SKIN BLISTERING DISEASES

EDITED BY: Ralf J. Ludwig, Philippe Musette, Cristina Has, Dedee Murrell and Kyle T. Amber

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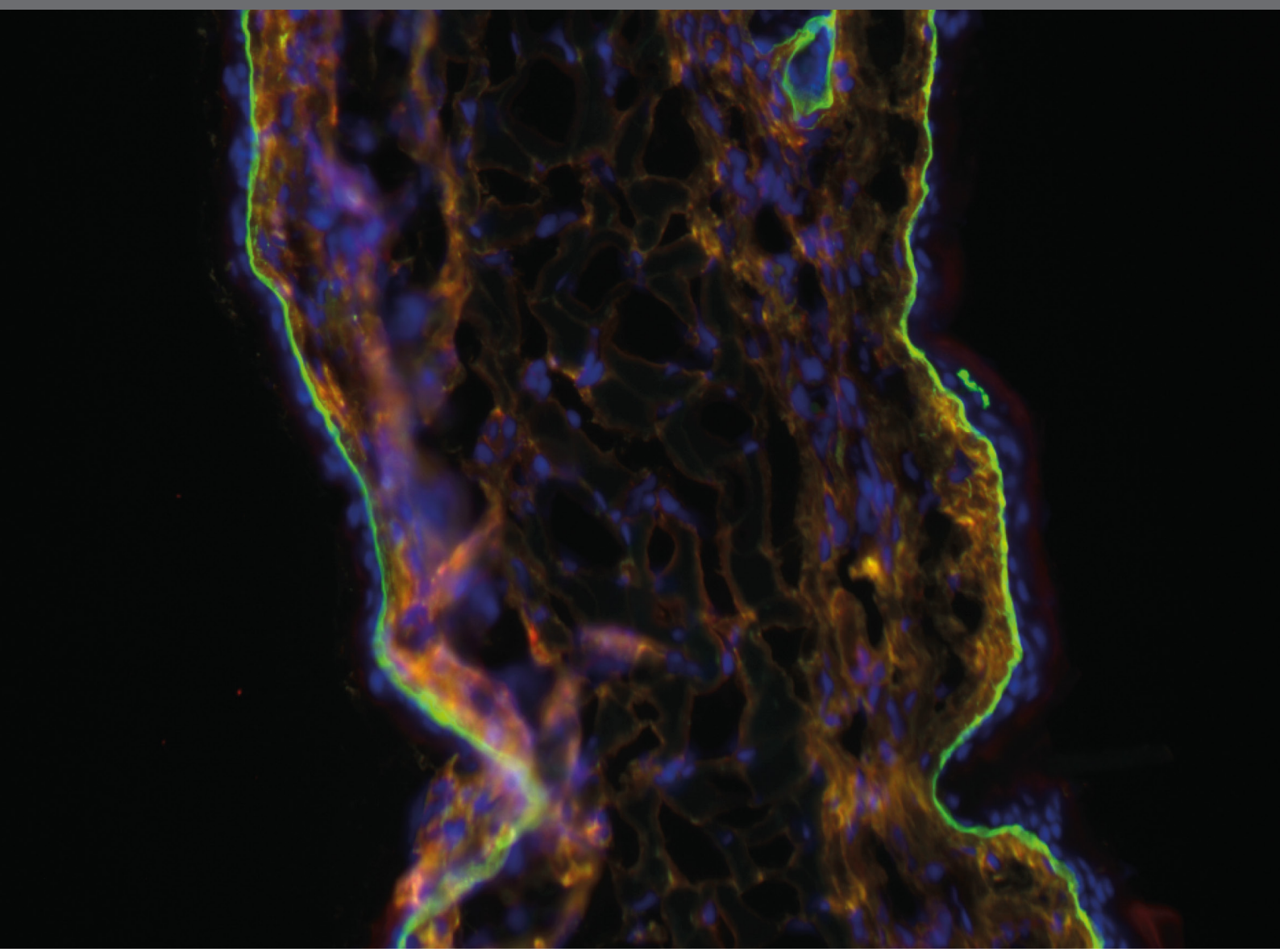

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SKIN BLISTERING DISEASES

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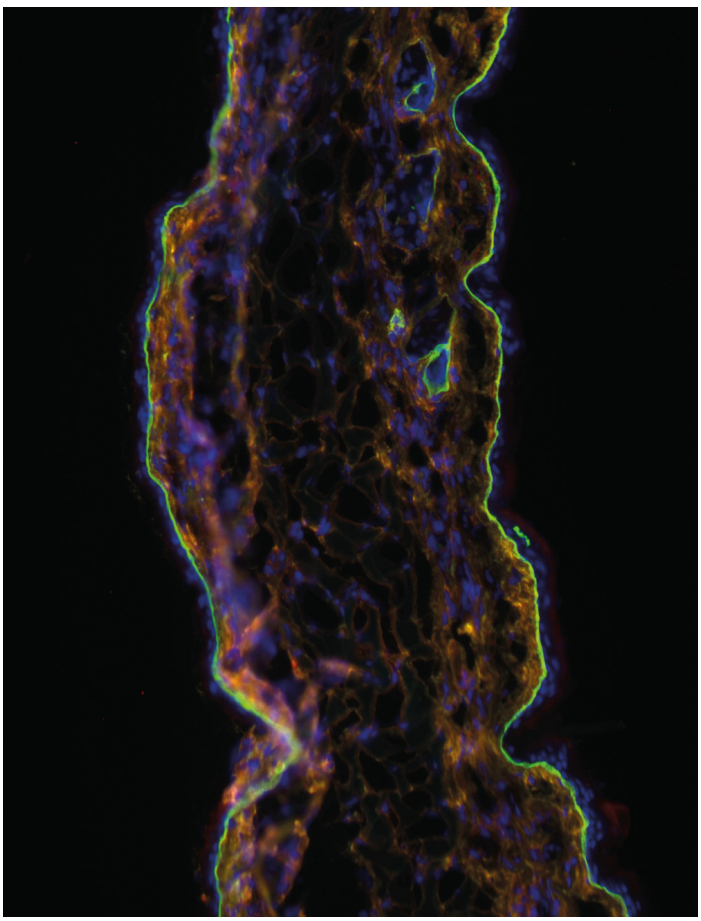

IgG (green) and C3 (red) staining of skin from experimental pemphigoid disease.
Counterstaining of nuclei using DAPI.
Image: Ralf Ludwig.

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EDITORIAL
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Editorial: Skin Blistering Diseases

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Keywords: skin, autoimmunity, hereditary diseases, pemphigoid, pemphigus, epidermolysis bollosa

Editorial on the Research Topic

Skin Blistering Diseases

Skin blistering is commonly caused by mechanical, physical, or infectious insults. Less often, mutations of structural components of the skin (1) or autoimmunity (2) directed against those structural components (3) lead to skin blistering. Albeit among the less frequent causes of skin blistering, understanding of the pathomechanisms of hereditary and autoimmune skin blistering has provided detailed insights into cutaneous biology and (auto)immunity. More specifically, in recent years, genetics, definition of autoantigens, model systems, and clinical research have led to a tremendous improvement for both diagnosis and treatment for patients suffering from skin blistering diseases. This is well-reflected by the article within the Research Topic Skin Blistering Diseases: Regarding genetics Vodo et al. herein review the genetics of pemphigus vulgaris (PV). As for many autoimmune diseases, the strongest association is observed for the HLA-locus. Thus far, 3 genome-wide association studies (GWAS) for PV, have found ST18 and TAP2 to be associated with PV (4–6). PV is a potentially fatal autoimmune blistering disease, characterized, and caused by autoantibodies targeting epithelial desmosomal antigens. The autoantibodies in PV are mainly directed against desmoglein (Dsg) 3 and −1. However, as highlighted by Sinha and Sajda in the Research Topic Skin Blistering Diseases, the anti-Dsg1/3 immune response does not explain the disease heterogeneity in PV. Indeed, the advance of technology, such as protein microarrays, has identified a number of additional autoantibodies in PV patients (7). Based on these observations, they here propose a super-compensation hypothesis, whereby the mixture, specificity and pathogenicity of PV-specific autoantibodies determine the clinical disease presentation. Whereas in pemphigus, and other autoimmune skin blistering diseases, genetics is mostly used to obtain insights into disease pathogenesis, genetic research in hereditary blistering diseases is far advanced. As reported by Condrat et al. in the Research Topic Skin Blistering Diseases, allelic heterogeneity and mutation status are at the verge of being employed for molecular-targeted, personalized treatment for patients with junctional epidermolysis bullosa (JEB), caused by reduced dermal-epidermal adhesion due to deficiencies of specific hemi-desmosomal adhesion proteins, such as type XVII collagen or laminin-332 (8).

Insights into the pathogenesis of skin blistering diseases have been reviewed in detail elsewhere (9–11). Within the Research Topic Skin Blistering Diseases, the role of eosinophils in bullous pemphigoid (BP) has been highlighted. BP is the most common subepidermal autoimmune skin blistering disease (12), with a variable clinical presentation, caused by autoantibodies targeting BP180 (9) and/or BP230 (13, 14). The contribution of eosinophils, albeit constituting the majority of cells observed in the dermal infiltrate (15), to BP pathogenesis remains uncertain. In their review, Amber et al. review the evidence for a pro-inflammatory role of eosinophils in BP, demonstrating pathways both dependent and independent of IgE autoantibodies targeting the major autoantigen (BP180) (16).

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During the past decades, the improved diagnosis of autoimmune skin blistering has partially contributed to the observed increase in the incidence of those diseases, especially BP. With the availability of commercial test systems for the diagnosis of epidermolysis bullosa acquisita (EBA), in line with an increased awareness for this rare autoimmune skin blistering disease (17), we also expect that EBA will be diagnosed more often. Two original articles in the Research Topic Skin Blistering Diseases addressed the significance of indirect immunofluorescence (IF) microscopy for the diagnosis of autoimmune skin blistering. Kridin and Bergman demonstrate that the predictive value of negative finding in IF microscopy using monkey esophagus as a substrate is reliable diagnostic test to exclude the diagnosis of pemphigus. In mucous membrane pemphigoid (MMP), an autoimmune blistering disease with predominant mucosal involvement, caused by a variety of autoantibodies (15), diagnosis is often difficult due to non-specific destruction of the mucosal biopsies and due to the low titers of circulating autoantibodies. By performing direct IF microscopy of peri-lesional mucosal biopsies from MMP patients, Kamaguchi et al. demonstrated a better sensitivity and specificity compared to H&E staining and serology. In addition to laboratory testing, careful clinical examination will provide important clues for diagnosis. In the article by Pietkiewicz et al. the frequency of nail involvement in pemphigus patients was determined, as this had been published on a case report basis and prospective data had been not available. Collectively, they demonstrated a low frequency of nail involvement in pemphigus.

Based on these insights into disease pathogenesis, many novel treatments have emerged, which were jointly presented and discussed among patients, clinicians, and researchers at the 5th International Pemphigus and Pemphigoid Foundation Scientific Conference held in Orlando, Florida, on May 15–16, 2018. In the flanking Perspectives Article, Lee et al. have summarized the emerging treatments for pemphigus and pemphigoid diseases. As highlighted by 2 case reports, one focusing on the treatment of paraneoplastic MMP, and one on the treatment of pemphigus with rituximab, current treatment still relies on systemic immunosuppression, which causes significant patient morbidity and mortality. In addition, long-term immunosuppression also fails to induce remission, especially in EBA (18–20). Hence, novel treatments are urgently needed to meet this unmet medical need (21). With the advent of consensus definitions (22–24) and the development and validation of objective (25, 26) and subjective (27) scoring systems to measure treatment responses objectively, we have entered the era of sponsored clinical trials of new treatments for these orphan blistering diseases.

As highlighted by Bech et al. the treatment of autoimmune skin blistering diseases, is becoming more challenging due to the observed co-morbidity. They highlight that in BP the high incidence of cardiovascular and neurological co-morbidity must be considered when selecting treatments for BP. Use of high doses of corticosteroids, the backbone of BP treatment, may aggravate cardiovascular co-morbidity, and be at least partially responsible for the increased cardiovascular mortality in BP patients. Furthermore, co-occurrence of chronic inflammatory diseases, such as psoriasis and autoimmune skin blistering diseases, need to be taken into consideration when selecting treatments. However, the Research Topic Skin Blistering Diseases Kridin et al. demonstrate that the anecdotal association of pemphigus with thyroid diseases can only be partially confirmed in a large cohort of pemphigus patients. The authors show that Hashimoto's thyroiditis is associated with pemphigus in male patients, while no association of pemphigus with Graves' disease or thyroid cancer was detected.

With the Research Topic Skin Blistering Diseases we aim to increase awareness for these diseases, present the state-of-the art diagnosis and treatment, and (maybe most importantly) stimulate further basic, translational and clinical research in the field.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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CASE REPORT
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Severe Refractory Paraneoplastic Mucous Membrane Pemphigoid Successfully Treated With Rituximab

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Mucous membrane pemphigoid (MMP) is a rare autoimmune bullous disease of the mucous membranes, which can cause irreversible scarring and is discussed to be associated with cancer, if laminin-332-autoantibodies are present. MMP with severe ocular and laryngeal involvement is difficult to treat and can be treatment-refractory to conventional immunosuppressant therapy. A 67-year-old man with a history of prostate cancer presented to our clinic with sore throat, intraoral bullae, odynophagia, dysphonia, exertional dyspnea, and erosions of the glans penis. Clinical examination confirmed a laryngo-pharyngitis with involvement of the epiglottis and bilateral symblepharon. Diagnostics comprising multiple biopsies, direct and indirect immunofluorescence, serology analysis, and immunoblotting confirmed the diagnosis of a paraneoplastic MMP by showing a subepithelial split in histology and the presence of anti-laminin-332-antibodies. Despite combined systemic treatment with prednisolone and either dapsone or azathioprine, a progress of the disease occurred leading to severe ocular and laryngeal complications. Two month after rituximab treatment, complete disease control was achieved. This case report shows a severe ocular and life threatening laryngeal involvement of therapy-refractory paraneoplastic MMP highlighting the importance of interdisciplinary management and difficulty of diagnosing MMP despite repeated diagnostic testing.

Keywords: mucous membrane pemphigoid, rituxmab, paraneoplastic, laminin 332, therapy refractoriness

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BACKGROUND

Mucous membrane pemphigoid (MMP) is defined as a heterogeneous group of autoimmune, chronic inflammatory blistering diseases, which lead to subepithelial bullae predominantly of the mucous membranes and occasionally the skin (1–3). The most common affected sites are the oral and ocular mucosae, but an involvement of the nasopharynx, esophagus, larynx, and anogenital region may also occur. The underlying pathophysiology is characterized by a linear deposition of IgG, IgA, or C3 along the epithelial basement membrane zone (1). If MMP is suspected clinically, diagnostic testing and treatment is required without delay in order to prevent complications like irreversible scarring potentially leading to blindness, airway stenosis, esophageal, and anogenital stricture (3). Smaller studies and case reports suggest positive laminin-332 (laminin-5)-autoantibodies to be associated with a paraneoplastic manifestation of MMP (4–7).

Epidemiological studies of MMP are rare. Thus, the real world incidence of MMP remains unknown. In the literature the incidence in United Kingdom of cicatricial conjunctivitis was

calculated as 0.8 per million, whereas the incidence of MMP in France and Germany was estimated to be 1.3–2.0 per million per year (8–10).

Therapy of MMP is mainly dependent on the classification of high and low risk disease. Low risk MMP (involvement of oral mucosae and skin) should be treated initially by topical steroids whereas it is recommended to treat high risk MMP (involvement of the eyes, esophagus, larynx, urogenital region) by systemic corticosteroids. In case of incomplete disease control, dapsone in combination with immunosuppressive therapies like azathioprine, cyclophosphamide, or mycophenolate mofetil should be applied (1). According to the European guideline for management and treatment of bullous pemphigoid, rituximab is recommended as third-line therapy, if conventional immunosuppressive drugs were not effective, contraindicated, or showed unacceptable side effects (11). In the literature, rituximab has been described effective as treatment in therapy-recalcitrant MMP (12–16). However, relapse is frequent and only a few studies including a small quantity of patients are available (12–16).

Herewith we present a case of a MMP with a positive history of cancer, severe laryngeal, ocular, and genital involvement showing a refractory course of the disease on azathioprine and dapsone immunosuppressive treatment. Given the severe involvement of the eyes and epiglottis we emphasize the indispensable multidisciplinary management of paraneoplastic MMP.

CASE PRESENTATION

A 67-year-old caucasian male patient presented first to the Clinic for Dermatology in August 2017 suffering since March 2017 from sore throat, intraoral bullae, odynophagia, dysphonia, exertional dyspnea, and erosions of the glans penis. He was first treated by his general practitioner for a suspected oral herpes infection with antiviral medication without improvement. At the onset of the symptoms the patient had been retired.

The medical history of the patient revealed a history of prostate cancer diagnosed and treated by radical prostatectomy ∼1 year before the onset of symptoms, epilepsy treated with levetiracetam since 2002, asthma and a chronic rhinosinusitis since 1988 treated with surgery.

The clinical examination revealed dry mucuous membranes in the oral cavity with erosions and swellings of the buccal mucosa and the hard palate. Inspection of the pharynx showed a distinct laryngo-pharyngitis with involvement of the epiglottis. To exclude an involvement of trachea a bronchoscopy was done revealing multiple ulcers of the pharynx, highly vulnerable mucous membranes and granulomatous changes of the vocal cords (**Figure 1**).

A biopsy, taken shortly before the first presentation to our clinic in an external hospital showed a subepithelial split together with an inflammatory cell infiltration comprising monocytes and granulocytes. The DIF analysis was negative. In our clinic an additional biopsy of the oral mucous membrane stained with haematoxylin and eosin staining was done. The result was negative for MMP showing an increase of collagen fibers with lymphohistiocytic infiltrate and an increased amount of plasma cells in the corium. The DIF analysis revealed unspecific perivascular C3 deposits. Consistent with the first biopsy, a third biopsy with haematoxylin and eosin staining, showed a subepithelial split (**Table 1**). Indirect immunofluorescence using both monkey esophagus and human salt-split skin did not detect circulating IgG- or IgA-autoantibodies. In addition, serum analysis using ELISA with recombinant BP180 NC16A, BP180, BP230, and desmoglein 1 and 3 was negative (**Table 2**). As serology testings were negative, immunoblotting of extracellular matrix was performed, which was positive for circulating IgG4-autoantibodies to γ2-chain of laminin-332 (**Figure 2**). The differential diagnosis of Behçet's disease presenting orogenital ulceration was unlikely as the patient only fulfilled one minor criteria, did not show characteristic histological changes for Behçet's disease or any other major or minor criteria for Behçet's disease. Accordingly, clinical criteria such as uveitis or retinal

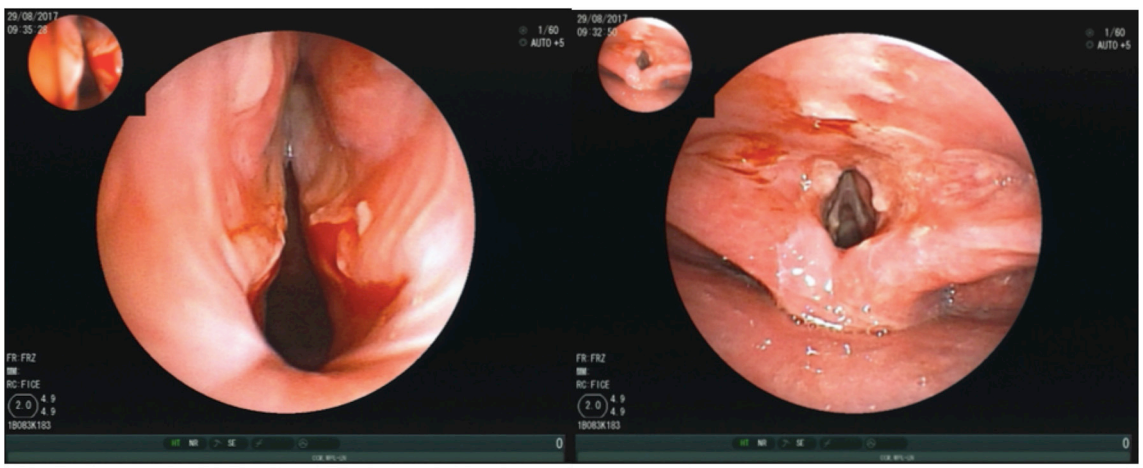

FIGURE 1 | Bronchoscopy showing multiple ulcers of the pharynx, highly vulnerable mucous membranes and granulomatous changes of the vocal cords.

TABLE 1 | Histological findings of performed biopsies.

Biopsy No.	Hematoxylin and eosin staining	DIF analysis
1	Subepithelial split together with an inflammatory cell infiltration comprising monocytes and granulocytes	Negative
2	Increase of collagen fibers with lymphohistiocytic infiltrate and an increased amount of plasma cells in the corium	Negative
3	Subepithelial split	Not done

TABLE 2 | Laboratory findings.

Method	Substrate analyzed	Positive or negative result
ELISA	Recombinant proteins: BP180 NC16A, BP180, BP230, desmoglein 1, desmoglein 3	Negative
Immunoblot	Extracellular matrix	Positive for γ2-chain of laminin-332-autoantibodies
Indirect immunofluorescence	Monkey esophagus and human salt-split skin	Negative

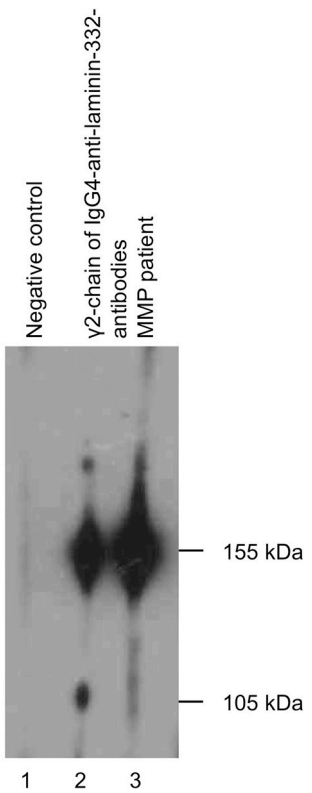

FIGURE 2 | Immunoblotting of extracellular matrix showing circulating IgG4-autoantibodies to γ2-chain of laminin-332.

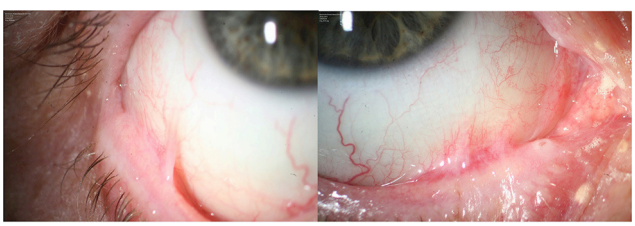

FIGURE 3 | Active bilateral stage 4 symblepharon according to Tauber und Foster classification.

vasculitis, characteristic skin lesions, HLA-typing for B51 and pathergy test were negative. The differential diagnosis of a cytotoxic-mediated disease like Stevens-Johnson-Syndrome was rather unlikely, given the course of the disease, the affected sites, the lack of a possible trigger and the histological findings without signs of a CD8+-mediated reaction like an interface dermatitis or necrotic keratinocytes. An oral candida infection was excluded by a swap. Given the positive history for prostate cancer we performed a tumor staging. The chest-x-ray, ultrasound of the abdomen and PSA-value (0.1 µg/l) were within normal limits. Based on the clinical course, the histological finding and the immunoblot positive for laminin-332-autoantibodies, we suspected a paraneoplastic MMP.

Due to an acute exacerbation with progressive exertional dyspnea, anxiety choking, dry cough, hoarseness and ocular irritation a chest-x-ray, and body plethysmography were performed to exclude an acute exacerbation of asthma. Because of exertional dyspnea a laryngoscopy was performed which revealed progressive oral ulcers as well as a synechia of the first third of the vocal cords.

Even though the diagnosis could not be confirmed by immunohistological criteria at the time of the first symptoms, a paraneoplastic MMP was suspected based on the clinical manifestation with the positive cancer history. Given both, the critical laryngal involvement causing dyspnoea and the ocular bilateral stage 4 symblepharon according to Tauber und Foster classification (17) (**Figure 3**), systemic treatment was initiated. Intravenous methylprednisolone was applied (250 mg/day) at 3 consecutive days. The pulse therapy was repeated for three times every 4 weeks. Oral therapy with dapsone (100 mg/day), which had been initiated after the first pulse therapy was discontinued by the general practitioner due to methemoglobinemia, cyanosis of the lips, and dyspnoea. Instead a combined oral therapy comprising azathioprine (100 mg/day) and prednisolone (50 mg/day) was given. Prednisolone was consecutively reduced to 10 mg per day. Topical treatment included Hylogel due to ocular involvement, inhalation of Tacholiquin 1% and a prednisolone-dexpanthenol solution. Hereafter disease control was achieved with reduction of hoarsness and dyspnoea. Azathioprine was discontinued after 4 month due to elevated values of gamma-glutamyltransferase.

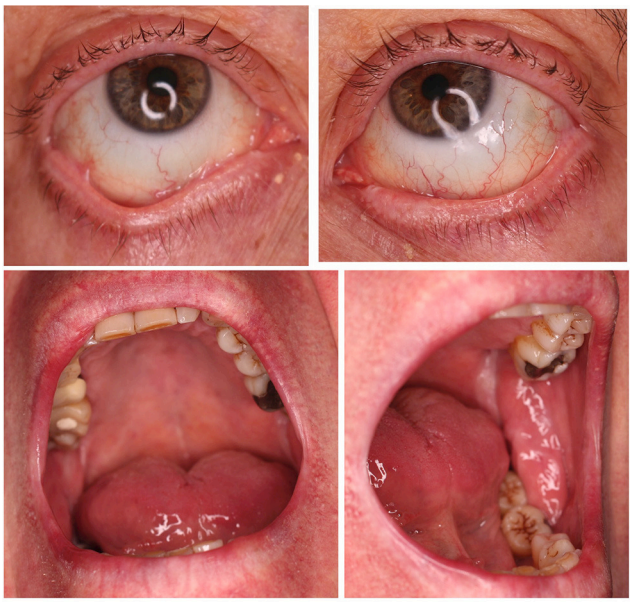

FIGURE 4 | Post-treatment pictures showing a stable ocular involvement and no new enoral lesions 5 month after rituximab treatment.

Due to an acute laryngotracheitis with acute dyspnea as well as inspiratory and expiratory stridor, a microlaryngoscopy with division of the synechia of the anterior commissure was performed in the clinic for ear, nose, and throat followed by a fixation of a silicone sheet.

Given both systemic treatments with azathioprine and dapsone had to be discontinued due to adverse effects, therapy with rituximab 1,000 mg was initiated twice in a 14-days interval. The follow-up examination 8 weeks later revealed a stable disease with no new oral lesions (**Figure 4**). According to the patient dyspnea did not appear since the start of rituximab treatment. The ocular manifestation of the MMP was assessed stable by the ophthalmologists. During the latest check-up for cancer no signs of relapse were detected. Differential white blood cell count was taken during and after the treatment with rituximab. Initially, total leukocytes and lymphocytes were within normal limits (Leukocytes: 6.75/nl, lymphocytes: 1.13/nl). 7 weeks after the second treatment with rituximab a lymphocytopenia was detected (0.60/nl). Leukocytes and lymphocytes before and after radical prostatectomy were normal (leukocytes: before 6.44/nl, after 9.90/nl, lymphocytes: before 1.12/nl, after 1.24/nl)

DISCUSSION

MMP is a rare autoimmune blistering disease, which predominantly affects the mucous membranes and is characterized by the linear deposition of IgG, IgA, or C3 along the epithelial basement membrane zone (1). The diagnosis of MMP is mainly based on clinical manifestation, immunohistological examination as well as histopathological and serology analysis like indirect immunofluorescence, immunoblotting, or immunoprecipitation techniques (3). According to the consensus conference the clinical course of disease as well as the DIF analysis are crucial criteria to diagnose a MMP (1).

Diagnosing MMP can be challenging (18). The diagnosis of MMP in our patient at the time of the first hospitalization was made by clinical features only, as the first diagnostic workup including histology, serology, and immunohistology remained negative. As the DIF of the biopsy taken in an external hospital did not show specific changes for MMP, an additional biopsy was taken from the buccal mucosa. Again, the histology and DIF analysis did not show characteristic changes for MMP. According to the literature false-negative results at the first diagnostic workup of MMP are not rare (18). Repeated testing is highly recommended to increase the sensitivity of the DIF analysis for MMP diagnosis (18). However, DIF analysis can show false-negative results especially in patients with only ocular mucous membrane involvement or when longstanding lesions are being analyzed (3, 19). A third biopsy taken of the laryngeal mucosa showed a subepithelial split in hematoxylin and eosin staining consistent with the diagnosis of a MMP. Unfortunately an additional DIF analysis of the third biopsy has not been arranged by the ENT department. Shimanovich et al. state, that despite repeated testing up to 5% of the biopsies taken from patients with MMP remain negative (18). Consistent with our report, the diagnosis of MMP could be confirmed by the detection of circulating autoantibodies against bullous pemphigoid antigen 180 or laminin-332 (18).

For ocular MMP Labowsky et al. have shown that patients showing a linear deposition of IgG, IgA, or C3 at the basement membrane in a DIF biopsy were more likely to be treated with systemic immunosuppression compared to patients showing no linear immunologic deposits at the basement membrane zone in a DIF biopsy (19). They conclude that even without confirming the diagnosis by DIF, treatment with systemic immunosuppression should be initiated (19). In our case systemic treatment was initiated due to the reduced general condition of the patient as well as the risk of irreversible complications due to a delay of intervention. Due to progressive disease with severe dyspnea and the need of discontinuation of systemic dapsone and azathioprine therapy, rituximab treatment was initiated. In the literature, large randomized controlled studies analyzing rituximab treatment in recalcitrant MMP are sparse. Lamberts et al. investigated the effectiveness and safety of rituximab in 28 patients with recalcitrant pemphigoid diseases (13). Disease control was achieved in the majority of the cases (67.9%). However, during follow-up, 66.7% patients relapsed. Repeated treatment with rituximab was effective in 85.7% of retreated cases. Interestingly, MMP patients showed the most benefit of rituximab (disease control in 85.7%) compared to other pemphigoid diseases. However, the rate of relapses was high (75%). The best outcome was achieved using the high dose protocol (1,000 mg rituximab at days 1 and 15) compared to the low dose protocol (500 mg rituximab) (13). In comparison to IgG-dominant pemphigoid diseases, rituximab was less effective in IgA-dominant pemphigoid diseases, representing an unresponsiveness of IgA positive plasma cells to rituximab (13).

During the follow-up, 3 patients died of which one was probably treatment related. Rübsam et al. analyzed 6 MMP patients with ocular involvement after being treated with rituximab (14). Using the high dose protocol all patients responded to rituximab. However, relapse occurred in 83.3% of the cases. Consistent with the results of Lamberts et al. repeated treatment with rituximab lead to remission in all patients (14). Two patients died probably not related to the treatment (14). Heelan et al. investigated 8 patients with MMP being treated with 1,000 mg rituximab at days 1 and 15 (12). After disease control was achieved in all patients, a relapse occurred in 100% of the cases. Retreatment with rituximab lead to a 100% response rate (12). Shetty et al. published a retrospective study including case series and case reports of 28 patients with MMP (15). In this study, different protocols of rituximab treatment were applied. Consistent with the previous studies a high response and relapse rate was described (disease control 82.1%, relapse in >50%) (15). Tomsitz et al. investigated a cohort of 22 patients with recalcitrant autoimmune blistering diseases using the high dose rituximab protocol (16). Seventy-two percent of the patients showed a partial or complete remission after the first cycle. However, unlike the findings of Lamberts et al. the response rate in patients with MMP was low (40%) (16). In our case, disease control was achieved 2 month after administering rituximab using a high dose protocol. Since 5 months after the treatment no relapse of MMP or adverse effects have been reported.

Adjuvant treatment with intravenous immunoglobulin or immunoadsorption in combination with rituximab has been described effective to treat therapy-refractory MMP (5, 20–22). Protein A immunoadsorption describes a method to selectively remove circulating antibodies from the blood in an extracorporeal circuit (23). It is recommended to be used as first line treatment in pemphigus vulgaris, pemphigus foeliaceus, paraneoplastic pemphigus vulgaris, or epidermiolysis bullosa aquisita (24). Albeit complete disease control was achieved in our case by rituximab monotherapy, relapse of the disease after rituximab treatment is frequent in ocular MMP (12). Thus, protein A immunoadsorption or immunoglobulin treatment represent a promising alternative to rituximab in case of recurrence.

The patient presented here had circulating IgG4-autoantibodies to the γ 2-chain of laminin-332-autoantibodies determined by immunoblotting analysis. Even though studies and case reports suggest an association between detection of laminin-332-autoantibodies and paraneoplastic MMP, Bernard et al. could not detect a significant relationship in a multicenter retrospective study (4, 6, 7, 25). The patient's history was positive for a prostate cancer diagnosed and treated ∼1 year before the onset of the first symptoms. A staging examination remained negative, therefore a direct link between progressive oncological disease and MMP has not been verified in our case. Further investigations regarding the interaction of neoplasms and anti-laminin-332-antibodies are required.

Multidisciplinary management and treatment of the MMP is of the utmost importance, as different sites can be affected (1). Unlike the majority of the cases with involvement of the oral mucosae and the eyes, the patient presented with severe involvement of the larynx and epiglottis leading to an acute laryngo-pharyngitis, synechia of the vocal cords and recurrent episodes of severe dyspnea. On account of the close cooperation with the clinic for ear, nose, and throat, the division of the synechia was performed, leading to an improvement of the symptoms. Herewith we emphasize that the optimal outcome for the patient was achieved only due to multidisciplinary management including ENT specialists and ophthalmologists. A multidisciplinary follow-up is highly recommended to ensure best disease management.

For the publication of this case report written informed consent from the patient in accordance with the Declaration of Helsinki was obtained.

AUTHOR CONTRIBUTIONS

MW and MD writing of the manuscript, literature research, figures. MW revision of the manuscript, initiation of the publication.

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Junctional Epidermolysis Bullosa: Allelic Heterogeneity and Mutation Stratification for Precision Medicine

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Junctional epidermolysis bullosa (JEB) is a hereditary blistering disease caused by reduced dermal-epidermal adhesion due to deficiencies of one of the proteins, laminin-332, type XVII collagen, integrin α6β4 or integrin α3. Significant progress has been achieved in the development of therapies for EB, such as bone-marrow transplantation, local or systemic injections with fibroblasts or mesenchymal stromal cells, readthrough of premature termination codons, or exon skipping. These were tailored in particular for dystrophic EB, which is caused by type VII collagen deficiency and have not yet reached broad clinical practice. Recently, pioneering combined gene and stem cell therapy was successful in treating one boy with junctional EB. Beside these exclusive approaches, no specific therapy to amend the major clinical features, skin and mucosal blistering and non-healing wounds is available to date. Here we extend the mutational spectrum of junctional EB, provide a stratification of *COL17A1* mutations and discuss potential molecular therapeutic approaches.

Keywords: junctional epidermolysis bullosa, collagen XVII, *COL17A1*, mutation, premature termination codon, therapy

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INTRODUCTION

Inherited epidermolysis bullosa (EB) is a group of genetic diseases characterized by skin fragility and is caused by mutations in the genes encoding different proteins with roles in cell adhesion. Junctional EB (JEB) is a subtype of EB in which dermal-epidermal adhesion is reduced, due to deficiencies in one of the proteins laminin-332, type XVII collagen, integrin α6β4 or integrin α3 (1).

Type XVII collagen (also known as ERED, BP180, BPA-2, BPAG2, LAD-1, BA16H23.2) is expressed by basal epidermal keratinocytes and plays an important role in cell-matrix interactions as a transmembrane component of the hemidesmosomes. Structurally, type XVII collagen is a homotrimer, consisting of three collagen alpha-1(XVII) chains, each with a molecular weight of 180-kDa. It is a type II protein, which spans the cell membrane with two domains, an endodomain toward the cytosol and an ectodomain toward the lamina densa of the basement membrane. It links, together with integrin α6β4 and CD151, the inner hemidesmosomal plaque consisting of plectin and BPAG1 to the anchoring filaments buildup of laminin-332 (2). The primary, secondary and tertiary structures, post-translational modifications, as well as interactions and functions of type XVII collagen have been recently extensively reviewed (3, 4).

The main function of type XVII collagen as an adhesion molecule is assured by tight, but dynamic incorporation into hemidesmosomal multiprotein complexes in stratified, pseudostratified and transitional epithelia (e.g., skin, oral mucosa, ocular conjunctiva, epithelial basement membrane of the cornea, upper esophagus, transitional epithelium of the bladder). Besides this role in cell-matrix adhesion, type XVII collagen is responsible for maintenance of follicular stem cells, cell polarity, and migration.

Against this dogma, recent studies provide evidence for the presence of type XVII collagen between the basal keratinocytes, this type being known as the non-hemidesmosomal collagen XVII (4). Non-hemidesmosomal collagen XVII is not a part of the dermal-epidermal adhesion and after its discovery, questions emerged regarding its role. Molecular interactions and cross-talk with focal adhesions and the actin cytoskeleton may be implicated (5, 6). Watanabe and colleagues reported that reduction of non-hemidesmosomal collagen XVII in the interfollicular epidermis is involved in physiological aging, but further work is required in order to decode the exact mechanism (4, 5). Besides these findings, type XVII collagen is also found in the hair follicles stem cells and its proteolytic degradation grounds for age-related hair loss, making it a potential targeted candidate for age-related alopecia, explaining definitive hair loss in patients with JEB (7, 8).

Type XVII collagen is encoded by *COL17A1* which spans 52 kb of the genome and is located on the long arm of chromosome 10 (10q24.3). *COL17A1* consists of 56 exons and short introns. Mutations in this gene lead to a complete or partial loss-of-function of type XVII collagen in tissues and cause generalized or localized skin blistering, amelogenesis imperfecta, epithelial recurrent erosion dystrophy, alopecia and nail dystrophy (3). Although life expectancy is not reduced, patients with JEB due to *COL17A1* pathogenic variants experience extensive trauma-induced blistering resulting in multiple wounds that tend to heal slower with time, excessive caries, diffuse progressive irreversible alopecia, and have impaired quality of life (**Figure 1**).

Currently, EB research is focused on elucidating the disease mechanisms and development of therapies (9). Experimental therapeutic approaches include *ex vivo* gene therapy to correct *LAMB3* and *COL7A1* mutations in epidermal stem cells (10, 11), cell therapies for dystrophic EB, repurposed drugs with anti-inflammatory effects for dystrophic EB and EB simplex and topical agents aiming at improving wound healing. There is an urgent need for new treatments for JEB with *COL17A1* mutations for which no experimental therapies are presently available.

