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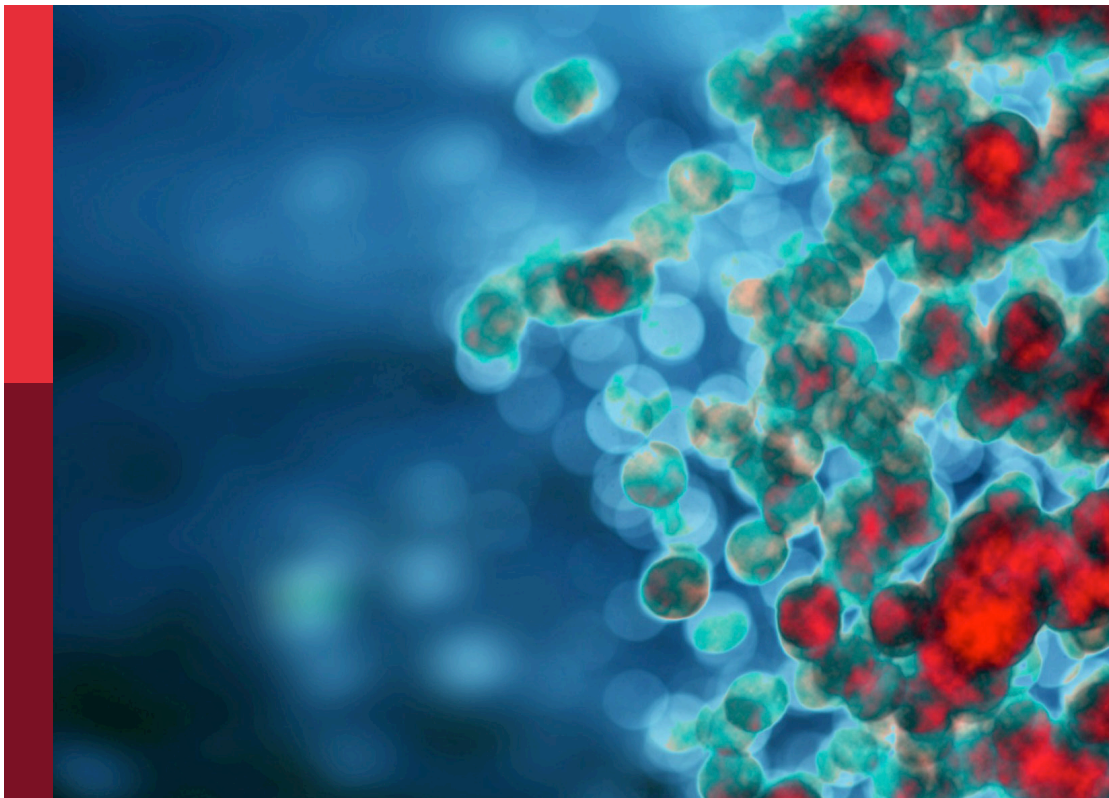
Pemphigus and pemphigoid diseases: In memoriam Detlef Zillikens, 2nd edition

Edited by
Ralf J. Ludwig, Frédéric Caux, Christian David Sadik, Enno Schmidt, Dedee Murrell, Takashi Hashimoto and Aimee S. Payne

Published in
Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-8325-5059-5
DOI 10.3389/978-2-8325-5059-5

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Pemphigus and pemphigoid diseases: In memoriam Detlef Zillikens, 2nd edition

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Citation

Ludwig, R. J., Caux, F., Sadik, C. D., Schmidt, E., Murrell, D., Hashimoto, T., Payne, A. S., eds. (2024). *Pemphigus and pemphigoid diseases: In memoriam Detlef Zillikens, 2nd edition*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-5059-5

Dr. Enno Schmidt receives grant funding from Euroimmun, Incyte, Dompe, Admirx, Synthon/biondis, Bayer, Pharmix, Alpine Immune, AstraZeneca, Sanofi, ArgenX, UCB, Novartis, Biotest, Fresenius Medical Care, and is consulting for Roche, Imevax, Thermo Fisher, Janssen, Topas, Bristol-Myers Squibb, Almirall, and Chugai. The other Topic Editors declare no potential competing interests in relation to the Research Topic theme.

Publisher's note: In this 2nd edition, the following article has been updated: van Beek N, Holtsche MM, Atefi I, Olbrich H, Schmitz MJ, Pruessmann J, Vorobyev A and Schmidt E (2024) State-of-the-art diagnosis of autoimmune blistering diseases. *Front. Immunol*. 15:1363032. doi: 10.3389/fimmu.2024.1363032

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frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 15 May 2024
DOI 10.3389/fimmu.2024.1426834

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OPEN ACCESS

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RECEIVED 02 May 2024
ACCEPTED 06 May 2024
PUBLISHED 15 May 2024

CITATION
Schmidt E, Ludwig RJ, Caux F, Payne AS, Sadik CD, Hashimoto T and Murrell DF (2024) Editorial: Pemphigus and pemphigoid diseases: in memoriam Detlef Zillikens. *Front. Immunol.* 15:1426834. doi: 10.3389/fimmu.2024.1426834

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Editorial: Pemphigus and pemphigoid diseases: in memoriam Detlef Zillikens

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KEYWORDS

autoimmune bullous diseases, pemphigoid, pemphigus, Detlef Zillikens, autoantibody

Editorial on the Research Topic
Pemphigus and pemphigoid diseases: in memoriam Detlef Zillikens

Introduction

This Research Topic is dedicated to Detlef Zillikens, MD, director and chair of the Department of Dermatology at the University of Lübeck, Lübeck, Germany, who died in office in September 2022. This article collection in the field of autoimmune blistering diseases (AIBD) was assembled by his long-standing research collaborator colleagues and friends. He intensively worked on AIBD during his entire career and built his department into one of the world's leading centers for these diseases. With his warm, integrative, open-minded, ever optimistic attitude he was a highly reliable colleague, mentor, and friend to many in the field including each of the editors.

He was a great motivator and an extraordinarily hard-working clinician and researcher who consistently followed his principles and goals. We will never forget his broad knowledge, his passion for research and his patience, his enthusiasm, his empathy, his smiling face and his warm-hearted character. Detlef Zillikens published more than 600 peer-reviewed papers including his most cited review about *Pemphigoid Diseases* in *The Lancet* and the posthumous publication of the identification of laminin β4 as target antigen of anti-p200 pemphigoid, a quest that had occupied him for more than two decades (1, 2).

Several obituaries have already highlighted and valued his scientific work (3–6). Here, within the Research Topic *Autoimmune Blistering Diseases: in memoriam Detlef Zillikens*, the lead article by Hundt et al., details his life and work from different angles and perspectives.

The present Research Topic comprises 36 additional articles on AIBD, many of them referring to and citing the work of Detlef Zillikens. This article collections contains 11 review and opinion articles, 7 case reports and case series, and 18 original research articles.

Review and opinions on AIBD

Rosi-Schumacher et al. summarize data on the prevalence, incidence, age, and gender of two major AIBD, bullous pemphigoid (BP) and pemphigus vulgaris. An overview about the state-of-the-art diagnosis of AIBD is delivered by (van Beek et al.). Here, clinical features, direct immunofluorescence microscopy and the increasing relevance of detection of serum autoantibodies are described. Huttelmaier et al. provide a systematic literature review about comorbidities in BP following the PRISMA criteria. Of the 48 eligible studies the authors included 34 and calculate associations with neurological and psychiatric diseases, metabolic disorders, and malignancies as well as autoimmune and inflammatory diseases. Patzelt and Schmidt summarize the available data about autoimmunity against laminin 332, a structural protein of the basal membrane zone (BMZ). Laminin 332 is, besides BP180 (type XVII collagen), a major target antigen in mucous membrane pemphigoid (MMP). Repeated injection of anti-laminin 332 IgG in C57BL/6 mice induced major clinical and immunopathological features including fibrosis. Oral lesions were subsequently shown to be ameliorated by dapsone, a first line therapy of MMP (7–9). Of note, following the initial observation by Egan et al. (10) several studies detailed in the present review have corroborated the finding that about a quarter of MMP patients with autoantibodies against laminin 332 have a malignancy. Since a standardized sensitive and specific assay for the detection of serum anti-laminin 332 reactivity is widely available, testing for anti-laminin 332 IgG in all MMP patients is recommended by the European S3 guideline on MMP (11–13). Recently, laminin 332 has also been described in orf-induced pemphigoid (14).

Anti-p200 pemphigoid is characterized by autoantibodies against a 200 kDa protein in the extract of human dermis (15). Subsequently, laminin γ1 and very recently, laminin β4 have been identified as target antigens (2, 16). Gao et al. analyzed the distribution of mucosal lesions, that are present in the majority of patients with anti-p200 pemphigoid, compared to anti-laminin 332 and anti-BP180 MMP. Data about the treatment of pemphigus foliaceus with rituximab and/or high-dose intravenous immunoglobulin are systematically analyzed by Carver et al. They found that both biologics resulted in complete remission in about 63% of patients with a higher relapse rate following rituximab administered by the lymphoma protocol (four times 375 mg/m^2) compared to two times 1 g.

The so far described mouse models for pemphigus are described in detail by (Emtenani et al.). Furthermore, data about CD4+ T lymphocytes in AIBD, heat shock proteins 90 and 70 in AIBD and COVID, and Janus kinase inhibitors in AIBD are summarized in this Research Topic. Finally, Dmochowski et al. propose a new nomenclature for AIBD incorporating the molecular identity of the target antigens divided into targets being structural proteins or enzymes; the article also highlights pemphigus vulgaris as occurring adjacent to body orifices. The latter were proposed to include atypical immune privileged site as 'orifices', including the hair follicles of the scalp, the nipples, and sweat glands in the axillae, genitals, and palms.

Case reports and case series on AIBD

In a case series of three patients, Foerster al. present the clinical and immunological findings in BP with concomitant HIV-1 infection and give an overview about similar cases in the literature. Three unusual cases of BP in young age, i.e. in a three-month-old boy as well as in association with COVID-19 vaccination in a 43-year old woman and with metastatic colon cancer in a 25-year-old male were described by Rechtien et al.. Corbella-Bagot et al. report on seven BP patients with localized diseases and local triggers such as burns, surgery, rosacea, edema, and paresthesia. A series of 15 BP patients with mucosal involvement not predominating skin lesions and without ocular disease are presented by (Janela et al.). All patients revealed serum IgG against BP180 NC16A with most of them also reacting with C-terminal parts of BP180. Li et al. describe a 55-year-old Chinese woman with multiple myeloma that developed pruritic flaccid blisters, erosions, and pustules on the skin without mucosal involvement. IgA deposits along the dermal-epidermal junction and in an intercellular pattern in the epidermis were seen by direct immunofluorescence of a perilesional biopsy. Unfortunately, the target antigen could not be identified. Furthermore, a case of MMP with multiple autoantibody specificities is reported by Liu et al., and a series of 12 patients with lichen planus pemphigoides with predominant mucosal involvement by Combemale et al.

Original research on AIBD

In this Research Topic, eighteen original publications are included covering epidemiology, diagnosis, pathophysiology, and treatment of AIBD.

Epidemiology and clinical phenotype

While Sollfrank et al. report on a retrospective single-center analysis of AIBD in Middle Franconia, Germany, Kridin et al. identified more than 30 diseases associated with epidermolysis bullosa acquisita based on routine data fueled in the TriNetX database including more than 100 million patients, among them 1,344 patients with latter AIBD. The epidermolysis bullosa acquisita patients showed an increased risk for metabolic and cardiovascular disease, and thrombosis, indicating that these patients should be rigorously investigated and treated to prevent such complications. Lupus erythematosus and lichen planus were the most frequent comorbidities in this entity. Jakowska et al., analyzed involvement of palms and soles that was found in 21 of 462 AIBD patients from Poland, finding no difference in the incidence of these sites between the pemphigus and pemphigoid group. Jukić et al. report HLA class II haplotypes associated in 30 pemphigus vulgaris patients from Croatia compared to 190 controls and showed HLA-DRB1*04:02 not only being highly prevalent in pemphigus vulgaris but also linked to patients with high serum levels of anti-desmoglein 3 IgG.

Diagnosis

Schauer et al. compared sensitivity and specificity of indirect immunofluorescence on monkey liver to monkey esophagus in the

detection of anti-endomysium IgA employing sera from 16 patients with dermatitis herpetiformis, 67 with coeliac disease, and 20 healthy controls. While sensitivities were comparable in dermatitis herpetiformis, specificity was surprisingly low with monkey esophagus (75%) compared to monkey liver (92%). Höcke et al. reported on a computer-aided classification of indirect immunofluorescence microscopy on primate esophagus and salt-split skin. The established deep network allows the automated recognition of reactivity of AIBD sera on these two substrates differentiating between anti-BMZ and intercellular epithelial binding on esophagus and epidermal and dermal binding on salt-split skin using miniature biochips as previously established for the routine diagnosis of AIBD with manual reading (17–19). This semi-automated algorithm will further facilitate interpretation of indirect immunofluorescence microscopy on these two substrates pivotal for AIBD diagnosis and will also open an avenue for high throughput analyses.

Pathophysiology

Schmitt et al. provided an update on the role of pathogenic epitopes within Dsg3 in pemphigus vulgaris using two epitope-specific monoclonal antibodies directed against Dsg3- EC domains EC-1 and EC-5. The data showed that the antibody against EC-5 was less effective in causing loss of cell adhesion, compared to AK23 against EC-1. Both autoantibodies had similar effects on keratin retraction and reduction of desmosome number but only AK23 induced Dsg3 depletion. Moreover, both antibodies induced phosphorylation of p38MAPK and Akt, whereas Src was phosphorylated upon treatment with AK23 only. All pathogenic effects were rescued by p38MAPK inhibition and AK23-mediated effects were also ameliorated by Src inhibition. These data suggest that specific p38MAP kinase inhibitors might be helpful in pemphigus, as was previously proposed. Koga et al. reported 22 patients with BP in whom IL-9 levels were found to be elevated in serum and skin, whereas they were not in patients with epidermolysis bullosa acquisita and normal controls. Four of the patients had serum IL-8 levels measured every 2 weeks; as their BP improved, so did the IL-9 levels, suggesting that IL-9 may play a pathophysiological role. Schinner et al. studied the expression of two transcription factors, T-bet and GATA-3, in skin biopsies from patients with lichen planus, BP, pemphigus vulgaris, and pemphigus foliaceus. They found more GATA-3 in the blistering diseases than lichen planus, whereas all 3 diseases had similar IL-17 signatures. An inhibitor of Fas Ligand, PC111, was tested by Lotti et al. in two human skin organ culture models and in the keratinocyte dissociation assay, showing that this inhibitor could reduce the severity of experimental pemphigus by about 50% and significantly more than normal saline.

Two groups employed the antibody transfer mouse model of epidermolysis bullosa acquisita (20). Akbarzadeh et al. showed that CCR2-deficient mice were significantly less diseased compared to wild type animals and that this effect was not mediated by monocytes, a prominent CCR2-expressing cell type. Seiler et al. demonstrated that mice with neutrophils deficient of C5aR2 were significantly protected confirming a previous study using mice with global deficiency of C5aR2 (21). In an *ex vivo* model of this disease, where incubation of cryosections of normal human skin with anti-type VII collagen IgG and subsequently, with leukocytes from healthy volunteer, induces dermal-epidermal separation (22), Yang et al. screened a natural product library of 800 compounds. Two of them, luteolin peracetate and gossypolone inhibited dermal-epidermal separation in this model.

Finally, Liu et al. investigated the gut microbiome in 66 BP patients compared with the same number of age-, sex-, and study center-matched controls with non-inflammatory skin diseases. They found an overall altered gut microbial community in the BP patients with decreased alpha diversity and reduced *Faecalibacterium prausnitzii* and a greater abundance of pathways related to GABA metabolism. While *F. prausnitzii* is a known microbiomarker for inflammatory diseases, GABA is involved e.g. in inhibition of itch. This study is complementing a multicenter prospective study on the skin microbiome of 228 BP and 190 control patients (23).

Treatment

Oren-Shabatai et al. reported the use of rituximab, omalizumab and dupilumab in 9 cases of recalcitrant BP. Rituximab was variably effective in 5/8 patients to get them to a lower dose of corticosteroids. None of these treatments proved very useful in recalcitrant BP but these treatments ought to be assessed in appropriately randomized new onset BP patients. The effect of dupilumab in BP was also investigated by (Yan et al.). In this multicenter retrospective cohort study, 20 BP patients received dupilumab with or without systemic corticosteroids, while 20 BP patients were treated with systemic corticosteroids alone. No differences in the clinical response was noted after 4 and 24 weeks between the dupilumab-treated and the dupilumab-naïve patients. Moreover, levels of serum anti-BP180 IgG did not significantly differ between these two groups, whereas the cumulative corticosteroid dose was significantly lower in the dupilumab arm. When comparing retrospectively the early initiation of corticosteroid-sparing drugs with their late use (after 28 days) in more than 250 BP patients, Fenne et al. did found a lower relapse rate as well as a reduced initial dose of corticosteroids and admission time in the patients with earlier initiation of corticosteroid-sparing medication.

In conclusion, with this Research Topic we would like to honor our mentor and friend, Detlef Zillikens, and will always remember him as kind, integrative, and highly knowledgeable hard-working clinician and researcher who not only left heavy footprints in the field of AIBD but also in our minds and souls.

Author contributions

ES: Conceptualization, Writing – original draft, Writing – review & editing. RJL: Writing – review & editing. FC: Writing – review & editing. ASP: Writing – review & editing. CDS: Writing – review & editing. TH: Writing – review & editing. DFM: Writing – original draft, Writing – review & editing.

Conflict of interest

ES has a scientific cooperation and holds patents with Euroimmun. ASP has equity and equity with Cabaletta and

received honoraria and research funding from this company. DFM received honoraria from Sanofi, Pfizer, and Genenteck/Roche.

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 15 December 2022
DOI 10.3389/fimmu.2022.1080786

Check for updates

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 26 October 2022
ACCEPTED 02 December 2022
PUBLISHED 15 December 2022

CITATION
Kasperkiewicz M and Tukaj S (2022) Targeting heat shock proteins 90 and 70: A promising remedy for both autoimmune bullous diseases and COVID-19. *Front. Immunol.* 13:1080786. doi: 10.3389/fimmu.2022.1080786

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Targeting heat shock proteins 90 and 70: A promising remedy for both autoimmune bullous diseases and COVID-19

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Heat shock proteins (Hsps), including Hsp90 and Hsp70, are intra- and extracellular molecules implicated in cellular homeostasis and immune processes and are induced by cell stress such as inflammation and infection. Autoimmune bullous disorders (AIBDs) and COVID-19 represent potentially life-threatening inflammatory and infectious diseases, respectively. A significant portion of AIBDs remain refractory to currently available immunosuppressive therapies, which may represent a risk factor for COVID-19, and suffer from treatment side-effects. Despite advances in vaccination, there is still a need to develop new therapeutic approaches targeting SARS-CoV-2, especially considering vaccine hesitancy, logistical distribution challenges, and breakthrough infections. In this mini review, we briefly summarize the role of targeting Hsp90/70 as a promising double-edged sword in the therapy of AIBDs and COVID-19.

KEYWORDS

autoimmune bullous disorders (AIBDs), heat shock proteins (HSPs), Hsp90, Hsp70, COVID-19, SARS-CoV-2

Introduction

The expression of heat shock proteins (Hsps), including Hsp90 and Hsp70, can be up-regulated by various stress factors including inflammation and infection. As chaperones, they classically interact with protein substrates and (co-)chaperones to maintain cellular homeostasis by participating in correct protein folding or stability, as well as cell differentiation, survival, and death. In addition, both intra- and extracellular Hsps have an integral role in inflammatory responses through active involvement in a wide range of immune processes (1).

Autoimmune bullous diseases (AIBDs), comprising pemphigus and pemphigoid, are potentially life-threatening blistering disorders of the skin and/or mucosa mediated by

autoantibodies against desmosomal and hemidesmosomal structures, respectively. Patients usually require long-term potent immunosuppressive treatments including systemic corticosteroids and adjuvant immunomodulators. A significant portion of cases remain refractory to currently available therapies and suffer from treatment side-effects (2, 3).

COVID-19 is a disease caused by the novel coronavirus SARS-CoV-2 which has led to a global devastating pandemic since March 2020. The clinical presentation ranges from mild or even asymptomatic courses to critical symptoms such as respiratory failure and multi-organ dysfunction. Despite advances in vaccination, there is still a need to develop new therapeutic approaches targeting SARS-CoV-2, especially considering vaccine hesitancy, logistical distribution challenges, and breakthrough infections (4).

Patients with AIBDs have faced considerable challenges during the COVID-19 outbreak, taking into account that their immunosuppressive treatments, particularly the B-cell depleting agent rituximab (a first-line drug approved for pemphigus), may predispose them to more severe COVID-19 and compromise vaccine immunogenicity (5, 6). In addition, there have been some reports on SARS-CoV-2 vaccine-induced/exacerbated AIBD cases (7). Therefore, an ideal treatment during this pandemic would be a new medication that covers both AIBDs and COVID-19.

In this mini review, which is dedicated to the memory of our honourable mentor Professor Detlef Zillikens, we briefly summarize the role of targeting Hsp90/70 as a promising double-edged sword in the therapy of AIBDs and COVID-19.

Hsp90/70 and AIBDs

Several lines of evidence suggest that Hsp90 is involved as a notable pathophysiological factor in AIBDs including epidermolysis bullosa acquisita (EBA) and bullous pemphigoid. The anti-inflammatory clinical and molecular effects of anti-Hsp90 treatment were mostly demonstrated in several EBA studies using corresponding *in vivo* mouse models, *ex vivo* human dermal-epidermal separation cryosection experiments, and *in vitro* rodent and human cell culture assays (8, 9). Hsp90 inhibitors, including 17-DMAG, 17-AAG, and TCBL-145, exhibited activity by potently affecting inflammatory disease pathways (e.g., suppression of effector T-cells, B-cells, and neutrophils; down-regulation of NF-κB activity; blunting of autoantibody, pro-inflammatory cytokine, and reactive oxygen species production; inhibition of matrix metalloproteinases; promotion of regulatory B cells) (10–13). With regards to bullous pemphigoid, human skin biopsy analyses as well as human cell culture and serology assays revealed that Hsp90 is aberrantly expressed and secreted in these patients and that its blockade modulates autoantibody-induced IL-8 production by cultured keratinocytes (14, 15).

Compared to Hsp90, the role of Hsp70 in AIBDs has been overall less studied (9). Two recent studies indicated that both extracellular Hsp70 and autoantibodies to Hsp70 display pro-inflammatory activities in the context of EBA development (16, 17). In the first study, the *in vivo* pathophysiological relevance of extracellular Hsp70 was demonstrated in mice with experimental EBA in which elevated blood levels of this chaperone were recorded. Hsp70-treated animals had a more pronounced clinical disease severity compared to controls which was paralleled by increased levels of cutaneous matrix metalloproteinase 9 and plasma hydrogen peroxide, with the latter observation being confirmed in an independent EBA-specific reactive oxygen species release assay. In addition, cell culture experiments using human naive peripheral blood mononuclear cells revealed that extracellular Hsp70 stimulated the secretion of the T-cell-derived pro-inflammatory cytokines IL-6 and IL-8 (16). In the second study, it was demonstrated that autoantibodies to Hsp70 may contribute to EBA development *via* enhanced neutrophil infiltration to the skin and activation of the NF-κB signalling pathway in an IFN-γ-associated manner (17).

Hsp90/70 and COVID-19

To initiate infection, SARS-CoV-2 enters the human host cell *via* binding of its spike protein to cell surface receptors including angiotensin-converting enzyme 2, followed by endocytosis or fusion with the surface membrane, viral gene transcription, translation, and replication (4). Of note, SARS-CoV-2 is dependent on host molecular chaperones, mainly Hsp90/70, to accomplish these entries and/or replication steps (18, 19).

Hsp90 has been shown to be over-expressed in the damaged lungs of COVID-19 subjects (20), and Hsp90 inhibitors (AT13387 and AUY-922) were able to prevent and repair SARS-CoV-2 spike protein-induced pulmonary microvascular endothelial dysfunction (21). Wyler et al. demonstrated that inhibition of Hsp90 activity by onalespib, ganetespib, or 17-AAG resulted in a reduction of both SARS-CoV-2 replication and expression of pro-inflammatory cytokines in primary human airway epithelial cells (22). Similar results were obtained by Goswami et al. using the Hsp90 inhibitor SNX-5422 (23). In addition, *in vitro* experiments revealed inhibition of SARS-CoV-2 replication by the Hsp90 inhibitors 17-AAG and tanespimycin, respectively (24, 25). Lowering of SARS-CoV-2 propagation by pharmacological Hsp90 inhibition was confirmed by a computational study of patient RNA sequencing data (26).

Two members of the Hsp70 family, HSPA1L and GRP78, have been implicated in the modulation of SARS-CoV-2 (27–30). It has been suggested that SARS-CoV-2-infected cells epigenetically up-regulate HSPA1L, leading to the over-production of Hsp70 to facilitate SARS-CoV-2 replication in host cells (27). In addition, computer modelling predictions and experimental studies have shown that GRP78 assists in the host cell recognition of SARS-CoV-2 spikes and viral entry (28–30).

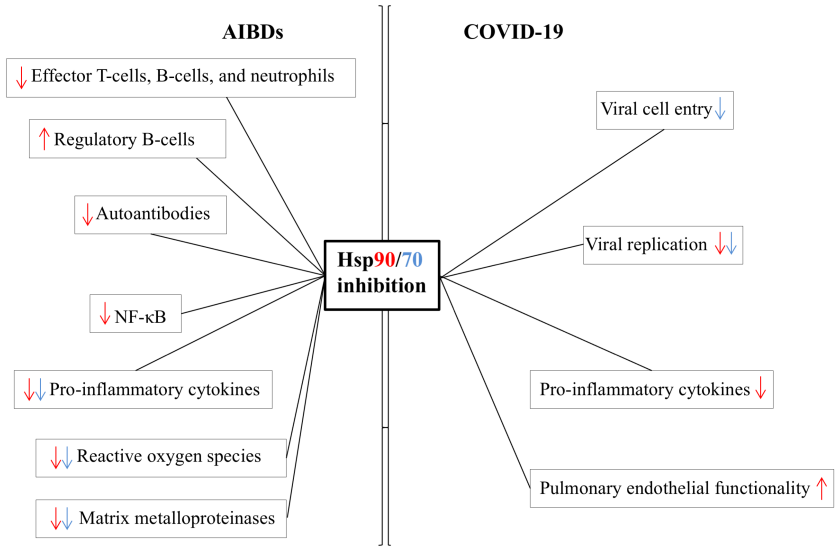

FIGURE 1
Proposed mode of action of targeting Hsp90/70 in AIBDs and COVID-19 based on current scientific evidence. AIBD data are derived from studies on epidermolysis bullosa acquisita and bullous pemphigoid. Although Hsp70 has been involved in the depicted pathophysiological factors/processes of AIBDs and COVID-19, data on the effects of true Hsp70 inhibition are limited to experiments on SARS-CoV-2 cell entry so far and remain speculative otherwise. The red and blue colours of arrows correspond to Hsp90 and Hsp70, respectively.

Role of molecular mimicry

A link between COVID-19 and the development of autoimmunity has been suggested, which is based on the assumption that molecular mimicry exists between immunogenic proteins of SARS-CoV-2 and human molecules including Hsps and autoantigens of AIBDs (31). This hypothesis has been recently disproven by two studies showing that autoantibodies to Hsp90/70/60 are not altered in anti-SARS-CoV-2 IgG-seropositive humans and that these circulating anti-SARS-CoV-2 antibodies do not cross-react with pemphigus or pemphigoid autoantigens (32, 33).

Conclusions

Hsps90/70 are involved in AIBDs and COVID-19 in many different ways and, thus, can be important therapeutic targets for both conditions, an ideal scenario during this pandemic (Figure 1). However, several questions remain open. Independent reports on the role of Hsps90/70 in either AIBDs or COVID-19 do not ultimately imply a direct correlation between these stress proteins and both diseases, which is hampered by the fact that all information is limited to preclinical studies so far (9, 18, 19). In addition, the family of Hsps is large, and the interaction between each other and their client proteins is complex (1). Thus, targeting one of the Hsps may lead to a ripple effect. As an example, inhibition of Hsp90 classically results in the activation of heat shock factor 1 and, consecutively, the over-expression of intracellular Hsp70. In contrast to extracellular Hsp70, intracellular Hsp70 has mostly anti-inflammatory properties by blocking NF-κB activation, a desirable effect for both AIBDs and COVID-19 (i.e., cytokine storm) (34). On the other hand, over-production of intracellular Hsp70 could potentially facilitate SARS-CoV-2 replication. Therefore, further exploration of the net effects of especially clinically available Hsp90 blockers in basic studies and clinical trials is needed.

Author contributions

MK drafted and ST edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Polish National Science Centre (NCN), grant no. 2020/39/B/NZ6/00357.

Conflict of interest

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TYPE Conceptual Analysis
PUBLISHED 19 December 2022
DOI 10.3389/fimmu.2022.1103375

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 20 November 2022
ACCEPTED 05 December 2022
PUBLISHED 19 December 2022

CITATION
Dmochowski M, Jałowska M and Bowszyc-Dmochowska M (2022) Issues occupying our minds: Nomenclature of autoimmune blistering diseases requires updating, pemphigus vulgaris propensity to affect areas adjacent to natural body orifices unifies seemingly diverse clinical features of this disease. *Front. Immunol.* 13:1103375. doi: 10.3389/fimmu.2022.1103375

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Issues occupying our minds: Nomenclature of autoimmune blistering diseases requires updating, pemphigus vulgaris propensity to affect areas adjacent to natural body orifices unifies seemingly diverse clinical features of this disease

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In this conceptual analysis, we present our concepts on two issues regarding autoimmune bullous diseases (AIBD), namely (i) current nomenclature of AIBD requires updating by incorporating molecular data and (ii) pemphigus vulgaris (PV) "likes" areas adjacent to natural body orifices. The problem of inadequacy of the currently used nomenclature was noticed recently by Zillikens, who proposed to form a group with the task of updating it. The early efforts by Dmochowski to update this nomenclature happened to be a daunting task. Nevertheless, the ideal nomenclature should retain the bulk of clinical data, which generations of dermatologists are accustomed to, including triggers if known, and incorporate molecular data revealing targets of autoimmune response and immunoglobulin isotypes involved. The natural body orifices affected by PV were previously described in numerous publications. However, these openings are described separately in these publications. Here, Dmochowski comes up with an intellectual concept that this propensity of PV unifies seemingly diverse clinical features of this disease.

KEYWORDS

autoimmune bullous diseases, nomenclature, pemphigus vulgaris, natural body, orifices

10.3389/fimmu.2022.1103375

Introduction

We participated in two meetings organized by the people from the Department of Dermatology, University of Lübeck led by Prof. Detlef Zillikens, namely International Pre IID 2013 Meeting on Autoimmune Bullous Diseases (MD) and Scientific Conference of International Pemphigus & Pemphigoid Foundation in 2017 (MD, MBD). We were impressed by the quality of experimental research conducted in this department and the extraordinary hospitality we enjoyed there. Saddened deeply by the news that Prof. Detlef Zillikens, one of the giants of modern dermatology, passed away prematurely, we decided to present two issues we are preoccupied with for years. The ideal nomenclature of autoimmune bullous diseases should retain the bulk of clinical data, which generations of dermatologists are accustomed to, including triggers if known, and incorporate molecular data revealing targets of autoimmune response. The intellectual concept conceived by Dmochowski which he now promotes is that propensity of pemphigus vulgaris (PV) to affect natural body orifices unifies seemingly diverse clinical features of this disease.

Current nomenclature of autoimmune blistering dermatoses requires updating by incorporating molecular data

It was Prof. Zillikens initiative communicated on September 30, 2021 to organize a working group within the activities of EADV task force on AIBD on the novel nomenclature of AIBD. He thought that the updated nomenclature should incorporate molecular data on the pathogenesis of those disorders. Dmochowski eagerly responded as he proposed his nomenclature of AIBD along this idea as early as 2003 (1). Unfortunately, this was published in the Polish language journal that was at that time unindexed by MEDLINE and thus went unnoticed by the wider dermatology community. Its newest version showing novel data (2) is shown in Figure 1. Hopefully, it

I. AIBD with autoimmunity to structural, predominantly, proteins (non-enzymes):

- **Desmosomal**

- **IgG pemphigus**
  1. **Pemphigus foliaceus circle (PF) (PF, endemic, seborrhoic, herpetiformis, paraneoplastic, drug-mediated)**
  2. **Pemphigus vulgaris circle (PV) (mucosal-dominant PV, mucocutaneous PV, vegetans, herpetiformis, paraneoplastic, drug-mediated)**
  3. **Pemphigus coexistent with other autoimmune (MAS, APS) and non-autoimmune diseases without malignancy present**
  4. **Transition from PV circle into PF circle, transition from PF circle into PV circle, transition within a circle**
  5. **Coexistence of PV circle with PF circle**
  6. **Paraneoplastic pemphigus without autoantibodies to DSG1 and/or DSG3**
- **IgA pemphigus (subcorneal pustular dermatosis type and intraepidermal neutrophilic type)**
- **IgG/IgA pemphigus (intercellular IgG/IgA dermatosis)**

- **Dermal-epidermal junction**

- **Bullous pemphigoid circle (BP) (numerous clinical BP proper presentations: 13 presentations-8,191; 15 presentations-32,767 mathematical combinations), BP230 BP, lichen planus pemphigoides, pemphigoid gestationis, linear IgA bullous dermatosis in adults or children /LABD/ lamina lucida type, immunoglobulin M pemphigoid)**
- **Mucous membrane pemphigoid, Brunsting-Perry pemphigoid**
- **Epidermolysis bullosa acquisita circle (EBA) (EBA, bullous systemic lupus erythematosus, LABD sublamina densa type)**
- **LABD without molecular links with either BP or EBA**
- **Anti - p200 pemphigoid (anti-laminin gamma1 pemphigoid)**

II. AIBD with autoimmunity to enzymes:

- **Dermatitis herpetiformis (including Cottini variant)**

III. Dermatoses outside points I, II and any coexistences of dermatoses from points I, II.

FIGURE 1
Current version of nomenclature of AIBD based on clinical-molecular features according to M. Dmochowski.

can serve as a contribution to considering the issue further. In total, just nine people responded from Europe, Israel and Singapore. The only online meeting took place on January 25, 2022 when group members were able to voice their opinions on the draft of new nomenclature put forward by Prof. Zillikens. For example, the nomenclature like pemphigus (IgG anti-DSG3) and further along that line was proposed in the draft. Still, in our opinion, there were important caveats in that draft.

Recently we followed-up a female middle-aged patient (Figure 2) with a mucocutaneous disease showing stubborn desquamative gingivitis and widespread itchy urticarial and bullous cutaneous lesions that plausibly were triggered by the treatment with nivolumab, an immune checkpoint inhibitor, for melanoma (3). The oral lesions appeared first on nivolumab treatment, whereas cutaneous lesions followed soon after the third dose of mRNA vaccine for COVID-19. The multiplex ELISA revealed grossly elevated level of anti-BP180 NC16A antibodies whereas IgG antibodies to laminin 332 were not detected with mosaic indirect immunofluorescence. The problem of nomenclature in this case is that the stubborn desquamative gingivitis would suggest the diagnosis of mucous membrane pemphigoid (MMP), whereas widespread cutaneous lesions would suggest bullous pemphigoid (BP). The rational diagnosis in this case would be, along the ideas put forward by Prof. Zillikens, mucous membrane pemphigoid/bullous pemphigoid (IgG anti-BP180 NC16A) abbreviated conveniently to MMP/BP (IgG anti-BP180 NC16A). Obviously, such cases evade the nomenclature of the nosological system currently used. Therefore, our opinion is that the ideal nomenclature should retain the clinical data, including triggers if known, combined with molecular data revealing targets of autoimmune response (4) and immunoglobulin isotypes involved. The nosology of AIBD should be both succinct and precise, and abbreviations/acronyms should be both catchy and user-friendly. These postulates seem to be trivial, but they should not be ignored. It should be noted in this respect that certain disease names coined "ages ago", like epidermolysis bullosa acquisita (EBA) or dermatitis herpetiformis (DH), are controversial now (5). The term EBA might wrongly suggest that epidermal lysis is taking place in this disease, whereas the term DH might imply a role of infection with herpes viruses in the pathogenesis of DH, which has never been proven to exist. Perhaps then, the term "gluten-sensitive IgA/neutrophil-mediated dermatitis" (the acronym GAND would sound nicely) suggested by us for the dermatosis described by Louis Adolphus Duhring would be more understandable for non-dermatologic medical/pharmaceutical/biotechnological community and lay public alike (6). The more recent term "paraneoplastic pemphigus" is also misleading as malignancy can coexist, albeit infrequently, with any AIBD with autoimmunity to predominantly structural proteins, regardless it has the word "paraneoplastic" in its name or not (7). The term "pemphigoid gestationis" which replaced the archaic term "herpes gestationis" unfortunately is also a misnomer, because this condition can manifest itself not only in pregnancy, but also in the postpartum period.

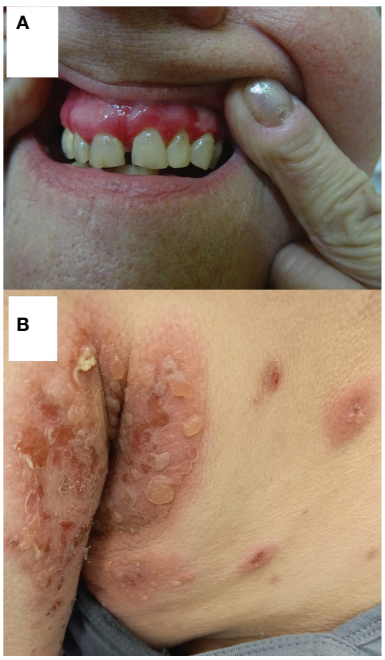

FIGURE 2
A middle-aged female with oral and cutaneous lesions plausibly related to nivolumab therapy for melanoma and mRNA vaccination for COVID-19 who could be diagnosed as mucous membrane pemphigoid/bullous pemphigoid (IgG anti-BP180 NC16A). Stubborn desquamative gingivitis **(A)**. Itchy urticarial and blistering lesions on armpit skin **(B)**. Reprinted/adapted with permission, Dmochowski M. Autoimmune bullous diseases associated with malignancy. 31st EADV Congress: Designing the Future of Dermatology and Venereology. Italy: Milan (2022) (3).

In the future, such a nomenclature incorporating molecular data should be particularly useful in the process of selecting AIBD patients for personalized therapies aiming at correcting exclusively patient-specific autoimmune responses.

Pemphigus vulgaris "likes" areas adjacent to natural body orifices: An intellectual concept unifying its seemingly diverse clinical features

Decades ago, at times when immunological methods of diagnosing pemphigus diseases were not as readily available as there are nowadays, the patients might have been referred to university dermatologists while on later stages of pemphigus, namely full-blown disease. Patients might have had spectacular lesions covering large parts of the body at the time of the referral, thus lacking initial presentations around natural body orifices. Therefore the propensity of PV lesions at early stages of the disease to natural body orifices might have gone unnoticed by dermatologists of that time. The initial observations on that

topic presenting monocentric clinical-laboratory experience were published by Dmochowski in 2007 in a Polish language journal again not covered by MEDLINE (8). Since then a number of papers and conference presentations containing relevant clinical-laboratory iconography that expand on that idea were prepared by Dmochowski et al. (9–15).

We think now that the areas around the natural body orifices (Figure 3) should be considered predilection sites/sites of privilege for PV. Moreover, most importantly, Dmochowski realized that the tendency of PV lesions to affect those sites should be considered a feature unifying diverse clinical presentations of PV. These sites are the scalp, the area around the medial canthus, the concha of the auricle and the external auditory canal, the anterior nostrils, lips, the nipple of both female and male breast, the umbilicus, sacral/pilonidal/spinal dimples, anus, and external female and male genitalia, as well as the periungual areas of fingernails and toenails in any combinations. PV lesions can even affect the palmar surface of hands, where the openings of acrosyringia of eccrine sweat gland are abundant, manifesting themselves as cheiropompholyx (17). The patient with severe mucocutaneous PV presenting also cheiropompholyx followed-up by us had no H+E examination of palmar lesions done that would reveal acantholysis there. Nevertheless, those lesions cleared up during immunosuppressive therapy suitable for treating PV and never appeared again. There are data that DSG1 is expressed in the eccrine glands, therefore it may be an explanation for this clinical feature (18). Obviously, the issue of cheiropompholyx in relation to the severity of PV requires further proof and patients' cohorts, ideally coming from diverse ethnicities, should be studied before arriving at the conclusion. Our opinion is that periorificial lesions should be a clinical hint for the clinicians of various specialties that they are dealing with a disease that requires imaging and biochemical-molecular immunopathological diagnostics and further patient management appropriate for the diagnosis of PV. The key question why PV "likes" the vicinity of natural body orifices requires an experimental explanation. The speculative explanations might be ideas that mechanosensing phenomena altering the expression and function of the desmosomal components (19) preferentially take place in such areas exposed to the intensive friction or that non-canonical functions of antibodies (20) more robustly manifest themselves in those sites. Mechanosensing describes the ability of a cell to sense mechanical cues of its microenvironment, including not only all components of force, stress, and strain but also substrate rigidity, topology, and adhesiveness (21). Therefore, mechanosensing may be relevant in the context the loss of adhesion characterizing PV, particularly in conjunction with the data that desmosomes are not merely structural proteins but also mediate keratinocyte transmembrane signalling (22). Non-canonical functions of antibodies such as antibody-mediated catalysis, receptor agonist activity of antibodies, antibodies acting as carriers and chaperones, and above all cell signalling modulated by antibodies (23) might be particularly relevant in PV at natural body orifices in relation to a periorificial microbiome. To sum up, we suspect that the microenvironment at any periorificial site facilitates the development of PV lesions.

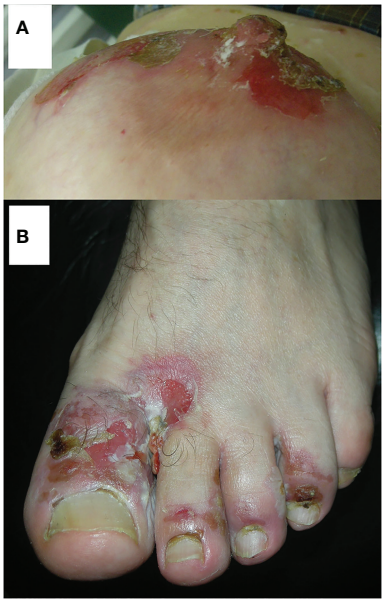

FIGURE 3
Representative PV patients having pemphigus lesions in less obvious locations adjacent to natural body orifices. A middle-aged female with cutaneous variety of PV having pemphigus lesions around the nipple of the breast **(A)**. Reprinted/adapted with permission, Dmochowski M. Pęcherzyca zwykła „lubi" okolice naturalnych otworów ciała/Pemphigus vulgaris "likes" areas around natural body orifices. X konferencja naukowo-szkoleniowa stowarzyszenia dermatologów wojska polskiego skóra 2022. Poland: Gdańsk (2022) (14). A middle-aged male with mucocutaneous variety of PV having pemphigus lesions on proximal nail folds of the toenails **(B)**. Reprinted/adapted with permission, Bowszyc-Dmochowska M, Pietkiewicz P, Gornowicz-Porowska J, Bartkiewicz P, Dmochowski M. Involvement of nail apparatus in pemphigus vulgaris in ethnic Slavs. 1st Congress of Trichoscopy. Symposium "All about hair & nails". Poland: Warsaw (2018) (16).

Author contributions

MD contributed to the conception and design of the article. MD wrote the first draft of the manuscript. MJ, MD, MB-D contributed to the writing—review, and editing. The formal analysis was done by MJ, MB-D. All authors contributed to the article and approved the submitted version.

Conflict of interest

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 25 January 2023
DOI 10.3389/fimmu.2023.1118846

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 08 December 2022
ACCEPTED 11 January 2023
PUBLISHED 25 January 2023

CITATION
Gao Y, Qian H, Hashimoto T and Li X (2023) Potential contribution of anti-p200 autoantibodies to mucosal lesions in anti-p200 pemphigoid.
Front. Immunol. 14:1118846.
doi: 10.3389/fimmu.2023.1118846

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Potential contribution of anti-p200 autoantibodies to mucosal lesions in anti-p200 pemphigoid

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Anti-p200 pemphigoid is a relatively rare subepidermal autoimmune bullous disease (AIBD), which was firstly reported by Detlef Zillikens, Takashi Hashimoto and others in 1996. Skin lesions are considered as the major clinical features of this disease, with occasional involvement of mucosal lesions. The mechanism of mucosal lesions involved in anti-p200 pemphigoid is still unclear. In the present study, we aimed to analyze published data on cases and case series of anti-p200 pemphigoid with mucosal lesions and explored the potential contribution of anti-p200 autoantibodies to mucosal lesions. A total of 32 papers that comprised 52 anti-p200 pemphigoid patients with various mucosal lesions were included in this review. Oral lesions were involved in 75.0% patients, followed by genital lesions (26.9%) and ocular lesions (11.54%). Only one patient had psoriasis, 26.9% patients had multiple mucosal lesions, and 30.8% cases had comorbidity of other AIBDs, particularly anti-laminin (LM) 332-type mucous membrane pemphigoid (MMP). In comparison with anti-LM332-type MMP, anti-BP180-type MMP and epidermolysis bullosa acquisita, higher frequency of genital lesions was identified as a unique character of anti-p200 pemphigoid with mucosal lesions. These results indicated that anti-p200 autoantibodies might contribute to mucosal lesions in a pattern different from other MMP-related autoantibodies, although its pathogenetic mechanisms are still unclear.

KEYWORDS

anti-p200 mucous membrane pemphigoid, anti-p200 pemphigoid, mucosal lesions, oral mucosa, genital mucosa

Introduction

Subepidermal autoimmune bullous diseases (AIBDs) are a group of rare autoimmune skin diseases characterized by autoantibodies against epidermal basement membrane zone (BMZ) proteins, which include bullous pemphigoid (BP), mucous membrane pemphigoid (MMP), anti-p200 pemphigoid, epidermolysis bullosa acquisita (EBA) and others (1). The

anti-BMZ autoantibodies disrupt the epidermal cell adhesive function and anchoring mechanism within epidermal-dermal junction, leading to the separation of skin and/or mucous membranes and the formation of blisters and/or erosions.

Anti-p200 pemphigoid was first reported in 1996 in two papers by Detlef Zillikens, Hashimoto and others and by Chen, Hashimoto and others, as a novel AIBD disease entity with IgG autoantibodies against a 200-kDa dermal protein (p200) (2, 3). Then, Kawahara, Detlef Zillikens, Hashimoto and others showed that p200 is localized in the lower lamina lucida of the epidermal BMZ (4). In 2009, Dainichi, Hashimoto and others reported that p200 is possibly LMγ1 (5, 6), although there might be other candidate molecules as p200.

Anti-p200 pemphigoid presents mainly skin lesions and occasionally mucosal lesions. The clinical presentation of anti-p200 pemphigoid is polymorphic, and may mimic to those of other subepidermal AIBDs, such as BP and EBA (7). A number of studies reported that anti-p200 pemphigoid is often associated with psoriasis (8). Histopathologically, lesional skin biopsy specimen shows subepidermal blister with prominent inflammatory infiltrations of neutrophils, followed by lymphocytes and eosinophils (4).

Diagnosis of anti-p200 pemphigoid is based on clinical, histopathological and immunological findings, particularly the presence of anti-p200 autoantibodies. In anti-p200 pemphigoid, direct immunofluorescence (DIF) using patient perilesional skin/mucosa usually shows linear deposits of IgG and C3 to the BMZ (9–13). Indirect immunofluorescence (IIF) using normal human skin detects circulating IgG antibodies to the BMZ (14–17), which reacts with dermal side of 1M NaCl-split normal human skin (ssIIF) (14, 18–21). Immunoblotting (IB) using normal human dermal extract detects IgG reactivity with p200 (14, 22–25).

In some cases of anti-p200 pemphigoid, additional autoantibodies to LM332, BP180, type VII collagen and BP230 were also detected (10, 22, 26–28).

Although autoantibodies in anti-p200 pemphigoid sera are considered to be pathogenic, their pathogenicity is not yet fully elucidated (29, 30).

MMP is a rare and chronic mucosal-dominant subepidermal AIBD. Known autoantigens of MMP include BP180, BP230, LM332, integrin α6β4 and type VII collagen (31). Recently, our group presented a unique AIBD case with only oral mucosal lesion, which was positive for p200 but negative for all known MMP-related autoantigens. We considered that the anti-p200 autoantibodies might cause the mucosal lesions, and suggested the new MMP subtype, anti-p200 MMP (14).

In the present study, we summarized the clinical, histopathological and immunological features of anti-p200 pemphigoid presented with mucosal lesions published either as sporadic case reports or in case series, for the better understanding of the potential contribution of anti-p200 autoantibodies to development of mucosal lesions.

Materials and methods

Literature review and article selection

A literature review was performed on PubMed (1996–2022) for anti-p200 pemphigoid cases by using the terms, p200, laminin gamma 1, lamγ1, LMγ1, anti-p200, anti-laminin gamma 1, anti-lamγ1, anti-LMγ1, anti-p200 pemphigoid, anti-laminin gamma 1 pemphigoid, anti-lamγ1 pemphigoid, anti-LMγ1 pemphigoid. Articles published in English were considered for eligibility. We also screened the references of the included studies for additional eligible publications. Then, a literature review on anti-p200 pemphigoid was conducted for several criteria between the cases with mucosal lesions and the cases only with skin lesions by statistical evaluation.

Selection criteria

All the studies reporting one or more cases with a diagnosis of anti-p200 pemphigoid were included. Criteria for the diagnosis were listed below:

i. clinical manifestations of subepidermal AIBD.
ii. linear deposition along the BMZ on DIF or showing positive signal along the dermal side of the split skin on ssIIF.
iii. positive signals for p200/LMγ1 by IB using human dermal extract or recombinant proteins of LMγ1.
iv. exclusion of other subepidermal AIBDs.

Data extraction

Each screened article was critically reviewed. The following variables were gathered as available: age, sex, location, final diagnosis, clinical features, histopathology, immunological profiles and associated comorbidities. According to the autoantigens detected, we classified the patients into two subgroups, “sole p200”, which reacted only with p200, and “multiple antigens (Ags)”, which reacted with p200 and other antigen(s).

Data analyses

The clinical, histopathological and immunological features of anti-p200 pemphigoid with mucosal lesions were summarized, and compared with anti-p200 pemphigoid only with skin lesions, other MMPs and EBA. The statistical analyses were performed using chi-square test, fisher exact test by SPSS software, version 23 (SPSS, Chicago, IL, USA). The p values less than 0.05 were considered as statistically significant.

Results

Demographic characteristics and diagnosis of the patients

From the literature, we selected total of 154 anti-p200 pemphigoid patients, among whom, 52 (33.8%) patients presented with mucosal lesions. The demographic characteristics of these 52 patients were summarized in Table 1. These 52 patients, reported from eight countries, were composed of 30 males (age, mean ± SD, 63.7 ± 16.0), 19 females (58.7 ± 21.8) and 3 with no gender information.

10.3389/fimmu.2023.1118846

TABLE 1 Demographic characteristics of the 52 cases of anti-p200 pemphigoid with mucosal lesions.

Items	Mean of ages (SD)	Number of cases (Percentage)
Gender		
Male	63.7 (16.0)	30 (57.7%)
Female	58.7 (21.8)	19 (36.5%)
Unknown	Unknown	3 (5.8%)
Geographical distribution of reported cases		
Germany	56.1 (21.7)	14 (26.9%)
France	75.1 (12.3)	11 (21.2%)
Japan	59.5 (16.8)	10 (19.2%)
India	58.2 (22.5)	9 (17.3%)
USA	65.7 (15.3)	3 (5.8%)
China	55.7 (7.0)	3 (5.8%)
Korea	49 (/)	1 (1.9%)
Poland	52 (/)	1 (1.9%)
Final diagnosis		
Anti-p200 pemphigoid	60.9 (20.1)	38 (73.1%)
Anti-p200 pemphigoid + anti-LM332-type MMP	63.2 (5.8)	8 (15.4%)
Anti-p200 pemphigoid + EBA	58.5 (9.2)	2 (3.8%)
Anti-p200 pemphigoid + anti-BP180-type MMP	87 (/)	1 (1.9%)
Anti-p200 pemphigoid + BP	68 (/)	1 (1.9%)
Anti-p200 pemphigoid + BP + anti-LM332-type MMP + anti-CNTN1-positive IP	77 (/)	1 (1.9%)
Anti-p200 MMP	49 (/)	1 (1.9%)

LM332, laminin 332; MMP, mucous membrane pemphigoid; BP, bullous pemphigoid; EBA, epidermolysis bullosa acquisita; anti-CNTN1-positive IP, anti-contactin-1-positive inflammatory polyneuropathy; "/", not applicable.

There are no statistical differences for ages of disease onset among different countries (all $p>0.05$). In these 52 cases, 38 (73.1%) were diagnosed as exclusively anti-p200 pemphigoid, while 14 (26.9%) were diagnosed as anti-p200 pemphigoid concurrent with other AIBDs or other autoantibodies, and 9 of the 14 patients were associated with anti-LM332-type MMP.

The clinical, histopathological and immunological features of the 52 cases of anti-p200 pemphigoid with mucosal lesions

Clinically, 52 cases had mucosal lesions on the oral (75.0%), genital (26.9%), ocular (11.5%), nasal (9.6%), pharyngeal (1.9%) and esophageal (1.9%) mucosae (Table 2). Fourteen (26.9%) cases showed lesions on multiple mucosal sites, including 3 cases on 4 sites, 2 cases on 3 sites and 9 cases on 2 sites (Table 3). In these 14 cases with multiple mucosal lesions, oral, genital, ocular, nasal lesions were found in 14, 10, 5 and 5 cases, respectively (Table 3).

Skin lesions were found in 51 of the 52 patients, however, only one patient was associated with psoriasis. In addition, there are two patients associated with malignancies, metastatic ovarian carcinoma and metastatic esophageal carcinoma (Table 2).

Histopathologically, 49 patients had pathological reports, which showed subepidermal blistering (98.0%), and inflammatory infiltrations of neutrophils (46.9%), eosinophils (38.8%) and lymphocytes (18.4%) (Table 2).

Concerning the results of immunofluorescence tests, the positive rates of IgG (Figure 1A) and C3 (Figure 1B) depositions to BMZ in DIF were 94.4% and 97.1%, respectively, positive IgG reactivity rate in IIF of normal human skin (Figure 1C) was 80.0%, and positive IgG reactivity with dermal side in ssIIF (Figure 1D) was 97.2%. A few patients showed IgA and/or IgM autoantibodies in these immunofluorescence tests (Table 2). By IB of human dermal extract, the positive rates of IgG against the 200 kDa p200 were 97.8%. In addition, anti-p200 autoantibodies were also detected by IB of various LMγ1 recombinant proteins in some cases (Table 2).

IgG autoantibodies targeting non-p200 autoantigens were reported in 30.8% patients. The autoantibodies against additional antigens, LM332 in 9 cases, BP180 in 6 cases, Type VII collagen in 2 cases and BP230 in one case were also detected. Moreover, for IgG autoantibodies against LM332, LMγ2 subunit was the most frequently recognized, followed by LMβ3 and LMα3 subunits (Table 2).

Because of the lack of detailed information for the treatments in most cases, we did not analyze the treatments in the present study.

The clinical, histopathological and immunological results of two subgroups of anti-p200 pemphigoid with mucosal lesions

According to the autoantigens detected, the 52 cases of anti-p200 pemphigoid with mucosal lesions were divided into two subgroups as sole p200 Ag-M (sole p200 antigen-anti-p200 pemphigoid with mucosal lesions) subgroup (36 cases), which reacted only with p200, and multiple antigens (Ags)-M (multiple antigens-anti-p200 pemphigoid with mucosal lesions) subgroup (16 cases), which reacted with p200 and other antigens. The detailed clinical, histopathological and immunological features of these two groups were summarized in Table 2.

Patients in sole p200 Ag-M and multiple Ags-M subgroups showed similar clinical, histopathological and immunological features, except that multiple Ags-M subgroup (25.0%) had higher ratio of nasal lesions than sole p200 Ag-M subgroup (2.8%) ($p<0.05$) (Tables 4 and 5).

Comparative analyses of the data of sole p200 Ag-M subgroup with two more subgroups of anti-p200 pemphigoid with only skin lesions

In the present study, the sole p200 Ag-M subgroup is the most important group to explore the potent contribution of anti-p200

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TABLE 2 Clinical, histopathological and immunological features of the 52 cases of anti-p200 pemphigoid with mucosal lesions.

Item			Sole p200 Ag-M (36) *	Multiple Ags-M (16) *	All cases (52)
Mucosal lesions	Oral	Erosion	9 in 36 (25.0%)	8 in 16 (50.0%)	17 in 52 (32.7%)
		Erythema	3 in 36 (8.3%)	0	3 in 52 (5.8%)
		Blister	5 in 36 (13.9%)	9 in 16 (56.3%)	14 in 52 (26.9%)
		Ulceration	1 in 36 (2.8%)	1 in 16 (6.3%)	2 in 52 (3.8%)
		Unknown	16 in 36 (44.4%)	2 in 16 (12.5%)	18 in 52 (34.6%)
		Total	27 in 36 (75.0%)	12 in 16 (75.0%)	39 in 52 (75%)
	Ocular	Hyperaemia	1 in 36 (2.8%)	0	1 in 52 (1.9%)
		Erosion	1 in 36 (2.8%)	1 in 16 (6.3%)	2 in 52 (3.8%)
		Blister	0	1 in 16 (6.3%)	1 in 52 (1.9%)
		Ulceration	0	1 in 16 (6.3%)	1 in 52 (1.9%)
		Scar	0	0	0
		Unknown	2 in 36 (5.6%)	1 in 16 (6.3%)	3 in 52 (5.8%)
		Total	3 in 36 (8.3%)	3 in 16 (18.8%)	6 in 52 (11.5%)
	Genital		8 in 36 (22.2%)	6 in 16 (37.5%)	14 in 52 (26.9%)
	Nasal		1 in 36 (2.8%)	4 in 16 (25.0%)	5 in 52 (9.6%)
	Esophageal		0	1 in 16 (6.3%)	1 in 52 (1.9%)
	Pharyngeal		0	1 in 16 (6.3%)	1 in 52 (1.9%)
	Laryngeal		0	0	0
	Unknown		5 in 36 (13.9%)	3 in 16 (18.8%)	8 in 52 (15.4%)
	Total		36 in 36 (100.0%)	16 in 16 (100.0%)	52 in 52 (100%)
Skin lesions			35 in 36 (97.2%)	16 in 16 (100.0%)	51 in 52 (98.1%)
Psoriasis			0	1 in 16 (6.3%)	1 in 52 (1.9%)
Malignancy	Metastatic ovarian carcinoma		0	1 in 16 (6.3%)	1 in 52 (1.9%)
	Metastatic esophageal cancer		1 in 36 (2.8%)	0	1 in 52 (1.9%)
	Total		1 in 36 (2.8%)	1 in 16 (6.3%)	2 in 52 (3.8%)
Histopathology feature	Subepidermal blistering		35 in 36 (97.2%)	13 in 13 (100.0%)	48 in 49 (98%)
	Neutrophils infiltration		15 in 36 (41.7%)	8 in 13 (61.5%)	23 in 49 (46.9%)
	Eosinophils infiltration		9 in 36 (25.0%)	10 in 13 (76.9%)	19 in 49 (38.8%)
	Lymphocytes infiltration		4 in 36 (11.1%)	5 in 13 (38.5%)	9 in 49 (18.4%)
Detection methods	DIF for BMZ (IgG)		23 in 24 (95.8%)	11 in 12 (91.7%)	34 in 36 (94.4%)
	DIF for BMZ (IgA)		5 in 7 (71.4%)	1 in 5 (20.0%)	6 in 12 (50%)
	DIF for BMZ (IgM)		0	1 in 5 (20.0%)	1 in 7 (14.3%)
	DIF for BMZ (C3)		23 in 24 (95.8%)	11 in 11 (100.0%)	34 in 35 (97.1%)
	IIF for BMZ (IgG)		4 in 5 (80.0%)	4 in 5 (80.0%)	8 in 10 (80.0%)
	IIF for BMZ (IgA)		1 in 1 (100.0%)	0	1 in 2 (50.0%)
	ssIIF reactive with both epidermal and dermal side (IgG)		0	4 in 4 (100.0%)	4 in 4 (100.0%)
	ssIIF reactive with dermal side (IgG)		26 in 27 (96.3%)	9 in 9 (100.0%)	35 in 36 (97.2%)
	ssIIF reactive with dermal side (IgA)		4 in 5 (80.0%)	1 in 1 (100.0%)	5 in 6 (83.3%)
	IB of normal human epidermal extracts for p200 (IgG)		6 in 9 (66.7%)	0	6 in 9 (66.7%)

(Continued)

TABLE 2 Continued

Item			Sole p200 Ag-M (36) *	Multiple Ags-M (16) *	All cases (52)
	IB of normal human dermal extract for p200 (IgG)		29 in 30 (96.7%)	15 in 15 (100.0%)	44 in 45 (97.8%)
	IB of normal human dermal extract for p200 (IgA)		1 in 1 (100.0%)	0	1 in 1 (100.0%)
	IB of LM521 RP for LMγ1 (IgG)		1 in 1 (100.0%)	1 in 1 (100.0%)	2 in 2 (100.0%)
	IB of LM111 RP for LMγ1 (IgG)		1 in 1 (100.0%)	1 in 1 (100.0%)	2 in 2 (100.0%)
	IB of LM411 RP for LMγ1 (IgG)		0	1 in 1 (100.0%)	1 in 1 (100.0%)
	IB of C-terminus of LMγ1 RP for LMγ1 (IgG)		9 in 11 (81.8%)	1 in 1 (100.0%)	10 in 12 (83.3%)
Combined with other antigens	LM332	α3 subunit	/	2 in 16 (12.5%)	2 in 52 (3.8%)
		β3 subunit	/	3 in 16 (18.8%)	3 in 52 (5.8%)
		γ2 subunit	/	4 in 16 (25.0%)	4 in 52 (7.7%)
		Unknown	/	3 in 16 (18.8%)	3 in 52 (5.8%)
		Total	/	9 in 16 (56.3%)	9 in 52 (17.3%)
	BP180		/	6 in 16 (37.5%)	6 in 52 (11.5%)
	Type VII collagen		/	2 in 16 (12.5%)	2 in 52 (3.8%)
	BP230		/	1 in 16 (6.3%)	1 in 52 (1.9%)
	Total#		/	16 in 16 (100.0%)	16 in 52 (30.8%)

LM332, laminin 332; Ag, antigen; *all 52 cases of anti-p200 pemphigoid with mucosal lesions were classified into 2 subgroups, (i) those with only p200 as autoantigen (sole p200 Ag-M subgroup) and (ii) those with p200 plus other autoantigens (multiple Ags-M subgroup); DIF, direct immunofluorescence; BMZ, basement membrane zone; IIF, indirect immunofluorescence; ssIIF, IIF using 1M NaCl-split skin; IB, immunoblotting; RP, recombinant protein; #, the total numbers of patients who combined with other antigens; "/", not applicable.

TABLE 3 Mucosal lesions distribution of 14 anti-p200 pemphigoid patients with multiple sites of mucosal lesions.

Case no.	Oral	Ocular	Genital	Nasal	Esophageal	Pharyngeal	Laryngeal	Total numbers of affected mucosae	References
1	+	+	+	+	–	–	–	4	Li et al., 2014 (32)
2	+	+	+	+	–	–	–	4	Mitsuya et al., 2008 (33)
3	+	–	+	+	–	+	–	4	Sarrazin et al., 2021 (27)
4	+	–	+	+	–	–	–	3	Liu et al., 2022 (34)
5	+	+	–	+	–	–	–	3	Gambichler et al., 2021 (35)
6	+	–	+	–	–	–	–	2	Kasperkiewicz et al., 2010 (36)
7	+	+	–	–	–	–	–	2	Cho and Kim, 2003 (37)
8	+	–	+	–	–	–	–	2	Meijer et al., 2016 (38)
9	+	–	+	–	–	–	–	2	Goetze et al., 2017 (16)
10	+	–	–	–	+	–	–	2	Yamada et al., 2006 (28)
11	+	–	+	–	–	–	–	2	Yamane et al., 2007 (39)
12	+	+	–	–	–	–	–	2	Iwata et al., 2009 (40)
13	+	–	+	–	–	–	–	2	Goto-Ohguchi et al., 2009 (41)
14	+	–	+	–	–	–	–	2	Alloo et al., 2014 (42)

"+", positive for indicated mucosal lesion; "-", negative for indicated mucosal lesion.

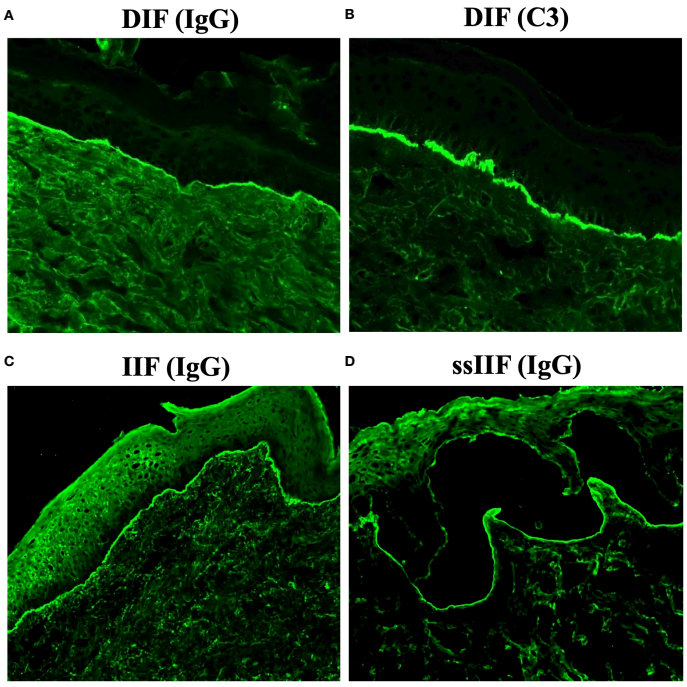

FIGURE 1
Representative results of various immunofluorescence tests for anti-p200 pemphigoid. **(A, B)** Direct immunofluorescence (DIF) for IgG **(A)** and C3 **(B)**. **(C)** Indirect immunofluorescence of normal human skin (IIF) for IgG. **(D)** Indirect immunofluorescence of 1M NaCl-split normal human skin (ssIIF) for IgG. All these representative results were originated from an anti-p200 pemphigoid patient with mucosal lesions.

autoantibodies to mucosal lesions. Therefore, sole p200 Ag-M subgroup was next employed to compare with two more subgroups of anti-p200 pemphigoid with only skin lesions, designated as sole p200 Ag-S (sole p200 antigen-anti-p200 pemphigoid only with skin lesions) subgroup (82 cases), which reacted only with p200, and as multiple Ags-S subgroup (multiple antigens-anti-p200 pemphigoid only with skin lesions) (20 cases), which reacted with p200 and other antigens.

Except for mucosal lesions, the sole p200 Ag-M subgroup (0%) contained a significantly lower percentage of psoriasis than the sole p200 Ag-S subgroup (31.7%) and multiple Ags-S subgroup (30%) (both p<0.05) (Tables 4 and 5).

Comparative analyses of the data of sole p200 Ag-M subgroup with two MMP groups and EBA group

The clinical and immunological features of anti-LM332-type MMP group (55 cases) (43), anti-BP180-type MMP group (332 cases) (44) and EBA group (105 cases) (45) were shown in Tables 4 and 5.

Compared with anti-LM332-type MMP group, sole p200 Ag-M subgroup had significantly lower ratios on oral, ocular, pharyngeal and laryngeal lesions (all $p<0.05$), and higher ratio on skin lesions ($p<0.05$).

Compared with anti-BP180-type MMP group, sole p200 Ag-M subgroup had significantly lower ratios on ocular and pharyngeal lesions (both $p<0.05$), and higher ratios on genital lesions, single mucosal site lesion and skin lesion (all $p<0.05$).

Compared with EBA group, sole p200 Ag-M subgroup had significantly higher ratios on genital and skin lesions (both $p<0.05$), and higher ratio for IgA reactivity to the dermal side on ssIIF ($p<0.05$).

Discussion

In the present study, 52 cases of anti-p200 pemphigoid with mucosal lesions from the literature were investigated for the potential contribution of anti-p200 autoantibodies to mucosal lesions. In these 52 cases, oral and genital lesions were the two most frequent mucosal lesions, 26.9% cases had lesions on multiple mucosal sites, in which oral and genital mucosae were also the most frequently affected mucosal sites.

Based on the results of autoantigens detected, the 52 cases of anti-p200 pemphigoid with mucosal lesions were classified into two subgroups, sole p200 Ag-M (36 cases), and multiple Ags-M (16 cases), both of which were similar in terms of clinical, histopathological and immunological features, except for higher ratio of nasal lesions appeared in multiple Ags-M subgroup.

Except for mucosal lesions, sole p200 Ag-M subgroup also showed lower frequency of psoriasis, when compared with other two subgroups of anti-p200 pemphigoid with only skin lesions, i.e., sole p200 Ag-S subgroup (82 cases) and multiple Ags-S subgroup (20 cases).

TABLE 4 Comparison of clinical features between anti-p200 pemphigoid with mucosal lesions and other AIBD subtypes.

Item		Anti-p200 pemphigoid				Anti-LM332-type MMP (55)	Anti-BP180-type MMP (332)	EBA (105)
		With mucosal lesions (sole p200 Ag-M, 36)	With mucosal lesions (multiple Ags-M, 16)	With only skin lesions (sole p200 Ag-S, 82)	With only skin lesions (multiple Ags-S, 20)			
Mucosal lesions	Oral	27 in 36 (75.0%)	12 in 16 (75.0%)	/	/	50 in 55 (90.9%) *	284 in 332 (85.5%)	66 in 105 (62.9%)
	Ocular	3 in 36 (8.3%)	3 in 16 (18.8%)	/	/	25 in 55 (45.5%) *	97 in 332 (29.2%) *	3 in 105 (2.9%)
	Genital	8 in 36 (22.2%)	6 in 16 (37.5%)	/	/	6 in 55 (10.9%)	32 in 332 (9.6%) *	7 in 105 (6.7%) *
	Nasal	1 in 36 (2.8%)	4 in 16 (25.0%) *	/	/	1 in 55 (1.8%)	11 in 332 (3.3%)	1 in 105 (1.0%)
	Esophageal	0	1 in 16 (6.3%)	/	/	3 in 55 (5.5%)	14 in 332 (4.2%)	1 in 105 (1.0%)
	Pharyngeal	0	1 in 16 (6.3%)	/	/	11 in 55 (20.0%) *	42 in 332 (12.7%) *	Unknown
	Laryngeal	0	0	/	/	10 in 55 (18.2%) *	35 in 332 (10.5%)	Unknown
Lesion on single mucosa		29 in 36 (80.6%)	9 in 16 (56.2%)	/	/	Unknown	127 in 200 (63.5%) *	Unknown
Lesion on multiple mucosa		7 in 36 (19.4%)	7 in 16 (43.8%)	/	/	Unknown	Unknown	Unknown
Skin lesions		35 in 36 (97.2%)	16 in 16 (100.0%)	82 in 82 (100.0%)	20 in 20 (100.0%)	37 in 55 (67.3%) *	132 in 332 (39.8%) *	69 in 105 (65.7%) *
Psoriasis		0	1 in 16 (6.3%)	26 in 82 (31.7%) *	6 in 20 (30.0%) *	Unknown	Unknown	Unknown
Malignancy		1 in 36 (2.8%)	1 in 16 (6.3%)	1 in 82 (1.2%)	0 in 20 (0.0%)	8 in 55 (14.5%)	21 in 332 (6.3%)	7 in 105 (6.7%)

LM332, laminin 332; MMP, mucous membrane pemphigoid; EBA, epidermolysis bullosa acquisita; Ag, antigen; all 52 cases of anti-p200 pemphigoid with mucosal lesions were classified into 2 subgroups,(i) those with only p200 as autoantigen (sole p200 Ag-M subgroup) and (ii) those with p200 plus other autoantigens (multiple Ags-M subgroup); all 102 cases of anti-p200 pemphigoid with only skin lesions were also classified into 2 subgroups, (i) those with only p200 as autoantigen (sole p200 Ag-S subgroup) and (ii) those with p200 plus other autoantigens (multiple Ags-S subgroup); "/", not applicable; *, $p < 0.05$ when compared with sole p200 Ag-M subgroup.

To determine the possible differences in the contribution to develop mucosal lesions between autoantibodies against p200 and other known MMP autoantigens, we next compared sole p200 Ag-M subgroup with anti-LM332-type MMP and anti-BP180-type MMP groups. The results indicated that anti-p200 autoantibodies might induce less frequently oral, ocular, pharyngeal and laryngeal lesions compared with anti-LM332 autoantibodies, while anti-p200 autoantibodies might produce less frequently ocular and pharyngeal lesions but more frequently genital lesions and single mucosa lesion, when compared with anti-BP180 autoantibodies.

Sole p200 Ag-M subgroup were also compared with EBA group, because both p200 and type VII collagen (EBA autoantigen) were located in dermal side of 1M NaCl-split skin. The results indicated that anti-p200 autoantibodies might induce more frequently genital and skin lesions when compared with anti-type VII collagen autoantibodies.

Importantly, anti-LM332-type MMP was the most commonly associated AIBD in anti-p200 pemphigoid with mucosal lesions, which further emphasize the necessity of detection of anti-LM332 autoantibodies in anti-p200 pemphigoid with mucosal lesions.

The results of the present study suggest that, in addition to LM332 and BP180, p200 is an important autoantigen of MMP. Therefore, anti-p200 pemphigoid with extensive mucosal lesions could be considered as anti-p200 MMP. Anti-p200 pemphigoid and anti-p200 MMP show the same autoantigen but different clinical features, which is the similar situation for BP and anti-BP180-type MMP.

IB of dermal extract is usually not used for the diagnosis of MMP, because this assay was mainly used for detection of antibodies to EBA antigen (type VII collagen) and p200. However, the results in the present review suggested the importance of IB of dermal extract for serological diagnosis of MMP, because p200 is also one of autoantigens of MMP. We occasionally encounter MMP cases, which do not show autoantibodies against known MMP-related autoantigens, such as LM332, BP180 and integrin $\alpha6\beta4$. In this situation, we would like to suggest detection of autoantibodies against p200.

This review was summarized and analyzed on the information from patients previously reported as case reports and case series. These published articles were not completely comprehensive, and some of them were only recorded with the clinical data and simple medications. Due to lack uniformity information, therapy profile was not analyzed. Additionally, 52 cases of anti-p200 pemphigoid with mucosal lesions in the literature were originated from only eight countries, which is possibly because very few countries have the

TABLE 5 Comparison of immunological features between anti-p200 pemphigoid with mucosal lesions and other AIBD subtypes.

Item		Anti-p200 pemphigoid				Anti-LM332-type MMP (55)	Anti-BP180-type MMP (332)	EBA (105)
		With mucosal lesions (sole p200 Ag-M, 36)	With mucosal lesions (multiple Ags-M, 16)	With only skin lesions (sole p200 Ag-S, 82)	With only skin lesions (multiple Ags-S, 20)			
DIF	BMZ (IgG)	23 in 24 (95.8%)	11 in 12 (91.7%)	17 in 17 (100.0%)	67 in 68 (98.6%)	55 in 55 (100.0%)	108 in 132 (81.8%)	80 in 83 (96.4%)
	BMZ (IgA)	5 in 7 (71.4%)	1 in 5 (20.0%)	1 in 5 (20.0%)	9 in 12 (75.0%)	Unknown	47 in 71 (66.2%)	9 in 15 (60.0%)
	BMZ (IgM)	0	1 in 4 (25.0%)	0	2 in 4 (50.0%)	Unknown	14 in 40 (35.0%)	Unknown
	BMZ (C3)	23 in 24 (95.8%)	11 in 11 (100.0%)	17 in 17 (100.0%)	70 in 70 (100.0%)	41 in 55 (74.5%)	110 in 127 (86.6%)	72 in 75 (96.0%)
IIF	BMZ (IgG)	4 in 5 (80.0%)	4 in 5 (80.0%)	8 in 8 (100.0%)	9 in 9 (100.0%)	23 in 55 (41.8%)	132 in 319 (41.4%)	96 in 105 (91.4%)
	BMZ (IgA)	1 in 1 (100.0%)	0	0	1 in 1 (100.0%)	Unknown	26 in 285 (9.1%)	3 in 48 (6.3%)
ssIIF	epidermal side (IgG)	0	0	0	1 in 1 (100.0%)	Unknown	207 in 317 (65.3%)	10 in 104 (9.6%)
	epidermal side (IgA)	0	0	1 in 1 (100.0%)	1 in 1 (100.0%)	Unknown	151 in 282 (53.55%)	9 in 45 (20.0%)
	both epidermal and dermal side (IgG)	0	4 in 4 (100.0%)	11 in 11 (100.0%)	0	Unknown	Unknown	10 in 104 (9.6%)
	both epidermal and dermal side (IgA)	0	0	0	1 in 1 (100.0%)	Unknown	Unknown	2 in 45 (4.4%)
	dermal side (IgG)	26 in 27 (96.3%)	9 in 9 (100.0%)	7 in 7 (100.0%)	58 in 58 (100.0%)	48 in 55 (87.3%)	62 in 317 (19.6%) *	93 in 104 (89.4%)
	dermal side (IgA)	4 in 5 (80.0%)	1 in 1 (100.0%)	0	2 in 3 (66.7%)	Unknown	4 in 282 (1.42%) *	3 in 45 (6.7%) *
IB	normal human epidermal extract (IgG)	6 in 9 (66.7%)	0	1 in 2 (50.0%)	3 in 4 (75.0%)	Unknown	Unknown	Unknown
	normal human dermal extract (IgG)	29 in 30 (96.7%)	15 in 15 (100.0%)	20 in 20 (100.0%)	57 in 57 (100.0%)	Unknown	Unknown	92 in 103 (89.3%)
	normal human dermal extract (IgA)	1 in 1 (100.0%)	0	0	1 in 2 (50.0%)	Unknown	Unknown	1 in 7 (14.3%)

LM332, laminin 332; MMP, mucous membrane pemphigoid; EBA, epidermolysis bullosa acquisita; Ag, antigen; all 52 cases of anti-p200 pemphigoid patients with mucosal lesions were classified into 2 subgroups, (i) those with only p200 as autoantigen (sole p200 Ag-M subgroup) and (ii) those with p200 plus other autoantigens (multiple Ags-M subgroup); all 102 cases of anti-p200 pemphigoid patients with only skin lesions were also classified into 2 subgroups, (i) those with only p200 as autoantigen (sole p200 Ag-S subgroup) and (ii) those with p200 plus other autoantigens (multiple Ags-S subgroup); DIF, direct immunofluorescence; BMZ, basement membrane zone; IIF, indirect immunofluorescence; ssIIF, IIF using 1M NaCl-split skin; IB, immunoblotting; *$p < 0.05$ when compared with sole p200 Ag-M subgroup.

abilities to diagnose this disease. Anti-BP180-type MMP group used in the present study is not a pure group with only anti-BP180 antibodies, which might influence the comparative results.

In summary, anti-p200 pemphigoid with mucosal lesions showed unique clinical features of higher frequency of genital lesions and very rare psoriasis, and distinct immunological features of high incidence of cooccurrence of autoantibodies against LM332. p200 is also a very important autoantigen of MMP. The epitopes and pathogenesis of anti-p200 autoantibodies were still inconclusive.

Author contributions

XL and TH designed this project and revised this manuscript. YG and HQ performed data collection, analysis and prepare the

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

Funding

This study was supported by JSPS KAKENHI Grant (Grant-in-Aid for Scientific Research (C)) (Number 21K08331) to TH.

Acknowledgments

Authors dedicate the present review in the memoriam of Professor Detlef Zillikens for his contribution to the field of AIBDs.

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 26 January 2023
DOI 10.3389/fimmu.2022.1103533

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 20 November 2022
ACCEPTED 28 December 2022
PUBLISHED 26 January 2023

CITATION
Kridin K, Vorobyev A, Papara C, De Luca DA, Bieber K and Ludwig RJ (2023) Risk factors and sequelae of epidermolysis bullosa acquisita: A propensity-matched global study in 1,344 patients.
Front. Immunol. 13:1103533.
doi: 10.3389/fimmu.2022.1103533

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Risk factors and sequelae of epidermolysis bullosa acquisita: A propensity-matched global study in 1,344 patients

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Identification of risk factors and sequelae of any given disease is of key importance. For common diseases, primary prevention and disease management are based on this knowledge. For orphan diseases, identification of risk factors and sequelae has been challenging. With the advent of large databases, e.g., TriNetX, this can now be addressed. We used TriNetX to identify risk factors and sequelae of epidermolysis bullosa acquisita (EBA), a severe and orphan autoimmune disease. To date, there is only enigmatic information on EBA comorbidity. We recruited 1,344 EBA patients in the Global Collaborative Network of TriNetX. Using the "explore outcomes" function we identified 55 diagnoses with a different prevalence between EBA and no-EBA patients. We next performed propensity-matched, retrospective cohort studies in which we determined the risk of EBA development following any of the identified 55 diseases. Here, 31/55 diseases were identified as risk factors for subsequent EBA. Importantly, the highest risk for EBA were other chronic inflammatory diseases (CID), especially lupus erythematosus and lichen planus. Lastly, we determined the risk to develop any of the identified diseases after EBA diagnosis. Here, 38/55 diseases were identified as sequelae. Notably, EBA patients showed an increased risk for metabolic and cardiovascular disease, and thrombosis. Furthermore, the risk for CIDs, especially lupus erythematosus and lichen planus, was elevated. These insights into risk factors and sequelae of EBA are not only of clinical relevance, e.g., optimizing cardiovascular disease risk, but in addition, point to shared pathogenetic pathways between EBA and other inflammatory diseases.

KEYWORDS

epidermolysis bullosa acquisita, TriNetX, risk factor, sequelae, systemic lupus erythematosus (SLE), inflammatory bowel disease, cardiovascular disease, lichen planus

10.3389/fimmu.2022.1103533

Introduction

Epidermolysis bullosa acquisita (EBA) is an orphan autoimmune disease that is characterized and caused by autoantibodies targeting type VII collagen (COL7). This short statement is grounded on research that was fronted by Detlef Zillikens: In 2005 his group demonstrated the induction of experimental EBA in mice following the transfer of antibodies targeting COL7 (1). At the same time, similar findings were published by the group of David Woodley (2). With these two landmark publications, the autoimmune pathogenesis of EBA had been firmly established. In parallel to this basic research on EBA, Detlef Zillikens also pursued epidemiological research questions, mainly aimed at the determination of the incidence and prevalence of autoimmune bullous dermatoses, such as EBA. Among other findings, he demonstrated a low EBA incidence and prevalence in Germany (3, 4).

Because EBA is such a rare disease, insights into risk factors and sequelae are sparse. Based on small cohorts, reviews of case reports and case report-series, an association between EBA and inflammatory bowel diseases (IBDs), such as ulcerative colitis (UC) and Crohn's disease (CD), as well as infectious, cardiovascular, metabolic, malignant, and neurological diseases have been reported (5–12). Whilst no association of EBA with systemic lupus erythematosus (SLE) was observed, antinuclear antibodies (ANAs), a hallmark of SLE, were described to occur at higher frequencies than expected in patients diagnosed with EBA (12). Without exception, these reports are based on case reports, case report series or a meta-analysis of these. Thus, the evidence provided is of rather limited validity. In addition, all previous publications on that topic had reported associations only, with no information of the sequence of events. Hence, insights into the sequence of events are missing.

Understanding risk factors and sequelae of EBA would, however, significantly improve patient outcomes because risk factors could be addressed to prevent disease onset, and screening for diseases that subsequently develop after EBA diagnosis may contribute to their early detection. This is, for example, exemplified in the implementation of screening for metabolic, psychiatric, and cardiovascular comorbidity in psoriasis (13). In the same line, the European League Against Rheumatism (EULAR) recently published recommendations concerning lifestyle behavior to prevent progression of rheumatic diseases. In the context of non-inflammatory diseases, such as cardiovascular diseases, preventive measures to normalize elevated blood pressure or cholesterol are common clinical practice (14, 15). These include smoking cession and weight reduction in obese patients because nicotine dependence and overweight/obesity are known risk factors for the development of rheumatic diseases, being often associated with a more severe disease and a reduced response to treatment (16).

To define the so far enigmatic risk factors and associations of EBA, we assessed the Global Collaborative Network of TriNetX that provides access to anonymized, longitudinal patient data from close to 115 million individuals. First, we determined differences in disease prevalence in patients with EBA to those without. Next, we performed several retrospective cohort studies in which we determined the risk of subsequent EBA development following the diagnosis of any of the diseases found to be different in prevalence between EBA and no-EBA patients. Lastly, we determined the risk to develop any of the identified diseases after the diagnosis of EBA.

Materials and methods

Study design and database

We performed a global population-based study with a propensity-matched retrospective cohort design. First, the Global Collaborative Network of TriNetX was used to compare differences regarding diagnoses of EBA patients (ICD10:L12.3) to those without EBA. For the latter group, individuals presenting for "Encounter for general examination without complaint, suspected or reported diagnosis" (ICD10CM:Z00) that had no diagnosis of EBA (ICD10CM:L12.3) were included. For the analysis, the "explore outcomes" function of TriNetX was used. Next, only diagnoses with a medium difference (2/3 bars) and a prevalence of 5% or more in EBA patients, and those with a high difference (3/3 bars) and a prevalence of 1% or more in EBA patients were considered further. We then performed the following retrospective cohort studies: First, the risk of EBA development following the diagnosis of any of the selected diseases was assessed. This was followed by the determination of development of any of the selected diseases following an EBA diagnosis (Figure 1).

Study population and definition of eligible patients

The data used in this study was collected between October 1st to 8th, 2022, from the TriNetX Global Collaborative Network, which, at the time of analysis, provided access to electronic medical records (diagnoses, procedures, medications, laboratory values, genomic information) from 113,986,635 million patients from 90 healthcare organizations (HCO). Propensity score matching was performed for the following variables: Age, sex, ethnicity, and race. TriNetX, LLC is compliant with the Health Insurance Portability and Accountability Act (HIPAA), the US federal law which protects the privacy and security of healthcare data, and any additional data privacy regulations applicable to the contributing HCO. TriNetX is certified to the ISO 27001:2013 standard and maintains an Information Security Management System (ISMS) to ensure the protection of the healthcare data it has access to and to meet the requirements of the HIPAA Security Rule, Any data displayed on the TriNetX Platform in aggregate form, or any patient level data provided in a data set generated by the TriNetX Platform, only contains de-identified data as per the de-identification standard

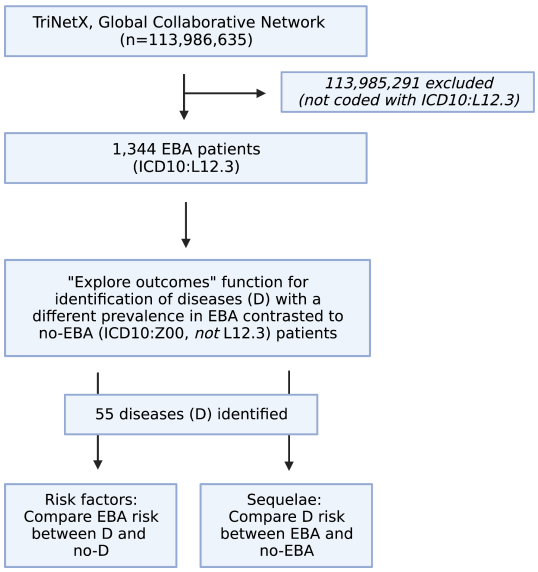

FIGURE 1
Study flow chart. Within the Global Collaborative Network of TriNetX that encompassed data from close to 115 million patients at the time of access (October 1st, 2022), 1,344 patients with the diagnosis of epidermolysis bullosa acquisita (EBA), defined by ICD10:L12.3 were identified. Patients without a diagnosis of EBA were excluded. Next, the "explore outcomes" function of TriNetX to identify diseases that are different in prevalence (at any given time) between EBA patients (defined by presence of ICD10:L12.3) those without EBA (defined as presence of ICD10:Z00 and absence of ICD10:L12.3). This identified 55 diseases with a different prevalence (at any given time) between EBA and no-EBA patients. Subsequently, 55 propensity-matched studies were performed to explore if any one of the identified diseases had an impact on subsequent EBA development. For this, patients with the disease, i.e., Crohn's disease (ICD10:K50) were compared to those without the disease, i.g., no-Crohn's disease (ICD10:Z00 and absence of ICD10:K50). Lastly, a propensity-matched study was performed to delineate the risk of EBA to develop any of the 55 identified diseases. Figure created with BioRender (https://biorender.com).

defined in Section §164,514(a) of the HIPAA Privacy Rule. The process by which the data is de-identified is attested to through a formal determination by a qualified expert as defined in Section §164,514(b)(1) of the HIPAA Privacy Rule. Because this study used only de-identified patient records and did not involve the collection, use, or transmittal of individually identifiable data, this study was exempted from Institutional Review Board approval.

Statistical analysis

Baseline characteristics were described by means and standard deviations (SDs) for continuous variables, and numbers and percentages for dichotomous variables. Continuous variables were compared using the student t-test, and dichotomous variables by Pearson chi-square test. Survival analyses were conducted by the Kaplan-Meier method. A log-rank test was run to determine if there were differences in the survival distribution for patients in the two investigated groups. Hazard ratios (HR)s for the study outcomes were obtained using the Cox regression model. Nelson-Aalen plots were utilized to test the proportional hazards assumption. Two-tailed P-values less than 0,05 were considered statistically significant.

Results

Cohort description and global results

For EBA-risk factors analysis, detailed cohort descriptions and results are displayed in Supplement Tables 1–55 and Table 1. Sample sizes for the risk factor analysis ranged from 11,573 (conjunctival scars) to 7,849,544 (hypertensive diseases), with a median of 1,370,800 (845,473 to 2.337,172, 25/75-percentile). For each disease, propensity matching (age, sex, race, and ethnicity) was performed for better comparability of the groups. Globally, 31/55 diseases were associated with a higher risk of subsequent EBA, 23/55 with no impact on EBA risk, while 1/55 disease led to a reduced risk of subsequent EBA (Figure 2 and Table 1). The corresponding data for the sequalae is found in Supplement Table 56 and Table 2. Overall, we included 1,344 EBA patients (cases) and a similar number of controls. For a better comparability between cases and controls, they were again propensity matched for age, sex, race, and ethnicity. Between groups, no differences regarding these variables were observed. Overall, 37/55 diseases developed more frequently than expected following EBA diagnosis, for 17/55 diseases EBA was not a risk factor, and EBA had a lower risk for 1/55 diseases.

Non-communicable inflammatory diseases are risk factors and sequelae of EBA

A total of 9 non-communicable inflammatory diseases (CID) were amongst the 55 diseases with a different prevalence contrasting EBA to no-EBA patients (Tables 1, 2 and Figure 2). Of these 9 CIDs, 6 were identified as risk factors for EBA. Considering that 23 diseases were identified as risk factors for EBA, CIDs are the most prevalent risk factor for subsequent EBA development. Lichen planus (HR 10.20, CI 2.39-43.50, p<0.0001), cutaneous lupus erythematosus (HR 26.96, CI 3.67-198.22, p<0.0001) and systemic lupus erythematosus (SLE, HR 3.10, CI 1.31-26.91, p=0.0087) were among the disease with the highest risk for future EBA development. Interestingly, "other chronic obstructive pulmonary disease" (COPD) was associated with a decreased risk of subsequent EBA development (HR 0.73, CI 0.55-0.98, p=0.0378).

Likewise, EBA was a risk factor for subsequent CID development. Of the 37 diseases for which EBA is a risk factor, 7 were CIDs. Again, lichen planus (HR 6.64, CI 1.74-25.28, p<0.0017), cutaneous lupus erythematosus (HR 15.55, CI

TABLE 1 Risk factors of epidermolysis bullosa acquisita (EBA).

		Cases			Controls					
Disease	ICD10 code	N of eligible participants	N of Outcomes	Risk, %	N of eligible participants*	N of Outcomes	Risk, %	Risk difference (95% confidence interval), %	Hazard ratio (95% confidence interval)	P value
Overweight and obesity	E66	6,923,027	280	0.004	6,923,063	245	0.004	0.001, 0-0.001	**1.257**, 1.059-1.493	0.0088
Type 2 diabetes mellitus	E11	5,637,675	240	0.004	5,637,665	255	0.005	–	–	n.s.
Hypothyroidism, unspecified	E03.9	3,402,569	145	0.004	3,402,556	133	0.004	–	–	n.s.
Malnutrition	E40-E46	919,769	46	0.005	919,811	33	0.004	0.001, 0-0.003	**2.363**, 1.505-3.71	0.0001
Systemic lupus erythematosus	M32	231,329	29	0.013	231,352	10	0.004	0.008, 0.003-0.014	**3.099**, 1.466-6.552	0.0018
Hypertensive diseases	I10-I16	7,840,452	284	0.004	7,840,405	307	0.004	–	–	n.s.
Heart failure	I50	2,460,148	84	0.003	2,460,164	113	0.005	–	–	n.s.
Atrial fibrillation and flutter	I48	2,440,224	84	0.003	2,440,208	113	0.005	–	–	n.s.
Nonrheumatic mitral valve disorders	I34	1,549,166	64	0.004	1,549,175	57	0.004	–	–	n.s.
Atherosclerosis	I70	1,246,293	57	0.005	1,246,328	50	0.004	–	–	n.s.
Acute embolism and thrombosis of deep veins of lower extremity	I82.4	845,427	53	0.006	845,451	24	0.003	0.003, 0.001-0.005	**2.628**, 1.622-4.259	< 0.0001
Hypotension	I95	1,837,108	89	0.005	1,837,163	84	0.005	0, -0.001-0.002	**1.395,** 1,035-1,882	0.0282
Cerebral infarction	I63	1,567,585	84	0.005	1,567,605	70	0.004	0.001, -0.001-0.002	**1,494**, 1,008-2,053	0.0126
Diverticular disease of intestine	K57	2,337,084	112	0.005	2,337,112	132	0.006	–	–	n.s.
Gastro-esophageal reflux disease	K21	6,973,564	295	0.004	6,973,597	251	0.004	–	–	n.s.
Ulcer of esophagus	K22.1	127,391	11	0.009	127,404	10	0.008	–	–	n.s.
Gastritis and duodenitis	K29	1,869,533	74	0.004	1,869,561	61	0.003	–	–	n.s.
Stomatitis and related lesions	K12	624,417	71	0.011	624,467	14	0.002	0.009, 0.006-0.012	**4.943**, 2.785-8.773	< 0.0001
Diaphragmatic hernia	K44	1,329,587	66	0.005	1,329,616	70	0.005	–	–	n.s.
Crohn's disease	K50	294,555	20	0.007	294,562	11	0.004	–	–	n.s.
Other chronic obstructive pulmonary disease	J44	2,317,942	72	0.003	2,317,939	114	0.005	-0.002, -0.003-0.001	0.732 0.545-0.984	0.0378
Sleep disorders	G47	5,970,685	251	0.004	5,970,732	216	0.004	0.001, 0-0.001	**1.231,** 1.026-1.477	0.0250

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TABLE 1 Continued

		Cases			Controls					
Disease	ICD10 code	N of eligible participants	N of Outcomes	Risk, %	N of eligible participants*	N of Outcomes	Risk, %	Risk difference (95% confidence interval), %	Hazard ratio (95% confidence interval)	P value
Acute kidney failure and chronic kidney disease	N17-N19	3,557,269	169	0.005	3,557,299	158	0.004	0, -0.001-0.001	**1.371**, 1.103-1.704	0.0043
Malignant melanoma of skin	C43	282,444	24	0.008	282,476	13	0.005	–	–	n.s.
Malignant neoplasm of liver and intrahepatic bile ducts	C22	594,423	38	0.006	594,476	21	0.004	0.003, 0-0.005	**3.037**, 1.737-5.309	< 0.0001
Melanocytic nevi	D22	1,370,655	40	0.003	1,370,781	46	0.003	–	–	n.s.
Other and unspecified malignant neoplasm of skin	C44	1,080,602	73	0.007	1,080,674	47	0.004	0.002, 0-0.004	**1.504**, 1.042-2.17	0.0282
Anemia, unspecified	D64.9	4,097,465	190	0.005	4,097,521	148	0.004	0.001, 0-0.002	**1.394,** 1.124-1.728	0.0024
Elevated white blood cell count	D72.82	1,554,558	94	0.006	1,554,612	61	0.004	0.002, 0.001-0.004	**2.174**, 1.57-3.009	< 0.0001
Purpura and other hemorrhagic conditions	D69	1,325,255	79	0.006	1,325,309	50	0.004	0.002, 0.001-0.004	**1.977**, 1.387-2.82	0.0001
Iron deficiency anemia	D50	2,055,291	84	0.004	2,055,347	64	0.003	0.001, 0-0.002	**1.473**, 1.064-2.04	0.0190
Age-related cataract	H25	1,663,835	111	0.007	1,663,880	83	0.005	–	–	n.s.
Other cataract	H26	1,223,272	99	0.008	1,223,308	50	0.004	0.004, 0.002-0.006	**1.663**, 1.183-2.339	0.0031
Visual disturbances and blindness	H53-H54	3,457,195	179	0.005	3,457,247	98	0.003	0.002, 0.001-0.003	**1.956**, 1.529-2.503	< 0.0001
Glaucoma	H40-H42	1,221,974	77	0.006	1,221,991	47	0.004	–	–	n.s.
Dry eye syndrome	H04.12	908,659	57	0.006	908,695	35	0.004	–	–	n.s.
Presbyopia	H52.4	878,532	77	0.009	878,566	41	0.005	0.004, 0.002-0.007	**1.569**, 1.074-2.294	0.0190
Conjunctival scars	H11.2	11,556	14	0.121	11,573	0	0	0.121, 0.058-0.185%	**n.a.**	0.0001
Keratitis	H16	362,090	32	0.009	362,108	17	0.005	–	–	n.s.
Disorders of choroid and retina	H30-H36	1,476,209	82	0.006	1,476,231	61	0.004	–	–	n.s.
Lupus erythematosus	L93	92,859	28	0.03	92,904	10	0.011	0.019, 0.006-0.032	**26.957**, 3.666,198.223	< 0.0001

(Continued)

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TABLE 1 Continued

		Cases			Controls					
Disease	ICD10 code	N of eligible participants	N of Outcomes	Risk, %	N of eligible participants*	N of Outcomes	Risk, %	Risk difference (95% confidence interval), %	Hazard ratio (95% confidence interval)	P value
Pressure ulcer	L89	520,244	25	0.005	520,276	23	0.004	0, -0.002-0.003	**1.828,** 1.035-3.228	0.0348
Pruritus	L29	1,719,598	180	0.01	1,719,763	56	0.003	0.007, 0.005-0.009	**3.507**, 2.598-4.735	< 0.0001
Seborrheic dermatitis	L21	667,636	44	0.007	667,680	14	0.002	0.004, 0.002-0.007	**2.893**, 1.585-5.283	0.0003
Lichen simplex chronicus and prurigo	L28	257,992	48	0.019	258,031	11	0.004	0.014, 0.009-0.02	**4.446**, 2.309-8.561	< 0.0001
Urticaria	L50	1,072,233	69	0.006	1,072,291	18	0.002	0.005, 0.003- 0.006	**4.094**, 2.437-6.88	< 0.0001
Lichen planus	L43	74,580	21	0.028	74,609	10	0.013	0.015, 0-0.029	**10.198**, 2.391-43.499	< 0.0001
Chronic pain, not elsewhere classified	G89.2	4,199,569	152	0.004	4,199,663	169	0.004	–	–	n.s.
Acute pain, not elsewhere classified	G89.1	1,261,295	61	0.005	1,261,333	34	0.003	0.002, 0.001-0.004	**2.371**, 1.554-3.617	< 0.0001
Polyneuropathy, unspecified	G62.9	1,013,058	74	0.007	1,013,111	40	0.004	0.003, 0.001-0.005	**2.45**, 1.647-3.644	< 0.0001
Benign prostatic hyperplasia	N40	1,593,540	70	0.004	1,593,550	84	0.005	–	–	n.s.
Candidiasis	B37	1,933,646	107	0.006	1,933,718	46	0.002	0.003, 0.002-0.004	**2.311**, 1.636-3.266	< 0.0001
Dermatophytosis	B35	1,676,899	105	0.006	1,676,983	59	0.004	0.003, 0.001-0.004	**1.565**, 1.137-2.153	0.0056
Sepsis, unspecified organism	A41.9	1,336,969	62	0.005	1,337,011	52	0.004	0.001, -0.001-0.002	**1.935** 1.333-2.808	0.0004
Nicotine dependence	F17	5,515,751	196	0.004	5,515,748	213	0.004	–	–	n.s.

The risk of EBA development in individuals diagnosed with any one of the listed diseases cases to those without these diagnoses controls. Data shown displays results from measures of association excluding patients with EBA prior to the time window. For the Hazard ratio. Kaplan-Meier Analysis again excluding patients with outcome prior to the time window with Log-Rank Test was performed. N, number; n.s., not significant; n.a., not applicable; Diseases where presence of any of the diseases increases the risk are highlighted in bold. Diseases where presence of the indicated disease decreases the risk are highlighted in blue and bold. Diseases that do not impose a risk for subsequent EBA development are displayed in gray. Please note that propensity score matching is re-run with each outcomes analysis so that the analysis uses the most current data available on the TriNetX network. Analysis was performed from October 3rd to 8th, 2022.

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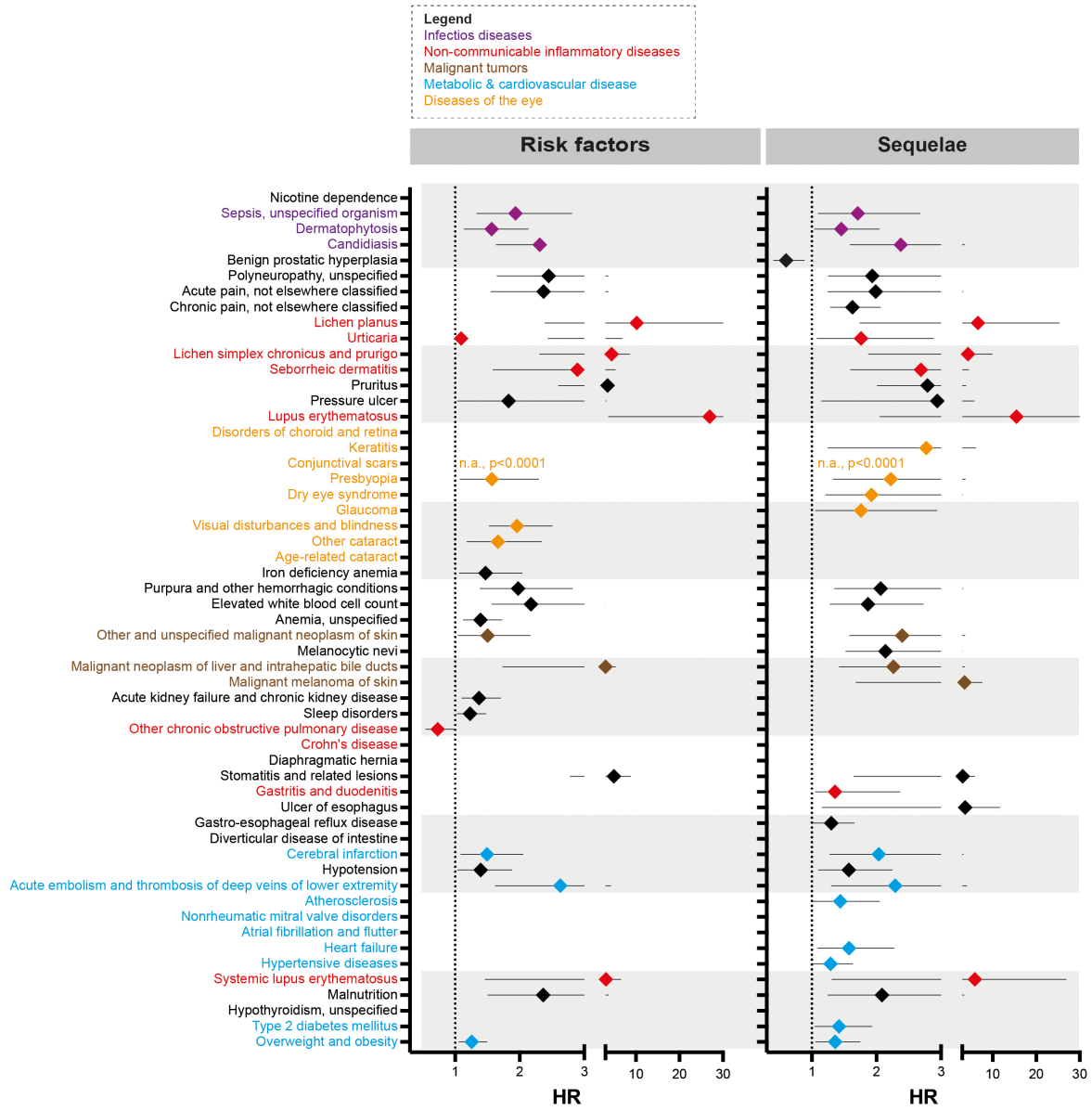

FIGURE 2
Risk factors and sequelae of epidermolysis bullosa acquisita. Hazard ratio (HR) event plots for the risk factors and sequelae of epidermolysis bullosa acquisita (EBA). Cohort descriptions and detailed results for risk factors are listed in Supplement Tables 1–55 and Table 1, respectively. For risk factors, these are shown in Supplement Table 56 and Table 2. Error bars indicate 5/95-percentiles. If no HR is shown, the indicated disease was not a risk factor / sequela of EBA.

2.05-117.69, p=0.0004) and SLE (HR 5.94, CI 1.47-6.55, p=0.0018) were among the diseases manifesting following an EBA diagnosis. Albeit not a classical CID, pruritus was found to be both a risk factor (HR 3.51, CI 2.60-4.74, p<0.0001) and a sequel of EBA (HR 2.79, CI 2.01-3.88, p<0.0001).

Of note, inflammatory bowel disease, such as ulcerative colitis and Crohn's disease, that had been previously assumed to be associated with EBA (12, 17–19) were not identified as risk factors or sequelae of EBA. Ulcerative colitis was not among the 55 diseases with a different prevalence when comparing EBA to no-EBA patients. Crohn's disease was identified to have a different prevalence in EBA compared to no-EBA patients. However, in neither of the two, propensity matched, case-control studies, Crohn's disease was identified as a risk factor or a sequela of EBA.

Infectious diseases are risk factors and sequelae of EBA

Sepsis, dermatophytosis and candidiasis were among the 55 diseases with a different prevalence between EBA and no-EBA

TABLE 2 Sequelae of epidermolysis bullosa acquisita (EBA).

		Cases			Controls					
Disease	ICD10 code	N of eligible participants	N of Outcomes	Risk, %	N of eligible participants*	N of Outcomes	Risk, %	Risk difference (95% confidence interval), %	Hazard ratio (95% confidence interval)	P value
Overweight and obesity	E66	1,031	128	12.415	1,175	130	11.064	1.351, -1.345-4.047	**1.360**, 1.059-1.747	0.0156
Type 2 diabetes mellitus	E11	1,048	93	8.874	1,138	80	7.030	1.844, -0.430-4.118	**1.420**, (1.045-1.931	0.0245
Hypothyroidism, unspecified	E03.9	1,180	44	3.729	1,222	65	5.319	–	–	n.s.
Malnutrition	E40-E46	1,285	44	1,335	1,335	23	1.723	1.701, 0.486-2.916	**2.088**, 1.250-3.467	0.0036
Systemic lupus erythematosus	M32	1,301	11	0.846	1,338	10	0.747	0.098, -0.581-0.777	**5.940**, 1.311-26.908	0.0087
Hypertensive diseases	I10-I16	729	145	19.890	809	147	18.171	1.720, -2.212-5.65	**1.289**, 1.017-1.632	0.0350
Heart failure	I50	1,241	70	5.641	1,286	54	4.199	1.442, -0.246-3.130	**1.575**, 1.090-2.275	0.0147
Atrial fibrillation and flutter	I48	1,234	47	3.809	1,256	49	3.901	–	–	n.s.
Nonrheumatic mitral valve disorders	I34	1,275	40	3.137	1,304	51	3.911	–	–	n.s.
Atherosclerosis	I70	1,284	73	5.685	1,306	60	4.594	1.091, -0.610-2.792	**1.441**, 1.014-2.050	0.0407
Acute embolism and thrombosis of deep veins of lower extremity	I82.4	1,283	38	2.962	1,330	19	1.429	1.533, 0.407-2.659	**2.291**, 1.301-4.035	0.0032
Hypotension	I95	1,235	73	5.911	1,313	57	4.341	1.570, -0.1460-3.286	**1.574**, 1.102-2.249	0.0119
Cerebral infarction	I63	1,255	51	4.064	1,313	31	2.361	1.703, 0.336-3.069	**2.036**, 1.280-3.238	0.0022
Diverticular disease of intestine	K57	1,226	83	6.770	1,285	106	8.249	–	–	n.s.
Gastro-esophageal reflux disease	K21	1,023	137	13.392	1,150	139	12.087	1.305, -1.507-4.117	**1.303**, 1.024-1.659	0.0312
Ulcer of esophagus	K22.1	1,327	12	0.904	1,344	10	0.744	0.160, -0.526-0.846	**3.677**, 1.156-11.691	0.0195
Gastritis and duodenitis	K29	1,265	57	4.506	1,315	47	3.574	0.932, -0.589-2.453	**1.578**, 1.053-2.365	0.0259
Stomatitis and related lesions	K12	1,251	36	2.878	1,339	14	1.046	1.832, 0.757- 2.907	**3.108**, 1.648-5.862	0.0002
Diaphragmatic hernia	K44	1,274	56	4.396	1,311	46	3.509	–	–	n.s.
Crohn's disease	K50	1,321	10	0.757	1,339	10	0.747	–	–	n.s.
Other chronic obstructive pulmonary disease	J44	1,261	49	3.886	1,287	52	4.040	–	–	n.s.
Sleep disorders	G47	1,069	124	11.6	1,193	126	10.562	–	–	n.s.

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TABLE 2 Continued

		Cases			Controls					
Disease	ICD10 code	N of eligible participants	N of Outcomes	Risk, %	N of eligible participants*	N of Outcomes	Risk, %	Risk difference (95% confidence interval), %	Hazard ratio (95% confidence interval)	P value
Acute kidney failure and chronic kidney disease	N17-N19	1,121	98	8.742	1,249	104	8.327	–	–	n.s.
Malignant melanoma of skin	C43	1,314	28	2.131	1,337	11	0.823	1.308, 0.389-2.227	**3.581**, 1.683-7.620	0.0004
Malignant neoplasm of liver and intrahepatic bile ducts	C22	1,311	54	4.119	1,338	32	2.392	1.727, 0.376- 3.079	**2.263**, (1.424-3.596	0.0004
Melanocytic nevi	D22	1,249	95	7.606	1,327	61	4.597	3.009, 1.157-4.862	**2.142**, 1.526-3.005	< 0.0001
Other and unspecified malignant neoplasm of skin	C44	1,244	69	5.547	1,308	40	3.058	2.489, 0.911-4.066	**2.398**, 1.582-3.636	< 0.0001
Anemia, unspecified	D64.9	1,126	119	10.568	1,261	129	10.23	–	–	n.s.
Elevated white blood cell count	D72.82	1,237	72	5.821	1,322	52	3.933	1.887, 0.214-3.561	**1.87**, 1.282-2.727	0.0010
Purpura and other hemorrhagic conditions	D69	1,246	61	4.896	1,310	36	2.748	2.148, 0.658-3.637	**2.064**, 1.348-3.16	0.0007
Iron deficiency anemia	D50	1,241	79	6.366	1,297	68	5.243	–	–	n.s.
Age-related cataract	H25	1,233	79	6.407	1,289	91	7.060	–	–	n.s.
Other cataract	H26	1,242	55	4.428	1,316	57	4.331	–	–	n.s.
Visual disturbances and blindness	H53-H54	1,151	85	7.385	1,270	90	7.087	–	–	n.s.
Glaucoma	H40-H42	1,266	38	3.002	1,303	30	2.302	0.699, -0.544-1.943	**1.762**, 1.057-2.940	0.0280
Dry eye syndrome	H04.12	1,286	49	3.81	1,325	34	2.566	1.244, -0.105-2.593	**1.923**, 1.213-3.05	0.0047
Presbyopia	H52.4	1,265	43	3.399	1,318	25	1.897	1.502, 0.262-2.743	**2.223**, 1.327-3.724	0.0019
Conjunctival scars	H11.2	1,327	13	0.98	1,344	0	0	0.98, 0.45-1.51	n.a.	0.0001
Keratitis	H16	1,311	22	1.678	1,339	10	0.747	0.931, 0.097-1.766	**2.77**, 1.247-6.156	0.0091
Disorders of choroid and retina	H30-H36	1,259	39	3.098	1,303	55	4.221	–	–	n.s.
Lupus erythematosus	L93	1,288	15	1.165	1,342	10	0.745	0.419, -0.326-1.164	**15.546**, 2.053-117.693	0.0004
Pressure ulcer	L89	1,310	32	2.443	1,336	13	0.973	1.470, 0.482-2.458	**2.942**, 1.513-5.723	0.0009

(Continued)

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TABLE 2 Continued

		Cases			Controls					
Disease	ICD10 code	N of eligible participants	N of Outcomes	Risk, %	N of eligible participants*	N of Outcomes	Risk, %	Risk difference (95% confidence interval), %	Hazard ratio (95% confidence interval)	P value
Pruritus	L29	1,111	112	10.081	1,330	61	4.586	5.495, 3.397-7.592	**2.791**, 2.010-3.877	< 0.0001
Seborrheic dermatitis	L21	1,269	49	3.861	1,334	20	1.499	2.362, 1.117-3.607	**2.688**, 1.597-4.525	0.0001
Lichen simplex chronicus and prurigo	L28	1,284	27	2.103	1,341	10	0.746	1.357, 0.447-2.267	**4.314**, 1.877-9.914	0.0002
Urticaria	L50	1,261	41	3.251	1,335	30	2.247	1.004, -0.257-2.260	**1.763**, 1.078-2.885	0.0222
Lichen planus	L43	1,308	15	1.147	1,342	10	0.745	0.402, -0.336-1.140	**6.640**, 1.744-25.278	0.0017
Chronic pain, not elsewhere classified	G89.2	1,188	164	13.805	1,260	142	11.270	2.535, -0.091-5.161	**1.631**, 1.290-2.062	< 0.0001
Acute pain, not elsewhere classified	G89.1	1,278	49	3.834	1,328	32	2.410	1.424, 0.087-2.762	**1.990**, 1.244-3.182	0.0035
Polyneuropathy, unspecified	G62.9	1,261	58	4.6	1,316	33	2.508	2.092, 0.66-3.524	**1.943**, 1.258-3.0	0.0023
Benign prostatic hyperplasia	N40	1,252	41	3.275	1,262	72	5.705	-2.43, -4.046- -0.815	**0.6**, 0.407-0.884	0.0090
Candidiasis	B37	1,219	73	5.989	1,311	40	3.051	2.937, 1.312-4.563	**2.377**, 1.592,3.55	< 0.0001
Dermatophytosis	B35	1,216	78	6.414	1,303	62	4.758	1.656, -0.142-3.454	**1.457**, 1.039-2.045	0.0283
Sepsis, unspecified organism	A41.9	1,270	51	4.016	1,326	32	2.413	1.602, 0.243-2.962	**1.713**, 1.097-2.676	0.0166
Nicotine dependence	F17	1,131	65	5.747	1,254	56	4.466	–	–	n.s.

The risk of individuals diagnosed with EBA cases to those without EBA controls to develop one of the listed diseases. Data shown displays results from measures of association excluding patients with outcome prior to the time window. For the Hazard ratio. Kaplan-Meier Analysis again excluding patients with outcome prior to the time window with Log-Rank Test was performed. N, number; n.s., not significant; n.a., not applicable; Diseases where presence of EBA increases the risk are highlighted in bold. Diseases where presence of EBA decreases the risk are highlighted in blue and bold. Diseases in which EBA does not impose a risk for subsequent disease development are displayed in gray letters. Please note that propensity score matching is re-run with each outcomes analysis so that the analysis uses the most current data available on the TriNetX network. Analysis was performed from October 1st to 3rd, 2022.

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patients (Tables 1, 2; Figure 2). Given that unspecific immunosuppression is the treatment of choice for EBA (12, 20–22), infectious diseases can be expected as sequelae of EBA. Indeed, sepsis, dermatophytosis and candidiasis were identified as sequelae of EBA. Interestingly, all were also risk factors for future EBA development: Sepsis (HR 1.94, CI 1.33-2.81, p=0.0004), dermatophytosis (HR 1.57, CI 1.14-2.15, p=0.0056) and candidiasis (HR 2.31, CI 1.64-3.27, p<0.0001).

Malignant melanoma, non-melanoma skin cancer and neoplasms of the liver and intrahepatic bile ducts are risk factors and sequelae of EBA

Regarding malignancy, 3/55 identified diagnoses fell into this category; namely, non-melanoma skin cancer, melanoma and malignant neoplasm of the liver and intrahepatic bile ducts. Whilst non-melanoma skin cancer and malignant neoplasm of the liver and intrahepatic bile ducts were both risk factors and sequelae of EBA, melanoma was not associated with a higher risk to develop EBA, but developed more frequently after an EBA diagnosis (Tables 1, 2 and Figure 2).

EBA has a considerable metabolic and cardiovascular disease risk

Metabolic and cardiovascular comorbidity significantly contributes to morbidity and mortality of CIDs (23–25). The potential metabolic and cardiovascular disease burden in EBA has, so far, not been addressed. Following the diagnosis of EBA, cerebral infarction (HR 2.04, CI 1.28-3.24, p=0.0004), deep vein thrombosis (HR 2.29, CI 11.30-4.04, p=0.0032), atherosclerosis (HR 1.44, CI 1.01-2.05, p=0.0407), heart failure (HR 1.58, CI 1.09-2.28, p=0.0147), hypertensive diseases (HR 1.29, CI 1.02-1.62, p=0.0350), type 2 diabetes mellitus (HR 1.42, CI 1.05-1.93, p=0.0245), and overweight & obesity (HR 1.36, CI 1.06-1.76, p=0.0156) developed more frequently than expected.

Discussion

We here determined risk factors and sequelae of EBA using a large-scale database. This led to the identification of so far unrecognized disease trajectories, as well as to the refutation of long-held beliefs, such as an association of inflammatory bowel disease with EBA.

Because EBA is such a rare disease, we believe that implementation of preventive measures based on the identification of risk factors is, for the most part, impracticable. For example, the identified EBA risk factors dermatophytosis and candidiasis are common diseases (26). The effort to screen for EBA in these patient populations would not justify the potential benefit. We, however, see some exceptions. These are pruritus, conjunctival scars, and visual disturbances. If in any of these, a definite cause cannot be determined, EBA should be excluded as a potential differential diagnosis following current diagnostic recommendations (20, 27). By contrast, the identified metabolic and cardiovascular risk profile of EBA patients warrants clinical implementation. Compared to controls, EBA patients were more prone to develop hypertensive diseases, atherosclerosis, cerebral infarction, heart failure, deep vein thrombosis, type 2 diabetes mellitus and overweight & obesity – for some, the risk was increased over 2-fold (Table 2 and Figure 2). This profile is in line with several other CIDs, for example SLE, rheumatoid arthritis and psoriasis (28–30). The finding of an increased frequency of deep vein thrombosis in EBA has also been made in bullous pemphigoid, where a prothrombotic state and an increased risk for venous thromboembolism had been noted (31, 32). Consequently, stringent control of known cardiovascular risk factors, and frequent screening for cardiovascular and metabolic disease should be implemented in the management of EBA patients.

We also found other CIDs to be risk factors and sequelae of EBA. Among these, lichen planus, SLE and cutaneous lupus were associated with the highest risk for both, future EBA development, and the probability of manifestation following an EBA diagnosis. Thus, it is tempting to speculate that EBA, lichen planus, SLE and cutaneous lupus share similar disease-driving pathomechanisms. These findings are also in line with previous clinical observations that noted a high prevalence of antinuclear autoantibodies in EBA patients (12, 33), and the co-occurrence of SLE with EBA, which coined the term bullous SLE (34–36).

Interestingly, inflammatory bowel diseases were not identified as risk factors or sequelae of EBA. An association of EBA with these had long been noted, with several subsequent publications supporting this assumption (5, 12, 17–19, 37). However, most of these observations were made before the establishment of the current diagnostic EBA criteria. Furthermore, these observations were based on single case reports, case report series and meta-analysis thereof. Considering the findings from our study, an association of EBA with inflammatory bowel disease seems rather unlikely.

Regarding malignancy, we think the increased risk of EBA patients to develop non-melanoma skin cancer and melanoma is due to regular and prolonged dermatological care. This is certainly not the case for malignant neoplasms of the liver and intrahepatic bile ducts, which were identified as a risk factor (HR 3.07, CI 1.74-5.31, p<0.0001) and a sequela of EBA (2.26, CI 1.42-3.60, p=0.0004). This necessitates regular screening for this malignancy, underscoring the need for a multidisciplinary care for EBA patients (38). The recently noted expression of COL7 in the liver (of mice) may explain this so far unnoticed association

(39) – albeit this finding is not sustained by other reports (40). In the human protein atlas, *COL7A1* expression has, however, been reported for the gallbladder – and to a minor extent also in the liver. Thus, autoantibody-induced inflammation in the bile ducts may promote the emergence of malignant neoplasms of the liver and intrahepatic bile ducts.

Our study has several limitations to be acknowledged. First, patient electronic health record data may suffer from misdiagnosis and/or miscoding and do not encompass all possible confounding factors. In our dataset this is underscored by the diagnosis of benign prostatic hyperplasia, where close to 0.5% of individuals with this diagnosis were coded to be female (Supplement Table 51). Second, the TriNetX database provides access to medical data from individuals who had medical encounters with healthcare systems. Thus, our analysis does not include patients with low access to healthcare facilities. Third, in the risk factor analysis, the results must be interpreted with caution if the number of EBA cases is low.

In conclusion, the use of TriNetx allowed to define risk factors and sequalae of an orphan disease, here exemplified by EBA. The identified interactions have clinical implications for the management of EBA and point towards shared pathogenic pathways among different CIDs. We envision that the here described methodology will serve as a blueprint to identify risk factors and sequelae for numerus orphan diseases.

This work has only been possible because of Detlef Zillikens. Since 2004, he continuously and methodically developed a research infrastructure for pemphigus and pemphigoid diseases at the University of Lübeck. In doing so, he spread his enthusiasm for pemphigus and pemphigoid research to improve the diagnosis and treatment of patients suffering from these diseases to those around him. All authors decided to come to Lübeck because of Detlef Zillikens. We truly miss him as an inspiring and always motivating mentor and friend, and now seek to continue his mission in pemphigus and pemphigoid.

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

Conceptualization: KK, RL; Investigation: All authors; Illustrations: AV, KB; Project Administration: RL; Resources: RL; Writing - Original Draft Preparation: RL; Writing - Review and Editing: All authors. All authors contributed to the article and approved the submitted version

Funding

This research was funded by the Cluster of Excellence "Precision Medicine in Chronic Inflammation" (EXC 2167), the Collaborative Research Centre "Pathomechanisms of Antibody-mediated Autoimmunity" (SFB 1526), the Research Training Group "Autoimmune Pre-Disease" (GRK 2633), all from the Deutsche Forschungsgemeinschaft and the Schleswig-Holstein Excellence-Chair Program from the State of Schleswig Holstein.

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.2022.1103533/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Case Report
PUBLISHED 10 February 2023
DOI 10.3389/fimmu.2023.1121380

Check for updates

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 11 December 2022
ACCEPTED 24 January 2023
PUBLISHED 10 February 2023

CITATION
Li T, Liu H and Li W (2023) Case report: Vesiculobullous eruption with an atypical IgA deposition pattern in a patient with multiple myeloma—A case report and literature review.
Front. Immunol. 14:1121380.
doi: 10.3389/fimmu.2023.1121380

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Case report: Vesiculobullous eruption with an atypical IgA deposition pattern in a patient with multiple myeloma—A case report and literature review

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Cutaneous vesiculobullous eruptions associated with multiple myeloma (MM) are rare. Although the development of blisters is mostly driven by amyloid deposits of paraproteins in the skin, autoimmunity may play a role. In this study, we report an unusual case of an MM patient with blisters who presented with both flaccid and tense vesicles and bullae. Direct immunofluorescence revealed IgA autoantibody deposits in the basement membrane zone (BMZ) and intercellular space of the epidermis, which displayed an atypical autoantibody deposition pattern. The patient showed rapid disease progression and died during follow-up. We performed a literature review of autoimmune bullous diseases (AIBDs) associated with MM or its precursors and found 17 previously reported cases. Together with the present case, cutaneous involvement of the skin folds was frequently reported, and mucous membranes were barely affected. IgA pemphigus, with consistent IgA monoclonality, was observed in half of the cases. Five patients displayed atypical autoantibody deposition patterns in the skin; the prognosis of these patients appeared to be poorer than that of other patients. We aim to increase our understanding of AIBDs associated with MM or its precursors.

KEYWORDS

multiple myeloma, autoimmune bullous disease, IgA autoantibodies, monoclonal gammopathy, direct immunofluorescence

Introduction

Multiple myeloma (MM) is a malignant disorder of plasma cells that typically manifests as bone pain, anemia, and renal failure in the presence of paraproteins. Skin symptoms are rarely observed in patients with this disorder. Clinically, skin rashes associated with MM can be classified as specific or non-specific. Although cutaneous plasmacytoma is a specific skin

condition associated with MM, cutaneous amyloidosis, Sweet's syndrome, and pyoderma gangrenosum (PG) are common non-specific skin findings in MM patients (1).

Vesiculobullous eruptions are a group of infrequent non-specific dermatoses associated with MM, and their etiology is generally ascribed to the existence of paraproteins (2). Although most cases of vesiculobullous eruptions are induced by amyloid deposits of paraproteins in the skin (3), autoimmunity reportedly plays a role. Autoantibodies against skin antigens can be observed in some patients with MM, resulting in autoimmune bullous diseases (AIBDs). Based on antigen localization, AIBDs can be divided into pemphigus (antigens found in the intercellular space of the epidermis) and pemphigoid [antigens found in the basement membrane zone (BMZ)] diseases. These two entities are considered completely different from each other owing to their distinct clinical features and autoantibody deposition patterns.

In this case report we describe a unique presentation of MM with multiple skin blisters. The patient presented with acute onset of vesiculobullous eruptions. A skin biopsy revealed subepidermal blisters with distinct leukocytoclastic vasculitis; direct immunofluorescence (DIF) revealed IgA autoantibodies in the epidermis and BMZ. Thus, it could not be categorized as pemphigus or pemphigoid disease. The patient showed rapid disease progression and died soon after the diagnosis. In addition, we performed a review of AIBDs associated with MM, or its precursors, and summarized the clinical features, DIF and hematoxylin–eosin (H&E) staining findings, and prognosis. This report aims to increase our understanding of the AIBDs associated with MM or its precursors, to raise clinicians' awareness of the development of monoclonal gammopathy in patients with IgA bullous dermatosis, and to reinforce the importance of closer follow-up in patients with atypical autoantibody deposition patterns in the skin.

Case report

A 55-year-old Chinese female was referred to the emergency room (ER) at the West China Hospital, Sichuan University. She had complained of an acute onset of pruritic vesiculobullous eruptions on her trunk and extremities 2 weeks before admission, and these eruptions originated primarily from the bilateral inframammary folds. The patient also complained of severe persistent pain in both thighs. Progressive asthenia, fatigue, and weight loss were also observed. She denied having fever, dyspnea, digestive disorders, or any other systemic symptoms. No other significant medical, family, genetic, psychosocial, or past histories were elicited. She had initially been diagnosed with eczema in the local hospital and treated with topical halometasone, but experienced minimal improvement. Physical examination revealed multiple flaccid vesicles, erosions, and scattered pustules on the inframammary folds, the hypochondrium area, and the left supraclavicular area. Some bullae resembled "half–half" blisters (Figures 1A–C). The Nikolsky sign for these lesions was positive. Scattered hemorrhagic bullae with negative Nikolsky signs, petechiae, and purpura were observed on her hands and lower legs (Figure 1D). No mucous membranes were involved. The muscle strength of both lower limbs was grade 4. No significant muscle tenderness or joint swelling was observed.

The skin biopsy of the hemorrhagic bulla revealed a subepidermal blister and distinct leukocytoclastic vasculitis, primarily affecting

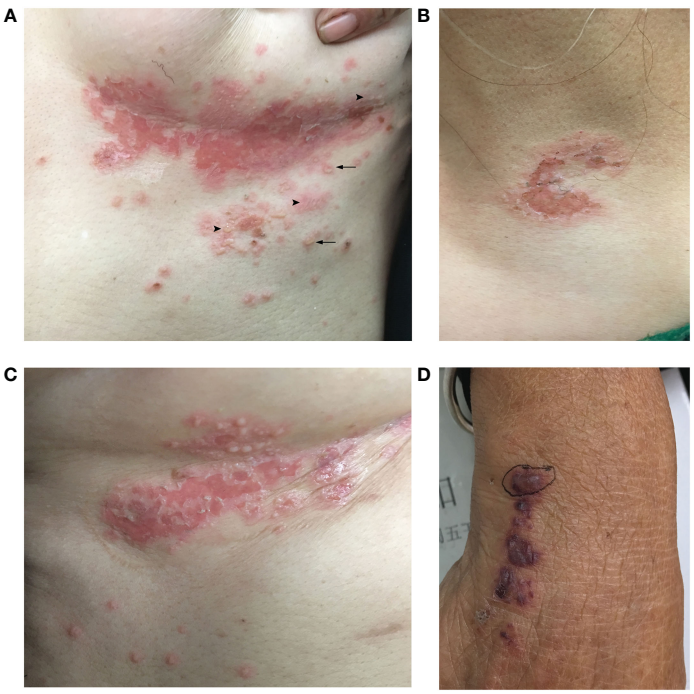

FIGURE 1
Clinical skin manifestation of the patient. **(A)** Multiple flaccid vesicles, pustules, and erosions on the left inframammary fold. Arrowhead: multiple flaccid vesicles and bullae. Arrow: a few pustules with the appearance of "half–half" blisters. **(B)** Erosion with erythema over the left supraclavicular area. **(C)** Multiple vesicles and erosions on the right inframammary fold. **(D)** Purpura and a hemorrhagic bulla on the right hand.

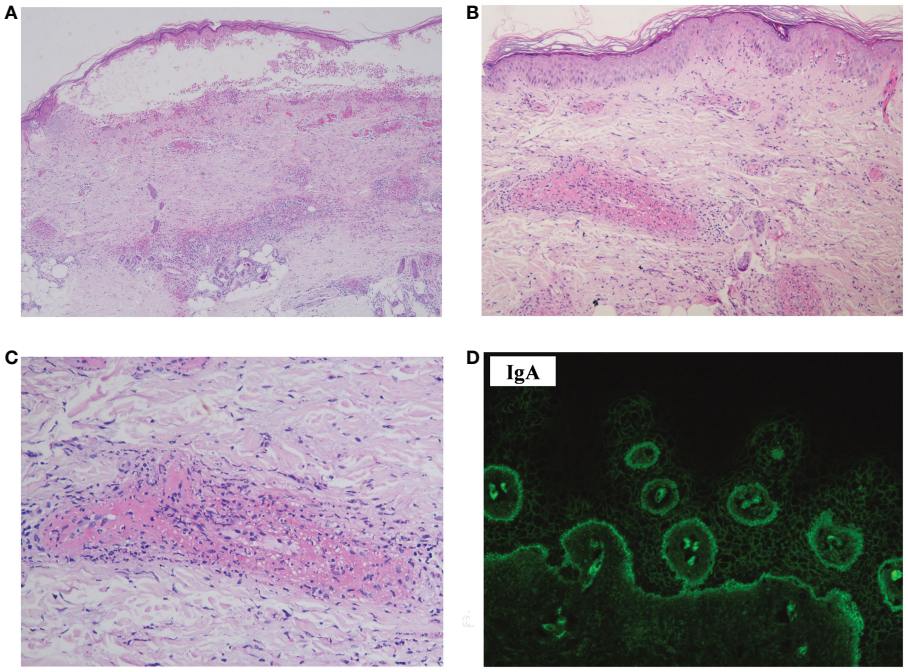

FIGURE 2
Histopathology, DIF, and immunohistopathology of the skin biopsy sample. **(A)** Hematoxylin–eosin staining showed a subepidermal blister, with perivascular infiltration of inflammatory cells in the dermis (×40). **(B)** Leukocytoclastic vasculitis affecting small-to-medium-sized vessels were observed in the dermis (×100). **(C)** Leukocytoclasis forming nuclear dust and fibrinoid necrosis of the vessels (×200). **(D)** DIF revealed deposits of IgA in the intercellular space of the epidermis and BMZ (×400). DIF, direct immunofluorescence.

small to medium-sized vessels in the dermis (Figures 2A–C). Acantholysis was not observed. DIF revealed deposits of IgA autoantibodies in the BMZ and the intercellular space of the epidermis (Figure 2D). However, these did not meet the diagnostic criteria for any specific type of IgA-associated bullous disease. IgM and C3 deposits were also detected in the BMZ; IgG deposits were not found (data not shown).

Laboratory investigations on the day she arrived suggested anemia; her hemoglobin level was 93 g/L, and this steadily decreased to 76 g/L over the next 4 days. Severe impairment of renal function was detected (with a serum creatinine level of 558 μmol/L). Circulating antibodies against either desmoglein (Dsg) 1/3 (Dsg1/Dsg3) or BP180 were not detected by enzyme-linked immunosorbent assay (ELISA) (MBL, Japan). Anti-nuclear antibody (ANA), extractable nuclear antigen (ENA), and anti-neutrophil cytoplasmic antibody (ANCA) panels were negative. Total protein (66.1 g/L; normal: 65.0–85.0 g/L) and globulin (34.1 g/L; normal: 20.0–40.0 g/L) levels were normal. Her IgA level was elevated (3,140.0 mg/L; normal: 836–2900 mg/L), whereas IgG (7.27 g/L; normal: 8–15.5 g/L) and IgM (670.0 mg/L; normal: 700–2,200 mg/L) levels were slightly lower than normal. Monoclonal IgA-κ light chains were detected in the serum by immunofixation electrophoresis. A computed tomography (CT) scan revealed mild pneumonia and multiple nodular lytic lesions in the thorax, axial skeleton, and pelvis. Following bone marrow aspiration and biopsy, the patient was diagnosed with IgA-κ MM. An acute kidney injury associated with MM was also suspected. Further laboratory investigations revealed monoclonal κ-light chains in the dermis, as

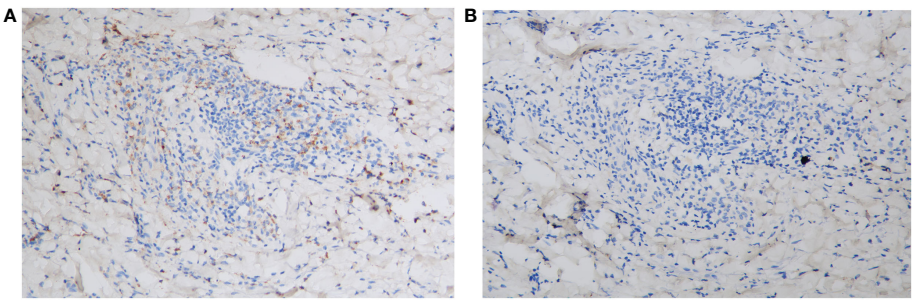

FIGURE 3
Immunohistopathology of the skin biopsy specimen. **(A)** The monoclonal κ-light chain was positive (×200). **(B)** The monoclonal λ chain was negative (×200).

detected by immunohistopathology (Figures 3A, B); amyloids were not detected with Congo red staining (data not shown). During her short stay (i.e., less than 1 week) in the ER, the patient received symptomatic treatments, including vigorous rehydration, electrolyte imbalance correction, and antibiotics (moxifloxacin) for pneumonia. However, after the diagnosis was confirmed, the patient refused a referral to the hematological department and instead asked for a transfer to the local hospital. She died 1 month later.

Discussion

In this case study we have reported an unusual presentation of vesiculobullous eruptions in a patient with MM. IgA autoantibodies were observed in both the epidermis and the BMZ, which is an atypical deposition pattern. To the best of our knowledge, only one report of MM has previously described a similar deposition pattern in the skin (4), and the IgA autoantibody was seen in the intercellular space of the basal layer, which was not completely the same as in our case.

Varied lesions were observed in this patient. Although the presence of vesicles and erosions suggested a bullous etiology, purpuras over her extremities, indicating an infarctive vascular etiology, were also observed. This may correspond to the histologic findings of distinct leukocytoclastic vasculitis, which have been reported in patients with MM (5). Although a KOH smear test and/or fungal cultures were not taken at the inframammary area, candidiasis and intertrigo were ruled out based on the cutaneous involvement of multiple skin areas and the classic morphology of "half–half" bullae, resembling IgA pemphigus (6). Another differential diagnosis considered was the paraneoplastic pemphigus (PNP) (7). The features of PNP mainly include intractable stomatitis, acantholysis accompanied by interface dermatitis and necrotic keratinocytes, deposition of IgG and C3 in the epidermis and BMZ, and anti-Dsg1/3 and anti-BP180 autoantibodies detected by ELISA (8). Given that our patient did not show the above features of PNP, this diagnosis could be ruled out.

To date, there have been approximately 70 case reports of vesiculobullous lesions associated with MM. The most common dermatosis is bullous amyloidosis, which is caused by cutaneous deposition of light chains originating from neoplastic plasma cells (3). AIBDs associated with MM are rare. We performed a literature review, using PubMed® (National Library of Medicine, Bethesda, MD, USA) and Google Scholar (Google, Mountain View, CA, USA), and found only 17 previously reported cases of AIBDs associated with MM (11/17) or its precursors (6/17), such as monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma (9) (Table 1). Together with our present case, the median age at which monoclonal gammopathy develops was 60.5 years (range 32–92 years), with approximately equal occurrence in males and females. Among these cases, cutaneous lesions mostly appeared as bullae, vesicles, pustules, and superficial erosions without signs of undermined ulcers. Only one study reported the coexistence of PG (10). In general, lesions can begin several months to years prior to monoclonal gammopathy. Skinfold (i.e., axillae, groin, and inframammary folds) involvement was frequently reported (8/18); mucous membranes were rarely affected (1/18). The IgA type of monoclonal gammopathy, with consistent IgA type of autoantibody deposition in the skin, was observed in 15 out of the 18 cases. Distinct infiltration of neutrophils was found in the skin lesions of all patients with IgA monoclonal gammopathy. Based on the autoantibody deposition pattern, IgA pemphigus was the most frequently reported (i.e., 9/18), followed by pemphigus vulgaris (i.e., 2/18), pemphigus foliaceous (i.e., 2/18), and linear IgA bullous dermatosis (i.e, 1/18). Five cases (4, 10, 16, 20), including ours, demonstrated an atypical IgA deposit pattern in the skin, which did not meet the diagnostic criteria for any specific type of IgA bullous disease. A common feature of these cases was that the patients either responded poorly to treatment (20) or presented with disease progression (4, 16), and all died during follow-up (Table 1). Owing to the small sample size, we cannot definitively state that the poor prognosis was related to the unusual autoantibody deposition pattern; further studies are still required.

The relationship between autoantibodies against the skin and paraproteins remains unclear. Generally, monoclonal immunoglobulins do not bind to any autologous antigens (18). However, Espana et al. (21) discovered in a patient with IgA pemphigus and IgA-κ MM that the autoantibodies against the skin were monoclonal IgA-κ, suggesting that paraproteins might play a role in the pathogenesis of AIBDs. As our patient presented with similar clinical features, we speculated that the κ-light chains found in the dermis might have the same origin as IgA in the epidermis and BMZ, that is, aberrant plasma cells. The specific antigens in the epidermis or BMZ, against which autoantibodies are formed, remain unclear. In one report, the authors discovered Dsg1/Dsg3 and desmocollin (Dsc) 1–3 autoantibodies for IgA with ELISA-based methods (25). However, the results of ELISAs for anti-Dsg1/Dsg3 and anti-BP180 were negative in our patient. This phenomenon may be explained by the fact that only IgG autoantibodies can be detected using commercial ELISA kits, whereas the main deposits in our patient's skin were IgA. Nevertheless, in two other studies, despite using multiple laboratory analyses, such as ELISA of Dsg1/Dsg3 and Dsc 1–3 for IgG/IgA and immunoblotting for IgA/IgG, no skin antigens were conclusively identified (13, 21). The major limitation of the present case report is that owing to the rapid progression of the patient's condition, we were not able to preserve the patient's serum in time for specialized laboratory tests, such as indirect immunofluorescence, immunoblotting, or immunoprecipitation. Further studies are needed to explore the molecular basis and pathogenesis of AIBDs associated with MM or its precursors.

According to our review, the autoantibodies deposited in the skin, and which display a typical or atypical pattern, are mostly of the IgA type. In addition, concomitant monoclonal gammopathy shows a predilection for the IgA-κ subtype in patients with MM. This implies that IgA-κ monoclonality, rather than other subtypes of monoclonality, may be more closely related to MM-associated IgA dermatosis (25). Histologically, distinct neutrophilic infiltration was observed in all these patients. A recent study found that IgA immune complexes can prime polymorphonuclear neutrophils and lower the threshold for Neutrophil extracellular traps release (NETosis) (26),

TABLE 1 Summary of the clinical features of AIBDs associated with MM or its precursors.

Study (first author and year of publication)	Age (years)	Sex	Onset of skin lesions	Distribution of lesions	Mucous membrane involvement	Manifestation of PG	Monoclonal gammopathy subtype	Location of Ig deposits	Dominant inflammatory cells	Diagnosis of lesions	Hematological disorders	Outcome
Wallach, et al., 1982 (10)	92	F	Almost at the same time	Face, limb, trunk, axillary and inguinal folds	No	Yes	IgA-κ	Linear IgA deposition pattern in the epidermis	Neutrophils	SPD	MGUS	Died in hospital
Burrows, et al., 1984 (11)	73	M	2 years prior to MM	Axillae, groin	No	No	IgA-κ	Intercellular IgA in the epidermis	Neutrophils	IgA pemphigus†	MGUS	Controlled
Iwatsuki, et al., 1988 (12)	58	F	8 years prior to MM	Axillae, trunk, and limbs	No	No	IgA	Intercellular IgA in the epidermis	Neutrophils	IgA pemphigus†	MGUS	Controlled
Takata et al., 1994 (13)	59	F	13 years prior to MM	Neck, axillae, groin	No	No	IgA-κ	Intercellular IgA in the epidermis	Neutrophils	IgA pemphigus†	MM	Complete remission
Miyagawa, et al., 1995 (14)	60	F	3 months prior to MGUS	Lateral trunk, dorsal hands, fingers	No	No	IgA-λ	Intercellular IgG, IgA in the epidermis	Neutrophils	Pemphigus foliaceus and IgA pemphigus	MGUS	Controlled
Aste, et al., 2003 (15)	45	M	3 years prior to MGUS	Trunk, extremities, and intertriginous area	No	No	IgA-λ	Intercellular IgA in the epidermis	neutrophils	IgA pemphigus	MGUS	Controlled
Wong et al., 1999 (16)	63	M	5 months prior to MM	Legs, sacrum, buttocks, extensor of the elbow	No	No	IgA-κ	IgA in the dermis	Neutrophils	Undefined	MM	Died 1 year after the first visit
Kurokawa et al., 2005 (17)	62	F	NR	Trunk and lower gingiva	Yes	No	IgG-λ	Intercellular IgG and C3 in the epidermis	Eosinophils	Pemphigus vulgaris	MM	Controlled
Barnadas et al., 2010 (4)	70	M	4 months prior to MM	Thighs and legs	No	No	IgA-λ	Intercellular IgA in the epidermis and BMZ	Neutrophils	Undefined	MM	Died 2 years after the first visit
Szturz et al., 2011 (18)	61	F	6 years prior to MM	Trunk and extremities	No	No	IgA-λ	Intercellular IgA in the epidermis	Neutrophils	IgA pemphigus	MM	Skin lesions improved with hematologic response

(Continued)

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TABLE 1 Continued

Study (first author and year of publication)	Age (years)	Sex	Onset of skin lesions	Distribution of lesions	Mucous membrane involvement	Manifestation of PG	Monoclonal gammopathy subtype	Location of Ig deposits	Dominant inflammatory cells	Diagnosis of lesions	Hematological disorders	Outcome
Sung et al., 2012 (19)	76	F	27 years prior to MM	Scalp, trunk, and extremities	No	No	IgA-κ	Intercellular IgA in the epidermis	Neutrophils	IgA pemphigus	MM	Controlled
Battistella, et al., 2013 (20)	87	F	Almost at the same time	Legs, trunk, hands, face, scalp	No	No	IgA-λ	IgA in the dermis	Neutrophils	Undefined	MM	Died 3 years after the first visit
Espana et al., 2015 (21)	32	M	2 years prior to MM	Trunk, limbs, and skinfolds	No	No	IgA-κ	Intercellular IgA in the epidermis	Neutrophils	IgA pemphigus	MM	Controlled
Yamaguchi, et al., 2017 (22)	73	M	Almost at the same time	Back, elbow, ankle	No	No	IgA-λ	IgA in the BMZ	Neutrophils	LABD	MGRS	Controlled
Lee et al., 2017 (23)	53	M	2 years prior to MM	Trunk and axilla	No	No	IgA-κ	Intercellular IgG in epidermis	NR	Pemphigus foliaceus-like exanthem	MM	Rapid improvement
Sendrasoa et al., 2018 (24)	55	M	2 months prior to MM	Trunk and scalp	No	No	IgG-λ	Intercellular IgG in the epidermis	Neutrophils and eosinophils	Pemphigus vulgaris	MM	Remission maintained for 12 months
Koga et al., 2022 (25)	42	F	7 years prior to MM	Trunk and lower leg	No	No	IgA-κ	Intercellular IgA in the epidermis	Neutrophils	IgA pemphigus	MM	Controlled
Present case	55	F	Almost at the same time	Trunk, limbs, and inframammary fold	No	No	IgA-κ	Intercellular IgA in the epidermis and BMZ	Neutrophils	Undefined	MM	Died 1 month after the visit

MM, multiple myeloma; BMZ, basement membrane zone; F, female; M, male; NR, not reported; PG, pyoderma gangrenosum; SPD, subcorneal pustular dermatosis; MGUS, monoclonal gammopathy of undetermined significance; MGRS, monoclonal gammopathy of renal significance; LABD, linear IgA bullous dermatosis.

† The cutaneous lesions in these cases were originally described as subcorneal pustular dermatosis (SPD). However, based on the patients' clinical manifestation and DIF results, the SPD subtype of IgA pemphigus can be considered.

10.3389/fimmu.2023.1121380

suggesting that a more active neutrophil chemotactic state may exist in patients with IgA MM.

Conclusion

The present case and our literature review can increase our understanding of the coexistence of AIBDs and MM or its precursors. Clinicians should be aware of the development of monoclonal gammopathy in patients with IgA bullous diseases. Furthermore, screening for AIBDs in patients with MM and vesiculobullous eruptions is of great importance. Meanwhile, for patients with MM and AIBDs, atypical autoantibody deposition patterns within the skin may require special attention and closer follow-up to evaluate their prognosis.

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

Ethics review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

Author contributions

TL was responsible for writing the article. HL was responsible for histopathology support. WL was responsible for article revision. All authors contributed to the article and approved the submitted version.

Funding

This study is supported by the grant from the Natural Science Foundation of China, Sichuan (Grant No. 2022NSFSC1582).

Acknowledgments

We would like to thank Prof. Gao and Prof. Jian from the Pathology Department of West China Hospital for their generous support.

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 16 February 2023
DOI 10.3389/fimmu.2023.1104360

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 21 November 2022
ACCEPTED 30 January 2023
PUBLISHED 16 February 2023

CITATION
Schauer F, Tasiopoulou G, Schuster D, Behrens M, Müller S and Kiritsi D (2023) Primate liver tissue substrate in indirect immunofluorescence diagnostics for patients with dermatitis herpetiformis and celiac disease.
Front. Immunol. 14:1104360.
doi: 10.3389/fimmu.2023.1104360

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Primate liver tissue substrate in indirect immunofluorescence diagnostics for patients with dermatitis herpetiformis and celiac disease

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Introduction: Dermatitis herpetiformis (DH) is a rare autoimmune, polymorphous blistering disorder, characterized by severe itch or burning sensation, which represents the cutaneous manifestation of celiac disease (CD). The current estimation of DH versus CD is around 1:8 and the affected individuals have a genetic predisposition. Pathogenetically, IgA autoantibodies against the epidermal transglutaminase, an essential constituent of the epidermis, cause DH and are reported to develop through cross-reaction with the tissue transglutaminase, with IgA auto-antibodies causing CD. Immunofluorescence techniques allow for a rapid diagnostics of the disease using patient sera. Evaluation of IgA endomysial deposition with indirect immunofluorescence on monkey oesophagus is highly specific, but moderately sensitive, with some operator-dependent variability. Recently, indirect immunofluorescence with monkey liver as a substrate has been proposed as an alternative, well-functioning diagnostic approach with higher sensitivity in CD.

Methods: The objective of our study was to evaluate whether monkey oesophagus or liver tissue shows advantage for diagnostics in patients with DH, compared to CD. To that end, sera of 103 patients with DH (n=16), CD (n=67) and 20 controls ere compared by 4 blinded experienced raters.

Results: For DH, we found a sensitivity of 94.2% for monkey liver (ML) compared to 96.2% in monkey oesophagus (ME), while specificity in ML was superior (91.6% versus 75%) to ME. In CD, ML had a sensitivity of 76.9% (ME 89.1%) and specificity of 98.3% (ME 94.1%).

Discussion: Our data show that ML substrate is well suitable for DH diagnostics.

KEYWORDS

monkey oesophagus, monkey liver, gluten, Duhring, transglutaminase

Introduction

Dermatitis herpetiformis (DH) is a polymorphic, subepidermal blistering disease with severe itch or burning sensation, considered to represent the cutaneous manifestation of celiac disease (CD) (1). The blistering mostly affects elbows, knees and buttocks (2). CD is a chronic, small-intestinal T-cell-mediated enteropathy, caused by reaction to dietary gluten and presenting with diarrhoea, unexpected weight lost and vague abdominal discomfort (3, 4). The disease typically occurs in genetically predisposed persons (*HLA-DQ2* or- *DQ8*) with frequent onset in childhood, while is has also been described as an immune therapy-related adverse event (5). Not all patients with CD suffer from DH, the current estimations are at 1 to 8 (6). In general, DH is a rare disease diagnosed in 11.2 to 75.3 per 100,000 people in the United States and Europe with an incidence of 0.4 to 3.5 per 100,000 people per year (7, 8). Whereas DH can manifest solely in the skin, most patients have some degree of histologic features of CD in their small bowel, while in most cases the gastroenterological symptoms are minor (8).

Pathogenetically, CD has been linked to development of the following main types of autoantibodies, namely IgA autoantibodies against tissue and epidermal transglutaminases (TG2, TG3), IgA- and IgG- autoantibodies against deaminated gliadine peptides (dGP) and IgA autoantibodies against the endomysium (EMA) (9). TG2 and IgA-EMA autoantibodies account for around 95% in serological screening; dGP IgA/IgG testing was shown to improve accuracy (10). TG2 is expressed in basal keratinocytes, dermal capillaries, as well as blood vessel walls and small bowel, while TG3 is found in epidermis, oesophagus, brain, the eyes and lowly expressed in the small intestine (2, https://www.proteinatlas.org/). TG2 modifies gluten to gliadin in colon (11), while the main function of TG3, the dominant autoantigen in DH, is the maintenance of the cornified envelope integrity (12). It shows homology to TG2 within the enzymatic active domains (13). The current view of the DH pathogenesis is that patients with DH develop autoantibodies against both TG2 and/or solely against TG3, while individuals with CD have mainly autoantibodies against TG2 (13–15). It has been suggested that DH develops as a result of prolonged gluten exposure and an untreated CD, however no data exist on why antibodies against TG2 and TG3 develop in parallel, or if TG3 merely becomes targeted *via* gradual loss of antigen specificity against TG2 in a subset of individuals with CD (14).

The diagnosis of DH is often difficult and prolonged. Studies revealed a mean time of 3 years between development of the rash and diagnosis, which is significantly increased for female patients (16). An important factor affecting this, is the duration time of undiagnosed preceding CD, partly misdiagnosed as irritable bowel disease (16). The DIF is gold standard with a sensitivity of around 94% (17). It shows micro-granular-fibrillar deposition of IgA (IgA- TG3 complexes), most prominently found at the tips of the dermal papillae, within vessels of the dermal papillae and along the dermoepidermal junction zone (DEJ) (2, 13). Other possible findings are isolated reactivity of C3 at DEJ or IgA deposits along the DEJ of the hair follicles (2). The biopsy site is of vital importance, since the pathognomonic IgA deposits are significantly increased in perilesional, non-affected skin (18). Nevertheless, false positivity of DIF can occur in CD patients without DH characteristic skin changes (2). Serological testing has gained an important role in diagnosis of DH, due to the high specificity and easier application (15). Specifically, immunofluorescence assays of antibodies against endomysium on primate, specifically monkey oesophagus (ME) sections represent the gold standard in serological testing for DH, but also CD (19). The reactivity focuses on the connective tissue layers around the smooth muscle fibers of the lamina muscularis mucosae and tunica muscularis, which highly express TGs. Recently, an immunofluorescence assay with primate liver tissue (monkey liver, ML) was proposed as an alternative, well-functioning diagnostic approach with higher sensitivity in CD (19). On this substrate the antibodies bind on the vessels of the liver, called the sinusoids (Vv. intralobulares) (19). The objective of our study was to evaluate whether ME or ML as substrates show advantage for diagnostics in patients with DH and CD and to gain insights if a correlation to the antibody titers against TG and gliadins exists when employing ME or ML, irrespective of the disease activity.

Materials and methods

Serum samples and ethic approval

In total 103 human sera of different patients (DH, n=16, CD, n=67, control=20) were collected between 2008 and 2022 in accordance with the principles of the Declaration of Helsinki and the Ethics Committee University of Freiburg. Blood from residual sera was used, which had no further diagnostic purposes and therefore informed consent was waived, based on Ethics Committee decision (reference no 235/15). In all DH patients diagnosis was made based on clinical picture, histology and IgA deposits on DIF, according to international S2k guidelines (2). Sera collection time point was variable: at initial presentation or at a follow up visit, however some disease activity had still to be present. Sera from the same patient at different time points were not used, in this case the serum from the initial presentation was analysed. Control sera were collected from patients with pruritic skin disorders. Data on age, sex and comorbidities, like diabetes mellitus type I or irritable bowel disease were not collected. The dietary status and specifically the gluten free intake had not been recorded for all patients and were thus not used in the evaluation.

Raters

Four raters with experience of at least 4 to 10 years in immunofluorescence diagnostics participated in this study and evaluated slides with ME and ML substrates in a blinded manner. Both tests were implemented to the routine already at the time of investigation. The individual results were correlated to serological data of celiac specific autoantibodies (anti-TG2 IgA, dGP IgG and dGP IgA) and defined as true positive (TP), true negative (TN) or false positive (FP) and false negative (FN) per rater.

ELISA

The levels of serum IgA autoantibodies against TG2 were assessed with anti-TG2 ELiA (Thermo Science, 14-5517-01) with

manufacturer's cut off value of > 10 U/ml. Cut off values of anti-dGP IgG and IgA are at > 10 U/ml (Thermo Science, ELiA GliadinDP IgG, 14-5538-01; ELiA GliadinDP IgA, 14-5539-01). All measurements were made using the programmable ELISA reader Phadia™ 250.

Indirect immunofluorescence

Cryostat sections for ME and ML were processed as described in manufacturer's manual (ME Inova; FC 1914-1005; ML Euroimmune, Lübeck, Germany). Immunofluorescence patterns were evaluated with Nikon Eclipse 80i microscope. For visualization, pictures were taken with the imaging software NIS-Element.

Statistics

Descriptive statistics was performed using Excel. Further, inductive statistical analyses were performed using R (R version 4.1.3) to compute sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of our results. Therefore, 95% confidence intervals (95% CI) for rater diagnostic statistics were computed using a Wilson Score interval. Fleiss' kappa was used to evaluate the interrater reliability of agreement among these two systems. Associations in the results between tests were assessed using McNemar's test for correlated proportions with continuity correction in case of low cell numbers. Level of significance was considered at $P < .05$.

Results

Dermatitis herpetiformis

Eight out of 16 DH (50%) sera were positive for anti-TG2 IgA, anti-dGP IgA and IgG autoantibodies. Three out of 16 (19%) patients showed anti-TG2 IgA and anti-dGP IgG positivity and anti-dGP IgA negativity, one serum (6%) was anti-TG2 IgA, anti-dGP IgA positive, one serum showed anti-TG2 IgA reactivity only, whereas three out of 16 (19%) sera were completely negative (see Table 1). The mean values of the ELISA analyses used are shown in Table 2.

Celiac disease

For the diagnostics 67 sera of different patients with CD were available. Sixteen out of 67 (24%) were positive for anti-TG2 IgA and anti-dGP IgA and IgG; fourteen sera (21%) had anti-TG2 IgA antibodies only; three sera (5%) were positive for anti-TG2 IgA and anti-dGP IgG and seven sera (10%) were positive for anti-TG2 IgA and anti-dGP IgA. Four sera (6%) were positive for anti-dGP IgA and IgG. Fourteen patients (21%) had solely anti-dGP IgA, and 9 patients (13%) had anti-dGP IgG antibodies only. We had no negative sera in our CD cohort (see Table 1).

The mean values of the ELISA analyses used are shown in Table 1. In general, the anti-TG2 IgA autoantibody titers were significantly higher (P=.0392) in the DH group than the CD group, probably because most of them were treatment naïve at sera collection. The titers of the other autoantibodies, however, were similarly distributed within both groups.

Control sera

Twenty control sera were used from patients with pruritic skin disorders. IIF with the ME and ML substrates showed negative results in all these samples. ELISA results are shown in Tables 1, 2. Note that besides anti-dGP IgA, which were found slightly increased in 3 patients (15%), all other patients (n=17, 85%) had negative ELISA results.

Rater decisions

In total, 332 decisions have been made for all DH/CD sera. All decisions have been summarized in Table 3. For all DH samples the raters had a Fleiss' kappa of 0.60 in ME and 0.63 in ML, which is a moderate and substantial agreement based on Landis and Koch,

TABLE 1 Number of examined sera used with the respective positive ELISA results for anti-TG2 IgA, anti-dGP IgG and/or anti-dGP IgA, and number of negative sera.

Parameter	DH (%)	CD (%)	Control (%)
No. of sera	16	67	20
TG2 IgA, dGP IgG, dGP IgA	8 (50)	16 (24)	–
TG2 IgA, dGP IgG	3 (19)	3 (4)	–
TG2 IgA, dGP IgA	1 (6)	7 (10)	–
TG2 IgA	1 (6)	14 (21)	–
dGP IgG, dGP IgA	–	4 (6)	–
dGP IgG	–	9 (13)	–
dGP IgA	–	14 (21)	3 (15)
negative	3 (19)	–	17 (85)

CD, celiac disease; DH, dermatitis herpetiformis; TG2, tissue transglutaminase; dGP, deaminated gliadine peptides.

TABLE 2 Mean ELISA levels of examined sera.

Parameter	DH	CD	Control
No. of sera	16	67	20
ELISA Score (U/ml)			
TG2 IgA (mean ± SD)	71.9 ± 52.1	41.9 ± 51.4	0.8 ± 0.9
dGP IgG (mean ± SD)	36.6 ± 43.1	33.8 ± 56.8	0.5 ± 0.3
dGP IgA (mean ± SD)	18.9 ± 18.7	24.7 ± 34.3	3.0 ± 4.8

CD, celiac disease; DH, dermatitis herpetiformis; SD, standard deviation; TG2, tissue transglutaminase; dGP, deaminated gliadine peptides.

respectively (20). The raters' results revealed a sensitivity of 96.2% for ME compared to 94.2% in ML (P=1). In contrast, the specificity was higher for ML evaluation compared to ME (ML: 91,7% versus ME: 75%, P = .48), although not significant. Rater decisions on control samples were not taken into consideration for this analysis, since they were all negative.

Interestingly, one serum that was rated positive by some raters showed a different pattern than the characteristic honeycomb-like, endomysial staining in the ME sections, this patient has been excluded from the further analysis (Figures 1A–C). This hints one limitation of the ME substrate, namely that SMA autoantibodies might easily be misinterpreted as EMA antibodies. The diagnostic sensitivity and specificity of ML and ME, as well as positive and negative predictive values for the analyses, are shown in Table 3.

Univariate anti-TG2 IgA positivity

When considering only anti-TG2 IgA positivity, our analysis showed that ML has generally fewer false positive results (Table 4). It shows that the specificity is significantly higher for ML compared to ME (ME: 0.812 (95% CI 0.57 - 0.934), ML: 1.0 (95% CI 0.806 - 1.0), P=.00). Further, Fleiss' kappa was 0.32 and 0.51 for ME and ML, respectively. This indicates a difference regarding rater accordance within the anti-TG2 IgA positivity subset. As a limitation, sera of DH were not considered separately, since sample size was only one serum. Thus, the main data were based on sera of CD. Generally, when assessing our data based only on anti-TG2 positivity, it appears that sensitivity is lower compared to the whole data set. Thus, there is a difference regarding the rater diagnostic metrics for the subset of positive IgA titers compared to all probes.

Positivity of anti-TG2 IgA combined with positive dGP IgG or positive dGP IgA

The subset analysis for probes with TG2 IgA positive and positive dGP IgG or positive dGP IgA are presented in Table 5. Sensitivity is generally a bit higher for this subset. Specificity cannot be computed, since all sera in the subset were truly positive. For CD, the sensitivity of ME is worse compared to ML (ME: 83.7% (95% CI 0.754 - 0.895), ML: 91.3% (95% CI 0.844 - 0.954), P=.06). For DH, the sensitivity of ME is significantly higher, although based on a small sample size. The Fleiss' kappa values are marginally higher for ML compared to ME, although both with a moderate accordance based on (20) with values of 0.51 and 0.45, respectively (20).

TABLE 3 Interrater agreement of 332 decisions; calculation of diagnostic sensitivity, specificity and predictive values of ME and ML, in relation to all ELISA results.

Parameter	DH		P	CD		P
	ME	ML		ME	ML	
Fleiss' kappa	0.60	0.63		0.73	0.72	
TP	50	49		139	120	
TN	9	11		106	110	
FP	3	1		6	2	
FN	2	3		17	36	
Sensitivity (95%CI)	0.962 (0.87 - 0.989)	0.942 (0.844 - 0.98)	1	0.891 (0.832 - 0.931)	0.769 (0.697 - 0.828)	<.00
Specificity (95%CI)	0.75 (0.468 - 0.911)	0.917 (0.646 - 0.985)	0.48	0.946 (0.888 - 0.975)	0.982 (0.937 - 0.995)	0.29
PPV (95%CI)	0.943 (0.846 - 0.981)	0.98 (0.895 - 0.996)		0.959 (0.913 - 0.981)	0.984 (0.942 - 0.995)	
NPV (95%CI)	0.818 (0.523 - 0.949)	0.786 (0.524 - 0.924)		0.862 (0.79 - 0.912)	0.753 (0.678 - 0.816)	

CD, celiac disease; CI, confidence interval; DH, dermatitis herpetiformis; TN, true negative; TP, true positive; FN, false negative; FP, false positive; PPN, positive predictive value; NPV, negative predictive value; ME, monkey oesophagus; ML, monkey liver.
*Level of significance P <.05.

