP180 autoantibodies among elderly patients with chronic disorders or non-inflammatory skin diseases and blood donors using a BP180 NC16A ELISAs (40). These results are conflicting, and further studies are needed.

Collagen disease

Several case reports showed that collagen diseases such as systemic lupus erythematosus, dermatomyositis, rheumatoid arthritis, or systemic sclerosis can co-exist with BP (65–73). Although it remains unclear whether collagen diseases are a risk factor for BP development, two studies explored preclinical anti-BP180 autoantibodies in a collagen disease population while evaluating the diagnostic performance of BP180 NC16A ELISAs (6, 16). These studies showed the prevalence of anti-BP180 autoantibodies to be 1.1–8.0% in collagen disease patients, which is comparable with the 1.5–2.0% in healthy controls.

Neurological disease

Previous epidemiological studies suggest that neurological diseases are a risk factor for BP development (74–76). A Finnish epidemiological study indicated that multiple sclerosis (MS) and Alzheimer's disease increase the risk of BP development (77, 78). As neurological diseases are associated with BP, several studies examined the prevalence of preclinical anti-BP180 autoantibodies in patients with neurological disorders (42, 43, 50, 51).

Messingham et al. investigated the prevalence of BP autoantibodies in patients with Parkinson's disease and dementia using a BP180 NC16A ELISA and immunoblotting using the BP180 ectodomain or intracellular domain (50). BP180 NC16A ELISA detected BP autoantibodies in 3.8% of the dementia patients but not in any of the Parkinson's disease patients nor in the control

TABLE 2 The prevalences of anti-BP180 autoantibodies in patients with various medical conditions.

Condition	Paper	Year	Subjects	Positive rates of anti-BP180 autoantibodies	Subject attributes	Assay
Aging or pruritus						
	Reickhoff-Catoni et al. (45)	1992	24	0.0%*	Control subjects	Immunoblotting using keratinocyte extracts including BP180 and BP230
			83	15.7%*	Patients with pruritic disorders	Immunoblotting using keratinocyte extracts including BP180 and BP230
	Hachisuka et al. (46)	1996	32	0.0%	Normal elderly individuals	Immunoblotting using human or guinea pig epidermal extracts
	Hofmann et al. (47)	2003	25	12%	Elderly patients with pruritic disorders	BP180 NC16A ELISA
	Feliciani et al. (48)	2009	25	0.0%	Elderly patients with immediate-type allergy	ELISA using BP180 aa490–812
			15	13.3%	Elderly patients with pruritic disorders	ELISA using BP180 aa490–812
			25	4.0%	Elderly patients with immediate-type allergy	ELISA using BP180 aa1048–1465
			15	13.3%	Elderly patients with pruritic disorders	ELISA using BP180 aa1048–1465
	Fania et al. (49)	2012	24	4.2%	Elderly patients with allergic disorders	ELISAs using BP180 aa490–812 and aa1048–1465
			21	23.8%	Elderly patients with pruritic dermatoses	ELISAs using BP180 aa490–812 and aa1048–1465
	van Beek et al. (40)	2014	50	0.0%	Blood donors	BP180 NC16A ELISA
			93	0.0%	Elderly patients with non-inflammatory skin diseases	BP180 NC16A ELISA
			78	1.3%	Elderly patients with chronic pruritic skin disorders	BP180 NC16A ELISA
			50	0.0%	Blood donors	BP180 tetrameric NC16A ELISA
			93	3.2%	Elderly patients with non-inflammatory skin diseases	BP180 tetrameric NC16A ELISA
			78	0.0%	Elderly patients with chronic pruritic skin disorders	BP180 tetrameric NC16A ELISA
Collagen diseases						
	Kobayashi et al. (6)	2002	336	1.5%	Normal individuals	BP180 NC16A ELISA
			91	1.1%	Collagen disease patients	BP180 NC16A ELISA
	Sitaru et al. (16)	2007	494	2.0%	Healthy donors	BP180 tetrameric NC16A ELISA
			50	4.0%	Systemic scleroderma	BP180 tetrameric NC16A ELISA
			72	1.4%	Systemic lupus erythematosus	BP180 tetrameric NC16A ELISA
			107	1.9%	Rheumatoid arthritis	BP180 tetrameric NC16A ELISA
			50	8.0%	Systemic scleroderma	BP180 NC16A ELISA
			72	4.2%	Systemic lupus erythematosus	BP180 NC16A ELISA
			107	2.0%	Rheumatoid arthritis	BP180 NC16A ELISA
Neurological diseases						
	Messingham et al. (50)	2016	23	0.0%	Control subjects	BP180 NC16A ELISA
			24	0.0%	Parkinson's disease	BP180 NC16A ELISA
			26	3.8%	Dementia	BP180 NC16A ELISA

(Continued)

TABLE 2 Continued

Condition	Paper	Year	Subjects	Positive rates of anti-BP180 autoantibodies	Subject attributes	Assay
			23	0.0%	Control subjects	Immunoblotting using the BP180 ectodomain
			24	29.2%	Parkinson's disease	Immunoblotting using the BP180 ectodomain
			26	3.8%	Dementia	Immunoblotting using the BP180 ectodomain
			23	0.0%	Control subjects	Immunoblotting using the BP180 intracellular domain
			24	8.3%	Parkinson's disease	Immunoblotting using the BP180 intracellular domain
			26	19.2%	Patients with dementia	Immunoblotting using the BP180 intracellular domain
	Recke et al. (42)	2016	75	2.7%	Healthy controls	BP180 NC16A ELISA
			65	1.5%	Non-inflammatory skin diseases	BP180 NC16A ELISA
			50	4.0%	Parkinson's disease	BP180 NC16A ELISA
			50	0.0%	Multiple sclerosis	BP180 NC16A ELISA
			75	0.0%	Parkinson's disease	BP180 NC16A ELISA
			75	6.7%	Other neurological diseases	BP180 NC16A ELISA
			65	1.5%	Healthy controls	BP180 NC16A ELISA
	Kokkonen et al. (51)	2017	38	2.6%	Healthy control subjects	BP180 NC16A ELISA
			111	18.0%	Alzheimer's disease	BP180 NC16A ELISA
	Tuusa et al. (43)	2019	140	1.4%	Healthy control subjects	BP180 NC16A ELISA
			143	7.7%	Multiple sclerosis	BP180 NC16A ELISA
			35	11.4%	Multiple sclerosis	Full-length BP180 ELISA
			111	6.3%	Alzheimer's disease	Full-length BP180 ELISA
			35	0.0%	Multiple sclerosis	Immunoblotting using BP180 NC16A
	Wang et al. (52)	2019	100	5.0%	Healthy control subjects	BP180 NC16A ELISA
			100	14.0%	Stroke	BP180 NC16A ELISA
	Wang et al. (53)	2020	50	8.0%	Healthy control subjects	BP180 NC16A ELISA
			48	47.9%	Alzheimer's disease	BP180 NC16A ELISA
Diabetes mellitus						
	Jedlickova et al. (54)	2008	20	10.0%	Non-diabetic patients without pruritus	ELISA using recombinant BP180
			31	9.7%	Non-diabetic patients with pruritus	ELISA using recombinant BP180
			18	0.0%	Diabetic patients without pruritus	ELISA using recombinant BP180
			21	9.5%	Diabetic patients with pruritus	ELISA using recombinant BP180
	Izumi et al. (55)	2019	54	0.0%	Diabetic patients without DPP4i	BP180 NC16A ELISA
			221	1.8%	Diabetic patients with DPP4i	BP180 NC16A ELISA
			54	5.6%	Diabetic patients without DPP4i	Full-length BP180 ELISA
			221	10.9%	Diabetic patients with DPP4i	Full-length BP180 ELISA
Other skin diseases						
	Powell et al. (37)	2005	166	3.0%	Control subjects	BP180 NC16A ELISA

(Continued)

TABLE 2 Continued

Condition	Paper	Year	Subjects	Positive rates of anti-BP180 autoantibodies	Subject attributes	Assay
			164	4.9%	PUPPP	BP180 NC16A ELISA
	Feliciani et al. (48)	2009	25	0.0%	Elderly patients with immediate-type allergy	ELISA using BP180 aa490–812
			15	13.3%	Elderly patients with pruritic disorders	ELISA using BP180 aa490–812
			25	4.0%	Elderly patients with immediate-type allergy	ELISA using BP180 aa1048–1465
			15	13.3%	Elderly patients with pruritic disorders	ELISA using BP180 aa1048–1465
	Tampoia et al. (24)	2012	40	0.0%	Healthy subjects	BIOCHIP
			54	0.0%	Psoriasis, discoid lupus erythematosus, lichen ruber planus	BIOCHIP
	van Beek et al. (22)	2012	100	1.0%	Healthy blood donors	BIOCHIP
			97	7.2%	Non-inflammatory skin diseases	BIOCHIP
	Fania et al. (49)	2012	24	4.2%	Elderly patients with allergic disorders	ELISAs using BP180 aa490–812 and aa1048–1465
			21	23.8%	Elderly patients with pruritic dermatoses	ELISAs using BP180 aa490–812 and aa1048–1465
	van Beek et al. (40)	2014	50	0.0%	Blood donors	BP180 NC16A ELISA
			93	0.0%	Non-inflammatory skin diseases	BP180 NC16A ELISA
			78	1.3%	Chronic pruritic skin disorders	BP180 NC16A ELISA
			50	0.0%	Blood donors	BP180 tetrameric NC16A ELISA
			93	3.2%	Non-inflammatory skin diseases	BP180 tetrameric NC16A ELISA
			78	0.0%	Chronic pruritic skin disorders	BP180 tetrameric NC16A ELISA

*These data include autoantibodies targeting BP180 and BP230.
PUPPP, pruritic urticarial papules and plaques of pregnancy.

subjects. Immunoblotting using the BP180 ectodomain found BP autoantibodies in 29.2% of the Parkinson's disease patients, whereas immunoblotting using the BP180 intracellular domain detected BP autoantibodies in 19.2% of the dementia patients. Tuusa et al. indicated that BP autoantibodies were detected in 11.8% of MS sera with the full-length BP180 ELISA, while BP autoantibodies were detected in 53.8% of MS sera with immunoblotting using the full-length BP180, suggesting that BP autoantibodies from MS sera preferentially react to the denatured form of BP180 but not to its native form (43). A conflicting result was reported by Recke et al. (42). Although BIOCHIP, BP180 NC16A ELISA, BP230 ELISA, and immunoblotting with the extracellular matrix of cultured human keratinocytes were used to detect anti-BMZ antibodies in MS and Parkinson's disease patients, MS and Parkinson's disease showed no significant increased prevalence of BP autoantibodies (42). Wang et al. recently reported that anti-BP180 NC16A autoantibodies were found in 14 (14.0%) of 100 patients with stroke, and the mean titer ± SD was 19.2 ± 6.1 (52). Among these patients, 12 (85.7%) of the 14 positive sera were also positive by immunoblotting using BP180 recombinant proteins. The prevalence of BP autoantibodies detected by BP180 NC16A ELISA was found to be 18.0% in Alzheimer's disease patients (51). Interestingly, subsequent analysis found the severity of dementia to correlate significantly with the titration of anti-BP180 NC16A autoantibodies in Alzheimer's disease (51). In addition, Wang et al. reported that 20 of 48 (47.9%) patients with Alzheimer's disease had anti-BP180 autoantibodies detected by BP180 NC16A ELISA, and the mean titer ± SD was 22.9 ± 20.9 (53). Among these patients, 9 (64.3%) of the 14 positive sera were also positive by immunoblotting using BP180 recombinant proteins.

Diabetes mellitus

BP may initially mimic other pruritic dermatoses, and it is known that DM is often associated with pruritic dermatoses (79). Therefore, the potential association between DM and

the early phase of BP has been discussed. To address this issue, Jedlickova tested the seropositivity of anti-BMZ antibodies by IIF and ELISAs using recombinant BP180, BP230, and Laminin 332 (54). There were no significant differences in the seropositivity of anti-BMZ autoantibodies among non-diabetic patients or diabetic patients with or without pruritus; however, the prevalence of anti-BMZ autoantibodies in all groups detected by IIF was relatively high (12.2%). Recently dipeptidyl peptidase-IV inhibitors (DPP4i), medications for type 2 diabetes treatment, have attracted interest as a causative drug of BP (80–86). Izumi et al. investigated the prevalence of anti-BMZ autoantibodies among 275 DM patients with or without DPP4i administration (55). BP180 NC16A ELISA, BP230 ELISA, and full-length BP180 ELISA detected BP autoantibodies in 1.8%, 2.2%, and 10.9% of 221 DM patients with DPP4i treatment, respectively, whereas these assays detected BP autoantibodies in 0%, 7.4%, and 5.6% of 54 DM patients without DPP4i treatment, respectively. Among these patients, 54.2% of full-length BP180 ELISA-positive sera were also positive by ssIIF. Although there were significant differences in the prevalence of anti-full-length BP180 IgG between DM cases with and without DPP4i treatment in this study, the anti-full-length BP180 autoantibody-positive cases are likely to have been significantly older than the anti-full-length BP180 IgG-negative cases in the DM cases with DPP4i.

Other skin diseases

For the validation of anti-BP180 autoantibody detection, several studies performed the detection of anti-BP180 autoantibodies in patients with various skin diseases as a negative control for the detection system (16, 76). van Beek et al. reported that 7.2% of patients with non-inflammatory skin diseases, including leg ulcers, basal cell carcinoma, and squamous cell carcinoma, had anti-BP180 autoantibodies detected by BIOCHIP, although the details were not mentioned (22). For investigating the prevalence of anti-BP180 autoantibodies in elderly patients with pruritic disorders, elderly patients with allergic disorders or with non-inflammatory skin diseases were used as a control group (48, 49). In these studies, positive rates of anti-BP180 autoantibodies were 0.0–4.2% in elderly patients with allergic disorders detected by ELISAs using BP180 aa490–812 and aa1048–1465 (48, 49), and 1.3–3.2% in elderly patients with non-inflammatory skin diseases (40).

In addition, Powell et al. examined whether BP180 NC16A ELISA was able to differentiate pemphigoid gestationis, which is a blistering autoimmune disease mediated by anti-BP180 autoantibodies during pregnancy, from polymorphic urticarial papules and plaques of pregnancy (PUPPP) (37). In this study, 4.9% of the PUPPP patients showed seropositivity with BP180 NC16A ELISA.

Prodromal BP

The diagnosis of BP is usually made by the combination of clinical, histopathological, and immunological findings described above. Typical tense blisters are an essential clue in diagnosing BP, and it is challenging to diagnose BP in cases without blisters. A prospective nationwide cohort study found that BP was diagnosed an average of 6.1 months after onset of the first symptoms (87). Cohort studies showed that 17–20% of BP presented with no apparent blisters at the time of BP diagnosis (2, 87). There is the concept of prodromal BP (62, 88–90). Retrospective studies showed that 31.8% and 36.8% of BP patients had non-bullous lesions before the appearance of typical blisters (90, 91). The duration before blister development ranged from 2 weeks to 19 years: That duration was up to 12 months for 82% of the patients in the Sun et al. study, and the mean duration before blister appearance was 15.9 months in the Zhang et al. study (90, 91). This non-bullous stage of BP may lead to a delayed diagnosis.

Also, a subtype called non-bullous pemphigoid has been reported (63, 92–94). In a systematic review of non-bullous pemphigoid described by Lamberts et al., the most common clinical presentations of patients with non-bullous pemphigoid were erythematous, urticarial plaques and papules/nodules, and 9.8% of the non-bullous pemphigoid patients developed bullae during the reported follow-up (63). In addition, an IgM autoantibody-mediated pemphigoid disease called IgM pemphigoid has been reported (95–97), and IgM autoantibodies targeting BP180 might play a pathogenic role in IgM pemphigoid (95, 96). It is debatable whether non-bullous pemphigoid and IgM pemphigoid are just prodromes of BP or are distinct pemphigoid variants.

The detection of anti-BP180 autoantibodies might be an indicator for subsequent BP development. Wang et al. retrospectively analyzed medical records from 2005 to 2015 from a single center and found 208 BP autoantibody-positive patients with BP180 and/or BP230 ELISA but not with DIF (98). Dermatitis was the most common diagnosis among preclinical BP autoantibody-positive patients; of note, four patients had positive DIF results upon repeating the tests, and a diagnosis of BP was made during follow-up (98). Raneses et al. showed that 4 of 18 BP patients had positive ani-BP180 or anti-BP230 autoantibodies 79 months (range: 0.1 to 217 months) before the onset of BP symptoms (9).

Concluding remarks

Certain patients with various medical conditions such as aging with pruritus, neurological diseases, and diabetes mellitus with the

administration of DPP4i can have anti-BP180 autoantibodies without BP development. However, it remains controversial whether the existence of anti-BP180 autoantibodies is a predictive factor for BP development. Further studies on preclinical BP autoantibodies promise to reveal whether the presence of anti-BP180 autoantibodies is a predictive marker for BP development and to identify the critical factors contributing to BP onset in the presence of BP autoantibodies.

Author contributions

YM, KI, and SM drafted the paper. HU supervised the writing of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Type XVII collagen: Relevance of distinct epitopes, complement-independent effects, and association with neurological disorders in pemphigoid disorders

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Pemphigoid diseases (PD) are autoimmune skin blistering diseases characterized by autoantibodies directed against proteins of the cutaneous basement membrane zone (BMZ). One of the major antigens is type XVII collagen (BP180), a transmembrane glycoprotein, which is targeted in four PDs: bullous pemphigoid, mucous membrane pemphigoid, linear IgA dermatosis, and pemphigoid gestationis. To date, different epitopes on BP180 have been described to be recognized by PD disease patients' autoantibodies. Different BP180 epitopes were associated with distinct clinical phenotypes while the underlying mechanisms are not yet fully understood. So far, the main effects of anti-BP180 reactivity are mediated by Fcγ-receptors on immune cells. More precisely, the autoantibody–antigen interaction leads to activation of complement at the BMZ and infiltration of immune cells into the upper dermis and, by the release of specific enzymes and reactive oxygen species, to the degradation of BP180 and other BMZ components, finally manifesting as blisters and erosions. On the other hand, inflammatory responses independent of Fcγ-receptors have also been reported, including the release of proinflammatory cytokines and internalization and depletion of BP180. Autoantibodies against BP180 can also be found in patients with neurological diseases. The assumption that the clinical expression of PD depends on epitope specificity in addition to target antigens, autoantibody isotypes, and antibody glycosylation is supported by the observation that epitopes of PD patients differ from those of PD patients. The aim of the present review is to describe the fine specificities of anti-BP180 autoantibodies in different PDs and highlight the associated clinical differences. Furthermore, the direct effects after binding of the autoantibodies to their target are summarized.

KEYWORDS

BP180, epitopes, pemphigoid, autoimmunity, bullous disease, autoantigen

Introduction

The skin is the largest human organ. It serves as a barrier and protects the body from environmental influences, including heat, cold, dehydration, UV radiation, and pathogens, as well as many toxic and immunogenic substances. The integrity of the skin is established by cell–cell contacts among keratinocytes and the adhesion of the epidermis to the dermis at the dermo-epidermal junction (DEJ). Proteins of these structures target antigens in various autoimmune bullous disorders, i.e., pemphigus and pemphigoid diseases (PD) (1, 2).

In addition to providing structural integrity, the DEJ has multiple functions and regulates epithelial–mesenchymal interaction during skin homeostasis, growth, and wound healing; serves as a permeability barrier; and participates in signal transduction (3–8). The individual layers of the DEJ can electron microscopically be subdivided in the lamina lucida, directly adjacent to the plasma membrane of basal keratinocytes, the lamina densa, and the sublamina densa. The DEJ is mainly composed of widely conserved molecules, including collagen type IV, laminins, nidogen, and perlecan (6). Within the DEJ-specialized cell-substratum adhesion sites, the so-called hemidesmosomes mediate the interaction between the cytoskeleton and, *via* anchoring filaments and anchoring fibrils, dermal collagens (5, 9). Hemidesmosomes of the epidermis and surface-close epithelia are composed of an intracellular plaque where the intermediated filaments keratins K5 and K14 link with the plectin isoform 1a (P1a) and the bullous pemphigoid antigen 1 isoform e (BPAG1e), also termed BP230. The latter two hemidesmosomal molecules interact with the two transmembrane molecules α6β4 integrin and BP180 (also called type XVII collagen or BPAG2). As the fifth hemidesmosomal protein, the transmembrane tetraspanin CD151 associates with the extracellular loop of α6 integrin. In the extracellular space, α6β4 integrin and BP180 connect with anchoring filaments, mainly consisting of laminin 332, laminin 331, and uncein. Anchoring filaments interact with anchoring fibrils, mainly consisting of type VII collagen, that finally links with dermal collagen (9–11).

In PD, autoantibodies are directed against proteins of the DEJ, primarily BP180, BP230, laminin 332, the p200 antigen (laminin γ1), and collagen type VII (12–15).

The present review focuses on BP180, the main autoantigen of the DEJ, and highlights the fine autoantibody specificities against this autoantigen targeted in four PD, i.e. bullous pemphigoid (BP), mucous membrane pemphigoid (MMP), linear IgA dermatosis, and pemphigoid gestationis (16, 17). In addition, this review summarizes the described direct effects immediately after binding of anti-BP180 antibodies to their cellular target.

Epidemiology and clinical features of pemphigoid diseases

By far, the most common PD is BP, which predominantly affects patients with a mean age of between 75 and 80 years at the time of diagnosis (18–21). The incidence of BP has recently been prospectively estimated to be 19.6 patients/million/year in Northern Germany (22). About the same incidence has been reported in France, while incidences of 7.63 patients/million/year and 7.1 patients/million/year were described in UK and Sweden based on national health registries (23, 24). In other populations, like in Tehran and Israel, pemphigus is more frequent than BP (25). Annual incidences of the other BP180-related diseases MMP, pemphigoid gestationis, and linear IgA dermatosis were reported to be 1.3 and 2.0/million/year, 0.8–2.0, and 0.25–1.0, respectively (21, 26–29).

BP clinically presents with intense pruritus and tense blisters and erosions. In some patients, urticarial plaques can predominate, and in 20% of patients, no blisters are found (30–32). Pemphigoid gestationis is a mainly transient disease occurring during pregnancy with intense itch and urticarial erythema; vesicles may or may not arise (33). In linear IgA disease, autoantibodies are mainly of the IgA isotype and blistering tends to occur at the edge of lesions, presenting as the so-called crown of jewels sign (34). In contrast to other pemphigoid disorders, in MMP, mucosal surfaces are predominantly involved, manifesting as erosions and crusts, mainly affecting the mouth, conjunctivae, nose, and genital area (26, 35–37).

Anti-BP230 reactivity in pemphigoid diseases

In 50%–60% of BP patients, in addition to BP180-specific antibodies, reactivity against BP230 is found (38–45). BP patients with autoantibodies restricted to BP230 are rare (40). In pemphigoid gestations, linear IgA diseases, and MMP, anti-BP230 reactivity is less frequent and nearly always accompanied by anti-BP180 antibodies (14, 15, 46). While the main pathogenic effect is mediated by autoantibodies against BP180 (47, 48), the pathogenic effect of anti-BP230 IgG has also recently been described *in vivo* (49).

Epitopes on BP180

BP180 is a homotrimeric, transmembrane, hemidesmosomal glycoprotein with type II orientation, meaning that the amino-terminal end is located in the cytoplasm while the carboxy-terminus is located in the extracellular space (3, 46) (Figures 1A, B). The protein consists of a globular intracellular domain and a large

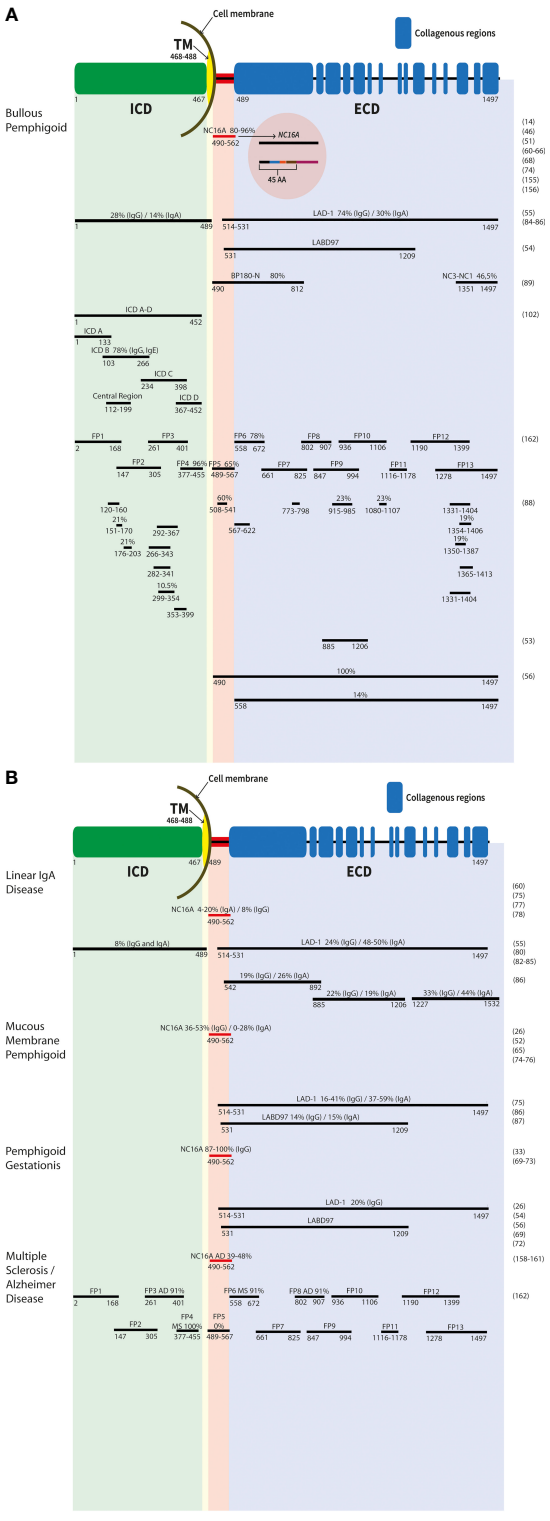

FIGURE 1
Schematic representation of the antigenic sites of the BP180 protein. The different epitopes of the molecule are shown. Numbers represent the first and last amino acid of each fragment/recombinat protein. The common names are listed above. The main targets are labelled with antibody reactivity (%) and IgG if not otherwise mentioned. Results in bullous pemphigoid **(A)**, linear IgA disease, mucous membrane pemphigoid, pemphigoid gestationis, as well multiple sclerosis and Alzheimer's disease **(B)** are depicetd. ECD, extracellular domain; ICD, intracellular domain; TM, transmembrane region.

extracellular domain (46, 50). Both domains contain multiple antigenic sites (51–56). The extracellular domain is composed of 15 collagenous domains interrupted by noncollagenous domains called NC1 to NC16 (3, 57, 58). The importance of BP180 for the structural integrity of the DEJ and the attachment of keratinocytes to the underlying dermis is highlighted by mutations in *COL17* encoding for BP180 and resulting in variants of junctional epidermolysis bullosa characterized by blisters and erosions present at birth or in early childhood (59).

In BP, the major antigenic site is the NC16A domain (40). The NC16A domain consists of 76 amino acids and is located immediately outside the transmembrane part (amino acids (aa) 490-562, Figure 1A) (46). Interestingly, all reactive sites recognized by autoantibodies are located within the N-terminal region of the NC16A domain and are recognized by IgG autoantibodies in 80%–96% of BP sera (14, 51, 60–65) (Figure 1A). For the detection of autoantibodies to BP180, highly sensitive and specific enzyme-linked immunosorbent assays using the NC16A domain as an antigenic target, have been developed recently (60, 61, 64). It has also been shown that disease activity in BP patients correlates with the serum levels of autoantibodies to BP180 NC16A in addition to IgG antibodies, also IgE and IgA autoantibodies are found in BP sera, mainly targeting the NC16A domain (46, 63, 66–68). The NC16A domain of BP180 is also the main target of autoantibodies in pemphigoid gestationis. In total, 87%–100% of cases exhibit IgG autoantibodies against this domain (33, 69–73). Reactivities for IgG and IgA with the NC16A domain were detected in 36%–53% and 0%–28% of MMP patients, respectively (26, 52, 65, 74–76). In LAD, 8% and 4%–20% of analyzed sera exhibit anti-BP180 NC16A IgG or IgA autoantibodies, respectively (75, 77, 78) (Figure 1B).

The BP180 ectodomain can be shed within the NC16A domain *via* a disintegrin and metalloprotease (ADAM) 9, 10, and 17 (79–81), resulting in a 120-kDa protein fragment named linear IgA bullous dermatosis autoantigen 1 (LAD-1) and a 97-kDa protein (97-kDa linear IgA bullous dermatosis autoantigen (LABD97)), both originally described as autoantigens in LAD (Figures 1A,B) (82–84). By this cleavage within the NC16A domain, different neoepitopes are induced, which might be important for the detection of autoantibodies in LAD, since it was shown that autoantibodies are preferentially bound to LAD-1 than to full-length BP180 (55, 80). By immunoblot analysis, sera from LAD patients showed IgA reactivity against keratinocyte-derived LAD-1 in 48%-50% and IgG reactivity in 24% of cases, whereas sera from BP patients had IgG against LAD-1 in 74% and IgA in 30% of cases (Figures 1A,B) (55, 85, 86). In addition, also patients with pemphigoid gestationis and MMP revealed serum reactivity with these two proteins (26, 54, 56, 72). In MMP, 16%–41% and 14% of analyzed sera contained IgG against LAD-1 and LABD97, respectively, and 37%–59% and 15% of sera exhibited IgA reactivity against LAD-1 and LABD97, respectively (Figure 1B) (75, 87).

Besides the soluble keratinocyte-derived proteins of the BP180 ectodomain, several recombinant fragments of the BP180 ectodomain have been generated by different investigators (Figures 1A,B) (53, 56, 88, 89). In total, 7.8%–47% of BP sera exhibit autoantibodies (IgG and IgA) that are directed against different recombinant proteins of the BP180 ectodomain, excluding the NC16A domain (46, 88–90). IgE against these epitopes has also been detected (91). Recently, in patients without frank blistering but erythematosus lesions, exclusive IgM autoantibodies against BP180 have been reported (92, 93). Boch et al. described three patients who had IgM reactivity against the DEJ by direct IF microscopy and serum IgM against the BP180 ectodomain BMZ. In one patient, additional IgM reactivity against the NC16A domain was present (93). Interestingly, in the last years, it has been reported, that patients with diabetes mellitus treated with dipeptidyl peptidase-4 inhibitors (DPP-4i or gliptins) develop a BP with autoantibodies against epitopes on the C-terminus of BP180 (94–96). In Japanese patients with DPP-4i-associated BP, a noninflammatory phenotype has been described and an association with HLA-DQB1_03:01 was suggested (94, 97).

In MMP, autoantibodies against C-terminal epitopes on BP180 outside NC16A were detected in 16%–53% (IgG) and 4%–37% (IgA) of patients, respectively (26, 46, 52, 74, 75, 98, 99). In pemphigoid gestationis, individual patients exhibit IgG and IgA extracellular epitopes outside NC16A (46, 72). In linear IgA disease, autoantibodies against C-terminal epitopes on BP180 outside NC16A were detected in 50%–100% (46, 55, 77, 80, 100, 101).

Antigenic regions targeted by pemphigoid patients' autoantibodies can also be found on the intracellular portion of BP180 (88, 102). Overall, 28%–82% of BP sera (IgG reactivity) can recognize at least one epitope of the intracellular domain (56, 88, 102, 103), and also IgE reactivity against this site was described (102). For MMP, serum IgG and IgA against the intracellular part of BP180 were observed in about one-fifth of patients (75). In a small cohort of pemphigoid gestationis patients, about half of the patients showed IgG reactivity with a least one recombinant protein of the intracellular domain of BP180 (72) and in linear IgA disease, IgG and IgA reactivity was detected in 8% (for both isotypes) of analyzed sera (85).

It is currently assumed that intracellular epitopes are only recognized by autoantibodies after there is already reactivity to extracellular domains. Internalization *via* macropinocytosis of a BP180 NC16A IgG immune complex has recently been described (104). Together with the observation that anti-BP230 IgG alone is pathogenic *in vivo* (49, 105), it may be speculated that even autoantibodies against the intracellular part of BP180 per se are pathogenic. Of note, the autoantibody profile in individual PD patients is dynamic and may change over time, a phenomenon called epitope spreading. So far, little is known about the pathogenic relevance of autoantibodies against the intracellular and non-NC16A epitopes on the BP180

ectodomain. Alike, clinical phenotypes within a distinct PD depending on the targeted epitopes have not yet been sufficiently explored. Setterfield et al., however, have found concomitant serum IgG and IgA reactivity against the DEJ to be associated with a more severe phenotype in MMP (106).

Fcγ-receptor-independent effects of BP180-specific antibodies

Tissue destruction in BP is mediated by a variety of Fcγ-receptor (FcγR)-mediated effects. The antigen-autoantibody complexes lead to the activation of complement, followed by the infiltration of inflammatory cells such as mast cells, macrophages, and neutrophils to the upper dermis (107–110). These infiltrating cells release reactive oxygen species and specific proteases which finally degrade the DEJ, clinically leading to blister formation (109, 111). Most data about the essential role of complement activation in BP stems from observations in the neonatal mouse model of this disease (107, 108, 112). These data were supported by the high impact of complement activation in the mouse models of EBA and MMP (113–115).

Of note, antibodies against BP180 also promote blister formation in a FcγR- and complement-independent manner (116). The binding of anti-BP180 antibodies led to reduced adhesion of keratinocytes to the substrate/basement membrane using cultures of human keratinocytes and skin by macropinocytosis/internalization (104, 117–119). In detail, it was shown that BP-IgG induced a 45%–50% depletion of BP180 from normal human keratinocytes and DJM-1 cells (malignant trichilemmal cyst cells) after 2 h of incubation and about 75% in DJM-1 cells and 85% in normal human keratinocytes after 6 h. BP patient's sera had the same depletion ability. A depletion of 80%–95% of BP180 from cells compared to normal control sera could be observed in seven of nine sera, the remaining two had a depletion activity of about 50%. The depletion of BP180 led to a loss of adhesion strength of about 40% in normal human keratinocytes and DJM-1 cells (119).

The internalization of BP180 upon binding of anti-BP180 IgE to cultured keratinocytes was also observed by confocal microscopy. After a time period of 15 min, the anti-BP180 IgE staining was detectable on the cell surface and 45 min later, the staining had moved from the periphery to the center, i.e., the cytoplasm of the cultured keratinocytes (117). The authors suggested that the possible blistering-inducing potential of anti-BP180 IgE is derived from its more potent cytokine-inducing potential compared to anti-BP180 IgG. This view was based on the observation that the relation of serum anti-BP180 IgE to total serum IgE was 30%–40% considerably higher compared to less than 1% of anti-BP180 IgG in relation to total serum IgG (117). These data are in line with the results Hiroyasu et al. observed for the internalization of BP IgG (118). They used BP-IgG to treat subconfluent/confluent normal human keratinocyte cultures that expressed green fluorescent protein-BP180 (GFP-BP180) and observed the beginning of an internalization, described as spot-like structures, from the surface into the cytoplasm after 20/105 min. By additionally labeling the BP-IgG with Fluor 647, it turned out that BP180 is internalized together with its antibody no matter if the antibodies are bound to the intracellular domain or extracellular domain (104). Internalization occurred *via* a non-inflammatory pathway and is FcγR-independent. This was tested using BP-IgG Fab fragments for cell stimulation, where an internalization of GFP-BP180 still occurred. By additionally treating the cells with EIPA, a Na+/H+ exchanger inhibitor, and cytochalasin D, an actin polymerization inhibitor, the internalization of GFP-BP180 failed. This indicates that internalization occurs *via* a macropinocytotic pathway (118). This process was suggested to be mediated by the protein kinase C pathway *via* phosphorylation of BP180 followed by the internalization and degradation of immune complexes, leading to a deficiency of BP180 in cell membranes and, consequently, loss of adhesion strength in keratinocytes (104). Furthermore, Kamaguchi and co-workers showed for MMP that the interaction between collagen type IV and BP180 is disrupted by MMP IgG directed against the BP180 C-terminus in keratinocytes of the oral mucosa, resulting in a reduction of the adhesion to the BMZ. However, the mechanism of lesion formation in the mucosa is still not fully understood. It has been shown that the expression level of BP180 is higher in the oral mucosa compared to the skin (120). This in turn is thought to compensate for the depletion of BP180 induced by anti-BP180 NC16A IgG.

The importance of the release of inflammatory cytokines like IL-6 and IL-8 in the pathogenesis of BP is supported by the finding of increased levels of both cytokines in the blister fluid of BP patients compared to suction blister controls (111, 121). Furthermore, a correlation between IL-6 and IL-8 serum levels and BP disease activity was shown (122). The release of IL-6 and IL-8 upon stimulation of cultured human keratinocytes with BP patient IgG indicates that FcγR-independent events might be relevant for disease development. Schmidt et al. observed a dose- and time-dependent release of the two cytokines. The elevated IL-6 and IL-8 levels in this approach were shown to be mediated by anti-BP180 IgG and were also observed at the mRNA level (123). Subsequently, the IL-8 release was shown to be significantly inhibited by dapsone but not doxycycline (124), two drugs regularly used in the treatment of BP (125–127). These data were corroborated by the observation that the treatment of cultured keratinocytes with IgE against BP180 also leads to the release of IL-6 and IL-8 (117). These cytokines may directly attract neutrophils without complement activation, leading to blister formation (128).

In addition, the injection of anti-BP180 IgG into humanized neonatal C3-deficient mice resulted in skin detachment by depletion and degradation of BP180 without complement deposits (107). This was somehow unexpected since, in C5-deficient mice, no dermal–epidermal separation occurred (107). Supporting the findings in C3-deficient animals, injection of F(ab′)2 fragments resulted in skin fragility without complement activation and neither skin detachment nor complement deposits in C5-deficient mice (107). Collectively, these data support the hypothesis that both FcγR- and complement-independent processes are involved in the pathophysiology of BP.

Anti-BP180 reactivity in neurological disease with and without concomitant bullous pemphigoid

BP is often associated with neurological diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis (129–133). Proteins associated with neurological diseases, like amyloidogenic proteins, were detected in human skin (134). In Alzheimer's disease patients, skin physiology was altered and their risk of developing BP was 2.6 times higher than in individuals without the neurological disease (134–136). On the other hand, patients with nonmelanoma skin cancer had a lower risk of developing Alzheimer's disease (137). One explanation for both observations is that the main antigen for BP, BP180, is expressed not only in the skin but also in the central nervous system (CNS) (138, 139). It has further been suggested that due to the neurodegenerative processes in neurological diseases the immune privilege of the CNS may be lost, i.e., that the blood–brain barrier no longer protects against harmful substances, pathogens, and toxins (140). This loss can then lead to the accessibility of the antigens in the brain and a subsequent autoimmune response against BP180 and BP230 (129, 133). More precisely, Seppänen et al. localized BP180 in the soma and proximal axons, but not in glial cells (139). BP180 was mostly found in the pyramidal cells of hippocampal regions, the ganglionic layer of the cerebral cortex, the hypoglossal nucleus (nucleus XII), oculomotor nucleus (nucleus III), nucleus basalis of Meynert, supraoptic nucleus, and subthalamic nuclei (139, 141). The observation that the neurological disease precedes BP by an average of 5.5 years supports the hypothesis that, indeed, the neurological disease triggers the autoimmune blistering disorder (130, 142). In fact, the relationship between BP and stroke, dementia, Parkinson's disease, multiple sclerosis, and epilepsy has been well established in various populations (129–131, 133, 142–153). Since both neurological diseases such as stroke or dementia increase in the elderly and an age-related impairment of the blood-brain barrier has been described, this may also explain the striking occurrence of BP above the age of 70 years (140, 154–158).

To provide further experimental evidence for the generation of anti-BP180 IgG in patients with neurological diseases, Kokkonen and co-workers tested 115 Alzheimer's disease patients and 40 neurologically healthy controls for anti-BP180 IgG. 21% of the patients with Alzheimer's disease but only 7.5% of the controls showed IgG against BP180 NC16A by ELISA (159). All positive samples were further analyzed by immunoblotting against recombinant full-length BP180. In total, 18% of Alzheimer's disease patients revealed IgG against NC16A and full-length BP180 in contrast to 3% in controls. Interestingly, Alzheimer's disease patients with higher serum anti-BP180 IgG levels had more severe dementia (159). Comparable results were obtained by Wang et al., who found NC16A ELISA reactivity in 48% of 23 Alzheimer's disease patients (160) (Figure 1B). In the sex- and age-matched healthy control group, only 8% of the tested 50 sera reacted with NC16A. Validation by immunoblot analyses with recombinant full-length BP180 or NC16A showed that nine of the 23 ELISA-positive Alzheimer's disease patients (39%) and one of four (25%) controls reacted with the proteins (Figure 1B). In addition, 11 of the 23 ELISA-positive Alzheimer's disease sera reacted with a 180-kDa protein from the human brain extract, but none of the controls did. The brain extract was obtained from the human hippocampus, which is a known BP180 expression locus (160). In contrast, Recke et al. found no significant increase in autoreactivity of autoantibodies against BP180 in patients with multiple sclerosis and Parkinson's disease (161). The latter study is compatible with further work by Tuusa et al. that detected serum anti-BP180 NC16A IgG in only eight of 143 (5.6%) patients with multiple sclerosis and two of 140 (1.4%) neurologically healthy controls by ELISA, while none of these sera reacted with a glutathione-*S*-transferase NC16A fusion protein by immunoblotting (162). When different fusion proteins (FP1-FP13) covering BP180 (Figures 1A,B) were employed, BP sera preferentially reacted with the extracellular fragment FP5, corresponding to the NC16A domain, while there was no reactivity in sera of patients with multiple sclerosis, Alzheimer's disease and healthy controls. Of note, patients with multiple sclerosis primarily reacted with the intracellular fragment FP4 (in 100%) and the extracellular fragments FP6 outside the NC16A domain (in 91%). Alzheimer's disease sera predominantly recognized the intracellular fragment FP3 (in 91%) and the extracellular fragments FP8 outside the NC16A domain (in 91%; Figure 1) (162). Collectively, these data indicate that autoantibodies against different epitopes in BP180 are associated with different pathologies and as such, it can be expected that the pathogenic effect of anti-BP180 autoantibodies not only depends on their complement-activating potential, their glycosylation status, and isotype but also on the targeted epitope.

Outlook and conclusions

It has been well documented that different epitopes on BP180 are targeted in different PD as well as in patients with neurodegenerative disorders. The main pathogenic effect of anti-BP180 autoantibodies appears to be mediated by FcγR. Correspondingly, the pathogenicity of anti-BP180 autoantibodies was shown to depend on the autoantibody isotype, IgG subclass, and glycosylation status, leading to a varying extent of complement activation at the DEJ and attraction of inflammatory cells to the upper dermis. A more puzzling observation was that in different PDs, different regions of BP180 are predominantly targeted. In addition, most *in vitro* and all animal models have shown pathogenicity only against the NC16A domain and its murine homolog, not against intracellular or extracellular epitopes outside of this domain. In line, only serum levels of antibodies against the NC16A domain were shown to correlate with the disease activity of patients. As such, different epitopes on BP180 may convey different pathologies. This hypothesis, however, cannot be well explained by FcγR-dependent mechanisms alone.

Increasing evidence is provided for FcγR-independent mechanisms of anti-BP180 IgG in different *in vitro* and *in vivo* models. These mechanisms would allow us to more closely investigate the role of different epitopes in the pathophysiology of PD. The targeted epitope(s) may vary among the different disease stages and may also play a role in the transition of pemphigoid predisease, i.e., when autoantibodies against the DEJ are present but no skin or mucosal lesions have yet evolved. The in-depth characterization of epitope-dependent disease mechanisms in PD will not only allow a better understanding of autoantibody-triggered inflammation but may also provide novel therapeutic approaches that are urgently needed.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The relevance of complement in pemphigoid diseases: A critical appraisal

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Pemphigoid diseases are autoimmune chronic inflammatory skin diseases, which are characterized by blistering of the skin and/or mucous membranes, and circulating and tissue-bound autoantibodies. The well-established pathomechanisms comprise autoantibodies targeting various structural proteins located at the dermal-epidermal junction, leading to complement factor binding and activation. Several effector cells are thus attracted and activated, which in turn inflict characteristic tissue damage and subepidermal blistering. Moreover, the detection of linear complement deposits in the skin is a diagnostic hallmark of all pemphigoid diseases. However, recent studies showed that blistering might also occur independently of complement. This review reassesses the importance of complement in pemphigoid diseases based on current research by contrasting and contextualizing data from *in vitro*, murine and human studies.

KEYWORDS

complement, relevance, pemphigoid, bullous pemphigoid, BP, EBA, MMP, pathophysiology

1 Introduction

The complement system is a complex network of more than 50 proteins, which hold crucial roles in host defense against invading microorganisms as well as in tissue homeostasis, thus representing a fundamental component of the innate immune system. There are three distinct pathways of complement activation: the classical (CP), lectin (LP), and alternative pathway (AP) (Figure 1). Under physiological circumstances, nonself recognition in an immunoglobulin (Ig)-dependent or -independent manner triggers various proteolytic cascades that result in the activation of complement component C3. All three pathways generate a C3 convertase which elicits the immediate (C3a) and downstream (C5a) release of potent proinflammatory peptides (i. e., C5a, C3a), the opsonization of susceptible pathogens (*via* C3b and C4b), and ultimately, the lysis of microorganisms by the assembly of the membrane attack complex (MAC; composed of C5b6789) (2, 3), thereby contributing to their efficient elimination. Several soluble and membrane-bound factors regulate complement activation on different levels to protect healthy tissue from undesired damage. C1-esterase inhibitor (C1-INH), C4b-binding protein (C4BP), carboxypeptidase N (CPN1), factor H (FH), factor I (FI), protein S, and clusterin are soluble regulators of complement activation, whereas complement receptor of the immunoglobulin superfamily (CRIg), complement receptor 1 (CR1/CD35), decay-accelerating factor (DAF/CD55), membrane-cofactor protein (MCP/CD46), and protectin (CD59) mediate complement activation and regulation on the cell membrane (4).

Deficiencies in complement components or impaired complement activation pathways hinder efficient host defense responses, resulting in an increased susceptibility to infections: properdin deficiency and defects in the MAC formation are associated with an increased risk of meningococcal infections, whereas C3 deficiency is linked to recurrent infections with Streptococcus pneumoniae (5–7). In contrast, C4 deficiency is associated with systemic lupus erythematosus (SLE), but also with repeated severe herpes infections (8, 9). In children, deficiencies in mannose-binding lectin (MBL) result in recurrent bacterial respiratory infections (10). Furthermore, a defective C3-dependent opsonization increases the risk of infections with Streptococcus pneumoniae and Haemophilus influenzae, while a lack of CR3 enhances the likelihood of recurrent skin infections (2, 11).

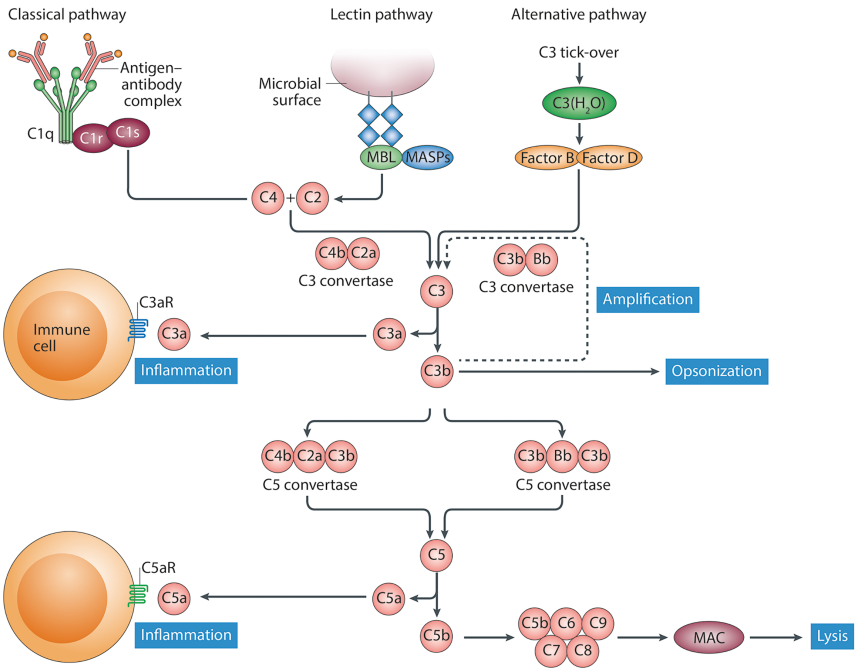

FIGURE 1
Schematic representation of the complement system. The classical pathway of complement activation is activated following to binding of the recognition molecule C1q to ligands such as immune complexes. The lectin pathway is activated following to binding of recognition molecules, such as mannose-binding lectin (MBL), collectins or ficolins, to their ligands, which include carbohydrate structures. Although the alternative pathway is initiated spontaneously, properdin (not shown) might also serve as a recognition molecule for directing activation of this pathway. Following activation *via* the initiating molecules a cascade of proteolytic activation steps leads to the formation of C3 convertases that cleave C3 into the anaphylatoxin C3a and the opsonin C3b. Next, C5 convertases generate the potent pro-inflammatory anaphylatoxins C5a and C5b, the latter of which, together with C6–C9, forms the membrane attack complex (MAC). This figure was obtained with permission from Trouw, L.A., Pickering, M.C., & Blom, A.M. Nat Rev Rheumatol. 9, 538–547 (2017), (1).

Impaired complement system activation or regulation has been observed in many dermatological diseases, such as hereditary (12) and acquired angioedema (13), cutaneous small vessel (14, 15) and hypocomplementemic urticarial vasculitis (16), SLE (17, 18), psoriasis (19, 20), acne vulgaris (21, 22) and hidradenitis suppurativa (23). Moreover, the complement system is also involved in the pathogenesis of autoimmune blistering dermatoses (AIBD), in particular the pemphigoid group, including bullous pemphigoid (BP), epidermolysis bullosa acquisita (EBA), mucous membrane pemphigoid (MMP), pemphigoid gestationis (PG), and, to a lesser degree, the pemphigus group (24–28). C3 deposits along the dermal-epidermal junction (DEJ) are observed in approximately 90% of patients with pemphigoid diseases (PDs) (29–32). Interestingly, complement activation seems to be mainly restricted to the skin (33). In fact, the detection of C3 by direct immunofluorescence (DIF) microscopy of perilesional skin is a highly valuable diagnostic marker of all PDs. In BP, the activation of complement at the DEJ, as a result of pathogenic autoantibodies binding to collagen type XVII (BP180), initiates and maintains inflammatory processes resulting in characteristic subepidermal blistering (34). Consequently, complement components of both the CP and AP have been detected at the DEJ and blister fluid. Similarly, complement-induced separation at the DEJ has also been demonstrated in EBA, MMP and PG (35, 36). In addition, complement-fixating antibodies along the DEJ represent a characteristic feature of PG, and DIF microscopy often demonstrates rather linear C3 than IgG deposition along the basement membrane (31, 37). Regarding pemphigus, DIF microscopy shows intraepidermal deposition of IgG and/or C3, and the pathogenic IgG autoantibodies belong to the IgG1 subclass, which is a notable complement activator, but also to IgG4 (38).

By contrasting and contextualizing seemingly contradictory data from *in vitro*-, murine- and human studies we will corroborate the central role of complement, particularly in the effector phase of PDs, with a special focus on BP, EBA and MMP as prototypic PDs.

2 Complement-dependent pathogenic pathways in PDs

2.1 Experimental lines of evidence

2.1.1 BP

BP is the most common autoimmune bullous disease that mainly affects the elderly, usually in the 7th decade of life (24). It is caused by autoantibodies targeting two hemidesmosomal proteins, BP180 (collagen XVII, BPAG2) and BP230 (BPAG1), resulting in characteristic subepidermal blister formation (39). Clinical hallmarks range from generalized, pruritic, large skin blisters to eczematous and urticarial lesions (24).

Chorzelski and Cormane were the first to demonstrate that complement binds *in vivo* to the basement membrane of BP patients' skin (40), followed by the substantial work of Jordon et al., which significantly contributed to our current understanding of the role of complement activation in the pathogenesis of BP. By using DIF microscopy, Jordon and co-workers showed that BP autoantibodies are able to fix not only C3 (41–43), C1q and C4 (44), but also factor B (FB) and properdin (45, 46), thus underlining the involvement of both classical and alternative complement activation pathways. Remarkably, C3 deposition was also seen in the absence of skin-bound BP autoantibodies (47), indicating a high sensitivity of the C3 staining. Furthermore, the same group demonstrated that BP blister fluids exhibited both eosinophil and neutrophil chemotactic activity, with the latter being inhibited by the use of an anti-C5 antiserum, therefore suggesting for the first time the role of complement, namely C5a, in the effector phase of BP (48). Several studies followed that corroborated these findings, to the extent that the detection of complement deposits in the skin is now considered a highly valuable and important diagnostic hallmark of BP (49–51).

Later on, Liu et al. provided experimental evidence by transferring polyclonal rabbit antibodies against murine NC14A, a homolog of the human NC16A domain of BP180, into neonatal BALB/c mice, showing that subepidermal blistering did not occur if either (i) serum complement was depleted by cobra venom factor, (ii) mice were C5-deficient, or (iii) F(ab′)2-fragments derived from the anti-murine BP180 antibody were used (52, 53). Similar results were observed in the antibody transfer model of BP in neonatal hamsters (54). These findings suggest that complement activation is indispensable for blister formation in experimental antibody transfer models of BP. Correspondingly, Nelson et al. demonstrated, in the same model, that C4-deficient mice and wild-type (WT) mice pretreated with anti-C1q-antibody were protected from disease development, thereby underscoring the importance of the classical pathway activation in the pathogenesis of PDs (55). Interestingly, the same study showed that FB-deficient mice developed delayed and less intense subepidermal blisters, suggesting a minor role of complement activation *via* the alternative pathway in experimental BP.

To reduce the shortcomings of the previously mentioned antibody transfer mouse models, both Nishie et al. and Liu et al. established humanized mouse models of BP, introducing either human BP180 (56) or replacing murine BP180NC14A with the homologous human BP180NC16A epitope cluster region (57). The CP of the complement system appeared to be pivotal also in these humanized mouse models: F(ab′)2-fragments derived from pathogenic antibodies did not induce blisters, complement C3 depletion with cobra venom factor protected from disease development, and transfer of humanized IgG1 against BP180NC16A that were previously mutated at the C1q binding site resulting in reduced C1q binding, induced less blisters compared to the unmutated humanized antigen-specific IgG1 (57, 58).

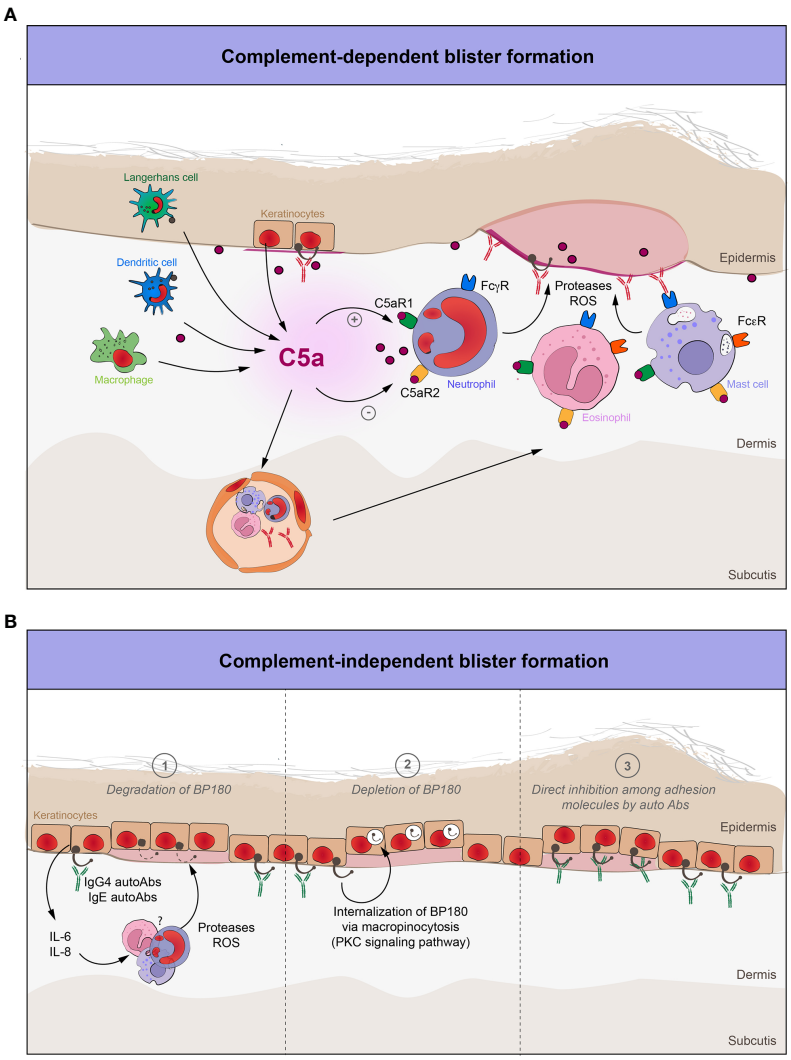

FIGURE 2
The role of complement in blister formation in pemphigoid diseases. **(A)** Complement-dependent pathways in pemphigoid diseases. Complement-fixing autoantibodies target various structural antigens of the basement membrane. The complement system is activated, thus leading to the recruitment and activation of various effector cells. C5a, which is the most potent proinflammatory peptide of the complement cascade, will interact with these cells through their C5a receptors (C5aR1, C5aR2), enabling them to release several proteases and ROS, thereby inflicting tissue damage and characteristic subepidermal blistering. **(B)** Complement-independent pathways in pemphigoid diseases. Non-complement fixing autoantibodies (IgG4) and IgE against BP180 bind to the dermal-epidermal junction and (1) degrade BP180 by increasing the expression of IL-6 and IL-8, which in turn will attract and activate neutrophils, as a major source of various proteases and ROS; (2) decrease the BP180 hemidesmosomal content by internalization of the IgG-BP180 immune complex *via* macropinocytosis; (3) directly inhibit the adhesion molecules of the dermal-epidermal junction.

Another crucial pathway underlining the importance of complement activation in BP is the interaction between C5 and its receptors, C5a receptor 1 (C5aR1) and C5a receptor 2 (C5aR2) (Figure 2A). By the injection of anti-BP180 IgG in adult mice, Karsten et al. recently demonstrated that C5 knockout mice had an up to 50% reduction in disease activity when compared to corresponding WT-mice (59). Unlike previous findings, this intriguing result could be explained by the use of exclusively murine models, as well as by the opposing roles of the two receptors: C5aR1-deficient mice were protected from disease development, whereas C5aR2-deficient were rather prone to develop inflammatory skin lesions (59). In addition, the pharmacological inhibition of C5aR1 by the PMX53 peptide mitigated disease development, but only when applied in a preventive setting (59).

These findings emphasize the role of complement activation in the early effector phase of the disease when neutrophils, eosinophils and mast cells are attracted by C5a to the DEJ to inflict characteristic tissue damage and subsequent blister

formation (60–63). Remarkably, the depletion of neutrophils protected against BP in WT-mice, whereas the use of C5a and interleukin (IL)-8 in C5-deficient mice made them susceptible for disease development (64).

In fact, complement-dependent neutrophil infiltration in BP is contingent on prior mast cell degranulation (65), which is also induced by the activation of complement: upon the interaction between C5a and C5aR1, mast cells degranulate and release various pro-inflammatory cytokines that in turn attract neutrophils and eosinophils, as well as proteases, including the mouse mast cell protease-4 (mMCP-4) (63). The latter activates the key neutrophil protease, matrix metalloproteinase-9, both cleaving BP180 and thus, leading to blister formation (66, 67). Given this, mice deficient in mast cells, mMCP-4, or C5aR1 failed to develop BP (63, 65, 66).

In addition to neutrophils and mast cells, eosinophils appear to be involved in, at least, IgE-mediated pathology in BP, which may occur independently of neutrophils and relies on prior mast cell degranulation (61, 68). In this regard, eosinophils from most BP patients do highly express the high-affinity IgE receptor (FcεRI) (69), and, as previously shown in a humanized FcεRI mouse model of BP, human anti-BP180 IgE autoantibodies recruit and activate eosinophils *via* FcεRI, resulting in eosinophil degranulation and subsequent blister formation (61). Of note, anaphylatoxins C5a and C3a can however directly induce degranulation of eosinophils *via* selective, receptor-mediated processes (70, 71), potentially connecting complement and eosinophils in human disease.

Taken together, all these data suggest that infiltration and activation of specific effector cells responsible for the characteristic BP skin pathology depend, at least indirectly, on the activated complement cascade.

2.1.2 EBA

EBA is a rare autoimmune blistering disease, which is characterized by autoantibodies against type VII collagen (COL7) (72). Clinically, it can present with various phenotypes, with the classical/mechano-bullous and the non-classical/non-mechano-bullous variant being the most common forms.

Our current understanding of the role of complement in the pathogenesis of EBA is mainly relying on the use of experimental murine models which, by design, mimic human disease clinically, immunologically and histologically. EBA can be induced in mice by either transfer of human anti-COL7 IgG or rabbit anti-mouse-COL7 IgG, or by immunization of mice with an immunodominant fragment of the murine COL7 antigen (73).

Sitaru et al. showed in the active EBA model, that diseased mice presented with significantly higher C3 skin deposition compared to non-diseased animals (36). Furthermore, the complement-fixing IgG2a and IgG2b autoantibody subclasses were considerably elevated in the diseased group (36), whereas F(ab′)2-fragments of pathogenic rabbit anti-COL7 IgG did not induce blistering in the passive EBA mouse model (74). Similar results were obtained for F(ab′)2-fragments generated from affinity-purified anti-COL7 antibodies from EBA patients' sera (75), thus underlining a key role of the IgG-fragment crystallizable region (Fc), likely mediating inflammation *via* C1q interaction and CP complement activation in these EBA models.

Experimental evidence also illustrates the involvement of the AP in passive EBA. Mihai et al. demonstrated that FB-deficient mice as well as WT-mice treated with an anti-FB antibody developed a delayed and significantly less severe blistering phenotype when compared to controls (76, 77). In contrast, C1q-, C6- and MBL-deficient mice did develop the disease, implying that the MAC formation and the LP may be dispensable for blister formation. The rather unexpected finding that C1q-deficient mice were not fully protected from disease could be explained by either the presence of non-canonical complement activation or by different Fc N-glycosylation autoantibody patterns associated with proinflammatory responses and high neutrophil activating potential and ROS release (78–80). Moreover, the interaction between T cells and antigen-presenting cells was shown to unexpectedly induce the secretion of C3 or C5, as well as the formation of their corresponding convertases, thereby generating activated complement fragments and yielding characteristic effector responses without depending on CP or AP (i.e., non-canonical complement activation) (81). Correspondingly, macrophage-generated C4 restored the altered humoral response against tumor antigens in C4-deficient mice (82). Interestingly, it was previously shown that C5a can also be generated in the absence of C3-dependent convertases (83). In addition, human keratinocytes are able to express and produce various complement proteins, including C3 (84).

Similarly to murine BP models, the C5-C5aR1 axis is engaged in downstream tissue damage and subsequent blister formation in EBA: C5-deficient mice were partially or fully protected from developing EBA in the antibody transfer model (74, 85), while the application of an anti-murine-C5 monoclonal antibody significantly reduced the blistering phenotype in this system (77). Moreover, C5aR1-deficient mice did not develop disease, and the pharmacological blockade of this receptor also led to a notable improvement of the blistering phenotype (77, 86). In contrast to the protective role of C5aR2 in experimental BP, C5aR2-deficient mice developed an attenuated disease phenotype in the antibody transfer mouse model of EBA, with C5aR2 being essential for neutrophil activation and recruitment by regulating Fcγ receptor (FcγR) expression levels in this model (59, 87). In line with these findings, Sezin et al. demonstrated that dual inhibition of both C5 and leukotriene B4 (LTB_4) with coversin (i.e., nomacopan)

suppressed disease in passive EBA much more efficiently than the inhibitor of LTB_4 alone (88).

2.1.3 MMP

MMP is a group of pemphigoid diseases with predominant mucous membrane involvement and a significant tendency towards scarring (89). Autoantibodies target various antigens, mainly BP180 and laminin 332. The mechanisms of blistering in MMP were also studied in different animal models (73). In contrast to BP and EBA, no immunization-induced mouse models have been yet described, but are in the pipeline (89). Since initial antibody transfer mouse models developed in the 90s were not completely replicating human disease, Heppe et al. recently introduced a novel model by transferring rabbit IgG against the middle and the C-terminal part of the murine laminin α3 chain, that reproduced disease clinically and immunopathologically (90–92).