Extensive genetic testing and research has provided a comprehensive database (Human Gene Mutation Database® Professional 2018.2) with over 100 distinct mutations encountered in *COL17A1*, but the database constant development is an ongoing task. The need for such mutational and patients databases is crucial for patients' counseling and prognosis. Development of therapies and selection of patients for clinical trials also depends on their individual mutations.

Abbreviations: EB, epidermolysis bullosa; JEB, junctional epidermolysis bullosa; PTC, premature termination codon; NMD, nonsense mediated decay.

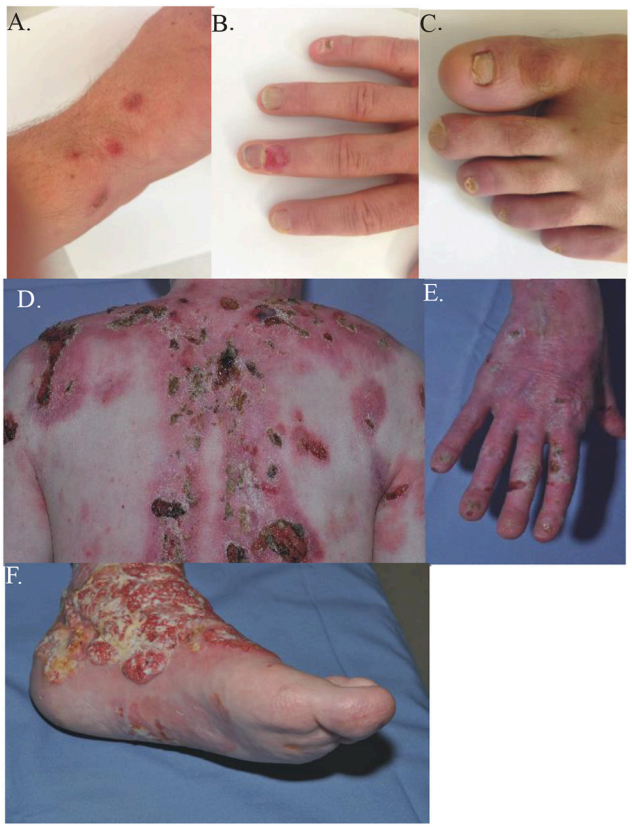

FIGURE 1 | Clinical presentation and different phenotypes in JEB. The clinical manifestations of two JEB patients, both with *COL17A1* mutations. **(A–C)** Case 66, compound heterozygous with a missense mutation c.3908G > A and a deletion c.4100_4101delTT, mildly affected. **(A)** discrete erosions and blisters on upper hand; **(B)** nail dystrophy and crusts on the hand; **(C)** toe-nail dystrophy. **(D–F)** Case 68, a 45 y-old male compound heterozygous with a missense mutation c.2T > A and a deletion c.3164delT, severely affected. **(D)** Extensive crusts and scarring on the upper posterior thorax. **(E)** Nail dystrophy, erosions and crusts on the right hand. **(F)** Squamous cell carcinoma on the lower left leg.

Here, we review our cohort comprising of 68 JEB patients with *COL17A1* mutations diagnosed over the past 15 years, report novel mutations, genotype-phenotype correlations and finally, discuss potential experimental therapies for these patients.

METHODS

The study was conducted according to the Principles of Helsinki and was approved by the Ethics Committee of the University of Freiburg. Written informed consent was obtained from the participants for the publication of the case reports and identifiable images included in this article.

Mutation Detection

After informed consent genomic DNA was extracted from EDTA-blood using the Qiagen blood minikit (Qiagen, Hilden Germany). In most cases, mutation analysis was performed by

bidirectional Sanger sequencing as reported before (12). Targeted next generation sequencing (NGS) of EB genes was performed since 2016 as described (13). In one case clinical exome analysis was performed (http://www.humangenetik-freiburg.de/).

Cell Culture

Human primary keratinocytes were isolated from the skin of the patients and immortalized with the E6E7 genes as previously described (14). Keratinocyte lines were cultured at 37°C in 5% CO_2 in serum-free medium containing epidermal growth factor and bovine pituitary extract (Invitrogen).

RNA Isolation and RT-PCR

Isolation of total RNA from confluent cell monolayers was performed using the RNeasy Plus Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One Microgram of isolated RNA was used for cDNA synthesis with the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Wilmington, USA) in a volume of 100 μl.

RT-PCR was then performed with 5 μl of the cDNA in a 50 μl mix containing 10x Puffer, nucleoside diphosphatase (dNTP), Taq DNA Polymerase (Sigma) and primers. Primers for *COL17A1* cDNA were F: TACCATGTACGTGTCAGGCC and R: TGATGCTGGACCACACATTG. The annealing temperature was calculated with UCSC *in-silico* PCR (https://genome.ucsc.edu/cgi-bin/hgPcr).

Immunofluorescence Staining of Skin Sections

Biopsy technique, tissue processions, antigen-antibody interaction, and afterwards visualization were previously described (15).

Protein Isolation and Immunoblotting

For protein extraction, cells were lysed on ice for 20 min in lysis buffer, proteinase inhibitors and phosphatase inhibitors and centrifuged for 20 min at 14,000 rpm. Extracts were subsequently heated to 95°C for 5 min. Bradford assay (BioRad) was used to determine the protein concentration. For collagen XVII immunodetection (case 61), 35 μg protein was run on a 8% SDS Page for 1.5 h and transferred to a nitrocellulose membrane at 300 mA for 1.5 h. For detection, collagen XVII-specific antibodies were used: NC16A, against epitope within the NC16A region, at a dilution of 1:1000 (16).

RESULTS

The results of the genetic and molecular analysis of the cohort are described below. Also, novel mutations, genotype-phenotype correlations, and immunofluorescence mapping are reported in **Table 1**.

Novel *COL17A1* Mutations and Genotype-Phenotype Correlations

This study contributes to the *COL17A1* mutational database with 9 mutations that, to the best of our knowledge, have not been reported before (**Table 1**, **Figure 2**). These include two nonsense mutations c.1750C > T, p.R584* and c.4153C > T, p.Q1385*, five frameshift mutations, one mutation of the translation initiation codon, and one splice site mutation. Several of these cases demonstrated interesting genotype-phenotype correlations or clinical course of the disease and will be highlighted here.

Case 61 was a female patient suffering from mild skin blistering since birth. Epidermolysis bullosa simplex was suspected and several candidate genes analyzed, but no mutations were found. Clinically, skin blisters and erosions were present on the face, upper posterior thorax, and lower limbs (**Figure 3**). As the girl grew older, nails became dystrophic and were progressively lost, suggesting the diagnosis of JEB. She was found to be compound heterozygous for the *COL17A1* mutations c.1750C > T, p.R584* and c.3509-1G > C. To the best of our knowledge both mutations were previously unreported. The mutation c.3509-1G > C is located in intron 49 of *COL17A1* and alters the conserved acceptor splice site. RT-PCR and sequencing of the RNA extracted from keratinocytes of the patient revealed that an alternative splice site was used, 33 nucleotides downstream in the middle of exon 50, thus restoring the reading frame and allowing the synthesis of an eleven amino acids shorter truncated polypeptide. Intriguingly, in immunofluorescence mapping, the immunoreactivity for collagen XVII with the commercial monoclonal antibody NC16A3 (Abcam) was comparable to the control skin (**Figure 3B**). The polyclonal sera NC16A (17) demonstrated reduced immunoreactivity in the skin of the patient and a decreased amount of collagen XVII in lysates from patient's keratinocytes as compared to the controls (**Figure 3B**), explaining the mild phenotype.

Case 66 was a 22-year-old male who presented with acral blistering that had started at the age of 16 and was induced by sport activities. Additionally, he displayed nail dystrophy (**Figures 1A–C**). Immunofluorescence microscopy demonstrated a broadened staining pattern of type XVII collagen and no skin split. Clinical exome sequencing demonstrated that he was compound heterozygous for the *COL17A1* mutations c.3908G>A, and the frame shift deletion c.4100_4101delTT, p.F1367Cfs*8, that truncates the last 130 amino acids in the C-terminus of the α1(XVII)-chain.

The severe clinical picture of generalized intermediate JEB is illustrated in case 68 from our cohort, a 45-year-old male, compound heterozygous for the previously unpublished mutation in the translation initiation codon, c.2T>A, p.?, and the also novel frame-shift deletion c.3164delT, p.F1055Sfs*11. Generalized blistering resulted in multiple chronic non-healing wounds at sites of permanent trauma (**Figures 1D,E**); on such a wound on the lower left leg, he developed a squamous cell carcinoma at the age of 44 (**Figure 1F**). Although initially the histological aspect was that of a verrucous carcinoma, recurrence occurred rapidly with local invasion, necessitating amputation. This is the only patient of this cohort who developed squamous cell carcinoma during the observation period. Squamous cell carcinoma has been reported in JEB patients from The Netherlands (18), but the precise risk for developing this complication in JEB remains unknown.

TABLE 1 | Mutational analysis, immunofluorescence mapping and genotype-phenotype correlations of the patients previously unpublished.

Case	Mutations c.DNA level	Mutation Protein level	Immunofluorescence	Clinical phenotype (age)
44	**c.4153C>T**	**p.Q1385***	Not available	Not available
45	c.2861delG	p.G954Afs*112	Not available	Not available
49	c.2062C>T	p.R688*	Negative staining for collagen XVII	Severely affected with generalized blistering, nail dystrophy (age 1)
50	c.2407G>T	p.G803*	Reduced immunoreactivity for collagen XVII	Multiple blisters and erosions, nail loss, dental abnormalities (age 3)
51	c.[3487G>T;4319dup]	p.[E1163*;G1441Wfs*14]	Reduced immunoreactivity for collagen XVII	Mildly affected, nail dystrophy (age 1)
52	c.2237del	p.G746Afs*53	Reduced immunoreactivity for collagen XVII, almost negative	Severely affected, extensive blisters and erosion, nail loss (1 week old)
58	c.418_419delAG	p.S140*	Reduced immunoreactivity for collagen XVII, almost negative	Not available
59	c.[2237delG;3198C>T]	p.[G746Afs*53;S1066S]	Strongly reduced immunoreactivity for collagen XVII at the blister roof	Mildly affected
60	c.2062C>T	R688*	Negative staining for collagen XVII	Not available
61	**c.[1750C>T;3509-1G>C]**	**p.[R584*;?]**	Reduced immunoreactivity for collagen XVII at blister floor (**Figure 3**)	Mildly affected, nail dystrophy and loss (age 2) (**Figure 3**)
62	**c.2282_2283delGG**	**p.G761Dfs*40**	Reduced immunoreactivity for collagen XVII, almost negative	Not available
64	**c.[779delC;3569dupG]**	**p.[P260Qfs*32;N1191Qfs*51]**	Reduced immunoreactivity for collagen XVII at blister roof	Skin blisters, alopecia, nail loss
65	c.2062C>T	p.R688*	Negative staining for collagen XVII	Flaccid serous and hemorrhagic blisters, alopecia, poikiloderma, multiple hypopigmented scarred areas, mucosal involvement, generalized enamel defects
66	c.[3908G>A;**4100_4101delTT**]	p.[R1303Q;F1367Cfs*8]	Collagen XVII is present at blister roof	Mildly affected (see **Figure 1**)
68	**c.[2T>A;3164delT]**	p.[?;F1055Sfs*11]	Not available	Severely affected (see **Figure 1**) and squamous cell carcinoma

*Mutations in bold have not been reported before. *if only one mutation mentioned, then homozygous.*

Stratification of *COL17A1* Mutations

Stratified medicine, and in particular stratification of mutations, focuses on establishing a therapeutic approach tailored to the specific needs of a patient. Therefore, determining the most prevalent mutations and addressing their consequences is a practical and feasible concept, with previous work in cystic fibrosis supporting this aim (19).Currently, no international database of patients with JEB and *COL17A1* mutations is available, although many countries have established registries with clinical, molecular and follow up data for EB patients. Over 100 distinct *COL17A1* mutations have been reported in the literature (HGMD professional as of 1/15/2018) including 24 nonsense mutations, 14 missense mutations, 24 splicing mutations, 27 small deletions, 20 small insertions, one small indel, one gross deletion, and one gross insertion. Even though *COL17A1* is the only gene involved, phenotypes may differ in severity, so that mutations leading to complete loss of type XVII collagen will result in a more severe phenotype as compared to mutations that allow some degree of protein synthesis and function.

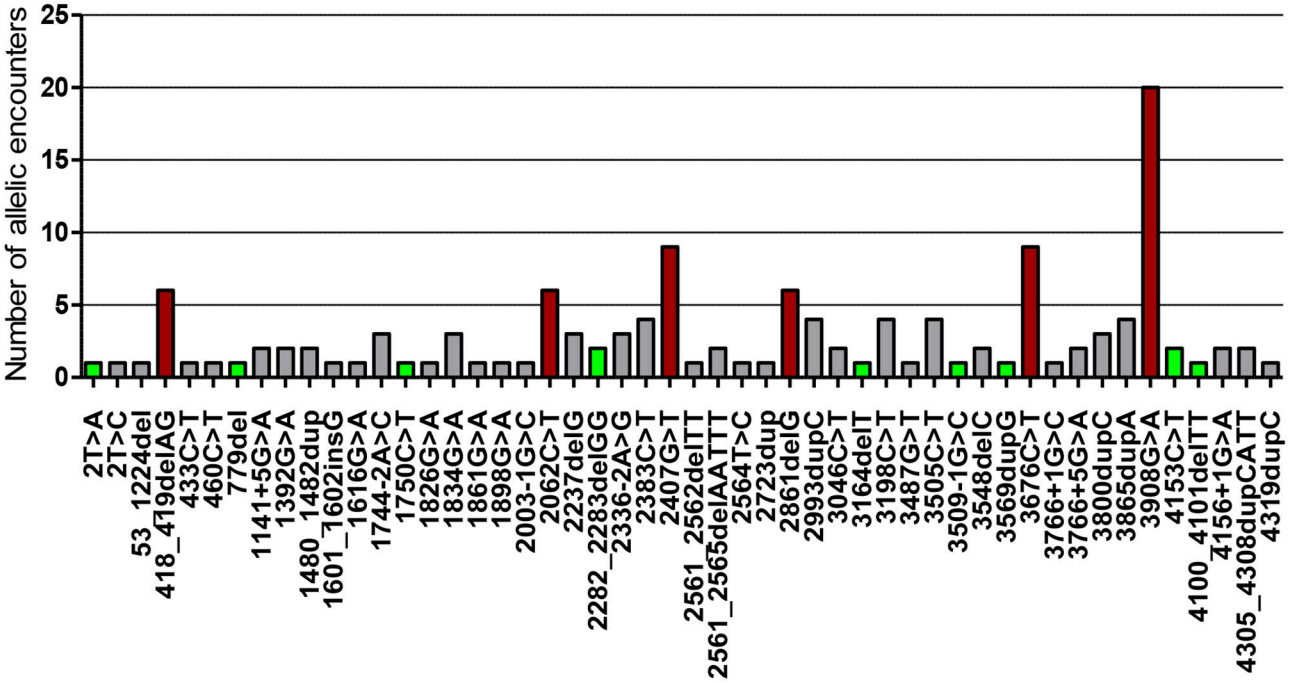

FIGURE 2 | Illustration of all 50 distinct mutations (both previously published and not) and their allelic mutation frequency, included in the article. The mutations have been sorted based on their position on the *COL17A1* gene. Mutations are at cDNA levels. Novel mutations are highlighted in green squares and the most frequent are shown in dark red squares. The Y axis represents numbers of allelic encounters in our cohort.

In the past 15 years we have systematically performed molecular diagnostics for EB employing immunofluorescence mapping and mutation detection (13, 15). Altogether, we identified 68 patients with JEB with *COL17A1* mutations, with a mean of about 5 new cases per year.

Fifty distinct *COL17A1* mutations were identified (**Table 2**). Evaluation and stratification of these mutations was performed based on the number of allelic encounters and mutation type (**Figure 2**). As shown in **Figure 2**, most mutations are unique, occurring in single cases / families and only few are recurrent. Approximately 33% of *COL17A1* mutant alleles are nonsense mutations, and another 33% are frame shift mutations leading to formation of premature termination codons (PTC). Splice site mutations account for 10% of mutant alleles.

In this cohort, the most frequent recurrent mutation was c.3908G > A, p.R1303Q detected in 16.17% of cases and 14.70% of the mutant alleles (in 9 patients in a homozygous state and in 2 patients in heterozygous manner), respectively. This missense mutation changes the amino acid arginine with glutamine and is located in the fourth non-collagenous domain (NC4) of the α1(XVII)-chain, which is part of the putative laminin-332 binding region in type XVII collagen (20). Secondary protein structure analysis and predicted changes in the structural integrity within this binding region suggested that these modifications result in abnormal laminin-332 binding (21). This mutation also appears to hamper the physiological C-terminal cleavage of type XVII collagen. Consequently, non-cleaved type XVII collagen ectodomain remnants induce the aberrant deposition of laminin-332 in the extracellular matrix (12, 22). Clinically, p.R1303Q is associated with a mild, Kindler-syndrome-like phenotype, manifesting with late onset skin blistering, progressive skin atrophy and sclerosis, loss of dermatoglyphics, scarring, and nail anomalies. Typically, type XVII collagen is deposited at the dermal-epidermal junction in a broad irregular pattern (13, 16).

Second most common mutation was c.2407G > T, representing 6.61% of the mutant alleles in 10.29% of cases (in 5 patients in a heterozygous state and in 2 homozygous patients). This mutation located in exon 34, where it converts a glycine residue to a premature termination codon (PTC), p.G803*,was also frequent in patients with Austrian (18) and Finish background (23). With the same allele frequency there was also c. 3676C > T, found in 11.76% of patients. It also translates into a PTC, p.R1226*. This recurrent mutation has also been previously reported in Dutch population (24) and leads to nonsense mediated mRNA decay, demonstrated by absence of full length collagen XVII (12, 25). Next, we found c.2062C > T, p.R688*, c.2861delG, p.G954Afs*112 and 418_419delAG, p.S140* to represent 4.41% of the mutant alleles in 4.31% of our patients. All these null-mutations lead to absence of type XVII collagen and generalized intermediate JEB (previously known as generalized atrophic benign epidermolysis bullosa (GABEB) and

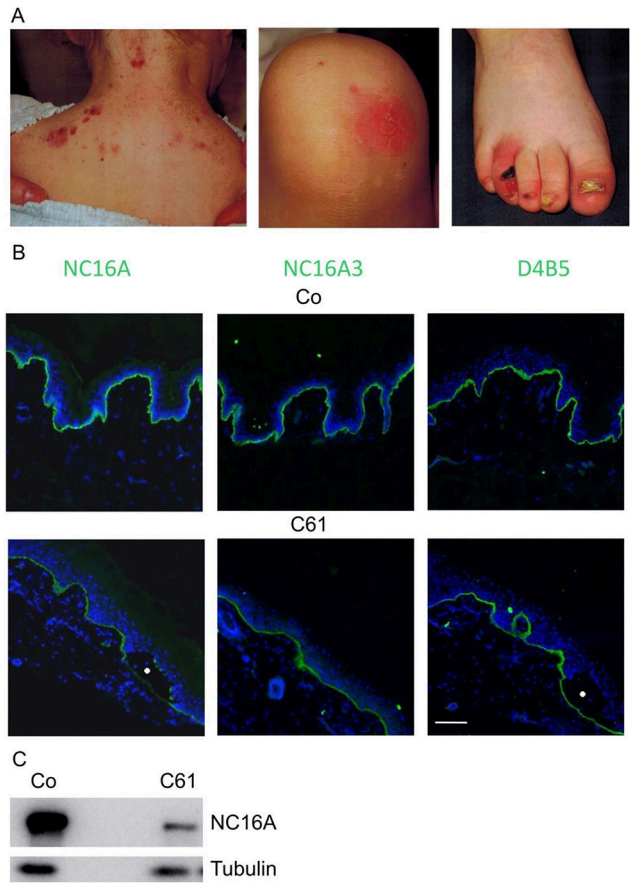

FIGURE 3 | Case 61, a 2 y-old female JEB patient, mildly affected. **(A)** Clinical manifestations include skin blisters on the upper posterior thorax, erosions on the lower limb and nail dystrophy on lower limbs. **(B)** Immunofluorescence antigen mapping performed with domain-specific antibodies against collagen XVII (NC16A, NC16A3) and collagen IV (D4B5) on normal control human skin (Co, upper pictures) and case 61 (C61, lower pictures) with JEB. The antibodies used were NC16A rabbit polyclonal (Abcam), NC16A3 mouse monoclonal (Medimabs), and D4B5 mouse monoclonal for collagen IV (Abcam). Collagen XVII and collagen IV appear in green and nuclei appear in blue. Blisters are marked with circles. **(C)** Immunoblot analysis with lysates from control normal keratinocytes and from Case 61 with NC16A and antibodies to tubulin, as a loading control. Scale bar for all panels 100 μm.

later as non-Herlitz JEB). This most severe *COL17A1*-associated phenotype was the first to be recognized (12, 17, 22). The main clinical manifestation is represented by mechanically induced skin blistering that starts at birth, is generalized and persists lifelong, without spontaneous improvement. Repeated wound and healing episodes result in skin scarring and atrophy. Nails become dystrophic and are progressively lost, as are scalp and body hairs. Teeth exhibit amelogenesis imperfecta which leads to excessive and premature caries and loss of dentition.

Such stratification of mutations endorses the high frequency of nonsense mutations or frame-shift leading to premature termination codons and the need for therapy of JEB patients. Moreover, the recurrence rates gives perspective to the possible similarities between different cultural backgrounds or to the extent in which external factors may impact this disease.

DISCUSSIONS

Consequences of *COL17A1* Mutations and Potential Therapeutic Strategies

Potential therapeutic strategies emerge from analysis of the mutations, their consequences and from the resulting phenotypes (**Table 2**). Patients with acral or late onset JEB, like our cases 61 and 66, have limited disease burden and can be treated symptomatically. In contrast, severely affected patients, like our case 68 require effective therapeutic strategies for their chronic wounds. There is also no existing treatment for other disease manifestations, such as: (i) hair loss, which is definitive; (ii) nail dystrophy or loss, which are regarded as cosmetic problems; (iii) amelogenesis imperfecta leading to increased caries and premature destruction of teeth, which require careful and sustained dental care and treatment. The analysis of genotype-phenotype correlations in patients the JEB suggests that 12–25% of the normal type XVII collagen levels are sufficient to provide a certain degree of skin stability and consequently ameliorate the phenotype (3).

Taking into account the large number of *COL17A1* PTC-mutations, therapies to restore the mutant gene seem to be appropriate in this EB subtype. In our study 33% of mutations are nonsense mutations. These relatively high figures render the thoroughfare for an efficient therapeutic approach directed against these particular types of mutations. *Ex-vivo* gene therapy, where the defective gene is corrected, transduced in cells and later grafted to the patient is highly complex and expensive. As an alternative approach, RNA trans-splicing was developed and showed expression of the replaced exon 52 in up to 61% assayed cells *in vitro* (26).

Revertant mosaicism is frequent in patients with JEB-*COL17A1* (18), but GMP-cultivation and expansion of revertant epidermal stem cells is not yet broadly available. Because type XVII is solely synthetized by keratinocytes and must be correctly assembled as a transmembrane protein in a supramolecular complex, fibroblast / mesenchymal stem cell therapies and protein therapy are not feasible.

Type XVII collagen has a modular collagenous structure, and *COL17A1* has 54 out of 56 exons in-frame, except of the first and last. In-frame truncations seem to be tolerated, and account for the presence of molecules which are stable and partially functional, such in our case 61 and in other cases reported in the literature (27, 28). Thus, antisense-mediated skipping of exons with PTCs might also be an alternative strategy for *COL17A1* mutations (29).

Nevertheless, drug-based therapeutic strategies that aim to modify either the synthesis of protein by inducing readthrough or the mechanisms that lay the ground for RNA translation might be easier available. Suppression of stop codons and enabling readthrough in translation can be effective for several genetic disorders, including EB (24, 27, 30, 31).

Readthrough therapy modifies the response of the translational ribosome to nonsense mutations, by allowing a near-cognate aminoacyl tRNA to step into the spot on the stop codon and thus proceeding toward protein synthesis and allowing a functional protein to be produced. In order to restore

TABLE 2 | Cohort description with position of mutations on *COL17A1* gene and proposed treatment based on the type of mutation.

COL17A1 mutations cDNA	Location	Mutation protein	Proposed treatment	Comment
2T>A	2	?	Gene therapy	
2T>C	2	?	Gene therapy	
53_1224del	3	?	Gene therapy	
418_419delAG	8	S140*	Readthrough	
433C>T	8	R145*	Readthrough	
460C>T	8	R154*	Readthrough	
779del	11	P260Qfs*32	Gene therapy	
1141+5G>A	IVS14	Residual expression of full-length collagen XVII due to leaky splice site	Symptom reliefing therapies	Moderate disease severity
1392G>A	17	W464*	Readthrough	
1480_1482dup	18	K494dup	Symptom reliefing therapies	Moderate disease severity
1601_1602insG	18	S534Efs*10	Exon 18 in frame skipping	
1616G>A	18	G539E	Symptom reliefing therapies	Moderate disease severity
1744-2A>C	IVS20	In frame skipping of exons 21 and 22 or partial skipping of exon 21 leading to residual expression of collagen XVII	Symptom reliefing therapies	Moderate disease severity
1750C>T	21	R584*	Readthrough	
1826G>A	22	G609D	Symptom reliefing therapies	Moderate disease severity
1834G>A	22	G612R	Symptom reliefing therapies	Moderate disease severity
1861G>A	23	G621S	Symptom reliefing therapies	Moderate disease severity
1898G>A	23	G633D	Symptom reliefing therapies	Moderate disease severity
2003-1G>C	IVS24	?	Gene therapy	
2062C>T	26	R688*	Readthrough	
2237delG	30	G746Afs*53	Exon 30 in frame skipping	
2282_2283delGG	31	G761Dfs*40	Exon 31 in frameskipping	
2336-2A>G	IVS31	In frame skipping of exon 32 leading to expression of truncated protein	Symptom reliefing therapies	Moderate disease severity
2383C>T	33	R795*	Readthrough	
2407G>T	34	G803*	Readthrough	
2561_2565delAATTT	37	N854Tfs*109	Exon 37 in frame skipping	
2561_2562delTT	37	N854Ifs*110	Exon 37 in frame skipping	
2564T>G	37	L855*	Readthrough	
2723dup	40	G909Rfs*56	Exon 40 in frame skipping	
2861delG	43	G954Afs*112	Exon 43 in frame skipping	
2993dupC	44	G999Wfs*22	Exon 44 in frame skipping	
3046C>T	45	Q1016*	Readthrough	
3164delT	46	F1055Sfs*11	Exon 46 in frame skipping	
3198C>T	46	Residual expression of full-length collagen XVII due to leaky splice site	Symptom reliefing therapies	Moderate disease severity
3487G>T	49	E1163*	Readthrough	
3505C>T	49	R1169*	Readthrough	
3509-1G>C	IVS49	In frame skipping of part of exon 50 leading to truncated protein expression	Symptom reliefing therapies	Moderate disease severity
3548delC	50	P1183Rfs*68	Exon 50 in frame skipping	
3569dupG	50	N1191Qfs*51	Exon 50 in frame skipping	
3676C>T	51	R1226*	Readthrough	Recurrent mutation
3766+1G>C	IVS51	Out of frame and absence of collagen XVII	Gene therapy	
3766+5G>A	IVS51	Absence of collagen XVII	Gene therapy	
3800dupC	52	G1268Rfs*25	Exon 52 in frame skipping	
3865dupA	52	S1289Kfs*4	Exon 52 in frame skipping	

(Continued)

TABLE 2 | Continued

COL17A1 mutations cDNA	Location	Mutation protein	Proposed treatment	Comment
3908G>A	52	R1303Q	Symptom reliefing therapies	Late onset and moderate disease severity
4153C>T	52	Q1385*	Readthrough	
4100_4101delTT	52	F1367Cfs*8	Exon 52 in frame skipping	
4156+1G>A	IVS52	In frame skipping of exon 52	Symptom reliefing therapies	Moderate disease severity
4305_4308dupCATT	54	Q1437Hfs*19	Symptom reliefing therapies	Moderate disease severity
4319dupC	54	G1441Wfs*14	Symptom reliefing therapies	Moderate disease severity

**stands for a premature termination codon.*

the function of nonsense mutations carrying genes several compounds have been identified in the last decades. The PTCs are normally recognized by a mechanism called nonsense-mediated mRNA decay (NMD), which tries to eliminate them in order to reduce errors in the expression of a particular gene. NMD efficiency can influence the response of readthrough by several factors (32). Particularly, when some key factors in NMD pathway are inhibited, such as ATP dependent RNA helicase upframeshift 1 (UPF1) and upframeshift 2 (UPF2), a better function of the gene implicated in the pathology of cystic fibrosis was noticed (32). On the contrary, in recessive dystrophic epidermolysis bullosa, it has been shown that a better response to readthrough was associated with an increase in the factor UPF1 in all cells treated, results which support that the more stable this UPF1 factor is, the more inhibited NMD pathway is (33). Another factor that can be limiting in the response of readthrough could be the PTC bearing transcripts of patients. These may vary among individuals and could be beneficial to assess before treatment (32).

CONCLUSIONS

We report here nine previously unpublished mutations, which will enhance the already established database with *COL17A1* mutations. The additionally reported genotype-phenotype correlations will help in diagnosis and genetic counseling, thus providing advantage in the clinical setting. The stratification of the mutations and their consequences should also provide insights for mutation analysis strategies and potential therapies.

As far as therapeutic strategies are regarded for *COL17A1* PTC-mutations, readthrough compounds and exon skipping-approaches seem to be reliable and aim at increasing stability of the skin, wound healing and improving the quality of life. Progress still needs to be made for the other cutting-edge therapeutic possibilities.

AUTHOR CONTRIBUTIONS

CH has molecularly characterized the cohort of patients and has written the final version of the manuscript. IC has analyzed the data, performed the molecular analyses in patient 61, the characterization of the cell lines and has drafted the manuscript and figures. YH has analyzed data. RC has contributed with the phenotypes analysis. All authors have read and corrected the manuscript.

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Epidermolysis Bullosa Acquisita: The 2019 Update

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Epidermolysis bullosa acquisita (EBA) is an orphan autoimmune disease. Patients with EBA suffer from chronic inflammation as well as blistering and scarring of the skin and mucous membranes. Current treatment options rely on non-specific immunosuppression, which in many cases, does not lead to a remission of treatment. Hence, novel treatment options are urgently needed for the care of EBA patients. During the past decade, decisive clinical observations, and frequent use of pre-clinical model systems have tremendously increased our understanding of EBA pathogenesis. Herein, we review all of the aspects of EBA, starting with a detailed description of epidemiology, clinical presentation, diagnosis, and current treatment options. Of note, pattern analysis via direct immunofluorescence microscopy of a perilesional skin lesion and novel serological test systems have significantly facilitated diagnosis of the disease. Next, a state-of the art review of the current understanding of EBA pathogenesis, emerging treatments and future perspectives is provided. Based on pre-clinical model systems, cytokines and kinases are among the most promising therapeutic targets, whereas high doses of IgG (IVIG) and the anti-CD20 antibody rituximab are among the most promising "established" EBA therapeutics. We also aim to raise awareness of EBA, as well as initiate basic and clinical research in this field, to further improve the already improved but still unsatisfactory conditions for those diagnosed with this condition.

Keywords: epidermolysis bullosa acquisita, animal models, diagnosis, treatment, pathogenesis

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EPIDEMIOLOGY

The incidence of most autoimmune blistering diseases is increasing. Although the incidence of epidermolysis bullosa acquisita (EBA) is not known in detail, it is estimated to be rare. The most common autoimmune subepidermal blistering disease, bullous pemphigoid (BP), is reported to have an annual estimated incidence between 2.4 and 21.7 per million (1, 2). By contrast, the estimated incidence of EBA is reported to be <0.5 per million (3–7). In South Korea, the incidence and prevalence of EBA is estimated to be higher than that of previous reports (8), but the exact epidemiologic data have not been surveyed. In Germany, the EBA prevalence has recently been determined to be 2.8 cases per million (9). This ethnical difference may be due to the reported association of EBA with the human leukocyte antigen (HLA) class II (10–12). EBA occurs at any age; the onset age in previous case reports exhibit a wide range from 1 to 94 years old (13–15). Two onset age peaks are reported; the second and seventh decades (9).

CLINICAL PRESENTATION

Several clinical EBA manifestations have been described: (i) the classical/mechano-bullous form and (ii) the non-classical/non-mechano-bullous forms (16). The latter includes BP-like EBA that meets the clinical criteria of both EBA and BP, mucous membrane (MM)-EBA that is clinically defined by predominant mucous membrane lesions, IgA-EBA that is defined by the IgA class of immune deposits, and Brunsting-Perry-like EBA (**Figure 1**). Few patients may have a MM-IgA-EBA. The relative frequencies of these different clinical forms of EBA reported in the few series in the literature (17–21) depend on the morphological and/or serological diagnostic means available to the authors (**Table 1**). The two most common presentations of EBA are the classical/mechano-bullous and the BP-like forms.

It should be recognized that in an individual EBA patient, clinical presentation may change over time. Notably, patients may switch from a BP-like form to a classical/mechanobullous form or when mucous membrane lesions appear secondarily from a BP-like form to a MM-EBA (22).

Regardless of the clinical form, patients present with cutaneous-mucous fragility, which is easily suspected when the lesions are on trauma-prone areas. Bullous lesions or erosions that are linear or with angular contours can also provide evidence of this fragility (**Figure 2**). Questioning the patient can confirm that bullous lesions appear immediately or a few hours after a trauma which can be minimal. This fragility can be quantified by applying an analogical visual scale. Because in EBA the subepidermal cleavage is deep on the dermal side of the basement membrane zone (BMZ), the cutaneous blisters can persist for a long time, collapsing and becoming flaccid before their rupture; they can also be haemorrhagic.

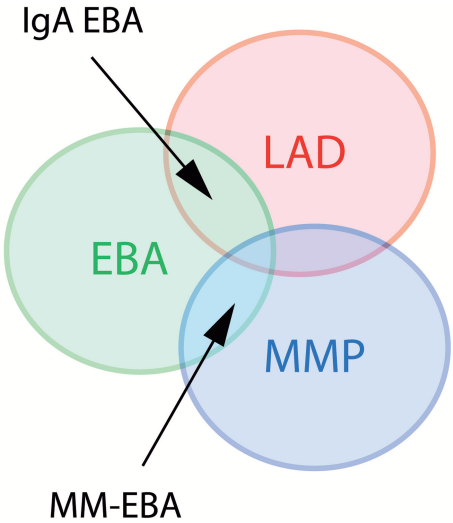

FIGURE 1 | Diagram of different clinical forms of EBA. LAD, linear IgA disease. MMP, mucous membrane pemphigoid; EBA, epidermolysis bullosa acquisita; MM-EBA, mucous membrane EBA.

CLASSICAL/MECHANO-BULLOUS EBA

Two cases of an adult-onset, acquired blistering disease resembling patients with hereditary dystrophic epidermolysis bullosa were reported by Elliott (23). Other similar cases were described in early decades of the twentieth century (24, 25). The first to actually coin the term "epidermolysis bullosa acquisita" was probably Hundley and Smith (26). A landmark paper by Roenigk and colleagues was published in 1971, who described three new cases of EBA, reviewed the world literature and proposed the first diagnostic criteria for classical/mechano-bullous EBA (27).

The criteria of Roenigk were modified once immunological tests for EBA diagnosis had been developed. Advanced clinical criteria of the classical/mechanobullous form have been published in 2017 (16). Current clinical diagnostic criteria for the classical/mechanobullous form are skin fragility, blisters, or erosions on non-inflamed or scarred skin, scarring and milium formation, preferably located at trauma-prone sites and the extensor skin surface (dorsal hands, elbows, knees, Achilles tendon, feet) with possible nail dystrophy and scarring alopecia (**Figure 2**). Fibrosis of the hands and fingers leading to a mitten-like deformity may occur in patients with severe disease reminiscent of hereditary dystrophic epidermolysis bullosa. Mucosal involvement may also occur, but it is not predominant.

BP-LIKE EBA

BP-like form of EBA was first described by Gammon et al. (28). This occurred shortly after the demonstration by Nieboer

Abbreviations: 17-AAG, tanespimycin; 17-DMAG, 17-imethylaminoethylamino-17-demethoxygeldanamycin; ABQOL, autoimmune bullous disease quality of life; AIBD, autoimmune blistering disease; AKT, protein kinase B; APC, antigen-presenting cell; AZA, azathioprine; BLT, leukotriene B4 receptor; BMZ, basement membrane zone; BP, bullous pemphigoid; C, complement factor; CARD9, caspase recruitment domain-containing protein 9; CD, cluster of differentiation; CDNP, cell-derived nanoparticles; COL, collagen; CPA, cyclophosphamide; CR, complete remission; CSA, cyclosporine; CXCR, CXC-chemokin receptor; DDS, diaminodiphenyl sulfone; DEJ, dermal-epidermal junction; DIF, direct immunofluorescence; DMF, dimethylfumarate; EBA, epidermolysis bullosa acquisita; ECP, extracorporeal photochemotherapy; ELISA, enzyme-linked immunosorbent assay; EndoS, endoglycosidase S; ERK, extracellular signal-regulated kinase; FcgR, Fc gamma receptor; FcRn, neonatal Fc receptor; Flii, flightless I; FOAM, fluorescent overlay antigen mapping; G0, agalactosylated antibodies; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human leukocyte antigen; Hsp, heat-shock protein; i.p., intraperitoneal; IA, immunoadsorption; IC, immune complexes; IEM, immunoelectron microscopy; IFN, interferon; Ig, immunoglobulin; IIF, indirect immunofluorescence microscopy; IL, interleukin; IVIG, high-dose intravenous immunoglobulin; JAK2, janus kinase 2; LAD, linear IgA bullous disease; LTB4, leukotriene B4; MHC, major histocompatibility complex; MIP1a, macrophage inflammatory protein1a; MM-EBA, mucous membrane EBA; MMF, mycophenolate mofetil; MMP, mucous membrane pemphigoid; MMPs, matrix metalloproteases; MTX, methotrexate; NADPH, nicotinamide adenine dinucleotide phosphate; NC, non-collagenous; NCF1, neutrophil cytosolic factor 1; NKT, natural killer T cells; p.o., per os; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PR, partial remission; RORa, retinoid-related orphan receptor-alpha; ROS, reactive oxygen species; RTX, rituximab; SLE, systemic lupus erythematodes; SSS, NaCl-split skin; SYK, Spleen tyrosine kinase; TABQOL, treatment-based autoimmune bullous disease quality of life; Th, T-helper; TNF-α, tumor necrosis factor α; Treg, regulatory T cells; Trem1, triggering receptor expressed on myeloid cells-1.

TABLE 1 | Clinical variants of EBA in the series of the literature.