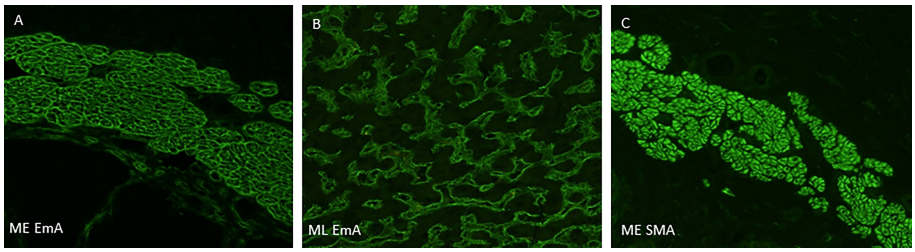

FIGURE 1
Representative staining pattern of the indirect immunofluorescence-based primate monkey oesophagus and monkey liver assays. **(A)** Serum of a patient with suspected DH shows IgA autoantibodies with typical honeycomb-like, endomysial staining on monkey oesophagus section. **(B)** Serum of the same patients shows endomysial autoantibodies within the liver sinusoids (Vv. intralobulares). **(C)** Serum of a patient with only anti-SMA antibodies shows staining of smooth muscle fibres. *DH, dermatitis herpetiformis; CD, celiac disease; SMA, smooth muscle antibodies; ME, monkey oesophagus; ML, monkey liver.*

Discussion

IgA-based indirect immunofluorescence and enzyme linked immune sorbent assays (ELISA) are the main serological methods for DH diagnostics. The microscopic detection of EMA in the sera of the patients is a semi-quantitative analysis. However, experienced diagnosticians are needed for a high quality evaluation to avoid false negative results (21). Current guidelines recommend using cryosections from ME for indirect IF (IIF) diagnostics, although other substrates such as human umbilical cord, human appendix, monkey uterus or rabbit oesophagus may also be considered as smooth muscle fibre substrates (2). The sensitivity is described between 60-90%, with specificity of up to 100% in untreated DH cases (10, 22).

We here used primate ML tissue as substrate for IIF detection of EMA antibodies to evaluate whether by its use we can increase sensitivity of EMA IIF diagnostics. IgA EMA antibodies on ML have been reported to represent the reticulin antibody binding pattern in CD (23). Reticulin antibodies were among the first antibodies described in CD in the early 1970s and were reported to have excellent specificity, but poor sensitivity. Being found in only 40-60% of cases of active CD, the test method was displaced (24). In an unselected, small DH cohort IgA reticulin autoantibodies detected by IIF with ML were present in 10-40% of the patients with increasing incidence corresponding to the severity of the jejunal abnormalities (23, 25, 26).

We compared ML and ME diagnostics in both DH and CD patients in a blinded manner and thereafter categorized them, based on their antibody profile. In our DH cohort, 75% (12/16) of the sera were positive in ML IIF. In CD an inaccordance (FN) in eight out of 67 sera (12%) was detected, interestingly in patients with anti-TG2 IgA below 20 U/ml. Considering that levels of TG2 autoantibodies can be affected by diet status, a limitation of the study is the lack of knowledge on the gluten intake of our patients. In a retrospective pediatric study a decrease of around 70% in the anti-TG2 levels within 3 months of a gluten-free diet was shown, while around 80% of the children were sero-negative for anti-TG2 and in the IIF diagnostics only after 2 years of the diet (27). Twenty-seven sera of the CD cohort (40%) showed antibodies against anti-dGP IgA and IgG in combination with anti-dGP IgA or anti-dGP IgG antibodies only and were taken at different time points during clinical course. An isolated positive anti-dGP IgG test in absence of anti-TG2 IgA

TABLE 4 Calculations of diagnostic sensitivity, specificity and predictive values of ME and ML in relation to subset of anti-TG2 IgA positive sera. CD and DH combined, since DH has only 2 samples.

Parameter	All (DH and CD)		*P*
	ME	ML	
Fleiss kappa	0.32	0.51	
TP	34	28	
TN	13	16	
FP	3	0	
FN	10	16	
Sensitivity (95%CI)	0.773 (0.63 - 0.872)	0.636 (0.489 - 0.762)	0.11
Specificity (95%CI)	0.812 (0.57 - 0.934)	1.0 (0.806 - 1.0)	*>.00
PPV (95%CI)	0.919 (0.787 - 0.972)	1.0 (0.879 - 1.0)	*>.00
NPV (95%CI)	0.565 (0.368 - 0.744)	0.5 (0.336 - 0.664)	1

CD, celiac disease; CI, confidence interval; DH, dermatitis herpetiformis; TN, true negative; TP, true positive; FN, false negative; FP, false positive; PPN, positive predictive value; NPV, negative predictive value; ME, monkey oesophagus; ML, monkey liver.
*Level of significance P <.05.

TABLE 5 Calculations of diagnostic sensitivity, specificity and predictive values of ME and ML in relation to subset of anti-TG2 IgA positive sera combined with either a positive anti-dGP IgG or a positive anti-dGP IgA.

Parameter	DH		*P*	CD		*P*
	ME	ML		ME	ML	
Fleiss' kappa	–	–		0.45	0.51	
TP	48	46		95	87	
TN	0	0		0	0	
FP	0	0		0	0	
FN	0	2		9	17	
Sensitivity (95%CI)	1.0 (0.926 - 1.0)	0.958 (0.86 -0.988)	*<.00	0.837 (0.754 - 0.895)	0.913 (0.844 - 0.954)	.06
Specificity (95%CI)	–	–		–	–	
PPV (95%CI)	1.0 (0.926 - 1.0)	1.0 (0.923 - 1.0)	1	1.0 (0.961 - 1.0)	1.0 (0.958 - 1.0)	1
NPV (95%CI)	–	0.0 (0.0 - 0.658)		0.0 (0.0 - 0.299)	0.0 (0.0 - 0.184)	1

CD, celiac disease; CI, confidence interval; DH, dermatitis herpetiformis; TN, true negative; TP, true positive; FN, false negative; FP, false positive; PPN, positive predictive value; NPV, negative predictive value; ME, monkey oesophagus; ML, monkey liver.
*Level of significance *P* <.05.

antibodies at initial diagnostics may be nonspecific and often FP in infancy. It therefore has no predictive value for CD (28). Isolated searching for IgA antibodies against dGP also has low specificity and is not recommended in childhood (29). Although the dietary status of our patients is unknown, all had some signs of disease activity or persistence, when the sera were taken.

Our initial data indicate that ML IIF is useful as primary or confirmatory assays in DH with positive anti-TG2 IgA at initial diagnosis, but might be less suitable as a follow-up parameter. The results also indicate an equivalence for ML and ME, as no statistically significant differences were found. Due to low sample size, confidence intervals for DH are quite wide. Both sensitivity and specificity seem to perform slightly better for ML in DH. The bigger sample size for CD leads to a narrower confidence interval and a better interpretability of results. Regarding sensitivity ME seems to be slightly better, which is statistically significant (P <.00). Regarding specificity ML performed slightly better, but not significantly. Further analyses in larger DH cohorts would be desirable to confirm our results.

In our cohort we did not test for TG3 autoantibodies, which are reported to be the autoantigen for DH, this is a limitation that should be addressed in future studies. In future, additional immunoblot (IB) testing with bianalyte detection of IgA against TG2 and nanopeptides of gliadin could be of interest. In a recently published cohort, IB showed 78% sensitivity, 100% specificity, 100% positive predictive value, and 82% negative predictive value in relation to ELISA against TG2 alone. Henceforth, a multiplex approach for DH diagnostics with multianalyte IIF and multivariant ELISA profile will probably replace monoparametric diagnostics (30).

Conclusion

The results show that ML substrate is suitable for EMA diagnostics in DH. It appears to be slightly easier for the raters to evaluate and might be interesting for inexperienced raters, since misinterpretation occurring in ME diagnostics can be avoided. The study is limited by its retrospective approach and the fact that sera were taken at different time points considering diet status and disease activity. Also, data on anti-TG3 IgA ELISA diagnostics in DH are not available and were not considered. Since DH and CD are rare disorders and we here present a single-centre study, statistical significance of the data was difficult to achieve in this cohort.

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 Human Ethics Committee University of Freiburg (reference No.235/15). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

The idea for this research topic was brought up by FS and DK, who also performed data acquisition and sorting. FS, DK, GT and DS were blinded evaluators of the stainings. MB performed statistical analysis. SM provided patient sera and revised the manuscript. The manuscript was drafted and designed by FS and DK. All authors contributed to the article and approved the submitted version.

Funding

DK is funded by the German Research Foundation (DFG) through SFB1160 project B03, SFB-1479 – Project ID: 441891347 and KI1795/2-1 and the Fritz Thyssen Foundation. FS reported funding from the Berta-Ottenstein-Programme for Advanced Clinician Scientists, Faculty of Medicine, University of Freiburg.

Acknowledgments

We thank our fellow colleagues, who diagnosed and treated the patients in our Department. We also thank Kaethe Thoma and Annegret Bedorf for expert technical assistance.

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 Technology and Code
PUBLISHED 28 February 2023
DOI 10.3389/fimmu.2023.1111172

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 29 November 2022
ACCEPTED 13 February 2023
PUBLISHED 28 February 2023

CITATION
Hocke J, Krauth J, Krause C, Gerlach S, Warnemünde N, Affeldt K, van Beek N, Schmidt E and Voigt J (2023) Computer-aided classification of indirect immunofluorescence patterns on esophagus and split skin for the detection of autoimmune dermatoses.
Front. Immunol. 14:1111172.
doi: 10.3389/fimmu.2023.1111172

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Computer-aided classification of indirect immunofluorescence patterns on esophagus and split skin for the detection of autoimmune dermatoses

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Autoimmune bullous dermatoses (AIBD) are rare diseases that affect human skin and mucous membranes. Clinically, they are characterized by blister formation and/or erosions. Depending on the structures involved and the depth of blister formation, they are grouped into pemphigus diseases, pemphigoid diseases, and dermatitis herpetiformis. Classification of AIBD into their sub-entities is crucial to guide treatment decisions. One of the most sensitive screening methods for initial differentiation of AIBD is the indirect immunofluorescence (IIF) microscopy on tissue sections of monkey esophagus and primate salt-split skin, which are used to detect disease-specific autoantibodies. Interpretation of IIF patterns requires a detailed examination of the image by trained professionals automating this process is a challenging task with these highly complex tissue substrates, but offers the great advantage of an objective result. Here, we present computer-aided classification of esophagus and salt-split skin IIF images. We show how deep networks can be adapted to the specifics and challenges of IIF image analysis by incorporating segmentation of relevant regions into the prediction process, and demonstrate their high accuracy. Using this semi-automatic extension can reduce the workload of professionals when reading tissue sections in IIF testing. Furthermore, these results on highly complex tissue sections show that further integration of semi-automated workflows into the daily workflow of diagnostic laboratories is promising.

KEYWORDS

autoimmune dermatoses, neural networks, immunofluorescence tests, tissue classification, deep learning

1 Introduction

Autoimmune bullous diseases (AIBD) are a highly heterogenous group of autoantibody-driven diseases in which autoantibodies against various proteins of the desmosomes and basement membrane zone (BMZ) of the skin and surface epithelia (1–5). Depending on the antigens involved, blister formation either occurs intra-epidermally in pemphigus diseases or sub-epidermally in pemphigoid diseases. Deciphering the affected structures in this heterogeneous group of disorders is essential for both prognosis and treatment, as immunosuppressive therapy varies according to disease entity. Initial differentiation can be achieved by indirect immunofluorescence (IIF) microscopy on monkey esophagus for pemphigus diseases and primate salt-split skin for pemphigoid diseases as the most sensitive screening methods for autoimmune bullous diseases (6, 7). IIF on monkey esophagus can detect circulating autoantibodies against the epithelial and endomysial autoantigens. IIF on primate salt-split skin discriminates autoantibodies against the BMZ. Additional testing with, for example, recombinant desmoglein 1 (DSG1), DSG3, BP180 (type XVII collagen), BP230, laminin 332, type VII collagen, and deamidated gliadin peptides by IIF, enzyme-linked immunosorbent assay (ELISA), or immunoblot analysis (2–4, 8) can be performed to identify target antigens.

Previously, BIOCHIP® mosaics have been developed that allow the simultaneous detection of different autoantibody specificities on a routine laboratory slide by placing multiple miniature so-called biochips in a single incubation field. In addition to recombinant proteins such as BP180 NC16A and cells recombinantly expressing DSG1, DSG3, BP230, type VII collagen, and laminin 332, tissue substrates such as monkey esophagus and primate salt-split skin can also be used (6, 9–11). The BIOCHIP® technology is increasingly used for routine diagnostics of autoimmune blistering diseases (AIBD) (12–22).

Circulating autoantibodies in pemphigus characteristically bind to the epithelium of esophagus in an intercellular pattern (Figure 1A). The pattern is seen as smooth linear fluorescence along the borders of the epithelial cells with a mesh-like appearance. The presented algorithm maps this pattern as 'Intercellular'. Anti-BMZ reactivity in pemphigoid diseases, e.g. directed against BP180, BP230 or type VII collagen can also be visualized in this substrate (Figure 1B) revealing to a smooth linear fluorescence along the BMZ. This pattern is subsequently referred to as 'BMZ' pattern. Furthermore, in celiac disease and dermatitis herpetiformis, IgA autoantibodies label the endomysium in a characteristic pattern (11) which appears as a honeycombed pattern within the lamina muscularis mucosae. Here, we focused on 'Intercellular' and 'BMZ' pattern and as such endomysial pattern is not reported. In the diagnosis of AIBDs, other histopathological regions of the esophagus are not relevant.

Separating dermis and epidermis of primate skin with 1M NaCl results in split formation within the lamina lucida, which allows differentiation between two linear IF patterns in pemphigoid diseases. Antibodies against BP180, BP230, and α6β4 integrin stain along the epidermal side of the artificial split (Figure 1C). For the computer-generated outputs, we refer to this binding as 'epidermal'. In contrast, autoantibodies against the p200 antigen, laminin γ1, laminin 332, and type VII collagen bind along the dermal side (23), a pattern that is referred to as "dermal" in this manuscript (Figure 1D). In areas where the tissue is still connected, linear fluorescence is seen.

Reading IIF patterns is done by visual evaluation of microscope images and is not standardized. Hence, the results strongly depend

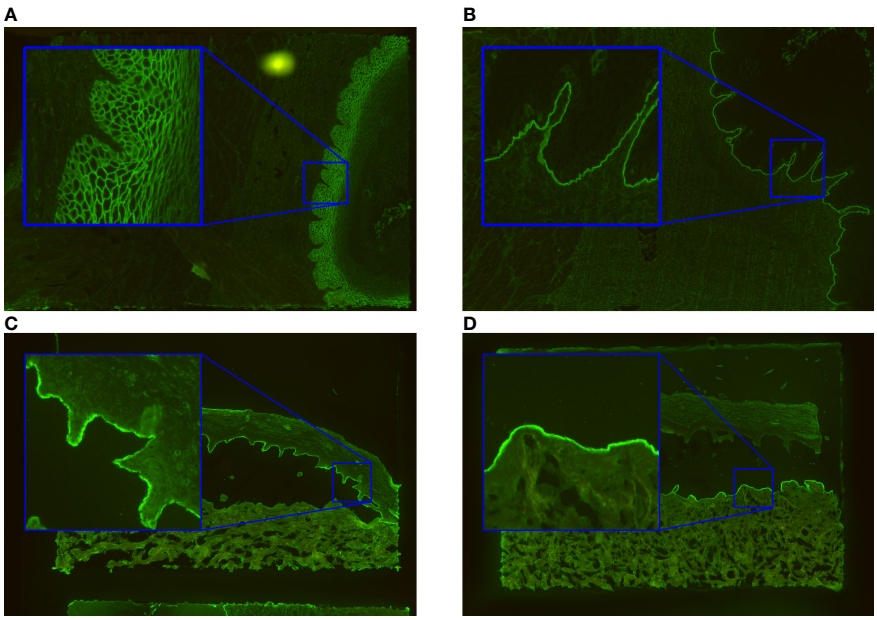

FIGURE 1
Exemplary immunofluorescence images of the incubated substrates esophagus and salt-split skin. The 'intercellular' pattern seen by intercellular labelling of monkey esophagus in pemphigus vulgaris/foliaceus is shown in **(A)**. In **(B)**, the basal membrane zone ('BMZ') pattern typical for pemphigoid diseases is indicated. The patterns found in salt-split skin are 'epidermal' **(C)** and 'dermal' **(D)**. They arise by binding of autoantibodies in pemphigoid patients to the epidermal or dermal side of the artificial split.

on the practical experience of the professional and are subjective. The high variance in the appearance of tissue segments complicates the evaluation leading to an undesirably high variability of the results. Computer-aided evaluation of IIF became popular both because of the automated workflow and more standardized objective evaluation of IIF (24–26). Computer-aided diagnostics rapidly evolved as deep learning became a state-of-the-art method for computer vision tasks. Deep learning enables the solution of complex tasks such as image segmentation (27), object detection (28, 29), and image classification (30). In the medical field, deep learning has been successfully used for the detection of skin cancer (31), lung nodules (32), and diabetic retinopathy (33).

Here, we show how deep learning can be used for computer-aided evaluation of IIF images of highly complex tissue substrates. The images are from slides containing millimeter-sized fragments of coated cover glasses with biological substrates called biochips (EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany). The primary esophagus and primate salt-split skin each cover large areas of the biochip, while only small structures on specific parts of the tissue are decisive for the classification result. Standard deep networks are not suitable for processing these images due to limitations in computer memory and the number of images available for training. We describe two architectures, both of which use segmentation to aid the training process of a classification network. We demonstrate their effectiveness on real data acquired with a EUROPattern® Microscope (EPM) 1.5 microscope (EUROIMMUN) by comparing their outputs with the results of visual evaluation of the same images by a professional.

In the present study, we would also commemorate Detlef Zillikens, director and chair of the Department of Dermatology, University of Lübeck, Lübeck, Germany. Detlef passed away in September 2022 after short and severe disease. He has been one of the leading clinician scientists in the field of AIBD who authored more than 600 articles on this subject. Detlef Zillikens has inspired, motivated, and mentored numerous colleagues including N.v.B. and E.S. E.S. was amongst his first students performing his medical thesis about the presence of inflammatory mediators in sera and blister fluids of bullous pemphigoid patients compared to sera and suction blisters of healthy volunteers in 1993 (34–37). This fruitful and passionate cooperation has been functional for 25 years bringing to light more than 250 articles and book chapters about AIBD. N.v.B. and E.S. are greatly indebted to Detlef Zillikens and thankful for his wise, winning, and kind mentoring and his friendship. All authors are sad and affected about the early death of this exceptional clinician and researcher.

Overall, we present classifiers for the detection and analysis of these IIF patterns. In addition to the classification of circulating antibodies as positive or negative, a brightness score is also returned for titer estimation.

2 Materials and methods

2.1 Human sera

Serum samples from patients were collected for routine diagnostic analysis by the Immunological Laboratory (Lübeck, Germany) and the Department of Dermatology, University of Lübeck. Samples were anonymized for analysis and stored at -20°C until use. Left-over patient samples were used for this study after completion of all diagnostic procedures. Serum samples from healthy blood donors served as controls. The study performed in accordance with the ethical guideline stated in the Declaration of Helsinki and positively evaluated by the ethics committee of the University of Lübeck (12–178).

2.2 Salt-split skin and esophagus dataset

Data were acquired using the EUROPattern® Suite (EUROIMMUN), a system of hardware and software components for computer-aided IIF. Images were acquired using an EPM 1.5 microscope at 10x magnification. To picture an entire biochip, four images are combined. This results in images with slightly varying sizes of about 4,500 x 3,400 pixels.

To generate the training dataset, the biochips were incubated at various dilutions from 1:10 to 1:10,000 to ensure distinction of weak patterns for AIBD from negative samples. The salt-split skin training set consisted of 3,428 images, of which 1,040 images showed a positive reaction in epidermal tissue and 326 showed a positive reaction in dermal tissue. 2,076 of these images showed no positive reaction. The esophagus dataset consisted of 7,022 training images with 1,399 images with intercellular reactivity in the epithelium, 2,539 with anti-BMZ reactivity, and 3,166 images with no or unspecific reactions. The dataset also contained images with patterns caused by a variety of confounder antibodies (e.g., antinuclear antibodies), not associated with AIBD resulting in images with multiple patterns.

To generate the validation dataset, 110 patient samples were incubated at 1:10, 1:32, and 1:100 dilutions to test for correct assignment including weak specific patterns, resulting in three images of esophagus and salt-split skin per patient. For the 52 samples from healthy controls, only a 1:10 dilution was incubated, resulting in one image of esophagus and salt-split skin per control. For the validation dataset, 26 patients with serologically tested epidermal binding to salt-split skin and 8 patients with serologically tested dermal binding were used. 30 patients with serologically tested bullous pemphigoid showed linear binding at the BMZ on monkey esophagus, and 20 patients with serologically tested pemphigus vulgaris/foliaceus stained monkey epithelium with an intercellular pattern. One patient reacted positively to both epidermal BMZ and epithelial desmosomes. In addition, 52 substrates with no or with unspecific reactivity were used, incubated with sera from control subjects or patients with, for example, pemphigus on salt-split primate skin.

The collected training and validation data were read manually by IF professionals. The readers were medical technicians with at least 5 years of experience in reading IF tests including monkey esophagus and salt-split skin.

2.3 Salt-split skin algorithm

The number of salt-split skin images for the training dataset and the memory required to process images of a given size with a deep

network is limited. In addition, generalization to unseen data is difficult to achieve due to the large number of parameters needed in a suitable network. Therefore, we chose to first identify relevant subregions of the image and then analyze only those subregions. This approach addresses both memory requirements and generalization. The algorithm includes multiple steps of image processing (Figure 2). First, the image is segmented to identify the relevant subregions of the tissue. Based on the segmented regions, lines of attention are computed. Image patches are sampled along these lines and then processed individually by a deep neural network. The individual outputs are aggregated to obtain the final result.

Only the green channel is used for segmentation. The image is downscaled to a resolution of 512 x 512 pixels and then fed into a U-Net type deep network for segmentation. This type of network is known to work well in segmenting microscopy images (27). Here, we used a Fusion-Net, which incorporates recent developments in deep learning and further improves performance (38). The individual outputs are divided into roof (epidermal), floor (dermal), interspace, and background. Here, the interspace is the empty area between the roof and the floor.

The attention regions are then computed. The image content relevant for analysis is located along these attention lines. They are computed from the overlap of the segmented areas expanded by dilation (DIL) (Figure 2) by a disc of 3 pixels in diameter. The extended roof (R), floor (F) and interspace (I) subregions are interpreted as sets in the following. The lines L on the epidermal (L_{Roof}) and dermal (L_{Floor}) substrate are computed as

$$L_{Roof} = \mathrm{DIL}(R) \cap \quad (\mathrm{DIL}(F) \cup \quad \mathrm{DIL}(I)),$$

$$L_{Floor} = DIL(F) \cap \quad (DIL(R) \cup \quad DIL(I)) .$$

To focus on the relevant image content, image patches of size 64 x 64 pixels are extracted along the attention lines. They are taken from a downscaled version of the original image with a size of 2,048 x 2,048 pixels. To achieve a uniform distribution of the patches along the lines, Poisson disc sampling is used (39). For each line, 40 patches are extracted. The following inference steps are performed separately for roof and floor.

Each patch is then processed separately by a deep neural network to obtain local results (Figure 3). This is a very small network with only a few layers, so only a few parameters need to be optimized. The network has two outputs: a probability for a label and a brightness score for roof or floor. The label values are 'positive', 'negative', 'background', or 'unclear'. The 'unclear' label is needed because not all sections along the attention lines provide the information needed for classification. The 'background' label was only used during training to indicate empty patches.

Finally, the labels of the patches are aggregated to obtain a result for the entire attention line and thus for the entire image. Every patch x_i is processed by a deep network f. For every index $i \in N$ the network returns 'negative' as the label with the highest probability. Accordingly, $i \in P$ if 'positive' is the label with highest probability. The other labels 'background' and 'unclear' are ignored during aggregation. Therefore, these regions do not influence the result. The result $y \in [0,1]$ is given by

$$y = \frac{1 + y_{pos} - y_{neg}}{2} \text{ , } where \;\; y_{neg} = \frac{\sum_{i \in N} f(x_i)}{|N|}, \;\; y_{pos} = \frac{\sum_{i \in P} f(x_i)}{|P|}.$$

The brightness $b \in \mathbb{R}$ is obtained from the mean of the three samples P_3 with the highest probability value for the label 'positive':

$$b = \frac{\sum_{i \in P_3} b_{i,}}{|P_3|}.$$

These brightness scores are then combined for images of different dilutions to estimate the titer.

2.3.1 Training of the salt-split skin neural networks

Both the segmentation network and the network for processing patches were optimized using adaptive moment estimation (ADAM) (40). For the segmentation network, a generalized dice loss (41) was used due to its robustness to imbalanced region sizes. The patch analysis network has two outputs, containing classification and regression output. Cross entropy was used to derive the classification output, and mean squared error was used to derive the regression output. Both were weighted equally. Separate analysis networks were trained for roof and floor to achieve a better adaption to the pattern features. In total, there are three networks: one for segmentation, one for the analysis of the roof, and one for the analysis of the floor.

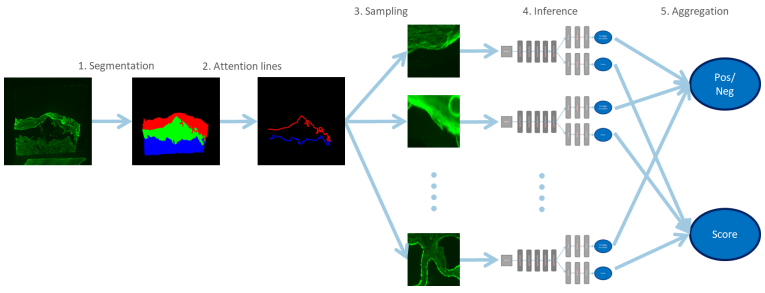

FIGURE 2
Overview of the steps for processing salt-split skin images. Segmentation is used to identify relevant parts of the image. Samples of these subregions are then analyzed *via* deep networks and the results are aggregated. This approach ensures low memory requirements and good generalization.

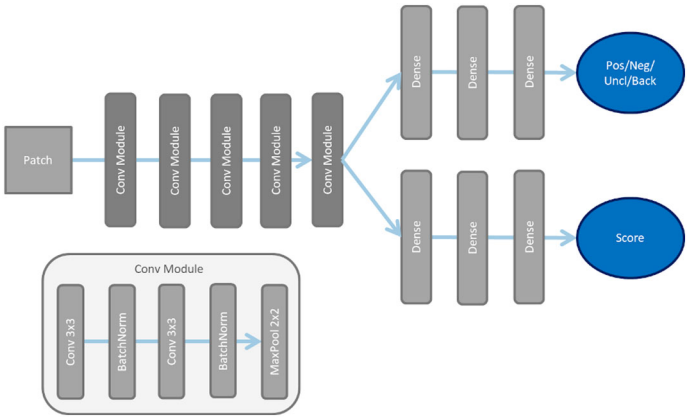

FIGURE 3
For processing the samples of the subregions of the salt-split skin substrate, the displayed deep network architecture is used. After processing by convolution module shown below, the processing is divided into two branches of dense layers for the different outputs.

Several augmentations were applied to improve the generalization of these networks. When training the segmentation network, flip and rotation were applied to the entire image. For the analysis networks, the used augmentations flip and Gaussian noise were applied only to the patches.

As mentioned earlier, when training the analysis networks, empty patches are labeled as 'background' for technical reasons. It is easier to train the networks with a fixed number of patches per image, but since the size of the salt-split skin segment varies, the number of extracted patches also varies. Therefore, a fixed, generous number of patches is assumed for each image, and missing patches are inserted as empty ones.

2.4 Esophagus algorithms

For the diagnosis of AIBDs, the detection of circulating autoantibodies against epidermal/epithelial antigens is essential. Monkey esophagus is the most sensitive tissue substrate for serum autoantibodies in pemphigus vulgaris/foliaceus. In pemphigoid diseases, autoantibodies label the BMZ of monkey esophagus, but with a lower sensitivity compared with primate salt-split skin (15, 42–45). The algorithm for classification of the immunofluorescence (IF) patterns on esophagus tissue is divided into two subtasks.

The first task is to localize and segment the tissue segments in the image. An adapted version of the U-Net model (27) is trained on an esophagus dataset containing image pairs of IF images and segmentation ground truth images. The segmentation images consist of seven segments representing the esophagus tissue sections longitudinal muscle, circular muscle, muscular mucous membrane, BMZ, lamina propria, epithelium, and background. The trained neural network can process unseen IF images of the esophagus and assigns one of the aforementioned sections to each pixel of the image. The key information of the segmentation result is the spatial information of the epidermal BMZ that could be used in the following subtask.

The second task is the classification of the IF image into the classes 'BMZ', 'intercellular' and 'negative'. One approach to achieve this goal is a whole-image classification neural network that is trained on a dataset of esophagus IF images and corresponding target labels, assigning a probability between 0 and 1 for each class to each image. However, the neural network had low accuracy in predicting the 'BMZ' class. Presumably, the sparse information of the very thin epidermal BMZ compared with the large number of other pixels occurring in the image and the high variance of tissue morphologies prevented the neural network from focusing on the crucial image regions and features. To bypass this disadvantage, the spatial information of the epidermal BMZ processed in the first step can be utilized. The classification neural network is designed to receive two input images: The first input is the original IF image, and the second is a post-processed mask based on the segmentation result. The post-processing drops all segmentation information except the epidermal BMZ and converts the remaining image to a binary image. The binary image is used as attention mechanism input and is intended to hint the neural network to pay special attention to the masked region (Figure 4). This approach dramatically increased the accuracy in our classification experiments.

At last, not only the classification with a probability between 0 and 1 for each class, but also a quantification of pattern intensity, which represents the titer of the autoantibodies in patient serum, is valuable information for the evaluation of IIF images, in particular, if more than one pattern is detected. Hence, the algorithm extracts the intensity of the pattern in the regions where the pattern occurs and predicts its titer. For a positive 'BMZ' pattern, the intensity extraction is straightforward, when the segmentation information of the esophagus is given. However, for a positive 'intercellular' pattern, more post-processing is required to find the relevant region, since the desmosome region in the epithelium is in close proximity to the BMZ and the segmentation contains the epithelium as a whole region. Therefore, post-processing with convolution operations is calculated on the segmentation image to predict the region adjacent to the BMZ that extends into the

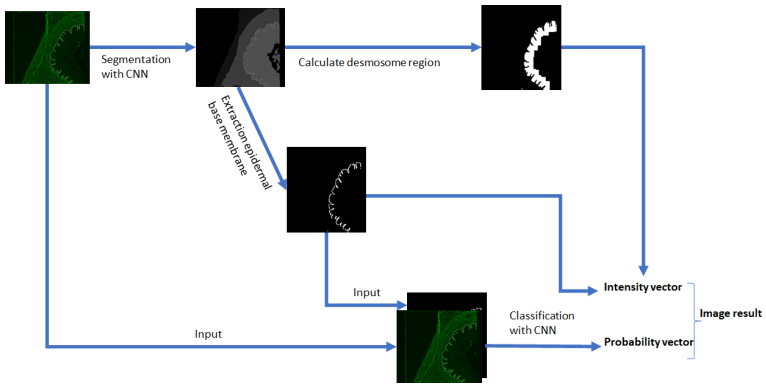

FIGURE 4
Steps for processing esophagus images. The first processing path segments the image with a convolutional neural network (CNN) into six tissue segments plus background and extracts the epidermal basement membrane as input to the classification path. The segmentation mask is post-processed to calculate the desmosome region. The spatial information is used for intensity estimation of found pattern.

epithelium (Figure 1A). In the case of a positive reaction, the 'intercellular' staining appears as the brightest part in that region and can be extracted using a previously defined quantile of the occurring pixel intensities.

2.4.1 Training of the esophagus neural networks

The segmentation neural network for esophagus tissue images was trained on preprocessed and downscaled images of size 512 x 512 pixels. This ensures rapid and memory-optimized segmentation of the images, with resolution high enough to distinguish the different tissue regions that appear. In addition, the images were augmented by horizontal and vertical flipping, random rotation and zooming, resulting in a more generalized network. The segmentation network has seven outputs corresponding to the tissue segments longitudinal muscle, circular muscle, muscular mucous membrane, epidermal basement membrane, lamina propria, epithelium, and background. The network was optimized using the ADAM optimizer and categorical cross-entropy.

The classification neural network was trained on preprocessed images of size 2,048 x 2,048, preserving all the crucial information available in positive reactions. For each image, the previously trained segmentation network processes a segmentation map on the fly, extracting the BMZ, converting it to a binary image and upscales it to the size of 2,048 x 2,048 pixels, building an image input pair of original tissue image and binary map of the epidermal BMZ. The image pairs were also augmented by horizontal and vertical flipping, random rotation and zooming to increase the variance of the training dataset. The classification network also used the ADAM optimizer. The employed optimization function was binary cross-entropy.

2.5 Titer estimation

The titer value is computed for the positive IF patterns of each patient to estimate the amount of serum autoantibodies. Titer values are taken from the dilution series(1:10,1:32,1:100,1:320,…). An image of dilution d_k has the kth dilution from that series. For a single image, the brightness score $s \in [1,5]$of a detected pattern is converted to the titer by addition: d_{k+s-1}. If multiple images are available, the lowest-dilution image with a negative pattern is defining the total dilution. If there is no image with a negative pattern, the highest-dilution image is taken, and the total dilution is computed in the same way as the single image.

3 Results

The esophagus and salt-split skin classification algorithms were used to process the samples of the validation dataset. Therefore, each individual image was processed. For sera with several dilutions, each individual image was classified. The identified pattern and intensities were used to compile a patient-based result including the predicted titer. For sera with only one dilution, the result depended solely on the result of that image.

3.1 Salt-split skin classification results

The trained salt-split skin models were evaluated using the validation dataset. One patient had to be discarded from this analysis due to a defect in the substrate image. Thus, the validation dataset consisted of 109 patient sera for analysis (Table 1). A pattern detection accuracy of 96% was achieved for the salt-split skin 'epidermal binding' algorithm. Both positive percent agreement (PPA) and negative percent agreement (NPA) were 96%. Here, the results of one patient serum with a titer of 1:10 gave a barely positive IF result and were incorrectly classified as negative. The salt-split skin 'dermal binding" algorithm achieved 97% accuracy, with 100% PPA and 97% NPA. Of note, only eight patients showed dermal binding. The classification errors are exemplified in Figure 5. These errors occurred mostly in borderline cases where luminance was low and sometimes unspecific. When only 'binding' was evaluated, regardless of epidermal or dermal localization, binding was detected with 95%

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TABLE 1 Performance of the salt-split skin classifier (EPa-Classifier) compared to the conventional evaluation by an IIF professional.

		EUROPattern® visual mode								
		Combined			'epidermal'			'dermal'		
		positive	negative	total	found	not found	total	found	not found	total
EPa Classifier	positive	31	4	35	25	3	28	8	3	11
	negative	1	73	74	1	80	81	0	98	98
	total	32	77	109	26	83	109	8	101	109
			Accuracy:	95.4%		Accuracy:	96.3%		Accuracy:	97.3%
			PPA:	96.9%		PPA:	96.2%		PPA:	100.0%
			NPA:	94.8%		NPA:	96.4%		NPA:	97.0%

The combined section shows the overall result when any positive pattern is counted as positive. Samples were present that were positive in both patterns. 'Epidermal' and 'dermal' indicate reactivities detected along the epidermal and dermal side of the artificial split by IIF on primate salt-split skin.

accuracy, with 97% PPA and 95% NPA. Titer estimates were almost all in the +/-1 range for both patterns.

3.2 Esophagus classification results

The algorithm detected 46 of 49 positive patterns. The algorithm classified no patient serum as false positive and 61 patient samples as true negatives. This resulted in an overall agreement of 97%, a PPA of 94% and a 100% NPA (Table 2). Of note, 3 sera that were classified as false negative by the algorithm were identified by visual classification as marginally positive with a titer of 1:10.

Regarding the pattern recognition for the 'BMZ' pattern, 27 of 30 patient sera were classified by the algorithm as true positive reactions, three patient sera were classified as false negative (the three marginally positive sera, example in Figure 6A), and two patient sera were classified with a positive pattern, but instead of a 'BMZ' the algorithm predicted an 'intercellular' pattern (an example is shown in Figure 6B). The algorithm predicted 78 patients as true negative samples to BMZ and two patients as false positive samples. The overall agreement for the pattern is 96% compared to the manual reading with a PPA of 90% and NPA of 98%. The agreement of titer prediction for BMZ, calculated only for the true positive samples, agreed in 70% of patients. 30% of the

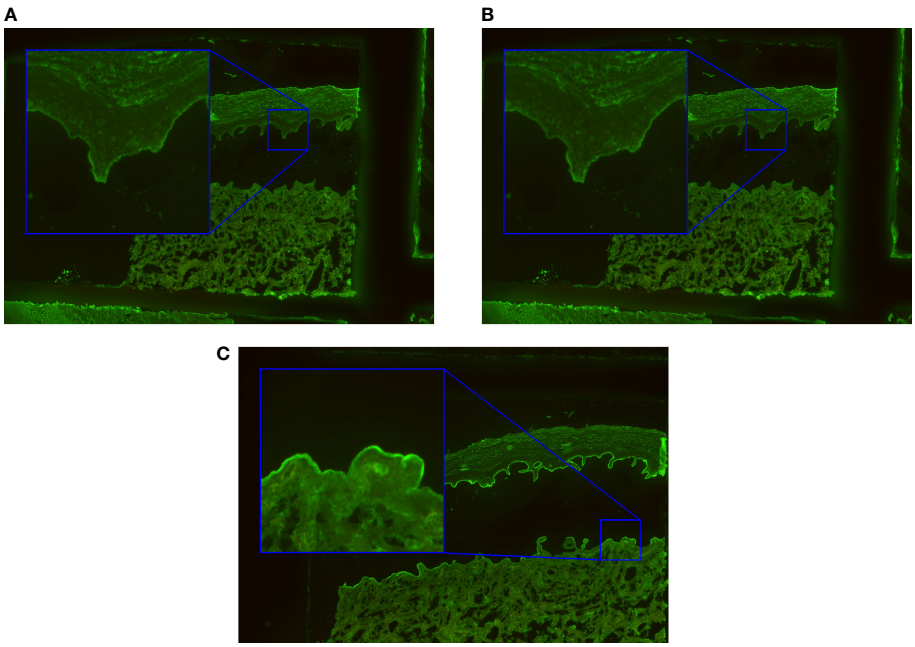

FIGURE 5
Classification errors usually occur in borderline cases. **(A)** The roof was incorrectly classified as positive due to unspecific immunofluorescence. **(B)** A false negative result was caused by weak staining along the epidermal side of the artificial split. **(C)** The false positive reactivity along the dermal side was the result of unspecific labelling which may occur with strong staining along the blister roof.

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TABLE 2 Performance of the esophagus classifier (EPa-Classifier) compared to the conventional evaluation by an IIF professional.

		EUROPattern® visual mode								
		Combined			'BMZ'			'Intercellular'		
EPa Classifier		positive	negative	total	found	not found	total	found	not found	total
	positive	46	0	46	27	2	29	20	0	23
	negative	3	61	64	3	78	81	0	90	87
	total	49	61	110	30	80	110	20	90	110
			Accuracy:	97.3%		Accuracy:	95.5%		Accuracy:	100.0%
			PPA:	93.9%		PPA:	90.0%		PPA:	100.0%
			NPA:	100.0%		NPA:	97.5%		NPA:	100.0%

The combined section shows the overall result when any positive pattern is counted as positive. Samples were present that were positive in both patterns. The basal membrane zone ('BMZ') and 'intercellular' pattern reflect IF reactivity against the BMZ and intercellular staining of the epithelium as seen with pemphigoid and pemphigus patients, respectively.

predictions differed by +/-1 titer step. There were no deviations greater than this (Figure 7).

For the 'intercellular' pattern, all 20 patient sera were correctly identified by the algorithm and all 90 sera without this pattern were correctly classified as negative pattern. Thus, the overall agreement for the 'intercellular' pattern was 100% with a PPA of 100% and NPA of 100%. The titer prediction for the pattern had a 60% match rate for the true positive patient samples. 40% of the titer predictions had an error of +/- 1 titer step. As with the 'BMZ' pattern, no deviations greater than this were detected (Figure 7).

4 Discussion

The diagnostics of AIBD is based on the detection of circulating and tissue-bound autoantibodies, with the latter remaining the gold standard. However, detection of circulating autoantibodies allows further differentiation of disease entities, which is relevant for both prognosis and choice of treatment. IIF testing on monkey esophagus and salt-split skin is commonly used as screening test and provides initial insight into likely disease entities (46). Delayed testing for AIBD and delayed treatment can lead to adverse outcomes with permanent impairment or even fatality (47, 48). While monkey esophagus epithelium has the highest sensitivity for autoantibodies against desmosomal proteins, autoantibodies against BMZ proteins can also be detected (5, 48–50). For the detection of BMZ structures, sensitivity is higher when salt-split skin is used as substrate, which has the advantage that epidermal-binding can also be distinguished from dermal-binding AIBD types (5, 50–52). Analysis of IIF on these substrates requires experienced readers. In this study, automated reading of IIF on primate esophagus and salt-split skin was evaluated. The presented algorithms for classification of both monkey esophagus and salt-split skin tissue sections for the detection of autoantibodies specific for AIBD showed a high accuracy with over 95% agreement compared to the results of conventional reading by an IIF professional. The PPA was above 97% for all positive IF patterns, on both the esophagus and salt-split skin. The NPA was at least 95% for all patterns. The results showed a slightly higher agreement for blister floor staining on salt-split skin and for intercellular staining on monkey esophagus, whereas for blister roof staining on salt-split skin and BMZ staining on monkey esophagus, some samples with low staining intensity were not detected and some samples with unspecific staining were classified as positives.

The confounder autoantibodies described previously were present on both positive and negative samples of the training dataset. The deep learning algorithm was trained to assign a negative label to the patterns caused by the confounder antibodies

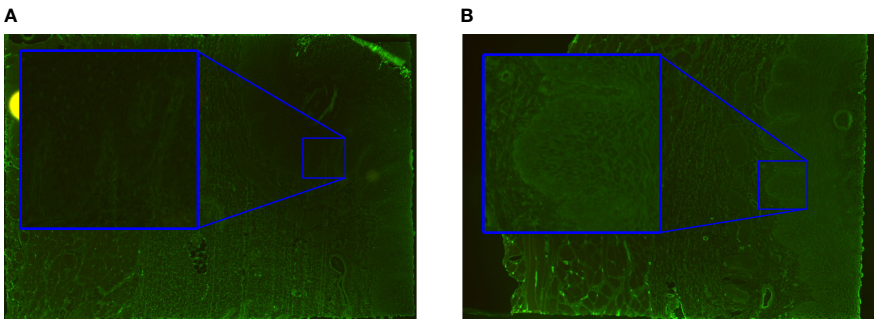

FIGURE 6
Classification errors on esophagus with faint staining. **(A)** 'BMZ' pattern incorrectly classified as 'negative'. **(B)** 'Intercellular' pattern incorrectly classified by the algorithm as 'BMZ' in a patient with pemphigoid disease.

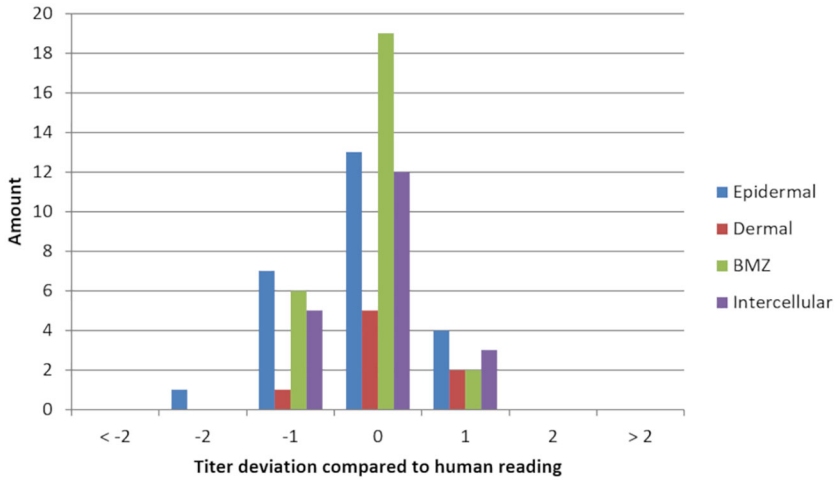

FIGURE 7
Titer deviation of each pattern compared to the evaluation by an experienced reader of indirect immunofluorescence images. Titer deviation was only calculated in samples where the algorithm and conventional reading detected the same pattern. The reading by experienced personnel was used as a reference. A negative deviation indicates an underestimation of the titer steps, a positive deviation an overestimation by the algorithm.

on images if only confounder patterns are present. When both a cofounder pattern and an AIBD-relevant pattern, i.e. "BMZ", "intercellular", "epidermal", or "dermal" were present, the confounder pattern was suppressed and only the AIBD-relevant pattern indicated. Therefore, the algorithm learned that the patterns caused by confounder antibodies are irrelevant for the desired outcome and can be ignored.

Both algorithms can assist laboratory staff in the challenging task of evaluating IF patterns on tissues. The classifiers are an excellent extension of the screening methods for AIBD. Automation of IIF evaluation has already been successfully used in rheumatology for the detection of antinuclear antibodies. Several commercially available systems can be used here [reviewed in (53)]. In the latter review, the authors conclude that higher standardization of results is achieved by less subjectivity and less influence by expertise and that automated workflows are more efficient due to higher throughput. We think standardization of reading IF patterns, especially tissues can be prone to have a high variance between the readings of different personnel. Computer-aided classification, therefore offers a second opinion in a deterministic manner. However, the limitation of our algorithm lies in the design of a pattern proposal system that only can give hints on learnt patterns. For rare patterns that were not contained in the training dataset, the algorithm will likely fail with its proposal and the IF professional must act independently. Also, our study revealed some discrepancies between the algorithm and the reading of the IF professionals. In these cases, the IF professional must take the final decision. Obviously, the benefit of having a high-throughput systems only takes effect in laboratories with a high number of sera subjected to IIF on esophagus and salt-split skin.

Software solutions for computer-aided diagnostics of IIF are already available for other substrates, using the EUROPattern® Microscope (EUROIMMUN), which was also used in the present study (54). The EUROPattern® Suite software is capable of classifying images of HEp-2/HEp-2010 cells for the detection of anti-nuclear antibodies (ANA) (25, 55), anti-neutrophil cytoplasmatic antibodies (ANCA) (56), anti-mitochcondrial antibody (AMA), anti-Epstein- Barr virus (EBV) antibodies, anti-dsDNA antibodies using *Crithidia luciliae*-based IIF (26), recombinant cell-based assays for multiparametric serological testing in autoimmune encephalitis, e.g., with recombinant cells (54), and rat liver and kidney for detection of reactivity against liver-kidney microsomes (LKM). In the present study, the main challenge was that the small structures relevant for classification were present only on certain parts of the large area covered by the tissue on the biochip. This prevented the use of standard deep networks. We applied segmentation to focus the attention of the classification networks to the crucial regions. For salt-split skin substrate, unspecific and low luminance borderline cases led to the most difficulty because there is no clear decision boundary. The segmentation of esophagus tissue was particularly challenging due to its naturally high variance in tissue layout. Specifically, segmentation and thus detection of the BMZ, which is very thin compared to other sections of the tissue, is a challenging task. Furthermore, classification of the BMZ is complex because it lacks specific structural features. Besides the challenging algorithmic processing, collecting a sufficient amount of training data containing hand-labeled ground truth masks for the segmentation neural network is a tedious and time-consuming manual task.

Our results show that deep learning as a method for computer-aided diagnostics on microscopy images has evolved into a state-of-the-art method besides traditional computer vision. Segmentation and classification of neural networks showed good results in this work. Even very complex microscopy images with tissue layers that are difficult for professionals to evaluate can reliably be segmented and classified *via* computer-aided algorithms.

The present study has several limitations. Only a limited number of sera with salt-split skin dermal binding pattern were applied. Therefore, the accuracy for this pattern is of lower significance. A larger study focusing on the clinical application of the classifiers will certainly

improve this aspect and is anticipated. Also, the algorithm only helps to find the expressing patterns for IgG autoantibodies in pemphigus and pemphigoid diseases. Patterns due to IgA autoantibodies are not expected to greatly differ between the ones by IgG reactivity but have not been formally employed in the present study. Currently, there is also no automated assignment of the identified patterns to a specific AIBD. Additional algorithms for advanced diagnostics of AIBD using BIOCHIP® mosaic-based IIF, including classifiers for recombinant BP180 as well as for cell-based IIF with recombinant BP230, DSG1, and DSG3 classifiers, are under development to detect autoantibodies specific for these disorders. The great advantage is that the results of IF image evaluation will be automatically proposed for verification and approval by laboratory personnel. In conclusion, the presented classifiers and algorithms allow the semi-automated assessment of autoantibody binding on monkey esophagus and primate salt-split skin in routine diagnostics of pemphigus and pemphigoid diseases. This innovation will further improve and facilitate the diagnosis of these rare autoimmune disorders.

Data availability statement

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics committee of the University of Lübeck. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

JK and JH contributed equally to this work and share first authorship. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by structural funding through the Cluster of Excellence 2167 and the Collaborative Research Center 1526 (Project A01 to E.S.).

Conflict of interest

JV, JH, SG, JK, CK, NW, and KA are employees of EUROIMMUN Medizinische Labordiagnostika AG. ES has a scientific cooperation with EUROIMMUN. JV, JH, SG, JK, CK, MH, ES, and NB are inventors of the patent application number 22199236 that has been filed by EUROIMMUN Medizinische Labordiagnostika AG on 30th Sept 2022. This patent application is pending and covers the algorithmic processing of the salt-split skin substrate.

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frontiers | Frontiers in Immunology

TYPE Case Report
PUBLISHED 09 March 2023
DOI 10.3389/fimmu.2023.1149119

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders: Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 21 January 2023
ACCEPTED 27 February 2023
PUBLISHED 09 March 2023

CITATION
Liu W, Li H, Jin Y, Cheng L, Shi L, Gao Y, Zhou Z, Feng S, Qian H, Hashimoto T and Li X (2023) Case report: Mucous membrane pemphigoid with complicated autoantibody profile indicating the necessity of comprehensive diagnostic methods and the contribution of IgA autoantibodies.
Front. Immunol. 14:1149119.
doi: 10.3389/fimmu.2023.1149119

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Case report: Mucous membrane pemphigoid with complicated autoantibody profile indicating the necessity of comprehensive diagnostic methods and the contribution of IgA autoantibodies

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Mucous membrane pemphigoid (MMP) is a type of subepithelial autoimmune bullous disease, affecting various mucosae, occasionally with skin lesions. Both diagnosis and treatment of MMP are difficult. Although multiple autoantigens have been identified for MMP, the pathogenesis of MMP is still unclear. In this study, we presented a female MMP case with extensive oral mucosal lesions and skin lesions, particularly on the extremities. IgG and IgA autoantibodies against multiple autoantigens including BP180, laminin 332, integrinα6β4 and desmoglein 3, and IgM autoantibodies against BP180 were identified during the disease course. Compared with IgG autoantibodies, the levels of IgA autoantibodies against various autoantigens decreased more significantly with improvement of clinical features after the initiation of treatments. Our findings indicated the importance of comprehensive autoantibody screening for different immunoglobulin types and autoantigens at multiple time points for the precise diagnosis of various autoimmune bullous diseases, and the significant involvement of IgA autoantibodies into the pathogenesis of MMP.

KEYWORDS

mucous membrane pemphigoid, IgA autoantibody, lesions on the extremities, bronchiolitis obliterans, integrin α6β4, laminin 332, BP180

Introduction

Mucous membrane pemphigoid (MMP) is a subgroup of chronic, inflammatory, subepithelial autoimmune bullous disease (AIBD), mainly affecting various mucosal membranes, particularly oral and ocular mucosae, with or without skin lesions (1–3). Identified MMP-related autoantigens include BP180, BP230, laminin (LM) 332, type VII collagen, integrin (ITG) α6β4 and LMγ1 (1, 4, 5), among which autoantibodies against BP180 and LM332 have been most frequently reported (1–3, 6–8). MMP-related autoantibodies are mainly IgG type and less frequently IgA type, and MMP cases occasionally showed multiple autoantibodies (1–4). Our group has continuously focused on the research of MMP cases and attempted to find autoantibodies relevant for the diagnosis and pathogenesis by analyzing the clinical features and the autoantibody profiles of each MMP case during the disease course.

In this study, we presented a unique MMP case with autoantibodies of different immunoglobulin types against multiple autoantigens, which emphasized the diagnostic and pathogenic values of IgA autoantibodies in MMP. In addition, the findings in the present case suggested the potential relationship between predominant lesions on the extremities and the presence of anti-BP180 autoantibodies, which we hypothesized in our previous study (9).

Case description

An 80-year-old female presented with scattered erythema, blisters, erosions and bloody crusts with itch on the head, trunk and limbs (Figures 1A–E), accompanied with extensive oral mucosal lesions (Figure 1F). Histopathology of biopsy from lesional skin on the left forearm showed subepidermal blister and infiltration of lymphocytes and eosinophils (Figure 2A). Direct immunofluorescence showed only linear basement membrane zone (BMZ) deposition of IgG (Figure 2B), but not of IgA, IgM and C3 (data not shown), without depositions to keratinocyte cell surfaces.

By indirect immunofluorescence (IIF) using normal human skin, the patient serum taken at Day 0 showed IgG and IgA anti-BMZ antibodies, without antibodies to keratinocyte cell surfaces (Figure 2C), while the serum of Day 11 showed only IgG anti-BMZ antibodies (Figure 2C). By IIF using 1M NaCl-split normal human skin (ssIIF), the patient serum of Day 0 showed IgG and IgA antibodies reactive with the epidermal side of the split skin (Figure 2D), while the serum of Day 11 showed strong IgG and weaker IgA reactivities to the epidermal side of the split skin (Figure 2D). IIF using rat bladder tissue showed IgG staining on transitional epithelia weakly for the serum of Day 0 and strongly for the serum of Day 11 (Figure 2E).

FIGURE 1
Clinical features of this patient. **(A, B)** Erythema, blisters and bloody crusts on the knees and lower limbs **(A)** and on the hands **(B)**. **(C)** Erythema and blisters on the buttocks and thighs. **(D)** Blisters on the face and bloody crusts around the eye. **(E)** Bloody crusts around the ear. **(F)** Sever mucosal lesions on the lips and in the oral cavity.

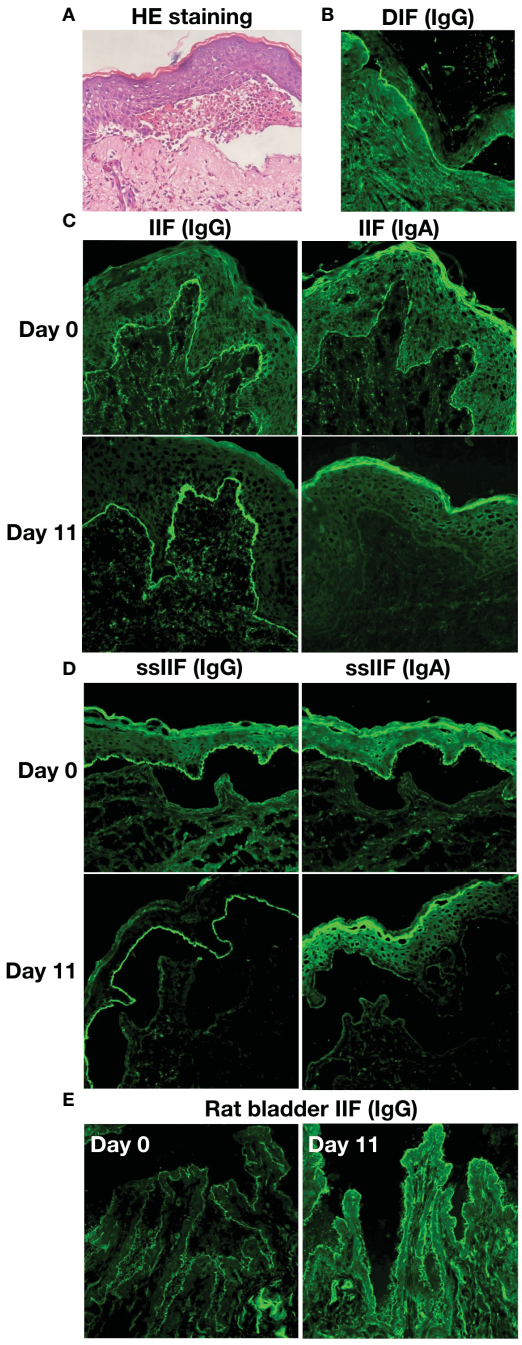

FIGURE 2
The results of histopathological and immunofluorescence tests of this patient. **(A)** Histopathological features of biopsy from lesioned skin on the left forearm (HE staining, original magnification, x200). **(B)** Direct immunofluorescence (DIF) for IgG. **(C)** Indirect immunofluorescence with normal human skin (IIF) for IgG and IgA antibodies on Days 0 and 11. **(D)** IIF using 1 M NaCl-split normal human skin (ssIIF) for IgG and IgA antibodies on Days 0 and 11. **(E)** IIF using rat bladder tissue (Rat bladder IIF) for IgG antibodies on Days 0 and 11.

Clinical features and treatments during the disease course are summarized in Table 1.

We next performed various immunoblotting tests and ELISAs for this patient sera taken at Days 0, 11 and/or 57 (Table S1). By immunoblotting of normal human epidermal extract, the sera of Days 0 and 11 showed IgG autoantibodies against BP230, BP180 and LAD-1, and IgA autoantibodies against envoplakin, periplakin and desmoglein (Dsg) 3, with relatively stronger IgA reactivities for the serum of Day 11(Figure 3A). By immunoblotting of LM332 recombinant protein (RP), the serum of Day 11, but not of Day 0, showed IgA autoantibodies against LMβ3 (Figure 3B). By immunoblotting of RP of extracellular domain (ECD) of ITGα6β4, the sera of both Days 0 and 11 showed IgA autoantibodies against ITGα6 and ITGβ4 (Figure 3C).

By ELISAs using various RPs, IgG autoantibodies against BP180 NC16a-domain were positive for all the sera of Days 0, 11 and 57,

TABLE 1 Clinical features and treatment during the disease course.

Days	Clinical features	Treatment
Before visit to our hospital	Erythema and blisters with itch on the head, trunk and limbs, with oral mucosal lesions for 20 days	No treatments
Day 0	Erythema, blisters, erosions and bloody crusts with itch on the head, trunk and limbs	Prednisone 15 mg/day, minocycline hydrochloride 100 mg/day, thalidomide 50 mg/da
Day 11	There were no new erythema or blisters or erythema, and no itching. New oral mucosal lesions appeared.	Prednisone 15 mg/day, minocycline hydrochloride 100 mg/day, thalidomide 50 mg/day
Day 57	There were no new lesions and no itching. Dyspnea developed	Prednisone 10 mg/day, minocycline hydrochloride 100 mg/day, thalidomide 50 mg/day
Day 105	There were no new lesions, and no itching. Diagnosis of bronchiolitis obliterans was made.	Prednisone 5 mg/day, minocycline hydrochloride 100 mg/day, thalidomide 50 mg/day

IgG autoantibodies against RPs of ECDs of ITGα6β4 and ITGβ4 were weakly positive for only the serum of Day 11, while all the sera were negative for IgG autoantibodies against Dsg1, Dsg3 and LM332 (Figure 3D).

By ELISAs of BP180 NC16a-domain RP, IgA autoantibodies were positive at Days 0, 11 and 57 with decreasing levels with time, and IgM autoantibodies were positive at Days 0 and 11, but negative at Day 57, while IgE autoantibodies were continuously negative (Figure 3E). By ELISA of LM332 RP, IgA autoantibodies against LM332 was positive at Day 11, but negative at Days 0 and 57 (Figure 3E).

By ELISA of peptides of various autoantigens, IgG autoantibodies against Dsg1 and BP180, but not Dsg3 and ITGβ4, were positive at Days 0, 11 and 57, and IgA autoantibodies against Dsg3, BP180 and ITGβ4, but not Dsg1, were positive at Days 0, 11 and 57, although their levels changed with time (Figure 3F).

The sera of this patient showed negative results in other tests, which are routinely performed in our laboratory for detection of other known autoantibodies in AIBDs.

Based on the clinical, histopathological features and immunological findings, this patient was diagnosed as MMP with IgG autoantibodies against BP230, BP180, LAD-1, Dsg1, ITGα6β4 and IgA autoantibodies against BP180, LM332, ITGα6β4, Dsg3, envoplakin and periplakin.

The treatment with prednisone 15mg/day was initiated with other drugs described in Table 1. The oral mucosal lesions were most severe at Day 11, and then improved with therapy. The HSV DNA was detected in the oral secretions at Day 11. The dose of prednisone tapered to 10 mg/day at Day 57, when no new mucocutaneous lesions developed (Table 1). In addition, the patient was developing dyspnea during the disease course and diagnosis of bronchiolitis obliterans was made at Day 105 (Table 1).

Discussion

This case showed very complicated immunological profile of IgG and IgA autoantibodies to multiple autoantigens during the disease course, although this case might be simply diagnosed as bullous pemphigoid or anti-BP180-type MMP, if the comprehensive autoantibody screening was not performed. The results in this study emphasized the importance of comprehensive autoantibody screening with different immunoglobulin types for multiple time points (4, 10).

Compared with Day 0, the serum of Day 11 showed relatively lower IgA reactivities in IIF and ssIIF, which was consistent with the levels of IgA anti-BP180 NC16a-domain autoantibodies detected by ELISA. However, compared with Day 0, the serum of Day 11 had increased levels of IgA autoantibodies against BP180, Dsg3, LM332 and ITGα6β4 detected by immunoblotting and ELISAs of RPs, and increased levels of IgA autoantibodies against Dsg3, BP180 and ITGβ4 detected by ELISAs of peptides, which implied that those increased IgA autoantibodies might be responsible for the severer oral mucosal lesions at Day 11. Compared with Day 11, the serum of Day 57 showed decreased levels of IgA autoantibodies against Dsg3 and ITGβ4 detected by ELISAs of peptides. Although various IgA autoantibodies in the present case showed distinct pattern of intensities, their levels were finally decreased with accordance of improvement of clinical features after the treatments. In contrast, the levels of IgG against multiple autoantigens showed no significant decreases with therapy. These results implied that IgA autoantibodies in this patient might be the major pathogenic autoantibodies.

The complicated dynamics of various autoantibodies appearance in the disease course of this patient could be justified by epitope spreading phenomena (11).

This case showed positive IgG signals on transitional epithelia in rat bladder IIF, demonstrated autoantibodies against envoplakin and periplakin in immunoblotting of normal human epidermal extract, and was diagnosed with bronchiolitis obliterans, which suggested the possible diagnosis of paraneoplastic pemphigus (PNP) (9). However, this case showed prominent reactivity with BMZ in both direct and indirect immunofluorescence tests, strongly reacted with various MMP-related autoantigens, and showed no tumors. Therefore, we finally diagnosed this case as MMP, rather than PNP (sine neoplasia). Various findings in this case strongly suggested the possible relationship between MMP and PNP.

We have previously reported that skin lesions on the extremities were seen significantly more frequently in PNP patients with positive anti-BP180 autoantibodies (9). The clinical features and autoantibody profile of the present case also support this conclusion.

10.3389/fimmu.2023.1149119

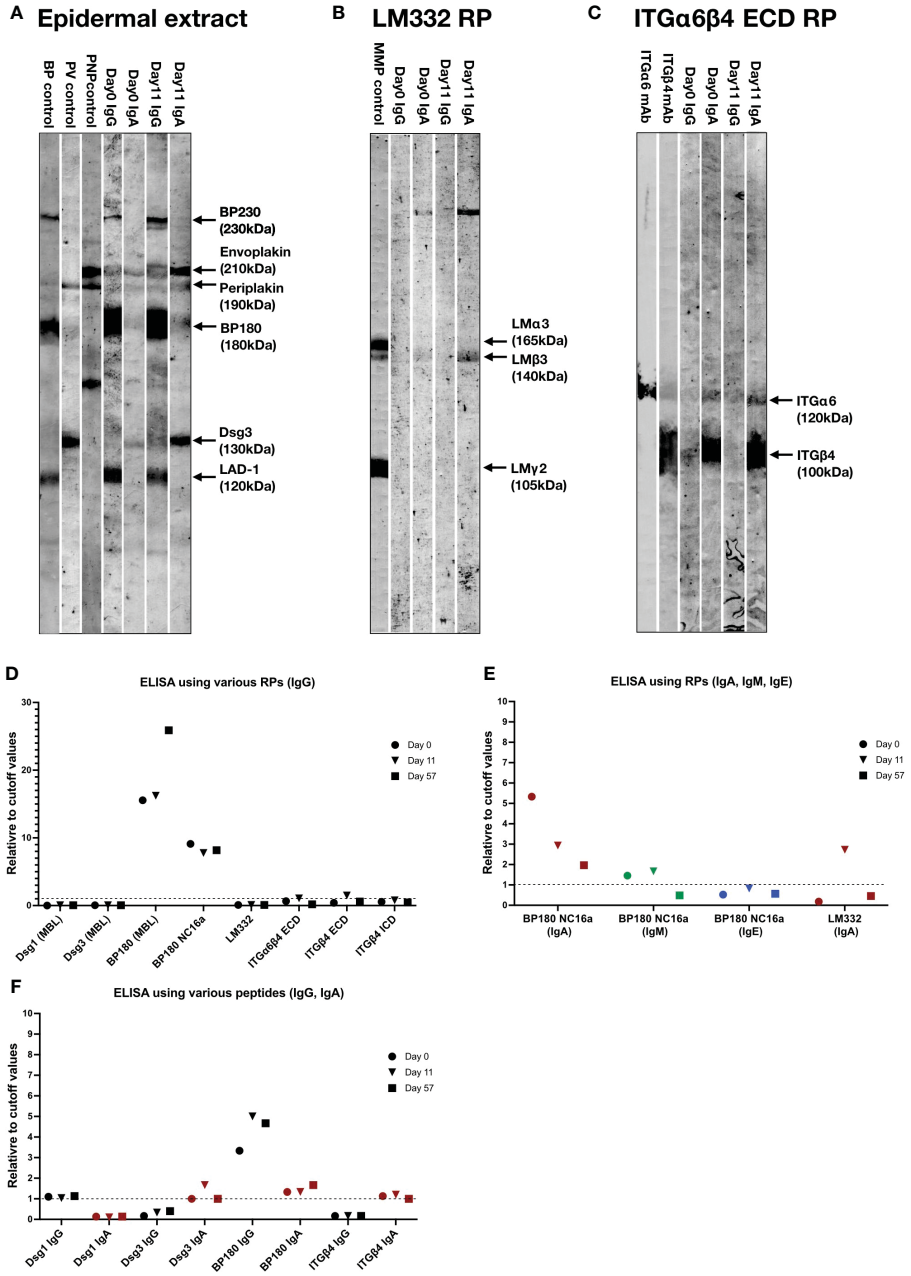

FIGURE 3
The results of immunoblotting tests and ELISAs of this patient. **(A–C)** Immunoblotting using normal human epidermal extract **(A)**, recombinant proteins (RP) of laminin (LM) 332 **(B)** and RP of extracellular domain (ECD) of integrin (ITG) α6β4 **(C)**. IgG antibodies of bullous pemphigoid (BP) control serum were positive for BP230, BP180 and LAD-1, IgG antibodies of pemphigus vulgaris (PV) control serum were positive for desmoglein (Dsg) 1 and Dsg3, IgG antibodies of paraneoplastic pemphigus (PNP) control serum were positive for envoplakin and periplakin. IgG antibodies of anti-LM332-type mucous membrane pemphigoid (MMP) control serum were positive for three subunits of LM332. mAb, monoclonal antibody. The RPs of LM332 and ITGα6β4 ECD were purchased from Biolamina (Sundbyberg, Sweden) and R&D systems (Minneapolis, MN, USA), respectively. **(D)** ELISAs using various RPs, including three commercially available ELSIA kits (MBL, Tokyo, Japan) and other in-house ELISAs for detection of IgG autoantibodies against corresponding autoantigens. **(E)** ELISAs using BP180 NC16a-domain and LM332 RPs for detection of IgA, IgM or IgE autoantibodies. **(F)** ELISAs using peptides of indicated autoantigens for detection of corresponding IgG and IgA autoantibodies. Dot lines indicate the cutoff values.

In addition, autoantibodies against envoplakin and periplakin were important diagnostic markers for PNP, and a considerable number of PNP patients would later develop bronchiolitis obliterans (9). Therefore, we speculate that autoantibodies against envoplakin and periplakin might also be predictive markers for bronchiolitis obliterans. In the present case, autoantibodies against envoplakin and periplakin were detected in the patient serum of Day 11, and bronchiolitis obliterans was finally confirmed at Day 105, which suggested the possible relationship between anti-envoplakin/periplakin autoantibodies and bronchiolitis obliterans.

We recently investigated the correlation of HSA infection and pemphigus vulgaris (PV), and found that the significantly elevated

prevalence of HSV infection in PV patients might mainly be caused by ruptured barrier of oral mucosa (12). HSV infection in MMP patients was rarely reported (13), and its potential participation mechanism in the present MMP case is still unclear.

In the present MMP case, prednisone combined with minocycline hydrochloride, and thalidomide had a good therapeutic effect.

In summary, we reported a MMP case with complicated IgG and IgA autoantibody profile against against multiple autoantigens. The results in the present report will benefit for future pathogenesis studies of MMP, particularly on IgA autoantibodies and involvement of multiple autoantibodies.

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

This study was performed according to Declaration of Helsinki and approved by ethics committee of Dalian 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

WL, HQ, TH and XL had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. HQ, TH and XL conceived and designed the project. WL, HL, YJ, LC, LS, YG, ZZ, SF, HQ, TH, XL collected case information and laboratory data, and analyzed the data. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by JSPS KAKENHI Grant (Grant-in-Aid for Scientific Research (C)) (Number 21K08331) to TH, by "Research on Measures for Intractable Diseases" from the Ministry of Health, Labor and Welfare Project to TH, and by a grant from Takeda Science Foundation.

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.2023.1149119/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 15 March 2023
DOI 10.3389/fimmu.2023.1134720

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 30 December 2022
ACCEPTED 17 February 2023
PUBLISHED 15 March 2023

CITATION
Janela R, Ishii N, Castel M, Jouen F, Cellier L, Courville P, Joly P and Hébert V (2023) Mixed muco-cutaneous pemphigoid: Clinical and immunological features of 15 cases.
Front. Immunol. 14:1134720.
doi: 10.3389/fimmu.2023.1134720

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Mixed muco-cutaneous pemphigoid: Clinical and immunological features of 15 cases

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Introduction: We describe a series of patients whose auto-immune bullous skin disease (AIBD) of the dermal-epidermal junction (DEJ) was characterized by clinical, immunological and ultrastructural features intermediate between bullous pemphigoid (BP) and mucous membrane pemphigoid (MMP), and a recalcitrant course.

Patients and Methods: From the database of the French reference centre for AIBD, we screened all the patients who were referred for an AIBD of the DEJ with a mucosal involvement, who neither met the diagnostic criteria for the diagnosis of BP, nor were typical of MMP. Sera were analysed by NC16A-ELISA and immunobloting against the C-terminal and LAD-1 parts of BP180. Skin biopsies were studied by direct immunoelectron microscopy (IEM).

Results: Fifteen patients (4 males, 11 females) of mean age 70.8 ± 11.8 years were included. The mucosal involvement was localized in oral cavity in all cases and in pharyngeal/laryngeal or genital area in 8 (53%), and 6 patients (40%), respectively. No patient had ocular involvement, nor atrophic or fibrosing scars. All patients had extensive skin lesions (mean BPDAI score =65.9 ± 24.4), which predominated on the upper body part. Direct IEM performed on 8 patients showed IgG deposits on the lamina lucida in all cases, and the lamina densa in 5 cases. All sera recognized NC16A, while none recognized BP-230 in ELISA. 10 out of the 13 tested sera (76.9%) contained IgG which recognized the C-terminal domain of BP180 and 10 sera (76.9%) the LAD-1 domain of BP180. Patients poorly responded to super potent topical corticosteroids and were treated with oral corticosteroids ± immunosuppressant in 13 cases (86.6%).

10.3389/fimmu.2023.1134720

Conclusion: This mixed muco-cutaneous pemphigoid differs from BP by the younger age of patients, multiple mucosae involvement, circulating antibodies against both the C- and N-terminal part of BP180, and very poor response to topical CS. It differs from MMP by extensive inflammatory skin lesions, absence of ocular involvement and atrophic/fibrosing scars.

KEYWORDS

bullous pemphigoid, mucous membrane pemphigoid, new entity, immunoblot (western blot), direct electro immunomicroscopy

Highlights

- Some patients with junctional autoimmune bullous disease have clinical atypical involvements as well as serological profile that overlap between bullous pemphigoid (BP) and mucous membrane pemphigoid (MMP).
- We described patients who differ from BP by the younger age of patients, deep mucosae involvement but from MMP by extensive inflammatory skin lesions, absence of ocular involvement and atrophic/fibrosing scars. Histologically and serologically, patients had findings that could observed both in BP and MMP.

Introduction

Bullous pemphigoid (BP) and Membrane Mucous Pemphigoid (MMP) are the two most common autoimmune bullous skin diseases (AIBD) of the dermal epidermal junction (DEJ).

BP classically occurs in the elderly and is characterized by a severe pruritic blistering eruption. BP is associated with autoantibodies targeting the NC16-A domain of BP180 and BP230, which are two components of the hemidesmosomes (1–7). Clinical criteria have been proposed in 1998 to differentiate BP from other AIBD (5), that have been re-evaluated in 2004 using immunoblot analysis (8). The presence of at least 3 of these 4 criteria: no mucosal lesion, no predominant damage to the head and neck, age older than 70 years, no atrophic scar; allows to predict the diagnosis of BP with a 86% sensitivity and a 90% specificity (5).

One the other hand, MMP describes a group of chronic AIBD of the chorio-epithelial or DEJ. Typical presentation is characterized by predominant or exclusive mucosal lesions which can involve oral, naso-pharyngeal, laryngo-tracheal, genital, esophageal, anal and ocular mucous membranes (9–11). Skin lesions are often limited and evolve to atrophic scars. MMP is mediated by autoantibodies directed against various antigens of the basement membrane zone (BMZ) including BP180, laminin 332, and type 7 collagen (12).

Numerous atypical forms of BP have been described, which account for around 20% of BP patients (13–15). A few atypical forms of MMP with extensive skin lesions on inflammatory skin have also been reported (16, 17). We recently treated patients with clinical features intermediate between BP and MMP. These patients were younger than the usual age of patients with the classical type of BP, and had both oral and laryngeal/pharyngeal involvement, and extensive inflammatory skin lesions. They had a common histological and immunological profile characterized by a subepidermal blister with polynuclear eosinophils, linear IgG deposits on the DEJ by DIF and the presence of anti-BP180 Abs using the commercially available ELISA, which detects antibodies against the NC16-A region (18, 19). To assess whether the clinical and immunological features of these patients were anecdotal or corresponded to a rare subgroup of AIBD of the DEJ, we screened all the patients who were referred to our centre for an AIBD of the DEJ with mucosal involvement, who neither met the diagnostic criteria proposed by Vaillant et al. for the diagnosis of BP, nor were typical of MMP.

We found 15 patients with the same clinical phenotype, overlapping between BP and MMP. These patients whose mean age was more than 10 years younger than "classical" BP patients presented with extensive inflammatory skin lesions, which predominated on the upper part of the body in many of them, and were associated with almost constant oral and extra-oral mucosal involvement. However, none of them had ocular involvement, nor atrophic or fibrosing scars. These clinical features which were reminiscent of both BP and MMP, were associated with: i) the detection of circulating antibodies which recognized both the NC16A domain and the C-terminal part of BP180, with no anti-BP230 antibodies, ii) the *in vivo* localization of IgG deposits on both the lamina lucida and the lamina densa in half of the cases and iii) a refractory course with poor response to super potent topical corticosteroids. Based on these characteristics, we suggest that it is a new entity that overlaps between BP and MMP as a mixed muco-cutaneous pemphigoid (MMCP).

Patients and methods

Inclusion criteria

Inclusion criteria were: i) skin lesions suggestive of AIBD of the DEJ with large tense blisters associated with mucosal involvement,

which however did not meet 3 of the 4 clinical criteria proposed for the diagnosis of BP (5, 8); ii) ii) a histological picture of subepidermal blister with linear IgG and/or C3 deposits on the DEJ by direct immunofluorescence (DIF), and iii) labelling of the epidermal side of the detachment by indirect examination of patients' serum on NaCl-split skin. Exclusion criteria were: i) the presence of IgA deposits on the DEJ by DIF, ii) a dermal labelling by IIF on salt split skin, iii) the recognition of collagen 7 or laminin γ1 by ELISA and/or immunoblot on dermal extract.

Clinical data

The following clinical and biological data were recorded at the time of diagnosis: gender, age, drug intake, number of daily new blisters (< or ≥ 10), disease severity as measured by the bullous pemphigoid disease area index (BPDAI) (20, 21), and blood eosinophil count.

During patients' follow-up, treatment efficacy and safety outcomes were recorded: proportion of patients who achieved disease control at Day 21, and one-year complete remission, and one-year mortality rate.

Serological assays

Anti-BP180 and anti-BP230 autoantibody detection

Anti-BP180 and anti-BP230 autoantibodies were detected in serum using specific enzyme-linked immunosorbent assays (ELISA EUROIMMUN®) in the Immunology laboratory of the University Hospital of Rouen. No further dilution was performed when titres were ≥ 200 U/mL.

Detection of antibodies directed against non-NC16A parts of BP-180

Patient's sera were also analysed by Dr Ishii from the Kurume University Hospital of Medicine, Japan using immunobloting on recombinant proteins directed against the LAD-1 (524-1497aa of human BP180) and the C-terminal (1193-1497aa of human BP180) part of BP180 in order to detect autoantibodies targeting epitopes other than NC16A. Out of the 13 patients, 11 sera were analysed, since sera from two patients were not available.

Histological analysis and direct immunoelectron microscopy

All examinations have been performed in the pathology laboratory of the Rouen University Hospital. Histological analysis by hematoxylin-eosin staining was performed in all patients. Direct immunoelectron microscopy examination of skin biopsies from 8 patients. Skin biopsies were collected in a Hanks' balanced salt solution. Thereafter, fragments were incubated with anti-IgG, peroxidase-coupled antibodies. After fixation in Karnovsky's fixative, revelation in DAB and post-fixation in osmium, the samples were included in epoxy resin. Semi-thin (± ultrathin) sections were made to demonstrate immune-staining by electron microscopy (22).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.0 (San Diego, CA, USA).

Results

Patients' characteristics

Clinical characteristics of patients

From January 2010 to December 2020, 233 patients were referred to our centre for an AIBD of the DEJ. Of them, 15 patients (6.4%) corresponded to the inclusion criteria and were analysed.

Clinical characteristics of the patients are described in Table 1. The male/female ratio was 0.36, and mean age 70.8 ± 11.8 years. Eight of the 15 patients were < 70 years old. All patients had oral mucosal involvement (Figure 1A) and 12 of 15 had an extra-oral involvement: 8 had a deep pharyngeal or laryngeal involvement (53%), and 6 had genital involvement (40%) (Figure 1B). No patient had ocular involvement. The mean BPDAI mucosal subscore was 13.3 ± 7. 5. Fourteen patients (93.3%) had an extensive skin involvement at the time of diagnosis, with a mean BPDAI total activity score of 65.9 ± 29.4, and a mean skin subscore of 52.6 ± 21.2. In particular, the blisters/erosions subscore was particularly high: 35± 12.5 out of 120. Ten patients (66.6%) had severe skin lesions on the head, neck and/or upper trunk (Figure 2). None of the patients had cicatricial or scarring skin or mucosal lesions. Surprisingly, only three patients suffered from neurological disorders.

Biological, histological and immunological findings

Blood eosinophilia (>0.5G/L) was present in 6 patients. According to the inclusion and exclusion criteria, all sera showed a labelling of the epidermal side of the detachment by IIF on salt-split skin, while no serum labelled the dermal side. All sera contained anti-BP180 antibodies without anti- BP230 antibodies by ELISA. Ten patients (76.9%) had anti-BP180 antibody ELISA values higher than 200 U/mL, which corresponds to the highest value detected by the kit.

Thirteen sera were analysed by immunoblot. All sera contained IgG which recognized the recombinant protein corresponding to the NC16A domain. In addition, 10 sera (76.9%) contained IgG which recognized the C-terminal domain of BP180, and 10 sera (76.9%) had IgG against the LAD-1 domain of BP180.

Histological analysis performed in all patients didn't evidence a particular pattern. All patients had a typical subepidermal blister with polynuclear eosinophils. Polynuclear neutrophils were observed in 1 patient and lymphocytes infiltrate in 1 other patient.

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TABLE 1 Patients characteristics.

1. Clinical characteristics	
Number of patients (*n*)	15
Sex, n (%)	
Male	4 (26.7)
Female	11 (73.3)
Age, years	
Mean age ± SD	70.8 ± 11.8
Number of daily new blisters, n (%)	
< 10 blisters per day	1 (6.7)
≥ 10 blisters per day	14 (93.3)
BPDAI, mean ± SD	
BPDAI total	65.9 ± 29.4
Activity of total skin involvement	52.6 ± 21.2
Blisters/erosions	35 ± 12.5
Erythema/urticaria	17.6 ± 10.7
Activity of mucosal involvement	13.3 ± 7.5
2. Immunobiological characteristics	
Serum anti-BP180 NC16A ELISA	
Number of patients (*n*)	13
Positive	13
Number of patients with titer ≥ 200, n (%)	10 (76.9)
Eosinophilia value	
Number of patients (*n*)	13
Mean ± SD (G/L)	1 ± 0.9
≥ 0,7 G/L, n (%)	6 (46.2)
Indirect immunofluorescence	
Number of patients (*n*)	13
Epidermic positivity, n (%)	11 (84.6)
Dermic positivity, n (%)	0 (0)
Direct Immunoelectron Microscopy	
Number of patients (*n*)	8
Lamina lucida IgG deposits	8
Lamina densa sIgG deposits	6
3. Treatments efficacy	
Number of patients (*n*)	15
Control of disease at day 21 with topical CS alone	1 (6.6)
Use of at least one systemic treatment, n (%)	13 (86.6)
Mean number of lines of treatment ± SD	2.4 ± 1.2
Different treatment used, n (%)	
Oral corticosteroids	13 (46.2)
Cyclins	6 (46.2)
Methotrexate	5 (38.5)
Mycophénolate mofetil	4 (30.8)
Dapsone	2 (15.4)
Rituximab	2 (15.4)
Omalizumab	2 (15.4)

Abbreviations: SD, standard deviation; BPDAI, bullous pemphigoid disease area index; BP, bullous pemphigoid.

Skin biopsies from 8 patients were examined by direct immunoelectron microscopy. All eight patients had IgG deposits in the lamina lucida, and 5 patients also had a labelling of the lamina densa (Figure 3). No deposits were observed within the hemidesmosomes.

Patients' course and treatment efficacy

All patients were initially treated with super potent topical CS. Disease control was achieved by Day 21 in only one patient (6.6%) and was further achieved during the evolution in only 2 cases with topical CS alone. Thus, 13 patients (86.6%) needed a second-line treatment which corresponded to oral prednisone in all cases, either alone (n=3) or combined with an immunosuppressant (methotrexate, mycophenolate mofetil, rituximab) and/or immunomodulator (dapsone, cyclins, omalizumab) in 10 cases. The mean number of treatments lines was 2.4 ± 1.2. Despite the use of a second- or third-line treatment, only 6 out of the 15 patients (40%) achieved a complete remission after 12 months of follow-up.

Discussion

We report a series of 15 patients presenting an atypical form of AIBD of the DEJ characterized by clinical, immunological and ultrastructural features intermediate between BP and MMP Table 2. These patients were characterized clinically by a rather young age, multiple oral and extra-oral mucosal involvement, extensive skin lesions, and a recalcitrant course. Immunological analysis of these sera showed the presence of anti-BP180 antibodies without anti-BP230 antibodies in all cases. Interestingly, anti-BP180 antibodies were not only directed against the NC16A domain, but also against the COOH-terminal domain of BP 180. Accordingly, *in vivo* IgG deposits were localised in the lamina lucida and in the lamina densa in 5 of the 8 biopsies examined by direct IEM.

Despite the fact that mucosal involvement has been reported in 20% of BP patients (14, 15, 23), the clinical phenotype of the patients reported in the present study differed from the main characteristics of BP patients including i) a mean age more than 10-year younger (70.8 ± 11.8 years versus around 83 years in French series (24, 25), including half of the patients who were < 70 years old; ii) the frequent involvement of mucosae other than the oral cavity, since a deep pharyngeal or laryngeal involvement was observed in half of these patients, while it is rarely observed in

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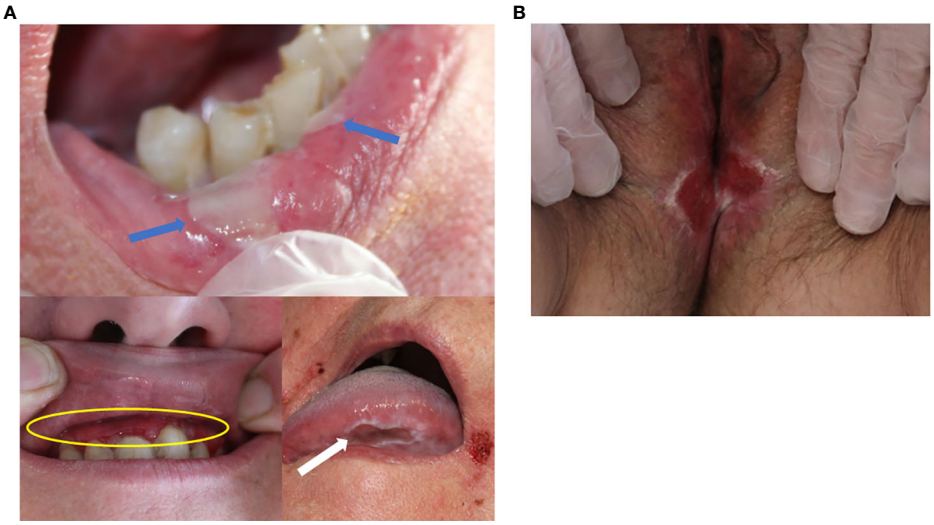

FIGURE 1
(A) Oral mucosal lesions. Blue arrows: labial blisters. Yellow circle: erosive gingivitis. White arrow: tongue ulceration. **(B)** Genital mucosal lesions. Erosive vulvitis.

FIGURE 2
Patients with unsual severe involvement of the face **(A, B)**, upper part of the trunk **(C)** and neck **(D, E)**.

patients with BP, and iii) the predominance of skin lesion on the head, neck and upper trunk in 66.6% of patients, which is reminiscent of MMP rather than BP. Moreover, only 3 out of the 15 patients had associated debilitating neurological disorders, as it has been classically reported in BP patients (26). Finally the serological profile of these patients, which was characterised by exclusive anti-BP180 antibodies with no anti-BP230 antibodies, differed from that of classical BP sera since only 26% of BP sera studied by ELISA in a large serological study had this BP180 positive/BP230 negative immunological profile (27).

The involvement of both the oral and extra oral mucosa, the predominance of skin lesion on the head and neck and upper part of the trunk in half of the patients, as well as the presence of circulating antibodies directed against BP180 with no anti-BP230 antibodies might suggest the diagnosis of MMP. However, all these patients had extensive inflammatory skin lesions with very high mean BPDAI skin activity

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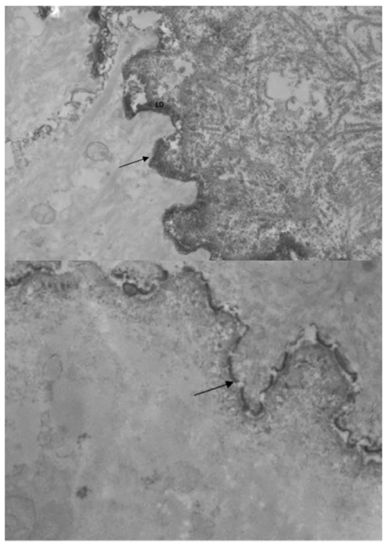

FIGURE 3
Electro immunomicroscopy IgG deposits on both lamina densa and lamina lucida in two patients.

TABLE 2 Characteristics of patients arguing against BP or MMP.

Against BP	Against MMP
Young age	Extensive cutaneous involvement
Head and neck involvement	Absence of opthalmic involvement
Deep mucosal involvement	Absence of scarring lesions
ELISA: absence of anti-BP230 Abs	ELISA: constant positivity of anti-BP180 Abs
Immunoblot: auto-Ab targeting the COOH-term domain of anti-BP180	
Poor response to conventionnal treatments	

sub-score of 52, which is extremely unusual in MMP. Interestingly, both the blister/erosion and the erythema/urticaria skin sub-scores were elevated, indicating the inflammatory character of skin lesions, which is not characteristic of MMP. Additionally, none of the patients had fibrosing nor atrophic scars, and none of them develop ocular involvement, which strongly argued against the diagnosis of MMP.

In accordance with the patients' clinical features which overlapped between BP and MMP, immunoblot analysis of the patients' sera using recombinant proteins corresponding to the C- and N-terminal parts of BP180 showed that 76.9% of these sera contained IgG antibodies directed against the LAD-1 and COOH-terminal parts of BP180, in addition to NC16A. These results are in accordance with the findings by Hoffman et al. who reported that 56% of sera from BP patients with mucosal involvement contained antibodies directed against the C-terminal domain of BP180, which confirms that this part of the protein seems implicated in the mucosal involvement of AIBDs of the DEJ, as observed in MMP (28). Indeed, some studies suggested that the presence of mucosal lesions in BP correlated with levels of IgG against the COOH-terminal epitope of BP180 that is located deeper than NC16A (19). Interestingly, whereas direct IEM examination of patients' skin showed *in vivo* antibody deposits on the lamina lucida in all the 8 biopsies examined, 5 patients whose serum contained antibodies directed against the C-terminal domain of BP180 also had *in vivo* IgG deposits on the lamina densa, in addition to the lamina lucida.

Finally, this particular clinical and immunological phenotype seems associated with a recalcitrant course since only 2 patients achieved disease control on topical CS alone whereas it usually allows it in 75% to 98% of cases (24, 25, 29, 30). The 13 others patients were secondarily treated with oral CS alone or combined with immunosuppressants.

Overall, while the overlapping distribution of antibodies directed against the C- and N-terminal part of BP80 has been described for many years in BP and MMP patients, we describe a corresponding particular clinical phenotype which is characterized by clinical, immunological and ultrastructural features intermediate between BP and MMP occurring in younger patients and associated with a recalcitrant course.

Data availability statement

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

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. 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

RJ: acquisition of data, drafting the manuscript NI: acquisition of data MC: acquisition of data FJ: acquisition of data LC: analysis of data PC: analysis of data PJ: design, draft and review of the manuscript VH: design, draft and review 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 constructed as a potential conflict of interest.

The handling editor DM declared a past co-authorship with the author PJ, VH.

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frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 17 April 2023
DOI 10.3389/fimmu.2023.1161927

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 08 February 2023
ACCEPTED 03 April 2023
PUBLISHED 17 April 2023

CITATION
Lee AY, Kim T and Kim JH (2023) Understanding CD4$^+$ T cells in autoimmune bullous diseases. *Front. Immunol.* 14:1161927. doi: 10.3389/fimmu.2023.1161927

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Understanding CD4$^+$ T cells in autoimmune bullous diseases

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Autoimmune bullous diseases (AIBDs) are a group of life-threatening blistering diseases caused by autoantibodies that target proteins in the skin and mucosa. Autoantibodies are the most important mediator in the pathogenesis of AIBDs, and various immune mechanisms contribute to the production of these pathogenic autoantibodies. Recently, significant progress has been made in understanding how CD4$^+$ T cells drive autoantibody production in these diseases. Here, we review the critical role of CD4$^+$ T cells in the production of pathogenic autoantibodies for the initiation and perpetuation of humoral response in AIBDs. To gain an in-depth understanding of CD4$^+$ T-cell pathogenicity, antigen specificity, and mechanisms of immune tolerance, this review covers comprehensive mouse and human studies of pemphigus and bullous pemphigoid. Further exploration of pathogenic CD4$^+$ T cells will potentially provide immune targets for improved treatment of AIBDs.

KEYWORDS

autoimmune bullous disease, pemphigus, bullous pemphigoid, pathogenicity, CD4 T cells

1 Introduction

Autoimmune bullous diseases (AIBDs) are a group of rare and serious skin diseases mediated by autoantibodies against adhesion molecules in the skin and/or mucous membrane, leading to the loss of cell-cell adhesion and blister formation. Autoantibodies produced by autoantigen-specific B cells cause blisters in AIBDs (1–3). However, reports suggests that CD4$^+$ T cells are also involved in the production of pathogenic autoantibodies and play a critical role in the pathogenesis of AIBDs. It is not yet known how pathogenic CD4$^+$ T cells are generated in AIBDs, but the studies of CD4$^+$ T cells may provide clues to understanding the development of these diseases. This review describes current views on the role of CD4$^+$ T cells in AIBDs, with a focus on pemphigus and bullous pemphigoid (BP).

2 Significance of CD4$^+$ T cells in pemphigus

2.1 Clinical features of pemphigus

Pemphigus is a representative type of AIBDs mediated by autoantibodies targeting desmoglein (DSG) 1 and 3, which chronically cause blisters and erosions on the skin and mucous membrane (4). The etymology of pemphigus, which comes from the Greek *pemphix*, means bubble. Pemphigus most commonly occurs between the ages of 50 and 60 years, and consists of several forms, the main three being pemphigus vulgaris (PV), pemphigus foliaceus (PF), and paraneoplastic pemphigus (PNP) (3). PV usually begins as oral mucosal blisters, but PF shows superficial skin blisters without mucosal involvement. PNP is mostly associated with neoplasia and is characterized by extensive stomatitis and polymorphous skin eruptions, including blisters (5). The main histologic features are intraepidermal blisters with acantholysis as a consequence of the loss of adhesion between keratinocytes. Immunofluorescence studies are able to detect the IgG autoantibodies directed against the surface of keratinocytes. While the current cornerstone of treatment is systemic corticosteroid, high dosage and long-term usage are usually required for disease control, which can result in multiple adverse effects (6). In that reason, steroid-sparing agents such as mycophenolate mofetil and azathioprine are often combined (7). Further, intravenous immunoglobulin was found to reduce disease activity in pemphigus (8). Rituximab, a monoclonal depleting antibody that targets CD20 on B cells, shows partial or complete remission achieved 3 to 6 months after treatment in 75% of patients with pemphigus (9–11), and recent studies showed that early administration of rituximab shortens the time to remission (12). Rituximab is currently used as a first-line treatment in the U.S. and Europe (13).

2.2 Evidence of CD4$^+$ T-cell pathogenicity in patients with pemphigus

B cells are the most important cells in induction of pemphigus, but there is growing evidence that autoreactive CD4$^+$ T cells play a pathogenic role. In particular, specific alleles of human leukocyte antigen (HLA) class II are associated with pemphigus. HLA-DRB1*04:02, HLA-DRB1*14 (14:01, 14:05, and 14:06), and HLA-DQB1*05:03 alleles are associated with PV, and HLA-DRB1*04 and HLA-DRB1*14 alleles are associated with PF and HLA-DRB1*01 alleles are especially associated with an endemic form of PF, also known as *fogo selvagem* (3, 14, 15). PNP is genetically associated with HLA-DRB1*03 (5, 16). These restricted types of HLA alleles differ among ethnic groups (17). DSG3-specific T-cell clones derived from patients with pemphigus vulgaris are proliferated following co-culture with a B-lymphoblastoid cell line expressing HLA-DRB1*14:01, HLA-DQB1*05:03, and HLA-DRB1*04:02 (18–20) (Figure 1). Although direct evidence for the production of pathogenic antibodies from DSG-specific B cells by these DSG-specific T cells is lacking, one study demonstrated that pathogenic anti-human DSG3 antibodies were induced by HLA-DRB1*04:02-restricted human DSG3-specific mouse T cells using humanized HLA-DRB1*04:02-transgenic mice (21).

The development of effective antibodies involves affinity maturation, a process by which B cells produce antibodies with higher affinity during subsequent responses to antigen. Affinity maturation is driven by somatic hypermutation and requires cognate-antigen interactions between B cells and CD4$^+$ T cells for initiation. In one study, more anti-DSG3 antibodies undergoing somatic hypermutation were detected in patients with pemphigus vulgaris after diagnosis compared to before disease onset, and these antibodies exhibited increased binding affinity for DSG3 and induced better dissociation of keratinocytes (22). These data further support the idea that DSG-specific CD4$^+$ T cells are pathogenic in pemphigus.

2.3 Pathogenicity, antigen specificity, and tolerance mechanism of CD4$^+$ T cells in murine pemphigus models

Many studies have attempted to understand the pathogenicity of CD4$^+$ T cells in pemphigus through use of active mouse models. These models are established by adoptive transfer of DSG3-immunized lymphocytes from *Dsg3*$^{-/-}$ mice into DSG3-expressing *Rag1*$^{-/-}$ or *Rag2*$^{-/-}$ mice (23, 24). Immunization is accomplished by grafting DSG3-expressing wild-type mouse skin into *Dsg3*$^{-/-}$ mice or by repeated injection of recombinant DSG3 protein into *Dsg3*$^{-/-}$ mice (23, 24). Recipient *Rag1*$^{-/-}$ or *Rag2*$^{-/-}$ mice develop acantholytic blisters and pathogenic anti-DSG3 IgG, consistent with the features of PV. In this model, the PV phenotype is induced after adoptive transfer of both B and CD4$^+$ T cells, but not after transfer of B cells alone (23). Specific subsets of pathogenic CD4$^+$ T cells were well described in another study that divided CD4$^+$ T cells into inducible costimulatory (ICOS)$^+$ T follicular helper (Tfh) cells, ICOS$^-$ Tfh cells, and non-Tfh cells. This study demonstrated that ICOS$^+$ Tfh cells are required for the induction of the PV phenotype (24). Tfh cells are the major cells that directly interact with B cells, and ICOS is an important co-stimulator that maintains the phenotype of Tfh cells and promotes the migration of Tfh cells to the germinal center (25, 26). Indeed, ICOS$^+$ Tfh cells have been found to be associated with B-cell differentiation in the PV mouse model (24) (Figure 1).

Since CD4$^+$ T cells help B cells with cognate antigen interaction, it is necessary to investigate the antigen specificity of T cells to evaluate their role in pemphigus. T-cell receptor (TCR) transgenic mice (Dsg3H1 mice) have been used to understand the role of DSG3-specific CD4$^+$ T cells in PV mouse models (27). After bone marrow transplantation from Dsg3H1 mice into *Dsg3*$^{-/-}$ mice, DSG3-specific TCR-transgenic CD4$^+$ T cells and *Dsg3*$^{-/-}$ B cells were co-transferred into *Rag2*$^{-/-}$ mice. In this model, DSG3-specific CD4$^+$ T cells induce acantholysis, suggesting that DSG3-specific CD4$^+$ T cells contribute to the generation of pathogenic antibodies in pemphigus. To analyze DSG3-specific CD4$^+$ T cells in their physiologic state, another study generated a major histocompatibility complex (MHC) class II tetramer (I-A^b/DSG3$_{516\text{-}530}$) in the pemphigus mouse model (24). This study demonstrated that DSG3-specific ICOS$^+$ Tfh cells captured by the

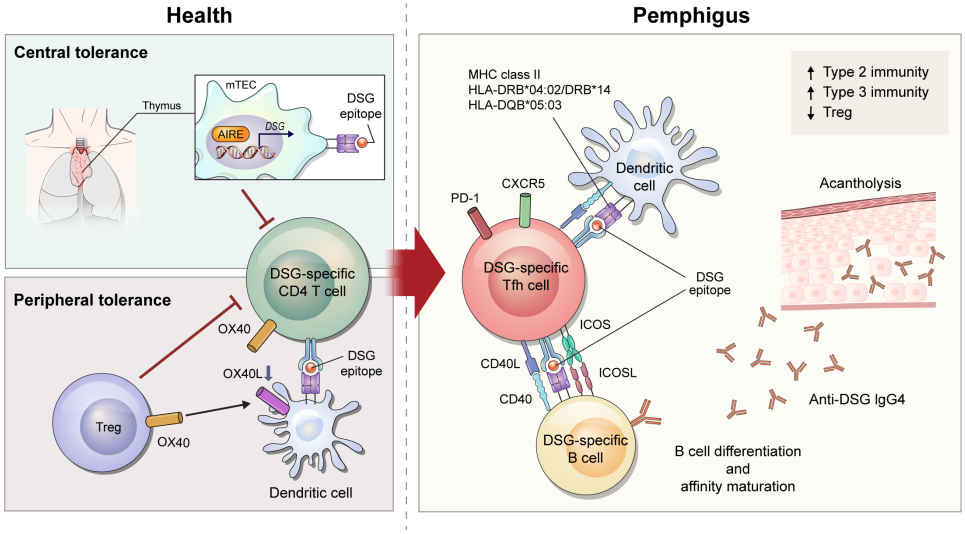

FIGURE 1
Schematic overview of immune mechanism of desmoglein (DSG)-specific CD4$^+$ T cells in pemphigus. After recognizing DSG epitope expressed on major histocompatibility complex class II from medullary thymic epithelial cells under the control of AIRE, DSG-specific T cells undergo deletion in the thymus. When DSG-specific T cells escape from central tolerance, Tregs control tolerance in peripheral regions *via* OX40L-OX40 signaling. In a diseased state of pemphigus, tolerance is broken and DSG-specific CD4$^+$ T cells become pathogenic. Of DSG-specific CD4$^+$ T cells, ICOS$^+$ Tfh cells interact with DSG-specific B cells to provide stimulatory signals including CD40L-CD40 interaction and cytokines. These signals lead to B-cell activation and proliferation, differentiation into plasma cells, and production of pathogenic DSG-specific IgG antibodies that induce acantholytic blister by loss of cell-cell adhesion in keratinocytes.

tetramer were proliferating and associated with the production of anti-DSG3 antibody (24). (Figure 1)

While this mouse model exhibits an immunopathology similar to that of human pemphigus, it has limitations for deciphering the breakdown of tolerance in autoimmunity. Immune tolerance consists of central and peripheral tolerance. Central tolerance of T cells occurs as negative selection in the thymus, where autoreactive T cells are deleted by autoantigen-presenting medullary thymic epithelial cells (mTECs). mTECs can express tissue-specific antigens induced by transcription factors including autoimmune regulator (AIRE). AIRE induces expression of DSGs in mTEC (28), and T cells help B cells to produce anti-DSG3 IgG in *Aire*$^{-/-}$ mice after adoptive transfer of *Dsg3*$^{-/-}$ B cells (29) (Figure 1). Regulatory T cells (Tregs) are a key player in controlling peripheral tolerance. Anti-DSG3 antibody production decreases after transfer of Tregs in the active PV mouse model (30). In a recent study, DSG3-sepcific TCR-transgenic CD4$^+$ T cells were able to overcome peripheral tolerance and induce dermatitis in depletion of regulatory T cell (DEREG) mice, but not wild-type mice. This study showed that OX40-OX40L signaling is important in disrupting Treg-dependent peripheral tolerance of DSG3-sepcific TCR-transgenic CD4$^+$ T cells (31). (Figure 1) In this context, mechanisms of T-cell tolerance should be considered to understand the disease etiology of pemphigus.

2.4 Immunophenotype and function of CD4$^+$ T cells in the human pemphigus

There are four subclasses of IgG (IgG1, IgG2, IgG3, and IgG4) in human, and IgG4 is the predominant subclass in patients with pemphigus (32). IgG4 is a well-known immunoglobulin that relies on a type 2 immune response. Thus, human T-cell studies have explored whether Th2 cells are involved in the pathogenesis of pemphigus. Early studies assessed the amount of DSG-reactive CD4$^+$ T cells by stimulating DSG protein in peripheral blood mononuclear cells (PBMCs). IL-4-secreting DSG3-reactive Th2 cells predominate in the active phase of PV, whereas IFN-γ-secreting DSG3-reactive Th1 cells predominate in the chronic phase of PV and in healthy conditions (20, 33). When evaluating CD4$^+$ T-cell subsets by observing expression of CXCR3 and CCR6, ICOS$^+$PD-1$^+$ circulating Tfh (cTfh) 2 cells, the Th2 phenotype of cTfh cells, were found to be associated with the production of autoantibodies against DSG3 in patients with PV (24). However, another study showed increased cTfh17 cells with responsiveness to DSGs in PBMCs from patients with pemphigus (34). In addition to an imbalance in conventional CD4$^+$ T-cell subsets, the amount of circulating regulatory T cells were found to be decreased in patients with pemphigus. Moreover, FoxP3$^+$CD25^{hi}CD4$^+$ T cells and DSG3-reactive IL-10$^+$CD4$^+$ T cells are reduced in blood from patients with pemphigus compared to healthy individuals (35, 36). Taken together, it is still unclear which subsets of CD4$^+$ T cells play a key pathogenic role in human pemphigus and thus, this area require further study (Figure 1).

Although pemphigus is an autoimmune disease mediated by the systemic immune system, tertiary lymphoid structures resembling B-cell follicles are occasionally discovered in skin blisters of patients with pemphigus (37). In these TLSs, differentiated B cells, including DSG-specific B cells, are detected suggesting that these structures contribute to the pathogenicity of pemphigus (38). There are a number of T cells in TLSs, and IL-21$^+$CD4$^+$ T cells are expanded and produce IL-17 in lesions with TLSs (38). However, the role of various T-cell subsets in skin TLSs of pemphigus has not yet been fully studied and thus, represents an interesting area for future research.

2.5 Targeting CD4$^+$ T cells in pemphigus

Many studies have been conducted to evaluate the efficacy of T-cell targeted therapy for the treatment of pemphigus. The CD40-CD40L interaction is essential for cognate antigen interaction of T and B cells. Indeed, anti-CD40L blocking antibody reduces disease induction in the PV mouse model (39). (Figure 1) In addition to the CD40-CD40L interaction, treatment of anti-ICOS blocking antibody *in vivo* also decreases disease progression (24). These therapeutic approaches can be used to control early disease provocation or prevent relapse, as these molecular interactions are required for disease initiation. Therefore, strategies to enhance the function of Tregs or expand Tregs can be applied to treat the active phase of the disease. Currently, adoptive polyclonal Treg cell therapy is being investigated in a phase I clinical trial for pemphigus (40).

3 Significance of CD4$^+$ T cells in bullous pemphigoid

3.1 Clinical features of BP

Bullous pemphigoid (BP) is an AIBD characterized by autoantibodies against bullous pemphigoid antigens (BPAG) 1 and 2, and is distinguished from pemphigus in that blisters develop in the subepidermal layer (41, 42). The mean age of onset is older than that of pemphigus, and the relative risk of BP increases with age. Mortality in patients with BP is quite high (approximately 20% of the first-year mortality) and may be due to comorbidities or secondary infections (43). Typically, BP presents with multiple tense bulla in urticarial lesions of the skin. Mucosal involvement may occur, but not as often as it does in pemphigus vulgaris. Pruritus is usually present and can be intense. Some medications, such as immune checkpoint inhibitors and dipeptidyl peptidase-4 (DPP4) inhibitors, have been reported to be associated with the development of BP (44, 45). Histologic examination reveals subepidermal blisters usually accompanied by eosinophilic infiltration. IgG autoantibodies deposited along the basement membrane can be detected by direct immunofluorescence. In order to distinguish BP from other pemphigoids, salt-split skin is used in an indirect immunofluorescence study, looking for the presence of autoantibodies binding along the epidermal side of blister. First-line treatment options are topical and systemic corticosteroids (46, 47). Doxycycline, azathioprine, mycophenolate mofetil, or other immunosuppressive agents may be used as steroid-sparing agents (48). In severe cases, intravenous immunoglobulin, rituximab, omalizumab, and dupilumab can be administered (49–51).

3.2 Evidence of CD4$^+$ T-cell pathogenicity in patients with BP

Although pathogenic autoreactive B cells undergoing affinity maturation have not been detected in patients with BP, there is indirect evidence that CD4$^+$ T cells contribute to the development of autoantigen-specific B cells in BP. Epitope spreading refers to the intra- or inter-molecular diversification of antibody epitopes possibly caused by antigen cross-reactivity or cognate B-T cell interaction, and frequently occurs in autoantigen responses of BP (52, 53). These phenomena suggests that BPAG1- and BPAG2-specific B cells might experience somatic hypermutation *via* the help of BPAG1- or BPAG2-specific CD4$^+$ T cells in BP. Further indirect evidence of this is the MHC class II restriction in patients with BP. Although the HLA genetic predisposition for BP exhibits a more diverse pattern across countries than does that of pemphigus, HLA-DQB1*03:01 is primarily associated with BP in many countries (54). These findings indicate that CD4$^+$ T cells may be involved in the development of autoantibodies in BP.

3.3 Pathogenicity and tolerance mechanism of CD4$^+$ T cells in murine BP models

An active mouse model of BP was established using BPAG2-humanized *Rag2*$^{-/-}$ mice (*Col17*$^{m-/-,h+}$*Rag2*$^{-/-}$) (55, 56). Similar to the active PV mouse model, splenocytes from wild-type mice immunized by grafting human BPAG2-expressing mouse skin are adoptively transferred into *Col17*$^{m-/-,h+}$*Rag2*$^{-/-}$ mice. These mice developed subepidermal blisters with deposition of anti-human BPAG2 antibody in the basement membrane zone. In this mouse model, the BP phenotype does not emerge after adoptive transfer of CD4$^+$ T-cell depleted splenocytes (55). Whether these pathogenic CD4$^+$ T cells directly help B cells and are specific for BPAG2 is not yet known, but the CD40-CD40L interaction is crucial for the early stages of the disease in the active BP mouse model (57). In addition, the intramolecular epitope spreading to NC16A domain of human BPAG2 is suppressed by blocking CD40L in the mouse model, suggesting that the cognate B-T interaction is responsible for epitope spreading to NC16A of BPAG2 (56) (Figure 2).

As seen in pemphigus, Tregs also are involved in the pathogenesis of BP. Interestingly, *Foxp3*-mutant scurfy mice spontaneously produce autoantibodies that target the basement membrane zone and contain pathogenic anti-BPAG1 autoantibodies that cause subepidermal blisters (58). In addition, when T-cell deficient nude mice were transferred with CD4$^+$ T cells from scurfy mice, subepidermal blisters positive for autoantibodies developed (58). These results indicate that autoreactive CD4$^+$ T cells are crucial to induce subepidermal blisters. These autoimmunity in scurfy mice is dependent on STAT6 pathway, a JAK-STAT signaling pathway known to be important in Th2 immunity, and Tfh cells are also decreased in the lymph nodes and spleen of *Stat6*$^{-/-}$ scurfy mice (59) (Figure 2).

3.4 Immunophenotype and function of CD4$^+$ T cells in the human BP

In patients with BP, the NC16A domain of BPAG2 protein is recognized as a major pathogenic epitope, and serum levels of anti-NC16A IgG correlate with clinical disease activity (60). Levels of serum anti-NC16A IgG positively correlates with the frequencies of

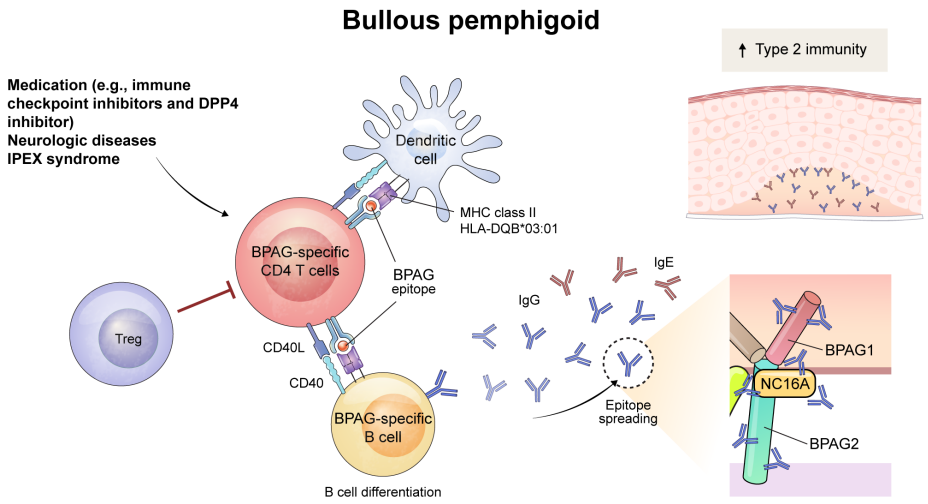

FIGURE 2
Schematic overview of immune mechanism of BPAG-specific CD4$^+$ T cells in bullous pemphigoid (BP). The triggering factor for BP can be medication (e.g., immune checkpoint inhibitors and dipeptidyl peptidase-4 inhibitor) or neurologic diseases. The process begins with the presentation of a BPAG epitope by dendritic cells through MHC class II, including HLA-DQB*03:01, which activates BPAG-specific CD4$^+$ T cells. Also, dysfunction of Treg cells play a role in the development of BP, as evidenced in patients with IPEX syndrome. This leads to interaction with BPAG-specific B cells through CD40-CD40L interaction, resulting in the differentiation of B cells and the epitope spreading of IgG and IgE autoantibodies. Among them, NC16A domain of BPAG2 is recognized as a major pathogenic epitope of autoantibodies. These autoantibodies bind to the basement membrane zone of the skin and mucous membranes, inducing subepidermal blistering in BP. The increased type 2 immunity was seen in BP and might contribute to the development of the diseases.

PD-1$^+$ and ICOS$^+$ circulating Tfh cells and serum IL-21 level in patients with BP (61). Besides anti-NC16A IgG, anti-NC16A IgE is known to be associated with the pathogenicity and disease severity of BP (62, 63). Since IgE is typically mediated by Th2 cells, it has been thought that Th2 immunity is mainly involved in BP. Indeed, BPAG2-reactive Th1 cells can be detected in both healthy individuals and patients with BP, but BPAG2-reactive Th2 cells are only observed in patients with BP (64). Several studies have used epitopes in the NC16A domain to detect BPAG2-reactive CD4$^+$ T cells in patients with BP (65–67), although the immunodominant epitopes of autoreactive CD4$^+$ T cells in BP varies among individuals (68),

Whether the frequency of Tregs is altered in peripheral blood and skin from patients with BP is controversial (69–73). However, a substantial role of Tregs in the disease pathogenesis of human BP can be confirmed by observing patients with immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) syndrome (59, 74). IPEX syndrome is a genetic disorder manifesting an absence or dysfunction of Tregs due to *FOXP3* mutation. Several cases of IPEX syndrome show features of BP and circulating autoantibodies to BPAG1 and/or BPAG2, suggesting that Tregs contribute to the suppression of autoantibody production in BP (59, 74, 75). Human CD25$^+$FoxP3$^+$ Tregs are subdivided into three populations based on CD45RA and FoxP3 expression; CD45RA$^+$FoxP^{low} naïve Treg, CD45RA$^-$FoxP3^{hi} effector Treg, and CD45RA$^-$FoxP3^{low} non-suppressive T cells (76). One study of these three subsets found that the frequency of effector Tregs correlated with disease severity in patients with conventional BP; however, in patients with BP induced by DPP4 inhibitors, Tregs did not correlate with disease severity (77). Therefore, the contribution of Tregs to disease pathogenesis may differ depending on the types of BP.

4 Conclusion

Although B-cell targeting therapies have been successfully used to treat AIBDs, curing the disease remains challenging. Therefore, many clinical trials are underway to develop more effective treatments for AIBDs (78). As shown in this review, a number of studies in mouse models and in humans have investigated the contribution of CD4$^+$ T cells to the pathophysiology of AIBDs. However, the underlying regulatory mechanisms and their precise immunological roles have not yet been fully described. A deeper understanding of autoreactive CD4$^+$ T cells and Tregs may support the identification of therapeutic targets to completely cure AIBDs. Furthermore, to develop optimal treatment strategies for AIBDs, it is necessary to elucidate the immune networks between antigen-presenting cells, autoreactive T and B cells, and Tregs through a comprehensive analysis.

Author contributions

AL and TK wrote the manuscript. JK wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by Korea Drug Development Fund funded by Ministry of Science and ICT, Ministry of Trade, Industry, and Energy, and Ministry of Health and Welfare (RS-2022-00165917, Republic of Korea).

Acknowledgments

The authors extend their heartfelt gratitude to the late Professor Detlef Zillikens for his invaluable contribution to the field of autoimmune bullous diseases and for his exceptional impact on their academic pursuits. The corresponding author, in particular, expresses deep gratitude for the opportunity to learn from such a remarkable professor and extends appreciation for his legacy in the field. Professor Zillikens will always be remembered as a kind and generous person who made every student and colleague feel welcomed and valued. The authors thank Medical Illustration & Design part of the Medical Research Support Services of Yonsei University College of Medicine, for artistic support related to this work.

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 18 April 2023
DOI 10.3389/fimmu.2023.1180150

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 05 March 2023
ACCEPTED 03 April 2023
PUBLISHED 18 April 2023

CITATION
Bamberger F, König IR, Gola D, Zillikens D and Sadik CD (2023) Retrospective analysis of the long-term therapeutic effectiveness and safety profile of rituximab in the treatment of mucous membrane pemphigoid in a German university center between 2008 and 2019.
Front. Immunol. 14:1180150.
doi: 10.3389/fimmu.2023.1180150

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Retrospective analysis of the long-term therapeutic effectiveness and safety profile of rituximab in the treatment of mucous membrane pemphigoid in a German university center between 2008 and 2019

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Background: The B-cell-depleting anti-CD20 antibody rituximab (RTX) is often used as an adjuvant drug for the treatment of refractory cases of mucous membrane pemphigoid (MMP).

Objective: This study aims to determine the therapeutic effectiveness and the safety profile of RTX in MMP.

Methods: The medical records of all cases of MMP treated with RTX between 2008 and 2019 in our university medical center located in northern Germany, which specialized in autoimmune blistering skin diseases, were retrieved and systemically analyzed for treatment responses and potential adverse events over a median period of 27 months.

Results: We identified 18 MMP patients who received at least one cycle of RTX to treat MMP. RTX was always used as an adjuvant treatment, and its application did not change concomitant treatments. Under treatment with RTX, 67% of the patients achieved an improvement in their disease activity within 6 months. This was also reflected in a statistically significant reduction in the *Mucous Membrane Pemphigoid Disease Index* (MMPDAI) activity score. The frequency of infections under RTX treatment increased only slightly.

Conclusions: The use of RTX is associated with an attenuation of MMP in a large proportion of MMP patients in our study. At the same time, its application

10.3389/fimmu.2023.1180150

was not found to further increase the susceptibility of the most strongly immunocompromised population of MMP patients to opportunistic infections. Collectively, our results suggest that the potential benefits of RTX outweigh its risks in patients with refractory MMP.

KEYWORDS

mucous membrane pemphigoid, rituximab, pemphigoid disease, autoantibodies, side-effects, B cell depletion, retrospective study, mucous membrane pemphigoid disease index

Introduction

Mucous membrane pemphigoid (MMP) is an antibody-mediated autoimmune disease. The most common autoantigens are the C-terminus of type XVII collagen (BP180), BP230, laminin 332, integrin α6β4, and type VII collagen (1). MMP differs from other pemphigoid diseases in that it primarily affects the mucosa of the conjunctiva, oral cavity, esophagus, nose, pharynx, larynx, trachea, anal canal, and genitalia (2). Scarring is another symptom of MMP unique to pemphigoid diseases. It mostly manifests at the eye and as strictures in the esophagus, pharynx, and larynx. Preventing the development of scarring is an important goal in the treatment of MMP and often requires a swiftly initiated, marked, and long-term maintained immunosuppression. Drugs used in the treatment of MMP, mostly in combination, include systemic and topical corticosteroids, mycophenolate mofetil, azathioprine, dapsone, intravenous immunoglobulins, cyclophosphamide, and rituximab (RTX) (2).

RTX is an anti-CD20 antibody designed to deplete B cells (3). In MMP, it is used off-label following the pharmacological rationale that depleting B cells to blunt the production of pathogenic autoantibodies should have beneficial effects. However, with clinical trials of adequate size on the impact of RTX on MMP still lacking, the effectiveness and safety of RTX in MMP have remained controversial. We have therefore retrospectively analyzed the long-term impact of rituximab on disease and adverse events in 18 MMP patients treated in our department specializing in pemphigoid diseases between 2008 and 2019.

Materials and methods

The study was approved by the ethics committee of the University of Lübeck (20-130A). The medical records of all patients treated for MMP at the University of Lübeck between 2008 and 2019 were retrieved. Patients who received RTX at least once were identified, and their medical history before and after the first administration of RTX was examined by analyzing all medical records of in- and outpatient visits available.

Disease activity was categorized as "active disease," "disease control," "partial remission," and "complete remission" according to the definitions of an international consensus conference (4). We modified this categorization to additionally distinguish "complete remission on therapy" and "partial remission on therapy" (Supplementary Table S1).

All side effects were recorded to detect potential adverse events associated with the administration of RTX. The period of 6 months after RTX administration was given special attention throughout all analyses of efficacy in MMP because the effect of RTX is supposed to reach its maximum at this time.

Mucous Membrane Pemphigoid Disease Area Index (MMPDAI) scores before and approximately 6 months after RTX were statistically analyzed using the Wilcoxon signed-rank test. Statistical significance was defined by a p-value of < 0.05. R version 4.1.1 was used for statistical analysis.

Results

A total of 77 patients were treated for MMP as inpatients in our department between April 2008 and December 2018. Approximately 21 patients (27%) received at least one cycle of RTX, and 18 were followed up after the administration of RTX for at least 5 months between April 2008 and September 2019 (Supplementary Table S2). Those 18 patients were included in our detailed analysis. Ten of them received one, while eight had two cycles of RTX using the "rheumatoid arthritis protocol," i.e., two administrations of 1 g of RTX within 2–4 weeks (5). Their demographics, pattern of organ involvement, autoantigens, and the treatments received prior to RTX are summarized in Table 1 and Supplementary Tables S3, S4.

The median time between the first diagnosis of MMP and the first cycle of RTX was 14 months (range, 2–59 months). In all 18 cases, RTX was applied as an adjuvant treatment together with corticosteroids. In total, 17 patients received at least one more immunosuppressive or immunomodulatory drug (Table 1; Supplementary Table S3).

The course of disease with the categorization of disease activity over a median period of 27 months after the first administration of RTX is compiled for each individual patient in Figure 1A. RTX was initiated in 15 patients with active disease and in three patients with controlled disease who showed no further improvement with first- and second-line therapy. In 12 patients, the administration of RTX

TABLE 1 Demographics, organ involvement, and therapies prior to RTX.

Gender (number of patients)	
Men	5 (27.8%)
Women	13 (72.2%)
Age (median)	
Age at diagnosis (years)	61.5 (range, 23–88)
Age at first RTX administration (years)	64.5 (range, 24–90)
MMP organ involvement (number and percentage of patients)	
Ocular involvement	6 (33.3%)
Oral involvement	15 (83.3%)
Nasal involvement	8 (44.4%)
Laryngeal involvement	1 (5.6%)
Genital involvement	4 (22.2%)
Anal involvement	2 (11.1%)
Cutaneous involvement	2 (11.1%)
Disease duration (median) before first RTX administration (months)	14.0 (range, 2–59)
Previous systemic therapies (number and percentage of patients)	
Corticosteroids	17 (94.4%)
Adjuvant therapy	16 (88.9%)
Mycophenolic acid	11 (61.1%)
Azathioprine	5 (27.8%)
Intravenous immunoglobulin	4 (22.2%)
Protein A immunoadsorption	1 (5.6%)
Dapsone	13 (72.2%)
Cyclophosphamide	2 (11.1%)
Cyclosporine A	1 (5.6%)

RTX, rituximab; MMP, mucous membrane pemphigoid.

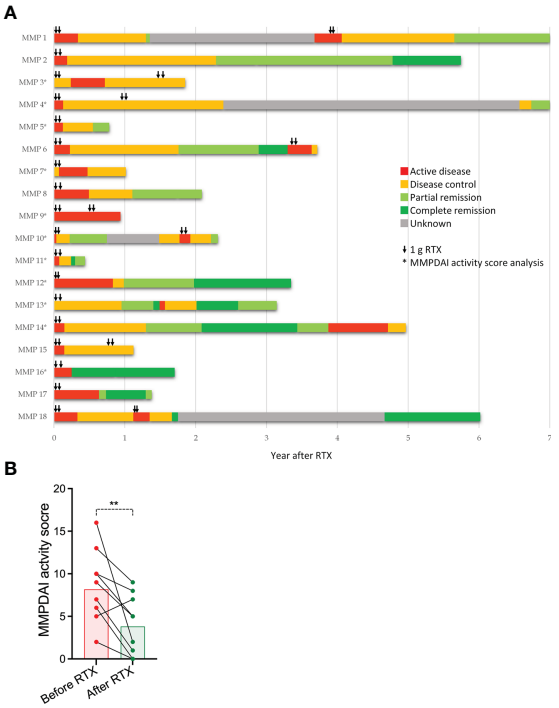

FIGURE 1
MMP disease activity before and in the years after the administration of RTX. **(A)** Individual course of MMP disease activity in 18 MMP patients starting with the first administration of RTX. Disease was categorized as "active disease," "disease control," "partial remission," "complete remission," and "unknown," following the categorization system described in the Materials and methods section. Black arrows indicate the time points of the administration of 1 g of RTX. **(B)** Comparison of the MMPDAI activity score at the initiation of RTX and 6 months later. Each dot represents an individual patient (n = 11). The black lines connect the MMPDAI activity scores before and after the RTX for the individual patient. The MMPDAI before and after RTX was compared by a Wilcoxon signed-rank test (p = 0.0073).

was followed by a decrease in disease activity within 6 months. In two patients (MMP3/7), the disease aggravated during this time. During the time of follow-up of up to 7 years, 14 patients exhibited periods of partial or complete remission. In three patients (MMP10/11/16), a partial or complete remission was achieved within 6 months after RTX (Figure 1A). This was also reflected in the discontinuation of the dexamethasone pulses applied to all patients at the time of the initiation of RTX or in the extension of the time intervals between the individual pulses 6 months after the administration of RTX (Supplementary Table S5). There was no significant association between the response to RTX and autoantibody specificity or body sites affected by MMP (results not shown).

In 11 patients, the MMPDAI activity score (4) was assessed repeatedly before and after treatment with RTX, allowing a more objective comparison of disease activity at the initiation of RTX therapy (day 0) and approximately 180 days thereafter. This comparison revealed a statistically significant decrease from a median of 9 to a median of 5 (Figure 1B).

To assess the safety profile of RTX in our patient cohort, we systemically reviewed the patients' records for medical conditions constituting potential RTX-induced adverse events. Our patient records allowed follow-up for up to 58 months before and 85 months after the first administration of RTX. This comparison showed that the frequency of potential adverse events did not change in our cohort after the administration of RTX (Table 2). Comparing the pattern and frequency of possible adverse events exclusively in the 12 months before and after the administration of RTX showed no differences except for a slight tendency towards more respiratory tract infections, with four patients developing newly respiratory tract infections in the first 12 months after RTX (Supplementary Table S1). Furthermore, only one patient (MMP8) developed a severe infection, specifically sepsis. None of the patients died in the year following the administration of RTX. No significant association was found between the number of infections during the 12 months after RTX and autoantibody specificity or sites affected by MMP (results not shown).

10.3389/fimmu.2023.1180150

TABLE 2 Frequency of potential side effects.

	Therapy before RTX (n = 15)	Therapy after RTX (n = 18)	
Median documented time (months)[a]	5.9 (range, 1–58)	26.8 (range, 5–85)	
Documented adverse events	Number of patients	Number of patients	Median TAE (months)
Infections	9 (60%)[ibefore]	12 (67%)[iafter]	5.0 (range, 1–64)
Eyes	1 (7%)	1 (6%)[e]	13
Conjunctivitis	–	1 (6%)	44
Meibomitis	1 (7%)	–	
Ocular bacterial inflammation[b]	–	1 (6%)	13
Respiratory system	6 (40%)	8 (44%)[i]	4.5 (range, 1–64)
Upper respiratory infection	4 (27%)[c]	6 (33%)	5.5 (range, 3–64)
Tracheitis	–	1 (6%)[d]	39
Bronchitis	2 (13%)	1 (6%)	2
Pneumonia	0 (0%)	1 (6%)	1
Gastrointestinal tract	1 (6%)	2 (9%)	49.0 (range, 10–88)
Gastrointestinal infection	1 (6%)	2 (9%)[e]	
Urinary tract	4 (27%)	4 (22%)	10.5 (range, 1–26)
Urinary tract infection	4 (27%)	4 (22%)	
Systemic	0 (0%)	1 (6%)	6
Sepsis	–	1 (6%)[f]	
Other	2 (13%)[j]	–	–
Hepatitis B infection	1 (7%)	–	
Genital herpes	1 (7%)	–	
Pulpitis	1 (7%)	–	
Liver	0 (0%)	1 (6%)	36
Steatosis hepatis	–	1 (6%)	
Musculoskeletal	0 (0%)	4 (22%)[k]	1.5 (range, 0–10)
Osteoporosis	–	2 (11%)	25.5 (range, 10–41)
Osteopenia	–	1 (6%)	2
Myalgia	–	1 (6%)	0
Fibromyalgia syndrome	–	1 (6%)	1
Arthrosis	–	1 (6%)	11
Neurological	1 (7%)[l]	2 (11%)	33.5 (range, 5–62)
Apoplex	–	1 (6%)	62
Cerebral aneurysm	1 (7%)	–	
Polyneuropathy	1 (7%)	–	
Muscular cramps	–	1 (6%)[g]	5
Mental disorders	0 (0%)	1 (6%)	26
Depressive episodes	–	1 (6%)	
Weight	0 (0%)	1 (6%)	28
Cachexia	–	1 (6%)	

(Continued)

TABLE 2 Continued

	Therapy before RTX (*n* = 15)	Therapy after RTX (*n* = 18)	
Median documented time (months)[a]	5.9 (range, 1–58)	26.8 (range, 5–85)	
Documented adverse events	Number of patients	Number of patients	Median TAE (months)
Cardiovascular system	1 (7%)	1 (6%)[m]	3
Reduced ejection fraction (30%)	–	1 (6%)	3
Orthostatic circulatory dysregulation	1 (7%)	–	
Left bundle branch block	–	1 (6%)	3
Eyes	0 (0%)	2 (11%)[n]	3.5 (range, 3–4)
Macular degeneration	–	1 (6%)	18
Macular foramen	–	1 (6%)	3
Vitreous opacity	–	1 (6%)	3
Progressive cataract formation	–	1 (6%)	4
Hypersensitivity	0 (0%)	1 (6%)	0
Asthma exacerbation	–	1 (6%)	
Respiration	0 (0%)	2 (11%)	6.5 (range, 0–13)
Dyspnoea	–	2 (11%)	

RTX, rituximab; TAE, time to adverse event (time until the first occurrence of the adverse event, based on the day of the first RTX administration of the first RTX cycle).
[a]Therapy before RTX: based on the first patient presentation at the Department of Dermatology, Allergology, and Venerology at the University Hospital Schleswig-Holstein (Lübeck) up to the first RTX administration. Therapy after RTX: based on the day of the first RTX administration of the first RTX cycle.
[b]Not more precisely defined.
[c]Includes ulcerating and granulating pharyngitis.
[d]With severe tracheal stenosis.
[e]Includes a patient with pangastritis and bulbitis.
[f]By port infection with Escherichia coli.
[g]Recurrent: (a) [ibefore], nine patients developed one or more infections: two patients developed bronchitis. Two patients developed a urinary tract infection. One patient developed an upper respiratory infection and a hepatitis B infection. One patient developed urinary tract infections and an upper respiratory infection. One patient developed meibomitis and a urinary tract infection. One patient developed genital herpes, an upper respiratory infection, gastrointestinal infection, and pulpitis. One patient developed an upper respiratory infection. (b) [iafter], 12 patients developed one or more infections: one patient developed an upper respiratory infection. One patient developed an upper respiratory infection. One patient developed an upper respiratory infection, a urinary tract infection, and a gastrointestinal infection. One patient developed pneumonia and an upper respiratory infection. One patient developed tracheitis with severe tracheal stenosis. One patient developed a gastrointestinal infection. One patient developed an upper respiratory infection and sepsis. Two patients developed a urinary tract infection. One patient developed bronchitis. One patient developed ocular bacterial inflammation and conjunctivitis. One patient developed urinary tract infections and an upper respiratory infection.
[h]One patient developed conjunctivitis some time after ocular bacterial inflammation.
[i]One patient developed an upper respiratory infection some time after pneumonia.
[j]One patient developed genital herpes and pulpitis in the documented time prior to RTX administration.
[k]One patient was diagnosed with arthrosis after osteoporosis. Another patient was found to have osteoporosis after myalgia.
[l]One patient developed a cerebral aneurysm and polyneuropathy in the documented time prior to RTX administration.
[m]In one patient, both a reduced ejection fraction and a left bundle branch block were found.
[n]Macular degeneration was found in one patient in addition to progressive cataract formation. Another patient was diagnosed with vitreous opacity in addition to a macular foramen. In some patients, several adverse events falling into one category have occurred. The adverse event with the shortest TAE among the respective patients was included in the calculation of the median TAE for the corresponding categories.

An analysis of 11 patients showed no difference in the peripheral blood leucocyte counts and the serum levels of the C-reactive protein (CRP) and liver enzymes 100 days before and after the first administration of RTX (results not shown).

Discussion

We retrospectively analyzed the course of the disease in 18 MMP patients who received at least one cycle of RTX for MMP. The cohort of our 18 MMP patients appears representative of MMP, with its demographics and pattern of organ affection closely resembling those of other MMP patient cohorts previously published. After 6 months, when RTX is considered to have had its maximal effect, the severity of MMP was significantly reduced in 12 patients (67%), which was also indicated for a subgroup of our cohort by the MMPDAI activity score. However, as other therapeutics were continued after the administration of RTX, it is not possible to clearly distinguish to what extent this improvement was a result of the RTX-mediated depletion of B cells. However, with the treatment before and after RTX administration not changing in most patients and most patients having already been treated for a long time prior to RTX, it is likely that RTX contributed to this improvement. In six patients (33%), disease activity did not decrease or barely decreased, indicating that RTX might only be effective in a subgroup of patients. This suggests that the RTX-mediated depletion of B cells either does not reach the pathogenic autoreactive B-cell population in all patients or that the continuous production of autoantibodies is not required in a subgroup of MMP patients to sustain disease activity.

With 67% of our cohort responding to RTX, our response rate was similar to that recently reported in retrospective studies from academic dermatological centers in France and the Netherlands as well as in a systematic review, which retrieved the reports on 112 MMP patients (6–9). The latter found that the administration of RTX was associated with a complete resolution of disease in 70.5% of MMP patients within 8.7 months (6). In the two Dutch studies, 64% and 75% of patients with refractory MMP responded to rituximab with partial or complete remission (7, 8).

Bohelay et al. retrospectively analyzed the response of 109 patients with severe and/or refractory MMP to RTX in a French center (9). The outcomes were defined in this study, like in ours, following the definitions of Murrell et al. (4). The standard treatment regimen used in the French center differed from our protocols, e.g., in that it included more regular administrations of RTX. More specifically, patients received RTX every 6 months until complete remission was achieved or failure was declared. Patients who did not show an improvement 3 months after the first administration of RTX received another cycle of RTX at this point. When complete remission was achieved, the patients received another cycle of RTX before the drug was discontinued. All patients received dapsone or salazopyrine, which was the only adjuvant therapy in 106 of the 109 patients. Under this regimen, 85.3% of patients achieved complete remission after a median of two cycles of rituximab, i.e., within 1 year after the initiation of RTX, but the disease relapsed in 38.7% of these patients. Thus, while the response to the first cycle of RTX reported in this study was similar to our finding, the response rate over time was higher and the remission was more stable after several cycles of RTX than the outcomes in our study. Collectively, this suggests that several regular cycles of RTX are beneficial for MMP patients and lead to better outcomes. Importantly, like our study, Bohelay et al. did not find a substantial increase in the rate of adverse events under RTX either. The most common adverse events reported were diverse infections.

A strength of our study is that we can compare the change in disease activity of 11 MMP patients before and after RTX using the MMPDAI activity score. Although calculating this score is laborious, it should become part of the clinical routine in all medical centers to extend the capabilities for insightful clinical studies in this field.

Our study did not detect any change in adverse events potentially associated with RTX, except for a slight increase in respiratory tract infections. This is important because MMP patients are predominantly elderly, with the disease mostly diagnosed in patients over 60 years, and are usually already under aggressive immunosuppression when RTX is applied (6). Our results suggest that RTX does not further add to the long-term risk of MMP patients for infections. Thus, the decision to use RTX in MMP patients is not a trade-off between safety and disease control. This good tolerability of RTX in MMP patients is in line with a recent retrospective study in patients with bullous pemphigoid (BP) who have similar demographics and are immunosuppressed to a degree similar to MMP patients. This study showed that the use of RTX in BP is associated with longer survival indicating a small risk for lethal infections due to RTX (10).

Collectively, considering that disease severity improved in 67% of MMP patients and that MMP is a debilitating, most difficult-to-treat disease, we therefore propose that there are more potential benefits in applying RTX to MMP patients than in withholding it. However, it must be noted that the retrospective design of our study limits its power to comprehensively determine the effectivity and risks of the use of rituximab in MMP patients.

Limitations

The limitations of this study are that it is a monocentric, retrospective study, including only 18 patients who received rituximab. MMP is a heterogeneous disease; thus, studies on the effect of RTX and its side effects would probably benefit from the stratification of patients with different patterns of organ affection. This, however, was not possible in this study with its low number of patients. Furthermore, the treatment of the patients over time did not follow a specific protocol, and there is no control group of MMP patients not receiving rituximab.

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 University of Lübeck. The patients/participants provided their written informed consent to participate in this study.

Author contributions

FB, DZ and CS conceived the study. FB retrieved and collected the data. FB, IK, DG and CS analyzed the results. FB and CS wrote the manuscript and prepared the figures. IK, DG and DZ edited the manuscript. All authors contributed to the article and approved the submitted version.

In memoriam

This article is part of the memorial collection for Detlef Zillikens. This research project was inspired by a discussion the first author and the senior author had with Detlef Zillikens during a ward round. This article was the last manuscript Detlef Zillikens worked on while being on sick leave in March 2022 due to an unknown condition, and at this point, he still had not anticipated that he would not return to work and would pass away only 6 months later.

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.2023.1180150/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 18 April 2023
DOI 10.3389/fimmu.2023.1163066

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 10 February 2023
ACCEPTED 31 March 2023
PUBLISHED 18 April 2023

CITATION
Schmitt T, Hudemann C, Moztarzadeh S, Hertl M, Tikkanen R and Waschke J (2023) Dsg3 epitope-specific signalling in pemphigus.
Front. Immunol. 14:1163066.
doi: 10.3389/fimmu.2023.1163066

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Dsg3 epitope-specific signalling in pemphigus

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Introduction: Pemphigus is an autoantibody driven disease that impairs the barrier function of the skin and mucosa by disrupting desmosomes and thereby impeding cellular cohesion. It is known that the different clinical phenotypes of pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are dependent on the autoantibody profile and target antigens that, amongst others, are primarily desmoglein (Dsg)1 and/or Dsg3 for PV and Dsg1 for PF. However, it was reported that autoantibodiesagainst different epitopes of Dsg1 and Dsg3 can be pathogenic or not. The underlying mechanisms are very complex and involve both direct inhibition of Dsg interactions and downstream signalling. The aim of this study was to find out whether there is target-epitope-specific Dsg3 signalling by comparing the effects of the two pathogenic murine IgGs, 2G4 and AK23.

Methods: Dispase-based dissociation assay, Western Blot analysis, Stimulated emission depletion microscopy, Fura-based Ca2+ flux measurements, Rho/Rac G-Protein-linked immunosorbent assay, Enzyme-linked immunosorbent assay.

Results: The IgGs are directed against the EC5 and EC1 domain of Dsg3, respectively. The data show that 2G4 was less effective in causing loss of cell adhesion, compared to AK23. STED imaging revealed that both autoantibodies had similar effects on keratin retraction and reduction of desmosome number whereas only AK23 induced Dsg3 depletion. Moreover, both antibodies induced phosphorylation of p38MAPK and Akt whereas Src was phosphorylated upon treatment with AK23 only. Interestingly, Src and Akt activation were p38MAPK-dependent. All pathogenic effects were rescued by p38MAPK inhibition and AK23-mediated effects were also ameliorated by Src inhibition.

Discussion: The results give first insights into pemphigus autoantibody-induced Dsg3 epitope-specific signalling which is involved in pathogenic events such as Dsg3 depletion.

KEYWORDS

pemphigus, autoimmune disease, skin, epidermis, desmosomes, adhesion, keratin, desmoglein (dsg)

Introduction

The skin and mucosa provide an effective barrier against external hazards such as pathogens or harmful chemicals. Pemphigus is an autoantibody driven disease that impairs barrier integrity by disturbing the turnover of desmosomes. Desmosomes provide mechanical cohesion between neighbouring epithelial cells (1). As a result, liquid-filled blisters are formed within the epithelia, which can affect a large portion of the skin or mucous membranes. The blisters can thus cause severe complications and high mortality, mostly due to barrier defects (2). Desmosomes consist of the cadherin adhesion molecules desmoglein (Dsg) and desmocollin (Dsc) subfamily. These are anchored to keratin filaments by adaptor proteins such as plakoglobin (Pg) and plakophilin (Pkp) and the linker protein desmoplakin (Dp). It is well known that the clinical pemphigus phenotypes correlate with the autoantibody profile. The most common pemphigus variants are pemphigus vulgaris (PV) characterized by autoantibodies against Dsg3, affecting the mucosa only or against Dsg1 and Dsg3, affecting the mucosa and epidermis and pemphigus foliaceus (PF) characterized by autoantibodies against Dsg1, affecting the epidermis exclusively (3–5). However, the pathomechanisms causing the loss of adhesion are quite complex (6). For anti-Dsg3 antibodies, it was shown that both direct inhibition of Dsg interaction (7–9) and signalling events induced by the autoantibody binding play a major role (10, 11). In this context, it was reported that several kinases and other signalling proteins relevant for pemphigus pathogenesis form complexes with desmosomal proteins (12, 13). Recently, interaction of Erk1 and p38MAPK with Dsg1 was confirmed by BIO-ID (14).

For anti-Dsg1 autoantibodies, direct inhibition was not found in all studies (7, 15–17). Frequently, autoantibodies against other antigens are also found (18). Autoantibodies against Dsc1, Dsc2 or Dsc3 were also reported to be pathogenic (19–26). However, the role of autoantibodies against other targets is less clear. It is hypothesised that they cause additive effects, thereby potentiating the pathogenic effects of the autoantibodies against the desmosomal cadherins (27–29).

It was reported that different anti-Dsg antibodies do not always show a similar degree of pathogenicity (7, 15–17). Dsg isoforms consist of five extracellular domains (EC1-EC5), similar to other cadherins. These share a high structural homology, except for the membrane proximal EC5 domain (30). Several studies reported that most pemphigus autoantibodies bind to the N-terminal EC1-domain, the major mediator of homo- and heterophilic interactions (31, 32). Approximately 50 % of PV patients exhibit detectable serum IgGs recognizing the EC1 domain, followed by EC2 (26 %), EC4 (21 %), EC5 (17%) and EC3 (15%) (33). Antibodies directed against EC1 or EC2 are known to exhibit pathogenic effects, whereas those targeting EC5 are reported to be mostly non-pathogenic (34, 35). It was reported that healthy individuals from endemic groups with an increased risk of PF onset often showed increased levels of non-pathogenic anti-Dsg1-IgGs (35–38). These were exclusively IgG2 whereas pathogenic autoantibodies were usually of IgG4 subtype (39). However, non-pathogenic and pathogenic antibodies can often share the same light or heavy chains (34). It is even hypothesised that pathogenic pemphigus autoantibodies can evolve from non-pathogenic antibodies via epitope spreading (34, 35, 40). Epitope spreading from Dsg3 to Dsg1 is described in the relatively frequent transformation from mucosal-dominant (anti-Dsg3-IgG only) to mucocutaneous PV (anti-Dsg1- and anti-Dsg3-IgG). The same is true for a less frequent transformation from PF (anti-Dsg1-IgG only) to PV (anti-Dsg1- and anti-Dsg3-IgG) (41, 42). A further piece of evidence is the observation that the introduction of a single Dsg3-reactive T-cell was sufficient to induce the expression of polyclonal anti-Dsg3 IgG in mice *in vivo* (43). Pemphigus patient sera usually display a complex polyclonal mixture of autoantibodies that in most, but not all cases show a good correlation with the disease severity (44, 45).

Due to their high prevalence in pemphigus patients and their high pathogenicity in several *in vitro* and *in vivo* studies, EC1-specific antibodies such as the mouse monoclonal IgG1 AK23 are typically used to investigate PV pathology (Figure 1A) (46). Recently, a pathogenic mouse monoclonal anti-Dsg3 IgG called 2G4, directed against the EC5 domain, was developed (47). The aim of this study was thus to directly compare the two antibodies to clarify if autoantibodies in PV can induce a Dsg3 epitope-specific signalling response.

Results

AK23 is more effective than 2G4 in reducing cell-cell adhesion

Using immunostaining and super resolution stimulated emission depletion (STED) microscopy showed that both 2G4 and AK23 produced a Dsg3 staining pattern comparable to a commercial polyclonal IgG directed against the intracellular domain of Dsg3 (Figure 1B). In the dispase-based dissociation assay, 2G4 induced a significant loss of adhesion, compared to the control using IgG purified from the serum of healthy volunteers (C-IgG). However, 2G4 showed a 2.54-fold weaker effect on the adhesion of HaCaT cells than AK23 (1.98-fold without subtracting background fragments under control conditions). The effects of both IgGs were ameliorated by inhibition of p38MAPK, a kinase central for pemphigus pathogenesis (6), using SB202190 (SB20) (Figures 1C, D) or EO1428 (EO) (Figures 1E, F). Inhibition of Src, using PP2, on the other hand was effective in reducing AK23-induced effects, but had no effect on fragment numbers under 2G4 treatment (Figures 1G, H).

To check if there are additive and polyclonal effects potentially increasing the loss of adhesion, we used mixtures of AK23 and 2G4 in dispase dissociation assays. A mixture of AK23 and 2G4 at a concentration of 75 µg/ml each did not lead to a significant increase in the number of fragments, compared to AK23 alone at a concentration of 75 µg/ml (as applied in all other experiments). A mixture of AK23 and 2G4 at a concentration of 37.5 µg/ml for both led to a number of fragments similar to the number of fragments induced by 2G4 alone at a concentration of 75 µg/ml (Figures 2A, B).

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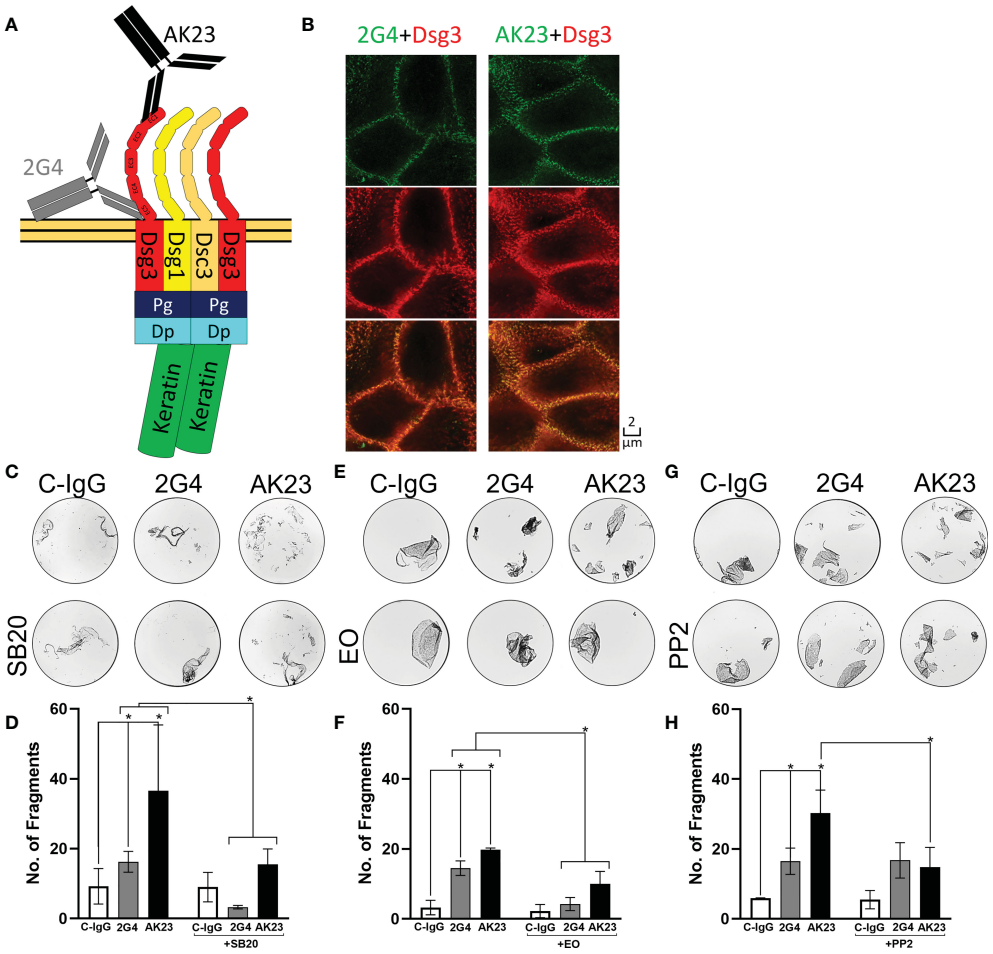

FIGURE 1
2G4 is a pathogenic Dsg3 specific IgG. **(A)** Schematic depiction of binding positions of AK23 (Black), directed against the EC1 domain and 2G4 (Grey) directed against the EC5 domain of Dsg3, on a desmosome-bound Dsg3. **(B)** Co-staining of either 2G4 or AK23 (green) with Dsg3 (intracellular domain, red) and an overlay of both channels in HaCaT cells after 24 hours incubation with the IgGs (1:50), imaged with the STED microscope (N=4). **(C)** Representative images of dispase-based dissociation assay results in HaCaT cells. Vehicle control (left) pre-treated with SB20 (60 µM, inhibiting p38MAPK, right). **(D)** Quantification of dispase-based dissociation assay results using SB20 (N=4). **(E)** Representative images of dispase-based dissociation assay results in HaCaT cells. Vehicle control (left) pre-treated with EO (40 nM, inhibiting p38MAPK, right). **(F)** Quantification of dispase-based dissociation assay results using EO (N=4). **(G)** Representative images of dispase-based dissociation assay results in HaCaT cells. Vehicle control (left) pre-treated with PP2 (10 µM, inhibiting Src, right). **(H)** Quantification of dispase-based dissociation assay results using PP2 (N=4). * indicates statistically significant differences in two-way-ANOVA with Sidaks correction for multiple comparisons p<0.05.

Effects of 2G4 and AK23 on the organization of keratin filaments and distribution of Dsg3 and Dp

Using STED microscopy, we observed that both 2G4 and AK23 caused fragmentation of Dsg3 staining along cell borders (Figure 3A, S1A). In addition, following incubation with AK23 the staining intensity of Dsg3 was significantly reduced, both along the cell borders and in the cytoplasm. This effect was not observed for 2G4, showing that AK23 led to a stronger redistribution and loss of Dsg3 than 2G4. The effects were ameliorated by inhibition of p38MAPK using either SB202190 (Figures 3A, D) or EO1428 (Figures S1A, D). p38MAPK inhibition with SB202190 under control conditions significantly increased Dsg3 staining intensity both at the cell border and in the cytoplasm whereas effects of EO1428 were not significant. This is indicating that p38MAPK is involved in Dsg3 turn-over under basal conditions as well (Figures 3A, D; S1A, D). Furthermore, a significant retraction of keratin filaments from the cell borders towards the nucleus, another typical pemphigus hallmark, was observed after incubation with either 2G4 or AK23. The effect was similar in extent for both IgGs and was also blocked by inhibition of p38MAPK with either SB202190 (Figures 3A, C) or EO1428 (Figures S1A, C).

In addition to Dsg3 and keratin filaments, the desmosomal plaque protein Dp was stained and investigated using STED microscopy. Neither of the two IgGs caused significant changes in desmosome morphology (Figures 3B; S1B). Due to the regular size and shape of desmosomal plaques, this staining was also utilized to determine the number of desmosomes per µm of cell border. Both 2G4 and AK23 caused a similar reduction of desmosome number,

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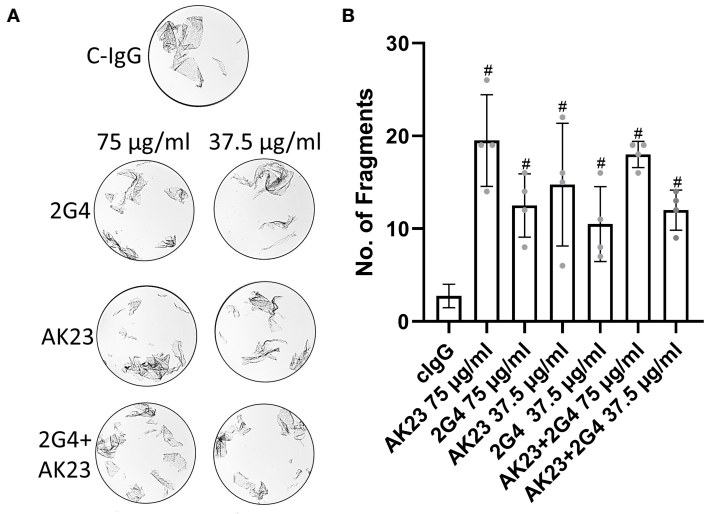

FIGURE 2
(A) Representative images of dispase-based dissociation assay results in HaCaT cells. Control, treated with C-IgG only (top), treated with either single 2G4, AK23 or a mixture of both at a concentration of 75 µg/ml eacht (left) and at half concentration of 37.5 µg/ml right. **(B)** Quantification of dispase-based dissociation assay (N=4). # indicates statistically significant differences towards control conditions, one-way-ANOVA with Dunett correction for multiple comparisons, $p<0.05$.

comparable to results for PV-patient skin (12). This effect was also abrogated by inhibition of p38MAPK with either SB202190 (Figure 3B, E) or EO1428 (Figure S1B, E).

2G4 and AK23 induce overlapping, yet distinct signalling responses

Next, we analysed the signalling pathways induced by 2G4 and AK23 respectively, using Western blot analyses after Triton X protein fractionation. Similar to previous studies, p38MAPK was significantly phosphorylated in the Triton X soluble (not cytoskeleton-bound fraction) and both IgGs induced a similar degree of p38MAPK phosphorylation. This effect was ameliorated upon treatment with the p38MAPK inhibitor SB202190 (Figures 4A, B) or EO (Figures S2A, N). Interestingly, Src, which is another well-known Dsg3-dependent signalling protein involved in loss of cell adhesion in pemphigus (48, 49), was significantly phosphorylated upon treatment with AK23 but not following 2G4 incubation. The effect was dependent on p38MAPK since treatment with SB202190 (Figures 4A, B) or EO 1428(Figures S2A, B) abolished this effect. As a third target of interest, Akt as downstream kinase of PI3-kinase, which is regulated by the Dsg3-dependent EGFR signalling pathway (6, 11, 50), was investigated. Akt phosphorylation was significantly increased to a similar degree after treatment with either 2G4 or AK23. Inhibition of p38MAPK with SB202190 (Figures 4A, B) or EO1428 (Figures S2A, B) abolished this effect. Neither of the two antibodies showed a significant impact on the activity of RhoA or Rac1 GTPases (Figure S3).

In addition to protein phosphorylation, the Ca^{2+}-influx after treatment with either 2G4 or AK23 was monitored. 2G4 induced a rapid influx of Ca^{2+} into the cells whereas AK23, similar to a previous report, did not (51) (Figure 5A). 2G4 did not show any signs of cross reactivity with Dsg1 (Figure S4), indicating a signalling mechanism dependent on Dsg3 in this case. This phenomenon was investigated further, employing mediators used in a previous study (51) (Table S2). However, in contrast to Dsg1-IgG-dependent signalling (51), inhibition of PI4K (with GSK-F1), PLC (with U-73122) or IP3R (with Xestospongin C) was not effective in preventing the 2G4-mediated Ca^{2+}-influx. Rather, inhibition of the downstream calcium release-activated channel (CRAC) (with BTP-2) blocked Ca^{2+}-influx, indicating that 2G4 induced a different pathway of Ca^{2+}-influx into the endoplasmic reticulum (Figure 5B). Next, we tested whether this Ca^{2+}-influx is involved in loss of cell adhesion caused by 2G4. However, none of the mediators including BTP-2 prevented any of the pathological changes, including loss of adhesion in the dispase-based dissociation assay (Figures 5C, D), keratin filament retraction and fragmentation of Dsg3 staining as revealed by STED microscopy (Figure 5E).

Discussion

Differences in the pathogenic effects between 2G4 and AK23

We found that 2G4 induced effects typical for pemphigus pathogenesis including loss of adhesion, keratin filament retraction and fragmentation of Dsg3 staining, which is in line with previous data (47). In addition, we observed that 2G4, similar to AK23, induced a decrease in the number of desmosomes per µm of cell border. This is important because loss of desmosomes is a hallmark in pemphigus patient lesions (12, 52). The same was observed by using transmission electron microscopy of human skin treated with PV-IgG *ex vivo*. However, AK23 did not cause significant loss of desmosomes in intact full skin (53). The results of the current study show that 2G4 was more than two times less effective than AK23 in inducing loss of cell adhesion. Despite this,

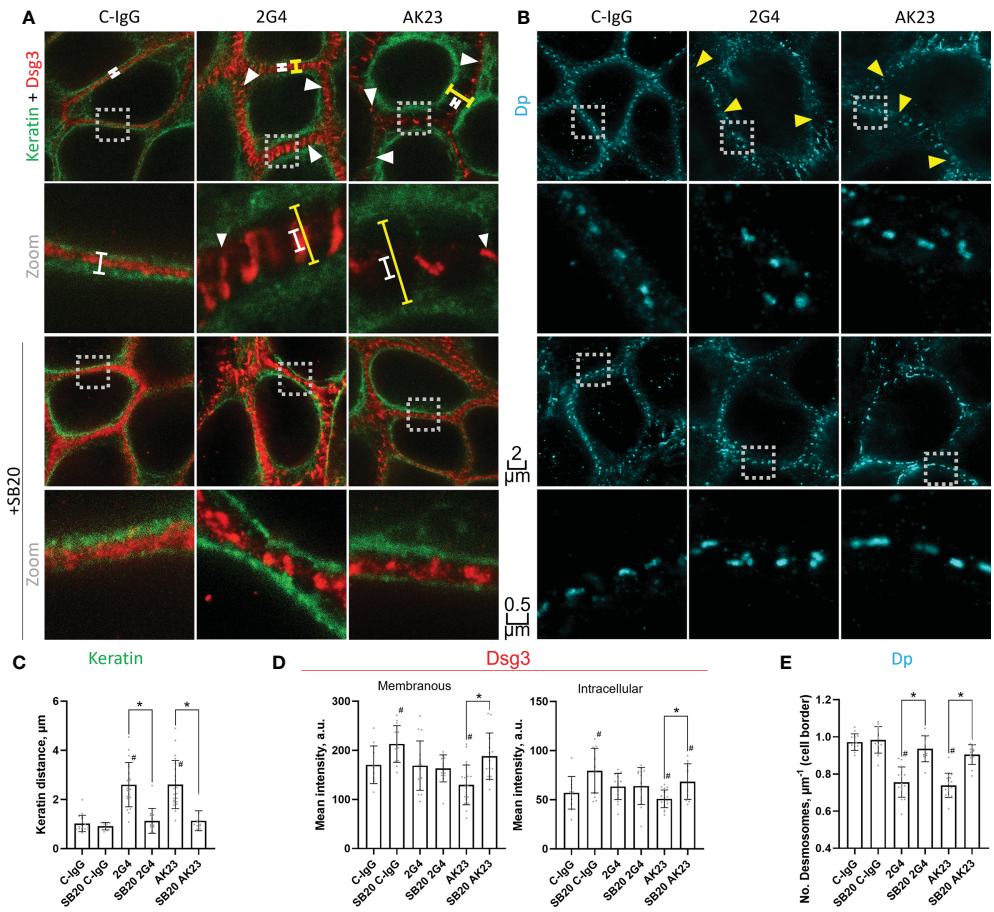

FIGURE 3
STED microscopy images reveal specific pathogenic effects of 2G4 and AK23. **(A)** Co-staining of Dsg3 (red) and pan-cytokeratin (green) in HaCaT cells treated with either C-IgG, AK23 or 2G4 for 24 h after 1 h preincubation with vehicle or SB20 (60 µM, inhibiting p38MAPK). Spans illustrate the degree of keratin filament retraction (yellow) compared to control (white). White arrowheads showcase the fragmented staining of Dsg3, which is mostly associated with keratin filaments. **(B)** Staining of Dp (with anti-Dp-pAb, A7169) after 24 h treatment with IgGs and 1 h preincubation with vehicle or SB20. Yellow arrowheads indicate reduced numbers of desmosomes per µm of cell border. Two different magnifications are shown for each condition (Scalebars as indicated, boxes with dotted outline indicate the area which was zoomed in on); Quantification of the STED images: **(C)** Keratin filament retraction form the cell borders towards the nucleus. **(D)** Dsg3 staining intensity at the cell borders/membranes (left) or in the cytoplasm (right). **(E)** Number of desmosomes per µm membrane (Experiments (N)=4-6, Evaluated images (n)=12-22). * marks statistically significant differences between the two conditions indicated, # indicates statistically significant differences towards control conditions, both in two-way-ANOVA with Sidaks correction for multiple comparisons, $p<0.05$.

STED imaging revealed a similar degree of keratin filament retraction and loss of desmosomes for the two IgGs. The difference may still be explained in part by the loss of Dsg3 staining caused by AK23, but not 2G4, both along the cell membrane and within the cytosol, indicating Dsg3 degradation. In fact, a complete lack of extradesmosomal Dsg3 following incubation with AK23 was observed, whereas in 2G4-treated cells, Dsg3 was present in linear streaks between adjacent cells. This is also in agreement with previous findings, indicating that the extradesmosomal pool of Dsg3 is affected first, without major changes in desmosome composition (12, 54–57). It also fits to the finding that the loss of adhesion and the widening of the intercellular distance is actually initiated in the spaces between the desmosomes (58, 59) and the desmosomes are affected only later (12, 60–63). Consistent with these reports, the morphology of the desmosomes did not appear to be altered.