Current evidence on the role of complement in experimental MMP models is scarce and contradictory at this point of time. Lazarova et al. showed that C5-deficient mice were not protected from disease development (90). Moreover, F(ab′)2-fragments from pathogenic rabbit anti-laminin 332 IgG elicited blistering in mice (91). Conversely, a recent study by Heppe et al., demonstrated that C5aR1- and FcγR-deficient mice developed little or no disease (92). The use of different mouse models for MMP could explain these discrepancies: the first study group induced MMP by passively transferring rabbit anti-laminin 332 IgG into neonatal mice, whereas the latter injected adult mice with rabbit anti-laminin 332 IgG against 2 immunodominant regions of laminin 332. Moreover, the latter mouse model was shown to fully replicate human disease, and dapsone, which is the first-choice in the therapeutic armamentarium of MMP, has proven effective in this passive MMP mouse model, thus further underlining its utility for the study of MMP (92, 93). Nevertheless, more experimental studies are needed to clarify the impact of complement in the pathogenesis of MMP.

2.2 Clinical lines of evidence

The detection of linear C3 deposits at the DEJ by DIF microscopy studies is routinely used to diagnose PDs and considered as the "gold standard" (31, 32, 94, 95).

In a large cohort study, Romeijn et al. demonstrated that over 80% of patients with BP showed C3c deposition along the DEJ in their (peri)lesional cutaneous biopsies (30). Moreover, this finding also significantly correlated with both clinical and serological disease activity (96). Similar results were also obtained by Ständer et al., who have shown that BP patients with C3 deposition had higher levels of seropositivity and autoantibodies (97).

Other complement components and activation factors, including C1q, C3, C3c, C3d, C4, C4d, C5, C5b-9, FB, FH, and properdin were also detected in the skin and blister fluid of BP patients, thus pointing towards the involvement of both CP and AP in the pathogenesis of human BP *in vivo* (42, 98). Interestingly, the detection of the two complement split products C3d and C4d at the DEJ by immunoperoxidase staining in formalin-fixed paraffin-embedded tissue proved as an effective novel diagnostic option for BP (99–103).

The pathogenic role of autoantibodies against BP180 is intimately intertwined with the activation of complement. Analysis of the IgG subclass distribution revealed that BP patients present a predominance of IgG1 antibodies in the skin (38). In addition, serum levels of IgG1 autoantibodies against the NC16A domain correlated with disease severity in these patients (104). Since IgG1 strongly binds complement *via* the C1q binding site of the Fc region, this adds further evidence to the decisive role of the CP in BP. Correspondingly, Sitaru et al. demonstrated that F(ab')2 fragments of pathogenic antibodies against BP180-NC16A, which lack the Fc tail required for complement activation, did not induce dermal-epidermal separation in cryosections of human skin (105). Furthermore, Chiorean et al. recently demonstrated that the functional complement activation capacity of autoantibodies *ex vivo* in BP correlates with disease severity and autoantibody levels (96).

An IgA autoimmune response to BP180 and BP230 was also demonstrated in PDs, especially in MMP (106). It was shown to induce dermal epidermal separation *via* FcαRI-mediated neutrophil activation (107). However, there is contradictory data regarding IgA's potential to activate complement (108, 109). With regard to PDs, neither IgA1 nor IgA2 were able to induce complement deposition at the DEJ in cryosections of human skin. However, they still could amplify the complement activation pathways *via* AP activation, which is a major source of the proinflammatory C5a (110, 111). This might explain the stronger ability of both IgA1 and IgA2 to activate neutrophils, as well as to induce blistering without the CP activation.

3 Complement-independent pathogenic pathways in PDs

3.1 Experimental lines of evidence

First evidence pointing towards direct, complement-independent blistering in PDs was presented by Kitajima et al.: Here, binding of anti-BP180 IgG to the lateral-apical cell surface of basal cells led to internalization of the BP180-IgG immune complex in both cultured keratinocytes and biopsy specimens from BP patients (112, 113). Furthermore, Iwata et al. corroborated this finding by demonstrating that pathogenic autoantibodies from BP patients not only significantly depleted the hemidesmosomal BP180 content from cultured keratinocytes, but also reduced their adhesive strength to the basement

membrane, as determined in a standardized detachment assay using vibration (114). Interestingly, the α6 and β4 integrin levels of hemidesmosomes were not altered, thus underlining the putative specificity of the anti-BP180 IgG-mediated effect. The mechanism behind the internalization of the whole immune complex is attributed to the macropinocytosis pathway via calcium-dependent phosphorylation of the intracellular domain of BP180 by the protein kinase C (115, 116) (Figure 2B).

In line, in a neonatal BP180-humanized mouse model, Natsuga et al. were able to show that transfer of rabbit and human F(ab′)2-fragments against the immunodominant human NC16A domain of BP180 induced dermal-epidermal separation by mechanical stress, and also reduced the expression of BP180 in mouse skin as shown by immunoblotting (117). However, not all mice injected with F(ab′)2-fragments against NC16A showed skin detachment, pointing towards several synergistic pathways including Fc-mediated complement activation, potentially contributing to autoantibody-induced tissue pathology in BP (118).

Further *in vivo* data revealed that BP180 internalization and degradation *via* the ubiquitin/proteasome pathway is sufficient to induce blister formation in a C3-deficient BP180-humanized mouse model (119). Mice injected with a recombinant human IgG4 monoclonal antibody against the human NC16A domain of BP180 developed blisters, even though the IgG4 subclass does not fix complement, and also a proteasome inhibitor was added simultaneously. These results imply that BP180 internalization with subsequent hemidesmosomal weakening, followed by BP180 degradation *via* proteasome pathway may suffice for blister formation. Surprisingly, IgG4 antibodies from BP patients were found to induce dermal-epidermal separation in human cryosections, although with a much lower potential compared to IgG1 antibodies (120). Conversely, Zuo et al. suggested a protective role for IgG4 anti-NC16A antibodies in BP. He showed that the transfer of human IgG4 anti-NC16A to humanized BP180 mice inhibited human IgG1 and IgG3 induced complement activation with subsequent neutrophil infiltration, preventing both clinical and histological blistering in a dose-dependent manner (121). Correspondingly, IgG4 mitigates allergic diseases by inhibiting the activity of IgE (122). Based on these findings, it is tempting to speculate that IgG4 anti-NC16A might abrogate complement-dependent blister formation in BP. More studies are needed to fully clarify the role of IgG4 antibodies in PDs (38, 123).

Previously cited studies were nonetheless performed in neonatal mice, making data interpretation difficult at times. It was shown that neonatal mice do not entirely reproduce the clinical disease, since the majority of patients with PDs are adults and elderly, and lesions develop only with the application of friction in neonatal mice (24, 124). Moreover, there are known immunological differences between species, as well as in neutrophil function and skin physiology between neonatal and adult mice, and between murine and human skin, respectively (124, 125).

3.2 Clinical lines of evidence

Dainichi et al. reported two unusual BP cases with no complement deposition at the DEJ (126). Both patients had predominant IgG4 antibody involvement, which has restricted complement activating abilities. In line, approximately 20% of BP patients did not show C3 deposition at the DEJ in a large cohort of patients, and of these, the majority had prevalent IgG4 antibodies (30). Moreover, BP patients presenting with the non-blistering phenotype showed also less C3 deposition and previous studies hinted towards a IgG4 predominance in C3-negative cases (30, 127). In addition, BP-IgG4 antibodies induced subepidermal split formation *ex vivo* (120). Therefore, Dainichi et al. proposed a new entity in PDs, the so called "C3-negative BP" or "IgG4-dominant BP" (128). Given that the detection of C3 at the basement membrane is highly more sensitive than IgG for the diagnosis of BP by DIF microscopy, C3-negative DIF may require additional diagnostic methods or reconsidering the initial diagnosis (39). On the other hand, Boch et al. recently described 3 PD patients with weak or no C3 deposition, but with exclusive IgM reactivity at the cutaneous basement membrane (129). Interestingly, these patients also manifested with pruritic erythematous lesions without macroscopic or microscopic blistering. Since IgM is usually a strong inducer of complement activation, these findings further suggest that the activation of complement at the DEJ is indeed required for blister formation in PDs (108).

Both IgG1 and IgG4 against the NC16A domain of BP180 antibodies prevail in BP, and their serum levels significantly correlate with disease severity and poor prognosis (38, 104, 123). Interestingly, the IgG4 subclass was the first to be detected as well as the most prevalent in the prodromal, papular and urticarial BP variant (130). Since almost half of the IgG4 positive BP patients had also other IgG subclasses, and that IgG4 prevented IgG1 and IgG3 induced complement activation and final blister formation in mice, one might easily assume that IgG4 antibodies may play an essential role in disease induction, especially in the non-blistering phase, exerting also potential inhibitory effects as shown by Zuo et al. (121, 130). Conversely, the antibody switch to complement-fixing IgG subclasses will promote characteristic blister formation in BP (38, 107). Despite the fact that IgG4 antibodies do not fix complement, all patients with the non-blistering phenotype and IgG4 predominance still presented C3 and/or C5-9 deposition, suggesting early complement activation even with less IgG deposition at the DEJ and without blister formation (130). These findings underscore the utility of complement detection in the diagnosis of PDs, even in earlier disease stages. Recent data

suggests that IgG4 might unexpectedly activate complement through both the CP and LP (131).

The report of a C4-deficient BP patient further questioned the role of complement as a prerequisite for blister development (132). Interestingly, this patient still showed linear C3 deposition at the basement membrane, implying non-canonical complement activation pathways. In contrast, C4-deficient neonatal mice were protected from BP development (55, 132). However, the administration of IL-8, which is an important polymorphonuclear neutrophil chemoattractant, restored disease susceptibility of these C4-deficient mice (55, 64). In line with this, cultured NHEKs with BP-IgG or -IgE anti-NC16A antibodies directly induced the expression of IL-6 and IL-8, whereas control IgG or BP180-deficient keratinocytes did not (133, 134). These findings suggest that the binding of BP180 IgG may induce blister formation without complement activation, rather by attracting neutrophils, which release various proteases and ROS that inflict characteristic tissue damage. Accordingly, neutrophils were shown to be indispensable for dermal-epidermal separation in both *ex vivo* murine cryosections and *in vivo* passive mouse models of PDs (64, 105, 134–136). In this sense, complement may at least maintain the inflammatory response in PD patients in a positive feedback loop pattern (39, 59, 117). On the other hand, it was shown that proinflammatory cytokines (i.e., IL-6) may actually activate complement (137, 138). More studies are needed to evaluate these complement-dependent and -independent pathways concomitantly, since one does not rule out the other, and most PD patients do feature complement fixation at the DEJ.

Notwithstanding the critical role of neutrophils in mice, eosinophil-predominant inflammatory infiltrates in the papillary dermis and eosinophilic spongiosis represent the main histological hallmarks of BP in humans (39). Furthermore, the majority of BP patients show also elevated levels of eosinophils and IgE antibodies in their sera, skin and blister fluid, respectively (139, 140). In addition, about 70% have specific IgE autoantibodies against both the NC16A and non-NC16A domains of BP180 (139, 141). Eosinophils are considered the liaison between IgE autoantibodies and skin blistering in BP. Lin et al. showed in a humanized IgE receptor mouse model of BP that IgE-mediated blistering relies on eosinophils, since eosinophil-deficient mice were protected from IgE-induced blister formation (61). Moreover, the degree of eosinophil infiltration correlated with disease severity. It seems that eosinophils are most prevalent in early urticarial lesions and that they degranulate at the basement membrane before blister formation (142). Given the fact that IgE antibodies are indicative of type I hypersensitivity responses, together with eosinophils they may be the driver of the prodromal, non-blistering phase of BP, which could not be unraveled by the clearly established IgG deposition-induced complement activation and immune effector cells recruitment paradigm. Even though IgE antibodies do not activate complement, both *in vitro* and *in vivo* data demonstrated that specific anti-BP180 IgE antibodies were still able to induce dermal-epidermal separation (62, 134, 143). However, many BP patients have both specific IgG and IgE antibodies in their sera, with the latter predominantly found on eosinophils and mast cells, and very rarely, in a discontinuous pattern along the basement membrane (144–146). Since eosinophils express the high-affinity IgE receptor FcεRI, this might explain how anti-BP180 IgE antibodies can activate them (69). On the other hand, *in vitro* studies showed that full-length Ig as well as F(ab')2 fragments from BP-IgG and -IgE are able to decrease the hemidesmosomes number by cytokine secretion, thereby contributing to blister formation in a FcR-independent manner (134). Moreover, BP-IgE antibodies seem to target the same NC16A domain as pathogenic IgG antibodies (146). Interestingly, Messingham et al. demonstrated by using a cryosection model of BP that eosinophil localization along the DEJ is dependent on IgG and complement deposition rather than on IgE (147). No subepidermal split was however observed in this study. To induce blister formation eosinophils required IL-5 mediated activation, also prompted by IgG deposition and complement fixation (148). Considering that most BP patients have both IgG and IgE antibodies, and that each of them may not fully explain all pathologies observed in blister formation, a potential way to integrate all the above-mentioned findings is the following: to induce blistering, specific anti-BP180 IgE autoantibodies require the presence of eosinophils, which are recruited and activated mainly as a result of IgG deposition and subsequent complement activation. Given these results, future studies should sequentially connect and integrate these distinct pathways rather than dissect and presume the existence of solely one in the intricate pathophysiology of blister formation in PDs.

4 Complement – useful or dispensable?

In the 90s numerous studies showed that complement is indispensable for blister formation in *ex vivo* cryosections and *in vivo* experimental mouse models of PDs (52, 53, 64, 90, 95, 149). Furthermore, the detection of C3 along the basement membrane by DIF was used since then as an important diagnostic hallmark of PDs (24, 30, 32). However, Iwata et al. questioned the necessity of complement by demonstrating that BP autoantibodies were able to deplete BP180 content in cultured keratinocytes, which in turn led to an increase in cells' detachment implicating loss of function (114). Further research showed potential complement-independent pathways also in experimental mouse models (117, 119). However, these studies were performed in neonatal and complement knock-out mice, with the former exhibiting many limitations when compared to adult mice, and the latter being not physiologic, since patients with PDs with an additional complement component deficiency are rather an exception than the rule. Notwithstanding that Natsuga et al. demonstrated that BP

antigen specific human or rabbit derived F(ab')2-fragments were still able to induce disease, this reflects merely a theoretical potential of antibodies than a suggestion of the dispensability of complement, considering that full, complement-activating Ig molecules are prevalent in human sera and tissue, whereas F(ab')2 fragments are artificial (117, 150).

Even though these data may refute the necessity of complement in PDs, they rather provide new insights into PD pathogenesis, suggesting that complement-dependent and -independent mechanisms may indeed coexist in patients at the same time. Even if complement is not absolutely indispensable to induce blister formation, we propose that it is essential in the amplification of characteristic inflammation and tissue damage, thus contributing to disease severity. Therefore, eliminating complement activation in PDs may significantly ameliorate disease, as data from *in vitro* and *in vivo* studies suggest (151, 152).

In view of this, different complement-targeting therapies have been specifically developed for PDs. Among these are sutimlimab and nomacopan. In a phase I trial, sutimlimab, a humanized monoclonal IgG4 antibody directed against the C1s subunit of human complement component C1, was shown to partially or completely abrogate C3 deposition along the DEJ in BP patients (153). Nomacopan (formerly known as coversin) is a bifunctional inhibitor of both C5 and leukotriene B4 (88, 154). EBA mice treated with coversin were almost completely protected from disease development mainly due to the C5-inhibitory effect of this compound (88). More recently, *Sadik et al.* demonstrated in a phase IIa clinical trial that nomacopan successfully reduced the clinical disease severity in BP patients, without any serious adverse event (152). Furthermore, several other complement-targeting treatments have been developed that so far have not been evaluated in preclinical and clinical settings (151, 154).

Piecing these data together, we conclude from the evidence published so far that complement remains an important and, in most cases, an indispensable hallmark of both human and experimental models of PDs and that complement-targeting therapies are effective and safe treatment strategies for these patients.

Author contributions

All authors contributed to the writing and review of this manuscript. All authors approved the final version of this manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Role of the hedgehog signaling pathway in rheumatic diseases: An overview

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Hedgehog (Hh) signaling pathway is an evolutionarily conserved signal transduction pathway that plays an important regulatory role during embryonic development, cell proliferation, and differentiation of vertebrates, and it is often inhibited in adult tissues. Recent evidence has shown that Hh signaling also plays a key role in rheumatic diseases, as alterations in their number or function have been identified in rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, systemic sclerosis, and Sjogren's Syndrome. As a result, emerging studies have focused on the blockade of this pathogenic axis as a promising therapeutic target in several autoimmune disorders; nevertheless, a greater understanding of its contribution still requires further investigation. This review aims to elucidate the most recent studies and literature data on the pathogenetic role of Hh signaling in rheumatic diseases.

KEYWORDS

hedgehog signaling pathway, SMO, GLI, inflammation, target treatment

Introduction: An overview of the Hh signaling pathway

The Hedgehog (Hh) gene was first discovered in Drosophila in 1980 by Nusslein-Volhard, C. and Wieschaus, E., and was named Hedgehog because its mutation developed Drosophila larvae into a hedgehog-like morphology, which was also confirmed in vertebrates lately (1). The Hh signaling pathway in vertebrates mainly consists of four parts, including Hh ligands, membrane protein receptors, nuclear transcription factors, and downstream target genes. There are three Hh genes that have been detected: the Sonic Hedgehog (Shh), Desert Hedgehog (Dhh), and Indian Hedgehog (Ihh), which encode the corresponding Shh, Dhh, and Ihh proteins. These three proteins are collectively known as Hh ligands. Among them, Shh is the most widely distributed with a high positive expression rate, which is also the most studied Hh protein in the literature (2); Dhh is an essential regulator in the development of reproductive system (3); Ihh is mainly produced and secreted by prehypertrophic chondrocytes to

regulate the growth process of growth plate cartilage (4). Ptched (Ptch) and Smoothened (Smo) are the receptors for Hh protein, existing on the target cell membrane. Ptch is a 12-fold transmembrane protein encoded by the tumor suppressor gene Ptched, which negatively regulates Hh signaling, and vertebrate has two forms of Ptch1 and Ptch2 (5). Smo is one of the family members of seven transmembranes G protein-coupled receptors and is a bioreceptor required for activation of Hh signaling. It is a highly conserved amino acid sequence in transmembrane regions with the N terminus located extracellular, and the C terminus intracellular (5). The nuclear transcription factors are homologous zinc finger structural transcription factors encoded by Gli genes. In vertebrates, there are three members of the Gli gene family: Gli1, Gli2, and Gli3. Gli proteins regulate the expression of target genes by directly binding to their promoters (6). Hh target genes are involved in cell cycle regulation, proliferation, apoptosis, and angiogenesis (7). Furthermore, suppressed fusion protein (Sufu) is a critical intracellular negative regulator of Hh signaling. Sufu inhibits Gli protein by preventing its translocation into the nucleus (8). It plays a crucial role in Gli stabilization and processing.

Activation of Hh signaling includes canonical and non-canonical pathways, the canonical pathway is the most extensively studied and clearly understood currently, which is also known as the ligand-dependent pathway (9). In the absence of Hh ligands, Ptch suppresses Smo, leaving Sufu free to bind to Gli activator (Glia), thus repressing it and keeping Hh target genes switch off. When the Hh ligand binds to Ptch, resulting in loss of inhibition of Smo and then inhibits Sufu, thereby releasing the nuclear translocation of Glia proteins. Activated Glia is transported from the cytoplasm to the nucleus to promote the transcription of Hh target genes (10)(Figure 1).

Numerous studies have shown that aberrant expression of Hh signaling may take part in a wide range of diseases, and current studies have confirmed the involvement of this pathway in a variety of tumors. Similarly, scholars have also explored the engagement of the Hh signaling pathway in the pathogenesis (Table 1) and targeted therapy (Table 2) of rheumatic diseases.

The role of Hh signaling pathway in rheumatic diseases

Rheumatoid arthritis

Rheumatoid arthritis (RA) is mainly characterized by chronic inflammation of the synovium and progressive joint destruction. Fibroblast-like synoviocytes (FLSs) are the key effectors that mediate synovitis and joint destruction in RA (27). Abnormal activation and proliferation of FLSs release a large number of cytokines, chemokines, and matrix-degrading enzymes, leading to joint destruction and bone erosion. The

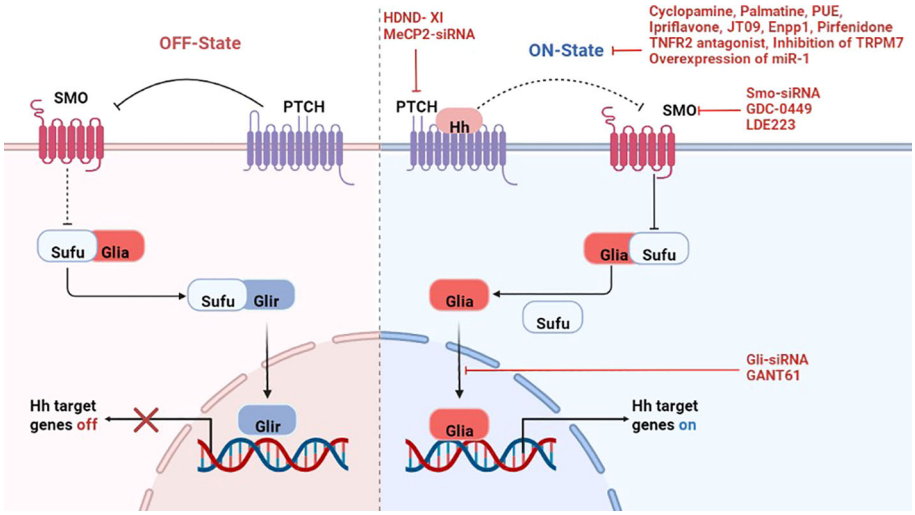

FIGURE 1
A simplified display of the canonical Hedgehog signaling pathway and treatment of rheumatic diseases by targeting the pathway. Left panel: In the absence of Hh ligands, Ptch suppresses any inactive Smo, leaving Sufu free to bind to Gli activator (Glia), thus repressing it and keeps Hh target genes switch off. Right panel: Binding of Hh ligands to Ptch leading to the dis-inhibition of Smo, which then inhibits Sufu, thereby releasing the nuclear translocation of Glia proteins. Activated Glia in the cytoplasm then translocate into the nucleus and promote transcription of Hh target genes. And there are inhibitors of different genes to suppress the activation of this pathway. Hh-Hedgehog; Ptch-Patched; Smo-Smoothened; Gli-glioma-associated oncogene; Glia-Gli activator; Glir-Gli repressor; Sufu-Suppressed fusion protein; HDND-XI-5-(4-Chlorophenethyl) imino-7-O-acetyl-4-chlorophenethylamine hesperetin; PUE-Phlomis umbrosa extract; Enpp1-Ectonucleotide pyrophosphatase/phosphodiesterase 1.

TABLE 1 The expression of Hedgehog signaling in rheumatic diseases.

Disease	Source	Result	Ref
RA	serum	Shh is increased compared with SLE, AS and health controls, it correlates with RF and anti-CCP Ab positively	(11)
	PBMCs	Shh and Gli1 mRNA are increased, and there is no difference in the expression of Ptch1 mRNA compared with health controls	(12)
	synovial tissue	Shh, Smo, and Gli1 protein are higher than health controls	(13)
	FLS	Shh, Ptch, Smo, and Gli are highly expressed	(11)
	chondrocyte	Shh, Ptch1, Smo, and Gli1 proteins are increased in AIA rats cartilage tissue, and the level of these proteins are proportional to the degree of chondrocyte damage	(14)
	endothelial cells	Smo expression is significantly elevated	(15)
OA	chondrocyte	Gli1, Ptch1 expression are increased	(16)
		Ihh is highly expressed	(16–23)
		Shh is expressed	(24)
SSc	skin	Shh, Ptch1, Ptch2, Gli1, Gli2 are increased	(25)
AS	serum	Ihh is higher than RA patients and healthy controls, and its expression is reduced after TNF-antagonist treatment	(26)

relationship between Hh signaling and RA has also been gradually increasing recently.

Compared with systemic lupus erythematosus (SLE), ankylosing spondylitis (AS) and healthy controls, the expression of Shh in serum of RA patients was significantly increased; the level of Shh in RA patients was correlated with rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (anti-CCP Ab) positively, but it had no correlation with erythrocyte sedimentation rate (ESR) (11). Similarly, Wang et al. detected the gene expression in PBMCs of 35 RA patients and found that the relative expression of Shh and Gli1 mRNA were higher than the control group, while there was no difference in the expression of Ptch1 mRNA (12). In addition, academics found the expression of Shh, Smo, and Gli1 protein in the synovial tissue of RA patients by immunohistochemistry were higher than health control (13). Shh is elevated in the plasma of RA patients with mild cognitive impairment (MCI), suggesting that it may be a biomarker for differentiating RA patients with MCI or without MCI (28). Furthermore, Shh signaling is activated both in the synovium of RA patients, in RA-FLS, and in rat RA synovial fibroblasts (RA-SF) (11).

In addition to the involvement of FLSs, damage of chondrocytes is also an important pathophysiological process in RA pathogenesis. In particular, cartilage damage is associated with irreversible disability in RA. Therefore, special attention should be given to the treatment of cartilage damage. The expression of Shh, Ptch1, Smo, and Gli1 proteins increased in AIA rats cartilage tissue, and the level of these proteins were proportional to the degree of chondrocyte damage (14). Moreover, angiogenesis is the fundamental cause of persistent synovial and chronic damage in RA. Apoptosis inhibition of microvascular endothelial cells may accelerate proliferation of synovial micro vessels. In the endothelial cells of RA synovial tissues, Smo expression was significantly elevated. The Shh pathway regulates apoptosis of endothelial cells through Smo protein (15).

There is also cross-linking between the Hh signaling and other pathways. Smo is associated with the proliferation of RA-FLSs and participates in the migration of RA-FLSs by activating the Rho GTPase signaling pathway (29). Shh signaling mediates proliferation and migration of RA-FLSs via the mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) signaling (30). Besides, there is an interaction between c-Jun N-terminal kinase (JNK) signal and Shh signal in RA-FLS. Shh activation promotes the phosphorylation of JNK and c-jun, on the contrary, blocking Shh inhibits the activation of JNK signal. In the presence of JNK inhibitor, c-jun phosphorylation stimulated by Shh agonists was inhibited, indicating that Shh promoted the proliferation, migration and invasion of FLSs in a JNK-dependent manner (31).

Osteoarthritis

Osteoarthritis (OA) is a degenerative disease characterized by pain and loss of joint function and is the most common cause of chronic disability in adults. Therefore, early diagnosis and effective treatment are crucial for OA. There is currently no effective treatment for OA. Therefore, a better understanding of the pathogenesis of OA will be beneficial to improve our knowledge of the disease (32). The pathophysiological changes of OA are mainly manifested in the morphological, biochemical, molecular, and biomechanical changes of cells and extracellular matrix (ECM), resulting in the gradual destruction of articular cartilage, inflammation of the synovium, and changes in the periarticular bone, and finally the formation of bone hyperplasia and subchondral bone sclerosis (33). Hypertrophic chondrocyte differentiation and degradation of the ECM are closely associated

TABLE 2 Treatment of rheumatic diseases by targeting the Hh signaling pathway.

Disease	Inhibitor	Target	Result	Ref
RA	Cyclopamine	–	reduces the proliferation and expression of Shh, Smo, Gli1 mRNA in RA-FLSs	(13)
			decreased the expression of Shh, Ptch1, Smo, and Gli1 in AIA rats, while reducing joint inflammation and cartilage damage, decreasing the levels of pro-inflammatory factors in serum, and increasing the levels of COII in articular cartilage.	(14)
			inhibits apoptosis of chondrocytes	(66)
			decreases EA.hy926 endothelial cell viability and survival, and promoted apoptosis.	(15)
	Smo-siRNA	Smo	inhibites the proliferation of endothelial cells and promotes its apoptosis	(15)
	GANT61	Gli	inhibites proliferation in a dose-dependent manner and increases the apoptosis rate of RA-FLSs	(11)
	GANT61	Gli	reduces the level of Shh protein and inhibited proliferation of FLSs in CIA rats	(67, 68)
	GDC-0449	Smo	suppresses the proliferation of FLSs	(69)
	MeCP2-siRNA	Ptch1	inhibites the activation of Hh pathway and decreases the expression of Gli1 and Shh	(70)
	5-(4-Chlorophenethyl) imino-7-O-acetyl-4-chlorophenethylamine hesperetin(HDND- XI)	Ptch1	attenuates AA-FLSs inflammation by reducing the methylation level of the Ptch1 gene	(71)
	TNFR2 antagonist	TL1A	reduces the expression of Ihh and its receptor Ptch1, 2 in RA-FLSs	(72)
	Inhibition of TRPM7	–	attenuate chondrocyte apoptosis and articular cartilage damage by modulating Ihh signaling	(73)
	AMSP-30m	HIF-1α	inhibited SFs proliferation and promoted its apoptosis.it inhibites the activation of Shh pathway	(74)
OA	Ihh knockdown	Ihh	prevents OA progression by inhibiting chondrocyte hypertrophy and collagen type X, MMP-13 and Runx2 expression	(17, 75)
	Smo-siRNA	Smo	reduce the severity of OA	(16)
	Inhibition of Gli1	Gli1	reduces subchondral local immune inflammatory responses and attenuates articular cartilage degeneration in TMJOA mouse model	(76)
	LDE223	Smo	inhibits chondrocyte differentiation, reduces the expression of type X collagen, and inhibits bone hyperplasia in mice	(77)
	Palmatine(Pal)	–	has a protective effect on cartilage and may inhibit MMPs by inhibiting the Hh signaling	(78)
	Phlomis umbrosa extract (PUE)	–	improves joint pathology in animal models of OA by modulating the Shh signaling pathway	(79, 80)
	Ipriflavone	–	attenuates the degeneration of cartilage by blocking the Ihh pathway	(81)
	JT09	kappa opioid receptor	reduces cartilage loss and prevented degenerative changes in joints, while reducing the expression of Hh signaling components in cartilage	(82)
	GANT-61 and indomethacin	–	reduces cartilage damage and decreases levels of TNF-α, IL-2, and IL-6 in OA	(83)
	Ectonucleotide pyrophosphatase/ phosphodiesterase 1 (Enpp1)	–	inhibits ectopic joint calcification and maintains articular chondrocytes by inhibiting Hh signaling	(84)
	Overexpression of miR-1	–	inhibits the development of OA by inhibiting Ihh signaling	(85)
SSc	Inhibition of Gli	Gli	reduces the expression of CLIC4 in fibroblasts	(86)
	HHAT knockdown	Shh	inhibits TGF-β-induced Hh signaling expression, and suppresses fibroblast activation and tissue fibrosis	(87)
	LDE223	Smo	lighten bleomycin-induced dermal fibrosis and inhibit the aberrant activation of Hh pathway	(88)
	Pirfenidone	–	reduces the Hh signaling expression	(89)

with the development and progression of OA. Hypertrophic chondrocytes upregulate the expression of extracellular matrix degrading enzymes MMP-13 and ADAMTS-5 (34).

The Hh signaling is activated in OA and thought to influence chondrocyte differentiation as well as osteoblastogenesis (35). Olex A et al. identified Hh as the most strongly regulated and metabolic pathway in OA disease development through genetic data analysis (36). Furthermore, Smo missense variants may be a new loci associated with OA. It regulates the transduction of Hh signaling by affecting the binding of cholesterol to its extracellular structure, thereby increasing the risk of hip OA (37). Ihh signaling protein expression is elevated in both human and mouse OA and positively correlates with OA severity (16). At the same time, Ihh promotes the hypertrophic chondrocyte phenotype and modulates the expression of canonical markers type X collagen and MMP-13 (17). The pathological score of FJOA is positively related with Ihh-related genes and hedgehog interacting protein (HHIP) (18).

Temporomandibular joint (TMJ) OA is another type of OA that exhibits degeneration of the articular cartilage and bone of the mandibular condyle and glenoid fossa/protrusion. In TMJOA rats, Ihh signal-related protein levels in cartilage were elevated and showed a time-dependent relationship with the severity of cartilage degeneration, suggesting that activation of Ihh signaling is associated with cartilage damage (19). Ihh is also associated with osteophytes and matrix mineralization in TMJOA (20). Shimoyama, A's results indicate that Ihh temporally and spatially regulates Runt-related transcription factor (RUNX)2, an essential transcription factor for osteoblastogenesis, through Gli2 and consequently stimulates osteoblast differentiation (21). Ihh signaling promotes collagen X transcription and expression of chondrocytes by binding Runx2, which subsequently calcifies chondrocytes and participates in the pathogenesis of OA (22). The Ihh–parathyroid hormone–related protein (PTHrP) axis maintains the stability of articular chondrocytes. PTH1R may have a protective effect on TMJOA cartilage after Ihh signaling inhibition (23). In addition, Shh signaling is also involved in cartilage damage in OA (24).

Interleukin-1β (IL-1β) is colocalized with MMP-13 to sites of cartilage degradation in OA joints and is a key mediator of OA pathogenesis (38). Inhibition of IL-1β may lead to enhanced expression of Shh and MMP-13 in OA (39). Interleukin 18 (IL-18) could be secreted by chondrocytes, which is a member of the IL-1 cytokine family (40). IL-1β stimulates IL-18 expression in chondrocytes (41). IL-18 exerts a pro-inflammatory effect on chondrocytes by affecting their response to matrix degrading enzymes and matrix components, with the Hh pathway being involved in this process (42). However, other *in vitro* studies have shown that Ihh does not cause degradation of ECM in healthy chondrocytes even in the presence of IL-1β and that IL-1β downregulated the expression of Ihh. Thus, there may be other factors other than IL-1β involved in the degradation of OA ECM by Hh. Primary cilia are involved in the development and maintenance of articular cartilage and regulated Hh signaling (43). Depletion of primary cilia in articular chondrocytes activates Hh signaling, resulting in symptoms of early OA with a reduced ratio of Gli3 repressor to activator (44). Cholesterol is involved in OA chondrocyte development. Hh signaling is involved in cholesterol metabolism in human and mouse cartilage. The level of Gli expression was positively correlated with the intracellular cholesterol accumulation level and the severity of OA. Blocking cholesterol may reduce the severity of OA (45). Furthermore, activation of Smo is dependent on binding to sterols (46). Smo mutations in humans affect its binding to cholesterol and are associated with a high risk of hip OA (37).

Systemic sclerosis

Systemic sclerosis (SSc) affects the skin and various internal organs including the lungs, the gastrointestinal tract and the heart. The accumulation of extracellular matrix (ECM) components in SSc is caused by increased production of ECM by activated fibroblasts (47). The profibrotic cytokine transforming growth factor-β (TGF-β) has been identified as a central mediator of fibroblast activation in SSc (48). The current study recognizes that TGF-β signaling and Wnt signaling are involved in the pathogenesis of SSc (49). Aberrant activation of these pathways in SSc potently stimulate fibroblast activation and collagen release, which results in tissue fibrosis. In addition, the Hh pathway is also activated in SSc. Hh is similar to TGF-β in that it induces myofibroblast differentiation and stimulates collagen release, which is also sufficient to induce fibrosis *in vivo* (25). These findings confirm the involvement of Hh signaling in the aberrant activation of SSc fibroblasts. However, the relationship between these intracellular signals that stimulate ECM production is not fully understood. In skin biopsy tissues of SSc patients, the expression of Shh, Ptch1, Ptch2, Gli1, and Gli2 are all up-regulated, suggesting the activation of Hh pathway. The activation of it can stimulate collagen deposition and myofibroblast differentiation, and promote the development of fibrosis. Meanwhile, TGF-β and Wnt signaling promote the activation of this signaling through interaction in the animal model of SSc (25). Subsequent studies have shown that both Hh and TGF-β pathways intersect to Gli2, which promotes tissue fibrosis by integrating these signals. Moreover, they found that Gli2 is an important downstream agent of the profibrotic effects of TGF-β (50). HOTAIR regulates Gli2 expression in SSc myofibroblasts, and Gli2 also mediates the expression of profibrotic markers in Notch signaling (51). These findings may have translational implications as non-selective inhibitors of Gli2 are in clinical use and selective molecules are currently in development.

Ankylosing spondylitis

Ankylosing spondylitis (AS) is characterized by inflammation and new bone formation (NBF) in the spine and joint (52). During the development of the vertebrate skeleton, chondrocytes are gradually replaced by bone. The Hh family is involved in ossification process (53), and Ihh is the main controller of endochondral ossification (54). Daoussis, D reported that the serum level of Ihh in AS patients was higher than RA patients and healthy controls, and its expression was reduced after TNF-antagonist treatment, suggesting that Ihh may be involved in the onset of AS and is important for the evaluation of AS prognosis. This finding may have both clinical and pathogenic implications (26). Relevant mechanistic studies have shown that Ihh is crucial for skeletal development and morphogenesis in vertebrates. It is mainly present in prehypertrophic chondrocytes and is involved in the proliferation and maturation of chondrocytes, but also in the maturation of osteoblasts in the inner cartilage layer (55). In addition, HLA-B27 is the most widely known genetic factor in AS, first reported in the early 1970s (56). HLA-B27 affects serum levels of key regulators of bone homeostasis. The serum Ihh level

was significantly higher in HLA-B27 carriers than negative controls (57). Besides Ihh, after knockdown of Ptch1, the Hh pathway in chondrocytes is activated, which induces spinal fusion and spinal deformity. This is caused by massive proliferation of Ptch1 negative chondrocytes and impaired chondrocyte maturation thus leading to defects in endochondral ossification (53). In summary, the Hh pathway is participated in the development of AS disease, but there is a lack of studies on animal models of AS, and there are no clinical data from large samples to confirm the effectiveness of targeted inhibition of this pathway in the treatment of AS.

Sjogren's syndrome

Sjogren's Syndrome (SS) is mainly involved in salivary glands and lacrimal glands. Several studies have demonstrated that Hh signaling participates in the morphogenesis of salivary glands and it has the potential to promote salivary glands (58, 59). Shh signaling is involved in the embryonic SMG branching morphogenesis. Cyclopamine decreased in branching and epithelial cell proliferation of SMG *in vitro* (60). In the developing mouse submandibular gland, activation of Shh promotes cell polarization and lumen formation of submandibular gland (61). The expression of Shh in epithelium of mice embryonic salivary glands is induced by Edar/NF-κB pathway (62). Hh signaling is activated during functional regeneration in salivary glands after duct ligation of adult mice (63). Fiaschi M revealed that Gli1 has the ability to regulate salivary gland differentiation and promote ductal epithelial cell proliferation (64). Finally, the investigators also confirmed that the expression patterns of Shh pathway are highly similar during SG development in humans and mice, suggesting that the molecular mechanism regulating SG morphogenesis may be conserved (65). Therefore, targeting inhibition of Hh signaling may be a new direction for the treatment of SS.

Treatment of rheumatic diseases by targeting the Hh signaling pathway

Rheumatoid arthritis

With the intensive study of the Hh signaling in RA, the targeted therapy is also gradually increasing. After the administration of cyclopamine, a specific inhibitor of Shh pathway, the proliferation and expression of Shh, Smo, Gli1 mRNA were both reduced in RA-FLSs (13). Gli-antagonist 61 (GANT61) is a Gli specific inhibitor, it inhibited proliferation in a dose-dependent manner and the apoptosis rate of RA-FLSs was increased as well (11). Likewise, GANT61 reduced the level of Shh protein and inhibited proliferation of FLSs in CIA rats (67, 68). GDC-0449 is a Smo antagonist, using it or Smo siRNA can suppress the proliferation of FLSs, and the cell cycle-related proteins changed (69). Indicating that Shh pathway regulates FLSs in a Smo-dependent manner, and Smo may be a new target for RA therapy in the future. Ptch1 regulates Hh signaling negatively and the methylation level of it can affect the proliferation of FLSs. Methyl-CpG-binding protein 2 (MeCP2) overexpression results in an increase in overall methylation levels (90). The Ptch1 gene methylation level was increased in FLSs of adjuvant arthritis (AA) rats, which was related to the activation and inflammation of FLSs, and its methylation level was down-regulated after the administration of DNA methylation inhibitor. Knockdown of MeCP2 using siRNA added Ptch1 expression in AA FLSs. Increased expression of Ptch1 inhibited the activation of Hh pathway and decreased the expression of Gli1 and Shh (70). 5-(4-Chlorophenethyl) imino-7-O-acetyl-4-chlorophenethylamine hesperetin, a species of dihydroflavone, attenuates AA-FLSs inflammation by reducing the methylation level of the Ptch1 gene (71). Given that hypermethylation of the Ptch1 gene is associated with sustained FLSs activation and inflammation in AA rats, reducing the methylation level of it can not only regulate the FLSs inflammatory response but also inhibit the excessive proliferation of FLSs. Tumor necrosis factor (TNF)-like ligand 1A (TL1A) is a member of the TNF superfamily (TNFSF) ligands, which could have an influence on RA-FLSs through binding to TNFR2. TL1A significantly increased the expression of Ihh and its receptor Ptch1, 2 in RA-FLSs. However, TNFR2 antagonists can reduce the above genes notably. The enhanced migratory property of FLSs after stimulating with TL1A suggests that TL1A regulates the migration and Ihh signaling of RA-FLSs by TNFR2 (72). These findings may introduce a potential therapeutic strategy for targeting Ihh/Ptch1, 2 axes and control RA-FLSs pathological synovial invasion and suppress their undesired action. Furthermore, they confirmed the importance of clinical usage of anti-TNF drugs in patients with RA.

As for chondrocytes, Cyclopamine effectively decreased the expression of Shh, Ptch1, Smo, and Gli1 genes in AIA rats (14), while reducing joint inflammation and cartilage damage, decreasing the levels of pro-inflammatory factors in serum, and increasing the levels of COII in articular cartilage. Furthermore, cyclopamine inhibits apoptosis of chondrocytes, which may be related to its mechanism of alleviating cartilage damage (66). Transient receptor potential melastatin-like seven channel (TRPM7) has been reported to be associated with apoptosis. Its expression is elevated in chondrocytes and articular cartilage of AA rats, and inhibition of it can attenuate chondrocyte apoptosis and articular cartilage damage by modulating Ihh signaling (73).

In the endothelial cells of RA synovial tissues, Cyclopamine significantly reduced the expression of Shh pathway-related proteins in EA.hy926 endothelial cells, decreased cell viability and survival, and promoted apoptosis. Similarly, Smo-siRNA inhibited the proliferation of endothelial cells and promoted its apoptosis (15). Scube proteins are associated with endothelial

cell inflammation and angiogenesis. More importantly, Scube promotes the activation of Hh signaling, and these features suggest that Scubes may be involved in inflammatory and angiogenesis of RA, and may be an ideal target for anti-angiogenic therapy in RA (91). Hypoxia-inducible factor 1 (HIF-1) α is also associated with RA synovitis and angiogenesis (92). AMSP-30m, a novel HIF-1 alpha inhibitor, inhibits angiogenesis in many tumor cells (93). In the AIA model, it had strong anti-arthritic and anti-inflammatory effects, inhibited SFs proliferation and promoted its apoptosis. AMSP-30m exerted its anti-angiogenic effect by reducing the expression of synovial vascular endothelial growth factor (VEGF) and the number of blood vessels. In addition, it inhibited the activation of Shh pathway (74). Collectively, the HIF-1α inhibitor AMSP-30m may exert an effective anti-arthritic effect by promoting synovial apoptosis, reducing angiogenesis and inhibiting the Shh pathway. Annexin a2 (Axna2) is an important mediator of pannus formation in RA. The expression of Axna2 and Axna2 receptor (Axna2R) is elevated in RA patients. In CIA model, overexpression of Axna2 promotes the development of arthritis, especially the formation of pannus. Meanwhile, Axna2 activates and amplifies the expression of Hh pathway and its downstream VEGF, angiopoietin-2 (Ang-2), and matrix metalloproteinase-2 (MMP-2) by binding to Axna2R, promoting the proliferation of HUVEC and ultimately leading to the formation of pannus (94). Therefore, inhibition of Axna2 may be a new potential measure for the treatment of RA.

Osteoarthritis

Given the important role of the Hh signaling in the development of OA, researchers have explored its use as a therapeutic target. Zhou, J's study demonstrates that knockdown of Ihh prevents OA progression by inhibiting chondrocyte hypertrophy and collagen type X, MMP-13 and Runx2 expression (75). The study also found that treatment with an Ihh inhibitor or Smo-siRNA could inhibit Hh signaling and reduce the severity of OA (16), consistenting with the results of Wei, F et al. (17). Activation of Notch signaling inhibits Hh signaling in OA. Studies have found that when Notch signaling is inhibited, Hh signaling is activated, thereby promoting chondrocyte hypertrophy and bone hyperplasia (95). Selective inhibition of Hh signaling in $Gli1^{+}$ osteoblastic progenitor cells reduces subchondral local immune inflammatory responses and attenuates articular cartilage degeneration in TMJOA mouse model, providing a potential method for TMJOA treatment (76).

There are already Hh inhibitors used in the treatment of tumors, which provides some basis for the use of Hh inhibitors in OA. LDE223 is a Smo-specific small molecule inhibitor that inhibits chondrocyte differentiation, reduces the expression of type X collagen, and inhibits bone hyperplasia in mice (77). Palmatine (Pal), a member of the protoberberine class of isoquinoline alkaloids, is a structural analog of berberine. It has a protective effect on cartilage and may inhibit MMPs by inhibiting the Hh signaling (78). *Phlomis umbrosa* extract (PUE) has been used in the treatment of RA (79). PUE also can improve joint pathology in animal models of OA by modulating the Shh signaling pathway (80). Ipriflavone also can attenuate the degeneration of cartilage by blocking the Ihh pathway (81). Prochondrocytes express the kappa opioid receptor (KOR), which prevents the onset of cartilage degeneration (96). It was found that the selective KOR agonist JT09 reduced cartilage loss and prevented degenerative changes in joints, while reducing the expression of Hh signaling components in cartilage (82). Combined treatment of GANT-61 and indomethacin reduced cartilage damage and decreased levels of TNF-α, IL-2, and IL-6 in OA (83). The ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1) is an inhibitor of pathological calcification that inhibits ectopic joint calcification and maintains articular chondrocytes by inhibiting Hh signaling, whereas Enpp1 expression is diminished in articular cartilage of human and mouse OA (84). miR-1 is lowly expressed in human OA joint tissues, and overexpression of it inhibits the development of OA by inhibiting Ihh signaling (85). The activation of Hh signaling plays an important role in the occurrence and development of OA, and a large number of studies have proved that targeting and blocking this pathway can treat OA (97, 98), laying a certain foundation for its future clinical translation.

Systemic sclerosis

As a driver of fibroblast activation, chloride intracellular channel 4 (CLIC4) expression is elevated in fibroblasts from SSc patients. It is associated with increased activation of TGF-β (99). Investigations revealed the expression of CLIC4 in normal dermal fibroblasts was driven by TGF-β/SMAD3 together with Wnt3a/β-catenin and Smo/Gli signaling, and inhibition of Gli reduced CLIC4 expression (86). The hedgehog acyltransferase (HHAT) catalyses the attachment of palmitate onto Shh (100). Knockdown of HHAT inhibits TGF-β-induced Hh signaling expression, while suppressing fibroblast activation and tissue fibrosis (87). Inhibition of Hh signaling by targeting HHAT may become a new approach for SSc antifibrotic therapy. LDE223 is a selective small-molecule inhibitor of Smo, which can lighten bleomycin-induced dermal fibrosis and inhibit the aberrant activation of Hh pathway (88). In lung tissue of SSc-ILD patients, Hh pathway is activated, pirfenidone intervention reduces its expression (89). In all, the Hh signaling contributes to the aberrant activation of SSc fibroblasts, and antifibrotic therapy targeting it is expected to improve the prognosis of SSc patients, however, there is still a long way to go for true clinical translation.

It is not difficult to see that there have been many studies on the targeting treatment of the pathway. However, it is worth noting that these studies are all animal or *in vitro* experiments,

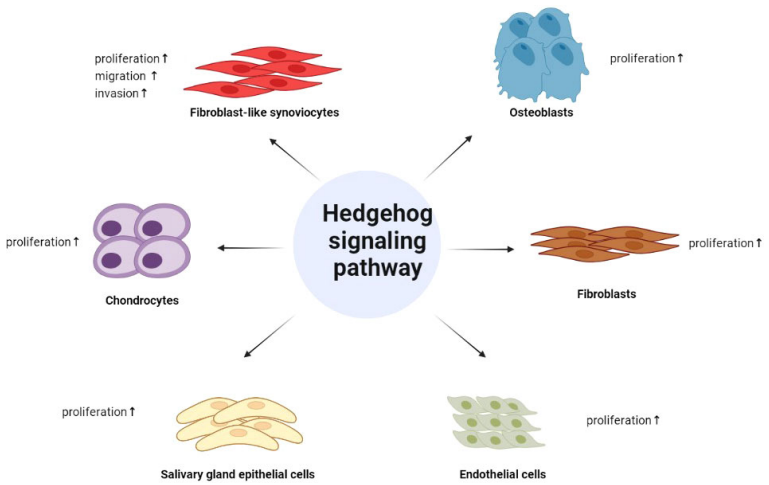

FIGURE 2
The role of Hedgehog signaling in rheumatic diseases. Hedgehog signaling pathway is involved in the inflammatory proliferation of FLSs, the differentiation and proliferation of chondrocytes, osteoblasts, and fibroblasts, and can stimulate the proliferation of endothelial cells and salivary gland epithelial cells.

and there is no relevant clinical research. Moreover, research on specific inhibitors of this pathway is still missing. Expression of target genes can be inhibited by si-RNA, thereby inhibiting the activation of this pathway, but this method is difficult to apply to patients. GANT61, GDC-0449 and LDE223 are specific small molecule inhibitors of this pathway, at the moment there is little/no knowledge on the pharmacokinetics (e.g. solubility, metabolism, etc.) of these agents and its toxicity. Cyclopamine is the most studied inhibitor of the Hh pathway, and its clinical use is limited due to its insolubility in water and organic solvents. The inhibitory effect of the above drugs on this pathway is not specific, and there is a lack of research on its adverse reactions in rheumatism.

Conclusion

With the deepening of the current research on the Hh signaling, its role in rheumatic diseases has also been verified. In conclusion, the aberrant activation of it is involved in the inflammatory proliferation of FLSs, the differentiation and proliferation of chondrocytes, osteoblasts, and fibroblasts, and can stimulate the proliferation of endothelial cells and salivary gland epithelial cells, providing a new perspective for the treatment of multiple rheumatic diseases (Figure 2). What is even more exciting is that some inhibitors of the Hh pathway have been applied in the clinical treatment of certain tumors, and many drugs have entered the clinical research of various tumor treatments, so the application of these drugs in rheumatic diseases will also be not far. At the same time, it is even more necessary for us to conduct in-depth research on Hh signaling pathway to provide new ways for the treatment of rheumatic diseases.

Author contributions

This article is mainly written by YS. HX wrote part of the manuscript and proofread the manuscript. JK and LB helped us collect literature information and draw pictures. LZ reviewed the manuscript and proposed final revisions. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Case report: Documentation of cutaneous only pemphigus vulgaris without history of mucosal lesions in North America

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Background: Pemphigus is a group of autoimmune blistering diseases including Pemphigus vulgaris (PV) and Pemphigus foliaceus (PF). These conditions exhibit lesions with mucosal or mucocutaneous (PV) or cutaneous (PF) morphology, as framed by the Desmoglein Compensation Hypothesis (DCH). However, some PV patients present with solely cutaneous disease (cPV), and growing evidence suggests the existence of a cPV subtype *without any history* of mucosal erosions/blisters (cPVwohm), neither of which are predicted by the DCH.

Methods: Participants were recruited from several outpatient clinical settings and patient support group meetings throughout the US. On intake, subjects provided blood samples and completed questionnaires regarding their disease status.

Results: We report three cases of clinically and histologically confirmed cPV without history of mucosal lesions (cPVwohm). Of these patients, two do not carry the most common PV associated HLA alleles, DRB1*0402 or DQB1*0503. The same two patients also tested negative for the primary PV associated autoantibodies, anti-desmoglein 3 and anti-desmoglein 1, while in active disease status.

Conclusion: We confirm the first documented individual cases of cPVwohm in North America, supporting the existence of PV patients that develop cutaneous disease without a history of mucosal lesions, challenging the fidelity of the DCH. Two of the 3 patients reported did not type for the common PV-associated HLA genes or display anti-desmoglein autoantibodies while in active disease, suggesting cPV patients may develop Pemphigus *via* genetic and immune mechanisms that differ from typical mucosal or mucocutaneous PV.

KEYWORDS

autoantibody, cutaneous, mucosal, North America, pemphigus vulgaris

Introduction

Pemphigus is a group of rare autoimmune blistering diseases that includes Pemphigus vulgaris (PV) and Pemphigus foliaceus (PF). Lesion morphology in PV and PF has been framed within the dictates of the desmoglein compensation hypothesis (DCH), an elegant hypothesis based on patient desmoglein-specific autoantibody profiles to predict lesion morphology. Central to this theory was the discovery of PV associated autoantibodies targeting the cadherin proteins Desmoglein-3 (Dsg3) and Desmoglein-1 (Dsg1), key components of desmosomal cell-cell attachments critical for epidermal integrity (1). According to the DCH, differences in lesion morphology in PV and PF are attributable to differential expression of the Dsg1 and Dsg3 proteins throughout the epidermis. Dsg1 is expressed at higher concentrations towards the superficial layers of the skin and mucosal epidermis, while Dsg3 is concentrated towards the basal layers. Non-mucosal skin notably expresses higher levels of Dsg1 throughout the epidermal layers, while Dsg3 is only present in the basal layers. Conversely, the mucosal membranes have higher concentrations of Dsg3 throughout the epidermis while Dsg1 is present in the superficial layers of the epidermis only.

The DCH posits that the differential expression of Dsg3 and Dsg1 proteins in mucosal vs. non-mucosal skin underlies the clinical presentation of 3 distinct classes of lesion morphology in Pemphigus: 1) PF is characterized by subcorneal skin dissociation, and is linked to the presence of only anti-Dsg1 autoantibodies. 2) Mucosal PV presents with suprabasilar blistering, hypothesized to be a result of only anti-Dsg3 autoantibodies. 3) Mucocutaneous PV is characterized by suprabasilar skin and mucosal lesions and is attributed to the presence of anti-Dsg1 and anti-Dsg3 autoantibodies (2).

While the DCH attempts to account for the pathology and clinical presentation in PF as well as mucosal and mucocutaneous PV, it cannot explain the cutaneous only manifestation of PV (cPV), where patients present with suprabasilar lesions on non-mucosal skin, including those that do not have *any history of* mucosal lesions. Yet, the cPV phenotype has been reported in several case studies (3–5). Currently, there are a limited number of reported cases of cPV and, upon review of the literature, most of these cases have been in Japanese patients. There have been only two reported Caucasian patients with cPV (6, 7). While no individual cases of cPV in the United States have been reported in literature, analysis of PV patient data collected in a registry initiated by the International Pemphigus and Pemphigoid Foundation revealed that 24.5% of patients reported only cutaneous lesions at the time of clinical screening (8).

A distinction that is often not made when assessing cPV is the patient's lesion history, namely if they have had mucosal lesions in the past, or if they have only ever presented with cutaneous lesions. One example where this information is noted is Nagasaka et al., 2005, in which the patient's lack of mucosal lesion history is explicitly stated (3). Thus, we note the distinction of two cPV subgroups: cPV with history of mucosal lesions (cPVwhm), and cPV without history of mucosal lesions (cPVwohm). Here, we identify 3 biopsy confirmed PV patients with cPVwohm. We further examine the HLA type and anti-Dsg3 and anti-Dsg1 titers of these patients.

Materials and methods

Participants were recruited to the study from the Dermatology outpatient clinics at the University at Buffalo, Weill Cornell Medical College, and Michigan State University, as well as annual meetings hosted by the International Pemphigus and Pemphigoid Foundation (IPPF) for enrollment to our autoimmune blistering disorder biorepository. The study was approved by the institutional review boards of the respective academic institutions and followed all ethical guidelines, including written informed consent prior to study enrollment.

Patients provided information about their demographics, disease history, disease classification, lesion morphology, past medical history, and family history. Venous blood samples were collected from which serum was isolated and used immediately or stored at -80°C for future analysis. Patients with more than 1 visit provided venous blood and current clinical information at every visit when possible.

Of a total of 408 PV patients analyzed for this study, 34 patients reported a biopsy-confirmed case of PV and presented with active cutaneous lesions only (cPV) at time of enrollment. These patients, while not featured in this report, are presented in the context of a larger study presenting PV cases that violate the postulates of the DCH (Sielski et al.).

Of these patients, 7 reported no history of mucosal lesions. Three of those 7 patients were able to provide biopsies confirming their diagnosis of PV as well as confirm that they did not have a history of mucosal lesions. Patients were considered active if they met the criteria of 3 or more non-transient lesions and/or extension of current lesions in the past month (9). For the purposes of this study, the diagnosis of PV was determined by histopathological findings (suprabasilar acantholysis) and DIF (IgG and C3 deposition in intercellular epidermis).

High resolution HLA typing was performed *via* PCR amplification using sequence specific primers at the Histocompatibility and Immunogenetics Laboratory at Michigan State University employing commercial kits (One lambda, Thermo Fisher Scientific) (10). Patients were deemed "HLA-positive" if they possessed one or both of the PV-associated HLA alleles, DRB1*0402 and DQB1*0503. Patients that did not have either of these alleles were considered "HLA-negative".

Anti-Dsg 3 and -1 levels were detected by ELISA (MBL Intl. (RG-M7593-D) as per manufacturer's protocol with a 1:101 serum dilution. These kits detect immunoglobulin G (IgG) antibodies directed against Dsg1 and Dsg3, but do not distinguish between IgG subclasses. Antibody positivity was defined as ELISA levels of >20U/mL for both anti-Dsg1 and anti-Dsg3. The cutoff of 20U/mL was used as it was previously used by the manufacturer and has been used as a cutoff in other studies into PV (11).

Results

Patient 1 is a 64-year-old male of South Asian descent. This patient initially presented with eroded lesions on the scalp. He was not receiving any immunotherapy at the time of presentation. His biopsy showed suprabasilar acantholysis, with DIF showing intraepidermal separation and weak IgG and C3 deposition in the intracellular epidermis consistent with a diagnosis of Pemphigus vulgaris. A blood draw two weeks after the initial biopsy revealed elevated anti-Dsg1 (61 U/ml), but not anti-Dsg3 levels. Additionally, the patient carries a known PV-associated HLA risk allele, DQB1*0503. Over the course of the past 6 years and as of writing of this report, the patient has never experienced mucosal lesions (Table 1).

Patient 2 is a 68-year-old male of Caucasian descent. He presented with blisters on the waist, legs, and both arms (Figure 1). His initial biopsy showed acantholysis in the mid-upper epidermal layer. DIF revealed IgG and C3 intercellular deposits along the lower aspects of the epidermal layer. A later biopsy showed suprabasilar and intragranular acantholysis. He did not carry either of the reported PV-associated HLA alleles

TABLE 1 Patient Demographics and Clinical Information.

	Patient 1	Patient 2	Patient 3
Patient ID	PV 386	PV 445	PV 463
Sex	Male	Male	Female
Ethnicity	South Asian	Caucasian	Caucasian
Age of onset	64	68	59
HLA-Association			
DRB1	13:01 and 14:04	16:01 and 16:02	03:01 and 16:01
DQB1	**0503** and 06:03	05:02 and 15:02	02:01 and 05:02
HLA status	"Positive"	"Negative"	"Negative"
Biopsy Findings			
Date of Biopsy	Oct. 1, 2014	Feb. 8, 2017	Dec. 27, 2016
Histology	**Suprabasilar** Acantholysis	Acantholysis in mid-upper epidermal layer.	**Suprabasilar acantholysis** and tombstoning
Direct Immuno-fluorescence (DIF)	DIF shows perilesional skin with intraepidermally separated epidermis. Weak IgG and C3 deposits in intercellular epidermis	DIF distributed along **lower 3/4 of epidermal thickness**. Antibodies to IgG and C3 show intercellular deposits	IgG and C3 intercellular distribution
Date of Repeat Biopsy		Nov. 19, 2019	
Histology		**Suprabasilar** and intergranular **acantholysis** with scale crust and parakeratosis consistent with pemphigus	
Serum Antibodies and Clinical Information			
Date of Blood Draw	Oct. 15, 2014	May 8, 2017	Oct. 13, 2018
Anti-DSG1	**61 U/ml**	3.7 U/ml	4.7 U/ml
Anti-DSG3	0.8 U/ml	1.5 U/ml	0 U/ml
Lesion Morphology at Time of Draw	Eroded lesions on scalp, one quadrant	2 blisters above waist, 1 on right tricep, 1 on leg, 1 on left shoulder	Multiple lesions on back, arms, and legs
Activity PDAI at Time of Draw	1	4	5
Medications at Time of Draw	No immunotherapy	Mycophenolate 1g BID, rituximab infusion 3 weeks ago, 40 mg prednisone/day, IVIG infusion 2 weeks ago	Mycophenolate 1g/day, rituximab infusion 2 months ago

Relevant data include Histological and DIF finding, serum autoantibodies and associated morphology at time of blood draw. "HLA Positive" describes a carrier of the PV -susceptibility alleles DRB1*0402 and/or DQB1*0503; "HLA Negative" describes a subject that does not carry either of the PV -susceptibility alleles DRB1*0402 and/or DQB1*0503.
Certain values were bolded in Table 1 in order to draw the attention of the reader to some of the pertinent findings.

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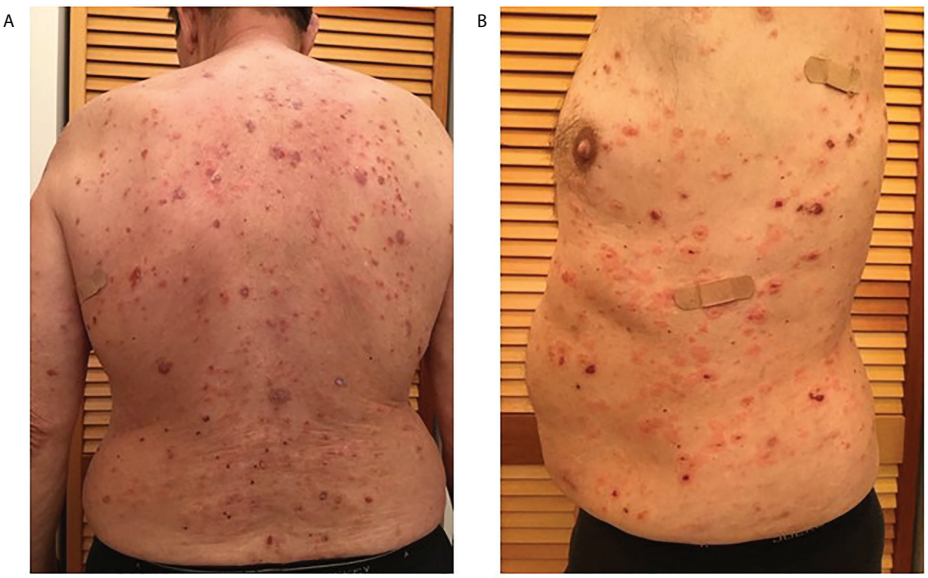

FIGURE 1
Multiple scattered blisters and erosions in various stages of healing observed over the entire torso **(A.** back, and **B.** left side) of patient 2 prior to original biopsy (February 2017).

DRB1*0402 or DQB1*0503. Upon presentation to our group 3 months after the initial biopsy, the patient had received rituximab (1g i.v. infusion 3 weeks and 5 weeks prior), and IVIg infusion (2g/kg over 3 days; 2 weeks prior) and was on mycophenolate mofetil 1g BID and, 40mg prednisone daily, but was still presenting with active disease (erosions on torso and extremities). Anti-Dsg1 antibodies recorded at low levels (3.7U/ml respectively) but well below the manufacturer-set cutoff for positivity in this patient presenting in active disease. There is no documented history of mucosal lesions (Table 1).

Patient 3 is a 59-year-old female of Caucasian descent. Her diagnostic biopsy revealed suprabasilar acantholysis and tombstoning consistent with PV. Her DIF also showed intercellular deposition of IgG and C3. She did not type for either PV associated risk alleles, DRB1*0402 or DQB1*0503. Upon intake and blood draw, roughly 2 years after her initial diagnosis, she presented with multiple skin lesions on her back, arms, and legs, without history of mucosal disease. At the time of evaluation, she was receiving mycophenolate mofetil 1g/daily, and her last rituximab infusion (1g x 2, 2 weeks apart) was 2 months prior. Despite showing disease activity with a PDAI of 5, her anti-Dsg3 was undetectable at 0U/ml and anti-Dsg1 was recorded well below the cutoff for positivity at 4.7 U/ml (Table 1).

Discussion

Pemphigus encompasses a group of autoimmune blistering disorders in which the precise location of epidermal splitting is key to accurate diagnoses. The DCH categorizes patients into one of three groups based on the level of the intradermal split (suprabasilar acantholysis in the mucosa for mucosal PV, suprabasilar acantholysis in skin and mucosa for mucocutaneous PV, and subcorneal acantholysis in nonmucosal skin only for PF), but does not predict the existence of PV patients with cutaneous lesions in the absence of mucosal lesions (1, 4, 8, 12). A cutaneous only expression of PV (cPV) as reported in the literature, (Table 2), including those patients without any history of mucosal lesions (cPVwohm), cannot be accommodated within the current paradigms. Modified models of disease will be required to better account for the full spectrum of clinical presentation in pemphigus.