References	Years of study	*n*	Classica/ mechano-bullous	Brunsting-Perry like	Bullous pemphigoid like	Mucous membrane EBA	IgA EBA
Briggaman (17)	<1985	12	4 (30%)	0	5 (40%)	1 (8%)	2 (17%)
Kim (18)	1994–2009	30	11 (36.7%)	2 (6.7%)	14 (46.7%)	2 (6.7%)	1 (3.3%)
Buijsrogge (19)	2002–2008	38	13 (34%)	1 (2.6%)	13 (34%)	2 (5.2%)	9 (24%)
Iranzo (20)	1985–2012	12	5 (42%)	1 (8.3%)		4[a] (33%)	
Seta (21)	1983–2013	77	42 (56%)	1 (1.2%)	21[b] (27%)	11[c] (14%)	2 (2.4%)

[a]*2 patients had the mixed form,* [b]*1 had a prurigo-like form,* [c]*11 had mucous membrane-EBA, including 2 with isolated IgA deposits.*

et al. (29) and Yaoita et al. (30) that EBA autoantibodies are deposited in the anchor fibrils zone, which allowed for a definite diagnosis by immunoelectron microscopy (IEM), even though the clinic was (based on the criteria by Roenigk) atypical. Indeed, patients with a BP-like form of EBA have generally profuse skin lesions suggestive of a BP in some areas and an EBA in others (17, 31, 32). The patients have pruritus, tense bullae and erosions on inflamed erythematous or urticarial skin as well as trauma-induced bullous lesions surrounded by normal skin (**Figure 2**). The lesions are on the trunk and folds but also on limb extensor areas and distal extremities. The face can be affected. Mucosal involvement is also possible, but it is not predominant. Finally, lesions heal most often leaving atrophic scars and milia cysts as in the mechanobullous form.

MM-EBA

The high frequency of mucosal lesions in EBA, in particular tongue and lip involvement, was highlighted by Dahl (33). Currently, MM-EBA cases are defined as EBA that mainly affects mucous membranes with a squamous epithelium (16, 33–35), such as the mucous membrane of the mouth, pharynx, esophagus, epiglottis, conjunctiva, genitalia, anus, and respiratory tract in malpighian metaplasia, especially the trachea and bronchi. Only one of these sites can be involved and remain so for a long time before a second localization appears in the case of inadvertent discontinuation of the treatment, a decrease in dose or no treatment (36). These cases are frequently misdiagnosed.

As on the skin, bullous lesions of mucous membranes rupture late in MM-EBA. Thus, intact blisters are frequently seen on mucous membranes in comparison to mucous membrane pemphigoid (MMP), in which they are rare (**Figure 2**). The erosions on the mucous membranes are similar in MM-EBA and classical MMPs, except in the esophagus where they can be linear, caused by mucous membrane fragility and the passage of the fibroscope (37). The cicatricial lesions (atrophic scars, synechiae, and stenosis) are identical in MM-EBA and MMP. The cicatricial lesions have mild consequences in the mouth, genitals and anus, but cause severe impairment in the esophagus, larynx, trachea, bronchi, and conjunctiva, which dictate more aggressive treatment and multidisciplinary management.

Esophageal stenosis, usually as a web located at the upper esophagus, causes the most severe damage to the esophagus. Esophageal stenosis causes dysphagia, weight loss and, at worst, malnutrition and/or false routes and pulmonary infection (34, 36–50).

Severe lesions at the nose and throat are perforation of the nasal septum and/or stenosis of nostrils, choanal, pharynx, and larynx (39, 51–53). Involvement of the trachea and bronchi may also rarely occur (51, 54). Scarring of the larynx or trachea are potentially life-threatening because this may lead to asphyxiation if tracheostomy is not performed. In general, mucous membrane lesions in EBA patients are, however, asymptomatic in 30% of cases (51).

Few case reports and small series of ocular involvement in EBA have been reported (13, 33, 34, 55–64). Patients presented the involvement of at least two other sites. Interestingly, in MM-EBA, immune deposits of IgA are present in half of the cases and the only Ig class in a third. Patients displayed a fibrosing conjunctivitis that might worsen and eventually cause blindness.

The frequency of esophageal, nasal and throat, and conjunctival involvement was, respectively, 6, 11, and 25% of 39 EBA cases in a French series (65). Bladder involvement has also been reported in one case (66).

In addition to MM-EBA, the involvement of mucous membranes in "skin-predominant" EBA is common (34). Hence, after the diagnosis of EBA, an interdisciplinary approach is needed for both diagnosis and treatment.

IGA-EBA

Currently, IgA-EBA is defined as an EBA that presents with linear IgA deposits at the BMZ. IgA-EBA may resemble linear IgA bullous disease with erythematous cutaneous arciform lesions and a few scars and milium cysts, in particular in children. However, it IgA-EBA may also develop into a more severe clinical manifestation, especially with scarring at mucosal

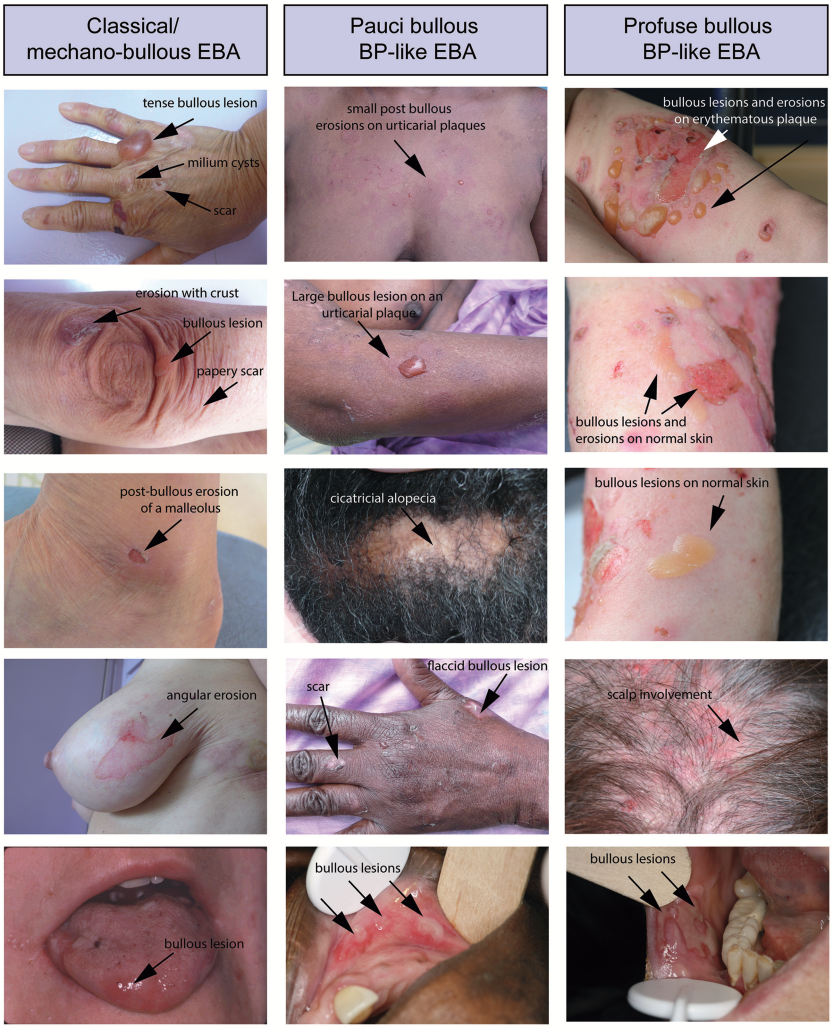

FIGURE 2 | Different clinical forms of EBA. All patients were documented at the first visit in a center for auto-immune bullous disease. **(Left)** Patient with a classical/mechanobullous form of EBA: lesions are preferably localized to the extensor skin surfaces and trauma-prone sites, i.e., dorsal hands knees elbows and ankles. Tense or flaccid bullous lesions are surrounded by non-inflamed skin; erosions are covered or not by crusts; one erosion with angular contours had been induced by adhesive plaster; old lesions have healed with milium formation and/or are atrophic papery scar. **(Middle)** Patient with a BP-like form of EBA, with little blistering: urticarial plaques with small or large bullous lesions as in BP, but location of lesions on extensor areas of limbs, hands and scalp, and scars and extensor areas of the face (not shown) and limbs (atypical for BP). **(Right)** Patient with a BP-like form of EBA, with extensive blistering: bullous lesions and erosions on erythematous skin in flexural areas of limbs (tight and arm) as in BP but also bullous lesions and erosions on normal skin and involvement of extensor area of the limbs and scalp, atypical for a BP involvement of the scalp (not shown) and both flexural and extensor areas of limbs extremities with bullous lesions and erosions on erythematous but also normal skin. The tongue and the lips are the most frequent sites of mucosal lesions in all EBA variants. Other mucosal lesions (not shown) are possible regardless of the variant of EBA The involvement of nasal and buccal mucous membrane are visible in all EBA variants.

sites, as reported in 30% of the 82 cases in the Vodegel literature review, including 4% with severe ocular involvement (16, 19, 67, 68).

BRUNSTING-PERRY TYPE EBA

Patients with Brunsting-Perry type EBA present only cutaneous lesions, without erythematous or urticarial plaques, which predominate in the head and neck and heal leaving very atrophic scars. Review of seven of the eight cases reported in the literature has recently been published by Asfour et al. (69–76).

NON-INFLAMMATORY VS. INFLAMMATORY FORMS OF EBA

The definition of inflammatory and non-inflammatory forms of EBA varies with the authors in the literature (17–21): (i) for most of them but one, the non-inflammatory form of EBA overlays the classical/non-mechano-bullous form; (ii) Buijsrogge et al. included the Brunsting-Perry like type in the mechano-bullous phenotype (19); (iii) for Briggaman et al. (17) who were the first to describe the inflammatory form of EBA, and for Kim et al. (18), the inflammatory form of EBA is synonymous to the BP-like

form; (iv) for Buijsrogge et al. (19); and Iranzo et al. (20), all the patients who have not a mechano-bullous phenotype form, have an inflammatory phenotype and (v), for Seta et al. (21), inflammatory lesions are characteristic of the BP-like form of EBA but can also be seen in some patients with MM-EBA or IgA EBA.

The authors of the consensus conference (16) agreed on the following: (i) BP-like EBA are usually inflammatory forms of EBA, (ii) MM-EBA may be inflammatory forms of EBA, (iii) IgA-EBA may be inflammatory forms of EBA, (iv) Brunsting-Perry-like EBA are usually non-inflammatory forms of EBA, and (v) Brunsting-Perry-like EBA are not classical/mechano-bullous forms of EBA.

QUALITY OF LIFE IN EBA AND ASSOCIATED DISORDERS

In general, EBA has a significant impact on the quality of life, which is now to be measured by generalized scores and the "autoimmune bullous disease quality of life" (ABQOL) and "treatment-based autoimmune bullous disease quality of life" (TABQOL) scores created specifically for autoimmune bullous diseases (77, 78).

Many systemic diseases have been reported to be associated with EBA, such as amyloidosis, thyroiditis, multiple endocrinopathy syndrome, rheumatoid arthritis, pulmonary fibrosis, chronic lymphocytic leukemia, thymoma, and diabetes [review in Gupta et al. (22)]. Most of these reports are, however, anecdotal. The only unarguable association of EBA with other diseases is with chronic inflammatory bowel diseases, in particular Crohn's disease, which has been reported to be present in 25% of EBA patients (65, 79). In B-cell lymphomas, presence of circulating and tissue-bound auto-antibodies to type VII collagen (COL7) has also been described in association with a frequency of 6% in 100 EBA cases, but the patients did not have clinical features suggestive of EBA (80). Furthermore, EBA associated with systemic lupus erythematosus, but not fulfilling the criteria of bullous erythematosus systemic lupus, are described (81, 82).

DIAGNOSIS

If clinically suspected, the minimal diagnostic criteria for EBA diagnosis are the detection of linear immunoglobulin- or C3-deposits along the dermal-epidermal junction in a perilesional skin biopsy with detection of a u-serrated pattern of Ig-binding.

Routine histopathology from a lesional skin (or mucous membrane) biopsy does not allow to distinguish EBA from other subepidermal AIBD. It shows: (i) initially, papillary oedema and vacuolar alteration along the dermo-epidermal junction and at a later stage, a subepidermal or subepithelial cleavage, (ii) a great variability in the magnitude and/or quality of the inflammatory infiltrate, (iii) milia cysts and fibrosis in older lesions (**Figure 3**).

Definite diagnosis can be performed by either of the following methods: (i) serration pattern analysis of linear immunoglobulin deposits in the perilesional skin biopsy, (ii) fluorescent overlay antigen mapping (FOAM), (iii) immunoelectron microscopy, and/or (iv) detection of circulating antibodies against COL7.

If possible and needed, more than one of the above may be used to diagnose or exclude EBA. In addition, serology, i.e., detection of circulating anti-COL7 antibodies, should be performed, and, if positive, can serve as a biomarker of disease severity (83).

Diagnosis of EBA can be made by indirect immunofluorescence microscopy (IIF) using 1 M NaCl-split skin (SSS) as a substrate (84). Here, binding of antibodies to the dermal site (floor) of the blister is observed. By immunoblot analysis, binding to the 290-kDa antigen by the patient IgG is detected. The newly developed COL7 ELISA has a sensitivity of 45%. Combining SSS and ELISA reaches a sensitivity of 50%. Thus, half of the patients with EBA are sero-negative (85), and thus a negative serological finding does not exclude EBA as a differential diagnosis.

In serological negative cases, direct immunofluorescence (DIF) on sodium chloride-separated skin biopsy might reveal the diagnosis. Specifically, the diagnosis can be made using DIF serration pattern analysis, which shows distinct, EBA-specific, linear u-serrated immune-depositions at the BMZ (86). DIF serration pattern analysis by n-vs.-u may consider require expertise, which can be studied online: "n-vs.-u UMCG" (https://www.umcg.nl/NL/UMCG/Afdelingen/dermatologie/Wetenschappelijk_Onderzoek/NversusU/Paginas/default.aspx.

Direct Immunodetection of Perilesional Skin Biopsies

All pemphigoid diseases are characterized by a linear deposition of immunoglobulins and/or complement along the epidermal basement membrane zone (**Figure 4A**). These antibodies are directed against various hemidesmosomal proteins: (i) type XVII collagen (BP180) in BP, MMP, pemphigoid gestationis, lichen planus pemphigoides, and LAD, (ii) BP230 in BP, (iii) laminin-332 in anti-laminin-332 pemphigoid, (iv) integrin β4 in ocular MMP, and (v) p200 in anti-p200 pemphigoid. Moreover, in EBA and bullous systemic lupus erythematosus (SLE), antibodies against COL7, present in the sublamina densa, also give rise to a linear deposition pattern (87).

If in suspected pemphigoid disease, a linear Ig- and/or C3- deposition is observed, it is important to determine the targeted autoantigen. In most variants of BP and in EBA, the deposits consist of IgG and complement. Mixed IgG/IgA depositions are usually encountered, especially in mucosal dominant pemphigoid. In some patients, IgA is the only present Ig-subtype, leading to a diagnosis of LAD or IgA EBA (67, 88, 89). However, in mucosal dominant pemphigoid with mixed IgA/IgG depositions, the IgG component may be faint, which sometimes result in a misdiagnosis of LAD. In very few patients, linear IgM deposition may be present in addition to IgG and C3. Even less cases have been described with linear IgM deposition only (90, 91).

Bullous SLE is characterized by antibodies against COL7 in patients fulfilling the diagnostic criteria for SLE. In bullous SLE, in addition to, or superimposed on a linear IgG deposition,

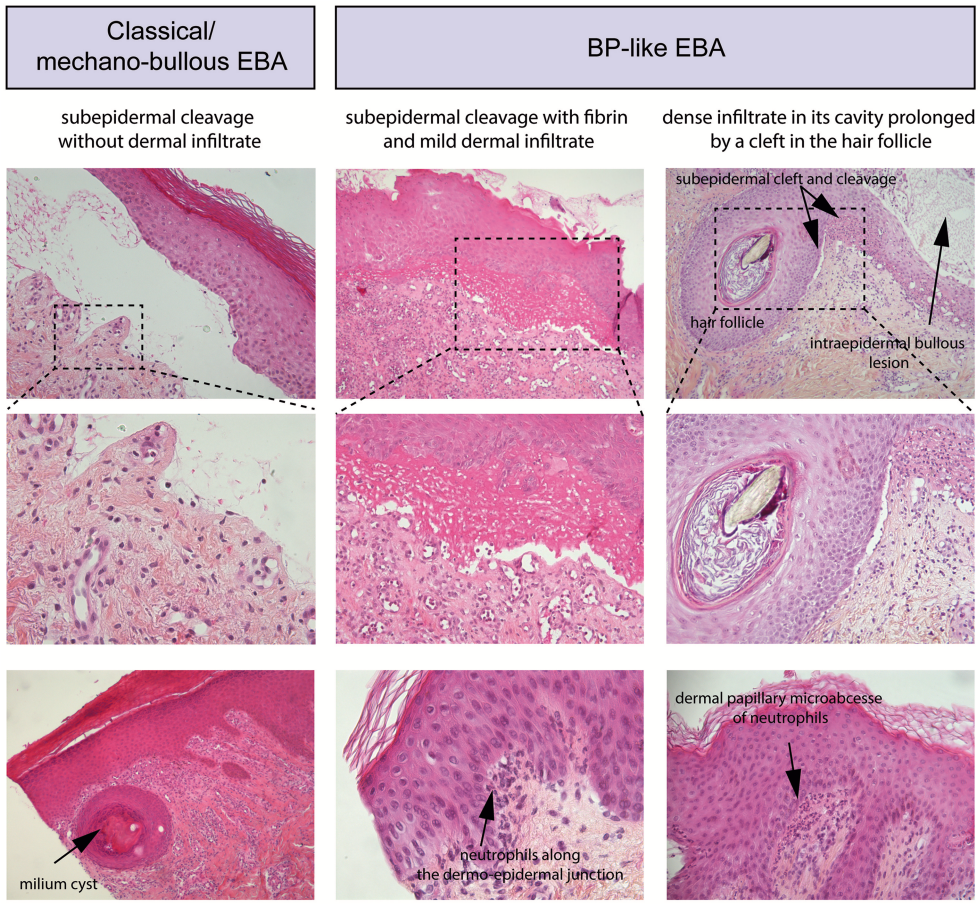

FIGURE 3 | Standard histology. **(Left)** Histological study of lesional skin biopsies in a patient with a classical/mechanobullous form of EBA, subepidermal cleavage without dermal infiltrate; bottom, milium cyst in the dermis. **(Middle)** Patient with a BP-like form of EBA, subepidermal cleavage with fibrin in the blister cavity and mild dermal infiltrate. Bottom, Neutrophils along the dermo-epidermal junction **(Right)** Patient with a BP-like form of EBA, one intraepidermal bullous lesion and one subepidermal bullous lesion with dense infiltrate in its cavity prolonged by a cleft in the hair follicle; bottom, a dermal papillary microabcesse of neutrophils.

a biopsy might show a lupus band, which is characterized by granular deposition of immunoglobulins and complement, in coincidence with epidermal anti-nuclear antibodies.

In most pemphigoid patients, a linear-serrated pattern can be discerned in direct IF microscopy of a peri-lesional skin biopsy. This serration pattern can be divided into an n-serrated and a u-serrated pattern (**Figure 4A**). The identification of these particular patterns allows to differentiate between (i) sublamina densa binding diseases caused by autoantibodies against COL7, e.g., EBA and bullous SLE and (ii) blistering diseases with binding above the lamina densa with antibodies against hemidesmosomal components, e.g., BP, pemphigoid gestationis, MMP, anti-p200 pemphigoid, and anti-laminin 332 pemphigoid. The u- and n-serrated patterns form based on the molecular architecture of the dermal-epidermal junction (DEJ): Specifically, if autoantibodies against COL7 are present, the immunodeposits are located between the rootlets of the basal keratinocytes, leading to the EBA-associated u-serrated pattern (**Figure 4A**). If, however, immune-depositions are located above the lamina densa, they trail the plasma membrane in the basal cell rootlets, resulting in the n-serrated pattern (**Figure 4A**). If it is not possible to determine the serration pattern, which occurs in few cases, it is recommended wise to cut thinner sections or obtain an additional peri-lesional skin biopsy.

However, if diagnosis of EBA cannot be established based on the serration pattern, the level of antibody deposition can be determined by fluorescent overlay antigen mapping (FOAM), which is based on the visualization of a targeted antigen relative to a known topographic marker. For instance, in **Figure 4B**, red staining is used for BP180 as a topographic reference, whereas green staining corresponds to IgA deposits. In the case of IgA-EBA, separate patterns of IgA deposits (green) and BP180 (red) can be seen with red staining on the dermal side (89). FOAM can be performed using a standard immunofluorescence microscope. However, confocal microscopy usually yields better results.

Direct immunoelectron microscopy provides a more detailed location of the deposition site, i.e., the lamina densa and/or sublamina densa at the DEJ (29). This location is distinct from that in other AIBDs (29, 30, 92).

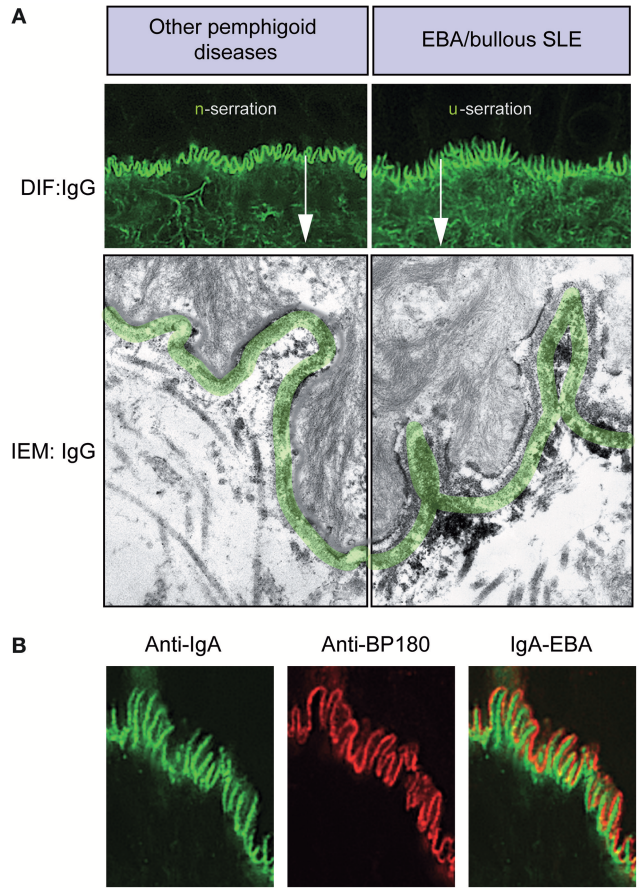

FIGURE 4 | Diagnosis of EBA by direct immunofluorescence. **(A)** All pemphigoid variants are characterized by linear deposition of immunoglobulins and/or complement along the epidermal basement membrane zone. Serration pattern can be separated in an n-serrated pattern (blistering diseases with binding above the lamina densa with antibodies against hemidesmosomal components, e.g., BP, pemphigoid gestationis, mucous membrane pemphigoid, anti-p200 pemphigoid, and anti-laminin 332 pemphigoid, exept MM-EBA) and a u-serrated pattern (sublamina densa binding diseases caused by autoantibodies against COL7, e.g., EBA and bullous SLE). **(B)** In some cases, it is not possible to determine the serration pattern, especially in mucosal biopsies. In these cases, the level of the deposition of antibodies can be determined by fluorescent overlay antigen mapping (FOAM). FOAM is a technique that is based on the possibility of visualizing a targeted antigen relative to a topographic marker. For instance, using red staining, it is used for BP180 as a topographic reference marker, and green staining is used for IgA deposits. In the case of IgA-EBA, separate patterns of IgA deposits (green) and BP180 (red) can be observed with red staining on the dermal side.

Indirect Immunofluorescence on Salt-Split Skin

Diagnosis of EBA can be made by indirect immunofluorescence (IIF) using 1 M NaCl-split skin (SSS) as a substrate. Here, antibody binding of to the dermal site (floor) of the blister is observed. By immunoblot analysis binding to the 290-kDa antigen of patient immunoglobulin is detected (84). Using SSS as a substrate, IIF shows IgG binding on the dermal side of the split. IgG binding can therefore be easily distinguished from BP and, in some cases, MMP, in which the immunoglobulins bind on the epidermal side of the split.

COL7-SPECIFIC SEROLOGICAL ANALYSES BY ELISA

For the serological diagnosis of EBA, three different assays are available: (i) an enzyme-linked immunosorbent assay (ELISA) that uses the non-collagenous (NC)1 and NC2 domains of COL7 (15, 93–95), (ii) an ELISA that is based on the NC1 domain alone (83), and (iii) an indirect IF test employing the NC1 domain (83). If they can be detected, serum levels anti-COL7 IgG correlate with disease activity (96). However, no correlation has been detected between the antibody specificity and the clinical phenotype (97).

THERAPY

First, the treatment of EBA remains challenging because no randomized control trials have been documented due to its rare prevalence (98, 99). Similar to other AIBDs, systemic corticosteroids are widely accepted as a first choice in the treatment of EBA. Initial doses range from 0.5 to 2.0 mg/kg/day (87). With systemic corticosteroids, steroid-sparing agents, including colchicine, diaminodiphenyl sulfone (DDS, dapsone), methotrexate (MTX), azathioprine (AZA), cyclosporine (CSA), mycophenolate mofetil (MMF), and cyclophosphamide (CPA), have been reported in treatment of EBA. Other therapeutic options, including high-dose intravenous Immunoglobulin (IVIG), rituximab (RTX), plasmapheresis and immunoadsorption (IA), and extracorporeal photochemotherapy (ECP) also have been reported (98, 100).

A retrospective analysis included 30 cases from the EBA cohort study regarding treatment and outcome (18). In this cohort study, the median time to remission was 9 months, and complete remission (CR) was observed in 33, 33, and 45% at 1 year, 3 years, and 6 years follow-up, respectively. Relapse sometimes occurs while receiving therapy, while a rate of relapse has not been reported in cohort studies.

Very recently, we collected information that included the treatment of EBA cases who met current diagnostic criteria published between 1971 and 2016 (101). Among all the reported treatments, we found IVIG and RTX to be associated with CR. Based on this retrospective analysis and previous reports in other autoimmune bullous diseases as a reference, each treatment is shortly summarized below.

COLCHICINE

In EBA, colchicine is usually used at 1–2 mg/day. Of note, colchicine monotherapy (2 mg/day) has been reported (20, 102). By some experts, colchicine is considered a first line EBA treatment, especially for mild cases due to the relatively minor side effects compared with other therapeutic choices (103–105). There is currently no controlled study reporting the efficacy of this treatment, and no experimental data are available.

Hence, colchicine is a triable treatment for mild cases, and it should be considered if other immunosuppressant treatments are ineffective.

DIAMINODIPHENYL SULFONE (DDS, KNOWN AS DAPSONE)

Based on expert opinion, DDS is considered a safe and relatively effective first line treatment (18). Although DDS monotherapy (100 mg/day) has been reported with CR in single IgA-EBA cases (67), 25–150 mg/day of DDS is usually used as an adjuvant therapy with systemic corticosteroids. Adverse effects, haemolysis, methemoglobinemia, agranulocytosis, peripheral neuropathy, and psychosis might be observed (106). A rare adverse effect called "dapsone syndrome" should be considered. Some experts recommend that this therapy can be effective (105, 107). Similar to BP (108), DDS appears to have a corticosteroid-sparing effect in EBA.

METHOTREXATE (MTX)

Usually, MTX is used in combination with systemic corticosteroid with/without other immunosuppressants at 20–25 mg weekly. A retrospective review for MTX in the treatment of pemphigus and pemphigoid has reported the use of 5–50 mg weekly of MTX. Adverse effects, including nausea, anemia, and infection sometimes led to the discontinuation of treatment (109). In EBA, no case-series study has focused on the efficacy of MTX. Based on the efficacy in BP (109), MTX may be a viable treatment for EBA as a corticosteroid-sparing agent.

AZATHIOPRINE (AZA)

In EBA, AZA was the most frequently used immunosuppressant as an adjuvant therapy (101). Two types of adverse effects have been reported; non-dose-related adverse effects, including pancreatitis, fever, rash, malaise, nausea, diarrhea, and hepatitis, and dose-related adverse effects, including leucopoenia and some forms of hepatitis (110). Like MTX, no case-series study has focused on the efficacy of AZA in EBA. Based on the efficacy in BP (108), AZA may not be a beneficial treatment for EBA, and further analysis is needed.

CYCLOSPORINE (CSA)

CSA is mainly used as an adjuvant therapy (101). Renal dysfunction, hypertension, headache, tremor, paraesthesia, hypertrichosis, and hyperlipidaemia may be observed as adverse effects, and most persistent renal dysfunction is related to prolonged therapy or doses >5 mg/kg/day (111).

MYCOPHENOLATE MOFETIL (MMF)

In our recent, retrospective analysis (101), 1–3 g/day of MMF was used in combination with systemic corticosteroids. A randomized clinical trial of methylprednisolone plus-AZA vs. -MMF therapy in pemphigus showed a slightly lower frequency of adverse effects, including hypertension, hyperglycaemia, and infection in the MMF-treated group, although it was not a significant difference (112). The results from the randomized control study in pemphigus suggested that 2 g/day offered a better risk–benefit profile than 3 g/day (113). In a case-series study, EBA cases were successfully treated with MMF as a steroid-sparing agent (114). Hence, MMF could be a steroid-sparing agent in EBA.

CYCLOPHOSPHAMIDE (CPA)

There has been no case-series study focused on the efficacy of CPA in EBA, and relativity fewer reports have examined treatment with CPA compared with other immunosuppressants. Therefore, CPA appears to be a therapeutic option when other immunosuppressants cannot control the disease, but limited data is available (101).

HIGH-DOSE INTRAVENOUS IMMUNOGLOBULIN (IVIG)

In our recent, retrospective analysis (101), 31 EBA cases were treated with IVIG, among which 24 cases providing information on the regimen of IVIG and outcome are summarized in **Table 2**. Ahmed et al. reported 10 cases treated with 2 g/kg/cycle (divided into 3 consecutive days) of IVIG in EBA with severe disease and non-responsive to conventional therapies (118). After 16–22 cycles of IVIG therapy, clinical remission was observed from 29 to 123 (mean 53.9) months without any other therapies. The main adverse effect was headache, which increased the intervals of each infusion in two cases, although no severe adverse effects were observed. These two cases are also well summarized in previous reports of IVIG treatment in EBA. In most cases, 2 g/kg/cycle for 3 days or 400 mg/kg/day for 5 sequential days were used with clinical improvement. In experimental mouse studies of EBA and BP, beneficial effects of IVIG have been demonstrated (128–131). Recently, a randomized double-blind trial of IVIG was reported in BP showing a lower disease activity score in the IVIG-treated group compared with placebo (132). Therefore, IVIG is an effective treatment in severe or intractable cases of EBA.

RITUXIMAB (RTX)

Rituximab, a humanized anti-CD20 monoclonal antibody, was used in 10 cases with a protocol of 375 mg/m^2 weekly for 4 weeks (in most cases) and was associated with CR in our recent, retrospective analysis (101), which is summarized in **Table 3**. In pemphigus, another regimen, 1,000 mg every 2 weeks on day 0 and day 14 twice and 500 mg at 12 and 18 months, showed good efficacy in an open-label randomized trial (140). A recent study reported 4 EBA patients treated with 1,000 mg of RTX every 2 weeks twice, similar to the regimen in pemphigus with PR and CR outcomes in each case and no response in 2

TABLE 2 | Reports of IVIG treatment in EBA.

Reported year	Reference	Age	Sex	Phenotype of EBA	Treatments prior to IVIG	Regimen of IVIG	Concomitant-started treatment	Outcome
2013	(115)	37	f	BP-like	Corticosteroid, colchicine, DDS	2 g/kg/cycle		No response
2013	(116)	20	f	BP-like	Corticosteroid, DDS	500 mg/kg/day, 4 days	colchicine (1 mg/day)	CR
2013	(117)	2	m	BP-like	Corticosteroid	400 mg/kg/day, 4 days	DDS (1 mg/kg/day)	PR
2011	(118)	55	f	BP-like	Corticosteroid, AZA	2 g/kg/cycle divided in 3 days		CR
2011	(118)	61	m	Mechanobullous	Corticosteroid, DDS	2 g/kg/cycle divided in 3 days		CR
2011	(118)	37	m	Mechanobullous	Corticosteroid, DDS	2 g/kg/cycle divided in 3 days		CR
2011	(118)	55	f	Mechanobullous	Corticosteroid, DDS	2 g/kg/cycle divided in 3 days		CR
2011	(118)	47	f	Mechanobullous	Corticosteroid	2 g/kg/cycle divided in 3 days		CR
2011	(118)	50	f	Mechanobullous	DDS, MMF, MTX	2 g/kg/cycle divided in 3 days		CR
2011	(118)	73	m	Mechanobullous	Corticosteroid, DDS	2 g/kg/cycle divided in 3 days		CR
2011	(118)	75	f	Mechanobullous	Corticosteroid, DDS	2 g/kg/cycle divided in 3 days		CR
2011	(118)	59	m	BP-like	Colchicine, CSA	2 g/kg/cycle divided in 3 days		CR
2011	(118)	62	f	BP-like	Colchicine, MTX, MFM	2 g/kg/cycle divided in 3 days		CR
2007	(119)	70	m	Unknown	Corticosteroid, AZA, DDS, CSA	2 g/kg/cycles		PR
2007	(120)	65	m	Mechanobullous (+p200 pemphigoid)	Corticosteroid, DDS, CSA, MMF	400 mg/kg/day, 5 days		PR
2007	(121)	58	f	Unknown	Corticosteroid, AZA, MMF, CSA	2 g/kg/cycles		no response
2006	(63)	22	m	Unknown	Corticosteroid, DDS	2 g/kg/cycle divided in 3 days		PR (4 cycles later)
2006	(122)	54	f	Mechanobullous	Corticosteroid, AZA, colchicine,	2 g/kg/cycle divided in 5 days		PR (4 cycles later)
2002	(123)	43	f	Both	None	400 mg/kg/day, 5 days		CR (PR after 1 cycle)
2000	(124)	37	m	BP-like	Corticosteroid	1.2 g/kg/cycle divided in 2–3 days		CR (after 9 months)
1998	(46)	59	m	Mechanobullous	Corticosteroid, DDS, MTX, CSA, CPA, IA	400 mg/kg/day, 5 days		CR
1997	(125)	29	m	BP-like	Corticosteroid, AZA, DDS, PE, colchicine, CSA	40 mg/kg/day, 5 days		CR (after 4 cycles)
1995	(126)	55	m	BP-like	Corticosteroid, AZA, DDS, colchicine	400 mg/kg/day, 5 days		CR (after 9 cycles)
1993	(127)	16	m	Both	Corticosteroid, CSA	400 mg/kg/day, 4 days every 2 weeks		CR

BP, bullous pemphigoid; DDS, diaminodiphenyl sulfone; MTX, methotrexate; AZA, azathioprine; CSA, cyclosporine; MMF, mycophenolate mofetil; CPA, cyclophosphamide; IVIG, high-dose intravenous immunoglobulin; PE, plasma exchange; CR, complete remission; PR, partial remission.

cases (141). In EBA animal models, depletion of B cells at the induction of experimental disease showed that B cells, in addition to developing into plasma cells, serve as important antigen-presenting cells. Specifically, if anti-CD20 treatment was applied at the time of immunization, development of antigen-specific CD4+ T cells was significantly hampered in immunization-induced EBA (142). Hence, RTX seems to be a promising treatment option for EBA. Further controlled clinical studies are

TABLE 3 | Reports of RTX in EBA.

Reported year	Reference	Age	Sex	Phenotype of EBA	Treatments prior to RTX	Regimen of IRTX	Concomitant-started treatment	Outcome
2013	(133)	71	f	Unknown	corticosteroid, DDS	375 mg/m^2, every week, 4 w, 4 cycles	IA	CR (after 18 weeks)
2012	(134)	68	f	Mechanobullous	Corticosteroid, colchicine, MTX, DDS, AZA	375 mg/m^2, every week, 4 w		CR (after 16 weeks)
2010	(43)	50	m	Mechanobullous	Corticosteroid, MMF, colchicine, IVIG, AZA	375 mg/m^2, every week, 4 w		CR (over 4 months)
2010	(135)	71	f	BP-like	Corticosteroid, DDS, colchicine	375 mg/m^2 every week, 4 w, 1 cycle	IA	CR (within 16 weeks)
2009	(136)	54	f	Mechanobullous	Corticosteroid, dapsone, aza, CSA, CPA, IVIG, MMF	375 mg/m^2, every week, 4 w, 3 cycles		PR (CR for skin involvements)
2007	(137)	75	f	BP-like	Corticosteroid, AZA, MMF	375 mg/m^2, every week, 4 w		PR (for 10 months)
2007	(138)	67	m	Mechanobullous	Corticosteroid, AZA, CSA, DDS, MTX, CPA, ECP	375 mg/m^2, every week, 4 w	IA	PR
2007	(138)	42	m	Mechanobullous	Corticosteroid, AZA, CSA, DDS, MTX, CPA, IVIG	375 mg/m^2, every week, 4 w	IA	PR
2007	(121)	58	f	Mechanobullous	Corticosteroid, AZA, MMF, CSA, IVIG	375 mg/m^2, every week, 4 w		PR (after 1 week)
2006	(139)	46	m	BP-like	Corticosteroid, DDS, AZA, IA, colchicine	375 mg/m^2, every week, 4 w		CR (after 11 weeks)

DDS, diaminodiphenyl sulfone; MTX, methotrexate; AZA, azathioprine; CSA, cyclosporine; MMF, mycophenolate mofetil; CPA, cyclophosphamide; IVIG, high-dose intravenous immunoglobulin; RTX, rituximab; PE, plasma exchange; IA, immunoadsorption; ECP, extracorporeal photochemotherapy, CR, complete remission; PR, partial remission.

required to determine which regimen of RTX is most effective in EBA.

PLASMAPHERESIS AND IMMUNOADSORPTION (IA)

Although plasmapheresis has been used for the treatment of pemphigus and pemphigoid, including EBA, it makes the shift to IA because of its advantages compared with plasmapheresis: (i) selective removal of immunoglobulin from the circulation; (ii) no requirement for the substitution of plasma components, such as human albumin or fresh frozen plasma; (iii) two to three times more processing capacity per treatment session than plasmapheresis; and (iv) fewer side-effects, such as infections and allergic reactions (90). Interestingly, there are several reports of combination therapy with IA and RTX in EBA that might provide an effective treatment protocol in EBA (133, 135, 138). Kolesnik et al. (133) and Kubisch et al. (135) reported that each patient was treated with IA for 3 consecutive days followed by IA every week and 375 mg/m^2 of RTX on the day after IA for 4 weeks, leading CR after 18 months and CR within 16 weeks, respectively. Niedermeier et al. (138) reported 2 intractable cases treated with 2 cycles of IA for 4 consecutive days at 4-week intervals followed by RTX (375 mg/m^2, every week for 4 w), leading to PR. Interestingly, antigen-specific immunoadsorption, i.e., where only autoantibodies specific for the respective autoantigen are removed from the circulation, are in pre-clinical development (143, 144).