The fact that 2G4 induces the typical hallmarks of pemphigus pathology despite being directed against the EC5 domain is interesting because antibodies against most other domains than EC1 or EC2 and particularly those targeting the EC5 domain of Dsg3 or Dsg1, were reported to be mostly non-pathogenic (32, 34, 35, 64). For anti-Dsg1-IgGs found in individuals from an endemic population with high risk to develop PF, EC5-specific IgGs were reported to cause no symptoms. Individuals carrying anti-EC1 or anti-EC2 IgGs on the other hand suffered from clinically evident PF (39). Moreover, anti-Dsg3-IgGs isolated from two patients also demonstrated no pathogenic effects for any of the IgG fractions recognizing only EC5 (32). Furthermore, it was reported that IgGs

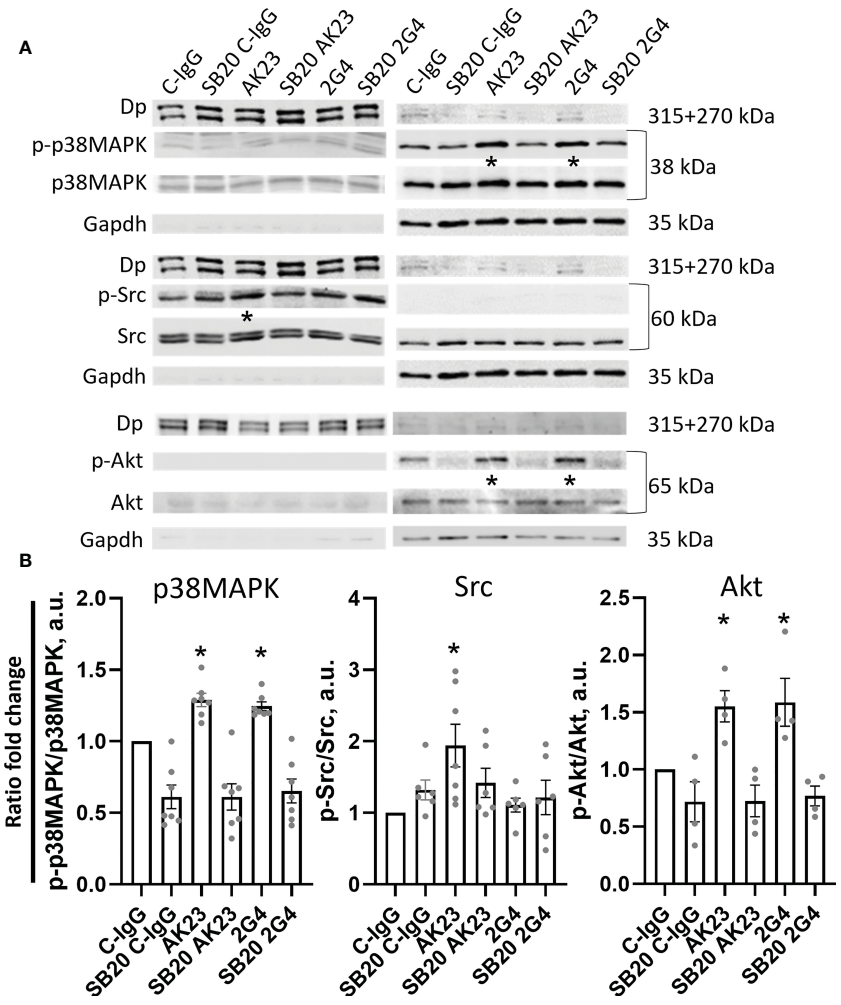

FIGURE 4
Western blot analysis reveals anti-Dsg3 epitope-specific kinase phosphorylation. **(A)** Representative Western blot images, with respective controls for Triton-soluble (membranous+cytosolic fraction, right) Gapdh and Triton-insoluble (cytoskeleton/desmosome-bound fraction, left) Dp. The cells were treated with either C-IgG, AK23 or 2G4 with or without pre-treatment with SB20 for 1 h, to inhibit p38 MAPK. **(B)** Quantification of differences in phosphorylation of the investigated proteins from the WB results. Significant changes were observed and quantified in the soluble fraction for p38MAPK and Akt and the insoluble fraction for Src (N=4-7). * marks statistically significant differences between the two conditions indicated in two-way-ANOVA with Sidaks correction for multiple comparisons, $p<0.05$.

against EC4 and EC5 are rarely found in clinically evident cases and only appear in rare atypical pemphigus cases, if at all. No patients were described to exclusively have IgGs against the EC5 domain, while patients with IgGs exclusively or mostly targeting EC1 and/or EC2 are common (33, 65). The lack of EC4 and EC5-specific IgGs in patients may be explained by the lower accessibility of these domains since the regions closer to the membrane are less exposed and are thus less likely to be subject to immunological processes, at least within mature desmosomes.

Differences in the pathogenic effects between 2G4 and AK23 are potentially caused by epitope-specific Dsg3 signalling

Signalling is known to be a major mechanism involved in pemphigus pathogenesis and was found to significantly contribute to blistering in human epidermis (66, 67). Stabilizing keratinocyte desmosomal adhesion may thus represent a novel additive treatment paradigm for pemphigus (68, 69). We proposed previously that autoantibody-specific signalling may contribute to the different clinical phenotypes of pemphigus (6). In line with this, we observed that 2G4 and AK23 induced p38MAPK activation. Akt as downstream target of EGFR and PI3-kinase was also phosphorylated and can thus also be considered to be activated in a Dsg3-dependent manner.

In addition to that, for the first time, we observed that Dsg3-mediated signalling is in part epitope-specific. Src was phosphorylated only after incubation with AK23 but was unaffected by 2G4. This difference in signalling response might also provide an explanation for the observed differences between the two IgGs in degree of loss of adhesion and their effect on the Dsg3 distribution and dynamics. It is likely that extradesmosomal Dsg complexes are degraded in response to AK23, since Dsg3, E-

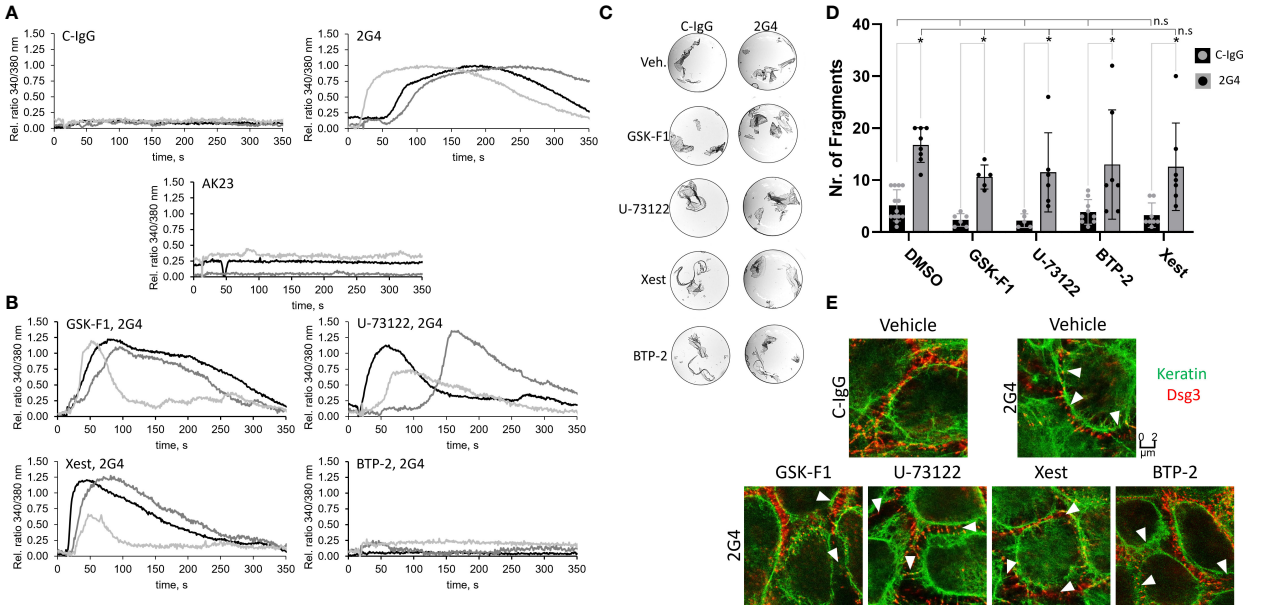

FIGURE 5
Alterations of intracellular Ca^{2+} signalling induced by 2G4. **(A)** Changes in intracellular Ca^{2+}-concentration determined in HaCaT cells, after addition of 2G4, AK23 or C-IgG, by a measured change in ratio of 340/380 nm fluorescence of intracellular FURA-2AM upon Ca^{2+} binding (One curve represents one independent experiment, N=3, obtained from the mean of 8 cells, n=8). **(B)** Changes in intracellular Ca^{2+}-concentration after addition of 2G4 to HaCaT cells previously treated with inhibitors of the anti-Dsg1-IgG-dependent Ca-influx pathway (see Table S2). **(C)** Representative images of dispase-based dissociation assay results after pre-treatment of HaCaT cells with Ca^{2+}-influx pathway mediators for 1 h. **(D)** quantification of the dispase-based dissociation assay results (N=5-13, * makrs statistically significant differences between the two conditions indicated, n.s. (not significant) indicates, that there are no significant differences to the corresponding C-IgG (negative) or 2G4 (positive) control condition, both in two-way-ANOVA with Sidaks correction for multiple comparisons, $p<0.05$). **(E)** STED images of cells pre-treated with vehicle or Ca^{2+}-influx pathway mediators for 1 h and C-IgG or 2G4 for 24 h. White arrowheads indicate fragmented Dsg3 staining.

Cadherin and Pg containing complexes were reported to be regulated in a Src-dependent manner (48, 70, 71). This would impact adhesion via alterations in the actin cytoskeleton which was reported to stabilize extradesmosomal Dsg3 binding events (72).

Interestingly, the phosphorylation of both Src and Akt was dependent on p38MAPK activity. However, previous reports indicated that p38MAPK can also be regulated by Src, indicating a feedback loop (49, 73). Src seems to regulate the actin dynamics (48, 70, 71), p38MAPK on the other hand was reported to regulate keratin dynamics (74–77). However, p38MAPK was mostly found to be located in the soluble (non-desmosome-bound) pool and to be activated by depletion of Pg. Src on the other hand was confined mostly to the cytoskeleton bound (desmosomal) fraction, as also shown previously (78). Since it was proposed that autoantibodies in pemphigus first induce signalling via extradesmosomal Dsg molecules (79), it may be that Src activity is regulated by p38MAPK activity via keratin restructuring and p38MAPK activity at least in part by Src activity via actin restructuring.

These data support the hypothesis that p38MAPK is a central pathway in pemphigus pathogenesis, which in epidermal keratinocytes orchestrates other signalling events involved in skin blistering. The current results together with previous finding may explain, why p38MAPK inhibition alone was effective to abrogate PV-IgG-induced skin blistering *in vivo* and in human skin ex vivo (53, 80). Inhibition of Src alone on the other hand was protective against the effects of AK23 but not PV-IgG in human epidermis (49). This might be a result of the activation of Dsg1-dependent pathways via anti Dsg1-IgG, but it is now clear, that this effect could also be epitope specific and dependent on the presence of the right mixture of polyclonal IgGs. In contrast to that, for the formation of mucosal erosions, p38MAPK appears to not be critical. Inhibition of p38MAPK alone was not sufficient to prevent blister formation in human mucosa *ex vivo* (81). This indicates that the interplay of several different pathways is important for a significant loss of adhesion and blister formation *in vivo*. Both p38MAPK and Src are major players in this context and seem to be interdependent at least to some degree but can possibly also act independent of each other. This dynamic seems to be regulated at least in part in an epitope specific manner.

The second example for epitope-specific signalling we observed was that 2G4 induced a significant degree of rapid Ca^{2+}-influx, whereas AK23, similar to previous findings, did not (17, 51). Since 2G4 showed no cross-reactivity with Dsg1, this most likely is a Dsg3-dependent mechanism. Inhibitors of the known Dsg1-dependent Ca^{2+}-influx pathway revealed that CRAC was involved in this effect whereas PI4K, PLC and IP3R were not. Because inhibition of CRAC did not show any protective effect on 2G4-mediated loss of cell adhesion, we conclude that Ca^{2+}-influx by this

mechanism does not play a major role in pemphigus pathogenesis, which is different to the anti-Dsg1-induced Ca^{2+}signalling, which reduced cell adhesion *via* PI4K, PLC, IP3R and CRAC (51).

Previous studies reported that the role of p38MAPK for loss of keratinocyte adhesion and epidermal blister formation might be dependent on polyclonal effects of anti-Dsg1- and/or anti-Dsg3-IgGs (82, 83). At least in combination with the bacterial exfoliative toxin A, which specifically cleaves Dsg1, much lower concentrations of AK23 of 10-15 µg/ml (compared to the 75 µg/ml used in the current study) were sufficient to cause loss of adhesion in a p38MAPK- and tyrosine kinase-independent manner. Moreover, a monoclonal PF-IgG was sufficient to cause epidermal blistering in a p38MAPK-independent manner. Therefore, we tested whether additive or polyclonal effects of AK23 and 2G4 on loss of cell adhesion were present when both antibodies were applied in combination. However, effects were similar when either the full or half of the concentration was used for both autoantibodies. These results indicate that polyclonal mixtures of these two antibodies did not induce additive effects on loss of adhesion and that the concentrations used are effective to induce maximal loss keratinocyte adhesion which is blunted by p38MAPK inhibition. Moreover, a single pathogenic autoantibody directed against Dsg3 is sufficient to induce an epitope-specific signalling response, at least at the concentrations used in this study. However, this does not rule out that combinations with other autoantibodies, which alone are considered to be non-pathogenic, may alter the signalling response or the extent of Dsg1/Dsg3 clustering and depletion (55, 82, 83).

Conclusion

The study supports the notion that monoclonal Dsg3-specific autoantibodies such as 2G4 and AK23 are pathogenic (47, 84) and are effective to cause loss of keratinocyte adhesion and induce reorganization of keratin filaments and loss of desmosomes. However, the pathogenicity is dependent on the target epitope and maybe explained in part by epitope-specific signalling including Src which may be involved in Dsg3 degradation. Further studies are required to determine how epitope-specific signalling is elicited upon autoantibody binding.

Materials and methods

Cell culture

The human epidermal keratinocyte cell line HaCaT was used for all experiments. The cells were cultivated in a humidified, 5% CO_2 atmosphere at 37°C in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, CA) with 10 % FCS (Biochrom, DE) and 50 U/ml penicillin and 50 µg/ml streptomycin (both AppliChem, DE). They were passaged using a trypsin-EDTA solution (Merck, DE). For experiments, the cells were pre-incubated with the mediators (Table S2) for 1 h and with the IgGs for 1 h for signalling or 24 h for other experiments. Both AK23 and 2G4 were used in a concentration of 75 µg/ml, IgG purified from healthy volunteers was used 1:50.

Ratiometric intracellular Ca^{2+} measurements

Fura-2AM (Thermo Fisher, USA) was used to measure intracellular Ca^{2+} in real time. The cells were grown in an 8-well µ-slide (Ibidi, DE). Mediators (Table S2) were contained in all incubation steps and during the measurement. A mix of 1 µmol/l Fura-2AM and 0.02 % pluronic (Thermo Fisher, USA) was applied for 20 min in measurement buffer (140 mmol/l NaCl, 3.6 mmol/l KCl, 2.6 mmol/l $CaCl_2(H_2O)_2$, 0.5 mmol/l $MgSO_4$, 0.5 mmol/l $NaH_2PO_4(H_2O)_2$, 2 mmol/l $NaHCO_3$, 5 mmol/l HEPES and 5 mmol/l D+Glucose, pH 7.35) at 37°C. The cells were washed twice with measurement buffer. Measurements were performed using MetaFluor (Moleculardevices, USA) on an Axio Observer A1 (Zeiss, DE) with a Polychrome V (Till Photonics, DE), a CoolSNAP-Hq2 digital camera (Photometrics, USA) and a Fura-2 filter set. For each independent experiment, the signals from 8 out of 15 randomly selected cells were evaluated (occasional non-responding cells were not included, very rare oscillating cells and weak responders were included, N = 4).

Cell lysis, gel electrophoresis and Western blotting

Cells were cultured in 24-well-plates. Lysates were fractioned into a soluble and insoluble fraction using Triton-extraction-buffer (0.5% Triton X-100, 50 mmol/l MES, 25 mmol/l EGTA, 5 mmol/l $MgCl_2$, pH 6.8, 0.1% pepstatin+aprotinin+leupeptin, 1% Phenylmethylsulfonylfluorid) for 10 min on ice under gentle shaking. The pellet was separated at 14.000 rpm for 10 min at 4°C and the supernatant was retrieved. The pellet was washed once and lysed with ultrasound in SDS lysis buffer (25 mmol/l HEPES, 2 mmol/l EDTA, 25 mmol/l NaF, 1% SDS, pH 7.6, complete™ (Merk, USA)). The protein amount was determined with a commercial Pierce BCA protein assay kit. Western-blotting was performed, using a standard wet blotting protocol on nitrocellulose membranes (Life Technologies, USA). Membranes were blocked with ROTI®Block (Carl Roth, DE) 1:10 in Tris-buffered saline with 0.05% tween (TBST) for 1 h. The Antibodies (Table S1) were used overnight at 4°C in 5% BSA in TBST anti-rabbit/mouse horseradish-peroxidase-coupled secondary antibodies (Dianova, DE) were used 1:10.000 in TBST for 1 h and visualized with self-made ECL solution on a FluorchemE developer (Protein Simple, USA).

Dispase-based dissociation assay

After incubation as described above, confluent cell monolayers were washed with Hank´s buffered saline solution (HBSS) and

subjected to 2.4 U/ml dispase II (Sigma Aldrich, USA) in HBSS for 20 min at 37 °C, 95% humidity and 5% CO_2. After detachment of the monolayer the reaction was stopped by adding 200 µl HBSS. Defined shear stress was applied with an electrical pipette. Images for fragment counting were taken using a binocular microscope (Leica, DE) and an EOS 600D camera (Canon, Japan).

Immunostaining

Samples were washed and fixed with ethanol (-20°C) shaking on ice for 30 min followed by acetone (-20°C) for 3 min. The samples were blocked with 3% bovine serum albumin (BSA) and 1% normal goat serum in PBS for 30 min. Primary antibodies were applied over 3 h at RT STAR-RED- or Alexa 594-coupled goat-anti-rabbit/mouse secondary antibodies (Abberior GmbH, DE) were incubated for 1 h and DAPI 1:10.000 for 15 min. The coverslips were mounted on glass slides using Prolong™ Diamond Antifade Mountant (Thermo Fisher GmbH, DE).

STED microscopy

The samples were imaged using an Abberior 3D Stimulated emission depletion (STED) confocal microscope with IMMOIL-F30CC (Olympus GmbH, DE) Star Red and Alexa 594 were excited at 638 nm and 594 nm respectively, using pulsed diode lasers (PDL 594, Abberior Instruments; PiL063X, Advanced Laser Diode Systems). Fluorescent molecules were depleted at 775 nm with a pulsed fibre laser (PFL-P-30-775B1R, MPB Communications) and the emission was detected with an avalanche photodiode detector at 605-625 and 650-720 nm range.

Statistical analysis

Data were analysed using two-way-ANOVA followed by Sidak-post-hoc-test for multiple comparison or one-way-ANOVA followed by Dunnett-post-hoc-test for multiple comparison, using Graphpad Prism (Graphpad Software, USA). Error bars represent SEM. Significance was assumed with $p \leq 0.05$. Data are shown as mean ± SEM. Each N represents one independent experiment, each n one technical replicate.

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

TS: 65%, conceptualizing the project, performing experiments, performing data evaluation writing the manuscript, conceptualizing and designing the Figures, revising the manuscript. CH: 18%, providing materials, performing experiments, performing data evaluation, checking the manuscript, revising the manuscript. SM: 3%, performing experiments, performing data evaluation. MH: 2%, providing materials, checking the manuscript. RT: 2%, providing materials, checking the manuscript. JW: 10%, providing funding, conceptualizing the project, checking the manuscript, revising the manuscript. All authors contributed to the article and approved the submitted version.

Funding

The research was supported by the Deutsche Forschungsgemeinschaft (DFG) FOR 2497/TP04 to RT (TI 291/10-2)/TP05 to JW and TP08 to MH (HE 1602/16-2) is acknowledged.

Acknowledgments

We thank Silke Gotschy, Martina Hitzenbichler and Manuel Schulze-Dasbeck for their excellent technical assistance. Special thanks also to Mariya Radeva for help with checking Figures.

In memoriam of Detlef Zilikens

The authors all knew Detlef Zilikens as an inspiring scientist. Jens Waschke (JW) listened to his lectures in dermatology when he was a student at Würzburg medical school where he found professor Zillikens to be a very student-friendly and relaxed teacher, which was not applying to all professors he met before. During these lectures, JW heard for the first time about pemphigus. He felt that for this disease the pathogenesis seemed rather simple. It was known that autoantibodies targeting adhesion molecules in desmosomes, the latter he knew well from his anatomy classes, were pathogenic. During his internship in internal medicine in 2001, JW again met Detlef Zillikens to initiate a research project together with Enno Schmidt on the pathogenic effects of autoantibodies in pemphigus. Soon, everything turned out not to be that simple and the pathogenesis of pemphigus not completely clarified. Since the background in the anatomy group on pemphigus was missing, the collaboration with Enno Schmidt, which is still continuing today, under the supervision of Detlef Zillikens was pivotal to complete our first studies and to get introduced to the pemphigus research community. JW remembers when Detlef was talking with him and his doctoral student Volker Spindler at the International Dermatology Meeting in Los Angeles in 2007, where we were presenting our first poster on pemphigus and he gave us the impression to talk among equal experts. At this occasion, he introduced us to the pioneers of pemphigus research. Afterwards, many discussions and also private talks followed at pemphigus meetings over the years, where Detlef Zillikens advised JW about the strengths and weaknesses of the manuscripts we published with him. For us, Detlef Zillikens was much more than a collaborator and without him and Enno Schmidt, we would not have completed a single study on pemphigus.

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.2023.1163066/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 25 April 2023
DOI 10.3389/fimmu.2023.1159351

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders: Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 05 February 2023
ACCEPTED 03 April 2023
PUBLISHED 25 April 2023

CITATION
Rosi-Schumacher M, Baker J, Waris J, Seiffert-Sinha K and Sinha AA (2023) Worldwide epidemiologic factors in pemphigus vulgaris and bullous pemphigoid.
Front. Immunol. 14:1159351.
doi: 10.3389/fimmu.2023.1159351

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Worldwide epidemiologic factors in pemphigus vulgaris and bullous pemphigoid

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Autoimmune blistering diseases such as bullous pemphigoid (BP) and pemphigus vulgaris (PV) are complex, multifactorial, and polygenic diseases, whose exact pathogenesis is difficult to pinpoint. Research aimed at elucidating the associated epidemiologic risk factors of these two diseases has been hampered by their rare disease status. Further, a lack of centralization and standardization of available data makes the practical application of this information challenging. In order to collate and clarify the available literature we comprehensively reviewed 61 PV articles from 37 different countries and 35 BP articles from 16 different countries addressing a range of disease relevant clinical parameters including age of onset, sex, incidence, prevalence, and HLA allele association. The reported incidence of PV ranged from 0.098 to 5 patients per 100,000 people, while BP ranged from 0.21 to 7.63 patients per 100,000. Prevalence of PV ranged from 0.38 to 30 per 100,000 people and BP ranged from 1.46 to 47.99 per 100,000. The mean age of onset in patients ranged from 36.5 to 71 years for PV and 64 to 82.6 years for BP. Female-to-male ratios ranged from 0.46 to 4.4 in PV and 1.01 to 5.1 in BP. Our analysis provides support for the reported linkage disequilibrium of HLA DRB1*0402 (an allele previously shown to be associated with PV) and DQB1*0302 alleles in Europe, North America, and South America. Our data also highlight that HLA DQB1*0503 (also known to be associated with PV) appears in linkage disequilibrium with DRB1*1404 and DRB1*1401, mainly in Europe, the Middle East, and Asian countries. The HLA DRB1*0804 allele was only associated with PV in patients of Brazilian and Egyptian descent. Only two HLA alleles were reported as associated with BP more than twice in our review, DQB1*0301 and DQA1*0505. Collectively, our findings provide detailed insights into the variation of disease parameters relevant to PV and BP that can be expected to inform future work aimed at unraveling the complex pathogenesis of these conditions across the globe.

KEYWORDS

pemphigus vulgaris, bullous pemphigoid, prevalence, incidence, gender, age of onset, HLA-association

1 Introduction

Pemphigus vulgaris (PV) and bullous pemphigoid (BP) are rare autoimmune skin diseases associated with high morbidity and mortality. PV is characterized by flaccid blisters and erosions of the skin and mucous membranes due to the intra-epidermal separation of keratinocytes. Autoantibodies specific for desmosomal (desmoglein 1 and desmoglein 3) and potentially additional non-desmosomal (thyroid peroxidase, thyroglobulin, and more) moieties lead to a breakdown of intercellular adhesion structures responsible for epithelial integrity (1). On the other hand, BP is characterized by tense bullae of the skin and less oral involvement due to autoantibodies targeting hemi-desmosomal proteins (BP180 and BP230) anchoring the epidermis (2).

The etiopathogenesis of pemphigus and pemphigoid diseases is complex; in other words, it involves largely unknown environmental and polygenic genetic risk elements. One key genetic component that is required, but insufficient for disease development links to HLA class II molecules. In PV, the alleles DRB1*0402 and DQB1*0503 have been identified as PV-associated in the Ashkenazi Jewish and Caucasian population (3). While investigation into HLA associations in BP have been less extensive, DQB1*0301 has been highlighted in the literature multiple times as being associated with BP (4–7).

The accurate assessment of globally associated epidemiologic/genetic risk factors for pemphigus/pemphigoid diseases is hindered by the rare nature of these diseases, the complex nature of disease risk and development, the heterogeneity and individual variability of disease expression, as well as a lack of centralization and standardization of the available epidemiological, clinical, genetic data. A critical evaluation of the current status and fault lines in the informational landscape is required to formulate clear-eyed strategies aimed to advance our understanding of disease relevant etiopathogenic factors and ultimately improve patient access, care and outcomes.

The objective of the present study was to comprehensively evaluate and integrate information regarding the epidemiological features of patients with PV and BP across countries worldwide to provide enhanced insights regarding the presentation, demographics and genetic basis of disease. The parameters investigated included age of onset, sex, prevalence, incidence, and HLA associations.

As a note of context, Dr. Detlef Zillikens devoted his career to the investigation of autoimmune bullous disorders. His extensive track record of original research has served to provide many deep and original insights into the etiology, pathogenesis and management of these often devastating conditions. Dr. Zillikens has left an impressive legacy not only based on his individual accomplishments and collegiality, but perhaps most importantly due to his selfless commitment to his outstanding academic and clinical program at the University of Luebeck, world-wide collaborations, and his mentorship of countless junior faculty, fellows and students who will continue to carry the torch in our field. A number of the studies reviewed in this report were co-authored by Dr. Zillikens and members of his group (8–16). Several of the themes explored in this report are a direct extension of areas of inquiry initiated by Dr. Zillikens.

2 Methods

2.1 Pemphigus vulgaris

The data for the study was compiled by a thorough review of the literature. A total of 63 articles from 37 different countries published between 1974 to 2020 were mined from a PubMed online search for mention of search terms “Pemphigus and Incidence” and “Pemphigus and Prevalence” during July 2016 and August 2021 and “Pemphigus and HLA” during December 2016 and August 2021. Studies included were limited to English language only. Map visualization was achieved using the program www.mapchart.net.

Some studies were excluded from analysis due to the study populations having a mixture of PV and PF patients. We elected to exclude six studies that had populations less than 70% PV patients (17–22). While information on pemphigus foliaceous is often reported in conjunction with PV, we elected to focus on PV for this paper as the data on PF was often found in the context of “Pemphigus” as a whole, rather than separated on its own. Further, much of the information on solely PF was in the context of fogo selvagem, or endemic PF, which has unique features compared to PF. While a subject of interest, unfortunately most studies used in this manuscript were not derived from national registries, but rather from independent institutions or regional groups of institutions. National registries may, in the future, serve as an advantageous tool for collecting information on these rare diseases.

2.2 Bullous pemphigoid

Data for this study were obtained *via* a comprehensive review of the literature. A total of 35 articles from 16 different countries between 1992 and 2021 were obtained from an online search of PubMed. Terms searched included “Bullous Pemphigoid and Incidence”, “Bullous Pemphigoid and Prevalence”, “Bullous Pemphigoid and Epidemiology”, and “Bullous Pemphigoid and HLA” between September 2021 and January 2022. Studies were limited to those written in the English language. Maps were created using the website www.mapchart.net.

3 Results and discussion

3.1 Prevalence

Six studies reported on the prevalence of PV in Iran (23), Italy (24), Romania (8), Saudi Arabia (25), Germany (12), and Bulgaria (26). The highest prevalence was found in Iran (23) with 30 per 100,000 people per year, while the lowest prevalence was found in Bulgaria (26) with 0.38 per 100,000 people per year (Figure 1A and Table 1A).

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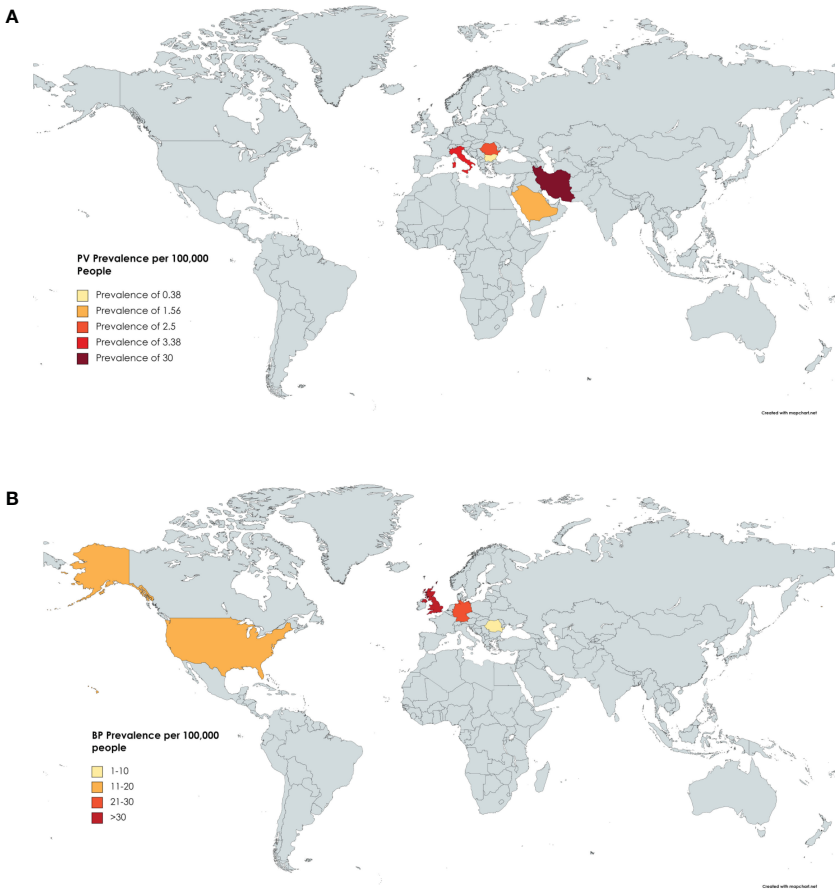

FIGURE 1
Prevalence by Country. **(A)** Countries include Iran, Italy, Germany, Romania, Saudi Arabia, and Bulgaria. Darker shading signifies higher PV prevalence. Specific values can be found in Table 1A. **(B)** Countries reporting BP prevalence include the USA, UK, Romania, and Germany. Specific Values can be found in Table 1B.

Four studies reported on the prevalence of BP in Romania (8), Germany (12), the UK (27), and the USA (28). The highest prevalence was seen in the UK with 47.99 cases per 100,000 people per year (27). The lowest prevalence was seen in Romania at 1.46 cases per 100,000 people per year (8). The mean prevalence was 21.84 per 100,000 people, but the relatively small number of studies included in this analysis may serve to obnubilate the significance of this mean (Figure 1B and Table 1B).

Figures on disease prevalence were the least available form of epidemiological data reported in the literature, likely due to the fact that prevalence data can be difficult to estimate in large populations in the case of rare diseases. Two factors that likely contribute to the variations in reported disease prevalence include regional discrepancies in (the largely unknown) disease predisposing genetic and/or environmental elements between different ethnic groups, as well as additional disease modifying factors, such as access to specialized health care facilities and expert care, that impact disease reporting practices in differing countries. Creating broad disease registries, coordinated to encompass standard reporting criteria implemented internationally could prove valuable in acquiring more accurate prevalence values.

TABLE 1A Prevalence of PV by Country.

Country	Prevalence of PV per 100,000 per year	Citation
Bulgaria	0.38	(26)
Saudi Arabia	1.56	(25)
Romania	2.5	(8)
Italy	3.38	(24)
Germany	9.48	(12)
Iran	30	(23)

TABLE 1B Prevalence of BP by Country.

Country	Prevalence of BP per 100,000 per year	Citation
Romania	1.46	(8)
USA	12	(28)
Germany	25.9	(12)
UK	47.99	(27)

3.2 Incidence

Twenty-one papers from 1974 to 2018 reported on the incidence of pemphigus vulgaris (8, 11, 23–26, 29–44). The overall incidence of PV varied significantly in various ethnic populations. The highest incidence was found in Iran with 5 per 100,000 people per year (31). The lowest incidence was found in Germany with 0.098 per 100,000 people per year (11). The highest incidences seem to cluster around the Middle East, with higher incidence trending south on the map towards the Tropic of Cancer (Figure 2A and Table 2A).

Seventeen papers published between 1995 and 2022 reported on the incidence of BP (8, 9, 16, 20, 27, 36, 38, 45–55) which was highest in the UK with one study reporting an incidence of 7.63 cases per 100,000 people per year (27) (Figure 2B and Table 2B). Kuwait had the lowest incidence at 0.21 cases per 100,000 people per year (45). The mean incidence worldwide was 2 cases per 100,000 people per year.

While there is more data available for incidence than prevalence in both of these diseases, there still exists both a paucity and notable variance in the worldwide data. Although there are reports of increasing rates of autoimmune diseases worldwide, there are

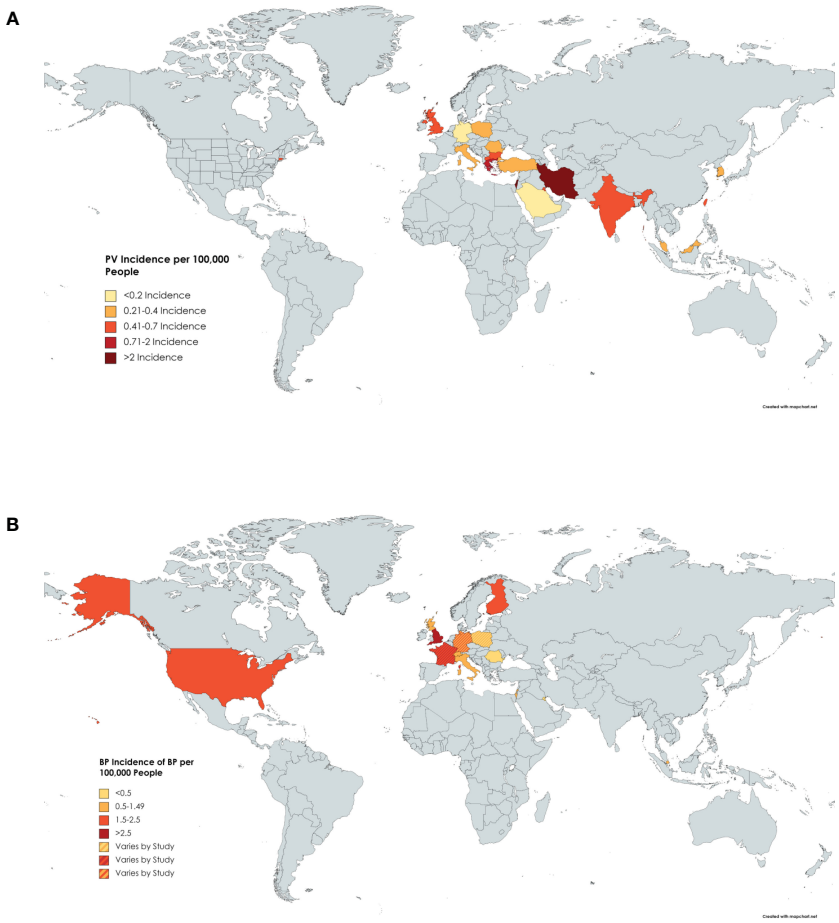

FIGURE 2
Incidence of PV by Country. **(A)** Darker shading of countries signifies higher incidence of pemphigus vulgaris. Specific incidence values can be found in Table 2A. **(B)** Darker shading of countries signifies higher incidence of BP. Specific incidence values can be found in Table 2B.

TABLE 2A Incidence of PV by Country.

Country	Incidence of PV per 100,000 per year	Citation
Germany, Lower Franconia and Mannheim	0.098	(11)
Macedonia, Ethnic Albanians	0.1	(43)
Saudi Arabia	0.16	(25)
Malaysia	0.2	(29)
South Korea	0.21	(37)
Poland	0.24	(44)
Turkey	0.24	(42)
Italy	0.25	(24)
Romania	0.4	(8)
Hartford, Connecticut, US, overall adults	0.42	(41)
India (Thrissur District)	0.44	(34)
Macedonia	0.44	(43)
South India	0.44	(34)
Kuwait	0.46	(38)
Bulgaria	0.47	(26)
Taiwan	0.47	(33)
Macedonia, Ethnic Macedonians	0.51	(43)
Jerusalem, Non-Ashkenazi	0.61	(39)
Iran	0.67	(40)
Guadeloupe	0.696	(32)
UK	0.7	(36)
Macedonia during unrest	0.89	(43)
Greece	0.93	(35)
Iran	1	(23)
Jerusalem Jews	1.61	(39)
Macedonia, Roma (Gypsies)	2.4	(43)
Jerusalem, Ashkenazi	2.7	(39)
Hartford,Connecticut, US, Jewish Adults	3.2	(41)
Iran	5	(31)

currently no reports endorsing this phenomenon in PV (56). BP, however, has displayed an increase in incidence over the past 2 decades. Explanations for this include increasing diagnostic capability and increased life expectancy (57).

Both PV and BP are known to be multifactorial in origin and the wide fluctuation among data worldwide supports the hypothesis that geographic location, and associated racial and ethnic population differences linked to genetic risk factors can influence the incidence of these diseases. Other confounding variances in socioeconomic status, access to healthcare, and the role of specific environmental and "exposome" factors are no doubt impactful as well, but cannot easily be accounted for. Nevertheless, broad trends in the data connote that careful analysis in future work focused on isolating the role of specific genetic and environmental risk elements within specific ethnic groups and geographic locations will be essential in lifting the veil on the fuller scope of meaningful agents that undergird and shape the spectrum of disease.

3.3 Gender

Twenty-five papers were reviewed that reported on the gender ratio of females to males in PV (10, 23–26, 31, 34, 36, 40, 42, 43, 58–72). The lowest ratio, less than one, indicating there were more

TABLE 2B Incidence of BP by Country.

Country	Incidence of BP per 100,000 per year	Citation
Kuwait	0.21	(38)
Romania	0.25	(8)
Kuwait	0.26	(45)
Poland	0.447	(46)
Poland	0.738	(47)
Singapore	0.76	(48)
Italy	1	(49)
Israel	1.14	(50)
Switzerland	1.21	(20)
Germany	1.34	(9)
Scotland	1.4	(51)
Finland	1.7	(52)
Germany	1.96	(16)
France	2.17	(53)
Olmstead County, Minnesota, US	2.4	(54)
UK	4.3	(36)
France	7	(55)
UK	7.63	(27)

males affected than females, was found in Saudi Arabia with a ratio of 0.46 (25). All other ratios were greater than one, indicating most studies found that more females were affected than males. The greatest ratios were found in Mexico with a ratio of 4.4 (72). The mean of all the ratios from the studies was 1.74 (Figure 3A).

For BP, we reviewed twenty papers reporting on the gender ratio (8, 9, 14, 18, 20, 36, 38, 42, 45, 47–54, 73–75). Finland had the lowest female to male gender ratio of 1.01 (52), while two separate studies in Kuwait reported the highest gender ratios of 5.1 (45) and 5.75 (38). The mean female to male ratio worldwide was 1.87 (Figure 3B).

As noted, Kuwait displayed by far the highest female to male ratio in BP. In two separate studies, the female to male ratio was recorded as being over 5 (38, 45). The next highest BP female to male gender ratio was 2.5, found in Germany by Ständer et al. (14) in a small subgroup of patients with localized BP. The extent to which this elevated gender ratio in Kuwait is a reflection of true regionally linked differences in reported ratios from other worldwide locations remains to be determined. Unfortunately, there are no studies from surrounding countries in proximity to Kuwait that could be used for comparison. However, the fact that two separate studies from two years apart reported similarly high female to male ratios in Kuwait lends credence to their validity, rather than being a statistical anomaly. Additional studies on gender ratio from countries in the Middle East would help to corroborate this data, whose potential disease significance awaits further exploration.

Overall, both PV and BP, like the majority of autoimmune diseases, show a consistent bias towards females. There are multiple theories that posit explanations for this bias including effects from the menstrual cycle, hormonal effects on the microbiome, and immune-related genes located on the X-chromosome (76). However, the specific mechanisms of female skewing in autoimmune blistering diseases, and autoimmunity in general remain enigmatic. It is also unclear the extent to which female patients differ from males in terms of key disease relevant factors such as genetic predisposition, immune dysregulation including autoantibody levels, and clinical course. However, within the context of PV specifically, Naseer et al. found that female patients had a later age of onset as well as higher rates of mucosal disease than male patients (77). Further investigation *via* retrospective as well as longitudinal prospective analyses are needed to better define gender specific differences regarding autoimmune risk and expression.

3.4 Age of onset

Fourteen papers were reviewed that presented information on the mean age of onset in PV patients enrolled in their study (8, 23–

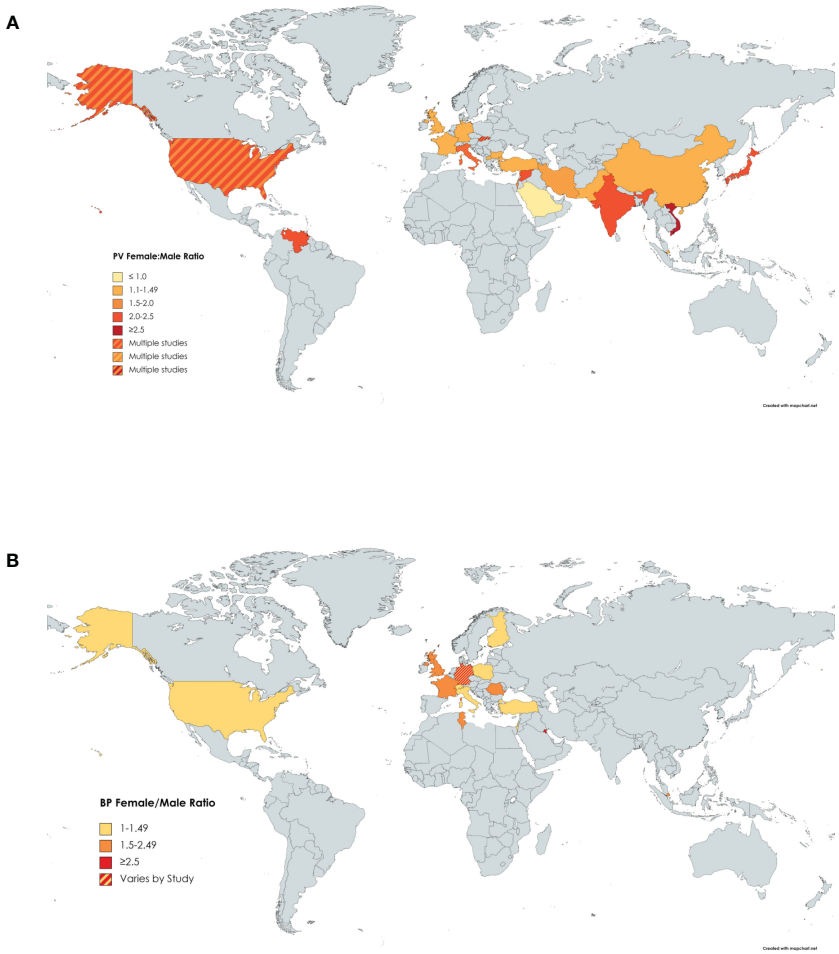

FIGURE 3
Gender Ratio of PV by Country. Darker shading signifies higher female:male gender ratio of **(A)** PV or **(B)** BP. Countries with more than one shaded color signify that more than one study had results that fell into different categories.

26, 31, 36, 38, 40, 42, 43, 58, 64, 71). The ages ranged from 36.5 years in Kuwait (38) to 71 years in the United Kingdom (36), with the average age across these studies being 49.8 years (Figure 4A). The average age of onset of PV was higher than several autoimmune diseases such as SLE, systemic sclerosis, and psoriasis (78). PV shared a similar age of onset to autoimmune diseases such as rheumatoid arthritis and Sjogren's syndrome which tend to occur in the mid to late 40's (78).

We reviewed 16 papers that reported the mean age of onset of BP patients enrolled in investigative studies (8, 9, 18, 20, 36, 38, 42, 45, 48, 50–54, 79, 80). Ages of onset ranged from 64 (42) to 82.6 (53) years with an average of 73.4 years (Figure 4B). This later age of onset in BP relative to PV is consistent with the fact that BP is known as a disease of the elderly.

Interestingly, both PV and BP both tend to have lower average ages of onset in southwestern Asia. PV was also found to generally have a higher incidence and prevalence in this region, pointing towards the possibility that there are distinct genetic risk factors specific to populations in that region. On the other hand, there is minimal data on BP incidence and prevalence in this region of southwest Asia.

The genetic, environmental, and ultimately the immunologic factors that arbitrate the generally late onset of the majority of autoimmune conditions, and specifically the predominant age of onset window in PV and especially BP are nebulous. Potential explanations include the erosion of immune tolerance over time, and an accumulating environmental burden that evolve a patient's "umwelt" in later years.

3.5 HLA association

Analysis of the aggregated HLA data on PV revealed several trends, one of which highlights the evident linkage disequilibrium between HLA DRB1*0402 (10, 59–61, 63, 66, 68, 81–90) and DQB1*0302 alleles (60, 83, 86, 89, 91–94). HLA DRB1*0402 shows clustering in the Middle East (10, 59, 61, 68, 88, 89) and around Europe (10, 60, 85–87, 90, 95), with migratory spread into

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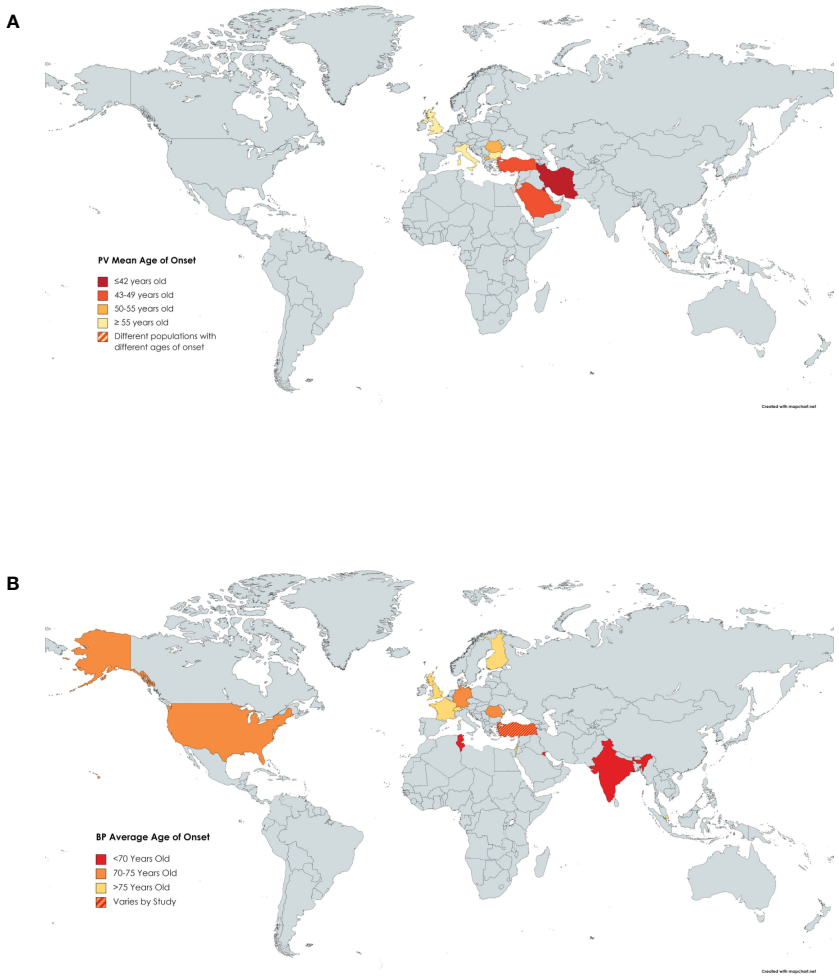

FIGURE 4
Age of Onset of PV by Country. Darker shading signifies a younger age of onset of **(A)** PV or **(B)** BP. Countries with more than one shaded color signify that more than one study had results that fell into different categories.

North (66) and South America (81, 82, 84) (Figure 5A). This genetic undercurrent is consistent with historic Ashkenazi Jewish and Mediterranean population migration to North America. DQB1*0302 allele distribution echoes similarly worldwide, promulgating support for the actuality of linkage disequilibrium between the DRB1*0402 and DQB1*0302 alleles (69) (Figure 5B). DQB1*0302 alleles are also prevalent in Asian countries such as China and Japan.

HLA DQB1*0503 (60, 67, 71, 84, 86, 92, 93, 95–98) shows clustering in western Europe (60, 86, 93, 95), North (67) and South America (84) as well as South and East Asia (India, Pakistan, China, Japan) (71, 96–99) (Figure 5C). A similar distribution is adhered to by DRB1*1404 and DRB1*1401 (10, 59, 60, 65, 68, 81, 82, 84, 89, 90, 93, 95–97, 100, 101) as seen in Figure 5D, again likely as a reflection of linkage disequilibrium between the DQB1*0503 and DRB1*14 alleles. DRB1*14 alleles are also found in the Middle East (10, 59, 68, 89, 96) and Brazil (81).

The HLA allele DRB1*0804 is predominantly detected in the Brazilian (102) and Egyptian (10) populations (Figure 5E). Both DRB1*0804 and DRB1*14 have been reported among PV patients in Brazil and Egypt, although further analyses will be required to certify the strength of these proclaimed diseases linkages.

Fewer studies were available on HLA and BP; we reviewed 9 papers that reported on HLA predominance among BP patients (Figure 6) (4–6, 13, 103–107). Of the nine articles that include information on HLA alleles of BP patients, only 2 HLA alleles were repeated more than twice across studies: DQB1*0301 and DQA1*0505.

DQB1*0301 came up as being associated with BP as early as 1996 and was reported in five countries. DQB1*03:01 has been included in several publications as an implicated factor in drug-induced BP. Antigen presentation by the DQB1*03:01 molecule has also been shown to boost T-cell avidity to several epitopes of BP180, a major autoantigen associated with bullous pemphigoid (108).

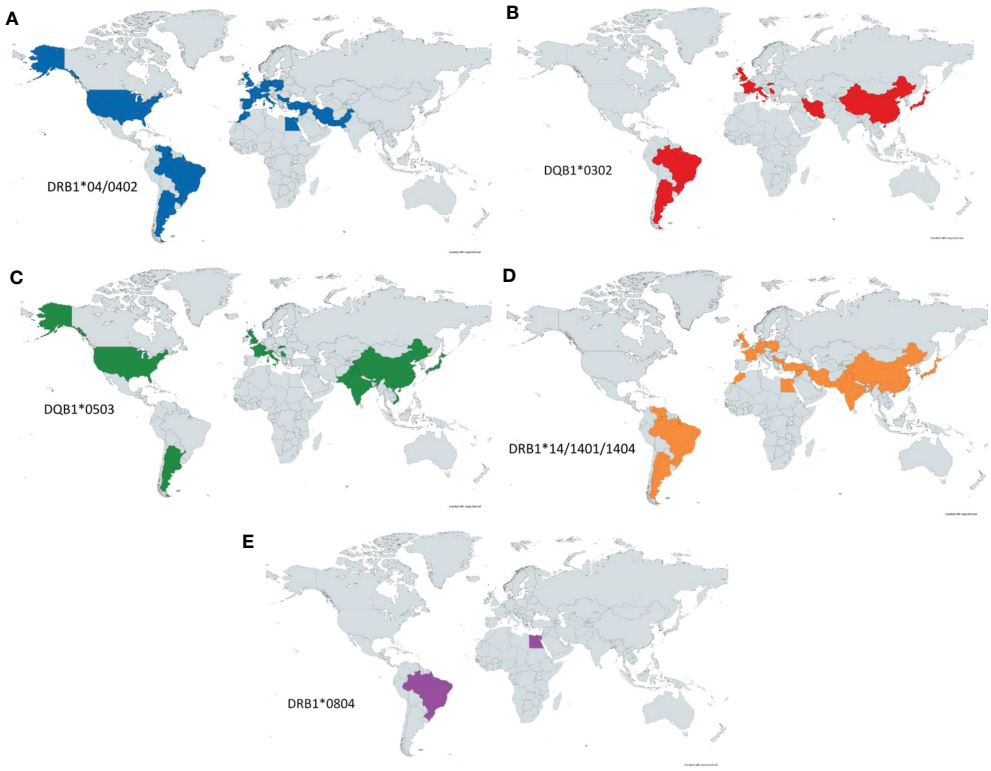

FIGURE 5
PV-HLA Associations Reported by Country. Known HLA associated alleles as reported by country. Significant overlap is seen between DRB1*0402 **(A)** and DQB1*0302 **(B)** as these alleles have previously been shown to be in linkage disequilibrium. DQB1*0503 **(C)** is seen throughout the world but is notably concentrated in eastern and southern Asia. DRB1*14 **(D)** is seen diffusely throughout the eastern hemisphere as well as South America. Lastly, DRB1*0804 **(E)** has been more rarely reported in PV but has been noted in Egypt and Brazil.

Regarding the significance of DQA1*05:05 being overrepresented in BP patients, there is a need for follow-up inquest.

HLA genetics, global allele distribution and migrations, ethnic and disease associations are immensely complex. Yet the importance of HLA in determining disease risk, the specificity of the autoimmune attack and the final clinical presentation of disease cannot be overstated. A clearer discernment of true HLA-disease associations in autoimmune blistering diseases would benefit from robust, multinational databases that accelerate the collection of epidemiologic and genetic information at a larger scale in order to sufficiently power detailed multivariate analyses and improve the calculation of disease risk. Ultimately, studying the physical structure of each of the key HLA molecules associated with these diseases will be required to understand how a patient's genetic profile translates to immune (dys)functionality to induce aberrant immune responses resulting in skin pathology.

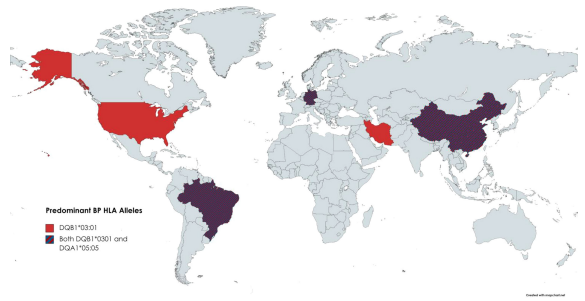

FIGURE 6
BP-HLA Associations reported by Country. Known HLA associations reported by country. The only two alleles repeated more than twice were DQB1*0301 and DQA1*0505.

4 Conclusions

At present, available epidemiologic information on PV and BP is sparse worldwide. Existing data on the overall ranges of prevalence, incidence, gender ratio, and age of onset for PV, BP, and pemphigus foliaceous (PF) are summarized in Figure 7 (8, 11, 12, 15, 17, 23, 25–27, 31, 36, 38, 42, 52, 53, 71, 109, 110). PF data were added as this condition closely relates to PV in terms of etiology and clinical expression, and the two are often reported together in studies. Yet, there remain gaps regarding epidemiology, and even HLA associations for both pemphigus and pemphigoid disease.

Moving forward, it will be important to develop concerted and coordinated efforts to compile more complete, comprehensive global datasets to hurdle current barriers impeding the study of ethnicity,

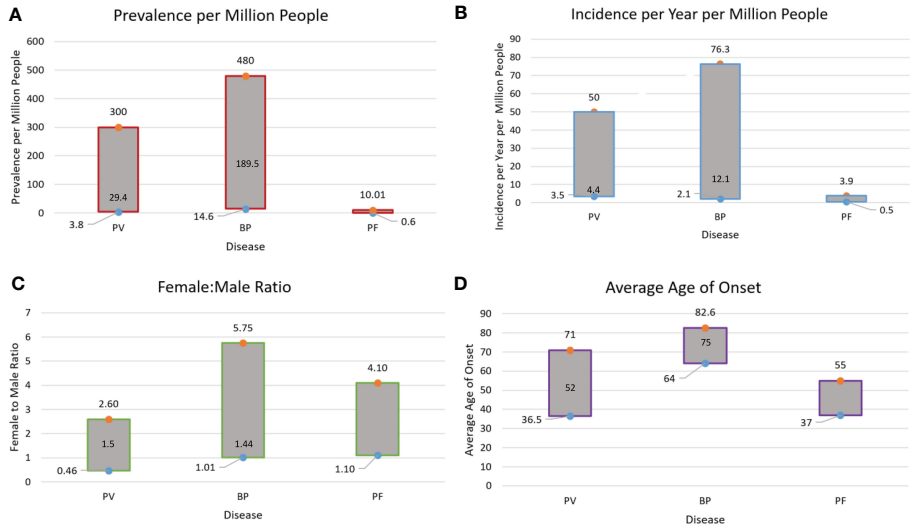

FIGURE 7
Summary of Prevalence **(A)**, Incidence **(B)**, Gender Ratio **(C)**, and Age of Onset **(D)** Ranges for PV, BP and Pemphigus Foliaceous (PF). Data on PF incidence and prevalence are sparse in the literature. This is due to the fact that PF is seldomly studied independently, while studies that report on Pemphigus as a singular disease category (including PV and PF) do not provide data individually on disease subtypes. The median values for age of onset, gender ratio, prevalence, and incidence in PV and BP are noted within the bars.

genetic background, geographic location and environmental elements that impact disease risk and expression. While envisioning and effecting such an endeavor is undoubtedly challenging, we are at the precipice of emerging technology conceived to leverage digital health care information gathering and communication platforms, including mobile applications, with the potential to escalate clinical investigation to previously unattainable levels and surmount the difficulties of unpacking the complexities of multifactorial diseases. On this basis, we can expect to further uncover the mechanisms of disease risk and clinical heterogeneity, and instruct the formulation of more informed genetic and immunologic research that upraises the development of superior strategies to avert and remedy the autoimmune condition.

Author contributions

AS and KS-S devised the project and guided data collection. MR-S, JW, and JB performed data selection and analysis. All authors contributed to writing and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

We would like to acknowledge Dr. Zillikens and his group for their tremendous impact on the field of autoimmune bullous diseases.

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 27 April 2023
DOI 10.3389/fimmu.2023.1157250

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OPEN ACCESS

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RECEIVED 02 February 2023
ACCEPTED 11 April 2023
PUBLISHED 27 April 2023

CITATION
Oren-Shabtai M, Mimouni D, Nosrati A, Atzmony L, Kaplan B, Barzilai A and Baum S (2023) Biological treatment for bullous pemphigoid.
Front. Immunol. 14:1157250.
doi: 10.3389/fimmu.2023.1157250

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Biological treatment for bullous pemphigoid

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Background: Bullous pemphigoid (BP) is the most common autoimmune subepidermal bullous disease. Topical or systemic corticosteroids are often used as the first-line treatment. However, long-term corticosteroid use may lead to significant side effects. Therefore, various adjuvant immunosuppressant therapies are used as steroid-sparing agents, with accumulating reports of biological treatments for severely recalcitrant BP.

Objective: To describe the clinical and immunological features of a series of patients with recalcitrant BP treated with immunobiological therapies. To assess the efficacy and safety of their therapies.

Methods: Patients receiving biological treatment for BP from two centers were assessed. Here, we described the clinical, immunopathological, and immunofluorescence findings of adult patients with BP and analyzed the clinical response and adverse events associated with various biological therapies.

Results: We identified nine eligible patients treated with rituximab (seven), omalizumab (three), or dupilumab (one). The mean age at diagnosis was 60.4 years, the average BP duration before biologic initiation was 1.9 years, and the average previous treatment failure was 2.11 therapies. The mean follow-up period from the first biological treatment to the last visit was 29.3 months. Satisfactory response, defined as clinical improvement, was achieved in 78% (7) of the patients, and total BP clearance was achieved in 55% (5) of the patients at the last follow-up visit. Additional rituximab courses improved the disease outcomes. No adverse events were reported.

Conclusions: Efficient and safe novel therapies can be considered in recalcitrant steroid-dependent BP non-responsive to conventional immunosuppressant therapies.

KEYWORDS

bullous pemphigoid, autoimmune subepidermal bullous disease, rituximab, dupilumab, omalizumab, corticosteroids, steroid-sparing immunomodulatory agents

1 Introduction

Bullous pemphigoid (BP) is the most common autoimmune blistering skin disease worldwide, typically affecting the elderly population (over 70 years). BP is chronic, accompanied by significant morbidities, clinically characterized by pruritic tense blisters imposed on normal skin or erythematous urticaria-like plaques, and is caused by circulating autoantibodies targeted at structural dermal-epidermal junction components, such as bullous pemphigoid antigen-1 (BP230) and bullous pemphigoid antigen-2 (collagen XVII, BP180) (1–3). Histologically, subepidermal separation is observed along with an inflammatory infiltrate, often containing eosinophils (4). Linear depositions of IgG +/- C3 are observed along the basement membrane using a direct immunofluorescence assay (5), and autoantibodies are detected in the serum of 60–80% of patients (6). In addition, an indirect fluorescence assay of patient serum with a normal human skin salt split (1 Mol NaCl) demonstrates autoantibodies binding to the epidermal side of the sample (5).

Systemic corticosteroids (SCS) are the mainstream BP therapy; however, their long-term use is associated with significant adverse effects, especially in the elderly (7, 8). Whole-body super-potent topical corticosteroids (TCS) application is effective in patients with BP without the morbidity and mortality associated with systemic administration (9, 10).

Patients who experience a resistant disease and are dependent on long-term steroid treatment require a steroid-sparing immunomodulatory agent, such as tetracycline, intravenous immunoglobulin (IVIG), nicotinamide or dapsone, or immunosuppressive medications (azathioprine, mycophenolate mofetil, cyclosporine, methotrexate, and cyclophosphamide) (11–14). Cumulative data on biological therapies, such as rituximab (RTX)and omalizumab, indicate their clinical benefits in patients with BP (15). In addition, emerging data on BP treatment with dupilumab (DUPI)indicate that it may be an additional immunomodulatory treatment that efficiently controls disease while maintaining a steroid-sparing effect, further expanding the limited existing armamentarium (16, 17).

The exact mechanism by which RTX, omalizumab, and DUPI induce clinical remission in BP has been previously postulated. RTX is a chimeric monoclonal antibody aimed at the CD20 surface protein expressed on B-cell, which produce pathogenic autoantibodies in BP. Immediate lysis of antibody-producing B-cells by effector cells leads to BP remission. During repopulation, nonpathogenic B-cells are generated, which may contribute to long-term clinical remission (18).

Omalizumab is a recombinant humanized anti-immunoglobulin E (IgE) antibody. IgE autoantibodies play an essential role in BP pathogenesis and contribute to tissue damage. IgE autoantibodies directed against the extracellular domain of BP180 bind to FcεRI on mast cells and eosinophils, causing degranulation and initiating an inflammatory cascade. The binding of specific anti-BP180 IgE to the ectodomain expressed on the basal cells leads to BP180 internalization, followed by adhesion loss and blister formation (19, 20).

DUPI is an IL-4 receptor alpha antagonist, and its efficacy is related to the Th2 inflammatory axis involved in BP pathogenesis. DUPI directly inhibits the activity of IL-4 and IL-13; however, it also downregulates eosinophil chemotaxis and inhibits preactivated B-cell proliferation (16, 21).

In the following case series, we described the clinical and immunopathological features of nine refractory BP patients treated with various biological therapies (RTX, Omalizumab, and DUPI). Additionally, responses to treatment and adverse effects are presented.

2 Materials and methods

2.1 Patients

Patients from two tertiary medical centers (Rabin and Sheba Medical Centers) in Israel were included in this study under the following criteria (22):

1. Clinical presentation: characteristic pruritic eruption composed of tense bullae and erosions on normal or erythematous skin.
2. Histological findings: lesional skin biopsy stained with hematoxylin-eosin demonstrating subepidermal blister formation and eosinophilic infiltrate.
3. Immunofluorescent assays: one or more of the following:

I. Direct immunofluorescence (DIF) of normal perilesional skin demonstrated linear IgG deposits with or without C3 along the basement membrane zone.

II. Indirect immunofluorescence (IIF) microscopy of patients' sera with 1 M NaCl-split normal human skin exhibiting IgG autoantibodies on the epidermal aspect of the separated skin.

Patients with other subepidermal blistering diseases were excluded. Patient medical records were reviewed to extract data regarding clinical response and adverse events.

2.2 Therapy

Patients were treated non-exclusively with various biologic therapies, including RTX, omalizumab, and DUPI, without any enforced washout period. Patients treated with intravenous (IV) RTX received either the rheumatoid arthritis protocol (2 infusions 1000 mg each, administered 2 weeks apart) or the modified lymphoma protocol (4 infusions of 375 mg/m^2 1 week apart), as in pemphigus vulgaris (23). Patients treated with subcutaneous (SC) omalizumab received 300 mg every 4 weeks (24). DUPI administered SC in the regimen approved for atopic dermatitis (AD): 600 mg SC initially, followed by 300 mg SC every other week (25) unless otherwise stated.

Clinical response was assessed using the degree of skin lesions and pruritus, according to the recommendations of an international

panel of experts for outcome measures in bullous pemphigoid (26). Disease control was achieved when new lesion formation ceased, established lesions began to heal, or pruritic symptoms began to abate. Complete remission (CR) on minimal therapy is the absence of new or established lesions or pruritus while the patient is receiving minimal therapy for at least 2 months (≤0.1 mg/kg/d of prednisone or minimal adjuvant or maintenance therapy). On the contrary, CR off therapy was without BP therapy for at least 2 months. Partial remission (PR) was defined as a transient new lesion occurrence that healed within 1 week of minimal or off therapy in the same manner. Relapse was defined as the appearance of three or more new lesions in 1 month or at least one large (>10 cm diameter) eczematous lesion or urticarial plaque that did not heal within 1 week in patients who achieved disease control.

3 Results

3.1 Index case

The 8th patient was a 75-year-old Caucasian male with known dyslipidemia treated with atorvastatin, recently diagnosed with a 6-mm nodular lesion using computed tomography (CT) scan, suspected of renal cell carcinoma. He presented with multiple extremely pruritic erythematous papules and patches (Figure 1A), followed by progressive bullae on the trunk and limbs (Figure 1B), which developed 4 months prior to his admission to our department. BP was diagnosed based on characteristic clinical features, consistent skin lesion biopsy and histopathological findings (subepidermal blistering with a dermal eosinophilic cell infiltrate), and positive DIF assessment of a perilesional sample (linear IgG and C3 deposition along the basement membrane zone). IIF was negative, yet serological examination *via* a biochip dermatology mosaic 7 (by EUROIMMUN medizinische labordiagnostika, Lübeck, Germany) indicated BP180 positivity on the salt-split normal human skin epidermis. At presentation, his serum IgE level increased to 235 IU/mL (normal range 0–100 IU/mL). The blood eosinophil count was normal, yet it increased later to 3.5 K/μL (normal range 0–0.8 K/μL).

The patient did not respond adequately to twice-daily high-potency TCS whole-body application and oral antihistamines. Thus, he was administered 1 mg/kg prednisone (80 mg/day), with satisfactory clinical response and pruritus alleviation. Nonetheless, the patient repeatedly flared while several SCS tapering attempts were made. After 3 years of intermittently SCS courses, he was diagnosed with iatrogenic osteoporosis, necessitating bisphosphonate treatment and a corticosteroid-sparing agent. The patient responded poorly or suffered from side effects due to prior standard treatment regimens, including doxycycline (100 mg twice daily for 6 months), yielding no efficacy, dapsone (up to 100 mg/day for 6 months), causing significant hemolytic anemia, and non-responsiveness to a lower dose of 50 mg/day and an inefficient azathioprine trial (up to 200 mg/day for 9 months). Therefore, omalizumab was administered in the aforementioned regimen, with no response after 2 months of therapy, and thus ceased.

RTX treatment was administered to achieve disease control. An uro-oncology consultation, based on a repeated CT scan, concluded that there was no contraindication for treatment and that stable solid malignancy requires monitoring only. The patient received his first RTX course at the rheumatological regimen one year after his BP diagnosis while on <0.5 mg/kg/day prednisone. Subsequently, at the 3-month follow-up visit, he reported transient lesions with a minimal itch, and thus concluded as a PR on minimal therapy (prednisone 7.5 mg). Six months after the first course, he experienced a severe relapse, with a disseminated bullous rash and intractable pruritus. A second RTX course was administered, which was soon followed by a good clinical outcome of PR on minimal therapy (prednisone 7.5 mg). After a long remission period of 14 months, another relapse occurred. The third RTX course was administered with complete clearance of skin lesions and no

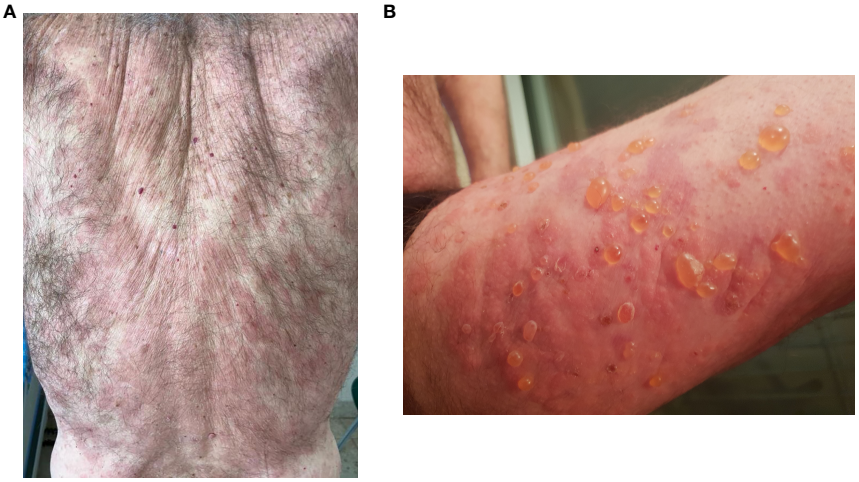

FIGURE 1
Bullous pemphigoid before rituximab in the index case, the 8th patient. **(A)** Extensive erythematous patches and plaques on the back. **(B)** Tense bullae on erythematous skin on the right thigh.

pruritus. Thus, CR was concluded on minimal therapy (prednisone 5 mg). Three months later, the patient maintained this condition (Figures 2A, B).

3.2 Patient characteristics

Nine patients with confirmed BP diagnosis, according to the clinical and immunopathological features stated previously, were treated with novel biological therapies and included in this case series. The patient demographics and clinical features are outlined in Table 1. The mean age of the patients at diagnosis was 60.4 years. None were treated with dipeptidyl peptidase-4 inhibitor (DPP4i).The seventh patient in the series had BP secondary to pembrolizumab treatment due to lung adenocarcinoma, which was crucial and could not be ceased. Other patients received drugs associated with BP. However, the timeline did not indicate it as a possible trigger or exacerbating factor (the third and seventh patients received diuretics, and the fourth received angiotensin-converting-enzyme inhibitors).

Notably, some patients exhibited distinct characteristics. The 5th patient was diagnosed with Noonan syndrome and presented with erythroderma followed by a pruritic disseminated bullous rash diagnosed as BP at the age of 19. However, no association between Noonan syndrome and BP has been reported.

The 9th patient was diagnosed with leaky severe combined immunodeficiecy syndrome due to heterozygous BCL11B mutation. His clinical presentation included marphanoid features, severe asthma (leading to chronic steroid dependence causing iatrogenic diabetes mellitus and osteoporosis), AD, elevated IgE levels (8000 IU/mL), developmental delay, and recurrent skin and respiratory tract infections. He was presented at our department, at 38 years, with a new onset disseminated bullous rash that occurred while he was undergoing SCS treatment, consisting of figurate oval erythematous plaques with tense bullae along the surrounding border. Histology and direct and indirect immunofluorescence analysis were consistent with BP. BCL11B is related to a neurodevelopmental disorder, severe combined immunodeficiency, and AD-like skin inflammation phenotype (27, 28). To date, no known associations with BP have been reported.

3.3 Response to therapy

The therapeutic characteristics of patients are presented in Table 2. All the patients had refractory diseases which failed to respond to numerous conventional therapies (an average of 2.11 therapies). The average BP duration before biological therapy initiation was 1.9 years.

Seven patients received at least one RTX course. Five patients received two RTX courses, and the remaining two received three. Omalizumab was administered to 3 patients, and 1 received DUPI every other week, followed by once weekly administration due to poor response of his asthma and AD. Satisfactory response to biological therapy, implying clinical improvement, was achieved in 78% (seven of nine) of patients at the first 3-month follow-up visit. Three patients maintained their clinical outcome up to the last follow-up visit, while three improved their clinical response, with additional RTX courses administered at that time interval. The last patient with a satisfactory response was the ninth, who maintained a good response on weekly DUPI. However, this regimen was changed due to poor control of his prior steroid-dependent asthma (see below). During the last visit, seven patients achieved a satisfactory response.

Two patients were defined as non-responders at the first 3-month follow-up visit, consisting of the 5th and 6th patients who received a rheumatological regimen of RTX. The 6th patient did not respond to RTX or omalizumab, and yet, was considered to achieve CR off therapy at the last follow-up visit, as prurigo nodularis lesions were observed, with no signs of bullae or erythematous/eczematous plaques. Additionally, histological and DIF analyses were negative for BP. The 8th patient (*index case*) received omalizumab for 2 months without response. Thus, RTX treatment was applied with significant clinical improvement (*the data in* Table 2 *solely refer to the RTX treatment*).

The mean follow-up period from the first biological treatment to the last recorded visit was 29.3 months. Notably, on evaluating the last visit, 5 patients achieved CR (55%), of which, 4 (44%) achieved CR off therapy, all treated with RTX (4 of 7 patients, 57%). In addition, one patient received a single course, and two received two courses. The sixth patient was treated with omalizumab after a lack of response to the first RTX course (see above, the fifth patient).

FIGURE 2
Clinical remission of the bullous pemphigoid three months after the third rituximab infusion in the index case, the 8th patient. No evident skin lesions on the **(A)** front torso or **(B)** back torso.

TABLE 1 Demographic, clinical, and immunopathological characteristics of patients.

Patient	Sex	Age (years)	Comorbidities	Age at diagnosis (years)	Cutaneous presentation	Mucosal involvement	Diagnosis confirmation
1	M	84	Parkinson, dementia, HTN, BPH	81	Multiple pruritic bullae on the limbs	None	Histology, DIF
2	F	62	Hypothyroidism	57	Multiple pruritic bullae on the trunk and limbs	None	Histology, DIF
3	M	61	Heart failure, DM, HTN, renal failure	52	Multiple pruritic bullae on the face, trunk, and limbs	None	Histology, DIF, IIF
4	F	89	DM, HTN, hypothyroidism, dyslipidemia	79	Multiple pruritic bullae on the scalp, trunk, limbs, and palms	None	Histology, DIF
5	M	22	Noonan syndrome	19	Near erythroderma followed by multiple pruritic bullae on the trunk and limbs		Histology, DIF
6	F	70	Hypothyroidism, depression, and anxiety disorder	62	Multiple pruritic, erythematous papules on the trunk and limbs followed by bullae formation	None	Histology, DIF, IIF
7	F	81	HTN, DM, dyslipidemia, hypothyroidism; Adenocarcinoma of the lung (treated with Pembrolizumab); Chronic urticaria (treated with Omalizumab)	81	Multiple pruritic bullae on the trunk and limbs	Oral and genital	Histology, DIF
8 *Index case*	M	78	Dyslipidemia; Renal cell carcinoma (*requiring follow-up only*)	75	Multiple pruritic, erythematous papules and patches followed by bullae formation on the trunk and limbs	None	Histology, DIF
9	M	42	BCL11B mutation - severe asthma, atopic dermatitis, IgE ~ 8000 IU/mL, developmental delay, recurrent infections. Iatrogenic DM and osteoporosis (steroid dependent).	38	Multiple pruritic bullae on the trunk and limbs	None	Histology, DIF, IIF

DIF, direct immunofluorescence; IIF, Indirect immunofluorescence; HTN, hypertension; BPH, benign prostatic hypertrophy; DM, diabetes mellitus.

Only one patient received RTX as a hematological regimen. Therefore, it was impossible to establish a difference in the effectiveness between both regimens with regard to BP. In 3 patients, additional RTX courses improved the clinical outcome; the 2nd and 4th patients experienced CR on minimal therapy at the 3-month follow-up visit after the first RTX course and were concluded to have CR off therapy by the end of the follow-up after the second course. The 8th patient attained PR on minimal therapy (prednisone 7.5 mg) after the 1st and 2nd RTX courses. After the third course, he achieved CR. Consequently, his daily prednisone dose was lowered to 5 mg.

Two patients required adjuvant therapy with mycophenolate mofetil (MMF) 2 g/day. The 3rd patient was also administered 5 mg prednisone daily, attaining PR on therapy. The fifth patient remained a non-responder. At the last visit, 2 other patients were treated solely with a residual 5 mg of prednisone. The eighth patient *(index case)* treated with omalizumab, followed by three courses of RTX, achieved CR on minimal therapy. Additionally, the seventh patient suffered from chronic urticaria a decade prior to her current BP diagnosis. She was treated with omalizumab for 5 months after the urticarial rash ceased. Soon after the first treatment with pembrolizumab, she developed pruritus followed by a disseminated bullous rash involving the oral and genital mucosal surfaces. SCS provided temporary relief, and doxycycline 100 mg twice daily for 6 months led to minor improvement, yet the pruritus persisted. On the second omalizumab dose, she experienced a significant improvement in pruritus, and on the last visit, she described minor palmoplantar itch, treated with an additional antihistamine, and concluded as PR on minimal therapy. The patient is still receiving lifesaving pembrolizumab therapy, and omalizumab allows her to maintain a good quality of life with minimal pruritus and no blistering.

The ninth patient suffered from the complexity of severe uncontrolled steroid-dependent asthma, recurrent AD, and BP flares, with no treatment thus far to adequately control the three conditions. He was initially treated with 300 mg of DUPI once weekly. Frequent administration was recommended due to the poorly controlled asthma for 5 months, with BP partial remission. Nonetheless, AD and asthma exacerbations led to treatment cessation. Subsequently, the patient received 15 mg of

TABLE 2 Therapeutic characteristics of patients.

Patient	Initial prednisone dose (mg) [Maximal at diagnosis]	Previous systemic BP therapies (other than corticosteroids)	Time from BP diagnosis to biological therapy initiation (years)	Biological therapeutic agent for BP	Biological therapy dosing*	Treatment response at 3-month Follow-up	Time from 1st treatment to relapse and 2nd treatment (months)	2nd treatment response at 3-month Follow-up	Time from 2nd treatment to relapse and 3rd RTX treatment (months)	3rd treatment response/ clinical status at the last visit	FU time from diagnosis to last visit (years)	FU time from 1st biological treatment to last visit (months)
1	30	MINO, DOXY, MMF	0	RTX	Rheumatological	CR off therapy				CR off therapy	0.89	2.67
2	40	MINO, MMF	2	RTX	Rheumatological	PR on minimal therapy (*prednisone 5 mg*)	1.76	CR on minimal therapy (*prednisone 2.5 mg*)		CR off therapy	3.72	26.03
3	100	MINO, MMF	2	RTX	Rheumatological	PR on therapy (*prednisone 20 mg*)	12.17	PR on minimal therapy (*prednisone 10 mg*)	22.3	PR on therapy (*prednisone 5 mg and MMF 2g*)	7.18	70.10
4	40	MINO, MTX	1	RTX	Hematological	PR on minimal therapy (*prednisone 5 mg*)	48.70	CR on minimal therapy (*prednisone 5 mg*)		CR off therapy	8.46	89.77
5	50	MINO, DOXY	3	RTX	Rheumatological	NR				NR (*MMF 2g*)	3.47	3.47
6	40	MTX, MMF, MINO	4	RTX > Omalizumab	Rheumatological	NR	7.13 **	NR		CR off therapy; Prurigo nodularis lesions were seen at the last FU visit. Histology and DIF were negative for BP	7.49	42.43
7	30	DOXY	0	Omalizumab	300 mg every 4 weeks	PR on minimal therapy (prednisone 5 mg)				PR on minimal therapy (*prednisone 5mg*)	0.73	4.87
8 *Index case*	80	DOXY, dapsone, azathioprine	1	Omalizumab > RTX	Rheumatological	PR on minimal therapy	6.07	PR on minimal therapy	14.2	CR on minimal therapy (*prednisone 5mg*)	3.16	21.13

(Continued)

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TABLE 2 Continued

Patient	Initial prednisone dose (mg) [Maximal at diagnosis]	Previous systemic BP therapies (other than corticosteroids)	Time from BP diagnosis to biological therapy initiation (years)	Biological therapeutic agent for BP	Biological therapy dosing*	Treatment response at 3-month Follow-up	Time from 1st treatment to relapse and 2nd treatment (months)	2nd treatment response at 3-month Follow-up	Time from 2nd treatment to relapse and 3rd RTX treatment (months)	3rd treatment response/ clinical status at the last visit	FU time from diagnosis to last visit (years)	FU time from 1st biological treatment to last visit (months)
						(prednisone 7.5 mg)		*(prednisone 7.5 mg)*				
9	60	DOXY	4	DUPI	600 mg initially followed by 300 mg weekly	PR on therapy *(prednisone 20 mg, due to steroid-dependent asthma)*	No BP relapse			Treated with 300 mg DUPI weekly for 5 months with relatively good BP control, yet AD and asthma exacerbations led to treatment cessation. Patient received 15 mg of Upadacitinib, leading to AD alleviation. One month later, DUPI 300 mg bimonthly was added due to poor respiratory control. After 3 months, on the last visit, his BP flared.	4.10	3.20

*RTX dosing - rheumatoid arthritis protocol (two 1000 mg infusions each, administered 2 weeks apart) or the modified lymphoma protocol (four infusions of 375 mg/m^2 1 week apart).
**Initiation of Omalizumab 300 mg every 4 weeks.
MINO, minocycline; DOXY, doxycycline; CR, complete remission; MMF, mycophenolate mofetil; PR, partial remission; MTX, methotrexate; NR, no response; RTX, rituximab; FU, follow-up; DUPI, dupilumab; AD, atopic dermatitis.

10.3389/fimmu.2023.1157250

upadacitinib (for 6 months), leading to AD alleviation. One month later, DUPI 300 mg bimonthly was added to achieve respiratory control. Unfortunately, 3 months later, at the last follow-up visit, his BP flared. Notably, the 1st course of DUPI, administered at 300 mg weekly, maintained his severe BP under partial remission. Nonetheless, the asthma was unstable and required a therapeutic change.

Looking at clinical outcomes according to biological treatment, RTX was given to 7 patients (cases 1-6 and 8), among which by the last visit, 3 achieved CR off therapy and one on minimal therapy, two patients required adjuvant therapy with MMF and one did not respond at all, leading to a trial of omalizumab. Among the 3 patients receiving omalizumab (cases 6-8), one maintained CR off therapy at the last visit, and the other PR on minimal therapy, while the third did not respond, thus a RTX course was attempted. DUPI was given to one patient only, concluded to relapse by the last follow-up visit.

The medical records of all the patients were reviewed for adverse events. No adverse events were reported, including infections or allergic/drug reactions. None of the patients discontinued the treatment due to adverse reactions.

4 Discussion

This case series describes nine patients diagnosed with recalcitrant BP, necessitating an efficient steroid-sparing agent. Of these, seven patients received RTX treatment. Three were treated with omalizumab and one with DUPI. Satisfactory response, with good control of pruritus and bullae formation, was achieved in 78% (7 of 9) of patients at the first 3-month follow-up visit. At the last follow-up, CR was achieved in 55% of patients (5 of 9), of which, 4 were off therapy, 3 were treated with RTX, and 1 with omalizumab. Additional RTX courses improved the clinical outcomes and enabled further prednisone tapering. Only 1 patient was treated with DUPI 300 mg weekly and achieved good disease control through a 5-month course. The biological treatments were well-tolerated with no reported adverse events.

Recently, Cao et al. evaluated the treatment outcomes of RTX, omalizumab, and DUPI for BP *via* a systematic review of 75 publications, including 211 patients. The mean age of the patients was 68. RTX (122 patients), omalizumab (53 patients), and DUPI (36 patients) had similar clinical benefits in treating BP, with complete remission rates of 70.5%, 67.9%, and 66.7%, respectively. The recurrence rate in the RTX group was higher than that in the omalizumab and DUPI groups. However, the disease duration before treatment initiation was longer. The absence of adverse events in the DUPI group was higher than that in the omalizumab and RTX groups. However, it was similar between the omalizumab and RTX groups (29). This was consistent with a previous systematic review by Kremer et al., which indicated a similar adverse event rate between the two therapeutic agents of 20% and 24%, respectively (15). The main adverse event in RTX treatment was infection (6.6%, 8 of 122), probably related to the mechanism of depleting the B-cell population expressing CD20. Infections were not reported in the other treatment groups; however, the RTX group was significantly larger than the omalizumab or DUPI groups, and the mean age of patients in each group was not stated (29).

Previous studies have indicated RTX as a promising treatment for BP with increased remission rates and steroid-sparing activity (30, 31). Furthermore, Polansky et al. reported that 75% of patients achieved a durable remission after RTX treatment with fewer adverse events and infections after taking RTX than before, suggesting RTX as an alternative treatment for recalcitrant bullous pemphigoid (32).

Omalizumab directly interferes with IgE binding to the cell surface, FcεRI, leading to BP remission. A subset of patients responds to omalizumab therapy, and biomarkers for predicting good clinical outcomes are currently under investigation (19). The total IgE level correlated with disease severity, yet inconclusively. Furthermore, patients may respond to omalizumab without significantly increasing IgE levels (33). Our index case had mildly elevated IgE levels, though specific anti-BP180 IgE levels were not examined. The patient did not respond to omalizumab. Nonetheless, it might have been too early to conclude, as he was treated for 2 months, with previous reports indicating good outcomes after 4 months of treatment (19) and a mean time to remission of 6.6 months, according to the previously mentioned systematic review (29).

Interestingly, DUPI may improve pruritus by decreasing peripheral itch sensory neuron signaling through its inhibitory effect on IL-4, IL-13, and eosinophils, leading to decreased IL-31 secretion (34).

The seventh patient in this series developed BP secondary to immunotherapy. Cutaneous adverse reaction related with PD-1/PD-L1 inhibitors are common and being increasingly reported. Lopez et al. further characterized immunotherapy-induced BP, stating that pruritus and non-bullous cutaneous findings may be the only symptoms, necessitating awareness and prompt immunofluorescence assays to establish the diagnosis (35).

The most effective treatment of immunotherapy-induced BP has yet to be established. The primary goal is to continue with the immunotherapy treatment and to improve the cutaneous symptoms while enabling a good quality of life. New lesions can appear for months following PD-1/PD-L1 cessation. In the same report by Lopez *at al.*, development of BP required discontinuation of immunotherapy in 76% (16/21) of cases, while the remaining five patients continued the immunotherapy with an additional designated BP treatment. In most cases SCS were the main component of the treatment regimen (35). Muntyanu et al. review the management strategies and recommendations for cutaneous immune-related adverse events of immune checkpoint inhibitors, suggesting starting with whole body application of topical potent corticosteroids twice daily, followed by SCS (0.5-1 mg/kg/day prednisone) intermittently for short periods as possible (36). The optional efficacy reduction of immunotherapy by a concurrent treatment with SCS was postulated, yet not established and further investigation is needed (37).

Other treatment options include dapsone, methotrexate and IVIG. Azathioprine, MMF and cyclosporine should be avoided due to an immunosuppressive effect (36, 38). Antibiotics, such as tetracyclines, should be used with caution. A possible impairment of immunotherapy's efficiency by an 1-2-months antibiotic course

prior to the treatment initiation was postulated (39). At Lopez et al. review five patients were treated with doxycycline with variable cancer outcome, without a causal connection that can be established between the antibiotics and disease progression (35).

Biological therapies, RTX and omalizumab, are also among the treatment recommendations (36, 38). Several refractory immunotherapy-induced BP cases were treated with RTX, experiencing clinical improvement with no established detrimental effect on the malignancy, and even some achieved oncological remission (40–42). Rituximab has been shown to be safe in some malignancies and can act synergistically to other chemotherapeutic agents (43), yet this requires further investigation to a wide range of solid malignancies and combination with immunotherapy specifically. Omalizumab was efficacious as a steroid-sparing agent in a case of a nivolumab-induced BP and was preferred due to evidence of elevated IgE levels. The malignancy outcome is unknown (44).

The limitations of this study include its retrospective nature, small sample size, and the lack of a control group or standardized clinical assessment tool. The mean age of the patients at diagnosis was slightly lower than that reported for BP, and the patients had recalcitrant BP. The group was heterogenic in its medical background, exhibiting unique syndromes and drug associations, which could affect the response to therapies, especially in BCL11B mutation with an atopic predilection, treated with DUPI. Finally, the patients were treated with three biological therapies, which limited the ability to discuss the actual efficacy of each therapeutic agent.

Our study suggests that treatment with biological therapies in severe recalcitrant BP can lead to a satisfactory clinical response, which is significant in persistent cases. Future research should focus on more extensive prospective, randomized controlled trials to evaluate the individual effectiveness and safety of the biological treatment to expand the treatment armamentarium for this disabling disease.

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

Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

Author contributions

DM and SB conceived of the presented idea for this case series. MO-S and AN extracted data from the medical registries and MO-S analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

We wish to honor dear Professor Detlef Zillikens who was a truly remarkable person with a profound impact on our academic pursuits and the field of autoimmune bullous diseases. We are deeply grateful for the time spent learning from him and his passion for teaching, through his unique way of making every student or colleague feel welcome and valued. We will always remember him as a kind and generous person, and we will be forever thankful for the opportunity to learn from such an honorable professor.

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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frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 27 April 2023
DOI 10.3389/fimmu.2023.1169947

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OPEN ACCESS

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RECEIVED 20 February 2023
ACCEPTED 10 April 2023
PUBLISHED 27 April 2023

CITATION
Emtenani S, Hertl M, Schmidt E and Hudemann C (2023) Mouse models of pemphigus: valuable tools to investigate pathomechanisms and novel therapeutic interventions.
Front. Immunol. 14:1169947.
doi: 10.3389/fimmu.2023.1169947

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Mouse models of pemphigus: valuable tools to investigate pathomechanisms and novel therapeutic interventions

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Autoimmune blistering diseases (AIBD) are paradigms of autoantibody-mediated organ-specific autoimmune disorders that involve skin and/or mucous membranes. Compared to other autoimmune diseases, the pathogenicity of autoantibodies in AIBD is relatively well described. Pemphigus is a potentially lethal autoantibody driven autoimmune disorder with a strong HLA class II association. It is mainly characterized by IgG against the desmosomal adhesion molecules desmoglein 3 (Dsg3) and Dsg1. Several murine pemphigus models were developed subsequently, each allowing the analysis of a characteristic feature, such as pathogenic IgG or Dsg3-specific T or B cells. Thus, the models can be employed to preclinically evaluate potentially novel therapies. We here thoroughly summarize past and recent efforts in developing and utilizing pemphigus mouse models for pathomechanistic investigation and therapeutic interventions.

KEYWORDS

pemphigus, desmoglein, autoantibody, mouse model, autoimme diseases

Introduction

Pemphigus is a severe autoimmune blistering skin disease characterized by disruption of desmosomes, thereby affecting the epidermis of the skin and surface-close mucosal epithelia (1, 2). Depending on their immunohistological manifestations, three major forms of pemphigus are distinguished. The mucocutaneous pemphigus vulgaris (PV) presents in both mucosal epithelia and the epidermis and is characterized by IgG autoantibodies (autoabs) directed against desmoglein 1 (Dsg1) and Dsg3 antigens, which were identified over three decades ago (3). Pemphigus foliaceus (PF) is triggered by anti-Dsg1 IgG leading to intraepidermal blistering limited to the skin. Paraneoplastic pemphigus (PNP) is

characterized by polymorphic mucocutaneous eruptions and originates from a pool of auto-abs mainly against plakin proteins such as envoplakin, periplakin, desmoplakins, epiplakin, desmocollins, and BP230 as well as against α2 macroglobulin-like 1 and Dsg (4, 5). In addition to Dsg3 and/or Dsg1 auto-abs, IgG auto-abs against several target proteins other than Dsgs such as desmocollin 3 were identified in pemphigus patients, raising speculations about potential synergic effects eventually triggering acantholysis (6, 7).

Autoreactive B cells are key players in the production of pathogenic antigen-specific IgG in specific organs such as the skin (8). Pathogenic auto-abs can be generated after clonal expansion of autoreactive B cells in secondary lymphoid organs, but also in the skin (9). During T-dependent and T-independent responses to Dsg3, Dsg3-specific memory B cells are generated which can remain in immunological niches and become reactivated (10). In remission following immunosuppressive therapy, these cells might potentially lead to reencounter of the clinical symptoms (11). The critical role of auto-abs in pemphigus pathogenesis is supported by the observations that i.) the level of IgG auto-abs frequently correlates with the severity of the disease (12), ii.) pemphigus-associated blister formation can be induced by passive transfer of IgG from pemphigus patients into neonatal mice (13), and iii.) transplacental transmission of pemphigus IgG antibodies from diseased mothers to neonates leading to temporal neonatal pemphigus (14).

Despite the direct pathogenic role of B cells in pemphigus, recent studies shed light on the so far largely neglected autoimmune block, the T cells. While several T-cell subsets with a strong Th2 and Th17 polarization are undoubtedly involved in the pathogenesis of pemphigus (15, 16), identifying immunodominant self-peptides and characterization of autoreactive T cells is still challenging. Indirect characterization via activation markers such as CD154 presents as a reliable method to detect antigen-reactive T cells, while other methods such as ELISPOT or incorporation of ^{3}H-thymidine are unable to distinguish specific cell populations (^{3}H-T, ELISPOT) or focus on a single cytokine secreted by activated T cells (ELISPOT) (17). With the advent of MHC class II–peptide technology a more comprehensive understanding of antigen-specific T cells in the immune pathogenesis of several HLA class II–linked autoimmune disorders is now achievable (18).

Considering the potential individual contributions of loss of tolerance, arising antigen-specific T and B cells as well as antigen-specific IgG as key elements of pemphigus pathogenesis, we next summarize the development of pemphigus-related mouse models focusing on those different aspects of the disease with each of the advantages and disadvantages (Table 1).

Animal models of pemphigus

Depending on the desired complexity of parameter induction, pemphigus-associated blister research brought forth different mouse models of pemphigus during the last three decades. *In vivo* models of pemphigus are classified as follows (Figure 1) (19, 28, 29):

1. Antibody transfer-induced ("passive") models based on the injection of pemphigus auto-abs into mice to reproduce a transient disease phenotype
2. Lymphocyte transfer-induced disease models based on the transfer of autoreactive lymphocytes into mice, using transgenic models that induce a complex auto-ab-driven clinical phenotype
3. Antigen-specific immunization of mice to induce B and T cell-specific autoimmune responses including induction of pathogenic auto-abs

TABLE 1 Advantages and disadvantages of pemphigus mouse models.

Model	Mice	Advantages	Disadvantages	References
Antibody transfer-induced ("passive")	Neonatal mice	• Faster and easier completion time • Less antibody required to induce disease • Reduction of animal usage in research	• Inability to follow up for a long period of time • Inability to produce their own auto-abs • No finalized epidermal morphogenesis • Does not allow to study of lesions in mature hair follicles and stem cell niche • No possibility of applying a systemic therapeutic approach	(13, 19, 20)
	Adult mice	• Better resembling the clinical situation • Drug screening in a therapeutic approach	• Requirement of higher amounts of antibodies to induce disease • No possibility of evaluating the role and targeted therapy of autoreactive T cells or B cells	(19, 21)
	SCID	• Investigation of autoantibody-induced pathology in human tissue	• Labor-intensive and time-consuming • Complex surgical intervention • Immunological differences between human and mouse • No further validation	(22, 23)
Lymphocyte transfer-induced	Adult mice	• Solid inducible phenotype • 10^{7} splenocytes/transfer allows a reasonable amount of recipient mice	• Artificial system due to using immune-deficient Rag2$^{-/-}$ recipient mice	(24, 25)
Immunization-induced	Adult mice	• Active antigen-specific immune modulation • Possibility of immune interventions in both preventive and therapeutic setups	• While antigen-specific IgG total titers can be affected, no clinical phenotype is inducible so far	(26, 27)

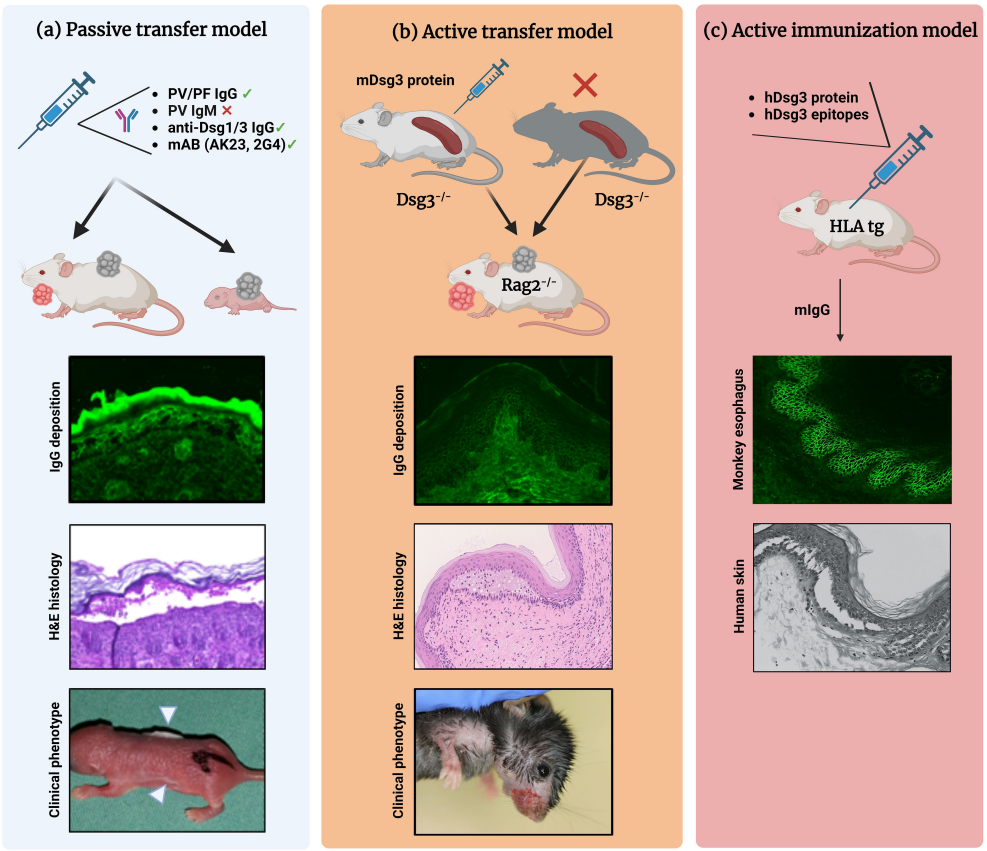

FIGURE 1
Different mouse models of pemphigus. **(A)** Antibody transfer-induced ("passive") models. Total pemphigus vulgaris (PV) or pemphigus foliaceus (PF) IgG fractions, anti-desmoglein (Dsg) 1/3 IgG isolated from patients' sera, or monoclonal anti-Dsg3 antibodies (e.g., AK23, 2G4) are injected into adult or neonatal mice leading to blister formation. Direct immunofluorescence (IF) microscopy, hematoxylin and eosin (H&E)-stained histology, and clinical presentation (white arrowheads indicate the blister sites) of a neonatal mouse injected with purified IgG from a patient with PV. **(B)** Lymphocyte transfer-induced models. Following adoptive transfer of naive lymphocytes from $Dsg3^{-/-}$ mice or splenocytes from $Dsg3^{-/-}$ mice immunized with recombinant Dsg3 protein, $Rag2^{-/-}$ immunodeficient mice produce anti-Dsg3 IgG antibodies and display the PV phenotype, allowing us to study the loss of tolerance. **(C)** Immunization-induced models. Immunization of humanized HLA-DBR1*04:02 transgenic mice with human Dsg3 recapitulates the effector phase of T and B cells followed by the formation of pathogenic Dsg3-specific IgG formation. Binding of the murine IgG from immunized mice to the keratinocyte cell surface was confirmed by indirect IF staining on monkey esophagus. Injection into human skin with murine IgG resulted in intraepidermal acantholysis and typical pathology. Figure was created with BioRender.com.

Antibody transfer-induced ("passive") mouse models

Transfer of auto-abs into neonatal mice

The direct pathogenicity of pemphigus auto-abs has been confirmed by their passive transfer into mice. The first experimental model for pemphigus was the passive transfer model described by Anhalt and coworkers in 1982 (13). Intraperitoneal (i.p.) administration of IgG from sera of PV patients at doses of 1.5 to 16 mg/g/day into neonatal BALB/c mice resulted in cutaneous blisters and erosions with clinical, histological, ultrastructural, and immunological features of human pathology. In the course of 18-72 h following IgG administration, mice developed skin lesions in a dose- and titer-dependent manner. Continuous IgG administration led to the development of new erosions and crusts; however, skin lesions healed when the IgG injections were discontinued. This model further allowed mechanistical investigations of of auto-ab-induced acantholysis in pemphigus. In a time-course study, the ultrastructural immunological changes in the epidermis of mice injected with PV IgG were examined by electron microscopy (30). Early detachment of the epidermis was observed 1 h post IgG injection as a widening of the interdesmosomal spaces, followed by splitting of the desmosomes and complete cell detachment in the suprabasal epidermal layers within 6 h. Of note, basal cells remained unaffected in a tombstone-like pattern. Anti-Dsg3 auto-abs purified from PNP sera were also pathogenic and caused blistering in neonatal mice (31).

The principle of disease induction by passive transfer of pathogenic PV IgG into neonatal mice has also been successfully

applied to other pemphigus variants. Roscoe et al., in 1985 published the first *in vivo* study on the pathogenic role of anti-Dsg1 antibodies (32). When injected into neonatal mice, PF IgG (at 10 mg/g/day for a total of four injections) induced superficial skin blisters reproducing the clinical and immunopathological findings of human disease. Similar to the PV model (13), there was a close correlation between disease severity and the amount of injected IgG. In contrast to PV IgG, PF IgG did not cause suprabasal acantholysis in the epidermis but rather a superficial loss of epidermal adhesion, suggesting that PV and PF are mediated by different auto-abs specificities. Further studies showed that the pathogenic auto-abs in pemphigus are predominantly of the IgG4 subclass (33, 34). In contrast, some PF patients only had IgG1 auto-abs, which caused acantholysis in mice (35).

Autoantibodies alone cause skin pathology in pemphigus

Unlike the pemphigoids, IgG-induced acantholysis in pemphigus is independent of complement activation or Fc-effector function. In an experimental passive transfer model, i.p. injection of intact pathogenic IgG and F(ab′)2 from PV sera led to the same extensive blistering, while the latter failed to induce C3 deposition. In addition, blistering was observed in C5-deficient or cobra venom factor (a structural analog of complement component C3) pretreated neonatal mice following administration of PV IgG (36). Hence, it was speculated that crosslinking of PV antigen on the surface of keratinocytes by bivalent PV auto-abs may be a necessary step for acantholysis.

Another study showed that subcutaneous (s.c.) injection of F(ab′)2 and Fab fragments caused blistering in mice (37). Pemphigus-like lesions have also been induced in neonatal mice by passive transfer of F(ab′)2 and Fab′ fragments purified from PF IgG (33).

These early studies relied predominantly on polyclonal IgG or its cleavage products. Although patient's IgG is certainly an excellent tool for investigation of pemphigus pathomechanisms, its limited availability and heterogeneity over the course of the disease and between individuals may complicate systematic studies (38, 39). Monoclonal antibodies (mAbs), developed by immunization of mice with the recombinant mouse Dsg3 ectodomain, such as AK23, or isolated by phage display from active pemphigus patients, are now being extensively used to model PV pathogenesis (40–44). Payne et al. cloned anti-Dsg3 mAbs as single-chain variable fragments (scFvs) from a mucocutaneous PV patient using antibody phage display, which induced typical PV lesions in neonatal mice (40). Using the same technique, Ishii et al., isolated anti-Dsg1 scFv mAbs from a PF patient that were pathogenic in mouse and human skin (44). While most pathogenic Dsg3-related mAbs bind to the membrane distal EC-1/2 regions of the Dsg3 ectodomain, a novel Dsg3-EC5 binding mAb 2G4 allows the characterization of membrane proximal pathogenic binding (45). Additionally, experimental pemphigus can also be induced by Dsg3-hybridoma cell lines. Passive transfer of AK23-IgG hybridoma derived from splenocytes of the PV model caused blistering in neonatal mice (41). The practical advantage of hybridoma technology is that once stable somatic cell lines are established, they can be used to produce sensitive and specific mAbs in unlimited quantities. Furthermore, this technique preserves the native pairing of antibody variable and constant regions (46). Despite these advantages, hybridoma cells are limited by long generation times and the difficulty of controlling the epitopes against which antibodies are produced. Immunoadsorption of pathologic auto-abs from PV sera using the entire EC domains of Dsg1 and Dsg3 abolished the blister-inducing ability of IgG fractions in mice, suggesting that anti-Dsg1/3 IgG alone is pathogenic; other factors such as additional non-desmoglein PV auto-abs alone or in combination are not required (47, 48). In line with these observations, adjuvant immunoadsorption has been successfully applied in patients with severe PV and PF to efficiently reduce circulating pathogenic auto-abs (49–51).

The possible involvement of IgM in the pathogenesis of PV has also been investigated. In experimental PV, in contrast to AK23 IgG, administration of its AK23-IgM hybridoma cells isotype did not induce clinically overt blisters (52). As monovalent (Fab), bivalent (F(ab')2), or scFv fragments of anti-Dsg auto-abs induce keratinocyte dissociation *in vitro* and *in vivo*, the Fc portion of pathogenic pemphigus auto-abs is probably of minor significance (53).

Taken together, these results demonstrate that Fc-mediated mechanisms are not required for blister formation in PV and PF. Accordingly, research in pemphigus has focused on characterizing anti-Dsg B cells and antibodies as well as signaling pathways that modulate the pathogenic effects of auto-abs.

Desmoglein compensation model explains the localization of lesions

Dsg1 or Dsg3 can, at least in part, compensate for the adhesive functions of each other (1, 54). The desmoglein compensation model has been supported by several experimental studies. Passive transfer of PF IgG to neonatal mice caused loss of adhesion in the superficial epidermis (55). Consistent with this finding, exfoliative toxins produced by *Staphylococcus aureus* specifically cleave Dsg1, resulting in blistering just below the stratum corneum of neonatal mice (56, 57). On the other hand, forced superficial expression of Dsg3 in transgenic mice protected them from PF antibody-induced blistering. Another proof comes from the passive transfer of pemphigus IgG to normal and neonatal Dsg3^{null} mice. Basically, Dsg3^{null} neonatal mice do not present skin blisters because Dsg1 is present throughout the epidermis to compensate for the genetic loss of Dsg3. However, challenging these mice with anti-Dsg1 IgG led to severe PV-like blistering similar to cutaneous PV patients (54, 58). Additionally, the telogen hair sheds early in Dsg3$^{-/-}$ mice leading to an alopecia areata-like phenotype, whereas transgenic Dsg1 over-expression in the telogen club leads to a decreased or delayed balding phenotype (59).

Emerging novel therapeutic targets for pemphigus

Targeting signaling pathways

Beyond steric hindrance, signaling pathways induced by binding of pemphigus IgG have been proposed to indirectly trigger acantholysis (60–62). Pharmacological modulation of signaling molecules blocked blister formation in the passive transfer mouse models for pemphigus. Consequently, some of the therapies derived from treating these experimental models might be translatable to human disease. For instance, 38 mitogen-activated protein kinase (p38MAPK) was shown to be activated by polyclonal PV (63, 64) and PF IgG (65) *in vivo*. Furthermore, pretreatment with p38MAPK inhibitors (i.e., SB202190 and SB203580) blocked PV and PF IgG-induced blistering in mice, suggesting a pivotal role for p38MAPK signaling in acantholysis (64–66). On the other hand, blisters induced by mAbs isolated from PV patients were not affected by p38 or MAPK-activated protein kinase 2 (MK2) inhibition, pointing to the significance of steric hindrance mechanisms (42, 67). Application of a Dsg3-specific tandem peptide stabilized adhesion inhibited PV IgG-mediated activation of p38MAPK and skin blistering (68). Unfortunately, clinical trials with a p38MAPK inhibitor (NCT00606749) in PV patients were terminated due to its dose-limiting hepatotoxicity (69). Downstream of p38MAPK, epidermal growth factor receptor (EGFR) signaling was shown to be activated in PV IgG-treated keratinocytes. In line, treatment with AG1478, an inhibitor of EGFR signaling, blocked blister formation in mice (70). Moreover, other clinically approved EGFR (Erlotinib) and dual EGFR/ErbB2 (Lapatinib) inhibitors prevented blistering in a neonatal PV mouse model (71). ADAM10 together with Src regulates signaling downstream of Dsg3 which may also include EGFR signaling. It was shown that PV IgG increased ADAM10 activation in a Src-dependent manner. Thus, inhibition of ADAM10 prevented histological acantholysis and skin blistering in mice injected with PV IgG (72). In addition, in neonatal mice injected with PV IgG, enhanced phosphorylation of mammalian target of rapamycin (m-TOR) and Src as well as increased blister formation and apoptosis were observed, all of which were eliminated or reduced by focal adhesion kinase (FAK) inhibition (73). In another experimental setting, modulation of mTOR with sirolimus (or rapamycin) and Src with PP1 abolished acantholysis (74). Additionally, Src blockade was protective against AK23-induced skin blistering (75). In a neonatal PV model, pharmacological inhibition of cyclin-dependent kinase 2 (Cdk2) by roscovitine prevented blister formation (76). Pretreatment of mice with inhibitors of tyrosine-kinase (TK) completely prevented the clinical and histological findings of PV (77). PV IgG was demonstrated to induce protein kinase C (PKC) activation as well. In line with these findings, PKC inhibition reduced PV IgG-induced skin blistering *in vivo* (78, 79). Finally, elevated levels of cyclic adenosine monophosphate (cAMP) have been shown to interfere with signaling pathways and prevent blister formation *in vivo* (80). A recent study showed that the phosphodiesterase 4 (PDE4) inhibitor apremilast inhibited blister formation in a passive transfer PV model via protective cAMP signaling in keratinocytes. Hence, apremilast offers a promising approach to target loss of desmosomal adhesion in pemphigus patients (81). PV IgG not only induces direct signaling effects but also changes gene expression patterns, which may contribute to pemphigus pathogenesis. In contrast, corticosteroids antagonistically upregulate *DSG3* transcription through inhibition of the transcription factor Stat3 (82, 83). Additionally, it has been demonstrated that methylprednisolone reduces the extent of PV IgG-induced acantholysis in the skin of neonatal mice.

Targeting Fas ligand

Several lines of evidence indicate that certain mediators involved in apoptosis contribute to the pathological mechanisms of pemphigus, while apoptosis itself is not apparent in pemphigus lesions nor required for acantholysis *in vitro* (84–86). In particular, cleaved caspase-8 and -3 were detected in pemphigus lesions, and upregulation of Fas ligand (FasL) was observed in keratinocytes co-cultured with PV IgG (87–89). Accordingly, a study by Lotti et al., showed that an anti-FasL antibody blocked blister formation in a neonatal mouse model of PV. Following PV IgG injection, $sFasL^{-/-}$ mice (lacking secreted soluble form of FasL) revealed reduced acantholytic area, whereas $mFasL^{-/-}$ mice (lacking membrane-bound form of FasL) developed blisters. These observations indicate that sFasL, which is upregulated and released from keratinocytes, plays a critical role in pemphigus pathogenesis (90). In a neonatal PF model, the administration of caspase inhibitors blocked intraepidermal blistering (91). In line, a novel anti-sFasL human mAb (PC111) has been tested for pemphigus therapy due to low its potential for immunogenicity, favorable chemical and physical stability, and high binding affinity of the compound (92).

Targeting neonatal Fc Receptor

Several studies have shown a pivotal role for FcRn in pemphigus. Li et al., demonstrated that FcRn-deficient mice were resistant to experimental PV and PF, in which circulating auto-ab levels were significantly reduced as compared to wild type mice. Besides, the therapeutic efficacy of high-dose intravenous immunoglobulins (IVIg) might be attributed to FcRn saturation and increased catabolism of pathogenic IgG (93). Of note, IVIg protected target cells from apoptosis by interfering with signaling pathways and increasing the sensitivity to corticosteroids (89). In an auto-ab-induced model of PV, IVIg selectively inhibited anti-Dsg3 antibodies, decreased the number of circulating auto-abs, and reduced blister formation (94). Additionally, disease-specific IVIg was shown to be more effective in neutralizing pathogenic antibodies and preventing blister formation *in vivo* than commercial IVIg (95). Clinical trials are currently evaluating the safety and efficacy of several FcRn-targeting compounds (e.g., efgartigimod (ARGX-113) and SYNT001 (ALXN1830)) in pemphigus (96–98). Showing unexpected effects for efgartigimod

in vitro, a recent study additionally suggested that efgartigimod-induced blockade of FcRn may have functions on B cell homeostasis beyond IgG recycling. Improvement of keratinocyte adhesion by FcRn blockade may provide a novel treatment option for pemphigus (39). However, the antibody-induced model is not suitable to study the role and specific targeting of autoreactive T cells or B cells.

Transfer of autoantibodies into adult mice

Due to incomplete epidermal development in neonates, the principle of autoantibody transfer has been further adapted to adult mice. Experimental PV was induced in adult C57Bl/6J or $Rag2^{-/-}$ mice by passive transfer of a murine pathogenic Dsg3 mAb, AK23 (21). This model has proved useful in exploring non-apoptotic aspects of pemphigus related signaling. For instance, EGFR was shown to be activated in response to AK23, followed by an increase in c-myc levels. Adult passive transfer model of pemphigus was also applied in fully humanized Dsg3 (hDsg3) mice, showing that the administration of anti-Dsg3 serum IgG from patients with mucosal PV (mPV) caused IgG deposits on the surface of epidermal keratinocytes and suprabasilar acantholysis in mucosal tissues of hDsg3 mice, but not in WT mice. This finding confirms the *in vivo* pathogenicity of mPV anti-hDsg3 IgG (99).

Transfer of autoantibodies into human skin grafted onto mice

An alternative approach for studying the pathogenesis of human auto-abs in mice is to passively transfer pemphigus serum into athymic nude mice grafted with human oral mucosa. This model combines a mouse model with human skin allowing *in vivo* studies with pathogenic anti-human Dsg3 auto-abs (22, 100). A low degree of epithelial cell detachment was observed in this mucosal graft model, whereas human IgG was detected in the skin of all mice, and nearly two-thirds of the transplants showed basal epithelial edema (23). When full-thickness human skin was grafted onto the backs of severe combined immunodeficient (SCID) mice followed by injection of PF and PV IgG, subcorneal and suprabasal loss of adhesion as well as intercellular IgG deposits in the upper and lower layers of the epidermis, respectively, was seen (22). Because the xenograft model in pemphigus is complex and adds little to a classical passive model, few experiences have been gained with these models.

Lymphocyte transfer-induced disease models based on the transfer of autoreactive lymphocytes into mice

Considering the half-life of serum IgG, passive transfer models can only be used and analyzed for a limited amount of time. The main obstacle to an active immunization model is immunological self-tolerance. The usage of pemphigus mouse models was transformed by the development of $Dsg3^{-/-}$ knockout mice. These mice show a loss of keratinocyte cell adhesion mainly in oral mucous membranes, a phenotype that resembles that of patients with PV (24). These erosions, also occasionally found in the skin with suprabasal acantholysis demonstrated the importance of Dsg3 *in vivo*, however, no immunological inherent components were involved. Immunization with murine Dsg3 induced a more severe phenotype in terms of affected skin area and eosinophilic spongiosis (25). To further pinpoint Dsg3-specific immune modulation, immunization of $Dsg3^{-/-}$ mice with recombinant murine Dsg3 was followed by the intravenous transfer of the splenocyte pool into immunodeficient $Rag2^{-/-}$ $Dsg3^{+/+}$ recipient mice (25). Newly formed antigen-specific T and B cells then induced a Dsg3-specific autoimmune response presenting with suprabasal acantholysis, rows of tombstone basal keratinocytes, and half-desmosomes (101). Interestingly, antigen-specific T cell infiltration into Dsg3-expressing tissues led to interface dermatitis, a distinct form of T cell-mediated autoimmunity causing acantholysis and can be found in autoimmune skin diseases such as PNP (102). Establishing a retroviral transduction system, Takahashi et al., generated a C57BL/6J-Tg (Dsg3TCR140) mouse line which enabled them to show that while tolerized Dsg3H1 T cells could induce interface dermatitis, but not PV, non-tolerized Dsg3H1 T cells induced both anti-Dsg3 IgG production and interface dermatitis. These results demonstrated that induced anti-Dsg3 auto-abs potentially interfere with cell-cell adhesion of keratinocytes in the PV model. Based on those studies, a series of human, murine, and bispecific T cell clones were generated. Further characterization indicated that although an individual anti-Dsg3 IgG is not sufficient to cause acantholysis in adult mice, several clones together can induce a pemphigus phenotype (43). An array of various immunosuppressive agents frequently used in PV patient treatment was evaluated by means of ELISA, weight, and PV severity score (103). While cyclophosphamide displayed the strongest immunosuppressive properties, other agents such as azathioprine were shown to be less effective or not suppressive at all such as methylprednisolone and dexamethasone. Those findings are partially in contrast to the experience in humans (104), showing that observations in preclinical mouse models cannot be directly transferred to the human situation. Unfortunately, no follow-up studies applying novel treatment modalities in this model have been published so far.

Another modification of the pemphigus mouse model involved the transfer of naïve splenocytes from $Dsg3^{-/-}$ mice into $Rag2^{-/-}$ $Dsg3^{+/+}$ recipients (105). Here, Dsg3-specific naïve lymphocytes in $Dsg3^{-/-}$ mice can be primed and activated by endogenous Dsg3 in recipient mice to ultimately induce pathogenic anti-Dsg3 IgG without active immunization. While the time and overall levels of IgG and phenotype induction were delayed by two weeks, once the phenotype developed, no apparent differences in disease severity between $Rag2^{-/-}$ recipients of naïve or immunized splenocytes were found (105). Immunosuppressive drugs such as cyclophosphamide successfully inhibited disease development in a preventive setting, and mice were free of symptoms 35 days after discontinuing the treatment (106). To further pinpoint crucial $CD4^{+}$ subpopulations

for anti-Dsg3 IgG formation, Kim et al., developed a modified murine transfer protocol based on Rag1$^{-/-}$ recipient mice and provided compelling data that Dsg3-specific ICOS^{+} T follicular helper (Tfh) cells play a pivotal role in pathogenic humoral immunity in PV (107). Since alterations in the Tfh compartment have previously been found in PV (108), targeting ICOS represents a valid therapeutic option for the treatment of PV.

Antigen-specific immunization of mice to induce B and T cell-specific autoimmune responses including induction of pathogenic antibodies

PV is an oligoparametric disease, arising from a combination of different environmental factors in predisposed individuals carrying individual susceptibility genes. While genome-wide association studies are lacking to complete the picture of protective, susceptible, or neutral alleles (109), two highly associated class II alleles have been found in PV patients (DQB1*0503 and DRB1*0402) (110). PV patients carrying these alleles showed Dsg3-specific auto-aggressive Th2 cells, while healthy carriers preferentially displayed an autoreactive Th1 response (111). This prompted us to develop an active mouse model using a C57B/6J transgenic mouse line with the respective alleles HLA-DQB1*04:02 in a linkage disequilibrium with HLA-DQB1*03:02 that additionally expresses the human CD4 co-receptor while lacking a functional murine major histocompatibility class II molecule (I-Aβ−/−) (26). Antigen presentation after Dsg3 immunization is therefore restricted to human alleles which are presented in association with these human HLA class II alleles to CD4+ T cells. Intraperitoneal immunization with human Dsg3 protein or with a set of immunodominant Dsg3-peptides (50 µg/mouse day 0 and day 14), which share a positively charged anchor motif for HLA-DRB1*04:02, led to the formation of Dsg3-reactive CD4^{+} T cells followed by a profound and lasting induction of anti-Dsg3 IgG (26). This model was used to show that T and B cell interaction is crucial for pemphigus pathology. Blockade of this interaction by anti-CD40L mab completely prevented anti-Dsg3 IgG formation. Furthermore, the induction of T regulatory cells by the superagonistic anti-CD28 antibody D665 also significantly reduced anti-Dsg3 IgG induction (112).

Dsg3-specific immunization of the HLA tg mice allows cellular characterization and therapeutic intervention studies in a preclinical setting (i.e., during the formation of antigen-specific T and B cells and subsequent auto-Ab formation) or a therapeutic setting (i.e., after the onset of immunization with an ongoing auto-Ab formation as in human PV). Using this model, CD4+ T cell-specific tolerance could be induced and analyzed based on the application of a set of immunodominant Dsg3 CD4+ T cell epitopes linked to nanoparticles (113). For identification and characterization of low-frequent Dsg3-specific CD4+ T cells in lymphatic tissues, a novel detection based on DRB1*04:02 HLA class II specific dextramers loaded with the aforementioned immunodominant Dsg3-T cell epitopes was developed. CD4+ T cells were primarily detected in lymphoid tissue after initial immunization (4% Dsg3+ CD4+ CD19+ cells) which steadily decreased thereafter. Additionally, applying IVIg as an established treatment for pemphigus was shown to modulate antigen-specific T and B cell formation (114). A significant reduction of Dsg3-specific serum IgG correlated with an upregulation of regulatory T cells.

Recent endeavors to break tolerance against Dsg3 in various strains of Dsg3-expressing mice employing different immunization protocols with recombinant human and murine Dsg3/Dsg1 forms did not elicit a clinical phenotype while non-pathogenic anti-Dsg3/Dsg1 IgG was induced (115). However, unpublished work currently focuses on the establishment of a Dsg3-transgenic mouse model displaying the formation of HLA-dependent antigen-specific T and B cells as well as a solid antigen-specific IgG as a basis of a lasting clinical phenotype. This model will be suitable for in-depth analysis of autoreactive B and T cells and IgG formation as well as a preclinical model for testing novel specific immune interventions in pemphigus.

Conclusions

Even though our understanding of T- and B-cell-related induction of antigen-specific IgG in AIBD PV and PF has dramatically improved over the last decades, we still lack a proper mouse model that reflects the "grande picture" of patient-relevant characteristics. The interplay between blister formation based on steric hindrance by auto-abs against Dsg3 and prominent induced signaling pathways such as p38MAPK or ERK is still under debate. The identification of novel PV-related antigens allows the further distinction of novel clinical subtypes with specific clinical outcomes. Different preclinical models reflect distinct hallmarks and pathways of PV immune pathogenesis, i.e., induction of autoreactive T and B cells and auto-ab-induced acantholysis. They are constantly improved and reflect, so far, only a few PV-related parameters. Novel therapeutic interventions in PV address these parameters and be further characterized *in vivo*. Nonetheless, a mouse model reproducing PV pathology based on HLA-dependent T- and B-cell mediated antibody formation leading to mucosal acantholysis would be a desired gold standard.

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.

Funding

This work received support through the Deutsche Forschungsgemeinschaft (DFG) FOR 2497/TP01 to CH and TP08 to MH (HE 1602/16-2), the Collaborative Research Center CRC 1526 (Project-ID 454193335 to ES), the Schleswig-Holstein

Excellence Cluster Precision Medicine in Chronic Inflammation (DFG EXC 2167/1 to ES), and the research grant from the University of Lübeck (J13-2022 to SE). Open Access funding is provided to CH by the Open Access Publication Fund of Philipps-Universität Marburg with support of the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation).

Acknowledgments

We are highly grateful to Dr. Masayuki Amagai and Dr. Hayato Takahashi (Keio University School of Medicine, Tokyo, Japan) for providing the murine images of the active transfer model, and Dr. Rüdiger Eming (Philipps-Universität Marburg, Marburg, Germany) for the human skin histology after transfer of pathogenic murine IgG.

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 19 May 2023
DOI 10.3389/fimmu.2023.1197709

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OPEN ACCESS

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RECEIVED 31 March 2023
ACCEPTED 02 May 2023
PUBLISHED 19 May 2023

CITATION
Seiler DL, Kähler KH, Kleingarn M, Sadik CD, Bieber K, Köhl J, Ludwig RJ and Karsten CM (2023) The complement receptor C5aR2 regulates neutrophil activation and function contributing to neutrophil-driven epidermolysis bullosa acquisita. *Front. Immunol.* 14:1197709. doi: 10.3389/fimmu.2023.1197709

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The complement receptor C5aR2 regulates neutrophil activation and function contributing to neutrophil-driven epidermolysis bullosa acquisita

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Introduction: The function of the second receptor for the complement cleavage product C5a, C5aR2, is poorly understood and often neglected in the immunological context. Using mice with a global deficiency of *C5aR2*, we have previously reported an important role of this receptor in the pathogenesis of the neutrophil-driven autoimmune disease *epidermolysis bullosa acquisita* (EBA). Based on *in vitro* analyses, we hypothesized that the absence of C5aR2 specifically on neutrophils is the cause of the observed differences. Here, we report the generation of a new mouse line with a LysM-specific deficiency of *C5aR2*.

Methods: LysM-specific deletion of *C5aR2* was achieved by crossing *LysM*^{cre} mice with *tdTomato-C5ar2*$^{fl/fl}$ mice in which the *tdTomato-C5ar2* gene is flanked by loxP sites. Passive EBA was induced by subcutaneous injection of rabbit anti-mouse collagen type VII IgG. The effects of targeted deletion of *C5ar2* on C5a-induced effector functions of neutrophils were examined in *in vitro* assays.

Results: We confirm the successful deletion of C5aR2 at both the genetic and protein levels in neutrophils. The mice appeared healthy and the expression of C5aR1 in bone marrow and blood neutrophils was not negatively affected by LysM-specific deletion of C5aR2. Using the antibody transfer mouse model of EBA, we found that the absence of *C5aR2* in LysM-positive cells resulted in an overall amelioration of disease progression, similar to what we had previously found in mice with global deficiency of *C5aR2*. Neutrophils lacking C5aR2 showed decreased activation after C5a stimulation and increased expression of the inhibitory Fcγ receptor FcγRIIb.

Discussion: Overall, with the data presented here, we confirm and extend our previous findings and show that C5aR2 in neutrophils regulates their activation and function in response to C5a by potentially affecting the expression of Fcγ receptors and CD11b. Thus, C5aR2 regulates the finely tuned interaction network

between immune complexes, Fcγ receptors, CD11b, and C5aR1 that is important for neutrophil recruitment and sustained activation. This underscores the importance of C5aR2 in the pathogenesis of neutrophil-mediated autoimmune diseases.

KEYWORDS

C5aR2, neutrophil, FcgRIIB, EBA, C5a, CD11b, Mac-1, Fcγ receptor (FcγR)

1 Introduction

The potent anaphylatoxin C5a plays a critical role in promoting and sustaining inflammation by recruiting and activating immune cells that express C5a receptors (C5aRs; 1–4). In plasma or tissues, the C-terminal arginine (Arg) residue of C5a is readily cleaved by the proteolytic activity of carboxypeptidases, resulting in $C5a^{desArg}$ (5–7). C5a and to a lesser extent $C5a^{desArg}$ can bind to two receptors, C5aR1 (CD88) and C5aR2 (C5L2, GPR77). Both receptors contain seven transmembrane domains and therefore belong to the group of G protein-coupled receptors (GPCRs). However, despite their classification as GPCRs, only C5aR1, but not C5aR2, can signal through G proteins (8, 9). This difference is mainly attributed to three factors: (I) the replacement of an arginine residue by a leucine residue in the DRY motif, which is critical for Gα-protein coupling, (II) the absence of serine/threonine residues in the third intracellular loop mediating G-protein recognition in C5aR1, and (III) a modification in the NPXXY motif of the seventh transmembrane helix, acting as an important signal transduction sequence in GPCRs (8, 10–15). However, it has been shown that C5aR2 can modulate the recruitment of β-arrestin to C5aR1, and also recruit β-arrestin itself (16–18). It has therefore been suggested that it should be correctly termed an arrestin-coupling receptor (ACR; 9). Interestingly, this study also showed that the site of phosphorylation is dependent on the ligand, the ligand-binding receptor, and the cells expressing these receptors. This underscores an important finding from previous studies that downstream signaling and receptor internalization of C5a receptors and other GPCRs can be precisely controlled by the number and location of phosphorylations (9, 16, 19). Another potential mode of regulation of downstream signaling has been described at the level of β-arrestins themselves, as different binding modes and conformations of β-arrestins upon binding to a GPCR or ACR have been associated with their multifunctionality and functional diversity (9, 20–23). This might partially explain the controversy over the biological and pathological function of C5aR2, with both pro- and anti-inflammatory properties reported. Studies of LPS- (24) and IC-mediated lung injury (25), allergic contact dermatitis (26), and intestinal ischemia-reperfusion injury (27) suggest anti-inflammatory properties of C5aR2. By contrast, studies in sepsis (28), experimental allergic asthma (29), thioglycolate-induced peritonitis and air-pouch inflammation (30), and renal ischemia-reperfusion injury (31, 32), among others, suggest pro-inflammatory functions for C5aR2. Moreover, in a disease model of immune complex (IC)-induced arthritis, C5aR2 has been shown to be required for the transport of C5a into the blood vessel lumen and therefore critically involved in the recruitment and adhesion of circulating leukocytes, a fundamental requirement for tissue inflammation (33, 34). In line with these latter findings, we recently reported a net pro-inflammatory contribution of C5aR2 to the pathogenesis of *epidermolysis bullosa acquisita* (EBA; 35). EBA is a rare autoimmune disease of the skin that belongs to the group of pemphigoid diseases and is characterized by tense blisters and erosions. The autoantibodies in EBA are directed against type VII collagen (COL7) an important structural protein that stabilizes the dermal-epidermal junction zone (36). The ICs formed by the binding of the autoantibodies to their target antigen activate the complement system, mainly through the classical and alternative pathways (36, 37). Activation of the complement system leads to exponential local generation of the anaphylatoxin C5a, which has been identified as critical for EBA pathogenesis by inducing leukocyte recruitment and activation (38, 39). Previously, global C5aR1-deficient mice have been shown to be almost completely protected from disease development in a mouse model that closely resembles the effector phase (39–41). In the same mouse model, we recently showed that global C5aR2 deficiency significantly ameliorated the disease development, although the effect was not as pronounced as with global C5aR1 deficiency (35).

Interestingly, when we examined the activation potential of neutrophils from mice with global deficiency in *C5ar2 in vitro*, we found reduced CD11b up-regulation after C5a stimulation (35). CD11b is a unique α-subunit (α_m) that non-covalently couples to a common β_2-subunit (CD18) to form Mac-1 (macrophage-1 antigen, CD11b/CD18, CR3), a member of the CD18 family of integrins found mainly on granulocytes and monocytes/macrophages (42). Using mice deficient in Mac-1, it has been shown that this integrin supports adhesive functions that contribute to leukocyte recruitment and spreading as well as pathogen clearance by phagocytosis and reactive oxygen species (ROS) generation (43, 44). Ligands for Mac-1 include the intracellular adhesion molecule-1 (ICAM-1), a leukocyte adhesion receptor found on endothelial cells, the inactivation product of C3b, iC3b, the matrix molecule heparin, and factors of the coagulation system such as fibrinogen (45). Unlike other members of the CD18 family, such as lymphocyte function-associated antigen 1 (LFA-1), Mac-1 is stored in secretory vesicles in neutrophils that are

transported to the cell surface once the cell receives an activating stimulus (46), e.g., through the C5a/C5aR1-axis.

The importance of Mac-1 for neutrophil recruitment to the skin and subsequent disease development has also been demonstrated in the pathogenesis of EBA and bullous pemphigoid (BP), another pemphigoid disease. *Cd18*$^{-/-}$ mice exhibited defective recruitment of neutrophils to the skin and were resistant to disease development in the antibody transfer model of EBA (47). Using the same mice as well as specific antibody blockade of CD18, an elegant study showed that IC-mediated neutrophil adhesion to target tissues is mediated by CD18 and creates a closed, protected space in which proteinases and ROS can exert their tissue-damaging effects (48). In a neonatal antibody transfer mouse model of BP, neutrophil infiltration was also significantly reduced in Mac-1-deficient mice or when CD11b or CD18 was targeted by neutralizing antibodies (49). The latter study also found that neutrophil degranulation, particularly release of the proteases neutrophil elastase and matrix metallopeptidase 9 (MMP-9), was impaired in mice lacking Mac-1 (49). These studies illustrate that Mac-1 is not only involved in neutrophil migration in these disease settings, but also affects important effector functions of neutrophils.

Since neutrophils are the major effector cells in EBA that are recruited and activated, releasing ROS and proteases that ultimately lead to tissue damage, based on our *in vitro* activation assays, we previously hypothesized that the effects of global *C5ar2* deficiency observed in the EBA mouse model were due to impaired neutrophil function in the absence of C5aR2. Here, we report the generation of a new mouse line with a myeloid-specific deletion of *C5aR2*. Mice of this strain were then used for the EBA mouse model, the results of which we also report here to further support our previously published data and hypotheses.

2 Materials and methods

2.1 Mice and study approval

Mice of both sexes of the mouse strains *C57BL/6J* (WT) as well as *C5ar2-tdTomato*$^{fl/fl}$ and *LysM*^{cre}-*C5ar2*$^{-/-}$, the latter both on the genetic background *C57BL/6J*, were used for this study. Mice were bred and housed in the animal facility of the University of Lübeck on a 12-hour light-dark cycle. Injections were performed on 8- to 12-week-old animals after anesthesia. All animal experiments were performed according to the guidelines of the German Society for Laboratory Animal Science and the European Health Law of the Federation of Laboratory Animal Science Associations. The approval number of the local ethics committees for animal experiments of the state of Schleswig-Holstein (Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Digitalisierung des Landes Schleswig-Holstein) was 106-10/19.

2.2 PCR-based identification of the floxed *C5ar2-tdTomato* gene in sorted immune cells

The PCR-based identification of the floxed *C5ar2-tdTomato* gene in sorted immune cell populations was performed as previously described (50). Briefly, DNA from 1 × 10^5 sorted immune cells was extracted using the KAPA Express Extract Kit (Peqlab), according to manufacturer's protocol. To amplify the different DNA fragments, the following primers were used: GK91: 5'-CAAATGTTGCTTGTCTGGTG-3', GK92: 5'-GTCAGTCGAGTGCACAGTTT-3', GK360: 5'-TGTCAGCCCGG GACCTTTA-3', GK361: 5'-CTTATCACGTCCTGCGGGTAA-3' (Eurofins Scientific). PCRs were run using the following conditions: 95°C for 3 min, followed by 35 cycles at 95°C for 15 sec, 67°C for 15 sec, and 72°C for 10 sec, followed by 72°C for 120 sec. The primer pair GK91/GK92 amplifies a 206-bp DNA fragment of the TCR delta chain gene on chromosome 14 as an internal template control in samples from all strains. The primer pair GK360/GK361 amplifies a 478-bp fragment of the floxed *C5ar2-tdTomato* gene (from exon 2 and the 3'-UTR) including the fragment encoding the *loxP* site. The samples were transferred to a 1.0% sodium borate agarose gel, which was stained for amplification products with GelRed (Biotrend Chemikalien GmbH).

2.3 Antibodies

For the antibody transfer model, rabbit anti-murine type VII collagen IgG was used, which was produced and purified as described (51, 52). For flow cytometric analyses, dead cells were stained with the amine-reactive fixable viability dye eFluor 780 (Life Technologies). FcγRs were blocked with anti-mouse CD16/CD32 (5 µg/ml; 93, Life Technologies). Fluorescently labeled antibodies used for flow cytometric analysis or fluorescence-activated cell sorting (FACS) are listed in Supplementary Table 1.

2.4 Antibody transfer-induced EBA mouse model

WT, *C5ar2-tdTomato*$^{fl/fl}$, and *LysM*^{cre}-*C5ar2*$^{-/-}$ mice of either sex were anesthetized and injected subcutaneously with 100 µg affinity-purified rabbit anti-mCOL7 IgG on each days 0, 2, and 4. From day 0, mice were observed every other day for weight, general well-being, and signs of skin lesions (i.e., erythema, blisters, erosions, and crusts), which were assessed as previously described (52). On day 12, tissue biopsies (ear skin, lesions and perilesional skin stored at -80°C) were collected for histopathologic analysis. Immune cells from blood and bone marrow (BM) were isolated for quantitative immunophenotyping by flow cytometry, the latter were also used for *in vitro* stimulation assays.

2.5 Histopathology

Ear skin samples were sectioned (6 µm) and differentially stained according to the manufacturer's protocol of the Kwik-Diff™ Stain Kit (Thermo Scientific, Kalamazoo, MI, USA). Images of the stained tissue sections were acquired and analyzed using the Keyence BZ-X810 all-in-one fluorescence microscope

(Keyence, Neu-Isenburg, Germany) and BZ-X800 viewer software after applying white balance to the entire image.

2.6 Bone marrow cell preparation

Isolation of mouse BM cells was performed as previously described (53). Briefly, femurs and tibias were rinsed with DPBS containing 2 mM EDTA using a 27G needle. To obtain a single cell suspension and exclude bone fragments, the suspension was filtered through a 40-µm cell strainer. Subsequently, a hypotonic erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA at pH 7.2) was used to lyse the contained erythrocytes. For subsequent experiments, the isolated BM cells were stored in DPBS or complete RPMI 1640 medium (RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin).

2.7 Flow cytometry-based immunophenotyping and fluorescence-activated cell sorting

For flow cytometric immunophenotyping and fluorescence-activated cell sorting (FACS) of isolated cells, cells were stained with a fixable viability dye (eF780; Life Technologies) and then incubated with the respective antibodies (see Supplementary Table 1) for 15 min at 4°C in the dark. For samples in which Fcγ receptor expression was not of interest, nonspecific binding of antibodies to the Fcγ receptors was prevented by blocking the FcγRs with F_c-block (unlabeled anti-CD16/CD32 antibody) for 15 min at 4°C. Stained cells were washed and then resuspended in DPBS/1%BSA for flow cytometric analysis on a BD LSRII conventional flow cytometer or a Cytek Aurora spectral analyzer or sorted on a BD AriaIII sorter. Sorting was performed using a 70 µm nozzle.

2.8 Assessment of intracellular calcium changes in BM neutrophils

To measure the increase in intracellular calcium $(Ca^{2+})_i$ concentration, neutrophils ($Ly6G^+$ cells) from isolated BM cells of WT and *LysM^{cre}-C5ar2$^{-/-}$* mice were stimulated with recombinant C5a (0.2 nM; Hycult Biotech, Uden, Netherlands), as previously described (53). In brief, BM cells were first stained for neutrophil surface markers, resuspended in DPBS to a final concentration of 1×10^7 cells/mL, and then incubated with 10 µM Fluo-4 AM for 30 minutes at room temperature in the dark. After a wash and repeated incubation step, the fluorescent compound remained in the cytoplasm as intracellular esterases hydrolyzed the acetoxymethyl, exposing the negatively charged carboxylate groups and rendering Fluo-4 cell-impermeant. By measuring the fluorescence signal of Fluo-4 in neutrophils before and after the addition of C5a with a BD LSR II flow cytometer and calculating the relative increase in the AUC of the fluorescence signal with the Kinetics tool in FlowJo software (version 10; Tree Star, Ashland, OR), the increase in $(Ca^{2+})_i$ concentration was quantified.

2.9 Neutrophil CD11b upregulation assay

Differential expression of the integrin CD11b on neutrophils from WT, *C5ar2-tdTomato$^{fl/fl}$*, and *LysM^{cre}-C5ar2$^{-/-}$* mice after stimulation with C5a served as an indicator of cellular activation and was measured by flow cytometry. Briefly, BM cells were incubated in complete RPMI 1640 medium for 30 minutes at 37°C, 5% CO_2 supplemented with or without 2.5 nM C5a. After washing and blocking FcγRs with anti-CD16/32 antibodies, cells were stained for Ly6G and CD11b. Surface expression of CD11b on neutrophils ($Ly6G^+$ cells) was determined using a BD LSRII flow cytometer. Calculation of the relative difference in the geometric mean fluorescence intensity (gMFI) of CD11b expression from stimulated and unstimulated neutrophils then revealed the relative, C5a-induced upregulation of CD11b on neutrophils.

2.10 Neutrophil chemotaxis assay

Chemotaxis of neutrophils from WT, *C5ar2-tdTomato$^{fl/fl}$*, and *LysM^{cre}-C5ar2$^{-/-}$* mice toward C5a was determined using a transwell assay as previously described (54). In brief, 2×10^6 BM cells were transferred to an insert of a 96-well transwell plate with a pore size of 3 µm (Corning Inc., Kennebunk, ME, USA). The bottom wells of the samples contained 12.5 nM C5a in complete RPMI 1640 medium, whereas the control wells contained only complete RPMI 1640 medium. Cells were incubated at 37°C, 5% CO_2 for 30 minutes and then collected separately from the transwell insert (non-migrated cells) and the bottom wells (migrated cells). After staining for Ly6G and CD11b, neutrophils were quantified using a Cytek Aurora spectral flow cytometer. To obtain the relative amount of chemotactic neutrophils, the number of migrated neutrophils was divided by the total number of neutrophils recovered (non-migrated and migrated). In addition, the values obtained were corrected for the number of neutrophils that migrated through the pores passively due to chemokinesis.

2.11 Statistical analysis

GraphPad Prism software (version 9.5.1; GraphPad Software, San Diego, CA, USA, www.graphpad.com) was used for statistical analysis of the data. All plots show mean values ± standard error of the mean (SEM). Data sets were analyzed for outliers using the ROUT method with a false discovery rate (FDR) of Q = 5%. Outliers were excluded from the analysis. The unpaired Mann-Whitney test was used to determine the *p*-values of comparisons between two independent groups. For comparisons between more than two independent groups, the Kruskal-Wallis test with Dunn's multiple comparison test was used, and for comparisons involving different time points or different subpopulations, the two-way ANOVA with Holm-Šídák multiple comparison test was used. Significance

wasassumed when the p-value was < 0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

3 Results

3.1 Generation and characterization of a neutrophil-specific *C5aR2* knock-out mouse line

We generated a LysM-specific *C5aR2* knock-out mouse line by *in vitro* fertilization of female *C5ar2-tdTomato*$^{fl/fl}$ mice with sperm of *LysM*^{cre} mice and subsequent breeding of a homozygous *LysM*^{cre}-*C5ar2*$^{-/-}$ mouse line. In *C5ar2-tdTomato*$^{fl/fl}$ mice a sequence encoding the reporter protein tandem dimer Tomato (tdTomato) has been inserted in-frame with the coding sequence of *C5ar2* immediately following the splice acceptor of exon 2 (4). Because the coding sequences of *tdTomato* and *C5ar2* are separated by a sequence encoding the self-cleaving peptide from porcine teschovirus-1 (P2A), both the reporter protein tdTomato and C5aR2 are expressed as individual protein products in this mouse once the corresponding mRNA is transcribed (4). In addition, the entire gene cassette is floxed with loxP sites, allowing specific knockout of the entire locus by cell-specific expression of Cre recombinase (55). We sorted BM neutrophils (Ly6G$^+$, CD11b$^+$), BM CD11b$^+$ cells (Ly6G$^-$, CD11b$^+$), splenic B cells (CD19$^+$, CD45R$^+$) and splenic T cells (CD3$^+$; Supplementary Figures 1A, B) and analyzed the cell-specific deletion of the *C5ar2* gene by genotyping. We found a signal corresponding to the floxed *C5ar2-tdTomato*$^{fl/fl}$ gene construct (478 bp) in splenic B and T cells as well as in BM Ly6G$^-$/CD11b$^+$ cells but could not detect this sequence in BM neutrophils (Figure 1A).

We also checked for C5aR2 expression in WT, *C5ar2-tdTomato*$^{fl/fl}$, and *LysM*^{cre}-*C5ar2*$^{-/-}$ mice by analyzing the surrogate marker tdTomato signal in BM and blood neutrophils (Ly6G$^+$, CD11b$^+$) by flow cytometry (Figure 1B). We found a clear tdTomato signal in BM (approximately 75% tdTomato$^+$ neutrophils) and particularly in blood neutrophils (>95% tdTomato$^+$ neutrophils) from *C5ar2-tdTomato*$^{fl/fl}$ mice, whereas the tdTomato signal in neutrophils from *LysM*^{cre}-*C5ar2*$^{-/-}$ mice corresponded to the negative tdTomato signal in neutrophils from WT animals. In Ly6G$^-$, CD11b$^+$ cells, we did not detect differences between *LysM*^{cre}-*C5ar2*$^{-/-}$ and *C5ar2-tdTomato*$^{fl/fl}$ control mice, with only a subfraction of cells staining positive for tdTomato in each case. Overall, this confirms the cell-specific deletion of the *C5ar2* gene also at the protein level.

Phenotypically, the newly created *LysM*^{cre}-*C5ar2*$^{-/-}$ mice appeared healthy and were not different from either *C5ar2-tdTomato*$^{fl/fl}$ or WT mice. However, as both the *C5ar1* and *C5ar2* gene loci are located on chromosome 7 in close proximity (only about 15 kb apart), we additionally compared the C5aR1 expression pattern in WT; *C5ar2-tdTomato*$^{fl/fl}$ and *LysM*^{cre}-*C5ar2*$^{-/-}$ mice. In both BM and blood neutrophils, >99% of cells stained positive for C5aR1. At steady state, surface expression of C5aR1 was comparable high in BM and blood neutrophils (Ly6G$^+$ cells) from *LysM*^{cre}-*C5ar2*$^{-/-}$ mice compared with respective cells from *C5ar2-tdTomato*$^{fl/fl}$ and WT mice (Figures 1C, D). Of note, we detected a significant difference in surface expression of C5aR1 between neutrophils from *C5ar2-tdTomato*$^{fl/fl}$ and WT mice based on the gMFI. We additionally checked whether neutrophil development in the BM niche may be influenced by Cre recombinase activity under the control of the *LysM* promoter and cell-specific deletion of *C5ar2* in neutrophils. Based on the percentage of detected neutrophil precursors (pre neutros), immature neutrophils (immature neutros), and mature neutrophils (mature neutros), no differences in neutrophil development were detected (Figure 1E).

3.2 *C5aR2* deficiency in neutrophils ameliorates pathogenesis in an experimental model of EBA

We used the established *LysM*^{cre}-*C5ar2*$^{-/-}$ mouse line in the antibody transfer model of EBA (Figure 2A) to confirm our previous hypothesis that the lack of *C5ar2* in neutrophils is responsible for the decreased disease severity reported in mice with a global *C5aR2* deficiency. In these experiments *C5ar2-tdTomato*$^{fl/fl}$ mice served as a control.

Signs of skin blistering (erosion, lesions, and/or crusts) were detectable as early as 2 days post anti-mCOL7 IgG injection in mice of both genotypes. Of note, similar to mice with global *C5aR2* deficiency (35), *LysM*^{cre}-*C5ar2*$^{-/-}$ mice showed an overall ameliorated disease progression (Figure 2B). Interestingly, however, and in contrast to *C5ar2*$^{-/-}$ mice disease onset was similar in *LysM*^{cre}-*C5ar2*$^{-/-}$ mice when compared to *C5ar2-tdTomato*$^{fl/fl}$ mice and was slowed only after day 6. By day 12, significantly less areas of blister-affected skin (7.5 ± 0.8%) were detected in *LysM*^{cre}-*C5ar2*$^{-/-}$ mice compared to *C5ar2-tdTomato*$^{fl/fl}$ mice (11.6 ± 1.0%), which showed a similar disease progression as we previously reported for WT mice (35).

The significant difference in the affected body surface area found on day 12 is exemplified by representative photographs of mice from both genotypes showing that the distribution of skin lesions on the body surface was comparable, but numbers and severity of blisters were clearly higher in *C5ar2-tdTomato*$^{fl/fl}$ control mice (Figures 2C, E). In addition, histological analysis of skin sections of the ear showed evident detachment of the epidermis from the dermis at the dermal-epidermal junction (DEJ), swelling of the tissue, and cellular infiltration in samples from *C5ar2-tdTomato*$^{fl/fl}$ control mice but not in samples from *LysM*^{cre}-*C5ar2*$^{-/-}$ mice, suggesting ameliorated disease in the latter (Figures 2D, F). Taken together, these data demonstrate that the deficiency of *C5aR2* in neutrophils leads to an ameliorated disease phenotype in the antibody transfer model of EBA when compared with *C5ar2-tdTomato*$^{fl/fl}$ control mice.

3.3 *In vitro* chemotaxis of neutrophils from *LysM*^{cre}-*C5ar2*$^{-/-}$ mice toward C5a is unchanged

Because we previously observed reduced C5a-mediated chemotaxis of BM neutrophils from *C5ar2*$^{-/-}$ mice compared to

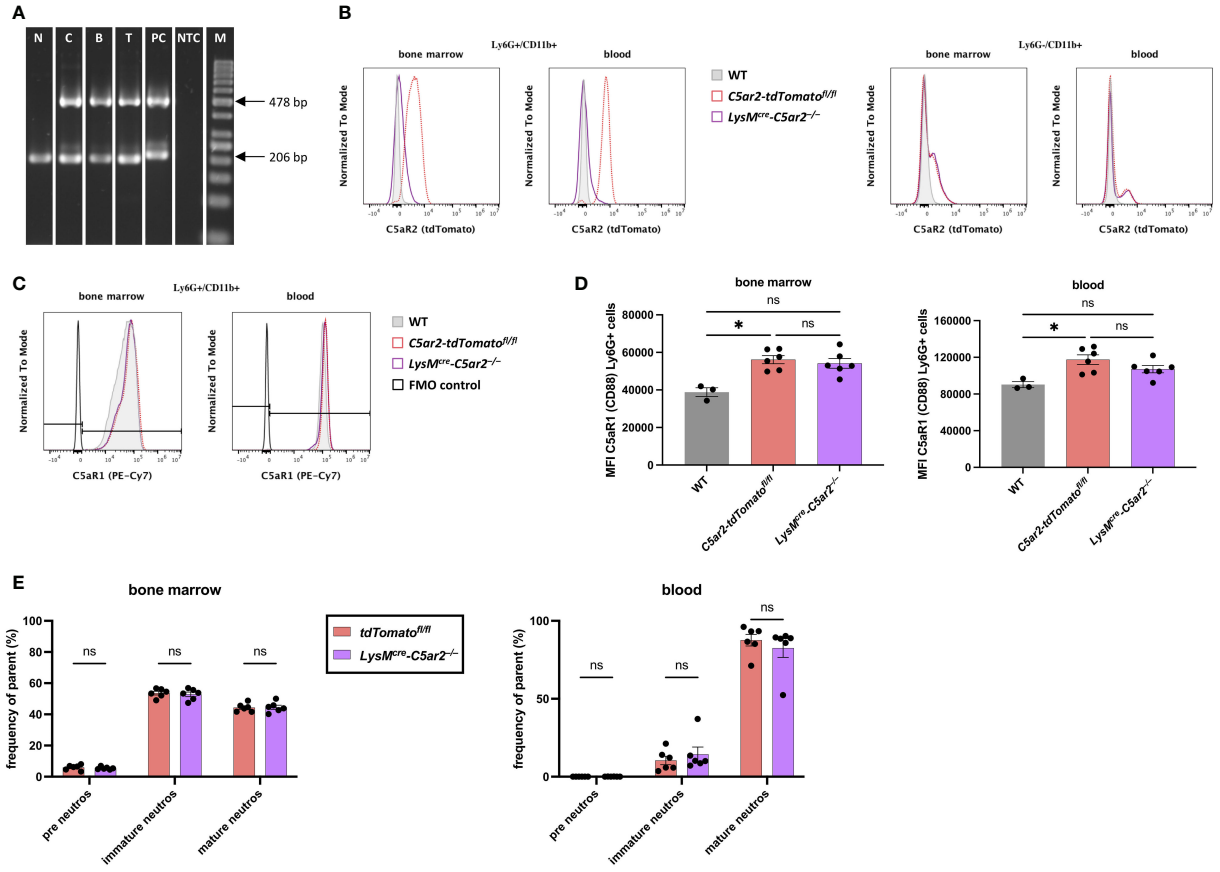

FIGURE 1
Characterization of the cell-specific C5aR2 knock-out mouse line. **(A)** PCR-based detection of the floxed *tdTomato-C5ar2* gene cassette in BM neutrophils (N), BM Ly6G$^-$/CD11b$^+$ cells (C), splenic B (B) and T cells (T) from *LysM^{cre}-C5ar2$^{-/-}$* mice. Positive control from *C5ar2-tdTomato$^{fl/fl}$* mice (PC) and No Template Control (NTC) are shown. M: GeneRuler 50 bp DNA Ladder. **(B)** Histogram overlay of tdTomato reporter signal indicating C5aR2 expression in BM and blood neutrophils (Ly6G$^+$/CD11b$^+$) and Ly6G$^-$/CD11b$^+$ cells from *LysM^{cre}-C5ar2$^{-/-}$* (purple) and *C5ar2-tdTomato$^{fl/fl}$* (red) mice. The tdTomato signal of corresponding cells from WT (grey) mice served as a negative control. **(C)** Histogram overlay indicating C5aR1 expression in BM and blood neutrophils from *LysM^{cre}-C5ar2$^{-/-}$* (purple), *C5ar2-tdTomato$^{fl/fl}$* (red), and WT (grey) mice. The signal corresponding to the FMO control is shown in black. **(D)** C5aR1 expression in BM and blood neutrophils (Ly6G$^+$ cells) from *LysM^{cre}-C5ar2$^{-/-}$*, *C5ar2-tdTomato$^{fl/fl}$*, and WT mice quantified by the gMFI. **(E)** Frequency (of parent) of neutrophil subsets in the BM and blood of naïve *LysM^{cre}-C5ar2$^{-/-}$* and *C5ar2-tdTomato$^{fl/fl}$* control mice (n = 6/group). * $p < 0.05$; ns, not significant.

neutrophils from WT mice *in vitro*, we aimed to verify this by employing BM neutrophils from *LysM^{cre}-C5ar2$^{-/-}$* mice to the same chemotaxis assay. Here, we did not detect a significant difference in chemotaxis toward C5a between BM neutrophils from *LysM^{cre}-C5ar2$^{-/-}$* and *C5ar2-tdTomato$^{fl/fl}$* or WT control mice, although there was a non-significant trend toward lower chemotaxis of neutrophils from *LysM^{cre}-C5ar2$^{-/-}$* mice (Figure 3A).

In addition to the *in vitro* chemotaxis assay we also checked for neutrophil numbers in the blood of diseased *LysM^{cre}-C5ar2$^{-/-}$* and *C5ar2-tdTomato$^{fl/fl}$* control mice. We did not detect any differences in the number of neutrophils found in the blood being in line with our previous report (35; Figure 3B).

3.4 C5aR2 deficiency on neutrophils reduces C5a-mediated cellular activation

We reported previously that BM neutrophils from *C5ar2$^{-/-}$* mice exhibit reduced cellular activation following stimulation with C5a (and C5a^{desArg}; 15), which prompted us to hypothesize that this is partly the reason for the ameliorated disease phenotype observed in the EBA mouse model with these mice (35). In line with this hypothesis, we here found a significantly ameliorated disease phenotype for *LysM^{cre}-C5ar2$^{-/-}$* mice. However, as C5a-induced chemotaxis of neutrophils was not affected by the cell-specific deletion of *C5ar2* in these mice, we tested for the cellular activation of BM neutrophils from *LysM^{cre}-C5ar2$^{-/-}$* mice after C5a stimulation. C5a-induced up-regulation of CD11b surface expression was significantly diminished in BM neutrophils from *LysM^{cre}-C5ar2$^{-/-}$* mice compared with WT but not *C5ar2-tdTomato$^{fl/fl}$* control mice (Figure 3C). The mean up-regulation of CD11b surface expression was 172.3 ± 9.7% in neutrophils from *LysM^{cre}-C5ar2$^{-/-}$* mice, whereas surface expression of CD11b increased by 190.3 ± 9.5% and 215.1 ± 10.0% in neutrophils from *C5ar2-tdTomato$^{fl/fl}$* and WT control mice, respectively. We also checked for C5a-induced calcium flux in neutrophils from *LysM^{cre}-C5ar2$^{-/-}$* and WT mice. The mean increase in the calcium flux, as measured by the calcium-sensitive dye Fluo-4 AM, was 85.9 ± 6.2%

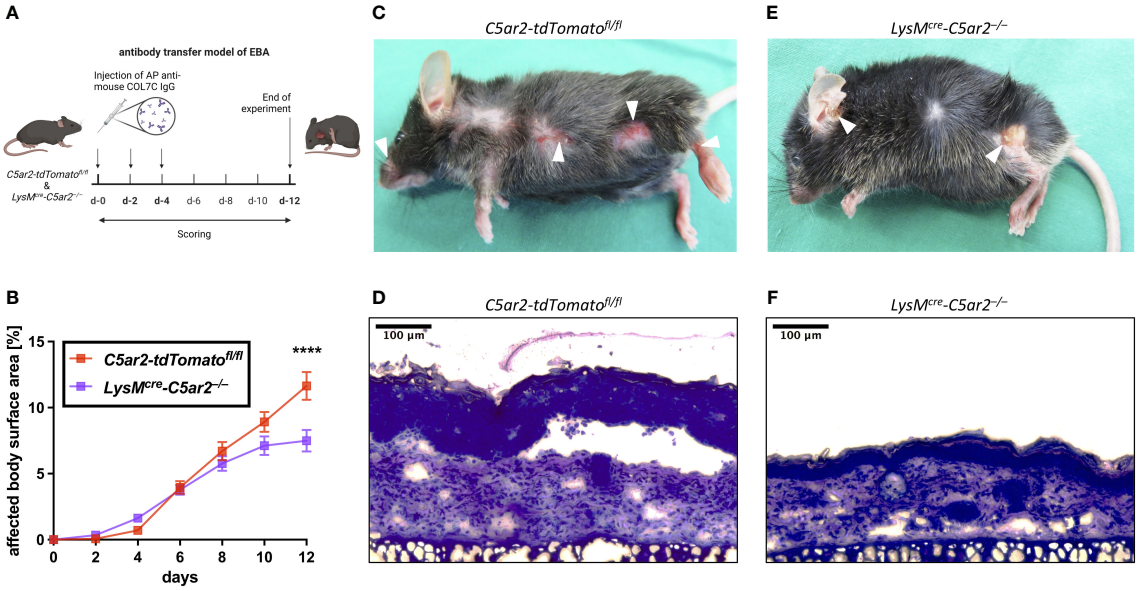

FIGURE 2
C5aR2 deficiency in neutrophils ameliorates pathogenesis in an experimental model of EBA. **(A)** Schematic representation of the antibody transfer mouse model of EBA. Subepidermal blistering is induced by subcutaneous injection of affinity-purified rabbit anti-mCOL7 IgG on days (d) 0, 2, and 4. Cutaneous lesions are scored every other day. Mice are sacrificed on d-12 (figure created with BioRender.com). **(B)** Development of the clinical disease phenotype as indicated by the percentage of body surface area affected by lesions, erosions, and blisters in *LysM^{cre}-C5ar2$^{-/-}$* and *C5ar2-tdTomato$^{fl/fl}$* control mice (n = 15 per group). Representative pictures of clinical lesions found in **(C)** *C5ar2-tdTomato$^{fl/fl}$* and **(E)** *LysM^{cre}-C5ar2$^{-/-}$* mice. Example photographs of histopathologically examined skin sections from **(D)** *C5ar2-tdTomato$^{fl/fl}$* and **(F)** *LysM^{cre}-C5ar2$^{-/-}$* mice. **** $p < 0.0001$.

in neutrophils from *LysM^{cre}-C5ar2$^{-/-}$* mice, while neutrophils from WT control mice showed a mean increase of 162.8 ± 11.8% after C5a stimulation (Supplementary Figure S2).