We report here 3 additional cases of cPV that are at odds with the postulates of the DCH which predicts suprabasal blistering in the skin only when accompanied by lesions in the oral mucosa. These are the first individually reported cases of cPV in North America, as well as examples of the subgroup cPVwohm, presenting with no history of mucosal lesions. One limitation faced in diagnosing this subgroup is the reliance on patient provided data. Many patients face delays in diagnosis making it potentially difficult for individuals to accurately recall isolated mucosal lesions, despite their strong assertions regarding the extent of their clinical lesions along with extensive interrogation of their clinical history for this study by trained clinical investigators (8). It should also be noted that while Patient 1 was classified as biopsy-proven pemphigus vulgaris with suprabasilar acantholysis, his lesions on

TABLE 2 Cases of cutaneous only PV reported in the literature.

Cases	Age (y)	Sex	Skin Lesion	Mucosal Lesion	Duration of cPV	Phenotype	Supra-basilar acantholysis	Superficial acantholysis	Skin DIF	Anti-Dsg1 (index values)	Anti-Dsg3 (index values)
Müller et al. (2002) (7)											
#1	51	M	Positive	–	ND	cPV	Positive	Positive	Positive	192.2	31.1
Yoshida et al. (2005) (4)											
#2	52	F	Positive	–	3 mo	cPV	Positive	–	Positive	2160	301.8
#3	52	M	Positive	–	10 y	cPV	Positive	Positive	Positive	459.6	82.5
#4	58	F	Positive	–	8 mo	mcPV-R-cPV	Positive	–	Positive	651.6	462.8
#5	57	F	Positive	–	7 mo	cPV	Positive	–	Positive	140.2	90.6
Nagasaka et al. (2005) (3)											
#6	45	F	Positive	–	ND	cPV	Positive	–	Positive	114	42
Shinkuma et al.(2008) (5)											
#7	50	M	Positive	–	2 weeks	cPV	Positive	Positive	Positive (skin and oral mucosa)	680	220
Bello et al. (2013) (12)											
#8	30	M	Positive	–	5 months	cPV	Positive	–	Positive	Not Assessed	Not Assessed
Carew et al. (2014) (6)											
#9	79	M	Positive	–	6 weeks	cPV	Positive	–	Positive	10	160

A total of nine cases of cPV have been reported to date between 2002-2014. With the exception of Nakasaka et al., no other study documents whether mucosal lesions had been present in the past. Studies have used varying criteria when reporting cPV, including one patient (Patient #3) who displayed both suprabasilar acantholysis (diagnostic of PV) and superficial acantholysis (diagnostic of PF) (4). ND, Not Determined; cPV, Cutaneous type Pemphigus Vulgaris; mcPV, mucocutaneous Pemphigus Vulgaris; R, Remission.

presentation were generally superficial erosions and he had elevated anti-Dsg1 levels without anti-Dsg3 antibodies. As such, it cannot be ruled out that this case could represent atypical PF rather than atypical PV (in a patient of South Asian descent carrying the PV-associated HLA DQB1*0503 allele with a predominantly cutaneous profile as discussed below). In any case, this patient illuminates the complexities of assigning subtypes of pemphigus, and reinforces the point that our current diagnostic paradigms for PV and PF may be limited and oversimplified under the stricture of the DCH as currently formulated.

A strong association with the HLA-alleles DRB1*0402 and DQB1*0503 has been shown for PV, with the vast majority of Caucasian and Ashkenazi Jewish patients carrying one or both of these alleles (13–15). Only one of the three cPV patients reported in this study carried the known PV-associated HLA risk allele DQB1*0503 and presented with an elevated anti-Dsg1 antibody level. This patient's presentation is in line with reports in the literature of PV patients of South Asian descent carrying DQB1*0503 as well as having higher levels of anti-Dsg1 and higher rates of cutaneous disease (16). Of note, two of the three patients presented here did *not* carry the primary PV-associated HLA alleles, and thus may be carriers of non-Dsg antibodies such as anti-desmocollin or others. These same two patients did not meet the threshold for positivity for the key PV associated autoantibodies, anti-Dsg3 and anti-Dsg1, despite being in the active phase of disease at the time of evaluation. While the two patients did have some level of detectable anti-Dsg1 (albeit well below the manufacturer-set level of detectability), this could reflect residual autoantibodies in a previously antibody positive patient, or represent highly reactive autoantibodies. We acknowledge that in these cases serum was sampled after treatment with the anti-CD20 antibody Rituximab (either 3 weeks or 2 months prior to blood draw) which has been

shown to lead to a reduction in anti-desmoglein antibodies (17). Nonetheless, even without detectable anti-Dsg3 and anti-Dsg1 antibodies, both patients still presented with active lesions (PDAI score of 4 and 5, respectively) and showed IgG and C3 deposition by DIF, consistent with the diagnosis of PV, but also at odds with the DCH. While anti-Dsg3 and anti-Dsg1 autoantibodies are believed to be the primary mediators of lesional activity in PV, the literature does include studies reporting patients with biopsy proven PV in active disease with negative autoantibody levels (18). These findings lend support for the possibility that other factors, potentially including non-desmoglein autoantibodies, are relevant for blister formation in PV patients, perhaps particularly in cPV cases (19–21). Interestingly, for patient 2, the DIF finding of antibody distribution "along the lower ¾ of epidermal thickness" may indicate the presence of non-Dsg antibodies with a distribution in those areas such as antibodies against desmocollin 2 or -3 (22).

The HLA negative status of 2/3 cPV patients reported here along with the absence of anti-Dsg3 and anti-Dsg1 antibodies suggests that differences in HLA genetics may be linked to distinct autoantibody profiles, that are in turn linked to variance in clinical presentation (mucosal PV vs. mucocutaneous PV vs. cutaneous PV). Of note, the majority of cPV cases reported in the literature are from Japan, a population in which Pemphigus is not as tightly linked to the PV-associated HLA alleles DRB1*0402 and DQB1*0503 (23). The further exploration of the genetic and immune profiles of uncommon PV clinical subgroups such as cPV patients, including cPVwohm, can be expected to deepen our understanding of disease risk, disease mechanisms and disease presentation, and ultimately allow us to better predict, prognose and manage the full range of pemphigus cases.

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 Institutional Review Board, Jacobs School of Medicine and Biomedical Sciences; Institutional Review Board, Michigan State University; Institutional Review Board, Weill Medical College of Cornell University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JB, KS-S, and AS devised the project. KS-S and AS collected patient samples and associated clinical data. JB performed the analysis, and drafted the first version of the article. KS-S and AS performed critical revision of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Serum proteomic networks associate with pre-clinical rheumatoid arthritis autoantibodies and longitudinal outcomes

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Objectives: The development of autoantibody directed towards citrullinated proteins (ACPA) are predictive of RA in at-risk individuals. The biological events that underpin loss of immune tolerance and progression into inflammatory arthritis are not known. We sought to identify serum proteomic alterations that drive autoantibody formation, persistence and progression into inflammatory arthritis in a cohort of first-degree relatives (FDR) of RA patients.

Methods: We studied baseline serum samples from a cohort of Indigenous FDR (n = 147) and quantified serum proteins using a 48-plex platform. Longitudinal outcomes were defined on the basis of ACPA status and progression into inflammatory arthritis (IA). K-means clustering, differential expression, and principal components analyze group differences. A co-expression module analysis was used to identify enriched networks. Random forest was used to classify ACPA positive samples, while network analysis was used to understand underlying biological processes based on protein expression.

Results: We defined 6 proteomic clusters, with enrichment of ACPA positive samples in one of the clusters. 23 of 24 differentially expressed proteins in ACPA positive samples were upregulated. A co-expression network was enriched in ACPA positive sera and individuals who progressed into IA. Random Forest achieved an area under the curve of 0.767 to classify ACPA positive sera in a test dataset. Network analysis revealed upregulation of JAK-STAT signalling as being activated in those at highest risk to develop future IA.

Conclusions: The serum proteome provides a rich dataset to understand biological processes in ACPA seropositive individuals. A combination of serum biomarkers, including ACPA, may predict future arthritis onset in at-risk individuals.

KEYWORDS

rheumatoid arthritis, proteomics, pre-clinical, jak-stat, prevention, machine learning

Key Messages

- Serum proteomics classifies ACPA positive from ACPA negative first-degree relatives
- Protein co-expression network associates with ACPA status and progression into inflammatory arthritis
- JAK-STAT signalling is upregulated in those at highest risk to develop inflammatory arthritis

Introduction

Rheumatoid Arthritis (RA) is a common, systemic autoimmune disease that primarily targets the synovial joints (1). Without immunosuppressive treatment, RA leads to chronic pain and joint damage which ultimately impacts on functional status and quality of life (2). Despite decades of research, the etiology of RA remains elusive. The study of pre-clinical RA, the stage prior to the onset of clinically detectable joint inflammation, has provided key insights into how a state of subclinical systemic autoimmunity transitions to an immune mediated inflammatory arthritis (3, 4). The hallmark of this pre-clinical RA period is the detection of specific autoantibodies, particularly those directed towards citrullinated peptide epitopes (anti-citrullinated protein antibodies; ACPA), along with autoantibodies directed towards other post-translational modifications (3, 5, 6). These autoantibodies, most of which ultimately persist throughout the course of this chronic disease, can be detected months and even years prior to disease development (7).

Despite these insights, the complex biological events that initiate, propagate, sustain, and amplify pre-clinical RA autoimmunity remain poorly understood. It has now been clearly shown by multiple groups that ACPA seropositivity in an otherwise unaffected individual is a strong risk factor for future RA development (5, 8, 9). Importantly, we have also shown in a prospective longitudinal cohort of at-risk, but unaffected first-degree relatives (FDR) of Indigenous North Americans (INA) that only a proportion of individuals in whom ACPA are detected at any specific timepoint will actually go on to develop RA, and indeed many seropositive individuals become seronegative over time (5). These observations suggest that subsequent to an initial break in immune tolerance characterized by the development of ACPA, and possibly other anti-modified protein antibodies (AMPA), there are regulatory mechanisms that may serve to control these potentially harmful processes, and that these mechanisms are effective in preventing the progression towards pathogenic autoimmunity in a substantial proportion of individuals. Indeed, it can be hypothesized that the development of this autoimmune disease potentially represents a failure of these regulatory mechanisms.

Networks of cytokines that regulate broad inflammatory processes are implicated in the pathogenesis of RA (10). Numerous studies have demonstrated the upregulation of these networks in both blood and the synovium of RA patients (11). These proteins promote several key aspects of RA pathogenesis, including the formation of autoantibodies, sustaining inflammatory arthritis and promoting joint damage. Indeed, cytokine networks are implicated in the function of lymphocytes (both T (12) and B (13) cells), innate immune cells (macrophages (14), neutrophils) and stromal cells such as fibroblast-like synoviocytes (15), responsible for local joint invasion and destruction of cartilage. Importantly, targeting these specific cytokine networks has led to the development of therapeutics that have altered patient outcomes drastically (16). However, precisely when these cytokine networks initiate, and how they contribute to pre-clinical RA pathogenesis is not known. Defining functional modules (17) and disease-specific cytokine hierarchies across all stages of RA will help provide key insights into therapeutic targets, disease pathogenesis and personalized medicine approaches.

Our prospective longitudinal cohort study of the FDR of INA RA patients has afforded us the opportunity to explore the pre-clinical period of individuals who ultimately developed seropositive RA, comparing them to individuals in whom autoantibodies were not detected, and particularly to ACPA seropositive individuals in whom the autoimmune processes did not appear to progress during the observation period (5). As such, we have characterized the ACPA themselves and shown that epitope spreading (5), and a high degree of variable region glycosylation (18) were both associated with clinical disease development. Moreover, we used exploratory broad-based proteomic approaches to detect proteomic signatures that predicted RA development with a high degree of accuracy (19). These signatures were enriched for proteins associated with innate immune mechanisms. In the current study we present data regarding the circulating cytokine/chemokine profiles of these groups of individuals, and how these profiles relate both to the RA autoantibodies, and to the ultimate development of RA.

Methods

Cohort overview and sample selection

Methods and protocols for patient recruitment for this study were described in our group's previous work (20). In brief, INA RA probands who met the 2010 ACR/EULAR criteria (21) were approached to help recruit their eligible FDR for longitudinal follow up. We expected that a proportion of these patients would go on to develop inflammatory arthritis. Both RA probands and

FDRs were required to have at least 3 grandparents with INA ethnicity by self-report. Participants had to be 18 years of age or older. At baseline, all FDR were examined by a rheumatologist to confirm the absence of clinical synovitis. Participants then entered the study and underwent annual examination for the presence of clinical synovitis. Between the annual evaluations, FDRs were instructed to report any new symptoms suggestive of arthritis. Clinical assessment by a member of the research team took place as soon as possible, to assess reported symptoms. If synovitis was unequivocally detected in one or more joints by a rheumatologist, the individual was deemed as having "progressed" to having inflammatory arthritis. Serum samples were collected at all study visits and stored at -20°C for future studies. Anti-CCP antibodies were detected using CCP3 ELISA (Inova), using manufacturer's cut-off values to determine positivity. We assigned longitudinal outcomes in ACPA positive samples as: 1. Seroconversion (ACPA negative after follow-up) 2. ACPA positive persistence or 3. ACPA positive with progression into inflammatory arthritis. These pre-clinical baseline states were chosen on the basis of increasing risk for future development of seropositive RA.

Ethics

All study participants provided informed consent in accordance with the Declaration of Helsinki. The Biomedical Research Ethics Board of the University of Manitoba approved all aspects of the study (Board approval number HS14453). Specific community research agreements were put in place with the study communities. Consistent with the guidelines from the Canadian Institutes of Health Research for conducting research involving indigenous people in Canada, we established an arthritis advisory board, to provide indigenous oversight for the study.

Serum proteomics

Serum proteins were analyzed using a Luminex xMAP 48-plex cytokine/chemokine/growth factor panel (Millipore, HCYTA-60K) which included: IL1A, CXCL1, IFNA2, IFNG, IL1B, CCL3, CCL4, TGFA, TNF, LTA, CXCL9, IL25, PDGFB, FGF2, FLT3LG, CSF3, IL1RN, IL2, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12B, IL13, IL15, IL17A, IL17F, IL27, CCL2, CCL7, MCSF, PDGFA, VEGFA, IL12A, CXCL10, IL18, IL22, CCL5, CSF2, IL3, MDC, IL25, CX3CL1, EGF and CD40LG. Standard curves for all analytes were generated using 5-parameter logistic regression on 8 standard samples. Individual analyte sensitivity values are available in the MILLIPLEX® MAP protocol provided with the manufacturer's kit. Samples were analyzed using a Luminex 200 luminometer and the concentration of analytes was reported in pg/mL. Gene names were used to annotate proteins for readability. Protein expression was log-transformed to normalize, and assessed for distribution/quality control (Figure S1) and proteins (CCL5, CSF2, IL3) were removed if > 25% of the samples fell outside of the assay range.

ACPA N-linked variable domain glycan

Methods to extract ACPA and quantify glycan composition of the variable domain have been published previously (22). In brief, ACPA and non-ACPA IgG are captured and eluted. Glycans are released, purified and profiled using ultra-high performance liquid chromatography (UHPLC) and custom software. The Fab glycosylation is expressed as a % of Fc glycans for each IgG sample. Data for Fab glycosylation of ACPA was available for 17 samples in this study.

Data analysis

All data was analyzed in the R (v4.0.2) environment using RStudio (2022.02.0 Build 443). Dichotomous group differences were assessed using chi-square test, while continuous outcomes were analyzed using Wilcoxon rank sum test for non-parametric assessments, and t-test for parametric assessments. Dunn's was used to evaluate non-parametric differences between multiple groups, adjusted for multiple comparisons (Holm). Correlation between two continuous variables was evaluated using spearman rank correlation. A correlation matrix of protein expression was created using Pearson correlation, adjusted for multiple comparisons (Bonferroni). Principle components analysis was undertaken using multi-dimensional scaling (MDS). Consensus clustering (*ConsensusClusterPlus)* was used to identify sample clusters using k-means clustering, anchored to cluster optimization based on the area under the cumulative distribution function (CDF) curve. The clinical data collected that was used for the analysis was complete and without any missing values.

Differentially expressed proteins (DEPs) were determined using pairwise t-test with statistical adjustment for multiple comparisons (Benjamini-Hochberg). Proteins considered significant required a fold change of greater than 2 or less than 0.5, along with an adjusted p-value of < 0.05. Weighted gene correlation network analysis [*WGCNA* (23)] was used to identify protein modules based on hierarchical clustering (23). The following parameters were used to define modules: power = 10, deepSplit = 2, maxCutHeight = 0.5. The module score was created by extracting the scaled protein expression data, multiplying each protein by the correlation value (to weight towards strong module members) and summing each protein.

Network analysis was undertaken using *clusterprofiler* (24) and enriched for gene ontology, while drug/disease data was extracted from EnrichR's web tool (25). Drug data was

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annotated manually. In brief, drug networks containing at least 6 protein module members with a p-value of < 4.0 x 10^{-5} were selected then classified based on literature review. Visualizations were undertaken using the packages *ggplot2* (26), *corrplot* (27)= and *pheatmap* (28). A random forest classification model was trained on 75% of the cohort samples, with the intent of classifying ACPA positivity samples from ACPA negative, using the *tidymodels* (29) package in R (trees = 1000). Variable importance was extracted from the model using the package *vip* (30).

Results

Upregulation of soluble mediators in ACPA positive compared to ACPA negative FDR

Using a 48-plex cytokine/chemokine array as outlined in the Methods, we initially sought to determine whether there were group differences in the circulating profile of ACPA positive FDRs (n = 48) compared to ACPA negative FDRs (n = 99), irrespective of the outcome in the ACPA positive group. We selected serum samples from 117 individuals (n = 147 serum samples) to balance age and sex, and to include participants with well-defined longitudinal outcomes (Table 1). Importantly, all sera analyzed were from the pre-clinical phase of the longitudinal cohort study and none of the participants had inflammatory arthritis. Principle components analysis (PCA) performed using all of the measured proteins revealed substantial overlap between ACPA negative and ACPA positive samples (Figure 1A).

K-means clustering identified 6 clusters (Figure S2) of samples, which separated on the basis of protein expression in PCA space (Figure 1A). Interestingly, cluster enrichment was observed on the basis of ACPA status, with Cluster 4 containing a significant proportion of ACPA negative samples (38.4% overall, p = 0.002, 86.3% within cluster) and Cluster 6 containing a significant proportion of ACPA positive samples (25.0% overall, p < 0.001, Figure 1B, 92.8% within cluster), and individuals who progressed into inflammatory arthritis (50.0% overall, 42.9% within cluster, p = 0.0003). To determine other factors that may influence circulating cytokines expression, we analyzed cluster assignment differences in Sex, Age and BMI (Table 2). Sex differences did not reach statistical significance; however, the proportion of Females in Cluster 6 (enriched in ACPA positive samples) was highest amongst the 6 clusters (92.8%). Age was the lowest in cluster 5 (27.9), which reached significance when compared to cluster 2 (46.4), cluster 3 (49.3) and cluster 4 (46.0). CCP3 level was highest in Cluster 6 and reach statistical significance in comparison to Cluster 1 and 4. BMI showed no significant differences based on cluster assignment.

TABLE 1 Demographics of serum samples.

	ACPA negative (n = 99)	ACPA positive (n = 48)	p-value
% Female (n)	68.7 (68)	77.1 (37)	0.389
Age (IQR)	43.8 (22.8)	35.9 (25.0)	0.239
BMI (IQR)	28 (8.9)	28.7 (8.9)	0.574
CCP3 (IQR)	2.6 (5.9)	141.0 (224)	p < 0.0001

BMI, body mass index. Analyzed by Wilcoxon rank sum test and chi-square.
CCP3: third generation cyclic citrullinated protein antibodies.

Next, differential expression of proteins (DEPs) was determined between ACPA positive and negative samples, which identified 13 DEPs, all of which were upregulated after adjusting for multiple comparisons (Table S1; Figure 1C). The highest upregulated proteins in ACPA positive samples included CCL11, IFNG, IL1B and IL1A (Figure 1D). These data suggest that differentially expressed soluble mediators in ACPA positive sera are nearly exclusively upregulated, suggestive of a pro-inflammatory state that is synchronous with subclinical RA autoimmunity.

Longitudinal outcomes differ on the basis of cytokine profiles in FDR of RA patients

Given the DEPs identified between ACPA positive and negative individuals, we next sought to understand if the upregulation of cytokines/chemokines varied on the basis of longitudinal outcomes. As indicated above, we have previously observed that a proportion of individuals who are ACPA positive will seroconvert to an ACPA negative state over follow up (5). We classified longitudinal outcomes in ACPA positive samples as: 1. Seroconversion (ACPA negative after follow-up) 2. ACPA positive persistence or 3. ACPA positive progression into inflammatory arthritis (Table S2, see Methods). Prior to analyzing differences between these groups, we sought to determine the longitudinal stability of the levels of the analytes in samples from persistently ACPA negative individuals. This analysis revealed no statistically significant differences in any of the proteins after a mean of 782 days between sample collection, suggesting protein expression is stable over time in individuals whose autoantibody and clinical status did not change (Figure S3; Table S3).

We then compared the expression levels in the groups outlined above. We observed substantial differences in multiple proteins based on this clinical classification. IL2, IFNG, IL1A, IL1B all displayed a similar pattern of expression, where individuals who subsequently progressed to develop IA had the highest levels, while individuals whose seropositivity disappeared over time were no different

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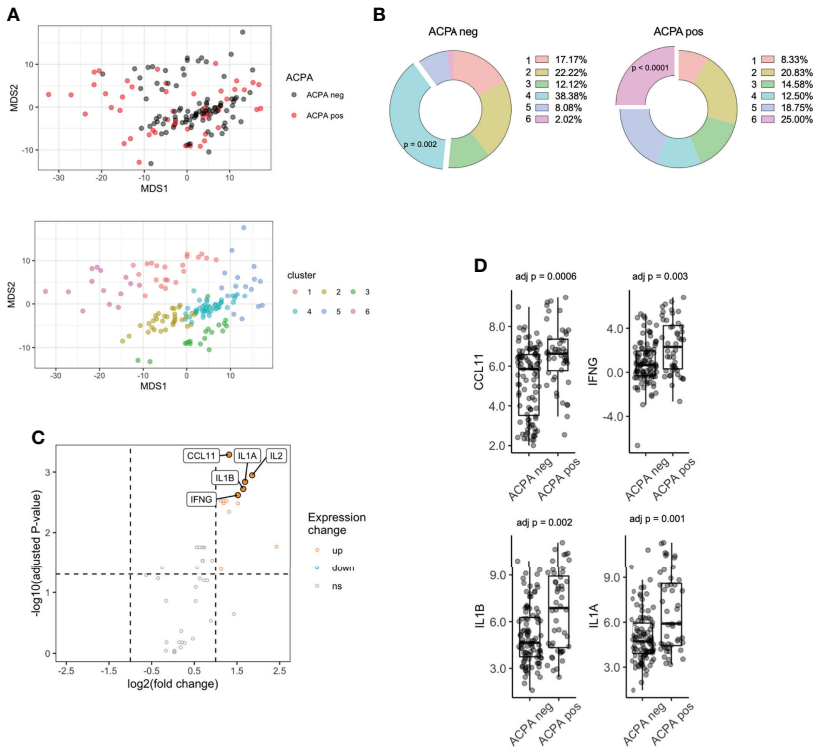

FIGURE 1
Serum proteomics differences between ACPA positive and ACPA negative FDR of RA patients. **(A)** Multi-dimensional scaling (MDS) plot of FDR sera based on 48-plex proteomics coloured by ACPA status (top) or consensus cluster (bottom, n = 6). **(B)** Cluster assignment enrichment based on ACPA status. Clusters analyzed by chi-square test. **(C)** Volcano plot of differentially expressed proteins, colored by upregulated (fold change > 2) and downregulated (fold change < 0.5) in ACPA positive group. Analyzed by pairwise t-test adjusted by Benjamini-Hochberg. **(D)** Box plots of CCL11, IFNG, IL1B, IL1A comparing ACPA positive and ACPA negative groups. ACPA, anti-citrullinated protein antibodies; FDR, first-degree relatives; CCL11, C-C Motif Chemokine Ligand 11; IFNG, Interferon gamma; IL1B, Interleukin 1 Beta; IL1A, Interleukin 1 Alpha. ns = not significant.

compared to persistently ACPA negative controls (Figure 2A; Supplementary Files). Although this was observed for these pro-inflammatory cytokines, some proteins, for example CCL11, showed the opposite pattern, with the highest expression in individuals whose seropositivity resolved rather than the IA progressor group.

Since we (22), and others (18), had previously demonstrated that individuals in whom ACPA exhibited high levels of Fab glycosylation were most likely to progress to IA, we next sought to determine if there was a relationship between cytokine/chemokine expression patterns and Fab glycosylation levels of ACPA. We observed a significant positive correlation between

TABLE 2 Demographics of serum samples split by cluster assignment.

	Cluster 1 (n = 21)	Cluster 2 (n = 32)	Cluster 3 (n = 19)	Cluster 4 (n = 44)	Cluster 5 (n = 17)	Cluster 6 (n = 14)	p-value
% Female (n)	71.4 (15)	81.3 (26)	68.4 (13)	68.2 (30)	47.1 (8)	92.8 (13)	p = 0.075
Age (IQR)	34.1 (24.4)	46.4 (18.4)	49.3 (21.6)	46.0 (25.2)	**27.9 (10.8)**	39.1 (23.6)	5 vs 2 (p = 0.002) 5 vs 3 (p = 0.01) 5 vs 4 (p = 0.008)
BMI (IQR)	31.1 (13.3)	28.0 (10.3)	27.6 (7.6)	29.1 (7.5)	27.4 (8.4)	27.3 (8.7)	ns
% Progression (n)	0 (0)	9.4 (3)	10.5 (2)	0 (0)	5.9 (1)	**42.9 (6)**	p = 0.0003
CCP3 (IQR)	2.6 (16.5)	5.2 (107.0)	6.5 (87.6)	4.45 (7.29)	66.8 (107)	**132.0 (252.0)**	6 vs 1 (p = 0.01), 6 vs 4 (p = 0.002)

CCP3, third generation cyclic citrullinated protein antibodies; BMI, body mass index; ACPA, anti-citrullinated protein antibodies; ACPA, ACPA negative; ACPA seroconv, ACPA seroconversion, ACPA +: ACPA positive, ACPA + prog: ACPA positive progressor. Analyzed by Dunn's test and chi-square; corrected for multiple comparisons with Benjamini-Hochberg. Bold, means statistically significant vs multiple clusters. ns = not significant.

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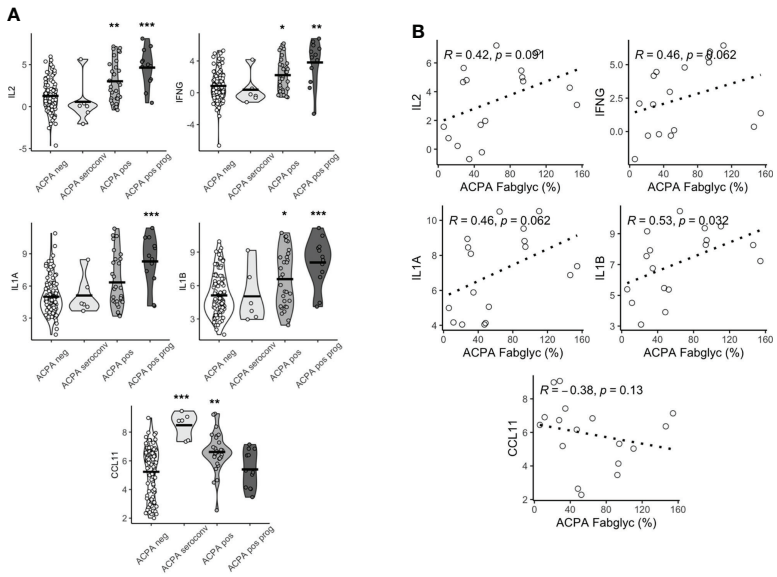

FIGURE 2
Baseline serum proteomics varies based on longitudinal outcomes. **(A)** Violin plots of CCL11, IFNG, IL2, IL1A, IL1B based on longitudinal outcomes, ACPA negative, ACPA seroconversion, ACPA positive and ACPA positive progression (prog). Analyzed by Dunn's test adjusted by holm. ***p < 0.001, **p < 0.01, *p < 0.05. **(B)** Spearman correlation plots of IL2, IFNG, IL1A, IL1B and CCL11 and ACPA Fab glycosylation. ACPA, anti-citrullinated protein antibodies; Fab, variable domain region of IgG; CCL11, C-C Motif Chemokine Ligand 11; IFNG, Interferon gamma; IL1B, Interleukin 1 Beta; IL1A, Interleukin 1 Alpha; IL2, Interleukin 2.

the highest DEPs (IL2, IFNG, IL1A and IL1B) and Fab ACPA glycosylation (Figure 2B), which was only available for 17 samples. We also observed a negative but non-significant correlation between CCL11 and Fab ACPA glycosylation. This suggests that a specific serum cytokine/chemokine expression pattern is associated with heavily Fab glycosylated ACPA, a demonstrated major predictor of future RA.

A co-ordinated serum protein network in pre-clinical RA is associated with arthritis onset.

Given that several individual circulating cytokine/chemokine proteins were associated with a high risk of RA development, we next sought to define the co-ordination of the serum cytokine/chemokine proteome. Using a network algorithm (WGCNA), protein co-expression was split into 2 modules (Grey, n=22 and Teal, n = 23) on the basis of hierarchical clustering. The relationship between module eigengene expression score and predefined clinical traits was positive for the Teal module, with a significant correlation for both ACPA seropositivity (Figure 3A, R = 0.34, p < 0.0001) and longitudinal clinical outcomes (state; R = 0.44, p < 0.0001). Correlation between module members was indeed strong (range 0.27 to 0.92) as visualized in a correlation matrix (Figure 3B). A module score was calculated and found to be higher in ACPA positive FDR compared to ACPA negative FDR (7.15 vs -3.46, p = 0.0005, Figure 3C). When categorized into longitudinal clinical outcomes, compared to ACPA negative FDR, those who eventually developed arthritis (Progression) had the highest baseline module score (19.8 vs -3.46). Interestingly, there was no difference in serum module score between individuals who underwent ACPA seroconversion, and ACPA negative FDR. Numerically, modules scores increased based on clinical state, from ACPA negative (-3.46) and ACPA seroconversion (-12.8), to persistent ACPA positivity (6.06) and Progression into inflammatory arthritis (19.8, Figure 3D). Furthermore, module score correlated significantly with ACPA Fab glycosylation (Figure 3E, R = 0.49, p = 0.049). These data suggest that a co-ordinated protein network associates with key longitudinal outcomes in at-risk FDR of RA patients.

Machine learning to classify ACPA positive from ACPA negative FDR

ACPA seropositivity remains a key biomarker in predicting future RA. We were able to identify serum proteins that associated with ACPA status, however the utility of serum proteomics to classify pre-clinical autoantibody positive individuals is not clear. Using chi-square test, hierarchical clustering with DEP's enriched in ACPA positive pre-clinical samples was able to classify

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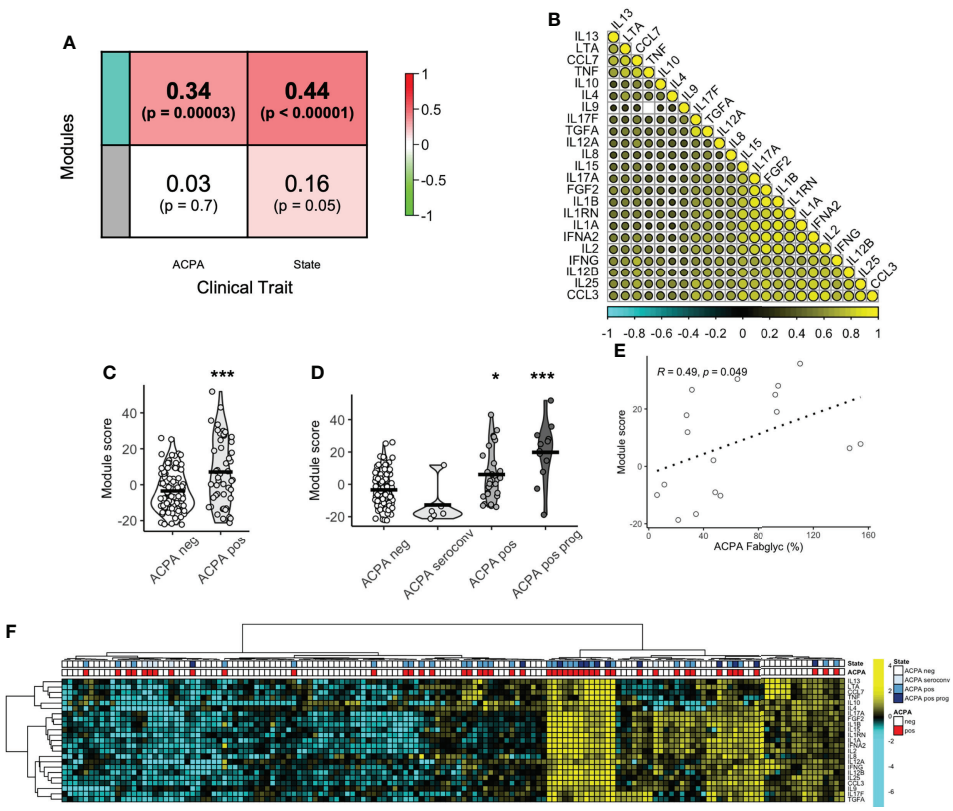

FIGURE 3
Protein co-expression network enriched in ACPA positive FDR. **(A)** Correlation coefficient between protein co-expression modules (Teal, Grey) and ACPA or longitudinal outcome (state), colored based on strength of association. **(B)** Correlation matrix of protein module members (Teal) colored based on strength of correlation (range -1 to 1). **(C)** Box plots of module score comparing ACPA positive and ACPA negative groups. Analyzed by t-test, *** $p < 0.001$. **(D)** Violin plots of module score based on longitudinal outcomes, ACPA negative, ACPA seroconversion, ACPA positive and ACPA positive progression (prog). Analyzed by Dunn's test adjusted by holm. ***$p < 0.001$, *$p < 0.05$. **(E)** Spearman correlation plot of module score and ACPA Fab glycosylation. **(F)** Heatmap of co-expression network protein members ordered by hierarchical clustering and colored by ACPA status and longitudinal outcomes. ACPA, anti-citrullinated protein antibodies; Fab, variable domain region of IgG.

ACPA positive individuals from ACPA negative (Figure 3F, p = 0.008). However, clustering performed poorly as a diagnostic test to identify ACPA positive samples even without the stringency of a train-test approach (Accuracy 58.5%, Sensitivity 72.9%, Specificity 51.5%). Thus, we next sought to determine the value of combining machine learning with serum proteomics to classify FDR with pre-clinical ACPA. Random forest (RF) classification achieved 100% accuracy in the training cohort (n = 104), and 80.5% accuracy in the test cohort (n = 36, Sensitivity 54.6%, Specificity 92.0%, Figure 4A). In the training cohort the RF model provided an area under the curve (AUC) value of 0.767 (Figure 4B). Model variable importance included identified predominant members of the protein module network that was identified previously (Figure 4C). These results suggest that ACPA status may be classifiable on the basis of serum proteomics, further strengthening the evidence that autoantibody positive individuals display a measurable co-ordinated serum protein expression profile that is distinct from FDR who are ACPA negative.

Serum protein network proteins are linked to JAK-STAT activation and pharmaceutical targets

We next aimed to determine the underlying biological pathways of the module protein members, from the WGCNA analysis. Gene enrichment identified several pro-inflammatory pathways that were upregulated on the basis of module protein members. Notably, the pathway *positive regulation of cytokine production* contained 16 of 23 module members and was found to the most significantly enriched pathway (Figure 5A). The remaining top pathways all included references to JAK-STAT enrichment, a pathway known to be active in individuals with established RA (31). Indeed, *JAK-STAT activation* connected

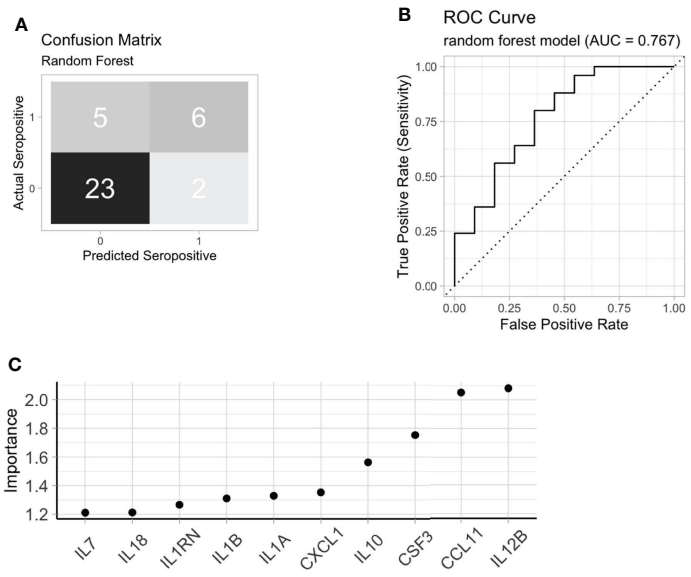

FIGURE 4
Machine learning to identify ACPA positive FDR. **(A)** A random forest model confusion matrix on a test cohort (n = 36). **(B)** Receiver operating curve (ROC) of random forest model to classify ACPA positive FDR from ACPA negative FDR based on test cohort. **(C)** Variable importance extracted from the random forest model. FDR: first-degree relatives, ACPA: anti-citrullinated protein antibodies.

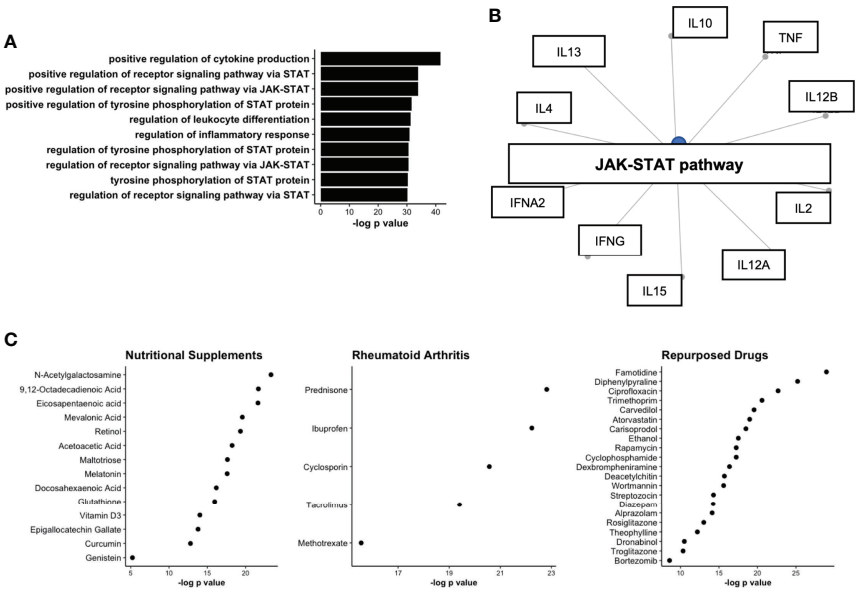

FIGURE 5
Network analysis of protein co-expression module upregulated in ACPA positive FDR. **(A)** Upregulated GO pathways based on proteins identified in co-expression network. **(B)** Gene concept plot displaying common node (JAK-STAT), connecting protein module members. **(C)** Drug network analysis split by broad classes of pharmaceutical including nutritional supplements, Rheumatoid Arthritis treatments, and drugs approved for other diseases/conditions (Repurposed). STAT, signal transducer and activator of transcription; JAK, Janus Kinase; ACPA, anti-citrullinated protein antibodies.

several module members, suggestive of a common node that might be responsible for the co-ordinated serum protein network (Figure 5B). We next sought to determine drug targets that could be deployed to disrupt this network and generate hypothetical treatment options for RA prevention studies. The Drug Signatures Database (*EnrichR*) identified 1030 drugs associated with expression of module proteins after adjustment (Table S4). Following strict filtering, 3 broad categories of drugs were identified 1. Nutritional Supplements 2. Drugs commonly used to treat RA (*Rheumatoid Arthritis*) and 3. Drugs that are commonly used in medical practise, but not typically for treatment of RA (*Repurposed*, Figure 5C). Notably, nutritional supplements identified included: Vitamin D3 and Omega-3 fatty acids, which have recently been shown to reduce incident autoimmune disease in a healthy population (32). These results suggest that serum protein networks upregulated in pre-clinical RA may be modulated with medications that target JAK-STAT signalling.

Discussion

The biological processes that precede the onset of clinically detectable RA remain unclear despite extensive documentation by many groups after a prolonged pre-clinical period (5, 8, 33). This preclinical period is defined primarily by the presence of ACPA and other RA associated autoantibodies in otherwise unaffected individuals (33). Although the detection of ACPA remains a strong predictor of future RA development, we have previously demonstrated that a substantial proportion of prospectively followed ACPA positive FDR of RA patients do not develop RA, and indeed not uncommonly seroconvert to an ACPA negative state (5). This observation suggests that breaking immune tolerance to citrullinated endogenous autoantigens is not an irreversible process that inexorably leads to pathogenic autoimmunity and RA. As such, it of considerable interest to identify immunological process that not only associate with ACPA development, but also with the evolution and maturation of this autoimmunity to become pathogenic. In the current study we have focused on the circulating cytokine/chemokine networks that are cross-sectionally associated with ACPA seropositivity, and particularly on the utility of these networks in predicting future RA onset in individuals who ultimately developed RA. We demonstrate that these networks are enriched in proteins associated with activation of the JAK-STAT pathway, which is known to play a key role in RA pathogenesis.

Several studies of ACPA positive individuals who ultimately developed RA have shown that as RA onset approaches, the ACPA response exhibits epitope spreading with expansion of the targeted autoantigens and increasing levels of glycosylation in the variable region domains, both of which are associated with increases in the circulating levels of these autoantibodies (5, 18). These observations are consistent with an immunological maturation of the autoimmune response that requires close interactions between B and T lymphocytes, a process that typically takes place in germinal centers located in lymphoid tissues (1). It remains abundantly clear that sustainment and maturation of autoantibody production in pre-clinical RA is representative of a key step towards the onset of clinically apparent disease. Interrupting this process may be crucial in the prevention of RA in at-risk, clinically quiescent individuals (4).

We identified a co-expression network of serum proteins that associated with both baseline seropositivity status and longitudinal outcomes in this at-risk cohort. The network, made up of roughly 50% of our measured proteins, was enriched for biological for processes suggestive of JAK-STAT activation. It is well established that the JAK-STAT pathway plays a key role in orchestrating several important immune responses, many of which are upregulated in those with established RA (31). Perhaps the most convincing evidence of JAK-STAT activation in RA, is the robust efficacy of JAK inhibitors to control RA-related disease activity, achieving response rates that exceed those of TNF inhibitors, the gold standard of initial RA biologic therapy (34). The family of JAKS includes 4 tyrosine kinases including JAK1, JAK2, JAK3 and TYK2, which govern essential cytokine responses including IL-6, and type 1/2 interferons. Binding of a cytokine/chemokine to a respective receptor initiates an intracellular cascade of events, most importantly phosphorylation of JAK proteins, phosphorylation and dimerization of STAT, and translocation of STAT to the nucleus to initiate transcription of target genes (31). Despite its prominent role in RA, the role of JAK-STAT signalling in pre-clinical RA is not known, and our data is the first to associate JAK-STAT activation in pre-clinical autoantibody development and arthritis onset.

The interplay between autoantibody development and other dysregulated features of the immune system remains relatively unexplored, however we have previously observed innate immune protein signatures that associate with progression into RA (19). The present study builds off of this work, by seeking to address differences between FDR who are ACPA positive, compared to those that are not. Past studies have also indicated familial clustering of cytokines (35) and upregulation of type-1 interferon (36) in pre-clinical RA. It is entirely unclear if cytokines/chemokines drive autoantibody formation, or if ACPA themselves lead to dysregulated immune responses, which has been show in established RA (37, 38). Identifying activated biological pathways that facilitate autoantibody formation may provide an opportunity to deploy targeted therapies to modulate pre-clinical autoimmunity, with the intention of preventing future RA in seropositive individuals (39). Indeed, our data and others (36), suggests that key protein networks activated in pre-clinical RA such as JAK-STAT signalling, may be modulated by certain nutritional supplements, with the possible added benefit a favorable safety profile for deployment in prevention studies (32).

In conclusion, these data suggest that serum proteomics differ significantly on the basis of autoantibody status amongst

FDR of RA patients, and a co-expression network of proteins associates with longitudinal outcomes in pre-clinical RA. Replication of these data in future studies will help establish a working model for predicting incident RA in at-risk populations using a combination of serum and clinical biomarkers.

Data availability statement

The data presented in the study are deposited in the Mendeley Data Repository, doi: 10.17632/rf3vbm4ykm.1

Ethics statement

The studies involving human participants were reviewed and approved by University of Manitoba REB. The patients/participants provided their written informed consent to participate in this study.

Author contributions

LO'N conceptualized study, performed data analysis and wrote the manuscript. XM performed all CCP testing. CM maintains cohort, recruitment, clinical data. MF generated all of the proteomic data. HE=G conceptualized study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2022.958145/full#supplementary-material

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Detection of rare autoreactive T cell subsets in patients with pemphigus vulgaris

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Analysis of T lymphocyte proliferation and activation after antigenic or mitogenic stimulation is a vital parameter used in the diagnosis of various immuno-deficiencies and during the monitoring of treatment responses. Most applied techniques are based on the incorporation of tritiated thymidine (^{3}H-TdR) or ELISPOT analysis, both rely on rather time-consuming/-intensive *ex vivo* protocols or encompass inherent drawbacks such as the inability to distinguish specific cell populations (^{3}H-TdR, ELISPOT) or focus on a single cytokine (ELISPOT). Here we aimed at characterizing the rapid expression of intracellular CD154 (CD40L) as a marker for rare antigen-specific CD4+ T cells in pemphigus vulgaris (PV). Upon stimulation with human desmoglein (Dsg) 3, the major autoantigen in PV, the expression of CD154 was significantly increased in PV patients compared to healthy controls (HC) and correlated with anti-Dsg3 IgG titers. Patients with active disease showed higher numbers of Dsg3-reactive CD4+ T cells in CXCR5+ T follicular helper cells. In remittent PV and HC, CXCR5+CD4+ T cells remained largely unaffected by Dsg3. IL-17 and IL-21 expression were significantly induced only in CD154+CD4+ T cells from PV patients, lending themselves as potential novel treatment targets. Additionally, stimulation with immunodominant Dsg3-derived epitopes strongly induced a CD4+ T cell response *via* CD40-CD154 interaction similar to the human Dsg3 protein. We here established a rapid *ex vivo* assay allowing the detection of Dsg3-reactive CD4+ T cells from activated systemically available PBMCs, which further supports the crucial concept of antigen-specific T cells in the pathogenesis of PV.

KEYWORDS

CD154, CD40L, CD4+ T cells, pemphigus, autoreactive, desmoglein, autoimmunity

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Introduction

Pemphigus vulgaris (PV) is a rare and potentially life-threatening autoimmune disease, characterized by a production of autoantibodies targeting desmosomal proteins, namely desmoglein (Dsg)3 and/or Dsg1 (1). Binding results in a loss of adhesion between keratinocytes (referred to as acantholysis), leading to the occurrence of intraepidermal blisters and erosions of skin and/or mucous membranes (2). Different expression patterns of Dsg3 and Dsg1 give rise to either the mucosal-dominant PV (Dsg3) or the primarily skin-associated pemphigus foliaceus (PF; Dsg1) (1, 3). Pathogenic anti-Dsg3 and -Dsg1 IgG autoantibodies recognize both N- and COOH-terminal epitopes of the human Dsg3 ectodomain and are widely known to play a crucial role in the manifestation of PV (4, 5). Autoantibody formation originates in the activation of Dsg3-reactive CD4+ T cells by Dsg3 peptides binding to HLA class II and *via* CD40-CD40L-dependent T cell-B cell interaction. This Th2/Th17 driven process is critical for the induction and maintenance of autoreactive memory B cells as precursors of autoantibody-producing plasma cells (6–9). It is widely regarded that the HLA class II alleles HLA-DRB1*04:02 and HLA-DQB1*05:03 confer susceptibility to PV (10) and Dsg3-reactive CD4+ T cell responses against the ectodomain of Dsg3 were identified in PV patients (11). So far, several Dsg3 peptides were identified as potential immunodominant Dsg3 epitopes recognized by CD4+ T cells, which in the context of the PV-associated HLA-alleles, were shown to induce the formation of Dsg3-specific IgG in a humanized HLA-transgenic mouse model (12).

Analysis of T lymphocyte proliferative responses to antigenic or mitogenic stimuli is a vital parameter used in both diagnosis and monitoring of a variety of immune responses. Most commonly applied techniques are based on the incorporation of tritiated thymidine (^{3}H-TdR) or enzyme-linked immunospot (ELISPOT) analysis (13, 14). Both rely on a rather long *ex vivo* expansion periods and stimulation protocols (up to 11 days). Additionally, these methods contain inherent drawbacks such as the inability to distinguish specific cell populations (3H-TdR, ELISPOT) using peripheral blood cells as well as the assumption of the importance of certain cytokines (ELISPOT) (15–17). Rapid identification and quantification of rare antigen-specific T cell numbers as a marker for treatment-response is of importance in diseases such as PV (18). To overcome this obstacle, here we introduce a concept for the assessment of antigen-specific CD4+ T cells based on CD154 expression after short-term *ex vivo* stimulation with defined antigens or epitopes. CD154 (CD40 ligand; CD40L) is an activation-induced T cell surface glycoprotein belonging to the tumor necrosis factor receptor (TNFR) superfamily. In T cell-mediated immune responses and inflammation, interactions between CD154 and CD40 provide essential signals in T cell priming and T cell effector functions and subsequently induce B cell proliferation and differentiation, isotype-switching, and formation of memory B cells (19–21). CD154 is transiently expressed on activated, but not resting CD4+ T cells, therefore, it cannot be used in long-term assays for the analysis of antigen-specific CD4+ T cells (20). Its expression is directly correlated to CD4+ T cells specific for defined antigens in several diseases such as pathogenic infection, viral immunity, and self-tolerance (22–24). Its increased expression was observed as a marker of disease activity in rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus (25–27). Moreover, in a pemphigus mouse model, administration of anti-CD154 mAb blocked anti-Dsg3 IgG production and prevented blister formation (28). The latter indicates the quintessence of CD40-CD154 interaction for the induction of pathogenic IgG anti-Dsg3 antibodies and, when blocked, induction of tolerance.

In this study, we sought to analyze the presence of antigen-specific CD4+ T cells with regards to an activation marker CD154 combined with previously suggested cytokine analysis in PV patients using multicolor flow cytometry. By using full-length human Dsg3 or Dsg3-specific epitopes for *ex vivo* stimulation, we show that expression of the activating CD154 of PBMCs from PV patients can be found in CXCR5+ T follicular helper cells associated with IL-21 expression, further supporting the concept that antigen-specific T cells are central for the pathogenesis of PV. These findings underline the concept that activating T cell responses against Dsg3 may be critical in driving the IgG-dependent immune pathogenesis and identify IL-21 as a potential target in pemphigus.

Materials and methods

Study participants

A total of 33 patients with PV were included in this study by the Department of Dermatology and Allergology at the University Hospital Giessen and Marburg and the Department of Dermatology, Venereology and Allergology at the Charité–Universitätsmedizin Berlin (Table 1). This study was approved by the Ethics Committees of the Medical Faculty of the Philipps-Universität Marburg (Az. 20/14) and the Charité Berlin (AZ: EA4/194/19) and was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants. Inclusion of all tested subjects, apart from clinical presentation of erosions and flaccid blisters on the skin and/or mucous membrane, was made using at least one of the following diagnostic criteria: serological analysis of titers of circulating anti-Dsg1 and anti-Dsg3 IgG autoantibodies, histopathological evidence of intraepidermal acantholysis and/or epidermal intracellular deposition of IgG and/or complement factor C3 using direct immunofluorescence of perilesional skin biopsy and/or indirect immunofluorescence using monkey

esophagus. To distinguish mucocutaneous from the mucosal-dominant pemphigus phenotype, serological autoantibody titers were analyzed in serum using both the commercially available anti-Dsg1 and anti-Dsg3-ELISA (Euroimmun, Lübeck, Germany). PV patients were further classified into disease stages of relapse (active) and remission with respect to their clinical disease activity and systemic therapy (29). Epidemiological data, clinical status, therapy and Dsg1 or 3 titers at the time of analysis are shown in the Supplementary Table 1.

Healthy controls (HC; n=23) were recruited at the Department of Dermatology and Allergology and had no history of autoimmune skin diseases (Supplementary Table 2).

Human Dsg3 and peptides

The extracellular domain of human Dsg3 was produced in baculovirus-infected insect cells (High Five, Carlsbad, CA, USA) as described previously (30). Proteins were purified by affinity chromatography using nickel-nitrilotriacetic agarose beads (Quiagen, Hilden, Germany) according to the manufacturer's instructions. Purified protein was gradually dialyzed against PBS supplemented with 0.5mmol/L $CaCl_2$. Quality control was done by coomassie staining, ELISA with patients' sera and western-blot analysis with Dsg3-specific antibodies. Fifteen-mer Dsg3 peptides used for *ex vivo* stimulation were synthesized by ProImmune (thinkpeptides, Oxford, UK; Figure 3A).

Antigen stimulation

Human peripheral blood mononuclear cells (PBMCs) were isolated from citrate-phosphate-dextrose-adenine (CPDA)-containing peripheral blood samples by density gradient sedimentation using Lymphocyte Separation Medium (Capricorn Scientific, Ebsdorfergrund, Germany), washed twice in PBS and plated in round-bottom 96-well plates at a concentration of 3 x 10^5 cells/200µl in a complete RPMI 1640 medium (Capricorn Scientific, Ebsdorfergrund, Germany) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO). Freshly isolated cells were immediately stimulated after PBMC isolation. Thawed PBMCs were first rested for 22 hours at 37°C and 5% humidified CO_2 to improve antigen-induced CD154 expression. Subsequently, PBMCs were stimulated with one of the following: recombinant human $Dsg3_{1-566}$ (10-20 µg/ml), four Dsg3 epitopes ($P1_{190-204}$, $P2_{206-220}$, $P3_{251-265}$, $P4_{375-391}$; 10 µg/ml), PHA (phytohemagglutinin, 10 µg/ml, Sigma-Aldrich, St. Louis, MO) used as a positive control and unstimulated PBMCs served as a negative control. All samples were treated with Brefeldin A (10 µg/ml, Invitrogen by Thermo Fisher Scientific, Waltham, Massachusetts) 4 hours prior to analysis. Subsequently, cells were stained for flow cytometry as described below.

Flow cytometry staining and data analysis

For flow cytometry, cells were coated with Fc-block (10 µl of 1: 100 diluted TruStain Fc block, Biolegend, San Diego, CA) for 10 minutes and then stained with fluorescently labeled antibodies for 30 minutes at room temperature. For extracellular staining, the following monoclonal antibodies were used: CD4-AF 700 (RPA-T4, BD Biosciences, San Jose, CA, USA), CD8-PE-Cy7 (SK1), CXCR3-BV421 (G025H7), CD4-BV510 (RPA-T4), CD45RA-FITC (HI100), CD3-PerCP-Cy5.5 (SK7), CXCR5-PE (J252D4), CCR6-APC (G034E3; all BioLegend). For intracellular staining, cells were fixed and permeabilized using fixation/permeabilization buffer (eBioscience FOXP3/Transcription; Invitrogen, San Diego, CA) and stained intracellularly for 20 minutes at 4°C for functional readouts in permwash buffer (eBioscience FOXP3/Transcription; Invitrogen, San Diego, CA). For intracellular staining, the following monoclonal antibodies were used: CD154-BV421 (24-31), CD154-PE/Cyanine7 (24-31; both BioLegend), IL-4-BV786 (MP4-25D2), IFN-γ-FITC (B27), IL-17A-BV650 (N49-653), IL-21-PE (3A3-N2.1; all BD Biosciences, San Jose, CA, USA). Samples were acquired on the BD FACS LSR Fortessa equipped with four lasers (BD Biosciences, San Jose, CA). Standard flow cytometry data analysis was performed using BD FACSDiva Software (BD Biosciences, San Jose, CA, USA) and FlowJo version 10.8 (BD Biosciences, San Jose, CA).

Statistical analysis

Statistical analysis was conducted using Graph Pad Prism version 8 software (GraphPad Software, La Jolla, CA). Statistical significance was calculated using a nonparametric, two-tailed unpaired Mann-Whitney U test and for multiple comparisons Kruskal-Wallis test followed by Dunn's multiple comparisons; *p<0.05, **p<0.01, ***p<0.001.

Results

Kinetic *ex vivo* analysis of CD154 expression

To establish optimal conditions for high CD154 expression, PBMCs were polyclonally stimulated *ex vivo* with PHA for 2, 6, 12, 16 and 22 hours. Using flow cytometry, relative frequencies of CD154-expressing cells out of the respective parent population were identified and followingly 5 major cell types were analyzed, namely: (1) CD3+ T cells, (2) CD3+CD4- T cells, (3) CD3+CD4+ T cells. To determine different CD4+ T cell subsets, we further stratified our gating strategy based on the

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surface expression of the CXCR5 chemokine that mediates T cell mobilization and homing into secondary lymphoid organs (31) (Figures 1A, B). This allows discrimination between (4) CD3+CD4+CXCR5- T helper (Th) and (5) CD3+CD4+CXCR5+ T follicular helper (Tfh) cells. Fresh PBMCs were, as compared to previous storage in liquid nitrogen, more responsive in their ability to produce CD154 expression (Supplementary Figure 1). With prolonged periods of *ex vivo* stimulation, an increasing CD154 expression up to 16 hours in CD4+ T cells was observed (Figure 1C), however, after 16 hours a notable decrease of CD154 expression with a decrease of viable cells was found (not shown). Based on these results, a 16-hour stimulation period of PBMCs was further applied. Analysis of CD8+ and CD4+ T cell subpopulations in view of their ability to express CD154 after specific (Dsg3) and nonspecific (PHA) stimulation showed that even though CD8+ T cells express CD154, it remained unchanged after stimulation (Figure 1D). In contrast, CD4+ T cells display a significant increase in CD154 expression after both antigen-specific (Dsg3) and polyclonal (PHA) stimulation (Figure 1D). In summary, a 16-hour stimulation interval of fresh PBMCs was found optimal for evaluation of CD154-expressing T cell responsiveness to stimuli.

CD154 marker allows for detection of autoreactive T cells in patients with pemphigus vulgaris

Next, a total of 25 PV patients and 22 HC (Supplementary Table 1) were further analyzed by applying the previously established optimal readout conditions. Patients were stratified into acute and remittent disease stage based on ABSIS score and

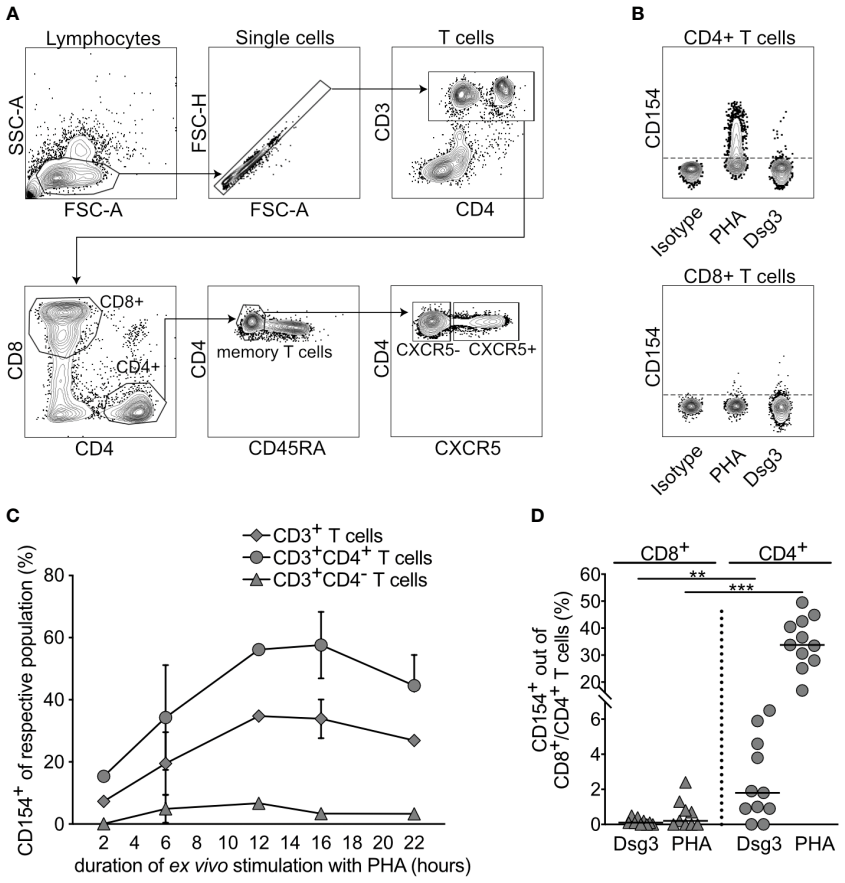


FIGURE 1
CD154 expression of CD3+ T cells and their subpopulations upon *ex vivo* stimulation. **(A)** Gating strategy to identify lymphocytes, single cells, T cells in peripheral blood, CD4+ and CD8+ T cells, memory T cells, CXCR5- and CXCR5+CD4+ T cells. FSC-A, forward scatter area; SSC-A, side scatter area; FSC-H, forward scatter height. **(B)** Representative flow cytometry plots portraying CD154 expression by CD4+ T cells upon antigenic (Dsg3) and mitogenic (PHA) *ex vivo* stimulation for 16 hours. **(C)** Kinetic analysis of relative CD154 frequency, identified by flow cytometry in peripheral blood, upon polyclonal stimulation out of respective parent population, i.e. CD3+ T cells, CD3+CD4+ T cells, CD3+CD4- T cells, CD3+CD4+CXCR5- T helper and CD3+CD4+CXCR5+T follicular helper cells, respectively. (n=1-3). **(D)** Relative frequency of CD154 expression on CD8+ and CD4+ T cells in peripheral blood of PV patients upon specific (Dsg3) or nonspecific (PHA) *ex vivo* stimulation for 16 hours (n=9-11). p<0.01 **, p<0.001 ***.

appearance of new clinical lesions for ≥ 3 month (29). Polyclonal T cell reactivity with PHA was used as positive control for each subject. To calculate changes in CD154 expression upon Dsg3 stimulation within the CD4+ T cells, relative frequencies of CD154 expression by unstimulated PBMCs were subtracted from the stimulated samples.

Stimulation with Dsg3 significantly increased CD154 expression in PBMCs derived from PV patients compared to HC (Figure 2A). Moreover, expression of CD154 on CD4+ T cells correlated positively with Dsg3 titers in pemphigus patients as shown by Spearman rank correlation (correlation coefficient 0.4634, p= 0.0197; Figure 2B). CD154+CD4+ T cells derived from PV patients were expressing significantly higher levels of IL-17 and IL-21 upon Dsg3 stimulation in contrast to HC and CD154-CD4+ T cells (Figures 2C, D). To further characterize CD4+ T cell subpopulations, the chemokine receptor CXCR5 was introduced, dividing CD4+ T cells into CXCR5-CD4+ Th and CXCR5+CD4+ Tfh cells (Figure 2E). Antigen-specific stimulation with Dsg3 resulted in a significant increase in CD154 expression on Th and Tfh cells alike, however, particularly in acute PV patients compared to HC and to a lesser extent in patients in clinical remission (Figure 2F). Taken together, CD154 marker expression associates with IL-17 and IL-21 expression specifically in PV patients, CXCR5+ Tfh cells react significantly upon Dsg3 stimulation allowing the detection of Dsg3-reactive CD4+ T cells in patients with PV.

Dsg3-derived peptide P2 stimulated CD4+ T cells comparably with Dsg3

The stimulatory effects using full protein might mask epitopes that are not recognized in a 3-dimensional structure. Besides the antigen-specific *ex vivo* stimulation with the full length Dsg3 and respective unstimulated control, we therefore analyzed the effects of a set of distinct Dsg3 epitopes for *ex vivo* stimulation. They all share a positively charged arginine (R) or lysine (K) at position 4 and are known to induce a proliferative *in vitro* response of peripheral T cells from PV patients. They are originally based on a peptide-binding algorithm for HLA-DRB1*04:02, which shares similar binding motifs to HLA-DQB1*05:03 (13). Human Dsg3 consists of 5 extracellular domains, two of which, namely EC1 and EC2, contain the immunodominant epitopes P1-P4 analyzed in this study in terms of their activating potency. Positions and aminoacidic sequences of P1-P4 are outlined in the Figure 3A. While the general trend in pemphigus patients was increased compared to HC, stimulation with P2 appeared to be the most potent (p=0.16; Figure 3B). Additionally, no statistical difference was found between the increase of CD154 expression induced by desmoglein 3 or $P2_{206-220}$ (Figure 3C). Considering T cell activation critically depends on the recognition of epitopes of the Dsg3 ectodomain, T cell epitopes of Dsg3 (specifically P2) may be sufficient to induce a robust CD4+ T and B cell response *via* CD40-CD154 against human Dsg3 leading to the production of pathogenic Dsg3 IgG antibodies.