EXTRACORPOREAL PHOTOCHEMOTHERAPY (ECP)

ECP has been reported in the treatment of Sezary syndrome, mycosis fungoides, and autoimmune bullous diseases (145). There are several reports of the use of ECP in refractory EBA with outcomes of CR and PR in 3 cases, respectively, and no response in 1 case (61, 100, 146, 147). The mode of action of ECP in the treatment of EBA is still unknown, although one report has shown a decrease in circulating antibody detected by immunofluorescence and an increase in suction blister time (100). Despite the low number of published EBA patients, due to the reported success rates, ECP should be considered a therapeutic option in patients with refractory EBA.

OTHERS

Daclizumab, a humanized monoclonal antibody against the a-subunit of the high-affinity interleukin-2 receptor also known as the Tac antigen or CD25, was reported in the treatment of EBA (148), in which only one of 3 cases showed clinical improvement. Sulfasalazine was used in a patient with EBA associated with Crohn's disease, resulting in no improvement of the skin lesion (149). The usefulness of doxycycline has been reported in BP (150). Doxycycline and another tetracycline, minocycline, were found in the literature on EBA cases, although its usefulness remains unclear in EBA treatment (151–153).

PROGNOSIS

EBA is a chronic disease characterized by exacerbations and remissions over the course of months to years. Although data on the prognostic factors in EBA are lacking in the literature, the experts admit that the prognosis of EBA depends on its severity at the time of diagnosis and propose treatment accordingly.

Analogous to BP (154, 155) and MMP (35), an EBA is considered severe if the patient has 10 or more cutaneous bullous lesions and/or 3 or more instances of mucosal sites and/or conjunctival, laryngo-tracheal or esophageal involvement. Otherwise, the EBA is classified as moderate or minimal. The MMP-DAI (disease activity index) score (156) can be used to quantify the extent of the disease, but the cut-offs between the severe, moderate, and minimal forms of EBA have not been established to date.

The goal of the treatment is to obtain control of the disease followed by CR, i.e., the absence of active lesions (erythema, urticaria, bullous lesions, and erosions) without worsening of the cicatricial lesions, which are irreversible.

A CR off treatment of EBA is not possible since a long-term maintenance treatment is recommended. CR under minimal treatment may occur after months to years in mild or moderate forms (unpublished data), but minimal skin fragility without bullous lesions can persist for several months to years. The milium cysts may eventually disappear.

The prognosis has been reserved in severe forms, as evidenced by the publication of numerous case reports in therapeutic failure. Indeed, in some patients, the disease may progress quickly with periods of severe exacerbation and rapid scarring. The cicatricial lesions (synechiae, stenosis, joint contractures) may engage the functional prognosis and be life-threatening. In a retrospective study of 30 patients with EBA, all of whom were initially treated with a combination of methylprednisolone, dapsone, and colchicine (six who did not respond were subsequently treated with other immunosuppressants), 8 of 24 patients (33 percent) achieved complete remission and 5 of 24 (21 percent) achieved partial remission within 1 year (18). The prognosis of these severe forms could improve because of recent publications demonstrating the therapeutic success of intravenous immunoglobulins and rituximab (see above). The overall prognosis and response to treatment may be more favorable in children than in adults (157, 158).

Taken together, these findings underline the need for an early diagnosis, multidisciplinary care by experienced practitioner and prompt implementation of appropriate treatment to improve the prognosis of EBA.

PATHOGENESIS

COL7 as the Autoantigen in EBA

Nearly a century after the first description of EBA, the carboxyl terminus of COL7 was identified as the autoantigen in EBA. Since that time, it has been shown that most patients develop autoantibodies that bind to epitopes located within the NC1 domain of COL7 (159–162), whereas antibody reactivity to either the collagenous domain (163) or the NC2 domain (164) is detected in a very small minority of patients. No correlation was detected between antibody specificity and clinical phenotype (159). In a recent multicentre study with 95 EBA patients, NC1/NC2 ELISA showed a higher sensitivity (97.9%) than NC1 ELISA (89.5%), supporting a considerable number of patients with antibodies against NC2 (95).

Interestingly, the humoral autoimmune response toward COL7 encompasses almost all IgG subclasses. Most commonly, COL7 autoantibodies are IgG, but in ∼10% of EBA patients, IgA autoantibodies against COL7 are detected. Few cases of IgE- and IgM-COL7-reactive immunoglobulins have been described (101). The nature and/or cause of this broad immunoglobulin isotype reactivity against COL7 is, however, unknown.

GENETIC AND ENVIRONMENTAL FACTORS CONTRIBUTE TO TOLERANCE LOSS IN EBA

As with most autoimmune diseases, the exact cause of the disease is unknown. With regard to EBA, the data indicate a certain genetic predisposition as well as a contribution of environmental factors to EBA pathogenesis. Due to the small number of EBA cases, it is difficult to study the influence of certain environmental factors or infections. EBA susceptibility is associated with genes in and outside the major histocompatibility complex (MHC) locus. Specifically, an association with the MHC locus (HLA-DR2) has been documented in humans in two independent studies (10, 11). The association with the MHC locus is also supported by animal studies, where an association of susceptibility to immunization-induced EBA is linked to the H2s locus (165). Evidence for the involvement of genes outside the MHC locus arises from one case of coincident EBA in members of a family provided further support for the genetic control of EBA (166). The contribution of genes outside the MHC locus is again underscored by corresponding observations in experimental EBA (165, 167, 168). First, when C57Bl6/J mice are immunized with COL7, they develop autoantibodies but no clinical disease. When mice on the same genetic background lack expression of the inhibitory Fc gamma receptor (FcγR) IIB, they also develop clinically overt blistering (165, 168). Similarly, mice carrying the EBA-associated H2s allele develop severe clinical disease when on the B6 genetic background but only moderate disease when on the C57BL/10 background (165, 169). To pinpoint the mutations associated with EBA susceptibility, mice of an advanced, autoimmune-prone intercross line were immunized with COL7. Herein, one third of the mice developed clinical disease, while the remaining mice remained phenotypically healthy (170, 171) Classical quantitative trait loci mapping identified several genes outside the MHC that were associated with either the onset or severity of clinical disease (170). However, the number of genes is still too large to pinpoint the association with clinical disease to single genes, yet in a nutshell, it provides evidence for a genetic basis of EBA susceptibility.

In addition to genetic factors, animal models of EBA clearly indicated an influence of resident microbial communities in disease pathogenesis (172, 173) (**Figure 5**). By the use of outbred mice in immunization-induced EBA, it could be shown that Firmicutes were the most abundant (54%), followed by

Proteobacteria (21%), Actinobacteria (12%), and Bacteroidetes (6%), which is similar to previous studies in the skin. At the genus level, Staphylococcus (36%), Corynebacterium (9%), and Ralstonia (8%) were most abundant (172). Ellebrecht et al. used the same model to show skin community changes before and after immunization. Among SJL/J mice that were immunized with COL7, only 80% of the mice developed disease, whereas the others remained healthy. Interestingly, the specific antibody concentrations and binding of antibodies to the DEJ were unaffected. By contrast, immunized mice that did not develop clinical phenotypes showed a greater alpha diversity, compared to mice that developed EBA symptoms after immunization (173).

THE AFFERENT PHASE: CD4 T CELL-DEPENDENT PRODUCTION OF AUTO-ANTIBODIES AGAINST COL7

EBA is characterized and causes autoantibodies directed against COL7. Unfortunately, little human data are available for this relevant and complex phase of pathogenesis, which includes the interaction of various immune cells, such as antigen-presenting cells, autoreactive B cells, T cells and neutrophils, and subsequently leads to antibody production. Regarding human data, COL7-specific T cells (isolated from the blood) can be detected EBA patients (174, 175), but most data concerning the interaction of these cell in the afferent phase has been derived from animal models (167).

In the immunization-induced EBA model, T-cell-deficient mice do not develop COL7-specific antibodies and, consequently, clinical disease development, indicating that autoantibody production in this model is T cell-dependent. Furthermore, disease susceptibility could be restored in T cell-deficient SJL/J mice by T cells transfer from COL7-immunized wild-type mice (176). To delineate T-cell subsets involved in the generation of anti-COL7 antibodies in this model of immunization-induced EBA, CD4+ and CD8+ T cells were depleted for 2 weeks, starting at immunization. Depletion of CD4+ T cells led to a delay of both autoantibody production and the clinical disease onset. By contrast, CD8+ T cells depletion at the same time period did not impact production of COL7-specific autoantibodies or clinical disease (142). Therefore, in experimental EBA, CD4+ T are required for induction of autoantibody production. Since only few and specific inbred mouse strains developed clinically overt disease after COL7 immunization, the autoantibody response of clinically healthy vs. diseased mice after immunization was contrasted. Here, complement-fixing antibodies were linked to clinical EBA manifestation (169). Furthermore, by determination of the IgG isotype of the autoantibodies, a Th1 polarization of the immune response was noted. In addition, an increase in the Interferon (IFN)-γ/Interleukin (IL)-4 ratio in the draining lymph nodes of EBA-susceptible mice compared with EBA-resistant strains was observed (169). Regarding the involvement of neutrophils in autoantibody production, GM-CSF-deficient mice generated less COL7-specific autoantibodies, which was paralleled by reduced neutrophil numbers in peripheral lymph nodes, draining the immunized site. The same effect was observed in neutrophil-depleted wild-type mice (177).

To further address which antigen-presenting cells are required the formation of antigen-specific CD4+ T cells, B cells were depleted in mice and subsequently immunized with COL7. In the absence of B cells, the antigen-specific CD4+ T-cell response was completely abolished. Furthermore, depletion of dendritic cells and macrophages had similar effects. Hence, the development of COL7-specific CD4+ T cells requires the presence of APC—specifically B cells, dendritic cells, and macrophages (142) (**Figure 5**). More interestingly, the absence of T_{reg} cells in scurfy mice cells led to blistering via the formation of pathogenic autoantibodies, demonstrating a critical involvement of these cell types in the afferent phase of EBA (178, 179).

In contrast to T cells, autoreactive B cells are almost exclusively found in the peripheral lymph nodes in the immunization-induced EBA mouse model (180, 181), which may be due to missing expression of homing-associated CXCR3 and CXCR4 chemokine receptors. In immunization-induced EBA, COL7-specific plasma cells have a half-life of ∼7 weeks. This resembles an intermediate between short- and long-living plasma cells (180). A similar intermediate plasma cell type is most likely also present in patients because autoantibody titers in patients with autoimmune bullous diseases slowly decline over a period of 8–12 weeks after B cell-depleting rituximab treatment (182).

An important molecular requirement for autoantibody production in experimental EBA is heat-shock protein 90 (Hsp90). Specifically, pharmacological HSP90 inhibition suppressed autoantibody production in immunization-induced EBA. In the same model, HSP90 blockade impaired the onset of clinical disease manifestation when injected prior to immunization with COL7. Furthermore, clinical disease progression was ameliorated when the compounds were applied in therapeutic experimental settings. Interestingly, B cell development was unaffected by the HSP90 inhibition, while T-cell proliferation was impaired. Overall, this identified T cells as targets of HSP90 inhibition in experimental EBA (174).

CIRCULATION AND PATHOGENICITY OF AUTOANTIBODIES AGAINST COL7

Targeting the Half-Life of Anti-COL7 Autoantibodies

After (auto)-antibodies are present in the circulation, their half-life is controlled by the neonatal Fc receptor (FcRn). FcRn is constructed as a heterodimer, consisting of an alpha-chain and a beta-2-microglobulin light chain (183). Among other functions, FcRn protects IgG from catabolism (184). Inhibition of the FcRn leads to the enhanced clearance of IgG, including autoantibodies. In antibody transfer-induced animal models of EBA (167), disease induction in mice is completely blocked (128, 185). However, this protection can be overridden by the transfer of large amounts of antibodies (185). Similarly, treatment of experimental EBA or other pemphigoid diseases with high doses of IgG (IVIG), which by saturation also inhibits the FcRn, reduces circulating autoantibody titers and leads to disease improvement

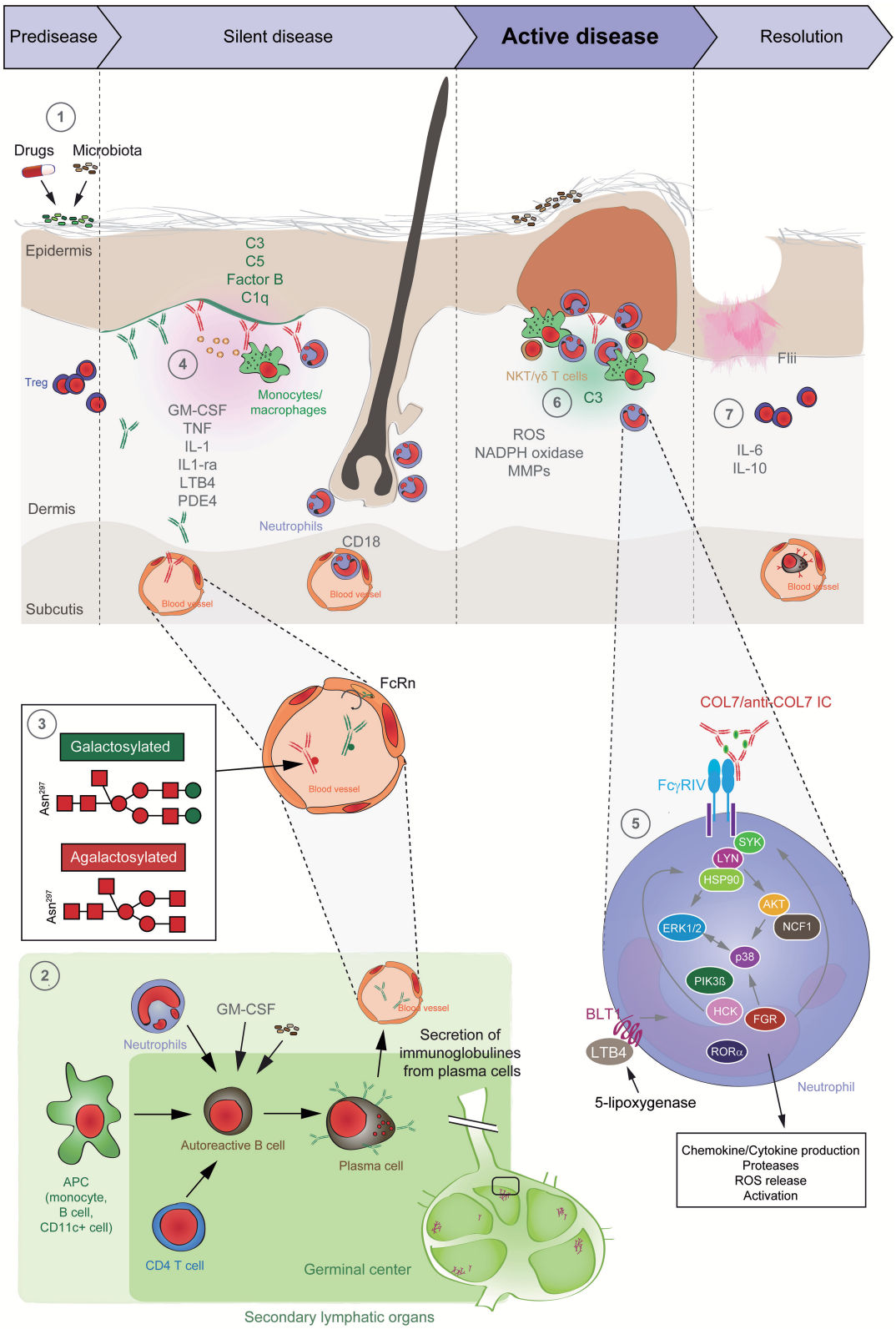

FIGURE 5 | Pathogenesis of EBA. (1) Genetic factors and the skin microbiome promote a tolerance loss. (2) This phenomenon is mediated by the interaction of APCs with autoreactive B and T cells, leading to clonal expansion and differentiation into plasma cells. Autoantibodies against COL7 are released into the blood circulation and effector organs. (3) During inflammation, galactosylation of antibodies may differ. High galactosylation of IgG is crucial for these anti-inflammatory properties,

(Continued)

FIGURE 5 | whereas low galactosylation is pro-inflammatory. (4) Binding of autoantibodies to DEJ in the skin induces complement deposition, pro-inflammatory cytokine and mediator release and subsequently leukocyte extravasation. (5) Immune complexes bind in a Fc-dependent manner to neutrophils and induce a signaling cascade leading to activation, including the (6) release of ROS and matrix metalloproteases. In addition to neutrophils, other cell types are involved in split formation, as shown for monocytes/macrophages, NKT and γδ T cells. By contrast, T_{reg} cells have an inhibitory effect on EBA progression. (7) Resolution of autoantibody-induced tissue injury. T_{reg}, regulatory T cell; NKT, natural killer cell; C, complement; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; LTB4, leukotriene B4; PDE4, phosphodiesterase 4; ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate; MMPs, matrix metalloproteinases; APC, antigen-presenting cell; CD, cluster of differentiation; SYK, spleen tyrosine kinase; Lyn, tyrosine-Protein Kinase Lyn; HSP, heat shock protein; AKT, protein kinase B; NCF1, neutrophil cytosolic factor 1; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; HCK, tyrosine-protein kinase HCK; FGR, tyrosine-protein kinase FGR; RORα, retinoid-related orphan receptor-alpha; BLT1, leukotriene B4 receptor 1; LTB4, leukotriene B4.

(128, 129, 186, 187), although FcRn inhibition is most likely not the only mode of action of IVIG in EBA (131, 188, 189).

Interestingly, two clinical trials are currently being conducted to evaluate the safety and efficacy of FcRn inhibitory treatments in patients with other autoimmune skin blistering diseases (NCT03334058) (190). Hence, this may become a treatment option for EBA patients in the not too distant future (167).

Targeting the Pathogenicity of Anti-COL7 Autoantibodies

In addition to its half-life, the pathogenicity of an antibody can also affect disease progression. In general, IgG antibodies have one conserved N-glycosylation site in each of their constant heavy chain regions. These Fc glycans have a major impact on their structure as well as their effector functions. Non-galactosylated (agalactosylated; G0) IgG antibodies have long been thought to have pro-inflammatory effector functions in autoimmune patients with rheumatoid arthritis. In contrast, sialylated IgGs mediate anti-inflammatory effects. Recent evidence also suggests that pro-inflammatory immune responses, including autoimmune reactions, mainly induce antigen-specific G0 IgGs, whereas tolerance leads to the generation of immunosuppressive, galactosylated, and sialylated IgGs. Under normal conditions, differentially glycosylated IgGs clearly mediate their pro- or anti-inflammatory effector functions as immune complexes in an antigen-specific manner (191) (**Figure 5**). In agreement with these findings, the use of EndoS, an endoglycosidase derived from Streptococcus pyogenes that selectively hydrolyses the N-linked glycan of native IgG, impaired split formation at the DEJ in skin cryosections. In EBA mouse models, EndoS abrogated clinical disease induction in mice (192, 193). This observation raises the possibility that the glycosylation status of IgG can also affect the onset, severity and progression of disease.

THE EFFERENT PHASE OF EBA: ATTRACTION AND ACTIVATION OF LEUKOCYTES LEADS TO BLISTER FORMATION

Based on the current understanding of EBA pathogenesis (**Figure 5**), the effector phase of EBA, i.e., autoantibody-induced inflammation and blistering, can be divided into (**i**) autoantibody binding to COL7, (**ii**) complement activation and the formation of a pro-inflammatory milieu, (**iii**) leukocyte extravasation, (**iv**) activation by Fcγ receptors, and (**v**) tissue damage. Mechanisms leading to non-inflammatory blistering in EBA are, in contrast, poorly understood. With the increased understanding of pathomechanisms of epitope-dependent pathogenicity-associated (194), non-inflammatory BP, new insights into mechanobullous EBA can be expected. However, due to a lack of data on non-inflammatory mechanisms of blistering in EBA, the following text relates to inflammatory EBA.

Autoantibody-induced tissue injury in EBA is initiated by **(i) the deposition of autoantibodies at the DEJ**. Apart from the skin anti-COL7 antibodies bind to the esophagus, stomach, small intestine, and colon because of the autoantigen expression at these sites (195, 196). Yet, not all isotypes of anti-COL7 have the potential to induce dermal-epidermal separation: *Ex vivo*, only human IgG1 and IgG3, but not IgG2 and IgG4, are capable to cause blistering (197). Furthermore, immune complexes containing IgA1 or IgA2 COL7 autoantibodies activate neutrophils and also induce subepidermal blistering when in cryosections of human skin. Of note, and in contrast to IgG1 autoantibodies, neither IgA1 nor IgA2 leads to complement deposition at the dermal-epidermal junction. Because complement activation has traditionally been thought a prerequisite for blister induction, this may be compensated by so far unknown soluble factors and/or by a stronger activation of neutrophil granulocytes when engaging IgA immune complexes (198).

Thereafter, **(ii) a pro-inflammatory milieu is generated** in the skin, which includes activation of the complement system (199). The complement system consists of circulating proteins that, upon activation, initiate a highly controlled cascade that is an integral part of the innate humoral immune response (200). C5-deficient mice (168) are either completely or (168) partially (201) protected from induction of experimental EBA by antibody transfer.

Dissecting the specific role of each complement activation pathway (classical, lectin, and alternative pathway) showed the following: MBL deficient mice showed a similar EBA phenotype to the wild-type controls. C1q-deficient mice showed weak and partial protection, while factor-B-deficient mice showed clinically relevant protection from EBA induction by antibody transfer (199). This identified the alternative pathway of the complement system as a main driver of skin blistering and inflammation in antibody transfer-induced EBA (202). Downstream of C5, C5ar1-deficient mice are significantly protected from experimental EBA, whereas C6-deficient

mice developed widespread blistering disease, excluding the membrane attack complex as a pharmacological target for EBA. In line, pharmacological blockade of C5, factor B, or C5aR1 led to a significant improvement of the blistering phenotype in antibody transfer-induced EBA (202, 203).

In addition to the complement system, the lipid mediator leukotriene B4 (LTB4) is a potent granulocyte chemoattractant (204, 205) and activator (206) and is abundant in the blister fluids of bullous pemphigoid patients, but its pathogenic significance for pemphigoid diseases had been unknown until recently. LTB4 is biosynthesized from arachidonic acid through sequential enzymatic conversion by 5-lipoxygenase and LTA4-hydrolase. The 5-lipoxygenase is most abundant in neutrophils, and it is activated upon cell stimulation by, for example, immune complexes or the complement fragment C5a (207). Mice deficient in 5-lipoxygenase, a key enzyme in LTB4 biosynthesis, or BLT1-deficient mice are completely resistant to the induction of experimental EBA by antibody transfer (208). In addition to complement and lipid mediators, several cytokines have been identified to modulate the effector phase of EBA. Cytokines that are differentially regulated in experimental EBA (209) or cytokines associated with those functional data (210) are summarized in **Table 4** (171, 224).

The pro-inflammatory milieu induces the **(iii) attraction of different leukocyte populations** (**Table 5**). Unfortunately, the composition of these cells has not been investigated thus far in EBA patients, but it is known from BP patients that the infiltrate includes cells such as lymphocytes, histiocytes, eosinophils, neutrophils, and mast cells (228, 229). Subsequent mechanistic studies using the antibody transfer-induced model have uncovered neutrophils as the major culprits responsible for blister formation (208). The recruitment of neutrophils into the skin is mediated by CD18- and ICAM-1 (216, 230–232). In addition, CD18 crucially regulates neutrophil adhesion as an indispensable step leading to tissue damage (233).

Concerning the functional role of T cells, it was recently demonstrated that T cells can enhance neutrophil recruitment into the site of inflammation by modulating the expression of the cell surface integrin CD18 on neutrophils. Interestingly, this effect was neither mediated by CD4 nor CD8 cells, but rather γδT and NKT cells (225). Interestingly, blockade of T_{reg} led to a dramatic worsening of the clinical disease manifestation in antibody transfer-induced EBA (224).

In addition to granulocytes and lymphocytes, macrophages/monocytes may be involved in the blister formation in EBA. However, the blockade of these cells is technically difficult and does not effectively impair disease progression (142). Nevertheless, more studies are needed because macrophages/monocytes are able to produce high amounts of reactive oxygen species (ROS) after immune complex stimulation and induce *ex vivo* split formation in human skin sections (214). Furthermore, the application of high concentrations of anti-COL7 IgG has been shown to induce mast cell activation, but mast cell deficient mice develop experimental EBA just like wild type animals, indicating that mast cells do not contribute to the immune-mediated tissue injury (234–236). Concerning the role of additional cell types in inflammation, a possible role of plasma cells (227) has been discussed, but further studies are needed to unravel the cellular orchestration responsible for the lesional sites.

After extravasation from the blood into the skin, **(iv) myeloid effector cells bind to the skin-bound immune complexes** in a FcγR-dependent fashion (**Table 6**). In EBA the full IgG molecule of the autoantibodies, but not their corresponding F(ab)2 fragments, are pathogenic. Specifically, only the full anti-COL7 IgG elicits dermal-epidermal separation when, together with PMN, incubated on cryosections of human skin (248). Likewise, and unlike the full IgG, F(ab′)2 fragments of anti-COL7 IgG do not induce clinical EBA manifestation when injected into mice (168). The central role of these Fc-FcγR interactions for mediating skin inflammation and subepidermal blistering in experimental EBA is further supported by the complete protection of mice toward EBA induction when injected with chicken anti-mouse COL7 IgY, which is known not bind to murine complement and Fc receptors (249). In addition, the therapeutic effects observed when blocking these interactions, i.e., using soluble CD32/SM101 (231), highlights the key role of Fc-FcγR interactions in EBA pathogenesis. Furthermore, IgG glycosylation has been shown to have preventive and therapeutic effects in mouse models of chronic inflammatory diseases, including EBA (191). Further studies eluted on the differential contribution of the different FcγR (250). In mice, three different activating FcγR and one inhibitory FcγR are described: FcγRI, FcγRIII, and FcγRIV are activating FcγR, all with specific binding avidities toward IgG. The FcγRIIB is the only inhibitory FcγR (250). Of note, an increased expression of FcγRIV has been demonstrated in the skin of mice with experimental EBA (181). Subsequent functional studies identified the FcγRIV as the key mediator of tissue injury in EBA. By contrast, blockade of FcγRI, FcγRIII, or both receptors in combination had no effect on the induction of experimental EBA by antibody transfer. In FcγRIIB deficient mice enhanced blistering was observed in antibody transfer-induced EBA, as well as BP (181, 251), indicating a protective role of this FcRγ in experimental EBA. In human *ex vivo* models of BP, FcγRIIA, and FcγRIIIB contributed to the autoantibody-induced tissue damage (252). Once the neutrophils are bound to the immune complexes, a multifaceted signaling cascade is initiated (**Table 6**). This involves activation of the retinoid-related orphan receptor (ROR)α (230), heat shock protein (HSP)90 (241), phosphodiesterase 4 (240), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) β and δ (238, 239), p38, AKT, ERK1/2 (244), the spleen tyrosine kinase SYK (171, 245), and src kinases (247), as well as CARD9 (246)—which have been reviewed in detail elsewhere (253). The exact temporal and spatial order of these signaling events is currently unknown. Ultimately, the signaling cascade leads to the activation of myeloid effector cells, specifically release of ROS and proteases, both of which are required for subepidermal blistering in EBA (216, 225).

Recently the contribution of cytokines in EBA pathogenesis has been eluted both on the morphological, as well as the functional level (254). Because biologics targeting cytokines are already in clinical use—although, with few exceptions such

TABLE 4 | Mediators of the EBA effector phase.

Target	Function	References
C5	C5-deficient mice are partially or completely protected from EBA inducing by antibody transfer	(201, 211)
C1q/factor B	Respective knock-out mice are partially protected from EBA inducing by antibody transfer	(203)
IgG glycosylation	Enzymatic removal of terminal IgG N-glycosylation renders anti-COL7 antibodies non-pathogenic in antibody transfer-induced EBA	(192, 193)
Galactosylated IgG	Immune complexes with highly galactosylated immune complexes inhibit pro-inflammatory signaling of the C5aR1 through dectin-1 and Fc gamma receptor IIB, resulting in a protection from antibody transfer-induced EBA	(202)
IL-6	In antibody transfer-induced EBA, IL-6 has anti-inflammatory effects, through up-regulation of IL-1ra	(209)
CXCR-1/2	Blockade of the CXCR-1/2 ligands impairs induction of EBA by antibody transfer and slows disease progression when applied in therapeutic settings in immunization-induced EBA	(212)
GM-CSF	Blockade of GM-CSF impairs induction of EBA by antibody transfer and slows disease progression when applied in therapeutic settings in immunization-induced EBA	(177)
IL-1/IL-1ra	Both anti-IL1β or IL-1ra (anakinra) treatment impair the induction of EBA by antibody transfer. Additionally, anakinra halts disease progression when applied therapeutically in immunization-induced EBA	(213)
TNFa	Blockade of TNF impairs induction of EBA by antibody transfer and halts disease progression when applied in therapeutic settings in immunization-induced EBA	(214)
LTB4	Blockade of either LTB4 biosynthesis or its' receptor completely protects mice from EBA induction by antibody transfer	(208)
IL-17A/E	IL17R-deficient mice are partially protected from EBA inducing by antibody transfer	(215)
NADPH oxidase	Neutrophil cytosolic factor 1-deficient mice, lacking functional NADPH oxidase, -deficient mice are completely protected from EBA inducing by antibody transfer	(216)
Elastase	Elastase is required for the induction of subepidermal blisters *ex vivo*	(217)
Flii	Blockade of Flii protects mice from EBA induction by antibody transfer	(218–220)
MIP1α	Increased expression, but no effect on clinical phenotype	(221)
S100	Increased expression, but no effect on clinical phenotype	(222)
Trem1	Increased expression, but no effect on clinical phenotype	(223)

C, complement factor; IgG, immunoglobulin G; IL, interleukin; COL7, type VII collagen; CXCR, CXC-chemokin receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF, tumor necrosis factor; LTB, leukotriene; NADPH, nicotinamide adenine dinucleotide phosphate; Flii, flightless I; MIP1α, macrophage inflammatory protein1α; Trem1, Triggering receptor expressed on myeloid cells-1.

TABLE 5 | Cell lineage in the effector phase.

Cell type	Function	References
Neutrophils	Neutrophil depletion partially protects from EBA induction by antibody transfer	(208, 216)
NKT/γδ T cells	Depletion of NK or γδ T cells partially protects from EBA induction by antibody transfer	(225)
T_{regs}	Depletion of T_{regs} worsens the clinical disease manifestation in antibody transfer-induced EBA	(224)
Keratinocytes	Upon binding of COL7 antibodies, pro-inflammatory mediators are released from keratinocytes	(209, 226)
IL-10^+ B lineage cells	IL-10, derived from IL-10+ B cells impairs neutrophil functions and impairs clinical disease manifestation in immunization-induced EBA	(227)
Monocytes/macrophages	Monocytes/macrophages induce subpeidermal splits *ex vivo*, and depletion of all myeloid cells completely protects from EBA induction by passive antibody transfer, while selective depletion of neutrophils confers partial protection	(214, 216)

NKT, natural killer T cells; T_{regs}, regulatory T cells; IL, interleukin.

as rituximab and omalizumab, for other indications (255)—we here highlight their contribution for EBA pathogenesis. Several cytokines have been shown to contribute to blister formation in experimental EBA (**Table 4**). IL-6 acts as a pro-inflammatory cytokine in various autoimmune diseases (256, 257). Hence, IL-6 has emerged as a potential target for the treatment of autoimmune diseases, and IL-6-targeting therapies are licensed for rheumatoid arthritis and juvenile idiopathic arthritis (256). In EBA patients and in experimental EBA models, serum IL-6 levels are elevated. Unexpectedly, mice lacking IL-6 expression show an increased clinical phenotype in experimental EBA compared with controls. Furthermore, treatment with recombinant IL-6 leads to a dose-dependent reduction of clinical disease manifestation in EBA induced by antibody transfer. Thus, in experimental EBA, IL-6 has a profound anti-inflammatory activity (209). At the molecular level, the protective effect of IL-6 is mediated by the IL-6-dependent release of IL-1ra (213). In line, IL-1R–deficient mice or wild-type mice treated with either the

TABLE 6 | Receptors and signaling in the efferent phase of EBA.

Target	Function	References
FcγRs	Activating FcγR promote skin inflammation in experimental EBA, while the inhibitory FcγRIIB confers protection from induction of EBA by antibody transfer	(181)
FcRn	The FcRn controls the half-live of IgG. This FcRn-deficient mice are partially protected from EBA induction by antibody transfer	(185)
CD18	CD18-deficient mice are completely protected from EBA induced by antibody transfer	(216)
CD11b	CD11b-deficient develop a more severe clinical phenotype in antibody transfer-induced EBA.	(237)
C5aR1	C5aR1-deficient mice are almost completely protected from EBA induced by antibody transfer	(202, 203)
CXCR1/2	Pharmacological CXCR1/2 inhibition prevents disease progression in immunization-induced EBA	(212)
BLT1	BLT41-deficient mice are almost completely protected from EBA induced by antibody transfer	(208)
PI3Kß	PI3Kβ-deficient mice are partially protected from EBA induction by antibody transfer, through inhibition of neutrophil activation	(238)
PI3Kδ	Pharmacological inhibition of PI3Kδ impairs induction of EBA by antibody transfer and has therapeutic effects in immunization-induced EBA	(239)
Phosphodiesterase 4	Pharmacological inhibition of PDE4 impairs induction of EBA by antibody transfer and has therapeutic effects in immunization-induced EBA	(240)
RORα	RORα-deficient mice are completely protected from EBA induced by antibody transfer	(230)
HSP90	HSP90 is involved in both loss of tolerance to COL7, as well as to antibody-induced tissue damage in experimental EBA	(241, 242)
JAK2	Pharmacological inhibition of JAK2 impairs induction of EBA by antibody transfer and has therapeutic effects in immunization-induced EBA	(243)
AKT, ERK, p38	Pharmacological inhibition of these targets impairs induction of EBA by antibody transfer (ERK, p38) or impairs subepidermal splitting *ex vivo* (all)	(244)
SYK	Pharmacological inhibition of SYK or SYK-deficient mice are completely protected from EBA induction by antibody transfer	(171, 245)
CARD9	CARD9-deficient mice are partially protected from EBA induction by antibody transfer	(246)
Src kinases	Hck, Fgr and Lyn-tripple-deficient mice are partially protected from EBA induction by antibody transfer	(247)
TREM1	See **Table 5**	(223)
Caspase 1	Caspase-1/11-deficient mice develop antibody transfer-induced EBA similarity to wild type littermate controls	(213)

FcγR, Fc gamma receptor; FcRn, neonatal Fc receptor; BLT, leukotriene B4 receptor; CXCR, CXC-chemokin receptor; PI3K, Phosphatidylinositol-4,5-Bisphosphate 3-kinase; RORα, retinoid-related orphan receptor-alpha; HSP90, heat-shock protein 90; JAK2, Janus kinase 2; AKT, protein kinase B; ERK, extracellular signal-regulated kinase; SYK, spleen tyrosine kinase; CARD9, Caspase recruitment domain-containing protein 9; SRC, tyrosine-protein kinase SRC; HCK, tyrosine-protein kinase HCK; LYN, tyrosine-protein kinase LYN; TREM1, Triggering receptor expressed on myeloid cells-1.

IL-1R antagonist Anakinra or a IL-1β function blocking antibody are also partially protected from the EBA-inducing effects of anti-COL7 IgG. Mechanistically, IL-1β increases ICAM-1 expression on endothelial cells, suggesting that IL-1β supports recruitment of inflammatory cells into the skin. Interestingly, these effects of IL-1 were independent of caspase-1 because of caspase-1/11–deficient mice showed a similar phenotype to wild type control animals when injected with anti-COL7 IgG (213).

Additionally, the evaluation of cytokines affecting neutrophil functions, such as IL-8 (CXCL1 and CXCL2 in the mouse) and GM-CSF in experimental EBA, showed an increased expression. To evaluate if the increased expression is of functional relevance these cytokines were inhibited by either antibodies of mice lacking expression of the respective cytokine(s). Treatment with allosteric CXCR1 and 2 inhibitors (DF2156A) impaired the induction of skin blistering in antibody transfer-induced EBA.

In a therapeutic setting, the administration of DF2156A improves the clinical manifestation of EBA after disease onset in immunization-induced EBA (212). For the evaluation of GM-CSF, anti-COL7 IgG was injected into GM-CSF-deficient mice or wild type mice treated with a function-blocking GM-CSF antibody. The induction of experimental EBA was impaired if the function of GM-CSF was blocked in comparison to appropriate controls. *In vitro* studies have demonstrated the requirement of GM-CSF for neutrophil recruitment from bone marrow into the blood and from the blood into the skin. Furthermore, GM-CSF preactivates neutrophils, leading to an enhancement of immune complex-induced neutrophil activation. In a therapeutic setting, the blockade of GM-CSF in mice with already established immunization-induced EBA has demonstrated beneficial therapeutic effects (177, 213). In addition to these cytokines, increased expression of TNF has been observed in experimental EBA, and prophylactic blockade of TNF and therapeutic use of etanercept in the immunization-induced EBA model impair the induction and progression of experimental EBA (214). By contrast, increased expression of CCL3/MIP1α is a mandatory cytokine for disease development (221).

THE RESOLUTION PHASE OF EBA

For most autoimmune bullous diseases, the location of blisters differs over time, indicating a frequent, but unfortunately overall insufficient, healing process in the skin (**Figure 5**). Flightless I (Flii), an actin remodeling protein, has been shown to modulate the resolution of skin blistering in experimental models of EBA. *In vivo*, the induction of EBA leads to increased cutaneous Flii expression, resulting in impaired Claudin-1 and Claudin-4 tight junction protein expression, as well as a delay in the recovery from blistering (218, 258). Overexpression of Flii produces severe blistering post-induction of EBA, while decreased Flii reduces blister severity, elevates integrin expression, and improves COL7 production. In addition, topically applied Flii neutralizing antibodies improve the healing of blistered skin in murine EBA (218–220, 259).