3.5 Fcγ receptor expression is altered in neutrophils with C5aR2 deficiency

Recently, we published that neutrophils isolated from *C5ar2$^{-/-}$* mice exhibit a changed expression pattern in Fcγ receptors, resulting in a decreased release of ROS – critical drivers of tissue destruction in EBA pathogenesis (47, 56) – after stimulation with ICs consisting of murine COL7C and rabbit anti-mouse COL7 IgGs (35). Using *LysM^{cre}-C5ar2$^{-/-}$* mice we tested if the change in FcγR expression is a direct effect resulting from the deficiency in *C5ar2* in neutrophils or whether this is a consequence of global deficiency in *C5ar2*. Only 10%, 27%, and 15% of BM neutrophils from *LysM^{cre}-C5ar2$^{-/-}$*, *C5ar2-tdTomato$^{fl/fl}$*, and WT mice, respectively, stained positive for FcγRI Figure 4A. Surface expression of FcγRI in these cells quantified by the relative gMFI was low. However, there was a significant difference in the relative gMFI for FcγRI between cells from *LysM^{cre}-C5ar2$^{-/-}$* and *C5ar2-tdTomato$^{fl/fl}$* control mice, with cells from *C5ar2-tdTomato$^{fl/fl}$* control mice showing an increased relative gMFI. Almost all blood neutrophils (98.5-99.5%) were negative for FcγRI, regardless of the

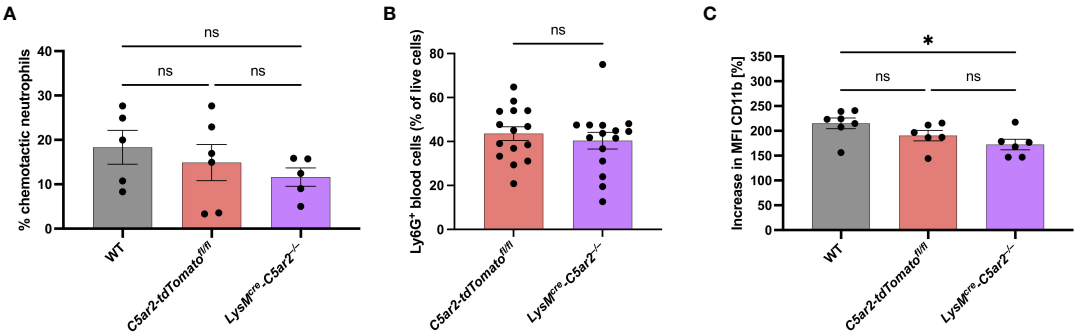

FIGURE 3
C5a-mediated *in vitro* activation of neutrophils from *LysM^{cre}-C5ar2$^{-/-}$* mice. **(A)** *In vitro* chemotaxis of BM cells of *LysM^{cre}-C5ar2$^{-/-}$*, *C5ar2-tdTomato$^{fl/fl}$* and WT control mice towards C5a (n ≥ 5/group). **(B)** Neutrophil frequency in the blood of diseased (EBA) *LysM^{cre}-C5ar2$^{-/-}$* and *C5ar2-tdTomato$^{fl/fl}$* control mice (n = 15/group). **(C)** C5a-induced increase in CD11b surface expression of *LysM^{cre}-C5ar2$^{-/-}$*, *C5ar2-tdTomato$^{fl/fl}$* and WT control mice (n ≥ 6/group). * $p < 0.05$; ns, not significant.

genotype from which these cells were isolated (Figure 4B). FcγRIIb and FcγRIII were expressed on >99.5% and >98.5%, respectively, of all BM neutrophils from mice of all three genotypes. Interestingly, based on the relative gMFI, surface expression of inhibitory FcγRIIb was significantly increased in BM neutrophils from *LysM*^{cre}-*C5ar2*$^{-/-}$ mice compared with neutrophils isolated from *C5ar2-tdTomato*$^{fl/fl}$ or WT mice (Figures 4C). Surface expression of FcγRIIb was lowest in neutrophils from *C5ar2-tdTomato*$^{fl/fl}$ mice. Although the surface expression of FcγRIIb on neutrophils decreased once neutrophils transitioned from the BM to the blood, ≥99.5% of cells still expressed FcγRIIb, and the effect of significantly increased expression on the surface of neutrophils from *LysM*^{cre}-*C5ar2*$^{-/-}$ mice persisted (Figure 4D). FcγRIII expression on blood neutrophils was similar to BM neutrophils. Surface expression of FcγRIV was found on the majority of BM and blood neutrophils, regardless of the genotype from which the cells were isolated. Based on the relative gMFI, FcγRIV surface expression on blood neutrophils was increased compared with their BM counterparts. However, no difference in surface expression of FcγRIV was detected between *LysM*^{cre}-*C5ar2*$^{-/-}$, *C5ar2-tdTomato*$^{fl/fl}$, and WT mice (Figure 4A).

Taken together, these data confirm that deficiency of *C5ar2* on neutrophils alters the expression level of Fcγ receptors, leading in particular to increased surface expression of inhibitory FcγRIIb.

4 Discussion

We recently reported that *C5ar2*$^{-/-}$ mice have an ameliorated disease phenotype in the antibody-transfer model of EBA. Based on the critical role of neutrophils in EBA pathogenesis (47, 56–58) and the results of our *in vitro* data analyzing the C5a and Fcγ receptor-mediated activation of neutrophils, we hypothesized that a deficiency of *C5ar2* in neutrophils is the leading cause for the ameliorated disease phenotype. Here, we confirmed our previous hypothesis by generating a mouse line, *LysM*^{cre}-*C5ar2*$^{-/-}$, in which *C5ar2* is specifically deleted in *LysM*-expressing cells. Like mice with a global deficiency in *C5ar2*, mice of this newly generated mouse line showed an ameliorated disease phenotype in the antibody transfer mouse model of EBA. However, a limitation of using the *LysM*^{cre} mice is that the *LysM*/*Lyz2* promoter is expressed in cells of the myeloid lineage, including not only neutrophils but also monocytes/macrophages (59, 60). And although we still detected the tdTomato coding sequence in Ly6G^{-}/CD11b^{+} BM cells (Figure 1B), it is still reasonable to assume that some of the observations made with these mice using the antibody transfer mouse model of EBA are due to altered monocyte/macrophage function (34). Yet, there is limited evidence for a functional role of monocyte-derived cells in the pathogenesis of EBA (61, 62). Comparison of the use of anti-Ly6G (1A8) and anti-Ly6C/G (RB6-8C5) depleting antibodies suggests that monocyte depletion has an additional beneficial effect in experimental EBA (47, 58, 62). Moreover, monocytes have been shown to induce subepidermal blisters in an *ex vivo* model of EBA (61). Therefore, it cannot be completely excluded that monocytes or monocyte-derived cells that have been shown to express *C5ar2* (4, 63) also contribute in part to the attenuated disease phenotype observed in global and cell-specific *C5ar2* knockout mice, although the main drivers of the disease are clearly neutrophils (34). In addition, we used *C5ar2-tdTomato*$^{fl/fl}$ mice but not *LysM*^{cre} mice as controls. In *LysM*^{cre} mice, the function of the endogenous *LysM* gene (chromosome 10) is abolished (59, 64). Although an effect of lysozyme, a glycoside hydrolase that catalyzes the hydrolysis of the β-1,4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine, on the pathogenesis of EBA seems unlikely, and *LysM*^{cre} mice showed a similar response to WT animals in an LPS-induced model of sterile acute lung injury (64), an influence of the *LysM*^{cre} allele on disease progression in the EBA model cannot be completely excluded.

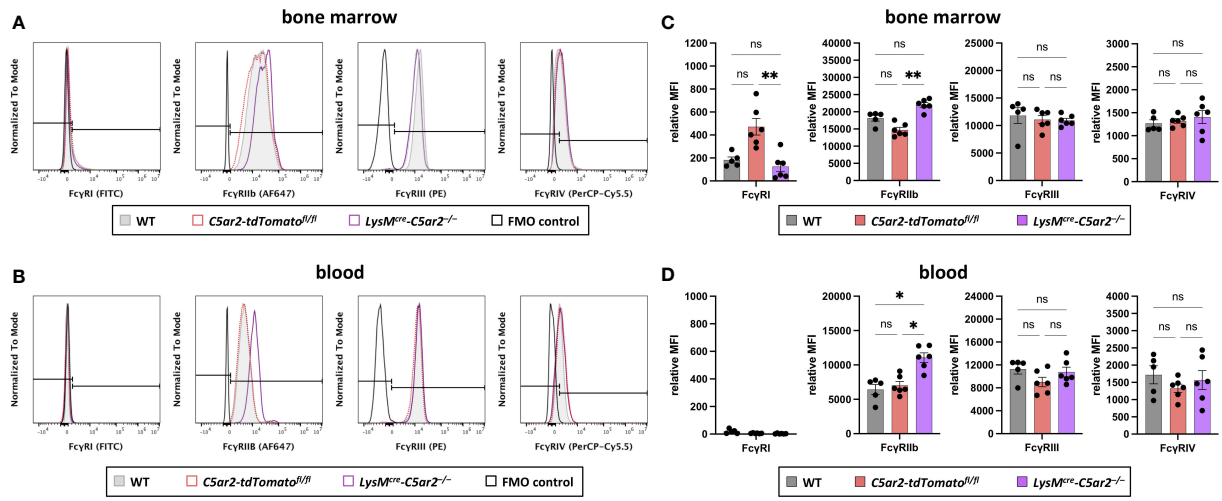

FIGURE 4
Fcγ receptor expression on BM and blood neutrophils. Histogram overlays of surface expression of FcγRs (FcγRI, FcγRIIb, FcγRIII, FcγRIV) on neutrophils from **(A)** BM and **(B)** blood of WT, *C5ar2-tdTomato*$^{fl/fl}$, and *LysM*^{cre}-*C5ar2*$^{-/-}$ mice. The signal corresponding to the FMO control is shown in black. Relative gMFI of FcγRs corresponding to surface expression on **(C)** BM and **(D)** blood neutrophils of WT, *C5ar2-tdTomato*$^{fl/fl}$, and *LysM*^{cre}-*C5ar2*$^{-/-}$ mice. * $p < 0.05$, ** $p < 0.01$; ns, not significant.

Remarkably, the differences in disease progression between WT and *LysM*^{cre}-*C5ar2*$^{-/-}$ mice began after an initiation phase that was similar in both mouse strains. Because this effect was also found when comparing WT and *C5ar2*$^{-/-}$ mice (35), we hypothesize that this is an effect caused by the sustainment of neutrophil recruitment and activation, possibly controlled by CD11b and FcγRs. In turn, the expression of CD11b and FcγRs appears to be influenced by C5a receptor expression and/or signaling. Consistent with this and our results in the chemotaxis assay, Chen et al. found no difference in neutrophil recruitment to the peritoneum between WT and *C5ar2*$^{-/-}$ mice after *i.p.* injection of C5a. However, using an air-pouch model, they showed that after injection of C5a alone or together with thioglycolate into the air-pouches, sustained neutrophil recruitment was significantly reduced in *C5ar2*$^{-/-}$ mice compared with WT mice (30). This suggests that although C5aR2 deficiency does not affect short-term neutrophil recruitment, it significantly reduces long-term sustained neutrophil recruitment, which may influence disease progression in the antibody transfer model of EBA.

In our previous publication, we reported reduced cellular activation in BM neutrophils from *C5ar2*$^{-/-}$ mice that was dependent on the presence of C5aR1 (35). We have shown that C5a-induced up-regulation of CD11b is reduced in neutrophils from *C5ar2*$^{-/-}$ mice compared with neutrophils from WT animals and is completely absent in neutrophils from *C5ar1*$^{-/-}$ mice. In addition, we found an altered expression pattern of FcγRs, favoring a more anti-inflammatory phenotype in neutrophils from *C5ar2*$^{-/-}$ mice. Here, we confirm our previous findings by using mice with LysM-specific *C5ar2* deficiency. Neutrophils from these mice also exhibited reduced C5a-induced CD11b up-regulation and an altered expression pattern of FcγRs characterized by increased expression of the inhibitory Fcγ receptor FcγRIIb. This was an interesting finding as it links the function of C5aR2 to both Mac-1 (CD11b/CD18) and FcγRs.

In experimental EBA, the activating Fcγ receptors including FcγRIII and FcγRIV promote disease development, whereas the inhibitory FcγRIIb has been shown to convey some level of protection (65, 66). A bi-directional crosstalk between C5a and Fcγ receptors has been described earlier, suggesting that IC-induced C5a tips the balance between activating and inhibitory FcγRs (A/I ratio) toward the activating phenotype (40, 67–70). This results in a self-amplifying feedback loop sustaining C5a generation and priming the cells for an inflammatory response (69–72). Moreover, in addition to neutrophil activation it has been proposed that the crosstalk between C5a and Fcγ receptors also sustains neutrophil recruitment *in vivo*, which relied on C5a-induced secretion of leukotriene B4 (LTB_4) by neutrophils (73).

Intriguingly, the C5a/C5aR1-axis has previously been shown to play an important role in the transition of neutrophils from firm arrest to spreading and crawling to enable subsequent extravasation in a LTB_4/BLT1-dependent manner (74). Importantly, this process was dependent on the crosstalk with Mac-1 and LFA-1, indicating that both β_2-integrins are activated by C5a-stimulation in neutrophils and that this is an important regulatory step in C5a-triggered diapedesis into inflamed tissue (74).

In addition, Mac-1 has also been described to interact with several other immune receptors on neutrophils, including Fcγ receptors, CD14, Dectin-1, and TLRs, which might explain why Mac-1 is not only involved in neutrophil migration but also affects important effector functions of neutrophils (42). Considering IC-mediated autoimmune diseases such as EBA, crosstalk with Fcγ receptors is of particular interest, as it has been shown that the activating Fcγ receptors in mice are critical for neutrophil accumulation in models of IC-induced inflammation using mice lacking the common γ-chain (42). It has been shown in the past that Mac-1 is not essential for the initial FcγR-mediated binding to ICs but is required for sustained firm interaction with and activation of neutrophils by these ICs, which likely involves paxilin-induced cytoskeleton remodeling (45, 48, 75–78). Moreover, in an antibody transfer model of acute anti-glomerular basement membrane glomerulonephritis, the sustainment of neutrophil interaction with deposited ICs was abolished in the absence of Mac-1 (45, 77) and prevented the local release of ROS associated with renal damage in this model (42, 77). Moreover, blockade of CD18 has been shown not to affect ROS production or neutrophil elastase release from IC-activated neutrophils *in vitro* (48). However, IC-induced neutrophil adhesion, which limits protease inhibitor access and is critical for anti-COL7 IgG-mediated tissue injury in an *ex vivo* model, was significantly affected by blockade of CD18 *in vitro* and *in vivo* (48). Accordingly, we hypothesize that the altered expression pattern of FcγRs and the lower C5a-induced expression of CD11b on neutrophils from *LysM*^{cre}-*C5ar2*$^{-/-}$ mice affects the IC-mediated neutrophil adhesion and, consequently, the duration rather than the magnitude of local neutrophil activation in tissues, particularly contributing to the late phase of disease progression.

Based on these previous findings and our results, we propose an important interaction network between ICs, FcγRs, C5a receptors, and β_2-integrins (including Mac-1 and potentially also LFA-1) that appears to be critical for disease development in experimental EBA. In this model, deposition of ICs in the skin leads to local activation of complement, in particular C3b and its degradation products, which deposit along the DEJ, and C5a, which causes mobilization and activation (up-regulation of CD11b, increase in the A/I-ratio) of neutrophils. Activated neutrophils migrate to the site of inflammation, a process promoted by β_2-integrins and FcγRs and supported by chemokines such as LTB_4. In the skin, neutrophils accumulate at the DEJ, where sustained interaction with and activation by deposited ICs and C3b is achieved by FcγR/Mac-1 interaction, resulting in local adhesion and the continuous release of ROS and proteases that destroy the tissue in an enclosed space protected from ubiquitously expressed proteinase inhibitors (48). In tissue, activated neutrophils also release IL-1β, which stimulates fibroblasts to release cytokines that contribute to the sustainment of neutrophil influx and inflammation (74). Accordingly, anything that disturbs this interaction network also has a critical impact on disease development: IC deposition at the DEJ is a prerequisite for complement activation and neutrophil recruitment to trigger subepidermal blister formation (38, 79, 80); deficiency of the activating Fcγ receptor FcγRIV (and FcγRIII) protects from disease development, whereas deficiency of FcγRIIb significantly exacerbates disease (65, 66); deficiency of CD18 abrogates infiltration of neutrophils into the skin and local adhesion to deposited ICs, thus preventing disease development (47, 48);

deficiency in C5aR1 protects from disease development (40), whereas deficiency in C5aR2 significantly ameliorates pathogenesis of EBA, despite similar blood neutrophilia and similar amounts of ICs deposited at the DEJ (35).

Consistent with this view, neutrophils from *C5ar1*$^{-/-}$ mice show neither C5a-induced cellular activation (intracellular calcium flux, up-regulation of CD11b) nor directional chemotaxis toward C5a *in vitro* (35). Moreover, we showed that neutrophils from mice with a global (*C5ar2*$^{-/-}$) or neutrophil-specific (*LysM*^{cre}-*C5ar2*$^{-/-}$) deficiency of C5aR2 exhibit a partial inhibition of C5a-induced up-regulation of CD11b, an A/I-ratio of FcγRs shifted to a more inhibitory phenotype, and reduced chemotaxis toward C5a and C5a^{desArg} (15, 35). Thus, signaling through C5a receptors induces a local inflammatory milieu, primes neutrophils for an inflammatory response, and facilitates directional chemotaxis within the tissue. All of this appears to be primarily dependent on the availability of C5aR1, whose function is promoted by C5aR2 in these settings (34).

In summary, the findings presented here confirm our previous hypothesis that C5aR2 plays a critical role in regulating neutrophil activation and function that contributes to neutrophil-driven autoimmune diseases such as EBA. However, although we have shown that *C5aR2* expression in LysM-positive cells appears to be determinant of disease progression, and mechanistically this appears to be related to the C5a-dependent control of CD11b and FcγRIIb expression, further experiments are needed to fully understand the still enigmatic role of C5aR2 in the regulation of the neutrophil-driven immune response.

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 animal study was reviewed and approved by Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Digitalisierung des Landes Schleswig-Holstein.

Author contributions

Conceptualization: CK, DS, JK. Funding Acquisition: CK. Investigation: DS, KK, MK. Project Administration: DS, CK. Resources: RL, KB, JK. Supervision: DL, CK. Writing – Original Draft Preparation: DS. Writing – Review and Editing: DS, KK, MK, CS, RL, JK, CK. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by Deutsche Forschungsgemeinschaft through CRC 1526 Pathomechanisms of Antibody-mediated Autoimmunity (PANTAU), project 413535489 (A04).

Acknowledgments

We are grateful and acknowledge Detlef Zillikens' pioneering work in pemphigoid disease research that has made a significant impact on patient's lives worldwide, inspiring and guiding future generations of researchers and clinicians in the field. We acknowledge support by the Cell Analysis Core Facility (CAnaCore) of the University of Lübeck.

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.2023.1197709/full#supplementary-material

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TYPE Case Report
PUBLISHED 24 May 2023
DOI 10.3389/fimmu.2023.1179294

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RECEIVED 03 March 2023
ACCEPTED 09 May 2023
PUBLISHED 24 May 2023

CITATION
Foerster Y, Sollfrank L, Rechtien L, Harrer T, Berking C and Sticherling M (2023) Case report: Bullous pemphigoid in HIV-1-positive patients: interplay or coincidence? A case series and review of the literature. *Front. Immunol.* 14:1179294. doi: 10.3389/fimmu.2023.1179294

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Case report: Bullous pemphigoid in HIV-1-positive patients: interplay or coincidence? A case series and review of the literature

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Bullous pemphigoid (BP) is an autoimmune inflammatory skin disease, mostly affecting the elderly population. Therefore, patients often have multiple comorbidities, but there is inconsistent data regarding the relationship between HIV-1 infection and BP, which has been rarely reported in combination. Herein, we describe three patients who presented with BP and concomitant HIV-1 infection that was well controlled with modern combined antiretroviral therapy. All patients received topical and oral corticosteroids. Depending on the individual severity, further add-on therapeutics, such as azathioprine, dapsone, doxycycline and the interleukin 4/13 antibody dupilumab, were added to the therapy regimen. All patients recovered from pruritic skin lesions and blistering. The cases are further discussed in the context of the current study landscape. In conclusion, HIV-1 infection shifts the cytokine profile from T-helper type 1 (TH1) towards T-helper type 2 (TH2), resulting in the excessive secretion of distinct cytokines, such as interleukin 4 (IL-4) and interleukin 10 (IL-10). With IL-4 being a main driver in the pathogenesis of BP, HIV-1-positive patients may benefit greatly from targeting IL-4 with monoclonal antibodies.

KEYWORDS

bullous pemphigoid, HIV-1, aids, immune reconstitution inflammatory syndrome, iris, dupilumab, COVID-19

1 Introduction

Bullous pemphigoid (BP) is the most frequent autoimmune blistering disease with a prevalence of 0.13% in Europe, mostly affecting the elderly population (age > 60 years) (1). The disease is caused by autoantibodies against the hemidesmosomal proteins, BP180 and BP230, which are crucial for the attachment of epidermal cells to the basal lamina (2). In

consequence, patients typically present with tense subepidermal blisters on erythematous skin accompanied by strong pruritus. However, clinical presentation is highly pleomorphic, ranging from eczema-like to multiform or urticarial lesions (3). Most cases occur spontaneously, but there are several trigger factors, such as drugs (oral antidiabetics, antibiotics, gold), human leukocyte antigen (HLA) polymorphisms (HLA-DQB1*0301), infections, vaccinations or malignant neoplasms that may induce or exacerbate BP (4). BP is also associated with different autoinflammatory diseases, and, among other factors, the TH1/TH2 and TH17/Treg homeostasis is considered to play a key role in the development of BP and other autoimmune diseases (5). Human immunodeficiency virus 1 (HIV-1) infection and acquired immunodeficiency syndrome (AIDS) lead to a T cell imbalance not only as a result of CD4+ T cell depletion, but also due to reconstitution of the immune system after initiation of a highly effective antiretroviral therapy (HAART) (6). Immune reconstitution inflammatory syndrome (IRIS) therefore contributes to the development of a variety of autoimmune disorders including Sjogren's syndrome, psoriasis, systemic lupus erythematosus (SLE) and uveitis (6–8). However, there is inconsistent data regarding the association between HIV-1 infection, IRIS and BP. Herein, we describe three HIV-1-positive patients who presented with severe BP and we review the current literature to clarify the relationship between HIV-1 infection and BP.

2 Case presentation

2.1 Case 1

In October 2022, a 60-year-old man presented with a two-week history of pruritus, erythema and tender blisters on his body. Before admission, he had been treated with intravenous steroids and antihistamines once for suspected acute urticaria. However, symptoms worsened over the following days. The patient had a history of HIV-1 CDC A2 (classification system according to the Center for Disease Control and Prevention) infection, which had been diagnosed in 2004 and was well controlled with antiretroviral triple therapy (bictegravir 50 mg, emtricitabine 200 mg and tenofovir alafenamide 25 mg) once daily. Viral load was <20 copies/mL and CD4+ count was 418/µL. He had received a third dose of an mRNA-based COVID-19 vaccine in July 2022 and reported no prior history of COVID-19 infection.

At physical examination, multiple blisters ranging in size from millimeters to several centimeters and crusted lesions on erythematous skin were found on his whole body with accentuation on his thighs and upper body (Figure 1A). Differential blood count revealed 11% (Ref. 2-4%) of eosinophilic granulocytes (1716/µL total). Total IgE level was 810 kU/L (Ref. <200 kU/L). However, the patient did not have a history of atopic dermatitis, asthma or chronic rhinitis. Highly elevated serum levels of anti-BP180 autoantibodies (147.7 U/mL, Ref. 0-9 U/mL) and linear IgG, IgM and C3 deposits at the dermal-epidermal junction (DEJ) confirmed the diagnosis of BP. For induction therapy, topical mometasone twice daily and oral therapy with prednisolone 80 mg (1 mg/kg body weight) once daily were administered. Oral doxycycline 100 mg twice daily was added to the initial therapy regimen and switched to dapsone up to 150 mg once daily after 7 days. After two weeks, the patient achieved partial remission, but still developed new lesions with blistering, so he additionally received a total of 160 g (2 g/kg body weight) of intravenous immunoglobulin (IVIG) over 4 days. During the following days no new blisters appeared and he was discharged from hospital after 18 days of treatment. Three weeks later the patient presented with a new flare of pruritic skin erosions and blistering (Figure 1B). At this time oral prednisolone was tapered (10-20 mg per week) to a dose of 30 mg once daily. Hence, dupilumab 600 mg was administered subcutaneously for induction and followed by 300 mg every two weeks for maintenance. Also, the dose of oral prednisolone was increased up to 80mg (1 mg/kg body weight) per day. He continued with dapsone 150 mg once daily and oral prednisolone was again tapered over the following weeks (10 mg per week) and then stopped completely due to significant improvement of the skin. At the 2-month follow-up visit in January 2023, the patient reported major relief with no new pruritic lesions or blistering (Figure 1C).

FIGURE 1
Case 1: In October 2023, a 60-year-old male presented to us with multiple blisters on erythematous skin **(A)**. A treatment with topical and oral steroids was administered plus intravenous immunoglobulins and dapsone as add-on therapy. Three weeks later, he presented again with pruritus, erythema and multiple bullae **(B)**, hence dupilumab was added to the treatment with dapsone and prednisolone. The skin continuously improved over the following weeks and oral steroids were tapered. At the 2-month follow-up, the patient did not show any new pruritic lesions or blistering **(C)**.

2.2 Case 2

A 62-year-old man, who had been diagnosed with HIV-1 CDC B3 in 2006, presented to us with pruritic urticarial lesions on his arms in February 2022. HIV-1 infection was well controlled with antiretroviral therapy (elvitegravir 150 mg, cobicistat 150 mg, emtricitabine 200mg and tenofovir alafenamide 10 mg). His viral load was <20 copies/mL and the CD4+ count 318/µL. In addition, he had a prior history of currently asymptomatic coronary heart disease, arterial hypertension, chronic kidney disease, hyperuricemia, steatosis hepatis, nicotine dependance (60 pack years) and alcohol abuse (3 liters of beer per day plus liquor). He also had a five-year history of hyperkeratotic papules on his right chest and the diagnosis of Darier's disease was confirmed in 2021. Concomitant medications were acetyl salicylic acid, allopurinol, fluvastatin, ramipril, amlodipine, bisoprolol, folic acid and torasemide. He had a history of mild COVID-19 infection in May 2021 and received his second dose of an mRNA-based COVID-19 vaccine in December 2021. Biopsy revealed subepidermal blistering and eosinophilic infiltration accompanied by linear deposition of IgG and C3 at the DEJ. Anti-BP180 autoantibodies were 1735.4 U/mL. Upon hospitalization, topical mometasone twice daily, oral prednisolone 60 mg (0,5 mg/kg body weight) and dapsone 50 mg once daily were initiated. In clinical remission the patient was discharged from hospital after 9 days. Under treatment with oral dapsone over the following 6 weeks, hemoglobin concentration (Hb) fell from 14,6 g/dL (Ref. 13,5-17,5 g/dL) to 12,2 g/dL. Methemoglobin level also increased from 1,1% (Ref. <1%) to 3,2% and the patient complained about progressive dyspnea. Therefore, dapsone had to be discontinued, which resulted in a relapse of pruritic urticarial lesions and blisters. Consecutively, the patient received azathioprine 100 mg/day and prednisolone was tapered over the following weeks (10mg per week). As the patient developed pulmonary embolism, he received anticoagulation with apixaban and his antiviral medication was switched from elvitegravir/cobicistat to bictegravir, also in combination with emtricitabine and tenofovir alafenamide. The cutaneous lesions gradually improved over the following months, formation of new blisters ceased and erythema faded. After six months of treatment azathioprine had to be stopped in December 2022 because of pancytopenia (Hb: 10 g/dL, Ref. 13,5 – 17,5 g/dL; Leukocytes: 2990/µL, Ref. 4000-10000/µL; Thrombocytes 127000/µL, Ref. 150000-300000/µL) and general malaise. So far the patient did not develop any new pruritic lesions or bullae off-therapy, though anti-BP180 autoantibodies were still detectable at a concentration of 241.8 U/mL. However, since Darier's disease severely deteriorated with plaque formation, acitretin 10 mg once daily was started in January 2023.

2.3 Case 3

In August 2021, a 74-year-old female presented with a three-week history of pruritic exanthema and flaccid blisters on her abdomen and both arms. She had been diagnosed with HIV-1 CDC C3 in 2003, which was well controlled with emtricitabine 200 mg, tenofovir disoproxil 245 mg and efavirenz 600 mg once daily. At the time of skin disease, viral load was 20 copies/mL and CD4+ count was 383/µL. She had a history of chronic hepatitis C (diagnosed 2003) which had successfully been treated with ribavirin and pegylated interferon-alpha in 2006, coronary heart disease with coronary artery bypass graft (ACBG), biological aortic valve replacement, mitral valve repair, chronic obstructive pulmonary disease (COPD), first-degree atrioventricular block and nicotine abuse (30 packyears). The patient had no history of COVID-19 infection or vaccination and no COVID-19 antibodies were detectable. Direct immunofluorescence showed linear C3 deposits at the DEJ. Elevated levels of anti-BP180 (392.2 U/mL) and anti-BP230 (49.9 U/mL) autoantibodies confirmed the diagnosis of a BP. When topical treatment with mometasone twice daily was administered with only partial response and little impact on pruritus, oral prednisolone with 30 mg daily per os (0,5 mg/kg body weight) was added. To achieve full disease control, the patient was given doxycycline 200 mg once daily as add-on therapy. Under treatment with oral doxycycline the patient did well without any recurrent lesions and she was discharged from hospital after seven days. The patient did not attend to a follow-up visit. Overview of cases 1-3 are given in Table 1.

3 Discussion

The relationship between dysregulation of the immune system and HIV-1 infection is complex and has not yet been fully understood. Until now, almost 40 years have elapsed since the onset of the HIV-1 epidemic in the mid-1980s (9). Since the introduction of antiretroviral therapy, the disease has changed from a life-limiting disorder to a chronic one, leading to the arise of dominant comorbidities, such as autoimmune diseases or sarcoidosis (6). With regard to the three cases presented here, the following scenarios of HIV-1-BP relation may be discussed: 1. An increased rate of BP autoantibodies in HIV-1 infection, 2. A modulation of BP autoantibodies by HAART, 3. A distinct role of IL-4 in both diseases and 4. The impact of COVID-19 or vaccination.

There are various autoantibodies that are detected more or less frequently in HIV-1-infected people than in HIV-1-negative controls, but only limited data exist on HIV-1 infection and BP autoantibodies anti-BP180 and anti-BP230. A study by Touzeau-Roemer et al. showed comparably low prevalence of anti-BP180 and anti-BP230 autoantibodies in HIV-1-infected patients and HIV-1-negative controls with 6.23% (anti-BP180) and 5.72% (anti-BP230) of 594 HIV-infected patients, respectively, compared with 5.24% (anti-BP180) and 4.03% (anti-BP230) of 248 negative controls (10). Another study of the pre-HAART era by Kinloch-de Loës et al. revealed that the incidence of BP autoantibodies is significantly higher in HIV-1-positive than in HIV-1-negative persons (11). Within this group, circulating BP autoantibodies were found in the serum of 38% of 90 HIV-1-infected patients compared to only 21% in the serum of 21 negative controls suffering from chronic pruritus. The frequency was also dependent on the duration of the HIV-1 infection, increasing from 21% in early disease to 40% in advanced stages (11). To our knowledge, only three case reports of patients with both HIV-1 infection and BP have been published so far (Table 2). In line with the findings of the previous studies, two

TABLE 1 Overview of cases.

Case	Age	Gender	HIV type	HIV-1 diagnosis	CDC stage	HIV-1 treatment	BP diagnosis	anti-BP180 [U/mL]	Therapy	COVID-19 disease	COVID-19 vaccination
1	60	male	HIV-1	2004	A2	emtricitabine, tenofovir-alafenamide, bictegravir	Oct 22	147.7	Topical mometasone, prednisolone, doxycycline, IVIG, dupilumab		3rd in July 2022 (mRNA based)
2	62	male	HIV-1	2006	B3	emtricitabine, tenofovir-alafenamide, elvitegravir, cobicistat (7/22 evg/c switched to bictegravir)	Feb 22	1735.4	Topical mometasone, prednisolone, dapsone, azathioprine	May 2021	2nd in December 2021 (mRNA based)
3	74	female	HIV-1	2003	C3	emtricitabine, tenofovir-disoproxil, efavirenz	Aug 21	392.2	Topical mometasone, prednisolone, doxycycline		

HIV, human immunodeficiency virus; CDC, Center for Disease Control and Prevention; BP, bullous pemphigoid; evg, elvitegravir; c, cobicistat.

case reports are from the pre-HAART era (12–14). These findings suggest that subsequent reconstitution of the immune system by HAART may reduce the risk of developing autoantibodies. Accordingly, recent studies have shown that there is an inverse correlation between the presence of antinuclear antibodies (ANA), anti-extractable nuclear antigens (anti-ENA), antineutrophil cytoplasmic antibodies (ANCA) and CD4+ count due to successful HAART (15).

On the other hand, successful antiretroviral treatment may lead to alterations of the T cell balance with excessive immune response and development of autoimmune disorders. For example, in patients with chronic HIV-1 infection increased levels of FoxP3-positive regulatory T cells (Tregs) are found, which upon successful treatment of the viral infection leads to transformation of the Tregs into interleukin 17 (IL-17)-producing T cells. These may in turn induce novel or exacerbate a pre-existing psoriasis (16–18).

Besides immunoglobulin G (IgG)-induced inflammation at the epidermal basement membrane, almost 50% of patients with BP have elevated serum levels of immunoglobulin E (IgE) and blood eosinophilia. This indicates that a T-helper type 2 (TH2)-polarized

TABLE 2 Published cases of patients with bullous pemphigoid (BP) and concomitant infection with human immunodeficiency virus (HIV).

Case	Published	Gender	Age	HIV diagnosis	HIV treatment	BP diagnosis	Clinical findings	Diagnostic findings	Therapy	Outcome
Levy et al. (12)	1986	male	58	1983	none	Nov 84	pruritus, erythematous papules	eosinophilia (16%), linear IgG at DEJ	ritodrine	pruritus completely resolved after 4 weeks, promptly returned after discontinuation of ritodrine
Bull et al. (13)	1993	male	58	1985	none	Sep 87	tense blisters on legs	histological examination with subepidermal blistering, linear IgG at DEJ	oral prednisolone	died due to pneumocystis pneumonia in 1990
De et al. (14)	2008	male	30	year NA, HIV diagnosis 10 years before onset of BP	nevirapine, lamivudine, stavudine (started 6 weeks before onset of BP)	NA	pruritus, tense blisters, erosions	histological examination with subepidermal blistering and eosinophilic infiltration	oral prednisolone	all lesions completely disappeared and prednisolone was tapered

There is no information available regarding the HIV subtypes. NA, not available; IgG, immunoglobulin G; DEJ, dermal-epidermal junction.

autoimmune response plays an important role in the pathogenesis of BP (19). It is known that during the course of HIV-1 infection secretion of TH1 cytokines, such as interleukin 2 (IL-2), and interferon γ (IFNγ), is generally decreased, whereas production of TH2 cytokines, interleukin 4 (IL-4), interleukin 10 (IL-10) and interleukin 13 (IL-13), is increased (20, 21). This observation suggests that TH2-mediated immune response with excessive release of IL-4, IL-10 and IL-13 may be the predominant pathomechanism in HIV-1-positive patients with BP. This may also explain why BP autoantibodies increase from early to advanced stages of the HIV-1 infection as seen in the study by Kinloch-de Loës et al. A successful antiretroviral therapy counteracts this TH1/TH2 and cytokine shift, thus reducing the seroprevalence of circulating anti-BP180 and anti-BP230 autoantibodies in HIV-1-positive patients who receive HAART. However, there is evidence that serum levels of TH2 cytokines decrease after initiation of HAART, but still persist at higher levels compared to HIV-1 negative controls (22).

If IL-4/IL-13 is assumed to be the main driver of BP especially in HIV-1-positive patients, it may also explain why patient 1 responded so well to the treatment with the monoclonal IL-4/IL13 antibody dupilumab. Dupilumab as a possible treatment option for patients suffering from moderate to severe BP has also been described in few case reports and case series (23–25) and is currently evaluated in ongoing controlled clinical studies.

Remarkably, three HIV-1-positive patients with BP presented to our department in 2021 and 2022 whereas over a long time before, no single patient has been seen. Two patients developed blistering in temporal relationship to mRNA-based vaccination against COVID-19, and there is an ongoing discussion that SARS-CoV-2 vaccines might play a role in BP initiation in healthy adults (26). The induction of new or exacerbation of existing BP by COVID-19 or respective vaccination is currently controversially debated, yet available data do not support a clear relation different from other viral infections. As known for different chronic inflammatory diseases, an induction or exacerbation may be anticipated in patients with appropriate immunogenetic background. In fact, a large population-based study by Birabaharan et al. evaluated available health data of over 70 million people who had received mRNA-based vaccines between December 2020 and June 2021. No difference in risk of new-onset BP was seen among patients who had received mRNA COVID-19 vaccine compared to the control cohort (27). However, recent studies report the development of immune reconstitution inflammatory syndrome (IRIS) in HIV-1-positive patients following COVID-19 infection due to dysregulated innate immune responses generated in the absence of effective adaptive immune responses (28). Stevenson et al. recently reported that the COVID-19 mRNA vaccine BNT162b2 leads to transcription of HIV proviruses with consecutively enhanced T-cell induction (29). This in turn may also affect autoimmunity, suggesting mRNA vaccination may act as a cofactor in the pathogenesis of autoimmune disorders in HIV-positive patients. In conclusion, the risk for BP may increase over the course of HIV-1 infection either following disbalance of T-cell responses or due to enhanced TH2-mediated immune response, leading to altered cytokine profiles with high levels of IL-4 and IL-10. Targeting of IL-4 and IL-10 by monoclonal antibodies may therefore be very effective for patients suffering from both HIV-1 and BP. However, due to the limited number of cases and published data, further prospective studies must evaluate serum levels of BP autoantibodies over the course of HIV-1 infection. Randomized controlled trials evaluating dupilumab as a treatment for BP should also focus on patients with concomitant HIV-1 infection to prove its effectiveness for this special patient group.

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

Written informed consent was obtained from the individual(s) for the publication of any identifiable images or data included in this article.

Author contributions

YF, LS, LR, TH, CB and MS participated in the examination and treatment of one or more of the patients. YF and MS collected the data and wrote the manuscript. CB, TH, LS and LR reviewed the manuscript and aided with language prettification. All authors contributed to the article and approved the submitted version.

Funding

We acknowledge financial support by Deutsche Forschungsgemeinschaft and Friedrich-Alexander-Universität Erlangen-Nürnberg within the funding programme.

Conflict of interest

CB has received honoraria from Sanofi. MS has participated in clinical studies conducted by Sanofi and Regeneron. MS also participated in advisory boards by Sanofi.

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

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frontiers | Frontiers in Immunology

TYPE Case Report
PUBLISHED 02 June 2023
DOI 10.3389/fimmu.2023.1160779

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OPEN ACCESS

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RECEIVED 07 February 2023
ACCEPTED 17 May 2023
PUBLISHED 02 June 2023

CITATION
Corbella-Bagot L, Gil-Lianes J, Fernández-Vela J, Martí-Martí I, Alegre-Fernández M, Fuertes I, Garbayo-Salmons P, Bosch-Amate X, Guilabert A and Mascaró JM Jr (2023) Case Report: Localized bullous pemphigoid induced by local triggers: a case series and a proposal for diagnostic criteria based on a literature review.
Front. Immunol. 14:1160779.
doi: 10.3389/fimmu.2023.1160779

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Case Report: Localized bullous pemphigoid induced by local triggers: a case series and a proposal for diagnostic criteria based on a literature review

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Introduction: Localized bullous pemphigoid (LBP) is an infrequent bullous pemphigoid (BP) variant restricted to a body region. According to the most compelling evidence, LBP occurs in patients with pre-existent serum antibodies against the basement membrane zone, which occasionally acquire the capacity to induce disease after the influence of different local factors acting as triggers.

Methods: We hereby present a multicenter cohort of 7 patients with LBP developed after local triggers: radiotherapy, thermal burns, surgery, rosacea, edema and a paretic leg. In addition, we conducted a review of the literature, and we propose a set of diagnostic criteria for LBP, also based on our case series and the 2022 BP guidelines from the European Academy of Dermatology and Venereology.

Results: During follow-up, three of the patients from our series evolved to a generalized BP, with only one requiring hospitalization. Our literature search retrieved 47 articles including a total of 108 patients with LBP, with a 63% with a potential local precipitating factor previous to their diagnosis. LBP mostly affected older females, and a subsequent generalized progression occurred in 16.7% of the cases. The most frequently involved areas were the lower limbs. Radiation therapy and surgery were responsible for the inducement of nearly 2 in 3 cases of LBP. We observed a significantly higher risk of generalization in cases where the trigger led to the developing of LBP earlier (p=0.016). Our statistical analysis did not detect any other prognosis factor for generalization when assessing direct immunofluorescence, histological and serological results, or other patient related factors.

Conclusion: LBP should be suspected in patients with recurrent localized bullous eruptions. The presence of a trauma history in the same anatomic area is reported in most cases.

KEYWORDS

bullous pemphigoid, localized bullous pemphigoid, criteria, precipitating factors, triggers, case report, radiotherapy, rosacea

Introduction

Bullous pemphigoid (BP) is the most frequent autoimmune bullous disease, caused by autoantibodies targeting the hemidesmosomal proteins BP180 and/or BP230. It mostly affects the elderly, patients with neurologic conditions, and users of certain medications such as gliptins and anti-programmed cell death protein 1 (anti-PD1) antibodies (1).

BP usually presents with a generalized bullous eruption. In fewer cases (2.5%-29%) lesions appear confined to a particular area: the so-called localized bullous pemphigoid (LBP) (2–4). In 1990, Domloge-Hultsch et al. corroborated that LBP shared the same 230-kd antigen and was a BP subtype (5). The pathophysiology of LBP is not fully understood. Sometimes LBP affects an area with a previous trauma or damage, which would induce the disease. Radiation therapy, surgeries, local burns, ultraviolet radiation and photodynamic therapy are some reported triggers. Latency can range from a few days to several years (6). LBP prognosis is highly varied, from complete resolution to generalization of the lesions.

LBP is however an ill-defined term. Some authors include cases affecting up to 3 body areas (7), while others consider that lesions need to be confined to a single area (8).

Clinical, pathological, serological and immunofluorescence findings resemble those of generalized BP forms. However, there is a lack of standardization regarding its diagnosis criteria. Diagnosis is often delayed as it can mimic other localized bullous diseases. LBP tends to have lower mortality rates compared to generalized BP, and often responds to topical corticosteroids (9). Systemic steroids and immunosuppressants are generally reserved as second-line therapies.

We hereby report 7 cases of LBP, all of them presenting with potential local triggers. We also performed a review of the literature and propose diagnostic criteria for LBP, based on the 2022 BP guidelines from the European Academy of Dermatology and Venereology.

Materials and methods

A case description of a multicenter cohort and a review of the literature were carried out. We performed a literature search with Pubmed from January-1976 to December-2022 using the keywords "localized" and "bullous pemphigoid". The search strategy was restricted to English language articles. Reports and reviews describing localized forms of bullous pemphigoid were analyzed. We included LBP patients with lesions restricted to a single cutaneous area. We also included cases of bilateral involvement in limbs, and cases with two contiguous body areas involved at the site of a previous trigger. Importantly, generalized BP occasionally presents with initially localized lesions. In line with the proposal from Ständer et al., we excluded cases where generalization had been reported prior to 3 months after the onset of the localized disease (4). We also excluded cases of dyshidrosiform BP, localized mucosal lesions, localized cicatricial pemphigoid (Brunsting-Perry), localized forms in patients with previous generalized BP, cases with insufficient data or without a closed diagnosis, and cases described as "localized" but with several anatomical areas involved.

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS). Quantitative variables were summarized with means or medians and standard deviations or interquartile ranges. Categoric variables were reported as percentages. Comparisons between groups to identify predictor variables for generalized BP were performed using a Chi-squared or a Fisher's exact test. P values < 0.05 were considered statistically significant.

Results

Our series includes 7 patients with a mean age of 72.3 (± 9.1) years and a male predominance (71.4%). LBP was attributed to radiation therapy in 28.6% of the cases. Mean latency from the trigger was 12.8 (± 18.3) months. Chest (42.9%) and lower limbs (28.6%) were the most frequent locations. Subsequent generalization of BP took place in 42.9% of the patients. In all cases in which histology and immunofluorescence were performed, positive results were observed, while anti-BP180 and anti-BP230 had a positivity rate of 75% and 25% among patients tested, respectively (Table 1).

Case 1

A 72-year-old woman, with a history of diabetes managed with sitagliptin/metformin, was treated with tumorectomy and adjuvant

TABLE 1 Cohort of localized bullous pemphigoid patients from the present study.

N.	Age, yrs.	Sex	Local trigger	Latency	Other contributing factors	Affected Area	Treatment	Generalization of BP (time elapsed from LBP onset)	Histology	ELISA	DIF	IIF
1	72	F	Radiation therapy	1 week after last RT session	Sitagliptin	Right chest	Topical and systemic corticoids. Topical antibiotics	Yes (10 months)	Subepidermal blister with abundant eosinophils.	Anti-BP180:+ Anti-BP230:+	Linear C3 deposits along the BMZ.	Circulating IgG autoantibodies against the BMZ on monkey esophagus, which also bound the epidermal side of 1M salt-split-skin BMZ.
2	87	M	Thermal burns	1-2 months	None	Abdomen	Topical corticoids	No	Subepidermal blister with a dermal infiltrate composed of eosinophils and lymphocytes.	Not performed	Linear IgG and C3 deposits along the BMZ.	Not performed
3	76	M	Vascular access device implantation.	3-4 weeks	Anti-PD1 drug.	Right chest	Topical antibiotics and corticoids	No	Subepidermal blister with presence of eosinophils.	Not performed	Linear IgG, C3 and mild IgM deposits along the BMZ.	Not performed
4	57	M	Radiation therapy	49 months	Anti-PD1 drug.	Right chest	Topical corticoids (initially)	Yes (14 months)	Subepidermal blister with presence of eosinophils.	Anti-BP180:+ Anti-BP230:-	Linear IgG, C3 and mild IgA deposits along the BMZ with an n-serrated pattern.	Positive, circulating IgG antibodies against the epidermal side of the BMZ.
5	68	F	Orthopedic surgery	1 month	Parkinson's disease	Lower left limb	Doxycycline, oral prednisone, methotrexate	Yes (3 months)	Suggestive of BP.	Anti-BP180:+ Anti-BP230:-	Linear deposits of IgG and C3 along the BMZ with areas of subepidermal vesiculation. IgG deposits on the epidermal side of the blister.	Not performed
6	78	M	Rosacea	NA	None	Nose	Topical corticoids	No	Subepidermal blister with a predominance of dermal eosinophils.	Anti-BP180:- Anti-BP230:-	Linear deposits of IgA (+), IgG (++), and C3 (+++), with a higher intensity on the epidermal side of the BMZ.	Not performed
7	82	M	Paretic leg	2 years	None	Lower right limb	Topical corticoids	No	Subepidermal blister with a predominance of eosinophils.	Not performed	Linear deposits of IgG and C3 along the BMZ.	Not performed

N., Case number; BP, Bullous pemphigoid; M, Male; F, Female; RT, Radiotherapy; BMZ, Basement membrane zone; DIF, Direct immunofluorescence; IIF, Indirect immunofluorescence; LBP, Localized Bullous Pemphigoid; NA, Not Available.

10.3389/fimmu.2023.1160779

radiotherapy for a breast cancer. One week after radiotherapy, she presented painful erosions, crusts, and a large bulla confined to the irradiated breast (Figures 1A, B). Histology revealed a subepidermal blister with abundant eosinophils. Direct immunofluorescence examination (DIF) showed linear deposition of C3 along the basement membrane zone (BMZ). Indirect immunofluorescence examination (IIF) showed the presence of circulating IgG autoantibodies against the BMZ, which bound the epidermal side of 1M salt-split-skin BMZ (Figures 2A, B). ELISA studies detected autoantibodies against both BP180 (234 U/mL, normal values <20 U/mL) and BP230 (70 U/mL, normal values <20 U/mL). Sitagliptin was withdrawn and systemic and topical corticoids were administered. Three months after the onset of the bullous eruption, the lesions epithelized, and oral corticosteroids could be successfully withdrawn. The patient remained free of oral corticosteroids and with clinical stability for a total of 4 consecutive months, until a new localized flare in the same area required their restart. Few days after this second course of corticosteroids was progressively tapered to 10 mg daily, the patient developed a generalization of her BP that required hospitalization. At follow up, she has presented new flares mainly localized to her right breast.

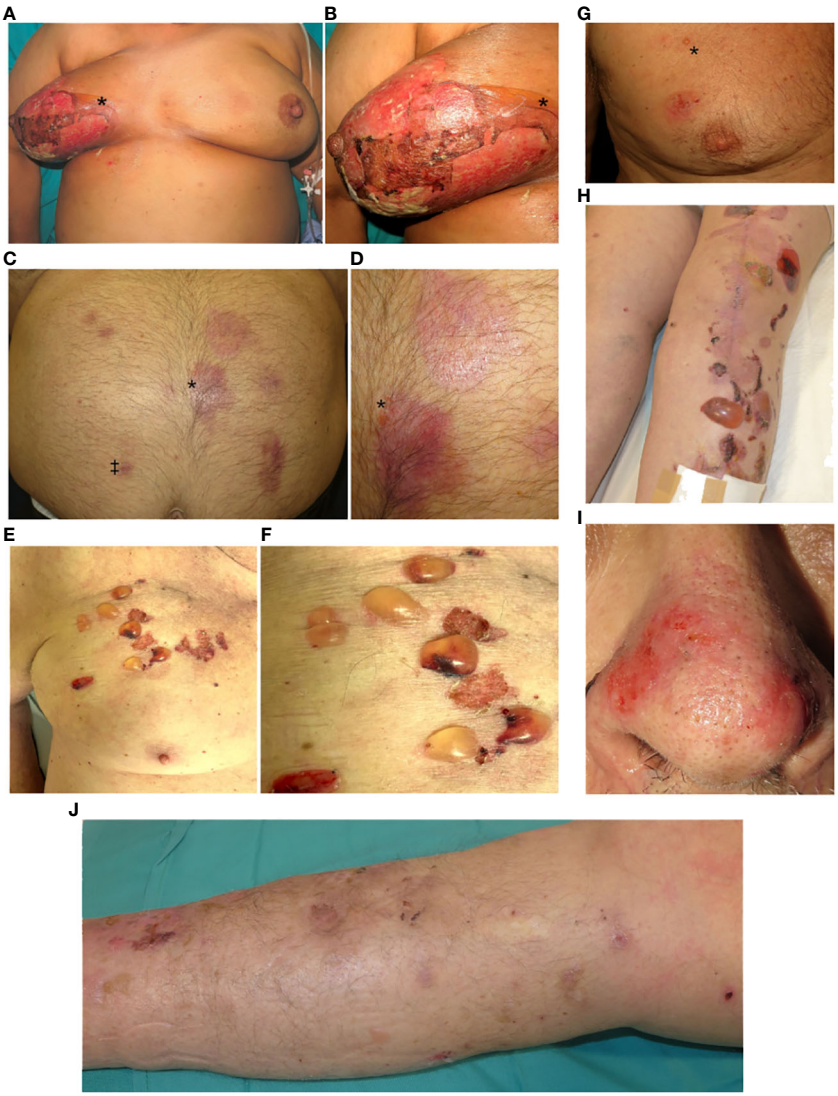

FIGURE 1
Localized bullous pemphigoid (LBP). **(A, B)** Case 1. Radiation-induced LBP: Multiple skin erosions confined to the right breast. On the upper medial quadrant a flaccid blister can be distinguished (*). **(C, D)** Case 2. Thermal burns-induced LBP: Multiple plaques in the abdomen 6 months after an accidental burn. Note the presence of milia cysts (‡) and a small tense vesicle (*). **(E, F)** Case 3. LBP induced by Central venous port insertion: Serous and sero-hemorrhagic tense blisters confined to the upper right chest. Note a small protuberance near the scar corresponding to the Port-a-Cath® device. **(G)** Case 4. Radiation-induced LBP: Presence of erosions and a tense vesicle localized on the right chest (*). **(H)** Case 5. LBP induced by orthopedic surgery: Tense bullae, erosions and crusting over an erythematous base located predominantly over the surgical scar. **(I)** Case 6. LBP induced by rosacea: Multiple erosions over an erythematous base on the nasal tip. **(J)** Case 7. LBP induced by right hemiparesis: Presence of erosions, crusting and post-inflammatory pigmentation on the right lower limb.

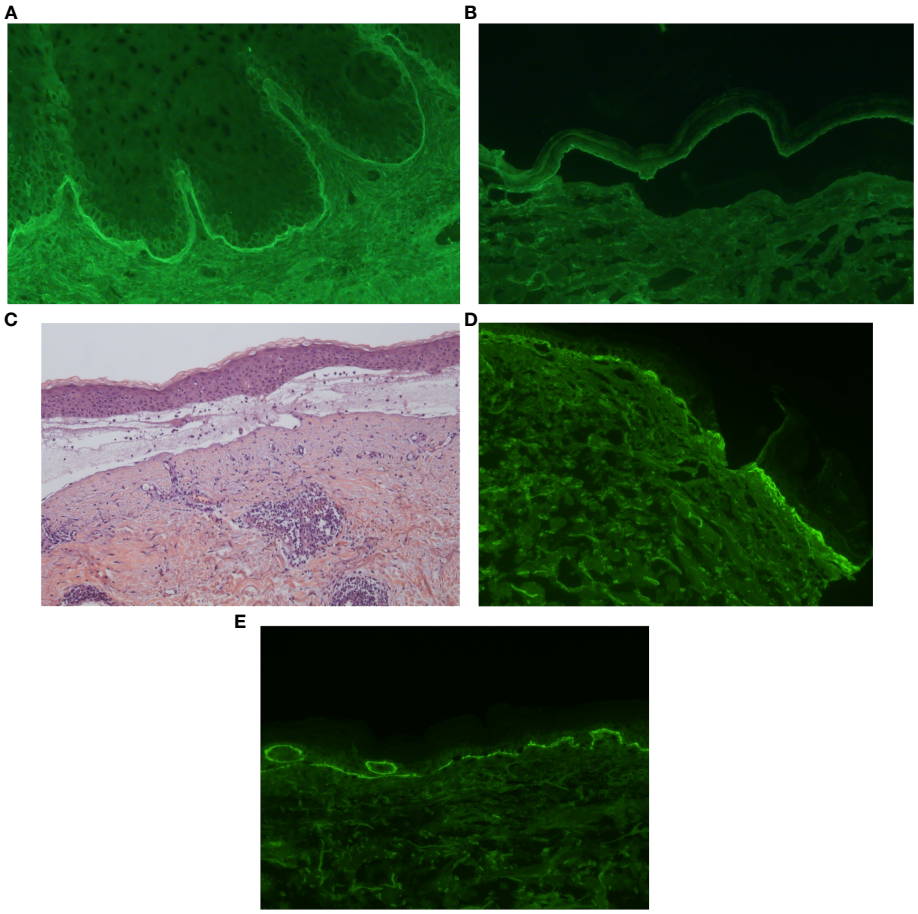

FIGURE 2
Histology and immunofluorescence studies of localized bullous pemphigoid (LBP). **(A, B)** Indirect immunofluorescence of radiation-induced LBP: IgG autoantibodies bound to the basement membrane zone (BMZ) of monkey esophagus **(A)**. These IgG autoantibodies also bound to the epidermal side of 1M salt-split-skin BMZ **(B)**. **(C–E)** Histology and direct immunofluorescence (DIF) of LBP induced by thermal burns. Images show a subepidermal blister with superficial dermal mixed inflammatory infiltrate composed of eosinophils and lymphocytes **(C)**. Direct immunofluorescence showed linear IgG **(D)** and C3 **(E)** deposits on the BMZ.

Case 2

An 87-year-old male with a history of cardiopathy suffered an accidental scalding in the abdomen. One month later, he started with asymptomatic recurrent vesicles on the previously wounded area. Physical exam revealed multiple erythematous to violaceous plaques, milium cysts and a vesicle on the edge of a plaque (Figures 1C, D). Histology showed a subepidermal blister with a mixed lympho-eosinophilic infiltrate (Figure 2C). DIF revealed lineal deposition of IgG and C3 along the BMZ (Figures 2D, E). IIF and ELISA assays were not performed. Topical corticosteroids were started with a complete remission at follow-up.

Case 3

A 76-year-old male had a history of metastatic lung adenocarcinoma. A vascular access device with a reservoir (Port-a-Cath®) was implanted for the administration of pembrolizumab. A few days later, an erythematous plaque with vesicular lesions appeared restricted to the skin under the adhesive tape. Contact dermatitis triggered by adhesive tape was suspected, and presented remission when the tape was removed. However, after 3 weeks the patient started presenting serohemorrhagic tense bullae and several erosions on the same area (Figures 1E, F). Histology showed a subepidermal blister with presence of eosinophils, and DIF showed linear IgG, C3 and mild IgM deposition along the BMZ. IIF and ELISA assays were not performed. Topical corticosteroids were prescribed with a complete remission. Immunotherapy was not interrupted. No relapses have been detected after 6 months of follow-up.

Case 4

A 57-year-old male had a history of lung adenocarcinoma treated with surgery, chemotherapy, and radiation therapy. Twenty months later, nivolumab was initiated due to metastatic

progression. After 29 months, the patient presented with a tense vesicle, multiple erosions and scars on the irradiated right pectoral area (Figure 1G). A subepidermal vesicle with eosinophils was observed in the biopsy, and linear IgG, C3 and IgA deposits along the BMZ were shown on DIF. ELISA studies detected autoantibodies against BP180 (74 U/mL, normal values <20 U/mL). IIF showed circulating linear IgG deposits against the epidermal side of the BMZ. Topical corticosteroids were started, with a complete remission. On follow-up, nivolumab was interrupted and oral prednisone was started due to an immune-mediated pneumonitis. A generalized BP flare occurred during the corticoids tapering, which required treatment with prednisone 0.5 mg/kg/day, with no further relapses after 3 years.

Case 5

A 66-year-old woman with Parkinson's disease underwent knee replacement surgery. One month later, she developed bullous lesions on the operated leg, predominantly over the surgical scar (Figure 1H). Histology was compatible with BP. DIF showed linear deposits of IgG and C3 along the BMZ, with IgG deposits located in the epidermal side of the blister. IIF was not performed. ELISA study showed BP180 autoantibodies (79 U/mL, normal values <20 U/mL). Doxycycline with gradually tapered systemic corticosteroids were administered with a good initial response. Three months after the eruption, the patient presented scattered lesions in the arms that responded to topical corticosteroids and a short course of methotrexate. The patient did not present new lesions over one year of follow-up.

Case 6

A 78-year-old man with papulopustular rosacea with nasal involvement presented with a 2-year history of vesicular lesions with erosions and crusting on the nasal tip (Figure 1I). Histology showed a subepidermal blister with abundant dermal eosinophils. DIF revealed linear deposits of IgA (+), IgG (++), and C3 (+++) along the BMZ with a higher intensity in the epidermal side of the blister. IIF was not performed. ELISA exam was negative. The patient presented a complete response to topical corticosteroids.

Case 7

A 77-year-old male with a history of ischemic stroke with residual right hemiparesis, presented two years later with pruritic papules and blisters restricted to the hemiparetic right lower extremity (Figure 1J). Histology showed a subepidermal blister with a mixed infiltrate composed predominantly of eosinophils. DIF revealed linear deposits of IgG and C3 along the BMZ. Serologic studies were not performed. Topical corticosteroids were started with a good response without relapse over the next 6 months.

Cases from a literature review

Of a total of 239 reviewed articles, we selected 47 articles with 101 patients matching the inclusion criteria. With our case series, 108 patients were included (Supplementary Table 1).

The mean age at diagnosis was 71.2 (± 14.4) years, with a female predominance (66.7%). A local trigger was identified in 63% of the cases, being radiation therapy (32.3%) and surgery (29.4%) the most frequently reported. Median latency was 150 days. Other contributing factors were reported in 26.9% of the cases. The most frequently affected areas were the lower limbs (43.9%). Topical corticosteroids were administered as the exclusive treatment in 46.6% of the cases. Generalization of BP was reported in 16.7% of the patients, after a median of 5 months from the onset of symptoms. Histological analysis yielded positive results in 86.8% of the cases, defined as a subepidermal blister with eosinophils, or alternatively a description of a "positive" or "compatible" biopsy. DIF and IIF reported positive results in 94.4% and 85.1% of the cases, respectively. Other serological analysis testing for the presence of anti-BP180 and anti-BP230 antibodies were reported in 38.9% of the cases, with a positivity of 76.2% (anti-BP180 48.7%, anti-BP230 41.0%) (Table 2). Statistical analysis detected a significant relationship between the latency time and the risk of generalization

TABLE 2 Literature review – clinical presentation, diagnosis, treatment and prognosis of localized bullous pemphigoid retrieved from Pubmed search engine and our case series (n=108).

	N (%)
Sex	
Male	70 (33.3%)
Female	35 (66.7%)
Age	
Mean years (± SD)	71.2 (± 14.4)
Local trigger	
Yes	68 (63.0%)
Radiation therapy	22 (32.3%)
Surgery	20 (29.4%)
Stoma	6 (30%)
Hemodialysis fistula	2 (10%)
UV exposure	4 (5.9%)
Phototherapy	1 (25%)
Infection	3 (4.4%)
Edema/lymphedema	2 (2.9%)
Thermal burns	2 (2.9%)
Injury	2 (2.9%)
Other	13 (19.1%)
No	40 (37.0%)

(Continued)

10.3389/fimmu.2023.1160779

TABLE 2 Continued

	N (%)
Latency	
Median months (IQR)	5 (47.9)
Affected area	
Lower limb(s)	47 (43.9%)
Chest	21 (19.7%)
Chest + other contiguous areas	2 (10.5%)
Upper limb(s)	14 (13.1%)
Abdomen	9 (8.4%)
Genital	6 (5.6%)
Facial	4 (3.7%)
Axillar	2 (1.8%)
Other	4 (3.8%)
Treatment	
Topical corticosteroids in monotherapy	34 (46.6%)
Oral corticosteroids in monotherapy	7 (9.6%)
Combination of topical and oral corticosteroids	10 (13.7%)
Combination of topical, intralesional and topical corticosteroids	2 (2.7%)
Oral corticosteroids and topical tacrolimus	2 (2.7%)
Tetracyclines-based regimens	5 (6.8%)
Niacinamide-based regimens	1 (1.4%)
Niacinamide and tetracyclines in combination	2 (2.7%)
Others	10 (13.7%)
Subsequent generalization	
Yes	18 (16.7%)
No/Not referred	90 (83.3%)
Time elapsed from LBP onset to BP diffuse progression	
Median months (IQR)	5 (6)
Histology	
Performed	68 (63.0%)
Subepidermal blister with eosinophils/Positive (description unavailable)	59 (86.8%)
Negative/Other findings	9 (13.2%)
Not performed/not mentioned	40 (37.0%)
Serological presence of anti-BP180 and anti-BP230 antibodies	
Performed	42 (38.9%)
Anti-BP180 positivity only	15 (35.7%)
Anti-BP230 positivity only	12 (28.6%)
Anti-BP180 and anti-BP230 positivity	4 (9.5%)
Positive (not specified)	1 (2.4%)
Negative	10 (23.8%)
Not performed/not mentioned	66 (61.1%)
DIF	
Performed	90 (83.3%)
Positive	85 (94.4%)
Negative	5 (5.6%)
Not performed/not mentioned	18 (16.7%)
IIF	
Performed	67 (62.0%)
Positive	57 (85.1%)
Negative	10 (14.9%)
Not performed/not mentioned	41 (38.0%)

BP, Bullous pemphigoid; LBP, Localized bullous pemphigoid; SD, standard deviation; IQR, interquartile range.

(p=0.016), showing a higher risk in cases in which the local factor quickly triggered the disease. No association was observed between the risk of progression and age, sex, affected area, presence or type of local trigger, compatible histology; or positive DIF, IIF, BP180 and/or BP230 antibodies (Table 3).

Discussion

LBP is a variant of BP confined to a single body site, with similar clinical and immunopathologic features to generalized forms, yet with some singularities: a high prevalence of localized triggers, a better prognosis (9), higher chances of therapeutic success with topical corticosteroids, and a risk of generalization.

All cases from our series involved potential triggers. In Case 1 the patient was under gliptins, a pharmacological trigger for BP. She developed LBP one week after completing radiotherapy in her right breast. In this presentation, generalized pruritus and bulla formation were decisive to differentiate BP from radiodermatitis. Oral corticosteroids were required since the affected breast was almost completely denudated and topical treatment would have most likely been insufficient. In our review, radiotherapy was the most frequent trigger reported in LBP, being breast cancer the most frequent neoplasm. In 69% of the cases, LBP appears within the first 6 months after radiotherapy and in 38%, irradiated LBP evolved to generalized BP (10), as it occurred in Cases 1 and 4. It has been observed that radiation enhances 2 to 3-fold autoantibody binding to the BMZ (11). Radiotherapy induces tumor and epidermal cell death, but Langerhans cells have shown resistance to radiation-induced apoptosis. Hence, these cells would present the exposed antigens to CD4+ T cells, eventually leading to autoantibody formation, complement activation and proinflammatory cytokine secretion.

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TABLE 3 Literature review – Statistical analysis by means of a Chi-squared test to assess potential associations to the risk of subsequent generalization in the reported sample (n=108).

Variable	No/non referred subsequent generalization	With subsequent generalization	Difference (%)	p-value (CI 95%)
Sex			-13.4%	0.272 (-0.0827, 0.351)
Female	56 (64.4%)	14 (77.8%)		
Male	31 (35.6%)	4 (22.2%)		
Age (years)			-6.7%	0.411 (-0.250, 0.116)
≤50	9 (10.0%)	3 (16.7%)		
>50	81 (90.0%)	15 (83.3%)		
Local trigger			-17.8%	0.154 (-0.395, 0.0393)
Yes	54 (60.0%)	14 (77.8%)		
No	36 (40.0%)	4 (22.2%)		
Local trigger				0.294
RT	17 (31.5%)	5 (35.7%)	-4.2%	
Surgery	14 (25.9%)	6 (42.9%)	-27.0%	
Other	23 (42.6%)	3 (21.4%)	21.2%	
Latency (days)				0.016
≤15	4 (11.8%)	6 (50.0%)	-38.2%	
16-60	9 (26.5%)	3 (25.0%)	1.5%	
>60	21 (61.8%)	3 (25.0%)	36.8%	
Affected area				0.215*
Chest (exclusively)	16 (18.0%)	3 (16.7%)	1.3%	
Abdomen	8 (9.0%)	1 (5.6%)	3.4%	
Axilla	2 (2.2%)	0 (0%)	2.2%	
Upper limb	12 (13.5%)	2 (11.1%)	2.4%	
Lower limb	39 (43.8%)	8 (44.4%)	-0.6%	
Site of irradiation	1 (1.1%)	0 (0%)	1.1%	
Genital	6 (6.7%)	0 (0%)	6.7%	
Chest and other contiguous areas	2 (2.2%)	0 (0%)	2.2%	
Inguinal	0 (0%)	1 (5.6%)	-5.6%	
Facial	3 (3.4%)	1 (5.6%)	-2.2%	
Back	0 (0%)	1 (5.6%)	-5.6%	
Neck	0 (0%)	1 (5.6%)	-5.6%	
Treatment				0.723*
Topical corticosteroids	27 (45.8%)	7 (50.0%)	-4.2%	
Oral corticosteroids	5 (8.5%)	2 (14.3%)	-5.8%	
Topical and oral corticosteroids	6 (10.2%)	4 (28.6%)	-18.4%	
Niacinamide and tetracycline	2 (3.4%)	0 (0%)	3.4%	
Topical, systemic and IL corticoids	2 (3.4%)	0 (0%)	3.4%	

(Continued)

TABLE 3 Continued

Variable	No/non referred subsequent generalization	With subsequent generalization	Difference (%)	p-value (CI 95%)
Oral corticoids and topical tacrolimus	2 (3.4%)	0 (0%)	3.4%	
Other regimens Other niacinamide-based regimens	9 (15.3%) 1 (1.7%)	1 (7.1%) 0 (0%)	8.2% 1.7%	
Other tetracycline-based regimens	5 (8.5%)	0 (0%)	8.5%	
Histology			-6.0%	0.581 (-0.122, 0.241)
Positive	48 (85.7%)	11 (91.7%)		
Negative	8 (14.3%)	1 (8.3%)		
Anti-BP180 and anti-BP230 antibodies			-9.8%	0.609 (-0.253, 0.431)
Positive	25 (73.5%)	5 (83.3%)		
Negative	10 (26.5%)	1 (16.7%)		
Direct immunofluorescence			-6.2%	0.340 (0.00932, 0.114)
Positive	76 (93.8%)	14 (100%)		
Negative	5 (6.2%)	0 (0%)		
Indirect immunofluorescence			0.9%	0.939 (-0.226, 0.209)
Positive	47 (85.5%)	11 (84.6%)		
Negative	8 (14.5%)	2 (15.4%)		

BP, Bullous pemphigoid; LBP, Localized bullous pemphigoid; IL, Intralesional; SD, standard deviation; IQR, interquartile range; CI, Confidence Intervale; RT, Radiotherapy. * Calculation performed by means of Fisher's exact test.

Breast cancer cells have shown to express hemidesmosomes *in vitro*. Thus, radiotherapy-induced apoptosis could release BP180 and BP230 antigens, which may become immunogenic and lead to autoantibody production (10).

Case 2 was triggered by thermal burns, which have been reported as rare inductors of BP and pemphigus vulgaris (12). Cases 3 and 5 were triggered by surgeries, one of the most frequent triggers of LBP, although we have not found any report of LBP triggered by Port-a-Cath® implant surgery in the literature. We believe that in Case 3 the prior diagnosis of contact dermatitis was in fact an early manifestation of LBP. Due to their clinical similarities, early manifestations of LBP have been misdiagnosed as contact dermatitis in several cases (13), which is an infrequent but also reported cause of LBP (14). Pembrolizumab (Case 3) and nivolumab (Case 4) are anti-PD1 monoclonal antibodies with the potential to induce LBP. However, both patients did not develop new lesions with subsequent drug administrations. Even though some inflammatory cutaneous diseases have been described as triggers for LBP (13, 15, 16), Case 6 is to our knowledge the first reported case secondary to rosacea. In rosacea, DIF may be not specific of LBP, since an IgG, IgM or C3 band, with or without dermal deposits, have been observed in 39% of the cases (17) and histological features of BP would be mandatory for the diagnosis. In Case 7, skin lesions were confined to the hemiparetic limb, a rare but reported presentation of LBP (18–22). Association between neurologic disorders and BP is well known. It has been proposed that local neuromuscular and vascular changes in the paretic leg, together with immobility and scratching can contribute to the onset of LBP lesions (19). Moreover, an injured cutaneous site, such as the lymphedematous paretic limb, may become an immunocompromised district in which the neuro-immunocutaneous system would become destabilized, predisposing the area to the development of secondary diseases, including autoimmune disorders (22). This mechanism has been proposed to act more intensely on late-onset induced LBPs (23). Differential diagnosis of LBP includes *bullosis diabeticorum*, bullous drug eruption, insect bite reaction, other autoimmune bullous diseases and viral infections. Potent topical corticosteroids usually suffice for disease control. For extended or refractory cases, systemic corticosteroids and/or other immunosuppressants can be considered.

The mechanisms underlying how LBP is triggered are not fully understood. It has been demonstrated with mouse models with epidermolysis bullosa acquisita how skin areas exposed to mechanical irritation showed increased autoantibody binding along the dermoepidermal junction and severe clinical manifestations (24).

According to one hypothesis evaluated ex-vivo, LBP would present in initially asymptomatic, genetically susceptible individuals with pre-existent serum antibodies against the BMZ. These autoantibodies would bind to BMZ proteins without inducing the disease. After tissue destruction, the wound remodeling process would stimulate the synthesis of vascular endothelial growth factor (VEGF), increasing vascular permeability, leading the migration of granulocytes and other

inflammatory cells, and enhancing anti-BP180 and anti-BP230 circulation. The presence of granulocytes in the BMZ would trigger the disease by binding to these autoantibodies, activating the complement system (6).

The results from our literature review show a clear female preponderance in LBP with twice as many cases in females than in males, which is consistent with a female-to-male ratio of 1.04-5.1 reported in BP (25). Cases triggered by radiation therapy in breast cancer and the female predominance among the elderly population can explain these results. Radiation therapy and surgery were the most frequent triggers in LBP, though these patients can be overrepresented considering that some case series included were restricted to patients with these triggers. Interestingly, we found that 16.7% of the cases presented generalization of lesions. The most frequently affected areas were the lower limbs. In line with Kohroh et al. (26), we believe that increased hydrostatic pressure, friction due to socks and pretibial microtrauma can play a role in LBP development in these regions. Latency from the local trigger to the onset of the bullous eruption ranged from 2 days to 47 years. Even though lower rates of positive results in DIF and in anti-BP180 ELISA compared to generalized BP had been detected in previous studies (8, 27), we found a 94.4% positivity in DIF and 76.2% in ELISA studies. Interestingly, a study showed an increased auto-reactivity in LBP to BP230 IgG compared to classic BP (28). We only found higher risk for generalization in cases in which the local trigger induced the disease within the first 15 days. On the other hand, cases induced by a trigger with more than 60 days of latency had a lower risk for generalization. As mentioned previously, it has been proposed that LBP could be induced by different mechanisms in early and late-onset triggered LBP. Thus, the generalization risk could differ too. Further research is needed to elucidate the implications of this association. The lack of differences in test results compared to cases that remain localized support the theory that LBP diagnosis should not be retrospectively discarded in cases which get generalized after at least 3 months. Thus, we believe that both these subtypes correspond to the same variant of BP.

Limitations of this study include the variable follow-up time and the designation of triggers with a temporal and spatial relationship with LBP onset which may not always be responsible for LBP. In articles where the performance of serological, histological or DIF exams was not mentioned, we could not discern reliably between negative and not performed results. The prerequisite of 3 months of localized activity to accept the diagnosis and discard an early classic form of BP is arbitrary, and could require adjustments in further studies as more knowledge on the field is available. Due to the retrospective nature of the study, results shown can also be influenced by a publication bias.

Diagnostic criteria for Localized Bullous Pemphigoid

Diagnosis of LBP is often delayed, and misdiagnosis is not rare (29). With the purpose to contribute to early diagnosis, we propose that LBP diagnosis could be based on an adaptation of the recently published criteria for BP (30). LBP will be confirmed by the fulfillment of three of the following four criteria, with the clinical criteria being mandatory:

1. **Clinical criteria**:

Presence of a bullous eruption confined to a single anatomic region, sometimes preceded by a predisposing factor ("triggered LBP"), without a previous history of generalized BP. In the event of a subsequent generalization, lesions must have remained localized for at least 3 months and patients should not have received systemic corticosteroids in the intervening period. *

2. **DIF criteria**:

Positive DIF with linear deposits of IgG and/or C3 along the BMZ (preferably with an n-serrated pattern). Sometimes IgA and IgE with a similar pattern.

3. **Serological criteria**:

Positive IgG antibodies against the epidermal side of BMZ by IIF.

and/or

Positive IgG antibodies reacting with BP180 and/or BP230 by ELISA, IIF, immunoblot, or immunoprecipitation.

4. **Histological criteria**:

Subepidermal blister with the presence of eosinophils.

*In localized lesions with absence of blisters, LBP can be accepted in patients fulfilling both DIF and serological criteria.

Conclusion

LBP should be considered in patients presenting recurrent local bullous eruptions, especially in patients with a history of exposure to any known trigger, which are responsible for 63% of LBP. Diagnostic confirmation using DIF, serology and histology is required as in other variants of BP. Even though we have not found any prognosis factor for generalization in LBP among complementary tests, we believe that serologic exams and histology are crucial for a reliable diagnosis. We propose new diagnostic criteria for LBP to aid rapid diagnosis and direct treatment. Topical corticosteroids are enough for the control of milder forms of LBP, but at least half of the patients will require systemic therapies at some point of their disease.

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

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and

institutional requirements. 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

LC-B wrote the first draft, performed the review of the literature and statistical analysis, and wrote the final version. JM contributed to conception of the work, and leaded in the writing. JG-L contributed in the design of the study and statistical analysis and performed multiple reviews of the text. JF-V wrote sections of the manuscript. XB-A contributed in the design of the study and performed multiple reviews of the text. AG leaded in the description of the patients from Hospital de Granollers and in the design of the study. All authors (LC-B, JM, JG-L, JF-V, XB-A, AG, PG-S, IM-M, MA-F, IF) participated in the inclusion of patients and their data submission to our case series. 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1160779/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Brief Research Report
PUBLISHED 15 June 2023
DOI 10.3389/fimmu.2023.1135002

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OPEN ACCESS

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RECEIVED 31 December 2022
ACCEPTED 30 May 2023
PUBLISHED 15 June 2023

CITATION
Koga H, Teye K, Sugawara A, Tsutsumi M, Ishii N and Nakama T (2023) Elevated levels of interleukin-9 in the serum of bullous pemphigoid: possible association with the pathogenicity of bullous pemphigoid. *Front. Immunol.* 14:1135002. doi: 10.3389/fimmu.2023.1135002

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Elevated levels of interleukin-9 in the serum of bullous pemphigoid: possible association with the pathogenicity of bullous pemphigoid

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Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease (sAIBD). In addition to disease causing autoantibodies, several leukocyte subsets, including mast cells and eosinophils, play key roles in mediating skin inflammation. Detailed immunophenotyping and, more recently, the therapeutic effects of interleukin-4 (IL-4) receptor alpha inhibition in BP pointed to a prominent role of T helper 2 (Th2) cells. Among other cell types, IL-9 is expressed by Th2 and mast cells and potentially drives allergic, Th2-dominated inflammation. Although cytokines in BP have been relatively well investigated, the role of IL-9 has remained enigmatic. This study aimed to evaluate the effect of IL-9 in BP. Serum IL-9 levels were significantly elevated in patients with BP and decreased upon induction of remission. Serum IL-9 levels were not elevated in epidermolysis bullosa acquisita, another sAIBD. The time-course analysis using serum sets from four patients with BP revealed that serum IL-9 was a sensitive biomarker of BP. IL-9–positive cells infiltrated dominantly in BP lesions, especially in the blister fluid, and Th9 cells were abundant. Therefore, IL-9 was elevated in the serum and lesions of BP, which could be a biomarker of BP.

KEYWORDS

autoimmune bullous disease, autoantibody, basement membrane, cytokine, pemphigoid

Introduction

Bullous pemphigoid (BP), an autoimmune bullous disease, is characterized immunologically by the presence of circulating autoantibodies directed to BP180 (type XVII collagen) and BP230, which are components of hemidesmosomes at the basement membrane zone (BMZ) of the skin. Antibody binding to target proteins, especially the

NC16A domain of BP180, at the BMZ is necessary for blister formation, whereas mast cell degranulation plays a key role in inflammation (1, 2). In addition to neutrophils, macrophages showed pathogenic roles *in vivo* (1). The histopathology of a lesional biopsy from patients with BP shows subepidermal splitting and a moderate-to-dense inflammatory infiltration composed of lymphocytes, neutrophils, and, most characteristically, eosinophils (1). Moreover, eosinophils can cause blistering *ex vivo* (3). Other than Immunoglobulin (Ig) G, IgE direct to BP180 and elevation of total serum IgE have been reported in BP (4). Interestingly, IgE deposition at the BMZ was associated with disease severity and treatment efficacy for BP (5). Thus, eosinophils, neutrophils, and mast cells activated by autoantibodies binding to the BMZ and cytokines that have IgG shift to IgE appear to be involved in the pathogenicity of BP. Interleukin-9 (IL-9) is expressed by T helper (Th) cell subsets, including Th2 and Th17, as well as regulatory T (Treg) cells, natural killer T cells, and mast cells. A preferential IL-9 producing Th subset was discovered and named Th9 cell (6). IL-9 exerts various effects, including the promotion of mast cell growth and activation, Th17 proliferation, transforming factor-β (TGF-β) production, and downregulation of IL-12 production in antigen-presenting cells (7, 8). Meanwhile, IL-9R knockout mice showed less suppressive effects on Treg cells and severe inflammation compared with wild-type ones in the experimental autoimmune encephalomyelitis (EAE) model (9). Some studies showed that IL-9 was involved in IgE class switch (10). IL-9 is thought to have detrimental roles in allergy such as asthma; autoimmunity, including EAE; type I diabetes; and parasitism. Recently, the anti-tumor effect of IL-9, especially to melanoma, was also reported (11). Regarding Th cell subsets and cytokines in BP, Th2, Th17, and their related cytokines, including IL-4, IL-5, IL-13, IL-17, and IL-21, have been reported (12). To the best of our knowledge, the influence of IL-9 on the pathogenesis of BP has not yet been investigated.

Method

Patients

Twenty-two patients with BP who were admitted to our hospital from 2013 to 2016 were enrolled in this study. Patients with BP met the following criteria: i) clinical manifestations (blistering or erythematous/urticarial plaques on the skin and/or mucosa), ii) subepidermal blistering confirmed by histopathology, iii) linear deposition of IgG and/or C3 at the BMZ as determined by direct immunofluorescence using standard procedures, and iv) positive for BP180 enzyme-linked immunosorbent assay (ELISA)/chemiluminescent enzyme immunoassay (CLEIA). Sera from patients with BP were taken at admission. Sera taken at the remission stage were also collected from the same patients. Twenty patients with epidermolysis bullosa acquisita (EBA) were enrolled in this study based on the following: i) compatible clinical features, ii) IgG reaction on the dermal side by indirect immunofluorescence of salt-split skin, and iii) detection of IgG antibodies to type VII collagen by Western blotting using a dermal extract of human skin and/or ELISA. The ethics committee of Kurume University (22008) approved the study, which was carried out in accordance with the Declaration of Helsinki guidelines.

ELISA

Serum levels of IL-9 and total IgE were measured using the LEGEND MAX™ Human IL-9 ELISA Kit (Biolegend, San Diego, CA) and the Human IgE ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's instructions, respectively. IgE antibodies directed to BP180 were measured as described previously (13). Four patients with BP who showed higher serum levels of IL-9 were selected for the time-course analysis.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) skin samples taken from skin lesions of patients with BP, atopic dermatitis, and psoriasis vulgaris and from the excision margin of skin tumors were used. FFPE sections were deparaffinized and rehydrated in xylene and 100% graded ethanol, respectively. FFPE sections were labeled with the rabbit polyclonal anti–IL-9 antibody (dilution, 1:200; ab181397, Abcam, Cambridge, MA, USA) using the Bond-III autostainer (Leica Microsystems, Newcastle, UK). Briefly, IL-9 antigen retrievals were heat-treated using epitope retrieval solution 2 (pH 9.0) for 30 min at 99°C and incubated with this antibody for 30 min at room temperature (RT). This automated system used the Refine Polymer Detection System (Leica Microsystems, Newcastle, UK) with a horseradish peroxidase polymer as a secondary antibody and 3,3′-diaminobenzidine (DAB) as the chromogen. The slides were visualized using DAB.

Immunofluorescence

FFPE skin samples taken from the skin lesions of one patient with BP were used. FFPE skin samples taken from the lesional skin of a patient with BP were subjected to deparaffinization and dehydration by passage through xylene and an alcohol series, followed by antigen retrieval with 10 mM sodium citrate, pH 6.0 (autoclave at 120°C for 20 min). The sections were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. After blocking with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 10% goat serum for 20 min, the cells were incubated with rabbit polyclonal anti–IL-9 antibody (ab181397, Abcam, Cambridge, MA) and anti-mast cell tryptase antibody (clone AA1; sc-59587, Santa Cruz Biotechnology, Dallas, TX), anti-forkhead box protein 3 (FOXP3) antibody (clone 236A/E7; Abcam, Cambridge, MA), anti-GATA binding protein 3 (GATA3) antibody (clone HG3-31; sc-268, Santa Cruz Biotechnology, Dallas, TX), anti-retinoic acid-related orphan receptor γt antibody (clone AFKJS-9; eBioscience, San Diego, CA), or anti-PU.1 antibody (clone G148-74; BD Pharmingen, San Diego,

CA) for 60 min RT, followed by Alexa 488 anti-mouse IgG and Alexa 568 anti-rabbit IgG (Invitrogen, Carlsbad, CA) for 30 min RT.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical calculations were performed using GraphPad Prism (version 6.05; GraphPad Software). The tests used are indicated in the figure legends. A p-value of 0.05 was considered statistically significant.

Results

Elevation of IL-9 levels in the serum of BP, not of EBA

The IL-9 concentration in 22 serum samples from patients with BP and serum from healthy volunteers [normal control (NC)] was measured by commercial ELISA. Serum IL-9 levels were significantly elevated in patients with BP (Figure 1A). During remission, serum IL-9 levels in patients with BP decreased (Figure 1B). EBA is a rare and another type of AIBD whose autoantibodies target type VII collagen, a component of anchoring fibril, and share some aspects seen in BP. Notably, serum IL-9 levels in EBA were not elevated and were comparable to that in NC (Figure 1A).

Serum levels of total IgE and specific IgE directed to BP180 are not related to serum IL-9 levels in BP

The relationship between serum total IgE and IL-9 levels in BP is yet to be investigated, although previous research indicated that IL-9 was involved in the IgE class switch (10). Therefore, serum levels of total IgE and IgE anti-BP180 antibodies were determined. In the analysis of the Pearson correlation test, we did not observe any significant correlations between serum total IgE and IL-9 ($r = 0.063$, $p = 0.284$) or serum IgE anti-BP180 and IL-9 ($r = 0.003$, $p = 0.797$) in BP sera (Figures 1C, D).

Sensitivity of serum IL-9 as a biomarker for BP

The time-course analysis of serum IL-9 level was conducted using sera from four patients with BP, wherein sera were taken every week from admission. The peripheral number of eosinophils and titers for anti-BP180 IgGs was previously reported as

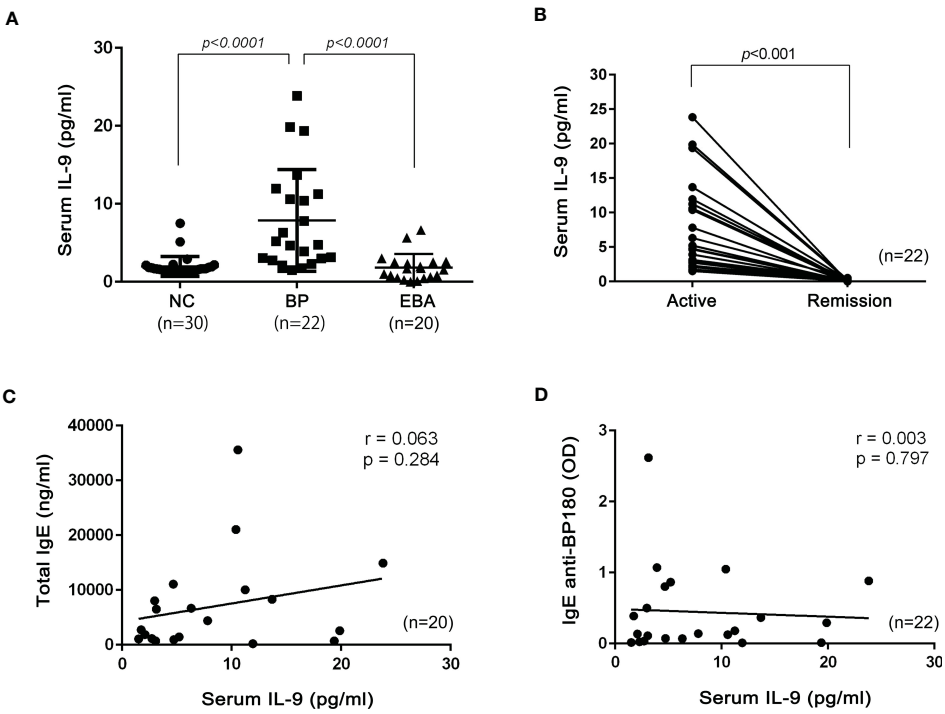

FIGURE 1
Serum IL-9 in patients with bullous pemphigoid (BP) and epidermolysis bullosa acquisita (EBA). **(A)** Serum IL-9 concentrations in normal control (NC; n = 30), BP (n = 22), and EBA (n = 20). One-way ANOVA followed by Kruskal–Wallis test was performed. **(B)** Comparison of IL-9 concentrations between patients in the active stage and in the remission stage of BP (n = 22). The Mann–Whitney test was used. The Pearson correlation test was performed with serum total IgE (**C**; n = 20) or serum IgE anti-BP180 (**D**; n = 22) and serum IL-9.

biomarkers for BP (14). In all four cases, serum IL-9 levels rapidly decreased, which was faster than the peripheral number of eosinophils and titers for anti-BP180 IgGs (Figures 2A–D). Interestingly, one patient (Figure 2A) had relapsed clinically during the disease course and required a high intravenous dose of methylprednisolone (mPSL) pulse therapy. At the time, the serum IL-9 levels and peripheral number of eosinophils were elevated again but not the titer for anti-BP180 IgG.

IL-9–positive cells infiltrated predominantly in BP skin lesions and blister fluid

To visualize whether IL-9–positive cells were predominant in BP lesions, immunohistochemistry analysis with anti–IL-9 antibody was conducted. Among skin diseases, it was reported that the patient's skin with psoriasis expressed a higher number of IL-9R–positive cells and the patient's skin with atopic dermatitis expressed higher IL-9 mRNA compared with the normal skin (15, 16). With these findings, IL-9–positive cells infiltrated the dermis of the patients' skin with atopic dermatitis and psoriasis, although they were insignificant. Interestingly, IL-9–positive cells infiltrated the skin, particularly in the blister fluid of patients with BP, with significance compared with NC ($p < 0.0001$) (Figures 3A, B).

Th9 cells are the main source of IL-9 in the blister fluid of BP

IL-9 is released by Th9, Th2, Th17, Treg, and mast cells (6). To clarify which cells released IL-9 in the blister fluid of BP, lesional skin samples from patients with BP were stained with transcription factors: PU.1, GATA3, RORγt, FOXP3, tryptase, and IL-9 (Figure 4). IL-9–positive cells strongly expressed PU.1, the transcription factor of Th9 cell, but not GATA3, RORγt, FOXP3, and tryptase. This result revealed that Th9 cells were the main cell sources of IL-9 in the blister fluid of BP.

Discussion

Previous studies showed the IL-9 level elevation in several dermatological diseases, including atopic dermatitis, psoriasis vulgaris, and cutaneous T-cell lymphoma (17). In atopic dermatitis, IL-9 levels correlated with clinical severity, IgE levels, and C-C motif chemokine ligand 17 (CCL17) levels (16). In psoriasis, IL-9 in induced Th17-dependent psoriasis-like skin inflammation and angiogenesis was reported (15). In mycosis fungoides, the most common form of cutaneous T-cell lymphoma, malignant and reactive T cells produce IL-9 in skin lesions and the number of producing cells decreased following a successful therapy (18). This

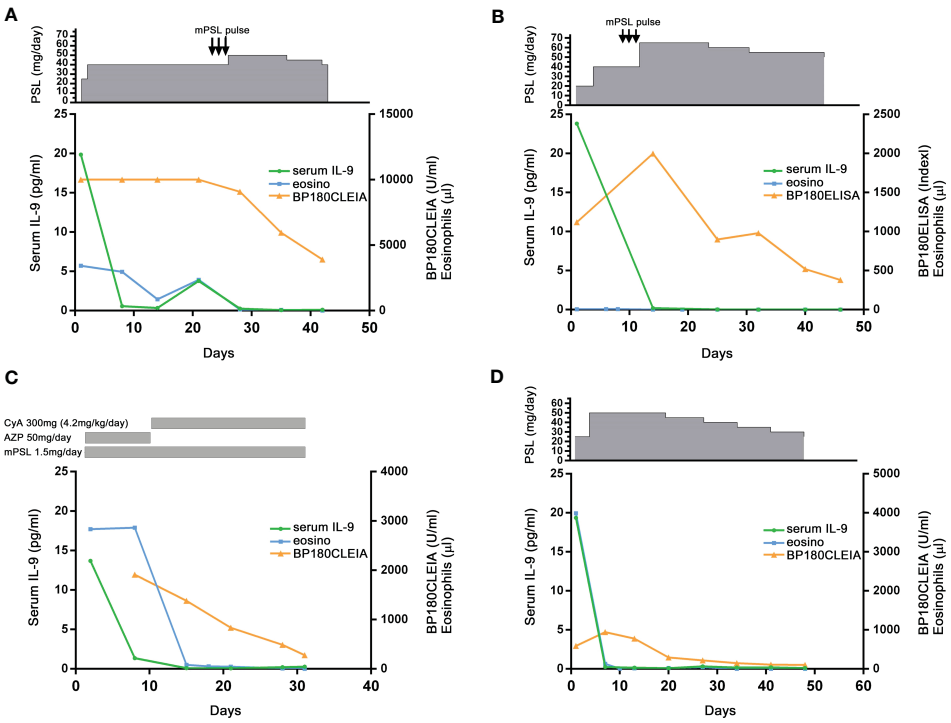

FIGURE 2
Time-course analysis of serum IL-9 levels during treatment for bullous pemphigoid (BP). In four patients with BP **(A–D)**, serum IL-9, anti-BP180 titer (ELISA or CLEIA), and the absolute count of peripheral eosinophils were measured at the initiation of the treatment every week (except case **C**). The treatments were informed on the upper section of each figure. PSL, prednisolone; mPSL, methylprednisolone; mPSL pulse, mPSL pulse therapy (1,000 mg of mPSL for 3 days); CyA, cyclosporin A; AZP, azathioprine.

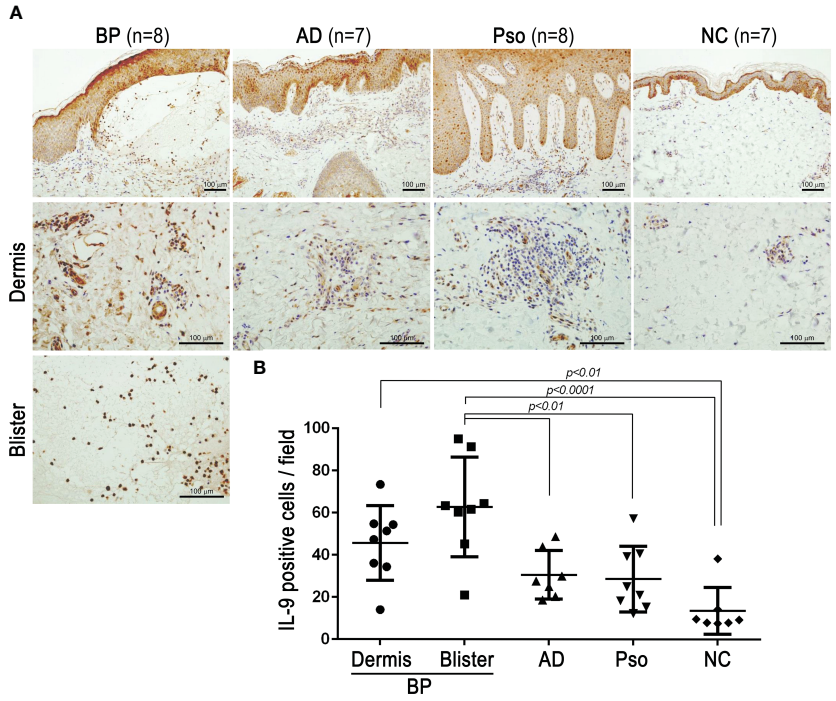

FIGURE 3
IL-9 expressions in the lesional skins of bullous pemphigoid (BP), atopic dermatitis (AD), and psoriasis vulgaris (Pso). Biopsied skin sections from BP (n = 8), AD (n = 7), Pso (n = 8), and normal control (NC; n = 7) were stained with anti-human IL-9 antibodies by immunohistochemistry. **(A)** Representative pictures with low magnification (upper panels) and high magnification in the dermis (middle panels) or blisters (lower panel) (bar = 100 μm). **(B)** IL-9–positive cells were counted at ×200 magnification. The average of counts from three fields at ×200 magnification was evaluated in each section. Data were analyzed by one-way ANOVA followed by Tukey's test.

study revealed that IL-9 levels were elevated in the serum, the skin lesion, and the blister fluid of BP and decreased in the remission stage following a successful therapy. This study revealed that Th9 cells are the main sources of IL-9 in the blister fluid of BP. Th9 cells are an important source of IL-9 and promote mast cell accumulation (8). The pathogenic role of mast cells has been reported in BP but not in EBA. The experimental EBA mice model by the injection of murine type VII collagen IgG could induce similar severity of the disease in

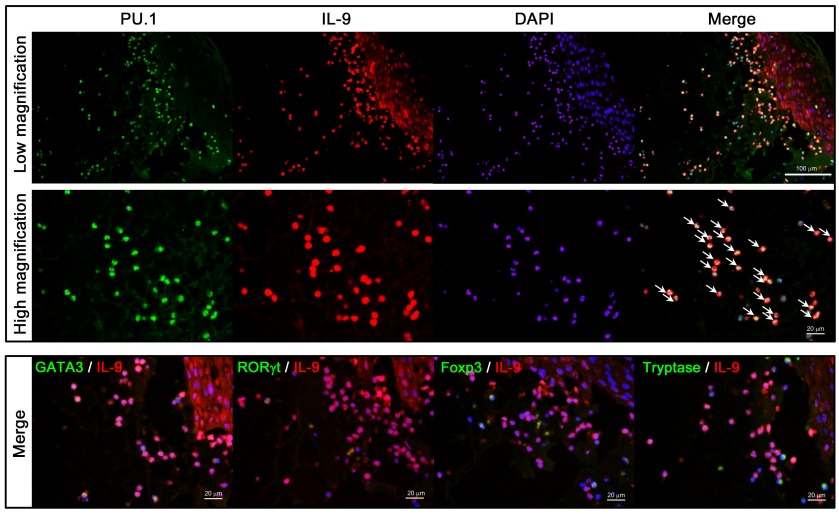

FIGURE 4
Double staining of IL-9 and transcription factors for Th2, Th17, Th9, or tryptase. A biopsied specimen from lesional BP skin was stained with combinations of anti–IL-9 (red) and the following antibodies (green): anti-PU.1 for Th9 cells (upper and middle panels), anti-GATA3 for Th2 cells, anti-RORγt for Th17 cells, anti-Foxp3 for regulatory T cells, and anti-tryptase for mast cells (lower panels), followed by 4', 6-diamidino-2-phenylindole (DAPI) to counterstain the nucleus (blue). Double-positive cells are highlighted with arrows.

both wild-type and mast cell–deficient mice (19, 20). Although BP and EBA share various similarities clinically and pathologically, the states of Th9 cells and releasing of IL-9, resulting in the accumulation of mast cells, appear different between these diseases. Compared with BP, cytokine balance in EBA is barely explored. The finding that blockades of IL-1/IL-1Ra, tumor necrosis factor-α (TNF-α), and IL-17R were impaired the disease in EBA mice model indicated that these cytokines were involved in the pathogenesis of EBA (21). Niebuhr et al. reported Th1 dominance in scratch-induced skin wounds in the EBA mice model (22). This study clearly indicated that IL-9 was not involved in EBA, and the distinctive trend in IL-9 level might be useful when EBA should be distinguished from BP in clinical practice.

Singh et al. reported that intradermal injection of IL-9 caused epidermal hyperplasia and dermal infiltration of $CD3^+$ T cells, $CD68^+$ monocytes/macrophages, and mast cells that were suppressed by pre-anti–IL-17 administration in mice. Furthermore, IL-9 injection enhanced the Th17 pathway in the psoriasis mice model (15). These results suggested that IL-9 was involved in the Th17 pathway, and Th17 cells were the main sources of IL-9 in psoriasis. Although Th17 cells and IL-17 were reported to be involved in the pathomechanism of BP (23, 24), this study showed that Th17 cells rarely produced IL-9 in the blister fluid of BP, suggesting that the pathogenic role of IL-9 differs between BP and psoriasis. Our study found no association between serum total IgE or serum anti-BP180 IgE and serum IL-9 in patients with BP, although a correlation between IL-9 and IgE levels in atopic dermatitis was reported (16). These findings suggested that IL-9 was not the primary inducer of high serum total IgE levels and the presence of IgE anti-BP180 in BP.

This study revealed that serum IL-9 level is an available biomarker to evaluate the disease activity in patients with BP. BP180 IgG and IgE levels and absolute peripheral eosinophil count are known biomarkers of BP. Among other cytokines, chemokines, and coagulation markers, eosinophil cationic protein, CXC chemokine–interleukin-8 (CXCL-8), CCL17, IL-5, IL-10, D-dimer, and F1 + 2 were also reported (14). Following treatment, serum IL-9 levels significantly decreased in patients with BP. Moreover, our fine time-course analysis showed that serum IL-9 levels rapidly decreased in all four patients after the initial treatment for treatment-naïve patients. The serum IL-9 levels and absolute peripheral eosinophil count, but not BP180 antibody, reincreased in one patient (Figure 2A) who experienced a flare that required additional treatment of mPSL pulse therapy. These results suggested that serum IL-9 is an incisive marker during the treatment for BP. However, further studies are needed to confirm this.

In conclusion, this study revealed that IL-9 was elevated in BP, but not in EBA, and that it is released by Th9 cells in the blister fluid. Moreover, the serum IL-9 level decreased in the remission stage of BP, suggesting its pathogenic role in BP. The distinct profile of serum IL-9 level may explain different pathomechanisms between BP and EBA. Two clinical subtypes exist in both BP and EBA, i.e., inflammatory type and non-inflammatory type (21, 25). This study could not address whether IL-9 and Th-9 cells are involved in either or both. Although IL-9 may play a role in the pathogenesis of BP, further large-scale studies are needed to confirm these findings and their clinical significance.

Data availability statement

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

Ethics statement

The ethics committee of Kurume University (22008) approved the study, which was carried out in accordance with the Declaration of Helsinki guidelines. Written informed consent to use serum and histopathological samples for research purposes had previously been obtained from all patients and an opportunity for refusal to participate in this research was guaranteed by an opt-out manner.

Author contributions

HK had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: HK. Acquisition of data: HK, KT, AS, and MT. Analysis and interpretation of data: HK, NI, and TN. Drafting of the manuscript: HK. Statistical analysis: HK. Obtained funding: HK. Study supervision: HK and TN. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by grants from the Kaibara Morikazu Medical Science Promotion Foundation.

Acknowledgments

The authors would like to thank Prof. Jun Akiba of the Department of Diagnostic Pathology, Kurume University Hospital, for his technical support with the immunohistochemical staining. The authors would also like to thank Dr. Norihiro Yoshimoto and Prof. Hideyuki Ujiie of the Department of Dermatology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, for their valuable insights through the discussions.

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 20 June 2023
DOI 10.3389/fimmu.2023.1203776

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RECEIVED 11 April 2023
ACCEPTED 30 May 2023
PUBLISHED 20 June 2023

CITATION
Schinner J, Cunha T, Mayer JU, Hörster S, Kind P, Didona D, Keber C, Hertl M, Worzfeld T and Juratli HA (2023) Skin-infiltrating T cells display distinct inflammatory signatures in lichen planus, bullous pemphigoid and pemphigus vulgaris.
Front. Immunol. 14:1203776.
doi: 10.3389/fimmu.2023.1203776

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Skin-infiltrating T cells display distinct inflammatory signatures in lichen planus, bullous pemphigoid and pemphigus vulgaris

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Introduction: We here thought to dissect the inflammatory signature in lesions of three skin disorders, which show a common adaptive immune response against autoantigens of the skin but are characterized by diverging clinical phenotypes. Pemphigus vulgaris (PV) and bullous pemphigoid (BP) are type-2-dependent, IgG autoantibody-driven blistering disorders of mucous membranes and skin, which target desmoglein (Dsg)3 and bullous pemphigoid (BP)180, respectively. In contrast, lichen planus (LP) is a common chronic inflammatory disease of the skin and mucous membranes with a pronounced dermal T cell infiltrate. We previously identified peripheral type 1 and 17 T cell responses against Dsg3 and BP180 in a cohort of LP patients strongly suggesting that the underlying inflammatory T cell signature may drive the evolving phenotype.

Methods: Paraffin-embedded skin biopsies from well-characterized patients with LP (n=31), BP (n=19), PV (n=9), and pemphigus foliaceus (PF) (n=2) were analysed. Areas with the most prominent inflammatory infiltrate were excised with punch biopsies and tissue microarrays (TMA) containing multiple biopsies were created. Using multicolor immunofluorescence, the inflammatory infiltrate was stained with antibodies against multiple cellular markers, i. e. CD3ϵ, CD4, CD15, TCR-δ, the cytokine IL-17A, and the transcription factors, T-bet and GATA-3.

Results: In LP, there was a higher number of CD4+ T cells expressing T-bet compared to GATA-3. In contrast, CD4+ T cells in PV and BP skin lesions more frequently expressed GATA-3 than T-bet. IL-17A+ cells and IL-17A+ T cells were found to a similar extent in all the three disorders. IL-17A+ granulocytes were more predominant in BP than in LP or PV. Of note, the majority of IL-17A+ cells in LP were neither T cells nor granulocytes.

Discussion: Our findings in inflammatory skin infiltrates clearly show a predominant type 1 signature in LP in contrast to a preponderance of type 2 T cells in PV and BP. In contrast to LP, granulocytes and to a much lesser extent CD3+ T cells were a cellular source of IL-17A in BP and PV. These data strongly suggest that different inflammatory cell signatures drive evolving clinically diverse phenotypes of LP, PV and BP despite common target antigens of the skin.

KEYWORDS

lichen planus, bullous pemphigoid, pemphigus vulgaris, T cells, immunological signature, Th1/Th2, IL-17, blistering disorders

Introduction

Chronic inflammatory skin disorders associated with autoimmune responses remain poorly understood, due to a lack of knowledge about their specific target antigens, disease variability and co-morbidities. Immunologically, they can be grouped into disorders associated with T cell-mediated and with antibody-mediated autoimmune responses, both of which require the activation of autoreactive T cell subsets against target proteins expressed in the epidermis and dermis.

Lichen planus (LP) is one of the most common T cell-mediated autoimmune diseases of the skin and mucous membranes, and affects 1-2% of the general population (1–3). Clinically, it presents as reticular, purple papules and plaques of the skin and white papules and erosions of mucous membranes, and follows a chronic-relapsing course (4–6). LP mucosal and skin lesions are infiltrated by T cells which include CD8+ and CD4+ populations. Most relevant for the direct pathology of LP are pro-inflammatory CD8 + T cells, which are mainly located around the basal layer of the epidermis where they trigger apoptosis of epidermal keratinocytes (1, 2, 6–8). While the targeted autoantigens have not been fully identified, self-peptides of keratinocyte-derived proteins presented on HLA class I and II alleles may play a central role in disease pathogenesis (4, 7). Several type 1 and recently type 17 cytokines such as IFN-γ, TNF-α , IL-17A and IL-22 have been detected in LP lesions, as well as in patient sera, supporting the concept that LP is a highly inflammatory skin disorder (8, 9). These cytokines are thought to be mainly produced by CD8+ T cells, and to a lesser extent, by CD4+ T helper cell populations (10), which display a Th1 and Th17 phenotype. While their recruitment and activation mechanisms remain to be elucidated, pro-inflammatory cytokines produced by these populations play an important role in disease pathogenesis, and initial studies using small molecules or anti-cytokine monoclonal antibodies have shown promising results (11–14).

Autoantibody-mediated autoimmune diseases, such as pemphigus vulgaris (PV) and bullous pemphigoid (BP), share clinical similarities with mucosal LP. PV and BP are characterized by blisters and erosions of the skin and mucous membranes, which are caused by IgG autoantibodies against distinct adhesion proteins of desmosomes and hemidesmosomes. In PV and pemphigus foliaceus (PF), IgG autoantibodies are directed against components of desmosomes, namely desmoglein (Dsg) 3 and Dsg1. In BP, IgG targeting hemidesmosomal antigens, namely bullous pemphigoid antigen 1 (BP230) and bullous pemphigoid antigen 2 (BP180) induce the influx of innate immune cells in the skin leading to pronounced inflammation with pruritus and tense blisters.

The cellular infiltrate in BP and PV skin lesions is very heterogeneous and consists primarily of neutrophilic granulocytes (BP), eosinophils, CD4+ T cells and B lymphocytes, yet is less inflammatory than LP. Autoreactive T and B lymphocytes play a central role in the pathogenesis of both, PV and BP, and pro-inflammatory autoreactive Th2 cells are observed at a higher ratio in PV and BP than in LP (15).

A recent single-cell RNA-sequencing study in a small number of LP and BP patients showed that both diseases lack infiltrating myeloid derived populations, when compared to healthy controls (HC), atopic dermatitis or psoriasis lesions, yet display distinct T cell signatures (16). This correlates with our own recent findings which showed that patients with LP exhibit IFN-γ- and IL-17A-dominated peripheral blood T cell responses, while BP patients display a higher number of peripheral type 2 autoimmune T cell responses (15). In LP, BP180-specific peripheral Th17 cell responses correlated with a distinct Th17 cell infiltrate along the dermal-epidermal basement membrane zone in LP skin lesions. This observation strongly suggests that IL-17A is a major driver of disease pathogenesis. IL-17A polymorphism and increased serum levels of IL-17A are indeed associated with LP (17, 18), not only affecting local infiltration, but also the microbiome of oral LP patients (19). Therapeutic targeting of IL-17 or IL-23 (which is involved in Th17 development) by monoclonal antibodies has also shown positive effects in several case series (12, 14), suggesting that IL-17A producing CD4+ or CD8+ T cells play an important role in LP.

Abbreviations: Dsg, desmoglein; BP180, bullous pemphigoid 180; HC, healthy controls; LP, lichen planus; PV, pemphigus vulgaris; BP, bullous pemphigoid; TMA, tissue microarray; Cd, cluster of differentiation; IL, Interleukin; IgG, Immunoglobulin G; Th cell, T helper cell, Tc cell, cytotoxic T cell; TCR, T cell receptor; DAPI, 4',6'-diamidino-2-phenylindole; IIF, indirect immunofluorescence; DIF, direct immunofluorescence; min, minutes; FCS, fetal calf serum; PBS, phosphate-buffered saline.

In light of the above observations, we hypothesized that LP, BP, and PV are inflammatory disorders which target identical autoantigens of the skin although with a different T cell signature. This led previous research to study whether functional differences of the evolving inflammatory skin infiltrate may eventually pave the way for the distinct clinical phenotypes that we see in LP, PV and BP in humans. A previous study has shown that mice with a transgenic T cell receptor for desmoglein 3 develop a type 1 inflammatory skin infiltrate resulting in a lichenoid phenotype with a dense band-like lymphocytic infiltrate resembling LP (20). These findings are in support of our initial hypothesis that LP is a type 1 variant of PV and BP, respectively. So far, thorough characterization of the inflammatory T cell responses in these disorders was mainly performed with peripheral blood lymphocytes. To characterize the immune signature of skin-infiltrating cells in more detail, we analyzed key transcription factors involved in type 1 and type 2 cell development, as well as IL-17A production by T cells and granulocytes in PV, BP and LP on tissue microarrays (TMA) of skin lesions using immunofluorescent antibody staining. Our findings link LP to a type 1-regulated inflammatory skin pathology while skin lesions from PV and BP patients show a more pronounced type 2 CD4+ T cell response.

Results

Dichotomy of type 1 CD4+ T cells in LP versus type 2 T cells in BP and PV

To characterize the immune signatures of the inflammatory CD4+ skin infiltrate in LP, BP, and pemphigus patients we collected paraffin-embedded skin biopsies. In total, the cohort comprised samples from 31 LP, 19 BP and 11 pemphigus patients. Within the pemphigus group, both PV (n=9) and pemphigus foliaceus (PF; n=2) were included. However, for statistical analyses, the PF samples were omitted due to the low sample number. All paraffin-embedded skin biopsies were re-embedded to generate tissue microarrays (TMAs) (Figure 1). The TMAs were then subjected to immunostaining with antibodies against distinct immune cell subsets.

It is known that CD4+ T helper (Th) cells play a pivotal role in inflammatory skin disorders. However, the predominant differentiation signatures of CD4+ T helper cells in the skin infiltrates in LP, BP and PV remain unclear. We therefore aimed at the visualization and quantification of Th1 and Th2 cells. To do so, we performed co-immunostaining for the Th cell marker CD4

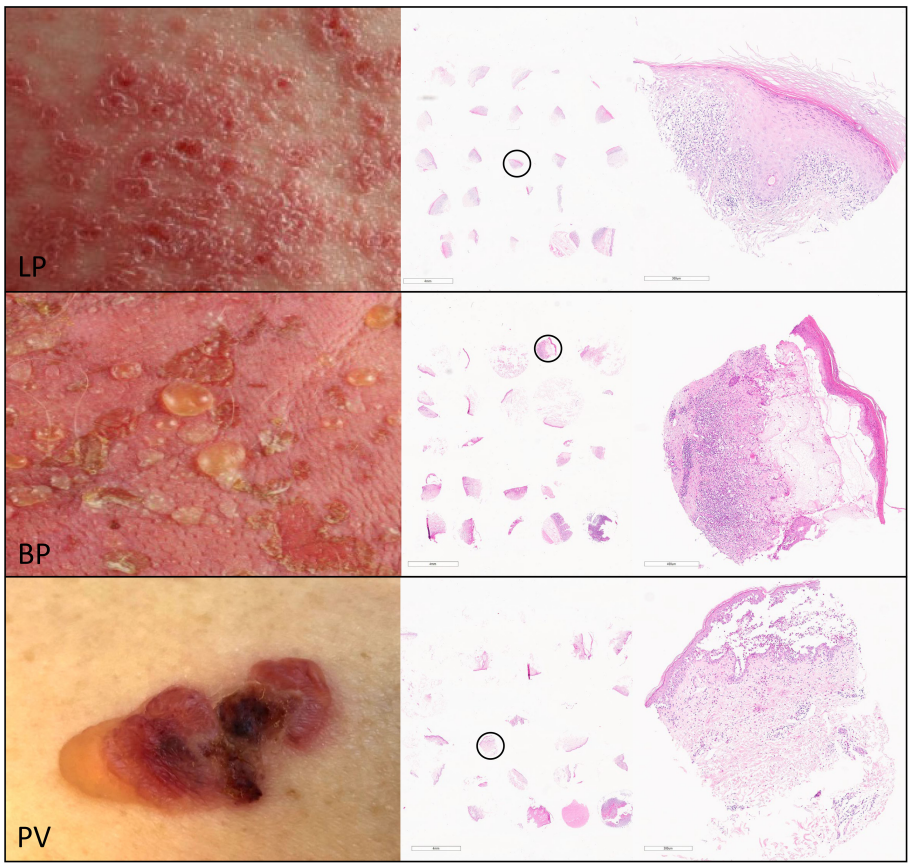

FIGURE 1
For the characterization of the inflammatory infiltrate of skin lesions in lichen planus (LP), bullous pemphigoid (BP) and pemphigus vulgaris (PV) we generated tissue micro arrays (TMAs) using punch biopsies from the inflammatory epidermal/dermal infiltrate of paraffin-embedded skin lesions from patients with LP (upper panel), BP (middle panel) and PV (lower panel). The right column shows representative hematoxylin-eosin staining of TMA sections from LP, BP and PV with a magnification of the circled punch biopsy.

together with T-bet and GATA-3, the master transcription factors for Th1 and Th2 cells, respectively (Figures 2A–D). In LP we found a significantly higher number of both CD4+/T-bet+ cells as well as CD4+/GATA-3+ cells compared to BP with a median of 44 CD4+/T-bet+ cells in LP and a median of 6.5 cells in BP ($p < 0.0001$) and a median of 19 CD4+/GATA-3+ cells in LP compared to a median of 13.5 cells in BP ($p = 0.025$). There was a significantly higher median of 44 CD4+/T-bet+ cells in LP compared to 5.2 cells in PV ($p = 0.001$) and no significant differences between BP and PV for both populations were observed (Figures 2E, F). Of note, Th1/Th2 cell ratios were highly significantly different between the three groups of LP, PV and BP patients: While LP skin lesions showed

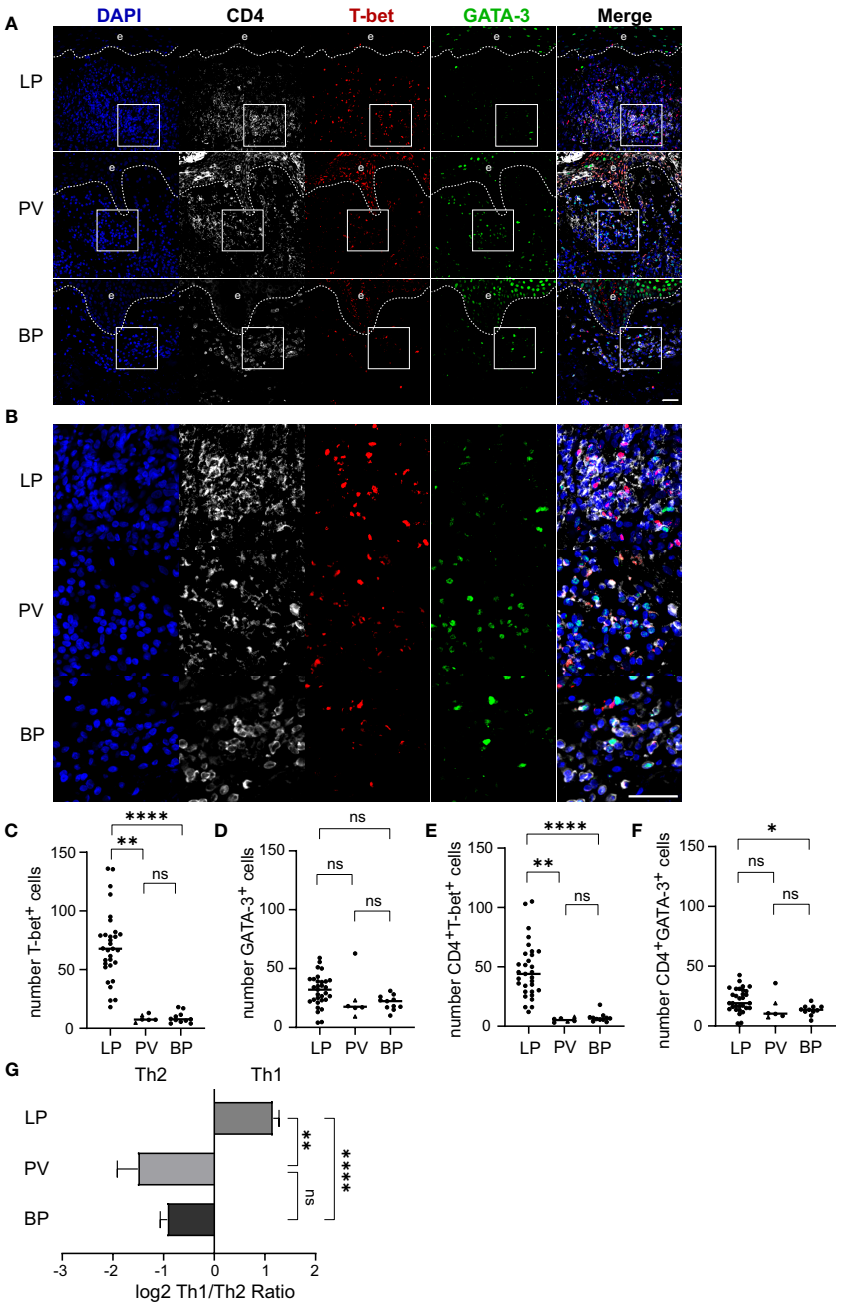

FIGURE 2
Th1/Th2 ratio in dermal immune cell infiltrates of LP, PV and BP patients. **(A, B)** Representative confocal images of immunostaining of tissue microarrays using anti-CD4 (white), T-bet (red) and GATA-3 (green) antibodies. Dashed lines in **(A)** indicate the border between epidermis **(e)** and dermis. Boxed areas in **(A)** are magnified in **(B)**. Blue: 4′,6-diamidino-2-phenylindole (DAPI). Scale bars represent 40 µm. **(C–F)** Quantification of **(C)** T-bet-positive, **(D)** GATA-3-positive, **(E)** CD4-/T-bet-double-positive and **(F)** CD4-/GATA-3-double-positive cells per tissue section. Horizontal bars indicate median values and individual points represent a biopsy from a different patient with LP (n = 31), PV (n = 4) and BP (n = 11). Triangles in the PV-Group represent biopsies from patients with PF (n = 2), which were excluded from statistical analysis. **(G)** Quantification of the log2 transformed ratio between CD4-/T-bet-double-positive (Th1) and CD4-/GATA-3-double-positive (Th2) cells. Shown are mean values + S.E.M. Statistical significance was assessed using Kruskal-Wallis test with Dunn's correction for multiple comparison. *$p \leq 0.05$, **$p \leq 0.01$, and ****$p \leq 0.0001$. n.s., not significant.

a preponderance of Th1 cells, PV and BP skin lesions predominantly contained Th2 cells (Figure 2G).

Type 17 T cells in skin lesions from LP, BP and PV/PF patients

In addition to Th1 and Th2 cells, IL-17 producing T cells have been shown to be of major pathophysiological relevance in inflammatory skin diseases (21, 22). Whether T cell receptor (TCR)-α/β+ T cells or TCR-γ/δ+ T cells serve as IL-17A producers in the inflammatory skin infiltrate of LP, BP and/or PV is ill-defined. We therefore immunostained skin lesions for CD3ε (CD3 as a pan-T cell marker), TCR-δ chain (a marker for γδ T cells), and IL-17A (Figures 3A, B). As expected, the highest median of 312 CD3ε+ cells was found in LP skin lesions with significant differences compared to the median of 84 CD3ε+ cells in PV ($p = 0.03$) and 51 CD3ε+ cells in BP ($p < 0.0001$) (Figure 3C). In LP a median of 1.5% of the total T cell infiltrate were γδ T cells which was a significantly higher fraction compared to the median of 0% in both PV ($p = 0.004$) and BP ($p = 0.02$) (Figures 3D, E).

In the analyzed skin disorders, IL-17A+ cells were observed in similar amounts, with a median of 3 IL-17A+ cells in LP, 5.5 in PV, and 5 in BP (Figure 3F). However, when normalized to the CD3ε+ infiltrate, there were significantly more IL-17A+ cells in PV ($p = 0.02$) and BP ($p = 0.001$) compared to LP (Figure 3G). Only a few CD3ε+/IL-17A+ cells were found, with no significant differences in both the absolute numbers and fraction of total T cells (Figures 3H, I). It is noteworthy that most IL-17A+ cells were negative for CD3ε, indicating that they were not T cells (compare Figures 3F, H), and none of the identified γδ T cells expressed IL-17A (data not shown). This prompted us to study inflammatory cells other than T cells as a potential source of IL-17A.

IL-17-producing granulocytes are predominant in BP

Our finding that the majority of IL-17A+ cells are no T cells prompted us to investigate the identity of the inflammatory cell populations other than T cells expressing IL-17A. Given that neutrophils, the most abundant of the granulocytes, are a common source of IL-17, we focused on CD15+ granulocytes in the skin infiltrates of LP, BP, and PV patients (Figures 4A, B). The total number of CD15+ granulocytes and CD15+/IL-17A+ cells were significantly greater in BP patients than in LP patients (Figures 4C, D). The median number of IL-17A+ granulocytes was 18.75 in BP and only 1 in LP ($p < 0.0001$). There was also a trend towards a higher median of granulocytes in PV compared to LP (Figures 4C, D). The medians of IL-17A+ cells that were negative for CD15 did not differ significantly between the three groups (Figure 4E). In LP, the majority of IL-17A+ cells were neither granulocytes nor T cells, whereas in BP and PV, most IL-17A+ cells were CD15+ granulocytes (Figures 4F, G).

Monoclonal antibodies against IL-17 ameliorate skin lesions in lichen planus

To gain insight into the functional consequences of the distinct inflammatory signatures in LP, we studied the clinical effect of therapeutic cytokine inhibition in a patient with LP. As the majority of skin-derived IL-17A+ cells in LP neither belong to T cells nor to granulocytes, we sought to determine the functional impact of IL-17A+ cells on LP pathogenesis in general. In a 28-year-old female patient with extensive cutaneous LP, we took advantage of the availability of Secukinumab, an anti-IL-17A monoclonal antibody which is approved for the treatment of psoriasis vulgaris. The LP patient was not sufficiently responsive to topical steroids and was treated in a compassionate trial with Secukinumab at 300 mg s.c. weekly for four weeks and then 300 mg every four weeks. At week 12 of treatment with Secukinumab, the initially reddish erythematous plaques had completely disappeared leaving brownish hyperpigmentations (Figure 5).

Discussion

The present study provides a comprehensive analysis of the inflammatory signature of skin lesions in three inflammatory skin disorders linked to T cell recognition of well-known autoantigens of the skin and mucous membranes. PV/PF and BP are IgG-driven autoimmune bullous skin disorders targeting the cutaneous autoantigens, Dsg3/Dsg1 and BP180, respectively. Previous studies by our group and others had identified mainly type 2 peripheral blood autoreactive T cells in both disorders (23–26). In contrast, we had identified type 1 and type 17 peripheral blood T cells reactive with Dsg3 and BP180 in patients with mucosal and cutaneous LP (15). As these studies have mainly focused on the peripheral blood, we here sought to investigate whether these peripheral signatures correspond to the signatures within the skin lesions that have remained largely unknown.

The major aim of the present study was therefore to study skin sections from a cohort of PV, BP and LP patients in order to investigate whether a similar polarized CD4+ T cellular signature could be identified in lesions of these inflammatory skin disorders. We generated TMAs allowing us to perform a comparative analysis of skin infiltrates with greater patient numbers and small inter-assay variability. Simultaneous staining of TMA containing several skin sections provides a practicable and suitable research instrument for immune profiling studies. Based on the ratio of CD4+/T-bet+ (Th1) and CD4+/GATA-3+ (Th2) cells, we clearly identified a type 1 inflammatory signature in LP skin lesions in contrast to a predominant type 2 inflammatory signature in BP and PV skin lesions.

It is well-known that LP lesions are infiltrated by large numbers of CD8+ and CD4+ T cells with a pro-inflammatory signature (4). Cytokines produced by these cells include IFN-γ, TNF-α, IL-6, IL-17, IL-22, which are not only observed in skin lesions but can also be detected in patients` sera (9, 27). While cytotoxic CD8+ T cells are presumably the main drivers of disease and target basal keratinocytes,

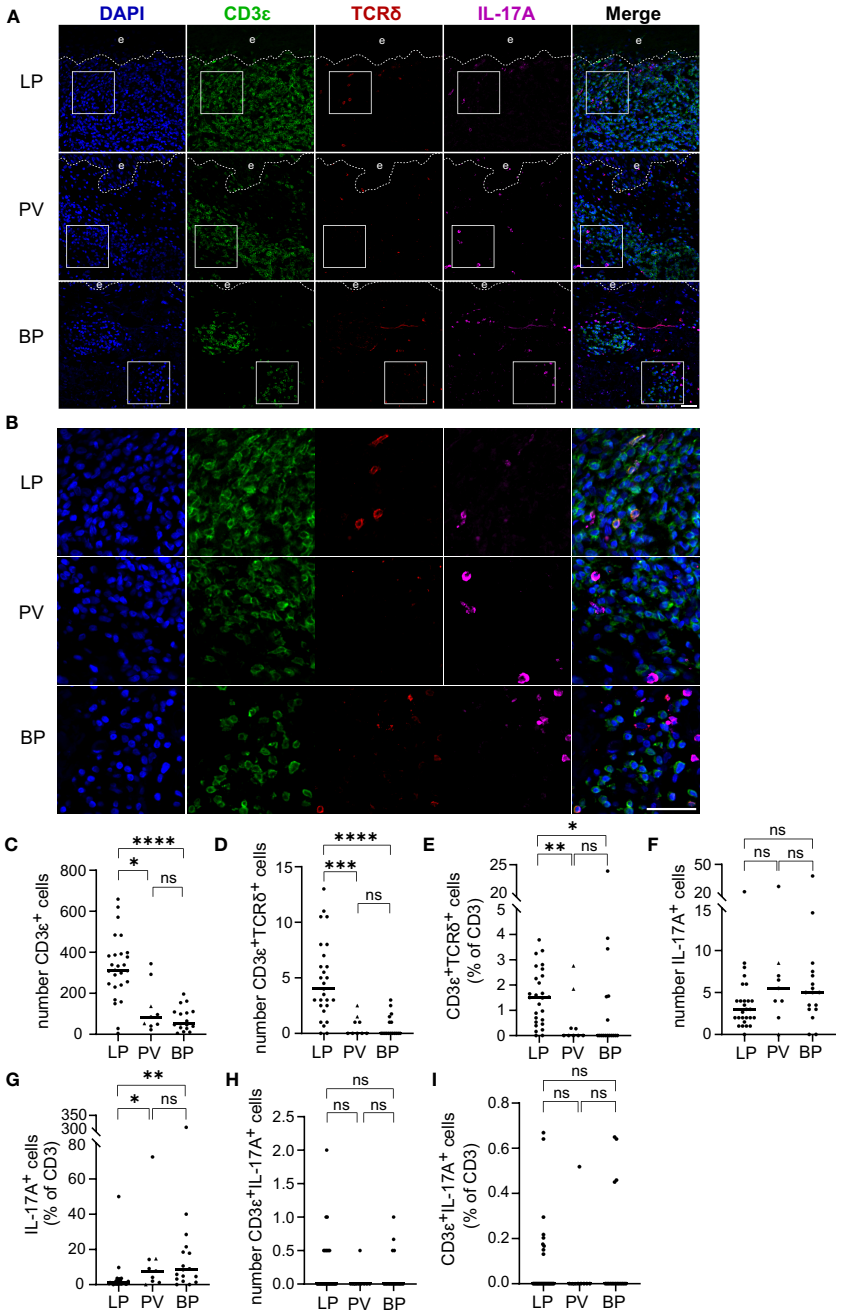

FIGURE 3
T cell infiltrate, γδ T cell proportion and IL-17A producing cells in LP, PV and BP patients. **(A, B)** Representative confocal images of immunostaining of tissue microarrays using anti-CD3ε (green), TCRδ (red) and IL-17A (purple) antibodies. Dashed lines in **(A)** indicate the border between epidermis **(e)** and dermis. Boxed areas in **(A)** are magnified in **(B)**. Blue: 4′,6-diamidino-2-phenylindole (DAPI). Scale bars represent 40 µm. **(C–E)** Quantification of **(C)** CD3ε-positive cells per tissue section, **(D)** CD3ε-/TCRδ-double-positive cells per tissue section and **(E)** proportion of CD3ε-/TCRδ-double-positive cells as percentage of total CD3ε-positive cells. **(F, G)** Quantification of **(F)** IL-17A-positive cells per tissue section and **(G)** IL-17A-positive cells compared to the CD3ε-positive cells expressed as percentage. **(H, I)** Quantification of **(H)** CD3ε-/IL-17A-double-positive cells per tissue section and **(I)** fraction of CD3ε-/IL-17A-double-positive cells as percentage of total CD3ε-positive cells. Horizontal bars indicate median values and individual points represent a biopsy from a different patient with LP (n = 27), PV (n = 7) and BP (n = 17). Triangles in the PV-Group represent biopsies from patients with PF (n = 2), which were excluded from statistical analysis. Statistical significance was assessed using Kruskal-Wallis test with Dunn's correction for multiple comparison. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001. n.s., not significant.

CD4+ T cells produce the majority of proinflammatory cytokines. The spectrum of (auto)antigens which are recognized by these T cells and how they are recruited to LP skin lesions remains to be elucidated. Candidate target antigens of epidermal keratinocytes and components of the dermal-epidermal junction, such as Dsg3, BP180 and others including microbial peptides have been recently identified (19, 28). It is also possible that these cytokines play an important role in disease progression, as proinflammatory cytokines reduce wound healing, impact peripheral tolerance and enhance blister formation (10).

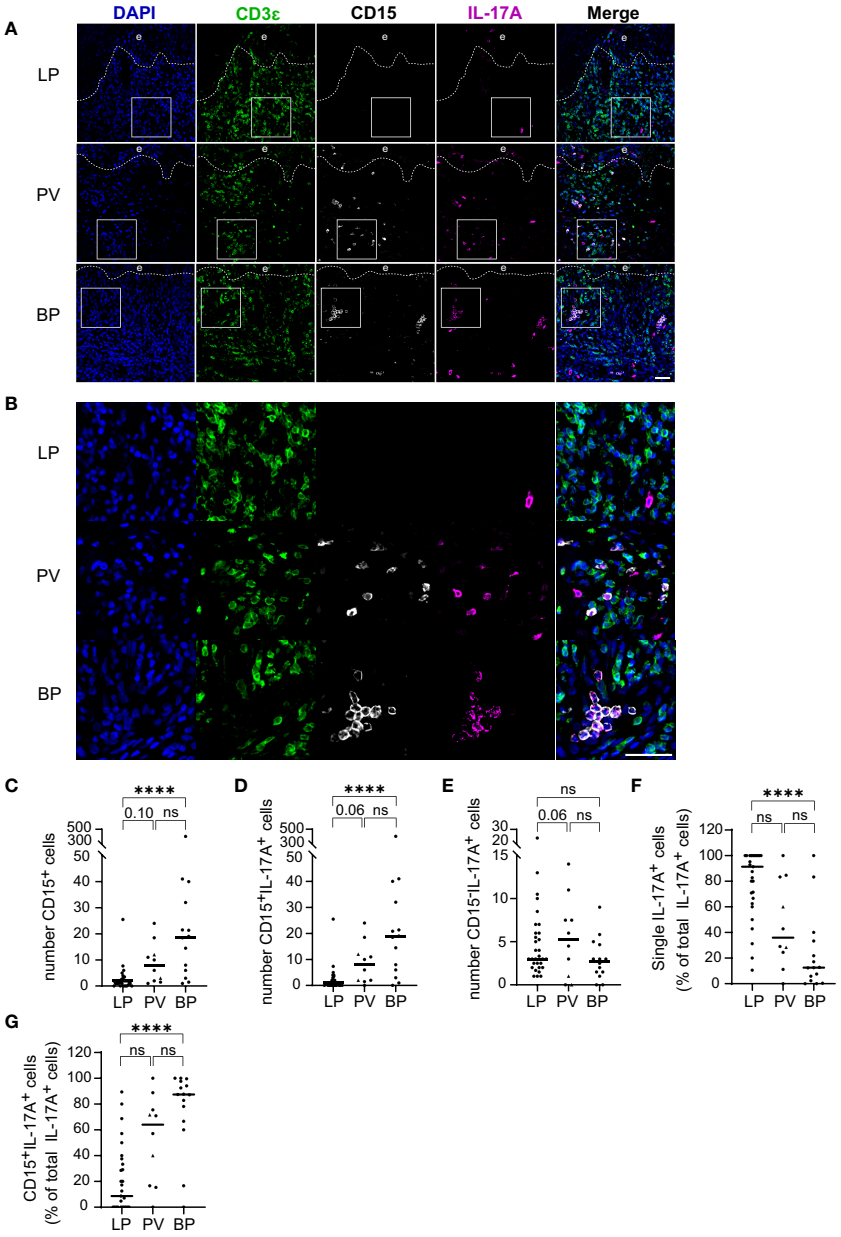

FIGURE 4
IL-17A producing Granulocytes in LP, PV and BP patients. **(A, B)** Representative confocal images of immunostaining of tissue microarrays using anti-CD3ε (green), CD15 (gray) and IL-17A (purple) antibodies. Dashed lines in **(A)** indicate the border between epidermis **(e)** and dermis. Boxed areas in **(A)** are magnified in **(B)**. Blue: 4′,6-diamidino-2-phenylindole (DAPI). Scale bars represent 40 µm. **(C–E)** Quantification of **(C)** CD15-positive **(D)** CD15-/IL-17A-double-positive and **(E)** IL-17A-positive/CD15-negative cells per tissue section. **(F, G)** Quantification of the proportion of **(F)** single IL-17A-positive (CD3ε-negative/CD15-negative) cells and **(G)** CD15-/IL-17A-double-positive cells as percentage of total IL-17A-positive cells. Horizontal bars indicate median values and individual points represent a biopsy from a different patient with LP (n = 31), PV (n = 8) and BP (n = 14). Triangles in the PV-Group represent biopsies from patients with PF (n = 2), which were excluded from statistical analysis. Statistical significance was assessed using Kruskal-Wallis test with Dunn's correction for multiple comparison. ****p ≤ 0.0001. n.s., not significant.

In contrast to LP, T cell infiltrates are much less pronounced in BP and PV skin lesions as confirmed in the present study and already strongly suggested by previous work (15, 29, 30). This is primarily linked to the distinct immunopathology associated with these diseases, as BP and PV are driven by autoantibodies whereas LP is mainly associated with effector T cells. While it is hard to identify autoreactive B cells in BP and PV skin lesions, the draining lymph nodes or the bone marrow, IgG autoantibodies can readily be detected in patients' sera, indicating their systemic distribution (31).

Autoantibody-mediated acantholysis in PV is presumably largely independent from immune cell infiltration and can readily be achieved *in vitro* and *in vivo* in preclinical animal models (32), suggesting that inflammatory immune cells are not directly required for disease progression. In contrast, BP which is characterized by subepidermal loss of adhesion largely depends on complement

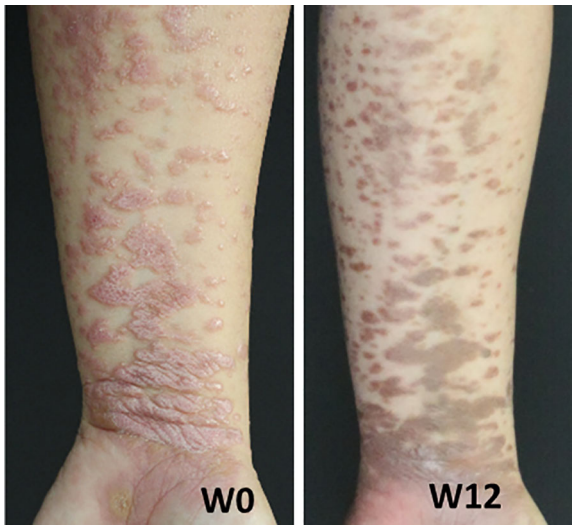

FIGURE 5
Lichen planus patient was treated with the anti-IL-17A monoclonal antibody Secukinumab at 300 mg administered weekly for 5 weeks starting at week 0, followed by monthly doses. Shown are images of the patient's left forearm at week 0 (W0) and week 12 (W12).

fixing IgG autoantibodies which lead to an influx of innate immune cells such as neutrophils and macrophages, which secrete proteolytic enzymes eventually leading to blister formation. While BP and PV blisters display a mild to moderate inflammatory phenotype, innate inflammation is not a key driver of disease and presumably more likely a secondary inflammatory response to epidermal cell death, barrier disruption and exposure to infiltrating microbes (33). The T cell signature in BP and PV is therefore less proinflammatory than in LP, and CD8+ T cells are observed at a much lower frequency. A higher ratio of anti-inflammatory GATA-3+ Th2 cells, which are associated with wound healing, barrier homeostasis and B cell activation can also be observed in BP and PV lesions (12, 15). However, type 2 T cells can also induce proinflammatory responses, cause itch, recruit eosinophils, basophils and cause their activation (34). In our opinion, the present approach to directly identify polarized T cell responses, i.e. Th1 versus Th2 versus IL-17A producing T cells extends previous studies looking at cytokine expression in skin lesions from patients with LP, BP and PV (35, 36). In contrast to these previous studies, the present study allows to directly identify and quantitate defined T cell subsets in the skin infiltrate of these inflammatory skin disorders.

It is still a matter of debate as to whether type 2 cells play an anti- or pro-inflammatory role in BP and PV, but treatment with Dupilumab, an anti-IL-4Ra antibody, has shown positive results in a small cohort of BP patients with over 50% of patients showing complete clearance of disease (37). The T cell dichotomy between Th1 and Th2 cells observed in LP vs BP/PV might therefore have important clinical implications for targeted therapies that reduce disease pathogenesis.

In recent years, a distinct T cell population residing in human epithelia, characterized by the expression of IL-17 has received major attention, as these cells may possess pro-inflammatory function thereby promoting the pathology of several skin diseases and chronic inflammatory immune responses (22, 38). IL-17+ Th17 and Tc17 cells have been now identified as suitable therapeutic targets in psoriasis vulgaris and additional inflammatory skin disorders (Table 1). In the present study, IL-17A+ cells were found in all three skin disorders to a similar extent. While LP showed a few IL-17A+ T cells, a significant number of IL-17A+ cells were neither T cells nor granulocytes. Of note, γδ T cells, which were more common in LP than in BP or PV are potential IL-17A producers. Skin lesions from patients with BP contained to the greatest extent IL-17A-producing granulocytes. Moreover, PV skin lesions contained IL-17A+ T cells and granulocytes in addition to IL-17A+ cells of unknown origin.

Similarly to LP and BP, IL-17A+ T cells were present in skin lesions from PV patients which confirms previous findings (39). Still, their pro- or anti-inflammatory function remains to be elucidated. It is noteworthy that genetic deficiency of Dsg1, the autoantigen of PF, leads to a Th17 signature as do antibodies against Dsg1 (40). Th17 cells and related cytokines are expressed in PV/PF and BP skin lesions. Their direct impact on the immune pathogenesis, e.g. the local production of IgG autoantibodies, has not yet been demonstrated (41, 42). In contrast, the majority of IL-17+ inflammatory cells in LP skin lesions do not belong to the T cell or granulocyte compartment. This finding is novel and of potential pathophysiological importance since monoclonal antibodies against IL-17 have been shown to significantly improve LP skin lesions (15). Finally, the third cellular source of IL-17 are neutrophilic granulocytes, which are a major inflammatory cell subset in BP skin lesions. IL-17 dependent activation of IL-1ß associated inflammasome augments the innate autoinflammatory immune responses inherent to BP (43). Moreover, blockade of IL-17-induced neutrophil recruitment into BP skin leads to amelioration of skin symptoms (44).

In LP, serum levels of IL-17 were readily detectable and IL-17+ CD4+ and CD8+ T cells were observed in LP lesions (8, 17). A recent meta-analysis including more than 600 patients with oral LP (OLP) observed that increased serum levels of IL-17 are strongly associated with the clinical disease severity of LP (45). Under inflammatory conditions, IL-17 induced chemokines lead to the recruitment of pro-inflammatory effector cells in the skin and the proliferation of keratinocytes (22). Keratinocyte proliferation might further increase the pathological autoantigen pool in LP, leading to further disease progression. While the major producers of IL-17 include CD4+ Th17 cells, which express the transcription factor ROR-γt, other IL-17 immune cell populations have also been identified in inflamed skin (see Table 1). These include IL-17-producing CD8+ T cells, also known as Tc17 cells, γδ T cells, as well as IL-17+ neutrophilic granulocytes, all of which infiltrate the epidermis or the subepithelial layer. While the development of Th17 cells requires IL-23 signaling, which is produced by dendritic cells during T cell priming, it is unclear which factors trigger the production of IL-17 in other cell types (22). Several monoclonal antibodies against IL-17, as well as IL-23, have been developed and have been shown to improve inflammatory skin diseases such as psoriasis (22). Individual LP patients have already been treated with monoclonal antibodies against IL-17 and IL-23 (12, 14), which has resulted in a clinical as well as a histological improvement of disease. Shown here is a patient

TABLE 1 Interleukin-17 (IL-17) producing cells in human skin.

Cell type	Disease	Location	Publication
CD3+ CD4+	Psoriasis Vulgaris	subepithelial layer	10.1046/j.1523-1747.1998. 00347.x
Th17 cells			10.1371/journal.pone.0014108
			10.1007/s10875-012-9716-x
			10.1007/s11596-007-0329-1
			10.1038/jid.2008.85
	Atopic Dermatitis	epidermis	10.1038/jid.2008.111
	Allergic contact dermatitis	epidermis	10.1111/j.1365-2133.2009. 09400.x
		subepithelial layer	10.4049/jimmunol.0901767
			10.1111/j.1365-2133.2009. 09400.x
			10.1111/cod.12043
			10.1111/all.12351
	Lichen Planus	subepithelial layer	doi/10.1111/jop.12898
			10.3389/fimmu.2019.01808
	Bullous Pemphigoid	subepithelial layer	10.1016/j.jaut.2018.09.003
			10.1038/srep18001
			10.1155/2013/967987
			10.1111/j.1600-0625.2011.01378.x
	Vitiligo	epidermis	10.1111/j.1365-2230.2010. 03972.x
	Cutaneous lupus erythematosus	epidermis	10.1111/j.1365-2230.2010. 03996. x
	Cutaneous T-Cell Lymphoma	dermis	10.1111/j.1365-2133.2011. 10647.x
	Basal cell carcinoma	epidermis	10.1002/eji.201445052
	Hidradenitis suppurativa	epidermis	10.1111/bjd.14075
	Cutaneous Candidiasis	epidermis	10.1007/s12026-011-8226-x
			10.3390/pathogens4030606
CD3+ CD8+	Psoriasis Vulgaris	subepithelial layer	10.1046/j.1523-1747.1998. 00347.x
Tc17 cells			10.1371/journal.pone.0014108
			10.1007/s10875-012-9716-x
			10.1007/s11596-007-0329-1
			10.1038/jid.2008.85
	Lichen Planus	epidermis	10.1111/jop.12898
	Atopic Dermatitis	stratum corneum	10.1038/jid.2012.456
	Allergic contact dermatitis	epidermis	10.1111/j.1365-2133.2009. 09400.x
	Graft-Versus-Host Disease	dermis	10.1007/s00005-019-00549-2
TCR−γ/δ+	Lichen Planus	epidermis	10.1111/1523-1747.ep12394904
T cells	Atopic Dermatitis	epidermis	10.1111/ijd.16364
	Psoriasis Vulgaris	epidermis	10.1016/j.immuni.2011.08.001
			10.1016/j.ebiom.2022.104136
			10.1038/nm.3016
			10.1007/s10875-008-9233-0

(Continued)

TABLE 1 Continued

Cell type	Disease	Location	Publication
Neutrophilic granulocytes	Psoriasis Vulgaris	dermis	10.4049/jimmunol.1100123
			10.1111/bjd.15533
	Cutaneous T-Cell Lymphoma	dermis	10.1111/j.1365-2133.2011. 10647.x
	Hidradenitis suppurativa	epidermis	10.1111/bjd.14214
	Bullous pemphigoid	dermis	10.1038/jid.2014.263

with extensive LP of the skin who showed an excellent therapeutic response, i.e. fast regression of skin lesions upon treatment with the IL-17A blocker Secukinumab. However, a larger cohort size and controlled setting as part of a clinical trial will be necessary to determine the efficacy of IL-17 blockade in LP and its clinical variants.

In summary, our findings support the concept that LP is a type 1 T cell-associated skin disorder with a type 17 component while PV and BP show type 2 cells with a strong autoantibody-driven pathogenesis. These findings are in line with the characterization of peripheral blood type 1/type 17 signatures in LP and a type 2 T cell response in PV. These immunological signatures may pave the way for the treatment of LP using antibodies targeting Th1-associated cytokines, as well as for PV and BP using antibodies targeting Th2-associated cytokines.

Material and methods

Human subjects

Formalin-fixed paraffin-embedded (FFPE) skin tissues from patients with LP (n=31), BP (n =19), PV (n=9) and PF (n=2) were included in the study. The cohorts were kindly provided by the Center for Dermatopathology in Freiburg (S. Hörster) and Dermatopathology (P. Kind) in Offenbach (Supplementary Table 1). The samples were processed, pseudonymized and included in the present study based on their diagnoses. The LP samples were included based on histologic criteria and the BP and PV samples were included based on histologic, direct immunofluorescence and immune serological criteria. The disease classification criteria were as follows:

1. Lichen planus: clinical suspicion (polygonal reddish papules on the skin) and histopathology showing a band-like subepidermal lymphocytic infiltrate in the upper dermis and sawtoothed rete ridges.
2. Bullous pemphigoid: clinical suspicion (tense blisters/erosions or polymorphic pruritic lesions of the skin); histopathology: subepidermal loss of adhesion and DIF: IgG and/or C3 at the dermal-epidermal junction or IIF: anti-basement membrane serum antibodies on monkey esophagus or ELISA: anti-BP180/BP230 serum IgG
3. Pemphigus vulgaris/foliaceus: clinical suspicion (flaccid blisters/erosions of the oral mucosa and/or skin); histopathology: intraepidermal loss of adhesion and DIF: IgG and/or C3 on the surface of epidermal keratinocytes or IIF: anti-epithelial serum antibodies on monkey esophagus or ELISA: anti-desmoglein 3/desmoglein 1 serum IgG.

This study was approved by the Ethics Committee of the Medical Faculty, Philipps University Marburg (165/19).

Tissue microarrays

To generate tissue microarrays (TMA), 2 mm punch biopsies were taken from FFPE tissues utilizing a Manual tissue arrayer (MTA-1) from Alphametrix Biotech, within regions of infiltrating cells. The excised tissues were combined and re-embedded in a newly arrayed recipient paraffin block, according to standard procedures of the Comprehensive Biomaterial Bank Marburg (CBBM).

Immunofluorescence staining

4 µm sections of TMAs were deparaffinized and rehydrated by two 5 min immersions in Xylene followed by 5 min in 99% ethanol and consecutive immersions in 95%-, 80%- and 70%-ethanol for 3 min each. After two 3 min washes in ddH_2O heat-induced antigen retrieval was performed in 10 mM Citrate Buffer pH 6.0 for 10 min using a pressure cocker. After 45 min cool down and a following 1 min wash in ddH_2O the sections were permeabilized by immersion in 0.2% Triton X-100 in PBS (PBST) for 10 min. Sections were blocked with 10% FCS in PBST for 1 h, primary antibodies were added in 2% FCS in PBST and incubated over night at 4°C in a humidified chamber. On the next day sections were washed three times with PBS for 10 min each followed by 2 h incubation with secondary antibodies in 1.5% FCS in PBST together with 4′,6-Diamidin-2-phenylindol (DAPI) in a humidified chamber at room temperature. After three consecutive washes in PBS the slides were mounted with Dako fluorescence mounting medium. The primary and secondary antibodies used as well as the dilutions can be found in Supplementary Table 2.

Image acquisition and cell counting

A Zeiss LSM 700 confocal microscope with a 20x objective was used to acquire 320 µm x 320 µm images of the immune cell infiltrate at the dermal-epidermal junction. The immunofluorescence images were analyzed using ImageJ (v 1.53c). Positively stained cells were manually counted when CD3ϵ, TCR-δ, CD4 or CD15 showed signals at the cell membrane around the DAPI+ nucleus. Since T-bet and GATA-3 are transcription factors positive cells were counted when the signal showed an overlap with DAPI. Cells were considered IL-17A positive when the signal was between the nucleus and the cell membrane.

Statistical analysis

Kruskal-Wallis test with Dunn's correction for multiple comparison was used to test for statistical significance between groups. Level of significance is indicated by asterixis with *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$, whereas ns stands for not significant. The analysis was performed using GraphPad Prism Version 9.5.0.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Medical Faculty, Philipps University Marburg (165/19). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. 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

HJ: study concept and design HJ, SH, PK and TC: acquisition of samples JS: immunohistochemistry staining and analysis JS, TC and HJ: immunohistochemistry analysis TC and JS: drafting of the manuscript JS: statistical analysis CK: technical and material support MH and TW: study supervision All authors: acquisition, analysis and interpretation of data and critical revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the Deutsche Forschungsgemeinschaft (FOR 2497 Pegasus; TP8 to MH). DD is supported by a Clinician Scientist Program of the Medical Faculty at the University of Marburg.

Acknowledgments

This paper is dedicated to the memory of Prof. Detlef Zillikens. Detlef was a world leader in autoimmune bullous disease research and largely contributed to much improved diagnostics and therapeutic options in pemphigus and the pemphigoids. He has encouraged many young scientists to pursue an academic career in this field some of whom have already become academic leaders. We are grateful to the patients of the study, to the medical doctors taking skin biopsies, and the technicians and the pathology unit of the Comprehensive Biobank Marburg (CBBM) for embedding the samples and creating the TMA.

Conflict of interest

MH is Coordinating PI of an ongoing phase 2 multicenter trial with guselkumab in lichen planus EutraCT 2021-000271-36 and has received an unrestricted grant from Janssen Cilag. TC and JM are investigators involved in an ongoing phase 2 multicenter trial with guselkumab in lichen planus EutraCT 2021-000271-36. MH has been Coordinating PI German study centers of a clinical phase 2 trial with secukinumab in lichen planus PRELUDE; Novartis Pharmaceuticals.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed 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.2023.1203776/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Secondary Antibody control, peritumoral skin control, positive T17 control. **(A)** Representative confocal images of immunostaining of peritumoral skin using only secondary antibodies anti-goat Alexa Fluor 488 (green), anti-mouse Alexa Fluor 555 (red) and anti-rabbit Alexa Fluor 647 (purple). **(B)** Representative confocal images of immunostaining of peritumoral skin using anti-CD3ϵ (green), anti-TCRδ (red) and anti-IL-17A (purple). **(C)** Representative confocal images of immunostaining of Psoriasis pustulosa using anti-CD3ϵ (green) and anti-IL-17A (purple) as a positive control for IL-17A producing T cells. Dashed lines indicate the border between epidermis **(e)** and dermis. Boxed areas in **(B, C)** are magnified in corresponding second row. Blue: 4′,6-diamidino-2-phenylindole (DAPI). Scale bars represent 40 µm.

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frontiers | Frontiers in Immunology

TYPE Systematic Review
PUBLISHED 29 June 2023
DOI 10.3389/fimmu.2023.1196999

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OPEN ACCESS

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RECEIVED 30 March 2023
ACCEPTED 19 May 2023
PUBLISHED 29 June 2023

CITATION
Huttelmaier J, Benoit S and Goebeler M (2023) Comorbidity in bullous pemphigoid: up-date and clinical implications. *Front. Immunol.* 14:1196999. doi: 10.3389/fimmu.2023.1196999

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Comorbidity in bullous pemphigoid: up-date and clinical implications

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Bullous pemphigoid is the most common autoimmune blistering disease in industrialized countries and particularly affects the elderly. In this patient population, comorbid diseases are frequent and may complicate management and treatment of bullous pemphigoid. A better understanding why distinct diseases are more frequent in bullous pemphigoid patients may lead to new pathophysiological insights and - as a consequence - result in better patient care. The association of bullous pemphigoid with neurological and psychiatric diseases is well known and confirmed by several case-control studies. Association with further diseases such as malignancy and metabolic diseases are still discussed controversially. In recent years new relationships between bullous pemphigoid and autoimmune as well as inflammatory skin diseases have been reported. This review provides a systematic overview on studies addressing comorbidity in bullous pemphigoid patients. Increasing the awareness of both, common and rare comorbid diseases, may enable clinicians to optimize patient support and individualized treatment of bullous pemphigoid.

KEYWORDS

bullous pemphigoid, autoimmune skin blistering disease, comorbidity, neurologic disease, metabolic disease, malignancy, inflammatory disease

Introduction

Bullous pemphigoid (BP) is the most common autoimmune blistering disease (AIBD) with an incidence of approximately 20 cases per million people in Germany (1). In recent years the incidence has been rising, presumably due to an ageing population and increasing prescription of certain drugs (2).

BP patients typically present with urticarial plaques, vesicles, blisters and erosions and often suffer from intense pruritus (1). In approximately 20% of patients itching is the main symptom, often being associated with non-bullous prurigo-like nodules. In severe cases the mucous membranes may be affected as well. BP is characterized by autoantibodies directed against structural proteins of the dermal–epidermal junction, especially BP-180. The latter connects keratinocytes to the basement membrane. Binding of specific autoantibodies leads to inflammation with subsequent disruption of the dermal-epidermal junction and blister formation (3).

The mean age of onset of BP is 78 years (1). As the incidence of BP is increasing with age patients often suffer from a variety of comorbid diseases. Comorbidity is defined as the coexistence of different diseases in one individual with an index disease, e.g. BP. Importantly, comorbidity is not a direct result or complication of the index disease itself but is defined by multiple diseases existing simultaneously in one individual regardless of their causal relationship (4). Comorbidity and resulting polypharmacy may delay and complicate treatment of BP, thus awareness of both common and rare comorbid diseases of BP may enable clinicians to optimize patient support and treatment.

In 2019 a 1-year combined mortality rate in patients with BP of 26.7% in Europe and 15.1% in the United States was reported (5). Recently, a retrospective cohort study including 148 Romanian patients with BP showed similar results with overall survival rates of 74.2% after one year, 53.4% after three years, 43.6% after five years and 31.3% after 10 years (6). Among others, advanced age, neurological disease, valvular heart disease and malignancies were associated with higher mortality rates, however, results were not validated by comparison to a control group. A retrospective analysis of patients with BP in Korea showed a 1.83-fold increased standardized mortality ratio as compared to age- and sex-matched controls (7). The authors identified age over 70 years, cardiac and renal disease as risk factors for increased mortality. However, other associated diseases such as hypertension, stroke, neurological disease, pulmonary disease, dementia and malignant diseases showed no significant differences in mortality rates.

Most studies assessing comorbid diseases in BP focus on a single disease or a group of diseases; reports on the overall prevalence of comorbidities are sparse. A Spanish retrospective observational study including 5,424 patients with BP reported an age-adjusted Charlson Comorbidity Index with significantly higher scores in the pemphigoid compared to the pemphigus group (6.0 vs. 4.7, $p < 0.001$) indicating a higher prevalence for comorbidity in these patients (2). In line, increased prevalence of arterial hypertension, kidney disease, diabetes, heart failure, dementia, chronic obstructive pulmonary disease and Parkinson's disease was observed.

The association of BP with neurological and psychiatric diseases is well known and has been confirmed by several case-control or cohort studies (8–13). Association with malignancy and metabolic diseases is still discussed controversially (13–18). In recent years new relationships between BP and autoimmune as well as inflammatory skin diseases have been reported (19–21).

Given the heterogeneity of studied patient cohorts and variable study designs, comparison and interpretation of published observations is quite challenging. Nevertheless, we herein provide a comprehensive overview of comorbid diseases in BP patients.

Methods

Searching strategy and selection criteria

Literature search was performed using the PubMed database for reports published between January 1990 and November 2022. Searches were limited to publications in English and German language. Medical subject heading terms used in the literature search included ''bullous pemphigoid'' AND "comorbidities", ''bullous pemphigoid'' AND "comorbidity", "subepidermal autoimmune blistering" AND "comorbidities" and "subepidermal autoimmune blistering" AND "comorbidity". Additionally, 11 studies were identified *via* citation in other publications.

Results

Altogether, our search strategy revealed 170 articles dealing with BP and comorbid diseases (Figure 1). Of those, 48 were original articles with 23 case-control, 21 cohort or register studies, two cross-sectional studies and two meta-analyses of cross-sectional, case-control and cohort studies.

Neurologic and psychiatric diseases

Of the considered 48 studies 17 studies discussed neurologic and psychiatric comorbidities in patients with BP (Table 1). The association of BP with neurologic and psychiatric diseases is well acknowledged and confirmed by several case-control and cohort studies (8–13). In 26.4 – 55.8% of patients with BP at least one neurological disease was documented compared to 9.1 – 20.5% in controls depending on the study criteria and designs (9, 24, 28). We recently published a case-control study including 300 patients and 598 age- and sex-matched controls evaluating neurologic and psychiatric comorbidities in BP with a documented neurologic disease in 49.0% and psychiatric disease in 20.3% of BP patients compared to 23.0% and 9.3% in controls, respectively (13). A Taiwanese population-based study identified stroke (36.8%), dementia (17.7%), Parkinson's disease (11.9%) and epilepsy (5.8%) as the most commonly associated neurologic diseases (20). A 3.63-fold increased risk for any neurological disease preceding the diagnosis of BP was calculated. Interestingly, multiple sclerosis (MS) was not diagnosed in any of the 3,485 evaluated patients. However, various other cohort and case-control studies identified MS to be strongly associated with BP (10, 13, 27). A study analyzing the Finnish Care Register identified MS to be the strongest risk factor for developing BP [Odds ratio (OR) 5.9; 95% CI 3.9-8.5] (8). A Danish register-based cohort study including 3,281 patients with BP described an increased prevalence of MS at time of diagnosis (OR 9.7; 95% CI 6.0–15.6) (10). Noteworthy, during the follow-up period the hazard ratio (HR) for development of MS was significantly higher in patients with BP than in controls (HR 9.4; 95% CI 4.9–18.0).

Dementia is also commonly reported in patients with BP. Nevertheless, studies are inconsistent and partially incomparable as the subtypes of dementia are often not differentiated and reported. Analysis of hospitalized patients with BP in the United States of America showed an association of BP with numerous forms of dementia such as presenile dementia (OR 2.63; 95% CI 1.34-5.18), vascular dementia (OR 1.67; 95% CI 1.40–2.00), senile dementia (OR 1.67; 95% CI 1.41–1.98) and other dementias (OR 1.71; 95% CI 1.51–1.94) (27). A German retrospective population-

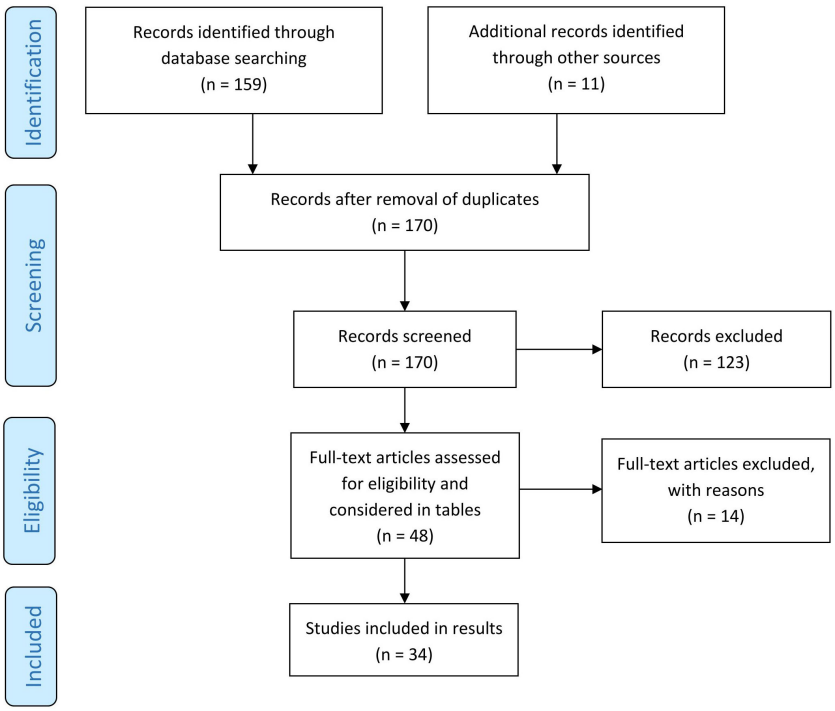

FIGURE 1
Screening method for published studies. [adapted according to the PRISMA statement (22)].

based study comprising 1,743 patients with BP who were matched with 10,141 controls showed a significant association with Alzheimer's disease (OR 2.11; 95% CI 1.73–2.57; P < 0.001) (11). The same study identified a significantly increased life-time prevalence for Parkinson's disease in patients with BP compared to age- and sex-matched controls (OR 2.71; 95% CI 2.19–3.35; p < 0.001). This observation is consistent with studies from Taiwan and the United States that showed an OR of 3.49 and 1.86, respectively. Interestingly, a case-control study from Portugal including 77 patients with BP identified an OR of 4.91 for Parkinson's disease; however, after multivariate analysis the association with Parkinson's disease did not prove to be statistically significant (24). This was possibly due to the small number of patients.

The above mentioned German retrospective population-based study including 1,743 patients with BP identified an increased life-time prevalence for stroke (OR 1.84; 95% CI 1.55–2.19; p < 0.001) (11). In line with these results, a population-based follow-up study from Taiwan including 390 patients and 1,950 matched controls observed 89 events of stroke (22.8%) in patients with BP compared to 223 events (11.4%) in controls during a 3-year follow-up period (p < 0.001) (23). The calculated HR for stroke in patients with BP was 2.37 (95% CI 1.78-3.15; p < 0.001) after multivariate adjustment for confounders such as hypertension, diabetes, hyperlipidemia, heart failure, atrial fibrillation and coronary heart disease.

Not only neurological but also psychiatric disorders seem to be associated with BP as mentioned above. A Danish register-based cohort study focusing on psychiatric disorders identified a significantly elevated risk for psychiatric disorders (HR 1.37; 95% CI 1.15–1.63) and specifically for organic disorders (ICD-10 Code F0) (HR 1.27; 95% CI 1.04–1.54) after adjusting for medication (12). In patients with a diagnosed psychiatric disorder, the risk of development of BP was increased (HR 1.79; 95% CI 1.61–1.99) with a mean latency of 14.6 years. Subgroup analysis identified significantly higher risks for individuals with prior intellectual disorder (ICD-10 code F7) (4.18-fold), substance abuse (ICD-10 code F1) (2.32-fold), schizophrenia (ICD-10 code F2) (2.01-fold), personality disorders (ICD-10 code F60) (2.01-fold), organic disorders (ICD-10 code F0) (1.92-fold) and neurotic disorders (ICD-10 code F4) (1.85-fold). In contrast, a recently published meta-analysis of cohort studies calculated that patients with BP exhibited no significantly increased risk for depression (HR 1.09; 95% CI 0.94-1.26) and schizophrenia (HR 1.35; 95% CI 0.76-2.39) (32). The authors hypothesized that these contradictory results are possibly due to an induction of psychiatric disorders like depression by steroid treatment of BP. Another Taiwanese case-control study reported that the association of BP with schizophrenia occurred predominantly in female patients, which may also explain these inconsistencies (20).