Discussion

The CD40/CD40 ligand (CD40L; CD154) co-stimulatory system, which amplifies immune responses and potentially induces inflammation, is considered to have a prominent role in autoimmune skin manifestations (32). Specific T cell recognition is increasingly considered a key component in the disease control and maintaining efficient cellular immune responses. Detection and characterization of antigen-specific CD4+ T cells has extensively improved over the last years. Most applied techniques based on the incorporation of 3H-TdR or ELISPOT analysis, are, however, lengthy, single-cell-cytokine-driven or do not allow the assessment of which specific cell subpopulation is proliferating in response to Ag-specific stimulation (15–17). Current state-of-the-art technologies based on the usage of peptide-MHC multimers, such as dextramers, that directly assess antigen-specific Th cells and are not dependent on activation, may potentially miss functional T-cells that bear T-cell receptors (TCRs) with low affinity for a cognate antigen (33, 34). Moreover, the process of manufacturing particular reagents is costly and MHC alleles as well as immunodominant peptide epitopes must be defined in advance of manufacturing (35, 36). A potential improvement of the detection of autoreactive T cells can be achieved by *ex vivo* expansion prior to MHC multimer labeling, which on the downside, is very time-consuming. Therefore, the present study aimed at developing a specific, sensitive, and rapid method for the analysis of Dsg3-specific CD4+ T cells in whole PBMCs from PV patients by using CD154 activation marker. Unlike peptide-MHC multimers, CD154 *ex vivo* assay allows access to the whole population of CD4+ T cells specific for Dsg3, independent of MHC alleles or immunodominant peptide epitopes (20, 34).

Potent B cell activation followed by antibody production requires stimulation through the antigen receptor along with co-stimulation. CD40 receptor and its ligand CD154 (CD40L) are co-stimulatory molecules belonging to the TNFR superfamily. Binding of CD154 on CD4+ T cells to CD40 is a prerequisite for activation of B cells and other APCs (37, 38). T cell–dependent B cell activation is, in the immune pathogenesis of PV, critical for the induction of pathogenic IgG Abs, which directly induce epidermal loss of adhesion (12). Therefore, B cell depletion by the monoclonal anti-CD20 mAb rituximab in PV patients led to a decreased anti-Dsg3 IgG serum correlating with a marked downregulation of Dsg3-specific T cells (39). This strongly suggests that Dsg3-reactive T cells depend, to a great extent, on B cells as APCs (40, 41). Of note, when patients' peripheral lymphocytes were depleted of CD4+ T cells, anti-Dsg3 IgG–producing B cells were no longer detectable. Two independent

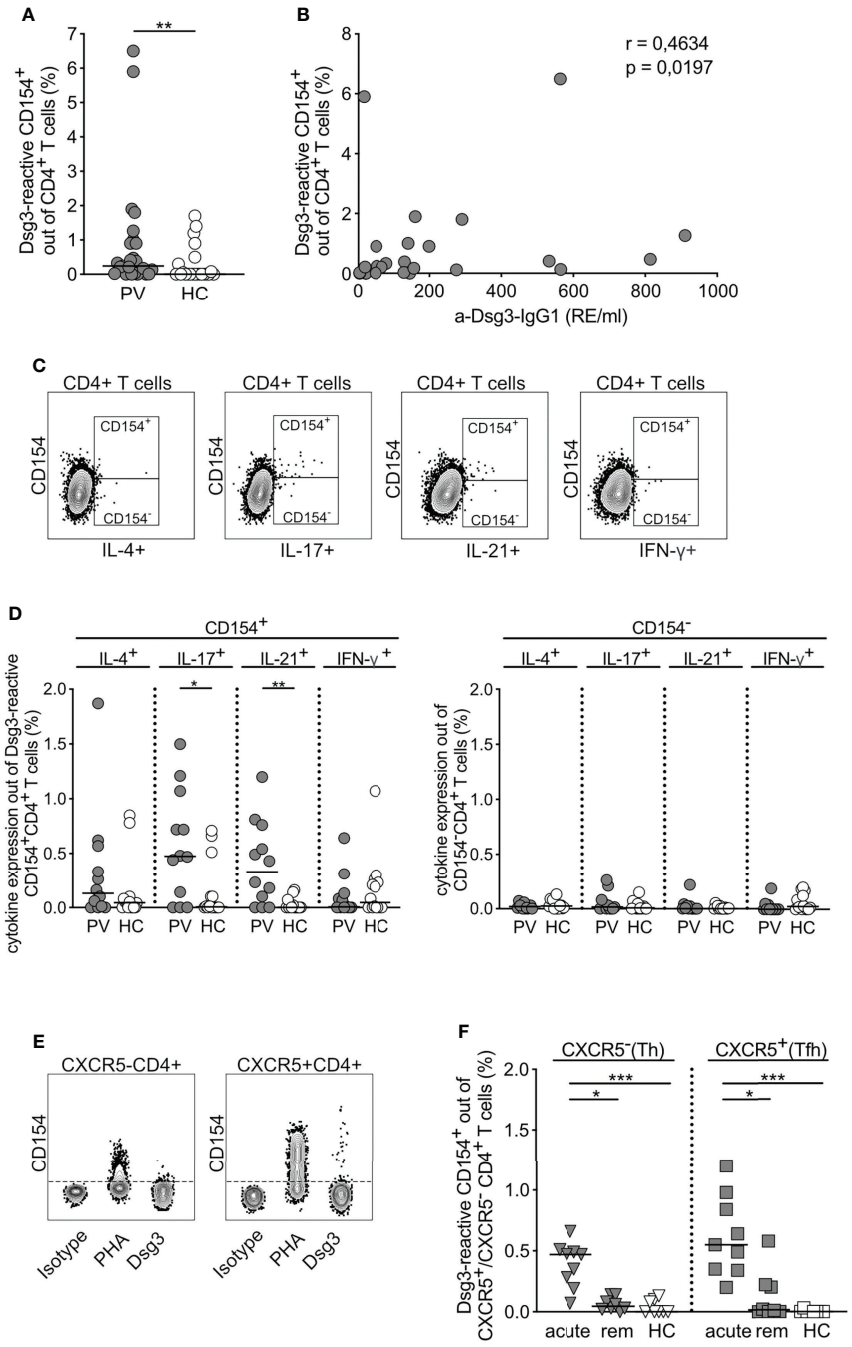
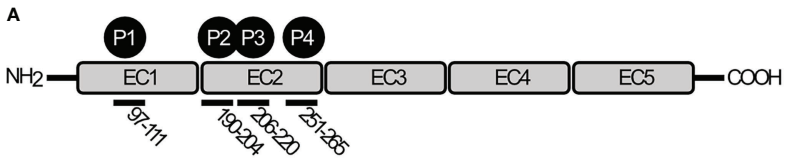


FIGURE 2
Characteristics of Dsg3-reactive CD154+ CD4+ T cells. **(A)** Relative frequency of CD154 expression upon *ex vivo* stimulation with Dsg3 in PV vs HC (PV n=25; HC n=22). **(B)** Correlation of anti-Dsg3-IgG1 serum concentration with relative frequencies of Dsg3-reactive CD154+CD4+ T cells in PV patients (n=25). Spearman's rank correlation coefficient r=0.4634 and p=0.0197. **(C)** Representative flow cytometry plots depicting CD154- and CD154+ CD4+ T cells upon specific Dsg3 stimulation and their respective cytokine secretion of IL-4, IL-17, IL-21 and IFN-γ. **(D)** Relative frequency of IL-4, IL-17, IL-21 or IFN-γ cytokine expression in Dsg3-reactive CD154+CD4+ T cells on the left and CD154-CD4+ T cells (right; PV n=12; HC n=14). **(E)** Representative flow cytometry plots depicting CXCR5 expression in CD4+ T cells upon respective stimulation. **(F)** Relative frequencies of CD154 on CXCR5-CD4+ T (Th) cells and CXCR5+CD4+ T (Tfh) cells for acute PV, remittent PV patients and HC upon specific *ex vivo* stimulation with Dsg3 (acute PV, n=9; remittent PV, n=8; HC, n=8). **(D)** p<0.05 *, p<0.01 **, p<0.001 ***.



Peptides	Peptide sequence	Peptide position
P1	FGIFVVDKNTGDINI	97-111
P2	LNSKIAFKIVSQEPA	190-204
P3	TPMFLLSRNTGEVRT	206-220
P4	CECNIKVKDVNDNFP	251-265

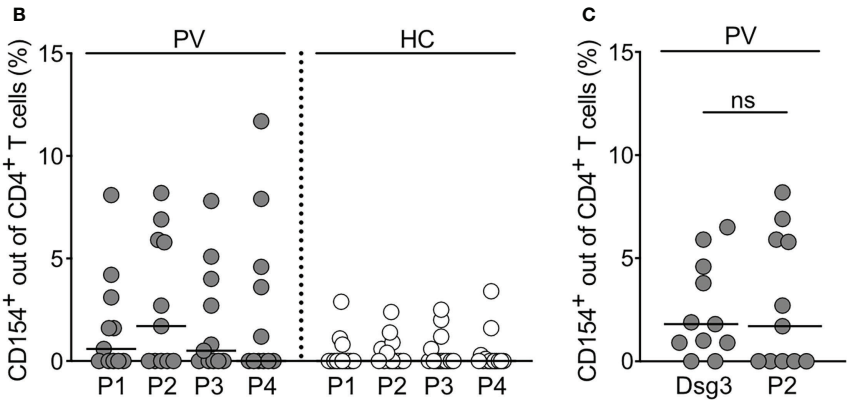


FIGURE 3
Induction of CD154 expression by Dsg3-specific T cell epitopes. **(A)** Summary of Dsg3-specific epitopes used for *ex vivo* stimulation. **(B)** Relative frequencies of CD154 expression in CD4+ T cells upon *ex vivo* stimulation with Dsg3-derived peptides (P1, P2, P3, P4) (PV n=11; HC n=14). **(C)** Relative frequency of CD154 expression out of CD4+ T cells upon *ex vivo* stimulation with Dsg3-derived peptide P2 and Dsg3 in PV patients (PV n=11).

groups showed in PV animal models that a single Dsg3-reactive T cell clone was sufficient to prime naive B cells to produce Dsg3-specific pathogenic IgG autoantibodies (42, 43). Therefore, using an analytic tool to detect T lymphocyte proliferative responses is of great importance in PV for both diagnosis and monitoring of treatment response.

The present study analyzes the expression of *de novo* synthesized CD154 after short-term activation. Kinetic analysis of optimal duration of *ex vivo* stimulation was 16 hours, which corresponds to related studies (44). The transient nature of CD154 expression requires its intracellular stabilization using Brefeldin A, which blocks protein degradation by preventing transport processes during cell activation, allowing the combined identification of rare Dsg3-specific and cytokine producing T cells in PV (45). Subsequent analysis revealed that after antigen-specific T cell stimulation, CD154 was significantly increased in CD4+ T cells from PV patients compared to HC, and directly correlated to anti-Dsg3 Ab-titers. Studies on T cells show that T follicular helper (Tfh) cells are critical for initiating autoreactive B cell responses (46–48). Moreover, Dsg3-specific Th2 lymphocytes were found in different disease stages of PV disease (acute/clinically active, chronic and remittent (clinically healed) (49). Using CXCR5, marker for homing into follicles of secondary lymphoid organs, we found a significant induction only in acute patients in both Th and Tfh CD4+ subpopulations compared to remittent and HC. Additional analysis of co-expression of CD154 with cytokines allows more detailed cellular activation profiling. Previous studies of T cells in the skin of PV patients reported elevated concentrations of IL-4 and IFN-y as well as, more recently, IL-17A and IL-21 (50, 51). We found a significant induction of IL-17 and IL-21 in CD154+CD4+ T cells from PV patients compared to HC, meanwhile CD154- CD4+ T cells remained mainly unaffected regarding their cytokine expression after antigenic stimulation. This is in line with results provided by Holstein et al. showing that peripheral blood T cell subsets of patients with active pemphigus are dominated by IL-17-producing Th and Tfh cell subsets (7). IL-17A was previously found to play a significant role at epithelial barrier sites by inducing the expression of other proinflammatory cytokines and chemokines. Additionally, IL-17 improves epithelial infiltration

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TABLE 1 Characteristics of pemphigus patients and HC.

		Individuals
Healthy control group size	N	23
Demographics	Age, median (range)	33 (22-58)
	Male (n, %)	15 (65%)
	Female (n, %)	8 (35%)
Pemphigus patients group size	N	33
Demographics	Age, median (range)	60 (41-83)
	Male (n, %)	16 (48%)
	Female (n, %)	17 (52%)
Clinical status	Active	9 (27%)
	Remission	24 (73%)
Clinical phenotype	Mucosal	9 (30%)
	Cutaneous	4 (13%)
	Mucocutaneous	8 (27%)
	None	9 (30%)
Pemphigus specific HLA-types	HR DRβ1*0402	5 (25%)
	HR DQβ1*0503	7 (35%)
	Both	5 (25%)
	None	3 (15%)

Demographics among PV patients include clinical status, clinical phenotype and pemphigus specific HLA-type.

and thereby pushing further ongoing inflammation of local autoimmune reactions, also by triggering a positive-feedback loop *via* an IL-6 induction (52). IL-21 not only induces B cell dependent antibody formation (53), but also promotes Th17 maturation in naïve CD4+ T cells (54). Therefore, co-expression of CD154 in CD4+ T cells with IL-17 and IL-21 only further emphasizes the concept of CD154 as a specific activation marker in PV.

Several studies have by now provided evidence that PV-associated HLA class II alleles are involved in the activation of Dsg3-reactive CD4+ T cells. Moreover, even CD4+ T cell responses against specific peptides of the Dsg3 ectodomain were identified in PV patients (11, 55) and HLA-DRB1*04:02–binding Dsg3 T cell epitopes in a corresponding mouse model (12). This prompted us to inquire whether stimulation with Dsg3 specific epitopes would suffice to induce CD154 expression in antigen-specific CD4+ T cells. According to the published sequence of Dsg3, position and amino acid sequences of used immuno-reactive epitopes (P1-P4) are outlined in the Figure 3A. Dsg3 peptide $P2_{(206-220)}$ was found to induce CD4+ T cells most effectively in PV patients compared to HC and to the similar extend as the full Dsg3 protein. In *in silico* modeling, P2 was found to be capable of binding only to DRB1*0402 (56), however, a respective clone displays a strong PV characteristic polarization towards Th2 by the secretion of IL-4 and IL-10 after stimulation with Dsg3 (11). This further highlights that this epitope alone could suffice for the detection of Dsg3-specific CD4+ T cells in PBMCs of PV.

To this end, we developed a method in which CD4+ T cells from fresh PBMCs could be *ex vivo* stimulated with either whole protein antigen (Dsg3) or immunodominant peptides (P1-P4) and after stimulation analyzed for the activation marker CD154 (CD40L). The presented methodological approach could be used to further advance our knowledge about the actual frequency and characteristics of CD4+ T cell reactivity in PV patients. Positive IL-17 and IL-21 co-expression as well as correlation of anti-Dsg3 titers with CD154 expression in PV patients compared to healthy controls further supports the concept that immune activity in PV relies, at least partially, on Dsg3 reactive Th17 T cell subsets. These findings strongly suggest that CD154 as a specific activation marker in PV, expressed by antigen-specific CD4+ T cells, is critical during the pathogenesis of PV. Moreover, upregulation of IL-21 and IL-17 were seen to associate with antigen-specific activation, therefore lend themselves as potential therapeutic targets in pemphigus.

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 Ethics Committees of the Medical Faculty of the Philipps-Universität Marburg (Az. 20/14) and the Charité Berlin (AZ: EA4/194/19) and was conducted according to the principles of the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: CH. Data curation: AP, LK, and AI. Formal analysis: AP and CH. Funding acquisition: MH and CM. Investigation: CH, AP, LK, and AI. Project administration: CH. Supervision: CH and CM. Patient recruitment: DD and FS. Writing original draft: AP and CH. Writing – review & editing: AP, LK, AI, MH, CM, CH, DD, FS, and KG. All authors read and approved the final version of the manuscript.

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Conflict of interest

MH is a recipient of an unrestricted grant from TOPAS Therapeutics relating to a collaboration aimed at inducing therapeutic T cell tolerance in pemphigus.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2022.979277/full#supplementary-material

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Desmoglein compensation hypothesis fidelity assessment in Pemphigus

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The pemphigus group of autoimmune blistering diseases encompasses pemphigus vulgaris (PV) and pemphigus foliaceus (PF). Lesion location in pemphigus has been elegantly postulated by the Desmoglein Compensation Hypothesis (DCH), which references the distribution of desmoglein (Dsg) proteins in the epidermis along with a patient's autoantibody profile to describe three different lesion phenotypes: PF is characterized by subcorneal lesions in the presence of anti-Dsg1 antibodies only, while lesions in PV are suprabasilar and accompanied by anti-Dsg3 antibodies only in mucosal PV, or both anti-Dsg3 and anti-Dsg1 in the case of mucocutaneous PV. While the validity of this hypothesis has been supported by several studies and is prominently featured in textbooks of dermatology, a number of logical inconsistencies have been noted and exceptions have been published in several small-scale studies. We sought to comprehensively assess the extent to which patient clinical and autoantibody profiles contradict the DCH, and characterize these contradictions in a large sample size of 266 pemphigus patients. Remarkably, we find that roughly half of active PV and PF patients surveyed present with a combination of lesion morphology and anti-Dsg3/1 levels that contradict the DCH, including: patients with a cutaneous only PV presentation, mucocutaneous disease in the absence of either Dsg3, Dsg1, or both, and mucosal disease in the absence of Dsg3 or presence of Dsg1. We also find stark differences in fidelity to the DCH based on ethnicity and HLA-association, with the lowest proportion of adherence in previously understudied populations. These findings underscore the need to expand our understanding of pemphigus morphology beyond the DCH, in particular for populations that have not been a focus in previous investigation.

KEYWORDS

desmoglein compensation hypothesis, pemphigus, autoantibodies, morphology, fidelity

Introduction

Pemphigus is a group of rare autoimmune skin blistering diseases characterized by mucosal or oral lesions due to the presence of autoantibodies against desmosomal cadherin proteins involved in cell-adhesion. The Desmoglein Compensation Hypothesis (DCH) is an elegant theory first proposed by Stanley and Amagai that correlates clinical presentation of pemphigus with the profile of autoantibodies directed against the cadherins desmoglein (Dsg)3 and -1 as the drivers of site-specific loss of cell-cell adhesion and blister formation (1, 2). The DHC references the distribution and expression of Dsg3 and -1 proteins within epidermal tissues to explain lesion site (cutaneous vs. mucosal) and lesion depth (suprabasal vs. subcorneal). Dsg1 is found in higher concentrations towards the superficial layers of skin or mucosa, while Dsg3 is found in higher concentrations towards the basal layers of skin or mucosa. Non-mucosal skin has a higher expression of Dsg1 throughout the epithelium, while Dsg3 is concentrated in the basal epithelium. Conversely, the mucosa has a greater expression of Dsg3 throughout the epithelium, while Dsg1 is only expressed in the superficial epithelium. Based on this distribution pattern of the Dsg3 and Dsg1 proteins, the DCH postulates 3 subtypes of pemphigus: Pemphigus foliaceus (PF), mucosal-limited Pemphigus vulgaris (PV), or mucocutaneous PV (3).

In this framework, PF is characterized by the presence of anti-Dsg1 antibodies only and presents with subcorneal skin ulcerations on cutaneous surfaces only, as the high concentration of Dsg3 in the mucosa is thought to compensate for the lack of functional Dsg1. PV, on the other hand presents with deeper, suprabasal blister formation due to the presence of anti-Dsg3 antibodies. The mucosal-limited subtype of PV is characterized by the presence of anti-Dsg3 alone, as the suprabasilar Dsg1 in the skin compensates for the lack of Dsg3. The mucocutaneous subtype of PV is characterized by both anti-Dsg3 and anti-Dsg1 antibodies and presents with suprabasilar mucosal and cutaneous lesions, since neither Dsg3 or Dsg1 are able to compensate for the inactivation of the other.

While many studies support the validity of the hypothesis (1, 3–5) and it is prominently featured in textbooks of Dermatology, numerous researchers have pointed out discrepancies in the theory. These contradictions include the presence of anti-Dsg antibodies in the absence of skin or mucosal lesions (i.e. clinical remission) (6, 7), or conversely, the absence of anti-Dsg antibodies in active disease (8, 9). They also include discrepancies between lesion location and anti-Dsg3/1 profiles, such as the presence of either anti-Dsg3 or -1 antibodies in mucocutaneous disease (9), elevated anti-Dsg1 levels without anti-Dsg3 antibodies in mucosal PV (10, 11), and the presence of only suprabasilar cutaneous lesions without mucosal lesions (11–13). Importantly, the DCH also cannot account for the clinical observation that PV patients with elevated anti-Dsg 1 that do not develop subcorneal blisters, as might be predicted, and as PF patients do, despite having the necessary antibody correlated with this level of intraepidermal split (7).

Though numerous previous studies have noted patients whose clinical presentation and antibody profiles contradict the DCH, these studies were primarily case studies with a limited patient population, review papers, or posed questions regarding the DCH in the context of other experiments (6, 7, 9–13). Here, in a larger patient sample size consisting of 253 PV and 13 PF patients, some with longitudinal sampling dates, we sought to determine (a) how often patients' phenotype and antibody profile contradict the postulates of the DCH, (b) in which way these patients' data contradict the DCH, and (c) if there are additional demographic or genetic factors that affect DCH conformity. We present evidence indicating that while the DCH can explain approximately 50% of PV phenotypes, it does not account for the clinical presentation in the other half of cases, making a strong case for the need to modify/expand the hypothesis.

Materials and methods

Patient population

Patients were recruited from the Dermatology outpatient clinics at the University at Buffalo (IRB 456887), Michigan State University (IRB 05-1034) and Weill Cornell Medical College (IRB 0998-398), in addition to annual meetings of the International Pemphigus and Pemphigoid Foundation (IPPF) between 2001 and 2018. A written informed consent from every patient was obtained at the time of enrollment. At all visits, patients were seen in person by medical staff with extensive experience in assessing Pemphigus based on histological, clinical and serological criteria.

For all PV and PF patients included in the study, disease diagnosis was verified using established clinical and histopathologic criteria. Specifically, the diagnosis of PV and PF was determined by histopathological findings (suprabasilar acantholysis vs. subcorneal acantholysis, respectively) and DIF (IgG and C3 deposition in intercellular epidermis). Patients were also asked to provide information regarding their demographics, disease course, medical history, and family history. Current lesion location was assessed at the time of intake and patients were classified as having mucosal only-, mucocutaneous-, or cutaneous only lesions. Subsequently, venous blood samples were obtained, and serum was isolated *via* centrifugation and stored at -80°C for future analysis. Patients with multiple visits had venous blood and clinical information taken at all visits when possible. The maximum number of repeat samples from a single patient was 6, and the average number was 1.4 samples per patient. Additionally, 221 healthy controls were enrolled, including 58 individuals with and 163 without a family history of pemphigus. All study

procedures were identical between controls, PV patients, and PF patients.

For this study, we enrolled 253 PV patients, 13 PF patients, and 221 healthy control subjects. As patients could present multiple times and in different phases of disease, we collected 159 samples from 142 PV patients in the active phase of disease, 235 samples from 146 patients in remission, and 246 samples from 221 healthy control subjects. PF patient all presented in the active phase of disease. Our study population demographic data is summarized in Supplemental Table 1. Disease activity was defined for each patient using consensus guidelines developed by the International Pemphigus Committee (14). Patients were deemed to be active if they had three or more non-transient lesions (lasting more than 1 week) and/or extension of existing lesions. Patients were considered to be in complete remission if they experienced an absence of new or established lesions for at least 2 months. In a modification from the consensus guidelines, patients with transient lesions only (lasting less than 1 week) were classified as being in partial remission. In addition, patients in remission were assigned to one of two groups depending on the length of time they maintained clinical remission: newly remittent (2-6 months) and long-term remittent (>6 months). We refer to this expanded clinical subgrouping as "disease phase." Information used to assign disease phase classifications was obtained from clinical assessment at the time of the visit and supplemented by patient history. At each study visit, patients were asked detailed questions about the time course of their lesions to assist in classification.

Therapy regimens in PV and PF vary considerably for each patient. To simplify, each patient was assigned a therapy status based on consensus guidelines (14) at each blood draw. "Minimal" therapy was defined by prednisone doses of ≤10mg/day and/or minimal adjuvant therapy for at least 2 months. Patients receiving >10mg/day of prednisone, IVIg, cyclosporin, dapsone, rituximab or other biologic agents were defined as "more than minimal" therapy. "Off" therapy was reserved for patients that were not receiving any systemic therapy. Our group has previously shown that therapy status defined as above does not significantly affect anti-Dsg levels in clinically active patients (6). Nevertheless, in order to reassess a potential effect of this extrinsic variable on autoantibody levels in our larger patient population, we determined anti-Dsg3 and -1 levels in patients classified according to disease activity and treatment status in this study population. We did not see any significant differences between anti-Dsg3/1 levels and treatment status in PV subjects in any phase of disease activity with the exception of patients in partial remission where anti-Dsg3 levels were found to be higher in the more than minimal group compared to the off-therapy group. ($p = 0.02$) (Supplementary Figures 1A-C). Thus, the analyses presented in this manuscript include patients on more than minimal, minimal and off therapy.

Detection of anti-desmoglein 3 and 1 levels

Anti-Dsg ELISA was performed *via* standard protocols using Dsg 3 and Dsg 1 test systems by MBL Intl. (RG-M7593-D) with a 1:101 serum dilution (or 1:1000 in a limited number of samples with antibody levels over 140 IU/ml at 1:101 dilutions, which were considered too high for accurate detection at that dilution). The kits detect immunoglobulin G (IgG) antibodies against Dsg 3 and Dsg 1 and do not distinguish between subclass of IgG. Antibody positivity was defined at three separate ELISA levels of >36/37 IU/mL, >20 IU/mL, and >10 IU/mL for both anti-Dsg3 and anti-Dsg1. The >36/37 IU/mL cutoff was as per current manufacturer recommendations, while the >20 IU/mL cutoff was recommended by the manufacturer prior to 10/31/14. However, from years of experience using these ELISA kits, we felt that both these cut-offs are too stringent and exclude patients with lower antibody levels that are still clinically relevant. Thus, we determined the mean and standard deviation of anti-Dsg3 and anti-Dsg1 levels amongst healthy controls that did not carry the pemphigus associated HLA alleles DRB1*0402 and DQB1*0503 and had no family history of disease (n=96, mean anti-Dsg3 levels = 1.64 ± 4.57 IU/mL, mean anti-Dsg1 levels = 1.97 ± 4.12 IU/mL). These individuals were excluded to eliminate the possibility of elevated anti-Dsg3 and -1 levels in genetically susceptible but healthy individuals that might skew the determination of the threshold for anti-Dsg3 and -Dsg 1 positivity. We additionally excluded control subjects with family history of disease to also control for the presence of rarer PV-associated alleles in the control population that may similarly lead to elevated autoantibody values in healthy individuals. We then added two standard deviations to each mean (anti-Dsg3: 10.78 IU/mL, anti-Dsg1: 10.21 IU/mL) to establish a lower cutoff of 10 IU/ml to be used in addition to the manufacturer recommended levels. We present our data for each cut off separately.

Detection of anti-thyroid peroxidase levels

Anti-thyroid peroxidase (TPO) levels were detected by ELISA as per manufacturer's recommendation (GenWay Biotech, GWB-521202). The kit detects immunoglobulin G (IgG) antibodies against TPO and does not distinguish between subclasses of IgG. Antibody positivity was defined as >20 IU/ml. Given that the frequency of anti-TPO antibody positivity in healthy euthyroid individuals has been estimated to be around 8% (15), this cutoff was determined from referencing other studys' cutoffs for anti-TPO (16), and choosing the one that resulted in 7.6% of our control population being positive for anti-TPO.

Detection of anti-thyroglobulin levels

Anti-thyroglobulin (Tg) levels were detected by ELISA as per manufacturer's recommendation (GenWay Biotech, GWB-521201). The kit detects immunoglobulin G (IgG) antibodies against Tg and does not distinguish between subclasses of IgG. Antibody positivity was defined as >5 IU/mL by first determining the mean and standard deviation of anti-Tg levels amongst healthy controls (1.18 ± 2.03 IU/mL) and then adding two standard deviations to the mean (5.24 IU/mL).

HLA typing

High resolution HLA typing was performed by PCR amplification with sequence specific primers (17, 18) at the Histocompatibility and Immunogenetics Laboratory at Michigan State University using commercial kits (One lambda, Thermo Fisher Scientific). "HLA-positivity" (HLA^+) was defined as the presence of one or both of the PV-associated HLA alleles, DRB1*0402 and DQB1*0503 (19). Patients not carrying either of these alleles were labeled as "HLA-negative" (HLA^-).

Statistical analysis

In order to assess variance in the anti-Dsg3/1 ELISA levels across disease activities (active, partial remission, complete remission) and vs. control subjects and across treatment status (more than minimal, minimal, and off therapy) we used Kruskal-Wallis testing. The Kruskal-Wallis test was initially performed across all subgroups for disease activity and treatment status. If the broader testing of all subgroups resulted in a value of $p \leq 0.05$, further analysis of each individual subgroup was completed. P-values of ≤ 0.05 were considered to be significant.

In order to elucidate disease modifying factors, we compared the proportion of contradiction to the DCH among different ethnicities, HLA status, anti-TPO or anti-Tg positivity/negativity using chi-squared analyses. The proportion of DCH conformity and contradiction in the Ashkenazi Jewish population was compared to that of each of the remaining ethnicities, as this group had the highest rate of conformity and was the classic group studied in pemphigus. For HLA status, the percentages of conformity and contradiction were compared between subjects carrying the PV-associated HLA alleles DRB1*0402 and/or DQB1*0503 ("HLA-positive") and those that did not carry the aforementioned alleles ("HLA-negative"). We also compared the proportion of DCH conformity and contradiction among anti-TPO or -Tg positive, anti-TPO or -Tg negative, and all PV subjects. P-values of ≤ 0.05 were considered to be significant.

Results

Both anti-Dsg3 and anti-Dsg1 antibodies can be detected in patients in disease remission

Numerous studies have shown that anti-Dsg antibody levels rise and fall in parallel to levels of disease activity (20, 21; K. E. 22; V. K. 23). However, it has also been noted that anti-Dsg1 and particularly anti-Dsg3 levels can remain elevated in states of disease remission (K.E. 6, 24). In order to assess the degree to which antibody profiles adhere to the postulate that lesions only appear in the presence of anti-Dsg3 and -1 antibodies in our PV patient population (n=253), we determined the proportion of patients that had positive anti-Dsg3 and -1 levels in active disease, in partial remission, and in complete remission. Anti-Dsg3 and -1 positivity was determined using three different cutoffs, two recommended by the manufacturer at different time points and one calculated in our laboratory based on comparison with a large set of healthy volunteers (see Materials and Methods). We show that among PV subjects that were clinically active (n=159), 77.99% were anti-$Dsg3^+$, and 34.59% were anti-$Dsg1^+$. However, despite exhibiting active disease, 18.24% carried neither anti-Dsg3 nor anti-Dsg1 autoantibodies when using the positivity cutoff of 20 IU/mL (see Materials and Methods) (similar percentages were seen across all cutoffs, Table 1), indicating that anti-Dsg3/1 antibodies are not the sole drivers of lesional activity. Depending on the cut-off value chosen, in PV subjects that were clinically in partial remission (i.e. presence of transient lesions only), 59.68-66.13% subjects were anti-$Dsg3^+$, and 16.13-24.19% were anti-$Dsg1^+$. Among PV patients that were clinically in complete remission, 7.30-15.73% were still anti-$Dsg1^+$ and 44.38-61.24% were still anti-$Dsg3^+$ (Table 1). As expected, in the healthy control population (n=246), anti-Dsg3 and anti-Dsg1 positivity was low to undetectable (anti-Dsg3 with a 0.40-1.59% positivity rate; anti-Dsg1 with a 0.79-4.37% positivity rate). Consistent with a previous study by our group performed in a slightly smaller patient cohort (197 PV patients), mean anti-Dsg1 levels significantly decreased from active to complete remission ($p < 0.001$) to levels below the threshold set by most studies for positivity (i.e. 20 IU/mL), while mean anti-Dsg3 levels decreased significantly from active to complete remission ($p < 0.001$), but often remained highly elevated even in remission (Supplementary Figure 2).

Anti-Dsg antibody positivity and lesion location do not follow the phenotype predicted by the DCH in more than half of active PV patients

In order to determine the degree to which the disease phenotype of active patients follows the postulates of the DCH, we compared the patient's lesion location and corresponding anti-Dsg3 and -1

TABLE 1 Presence of anti-Dsg3/1 antibodies in patients of varying states of disease activity.

A. Anti-Dsg1.	Active (n = 159)	Partial remission (n = 62)	Complete remission (n = 178)	Control (n = 246)
Anti-Dsg1 Median (IQR) (IU/ml)	5.88 (48.81)	2.92 (7.80)	2.11 (5.42)	1.28 (2.78)
Anti-Dsg1$^+$ patients (cut off >36 IU/ml)	30.19%	16.13%	7.30%	0.81%
Anti-Dsg1$^+$ patients (cut off >20 IU/ml)	34.59%	17.74%	8.99%	2.44%
Anti-Dsg1$^+$ patients (cut off >10 IU/ml)	40.25%	24.19%	15.73%	4.07%
B. Anti-Dsg3.	**Active (n=159)**	**Partial remission (n = 62)**	**Complete remission (n = 178)**	**Control (n = 246)**
Continued				
Anti-Dsg3 Median (IQR) (IU/ml)	105.5 (128.50)	76.2 (126.87)	24.47 (107.22)	0.61 (1.67)
Anti-Dsg3$^+$ patients (cut off >37 IU/ml)	74.84%	59.68%	44.38%	0.41%
Anti-Dsg3$^+$ patients (cut off >20 IU/ml)	77.99%	59.68%	51.12%	0.81%
Anti-Dsg3$^+$ patients (cut off >10 IU/ml)	81.76%	66.13%	61.24%	2.03%

IU, International Units; Dsg, desmoglein.
Antibodies are presented as median and interquartile range (IQR) as well as percent positive at different cut-off levels.

levels at the time of blood sampling. In order to conform to the postulates of the DCH, PF patients would be expected to only have detectable anti-Dsg1 antibodies, mucosal PV patients should only have detectable anti-Dsg3 antibodies, and mucocutaneous PV would be expected to harbor both anti-Dsg3 and anti-Dsg1 antibodies. Of note, the presence of patients with cutaneous PV (cPV), i.e. presence of suprabasal acantholysis limited to cutaneous lesions, inherently does not follow the DCH.

Surprisingly, we found that for active PV patients visits (n=159), over 50% of the subjects displayed lesion morphology and corresponding anti-Dsg3 and -1 profiles that contradict the postulates of the DCH regardless of the cut-off value for antibody positivity chosen (ranging from 52.83% for the lowest cut-off of 10IU/ml to 54.72% for the cut-off of 36/37 IU/ml currently suggested by the manufacturer) (Figure 1A). In contrast, in the active PF patient group (n=13), only 15.38% of patients were found to contradict the DCH (Figure 1B).

Among multiple observed contradictions to the DCH, the presence of "cutaneous only" PV is the most common

To define the extent and range of contradictions to the DCH, we differentiated multiple clinical subgroups based on a combination of morphology and anti-Dsg3 and -1 antibody levels. We found several categories of patients that would not be predicted to occur according to the DCH. We observed eight permutations of contradictions to the DCH within PV patients: i) mucocutaneous PV in the presence of the following antibody patterns: a) anti-Dsg3+/1-, b) anti-Dsg3-/1+, and c) anti-Dsg3-/1-, ii) mucosal only PV carrying a) anti-Dsg3-/1-, b) anti-Dsg3-/1+, or c) anti-Dsg3+/1+, iii) cutaneous only PV (with any autoantibody pattern), and iv) a PF phenotype in the absence of anti-Dsg3 and -1 (Figure 1).

Among these contradictions, the cutaneous PV clinical phenotype was found to be the most common violation of the DCH (41.67% using the 20 IU/mL cutoff), with 84 subjects presenting with cutaneous only lesions PV at the time of visit (Figure 1). Seven of the cutaneous only PV (cPV) subjects were identified without having had any *history of* mucosal lesions (cPVwohm). The anti-Dsg levels for cutaneous only PV subjects were approximately equally distributed across categories of patients carrying anti-Dsg3$^+$/anti-Dsg1$^+$, anti-Dsg3$^-$/anti-Dsg1$^+$, anti-Dsg3$^+$/anti-Dsg1$^-$, and anti-Dsg3$^-$/anti-Dsg1$^-$ antibody profiles (Supplementary Figure 3). Cutaneous only PV was followed in frequency in contradiction to the DCH by mucocutaneous PV in the presence of anti-Dsg3 antibodies only (22.62%), mucosal PV with anti-Dsg3$^-$/anti-Dsg1$^-$ (17.86%), mucosal PV with anti-Dsg3$^+$/anti-Dsg1$^+$ (10.71%), mucocutaneous with anti- Dsg3$^-$/anti-Dsg1$^-$ (4.76%), and equal numbers of mucocutaneous with anti-Dsg3$^-$/anti-Dsg1$^+$ (1.19%) or mucosal with anti-Dsg3$^-$/anti-Dsg1$^+$ (1.19%) (Figure 1A). The only contradiction type found in the PF population was the absence of both anti-Dsg3 and -1 antibodies (Figure 1B)

Contradictions to the DCH are more prevalent in non-Ashkenazi Jewish ethnicities and in HLA negative PV subjects

It has been reported that clinical phenotypes as well as HLA distribution vary among PV patients of different ethnicities (24).

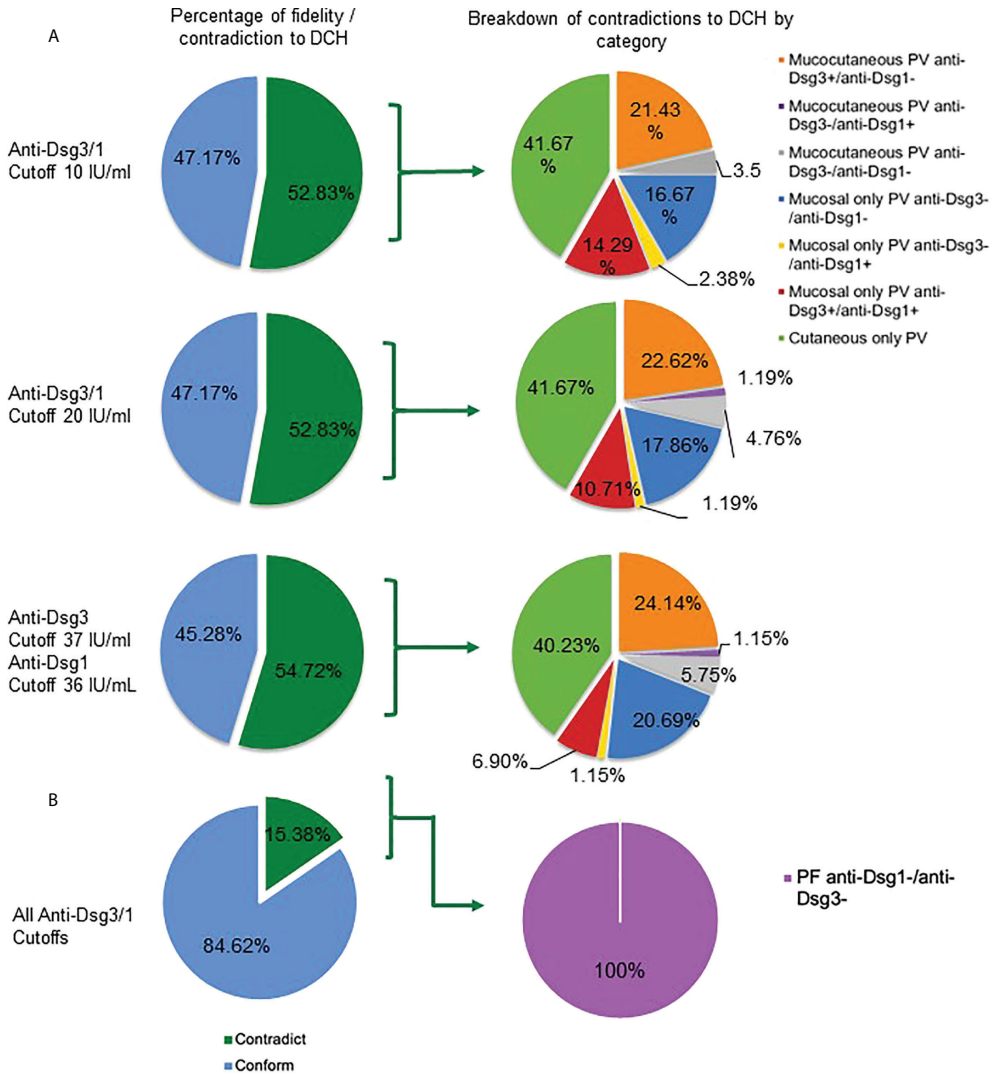


FIGURE 1
Contradictions to the DCH are consistent between different antibody cutoff values. **(A)** In active PV subjects (n = 159), more than half contradict the DCH based on their clinical lesions and autoantibody profile across all cutoffs for anti-Dsg3 and -1 positivity (10 IU/mL, 52.83%; 20 IU/mL, 52.83%; 37&36 IU/mL, 54.72%) (dark green shading in left column at a cut-off of 10 IU/ml, 20 IU/ml, and 36/37 IU/ml). Subjects that contradict the DCH were classified into seven categories based on how a PV subject may contradict the DCH (right column): i) mucocutaneous PV in the presence of the following antibody patterns: a) anti-Dsg1-/3+, b) anti-Dsg1+/3-, and c) anti-Dsg1-/3-, ii) mucosal only PV carrying a) anti-Dsg1-/3-, b) anti-Dsg1+/3-, and c) anti-Dsg1+/3+, and iii) cutaneous only PV (with any antibody pattern). **(B)** For active PF subjects (n = 13), we found that 15.38% across all cutoffs do not follow the DCH according to their lesion location and anti-Dsg1 and -3 positivity at the same visit. The only contradiction seen in the PF group was the absence of both anti-Dsg1 and anti-Dsg3.

In order to analyze whether ethnicity plays a role in a patient's fidelity to the postulates of the DCH we sub-grouped all active PV subjects by ethnicity and determined the combination of disease phenotypes and anti-Dsg levels that conformed with the DCH (n=75, 20 IU/mL cutoff) to those that did not (n=84, 20 IU/mL cutoff). We found that the lowest proportion of contradictions to the DCH by ethnic group are observed in the Ashkenazi Jewish (40.8%, n=49) and Caucasian populations (52.6%, n=57). We see even greater deviation from the DCH in the Latino (66.7%, n=15), South Asian (64.3%, n=14), African-American (66.7%, n=12), and East Asian (83.3%, n=6) populations. In six patients defined as "other" (multiracial), only 16.7% (n = 6) contradicted the DHC (Figure 2A). Thus, the Ashkenazi Jewish population had a noticeably smaller proportion of subjects that contradicted the DCH compared to those of Caucasian, South Asian, African American, Latino, and East Asian ethnicities (p=0.09). While this difference just missed the threshold of statistical significance at p=0.05, it is possible

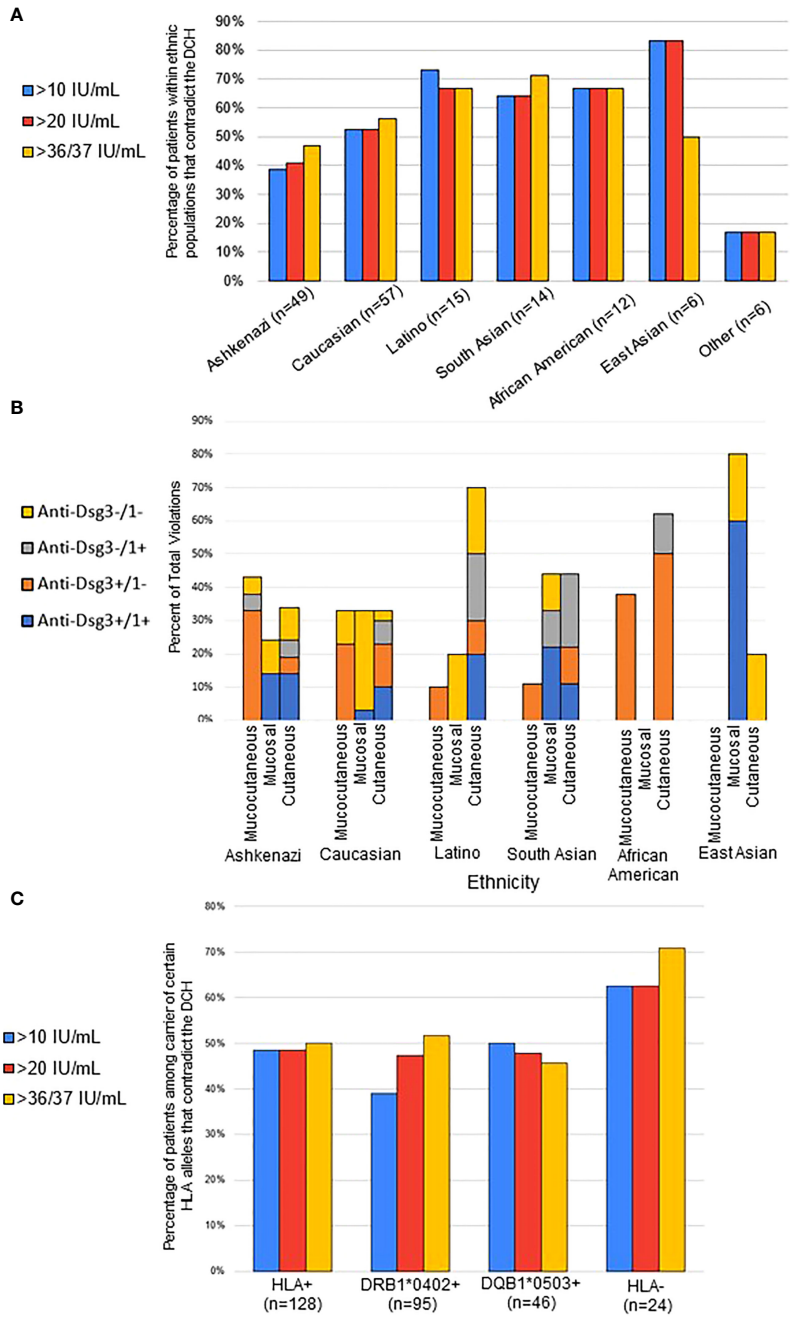


FIGURE 2
Contradictions to the DCH differ by ethnicity and HLA association. **(A)** For each ethnicity, the proportion of conformity and contradiction to the DCH was determined based on their lesion phenotype and anti-Dsg3/1 positivity for each visit. The percentage of contradiction is presented using the 10-, 20-, and 37/36 IU/mL anti-Dsg cutoff, with similar percentages seen in all cutoffs. The Ashkenazi Jewish population had a noticeably smaller proportion of subjects that contradicted the DCH compared to those of Caucasian, South Asian, African American, Latino, and East Asian ethnicities (p=0.09). **(B)** Within each ethnic group, the type of violation of the DHC was broken down into specific lesion morphology and associated anti-Dsg profile. Results are presented as percent of violation within the specific ethnic group. **(C)** Subjects were considered "HLA-positive" (HLA^+) if they carried either of or both the previously described PV-associated susceptibility HLA alleles DRB1*0402 and/or DQB1*0503. All active PV patients were classified as either HLA^+, only DQB1*0402-positive, only DQB1*0503-positive, or negative for either allele (HLA^-). We found that 48.44% (n = 128) of those that are HLA+ contradict, 47.37% (n = 95) that are $DRB0402^+$ contradict, 47.83% (n = 46) that are $DQB0503^+$ contradict, and 62.50% (n = 24) that are HLA^- for PV alleles contradict (p=0.06 when using chi-square with the anti-Dsg3/1 cutoffs of 37 and 36 respectively).

that the small sample sizes of non-Ashkenazi and non-Caucasian ethnic groups are masking true significant relevance of population differences.

Given the different rates of violating the DCH between different ethnicities, we then analyzed the types of DCH violations displayed by different ethnic groups (Figure 2B). Notably, we found that the two populations with the least contradictions of the DCH, Ashkenazi Jewish and Non-Jewish Caucasian, had the least variation in lesion morphology in DCH violations, where the other populations had bias towards specific lesion morphologies, particularly the cutaneous only phenotype for African Americans as well as Latinos. We also note that the African American population violations were overwhelmingly anti-Dsg3+/1-, regardless of lesion morphology, while over half of all violations in Caucasian patients presented with a double negative anti-Dsg3-/1- antibody profile.

The strong correlation between certain HLA types, particularly DRB1*0402 or DQB1*0503, and PV is well accepted (25). Thus, we also examined the relationship between HLA status and DCH adherence. The rate of contradiction to the DCH was determined for PV subjects that carried either: i) DRB1*0402, or DQB1*0503, or both alleles together ("HLA-positive", HLA^+), ii) only DRB1*0402, iii) only DQB1*0503, or iv) neither of these alleles ("HLA-negative", HLA^-). Approximately half of all active PV patients that carry at least one of the PV-associated HLA susceptibility alleles follow the postulates of the DCH. On the other hand, a higher number (62.5%) of active PV patients that are "HLA-negative "violate the DCH compared to "HLA-positive" patients (p=0.06 when using chi-square with the anti-Dsg3/1 cutoffs of 37 & 36 respectively) (Figure 2C).

Autoantibody profiles differ by ethnicity

Given that we found a higher proportion of contradictions to the DCH in non-Ashkenazi ethnicities compared to the Ashkenazi Jewish PV population, we wanted to assess whether autoantibody profiles of each ethnicity in our study population differed as well. We examined all active PV subjects according to their ethnicity, regardless of whether they contradict or conform to the DCH, and classified their autoantibody profile from each visit's blood draw into one of four subgroups; i) anti-$Dsg1^+/3^+$, ii) anti-$Dsg1^-/3^+$, iii) anti-$Dsg1^+/3^-$, and iv) anti-$Dsg1^-/3^-$, using the 20 IU/mL cutoff for anti-Dsg1/3 positivity. Similar to a previous study by Harman et al. (24), we found that South Asian PV subjects showed a greater proportion of anti-Dsg1 positivity (n=14, 71.4%) compared to Ashkenazi Jewish (n=49, 32.5%) and Caucasian (n=57, 26.3%) groups (Figures 3A–C). Conversely, of these 3 populations, Ashkenazi Jewish patients carry the highest percentage of anti-Dsg3 antibodies (85.7%, Figure 3A). The overall anti-Dsg3 and -1 profile in the Latino population appears similar to that of the Caucasian population, with slightly more anti-$Dsg1^+/3^-$ and less anti-$Dsg1^-/3^+$ (Figure 3F). Interestingly, African American patients show the highest percentage of anti-Dsg3 positivity (Figure 3E). The interpretation of these data is limited by the small sample sizes, particularly in the East Asian population (Figure 3D).

Subjects with detectable levels of anti-thyroid peroxidase and anti-thyroglobulin antibodies show a greater percentage of contradiction to the DCH than subjects not carrying these antibodies

Previous studies from our group have established that non-Dsg antibodies, such as anti-TPO and anti-Tg are found at elevated rates in PV patients, particularly in patients with no detectable levels of anti-Dsg3 and -1 antibodies (26). In order to investigate whether these antibodies play a discernable role in a patient's adherence/non-adherence to the DCH, we analyzed subjects who were carriers of either anti-TPO or anti-Tg and compared their proportion of contradiction to the DCH with that of anti-TPO negative or anti-Tg negative, and all active PV subjects. We found a higher, albeit non-significant, percentage of contradiction among anti-TPO^+ PV subjects (n=20) across all cutoffs compared to all anti-TPO^- subjects (n=101) and the entire active PV study population (n=159) (Figure 4A). Similarly, a higher proportion of anti-Tg^+ PV subjects (n=14) contradict the DCH than anti-Tg^- subjects (n=96) and all PV subjects (n=159) across all cutoffs (trending towards significance with Chi Square of p=0.086) (Figure 4B). The breakdown of lesion location was approximately equal in the TPO+ population that contradicted the DCH (5 cutaneous, 5 mucocutaneous and 5 mucosal), while it skewed toward cutaneous presentation for the Tg+ population (5 cutaneous, 2 mucocutaneous and 3 mucosal). Of note, there was one PF subject that was anti-TPO^+ and this subject contradicted the DCH across all cutoffs.

Discussion

The Desmoglein Compensation Hypothesis is an elegant theory that attempts to explain lesion morphology of pemphigus patients based on anti-Dsg3/1 antibody profiles. Original studies underlying this hypothesis were based on the observation that autoantibodies in PV patients target intercellular adhesion molecules (1), and that patients with distinct clinical presentations (mucosal dominant PV, mucocutaneous PV, and PF) display different autoantibody profiles (2, 3). These findings along with the known distribution of desmoglein proteins within the epithelium led to the creation of the DCH. Early animal studies as well as human *in vitro* analyses lent support to the hypothesis (3, 4, 27–29) and led to the DCH becoming a widely

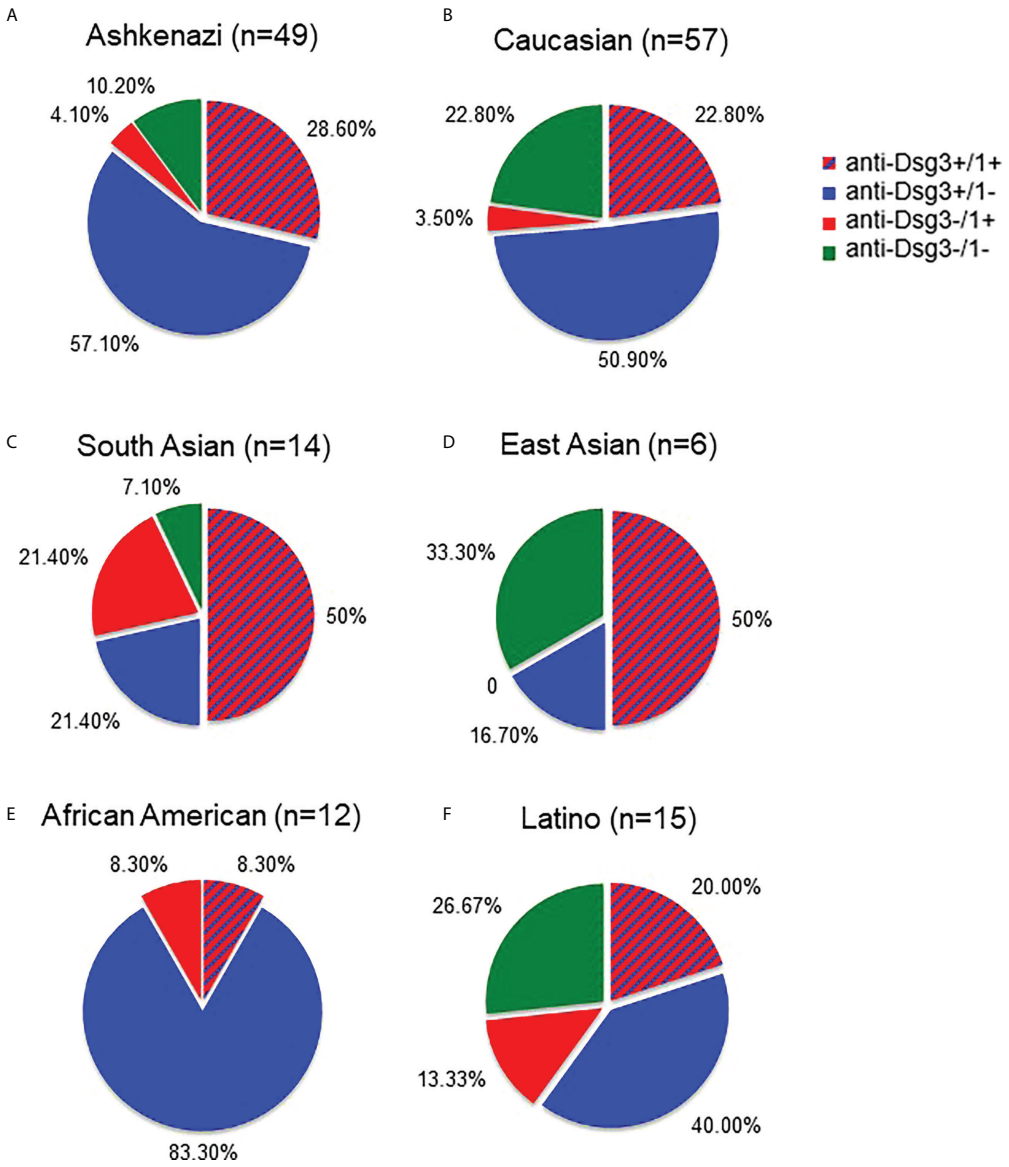


FIGURE 3
Autoantibody profiles differ between Ashkenazi and non-Ashkenazi ethnicities. We compared the anti-Dsg3/1 autoantibody profiles of all active PV subjects according to their self-identified ethnicity. The Ashkenazi Jewish population **(A)** was found to have a predominantly anti-Dsg3$^+$/1$^-$ profile. The Caucasian population **(B)** showed a similar profile to that seen in the Ashkenazi ethnicity, though with slightly less anti-Dsg3$^+$/1$^-$ and greater anti-Dsg3$^-$/1$^-$ percentages. The South Asian **(C)** antibody profile showed a greater proportion of anti-Dsg3$^-$/1$^+$ (21.4%) with a greater total percentage of total anti-Dsg1$^+$ (71.4%) than seen in the Ashkenazi and Caucasian populations (32.7% and 26.3%, respectively). Additionally, the East Asian population **(D)** shows predominantly anti-Dsg3$^+$/1$^+$ and anti-Dsg3$^-$/1$^-$ patterns. The African American population **(E)** shows a largely anti-Dsg1$^-$/3$^+$ profile. The Latino population **(F)** shows a relatively even distribution across anti-Dsg patterns, with slightly more anti-Dsg3$^+$/1$^-$ seen.

accepted theory for pemphigus pathogenesis. However, cases of "atypical" pemphigus were soon noted in the literature.

There are several instances in which the DCH is not followed in PV/PF, including (i) the presence of anti-Dsg antibodies without clinical disease (30), (ii) the absence of anti-Dsg1 or -3 antibodies in active disease (8, 9, 31), (iii) cases in which there is a mismatch of anti-Dsg levels and lesion type according to DCH postulates (10, 32), and (iv) the presence of cutaneous only PV (11, 33). To expand on these case studies, we performed a systematic and comprehensive analysis of the fidelity of the DHC in our large patient repository associated with carefully annotated clinical and epidemiologic data. We were able to confirm the presence of all potential contradictions to the DCH seen in previous studies. We also confirmed previous data from our group (6) generated from a

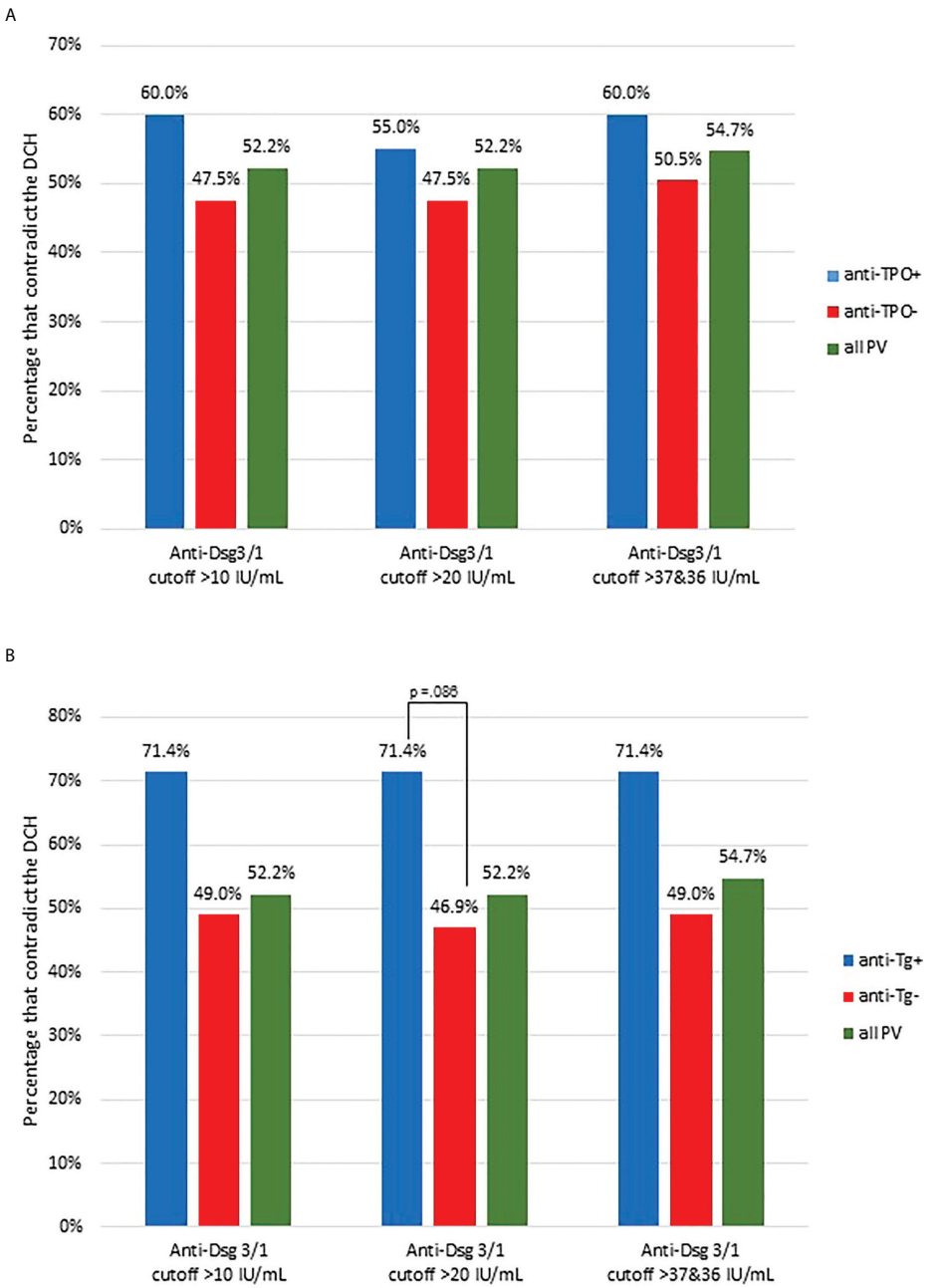


FIGURE 4
Proportion of contradictions to the DCH are slightly increased in PV patients carrying thyroid disease associated antibodies. **(A)** The proportion of conformity and contradiction to the DCH was assessed in all active PV subjects that had positive anti-TPO antibodies (anti-TPO+, n=20) and compared this proportion to those with negative anti-TPO levels (anti-TPO-, n=101) and all active PV subjects (all PV, n=159). The percentage of contradiction is presented using the 10-, 20-, and 36/36 IU/mL cutoff for anti-Dsg1/3. **(B)** Anti-Tg+ PV subjects had a higher proportion of contradiction to the DCH than anti-Tg$^-$ and all PV subjects. We assessed the proportion of conformity and contradiction in all active PV subjects that had positive anti-Tg positive antibodies (n=14) and compared this proportion to those with negative anti-Tg levels (n=96) and all active PV subjects (n=159). The percentage of contradiction is presented using the 10-, 20-, and 36/36 IU/mL cutoff for anti-Dsg1/3. The difference between anti-Tg+ and anti-Tg- subjects is trending towards significance (p=0.086).