An interesting experiment could show a protective effect of IL-10-positive plasma cells toward the neutrophil-dependent inflammation in EBA. After the start of skin inflammation, plasmacytosis is induced by injection of goat anti-mouse IgD serum and provides protection from skin inflammation and neutrophil infiltration for at least another 3 weeks. Suppression of EBA skin inflammation is abrogated by the co-injection of a neutralizing IL-10 receptor antibody. Despite its anti-inflammatory effect, plasmacytosis neither reduces the numbers of autoreactive COL7-specific plasma cells nor the autoantibodies that trigger the disease (227). These observations point toward a significant contribution of pathways that are involved in the resolution of cutaneous involvement. Therefore, EBA may manifest not only when many pro-inflammatory stimuli are present but also when the balance of pro-inflammatory, anti-inflammatory, and resolving pathways are unbalanced, driving the cells toward pro-inflammatory mechanisms.

CD11b is an integrin family member that pairs with CD18 to form the CR3 heterodimer. CD11b is expressed on the surface of many leukocytes, including monocytes, neutrophils, natural killer cells, granulocytes, and macrophages. Unexpectedly, in the antibody transfer-induced model of EBA, CD11b-deficient mice develop more severe disease symptoms than wild-type mice in the late phase of the disease. Furthermore, compared to wild-type controls, CD11b-deficient mice express increased levels of circulating IFN-γ and IL-4, suggesting an anti-inflammatory role for CD11b in the resolution phase of experimental autoimmune diseases, such as EBA (237).

EMERGING TREATMENTS

The current (unsatisfactory) treatment options for EBA have been outlined above. An increasing understanding of EBA pathogenesis indicates new potential therapeutic targets that interfere in different phases of disease progression, including the generation of autoantibodies, maintaining autoantibodies in the circulation, and autoantibody-induced tissue injury.

Due to the lack of clinical studies, we categorized the emerging treatments into three categories:

TREATMENTS DESCRIBED IN CASE REPORTS

Several case reports are known for the treatment of EBA using ECP (145) and Daclizumab (148). Furthermore, anti-C1s mAb, a mouse monoclonal IgG2a antibody, inhibits the activation of C1s, which is a serine protease of the classical complement system. TNT003 completely blocks complement classical pathway activation by the reduction of C4a and C5a production induced by incubation of the sera from patients with BP on cryosections of human skin (260). The disease induction of experimental EBA is also complement-dependent. This finding suggests that TNT003 might work even in EBA. A "humanized version" of TNT003, TNT009 is currently evaluated in a phase I clinical trial in BP patients (NCT02502903) and data reported on congresses indicates a favorable safety, as well as efficacy in some patients. Sulfasalazine was used in a patient with EBA associated with Crohn's disease, resulting in no improvement of the skin lesion (149). The utility of doxycycline has been reported in BP (150). Doxycycline and another tetracycline, minocycline, have been described in the literature in EBA cases, although their usefulness remains unclear (151–153).

TREATMENTS APPLIED IN THERAPEUTIC SETTINGS IN EXPERIMENTAL EBA

Due to the low incidence, clinical studies with EBA patients are not performed. Therefore, animal models are indispensable for investigating the pathogenesis of EBA and other autoimmune blistering diseases and to test new target substances for treatment; these models include the transfer of autoantibodies to experimental animals, adoptive transfer of autoantibody-producing B cells to immune-deficient mice and construction of transgenic mice that produce autoantibodies (167). Although animal models have many benefits, as described above, both the advantages and disadvantages of each model should be considered. Although the models duplicate the clinical, histopathological, ultrastructural, and immunological features of the human disease, these systems are mostly completely murine, and it is possible that findings in mice will not translate to humans.

The most important mouse model to test treatments applied in therapeutic settings is the immunization-induced EBA model (167, 168). Immunization with parts of the NC1 domain of COL7 with an adjuvant induces anti-COL7 IgG production in mice. The susceptibility of experimental EBA is closely associated with the H2s haplotype in mice (165). During a time period of 4–6 weeks, the mice develop antibodies against mCOL7 that bind to the DEJ and induce neutrophil activation and blistering. Using the immunization-induced EBA model, the pathomechanisms of blistering and potential therapeutic options have been studied, and more importantly, this model is used to test potential new drug candidates and therapeutics (167). Successfully applied drugs are summarized in **Table 7**.

TABLE 7 | Experimental treatments in pre-clinical immunization-induced EBA.

Medication	Target	Impact on disease	Application	References
Methylprednisolone	Multiple	Impair EBA progression	i.p.	(129, 212)
DF2156A	CXCR1/2	Halting EBA progression	p.o.	(212)
IVIG	Multiple	Impair EBA progression	i.p.	(129)
Anti-GM-CSF	GM-CSF	Impair EBA progression	i.p.	(177)
Etanercept	TNFα	Impair EBA progression	i.p.	(214)
EndoS	Fc glycosylation	Halting EBA progression	i.p.	(192)
sCD32/SM101	FcγR	Impair EBA progression	i.p	(231)
Anakinra	IL-1	Improvement	i.p.	(213)
17-DMAG	HSP90	Improvement	i.p.	(174)
17-AAG	HSP90	Impair EBA progression	top	(242)
TCBL-145	HSP90	Improvement	i.p.	(174)
Dimethylfumarate	Multiple	Improvement	p.o.	(261)
goat anti-mouse IgD serum	Induction of IL10 plasma cells	Impair EBA progression	i.p.	(227)
Calcitriol treatment	Vitamin D	Impair EBA progression	p.o.	(262)
LAS191954	PI3Kδ	Improvement	p.o.	(239)
Roflumilast	PDE4	Halting EBA progression	p.o.	(240)

CXCR1/2, CXC-chemokin receptor; GM-CSF; granulocyte-macrophage colony-stimulating factor; TNF, tumor necrosis factor; EndoS, endoglycosidase S; FcγR, Fc gamma receptor; IL, interleukin; 17-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin; HSP90, heat shock protein 90; 17-AAG, Tanespimycin (17-N-allylamino-17-demethoxygeldanamycin); TCBL-145, D-Tyr-Phe-D-Trp-Leu-AMB (AMB: NH-CH_2-CH_2-$CH(CH_3)_2$); PI3K, phosphoinositide 3-kinase; p.o., per os; i.p., intraperitoneal; top, topical.

TREATMENTS APPLIED IN PREVENTIVE SETTINGS IN EXPERIMENTAL EBA

Beneath the validation of potential drug candidates in the therapeutic setting in immunization-induced EBA, some authors have also investigated several drugs in antibody transfer-induced EBA. In this model, rabbit sera or whole rabbit IgG immunized with COL7 NC1 domain are injected into mice. These mice develop clinical and histological subepidermal blistering within a few days. The antibody transfer models are used to investigate the cellular and molecular basis of blistering and to investigate preventive effects of several therapeutic options. The use of antibody transfer models enables examination of the effector phase of EBA. Testing novel drugs or investigating the pathogenesis of EBA is straightforwardly done because clinical symptoms are visible days after starting the experiment. However, similar to most animal models, the situation in human patients is only partially reflected—mostly, because the signaling cascade and the interaction of different cell types vary among human and mice. Additionally, induced disease may last less than a few days after the transfer of antibodies. Furthermore, the loss of tolerance and the generation of autoantibodies cannot be studied in models based on antibody transfer. Therefore, the investigation of potential drug candidates should always be performed in addition to immunization-induced EBA in a therapeutic setting.

Complement

Complement activation has been described as a prerequisite for blister formation. In the antibody-transfer model, blister induction is completely dependent on complement C5 because C5-deficient mice or mice injected with F(ab)2 fragments of the immune IgGs are devoid of blisters. This finding indicates that the complement system can be targeted in animal models. Targeting therapies for complement system factor B, C5, and C5R ameliorate disease severity in antibody-transfer models (199, 201, 203, 211, 260). Interestingly, in antibody transfer-induced BP, C5ar1$^{(-/-)}$ mice are protected from disease development, whereas the extent of skin lesions is increased in C5ar2$^{(-/-)}$ animals.

SYK

SYK is a non-receptor cytoplasmic enzyme that is mainly expressed in hematopoietic cells. SYK regulates cellular responses to extracellular antigens or antigen-immunoglobulin complexes. In EBA pathogenesis, myeloid SYK is a central player in driving inflammation in prototypical autoantibody-transfer models. The SYK inhibitor (BAY61-3606) protects mice from inflammation in antibody-transfer EBA (171, 245).

Cell-Derived Nanoparticles (CDNPs)

CDNPs are intercellular protein complexes, such as annexin A1, annexin A5, actin, *14-3-3ε*, *14-3-3ζ*, galectin-3, and heat-shock proteins 27 and 70. CDNPs play a therapeutic role against viral infections, cancer and in the experimental EBA model. Mice that receive anti-COL7 IgG develop skin lesions. Treatment with CDNPs significantly reduces the affected skin lesions and increases IL-4 expression (263).

LTB4

LTB4 is a potent chemoattractant and activator of myeloid cells, particularly of neutrophils. The 5-lipoxygenase–deficient and BLT1-deficient mice exhibit neither any clinical nor histological signs of disease in the antibody-transfer model. The 5-lipoxygenase inhibitor (zileuton) targeting the LTB4/BLT1

pathway ameliorates disease severity by ∼50% in antibody-transfer models (208).

Flii I

Flii is a member of the gelsolin family of actin remodeling proteins in regulating cell adhesion and intracellular signaling. Flii expression is increased in the epidermis during development and during epidermal stratification to maintain barrier functions, such as tight junctions. Topical application of Flii-neutralizing antibody significantly reduces the clinical severity of blistering and histological separations in antibody-transfer EBA mice. In addition, the barrier function measured by transepidermal water loss (TEWL) is significantly decreased in Flii-neutralizing antibody-treated mice (218, 219).

IL-6 (Protective)

IL-6 is a proinflammatory cytokine and plays a role during the transition from innate to acquired immunity. In the initial immune response to pathogen, IL-6 attracts neutrophils to the affected tissue. In addition, IL-6 plays a crucial role in B- and T-cell differentiation. Elevated IL-6 concentrations are observed in many inflammatory diseases and often correlate with disease activity. IL-6 expression is increased in the skin of EBA patients. Interestingly, the EBA severity is enhanced in IL-6-deficient mice in an experimental model. Furthermore, treatment with recombinant IL-6 dose-dependently impairs the induction of experimental EBA (209).

Galactosylated Immunoglobulins

Antibody-transfer EBA model activation is FcgR-dependent in blister formation. Immune-complexes induce the classical complement pathway via Fc fragment. The mouse IgG1 subclass preferentially binds to inhibitory FcgRIIB and suppresses the inflammatory response. Glycosylation of Asn297 in the IgG Fc fragment plays an important role in complement activation. Highly galactosylated immune-complex treatment reduces the development of cutaneous lesions in an antibody-transfer model (202).

CARD9

Caspase recruitment domain-containing protein 9 (CARD9) is an intracellular adapter protein that is expressed in myeloid-lineage cells. CARD9 plays a critical role in host defense against pathogens in both mice and humans. Card9 deficient mice are significantly protected against skin blistering in antibody-transfer EBA. In this setting, CARD9 is required for development of the inflammatory response. These pieces of evidence suggest a therapeutic target in EBA (246).

Signal Transduction by Scr Kinases

Src family kinases, such as Hck, Fgr, and Lyn, play roles in malignant transformation and tumor progression, and therefore they can be targets of cancer therapy. In addition, Src family kinases are present in many types of immune cells. It is known that Src family kinases have a role in integrin signal transduction in neutrophils and macrophages. Hck, Fgr, and Lyn-deficient mice are completely protected in antibody-transfer EBA (247).

T Cells

T_{regs} are of importance in modulating host responses to tumors and infections and in inhibiting the development of autoimmunity and allergies. T cell-deficient mice are protected from induction of skin lesions in antibody-transfer EBA. In particular, specific depletion of T_{regs} increases disease progression in antibody transfer-induced EBA. In a similar experimental setting, NKT-deficient mice and γδT cell-deficient mice are protected against the induction of experimental EBA (224, 225).

Signal Transduction by p38, MAPK AKT

In the antibody-transfer model, neutrophils are crucial for inducing clinical disease. Neutrophil activation is mediated by the phosphorylation of ERK1/2, p38 MAPK, and Akt. Methylprednisolone inhibits the phosphorylation of Akt, ERK1/2, and p38 MAPKs in neutrophils. Chemical inhibitors of Akt (Akt inhibitor VIII), ERK1/2 (U0126), and p38 MAPK (SB203580) statistically suppress *ex-vivo* dermal-epidermal separation. In addition, ERK1/2 (U0126), and p38 MAPK (SB203580) demonstrate an ∼10% reduction of disease severity compared with the control (244).

RORα

RORα is a steroid nuclear hormone receptor and a transcription factor. Experimental EBA shows strain-dependent disease severity. To elucidate the strain-dependency, RORα is found to be a risk gene for the antibody-transfer model. The RORα agonist SR3335 impairs blister formation in both types of antibody-transfer EBA (230).

Signal Transduction by PI3Kβ

PI3Ks play major roles in the signaling pathways that link cell surface receptors to the control of cell function. There are four defined isoforms of PI3Ks. In particular, PI3Kβ is widely expressed and blocks arterial thrombus formation. PI3Kβ-deficient mice are protected from the development of blisters in the antibody-transfer EBA model.

Others

This antibody-transfer mouse model can be used to validate or, at least as important exclude novel therapeutic targets in EBA. For example, TREM1, MIP1a, CD11b, caspase 1/11, and S100, which are all increasingly expressed in experimental EBA, have no impact on disease manifestation (213, 221–223, 237).

CONCLUSIONS

Since the first description of EBA over 100 years ago, our understanding of this serious disease has dramatically improved. The knowledge of the cellular and molecular mechanisms led to the development of new therapies, some of which have been successfully tested in preclinical models and in concept studies in bullous pemphigoid patients. In the future, new drug targets will

evolve to improve the treatment and living standards of people with EBA.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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CASE REPORT
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Pemphigus Foliaceus—Repeated Treatment With Rituximab 7 Years After Initial Response: A Case Report

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Pemphigus foliaceus is an autoimmune skin disease mediated by autoantibodies directed against desmoglein-1 located in the upper epidermal layer. Rituximab, a monoclonal anit-CD20 antibody depleting b-cells, offers an effective treatment possibility for therapy-resistant pemphigus foliaceus. Here, we present the case of 55-year-old man who did not respond sufficiently to conventional treatment with prednisolone, azathioprine, and cyclophosphamide, but underwent almost complete remission after rituximab treatment. The patient relapsed 7 years later, and a repeated course of rituximab infusions led to a partial remission.

Keywords: pemphigus foliaceus, rituximab, anti-CD20, b-cell depletion, autoimmune blistering diseases

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INTRODUCTION

Pemphigus foliaceus is a rare, autoimmune blistering skin disease with an estimated incidence of <1 million individuals in the USA and Europe (1). It is caused by autoantibodies directed against desmoglein-1 (Dsg-1), a glycoprotein important for intercellular adhesion between keratinocytes (2, 3). The disease manifests as erythematous papules, plaques, and erosions with scaly crusts. The involvement of seborrheic skin areas is typical for the disease. The loss of adhesion occurs in the upper epidermal layers and is limited to the skin with no mucosal involvement; this is attributable to the expression pattern of Dsg-1 and distinguishes pemphigus foliaceus from the more common pemphigus vulgaris (4, 5).

Glucocorticoids are the first-line treatment for pemphigus foliaceus. Treatment with other immunosuppressants such as azathioprine, mycophenolate mofetil or methotrexate, is also well-established (6). Recent data suggest that a b-cell-depleting therapy with rituximab is highly effective in treating pemphigus vulgaris, but also pemphigus foliaceus (7–10). Here, we describe the case of a patient suffering from a therapy-resistant pemphigus foliaceus; the patient was in remission for 7 years after initial rituximab treatment and responded well to the repeated treatment.

CASE PRESENTATION

A 55-year-old man with a history of progressing skin lesions over the past 8 months visited our department for the first time in spring 2011. The clinical examination revealed multiple erythematous papules and plaques with crusts on his back, chest, face, and scalp (about 40% of body surface area was involved) with no mucosal involvement (**Figure 1**). The patient presented no other symptoms and had no chronic diseases or allergies. His blood tests revealed a highly elevated Dsg1 antibody level (130 U/ml; normal range < 20 U/ml) and a slightly elevated γ-glutamyltransferase level. Differential blood count, liver enzymes, creatinine, and Dsg3 antibody level were within the normal range. Histological examination of the patient's skin biopsy revealed an inflammatory infiltrate, eosinophilic spongiosis, and superficial epidermal blister formation.

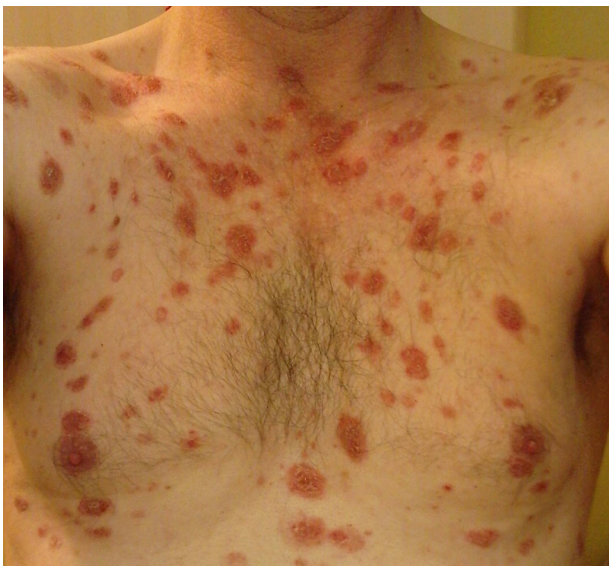

FIGURE 1 | Skin lesions before rituximab treatment.

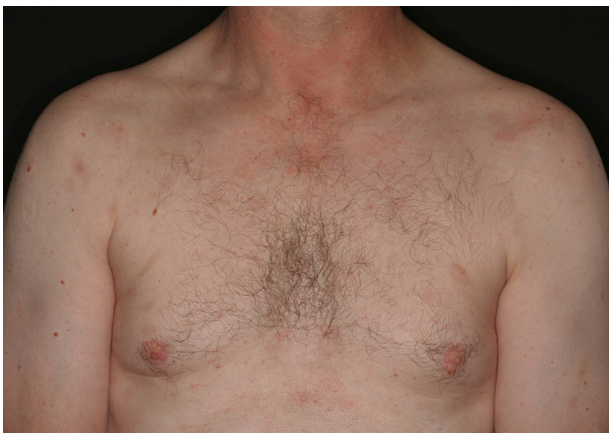

FIGURE 2 | Clinical picture eight months after initial rituximab treatment.

Based on the findings, pemphigus foliaceus was diagnosed and a treatment with prednisolone (10 mg/day) and azathioprine (100 mg/day) was started. Topical therapy with clobetasol propionate and chlorhexidine was also initiated. Furthermore, methylprednisolone infusions (750 mg) were administered once a month for 3 months. This treatment did not result in complete remission; thus, methylprednisolone was replaced with dexamethasone (300 mg) and cyclophosphamide infusions (500 mg) once a month. Azathioprine had to be discontinued due to increasing liver enzymes. The treatment with cyclophosphamide and glucocorticoids was discontinued after 5 months without achieving remission. Hence, we next treated the patient with rituximab. Therefore, two rituximab infusions (1 g each) were administered 2 weeks apart leading to a near-complete b-cell depletion in peripheral blood, a decrease in Dsg1 antibody levels (below the detection range), and an almost complete remission of the skin lesions within the next year (**Figure 2**). Consecutively, therapy with prednisolone (10 mg/day) and topical mometasone furoate was continued and in the following 2 years, the prednisolone dose was reduced to 5 mg/day. The patient remained in remission for 7 years with this therapy (with Dsg1 antibody levels continuously within the normal range). However, in autumn 2017, skin lesions reappeared, which was accompanied by an increase in the Dsg1 antibody levels (75 U/ml). The prednisolone dosage was increased (temporarily up to 60 mg/day), but it was not sufficient to control the disease. Therefore, rituximab infusions (2×1 g within 14 days) were readministered, which led to slow continuous healing of the skin lesions.

DISCUSSION

Numerous case reports and studies have reported on the efficacy of rituximab in treating pemphigus vulgaris but also pemphigus foliaceus (1, 7–11). Recently, Joly et al. (10) published a randomized, multi-center, open-labeled clinical study comparing rituximab and prednisolone as the first line of treatment for pemphigus. The study subjects were treated with 1,000 mg rituximab on days 0 and 14, followed by 500 mg rituximab after 12 and 18 months and 0.5–1 mg/kg prednisolone tapered over 3–6 months. The control group was treated with 1–1.5 mg/kg prednisolone tapered over 12–18 months. After 2 years, the achieved remission rates were 89 and 34% for the rituximab and the control group, respectively. In the control group, the rate of serious adverse events was twice as high as that among the rituximab-treated group.

Thus, rituximab is effective in treating pemphigus foliaceus; however, its ideal dosage regimen is still unknown. In most cases, either 375 mg/m² rituximab is administered once a week for 4 weeks (lymphoma protocol) or two doses of 1,000 mg rituximab are administered 2 weeks apart (rheumatoid arthritis protocol). In their comprehensive review, Ahmed and Shetty (11) reported that responder rates (in pemphigus vulgaris) for both protocols were similar. Because of the low B-cell burden in autoimmune diseases, some authors proposed a low dosage regimen (12–14).

Relapse of pemphigus after rituximab treatment is common (15). In majority of the patients, remission can be maintained up to a few years. A 7-year remission duration, as observed in the current study, is not frequent. Joly et al. reported that 25% of rituximab treated patients relapsed within 2 years (10). Colliou et al. followed 21 rituximab treated patients for 7 years (16). The relapse occurred in 81% of patients and was associated with either persisting or, as in our case, reincreasing Dsg antibody levels. The factors influencing the remission time are not well-characterized (17). The associations between prolonged time to relapse and either higher number of rituximab cycles or a high-dose regiment were described (7, 15, 17, 18). On the other hand, prophylactic administration of an additional rituximab infusion in patients in remission was shown not to be beneficial (19). Saleh (20) reported a correlation between Dsg1 antibody levels at baseline and time to relapse: patients with high levels of Dsg1 antibodies relapsed usually within one year after rituximab treatment, while patients

with low Dsg1 antibody levels stayed in remission for about 2 years. Interestingly there was no association between time to relapse and Dsg3 antibody levels or clinical severity at the time of rituximab treatment.

In a recent small study, Keeley et al. reported that a low-dose maintenance immunomodulatory treatment after rituximab therapy might prevent a relapse (21). Ahmed et al. observed no relapses in ten patients treated with immunoglobulins and rituximab (22). The adjuvant immunoglobulins were administered in average for 34 months following the rituximab treatment and a long-lasting remission was achieved in all patients [average follow up was 7 years after the initial rituximab treatment (22)]. In our patient, it is probable that the prolonged remission was supported by the continuous low-dose prednisolone therapy.

As here observed, treatment with rituximab is successful in the majority of the patients after relapse (15, 18, 23) so that repeated treatment can be recommended for these patients.

Serious adverse event following rituximab treatment are rare. Those are mostly infections (e.g., viral hepatitis reactivation, or herpes virus infections) (24). Few cases of infections with lethal outcomes after rituximab treatment have been reported (24); thus, patients and physicians should be aware of this risk and immediately act in case of suspected adverse event.

CONCLUDING REMARKS

Our case report demonstrates that rituximab can be effective in treating therapy-resistant pemphigus and long-lasting remission may be achieved. A low-dose maintenance immunomodulatory treatment after rituximab therapy may prolong the remission stage. In case of a relapse, repeated treatment with rituximab is usually successful. However, this therapy is limited due to the high cost of rituximab and the risk of rare but severe side effects.

AUTHOR CONTRIBUTIONS

MK wrote the manuscript. MW contributed to conception of the manuscript and revised it critically.

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PERSPECTIVE
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Perspective From the 5th International Pemphigus and Pemphigoid Foundation Scientific Conference

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The 5th Scientific Conference of the International Pemphigus and Pemphigoid Foundation (IPPF), "Pemphigus and Pemphigoid: A New Era of Clinical and Translational Science" was held in Orlando, Florida, on May 15–16, 2018. Scientific sessions covered recent, ongoing, and future clinical trials in pemphigus and bullous pemphigoid, disease activity and quality of life instruments, and the IPPF Natural History Study. Furthermore, the meeting provided an opportunity to hear firsthand from patients, investigators, and industry about their experience enrolling for clinical trials.

Keywords: rituximab, Btk, FcRn, eotaxin, T-cell, fumarate, leukotriene, doxycycline

INTRODUCTION

Following successful meetings in Bethesda, Maryland, USA in 2001, 2005, and 2010, and Lübeck, Germany in 2017, the 5th International Pemphigus and Pemphigoid Foundation (IPPF) Scientific Conference was the first meeting to focus on clinical trials in pemphigus and pemphigoid and their methodologies. The meeting was organized by Drs. Aimee S. Payne, Victoria P. Werth, Russell P. Hall III, and IPPF Director Marc L. Yale and brought together over 175 researchers, clinicians, industry representatives, and patients representing 13 different countries to discuss the latest scientific and clinical data from technologies advancing to clinical trials in pemphigus and pemphigoid.

WHAT'S NEW IN PEMPHIGUS AND PEMPHIGOID CLINICAL TRIALS?

The first session of the meeting focused on results of ongoing and recently completed clinical trials in pemphigus, moderated by Drs. John Stanley (University of Pennsylvania, Philadelphia, PA, USA) and Ron Feldman (Emory University, Atlanta, GA, USA), as well as pemphigoid, moderated by Drs. David Woodley (University of Southern California, Los Angeles, CA, USA) and Enno Schmidt (University of Lübeck, Lübeck, Germany).

Pemphigus

B cells are major effector cells in autoimmunity, both through autoantibody production as well as cellular tolerance mechanisms. Dr. Philippe Musette (Rouen University Hospital, Rouen, France) spoke about major advances being made in refractory pemphigus patients by using B cell depleting therapies, in particular the anti-CD20 monoclonal antibody rituximab. A recent clinical trial (NCT00784589) supports rituximab usage as a first line therapy in pemphigus patients (1). Additionally, B cell depletion in patients represents an opportunity to better understand pemphigus pathophysiology and human B cell biology. Dr. Musette reported that rituximab induces a prolonged and continuous repopulation of naive B cells with a new repertoire after the initial B cell depletion, whereas the reappearance of memory B cells is markedly delayed (2). IL-10-producing regulatory B cells are also expanded and are capable of downregulating inflammation, making them potentially important for maintenance of tolerance. This delay of B cell maturation is associated with a blockage of the auto-reactive IgM to IgG class switching process. Thus, B cell depletion induces a two-step mechanism of immunosuppression in pemphigus by eliminating the autoreactive B cells involved in the production of pathogenic IgG+ autoantibodies and by promoting the appearance of regulatory B cells that can maintain long term immune tolerance.

Inhibition of Bruton's tyrosine kinase (BTK), a protein essential for B cell development, is an appealing therapeutic strategy for pemphigus based on the dual mechanism of action to block autoantibody production as well as to quickly dampen inflammation by inhibiting B cell activation. Dr. Dedee Murrell (University of New South Wales, Sydney, Australia) reported on an open label study of the oral BTK inhibitor, PRN1008 (NCT02704429), which was previously tested in healthy volunteers (3). In the current study, safety and efficacy was demonstrated in 21 pemphigus patients with mild to moderate disease (Pemphigus Disease Area Index score of 8–39), with a disease duration between 0 and 20 years. The primary endpoint of control of disease activity after 4 weeks treatment with PRN1008 was met in 61% of patients, who were concomitantly using a daily prednisone dose between 0 and 30 mg. PRN1008 will shortly commence a large, global, placebo-controlled, randomized phase 3 study in patients with pemphigus.

Inhibition of the neonatal Fc receptor (FcRn) can promote protection from autoimmunity by reducing serum antibody levels and blocking pro-inflammatory immune pathways. Recently, a humanized IgG4 monoclonal antibody was developed that specifically inhibits FcRn function (SYNT001) (4). This antibody has been evaluated in a phase 1b open label safety, tolerability, and activity study to treat subjects with pemphigus (NCT03075904). Dr. Russell Hall (Duke University, Durham, NC, USA) presented results of the phase 1b study showing that infusion of SYNT001 in human subjects resulted in a rapid lowering of circulating levels of IgG and was safe and well-tolerated. Subjects were found to have a mean total IgG reduction of 56% by Day 30, and 5 of 7 subjects showed reduction in disease activity by day 42 (5). Further studies are ongoing to evaluate the efficacy and safety of FcRn blockade in the treatment of pemphigus.

Pemphigoid

Dr. Karen Harman (University of Nottingham, Nottingham, UK) explained the characteristics of pragmatic and non-inferiority trials, illustrated by the BLISTER (bullous pemphigoid steroids and tetracyclines) study (ISRCTN13704604). A pragmatic trial is designed to reflect everyday clinical practice and represents the opposite end of the spectrum to an explanatory trial, performed under ideal conditions. The degree of pragmatism is measured over 9 domains using the PRECIS-2 tool. A non-inferiority trial is used to assess whether a test treatment is not clinically worse than standard care (control) by more than an acceptable predetermined margin (the non-inferiority margin) and comes from the concept of an acceptable alternative that may not be quite as effective standard care but may have other advantages such as cost, convenience or side-effects. The BLISTER study concluded that doxycycline is non-inferior to oral prednisolone for short-term disease control in bullous pemphigoid and significantly safer in the long-term (6).

Since bullous pemphigoid is characterized by a predominance of eosinophils both in the skin and in the blood, it is logical to target the eosinophil by treating with an antibody directed against eotaxin, a chemokine whose levels are elevated in the skin and blood of patients with BP. Dr. Neil Korman (Case Western Reserve University, Cleveland, OH, USA) discussed the results of a pilot phase 2a study of the safety and efficacy of bertilimumab, an anti-eotaxin-1 antibody, in the treatment of patients with bullous pemphigoid (NCT02226146). The results demonstrated that bertilimumab was safe and efficacious in the treatment of bullous pemphigoid. Despite only receiving three bertilimumab infusions and low doses of prednisone that were rapidly tapered, the nine subjects in this study showed rapid and durable improvement in disease activity with an 81% reduction in disease severity, along with a significant steroid-sparing effect. These promising preliminary findings should be followed up with larger controlled trial of longer duration.

Dr. Janet Fairley (University of Iowa, Iowa City, IA, USA) reviewed the current state of therapeutic clinical trials in bullous pemphigoid and studies of the mechanisms of lesion formation that could lead to new treatments. Currently there are no approved drugs for pemphigoid, and the advanced age of these patients and their co-morbidities have made clinical trials challenging. However, recently a number of new treatment options have been identified. Potential targets

include complement, eosinophil chemotaxis and activation, polymorphonuclear cells, IgE and IgE receptors, IgG turnover, and cytokines. Several of these therapeutic strategies have shown promise in pre-clinical trials and case reports or case series. Larger controlled trials may lead to better treatments for pemphigoid patients.

CLINICAL TRIAL ENROLLMENT AND OUTCOMES

The second session of the meeting discussed data using disease-specific instruments that may help to determine clinical trial enrollment and outcomes, moderated by Drs. Katerina Patsatsi (Aristotle University School of Medicine, Thessaloniki, Greece) and Michael Hertl (Philipps-Universität Marburg, Marburg, Germany).

Dr. Jun Yamagami (Keio University, Tokyo, Japan) discussed a longitudinal study to quantify disease extent throughout the course of pemphigus and pemphigoid therapy, in order to better understand how the change in PDAI (Pemphigus Disease Area Index) or BPDAI (Bullous Pemphigoid Disease Area Index) score within the first 2 weeks from the initial treatment predicts whether the patient will require additional treatment. The study found significant change in the PDAI/BPDAI scores from baseline to 2 weeks between patients who needed additional treatment and those who did not, indicating that the ratio of PDAI/BPDAI scores could be useful as an objective parameter to determine the necessity of additional treatments.

Dr. Victoria Werth (University of Pennsylvania, Philadelphia, USA) discussed assessing the "quality" of quality of life surveys in autoimmune blistering disease. There are currently a number of skin-specific and one disease-specific autoimmune blistering disease quality of life instruments. A meta-analysis of health related quality of life in pemphigus identified at least 16 quality of life studies using 8 different instruments. It is important to determine which of the main instruments used capture the impact of disease on patients for both epidemiologic and therapeutic studies. This study sought to compare the change in three quality of life measures, including the autoimmune bullous diseases quality of life (ABQOL) questionnaire, the Dermatology Life Quality Index (DLQI), and the Skindex-29 (the latter of which has three subscores for symptoms, function, and emotion), with change in disease severity (measured in pemphigus with the Pemphigus Disease Area Index (PDAI) and Autoimmune Bullous Skin Disorder Intensity Score (ABSIS), and in bullous pemphigoid with the Bullous Pemphigoid Disease Area Index (BPDAI) and ABSIS) (7). Twenty three patients with mucosal involvement and 27 without mucosal involvement were enrolled and followed prospectively for a mean of 4.6 months. Sixty eight percent had pemphigus vulgaris, 12% had pemphigus foliaceus, and 20% had bullous pemphigoid. The Skindex-29 symptoms subscore correlated best with the change in disease severity ($r = 0.75$) relative to the ABQOL and DLQI ($r = 0.65$ and 0.68). The ABQOL had the best correlation with change in mucosal disease severity relative to Skindex-29 symptoms and DLQI ($r = 0.77$ vs. 0.66 and 0.56), and the Skindex-29 symptoms relative to ABQOL and DLQI had the best correlation with change in skin disease severity ($r = 0.81$ vs. 0.0.59 and 0.75). Overall the change in BPDAI and PDAI correlated more strongly with quality of life measurements than the ABSIS. Dr. Werth noted that the patients enrolled in the study had relatively mild disease, and higher correlations with the PDAI and BPDAI may reflect the ability to capture low disease activity accurately with these instruments.

PATIENT REPORTED OUTCOMES

The third session of the meeting focused on patient-reported outcomes, moderated by Drs. Meng Pan (Shanghai Jiao Tong University, Shanghai, China) and Animesh Sinha (University at Buffalo, Buffalo, NY, USA).

On behalf of the International Pemphigus and Pemphigoid Foundation (IPPF), Marc Yale (IPPF, Sacramento, CA, USA) summarized the Natural History Study being conducted by the IPPF. The IPPF Natural History Study is a new patient registry sponsored by the National Organization for Rare Disorders (NORD) and the US Food and Drug Administration (FDA). This online data system collects, stores, and retrieves patient data for analysis in research studies (https://pemphigus.iamrare.org/). The IPPF Natural History Study serves to provide a convenient online platform for patients or their legally authorized representative to report cases of pemphigus and pemphigoid, conduct a prospectively-planned natural history study that will result in the most comprehensive understanding of both diseases and their progression over time, and characterize and describe the pemphigus and pemphigoid population as a whole. In addition, it also serves to assist the pemphigus and pemphigoid community with the development of recommendations for standards of care, assist researchers studying the pathophysiology of pemphigus and pemphigoid, and support the design of clinical trials that explore new pemphigus and pemphigoid treatments. The IPPF Natural History Study is designed to help the medical and research community understand illness trends, treatment outcomes, disease burden, and some important demographic information about patient age and gender. With this vital information from large numbers of pemphigus and pemphigoid patients, the IPPF can better advocate for resources to improve patient support, education and outreach, as well as accelerate research.

Subsequently, Odette Miller (pemphigus patient, New Jersey, USA), Jeff Weisgerber (pemphigus patient, North Carolina, USA), Dr. Diana Chen (Genentech, South San Francisco, CA, USA), Ann Neale (Principia Biopharma, South San Francisco, CA, USA), and Dr. Donna Culton (University of North Carolina, Chapel Hill, NC, USA) led a panel discussion to allow patients who have participated in recent trials, as well as pharmaceuticals and investigators who have enrolled advanced phase clinical trials in pemphigus and pemphigoid to share their experiences.

COLLABORATIVE NETWORKS FOR RARE DISEASE RESEARCH

The co-founder of the Castleman Disease Collaborative Network, Dr. David Fajgenbaum (University of Pennsylvania, Philadelphia,

PA, USA) gave a keynote speech on the "collaborative network approach" that has helped to spearhead to accelerate research and drug discovery for Castleman disease (8). This information is significant because Castleman disease is one of the most common causes of paraneoplastic pemphigus, a highly deadly form of pemphigus. As rare diseases share many of the same hurdles in the way of drug discovery, there are great opportunities to leverage aspects of the collaborative network approach to make progress for pemphigus and other rare diseases.

CLINICAL TRIALS OF NOVEL CELLULAR THERAPIES FOR PEMPHIGUS

The fifth session of the meeting focused on future clinical trials in pemphigus, moderated by Drs. Annette Czernik (Icahn School of Medicine at Mount Sinai, New York, NY, USA) and Donna Culton (University of North Carolina, Chapel Hill, NC, USA).

In the pathogenesis of pemphigus, loss of immune tolerance to the major autoantigen desmoglein (Dsg) 3 is the key event leading to the production of Dsg-reactive autoantibodies. Dr. Rüdiger Eming (Philipps-Universität Marburg, Marburg, Germany) described strategies to restore immune tolerance to Dsg3 in the CD4+ T cell compartment by applying an HLA-transgenic mouse model of pemphigus. The study has shown that injecting animals with a set of immunodominant HLA-DRß1*04:02-binding Dsg3 peptides that are linked either to cellular carriers (splenocytes) or to defined particles (nanoparticles) prevents the induction of Dsg3-specific IgG antibodies upon Dsg3 immunization. Moreover, T cell reactivity to Dsg3 *in vitro* is markedly reduced in mice that previously received immunodominant Dsg3 peptides under tolerizing conditions. These results in the preclinical model provide the rationale for the development of a future phase I/II clinical trial to restore immune tolerance to Dsg3 in pemphigus vulgaris (PV) patients.

Adapted from a groundbreaking gene-engineered chimeric antigen receptor (CAR) T cell therapy that has led to long-lasting remissions of previously refractory B cell leukemia and lymphoma, Dsg3 chimeric autoantibody receptor T cell (DSG3-CAART) therapy has been shown to induce histologic and serologic remission of experimental pemphigus without detectable off-target toxicity in preclinical mouse models (9). Dr. Aimee Payne (University of Pennsylvania, Philadelphia, PA, USA) discussed strategies to move CAART technology forward to clinical trials in both mucosal PV, caused by antibodies to Dsg3, and in mucocutaneous PV, characterized by autoantibodies to Dsg3 and the homologous protein Dsg1. Dr. Payne reported that DSG1-CAART alone, and combined DSG1- and DSG3-CAART cells showed specific cytolysis of anti-DSG B cells, and no detectable toxicity to human skin xenografts *in vivo*. A first-in-human phase 1 clinical trial of DSG3-CAART in PV is planned to evaluate its safety and therapeutic potential.