Metabolic diseases

Eighteen of the selected 48 studies discussed metabolic diseases in patients with BP (Table 2). Apart from neurologic disorders metabolic diseases such as diabetes mellitus, arterial hypertension, cardiac diseases and renal impairment appear to be associated with BP (7, 40, 42, 43). Individuals suffering from BP showed a significantly higher prevalence of metabolic syndrome than

TABLE 1 Association between bullous pemphigoid and neurologic and psychiatric diseases.

Author	Year	Location	Study design	Reference cohort	N (case/control)	Neurologic/psychiatric comorbidities in individuals with BP	OR/*HR*	95% CI	*p*
Chen Y.J., et al. (20)	2011	Taiwan	Case-control	Age- and sex-matched controls	3,485/17,425	**Dementia** **Epilepsy** **Parkinson's disease** **Stroke** **Schizophrenia**	**4.81** **3.97** **3.49** **3.30** **2.56**	**4.26–5.42** **3.28-4.81** **3.05-3.98** **3.03-3.60** **1.56-4.30**	**< 0,003** **< 0,003** **< 0,003** **< 0,003** **< 0,003**
Yang Y.W., et al. (23)	2011	Taiwan	Cohort-study	Age- and sex-matched controls	390/1,950	**Stroke**	**2.37**	**1.78-3.15**	**<0.001**
Teixeira V. B., et al. (24)	2014	Portugal	Case-control	Age- and sex-matched controls	77/178	**Stroke** **Dementia**	**5.22** **4.13**	**1.65-15.51** **1.42-14.5**	**<0.001** **<0.001**
Kwan Z., et al. (25)	2015	Malaysia	Case-control	Age-, sex- and ethnicity-matched controls	43/43	**Neurological disorder** **Dementia** Stroke Parkinson's disease Epilepsy	**3.5** **n.a.** 2.1 n.a. n.a.	**1.2-10.2** **n.a.** 0.6-7.2 n.a. n.a.	**0.026** **0.002** 0.237 1.0 0.494
Försti A.K., et al. (8)	2016	Finnland	Cohort study	Age- and sex-matched controls	4,524/66,138	**Schizophrenia** **Personality disorders**	**2.7** **2.2**	**2.0-3.5** **1.3-3.3**	**<0.05** **<0.05**
Sim B., et al. (26)	2017	Singapore	Case-control	Age- and sex-matched controls	105/315	**Parkinson's disease** **Stroke** **Dementia** **Epilepsy** **Depression** Schizophrenia	**20.59** **6.24** **5.89** **3.75** **3.73** 4.37	**4.69-90.5** **3.48-11.2** **2.87-12.0** **1.01-13.9** **1.49-9.37** 0.37-51.3	**<0.001** **<0.001** **<0.001** **0.049** **0.005** 0.24
Daneshpazhooh M., et al. (9)	2017	Iran	Case-control	Age- and sex-matched controls	160/317	**Stroke** **Any neurological disease** **Dementia** Epilepsy Parkinson's disease Multiple sclerosis	**4.96** **3.53** **3.09** 4.04 1.14 1.00	**2.49–9.88** **2.1–5.90** **1.08–8.84** 0.73-22.3 0.33-3.94 0.98-1.01	**< 0.001** **< 0.001** **< 0.03** 0,08 0,84 0,5
Kibsgaard L., et al. (10)	2017	Denmark	Matched Cohort study	n.a.	3,281/32,213	**Multiple sclerosis** **Parkinson's disease** **Stroke** **Alzheimer's disease**	***9.7*** ***4.2*** ***2.7*** ***2.6***	**6.0 – 15.6** **3.1 – 5.8** **2.4 – 2.9** **1.8 – 3.5**	**<0.05** **<0.05** **<0.05** **<0.05**
Ren Z., et al. (27)	2017	USA	Register study of hospitalized adult patients in the USA	n.a.	2,108 BP primary diagnosis 11,234 BP secondary diagnosis 72,108,077 total hospital discharges	**Multiple sclerosis** **Demyelinating disorders** **Presenile dementia** **Paralysis** **Other neurological disorders** **Parkinson's disease**	**4.07** **3.57** **2.63** **2.52** **1.98** **1.86**	**3.22-5.14** **1.48-8.61** **1.34-5.18** **2.30-2.77** **1.65-2.10** **1.86-2.11**	**0.0003** **0.01** **0.01** **0.0003** **0.0003** **0.0003**

(Continued)

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TABLE 1 Continued

Author	Year	Location	Study design	Reference cohort	N (case/control)	Neurologic/psychiatric comorbidities in individuals with BP	OR/*HR*	95% CI	*p*
						Other dementias	**1.71**	**1.51-1.94**	**0.0003**
						Vascular dementia	**1.67**	**1.40-2.00**	**0.0003**
						Senile dementia	**1.67**	**1.41-1.98**	**0.0003**
						Epilepsy	**1.67**	**1.41-1.98**	**0.0003**
						Psychoses	**1.25**	**1.12-1.39**	**0.0003**
						Depression	**1.19**	**1.11-1.28**	**0.0003**
Kalińska-Bienias A., et al. (28)	2019	Poland	Case-control	Age- and sex-matched controls	218/168	**Dementia**	**7.89**	**2.99-20.85**	**<0.001**
						Neurologic disease	**3.76**	**2.13-6.65**	**<0.001**
Papakonstantinou E., et al. (29)	2019	Germany	Cohort-study	Age- and sex-matched controls	183/348	**Neurological disorder**	**10.8**	**6.9–16.7**	**<0.001**
						Dementia	**9.9**	**5.4-17.8**	**<0.001**
						Stroke	**4.1**	**1.7-9.7**	**0.0015**
						Parkinson's disease	**2.9**	**1.0-8.4**	**0.0434**
						Alzheimer's disease	0.3	0.0-7.9	0.5303
Rania M, et al. (12)	2020	Denmark	Cohort study (national register)	Adjustment for medication	2,892/6,470,450	Subsequent BP in			
						- **Any psychiatric disorder**	***1.4***	**1.25-1.56**	**n.a.**
						- **Organic disorder**	***1.55***	**1.32-1.81**	**n.a.**
						- **Substance use disorder**	***1.70***	**1.35-2.13**	**n.a.**
						- **Schizophrenia**	***1.57***	**1.21-2.02**	**n.a.**
						- **neurotic disorder**	***1.41***	**1.16-1.71**	**n.a.**
						- **personality disorder**	***1.53***	**1.22-1.92**	**n.a.**
						- **intellectual disorder**	***2.96***	**1.58-5.51**	**n.a.**
Kridin K., et al. (11)	2021	Germany	Cross-sectional study	Age- and sex-matched controls	1,743/10,141	**Parkinson's disease**	**2.71**	**2.19-3.35**	**<0.001**
						Epilepsy	**2.18**	**1.72-2.77**	**<0.001**
						Alzheimer's disease	**2.11**	**1.73-2.57**	**<0.001**
						Stroke	**1.84**	**1.55-2.19**	**<0.001**
Kilic Sayar S., et al. (30)	2021	Turkey	Case-control	Age- and sex-matched controls	145/310	**Stroke**	**2.29**	**1.15-4.56**	**0.017**
						Neurological disorder	**1.94**	**1.12-3.35**	**0.017**
						Parkinson's disease	1.28	0.30-5.46	0.731
						Alzheimer's disease	1.69	0.61-4.64	0.304
						Dementia	2.20	0.81-5.99	0.122
Sánchez-García V., et al. (2)	2022	Spain	Retrospective observational study	Compared to pemphigus patients	5,424/1,950	Higher prevalence in BP compared to pemphigus			
						- **Dementia**	**n.a.**	**n.a.**	**<0.001**
						- **Parkinson's disease**	**n.a.**	**n.a.**	**0.043**
						- Cerebrovascular disease	n.a.	n.a.	0.057
						- Depression	n.a.	n.a.	0.087
						- Epilepsy	n.a.	n.a.	0.09
						- Multiple sclerosis	n.a.	n.a.	0.074
Martin E., et al. (13)	2022	Germany	Case-control	Age- and sex-matched controls	300/583	**Multiple sclerosis**	**10.00**	**1.16–85.59**	**<0.05**
						Dementia	**7.47**	**4.45–12.54**	**<0.0005**

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TABLE 1 Continued

Author	Year	Location	Study design	Reference cohort	N (case/control)	Neurologic/psychiatric comorbidities in individuals with BP	OR/*HR*	95% CI	*p*
						Organic brain syndrome	**5.50**	**1.75–17.27**	**<0.05**
						Neurological disease	**3.59**	**2.56–5.02**	**<0.0005**
						Parkinson's disease	**3.03**	**1.51–6.08**	**<0.05**
						Psychiatric disease	**2.441**	**1.64-3.61**	**<0.0005**
						Epilepsy	**2.397**	**1.11–5.14**	**<0.05**
						Stroke	**2.355**	**1.60–3.45**	**<0.0005**
						Depression	**1.799**	**1.15–2.80**	**<0.05**
Titou H., et al. (31)	2022	Morocco	Case-control	Age- and sex-matched controls	81/162	**Significant association of BP with any neurological disease and stroke**	**n.a.**	**n.a.**	**0.001**

Data in bold indicate statistically significant results.
BP, bullous pemphigoid; OR, odds ratio; HR, hazard ratio; CI, confidence interval; n.a, not available.

matched controls (35.2% vs. 14.8%; $p < 0.001$) (43). BP patients with metabolic syndrome were older at time of diagnosis than BP patients without metabolic syndrome (72.0 vs. 60.0 years, $p = 0.006$). Overall, patients with BP have a higher risk for cardiovascular mortality compared to matched controls after one, three and five years (7.9 vs. 1.3%; 11.1 vs. 2.4% and 12.3 vs. 3.9%), respectively (42). Surprisingly, the mortality risk was higher in patients without a prior history of hypertension, cardiovascular disease and diuretic medication (HR 7.28, HR 6.59 and HR 5.75). Compared to other autoimmune bullous diseases such as pemphigus, BP shows the highest risk for hypertension, diabetes and cardiovascular disease (36).

A case-control study including 91 patients and 546 age- and sex-matched controls in the United States showed a significant association of BP with arterial hypertension (OR 2.03; 95% CI 1.24–3.32), diabetes mellitus (OR 2.59; 95% CI 1.60–4.19), chronic kidney disease (OR 2.29; 95% CI 1.19–4.40) and end-stage renal disease (OR 3.82; 95% CI 1.48–9.85) (40). Importantly, anti-diabetic medications, especially dipeptidyl peptidase-4 (DPP-4) inhibitors (HR 2.15; 95% CI 1.18-3.91, $p = 0.01$) and metformin (HR 1.93; $p = 0.006$), increase the risk for BP (41) rising the chicken-and-egg-question whether diabetes itself or antidiabetic medication increases the risk for BP. A Finnish registry-based study confirmed that 19.6% of patients with BP suffer from diabetes but subanalyses revealed that oral antidiabetic medication such as sulfonylurea, thiazolidinedione and glucose-like-peptid-1 analogues were not associated with an increased risk for BP (38). Previously, the same study group demonstrated that the use of DPP-4-inhibitors was significantly associated with an increased risk of developing BP (OR 2.19, 95% CI 1.55-3.11), whereas the association with metformin was not significant (OR 1.49; 95% CI 1.32-1.68) (37). These results are in line with a Taiwanese cohort study which observed that the cumulative incidence of BP was significantly higher in a DPP-4-inhibitor-treated cohort than in the non-DPP-4-inhibitor control group over a period of 6 years (0.74 per 1000 vs 0.38 per 1000, $p = 0.001$) (41). A case-control study from Israel reported a 3-fold increased risk for developing BP in patients treated with DPP-4-inhibitors (OR 3.2; 95% CI 1.9-5.4) (44).

The reported association of BP with chronic kidney disease may be responsible for a strong association of BP with anemia that we recently demonstrated in a case-control study (OR 2.13; CI 1.53–2.95, $p < 0.0005$) (13). Further analysis revealed that normocytic normochromic anemia, as seen in patients with chronic kidney disease (45), was the predominant form of anemia observed in BP patients. Similar to anemia, osteoporosis is associated with chronic kidney disease (46). A cross-sectional study from the United States that included 8,864 patients with BP showed an increased OR for osteoporosis (1.55; 95% CI 1.39-1.73) and pathological fractures (1.52; 95% CI 1.22-1.88) (39). Patients with long-term systemic steroid treatment were at even higher risk for osteoporosis. Possible pathogenetic links between BP and osteoporosis next to systemic steroid therapy and chronic kidney disease are chronic systemic inflammation with enhanced bone resorption, decreased physical activity and hypovitaminosis D (39). 25-hydroxy-vitamin D (25-OHD), the precursor of vitamin D, is determined in the blood to assess the vitamin D status. In patients with BP lower 25-OHD

TABLE 2 Association of bullous pemphigoid with metabolic, cardiovascular and kidney diseases and their medication.

Author	Year	Location	Study design	Reference cohort	N (case/ control)	Metabolic comorbidities and their medication in individuals with BP	OR/*HR*	95% CI	*p*
Marzano A.V., et al. (33)	2012	Italy	Case-control	age-, body mass index- and sex-matched controls	15/28	**Lower 25-OHD-levels**	**n.a.**	**n.a.**	**0.01**
Sarre M.E., et al. (34)	2016	France	Case-control	n.a.	31/59	**Hypovitaminosis D**	**3.7**	**n.a.**	**0.046**
Karabay E. A., et al. (35)	2017	Turkey	Cohort-study	n.a.	62/217	**Patients with BP with higher incidence of** **- Hypertension** **- Diabetes** **- Coronary artery disease**	 **n.a.** **n.a.** **n.a.**	 **n.a.** **n.a.** **n.a.**	 **<0.01** **<0.01** **0.001**
Ren Z., et al. (27)	2017	USA	Register study of hospitalized adult patients in the USA	n.a.	2,108 BP primary diagnosis 11,234 BP secondary diagnosis 72,108,077 total hospital discharges	**Myocarditis** **Diabetes** **Obesity** **Congestive heart failure** **Pulmonary circulation disorders** **Hypertension**	**6.29** **1.57** **1.56** **1.43** **1.38** **0.98**	**1.57-25.1** **1.35-1.82** **1.44-1.70** **1.35-1.52** **1.22-1.58** **0.93-1.02**	**0.02** **0.003** **0.003** **0.003** **0.003** **0.003**
Kwa M. C., et al. (36)	2017	USA	Register study of hospitalized adult patients in the USA	n.a.	13,342/ 72,651,487	**Congestive heart failure** **Pulmonary circulation disorder** **Hypertension** **Peripheral vascular disease** **Type 2 Diabetes** **Coronary artery disease**	**2.58** **1.92** **1.80** **1.68** **1.45** **1.17**	**2.42-2.74** **1.69-2.19** **1.71-1.90** **1.54-1.83** **1.24-1.69** **1.10-1.24**	**<0.001** **<0.001** **<0.001** **<0.001** **<0.001** **<0.001**
Sim B., et al. (26)	2017	Singapore	Case-control	Age- and sex-matched controls	105/315	**Hypertension** Diabetes Ischemic heart disease Hyperlipidemia Kidney disease	**2.37** 1.23 1.12 0.24 0.44	**1.18-4.77** 0.78-1.96 0.68-1.82 0.1-0.49 0.2-0.99	**0.015** 0.375 0.66 0.001 0.049
Jeon H.W., et al. (7)	2018	Korea	Cohort study	Age- and sex-matched general population of Korea	103/n.a.	**Standardized mortality ratio** **- 70-79 years** **- >80 years** **- total** Prevalence of **Diabetes** Arterial hypertension Chronic kidney disease	 ***2.35*** ***2.18*** ***1.83*** **1.64** 0.63 0.56	 **1.52-3.47** **1.31-3.42** **1.34-2.46** **1.06-2.55** 0.42-0.95 0.30-1.08	 **<0.05** **<0.05** **<0.05** **0.026** 0.036 0.109

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TABLE 2 Continued

Author	Year	Location	Study design	Reference cohort	N (case/ control)	Metabolic comorbidities and their medication in individuals with BP	OR/*HR*	95% CI	*p*
Varpuluoma O., et al. (37)	2018	Finnland	Register study	Age-, sex and year of diagnosis matched controls	3,397/12,941	Increased risk for BP after adjustment for diabetes and several neurologic disorders - **DPP-4-inhibitors** - Metformin	 **2.19** 1.05	 **1.55-3.11** 0.88-1.24	 **<0.05** >0.05
Varpuluoma O., et al. (38)	2018	Finnland	Register case-control study	Age-, sex and year of diagnosis matched controls	3,397/12,941	- Diabetes prevalence of 19.6% in BP patients - No association of anti-diabetic medication and BP (excluding DPP4-inhibitos, metformin and insulin)	n.a.	n.a.	>0.05
Kalińska-Bienias A., et al. (28)	2019	Poland	Case-control	Age- and sex-matched controls	218/168	**Arterial hypertension**	**2.17**	**1.35-3.49**	**<0.001**
Chovatiya R., et al. (39)	2020	USA	Register study	n.a.	8,864/ 198,102,435	**Osteoporosis** **Pathological fractures**	**1.55** **1.52**	**1.39-1.73** **1.22-1.88**	**<0.0001** **0.0002**
Lee S., et al. (40)	2021	USA	Case-control	Age- and sex-matched controls	91/546	**End-stage renal disease** **Diabetes mellitus** **Chronic kidney disease** **Hypertension**	**3.82** **2.59** **2.29** **2.03**	**2.45-11.1** **1.60-4.19** **1.19-4.40** **1.24-3.32**	**0.0235** **0.0011** **0.0473** **0.0235**
Wu C.Y., et al. (41)	2021	Taiwan	Cohort-study of diabetic patients with and without DPP4-i treatment	Matched by age, sex, duration of diabetes, insulin usage, and propensity score-matching of comorbidities	124,619 DPP4-I/ 124,619 Non-DPP4-I	**Increased risk for BP by** - **Renal disease** - **DPP4-Inhibitor treatment** - **Metformin**	 **2.32** **2.15** **1.93**	 **n.a.** **1.18-3.91** **n.a.**	 **<0.001** **0.01** **0.006**
Sánchez-García V., et al. (2)	2022	Spain	Retrospective observational study	Compared to pemphigus patients	5,424/1,950	Higher prevalence in BP compared to pemphigus - **Arterial hypertension** - **Kidney disease** - **Heart failure** - **Chronic obstructive pulmonary disease** - **Diabetes** - **Osteoporosis**	 **n.a.** **n.a.** **n.a.** **n.a.** **n.a.** **n.a.**	 **n.a.** **n.a.** **n.a.** **n.a.** **n.a.** **n.a.**	 **0.004** **<0.001** **<0.001** **<0.001** **<0.001** **0.003**
Martin E., et al. (13)	2022	Germany	Case-control	Age- and sex-matched controls	300/583	**Renal impairment** **Anemia** **Diabetes mellitus**	**2.21** **2.12** **1.41**	**1.64-2.99** **1.53-2.95** **1.03-1.93**	**<0.0005** **<0.0005** **0.029**
Titou H., et al. (31)	2022	Morocco	Case-control	Age- and sex-matched controls	81/162	No significant association of BP with - Hypertension - Type 2 diabetes - Dyslipidemia	 n.a. n.a. n.a.	 n.a. n.a. n.a.	 0.891 0.062 0.643

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TABLE 2 Continued

Author	Year	Location	Study design	Reference cohort	N (case/ control)	Metabolic comorbidities and their medication in individuals with BP	OR/*HR*	95% CI	*p*
Shen W.C., et al. (42)	2022	Taiwan	Cohort study	Age- and sex- matched controls	252/1,008	**Cardiovascular mortality**	**4.8**	3.07-7.59	**<0.05**
Zhang B. et al. (43)	2022	China	Case-control	Age- and sex-matched controls	162/162	**Diabetes** **Obesity** Hypertension	**1.87** **1.80** 0.75	**1.02-3.39** **1.02-3.18** 0.44-1.12	**0.001** **<0.001** 0.200

Data in bold indicate statistically significant results.
BP, bullous pemphigoid; OR, odds ratio; HR, hazard ratio; CI, confidence interval; n.a, not available.

levels (mean ± standard deviation (SD): 9.6 ± 7.2 ng mL(-1); controls: 22.6 ± 18.7 ng mL(-1), p = 0,022) and a higher prevalence of severe hypovitaminosis D than in controls (73% vs. 27%, p = 0.01) were recorded (33).

Malignancies

Thirteen of the selected 48 studies focused on malignant diseases in patients with BP (Table 3). The association of BP with malignancies has been discussed for a long time. First reports debating a relationship date back to the year 1968. Twelve out of 103 patients with estimated BP subsequently developed malignancy (50). In the following decades several studies addressed this association with contradictory results. In 2021, a Turkish case-control study with 145 BP patients showed higher rates of malignancies compared to controls (OR 2.597; 95% CI 1.328-5.076; p = 0.005). The type of malignancy was not reported but skin cancers were excluded and subgroup analysis found men to be at higher risk than women (OR 4.347; 95% CI 1.488–12.658; p = 0.004) (30).

In contrast, six studies and a meta-analysis observed no significantly increased overall risk for malignancy or cancer-related mortality in patients with BP (13, 15–17, 47, 48, 51). For example, an Asian study including 359 patients with BP reported no increased standard incidence ratio (IR) of malignant diseases compared to sex- and age-matched controls (IR 0.97; 95% CI 0.53-1.62) (48). In line, a British cohort study observed no increased risk for developing BP in patients with a prior history of malignant diseases (risk ratio (RR) 0.96; 95% CI 0.88–1.04) (47). Interestingly, in sub-analyses patients with a history of kidney cancer (RR 2.23; 95% CI 1.48–3.24, p < 0.001), laryngeal cancer (RR 2.22; 95% CI 1.21–3.75; p = 0.004) or lymphoid leukemia (RR 2.27; 95% CI 1.21–3.89, p = 0.005) were at higher risk for developing BP. A German cohort study observed an association of BP with hematological malignancies (OR 2.55; 95% CI 2.07-3.13; p < 0.001) but not with non-hematological malignancies (14). Further analyses found a strong association with Morbus Hodgkin (OR 4.2; 95% CI 1.90–8.9; p < 0.01), non-follicular lymphoma (OR 3.80; 95% CI 1.90–7.5; p < 0.01), mature T/NK-cell lymphomas (OR 6.90; 95% CI 3.1–16.0; p < 0.001), unspecified types of Non-Hodgkin's lymphoma (OR 2.60; 95% CI 1.60–3.9; p < 0.001), myeloid leukemia (OR 5.70; 95% CI 1.90–17.0; p = 0.01), and leukemia of unspecified cell type (OR 2.70; 95% CI 1.40–5.1; p = 0.03). However, the authors assumed it to be unlikely for the hematological malignancy to have triggered BP as the malignancy preceded BP in only about half of the cases.

A population-based cohort study with an added case-control design found an increased risk of 50% for patients with preexisting melanoma to develop BP (OR 1.53; 95% CI 1.14–2.06) (49). Males (OR 1.66; 95% CI 1.09–2.54) and patients older than 80 years (OR 1.63; 95% CI 1.11–2.38) were at higher risk. Vice versa, the risk for melanoma in patients with BP was elevated but did not reach significance. The increased risk for BP in patients with melanoma persisted even after adjustment for confounders such as therapy by programmed cell death-1/programmed cell death ligand-1 (PD-1/PDL-1) inhibitors.

TABLE 3 Association of bullous pemphigoid and malignancy.

Author	Year	Location	Study design	Reference cohort	N (case/ control)	Malignant comorbidities in individuals with BP	OR/*HR*	95% CI	*p*
Lindelöf B., et al. (17)	1990	Sweden	Register study	Age- and sex-matched reference cohort	497	Expected number of incident cancer diagnosis in individuals with BP compared to matched standard incidence ratio showed no significant difference	n.a.	n.a.	n.a.
Ong E., et al. (47)	2011	England	Cohort-study of hospital admission data	reference cohort without known association with either cancer or BP 4,720 individuals with BP	2,873,729 individuals with cancer	Risk of BP in individuals with vs. without cancer - **with lymphoid leukaemia** - **with kidney cancer** - **with laryngeal cancer** Risk of cancer in individuals with BP	0.96 **2.27** **2.23** **2.22** 1.00	0.88-1.04 **1.21-3.89** **1.48-3.24** **1.21-3.75** 0.92-1.09	>0.05 **<0.05** **<0.05** **<0.05** >0.05
Cai S.C.S., et al. (48)	2015	Singapore	Cohort-study	Age- and sex-matched general population	359/n.a.	Expected number of incident cancer diagnosis after BP compared to matched standard incidence ratio	0.97	0.53-1.62	n.a.
Schulze F., et al. (14)	2015	Germany	Cohort-study	Age- and sex-matched controls	1,743/10,141	**Mature T/NK-cell lymphoma** **Myeloid leukemia** **Hodgkin disease** **Non-follicular lymphoma** **Leukemia of unspec. cell type** **Unspec. non-Hodgkin lymphoma**	**6.9** **5.7** **4.2** **3.8** **2.7** **2.6**	**3.1-16.0** **1.90-17.0** **1.90-8.9** **1.90-7.5** **1.40-5.1** **1.60-3.9**	**<0.001** **<0.001** **<0.01** **<0.01** **0.03** **<0.01**
Atzmony L., et al. (15)	2017	n.a.	Meta-analysis of cross-sectional, case-control and cohort studies	n.a.	n.a.	**Pooled analysis of cross-sectional studies found significant association of BP and hematologic malignancies but not with overall cancer**	**n.a.**	**n.a.**	**n.a.**
Ren Z., et al. (27)	2017	USA	Register study of hospitalized adult patients in the USA	n.a.	2,108 BP primary diagnosis 11,234 BP secondary diagnosis 72,108,077 total hospital discharges	BP was not associated with higher odds of any solid-organ or hematological malignancies	n.a.	n.a.	>0.1
Kalińska-Bienias A., et al. (28)	2019	Poland	Case-control	Age- and sex-matched controls	218/168	Malignancy	1.74	0.84-3.65	0.13
Kridin K., et al. (16)	2021	Israel	Cohort-study	Age-, sex- and ethnicity-matched controls	3,924/19,280	**Risk of uterine cancer** Risk of solid malignancies History of malignancy	***2.56*** *0.9* 1.00	**1.39-4.72** 0.77-1.05 0.90-1.10	**0.003** n.a. 0.469
Kilic Sayar S., et al. (30)	2021	Turkey	Case-control	Age- and sex-matched controls	145/310	**Preexisting Malignancy**	**2.59**	**1.32-5.07**	**0.005**
Martin E., et al. (13)	2022	Germany	Case-control	Age- and sex-matched controls	300/583	No association between malignancies and BP was observed	n.a.	n.a.	>0.05

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TABLE 3 Continued

Author	Year	Location	Study design	Reference cohort	N (case/control)	Malignant comorbidities in individuals with BP	OR/*HR*	95% CI	*p*
Kridin K., et al. (49)	2022	Israel	Case-control	Age-, sex- and ethnicity-matched controls	3,924/19,280	**History of melanoma** Risk of melanoma **Higher prevalence of melanoma in BP patients**	**1.53** 1.13 **n.a.**	**1.14-2.06** 0.73-1.74 **n.a.**	**0.004** 0.587 **0.004**
Shen W.C., et al. (42)	2022	Taiwan	Cohort-study	Age- and sex-matched controls	252/1,008	**Cancer mortality**	***1.87***	**1.01-3.45**	**<0.05**
Titou H., et al. (31)	2022	Morocco	Case-control	Age- and sex-matched controls	81/162	**Significant association of BP with Malignancy**	**n.a.**	**n.a.**	**0.017**

Data in bold indicate statistically significant results.
BP, bullous pemphigoid; OR, odds ratio; HR, hazard ratio; CI, confidence interval; n.a, not available.

Immunomodulatory therapy of tumor diseases can also trigger BP. Since the beginning of immune checkpoint inhibitor therapy, many cases and case-series with PD-1/PDL-1 inhibitor-induced BP have been published (52). Unlike immune checkpoint inhibitor-induced maculopapular rash, first symptoms of BP, e.g. pruritus, often occur months after the start of immunotherapy or even after discontinuation (53). In the majority of published cases development of BP led to a stop of immunotherapy (54).

Autoimmune and inflammatory diseases

Eleven of 48 studies discussed autoimmune and inflammatory diseases in patients with BP (Table 4). The association of BP with MS is well established as described above. In 1981, a case report of concomitant BP and rheumatoid arthritis initiated an ongoing discussion about the association of BP and autoimmune or inflammatory diseases (64). A case-control study published in 1993 focused on the association between autoimmune disorders such as diabetes mellitus type 1, rheumatoid arthritis, alopecia areata and BP. No differences were detected between BP patients and age- and sex-matched controls (55).

In the last few years several studies investigating the association of psoriasis and BP have been published. A meta-analysis of four case-controls studies including 4,035 BP patients and 19,215 controls revealed a significantly higher prevalence of psoriasis in patients with BP than in controls (2.6% vs. 1.1%; OR 2.5; 95% CI 1.4-4.6), predominantly in males compared to females (3.0% vs. 1.9%; OR 1.75; 95% CI 1.1-2.7) (58). A large population-based retrospective cohort study including 3,924 patients with BP with age-, sex- and ethnicity-matched controls reported a higher prevalence of preexisting psoriasis in patients with BP than in controls (1.7 vs. 1.1%, $p < 0.001$) (21). Additionally, patients with a history of psoriasis had a higher risk of developing BP (OR 1.53; 95% CI 1.17–2.02) and patients with BP had a 2.6-fold increased risk of developing psoriasis as compared to controls (HR 2.60; 95% CI 1.59–4.27).

Next to psoriasis, patients with a preexisting diagnosis of atopic dermatitis or allergic rhinitis show an increased risk for subsequent BP (OR 1.76; 95% CI 1.44-2.15; $p < 0.001$ and OR 1.13; 95% CI 1.01-1.28; $p = 0.047$) (62). These findings are in line with a recent large population-based case-control study that identified atopic dermatitis to be a significant risk factor for BP compared to age-, sex-, and comorbidity-matched controls (OR 1,76, 95% CI 1,55–2,00) (19). Next to atopic dermatitis and allergic rhinitis asthma is associated with a 50% increased risk of developing BP (OR 1.45; 95% CI 1.30–1.62) and the prevalence of preexisting asthma was higher in patients with BP than in controls (11.1 vs. 7.9%; $p < 0.001$) (60).

Inflammatory bowel disease is characterized by a non-infectious inflammation mediated by inflammatory cytokines that lead to mucosal destruction. Skin diseases such as erythema nodosum and pyoderma gangraenosum are well-known to occur more frequently in patients with inflammatory bowel disease. Only few data regarding the association of Inflammatory bowel disease and BP have been published. In 2020, a population-based case-control

TABLE 4 Association of bullous pemphigoid with autoimmune and inflammatory diseases.

Author	Year	Location	Study design	Reference cohort	N (case/ control)	Autoimmune and autoinflammatory comorbidities in individuals with BP	OR	95% CI	*p*
Taylor G., et al. (55)	1993	United Kingdom	Case-control	Age- and sex-matched controls	108/108	- Incidence of autoimmune disorders between patients and controls was not significant - The haplotypes of patients with BP were similar to those of a locally drawn population	n.a.	n.a.	n.a.
Ameri P., et al. (56)	2013	Italy	Retrospective cohort-study	n.a.	23/46	**- Thyroid autoantibody positivity** **- anti-TPO autoantibody positivity**	**6.75** **6.27**	**1.55-31.5** **1.23-35.5**	**<0.01** **<0.05**
Kridin K., et al. (57)	2017	Israel	Case-control study	Age-and sex- and ethnicity-matched controls	287/1,373	**Psoriasis**	**4.4**	**2.2-8.9**	**<0.0001**
Phan K., et al. (58)	2019	Australia	Meta-analysis of case-control studies	n.a.	4,035/ 19,215	**Psoriasis**	**2.5**	**1.4-4.6**	**0.003**
Kridin K., et al. (21)	2020	Israel	Retrospective population-based cohort-study and case-control study	Age-, sex- and ethnicity-matched controls	3,924/ 19,280	**Psoriasis in individuals with BP** **BP in individuals with psoriasis**	**2.60** **1.53**	**1.59-4.27** **1.17-2.02**	**<0.001** **0.002**
Chen Y.J., et al. (59)	2020	Taiwan	Case-control study using a nationwide database	age-, gender- and hospital visit number-matched controls	5,263/ 21,052	**Ulcerative colitis**	**3.60**	**1.91-6.77**	**<0.001**
Kridin K., et al. (60)	2021	Israel	Population-based -study	Age-, sex- and ethnicity-matched controls	3,924/ 19,280	**History of Asthma**	**1.43**	**1.30-162**	**<0.001**
Ständer S., et al. (61)	2021	Germany	Retrospective cohort study	Comparison of BP patients with and without psoriasis	274 (11/ 262)	Prevalence of psoriasis 4% **- younger age at onset of BP** **- milder erosive phenotype** **- lower levels of BP-180 antibodies**	n.a.	n.a.	n.a. **0.023** **0.025** **0.008**
Kridin K., et al. (62)	2022	Israel	Population-based cohort-study and case-control study	Age-, sex- and ethnicity-matched controls	3,924/ 19,280	**History of atopic dermatitis (AD)** **History of allergic rhinitis (AR)** **Risk of AD** Risk of subsequent AR	**1.76** **1.13** **2.00** 1.00	**1.44-2.15** **1.01-1.28** **1.60-2.51** 0.83-1.20	**<0.001** **0.047** **<0.001** 0.997
Wu P.C., et al. (19)	2022	Taiwan	Two case-control studies using a nationwide database	1) age- and sex-matched controls 2) age-, sex-, and propensity score of comorbidities matched controls	1) 9,344/ 18,688 2) 7,196/ 14,392	**1)** **Psoriasis** **Atopic dermatitis** **2)** **Atopic dermatitis**	 **2.83** **1.71** **1.76**	 **2.27-3.51** **1.50-1.95** **1.55-2.00**	 **<0.001** **<0.001** **<0.001**
Kridin K., et al. (63)	2022	Israel	Population-based cross-sectional study	Age- and sex-matched controls	1,743/ 10,141	**Thyreoiditis**	**1.98**	**1.18-3.35**	**0.010**

Data in bold indicate statistically significant results.
BP, bullous pemphigoid; OR, odds ratio; HR, hazard ratio; CI, confidence interval; n.a, not available.

10.3389/fimmu.2023.1196999

study reported a significant association of BP with ulcerative colitis (OR 3.60, 95% CI 1.91–6.77, p < 0.001) but not Crohn's disease (59).

Inflammatory diseases of the thyroid including Graves' disease and Hashimoto thyroiditis are the most common autoimmune disorders with a prevalence of 2-5% predominantly affecting women (65). Several case reports of patients with autoimmune thyroid diseases who later developed BP have been published suggesting an association of these autoimmune disorders (66, 67). Recently, a significant association between thyroiditis and BP (OR 1.98; 95% CI 1.18-3.35; p = 0.01) has been reported (63). An Italian hospital-based study observed significantly higher anti-TPO-autoantibody levels in patients with BP but not in patients with pemphigus vulgaris compared to controls (56).

Discussion

Neurologic and psychiatric diseases

The association of BP with neurologic and psychiatric diseases is well acknowledged and confirmed by several case-control and cohort studies (8–13). BP-180, the main autoantigen in BP, is also expressed in the central nervous system (68). A prospective multicenter study including 499 patients with BP found an association between neuropsychiatric disease and increased BP-180 autoantibody seropositivity (69). Likewise in sera of 115 patients with Alzheimer's disease levels of BP-180 autoantibodies were significantly increased compared to controls, however, these antibodies failed to react with cutaneous basement membrane in indirect immunofluorescence analysis and none of the patients had clinical symptoms of BP (70). Next to BP-180, BP-230 is another major autoantigen in BP. Several isoforms of BP-230 are existing; among others the epithelial isoform BP-230-e connects hemidesmosomes to keratin filaments. BP-230-a is detected in the nervous system, maintaining the organization of the microtubule network in neurons. Possibly, patients with MS can develop an autoantibody response to neuronal variants of BP-230 (71). Due to the degeneration of nerves and their sheaths, structural proteins such as BP-230 may be recognized by the immune system and trigger autoinflammation. This may result in an autoimmune response against the epithelial isoform of BP-230 and development of BP (72). In mouse models sera of patients with both BP and neurologic diseases reacted with BP-230 proteins from mouse brain extract (73). In line with these results, a single-center retrospective case–control study from Turkey observed significantly higher BP-230 and BP-180 autoantibody titers at initial diagnosis of BP in patients with preexisting neurological disorders than without (30). Similar results were observed in a German retrospective study that reported a higher BP-230 autoantibody seropositivity in BP patients with comorbid neuropsychiatric disease than in those without (67.7% vs. 36.5%; P = 0.006); moreover, coexistence of BP and neuropsychiatric disease was significantly associated with anti-BP-230 seropositivity (OR 3.43; 95% CI 1.24-9.52; p = 0.018) (74). This possible pathomechanism suggests that the degeneration of nerves fosters the development of autoantibodies that cross-react with the skin. However, in the follow-up phase of the Danish registry-based cohort study mentioned above (10), the HR for the development of MS was significantly higher in patients with BP than in control subjects, raising the question of whether there are other, yet unknown mechanisms linking the two diseases.

Cardiovascular and metabolic diseases

Similar to BP, inflammation is the major trigger of the early phases of the atherosclerotic process and inflammatory cytokines are associated with a higher risk for developing cardiovascular diseases (75). Next to BP-230-a and BP-230-e, a third isoform named BP-230-b is known (76). This isoform is mainly expressed in the myocardium and skeletal muscle of mice (77). It seems likely that, similar to neurologic diseases, cross-reactivity of autoantibodies to exposed structural proteins may promote the development of BP, but evidence is still lacking. On the contrary, a prospective multicenter study including 499 patients with BP found no association of elevated BP-180 autoantibody serum levels with metabolic disorders (including diabetes mellitus type 2) and arterial hypertension (69). Interestingly, medication with DPP-4-inhibitors in patients with BP was associated with lower BP-180 and BP-230 autoantibody seropositivity compared to BP patients without DPP-4-inhibitor intake (69). Several studies reported clinical and serologic differences in DPP-4 inhibitor-induced BP compared to classic BP, such as they were more likely to show a noninflammatory phenotype, lower eosinophil counts and lower autoantibody seropositivity (78, 79). Although the exact mechanisms of induction of BP by DPP-4-inhibitors have not been finally elucidated yet, these drugs should only be used with caution in BP patients especially if alternative therapies are available. As patients with BP seem to be at higher risk for osteoporosis, pathological fractures and hypovitaminosis D they should be encouraged to be physically active and to substitute vitamin D if necessary.

Altogether, study data are inconclusive with respect to associations between BP and metabolic diseases as well as chronic kidney disease and arterial hypertension; however, since we are facing an older patient population, these diseases should certainly be considered.

Malignancies

The association of BP and malignancy is not conclusively proven or disproven. Interpretation of the data remains challenging since multiple confounders such as ethnicity (including variability in HLA genotypes (80, 81)), age, gender and lifestyle must be considered regarding the association of malignancy and BP. New data suggests associations of BP and melanoma (49); however, it should be kept in mind that both patients with BP and melanoma are usually examined by dermatologists, thus, the latter may be detected earlier and more frequently.

As BP affects an elderly population, malignancy is common but might not be necessarily associated with the skin disease by itself.

Autoimmune and inflammatory diseases

An ongoing discussion about the association of BP and autoimmune or inflammatory diseases already started in 1981 (64). In recent years new evidence reignited the discussion when inflammatory skin diseases such as psoriasis and atopic dermatitis were reported to be associated with BP.

Interestingly, patients with BP and comorbid psoriasis were significantly younger, presented with a milder erosive phenotype and lower levels of BP-180 autoantibodies (61). This leads to the question of how BP and psoriasis influence each other. Multiple hypotheses have been postulated addressing this question; it has been suggested that (i) impairment of the basal membrane in psoriasis may lower the threshold for the generation of anti-basal membrane autoantibodies, (ii) shared pathogenetic features such as inflammation mediated by neutrophils, interleukin-1 (IL-1) and -17 (IL-17) and (iii) anti-psoriatic medication may trigger BP. Degradation of proteins in the basal membrane such as laminin 1 in psoriasis can modify the antigenicity of the basal membrane and therefore possibly enhance the formation of autoantibodies that are found in BP (82). Neutrophils are present in lesional skin of both diseases and may promote destruction of matrix proteins of the basal membrane *via* secretion of metalloproteinases exposing antigens of the dermal-epidermal junction and thus contributing to the generation of autoantibodies (83). IL-1, IL-17 and T-helper 17 (Th-17) cells play a major role in the pathogenesis of psoriasis; interestingly, IL-1 beta was found in blister fluid of BP (84) and Th-17 cells were significantly increased in conjunctival biopsies of patients with ocular cicatricial pemphigoid (85). Whether these findings are causal or merely coincidental must be clarified by further investigations. On the other hand, treatment of psoriasis may trigger BP as several case reports described development of BP after treatment with PUVA or biologicals such as adalimumab, efalizumab, etanercept, ustekinumab and secukinumab, respectively (86, 87).

BP also shares pathogenetic aspects with atopic dermatitis, allergic rhinitis and asthma such as elevated serum levels of immunoglobulin E (IgE), eosinophilia and a Th-2 response (88, 89). Additionally, dupilumab, a monoclonal antibody inhibiting Th-2 cytokines IL-4 and IL-13, has been successfully applied for treatment of BP (90).

Conclusion

BP is frequently accompanied by comorbid diseases. For some conditions, e.g. neuropsychiatric disorders, a clear association with BP has consistently been demonstrated in several studies suggesting that they share common pathomechanistic attributes. For other diseases the data situation is ambiguous and further, ideally prospective investigations are necessary to more reliable estimate an association with BP.

Several observations point to a pathomechanistically based association between BP and other diseases and conditions. These include: (i) Formation of autoantibodies due to augmented exposure to human structural proteins following cell damage (due to degenerative diseases, malignancies, inflammation); (ii) underlying common canonical inflammatory pathways of BP and other autoimmune and inflammatory diseases; (III) exposure to medication that subsequently facilitates formation of autoantibodies.

In our opinion, the results of the available studies do not currently allow a general screening recommendation for comorbid diseases in BP beyond a thorough work-up including but not limited to a detailed medical history, surveying comedication and vital signs, medical examination and routine laboratory testing. Hereby, most of the commonly associated comorbidities should be detected. In case of doubt, referral to a specialist such as a neurologist should be considered. Treating physicians should be aware of commonly associated diseases such as neurological, cardiovascular, malignant and inflammatory disorders in order to treat the patient in the best possible way and to avoid therapy-associated complications.

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

JH conducted literature searches, drafted the manuscript and finalized the manuscript for submission. SB and MG edited drafts of the manuscript and finalized the manuscript for submission. MG initiated the review. All authors contributed to the article and approved the submitted version.

Funding

This publication was supported by the Open Access Publication Fund of the University of Würzburg, Würzburg, Germany, and generated within the European Reference Network for Rare Skin Diseases (ERN Skin).

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 06 July 2023
DOI 10.3389/fimmu.2023.1176284

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OPEN ACCESS

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RECEIVED 28 February 2023
ACCEPTED 15 June 2023
PUBLISHED 06 July 2023

CITATION
Fenne IJ, Askildsen Oftebro G, Vestergaard C, Frølunde AS and Bech R (2023) Effect of early initiation of steroid-sparing drugs in patients with bullous pemphigoid.
Front. Immunol. 14:1176284.
doi: 10.3389/fimmu.2023.1176284

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Effect of early initiation of steroid-sparing drugs in patients with bullous pemphigoid

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Introduction: Bullous pemphigoid (BP) can be treated using systemic and topical glucocorticoids and/or other immunomodulatory agents. However, the long-term use of systemic glucocorticoids causes severe adverse side effects. This study was aimed at investigating whether the early initiation of corticosteroid-sparing therapy (CST) in BP patients results in better outcomes than late or no CST.

Method: We retrospectively identified all BP patients referred to the tertiary center, of the Department of Dermatology and Venerology, Aarhus University Hospital, Denmark, from 2015 to 2021. Patients' demographics, comorbidities, treatment, remission of BP, length of admission, relapse, and 1-year mortality were recorded. All patients who received CST were dichotomised into two groups: initiated with CST <28 or >28 days. The groups were compared using t-tests. Additionally, all patients who received CST were compared with those who received systemic glucocorticoids alone. Our cohort was compared with that of a previous study (2006–2013) performed in our department. In 2015, we revised our BP treatment guidelines to include the early initiation of CST.

Results: On comparing the group of patients initiated with CST <28 versus >28 days, we found no significant differences in the complications or mortality between the groups (p = 0.63 and p=0.79, respectively). The <28 days group had a lower rate of relapse (p < 0.05). On comparing data from this study with those from the previous study, conducted before we revised our treatment guideline, we found a reduced initial dose of prednisolone and reduced admission time in this study. No significant differences were found between patients treated with CST and those treated with systemic glucocorticoids alone.

Conclusion: The rate of complications and 1-year mortality did not differ significantly between the two subgroups in this study. The relapse rate was lower in the CST <28 days group than in the CST >28 days group. The initial dose of prednisolone and admission time were reduced in this study compared with those in the previous study performed before the implementation of a local treatment guideline recommending the early initiation of CST.

KEYWORDS

bullous pemphigoid, steroid-sparing treatment, immunomodulatory treatment, glucocorticoids, relapse, mortality, cardiac disease

1 Introduction

Bullous pemphigoid (BP) is an autoimmune blistering disease that predominantly affects individuals aged >65 years[1] and is characterized by tense subepithelial blisters and generalized pruritic urticarial plaques arising from normal or erythematosus skin (1) (Figure 1). In one-third of BP patients, the oral and/or anogenital mucosa is also affected. However, one-fifth of patients do not develop blisters; in such patients, the diagnosis is made by direct immunofluorescence of skin biopsy staining for deposits of IgG at the dermo-epidermal border1. Standard therapies include topical and systemic corticosteroids and other immunomodulatory agents. The outcome and prognosis of the disease are correlated with the disease severity. Long-term monitoring is often required, as well as hospitalization in severe cases.

BP is caused by autoantibodies targeting the hemidesmosomal proteins, BP230 (BPAG1) and BP180 (also termed BPAG2 and type XVII collagen). These proteins are components of the dermo-epidermal junction and are important components of the adhesion complexes that promote epithelial-stromal adhesion (2). The binding of antibodies to antigens promotes inflammation by activating the complement system and mast cells. Activated inflammatory cells produce proteolytic enzymes that damage the dermo-epidermal junction. Consequently, subepithelial blisters develop.

The prodromal phase of BP is characterized by pruritus and/or urticarial lesions and detection of circulating autoantibodies in the blood1. As the disease progresses, bullae may develop, which are often located on the flexor surface of the trunk, abdomen, forearms, and medial thighs and in the axillae. The fluid in the bullae is clear, and the bullae can persist for days before leaving erosions and crusts that heal without scarring. Unlike other blistering diseases, such as pemphigus vulgaris, the Nikolsky sign is negative in typical cases of BP whereas the Asboe–Hansen sign is positive (3). Damaged skin

FIGURE 1
Admitted BP patient with characteristic tense subepithelial blisters and pruritic urticarial plaques.

1 Lægehåndbogen at Sundhed.dk. Pemfigoid (2022). https://www.sundhed.dk/sundhedsfaglig/laegehaandbogen/hud/tilstande-og-sygdomme/bulloese-tilstande/pemfigoid/ [Accessed May 1, 2022].

can lead to secondary *Staphylococcus* or *Streptococcus* infections, which can be fatal in elderly patients, in case of sepsis1.

The first-line treatment for BP is systemic corticosteroids; however, the choice of treatment also depends on the disease severity and patients' comorbidities. In mild cases, potent topical steroids are sufficient, whereas in moderate-to-severe disease, systemic immunosuppressive treatments may be needed, including systemic corticosteroids at dosages of up to 0.5 to 0.75 mg/kg/day for the severe form of BP. This consensus on BP treatment with lower doses of systemic corticosteroids was developed by an international panel of experts in the field of autoimmune blistering disorders (4). Higher doses of glucocorticoids may be more effective in disease control but are not advisable because of the associated risk of adverse effects and increased 1-year mortality (5, 6).

A Finnish study from 2016 indicated that an early initiation of corticosteroid-sparing therapy (CST) and limitation of the systemic steroid dose to a maximum of 0.75 mg/kg/day may increase the survival of BP patients (7). The local guidelines at the Department of Dermatology at Aarhus University Hospital were revised in 2015 to recommend the systematic introduction of CST to BP patients within the first day of admission, in addition to the regular dose of prednisolone 0.5–0.75 mg/kg/day. If the patient did not tolerate prednisolone, tetracycline was administered. Azathioprine 0.5–2.5 mg/kg/day is often used as a steroid-sparing drug. Alternatively, other steroid-sparing drugs such as methotrexate, mycophenolate mofetil, or cyclosporine may be used depending on the individual patient and discretion of the treating doctor. In treatment-refractory cases, intravenous immunoglobulins, plasmapheresis, or rituximab may be administered.

To differentiate between different clinical severities of BP, two scoring systems have been developed: the Autoimmune Bullous Skin Disorder Intensity Score (ABSIS) and the Bullous Pemphigoid Disease Area Index (BPDAI) (8, 9). The BPDAI was developed by the International Pemphigoid Committee and is based on three subcomponents: cutaneous blisters, erythema/urticaria, and mucosal blisters/erosions (8). ABSIS uses a combination of the percentage of body surface involved and a subsequent weighting factor based on whether a lesion is exudative, dry, or re-epithelialized (9). However, these two scoring systems are not always employed in clinics as they are rather comprehensive and, therefore, more suitable for research purposes.

This study aimed to retrospectively describe the demographics, comorbidities, treatment (pre-hospitalization medications; glucocorticoids, including doses; and steroid-sparing drugs), and treatment outcomes (remission, length of admission, relapse, and 1-year mortality) in a cohort of all BP patients referred to a tertiary centre (Department of Dermatology, Aarhus University Hospital) from 2015 to 2021. We hypothesized that consistent application of the new local guidelines (early initiation of CST) in the department would improve the survival rate, lower the rate of complications, and lower the rate of relapse, thereby lowering the number of readmission and length of admission. In this manner, the new guidelines can be evaluated to provide valuable information about the effects of current steroid-sparing treatments. Our secondary objective was to compare our findings regarding comorbidities and pre-hospitalization medications with existing literature to contribute to the understanding of possible associations and epidemiology of BP.

2 Materials and methods

2.1 Design and patients

In this retrospective case-series study, the medical records of all referred patients diagnosed with BP at the Department of Dermatology and Venerology, Aarhus University Hospital, from 2015 to 2021 were investigated (Figure 2). The cohort was a heterogeneous group of patients, some referred from general practitioners as having suspected BP and some from private dermatology practitioners. Patients were identified using the ICD10 system. The diagnoses included in this study were BP (DL 12.0), unspecified pemphigoid (DL 12.9), and other pemphigoids (DL 12.8). The project was reported to the Regional (Region Midtjylland) Record of Research Projects (registration number: 1-16-02-44-22).

Patient characteristics (age, comorbidities, smoking, and prescribed medicines), treatment during admission, and treatment outcomes (admission length, remission, relapse, and mortality) were recorded using the Research Electronic Data Capture (REDCap) data management platform. Patients were included if characteristic clinical presentations such as bullae and pruritus were found; skin biopsies revealed subepidermal clusters of eosinophils and/or bullae; and/or direct immunofluorescence (IF) showed linear deposits of IgG, IgA, or complement C3 along the basement membrane zone. Patients with primary mucosal involvement were excluded, as this suggested a cicatricial pemphigoid.

The treatment of the referred patients was based on the local guidelines of the Department of Dermatology at Aarhus University Hospital. First-line therapies included potent topical glucocorticoids in combination with prednisolone if needed. Prednisolone was usually administered as 0.5–0.75 mg/kg/day and updosed until effect (no appearance of new blisters). If the patient did not tolerate systemic corticosteroids, tetracycline was administered. Local treatment guidelines recommend the initiation of CST on the day of admission. According to these guidelines, CST can include azathioprine, methotrexate, mycophenolate mofetil, or cyclosporine. In treatment-refractory cases, intravenous immunoglobulins, plasmapheresis, or rituximab can be administered.

2.2 Variables

To assess the outcomes, multiple data from medical records were included in our study. Data such as treatment and dosage, length of admission, readmission, relapse, remission, complications after diagnosis (sepsis and/or diabetes), and 1-year mortality were included. The length of admission was used as a proxy for the disease course, as patients were normally discharged when new bullae did not appear for a minimum of 2 days and primary skin

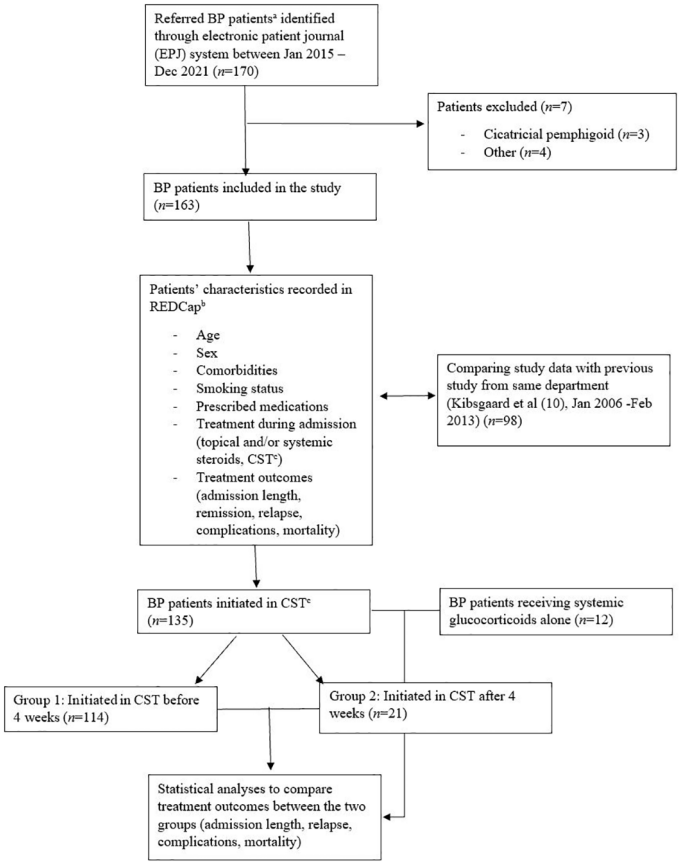

FIGURE 2
Flow chart of study design and patients. [a]Referred to the Department of Dermatology and Venerology at Aarhus University Hospital; [b]The Research Electronic Data Capture; [c]Corticosteroid-sparing therapy (CST).

lesions started to re-epithelialize. Frequent clinical control following a fixed scheme after discharge was common practice, and in this manner, relapse or remission was recorded. Remission was defined as the absence of bullae and/or urticaria. Readmission within the first year of diagnosis was interpreted as relapse of BP.

2.3 Data analysis

We used means and standard deviations (SDs) for all continuous variables and percentages for all categorical variables. To test our hypothesis that the early initiation of CST is related to treatment outcomes, all patients that received CST were secondarily dichotomised into two groups. The first group included patients receiving CST within 4 weeks (<28 days), and the second group included patients who received CST after 4 weeks (>28 days). We investigated the association between earlier initiation of CST and treatment outcomes versus late initiation. Furthermore, we compared all patients receiving CST with those who received prednisolone alone. The treatment outcomes were defined as the number of days admitted, relapse rate, rate of complications (sepsis and/or diabetes), and 1-year mortality. To compare the groups (CST before versus after 4 weeks groups and CST versus prednisolone alone group), we used *t*-tests, which was a two-sample equal variance (homoscedastic) test based on the assumption that the groups originated from the same population. *p*-values <0.05 were considered statistically significant.

3 Results

3.1 Patients

Between 01/01/2015 and 31/12/2021, a total of 170 patients were identified. Of these patients, three who were retrospectively diagnosed with cicatricial pemphigoid were excluded, along with four patients who were excluded for other reasons. Finally, 163 patients were included in the study (Figure 2).

The age at BP diagnosis ranged from 27 to 100 years with a mean ± SD age of 79 ± 10 years. Of the 163 patients, 94 (58%) were men and 69 (42%) were women (Table 1).

BP diagnosis was based on clinical, histological, and immunofluorescence in 143 (88%) of the 163 patients.

As for comorbidities, the most common comorbidity was cardiovascular diseases (CVDs), which was present in 75 patients (46%), followed by type II diabetes mellitus (DM), which was present in 48 patients (29%), and neurological disorders, including Alzheimer's disease, Parkinson's disease, multiple

TABLE 1 Baseline characteristics of patients admitted with bullous pemphigoid (n = 163) during the study period (Jan 2015–Dec 2021).

Characteristics	Patients
Sex, %	
Male	58
Female	42
Age at onset of BP, years, mean ± SD	79 ± 10
Diagnosis methods, %	
Objective	2
+ Histology	10
+ Immunofluorescence	88
Comorbidities, %	
Cardiovascular disease	46
Type II diabetes mellitus	29
Cancer	25
Neurologic disorders[a]	21
Type I diabetes mellitus	1
Other	26
Pre-hospitalisation medications[b], %	
Paracetamol	41
Acetylsalicylic acid	23
ACE inhibitors	21
Furosemide	17
Gliptins[c]	12
NSAIDs	4
None of the above	31

[a]Stroke (n = 24), Alzheimer's disease (n = 5), Parkinson's disease (n = 3), and multiple sclerosis (n = 3).
[b]Not included in the table: none of the patients (n = 0) received erlotinib, etanercept, anti-TNF-alfa, rifampicin, nivolumab/pembrolizumab, sirolimus, tiobutarit, or alogliptin. One patient received everolimus. Five patients did not use any medicines at all.
[c]A total of 18 patients received vildagliptin, one received linagliptin, and one received sitagliptin.

sclerosis, and stroke, which was present in 35 patients (21%) (Table 1).

Before hospitalization, the patients were most commonly treated with paracetamol (41%), acetylsalicylic acid (23%), angiotensin-converting-enzyme (ACE) inhibitors (21%), furosemide (17%), and gliptins (12%). None of the included patients were treated with nivolumab, erlotinib, etanercept, rifampicin, or sirolimus at the time of admission (Table 1). These drugs have been associated with the development of BP (2).

3.2 Treatment outcomes

Of the 163 patients, 106 (65%) were initiated with systemic glucocorticoids. The initial doses ranged from 5 to 50 mg, with a mean ± SD of 25 ± 11 mg (Table 2). Of the remaining 57 patients who were not initiated on systemic glucocorticoids at time of the first visit, 23 (40%) were initiated subsequently. We found that 131 (80%) of the 163 patients were initially treated with topical glucocorticoid group 4; 30 and 20% were treated with group 3 and 2 steroids, respectively. Whether the topical treatment was used only on the lesions or whole body (except face and anogenital region) varied based on clinical severity and doctor's discretion. In addition to topical steroids, some patients also received systemic steroids and/or CST.

Of the 163 patients, 135 (83%) were initiated on CST. The choice of CST was based on an individual assessment by taking into consideration comorbidities (CVD or existing/prior neoplasm), kidney and liver function, age, and performance status. Azathioprine was the most frequently used CST (n = 55), followed by doxycycline (n = 37) and methotrexate (n = 33; Table 2). Eight patients received dapsone as adjuvant CST. One patient was treated with mycophenolate mofetil and one with plasmapheresis. None of the patients were treated with rituximab during the study period. The number of days from diagnosis until the initiation of CST ranged from 0 to 719 days, with a mean of 3 days. For admitted patients, the number of days ranged from 0 to 120 days, with a mean of 1 day. For the non-admitted patients, the number of days ranged from 0 to 719 days, with a mean of 8 days (Figure 3).

At the time of diagnosis 62 (38%) out of the 163 patients were admitted to the hospital because of the severity of their BP disease. The number of days admitted ranged from 1 to 26, days with a mean ± SD of 8 ± 5 days (Table 2). Within the first year after BP diagnosis, 15 (24%) of the 62 admitted patients were readmitted. Of the 163 patients, 151 (93%) were classified as having remission of the disease within the first year and 141 (87%) did not have complications such as sepsis or diabetes after diagnosis. Overall, 17% of the patients died within the first year after diagnosis.

We found that 114 (84%) of those 135 patients initiated on CST were initiated on CST within 28 days (early initiation of CST) and 21 (16%) were initiated after 28 days (late initiation of CST).

In the CST <28 days group, the number of days admitted ranged from 1 to 26 days, with a mean ± SD of 8 ± 5 days, whereas in the CST >28 days group, this number ranged from 1 to 13 days, with a mean ± SD of 8 ± 5 days (p = 0.49). The rates of complications (sepsis and/or diabetes 1 year after diagnosis) were 15% and 19% (p = 0.63), the relapse rates were 38% and 62% ($p < 0.05$), and the 1-year mortality was 17% and 14% (p = 0.79) in the CST <28 days and CST >28 days groups, respectively (Table 3).

Only 12 patients (7%) were treated with prednisolone alone, whereas 135 (83%) were treated with CST. Compared with the CST group in which 46% of the patients were admitted to the hospital, none of those treated with prednisolone alone were admitted. The relapse rates were 33% and 42% (p = 0.55), the rates of complications were 8% and 16% (p = 0.50), and the 1-year mortality was 33% and 16% (p = 0.14) in the prednisolone alone and CST groups, respectively. None of the abovementioned data were statistically different between the prednisolone alone and CST groups.

10.3389/fimmu.2023.1176284

TABLE 2 Comparison of treatment and treatment outcomes in patients with bullous pemphigoid at the Department of Dermatology and Venereology at Aarhus University Hospital in two different time periods.

Jan 2006–Feb 2013 (n = 98)*		Jan 2015–Dec 2021 (n = 163)	
Treatment and outcomes	Patients	Treatment and outcomes	Patients
Length of primary admission, days mean ± SD	14 ± 9	Length of primary admission, days mean ± SD	8 ± 5
Dosage of systemic glucocorticoid at admission, mg, mean ± SD[a]	44 ± 15	Dosage of systemic glucocorticoid at admission, mg, mean ± SD[a]	25 ± 11
Corticosteroid-sparing therapy[b], patients, n		Corticosteroid-sparing therapy[b], patients, n	
Azathioprine	80	Azathioprine	55
Methotrexate	11	Doxycykline[c]	37
Mycophenolate mofetil	3	Methotrexate	33
Dapsone	1	Dapsone	8
Rituximab	1	Mycophenolate mofetil	1
		Plasmapheresis	1
		Rituximab	0
		Other treatments	0
6-week remission, %	74	1 year remission, %	93
Relapse[d], %	34	Relapse[d], %	38
		Complications[e], %	13
		1-year mortality, %	17

*Data from Kibsgaard et al. (10).
a2006-2013: Nine patients did not receive systemic glucocorticoid and did not contribute to this value (n=89). 2015-2021: 57 patients did not receive systemic glucocorticoids (n=106); b2006-2013: 12 of 98 patients did not receive CST for >1 week (n=86). 2015-2021: 28 of 163 patients did not receive CST (n=135); cTetracycline indicated when oral glucocorticoid treatment is contraindicated; d2006-2013: At any time of the study period. 2015-2021: 1 year after diagnosis. eDefined as diagnosis of sepsis and/or diabetes 1 year after BP diagnosis.

4 Discussion

The rate of complications and 1-year mortality did not differ significantly between the groups that initiated early (<28 weeks) or late (>28 weeks) corticosteroid-sparing therapy (CST). Instead, we found a minor increase in 1-year mortality in the <28-days group (17% *vs.* 14%, $p = 0.79$). However, the relapse rate was significantly different between the groups ($p < 0.05$), where those initiated early on CST had fewer relapses. This suggests a positive effect of the early initiation of steroid-sparing drugs. However, the relapse rate may be

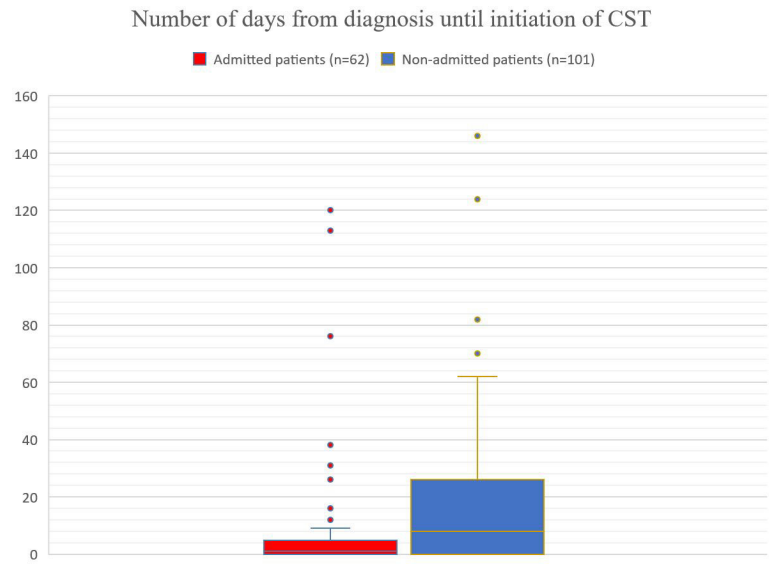

FIGURE 3
Admitted patients (red): mean of 1 day, number of days ranged from 0 to 120 days, with an upper quartile (75th percentile) of 5 days. Non-admitted patients (blue): mean of 8 days, number of days ranged from 0 to 719 days, with an upper quartile (75th percentile) of 26 days.

TABLE 3 Treatment outcomes stratified by the number of days until the initiation of CST.

All patients[a]			
	<28 days (n = 114)	>28 days (n = 21)	*p*-value
Number of days admitted, mean ± SD	8 ± 5	8 ± 5	0.49
Complications[b], %	15	19	0.63
Relapse, %	38	62	<0.05
1-year mortality, %	17	14	0.79

[a]Of 163 patients, 28 were not initiated on CST (n=135).
[b]Defined as diagnosis of sepsis and/or diabetes 1 year after BP diagnosis. During the study period only one patient was diagnosed with diabetes mellitus.

biased by patient compliance and disease severity; therefore, the outcome must be interpreted with caution.

Considering our cohort and its rather small size (compared with other cohorts of more prevalent diseases), finding significant results can be challenging. By performing a quick power calculation of our two subgroups and outcome relapse, the minimum powerful sample size was 134. As we had a cohort of 135 patients initiated on CST, there were theoretically enough patients to provide our study with statistical power.

There were 163 BP patients referred to the Department of Dermatology and Venerology from 2015 to 2021. This is an increase compared with an almost identical study by Kibsgaard et al. conducted from 2006 to 2013 within the same department (10). Kibsgaard et al. reported that 98 BP patients were referred to the Department of Dermatology during a 7-year study period from 2006 to 2013 (10). The increased number of BP patients could be due to several factors, including a continuously increasing proportion of the elderly population and/or better diagnostics of BP. Additionally, the same trend of an increasing incidence of BP patients has been observed in several other countries the last decades (11, 12). Moreover, other causal factors such as a higher prevalence of neurological diseases have been shown to increase the risk of developing BP (13). The same inclusion criteria were used in Kibsgaard et al.'s study and this study. Therefore, the increased number of referred BP patients may reflect an actual increase in the incidence of BP in the Danish population.

In this study, 38% of the referred patients were admitted to the hospital because of the severity of their BP disease. This was not only due to high disease activity and need for intensive therapy with intravenous treatments in some cases but also because of comorbidities, as all the admitted patients were initiated on CST to reduce the use of systemic steroids.

The time from BP diagnosis to the initiation of CST varied from 0 to 719 days, with a mean of 3 days (Figure 3). In the group of admitted patients, the mean was only 1 day, and 75% of the admitted patients were initiated on CST within 5 days. On the other hand, in the group of non-admitted patients, the mean was 8 days, and 75% of the patients were initiated within almost 4 weeks (26 days). Thus, the admitted patients experienced earlier initiation of CST. This supports more strictly application of our treatment guidelines in patients admitted to our inpatient ward compared with those treated in our outpatient clinic. There should be no differences in the handling of admitted/non-admitted patients.

We found no difference in treatment outcomes when comparing the group of BP patients treated with prednisolone alone and those treated with prednisolone in combination with CST (early and late initiation). Although it was a higher mortality in the prednisolone alone group, this was of no significance ($p = 0.14$). This might reflect the fact that only 12 patients were treated with prednisolone alone, and it was impossible to obtain statistical significance. On the other hand, this again reflects that we adhered to our local treatment guidelines for the initiation of CST in BP patients.

The time of admission was shorter in this study (mean [± SD]: 8 [± 5] days) than in the study by Kibsgaard et al. (mean [± SD]: 14 [± 9] days) (Table 2). This reduction in admission time may indicate more effective management of the acute phase of the disease. Furthermore, we found a decreased initial dose of prednisolone (from a mean ± SD of 44 ± 15 mg in Kibsgaard et al.'s study to 25 ± 11 mg in this study). However, we did not find any changes in the overall remission or relapse (34% in Kibsgaard et al.'s study, 38% in this study) of BP between the two study periods.

The 1-year mortality of 17.2% in this study was similar to that of 16.7% reported in a study from Finland conducted by Försti et al. (7). Finland is comparable with Denmark in several aspects, particularly demographics (for instance, having an older age structure), the incidence of BP, and treatment methods for BP. The 1-year mortality for citizens aged >70 years was approximately 5.4% in 2018 in Denmark[2]. Thus, BP patients have an increased mortality rate compared with the general population of the same age group, which has been confirmed in several studies, including this study (14–16).

Considering the epidemiological aspects of this study, there was a decreased rate of CVDs of 46% versus 70% in Kibsgaard et al.'s study (10). Few studies have demonstrated an association between CVD and BP. However, Kibsgaard et al.'s study and Försti et al.'s of 198 BP patients (7) both found CVD as the most common comorbidity. This was also observed in the present study. Recent studies have demonstrated BP association with hypertension (17, 18). Lee et al. found a significant adjusted odds ratio of 2.03 [95% CI 1.24–3.32] of BP when having hypertension (18). The present study did not differentiate between different types of CVD; therefore, the proportion of patients with hypertension was unknown. However,

2 «Number of deaths» from Statistic Denmark. (2018). https://www.dst.dk/en/Statistik/emner/borgere/befolkning/doedsfald [Accessed February 23, 2023].

the possible association between specific forms of CVD and BP is an interesting aim for further research.

In this study, CVD was the most common comorbidity, followed by type II DM (29%), cancer (25%), and neurological diseases (21%). There are hypotheses of cross-reactions against BP230 isotopes, where the autoantibody binds to both antigens expressed in neurological tissues and the basement membrane of human skin (19). This suggests an association between BP and neurodegenerative disorders. A larger case–control study from Denmark revealed a significantly increased frequency of multiple sclerosis (MS) among BP patients, with a risk of developing MS more than five times higher than that in the background population (19). As this was a retrospective descriptive study and not a follow-up study, we could not conclude any association between MS and BP based on our findings. Three patients of our cohort had MS as a comorbidity at the time of BP diagnosis.

As mentioned earlier, several drugs have been reported to increase the risk of developing BP, including antihypertensive drugs, anti-inflammatory drugs, diuretics, antirheumatic drugs, vaccines, and gliptins. The most frequent drugs used in our cohort were paracetamol (41%), acetylsalicylic acid (23%), and ACE inhibitors (21%). This may be explained by the advanced age and multiple comorbidities of our patients. In our cohort, 12% of the patients used gliptins, in total 18 received vildagliptin, one received linagliptin, and one received sitagliptin. In the last years of BP research, the use of dipeptidyl peptidase-4 inhibitors (particularly vildagliptin and linagliptin) for the treatment of DM has been investigated and has shown to be significantly associated with BP (20). The Danish Endocrine Society suggests alogliptin, linagliptin, and sitagliptin as first-line drugs if there is indication for using gliptins in the treatment of DM. Therefore, it is interesting that 18 out of 20 of our gliptin users were prescribed vildagliptin. Vildagliptin has in several studies been shown to have the highest association with BP compared with other gliptins (21). Lastly, there has been an increase in the use of non-insulin, glucose-lowering drugs such as gliptins the last decade in Denmark, and in 2021, 7.5% of the type II DM population in Denmark used gliptins (22). In comparison, we found 42% of our type II DM BP patients using gliptins. In conclusion, our results may support the rising evidence of gliptin-associated BP.

The strengths of this study were a long study period and the substantial percentage of patients (almost 90%) with a BP diagnosis based on histology, direct IF, and clinical presentation. The limitations of this study were the lack of assessment of BP severity, for example, using the ABSIS or BPDAI disease scores, which could have provided more specific information on the disease stage, and hence, the possible effect of early initiation of CST. Therefore, the results must be interpreted with caution. Furthermore, we could have confirmed the BP diagnosis by performing serological tests for autoantibodies against BP180 and/or BP230. Consequently, we have now adapted this as a routine practice in our department. Another limitation that may have affected our results was selection bias, as our department consulted the most severe cases within a limited geographic region. However, the fact that the same standard guidelines were used and that, in this study, these guidelines were followed quite consequently by the department specialists indicates the strength of this study and its results.

5 Conclusion

The rate of complications and 1-year mortality did not differ significantly between the groups that initiated early (<28 weeks) or late (>28 weeks) CST. However, the rate of relapse was lower in the group with early initiation of CST. When comparing the outcomes of patients treated with prednisolone alone with those of patients treated with CST, we found no difference.

Furthermore, we found that the initial dose of prednisolone and admission time were reduced in this study compared with those reported in a similar study conducted before the implementation of a local treatment guideline recommending the early initiation of CST. Thus, the initial prednisolone dose was reduced after a change in the treatment guidelines for BP patients; however, further studies are needed to clarify whether the reduced prednisolone dose lowers the risk of adverse outcomes and mortality in these 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

Ethical review and approval was 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. 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

RB, AF, CV conceived of the study, made the design, and coordinated the study. AF provided all patient data. GO collected the epidemiological data. GO and IF performed the interpretation of data. IF performed the statistical analysis, interpreted the results, and composed the discussion. RB, AF, CV contributed to drafting and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

We highly appreciate all contributors to this project at the Department of Dermato-Venereology at Aarhus University Hospital. We also deeply appreciate the work of doctor Detlef

Zillikens, and his mutual contribution to our understanding and handling of autoimmune blistering disorders of the skin.

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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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 10 July 2023
DOI 10.3389/fimmu.2023.1220887

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OPEN ACCESS

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RECEIVED 11 May 2023
ACCEPTED 27 June 2023
PUBLISHED 10 July 2023

CITATION
Huang D, Zhang Y, Kong L, Lu J and Shi Y (2023) Janus kinase inhibitors in autoimmune bullous diseases. *Front. Immunol.* 14:1220887. doi: 10.3389/fimmu.2023.1220887

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Janus kinase inhibitors in autoimmune bullous diseases

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Autoimmune bullous disease (AIBD) is a severe skin disorder caused by autoantibodies that target intercellular or cell-matrix adhesion proteins. Currently, the preferred treatment for AIBD involves the use of glucocorticoids or traditional immunosuppressants. Additionally, the utilization of biological agents such as rituximab, omalizumab, and dupilumab is on the rise. However, effectively managing AIBD remains a challenge. The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway has been implicated in various inflammatory diseases. In recent years, a range of drugs known as JAK inhibitors, which target this pathway, have been developed. Several studies have explored the efficacy and safety of JAK inhibitors for treating AIBD. Consequently, this review begins by examining the role of the JAK/STAT pathway in AIBD, summarizing the application of different JAK inhibitors in AIBD treatment, and emphasizing the importance of disease management in treating AIBD with JAK inhibitors. Furthermore, it highlights the need for a better understanding of the JAK/STAT pathway's role in AIBD, as well as the effectiveness and safety of JAK inhibitors for treating this disease.

KEYWORDS

autoimmune bullous disease, Janus kinase inhibitor, pemphigus, pemphigoid, JAK-STAT signaling pathway

1 Introduction

Autoimmune bullous diseases (AIBDs) are rare conditions characterized by erosion, blistering, and bullous lesions on the skin and mucous membranes. These diseases arise from the production of autoantibodies that target proteins responsible for maintaining cell-to-cell and cell-to-matrix adhesion (1, 2). AIBDs can be categorized into two groups: pemphigus disease (intraepidermal immunobullous disease) and pemphigoid disease (subepidermal immunobullous disease). Pemphigus diseases encompass pemphigus vulgaris (PV), pemphigus foliaceus (PF), paraneoplastic pemphigus (PNP), immunoglobulin A (IgA) pemphigus, and others. Pemphigoid diseases include bullous pemphigoid (BP), epidermolysis bullosa acquisita (EBA), anti-lamininγ1 pemphigoid, mucous membrane pemphigoid (MMP), dermatitis

herpetiformis (DH), linear IgA bullous dermatosis, pemphigoid gestationis, lichen planus pemphigoid (LPP), and bullous systemic lupus erythematosus (BSLE).

Since the availability of glucocorticoids and, later, non-steroidal immunosuppressive drugs, from the late 1960s, the mortality rate of AIBD patients has significantly decreased. However, as critical dermatological diseases, AIBDs are still associated with a high mortality rate (3, 4). In recent years, treatment modalities have gradually shifted toward more precise and targeted immune suppression and/or immune regulation. In particular, immunotherapy with biologics, such as rituximab, dupilumab, and omalizumab, is being clinically applied to treat various types of AIBDs with relatively good efficacy (5–9). Nevertheless, despite the availability of various treatment options, including glucocorticoids, traditional immunosuppressive drugs, and biological reagents, some patients still exhibit poor treatment responses or experience serious drug-related complications. Hence, there is a need for new therapeutic approaches that offer improved efficacy and fewer adverse effects (AEs).

The Janus kinase/signal transducers and activators of the transcription (JAK/STAT) pathway is vital for immune regulation, cell differentiation, apoptosis, and proliferation, affecting various cytokines and growth factors (10). This signaling pathway is associated with the pathophysiology of several autoimmune and autoinflammatory diseases. Therefore, blocking the JAK/STAT pathway has become an attractive approach for treating these diseases. In recent years, a variety of JAK pathway inhibitors have been used for the treatment of skin diseases such as psoriasis (11), alopecia areata (12), and atopic dermatitis (13). Since the JAK/STAT pathway is thought to play a role in the development of AIBDs, inhibiting this pathway could be a promising therapeutic strategy for these diseases.

This review aims to provide a concise overview of the potential involvement of the JAK/STAT pathway in the development of AIBDs and list the reported JAK inhibitors that have been used to treat AIBD. This information will serve as a foundation for further research on the pathogenesis, clinical diagnosis, and treatment of AIBDs.

2 The JAK/STAT pathway

To the best of our knowledge, only JAK inhibitors have been reported for the treatment of AIBD, while STAT inhibitors have not been similarly reported. Therefore, we will just summarize the function of JAK proteins. The JAK family comprises four proteins called TYK2, JAK1, JAK2, and JAK3 (14). JAK-dependent cytokine receptors transmit signals through various JAKs (Table 1), with each receptor having multiple subunits associated with JAK. A primary function of protein kinases is to transfer phosphate groups from guanosine triphosphate or adenosine triphosphate to amino acid hydroxyl groups (15). This fundamental mechanism is essential for receptors that lack intrinsic enzyme activity to facilitate biological processes. As a non-receptor tyrosine protein kinase, JAK can also conduct signal transduction through this mechanism. Typically, cytokine binding to their receptors triggers inflammatory signals. Receptors of type I and type II cytokines, including interferon (IFN), interleukin (IL)-2, IL-6, IL-12, IL-23, etc., are intrinsically deficient in enzyme activity and highly dependent on JAK for signal transduction (16). The JAKs initiate signaling pathways in the cell membrane that ultimately reach the nucleus after cytokines bind to their receptors (17). As part of this reaction, type I and II cytokine receptors undergo oligomerization to recruit JAKs and phosphorylate tyrosine residues, including tyrosine residues within the receptor chain. STAT proteins are then recruited, regulating the expression of related genes, resulting in antibody production, lymphocyte differentiation, increased inflammation, blister formation, and other pathophysiological processes.

3 The JAK/STAT pathway in AIBD

Multiple immune mechanisms, including cellular and humoral immunity, contribute to the development of pemphigus diseases. Researches have demonstrated that serum levels of tumor necrosis factor-α (TNF-α) and several type I and II cytokines, such as IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, and IL-15, are increased in patients with PV, while levels of IFN-γ are decreased (18–20). Furthermore, desmoglein (Dsg)3-reactive helper T cell (Th)1 and Th2 cells are found in patients with pemphigus disease. The presence of immunoglobulin (Ig)G4 and IgG1 antibodies against Dsg3 is directly related to the ratio of Dsg3 reactive Th1/Th2 cells (21). Thus, both Th1 and Th2 cells play an integral role in the pathogenesis of PV. Th1 cells overexpress IFN-γ to further mediate the immune response, while IL4, as a Th2-derived cytokine, regulates immunoglobulin conversion and antibody production by stimulating B cell proliferation (22). Cytokines such as IFN and IL-4 have been shown to play a biological role

TABLE 1 Summary of the cytokines associated with different JAK proteins.

JAKs	Cytokines/hormones
JAK1	IL-2, IL-4, IL-6 IL-7, IL-9, IL-10, IL-11, IL-13, IL-15, IL-19, IL-20, IL-21, IL-22, IL-24, IL-27, IL-28, IL-29, IFN-α, IFN-β, IFN-γ
JAK2	IL-3, IL-5, IL-6, IL-10, IL-11, IL-12, IL-13, IL-19, IL-20, IL-22, IL-23, IL-27, G-CSF, GM-CSF, GH, EPO, TPO, Leptin
JAK3	IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, IL-21
TYK2	IL-6, IL-10, IL-11, IL-12, IL-13, IL-19, IL-20, IL-22, IL-27, IL-28, IL-29, IFN-α, IFN-β, IFN-γ

JAK, Janus kinase; IL, interleukin; IFN, interferon; GH, growth hormone; GM-CSF, granulocyte-macrophage colony stimulating factor; G-CSF, granulocyte-colony stimulating factor; EPO, Erythropoietin; TPO, thrombopoietin; TYK, tyrosine kinase.

through JAK proteins. JAK1, JAK3, STAT6, and IL-4 signaling pathways are also important for the differentiation and proliferation of helper T cells (18). These discoveries suggest that the JAK/STAT signaling pathway plays a significant role in the pathogenesis of pemphigus diseases.

BP, a subepidermal form of AIBDs, is associated with autoantibodies targeting hemidesmosome components BP180 and BP230 (1). Studies using immunohistochemistry and western blotting analysis have shown that the expression of JAK/STAT proteins is significantly higher in BP-associated skin lesions compared to surrounding skin and healthy individuals (23). Furthermore, an increasing number of studies have demonstrated that Th2 cells play an important role in the production of antibodies in BP. It is worth noting that the chemotactic attraction of the Th2-like cytokine IL-4 towards eosinophils is very strong. IL-4 also takes part in the maturation and functional activity of eosinophils, as well as antibody production and the autoimmune response to BP (24, 25). The role of IL-17 in BP progression is evidenced by its upregulation of proteases involved in blister formation such as matrix metalloproteinase 9 and neutrophil elastase (26). The JAK signaling pathway has been proven to be closely related to the function of IL-4 and IL-17 (27, 28), which warrants further exploration of the role of the JAK signaling pathway in BP. Additionally, many JAK-dependent cytokines such as IL-6, IL-8, and IL-23 have been implicated in BP (29).

The role of JAK in other pemphigoid diseases, such as DH, MMP, and EBA, is similar to that observed for BP. For example, in DH and MMP, the expression of JAK/STAT-related proteins in the skin lesions of patients was significantly elevated (23). In the case of DH, eosinophilin and Th2 cytokines, such as IL-13, IL-4, and IL-5, also play an important role in the disease development (30). Additionally, a decrease in Treg cells and IL-10 has been observed in DH lesions (31), and the IL-31 concentration differs between healthy individuals and those with DH (32). This change in IL-31 concentration may be related to the intense itching typically associated with DH. In MMP, IL-4 and IL-13 not only affect disease onset but also influence the function of conjunctival fibroblasts, regulate scar formation, and thus, affect the prognosis of ocular MMP (33, 34). MMP is also associated with an increased localization of Th17 lymphocytes in lesions, especially in the conjunctiva, as well as local overexpression of IL-6, IL-12, and IL-17 (35). In EBA, high levels of IL-1β, IL-2, IL-6, IL-10, IL-21, TNF-β, and IFN-γ have been detected in serum samples (36). Similar to BP, in EBA, the Th2 pathway plays a key role in pathogenesis and itching. Consequently, the Th2 signaling pathway has received increasing attention in studies on pemphigoid diseases. Regarding treatment, an increasing number of reports have shown that the IL-4R monoclonal antibody dupilumab exhibits good efficacy in treating pemphigoid diseases (37). In addition to IL-4, the JAK/STAT pathway plays an important role in the function of other Th2 cytokines. In other words, inhibiting the JAK/STAT pathway can affect multiple Th2 cytokines. Therefore, evaluating the role of JAK/STAT in AIBDs is important for further research on pathogenesis and the development of therapeutic targets.

Animal models play a crucial role in understanding the mechanism of JAK inhibitors in treating AIBDs. However, there is a lack of research specifically using JAK inhibitors to treat AIBDs in mouse models, despite the existence of numerous reports on AIBDs in mouse models induced by antibody transfer, lymphocyte transfer, and immunization (38, 39). Interestingly, in veterinary medicine, oclacitinib, a selective JAK1 inhibitor for dogs, has shown promise in treating AIBDs like PF (40, 41). These observations suggest that JAK inhibitors may be beneficial for animals with AIBDs. It is still necessary to conduct animal model experiments focusing on AIBDs to gain further insights into the role of JAK inhibitors, representing an important avenue for future research.

4 Application of JAK inhibitors in AIBDs

4.1 Baricitinib

Baricitinib, which belongs to the class of JAK inhibitors, functions by blocking the JAK1 and JAK2-STAT signaling pathways. Additionally, it also inhibits the activity of IL-6, IL-12, and IL-23, thereby suppressing the differentiation of pathogenic Th17 cells. Baricitinib is used for treating rheumatoid arthritis, alopecia areata, and COVID-19, and has also been recommended for the treatment of psoriasis and other inflammatory-mediated diseases due to its considerable efficacy in controlling exaggerated inflammatory responses (42). Furthermore, some researchers have explored the potential of baricitinib as an off-label treatment for AIBDs (Table 2).

Baricitinib was initially utilized for the treatment of MMP in individual cases reported by Sarny et al. (43) and Burningham et al. (44). According to their reports, recalcitrant MMP was successfully managed with systemic glucocorticoids, methotrexate, and baricitinib. The favorable side effects of baricitinib and its oral administration make it a promising alternative to current interventions for MMP. Nevertheless, prospective studies are necessary to assess baricitinib's effectiveness and establish its position within the treatment approach for MMP.

In the case of BP, Xiao et al. first reported a case of aggressive BP alongside plaque psoriasis that was effectively managed using baricitinib (45). The co-existence of psoriasis and the poor health conditions of the patient made the use of systematic glucocorticoids difficult, so baricitinib was chosen as an alternative. This report underscores the potential of baricitinib as a promising alternative treatment for co-existing plaque psoriasis and BP, or either condition individually.

Moussa et al. successfully treated a case of refractory LPP with baricitinib, highlighting its potential as an effective therapy for persistent cases (46). The limited availability of treatment options for LPP makes baricitinib a valuable addition. Concerning the underlying mechanism, Shao et al. discovered that in LPP, cytotoxic T-cell-mediated injury in keratinocytes was dependent on JAK2 and STAT1 signaling, and was inhibited by the JAK1/2 inhibitor baricitinib (47). These findings suggest that baricitinib may be able to alleviate the lichenoid tissue reaction in LPP.

One case report described a patient with epidermolysis bullosa pruriginosa (EBP) who had severe skin lesions and intense itching. Treatment with baricitinib resulted in a marked improvement in the patient's condition (48).

TABLE 2 The use of baricitinib in treating autoimmune bullous disease.

	Age/ gender	Types of AIBD	Complication	Previous therapies	Treatment	Efficacy of initial use of JAK inhibitors	Tapering of JAK inhibitors	Efficacy of maintenance treatment	AE	Relapse
Sarny et al., 2018 (43)	43/M	MMP	Psoriasis	MTX, CP, MMF, RTX, IVIG, ADM, PSL	Baricitinib 4 mg/d, MTX 25mg/w, PSL 6mg/d	The disease was improved within two months	Baricitinib was stopped due to decreasing of neutrophils for 2 weeks. Subsequently, baricitinib was taken each day.	Progression of end-stage MMP seemed stopped.	None	NA
Burningham et al., 2022 (44)	69/F	MMP	T2DM, breast cancer	TCS, MTX, prednisone, IVIG, RTX, MMF, CP.	Baricitinib 2 mg/d, prednisone, MTX 12.5 mg/w, then increased to 20 mg/w.	Conditions of ocular, oral and esophageal were improved.	NA	The patient continued to improved.	NA	NA.
Xiao et al., 2022 (45)	83/M	BP	Psoriasis, stage III HT, postoperative lung cancer	Compound glycyrrhizin, TCS	Baricitinib 4 mg/d, Halometasone cream 0.5g/d	Skin lesions and pruritus improved significantly.	Baricitinib was halved and continued for 12 weeks	Both bullous and psoriatic lesions were in complete remission	None	A few new blisters were noted but faded away 3 days later.
Moussa et al., 2022 (46)	36/M	LPP	NA	TCS, SCS, MTX, AZP, ciclosporin, doxycycline, PUVA	Baricitinib 6.8 mg/d.	A significant improvement in pruritus and LPP lesions.	Baricitinib reduced to 3.4 mg/d	LPP almost completely relieved	NA	NA

AIBD, autoimmune bullous disease; JAKi, janus kinase inhibitors; AE, adverse effect; MMP, mucous membrane pemphigoid; BP, bullous pemphigoid; LPP, Lichen planus pemphigoides; M, male; F, female; MTX, methotrexate; CP, cyclophosphamide; MMF, mycophenolate mofetil; AZP, azathioprine; RTX, rituximab; IVIG, intravenous immunoglobulin; ADM, Adalimumab; PSL, prednisolone; TCS, topical corticosteroids; SCS, systemic corticosteroids; PUVA, psoralen plus ultraviolet A; T2DM, type 2 diabetes mellitus; HT, hypertension; NA, not available; mg/d, mg/day; mg/w, mg/week.

4.2 Tofacitinib

Tofacitinib, an oral inhibitor targeting JAK1/3, has received U.S. Food and Drug Administration (FDA) approval for treating moderate-to-severe rheumatoid arthritis, active psoriatic arthritis, and ulcerative colitis in adult patients (49), as well as polyarticular juvenile idiopathic arthritis in children. *In vitro* studies have indicated that tofacitinib is capable of reducing TNF, IL-1β, and type I IFN production in dendritic cells derived from monocytes stimulated with antigenic lipopolysaccharide (50). Further, the use of tofacitinib for treating DH, MMP, EBA, BP, and PV has been reported in the literature, as described below (Table 3).

Tofacitinib was initially used for DH that did not respond to conventional treatment. Kahn et al. presented a DH patient treated with tofacitinib, observing notable clinical improvement and inhibition of new lesion development (51). These findings suggest that tofacitinib could be a potentially effective alternative for managing DH in patients who are unable to adhere to a gluten-free diet or have contraindications to dapsone, or in cases where these approaches prove unsuccessful.

Tofacitinib has also shown promising results as a treatment for MMP, as reported in some cases (52). Two patients suffering from ocular MMP had failed to respond to several therapies and were treated with tofacitinib, resulting in long-term control of conjunctival inflammation and no observed progression of sub-conjunctival fibrosis.

Fan et al. described a recurrent EBA patient that responded well to tofacitinib treatment (53). In this patient, they observed a decrease in circulating neutrophil counts, although their association with the clinical response is debated, as well as the anti-COL-7 IgG titer. These observations indicate the potential therapeutic value of JAK inhibitors for EBA.

With regard to BP, Youssef et al. found that tofacitinib was effective in treating two cases of BP, achieving treatment goals while avoiding the side effects of standard therapies, and improving itch control (54). This study represents the initial report highlighting the benefits of oral tofacitinib in the management of BP. Fan et al. subsequently published a case series that supported these findings (56), reporting that all patients were relieved of itching after one week of tofacitinib treatment, and levels of serum autoantibodies, eosinophils, IL-6, IL-17, and TNF-α were lower after tofacitinib administration compared to before. These findings indicate that tofacitinib has good therapeutic prospects in BP.

In 2018, a review indicated that tofacitinib could potentially serve as an alternative treatment for pemphigus diseases (57). It was suggested that both systemic and topical tofacitinib could have positive therapeutic effects on pemphigus diseases. However, determining the ideal dosage requires further exploration through clinical trials. Compared to rituximab, tofacitinib offers the advantage of being available in both oral and topical forms, whereas rituximab can only be administered intravenously. Furthermore, tofacitinib may be more efficacious than rituximab

TABLE 3 The use of tofacitinib in treating autoimmune bullous disease.

	Age/ gender	Types of AIBD	Complication	Previous therapies	Treatment	Efficacy of initial JAK inhibitors	Tapering of JAK inhibitors	Efficacy of maintenance treatment	AE	Relapse
Kahn et al., 2021 (51)	76/M	DH	Celiac disease, HT	Dapsone, SAS	Tofacitinib 10mg/d	Significant improvement in pruritus, existing lesions and new lesions.	NA	Significant improved.	None	NA
James et al., 2021 (52)	79/F	MMP	HT, hyperlipidemia	MTX, MMF, RTX, CP, IVIG	Tofacitinib 11 mg/d, IVIG	Significant improvement in ocular inflammation.	NA	Free of further conjunctival inflammation.	None	NA
James et al., 2021 (52)	70/M	MMP	T2DM, myocardial infarction with subsequent stenting	Erythromycin ophthalmic ointment, 0.05% cyclosporine ophthalmic emulsion, MTX, MMF, CP, RTX	Tofacitinib 11 mg/d, MMF 2g/d	Ocular inflammation, nasal and oral ulcers resolved.	Stopped tofacitinib for over a month due to cost.	Rash recurred after tofacitinib withdrawal and improved after another oral dose.	None	Relapse due to withdrawal of medication
Fan et al., 2023 (53)	58/M	EBA	Pneumocystosis pneumonia, osteoporosis	PSL, MTX, dapsone, TCS	Tofacitinib 10 mg/d, PSL 8 mg/d	The disease was improved within one month.	Tofacitinib was tapered to 5 mg/d.	Blisters and erosions were almost disappeared.	None	None
Youssef et al., 2022 (54)	65/F	BP	Post-arthroplasty of the right patellar tendon rupture, hypothyroidism, hypercholesterolemia, HT, obesity, osteoarthritis, seronegative spondyloarthropathy	Prednisone, doxycycline, niacinamide.	Tofacitinib 20mg/d	BP dramatically improved.	Tofacitinib was cut to 10 mg/d due to sinus pain.	After tapering, she presented with recurrent pruritus and put tofacitinib back on 20 mg/d.	Sinus pain	Recurrent pruritus occurred due to the halving of dose.
Youssef et al., 2022 (54)	76/M	BP	Degenerative disc disease, steroid-induced atrial fibrillation	Prednisone, mycophenolate, dupilumab, RTX	Tofacitinib 20mg/d, dupilumab	Skin and pruritus were complete clearance.	NA	NA	NA	NA
Vander et al., 2022 (55)	34/F	PV	UC, controlled prolactinoma	Prednisone, topical clobetasol	Tofacitinib 10mg/d, RTX	Both paronychia and the quality of life have improved.	NA	NA	NA	NA

AIBD, autoimmune bullous disease; AE, adverse effect; DH, dermatitis herpetiformis; MMP, mucous membrane pemphigoid; EBA, epidermolysis bullosa acquisita; BP, bullous pemphigoid; PV, pemphigus vulgaris; M, male; F, female; TCS, topical corticosteroids; SCS, systemic corticosteroids; SAS, sulfasalazine; MTX, methotrexate; MMF, mycophenolate mofetil; RTX, rituximab; CP, cyclophosphamide; IVIG, intravenous immunoglobulin; PSL, prednisolone; HT, hypertension; T2DM, type 2 diabetes mellitus; UC, ulcerative colitis; NA, not available; mg/d, mg/day; mg/w, mg/week.

10.3389/fimmu.2023.1220887

because it targets both T cells and B cells, while rituximab mainly affects B cells. In 2022, Vander et al. reported a case of a female patient presenting with mild to moderate PV with nail involvement (55). The combination of oral tofacitinib and rituximab infusions resulted in a strikingly rapid improvement in her nail symptoms. Although rituximab has been shown to achieve long-lasting remission in pemphigus patients, its onset of action is slow. Therefore, tofacitinib may have contributed to the swift symptom improvement in the Vander et al. case report. The case findings suggest that combining tofacitinib with rituximab could potentially lead to rapid disease improvement and long-term remission.

The treatment of EBP with tofacitinib has also been reported (58), and it has been found to inhibit the inflammatory response, relieve pruritus, and decrease the recurrent onset.

4.3 Ruxolitinib

Ruxolitinib is an oral JAK1/2 inhibitor approved for the treatment of polycythemia vera, myelofibrosis, vitiligo, and steroid-refractory graft-versus-host disease (SR-GVHD). It has shown efficacy in treating dermatologic diseases like psoriasis and alopecia areata as an oral or topical agent. In SR-GVHD, ruxolitinib is used as a salvage therapy, particularly for cases with oral involvement and bronchiolitis obliterans (BO). This is because ruxolitinib plays a critical role in inflammation and T-cell activation (59). Some studies report high efficacy and survival rates with ruxolitinib for treating SR-GVHD. Based on the similarities in pathogenesis and clinical features of PNP and GVHD, ruxolitinib was considered a potential option for managing persistent stomatitis and BO in a female PNP patient (60) (Table 4). Despite the patient experiencing consistent healing of the skin with prednisolone, azithromycin, and cyclosporine, there was no noticeable improvement in oral lesions and respiratory function until ruxolitinib was added. The potent immunosuppressive and anti-inflammatory activities of JAK inhibitors were considered valuable for managing BO in this refractory PNP case.

4.4 Upadacitinib

Upadacitinib is a selective small molecule that functions as a JAK1 inhibitor. It has been approved for treating several medical conditions, including Crohn's disease, psoriatic arthritis, atopic dermatitis, ulcerative colitis, and rheumatoid arthritis. Regarding AIBDs, researchers have used upadacitinib to treat BP (Table 4).

Gresham et al. presented a 74-year-old woman with recurrent squamous cell carcinoma of the head and neck who developed drug-induced BP while undergoing immunotherapy with a novel immunoglobulin-like transcript 4 inhibitor (MK-4830) and pembrolizumab (61). As her condition worsened, the patient decided to transition to end-of-life care. Upadacitinib was used to manage the BP symptoms, and after four weeks of treatment, the patient responded well, suggesting the potential therapeutic efficacy of upadacitinib in BP. It is essential to emphasize that this case involved the use of upadacitinib under exceptional circumstances to provide palliative care for skin symptoms in a patient receiving end-of-life care for metastatic cancer.

In a case study presented by Nash et al., an 81-year-old woman with BP showed an incomplete response to prednisone but achieved complete resolution with upadacitinib (62). BP is commonly observed in elderly patients who have multiple systemic comorbidities; therefore, it is essential to investigate the use of safer drugs for such individuals. Conducting further research on JAK inhibitors like upadacitinib would be useful in expanding the treatment options for this condition.

Kim et al. reported a case study of a patient suffering from EBP. The patient experienced intense itching and had widespread lesions. Treatment with upadacitinib led to a substantial reduction in itching and lesions without any adverse effects (63).

TABLE 4 The use of ruxolitinib and upadacitinib in treating autoimmune bullous disease.

	Age/ gender	Types of AIBD	Complica-tion	Previous therapies	Treatment	Efficacy of initial JAK inhibitors	Tapering of JAK inhibitors	Efficacy of maintenance treatment	AE	Relapse
Fan et al., 2022 (60)	31/F	PNP	Castleman's disease	Prednisolone, cyclosporine, azithromycin	Ruxolitinib 5mg/d, prednisolone 10mg/d, cyclosporine 100mg/d	The symptoms were improved.	NA	Clinical presentation and laboratory tests improved.	NA	None
Gresham et al., 2023 (61)	74/F	DIBP	SCCHN, malignant melanoma	Prednisone, TCS	Upadacitinib, 15 mg/d	The patient demonstrate response to upadacitinib	NA	NA	NA	NA
Nash et al., 2023 (62)	81/F	BP	HT, dyslipidemia, osteoarthritis, endometriosis	Prednisone	Upadacitinib, 15 mg/d, prednisone	Complete resolution of disease.	NA	Continued efficacy with further healing of the skin and complete resolution of the disease.	None	None

AIBD, autoimmune bullous disease; AE, adverse effect; PNP, paraneoplastic pemphigus; DIBP, drug-induced bullous pemphigoid; BP, bullous pemphigoid; SCCHN, squamous cell carcinoma of the head and neck; HT, hypertension; TCS, topical corticosteroids; M, male; F, female; NA, not available; mg/d, mg/day.

5 Systematic management of AIBDs

AIBDs are chronic conditions that can persist for several years or a lifetime with a high likelihood of relapse. The primary aim of treatment is to facilitate the healing of bullous and erosive cutaneous and/or mucous lesions, with the additional objectives of reducing pruritus, preventing or minimizing the recurrence of blistering eruptions, enhancing the quality of life of patients, and promptly identifying serious adverse effects associated with long-term therapy, especially in the elderly. Glucocorticoids and immunosuppressants are still the main treatment agents for AIBDs. Furthermore, the emergence of monoclonal antibody drugs has provided more options for the treatment of AIBDs. However, in clinical practice, doctors may still encounter patients for whom none of these treatments work. Therefore, discovering newer, safer, and more effective drugs has become a future trend in AIBDs therapy. JAK inhibitors interfere with the function of various inflammatory cytokines by targeting the JAK/STAT pathway, and thus, they have become new therapeutic agents for AIBDs. However, until now, only individual cases treated with JAK inhibitors have been reported, and no large-scale prospective clinical studies have been conducted to prove their efficacy. Based on the case reports available, JAK inhibitors are highly effective in patients who have failed to respond to treatment with glucocorticoids, immunosuppressive agents, and biologics. Therefore, it appears that, in general, JAK inhibitors are an attractive option for the treatment of AIBDs.

In addition to effectiveness, safety is also a significant concern regarding the current treatment options for AIBDs. The adverse effects of glucocorticoids include skin atrophy, fat redistribution, acne, weight gain, impaired glucose tolerance, insomnia, secondary infections, and femoral head necrosis (64). Conventional immunosuppressants may have side effects on liver and kidney function, and the potential consequences of systemic immunosuppression cannot be overlooked either. In recent years, advancements in immunological research have led to the development of new immunosuppressants, with rituximab being a representative example for treating AIBDs. However, it could lead to serious infections resulting from immunosuppression, and the invasiveness of its intravenous route of administration may also carry additional risks (65). Although JAK inhibitors have shown promising efficacy in clinical reports, their safety issues should not be ignored. For instance, one of the serious adverse effects of most JAK inhibitors is the activation of infections, including tuberculosis, herpes zoster, and hepatitis B (66). In addition, JAK inhibitors are associated with the risk of malignant tumors, such as lymphoma, which may be induced with the use of baricitinib, especially in patients with cancer (67).

Furthermore, attention should be paid to vascular events and cardiovascular risks, as several studies have shown that JAK inhibitors may cause thrombosis and platelet loss (68). Since the FDA added a black box warning to tofacitinib in 2019 and warned about the safety of JAK inhibitors, there has been ongoing controversy over cardiovascular events and thrombosis caused by JAK inhibitors. Some *post-hoc* analyses have shown that certain cardiovascular events and thrombosis are attributable to the use of baricitinib and tofacitinib (69, 70), but multiple meta-analyses have failed to confirm that the use of JAK inhibitors increases the risk of cardiovascular events (71, 72). Large, multi-center clinical studies of two highly selective JAK1 inhibitors (abrocitinib and upadacitinib) have also shown a very high safety profile, with no reported increase in cardiovascular events or higher thromboembolic risk (73, 74). This seems to imply that highly selective JAK inhibitors may have a lower cardiovascular risk. However, studies of abrocitinib and upadacitinib have mostly been conducted in patients with AD, who have a lower median age and have not been followed for a long duration.

In general, the safety of JAK inhibitors is a matter of debate. Considering that BP patients are predominantly elderly and prone to cardiovascular and metabolic diseases (1), the pros and cons of JAK inhibitors need to be carefully weighed before treatment. However, it is essential to note that the mechanisms by which JAK inhibitors cause cardiovascular events and thromboembolism risk are still unknown, so all patients should be educated and monitored. Despite this, based on current case reports, there have been no instances of severe adverse reactions to JAK inhibitor treatment for AIBDs. However, it cannot be ruled out that patients with severe adverse reactions have not been reported. Due to the potential adverse effects of JAK inhibitors, it is crucial to screen patients before administering them and to closely monitor them during treatment. Additionally, timely management of any adverse effects that may occur is vital when using JAK inhibitors.

6 Conclusions

We have reviewed the literature on the effectiveness of several JAK inhibitors in the treatment of AIBDs, including PV, PNP, BP, MMP, EBA, LPP, and DH. Based on the reported findings, we recommend careful monitoring, screening, and management of adverse effects on patients during the treatment of AIBDs with these agents. Regarding the gaps in the literature, the specific mechanisms of JAK/STAT in the pathogenesis and progression of AIBDs remain unclear, and more experimental studies are needed to further explore the role of this pathway in this group of diseases. Moreover, no clinical trials have been conducted to test the efficacy and safety of JAK inhibitors in the treatment of AIBDs. Larger and higher-quality long-term follow-up studies are essential to determine the efficacy and safety of JAK inhibitors in treating AIBDs. However, it must be mentioned that this review is a narrative review rather than a systematic review, and due to the scarcity of studies and lack of reports in the relevant fields, the conclusions are largely dependent on theoretical hypotheses and case reports. Therefore, the clinical use of JAK inhibitors in AIBDs should be concluded after joint discussions with patients.

Author contributions

DH: writing – original draft. YZ: writing – original draft. LK: writing – original draft. JL: resources, methodology, writing – review & editing. YS: writing – review & editing. All authors contributed to the article and approved to the published version of the manuscript.

Funding

This work was sponsored by grants from National Natural Science Foundation of China (No. 82073429, 82273510, 82003335), Innovation Program of Shanghai Municipal Education Commission (No.2019-01-07-00-07-E00046), Clinical Research Plan of SHDC (No. SHDC2020CR1014B) and Program of Shanghai Academic Research Leader (No. 20XD1403300).

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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frontiers | Frontiers in Immunology

TYPE Brief Research Report
PUBLISHED 12 July 2023
DOI 10.3389/fimmu.2023.1193032

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OPEN ACCESS

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RECEIVED 24 March 2023
ACCEPTED 26 June 2023
PUBLISHED 12 July 2023

CITATION
Lotti R, Hundt JE, Ludwig RJ, Hammers CM, Bennett B, Amato A, Marconi A and Pincelli C (2023) Blocking soluble Fas Ligand ameliorates pemphigus: PC111 efficacy in ex-vivo human pemphigus models.
Front. Immunol. 14:1193032.
doi: 10.3389/fimmu.2023.1193032

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Blocking soluble Fas Ligand ameliorates pemphigus: PC111 efficacy in ex-vivo human pemphigus models

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Pemphigus is a life-threatening, chronic, autoimmune bullous disease affecting both the skin and the mucous membranes. Based on the mainstream concept that blister formation occurs upon binding of autoantibodies to their antigen proteins (desmoglein1, DSG1 and desmoglein3, DSG3), current therapies mostly aim to suppress the immune system. To avoid the severe side effects associated with the chronic use of immunosuppressive treatments, we have developed PC111, a fully human monoclonal antibody targeting human Fas ligand (FasL). We have provided a number of *in vitro* and *in vivo* evidences showing that soluble FasL induces keratinocyte apoptosis followed by acantholysis. An anti-murine FasL prevents blister formation in the pemphigus neonatal mouse model. To confirm the mechanism of action (MoA) and the efficacy of PC111 in a human pemphigus context, we used the keratinocyte dissociation assay and two independent Human Skin Organ Cultures (HSOC) pemphigus models. PC111 reduced acantholysis *in vitro*, as shown by the dose-dependent reduction of fragments in the monolayer cultures. In the first HSOC model, normal human skin was subcutaneously injected with a scFv antibody fragment directed against DSG1 and DSG3, resulting in a severe acantholysis (70-100%) after 24 hours. PC111 inhibited blister formation to around 50% of control. In the second model, normal human skin was injected with a mixture of pemphigus patients' autoantibodies resulting in a less severe acantholysis (20-30%). PC111 significantly suppressed blister formation to more than 75% up to 72 hours. These results confirm PC111 MoA and demonstrates the efficacy of the anti-FasL antibody also in a pemphigus setting.

KEYWORDS

pemphigus, FasL, acantholysis, drug development, ex-vivo model

1 Introduction

Pemphigus is a chronic, autoimmune blistering disease, characterized by loss of keratinocyte adhesion (acantholysis) leading to the formation of the blisters. Pemphigus affects both the skin and the mucous membranes where flaccid bullae and erosions are normally observed. Patients' autoantibodies (generally named PVIgG) binding to the main desmosome target antigens (DSG1 and DSG3) has long been considered to directly induce blister formation (1). Yet, it has now become clear that additional mechanisms following antibody binding contribute to skin blistering in pemphigus. Pathogenic IgGs activate phosphatidylcholine specific phospholipase C (PLC), which in turn elevates intracellular free calcium, and activates various kinases including p38MAP Kinase, Akt, Src, epidermal growth factor receptor kinase (EFGRK), and protein kinase C (PKC) (2, 3). Moreover, several lines of evidence indicate that keratinocyte apoptosis is involved in the pathological mechanisms of pemphigus [reviewed by Grando et al. (4)]. In particular, cleaved caspase-8 and -3 are detected in keratinocytes of pemphigus lesions, and caspase-8-positive keratinocytes display increased Fas Ligand expression (5).

Fas Ligand is a transmembrane protein (mFasL) that can be proteolytically cleaved to generate its soluble form of 26 kDa (sFasL) (6). Both forms of FasL can bind to their receptor, Fas, also known as CD95 or Apo1. FasL is normally stored within intracellular secretion vesicles, which, upon a certain stimulus (i.e. recognition of a target cells in the case of T cells and NK), fuse to the cell membrane resulting in the surface expression of FasL (7). Binding of mFasL to Fas receptor triggers the extrinsic apoptotic pathway through the activation of caspase-8 (8). On the other hand, sFasL appears to have both non-apoptotic and pro-inflammatory activities in immune cells (9, 10). In healthy skin, FasL is homogeneously stored in vesicles within the cytoplasm, in association to intermediate filaments of keratinocytes localized in the basal and first suprabasal layers of the epidermis (11). It has been reported that PVIgG are able to upregulate FasL expression, at the mRNA and protein level, and increase its translocation to the plasma membrane of human keratinocytes (12). Accordingly, sera from pemphigus patients contain abnormally elevated levels of sFasL (13). Anti-FasL antibodies (Ab) prevent PVIgG-induced caspase-8 activation and DSG cleavage in human keratinocytes *in vitro* (4) as well as blister formation in an *in vivo* mouse model of pemphigus (14). Finally, strong evidence of the critical role of sFasL in blister formation in pemphigus has been shown using two mutant mice selectively lacking either sFasL (FasLΔs/Δs) or mFasL (FasLΔm/Δm). Indeed, only the FasLΔs/Δs animals showed negligible levels of acantholysis induced by PVIgG injection, clearly indicating that soluble FasL, and not the membrane form, plays a crucial role in the mechanism of blister formation (14).

Currently, pemphigus therapy is based on chronic and systemic immunosuppression that is often associated with severe adverse events. Therefore, there is still need for novel treatments directed to altered pathways downstream of the antibody binding to the target antigens. Given the critical role of FasL in the signaling leading to blister formation in pemphigus, we have developed PC111, a fully human monoclonal antibody directed against the soluble form of FasL. Despite the body of evidence on the activity of anti-FasL Ab, we wanted to confirm the mechanism of action and the efficacy of PC111 in human contexts. To this purpose, we have used the *in vitro* keratinocyte dissociation assay and two 3D ex-vivo models, showing that neutralizing human FasL blocks acantholysis.

2 Materials and methods

2.1 Human biomaterial collection

All experiments with human samples were approved either by the Ethical Committee of Area Vasta Emilia Nord Policlinico of Modena, Italy (Protocol number 1186/2019) or by the Ethical Committee of the Medical Faculty of the University of Lubeck (ref number: 12-178/06-109). All subjects gave written informed consent in accordance with the Declaration of Helsinki. For sera collection, patients with suspected autoimmune bullous disease were enrolled during their first access to the Dermatology Unit, Rare Disease Room, of the Policlinico of Modena. Diagnosis of pemphigus was confirmed by routine laboratory tests (Indirect immunofluorescence and DSG ELISA, MBL International Corp., Nagoya, Japan). Patients negative for the diagnosis of pemphigus or any other skin bullous autoimmune disease were inserted in negative control group. Sera were then taken from the renaming volume after the routine diagnosis. For this study, we enrolled a total of 15 patients with pemphigus (PV, mucocutaneous pemphigus, and PF) and 8 negative patients.

Healthy human skin was obtained from discarded material of healthy patients undergoing cosmetic plastic surgery, usually for abdominal or breast reduction.

2.2 Reagent preparation

The HA-tagged scFv directed against DSG1 and DSG3 (also termed Px4-3) (15, 16) was produced in *Escherichia Coli* and purified as previously described (15).

Pemphigus autoantibodies (generally named PVIgG) and normal human IgG (NIgG) were purified by affinity binding on a HiTrapProtein G HP column (GE Healthcare Bio-Science, Piscataway, NJ, USA) as previously described (14). Sera were pooled and loaded on the column after dilution in binding buffer. Bound IgG was eluted with elution buffer (0.1 M glycine/HCl, pH 2.7) and immediately neutralized by Tris 1 M (pH 9). After dialysis against PBS and concentration by ultrafiltration (using Amicon, Beverly, MA, USA), IgG were filter-sterilized, and stored at +4°C until use. Protein concentration was determined by Bradford assay using protein Standard I (BioRad, Hercules, CA, USA). In general, PVIgG were purified from a mix of patients' sera collected from patients with PV, mucocutaneous pemphigus, and PF and pooled for IgG purification (ELISA titers of pooled samples: DSG1: 116.06 U/ml; DSG3: 179.27 U/ml);, while NIgG were isolated from sera from control patients (ELISA titers of pooled samples: DSG1: 5.05 U/ml; DSG3: 3.15 U/ml).

2.3 Keratinocyte dissociation assay

Primary cultured normal human keratinocytes (NHK) were obtained and expanded as previously described (14). Keratinocytes were seeded in 12-wells plate and maintained in a defined serum-free medium (KGM, Lonza Walkersville Inc., Walkersville, MD, USA). When cells reached 100% confluency, the calcium concentration was switched to 1.8 mM for at least 24 hours, to induce keratinocyte differentiation and desmosome maturation. NIgG or PVIgG were added at a concentration of 2 mg/ml in KBM. PVIgG were also used in combination with increasing doses of PC111 (from 0.001 to10 µg/ml) administered 2 hrs after patients' IgG and incubated at 37°C for 72 hours. As a control, we also used NIgG + PC111 at the highest dose 10 µg/ml. NHK were then incubated with dispase II (>2.4 U/ml; Roche®, Basel, Switzerland) to release monolayers that were in turn exposed to mechanical stress induced by repeated pipetting. Fragments were then fixed and stained with 1% Rhodamine B solution (Sigma-Aldrich, Taufkirchen, Germany). Dissociation score was calculated per each sample applying the formula below, considering N as the number of cell sheets in each well:

$$Dissociation\ Score = \frac{N - N\text{NIgG}}{N\text{PVIgG} - N\text{NIgG}} \times 100$$

2.4 scFv-induced pemphigus human skin organ culture model

The protocol used for the scFv-induced pemphigus HSOC has previously been described in detail (17). Briefly, healthy human skin (used within 24 h after collection) was cut into 1cm^2 sections and stored in William's E medium (Gibco) on ice until further use. Defined keratinocyte serum-free medium (D-K-SFM; Gibco) was added to the wells of a transwell cell culture insert plate (Sigma-Aldrich) maintaining the air-liquid interface between the epidermis and the air. To induce acantholysis, a human single-chain variable fragment (scFv) directed against DSG3 and DSG1 (also termed Px4-3) was used, while human intravenous immunoglobulins (IVIgG) or DPBS were injected as a negative control. The scFv was injected at a concentration of 3.75 µg/µl. PC111 was subcutaneously (s.c.) injected at 3 different concentrations (1, 10, or 50 µg) in a total volume of 50 µl 2 hours after scFv injection. Skin specimens were allocated by randomization to the different groups. Afterwards, the transwell plates were maintained in the incubator for 24 hours. Skin samples were then harvested and fixed in 4% Histofix solution for subsequent paraffin embedding and hematoxylin eosin (H&E) staining. Slices (5–10) were analyzed for each sample in order to cover the entire skin sample.

2.5 PVIgG-induced pemphigus human skin organ culture model

A second pemphigus HSOC model was set up, based on the model published by Egu and co-workers (18), using purified pemphigus antibodies mixture (PVIgG). Healthy human skin (<24 hours post-surgery) was cut into cubic sections of 0.5 x 0.5 cm. Skin specimens were allocated by randomization to the different groups. Purified PVIgG or NIgG were injected s.c. into the dermis of each tissue cube using an insulin syringe (30G gauge) in a total maximum volume of 50 µl. We tested different concentrations of PVIgG (PV1000: 1000 µg; PV1500: 1500 µg) in order to define the pathogenic concentration able to induce clear acantholysis at the histological level. PC111 was s.c. injected 2 hours after PVIgG treatment at 3 different concentrations (10, 50, or 100 µg). After injection, skin specimens were kept in organ culture in DMEM + 1% Pen/Strep in a 12 multi-wells plate and harvested after 24- and 72-hours treatment. Samples were then formalin fixed and paraffin embedded for histology analysis. We analyzed all of the skin biopsy by means of sequential cutting, with steps of 200-250 µm between each cut; approximately 8-10 slices were analyzed for each biopsy. Slices were stained by H&E and captured by D-Sight 2.0 software (Menarini Diagnostics, Italy) at 10X magnification for acantholysis evaluation.

2.6 Direct immunofluorescence staining

To show the binding of scFv to the treated epidermis, cryosections of scFv-treated HSOC skin pieces were stained using a Rat Anti-HA High-Affinity monoclonal Antibody (Roche). After few washes, samples were incubated with Alexa Fluor 594-conjugated goat anti-rat IgG (Life Technologies, Carlsbad, USA). Samples were then mounted in fluoromount-G and coverslips for the analysis. On the other hand, to show the binding of human IgG to the treated epidermis, sections of NIgG- or PVIgG-treated HSOC skin pieces were stained using polyclonal rabbit anti-human IgG/FITC conjugated antibodies (Dako, Glostrup, Denmark). After several washes, samples were mounted in fluoromount-G and coverslips for the analysis. Pictures were captured by ZOE Fluorescent Cell Imager (BioRad).

2.7 Acantholysis evaluation

Using the ImageJ software, the percentage of epidermal split formation, identified as areas of the epidermis in which suprabasal cell detachment spreads along more than four adjacent basal cells, was quantified by an investigator blinded to sample treatments. Splits were measured in each visual field as % of the skin coverage with 100% representing acantholysis covering the entire skin section.

2.8 Statistical analysis

If not otherwise indicated, data are presented as mean ± SEM. For statistical analysis, we used Prism Software (version 9; GraphPad; San Diego, CA, USA). One-way or two-way ANOVA was used for multiple comparisons. A value of $p < 0.05$ or less was assumed to indicate a statistically significant difference in the

compared parameters. Each statistical analysis performed was detailed in the figure legend.

3 Results and discussion

3.1 PC111 inhibits human keratinocyte detachment *in vitro*

Pemphigus is characterized by keratinocyte detachment at the desmosome level resulting in acantholysis. An *in vitro* keratinocyte dissociation assay was established to evaluate the pathogenicity of pemphigus autoantibodies (19). Using the keratinocyte dissociation assay, we demonstrated that PC111, administered few hours after pemphigus autoantibody stimulus, reduced acantholysis *in vitro*, as shown by the dose-dependent reduction of fragments in the monolayer cultures (Figure 1A). This was confirmed by the dissociation score in a statistically significant manner (Figures 1B, C). Indeed, both ordinary one-way ANOVA test plus multiple comparison (Figure 1B) and the 95% Confidence Interval on Mean differences (Dunnett's) (Figure 1C) highlighted a statistical difference in all PC111-treated groups versus the group stimulated with PVIgG only. We also calculated the IC50 of PC111 and determined that a concentration as low as 0.0097 µg/ml was sufficient to inhibit 50% of acantholysis *in vitro* (Figure 1D). No changes in the monolayer cultures were obtained when either NIgG or NigG+PC111 were added to the cells (Figures 1A, B). These findings are in line with and further confirm previous findings on the role of FasL in blister formation (14). In addition, pemphigus autoantibodies induce an upregulation of FasL and a release of FasL from keratinocytes upon binding to their antigens (5). Cells silenced for FasL expression are protected from PVIgG-induced acantholysis, and the use of an anti-FasL neutralizing antibody reduces the acantholytic effect *in vitro* (14). Here, we validated the use of the new monoclonal antibody PC111 in a "curative" protocol, in which PC111 displays a very strong effect in the amelioration of blisters *in vitro* till 72 hrs of incubation. Indeed, we used a 72 hrs incubation period, slightly modifying the original method (described in 19), given that our aim was to evaluate the effective and prolonged curative potential of PC111, in an already validated setting (14).

3.2 PC111 prevents blister formation in two independent HSOC models

In order to confirm the efficacy of PC111 in a human context, we used two independent protocols of pemphigus in human skin organ culture (HSOC) models. While the startup PinCell was in the process of refining the proof of concept on the effect of PC111 in human pemphigus, Prof. Detlef Zillikens, member of PinCell Scientific Advisory Board, inspired us by strongly recommending the use of 3D ex-vivo human models given that they are optimal complementary tools for *in vivo* pemphigus models (20).

The first model was performed at the University of Lubeck, where the scFv-induced pemphigus HSCO model has been developed. This method was published by the Zillikens's group from the department of Dermatology in Lubeck a few years ago (17). In this model, normal full thickness human skin was injected s.c. with a pathogenic anti-DSG1 and DSG3 single-chain variable fragment scFv (also formerly named Px4-3, as described in 15 and 16) in a dose able to induce 75-100% acantholysis after 24 hours of treatment (Scheme in Figure 2A). IVIg were used as negative control. PC111 was s.c. injected 2 hours after scFv, to mimic a curative and not a preventive treatment, at 3 different doses (1, 10 and 50 µg). After 24 hours, scFv-injected skin samples developed intraepidermal split formation at the suprabasal level, while a substantial inhibition of blister formation was observed with the highest dose of PC111, as shown by H&E staining (Figure 2B). By direct immunofluorescence, we effectively demonstrated the deposition of the scFv injected fragments at the inter-keratinocyte level only in scFv-injected skin samples (Figure 2C). Measurement of the epidermal splits (blisters) demonstrated that scFv induced a mean of 80% acantholysis, while no acantholytic effect was observed after IVIg injection. PC111 significantly reduced blister formation to around 50% of the scFv-treated controls (Figure 2D). We confirmed a statistical difference by calculating the 95% Confidence Interval on Mean differences (Dunnett's) comparing PC111 treated groups versus scFv alone (Figure 2E).

In the second model (inspired by 18), normal human skin was injected with two different amounts of purified pemphigus IgG mixture (PVIgG: PV1000 and PV1500) directed against both DSG1 and DSG3, and acantholysis was evaluated at 24 and 72 hours (Scheme in Figure 3A). Both PV1000 and PV1500 induced split formation at the suprabasal level, as shown by epidermal split in H&E staining, while the addition of PC111 2 hrs after pathogenic IgG injection prevented blister formation (Figure 3B). We then confirmed the deposition of the injected pemphigus IgG at the inter-keratinocyte level in both PV1000 and PV1500 treated skin samples (Figure 3C). The measurement of split length show that PV1000 induced 10 and 20% acantholysis at 24 and 72 hours, respectively. At 24 hours, PC111 tended to reduce blister formation, but not in a statistically significant manner. On the other hand, at 72 hours, when more severe acantholysis had developed, PC111 treatment induced a dose-dependent and statistically significant inhibition of blisters (Figure 3D). At 24 hrs blister formation is mild, possibly due to the fact that more incubation time is needed to induce a frank acantholysis at low concentrations of pemphigus antibodies, as formerly hypothesized by Schiltz and Michel (21). On the other hand, PV1500 induced 30% acantholysis at both 24 and 72 hours. In this context, PC111 significantly reduced blister formation in a dose-dependent manner both at 24 and 72 hours, as confirmed also by the 95% CI analysis (Figure 3E). In general, also the negative control sample of HSOC models maintained in culture for up to 72 hrs did not show any alterations (personal observations). These findings are in line with data presented in the pioneer study on ex vivo model of pemphigus performed in 1976 by Schiltz and Michel. Indeed, they demonstrated that the relative incorporation of H3uridine by the control samples was fairly constant during the first 67 hrs in culture. This indicates that our model is valid and reliable.

The MoA of PC111 was confirmed in two different HSOC models in order to reproduce pemphigus with diverse phenotypes

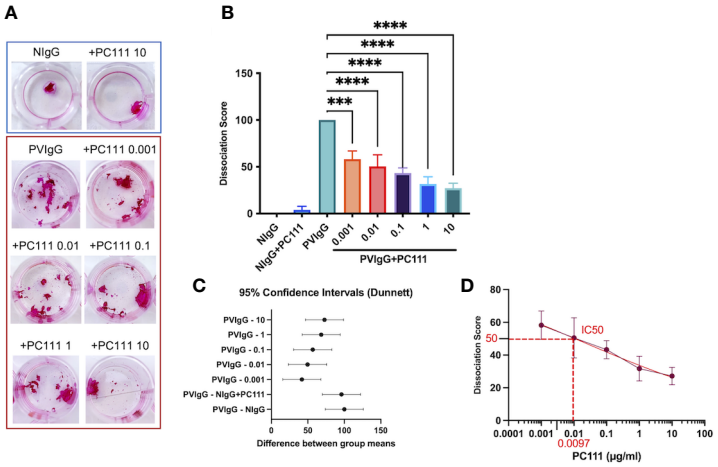

FIGURE 1
PC111 prevents keratinocyte acantholysis *in vitro*. Keratinocytes were cultured with purified normal human IgG (NIgG) or pemphigus autoantibodies (PVIgG) in the presence or absence of PC111 (from 0.001 to 10 µg/ml), and dispase-based dissociation assay was performed. **(A)** Representative images of cell sheet fragmentation, after staining with Rhodamine B. **(B)** Quantification obtained by counting the number of fragments of cell sheets and represented as dissociation score (means ± SEM). In general P< 0.0001 for ordinary one-way ANOVA for differences among groups; ***P< 0.001 and ****P< 0.0001 for Dunnett's multiple comparisons test versus PVIgG (n = 5 independent experiments). **(C)** Graph representing differences between group means and 95% Confidence Intervals (according to multiple comparisons with Dunnett analysis). **(D)** Dose response curve with absolute IC50 calculation.

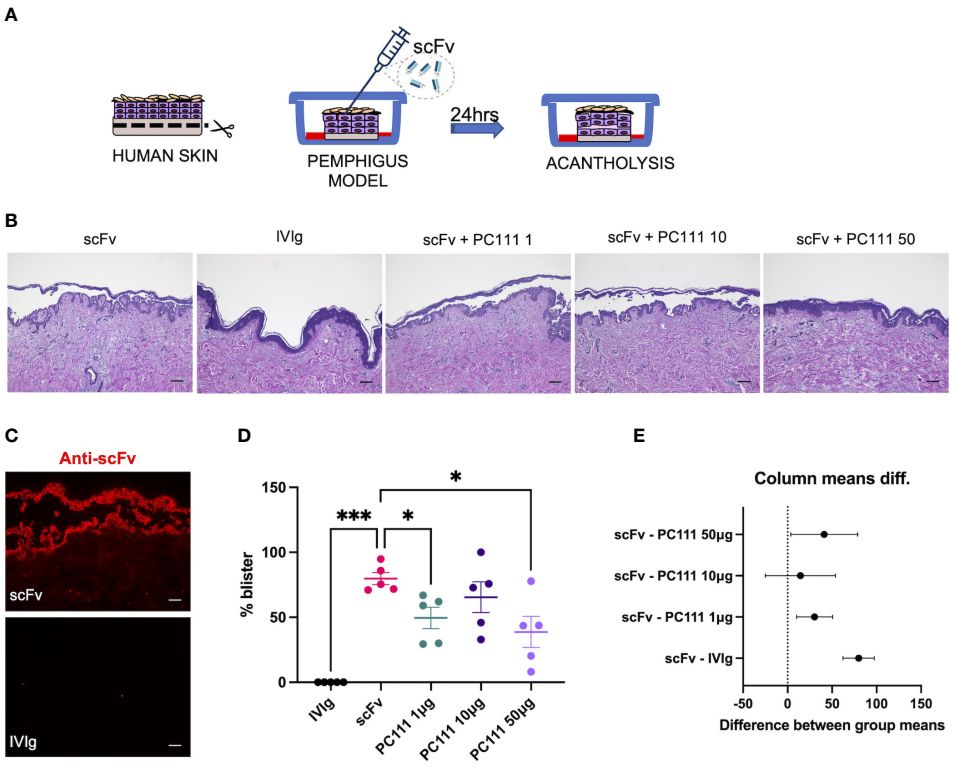

FIGURE 2
PC111 inhibits blister formation in scFv-induced ex vivo pemphigus model. **(A)** Schematic representation of the method used. **(B)** Exemplary pictures of the H&E staining of the 24 hrs cultures. PC111 (1-10-50 µg) was injected s.c. 2 hrs after scFv. IVIg were used as negative control. Scale bar = 100 µm. **(C)** Pictures showing direct immunofluorescence microscopy against scFv. Scale bar = 50 µm **(D)** Evaluation of the length of the blister expressed as % of the entire skin analyzed. Results are presented as mean ± SEM of 5 different experiments. In general, P =0.0001 for ordinary one-way ANOVA for differences among groups; *P<0.05; ***P< 0.001 for Dunnett's multiple comparisons test versus scFv. **(E)** Graph representing differences between group means and 95% Confidence Intervals (according to multiple comparisons with Dunnett analysis).

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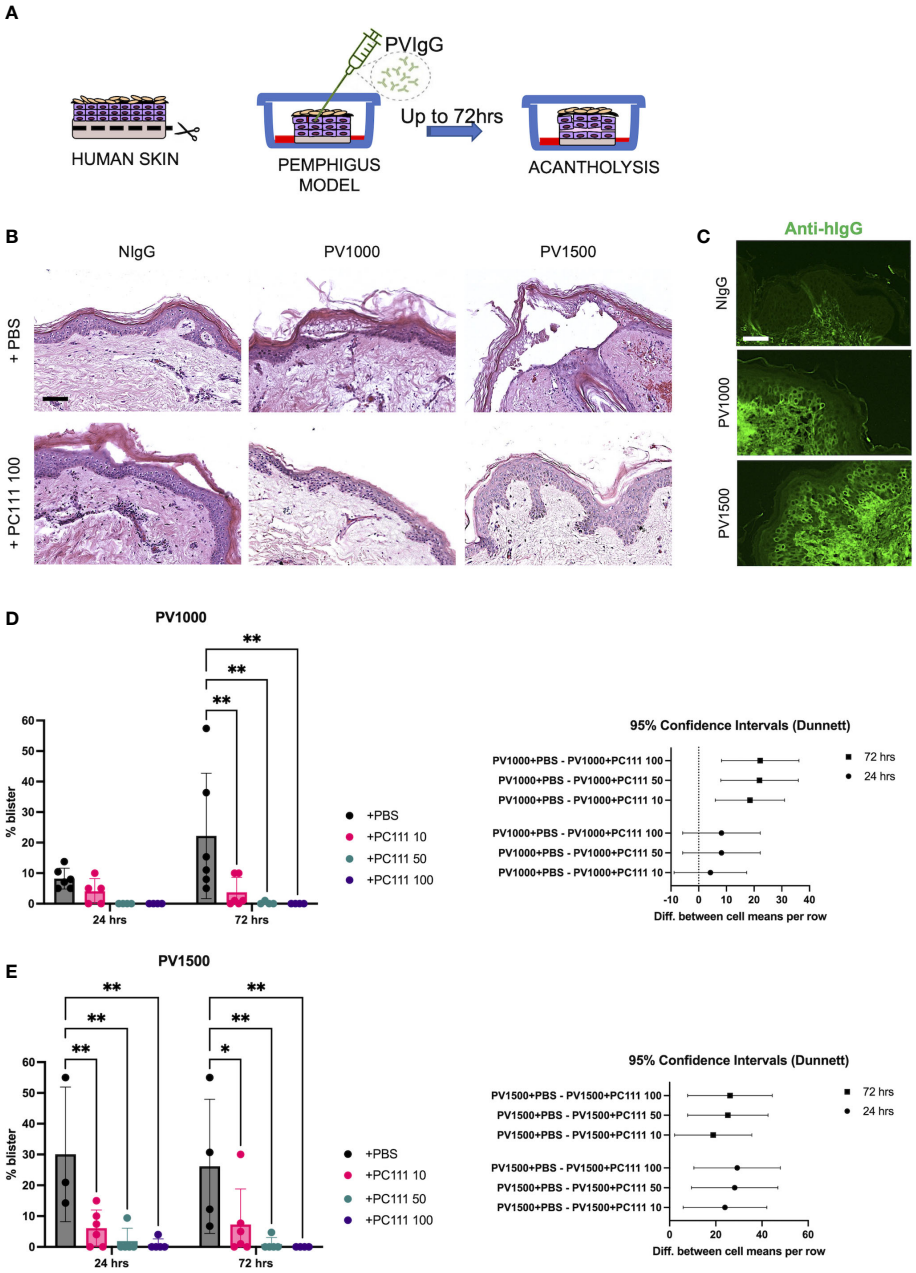

FIGURE 3
PC111 inhibits blister formation in PVIgG-induced ex vivo pemphigus model. **(A)** Schematic representation of the method used. **(B)** Exemplary pictures of the H&E staining of the 24 hrs cultures of skin injected s.c. with NIgG as negative control (1000 µg), PVIgG 1000 µg (PV1000) or PVIgG 1500 µg (PV1500) injected with diluent (PBS) or treated with PC111 100 µg (PC111 100). Scale bar = 100 µm. **(C)** Representative pictures showing direct immunofluorescence microscopy against hIgG in samples treated with NIgG, PVIgG 1000 µg (PV1000) or PVIgG 1500 µg (PV1500) injected with diluent (PBS). Scale bar = 50 µm. **(D)** On the left, evaluation of the length of the blister induced by PVIgG 1000 µg (PV1000) of samples injected 2hrs after PVIgG with PC111 (10-50-100 µg) expressed as % of the entire skin analyzed. Results are presented as mean ± SEM of 4 different samples. Two-way ANOVA test was followed by multiple comparison analysis. **P<0.01 per Dunnett's multiple comparisons test versus PVIgG. On the right, graph representing differences between group means at 24 (circles) and 72 (squares) hrs and 95% Confidence Intervals (according to multiple comparisons with Dunnett analysis). **(E)** On the left, evaluation of the length of the blister induced by PVIgG 1500 µg (PV1500) of samples injected 2hrs after PVIgG with PC111 (10-50-100 µg) expressed as % of the entire skin analyzed. Results are presented as mean ± SEM of 4 different samples. Two-way ANOVA test was followed by multiple comparison analysis. *P<0.05 and **P<0.01 per Dunnett's multiple comparisons test versus PVIgG. On the right, graph representing differences between group means at 24 (circles) and 72 (squares) hrs and 95% Confidence Intervals (according to multiple comparisons with Dunnett analysis).

and severity. Although we were not able to directly quantify the effective upregulation and release of sFasL in HSOC models, here we indirectly confirmed that the binding of autoantibodies to DSGs induce the release of sFasL from target keratinocytes, as previously postulated (5, 14), also in a more complex system. Our approach will allow a broad use of the new anti-FasL antibody. Indeed, scFv-induced pemphigus seems to reflect an extremely severe kind of pemphigus in humans, characterized by the almost complete detachment of the epidermis, while the PVIgG-induced model recapitulates a milder disease. In any case, the efficacy of PC111

in blocking blister formation was confirmed by two independent laboratories with two different models.

4 Conclusions

sFasL plays a critical role in the pathomechanisms underlying the formation of the blister in pemphigus (14, 22). We have been developing a human anti-FasL monoclonal antibody (PC111) that, by targeting sFasL in the skin at the keratinocyte level, downstream of autoantibodies binding to DSGs, does not affect the immune system, thus potentially avoiding the severe side effects associated with immunosuppressive treatments currently used in pemphigus. We had shown previously that FasL siRNA prevented DSG cleavage (14), while an anti-FasL antibody blocked blister formation in the passive neonatal pemphigus mouse model in a dose-dependent manner (14). Recently, using a modified version of the original model by Ohyama and co-workers (23), we have generated an active pemphigus mouse model that allows tracking of disease progression in adult animals for several weeks. The intraperitoneal injection of an anti-FasL antibody significantly reduced PV score up to 8 weeks, while restoring the weight loss and prolonging mice survival (unpublished data). While the proof of concept for FasL inhibition in the treatment of pemphigus is compelling, based on the body of *in vitro* and *in vivo* evidence, the data presented here confirm that the human anti-FasL antibody PC111 blocked blister formation in different human environments. In fact, PC111 inhibited acantholysis in the human keratinocyte dissociation assay and blocked blister formation in two independent and slightly diverse 3D ex-vivo pemphigus skin models (HSOC). Recently, HSOC model was successfully validated for the screening of novel treatment targets for pemphigus by simultaneously injecting scFv and the therapeutic compounds (24). In contrast, in both HSOC experiments presented here, PC111 was injected after the induction of the blisters, reflecting the temporal reality of diagnosis followed by treatment in human pemphigus. While scFv induced a marked and widespread epidermal detachment, mimicking an extremely severe form of pemphigus, PVIgG produced a milder acantholysis, possibly more similar to the pemphigus patients' situation. Nevertheless, PC111 significantly inhibited blister formation in both HSOC methods under a therapeutic protocol of administration. Currently, the most effective treatments for pemphigus target the production of antibodies by B-cells, such as anti-CD20 Abs, while inhibiting intracellular signaling pathways has not been fully investigated in clinical settings. Anti-B-cell treatment normally takes a few weeks to ameliorate symptoms and is potentially associated with immunosuppression-related side effects. On the other hand, PC111 displays a rapid onset of action, without affecting the immune system, possibly spearing/avoiding the use of steroids.

In conclusion, here we provide further evidence on the efficacy of specifically targeting sFasL in pemphigus and propose PC111 as a potential non-immunosuppressive treatment for this severe and life-threatening disease, opening the way to further development of this approach in clinical settings.

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 Ethic Committee of Area Vasta Emilia Nord Policlinico of Modena, Italy and Ethical Committee of the Medical Faculty of the University of Lubeck. The patients/participants provided their written informed consent to participate in this study.

Author contributions

RL, BB, JH, AM, and CP contributed to conception and design of the study. RL and JH performed experiments and data collection. JH and RL analysed data. CMH provided scFv. RL performed the statistical analysis. RL, AM, CP, AA, JH and RJL have discussed and interpreted the data. RL and CP wrote the first draft of the manuscript. RJL, BB, AA and AM wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study received funding from PinCell. The funder had the following involvement with the study: participated in the study design, research, analysis, data collection, interpretation of data, and review and approval of the publication.

Conflict of interest

RL, BB, AA and CP served as consultants for PinCell. JH had a collaborative research agreement with PinCell. AM and CP are co-founders PinCell s.r.l. This study received funding from PinCell. The funder had the following involvement with the study: participated in the study design, research, analysis, data collection, interpretation of data, and review and approval of the publication.

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 17 July 2023
DOI 10.3389/fimmu.2023.1200992

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RECEIVED 05 April 2023
ACCEPTED 30 June 2023
PUBLISHED 17 July 2023

CITATION
Lakoš Jukić I, Mokos M and Marinović B (2023) HLA class II antigens in Croatian patients with pemphigus vulgaris and their correlation with anti-desmoglein antibodies.
Front. Immunol. 14:1200992.
doi: 10.3389/fimmu.2023.1200992

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HLA class II antigens in Croatian patients with pemphigus vulgaris and their correlation with anti-desmoglein antibodies

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Pemphigus vulgaris (PV) is an acquired autoimmune blistering disease characterized by the production of autoantibodies targeting desmosomal cadherins, primarily desmoglein 1 and desmoglein 3, leading to acantholysis. The etiology of PV is multifactorial, including genetic susceptibility. This retrospective study aimed to evaluate the association of HLA class II alleles and PV and to examine the impact of PV-associated HLA class II alleles on the concentration of anti-desmoglein antibodies. The study group included 30 patients in whom the diagnosis of PV was confirmed by histopathological analysis, immunofluorescence findings, and ELISA testing for detecting antibodies against desmoglein 1 and desmoglein 3. HLA class II alleles were typed by polymerase chain reaction with sequence-specific primers (PCR-SSP). The control group consisted of 190 healthy volunteer blood donors. Data analysis revealed a significantly higher frequency of HLA class II alleles in our population of patients with PV, including HLA-DRB1*04:02, HLA-DRB1*14:54, HLA-DQB1*03:02, HLA-DQB1*05:03, HLA- DQA1*03:01, and HLA-DQA1*01:04, as well as a significantly lower frequency of HLA-DQA1*05:01 compared to the control group. We have also investigated the influence of risk alleles for PV, recognized in almost all study populations, HLA-DRB1*04:02 and HLA-DQB1*05:03, on the concentration of antibodies against desmogleins 1 and 3 in relation to the presence of these alleles. The results showed significantly higher levels of antibodies directed against desmoglein 3 among patients with DRB1*04:02 compared to patients without this allele. No difference was found for anti-desmoglein 1 antibodies. Regarding DQB1*05:03 allele, statistical analysis showed no differences in the concentration of anti-desmoglein antibodies in patients carrying this allele versus those without it.

KEYWORDS

pemphigus vulgaris, genetics, HLA class II allele, allele frequency, desmoglein

1 Introduction

Pemphigus vulgaris (PV) is a rare disease with an estimated incidence in Croatia of 3.7 new patients per million inhabitants per year (1). Although rare, PV prevalence and incidence differ significantly among different populations, ranging from 0.76 in Finland to 16.1 per million inhabitants in Israel, indicating that genetic factors are involved in the complex pathophysiology of the disease (2, 3). The estimated worldwide incidence is 0.75 to 5 new cases per million annually (4). The highest incidence of 32 new cases was observed in the study of Simon et al. in North America in people of Jewish descent compared to 4.2 new cases in non-Jewish adults (5). PV is more common in the Jewish populations, particularly of Ashkenazi origin, and in the Mediterranean (6, 7).

Hahn-Ristic et al. reported more than an eight-fold higher incidence of PV in foreigners, mainly Turks and Italians, living in Germany than in native Germans (8). The diversity of incidences of PV among different populations and ethnic groups is most likely connected to diverse genetic backgrounds and trigger factors. Population studies have shown a strong association between particular HLA class II alleles and PV. The two most common PV-associated alleles are DQB1*05:03 and DRB1*04:02, as reported in studies from Spain, France, Italy, Slovakia, North America, and Brazil (9–16). According to Kridin et al., most patients with PV express one of these two alleles (17). HLA DRB1*04:02 allele was found in 92% of Ashkenazi Jews patients with PV (18). A statistically significant association was found in the Jewish population with the DQB1*03:02 allele (19). Another DRB1 allele, DRB1*14:01, was positively associated with PV in Japanese, Italian, Pakistani, and Spanish patients (13, 20–22). In white European patients with PV, DRB1*14:54 has been significantly more expressed (16, 23).

The most commonly PV-associated alleles, DQB1*05:03 and DRB1*04:02, are considered to be not only genetic markers for susceptibility but can also influence the severity of the disease. Dhanda et al. have reported that anti-desmoglein 3 autoantibodies levels were significantly higher in patients who carried one or both of these alleles (24).

This study aimed to evaluate the association of HLA class II alleles in our group of patients with PV, compared with healthy subjects, and to determine the influence of PV-associated alleles on the levels of anti-desmoglein antibodies, which correlate with disease severity.

2 Materials and methods

2.1 Patients and controls

This retrospective study sampled 30 unrelated patients (20 female and 10 male) treated for PV at the Department of Dermatovenereology, University Hospital Centre Zagreb. The mean age of patients was 54 years, with a range of 33-82 years. The diagnosis of PV was based on clinical, histological, and immunofluorescence studies and ELISA testing to detect serum antibodies against Dsg1 and Dsg3 (MESACUP Desmoglein test, Nagano, Japan). Additionally, serum samples were collected for HLA typing, for which all patients signed informed consent. The control group consisted of 190 (41 female and 149 male) healthy volunteer blood donors with a mean age of 40 years and a range of 18-71 years. The study protocol has been approved by the Committee of Ethics of the University Hospital Centre Zagreb and was conducted in accordance with the Declaration of Helsinki.

2.2 DNA extraction and HLA typing

Genomic DNA was extracted from peripheral blood (3 ml) using QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany). HLA class II alleles were typed by polymerase chain reaction with sequence-specific primers (PCR-SSP). Detection of products of PCR amplification was performed by electrophoresis on 1.5% agarose gel. Gel images were analyzed with standardized tables or with HELMBERG SCORE software.

2.3 Statistical analysis

The frequencies of HLA alleles were directly extracted from the genotyping data. The statistical analysis was performed using the STATISTICA software package version 12 (www.statsoft.com. StatSoft, Inc. Tulsa, OK, USA) and the VassarStats website for Statistical Computation (http://vassarstats.net/odds2x2.html). The categorical (qualitative) variables were presented as frequencies and percentages and compared between groups (PV and control) using Fisher's exact test. The normality of antibody concentration distribution was assessed using the Kolmogorov-Smirnov test, and antibody concentrations were presented as the median, interquartile range (IQR), and range. The comparison between groups regarding the presence of HLA DRB1*04:02 and/or HLA DQB1*05:03 was performed using the Mann-Whitney U test. The association between HLA alleles and PV was determined using the odds ratio (OR) and 95% confidence intervals (95%CI). The Benjamini-Hochberg method was used to adjust for multiple comparisons. A two-tailed P-value (or adjusted P-value) <0.05 was considered statistically significant.

3 Results

The frequencies of HLA-DRB1 allele in pemphigus patients and control group are shown in Table 1. The presence of DRB1*04:02 (OR=24.69; 95%CI=9.80-62.25; P=2.55*10^{-11}) and DRB1*14:54 (OR=27.07; 95%CI=3.35-218,77; P=0.0011) was significantly higher in group of PV patients compared to controls. There were no significant differences for other alleles in DR region.

HLA-DQB1 typing results of PV patients and the control group are presented in Table 2. Frequencies of DQB1*03:02 (OR=8.81; 95%CI=4.46-17.42; Padj=3.76x10^{-8}) and DQB1*05:03 (OR=5.19; 95%CI=2.11-12.76; Padj=0.0068) were significantly increased in PV patients versus controls.

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TABLE 1 Frequencies of HLA-DRB1 alleles in patients with PV and control group.

	PV		Control						
DRB1	N	%	N	%	*P*	P_{adj}	OR	-95%CI	+95%CI
***0402**	**19**	**31,67**	**7**	**1,84**	**$7,29x10^{-13}$**	**$2,55x10^{-11}$**	**24,693**	**9,795**	**62,252**
***1104**	6	10,00	29	7,63	0,605496	1	1,345	0,534	3,390
***1601**	6	10,00	37	9,74	1	1	1,030	0,415	2,557
***1401**	4	6,67	13	3,42	0,268546	0,854465	2,017	0,635	6,403
***1454**	**4**	**6,67**	**0**	**0,00**	**6,59E-05**	**0,001153**	**27,071**	**3,350**	**218,773**
***1101**	3	5,00	38	10,00	0,245455	0,854465	0,474	0,142	1,586
***1602**	3	5,00	6	1,58	0,11095	0,63188	3,281	0,798	13,487
***0101**	3	5,00	36	9,47	0,333431	0,972506	0,503	0,150	1,688
***0701**	3	5,00	24	6,32	0,784735	1	0,781	0,228	2,677
***0401**	2	3,33	13	3,42	1	1	0,991	0,218	4,505
***1501**	1	1,67	34	8,95	0,068093	0,595815	0,173	0,023	1,284
***1301**	1	1,67	24	6,32	0,228298	0,854465	0,251	0,033	1,894
***0801**	1	1,67	13	3,42	0,703748	1	0,479	0,061	3,726
***1404**	1	1,67	0	0,00	0,136364	0,63188	Inf	NA	Inf
***0804**	1	1,67	6	1,58	1	1	1,057	0,125	8,933
***0302**	1	1,67	0	0,00	0,136364	0,63188	Inf	NA	Inf
***1201**	1	1,67	8	2,11	1	1	0,788	0,097	6,417
***0102**	0	0,00	5	1,32	0,61804	1	0	0	NA
***0103**	0	0,00	3	0,79	1	1	0	0	NA
***0301**	0	0,00	37	9,74	0,009396	0,109621	0	0	NA
***0403**	0	0,00	2	0,53	1	1	0	0	NA
***0404**	0	0,00	7	1,84	0,60062	1	0	0	NA
***0405**	0	0,00	1	0,26	1	1	0	0	NA
***0407**	0	0,00	1	0,26	1	1	0	0	NA
***0812**	0	0,00	1	0,26	1	1	0	0	NA
***1001**	0	0,00	6	1,58	0,603815	1	0	0	NA
***1103**	0	0,00	3	0,79	1	1	0	0	NA
***1111**	0	0,00	1	0,26	1	1	0	0	NA
***1302**	0	0,00	16	4,21	0,14443	0,63188	0	0	NA
***1303**	0	0,00	2	0,53	1	1	0	0	NA
***1305**	0	0,00	1	0,26	1	1	0	0	NA
***1405**	0	0,00	1	0,26	1	1	0	0	NA
***1408**	0	0,00	1	0,26	1	1	0	0	NA
***1502**	0	0,00	3	0,79	1	1	0	0	NA
***0901**	0	0,00	1	0,26	1	1	0	0	NA
Total	**60**	**100,00**	**380**	**100,00**					

N, frequency of allele; %, percentage; P, P value; P_{adj}, adjusted P value; OR, odds ratio; Cl, confidence interval; NA, not available; inf, infinite. Alleles with significant frequency differences between the examined groups have been highlighted in bold.

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TABLE 2 Frequencies of HLA-DQB1 alleles in patients with PV and control group.

	PV		Control						
DQB1	N	%	N	%	*P*	P_{adj}	OR	-95%CI	+95%CI
0302	**21**	**36,21**	**23**	**6,05**	**$2,35x10^{-9}$**	**$3,76x10^{-8}$**	**8,809**	**4,456**	**17,417**
0503	**9**	**15,52**	**13**	**3,42**	**0,000853**	**0,006821**	**5,185**	**2,107**	**12,762**
0502	9	15,52	42	11,05	0,376976	0,502635	1,478	0,678	3,224
0301	8	13,79	93	24,47	0,093052	0,248139	0,494	0,226	1,079
0501	3	5,17	51	13,42	0,086506	0,248139	0,352	0,106	1,17
0402	2	3,45	12	3,16	1	1	1,095	0,239	5,024
0303	2	3,45	5	1,32	0,234091	0,374546	2,679	0,507	14,140
0602	1	1,72	33	8,68	0,067471	0,248139	0,185	0,025	1,376
0603	1	1,72	26	6,84	0,154532	0,309065	0,239	0,032	1,795
0321	1	1,72	0	0,00	0,13242	0,302675	Inf	NA	Inf
0202	1	1,72	20	5,26	0,335955	0,488662	0,316	0,042	2,399
0201	0	0,00	39	10,26	0,010321	0,055045	0	0	NA
0304	0	0,00	4	1,05	1	1	0	0	NA
0307	0	0,00	2	0,53	1	1	0	0	NA
0601	0	0,00	3	0,79	1	1	0	0	NA
0604	0	0,00	14	3,68	0,232458	0,374546	0	0	NA
Total	**58**	**100,00**	**380**	**100,00**					

N, frequency of allele; %, percentage; P, P value; P_{adj}, adjusted P value; OR, odds ratio; Cl, confidence interval; NA, not available; inf, infinite. Alleles with significant frequency differences between the examined groups have been highlighted in bold.

As shown in Table 3, within HLA-DQA1* locus, a significantly higher frequency of the HLA-DQA1*03:01 allele (OR=7.76; 95% CI=3.88-15.53; Padj=$6.66x10^{-7}$) and HLA-DQA1*01:04 allele (OR=4.27; 95%CI=1.79-10.19; Padj=0.01198) were found among PV patients, compared to the control group. The frequency of DQA1*05:01 allele was significantly decreased in PV group versus the control (OR=<0.01; 95%CI=<0.001-<0.05; Padj=0,00226).

The association between concentration of anti-desmoglein 3 (anti-Dsg3) and anti-desmoglein 1 (anti-Dsg1) antibodies in relation to the presence or absence of DRB1*04:02 allele is presented in Table 4. There was a significantly increased concentration of anti-Dsg3 antibodies in patients who had DRB1*04:02 compared to patients without it (Mann-Whitney test; z=-3.701; p=0.00). No difference was observed for anti-Dsg1 antibodies. Table 5 shows the association of DQB1*05:03 allele and anti-Dsg3 and anti-Dsg1 antibodies. Statistical analysis showed no difference in the concentration of anti-Dsg1 and anti-Dsg3 antibodies in patients who carry this allele versus those without it.

4 Discussion

Pemphigus vulgaris is a chronic autoimmune bullous disease clinically characterized by erosions on the skin and mucous membranes and immunologically by autoantibodies targeting transmembrane components of desmosome, mainly desmogleins 1 and 3. Even though PV is a rare disease, its pathogenesis is constantly in the focus of research in dermatology, both due to its severe clinical course and significant impact on quality of life.

Different data on the prevalence of PV in relation to gender are reported in the literature. Polman et al. reported an equal representation of genders in PV patients, which is inconsistent with the fact that autoimmune diseases are more common among women (25, 26). Even more, the male preponderance has been reported in two reports from Kuwait and Saudi Arabia (27, 28). In this study, the sample included 20 women and 10 men, corresponding to 2:1 ratio, what is consistent with the recent report regarding the epidemiology of PV, which shows a female predominance with the female-to-male ratio ranging between 1 and 2, with 5 in the study of Simon et al. (5, 17). It is also expected, given the autoimmune nature of PV.

PV is a multifactorial disease resulting from the interaction of genetic and environmental factors (29). Although the predisposition to PV is genetically determined, the way of inheritance is still not clear (19). There are reports about the familial aggregation of the disease, but they are only sporadic, indicating PV is not inherited by a Mendelian pattern (29–32). Still, the presence of anti-Dsg3 antibodies in healthy first-degree relatives of PV patients points to dominant inheritance (29, 33, 34).

The association between PV occurrence and specific HLA antigens has been observed for almost sixty years, with the first report of Krain et al. (35). The role of the HLA system in the

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TABLE 3 Frequencies of HLA-DQA1 alleles in patients with PV and control group.

	PV		Control						
DQA1	N	%	N	%	*P*	P_{adj}	OR	-95%CI	+95%CI
0301	**19**	**33,33**	**23**	**6,05**	**$4,16x10^{-8}$**	**$6,66x10^{-7}$**	**7,761**	**3,879**	**15,529**
0102	9	15,79	95	25,00	0,137081	0,438659	0,563	0,266	1,189
0104	**9**	**15,79**	**16**	**4,21**	**0,002247**	**0,011981**	**4,266**	**1,786**	**10,185**
0505	8	14,04	40	10,53	0,493584	0,987167	1,388	0,614	3,139
0101	3	5,26	42	11,05	0,24322	0,648587	0,447	0,134	1,493
0201	3	5,26	24	6,32	1	1	0,824	0,240	2,830
0103	2	3,51	26	6,84	0,406944	0,930158	0,495	0,114	2,145
0401	2	3,51	14	3,68	1	1	0,951	0,210	4,297
0402	1	1,75	0	0,00	0,130435	0,438659	Inf	NA	Inf
0303	1	1,75	10	2,63	1	1	0,661	0,083	5,261
0105	0	0,00	6	1,58	0,60776	1	0	0	NA
0302	0	0,00	1	0,26	1	1	0	0	NA
0501	**0**	**0,00**	**80**	**21,05**	**0,000283**	**0,00226**	**<0.01**	**<0.001**	**<0.05**
0502	0	0,00	1	0,26	1	1	0	0	NA
0503	0	0,00	1	0,26	1	1	0	0	NA
0601	0	0,00	1	0,26	1	1	0	0	NA
Total	**57**	**100,00**	**380**	**100,00**					

N, frequency of allele; %, percentage; P, P value; P_{adj}, adjusted P value; OR, odds ratio; Cl, confidence interval; NA, not available; inf, infinite. Alleles with significant frequency differences between the examined groups have been highlighted in bold.

TABLE 4 The concentration of anti-Dsg1 and anti-Dsg3 antibodies in correlation with the presence or absence of DRB1*04:02 allele.

antibody	DRB1*04:02	N	Median	Minimum	Maximum	IQR
anti-Dsg1	NEG	11	36,52	1,27	168,83	12,05-79.90
anti-Dsg1	POS	19	29,92	0,42	156,71	11,42-92,30
anti-Dsg3	NEG	11	76,62	0,92	156,71	2,49-25,76
anti-Dsg3	POS	19	180,68	38,42	298,92	148,93-201,22

N, number of patients with DRB1*04:02 allele; IQR, interquartile range.

TABLE 5 The concentration of anti-Dsg1 and anti-Dsg3 antibodies in correlation with the presence or absence of DQB1*05:03 allele.

antibody	DQB1*05:03	N	Median	Minimum	Maximum	IQR
anti-Dsg1	NEG	21	35,45	1,38	168,83	17,95-92,30
anti-Dsg1	POS	9	22,55	0,42	150,23	1,27.56,62
anti-Dsg3	NEG	21	156,71	0,92	298,92	91,28-196,83
anti-Dsg3	POS	9	131,85	34,66	185,98	60,50-153,63

N, number of patients with DQB1*05:03 allele; IQR, interquartile range.

pathogenesis of PV corresponds with its direct involvement in immune reactions.

When comparing the frequency of HLA class II alleles in different populations, in groups of PV patients to healthy individuals, a significantly higher frequency of multiple alleles was observed among PV patients. In a meta-analysis of 18 studies published about the association of PV and DRB1* locus, DRB1*04, DRB1*08, and DRB1*14 were significantly

overexpressed (36). From all of these, the importance of DRB1*04:02 is by far the greatest. Namely, a significantly higher frequency of this allele has been demonstrated among PV patients compared to healthy individuals in almost all populations (9). The data from our study is in accordance with the aforementioned; a significantly higher frequency of the DRB1*04:02 allele (OR=24.69) was observed in the group of patients with PV compared to the control group. The frequency of this allele within a locus was 31.67% in the patient group, while the same allele had a frequency of 1.84% among healthy individuals. Since there were no patients homozygous for this allele in our study, the frequency of DRB1*04:02 per patient was 63.3%. This result is similar to the recently published report of 293 patients with PV, of which 60% carried DRB1*04:02 allele (37). We have also observed a significantly increased frequency of DRB1*14:54 allele in the PV group (OR=27.071). The association between this allele and PV has been reported in white European individuals in the United Kingdom as well as in Slovakia (16, 23). The association of DRB1*14:54 allele with PV in other populations is probably underreported because previously PV-associated allele DRB1*14:01 may actually represent DRB1*14:54 (10, 12, 20, 38). These alleles differ only in exon 3, at position 112, due to amino replacement (23). In Brazilian patients with PV, there is an increased frequency of the HLA-DRB1*08:04 allele compared to the control group. Since this allele was associated in haplotypes with alleles that do not carry a risk for PV, it was concluded HLA-DRB1*08:04 allele is associated with PV (39). Increased frequency of this allele has also been reported in African American patients with PV (37).

Within the HLA-DQB1* gene, the association of HLA-DQB1*05:03 and DQB1*03:02 alleles with PV has been confirmed by a meta-analysis of 18 population studies (40). However, due to strong linkage disequilibrium between DR and DQ loci, such as the association of DRB1*14:01 and DQB1*05:03 or DRB1*04 and DRB1*03, the question arises as to which of these alleles represents a risk for PV. In their study, Lee et al. found that haplotypes DRB1*04:02-DQB1*03:02 and DRB1*04:02 without DQB1*03:02 are more frequently expressed in patients with PV (14). After separating patients carrying the HLA-DRB1*04:02 allele, they did not find a significant difference in the frequency of the DQB1*03:02 allele. A similar finding was obtained in the study by Saenz-Cantele et al. among PV patients in Venezuela (41). Lee et al. also observed that HLA-DRB1*14:01 alone does not carry a risk for PV but is often found together with HLA-DQB1*05:03 due to the imbalance of association (14). Nowadays, HLA-DRB1*04:02 and HLA-DQB1*05:03 are considered to be the key alleles directly involved in the immunopathogenesis of PV (17, 36, 40). Veldman et al. reported that only antigen-presenting cells expressing HLA-DRB1*04:02 and HLA-DQ1*05:03 could present desmoglein 3 to autoreactive CD4+ T lymphocytes (42). Among our participants, we found a significantly higher frequency of the HLA-DQB1*03:02 allele (OR=8.81) and HLA-DQB1*05:03 allele (OR=5.19) in patients with PV compared to the control group. The higher frequency of DQB1*03:02 can be explained as a result of linkage disequilibrium with DRB1*04:02. HLA-DQB1*05:03 allele was seen in 9 of our PV patients with a frequency of 30% per patient. The same result has been reported in the study of Baker at al. (37). In that study this allele was predominant in the South Asian population with PV; 82% of them carried DQB1*05:03. No significant difference in distribution was found for other alleles of the HLA-DQB1* locus.

In our study, a significantly higher frequency of the HLA-DQA1*03:01 allele (OR=7.76) and HLA-DQA1*01:04 allele (OR=4.27) within the HLA-DQA1* locus was found among PV patients, compared to the control group. This result aligns with similar studies conducted in Iran and Brazil (39, 43). Given that loci DRB1* and DQB1* are in the focus of research due to their role in the pathogenesis of PV, there are scarce literary reports on the association of DQA1* locus genes and PV. Our study demonstrated a significantly lower frequency of the HLA-DQA1*05:01 allele (OR=<0.01) among PV patients compared to the control group. This result suggests a possible protective role of this allele in terms of PV occurrence. A negative association of this allele with psoriasis was also described (44).