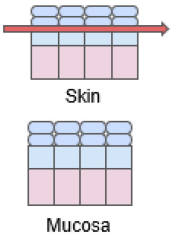
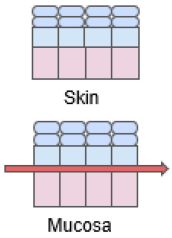
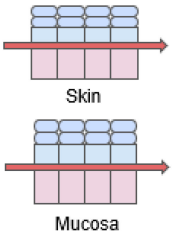
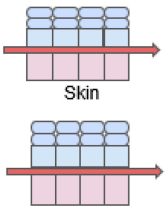
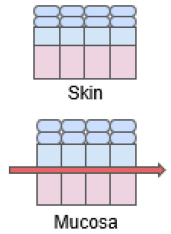
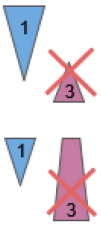
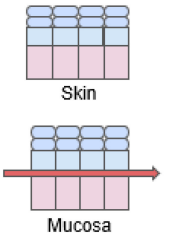
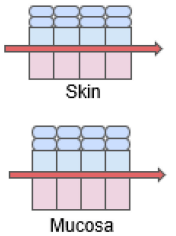
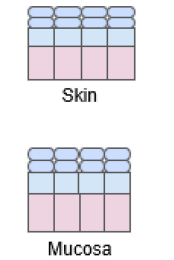
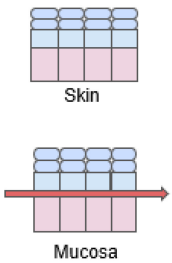
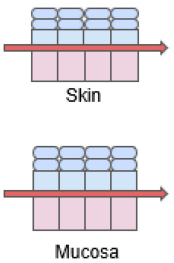
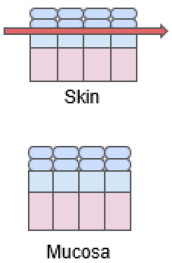
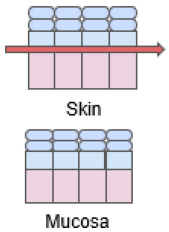
smaller subset of the data we presented here showing that while anti-Dsg3 and -1 levels generally decline with diminishing disease activity, a sizeable number of patients (7.30-15.73%) still show the presence of anti-Dsg1 antibodies in complete clinical remission, and about half of patients in remission continue to exhibit positive levels of anti-Dsg3 antibodies. On the other hand, we also observed clinically active patients with negative anti-Dsg3 and -1 levels in mucocutaneous disease, negative anti-Dsg3 levels in mucosal disease, and negative anti-Dsg1 levels in PF, all scenarios that do not conform to the postulates of the DCH. Strikingly, greater than 50% of visits overall in active PV subjects and approximately 15% of visits in active PF subjects were found to contradict the DCH based on their clinical lesions and anti-Dsg profile. Of note, nearly two decades ago the Bystryn group found strikingly similar discrepancies with only 50% of patients with exclusively mucosal lesions carrying anti-Dsg3, only 53% of patients with both skin and oral lesions carrying anti-Dsg3$^+$/1$^+$, and just 72% of patients with exclusively skin lesions carrying anti-Dsg1$^+$ (32). In our study, patients contradicted the predicted lesion morphology in a variety of ways (summarized in Figure 1), with the most prevalent contradiction being the cutaneous only manifestation in PV. A cutaneous only presentation of PV is in violation of the postulates of the DHC per definition, as suprabasilar acantholysis of the skin in the absence of mucosal lesions is not predicted by any autoantibody profile. Table 2 summarizes the antibody patterns observed in patients enrolled in the study and contrasts the expected morphology predicted by the postulates of the DHC with their actual clinical presentations.

One possible limitation of this study is the use of ELISA for quantification of anti-Dsg antibodies. While ELISA has been the standard of anti-Dsg antibody detection for over 20 years, it has been found that ELISA kits coated with baculovirus-made Dsg3 and -1 use recombinant molecules that contain both mature protein and immature proprotein (34, 35). This has raised the concern the binding of unrelated antibodies to Dsg-proprotein could lead to false positive detection of anti-Dsg3 or 1 antibodies, as has been shown for anti-Dsg1 in healthy controls and patients with the unrelated autoimmune disease thrombotic thrombocytopenic purpura (35). Conversely, it has been shown that custom made mature-Dsg3-only ELISA plates that were created by treating the baculovirus-made recombinant Dsg3 with furin to convert Dsg3 pro-peptide to mature Dsg3 resulted in increased mean serum index values for anti-Dsg3 when compared to plates coated with baculovirus-made recombinant Dsg3 ELISA plates (34). However, no difference in diagnostic results was found when comparing these two methodologies (34). Consequently, in 2009, the manufacturer of the most commonly used anti-Dsg3/1 ELISA kit (MBL International), the system that was also used in this study, began treating baculoviral-expressed Dsg3 and Dsg1 with furin to increase the ratio of mature protein to proprotein on their ELISA plates. In September 2013, MBL International switched to Dsg proteins made in mammalian systems with CHO cells with highly efficient post-translational modification systems, thus reducing the presence of proproteins (personal communication). In any case, the early studies that supported the DCH in fact used baculovirus-made recombinant Dsg to detect anti-Dsg antibodies, generating clear patterns in patient antibody-profiles in relation to lesion morphology that were used to develop the DCH (2–4). If the presence of proprotein would be altering antibody reactivity in the work presented in our study, this would have also been the case in the early studies used to create the DCH. Another potential limitation of this study is that we do not have concomitant DIF/IIF data for the sample time points presented. The phenomenon of DIF/IIF positivity with negative serum antibody ELISA is a theoretical (but unlikely) possibility, and this scenario in itself would further bring into question the validity of the DCH as it would support the notion of non-Dsg antibodies as contributors to lesional activity.

There is the theoretical possibility that a given non-DCH conforming patient could present at an undetermined later time point with different lesion locations (mucosal only vs. mucocutaneous vs. cutaneous only) that render the patient conforming. However, the DCH by definition was formulated to explain current lesion location by matching with the current antibody profile, and the data presented in this manuscript clearly shows that there are limitations to this hypothesis. Potentially, future studies could be designed to include a well-defined (but unknown) follow-up period to assess the possibility of a transition to a DCH-conforming phenotype in patients previously non-conforming. In any case, the exact cut off allowing for a transitional time period would need to be determined and agreed upon, and any such cases would still command an adjustment to the DCH as currently framed.

Factors that have been proposed to help explain contradictions to the DCH include the differences in pathogenicity of autoantibodies (36), transient phenotypes (11), ethnicity (24), HLA status (37), and other non-Dsg autoantibodies in the context of the multipathogenic theory of pemphigus pathophysiology (8, 9, 37–39). We investigated three of these potential explanations, i.e whether contradictions occur at a higher rate according to ethnicity, HLA status, and the presence of other auto-antibodies such as anti-TPO and anti-Tg. Our study supports the assertion that ethnicity is one of the main drivers of autoantibody selection as well as adherence or non-adherence to the DCH, and also that DCH adherence is influenced by HLA type. The groups most likely to contradict the DCH included non-Ashkenazi Jewish ethnicities, populations not expressing the accepted PV-susceptibility alleles, and anti-Tg positive subjects. It is conceivable that many of the early studies leading to the formulation of the DCH were done in patients of Ashkenazi Jewish descent, as this ethnic group has one of the highest rates of PV (40) and comprises an overwhelming majority of Pemphigus patients not just in Israel, but also in the US. The higher DCH conformity rates in the Ashkenazi Jewish population as observed in our study could have skewed our understanding of

TABLE 2 Expected vs. observed lesion morphology based on antibody profile.

Antibody pattern	Lesion location + phenotype	
observed	*Predicted phenotypes*	*Additional observed phenotypes*
anti-Dsg1+ / anti-Dsg3- 	PF 	Mucosal PV  Mucocutaneous PV 
anti-Dsg1+ / anti-Dsg3+ 	Mucocutaneous PV 	Mucosal PV 
anti-Dsg1- / anti-Dsg3+ 	Mucosal PV 	Mucocutaneous PV 
anti-Dsg1- / anti-Dsg3- 	No lesions 	Mucosal PV  Mucocutaneous PV  PF 
anti-Dsg1+ / anti-Dsg3-	PF	cutaneous only PV 
anti-Dsg1+ / anti-Dsg3+	Mucocutaneous PV	
anti-Dsg1- / anti-Dsg3+	Mucosal PV	
anti-Dsg1- / anti-Dsg3-	No lesions	

The left column displays the potential antibody combinations of anti-Dsg3 and anti-Dsg1 specificities in pemphigus patients.
The expected phenotypes with a given antibody profile as expected per the DCH are noted in the middle column. The right column displays additional phenotypes observed in this study and by others that are not predicted by the DCH. Notably, cutaneous only PV, which is not predicted by the DCH, was seen with every combination of autoantibodies.

disease towards a perspective from its presentation in this specific population, rather than that inclusive of broader and diverse ethnicities. Others have also noted that non-Ashkenazi populations, such as South Asians, exhibit an overall different anti-Dsg autoantibody profile than white ethnicities. In particular, South Asian PV patients have been reported as having a higher proportion of anti-Dsg1 positivity (24) and had a longer disease course than their white counterparts (41). Similarly, we found anti-Dsg profiles in South Asian subjects incorporated a higher percentage of anti-Dsg1 antibodies than their Ashkenazi Jewish counterparts, while Ashkenazi Jewish patients showed a higher percentage of anti-Dsg3 antibodies. Further studies with a larger sample size of previously underrepresented ethnicities are necessary to better elucidate these differences, especially in the East Asian and African American populations. Nonetheless, even among the Ashkenazi population, a large number of patients contradicted the postulates of the DHC, indicating that factors outside of ethnicity affect autoantibody selection and disease manifestation.

One of the factors that is related to, but also independent of ethnicity is the expression of the specific HLA alleles in a Pemphigus patient. The HLA system is a complex of genes that encodes the major histocompatibility complex in humans, the most polymorphic region of the human genome. Numerous HLA genes have been linked to autoimmune diseases; Pemphigus vulgaris has one of the strongest HLA associations of all autoimmune disease. Our group and others have shown that HLA genetic susceptibility in PV strongly, but not exclusively, maps to the HLA class II genes DRB1*0402 and DQB1*0503, with >80% of North American patients expressing one or both of these genes (19, 42). When comparing patients that are carriers of either one or both of these two alleles (termed "HLA^+") to patients that do not carry these alleles (termed "HLA^-"), we found a higher proportion of DCH contradiction in the HLA^- group relative to the HLA^+ group.

HLA molecules are a requirement for the initiation of disease *via* the presentation of as yet not clearly clarified (auto)antigens to CD4 cells that then prime B cells for autoantibody production (43, 44). Our data suggests that some of the patients who contradict the DCH carry HLA alleles that bind and present a different set of triggering (auto)antigens than the commonly PV associated HLA molecules DRB1*0402 an DQB1*0503. Recently, our group has shown that anti-TPO activity is heightened in the serum of North American PV patients and driven by HLA status as well as the absence of anti-Dsg3/1 reactivity (26). The presence of higher levels and rates of positivity of anti-TPO and anti-Tg antibodies in pemphigus patients compared to healthy controls was recently confirmed in the Chinese population (45). Interestingly, we found that both anti-TPO^+ and anti-Tg^+ subjects had a higher proportion of contradiction to the DCH compared to anti-TPO^- and anti-Tg^- subjects, respectively, suggesting that these antibodies may be involved in disease pathogenesis in certain cases not explained by the DCH, such as those not exhibiting detectable levels of anti-Dsg antibodies and/or PV in underrepresented HLA haplotypes.

Based on the accumulating evidence for the potential role of multiple non-Dsg autoantibodies in pemphigus pathogenesis, we further acknowledge that non-Dsg autoantibodies other than anti-TPO and anti-Tg could also be of relevance in cases that are nonadherent to the DCH. A number of groups have detected non-Dsg antibodies in the serum of PV patients including those directed against acetylcholine receptors, desmocollin, and mitochondria (9, 39, 44, 46, 47) that may provide an explanation of as to why patients with non-detectable anti-Dsg antibodies can nonetheless present in an active state of disease. Careful analysis of these autoantibodies and their impact on the expression and progression of clinical disease is an important area of future research.

Finally, there is also the possibility that antibody levels are not the only disease relevant factor in PV and that other, as yet undiscovered, genetic and/or skin structural factors contribute to disease presentation. Our results demonstrate a need to readjust the DCH as the standard explanation for disease expression in pemphigus, highlighting the complexity of autoantibody involvement and clinical disease. Unraveling this complexity will be a required step in deepening our understanding of disease mechanisms and developing increasingly targeted and individualized therapies across the spectrum of pemphigus.

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 Institutional Review Board, University at Buffalo; Institutional Review Board, Michigan State University; Institutional Review Board, Weill Medical College of Cornell University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

KS-S and AS devised the study and collected patient samples and associated clinical data; JB, LS, and MD performed the data analysis, JB, LS, KS-S, and AS drafted the article. KA provided statistical analysis of data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2022.969278/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Anti-Dsg Levels Across Treatment Status. Anti-Dsg3/1 levels in patients are shown based on based on level of therapy in active disease **(A)**, partial remission **(B)**, and complete remission **(C)**.

SUPPLEMENTARY FIGURE 2
Anti-Dsg Levels Across Disease Activity. Anti-Dsg1 levels **(A)** and anti-Dsg3 levels **(B)** are shown across varying levels of disease activity in patients as well as in the healthy control group.

SUPPLEMENTARY FIGURE 3
Contradictions in the DHC observed in active patients. The most common contradiction to the DCH observed in active PV is the cutaneous only variant of disease (cPV) (green shading in left column at a cut-off of 10 IU/ml, 20 IU/ml, and 36/37 IU/ml). A breakdown of contradictions within the cutaneous only manifestation of PV (right column) identifies all possible combinations of anti-Dsg3/Dsg1 expression.

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Detection of anti-desmoglein antibodies in oral lichen planus: What do we know so far

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Oral lichen planus (OLP) is an inflammatory disease of the oral mucosa. Clinically, two main subsets are described, namely non-erosive and erosive OLP. While non-erosive OLP is usually responsive to local therapies, erosive OLP is often refractory also to systemic therapies and extremely reduces the quality of life of the patients. Furthermore, in some erosive OLP cases different autoantibodies have been detected, including anti-desmoglein 1 and 3 autoantibodies, and anti-bullous pemphigoid 180 and 230 autoantibodies. However, their potential role is still not clear. In this paper, we reviewed the literature about the detection of autoantibodies against desmoglein 1 and 3, the main target antigens of pemphigus vulgaris, in patient with OLP, summarizing the more recent insights on this topic.

KEYWORDS

autoantibodies, desmoglein, epitope spreading, oral lichen planus, pemphigus vulgaris

Introduction

Oral lichen planus (OLP) is an inflammatory disease of the oral mucosa with a chronic course, that affects more often women in the fourth decade (1, 2). Classically, two main subtypes of OLP are described, namely non-erosive and erosive OLP (1, 2). The non-erosive type is clinically characterized by white streaks (Wickham´s striae), white plaques, and erythematous lesions, while the erosive type shows multiple painful erosions and ulcerations without well-defined borders and a necrotic base, that impair the quality of life of the patients (1, 2). Moreover, non-erosive OLP is usually asymptomatic and mostly responsive to topical therapies with corticosteroid or calcineurin inhibitors (3). By contrast, the therapy of erosive OLP is extremely tricky. Indeed, topical therapies are usually ineffective and patients need systemic therapies, which are mostly off-label,

including methotrexate, hydroxychloroquine, and interleukin (IL) antagonists, such as guselkumab (anti-IL23 agent) or secukinumab (anti-IL 17 agent) (3, 4). In addition, in erosive OLP a malignant transformation has been reported in up to 5% of the patients (5). Several risk factors for malignant transformations have been proposed, including ulcerations of the tongue, female gender, and old age (5).

The pathogenesis of OLP is still unclear (6). The cellular-mediated immunity, triggered by several factors (e.g. genetic background, oral flora, and trauma), plays a pivotal role, resulting in the production of several proinflammatory molecules, including tumor necrosis factor (TNF)-alpha and interferon (IFN)-gamma (2). Indeed, the interaction between CD4+ T cells and CD8+ T cells, which causes the activation of cytotoxic activity by CD8+ T cells, represents the main factor in the pathogenesis of OLP (6). Furthermore, Th17/Tc17 cells and T cell-derived IL-17A have been described as important players in OLP, because they support the perpetuation of the inflammatory response in OLP, that is partially responsible for the chronic course of the disease (4).

Because of its clinical features, erosive OLP should be differentiated from pemphigus vulgaris (PV), a rare autoimmune disease that can affect both skin and oral mucosa (7). Indeed, the oral mucosa of PV patients shows painful ulcerations, which resemble the ones that clinically characterized erosive OLP. Furthermore, PV patients develop oral large erosions that usually impair massively the food intake (8). However, patients affected by PV can also develop flaccid blisters on the skin and cutaneous erosions (7), which are not detected in OLP patients (2). Furthermore, esophageal involvement in OLP is usually asymptomatic and can be incidentally detected by esophagogastroduodenoscopy (2), while PV patients describe odynophagia in case of esophageal erosions (8). PV is serologically characterized by the presence of IgG autoantibodies directed against desmoglein (Dsg) 3 and, in case of involvement of the skin, also against Dsg1 (7). These autoantibodies can be detected in serum of PV patients by enzyme-linked immunosorbent assay (ELISA) and also in tissue by direct immunofluorescence (DIF) (9). Dsg, which are variable responsible of the cell-cell interaction in the skin and oral mucosa, show four cadherin repeats in their extracellular (EC) domains and a membrane-proximal extracellular anchor domain (7). The amino-terminal EC1 and EC2 domains are usually targeted by PV autoantibodies, which are necessary and sufficient to cause PV (9).

Rarely, both anti-Dgs1 and anti-Dgs3 autoantibodies have been detected in patients with erosive OLP (10, 11). However, their possible role in the pathogenesis of OLP still needs to be elucidated. In this paper, we reviewed the current literature on the detection of anti-Dsg autoantibodies in OLP, summarizing the most important aspects known so far.

Discussion

In 2006, the group of Kusic analysed a cohort of 57 patients with OLP (11). The diagnosis was confirmed by histology, and other erosive dermatoses of the oral mucosa were ruled out by DIF. Using ELISA, the authors found out in this retrospective study that the level of both anti-Dsg1 and anti-Dsg3 IgG antibodies was significantly higher in patients with erosive OLP compared to healthy control (HC), patients with recurrent aphthous ulceration, and patients with non-erosive OLP (11). Furthermore, these findings were confirmed by indirect immunofluorescence (IIF) (11). The authors concluded that anti-Dsg IgG autoantibodies could play a role in the pathogenesis of erosive OLP, which could be different to the pathogenesis of non-erosive OLP (11). However, they could not demonstrate the pathogenicity of these autoantibodies and the anti-Dsg1 and 3 autoantibodies values in all patients were below the cut-off level of the ELISA (11).

Several years later, a retrospective study on a cohort of 22 patients with OLP was conducted (12). In this cohort, 15 patients suffered by erosive OLP (12). Using a commercial ELISA (MBL International Corp.), they detected IgG autoantibodies against Dsg3 in a 61-year-old woman with erosive OLP (12). However, none of the 22 patients showed anti-Dsg1 autoantibodies (12). The clinical diagnosis of erosive OLP was confirmed histologically and the DIF did not detect the typical honeycomb IgG pattern of PV (12). In addition, a paraneoplastic pemphigus was ruled out based on the longstanding character of the mucosal erosions, the lack of consistent immune depositions by DIF, the negativity of the IIF on rat bladder, and a negative screening for neoplasia (12). Interestingly, levels of anti-Dsg3 antibodies were related to the clinical activity of the disease, that was evaluated using the Autoimmune Bullous Skin Disorder Intensity Score (ABSIS) (13). Because of the refractory course of the disease, the patient was treated with topical fluocinolone 0.05%, oral methylprednisolone, and mycophenolic acid (12). The authors concluded that the production of anti-Dsg3 IgG autoantibodies was probably stimulated by the exposition of intercellular proteins due to the intense and chronic inflammation (12).

In 2014, the case of a 43-year-old man with a six-month history of refractory oral erosions was described (14). By ELISA, IgG autoantibodies against both Dsg1 and 3 were detected (14). The diagnosis of erosive OLP was confirmed by histology and a PV was ruled out because of the negative findings by DIF and IIF (14). In this case, an improvement of the oral lesions was reported after a therapy with topical tacrolimus (14). Furthermore, the Dsg1 and 3 levels were not correlated to the clinical findings (14). Indeed, IgG autoantibodies against Dgs1 and 3 were detected also after the complete clinical remission (14). Also in this case report, the authors concluded that the

severe damage of keratinocytes could induce the production of anti-Dsg antibodies (14).

Two years later, two patients with erosive OLP with high levels of anti-Dsg1 and 3 autoantibodies were described in a case report (15). Actually, one patient, a 68-year-old Japanese woman with a three-month history of painful oral ulceration, showed both anti-Dsg 1 and 3 IgG autoantibodies by ELISA, while the other one, an 85-year-old Japanese woman with a 15-year history of painful oral ulcerations, showed only IgG autoantibodies against Dsg3 by ELISA (15). The diagnosis of erosive OLP was confirmed by histology and by the absence of positive findings by DIF and IIF (15). To better characterise the role of the anti-Dsg3 antibodies, the authors performed ELISA with or and without pre-treatment of the substrate with ethylenediaminetetraacetic acid (EDTA) (15). The ELISA index values for IgG autoantibodies against Dsg3 were not markedly different between EDTA-treated ELISA and untreated ELISA, suggesting that the autoantibodies against Dsg3 were Ca2+-independent and non-pathogenic in both cases (15). Furthermore, the authors performed an ELISA with the precursor and the mature forms of Dsg3 as substrates, showing that the autoantibodies targeted epitopes of prosequence-possessing Dsg3, which explained partially why *in vivo* deposition was not detected by IIF and DIF (15). Both patients were treated with topical tacrolimus and showed an improvement of the oral lesions, but a correlation between the clinical features and the serological levels of anti-Dsg1 and 3 autoantibodies was not evaluated (15).

In 2017, a retrospective study was conducted on a cohort of 113 individuals, including 24 patients with erosive OLP, 29 with non-erosive OLP, 30 patients with cutaneous lichen planus (CLP), and 30 HC (16). The diagnosis was based on clinical features and was confirmed by histology. Levels of circulating autoantibodies against Dsg1 and 3 were determined by ELISA on serum samples (EUROIMMUN, Medizinische Labordingnostika AG, Lubeck, Germany) (16). Furthermore, the activity of OLP was evaluated using Reticulation, Erosion and Ulceration (REU) scoring system (17). Although in all cases serum levels of anti-Dsg3 antibodies were in the normal range, a difference was detected between the level of anti-Dsg3 in erosive OLP and HC (p value=0.005) using the Mann–Whitney test (16). Regarding the serological concentration of anti-Dgs1, no difference between the four groups (p value = 0.748) was found using the Kruskal–Wallis analysis (16). Furthermore, no correlation was detected between clinical activity and anti-Dsg1 and 3 serum levels in patients OLP (16). Based on these findings, the authors concluded that serum levels of anti-Dsg3 antibodies in patients with erosive OLP were significantly increased in comparison with HC, although the serum levels of the anti-Dsg3 autoantibodies were under cut-off values (16).

A cross–sectional epidemiological study on the prevalence of autoantibodies in patients with several forms of lichen planus was conducted in Mumbai (10). In this study, 100 patients with CLP and OLP were tested for several circulating autoantibodies by IIF, including anti–Dsg1 and 3 too (10). In this cohort, 13 patients had OLP and ten were affected by both CLP and OLP, and nine out of these ten patients showed erosions of the oral mucosa (10). Anti–Dsg1 and 3 autoantibodies were reported respectively in 19% and 16% of the whole cohort of 100 patients, without specifying which patients showed these antibodies (10). Therefore, this study did not help to better understand the possible role of anti-Dsg1 and 3 autoantibodies in OLP.

Also our group evaluated the possible role of autoimmunity in the pathogenesis of OLP (18). Indeed, we reported that patients with OLP and with both CLP and OLP showed an increased peripheral blood Th1-dominated cell response against Dsg3 by ELISpot assay (18). Moreover, we showed in this perspective study that both OLP and CLP are characterized by a peripheral blood Th1/Th17-dominated cell response, which identifies the Dsg3 as target of the inflammation (18).

Because of lack of more detailed studies and due to the limited number of OLP patients with serological evidence of IgG autoantibodies against Dsg1 and 3, a clear role of these autoantibodies in the pathogenesis of OLP cannot be confirmed. Indeed, only in one paper the potential pathogenicity of IgG autoantibodies against Dsg3 was evaluated, showing that they were not pathogenetic (15).

Regarding the production of anti-Dsg1 and 3 autoantibodies in OLP, it seems possible that the humoral epitope spreading (ES) plays a pivotal role. ES consists in the diversification of B and/or T-cell response from an initial dominant epitope to a secondary epitope over the time (19). Two different subtypes of humoral ES have been described, namely the intramolecular and the intermolecular ES (19). In the first case, the diversification of immune response occurs in the same autoantigen, while the intermolecular ES involves different antigens of a single complex or that co-localize in the same anatomical site (19). Because it has been widely reported that chronic inflammation (as in erosive OLP) can lead to ES and several cases of ES have been described in autoimmune blistering diseases (e.g. PV and bullous pemphigoid), it is reasonable that humoral ES can lead to the production of IgG autoantibodies against Dsg1 and 3 in patients affected by erosive OLP (19).

Conclusion

The detection of IgG antibodies against Dsg1 and 3 is extremely useful to confirm the diagnosis of autoimmune bullous diseases of the pemphigus group (7). Noteworthy, these autoantibodies have been reported also in patient with OLP (10–12, 14–16). At present, the pathogenicity of IgG autoantibodies against Dsg1 and 3 in OLP has been neither in animal models nor *in vitro* evaluated. Indeed, the scientific literature on this topic is limited to case reports and

retrospective studies. Further studies are needed to better clarify this point, especially in view of the potential role of detection of IgG antibodies against Dsg1 and 3 for the diagnose and treatment of OLP. Indeed, the presence of these autoantibodies in OLP may be linked to a refractory disease that could be treated with systemic therapies.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Immunogenic cell death as driver of autoimmunity in granulomatosis with polyangiitis

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Cell death and dysregulated clearance of dead cells play essential roles in the induction of chronic inflammatory processes and autoimmune diseases. Granulomatosis with polyangiitis (GPA), a neutrophil-driven autoimmune disorder, is characterized by necrotizing inflammation predominantly of the respiratory tract and an anti-neutrophil cytoplasmic autoantibody (ANCA)-associated systemic necrotizing vasculitis. Defective regulation of neutrophil homeostasis and cell death mechanisms have been demonstrated in GPA. Disturbed efferocytosis (*i.e.*, phagocytosis of apoptotic neutrophils by macrophages) as well as cell death-related release of damage-associated molecular patterns (DAMP) such as high mobility group box 1 (HMGB1) contribute to chronic non-resolving inflammation in GPA. DAMP have been shown to induce innate as well as adaptive cellular responses thereby creating a prerequisite for the development of pathogenic autoimmunity. In this review, we discuss factors contributing to as well as the impact of regulated cell death (RCD) accompanied by DAMP-release as early drivers of the granulomatous tissue inflammation and autoimmune responses in GPA.

KEYWORDS

immunogenic cell death, autoimmunity, neutrophils, vasculitis, granulomatosis with polyangiitis, damage-associated molecular pattern (DAMP)

Introduction

Granulomatosis with polyangiitis (GPA) is an anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV). GPA is a rare and severe autoimmune disease characterized by necrotizing granulomatous inflammation localized predominantly in the upper and/or lower respiratory tract and ANCA-induced systemic necrotizing small vessel vasculitis (1). Its multifactorial etiopathogenesis is autoantigen- and pathogen-triggered, but still not fully understood. In more detail, predisposing genetic factors, environmental impact as well as immune-modulating pathways within the tissue microenvironment serve as initial trigger for the recruitment and activation of extravascular primed neutrophils forming neutrophil-rich

microabscesses (2, 3). These microabcesses over time begin to form ill-defined extravascular granulomas accompanied by large areas of geographic necrosis and typical innate immune cell infiltrates, which, together with the necrotizing vasculitis, mark the classical histomorphologic triad of GPA (4, 5). A central pathophysiological feature related to the necrotizing, granulomatous inflammation in GPA is the dysregulation of neutrophil homeostasis on genetic, molecular, and cellular level that is associated with an increased and sustained exposure of proteinase 3 (PR3), the main target antigen of ANCA in GPA. In particular, cell death mechanisms (dysregulated apoptosis, necrosis, necroptosis, and NETosis) lead to the release of damage-associated molecular patterns (DAMP), contribute to increased availability of (altered) PR3 and activate the local adaptive immune system favoring the formation of ectopic or tertiary lymphoid structures (ELS or TLS) (3). Cellular defects, *e.g.* in the regulation of the inhibitory immune checkpoint molecule programmed death-ligand 1 (PD-L1) in monocytes promote the development of highly activated T cells in AAV (6). Along with this, the continuous and/or altered presentation of PR3 to highly activated T cells accompanied by local inflammation-induced B and plasma cell survival, this sets the prerequisite for the loss of tolerance towards PR3 with subsequent production of ANCA in GPA (7, 8). Tissue damage occurs as a result of ANCA-induced systemic vasculitis and inflammation-mediated local tissue destruction predominantly in the upper respiratory tract (1, 9). Since therapeutic approaches are mainly symptomatic, *e.g.* immunosuppression, B cell depletion or complement-inhibition (10, 11), a future goal would be an intervention in early pathophysiological events from which the loss of tolerance against PR3 emerges. Therefore, in this review we discuss the role of immunogenic cell death as an early pathophysiological event in GPA involved in the development of the granulomatous inflammation possibly before the autoimmune response occurs.

Cell death mechanisms

Cell death is a ubiquitous process that can be either immunostimulatory or immunologically silent depending on the activated cellular components and on the signals from the surrounding tissue. On this basis, cell death can generally be divided into unregulated necrosis and several forms of regulated cell death (RCD). RCD is further differentiated into caspase-dependent and independent forms or based on the immunogenic outcome. Caspase-dependent forms of RCD include apoptosis, which is characterized by nuclear fragmentation (karyorrhexis), cytoplasmic shrinkage, chromatin condensation (pyknosis) and plasma membrane blebbing releasing small cell vesicles with intact cell membranes. Apoptosis is usually immunogenic silent under physiological conditions (12). Apoptosis is initiated either *via* intra- and extracellular perturbations (intrinsic apoptosis) or *via* death-receptor activation (extrinsic apoptosis) leading to activation of a cascade of several caspases with caspase 3 as the main executioner caspase (12). In addition to apoptosis, pyroptosis is another caspase-dependent cell death pathway, that has important functions in host defense and inflammation. Pyroptosis relies on the inflammatory activation of several caspases, such as caspase 1 or 11, that can be part of a multiprotein platform known as the inflammasome. Inflammasome-dependent pyroptosis controls the production of important pro-inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18 and critically depends on the formation of plasma membrane pores by members of the gasdermin protein family (13).

A caspase-independent RCD is necroptosis, which has become an increasing focus of inflammation research in recent years (14, 15). In contrast to previous assumptions that necrosis occurs as a purely uncontrolled event due to physiochemical stress, osmotic shock or freeze/thaw, necroptosis is a form of regulated necrosis. Necroptosis is a receptor-mediated RCD characterized by a complex interplay of receptor-interacting serine/threonine protein kinase (RIPK) 1, RIPK3, and mixed lineage kinase domain-like pseudokinase (MLKL), among others. Necroptosis is associated with loss of membrane integrity and the release of several DAMP (15, 16). Triggered similarly to apoptosis, necroptosis can be induced by activation of various death receptors of the tumor necrosis factor (TNF) superfamily, but also toll-like receptor (TLR) 3, TLR4, or interferon receptors. In fact, the initial steps to induce apoptosis or necroptosis are overlapping. However, the decision as to which of the two types of cell death is ultimately executed depends on the cell type, the nature of the stimulus, and the activity or inhibition of other intracellular factors (14, 17).

A more atypical form of cell death related to neutrophils is NETosis, that is morphologically distinct from apoptosis and necrosis (18). During NETosis, neutrophils extrude in a reactive oxygen species (ROS)-dependent manner so called neutrophil extracellular traps (NET), *i.e.*, long strands of nuclear DNA carrying cytoplasmic and granular proteins with antimicrobial and proinflammatory characteristics, initially described as a defense mechanism against bacteria (19).

Damage-associated molecular patterns

Cell death is a process important during normal cell turnover, elimination of pathogens or promotion of wound healing. The question is, therefore, how the immune system determines whether cell death is immunogenic or tolerogenic. In this context, Polly Matzinger introduced the concept of the danger model, stating that an immune response is induced by

endogenous and exogenous danger signals from injured or stressed cells (20). According to this concept, autoimmunity would arise from altered or increased presentation of antigen in the presence of persistent or chronic release of danger signals (21). These danger signals have been termed damage-associated molecular patterns (DAMP), referring to the ability of DAMP to elicit similar responses and to utilize the same receptors as their exogenous counterparts, the pathogen-associated molecular patterns (PAMP) (22, 23). The growing list of DAMP include nuclear, mitochondrial, or cytosolic factors with physiological roles within the cell that converts into danger signals when released to the extracellular milieu during inflammation or cell death (24). DAMP are then sensed by a broad range of receptors, e.g. Toll-like receptors (TLR) or NOD-like receptors (NLR) to alert the immune system by inducing immune cell migration, increasing phagocytosis by macrophages and dendritic cells (DC), stimulating the production of pro-inflammatory cytokines or contributing to the maturation of DC (25, 26). Several DAMP are already well characterized regarding the associated type of cell death and their role in the immune system (23).

Cell death and danger signaling in autoimmune diseases

For the maintenance of tissue homeostasis, a balance between cell proliferation, differentiation and death is crucial. In this regard, the immune system is routinely exposed to dead cells during normal cell turnover, injury, and infection. However, dysregulation of cell death mechanisms such as increased cell death rate, (genetic) alterations of cell death-related molecules or defective clearance of dead cells accompanied by massive release of DAMP can lead to uncontrolled excessive and/or prolonged inflammatory responses with subsequent tissue damage, thereby contributing to the pathogenesis of several pathologies, such as autoimmune diseases.

Apoptosis is a cell death program mostly related to non-inflammatory outcomes and likely to take major role in the maintenance of homeostasis by silently eliminating unwanted or damaged cells. However, defects in mechanisms of apoptotic cell clearance are linked to autoimmunity disorders, including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), likely due to the increased risk of loss of cell integrity with the consequent release of DAMP and increased availability of circulating self-antigens (23, 27).

Necroptosis has a profound proinflammatory effect, described in various inflammatory and autoimmune disorders, such as inflammatory bowel disease (IBD), RA, multiple sclerosis (MS) and SLE. Increased expression or phosphorylation of RIPK3 or MLKL and reduced expression of Caspase 8, have been detected in pathological samples (15, 28).

A role for inflammasome activation during pyroptosis in autoimmune diseases is likely, considering the wide spectrum of endogenous danger signals that activate NLR and the role of IL-1β and IL-18 in shaping adaptive immunity. More precisely, IL-1β and IL-18 amplify T and B cell responses and might serve as a crucial link translating NLR activation into adaptive immune responses. SNP in NLRP3 gene and an effect of pharmacological IL-1 blockade underline the clinical relevance for pyroptosis in autoimmunity, *e.g.*, in SLE, MS and type-1 diabetes (T1D) (29).

NET formation has been identified as a link between innate and adaptive immune responses in autoimmunity. Autoantigens including neutrophil granular proteins and post-translationally modified proteins, *e.g.*, citrullinated proteins, localize on NET. NET promote inflammation and provide stimuli to dendritic cells and can directly activate B and T cells, thereby potentiating adaptive autoimmune responses and induction autoreactivity against NET-associated antigens. NET have been detected *in vivo* in SLE, RA, IBD and AAV (18, 30).

As mentioned above, necrosis and RCD is accompanied by cell death-related release of DAMP. The best characterized and clinically relevant DAMP include high-mobility group box 1 (HMGB1), S100 proteins S100A8/A9/A12, heat shock proteins HSP60 and HSP70, β-defensins, and the cathelicidin LL-37, turning them into attractive therapeutic targets of chronic inflammatory and autoimmune diseases (31).

Immunogenic cell death in GPA

Necrotizing granulomatous inflammation is rich in neutrophils. In early lesions, numerous microabscesses formed by neutrophils are conspicuous, which presumably represent the starting point of the later necrosis areas. While ANCA-induced vasculitis is well studied, the pathogenetic mechanisms underlying necrotizing inflammation in GPA are poorly understood. It has been postulated that defects in the cell death machinery may contribute to the inflammatory response and autoimmunity in GPA (2, 3).

In this context, apoptosis has been the most and best studied cell death in GPA. On genetic level, an association with apoptosis-related genes was found in GPA, suggesting a shift in the balance of apoptosis (32). On cellular level, dysregulation of apoptosis in isolated neutrophils has been observed. Prolonged survival and/or a delay in apoptosis rate bear the risk of insufficient elimination of apoptotic neutrophils, that may lead to secondary necrosis releasing the intracellular content including proteases, pro-inflammatory cytokines, DAMP, and potential autoantigens into the tissue (33, 34). There is evidence that the autoantigen PR3 itself plays an essential role in this

context. On the one hand, PR3 was constitutively expressed at the plasma membrane of GPA neutrophils, and the percentage of neutrophils expressing PR3 was increased in GPA, demonstrating an abnormal increased availability of PR3 (35). On the other hand, PR3 co-externalized and interfered with "eat-me"-signals during apoptosis, thus impairing phagocytosis by macrophages (35). Moreover, we and others have shown that membrane expression of PR3 on apoptotic cells induced a pro-inflammatory response in macrophages and impaired their anti-inflammatory reprogramming following efferocytosis, indicating the impact of PR3 to alter the immunosuppressive effect of apoptotic cell efferocytosis and to promote sustained inflammation in GPA (35, 36).

In addition to (dysregulated) apoptosis, NET formation was initially observed in response to ANCA-stimulation in AAV including GPA (37). The authors further identified NET deposition in biopsies of inflamed kidney in active disease, demonstrating an *in vivo* relevance for NETosis in AAV. Since then, numerous studies have been conducted on the role of NET in AAV. In particular, NET can activate the alternative complement pathway with the production of C5a, that in turn primes and recruits more neutrophils, thereby amplifying the inflammatory process in AAV (38–40). Moreover, NET and NET-associated proteins were increased in the circulation of AAV patients, they contribute to vessel inflammation and act as a link between the innate and adaptive immune system by possessing a high immunogenicity and possibly promoting autoimmune responses against neutrophil components such as PR3 or MPO, thus inducing pathogenic ANCA production (41). In the context of GPA, PR3 shows unique structural and functional properties, in particular anchorage into the plasma membrane *via* a hydrophobic patch, which might render PR3 the favored autoantigen in GPA (42).

Furthermore, there is evidence that inflammasome-dependent and independent IL-1β processing and secretion is involved in GPA pathogenesis. IL-1β positive cells were found in renal biopsies from patients with AAV including GPA (43). As described above, processing of IL-1β in neutrophils and monocytes can be mediated by the NLRP3-inflammasome. In accordance with this, Factor H related protein 1 (FHR1) was found to be elevated in AAV serum. FHR1 was able to activate the NLRP3 inflammasome and IL-1β release from monocytes when bound to necrotic cells in AAV (44). In contrast, stimulation of human monocytes and neutrophils with monoclonal antibodies to PR3 or myeloperoxidase (MPO, the main autoantigen of microscopic polyangiitis, another AAV) or with human ANCA IgG, led to IL-1β generation and release in an inflammasome-independent manner. Instead, the authors of this study found that PR3 is the main protease involved in IL-1β maturation (45). In line with this, another study showed that neutrophils phagocytosing *Staphylococcus aureus* triggered a RIPK3-mediated activation of serine proteases in neutrophils independent of the NLRP3-inflammasome. Processing and secretion of IL-1β as well as cleavage of gasdermin D was PR3-dependent, leading to membrane pore formation and subsequent lytic cell death of neutrophils (46, 47). In addition, we identified a gene signature related to the inflammasome pathway (NLRP3, IL-1b, IL-18) as well as a significant enrichment of pathways consistent with immune responses triggered by *S. aureus* on the transcriptomic level in sorted T cells in GPA (48). *S. aureus* has been implicated in the induction and modulation of AAV including GPA. Studies have demonstrated that chronic nasal carriage of *S. aureus* was associated with higher relapse risk and endonasal activity in GPA (49, 50). Notably, *S. aureus* infection was linked to necroptosis in neutrophils and macrophages as well as with interference of efferocytosis, thereby contributing to inflammatory pathology (51, 52). Indeed, a functional link between RIP3-dependent necroptosis and ANCA-induced NET formation was discovered in murine MPO-AAV models as well as in human kidney biopsies of ANCA-associated necrotizing crescentic glomerulonephritis (30). These data suggest that infection-triggered necroptosis might be a relevant immunogenic cell death in GPA, as well.

DAMP signaling in AAV

Cell death-related DAMP signaling has been described in several studies in AAV. One of the best characterized DAMP in this regard is HMGB1. HMGB1 is a ubiquitously expressed nuclear protein with structural function such as stabilizing nucleosome structure and DNA bending (53). During activation or cell death, HMGB1 can be released from various cells mediating inflammation and acting as endogenous adjuvant, thus inducing autoimmunity (54, 55). There in increasing evidence that HMGB1 could represent a biomarker for disease activity, might act as pro-inflammatory mediator as well as inducer of autoimmunity in GPA.

In this context, we and others have shown a correlation between elevated serum HMGB1 with both disease activity and pulmonary granuloma. Therefore, HMGB1 may be used as a marker of the burden of granulomatous inflammation in GPA (56–58). MPO-positive microparticles (MPO+MPs), that could originate from activated or apoptotic neutrophils, are increased in plasma from patients with AAV. HMGB1 expressing MPO +MPs were associated with disease activity in AAV (59). HMGB1 could prime neutrophils by increasing ANCA antigens translocation, and the primed neutrophils could be further induced by ANCA, resulting in the respiratory burst and degranulation in a TLR4- and RAGE-dependent manner (60). Furthermore, the interaction between HMGB1 and C5a plays an important role in ANCA-induced neutrophil activation. This was shown by inhibition of HMGB1 leading to a decreased C5a-mediated translocation of ANCA antigens, as well as ANCA-induced respiratory burst and degranulation of C5a-primed

neutrophils (60). HMGB1 released by C5a-primed neutrophils can further induce ANCA antigen expression in an amplificatory manner, thus resulting in more intense activation of neutrophils (61). In addition, HMGB1 could enhance TLR9 expression in plasma cells and B cell proliferation and TLR9 expression in plasma cells was associated with disease activity in AAV (62). Plasma levels of HMGB1 correlated with endothelial cell activation in AAV patients. Further, HMGB1 amplified neutrophil activation, increased expression of intercellular adhesion molecules as well as the injury of glomerular endothelial cells in the presence of ANCA (63). The receptor of advanced glycation end products (RAGE), one of the main receptors of HMGB1, was highly expressed on cells infiltrating AAV kidney and lung biopsy tissue and mRNA expression was enhanced in PBMCs from active AAV patients (64).

These data demonstrate, that DAMP-signaling, here HMGB1, participates in neutrophil activation, B cell proliferation, endothelial cell activation, receptor-mediated inflammation as well as increased ANCA antigen translocation, thereby playing an essential role in inflammatory and autoimmune processes in GPA.

Discussion

In this review we provide an overview about factors contributing to and the consequences of immunogenic cell death in GPA. As summarized in Figure 1, a combination of predisposing genetic factors as well as exogenous and endogenous immune-stimulatory patterns such as infections (e.g., *S. aureus*), altered microbiome, and sterile inflammation serve as initial trigger for the recruitment and activation of extravascular primed neutrophils forming neutrophil-rich microabscesses. Immunogenic cell death (dysregulated apoptosis, pyroptosis, necroptosis, NETosis) leads to the release of DAMP, such as HMGB1, contributes to increased availability of the (altered) autoantigen PR3 and participates in the disruption of immune silencing due to disturbed efferocytosis. Concomitant pro-inflammatory cytokines, chemokines as well as complement drive recruitment of further immune cells, promoting the formation of inflammation-related ectopic lymphoid structures. Subsequent activation of the adaptive immune system set the prerequisite for the loss of tolerance against the autoantigen PR3 that is continuously displayed by high numbers of (dying) neutrophils and monocytes within the granulomatous lesion. ANCA induce systemic necrotizing vasculitis with further tissue damage, creating a self-amplificatory inflammation loop leading to chronification of inflammation (non-resolving inflammation) with tissue destruction and even organ failure. Therefore, a therapeutic intervention in cell death mechanisms could be a beneficial approach to prevent the chronification and autoimmune reactions at an early time

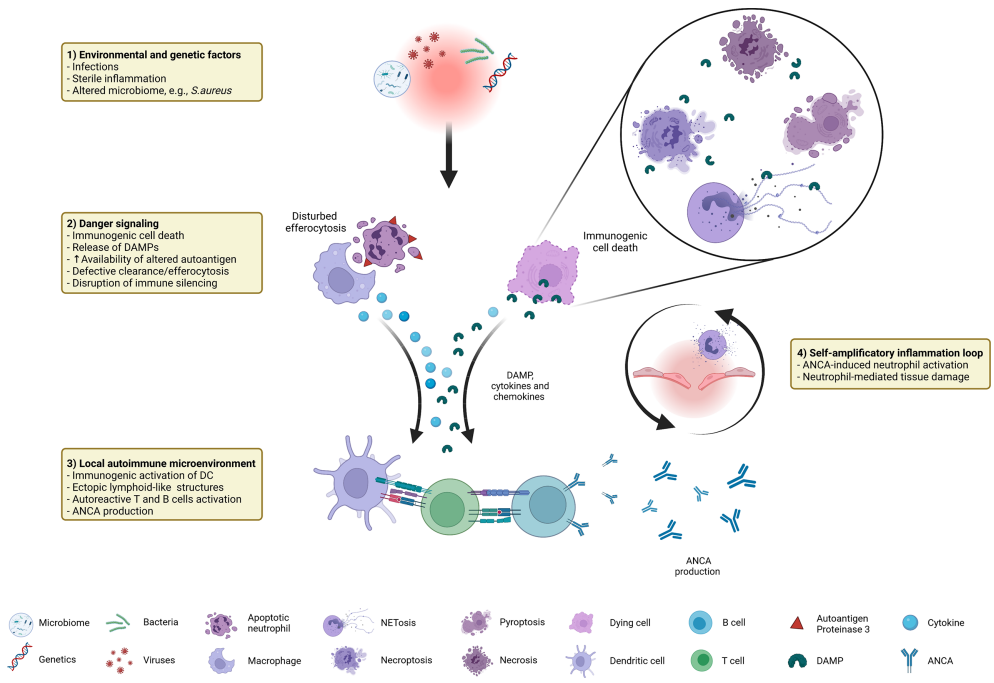

FIGURE 1
Immunogenic cell death in GPA at a glance: distinctive factors and mechanisms associated with immunogenic cell death contribute to autoimmune reactions in GPA.

point, possibly before the break of tolerance against PR3 occurs in GPA.

Author contributions

CB and AK-S designed and wrote the manuscript. PL corrected and advised the writing process. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Distinct CD4+ T cell signature in ANA-positive young adult patients

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Failure of immune tolerance can lead to autoantibody production resulting in autoimmune diseases, a broad spectrum of organ-specific or systemic disorders. Immune tolerance mechanisms regulate autoreactive T and B cells, yet some lymphocytes escape and promote autoantibody production. CD4+ T cell dysregulation, characterized by decreased or impaired regulatory cells (Tregs) and/or accumulation of memory and effector T cells such as TH17, plays a crucial role in the pathogenesis of these diseases. Antinuclear antibody (ANAs) testing is used as a first step for the diagnosis of autoimmune disorders, although most ANA-positive individuals do not have nor will develop an autoimmune disease. Studying the differences of T cell compartment among healthy blood donors, ANA-negative patients and ANA-positive patients, in which loss of tolerance have not led to autoimmunity, may improve our understanding on how tolerance mechanisms fail. Herein, we report that ANA-positive patients exhibit a distinct distribution of T cell subsets: significantly reduced frequencies of recent thymic emigrants (RTE) and naïve T cells, and significantly increased frequencies of central memory T cells, TH2 and TH17 cells; modulations within the T cell compartment are most profound within the 18-40 year age range. Moreover, CD4+ T cells in ANA-positive patients are metabolically active, as determined by a significant increase in mTORC1 and mTORC2 signals, compared to ANA-negative patients and healthy blood donors. No significant impairment of Treg numbers or pro-inflammatory cytokine production was observed. These results identify a unique T cell signature associated with autoantibody production in the absence of autoimmune disease.

KEYWORDS

ANA+, recent thymic emigrants (RTE), effector helper T cells (TH), mTOR, pre-autoimmune disease, ANA-, tolerance

Introduction

Establishment and maintenance of immunological tolerance prevents development of autoimmune disease. Tolerance induction involves many mechanisms (e.g., deletion, anergy, etc.) and occurs in primary and secondary lymphoid organs. In most instances, autoreactive T and B cells are eliminated, become anergic, develop into regulatory T cells (Tregs) or are actively suppressed by Tregs (1, 2). When autoreactive lymphocytes escape tolerance mechanisms, it can lead to autoimmune pathology such as autoantibody production. Presence of abnormal levels of autoantibodies before disease onset can be clinically useful for the diagnosis of autoimmune disorders (3). Antinuclear antibodies (ANAs) are detected in up to 20% of the general population and are associated with autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome (SjD) and multiple sclerosis (MS) (4–7). Although most ANA-positive individuals do not have an autoimmune disease and the probability of developing one during their lifetime is low, ANAs can appear many years prior to associated clinical features of disease (8–12). Because most cases of ANA positivity are not associated with pathology, studying immune cells from ANA-positive individuals may provide insight into how tolerance mechanisms to autoantigens fail.

T cell dysregulation is often associated with autoimmune diseases. Despite central and peripheral tolerance mechanisms, autoreactive T cells persist in the repertoire. Failure of T cell tolerance, and thereby initiation of autoimmune disease, can be due to environmental triggers, defective regulation, and/or genetic factors; these events have profound effects on the T cell compartment (13–15). For instance, decreased numbers or impaired function of Tregs can promote expansion of proinflammatory TH17 CD4+ T cells. Given their antagonistic functions, an imbalance of the Treg : TH17 CD4+ T cell ratio can signal active autoimmune disease; increased frequencies of TH17 cells is associated with autoimmune diseases due to their ability to secrete proinflammatory IL-17, promote B cell activation and elicit autoantibody production (16). Other T cell correlates with autoimmune disease include reduced frequencies of naïve T cells, accumulation of effector and memory T cells, mTOR activation, and elevated levels of inflammatory cytokines (17–21). Lastly, the aging process itself is associated with T cell dysregulation and increased risk of autoimmunity (22–25). Aging is accompanied by marked changes in the T cell compartment and chronic low levels of inflammation. Of note, age-related thymic involution leads to significant reductions in newly-generated naïve T cells, which stimulates compensatory peripheral expansion of T cells to maintain stable T cell levels (26, 27); this process may contribute to autoimmunity by inappropriately activating autoreactive T cell clones (28, 29). Additionally, thymic atrophy impairs efficient negative selection, leading to release of autoreactive T cells into the periphery (30). The cumulative effects of these changes within the T cell compartment have been characterized in autoimmune disease (17, 31); however, it is unclear how loss of humoral tolerance to autoantigens, in the absence of active autoimmune disease, affects the T cell compartment.

In many cases, production of ANAs is CD4+ T cell-dependent as autoreactive B cells have undergone antigen-driven clonal expansion and somatic mutation (32). Herein, we test the hypothesis that CD4+ T cells from ANA-positive patients are distinct from ANA-negative patients and healthy blood donors. To test this hypothesis, we analyzed and compared CD4+ T cells collected from ANA-positive patients, ANA-negative patients, and healthy blood donors. Because of age-related changes to the T cell compartment, data was analyzed based on age ranges. The most profound effects of ANA-positivity were observed in the 18-40 year age range whereby ANA-positive patients had significantly reduced frequencies of recent thymic emigrants (RTEs) and naïve T cells, compared to ANA-negative patients and healthy blood donors. In contrast, there was a significant increase in the frequencies of central memory, TH17, and TH2 CD4+ T cells detected in samples from ANA-positive patients, along with elevated plasma levels of IFNβ and IL-17, compared to controls. Unexpectedly, there were no differences observed in the frequency of Tregs or the ratio of Tregs to TH17 T cells. Finally, irrespective of age, CD4+ T cells from ANA-positive patients were significantly more metabolically active, compared to controls. Together, these data identify a T cell signature associated with production of ANAs that is distinct from age-related changes and unassociated with hospital admission.

Materials and methods

Human peripheral blood samples

Anonymized residual whole blood samples from individuals aged ≥ 18 years old were collected in EDTA and received from the Center for Advanced Laboratory Medicine at Columbia University or from consented healthy blood donors in an ongoing clinical trial (ClinicalTrials.gov #NCT02889133). Samples were de-identified and only age, sex, race, and ICD10 codes were collected (Table 1 and Supplementary Figure 1).

TABLE 1 Demographic characteristics of ANA-positive (ANA+), healthy blood donor (Healthy) and ANA-negative (ANA-) groups. Mean ± SEM was used.

	ANA+	Healthy	ANA-
Age (mean±SEM)	52±3.6	47±3	57±3.7
Sex (F/M)	9/11	15/17	10/10

Human subject participation was approved by the Columbia University Institutional Review Board.

Sample processing and flow cytometry

Samples were spun at 400 x g for 10 minutes to separate plasma from white blood cells (WBCs) and red blood cells (RBCs). Plasma was collected for detection of cytokines (IL-1β, IL-6, IL12(p70), IL-8, IL-10, GM-CSF, TNFα, IFNγ, IFNα2, IFNβ, IFNλ1 and IFNλ2-3: LEGENDplex™ Biolegend #740390, and for IL-17A/F: Legend Max Biolegend #435807). Plasma samples were diluted 2-fold and analyzed per manufacturer's instructions. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll density gradient centrifugation (Ficoll-Paque™ PLUS GE Healthcare #17-1440-02). Samples were washed with FACS buffer (phosphate buffered saline (PBS) supplemented with 2% fetal bovine serum and 0.4% of Ethylenediaminetetraacetic acid (EDTA)), stained for surface and intracellular markers for 30 minutes at 4°C with antibodies specific for CD4 (RPA-t4), CD3 (SK7), CD45RA (HI100), CD45RO (UCHL1), CD25 (CD25-4E3, BC96, M-A251), CCR7 (G043H7), CD31 (WM-59), FoxP3 (PCH101), CD194 (CXCR4;1G1), CD183 (CXCR3;1C6), CD196 (CCR6;11A9), CCR10 (1B5), CD185 (CXCR5;MU5UBEE), CD279 (PD1;J105), CD278 (ICOS;ISA3), p-Akt (SDRNR) and p-S6 (D57.2.2E). Staining for intracellular FoxP3, p-Akt and p-S6 was performed according to the protocol from Invitrogen using eBioscience™ FOXP3/Transcription kit (#00552300). Samples were collected using an Attune NxT flow cytometer (ThermoFisher) and data were analyzed with FlowJo software (BD Biosciences).

Statistical analysis

Subjects were divided into age groups (18-40, 40-60 and 60-80 years old) *a priori* due to the acceleration of thymic atrophy and cessation of active thymopoiesis after 40 years of age (33, 34) and the increase in immunosenescence that can alter T cell functionality after 60 years of age (35, 36). Between-group differences were compared using an analysis of variance (ANOVA) with Sidak's multiple comparison test and age as a categorical variable. Analyses were performed using Prism, version 9 (GraphPad Software, Inc.). For added rigor, the frequency of each T cell subset was also analyzed by multivariable linear regression incorporating age as a continuous variable and patient type (i.e., ANA-positive, ANA-negative and Healthy blood donors) as a categorical variable using SAS studio version 3.8 (SAS Institute Inc.). For comparisons that yielded different statistical results between ANOVA and multivariable linear regression analyses, additional supplemental figures were included. A p-value less than 0.05 was considered significant.

Results

Reduced frequencies of detectable recent thymic emigrants (RTEs) in young ANA-positive patients

Blood samples were collected from patients previously screened for antinuclear autoantibodies (ANAs) (N=40; 20 each of ANA-positive and ANA-negative) and healthy blood donors (N=32; Table 1 and Supplemental Tables S1, 2). T cell tolerance prevents many autoimmune diseases; thus, we hypothesized that T cell dysregulation would be evident in patients with prior ANA-positive results. Peripheral blood mononuclear cells (PBMCs), isolated from whole blood, were stained to identify CD4+ T cells. Absolute numbers of CD4+ T cells were unchanged in ANA-positive, compared to ANA-negative and healthy blood donors (Figure 1A). RTEs are the youngest peripheral T cells susceptible to peripheral T cell tolerance mechanisms. Due to thymic involution, the number of RTEs decrease with age, with a notable reduction in thymic output after 40 years of age (34, 37); as such, samples were evaluated in groups based on age ranges: (i) 18 to 40 years (young), (ii) 40 to 60 years (middle), and (iii) 60 to 80 years (old). RTEs, defined phenotypically as CD4+CD3+CD45RA+CD45RO-CD31+CD25-, were readily identified by flow cytometry (Figure 1B, gating strategy Supplemental Figure 1). Young ANA-positive patients had significantly fewer RTEs, compared ANA-negative patients and healthy controls (Figure 1C, $p<0.05$). To increase the statistical rigor, ANA-positive, ANA-negative and healthy controls were analysed with a multiple regression analysis with age as a continuous variable. ANA-positive had a significant reduction in the frequency of RTEs compared to healthy controls (statistical analysis Figure 1D). No significant differences in RTEs were noted with either statistical analysis approach between young ANA-negative patients and healthy controls. Consistent with prior observations (38, 39), the frequency of RTEs declined with age in ANA-positive patients, ANA-negative patients and healthy blood donors (Figure 1D; respectively $R^2 = 0.44$, $R^2 = 0.54$ and $R^2 = 0.83$). These data demonstrate that the RTE population in patients with ANAs is reduced compared to healthy controls, suggesting these patients may have perturbed differentiation into naïve, effector, and memory CD4+ T cell subsets.

Altered peripheral T cell populations detected in ANA-positive patients

In response to antigen exposure with co-stimulation, naïve T cells proliferate and differentiate into effector and memory T cells (40). To determine how generation of ANAs effected T cell

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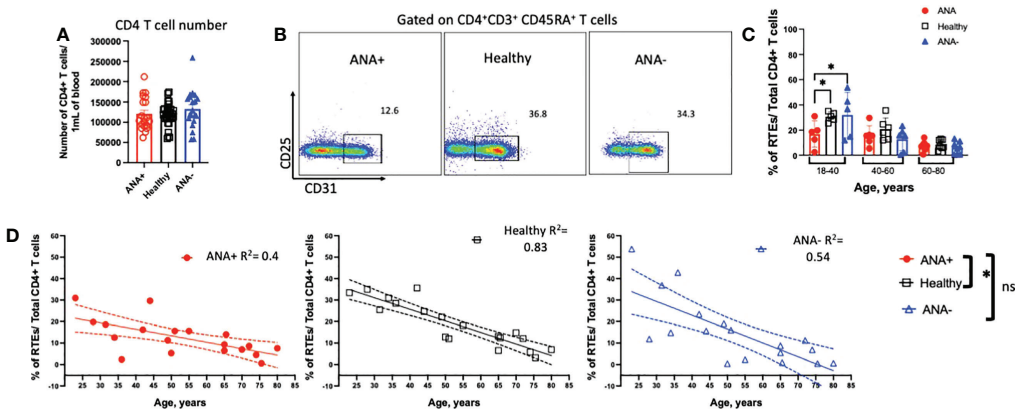

FIGURE 1
Young ANA-positive patients have fewer RTEs. PBMCs were isolated from whole blood and stained with antibodies to identify recenty thymic emigrants (RTEs). **(A)** The absolute number of CD4+ T cells per 1mL of blood was determined. **(B)** Representative flow plots of CD4+CD3+CD45RA+CD45RO-CD31+CD25- RTEs in ANA-positive patients, healthy blood donors, and ANA-negative patients. Subjects were analyzed in groups based on age ranges: 18-40 (young), 40-60 (middle), and 60-80 (old) years old. **(C)** The frequency of RTEs of total CD4+ T cells was calculated and data are shown as mean ± SEM and analyzed by a one-way ANOVA test followed by Tukey's multiple comparison test; *p<0.05. **(D)** The correlation between the frequency of RTEs and age was plotted. Linear regression analysis was used for all three groups and R2 are reported. Each data point reflects one subject (ANA-positive = 20, ANA-negative = 20, healthy blood donors = 32). Subjects were analysed using multiple regression analysis incorporating age as a continuous variable and type of blood donors (ANA-positive, ANA-negative, healthy blood donors) as a categorical variable followed by Tukey's multiple comparison test; *p<0.05; ns, non significant.

subsets, CCR7 and CD45RA were used to discriminate naïve (CD45RA+CCR7+), central memory (TCM; CD45RA-CCR7+), effector (TEF; CD45RA-CCR7-) and terminally differentiated effector memory (TEMRA; CD45RA+CCR7-) CD4+ T cell subsets (Figure 2A) (41). Young ANA-positive patients had significant reductions in the frequency of circulating naïve T cells and increased percentages of TCM, compared to ANA-negative patients and healthy blood donors (Figures 2B, C, $p<0.05$). Based on prior reports (42), we expected the frequency of TEMRA cells T cells to increase with age. In ANA-positive and healthy blood donors, TEMRA T cells showed moderate correlation with age (Figure 2D and Supplemental Figure 2; $R^2 = 0.44$ and $R^2 = 0.38$, respectively). No correlation between TEMRA T cells and age was observed in samples from ANA-negative patients ($R^2 = 0.09$). Comparing the frequency of TEMRA T cells in older individuals, the percentages were significantly increased in ANA-positive patients, compared to ANA-negative patients and healthy blood donors (Figure 2D). Analysis of other differentiated T cell subsets revealed no differences in the percentages of TEF or T follicular helper (TFH) CD4+ T cells (Figures 2E, F, gating strategy shown in Supplemental Figure 3).

Qualitative differences in TEF and TFH CD4+ T cells from ANA-positive patients

Upon activation, CD4+ T cells differentiate into specialized effector T helper (TH) subsets, characterized by distinct cytokine profiles and function. To test for differences in the distribution of TH subsets, TH1 (CCR4-CXCR3+CCR10-CCR6-), TH2 (CCR4+CXCR3-CCR10-CCR6-), and TH17 (CCR4+CXCR3-CCR10-CCR6+) CD4+ T cells were identified in PBMCs (gating strategy shown in Figure 3A). In all age groups, similar frequencies of TH1 CD4+ T cells were observed between ANA-positive patients, ANA-negative patients, and healthy blood donors (Figure 3B). In contrast, increased frequencies of TH2 and TH17 CD4+ T cells were observed in young ANA-positive patients, compared to ANA-negative patients and healthy blood donors (Figures 3C, D). Differences in TH subset distribution was not correlated with a particular disease state at the time of whole blood collection (Supplemental Figure 4). Finally, a specialized subset of circulating TFH CD4+ T cells, co-expressing PD-1 and ICOS, has been shown to reflect activated memory TFH and can facilitate B cell differentiation into plasma and memory cells (43). Young ANA-positive patients also had significantly higher percentages of PD1+ICOS+ TFH cells, compared to ANA-negative patients and healthy controls (Figure 3E, gating strategy shown in Supplemental Figure 3).

The mTOR pathway plays a key role in shaping T cell differentiation by differential activation of mTORC1 and mTORC2. And, mTOR activation in T cells has been noted during the development of several autoimmune diseases such as SLE, MS and RA (19, 44–47). To test whether CD4+ T cells were metabolically active, mTORC1 and mTORC2 activation was determined by detection of phosphorylated (p) -S6 and -Akt, respectively. Young ANA-positive patients had significantly

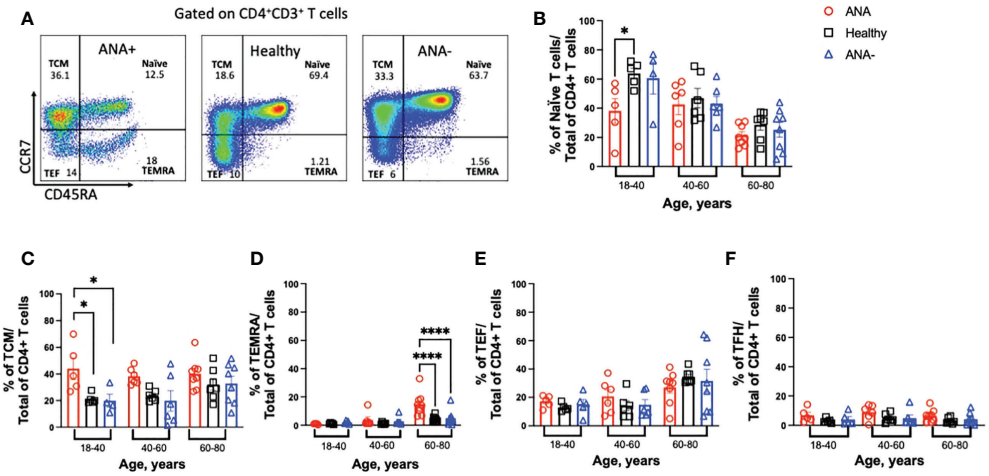

FIGURE 2
Young ANA-positive patients have reduced naïve and increased differentiated CD4+ T cells. PBMCs were isolated from whole blood and stained with antibodies to identify T cell subsets. **(A)** Gating strategy to visualize naïve, T central memory (TCM), terminally differentiated effector memory (TEMRA), and T effector (TEF) CD4+ T cells by flow cytometry. The frequencies of **(B)** naïve, **(C)** TCM, **(D)** TEMRA, **(E)** TEF, and **(F)** T follicular helper (TFH) T cell subsets of total CD4+ T cells was determined in ANA-positive patients, ANA-negative patients, and healthy blood donors. Each data point reflects one subject (ANA-positive = 20, ANA-negative = 20, healthy blood donors = 32). Data are shown as mean ± SEM and analyzed by a one-way ANOVA test followed by Sidak's multiple comparison test; *p<0.05, ****p<0.0001.

higher frequencies of p-S6, compared to ANA-negative patients and healthy blood donors (Figure 3F); no differences were noted in older cohorts. Frequencies of TH2 cells and mTORC1 in ANA-positive patients were significantly increased only compared healthy controls, after multiple linear regression analysis with age as a continuous variable were performed. In contrast to ANA-positive patients, TH2 cells and mTORC1 accumulate with age in ANA-negative patients (Supplemental Figures 5A, B). CD4+ T cells from ANA-positive patients in all age groups had significantly elevated frequencies of p-Akt, compared to ANA-negative and healthy blood donors (Figure 3G). Together, these data demonstrate that CD4+ T cells from ANA-positive patients are metabolically activated and have preferentially differentiated into TH17 effector T cells.