Dr. Haley Naik (University of California San Francisco, San Francisco, CA, USA) reported on an autologous polyclonal regulatory T cell therapy as a strategy for limiting autoantibody production by augmenting immune regulation and re-establishing immune tolerance. A phase 1 open-label multicenter trial of autologous polyclonal regulatory T cells in patients with active pemphigus vulgaris and pemphigus foliaceus is being conducted toward this end (NCT03239470). In addition to clinical evaluation for safety, this study also aims to assess the presence and persistence of transferred regulatory T cells in blood and skin, alterations in the tissue immunologic milieu, and disease-specific and immunologic biomarkers in blood and skin. Safety and efficacy data generated from this study will lay the foundation for future studies using antigen-specific regulatory T cell therapy.

EMERGING THERAPIES IN CLINICAL DEVELOPMENT FOR PEMPHIGOID

The final invited speaker session covered future clinical trials in pemphigoid, moderated by Drs. Peter Marinkovich Stanford University, Stanford, CA, USA) and Soo-Chan Kim (Yonsei University, Seoul, Korea).

Dr. Marcel Jonkman (University of Groningen, Groningen, Netherlands) summarized an international survey on unmet needs in pemphigoid diseases to explore and prioritize unmet needs from the perspectives of patients, clinicians, and researchers. The priority need for patients is a quicker diagnosis, for clinicians labeling of new drugs, and for researchers more head-to-head randomized controlled trials. All surveyed groups agreed on a high need for improvement of current treatment options, and future research should focus on this unmet need.

Dr. Ralf Ludwig (University of Lübeck, Lübeck, Germany) reported on an upcoming clinical trial evaluating the safety and efficacy of dimethyl fumarate (DMF) in bullous pemphigoid, which will enroll the first patients this year in France, Germany, Poland and Turkey (DPem Trial). This trial was based on findings in preclinical animal models of epidermolysis bullosa acquisita (EBA) (10), where DMF reduced clinical disease severity in mice with already clinically manifest skin lesions. In addition to DMF, preclinical EBA models (11) have defined several new therapeutic targets, i.e., SYK (12, 13) and novel compounds (14).

Dr. Christian Sadik (University of Lübeck, Lübeck, Germany) presented the topic of the C5a-LTB_4 axis in bullous pemphigoid diseases (15). The eicosanoid leukotriene B_4 (LTB_4) and the complement factor C5, the precursor of the anaphylatoxin C5a, are both abundant in lesional skin of bullous pemphigoid patients, but their significance for the pathogenesis of bullous pemphigoid or other pemphigoid diseases is still largely elusive. In his talk, Dr. Christian Sadik demonstrated preclinical results from mouse models of pemphigoid diseases pointing at a critical role of LTB_4 in the recruitment of granulocytes to the dermal-epidermal junction (16). He discussed evidence that LTB_4 may closely interact with C5a in the regulation of skin inflammation and that hence, inhibiting these two factors individually or in parallel may be effective in the treatment of pemphigoid diseases.

CONCLUSIONS

The next decade offers exciting promise for an increasing number of clinical trials in pemphigus and pemphigoid as new preclinical

programs advance to clinic and existing clinical stage companies apply their technologies to the treatment of pemphigus and pemphigoid. The patients, physicians, and researchers in the pemphigus and pemphigoid community remain committed to advocating for treatment options that improve the health and quality of life for pemphigus and pemphigoid patients.

AUTHOR CONTRIBUTIONS

JL wrote the first draft of the manuscript; VW, RH, RE, JF, DF, KH, MJ, NK, RL, DM, PM, HN, CS, JY, MY, and AP wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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Diagnosis of Autoimmune Blistering Diseases

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Autoimmune skin blistering diseases (AIBD) are characterized by autoantibodies that are directed against structural proteins in the skin and adjacent mucous membranes. Some clinical signs are typical for a specific AIBD, however, correct diagnosis requires the detection of tissue-bound or circulating autoantibodies. The gold standard for diagnosis of AIBD is the detection of autoantibodies or complement component 3 by direct immunofluorescence (DIF) microscopy of a perilesional biopsy. Circulating antibodies can be detected via indirect immunofluorescence (IIF) microscopy of different tissue substrates including human skin, monkey esophagus, and more recently, recombinant forms of the different target antigens. Latter are also employed in various commercial ELISA systems and by immunoblotting in in-house assays available in specialized laboratories. ELISA systems are also particularly valuable for monitoring of the disease activity during the disease course which can be helpful for treatment decisions. Exact diagnosis is essential for both treatment and prognosis, since some AIBD are associated with malign tumors such as paraneoplastic pemphigus and anti-laminin 332 mucous membrane pemphigoid. This review presents clinical and immunopathological features of AIBD for the state-of the art diagnosis of these disorders.

Keywords: autoantibody, biochip, immunofluorescence, ELISA, pemphigus, pemphigoid, epidermolysis bullosa acquisita, dermatitis herpetiformis

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INTRODUCTION

Autoimmune skin blistering diseases (AIBD) are a diverse group of dermatoses that are characterized by autoantibodies binding to antigens in the skin and mucous membranes. They can be subdivided into pemphigoid diseases (PD), with subepidermal split formation and autoantibody binding to structural components of the dermal-epidermal junction (DEJ), and pemphigus, with autoantibodies directed against desmosomal proteins that connect neighboring keratinocytes (1, 2). A special type of AIBD is dermatitis herpetiformis, with autoantibodies directed against the tissue and epidermal transglutaminase. In this review, we will provide a comprehensive overview about the clinical features and current diagnosis of AIBD extending and updating previous work (3, 4).

EPIDEMIOLOGY

Bullous pemphigoid (BP) is the most frequent AIBD in Central Europe. Its incidence reaches around 20/million/year. BP is followed by mucous membrane pemphigoid (MMP) and pemphigoid gestationis, with incidences of 2/million/year, respectively (5–8). Higher incidences of BP have been reported in Great Britain (9). In contrast to other autoimmune diseases, the incidence of BP is increasing with age. Regarding this matter, its annual incidence in people older than 80

years reaches 150–180/million/year (5, 6). Like other autoimmune diseases, the incidence of BP is constantly increasing and has nearly doubled in the last decade (7–11). This is partly due to the rising life expectancy of the general population, increasing awareness, and enhanced diagnostic tests. Further, the close association between BP and neurological diseases [reviewed in (12)], whose incidences are also rising, may contribute to the increased occurrence of BP. This rise in BP incidence is reflected by hospitalization numbers of BP patients that increased by 26% for a primary diagnosis and by 62% for a secondary diagnosis to 3,260/million inpatients between 2002 and 2012 in the USA (13).

In pemphigus, the incidence depends on the geographical region. In Central Europe and the United States, its incidence is estimated between 1 and 7 new patients/million/year (9, 14). Generally, PV is more common than pemphigus foliaceus (PF), with ratios ranging from 4:1 to 9:1 (15). In Tunisia and Brazil, endemic forms of PF with much higher incidences are present (16, 17).

The prevalence of AIBD in Germany have recently been calculated based on the ICD-coding-based dataset of the country's largest health insurance. The study revealed about 40,000 AIBD patients including 21,000 patients with BP, 7,700 with PV, and around 2,000 with MMP (18).

HISTORICAL BACKGROUND

The term pemphigus was first used by Hippocrates in 460–370 B.C. (19). However, the differentiation between pemphigus and BP was first made by Walter Lever in 1953 based on lesional histopathology (20). In 1964 and 1967, detection of autoantibodies in serum and skin were reported for pemphigus and BP (21, 22), providing milestones for the diagnosis of AIBD. Diagnosis of the different AIBD entities became subsequently possible by the molecular identification of target antigens (23). In parallel it became clear that the autoantibodies used for the diagnosis of AIBDs may be directly pathogenic (24–29), reviewed in (1, 30–34).

DIRECT IMMUNOFLUORESCENCE MICROSCOPY

The diagnosis of AIBDs is based on the combination of the clinical presentation and detection of tissue-bound and/or circulating autoantibodies. Tissue-bound autoantibodies can be detected via direct immunofluorescence (DIF) microscopy, which is the diagnostic gold standard for AIBD. For DIF microscopy, cryosections of perilesional biopsies are required and need to be snap frozen and stored at −20°C or conserved in isotonic NaCl or modified Michels medium until processed (35, 36).

DIF microscopy only provides limited information about the target antigen(s), however the diagnosis can be narrowed down according to the immunoglobulin subclass and binding pattern. In pemphigus, DIF microscopy reveals intercellular binding of IgG and/or C3 within the epidermis and/or epithelium. In pemphigoid diseases, a linear deposition of IgG and/or C3 at the DEJ can be observed (**Figures 1**, **2**). Linear staining at the DEJ can further be differentiated into n-serrated and u-serrated patterns. In an n-serration pattern, arches are closed at the top (**Figure 3** left) and in a u-serrated staining pattern, arches are closed at the bottom appearing like "growing grass" (**Figure 3** right). While u-serration is unique for antibody binding to type VII collagen and can been seen in epidermolysis bullosa acquisita (EBA), n-serration is found in all other pemphigoid diseases (37–39). Serration pattern analysis can be performed in any routine immunofluorescence (IF) laboratory and is best performed in 6 μm sections and 400- or 600-fold magnification without oil (38, 39). IF pictures for training of serration pattern analysis are freely available (www.nversusu.umcg.nl).

In dermatitis herpetiformis, DIF microscopy reveals granular deposition of IgA at the dermal papillae and along the DEJ. An automated staining for DIF microscopy sections has recently been developed and revealed more intensive IF staining and reduced background compared to the manual procedure due to continuous movement and overhead incubation (40). A flowchart navigating through differential diagnoses using DIF microscopy is shown in **Figure 1**. For further differentiation of the target antigen(s) serological analyses is required.

INDIRECT IMMUNOFLUORESCENCE MICROSCOPY USING TISSUE SUBSTRATES

Several tissues can be employed by indirect IF (IIF) microscopy to screen for serum autoantibodies in AIBD including monkey, rabbit, guinea pig, and human esophagus (for pemphigus and pemphigoid diseases), monkey and rat bladder (for paraneoplastic pemphigus), and amnion epithelium (for BP and PV). In one study, monkey esophagus was the most sensitive substrate for pemphigus; another study showed that monkey esophagus is more sensitive for PV and human esophagus is more sensitive for PF (41–44). The most frequently used substrates are monkey esophagus and human split skin. On monkey esophagus, autoantibodies in pemphigus reveal intercellular labeling of the epithelium and linear staining of the DEJ in pemphigoid diseases (**Figure 2**). Sensitivities of 90% and 73.2% have been reported for pemphigus and BP, respectively (41, 43). In dermatitis herpetiformis, IgA binds to the endomysium. The tissue substrate with the highest sensitivity for autoantibodies in pemphigoid diseases is 1M NaCl split human skin. Here, antibodies bind either to the epidermal ("roof") or dermal ("floor") side of the artificial blister (**Figure 2**, left and right panel, respectively). "Floor"-binding antibodies can be detected in EBA, anti-p200/laminin γ1 pemphigoid, and anti-laminin 332 MMP. "Roof"-binding antibodies target BP180 and BP230 and are observed in BP, linear IgA-disease, pemphigoid gestationis, and anti-BP180-type mucous membrane pemphigoid. Sensitivities for BP range between 73 and 84% (41, 45).

The most sensitive substrates for the detection of anti-plakin reactivity are monkey and rat bladder epithelium. In pemphigoid

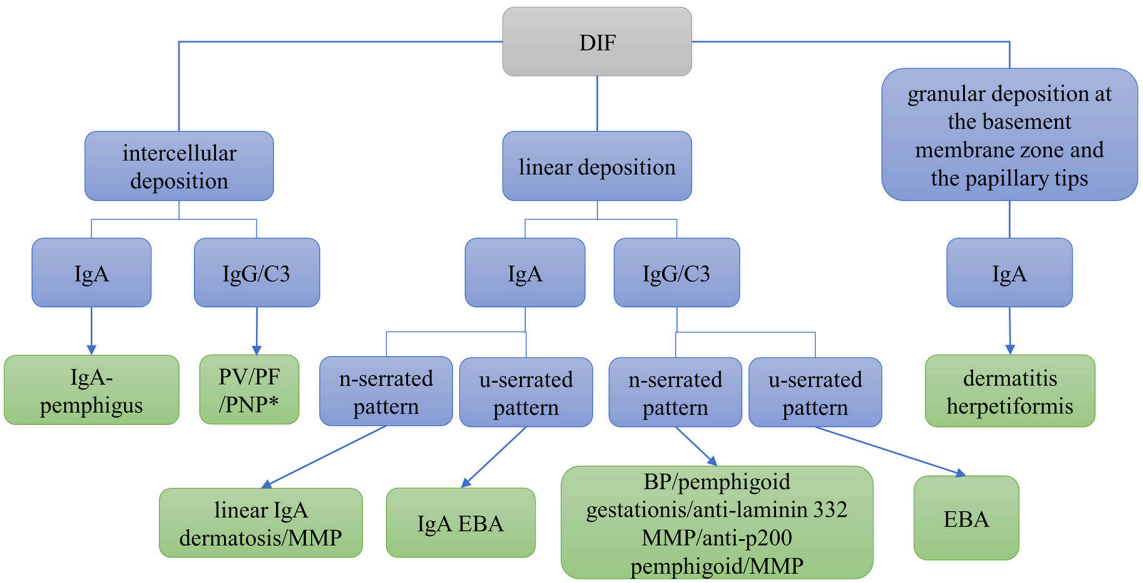

FIGURE 1 | Differential diagnosis of autoimmune skin blistering diseases based on direct immunofluorescence (DIF) microscopy of a perilesional biopsy. BP, bullous pemphigoid; EBA, epidermolysis bullosa acquisita; MMP, mucous membrane pemphigoid; PF, pemphigus foliaceus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris; *may be combined with linear deposition.

gestationis, the complement fixation test detects complement-fixing IgG on human salt-split skin. For definite diagnosis of most AIBD refined analysis of serum autoantibodies can be performed, employing recombinant or cell-derived antigens. A flowchart depicting the serological diagnosis of autoimmune blistering diseases is shown in **Figure 4**.

TARGET ANTIGEN-SPECIFIC ANALYSIS OF SERUM AUTOANTIBODIES

For the identification of the target antigen, three main systems have been described: (i) Enzyme-linked immunosorbent assay (ELISA), (ii) IIF microscopy, and (iii) immunoblot/immunoprecipitation.

(i) ELISA systems allow the identification and quantification of autoantibodies against specific autoantigens. They are applied for both diagnosis and monitoring of the activity of the disease during the disease process (46). For pemphigoid diseases, commercial ELISA systems include BP180 NC16A, BP230, and type VII collagen, which employ recombinant protein, respectively (MBL, Euroimmun) (47–52). The sensitivity of the BP180 NC16A ELISA ranges between 84 and 89% in BP (47, 49, 53) and between 96 and 97% in pemphigoid gestations (54, 55). The sensitivity in BP can be increased by the additional use of the BP230 ELISA by about 5% (52, 56, 57). For pemphigus, ELISA systems employ the ectodomains of Dsg1 and Dsg3 recombinantly expressed in HEK293 cells (Euroimmun) or baculovirus (MBL, Nagoya, Japan) (46, 58, 59). For paraneoplastic pemphigus, an ELISA system for autoantibodies against envoplakin has been developed (Euroimmun) (60). For dermatitis herpetiformis, ELISA systems for the detection of coeliac-specific gliadin IgG and IgA autoantibodies as well as anti-transglutaminase 2 and 3-antibodies are available (61). ELISA systems that are less standardized and only available in specialized laboratories include desmocollin (62, 63), laminin γ1 (64, 65), the ectodomain of BP180 (66), full-length BP180 (67), laminin 332 (68, 69) and BP180 NC16A-IgE-ELISA (70–72) as well as other forms of BP180 (73, 74).

In addition, two multivariant ELISA systems compiled of the individual assays include recombinant Dsg 1 and 3, BP180 NC16A, BP230, type VII collagen, and only in one system, envoplakin, are widely available (75, 76).

(ii) IIF-based assays employing recombinant forms of the target antigens are available as multivariant assays and thus, offer a single-step method for the diagnosis of AIBDs. These assays are based on the BIOCHIP® mosaic technology using normally-sized laboratory slides with 5–10 incubation fields. The serum sample is loaded onto an incubation field, consisting of several miniature biochips coated with different substrates (e.g., monkey esophagus, salt-split skin, recombinant BP180 NC16A or HEK293 cells recombinantly expressing Dsg1, Dsg3, or BP230). We have shown that the sensitivity and specificity of BIOCHIP® mosaic analysis is comparable to that of ELISA systems regarding AIBDs (77). Meanwhile, this technology has been applied in different routine laboratories worldwide (78–80). More recently, a mosaic comprising 4 biochips coated with recombinant BP180 NC16A, HEK cells expressing BP230, salt-split skin and monkey esophagus, respectively, showed a sensitivity

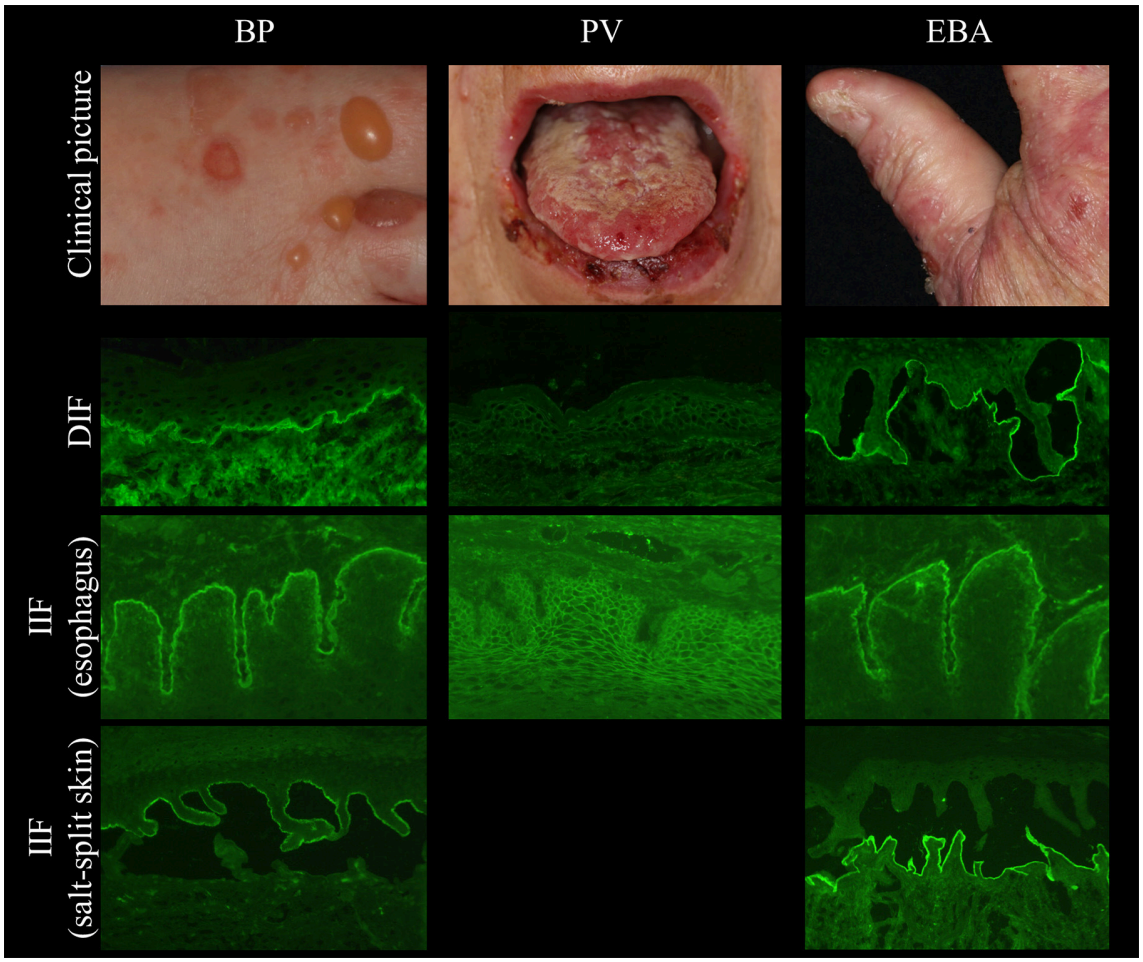

FIGURE 2 | Clinical and immunopathological characteristics in bullous pemphigoid (BP, **left**), pemphigus vulgaris (PV, **middle**), and epidermolysis bullosa acquisita (EBA, **right**).

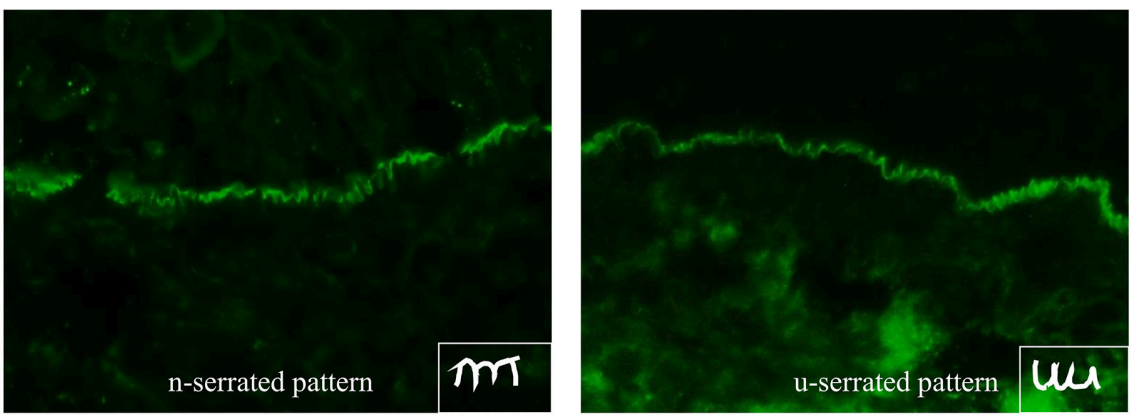

FIGURE 3 | n-serrated **(left)** and u-serrated pattern **(right)** of basement membrane zone staining in pemphigoid diseases detected by direct immunofluorescence microscopy. While an u-serrated pattern is exclusively seen in epidermolysis bullosa acquisita, an n-serrated pattern can be detected in all other pemphigoid diseases.

of 100% when testing with pemphigoid gestationis sera (79). A BIOCHIP® mosaic including the immunodominant NC1 domain of type VII collagen yielded sensitivities of 92 and 100% (50, 81), indicating that BIOCHIP® technology is a valuable tool in the routine diagnosis of pemphigoid gestationis and EBA. As for desmocollins, anti-desmocollin

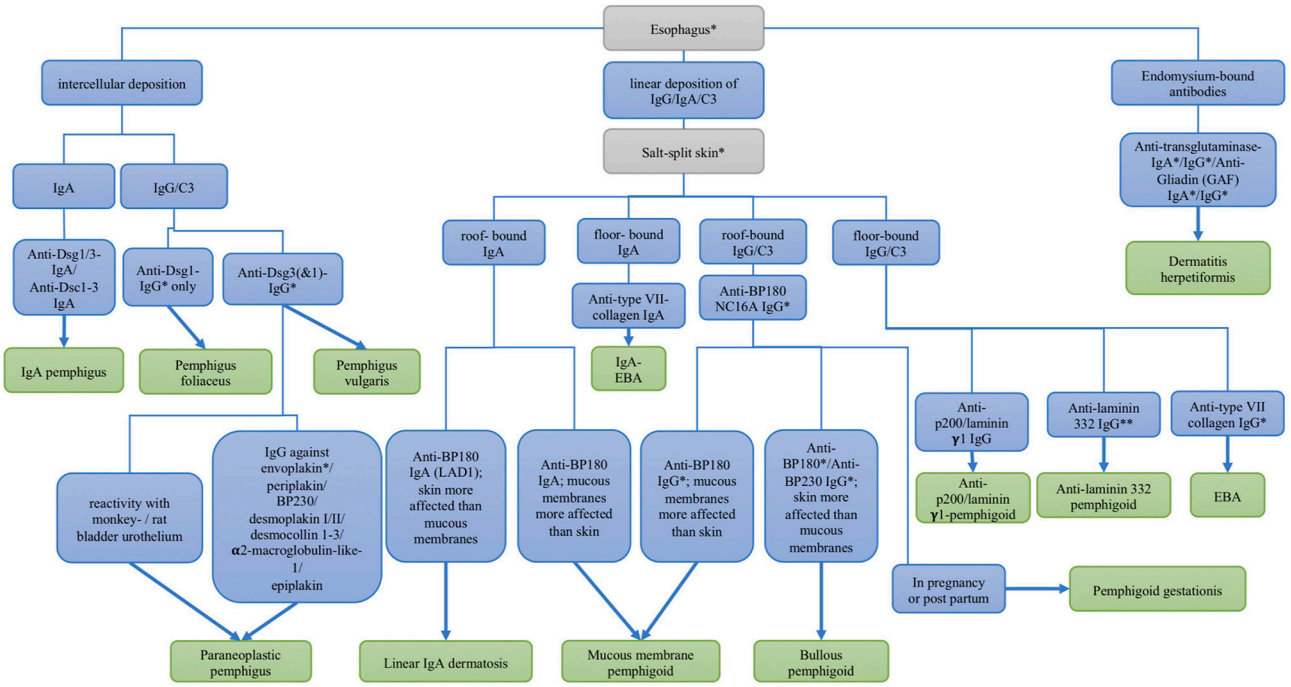

FIGURE 4 | Differential diagnosis of autoimmune skin blistering diseases based on the serological detection of autoantibodies. Dsc, desmocollin; Dsg, desmoglein; EBA, epidermolysis bullosa acquisita; * commercial assays are available; ** available by the end of 2018.

IgG and/or IgA reactivity was only found in about 3% of around 400 pemphigus sera, using a BIOCHIP® mosaic containing recombinant forms of desmocollin 1, −2, and −3 (82). Therefore, according to the guidelines of the German Dermatological Society, the analysis for anti-desmocollin reactivity is only recommended in patients with IgA pemphigus, pemphigus vegetans, atypical pemphigus, and the rare patients with pemphigus vulgaris/foliaceus without anti-Dsg reactivity (36). Most recently, a BIOCHIP® mosaic was developed containing recombinant chains of laminin 332 for the diagnosis of anti-laminin 332-pemphigoid (83) (**Figure 5**). This BIOCHIP® mosaic yields sensitivities between 75 and 85% with a specificity of nearly 100% (83).

(iii) Immunoblotting and immunoprecipitation are performed using recombinant proteins or extracts of dermis, epidermis, bovine gingiva, amnion membrane or cultured keratinocytes (80–86). These systems are part of the diagnostic algorithm for AIBD in some laboratories. They can be used for the detection of anti-p200 autoantibodies (**Figure 6**), anti-laminin γ1 autoantibodies, antibodies against C-terminal stretches of BP180, and the soluble ectodomain of BP180 (LAD-1; **Figure 7**), as well as autoantibodies against cell-derived forms of envoplakin, periplakin, desmoplakin, BP180, BP230, α4β6-integrin, laminin 332, and type VII collagen (**Figure 6**) (87). Latter test systems are, however, only available in specialized laboratories including the autoimmune laboratory of the Department of Dermatology, Lübeck, Germany (88). The laboratory has been accredited by Deutsche Akkreditierungsstelle (DAkkS D-ML-13069-06-00) and is also involved in the development of novel assay systems (www.uksh.de/dermatologie-luebeck/Infos+für+Ärzte+und+Einsender/Autoimmunlabor.html). The main diagnostic algorithm of our laboratory is shown in **Figure 4** and further detailed in Schmidt et al. (3).

DIAGNOSTICALLY RELEVANT CLINICAL AND IMMUNOPATHOLOGICAL CHARACTERISTICS OF MAJOR AIBD

Pemphigus Vulgaris and Pemphigus Foliaceus

Pemphigus can be divided in two major clinical subtypes, PV and PF. Autoantibodies in pemphigus are directed against epidermal desmosomes, mainly desmoglein (Dsg) 1 and 3. A common clinical finding is a positive Nikolsky sign. Here, mechanical friction of perilesional skin results in exfoliation of the outermost skin layer. The Nikolsky sign moderately sensitive, but highly specific in the diagnosis of pemphigus (89). PF is clinically characterized by flaccid, superficial erosions preferentially in seborrheic areas. The erosions are usually covered by scaling, which is due to the detachment of the superficial layers of the epidermis (90). In PF, mucous membranes are completely spared (3). Autoantibodies in PF are directed against Dsg1 and can be detected by ELISA or IIF microscopy (46, 88, 91) (**Figure 4**). In nearly all PF patients, anti-Dsg1 serum levels closely correlate with disease activity (46).

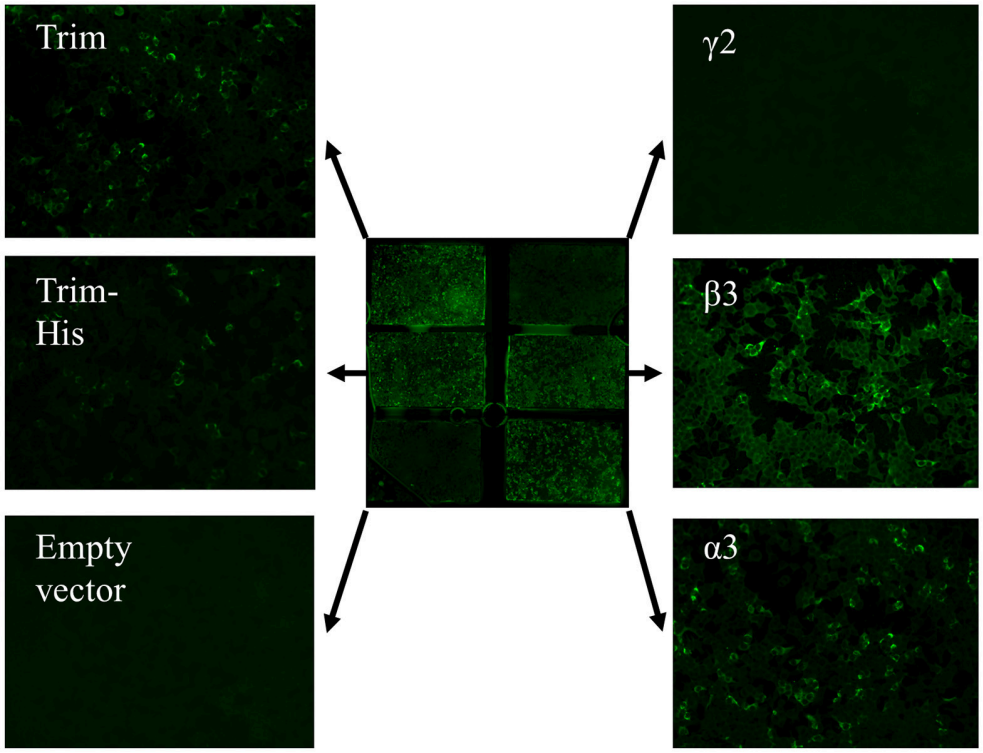

FIGURE 5 | Anti-laminin 332 BIOCHIP® mosaic. The mosaic contains HEK293 cells expressing the recombinant laminin 332 heterotrimer (Trim; upper left and middle left), the recombinant γ2- t β3-, and α3-chains of laminin 332 as well as negative control, cells transfected with the empty vector. Here, antibody binding is seen with the heterotrimer and the β3- and α3-chains in a patient with anti-laminin 332 mucous membrane pemphigoid.

In contrast to PF, patients with PV always suffer from mucous membrane lesions (**Figure 2** middle). These are accompanied to a variable extent with blisters and/or erosions of the skin. Autoantibodies in PV are directed against Dsg 3 (92). When in addition to mucosal involvement, lesions are also present on the skin, patients with PV also have autoantibodies against Dsg1 (1). According to the extent of affected skin, three types of PV can be distinguished: (i) the mucosal-dominant type with limited cutaneous involvement (Dsg 3-autoantibodies are predominant), (ii) the mucocutaneous type with both mucosal and cutaneous involvement (Dsg3- and Dsg1-autoantibodies are equally predominant) and the cutaneous type with predominant anti-Dsg1 and pathogenically weak anti-Dsg3 autoantibodies (1). Alike Dsg1-specific autoantibodies, anti-Dsg3-autoantibodies can be detected by ELISA (46). Both Dsg1- and 3-autoantibody levels correlate with the disease activity and can therefore be used as disease activity marker (30, 46).

Paraneoplastic Pemphigus

Paraneoplastic pemphigus (PNP) is an AIBD that is characterized by its association with malignant (or rarely benign) neoplasms. The most frequently associated neoplasms are B-cell lymphoma, Castleman disease, chronic lymphocytic leukemia, thymoma, and Waldenstrom macroglobulinemia (84, 93, 94). The clinical phenotype is diverse. First, PNP mainly affects the oral mucosa with other mucous membranes less frequently involved (95–97). Cutaneous lesions may arise on any part of the skin and may include: (i) pemphigus-like lesions with flaccid blisters, erosions, erythema and crusts; (ii) BP-like lesions such as urticarial lesions and tense blisters (96); (iii) erythema multiforme-like lesions and (iv) lichen planus-like lesions presenting as flat scaly papules and intense mucous membrane involvement (95). Furthermore, pulmonary destruction leading to bronchiolitis obliterans was noticed in many PNP-patients (98).

Apart from Dsg3, the autoantibodies may be directed against plakins such as BP230, periplakin, envoplakin, desmoplakin 1 and 2, and plectin (84). More recently, antibodies against desmocollins, α2 macroglobulin-like 1, and epiplakin have been described (62, 99, 100) (**Figure 4**). Antibodies against envoplakin and periplakin are most frequent (60, 101, 102). They can be detected via Western blotting or immunoprecipitation of extracts from keratinocytes (84), and, more conveniently, by a commercial ELISA employing the recombinant N-terminus of envoplakin (60).

Bullous Pemphigoid

In BP, autoantibodies are directed against a 180 kDa-sized (BP180/BPAG2/XVII collagen) and/or a 230 kDa-sized (BP230/BPAG1) antigen, which are essential for dermal-epidermal adhesion (103) (**Figure 2** left). The disease is mainly diagnosed in people aged between 75 and 80 years (18). It rarely occurs in people under the age of 50 years with few children

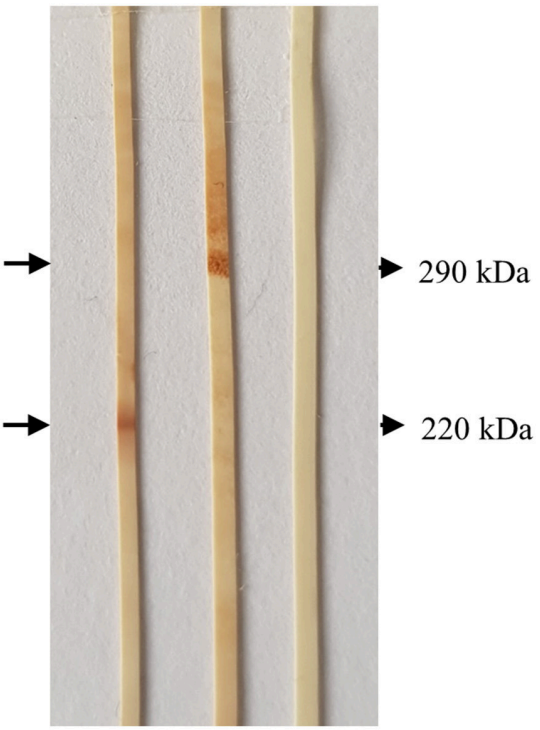

FIGURE 6 | Detection of serum autoantibodies against the p200 protein and type VII collagen by Western blotting with extract of human dermis. Sera from a patient with anti-p200/laminin γ1 pemphigoid and epidermolysis bullosa acquisita (EBA), respectively, showed IgG4 reactivity against the p200 antigen **(lower arrow)** and type VII collagen **(upper arrow)**. In the right lane, a normal human serum (NHS) was applied.

described with BP (104). In nearly all patients with BP, intense pruritus is present (105). Classically, BP presents with tense blisters and erosions. In contrast to pemphigus, the Nikolsky sign is negative. Alternatively or additionally, urticarial and erythematous non-bullous lesions are present (106). In fact, about 20% of patients present with non-bullous variants with excoriations, erythematous, or urticarial lesions (107). Non-bullous lesions also usually develop during a prodromal stage that may last for several months. Mucosal lesions, which occur in 15–20% of the BP patients, are associated with high disease severity and with absence of anti-BP230 antibodies (108).

BP180 is a collagen-type transmembrane glycoprotein of about 1,500 amino acids. It is a heterotrimer, consisting of a globular intracellular domain, a short transmembranous segment, and an extracellular C-terminal domain composed of 15 collagen repeats that are separated by 16 noncollagenous (NC) subdomains (109). The C-terminal domain forms a loop structure as it goes through the lamina lucida, spans the lamina densa, and then bends back into the lamina lucida (110). The 16th of the extracellular non-collagenous subdomains, NC16A, is the immunodominant region in BP (111). It is used in two ELISA systems (47, 48) (**Figure 4**), which on the one hand provide a sensitive and specific diagnostic tool for the routine diagnosis of BP and on the other hand are used to monitor the serum levels of anti-BP180 NC16A antibodies during the course of the disease (112). Alternatively, an IIF test using recombinant NC16A is widely available (77) (**Figure 4**). In most of the BP patients, autoantibodies are also directed against BP180-epitopes outside the NC16A-domain (113). In the 10–15% of BP patients with no reactivity against NC16A, testing for those antibodies is recommended; however, no commercial assay is available so far. In addition to several recombinant fragments of the C-terminal part of BP180, the cell-derived 120 kDa shed ectodomain present in the conditioned concentrated medium of cultures keratinocytes can be applied by immunoblotting for the detection of non-NC16A-reactive sera.

The major immunoglobulin class in BP is IgG. However, it has been shown that some patients also develop anti-BP180 IgA and IgE autoantibodies (114). In fact, most of the BP sera contain both IgG and IgA autoantibodies to BP180 (114, 115). Anti-BP180 IgE antibodies can be detected in 30–95% of the BP patients, and their detection corresponds to a high disease severity (70, 116).

BP230 is an intracellular component of the hemidesmosomal anchoring complex. It is a member of the plakin family. Anti-BP230 IgG can be detected in the serum of 40–60% of the BP patients. Like for BP180, commercial ELISA systems are available for the detection of anti-BP230 antibodies, which can be used for the diagnosis of BP (49, 52) (**Figure 4**). 80% of the anti-BP230 autoantibodies found in the sera of BP patients most frequently target the globular C-terminal domain of BP230 that interacts with keratin filaments (117, 118). However, it remains unclear whether anti-BP230 antibodies are directly pathogenic (119). Also, unlike anti-BP180, serum levels of anti-BP230-antibodies do not correlate with the disease activity in BP patients (120). However, the detection of anti-BP230 autoantibodies remains a useful tool for the diagnosis of BP. Regarding this matter, the combined use of the BP180 and BP230 ELISA system provides a sensitivity of around 90% for the detection of circulating autoantibodies (52, 56, 57).