The clinical presentation of PV is diverse, depending on the affection of mucous membranes and skin, the course of the disease, the number of lesions, and the effectiveness of treatment. The course of the disease cannot be predicted. In the study by Dhandha et al., patients with mucous membrane lesions and those with both mucous membrane and skin lesions had significantly higher levels of antibodies directed against Dsg3 than patients with only skin changes. Further research reported that the levels of these antibodies were significantly higher in patients who had either the HLA-DRB1*04:02 or HLA-DQB1*05:03 allele (24). The report of Svecova et al. documented a correlation between HLA-DRB1*04:02 and severe mucocutaneous PV (45). HLA-DQB1*03:02 was associated with a more severe form of PV only among female patients. The role of HLA-DRB1*04:02 or HLA-DQB1*05:03 allele in pathophysiology of PV has been additionally highlighted in studies focused on Dsg3 specific CD4 T lymphocyte response, restricted by these two alleles (46–50). Eming et al. demonstrated in an experiment with an HLA- DRB1*04:02 transgenic mouse model that this antigen is involved in activating specific auto-aggressive CD4+ T lymphocytes (50). These lymphocytes have a crucial role in the induction and maintenance of B lymphocytes or plasma cells, producing antibodies directed against Dsg3. Such a T lymphocyte response in patients with PV has been demonstrated in several studies (42, 51, 52). Based on the above, PV can be considered a T-cell, more precisely Th2, mediated autoimmune disease, which certainly opens new treatment possibilities (50).

In our study, we have demonstrated significantly higher levels of antibodies directed against desmoglein 3 in patients who have the HLA- DRB1*04:02 allele compared to those who do not have this allele. There was no difference in the concentration of anti-Dsg1 antibodies. Regarding DQB1*05:03 allele, statistical analysis showed no differences in the concentration of anti-desmoglein antibodies in patients carrying this allele versus those without it. Baker et al. reported higher levels of anti-Dsg1 antibodies in patients carrying DQB1*05:03 allele compared to DRB1*04:02 and DRB1*08:04 positive patients (37). Our result may be the consequence of the small sample size since DQB1*05:03 was expressed in only 9

patients. Given the literature reports of a correlation between antibody concentration and clinical presentation, and the fact that desmoglein 3 is the main autoantigen in PV, it is reasonable to conclude that HLA- DRB1*04:02 may impact the disease severity in our study population (53–55). Unfortunately, due to our study's retrospective nature, we could not perform further analysis of disease severity grading (ABSIS - Autoimmune Bullous Skin Disorder Intensity Score) in our patients. Lastly, 4 of our 30 patients did not express DRB1*04:02 or DQB1*05:03. Since these alleles have a crucial role in initiating an autoimmune response against desmogleins, further studies will be needed to elucidate the role of other, non-desmoglein autoantibodies in pathophysiology of PV.

5 Conclusion

Pemphigus vulgaris is probably a polygenic disease whose phenotype results from complex interactions between more than one gene and environmental factors. It is reasonable to assume that HLA class II genes, especially DRB1*04:2 and DQB1*05:03 are just one piece of the complex puzzle of PV pathophysiology. To the best of our knowledge, this is the first study investigating the association of specific HLA class II alleles with the occurrence of PV in the Republic of Croatia. Through data analysis, we found a significantly higher frequency of HLA class II alleles in our population of patients with PV, including HLA- DRB1*04:02, HLA-DRB1*14:54, HLA-DQB1*03:02, HLA-DQB1*05:03, HLA- DQA1*03:01, and HLA-DQA1*01:04, as well as a significantly lower frequency of HLA-DQA1*05:01 compared to the control group. The significance of this "negatively" associated allele with PV occurrence is unknown. Regarding the fact that pemphigus vulgaris-relevant HLA alleles are restricted to the DRB and DQB loci and strong linkage disequilibrium of DRB1*04:02 with DQB1*03:02 and of DQB1*05:03 with DRB1*14:54, we can conclude that DRB1*04:02 and, to a lesser extent, DQB1*05:03 are risk alleles for PV in Croatia. We have also demonstrated significantly higher levels of antibodies directed against desmoglein 3 in patients with the HLA- DRB1*04:02 compared to those who do not have this allele. Our findings support the previously reported hypothesis that HLA-DRB1*04:02 allele is not only genetic marker for development of PV, but can also influence activity and severity of the disease.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: They are archived in the hospital's patient databases. Requests to access these datasets should be directed to branka.marinovic@kbc-zagreb.hr.

Ethics statement

The studies involving human participants were reviewed and approved by Committee of Ethics of the University Hospital Centre Zagreb. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ILJ, MM, and BM contributed actively to the preparation of the manuscript. Conceptualization: ILJ; Writing - original draft preparation: ILJ, MM, and BM; Writing - review and editing: ILJ, MM, and BM; Tables: ILJ; Collecting sources of information: ILJ and BM; Supervision: BM. 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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 27 July 2023
DOI 10.3389/fimmu.2023.1194088

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OPEN ACCESS

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RECEIVED 26 March 2023
ACCEPTED 13 July 2023
PUBLISHED 27 July 2023

CITATION
Yan T, Xie Y, Liu Y, Shan Y, Wu X, Wang J, Zuo Y-G and Zhang Z (2023) Dupilumab effectively and rapidly treats bullous pemphigoid by inhibiting the activities of multiple cell types. *Front. Immunol.* 14:1194088. doi: 10.3389/fimmu.2023.1194088

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Dupilumab effectively and rapidly treats bullous pemphigoid by inhibiting the activities of multiple cell types

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Background: Bullous pemphigoid (BP) is an autoimmune skin-blistering disease. Systemic corticosteroids remain the first line treatment for moderate-to-severe BP with the potential for severe adverse events. Dupilumab has emerged as an alternative option for BP patients.

Objective: We evaluated the efficiency and safety of dupilumab on BP treatment and explored a mode of drug action in depth.

Methods and results: A multicenter retrospective cohort included 20 BP patients who received dupilumab with or without systemic corticosteroid in dupilumab group, and 20 matched BP patients who received corticosteroid alone in conventional group. Serum samples were collected from 20 patients (10 from dupilumab group and 10 from conventional group) at baseline and week 4. Compared to systemic corticosteroid alone, dupilumab with or without systemic corticosteroid was similarly efficacious in clinical remission at week4 (complete remission plus partial remission: 100%) and week24 (complete remission plus partial remission:100%), but allowing significant decreases in the cumulative doses of corticosteroids with reducing the incidence of adverse events. However, dupilumab did not decrease BP180 antibody despite an obvious clinical improvement. Comparative plasma proteomic analysis performed before and after treatment in 3 BP patients from dupilumab group revealed that drug use was associated with 30 differentially expressed proteins, including 26 down-regulated and 4 up-regulated proteins. The former consisted of immune related proteins involved in T/B cell interactions (inducible T-cell co-stimulator ligand, ICOSL) and in the activation of eosinophils (PRG2), mast cells

(S100A12), and complement (CR2). TARC and ICOSL levels correlated with BP severity in patients who received either dupilumab or conventional treatment.

Conclusion: Dupilumab has similar efficacy in treating BP as conventional drugs, by inhibiting the activities of many types of immune cells and complement, and regulating the interactions between T and B cells.

KEYWORDS

bullous pemphigoid, dupilumab, eosinophil, TARC, ICOSL

1 Introduction

Bullous pemphigoid (BP) is an autoimmune skin disease and more frequently observed in the elderly. Its incidence is increasing as populations age (1). The first-line treatment for severe BP remains a systemic corticosteroid (2), the use of which is associated with increased risks of adverse events (AEs), especially in older patients with cardiovascular, cerebrovascular, gastrointestinal disease, or diabetes mellitus (3). Antibody-producing B cells, T cell subpopulations (e.g., Th2 cells, eosinophils, and mast cells), the complement system, and certain inflammatory factors are involved in the pathogenesis of BP (4–7). Antibodies or small molecular drugs targeting Th2 cells, eosinophils, mast cells, and complement have recently been used to treat BP (NCT04563923, NCT04035733, and NCT02226146) (8–10). Among them, dupilumab, an antibody targeting interleukin (IL)-4 and IL-13 produced by Th2 cells, afforded clear clinical improvement (9, 11). However, more clinical evidence is required.

Corticosteroids can inhibit the abnormal immune reactions of BP by reducing B cell proliferation, antibody production, eosinophil generation and survival, as well as the levels of cytokines produced by T cells. BP180 antibodies produced by B cells are the key pathogenic factor in BP development (12), whose level usually falls on corticosteroid treatment. Also, an increased eosinophil in plasma and skin is a prominent feature of BP (13). Toxic granule proteins (eosinophil cationic protein [ECP], major basic protein [MBP], or eosinophil peroxidase [EPO]) and a cytokine (matrix metalloproteinase 9 [MMP9])released by eosinophils promote keratinocyte separation (6). Eosinophil counts, which fall on corticosteroid treatment, correlate with BP activity and may be prognostic in certain patients (14, 15). In addition, the plasma level of thymus and activation regulated chemokine (TARC), an important Th2 chemotactic agent, correlated positively with the Bullous Pemphigoid Disease Area Index (BPDAI) score of patients on systemic corticosteroids (16). IL-4 and IL-13 produced by Th2 cells participate in eosinophil recruitment (17) and anti-BP180 antibody production (18). IL-4 modulates TARC expression when the PI3K pathway is directly activated by STAT6 (19). It is not yet clear whether the actions of dupilumab are similar to those of corticosteroids. We therefore performed a multicenter, retrospective cohort study to explore the efficacy and safety of dupilumab for BP patients. As a novel treatment option, we aimed to investigate the possible mechanism of dupilumab application in treating pemphigoid. Therefore, we further compared plasma proteomes profile of 3 BP patients from dupilumab group before and after dupilumab injections. The eosinophils, TARC, and inducible T-cell co-stimulator ligand (ICOSL) data efficiently revealed the responses of BP patients to both dupilumab and corticosteroids.

Abbreviations: BP, Bullous pemphigoid; AE, adverse events; IL, interleukin; MMP9, matrix metalloproteinase 9; BPDAI, Bullous Pemphigoid Disease Area Index; ICOSL, inducible T-cell co-stimulator ligand; BSA, body surface area; CR, complete remission; PR, partial remission; DEP, differentially expressed protein; TMT, tandem mass tagging; LC–MS/MS, liquid chromatography and tandem mass spectrometry; ELISA, Enzyme-linked immunosorbent assays; PPI, Protein-protein interaction; KEGG, Kyoto Encyclopedia of Genes and Genomes; NET, neutrophil extracellular trap; Go, Gene ontology; BMZ, basement membrane zone.

2 Methods

2.1 Patient information

This was a retrospective cohort study conducted from April 2020 to December 2021. BP patient data were collected from the Eighth Affiliated Hospital of Sun Yat-sen University, Peking Union Medical College Hospital, and the University of Hong Kong Shenzhen Hospital. All BP patients fulfilled the recognized diagnostic criteria (20) based on clinical presentation, histopathological findings, and direct or indirect immuno fluorescence test results. Moderate-to-severe BP was defined as an affected body surface area (BSA) > 10% or a skin erosion/blister total score on the BPDAI > 15, a urticaria/erythema score on the BPDAI > 20 (21). According to the medical record, patients receiving dupilumab with or without systemic corticosteroid were included in the dupilumab group, while patients receiving systemic corticosteroid only were included in the conventional group. Patients without regular follow-up for 24 weeks were excluded. Medical records and photographs were reviewed. We retrieved demographic characteristics, any underlying diseases, the lesional features, histological and immunological findings, laboratory data, doppler vascular ultrasonography, bone densitometry, the drugs

used, the responses at weeks 4 and 24, and AEs during a 24-week regimen. Initially missing data were obtained by calling patients or their guardians. This study was approved by the Ethics Committee Institutional Board of our local hospital. Written informed consent was obtained from all participants.

2.2 Dupilumab efficacy and safety in BP patients

2.2.1 Primary outcomes

The clinical remission (complete + partial) rate at week 4 (20).

- Complete remission (CR): The absence of new or established lesions (blisters, eczematous lesions, urticarial plaques, or mucosal lesions) and pruritus.
- Partial remission (PR): The presence of transient new lesions that healed within 1 week.
- Mild new activity: Less than three lesions/month that did not heal within 1 week or an extension of established lesions or pruritus once weekly but less than daily in a patient who had achieved disease control; these lesions healed within 2 weeks.
- Relapse/flare: Appearance of at least three new lesions/month, or at least one large (> 10 cm in diameter) eczematous lesion, or urticarial plaques that did not heal within 1 week, or extension of established lesions or daily pruritus in a patient who had achieved disease control.

2.2.2 Secondary outcomes

- The clinical remission (CR + PR) rates at weeks 12 and 24.
- AEs during the 24-week regimen. The severity of AE was classed according to Common Terminology Criteria for Adverse Events (CTCAE) v4.0.
- Cumulative doses of corticosteroids taken to week 4.
- Anti-BP180 antibody titers at weeks 0 and 4.
- Eosinophil percentages (EOS%) in peripheral blood at weeks 0 and 4.

2.3 Proteome profiling

Six plasma samples from three BP patients in the dupilumab group were collected before and after dupilumab injections. Patient 1 received both a dupilumab injection and a maintenance dose of corticosteroids after BP recurrence. Patients 2 and 3 were treated with dupilumab alone after primary BP attacks (details were summarized in supplemental patient information). Differentially expressed proteins (DEPs) were analyzed. Tandem mass tagging (TMT) coupled with liquid chromatography and tandem mass spectrometry (LC–MS/MS) were used to explore the proteomes. Supplementary Methods 1 provides detailed information on plasma collection and purification, exosome depletion, peptide labeling, LC–MS/MS and bioinformatics analyses, and database searching.

2.4 Enzyme-linked immunosorbent assays

Serum BP180 IgG antibody, TARC, ICOSL and S100A12 levels in 40 plasma samples were quantitated by ELISAs in 20 BP patients (10 in the dupilumab group and 10in the conventional group) at baseline and at week 4 aftertreatment. All ELISAs followed the manufacturers' instructions [BP180 (MBL, Nagoya, Japan), TARC (R&D Systems, Minneapolis, MN, USA), ICOSL (Cusabio Biotech Co., Wuhan, China), and S100A12 (Cusabio Biotech Co.)].

2.5 Statistical analysis

A Student's t-test, Chi-Squared T test, Mann-Whitney U test, and Wilcoxon test, were performed as appropriate using SPSS ver. 26.0 software (IBM Corp., Armonk, NY, USA). A p-value < 0.05 was considered statistically significant.

3 Results

3.1 Dupilumab rapidly and effectively controlled BP without severe adverse events

Twenty patients with moderate-to-severe BP who received dupilumab alone or combined with conventional drugs and 20 age-, sex-, and disease severity-matched BP patients who received corticosteroid were recruited. All patients had been prescribed a background topical corticosteroid more than 1 week with poor clinical improvement. Among 40 patients, 22patients are newly diagnosed BP patients, and 18patients are recurrent ones. In conventional group, 10 of 20 patients received prior treatment with systemic corticosteroids before this recurrence, while in dupilumab group, 9 of 20 patients were treated with systemic corticosteroids or IVIG before. Notably, ten patients received dupilumab alone in the dupilumab group. Six patients in dupilumab group receiving systemic corticosteroid got poor outcomes after 1 week treatment and then received injections of dupilumab. The initial dupilumab dose was 600 mg followed by 300 mg weekly or every 1–4 weeks on 4 to 10 occasions, and the initial dosage of corticosteroid varied from 0.05mg/Kg to 2mg/Kg. By contrast, the initial dosage of corticosteroid in conventional group was varied from 0.2mg/Kg to 1mg/Kg prednisone depending on the disease severity and then was decreased at 2.5-10mg every 1-8 weeks in the follow-up. The baseline data are listed in Table 1. The details of dupilumab or prednisolone treatment protocol were listed in supplemental patient information.

Of the 20 patients in the dupilumab group, twelve attained a CR with six on dupilumab alone. Eight patients achieved a PR with four on dupilumab alone (Figure 1A). In the conventional group, ten

TABLE 1 The baseline information of study population.

	Dupilumab group	Conventional group	All patients
Male	11	10	21
Female	9	10	19
Age (year)	73.4 ± 10.65	67.70 ± 11.76	70.55 ± 11.45
Duration before admission (month)	10.25 ± 16.91	9.95 ± 13.66	10.10 ± 15.18
EOS% within the normal range			
Yes	6	10	16
No	13*	9*	22
Underlying disease			
Cardiovascular disease	12	9	21
Neurologic disorders	7	4	11
Diabetes	6	3	9
others	7	4	11
Initial treatment	11	11	22
Recurrence treatment	9	9	18

*Both groups have one patient without eosinophilic data.

patients attained a CR and ten attained a PR (Supplemental Figure 1). The therapeutic efficacy did not differ between the two groups (p =0.751) (Figure 1B). Notably, dupilumab alone efficiently treated both initial episode and recurrences of pemphigoid. In the 20 patients on dupilumab, a CR was attained by 16/20, a PR by 4/20 at weeks 12 and a CR was attained by 17/20, a PR by 3/20 at 24 respectively. While in the conventional group, a CR was attained by 15/20 patients, a PR by 5/20 at week 12and a CR by 20/20 at week 24.

The cumulative corticosteroid dose decreased in the dupilumab group by week 4 when a CR or PR was attained (dupilumab group222.75 ± 454.48mg; conventional group 859.63 ± 379.32mg, p<0.01) (Figure 1C), and by week 24 (dupilumab group 835.25 ± 1373.31mg; conventional group 3189.33 ± 999.69mg, p<0.01). It is important to highlight that 12 of 20 in dupilumab group received dupilumab without corticosteroid. Further details are provided in supplemental patient information. The AE rate in the conventional group (26 AEs in fourteen patients) was significantly higher than in the dupilumab group (6 AEs in five patients) during the 24 weeks of treatment (Figure 1D). What's more, AEs in the conventional group were more severe than that in the dupilumab group. According to CTCAE criteria, among 26AEs in the conventional group, twelve AEs were classified as grade 1, eleven were grade 2, and three were grade 3, while in the dupilumab group, two AEs were classified as grade 1, and four were grade 2. AEs were summarized in supplemental patient information.

3.2 The percentage of eosinophils but not the anti-BP180 IgG level decreased after dupilumab injection

The percentage of eosinophils (EOS%) data at baseline and week4 were collected from 10 individuals in the dupilumab group and 10 in the conventional group whose serum anti-BP180 IgG antibody were detected. The EOS% decreased as BP became controlled after 4 weeks in both groups. In dupilumab group, the EOS% were15.25 ± 15.41% and 0.59 ± 0.95% before and after treatment, p = 0.013. In conventional group, the EOS% were 10.24 ± 11.50% and 0.28 ± 0.37% before and after treatment, respectively, p = 0.021. There EOS% level at baseline (p=0.430) and the reduction from baseline at week 4 (p=0.452) were similar between the groups. However, eight patients (five in the conventional group and three in the dupilumab group) evidenced normal eosinophil counts at baseline. The anti-BP180 antibody level decreased when a CR was attained at 4 weeks of treatment with conventional agents (before 102.70 ± 34.37 U/mL and after 48.60 ± 35.74U/mL, p <0.001) but not in the dupilumab group (before 119.73 ± 48.65 U/mL and after 116.66 ± 50.56 U/mL, p = 0.66) (Figure 2A). There was no statistically significant difference of anti-BP180 antibody level in the baseline between the groups (p=0.378). In the conventional group, the reduction from baseline of anti-BP180 antibody level was greater than in the dupilumab group at week 4 (p<0.001). Thus, dupilumab and corticosteroids have similarly effect in decreasing the EOS%.

10.3389/fimmu.2023.1194088

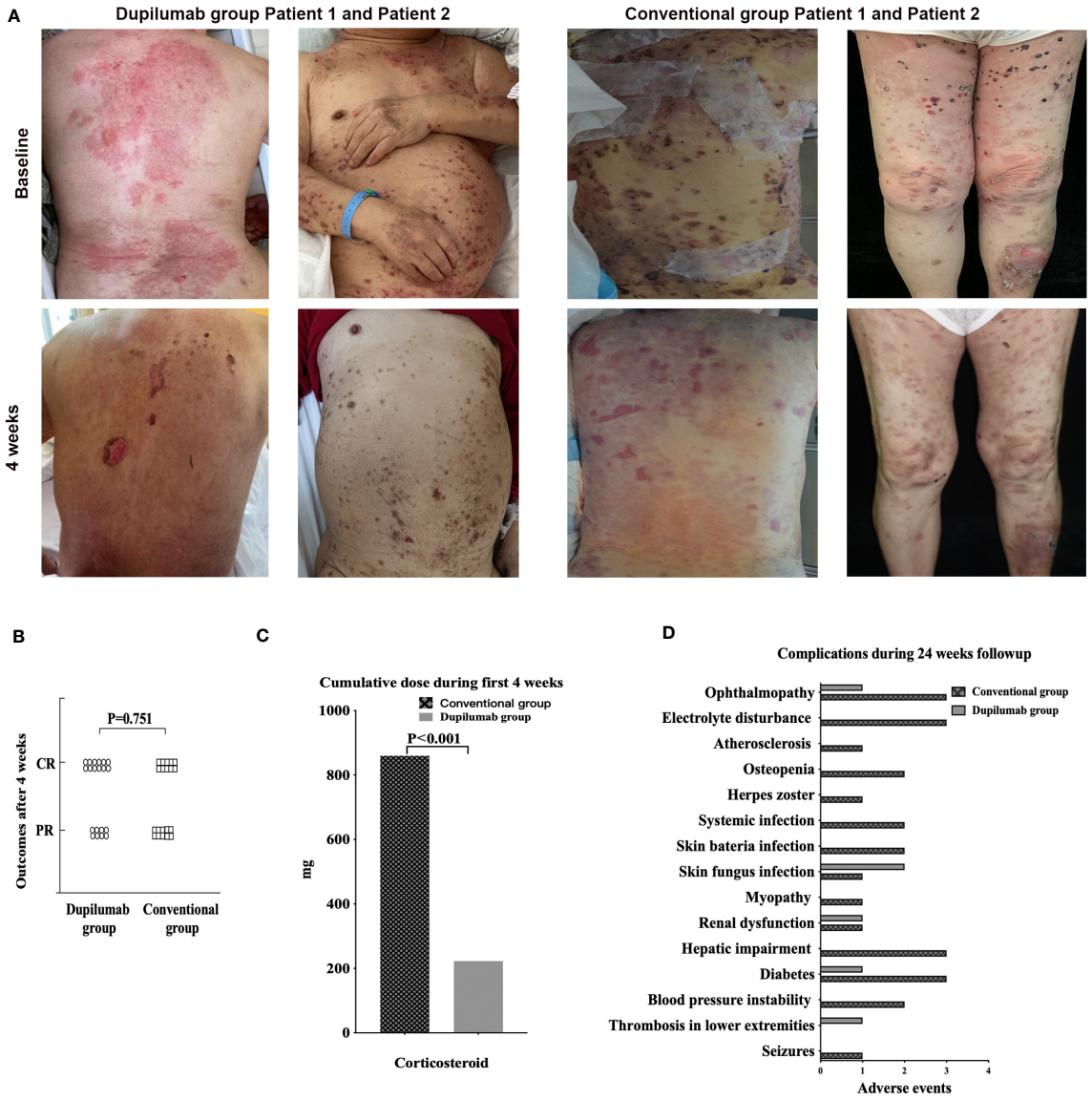

FIGURE 1
(A) Dupilumab effectively controlled BP without severe side effects. Clinical photographs taken at baseline and after 4 weeks from patients in dupilumab group and conventional group. **(B)** Primary outcomes of the dupilumab and conventional groups. In the former group, twelve patients achieved a CR and eight a PR; the figures for the conventional group were ten and ten. There was no significant difference between the two groups. **(C)** Cumulative doses of systemic corticosteroids at week 4, at which time BP was controlled in both the dupilumab and conventional groups. **(D)** Complications of BP patients treated with dupilumab or conventional drugs for 24 weeks. BP, bullous pemphigoid; CR, complete remission; PR, partial remission.

3.3 Dupilumab inhibits the activities of multiple cell types

Proteome profiling of six samples from 3 BP patients in dupilumab group (three samples before and three after treatment) revealed 879 unique proteins. Compared to the baseline, we detected 26 down-regulated and 4 up-regulated proteins at week 4 (Supplementary identified DEPs information). The roles played by several significantly down-regulated proteins, including ICOSL (before 109.65 and after 90.08, $p = 0.025$), CR2 (before 110.38 and after 89.67, $p = 0.03$), PRG2 (before 118.70 and after 81.40, $p = 0.013$), and S100A12 (before 110.31 and after 90.08, $p = 0.01$) are well understood (Figure 3D).

Gene ontology (Go) analysis was performed to comprehensively evaluate the plasma DEPs after dupilumab therapy. We explored molecular functions, the biological processes involved, and cellular components. Molecular function analysis revealed that some DEPs were associated with heterodimerization (H4C1, H2BC1, H3-3A, and H2AC20) or homodimerization (JUN, CR2, ADIPOQ, and TKT), calcium ion binding (H1-4, DSG1, TKT, and S100A12), and nucleosomal DNA binding (H3-3A and H1-4). Biological process analysis revealed that other DEPs were involved in nucleosome

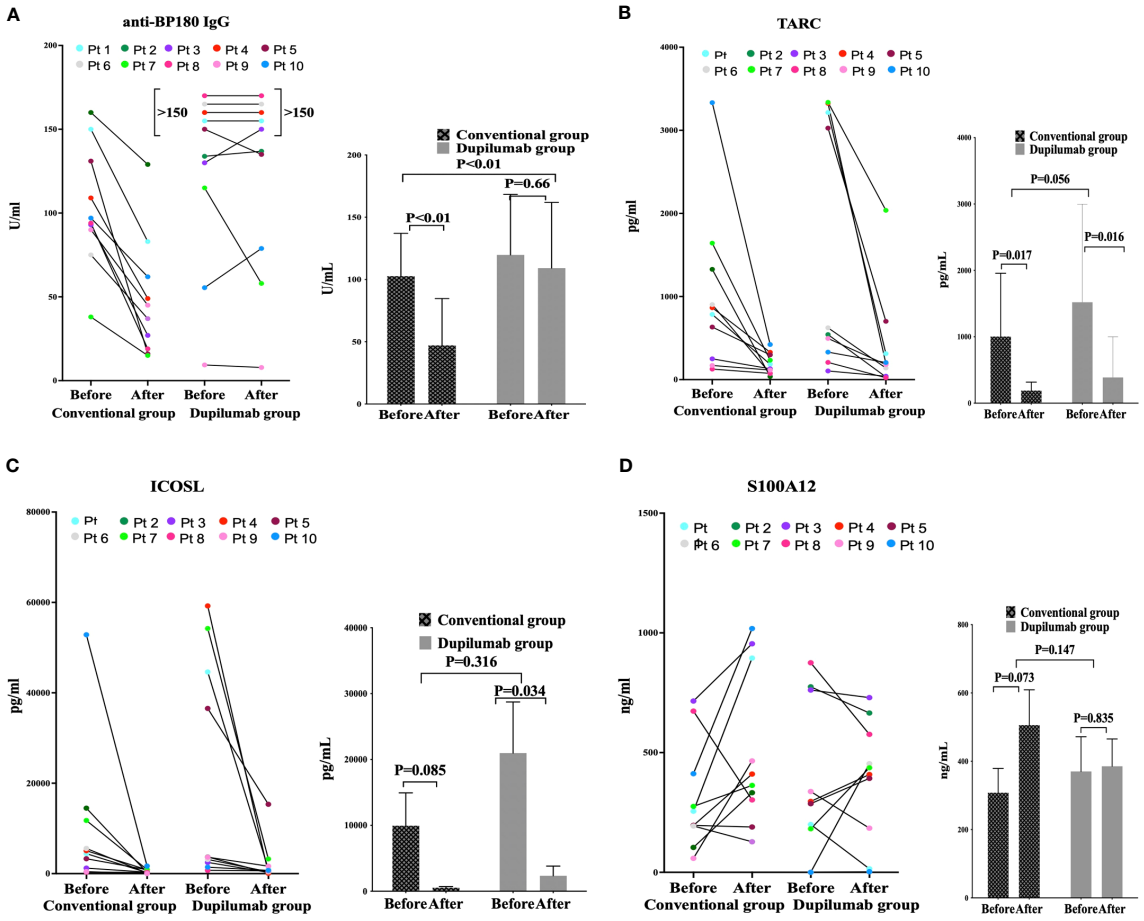

FIGURE 2
The enzyme-linked immunosorbent assay-based plasma levels of anti-BP180 IgG **(A)**, CCL17 **(B)**, ICOSL **(C)**, and S100A12 **(D)** before and after treatment in the conventional drug and dupilumab groups.

assembly (H2BC21, H1-4, and H3-3A), the antimicrobial humoral immune response (REG1A, GAPDH, S100A12, and H2BC21), and antibacterial defense (LYZ, S100A12, CHGA, PRG2, and GHV1-69D). Cellular component analysis revealed that some DEPs were enriched in extracellular exosomes (HABC21, PRG2, KPRP, REG1A, H3-3Q, H2AC20, OMD, TKT, UBA52, KRT78, JUP, GAPDH, LYZ, PYGB, FGB, CR2, ICOSL, TALDO1, GGH, PTPRJ, and ALDOB) which engage in intracellular molecule transportation (22), extracellular spaces (FGB, CR2, S100A12, POSTN, GGH, ADIPOQ, CHGA, H2BC21, REG1A, OMD, UBA52, KRT78, and LYZ), extracellular regions (S100A12, GGH, ADIPOQ, CHGA, H1-4, H3-3A, OMD, JUN, LYZ, PYGB, and FGB), the collagen-containing extracellular matrix (PRG2, OMD, FGB, POSTN, and ADIPOQ), and the lumen of a specific type of granule (JUN, LYZ, and GGH) (Figure 3B). Protein-protein interaction (PPI) analysis of all DEPs, performed with the aid of STRING, revealed that the top six categories were the extracellular exosome, nucleosome, extracellular space, killing of non-self-cells, nucleosome assembly, and defense against fungi (Figure 3A).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis disclosed that some DEPs affected neutrophil extracellular trap (NET) formation (H4C1, H2BC21, H3-3A, H2AC20, and FGB), the complement and coagulation cascades (CR2 and FGB), pentose phosphate metabolism (TKT, TALDO1, and ALDOB), and antifolate resistance (GGH) (Figure 3C). NET formation and the complement and coagulation cascades may play important roles in BP pathogenesis by promoting antibody production and blister formation (4, 23).

3.4 TARC and ICOSL are significantly down-regulated by dupilumab and corticosteroid

Compared to the pretreatment levels, the TARC levels decreased after treatment in both groups (dupilumab group: before 1,520.92 ± 1,477.20 pg/mL and after 386.86 ± 612.89 pg/mL, p = 0.016; conventional group: before 1,003.58 ± 953.50 pg/mL and after 190.22 ± 127.3 pg/mL, p = 0.017). The TARC levels at baseline and the reductions after treatment were similar in both groups (p = 0.056) (Figure 2B). Compared to before treatment, the ICOSL level decreased in both the conventional and dupilumab groups (dupilumab group: before 20.97 ± 24.55 ng/mL and after 2.35 ± 4.67 ng/mL, p = 0.034; conventional group: before 9.95 ± 15.78 ng/mL and after 0.544 ± 0.51 ng/mL, p = 0.085). The ICOSL levels at baseline and the reductions from baseline were similar in

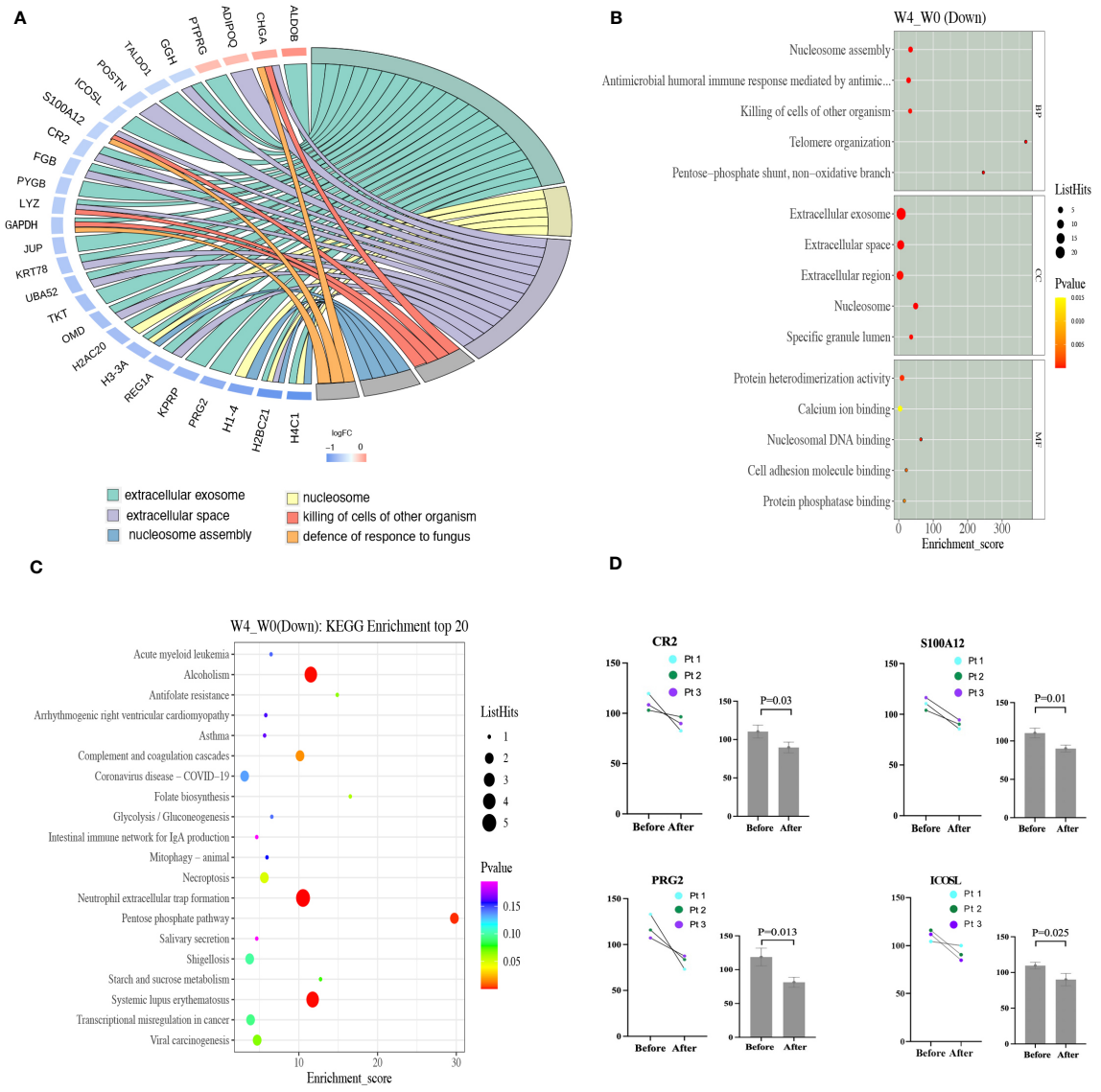

FIGURE 3
(A) The PPI network of DEPs based on STRING database analysis. Proteins up-regulated after dupilumab 4-week treatment are shown in orange and down-regulated proteins in blue (color of the outer circle). **(B)** The top 15 GO enrichment terms of the down-regulated DEPs are listed. The size of each bubble indicates the number of proteins it contains, and its color changes based on its p-value, with yellow representing the highest value and red representing the lowest. **(C)** Scatter plot of the top 20 pathways on KEGG enrichment of down-regulated DEPs. Larger bubbles indicate greater differences in the number of proteins, with their color transitioning from red to green, blue, and purple as the p-value increase and the difference becomes less significant. **(D)** The plasma levels of CR2, S100A12, PRG2, and ICOSL before and after 4-week dupilumab treatment in three patients as revealed by proteomic profiling. DEPs, different expressed proteins; GO, gene ontology.

both groups (p = 0.316) (Figure 2C). The S100A12 levels before and after treatment did not differ between the two groups (dupilumab group: before 370.32 ± 321.04 ng/mL and after 385.49 ± 252.28 ng/mL, p = 0.835; conventional group: before 307.91 ± 225.15 ng/mL and after 505.83 ± 326.75 ng/mL, p = 0.073). The baseline S100A12 levels did not differ between the two groups (p = 0.147) (Figure 2D).

4 Discussion

In recent years, rituximab (an anti-CD20 antibody), dupilumab, and omalizumab (an anti-IgE antibody) have become alternative BP therapies (24). Dupilumab was efficacious, shortened the hospitalization time, allowed for corticosteroid tapering, and reduced complications (9, 11). Our results showed that dupilumab alone or combined with corticosteroid was competitive with corticosteroids in the treatment of BP with good tolerance, which were consistent with previous reports (25–30). Notably, dupilumab alone can control BP as early as week 4, reduced the cumulative doses of corticosteroids and fewer drug-associated AEs. What's more, patients in dupilumab group had more health problems such as diabetes, neurologic disease, heart disease before treatment, but achieved less AEs than patients in conventional group. We recorded no conjunctivitis,

parasiticinfections, or eosinophilia; all are common during dupilumab therapy.

The pathogenesis of BP involves innate immunity [eosinophils (6), mast cells (31), and the complement system (4)], adaptive immunity [B (32), Th2 (5), Treg (33), and Tfh cells (34)], and complement-dependent and -independent pathways (4, 35–37). The complement-dependent pathway is initiated by antigen-antibody complexes in the basement membrane zone; these complexes activate complement (36–39). The complement-independent pathway is induced principally by antibodies and eosinophils, which results in blister formation regardless of complement activation (4, 13, 40–43). Plasma proteome profiling before and after dupilumab treatment revealed 26down-regulated proteins, such as ICOSL/B7H2, PRG2, S100A12, and CD21/CR2, which involved in T/B cell and B cell/complement interactions, eosinophil degranulation, and mast cell activation (44–47). ICOSL, a member of the B7 family of immune regulatory ligands, plays a vital role in the selection of high-affinity plasma cells, thus serving as a molecular linker of T and B cell interactions (44). CR2 recognizes complement 3 cleavage products bound to antigens and acts in conjunction with the B cell antigen receptor to lower the activation threshold and overcome B cell anergy (45). PRG2 participates in eosinophil activation, which is important in terms of BP pathogenesis (46). S100A12, a member of the S100 family of acidic calcium-binding proteins, may trigger mast cell degranulation and activation, in turn inducing the release of inflammatory mediators (47).

To investigate the effects of dupilumab on the activities of Th2 and mast cells, and T/B cell interactions, we measured the serum levels of TARC, S100A12, and ICOSL in 20 BP patients before and after treatment. The TARC and ICOSL levels decreased when BP was controlled. Surprisingly, the decreases occurred much earlier than the level of anti-BP180 antibody. Suzuki (16) found that the TARC level rather than the anti-BP180 level correlated with the BPDAI in BP patients on corticosteroids, consistent with our results. Similarly, there are decrease of TARC or eosinophils in peripheral blood and TARC, S100A12 or ICOS in skin lesions in atopic dermatitis patients after dupilumab treatment (48–52).

Bieber found that the S100A12 level fell in BP patients after 61 ± 40 weeks of therapy, as did the anti-BP180 level (53). In our plasma proteome profiling result from 3 patients in dupilumab group, S100A12 decreased after 4 weeks treatment. However, when we enlarged the samples to 10 patients, we found no decrease in S100A12 by week 4, which might indicate that mast cell activity differs between the early and late stages of BP remission.

The levels of eosinophils and anti-BP180 reflect disease severity in most BP patients (54, 55). In both groups, eosinophil levels clearly fell on entry into BP remission, in agreement with previous reports. However, dupilumab did not decrease the anti-BP180 antibody level despite the obvious clinical improvement, suggesting that corticosteroids and dupilumab act differently (56). Similar findings were reported in BP patients who received omalizumab. Balakirski et al. found no change in the level of anti-BP180 circulating autoantibodies after 6 months of omalizumab treatment (57). Yu et al. found that omalizumab slightly reduced the levels of anti-BP180 antibodies after disease remission (58). We speculate that dupilumab as well as omalizumab could not affect the production of IgG antibody directly but other pathways in pemphigoid disease.

In conclusion, dupilumab alone or combined with conventional drugs can control BP rapidly and safely over 24 weeks of treatment. Dupilumab may inhibit T/B cell interaction, and/or activate eosinophils, mast cells, neutrophils, and the complement system. Additional large-scale studies are required. Limited by sample size, missing data, and recall bias, further large-scale studies with prospective designs are warranted for the conclusion.

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 The university of Hong Kong-Shenzhen hospital. 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

TY analyzed the whole data and wrote the article. YX collected the information of BP patients in Peking Union Medical College Hospital and conducted the ELISA test. YL and XW collected the information of BP patients in The University of Hong Kong Shenzhen Hospital and The Eighth Affiliated hospital of Sun Yat-sen University and helped conducting the proteome profiling test. YS helped collected the information of BP patients in PUMCH. JW helped collecting the information of BP patients in Eighth Affiliated hospital of Sun Yat-sen University. ZZ and Y-GZ designed the research and revised the article. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the National Natural Science Foundation of China (grant number 81972944), National High Level Hospital Clinical Research Funding (2022-PUMCH-B-092) and Beijing Municipal Natural Science Foundation (7232118).

Acknowledgments

We are grateful to Yizhen Jia and Yuan Ren for her assistance in statistical analysis.

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.2023.1194088/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 10 August 2023
DOI 10.3389/fimmu.2023.1250115

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OPEN ACCESS

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RECEIVED 29 June 2023
ACCEPTED 24 July 2023
PUBLISHED 10 August 2023

CITATION
Patzelt S and Schmidt E (2023) Autoimmunity against laminin 332. *Front. Immunol.* 14:1250115. doi: 10.3389/fimmu.2023.1250115

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Autoimmunity against laminin 332

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Laminin 332 is a heterotrimeric structural protein of the basal membrane zone (BMZ) of the skin and adjacent mucosal tissues. The importance of laminin 332 for the structural integrity of the BMZ is demonstrated by mutations in any of the three genes encoding for its three chains causing variants of junctional epidermolysis bullosa. Autoimmunity against laminin 332 is observed in mucous membrane pemphigoid (MMP) and in the rare patients with orf-induced pemphigoid. MMP is an autoimmune blistering disease with predominant mucosal manifestations and autoantibodies against the BMZ of the skin and orifice-close mucous membranes. The main autoantigens of MMP are type XVII collagen (BP180) and laminin 332 targeted in about 80% and 10-20% of patients, respectively. An increasing number of studies has highlighted the association of anti-laminin 332 MMP and malignancies that can be revealed in about a quarter of these patients. This data has led to the recommendation of current guidelines to assay for anti-laminin 332 reactivity in all MMP patients. The present review focuses on anti-laminin 332 MMP describing clinical features, its pathophysiology, and detection of serum anti-laminin 332 IgG. In addition, the available data about the occurrence of malignancies in anti-laminin 332 MMP, the underlying tumor entities, and its biology are detailed.

KEYWORDS

autoimmunity, diagnosis, malignancy, immunofluorescence, guidelines, BP180, type VII collagen, laminin 332

Introduction

Laminin 332 is a heterotrimer and essential structural protein of the basal membrane zone (BMZ) of the skin, adjacent mucosal tissues including the mouth, pharynx, larynx, trachea, esophagus but also kidney, lung, and small intestine (1). The importance of laminin 332 for the structural integrity of the BMZ is demonstrated by mutations in any of the three genes *LAMA3*, *LAMB3* and *LAMC2*, that cause a variant of junctional epidermolysis bullosa (2, 3). Autoimmunity against laminin 332 is observed in the autoimmune blistering disease mucous membrane pemphigoid (MMP) and in the very rare patients with orf-induced pemphigoid (4, 5). Furthermore, autoantibodies against laminin 332 have been described in individual patients with bullous pemphigoid, anti-p200 pemphigoid, and epidermolysis bullosa acquisita in addition to the disease-typical

autoantibodies against, i.e. BP180/type XVII collagen, p200 protein, and type VII collagen, respectively (6–11). The present review focuses on anti-laminin 332 MMP summarizing clinical features, its pathophysiology, and detection of serum anti-laminin 332 IgG. In addition, the current data about the association between anti-laminin 332 MMP and malignancies are highlighted.

The current review is dedicated to the late Detlef Zillikens, director and chair of the Department of Dermatology, University of Lübeck, Germany. Detlef Zillikens has been one of the leading experts on autoimmune blistering diseases. With an enormous workload and his friendly, optimistic, supportive, and caring nature he has established in Lübeck one of the world largest research hubs for these disorders. As one of his first students in 1993, close collaborator, mentee, and friend, E.S. owes him the greatest thanks for constant support, motivation, and fruitful discussions. S.P. got to know Detlef Zillikens in 2016 when starting her PhD thesis and owes him the greatest respect and thanks for his support of a young scientist and incessantly enjoyment of research. He was able to close the gap between science and clinic due to his dedication for both disciplines and his view of the entire picture. Both authors will strive to continue Detlef's work and guard the best memories of him.

Laminin 332

Laminins are cross- or T-shaped heterotrimers of an α, β and γ chain with three short arms (single chains) and one long arm formed by all three chains (12). Laminins are integral proteins of the BMZ of the skin and surface-close mucosal tissues. Here, they are essential components of the anchoring filaments connecting the hemidesmosome with type VII collagen (13). Their physiological functions include adhesion of the epidermis to the dermis and epithelium to the lamina propria, respectively, cell migration and, cell signaling (12).

Laminin 332, previously termed laminin 5, epiligrin, nicein, and kalinin is composed of the α3, β3 and γ2 chains and expressed in the BMZ of e.g. oral mucosa, conjunctiva, skin, kidney, lung, and small intestine (1). In the skin, laminin 332 is synthesized by keratinocytes as a 460 kDa precursor protein that is extracellularly cleaved by proteases. As such, the α3 chain (190-200 kDa) is processed into a 165 kDa fragment, the 155 kDa γ2 chain in a 105 kDa fragment, while the 140 kDa β3 chain remains uncleaved (13). Laminin 332 interacts with BP180 (type XVII collagen), the NC-1 domain of type VII collagen (14, 15), with α3β1, α6β4, and α6β1 integrin as well as with syndecan-1 and syndecan-4 (16, 17).

Mucous membrane pemphigoid

MMP is a clinically and immunopathologically heterogeneous disease defined as pemphigoid disorder with prevailing involvement of orifice-close mucosal tissues (18). As a pemphigoid disorder, MMP is characterized by autoantibodies that bind to the BMZ of the skin and/or mucosa (19, 20). Clinical heterogenicity is reflected by the involvement of different mucosal sites, most frequently the mouth (in about three quarters of patients) and conjunctivae (in about 50-65% of patients) followed by nasopharynx and genitalia, and more rarely, larynx, esophagus, and trachea. In about a quarter of patients, in addition to mucosal manifestations, skin lesions are present (Figure 1) (21, 22). The high disease burden of MMP is due to frequently painful oral and genital lesions, life-threatening complications such as airway obstruction and esophageal strictures, conjunctival disease leading to vision impairment and finally, blindness, and the association with a malignancy in about a quarter of patients with anti-laminin 332 reactivity (22).

Immunopathological heterogenicity stems from the different target antigens and the autoantibody isotype. While in most MMP patients, autoantibodies belong predominantly to the IgG isotype, the majority of patients also reveal IgA autoantibodies, and in some, the autoantibody response is restricted to IgA (22–24). BP180 (type XVII collagen) as main target antigen in MMP is recognized by about 70-80% of patients followed by laminin 332 in 10-20% of patients. In less than 5% of MMP patients, type VII collagen is recognized. Reactivity against BP230, that can be found in 10-30% of cases, is nearly always accompanied by autoantibodies against one of the three other target antigens (21, 24). In some MMP patients, autoantibodies against α6β4 integrin have been described (25–28). The relevance of these α6β4 integrin-specific antibodies in MMP is, however disputed (24, 29). Patients with mostly mucosal manifestation and predominant IgA reactivity, that previously may have been classified as linear IgA disease, and those with autoantibodies against type VII collagen previously diagnosed as epidermolysis bullosa acquisita, are now regarded within the spectrum of MMP (21).

Few data about the frequency of MMP are available. With an incidence between 1.3 and 2.0/million/year in France and Germany, respectively, and a prevalence of 24.6 patients/million in Germany, MMP is certainly a rare disease (30–33). MMP arises independently of ethnicity and geographical region, mainly affects individuals in the 7^{th} and 8^{th} decennium, and appears to be more frequent in females (22).

Diagnosis of MMP, like in all autoimmune blistering diseases, is grounded on three pillars; clinical manifestations, direct immunofluorescence (IF) microscopy, and serology (20, 24). The clinical prerequisite is predominant mucosal involvement. Direct IF reveals linear deposits of IgG, IgA, and or C3 at the cutaneous or mucosal BMZ in a non-lesional biopsy (Figure 2). Since the initial biopsy only provides a sensitivity of 50-70% depending on the biopsy site, current guidelines recommend to repeat the biopsy for direct IF at least once after an initially negative result (24, 29).

Detecting of circulating autoantibodies against the above-mentioned antigens is complex, mainly based on in-house assays, and reviewed elsewhere (22, 24). The detection of anti-laminin 332 IgG is detailed below.

Treatment of MMP is greatly hampered by the lack of randomized controlled studies. National and international guidelines propose treatment regimens (24, 34–37). The S3 European guidelines included a systematic literature review and recommend dapsone, methotrexate, tetracycline, and topical corticosteroids as first line treatment for mild and moderate MMP. For severe MMP, dapsone plus cyclophosphamide and/or

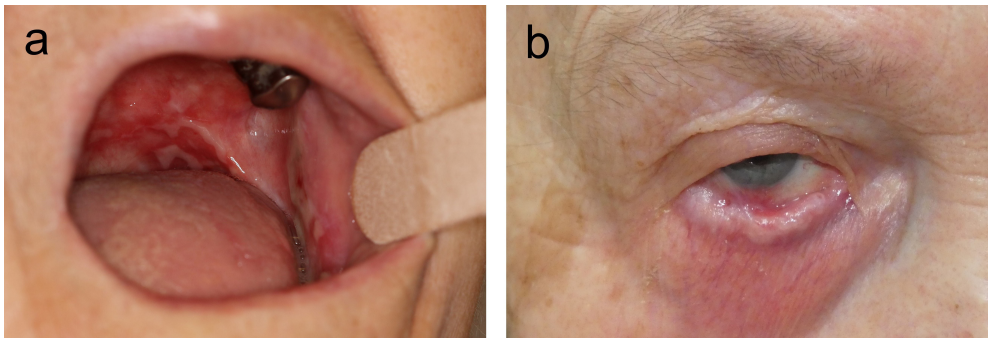

FIGURE 1
Clinical manifestations of mucous membrane pemphigoid. Extensive oral lesions in an 83-year-old female patient **(A)** and symblephara and shortening of the inferior fornix in a 72-year-old female **(B)**.

oral corticosteroids are suggested and, if not successful, dapsone plus rituximab followed by latter two drugs combined with high-dose intravenous immunoglobulin (24). A slightly different step-ladder approach was published in the recent German S2k guideline (29).

Anti-laminin 332 mucous membrane pemphigoid

In 1992, laminin 332 has been described as a target antigen in MMP by Kim Yancey and co-workers (4). Since then, numerous case reports and case series have reported IgG serum autoantibodies against this protein. It was only in 2019, when a highly standardized and specific assay for serum anti-laminin 332 IgG became widely available (38).

Clinical appearance of anti-laminin 332 mucous membrane pemphigoid

A patient with anti-laminin 332 MMP can clinically not be differentiated from a MMP patient with autoimmunity against BP180 or type VII collagen. In a systematic review of published cases and cohorts, Amber et al. reported significantly more pharyngo-laryngeal and oro-pharyngo-laryngeal involvement in MMP patients with reactivity against laminin 332 (39). In the so far largest study with 133 anti-laminin 332 MMP patients from Kurume, Japan, the oral cavity was the by far most frequently affected mucosal site (in 89% of patients) followed by conjunctivae (in 43%), pharynx (in 19%), larynx (15%), genital mucosa (in 11%), nasal mucosa (in 6%), and esophagus (in 3%) (40). Compared with MMP patients independent of the target antigen as recently reported in 154 MMP patient and as reviewed by Du et al., nasal lesions appear to occur less frequently in anti-laminin 332 MMP compared to 20-40% in all MMP patients, while oral lesions may be slightly more prominent (in 80-85% of all patients) (22, 41). These differences have, however, not been systematically evaluated and may also be related to the different ethnicity or other so far unrecognized factors.

Recently, a significant association of laminin 332-reactive MMP with male sex was reported (41). The most striking and clinically relevant feature that differentiates anti-laminin 332 MMP from MMP with other autoantibody reactivities, the association with malignancies in about a quarter of patients, is detailed below.

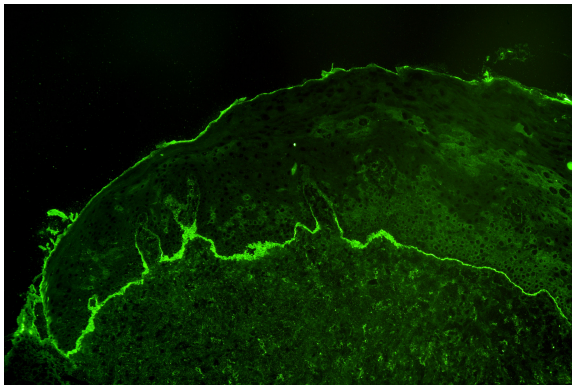

FIGURE 2
Linear deposits of complement C3 at the basement membrane zone by direct immunofluorescence microscopy of a perilesional biopsy in a patient with mucous membrane pemphigoid.

Detection of anti-laminin 332 reactivity

Several methods have been applied to detect anti-laminin 332 reactivity in skin and mucosal biopsies as well as in serum. Direct and indirect immunogold electron microscopy show deposits of immunoreactants at the lamina lucida/lamina densa interface of the BMZ in anti-laminin 332 MMP. In patients with autoantibodies against BP180 or type VII collagen, immunoreactants label the lamina lucida or the subbasal lamina-anchoring fibril zone, respectively (4, 42–45). Direct immunogold electron microscopy requires, however, fresh biopsy material that needs to be processed within hours and is only performed in few centers worldwide (46).

For the detection of serum autoantibodies against laminin 332, indirect immunogold electron microscopy is unpractical and as such, several in-house assays have been described including (i) immunoprecipitation of radiolabeled keratinocytes that was also

applied in the original report of anti-laminin 332 IgG in MMP (4, 47), (ii) immunoblotting with various substrates such as (a) conditioned media of cultured SCC-25 cells (48), (b) cultured primary human keratinocytes (47, 49), (c) cultured HaCaT keratinocytes (50), (d) cultured A-431 human epidermoid carcinoma cells (50), (e) extracts of human epidermal sheets (50), (f) extracellular matrix of cultured human keratinocytes (45, 50), (g) extract of human placental amnion (51), (h) recombinant fragments of the α3 chain (52), (i) human laminin 332 purified from cultured human keratinocytes (53), (j) primary human oral mucosal keratinocytes (54), and (k) immortalized human oral mucosal keratinocytes (54), and (iii) ELISA. When immunoprecipitation was compared to immunoblotting with five different substrates, i.e. (b-f), immunoprecipitation was identified as the most sensitive method followed by Western blotting with extracellular matrix of cultured human keratinocytes (II f) (50).

For ELISA, purified laminin 332 from conditioned medium of cultured SCC-25 cells (47, 55, 56), recombinant laminin 332 (57), laminin 332 purified from supernatant of cultured primary human keratinocytes (57), or extracellular matrix of cultured HaCaT keratinocytes were used (57). In particular the ELISA employing purified laminin 332 from conditioned medium of cultured SCC-25 cells has subsequently revealed conflicting results. Bekou et al. reported anti-laminin 332 IgG in 40% of bullous pemphigoid sera, although anti-laminin 332 reactivity is not present in latter patients (38, 55, 58, 59). Bernard et al. described serum anti-laminin 332 IgG in 31 of 154 MMP patients; when 19 of the 31 laminin 332-reactive sera were retested, anti-laminin 332 reactivity was only confirmed in 4 of the 19 sera (60).

In sera with reactivity against the cutaneous BMZ by indirect IF microscopy on human skin, indirect IF on laminin 332-deficient skin from patients with junctional epidermolysis bullosa (being unreactive on latter substrate) as well as the fluorescence overlay antigen mapping on human salt-split skin are elegant methods to determine autoantibodies against laminin 332 (61). Another test based on indirect IF, the so-called footprint assay, demonstrated that anti-laminin 332 serum IgG can be detected in the extracellular matrix of cultured primary keratinocytes after removal of the cells from the glass coverslips. Here, the extracellular matrix of the removed individual keratinocytes appear as traces or "footprints" that can be visualized by anti-laminin 332 antibodies followed by FITC labelling (59).

A breakthrough was achieved by Goletz et al. who described an indirect IF test based on the HEK293 cells that recombinantly express the laminin 332 trimer on their cell surface (Figure 3). As negative control, HEK293 cells transfected with an empty vector are used. These cells are applied using the BIOCHIP® mosaic technology, i.e. several substrates are placed together in a single incubation field of a laboratory slide (62–65). When in an international multicenter study, 93 anti-laminin 332 MMP patient sera and 315 sera from other autoimmune blistering diseases including 153 sera from anti-laminin 332 negative MMP patients, non-inflammatory dermatoses, and heathy blood donors were probed, a sensitivity of 84% and a specificity of 99.6% were observed (38). This assay has subsequently been validated by other groups (66, 67). When the BIOCHIP® technology-based assay has recently been compared with the footprint assay using 54 anti-laminin 332 MMP sera and together 50 sera from patients with pemphigus vulgaris and healthy blood donors, both assays revealed a specificity of 100% with a slightly higher sensitivity of the footprint assay (100% versus 96.3%) (60). When 35 sera of originally laminin 332-unreactive sera were subjected to both IF tests, 3 were reactive in the BIOCHIP® assay and 7 in the footprint assay. These data show that the footprint test may be more sensitive, whereas the advantage of the BIOCHIP® assay is its high standardization and wide availability (60).

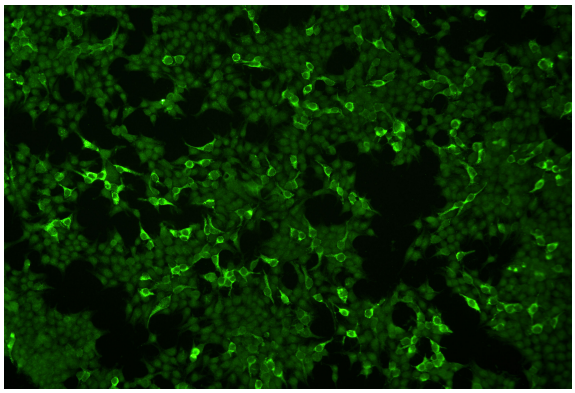

FIGURE 3
Indirect immunofluorescence microscopy of HEK293 cells that recombinantly express laminin 332 on the cells surface employing the Biochip™ technology. A serum of a patient with mucous membrane pemphigoid labels laminin 332-expressing cells. Non-transfected cells serve as internal negative control.

Reactivity against the different laminin chains varied considerably between studies. In 113 Japanese patients with anti-laminin 332 MMP, the γ2 chain was most frequently recognized (in 58% of patients) followed by α3 and β3 targeted in 49% and 36% of patients, respectively (40). In contrast, Goletz et al., using the BIOCHIP® technology-based IF assay in an international multicenter study with 93 sera, reported IgG4 reactivities against the α3, β3, and γ2 in 43%, 41%, and 13% of patients (38). These discrepancies maybe most likely due to the different study populations or detection methods.

In individual MMP patients, IgA and IgE antibodies against laminin 332 have also been reported (68, 69).

Since anti-laminin 332 MMP is associated with a malignancy in about a quarter of patients as detailed below, national and international guidelines recommend the detection of anti-laminin 332 serum IgG in all patients that show dermal binding by indirect IF on human salt-split skin or were unreactive in this assay (24, 29). A suggested diagnostic pathway for anti-laminin 332 MMP is depicted in Figure 4.

Association of anti-laminin 332 mucous membrane pemphigoid with malignancies

Gibson et al., have observed the first patient with anti-laminin 332 MMP and a malignancy, a lung carcinoma, in 1997 followed by Leverkus et al., who reported solid malignancies in 2 of 5 MMP

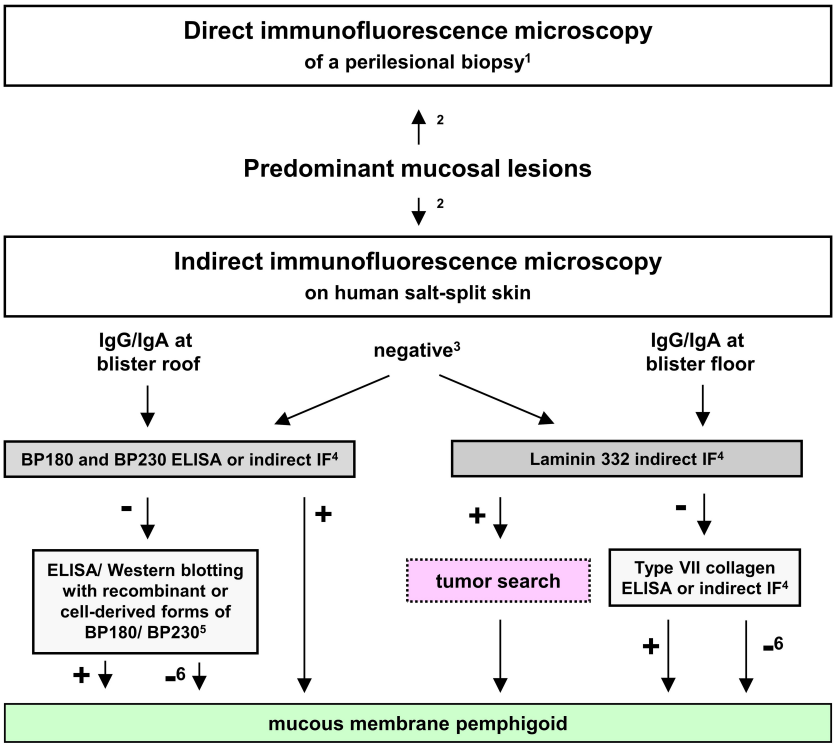

FIGURE 4
Proposed diagnostic algorithm for mucous membrane pemphigoid (MMP). Adopted from (22, 24, 70). [1]in the oral cavity, a non-lesional biopsy is equally sensitive compared to a perilesional; [2]recommended to be performed in parallel; [3]in 30-50% of MMP sera; [4]commercially available (for IgG); [5]only available in specialized laboratories as in-house assays; [6]only with positive direct and/or indirect IF microscopy.

patients with serum reactivity against laminin 332 (71, 72). The association was first noted by Egan et al. who described malignancies in 10 of 35 (29%) anti-laminin 332 MMP patients (73). When all nine subsequent studies with more than three anti-laminin 332 MMP patients were evaluated, a clear association with malignancies was evident. In fact, 57 of 253 (23%) patients with anti-laminin 332 reactivity had a malignancy (Table 1). These data align well with a recent review in which Shi et al., retrieved 344 reported cases of anti-laminin 332 MMP from the literature, of whom in 75 (22%), a malignancy was described. Van Beek et al.

TABLE 1 Association of anti-laminin 332 MMP with malignancies[1] .

Study	Year of publication	Origin of patients	No. of patients	No. of patients with malignancy
Leverkus et al. (72)	1999	Germany	5	2 (40%)
Egan et al. (73)	2001	USA	15	5 (29%)
Matsushima et al. (74)[2]	2004	Japan	16	5 (31%)
Terra et al. (47)	2011	Netherlands	10	2 (20%)
Bernard et al. (56) Goletz et al., (60)	2013	France	[31] 4	[2 (6%)][3] 2 (50%)[3]
Hayakawa et al., (75)	2014	Japan	4	2 (50%)
Goletz et al. (38)	2019	Germany, Japan, France, Italy, USA	53[4]	13 (25%)
Li et al., (76)	2021	Japan	[55][4,5]	[8 (14%)][5]
Qian et al. (40)	2021	Japan	133[4]	22 (17%)
van Beek et al., (41)	2021	Germany	13[4]	4 (31%)
Total			**253**	**57 (23%)**

[1]only studies with more than 3 patients are indicated; [2]review of Japanese cases; [3]when 17 of the 31 reported sera were re-analyzed by the Biochip®-based indirect IF assay only 4 reacted with laminin 332. Of these 4 sera, 2 had a malignancy (60). As such, here, only latter data were included; [4] some patients may have also been included in other studies listed here; 5 data of this study were not included in the total numbers since all patients also appeared in the study of the same group by Qian et al. (40). Total numbers are shown in bold.

calculated the risk for malignant neoplasms in anti-laminin 332 MMP to be 6.8-fold higher compared to the general population (41).

In the recent review by Shi et al., the most frequent tumor in 84 malignancy-associated anti-laminin 332 MMP patients retrieved from the literature, were lung carcinomas (in 23% of patients) followed by gastric (in 17%), uterine (in 13%), pancreatic (8%), colon (8%), ovary (7%), prostate (5%), and thyroid carcinoma (5%) (77). No relation between the recognized laminin chain and the tumor entity was found (77). Of the 12 malignancy-associated anti-laminin 332 MMP patients reported by Goletz et al., 3 (25%) had a lung and 2 (17%) a uterine/cervix carcinoma compatible with the data reported by Shi et al., while 2 (17%) revealed a urothel carcinoma and none has a gastric malignancy (38, 77). These data suggest that in anti-laminin 332 MMP, solid malignancies predominate with lung and uterine/cervix cancers being among the most prevalent entities, while the distribution of other solid malignancies may also depend on the population.

Interestingly, in patients with serum reactivity against α6β4 integrin, no higher rate of malignancies was found alike in MMP patients in general irrespective of the target antigen (78–80).

The exact reason for the association of ani-laminin 332 reactivity and solid cancers has not been fully elucidated yet. It is well known that laminin 332 is relevant for tumor proliferation and migration (81–83). Some solid tumors may produce excessive amounts of laminin 332 and an imbalance of extracellular matrix proteins including laminin 332 was shown to promote tumor cell migration via the Pi3-akt pathway as well as the differentiation of tumor-associated fibroblasts and tumor angiogenesis (84–86). As such, it may be hypothesized that an imbalance in laminin 332 expression during carcinogenesis induces an autoimmune response that leads to laminin 332-specific autoimmunity including anti-laminin 332 antibodies (87–89). This view is supported by the observation that MMP can regress after excision of the tumor (87, 90, 91).

Pathophysiology of anti-laminin 332 pemphigoid

Preliminary evidence for the pathogenic relevance of anti-laminin 332 IgG stems from the intraindividual correlation of anti-laminin 332 IgG serum levels with disease activity (38). Apart from *in-vitro* organ culture models of MMP employing normal human conjunctiva (25, 92–94), two mouse models of anti-laminin 332 MMP have been developed. One model reflects the inflammatory-poor variant of MMP and lesions develop independently of complement activation and the infiltration of inflammatory cells in the tissues, while the other model shows, oral, conjunctival, and skin lesions with inflammatory infiltrates and requires the involvement of the Fcγ-receptor and activation of C5aR1 (95–97). In latter model, dapsone has recently been shown to be effective supporting the notion that this model recapitulates important features of the human disease (98). Because most recent publications used the latter mouse model, a detailed description is depicted in Figure 5. In line with previous findings, methylprednisolone as another first-line therapy for MMP, was also able to reduce the severity of skin, although not oral lesions in this mouse model. In this study, Ghorbanalipoor et al. also showed that parsaclisib, a selective inhibitor of phosphoinositide 3-kinase delta (PI3Kδ) significantly reduced skin and oral mucosal lesions (99, 100). With regard to the characteristic symptom of scarring, typically occurring at the eyes of anti-laminin 332 MMP patients, this mouse model may also be suitable to unravel signaling pathways that contributes to this specific immunopathogenesis. Biopsies of the palpebral conjunctiva and the skin collected 28 days after the initiation of this model revealed highly condensed collagen fibrils in picro-sirius red staining and trichrome histological staining. In addition, biochemical analysis provided results on altered collagen-cross-linking signaling pathways in these tissues that are associated with fibrosis (101). Furthermore, the previously published upregulation of aldehyde dehydrogenase (ALDH1) in conjunctiva and in fibroblasts isolated from MMP patients with severe eye involvement, could be verified by transcriptome analysis of perilesional skin from this model (102). The inhibition of ALDH1 by disulfiram decreased disease severity in a mouse model for allergic eye disease (102). However, disulfiram was not effective in the anti-laminin 332 mouse model. Here, the same dosage and application of disulfiram was not able to reduce the severity of the conjunctival lesions (101).

In-vitro models specific for anti-laminin 332 pemphigoid are rare. Recently Bao et al. published results about anti-laminin 332 MMP patient antibodies that were sufficient to release inflammatory mediators upon binding to keratinocytes without the presence of inflammatory cells and as such without the usage of Fc-receptors. Thus, arising the question whether blistering may be a consequence of just the binding of the anti-laminin 332 IgG and whether the complement system has a nonobligatory role in the initiation of the inflammatory response (103, 104).

Anti-laminin 332 reactivity in other pemphigoid diseases

Outside MMP, antibodies against laminin 332 have been detected in individual patients with bullous pemphigoid, anti-p200 pemphigoid, and epidermolysis bullosa acquisita in addition to the autoantibodies against BP180, p200 protein, and type VII collagen, respectively (6–11). The report of anti-laminin 332 reactivity in about 40% of bullous pemphigoid sera was not confirmed in subsequent studies (38, 55, 58, 59). When Holtsche et al. investigated the specificities of serum autoantibodies in anti-p200 pemphigoid, anti-laminin 332 IgG was observed in 43 (18%) of 239 patients in addition to reactivity against the p200 protein and/or laminin γ1 (10).

Autoantibody reactivity in the very rare entity orf-induced pemphigoid has puzzled investigators for many years. Recently, Yilmaz et al, showed that the major target antigen in orf-induced pemphigoid is laminin 332 (5). Of note, while a single patient with orf-induced MMP has been described, all other cases associated with orf did not show predominant mucosal involvement and, consequently may be termed orf-induced pemphigoid when

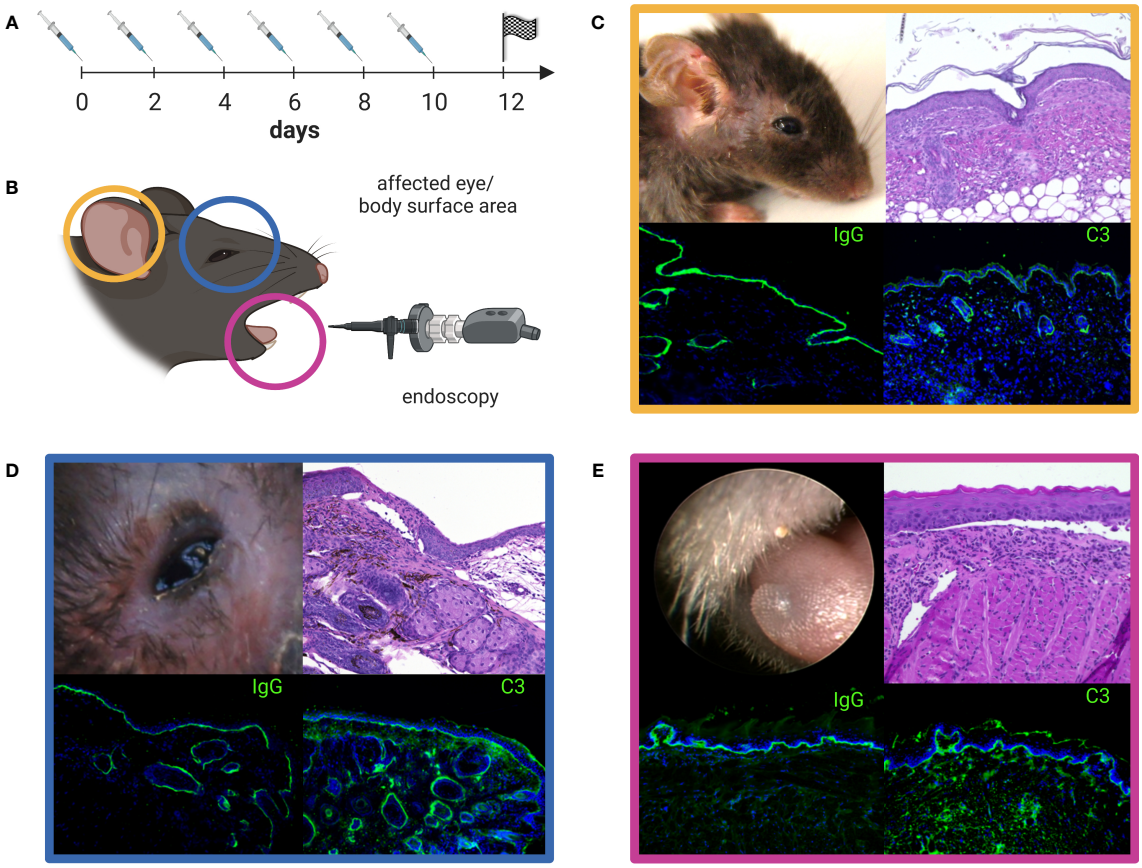

FIGURE 5
Anti-laminin α3 mucous membrane pemphigoid (MMP) mouse model. Rabbit anti-murine laminin α3 IgG is injected subcutaneously (s.c.) into adult C57Bl/6 mice every other day over a time period of 10 days **(A)**. The clinical manifestation of the mouse model seen on experimental day 12 can be quantified by the use of a validated scoring system comprising the affected body surface area (yellow, **C**), the affected eye-area (blue, **D**), and the severity of oral lesions as examined by endoscopy (pink, **E**) **(B)**. The color-framed boxes **(C-E)** show the clinical presentation (upper left panel), H&E stained lesional histopathology with an inflammatory infiltrate and split formation of the dermal-epidermal/epithelial junction (upper right panel) and, linear deposits of IgG (lower left panel) and C3 (lower right panel) along the basal membrane zone by direct immunofluorescence microscopy. Lesions, crusts and erosions of the skin are mostly restricted to the head, neck, and upper back of mice **(C)**. The image was created with BioRender.com.

antibodies against laminin 332 are detected or orf-induced epidermolysis bullosa acquisita in case of type VII collagen-specific antibodies (5, 105, 106). The reason why autoimmunity against laminin 332 is not associated with predominant mucosal manifestations when induced by an orf infection is enigmatic. It may be speculated that an underlying molecular mimicry between an orf virus protein and laminin 332 leads to autoantibodies against distinct epitopes on laminin 332 different from those targeted in anti-laminin 332 MMP. Of note, autoantibodies in orf-induced pemphigoid are predominantly of the IgG2 and IgG3 subclasses compared to IgG4 in anti-laminin 332 MMP (38, 107).

Conclusion

After diagnosis of MMP, testing for serum antibodies against laminin 332 and, when present, a search for the most prevalent solid tumors including chest, abdominal, and pelvic CT, gastroscopy, coloscopy, as well as urological and gynecological examinations appears to be mandatory. The anti-laminin α3 mouse model of MMP may be helpful to decipher key molecules and pathways in the pathophysiology of MMP. Only after definite preclinical data have been generated a randomized controlled treatment study will be initiated and open new therapeutic avenues for patients with this rare and frequently detrimental disorder.

Author contributions

Supervision: ES. Visualization: ES, SP. Writing - Review and Editing: ES, SP. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by structural funding from the Schleswig-Holstein Excellence Cluster *Precision Medicine in Chronic Inflammation* (DFG EXC 2167/1) and the CRC 1526 *Pathomechanisms of Antibody-mediated Autoimmunity: Insights from Pemphigoid Diseases (A01)*.

Conflict of interest

ES has research grants with Admirx, ArgenX, AstraZeneca, Biotest, Dompe, Euroimmun, CSL, Alpine Immune, and Fresenius Medical Care and in the last three years, received consulting fees and/or honoraria from Almirall, ArgenX, AstraZeneca, Janssen, Bristol-Myers Squibb, Chugai, Leo, and Sanofi. SP and ES have a patent application with Dompe.

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 01 September 2023
DOI 10.3389/fimmu.2023.1196116

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OPEN ACCESS

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RECEIVED 29 March 2023
ACCEPTED 15 August 2023
PUBLISHED 01 September 2023

CITATION
Yang K, Yin J, Yue X, Bieber K, Riemekasten G, Ludwig RJ, Petersen F and Yu X (2023) Luteolin peracetate and gossypolone inhibit immune complex-mediated neutrophil activation *in vitro* and dermal-epidermal separation in an *ex vivo* model of epidermolysis bullosa acquisita. *Front. Immunol.* 14:1196116. doi: 10.3389/fimmu.2023.1196116

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Luteolin peracetate and gossypolone inhibit immune complex-mediated neutrophil activation *in vitro* and dermal-epidermal separation in an *ex vivo* model of epidermolysis bullosa acquisita

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Introduction: Natural products have been shown to an important source of therapeutics for human disease. In this study, we aimed to identify natural compounds as potential therapeutics for epidermolysis bullosa acquisita (EBA), an autoimmune disease caused by autoantibodies to type VII collagen (COL7).

Methods: Utilizing an *in vitro* experimental system, we screened a natural product library composed of 800 pure compounds for their inhibitory effect on COL7-anti-COL7 IgG immune complex (IC)-mediated neutrophil activation and on neutrophil-mediated tissue damage.

Results: Three natural compounds, namely luteolin peracetate, gossypol, and gossypolone were capable in inhibiting the IC-induced neutrophil adhesion and oxygen burst *in vitro*. Furthermore, luteolin peracetate and gossypolone were able to inhibit the anti-COL7 IgG induced dermal-epidermal separation in an *ex vivo* model for EBA.

Discussion: In summary, this study demonstrates that luteolin peracetate and gossypolone are potential therapeutics for experimental EBA, which deserves further investigation.

KEYWORDS

epidermolysis bullosa acquisita, neutrophils, natural products, luteolin peracetate, gossypolone

Introduction

Since more than two decades, Detlef Zillikens (†2022) and his group has worked on the fundamental pathological principles of Epidermolysis bullosa acquisita (EBA), a rare autoimmune skin blistering disease caused by autoantibodies targeting type VII collagen (COL7) (1, 2). It is characterized by subepithelial blistering of the skin at the dermal-epidermal junction (DEJ), a key structure to keep the integrity of the skin (3, 4). Anchoring fibrils, a main component of DEJ, is composed of COL7 and plays a crucial role in tethering the basal lamina to the underlying dermis function of DEJ (4). Once the circulating autoantibodies to COL7 migrate into the skin, the binding of those autoantibodies to the autoantigen at the DEJ is capable to mediate disease manifestations *via* inflammation dependent and independent pathways. In inflammatory EBA, binding of autoantibodies to COL7 leads to the formation of immune complexes (IC) which activate the complement system and mediate subsequent inflammatory cell infiltration leading to tissue destruction (5, 6). By contrast, non-inflammatory EBA is characterized by the lack of an autoantibody-mediated inflammatory responses and the development of skin symptoms appears to depend on non-inflammatory mechanisms (7, 8).

Accumulating evidence by Zillikens and others suggests that neutrophils play an essential role in the development of EBA and its experimental models (9–11). As a complicated process, neutrophil-mediated tissue damage consists of several essential steps, including recruitment of neutrophils into the skin and their binding to IC leading to cell activation and subsequent tissue damage (5, 6, 10, 11). It has been shown that oxygen radical formation and neutrophil degranulation are essential steps involved in the anti-COL7 IgG mediated tissue damage in experimental EBA (1, 11, 12). Previously, our group has demonstrated that anti-CD18 blocking antibodies could prevent the dermal-epidermal separation induced by anti-COL7 IgG, without affecting production of reactive oxygen species (ROS) or neutrophil elastase release (13). These findings clearly demonstrate that IC-induced neutrophil adhesion plays an indispensable role in tissue damage in experimental EBA, providing a potential novel target for the development of new therapies for the disease.

Natural products are defined as chemicals that are biosynthesized as secondary metabolites in various organism, including plants, animals, fungus as well as organisms from the marine environment (14, 15). Since the earliest record of natural products for treating diseases in the Ebers Papyrus (2900 B.E.C.), natural products have been proven to be the most prosperous and significant resource for the new medicines (16). Therefore, here we hypothesized that the tissue damage in experimental EBA can be attenuated by natural compounds which are capable of inhibiting IC-mediated neutrophils adhesion.

Materials and methods

Materials

The Natural product library was obtained from TimTec, Germany (NPL-800). The library contains 800 pure natural compounds, mainly of plant origin but also from animal, bacterial, and fungal sources. Rabbit anti-COL7 IgG was generated and purified as described previously (17). Immobilized immune complexes (IC) were formed from the binding of rabbit anti-mouse COL7 IgG to recombinant mouse COL7 pre-coated on the cell culture plate.

Preparation of neutrophils and ethic approval

Neutrophils were isolated from peripheral blood of healthy donors by sedimentation and density gradient centrifugation as previously described (18). The purity and viability of neutrophils was determined by the morphological analysis of Jenner-Giemsa stained cytospins and Trypan Blue exclusion method, respectively (19). For all *in vitro* and *ex vivo* assays, neutrophils were cultured in CL medium (RPMI 1640 buffered with 25 mM HEPES without phenol red; Biochrom, Berlin, Germany) supplemented with 1% L-glutamine. This study was performed in accordance with the 1964 Helsinki Declaration, and the approval was obtained from the institutional ethics committee of the University of Lübeck (Number: Az 12-202A, from April 25, 2019). All donors agreed by written informed consent.

IC-induced neutrophil adhesion

IC-induced neutrophil adhesion was measured by an electronic impedance-based assay using the xCELLigence® Real Time Cell Analyzer (RTCA) which allows a real-time monitoring of the adhesion properties of cells *in vitro* in a non-invasive label-free manner (20, 21). Immobilized IC was prepared on wells of E-plate and freshly prepared neutrophils were added to wells in presence or absence of natural compounds. During the measurement, the electronic readout of cell sensor impedance was determined in real-time converted into cell index (CI). Neutrophil adhesion was quantified and expressed as the relative area under the curve (AUC) of cell index where the values of AUC of positive control (neutrophils stimulated with IC in absence of a compound) and negative control (unstimulated neutrophil) were set as 100% and 0%, respectively.

Cell viability test

The cytotoxicity of natural compounds was determined by utilizing the Alamar Blue assay according to the protocol provided by the manufacture. Briefly, freshly isolated neutrophils at a final concentration of $1x10^6$/ml with a total volume of 150 µl in CL-medium supplemented with 1% L-glutamine were stimulated with immobilized IC in presence or absence of natural compounds on 96-well cell culture plates. Subsequently, 15 µl Alamar Blue reagent was added to each well, and spectrophotometric absorbance was determined every 60 mins for 7 hours. Cell viability was expressed as a relative value in which the value of positive control (unstimulated neutrophils) was set as 100%.

IC-induced ROS release from neutrophils

To determinate IC-induced neutrophil ROS production, immobilized COL7-anti-COL7 IgG IC was prepared on solid opaque 96-well plates. Freshly isolated neutrophils were suspended in 2×10^6/ml in CL medium supplemented with 1% L-glutamine and 5 µM luminol (5-Amino-2, 3-dihydro-1, 4-phthalazinedione; Roche Applied Science) and distributed in 200 µl aliquots into well microtiter plate coated with IC. After addition of neutrophils onto plates with immobilized immune complex, chemiluminescence was recorded by microplate luminometer (Berthhold Technologies GmbH, Germany) for 60 min in a real-time manner and data were expressed as relative light units (RLU).

Ex vivo model of EBA

The *ex vivo* model of EBA was performed as previously described (17), with slight modification. Briefly, cryosections of mouse skin biopsies were incubated with rabbit anti-murine COL7 IgG, and further incubated with neutrophils in CL-medium supplemented with 1% L-glutamine to induce dermal-epidermal separation. Sections were washed in PBS, fixed with acetone acid, and subsequently stained with hematoxylin and eosin. Skin dermal-epidermal separation was evaluated by light microscopy, and the extent of dermal-epidermal separation was analyzed in a blinded fashion by two persons. This study was approved by the Animal Research Ethics Board of the Ministry of Environment, Kiel, Germany.

Statistical analysis

All data are expressed as the mean ± SD (standard deviation) or mean ± SEM (standard error of mean). Quantitative data was firstly examined with Kolmogorov-Smirnov normality test. For the normally distributed data, statistical difference was determined by two-tailed paired Student's t test, otherwise Wilcoxon signed rank test was used. For the data with two or more independent variables, statistical analysis was performed with two-way ANOVA test. For the evaluation of dose response relationship, linear regression analysis was performed, and the F-test was applied to assess the statistical significance. P values less than 0.05 were considered as statistically significant.

Results

Blocking of IC-induced neutrophil adhesion

To identify potential candidate compounds that are able to inhibit IC-induced neutrophil adhesion, the Natural Products Library which contains 800 compounds was screened. Among the 800 tested natural products, 11 showed relative AUC values of lower than 50% (Figure 1). These 11 natural compounds were luteolin peracetate, luteolin, capsaicin, gossypol, gossypolone, digitonin, plumbagin, estradiol valerate, 2-Chloroadenosine, 3,3-Diindolylmethane and peracetate rutine. In addition, a few molecules showed relative AUC values of higher than 250%, including digoxigenin, sinomenine and emetine dihydrochloride hydrate.

After the primary screening, the 11 natural products which showed an inhibitory effect on IC-mediated neutrophil adhesion were selected for further evaluation. In this part, dose-dependency on the inhibition of neutrophil adhesion by the selected 11 natural products was determined in a range between 0.1 µM and 10 µM of the respective compound. The dose-response assay was performed in four independent experiments by using the xCELLigence RTCA system with neutrophils isolated from 4 healthy donors, respectively. Among the 11 tested natural products, 5 products, namely luteolin peracetate, plumbagin, digitonin, gossypol, and gossypolone showed significant dose-response relationships (Figure 2).

Cytotoxicity of compounds

To exclude that inhibition on neutrophil adhesion is a consequence of toxic effects, toxicity test was carried out for the five natural products. Toxicity was evaluated by determining the cell viability using the Alamar Blue cytotoxicity assay. Neutrophils were stimulated with immobilized IC in the presence or absence of

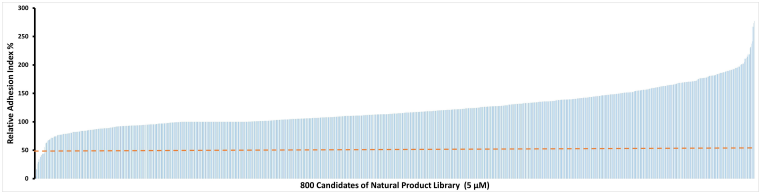

FIGURE 1
Effects of 800 natural products on IC-mediated neutrophil adhesion. Freshly isolated human neutrophils (2×10^6/ml) were stimulated with immobilized COL7-anti-COL7 IgG IC in the presence or absence of a natural product at a concentration of 5 µM. Neutrophil adhesion was determined by real-time impedance measurement for 90 minutes using xCELLigence RTCA and expressed as relative adhesion index where the value of AUC of positive control (neutrophils stimulated with IC in absence of the compound) and negative control (unstimulated neutrophils) were set as 100% and 0%, respectively. The X-axis stands for the 800 compounds of natural product library, and the Y-axis presents the mean relative adhesion index of two independent experiments. The dashed line represents 50% adhesion index of the positive control.

candidate natural products at a concentration of 1 μM or 10 μM, and Alamar Blue was added to cells 1 hour later to monitor the cell viability for 6 hours. At the concentration of 1 μM, plumbagin showed a strong and significant toxic effect on neutrophils, with a decrease of 50 percent in cell viability after 3h of incubation (Figure 3A). At the concentration of 10 μM, significant toxic effects were observed for both plumbagin and digitonin, with decreases of 60 percent in cell viability for both products after 3h of incubation (Figure 3B). In addition, Gossypolone at the concentration of 10 μM also showed a significant but mild toxic effect on neutrophils, with a decrease of roughly 10 percent in cell viability after 3h of incubation, but such effect was not observed at the concentration of 1 μM (Figure 3A). Therefore, luteolin peracetate, gossypol, and gossypolone were selected for further investigation.

Blocking of neutrophils ROS production

To figure out the effects of the 3 selected candidates on ROS production of neutrophil, freshly isolated neutrophils were stimulated with immobilized IC or control in presence or absence of the respective candidate compounds at a concentration of 10 μM. As shown in Figure 4A, time kinetics of ROS production showed that neutrophils immediately produced ROS when they attached to immobilized IC. The

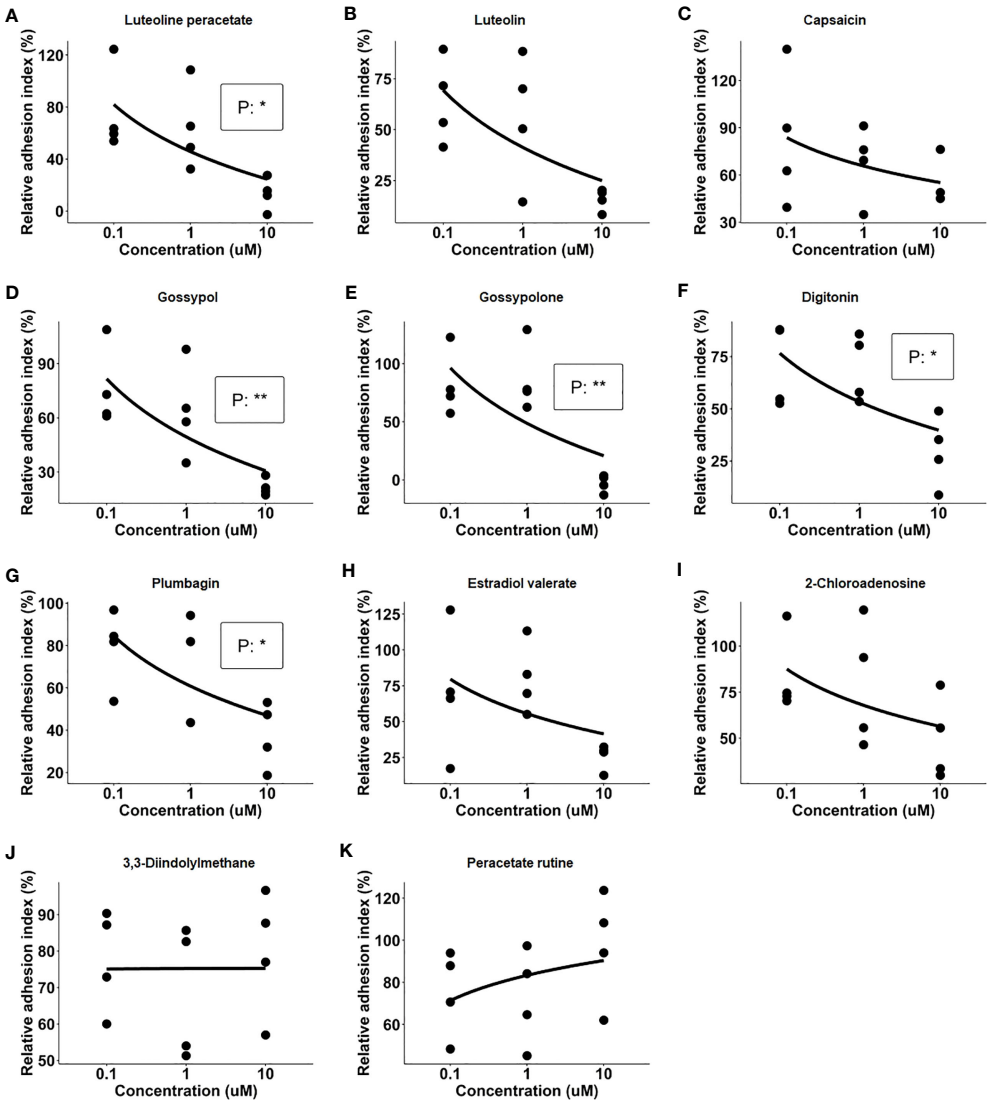

FIGURE 2
Dose-response relationships of 11 natural products. Freshly isolated neutrophils were stimulated with immobilized immune complex (IC) on wells of E-plate in presence of indicated concentrations of candidate compounds, including Luteolin peracetate **(A)**, Luteolin **(B)**, Capsaicin **(C)**, Gossypol **(D)**, Gossypolone **(E)**, Digitonin **(F)**, Plumbagin **(G)**, Estradiol valerate **(H)**, 2- Chloroadenosine **(I)**, 3,3-Diindolylmethane **(J)**, and Peracetate Rutine **(K)**. Cell indexes were recorded for 90 minutes, and AUC of Delta Cell Indexes was calculated. For further analysis, neutrophil adhesion was expressed as relative adhesion index where the value of AUC of positive control (neutrophils stimulated with IC in absence of the compound) and negative control (unstimulated neutrophils) were set as 100% and 0%, respectively. The X-axis presents doses of natural products, while Y-axis shows the relative adhesion indexes. Data from four independent experiments were used for analysis. Linear regression analysis was performed to evaluate the dose-response relationship, and the statistical significance was assessed using the F-test (*p<0.05, **p<0.01).

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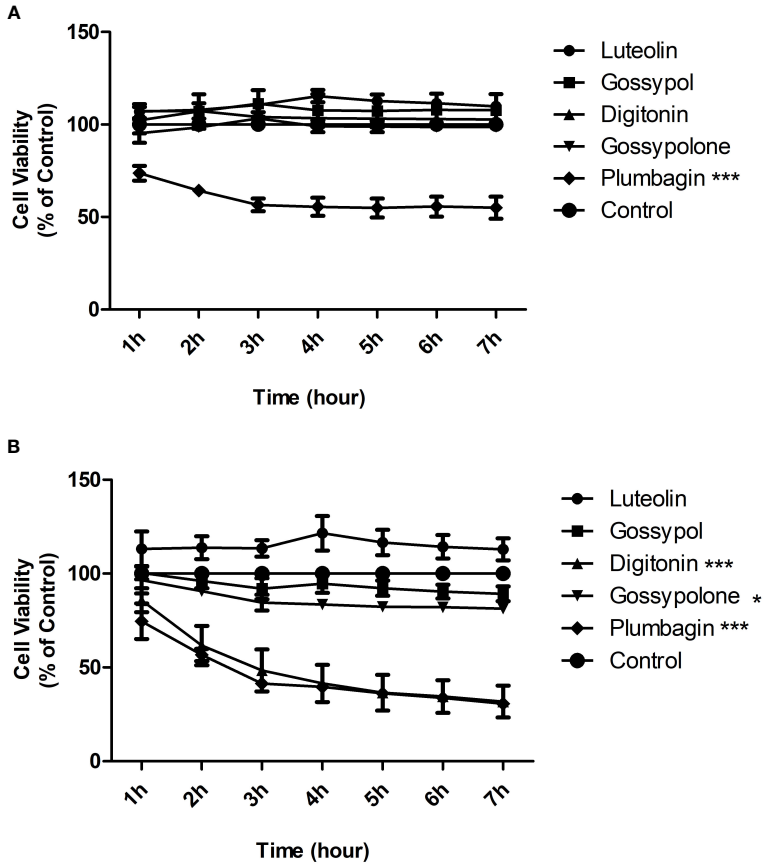

FIGURE 3
Cytotoxicity effects of 5 natural products on neutrophils. Freshly isolated human peripheral blood neutrophils were stimulated by immobilized IC in presence or absence (control) of indicated natural product at a concentration of 1 μM **(A)** or 10 μM **(B)**. Cell viability was assessed by using the Alamar Blue cytotoxicity assay. The Alamar Blue was added 1 hour after the cell incubation, and the absorbance was recorded every hour for 6 hours. Data are presented as mean ± SEM (standard error of the mean) of 4 independent experiments. Cell viability was expressed as a relative value where the value of control (unstimulated neutrophils) was set as 100%. The toxic effect of each natural product was evaluated by comparing the cell viability between product-treated neutrophils and controls using two-way ANOVA test (*p<0.05, ***p<0.001).

produced ROS reached a peak at approximately 10 minutes and returned to background level after 40 minutes. In contrast, neutrophils exposed to control surfaces did not show a significant ROS production. When neutrophils were exposed to immobilized IC in the presence of luteolin peracetate, gossypol or gossypolone, the IC-mediated ROS production was significantly decreased. Quantification of the ROS production in 6 experiments showed that all three natural products significantly inhibited the IC-induced ROS production from neutrophils (Figure 4B).

Inhibition of dermal-epidermal separation in an ex vivo model of EBA

Finally, we determined whether the three natural compounds are able to inhibit anti-COL7 IgG mediated tissue damage in an *ex vivo* model for EBA. In the experimental system, neutrophils activated by the COL7-anti-COL7 IC caused the dermal-epidermal separation of 33 ± 7.9%, while no separation was observed in the negative control in which anti-COL7 IgG was replaced by normal rabbit IgG (Figure 5). In the presence of 10 μM luteolin peracetate, the dermal-epidermal separation was significantly reduced to 4.9 ± 2.6% (P = 0.009). Significant and dramatic reduction in dermal-epidermal separation was also observed in the samples treated with 10 μM gossypolone (16.1 ± 8.4%, P=0.0091). In the presence of 10 μM gossypol, although the dermal-epidermal separation was also reduced to 12.5 ± 8.6%, this difference was not significant (P=0.0547). Taken together, these results suggest that both luteolin peracetate and gossypolone can inhibit tissue damage in the ex vivo model for EBA.

Discussion

In the present study, we screened a natural product library of 800 pure compounds for their inhibitory capability on anti-COL7 IgG mediated tissue damage. We demonstrated that two compounds, luteolin peracetate and gossypolone, showed a strong and significant inhibitory effect on anti-COL7 IgG mediated tissue damage in an ex vivo model for EBA. Two compounds, luteolin peracetate and gossypolone, showed a strong and significant inhibitory effect on IC-induced neutrophil tissue damage.

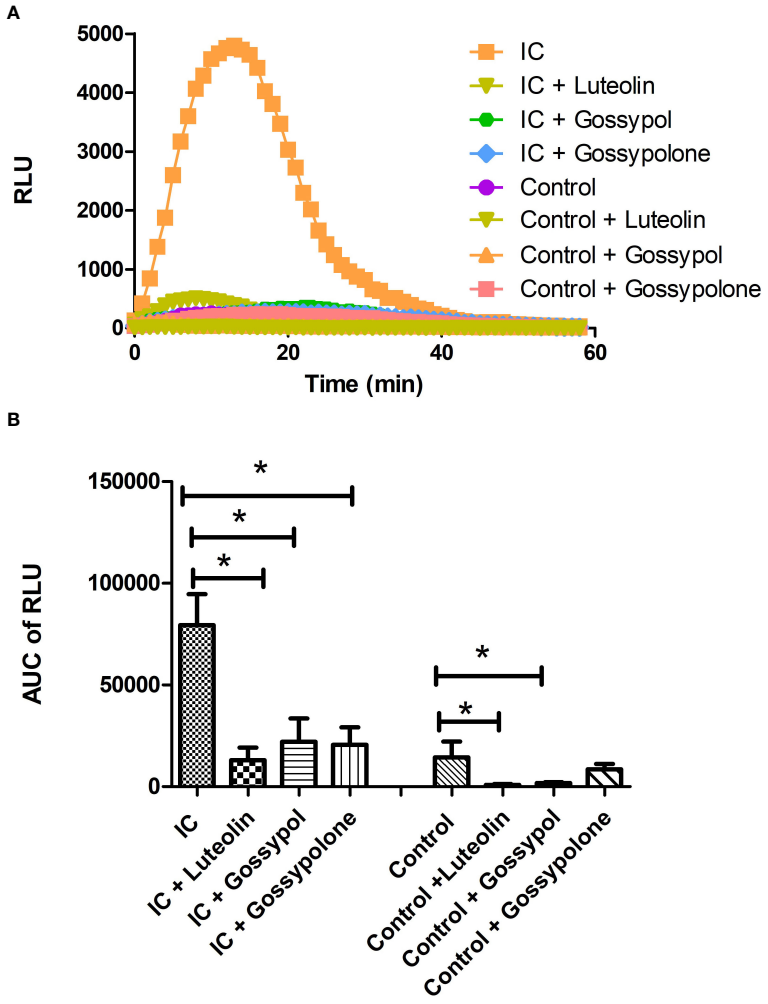

FIGURE 4
Effect of selected natural products on IC-induced ROS production of neutrophils. Freshly neutrophils were stimulated with immobilized IC or control in the presence or absence of a natural product at the concentration of 10 μM. Generation of ROS was determined by recording chemiluminescence using microplate luminometer for 60 min in a real-time manner and data were expressed as relative light units (RLU). **(A)** Representative kinetics of ROS production from neutrophils under indicated experimental conditions. **(B)** Kinetic data of IC-mediated ROS production from neutrophils were integrated and represented as the AUC (area under the curve) of RLU (relative light units). Data are presented as mean ± SD derived from 6 experiments, and statistical analysis was performed using Wilcoxon signed rank test (*p<0.05).

Importantly, both luteolin peracetate and gossypolone did not only inhibit the IC-mediated neutrophil adhesion, but also blocked IC-mediated ROS production of neutrophils, suggesting that both natural products represent inhibitors for neutrophil functions in general.

Luteolin peracetate is a flavonoid, with a typical structures with C6-C3-C6 (22). Although it is well established that flavonoids are featured by pharmacological properties of anti-inflammatory, immunomodulatory functions, antioxidant, anti-cancer and antimicrobial effects (23, 24), to our best knowledge the pharmacological property of luteolin peracetate has not been described so far. By contrast, luteolin, a flavonoid highly related to luteolin peracetate, has been well studied in the past (25). As one of the most common flavonoids, luteolin are found in around 300 plant species, including many edible plants, such as celery, broccoli, green pepper, parsley, thyme, dandelion, perilla, chamomile tea, carrots, olive oil, peppermint, rosemary, navel oranges, and oregano (25, 26). The current study showed that luteolin peracetate could inhibit neutrophil adhesion induced by immobilized IC. It has been reported that multiple flavonoids inhibit neutrophils migration both *in vitro* and *in vivo* by acting on multiple mechanisms, including decreasing the expression of β2-integrin expression of neutrophils (27). In addition, it has been demonstrated that the IC-induced neutrophil adhesion is dependent on β2-integrin (13). Therefore, luteolin might inhibit the IC-induced neutrophils adhesion and subsequent tissue damage by inhibiting the β2-integrin expression on neutrophils. However, this hypothesis needs to be validated in future studies. Besides its capacity to inhibit IC-induced neutrophil adhesion, luteolin peracetate was able to block anti-COL7 IgG-mediated tissue damage *via* decreasing IC-mediated neutrophil oxygen burst. In the current study, luteolin peracetate at the concentration of 10 μM significantly and considerably reduce the IC-induced production of ROS in neutrophils. This finding is in line with the result from a

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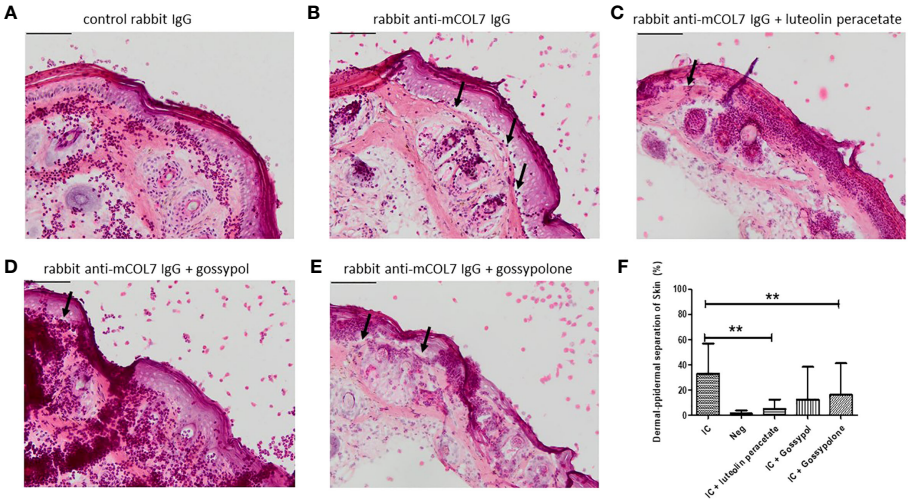

FIGURE 5
Effect of luteolin peracetate, gossypol and gossypolone on tissue damage in experimental EBA ex vivo. Mouse skin cryosections were incubated with 0.2 mg/ml rabbit control IgG **(A)** or rabbit-anti-mouse COL7 IgG **(B-E)** for 1 hour at 37°C. Subsequently, specimens were exposed to freshly isolated human neutrophils **(A, B)** or neutrophils in presence of 0.1% DMSO **(B)**, 10 µM luteolin peracetate **(C)**, 10 µM gossypol **(D)** or 10 µM gossypolone **(E)**. Images of the skin section were acquired using the NIS-Element D 3.0 software and an OLYMPUS Bx41 microscope. Micrographs of a representative experiment are shown, and black arrows indicate the dermal-epidermal separation. Scale bar = 100 µm. Skin separation was quantified as percentage of the length of epidermis detachment in relation to the length of the total dermal-epidermal zone **(F)**. Data are presented as mean ± SD of 9 independent experiments. Statically significant differences were performed using Wilcoxon signed rank test (**p<0.01).

previously study, where Oswald et al. reported that luteolin, a flavonoid highly related to luteolin peracetate, is able to suppress the ROS production from neutrophils stimulated with COL17-anti-COL17 IC in a dose-dependent manner (28).

In the *ex vivo* model for EBA used in this study, gossypolone inhibited significantly anti-COL7-induced tissue damage. These results suggest that gossypol and its derivatives could be potential therapeutics for EBA. As a natural occurring polyphenol, gossypol is isolated from the seed, roots, and stem of cotton (29). Due to its antifertility properties, it has long been known as a male contraceptive. Moreover, several biological effects such as anticancer, antivirus, antiparasitic and antimicrobial activities have been uncovered recently, making, Gossypol interesting as a potential drug for several cancer and chronic infectious diseases (29–31). By contrast, effects of gossypol and gossypolone on inflammation are much less extensively investigated. By administering gossypol as one potential antioxidant inhibitor orally and intrarectally, Fitzpatrick et al. discovered the anti-inflammatory activity of gossypol in an *in vivo* model of colitis around 30 years ago (32). Regarding the mechanism underlying the anti-inflammatory effect, an inhibition of lymphocyte proliferation, a suppression of inflammatory cytokines, and an antioxidative effect have been suggested (33–35). In the present study, gossypol and gossypolone are demonstrated to be able to inhibit IC-induced neutrophil adhesion and ROS production which is in line with results from previous studies. For example, Wang and colleagues showed that the hydroxyl groups of gossypol play a significant role in scavenging free radicals in various tumor cell lines (36). In addition, it has been reported that gossypol and gossypolone show antioxidant activity and have been suggested as potential therapeutics for psoriasis (33). By contrast, Benhaim et al. reported that pretreatment of gossypol could induce the superoxide production of human neutrophils in a time- and concentration-dependent manner, suggesting a pro-oxidant activity of gossypol (37). Therefore, the effect of gossypol and its derivatives on neutrophil ROS production might be condition-dependent, where they show pro-oxidant effects on unstimulated neutrophils and antioxidant effects on neutrophil activated by other stimuli.

Conclusion

In this study, we could demonstrate that luteolin peracetate and gossypolone are able to inhibit the anit-COL7 IgG-mediated tissue damage, providing novel potential therapeutics for EBA. However, the safety and efficacy of these compounds need to be further evaluated in animal studies *in vivo*. In addition, given shared mechanisms between EBA and bullous pemphigoid (BP), another autoimmune skin blistering disease caused by autoantibodies against COL17 (38, 39), it is conceivable to determine the effect of the two natural compounds on experimental BP.

This work has only been possible because of Prof. Dr. Detlef Zillikens. With his full support, in 2008 we started to our research in the field of autoimmune skin blistering diseases at the Research Center Borstel, Germany. We truly miss him as a motivating and supportive cooperation partner and friend.

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

XYu and FP conceived the study. XYu, FP and GR supervised the study. KY, JY, XYue and KB performed the experiments and analyzed the data. RL provided essential materials and methods. KY and XYu wrote the manuscript and all authors revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by funding from the German Research Foundation (DFG: RTG 1727, RTG 2633, YU 142/1-3 (No. 272606465), Excellence Cluster "Precision Medicine in Inflammation", TI4), the German Ministry of Education and Research (BMBF) *via* German Center for Lung Research (DZL).

Acknowledgments

We would like to thank the excellent technique support from Carola Schneider, Christine Engellenner, Cindy Jensen, and Diana Heinrich.

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 10 October 2023
DOI 10.3389/fimmu.2023.1256617

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OPEN ACCESS

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RECEIVED 11 July 2023
ACCEPTED 28 September 2023
PUBLISHED 10 October 2023

CITATION
Sollfrank L, Schönfelder V and Sticherling M (2023) Retrospective analysis of autoimmune bullous diseases in Middle Franconia. *Front. Immunol.* 14:1256617. doi: 10.3389/fimmu.2023.1256617

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Retrospective analysis of autoimmune bullous diseases in Middle Franconia

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Introduction: Autoimmune bullous diseases (AIBDs) are a group of rare cutaneous disorders affecting cornified skin and mucous membranes. They are characterized by tense or flaccid blistering and erosions due to autoantibodies against desmosomal and hemidesmosomal structural proteins of the skin. This group of disorders can be divided into those of pemphigoid and those of pemphigus diseases. If left untreated, these autoimmune diseases can cause serious or even life-threatening complications such as loss of fluid, superinfections or impaired food intake. Due to modern standardized serological assays, the diagnosis of AIBDs can usually be confirmed in combination with their clinical appearance. Whereas for a long time corticosteroids were the major players in the treatment of these diseases, with the approval of rituximab and other immunosuppressive agents, the therapy has increasingly improved.

Methods: In this study, we aimed to investigate epidemiologic and clinical features as well as diagnostics and therapy of bullous autoimmune diseases in Middle Franconia, a governorate within the German federal state of Bavaria. Patients diagnosed or treated because of a AIBDs between 01.04.2013 and 31.03.2019 at the dermatological department of the university hospital Erlangen were included in this retrospective study (n = 242). Patients were either diagnosed for the first time (n=176) or the diagnosis has been confirmed (n=66) at the department. The respective incidence was calculated among the 176 subjects who had been diagnosed at the center in this period. Data was taken from patient records and analyzed with Microsoft® Excel. The evaluation included the diagnoses of pemphigus vulgaris (PV), pemphigus foliaceus (PF), bullous pemphigoid (BP), mucous membrane pemphigoid (MMP), linear IgA dermatosis (LAD), epidermolysis bullosa acquisita (EBA), and dermatitis herpetiformis (DH).

Results: This study shows that the incidence of each AIBDs in Middle Franconia is low and comparable (PV, PF, LAD, EBA) or lower (BP, MMP, DH) than in other studies and regions. BP is the most common newly diagnosed AIBD in Middle Franconia.

Discussion: Due to the chronic and sometimes severe course of AIBDs, repeated in-house treatments are often necessary. To date, mainly topically and systemically applied corticosteroids in combination with immunomodulators are used as first-line therapy.

KEYWORDS

autoimmune bullous dermatoses, bullous dermatoses, pemphigus diseases, pemphigoid diseases, epidemiology, bullous pemphigod

1 Introduction

Autoimmune bullous diseases (AIBDs) are rare, autoimmune-induced, organ-specific diseases of the skin and skin-related mucous membranes induced by circulating autoantibodies against structural protein. Depending on the type of disease and its severity, the clinical features range from erythema on the skin and mucous membranes to excoriations, vesicles, blisters or even erosions. In patients with severe disease progression, old age or inadequate therapy, these diseases can lead to increased mortality (1, 2). A rough classification is made into the group of pemphigoid diseases showing erythema, eczema, tense blisters and erosions of the skin and pemphigus diseases presenting more flaccid blistering and erosions. This differentiation was first described clinically and histopathologically by Lever in 1953 (3). In pemphigoid disorders, autoantibodies are directed against proteins of the basement membrane connecting epidermis and dermis whilst in pemphigus diseases, antibodies are directed against desmosomal proteins connecting adjacent epithelial cells (4, 5). DH is a cutaneous manifestation of celiac disease induced by gluten and autoantibody response against the tissue transglutaminases 2 and 3. Clinical signs are polymorphic presenting vesicles, papules and macules (6). All of these diseases present diagnostic challenges due to their varied clinical presentations and overlapping histopathological features, and require a multifaceted approach to accurate assessment (7). Due to the often non-specific symptoms such as erythema, burning or itching at the beginning of the diseases, clinical suspicion of AIBD is frequently not raised and the use of specific immunoassays delayed. This leads to a significant delay in diagnosis, especially in AIBDs affecting the mucosa (8). As AIBDs have a significant impact on the quality of life of patients, there is a need for tools to assess the psychosocial health of those affected, underlining the need for comprehensive care beyond clinical assessment (9). The aim of this study is the comprehensive analysis of AIBDs at the Department of Dermatology of the University Hospital Erlangen with the catchment area of Middle Franconia, a governorate within the south-east German federal state of Bavaria. The epidemiology, clinical presentation, diagnostics and therapy of these diseases were examined.

1.1 Pemphigoid diseases

Pemphigoid diseases are characterized by the formation of subepidermal blisters along the dermo-epidermal junction zone. Autoantibodies are directed against hemidesmosomal structural proteins, which disrupts the connection of the basal keratinocytes with the basement membrane zone (5). BP is the most common AIBD in Germany with 6.6 to 13.4 newly diagnosed cases/million/year and rises with age of patients (10–12). Main clinical signs are tense blisters and erosions and in 10-20% mucosal involvement (13). In individuals older than 90 years the incidence was found at 87 new cases per 1 million residents for women and 398 new cases per 1 million residents for men. German Men as a group are more often affected than women by 1.9 times (14). In the UK, the incidence of BP was shown to be higher at 42.8 new cases/million/year (15). The other pemphigoid diseases occur less frequently. In Germany MMP has an incidence of 1.1 to 2.0 per million per year and occurs more frequently in women. As the name suggests, it predominantly affects mucous membranes, especially oral and conjunctival mucosa, eventually leading to blindness (16, 17). Approximately 30-40% of patients present symptoms on the skin with erythema, blisters or erosions (18). The mortality risk in BP is increased by a factor of 2.5, in patients with MMP by a factor of 1.7 (19). LAD is even less common with an incidence of 0.7 to 1.0 new cases/million/year (11, 12, 20). Its clinical appearance features tense, clear or hemorrhagic vesicles and blisters in the area of the skin and mucosa, typically in a linear, annular, “crowns of jewels” or rosette-like pattern (21). While BP and MMP mainly affect older patients, LAD is common in children and between the 6th-7th decades of life (22). With an incidence of 0.5 per million, EBA represents the rarest among the supepidermal blistering diseases of the pemphigoid group and has no sex-predilection (10, 11). It is characterized by skin fragility, leading to the presence of tense bullae and erosions. Individuals can be affected regardless of their age (23). The diagnosis of pemphigoid disorders is based on a combination of clinical criteria, serological and direct immunofluorescence microscopy results (5). The gold standard remains the detection of C3 and autoantibody deposition by direct immunoflourescence of a perilesional biopsy. Pemphigoid diseases present with linear deposits in the area of the dermal-epidermal junction. Furthermore, on monkey oesophagus and human saline split skin, linear deposits can also be shown by indirect immunofluorescence (7).

1.2 Pemphigus diseases

Pemphigus diseases, in contrast to pemphigoid diseases, are distinguished by intraepidermal and therefore more unstable blister

formation. The two most frequent representatives of this group are pemphigus vulgaris (PV) and pemphigus foliaceus (PF) (4). The incidence of PV in Germany ranges from 0.5 to 1.5 per 1 million inhabitants and year (11, 12). Again, a higher incidence in the UK is reported with 6.8 per million and year (15). In contrast to the pemphigoid disorders, the oral mucosa is affected as first manifestation in 75% of patients with PV. Erosions and enanthema are most common (24, 25). In 77,6%, skin involvement follows 4-5 months after the onset of disease, whereas in some rare cases, blistering of the skin is the only manifestation (26, 27). Due to frequent oral mucosal involvement, food intake is severely impaired in this disease group (4). At an incidence rate of 0.5 per million per year, PF is similarly rare in Germany (12). Since in patients with PF only autoantibodies against desmoglein (Dsg) 1, which is not present in mucous membranes, are produced, its only manifestation is on the skin. The intraepidermal blisters are due to the even thinner blister cover compared to PV more flaccid and fragile, sometimes leading to a total absence of blisters, but presence of erosions (28). With 5% of cases, paraneoplastic Pemphigus accounts for only a small proportion of pemphigus and presents a wide range of clinical manifestations because of its varying autoantibodies (Table 1) (29). IgA pemphigus, drug-induced pemphigus and the clinical variants of PV, pemphigus vegetans or pemphigus herpetiformis, are rarely diagnosed (30). The risk of mortality is increased by almost four times in patients with PV. In the paraneoplastic variant, the mortality risk is even increased by a factor of 8 (19). The mean age of onset of pemphigus diseases is around 60 years of age. While slightly more women develop PV, the opposite is true for PF (15, 31). As in pemphigoid diseases, the diagnosis is based on the clinical picture, as well as serologic and direct immunofluorescence microscopy results (4). In pemphigoid diseases, the deposition of C3 and autoantibodies in intraepidermal-epithelial can be observed. In indirect immunofluorescence, intracellular fluorescence can be detected (7).

TABLE 1 Bullous autoimmune dermatosis and their main target antigens.

Diseases with intraepidermal loss of adhesion Pemphigus diseases	Main target antigens
Pemphigus vulgaris	desmoglein 1, desmoglein 3 (4)
Pemphigus foliaceus	desmoglein 1 (4)
Paraneoplastic pemphigus	envoplakin, periplakin, desmoplakins I and II, plectin, BP230, desmoglein 1, desmoglein 3 (29)
Diseases with subepidermal loss of adhesion Pemphigoid diseases	**Main target antigens**
Bullous pemphigoid in the strict sense	BP180, BP230 (5)
Other pemphigoid diseases	p200, α6β4-Integrin (5)
Mucous membrane pemphigoid	BP180, laminin-5/laminin 332, α6β4-Integrin, laminin-6/laminin 311, type VII collagen (5)
Linear IgA dermatosis	LAD-1, BP230 (5)
Epidermolysis bullosa acquisita	Type VII collagen (5)

2 Methods

2.1 Patients

Data of patients presenting with AIBDs at the department from 04/01/2013 to 03/31/2019 were included. The electronic patient records were screened with regard to keywords of bullous autoimmune dermatoses. 464 patients were detected, of which the diagnosis of AIBDs was finally confirmed in 242. The diagnosis or confirmation of the diagnosis of AIBDs was made on the basis of the clinical presentation and - depending on availability – serologic and immunofluorescence microscopic results. If the diagnosis was previously made externally, it had to be reconfirmed again at the dermatology clinic. For calculation of incidence, all patients with first diagnosis of a AIBDs in the given period were included. This criterion was met by 176 patients. The data analysis was done with Microsoft® Excel.

2.2 Diagnostics

The description and localization of the patients' skin manifestations were taken from their initial presentation at the Department of Dermatology of the University Hospital Erlangen. Only the symptoms recorded by the treating physicians were included, whereas anamnestic data given by the patients were not considered. Skin biopsies were collected (peri-)lesionally and examined both histologically and by immunofluorescence. Serological tests were performed by indirect immunofluorescence (monkey oesophagus or salt-split human skin) and ELISA (MESACUP™). When DH and celiac disease were suspected, the patient's serum was tested for G-Transglutaminase-IgA antibodies.

2.3 Epidemiology

The catchment area of the Department of Dermatology at Erlangen University Hospital was determined on the basis of the postal codes of all patients (n = 464). Patients who came from a federal state other than Bavaria (n = 6) or from abroad (n = 2) were not taken into account. The catchment area is shown in Figure 1. The number of inhabitants of this defined area was 2,691,004 (50.8% women, 49.2% men). Cities from which more than two and counties from which more than four patients presented to the Department of Dermatology of Erlangen in the given period were included. The population data for the years 2013 to 2019 were provided by the Bavarian State Office for Statistics.

2.4 Statistics

Microsoft® Excel was used for the statistical analysis of the data as well as the creation of the diagrams. Continuous

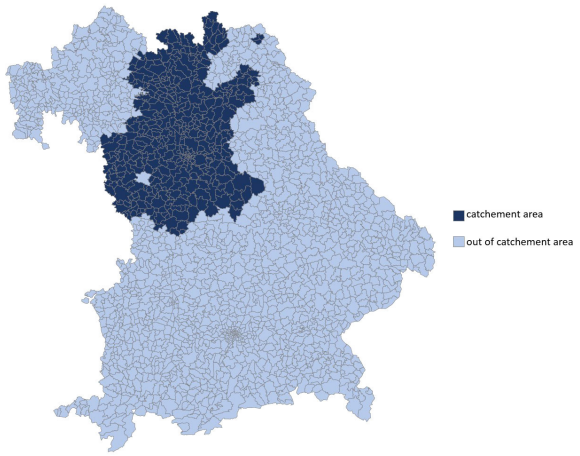

FIGURE 1
Catchment area of the Department of Dermatology at the University Hospital Erlangen within the federal state of Bavaria, determined on the basis of the Postal codes of all evaluated patients (n = 464). This covers the governorate of Middle Franconia and parts of Upper Franconia.

variables are presented with the arithmetic mean, median and standard deviation.

3 Results

In total, the electronic medical records of 464 patients were analyzed. For 242 of these patients, the final diagnosis of a AIBDs was made. The following results refer to data from these 242 patients.

In 75.2% (n = 182) the initial diagnosis was confirmed at the Department of Dermatology Erlangen. In 176 patients (72.7%), the initial diagnosis (ID) date was between 01.04.2013 and 31.03.2019. Based on this group of patients, the incidence calculation was performed. The frequency distribution of the individual AIBDs is shown in Figure 2. By far the most frequent diagnosis was BP (68.2%). Slightly less frequently pemphigus was diagnosed (25.6%).

The overall incidence of AIBDs was 10.9 per 1 million per year. The gender-related incidence was higher in males (11.5) than in females (10.4).

3.1 Pemphigus vulgaris

A total of 49 cases with PV were analyzed. The incidence of PV was 1.8 per 1 million and year (Table 2) and showed no relation to gender (m=48,3%; f=51,7%). Patients with PV were on average 51 ± 17 years old at first manifestation (Figure 3). The median age was also 51 years. Altogether, a wide age range was observed between 9 to 83 years.

Regarding clinical symptoms, 65.3% of patients reported pain and 14.3% reported pruritus (Figure 4). 18.4% observed weight loss as a result of mucosal changes. 81.6% of the cases showed oral involvement, 10.2% of the anogenital mucosa and in 4.1% of the conjunctiva. In 36.7%, only mucous membranes were involved. Patients with PV disease presented most frequently with erosions (87.8%), less frequently with crusts (36.7%), plaques (24.5%) or erythema (18.4%).

Skin biopsy with subsequent histopathologic examination was performed in 30 of the 49 patients (61.2%). The histopathological findings supported the diagnosis of PV in 83.3%. 79.6% of sera were tested on monkey esophagus by IIF and 29/39 showed positive test results. DIF examination was performed in 29 of the 49 patients (59.2%). In almost three-fourths of the cases (72.4%), the result was positive with the characteristic intercellular pattern. 50.0% of serological examinations showed elevated autoantibody concentrations against Dsg 1. Concentrations of autoantibodies against Dsg 3 were elevated in 95.7% (≥20 U/ml).

48 of the 49 patients (98.0%) received systemic therapy, all of them systemic corticosteroids while 87.8% were also started on an

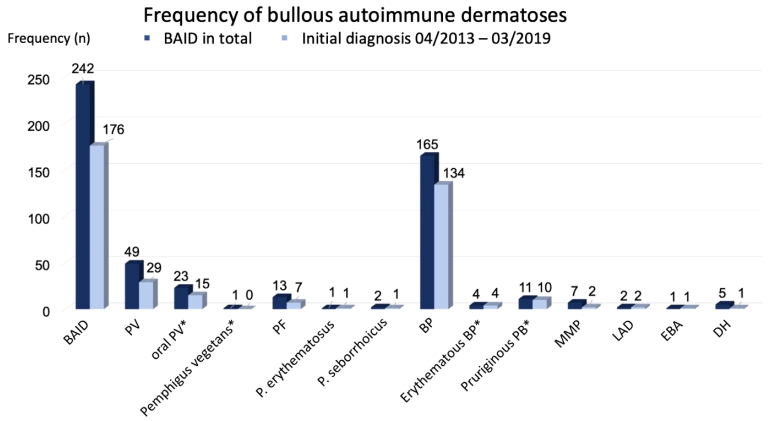

FIGURE 2
Frequency of bullous autoimmune dermatoses. Subgroups marked by * were included in main diagnose group (e.g. oral PV and PV).

immunomodulatory drug (azathioprine (AZA) 55.1%, mycophenolate mofetil (MMF) 22.4%, methotrexate (MTX) 8.1%, dapsone 2.0%).

3.2 Pemphigus foliaceus

PF was much less common, with an incidence of 0.4 per 1 million per year. The incidence in men (0.6; n=5) was three times higher than in women (0.2; n=2). At first manifestation, patients were 67± 19 years of age on average with a median of 72 years. 61.5% of patients reported pruritus in their anamnesis. Weight loss and mucosal involvement have not been observed. PF manifested as plaques and erythema in approximately three-quarters (76.9%) of patients. Scaling (61.5%), erosions (53.8%), crusts (38.5%) and blisters (15.4%) were also found. In 9 of the 13 patients (69.2%), a skin biopsy was taken for histopathological examination, which supported the diagnosis in 2/3. In 10 patients (76.9%), an IIF examination for monkey esophagus was performed, which resulted in a diagnosis in 70%. All patient specimens examined showed elevated anti-Dsg1 autoantibody concentrations. 84.6% of patients received systemic therapy (corticosteroids 76.9%; AZA 30.8%; MMF 15.4%; AZA + Dapsone 7.7%).

3.3 Bullous pemphigoid

BP was by far the most common diagnosis among the AIBDs studied (n=165). The incidence was 8.3 per million per year with no correlation to gender. The average age at the time of the first symptoms of the disease was 75 ± 12 years, the median age 78 years.

In BP, itching was the leading symptom in a large proportion of patients (81.8%). 10.9% suffered from pain and 2.4% reported weight loss. Oral involvement was diagnosed in 16.4% of patients. Approximately three quarters (72.1%) of the cases presented with firm blisters on the skin. Furthermore, erythema (62.4%), erosions (58.8%), plaque (38.8%), (scratch) excoriations (26.1%), and crusts (23.6%) were observed. In 152 of the 165 patients (92.1%), a tissue specimen was obtained and examined histopathologically. In 79.0%, the evaluation resulted in a diagnosis compatible with BP. IIF examination on monkey esophagus was performed in 142 of the 165 patients (86.1%). In 43.7%, a matching linear IgG deposit at the basement membrane could be found. An elevated anti-BP180 antibody concentration was detected in 79.3% of the sera tested and an elevated anti-BP230 antibody concentration in 54.8%. 73 of the patients (44.2%) showed increased autoantibody concentration against both BP180 and BP230. 74.6% received systemic corticosteroids, in 59.9% of patients combined with immunomodulatory drugs such as dapsone (48.5%) (50 -150 mg/d), azathioprine (9.6%) (0.5 - 2.0 mg/d), or MMF (1.8%) (2000 mg/d). 18 patients (11.5%) received monotherapy with dapsone and one (0.6%) received monotherapy with doxycycline.

3.4 Mucous membrane pemphigoid

The incidence of MMP was 0.1 per million per year. Patients presenting with MMP were on average 72 ± 8 years old. The median age was 71 years. The most common complaint was pain (71.4%), with only 14.3% reporting pruritus. In addition, nearly one-third (28.6%) of the patients reported weight loss. By definition, all patients had oral mucosal lesions. Additional involvement of the conjunctiva was documented in 57.1% and of the anogenital mucosa in 14.3%. In 85.7% a MMP manifested as erosions. Erythema (28.6%) and blisters (28.6%) were less common. Six of the seven patients (85.7%) had a biopsy taken for histopathologic examination, which supported the diagnosis in 50%. Similarly, in 50%, DIF examination could provide matching results. 71.4% were tested using IIF on monkey esophagus. With 100% positive results for IgG, this showed a higher sensitivity. In addition, four patient sera (66.7%) showed an increased concentration of autoantibodies against BP180. All 7 patients were systematically treated (85.7% corticosteroids; 42.9% AZA; 28.6% dapsone; 14.3% MMF; 14.3% cyclophosphamide).

3.5 Linear IgA dermatosis

LAD also had an incidence of 0.1 per million per year. The two male patients with LAD were 51 and 72 years old. Pruritus was the main symptom in both cases. One patient had oral and anogenital mucosal involvement. Both patients clinically showed mainly erythema on their skin. In one patient, the diagnosis of LAD was supported histopathologically. Evidence of linear IgA deposition in the DIF was conclusive for the diagnosis in both cases. Linear IgA deposits were also found in IIF. In addition, antibodies against BP180 were elevated. One patient was treated with internal corticosteroids while the other patient was administered dapsone.

3.6 Epidermolysis bullosa acquisita

During the observation period, only one 65-year-old man was diagnosed with EBA, resulting in an incidence of 0.1 per million per year. He suffered from acrally located blisters, erosions and crusts and reported itching and pain. Histopathologic evaluation of the specimen examined raised suspicion of EBA. Fluorescence microscopy showed a linear pattern along the basement membrane with IgG, IgM and C3 deposition. While IIF examination with monkey esophagus did not reveal any findings, salt split skin showed a linear IgG deposition along the blister basement. Autoantibodies against BP180, BP230, or collagen VII could not be detected in serum. The detection of collagen 7 antibodies is of high importance in the diagnosis of EBA. According to current recommendations, one of the following findings should be made when establishing the diagnosis. 1) u-serration pattern in DIF, 2) ELISA, indirect IF microscopy-based test, or immunoblotting for type VII collagen, 3) fluorescence overlay antigen mapping (FOAM) technique, or 4) direct immunogold electron microscopy (32). The investigation of the serration pattern, FOAM or direct immungold

electron microscopy was not performed in this case and was not repeated due to the retrospective study design. Although there was no clear diagnostic evidence in this case, based on the available data and the clinical presentation, the diagnosis EBA seemed most likely. Therapy was initiated with systematic steroids in combination with dapsone.

3.7 Dermatitis herpetiformis

In summary, four patients with DH were included in this study. However, only one male patient presented with newly diagnosed disease resulting in an incidence of 0.1 per million per year. DH showed the lowest mean age at first manifestation with 29 ± 11 years and a median at 27 years. The age range was from 16 to 47 years. The main symptom was pruritus (80%). Clinically, predominant erythema (60.0%), vesicles (40.0%), and erosions (40.0%) could be objectified. A skin biopsy was taken in 4/5 patients, which confirmed the diagnosis in 75%. DIF showed IgA deposits in 75%. Serologically, G-transglutaminase IgA antibodies were detected using ELISA in 75% of the patients investigated. As first-line therapy, all five patients (100.0%) received systemic therapy with dapsone (50 - 100 mg/d).

3.8 Malignancy

In 7 patients (2.9%), a malignant tumor was known at the time of AIBDs diagnosis. In addition, 20 patients (8.3%) were in remission from a malignant disease. One patient (2.0%) with PV suffered from prostate carcinoma at diagnosis, and another patient was in remission of colon and prostate carcinoma. Three patients (23.1%) with PF were in remission (2x breast carcinoma, 1x prostate carcinoma). Six patients with BP (3.6%) had a known malignancy at diagnosis (2x oral squamous cell carcinoma, 1x hepatocellular carcinoma, 1x renal cell carcinoma, 1x superficial spreading melanoma; 1x basal cell carcinoma). In addition, 16 patients with BP were in remission. Here, the most common diagnoses were breast carcinoma (31.3%), prostate carcinoma (18.8%) and urothelial carcinoma (12.5%). LAD, EBA and DH patients had no malignancy in their medical history.

4 Discussion

This study compiled relevant epidemiological data for AIBDs in a defined area of Germany. For PV, our study showed an incidence of 1.8 per million population per year. Somewhat lower incidence values between 0.5 - 1.5 for PV disease result from studies in Lower Franconia (2001 - 2002), Lübeck (2004 - 2007) as well as France (2004 - 2013) (11, 12, 30). In the UK, much higher incidence rates of 6.8 per million per year were reported, but these were calculated from a reporting system of general practitioners and could not be examined for duplicate reporting (11, 15). The mean age of 51 ± 17 years was comparable to a study from Japan, which showed a mean age of 52.8 years at first symptoms (24). Clinical symptoms were also similar to those reported in other researches (25, 33). PF showed a significantly lower incidence at 0.4 per million per year. Here, the studies from Lübeck and Lower Franconia showed slightly higher incidences of 0.6-0.7 (11, 12). BP is the most common autoimmune bullous disorder in Central Europe. A study from France showed a regional incidence of 7.4 new cases/million/year between 1984 to 1992 (34). Our calculation showed a slightly higher incidence of 8.3 per million per year. Other regional studies in Germany showed an even higher incidence of 13.4 - 16.8 per million population per year (11, 12).

TABLE 2 Incidence and total number of AIBDs. The incidence is given per million and per year. Data are provided both overall and by gender (f=female, m=male).

Diagnosis	Overall Incidence	Incidence (m)	Incidence (f)	Number	Number (m)	Number (f)
Total	10,9	11,5	10,4	176	91	85
PV	1,8	1,9	1,7	29	15	14
oral PV	0,9	1	0,9	15	8	7
Pemphigus vegetans	0	0	0	0	0	0
PF	0,4	0,6	0,2	7	5	2
Pemphigus erythematosus	0,1	0	0,1	1	0	1
Pemphigus seborrhoicus	0,1	0,1	0	1	1	0
BP	8,3	8,3	8,3	134	66	68
MMP	0,1	0,1	0,1	2	1	1
LAD	0,1	0,3	0	2	2	0
EBA	0,1	0,1	0	1	1	0
DH	0,1	0,1	0	1	1	0

BP occurs in higher age groups compared to PV. This was also shown in our study with an average of 75 ± 12 years at first symptoms. A retrospective analysis by Försti et al. on the development of the incidence of BP from 1985 to 2009 in Finland showed that it had increased 1.4-fold over the 24 years due to an aging population (35). This suggests that in the future, the incidence of these diseases will continue to increase due to higher life expectancy. The incidence of MMP in this study was significantly lower at 0.1 compared with data from France (1.25 new cases/million/year) and Lower Franconia (0.67) (10, 34). Regarding DH only one new case was diagnosed within the time limits of this investigation, leading to a low incidence of 0.1 per million and per year. The overall incidence of DH seems to vary widely depending on the region. While an incidence of 9.8 - 11 new cases per million per year has been reported in Sweden and the USA, the disease occurs only occasionally in Japan and Africa (36, 37).

LAD was also underrepresented in our study with only one case and also had an incidence of 0.1 per million per year. Studies from Lower Franconia showed a 10-fold higher incidence (11). A more frequent incidence was also reported in France with 0.48 new cases per million and year (34).

Concerning the case with EBA, we want to point out a certain diagnostic uncertainty. This again points to the need for further studies and guidelines in order to clarify this vagueness. One possibility would be to establish the examination and evaluation of the different immunodeposition patterns in DIF. Here it has already been shown that the pattern of U-serrated pattern distinguishes type VII collagen-targeted bullous diseases from other subepidermal bullous autoimmune diseases (38). A subsequent examination was not possible in our study due to the retrospective design.

In the present study, histopathological findings supported the diagnosis of PV in 83.3% and 66.6% in PF patients. This illustrates that histopathology plays an important, yet not exclusive role in the diagnostic process, but should not be used as the only method. The same is true for BP. Here, the suspicion of BP could be substantiated histopathologically by the subepidermal cleft formation in 79% of the patients (39). This needs to be confirmed by modern testing methods, such as ELISA, in the diagnosis of AIBDs.

We also demonstrated that AIBDs frequently occur in the context of malignant disease. The German and European guidelines also refers to this correlation (39–41). Therefore, it is even more important to ask for B-symptoms in patients with blistering diseases and to perform individually adapted age- and sex-specific screening examinations.

In our study, the majority of patients received internal systemic therapy with corticosteroids. Although these show rapid therapeutic success, they are accompanied by unavoidable long-term side effects. As shown in Table 3, different immunosuppressive or

TABLE 3 Duration of therapy in months given as mean, standard deviation and median.

Diagnoses		PV	PF	BP	MMP	LAD	EBA	DH
Systemic Corticosteroids (0,25 - 2,5 mg/kg/d)	data available (n)	46	10	127	6	1	1	0
	mean	32.6	34.8	13.0	26.0	2.0	6.0	–
	SD	34.3	39.2	23.2	36.9	0.0	0.0	–
	median	21.0	17.0	4.0	8.0	2.0	6.0	–
Dapsone (50 - 150 mg/d)	data available (n)	11	4	109	5.	1	1	5
	mean	54.4	30.0	12.2	5.0	16.0	14.0	77.0
	SD	55.4	31.1	18.8	4.3	0.0	0.0	100.2
	median	36.0	21.5	5.0	3.0	16.0	14.0	48.0
MMF (2000 mg/d)	data available (n)	22	4	22	2	0	0	0
	mean	53.7	52.8	31.8	53.0	–	–	–
	SD	66.0	36.0	35.2	13.0	–	–	–
	median	19.0	48.5	17.5	53.0	–	–	–
MTX s.c. (10 - 15mg/w)	data available (n)	2	0	3	0	0	0	0
	mean	27.0	–	4.7	–	–	–	–
	SD	2.0	–	3.3	–	–	–	–
	median	27.0	–	4.0	–	–	–	–
MTX p.o. (5 - 10 mg/w)	data available (n)	2	0	0	0	0	0	0
	mean	39.5	–	–	–	–	–	–
	SD	30.5	–	–	–	–	–	–

(Continued)

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TABLE 3 Continued

Diagnoses		PV	PF	BP	MMP	LAD	EBA	DH
	median	39.5	–	–	–	–	–	–
AZA (0,5 - 2,5 mg/kg/d)	data available (n)	28	5	33	3	1	0	0
	mean	23.6	56.0	12.6	26.3	34.0	–	–
	SD	31.9	63.8	17.2	35.8	0.0	–	–
	median	13.5	24.0	3.0	1.0	34.0	–	–
Doxycycline (200 mg/d)	data available (n)	0	0.0	7	0	0	0	0
	mean	–	–	3.4	–	–	–	–
	SD	–	–	2.9	–	–	–	–
	median	–	–	3.0	–	–	–	–
Cyclophosphamide (0,7 mg/kg/d)	data available (n)	0	0	0	1	0	0	0
	mean	–	–	–	19.0		–	–
	SD	–	–	–	0.0		–	–
	median	–	–	–	19.0		–	–
Ciclosporin (2,5 mg/kg/d)	data available (n)	2	1	0	0	0	0	0
	mean	43.5	13.0	–	–	–	–	–
	SD	19.5	0.0	–	–	–	–	–
	median	43.5	13.0	–	–	–	–	–
Doxycycline (200 mg/d) + Nicotinamid (1200 mg/d)	data available (n)	0	0	2	0	0	0	0
	mean	–	–	1.0	–	–	–	–
	SD	–	–	0.0	–	–	–	–
	median	–	–	1.0	–	–	–	–
Mycophenolic acid (1440 mg/d)	data available (n)	0	0	1	0	0	0	0
	mean	–	–	71.0	–	–	–	–
	SD	–	–	0.0	–	–	–	–
	median	–	–	71.0	–	–	–	–

immunomodulatory therapies are established depending on the AIBD. However, these are often associated with therapy-limiting complications or show primary efficacy failure. Rituximab is approved as first-line therapy for PV. In spite of its very good efficacy, it is associated with infusion-related side effects and, most notably, with more frequent infections (42). Furthermore, the significant influence of rituximab on the response to vaccination must be taken into consideration (43). Therefore, further research into new therapeutic options is inevitable. For example, the inhibition of B-cell activation by the BAFF inhibitor lanalumab is currently being investigated (44). Targeted therapy with efgartigimod, an antagonist of the neonatal Fc receptor, was able to significantly reduce anti-Dsg antibody levels in a phase 2 clinical trial (45). CAAR-T therapy could also be a promising treatment option in the future, in particular for patients who are refractory to therapy (46). In the case of pemphigoid diseases, dapsone is often used for systemic treatment. This requires close monitoring because of the risk of anaemia or methaemoglobulinaemia, especially in elderly and multimorbid patients. Novel treatment approaches are also being investigated in BP. First reports from studies on Dupilumab, an IL4/13 antibody, which has been used for many years in the treatment of atopic eczema, show promising efficacy and safety data (47).

The study shows some limitations, especially due to the retrospective monocentric design. The partly deviating incidences in the different studies mentioned can be explained by the overall low case numbers and different study designs. Even though the Department of Dermatology Erlangen is the only university hospital within a radius of 100 km, patients with AIBDs could have been diagnosed in other hospitals. In addition, cases may be underdiagnosed and not amply collected. To obtain more reliable data on the incidence of blistering diseases, large registries or the error-prone analysis of data from health insurance companies are needed. With regard to the

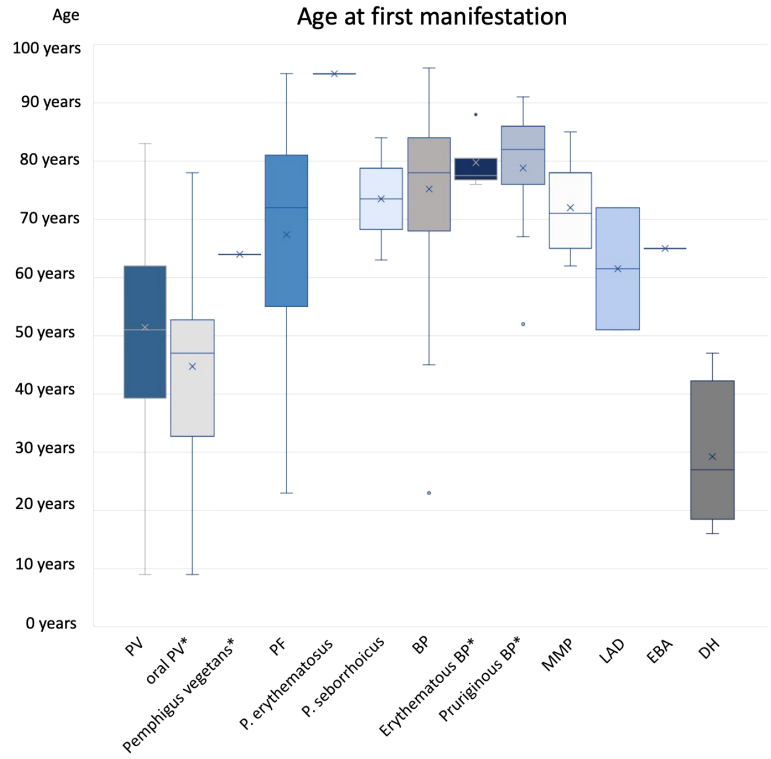

FIGURE 3
Age at first manifestation. Subgroups marked by * were included in main diagnose group (e.g. oral PV and PV).

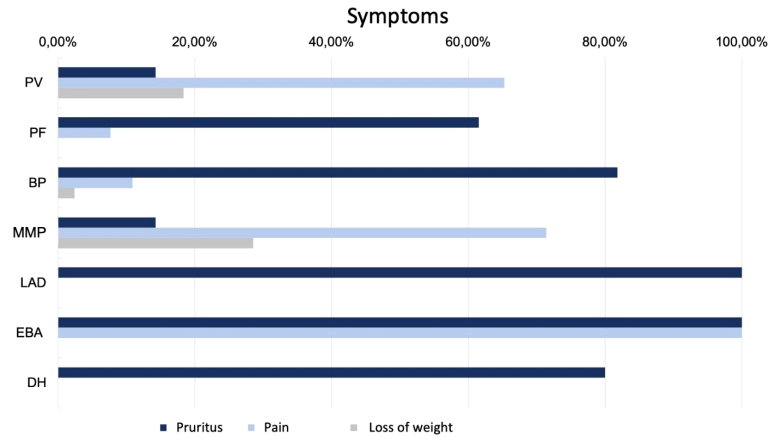

FIGURE 4
Symptoms in patients with AIBDs.

diagnostics, we did not repeat the DIF examination for sample biopsies that had already been performed externally if suitable circulating antibodies were detectable by IIF or ELISA. The diagnosis was always made according to the current guidelines by a board-certified dermatologist (40, 41).

In summary, this retrospective data analysis provided epidemiologic data similar to those obtained in other regional evaluations from central Europe. AIBDs often pose diagnostic as well as therapeutic challenges to both patients and treating physicians. Further research is needed for better understand this group of disorders and identify new treatment options.

Data availability statement

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Author contributions

LS: Writing – original draft. VS: Writing – original draft. MS: Writing – review & editing.

Funding

The authors declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

This study was performed as part of a medical doctoral thesis by VS.

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 10 October 2023
DOI 10.3389/fimmu.2023.1227855

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OPEN ACCESS

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RECEIVED 23 May 2023
ACCEPTED 19 September 2023
PUBLISHED 10 October 2023

CITATION
Jałowska M, Spałek M, Bowszyc-Dmochowska M, Gornowicz- Porowska J and Dmochowski M (2023) Involvement of palms and soles in patients with autoimmune bullous diseases: a comparative analysis of a diagnostically relevant localization. *Front. Immunol.* 14:1227855. doi: 10.3389/fimmu.2023.1227855

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Involvement of palms and soles in patients with autoimmune bullous diseases: a comparative analysis of a diagnostically relevant localization

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Introduction: The involvement of palms and soles is variable among disease entities belonging to autoimmune bullous diseases (AIBD). We present our own clinical-laboratory experience concerning presentations of skin lesions on palms and soles in the pemphigus diseases group, pemphigoid diseases group, epidermolysis bullosa acquisita (EBA), and lichen planus pemphigoides (LPP) and discuss the pertinent literature.

Methods: Lesions on palms and soles were assessed retrospectively on the basis of just photographic archives from the beginning of 2014 to March 2023. We comparatively evaluated 462 Slavic patients with AIBD.

Results: Palmoplantar involvement was observed in only 21 patients with AIBD (12 females and 9 males). There was no statistically significant difference between palmoplantar involvement in the pemphigus diseases group compared to the pemphigoid diseases group and no statistically significant difference between the pemphigus diseases group compared to the subepithelial AIBD.

Discussion: Nevertheless, particularly in LPP and EBA, and occasionally in pemphigus diseases and pemphigoid diseases groups of AIBD, localization on palms and soles may be diagnostically important at the clinical level.

KEYWORDS

palms, soles, autoimmune blistering dermatoses, pemphigus, pemphigoid

Introduction

Autoimmune bullous diseases (AIBD) are a heterogeneous group of dermatoses associated with autoimmunity directed against desmosomal structural proteins (pemphigus diseases group) or against dermal–epidermal junction structural proteins, namely, pemphigoid diseases group and epidermolysis bullosa acquisita (EBA), or with autoimmunity to enzymes-epidermal/tissue transglutaminases (dermatitis herpetiformis), clinically characterized by blisters and erosions on the skin and/or mucous membranes (1).

Pemphigus is a group of chronic, rare, potentially life-threatening AIBD of the skin and/or mucous membranes characterized most commonly by autoimmunity against desmoglein (DSG) proteins, namely, DSG3 alone, DSG1 alone, or both DSG3/DSG1, but other antibodies may be involved in immunization (2). Pemphigus vulgaris (PV), pemphigus foliaceus (PF), and paraneoplastic pemphigus (PNP) are the main subgroups of pemphigus (2). The skin lesions in PV typically can be found on the scalp, face, trunk, axilla, groin, and also pressure points (2). Oral and other mucosal lesions are seen in 80 to 90% of cases and may represent initial presentations of PV (2). PV "likes" areas adjacent to natural body orifices (1, 2).

The pemphigoid diseases group is the most common group of AIBD associated with tissue-bound and circulating autoantibodies most often directed against hemidesmosmal transmembrane protein (BP180, BPAG2, or type XVII collagen) and/or hemidesmosomal intracytoplasmic protein (BP230, BPAG1e—epithelial isoform); also, other antibodies may be involved in immunization (3). Pruritic tense subepidermal blisters usually develop in flexural areas that are proximally located, such as the axilla and the groin, inner thighs, and abdomen. The blisters can be localized or widespread (3).

Lichen planus pemphigoides (LPP) combines the features of lichen planus and bullous pemphigoid (BP). The hallmarks of this disease are lichen planus lesions with numerous bullae which develop in the context of autoantibodies targeting BP180 and/or BP230. LPP lesions are predominantly found on the extremities (4).

Epidermolysis bullosa acquisita (EBA) is a rare, clinically heterogenous, subepithelial AIBD which presents vesicle and bullae formation on the skin, and erosions on the mucous membranes associated autoantibodies to the VII collagen (coll7) (5). The disease has two main clinical forms: inflammatory and mechanobullous (classical or non-inflammatory). Patients with mechanobullous EBA develop non-inflammatory bullae and erosions at sites of trauma, while patients with the non-mechanobullous type develop inflammatory lesions which often mimic other blistering conditions including BP, linear IgA bullous disease, and mucous membrane pemphigoid (5). In the mechanobullous disease variant, skin fragility and blister formation occur with residual scarring and milia production on extensor surfaces of the skin and sites prone to trauma (6).

The acrally located blisters can present in one of two clinical scenarios. The first scenario occurs when the blisters can be localized only to the palms and soles with no additional bullous lesions. The second scenario involves the acral blisters preceding the development of blisters at other locations (7). Palmoplantar involvement is relatively common in patients with EBA and LPP, whereas palmoplantar involvement in the pemphigoid diseases group (excluding LPP) and pemphigus diseases group was very rarely described, and we do not know the exact frequency of occupation of these areas.

Aims

The aim of this study was to comparatively analyze the involvement of the palms and soles in AIBD mediated by autoimmunity to predominantly structural proteins in ethnic Poles in a setting of a single university referral center.

Materials and methods

This study was conducted in a setting of a Central European university dermatology department on 462 Slavic patients (males and females) with AIBD: pemphigoid diseases group (autoimmunity to BP180 BP230 or both), pemphigus diseases group (autoimmunity to DSG1, DSG3, or both), EBA (autoimmunity to coll7), and LPP (autoimmunity to BP180). We separated the group with LPP from the pemphigoid diseases group because, in this entity, involvement of the extremities is relatively very common. The group of patients studied was retrospectively analyzed for the presence of skin lesions on palms and soles. The lesions on palms and soles were assessed on the basis of just photographic archives in the period from the beginning of 2014 to March 2023. In this study, the involvement of palms and soles was analyzed jointly, as their microscopic features are similar. The AIBD appropriate treatment-naïve patients evaluated were as follows: 347 patients of the pemphigoid diseases group, 104 of the pemphigus diseases group, 8 with EBA, and 3 with LPP. The diagnoses of AIBD were based on the combination of clinical data, and DIF of perilesional tissue for evaluation of IgG, IgG1, IgG4,IgA, IgM, and C3 deposits, as well as monoanalyte ELISAs with DSG1, DSG3, BP180, and BP230 (initially MBL, Japan later Euroimmun, Germany) or multiplex ELISA with six different antigens (BP180, BP230, DSG1, DSG3, envoplakin, coll7) (Euroimmun, Germany) corroborated in some cases by the histopathological H+E examination, 6-substrate mosaic indirect immunofluorescence (IIF) (Euroimmun, Germany) or mosaic IIF with laminin 332 (Euroimmun Germany).

The monoanalyte ELISA kits manufactured by Euroimmun Germany utilized DSG1 and DSG3 recombinant proteins consisting of five subdomains of the extracellular domain of those molecules, as well as BP180 recombinant protein consisting of four copies of its most immunogenic domain called NC16A and BP230 recombinant protein consisting of a duplicated fragment of its C-terminal globular domain (manufacturer's cutoff values of 20 RU/ml). The recombinant DSG1, DSG3, BP180, and BP230 used in the multiplex ELISA system were the same as those in the monoanalyte ELISA kits. In multiplex ELISA, each antigen was coated in a separate well and a semiquantitative evaluation was carried out

with the manufacturer's cutoff ratio of 1. All measurements were made in the ELISA plate readers (Asys Expert 96 or Ledetect 96) equipped with MikroWin 2000 software by a single operator following the manufacturer's instructions.

Statistical analysis

The Z test for two independent proportions was used for our calculations. Statistical analysis was performed using the PQStat 1.8.2 program (PQStat Software, Plewiska, Poland). Statistical significance was $\alpha = 0.05$. A result was deemed statistically significant if $p < \alpha$.

Results

Diagnoses and statistics

The mean age of the patients with AIBD and palmoplantar involvement was 63 years. Palmoplantar involvement was observed in 21 of 462 patients with AIBD (11 females and 9 males). None of our patients had isolated skin lesions on the palms and soles. All of our patients had generalized blistering lesions with involvement of the palms and soles. Skin lesions on the palm and sole area were present in two patients in the pemphigus diseases group (1.92%), 10 in the pemphigoid diseases group (2.88%), 6 EBA patients (75%), and all patients with LPP (100%). The proportion of lesions on the palms and soles among the pemphigus diseases group (2 of 104, 1.92%) did not differ statistically significantly from the proportion in the subepithelial AIBD (pemphigoid diseases group, EBA and LPP patients) (19 of 357, 5.32%) ($p = 0.269$). The proportion of lesions on the palms and soles in patients from the pemphigus diseases group (1.92%) was not statistically significantly different from the proportion in patients from the pemphigoid diseases group (10 of 347, 2.88%) ($p = 0.853$). The LPP and EBA groups were too small to be separately reliably statistically evaluated. Clinical characteristics of AIBD patients with palm and sole involvement are shown in Table 1 and Figure 1.

Response to therapy

All but three patients with involvement of palms/soles exhibited favorable responses to the first-line therapeutic interventions involving systemic glucocorticosteroids (GCS) or potent topical GCS (Table 1, #21; this patient with LPP declined the systemic GCS treatment). One patient with mucocutaneous PV necessitated sequential therapy, which included oral GCS and four intravenous immunoglobulin infusions to achieve remission (Table 1, #2). Similarly, for another patient with the classic/mechanobullous form of EBA, a complex therapeutic approach was employed. This encompassed sequential administration of oral GCS, dapsone, azathioprine, methotrexate, cyclophosphamide, cyclosporine, five sessions of plasmapheresis, and 12 intravenous immunoglobulin infusions. Eventually, two infusions of rituximab (each 1 g) and intravenous GCS were used to bring the disease under partial control (Table 1, #7). In one patient with PF, a favorable response to therapy was achieved by adding orally administered cyclophosphamide to the initial intravenous and then oral GCS treatment (Table 1, #5). However, this intervention led to transient toxic liver damage, prompting the discontinuation of cyclophosphamide. Following the normalization of liver function parameters, dapsone at a daily dose of 50 mg was introduced. Despite these efforts, the cutaneous lesions continued to relapse, necessitating further treatment with intravenous GCS, which resulted in a partial reduction of skin lesions. Unfortunately, psychotic symptoms occurred. The administration of rituximab is scheduled.

Discussion

The single-center nature of this study, retrospective evaluation of just photographic archives available, and a small number of EBA and LPP cases are limitations of our research design. Importantly, diagnosing patients was done with biochemical–molecular techniques, not merely imaging ones.

The involvement of palms and soles is variable among disease entities belonging to AIBD. It was far more common in our EBA and LPP patients compared to the pemphigus diseases and pemphigoid diseases groups. Nevertheless, the proportions of patients with the involvement of palms and soles in the pemphigus diseases and pemphigoid diseases groups were small and did not differ statistically significantly.

Mechanical stress pressure could have triggered blisters in patients at clinically non-typical body sites (2). Another reason for the appearance of skin lesions on palms and soles could be localized trauma-induced koebnerization (9). The Koebner phenomenon (KP) may be classified into several different groups including true koebnerization (e.g., vitiligo, lichen planus, psoriasis), pseudo-koebnerization (occurs by seeding of infectious agents into traumatized skin), and localized trauma-induced koebnerization (10). Trauma-induced pemphigus has been reported after surgery, radiation, excessive sun exposure, and burns. In these cases, blister and bullae formation at the sites of injury were caused by localized exposure to self-antigens (10, 11). Rashid et al. described a patient with blisters which initially appeared around a surgical incision, 2 weeks following an appendectomy (12). Duick et al. presented a case of pemphigus that started in a Mohs surgical wound after excision of a squamous cell carcinoma (SCC) from a 49-year-old woman (13). Jetter et al. described pemphigus vegetans after cryosurgery for actinic keratosis (AKs) at the temple and forehead (14). In addition, minor procedures such as periodontal procedures, electrosurgery, Mantoux test, or laser surgery could also trigger pemphigus (10, 15). The explanation might be that epidermal injury may expose DSG 1 and 3 and lead to new autoantibody formation in genetically susceptible patients or to activation of preexisting antibodies already present in low (subclinical) titers (14, 16). Mechanical trauma and radiation are known also to trigger localized BP (17). Burns, phototherapy, X-ray irradiation, surgical incision sites, lymphedema, fistulas, and ostomies were described as an inducer of BP (10).

In the literature, we found a description of unilateral, localized BP in a patient with chronic venous stasis (18). The physical and

10.3389/fimmu.2023.1227855

TABLE 1 Clinical characteristics of AIBD patients with palm and sole involvement.

#	Age/ gender	Diagnosis	Treatment	Response to treatment
1	a/M	Pemphigoid diseases group/BP	GCS (M) tGCS	CR
2	y/M	Pemphigus diseases group/PV	GCS (M) tGCS IVIG	CR
3	a/F	EBA	GCS (M) tGCS DDS	CR
4	o/F	Pemphigoid diseases group/BP	GCS (M) tGCS	CR
5	a/F	Pemphigus diseases group/PF	iGCS (psychotic symptoms) GCS (M) tGCS CTX (toxic liver damage) DDS RTX is scheduled	PR
6	o/M	Pemphigoid diseases group/BP	DXC GCS (M) DDS CTX MTX	CR
7	o/F	EBA	GCS (M) tGCS DDS AZA MTX CTX RTX IVIG TP	PR, then lost to follow-up
8	i/M	Pemphigoid diseases group/BP	iGCS IVIG GCS (P)	CR
9	o/M	EBA	iGCS	CR
10	y/F	Pemphigoid diseases group/pemphigoid gestationis	GCS (P)	CR
11	o/F	EBA	GCS (M) tGCS	Initially PR, then lost to follow-up
12	o/F	EBA	GCS (M) tGCS DXC	CR
13	a/M	LPP	GCS (M) tGCS	CR
14	o/M	Pemphigoid diseases group/BP	DXC DDS GCS (M) IVIG	CR
15	o/F	Pemphigoid diseases group/BP	GCS (M) tGCS CTX DXC	CR
16	o/F	Pemphigoid diseases group/BP	GCS (M) tGCS	CR
17	a/F	EBA	GCS (M) tGCS	CR

(Continued)

TABLE 1 Continued

#	Age/ gender	Diagnosis	Treatment	Response to treatment
18	o/M	Pemphigoid diseases group/BP	DXC GCS (M) tGCS	CR
19	o/M	LPP	GCS (M) tGCS	CR
20	o/M	Pemphigoid diseases group/BP	GCS (M) tGCS	CR
21	o/F	LPP	tGCS	CR

Age range: i—infancy under 1 yr.; ch—childhood, 1–17 yrs.; y—youth 18–35 yrs.; a—adulthood, 36–64 yrs.; o—old age above 65 yrs.
tGCS, topical glucocorticosteroids; GCS, oral glucocorticosteroids: GCS (M) methylprednisolone, GCS (P) prednisone; iGCS, intravenous glucocorticosteroids pulses-methylprednisolone; AZA, azathioprine; MTX, methotrexate; CTX, cyclophosphamide; CSA, cyclosporine; DDS, dapsone; IVIG, intravenous immunoglobulin; DXC, doxycycline; RTX, rituximab; TP, therapeutic plasmapheresis.
A complete remission (CR) (8).
A partial remission (PR) (8).

immunologic changes associated with chronic venous stasis may have predisposed the patient to autoantigen presentation, generation of autoreactive T cells, and subsequent autoantibody-producing B cells (18). The anti-BP230^{+} and anti-BP180^{-} immunologic profile observed in this patient has also been described in the case of a patient who had localized BP in a bandlike pattern on the bilateral lower extremities (18). Furthermore, six in a series of eight patients with localized BP were found to be anti-BP230^{+} and anti-BP180^{-} in one study (19). Epidermal injury or inflammation is thought to uncover the antigen in predisposed individuals and results in new koebnerization lesions (20). Antibodies to the BP180 transmembrane protein are the primary mediators of BP. The intracellular location of BP230 in a hemidesmosomal plaque presumably makes it less prone to autoantibody attack. Autoantibodies directed against BP230 may result in a secondary phenomenon that is important in the pathogenesis of epitope spreading. However, in our patients with palmoplantar involvement in the pemphigoid diseases group, there was no patient with isolated anti-BP230 antibodies. In the pemphigoid diseases group, 50% of patients had anti-BP180 antibodies concomitant with antibodies against BP230, whereas in the remaining 50% of patients, only anti-BP180 antibodies were detected.

In the mechanobullous form of EBA, skin fragility and vesiculobullous lesions occur in areas that are more subject to pressure and trauma, especially the extensor surfaces of the acral regions (hands, feet, elbows, knees, and pretibial region) (5). We found numerous descriptions of the location of EBA on the palms and soles (21–23). They appear soon, or at most a few hours after trauma to the skin, which can be minimal (5). In the inflammatory form, lesions occur throughout the skin, not only in areas most often subject to trauma, and skin fragility is not so important. In LPP, lesions are predominantly found on the extremities (4, 24, 25), and involvement of palms and soles occurs more frequently in children (25). All our patients with LPP had palmoplantar involvement.

Another explanation for the location of skin lesions on the palms and soles may be the dyshidrotic form of AIBD. Dyshidrosiform BP, a less stereotypical form of BP, usually presents with pruritic blisters in elderly individuals; the hemorrhagic or purpuric lesions on the palms and soles can be the only manifestation of the disease (7).

FIGURE 1
Representative AIBD patients showing palmar or plantar involvement. **(A)** A 29-year-old male with mucocutaneous pemphigus vulgaris apparently induced by escitalopram treatment for depression having palmar lesions. Direct immunofluorescence examination—IgG4 (++) pemphigus epidermal deposits; multiplex ELISA—increased levels of IgG antibodies against DSG1 (1.8) and against DSG3 (4.4) (Table 1, #2). **(B)** A 71-year-old female with bullous pemphigoid showing plantar lesions. Direct immunofluorescence examination—deposits of IgG4 (+), C3 (++) with a pattern along the dermal–epidermal junction; multiplex ELISA—increased levels of IgG antibodies against BP180 (8.50) and against BP230 (4.98) (Table 1, #4). **(C)** A 73-year-old male with epidermolysis bullosa acquisita which developed following the initial diagnosis of Brunsting-Perry pemphigoid showing palmar lesions. Direct immunofluorescence examination—deposits of IgM (+), IgG (+/–), IgG1 (+), IgG4 (+++), and C3 (++) with a pattern along the dermal–epidermal junction; multiplex ELISA—increased level (4.54) of anti-coll7 IgG antibodies (Table 1, #9). **(D)** An 80-year-old female with lichen planus pemphigoides following nivolumab treatment for melanoma showing plantar lesions. Direct immunofluorescence examination—deposits of IgA, IgM, IgG, IgG1, IgG4, and C3 not found; multiplex ELISA—increased level (1.73) of anti-BP180 IgG antibodies (Table 1, #21).