ANA production does not correlate with reduction in regulatory T cells

An imbalance between regulatory and inflammatory immune cells and a pro-inflammatory environment are associated with autoimmune diseases (10, 17, 18, 21). To assess whether regulatory T cell (Treg) frequencies were altered in PBMCs from ANA-positive patients, Tregs were identified by staining with antibodies against CD25 and FoxP3. The percentage of Tregs was similar in every subject group and in all age ranges (Figure 4A). Because we observed an increased frequency of proinflammatory TH17 CD4+ T cells, we calculated the Treg: TH17 CD4+ T cell ratio. Indeed, a decreased ratio correlates with many autoimmune diseases (48, 49). No significant difference in the Treg : TH17 ratio was observed; however, there was a trend of decreasing ratios observed upon aging (Figure 4B). A distinctive subset of FoxP3+ Treg cells, defined by CXCR5+ expression, are bona fide circulating T follicular regulatory cells (TFR). Presence of TFR in blood is indicative of ongoing humoral activity and significantly increased in patients with Sjögren syndrome (50, 51). There was no significant difference in the ratio of TFR within TFH T cells for any age group (Figure 4C). However, multiple linear regression analysis showed a significant decreased in the ratio of TFR : TFH T cells in both type of patients (ANA-positive and ANA-negative) compared healthy blood donors (Supplemental Figure 5C). The environment was studied by quantifying circulating cytokines with protective or deleterious role. Of the cytokines analyzed, only IFNβ was significantly higher in ANA-positive patients, compared ANA-negative patients and healthy blood donors (Figure 4D). Elevated levels of IL-17 were detected in ANA-positive patients, compared to healthy blood donors. Of note, higher data points observed in ANA-positive and ANA-negative patients correspond to different individuals. Together, these data suggest that ANA-positive patients do not have decreased Treg subsets nor can they be defined by a particular inflammatory cytokine profile.

Discussion

The data presented herein demonstrate that the T cell compartment in ANA-positive patients, in which tolerance

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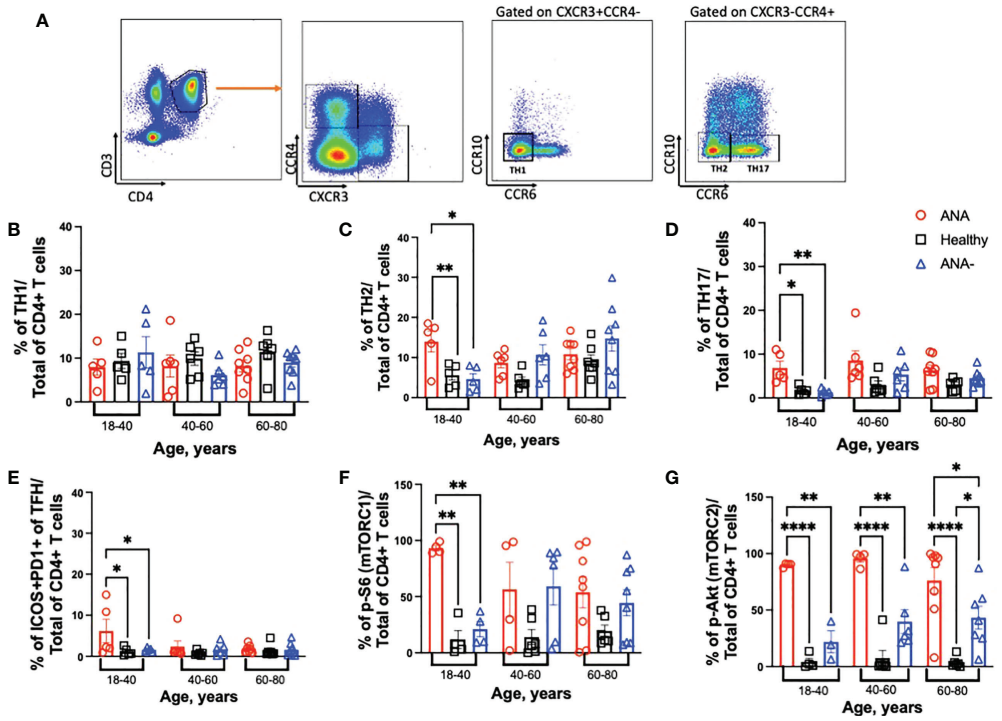

FIGURE 3
Young ANA-positive patients have increased frequencies of TH2 and TH17 CD4+ T cells that are metabolically active. PBMCs were isolated from whole blood collected from ANA-positive patients, ANA-negative patients, and healthy blood donors. Cells were stained with antibodies to identify CD4+ T helper (TH) subsets, activated TFH, and mTOR activation. **(A)** Representative gating strategy to visualize TH1, TH2 and TH17 CD4+ T cells. The frequencies of **(B)** TH1, **(C)** TH2, **(D)** TH17 and **(E)** ICOS+PD1+ TFH CD4+ T cells of total CD4+ T cells was determined. mTOR activation was evaluated by **(F)** p-S6 (marker for mTORC1) and **(G)** p-Akt (marker for mTORC2) on total CD4+ T cells. Each data point reflects one subject (ANA-positive = 20, ANA-negative = 20, healthy blood donors = 32). Data are shown as mean ± SEM and analyzed by one-way ANOVA test followed by Sidak's multiple comparison test; *p<0.05, **p<0.01, ****p<0.0001.

mechanisms have failed but have not led to active autoimmune disease, are distinct from ANA-negative patients and healthy blood donors. The most significant differences were observed in young 18-40 year old individuals whereby ANA-positive patients had significantly reduced numbers of naïve T cells and increased frequencies of effector T helper cell populations such as TH17 and TH2 CD4+ T cells. Unexpectedly no significant differences were observed in Tregs or the ratio of Treg : TH17 CD4+ T cells between groups. More generalizable to all age groups analyzed, ANA-positive patients had metabolically active CD4+ T cells and elevated levels of plasma IFNβ and IL-17 cytokines, compared to ANA-negative patients and healthy blood donors. Together, these data show that generation of ANAs, without active autoimmune disease, correlates with distinct changes in the CD4+ T cell compartment in young patients.

While age is not a significant predictor of autoantibody positivity (52), some studies report that the prevalence of ANAs increases with age in absence of autoimmune disease (4, 53). However, the aging process does siginificantly impact the T cell compartment due to antigen exposure, low-grade inflammation, cellular senescence and thymus involution (54). By analyzing subjects based on age ranges, significant changes within T cell subsets were observed. In particular, the frequency of RTEs progressively decreased with age in all groups, as expected (37). While age was a good predictor of the frequency of RTEs in healthy blood donors (i.e., $R^2 = 0.83$), it was not a strong correlate for ANA-positive or ANA-negative patients. This could be due to other, underlying medical conditions as these patients were currently in the hospital. Of interest, however, the presence of ANAs in young patients significantly reduced the frequency of CD4+ RTEs. RTEs are the youngest peripheral naïve T cells and are susceptible to tolerance induction (55); studies have shown that tolerance failure can induce autoimmune disease. Thus, the observed decreased frequencies of RTEs in ANA-positive patients may be due to premature differentiation into effector T cells and reflect tolerance failure. In general, aging also led to decreased frequencies of naïve T cells and an accumulation of TCM and TEF CD4+ T cells, as expected. However, consistent with the observation that young ANA-positive patients had decreased naïve T cell populations, these patients had a significant increase in TCM T cells; thus, there was no

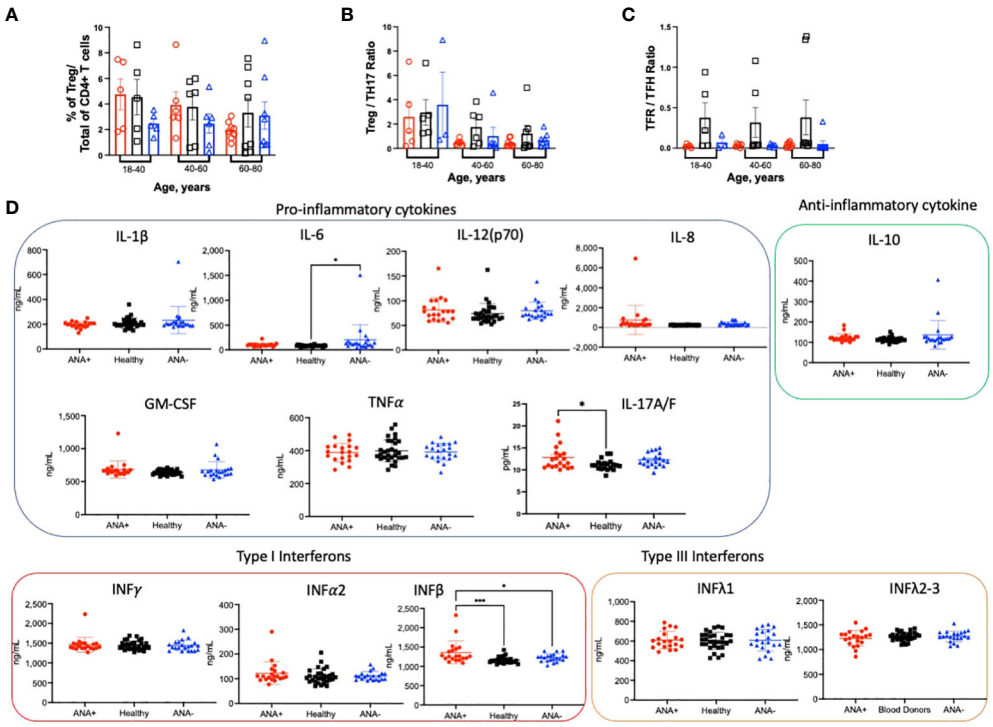

FIGURE 4
ANA-positivity does not correlate with changes in regulatory CD4+ T cells. PBMCs were isolated from whole blood collected from ANA-positive patients, ANA-negative patients, and healthy blood donors. Cells were stained with antibodies to identify **(A)** FoxP3+CD25+ regulatory T cells (Tregs). The ratios between **(B)** Tregs and TH17 and **(C)** T follicular regulatory (TFR) and TFH CD4+ T cells were calculated. **(D)** Plasma cytokines were measured by multiplex immunoassay. Each data point reflects one subject (ANA-positive = 20, ANA-negative = 20, healthy blood donors = 32). Data are shown as mean ± SEM and analyzed by a one-way ANOVA followed by Sidak's multiple comparison test; *p<0.05, ***p<0.001.

observed increase in TCM upon aging in this group. Finally, terminally differentiated effector memory (TEMRA) T cells that re-express CD45RA after antigen-stimulation have been documented to accumulate with age. TEMRA T cells exhibit multiple characteristics of senescent cells including DNA damage, low proliferative potential, high levels of reactive oxygen species (ROS) and can also be cytotoxic as it has the ability to secrete inflammatory cytokines such as IFNγ and TNFα (56, 57). In our study, the frequency of TEMRA cells slightly increase with age in all groups. However, older ANA-positive individuals have significantly increased percentages of TEMRA T cells, compared to ANA-negative patients and healthy controls; these data suggest that an accumulation of TEMRA T cells may be due to, or the result of, autoimmunity. And, although there is significant accumulation of differentiated T cell subsets in young ANA-positive patients, the data show that these T cells do not become senescent. Further studies will be required to investigate the role of ANAs in TEMRA T cell differentiation. Together, these data show that the presence of ANAs is associated with alterations in the T cell compartment, especially in 18-40 year old patients.

T cell lineage commitment can be influenced by the metabolic status of T cells. Antigen recognition triggers robust mTOR activation, which drives the differentiation of naive CD4+ T cells into the TH1, TH2 and TH17 cell effector lineages, while also inhibiting the induction of Tregs and T cell anergy (19, 58, 59). Our data showed that both mTOR complexes, mTORC1 and mTORC2, were significantly increased in CD4+ T cells of ANA-positive patients. While mTORC1 activation can promote expansion of TH1 and TH17 subsets, mTORC2 activation leads to TH2 differentiation; as increased frequencies of mTORC1 and mTORC2 activation was observed in CD4+ T cells from young ANA-positive patients, this could explain the elevated numbers of TH17 and TH2 T cell subsets. However, despite the fact that ANA-positive subjects had higher frequencies of TH2 cells and mTORC1 activation, compared healthy blood donors in all age groups, in ANA-negative patients these frequencies increased with age and are comparable to ANA-positive subjects suggesting that diseases state alone can influence T cell differentiation and activation. Increased frequencies of TH2 and TH17 CD4+ T cells are associated with autoimmune diseases such as SLE, RA and systemic sclerosis (60–62). The frequency of T helper cells can be altered by the presence of

other pathologies such as inflammation or immunodeficiency (63, 64). However, no significant correlation between diseases and distribution of T helper cells were observed. Despite the high frequencies of CD4+ T cells with mTOR activation, it was surprising that no overt alterations in Tregs were noted. However, Tregs were only determined by phenotype and were not assayed for functional capabilities. mTORC1 and mTORC2 can also promote differentiation of TFH cells (65–67), another specialized CD4+ T subset involved in the pathogenesis of autoimmune diseases (68). Both TFH and CD4+ helper T cells interact with and provide help to B cells to induce differentiation and antibody production (69). It has been shown that increased frequencies of circulating TFH are detectable in patients with autoimmune diseases (70). PD1 and ICOS are co-stimulatory molecules essential for the development and function of TFH. In our study, while the frequency of TFH cells was similar in all subjects, the frequency of circulating PD1+ICOS+ TFH cells in ANA-positive patients was higher than that in ANA-negative and healthy controls. Increased numbers of ICOS+ TFH cells in peripheral blood are detectable in patients with autoimmune diseases including SLE, SjD, RA, and autoimmune thyroid diseases (71, 72). ICOS plays an important pro-inflammatory role in the late effector phase and T memory-dependent B cell response (73) and PD1 is expressed on activated T cells (74). Taken together, we speculate that high levels of mTOR activation in T cells from ANA-positive patients contribute to autoantibody production by expanding effector T cells and PD1+ICOS+ TFH, which may eventually promote future development of autoimmune disease.

Dysregulated immunoregulation also contributes to failure of immune tolerance. Prior studies have shown that development of autoimmune diseases is influenced by decreased numbers or function of Tregs and a corresponding expansion of effector cells, reflecting an imbalance between immunoregulatory and inflammatory cells. The decreased ratios of Treg : TH17 and TFR : TFH have been proposed as useful metrics for active autoimmune diseases such as SjD and RA (50, 75, 76). However, there is disagreement between studies on whether these ratios differ between ANA-positive patients, ANA-negative patients, and patients with an autoimmune disease (77, 78). Our data showed no significant differences in the ratios of Treg : TH17 or TFR : TFH between ANA-positive and ANA-negative patients; both patient groups had significantly decreased ratio of TFR : TFH compared to healthy blood donors. Thus, the underlying factor(s) that promote naïve T cell differentiation into TCM or TEF remain unclear. Although no numerical differences were observed in Tregs from ANA-positive patients, there was a significant increase in proinflammatory TH17 T cells, an observation consistent with other publications (79, 80). TH17 T cells secrete proinflammatory cytokine IL-17, which is detectable in plasma of patients with autoimmune disease. Increased inflammatory cytokines are also reported in ANA-positive patients (77), albeit to a lesser degree than those found in patients with autoimmune disease (17, 77, 81). Of the cytokines measured in our study, only IFNβ and IL-17 were elevated in plasma from ANA-positive patients, compared to ANA-negative patients and healthy blood donor controls. The absence of an overt pro-inflammatory cytokine signature in plasma from ANA-positive patients is consistent with the lack of active autoimmune disease. Additionally, IFNβ was described with a protective role in acute viral infections and deleterious role in bacterial infection (82); in autoimmune diseases, IFNβ is used as an effective treatment to reduce recurrence in multiple sclerosis (83) by activating regulatory T cells thereby limiting the generation of TH17 response and modulating pro-inflammatory mediators (84). Moreover, IFNβ is involved in down-regulating the inflammatory response by inhibiting antigen presenting cells, increasing Treg activity, reducing the ability of B cells to present antigens (85) and shifting Th1/Th2/Th17 polarization to an anti-inflammatory state (86, 87), this could be a protective mechanism to prevent disease onset. Together, these data highlight that, although differences in the T cell compartment were evident in ANA-positive patients, there was no significant alteration observed in regulatory T cell subsets nor strong upregulation of multiple pro-inflammatory cytokines, as observed in active autoimmune disease.

The current study has several limitations. Each patient (both ANA -positive and -negative) was in the hospital for an organ transplant or had been diagnosed with other diseases (e.g., heart failure, cancers, hypertension, kidney failure, fever, and respiratory issues) which can, in and of itself, elicit different immune responses that impact T cells. Additionally, the samples analyzed in this study were de-identified and data regarding medical treatment, which would influence immune cells, were not collected. Moreover, although demographic data were collected, subjects were not matched by age, sex, or ethnicity. Finally, no patient was diagnosed with an autoimmune disease at the time of blood collection and, due to the nature of the study design, it is impossible to perform a long-term follow up to assess longlasting effects T cell compartment changes and determine the rate of autoimmune disease.

Herein, we show that CD4+ T cells from ANA-positive patients are distinct from ANA-negative patients and healthy blood donors. Although ANA-positive patients do not have an active autoimmune disease, the production of ANAs correlated with marked changes in the T cell compartment. As such, these data provide new insights on the distribution of T cell subsets, mTOR activation, and cytokine production in the absence of pathogenic autoimmune responses. Investigating these mechanisms in a longitudinal study of ANA-positive subjects would improve our knowledge in the development of T cell distribution at the onset and upon progression of autoimmune disease.

Data availability statement

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

Ethics statement

This study was reviewed and approved by the Columbia University Institutional Review Board. Written informed consent was obtained from all participants for their participation in this study.

Author contributions

FD and KH designed the studies and experiments. FD, CM, AQ, and AM collected and processed samples from subjects and volunteers. FD stained samples, collected data, and performed data analysis. FD and KH wrote the manuscript. All authors participated in data interpretation, revised the manuscript, and approved of the submitted version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Although unrelated to the contents of this manuscript, author KH has a sponsored research agreement with Alpine Immune Sciences.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2022.972127/full#supplementary-material

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Dysregulation and chronicity of pathogenic T cell responses in the pre-diseased stage of lupus

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In the normal immune system, T cell activation is tightly regulated and controlled at several levels to ensure that activation occurs in the right context to prevent the development of pathologic conditions such as autoimmunity or other harmful immune responses. $CD4^+FoxP3^+$ regulatory T cells (Treg) are crucial for the regulation of T cell responses in the peripheral lymphatic organs and thus for the prevention and control of autoimmunity. In systemic lupus erythematosus (SLE), a prototypic systemic autoimmune disease with complex etiology, a disbalance between Treg and pathogenic effector/memory $CD4^+$ T cells develops during disease progression indicating that gradual loss of control over T cell activation is an important event in the immune pathogenesis. This progressive failure to adequately regulate the activation of autoreactive T cells facilitates chronic activation and effector/memory differentiation of pathogenic T cells, which are considered to contribute significantly to the induction and perpetuation of autoimmune processes and tissue inflammation in SLE. However, in particular in humans, little is known about the factors which drive the escape from immune regulation and the chronicity of pathogenic T cell responses in an early stage of autoimmune disease when clinical symptoms are still unapparent. Here we briefly summarize important findings and discuss current views and models on the mechanisms related to the dysregulation of T cell responses which promotes chronicity and pathogenic memory differentiation with a focus on the early stage of disease in lupus-prone individuals.

KEYWORDS

systemic lupus erythematosus, autoimmunity, immune regulation, T cell signaling, metabolism, genetics, IL-2

Introduction

Systemic lupus erythematosus (SLE) is a severe multisystem autoimmune disease with complex pathogenesis which is characterized by immune dysregulation and chronic inflammation of various organs caused by the breach of immune tolerance predominantly towards nuclear autoantigens, such as double-stranded deoxyribonucleic acid (dsDNA). SLE primarily affects young women of childbearing age and clinical manifestations can range from relatively mild skin and joint involvement to life-threatening disease including renal, neurologic or cardiac inflammation (1, 2). Tissue inflammation and damage are mediated by the deposition of immune complexes and by the infiltration with autoreactive lymphocytes (2, 3). In particular, autoreactive $CD4^+$ T cells are considered to play a central role in the initiation and perpetuation of the pathologic immune responses in several ways: Follicular T helper (Tfh) cells, which represent a subset of $CD4^+$ T helper cells and which have the unique capability to migrate to the outer edge of the B cell follicles in the lymphatic organs in order to initiate germinal-center reactions (4), are essential for the activation and differentiation of autoreactive B cells in SLE (5, 6). Further, $CD4^+$ effector T cells invade the affected tissues and mediate tissue inflammation and damage by cell-cell interactions and by the production of inflammatory and cytotoxic cytokines such as interferon (IFN)-γ and IL-17 (6, 7). In addition, chronic activation of $CD4^+$ T cells and the generation of a robust autoimmune T cell memory may contribute to the recurrence of disease flares, the persistence of tissue inflammation and damage accrual and to treatment refractory disease states (6, 8). Despite the controlled deletion of most autoreactive T cells during T cell maturation in the thymus, T cells that can recognize autoantigens are still abundantly present in the healthy organism, but those do not become pathogenic when kept under check by intact mechanisms of peripheral self-tolerance (9). Regulatory $CD4^+$ T cells (Treg) expressing the linage specific transcription factor forkhead box P3 (FoxP3) are indispensable for the maintenance of peripheral self-tolerance and thus for the prevention and control of inflammation and autoimmunity throughout entire life. Predominantly derived from a predetermined T cell subpopulation in the thymus, $CD4^+FoxP3^+$ Treg mainly recognize auto-antigens *via* their T cell receptor and are required to regulate the activation and expansion of auto-reactive T cells and other harmful immune cells in the peripheral lymphatic organs (10–13). Given their crucial function in immunoregulation and peripheral tolerance, it appears obvious that disturbances in Treg biology contribute to the development of autoimmune diseases such as SLE (14, 15). Indeed, it was shown in murine and later also in human SLE that an acquired and progressive deficiency of the cytokine interleukin-2 (IL-2), an essential growth and survival factor for Treg, promotes an imbalance between Treg and effector/memory $CD4^+$ T cells, which was associated with accelerated disease activity (16–18). These pathophysiological findings have stimulated the successful translation of low-dose IL-2 therapy into clinical trials aiming to restore Treg activity in patients with active SLE (19–24).

Given the pivotal role of T cells and their dysregulation in SLE pathogenesis, it can be hypothesized that chronic T cell activation and memory differentiation in early disease are key events for the initiation and progression of autoimmunity in SLE.

Genetic basis of aberrant T cell activation in human and murine SLE

The development and progression of SLE involves a complex interaction of genetic risk, diet, environmental influences, and immune dysregulation (3, 25). Individuals with genetic risk alleles for SLE who are exposed to environmental risk factors during their lifetime could be more susceptible to autoimmune diseases where synergistic interactions facilitate the onset of pathogenic autoimmune responses. Up to now, several lines of evidence indicate that genetic factors contribute to the etiopathogenesis of SLE, supported by twin studies or familial aggregation investigations (26). Specifically, GWAS data have uncovered the involvement of several susceptible HLA and non-HLA genes supporting that altered T cell signal transduction and activation is important in SLE. Various proteins such as cytokines and kinases essential for regulating T cell activation, proliferation and differentiation, are encoded by these susceptible loci. The HLA region contains several genes encoding for molecules involved in antigen presentation or immune-related proteins (27). In SLE, expression of HLA-DR, which is an indicator of activated T cells, is elevated in circulating T cells and the frequency of HLA-DR-expressing $CD3^+$ T cells is associated with SLE disease severity (28). Besides HLA loci, genes outside the HLA region also appear to play an important role in SLE development. For instance, a mutation in the SLE risk gene *PTPN22* (R620W), encoding for a tyrosine phosphatase that regulates T cell signaling, is associated with aberrant receptor signaling function on effector and memory T cells as well as B cells (29). However, in polygenic diseases such as SLE genetic contribution could be distinctly involved in disease susceptibility at different ages (30) as suggested for pediatric and adult-onset SLE. Pediatric SLE patients, in particular those with monogenetic forms, are an inimitable group to highlight the importance of genetic contribution, as they develop disease earlier with a more severe disease manifestation and a higher frequency of family history (31). For example, a positive correlation between polymorphisms in HLA genes and the age of SLE diagnosis has been shown,

indicating that older patients have the higher genetic risk (32). Conversely, higher number of SLE-associated non-HLA polymorphisms are prevalent in the younger patients (32). Analysis of candidate genes in children with SLE and their parents has confirmed the involvement of SLE-associated genes, including SELP (P-selectin gene) and IRAK1 (interleukin-1 receptor-associated kinase 1 gene), that are overexpressed in $CD4^+$ Treg from patients with SLE (33, 34). Investigating both pediatric- and adult-onset patients with defined genetic defects could also provide valuable models to elucidate T cell dysregulation at the early phases of disease.

Parallel investigations over the last two decades indicated many similarities in the genetic basis for susceptibility to SLE between mice and men (3). In mice, the genetic involvement in SLE etiology is evidenced by various susceptible inbred mouse strains, which all develop a lupus-like disease, although to different extent, such as the (NZBxNZW) F1 (NZBW), NZM2410, and MRL–Fas^{lpr} strains (35). Similar to humans with SLE, GWAS has identified over 100 loci related to increased susceptibility for lupus-like disease in mice (36, 37). In the context of T cell-associated genetic alterations, the *Sle1a* gene segment in the NZM2410 lupus-prone strain is responsible for the increased activation of conventional $CD4^+$ T cells (Tcon) and for the low numbers of $CD4^+FoxP3^+$ Treg (38, 39). The *Sle1c2* sublocus is another lupus susceptibility gene segment, which contributes to an elevated $CD4^+$ T cell activation, a robust age-dependent expansion of IFN-γ-expressing Th1 cells, and a decrease in Treg counts (40). Signal transducer and activator of transcription (STAT) 4, a transcription factor engaged in the signal transduction of several cytokine receptors that plays a significant role in regulating T cell activation and differentiation is also a candidate gene for susceptibility to SLE. The deficiency of the *Stat4* gene in lupus-prone mouse models has confirmed its major effect on lupus severity, leading to reduced autoantibody production and T cell activation (41). Deficiency of *Fli1*, a transcription factor that is expressed by T cells, in MRL/lpr mice leads to diminished T cell activation, decreased expression of the Th1-associated chemokine receptor CXCR3 in T cells, and finally to reduced disease activity (42).

These findings in both humans and mice indicate that variants in several genes that are involved in T cell activation and differentiation are associated with SLE susceptibility and severity. In addition, $CD4^+$ T cells from patients with active SLE exhibit a global DNA hypomethylation (43, 44) which is likely to cause an overexpression of numerous relevant genes.

Abnormal T cell signaling in humans

Several studies have proven that T cells from SLE patients exhibit an abnormal signaling profile which can be detected already at the onset stage of disease (45, 46). One possible explanation for the excessive T cell response to antigen stimulation in SLE is related to an early abnormality in the molecular signaling pathway of T cells (3). In SLE patients, the complex of CD3 proteins, which is assembled with the TCR, shows defects in terms of a diminished expression of the CD3ζ chain, which is the only subunit that is both genetically and structurally distinct from the CD3δ, ϵ, and γ complex members (45, 47–49). In addition to the diminished expression of CD3ζ, it was shown that the functionally and structurally homologous Fc receptor gamma subunit (FcRγ) occupies the binding space of CD3ζ which may play a major role in the aberration of the antigen receptor-initiated signaling and therefore lead to a variety of pathogenic changes in the SLE T cell phenotype (46, 50). During the regular immune response in healthy individuals, CD3ζ recruits the tyrosine kinase zeta-chain-associated protein kinase-70 (ZAP-70), which ensures a controlled moderate calcium influx at the end of the signaling cascade (50, 51). In SLE T cells the replacement of CD3ζ with the FcRγ, induces the binding of the spleen tyrosine kinase (Syk) with high affinity instead of ZAP-70, which results in a much stronger calcium influx into the T cell cytoplasm and which in turn leads to a decreased activation threshold of $CD4^+$ T and B cells upon autoantigen recognition. The increased intracellular calcium content leads to upregulation of calcium-triggered calcium/calmodulin-dependent protein kinase IV (CaMK4) (52, 53), which mainly regulates various transcription factors through phosphorylation, such as the cAMP-responsive element modulator α (CREMα) (54, 55). CREMα is known to negatively regulate IL-2 transcription and to induce the expression of IL-17 (56, 57), which is likely to promote the disbalance between Treg and Tcon, as the growth and survival of Treg are severely impaired due to the limited availability of IL-2.

The mammalian target of rapamycin (mTOR), a serine-threonine kinase localized in the outer mitochondrial membrane, has been identified as a central regulator of T cell lineage specification, serving as a physiological sensor of mitochondrial dysfunction and ATP depletion in T cells (58). mTOR translates a variety of environmental information into signals that control either nutrient supply, cAMP levels, and osmotic stress, as well as cellular processes including protein biosynthesis and autophagy (59). mTOR complex 1 (mTORC1) is considered essential for Th1 and Th17 differentiation, whereas mTOR complex 2 (mTORC2) is important for Th2 differentiation in mice (60). Both complexes suppress the transcription factor FoxP3 and thus inhibit the differentiation of FoxP3+ Treg (61). In SLE patients, it was shown that mTOR activation in double negative (DN) T cells was increased and preceded disease flares (62). In more detail, mTORC1 activity was found to be increased, whereas mTORC2 activity was reduced accompanied by an increase in Th17 cells (63). Consistent with this, inhibition of mTORC1 by rapamycin promoted the expansion of the $CD4^+FoxP3^+$ Treg population and the suppression of Th17 cells, and was capable to decrease disease activity in patients with active SLE (63–65).

Similarly, also the serine-threonine kinases Rho-associated protein kinases (ROCK) 1 and 2 play an important role in SLE pathogenesis. Generally, ROCKs regulate migration, activation, and differentiation of T cells and are crucial for controlling cytoskeletal components including the ezrin/radixin/moesin (ERM) proteins (45, 66). ERMs are important for the association of plasma membrane proteins with actin filaments, and regulate migration and cell adhesion through association with the intracellular domain of CD44 (67). Signaling through ROCK2 also plays an important role in the differentiation of Th17 cells and Tfh cells (45). PBMC and T cells from patients with SLE show significantly higher activity of ROCK and ERM compared to healthy controls (68, 69) and expression levels of CD44 are strongly increased in T cells and correlate with disease activity (68, 70), suggesting that increased adhesion and migration of SLE T cells occurs due to the steady activation of the CD44-ROCK-ERM axis (45).

Interferons (IFNs), in particular type I IFNs, play a central role as initiators of the pathogenic immune response in SLE. Nucleic acids released from apoptotic cells and immune complexes trigger the production type-1 IFNs by tissue resident plasmacytoid dendritic cells. Type-1 IFNs exert stimulatory effects on a variety of immune cells including T cells through activation of the STAT1 pathway (71–73). Consistent with this, it was reported that the expression levels of STAT1 were increased in $CD4^+$ T cells from SLE patients and positively correlated with disease activity (74, 75). In addition, high levels of STAT1 phosphorylation were observed in activated Treg that were decreased in numbers, and it was shown that type-1 IFNs can induce apoptosis in Treg *via* the IRAK1 pathway (75, 76), indicating that type-1 IFNs negatively interfere with Treg homeostasis and survival.

Whether these abnormal signaling events are acquired during disease progression or genetically determined, however, remains to be determined.

Abnormal T cell phenotype in humans

Up to now the earliest time point for which data on the T cell phenotype from SLE patients are available is at the onset of disease. In SLE patients with established clinical manifestations, different subsets of T cells with an abnormal activation pattern can be identified, which mediate inappropriate inflammatory responses and support enhanced B cell activation (3, 6). The frequencies of $Ki67^+$, proliferating $CD4^+FoxP3^-$ conventional T cells (Tcon) is strongly increased in patients with active SLE and correlates with disease activity (17, 77), indicating that aberrant Tcon activation is associated with disease activity and severity. Th17 cells, a subset of $CD4^+$ T helper cells, show an overactivation and express increased levels of IL-17, which promotes inflammation and systemic tissue damage by recruiting neutrophils, monocytes and other immune cells to the inflamed tissues and by inducing autoantibody production (78, 79). Interestingly, the rarely present double negative (DN) T lymphocytes lacking the CD4 and CD8 co-receptors (<5% of T lymphocytes in healthy individuals) are increased in SLE patients and induce the production of anti-dsDNA antibodies by autoreactive B cells. These DN T cells differ in the secretion of cytokines such as IL-1β and IL-17 and are also found in cellular infiltrates in renal biopsies from patients with lupus nephritis (80). Similarly, $CD4^+$ T cells that express the Th1-associated chemokine receptor CXCR3 are abundantly present in the inflamed kidneys and their numbers in the urine are predictive for disease flares (81). More recently T follicular helper cells (Tfh) have been recognized as an important T cell population in SLE. Tfh cells are a heterogenous subset of $CD4^+$ T cells that have the capability to migrate into the lymphoid follicles *via* the chemokine receptor CXCR5 and to induce the activation and differentiation of autoreactive B cell as part of the germinal-center reaction (4). In peripheral blood of patients with SLE numbers of Tfh cells, in particular of so-called circulating precursor Tfh cells, are elevated and correlate with disease activity (5, 82). $CD4^+$ T cells lacking expression of the co-stimulatory receptor CD28 ($CD4^+CD28^{lo}$ cells), which are considered to represent chronically activated memory/effector $CD4^+$ T cells, where shown to be expanded and to produce IFN-γ in patients with moderately active SLE (83). In patients with juvenile-onset SLE, elevated $CD8^+$ effector memory T-cell frequencies indicated more persistently active disease over time. Active SLE is further characterized by a decline in $CD4^+FoxP3^+CD127^{lo}$ Treg that express high levels of CD25 ($CD25^{hi}$ Treg), a subset of Treg with a high suppressive capacity, and by an imbalanced proliferation between Treg and Tcon in favor of an enhanced Tcon proliferation (17). The reduced frequencies of $CD25^{hi}$ Treg and the Treg/Tcon proliferation imbalance, which both correlated with disease activity, are typical indicators of a low availability of IL-2 and constitute the most relevant defects in Treg biology in SLE, which, however, can be corrected by treatment with low doses of IL-2 (17, 19, 21). Nevertheless, although the central role of T cells in established SLE has been well recognized over the last decades, phenotypic alterations of immune cells in a pre-diseased state still remain poorly explored due to lack of material from humans.

Abnormal T cell phenotype in early murine SLE

Similar to other autoimmune diseases, it is proving difficult to study the origin and development of SLE before the onset of clinical symptoms in humans, and hence, mouse models of SLE provide valuable tools for the assessment of alterations at a cellular and molecular level before disease onset and during the progression of disease.

The NZBW mouse model is considered to authentically resemble most features of human SLE (35, 36). These mice spontaneously develop the lupus-like disease within 4 to 6 months of age, which provides a condition to study cellular and molecular changes of immune cells at the pre-diseased stage before disease onset. It has long been shown that immune responses such as the balance between T cell populations are altered by age in NZBW mice (84, 85). More recent studies indicated that increased $CD4^+$ T cell activation and memory differentiation are detectable in lymphoid organs a long time prior to the appearance of clinical manifestations and even before relevant titers of the autoantibodies can be measured in the plasma, suggesting that aberrant T cell activation is an early event in this autoimmune condition (16). We have investigated the phenotypic changes of conventional $CD4^+FoxP3^-$ T cells (Tcon) and $CD4^+FoxP3^+$ Treg during disease progression in NZBW mice including young clinically healthy mice as well mice at the disease onset and with established disease (16). $CD4^+$ Tcon from lymphoid organs of young, clinically healthy mice at an age between 8-12 weeks already showed signs of increased activation and memory formation evidenced by higher frequencies of $CD69^+$ and $CD44^+$ among $CD4^+$ Tcon compared to healthy BALB/c mice. Frequencies and numbers of activated and memory $CD4^+$ Tcon and IFN-γ producing Th1 cells further increased substantially during progression to disease onset and active disease. A lower prevalence of $CD4^+FoxP3^+$ Treg, which had an intact suppressive function, was already detectable in young pre-diseased NZBW mice compared to BALB/c mice. In parallel, these mice also had higher frequencies of $CD69^+$ and $CD44^+$ Treg suggesting that Treg activation with the attempt to counteract the increased Tcon activation occurs already early in disease development. Phenotypically, Treg from young mice still expressed normal levels of CD25 and IL-2 production by $CD4^+$ T cells was also not impaired in young mice indicating that, in contrast to the later disease stages, lack of IL-2 may not be responsible for the low prevalence of Treg which instead is rather genetically determined (16). This is supported by studies in congenic mouse strains related to the NZBW strain that indicated that the low prevalence of Treg was linked to the disease-related Sle1a locus (86). Alternatively, the Treg deficiency may be caused by an impaired thymic Treg generation, however, we found that the numbers and proliferation rates of thymic Treg were normal in young NZBW mice (16). A decrease in CD25 expression in Treg and a diminished proliferation ratio between Treg and Tcon, which are indicators of Treg exhaustion due to IL-2 deficiency, could be observed earliest at the onset stage of disease, when IL-2 production by $CD4^+$ T cells was also found to be significantly impaired (16), indicating that IL-2 deficiency is an acquired and potentially reversible phenomenon in SLE. Although the origins of IL-2 deficiency are certainly complex and not fully understood, we propose that it might be caused by the repression of IL-2 synthesis that occurs in chronically activated Tcon (21). Similar phenotypic alterations of Treg and Tcon could also be observed in the autoimmune susceptible NZB parental strain that develops a milder form of lupus, and to a lesser extent also in the clinically healthy NZW strain, suggesting that genetic alterations from both strains contribute to T cell hyperactivity in murine lupus (16).

Abnormal T cell signaling and metabolism in early murine SLE

As described previously in humans, murine SLE T cells also exhibit rewiring of their TCR, in which expression of the CD3ζ chain is reduced (45). This reduction or even complete deletion leads to a severe systemic inflammatory response in mice (87). However, the pathologic changes in T cell signaling are not limited to this single signaling pathway. Interestingly, the entire $CD4^+$ T cell life span from activation and proliferation to differentiation is strictly regulated by cellular metabolism (88, 89). A recent study in lupus-prone *B6.Sle123* and in SLE patients demonstrated that both aerobic glycolysis and mitochondrial oxidative phosphorylation are elevated in $CD4^+$ T cells (90). The pathophysiological relevance of these findings was confirmed by showing that the application of the glycolysis inhibitor 2-deoxy-D-glucose and of the mitochondrial metabolism inhibitor metformin, were capable to suppress autoimmunity, decrease IFN-γ and IL-17 production and restore IL-2 synthesis, indicating that an altered cellular metabolism contributes to chronic T cell activation in SLE (90, 91). These data were collected at the onset stage of disease; however, it is reasonable to assume that corresponding events also occur at the pre-diseased stage of SLE and serve as initiators for subsequent pathological changes in cellular metabolism. In addition, and similar to studies in humans, the mTOR inhibitor rapamycin was capable to restore T cell metabolism and to decrease disease activity in lupus-prone MRL/lpr mice (92, 93).

Proposed model of T cell dysregulation in SLE pathogenesis

Taking current knowledge into consideration, we propose a simplified model that may explain the gradual and progressive failure to adequately regulate the activation of autoreactive T cells in the immune pathogenesis of SLE which facilitates chronic activation of pathogenic T cells and promotes the generation of a robust autoimmune memory (Figure 1). In health there is a homeostatic balance between Treg

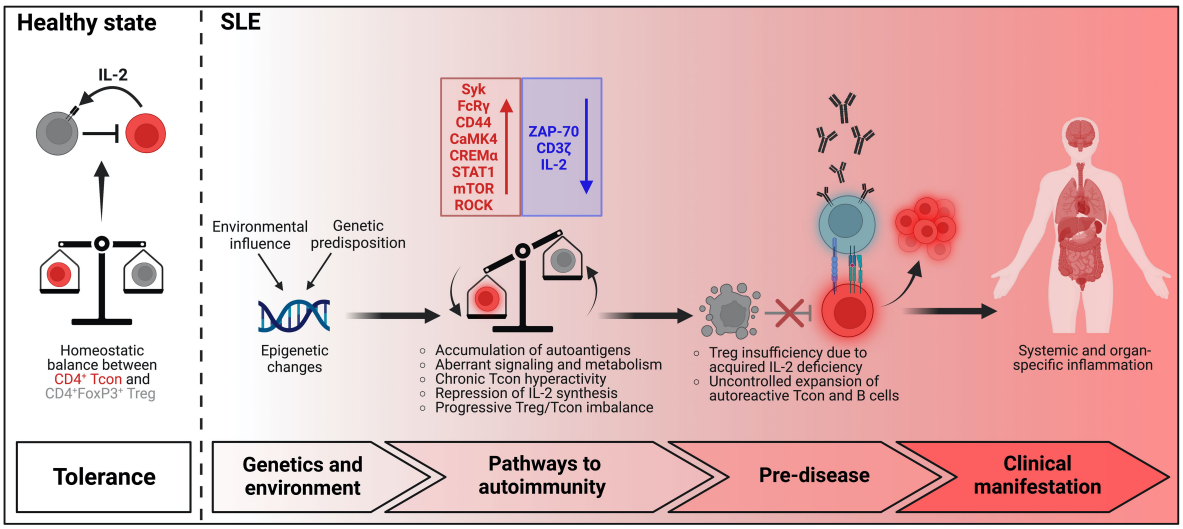

FIGURE 1
Model of T cell dysregulation in SLE pathogenesis.

and autoreactive Tcon that prevents the development of autoimmunity. In SLE distinct genetic alterations modified by environmental factors contribute to disease pathology. Initially, the accumulation of nuclear autoantigens which also serve as endogenous danger signals and induce the expression of pro-inflammatory cytokines, in particular of type-1 interferons, leads to the presentation of autoantigens by dendritic cells in an inflammatory context to autoreactive Tcon in the lymphoid tissues and consecutively to their activation and differentiation into effector/memory T cells and Tfh cells. In the early stage, the expansion of autoreactive T cell clones, which is facilitated by aberrant signaling and metabolism causing a lowered activation threshold, is partially counter-regulated by a functionally intact, yet already numerically restricted Treg population. The presence of pro-inflammatory cytokines upon antigen recognition also confers resistance in Tcon to Treg mediated suppression which further enhances the escape of autoreactive Tcon from immune regulation. During further progression of disease and due to the persistence of autoantigens and inflammatory signals, the pool of autoreactive effector/memory Tcon continuously expands, while their chronic and repetitive activation leads to the repression of IL-2 synthesis. The decreasing availability of IL-2 in turn impairs the adequate expansion of the Treg population in order to sufficiently counter-regulate the hyperactivity of Tcon which further facilitates the escape of autoreactive T cells and the chronicity of pathogenic T cell responses. This vicious cycle of a self-amplifying disruption of Treg homeostasis leading to progressive and chronic hyperactivity of autoreactive Tcon may continue for several years until numbers of Treg decline below a critical size and clinical manifestations emerge.

Perspective

While the role of T cells and their dysregulation in established SLE is currently relatively well understood, little is still known about the mechanisms and molecular pathways which drive the escape from immune regulation and the chronicity of pathogenic T cell responses in the very early stage of disease. Continuing research efforts in this field provide the unique opportunity to identify novel therapeutic targets that might be capable to prevent the occurrence of clinical manifestations or to induce long-lasting remission.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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IgG subclass and Fc glycosylation shifts are linked to the transition from pre- to inflammatory autoimmune conditions

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A crucial factor for the development of inflammatory autoimmune diseases is the occurrence of antibodies directed against self-tissues and structures, which leads to damage and inflammation. While little is known about the cause of the development of mis-directed, disease-specific T and B cells and resulting IgG autoantibody responses, there is increasing evidence that their induction can occur years before disease symptoms appear. However, a certain proportion of healthy individuals express specific IgG autoantibodies without disease symptoms and not all subjects who generate autoantibodies may develop disease symptoms. Thus, the development of inflammatory autoimmune diseases seems to involve two steps. Increasing evidence suggests that harmless self-directed T and B cell and resulting IgG autoantibody responses in the pre-autoimmune disease stage might switch to more inflammatory T and B cell and IgG autoantibody responses that trigger the inflammatory autoimmune disease stage. Here, we summarize findings on the transition from the pre-disease to the disease stage and vice versa, e.g. by pregnancy and treatment, with a focus on low-/anti-inflammatory versus pro-inflammatory IgG autoantibody responses, including IgG subclass and Fc glycosylation features. Characterization of biomarkers that identify the transition from the pre-disease to the disease stage might facilitate recognition of the ideal time point of treatment initiation and the development of therapeutic strategies for re-directing inflammatory autoimmune conditions.

KEYWORDS

autoimmunity, IgG subclass, IgG glycosylation, pre-autoimmune disease stage, inflammatory autoimmune disease stage

Introduction

Inflammatory autoimmune diseases are a worldwide threat to health and show an increasing prevalence (1). Although tumor-reactive IgG autoantibodies (autoAbs) can mediate beneficial roles in eliminating tumor cells, IgG autoantibodies are often key players in the induction of inflammatory autoimmune diseases. Accordingly, depletion of B cells with rituximab (monoclonal anti-CD20 Ab) often improves inflammatory autoimmune disease conditions (2). Interestingly, autoimmune patients can start to express IgG autoAbs years before developing specific clinical symptoms (3–5). Furthermore, a certain proportion of healthy individuals express specific IgG autoAbs without disease symptoms (6, 7).

The occurrence of IgG autoAbs at an early pre-disease stage was initially described for (seropositive) rheumatoid arthritis (RA) (Figure 1). Anti-citrullinated peptide IgG autoAbs can be detected years before RA disease symptoms develop (3).

Another example is diabetes mellitus type 1. In an interesting study, several thousand healthy infants without a first-degree family history of diabetes were screened for autoAbs typical of diabetes type-1 (4). A total of 155 of 7787 infants were repeatedly screened positive for such autoAbs. Several years later, 26 of these 155 autoAb-positive infants and only two of the autoAb-negative infants had developed diabetes type 1 (4).

These studies lead to two very important conclusions. First, individuals generating specific IgG autoAbs also have a higher risk of developing the specific inflammatory autoimmune disease (3, 4). This finding opens the possibility of identifying pre-disposed individuals before developing disease symptoms by close-meshed monitoring (5). Second, not all individuals with specific IgG autoAbs develop the respective inflammatory autoimmune disease, at least not at a short interval.

Accordingly, it is increasingly assumed that the development of inflammatory autoimmune diseases has to undergo two steps. In step one, and for incompletely understood reasons, tolerance mechanisms fail, and an autoantigen-specific T and B cell response leads to detectable IgG autoAbs. Several findings suggest that these IgG autoAbs, however, might be harmless in the pre-autoimmune disease stage and do not induce any clinical signs. In the second step, this specific immune response might shift in some but not all individuals to a more inflammatory T and B (T/B) cell and IgG Ab response that gives rise to various disease conditions (inflammatory autoimmune disease stage) often years after step one (Figure 1) (3, 4, 8–14). Such a shift might likely be dependent on genetic predispositions and

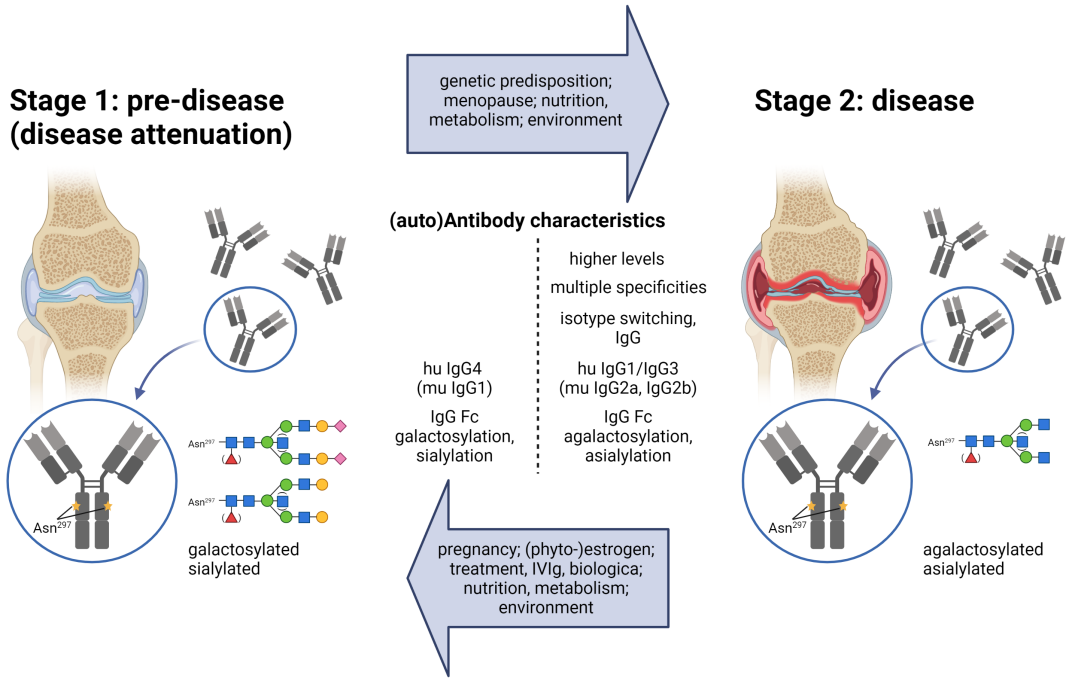

FIGURE 1
The two-stage model for the development of inflammatory autoimmune diseases. In Stage 1, the pre-disease state, autoAbs are present, but there are no disease symptoms. Due to reasons indicated in the upper arrow, the condition shifts to an inflammatory stage 2, where disease symptoms occur. Enhanced antibody titers, multiple specificities, IgG class switching, and shifts in IgG subclasses and Fc glycosylation patterns are described for autoantibodies in the disease state (described in the text). Factors shown in the lower arrow are confirmed, suggested, or discussed to redirect an inflammatory antibody response into the direction of the low-/non-inflammatory pre-disease state. IgG Fc glycosylation patterns: blue, *N*-acetylglucosamine (GlcNAcs); green, mannose; red, fucose, yellow, galactose; purple, sialic acid.

environmental factors determining inflammatory conditions. Steps one and two might also occur simultaneously.

Here, we summarize findings on the transition from the pre-disease to the disease stage and vice versa, e.g. by pregnancy and treatment, with a focus on low-/anti-inflammatory versus pro-inflammatory IgG autoAb responses.

Low versus high inflammatory IgG (auto) antibody responses

The inflammatory severity of IgG (auto)Abs may be dependent on the IgG autoAb-specific subclass and glycosylation pattern as well as the total IgG glycosylation pattern. Knowledge about these determinants will be described and discussed in the following paragraphs.

Activating versus inhibitory IgG subclasses

The following functional IgG subclass pairs between human and mouse have been identified: human (hu) IgG1 and murine (mu) IgG2a (IgG2c); hu IgG2 and mu IgG3; hu IgG3 and mu IgG2b; and hu IgG4 and mu IgG1 (15).

Human IgG1 and IgG3 as well as murine IgG2a and IgG2b show the highest affinities to the classical activating FcγRs and C1q, the starting molecule of the classical complement activation pathway (16–18). These IgG subclasses seem to be able to form hexamers facilitating the interaction with the six-arm C1q molecule (15, 19–23). Human IgG2 and murine IgG3 hardly interact with classical FcγRs and C1q and their effector functions are mostly unclear (16–18). They are induced for instance by T cell-independent antigens. Furthermore, recent studies have shown that murine IgG3 can form complexes that induce nephritis (24).

Human IgG4 and murine IgG1 show higher affinities to the only classical IgG inhibitory receptor FcγRIIB than to their classical activating FcγRs (16–18). Furthermore, human IgG4 and murine IgG1 cannot activate C1q, but seem to be able to disturb hexamer formation of the C1q-activating IgG subclasses (15). Murine IgG1 also inhibits the formation of murine IgG3 complexes (24). Furthermore, human IgG4 and murine IgG1 can generate Fab arm exchange meaning that heavy chains with different specificities can dimerize, which reduces their ability to form immune complexes (25).

Thus, human IgG1/IgG3 and murine IgG2a/IgG2b are the IgG subclasses with the highest potential to activate the immune system, whereas human IgG4 and murine IgG1 have less activating potential and can even inhibit the effector functions of human IgG1/IgG3 and murine IgG2a/IgG2b. Accordingly, inflammatory autoimmune diseases are often characterized by the appearance of the activating human IgG1/IgG3 and murine IgG2a/IgG2b subclasses, and the first studies showed that the autoantigen-specific IgG subclass shift from inhibitory to activating IgG subclasses is associated with higher inflammatory autoimmune conditions and vice versa that enrichment of the inhibitory human IgG4/murine IgG1 subclass can counteract inflammatory (auto)immune conditions (Figure 1) (10, 15, 24, 26). However, corresponding human studies are scarce and are needed to verify these observations. Other autoantigen-specific IgD, IgM, IgA and IgE isotypes might also be involved, an area that is less investigated and not the subject of this review. In addition, increased autoAb titers (14) and autoantigen specificities (4) might facilitate the transition from the pre- to the disease stage.

Pro-inflammatory versus lower-/anti-inflammatory IgG Fc *N*-glycosylation patterns

The effector functions of IgG molecules are additionally linked to their type of Fc *N*-glycosylation attached to asparagine 297 (Asn297, *N*) of both heavy chains in the IgG Ab Fc region (27). A highly conserved biantennary glycan core structure consisting of *N*-acetylglucosamines (GlcNAcs) and mannoses can be further modified with a fucose, a bisecting GlcNAc and one or two galactose residues, each of which can be further capped by a sialic acid (27, 28) (Figure 1).

Autoantigen-specific agalactosylated (non-galactosylated and non-sialylated; G0) IgG Abs are linked to pro-inflammatory conditions in inflammatory (auto-) immune diseases, whereas attachment of galactose and sialic acid is related to fewer inflammatory or even anti-inflammatory conditions (Figure 1) (3, 8, 11–13, 28–39). In most studies, IgG Fc bisection also correlates with pro-inflammatory conditions (28). IgG Fc afucosylation in particular has been connected to a high tumor fighting potential (16, 40), but shows different trends in distinct inflammatory autoimmune diseases (28, 41–43).

Mechanistically, afucosylated IgG Abs show an increased affinity to certain classical activating FcγRs (40). The effector function of IgG Fc bisection has rarely been investigated (28). Sialylated IgG Abs have shown a decreased affinity to classical (murine) activating FcγRs (27). The described functions of galactose are controversial as galactosylated IgG Abs show an enhanced interaction with C1q (44), whereas IgG agalactosylation increases the induction of the lectin and alternative complement pathways (32, 45–47). However, the *in vivo* functions of differently glycosylated IgG Abs seem to be much more complex because single terminal glycan residues can also interact with glycan binding receptors, e.g., of the galectin, siglec and C-type lectin receptor families (34–36, 48–50). *In vivo*, immune inhibitory functions have been described for sialylated as well as terminal galactosylated

antigen-specific and total IgG Abs (11, 27, 34–36, 38, 48–52). However, further studies are needed to solve the *in vivo* functions of differently glycosylated (auto)antigen-specific IgG (subclass) Abs.

Several studies have suggested that the transition from the pre-autoimmune disease stage to the inflammatory autoimmune disease stage is linked to decreasing IgG autoAb galactosylation and sialylation levels (3, 8, 9, 11–14). Interestingly, an increase in anti-citrullinated peptide IgG Fab glycosylation sites - very likely generated by somatic hypermutations - has recently been linked in addition to the shift from the pre-disease to the disease stage in the case of RA (53–55).

A reduction in autoantigen-specific human IgG4 and murine IgG1 galactosylation and sialylation levels seems to increase their inflammatory potential and might be an explanation for the appearance of autoantigen-specific IgG4-mediated inflammatory autoimmune diseases (15, 34, 36, 49, 52, 56–59).

Total IgG Fc *N*-glycosylation

In addition to autoantigen-specific serum IgG Fc glycosylation patterns, the corresponding total serum IgG Fc glycosylation patterns have been linked to inflammatory conditions, for instance in patients with RA. Autoantigen-specific IgG Abs not only enrich the total IgG, but rather the whole T and B cell responses seem to shift to a more inflammatory stage. Thus, low total serum IgG Fc galactosylation and sialylation levels correlate with severe inflammatory disease conditions in RA. In 2006, it was found in mouse studies that the therapeutic effect of IVIg (intravenous immunoglobulin; high amounts (2 g/kg/course) of pooled serum IgG from healthy donors to treat inflammatory diseases) is based on the sialylated total IgG subfraction (27, 28, 48, 60). Respectively, IVIg treatment may re-establish a lower inflammatory immune status.

The total IgG Fc glycosylation status seems to act as a huge immunological buffer system. Higher total IgG Fc sialylation levels seem to up-regulate classical inhibitory and down-regulate classical activating FcγRs (48, 52, 61), and further immune receptors might be affected. Accordingly, the total IgG Fc agalactosylation level is assumed to indicate the inflammatory status of each individual and increases with chronological and, in particular, biological age (62–65). The glycosylation pattern of total IgM and IgA Abs very likely also influences the immune status.

A change in total IgG Fc glycosylation during the transition from the pre-disease to the disease stage is controversial. A recent study described that low total IgG Fc galactosylation levels could also occur very early in the pre-disease stage of RA patients (66). However, total IgG Fc agalactosylation levels seem to be a risk factor for the development of RA.

Induction of IgG antibodies with low galactosylation and sialylation levels

Recent immunization studies have shown that different co-stimuli/adjuvants/inflammatory conditions induce distinct germinal center (GC) T and B cell responses that determine different expression levels of α2,6-sialyltransferase (St6gal1; the enzyme that adds sialic acid to IgG Fc parts) in GC-derived plasma cells (PCs) and corresponding IgG Fc sialylation levels (67). It is assumed that beta1,4-galactosyltransferase (B4galt1; the enzyme that adds galactose to IgG Fc parts) expression and corresponding IgG Fc galactosylation are regulated similarly in parallel (67). Accordingly, (auto)antigen-specific IgG Fc galactosylation and sialylation levels reflect the inflammatory immune status and can be used as biomarkers of the inflammatory potential of the running (auto)antigen-specific T and B cell response (67).

In this context and in the context of RA models, it has been shown that, in particular, IL-6, IL-27R-induced IFNγ-producing T_{FH1} and T_{FH17} cells contribute to the induction of low St6gal1 expression in GC B cells and corresponding PCs as well as low IgG Fc sialylation levels (12, 67). Abrogation of these signals has led to higher St6gal1 expression and higher IgG Fc galactosylation and sialylation levels (12, 67).

Reasons for the switch from pre- to inflammatory conditions

In step one, tolerance mechanisms fail, and an autoantigen-specific T and B cell response leads to detectable IgG autoAbs. This step is even less understood than step two. A certain portion of individuals expressing specific IgG autoAbs might never develop specific disease symptoms. Others can switch from the pre- to the inflammatory stage (Figure 1). The reasons are still unclear and might occur individually. Increasing evidence suggests that the T/B cell and IgG autoAb responses induced in step one do not have to be pathogenic, but can switch to inflammatory, pathogenic IgG autoAb responses initiating the disease stage (3, 4, 8–14).

Three scenarios seem to be most likely for the transition. First, unfavorable genetic predispositions such as certain MHC alleles might favor such a switch (14, 68). Nevertheless, there is only a small overlap of the disease appearance between monozygotic twins (69), suggesting that additional factors might play an important role. Second, a specific event such as a severe lung infection/inflammation might switch the inflammatory status of the whole immune system for some days, which may lead to the alteration of a harmless autoantigen-specific T/B cell response in the pre-stage to an inflammatory T/B cell response. Third, the switch might occur slowly over time. Aging and increasing BMIs, for instance, shift

the whole immune status as well as the total IgG Fc glycosylation level to a more inflammatory condition (62–65). Furthermore, unfavorable nutrition and a shift in the microbiome induced, for instance, by nutrition or antibiotics can influence the immune status (see also below). The accumulation of certain types of gut bacteria might then favor more inflammatory immune responses, e.g., by supporting the Th17 axis (70, 71). Slow shifts to more inflammatory immune conditions might also shift the autoantigen-specific T and B cell response to a more inflammatory state and induce the development of specific autoimmune disease symptoms.

Pregnancy and estrogen lead to a return to less inflammatory (auto) immune conditions

To understand the shift from the pre- to the inflammatory stage, it might be helpful to analyze conditions when the inflammatory autoimmune stage returns to a less inflammatory stage in the direction of the pre-stage.

The most prominent case is likely pregnancy. Women with RA show less disease symptoms during pregnancy, a tolerogenic status established to inhibit immune attacks against the fetus (72, 73). During pregnancy, total as well as autoantigen-specific IgG Abs shift to higher Fc galactosylation and sialylation levels (72, 73). Notably, Fab glycosylation does not change during pregnancy (74). Understanding the changes in T/B cell and Ab responses during pregnancy in patients with inflammatory autoimmune diseases, such as RA, will facilitate understanding and recognition of the switch from the pre- disease to the disease stage.

Appropriately, the level of the sex hormone estrogen, which is highly upregulated during pregnancy and downregulated during menopause, positively correlates with IgG Fc galactosylation and sialylation levels in males and females (75). Furthermore, application of estrogen or phytoestrogens reduced inflammatory conditions, up-regulated B4galt1 and St6gal1 expression and enhances IgG Fc galactosylation and sialylation levels (75–77). In addition, phytoestrogens have been described to exert anti-inflammatory effects (78, 79).

Early diagnosis

Healthy individuals with identified specific IgG autoAbs could be closely monitored to recognize any starting transition from the pre- to the inflammatory (auto)immune stage for starting therapies before clinical disease symptoms evolve. Therapies might then redirect the inflammatory autoantigen-specific T and B cell and Ab response back into the direction of the low-/non-inflammatory pre-stage (80). Total as well as autoantigen-specific IgG Fc galactosylation and sialylation levels seem to be promising biomarkers for characterizing any transition.

Discussion of existing and potential new therapies

There are increasing therapeutic tools that reduce inflammatory (auto)immune conditions. Some, e.g. rituximab, deplete central immune cells such as B cells, and others, such as IVIg or monoclonal anti-TNFα and anti-IL-6 Abs, redirect pro-inflammatory to less inflammatory conditions. Alhough different therapeutics are available and frequently used, their anti-inflammatory mechanisms are often not completely understood. In the following section, we will address the anti-inflammatory potential of some existing therapeutics and discuss further possibilities to redirect inflammatory immune conditions or to maintain the pre-disease stage.

IVIg

Different immune modulatory effects of IVIg have been described, one of which may be the re-establishment of the total IgG Fc glycosylation buffer system by increasing the proportion of the sialylated IgG Ab subfraction (27, 48, 81, 82). If a patient shows no response to IVIg therapy, higher amounts of IVIg might be necessary to re-establish a healthy/tolerogenic total IgG Fc galactosylation and sialylation level. In the meanwhile, there have been attempts to further modulate IVIg enzymatically by adding the maximal number of sialic acids (four) to one IgG molecule to enhance the anti-inflammatory properties and make the efficacy more consistent (83). However, when IVIg therapy is discussed, it must be mentioned that several anti-inflammatory mechanisms have been postulated for IVIg and that an anti-inflammatory effect of the sialylated IgG subfraction of IVIg remains to be confirmed in humans. It has for instance been described that IVIg might be contaminated with TGF-ß (84). Nevertheless, further analyses have revealed that TGF-ß contamination cannot explain most of the observed anti-inflammatory functions (85). Furthermore, the galactosylated subfraction of IVIg might also mediate anti-inflammatory functions (35, 50).

Blocking Abs/Biologica

Other used therapeutic tools are blocking Abs or Biologica that target pro-inflammatory cytokines or their receptors, such as TNFα, IL-6, IL-1, IL-12, IL-23 and IL-17.