Pemphigoid Gestationis

Pemphigoid gestationis (previously called herpes gestationis) is a dermatosis of pregnancy. It usually occurs during the third trimenon and less commonly, in the second trimenon or post partal period (121, 122). In contrast to BP, blisters are infrequent and usually small with predominating urticarial erythema frequently initiating around the umbilicus. Pemphigoid gestationis tends to recur in subsequent pregnancies, appearing earlier and with a more severe course. Serum autoantibodies to BP180 NC16A can be detected in >95% of the patients by ELISA, having a sensitivity of 97% (**Figure 4**) (47, 55). Recently, Sadik et al. detected NC16A reactivity in all of a large cohort of 65 pemphigoid gestationis sera using an IIF test based on the Biochip® mosaic technology (123). The main IgG subclasses are IgG1 and IgG3, explaining their high potential

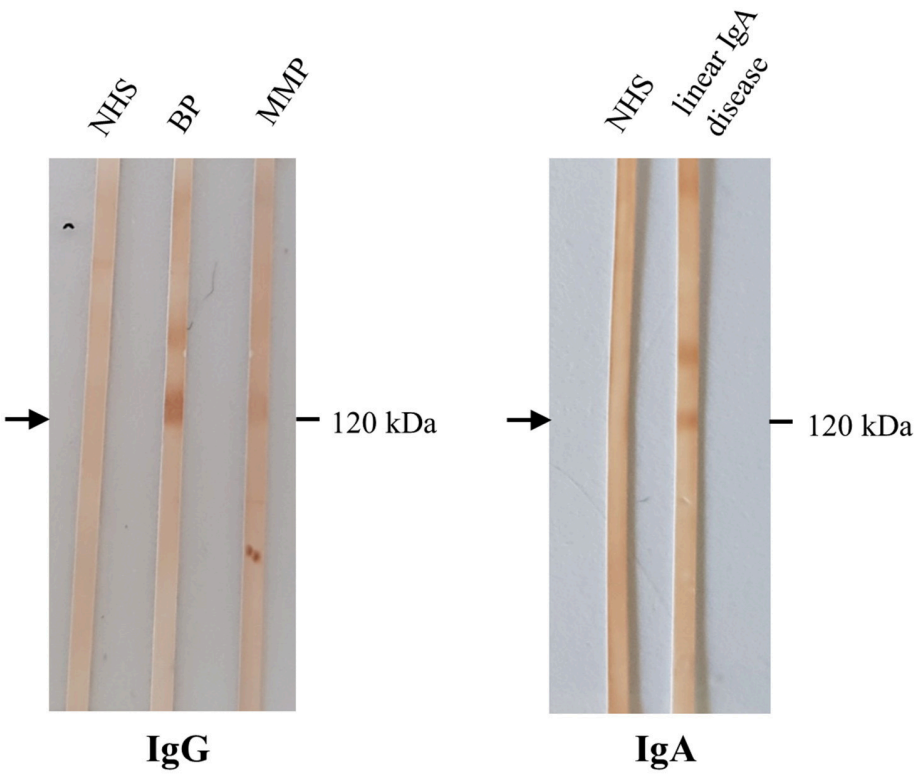

FIGURE 7 | Detection of IgG and IgA autoantibodies against LAD-1 by Western blotting with the concentrated conditioned medium of cultured human keratinocytes. Sera from patients with bullous pemphigoid (BP), mucous membrane pemphigoid (MMP), both without BP180 NC16A IgG by ELISA, showed IgG reactivity with the soluble ectodomain of BP180 (LAD-1; **left**, arrow). In serum of a patient with linear IgA disease, IgA antibodies against LAD-1 were present **(right)**. NHS, normal human serum.

for the fixation of complement (124), a feature that is exploited by the IIF complement binding test that visualizes complement-binding anti-basement membrane antibodies (**Figure 4**). Anti-BP230 reactivity is found in only 10% of sera (123).

Mucous Membrane Pemphigoid

MMP is defined as pemphigoid disease with predominant involvement of mucous membranes (125). It usually affects the mucous membranes of the mouth, eyes and genitals. Complications of the disease are conjunctival involvement and blindness, which may cause serious morbidity (**Figure 8**) (126, 127). Diagnosis is made by DIF microscopy of a perilesional biopsy, showing linear deposition of IgG and/or IgA and/or C3 along the DEJ (**Figure 1**) (126). In IIF microscopy, autoantibodies can only be detected in 50% of the MMP patients. Therefore, immunoprecipitation, Western blotting, and ELISA systems that employ cell-derived and recombinant proteins are essential diagnostic tests for MMP (**Figure 4**) (126). The main target antigen in MMP is BP180. However, in contrast to BP, the NC16A domain is only targeted in around 50%. More commonly, the autoantibodies target C-terminal epitopes of BP180 such as LAD-1, the soluble ectodomain of BP180 (128, 129). Those autoantibodies can be detected by Western blotting, using the respective recombinant fragments of the C-terminus of BP180. Both anti-BP180 IgG and IgA are predominant in anti-BP180-type MMP. Therefore, it is necessary to test for both isotypes (**Figure 4**) (128). Further antigens in MMP are laminin 332 as well as α6 and β4 integrin. Anti-laminin 332 antibodies can be detected in around 25% of the MMP-patients (128, 130). Laminin 332, which was formerly known as epiligrin or laminin 5, is a heterotrimeric protein composed of an α3, β3, and γ2 subunit. Anti-laminin 332 autoantibodies typically target the α3 chain (131). Since the presence of anti-laminin 332 autoantibodies is associated with development of malignancies in 25% (38, 132, 133), screening for anti-laminin 332 reactivity is strongly recommended in every MMP patient and in case of positivity, a tumor search is mandatory. Unfortunately, no detection system for serum antibodies against laminin 332 is widely available. A sensitive and highly specific assay for serum anti-laminin 332 IgG based on the Biochip® mosaic technology has recently been developed (83) and will be commercialized later in 2018. Antibodies against α6 and β4 integrin were detected in a few cases of oral and conjunctival MMP, respectively (134).

Linear IgA Disease

Linear IgA disease (LAD) is characterized by subepidermal blistering and linear deposition of predominantly IgA at the DEJ. The disease is characterized by its heterogenous phenotype that

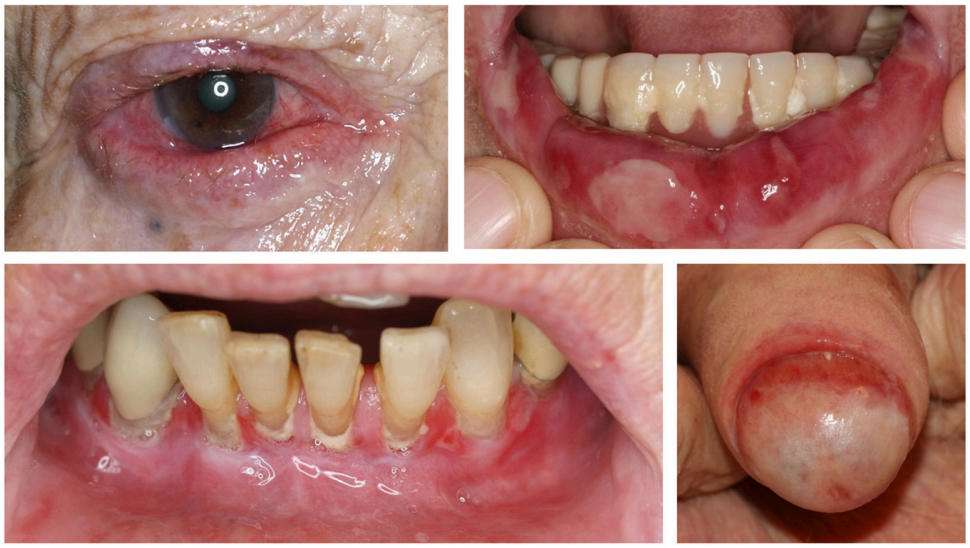

FIGURE 8 | Clinical findings in mucous membrane pemphigoid affecting the conjunctiva, lips, gingiva, and glans penis.

may be similar to other autoimmune skin blistering diseases. Mostly, the patients present with vesiculobullous lesions on the skin and adjacent mucous membranes (135, 136). Using DIF microscopy of a perilesional biopsy, a linear deposition of IgA autoantibodies along the DEJ can be detected (**Figure 1**). The autoantibodies bind to antigens of different molecular weights, including 97-, 120-, 180-, 200-, 230-, 280-, 285-, and 290-kDa proteins (137–139). According to the IIF findings, LAD can be divided into the lamina lucida type, where sera react with the epidermal side of salt-split skin and mostly with LAD-1 (140), and the sublamina densa type, that reveals serum antibodies against the dermal side of salt-split skin recognizing type VII collagen (138, 141, 142) (**Figure 4**). Interestingly, a recent study showed that type VII collagen is also the most common target antigen in vancomycine-induced LAD (143). Due to sematic overlap, patients with IgA reactivity against type VII collagen can also be classified as IgA EBA, a view that is supported by a recent consensus of an international expert panel (144).

Anti-P200/Anti-laminin γ 1 Pemphigoid

Anti-p200 pemphigoid is an autoimmune skin blistering disease with antibodies directed against a 200 kDa protein of the DEJ (145). Since laminin γ1 is the target antigen in 90% of the cases, it is also known as anti-laminin γ1 pemphigoid (65). Like LAD, the clinical presentation is heterogeneous and in most of the cases resembles BP and the inflammatory variant of EBA. The lesions heal without scarring or milia formation. Mucous membranes are involved in about 20% of the patients (146). Palmoplantar involvement seems to be more frequent compared to BP and a high association with psoriasis is seen in Japanese patients with anti-laminin 332 pemphigoid (146, 147). Antibodies against p200 bind to the floor of the artificial blister of salt-split skin using IIF microscopy (**Figure 4**). They can be detected by Western blotting with extracts of human dermis (**Figure 6**) (145). However, the preparation of those extracts is challenging and problematic. Therefore, an ELISA system was developed, using the recombinant C-terminus of laminin γ1, with a sensitivity of around 70% and a specificity of nearly 99% (64).

Epidermolysis Bullosa Acquisita

EBA affects skin and, to less extent, mucous membranes and is characterized by autoantibodies against type VII collagen (**Figure 2** right) (31, 148). There are two main clinical forms of EBA (144, 149, 150). The mechanobullous form represents the classical form of EBA. It is clinically characterized by skin fragility, tense blisters, vesicles, and erosions on non-inflamed skin in trauma-prone sites. Lesions may heal with scarring and milia formation (149, 151). In about two thirds of EBA, the inflammatory variant develops resembling BP, MMP, or LAD (149, 152). The diagnostic gold standards are direct immunogold electron microscopy, a methods nowadays only performed for this purpose in handful of centers, and more conveniently, DIF microscopy (144). By latter method, diagnosis of EBA can be made when a u-serrated binding pattern is present as detailed above (**Figures 1**, **3**). Type VII collagen-specific autoantibodies are deposited at the floor of the artificial blister of salt-split human skin using IIF microscopy (**Figure 2** right) and are mostly directed against the noncollagenous (NC)1 domain (153, 154). Autoantibodies can be detected via Western blotting, using an extract of the human dermis (**Figure 6**). Three assays for the diagnostic detection of serum IgG against type VII collagen are available; two ELISA system using the NC1 domain or both the NC1 and NC2 domains as well as an IIF test based on the Biochip mosaic technology employing human cells that express the recombinant NC1 domain (**Figure 4**) (50, 51, 75). Anti-type VII collagen ELISA values were shown to correlate with disease activity (155), thus, like in PV, PF, and BP, the respective

ELISA systems are useful tools not only for the diagnosis of the disease but also to guide treatment decisions during the course of the diseases. Patients with predominant or exclusive IgA reactivity against type VII collagen are usually classified as IgA EBA following the consensus of an international expert panel (144, 149, 152, 156, 157).

Dermatitis Herpetiformis

Dermatitis herpetiformis is an autoimmune disease that always occurs in combination with glutensensitive enteropathy (celiac disease). It is clinically characterized by grouped vesicles and papules and predominantly affects the elbows, buttocks, and knees (146, 147, 149, 158–160). The autoantigen is the epidermal transglutaminase, however antibodies against gliadin, endomysium, tissue transglutaminase (TG2), and epidermal transglutaminase (TG3) can be detected (161). The main antibody-subclass is IgA but can be IgG in some patients. In patients under treatment with dapsone or on gluten-restricted diet, autoantibodies against the epidermal transglutaminase are found more frequently than autoantibodies to tissue transglutaminase (162). The diagnosis is based on the DIF and IIF microscopy findings as well as commercial ELISA systems. Here, IgA (or IgG)-autoantibodies against the epidermal and tissue transglutaminase as well as the deaminated gliadin-analogous fusion (GAF) peptides can be detected (61, 162).

AUTHOR CONTRIBUTIONS

MW and ES performed the literature research, acquired and designed the figures and wrote the manuscript. DZ performed literature research and critically revised the manuscript.

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The Usefulness of Indirect Immunofluorescence in Pemphigus and the Natural History of Patients With Initial False-Positive Results: A Retrospective Cohort Study

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The specificity and the predictive values of indirect immunofluorescence (IIF) in real-life settings is yet to be firmly established. The natural history of patients with false-positive results has not been sufficiently elucidated. The primary aim of the current study is to evaluate the diagnostic value of IIF analysis on monkey esophagus in pemphigus, utilizing a large cohort arising from the real-life experience of a tertiary referral center. The secondary endpoint was to determine the clinical outcomes of patients with false-positive results. This was a retrospective cohort study including all patients who were tested for the presence of intercellular autoantibodies by IIF on monkey esophagus between 2000 and 2017. Overall, 770 sera from different individuals were tested by IIF microscopy. Of those, 176 patients had been diagnosed with pemphigus vulgaris (PV) and 29 patients with pemphigus foliaceus (PF). The sensitivity of this immunoassay was significantly higher for the diagnosis of PV (87.4%; 95% CI, 81.5–91.9%) as compared to PF (69.0%; 95% CI, 49.2–84.7%; $P = 0.018$). The specificity for the diagnosis of pemphigus was 93.5% (95% CI, 91.1–95.4%). Patients with false-positive results ($n = 37$) were followed for a median duration of 5.3 years contributing 280.8 person-years. Thirty patients (81.1%) were eventually diagnosed clinically and immunopathologically with subepidermal autoimmune bullous diseases, whereas the remaining patients (18.9%) were diagnosed clinically and histologically with other inflammatory dermatoses, but none of them developed pemphigus during the follow-up duration. Of note, 7.0% ($n = 23$) of all patients diagnosed with bullous pemphigoid (BP) in the same period ($n = 328$) were tested positive for IgG intercellular antibodies. Histopathological review of the biopsy specimens of these patients did not reveal acantholysis. In conclusion, the predictive value of negative test in IIF on monkey esophagus is particularly reliable to exclude a diagnosis of pemphigus. Individuals tested positive for intercellular antibodies without an initial overt pemphigus did not show an increased risk for developing pemphigus subsequently. A sizable fraction of patients with BP showed circulating intercellular autoantibodies by IIF, without a histopathological evidence for acantholysis.

Keywords: pemphigus, indirect immune fluorescence assay, monkey esophagus, pemphigus vulgaris, pemphigus foliaceus, false positive, sensitivity, specificity

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INTRODUCTION

Pemphigus is a rare, chronic, potentially life-threatening, autoimmune bullous disease of the skin and the mucous membranes. The two major subtypes of pemphigus are pemphigus vulgaris (PV) and pemphigus foliaceus (PF). The etiopathogenesis underlying the development of the disease is characterized by acantholysis and intraepidermal blister formation, resulting from IgG autoantibodies directed against desmoglein (Dsg) 3 (PV) and/or Dsg 1 (PF), two transmembrane desmosomal glycoproteins (1, 2).

An essential element of the diagnosis of pemphigus is the detection of circulating intercellular antibodies. Despite the detailed knowledge of pemphigus antigens and the development of increasing number of sensitive and specific assays for the detection of circulating autoantibodies, including Western blotting of cell-derived and recombinant forms of the target antigens, immunoprecipitation, and enzyme-linked immunosorbent assay (ELISA), the diagnosis of pemphigus in most laboratories still relies chiefly upon indirect—immunofluorescence (IIF) technique. Although a universally sensitive substrate has not been established, IIF on monkey esophagus has been elucidated as the most sensitive screening test (3–5). The value of IIF titers in disease monitoring has been a subject of debate, with conflicting results throughout the years. Although early studies suggested that intercellular antibodies levels measured by IIF were a useful marker of disease activity (6–8), later studies concluded that IIF titers did not always correlate with the disease severity, and are not consistent enough to serve as a guide for therapy or for monitoring the disease activity (9–12).

IIF is positive in approximately 70–90% of pemphigus patients but lacks the ability to differentiate definitively between PV and PF since both have IgG antibodies directed against keratinocyte cell surface (4, 13, 14). The sensitivity of this assay varies according to the specificity of the epithelial substrate the sera are incubated with (4, 14–17). To the best of our knowledge, the specificity and the predictive values of this immunological assay was not estimated in real-life settings in the past. Unlike controlled trials examining the diagnostic features of this immunoassay under optimal setting in the absence of confounding factors, real life studies inform on the effectiveness of an analysis when performed in routine circumstances, by including all patients with initial suspicion of autoimmune bullous disease and not only patients and healthy control subjects. Furthermore, the natural history of patients with false-positive results is yet to be fully elucidated.

The aim of the current study is to evaluate the sensitivity, specificity and predictive values of IIF analysis on monkey esophagus utilizing a large cohort extracted from the real-life experience of a tertiary center throughout an extended duration. Our secondary endpoint was to determine the clinical outcomes and histological features of patients tested positive for intercellular antibodies without an initial diagnosis of pemphigus (false-positive patients).

METHODS

This was a retrospective cohort study that included all patients who were tested for the presence of intercellular autoantibodies by (IIF) in Rambam Health Care Campus, Haifa, Israel, throughout the years 2000–2017. The current study was approved by the institutional ethical board of our medical center.

Patients and Case Definition

The clinical and immunopathological criteria for the diagnosis of PV were: (1) presence of skin blisters and/or erosions on mucous membranes; (2) suprabasal intraepidermal acantholysis on histopathological examination of skin and/or mucosa; and (3) intraepidermal intercellular IgG and/or C3 deposits by direct immunofluorescence (DIF); or intercellular circulating antibodies demonstrated by using monkey esophagus and a standard IIF technique; or the presence of anti-Dsg 3 ± anti-Dsg 1 autoantibodies, measured by ELISA (UROIMMUN Medizinische Labordiagnostika AG; Lübeck) or immunoblotting (on human dermal and epidermal extracts as antigenic substrate) (18).

The clinical and immunopathological criteria for the diagnosis of PF were: (1) presence of skin blisters or erosions; (2) lack of mucosal lesions; (3) intraepidermal acantholysis compatible with PF on histopathological examination; and (4) intraepidermal intercellular IgG and/or C3 deposits by DIF; or intercellular circulating antibodies demonstrated by a standard IIF technique; or presence of anti-Dsg 1 autoantibodies, with lack of anti-Dsg 3 autoantibodies, measured by ELISA or immunoblotting (18).

The differentiation between the different phenotypes of PV (isolated mucosal, isolated cutaneous, or mucocutaneous) was grounded on the clinical and endoscopic (in cases of laryngeal involvement) presentation at the onset of the disease, without referring to serological analyses to ascertain that those with the isolated mucosal disease had only anti-Dsg 3 antibodies, and those with mucocutaneous had both anti-Dsg 3 and anti-Dsg 1 antibodies.

Indirect Immunofluorescence

All sera were tested in serial dilution for intercellular antibodies by IIF. IIF assay using monkey esophagus as the substrate was performed according to a standard technique (18). Sera samples were incubated with monkey esophagus, and fluorescein-labeled goat anti-human IgG sera (Tago, Camarillo, CA) were added subsequently. Each serum sample was examined under fluorescence microscopy. Serum samples were considered to be positive if they stained epidermal intercellular spaces at a titer of $\geq$20.

Histopathology

Hematoxylin-eosin stained cutaneous biopsies of patients with false-positive results were re-examined for evidence of acantholysis.

Statistical Analysis

All continuous parameters were expressed as mean values ± standard deviation (SD). Categorical variables were expressed as proportions. Comparisons of percentages between different

patient groups were carried out using the Chi-square test. To determine the sensitivity and specificity of the IIF immunoassay, receiver operating curves (ROC) were analyzed. For sample proportions, 95% confidence interval (CI) was computed using the Clopper–Pearson exact binomial proportion interval method as fitting. CIs for the likelihood ratios were calculated using the "Log method." CIs for the predictive values were the standard logit CIs given by Mercaldo et al. (19). Figures with *P*-values< 0.05 were considered statistically significant. Statistical analysis was performed using IBM SPSS statistics software, version 23 (IBM Corp, Armonk, New York, USA) and MedCalc Statistical Software (version 16.4.3, MedCalc Software, Ostend, Belgium).

RESULTS

Clinical and Demographic Characteristics of the Study Participants

Overall, 770 sera from different individuals were tested for the presence of intercellular autoantibodies by IIF microscopy between the years 2000 and 2017. Of those, 176 patients had been diagnosed with PV and 29 patients with PF. IIF analysis was performed on the sera of 174 (98.9%) patients diagnosed with PV and all patients with PF before the initiation of any immunosuppressive therapy. With regard to patients with PV, the most frequent clinical phenotype was mucocutaneous ($n = 106$; 60.2%), followed by isolated mucosal disease ($n = 63$; 35.8%) and isolated cutaneous disease ($n = 7$; 4.0%). The demographic and clinical characteristics of the patients with pemphigus included in the analysis are demonstrated in **Table 1**.

The Diagnostic Value of IIF on Monkey Esophagus

Of the 174 sera from PV patients tested by IIF, 152 were positive for intercellular antibodies against monkey esophagus, corresponding to a sensitivity of 87.4% (95% CI, 81.5–91.9%; **Table 2**). When PV patients were divided according to the clinical phenotype, IIF sensitivity was comparable for those with isolated mucosal (87.1%; 95% CI, 76.2–94.3%) and mucocutaneous phenotypes (87.6%; 95% CI, 79.8-93.2%; $P = 0.925$). Patients with isolated cutaneous disease had lower sensitivity (57.1%; 95% CI, 18.4–90.1%), but the small size of this subgroup ($n = 7$) hinders drawing meaningful comparisons. The sensitivity of IIF on monkey esophagus in PF patients was only 69.0% (95% CI, 49.2–84.7%; **Table 2**). Taken together, the sensitivity of this immunoassay was significantly higher for the diagnosis of PV as compared to that of PF ($P = 0.018$).

Overall, 770 patients were tested by IIF microscopy, including 567 (73.6%) patients who did not have an established diagnosis of pemphigus. Of those, 37 (6.5%) were tested positive for intercellular antibodies by IIF. Altogether, the specificity of this immunoassay for the diagnosis of pemphigus was as high as 93.5% (95% CI, 91.1–95.4%).

The positive predictive value (PPV) of IIF on monkey esophagus for the diagnosis of PV was 80.4% (95% CI, 75.0–84.9%) and the negative predictive value (NPV) was 96.0% (95% CI, 94.2–97.3%). The positive likelihood ratio (PLR) was 13.4 (95% CI, 9.8–18.4), while the negative likelihood ratio (NLR) was 0.14 (95% CI, 0.1–0.2). With regard to PF, the PPV of this assay was 35.1% (95% CI, 26.7–44.5%), whereas the NPV was 98.3% (95% CI, 97.2–99.0%). PLR and NLR were 10.6 (95% CI, 7.1–15.7) and 0.33 (95% CI, 0.2–0.6), respectively (**Table 2**).

Characterization of False-Positive Patients

The clinical features of the 37 non-pemphigus patients tested positive for intercellular antibodies were analyzed. These patients were followed for a median duration of 5.3 years (range, 0.7–16.9 years), contributing 280.8 person-years.

Thirty patients (81.1%) were eventually diagnosed clinically and immunopathologically with subepidermal autoimmune bullous diseases (SAIBD); 23 patients (62.2%) with bullous pemphigoid (BP), of whom one patient had coexisting psoriasis, 4 patients (10.8%) with mucous membrane pemphigoid, 2 patients (5.4%) with linear IgA bullous dermatosis (had IgA intercellular antibodies), and one patient (2.7%) with lichen planus pemphigoides. Apart from one patient with BP having dual intercellular and anti-basement membrane zone (BMZ) antibodies, all the remaining 29 (96.7%) patients with SAIBD had isolated intercellular antibodies detected by IIF. The diagnosis of theses SAIBD was grounded on suggestive clinical presentation, compatible histopathology, and linear deposits of immunoreactants along the BMZ by DIF

TABLE 1 | Demographic and clinical characteristics of patients with pemphigus whose sera were tested by indirect immunofluorescence microscopy.

	Pemphigus vulgaris ($n = 174$)	Pemphigus foliaceus ($n = 29$)
AGE AT DIAGNOSIS		
Mean ± SD	55.5 ± 15.3	57.1 ± 21.4
Median (range)	55 (20–90)	63 (20–87)
% female (n)	65.9% (116)	34.5% (10)
CLINICAL PHENOTYPE, % (N)		
Isolated mucosal	35.6 (62)	NA
Mucocutaneous	60.4 (105)	NA
Isolated cutaneous	4.0 (7)	NA

n, number; SD, standard deviation.

TABLE 2 | Evaluation of indirect immunofluorescence immunoassay on monkey esophagus.

	Pemphigus vulgaris		Pemphigus foliaceus	
	Value (%)	95% confidence interval	Value (%)	95% confidence interval
Sensitivity	87.1	76.2–94.3%	69.0	49.2–84.7%
Positive predictive value	80.4	75.0–84.9%	35.1	26.7–44.5%
Negative predictive value	96.0	94.2–97.3%	98.3	97.2–99.0%
Positive likelihood ratio	13.4	9.8–18.4	10.6	7.1–15.7
Negative likelihood ratio	0.14	0.1–0.2	0.33	0.2–0.6

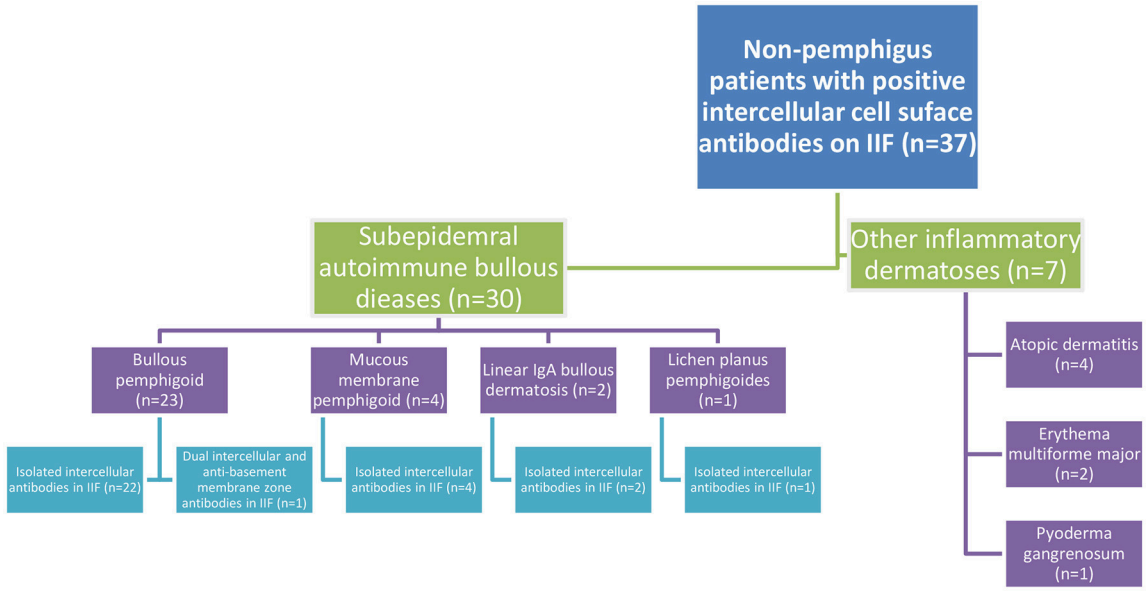

FIGURE 1 | Flowchart of the clinical characteristics of non-pemphigus patients tested positive for intercellular cell surface antibodies in IIF on monkey esophagus.

and/or the presence of circulating IgG antibodies against the immunodominant domain of BP180 (NC-16A) using ELISA (in cases of BP) (20). Intercellular tissue-bound antibodies was not detected in any of these patients, as DIF microscopy revealed isolated linear deposition along the BMZ in all 30 cases. Altogether, 7.0% ($n = 23$) of all patients diagnosed with (BP) in the same period ($n = 328$) were tested positive for IgG intercellular antibodies by IIF on monkey esophagus (**Figure 1**).

The remaining 7 patients were diagnosed clinically and histopathologically with various inflammatory dermatoses; 4 patients with atopic dermatitis, 2 patients with erythema multiforme major, and one patient with pyoderma gangrenosum (**Figure 1**).

None of the above 37 seropositive patients have subsequently developed pemphigus throughout the duration of follow up. Lesional biopsy specimens of 32 patients with the false-positive results were reviewed, and no histopathological sign of acantholysis was detected.

DISCUSSION

The results of the current study indicate that IIF microscopy on monkey esophagus is a more sensitive test for the detection of circulating intercellular antibodies in PV than in PF. The specificity of the assay is high when used in routine practice, and the predictive value of a negative test is particularly reliable to exclude the diagnosis of pemphigus. Individuals tested positive for intercellular antibodies by IIF, without initial overt pemphigus, did not show an increased risk for developing pemphigus subsequently. None of the false-positive patients had histological evidence of acantholysis.

The positivity rate of IIF in previous cohorts varied depending on the epithelial substrate which the sera were incubated with. In a recent German study, intercellular epithelial staining on monkey esophagus by IIF was observed in 100.0 and 98.0% of 65 patients with PV and 50 patients with PF, respectively (5). In an earlier British study, the diagnostic sensitivity of IIF on monkey esophagus was 100.0% in 20 patients with PV and 67.0% in 9 patients with PF (14). Jiao and Bysryn (4), examining 41 sera from PV patients and 22 sera from PF patients, had demonstrated that 87.0 and 86.0% of patients with PV and PF, respectively, were tested positive for intercellular antibodies by IIF when the assays were conducted on 2 different substrates simultaneously (monkey and guinea pig esophagus). In a small-scale study from Singapore, Ng PPL et al. (15) reported that all 13 PV patients and 11 of 12 PF patients had positive IIF on monkey esophagus resulting in sensitivities of 100.0 and 91.7%, respectively. In two previous serological studies performed in our center, the sensitivity of IIF on monkey esophagus was estimated at 96.0 and 81.0% for the diagnosis of PV in small cohorts of 25 and 32 patients, respectively (18, 21). Our study examined a 2- to 10-fold larger number of patients and found that the sensitivity for PV (87.4%) was lower than reported in most studies, whereas the sensitivity for PF (69.0%) was within the range of previous studies (4, 14). The predictive value of a negative test in our study (96.0%) was found to be particularly reliable to rule out a diagnosis of pemphigus. The large sample size in our study provides sufficient statistical power to exclude chance as the basis for the findings and sustains its external validity.

Several studies have estimated the the specificity of IIF by testing—healthy control subjects. A recent German study which examined 115 pemphigus patients estimated the specificity at 89.1% (5). Another study comprising 33 Chinese pemphigus patients depicted that the specificity of this immunoassay was

91.8% (22). A similar specificity (94.8%) was reported in a previous small-scale ($n = 25$) study from our center (18). A higher specificity of 100.0% was demonstrated by another serological study including 32 Israeli PV patients (21). Apart from the latter, the specificity revealed in our study (93.5%) is comparable with that reported in other studies. It is noteworthy that all the aforementioned studies enrolled healthy control subjects in order to identify the specificity rates, most of them characterized by low pretest probability. Conversely, our study was undertaken in a real-life setting and consisted of individuals whose clinical presentation raised suspicion for autoimmune bullous diseases. Thus, the pretest probability was moderate to high in most control subjects. Our findings, therefore, should represent the real diagnostic value of this immunoassay in the everyday clinical practice more efficiently than studies recruiting healthy participants.

Interpretation of Findings

The higher sensitivity of this immunoassay for the diagnosis of PV is conceivable in light of the fact that monkey esophagus is a mucosal substrate with high expression of Dsg3, the main autoantigen in PV (4, 9, 14). This substrate is less sensitive for PF patients with circulating anti-Dsg1 autoantibodies due to lower expression of Dsg1. IIF positivity depends on both the quantities of anti-Dsg1 and anti-Dsg3 antibodies in the test serum and the relative expression of Dsg1 and Dsg3 in the epithelial substrate. It was demonstrated that Dsg1-rich epithelial substrates like guinea pig esophagus and human skin were more sensitive than monkey esophagus for the diagnosis of PF (3, 4). One study showed that the sensitivity of IIF on human skin was greater than on monkey esophagus in patients with PF, whereas the sensitivity of IIF on monkey esophagus was higher than on human skin in patients with PV (14). Thus, some authors claim that the combination of a Dsg1-rich substrate, such as guinea pig esophagus or human skin, and a Dsg3-rich substrate, such as monkey esophagus, is crucial prerequisite to increase the sensitivity of IIF when screening the sera of pemphigus patients. It is noteworthy that other authors reported conflicting results suggesting that the sensitivity of IIF on monkey esophagus was comparable in PV and PF patients (5, 15). The reason for this discrepancy is unknown.

The predominance of SAIBDs among patients with false-positive assays may be ascribed to the "epitope spreading phenomenon"; a process in which a primary autoimmune or inflammatory cutaneous process may induce structural alterations in epidermal antigens (23, 24). The immune responses can spread over the disease course and recognize epitopes which are different from the original target. If it occurs in the same molecule, it is termed "intramolecular epitope spreading" (25), and if this immunological response involve epitopes on other proteins, it is then termed "intermolecular epitope spreading" (26). Regarding the relatively high false-positivity among patients with SAIBDs, it may postulated that intercellular cell-surface antigens that were previously concealed from the immune system became exposed, leading to the induction of a secondary autoimmune response that may be reflected by the production of non-pathogenic intercellular autoantibodies (24). A remarkable multicenter longitudinal study examined the profile of IgG autoantibody response to distinct BP180 and BP230 epitopes during the clinical course of 35 BP patients (27). Epitope spreading events were detected in up to 50% of cases, mainly intramolecular epitope spreading events consisting of early IgG reactivity with extracellular epitopes, which was followed by IgG reactivity with intracellular epitopes of BP180. This study did not investigate the development of intermolecular epitope spreading against Dsg1/3 (27).

Sami et al. (28) presented 13 patients with an initial immunopathological diagnosis of BP who subsequently demonstrated coexistent serological features of both BP and PV and failed to respond to conventional systemic therapy. IIF using monkey esophagus as substrate revealed high levels of intercellular cell surface antibodies in all patients (7 in conjunction with anti-BMZ antibodies and 6 without anti-BMZ antibodies). Additionally, all 13 patients had anti-Dsg3 antibodies and 9 had anti-Dsg1 antibodies on ELISA. The administration of intravenous immunoglobulin (IVIg) resulted in effective clinical response and the maintenance of prolonged clinical remission. In view of their findings, the authors recommended performing detailed serological re-evaluation and considering a dual diagnosis of BP and PV in patients with an initial diagnosis of BP who are nonresponsive to conventional therapy. Unlike these findings, the great majority of our "false-positive" BP patients had isolated intercellular antibodies without simultaneous detection of anti-BMZ antibodies by IIF. While Sami et al. (28) attributed a pathogenic role for intercellular antibodies detected in patients with BP which supposedly rendered them more recalcitrant for conventional therapy, we did not find any distinct clinical features for BP patients with intercellular antibodies. In addition, histological review of lesional biopsy specimens did not reveal acantholysis, thus arguing against a pathogenetic role for intercellular antibodies detected in IIF in this subgroup.

In 2001, Sami and Ahmed (29) reviewed the literature and summarized 17 reported patients with mutual features of both BP and PV. Of whom, 83% had serum antibodies typical of PV. The present study demonstrated that this phenomenon occurred in 7% of BP patients. A notable case of 26-years old woman presenting with tissue-bound and circulating antibodies suggestive of both pemphigoid gestationis (PG) and pemphigus in DIF and IIF, respectively, was reported (30). Clinically, she presented with erythematous eruption on the lower abdomen and thighs the day following delivery. The patient behaved clinically as a typical case of PG exhibiting a good response to moderate dose of oral corticosteroids, and lacked conventional clinical manifestation of pemphigus (30).

It was evidenced that IgG4 is the major subclass of autoantibodies in active pemphigus (31–33). Dsg-specific autoantibodies in pemphigus patients with active disease tend to preferentially associate with IgG4 subclass (33, 34). In both PV and PF, patients with active disease demonstrate Dsg-reactive IgG4 and IgG1, while patients in remission and some healthy relatives of patients with pemphigus can demonstrate only anti-Dsg IgG1 (34–36). A recent study had revealed that serum IgG4, but not other IgG subclasses, was enriched in

patients with pemphigus compared with unaffected individuals (32). Additionally, IgG4 depletion in PV sera diminished pathogenicity in a keratinocyte dissociation assay and depicted that affinity-purified IgG4 is more pathogenic than other serum IgG fractions (32). Another study found IgG4 to be the exclusive subclass that differentiates PV patient subgroups based on different disease morphologies and disease durations (33). Moreover, an IgG4-specific Dsg ELISA was verified to have greater sensitivity and specificity than a total IgG Dsg ELISA in identifying active disease in endemic PF, suggesting a more substantial clinical association of pathogenic antibodies with IgG4 rather than with other IgG subclasses (37). It is of great interest to explore whether false-positive patients had IgG4 anti-cell surface antibodies or, alternatively, IgG2 and IgG3 autoantibodies which have not been associated with a pathogenic role in pemphigus (38, 39). Given the retrospective data collection and the unavailability of the sera, characterization of the specific subclass of IgG antibodies could not be performed.

Strengths and Limitations

The sample size is large, and all the analyses were performed before the initiation of any immunosuppressive medications which could interfere with the results. Our study has some limitations to consider. First, the phenotypes of PV patients were not categorized according to the immunoserological profile. Second, although the immunoassays were performed in the same laboratory using the same substrate, at least 2 technicians analyzed the results of this subjective technique. The titers and the specific subclass of autoantibodies, as well as the specific pattern of deposition were not evaluated systematically in all patients. Thus, we could not investigate the association between autoantibodies levels and subclasses and the clinical characteristics.

It is noteworthy that a growing body of evidence accumulated in the last decade to signify the high sensitivity of Dsg 1 and Dsg 3-ELISA (40, 41). Many authors recommend utilizing this technique, available as mono-analyte or multi-analyte systems, as an easier technique. However, IIF is still widely used for the immunoserological diagnosis of pemphigus, specifically in low-income countries.