Dyshidrosiform pemphigus, however, is rarely seen (26). Palmoplantar involvement in PV is a poor prognostic indicator, and palm involvement was associated with aggressive behavior of the PV. The exact pathogenesis of dyshidrosiform pemphigus is unknown (26). Nevertheless, there are data showing that murine DSG1 is expressed in the eccrine glands; therefore, it may be an explanation for this clinical feature (27). A similar prognostic importance of palm involvement is also reported by Vaishnani et al. (28). Borradori et al. have reported PV presenting as pompholyx of the left foot (29). Bolling et al. (30) have reported a unique case of palmoplantar keratoderma and a pemphigus-like immunobullous disorder, with an antibody against desmocollin 3 (not against DSG 3). However, this case showed no classical dyshidrosiform involvement.

Long-lasting and recurring bullous skin lesions with palmoplantar involvement always require a detailed differential diagnosis. The morphology of palmar and plantar bullous lesions can mimic numerous conditions including dyshidrosiform dermatitis, allergic or irritant dermatitis, cutaneous T-cell lymphoma (vesicular palmoplantar variant), porphyria cutanea tarda, dystrophic EB, dermatophyte infection, erythema multiforme, impetigo bullous, lichen planus bullous, scabies bullous, bullous systemic lupus erythematosus, Sweet's syndrome, herpes simplex (HSV) type 1 and 2, and infectious diseases with blisters (2). Indeed, the possibility of the AIBD should be initially considered also in those individuals in whom blisters occur solely on their palms and soles (2).

Conclusion

Putting aside the frequency issues, the AIBD should be kept in mind in the differential diagnosis at the clinical level of bullous lesions on the palm and soles. The involvement of palms and soles is frequent in LPP and EBA, whereas it is occasional in pemphigus diseases and pemphigoid diseases groups. In patients, showing bullous palmoplantar lesions thorough immunopathological examinations as a part of differentiating laboratory workup may be required for the definitive AIBD diagnosis (31).

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

This study protocol was reviewed and approved by the local Polish Ethical Committee of the Poznan University of Medical Sciences, approval number (560/15). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individuals and the minor's next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

Conceptualization MD; methodology MJ; software MJ, MD, JG-P; validation JG-P, MB-D; MS formal analysis MJ, MB-D; investigation MD; resources MD, MJ, data curation MJ, JG-P; writing—original draft preparation MJ; writing—review and editing MJ, MD; visualization MD; supervision MD project administration MJ, MB-D, JG-P. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

We thank Ms Barbara Jastrzębska, a laboratory diagnostician, for skillful laboratory assistance, and Ms Agata Pruciak, a statistician, for performing the statistical analysis.

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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frontiers | Frontiers in Immunology

TYPE Systematic Review
PUBLISHED 12 October 2023
DOI 10.3389/fimmu.2023.1267668

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OPEN ACCESS

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RECEIVED 03 August 2023
ACCEPTED 26 September 2023
PUBLISHED 12 October 2023

CITATION
Carver CA, Kalesinskas M and Ahmed AR (2023) Current biologics in treatment of pemphigus foliaceus: a systematic review. *Front. Immunol.* 14:1267668. doi: 10.3389/fimmu.2023.1267668

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Current biologics in treatment of pemphigus foliaceus: a systematic review

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Background: Pemphigus foliaceus (PF) differs from pemphigus vulgaris (PV) in that it affects only the skin and mucous membranes are not involved. Pemphigus is commonly treated with systemic corticosteroids and immunosuppressive agents (ISAs). More recently, biologics have been used. The current literature on biologic therapy often combines treatment of PF with PV, hence it is often difficult for clinicians to isolate the treatment of PF from PV. The purpose of this review was to provide information regarding the use of current biological therapy, specifically in PF.

Materials and methods: A search of PubMed, Embase, and other databases was conducted using keywords pemphigus foliaceus (PF), rituximab (RTX), intravenous immunoglobulin (IVIg), and biologics. Forty-one studies were included in this review, which produced 105 patients with PF, treated with RTX, IVIg, or a combination of both. Eighty-five patients were treated with RTX, eight patients with IVIg, and 12 received both RTX and IVIg.

Results: Most patients in this review had PF that was nonresponsive to conventional immunosuppressive therapies (CIST), and had significant side effects from their use. RTX treatment resulted in complete remission (CR) in 63.2%, a relapse rate of 39.5%, an infection rate of 19.7%, and a mortality rate of 3.9%. Relapse was greater in the lymphoma (LP) protocol than the rheumatoid arthritis (RA) protocol ($p<0.0001$). IVIg led to CR in 62.5% of patients, with no relapses or infections. Patients receiving both biologics experienced better outcomes when RTX was first administered, then followed by IVIg. Follow-up durations for patients receiving RTX, IVIg, and both were 22.1, 24.8, and 35.7 months, respectively.

Discussion: In pemphigus foliaceus patients nonresponsive to conventional immunosuppressive therapy or in those with significant side effects from CIST, RTX and IVIg appear to be useful agents. Profile of clinical response, as well as relapse, infection, and mortality rates in PF patients treated with RTX were similar to those observed in PV patients. The data suggests that protocols specific for PF may produce better outcomes, less adverse effects, and improved quality of life.

KEYWORDS

pemphigus foliaceus, pemphigus vulgaris, rituximab, intravenous immunoglobulin, biologics, systemic corticosteroids, immunosuppressive agents

1 Introduction

Pemphigus is an autoimmune mucocutaneous blistering disease that has two major subsets. The more common Pemphigus vulgaris (PV) involves mucous membranes, in addition to the skin, and is characterized by intraepidermal vesicles, deposition of IgG and C3 on the cell surface of the entire epidermis, and autoantibodies to both desmoglein 1 and 3 (1, 2). Pemphigus foliaceus (PF) is limited to the skin. It is characterized by subcorneal vesicles on histology and IgG and C3 deposition in the superficial epidermis on direct immunofluorescence (DIF) (1, 2). Sera of PF patients has antibodies to desmoglein 1 only (1, 2). It needs to be emphasized that the incidence of PF is considerably lower than that of PV, and that PF is clinically distinct from PV.

The vast majority of studies on pemphigus have combined PF patients with PV patients. Since there are considerable differences between the two, the authors in this review have presented data on the clinical outcomes solely in PF patients. The important reason for this was, when a treating physician decides to use a biologic agent to treat PF, this systemic review may facilitate the decision making process.

Since the advent of systemic corticosteroids, pemphigus patients have been treated with these potent anti-inflammatory agents (1, 2). The significant side effects of systemic corticosteroids led to the use of immunosuppressive agents (ISAs), with the goal being to reduce autoantibody production and have a possible steroid-sparing effect. However, the combination of systemic corticosteroids and ISAs as conventional immunosuppressive therapy (CIST) contributed to profound immunosuppression, leading to opportunistic infections and death in some cases (2). Hence, newer therapeutics were needed.

In an attempt to reduce the adverse events associated with CIST, biologic agents have been introduced in treating pemphigus (1, 2). Biologic agents are pharmacological agents derived from human plasma or produced by cells *in vivo* in a laboratory using a spectrum of technologies of molecular biology. In this review, only use of two such agents are described. Intravenous immunoglobulin (IVIg) is derived from human plasma. Rituximab (RTX) is a half human, half mouse monoclonal antibody, which targets $CD20^{+}$ B-cells. Initially, IVIg was used. More recently, the use of RTX, a B-cell depleting agent, has been advocated by several authors as first line therapy for pemphigus (2). In these studies, the emphasis has been on PV, and the readership often does not get the opportunity to isolate specific responses in PF patients.

Therefore, in this review, the authors focus solely on presenting data on patients with PF, treated with RTX and IVIg. The main purpose of this review is to provide specific information on the clinical outcomes and adverse events associated with the use of only these two biologic agents in PF. As present in current literature, this could result in evidence-based clinical decisions that may possibly benefit physicians and patients.

2 Materials and methods

Collection of the data for this analysis is presented in the PRISMA flow diagram (Figure 1). A review of PubMed, Embase and other databases was conducted from January 1990 to July 2023,

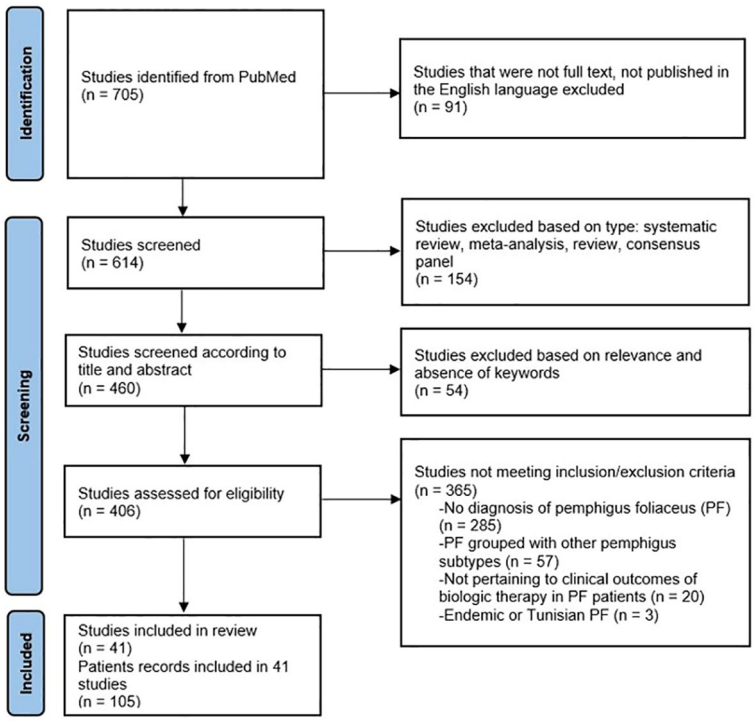

FIGURE 1
PRISMA flow diagram for systematic review of current biologics in treatment of pemphigus foliaceus (PF).

using keywords pemphigus foliaceus, rituximab, intravenous immunoglobulin (IVIg) and other biologic agents. Forty-one studies (3–43) were included in this review, totaling to 105 patients with PF, treated with a biologic agent/(s). Eighty-five adult patients received RTX, and eight received IVIg. Twelve patients received a combination of RTX and IVIg.

Inclusion criteria were (1) patients with PF diagnosed by clinical presentation, histology, and direct immunofluorescence, (2) patients treated with biologic agents, and (3) studies published in the English language. Exclusion criteria were (1) studies failing to differentiate pemphigus foliaceus from pemphigus vulgaris, and (2) studies where a joint diagnosis of PF and PV was made, (3) pediatric patients, (4) endemic PF, and (5) Tunisian PF patients.

Data extracted from each study, when present, included patient demographics, duration of disease prior to therapy, previous and concomitant therapies, treatment protocol, clinical outcomes, length of follow-up, relapse, adverse events, CD 20^+ B-cell studies, and anti-Dsg1 antibody levels. Statistical analyses were performed using MedCalc for Windows, version 22.009 (MedCalc Software, Ostend, Belgium).

3 Results

3.1 Patient characteristics

3.1.1 Rituximab group

Eighty-five patients were treated with RTX. Of the 85 patients, 50% were female and 50% were male. The mean age at initiation of RTX was 51.4 years (range 21-86). RTX was initiated at a mean of 55.4 months (range 0-540) after diagnosis of PF. Comorbidities were reported in eight (9.4%) patients. Of these, two had diabetes mellitus not attributed to previous PF treatment (3, 4), two were obese (3, 4), two had hypertension (3, 4), and two had Hepatitis C (5, 6). Other comorbidities included autoimmune hepatitis (7), hypothyroiditis (8), previously resected C-kit positive gastrointestinal stromal tumor and urothelial cell bladder cancer (8), tobacco use (9), gastroesophageal reflux disease (GERD) (9), dyslipidemia (10), history of pulmonary embolism (10), and anemia (11). Most patients had severe or recalcitrant disease or contraindications to conventional immunosuppressive therapy.

3.1.2 IVIg group

Eight patients were treated with IVIg. Of the 8 patients, 66.7% were female and 33.3% were male. The mean age at IVIg initiation was 50 years (range 31-66). Comorbidities were reported in one patient, who had Sjögren's syndrome and asthma (12). In all patients the indication for IVIg was disease nonresponsive to conventional immunosuppressive therapy and side effects from its use. The mean duration of PF prior to IVIg was 83 months (range 82-84).

3.1.3 RTX and IVIg group

Twelve patients received both RTX and IVIg. Of the 12 patients, 41.7% were female and 58.3% were male. The mean age of patients receiving RTX and IVIg was 58.8 years (range 38-77). The mean duration of PF at initiation of therapy was 52.5 months (range 12-132). No comorbidities were reported by the authors in the RTX and IVIg group.

The authors presume that the dermatologists that initially used IVIg, did so because it may be the only biologic agent available at that time. It is entirely possible that IVIg did not produce the desired clinical outcome. Now, as RTX was available, it was used. The authors who used RTX and IVIg simultaneously may have done so because they presumed that the combination would be clinically effective. The treating dermatologists who used RTX initially and subsequently used IVIg may have done so because the patient may have had a relapse after RTX use.

It needs to be highlighted that none of the authors of these publications provided any reasoning for the sequence in which they used these biologic agents.

3.2 Previous therapies

3.2.1 Rituximab group

Previous therapies are reported in 81 patients (95.3%) (Table 1). Seventy-four (91.3%) received corticosteroids. Of those, 66 (81.5%) received oral corticosteroids and 19 (23.5%) received intravenous corticosteroids. Sixty-two (76.5%) received both corticosteroids and immunosuppressive agents (ISAs). Of sixty-two (76.5%) patients treated with ISAs, 50 (61.7%) received azathioprine, 21 (25.9%) received mycophenolate mofetil, eight (9.9%) received cyclophosphamide, three (3.7%) received methotrexate. Anti-inflammatory agents were also used. One (1.2%) received gold (13), one (1.2%) received hydroxychloroquine (7), and dapsone was used in thirteen patients (16%).

Adverse effects of previous therapy were reported for 16 (19.8%) patients. Nine (11%) patients developed Cushing syndrome or steroid-induced diabetes. Osteoporosis, cytopenia, and hepatotoxicity were reported in four (4.9%) patients each. Additional side effects included gastrointestinal hemorrhage (10),

TABLE 1 Previous therapies in patients receiving rituximab or intravenous immunoglobulin.

Previous therapies	Rituximab N=81 (%)*	IVIg N=8 (%)
Corticosteroids	74 (91.4%)	8 (100%)
Azathioprine	50 (61.7%)	4 (50%)
Mycophenolate mofetil	21 (25.9%)	1 (12.5%)
Dapsone	13 (16%)	2 (25%)
Cyclophosphamide	8 (9.9%)	1 (12.5%)
Methotrexate	3 (3.7%)	2 (25%)
Gold	1 (1.2%)	None
Hydroxychloroquine	1 (1.2%)	1 (12.5%)

*In 4 patients treated with rituximab, the authors mentioned prior use of conventional immunosuppressive therapy (CIST), but did not provide details of which agent/(s) were used. Hence, the number of patients is n=81.
IVIg, intravenous immunoglobulin.

eczema herpeticum (4), ophthalmological disorders (14), myalgia (15), and trembling (15).

3.2.2 IVIg group

Previous therapies are presented in Table 1. All patients received oral corticosteroids and one (12.5%) received intravenous corticosteroids. Four (50%) were treated with ISAs, of which all received azathioprine. Two (25%) patients received methotrexate, two (25%) received dapsone (12, 16), one (12.5%) received mycophenolate mofetil (16), one (12.5%) received cyclophosphamide (12), and one (12.5%) received hydroxychloroquine (12). Tóth et al. reported one patient treated unsuccessfully with 10 courses of plasma exchange (12).

Adverse effects were reported for two (25%) patients, one (12.5%) who experienced gastrointestinal hemorrhage while receiving corticosteroid therapy (16), and one (12.5%) who developed anemia secondary to dapsone (17).

3.2.3 RTX and IVIg group

Prior to receiving both RTX and IVIg, all 12 patients were treated with systemic corticosteroids or ISAs, or a combination of both. ISAs used were azathioprine, mycophenolate mofetil, methotrexate, cyclophosphamide, and cyclosporine. Additional agents used were dapsone, hydroxychloroquine, gold, and plasmapheresis.

3.3 Treatment regimen

3.3.1 Rituximab group

Eighty-five PF patients received RTX therapy. Thirty-nine (45.9%) patients were treated with the rheumatoid arthritis (RA) protocol (two 1000 mg infusions two weeks apart) and 42 (49.4%) were treated with the lymphoma protocol (LP) (four 375 mg/m2 infusions one week apart). Two (2.4%) patients received RTX via an unspecified protocol (18) and two (2.4%) patients received RTX as maintenance therapy with a single 1000 mg infusion every six months (19). RTX was used as first-line therapy in four (4.9%) patients.

Concomitant therapy was reported in all 85 patients (100%) (Table 2). Systemic corticosteroids were given in 48 (56.5%) patients, azathioprine in 22 (25.9%) patients, and mycophenolate mofetil in eight (9.4%) patients. Three (3.5%) patients received dapsone (15, 20, 21) with RTX. Additional therapies given with RTX were protein A immunoadsorption (PAIA) (6, 21), cyclosporine (22), and imatinib (8).

3.3.2 IVIg group

Eight adult PF patients received IVIg therapy. Protocols varied, but IVIg was most-commonly given at 0.4 g/kg/day for five consecutive days (17, 23). The number of IVIg cycles was not frequently reported.

All eight (100%) patients received concomitant therapy with corticosteroids. Four (50%) received dapsone, and one (12.5%) received pulsed dexamethasone (12). (Table 2).

3.3.3 RTX and IVIg group

In those receiving both agents, two (16.7%) patients were given RTX prior to IVIg. One of these patients was treated with the LP protocol, and one was treated with an unspecified protocol. Two (16.7%) were given RTX and IVIg concomitantly. Both received the rheumatoid arthritis (RA) protocol. Eight (66.7%) patients were given IVIg prior to RTX. Five were treated with the LP protocol, one was treated with the RA protocol, and two received unspecified RTX protocols. All patients received additional therapy with conventional ISAs.

3.4 Clinical outcomes

3.4.1 Rituximab group

Clinical outcomes were analyzed for the 81 (95.3%) patients treated with RTX, using the LP or RA protocol. The course of these patients is presented in Table 3. Of these 81 patients, clinical outcomes were not reported for five patients. Thus, clinical outcomes were analyzed for 76 patients, of whom 74 (97.4%) patients experienced clinical improvement. Treatment failure was experienced by one (2.6%) patient receiving the RA protocol (10) and one (2.6%) patient receiving the LP protocol (13). Rates of complete remission (CR), CR off-therapy, CR on-therapy, nonresponse, and follow-up were statistically similar between the two (Table 3). Relapse occurred significantly more in the LP protocol than the RA protocol (68.4% vs 10.5%, $p<0.0001$). Forty-eight (63.2%) of all patients treated with RTX achieved CR. Remission status on or off-therapy was not reported for two patients treated with RTX via the RA protocol, however, both reached CR. Thus, these patients were included in the total number of patients achieving CR. Twenty-two (28.9%) achieved CR off-therapy and 24 (31.6%) achieved CR on-therapy (Table 3). Twenty-two (28.9%) reached partial remission (PR).

3.4.2 IVIg group

All patients reported clinical improvement. Five (62.5%) total patients reached CR, including four (50%) who reached CR on-therapy. One patient reached CR, but it was not specified if the patient was in remission on or off-therapy. Two (25%) patients reached PR. One patient had clinical improvement without meeting the criteria for PR or CR and is not included in Table 4. Clinical outcomes are reported in Table 4.

TABLE 2 Concomitant therapies with rituximab or intravenous immunoglobulin.

Concomitant therapies	Rituximab N=85 (%)	IVIg N=8 (%)
Corticosteroids	48 (56.5%)	8 (100%)
Azathioprine	22 (25.9%)	None
Mycophenolate mofetil	8 (9.4%)	None
Dapsone	3 (3.5%)	4 (50%)
Protein A Immunoadsorption (PAIA)	2 (2.4%)	None
Cyclosporine	1 (1.2%)	None
Imatinib	1 (1.2%)	None

IVIg, intravenous immunoglobulin.

TABLE 3 Clinical outcomes in 76 patients treated with rituximab, using the rheumatoid arthritis (RA) protocol or lymphoma protocol (LP).

Protocol (# patients)*	CR % (n) **	CR off % (n)	CR on % (n)	PR % (n)	Nonresponse/ failure (n)	Relapse % (n)	Follow-up, months (range)
RA (38)	60.5% (23)	23.7% (9)	31.6% (12)	28.9% (11)	2.6% (1)	10.5% (4)	21.9 (4-84)
LP (38)	65.8% (25)	34.2% (13)	31.6% (12)	28.9% (11)	2.6% (1)	68.4% (26)	22.2 (4-75)
P-value	NS	NS	NS	NS	NS	p<0.0001	NS

*Clinical outcomes were not reported in five out of 81 patients treated with RTX via the RA or LP protocols. Hence, data is provided only on 76 patients.
**Number of patients reaching complete remission (CR) included those reaching CR on or off-therapy, and those where on or off-therapy was not specified in reaching CR.
CR, complete remission; CR off, clinical remission off-therapy; CR on, clinical remission on-therapy; PR, partial remission; RA protocol, rheumatoid arthritis protocol; LP, lymphoma protocol; NS, not statistically significant.

3.4.3 RTX and IVIg group

Six (54.5%) patients had CR, and five (45.5%) had PR. Clinical outcome was not reported for one patient. Outcomes are reported in Table 5.

3.5 Follow-up duration

3.5.1 Rituximab group

Patients were followed for a mean of 22.1 months (range 2-84) (Table 4).

3.5.2 IVIg group

Patients were followed for a mean of 24.8 months (range 6-51) (Table 4).

3.5.3 RTX and IVIg group

Patients were followed for a mean of 35.7 months (range 5-110).

3.6 Relapse

3.6.1 Rituximab group

Thirty relapses occurred in 22 patients, with a relapse rate of 39.5%. In 18 relapses, time course was reported. Relapse occurred at a mean of 20.7 months (range 1.4-84) after last RTX infusion. In two patients, B-cell repopulation occurred at 6.5 and 10 months, followed by relapses at 9 and 13 months, respectively (24). Two patients relapsed when $CD20^+$ cells were less than 10% (22). In non-relapsing patients, time course for B-cell depletion was reported in four patients. Cianchini et al. reported two patients in whom B-cell depletion occurred within the first RTX infusion and remained undetectable for at least six months (25). Marzano et al. reported two patients in whom $CD20^+$ depletion occurred at three months and lasted for 15-18 months (26). Anti-Dsg1 levels were reported during relapse in two patients (8, 27). Antibodies in both were negative following therapy with RTX. In one, anti-Dsg1 level was 75 U/mL when relapse occurred after 84 months (27). In the other, anti-Dsg1 level was 27.25 U/mL when a relapse occurred after 42 months (8).

Treatment of relapse was reported for 15 of 22 patients. Nine (60%) received additional RTX, seven (46.7%) received corticosteroids, two (13.3%) received dapsone, and one (6.7%) received azathioprine. Following relapse, 14 patients (63.6%) again achieved disease control. Eight (36.4%) patients had CR and three (13.6%) had PR.

3.6.2 IVIg group

No relapses were observed in patients receiving IVIg therapy.

3.6.3 RTX and IVIg group

Four (33.3%) of 12 patients receiving both IVIg and RTX therapy relapsed (Table 5). All relapses occurred in patients who first received IVIg, followed by RTX. Patients who relapsed were all

TABLE 4 Clinical outcomes after rituximab, via the LP or RA protocols, or intravenous immunoglobulin.

Protocol (# patients)	CR % (n)	CR off % (n)	CR on % (n)	PR % (n)	Nonresponse/ failure % (n)	Relapse % (n)	Infection % (n)	Mean follow-up, months (range)
RTX (n=76) [$]	63.2% (48)*	28.9% (22)	31.6% (24)	28.9% (22)	2.6% (2)	39.5% (30)	19.7% (15)	22.1 (2-84)
IVIg (n=8)**	62.5% (5)	None	50% (4)	25% (2)	None	None	None	24.8 (6-51)
p-value	NS	p<0.0001	NS	NS	p<0.0001	p<0.0001	p<0.0001	NS

[$]Clinical outcomes were not reported in five out of 81 patients treated with RTX via the RA or LP protocols.
*The total number of patients reaching CR (48) included patients reaching CR-off and on therapy. In two reports, the authors of those reports did not specify if the patient was on or off therapy, and were thus included in the total value reaching CR.
**One patient only had clinical improvement, without meeting criteria for either CR or PR, and was thus not presented here.
CR, complete remission; CR off, clinical remission off-therapy; CR on, clinical remission on-therapy; PR, partial remission; RA protocol, rheumatoid arthritis protocol; LP, lymphoma protocol; NS, not statistically significant.

TABLE 5 Clinical outcomes after rituximab and intravenous immunoglobulin therapy.

Regimen	CR, n (%)	PR, n (%)	Relapse, n (%)	Infection, n (%)
CIST→RTX→IVIg* N=2	1 (50%)	1 (50%)	None	None
CIST→RTX+IVIg N=2	1 (50%)	1 (50%)	None	1 (50%)
CIST→IVIg→RTX N=8	4 (57.1%) *	3 (42.9%) *	4 (50%)	2 (25%)

*One patient did not report the final outcome as CR or PR, but only clinical improvement.
Hence, the number of patients in Table 3 is 11.
CIST, conventional immunosuppressive therapy; RTX, rituximab; IVIg, intravenous immunoglobulin; CR, complete response; PR, partial response.

given additional RTX. In one patient only partial response was achieved. In two, clinical remission off- therapy was reported. In patients who got IVIg after RTX and those who got both agents simultaneously, relapses were not reported during the follow-up period. Clinical response was not reported in one patient. B-cell studies were not reported in this group.

3.7 Adverse effects

3.7.1 Rituximab group

Side effects were reported in 18 (23.7%) patients. Of these side effects, 15 episodes of infection occurred in 13 patients (Table 6), resulting in an infection rate of 19.7% (Table 4). Infection occurred at a mean of 3.3 months (range 0-10) after treatment with RTX. All patients received corticosteroids during treatment. At the time of infection, nine (69.2%) patients were receiving either systemic corticosteroids or ISAs for maintenance therapy. Six (46.2%) were on systemic corticosteroids, four (30.8%) were on azathioprine, and one (7.7%) was on mycophenolate mofetil. Three (23.1%) patients were on both steroids and ISAs and two (15.4%) were on more than one ISAs when infection was reported (Table 6).

Mortality occurred in three (3.9%) patients treated with RTX. One patient had disseminated nocardiosis (10), and one had osteomyelitis and gastrointestinal hemorrhage (20). In one patient the specific cause of death was not reported (13). This patient had relapsed and received additional RTX and systemic corticosteroids during their course of therapy (13). It is important to note that gastrointestinal hemorrhage could be related to concomitant therapy, and not RTX therapy. Furthermore, osteomyelitis can occur as a consequence of RTX and concomitant therapy.

3.7.2 IVIg group

A mild infusion reaction occurred in one patient. Adverse effects and mortality were not reported in patients treated with IVIg. Maintenance therapy following IVIg was reported for six (75%) patients. Four (50%) were on systemic corticosteroids and three (37.5%) were on dapsone, with one (12.5%) receiving both dapsone and systemic steroids (17).

3.7.3 RTX and IVIg group

Infection was reported in three (25%) patients treated with RTX and IVIg (Table 5). Of these, one (8.3%) received RTX and IVIg simultaneously. This patient had both erysipelas and multiple HSV infections (15). One patient had a labial abscess and a VZV infection (15). One had tuberculous meningitis after RTX (13). These patients received both systemic corticosteroids and ISAs during treatment with biologics (13, 15).

4 Discussion

Clinical studies and reviews on the use of biologics in pemphigus, mix patients with PF and PV, though the two are clinically, histologically, and immunopathologically distinct. In order to better understand the role of these treatments in PF, the authors segregated patients with PF from those with PV and presented their clinical outcomes.

To analyze the clinical response to biologic agents in PF, patients were divided into three groups. Eighty-five patients were treated with rituximab (RTX). Eight patients were treated with intravenous immunoglobulin (IVIg). Twelve patients were treated with RTX and IVIg. Indications for the use of biologic therapy were disease that was nonresponsive to conventional immunosuppressive therapy (CIST) or previous adverse effects from CIST. In this review, only use of two such biologic agents were analyzed. In the literature, only two cases of the use of TNFα inhibitors in PF are reported (44, 45). Due to this limited number of patients, the authors did not include it as a major biologic agent to treat PF in this review. It is important to highlight that, like PF, these agents have been used in a very limited number of patients with PV also. Future studies of large cohorts of PF patients treated with other biologics, such as etanercept, are necessary to establish their efficacy in treatment of PF.

RTX resulted in complete remission in 48 patients (63.2%), although 30 patients (39.5%) relapsed, and the infection rate was 19.7%. The mortality rate was 3.9%. The clinical outcomes of the rheumatoid arthritis (RA) and lymphoma (LP) protocols were similar, although less relapses were reported when the RA protocol was used. The mean duration of follow-up for this group was 22.1 months (range 2-84).

Four patients received RTX as first line therapy (24, 28). Three of these patients relapsed and required additional RTX and systemic corticosteroids (24). Only one patient had sustained clinical remission (28). Treatment with IVIg resulted in complete remission in 5 patients (62.5%), which was sustained and without

10.3389/fimmu.2023.1267668

TABLE 6 Infections in 76 patients treated with rituximab via the LP or RA protocols.

Infection	Number of infections N=76 (%)[§]	Duration since last RTX	Concomitant therapies
Disseminated nocardiosis with widespread abscesses leading to death	1 (1.3%)	1 wk	pred
Pneumocystis jirovecii pneumonia	1 (1.3%)	6 m	psl, aza
Joint infection with septic shock	1 (1.3%)	2 wk	psl, aza
Osteomyelitis	1 (1.3%)	4 m	psl
Erysipelas	2 (2.6%)	3 wk, 10 wk	dap, aza
Staphylococcus aureus skin infection/impetigo	3 (3.9%)	NR	pred, dap
Herpes Simplex	1 (1.3%)	NR	NR
Upper respiratory tract infection	1 (1.3%)	NR	NR
Herpes Zoster	1 (1.3%)	0 wk	mmf
COVID-19 pneumonia	1 (1.3%)	10 m*, 2 m**	pred
Viral conjunctivitis	1 (1.3%)	NR	NR
Dental abscess	1 (1.3%)	4 m	psl, aza

[§]Clinical outcomes were not reported in five out of 81 patients treated with RTX via the RA or LP protocols.
*After initial rituximab.
**After repeat rituximab.
Psl, prednisolone; pred, prednisone; aza, azathioprine; mmf, mycophenolate mofetil; dap, dapsone; NR, not reported. *after initial rituximab, **after repeat rituximab.

adverse effects. No infection or mortality was reported in this group in a follow-up of 24.8 months. At the time of reporting, patients were still on IVIg therapy. Hence, a true relapse rate could not be determined from the available data.

Clinical outcomes of a combined protocol with RTX and IVIg varied depending on the order in which each biologic was used. In this group, relapse occurred only when RTX was used as the second biologic, after initial therapy with IVIg. Two out of three infections occurred when RTX was given as the second biologic, after IVIg. In contrast, when RTX was used as the first biologic agent, followed by the addition of IVIg, there was no relapse or infection. Duration of follow-up was 35.7 months (range 5-110).

Patients in all three groups were frequently treated with systemic corticosteroids and ISAs as concomitant therapy and post-biologics as maintenance therapy. Consequently, two issues emerged. The simultaneous use of CIST increased immunosuppression, which may have resulted in systemic infections and mortality. The use of CIST also made it difficult to isolate the direct effect of each biologic agent.

Chen et al. reported that 90% of PV patients treated with RTX and systemic corticosteroids reached clinical remission (CR) off-therapy (46). A 2015 review of 433 PV patients reported CR off-therapy in 57.6% with the LP protocol and 47.3% with the RA protocol (47). PV patients had relapse rates of 65% after the RA protocol and 40.7% after the LP protocol (47). Infections occurred in 5% (48), 16.9% (49), 25% (50), and 63% (51) of patients following RTX. Mortality rates as high as 5.2% were reported in pemphigus patients treated with RTX (48). In most, but not all, the cause of death was infection leading to septicemia.

Previous reports indicate that the relapse rate after RTX is dependent on the duration of follow-up (52). This was demonstrated in a French study of PV patients, wherein a relapse rate of 77.3% was reported in a follow-up of 6.6 years after RTX (52).

Adverse event profiles varied in other autoimmune diseases treated with RTX. Some examples are as follows. Serious infection occurred in 2.3% of rheumatoid arthritis patients (53), 15.4% of autoimmune vasculitis patients (54), and 23.7% of relapsing-remitting multiple sclerosis patients (55). In monoclonal hematologic disease, serious infection was reported in 58%, with a mortality rate of up to 35.4% following RTX therapy (56). Serious infections and death are of grave concern to physicians, patients, and their families.

IVIg has demonstrated clinical effectiveness with minimal adverse events in previous studies on PV (57). A 2001 study reported 15 patients with recalcitrant PV, treated with IVIg as monotherapy, resulting in sustained clinical remission without relapse or adverse effects over a follow-up of 20.4 months after IVIg (57). In a Consensus Developing Conference, which included 38 experts on blistering disease from the United States, Canada, and Europe, a succinct protocol was described for the use of IVIg in autoimmune blistering disease. According to these consensus guidelines, CIST may be discontinued, followed by IVIg as monotherapy for successfully achieving complete remission (58).

Previous studies have reported combination therapy with RTX and IVIg to successfully treat PV patients. A 2006 study described a protocol in which the combination of RTX and IVIg produced clinical remission in a cohort of patients with recalcitrant PV (59). Initially, multiple doses of RTX were used to deplete autoreactive clones of autoantibody producing B-cells. IVIg was then added for immunoprophylaxis and subsequently to promote immune balance and reconstitution (59). Patients had no infections, hospitalizations, and no mortality (59). In a subsequent publication by the same authors, these patients were drug-free, disease-free, and had sustained clinical remission 15 years later (60). A study of 10 patients with PV, in whom the use of systemic corticosteroids and ISAs was strongly contraindicated, reported the combination protocol of RTX and IVIg, used as first-line therapy (61). These patients remained in clinical remission with no disease and no drugs for 7.2 years after the last RTX infusion (61).

IVIg demonstrated beneficial outcomes in the treatment of PF in this study, and in previous studies on PV. Nonetheless, IVIg has been used considerably less than RTX. One of the reasons for the less frequent use of IVIg could be the perceived high cost associated with it. The high price is entirely due to the cost of producing the drug. However, a 2006 study described that the total cost of IVIg was considerably lower than the cost of CIST in treating autoimmune

bullous diseases (62). The authors created the paradigm that the total cost of a drug includes the cost of the drug itself, in addition to the cost of treating side effects it produces (62). If that paradigm is applied to comparing the cost of IVIg to RTX, the true cost of RTX would likely be higher, but impossible to fully assess. While determining the cost of adverse effects such as infection and associated hospitalization is possible, the cost of death can never be truly quantified. In a 2017 study by a French group in Lancet, RTX was reported as being less expensive than IVIg and was promoted as first-line therapy (63). The follow-up period of this study was very limited and coincided with recent RTX infusions (63). No deaths were reported by the authors. The study was funded by the manufacturer and therefore no cost was incurred by the patients (63). Furthermore, it was not clarified that healthcare in most of Europe is state funded, and patients frequently do not bear the full burden of treatment costs. In many European countries, permissions and authorizations for the use of biologic agents are made by local health authorities. Healthcare in the United States is funded by private health insurance companies and the State pays only for the elderly or disabled. Funding for health is variable worldwide. Hence, cost comparisons are likely to be effective only locally.

There were several limitations to this study. A major limitation was the lack of a control group. In a rare, potentially fatal disease, this may not be possible, and may be considered unethical. Since the prevalence of PF is low, patient cohorts were limited in size. Eighty-five patients were treated with RTX and only eight patients were treated with IVIg, therefore, a critical comparison of many parameters becomes difficult and can be biased based on size. Another major concern was the lack of detailed clinical information and a limited follow-up period. Additionally, infrequent reporting of $CD20^+$ B-cell counts before, during, and after RTX therapy limited the ability of the study to determine B-cell depletion and repopulation. The infrequent reporting of autoantibody titers to desmoglein-1 was also a significant limitation. Both of these aspects prevented the correlation of clinical response, and more importantly, relapse, with repopulation of B-cells and rise in autoantibody titers. In the final analysis, it was somewhat difficult to fully assess the impact of these new therapies on the B-cell biology and autoantibody production, besides the clinical course of disease.

The clinical presentation, histology, and immunopathology of PF is different from PV. Therefore, it would be reasonable to suggest that protocols using biologic agents or other systemic therapies to treat PF should be different from PV. Furthermore, since PF only involves the skin, it would be reasonable to propose that topical therapy be implemented in a manner that decreases the need for high doses of systemic therapy. In addition, most PF lesions occur due to involuntary trauma. Therefore, advising patients to avoid such trauma will lead to better disease control and improved quality of life. There is evidence that extensive UV exposure may also trigger the formation of PF lesions (64, 65). Hence, patients must be advised about photoprotection as an added precaution.

A combination of these conservative management strategies may, to some degree, limit the use of systemic therapies. When PF lesions are severe, widespread, or nonresponsive to conservative management, biologic therapy should be applied systematically. RTX is initially effective at depleting autoreactive B-cells that produce autoantibodies. Excessive immunosuppression may not be in the best interest of the patient. IVIg provides immunoprophylaxis and may be beneficial whenever its use is possible. Since there was such a vast disparity in the number of patients treated with RTX compared to IVIg, it would be unreasonable to provide an opinion on which biologic agent is preferably recommended. IVIg appears safe, has fewer adverse events, no infections and no relapses. However, it is often cost prohibitive. Randomized control trials (RCT) for the use of IVIg in PV or PF are unlikely because most of the IVIg produced is rapidly used. Not infrequently, it is often unavailable. There is no incentive to the manufacturers to do a RCT. In the U.S., its use in PV and PF is covered by Medicare and most private insurance companies. The authors recognize that in some developing countries, it may not be available. When available, it may not be affordable. Rituximab is cheaper but has its own baggage. According to the updated guidelines provided by the European Academy of Dermatology and Venereology (EADV) (66), the first-line treatments for PF include dapsone, corticosteroids, or RTX (RA protocol). However, these guidelines are intended for dermatologists treating PF in Europe. Treatment of patients in most European countries is state sponsored. This may not be the case for the rest of the world. Consequently, in the United States, as well as globally, there is no uniform policy or guidelines of how to treat PF. Hence, the treating dermatologist will need to consider multiple factors in deciding which agent to choose or to use both agents. Recognizing the rarity of PF, multicentered trials that provide statistically significant numbers of patients are needed to develop comprehensive therapeutic approaches and management protocols. Future research should target such a goal.

In conclusion, the data analysis in this review suggests that rates of clinical response, relapse, infection and mortality in PF patients, treated with RTX, are reasonably similar to the majority of PV patients treated with RTX. RTX is an effective agent for PF. In spite of differences between PV and PF, similar results were observed when RTX was used. There could be many reasons. One important reason is that both diseases are characterized by autoantibodies to desmogleins, produced by autoreactive B-cells. RTX kills these cells in both clinical entities with similar efficacy and efficiency. The RA and LP protocols led to similar rates of clinical response, although less relapses occurred with the RA protocol. Infection was common after RTX, suggesting that measures need to be taken to protect patients when this therapy is used. Although the cohort was limited, IVIg resulted in similar clinical efficacy to RTX with no relapses, infections, or hospitalizations. Considering these outcomes, it seems reasonable to recommend a combination of RTX and IVIg when PF is severe, widespread, or recalcitrant and combination therapy is possible or permitted.

This review reinforces that PF is clinically distinct from PV, in that it involves only the skin and not the mucous membranes. The histology and immunopathology is limited to the very upper layers of the epidermis. The predominant autoantibody targets only desmoglein-1. Therefore, it would be reasonable to presume that the autoreactive cells that produce the autoantibody may be different in PF than PV. Likewise, the molecular mechanisms in

the pathogenesis and in the microenvironment could be different in the two distinct forms of pemphigus.

As such, it would be in the best interest of both the patients and their physicians for protocols specific to PF, to be designed, developed, and effectively used. The main reason to suggest such an action is that only cutaneous disease may require less aggressive therapy. In PV patients, it is often that cutaneous disease responds more rapidly than mucosal disease. Cutaneous disease lends itself to topical therapy, which, if effective, may warrant less systemic therapy. It is entirely possible that PF patients may encounter less adverse effects. Using new ideas and creating new treatment protocols is progressive clinical medicine, which brings new advances and opens corridors for new thoughts and ideas.

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

CC: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Visualization, Writing – original draft, Writing – review and editing. MK: Conceptualization, Methodology, Project administration, Supervision, Visualization, Writing – review and editing, Validation, Formal Analysis, Investigation. AA: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review and editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported in part by an unrestricted educational grant from the Dysimmune Diseases Foundation, Plantation, FL.

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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frontiers | Frontiers in Immunology

TYPE Case Report
PUBLISHED 12 October 2023
DOI 10.3389/fimmu.2023.1272742

Check for updates

OPEN ACCESS

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RECEIVED 04 August 2023
ACCEPTED 12 September 2023
PUBLISHED 12 October 2023

CITATION
Rechtien L, Sollfrank L, Foerster Y, Berking C and Sticherling M (2023) Case Report: The many faces of bullous pemphigoid.
Front. Immunol. 14:1272742.
doi: 10.3389/fimmu.2023.1272742

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Case Report: The many faces of bullous pemphigoid

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The pemphigoid group comprises a number of bullous skin diseases with autoantibodies against different constituents of the basement membrane zone that result in subepidermal detachment and clinically characteristic tense blisters, erosions, urticarial erythema, and itching. Apart from the most frequent type of bullous pemphigoid with antibodies against BP180, which is found predominantly in elderly patients, the disease may present at other ages and different pathogenic conditions. Here, four cases are presented of young age (3 months and 25, 34, and 46 years) and in association with vaccination, pregnancy, or metastatic cancer. Though anti-BP180 was found in all cases, a different pathogenic background may be found in any of them, resulting in characteristic clinical manifestation, yet demanding specifically adapted therapeutic approaches.

KEYWORDS

bullous pemphigoid, juvenile pemphigoid, pemphigoid gestationis, bullous autoimmune disease, COVID-19 vaccination

Introduction

Bullous pemphigoid (BP) is the most common human autoimmune bullous skin disease with an incidence of 7/100,000 inhabitants/year. It occurs predominantly at the beginning of the 7th decade of life. Autoantibodies are found against various constituents of the basement membrane zone, which induce subepidermal detachment and an inflammatory cell infiltrate in the superficial dermis containing eosinophils (1). Clinically, BP is characterized by tense blisters on normal or erythematous surrounding skin, erosions, urticarial lesions, and itching and characterizes a set of diseases currently referred to as the pemphigoid group (2). Among other antigens like p200 and α4β6 laminin, bullous pemphigoid antigen 180 kD (BP180), representing collagen XVII, is the main immunogenic target. It characterizes the most frequent subset of pemphigoid diseases, BP in the strict sense (3). The different antibody subsets seem to characterize clinical manifestations, which vary regarding prognosis, predilection sites, associated malignancy, and therapeutic responses. Specifically, linear IgA dermatosis and pemphigoid gestationis are related to different immunoglobulin responses (IgA) or target structures different from the most immunogenic NC16A part of BP180, which characterizes classical BP. In this case series, rare subsets of pemphigoid disease are described and discussed, relating them to their role within the group of pemphigoid diseases.

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Case histories

The first case shows a 43-year-old female patient, Fitzpatrick skin type VI, who developed marked pruritus 5 days after her first COVID-19 vaccination (mRNA, Pfizer BioNTech). Multiple erythematous, partly excoriated papules as well as blisters and erosions were disseminated on the entire skin (Figure 1). Despite local therapy with mometasone furoate cream, the lesions progressed. The patient had no previous medical history, no allergies, no comedication or consumption of any other noxious substances. BP was confirmed by histologic and direct immunofluorescence as well as immunoserologic examinations. The tumor search including abdominal ultrasound, chest X-ray, and hemoccult remained without pathologic findings. Dapsone p.o. and prednisolone 1 mg/kg body weight was initiated, but had to be discontinued due to methemoglobinemia, and azathioprine p.o. was stopped due to toxic hepatitis. The patient ultimately received intravenous immunoglobulins (IVIGs) (Intratect®), mycophenolate mofetil p.o., and prednisolone p.o. During further course, the patient developed steroid-induced diabetes mellitus type 2, which was treated with metformin. Incipient arterial hypertension was controlled with ramipril. The patient is currently on mycophenolate mofetil 2 g/day and prednisolone 5 mg/day p.o. as well as clobetasol proprionate cream topically without any relapses. Because of the pronounced skin findings, we directly opted for a combined antibiotic and anti-inflammatory systemic treatment with dapsone and prednisolone to accompany the local therapy. The patient has been followed up since diagnosis, currently for 1 year and 10 months.

The second case presents a 25-year-old male patient with sudden onset of multiple blisters, wheals, and erosions on his trunk and extremities (Figure 2). Based on increased BP180 antibody levels and linear C3 immunofluorescence along the basement membrane zone, BP was diagnosed. Treatment with mometasone furoate cream (topical) and prednisolone 0.5 mg/kg body weight (systemic) initially improved the skin lesions. Because of the severity of BP, we decided to use antibiotic as well as anti-inflammatory systemic therapy with dapsone and prednisolone. When the skin lesions relapsed upon tapering of prednisolone, systemic therapy with dapsone 100 mg/day p.o. was initiated. Both dapsone and mycophenolate mofetil were stopped after a few weeks by the patient himself, and consequently, his skin condition markedly deteriorated. He was hospitalized, and dapsone and mometasone furoate cream were restarted. A colonoscopy was performed due to incipient diarrhea, which incidentally detected a tumor in the left colon. The histological analysis revealed a concealed perforated adenocarcinoma of the colon and tumor staging cT4b N1b M1a with pulmonary metastases. An extended left hemicolectomy with partial liver resection, gastric fundus resection, omenectomy, left partial diaphragmatic resection, left partial lung lobe resection, and splenectomy were performed. A few days after tumor resection, the skin lesions healed, and the patient has since been in remission (for 6 months) without any medication.

FIGURE 1
Multiple erythematous, partly excoriated papules, blisters, and erosions disseminated on the entire skin.

FIGURE 2
Multiple blisters, wheals, and erosions on the trunk and extremities.

The third case shows a 34-year-old female patient in the 34th week of her third pregnancy, who had already developed skin manifestations diagnosed as polymorphic eruption of pregnancy during her second pregnancy (Figure 3). Two weeks prior to consultation, she had developed widespread urticarial skin lesions on her trunk including the periumbilical region. Based on the positive results of direct immunofluorescence, and histopathologic and immunoserologic examinations, she was diagnosed with pemphigoid gestationis. The patient received systemic treatment with prednisolone 0.5 mg/kg body weight and local therapy with methylprednisolone aceponate cream. Antipruritic therapy consisted of clemastine fumarate p.o. and polidocanol cream topically. Owing to pregnancy, the patient received prednisolone exclusively as a systemic therapy concomitant to local therapy. Following a moderate improvement of her skin lesions, the patient was discharged. Two weeks later, the patient continued to suffer from pruritus but without any new skin lesions.

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FIGURE 3
Widespread urticarial skin lesions on the trunk including the periumbilical region.

Subsequently, the patient did not present to our hospital again, so she and her baby were lost to follow-up.

The fourth and last case is a 3-month-old boy with sudden onset of multiple wheals and small erosions on his trunk and extremities (Figure 4). The therapy and course of disease are described in detail by Holtsche et al. (submitted for publication). Two weeks earlier, the patient has had conjunctivitis, followed by small wheals and plantar blisters. During further course, lesions aggravated and disseminated over the entire body surface. The direct immunofluorescence examination of a skin biopsy showed linear deposits of C3 at the basement membrane zone in addition to circulating anti-BP180 antibodies in blood serum. The mother reported an unremarkable pregnancy and showed neither skin lesions nor elevated BP180/230 antibodies. In summary, juvenile pemphigoid was diagnosed. Initially, the patient received local therapy with prednicarbate cream. As skin lesions progressed, systemic therapy was initiated with 2 mg/kg prednisolone p.o., which was gradually tapered over 3 months. Because of the age of the patient, we initially decided to use anti-inflammatory systemic therapy with prednisolone. In addition, the patient received a systemic therapy with IVIG (2 g/kg body weight) (Intratect®) and anti-CD20 antibody rituximab (12.5 mg/kg body weight). Complete disease remission was achieved after 4 months and therapy could be discontinued. Since then, the patient remained without evidence of disease and is without any other medication.

FIGURE 4
Multiple wheals and small erosions on the trunk and extremities.

Discussion

BP is classically seen in patients above the age of 70 and only rarely definite causes are found. Yet, exceptions need to be anticipated and pemphigoid disease is found in unusual circumstances. Although BP is not a common side effect of mRNA-COVID vaccination, there are some individual case reports describing this occurrence (4–12). To date, no cross-reactivity between SARS-CoV-2 antigens and BP180 could be demonstrated (13). However, both inception and exacerbation of BP have been described with other vaccinations as well and may be related to immunological disturbances during vaccine reaction, including the induction of autoimmune mechanisms.

The incidence of BP depends on ethnicity and is more common in Caucasians than in dark-skinned populations (14). On the other side, skin color may influence the course of BP disease: people of darker skin often show higher disease activity with higher BPDAI scores, higher levels of anti-BP180/230 IgG, and increased pruritus compared to a Caucasian population, as shown in a monocentric study (15). Moreover, people with darker skin types more commonly suffer from pigmentary changes even if control of disease activity has been achieved (16). The diagnosis of BP in dark skin may be challenging as the characteristic erythema is not easily discernible and diseases of colored skin are underrepresented in dermatologic textbooks (17).

Paraneoplastic BP has long been described, yet in recent meta-analyses, this connection has been debated. Approximately 11% of cases are suspected to be associated with malignancies. In this context, hemato-oncological diseases, renal cell carcinoma, or laryngopharyngeal cancer has been mainly described, but there are also rarer cases with other solid tumors (18). The pathogenesis of paraneoplastic BP has not yet been clarified in detail. However, it is suspected that antibodies against tumor-specific antigens cross-react with antigens of the basement membrane and, thus, cause blistering (19). As laminin-332 is present in many types of cancers, it is suggested that BP with antibodies against laminin-332 may be related to malignancies (20). On the other hand, no increased cancer risk has been observed in patients with BP (21). According to the current German S2k guideline, “diagnostics and therapy of bullous pemphigoid”, a tumor screening that excludes a paraneoplastic etiology is not routinely recommended (22).

TABLE 1 Overview table (IIF= indirect immunofluorescence, DIF= direct immunofluorescence).

	classification	immunological features	clinical presentation	therapy
1. case	**bullous pemphigoid**	BP180 antibodies BP230 antibodies IIF: linear IgG fluorescence along BMZ (monkey oesophagus) DIF: linear IgG & C3 immunofluorescence along BMZ	multiple erythematous, partly excoriated papules, blisters, erosions disseminated on the entire skin	1. prednisolone + dapsone 2. prednisolone + azathioprine 1. & 2. were discontinued due to side effects 3. IVIGs + mycophenolate mofetil, prednisolone topically: clobetasol proprionate cream
2. case	**paraneoplastic bullous pemphigoid**	BP180 antibodies linear C3 immunofluorescence along BMZ	multiple blisters, wheals and erosions on the trunk and extremities	1. prednisolone + dapsone (stopped by patient himself) 2. prednisolone + mycophenolate mofetil (stopped by patient himself) 3. dapsone 4. excision of adenocarcinoma of the colon topically: mometasone furoate cream
3. case	**pemphigoid gestationis**	BP180 antibodies linear C3 immunofluorescence along BMZ	widespread urticarial skin lesions on her trunk including the periumbilical region	Prednisolone topically: methylprednisolone aceponate cream
4. case	**juvenile bullous pemphigoid**	BP180 antibodies linear C3 immunofluorescence along BMZ	multiple wheals and small erosions on his trunk and extremities	1. prednisolone 2. IVIGs 3. rituximab topically: prednicarbate cream

Nevertheless, a tumor screening should be performed if the patient does not show the usual clinical symptoms or if the disease course is refractory to therapy as described in our case.

Pemphigoid gestationis is a disease within the spectrum of BP with an incidence of 0.2/100,000 inhabitants/year. It occurs concomitantly with pregnancy, chorionic carcinoma, or bladder mole. The skin lesions usually disappear spontaneously after childbirth but may recur with a new pregnancy, perimenstrually, or with hormonal contraception. In addition to antipruritic treatment, therapy consists of high-potency topical glucocorticosteroids and may be escalated to systemic treatment with glucocorticosteroids (23). Skin lesions can also be observed in 10% of newborns due to placental transfer of maternal BP180 antibodies (24). Patients should refrain from hormonal contraceptives in the future to avoid disease recurrence. As BP180 is present in placental ectodermal chorionic and amniotic epithelium, pemphigoid gestationis may lead to placental insufficiency and preterm delivery or small-for-date babies (25). In our case, the patient already had pemphigoid gestationis during her second pregnancy, which was then misdiagnosed as polymorphous eruption in pregnancy.

Juvenile pemphigoid is a very rare disease with less than 100 described cases (26). Frequent acral involvement with self-limiting course and remission within 12 months is common (27). The relevant autoantibodies may be transferred from the mother via the placenta with clinically manifest or latent BP. These antibodies will disappear over the course of weeks to a few months after birth, necessitating only symptomatic, mainly topical treatment. Alternatively, autochthonous BP may be present with long-lasting disease and persistent autoantibodies requiring continued systemic immunosuppressive treatment.

The presented case series (Table 1) underline that BP should always be considered as an important differential diagnosis when it comes to blistering skin lesions regardless of the patient's age and conditions.

Apart from the most common type of BP, other manifestations and disease subsets are encountered. These cases highlight the various rare manifestations of pemphigoid disease that first need to be recognized and treated accordingly.

Data availability statement

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

Ethics statement

Written informed consent was obtained from the individual (s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

LR wrote the manuscript. LS, YF, and CB took part in drafting, revising, and critically reviewing the article. MS was responsible for the conceptualization as well as project administration and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

Funding

The authors declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

MS has performed clinical studies on bullous pemphigoid sponsored by Sanofi and Argenx.

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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frontiers | Frontiers in Immunology

TYPE Hypothesis and Theory
PUBLISHED 19 October 2023
DOI 10.3389/fimmu.2023.1291590

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OPEN ACCESS

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RECEIVED 09 September 2023
ACCEPTED 22 September 2023
PUBLISHED 19 October 2023

CITATION
Hundt JE, Sadik CD, van Beek N, Busch H, Caux F, Goebeler M, Hammers CM, Hartmann K, Hashimoto T, Ibrahim S, Kasperkiewicz M, Murrell DF, Recke A, Rose C, Schumacher N, Shimanovich I, Sitaru C, Terheyden P, Thaçi D, Ludwig RJ and Schmidt E (2023) A life for autoimmune blistering diseases: in memoriam Detlef Zillikens. *Front. Immunol.* 14:1291590. doi: 10.3389/fimmu.2023.1291590

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A life for autoimmune blistering diseases: in memoriam Detlef Zillikens

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Detlef Zillikens, MD, director and chair of the Department of Dermatology at the University of Lübeck, Lübeck, Germany, died in September 2022, aged only 64. He dedicated his professional life to autoimmune blistering diseases (AIBDs) and built his department into one of the world's leading centers for these diseases. Herein, his professional life and the impact on the field of AIBDs and the research landscape at the University of Lübeck are addressed. With his warm, integrative, open-minded, ever-optimistic attitude, he was a highly reliable colleague, mentor, and friend to many in the field including each of the authors. Combined with his in-depth knowledge of dermatology, interest in many fields of life science, and hard work, Detlef Zillikens initiated the founding of two independent research institutes, the Lübeck Institute of Experimental Dermatology and the Institute and Comprehensive Center for Inflammation Medicine. He was also instrumental in establishing the Center for Research on Inflammation of the Skin, where in a new research building, over 140 scientists pursue research questions related to skin inflammation. By inviting numerous researchers and clinicians to his department and hosting two large international meetings, he brought the field of AIBDs much closer together and inspired multiple national and international research initiatives. His ideas will live on and grow in many of his colleagues and mentees.

KEYWORDS

autoimmune blistering diseases, pemphigoid, pemphigus, autoantibody, immunofluorescence, dermatology, clinician scientists

10.3389/fimmu.2023.1291590

Introduction

Detlef Zillikens, MD, died on September 19, 2022, aged 64, after being diagnosed with a glioblastoma in March of that year (Figure 1). Following emergency surgery, he could not return to the office. Detlef Zillikens was the director and chair of the Department of Dermatology, Allergology and Venereology at the University of Lübeck, Lübeck, Germany. He was a leading expert in autoimmune blistering diseases (AIBDs) and has established the world's most prominent research center for these disorders in Lübeck. Detlef Zillikens was an optimistic, warm, integrative, and highly reliable colleague, mentor, teacher, and friend to us authors. He was a great motivator and an extraordinarily hard-working clinician and researcher who consistently followed his principles and goals. We will never forget his broad knowledge, passion for research, patience, enthusiasm, empathy, smiling face, and warm-hearted character. His death left all of us stunned and deeply saddened. We will now strive to continue and further develop the enormously successful path he has led us. Some part of Detlef Zillikens will always live on in our work and hearts. His life is honoured by the naming of the Clinician Scientist Academy of the Universities of Lübeck and Kiel after his person.

Several obituaries have already highlighted and valued his scientific work (1–4). Here, within the Research Topic *Autoimmune Blistering Diseases: in memoriam Detlef Zillikens*, numerous colleagues presented their research and how they were connected and inspired by Detlef Zillikens to focus on AIBDs.

FIGURE 1
Detlef Zillikens, July 15, 1958, to September 19, 2022.

Below, we take a closer look at his life and work from different angles and perspectives.

Curriculum vitae

Detlef Zillikens grew up in Wesseling near Bonn, Germany, with two sisters, his mother, who worked as a lab technician, and his father, who held a PhD in chemistry. After schooling in Wesseling and later in Bonn, he went to medical school at the Universities of Bonn and Heidelberg and, in his final year, to the University of Illinois at Champaign-Urbana, USA, where he gained his fine Midwestern American accent. His medical thesis (Dr. med.) was performed at the Institute of Clinical Chemistry, University of Heidelberg (Prof. H. Schmidt-Gayk), where he developed a radioimmunoassay to detect human parathyroid hormone. In 1986, he began a residency at the Department of General Surgery in Heppenheim, and he—fortunately—moved into Dermatology in 1988 to start his residency at the Department of Dermatology, University of Würzburg, Würzburg, Germany, under the guidance of Prof. Günther Burg. In 1992, the Department was chaired by Prof. Eva-Bettina Bröcker; just a week after passing his board examination, he became a consultant. In the same year, he published his first research papers about AIBDs (5, 6). After his board certification in allergology and phlebology, he habilitated in 1994 on "Cellular and humoral immune mechanisms in the pathogenesis of bullous pemphigoid and pemphigus", becoming an assistant professor. With a prestigious grant from the German Research Foundation (*Deutsche Forschungsgemeinschaft* (DFG)), he joined the laboratory of Prof. Luis Diaz and Dr. George Giudice, Medical College of Wisconsin, Milwaukee, USA, from 1994 to 1997. During this time, Detlef Zillikens characterized the fine specificities of autoantibodies against BP180 (type XVII collagen) in different pemphigoid diseases. Back in Würzburg, he became an associate professor in 2000 and full professor in 2003.

In April 2004, Detlef Zillikens was appointed director and chair of the Dermatology Department at the University of Lübeck. In parallel, while energetically restructuring and considerably enlarging the clinical department, he implemented his major field of interest, the AIBDs, in the research agenda of the University of Lübeck. Since 2007, he acted as principal investigator and member of the steering committee of the Cluster of Excellence 303 *Inflammation at Interfaces*, followed since 2019 by the Cluster of Excellence 2167 *Precision Medicine in Chronic Inflammation*. The Clusters of Excellence are the largest funding instruments of the German Research Foundation. Through this initiative, five additional professorships were taken by R.J.L. (2008), S.I. (2009), and Manfred Kunz (2009), followed by D.T. (2013) and then H.B. (2016) and J.H. (2018). Subsequently, two independent institutes were established, the Institute and Comprehensive Center for Inflammation Medicine (CCIM; 2009–2010, director, Manfred Kunz; 2010–2013, acting director, E.S.; since 2013, director, D.T.) and, in 2014, the Lübeck Institute of Experimental Dermatology (LIED) with its four divisions: *Model Systems*, director, R.L. and J.H.; *Genetics*, director, S.I.; *Translation*, director, E.S. (endowed professorship 2014); and *Systems Biology*, director, H.B.

Detlef Zillikens initiated and led several large research consortia, initially, the Focus Program *Autoimmunity* by internal funding (2007–2011) being the nucleus of the DFG-funded research consortia comprising the Research Training Group 1727 *Modulation of Autoimmunity* (2011–2020), the Clinical Research Group 303 *Pemphigoid Diseases* (2016–2021), and finally, starting in 2022, the Collaborative Research Center 1526 *Pathomechanisms of Antibody-mediated Autoimmunity: Insights from Pemphigoid Diseases*. By the CRC 303, another professorship taken by C.S. was brought to his department in 2016.

With his kind and integrative manner, Detlef Zillikens attracted the interest in AIBDs of other institutes at the University of Lübeck, such as the Departments of Anatomy, Microbiology, Pharmacology, Nephrology, and Immunology. Currently, more than 20 clinicians and scientists work in the field of AIBDs on the Lübeck Campus outside the Department of Dermatology, CCIM, and LIED. To bring these people together under one roof, he secured funding for a 2,500-m^2 research building in 2017, the *Center for Research on Inflammation of the Skin* (CRIS). Unfortunately, Detlef Zillikens could not enjoy the opening of this center scheduled for 2026.

Apart from basic science and clinical dermatology, two other areas were close to his heart, the development of diagnostic tools for AIBDs and the training of clinician scientists. The long-standing and very fruitful scientific cooperation with the company Euroimmun, Lübeck, resulted in the availability of various serological assays for AIBDs that have greatly facilitated the diagnosis of these disorders. For the training of clinician scientists, he acquired DFG funding that led to the foundation of the *Lübeck School for Clinician Scientists* and negotiated a 6-year curriculum with the board of physicians of Schleswig-Holstein combining a 4-year clinical training with a 2-year research rotation.

Detlef Zillikens served as an elected member of the review board for medicine of the DFG (2012–2020) and as vice president of the University of Lübeck responsible for scientific activities from 2013 to 2018. Detlef Zillikens received numerous prizes and honors and co-authored more than 600 articles and book chapters in the field of inflammatory dermatoses.

Detlef Zillikens leaves behind his wife Monika, a psychiatrist and psychotherapist, and two children, currently in dermatological resident training and medical school.

Department of Dermatology, University of Lübeck

Under the care of 57 current physicians, the Department of Dermatology operates 62 beds on two wards and a day-care clinic that serves more than 10,000 patients per year. In 2004, when he became chair of the Department, only 17 physicians and 32 beds were present. A continuous innovative educational program based on a rotation principle through five different inpatient and outpatient teams imparts all the essential learning content of dermatology. The expansion of the research structures was accompanied by the establishment of special outpatient clinics for AIBDs (four clinics/week), skin cancer, phlebology, and chronic wounds and a section for inflammatory disease with a focus on atopic dermatitis, psoriasis, and urticaria. The clinic has a certified skin cancer center and is part of the Lübeck University Vascular and Cancer Centers. Other important pillars of clinical care are the division of allergology, the dermatopathology laboratory, and the routine autoimmune laboratory. Detlef Zillikens has particularly promoted and always supported dermatopathology, a section that constantly expanded during his guidance. Linking the autoimmune serology, direct immunofluorescence, and histopathology, the detailed histopathological features of anti-p200 and anti-laminin 332 pemphigoid were described (7, 8). The autoimmune laboratory in 2010 was the first dermatology laboratory in Germany to be ISO 15189 certified and developed into a national and international reference center for AIBDs (9). In 2022, the laboratory received AIBD samples from more than 400 sites in Germany and abroad and processed 7,000 serum and more than 2,000 tissue samples for direct immunofluorescence. Detlef Zillikens signed all reports personally and educated and supervised a team of motivated residents and junior consultants who read the immunofluorescence slides. He was in constant exchange with the dedicated technical team that valued him highly.

Institute and Comprehensive Center for Inflammation Medicine

During the first period of the Excellence Cluster *Inflammation at Interfaces*, the idea of establishing a center of excellence in Lübeck was supported very early by Detlef Zillikens. In the beginning, it was organized to have a coordinating function between basic research activities and interdisciplinary scientific projects. Following the appointment of D.T. as its director in 2013 (Figure 2) under the constant support of Detlef Zillikens, CCIM grew into an interdisciplinary center of excellence in the management of patients with common chronic inflammatory skin diseases, e.g., psoriasis, hidradenitis suppurativa, or atopic dermatitis, and also more complex cases requiring highly specialized approaches in diagnosing and treatment. In interdisciplinary joined clinics, e.g., together with rheumatologists, all patients were screened for the existence of associated musculoskeletal diseases. Later, other disciplines joined like gastroenterology, nutrition medicines, and infectious diseases. Another focus of CCIM is patient-oriented clinical research including randomized controlled trials (10–12). In 2018, Detlef Zillikens supported the founding of a research institute for inflammation medicine and, within the Department of Dermatology, a section for inflammatory skin diseases. All of more than 40 coworkers of the research institute and section of dermatology considered him a strong supporter, visionary, and pioneer of modern dermatology.

Lübeck Institute of Experimental Dermatology

Detlef Zillikens' research agenda on AIBDs directly contributed to the scientific focus of the University of Lübeck, namely, infection and inflammation. With the acquisition of two professorships

FIGURE 2
Inauguration of Diamant Thaci (left) to CCIM in 2013 (right, Detlef Zillikens). CCIM, Comprehensive Center for Inflammation Medicine.

within the Excellence Cluster *Inflammation at Interfaces* and an endowed professorship, it became possible in 2014 to further boost research in this direction by founding this institute as a spin-off of the Department of Dermatology (Figure 3). Translational research on inflammation of the skin has been intensified ever since and extended to other inflammatory skin diseases such as psoriasis and atopic eczema. Together with two additional professorships established within the Excellence Cluster in 2016 and 2018, the LIED harbored five professorships, J.EH., H.B., S.I., R.J.L., and E.S., within its four divisions, *Model Systems* (R.J.L. and J.E.H.), *Genetics* (S.I.; until 2022), *Translation* (E.S.), and *Systems Biology* (H.B.).

Its research comprises a broad scope including various *in vitro*, *ex vivo*, and *in vivo* models, imaging, genetics, epidemiology, diagnostics of AIBDs, and systems biology. Research on LIED is centered around but not restricted to AIBDs.

LIED has established strong cooperation with the University of Lübeck and the Excellence Cluster *Inflammation at Interfaces* and its continuation *Precision Medicine in Chronic Inflammation* and has achieved national and international visibility. This development is reflected in the participation in various third-party funded consortia such as the Research Training Groups 1727 *Modulation of Autoimmunity*, 1743 *Genes, Environment, and Inflammation*, and 2633 *Defining and Targeting Autoimmune Pre-Disease*, as well as in the Clinical Research Group 303 *Pemphigoid Diseases* and its successor CRC 1526 *Pathomechanisms of Antibody-mediated Autoimmunity*. LIED also combines basic research with clinical research in close cooperation with the Department of Dermatology and the CCIM. The laboratories of LIED house more than 60 scientists, clinician scientists, PhD students, and technicians, being located at three sites on the campus. With the newly acquired CRIS building, the LIED members will be united under one roof in 2026.

Research consortia

Research Training Group 1727 Modulation of Autoimmunity

The interdisciplinary projects for this Research Training Group (RTG) have evolved from the Priority Program on Autoimmunity, which was funded by the University of Lübeck from 2006 to 2011. The central scientific question of the RTG addressed the loss of immune tolerance in autoimmune diseases and the modulation of the mechanisms contributing to this loss. The projects were divided into two areas: A) the development of novel therapeutic strategies for the treatment of autoimmune diseases and B) the identification of novel therapeutic targets. The scientists involved in this RTG have been collaborating on this topic at the Lübeck campus for more than 10 years. The breadth of the spectrum of methods used in this work also served as a training program for doctoral students and is supplemented by a central qualification and supervision concept with defined training modules. This included a twice-yearly retreat where all students present their respective projects and receive feedback from the group of subproject leaders and external advisors (Figure 4). Principal investigators came from the Departments of Dermatology, LIED, Rheumatology, Immunology, Anatomy, Microbiology, and Chemistry. During the funding period of 2015–2020, three generations of 13 Dr. rer. nat./PhD students

FIGURE 3
Founding professors and directors of the Lübeck Institute of Experimental Dermatology in 2014 (Saleh Ibrahim, Ralf Ludwig, Detlef Zillikens, and Enno Schmidt, from left to right).

were trained. In addition, six to 12 scholarships per year were provided for medical students who pause their medical studies for 6–12 months to focus exclusively on an experimental MD thesis. In total, 36 PhD and 30 associated PhD students funded by other third-party funds as well as 80 MD and associated MD students received their degrees through this RTG.

The speaker of this RTG was Detlef Zillikens; scientific coordinators in the first funding phase were R.J.L. and in the second funding phase J.E.H. This funding instrument has also been particularly successful in getting young medical students interested in experimental research on autoimmunity. A number of PhD students have joined the Clinician Scientist Program of the clinic in their later training or continued their research as postdocs at the Lübeck campus.

Clinical Research Group 303 Pemphigoid Diseases and Collaborative Research Center 1526 Pathomechanisms of Antibody-mediated Autoimmunity: Insights from Pemphigoid diseases

In 2012, Detlef Zillikens recruited C.D.S. from the Center of Immunology and Inflammatory Diseases of the Massachusetts General Hospital/Harvard Medical School in Boston to Lübeck. He had been working at this point on the mechanisms regulating the recruitment of granulocytes in autoimmune diseases for 4 years. The mechanisms recruiting granulocytes in pemphigoid diseases thus became more of the focus of the research community at Detlef Zillikens' Department. With Detlef Zillikens' support, C.D.S. drove

FIGURE 4
Meeting of the Research Training Group 1727 in Boltenhagen, Germany, in 2016.

the development of a DFG-funded research consortium focused on the mechanism regulating granulocyte recruitment in the early stages of the effector phase of pemphigoid diseases as paradigm diseases for antibody-induced granulocytic tissue inflammation. This research consortium, the Clinical Research Unit (CRU) 303 *Pemphigoid Diseases—Molecular Pathways and their therapeutic Potential*, ran from 2015 to 2022. It consisted of nine research projects and two service projects with researchers from multiple departments of the University of Lübeck as well as from the Max Planck Institute for Evolutionary Biology, Plön. Through the translational research program of the CRU 303, the clinic and the research of the Department of Dermatology in Lübeck became more closely intertwined with the CRU, providing the resources to profile and sample patients with pemphigoid diseases at clinical and molecular levels in a highly standardized manner and giving many clinical fellows of the department the chance to engage into research rotation in the labs participating in the CRU 303.

The CRC 1526 succeeded the CRU 303. CRCs are in general larger funding instruments of the DFG than CRUs. They are meant to run for 12 years and allow more projects and research sites to engage in a concerted research effort than CRUs do. The CRC 1526 addresses pemphigoid diseases and other autoantibody-induced diseases on a broader scope. The CRC 1526 became operative in January 2022 just 2 months before Detlef Zillikens was diagnosed as terminally ill.

Center for Research on Inflammation of the Skin

The CRIS is an interdisciplinary research center with a thematic focus on chronic inflammatory processes of the skin. The research program of CRIS includes the elucidation of the molecular mechanisms underlying these processes, the uncovering of differences in the mechanisms of the various types of chronic inflammation of the skin, and the development of novel, selective strategies to interrupt and reverse these mechanisms for therapeutic purposes. The CRIS research building is currently under construction on the campus of the University of Lübeck. Its completion and occupancy are planned for 2026. The building will provide 2,500 m^2 of space for 140 scientists to implement the CRIS research program. In addition to state-of-the-art laboratory space, the building will house a research clinic and animal facility and several high-end equipment including a CyTOF mass cytometer and a mass spectrometry imaging system.

A total of €30 million will be made available for the construction and furnishing of the CRIS research building. The funding commitment in 2017 was made from federal and state funds on the basis of a competitive, two-stage application procedure coordinated by the Science Council in accordance with Article 91b of the German Basic Law. This application procedure promotes innovative research consortia that are to develop into international beacons. The 10-member interdisciplinary applicant group of scientists from the Lübeck campus was led by Detlef Zillikens and managed by C.D.S.

Clinician scientist program

The training of clinician scientists was always paramount for Detlef Zillikens. As such, within the last 15 years, more than 25 clinician scientists including N.v.B, C.M.M, M.K., A.R., and I.S. received their dermatological education and were enabled to perform research projects on the Lübeck campus. During his time as vice president of the University of Lübeck, Detlef Zillikens initiated a competitive funding program for dedicated physicians of all clinical disciplines, which allowed several of these clinical scientists to perform their research rotations. Detlef Zillikens had negotiated the dermatological training program with the Schleswig-Holstein Medical Association. The 5-year, originally purely clinical dermatological training curriculum was extended to 6 years but integrates 2 years of protected time for research. Due to his longstanding and exceptional input in this subject and as a lasting memory, the *Clinician Scientist Academy* of the Universities of Lübeck and Kiel was named after him.

Cooperation with Euroimmun

Soon after Detlef Zillikens started his position in Lübeck, he sought contact with Euroimmun, an aspiring and rapidly expanding company for autoimmune diagnostics situated in Lübeck. In close cooperation with Winfried Stöcker, former owner and CEO of Euroimmun, Wolfgang Schlumberger, the former CEO, and highly motivated and forward-thinking group leaders, e.g., Christian Probst, Lars Komorowski, and Cornelia Dähnrich, a variety of serological assays for the diagnosis of AIBDs were developed and became widely available. As such, employing the recombinant immunodominant fragments sensitive and specific ELISA for the detection of serum autoantibodies against major AIBD target antigens were released comprising BP180 (type VII collagen), BP230, desmoglein 1 and 3, envoplakin, type VII collagen, tissue-type transglutaminase, and deamidated gliadin (13–18). Based on the Biochip® technology, in which multiple miniature substrates are placed in a single incubation field mounted to a standard laboratory slide, additional assays were developed, e.g., the Biochip® mosaic containing monkey esophagus, primate salt-split skin, recombinant BP180 NC16A, and human HEK293 cells that recombinantly express the immunodominant regions of desmoglein 1, desmoglein 3, and BP230 on their surface (19). This Biochip® mosaic is now widely used and has further been extended by cells that express type VII collagen and laminin 332 (20–28). Future studies will address the detection of serum IgG against further target antigens such as the p200 protein, the ectodomain of BP180, and IgA autoantibodies in AIBDs. The cooperation with Euroimmun reflected Detlef Zillikens's ability to interest others in his agenda, to build bridges, to create win–win situations, and to convey a constructive and warm atmosphere on an equal footing. This cooperation was always among his highest priorities and emphasized his strong translational thinking.

Research

Already during residency, Detlef Zillikens became interested in AIBDs. When he was appointed head of the routine autoimmune laboratory at the Department of Dermatology in Würzburg in 1992, immediately after receiving his board certification in dermatology, he started his own research group. Without funding and experimental background, apart from his activities as a medical student, this was built on medical students and situated in the three rooms of the routine autoimmune laboratory. Here, Andreas Ambach, Achim Zentner, and E.S. were among his first students who performed their medical theses (to receive the title M.D. that in Germany is not automatically given after the final medical exam). At that time, he was mainly interested in inflammatory mediators in the sera and blister fluids of patients with the most frequent AIBDs, bullous pemphigoid. Meanwhile, Detlef Zillikens was working full time as a clinical consultant spending his evening and weekend hours with research activities. The latter was only possible through the dedicated team of the routine autoimmune laboratory that also greatly supported the medical students and highly valued Detlef Zillikens. The data of these medical students were meticulously published and, given the limited resources at that time, reached considerable attention (5, 6, 29–32).

Supported by a prestigious research grant from the DFG, he spent 3 years (1994–1997) in the laboratory of Prof. Luis Diaz, Milwaukee, WI, USA. He initially aimed to study the neonatal mouse model of bullous pemphigoid, established in this lab in 1993 (33). To his great disappointment, this did not work out, and together with Dr. George Giudice, he instead characterized the fine specificities of autoantibodies against BP180 (type XVII collagen) in different pemphigoid diseases by the use of various recombinant fragments of the immunodominant NC16A domain (34–37).

Back in Würzburg, he re-established his research group that initially included the part-time technician Heike Schömig and E.S., who decided to start residency in dermatology with a 1-year research rotation as, what today would be called a clinician scientist. At this time, anti-BP180 IgG was shown to trigger a signal-transducing event leading to the release of IL-6 and IL-8 when incubated with cultured human keratinocytes (detailed below) (38). By Detlef Zillikens' winning, caring, and binding character, the laboratory was seen populated with a number of highly motivated and dedicated medical students, including I.S., Karin Obe, Karin Herzele, Rebeccah Döpp, Stanislav Reimer, Werner Kippes, Robert Kränsel, and Matthias Georgi (37, 39–49). These activities were soon supported by the implementation of a so-called "tandem" system. Here, Detlef Zillikens and M.G., another talented and aspiring consultant at that time and current director and chair of the Department of Dermatology, University of Würzburg, Germany, covered a single clinical position, allowing them to exclusively dedicate about 4 months per year to research. With this system, Prof. Eva-Bettina Bröcker fueled their careers and anticipated the *advanced clinician scientist* by nearly 20 years. When E.S. moved to the Department of Biotechnology for his PhD in 1999, C.S. took over as manager of Detlef Zillikens' research laboratory.

Detlef Zillikens was involved as a supervisor in the DFG-funded Graduate Program *Immunomodulation*, a training program for students of the Graduate School of Life Sciences (GSLS) and medical students of the University of Würzburg. C.S. and later Sidonia Mihai were recruited to Detlef's lab as fellows in this program. C.S. together with Katrin Müller-Blech as a technical assistant, Yoshi Hirako as a postdoc, and several active medical students worked on an *ex vivo* model of autoantibody-induced dermal–epidermal separation. This model was used in subsequent studies to address the effector phase of the autoimmune response to components of the epidermal basement membrane (50, 51). In parallel, animal models of autoimmunity to collagen VII were developed (detailed below) (52, 53).

In Lübeck, the research group considerably expanded with the postdocs and clinician scientists including I.S., Mircea Chiriac, Andreas Recke, Josep Herrero-González, Alina Sesarman, Amrei Dilling, and Bernie Gibbs. The results obtained in these projects were the basis for participation in the DFG Cluster of Excellence *Inflammation at Interfaces*, which started in 2007.

After R.J.L. and E.S. joined the Department of Dermatology in Lübeck in October 2007 and January 2008, respectively, Detlef Zillikens retracted from everyday research activities and focused on serving in various functions to foster his research ideas as detailed below. In addition, he initiated and steered the above-mentioned research consortia that enabled more than 70 third-party-funded clinician scientists and researchers at the campus Lübeck to work on AIBDs in 2022. Nevertheless, he attended the weekly major laboratory meetings, gave highly appreciated input in nearly every project, and reviewed the resulting manuscripts. At the same time, he fostered the work of clinician scientists represented here by M.K., A.R., and N.v.B. as further detailed in the obituary of C.M.H. (3). Detlef Zillikens personally selected them from a large number of applicants and closely mentored their careers.

Below, the authors have detailed those articles from his more than 600 publications that have been highly cited and/or are regarded as most relevant for the current research activities both in Lübeck and internationally. Among his many review articles, the three most cited were *Pemphigoid diseases* in *Lancet* 2013 (54), *Pemphigus* in *Nature Reviews Disease Primers* in 2017 (55), and *Modern diagnosis of autoimmune blistering diseases* in *Autoimmunity Reviews* in 2010 (56). He also co-authored highly cited consensus and management guidelines of AIBDs (57–68) and was instrumental in generating the guidelines for the use of immunoadsorption and rituximab. Consensus recommendations for the latter therapies, at this time, highly innovative therapeutic options for severe and/or refractory AIBDs, have greatly facilitated the reimbursement of the treatment costs in Germany (69, 70).

Selected publications

A novel subepidermal blistering disease with autoantibodies to a 200-kDa antigen of the basement membrane zone, and Anti-laminin γ1 pemphigoid

In 1996, Detlef Zillikens and T.H. described the first patient with anti-p200 pemphigoid (71). The patients clinically resembled

bullous pemphigoid and also showed linear deposits of IgG and C3 along the dermal–epidermal junction. In contrast to bullous pemphigoid, serum IgG bound to the floor of the artificial blister by indirect IF microscopy on human salt-split skin as previously described for epidermolysis bullosa acquisita. In contrast to the latter disorder, the patient's serum reacted with a specific 200-kDa band by immunoblotting with dermal extract. The molecular identity of the target antigen was only revealed in 2009 by the group of T.H. (72), who described laminin γ1 to be recognized by 90% of anti-p200 pemphigoid sera. Subsequently, it became clear that only 70%–90% of anti-p200 pemphigoid sera are reactive with laminin γ1 and that the pathogenicity of anti-laminin γ1 IgG *in vivo* could not be demonstrated (73, 74).

Tight clustering of extracellular BP180 epitopes recognized by bullous pemphigoid autoantibodies and A highly sensitive enzyme-linked immunosorbent assay for the detection of circulating anti-BP180 autoantibodies in patients with bullous pemphigoid and Serum levels of autoantibodies to BP180 correlate with disease activity in patients with bullous pemphigoid

During his postdoc period in the lab of Dr. G. Giudice, Milwaukee, WI, USA, Detlef Zillikens revealed that approximately 90% of patients with bullous pemphigoid have circulating autoantibodies against the 76-amino-acid-long 16th non-collagenous domain just outside the transmembrane domain of BP180 (35). Based on its recombinant NC16A domain, he developed a sensitive and specific ELISA system for serum autoantibodies against NC16A in patients with bullous pemphigoid and pemphigoid gestationis (34). Applying this ELISA, he was then the first to show that serum anti-BP180 NC16A IgG correlates with disease activity in bullous pemphigoid (43). These data were later the basis for the development of a widely available commercial BP180 NC16A ELISA (13).

Autoantibodies to BP180 associated with bullous pemphigoid release interleukin-6 and interleukin-8 from cultured human keratinocytes

For the first time, an FcγR-independent effect of anti-BP180 antibodies was demonstrated when a signal-inducing event leading to the secretion of IL-6 and IL-8 was observed upon the binding of BP180-specific IgG to cultured human primary keratinocytes (38). The release of IL-8 could subsequently be shown to be significantly inhibited by dapsone but not tetracycline (75).

IgG4 and IgE are the major immunoglobulins targeting the NC16A domain of BP180 in bullous pemphigoid: Serum levels of these immunoglobulins reflect disease activity and Correlation of serum levels of IgE autoantibodies against BP180 with bullous pemphigoid disease activity

The initial publication was the first to describe IgE reactivity against BP180 NC16A in patients with bullous pemphigoid (42). The article by van Beek et al. confirmed the previous finding that serum levels of IgE against BP180 NC16A parallel the extent of the disease interindividually, i.e., in an individual bullous pemphigoid patient during the course of the disease (76). Unlike serum anti-BP180 NC16A IgG, IgE against this domain does not correlate with disease activity per se. In addition, while anti-BP180 NC16A IgG is associated with the clinical phenotype, i.e., with the classical picture represented by blisters and erosions, IgE against this domain is unrelated to both the classical and urticarial/erythematous phenotypes.

Autoantibodies to bullous pemphigoid antigen 180 induce dermal-epidermal separation in cryosections of human skin and Granulocyte-derived elastase and gelatinase B are required for dermal-epidermal separation induced by autoantibodies from patients with epidermolysis bullosa acquisita and bullous pemphigoid

In this *ex vivo* model, autoantibodies against the NC16A domain of BP180 from patients with bullous pemphigoid bind in cryosections of human skin and activate normal human neutrophils, which results in dermal–epidermal separation (51). Subsequently, it was shown that the activation of neutrophils leads to the release of specific proteolytic enzymes, which are pathogenically involved in the autoantibody-induced granulocyte-dependent dermal–epidermal separation (77).

Protein A immunoadsorption: A novel and effective adjuvant treatment of severe pemphigus and Rituximab for treatment-refractory pemphigus and pemphigoid: A case series of 17 patients and Treatment of severe pemphigus with a combination of immunoadsorption, rituximab, pulsed dexamethasone and azathioprine/mycophenolate mofetil: A pilot study of 23 patients

Detlef Zillikens and his team, initially in Würzburg and then in Lübeck, were among the first to systematically apply immunoadsorption

and rituximab as novel treatment options for severe AIBDs (78). The protocol combines the short-term effects of immunoadsorption and i.v. dexamethasone pulses with the long-term effects of rituximab, in addition to azathioprine/mycophenolate mofetil, and became the new standard therapy for severe and/or refractory pemphigus patients in Lübeck and offered an improved side-effect profile compared with the former standard therapy of high-dose oral corticosteroids plus azathioprine/mycophenolate mofetil (65, 79, 80).

Induction of dermal-epidermal separation in mice by passive transfer of antibodies specific to type VII collagen

As another milestone in pemphigoid research, Detlef Zillikens provided *in vivo* proof of the autoimmune nature of epidermolysis bullosa acquisita (EBA). The story behind the paper illustrates that serendipity is sometimes a significant contributor to success: the initial aim of the project was the development of a mouse model for bullous pemphigoid. However, the murine BP180 NC15A domain was challenging to produce, while in a separate project, the production of proteins of the NC1 domain of murine collagen type VII (COL7) was successful. Hence, IgG from rabbits immunized with recombinant fragments of COL7, when injected into wild-type mice, induced clinical, histological, and immunological phenotypes duplicating human EBA (52). In parallel, similar observations were independently made by David Woodley at almost the same time (81). Ever since its initial description, this antibody transfer-induced ("passive") EBA mouse model, or variations thereof, has been extensively used by multiple laboratories for basic and translational research in EBA and pemphigoid diseases in general (82–89).

Anti-inflammatory activity of IgG1 mediated by Fc galactosylation and association of FcγRIIB and dectin-1

The requirement of C5 for the induction of experimental EBA was already described in the initial description of the antibody transfer-induced EBA mouse model (see above) (52). In cooperative work within the Excellence Cluster *Inflammation at Interfaces*, the groups of Detlef Zillikens and Jörg Köhl identified a novel pathway blocking C5aR1-driven inflammation. In this work, they demonstrated that i) C5aR1-deficient mice, like C5-deficient mice, are almost completely protected from clinical disease manifestations in antibody transfer-induced EBA; ii) engagement of the FcγRIIB and dectin-1 by highly galactosylated immune complexes blocks signaling downstream of the C5aR1; and iii) clinical disease manifestations can be ameliorated by treatment of mice with highly galactosylated immune complexes in the antibody transfer-induced EBA mouse model (90).

Serological diagnosis of autoimmune bullous skin diseases: prospective comparison of the BIOCHIP mosaic-based indirect immunofluorescence technique with the conventional multi-step single test strategy

Here, a multivariant indirect immunofluorescence test based on the Biochip® technology employed six miniature substrates, i.e., monkey esophagus, primate salt-split skin, recombinant BP180 NC16A, and HEK293 cells recombinantly expressing desmoglein 1, desmoglein 3, and BP230 on their cell surface in a single incubation field of a normal laboratory slide. This approach allowed the serological diagnosis of more than 90% of sera from patients with AIBDs (19). Subsequently, these findings could be corroborated using an extended Biochip® mosaic (24). Meanwhile, Biochips® with cells expressing type VII collagen and laminin 332 are widely available (20, 22).

Population-specific association between a polymorphic variant in ST18, encoding a pro-apoptotic molecule, and pemphigus vulgaris

In this study in cooperation with Prof. Eli Sprecher, Tel Aviv, the first non-HLA-related gene, ST18, associated with pemphigus was described in Israeli and Egyptian patients but not in patients of German ancestry (91).

Genome-wide mapping of gene-microbiota interactions in susceptibility to autoimmune skin blistering

In this collaborative study with John Baines, from the Max Planck Institute for Evolutionary Biology, Plön, Germany, the role of skin microbiota in the pathogenesis of AIBDs was first systemically studied (92). The study also provided evidence for host gene–microbiota interactions contributing to disease risk in a mouse model of EBA. The study was followed by several others (93, 94), eventually identifying multiple species involved in AIBDs and the metabolites mediating their effects.

The leukotriene B_4 and its receptor BLT1 act as critical drivers of neutrophil recruitment in murine bullous pemphigoid-like epidermolysis bullosa acquisita

In mouse models of bullous pemphigoid-like EBA and of bullous pemphigoid mice deficient in 5-lipoxygenase, a key

enzyme in LTB4 biosynthesis, or BLT1, was resistant to anti-COL7- and anti-COL17 IgG-mediated neutrophil recruitment and, consequently, skin inflammation (95). A pronounced parallel increase in LTB4 and neutrophils in the skin was seen, while eosinophils appeared to be dispensable for blister formation in the bullous pemphigoid-like EBA mouse model. These data highlighted LTB4/BLT1 as critical drivers of antibody-induced tissue destruction in experimental bullous pemphigoid-like EBA and bullous pemphigoid.

Experimental laminin 332 mucous membrane pemphigoid critically involves C5aR1 and reflects clinical and immunopathological characteristics of the human disease

This article describes the first mouse model for mucous membrane pemphigoid that reflects major clinical and immunological characteristics of the human disease (96). In this model, like in patients with mucous membrane pemphigoid, dapsone significantly reduced skin and oral lesions and ocular inflammation that leads to fibrosis (97, 98). This model will be instrumental in characterizing key inflammatory pathways and potential novel therapeutic agents for this devastating disease.

A sensitive and specific assay for the serological diagnosis of anti-laminin 332 mucous membrane pemphigoid

Based on the Biochip® technology, the first widely available detection system for serum autoantibodies against laminin 332 was developed and validated in a large multicenter study (22). This study corroborated the previous finding that anti-laminin 332 IgG in mucous membrane pemphigoid is associated with a malignancy in about a quarter of patients (99) and led to the recommendation of testing for anti-laminin IgG in all patients with mucous membrane pemphigoid in national and international guidelines (100, 101). Subsequently, N.v.B. et al. calculated a 6.8-fold higher risk of patients with anti-laminin 332 mucous membrane pemphigoid to develop malignancies than the age- and sex-matched general population (102). This association and the autoimmunity against laminin 332 are further detailed in another article on this research topic (103).

International relations

Detlef Zillikens spent several years in the laboratory of Luis Diaz at the Medical College of Wisconsin in Milwaukee, USA, where he met European postdoctoral fellows such as José Mascaro Jr. or F.C. He worked with scientists, namely, George Giudice, who cloned BP180, and Zhu Liu. At that time, he mapped epitopes on BP180 of bullous pemphigoid, pemphigoid gestationis, lichen planus pemphigoides, and linear IgA disease. He kept life-long connections with these colleagues from this period abroad.

Detlef Zillikens organized and hosted two large international meetings on AIBDs in Lübeck, the first in 2013 before the International Investigative Dermatology (IID) meeting in Edinburgh. A chartered aircraft, known as the "Detlef Zillikens Express", flew everyone from the local airport in Lubeck to Edinburgh. With Detlef Zillikens, nothing was impossible. The second took place in 2017 with the help of the American patients' association International Pemphigus & Pemphigoid Foundation (IPPF) (Figure 5). These reunions occurred in a friendly atmosphere, permitted many exchanges of ideas and contact between people, and are still well-remembered in the entire community.

Detlef Zillikens has always welcomed students from abroad so that they can learn or do research on AIBDs in his laboratory. He also regularly received visiting professors in his department for teaching and scientific fostering including DFM, F.C., and T.H.

From an early age, Detlef Zillikens had a passion for exploring the world and meeting people. He spent several months during his medical school at the University of Illinois in Urbana, USA, where he perfected his Midwestern American accent. He returned to the Midwest, to the University of Wisconsin, where he was mentored by another world traveler physician-scientist, Dr. Luis Diaz, originally from Peru. Through this thread, Detlef Zillikens became part of a far-reaching international team of mentees of Luis Diaz who were passionate about AIBDs. Once he had established his chairmanship in Lübeck, he was then able to draw in numerous German experts and also many of the best physician scientists from abroad with grant funding to support them including Norito Ishii (University of Kurume, Japan) Hiroaki Iwata (University of Gifu, Japan) and Kentaro Izumi (University of Sapporo, Japan).

Detlef Zillikens has always enjoyed networking and missed a few annual meetings of the German Society of Dermatological Research (ADF) and the European Society of Dermatological Research (Figure 6). He managed to find the time to lecture in far-flung corners of the world, including India and Iran, where he was constantly swamped by young dermatologists who were hoping to work with him.

Nevertheless, somehow, he also managed to fit in amazing trips to exotic locations with his family for their passion for swimming with tropical fish.

Personal notes and anecdotes

Detlef Zillikens has always been an extremely hard-working person who dedicated large parts of his spare time to his clinic and research on AIBDs. This may be exemplified when, at a time when he corrected manuscripts by hand on the printed version, he occasionally handed the reviewed manuscript back, which appeared slightly wrinkled and rippled. With an exclusive smile he added, "Oh, I am so sorry, I went to the sauna". It was rare, even when traveling with Detlef Zillikens, that he completely relaxed and took more than a few minutes to talk about private matters. E.S. always remembered one of these rare occasions when sitting in the

FIGURE 5
The International Pemphigus & Pemphigoid Foundation (IPPF) Meeting in Lübeck, Germany, 2017.

evening light in view of the Alhambra castle during the Autoimmune Symposium 2012 in Granada, Spain. Strangely enough, Detlef Zillikens mentioned this evening during our last encounter in the rehabilitation clinic in July 2022.

M.G. first met Detlef Zillikens in 1993 when he started his dermatology residency at the Department of Dermatology, University of Würzburg. Detlef Zillikens was his first attending: he introduced M.G. into general dermatology and stringently

FIGURE 6
Detlef Zillikens (right) with Enno Schmidt, Frédéric Caux, Dolca Thomas, Dedee Murrell, and Katerina Patsatsi (from left to right) at the annual meeting of the European Society of Dermatological Research, Bordeaux, France, 2017.

supervised his clinical activities and rigorously corrected his medical reports. M.G. remembers the daily medical rounds ("Mittagsvisiten") where Detlef, together with Prof. Eva-Bettina Bröcker (at that time chair of the department), Henning Hamm, Boris Bastian (now San Francisco, CA, USA), E.S. and C.R. (now both in Lübeck), and others contributed to an extremely dynamic working atmosphere that fostered intensive clinical and scientific discourse, in which M.G., among many others, was fortunate enough to complete his training. Detlef Zillikens inspired M.G. for AIBDs, and when Detlef Zillikens went to the USA, M.G. was allowed to represent him in this field in Würzburg. Returning to Würzburg, Detlef Zillikens expanded the clinic's autoimmunology diagnostic laboratory, introduced new therapeutic approaches for AIBDs such as immunoadsorption, and was one of the first to use rituximab for AIBDs. Detlef Zillikens and M.G. formed a clinical tandem, which gave both of us considerable freedom for our research interests. This concept was implemented by Prof. Eva-B. Bröcker, which she also introduced for her oncology senior physicians, preceded today's demand for academic support of specialists and senior physicians (now called advanced clinician scientists) by two decades and may have inspired Detlef Zillikens to introduce something comparable in Lübeck. When M.G. became chair of the Dermatology Department in Würzburg, both not only continued their long-standing scientific cooperation with Detlef but also regularly exchanged on the promotion of young scientists and discussed information on clinic matters and key operational figures of their departments. With the passing of Detlef Zillikens, M.G. has lost a passionate teacher and close friend to whom he owes a lot.

Detlef Zillikens has been the exemplary mentor of M.K. for clinical and experimental dermatology at the University of Lübeck for over a decade. With Detlef's most dedicated help, M.K. was able to achieve his academic goals starting from board certification in both dermatology and dermatopathology up to his current professorship at the University of Southern California, Los Angeles, CA, USA. The knowledge that M.K. gained from him concerning the diagnosis and treatment of AIBD patients has been most valuable, and M.K. feels honored to share it now with his new dermatology team including Prof. David Woodley (another well-known clinician/researcher in the field of AIBDs and close colleague of Detlef Zillikens). M.K. remembers well the moment when he started his career at the University of Lübeck. He initially asked Prof. Stefania Jabłońska, Warsaw, Poland, a pioneer in immunodermatology, for advice regarding a high-ranking academic place with a focus on immunobullous diseases. Her answer was, "Prof. Zillikens' clinic, but you would need to work hard". This hard work has been more than rewarding to M.K. M.K. will always mourn the loss of Detlef Zillikens and remember him as a monumental figure in his life.

Detlef Zillikens and T.H. were close friends for a long time starting in 1994 and intensified further following our landmark paper about the first case of anti-200 pemphigoid (see above) (71). Detlef Zillikens and T.H. have published 53 manuscripts together in total. T.H. still well remembers when visiting Detlef Zillikens in Würzburg, the dinner of delicious asparagus in the restaurant overlooking the city of Würzburg. Detlef Zillikens and his wife Monika also treated T.H. in their home with very nice German food. More recently, in 2017, T.H. had a 1-month sabbatical at Lübeck owing to Detlef Zillikens' help. T.H. prays that Detlef's soul rests in peace.

In conclusion, we have lost not only a giant in dermatology but also a wonderful and kind mentor and friend, Detlef Zillikens, to whom this Research Topic of *Frontiers in Immunology* is dedicated.

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

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

JEH: Conceptualization, Writing – review & editing. CDS: Writing – review & editing. NvB: Writing – review & editing. HB: Writing – review & editing. FC: Writing – review & editing. MG: Writing – review & editing. CMH: Writing – review & editing. KH: Writing – review & editing. TH: Writing – review & editing. SI: Writing – review & editing. MK: Writing – review & editing. DFM: Conceptualization, Writing – original draft, Writing – review & editing. AR: Writing – review & editing. CR: Writing – review & editing. NS: Writing – review & editing. IS: Writing – review & editing. CS: Writing – review & editing. PT: Writing – review & editing. DT: Writing – review & editing. RJL: Writing – review & editing. ES: Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

ES and AR have a scientific cooperation with Euroimmun. NB, CH and ES hold patents together with Euroimmun. AR received honoraria from Euroimmun.

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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TYPE Original Research
PUBLISHED 10 November 2023
DOI 10.3389/fimmu.2023.1212551

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RECEIVED 26 April 2023
ACCEPTED 18 October 2023
PUBLISHED 10 November 2023

CITATION
Liu X, van Beek N, Cepic A, Andreani NA, Chung CJ, Hermes BM, Yilmaz K, Benoit S, Drenovska K, Gerdes S, Gläser R, Goebeler M, Günther C, von Georg A, Hammers CM, Holtsche MM, Hübner F, Kiritsi D, Schauer F, Linnenmann B, Huilaja L, Tasanen-Määttä K, Vassileva S, Zillikens D, Sadik CD, Schmidt E, Ibrahim S and Baines JF (2023) The gut microbiome in bullous pemphigoid: implications of the gut-skin axis for disease susceptibility. *Front. Immunol.* 14:1212551. doi: 10.3389/fimmu.2023.1212551

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The gut microbiome in bullous pemphigoid: implications of the gut-skin axis for disease susceptibility

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Bullous pemphigoid (BP) is an autoimmune blistering disease that primarily affects the elderly. An altered skin microbiota in BP was recently revealed. Accumulating evidence points toward a link between the gut microbiota and skin diseases; however, the gut microbiota composition of BP patients remains largely underexplored, with only one pilot study to date, with a very limited sample size and no functional profiling of gut microbiota. To thoroughly investigate the composition and function of the gut microbiota in BP patients, and explore possible links between skin conditions and gut microbiota, we here investigated the gut microbiota of 66 patients (81.8% firstly diagnosed) suffering from BP and 66 age-, sex-, and study center-matched controls (CL) with non-inflammatory skin diseases (132 total participants), using 16S rRNA gene and shotgun sequencing data. Decreased alpha-diversity and an overall altered gut microbial community is observed in BP patients. Similar trends are observed in subclassifications of BP patients, including first diagnoses and relapsed cases. Furthermore, we observe a set of BP disease-associated gut microbial features, including reduced *Faecalibacterium prausnitzii* and greater abundance of

pathways related to gamma-aminobutyric acid (GABA) metabolism in BP patients. Interestingly, *F. prausnitzii* is a well-known microbiomarker of inflammatory diseases, which has been reported to be reduced in the gut microbiome of atopic dermatitis and psoriasis patients. Moreover, GABA plays multiple roles in maintaining skin health, including the inhibition of itching by acting as a neurotransmitter, attenuating skin lesions by balancing Th1 and Th2 levels, and maintaining skin elasticity by increasing the expression of type I collagen. These findings thus suggest that gut microbiota alterations present in BP may play a role in the disease, and certain key microbes and functions may contribute to the link between gut dysbiosis and BP disease activity. Further studies to investigate the underlying mechanisms of the gut-skin interaction are thus clearly warranted, which could aid in the development of potential therapeutic interventions.

KEYWORDS

bullous pemphigoid, skin, inflammation, gut microbiome, 16S rRNA gene, metagenome

Introduction

Bullous pemphigoid (BP) is the most common autoimmune blistering disorder in Europe, with an incidence of 0.0419 per 1000 person-years, which is increasing by time, and typically develops in individuals over the age of 70 years (1–5). It occurs when autoantibodies target the hemidesmosomal proteins BP180 (type XVII collagen) and/or BP230 in the epidermal basement zone (1, 6, 7). Moreover, BP is characterized by subepidermal blistering and is associated with a significantly decreased quality of life, numerous comorbidities, and a significantly increased mortality risk (6). Thus, the need to further understand the pathogenesis and factors associated with the initiation and progression of BP is instrumental to improve the lives of patients suffering from this disease.

Recently, the host microbiota is considered as a key factor affecting host skin health (8–10). The skin microbiome plays a curial role in the maintaining the skin homeostasis, defending the invasion of pathogens and modulating the immune system (11). There is evidence that microbiota composition of lesional skin, in diseases such as atopic dermatitis and psoriatic disease, show distinct differences compared to healthy skin (8, 12–16). Moreover, these alterations are considered to be involved in the development of several inflammatory dermatoses, which is illustrated e.g. by the predominance of *Staphylococcus aureus* in patients with atopic dermatitis before an outbreak (11). Similarly, studies have indicated a crucial role of skin microbiota in the pathogenesis of BP (17, 18). Of those, one large-scale study involving 228 BP patients thoroughly investigated the skin microbial indicators of BP, in which the abundance of *S. aureus*, an inflammation-promoting species, was reported to be increased in BP patients, suggesting a pathogenic role (17).

Apart from the skin microbiota, the role of gut microbiome in host skin health and disease is also gaining interest. Recent studies revealed intriguing links between gut microbiota and skin diseases (9, 19–22). Firstly, some dermatoses pose as comorbidities of gastrointestinal disorders. For example, 7–11% of patients with inflammatory bowel disease (IBD) also suffer from psoriasis (9). Moreover, multiple inflammatory skin disorders including psoriasis (19, 20, 23–25) and atopic dermatitis (26, 27), are accompanied by gut dysbiosis with altered diversity and composition of the gut microbiota. Decreased levels of *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* in the gut were observed in patients with psoriasis (23, 24, 28), while *Escherichia coli* was reported to be increased in patients with both psoriasis (24) and atopic dermatitis (27). Similarly, a recent study thoroughly characterized the alterations in the gut microbiota in pemphigus, another autoimmune bullous disease, whereby potentially pathogenic bacteria such as *E. coli* and *Bacteroides fragilis* were enriched in patients, while other anti-inflammatory and butyric acid-producing bacteria were significantly reduced (29). On the other hand, gut dysbiosis could increase host vulnerability and trigger an immunological response, resulting in skin imbalances (21, 30). This intricate, bidirectional link known as the "gut-skin axis", is also thought to involve microbial metabolites such as short chain fatty acids (SCFAs) and gamma-aminobutyric acid (GABA), with SCFAs enhancing epithelial barrier function and GABA inhibiting itch-signaling (30).

The gut microbiota in patients with BP, however, remains largely unexplored, with only a single study to date, with a small sample size and no functional shotgun sequencing data (31). To further investigate the composition and function of the gut microbiota in BP patients, and explore possible interaction between skin conditions and gut microbiota, we thus here conducted a larger study with 16S rRNA gene sequencing and functional shotgun metagenomic sequencing data of 132 individuals. By comparing 66 BP patients and their age-, sex-, and study center-matched controls, we reveal a decreased

community richness, altered beta diversity, as well as microbial and functional indicators for disease in BP patients, including *F. prausnitzii*, *Flavonifractor plautii*, and GABA metabolism-related pathways. We furthermore sub-grouped the patients and their corresponding controls according to their disease status (firstly diagnosed and relapsed BP), which reveals similar trends in both clinical conditions.

Materials and methods

Ethics statement

All procedures involving human subjects were conducted in accordance with the standards of the ethical policies and procedures approved by the ethics committee of the University of Lübeck (Approval no. 15–051, 18–046), and the respective committees of the study centers, adhering to the Declaration of Helsinki. All participants provided written, informed consent.

Participant recruitment

A total of 66 pairs of patients with BP and age-, sex-, and study center-matched controls (CL) with non-inflammatory skin diseases such as basal cell carcinoma or squamous cell carcinoma were included in this study. Participant pairs were recruited from seven study centers across Europe (Germany: Dresden (n=14), Freiburg (n=12), Kiel (n=3), Lübeck (n=7) and Würzburg (n=22); Finland: Oulu (n=3); Bulgaria: Sofia (n=5)) from June 2015 to July 2020. All participants were of European descent. Inclusion criteria of BP patients required a diagnosis according to national and international guidelines as described before (17), and no topical antiseptics usage within the prior two weeks and no antibiotic therapy four weeks prior to sampling. Patients can be further grouped into two sub-classifications according to their disease status, firstly diagnosed (BPF) and relapsed ones (BPR). Exclusion criteria for controls included the presence of inflammatory/infectious dermatoses and systemic antibiotics usage within the last four weeks as well. Metadata of BP patients and their controls are provided in Supplementary Table S1 and summarized demographics are provided in Supplementary Table S2.

Sample collection

Patient and control fecal samples were collected at each study center and immediately frozen at -80°C until further use. Specifically, fecal sampels were transferred with sterile CE-certified plastic spoons into sterile plastic containers (Sarstedt, Numbrecht, Germany) from stool not touching the collection container. DNA extraction from stool samples was performed with QIAamp PowerFecal DNA Kit (Qiagen) following the manufacturer's instructions. Briefly, a pea-size aliquot of the sample was resuspended in 750 µl of PowerBead Solution and moved to a sterile bead tube with a wide bore tip. Final elution volume was 100 µl and DNA was stored at -20°C until further use. DNA was diluted 1:10 before library preparation.

16S rRNA and data processing

The V1-V2 region of the 16S rRNA gene was amplified using 27F and 338R primers using a dual barcoding approach as described in our previous analysis of the skin microbiota in BP (17). The resulting library was sequenced on an Illumina Miseq sequencer (250PE). During the demultiplexing process, only barcodes with no mismatches were allowed (Casava, Illumina). Raw 16S rRNA gene sequencing data were processed using QIIME2 (v2022.8) (32). Specifically, sequences were firstly denoised by the embedded DADA2 (33) for trimming the first 7 or 9 bases from 5' end of forward or reverse read sequences, truncating the 230 or 200 bases from 3' end of forward or reverse sequences, and reads were also truncated at the first instance of a quality score smaller than or equal to 3. An abundance table of 16S rRNA amplicon sequence variants (ASVs) was generated, and the taxonomic annotation of ASVs was obtained using the SILVA 138 database (34). To normalize sequencing depth, the samples were randomly sub-sampled to 11,000 sequences. Subsequently, a total of 3,716 ASVs were identified and included in downstream analyses.

Metagenomic shotgun sequencing and data profiling

Paired-end metagenomic shotgun sequencing was performed on the Illumina NovaSeq platform. Raw metagenomic sequencing reads were subjected to quality control by KneadData v0.12.0 (https://github.com/biobakery/kneaddata), with usage of trimmomatic 0.39 (35) to filter and remove reads with low-quality and reads aligned to the host genome. The clean reads were then aligned to the database PlusPFP (v.20220908, https://benlangmead.github.io/aws-indexes/k2) using Kraken v2 2.1.2 (36, 37), and the taxonomic information at the species level was obtained with Bracken v2.8 (38). The eukaryotic reads (taxid 2759 in NCBI) in clean data were removed using report files produced by Kraken, and the functional profiling was conducted using the Uniref90 protein database in HUMAnN v3.6 (39) with concatenated paired fastq files. This yielded 2,938 species and 417 pathways that were used for downstream analyses.

Microbial diversity

Analyses of diversity indices were conducted in the R package vegan (R version: 4.2.2; vegan version: v2.6-4). Alpha diversity was assessed by the Chao1 index and Shannon indices, and differences between groups were determined by a non-parametric Wilcoxon

test. Statistical analyses of beta diversity based on Bray-Curtis dissimilarity were performed using a non-parametric multivariate analysis (PERMANOVA) (40), which accounts for the effects of confounding factors, including study centers, sex, and age, on microbiome composition. The test was conducted by using the "adonis" function, and the empirical p value was obtained by running 9,999 permutations. When appropriate, statistical p values were adjusted by the Benjamini-Hochberg procedure. For species detected by Kraken and Bracken in the shotgun data, samples were randomly sub-sampled to 1,015,546 reads for diversity analysis.

Disease-associated gut microbial feature identification

To identify candidate gut microbial features associated with BP, we first performed quality control filtering for taxonomic and functional features. Specifically, an ASV, a species or a pathway needed to have a minimum prevalence of 25%, and minimum relative abundance of 0.01% in at least 10% samples. Gut microbial features associated with BP disease were then determined using linear discriminant analysis effect size (LEfSe) analysis (41), with an absolute value of LDA score no less than 2, and a p value <0.05. The Multivariate analysis by linear models (MaAsLin2) (42, 43) was then applied to evaluate associations between BP disease status and microbial features, correcting for the effect of study center, sex and age, using the Maaslin2 package (v1.12.0) in R. The arcsine square root transformation was performed on relative abundances, and the centred log ratio normalization method in the "Maaslin2" function was performed. Following a similar approach to Kong et al. (43), we considered the intersection of results of LEfSe and MaAsLin2 analyses (abs (LDA) >2, and p value <0.05 in LEfSe analysis; and p value < 0.05 in MaAsLin2 analysis) as criteria for identifying differential microbial features in BP patients.

The Bullous Pemphigoid Disease Area Index (BPDAI) is a validated scoring system commonly used to evaluate the severity of BP disease (17). It takes into account disease activity assessments on both the skin and mucosal surfaces. To evaluate potential correlations between microbial features and BP disease severity, we used the BPDAI scores that were available for a subset of BP patients (44 out of 66). Spearman correlations, corrected for study center, were computed using the "cor.test" function in R. To avoid the influence of zero-inflation, only species or pathways with a prevalence of at least 50% were selected to be included in the correlation analysis. When appropriate, statistical p values were adjusted by the Benjamini-Hochberg procedure.

Results

A total of 66 pairs of patients with BP and their matched controls (CL) were recruited from seven study centers located in Germany (5/7; Würzburg, Dresden, Freiburg, Lübeck, and Kiel), Finland (1/7; Oulu), and Bulgaria (1/7; Sofia) between June 2015 and July 2020 (Tables S1, S2). Of the 66 BP patients, 54 (81.8%) were firstly diagnosed (BPF), 11 (16.7%) had relapsed (BPR), and one (1.5%) was unspecified. The average age of BP patients was 80.26 years (range: 63-98), while that of the 66 controls was 80.64 years (range: 62-100). Of the BP patients and controls, 66.7% were males and 33.3% were females (Tables S1, S2). Fecal samples were collected from BP patients and their corresponding controls, and bacterial genomic DNA was extracted from the samples. Subsequently, 16S rRNA gene sequencing and shotgun sequencing were conducted (Figure 1). To explore the potential impact of disease status on gut microbiota, the BP patients and their corresponding HC were sub-grouped into those who were firstly diagnosed and their matched controls (BPF and CLF) and those who had experienced relapses and their controls (BPR and CLR) (44) (Figure 1).

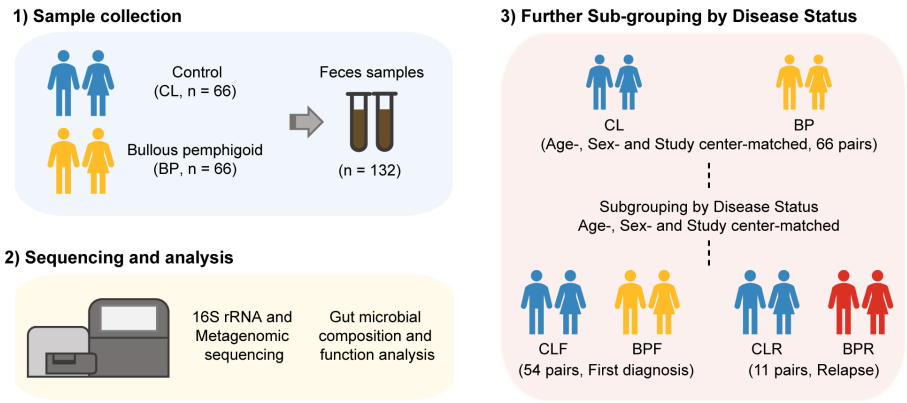

FIGURE 1
Workflow of investigation on gut microbiome in Bullous pemphigoid (BP) disease. Feces samples were collected from 66 BP patients and their age-, sex- and study center-matched controls (CL). 16S rRNA gene and metagenomic sequencing was then performed to get the profile of gut microbiome of BP patients. The participants were next sub-grouped by disease status of BP patients, that is, firstly diagnosis (CLF and BPF) and relapse (CLR and BPR).

Decreased alpha diversity and altered overall gut microbial composition in BP patients

We firstly utilized 16S rRNA gene sequencing data to investigate both alpha and beta diversity in BP patients at the level of amplicon sequence variants (ASVs), which were rarefied to 11,000 reads per sample. Alpha diversity was then assessed with Chao1 index and Shannon indices, which provide measure of richness and evenness of the microbiome, respectively. A significant decrease of the Chao1 index is observed in BP patients, while only a trend towards lower values of the Shannon index is observed (Figure 2A). Following the aforementioned sub-grouping, a significant decrease in the Chao1 index is also observed in BPR compared to the CLR group (Figure 2A).

Subsequently, beta diversity was analyzed based on Bray-Curtis dissimilarity, and a partial constrained principal coordinate analysis (PCoA) was conducted to examine clustering according to disease status (Figure 2B). This reveals a significant difference between BP patients and their controls (adonis R^2 = 0.0123, p =0.0004), as well as in a comparison of firstly diagnosed BP patients and their controls (adonis R^2 = 0.0139, p = 0.0017). No significant difference is observed between relapsed BP patients and their corresponding controls (adonis R^2 = 0.0528, p = 0.138), which may be attributed to sample size limitations. We next evaluated the extent to which confounding factors such as study center, age, and sex contributed to variance in microbial diversity. In line with the previous reports, our analysis indicates that microbial composition is significantly impacted by study center (44), in addition to disease status and age (PERMANOVA q value < 0.05) (Figure 2C). A parallel analysis was performed using shotgun data, which, consistent with the findings in 16S rRNA sequencing data, reveals a reduction in the Chao1 index and significant differences in beta-diversity according to disease status (Figure S1).

Disease-associated gut microbes in BP patients

Following the observation of altered patterns of diversity in BP patients, we performed analyses to identify individual taxa contributing to these patterns, both in the 16S rRNA gene and shotgun data. We first conducted linear discriminant analysis effect size (LEfSe) analysis (41) to identify differentially abundant microbial signatures in BP patients, as well as the two BP sub-groups. Multivariate association with linear models (MaAsLin2) (42) was then applied to control for potentially confounding factors, including study center, age and sex. The intersection of significantly differential features identified by LEfSe and MaAsLin2 were deemed as credible BP-associated gut microbial features, following similar previous studies [(43); see Methods]. Compared to the controls, a total of four ASVs are significantly more abundant in BP patients, whereas seven ASVs are significantly reduced (Figures S2A, B). Of the increased ASVs, ASV_219, is enriched in relapsed BP patients as well (Figures S2D, E), which primarily matches the genus *Flavonifractor* in the SILVA 138 database (34), and *Flavonifractor plautii* when queried in NCBI (E = 2e-154). Conversely, two out of seven ASVs reduced in BP groups belong to *Faecalibacterium*, together with one closely matching *Sutterella* (Figures S2A, B). Notably, ASVs matching *Faecalibacterium* are also reduced in both

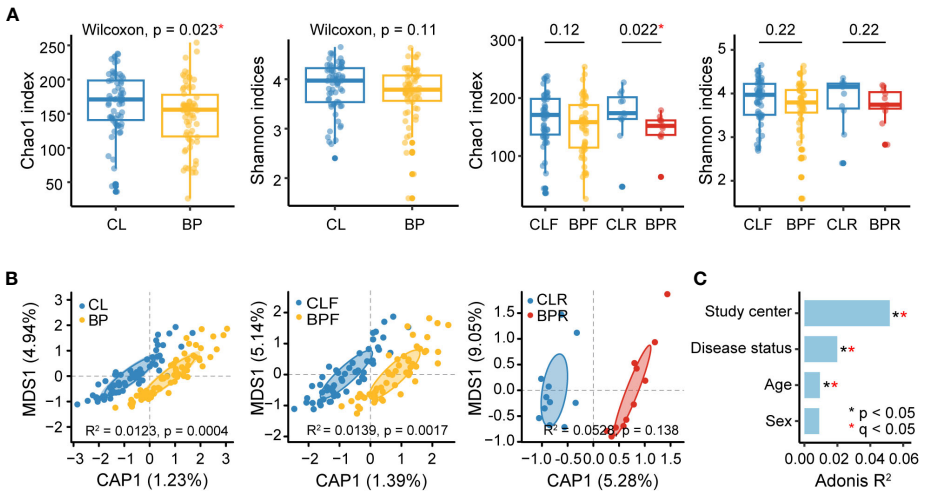

FIGURE 2
Diversities of gut microbiome of BP patients and their matched controls at the ASV level. **(A)** Alpha diversity of gut microbiome in BP, BPF, BPR and their controls. Abundances of amplicon sequence variants (ASVs) were computed and alpha diversity was assessed by richness (Chao1index) and evenness (Shannon indices). Difference between groups was measured by non-parametric Wilcoxon test. **(B)** Principal coordinates analyses (PCoA) of gut microbiome composition of BP, BPF, BPR groups and corresponding controls (CL, CLF, and CLR) by Bray-Curtis dissimilarity. Statistical significance of beta diversity difference between groups was computed by PERMANOVA in "adonis" function. **(C)** Effect size (Adonis R^2) of confounding factors and disease status significantly associated with gut microbial variations (Bray-Curtis dissimilarity, PERMANOVA). Statistical p values were adjusted by Benjamini-Hochberg procedure. The "*" means statistical significance based on nominal p value and the "*" in red refer to an adjusted p value (q) smaller than 0.05.

subgroups of BP patients (Figures S2C-E). Altogether, alterations in the genera *Flavonifractor* and *Faecalibacterium* appear to be associated with BP disease.

Next, we performed the same differential analyses using shotgun data at the species level. This reveals three species to be enriched in BP patients: *Ruthenibacterium lactatiformans*, *Anaerotruncus colihominis*, and *Eubacterium callanderi*. In contrast, *Prevotella copri*, *Faecalibacterium prausnitzii* and *Faecalibacterium* sp. *I417* are decreased in BP patients (Figures 3A, B). Of the three increased in BP, *R. lactatiformans* and *A. colihominis* are more abundant in BP patients who were newly diagnosed (BPF), along with *Bacteroides eggerthii* and *Bifidobacterium dentium* (Figures 3C, E). Conversely, *Sutterella wadsworthensis* was found to be decreased in the BPF group. The differential analysis between individuals with relapsed BP disease (BPR) and their controls (CLR) identifies a significant increase in six bacterial species, including *F. plautii*, along with a reduction in the abundance of three other species, such as *Alistipes shahii* (Figure 3D). Overall, the observed changes in gut microbial species, including *F. prausnitzii*, *F. plautii*, *R. lactatiformans*, and *A. colihominis*, are likely associated with BP. Notably, *F. plautii* appears to have a more prominent association with BP disease relapse, which is consistent with the findings from the 16S rRNA gene data.

Functional alterations in the gut microbiome of BP patients

To investigate differences in the functional potential in the gut microbiome of BP patients, we used the HUMAnN3 workflow [(39); see Methods], followed by the same differential analyses that were applied to the taxonomic data described above. A total of twelve gut microbial pathways are significantly altered in relation to BP. Among the nine pathways more represented in BP groups, two are related to pyridoxal 5'-phosphate biosynthesis [PYRIDOXSYN-PWY (pyridoxal 5'-phosphate biosynthesis I), PWY0-845 (superpathway of pyridoxal 5'-phosphate biosynthesis and salvage)]; three involve fatty acid biosynthesis, [PWY-5989 (stearate biosynthesis II (bacteria and plants)), FASYN-ELONG-

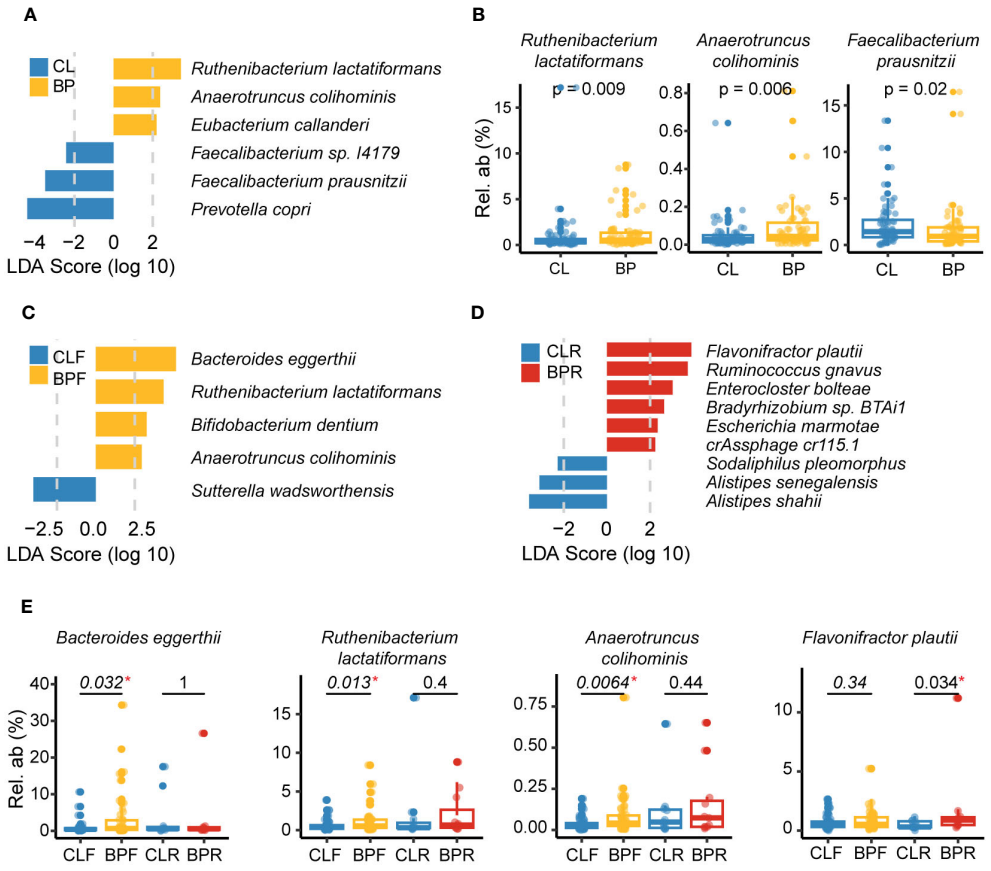

FIGURE 3
Gut microbes altered in BP disease at the species level. **(A)** Differential species identified by combination of linear discriminant analysis effect size (LEfSe) analysis and Multivariate analysis by linear models (MaAsLin2) in comparison of BP and CL groups based on the metagenomic sequencing. Specifically, the species with an absolute value of LDA score >2 and p <0.05 (calculated by non-parametric Wilcoxon test) in LEfSe analysis was identified as candidates of differential species, and MaAsLin2 was then applied to adjusted effect of study center, age and sex (p <0.05). The blue bars show the bacterial taxa with decreased relative abundance in BP group and the yellow ones refer to those species enriched in BP group after adjusting for the study center using MaAsLin2. **(B)** Representative species that were significantly changed in BP group. The P values were computed by non-parametric Wilcoxon test. **(C, D)** Differential species detected by comparing **(C)** BPF and their matched controls (CLF), or **(D)** BPR and their controls (CLR). Bars with different colors represent altered species in different groups. **(E)** Representative species that were significantly altered in firstly diagnosed or relapsed BP patients (BPF or BPR). The P values were computed by non-parametric Wilcoxon test. "*" means statistically significant in non-parametric Wilcoxon test.

PWY (fatty acid elongation – saturated), and PWY-6282 (palmitoleate biosynthesis I (from (5Z)-dodec-5-enoate))]; two are related to biotin biosynthesis [BIOTIN-BIOSYNTHESIS-PWY (biotin biosynthesis I) and PWY-6519 (8-amino-7-oxononanoate biosynthesis I), with amino-7-oxononanoate serving as a biotin precursor (https://metacyc.org)]. The remaining two are PWY-6305 (superpathway of putrescine biosynthesis) and GLUDEG-I-PWY (GABA shunt). In contrast, two pathways in relation to adenosine nucleotides biosynthesis [(PWY-6126 and PWY-7229, superpathway of adenosine nucleotides *de novo* biosynthesis II and I), and PWY1ZNC-1 (assimilatory sulfate reduction IV)] are reduced in BP patients (Figures 4A, B).

Notably, the pyridoxal 5'-phosphate serves as a critical cofactor for converting glutamate to GABA (45), which can be involved in the GABA shunt. GABA shunt is a pathway involving the conversion of L-glutamate into GABA, and next converting GABA into succinate with the presence of α-Ketoglutaric acid, which is then coupled with the tricarboxylic acid cycle (46). Additionally, putrescine, whose production was elevated in BP patients, was reported to be a disrupter of intestinal barrier function, with its oral administration increasing gut permeability in mice (47), and furthermore to have immunoregulatory potential such as increasing anti-inflammatory macrophages in the colon and positively correlating with the proinflammatory chemokine CXCL8 in psoriasis patients (48, 49). Moreover, putrescine metabolism also can be linked with GABA shunt, as it is a potential source of GABA to the brain, pancreas, and bacteria including *E.coli* (50–52). We therefore evaluated gut microbial candidate species contributing to PWY-6305 and GLUDEG-I-PWY in the shotgun metagenomic data using the HUMAnN3 workflow. Interestingly, this analysis identifies *E.coli* as the predominant species associated with these two pathways (Figure 4C).

Subsequent comparison between BPF and CLF groups revealed six altered pathways in the newly diagnosed BP patients, including three differential pathways identified in the BP group, namely PYRIDOXSYN-PWY, PWY0-845 and PWY1ZNC-1 (Figure 4D). Additionally, the other two pathways which were more represented in the BPF group are HSERMETANA-PWY (L-methionine biosynthesis III) and PWY0-1241 (ADP-L-glycero-β-D-manno-heptose biosynthesis). Conversely, the PWY-6527 (stachyose degradation) is one of two pathways reduced in BPF group. We next compared the abundance of pathways between BPR and CLR groups, and a total of ten differential pathways were identified. Notably, we observed that four out of five pathways enriched in the BP group are also significantly enriched in the BPR group (Figures 4E, F). Another enriched pathway is GALACTITOLCAT-PWY (galactitol degradation). Five pathways that were decreased in the BPR group are PWY-5705 (allantoin degradation to glyoxylate III), PWY-7242 (D-fructuronate degradation), ARGDEG-PWY (superpathway of L-arginine, putrescine, and 4-aminobutanoate degradation), ORNARGDEG-PWY (superpathway of L-arginine and L-

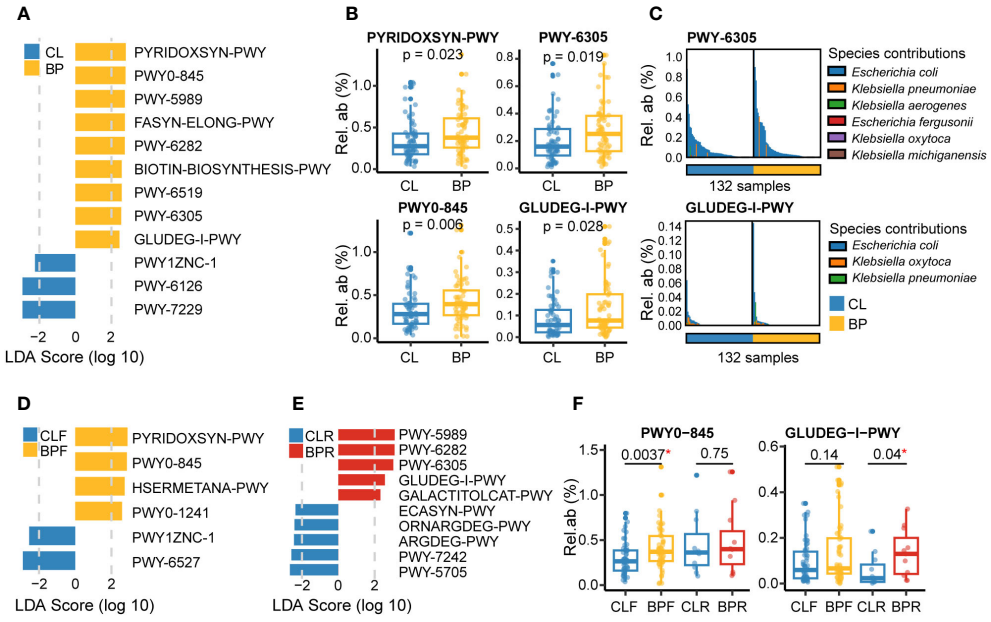

FIGURE 4
Gut microbial functions altered in BP disease based on the metagenomic sequencing. **(A)** Differential metagenomic pathways altered in BP patients versus their matched control (CL), which were identified by LEfSe analysis (absolute value of LDA score >2 and p <0.05; p values were calculated by non-parametric Wilcoxon test), with MaAsLin2 correcting effect of study center, age and sex (p <0.05). **(B)** Relative abundance of four representative pathways that were significantly changed in BP group. The P values were computed by non-parametric Wilcoxon test. **(C)** Dominant microbial species contributing to two representative pathways: PWY-6305 and GLUDEG-I-PWY, referring to superpathway of putrescine biosynthesis and GABA shunt, respectively. Bars with different colors means different species. **(D, E)** Differential gut microbial functions detected in the comparisons of **(D)** BPF versus their controls (CLF), or **(E)** BPR versus their controls (CLR). **(F)** Relative abundance of two representative pathways that were significantly altered in firstly diagnosed or relapsed BP patients (BPF or BPR). The PWY0-845 means superpathway of pyridoxal 5'-phosphate biosynthesis and salvage. The P values were computed by non-parametric Wilcoxon test. "*" means statistically significant in non-parametric Wilcoxon test.

ornithine degradation), and ECASYN-PWY (enterobacterial common antigen biosynthesis). Among them, ARGDEG-PWY is a pathway involved in GABA (4-aminobutanoate) degradation, and is hence also linked to the GABA shunt.

BP disease severity-associated gut microbial species and functions

The severity of BP disease is commonly assessed using the BPDAI score, a validated scoring system that incorporates disease activity assessments on both the skin and mucosal surfaces (17). To evaluate the potential relationship between gut microbial features and BP severity, we tested for correlations with BPDAI scores, which were available for a subset of BP patients (44 out of 66). The analysis was limited to species or pathways with a prevalence of 50% or greater. After calculating Spearman's correlation coefficients and correcting for the effect of study center, we identify 30 species and 49 pathways with nominally significant association to the BPDAI score ($p < 0.05$) (Figure S3). After correction for multiple testing, no association is significant (q value > 0.05), although we considered associations with q values < 0.25 as "possible" candidates. Among these, *E. coli* abundance displays the highest positive correlation with BP disease severity (Spearman's rho = 0.48, p = 0.001, q = 0.2). In terms of pathways, three out of 49 were negatively correlated with BPDAI scores, namely 1CMET2-PWY (folate transformations III (*E. coli*); Spearman's rho = -0.39, p = 0.0082, q = 0.15), DTDPRHAMSYN-PWY (dTDP-β -L-rhamnose biosynthesis; Spearman's rho = -0.39, p = 0.0089, q = 0.16), and PWY-6609 (adenine and adenosine salvage III; Spearman's rho = -0.35, p = 0.02, q = 0.24). Conversely, PWY-5723 displays the highest positive correlation with BPDAI scores (Rubisco shunt; Spearman's rho = -0.49, p = 0.00075, q = 0.14), together with the two differential pathways in relation to GABA metabolism, namely the GABA shunt (GLUDEG-I-PWY, Spearman's rho = -0.36, p = 0.017, q = 0.22), and PWY-6305 (Spearman's rho = -0.31, p = 0.038, q = 0.28, nominally associated). Overall, the positive correlations between GLUDEG-I-PWY, PWY-6305 and *E. coli* (the aforementioned dominant species involved in the GLUDEG-I-PWY and PWY-6305 pathways) with BP disease severity again suggest a potential link between the GABA shunt and BP.

Discussion

Our study provides a first thorough analysis of the gut microbiome in BP, at both the taxonomic and functional metagenomic level. We find significant alterations of diversity patterns and promising candidate pathways such as the GABA shunt. These findings are in agreement with previous studies reporting altered microbial diversity in various inflammatory and autoimmune diseases, and in particular represents an additional example of a skin disease associated with an altered gut microbiome (53–58).

Among the significant microbial features identified by 16S rRNA gene analysis, increases in abundance of the genus *Flavonifractor* and a decrease of *Faecalibacterium* are of particular interest. *F. prausnitzii* is a well-known microbiomarker of inflammatory diseases, including Crohn's disease (59, 60), irritable bowel syndrome (61), multiple sclerosis (44), rheumatic diseases (62), and even other inflammatory skin diseases, such as atopic dermatitis, psoriasis, and another autoimmune bullous disease, pemphigus (19, 21, 29). *F. prausnitzii* possesses anti-inflammatory properties, which could contribute to its potential protective effects in inflammatory diseases (63). A reduction of *F. prausnitzii* was also observed in the shotgun data, which adds further reliability to this result. Similarly, the shotgun data also confirms an increase of *F. plautii* in relapsed BP patients, and *F. plautii* was reported to be enriched in early-onset colorectal cancer (43, 64). It is thus intriguing that the increase of *Flavonifractor* and positive correlation between *Flavonifractor* and circulating inflammatory markers (C5a, IL-6, IL-8, IL-7, IL-1β and IL-21) were reported in pemphigus vulgaris, a subgroup of pemphigus (65). *Flavonifractor* was shown to have the ability to cleave quercetin, which has anti-oxidant and anti-inflammatory properties (66). It is thus possible that increased *Flavonifractor* could contribute to oxidative stress and inflammation in the host. Moreover, investigations on a potential immunoregulatory role of *F. plautii* based on mouse models revealed it to inhibit the Th2 immune response, TNF-α expression and interleukin (IL)-17 signaling, thus, potentially alleviating inflammatory responses in allergic diseases, adipose tissue in obesity, and gut inflammation, respectively (67–69). Given these diverse observations, *F. plautii* may contribute to the dysregulation of the immune system in BP leading to the onset or progression of disease. Further research is needed to fully understand the role of *F. plautii* in BP disease. Two other notable species of interest include *R. lactatiformans* and *A. colihominis*, which are increased in the BP and BPF groups; interestingly, both were reported to be associated with multiple sclerosis (44, 70).

Importantly, our inclusion of shotgun metagenomic sequence data provides critical insight into candidate functional pathways associated with BP. In particular, we identified the GABA shunt and related pathways, two involving the biosynthesis of a key cofactor (pyridoxal 5'-phosphate) in GABA conversion into succinate, and one involving biosynthesis of precursor (putrescine) of GABA, which points towards an underlying importance of the gut microbial GABA shunt in BP pathophysiology. Of note, the key metabolite in this bioprocess, GABA, known as an inhibitory neurotransmitter, has various biological activities, such as antioxidant and anti-inflammation (71), and plays multiple roles in maintaining skin health, including inhibition of itching by acting as an inhibitory neurotransmitter (9, 30), attenuating skin lesions by

balancing Th1 and Th2 levels and maintaining skin elasticity by increasing the expression of type I collagen (30). Interestingly, GABA metabolism was also reported to be altered in patients with atopic dermatitis, with degradation being significantly positively associated with reduction of disease severity and GABA biosynthesis from putrescine being positively associated with disease severity (72), which suggests a possible positive association between microbial-derived GABA and atopic dermatitis. Further, GABA or GABA receptor agonists were reported to alleviate induced atopic dermatitis or acute skin inflammation in mouse models (73, 74). In the current study, the GABA shunt was observed enriched in the BP patients, with the increased biosynthesis of a key cofactor in GABA shunt, and increased biosynthesis of a precursor of GABA. However, there is evidence that components and activity of GABA shunt can vary a lot even within the same genus (75), which suggests that more refined profiles on GABA metabolism in the gut microbiome need to be carried out to clearly investigate the changes in GABA, GABA metabolism and signaling in BP.

In addition to the GABA shunt and related pathways, other notable pathways, such as PWY-6282 (palmitoleate biosynthesis I (from (5Z)-dodec-5-enoate)), which is higher in BP, overlaps with a previous report showing its increase in individuals with irritable bowel syndrome (76). In contrast, a study investigating links between the human gut microbiome and inflammatory cytokine production reported anti-inflammatory properties of palmitoleic acid (or palmitoleate) by reducing monocyte-derived cytokines, such as IL-1β, TNFα, and IL-6 (77). Finally, the ADP-L-glycero-β-D-mano-heptose in PWY0-1241 (ADP-L-glycero-β-D-manno-heptose biosynthesis), which is more presented in the newly diagnosed BP patients, can be involved in the involved in the synthesis of bacterial lipopolysaccharides (LPS) (78).

Our study has limitations, including a comparatively small sample size and lack of longitudinal data and metabolome data. Further, our age and sex match controls included individuals with basal cell carcinoma or squamous cell carcinoma, thus, it is possible that differences observed in BP could also reflect elements of this type of cancer in the controls. However, the inclusion of these individuals as controls was motivated by the fact that the basal cell carcinoma and squamous cell carcinoma are mainly epidermal skin cancers, characterized by skin lesions or abnormalities, but only rarely metastasize (79, 80). We also excluded patients with any possible metastases to make sure the cancer was always restricted to the epidermis. Moreover, gut dysbiosis was not reported in patients with these cancers thus far (9), despite an altered gut microbiome being reported in other types of cancer, including melanoma, which in contrast can be characterized by rapid metastasis (81, 82). On the other hand, given that these cancers are so common within the elderly, it can be reasonably argued that they genuinely reflect the gut microbiome of this age group (83, 84).

Despite these limitations, our study contributes to a growing body of evidence of common dysbiotic features in the gut microbiome across inflammatory diseases (i.e. reduced alpha diversity, reduced *F. prausnitzii*, role for GABA-related pathways), including those afflicting the skin, and as such emphasize the importance of the gut-skin axis. Future studies including longitudinal data and experimental preclinical models are thus justified to help establish causality and to test microbiome-based intervention strategies.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://ncbi.nlm.nih.gov/ under the BioProject accession number PRJNA954584.

Ethics statement

The studies involving humans were approved by The ethics committee of the University of Lübeck and respective committees of the study centers, adhering to the Declaration of Helsinki. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, CS, ES, SI, and JB. Methodology, XL, NB, AC, NA, JB. Software, XL, AC. Validation, XL, AC, JB. Formal Analysis, XL, AC, NA. Investigation, NA, CC. Resources, NB, SB, KD, SG, RG, MG, CG, AG, CH, MH, FH, BL, LH, SV, CS, ES, SI, JB. Data Curation, XL, NB, AC, NA, CC. Writing – Original Draft Preparation, XL, BH, JB. Writing – Review & Editing, all authors. Visualization, XL, AC, JB. Supervision, JB. Project Administration, NB, ES, SI, JB. Funding Acquisition, CS, ES, SI, JB. Data curation, Writing – original draft, FS. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the German Research Foundation (DFG), through Clinical Research Unit 303, project number 269234613, subproject P2, jointly awarded to JB and ES, the Collaborative Research Center 1526, project number 454193335, subproject B07 jointly awarded to JB and SI, Research Unit 5042 “miTarget”, project number 426660215, subproject P10 awarded to JB, and the Cluster of Excellence 2167 ‘Precision Medicine in Chronic Inflammation (PMI)’ (grant no. EXC2167).

Acknowledgments

We thank Katja Cloppenborg-Schmidt for excellent technical assistance. We are grateful to Sarah Gaugel and Stephanie Freyher for technical assistance with sample storage, and Ana Luiza Lima and Onur Dikmen for assistance with sample storage and communication with study centers during various phases of the study.

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.2023.1212551/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 05 December 2023
DOI 10.3389/fimmu.2023.1241461

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OPEN ACCESS

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RECEIVED 16 June 2023
ACCEPTED 21 November 2023
PUBLISHED 05 December 2023

CITATION
Akbarzadeh R, Czyz C, Thomsen S-Y, Schilf P, Murthy S, Sadik CD and König P (2023) Monocyte populations are involved in the pathogenesis of experimental epidermolysis bullosa acquisita. *Front. Immunol.* 14:1241461. doi: 10.3389/fimmu.2023.1241461

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Monocyte populations are involved in the pathogenesis of experimental epidermolysis bullosa acquisita

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Monocytes play a significant role in the pathogenesis of most inflammatory diseases, including autoimmune diseases. Herein, different subpopulations of monocytes often play differential, partially antagonistic roles, in the regulation of tissue populations. Pemphigoid diseases constitute a group of autoimmune blistering skin diseases featuring a marked infiltration of the dermis with immune cells, including monocytes. The monocyte subsets infiltrating the skin, however, have largely remained elusive. Monocyte adhesion and recruitment into the inflamed tissues are regulated by chemokine receptors, most prominently by CCR2 and CX3CR1. To delineate the involvement of monocyte populations in autoimmune blistering skin diseases, we spatiotemporally monitored the dynamic spectrum of monocyte populations that infiltrate the inflamed skin using multiphoton intravital imaging and reporter mice for chemokine receptors. Experimental epidermolysis bullosa acquisita (EBA) was induced by injection of anti-murine type VII collagen (amCOLVII) IgG into the $Csf1r^{EGFP}$-reporter mice, where circulating myeloid cells, such as monocytes and neutrophils, express an EGFP. $EGFP^+$ cells, including neutrophils and monocytes, were present in the skin, immediately after the deposition of the amCOLVII antibody at the dermal-epidermal junction. To investigate the recruitment and involvement of different monocyte-derived cell populations in the disease course further, EBA was induced in $CCR2^{RFP/+}$-reporter and $CX3CR1^{GFP/+}$-reporter mice. A comparable distribution of red fluorescent protein $(RFP)^+$ or green fluorescent protein $(GFP)^+$ was found in both diseased mice and their respective controls over time, indicating the similar recruitment of monocytes into the skin following the binding of autoantibodies. Experiments were extended to the $CCR2^{RFP/RFP}$-deficient and $CX3CR1^{GFP/GFP}$-deficient mice to determine whether monocyte recruitment and disease severity are compromised in the absence of the receptor. A comparable pattern was seen in the recruitment of monocytes into the skin in both reporter and deficient mice. However, in contrast to similar disease severity between CX3CR1-deficient and reporter mice, CCR2-deficient mice developed significantly less disease than CCR2-

reporter mice, as indicated by the percentage of affected area of ears. Collectively, our observations indicate that while CCR2 and CX3CR1 receptors are not involved in the recruitment of monocytes into the skin, CCR2 deficiency is associated with improved disease outcomes in experimental EBA in mice.

KEYWORDS

autoimmune disease, monocyte, CCR2, CX3CR1, intravital imaging

Introduction

Epidermolysis bullosa acquisita (EBA), a rare autoimmune bullous disease, is characterized by the presence of autoantibodies directed against type VII collagen (COLVII) (1). Following the binding of autoantibodies to their antigen, a proinflammatory milieu is generated, in which the infiltration of effector cells is the key pathogenic process initiating and fueling skin inflammation, leading to blister formation (2). In contrast to other cell types, such as neutrophils that are associated with autoantibody-induced tissue damage (2, 3), infiltration kinetics and the contribution of monocytes in skin lesions of EBA in both patients and the experimental models have not been studied in detail.

Bearing wide immune-modulatory and inflammatory potency, monocytes and their infiltration into the inflamed tissue are the hallmarks of several autoimmune diseases, such as systemic sclerosis and rheumatoid arthritis (4–6). Circulating monocytes are heterogeneous, plastic populations that are categorized into at least two subpopulations, classical monocytes (lymphocyte antigen 6 complex locus C (Ly6C)^{hi} in mice, CD14^{+}CD16^{-} in humans) and non-classical monocytes (Ly6C^{lo} in mice, CD14^{dim}CD16^{+} in humans) with different biological functions (7, 8). The classical, or inflammatory monocytes migrate into inflamed tissue and differentiate further into pro-and anti-inflammatory macrophages that can promote tissue injury or remodeling (9). Conversely, non-classical, or patrolling monocytes scrutinize the blood vessel to maintain vascular homeostasis (10). Chemokines and their receptors play an essential role in the spatiotemporal dynamics of effector cells and their navigation into the peripheral tissues (11). Distinct expression of the chemokine receptors CC-chemokine receptor 2 (CCR2) and C-X3-C motif chemokine receptor 1 (CX3CR1) is associated with monocyte subsets, in which classical monocytes express predominantly CCR2 and the non-classical subsets express lower CCR2 and significantly higher CX3CR1 (12, 13). CCR2 and CX3CR1 have been applied as useful markers to distinguish classical monocytes (CCR2^{high}CX3CR1^{low}) and non-classical monocytes (CCR2^{low}CX3CR1^{high}) in both mice and men (14–16).

In the current study, we aimed to resolve the spatiotemporal migration of monocytes in emerging skin lesions. To this end, we used colony-stimulating factor 1 receptor (CSF1r)-reporter mice, where GFP is expressed on the circulating myeloid cells, such as monocytes and granulocytes (17). To uncover further the role of monocyte subsets in EBA disease and the specific function of CCR2 and CX3CR1 in the spatiotemporal dynamics of monocytes, we used CCR2$^{RFP/+}$ (CCR2-reporter) and CX3CR1$^{GFP/+}$ (CX3CR1-reporter) mice to monitor the recruitment of monocyte populations into the inflamed skin tissue. To underscore the specific function of CCR2 or CX3CR1, we generated mice with selective deletion of these chemokine receptors, i.e., CCR2$^{RFP/RFP}$ (CCR2-deficient) and CX3CR1$^{GFP/GFP}$ (CX3CR1-deficient), whose cells express red fluorescent protein (RFP) or green fluorescent protein (GFP) instead of CCR2 and CX3CR1, respectively, and are potentially good candidates to determine the importance of these receptors for the recruitment of monocytes into the tissue (18, 19). We show here that monocyte subtypes infiltrate the inflamed skin during the early stage of the disease in an experimental EBA mouse model, and CCR2 and CX3CR1 chemokine receptors are dispensable for their recruitment. Similarly, monocyte counts are reduced in the blood of human patients with bullous pemphigoid (BP) regardless of chemokine receptors. Although the chemokine receptors are not involved in the recruitment of monocytes into the skin, CCR2 deficiency is involved in protection against experimental EBA, indicating its influence on disease severity.

Materials and methods

Mice

B6.Cg-Tg(Csf1r-EGFP)1Hume/J (CSF1r^{EGFP+}, CSF1r-reporter) mice with the expression of enhanced green fluorescent protein (EGFP) under the control of the mouse Csf1r promoter, B6.129(Cg)-Ccr2$^{tm2.1Ifc}$/J (CCR2$^{RFP/RFP}$, CCR2-deficient) and B6.129P2(Cg)-Cx3cr1^{tm1Litt}/J (CX3CR1$^{GFP/GFP}$, CX3CR1-deficient) mice with a knock-in/knock-out homozygous expression of RFP and GFP replacing the coding sequence of the CCR2 and CX3CR1 loci respectively, as well as wildtype C57BL/6J mice (WT), were obtained from The Jackson Laboratories (Bar Harbor, ME) and imported into the animal facility at the University of Lübeck. CCR2-deficient and CX3CR1-deficient mice were crossed with WT mice to produce CCR2$^{RFP/+}$ (CCR2-reporter) and CX3CR1$^{GFP/+}$ (CX3CR1-reporter) mice respectively, which have the heterozygous expression of RFP and CCR2 or GFP and CX3CR1. Further, these reporter mice were crossed to produce CCR2$^{RFP/+}$×CX3CR1$^{GFP/+}$ double reporter (CCR2×CX3CR1-reporter) mice. Animal protocols were approved by local authorities of the Animal Care and Use Committee of the Federal Ministry of Food and Agriculture of Schleswig-Holstein (Kiel, Germany).

Human blood samples

This study enrolled 10 consecutive BP patients who were admitted to the Department of Dermatology, University of Lübeck between 2017 and 2019. BP disease was diagnosed based on typical clinical manifestations, detection of linear deposits of IgG and/or complement C3 at the dermal-epidermal junction (DEJ) by direct immunofluorescence of a perilesional skin biopsy, and presence of circulating IgG autoantibodies against BP180 non-collagenous (NC)16A by enzyme-linked immunosorbent assay (ELISA) (20, 21). The study also included a control group with 9 healthy individuals who were age- and sex-matched to the BP patient group. All volunteers gave written informed consent. Approval for this study was obtained from the Institutional Review Board at the University of Lübeck (Lübeck, Germany; ethical proposal numbers 18-046 and 15-051) according to the Declaration of Helsinki. Peripheral blood and biopsies from perilesional skin were collected, and samples with a lack of demographic data or inappropriate processing of initial laboratory specimens which could bias the study were excluded.

Antibodies

Pathogenic rabbit anti-murine type VII collagen (amCOLVII) IgG was generated as previously described (22, 23). Briefly, white New Zealand rabbits were immunized against the C epitope of COLVII. The IgG was purified, and indirect immunofluorescence analysis was applied to assess its reactivity to murine COLVII on murine skin sections as previously described (24). The amCOLVII antibody was subsequently conjugated with DyLight488 or DyLight594 using commercially available labeling kits (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions.

Alexa Fluor 647 anti-mouse Ly6G (clone: 1A8), PerCP/Cyanine5.5 anti-human CD14 (clone: 63D3), FITC anti-human CD192 (CCR2) (clone: K036C2), and PE/Cy7 anti-human HLA-DR (clone: L243) antibodies were obtained from BioLegend (San Diego, CA). APC anti-human CD16 (clone: REA423) and PE anti-human CX3CR1 (clone: 2A9-1) antibodies were purchased from Miltenyi Biotech (Bergisch Gladbach, Germany). FITC rat anti-mouse Ly-6C (clone: AL-21) antibody was acquired from BD Bioscience (Heidelberg, Germany). Mouse anti-CD68 antibody was purchased from Thermo Fisher Scientific Inc. (Waltham, MA), and rabbit anti-CD163 antibody was obtained from Abcam Inc. (Cambridge, MA). Alexa Fluor 488 AffiniPure donkey anti-mouse IgG and Alexa Fluor 594 AffiniPure donkey anti-rabbit IgG were received from Jackson ImmunoResearch Laboratories (Cambridgeshire, UK).

Flow cytometry

Peripheral blood of BP patients or controls was collected, and erythrocytes were lysed with erythrocyte lysis buffer (Buffer EL, Qiagen, Valencia, CA) before counting and staining. Prior to surface staining, 2×10^6 cells were incubated for 15 min at room temperature with Vioblility 405/452Fixable Dye (Miltenyi Biotech; 130-109-816) diluted 1:100 in phosphate-buffered saline (PBS) to label dead cells. Cells were washed with FC buffer (3% fetal calf serum in 0.01 M PBS, pH 7.2) and blocked with Human TruStain FcX™ (BioLegend, San Diego, CA) and stained for surface markers by incubation with titrated amounts of fluorochrome-conjugated monoclonal antibodies against CD16 APC (1:100), CD14 PerCP/Cyanine5.5 (1:100), HLA-DR PE/Cy7 (1:100), CCR2 FITC (1:40), and CX3CR1 PE (1:40) for 15 min in the dark at 4°C. After incubation, cells were washed two times with PBS before fixing with 4% paraformaldehyde solution (Roti-Histofix, Carl Roth, Germany) for 15 min at room temperature. Subsequently, cells were washed two times using perm wash buffer (BioLegend, San Diego, CA) and resuspended in 500 µL FC buffer before acquisition. All samples were analyzed using a BD FACSAria II flow cytometer (BD Biosciences) and analyzed with FlowJo software (version 10.6.1). The analysis started by gating for single and alive cells. Afterward, gating for CD16 and CD14 allowed the detection of the three different monocyte subsets and gates for the chemokine receptor expression of CCR2 and CX3CR1. To confirm the monocytic cell lineage, HLA-DR positivity was used (Supplementary Figure 1).

Immunohistochemistry and microscopy

For Ly6G/Ly6C staining of mouse skin, cryosections (200 µm thick) were cut from the ear skin of the WT C57BL/6 mice, which were sacrificed 24-, 48-, 72- and 96-hours post-amCOLVII injection. Briefly, hair on the mouse ears was removed by applying hair removal cream (GlaxoSmithKline, Bühl, Germany). Ears were then rinsed with 70% ethanol and amputated with scissors. Using a scalpel, a part of the cartilage was removed by a straight cut along the cranial side of the ear. Ears were then cut medially for better handling and staining. Sections were washed once in PBS to remove any remaining hairs, oriented upright in Tissue-Tek O.C.T. Compound (Sakura Tissue-Tek Xpress, Torrance, CA) in a metal mold, and frozen in liquid nitrogen. Samples were then stained with Alexa Fluor 647 anti-mouse Ly-6G antibody (1:1600) and FITC Rat anti-mouse Ly6C antibody (1:75) overnight at room temperature. After rinsing the sections with Tris-buffered saline (TBS), samples were incubated with a 1:10000 dilution of Hoechst 33342 in TBS for 10 min to stain cell nuclei. Sections were then washed and covered in buffered Mowiol, and mounted with coverslips. Three-dimensional (3D) stacks of stained sections were imaged using a Leica confocal microscope (SP5). FITC Rat anti-mouse Ly6C antibody (emission=490-530 nm) was excited at 488 nm by an Argon laser. Alexa Fluor 647 anti-mouse Ly6G antibody (emission=650-690 nm) was excited at 633 nm using a helium-neon (HeNe) laser. Twenty images of 2 µm thickness were taken for each 3D stack.

To stain the human monocytic cells, biopsies from the perilesional skin of BP patients, and sex- and age-matched healthy donors, were collected. Briefly, 6 µm paraffin sections were deparaffinized, subjected to heat-induced antigen retrieval in the presence of Tris (10 mM)-EDTA (1 mM) buffer (pH 9), washed

3 times with PBS, and blocked with AffiniPure F(ab')$_2$ Fragment donkey-anti-human IgG (Jackson ImmunoResearch Laboratories, Cambridgeshire, UK) in PBS, before blocking with 10% donkey serum (Jackson ImmunoResearch Laboratories, Cambridgeshire, UK). Mouse anti-CD68 (1:600) and rabbit anti-CD163 antibodies (1:800) were applied as primary antibodies. Alexa Fluor 488 donkey anti-mouse IgG (1:500) and Alexa Fluor 594 donkey anti-rabbit IgG (1:500) were used as the corresponding secondary antibodies. Mouse IgG (Thermo Fisher Scientific, Waltham, MA) and unspecific rabbit IgG (Emfret analytics, Würzburg, Germany) were used as corresponding isotype controls for anti-CD68 and anti-CD163 antibodies, respectively. After a washing step, all slides were mounted with 4′,6-diamidino-2-phenylindole (DAPI) Fluoromount-G (Southern Biotech, AL). The sections were visualized and analyzed using the BZ-9000E series microscope (Keyence, Osaka, Japan). Overlay of the signals was conducted by the analysis software BZ-II Analyzer (Keyence, Osaka, Japan). Scoring of positively labeled cells per defined-high power field was performed using Image-J-win64.

Antibody transfer model of EBA in mice and intravital imaging

The dermis of mice ears was visualized using intravital multi-photon microscopy to examine the binding of amCOLVII IgG at the DEJ, as well as the extent of cell infiltration into the skin. To induce the inflammation and blisters in an antibody transfer-induced EBA, 250 µg of DyLight 488- or DyLight 594-conjugated purified amCOLVII IgG was injected intravenously into the anesthetized mice on day 0. The ears of the mice were scratched lightly with tweezers to promote the extravasation of antibodies at a predefined location. For the long-duration imaging experiments, mice were anesthetized intraperitoneally using 10 µg/kg fentanyl, 16.8 mg/kg midazolam, and 1.7 mg/kg medetomidine hydrochloride. Subsequently, hair removal cream (GlaxoSmithKline, Bühl, Germany) was applied to remove ear hairs. The mouse was placed on a custom-made, heated holder and was kept in a prone position with the head tilted slightly so that the dorsal side of the ear was facing upwards. One ear was fixed lightly with double-sided tape (Tesa SE, Germany), and Vidisic gel (Bausch + Lomb Inc., Laval, CA) was placed in between the ear and a coverslip. The coverslip was fixed lightly with surgical tape (Durapore, Germany) to minimize pressure on the ear. Vital signs of the mouse (O_2 saturation, heart rate, temperature) were monitored. Images were captured on the outer posterior of the pinna where hair follicles are sparse. Imaging was performed using a TriM Scope II multiphoton microscope (LaVision BioTec GmbH, Bielefeld, Germany) equipped with tunable fs-pulsed lasers and XLPlanN 25×1.05 WMP Objective (Olympus, Hamburg, Germany). Vidisic gel was used as an immersion medium. GFP and DyLigh-488 fluorophores were excited at 920 nm and emissions were detected at 495-560 nm using a photomultiplier tube (PMT). RFP, DyLigh-594 fluorophores, as well as second harmonic generation (SHG) signals, were excited at 1100 nm, and emission signals were collected at >560 nm and 495-560 respectively by separate PMTs. Immediately following the injection of amCOLVII IgG, the visual areas of interest were selected under the microscope in proximity to blood vessels by monitoring of binding amCOLVII IgG to the DEJ and infiltration of the first cells into the skin. Images were acquired from the selected areas during the scan period of each frame with a total of 90 µM Z-stacks at each time point of the experiment for each mouse by starting 20 µM above the DEJ and ending 70 µm below it with a 3 µm thickness between stacks. Image dimensions were 350×350 µm with pixels of 520×520 and the photon frequency was set to 600 Hz. The laser intensity was optimized to reliably image the bright cells and reduce possible laser damage from the excitation of melanin. Image processing was performed using Imaris Software (Bitplane, Zürich, Switzerland). With the use of automatic 3D object tracking and Imaris Spots, the spatiotemporal coordinates of cells were documented. These data were imported into GraphPad Prism 9 for the quantification of cell counts.

Disease severity was scored by determining the percentage of the body surface area affected by skin lesions such as erythema, blisters, erosions, alopecia, and crusts (22). Cell recruitment was monitored on days 0, 1, 2, 4, and 10 in the case of CSF1r-reporter mice and on days 0, 2, 4, 9, and 18 for CCR2-reporter and CX3CR1-reporter, deficient, and double-reporter mice. Control mice were not injected with antibodies but were imaged alongside the diseased mice.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (La Jolla, CA). As means of separate groups were analyzed for differences, unpaired two-tailed Student's t-tests were used. Shapiro–Wilk test was applied to verify the normality of data distribution. Mann-Whitney U test was used to analyze datasets with the non-Gaussian distribution. Dunn's *post-hoc* test was applied for multiple comparisons between the cell counts of control and diseased mice from all backgrounds. Non-significance was indicated by the symbol "n.s.". Mean values are indicated on scatter plots. Data are presented as mean ± standard deviation (SD) or as median with interquartile range and $p < 0.05$ was considered statistically significant.

Results

Monocytes and neutrophils are present in the early stages of EBA disease onset in mice

To precisely spatiotemporally resolve inflammatory leukocyte migration in emerging skin lesions, we used CSF1r-reporter mice to chart the dynamics of cell recruitment into the skin of an antibody-transfer EBA mouse model by intravital multiphoton microscopy. To this end, we injected Alexa Fluor 594-labeled amCOLVII antibodies into CSF1r-reporter mice and monitored cell recruitment on 0, 1-, 2-, 4-, and 10 days post-injection. Control mice were not injected with antibodies but were imaged alongside the diseased mice. A gentle mechanical irritation was applied with tweezers on the mice's ears to amplify the extravasation of pathogenic antibodies (25).

This procedure did not visibly affect the epidermis. Shortly after injection, the amCOLVII antibody was observed at the DEJ in mice ears (Figure 1A). While few EGFP$^+$ cells are present in the skin, immediately after the localization of the amCOLVII antibody at the DEJ, these cells were recruited into the skin in the proximity of antibodies (Figures 1A, B). Migrated EGFP$^+$ cells form aggregations at several spots in the skin, and the cell counts in these spots increase over time (Figure 1B) as judged by the quantification of CSF1r^{EGFP+} cells (Figure 1C). Accumulation of these cells was observed over several days, reaching a 7-fold increase on day 10 (Figure 1D). Monitoring of the total EGFP$^+$ cells in control mice that did not receive pathogenic antibodies indicated a slight increase in the first four days (Figures 1A, D). This increase was, however, transient, and only a few scattered populations of cells were registered in the deeper part of the skin, mostly in the dermis.

Given the EGFP expression by both monocytes and neutrophils in CSF1r-reporter mice, recruited cells into the skin could be a mixture of both cells. To discriminate monocytes and neutrophils present in the skin during the early stages of the disease, Ly6G/Ly6C antibody staining was applied in the antibody transfer model of EBA in WT mice. The ear samples were collected from WT mice injected with amCOLVII at 1-, 2-, 3-, and 4 days post-injection. By staining for Ly6G, a marker for neutrophils, and Ly6C, a marker for monocytes, we demonstrated that both cell types are present during initial disease onset (Figure 1E). A comparison of the Ly6G$^+$ to Ly6C$^+$ cells showed that neutrophils are recruited in two waves, on days 1 and 4, whereas monocytes arrive in the greatest numbers on days 2 (Figures 1E, F). Together this data suggests that both monocytes and neutrophils are recruited into the skin during the early stages of EBA disease onset in mice.