Anti-TNFα therapy has probably been developed to reduce local inflammatory immune conditions. However, successful anti-TNFα therapy of RA patients has been shown to increase autoantigen-specific as well as total IgG galactosylation and sialylation levels (86), also assuming an effect on all current (GC) T and B cell responses. The involvement of TNFα in the proper formation of GCs is well known (87). However, the influence of

TNFα on certain T_{FH} cell subpopulations and corresponding glycosyltransferases in GC B cells has not yet been verified.

In addition to anti-TNFα application, it was found that treatment of RA patients with tocilizumab, an IL-6 blocking Ab, increased IgG Fc galactosylation levels (88). Recent mouse studies have shown that IL-6 is an important cytokine for inflammatory GC reactions with low B cell intrinsic St6gal1 expression leading to IgG Abs with low galactosylation and sialylation levels (67).

New blocking Abs target IL-12, IL-23 and IL-17, that might inhibit the generation of Th1 and Th17 cells as well as GC T_{FH1} and T_{FH17} cells, which have been shown to be necessary for the induction of IgG Abs with low galactosylation and sialylation levels (67).

Furthermore, whether these new or further cytokine blocking Abs can shift the IgG subclass composition to human IgG4/murine IgG1 to influence inflammatory conditions via this pathway has hardly been examined.

Treatment with corticosteroids also reduce inflammatory conditions and might influence IgG Fc subclass and/or glycosylation shifts.

Currently, the described treatments are applied when inflammatory (auto)immune disease conditions appear. However, in the future, treatments could start earlier when the starting point of the transition from the pre-disease to the inflammatory disease stage is monitored and recognized in IgG autoAb positive "healthy" individuals.

Nutrition/metabolism

Corticosteroids have unfavorable side effects, and biologic treatment is very expensive. What can autoAb-positive "healthy" individuals do to reduce the probability of undergoing the shift from the autoimmune pre-disease to the inflammatory disease stage (Figure 1)? The role of nutrition and metabolism regarding inflammatory conditions has been increasingly discussed lately and could therefore be one possibility to counteract such a shift.

Researchers have found that obesity, a known driver of inflammation (89), increases, whereas extensive weight loss decreases the IgG Fc agalactosylation level (65). Furthermore, a positive correlation between body mass index (BMI) and the IgG Fc agalactosylation level has been recently described in various studies (62, 64). Thus, the metabolic state of an individual seems to influence the inflammatory immune status.

Accordingly, a positive correlation between BMI and the development of several autoimmune diseases has been observed (90–92). Moreover, fasting intervals simultaneously decrease inflammatory disease symptoms and the IgG agalactosylation level in RA patients (93).

More targeted dietary changes also result in improvement of inflammatory (auto)immune diseases (94, 95) and for some inflammatory autoimmune diseases, an influence of diet on T/B cell responses has been described (96–99).

Secondary plant metabolites seem to be able to change an inflammatory state toward more tolerogenic conditions, such as certain phenolic acids that can modulate the production of pro-inflammatory cytokines (100). Moreover, polyunsaturated fatty acids (PUFAs) that occur not only in plants but also in eggs and fish have shown beneficial effects on inflammatory autoimmune diseases such as RA, SLE, multiple sclerosis and diabetes type-1 (101, 102).

Certain diets might also act on the gut bacterial composition and the generation of gut bacterial metabolites. Fasting versus Mediterranean diets change the microbiome composition in RA patients (103). For some of these microbial metabolites, like short chain fatty acids (SCFAs), it is well known that they mediate anti-inflammatory properties and can even influence T/B cell responses. The SCFA butyrate (C4), for instance, reduces IFNγ and inflammatory IL-17 levels (104) and promotes the differentiation of T follicular regulatory cells (105). Furthermore, butyrate induces the generation of IL-10^+ regulatory B cells (106) and PCs (107) and alters IgG subclass distributions toward less IgG2b (and a tendency toward less IgG2a) in mice (107).

Together, single nutrients and metabolites might have a strong potential to boost the transition to the inflammatory autoimmune disease stage, but others might have the capacity to re-direct inflammatory T/B cell responses or even to hold IgG autoAb-positive "healthy" individuals in the pre-disease stage (Figure 1).

Environment

Another interesting factor that should be considered in the context of inflammatory autoimmune diseases is the environment, such as stress. It is generally believed that stress is a potent inducer of inflammation (108, 109) and, even further, of inflammatory autoimmune diseases (110). In living conditions where stress-levels are generally high, such as shift work, there is growing evidence that the prevalence and disease onset of inflammatory autoimmune diseases is enhanced (111–113). Therefore, it is of interest to determine whether stress can influence the inflammatory status of the T/B cell and the Ab response. Recently, a study with rats investigated the effects of chronic stress on IgG Abs (114). Stress induced higher IgG2a and IgG2b agalactosylation levels in young female rats but higher IgG2b galactosylation levels in older female rats. In the future, addditional research is needed to investigate the influence of stress and other environmental factors on the inflammatory T/B cell and IgG Ab status.

Conclusion

Several lines of evidence suggest a two-step model for the development of inflammatory autoimmune diseases. In stage one low–/non–inflammatory T/B cell and IgG Ab responses occur

that can, but do not have to shift to more inflammatory T/B cell and IgG Ab responses inducing stage two with inflammatory autoimmune disease phenotypes. Early identification and observation of IgG autoAb positive "healthy" individuals might help to recognize changes in the T/B cell and IgG Ab response for starting anti-inflammatory treatments to abolish the transition into stage two. Healthy diets and agreeable environments might help to maintain the less inflammatory stage one. The IgG subclass distributions and IgG Fc glycosylation pattern might thereby act as suitable biomarkers to recognize the transition from stage one to stage two.

Author contributions

The conceptualization was done by JSB and ME. The review results from the discussion and the consensus of all authors. The review was written by JSB, MB, and ME. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Analysis of T cell repertoires of CD45RO CD4 T cells in cohorts of patients with bullous pemphigoid: A pilot study

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Autoimmune diseases develop over years - starting from a subclinical phenotype to clinically manifest autoimmune disease. The factors that drive this transition are ill-defined. To predict the turning point towards clinical disease and to intervene in the progress of autoimmune-mediated dysfunction, the establishment of new biomarkers is needed. Especially CD4 T cells are crucially involved in autoimmunity: first, during the initiation phase, because they lose their tolerance towards self-peptides, and second, by the subsequent ongoing presentation of self-peptides during the active autoimmune disease. Accordingly, changes in the degree of diversity of T cell receptor (TCR) repertoires in autoimmunity have been reported. These findings led to the hypothesis that transition from pre-disease to autoimmune disease is associated with an increase of abnormally expanded T cell clones that occupy large portions of the TCR repertoire. In this pilot study, we asked whether the ratio and the diversity of the TCR repertoires of circulating memory (CD45RO) and naïve (CD45RA) CD4 T cells could serve as a predictive factor for the development of autoimmunity. To find out, we analyzed the TCRβ repertoires of memory and naïve CD4 T cells in a small cohort of four gender- and age-matched elderly patients having the autoimmune blistering disease bullous pemphigoid or non-melanoma skin cancers. We found that the extent of clonal expansions in the TCRβ repertoires from the circulating memory and naïve CD4 populations did not differ between the patient groups. This result shows that the diversity of TCR repertoires from peripheral CD4 T cells does not reflect the manifestation of the skin-associated autoimmune disease BP and does not qualify as a prognostic factor. We propose that longitudinal TCR repertoire analysis of younger patients might be more informative.

KEYWORDS

naive and memory CD4 T cells, autoimmunity, skin diseases, T cell receptor, repertoire diversity, next generation sequencing

Introduction

The T cell receptor (TCR) repertoire is the sum of all T-cell antigen-binding sequences that are present within one individual. It is determined not only by the number of different TCR combinations but also by the number of T cells within one body. Recent data predicted 4 x 10^{11} circulating T cells in humans (1) with a potential diversity of 10^{6} -10^{8} different αβ TCR combinations (2, 3). A highly diverse TCR repertoire is the prerequisite for (i) mediating protection against the broad variety of pathogenic antigens, (ii) eliminating altered self to control neoplasias, and (iii) preventing recognition of self-antigens to cause autoimmunity. The diversity of the TCR repertoire changes during a lifetime. It is at its peak during young ages due to the constant thymic output of recently developed naïve T cells, and it decreases during aging as a result of the thymic involution, increased homeostatic proliferation, and the development of a T cell memory pool (4–6). Along with this shrinking diversity, the tendency to acquire inflammatory autoimmune diseases rises with aging. This holds true also for autoantibody-mediated diseases due to the prominent role of CD4 T cells in providing B cell help (7, 8). For example, the titers of antinuclear antibodies and rheumatoid factors increase after the age of 60 years in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), respectively. There is growing evidence that the clinical manifestations of autoimmune diseases correlate with changes in features of the TCR repertoire (9). Thus, it has been shown that not only the diversity of the TCR repertoire is significantly decreased in SLE patients but also the number of disease-associated T cell clones connects with disease severity in SLE and RA patients (10, 11). Additionally, the onset of clinical features in cases of SLE and RA correlates to changes in the specificity of the autoantibodies (12–14). The reasons for the shift in autoantibody-specificity and for turning the preclinical stage into clinical autoimmunity are not known. Considering the relationship between the TCR repertoire, aging, and the development of autoimmune diseases, we hypothesize that the diversity of the TCR repertoire might change profoundly in the process of development of the autoimmune disease from preclinical autoimmunity and could serve as predictive factor.

To find out we compared the peripheral TCRβ repertoire of small cohorts of four autoimmune patients and four patients with a non-autoimmune disease. Specifically, we focused on the circulating memory CD4 T cell population, and hypothesized that the degree of TCRβ repertoire diversity of this population might be lower in autoimmune patients when compared to non-autoimmune patients. Memory CD4 T cells emerge upon repeated contacts with their antigenic peptide presented in the MCHII complex. They are defined as lymphocytes that present fast recall responses to antigens and thereby provide immediate protection in peripheral tissues in case of pathogenic invasions. Thus, memory T cells are irreplaceable in combating infections and preventing pathogen-specific reinfections. However, if T cells lose their tolerance to self-peptides and differentiate into memory T cells, they bear the dangerous potential to induce autoimmunity. In contrast to pathogens, self-peptides cannot be dispelled, which leads to ongoing contacts between T cells and self-peptides resulting in chronic inflammations and might lead, as hypothesized in this study, to an accumulation of memory T cell clones specific to self-peptides. This contrasts with naïve T cells that exhibit no immediate effector functions because of the lack of antigen contacts and extensive clonal divisions (15). Memory T cells can be distinguished from naïve T cells by the expression of the surface markers CD45RO (CD45RAneg). CD45RO and CD45RA (CD45RApos) are isoforms of the CD45 tyrosine phosphatase, which is expressed on all T cells. Upon activation, T cells change the surface expression from the CD45RA to the CD45RO isoform. Therefore, CD45RO T cells are considered as memory T cell population including both recently activated effector and memory T cells (16, 17).

As autoimmune patient group, we chose patients diagnosed with the autoimmune blistering disease bullous pemphigoid (BP), which is mediated by autoantibodies directed against domains of type XVII collagen in the skin (18, 19). As control group, age- and gender-matched patients with non-melanoma skin cancers (NMSC) such as basal (BCC) and squamous skin cancer (SCC) were selected. By choosing NMSC patients as control group we focused on the availability of samples from elderly patients, who (i) were diagnosed with a completely different disease entity (autoimmune versus cancer), (ii) displayed inflammatory skin conditions, and (iii) were hospitalized at the same ward, to exclude potential effects of the surrounding microbiome. NMSC is characterized by a low metastatic rate from the host cells of the skin without involvement of autoreactive and systemic T and B cell responses (20, 21) (Supplementary Table 1). This contrasts with the inflammatory skin disease BP, which is induced by aberrant T and B cell responses including an accumulation of CD4 and CD8 T cells in skin lesions (22–25). In addition, skin lesions in BP patients appear body-wide, which indicates the systemic character of this autoimmune disease (18, 19).

In this study, we compared the number of T cells within the memory and naïve CD4 T cells population between both patient groups. Deep sequencing was used to identify their TCRβ repertoires for enumeration of TCRβ clonotypes and to compare the degree of the TCRβ repertoire diversity, the clonal overlap between the memory and naïve CD4 populations, and other TCR repertoire features such as the V/J

Abbreviations: BCC, basal cell carcinoma; BP, bullous pemphigoid; CD3, complementarity determining region 3; CD45RO CD45RA negative, memory; CD45RA, CD45positive, naïve; NMSC, non-melanoma skin cancers; PBMC, peripheral blood mononuclear cells; RA, rheumatoid arthritis; SCC, squamous cell carcinoma; SLE, systemic lupus erythematosus; TCR, T cell receptor; TRBJ, TCR beta joining genes; TRBV, TCR beta variable genes.

gene usage and the complementarity determining region 3 (CDR3) length of the antigen-binding regions. Unexpectedly, despite differences in the ratio of CD4 T cell numbers and TCRβ clonotypes, we found that the extent of clonal expansion of the circulating memory CD4 T cell population did not differ between the patient groups.

Material and methods

Patient samples

Patients with BP and age- and sex-matched biopsy-confirmed patients with NMSC were recruited at the University of Luebeck. The peripheral venous blood from participants was collected in the EDTA-containing tubes and the skin biopsy was taken as a punch biopsy and shock frozen in liquid nitrogen. The blood from 14 BP patients and six NMSC patients was used to enumerate naïve and memory CD4 T cells. Finally, sorted naïve and memory CD4 T cells from 4 BP and 4 NMSC patients as age- and gender-matched controls were subjected to deep sequencing for analysis of their TCRβ repertoire. BP was diagnosed based on the typical clinical manifestation of the disease, detection of the linear deposition of IgG at the dermal-epidermal junction in the direct immunofluorescence, detection of IgG on the blister roof on salt-split skin biopsies, or the enzyme-linked immunosorbent assay against BP180-NC16A (data not shown). The clinical characteristics of the BP and NMSC patients are summarized in Supplementary Table 1. The study was conducted based on the principles of the 1964 Helsinki declaration and its further amendments. All patients enrolled in this study provided written informed consent prior to their inclusion in this study. The study was approved by the ethical committee of the University of Luebeck (Approval ID: AZ 12-178).

Isolation of CD45RA positive and CD45RA negative CD4 T cells

Peripheral blood mononuclear cells (PBMC) were extracted from 9 ml EDTA blood samples using a Ficoll density gradient separation (Ficoll-Paque Plus; GE Healthcare Bio-Science, Sweden). The CD4 T cells were then negatively selected from the PBMC *via* a magnetic separation using a CD4 T cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer's protocol. The flow-through (CD4 T cells) were magnetically labeled with CD45RA MicroBeads. CD45RO (CD45RAneg) memory CD4 T cells were isolated as flow-through by negative selection and CD45RA CD4 T cells were released from microbeads based on the manufacturer's instruction (Miltenyi Biotec, Germany). An aliquot of the isolated cells was then stained using DAPI (4′,6-Diamidin-2-phenylindol; Thermo Fisher Scientific, USA), FITC-conjugated anti-human CD45RA (Clone HI100, Biolegend, USA), PE-conjugated anti-human CLA (Clone HECA-452, Miltenyl Biotec, Germany), PerCP/Cy5-conjugated anti-human CD4 (Clone RPA-T4, Biolegend, USA), APC-conjugated anti-human CD45RO (Clone UCHL1, Biolegend, USA) following standard procedures, and fluorescence-activated cell sorting measurements were performed on the MACSQuant X (Miltenyi Biotec, Germany) Flow Cytometer. Sample analysis was performed using FlowJo v10.8.1 (BD Biosciences). CD45RO and CD45RA CD4 T cells were identified as CD4/CD45RO co-expressing cells and CD4/CD45RA co-expressing cells, respectively.

Identification of TCRβ clonotypes

Total RNA isolation was performed for CD45RA CD4, CD45RO CD4 T cell populations, and the skin biopsy sample with the innuPREP RNA Mini Kit (Analytik Jena, Hildesheim, Germany). The cDNA preparation and the amplification of the antigen-binding site (CDR3β region) of the TCRβ chain were conducted as suggested by the manufacturer (iRepertoire, patent 7999092, 2011, Huntsville, USA) and prepared for pair-end sequencing using an Illumina Miseq system as previously reported (26). The MiXCR pipeline (v4.0) was used for the processing, alignment, assembling, correcting PCR errors, and exporting the data from the fastq files (27, 28). TCRβ clonotypes were then annotated as described before (29, 30). The TCRβ repertoire was further analyzed by employing the immunarch package (version 0.6.9) on the R programming language (R-4.4.1) (31). The diversity of TCRβ repertoire was estimated using the Inverse Simpson Index. The Jaccard Index was used to quantify the overlapping TCRβ clonotypes by normalizing their numbers. Both indices were calculated with the immunarch package. To avoid unpredictable PCR and sequencing errors, the default parameters ("eliminate these errors") were used. Additionally, to avoid artificial diversity due to PCR errors, all TCRβ clonotype sequences that appeared only once were removed. The number of total and unique TCRβ clonotypes are shown in Supplementary Table 2.

Statistical analysis

Statistical analyses were carried out using either R programming language or GraphPad Prism (Version 9.0, GraphPad Software Inc, USA). Statistical significance was evaluated by 2-way ANOVA with turkeys' multiple comparison test or Mann-Whitney U tests. The data were considered statistically significant at p values < 0.05.

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Results

Quantification of circulating memory and naïve CD4 T cell populations

In this study, we hypothesize that the clonal abundances of memory CD4 T cells would differ between autoimmune (BP) and non-autoimmune patients (NMSC). Peripheral blood (9ml) was collected for analyzing the peripheral TCRβ repertoire of circulating T cells. Because BP is a disease of the elderly, the average age of both groups is about 87 ± 2.5 years. Most patients show several comorbidities and are given long-term medications (Supplementary Table 1).

To identify memory CD4 T cells, CD4 and CD45RO T cells were sorted by two negative selection steps, after which the CD45RO CD4 T cells are clearly identifiable as distinct cell populations by flow cytometric analysis (Figure 1A, middle panel). All other CD4 T cells, which did bound to the magnetic beads that were coated with CD45RA-directed antibodies were defined as the CD45RA population. For the sake of simplicity and easier reading these CD45RA CD4 T cells are called naïve T cell population, even though this population is a heterogeneous mixture that contains CD4 T cells that are highly positive or almost dull for CD45RA (Figure 1A, right panel) (32). In all samples, the sum of the CD4 CD45RO and CD4 CD45RA was close to 100% (data not shown). Evaluation of cell numbers and percentages revealed that approximately 30% of the PBMC were CD4 positive and did not differ between the BP group and the NMSC group (Figure 1B). This was different for the CD45RA/RO populations. Here, we found that BP patients have almost equal numbers of memory and naïve CD4 T cells in contrast to NMSC patients, in which a significantly higher proportion of memory T cells was found. Calculation of the ratio of CD45RO to CD45RA shows a significant difference between BP patients and NMSC patients (Figures 1C, D).

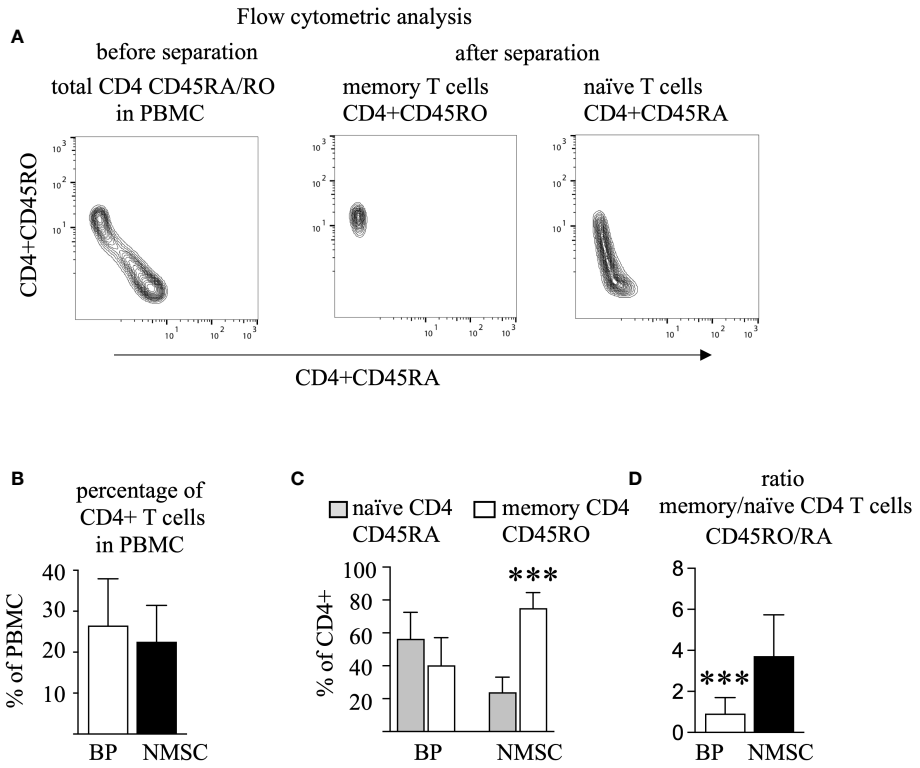

FIGURE 1
The number of memory T cells prevails in NMSC patients. PBMC from BP and NMSC patients were sorted for CD4 CD45RA and CD45RO T cells. **(A)** The gating strategy of one typical example is shown. Because CD45RO T cells were sorted by negative selection (middle panel), they appear as a specific population in contrast to the CD45RA population (right panel). **(B)** The size of the circulating CD4 T cell population is similar in BP and NMSC patients as shown as percentages of PBMC (Data are mean ± SD, p=0.43, not significant (n. s.) Mann-Whitney test). **(C)** NMSC patients harbor significantly more CD45RO T cells (Data are mean ± SD, ***p < 0.001, 2-way ANOVA with turkeys' multiple comparison test). **(D)** The ratio of the CD45RO/RA population is significantly lower in BP patients. (Data are mean ± SD, ***p < 0.001, Mann-Whitney U test), (in **B–D**, n=14 (BP) and n=6 (NMSC)).

No difference in the abundances of TCRβ clonotypes between the patient groups

For initial repertoire analysis, memory and naïve CD4 T cell populations from four typical patients of each group were chosen for identification of their CDR3 antigen-binding sequences. RNA was extracted from each T cell population, their TCRβ genes were amplified by multiplex PCR and the nucleotide sequence was identified by next-generation sequencing using the MiSeq™ Illumina system. Between 46'714 to 267'496 (mean ± SD = 127696 ± 67540) unique TCRβ clonotypes could be identified (Supplementary Table 2). Thereby, the memory CD4 T cell sample harbored less TCRβ clonotypes compared to the naïve CD4 T cell population in each patient regardless of the type of pathogenesis (Figures 2A, B). This difference in clonotype numbers might be caused by the enormous number of naïve CD4 T cells that never expanded due to the lack of antigen and therefore exist at low frequencies. Indeed, apportioning the TCRβ repertoires according to their clonal abundance in four groups (hyperexpanded > 0.001, high abundant 0.0001-0.001, medium abundant 0.00001-0.0001, and low abundant < 0.00001) revealed that the naïve population contained the highest number of low abundant clonotypes (yellow bars, Fig. 2C). Vice versa, the memory population is characterized by a higher number of hyperexpanded and highly abundant clonotypes (gray bars, Figure 2C). To quantify these observations, the Inverse Simpson Index was calculated that measures both the number of different TCRs and their clonal expansion. Due to the different numbers of TCRβ clonotypes per patient the values are variable. However, a trend towards a decreased Inverse Simpson Index in the memory populations could be observed, which indicates the higher number of expanded TCRβ clonotypes in this population compared to the naïve populations (Figure 2C). No difference was found between the patient groups (Figure 2D). This data contrasts with our hypothesis that the CD4 memory population of autoimmune patients would display more abundant clonotypes.

Similarity analysis and individual clone tracking reveal a high overlap between memory and naïve CD4 TCRβ clonotypes

Potential explanations for the lack of more abundant TCRβ clonotypes in the autoimmune group could be the presence of effector memory expressing CD45RA (terminally differentiated helper T lymphocytes, Temra) cells, which form by the conversion of CD45RO into CD45RA T cells (33, 34). To address a potential conversion of CD45RO to CD45RA T cells we identified the overlapping T cell clonotypes in both populations, within each patient. As shown in Figure 3A, the Jaccard Index, which reflects the similarity between samples based on the number of overlapping clonotypes after normalization to the overall number of clonotypes, revealed no significant differences between BP and NMSC patients. The identified means are 0.09 and 0.07 for the BP and NMSC patient group, respectively, when compared within one patient (intra-individually, Figure 3A). Naturally, since each patient has its individual TCR repertoire, these values are lower when compared to all other patients of the same group (inter-individually) (Figure 4A). By limiting the comparison of overlapping TCRβ clonotypes to the top 500 most expanded TCRβ clonotypes from the memory and naïve populations within one patient, the Jaccard Index increased significantly regardless of the patient group (Figure 3A). Strikingly, the top 20 most abundant TCRβ clonotypes from memory and naïve CD4 T cell population overlap almost completely within each patient. Figure 3B shows the presence and abundance of the top 20 TCRβ clonotypes from the CD45RA population within the CD45RO population of one typical patient (Figure 3B, left panel) and vice versa (Figure 3B, right panel). The data obtained for all other patients were comparable (data not shown). In conclusion, the high number of overlapping TCRβ clonotypes raises the possibility that memory CD4 T cells are converted to Temra cells by re-expressing CD45RA. However, there is no difference between the patient groups. Considering that this is just a pilot study with four individuals per group, further analysis with more detailed phenotypically selected CD4 cell subsets will be required to understand the high similarity between both T cell populations.

Characteristics of TCRβ repertoire: TRBV, TRBJ gene usage, and CDR3 length

This high overlap between the intra-individual memory and naïve CD4 T populations suggests that typical TCRβ repertoire features such as V/J usage and CDR3 length would be also highly similar within each patient. Indeed, most of the V genes were expressed in similar frequencies in both CD4 T cell populations within each patient and regardless of the type of disease (Figure 5A). Unexpectedly, also the pattern of the V/J gene expression was similar in all patients. For example, TCR beta variable gene (TRBV) 20-1 was expressed at a high frequency in all samples. Comparable results were found for the J genes (Supplementary Figure 1).

Studying the CDR3 length distribution revealed no difference between both CD4 T cell populations (Figure 4B). Instead, subtle shifts were observed between BP and NMSC patients. The TCRβ clonotypes of BP patients are in tendency one amino acid shorter than those of the NMSC controls.

In conclusion, our pilot study shows that neither the extent of clonal expansion nor the overlap of TCRβ clonotypes from circulating memory and naïve CD4 T cells differ between the

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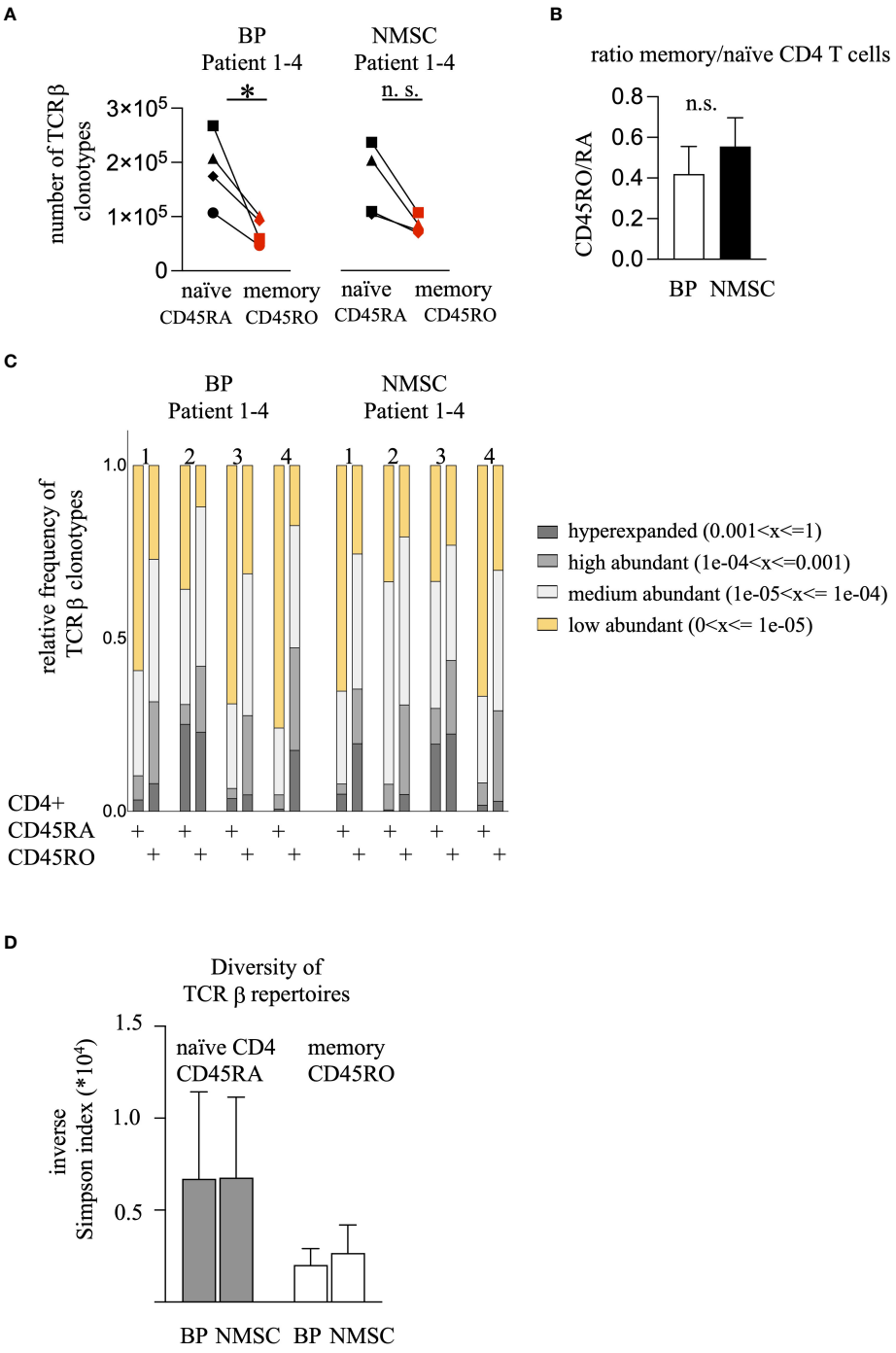

FIGURE 2
BP patients harbor fewer CD45RO clonotypes but the frequency of the high-abundant TCRβ clonotypes do not differ compared to the NMSC patients. **(A)** The numbers of the unique TCRβ clonotypes from the CD45RA (black symbols) and CD45RO (red symbols) CD4 T cell population are shown. The values obtained from the same patient are connected. (n = 4, *p<0.05, One-way ANOVA with turkeys' multiple comparison test). **(B)** The ratio of the unique TCRβ clonotypes from the CD45RO/RA population was calculated. Data are mean ± SEM. No significant difference between BP and NMSC patients was found. (n = 4, Mann-Whitney test). **(C)** The clonal distribution of the TCRβ clonotypes is shown. TCRβ clonotypes were divided regarding their clonal abundance. Each bar represents the clonotypes of the CD45RA and CD45RO population from one patient. Each bar is divided into 4 sections. Each section represents the fraction of clonotypes that exist at a certain frequency (yellow: > 1 x 10^{-5}, bright gray: 1 x 10^{-5} - 1 x 10^{-4}, gray: 1 x 10^{-4} - 1 x 10^{-3}, dark gray: < 1 x 10^{-3}). **(D)** Inverse Simpson Index shows no difference between the BP or NMSC patients. (Data are mean ± SEM, gray bars: CD45RA, white bars: CD45RO no significance, 2-way ANOVA).

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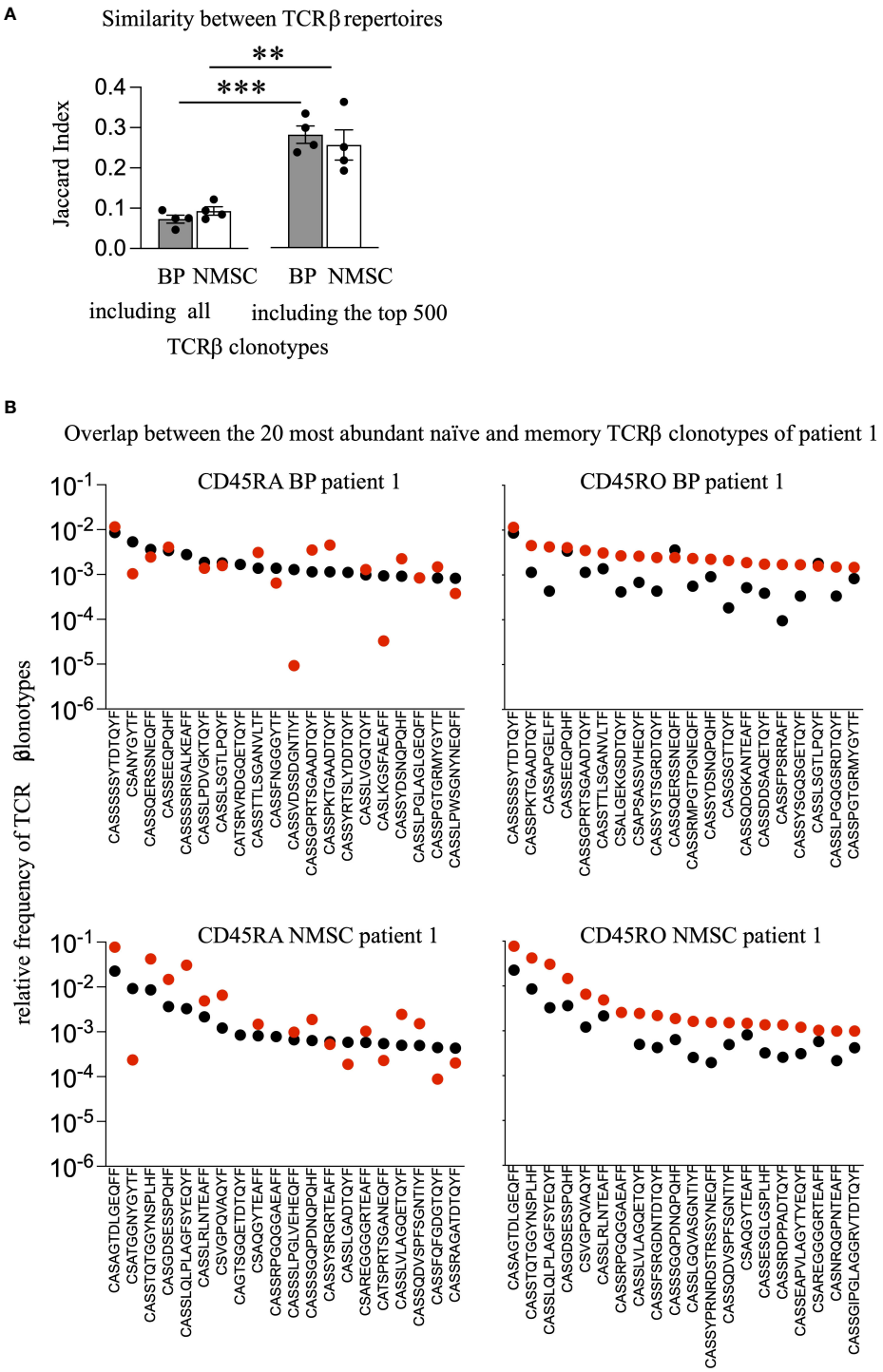

FIGURE 3
No difference in the degree of overlapping TCRβ clonotypes between BP and NMSC patients. **(A)** To assess the portion of overlapping TCRβ clonotypes between the CD45RO and CD45RA populations within each patient quantitatively, the Jaccard Index was calculated by either including all TCRβ clonotypes or including only the top 500 most abundant TCRβ clonotypes. (Data are mean ± SEM, ***p < 0.001, **p < 0.01, 2-way ANOVA with turkeys' multiple comparison test). **(B)** The 20 most frequent TCRβ clonotypes present in the CD45RO population (black dots, left panel) are compared to their presence in the CD45RA population (red dots, left panel) and vice versa, the 20 most frequent TCRβ clonotypes present in the CD45RA population (black dots, right panel) are compared to their presence in the CD45RO population (red dots, right panel) within BP patient 1 (upper panel) or NMSC patient 1 (lower panel). One representative comparison out of all patients is shown.

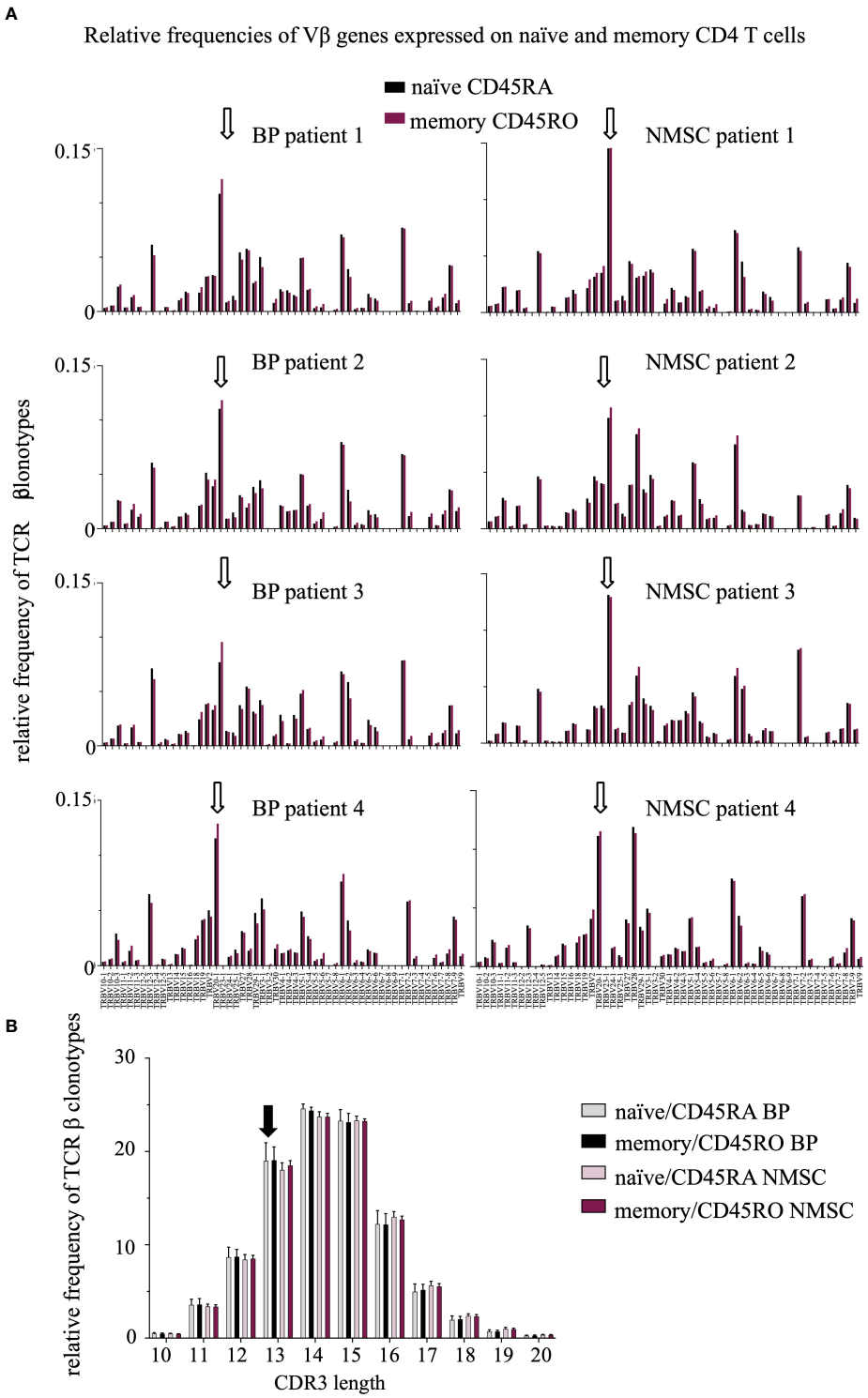

FIGURE 4
A trend toward a reduced length of the CDR3 region in BP patients was observed but no difference in the V gene usage between the patient groups. **(A)** Relative frequencies of Vβ genes were compared between the CD45RO (red bars) and CD45RA (black bars) population in BP patients (1-4, left) and NMSC patients (1-4, right). White arrows indicate the high expression of the Vβ genes TRB20-1 in all samples. The high usage of TRBV20-1 has been described previously and was associated with aging (35, 36). **(B)** Relative length distribution of the CDR3 regions of CD45RO and CD45RA TCRβ clonotypes were compared between BP and NMSC patients (Data are means ± SD). The black arrow indicates the increased frequency of TCRβ clonotypes with a CDR3 length of 13 amino acids in BP patients (black, gray bars) compared to NMSC patients (bright and dark red bars).

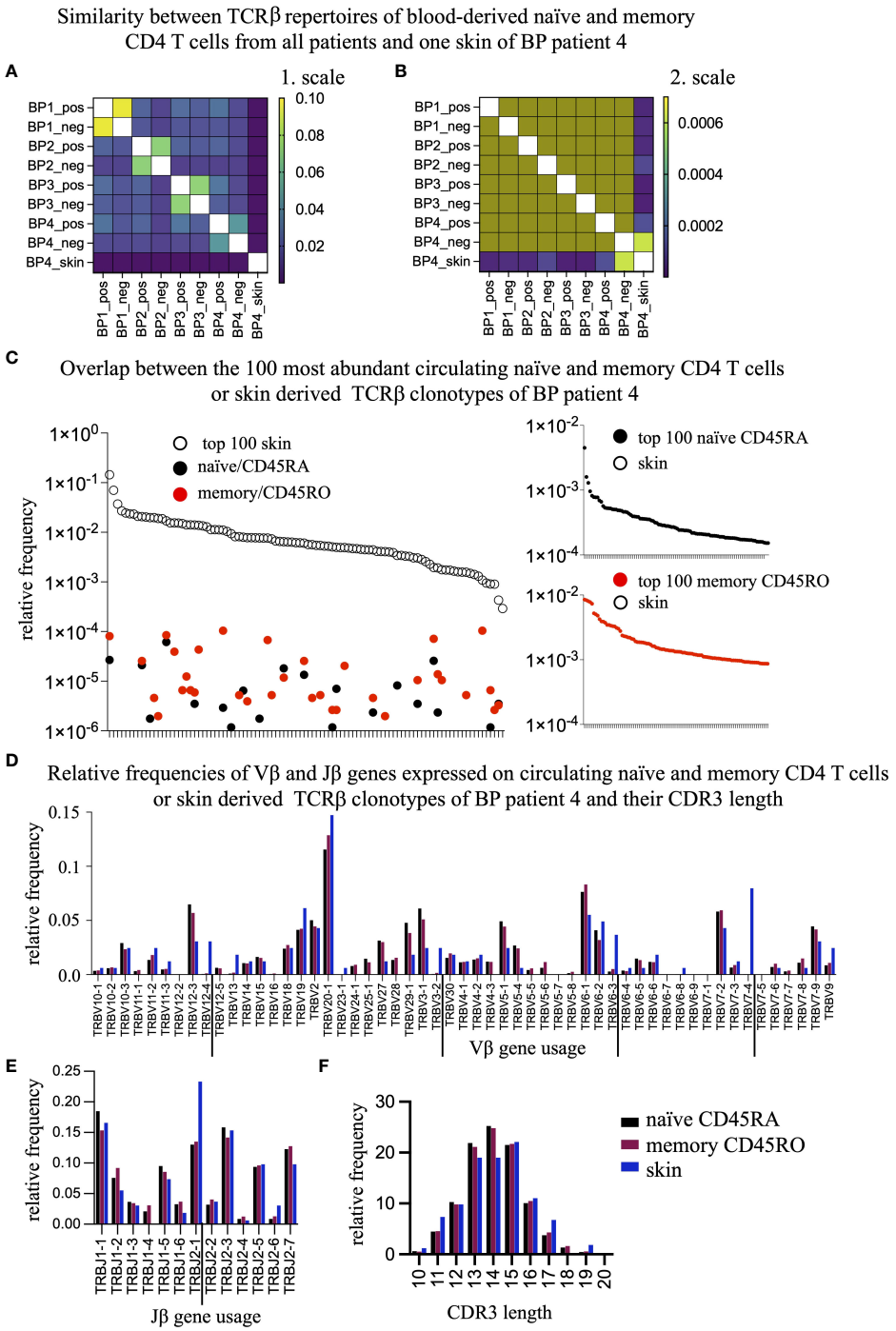

FIGURE 5
The overlap between the skin-residing and the circulating TCRβ clonotypes is negligible. **(A, B)** To assess the percentage of overlapping TCRβ clonotypes between the blood-derived CD45ROand CD45RA populations and the skin-resident T cell clones of BP patient 4, the Jaccard Index was calculated and displayed as a heat map with a range from 1e-006 to 0.1 **(A)** or from 1e-006 to 0.0007 **(B)**. **(C)** The top 100 most frequent TCRβ clonotypes present in the skin of BP4 (white dots, left panel) are compared to their presence in the CD45RA (black dots, left panel) or CD45RO population (red dots, left panel) of BP patient 4. Vice versa, the top 100 most frequent TCRβ clonotypes of in the CD45RA population (black dots, right upper panel) or the CD45RO population (red dots, right lower panel) are compared to their presence in the skin and could not be found. **(D, E)** Relative frequencies of Vβ genes **(D)** and Jβ genes **(E)** were compared between the skin (blue bar), the CD45RO (red bars), and CD45RA (black bars) populations of BP patient 4. The TRBV genes and TRVJ genes that are preferentially expressed in the skin are underlined. Indicated are means ± SEM. **(F)** Relative length distribution of the CDR3 regions of skin-residing TCRβ clonotypes were compared to the CD45RO and CD45RA populations of BP patients 4. (Data are means ± SEM). Differences can be observed at a length of 11, 13, 14, and 17 amino acids.

groups of autoimmune and non-autoimmune patients. Obviously, in contrast to our hypothesis, the memory CD4 T cell population was not expanded under autoimmune conditions.

TCRβ clonotypes in skin lesions overlap only marginally with the circulating CD4 T cell populations of the same donor

Next, we asked whether the circulating TCRβ clonotypes would be present in the skin lesions of BP patients (Figure 1C, Figure 2A). Because only one of the BP patients (BP4) agreed to provide a skin biopsy simultaneously with the collection of blood our analysis was restricted to this one sample. In the perilesional biopsy of BP4, we found 163 unique TCRβ clonotypes (Supplementary Table 2). As shown in Figure 5A, the Jaccard Index-based heat map reveals that the skin biopsy of BP4 has very low similarity to the circulating memory and naïve CD4 T cell populations from all patients. For better visualization, we changed the color codes (Figure 5B). Now, it is obvious that the TCRβ clonotypes from the skin and the memory CD4 T cells overlap highest in the same donor BP4 when compared to the T cell populations of all other patients (bright green square in the lower right corner, Figure 5B). Taking a closer look at the top 100 most abundant skin-derived TCRβ clonotypes, it becomes evident that they are present at very low frequencies only in the circulation irrespective of the memory or naïve population (between 0.01% and 0.0001% of the blood TCRβ repertoire, Figure 5C, left panel). Even more so, the top 100 most abundant circulating TCRβ clonotypes do not exist in the skin lesions at all (Figure 5C, CD45RA-derived clonotypes right, upper panel; CD45RO-derived clonotypes, right lower panel), which could suggest a skin-specific accumulation. In line, the TRBV and TRBJ gene usage from TCRβ clonotypes of the skin lesion and of circulating CD4 T cell population are different (Figures 5D, E). Especially, T cells expressing the TRBV genes 12-4, 3-2, 6-3, and 7-4 and the TRBJ gene 2-1, reside in the skin lesion but are only minor in blood. Moreover, the length of the CDR3 regions differs between blood- and skin-derived TCRβ clonotypes from the same patient (Figure 5F). These data indicate that T cell clones distribute differently between skin lesions and blood.

Discussion

This is the first study evaluating and comparing the circulating CD45RO (memory) and CD45RA (naïve) CD4 T cells in BP patients at the level of TCRβ clonotypes. We hypothesized that a persistent T cell activation under autoimmune condition would lead to a higher portion of high-abundant T cell clones in the circulating memory CD4 T cell population of BP patients when compared to age- and gender-matched non-autoimmune (NMSC) controls. Our data show that this is not the case (Figure 2). Furthermore, other features of TCR repertoires such as V/J gene usage have not been affected in BP patients (Figure 4). This contrasts with other autoimmune diseases such as SLE, RA, and type 1 diabetes mellitus, for which a reduced diversity of the TCR repertoire from the circulating CD4 T cells pool and changes in the V/J gene usages have been reported ((11) reviewed in (9)). These discrepancies might be caused by technical differences, but also by natural reasons such as the stage of the disease activity, the types of medication, and the type of the autoimmune disease. Another important difference between the above-mentioned studies of TCR repertoires in SLE, RA, and type 1 diabetes mellitus is the age of the patients. The average age of the BP patients in this study is 87 ± 2.5. Profound changes in the T cell repertoire have been described in individuals, who are older than 70 years (4). It is assumable that at older ages, the extent of the clonal expansions of the most abundant T cell clones might have such a high level, that any distinction between autoimmune and non-autoimmune patients is not assessable anymore. Further analysis will be required not only by including more patients but also by including additional markers such as CD62L, CCR7, CD27, and CD28 for detailed phenotyping. In line, comparative analysis of the TCR repertoire should be extended to include CD8 T cells, gamma-delta T cells, and especially follicular T helper cells in BP and NMSC patients. For example, a crucial role for CD8 T cells has been reported for pemphigus vulgaris (37). Gamma-delta T cells are involved in the pathogenesis of BP and pemphigus vulgaris (38, 39).

Besides the main finding that the extent of clonal expansion was not elevated in the BP group compared to the NMSC group (Figures 2B, C), we found that the ratio between circulating memory and naïve CD4 T cells was significantly lower in the BP group compared to the NMSC patients. The means of the ratios are 0.9 and 3.7 for BP and NMSC, respectively (Figures 1B, C). It is tempting to speculate that these simple ratios in cell numbers could serve as a prognostic factor during the development of autoimmunity. Longitudinal studies should answer this question in further analysis. Opposite results have been found in other autoimmune diseases, such as RA and SLE, and also in healthy donors (32, 40, 41). The question arises what causes the decreased ratio of memory to naïve CD4 T cells in BP patients? BP-specific CD45RO CD4 T cells have been isolated from the peripheral T cell pool of patients, which does not exclude that the majority of this population might reside in the skin (42–44). In line with the decreased ratio of memory to naïve CD4 T cells in BP patients, the ratio of the TCRβ clonotypes is also lower in BP patients. This difference was not so obvious anymore (Figure 2A), which might be due to the normalization of the RNA concentrations for all samples that were capped at 0.5 µg according to the protocol for library preparation in both T cell populations (iRepertoire, Inc., Huntsville, USA).

The identification of overlapping TCRβ clonotypes between the memory and naïve population revealed an extensive overlap within each patient (Figure 3) and a highly similar pattern of the V/J gene usages (Figure 4). Especially the top 500 TCRβ

clonotypes overlap between both CD4 T cell populations. Considering the well-established antigen-specificity of the memory CD4 T cell population, these data are difficult to interpret. The process of CD4 T cell activation is a multistep process (45), during which CD4 T cells lose their expression of CD45RA upon contact with antigen (16, 17). Thus, one would expect that TCR sequences from memory and naïve CD4 T cells would differ due to the proposed antigen-specificity of the memory T cell population. It will be interesting to find out whether this overlap reflects the presence of Temra cells, which re-express CD45RA and represent a terminal stage of effector differentiation (46). Especially, when considering the old age of both patient groups one could speculate that this high overlap between memory and naïve TCRβ clonotypes might be caused by conversions to Temra cells and might be typical for repertoires of the elderly. In line, a recent longitudinal study reported that the number of overlapping CD4 TCRβ and TCRα clonotypes increases with age (6). Additionally, the presence of high abundant TCR clonotypes in naïve T cell populations and their maintenance by homeostatic proliferation might contribute (8, 47). It is particularly noteworthy that especially the naïve CD4 population is not pure and contains transient double positive cells CD45RA+CD45RO+ (32) even though MACS sorting was performed carefully according to the manufacturer's protocol (Miltenyi Biotec). More selective markers such as CD27 or CD28 should be applied in further studies.

Finally, we tested whether T cells would preferentially accumulate in skin lesions of BP patients. Comparison of TCRβ sequences of the one skin that matched donor and time point of blood collection revealed a low similarity to the blood sample (Figure 5B). This finding agrees to the differential accumulation of T cell bearing TRBV 12-4, 3-2, 6-3, and 7-4 and the TRBJ 2-1 segment between skin and blood, which might not be surprising (Figures 5D–F). Considering that the collected blood samples reflect only 1-2% of the circulating blood volume and that up to 50% of memory CD4 T cells reside in lymphoid organs and do not enter the circulation (44), it is unlikely to find overlapping clonotypes. However, it is important to note that in this patient BP4, the similarity between the skin and memory T cell repertoires is higher than between skin and naïve T cell repertoires even though the naïve population harbors substantially more TCRβ clonotypes (Figure 5B). This opens the possibility of a skin-specific TCR repertoire in BP patients. A recent analysis of TCR repertoires revealed skin-specific T cell accumulations in healthy human skins. In line, skin-specific TCR repertoires have been described in the mouse model for pemphigoid diseases (22, 43). On the other side, it has been shown that topical treatments affect the number of BP reactive T cells in the blood, which suggests ongoing exchanges of T cells between blood and skin (24). Further approaches are required to assess the role of skin-immigrating T cells even though it might be difficult to recruit enough patients. Understandably, the willingness of the patients to donate biopsies from inflamed perilesional skin is rather low.

In summary, aberrant T cell responses have been reported in BP, even though it is an autoantibody-mediated disease. This pilot study shows that the ratio of circulating memory CD4 T cells to naïve CD4 T cells is lower in BP patients than in controls. Comparison of the TCRβ repertoires of these circulating T cell populations revealed no differences in diversity, the extent of clonal expansion, and V/J gene usage between the BP and NMSC patients. This data indicates that an enumeration of memory and naïve T cell subsets in BP patients might serve as prognostic parameters and should be followed up, especially in longitudinal studies.

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 below: https://www.ncbi.nlm.nih.gov/, bioproject # PRJNA867683.

Ethics statement

The studies involving human participants were reviewed and approved by The ethical committee of the University of Luebeck. The patients/participants provided their written informed consent to participate in this study.

Author contributions

CH, ES, KK, MN, SI contributed to conception and design of the study. CB, FB, MN, KB performed experiments. AF, KK, MN, FB analyzed the data. CH, ES recruited patients. KK, SI obtained funding. FB, KK wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2022.1006941/full#supplementary-material

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Potential effects of shift work on skin autoimmune diseases

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Shift work is associated with systemic chronic inflammation, impaired host and tumor defense and dysregulated immune responses to harmless antigens such as allergens or auto-antigens. Thus, shift workers are at higher risk to develop a systemic autoimmune disease and circadian disruption with sleep impairment seem to be the key underlying mechanisms. Presumably, disturbances of the sleep-wake cycle also drive skin-specific autoimmune diseases, but epidemiological and experimental evidence so far is scarce. This review summarizes the effects of shift work, circadian misalignment, poor sleep, and the effect of potential hormonal mediators such as stress mediators or melatonin on skin barrier functions and on innate and adaptive skin immunity. Human studies as well as animal models were considered. We will also address advantages and potential pitfalls in animal models of shift work, and possible confounders that could drive skin autoimmune diseases in shift workers such as adverse lifestyle habits and psychosocial influences. Finally, we will outline feasible countermeasures that may reduce the risk of systemic and skin autoimmunity in shift workers, as well as treatment options and highlight outstanding questions that should be addressed in future studies.

KEYWORDS

shift work, skin, sleep, circadian, autoimmune, cortisol, melatonin, inflammation

Abbreviations: ANA, Antinuclear antibody; BMAL1, Brain and muscle ARNT-Like1; BP, Bullous pemphigoid; BPDAI, Bullous pemphigoid disease area index; CLOCK, Circadian locomotor output cycles kaput; CRY, Cryptochrome; Dbp, D-site of albumin promoter binding protein; DLQI, Daily quality of life index; DTH, Delayed type hypersensitivity; EBA, Epidermolysis bullosa acquisita; ELISA, Enzyme-linked immunosorbent assay; h, hours; HPA-axis, Hypothalamus-pituitary-adrenal axis; IBD, Inflammatory bowel disease; Ig, Immunoglobulin; IL, Interleukin; NFIL3, Nuclear factor interleukin 3 regulated protein; NK, Natural killer cell; PER, Period; PSQI, Pittsburgh sleep quality index; RA, Rheumatoid arthritis; REM, Rapid eye movement; RORa, Retinoic acid-related orphan receptor-alpha; SCI, Systemic chronic inflammation; SCN, Suprachiasmatic nuclei; SLE, Systemic lupus erythematosus; SNS, Sympathetic nervous system; SpA, Spondyloarthritis; SSc, Systemic sclerosis; SWS, Slow wave sleep; TNF, Tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; Tregs, Regulatory T cells.

1 Introduction

Our 24/7 society leads to an increase in shift work with work schedules that fall outside the standard work hours from 7 AM to 6 PM. Shift work includes early morning, evening, or night shifts, as well as fixed or rotating shifts. About 15-25% of the world-wide population is working in shifts (1), in particular health care workers. In this latter population epidemiological studies provide alarming data on shift workers showing increased rates of longstanding illnesses (2). In addition, the Nurses' Health Study demonstrated enhanced mortality due to cardiovascular diseases or lung cancer (3). Shift workers are forced to be active and to sleep at time periods that are out of sync with their endogenous time-keeping system. This internal clock, the circadian system, is comprised of clock genes in many, if not all cells of the body. The molecular machinery of cellular clocks involves -among others- the core clock genes Brain and Muscle ARNT-Like1 (in humans *BMAL1*, in mice *Bmal1*) and Circadian Locomotor Output Cycles Kaput (*CLOCK, Clock*), which form dimers upon translation and initialize the transcription of Period (*PER, Per*) genes. These form a dimer with Cryptochrome (*CRY, Cry*) genes. This in turn inhibits the transcription of *BMAL1*. Moreover, the BMAL1-CLOCK-dimer is binding together with the dimer of *NFIL3* and *DBP* to the E box of *REVERB/Reverb* genes, initiating their transcription. In turn, *REVERB/Reverb* genes inhibit the transcription of *NFIL3/Nfil3*, forming another feedback-loop (4). With the interaction of other genes and proteins, a network of interconnected loops is formed, taking approximately 24 hours (h) to be executed. The master clock in the hypothalamic suprachiasmatic nuclei (SCN) is synchronized to external time cues like 24 h light-dark changes, a process that is called entrainment (4). In turn, the SCN entrain peripheral clocks by systemic signals such as core body temperature, mediators of the stress systems (such as the sympathetic nervous system (SNS) and the hypothalamus-pituitary-adrenal (HPA)-axis, as well as melatonin, the pineal hormone of darkness (5). The circadian system controls virtually all body functions like sleep and wakefulness, behavioral changes in physical activity and food intake, thermoregulation, cell proliferation and metabolism, the cardiovascular, the endocrine, the digestive, the reproductive and the immune system (6–16). Apart from light, several non-photic external time cues have been described that were summarized in a review by Mistlberger and Skene (17). Exemplarily, food is a non-photic external time cue that can feedback to the SCN to further entrain 24 h rhythms

When shift workers experience a mismatch of their internal clock with environmental cues and obligations this circadian misalignment can result in circadian disruption of body functions from the molecular to the behavioral level. Potential outcomes are poor sleep, chronic stress (18), burn out syndrome (19), social isolation (20) and adverse lifestyle habits like physical inactivity (18), unhealthy diet (21, 22), or substance abuse (23). All these processes can trigger systemic chronic inflammation (SCI) (24, 25) and a dysregulation of innate and adaptive immune responses. That alone or in combination may foster infectious, cardiovascular, metabolic and cancer diseases in shift workers (22, 26, 27). In addition, the STRESSJEM Study, which was conducted in France, analyzed mortality and cause-specific mortality due to night and shift work. Niedhammer et al. described a sex-specific association between night and shift work and cerebrovascular diseases, ischemic heart diseases, respiratory cancers and breast cancer as causes of mortality (27). Thus, a life against the internal clock can impair host defense against pathogens (28, 29) and tumors (30) and also seems to promote unwanted immune responses to harmless antigens, like allergens (31) or auto-antigens (32). While shift workers are at higher risk to develop systemic autoimmune diseases as shown for rheumatoid arthritis (RA) (33), data on associated skin manifestations or skin-specific autoimmune diseases is only limited. In the following, we will outline that shift work likely impairs skin physiology and immunity and thus could promote skin autoimmune diseases, a causality that, however, needs to be experimentally clarified in future studies.

In detail, we will first describe 24 h in the life of a physician during a night shift in a narrative in section 2 and outline (Table 1) experimental approaches to delineate the effects of shift work on the skin and the immune system in section 3. In section 4, we will then summarize epidemiological and experimental evidence in humans and animals indicating that shift work could promote skin autoimmune diseases, before we give more detailed reports on the cellular effects of shift work on skin physiology, skin innate immunity and skin adaptive immunity in section 5. In section 6, we will discuss candidate neuroendocrine mediators linking shift work with skin autoimmune diseases and in section 7 we will highlight potential countermeasures and therapeutic approaches to prevent, ameliorate or treat skin autoimmune diseases in shift workers. In the last part of our review, in section 8, we will summarize the outlined findings and give an outlook on outstanding questions that should be addressed in future experiments

2 24 hours in the life of Dr. S.W.

In a narrative describing 24 h in the life of an intensive care unit physician who we called Dr. S.W. on night shift, we would like to outline shift work-induced changes in behavior, neuroendocrine mediators, thermoregulation, skin physiology and immune functions in comparison to regular 24 h rhythms. We hypothesize that fine-tuned physiological rhythms in neuroendocrine-immune interactions foster skin barrier functions and that disturbances thereof could promote pathophysiological processes of skin autoimmune diseases. We chose a tabular form with 4 h intervals (Table 1) (34–55).

TABLE 1 24 hours in the life of Dr. S.W. on night shift.

1 PM	When Dr. S.W. went to bed after her second night shift it took a while until she fell asleep. Her thermoregulation is out of phase, and she consumed several cups of coffee. Both aspects likely interfere with sleep initiation, maintenance and deep sleep (34). Normally, she would have lunch now, her stress systems would be active, and the bright light of the midday sun would suppress her melatonin levels (35). In bed however, the light that breaks through the window sealings and the noise from outside fragment and shallow her sleep.
5 PM	Dr. S.W. woke up but does not feel well rested or refreshed. Presumably, she lacked slow wave sleep (SWS), the deepest form of sleep that normally helps to flush away metabolites from the brain parenchyma (36) and to clear sleep regulatory substances like adenosine, tumor necrosis factor (TNF) and interleukin-1 (IL-1) that induce fatigue and sleepiness (37). When she looks into the mirror, she notices that her skin is pale and that she has an unhealthy appearance. These are findings that also emerge after experimental sleep deprivation (38). Sleep supports anti-oxidative and regenerative processes and a lack thereof impairs skin integrity (39). Moreover, sleep loss is associated with systemic chronic inflammation (SCI) that is a likely mechanism of fatigue, sleepiness, bad mood, cognitive impairments and other feelings and symptoms of sickness (40). It is getting dark already and Dr. S.W.'s breakfast consists of three cups of coffee and, as she failed to buy groceries, a chocolate bar. She comforts herself that caffeine not only antagonizes the sleep-inducing substance adenosine (41) but may also counteract SCI that evolved due to sleep loss (42). Maybe she should take some vitamin D as well to fight against the inflammatory processes (43).
9 PM	Dr. S.W. heads to the clinic for her third night shift and takes the car instead of her bicycle. In the doctor's room she switches on all the lights and drinks a cup of coffee to become alert (34). Normally, her melatonin would rise, and her stress systems would calm down at this time of the day (35). These changes would induce an increase in skin temperature, a decrease in core body temperature and in this way her body would get prepared for the sleep period (34, 44, 45).
1 AM	In the patient rooms and the ward corridor the light is dimmed, and the volume of the alarm sounds were turned down. The intensive care unit (ICU) staff generally agrees that the patients should sleep at night to recover (46). On a regular wake-sleep cycle also Dr. S.W. would be in deep SWS now. Her immune system would be boosted by increases in growth hormone, prolactin and aldosterone and very low cortisol and catecholamine levels (35, 47, 48). These hormonal changes presumably also support anabolic processes like cell proliferation and cell growth, as well as anti-oxidative and regenerative processes (49, 50). However, Dr. S.W.'s hormone secretion is disturbed.
5 AM	Dr. S.W. is freezing. Normally, her core body temperature would be at minimum now and her internal clock would increase the propensity of rapid eye movement (REM) sleep. Neurotransmitters of the sympathetic nervous system (SNS) such as the catecholamines epinephrine and norepinephrine now would reach nadir levels (51). For the staff of the ICU this night, the opposite holds true. Dr. S.W. hears a red alarm. A patient has a cardiac arrest, and she starts resuscitation. After stabilizing the patient, the ICU team sits together, they drink coffee and eat potato chips. They agree that night shifts favor unhealthy diets (18, 52), substance abuse (23), social isolation (20) and TV time and that these adverse lifestyle habits may increase mortality (53).
9 AM	Dr. S.W.'s internal clock activated her stress systems, leading to reduced feelings of fatigue and sleepiness. She drives back home and reflects her life. She loves being at the ICU. It is a meaningful work and there is no doubt about the necessity of 24/7 shifts in contrast, to e.g., night shifts in the supermarket. However, increasing economic pressure in health care leads to displacements of routine procedures into the evening and night hours with adverse consequences for health care workers, their performance and their stress levels and for patient outcomes (54, 55). When she gets out of the car, she wonders whether seeking a specialization in dermatology or rheumatology would be a healthier career option.

3 Experimental approaches to delineate the effects of shift work on the skin and the immune system

In this chapter we aim to describe the different types of studies in humans as well as in animals, which can be used to assess the effects of shift work on the skin and the immune system.

3.1 Epidemiological and in-laboratory studies in humans

The circadian system controls skin physiology (56) as well as leukocyte ontogeny, differentiation, traffic, and function and thus various aspects of innate and adaptive immunity (57, 58).

Skin physiology in humans can be assessed *in vivo* non-invasively by inspection, by photo documentation, by imaging techniques like optical coherence tomography (59), as well as by measurements of skin temperature, skin pH, skin conductance, or transepidermal water loss (60). Skin physiology can also be measured invasively by harvesting suction blister fluid, by skin biopsies, or by injecting substances that induce an observable skin reaction.

Delayed type hypersensitivity (DTH) describes the cell mediated allergic immune reaction to a certain substance. This takes several days to develop as it involves antigen presenting cells as well as T helper 1 cells (Th1) and T helper 17 (Th17) cells. These recognize the antigen and release cytokines, attracting cytotoxic T cells, which kill the target cells (61, 62). A DTH-reaction also occurs in several autoimmune diseases such as RA where collagen is attacked as well as thyroiditis with the thyroglobulin antigen as a target (63, 64).

Immune parameters in humans are mainly assessed in blood (e.g., numbers of certain leukocyte subsets, levels of cytokines) and immune functions can be tested *in vitro* by using leukocyte cell lines, *ex vivo* by culturing freshly sampled blood leukocytes or *in vivo* by administering immunomodulatory substances and measuring the emerging immune response (e.g., antibody response to vaccination). The assessment of innate and adaptive immunity of the skin requires the invasive methods described above that allow the measurement of immune cells or mediators in fluids or tissues (e.g., histology, immunohistochemistry, immunofluorescence, fluorescent activated cell sorting, enzyme linked immunosorbant assay (ELISA), Western blotting).

The circadian system is closely linked to sleep that itself has manifold effects on skin health and skin ageing (65) and the immune system (47). 24 h rhythms of human behavior including sleep-wake behavior are monitored by questionnaires or wearables like actigraphy watches (66, 67). The latter can also track ambient light and temperature, heart rate, skin temperature and skin conductance. The gold standard to measure sleep with its different stages from light sleep to deep sleep (slow wave sleep (SWS)) and rapid eye movement (REM) sleep is polysomnography, encompassing electroencephalography for brain activity, electrooculography for eye movements and electromyography for muscle activity. It can be recorded with ambulatory devices in the home setting or in the sleep laboratory, where it can be combined with videotaping, monitoring of core body temperature and repeated blood sampling. The two-process model of sleep regulation describes the control of the onset, duration and quality of sleep, as well as increases in alertness and performance during wakefulness. The homeostatic "process S" involves sleep regulatory substances such as adenosine, tumor necrosis factor (TNF) and interleukin (IL)-1. This is combined with the circadian "process C", which regulates wakefulness by wake-promoting neurotransmitters such as catecholamines (68, 69). Interestingly, the likelihood to fall asleep is highest, when temperature of the distal skin regions (e.g., fingers and toes) is rising in the evening (44). Further, sleep can be deepened by passive warming of the skin (70). Apart from the circadian regulation of sleep by process C, sleep can feedback to the circadian system and impact body rhythms on the level of the SCN (71–73) and the periphery (74–78). Consequently, 24 h rhythms in skin physiology or a given immune parameter, could stem from the effects of the circadian system, from sleep, or both. Moreover, experimental manipulation of the circadian system likely changes sleep and *vice versa*, experimental manipulation of sleep can impact 24 h rhythms of skin and immune parameters.

Along this line, it has been shown in cross-sectional and longitudinal epidemiological studies that shift work induces both circadian disruption and sleep disturbances (79, 80). About 20-30% of shift workers even suffer from shift work disorder, a primary circadian rhythm sleep disorder with debilitating sleep disturbances and/or excessive sleepiness (81). Notably, also other primary sleep disorders (e.g., obstructive sleep apnea) and secondary sleep complaints due to comorbidities (e.g., depression) or medication (e.g., steroids) should be ruled out when studying shift work-autoimmune relationships (82, 83). To mimic shift work, circadian disruption can be induced in healthy volunteers experimentally by changes of the light-dark cycle, mistimed food intake, or mistimed sleep by delaying, depriving or fragmenting sleep (84, 85). Comparable experimental procedures in animals to directly manipulate the circadian system or sleep on the cellular level will be summarized in the next section.

3.2 Animal models to study interactions between the circadian system, sleep, and the immune system

Most animal experiments in biomedical research are performed in mice that are active at night and sleep during the day (86). Wild mice are orientating on *zeitgebers* such as the light/dark cycle, ambient temperature and seasonal dynamics (87). During the day, the mice are asleep and when the sun is downing and the temperature is lowering, this is the signal to get awake and be active (Figure 1). These wild mice are exposed to a completely different life and stressors (87) than their counterparts in the laboratories. They have many more options to explore, more space to run around and also larger territories than in a standard cage. The social groups form dynamically and are not gender specific (88). Wild mice are rarely disturbed during the rest phase; however, they have to cope with the stressors of predators, pathogens and limited access to food (87, 89, 90). Unfortunately, these natural conditions cannot be mimicked in the laboratory setting (91) (Figure 2 and Box 1) (92–98).

Laboratory mice do not have to scare predatory animals, but care takers and scientists are fulfilling this aspect sufficiently. It was shown in several experimental studies that handling and exchange or cleaning of cages is inducing stress in mice (92, 93), especially when male care takers and scientists are involved (99) (Figure 2 and Box 1). The laboratory mice cannot entirely follow their circadian rhythm as most experimental interventions take place during the work hours of the scientists and animal caretakers, thus in the rest period of these animals during the day. It therefore cannot be excluded that laboratory mice are constantly sleep deprived and suffer somehow from "shift work disorder". Keeping a mouse below its thermoneutral zone of 30° C ambient temperature may further activate the stress systems and interfere with circadian regulation (100), sleep and immunity (101). In most cases, studies in mice on the circadian system or sleep address these issues. In their experimental designs the light-dark cycle is changed on purpose (i.e., the dark period for the animals is during the daytime working hours of experimenters). Manipulations are in this way mainly scheduled to the active period of the animal and experimenters work in dim or red light that does not impact the SCN. As shown in Figure 1, mice in nature are exposed to circadian changes in ambient temperature, which are not present in laboratories, also leading to differences in sleep and circadian alignment (100, 102). However, the fact that warmer ambient temperature, the availability of nesting material and group housing support sleep in mice is taken into account in most laboratories (45).

Shift work can be mimicked experimentally in mice by changes in the light-dark schedule. As food intake is an important external time cue, time restricted feeding can be

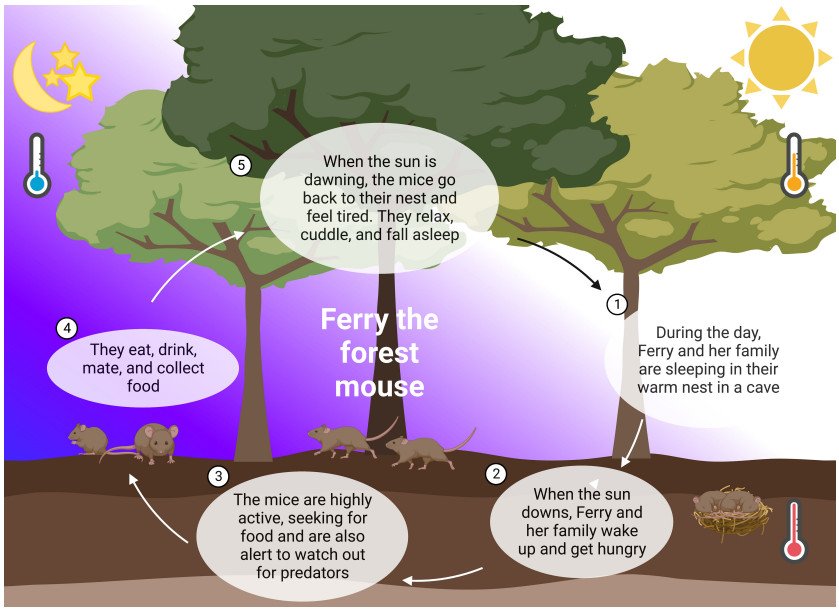

FIGURE 1
24 hours in the life of Ferry the forest mouse. In nature, mice follow their natural rhythm guided by abiotic *zeitgebers* such as light and temperature. They sleep during the day, cuddling in their warm nest in the soil (**1**). When the sun is downing, they wake up and leave the nest (**2**). The mice use the night and their highest alertness to seek for food and watch out for predators (**3**). A lot of running, climbing, and collecting of food during the night (**4**), releases them happily tired into the day and their sleep (**5**).

seen as another method to mimic shift work, in particular, when food is offered during the light phase, thus during the rest period of mice (103, 104). In *ad libitum* feeding protocols, food intake is often not controlled for, although it has a major impact on skin clocks (105) and on immune outcomes in response to circadian or sleep manipulations (106). Apart from changes in external time cues to mimic shift work, in animals the circadian system can be directly targeted by SCN lesions or genetic manipulations of clock genes in the germline or on a cell-specific level (e.g., *Clock* knock-out in particular immune cells, or knock-downs by adeno-associated viruses) (10, 107–110). Notably, these interventions might also induce sleep changes that should be controlled for (111). On the other hand, sleep can be manipulated by various more or less stressful techniques of sleep deprivation or fragmentation, or by optogenetics (112). However, it should be kept in mind, that mice cannot follow a constant routine to avoid confounding influences of physical activity during induced wakefulness. Mice are still the most common animal model although they are nocturnal animals with a complementary sleep-wake rhythm compared to humans. However, not all cellular or endocrine factors have complementary rhythms. Melatonin for example has a similar pattern in mice and humans, underlining its role as a dark-signal, which is downstream activating different pathways. Noteworthy, it is under debate for many mouse strains whether they are able to synthesize melatonin (113, 114). Despite this, melatonin was shown to affect mice regarding depressive-like and stress behavior and also circadian alignment (115–117).

On the other hand, mice offer plenty of possibilities to study sleep-wake behavior, e.g. by implanting electrodes or by assessing circadian locomotor activity patterns with electronic running wheels (even in group housed mice) (118). It is also possible to implant radiotelemetry transmitters to constantly measure the heart rate and blood pressure (119, 120) or using photobeam, and electroencephalograms. Metabolic feeding cages using indirect calorimetry are also a great option to follow the circadian metabolic patterns of mice (121). Genetic knock-outs, knock-downs and knock-ins make these animals a valuable tool to examine the effect of specific genes. It should be taken into account, however, that extended breeding, husbandry and genetic manipulation of lab mice resulted in profound changes in gene expression distancing them further from wild animals (89).

The murine immune system is only partly comparable to that of humans with various cellular and molecular differences (122, 123). Neutrophils display about 10-25% of the cells in the peripheral blood in mice whereas these are 50-70% in humans. Lymphocytes are the most abundant cell type in peripheral blood in mice with 75-90%, compared to only 30-50% in humans (122). Mice are also commonly used to assess skin biology. This is an interesting option to induce certain diseases

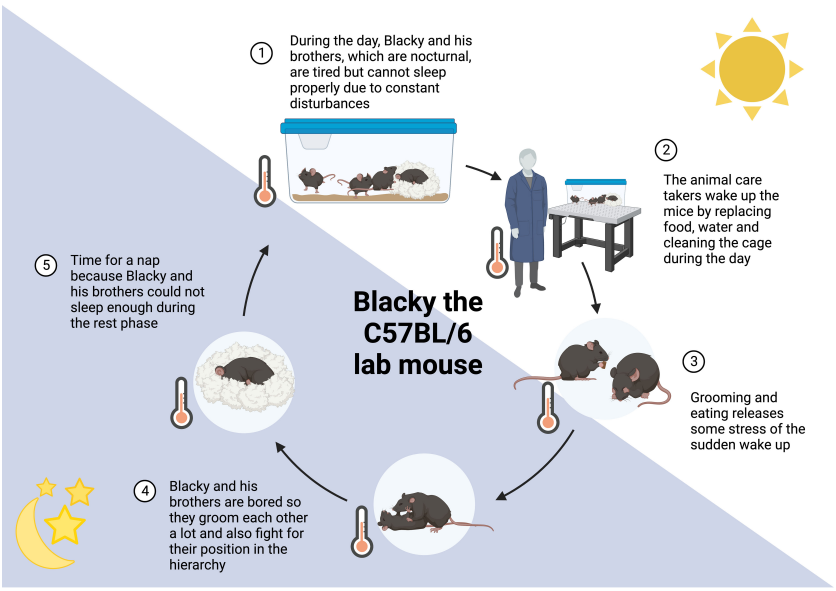

FIGURE 2
24 hours in the life of Blacky the lab mouse. Mice are nocturnal animals, being active during the night, which is opposite to humans. This leads to various interruptions of their rest period (**1**, **2**), accompanied by stress and changes in behavior. Grooming and eating alleviates some stress caused by sleep deprivation (**3**). The cage-environment is highly restricted in space, movement, and explorative options, resulting in coping behavior (**4**). Moreover, mice might catch up some sleep that was missed in the rest period (**5**). More information can be found in the Box 1.

such as epidermolysis bullosa acquisita (EBA) (124–126), and allows to take skin and organ biopsies in a degree that is not possible to gain from human patients. Human and murine skin are composed of the same layers. However, the thickness of human skin is much higher and more adherent to underlying tissues. Accordingly, mice have decreased barrier function and enhanced percutaneous absorption, which should be considered when using murine models for topic drug delivery. Moreover, mice have fur and therefore more hair follicles, which leads to differences in wound healing (127, 128). In contrast to humans, mice also show a subcutaneous layer called *panniculus carnosus*, which is a muscle layer, enabling skin contraction. This is of interest since large wounds require muscle contraction for healing, whereas in humans wound healing is achieved by formation of granulation tissue and reepithelization (129).

Even though rodents have the above-mentioned limitations, they are nevertheless a valuable and hitherto irreplaceable tool for studying effects of sleep and the circadian machinery on the immune system. Murine studies allow to explore the relations and connections between different organs and influences of the lifestyle, which cannot be shown in other models. This is either due to limitations in genetic manipulations in non-rodents or

BOX 1 Blacky, the lab mouse (Figure 2).
Blacky, a C57BL/6 mouse, is living with his brothers in a ventilated cage with 501 cm^2. Their home is comfortable with a fluffy nest. During the day, when the light is turned on, all brothers want to sleep and cuddle in their nest to stay warm and get some rest (**1**). It is not as cozy as it sounds because there is constantly noise from humans around them and the ambient temperature is below the brothers' thermoneutral zone of 30°C. The temperature of 22°C is constant for 24 hours, which makes it even more difficult to decide whether it is time to be awake or asleep, it is just always cold. From time to time, their entire home is picked up while they are asleep and put somewhere else or the nest is exchanged for a new nest. Somedays even all brothers are woken up and placed in a new home (**2**). These days are truly horrible and to compensate the stress, the brothers groom each other, even plucking off the whiskers or entire patches of fur. Some of the brothers also get hungry or feel the urge to chew to get rid of the stress (**3**) (92, 93). So, a little meal in between is very common and especially Black-Jack, the biggest brother who leads the group, starts accumulating excessive fat. When the light is turned off, the brothers' activity period starts. It is getting quiet around them, they do not hear the humans anymore, just other mice from other homes nearby. This is a good time to take a meal and luckily, they do not need to search long for their food, there is enough for everyone. Apart from food, their cage is boring because there is nothing to explore and barely anything to play with or space to run around. This is why Blacky and his brothers groom each other a lot (**4**) (94) causing small bold patches, sometimes even wounds and infections (95, 96). The grooming follows a strict hierarchy, and Black-Jack gives all the calls. If another brother has a different opinion, Black-Jack is showing him who is the boss by biting him. These fights and dominance behaviors alter neuroendocrine mediators such as corticosterone and tyrosine hydroxylase (97). After feeding, grooming and fighting, there is nothing much to do and since the brothers were woken up several times during their rest period, this is a good time to sleep again (**5**) (98). Unfortunately, this sleep is never as good as when the lights are on but who knows when to be active and when to sleep anyways?

because the model is not a full organism such as cell- or organ-cultures. Hopefully, future techniques are overcoming the need for murine studies and thereby offer new models without the above-mentioned drawbacks of mice. It is important to address difficulties with certain models to be able to overcome these. Nevertheless, there are various well-working mouse models, which delivered valuable results. Just to mention two out of many studies, we would like to outline the work of Toth et al. who studied the effects of shift work in a lupus mouse model, which resembled also typical human outcomes (130) as well as the work of Sadeghi et al. who used an EBA mouse model with an unbiased genetic approach to investigate inflammatory processes and discovered the role of the clock gene retinoic acid-related orphan receptor-alpha (*Rora)* in this disease (126).

Apart from invertebrates that cannot be used to assess skin diseases, other animal models are fish to study melanoma (131, 132) or mammals such as pigs to study skin and circadian regulation of the immune system (133, 134). However, porcine models are difficult to establish as the animals need a lot of space and require comparably long breeding spans, although some genetical modifications such as CRISPR/Cas are already well-established (135). Regarding non-human primates, pigs are ethically more accepted for experimental purposes and show higher numbers of offspring, allowing to gain sufficient animals for statistical analysis of the experiments.

4 Epidemiological and experimental evidence that shift work could promote skin autoimmune diseases

This section outlines the connection between shift work and skin autoimmune diseases. Firstly, described by shift works' general effect on the immune system and then depicting more detailed effects on certain skin autoimmune diseases.

4.1 Shift work induces systemic chronic inflammation, immunodeficiency, and dysregulation of adaptive immunity

Shift work drives SCI (25, 136, 137) that is associated with endothelial dysfunction, atherosclerosis, cardiovascular diseases, impaired glucose tolerance, metabolic syndrome, diabetes mellitus, obesity, mood disorders and neurodegenerative diseases (22, 24, 136, 138). Likewise, experimental circadian disruption as well as sleep deprivation in humans and animal models can induce an inflammatory response (40, 139, 140) and dysfunctions of cardiovascular processes (75, 141, 142), metabolism (143, 144), mood and cognition (145). Presumably, SCI in the periphery and a parallel neuroinflammatory response in the brain are the consequence of innate immune cells responding to sterile immune stimuli (e.g., reactive oxygen species, metabolites, danger/damage associated molecular patterns) (37, 146–148) and failures in counter-regulatory, anti-inflammatory mechanisms that normally regulate and resolve inflammation (e.g., IL-10, resolvins, M2 macrophages, regulatory T cells (Tregs)) (47, 149–151). While the innate immune system fights this unnecessary battle against sterile stimuli, targeted and protective immune responses are compromised. Thus, shift work or experimental circadian disruption, as well as insufficient sleep are associated with failures in innate and adaptive immunity against pathogens (152) and tumors (153–156) and with reduced vaccination-driven T cell and antibody responses (157–160), both in humans and in mice. On the other hand, shift work seems to boost unwanted adaptive immune responses to harmless antigens such as allergens and auto-antigens. In detail, shift work, circadian misalignment, clock gene polymorphisms or poor sleep are associated with a higher risk to develop allergic or autoimmune diseases (31, 161). The latter include (i) connective tissue diseases such as systemic lupus erythematosus (SLE) (162), systemic sclerosis (SSc) (161), and Sjögren's syndrome (163, 164), (ii) different forms of arthritis such as rheumatoid arthritis (RA) (165), and spondyloarthritis (SpA) (161, 166) including psoriasis arthritis (163), (iii) inflammatory bowel disease (IBD) (167–173), (iv) autoimmune thyroiditis (32, 174–176), and (v) multiple sclerosis (177). Undoubtedly, sleep is impaired in patients with systemic autoimmune diseases (178). Moreover, they show disturbances in 24 h rhythms of their stress systems (179, 180), cardiovascular functions (181), and melatonin (182–185). These changes may be the consequence rather than the cause of the autoimmune disease, as disease symptoms such as pain, itch, respiratory or gastrointestinal dysfunctions can heavily interfere with a regular sleep-wake behavior (186). However, lupus-prone mice show disturbed 24 h rhythms of corticosterone and melatonin already in asymptomatic phases, thus before manifestation of the disease (187). Moreover, experimental circadian disruption and/or sleep deprivation in animal models can promote autoimmune diseases like lupus (130, 188). It can also worsen colonic inflammation in murine models of IBD (189) or attenuate others such as experimental auto-immune encephalomyelitis (190). Thus, autoimmune processes and wake-sleep-disturbances most likely show bidirectional relationships that could feed into a vicious circle.

4.2 Does shift work boost skin manifestations of systemic autoimmune diseases or skin-specific autoimmune diseases?

4.2.1 Systemic autoimmune diseases and thyroiditis

Many systemic autoimmune diseases affect the barrier organs, thus the mucosa of the respiratory, gastrointestinal, or urogenital

tract or the epidermis, dermis and subcutis of the skin (Table 2) (191–260). Cutaneous manifestations are leading symptoms in SSc (e.g., puffy fingers, skin fibrosis and calcinosis cutis), or characteristic clinical presentations of SLE (e.g., malar 'butterfly' rash), of Sjögren's syndrome [e.g., dry skin called xerosis cutis or xeroderma (232, 233)], of RA [e.g., rheumatoid nodules (241)], of SpA, celiac disease or IBD (e.g., pyoderma gangrenosum) (242, 252), or of thyroiditis [e.g., myxedema (259)]. Connective tissue diseases, but also any other systemic autoimmune disease can lead to secondary Raynaud's phenomenon with an exaggerated cold-induced vasoconstriction of arteriovenous anastomoses in distal skin regions (e.g., fingers and toes). Currently, there is only limited data on the impact of the sleep-wake cycle on cutaneous symptoms of systemic autoimmune diseases or thyroiditis. The circadian system may be involved in photosensitivity in SLE (218). Moreover, poor sleep correlates with enhanced skin thickness in SSc (225, 227) and with genital ulcers in the vasculitis Behçet's disease (235, 236, 261). However, although shift work has been shown to impair skin health with respect to allergic and cancerous conditions (262, 263), to our knowledge epidemiological or experimental studies on skin manifestations of systemic autoimmune diseases are currently lacking. Even for Raynaud's phenomenon that involves a pathophysiology closely linked to circadian and sleep-dependent thermoregulation (which could be easily monitored by wearables), we did not find a single study that investigated this condition in shift workers.

4.2.2 Psoriasis

Likewise, studies on the association between shift work, circadian disruption or poor sleep on skin-specific autoimmune diseases are rare and mainly focus on psoriasis. Psoriasis typically presents with cutaneous erythematosquamous plaques and approximately 50% of patients develop typical nail changes. Rarely, pustular changes occur, which can affect the palms and soles, but also the entire body. One aspect of psoriasis research that has recently been investigated is the time-of-day variability in disease symptoms and severity with a peak of itch and psoriasis flares in the evening and at night. However, the reasons for this observation remain unclear (194). Related diseases such as SpA show similar peaks of symptoms at night (249).

Early circadian research on psoriasis investigated time-of-day-dependent changes in the epidermis. One study revealed no diurnal differences in mitotic index (264), whereas another one showed increased cell proliferation at 6 AM compared to healthy controls (265). A further study investigating circadian cell kinetics revealed a stable epidermal and dermal infiltrate cell proliferation over the day both in uninvolved and involved psoriatic skin, but with a circadian rhythm in epidermal DNA synthesis (266). However, in 1985, a 24 h rhythm of neutrophil migration in psoriatic skin with a peak at around 10 PM was detected that could not be shown in the skin of healthy controls (193). Further reports suggest a systemic circadian perturbation, with disruption of circadian rhythms of urinary and haematological parameters (192), of blood pressure and heart rate (237, 267) and of plasma melatonin levels (202) in psoriasis (and vasculitis) patients. In addition, lower urinary 24 h cortisol levels, lower serum cortisol at 8 AM and 5 PM were found in patients with psoriasis and IBD (251) compared to healthy controls suggesting an altered function of the HPA-axis (268).

To unravel the link between the circadian clock and psoriasis, a transcriptomic study showed a downregulation of *CRY1/2, REVERBA, CLOCK, BMAL1* and *RORA/C* in keratinocytes from psoriatic lesions (269). A recent study found changes in core clock genes and clock proteins in non-lesional and lesional human skin samples in psoriasis (270). In a mouse model, imiquimod-induced psoriasis-like dermatitis was ameliorated in mice with a loss of function mutation in the *Clock* gene compared to wild-type mice. Accordingly, in mice with a loss of function mutation in the *Per2* gene imiquimod-induced psoriasis-like dermatitis was exaggerated because PER2 inhibits CLOCK activity (271).

Based on the described investigations, few groups studied the application of chronotherapy in psoriasis. Balneotherapy (bathing therapy) has the highest efficiency when it is applied in the morning (272). Topical corticosteroid application in the evening has a higher efficiency than application in the morning after two days of treatment. However, this difference evened out after five treatment days (273). Discussed reasons for the higher effectiveness are an improved corticosteroid absorption in the evening due to a higher cutaneous perfusion and a higher skin barrier permeability or a higher therapeutic potential due to a rise in inflammation and cell proliferation in the skin during evening hours (273, 274). Recently, chronotherapy of maxacalcitol, a vitamin D analogue, was investigated in a mouse model of psoriasis in which the skin inflammation was induced by topical 12-O-tetradecanoylphorbol-13-acetate (TPA). In the skin of mice, expression of the nuclear vitamin D receptor exhibits a distinct daily variation with a peak in the middle of the active period. Accordingly, in TPA-mice application of maxacalcitol during early to middle of the active period had the highest therapeutic efficacy (275).

Various studies describe the occurrence of sleep disturbances and fatigue in patients with SLE, Sjögren's syndrome, vasculitis, psoriasis, and psoriatic arthritis (197, 198, 219, 238, 276–279). In both conditions, patients rate sleep disturbances as a factor severely impairing quality of life (280–283). Sleep disturbances in patients with psoriasis and psoriatic arthritis are caused not only by disease manifestations such as nocturnal itching or pain (20, 284), but also by gastroesophageal reflux disease, anxiety or depression (285, 286). In addition, an association between psoriasis and prevalence of sleep disorders, such as obstructive sleep apnea or restless legs syndrome, has been described. Data on the prevalence of sleep disorders in psoriasis and potential effects of psoriasis treatment with biologics on sleep and sleep disorders are presented in a recently published review (287).

TABLE 2 Effects of shift work, circadian or sleep disturbances on autoimmune (skin) diseases.

Disease	Skin manifestations	Findings in shift workers	Circadian findings	Sleep findings	Key cellular players	Involved neuroendocrine mediators
Skin-specific autoimmune diseases						
Psoriasis	Erythematosquamous plaques; rarely pustular changes; nail psoriasis in 50% of patients	Higher risk of development (191)	Generally disturbed 24 h rhythm (192); rhythmic regulation of neutrophil traffic (193); symptoms show diurnal pattern (163); symptom severity peaks in the evening and at night (194)	Pruritus disrupts sleep (195); systemic increase of proinflammatory cytokines by sleep deprivation in mice (196); increased frequency of sleep disturbances (197) and fatigue (198)	Neutrophils (193); gamma-delta T cells (199)	Dysfunctional HPA-axis with reduced cortisol levels; prolactin levels tend to be higher in psoriasis than in controls (200, 201); disturbed circadian rhythm of melatonin (202)
Vitiligo	Whitening due to destruction of melanin (203)	No data available	Blood rhythms in NK cell activity are shifted (204, 205), CD4 T cell number rhythm disrupted (206)	Sleep disturbances; poor sleepers show a higher risk of vitiligo (207, 208)	NK cells, CD4 T cells (204, 205)	Stress mediators and melatonin (209)
Pemphigus	Blisters in epidermis and mucous membranes; positive Nikolsky sign (210)	No data available	No data available	Bidirectional relationship (211)	Autoreactive B cells (212)	Corticosteroids are used as common symptomatic treatment; stress is able to trigger phemphigus flares and can worsen symptoms (213)
Pemphigoid diseases	Subepidermal blisters; negative Nikolsky sign (210)	No data available	No data available	Sleep disturbances due to symptoms peaking at night (66, 67, 214)	Autoreactive B cells; neutrophils (124, 215, 216)	Corticosteroids are used as common symptomatic treatment (217)
Systemic autoimmune diseases						
Connective tissue diseases						
Systemic lupus erythematosus	Malar rash, photosensitivity, alopecia, livedo reticularis (218)	Higher risk of development (162)	Photosensitivity (218); symptoms show diurnal pattern (163); excessive daytime-fatigue, especially in the morning (219)	Sleep disorders increase the risk of disease development (161)	B cells, plasma cells, Tregs (220); oxidative stress caused by Th17 cells (221)	Dysfunctional HPA-axis with reduced cortisol levels and changes in prolactin (222) and melatonin (221, 223); adrenal insuffiency (224)
Systemic Sclerosis	Puffy fingers, skin fibrosis, skin ulcers, calcinosis cutis, teleangiektasia (225)	No data available	Altered prolactin rhythms (226); symptoms show diurnal pattern (163)	Poor sleep leads to enhanced skin thickness (227); therapeutic sleep can be used to ameliorate skin symptoms (228); fatigue (225)	Th2 cells, B cells, macrophages (229)	Lower cortisol (230) and melatonin levels (231); altered prolactin rhythm (226)

(Continued)

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TABLE 2 Continued

Disease	Skin manifestations	Findings in shift workers	Circadian findings	Sleep findings	Key cellular players	Involved neuroendocrine mediators
Sjögren's syndrome	Xeroderma (232)	Higher risk of development (163)	Circadian disruption is enhancing disease onset and progression (164); symptoms show diurnal pattern (163); excessive daytime-fatigue (219)	Patients often suffer from excessive daytime sleepiness, fatigue, insomnia, nocturnal headaches and nocturnal sweats (178); sleep disorders increase the risk of disease development (161)	Memory B cells, marginal zone B cells, plasma blasts and plasma cells (233)	Hypofunctional HPA-axis resulting in lower basal ACTH and cortisol levels (234)
Vasculitis	Purpura; Behcets syndrome (235, 236)	No data available	Inadequate decrease of nocturnal blood pressure (237)	Fatigue (238)	Dendiritc cells, Th17 cells and macrophages (239) and B cells (240)	Melatonin is able to relieve vascular endothelial cell damage (185)
Arthritis						
Rheumatoid arthritis	Rheumatoid nodules, Felty syndrome, rheumatoid vasculitis, pyoderma gangrenosum, rheumatoid neutrophilic dermatosis, interstitial granulomatous dermatitis, palisaded neutrophilic dermatitis (241)	Higher risk of development (161); independency of higher risk for development from socioeconomic factors, health behavior or psychological distress (243); development of sero-positive rheumatoid arthritis was especially increased in rotating shift work and day-oriented shift work, whereas increasing duration of permanent night shift appears to be protective against rheumatoid arthritis (33)	Symptoms show diurnal pattern (163); peak of symptoms between 02 and 04 AM (244); excessive daytime-fatigue, especially in the morning (219); Disruption of the circadian clock results in aberrant expression of inflammatory cytokines (e.g. IL-6) disruptions of the peripheral chondrocyte clock promotes catabolic processes in cartilage (245)	Sleep disorders increase the risk of disease development (161); fatigue as common symptom (163); sleep disturbances, poor sleep quality and decreased total sleep time are common in rheumatoid arthritis; short sleep duration is causally linked to an increased disease risk (165)	T cells (Th1, Th2, Th17, Treg), cells of the B cell compartment, macrophages (246)	Dysfunctional HPA-axis and altered circadian rhythm of cortisol (248) and melatonin (183)
Spondyloarthritis	Pyoderma gangrenosum, hidradenitis suppurativa (242)	Higher risk of development (161)	Symptoms show diurnal pattern (163); major peaks in the morning between 06 AM and 09 AM (166)	Sleep disorders increase the risk of disease development (161); disease is also by sleep disturbances (178)	IL-17 is a key mediator which is produced by a variety of cells such as Th17 cells, neutrophils and macrophages (247)	Increase in backpain at midnight might be related to lower melatonin levels in spondyloarthritis patients compare to healthy controls (249)
Inflammatory bowel disease	Pyoderma gangrenosum, hidradenitis suppurativa (242)	Higher risk of development (167, 168); risk factor for surgery in Crohn's disease, but not in ulcerative colitis (172); extended and irregular shift work might be a risk for chronic inflammatory bowel disease (173)	Disruption of the circadian system increases the activity of the gut immune system and the release of inflammatory factors; diurnal oscillations of microbiota (170)	Sleep disturbances are risk factors for development of Crohn's disease in children (171); increased risk for ulcerative colitis in people sleeping less than 6 h and more than 9 h per day (173)	(169) Crohn's disease: Th2; ulcerative colitis: Th1; both: Th17, Treg (250)	Lower 24 h amplitude of plasma cortisol in ulcerative colitis (251), lower levels of melatonin in ulcerative colitis (184)

(Continued)

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TABLE 2 Continued

Disease	Skin manifestations	Findings in shift workers	Circadian findings	Sleep findings	Key cellular players	Involved neuroendocrine mediators
Celiac disease	Dermatitis herpetiformis; association to psoriasis, chronic urticaria, leukocytoclastic vasculitis, alopecia areata (252)	No data available	No data available	Data on the presence of insomnia and sleep disturbances in patients with celiac disease is heterogeneous (253–255). sleep disturbances as well as primary sleep disorders such as sleep apnoea improve under gluten-free diet (256, 257)	Th1 cells, cells of the B cell compartment (258)	No data on the role of neuroendocrine mediators in dermatitis herpetiformis
Thyroiditis	Myxedema (259)	Higher risk of development (32)	Downregulated *BMAL1* and *PER2* expression (175)	Often occurs together with obstructive sleep apnoea but unclear if cause or consequence (176)	Infiltration of T cells, which release inflammatory cytokines (260)	Significant decrease of serum melatonin levels (175)

Overview of autoimmune diseases and the effects of shift work and the circadian rhythm. Abbreviations in order of apearance: HPA, Hypothalamus-pituitary-adrenal; NK, natural killer; Th, T helper; Tregs, regulatory T cells; ACTH, adrenocorticotropic hormone; ACPA, anti-citrullinated protein antibody; IL, interleukin; UC, ulcerative colitis No relevant results were available for mixed connective tissue disease, polymyositis or dermatomyositis.

Until now, the effect of immunomodulatory and immunosuppressive therapeutics on sleep disturbances remains elusive (288–290). In addition, studies investigating the relationship between sleep quality and disease activity in psoriasis and psoriatic arthritis give contradictory results (276, 291–298). So far, studies on lifestyle interventions that affect sleep and investigate respective effects on disease activity are lacking (299). Impairment of sleep in patients with psoriasis, psoriatic arthritis and axial SpA increases the risk for psychiatric diseases, which themselves might impair sleep, resulting in a vicious circle (300–302). Moreover, patients with psoriasis and sleep disturbances have a higher risk of stroke and ischemic heart disease compared to psoriatic patients without sleep disturbances (303). Finally, jet-lag in patients with psoriasis experiencing a flight crossing at least two time-zones increases self-reported disease severity (304).

Circadian and diurnal variations as well as sleep disturbances are well investigated in psoriasis and psoriatic arthritis but only a single study investigated the influence of shift work on the risk of psoriasis. Li and colleagues published a study in 2013 showing that enhanced duration of rotating night shift work increases the risk of psoriasis independently of important behavioral risk factors for psoriasis, namely body mass index and smoking (191). Moreover, night shift work is associated with an increased risk of psoriasis comorbidities, e.g. myocardial infarction (305).

4.2.3 Other skin-specific autoimmune diseases

Apart from psoriasis further skin-specific autoimmune diseases are vitiligo, pemphigus, EBA, and bullous pemphigoid (BP). Poor sleepers show a higher risk of vitiligo (306, 307). There are also reports on sleep disturbances in patients with BP (67) or vitiligo (207, 208). For EBA, the clock gene *Rora* was found to be a genetic risk locus in the murine passive anti-collagen type VII transfer model (126). The core-loop of the circadian clock consists of *Bmal1/Clock* and *Per/Cry*. However, more genes interact and form additional loops. The transcription of this clock gene *Rora* is initiated by *Bmal1/Clock*, which itself fosters the transcription of *Bmal1* (308). Sadeghi and colleagues found that a knockout of *Rora* in mice diminished the skin lesions upon anti-COL7 challenge. They could further show that even a *Rora* blockade was able to reduce skin inflammation and blistering in this model (126). Patients with BP (mostly elderly) are often also diagnosed with neuro-psychiatric comorbidities years before skin manifestations of BP appear (309). It turned out that anti-BP230 (one of the autoantibodies emerging in BP), is an independent predictor of neuro-psychiatric illnesses in BP patients (310). In addition to the dermis, BP230 is also expressed in the central nervous system and an immunologic cross-reaction of the autoantibodies causing neuroinflammation might explain the BP-associated neuropsychiatric disorders (311). Circadian disruption (e.g by

aging or sleep disturbance) increases neuroinflammation in rats (312, 313) and humans (24, 314) and might amplify neuropsychiatric symptoms in BP patients. If this scenario is translatable to shift work remains to be elucidated. Symptoms of autoimmune skin diseases peak at night, as shown by actigraphy also in BP patients (66, 67). This could further disrupt sleep and circadian rhythms thus feeding into a vicious cycle.

Apart from autoimmune skin diseases, disturbances of the wake-sleep cycle seem to promote also infectious skin diseases such as bacterial invasions (315), allergic skin diseases such as atopic dermatitis (316), contact hypersensitivity (317), or skin cancers (318).

In general, skin diseases show distinct 24 h rhythms in symptoms (163), with a nocturnal peak in pain, pruritus and scratching that heavily interferes with sleep (195, 244, 319–321). The dermatology life quality index (DLQI) questionnaire is not considering sleep disturbances (322), whereas the bullous pemphigoid disease area index (BPDAI) explicitly queries sleep impairments to quantify the extend of pruritus (217, 323). These questionnaires are not standardized to span different (autoimmune) skin diseases, which renders it difficult to compare them with each other. The Pittsburg Sleep Quality Index (PSQI) covers various aspects of sleep, its duration and disruptions but does not include any aspects of autoimmunity besides unspecified pain at night (324). Sleep disturbances due to nocturnal symptoms should therefore more often be rated in clinical scores of skin diseases or by objective assessments using, e.g., wearables (66, 67).

5 Potential effects of shift work on skin and immune cells

So far, we described the manifold influences of the circadian system on sleep, behavior, thermoregulation, neuroendocrine mediators, the immune system, skin manifestations of systemic autoimmune diseases and skin-specific autoimmune diseases. In the following, we will outline how the circadian system and the sleep-wake cycle physiologically regulate skin and immune cells and how disturbances of this regulation, e.g. due to shift work, could drive the pathophysiology of these disorders.

5.1 Potential effects of shift work on skin cells

It is hypothesized that the circadian system evolved to protect proliferating cells from DNA damage due to UV light (10, 325, 326). Such a rhythmic adaptation to light or to other environmental stimuli like ambient temperature, moisture, or pathogens conceivably is most relevant in the skin, the major barrier between the outer and the inner world (Figure 3). Thus, multiple epidermal, dermal and hair follicle clocks tick in stem cells, keratinocytes, fibroblasts, and melanocytes and jointly seem to serve protection against environmental challenges during the activity period (49, 109, 327–332). Notably, clock genes also regulate cellular functions in human keratinocyte and melanoma cell lines (327). The subcutis with its fat depots and its dense sympathetic innervation and vascularization also seems to have a circadian regulation that might serve in particular metabolic and thermoregulatory functions (333). Presumably, this skin clockwork maintains skin physiology and skin integrity (39, 331). Thus, skin blood flow, temperature, pH, transepidermal water loss, and sebum excretion show 24 h rhythms in humans (334–336) and animals (337). The circadian system also impacts wound healing in mice (338) and hamsters (339). Experimental disruptions of this finetuned rhythmic skin regulation by circadian misalignment or sleep deprivation impairs skin integrity (65, 340), regeneration (341, 342), and wound healing (339, 343, 344), accelerates skin aging (345), enhances the activity of skin proteases (196), and leads to skin ulcers and hyperkeratosis (346). These outcomes prompt symptoms such as itching and pain. Interestingly, the phenomenon that pruritus and scratching peak at night, is presumably driven by the described rhythmic changes in skin barrier functions and by enhanced nocturnal skin temperature (214). Although pain perception shows conflicting results with respect to circadian regulation in humans (347, 348), it clearly increases upon circadian or sleep disturbances (349). Vice versa, as outlined above itching and pain at night are likely explanations of sleep disturbances in patients with skin diseases (277, 319). Thus, circadian disruption, sleep loss, enhanced scratching, and exaggerated pain perception likely feed into a vicious circle that further fosters skin barrier damage. Shift workers show disrupted rhythms in hair follicle cells and interfollicular epidermal cells (341, 350) and changes in pain perception (351). However, it is presently unknown, whether shift workers suffer from impairments of skin physiology or integrity. Itching leads to scratching, causing wounds that need to heal. In mice, wounds occurring during the rest phase healed less quickly than wounds that occurred during the active phase. Responsible for this is the rhythmic mobilization of fibroblasts by dynamic actin (338). Similarly, it was found in mice that sleep fragmentation delays wound healing (352). Likewise, difficulties in wound healing are commonly observed in patients with autoimmune diseases and sleep abnormalities (353).

5.2 Potential effects of shift work on innate immune cells in the skin

The skin is populated by transient and resident innate immune cells. Previous studies showed a sleep-wake cycle-dependent hematopoietic release of granulocytes and monocytes and

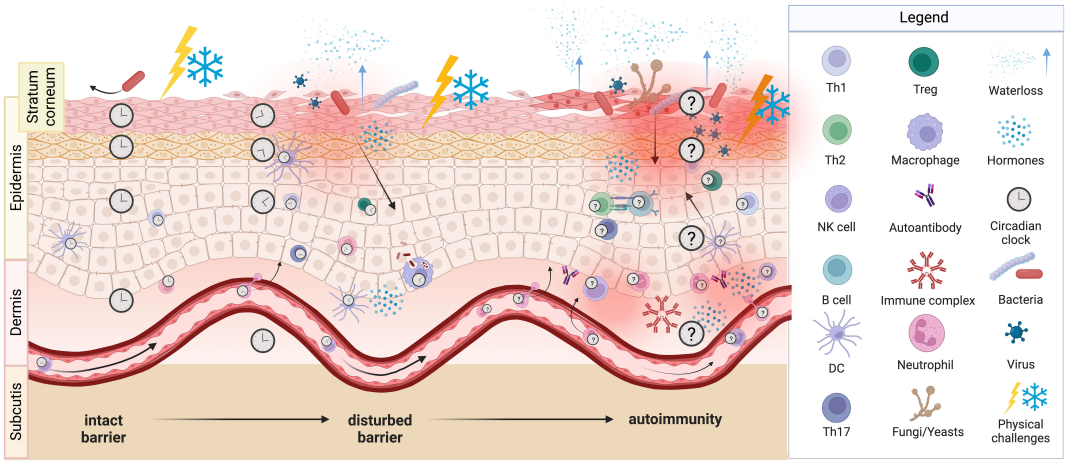

FIGURE 3
Potential consequences of shift work. Circadian misalignment and/or sleep impairments as a result of shift work presumably lead to changes in the skin barrier function. An intact barrier (left part) is able to block physical challenges as well as pathogens and prevents transepidermal water loss, whereas a disturbed barrier (middle part) is not able to do so. Noxi and intruders then can reach the epidermis, induce damage and activate immune cells and thus local inflammation can occur. A severely disturbed barrier (right part) shows breaches, through which bacteria, viruses and fungi enter the skin and cause inflammation with the attraction of various innate and adaptive immune cells. Likely, consequences are itching, scratching, and pain. Molecular clocks tick in skin cells, in innate and adaptive immune cells, as well as in endothelial cells and could be entrained by light, temperature, and neuroendocrine mediators such as cortisol, catecholamines and melatonin. We hypothesize, that this circadian system of the skin strengthens barrier functions during daytime and that shift work-induced changes favor the development of autoreactive T cells and autoantibodies resulting in autoimmune diseases.

subsequent traffic of these cells to various tissues (110, 354–356). Although rhythmic leukocyte homing to skin seems to be neglectable in the steady state (355), indirect evidence indicates that there is a circadian regulation of immune cell traffic to the skin upon wounding and upon microbial or antigenic challenges (357–362). Likewise, tissue-resident innate immune cells like dendritic cells (363, 364), mast cells (317, 365, 366) or macrophages (367–369) show circadian regulation that impacts cutaneous responses to antigens and allergens, respectively. Several genes controlling immune functions are rhythmically regulated in murine skin (331) and the expression of cytokines (e.g. TNF) and chemokines (e.g. IL-8 or C-X-C-motif ligand 1) in human skin are under clock control as well (370). Overall, disturbances of these fine-tuned rhythms in leukocyte traffic and function seem to result in unchecked innate immunity. This is a mechanism that could also contribute to systemic inflammatory responses with increases in blood leukocytes, neutrophils, monocytes, and C-reactive protein in shift workers (75, 136, 138, 371–376). Along this line, both experimental circadian disruption and sleep deprivation enhance the responsiveness of the innate immune system to inflammatory stimuli and trigger inflammation (75, 106, 139, 354, 356). The outlined interactions between the sleep-wake cycle and innate immunity may be relevant for skin diseases, as sleep deprivation induces systemic increases in pro-inflammatory cytokines also in a mouse model of psoriasis (196), and as neutrophil traffic into the skin of psoriatic patients shows rhythmic regulation (193). Apart from granulocytes, monocytes, dendritic cells, and mast cells, also natural killer (NK) cells show 24 h rhythms of their numbers and their activity in human blood (377–379) and rodent spleen (154, 380). Circadian and sleep manipulations alter these parameters (154, 340, 377, 378, 380–382). Likewise, experimentally simulated shift work in healthy individuals changed gene transcripts of NK cell-mediated immune responses (77) and shift workers show impaired NK cell-function (383, 384). Also, in patients with systemic autoimmune diseases (385) or with vitiligo (204, 205), altered rhythms of NK cell-activity in peripheral blood were described. Time-of-day dependent changes in NK cell numbers or functions in healthy or diseased skin, however, were not tested so far.

5.3 Potential effects of shift work on adaptive immune cells in the skin

T cells play key roles in a variety of autoimmune diseases (239, 240, 247, 250, 258, 260) and can enter and reside in the epidermis and dermis. Their recirculation between blood, lymphoid organs and other tissues is regulated by the circadian system (190, 382, 386) and by sleep (387, 388). This is presumably mediated by sleep-wake cycle dependent changes in T cell selectins, integrins, and chemokine receptors, and in corresponding ligands on endothelial cells and surrounding tissues (190, 386, 389, 390). Moreover, T cell functions like proliferation (151, 391), Th1-, Th2-, and Th17-differentiation or

cytokine production (392–398), and the activity of Tregs (151, 399, 400) are linked to the sleep-wake cycle. Rhythmic changes in T cell traffic and functions seem to be regulated by T cell intrinsic clocks such as *REVERBA* (398, 400, 401) and by effects of the SCN or sleep on neuroendocrine mediators (see next section) (387, 390, 402). In shift workers, increases in T cell numbers (384, 403) and impairments in T cell proliferation (28) were reported.

Joint effects of the circadian system and sleep on T cell immunity could also contribute to sleep-wake cycle dependent changes in cutaneous T cell responses and disturbances thereof in shift workers. Indeed, the T cell driven induction or recall of cutaneous DTH reactions show rhythmic modulation in humans (404, 405) and rats (406, 407). Primary DTH responses were impaired in stroke patients showing sleep rhythm disturbances (408) or in hamsters upon experimental circadian disruption (363) or light at night (409). In contrast, *Clock* mutant mice showed enhanced T cell driven contact hypersensitivity to allergens upon challenge (317). Likewise, constant light in mice enhanced allergic skin responses, while the development of immune tolerance and subsequent Treg infiltration in the challenged skin was impaired (410). Another mouse experiment demonstrated reductions in skin allograft rejection and in T cell infiltration of the graft by sleep deprivation (411). Overall, the picture suggests, that disturbances of the circadian system and/or sleep could impair developing but exaggerate established T cell responses in the skin. With respect to skin autoimmune diseases, one study reported disrupted rhythms in blood CD4 T cell numbers in patients with vitiligo (206). In mouse models of psoriasis, clock gene mutations changed skin inflammation by modulating IL-23 receptor expression in gamma delta T cells and subsequent IL-17 and IL-22 production (271) and treatment with a REVERB agonist suppressed IL-17 production in gamma-delta T cells and improved dermatitis (199). In humans, clock genes might likewise impact cutaneous T cell responses, although this was so far only elaborated in the context of skin cancer (412).

Clocks also tick in B cells of mice (413) and humans. There are 24 h rhythms in human B cell numbers in blood (414) and murine B cell numbers in spleens and lymph nodes (415), and in systemic levels of antibodies, so called immunoglobulins (Ig) of the three subtypes IgG, IgA, and IgM (416–418). In line with exaggerated allergic DTH responses that are driven by T cells, *Clock* mutant mice also show enhanced IgE reactions to allergens (317). There is evidence that day-night-shift rotations attenuate the release of the anti-inflammatory cytokine IL-10 by B cells (419). This lack of immunologic regulation could be deleterious as the IL-10 releasing B cells of shift workers were unable to inhibit the proliferation of T cells (419). It could also be shown that *CLOCK* expression in peripheral B cells of shift workers was higher, which leads to a reduced expression of transforming growth factor beta (a cytokine mainly released by Tregs) (420). *Cry 1/2* deficient mice showed an autoimmune phenotype with elevated levels of serum IgG, antinuclear antibodies (ANAs), and immune complexes, as *Cry* presumably regulates B cell development and B cell receptor signaling (421). On the other hand, the distribution of B cell subsets in spleen, lymph nodes and peritoneal cavity in wild type and *Bmal1* knockout mice did not differ. The maturation of B cells was also not influenced by the knockout of *Bmal1*. Moreover, there was minor circadian regulation of *Per2*, which was detected by a reporter mouse model. It seemed as if cell intrinsic circadian clocks did not affect the B cells. They were probably gated by cell-extrinsic circadian variations (422).

B cells generate long-lasting immunologic memory by becoming (auto-)antibody-producing plasma cells and being able to survive decades in niches in the bone marrow (423). Therefore, plasma cells can be key in autoimmunity. In several cutaneous autoimmune diseases, auto-antibodies are a common diagnostic criterion and B cells are also one of the most often targeted cell types in the treatment of autoimmune diseases (424). In accordance, disease severity of pemphigus, BP, and SSc correlated with the number of B cells infiltrating the skin (210, 212, 215, 216, 229, 425). Auto-antibodies can be developed against all kinds of self-molecules. For connective tissue diseases such as SLE and SSc, for example, ANAs are formed against contents of the cell nucleus (220). This content is presumably presented for extended time to the immune system due to insufficient clearance after cell death (426). Apoptosis is a natural process but can also be triggered by UV light, explaining the photosensitivity in SLE (218).

Plasma cells cannot only develop in primary and secondary lymphoid organs by the help of T cells but also in a T cell-independent manner in the skin. This phenomenon was observed in several autoimmune and inflammatory diseases and the effect of local auto-antibody secretion is believed to play a role in chronic inflammation (424). Unfortunately, the influence of the circadian system or sleep on these skin-resident B cells is currently unknown.

6 Candidate neuroendocrine mediators linking shift work with skin autoimmune diseases

This section concentrates on central and peripheral hormonal agents that connect circadian, neuronal, and immunologic mechanisms.

The exact contributions of the SCN (109) with their systemic signals (e.g., stress mediators or core body temperature), of external time cues that directly affect the skin (e.g., extraretinal photoreception by keratinocytes, changes in ambient temperature) (427, 428) or of sleep and associated changes in behavior (e.g., darkness, supine position, reduced physical activity, fasting) (333, 390) in the entrainment of skin clocks and in the circadian regulation of the skin remain to be elucidated (429) (Figure 4). Whatever the case, the stress

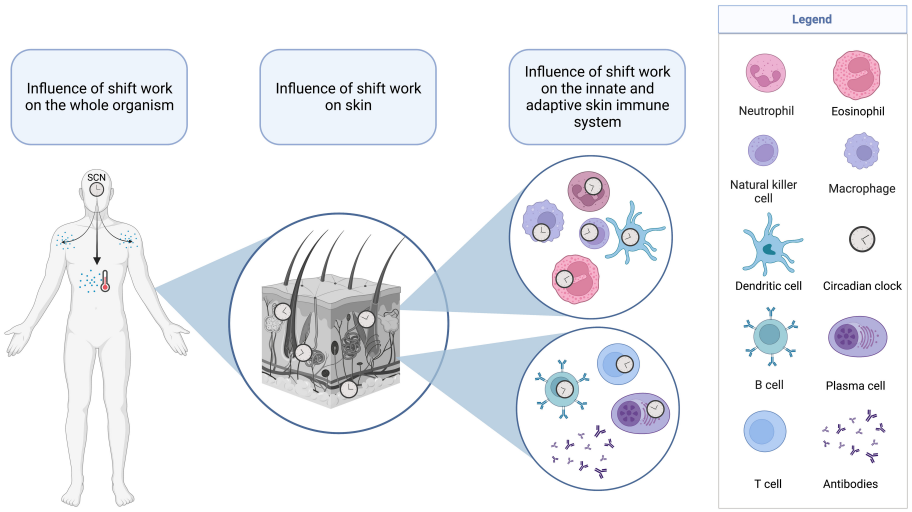

FIGURE 4
Layers of systems in the human body that are affected by shift work. Circadian disruptions due to shift work can affect the suprachiasmatic nuclei (SCN) and the major outputs of this master clock such as core body temperature and neuroendocrine mediators. These systemic influences as well as direct effects of ambient temperature and light can affect the circadian system in the skin and the cellular clocks of skin leukocytes of the innate (top) and adaptive (bottom) immune system.

systems and melatonin seem to be key, either as SCN outputs to the periphery, as signals in cutaneous light perception or thermoregulation or as mediators that change during sleep (22). In humans, blood levels of stress mediators and melatonin oscillate in anti-phase with peak levels during daytime activity for stress mediators and nocturnal sleep for melatonin, respectively (430, 431). These changes induce an increase in skin temperature in the evening that in turn essentially contributes to sleep onset (44). Sleep and its associated behavioral changes further reduce nocturnal levels of stress mediators and enhance nocturnal levels of melatonin. The peripheral hormone of the HPA-axis is cortisol that regulates human keratinocyte clock functions and suppresses proliferation of these epidermal cells (49). The mediators of the SNS are the catecholamines epinephrine and norepinephrine that together with melatonin are key in thermo- and vasoregulation. Thus, the supporting effects of melatonin intake on sleep initiation is assumed to be mediated by vasodilatatory effects of this hormone (432). Cortisol, catecholamines, and melatonin also impact immune cell clocks, traffic and functions in blood and various tissues (154, 381, 390), including the skin (363). They are not only released systemically, but also produced locally in the epidermis and dermis (433, 434). In mice and rats, the peripheral hormone of the HPA-axis is corticosterone that likewise increases sharply at the transition from the rest to the activity period (435, 436). For blood and tissue levels of catecholamines in rodents, rhythms and peak times were rather inconsistent (437–439). In contrast to humans, mice show highest melatonin levels during their activity period, thus in phase with corticosterone (114). As melatonin has many effects on skin physiology and immunity (440), it therefore seems not very straightforward to study the effects of the circadian system and sleep on skin immunity in mice. A counter argument could be that many laboratory mouse strains like C57BL/6 and Balb/C seem to be genetically incapable to synthesize sufficient amounts of systemic melatonin (441) or to express melatonin receptors. C57BL/6 mice assume to lack both melatonin receptors, yet scientists bred a strain of melatonin receptor (MT)1 and MT2 knockout mice and backcrossed them into a melatonin proficient strain to study the effects of each receptor separately (442). Recently, a melatonin proficient C57BL/6 strain was also developed (443). In sum, these different mouse strains could serve to study the effects of melatonin administrations on skin immunity, but experimental design needs to be chosen with caution.

Stress mediators and melatonin may play a role in SLE (221–224), SSc (230, 231, 444), psoriasis (196, 200–202, 268, 445), Sjögren's syndrome (234), and vitiligo (203, 209). A failure in the HPA-axis to control inflammation is discussed in autoimmune diseases (180, 248) and glucocorticoids are widely used therapeutically as immunosuppressants.

Vasodilatatory, anti-inflammatory, and melatonin-releasing effects of catecholamines are mediated by beta-adrenoceptors (389, 446). Beta-blockers therefore can have manifold unwanted effects on the circadian system, sleep, and the immune system and in this way may contribute to disease flares in psoriasis (447). Also other hormones and mediators such as growth hormone, prolactin, aldosterone, thyroid hormones, sex

hormones, ghrelin, leptin, prostaglandins, serotonin, histamine, adenosine, endorphins, αMSH, neuropeptides, and vitamin D are regulated by the wake-sleep cycle (22, 35, 47, 226, 389, 390, 448), are involved in autoimmune diseases (180, 449), can impact skin physiology, and immunity (205, 209, 450) and may therefore be of relevance in the etiopathology of skin autoimmune diseases in shift workers.

As itch and pain are key, interrelated symptoms in skin autoimmune diseases, underlying mediators could be of particular interest in studying effects of shift work on dermal cells and leukocytes. Neuronal pathways of itch and pain involve A- and C-fibers in the epidermis, which are activated by histamine, neuropeptides, and cytokines, as well as forwarding of the signals *via* the dorsal root ganglion to the brain. Beta-endorphins are able to act as analgesics by binding to opiod receptors, starting a cascade of interactions, which finally results in the inhibition of pain signalling (451). Histamine is often released at the site of inflammation in the skin (452) and plasma levels of beta-endorphin are enhanced in children with atopic dermatitis (453). In healthy humans, plasma levels of beta-endorphins (but not of histamine (454)) show rhythmic regulation being highest in the morning and reduced in the night (455–457). Ligand binding to histamine receptors on leukocytes likewise changes from night to day with complex patterns in healthy and atopic individuals (458). Moreover, in healthy individuals blood levels of cortisol and beta-endorphins are coupled, meaning that cortisol follows beta-endorphin with a lag-phase of ten minutes (459). Glucocorticoids are potent antipruritic drugs, but are also known to inhibit pain pathways (460). Therefore, not only low beta-endorphin levels but also low cortisol levels could explain why symptoms of itch and pain are most pronounced at night, as it was demonstrated for itch in patients with psoriasis (211, 461).

The effect of sex hormones is expected to explain the sexual dimorphism in autoimmune diseases that predominantly affect women (462). The effect of estrogen on sleep and circadian rhythms becomes also visible in women, when they reach the menopause and suffer from sleep disorders due to a decline or imbalance of this hormone (463).

Shift workers show enhanced average cortisol levels (25) and disrupted melatonin rhythms (464). However, until yet there is no data on catecholamines, histamines, endorphins, sex hormones, or other neuroendocrine mediators in shift workers.

7 Countermeasures to avoid skin immune dysregulation in shift work

Until now, this review focused on potential circadian drivers of autoimmunity. However, there are also some factors that might improve and stabilize the circadian system and thereby might be able to alleviate symptoms and negative effects of shift work.

Shift work is indispensable in healthcare, public protection and transportation. Some industries use 24 h schedules due to difficulties in stopping machines and production chains. However, shift work may also serve to maximize profit. From a health perspective, night shifts should only be demanded from workers when absolutely required. To minimize health issues, employers should use sophisticated shift work schedules (i) favoring a forward rotating system instead of a backwards rotating one, (ii) allowing rest periods of at least 11 h between two shifts, days off after night shifts and free weekends, (iii) avoiding early morning starts and long night-time working hours, and (iv) limiting the number of (consecutive) evening and night shifts (465, 466). Ideally, the chronotype of the workers should be assessed (i.e., being a morning or evening person) and whenever possible, this should be taken into account when scheduling shifts (e.g., avoiding night shifts in morning persons) (467). To facilitate alignment to night shifts and re-alignment to the regular sleep-wake cycle, bright light can help to suppress melatonin secretion and sleepiness during wake periods, whereas melatonin supplementation, as well as a cool and dark bedroom with a bedding that facilitates a suitable skin temperature might help to catch up on sleep (465, 468, 469).

Melatonin acts as dark signal, which is only to some degree able to facilitate sleep. Nevertheless, the idea of melatonin supplementation is to re-gain the circadian rhythm if it was disturbed. Even though the available data on this topic is limited, there are some studies on melatonin supplementation in shift workers and also as treatment for SLE. Nabatian-Asl and colleagues were able to show that 10 mg/day melatonin supplementation for 12 weeks is reducing serum malondialdehyde, which is a marker for oxidative stress. Oxidative stress levels are known to correlate with SLE-activity (221). A recent systematic review by Carriedo-Diez and colleagues investigated melatonin supplementation in shift workers. The investigated studies used between 1 and 10 mg melatonin and recognized improvements such as reduced day-time sleepiness and increased total sleep period (470). However, the studies varied in their design and group sizes and ages. More future work will hopefully shed light on this topic, also investigating other autoimmune diseases and effects as well as side-effects of melatonin.

As food intake entrains rhythms, there are some recommendations for meal timing and composition during shifts (465, 471). Caffeine and physical activity promote wakefulness and the latter seems to protect from shift work disorder (34, 81). Pharmacological interventions in shift workers with sleeping aids such as zopiclone or wake-promoting substances such as modafinil did not lead to clear improvement of sleep or alertness, respectively (472).

The psychosocial and socioeconomic situation of the workers cannot be neglected. Apart from physical consequences, shift workers may also suffer from emotional and mental health issues. The socioeconomic status differs among

shift workers and many report increased job stress (473) or social isolation (20). As social stress and isolation were shown to be associated with the conserved transcriptional response to adversity as an indicator of SCI (474, 475), they may mediate or influence the effects of shift work on skin autoimmune diseases. Older age, female gender, being married, or having children is increasing the risk of suffering from sleep related impairment in response to shift work (81). Medical surveillance, in particular in employees with these kind of risk factors, should also cover skin health and care. Considering working time preferences and giving employees shift schedules one month in advance can help them to plan activities with family and friends and thus to improve the work-life balance and social contacts (18, 465, 466). Further countermeasures to avoid negative health outcomes in shift workers are financial compensation, individual counselling, health education (e.g. dietary habits, physical activity, avoidance of substance abuse), and information about non-pharmacological interventions to reduce stress (e.g., mindfulness based stress reduction) and to ease sleep (e.g. sleep hygiene, napping) (466, 476).

In case that a systemic or skin-specific autoimmune disease has already occurred, physicians might recommend to avoid night shifts, as stress and stressful events could worsen symptoms and cause further disease flares (213). If this is not feasible, wise timing of topic and/or systemic drug treatments, thus during work hours or before bedtime and not at standard clock times might be advantageous (477).

8 Conclusions and future directions

Available evidence indicates that shift work by disrupting the circadian system and sleep impairs skin physiology and immunity and presumably contributes to skin autoimmune diseases. Circadian and sleep aspects should be considered in basic research on immunity in particular in experiments on nocturnal animals. Moreover, other animal models such as pigs should be considered in immunologic and chronobiologic studies.

The influences of shift work and disrupted circadian clocks are manifold, ranging from fatigue and metabolic disturbances over SCI to the development of autoimmune diseases. Innate and adaptive immune cells (as well as all other cells) show rhythmic regulation and may react adversely to different stimuli such as pathogens, allergens or (auto-)antigens if the rhythm is disrupted. The signaling of the SCN downwards to peripheral organs and cells is mediated by hormones like melatonin and cortisol. These hormones should be monitored in shift workers, to delineate their role in the development of autoimmune diseases. The skin as the largest human organ is in contact with the outer world and therefore an important barrier, which seems to be weakened by circadian disruption, sleep loss, or shift work. In our society, shift work is unavoidable, however, this review offers several opportunities to improve the health of shift workers.

Author contributions

All authors contributed in writing and revising the manuscript. TL and JH developed the concept, structure and both tables, SS prepared the figures and wrote paragraphs about animal studies and B cells, HG provided her clinical insight and knowledge especially regarding psoriasis, arthritis and inflammatory bowel disease and wrote these sections. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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