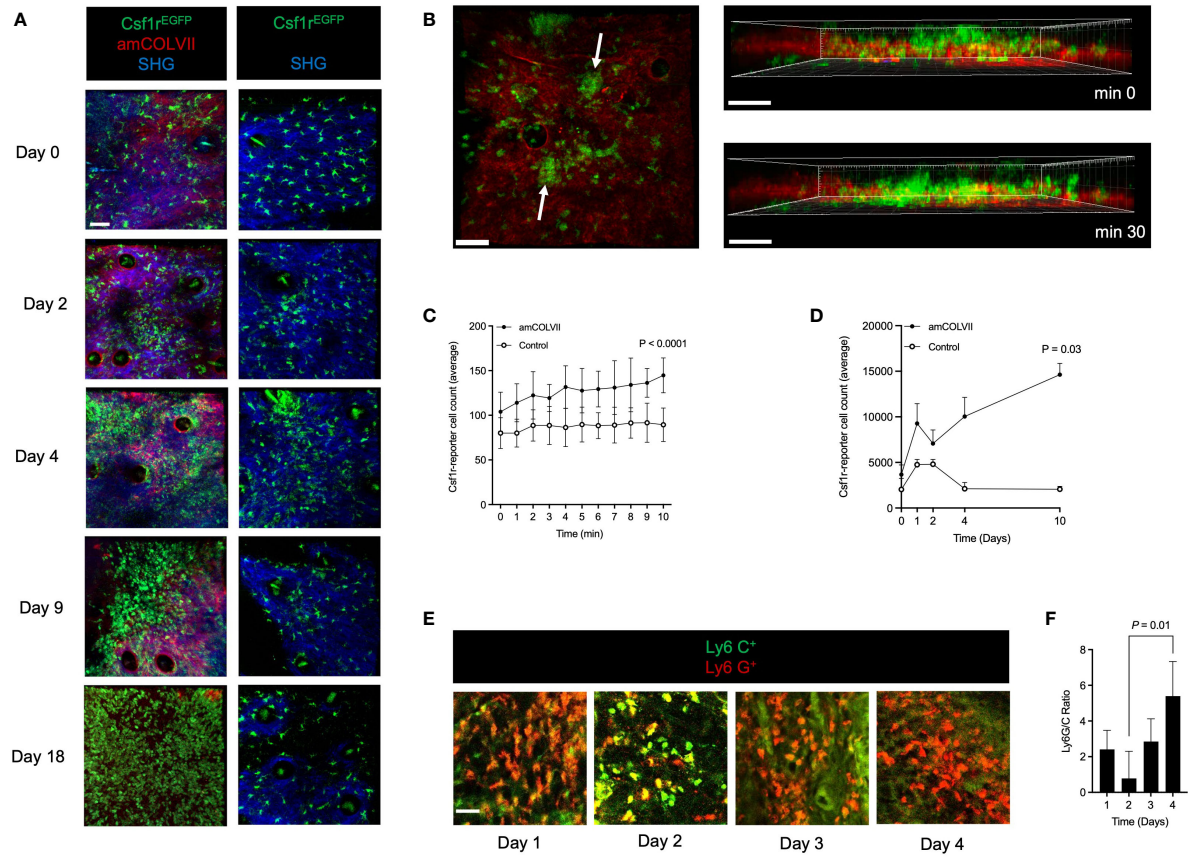

FIGURE 1
Monocytes and neutrophils are recruited into the skin of mice after amCOLVII antibody injection in an experimental EBA mouse model. **(A)** Representative multi-photon intravital microscopy images of CSF1r^{EGFP+} cells 0, 1-, 2-, 4- and 10-days post-injection with amColVII antibody to the Csf1r-reporter mice (n=8, left) and in control mice (n=3, right). The green color indicates CSF1r^{EGFP+} cells. The red indicates that amCOLVII is only present in diseased (left) mice and not in control (right) mice. SHG is shown in blue. Scale bar: 50 µM. **(B)** Following injection of amCOLVII, CSF1r^{EGFP+} cells infiltrate towards the EDJ in the mouse ear, where the pathogenic antibody is present. White arrows in the left panel indicate the heterogeneous accumulation of the GFP cells. A 3-dimensional image in the right panel shows the spatial orientation and proximity of accumulated antibodies and migrated immune cells in the first 30 minutes. **(C, D)** The average number of CSF1r^{EGFP+} cell count in diseased (amCOLVII) and healthy (control) mice over **(C)** 10 minutes on day 0 and **(D)** 10 days (0, 1-, 2-, 4-, and 10) post antibody injection. **(A–D)** Data presented here are derived from at least 3 monitored areas per mouse. **(E)** Confocal microscopy images of Ly6C$^+$(green) and Ly6G$^+$ (red) cells stained in ear skin of mice 1-, 2-, 3-, and 4-days post amCOLVII injections. **(F)** The ratio of Ly6G$^+$ (Ly6G^{high}) cells, which identify neutrophils, to Ly6C$^+$ (Ly6G^{low}) cells, which identify monocytes, in stained thick sections of C57BL/6 mice 1-, 2-, 3-, and 4-days post amCOLVII injection. **(E, F)** Data are derived from 4 mice per day with at least 3 stained samples. Scale bar: 50 µM. Data are presented as mean ± SD. Statistically significant differences were compared between groups using unpaired t-test analysis. Results of one representative experiment out of three to five independent experiments (ear samples) are shown. amCOLVII, anti-mouse Collagen VII; CSF1R, Colony-stimulating factor 1 receptor; SHG, Second-harmonic generation; SD, standard deviation.

10.3389/fimmu.2023.1241461

CCR2 and CX3CR1 are not involved in the recruitment of classical and nonclassical monocytes in the EBA model

Having identified monocytes to be present during EBA in mice throughout the disease progression, we sought to investigate the involvement of different monocyte-derived cell populations in the disease course. In mice, monocytes are categorized according to their expression levels of Ly6C and CCR2 as classical (Ly6C^{hi}/CCR2^{+}) and nonclassical (Ly6C^{low}/CCR2^{-}) monocytes, with the latter monocyte population typically expressing high levels of the receptor CX3CR1 (26). We investigated the recruitment of classical and nonclassical monocytes into the skin after amCOLVII injection in CCR2, CX3CR1, and CCR2×CX3CR1-reporter mice. Mice were imaged on days 0, 2, 4, 9, and 18 post amCOLVII antibody injection. Non-injected control mice were imaged alongside injected mice to provide information on baseline recruitment due to the mechanical irritation of the skin. As indicated in Figures 2A, B, some CCR2^{RFP+} cells were observed in the skin shortly after injection of pathogenic antibody or non-injected control mice (day 0), which was comparable between CCR2-reporter mice in the amCOLVII-injected and control group.

CCR2 is involved in the recruitment of the respective monocyte population into peripheral tissues. To scrutinize the influence of CCR2 molecules in the recruitment of monocytes, CCR2-deficient animals were used, in which the CCR2 locus was completely replaced by RFP expression. In contrast to the healthy mice with the recruitment of few cells until day 4, increased infiltration of CCR2$^{RFP/+}$ cells was seen in mice with or without the CCR2 chemokine receptor (Figures 2A, B) with the greatest on day 4 (Figures 2C, D). However, in both diseased and control mice, a comparison between CCR2-reporter and CCR2-deficient mice

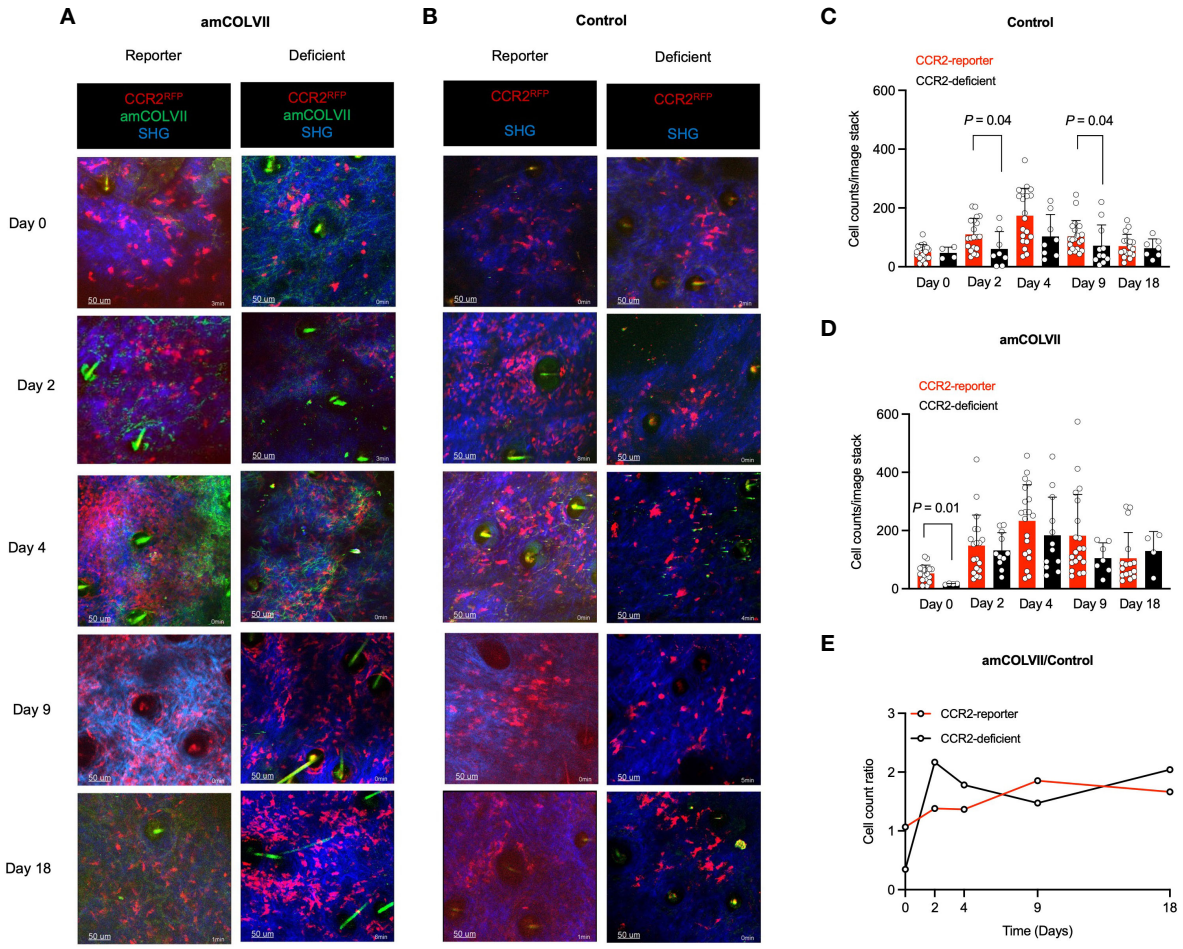

FIGURE 2
In vivo cell recruitment patterns of classical monocytes in mouse ears 0, 2-, 4-, 9- and 18 days post-injection with amCOLVII antibody. **(A, B)** Two-photon microscopy images of RFP^{+} (red) cells at sites of amCOLVII antibody (green) in CCR2-reporter (n=4), CCR2×CX3CR1-reporter (n=6), and CCR2-deficient (n=4) mouse ears injected with in comparison to control (same number of mice). SHG is shown in blue. CCR2^{RFP+} count in the ear skin control **(C)** and diseased **(D)** mice. **(E)** A ratio counts of CCR2^{RFP+} monocytes between diseased and control mice. Data presented here are derived from at least 3 monitored areas per mouse. Scale bar: 50 µM. Data are presented as mean ± SD. Statistically significant differences were compared between groups using the Mann-Whitney test analysis. Results of one representative experiment out of three to five independent experiments (ear samples) are shown. amCOLVII, anti-mouse Collagen VII; CCR2, CC chemokine receptor 2; SHG, Second-harmonic generation; SD, standard deviation.

displayed a similar pattern in the recruitment of classical monocytes into the skin (Figures 2A, B). Quantification of CCR2^{RFP+} monocytes does not indicate a significant difference between CCR2-reporter and CCR2-deficient mice (Figures 2C, D). To detect the effect of disease on the recruitment of classical monocytes, the ratio of cell counts was analyzed. Overall, comparable recruitment of CCR2^{RFP+} monocytes is seen in CCR2-reporter and CCR2-deficient mice (Figure 2E).

Through interaction with the CX3CR1 receptor, migration of CX3CR1-bearing cells such as monocytes, is facilitated. Hence, a possible effect of this chemokine receptor was considered in the recruitment of the CX3CR1^{+} monocyte population. To identify non-classical monocytes, CX3CR1-reporter mice were used and the involvement of CX3CR1 in the recruitment of CX3CR1^{GFP+} monocytes was analyzed. In contrast to classical monocytes, visualization of non-classical monocytes exhibited no difference in the recruitment of these cells between CX3CR1-reporter and CX3CR1-deficient mice neither in diseased mice nor in controls (Figures 3A, B). This was further confirmed by no significant differences in cell counts between control and injected mice (Figures 3C, D). Confirming these data, the ratio of CX3CR1-reporter and CX3CR1-deficient remains similar between diseased mice and controls (Figure 3E). Taken together, these data indicate that deficiency in chemokine receptors CCR2 and CX3CR1 does not play a role in the recruitment of classical and non-classical monocytes into the inflamed skin of an experimental model of EBA.

CCR2 deficiency, but not CX3CR1, contributes to partial protection against experimental EBA in mice

Classical CCR2^{+} and non-classical CX3CR1^{+} cells have been previously identified as inflammatory and resident monocytes, respectively, which play a role in inflammation and homeostatic

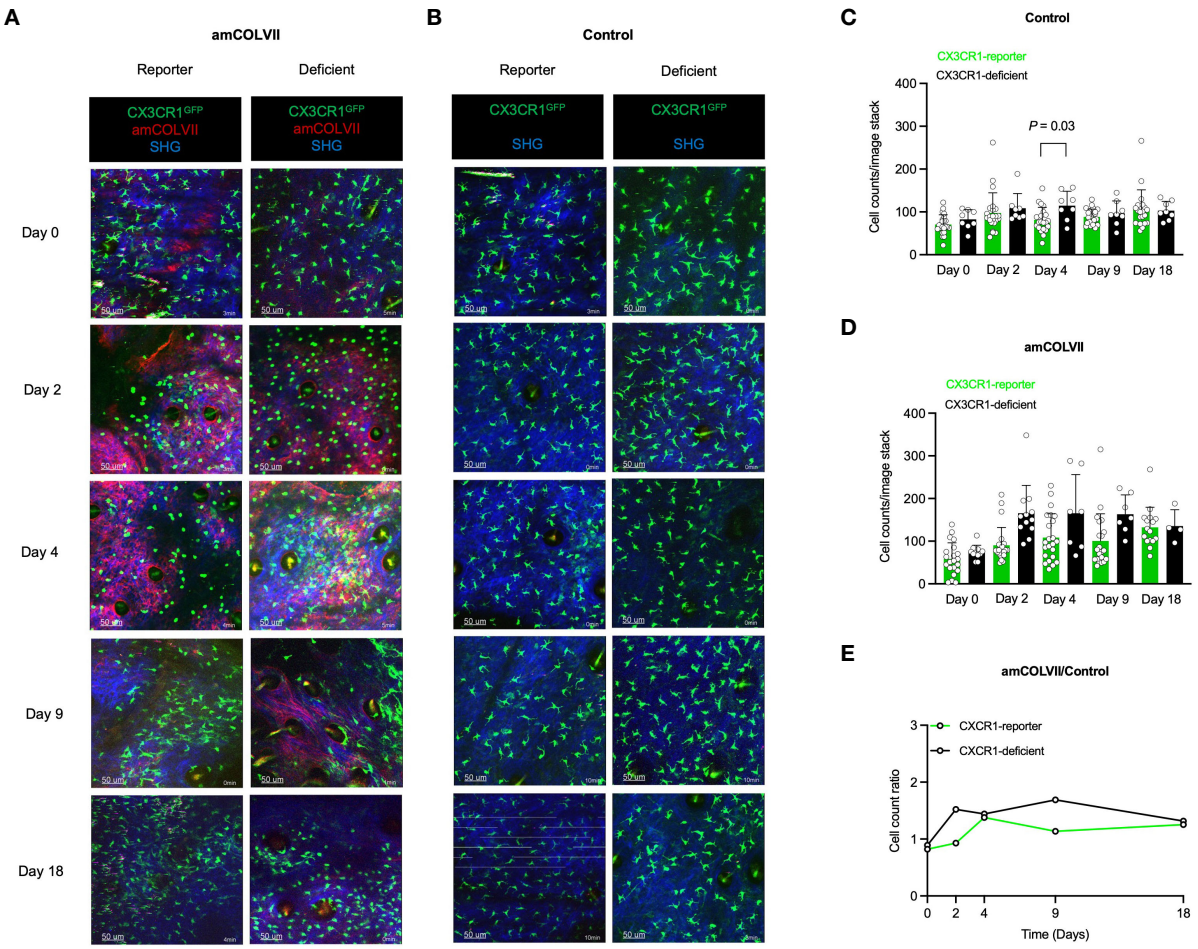

FIGURE 3
Intravital visualization of nonclassical monocyte recruitment in mouse ears 0, 2-, 4-, 9- and 18 days post-injection with amCOLVII antibody. **(A, B)** Images of CX3CR1-reporter (n=5), CCR2×CX3CR1-reporter (n=6), or CX3CR1-deficient (n=3) monocytes in mice injected with amCOLVII (red) displayed CX3CR1^{+} (green) cells in comparison to the control (same number of mice). SHG is shown in blue. The counts of CX3CR1^{GFP+} monocytes in the ear skin of control **(C)** and diseased **(D)** mice. **(E)** A ratio counts of CX3CR1^{GFP+} monocytes between diseased and control mice. Results of one representative experiment out of three to five independent experiments (ear samples) are shown. Data presented here are derived from at least 3 monitored areas per mouse. Scale bar: 50 µM. Data are presented as mean ± SD. Statistically significant differences were compared between groups using the Mann-Whitney test analysis. Results of one representative experiment out of three to five independent experiments (ear samples) are shown. amCOLVII, anti-mouse Collagen VII; CX3CR1, CX3C chemokine receptor 1; SHG, Second-harmonic generation; SD, standard deviation.

functions (14). Therefore, we hypothesized that the recruited inflammatory CCR2$^+$ monocytes could be involved in the pathogenesis of EBA disease and CCR2-deficient mice should show a less severe skin disease as compared to the WT. Since CCR2 and CX3CR1 are not functional in CCR2-deficient and CX3CR1-deficient mice, the influence of these chemokine receptors on the clinical phenotype was investigated in experimental EBA, where the deficient mice were compared with its corresponding reporter control. Both reporter and deficient mice started to develop clinical symptoms such as erosions, alopecia, and crusts on day 2 after the injection of the pathogenic amCOLVII antibodies (Figures 4A, B). Increased severity of disease based on the total area of affected ears was observed over time, with the strongest effect seen on day 9 in the EBA model (Figures 4A, B). Control mice without injection of pathogenic amCOLVII IgG did not develop the disease. While a significant difference was observed between CCR2-reporter and CCR2-deficient mice, disease severity remained similar between CX3CR1-deficient and reporter mice (Figures 4A–C). These data indicate that although the chemokine receptor CCR2 is dispensable in the recruitment of monocytes into the skin, it still plays a role in disease severity.

Circulating monocyte proportion regardless of chemokine receptors CCR2 and CX3CR1 is altered in BP in human

Since we observed the recruitment of classical monocytes in a mouse model of bullous pemphigoid (BP)-like EBA disease, we next investigated if monocytes are involved in human BP. Patients with EBA

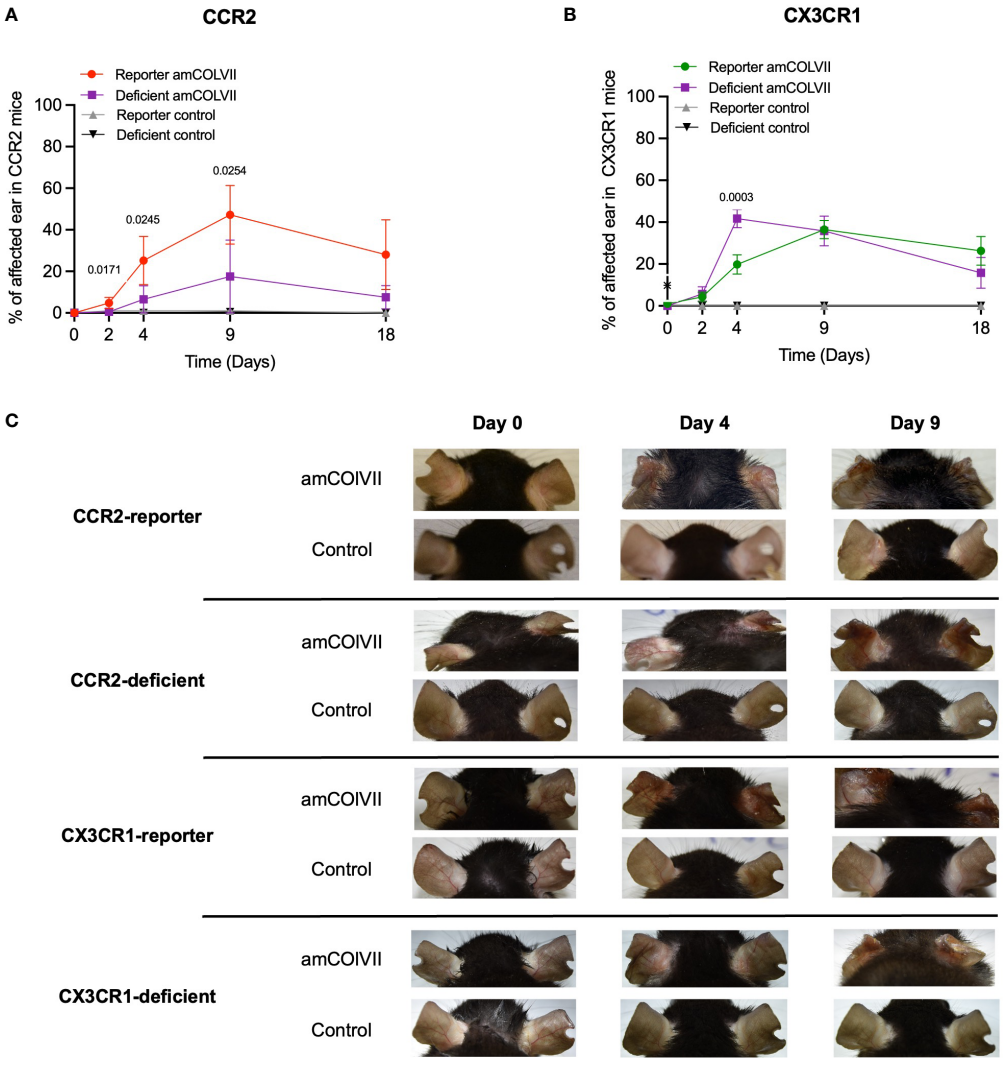

FIGURE 4
Disease phenotypes in CCR2 and CX3CR1 reporter and their respective deficient mice in an EBA mouse model after amCOLVII antibody injection. CCR2-reporter (n=4), CX3CR1-reporter (n=5), CCR2×CX3CR1-reporter (n=6), CCR2-deficient (n=4), CX3CR1-deficient (n=3) mice were injected with an amCOLVII antibody, and disease severity of the ears was assessed on 0, 2-, 4-, 9- and 10 days post-injection. Control mice were not injected. Disease phenotype development is shown as a percentage of ear skin surface area affected by blistering (crusting, erosions, and/or abrasions). **(A)** The time course of clinical disease development in CCR2-reporter and CCR2-deficient diseased and control mice. Data are presented as mean ± SD. Statistically significant differences were compared between groups using unpaired t-test analysis. **(B)** The time course of disease phenotype development in CX3CR1-reporter and CX3CR1-deficient diseased and control mice. **(C)** Ears of the CCR2-reporter and CCR2-deficient mice.

are extremely rare. The clinical, histologic, and immunohistologic features as well as immune profiles, binding pattern of autoantibodies, and mechanisms of cell recruitment in BP disease are like what is seen in EBA. We therefore analyzed the number of blood monocytes between the BP patients and healthy individuals. As indicated in Figure 5A, our data show that the percentages of monocytes are significantly lower in the blood of the patients compared to the control subjects. Further analysis of classical monocyte subpopulations indicated no difference in the percentages of $CCR2^+$, $CCR2^+CX3CR1^+$, and $CX3CR1^+$ monocytes between BP patients and healthy controls (Figure 5B). This data suggests that the egress of monocytes from the blood is not regulated by the expression of these chemokine receptors.

Monocytes and macrophages are infiltrating the inflamed skin of human BP patients

Since a reduced number of classical monocytes in the systemic blood circulation of BP patients was observed, we hypothesized that these monocytes are being recruited into the inflamed skin. Herein, monocytes/macrophages (MOMA) staining was applied on the human skin biopsies, where CD68, CD163, and double staining of CD68/CD163 were performed (Figures 5C, D). Of note, the number of activated MOMA, which are represented by the CD68-CD163 double-positive stained cells, is significantly increased in perilesional skin sections of BP patients compared to the control group. Compared to the healthy controls, the expression of CD163, a mononuclear phagocyte-restricted phagocytic receptor expressed on activated monocytes and macrophages, as well as CD68, a pan-macrophage marker, in diseased skin is consistent with the recruitment of monocytes into the inflamed tissue.

Discussion

The recruitment of effector cells into the injured tissue is the key pathogenic process initiating and fueling skin inflammation in many autoimmune diseases such as rheumatoid arthritis, systemic sclerosis, and pemphigoid diseases. In pemphigoid diseases, autoantibodies are considered the direct cause of disease pathology. Although the key

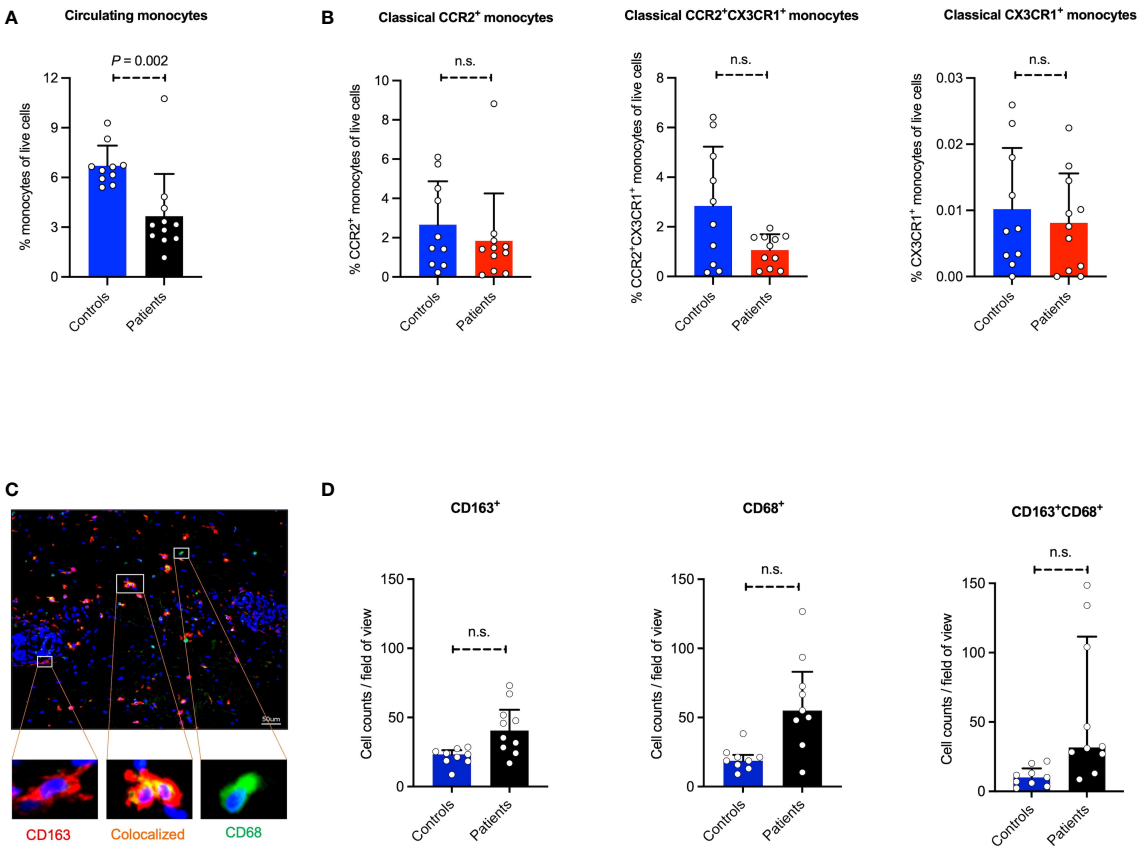

FIGURE 5
Prevalence of monocyte/monocyte-derived population in human blood and skin. Absolute cell counts of monocyte populations and subpopulations were determined in peripheral blood in 10 BP patients and 9 healthy controls. **(A, B)** The monocytes and monocyte subpopulations (classical $CCR2^+$, classical $CCR2^+CX3CR1^+$, and classical $CX3CR1^+$ monocytes) in human peripheral blood samples using flow cytometry measurement. **(C, D)** The human skin section performed immunostaining of CD68, CD163, and CD68/CD163. Skin biopsies were taken from the lesions and healthy skin, stained, and microscopically counted. The CD68 and CD163 single-positive, as well as double-positive cells, are indicated. Data are presented as mean ± SD. Statistically significant differences were compared between groups using the Mann-Whitney test analysis. Scale bar: 50 µm.

requirements for antibody-mediated diseases have so far been identified, the spatiotemporal dynamics of effector cell recruitment from the blood vessel lumen to the skin as well as the molecular cues choreographing this recruitment, are poorly understood. We investigated here whether these chemokine receptors are essential for monocyte recruitment into the skin, and contribute to the development of the disease manifestation in an experimental mouse model of EBA. Our results indicate a dispensable role of CCR2 or CX3CR1 chemokine receptors for monocyte recruitment to the site of antibody-binding and inflammation in mice. Our data derived from human BP samples also confirmed monocyte recruitment into the patient's skin compared to healthy individuals. However, deficiency in CCR2 molecules ameliorates the development of experimental EBA in mice.

Establishing tissue inflammation requires the directed migration of effector cells from the vessel lumen to the emerging inflammatory focus and the congregation of cells at this site. Monocyte recruitment is observed following various forms of tissue injury, which represents a characteristic hallmark of inflammation. They have been implicated in the pathogenesis of several autoimmune diseases, executing diverse, partially opposing functions in both the emergence and the resolution of tissue inflammation (5, 6). Within the context of pemphigoid diseases such as EBA, the role of monocytes has hardly been addressed. It has been shown that monocytes are the dominant cell type in early skin lesions of human BP (27), and $CD163^{+}CD206^{+}$ M2 macrophages and $Fc\varepsilon RI^{+}CD207^{+}$ Langerhans cells infiltrate lesional BP in significant numbers (28, 29). The C-X-C motif chemokine ligand 10 (CXCL10) has been suggested to drive BP by inducing the release of matrix metalloproteinase-9 (MMP-9) from monocytes and neutrophils (30). Recently, Graauw et al. confirmed that monocytes can increase neutrophil-induced blister formation in an ex vivo model of bullous pemphigoid (31). Albeit the presence of monocytes in the diseased tissues has so far been confirmed, these data are mostly based on immunostaining or *in vitro* and ex vivo analysis and do not provide precise information about the dynamics of monocyte populations in recruitment towards the injury. Using multi-photon microscopy, we charted infiltration of myeloid-derived cells such as monocytes and neutrophils in CSF1r-reporter mice. Since we have previously visualized the immediate and inhomogeneous distribution of amCOLVII autoantibodies along the DEJ (25), a local stimulation was performed on the mice ears to induce local antibody extravasation and restrict antibody binding to a defined skin area to facilitate visualization. Our imaging data indicate early recruitment of $CSF1r^{EGFP+}$ cells, predominantly neutrophils and monocytes, followed by a second wave of cell recruitment. Complemented with Ly6C/Ly6G staining for uncovering the identity of recruited cells, we show that both neutrophils and monocytes are rapidly infiltrated into the skin, where neutrophils and monocytes accumulate in the skin in their greatest number at 48 and 72 hours, respectively. In agreement with these data, we recently confirmed rapid dermal recruitment of neutrophils in an experimental EBA model in $LysM^{EGFP}$ mice (25), that express EGFP under the control of the endogenous lysozyme M promoter (32). These data suggest that both monocytes and neutrophils are recruited into the skin during the early stages of EBA disease onset in mice. The observation of two waves of cell recruitment agrees with the model of initial recruitment of limited numbers of cells possibly due to mechanical irritation that we used to induce localized antibody extravasation. These initially recruited cells then could bind to autoantibody at the DEJ which triggered the release of inflammatory mediators that led to the recruitment of larger numbers of cells (33). In accordance with this model, we have observed a rapid recruitment of lower numbers of GFP^{+} cells in control mice that did not receive autoantibody.

The directed migration of effector cells is a complex process consisting of several distinct steps, including trans-endothelial migration (TEM), interstitial tissue migration, and, finally, arrest before effector functions can be executed (34). Evidence has accumulated that effector cell migration is often choreographed by temporally and spatially organized networks of tissue environment- and pathogenic stimulus-dependent molecular guidance signals including chemokine-chemokine receptor interactions and cell adhesion molecules (34–38). Herein, CCR2 is believed to be involved in the migratory functions of monocyte subsets. It has been shown that following the binding of monocyte chemoattractant protein-1 (MCP-1) to its receptor CCR2, the MCP-1/CCR2 signaling pathway contributes to the recruitment of monocytes to the site of inflammation (39, 40). In line with these findings, other investigations have shown that CCR2-deficient monocytes do not migrate in response to MCP-1 and have less capability of adhering to the endothelium, indicating the involvement of CCR2 in firm adhesion of monocytes to the endothelium for exiting the blood vessels (41). In contrast to this data, in the present study, a comparable pattern was observed in the recruitment of CCR2-reporter and CCR2-deficient monocytes into the skin in the experimental model of EBA. These results can be interpreted by the different effects of CCR2 in monocyte release from bone marrow to the blood or migration of monocytes from blood into the tissue. Despite the existence of evidence that CCR2-signaling is important for monocyte egress from the bone marrow into the bloodstream (42), its contribution to the infiltration of monocytes into the tissue remains controversial. Challenging the traditional concept of CCR2 function, some observations indicate that a reduced number of CCR2-deficient monocytes in the inflamed tissue may not solely be due to the essential role of CCR2 in the recruitment of monocytes into the tissue, as there is a remarkable decrease in the ratio of CCR2-deficient to WT monocytes in circulation (43). Brühl et al. also indicated increased recruitment of monocytes into the joints after CCR2 blockade, suggesting that monocyte infiltration can be independent of CCR2 (44). Hence, our findings agree with previous data suggesting that CCR2 only indirectly influences monocyte infiltration into the infected tissues (45, 46).

To translate the effect of CCR2 in the migration of human monocytes in pemphigoid diseases, we further monitored the monocyte subsets in the blood and perilesional skin of BP patients. Our results indicated that although the absolute count of monocytes drops in patients compared with healthy individuals, the number of subpopulations in classical monocytes remains similar between BP patients and healthy controls. A comparable number of $CCR2^{+}$ classical monocytes compared to other monocyte subpopulations indicates that no selective monocyte draining from blood occurs based on chemokine receptors, suggesting that CCR2 is dispensable for the recruitment of monocytes to the skin. However, although the chemokine receptors CCR2 and CX3CR1 do not contribute to the recruitment of monocytes into the skin in our mouse model, we could

show that CCR2-deficient mice develop less disease than reporter mice, suggesting a role for CCR2 in disease severity. This could be due to the plastic nature of monocytes that are crossly differentiated into the different populations upon infiltration into the tissue. Circulating inflammatory monocytes infiltrate into inflamed tissue and are polarized into phagocytic pro-inflammatory macrophages at the early disease stages, which later switch their phenotype to an anti-inflammatory subset that helps the resolution of inflammation (10, 47, 48). CCR2 deficiency can alter the balance between inflammatory and anti-inflammatory macrophages, in which CCR2 deficiency blocks the excessive converting of macrophages to a proinflammatory phenotype (49). Hence, this is not beyond the expectation that CCR2-deficient monocytes might migrate into the skin and restore the macrophage polarization balance to favor anti-inflammatory cells, reducing the disease severity. Another possibility for disease attenuation could be the involvement of CCR2 signaling in cells other than monocytes and macrophages that play a role in disease development. For instance, in an animal model of allergic contact dermatitis, it has been shown that CCR2/CC-chemokine ligand 2 (CCL2) signaling is activated in skin neurons that promote itch behavior (50). Since mechanical irritation such as scratching and itching implicates the initiation phase of disease development in various autoimmune skin diseases such as EBA (51), CCR2 deficiency might decrease the scratching events and thereby ameliorate clinical disease manifestation. However, defining the exact role of CCR2 in EBA and BP requires further investigation.

We observed a significant increase in the number of activated monocytes/macrophages (MOMA), represented by the CD68-CD163 double-positive stained cells in perilesional skin. This increase in MOMA in the skin lesions of BP patients is compatible with the declining monocytes in the systemic circulation, where monocytes most likely leave the blood vessels to infiltrate the inflamed skin. Although MOMA markers do not show the exact composition of the specific monocyte/macrophage populations, it is reasonable to assume that the blood monocytes could have migrated into the skin and differentiated to activated macrophages or even function as effector cells. For example, it has been shown that $CD163^{+}$ activated tissue macrophages are increased in several autoimmune diseases such as BP, pemphigus vulgaris, and systemic sclerosis (52, 53). CD163 expression can be increased both by factors inducing the differentiation of monocytes to macrophages and/or by anti-inflammatory mediators such as interleukin (IL)-10 or glucocorticoids (54).

Our study is not without limitations. We could not resolve the spatiotemporal relationship between neutrophils and monocytes at the site of antibody binding. Using dual reporter mice with labeled neutrophils and classical or non-classical monocytes could be helpful in simultaneously charting the kinetics of cell migration in emerging skin lesions. The second limitation was the lack of samples from patients with the inflammatory form of EBA, as this is an extremely rare disease. However, the mouse model we used is very similar to BP with respect to immunologic mechanisms. Therefore, we anticipate that the results we obtained by analysis of samples of BP patients are comparable to results we would have obtained from patients with the inflammatory form of EBA. Despite these limitations, our findings demonstrate a substantial recruitment of monocytes in the skin following the binding of autoantibody to the DEJ. Although the role of monocytes in blistering diseases remains to be determined, they could serve as potential pharmacological targets. The observation that CCR2, despite being dispensable for monocyte recruitment, modulates disease severity, might help in developing potential therapeutic strategies targeting this receptor pathway in EBA and BP.

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

Approval for this study was obtained from the Institutional Review Board at the University of Lübeck (Lübeck, Germany; ethical proposal numbers 18-046 and 15-051). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Animal protocols were approved by local authorities of the Animal Care and Use Committee of the Federal Ministry of Food and Agriculture of Schleswig-Holstein (Kiel, Germany). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RA, CC, and S-YT participated in performing the experiments. RA, CC, S-YT, PS, and SM analyzed the data. RA, PK, and CS contributed to the conception and design of the study. RA and CC wrote the first draft of the manuscript. RA provided the final version of the manuscript, and PK and CS revised it. All authors contributed to the manuscript revision, read it, and approved the submitted version.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. German Research Foundation, Clinical Research Unit (CRU) 303 Pemphigoid Diseases (KO3916/1-2).

Acknowledgments

We would like to thank the Institute of Anatomy and Department of Dermatology scientific staff at the University of Lübeck for their excellent technical support.

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.2023.1241461/full#supplementary-material

SUPPLEMENTARY FIGURE

Flow cytometry gating strategy for monocyte subpopulations in human peripheral blood samples. Cell debris was excluded during measurement. Cells were separated from counting beads and gated in FSC/SSC, single cells gated in SSC-A/SSC-H. Cells were plotted for their CD14 and CD16 expression and three distinct subpopulations of monocytes, i.e., classical, non-classical, and intermediate were analyzed. Resulting populations were subsequently plotted for CCR2 and CX3CR1 expressions. And finally, cells were selected for HLA expression.

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 15 April 2024
DOI 10.3389/fimmu.2024.1243566

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OPEN ACCESS

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RECEIVED 20 June 2023
ACCEPTED 12 March 2024
PUBLISHED 15 April 2024

CITATION
Combemale L, Bohelay G, Sitbon I-Y, Ahouach B, Alexandre M, Martin A, Pascal F, Soued I, Doan S, Morin F, Grootenboer-Mignot S, Caux F, Prost-Squarcioni C and Le Roux-Villet C (2024) Lichen planus pemphigoides with predominant mucous membrane involvement: a series of 12 patients and a literature review.
Front. Immunol. 15:1243566.
doi: 10.3389/fimmu.2024.1243566

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Lichen planus pemphigoides with predominant mucous membrane involvement: a series of 12 patients and a literature review

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Background: Lichen planus pemphigoides (LPP), an association between lichen planus and bullous pemphigoid lesions, is a rare subepithelial autoimmune bullous disease. Mucous membrane involvement has been reported previously; however, it has never been specifically studied.

Methods: We report on 12 cases of LPP with predominant or exclusive mucous membrane involvement. The diagnosis of LPP was based on the presence of lichenoid infiltrates in histology and immune deposits in the basement membrane zone in direct immunofluorescence and/or immunoelectron microscopy. Our systematic review of the literature, performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines, highlights the clinical and immunological characteristics of LPP, with or without mucous membrane involvement.

Results: Corticosteroids are the most frequently used treatment, with better outcomes in LPP with skin involvement alone than in that with mucous membrane involvement. Our results suggest that immunomodulators represent an alternative first-line treatment for patients with predominant mucous membrane involvement.

KEYWORDS

lichen planus pemphigoides, mucous membrane pemphigoid, oral lichen planus, bullous pemphigoid, autoimmune blistering disease, autoimmune blistering dermatosis

1 Introduction

First described clinically by Kaposi (1), lichen planus pemphigoides (LPP) is commonly associated with lichen planus (LP) and bullous pemphigoid (BP). In addition to clinical (lichenoid lesions and tense blisters) and histological (lichenoid changes and subepidermal split) findings, the gold standard for the diagnosis of LPP is the detection of autoantibody deposits along the dermal–epidermal junction (DEJ) using direct immunofluorescence (DIF) on perilesional skin biopsies, as first reported by Stingl et al. (2). Circulating autoantibodies targeting type XVII collagen (COL17; molecular weight 180 kDa) in the sera of patients with LPP were first reported by Cognat et al. (3). Using immunoblotting (IB), Ogg et al. (4) demonstrated that these circulating anti-COL17 autoantibodies react with the membrane-proximal NC16A subdomain.

Additional studies suggested that LPP is not a simple association between LP and BP and highlighted its heterogeneity regarding targeted antigens. The primary antigenic target of LPP is COL17; however, other antigenic targets have also been identified. Zillikens et al. (5) showed that a novel epitope within the BP-NC16A domain, designated as MCW-4, is recognized by the serum autoantibodies in patients with LPP. Moreover, using recombinant proteins covering the entire NC16 domain of BP-180, the same team identified more subtle differences in the epitope specificity of circulating autoantibodies in the sera of patients with LPP compared with those with mucous membrane pemphigoid (MMP), BP, and pemphigoid gestationis (PG) (6). Other targets of autoantibodies described in LPP are the C-terminal domains of BP180 (7–10), BP230, LAD antigen (8, 11), desmoglein 1 (7, 8), and an antigen with a molecular weight of 200 kDa (12, 13).

Clinically, LPP is often described as the development of blisters on the skin, in not only areas with lichenoid changes but also uninvolved areas. Mucous membrane (MM) involvement has mainly been observed in the oral mucosa. Indeed, Zaraa et al. (14), in their review of literature published between 1980 and 2010, reported oral involvement in 28 of 78 (36%) cases, but rarely in other MM, and did not report LPP with predominant mucosal involvement.

Herein, we report 12 cases of LPP with exclusive or predominant MM involvement diagnosed at our center. To date, this is the largest reported study of LPP with predominant MM involvement. We described the clinical, histological, and immunological characteristics of these LPP cases and compared our findings with those of previous studies on LPP with and without MM involvement via a systematic review, according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

2 Patients and methods

This single-center, retrospective study was conducted in May 2022 and involved patients treated between 2001 and 2020 at Avicenne Hospital (Assistance Publique-Hôpitaux de Paris, Bobigny, France) using the computer database (eDBAI) of the referral center for autoimmune bullous diseases (AIBD). The information of all patients was systematically recorded and stored in a computerized medical chart standardized for AIBD after obtaining written informed consent from the patients. This study was approved by the institutional review board of Avicenne Hospital (approval number: CLEA-2022-240).

2.1 Patient selection

Among patients with AIBD monitored at our center, those with a confirmed diagnosis of LPP were included. The inclusion criteria were a diagnosis of (i) LP relying on clinical data (white network lesions of MM and/or pruritic violaceous papules on the skin) with histological confirmation (lichenoid interface dermatitis) and (ii) sub-epithelial/epidermal AIBD relying on clinical data (blisters), histological data (subepithelial/epidermal cleavage), and autoantibody deposits (IgA, IgG, and IgM) along the DEJ or chorioepithelial junction (CEJ) detected using DIF and/or direct immunoelectron microscopy (IEM).

2.2 Data collection

The patients were designated with numbers N°1–12 (Table 1), and the following data were collected from each patient's medical record:

a. Epidemiological characteristics, including sex, age, comorbidities, medications, and LPP diagnosis time.
b. Clinical findings on the physical examination of LP papules, LP MM white network, blisters, erosions, erythema, atrophy, and synechiae, as well as their locations on the MM and/or skin.
c. Histological and immunological findings of skin and/or MM biopsies at diagnosis, including lichenoid interface dermatitis (band-like lymphocytic infiltrate, degeneration of basal keratinocytes, with varying degrees of epithelial lymphocytic exocytosis and necrosis of basal keratinocytes) and subepithelial/epidermal cleavage, as well as Ig class(es) (IgA, IgG, and IgM), ± C3 deposits at the CEJ, and/or DEJ on DIF, and/or direct IEM (on semi-thin sections), and ultrastructural immune deposits' locations on direct IEM (on ultrathin sections).
d. Immunoserology results at diagnosis, including indirect immunofluorescence (IIF) with patient sera on rat and/or monkey oesophagus (BMD, Marne la Vallée, France, The Binding Site, Saint Egrève, France); IIF on 1 M NaCl-treated human or monkey salt-split skin (IIF-SSS) (Immco Diagnostics, Buffalo, USA); commercially available BP180-NC16A, BP230, and type 7 collagen enzyme-linked immunosorbent assays (ELISAs) (MBL, Nagoya, Japan); and immunoblotting (IB) using the human amniotic membrane (15).
e. Treatments, including topical steroids, dapsone, sulfasalazine, doxycycline, acitretin, systemic corticosteroids, and immunosuppressants (mycophenolate mofetil and rituximab).

TABLE 1 Clinical data of our patients with lichen planus pemphigoid predominant on mucous membrane.

Patient no.	Sex	Age (years)	Disease duration at LPP diagnosis (months)	Order of diagnosis between LP and AIBD	Sites involved at LPP diagnosis			Clinical data				
					Skin yes/no	MM n	MM sites	Cutaneous LP papules	MM LP network	Cutaneous blisters	MM blisters	Other lesions
1	F	86	15	LP first	yes	2	B, G	yes	no	yes	yes	atrophia, synechiae
2	F	43	18	LP first	yes	2	B, NT	yes	no	no	yes	atrophia
3	M	58	24	LP first	yes	3	B, G, A	yes	yes	no	yes	erosion
4	F	74	96	LP first	yes	2	B, G	no	yes	yes	yes	atrophia, erosion, synechiae
5	F	82	24	LP first	yes	3	B, G, NT	no	yes	yes	yes	atrophia, erosion, synechiae
6	M	69	156	LP first	no	3	B, G, A	no	yes	no	no	erosion
7	F	71	36	LP first	no	2	B, NT	no	yes	no	no	atrophia, erosion
8	M	35	228	LP first	no	2	B, G	no	yes	no	yes	atrophia, synechiae
9	F	58	8	AIBD first	yes	2	B, NT	no	yes	yes	yes	synechiae
10	F	65	48	AIBD first	no	2	G, C	no	no	no	no	synechiae
11	F	42	4	at once	yes	2	B, G	no	yes	yes	yes	erosion
12	F	66	nd	at once	yes	3	B, G, A	yes	yes	yes	no	atrophia, erosion,

LPP, Lichen Planus Pemphigoid; LP, Lichen Planus; AIBD, autoimmune bullous disease; n, number; MM, mucous membrane; F, female; B, buccal mucous membrane; G, genitalia; NT, nose and throat; M, male; A, anal; C, conjunctiva; nd, not determinated.

f. Follow-up and status at the last visit, including complete remission (CR), almost CR (aCR; transient new lesions that heal within 1 week), controlled disease, or active disease adapted from the consensus statement for MMP (16). The active lesions included erythema, erosion, and blisters. A persistent mucosal residual lichenoid network without inflammation was not considered active disease.

2.3 Literature review

The medical literature search was performed following the PRISMA guidelines (17). Bibliographical research was carried out using the PubMed database for the period between 1975 and May 2022 using the following keywords: "lichen planus" AND "pemphigoid." Cases of histologically proven LP and positive DIF, case reports and series, and manuscripts written in English or French were included in this systematic literature review.

2.4 Statistical analysis

Statistical analyses were performed using StatView (v5.0, SAS Institute Inc.). Quantitative variables are expressed as the medians and interquartile ranges or extreme values, as indicated, according to normality assessed using the Shapiro–Wilk test. Qualitative variables are presented as numbers and proportions. Quantitative variables were analyzed via univariate comparisons between subgroups using Mann–Whitney tests; qualitative variables were analyzed via univariate comparisons using Pearson's χ^2 tests, with or without Yate's continuity correction, or Fisher's exact tests, as appropriate, according to sample size.

3 Results

3.1 Patients and clinical findings

In total, 12 patients (nine females and three males, ratio 3:1), with a median age of 65.5 years (range: 35–86 years), were included in this study (Table 1). No patient had a medical history of other autoimmune diseases or chronic infections, such as diabetes or hepatitis. Two patients had a medical history of lymphoma: one had Hodgkin lymphoma a few years before LPP (N°3), and the other was diagnosed with LPP during the course of non-Hodgkin lymphoma therapy (N°11). The latter was the only patient among the 12 (8.3%) to have a plausible drug-induced LPP—the culprit drug being pembrolizumab.

10.3389/fimmu.2024.1243566

The median time between the first symptoms and diagnosis of LPP was 5.4 years (0.7–19.0 years). In eight patients (66%; N°1–8), the lichenoid component preceded the first evidence of autoimmune blistering components by 1.2–13.0 years (mean value: 4.7 years). In two patients (16.7%; N°9–10), typical blistering lesions involving MM preceded (clinically and histologically) typical lichenoid lesions (N°9–10). In the last two patients (N°11–12), LPP was suspected from the outset because of both typical LP lesions and blisters on the MM.

Upon clinical examination, all patients had mucosal involvement affecting one to three different MMs. The MMs involved were the oral (11 of 12), genital (nine of 12), nose and throat (NT; four of 12), anal (three of 12), and conjunctival (one of 12) mucosa; skin lesions were associated in eight of these (66%). Among the 12 patients, four had exclusive MM involvement; the remaining eight had predominant MM involvement, with at least two MM involvements. Moreover, 11 patients had typical LP lesions involving the skin and/or the MM (Figure 1). A white reticulated network in the oral and anal MM (Figure 1A) was observed in nine (N°3–9, 11, 12) and one (N°3) of the 12 patients, respectively. Cutaneous violaceous papules with a Wickham network (Figure 1B) were observed in four patients (N°1–3, 12), associated with nail involvement (pterygium; Figure 1C) in one patient (N°12). One patient (N°10) had typical characteristics of LP on histological examination, without typical clinical lesions of the lichen (see below). Nine patients (N°1–5, 8–9, 11–12) had blisters on the MM and/or skin, eight on the oral MM, and six on the skin (Figure 2). In addition to typical LP and autoimmune blistering disease (AIBD) lesions, all patients demonstrated non-specific clinical lesions that could be found in both LP and AIBD (erythema, erosion, atrophy, synechiae, and conjunctival fibrosis) on their MM [oral, eight of 12; genital, eight of 12; NT, two of 12; anal, two of 12; and/or conjunctival, one of 12, patient N°12, fibrosis of stage IIIA in Tauber and Foster's classification (18); Figure 3].

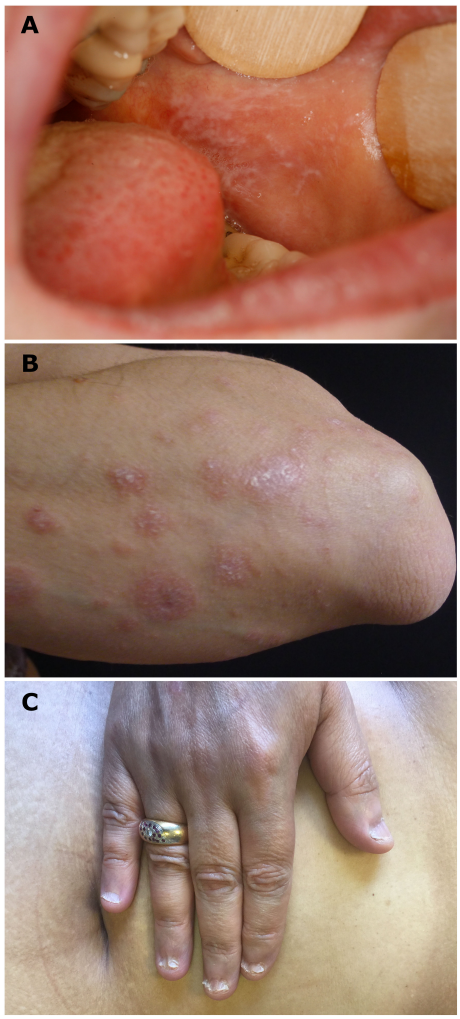

FIGURE 1
Typical clinical lichenoid lesions. **(A)** White reticulated network on buccal mucosa (patient N°9), **(B)** cutaneous violaceous papules with Wickham network on a left forearm (patient N°2), and **(C)** lichen planus onychodystrophy (pterygium) of one patient (patient N°12).

3.2 Histological and immunological data of skin and MM biopsies

Each patient underwent an average of two biopsies, including at least one mucosal biopsy, to confirm the LPP diagnosis. Most patients required separate biopsies of lesions suggestive of LP and MMP, with multiple biopsies necessary in some. Subepithelial/epidermal cleavage was observed in eight of the 12 patients (Table 2). All patients had the histological characteristics of LP, including subepidermal band-like lymphocytic infiltrate in 11 patients, necrotic keratinocytes in seven patients, and lymphocytic

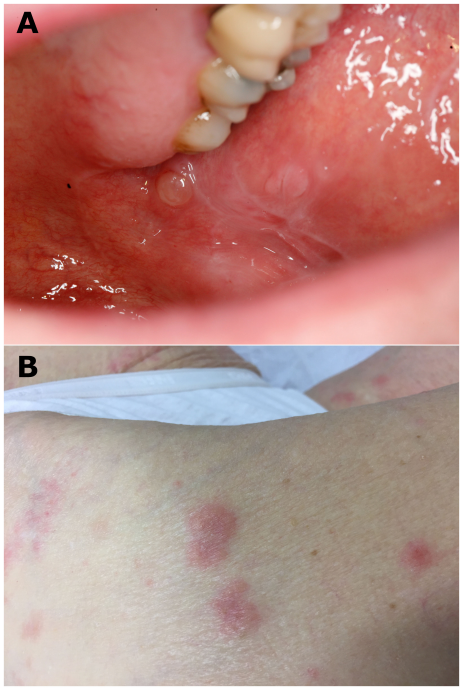

FIGURE 2
Typical clinical bullous lesions. **(A)** Blisters and white reticulated network observed on buccal mucosa (patient N°3) and **(B)** bullous and erythematous lesions on a leg (patient N°4).

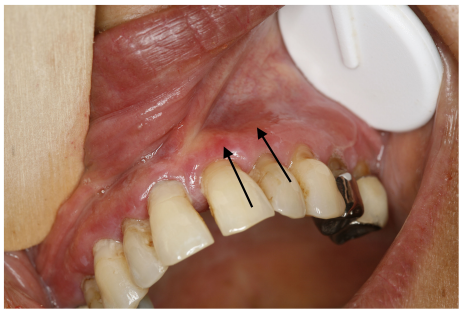

FIGURE 3
Erythema (black arrows), erosion, white lichenoid lesion, and atrophy on vestibular gingiva. Erythema could be a manifestation of lichen planus or mucous membrane pemphigoid. (patient N°12).

exocytosis in eight patients (Figure 4). Subepithelial cleavage and lichenoid reaction patterns could be observed in one section (Figures 4A, B), separately in two different sections of a unique biopsy sample (Figures 4C, D), or separately in two samples obtained from two different sites (Figures 4E, F).

DIF and/or direct IEM of semi-thin sections (Figures 4G, H, 5A) revealed linear deposits of autoantibodies (IgG and IgA) on JDE/JCE in all patients (Table 2). IEM on ultrathin sections performed in nine of the 11 patients exhibited autoantibody deposits (IgG and IgA) in all samples studied, of which four had a negative DIF. IgG and/or IgA deposits were located on hemidesmosomes (HD) or on HD and lamina lucida (LL) in four of the 11 patients or on the lamina densa (LD) or LD and LL in five of the 11 patients (Figures 5B, C). It was non-contributory in two of the 11 patients.

3.3 Circulating autoantibodies

Standard IIF on rat/monkey oesophagus and IIF-SSS were positive in the sera of three of 12 patients (N° 4, 9, 12), with labeling of the epidermal side on SSS (Table 2).

ELISA demonstrated the presence of circulating IgG autoantibodies directed against BP180-NC16A in six patients (50%; N° 1,4,5,8,11,12), BP230 in two patients (N° 8,12), and type VII collagen in one patient (N° 8) (Table 2). IB using amniotic membrane extracts was performed only on the sera of three patients; the serum of one patient (N°10) showed IgG autoantibodies with a 200-kDa band (Table 2).

3.4 Treatment and follow-up

All 12 patients received topical corticosteroids, notably awaiting confirmation of LPP diagnosis (Table 3). All but one patient received systemic treatment. Systemic therapies used in the first-line treatment mostly comprised immunomodulatory drugs (11 of 12), such as dapsone, doxycycline, and sulfasalazine, in combination with systemic corticosteroids for one patient. Acitretin was also administered to treat the LP component (three of 12 patients) as the first- or second-line treatment when LP lesions seemed particularly active. Dapsone was administered as a first-line treatment to nine of the 12 patients; six of the 12 patients received dapsone alone and three of the eight patients received it in combination with other drugs (systemic corticosteroids [N°5], sulfasalazine [N°11], and doxycycline and acitretin [N°12]). Six patients were in CR or aCR after first-line treatment, of whom five were treated with dapsone alone (N°3, 4, 6, 9, 10). The other patient was treated with dapsone combined with doxycycline (200 mg) and acitretin (0.3–0.5 mg/kg) (Table 3).

Among the 12 patients, five received more than one line of treatment (Table 3). The molecules administered as second-line treatments were immunomodulators, systemic corticosteroids, and/or immunosuppressants (mycophenolate mofetil or rituximab). All the patients received dapsone therapy. Two of the five patients achieved CR or aCR (N°1, 2), one had controlled disease (N°8), and the last two still presented with active disease (N°5, 11) at the last follow-up. One patient (N°6) in the CR group developed transient aplasia, and dapsone was discontinued.

Relapse occurred in 10 of the 12 patients, most of whom had lichenoid lesions; three of the 12 patients had frequent flares. Notably, relapse of bullous lesions occurred in four of the seven patients because of the decrease or discontinuation of immunomodulators.

At the last visit, after a median follow up of 5.5 years (range: 2–19 years), eight patients (66.7%) were in CR or aCR, one had a controlled disease, and three still had active disease (Table 3).

4 Literature review

Only 132 patients with LPP have been reported in 112 case reports or small series since the first description of autoimmune deposits on the basement membrane zone (BMZ) in LPP, 50 years ago by Sting et al. (2) (Annex 1). A predominance of females (60%, 80 females vs. 52 males) was observed, with a median age at diagnosis of 48.9 years, and 19 paediatric cases of LPP have been reported (14.4%). Comorbidities included diabetes in 17 patients (12%), chronic viral hepatitis in 4 (3%), and cancer in 23 (16%). LPP was considered as drug induced in 24 cases (17%), the suspect drugs mostly being anti-PD1 or anti-PDL1.

Among the reported cases, 71 patients (54%) had mucosal involvement (Table 4). Most of these patients were initially diagnosed with LP (75.4%); only a few cases were diagnosed with LPP at the outset (21.5%). Clinically, oral mucosa is the most frequent site of involvement (97%), followed by skin (82%), genitals (18.3%), eyes, and ENT (both 4.2%); one case of oesophageal involvement has been described (19). Blisters (77.5%) and lichenoid lesions (80.3%) were mostly observed on the skin. A total of 56 patients (78.9%) presented histological features of subepidermal AIBD. Several biopsies were required to diagnose LPP (mean number of 1.7 biopsies per patient). All DIF cases were positive, and cases without positive results were excluded from the literature review. Circulating autoantibodies were found in 83.7% of patients; IIF/IIF-SSS was positive in 73.9% of cases, labeling most frequently the roof (77.3%), as opposed to the floor (4.5%). Autoantibodies reacted more frequently with BP180-NC16A

TABLE 2 Histological and immunological data of our patients with lichen planus pemphigoid predominant on mucous membrane.

Patient no.	Lichenoid typical features	Subepidermal band-like lymphocytic infiltrate	Necrotic keratinocytes	Lymphocytic exocytosis	Sub-epithelial cleavage	DIF: BMZ (IgG, IgA, IgM, C3)	DIEM (SF): BMZ	DIEM (UF): HD, LL, LD	IIF/ IIF-SSS: BMZ/ roof, floor	ELISAs: BP180, BP230 COL VII	WB (amniotic extracts)
1	yes	yes	yes	no	yes	+ (G, C3)	+	LD, LL	neg	BP180 +/neg/nd	nd
2	yes	no	yes	no	yes	+ (G, A M, C3)	+	HD, LL	neg	neg/neg/neg	nd
3	yes	yes	yes	yes	yes	neg	+	LD, LL	neg	neg/neg/nd	nd
4	yes	yes	no	yes	no	+ (G, C3)	+	HD, LL	+/roof	BP180 +/neg/nd	nd
5	yes	yes	yes	yes	yes	+ (G, A)	+	LD	neg	BP180 +/neg/neg	neg
6	yes	yes	yes	yes	no	neg	+	LD, LL	neg	neg/neg/neg	nd
7	yes	yes	no	no	yes	neg	+	nd	neg	neg/neg/nd	nd
8	yes	yes	yes	no	yes	neg	+	HD	neg	BP180 +/BP230 +/Col VII +	nd
9	yes	yes	no	yes	no	+ (G, C3)	+	LD, LL	+/roof	neg/neg/nd	neg
10	yes	yes	no	yes	no	+ (C3)	nd	nd	neg	neg/neg/neg	200kDa band
11	yes	yes	yes	yes	yes	+ (G, C3)	+	nd	neg	BP180 +/neg/neg	nd
12	yes	yes	no	yes	yes	+ (G, C3)	+	HD, LL	+/roof	BP180 +/BP230 +/nd	nd

DIF, direct immunofluorescence; BMZ, basal membrane zone; Ig, immunoglobulin; C3, complement C3; DIEM, direct immunoelectron microscopy; SF, semi-thin section; UF, ultra-thin section; HD, hemidesmosome; LL, lamina lucida; LD, lamina densa; IIF, indirect immunofluorescence; IIF-SSS, immunofluorescence on salt-split skin; ELISA, enzyme-linked immunosorbent assay; BP, bullous pemphigoid antigen; col VII, type VII collagen; WB, Western blot assay; +, positive; neg, negative; nd, not determinated.

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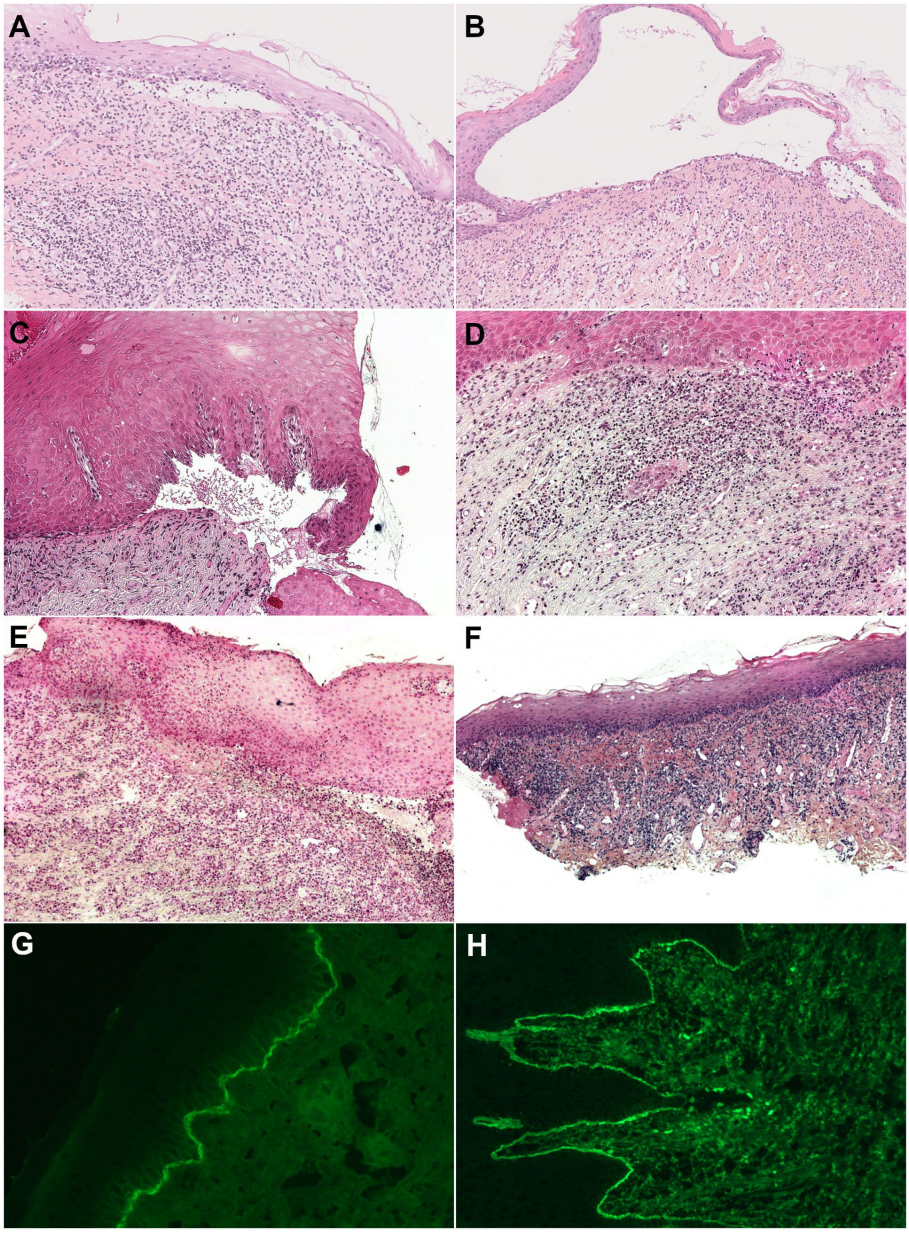

FIGURE 4
Histology of mucous membrane biopsy (Hematoxylin and eosin staining) and subepithelial cleavage and lichenoid reaction pattern (band-like lymphocytic infiltrate hugging the chorioepithelial junction with, depending on the patient, vacuolar degeneration of the basal layer of the epithelium, necrosis of individual keratinocytes, and lymphocytic exocytosis). **(A, B)** Both in one section of a biopsy (original magnification, 50×), with **(A)** discrete subepithelial cleavage and predominant lichenoid reaction (patient N° 8) and **(B)** large subepithelial cleavage and discrete lichenoid pattern with lymphocytic exocytosis (patient N° 12). **(C, D)** Separately in two different sections of one biopsy (original magnification, 100×), with **(C)** subepithelial cleavage and **(D)** a lichenoid reaction pattern (patient N°3). **(E, F)** Separately in two different biopsies (original magnification, 100×), with **(E)** subepithelial cleavage on gingiva biopsy and **(F)** a lichenoid reaction pattern on buccal mucosa biopsy (patient N°5). **(G, H)** Direct immunofluorescence, with **(G)** linear immune deposits of IgG on the DEJ (patient N°11) and **(H)** on the CEJ (patient N°4).

(88.5%) than with BP230 (26.7%) on ELISA. IB also detected anti-BP180 autoantibodies more frequently than anti-BP230 antibodies. In 10 patients in whom IB was specified, antibodies were directed against NC16A (six of 10) and the C-terminal region of BP180 (two of 10). Regarding treatment, corticosteroids were widely used as the first-line treatment (48.6% alone and 14.3% combined), immunosuppressive and immunomodulatory drugs were used in 11.4% and 8.6% of patients, respectively. Remission (CR and aCR) was achieved in 65% of cases at the end of follow up (median, 10.5 months). Subsequent lines of treatment included combined therapies, immunomodulators, and retinoids, and one patient also received rituximab.

Regarding purely cutaneous LPP (Table 5), 51 of 61 patients (83.6%) presented histological features of subepidermal AIBD. All

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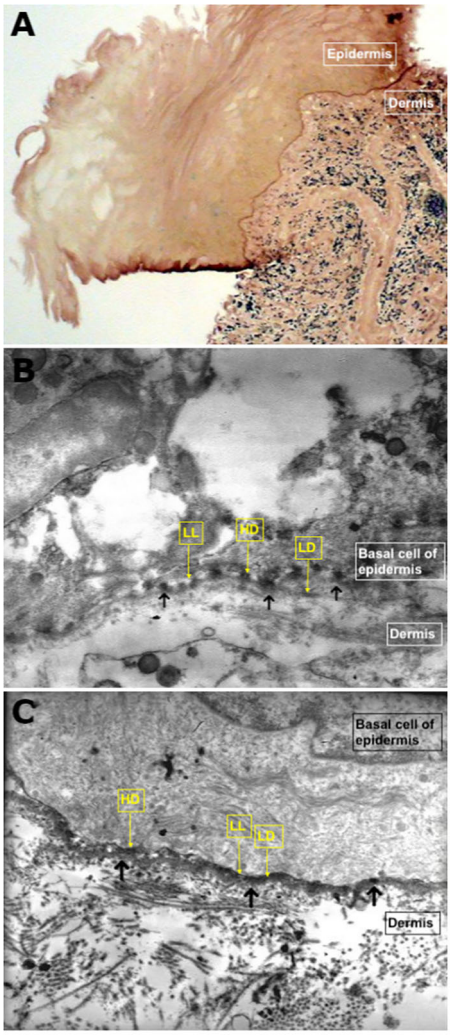

FIGURE 5
Direct immunoelectron microscopy. **(A)** Semi-thin section demonstrating linear deposits of autoantibodies on the chorioepithelial junction (original magnification, 40×; patient N°2). **(B)** Ultra-thin section demonstrating autoimmune deposits on hemidesmosomes or hemidesmosomes and lamina lucida (shown by the black arrows) (original magnification, 15,000×; patient N°4). **(C)** Ultra-thin section demonstrating thick immune deposits (shown by the black arrows) on lamina densa intermittently overlying the lamina lucida (original magnification, 15,000×; patient N°1).

DIF results were positive. Circulating autoantibodies were detected in 90.5% of patients, and the sera of 84.4% of patients were positive for IIF on skin and IIF-SSS. ELISA BP180NC16A was positive in 89.5% of patients who underwent the test, and ELISA BP230 was positive in 25% of patients. IB also showed more anti-BP180 autoantibodies (88.9%) than anti-BP230 (11.1%). Most first-line treatments were corticosteroids (54.2% alone and 18.6% in combination with other drugs); immunosuppressive and immunomodulatory drugs were used in 8.5% and 17% of patients, respectively, whereas drug cessation was reported in 93.3% of drug-induced skin LPP. Remission was achieved in 84.7% of cases at the end of follow-up (median duration, 9 months).

5 Discussion

LPP is rare, with an annual incidence in Kuwait (20) estimated at 0.3 per million. Epidemiological studies in France, Germany, Greece, Serbia, and Singapore have not identified any cases of LPP among patients with AIBD, because LPP is rare and not always considered to be a part of the classic list of AIBD (5, 21–25). However, patients with LPP are continuously identified and 132 case reports and small series have been published since 1975; moreover, LPP has been returning to the center stage because of antiPD1/PDL1 induced cases (26–31).

All patients diagnosed with LPP at our center had predominant or exclusive MM involvement. This may have been influenced by the specific recruitment of our referral AIBD center, which specializes in MMP. Therefore, we reported the largest case series of patients with LPP and exclusive or predominant MM involvement.

Our patients did not present any significant differences from those in the literature in terms of age, female predominance, or percentage of comorbidities (particularly cancer) (Tables 4, 5). Approximately half of the LPP cases with a history of malignancy were suspected to be induced by oncological treatment. In the remaining patients, the cancer was in remission, except in one case of LPP associated with multiple keratoacanthomas and colon carcinoma (32). Two patients in the literature review had anti-laminin 332 autoantibodies, which are often associated with cancer (9), but none were detected in the cases reported in this study.

Patients with LPP are typically young, with a median age of 48.9 years at LPP diagnosis, compared with patients with BP (median age, 80 years) (33) and MMP (median age, 60–80 years) (34). Among our cases, one was induced by pembrolizumab (anti-PD1), as observed in nine other recently reported cases of LPP with anti PD1/PDL1 immunotherapies in the literature (26–31).

In clinical presentation, our cases differed significantly from those reported in the literature because they had exclusive or predominant MM lesions (100% vs. 54%; at least two MM involved or MM lesions alone; 100% vs. 31%; Tables 4, 5; Annex 1). In comparison with LPP patients with MM involvement in the literature (Table 4), we observed more patients with three or more affected sites (50% vs. 15%), more genital involvement (75% vs. 18.3%), and more nasal or pharyngeal (33% vs. 4.2%) involvement. Our patients had less skin involvement (such as blisters and lichenoid lesions). The least frequently affected site in our series was conjunctiva, as also reported in the literature (three patients, 0.04%); however, we did not report any oesophageal involvement, whereas one such case was described in the literature (19, 35, 36) (Table 4). Significantly more non-specific lesions were observed in MM in our series compared with the literature, possibly because of the absence of data collection (Table 4).

All IEM performed in our study (11 of 12) revealed autoimmune deposits (IgG and/or IgA) on semi-thin sections. Notably, it enabled the detection of deposits not visualized on DIF in four patients, suggesting that IEM might be more sensitive than DIF and of particular interest in diagnosing LPP in patients with negative DIF (37). IEM sensitivity has never been clearly compared with DIF sensitivity, because this technique is usually

TABLE 3 Treatment and outcome data of our patients with lichen planus pemphigoid predominant on mucous membrane.

Patient	Treatment (maximum dose)		Clinical status (last visit)	Follow-up (years)	Relapses (number) and type of lesions	
no.	First-line	Second-line			Lichenoid	AIBD
1	DOX 200 mg	DDS 1 mg/kg	CR	5	0	3
2	DDS 1.3 mg/kg	DDS 1.3 mg/kg + MMF 3.5g + ACT 0.3 mg/kg	aCR	9	2	no CR
3	DDS 0.5 mg/kg		CR	6	0	0
4	DDS 2 mg/kg		aCR	6	6	3
5	DDS 0.6 mg/kg + sCS 0.3 mg/kg	DDS 0.6 mg/kg + sCS 0.3 mg/kg + RTX	AD	4	1	1
6	DDS 2.6 mg/kg	tCS	AD	7	0	1
7	tCS mouth wash		CR	2	2	0
8	DOX 200mg	DDS 1mg/kg + SSZ 1g	controlled	2	6	4
9	DDS 1 mg/kg		CR	9	1	1
10	DDS 1 mg/kg		CR	5	0	0
11	DDS 2 mg/kg + SSZ 1g	DDS 2 mg/kg + SSZ 1g + ACT 0,1 mg/kg + RTX 2 g	AD	2	0	no CR
12	DDS 2 mg/kg + DOX 200 mg, + ACT 0.3-0.5 mg/kg		aCR	19	10	2

AIBD, autoimmune bullous disease; DOX, Doxycycline; DDS, Dapsone; CR, Complete Remission; MMF, Mycophenolate Mofetil; ACT, Acitretin; aCR, Almost Complete Remission; sCS, Systemic Corticosteroids; RTX, Rituximab; AD, Active Disease; tCS, Topical Corticosteroids; SSZ, Sulfasalazine.

used to show ultrastructural localization of target antigens and provide an accurate diagnosis of subepithelial AIBD with positive DIF (38). However, two studies on ocular MMP suggested that IEM is more sensitive than DIF (100% sensitivity vs. 67% in a small series of MMP with ocular involvement, or 50% in cases of pure ocular MMP) (37, 39).

In nine of our cases and eight in the literature, ultrastructural deposits were found on the ultrathin sections, in 63% of cases on HD and LL, as in BP, and in 37% of cases on LD and LL, as in MMP (Table 2) (19, 40), showing that target antigens are heterogeneous in LPP, in both MMs and skin.

Regarding serological analyses, a significant difference was observed, with more positive skin IIF/IIF-SSS results in the literature compared with our study (73.9% vs. 25%). ELISAs showed the presence of circulating IgG autoantibodies against the BP180-NC16A epitope in six of the 12 (50%) patients in our study, a percentage significantly lower than that observed in the literature [88.5%] (Table 4). However, these results do not imply that LPP is the association of LP and BP; as demonstrated by Kromminga et al. (6), LPP sera generally show reactivity with different BP180 fragments, comprising D514–L565, and lack binding to E490–R507 in the NC16A domain, whereas the sera of most BP and PG patients bound to fragments representing amino acids E490–G532 also present in the NC16A domain. Furthermore, IB studies showed IgG autoantibodies to a 200 kDa molecular weight antigen compatible with the laminin 332 alpha 3 chain in one of our patients, as previously reported in two cases in the literature (9, 13). Finally, serological studies were negative in five of our patients. In the literature, ELISA revealed that some patients were negative for BP180–NC16A and/or IB for BP180 and/or BP180–NC16A recombinant protein, but positive in IB for the C-terminal portion, as found in some patients with MMP (41, 42). Unfortunately, we did not have the opportunity to perform IB using C-terminal BP180. Notably, three of the five seronegative patients had immune deposits on the LD of the IEM, consistent with autoantibodies directed against the C-terminal portion of BP180.

The median time between the first symptoms and the diagnosis of LPP in our case series was 5.4 years (range: 0.7–19 years). In most patients, the lichenoid component preceded the first evidence of autoimmune blistering (mean of 4.75 years). The delay between the first lesions and LPP diagnosis might be explained by the presence of mucosal non-specific elementary lesions that may result from LP and/or AIBD (erosions, erythema, atrophy, and synechiae), and LPP was not suspected until typical blisters appeared. Moreover, LPP diagnosis not only requires immunological findings with BMZ autoimmune deposits on DIF or IEM but also histological findings such as lichenoid infiltrates and ideally subepidermal blisters, which sometimes require multiple biopsies at two different sites, especially when AIBD and

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TABLE 4 Comparison between our series and the MM-LPP cases from the literature.

Variables	All cases N =83	MM-LPP our series N = 12	MM-LPP litterature N = 71	p-value
Female gender, N (%)	56 (67.5)	9 (75.0)	47 (66.2)	0.7426
Age at diagnosis (years), median (IQR)	58 (23.0)	65.5 (22.0)	57 (21.5)	0.0922
Diabetes (%)	9 (10.8)	0 (0)	9 (12.7)	0.3445
Chronic viral hepatitis (%)	3 (3.6)	0 (0)	3 (3.0)	>.9999
Cancer (%)	15 (18.1)	2 (16.7)	13 (18.3)	>.9999
Drug induction (%)	10 (12.0)	1 (8.3)	9 (12.7)	>.9999
Order of diagnosis (%) – available for n=	74 (89.2)	12 (100)	62 (87.3)	
LP first	57 (74.0)	8 (66.7)	49 (75.4)	0.4977
AIBD first	4 (5.2)	2 (16.7)	2 (3.1)	0.1124
At once	16 (20.8)	2 (16.7)	14 (21.5)	>.9999
Clinical presentation (%)				
Mouth	80 (96.4)	11 (91.7)	69 (97.2)	0.3779
Skin	66 (79.5)	8 (66.7)	58 (81.7)	0.2553
Genital	**22 (26.5)**	**9** (75.0)	**13** (18.3)	**0.0002**
Eyes	4 (4.8)	1 (8.3)	3 (4.2)	0.4713
NT	**7 (8.4)**	**4 (33.3)**	**3 (4.2)**	**0.0073**
Oesophagus	1 (1.2)	0 (0)	1 (1.4)	>.9999
Number of MM involved, median (IQR)	**2** (0)	**3** (1)	**2** (0)	**0.0018**
One site involved	8 (9.6)	0 (0)	8 (11.3)	0.5954
Two sites involved	56 (64.5)	5 (41.7)	51 (71.8)	0.0507
≥ 3 sites involved	**17 (20.5)**	**6 (50.0)**	**11 (15.5)**	**0.0184**
Blisters (%)				
Mouth	**26 (31.3)**	**8 (66.7)**	**18 (25.3)**	**0.0074**
Skin	**60 (72.3)**	**5 (41.7)**	**55 (77.5)**	**0.0265**
Genital	3 (3.6)	0 (0)	3 (4.2)	>.9999
LP lesions (%)				
Mouth	61 (73.5)	11 (91.7)	50 (70.4)	0.1681
Skin	**62 (74.7)**	**5 (41.7)**	**57 (80.3)**	**0.0127**
Genital	**9 (10.8)**	**4 (33.3)**	**5 (7.0)**	**0.0221**
LP or AIBD lesions (%)				
Mouth	**49 (60.5)**	**11 (91.7)**	**38 (55.1)**	**0.0230**
Genital	**15 (18.5)**	**7 (58.3)**	**8 (11.6)**	**0.0006**
Histology (%)				
AIBD	64 (77.1)	8 (66.7)	56 (78.9)	0.4570
LP	83 (100)	12 (100)	71 (100)	*na*
Circulating auto-antibodies (whatever the method) (%)				
Performed	67 (80.7)	12 (100)	55 (77.5)	0.1106
Positive	54 (80.6)	8 (66.7)	46 (83.7)	0.3421

(Continued)

TABLE 4 Continued

Variables	All cases N =83	MM-LPP our series N = 12	MM-LPP litterature N = 71	p-value
IFI/IFI-SSS (%)				
Performed	**58 (69.9)**	**12** (100)	**46 (64.8)**	**0.0147**
Positive	**37 (63.8)**	**3 (25.0)**	**34 (73.9)**	**0.0049**
Blot (%)				
Performed	19 (22.9)	3 (25.0)	16 (22.5)	>.9999
Full BP180 (n=19)	**14 (73.7)**	**0** (0)	**14 (87.5)**	**0.0103**
BP180 NC16A (n=10)	6 (60.0)	*nd*	6 (60)	*na*
BP180 Cterm (n=10)	2 (20.0)	*nd*	2 (20)	*na*
BP230 (n=19)	4 (21.1)	0 (0)	4 (25.0)	>.9999
ELISA (%)				
Performed	**38 (45.8)**	**12** (100)	**26 (36.6)**	**<0.0001**
BP180 NC16a (n=38)	**29 (76.3)**	**6 (50.0)**	**23 (88.5)**	**0.0164**
BP230 (n=27)	6 (22.2)	2 (16.7)	4 (26.7)	0.6618
First line treatment (%)	82 (98.8)	12 (100)	70 (98.6)	>.9999
Corticosteroids only	34 (41.5)	0 (0.0)	34 (48.6)	0.0010
Corticosteroids combined	11 (13.4)	1 (8.3)	10 (14.3)	>.9999
Immunosuppressive (ciclo, MTX, AZA, MMF, sirolimus)	8 (9.8)	0 (0)	8 (11.4)	0.5964
Immunomodulators (DDS/DOX)	**17 (20.7)**	**11 (91.7)**	**6 (8.6)**	**<0.0001**
First line treatment efficiency	50 (61.02)	7 (58.3)	43 (61.4)	>.9999
Last line treatment				
Corticosteroids only	**31 (37.8)**	**0** (0)	**31 (44.3)**	**0.0027**
Corticosteroids combined with IS	15 (18.3)	1 (8.3)	14 (20.0)	0.4509
Immunomodulators	**21 (25.6)**	**10 (83.3)**	**11 (15.7)**	**<0.0001**
Immunosuppressive	14 (17.1)	3 (25.0)	11 (15.7)	0.4216
Rituximab	3 (3.7)	2 (16.7)	1 (1.4)	0.0547
Retinoids	**5 (6.1)**	**3 (25.0)**	**2 (2.9)**	**0.0208**
Last line efficiency n=81	68 (84.0)	9 (75.0)	59 (85.5)	0.3975
Status	81	12	69	
CR	48 (59.3)	5 (41.7)	43 (62.3)	0.2130
aCR	5 (6.2)	3 (25.0)	2 (2.89)	0.0215
CD	14 (17.3)	1 (8.3)	13 (18.9)	0.6809
PR	3 (3.7)	0 (0)	3 (4.3)	>.9999
Failure	11 (13.6)	3 (25.0)	8 (11.6)	0.3549
Death reported	5 (6.2)	0 (0)	5 (7.3)	>.9999
Follow-up duration, median in months (IQR)	**15.0 (24.5)**	**66.0 (60.0)**	**10.5 (19.5)**	**<0.0001**

MMLPP, mucous membrane lichen planus pemphigoid; IQR, interquartile range; LP, Lichen Planus; AIBD auto-immune blistering disease; NT, nose and throat;MM, mucous membrane; IFI, indirect immunofluorescence; IFI-SSS, IFI on salt split skin; Blot; western-blot; ELISA, Enzyme-Linked Immunosorbent Assay; IS, immunosuppressive; Ciclo, ciclosporine; MTX, methotrexate; AZA, azathioprine; MMF, mycophenolate mofetil; DDS, dapsone; DOX, doxycycline; CR, complete remission; aCR, almost complete remission; CD, controlled disease; PR, partial remission. Bold values are statistically significant.

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TABLE 5 Comparison between the LP-MMP cases (our series + literature) and cutaneous LPP cases from the literature.

Variables	All patients N = 144	Mucous LPP our serie + litterature N = 83	Cutaneous LPP litterature N = 61	p-value
Female gender, N (%)	89 (61.8)	56 (64.5)	33 (54.1)	0.1027
Age at diagnosis (years), median (IQR)	55.0 (33.5)	58.0 (23.0)	47.0 (52.5)	0.0721
Diabetes (%)	17 (11.8)	9 (10.9)	8 (13.1)	0.8760
Chronic viral Hepatitis (%)	4 (2.8)	3 (3.6)	1 (1.6)	0.6375
Cancer (%)	23 (16.0)	15 (18.1)	8 (13.1)	0.5672
Drug induction (%)	24 (16.7)	10 (12.0)	14 (23.0)	0.0828
Order of diagnosis (%)	136 (94.4)	77 (92.8)	59 (96.7)	0.4673
LP first	98 (72.1)	57 (74.0)	41 (69.5)	0.5592
AIBD first	4 (2.9)	4 (5.2)	0 (0)	0.1326
At once	34 (25.0)	16 (20.8)	18 (30.5)	0.1941
Clinical presentation (%)				
Skin involvement	**127 (88.2)**	**66 (79.5)**	**61** (100)	**<0.0001**
Skin blisters (%)	**118 (81.9)**	**60 (72.3)**	**58 (95.1)**	**0.0003**
Skin LP lesion (%)	**122 (84.7)**	**62 (74.7)**	**60 (98.4)**	**<0.0001**
Histology (%)				
AIBD	115 (79.9)	64 (77.1)	51 (83.6)	0.3367
LP	144 (100)	83 (100)	61 (100)	na
Circulating antibodies (%)				
Performed	109 (75.7)	67 (80.7)	42 (68.9)	0.1008
Positive	92 (84.4)	54 (80.6)	38 (90.5)	0.1884
IFI/IFI-SSS (%)				
Performed	**90 (62.5)**	**58 (69.9)**	**32 (52.5)**	**0.0329**
Positive	64 (71.1)	37 (63.8)	27 (84.4)	0.0521
Blot Performed (%)	28 (19.4)	19 (22.9)	9 (14.8)	0.3144
Full BP180	22 (78.6)	14 (73.7)	8 (88.9)	0.6296
BP180 NC16A (n=16)	7 (50.0)	6 (60.0)	1 (25.0)	0.5594
BP Cterm (n=16)	4 (25.0)	2 (20.0)	2 (33.3)	0.6044
BP230	5 (17.8)	4 (21.1)	1 (11.1)	>.9999
ELISA Performed (%)	57 (39.6)	38 (45.8)	19 (31.1)	0.0760
BP180	45 (80.7)	29 (76.3)	17 (89.5)	0.3041
BP230	8 (22.9)	6 (22.2)	2 (25.0)	>.9999
First line treatment (%)	141 (97.9)	82 (98.8)	59 (96.7)	0.5741
Corticosteroids only	**66 (46.8)**	**34 (41.5)**	**32 (54.2)**	**0.1337**
CS combined	**22 (15.6)**	**11 (13.4)**	**11 (18.6)**	**0.3986**
Immunosuppressive (ciclo, MTX, AZA, MMF, sirolimus)	13 (9.2)	8 (9.8)	5 (8.5)	>.9999
Immunomodulateurs (DDS/DOX)	27 (19.1)	17 (20.7)	10 (17.0)	0.5734
First line treatment efficiency (%)	91 (64.1)	50 (61.0)	40 (67.8)	0.4057
Last line treatment	141	82	59	
Corticosteroids only	58 (41.1)	31 (37.8)	27 (45.8)	0.3435

(Continued)

TABLE 5 Continued

Variables	All patients N = 144	Mucous LPP our serie + litterature N = 83	Cutaneous LPP litterature N = 61	p-value
Corticosteroids combined with IS	30 (21.3)	15 (18.3)	15 (25.4)	0.3074
Immunomodulateurs	32 (22.7)	21 (25.6)	11 (18.6)	0.3300
Immunosuppressive	24 (17.0)	14 (17.1)	10 (17.0)	09846
Rituximab	4 (2.8)	3 (3.7)	1 (1.7)	0.6398
Retinoids	6 (4.3)	5 (6.1)	1 (1.7)	0.4008
Last line efficiency (n=140)	123 (87.9)	68 (84.0)	55 (93.2)	0.1199
Status (%)	140	48	44	
Follow-up duration	**12.0 (18.7)**	**15.0 (24.5)**	**9.0 (7.7)**	**0.0098**
CR	92 (65.7)	48 (59.3)	44 (74.6)	0.0594
aCR	11 (7.9)	5 (6.2)	6 (10.2)	0.5825
CR+ aCR	**103 (73.6)**	**53 (65.4)**	**50 (84.7)**	**0.0180**
CD	18 (12.9)	14 (17.3)	4 (6.8)	0.0776
PR	3 (2.1)	2 (2.4)	1 (1.6)	>.9999
Failure	**13 (9.3)**	**11 (13.6)**	**2 (3.4)**	**0.0441**
Death reported (%) n=140	6 (4.3)	5 (6.2)	1 (1.7)	0.4009

LPP, lichen planus pemphigoid; IQR, interquartile range; LP, Lichen Planus; AIBD auto-immune blistering disease; IFI, indirect immunofluorescence; IFI-SSS, IFI on salt split skin; Blot; western-blot; ELISA, Enzyme-Linked Immunosorbent Assay; Ciclo, ciclosporine; MTX, methotrexate; AZA, azathioprine; MMF, mycophenolate mofetil; DDS: dapsone; DOX, doxycycline; CR, complete remission; aCR, almost complete remission; CD, controlled disease; PR, partial remission.
Bold values are statistically significant.

lichenoid lesions are uncoupled. Therefore, diagnosis can be delayed if histological analyses are not performed on accurate lesions or if DIF or IEM are not systematically performed.

Given the lack of knowledge about the disease; the difficult clinical presentation, especially in case of MM involvement; the often long delay between the appearance of lichenoid and bullous lesions; the difficult diagnosis requiring multiple biopsies; and the low positive rate of complementary blood tests, LPP is probably under-diagnosed. The delay in diagnosis is also a consequence of the lack of awareness of the disease in general practice. In addition, the presence of LP before AIBD lesions, observed in most cases in our series and the literature, corroborates the hypothesis of exposure to normally unexposed BMZ antigens caused by lichenoid lesions. This immunopathological hypothesis regarding the onset of LPP, first proposed by Stingl (2), has been widely discussed in literature (2, 43). The emergence of autoantibodies in LPP appears to be associated with T-cell–mediated lichenoid inflammation, and the LPP phenotype could be the consequence of two kinds of immune responses to BP180, a Th1-response inducing lichenoid lesions and Th2-response inducing autoantibodies and bullous lesions (44). The mechanisms responsible for the relapse and lichenoid or bullous features have not been completely understood. As highlighted in our study, patients with lichenoid or bullous components experienced relapse. This suggests ongoing T- or B-cell–mediated autoimmunity and possibly individual differences in the pathophysiological pathways underpinning relapses. However, as patients received maintenance therapeutics, the differences in the components of relapse could be modulated by the higher efficacy of therapeutics on these pathways. Notably, the highest lichenoid-based relapse rates suggest a stronger therapeutic effect on the blistering component.

The different clinical pictures may be the result of the multiplicity of targeted antigens. Topical corticosteroids alone were not sufficient to control the disease in our series, except in one patient who was only treated with corticosteroid mouthwashes. As our patients shared similarities with patients with MMP because of the predominant mucosal picture, we used immunomodulators (such as dapsone, sulfasalazine, or doxycycline) in the first-line treatment. In non-responding cases, the first used immunomodulatory drug was switched to another or immunosuppressive drugs were added depending on extension/severity, as is usually performed to treat MMP. Among the 11 patients who received systemic drugs, two remained active despite receiving rituximab. Corticosteroids (topical or systemic) were mostly used as first-line treatment in the literature in patients with or without mucosal involvement. Dapsone is rarely prescribed in case reports or series, even in the presence of mucosal lesions. Patients without mucosal lesions were significantly more likely in remission (CR or aCR) than those with mucosal lesions at the end of follow up (84.7% vs. 65.4%), indicating that LPP with MM involvement is more difficult to treat. Mucosal LPP appears to respond well to both corticosteroid and immunomodulatory treatments. However, considering the need for long-term treatment and the well-known side effects of corticosteroids, immunomodulatory molecules appear to be more appropriate (Table 5). Most of our patients who received dapsone reached control or remission without systemic corticosteroids or immunosuppressive drugs (67%).

Immunomodulatory drugs could be considered a first-line treatment for active mucosal lesions, such as MMP, and as a steroid-sparing agent for this chronic disease (median follow-up duration of 5.5 years in our series). Two severe patients were treated by rituximab. The B-cell depletion therapy induced by rituximab might be of more interest in LPP considering the reported efficacy in AIBD, such as pemphigus and BP (45). Notably, rituximab was found to deplete circulating B cells and, thus, serum level of pathogenic auto-antibodies in pemphigus (46) by causing a reduction of B-cell and T-cell cross talk implicated in T-cell activation. Accordingly, rituximab was found to decrease circulating autoreactive T cells in pemphigus (47, 48), and long-lasting response to rituximab was found to rely on the decrease of DSG-specific T follicular helper cells participating in sustained depletion of memory auto-reactive B cells and DSG antibody-secreting cells (49). Whereas the pathogenic role of IgG anti-BP180 has been validated in BP (50), their pathogenicity is less demonstrated in MMP, but rituximab efficacy was reported in a large series (51). On the other hand, rituximab efficacy has been uncertain in small series of erosive lichen planus without AIBD, whose immunopathology is more T cell driven (52, 53). This may explain why the two patients who received rituximab still had active disease. It would appear that the indirect effect of rituximab on T cells is not sufficient to treat mediated T-cell lichenoid inflammation in LPP.

Thus, LPP with mucosal involvement can be managed as an MMP, which is supported by the nosology used by some authors. These authors diagnosed patients with clinical, histological, and immunological features similar to MMP because it fulfills the MMP criteria if the disease predominantly affects the MMs (54, 55).

The limitations of our study include its retrospective design and incomplete serological data, particularly the absence of IB using the C-terminal region of BP180.

In conclusion, LPP with mucosal lesions is clinically and immunologically heterogeneous and difficult to diagnose. Diagnosis often requires biopsy, particularly when lichenoid and bullous lesions are observed at different sites. Immunological studies have identified diverse target antigens, such as those in LPP with exclusive skin lesions. Our results suggest that immunomodulators represent an alternative first-line treatment for patients with predominant MM. Larger studies are necessary to clarify the accurate therapeutic strategies depending on disease severity.

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 humans were approved by Comité local d'éthique de l'hôpital Avicenne (CLEA)-2022-240. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

LC, CL-V, and CP-S contributed to the conception and the design of the study. LC, CL-V, GB, MA, FC, and CP-S handled the patients' daily care and dermatological data collection. IS was involved in ENT assessment and data collection. SD performed ophthalmological assessment and data collection. FP and I-YS performed stomatological assessment and data collection. FM and SG-M performed the serum immunological analyses. AM and BA performed histopathological and direct immunofluorescence analyses. CP-S performed direct immunoelectron microscopy. LC, GB, CL-V, and CP-S reviewed the charts of the patients and organized the databases. GB performed the statistical analysis. LC wrote the first draft of the manuscript and figures. All authors contributed to the article and approved the submitted version.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

We thank Nicole Lièvre, Michel Heller, and Gabrielle Villet for technical assistance.

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.2024.1243566/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 06 June 2024
DOI 10.3389/fimmu.2024.1363032

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OPEN ACCESS

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RECEIVED 29 December 2023
ACCEPTED 15 February 2024
PUBLISHED 06 June 2024

CITATION
van Beek N, Holtsche MM, Atefi I, Olbrich H, Schmitz MJ, Pruessmann J, Vorobyev A and Schmidt E (2024) State-of-the-art diagnosis of autoimmune blistering diseases. *Front. Immunol.* 15:1363032. doi: 10.3389/fimmu.2024.1363032

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State-of-the-art diagnosis of autoimmune blistering diseases

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Autoimmune blistering disorders (AIBDs) are a heterogeneous group of approximately a dozen entities comprising pemphigus and pemphigoid disorders and dermatitis herpetiformis. The exact diagnosis of AIBDs is critical for both prognosis and treatment and is based on the clinical appearance combined with the detection of tissue-bound and circulating autoantibodies. While blisters and erosions on the skin and/or inspectable mucosal surfaces are typical, lesions may be highly variable with erythematous, urticarial, prurigo-like, or eczematous manifestations. While direct immunofluorescence microscopy (IFM) of a perilesional biopsy is still the diagnostic gold standard, the molecular identification of the major target antigens opened novel therapeutic avenues. At present, most AIBDs can be diagnosed by the detection of autoantigen-specific serum antibodies by enzyme-linked immunosorbent assay (ELISA) or indirect IFM when the clinical picture is known. This is achieved by easily available and highly specific and sensitive assays employing recombinant immunodominant fragments of the major target antigens, i.e., desmoglein 1 (for pemphigus foliaceus), desmoglein 3 (for pemphigus vulgaris), envoplakin (for paraneoplastic pemphigus), BP180/type XVII collagen (for bullous pemphigoid, pemphigoid gestationis, and mucous membrane pemphigoid), laminin 332 (for mucous membrane pemphigoid), laminin β4 (for anti-p200 pemphigoid), type VII collagen (for epidermolysis bullosa acquisita and mucous membrane pemphigoid), and transglutaminase 3 (for dermatitis herpetiformis). Indirect IFM on tissue substrates and in-house ELISA and immunoblot tests are required to detect autoantibodies in some AIBD patients including those with linear IgA disease. Here, a straightforward modern approach to diagnosing AIBDs is presented including diagnostic criteria according to national and international guidelines supplemented by long-term in-house expertise.

KEYWORDS

autoimmune bullous disease, pemphigoid, pemphigus, autoantibody, immunofluorescence, epidermolysis, dermatitis herpetiformis, linear IgA dermatosis

Introduction

Autoimmune blistering diseases (AIBDs) are a heterogeneous group of mainly autoantibody-driven diseases presenting with blisters and/or erosions on the skin and/or mucous membranes (1). AIBDs can be categorized into pemphigus and pemphigoid diseases as well as dermatitis herpetiformis. Pemphigus diseases are immunopathologically characterized by intraepidermal split formation and acantholysis, i.e., keratinocytes that have lost cell–cell contact as well as autoantibodies against desmosomal proteins (2–4). In pemphigoid disorders, autoantibodies bind to structural proteins of the basement membrane zone (BMZ) of the skin and surface close epithelia inducing subepidermal/subepithelial splitting (5, 6). Dermatitis herpetiformis is also classified as subepidermal blistering AIBDs, while autoantibodies are directed against two enzymes, transglutaminase (TG) 2 and 3 (7, 8). In Western countries, bullous pemphigoid is the by far most common AIBDs with incidences ranging from 13 to 42 patients per million inhabitants per year (9–12). It affects mainly elderly patients with an increase in incidence from the age of 70 years (11, 13–15). In contrast, pemphigus diseases, mainly pemphigus vulgaris, are more common in Middle Eastern countries with incidences between 0.8 in Finland and 16.1 in Israel (9, 16, 17). In children, linear IgA disease is reported as the most frequent entity (18).

Therapy of AIBDs is based on different degrees of immunosuppression flanked by supportive therapy. While in bullous pemphigoid super-potent topical corticosteroids are preferred due to their favorable safety profile in this elderly patient population, in pemphigus, systemic corticosteroids at higher doses of 1–1.5 mg/kg body weight and the anti-CD20 antibody rituximab are recommended (19–21).

During the last decades, deciphering the molecular identity of the individual target antigens led not only to a better understanding of the pathophysiology but also to increasingly defined entities within AIBDs. These are associated with distinct clinical and/or pathological features such as needing long-term intensive immunosuppressive therapy in epidermolysis bullosa acquisita, an association with tumors in paraneoplastic pemphigus and anti-laminin 332 mucous membrane pemphigoid (MMP), or fast response to a gluten-free diet and dapsone treatment in dermatitis herpetiformis (1, 22–25). While direct immunofluorescence microscopy (IFM) is considered the gold standard for diagnosing AIBDs, for most targeted antigens, assays to detect the respective serum autoantibodies have been developed and allow exact identification of the disease entity. Here, we summarize the current state-of-the-art diagnostic approach for AIBDs.

This review is dedicated to Detlef Zillikens, professor and chair of the Department of Dermatology, University of Lübeck, Germany. He passed away in office after a short and severe disease in September 2022 (26). He established the routine autoimmune laboratory in Lübeck, inspired the introduction of new assays and methods, and enormously contributed to the field of diagnosis of AIBDs. All authors are greatly indebted to him; he has been our mentor and friend.

Clinical picture

Pemphigus diseases are clinically characterized by flaccid blisters and erosions on the skin and/or surface-close mucous membranes. In pemphigus vulgaris (PV), most patients initially develop painful and sometimes bleeding erosions of the oral mucosa that can lead to difficulties in food intake and consequently to weight loss (Figures 1A–C). Oral lesions are often localized on the palatine and buccal mucosa, but other mucous membranes of the nose, pharynx, hypopharynx, esophagus, and genitals may be involved as the disease progresses. Additionally, erosions on the skin surrounded by erythema are common, while frank blistering is rare due to the fragility of the blisters resulting from the intraepidermal splitting (Figures 1D–F). Skin lesions show a positive Nikolsky sign, i.e., epidermal separation on tangential mechanical pressure, and are usually less pruritic in contrast to pemphigoid diseases (1–3, 27). Pemphigus foliaceus (PF) does not involve mucous membranes and solely presents on the skin with cutaneous "puff pastry-" or "cornflakes-like" exfoliation, erosions, crusts, and scales. Skin lesions are often found in the seborrheic sites including the face and scalp as well as the upper chest and back (Figures 1G, H). The rare IgA pemphigus is categorized into a subcorneal pustular dermatosis type and an intraepidermal neutrophilic dermatosis type. Both subgroups present with predominantly cutaneous and often pruritic lesions with pustules and blisters configured in an annular or circinate pattern with central crusting; mucous membranes are occasionally involved (28). Paraneoplastic pemphigus (PNP) clinically resembles PV with a more multiform presentation including flaccid or tense blisters and pustules as well as often severe ulcerating oral lesions typically affecting the lips and tongue (23, 29, 30).

Bullous pemphigoid (BP) presents with intense pruritus and tense blisters of the skin and sometimes additionally mucous membranes (Figures 2A–C) (6, 31). In BP, an early pre-bullous stage can present with solely pruritus, eczema, or urticarious lesions (Figures 2D, E). With disease progression, tense blisters can form and persist for several days before eventual rupture leads to encrusted plaques and erosions (32, 33). In BP, mucous membranes are affected in 10%–20% of patients (34); however, mostly only mild oral lesions are seen. Pemphigoid gestationis clinically resembles the urticarial form of BP and is found in pregnant women in the second half of pregnancy or after delivery. Initially, in many patients, intensely pruritic plaques and, later, blisters form around the umbilical region and eventually disseminate across the trunk and extremities (Figure 2F) (6, 31, 35). The disease usually ceases within a few months but typically relapses during subsequent pregnancies.

MMP is defined as pemphigoid disease with predominant mucosal involvement (36). The mucosa can be affected at various sites as well as the skin. The most commonly affected location is the oral mucosa with erosions and blisters appearing primarily at the gingival and buccal regions that can consequently lead to scarring (Figures 3A) (31, 37, 38). The second most frequently involved site is the ocular conjunctiva. Patients typically present with reddened and irritated eyes with conjunctivitis that can lead to scarring and the formation of symblephara, culminating in severe mutilations that can result in blindness (Figures 3B, C). The nasal mucosa,

FIGURE 1
Clinical presentation of pemphigus. Classical presentation of pemphigus vulgaris with lesions of the oral mucosa, erosions on the soft palate **(A)**, crusts on the lips **(B)**, and erosions of the buccal mucosa **(C)**. Skin involvement shows erosions **(D, E)** and crusts with postinflammatory hyperpigmentation **(E)**. Pemphigus foliaceus with erythema and erosions predominantly in the seborrheic areas **(F)**. IgA pemphigus with pustules, erosions, and blisters **(G, H)**.

FIGURE 2
Clinical presentation of bullous pemphigoid **(A–E)** and pemphigoid gestationis **(F)**. Tense blisters and erythematous plaques on the trunk and arms **(A, B)**, fibrin-covered erosions of the buccal mucosa and palate **(C)**, and eczematous and urticarial lesions in bullous pemphigoid **(D, E)**. Pemphigoid gestationis in a pregnant woman with pruritic plaques and excoriations around the umbilical region **(F)**.

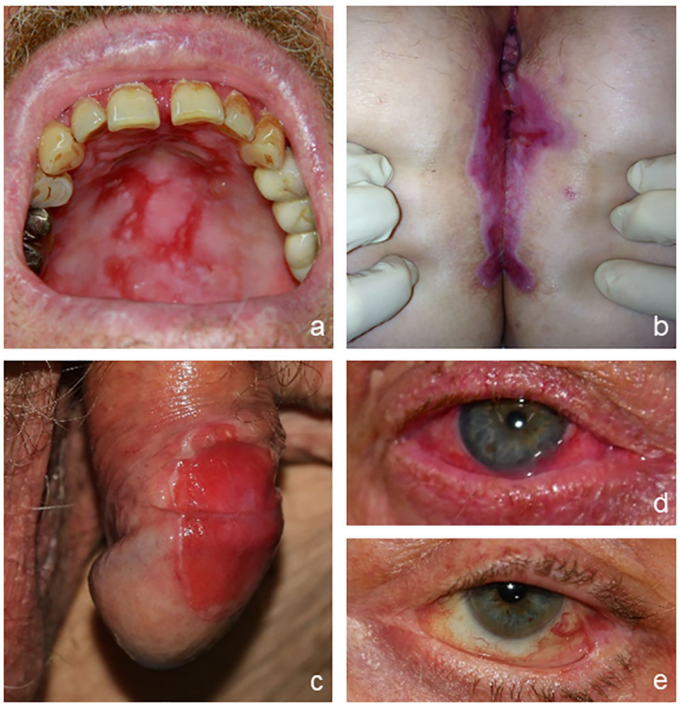

FIGURE 3
Clinical presentation of mucous membrane pemphigoid. Erosions on the hard palate **(A)**; vulva, perineal, and perianal regions **(B)**; and penis **(C)**. Conjunctival erythema **(D)** and symblepharon **(E)** as typical features of ocular involvement.

pharynx, larynx, trachea, esophagus, and genital mucosa can be involved and might result in dysfunctional scarring (Figures 3D, E) (39–41). Scarring, which occurs localized, mainly on the scalp, and may be associated with (subsequent) oral erosions, is referred to as Brunsting–Perry pemphigoid (42).

Linear IgA disease (LAD) presents with blisters arranged along the edges of round erythematous plaques in a typical annular pattern termed "string-of-pearls" (Figures 4A–C) (5). Additionally, oral, nasal, or genital lesions are often found. LAD is the most frequent AIBD in children and adolescents (18).

The clinical presentation of anti-p200 pemphigoid is diverse and mostly resembles BP. The LAD-like subtype is characterized by grouped blisters in an annular configuration. Palms and soles are frequently involved, whereas mucous membranes are affected in approximately 30%–40% of patients (Figures 4D–F), and an association with comorbid psoriasis, pronounced in Japanese patients, has been reported (43–46).

Epidermolysis bullosa acquisita (EBA) is clinically heterogeneous. In approximately one-third of patients, the mechanobullous (classical) form manifests as characterized by skin fragility and tense blisters forming upon minor traumata on exposed extensor skin locations without signs of inflammation. Healing is often associated with hyper-/hypopigmentation and scarring (Figures 4G, H) (6, 22). Nail dystrophy or scarring alopecia is frequently seen. In contrast, the inflammatory subtype manifests in two-thirds of patients and mimics the clinical features of BP or LAD. Clinical pictures of classical and inflammatory types of EBA can be present in the same patient, both simultaneously and sequentially. Mucous membranes are frequently involved, but when mucosal lesions predominate, MMP is diagnosed (22, 47).

Bullous systemic lupus erythematosus (SLE) is an AIBD occurring in SLE. The disease presents with eruptive evolution of tense bullae preferentially on sun-exposed skin with inflammatory erythematous plaques on the face, neck, upper trunk, and supraclavicular region, as well as axillary folds and oral and vulvar mucosa, but may erupt anywhere. The clinical phenotype is heterogeneous and can resemble other types of vesiculobullous diseases including BP, EBA, or dermatitis herpetiformis (48).

Rare variants of pemphigoid diseases include cicatricial pemphigoid characterized by scarring lesions without predominant mucosal involvement, IgM pemphigoid that usually manifests without frank blistering but with pruritic erythematous lesions, and lichen planus pemphigoides. In the latter pemphigoid disease, tense blisters arise also adjacent to lichen planus lesions. Furthermore, pemphigoid induced by orf virus infection has been described and linked to anti-laminin 332 autoantibodies (49–52).

Clinically distinct from pemphigoid diseases, subepidermal blistering in dermatitis herpetiformis leads to urticarial plaques, small vesicles, and papules arranged in a herpetiform pattern and located to the extensor skin surfaces, typically involving the buttocks, knees, and elbows. Dermatitis herpetiformis is characterized by intense pruritus and sometimes stinging sensations (8, 25, 53).

Direct immunofluorescence microscopy

Direct IFM of a perilesional biopsy is the gold standard in the diagnosis of AIBDs. This method is based on the detection of tissue-bound autoantibodies in the skin or mucous membranes of patients.

FIGURE 4
Clinical presentation of linear IgA disease (LAD), **(A–C)** anti-p200 pemphigoid, **(D, E)** and epidermolysis bullosa acquisita (EBA) **(F–H)**. LAD in a 4-year-old child with blisters along the edges of erythematous plaques (➔, "string-of-pearls", **A**). Annular pattern of lesions on the legs **(B, C)**. Anti-p200 pemphigoid with pruritic urticarial plaques and tense blisters **(D, E)**. Inflammatory variant of EBA presenting with erosions and postinflammatory hyperpigmentation on the back **(F)**. Mechanobullous form of EBA with high skin fragility and erosions after the removal of Band-Aids **(G)**. IgA EBA manifesting with eczema, excoriated papules, and erythematous plaques **(H)**.

Superior sensitivity of approximately 91% and specificity of approximately 98% of direct IFM compared to other diagnostic methods in different autoimmune diseases have been demonstrated in various studies (54–58). Direct IFM is performed on cryosections of a perilesional biopsy of the skin or mucous membranes. The tissue specimen must be snap-frozen and stored at −20°C or preserved in isotonic NaCl solution or modified Michel's medium until further processing (20).

Direct IFM allows the diagnosis of pemphigus and pemphigoid diseases as well as dermatitis herpetiformis by distinct binding patterns of immunoreactants. Direct IFM findings in dermatitis herpetiformis are mostly presented by granular deposits of IgA along the dermal–epidermal junction and/or at the tips of dermal papillae (Figure 5A) (25). In pemphigus diseases, IgG, IgA, and complement C3 bind to the desmosomes in the stratum spinosum of the epidermis/epithelium, forming intercellular net-like deposits (Figure 5B) (3, 20). In pemphigoid diseases, involved antigens are located at the dermal–epidermal junction, resulting in linear binding of IgG, IgA, and/or C3 and rarely of IgE and/or IgM at the BMZ (1, 6, 31). However, direct IFM only provides limited information on the involved target antigen. Discrimination between EBA and other pemphigoid diseases is possible using serration pattern analysis (59, 60). This is performed on thin (6-µm) cryosections of perilesional skin biopsies at 400–600-fold magnification using a conventional IF microscope. In EBA, immunoreactants demonstrate a "growing grass" or "upstanding arm" pattern with arches closed at the bottom, forming an "u" shape, which is termed u-serrated pattern (Figure 5C) (22). This pattern is pathognomonic for reactivity against type VII collagen, i.e., EBA and bullous SLE (54). In all other pemphigoid diseases, an n-serrated pattern with arches closed at the top is observed (Figure 5D) (61, 62). Differentiation between u- and n-serrated patterns only appeared feasible in approximately 75% of all samples and cannot be performed in mucosal biopsies (63, 64).

Particularly in MMP, it has been shown that the diagnostic sensitivity can be greatly increased by taking a new biopsy and repeating direct IFM (65, 66). In oral biopsies of PV and MMP, a non-lesional biopsy is equally sensitive than a perilesional (67). An overview of the evaluation of direct IFM for AIBDs is shown in Figure 6.

Serology

Indirect immunofluorescence microscopy on tissue substrates

Indirect IFM on tissue substrates is a screening method for circulating autoantibodies in AIBDs (69). Here, tissue substrates were incubated with patient sera followed in a second step, with fluorescence-labeled anti-human-IgG/IgA/and sometimes IgM antibodies. Monkey esophagus is the most sensitive tissue substrate for the detection of autoantibodies in PV and PF, displaying an intercellular net-like fluorescence within the epithelium (Figure 7A). For autoantibodies in PF, guinea pig esophagus has been reported with an even higher sensitivity. In the case of paraneoplastic pemphigus, plakin autoantibodies can be best detected in monkey or rat bladder (Figure 7B). IgA reactivity to the endomysium of monkey esophagus is a diagnostic hallmark of dermatitis herpetiformis and is also positive in patients with celiac disease alone (25) (Figure 7C). Pemphigoid diseases can also be diagnosed by indirect IFM using a monkey esophagus showing

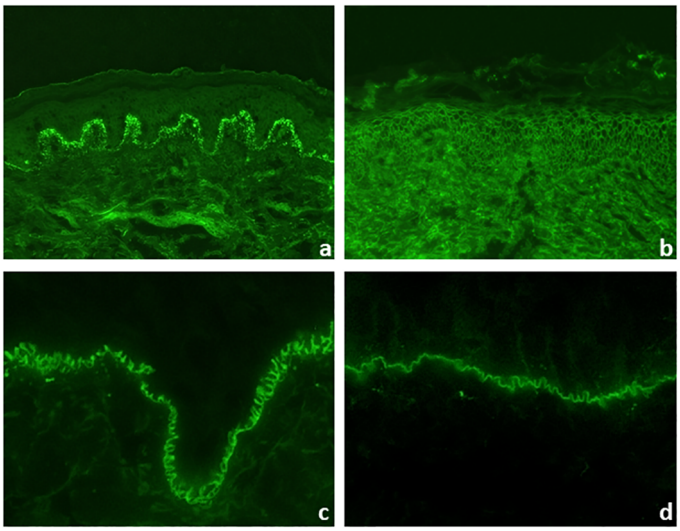

FIGURE 5
Direct immunofluorescence microscopy of perilesional biopsies. Granular deposits of IgA at the tips of dermal papillae and along the dermal–epidermal junction in dermatitis herpetiformis (×200 magnification) **(A)**. Intercellular deposits of IgG in the epidermis in pemphigus vulgaris (×200 magnification) **(B)**. In pemphigoid diseases, u- and n-serrated patterns of linear deposits at the dermal–epidermal junction can be distinguished. In epidermolysis bullosa acquisita, u-serrated pattern can be seen with arches closed at the bottom (×1,000 magnification) **(C)**. In all other pemphigoid diseases, n-serrated pattern is observed with arches closed at the top (×1,000 magnification) **(D)**.

linear anti-BMZ reactivity (Figure 7D) with a sensitivity of 60%–70% (69, 70) or normal human skin with a sensitivity of 80% (71). Sensitivity for pemphigoid autoantibodies is further increased by the use of human salt-split skin; i.e., normal human skin that is incubated with 1 M NaCl solution to induce artificial splitting within the lamina lucida (72, 73). Furthermore, NaCl-split skin allows the differentiation of pemphigoid autoantibodies in those with binding to the epidermal (against BP180 and BP230; Figure 7E) and dermal side (against laminin 332, p200 protein, and type VII collagen; Figure 7F). The diagnostic algorithm for indirect IFM on human salt-split skin is shown in Figure 8.

Since the main IgG subclasses in pemphigoid gestationis are IgG1 and IgG3, which are strongly complement activating, a complement source is added after incubating the serum on human salt-split skin, and by this, complement binding can be detected by a fluorescence-labeled anti-human-C3 antibody to visualize the respective autoantibody, previously termed "herpes gestationis factor" (74, 75).

Enzyme-linked immunosorbent assay

Following the molecular identification of most target antigens in AIBDs, enzyme-linked immunosorbent assay (ELISA) based on the immunodominant parts of the recombinant antigens has become widely available for specific and sensitive detection of circulating IgG against desmoglein 1, desmoglein 3, BP180 NC16A, BP230, and type VII collagen (76–83). Serum levels of anti-desmogleins 1 and 3 IgG as well as anti-BP180 NC16A IgG and type VII collagen were shown to correlate with disease activity in most PV, PF, BP, and EBA patients, respectively (78, 79, 82, 84–86). Regular evaluations of serum autoantibody levels at follow-ups are thus recommended in current guidelines for these diseases (19–21, 87). To enable a parallel identification of different autoantibodies, multivariant ELISA has been introduced, compiling the different target antigens, i.e., desmoglein 1, desmoglein 3, BP180 NC16A, BP230, type VII collagen, and envoplakin, on a single ELISA plate (81).

Patients with dermatitis herpetiformis develop IgA antibodies against gliadin, endomysium, tissue-type transglutaminase (TG2), and epidermal transglutaminase (TG3). Various specific and sensitive ELISA for serum IgA and IgG reactivity against TG2, TG3, and deamidated gliadin-analogous fusion peptides are widely available (25, 88).

Biochip-based indirect immunofluorescence microscopy

Biochips™ are miniature substrates that can be assembled together in a so-called mosaic in a single incubation field of a laboratory slide. An individual Biochip™ can be composed of a tissue substrate, transfected cells expressing the recombinant target antigens on the cell surface (Figure 9), or directly the spotted recombinant target antigen. For AIBDs, different Biochip™ mosaics have been developed, and the most widely used is composed of six Biochips™, i.e., primate salt-split skin, primate esophagus, recombinant BP180 NC16A, and HEK293 cells transfected with the c-globular domain of BP230, desmoglein 1, and desmoglein 3 (89–96). This approach allows simultaneous testing of autoantibodies against several antigens in a time and serum-saving manner, as reactivity to several antigens can be tested in parallel. Its diagnostic accuracy has been shown to be comparable to that of standard multistep approaches and allows the serological

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diagnosis of more than 90% of AIBDs (89–94). For this Biochip™ mosaic, an automated reading and evaluation based on deep learning algorithm has recently been developed and will further facilitate and speed up the diagnostic process for AIBDs at least for laboratories with a high throughput (97).

Further developments include Biochips™ with transfected HEK293 cells expressing desmocollins 1, 2, and 3; the laminin 332 heterotrimer (Figures 10A, B); the NC1 domain of type VII collagen; and most recently, laminin β4 (Figures 10C, D) (24, 98–103).

In-house assays

For the detection of some rare autoantibody specificities, no commercial tests are available. A few specialized laboratories have developed various in-house assays to overcome this problem. By using different cellular extracts or recombinant fragments of the target antigens and/or secondary antibodies for IgA reactivity, various additional autoantibodies can be detected by immunoblotting, ELISA, and immunoprecipitation. Until recently, the diagnosis of anti-p200 protein was based on the detection of IgG reactivity against the 200-kDa p200 protein in the extract of the human dermis or epidermis by immunoblotting (104–106) (Figure 11). The same assay can be used to detect IgG against the 290-kDa full-length type VII collagen (Figure 11). Alternatively, recombinant laminin γ1 applied by immunoblotting allowed the detection of serum autoantibodies in 70%–90% of anti-p200 pemphigoid patients (44, 45, 105, 107). Instead of recombinant laminin γ1, recombinant laminin trimers containing this subunit have been applied (71, 108–110).

The extracellular matrix of cultured human keratinocytes is used to detect antibodies against laminin 332 (111). In addition, reactivity against BP180 and BP230 can also be seen in this test (Figure 11). IgA autoantibodies against the LAD antigen-1 (LAD-1), which is the cell-derived soluble ectodomain of BP180 and primary target antigen in LAD, can be detected by immunoblotting with conditioned concentrated supernatant of cultured keratinocytes (112, 113). LAD-1 is also recognized by a subgroup of patients with BP (IgG) and MMP (IgG and/or IgA) (Figure 11). Furthermore, various recombinant fragments of the BP180 C-terminus were applied by immunoblotting and ELISA to describe autoantibodies in BP patients without anti-NC16A reactivity and in MMP (114). For IgA reactivity against the NC16A domain of BP180 in LAD and the NC1-domain of type VII collagen as well as the full-length type VII collagen in IgA EBA, immunoblotting can also be employed (115–117).

A dozen methods for the detection of anti-laminin 332 serum antibodies in MMP have been described including immunoprecipitation, immunoblotting, and ELISA with recombinant or cell-derived forms of this molecule (118–120). Recently, antibodies against laminin 332 have been determined by the so-called keratinocyte footprint assay, i.e., an indirect IFM where normal human keratinocytes are grown on glass coverslips and anti-laminin 332 IgG binds to the extracellular matrix in a characteristic pattern after removal of keratinocytes from the coverslips (121).

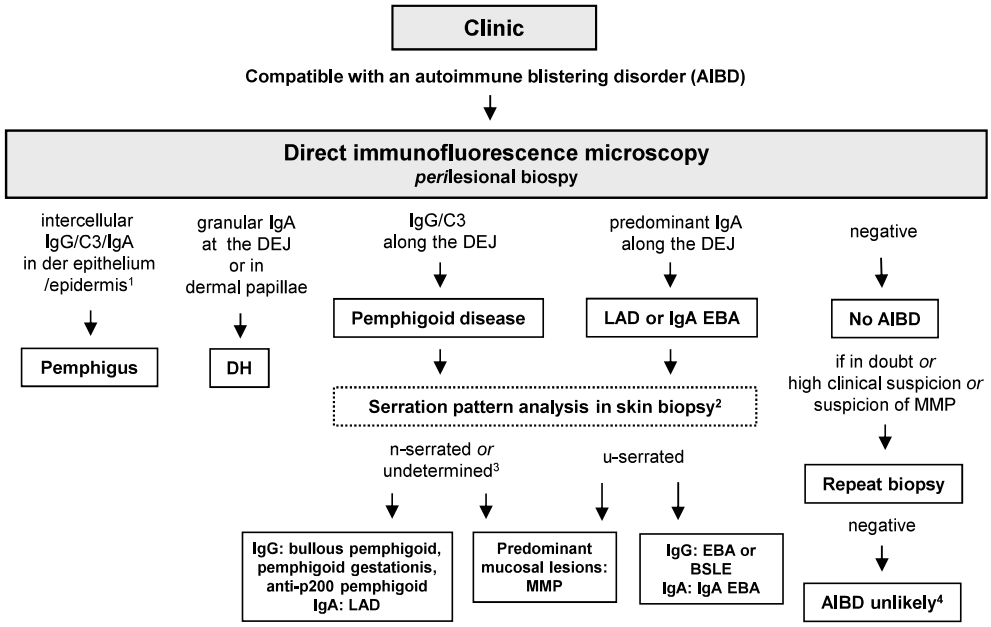

FIGURE 6
Diagnostic algorithm for direct immunofluorescence microscopy (IFM). 1, In paraneoplastic pemphigus, a combined pattern with anti-DEJ deposits may be present; 2, in mucosal biopsies, pattern analysis is not possible; 3, so far, in none of the undetermined cases, an EBA or BSLE has been diagnosed based on serum anti-type VII collagen IgG/IgA (64); 4, unless in ocular MMP, where direct IFM can be repeatedly negative; in these cases, diagnosis of MMP can be made based on the clinical picture and the clinical and histopathological exclusion of differential diagnoses (65, 68). BSLE, bullous systemic lupus erythematosus; EBA; epidermolysis bullosa acquisita; DH, dermatitis herpetiformis; DEJ, dermal–epidermal junction; LAD, linear IgA disease; MMP, mucous membrane pemphigoid.

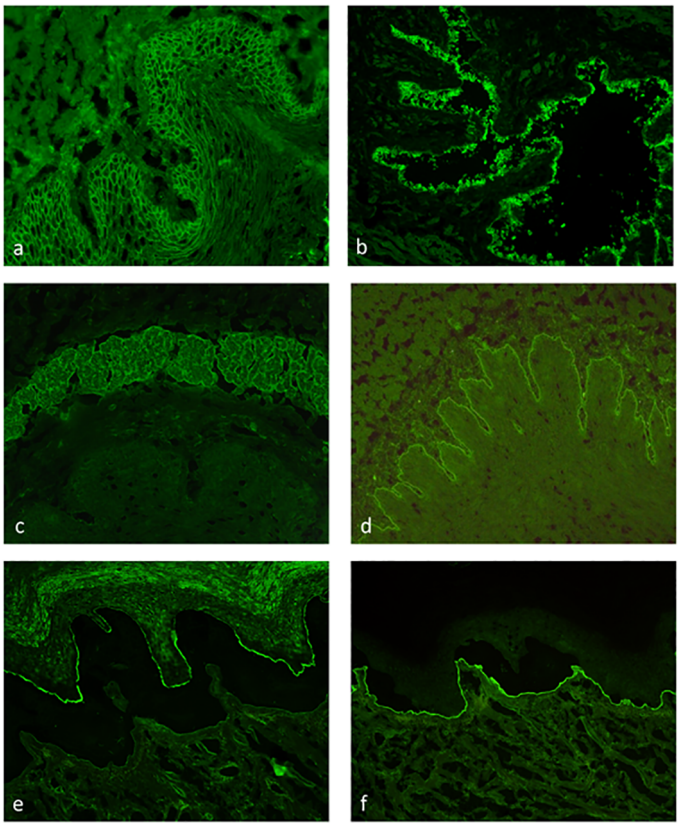

FIGURE 7
Indirect immunofluorescence on monkey esophagus, rat bladder, and human salt-split skin. Detection of IgG binding with an intercellular pattern in the epithelium of monkey esophagus pemphigus vulgaris **(A)**. IgG reactivity to rat bladder epithelium typical for paraneoplastic pemphigus **(B)**. IgA binding to the endomysium of monkey esophagus in dermatitis herpetiformis **(C)**. Linear IgG binding on monkey esophagus **(D)** and along the roof of the artificial blister of salt-split human skin **(E)** in a bullous pemphigoid patient. IgG reactivity along the blister floor of salt-split human skin, which can be seen in anti-laminin 332 mucous membrane pemphigoid, epidermolysis bullosa acquisita, and anti-p200 pemphigoid **(F)**.

In paraneoplastic pemphigus, detection of autoantibodies against periplakin, epiplakin, plectin, desmocollin, and α2-macroglobulin-like 1 has been achieved by various sophisticated in-house assays (3, 29, 100, 122–126). So far, only a few studies have shown reactivity against α6β4 integrin in a subgroup of patients with MMP (127, 128).

ELISA detected IgE autoantibodies against BP180 NC16A in 40%–70% of BP patients, but without increasing diagnostic sensitivity (129–133). In the case of MMP and the mucosal variant of PV, BP180 NC16A and desmoglein 3-specific ELISA using salivary samples have been described to detect IgA/IgG reactivity (134, 135).

Histopathology

For histopathology of AIBDs, lesional skin biopsies were investigated. Here, a differentiation between intraepidermal split formation with acantholysis in pemphigus diseases and subepidermal splitting in pemphigoid diseases and dermatitis herpetiformis can be achieved (136). Pemphigus samples present with subcorneal splitting in PF and PV, with acantholysis and suprabasal splitting, typically in a so-called tombstone pattern in PV, i.e., with remaining keratinocytes attached to the BMZ (2, 3). In paraneoplastic pemphigus, additional dyskeratosis, degeneration of the BMZ, and lichenoid lymphocytic infiltrates of the adjacent upper dermis can be seen (23). In contrast, IgA pemphigus shows subcorneal clefts with neutrophilic infiltrates and, if captured, subcorneal acantholysis (28). In pemphigoid diseases, subepidermal blister formation accompanied by an eosinophilic infiltrate is typical, but split formation might be lacking in pemphigoid gestationis and non-bullous forms of BP (137). Even in BP alone, lymphocyte-rich eosinophil-dominated and neutrophil-rich patterns can be observed (138). Classical patterns of LAD and dermatitis herpetiformis may present with neutrophil-dominated infiltrate along the dermoepidermal junction and in the dermal papillae forming microabscesses. Accompanying eosinophils and subepidermal blister formation may be present (139, 140). However, a clear differentiation based on histopathology alone is not feasible in pemphigoid disorders and dermatitis herpetiformis (53, 141, 142).

In pemphigoid diseases, staining for C3d/C4d reveals linear deposits at the BMZ (143–146). However, this method is hampered by its low sensitivity and specificity, most likely due to the lesional biopsy that favors unspecific labeling along the degraded BMZ and the degradation of immunoreactants by activated tissue proteases.

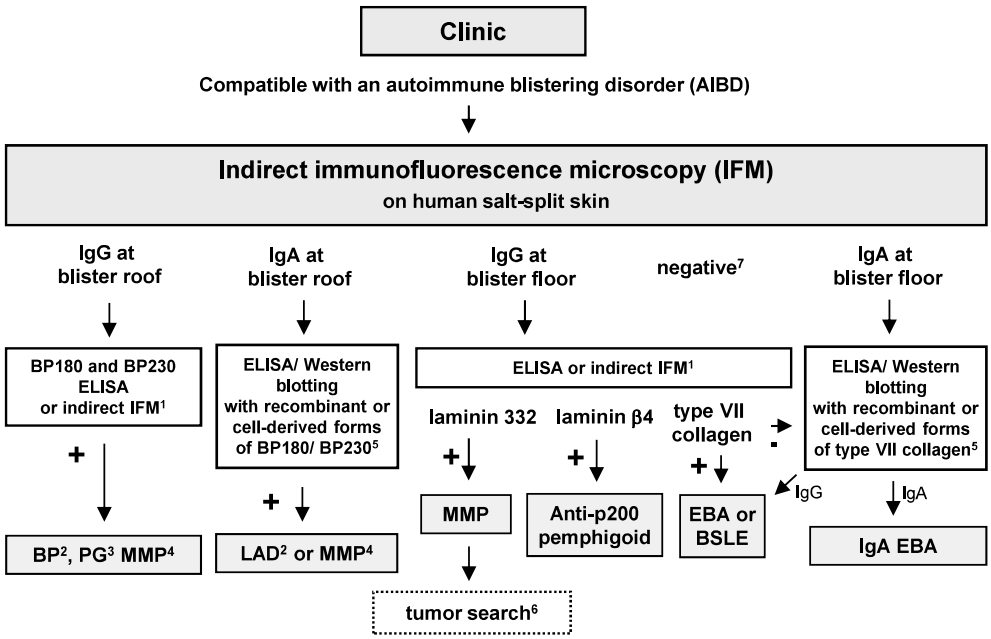

FIGURE 8
Diagnostic algorithm for serum autoantibodies in pemphigoid diseases. 1, Commercial assays are available; 2, with predominant skin lesions; 3, in pregnancy; 4, with predominant mucosal lesions; 5, in-house assays only available in specialized laboratories; 6, in approximately 25% of MMP patients with anti-laminin 32 reactivity, a malignancy was found; 7, on high clinical suspicion of BP, BP180 NC16A-specific ELISA/indirect IFM is recommended; on suspicion of MMP, additional serological tests in particular for IgG reactivity against laminin 332 are recommended; on suspicion of EBA, type VII collagen-specific ELISA/indirect IFM is recommended. BP, bullous pemphigoid; BSLE, bullous systemic lupus erythematosus; EBA; epidermolysis bullosa acquisita; DH, dermatitis herpetiformis; DEJ, dermal–epidermal junction; LAD, linear IgA disease; MMP, mucous membrane pemphigoid; PG, pemphigoid gestationis.

As such, this method is only suitable in individual cases when no direct IFM and serology are available (147).

In summary, lesional histopathology is still recommended in every patient with suspicion of an AIBD (19, 20, 25, 29, 65, 68, 148). Its true value lies in its use to propose differential diagnoses when direct IFM and serology are unreactive. However, profound knowledge of the histopathological spectra of AIBDs is essential for every (dermato-) pathologist to recommend or initiate direct IFM and serology when such a disorder is suspected.

Diagnostic criteria

Pemphigus vulgaris and pemphigus foliaceus

The diagnosis of PV and PF is based on refs. (19, 21)

- clinical appearance with flaccid blisters erosions and
- intercellular net-like deposits of IgG/C3 in the epithelium in direct IFM and/or
- intercellular fluorescence on the epithelium of monkey esophagus in indirect IF and/or
- detection of anti-desmoglein 1 IgG (PF), anti-desmoglein 3 IgG (mucosal PV), or both (mucocutaneous PV) and/or
- suprabasal (PV) or subcorneal splitting (PF) with acantholysis in histopathology

Direct IFM establishes the diagnosis of a pemphigus disease by intercellular (sometimes granular appearing) epithelial staining. Indirect IFM testing on monkey esophagus as the most sensitive substrate uncovers circulating autoantibodies that cause intercellular staining of the epithelium (1–3). However, intercellular reactivity on monkey esophagus alone is not sufficient to establish the diagnosis of pemphigus, as it may be unspecific, e.g., by cross-reacting anti-blood group autoantibodies. Identification of the target antigens is performed by either ELISA or indirect IF-based Biochip™ technology (78, 79, 149). Levels of anti-desmoglein 1 and 3 serum IgG measured by ELISA usually correlate with disease severity and have been established to monitor the course of the disease as recommended by national and international guidelines to guide treatment decisions (20, 21, 78, 79).

If direct IFM is not available, detection of medium to high levels of circulating anti-desmoglein antibodies, compatible histological appearance, and a typical clinical presentation may suffice for diagnosis (20).

IgA pemphigus

The diagnosis of IgA pemphigus can be established by

- clinical appearance with pustules and
- intercellular net-like deposits of IgA/C3 in the epithelium by direct IFM and/or

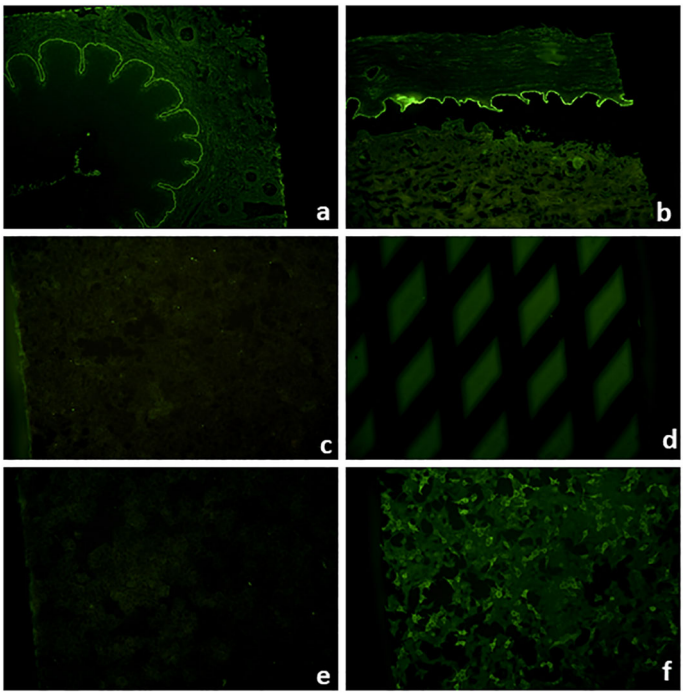

FIGURE 9
Biochip™ mosaic with six substrates that allow the serological diagnosis of approximately 90% of autoimmune blistering diseases when the clinical picture is known (89). Linear IgG binding on monkey esophagus **(A)**, primate salt-split skin blister roof **(B)**, positive IgG staining on recombinant BP180 NC16A **(D)**, and positive fluorescence of HEK293 cells transfected with BP230 **(F)**, while HEK293 cells transfected with desmogleins 1 **(C)** and 3 **(E)** show no fluorescence compatible with bullous pemphigoid.

- intercellular fluorescence with IgA on the epithelium of monkey esophagus in indirect IFM and/or
- detection of anti-desmoglein 1 and/or 3 and/or anti-desmocollin IgA autoantibodies and/or
- subcorneal neutrophilic infiltrate with vesicles in histopathology

Autoantibodies of patients with the rare IgA pemphigus target desmocollins 1, 2, or 3 or desmogleins 1 and 3. Circulating IgA antibodies may be detected by indirect IFM on monkey esophagus and in-house Biochips™ or ELISA using the recombinant forms of these antigens (28, 100, 150).

Unlike PF and PV, lesional histopathology reveals marked infiltration with neutrophils, which causes the formation of

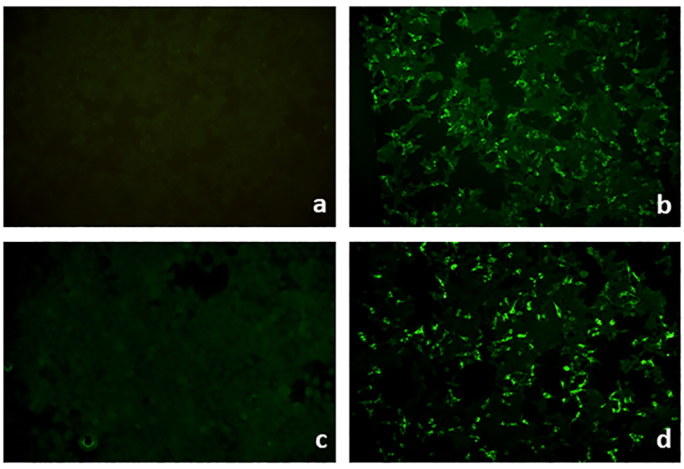

FIGURE 10
Biochips™ for serum IgG against laminin 332 and laminin β4. Biochips™ with HEK293 cells transfected with either the heterotrimer of laminin 332 **(A, B)** or laminin β4 **(C, D)**. IgG reactivity is seen in a patient with anti-laminin 332 mucous membrane pemphigoid **(B)** and a patient with anti-p200 pemphigoid **(D)**, while the corresponding negative controls show no reactivity **(A, C)**, respectively.

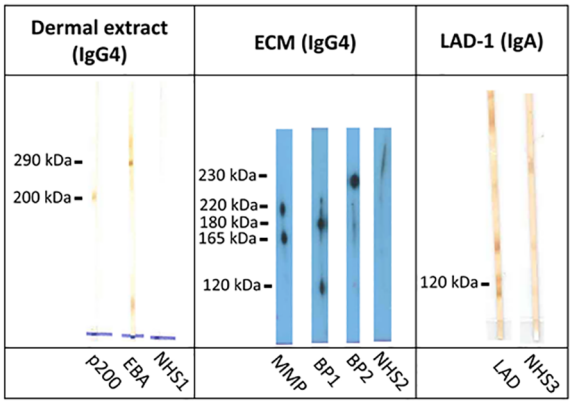

FIGURE 11
Immunoblotting with dermal extract, extracellular matrix of cultured human keratinocytes, and conditioned concentrated medium of cultured human keratinocytes. Immunoblot with extract of human dermis shows reactivity against the 200-kDa p200 protein in anti-p200 pemphigoid (p200) and the 290-kDa full-length type VII collagen in epidermolysis bullosa acquisita (EBA). IgG4 reactivity with the α3 chain of laminin 332 in mucous membrane pemphigoid (MMP; 220-kDa unprocessed and 165-kDa processed forms) as well as with BP180 (180-kDa full-length and 120-kDa processed forms) and with BP230 (230 kDa) in patients with bullous pemphigoid without reactivity against the immunodominant NC16A domain (BP1 and BP2) by immunoblotting with extracellular matrix of cultured human keratinocytes. Immunoblot with conditioned concentrated medium of cultured human keratinocytes for detection of IgA antibodies against the soluble ectodomain of BP180 [linear IgA disease antigen 1; LAD-1 in linear IgA diseases (LAD)]. Normal human sera (NHS1–3) were used as controls.

pustules in initially appearing vesicles. In a perilesional skin biopsy, IgA deposits are seen with an intercellular pattern in the epidermis by direct IFM (21, 28, 151).

Paraneoplastic pemphigus

The diagnosis of paraneoplastic pemphigus can be established by

- association with a malignancy
- clinical appearance with severe stomatitis and lip involvement
- intercellular net-like and additional linear deposits of IgG/IgA/C3 in direct IFM and/or
- IgG against urothelium by indirect IFM on monkey/rat bladder and/or
- detection of anti-envoplakin IgG by ELISA and/or
- detection of anti-plakin and/or anti-α2 macroglobulin like 1 autoantibodies and/or
- acantholysis, dyskeratosis, vascular degeneration of the BMZ, and lichenoid infiltrates in the upper dermis by lesional histopathology

Paraneoplastic pemphigus commonly leads to severe stomatitis including erosions on the tongue and lips. An underlying malignant disease, often hematological malignancies, thymoma, or Castleman disease, is a prerequisite for the diagnosis, but in some patients, it only manifests during the course of the disease. Direct IFM often uncovers linear IgG deposits at the dermal–epidermal junction in addition to a pemphigus-typical epidermal/epithelial intercellular distribution (2, 3, 21, 23). Histopathologically, lesions usually show acantholysis, dyskeratosis, vascular degeneration of the BMZ, and lichenoid infiltrates in the upper dermis (20, 23, 65). A broad variety of specific IgG antibodies may be found in serum, frequently directed against different plakins but also plectin, α2-macroglobulin-like 1, desmocollins 1–3, desmoglein 3, and BP180 (20, 21, 23, 30). The most relevant serological tests are indirect IM on monkey/rat bladder and the widely available envoplakin-specific ELISA (29). Additional in-house assays for the detection of autoantibodies periplakin, desmoplakin I/II, epiplakin, plectin, α2 macroglobulin like 1, and desmocollins 1, 2, and 3 are available in specialized laboratories.

Bullous pemphigoid

The diagnosis of BP is based on refs. (19, 21)

- the clinical picture with blisters and erosions and/or urticarial plaques and erythema predominantly at the skin and
- linear deposits of IgG/C3 and sometimes additionally IgA in direct IFM and/or
- blister roof staining on salt-split skin by indirect IFM and/or
- detection of BP180/BP230 IgG by ELISA or indirect IFM and/or
- subepidermal blisters with eosinophilic–lymphocytic or neutrophilic infiltrates by lesional histopathology

By direct IFM linear depositions of IgG, C3 and sometimes additional IgA and IgM at the BMZ in an n-serrated pattern can be seen (1, 6, 60, 152). A further distinction from other AIBDs is required by serology, which displays epidermal binding of predominant IgG in salt-split skin and/or reactivity to recombinant BP180 NC16A and/or BP230 by ELISA or indirect IFM (1, 6) (Table 1).

In case of epidermal binding in salt-split skin but no reactivity with the immunodominant NC16A region of BP180 or with BP230, other epitopes of BP180 might be targeted like the soluble cell-derived ectodomain or the C-terminal part of BP180. If no blister formation is present clinically, the European guideline recommends diagnosis based on compatible direct IFM findings and binding of circulating IgG to the epidermal side of salt-split skin by indirect IFM (19). In case of negative or unavailable direct IFM, diagnosis is concluded in case of a compatible clinical picture and consistent histology presenting with subepidermal blisters and eosinophilic infiltrates combined with serum IgG against BP180, BP230, and/or the epidermal side of salt-split skin by indirect IFM (19, 21). In the case of a classical clinical picture, the detection of serum IgG against BP180 NC16A in levels greater than threefold of the lower detection limit of the ELISA is sufficient for the diagnosis of BP (148).

10.3389/fimmu.2024.1363032

TABLE 1 Autoantibody specificities and diagnostically relevant clinical signs.

Disease	Target antigen	Clinical signs of diagnostic relevance*
Pemphigus vulgaris	***Dsg 3***, *Dsg 1*	Erosions and flaccid blisters of mucous membranes, ± flaccid blisters and erosions on skin
Pemphigus foliaceus	***Dsg 1***	Flaccid blisters, erosions, scaling; no involvement of mucous membranes
Paraneoplastic pemphigus	***Dsg 3***, ***envoplakin***, periplakin, desmoplakin I/II, α 2 macroglobulin-like 1, plectin, epiplakin, Dsc 1, 2, 3	Severe stomatitis, erosions of lips, neoplasia (hematological malignancies, thymoma, Castleman disease)
IgA pemphigus	**Dsc 1–3** Dsg 1, Dsg 3	Mostly pustules and erosions
Bullous pemphigoid	***BP180 NC16A***, *BP230*	Tense blisters, erosions, urticarial plaques, severe pruritus, old age (>75 years)
Mucous membrane pemphigoid	**Non-BP180 NC16A (different epitopes)**, *BP180 NC16A*, ***laminin 332***, *BP230*, α6β4 integrin	Predominant mucosal involvement; oral and ocular mucosae are most frequently affected
Linear IgA disease	**LAD-1**, BP230 (IgA)	Tense blisters/vesicles, erosions, annular orientation of lesions ("string of pearls")
Pemphigoid gestationis	***BP180 NC16A***, *BP230*	Erythema, papules, rarely vesicles, intense pruritus; pregnancy or post-partum period
Anti-p200 pemphigoid	***Laminin β4***, laminin γ1	Tense blisters, erosions
Epidermolysis bullosa acquisita	***Type VII collagen***	Mechanobullous and inflammatory variant (resembling BP or LAD)
Bullous systemic lupus erythematosus	*Type I:* ***type VII collagen*** type II: *BP180*, *BP230*, *laminin 332*	SLE present; tense blisters, erosions
Lichen planus pemphigoides	***BP180 NC16A***, *BP230*	Tense blisters independent of lichen planus lesions
Cicatricial pemphigoid	*BP180*, *BP230*, *laminin 332*	Skin blisters and erosions that heal with scarring and/or milia formation
IgM pemphigoid	BP180 (IgM)	Erythema and urticaria, excoriated papules/plaques, and lichenification
Dermatitis herpetiformis	***Epidermal TG***, ***tissue-type TG***, ***endomysium*** (IgA)	Severe pruritus, vesicles, erythematous excoriated papules on extremities and the gluteal region

Main target antigens are shown in bold; target antigens in italics indicate the wide availability of sensitive and specific assays.
Dsg, desmoglein; Dsc, desmocollin; TG, transglutaminase; LAD, linear IgA disease; BP, bullous pemphigoid; SLE, systemic lupus erythematosus.
*If not stated otherwise, skin involvement is predominant.

Mucous membrane pemphigoid

To diagnose MMP, a clinical presentation of erosions or blisters predominantly at mucous membranes is required. However, affection of mucous membranes as well as the targeted autoantigens are highly variable. Establishing the diagnosis is thus based on the following refs. (41, 65, 149):

- predominant mucosal blisters and/or erosions and
- linear deposits of IgG/IgA/C3 in direct IFM and/or
- linear staining at either blister roof or blister floor of salt-split skin by indirect IFM and/or
- serum reactivity against BP180, laminin 332, and type VII collagen

Diagnostics is further complicated by a lower positive rate of direct IFM samples ranging from 60% to 90% (38, 39, 65, 153). This issue can be circumvented in part by testing additional biopsies (66). As such, guidelines recommend in case of an initial negative direct IFM to repeat the testing with at least one additional biopsy (65, 68).

Serological diagnosis for MMP includes indirect IFM on salt-split skin for both IgA and IgG autoantibodies, which direct to subsequent assays for autoantibodies binding to the epidermal side (i.e., BP180 and BP230) or dermal side (i.e., laminin 332 and type VII collagen) (38, 65, 153, 154) (Table 1). Typically, first ELISA and Biochip™ analyses are prompted, followed by immunoblot-specific in-house assays for more rare target antigens (37, 38) (Figure 8). In case of negative findings on salt-split skin, ELISA/immunoblotting or Biochip™ techniques should be employed following the expected frequency of autoantibodies directed against targeted structures (39). However, low frequencies of circulating autoantibodies, in particular in ocular MMP, pose a challenge to serologic diagnostics (38, 65, 153, 154). The diagnosis of ocular MMP can be established without positive findings by serology and direct if other causes of cicatrizing conjunctivitis have been ruled out both clinically and histopathologically (65).

Of note, anti-laminin 332 reactivity warrants a tumor search since up to 30% of MMP patients with laminin 332-specific antibodies develop a malignancy (24, 38, 39, 65). As such, assaying for anti-laminin 332 reactivity is recommended in all newly diagnosed patients with MMP without clear antibodies against BP180 or type VII collagen and in case of initially negative reactivity, also during the course of the disease (65, 68).

Antibodies against α6β4 integrin have been described in MMP (127, 128). Following a systematic literature search, the recent S3 guideline has not recommended testing for anti-α6β4 integrin until further data are available (65).

Pemphigoid gestationis

Gestational pemphigoid is a rare condition in pregnancy and needs to be differentiated from other pruritic eruptions in pregnancy. The diagnosis is based on the following ref. (35):

- compatible clinical picture with urticarial paraumbilical plaques and/or vesicles and in pregnancy or post partum period
- linear deposits of C3 and sometimes additional IgG in direct IFM and/or
- positive complement binding test and/or
- blister roof staining on salt-split skin by indirect IFM and/or
- reactivity with BP180 NC16A in antigen target-specific assays

Histopathology shows an eosinophilic infiltrate but is often unspecific, as the split formation is initially mostly lacking (155, 156). By direct IFM, mainly C3 deposits can be seen along the dermal–epidermal junction in a linear pattern, and only approximately one-third of patients show concomitant IgG deposition (155). Similarly, standard indirect IFM detecting IgG and IgA autoantibodies may be negative in 75% of patients. Adding a complement source (also referred to as complement binding test) may yield positive results of indirect IFM in most sera (155, 156). IgG-autoantibodies are mainly directed against the NC16A domain of BP180 and are mainly constituted of the IgG1 and 3 complement activating subclasses (75). Reactivity against recombinant BP180 NC16A can be detected by ELISA or indirect IFM in nearly all patients, and serum anti-BP180 NC16A levels correlate with disease activity (92, 156–159).

Linear IgA disease

LAD is defined by predominant IgA autoantibodies against proteins of the BMZ. The diagnosis relies on the following ref. (51):

- clinical picture with vesicles and blisters frequently in an annular pattern with vesicles at the edge of lesions and
- predominantly IgA deposits along the BMZ by direct IFM and/or
- linear staining of blister roof or, more rarely, of floor on salt-split skin with predominantly IgA and/or
- IgA reactivity with LAD-1 or another epitope on BP180 and/or
- subepidermal splitting with neutrophils along the BMZ and in dermal papillae in lesional histopathology

By direct IFM next to the predominant IgA deposition, reactivity of C3 and to a lesser extent IgG and IgM can be seen (160). Circulating IgA autoantibodies usually bind at the blister roof of salt-split skin, where the main target antigen, the soluble ectodomain of BP180 (LAD-1), is situated (6, 161). In the case of staining with IgA at the blister floor of salt-split skin, the target antigen may be type VII collagen; here, the term IgA EBA has been proposed (162), and the European guideline, which is currently underway, will adopt this differentiation between LAD and, in case of predominant IgA reactivity against type VII collagen, IgA EBA. Histopathology is very similar to dermatitis herpetiformis with neutrophilic infiltrate along the dermal–epidermal junction and in the dermal papillae forming microabscesses. Accompanying eosinophils and subepidermal blister formation may be present (139, 163).

Anti-p200 pemphigoid

Anti-p200 pemphigoid is the most recently recognized AIBD (106). It is defined by refs. (44, 45)

- predominant skin lesions with involvement of extremities, often the palms and soles, and
- linear deposits of IgG/C3 by direct IFM and/or
- blister floor staining on salt-split skin by indirect IFM with IgG and/or
- reactivity with the 200-kDa p200 protein by immunoblotting with dermal extract or
- reactivity with laminin β4 and/or laminin γ1
- subepidermal blistering with neutrophilic or mixed infiltrate in lesional histopathology

While by direct IFM and histopathology anti-p200 pemphigoid mainly resembles BP, its dermal reactivity on salt-split skin distinguishes it from BP (43, 45). Direct IFM serration pattern is n-serrated, thus excluding EBA as a differential diagnosis (60, 164). For exact diagnosis, deciphering the target antigen is required. Traditionally, this is performed by immunoblotting with dermal or epidermal extract (104, 106). Subsequently, laminin γ1 has been described as a target antigen and is recognized by 70%–90% of patients (45, 105, 165). Assays for the detection of anti-p200 and anti-laminin γ1 have only been available in specialized laboratories. Since the pathogenic relevance of anti-laminin γ1 IgG could not be shown *in vitro* and *in vivo*, it has been hypothesized that the true autoantigen of this disease is still to be discovered (166, 167). In fact, most recently, laminin β4 has been reported as a target antigen recognized by nearly all patients (168, 169). A Biochip™-based indirect IFM assay for the detection of circulating laminin β4 is widely available (169). The pathogenic potential of anti-laminin β4 IgG is currently being investigated.

Epidermolysis bullosa acquisita

EBA is caused by autoantibodies to type VII collagen, the major component of anchoring fibrils. Clinically and histologically EBA can resemble other pemphigoid diseases, so diagnosis of EBA based only on clinical picture and histology alone is not possible, in particular in the inflammatory variant.

Diagnosis of EBA is based on (170)

- u-serration pattern by direct IFM
- detection of circulating anti-type VII collagen autoantibodies by ELISA, indirect IFM (Biochip™), or immunoblot with dermal extract or the recombinant NC1 domain
- indirect IFM on type VII collagen-deficient skin (only applicable when serum antibodies are reactive against human/primate skin)
- direct immunoelectron microscopy
- fluorescence overlay antigen mapping (FOAM)

Direct IFM of perilesional biopsy demonstrates linear deposits of IgG, IgA, or complement along the dermal–epidermal junction. By close inspection of 4–6-µm-thick sections at ×400–×600 magnification, the characteristic u-serrated pattern can be observed (Figure 5C). This pattern is pathognomonic for skin-bound antibodies to type VII collagen and resembles "growing grass". However, differentiation between u- and n-serrated patterns is not possible in approximately one-quarter of samples as well as in mucosal biopsies (59, 60, 64).

Immunoelectron microscopy, which used to be the diagnostic gold standard for EBA, allows the detection of autoantibody deposits in anchoring fibrils in the sublamina densa, which is typical for EBA or bullous systemic lupus erythematosus (BSLE) (171, 172). Unfortunately, this method requires the processing of fresh biopsies and is only performed in a few laboratories in the world. Another technique that could distinguish EBA from other pemphigoid diseases is FOAM. This method is based on the coincubation of perilesional biopsy with antibodies against other antigens, such as BP180, laminin 332, and type VII collagen. Overlay of different pictures allows detection of colocalization of autoantibodies (173, 174).

While diagnosing AIBDs by detecting circulating autoantibodies has become a major diagnostic approach (6, 69, 175), serological diagnosis in EBA is limited by the relatively low rate of circulating antibodies in approximately 60% of patients (54, 55). Antibodies to type VII collagen bind along the BMZ on monkey esophagus and label the floor of the artificial blister in salt-split skin (Figure 7F), similar to anti-p200 and anti-laminin 332 antibodies. Usage of type VII collagen-deficient skin as the substrate for indirect IIF is an elegant approach but is limited by the availability of type VII collagen-deficient skin and the restriction to patients with reactivity against normal salt-split skin (176).

The most practical approach outside specialized laboratories to diagnose EBA is the determination of anti-type VII collagen autoantibodies by commercial ELISA or Biochip™-based indirect IFM employing the recombinant NC1 domain of type VII collagen (77, 101). Antibody levels have been shown to correlate with disease severity (177). In specialized laboratories, immunoblots and ELISA with various cell-derived and recombinant forms of type VII collagen are available (99, 178–181). Most recently, patients with predominant IgA reactivity against type VII collagen are differentiated from LAD based on the possibly more severe clinical manifestation and more refractory course compared to the anti-BP180 IgA-mediated classical LAD (115).

Bullous systemic lupus erythematosus

BSLE is a rare AIBD in patients with SLE. Diagnostic criteria for BSLE were first proposed in 1983 by Camisa and Sharma and later revised (46, 182–184). These revised criteria include

- diagnosis of SLE based on the ACR/EULAR criteria
- vesiculobullous skin lesions predominantly in sun-exposed areas
- histopathological changes compatible with dermatitis herpetiformis
- linear or granular deposits of IgG, IgA, IgM, and complement at the BMZ by direct IFM

Lesional histopathology resembles changes observed in dermatitis herpetiformis with neutrophil-rich infiltrate in the upper dermis, dermal edema, and fibrin and neutrophils in the cavity of subepidermal blisters (185). By direct IFM, most cases reveal deposits along the BMZ of more than one Ig isotype, in many cases IgG, IgA, or IgM, whereas complement components are mostly detected in lesional skin and rarely in clinically uninvolved skin (186). In patients with linear IFM deposits, circulating autoantibodies can be often detected in indirect IFM on human salt-split skin, in most cases showing dermal binding (48). Serum autoantibodies are mainly directed against type VII collagen. In some cases, reactivity to BP180, BP230, and laminin 332 has been described (48, 51, 185, 187).

Dermatitis herpetiformis

Diagnosis of dermatitis herpetiformis is based on the correlation of the typical clinical picture of dermatitis herpetiformis with positive direct IFM demonstrating granular deposits of IgA at the tips of dermal papillae or along the BMZ (25, 163). In the case of typical clinical manifestations but repeatedly negative direct IFM, additional minor criteria can be used to support the diagnosis. These minor criteria include the following (25):

- dermatitis herpetiformis-typical histopathological findings
- positivity of at least one of the serological tests for IgA against TG2, TG3, or endomysium
- histopathological findings in duodenal biopsy compatible with celiac disease
- HLA testing compatible with dermatitis herpetiformis/coeliac disease
- positive result of oral iodine challenge or cutaneous iodine path test
- positive therapeutic effect of a long-term gluten-free diet
- fast response to therapy with dapsone

Classical histological findings in dermatitis herpetiformis are neutrophilic microabscesses, nuclear dust, and fibrin in the

papillary dermis, often accompanied by edema of dermal papillae and subepidermal split formation. Neutrophils with fibrin and polymorphonuclear cells with a variable number of eosinophils can be observed in the lumen of subepidermal blisters and the papillary dermis (140, 188). However, histological changes do not allow definite differentiation of dermatitis herpetiformis from other subepidermal AIBDs. Several serological tests are available. Anti-endomysium antibodies can be found in 60%–90% of untreated dermatitis herpetiformis patients with almost 100% specificity either using indirect IFM on monkey esophagus or using ELISA with tissue-type TG (TG2) (189, 190). IgA antibodies against epidermal TG (TG3) are a part of IgA deposits in direct IFM of dermatitis herpetiformis patients. The majority of dermatitis herpetiformis patients also have circulating anti-TG3 IgA even if anti-TG2 antibodies are absent (191–193). In the so far largest study with 242 dermatitis herpetiformis patients, 84.3% and 78.5% of sera revealed IgA reactivity against endomysium and TG2 with specificities of 100% and 99.0%, respectively (194). The European guideline of dermatitis herpetiformis recommends performing at least one serological test, i.e., either anti-endomysium IgA or anti-TG2 IgA. Serum anti-TG2 IgA is a specific marker for gluten-induced enteropathy in both dermatitis herpetiformis and coeliac disease and is recommended by the guideline. Although TG3 is regarded as an autoantigen of dermatitis herpetiformis, determination of anti-TG3 IgA is only recommended in addition to anti-TG2 IgA (25).

Genome-wide association studies (GWASs) have detected human leukocyte antigen haplotypes as major risk loci with DQ2.5 (HLA-DQA1*05 and HLA-DQB1*02), DQ2.2 (HLA-DQA1*02 and HLA-DQB1*02), DQ7.5 (HLA-DQA1*05 without HLA-DQB1*02), or DQ8 (HLA-DQB1*03:02) haplotypes detected in over 99% of individuals with celiac disease and dermatitis herpetiformis (195). However, as the frequency of these haplotypes in the population reaches 60%, their predictive value is low, but their negative predictive value is very high, as the detection of another haplotype can confidently exclude the diagnosis (196). Furthermore, challenges with either potassium iodine on healthy skin inducing dermatitis herpetiformis lesions or a gluten-free diet alleviating gastrointestinal symptoms rapidly with skin lesions improving over weeks or several months are indicative of dermatitis herpetiformis. Indirect diagnostic evidence may also come from treatment with dapsone leading to resolving symptoms after only a few days (8, 197–201).

Future perspectives

The correct and timely diagnosis of AIBDs is paramount for the management of patients. Thus, the identification of the exact specificity of the autoantibodies including the isotype (IgG, IgA, IgM, and IgE) and target antigen is of increasing importance. Diagnosis of the exact AIBDs is relevant to initiate an appropriate therapy and to communicate the prognosis. For example, anti-p200 pemphigoid can usually be treated with lower doses of corticosteroids compared to BP, and when antibodies against laminin 332 are present in MMP, a tumor search is required. Although enormous progress has been made with respect to the development of widely available standardized assays for serum autoantibodies in AIBDs, relevant diagnostic gaps remain. As such, no test system for antibodies to the BP180 ectodomain outside the NC16A domain is available, and also test systems for the detection of IgA autoantibodies in pemphigoid diseases are missing. Implementation of the regulation (EU) 2017/746 on *in vitro* diagnostic regulation (IVDR) will be a major hurdle for both manufacturers of test systems for rare diseases, such as AIBDs, and specialized laboratories with in-house assays, also termed laboratory-developed tests. Further innovations in the field of AIBDs diagnostics can be expected regarding automation, multivariate systems, and artificial intelligence (AI)-based evaluation (202, 203).

Author contributions

NB: Conceptualization, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. MH: Visualization, Writing – original draft, Writing – review & editing. IA: Writing – original draft, Writing – review & editing, Visualization. HO: Visualization, Writing – original draft, Writing – review & editing. MS: Visualization, Writing – original draft, Writing – review & editing. JP: Visualization, Writing – original draft, Writing – review & editing. AV: Visualization, Writing – original draft, Writing – review & editing. ES: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by grants from the Deutsche Forschungsgemeinschaft through CRC 1526 and the Cluster of Excellence Precision Medicine in chronic Inflammation (EXC2167).

Acknowledgments

We thank Marina Kongsbak-Reim and Randolph Bodenstein for their help with the IFM and immunoblot pictures.

Conflict of interest

ES has a scientific cooperation with Euroimmun. NvB and ES have a pending patent together with Euroimmun. ES holds patents together with Euroimmun.

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.

ES declared that they was an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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