In conclusion, IIF microscopy on monkey esophagus is a more sensitive immunoassay for the detection of circulating intercellular autoantibodies in PV than in PF. The specificity of the assay is relatively high when used in real-life clinical settings, and the negative predictive value is particularly reliable to exclude the diagnosis of pemphigus. A notable proportion of patients with BP (7.0%) showed false-positive circulating intercellular autoantibodies by IIF. None of the patients with false-positive results demonstrated a histological evidence for acantholysis or developed pemphigus during the follow-up duration, which argues against a pathogenic role of intercellular antibodies in this subgroup.

ETHICS STATEMENT

This retrospective, non-interventional study was approved by the institutional ethical board of Rambam Health Care Campus, waiving patient written informed consent.

AUTHOR CONTRIBUTIONS

KK substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work. KK and RB drafting the work or revising it critically for important intellectual content. KK and RB final approval of the version to be published.

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CASE REPORT
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Mucous Membrane Pemphigoid, Bullous Pemphigoid, and Anti-programmed Death-1/ Programmed Death-Ligand 1: A Case Report of an Elderly Woman With Mucous Membrane Pemphigoid Developing After Pembrolizumab Therapy for Metastatic Melanoma and Review of the Literature

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An 83-year-old patient developed erosions and a blister of the gingival mucous membrane, 6 months after discontinuation of the anti-programmed death-1 (anti PD-1) pembrolizumab therapy administered for 10 months for a metastatic melanoma. A diagnosis of mild mucous membrane pemphigoid (MMP) was made. Complete remission of MMP was rapidly obtained with minimal therapy (doxycycline). MMP remained in complete remission after a 3-month follow-up since discontinuation of the doxycycline therapy and no evidence of relapse of the melanoma was observed after a 14-month follow-up since discontinuation of the pembrolizumab therapy. The widespread use of anti PD-1 and anti-programmed death-ligand-1 (PD-L1) in several malignancies reveals new adverse events. MMP describes a group of chronic, inflammatory, mucous membrane-predominant, subepithelial auto-immune blistering diseases. It is clinically distinct from bullous pemphigoid another autoimmune blistering disease but shares some immunological similarities with it. Twenty-nine cases of bullous pemphigoid associated with anti PD-1/PD-L1 have been reported in the literature and one of MMP. Here, we described the case of a MMP developed after pembrolizumab and discussed the accountability of anti PD-1/PD-L1 in our case and the previous reported bullous pemphigoid and MMP cases using the Begaud system scoring.

Keywords: mucous membrane pemphigoid, bullous pemphigoid, melanoma, anti-programmed-death-1/death-ligand-1, immune checkpoints inhibitors, pembrolizumab, drug accountability study, adverse drug reaction

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BACKGROUND

Immune checkpoint inhibitors against programmed death-1 (anti PD-1) and programmed death-ligand 1 (anti-PD-L1) agents have revolutionized the treatment of metastatic melanoma and have shown encouraging promise in advanced solid tumors and hematological malignancies. However, these agents are associated with immune-related adverse events (IrAEs) that affect mainly the skin, hormone glands, liver and gastrointestinal tracts.

Indeed, up to 20% of treated patients may develop dermatological IrAEs. They are predominantly non-specific rashes and pruritus (1). Toxicities on buccal mucous membrane (MM) have also been described, including xerostomia, lichenoid reactions, and dysgeusia (2). Since 2015, an association between a treatment with anti PD-1/PD-L1 and bullous pemphigoid (BP) has been reported in 29 cases. One mucous membrane pemphigoid (MMP) case, an autoimmune bullous disease (AIBD) similar to BP, has also been described after pembrolizumab therapy.

Here, we report a second case of MMP that occurred 16 months after initiation of pembrolizumab therapy for a metastatic melanoma, discuss the association between MMP and melanoma, and review the literature on BP and MMP associated with anti PD-1/PD-L1.

CASE PRESENTATION

In 2014, an 83-year-old woman with no history of known autoimmune disease was diagnosed as having a right leg superficial spreading melanoma, initially T2b N0 M0. Eight months later, she developed iterative local and in transit cutaneous metastases on the same leg and she underwent four times surgical excision.

In 2016, at the fourth recurrence, surgery was not chosen. Baseline full-body computed tomography revealed no other metastasis (T2 N0 M1a). Mutation tested on a tumor sample excluded the presence of any BRAF mutation. Administration of pembrolizumab therapy was started at 2 mg/kg every 3 weeks, resulting in complete remission (CR) within 3 months (cycle 4). In March 2017, after 14 cycles, she remained in CR, and the pembrolizumab therapy was stopped at her request.

In October 2017, 6 months after pembrolizumab discontinuation, she complained of oral pain and was referred to our hospital. Clinical examination revealed gingivitis with one tense blister, a large pseudomembrane-covered erosion with a tweezers sign, an atrophy and pseudo lichenoid lesions. Other MM and skin were not involved. Gingival biopsy showed a subepithelial cleavage with the overlying intact epithelium (**Figure 1A**). A moderate perivascular infiltration consisting of lymphocytes and histiocytes was observed, with no lichenoid infiltrates. Direct immunofluorescence (DIF) microscopy revealed linear IgG (++) and C3 (++) immune deposits along the basement membrane zone (BMZ) (**Figure 1B**). Standard indirect immunofluorescence (IIF) microscopy on rat esophagus failed to detect circulating anti-BMZ antibodies. A diagnosis of mild MMP was made. Further immunological investigations demonstrated that the immune deposits identified using direct immunoelectron microscopy (IEM) were strictly localized in the lamina densa (**Figure 2**), a site consistent with autoantibodies against the laminin 332 or the C-terminal extremity of BP180 antigen (BP180). IIF on salt-split skin, immunoblot using amniotic extracts, and enzyme-linked immunosorbent assays (ELISAs) with BP180-NC16A epitope and BP230 antigen (BP230) had negative results.

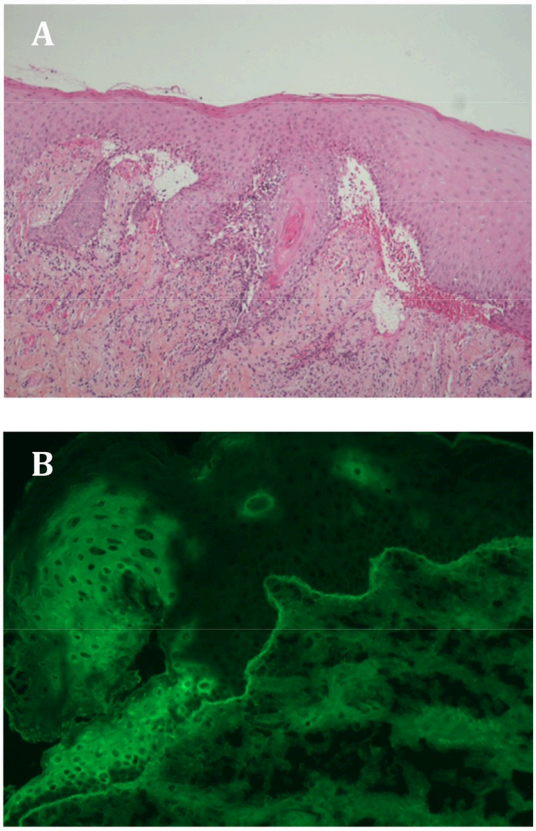

FIGURE 1 | (A) Histological examination of a gingival biopsy showing subepithelial cleavage with overlying intact epithelium. A moderate perivascular infiltration can be observed consisting of lymphocytes and histiocytes, and no lichenoid infiltrate. **(B)** Direct immunofluorescence microscopic image showing linear IgG (++) and C3 (++) immune deposits along the basement membrane zone on the gingival biopsy.

Doxycycline therapy (100 mg/day) and mouth washes with corticosteroid (betamethasone 2 mg) three times daily were initiated, which led to the control of the MMP within 2 weeks and CR under this minimal therapy in 6 weeks. After 3 months of treatment with doxycycline therapy, the patient decided on her own to discontinue it, and no MMP relapse had

Abbreviations: AIBD, autoimmune bullous disease; anti PD-1, anti-Programmed-Death-1; anti-PD-L1, anti-Programmed-Death-ligand-1; BMZ, basement membrane zone; BP, bullous pemphigoid; BP180, BP180 antigen; BP230, BP230 antigen; CR, complete remission; DIF, direct immunofluorescence; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; IEM, immunoelectron microscopy; IIF, indirect immunofluorescence; IrAEs, immune-related adverse events; MM, mucous membrane(s); MMP, mucous membrane pemphigoid.

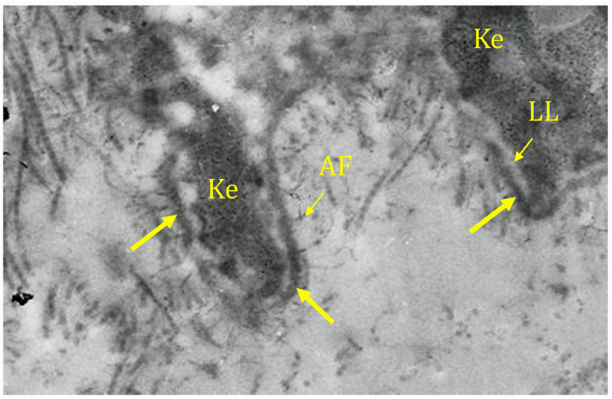

FIGURE 2 | Direct immunoelectron microscopy showing immune deposits (arrow) strictly localized in the lamina densa. Ke, keratinocyte; LL, lamina lucida; AF, anchoring fibril.

occurred 3 months later. No clinical or radiological evidence of relapse of the melanoma was observed on computed tomography imaging after a 14-month follow-up since discontinuation of the pembrolizumab therapy.

DISCUSSION

Our case raises the question of an association between MMP and melanoma, or between MMP/BP and pembrolizumab administration.

MMP encompasses a group of AIBDs clinically defined by the predominance of MM lesions over skin lesions (3, 4), and healing of its lesions leads to characteristic cicatricial scarring. The buccal involvement is the most frequent, followed in order of decreasing frequency by ocular, nasal, nasopharyngeal, anogenital, skin, laryngeal, and oesophageal involvements. The ocular, laryngeal, and oesophageal involvements can cause severe impairment or even death.

Our patient had a typical MMP, except the age at MMP onset, in a mild form because of the purely buccal involvement. Thus she was considered as a "low-risk patient" with few tendencies of scarring and required minimal therapy with doxycycline and topical steroids (3). Her MMP was controlled in 2 weeks, in CR on minimal therapy in 6 weeks, and in CR off treatment in 3 months. She developed mild cicatricial lesions of her gingival MM and did not relapse during follow-up. Rapid clinical improvement after only a short course of treatment is unusual in MMP.

MMP results from the activity of autoantibodies directed against BMZ antigens. The main autoantibody target is BP180, with the sera of most MMP patients reacting with its C-terminal domain (BP180-C term), combined or not with reactivity against the BP180-NC16A epitope and BP230 (5, 6). Other target antigens associated with a clinical MMP phenotype have been characterized molecularly, including the following: laminin 332, both α6β4-integrin subunits, and type VII collagen (7), respectively defining laminin 332-MMP, α6β4-integrin MMP, and MM epidermolysis bullosa acquisita. Autoreactive T lymphocytes are thought to also play a key role in the pathogenesis of MMP, particularly in the fibrosing process (8–15).

Our patient had a linear deposition of IgG/C3 along the epithelial BMZ on DIF microscopy and on the lamina densa on direct IEM, a location consistent with targeting of the C-terminal extremity of BP180 or laminin 332 by autoantibodies (16, 17). No circulating antibodies against BMZ antigens were detected by serological studies, notably BP180-NC16A ELISA, as in 49% of MMP in a recent series (18).

An association between laminin 332-MMP and malignancy was first reported in 1998 but is currently controversial. On one hand, 21 cases of laminin 332-MMP have been reported in association with cancer, including 15 reviewed by Sadler in 2007 (19) and six case reports after 2007 (20–25). An increased risk of solid cancers as compared with the general population was reported by two authors (26, 27), higher in the first year following the laminin 332-MMP diagnosis. On the other hand, no significant correlation was found between laminin 332 reactivity and the proportion of patients with an associated internal cancer in three recent serological studies of MMP (28–30).

Anyway, no association between laminin 332-MMP and melanoma has been reported (31).

In our patient, who had a possible laminin 332-MMP, a link between MMP and melanoma seems unlikely, as the first incidence occurred 3 years after the second and the latter was in CR.

We examined the intrinsic accountability of pembrolizumab therapy on MMP induction in our patient with metastatic melanoma because of its extrinsic accountability based on the following reports: (i) MMP and BP have immunological similarities (7); (ii) intrinsic accountability of anti PD-1/PD-L1 treatments on BP induction: 27 BP have been reported as case reports or short series (32–49) and two BPs listed as adverse drug reaction in two large trials with anti PD-1 (50, 51); (iii) recently, one pembrolizumab-associated MMP case report (52), and (iv) some of the anti PD-1/PD-L1-associated BPs had atypical clinical phenotypes (33–35, 42, 47).

Although the clinical characteristics of MMP differ from those of BP typified by the absence of MM lesions, absence of predominant head-and-neck involvement, absence of scars, and older age at onset (>70 years) (53), MMP and BP share physiopathological features; BP result from the activity of autoantibodies directed against BP230 and BP180, such as most MMPs. However, the sera of most patients with BP react with the BP180-NC16A epitope, contrary to those of patients with MMP.

The melanoma treatment has been revolutionized by innovative immunomodulation drugs that break tolerance. The main treatment targets for melanoma are cytotoxic T-lymphocyte antigen-4 and PD-1. Ipilimumab, a monoclonal antibody against cytotoxic T-lymphocyte antigen-4, was the first drug to demonstrate a benefit in overall survival in a randomized controlled phase 3 study of patients with advanced melanoma (54) and to be approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA). The anti-PD-1s and anti-PD-L1s are remarkably more effective in terms of response and overall survival rates than ipilimumab. The response rate with anti-PD-1 reaches 40% in melanoma, and the 5-year overall survival rate for naive patients is close

to 40% (55). Moreover, anti-PD-1s are less toxic in terms of IrAEs. In 2014, the FDA approved the anti-PD-1 antibodies pembrolizumab and nivolumab for advanced melanoma and, in 2015, the combination of ipilimumab and nivolumab.

PD-1 is a negative co-stimulatory receptor, which downregulates excessive immune responses by binding to its ligands, PD-L1 and PD-L2. This receptor is expressed mainly on activated T cells. In tumor tissue, binding of PD-1 inhibits effector T-cell function, which leads to exhausted T cells and suppression of the antitumor immune response (56). Pembrolizumab and nivolumab on one hand and atezolizumab and durvalumab on the other hand are selective humanized monoclonal antibodies that bind respectively to PD-1 and PD-L1 and thus block the interaction between PD-1 and its ligands, which leads to stimulatory effects on T cells.

B cells also express PD-1, and inhibition of PD-1 expression can directly activate B cells in a T-cell-independent manner (57). Lastly, it appears that anti PD-1 reduce regulatory T-cell activity (58), resulting in decreased tolerance and development of autoimmunity. All these mechanisms can be involved in the induction of MMP as soon as B and T cells played a central role in the MMP pathogenesis.

The potential drug induction of AIBD has been known for decades (18, 59–66). Recently, between 2015 and 2018, 18 case reports or small series described 27 patients who developed BP while receiving anti PD-1/PD-L1 therapy, including three with negative DIF (35, 37) or without DIF confirmation (47) but with typical clinical presentations (**Tables 1**, **2**). Fourteen patients received anti PD-1 nivolumab therapy (patients 2, 4, 7, 9, 10, 13, 15, 18, 20, 22, 23, and 25–27) (33, 35, 38, 42, 44, 46, 47, 49), 11 received anti PD-1 pembrolizumab therapy (patients 1, 5, 6, 8, 11, 12, 14,16, 19, 21, and 24) (32, 34–37, 39, 40, 43, 45, 48), one received anti-PD-L1 durvalumab therapy (patient 3) (33), and one received anti-PD-L1 atezolizumab therapy (patient 17) (41). Six of the 27 patients received ipilimumab therapy before anti PD-1, one received an association of ipilimumab and nivolumab therapy, and one received ipilimumab therapy after treatment with nivolumab. Two additional BP cases within a large series were simply mentioned, without any clinical description (50, 51). Recently, one MMP case treated with pembrolizumab therapy for a metastatic Merkel cell carcinoma has also been reported (53). Notably, the outcomes of the patients with melanoma were better [only 5 (33%) out of 15 cases in which the information were available, had a progressive disease] than reported in general anti-PD-1 treatment of melanoma (55). Other studies reported that objective response rate was significantly higher in patients with melanoma who experienced nivolumab-related adverse events (67).

The overall characteristics of these patients with anti PD-1/PD-L1-associated BPs were as follows: eight women and 19 men (female-to-male sex ratio, 0.4), median age of 68 years (35–90 years) at the time of BP diagnosis, and a median interval of 24 (6–84) weeks between starting anti PD-1/PD-L1 therapy (challenge) and BP onset. This time was significantly shorter (median, 16 weeks; range, 6–80 weeks) with nivolumab than with pembrolizumab (median, 27 weeks; range, 16–84 weeks) (Wilcoxon rank sum test with continuity correction: $p = 0.023$).

Pruritus was a prominent feature of most cases (68). Administration of anti PD-1/PD-L1 agents was discontinued (dechallenge) in 21 of the 27 patients (patients 1–14, 16–20, 22, 23, and 27) because of the evolution of BP and/or cancer and continued in five patients (no dechallenge, patients 1, 15, and 24–26). This information is unknown in one patient (patient 21). BP treatment with systemic steroids was required in all but four patients (patients 3, 5, 14, and 22). AntiPD-1/PD-L1 was reintroduced in two patients (rechallenge; patients 7 and 13). The reasons of the dechallenge, and the outcome after dechallenge or rechallenge are detailed in **Table 2**.

The comparison with the "usual" BPs (7, 69) highlights particularities in these anti PD-1/PD-L1-associated BPs as follows: a predominance of males (female-to-male sex ratio, 0.4 vs. 1.5), younger age [mean, 69 years; median, 68 years (range, 35–90 years) vs. mean, 83 years], no evidence of neurological disorders, more extensive diseases (52 vs. 41%), and no circulating autoantibodies against BP230 except in one patient (8 vs. 60–70%), while 91% of the tested sera had circulating autoantibodies against BP180-NC16A (vs. 80–90%). Moreover, seven cases had atypical clinical phenotypes, as head and neck involvement or mucosal lesions (33–35, 42, 47), raising doubts about the diagnosis of BP (53).

The patient with pembrolizumab-associated MMP reported by Haug et al. was a 62-year-old man who developed a pure buccal MMP after 13 weeks of pembrolizumab therapy. He had circulating autoantibodies targeting the C-terminal extremity of BP180. Pembrolizumab was discontinued at the time of MMP onset, and he was successfully treated with doxycycline and topical steroid, as in our case.

In our patient, MMP developed after 14 cycles and 24 weeks of pembrolizumab discontinuation, that is, 66 weeks after starting the treatment. Overall, IrAE induced by anti PD-1/PD-L1 agents usually appeared between 1 week and several months after starting immunotherapy (2). Among the 27 cases of anti PD-1/PD-L1-associated BPs, four had a long delay (>60 weeks) between starting anti PD-1/PD-L1 therapy and onset of BP (patients 5, 11, 17, and 18) (34, 36, 41, 42). Similarly to our patient, three patients developed a BP 4, 12, and 12 weeks after discontinuation of the anti PD-1 therapy (patients 1, 22, and 23, respectively) (32, 46, 47). This could be explained by the durable activity of anti PD-1/PD-L1 on immunity (70, 71).

Lastly, we assessed the intrinsic accountability score of anti PD-1/PD-L1 in AIBD induction for our patient with MMP, and *a posteriori* for the 27 BPs and MMP cases that have been reported using the Begaud scoring system (terms in bold type), updated in 2011 (72). The present **challenge** was the treatment of a malignancy by using an anti PD-1/PD-L1. Patients with a malignancy who started treatment with anti PD-1/PD-L1 before MMP/BP onset may have a **suggestive** or **compatible** challenge. The **dechallenge** was the discontinuation of the treatment. Outcome after **dechallenge** or **no dechallenge** may be **suggestive** (if BP is controlled with **dechallenge** or worsened **without dechallenge**), conversely **non-suggestive** (if BP worsened after **dechallenge** or controlled unless **without dechallenge**), and **inconclusive** (without details on BP evolution or continued treatment).

TABLE 1 | Case reports of anti-PD1/PD-L1-treated bullous pemphigoid patients.

Patients	First author, year (ref N°)	Sex/age (year)	Cancer type	Ipilimumab before anti-PD1	Other therapies before anti-PD1	Anti-PD1/-PD-L1	Dose[a]	1st anti PD1/PD-L1 dose to BP onset (weeks)
CASE REPORTS								
1	Carlos et al. (32)	M/75	Melanoma	Yes	Chemotherapy	Pembrolizumab	10 mg/kg	22
2	Naidoo et al. (33)	M/80	Melanoma	Yes	No	Nivolumab	NR	24
3		F/78	Melanoma	Yes	No	Durvalumab	NR	52
4		M/85	Lung SCC	No	Chemotherapy	Nivolumab	NR	18
5	Hwang et al. (34)	M/68	Melanoma	No	No	Pembrolizumab	10 mg/kg	78
6		M/72	Melanoma	No	No	Pembrolizumab	10 mg/kg	27
7	Jour et al. (35)	M/63	Tongue SCC	No	Radiation, chemotherapy, erlotinib	Nivolumab	3 mg/kg	8
8		M/68	Melanoma	No	No	Pembrolizumab[b]	2 mg/kg	16.4
9		F/74	Urothelial cancer	Yes + nivolumab	No	Nivolumab	3 mg/kg	16
10		F/73	Adenocarcinoma	No	Radiation, chemotherapy	Nivolumab	3 mg/kg	6
11	Mochel et al. (36)	M/63	Melanoma	No	No	Pembrolizumab	NR	84
12	Lomax et al. (37)	F/82	Melanoma	Yes	No	Pembrolizumab	NR	32
13	Damsky et al. (38)	F/77	Lung adenocarcinoma	No	No	Nivolumab	3 mg/kg	6
14	Bandino et al. (39)	M/73	Melanoma	No	No	Pembrolizumab	2 mg/kg	18
15		M/90	Melanoma	No	No	Pembrolizumab, nivolumab[c]	2 mg/kg, 3 mg/kg	24
16	Rofe et al. (40)	F/56	Melanoma	Yes	No	Pembrolizumab	2 mg/kg	24
17	Russo et al. (41)	M/58	Lung adenocarcinoma	No	Chemotherapy, bevacizumab	Atezolizumab	1200 mg	60
18	Sowerby et al. (42)	M/80	Lung adenocarcinoma	No	No	Nivolumab	3 mg/kg	80
19	Parakh et al. (43)	M/42	Melanoma	Yes	Radiation, chemotherapy, dabrafenib, trametinib	Pembrolizumab	2 mg/kg	44
20	Kwon et al. (44)	M/60	Renal cell carcinoma	No	Chemotherapy	Nivolumab	3 mg/kg	12
21	Wada et al. (45)	M/65	Melanoma	No	No	Pembrolizumab	2 mg/kg	51
22	Kuwatsuka et al. (46)	M/35	Melanoma	No[d]	No	Nivolumab[d]	NR	50
23	Anastasapoulou et al. (47)	M/48	Melanoma	No	No	Nivolumab	3 mg/kg	31
24	Amber et al. (48)	F/82	Melanoma	No	No	Pembrolizumab	2 mg/kg	27
25	Le Naour et al. (49)	M/66	Choroidal melanoma	No	No	Nivolumab	NR	28
26		M/78	Melanoma	No	No	Nivolumab	NR	16
27		F/68	Non-small-cell lung cancer	No	No	Nivolumab	NR	16
LARGE SERIES								
28	Muro et al. (50)	NR	Gastric cancer	NR	NR	Pembrolizumab	10 mg/kg	NR
29	El Khoueiry et al. (51)	NR	Hepatocellular carcinoma	NR	NR	Nivolumab	NR[e]	NR

[a] *Pembrolizumab, atezolizumab were administered every 3 weeks and nivolumab, durvalumab every 2 weeks.*
[b] *+ dabrafenib (150 mg) and trametinib (2 mg) after 3 cycles.*
[c] *Pembrolizumab 4 cycles switch to nivolumab + radiation.*
[d] *Bullous pemphigoid occured during ipilimumab, administered after nivolumab.*
[e] *Nivolumab 0.1-10 mg/kg every 2 weeks in the dose-escalation phase, nivolumab 3 mg/kg every 2 weeks in the dose-expansion phase.*
PD1, Programmed-Death-1; PD-L1, Programmed-Death-Ligand-1; SCC, squamous Cell Carcinoma; NR, Not reported.

TABLE 2 | Clinical, immunological, and evolutive data in 27 of the 29 anti-PD1/PD-L1-treated BP patients reported in the literature.

	Clinical data			Immunological data		Treatment			Dechallenge				Outcome at last visit	
	(Extensive/ moderate/ localized)	Atypical lesions (No/Yes, atypical site)	Neurological disorder (No/Yes)	DIF	ELISA BP180/ BP230	Systemic CS	Local CS	Others	Yes/ No	Time between BP onset and dechallenge (weeks)	Because of BP/cancer	Re-challenge Yes/No	BP	Cancer
1	Extensive	No	NR	+	NR	Yes	Yes	No	Yes	−4.3	No/ Yes, PD	No	Improvement	PD death
2	NR	Yes (buccal MM)	NR	+	+/−	Yes	Yes	Antihistamines, tacrolimus, oral ointment	Yes	28	Yes/ No	No	Improvement but peaked at each dose	CR
3	Moderate	Yes (buccal MM)	NR	+	+/+	No	Yes	No	Yes	0	Yes/ No	No	Improvement	prolonged PR
4	Extensive	No	NR	+	−/−	Yes	Yes	No	Yes	0	Yes/ No	No	Stable	prolonged SD
5	Extensive	Yes (buccal MM and face)	NR	+	NR	No	Yes	No	Yes	3	No/ Yes, prolonged PR	No	Initial response then relapse then NR	prolonged PR
6	Extensive	Yes (buccal MM and scalp)	NR	+	NR	Yes	Yes	Cyclins, methotrexate	Yes	>21	Yes/ Yes, PD	No	Initial response then relapse	PD death
7	Moderate	Yes (buccal MM, neck and face)	NR	+	NR	Yes	Yes	No	Yes	0	Yes/ No	Yes	Relapse at rechallenge—resolution at dechallenge	PD
8	Extensive	No	NR	+	NR	Yes	Yes	No	Yes	0	Yes/ No	No	Improvement	PD death
9	Extensive	No	NR	+	NR	Yes	Yes	No	Yes	0	Yes/ No	No	Remission	PR
10	Moderate	No	NR	−	NR	Yes	Yes	Cyclins, niacinamide	Yes	7	Yes/ Yes, PD	No	Improvement then relapse	PD
11	Moderate	No	No	+	+/−	Yes	Yes	No	Yes	12	No/ Yes, near CR	No	Improvement	CR
12	Extensive	No	NR	−	NR	Yes	Yes	Loratadine, prometazine	Yes	0	Yes/ No	No	Improvement but pruritus	CR
13	Extensive	No	depression	+	+/−	Yes	Yes	Omalizumab	Yes	0	Yes/ No	Yes	Controlled	NR
14	Moderate	No	NR	+	NR	No	No	Cyclins, niacinamide	Yes	6	No/ No	No	Slowly Improvement	NR
15	Localized	No	NR	+	NR	Yes	Yes	No	Yes	12	No/ Yes, CR	No	Improvement	CR
16	Extensive	No	NR	+	+/−	Yes (bolus)	Yes	Methotrexate	Yes	0	Yes/ Yes, PD	No	Improvement then relapse	CR
17	Extensive	No	NR	+	+/−	Yes	No	Cyclins	Yes	0	Yes/ No	No	Remission	CR

(Continued)

TABLE 2 | Continued

	Clinical data			Immunological data		Treatment			Dechallenge				Outcome at last visit	
	(Extensive/ moderate/ localized)	Atypical lesions (No/Yes, atypical site)	Neurological disorder (No/Yes)	DIF	ELISA BP180/ BP230	Systemic CS	Local CS	Others	Yes/ No	Time between BP onset and dechallenge (weeks)	Because of BP/cancer	Re-challenge Yes/No	BP	Cancer
18	Localized	Yes (buccal MM)	NR	+	+/–	Yes	No	No	Yes	0	Yes/ No	No	Remission after rituximab	CR
19	Extensive	No	Paraplegia	+	NR	Yes	Yes	Cyclins, nicotinamide	Yes	0	Yes/ No	No	Remission	PR
20	Extensive	No	NR	+	NR	Yes	Yes	No	Yes	0	Yes	No	No	Remission	NR
21	Moderate	No	NR	+	+/–	Yes	Yes	No	NR	NR	Nd/ Nd	No	Controlled	CR
22	Moderate	No	NR	+	+/–	No	Yes	No	Yes	<–6	No/ Yes,PD	No	Remission	NR
23	Extensive	Yes, (face and neck)	NR	Nd	NR	Yes	No	No	Yes	–12	No/ Yes, PD	No	Remission	PD
24	Localized (lower legs) then extensive	No	NR	+	+	Yes	Yes	No	No	No	Nd	Nd	No	Controlled	NR
25	Localized	No	NR	+	+	Yes	No	No	No	No	Nd	Nd	No	Controlled	PR
26	Localized	No	NR	+	NR	Yes	No	No	No	4	No	Yes, PD	No	Controlled	PD/palliative care
27	Extensive	No	NR	+	NR	Yes	Yes	No	No	0	No	PD	No	Remission	PD

PD1, Programmed Death-1; PD-L1, Programmed Death Ligand-1; BP, bullous pemphigoid; DIF, direct immunofluorescence; ELISA, enzyme-linked immunosorbent assay; BP180, BP180 antigen; BP230, BP 230 antigen; CS, corticosteroid; +, positive; –, negative; NR, not reported; PD, progression disease; MM, mucous membrane; CR, complete remission; PR, partial response; SD, stable disease; ND, not determinated.

The **rechallenge** was the reintroduction of the treatment. It may be **positive** (R+) or **negative** (R–) or **not done** (R0). The **chronological** scoring (combining status of **challenge**, **dechallenge**, and **rechallenge**) may be C1, **doubtful**; C2, **plausible**; and C3, **likely**. The **symptomatological** scoring may be S1, **doubtful**; S2, **plausible**; and S3, **likely**. Lastly, the **intrinsic accountability** scoring [combining **chronological** (C) and **symptomatological** (S) scores] may be I1 (C1S1), I2 (C1S2 or C2S1), I3 (C2S2), I4 (C1S3 or C3S1), I5 (C2S3 or C3S2), or I6 (C3S3) (see detailed results in **Supplementary Data**, **Table S1**).

The Begaud system scoring indicates that the possibility of anti PD-1/PD-L1 as a BP triggering factor is mostly low: only 10 (four with pembrolizumab, five with nivolumab, and one with durvalumab) of the 27 patients with BP were given high accountability scores [I5 for three patients (patients 2, 6, and 7), I4 for five (patients 8, 9, 14, 20, and 27), I3 for two (patients 3 and 5)], while 17 (seven with pembrolizumab, nine with nivolumab, and one with atezolizumab) had low accountability [I2 for six (patients 10, 12, 17, 18, 19, and 23) and I1 for 11 (patients 1, 4, 11, 13, 15, 16, 21, 22, and 24–26)]. One patient with BP had a positive rechallenge, and another had a negative rechallenge.

The Begaud system scoring indicates that the intrinsic accountability score was I4 for the MMP case that was reported but I1 for our patient with MMP.

In all the MMP/BP patients, the low score was essentially due to the long time between anti PD-1 introduction and AIBD onset (compatible challenge) and/or a non-suggestive dechallenge. Indeed, it could be the consequence of long delay of action of anti PD-1/PD-L1. A non-suggestive dechallenge may not be an argument against the accountability of a long-acting drug, and at the end, the Begaud scoring system may not be suitable for assessing the accountability of drugs with prolonged therapeutic effect.

In our patient, the eventuality of a rechallenge did not occur because the melanoma and MMP remained in CR. As an anti PD-1-induced MMP is possible, a rechallenge would theoretically expose her to a risk of MMP relapse in a potentially more serious form. Indeed, MMP can involve ocular, nasopharyngeal, laryngeal, esophageal, genital, or anal MM, sites that have a high likelihood of scarring, which is associated with loss of function. On the other hand and contrary to literatures on adverse drug reactions, a negative rechallenge with anti PD-1 has already been reported (38, 73).

In conclusion, we report the case of a patient who developed a mild MMP, possibly induced by anti PD-1 rather than by melanoma. We cannot also exclude that the MMP could be triggered by aging, malignancy, and pembrolizumab acting in concert or developed quite independently. MMP was rapidly controlled by a minimal treatment, raising the question of reintroduction of anti PD-1 if the melanoma relapses. With the increasing use of immunotherapies for various malignancies, clinicians should be alert for this new anti PD-1-induced IrAE, which is related to BP but potentially more severe. Long-term clinical follow-up is warranted owing to delayed adverse events, even after discontinuation of anti PD-1 inhibitors. Lastly the Begaud system scoring applied to our patient and previous reported cases with anti-PD-1/PD-L1 related BP/MMP indicates a low intrinsic accountability score in most of the patients suggesting it may not be suitable for assessing the accountability of drugs with prolonged therapeutic effect. Development of another specific assessment might be necessary.

ETHICS STATEMENT

A written consent has been obtained from the patient.

AUTHOR CONTRIBUTIONS

CZ and CP-S conceived and designed the study. CZ, MA, CL, PW, AG, and EM collected clinical data. AL and CP-S conducted the histological studies and FA and SM-G the immunological ones. CZ wrote the first draft of the manuscript. CP-S rewrote sections of the manuscript. EM and FC corrected the final version. All authors contributed to manuscript revision, and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2018.00268/full#supplementary-material

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Comorbidities and Treatment Strategies in Bullous Pemphigoid: An Appraisal of the Existing Litterature

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Bullous Pemphigoid is an autoimmune skin blistering disease. It is caused by deposition of auto antibodies along the dermal-epidermal border leading to inflammation. The antibodies are directed against anchoring filaments in the epidermis, but these antigens are also present in the neurological tissues and this has led to speculation of an association between multiple sclerosis and bullous pemphigoid. Additionally recent epidemiological studies have pointed at an increased risk of cardio-vascualr diseases and an increased moratality among the patients with bullous pemphigoid. In this mini review we present the recent findings in this area and as well as the treatment strategies when comorbidities are taken into consideration.

Keywords: Bullous pemphigoid, cardio-vascular diseases, multiple sclerois, neurodegenarative diseases, mortality

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INTRODUCTION

Bullous pemphigoid (BP) is an autoimmune blistering, and often intensely itching, blistering skin disease affecting especially the elderly generation with an incidence of 14–43 pr. million. Children are rarely afflicted by this disease. The blisters are thick walled, as opposed to the pemphigus diseases. The disease may present in a non-bullous form, i.e., pre-bullous pemphigoid. The incidence of BP is increasing in western countries, and patients have an increased mortality compared to healthy controls with a 1 year mortality increased six to seven times and increased hazard ratio, HR = 2.4, for death as shown in a Danish registry study. The increased mortality within the first year after diagnosis may have a combined background. Firstly, in many guidelines systemic corticosteroids are listed as the first line treatment and many BP patients suffer from declining health and do have several co-morbidities including diabetes (type I and II), and ischemic heart disease as well as hypertension at the time of diagnosis. These are diseases that, when the patients are subjugated to treatment with systemic corticosteroids, increases the risk of death. Interestingly, the incidence of diabetes is increased among BP patients within the first year of diagnosis compared to an age and gender matched control group.

The pathogenesis of BP is dependent on IgG auto antibodies directed against the hemidesmosomes of the epidermal cells in the basement membrane. The antigens are the BP230 (BPAG1/Dystonin) and the BP180 (BPAG2/Type XVII Collagen) that upon binding induces inflammation in the skin and causes the formation of the blisters. Histologically a split on the dermo-epidermal border is seen along with an eosinophil dominated inflammation. Using direct immunofluorescence a linear band of IgG and/or C3 along the dermo-epidermal border can be demonstrated, as can circulating antibodies against BPAG1 or BPAG2.

The BPAG-1 is also expressed by the neurons and Schwann cells of the central nervous system and peripheral nervous system. This is believed to be the molecular background for the strong association between BP and multiple sclerosis (MS), OR = 10 in several studies. Other neurological diseases such as Parkinson disease and Alzheimer's disease also have an increased prevalence among patients diagnosed with BP, yet the pathogenetical pathways for this are not as clear as for MS.

In this short review we will go through the evidence and studies on the association between BP and cardiovascular as well as neurological diseases, in order to show that this group of dermatological patients do have need for a multidisciplinary approach and special care when it comes to treatment.

BULLOUS PEMPHIGOID AND CARDIOVASCULAR DISEASES

There is some evidence for an association between BP and cardiovascular diseases. Kibsgaard et al. described this in a retrospective consecutive case-series study of inpatients admitted to Department of Dermatology and Venerology in Aarhus, Denmark, between 2006 and 2013 (1). A total of 69 patients (70%) suffered from cardiovascular diseases (as determined by ICD10 diagnoses: I00 to I99) of which 39 were hypertension. The remaining cardiovascular diseases were congestive heart failure, arrhythmias, prior acute myocardial infarcts, dilated cardiomyopathy, and valvular diseases. The prevalence of cardiovascular diseases in the studied patients was larger than the known prevalence of cardiovascular diseases in the Danish background population. This may indicate an association between BP and cardiovascular disease. Similarly, Försti et al. found that cardiovascular diseases were the most common comorbidities among 198 cases with BP. This study was a retrospective database study of all cases of BP diagnosed at the Department of Dermatology, Oulu University Hospital, Finland, between 1985 and 2012 (2).

In contrast, Lee et al. found that hypertension was less frequent among patients with BP (48.5%) compared to the general Korean population (65.7%). The study of Lee et al. was a retrospective evaluation of 103 patients diagnosed with BP at the Chonnam National University Hospital in Gwangju, Korea, between 2006 and 2013 (3). Yet, hypertension was the most frequent comorbidity among the Korean BP patients with a univariable hazard ratio (HR) of 1.23(0.67–2.25). However, the HR for hypertension was non-significant (p-value 0.505) when compared to the background population (3). This might be a result of the BP patients older age. Mean age of the BP patients in this study was 74.4 years at time of bp diagnosis.

Similarly, Banishahemi et al. found that hypertension is the most frequent comorbidity (22.9%) among patients with BP (4), and that the prevalence of hypertension was lower in the studied BP patients compared to the Iranian population older than 55 years of age. In this descriptive cross-sectional study of 122 Iranian patients the mean age of the BP patients was 69 years.

In a Danish population based cohort study of 3,500 BP patients a significantly increased OR (1.7) of hypertension at the time of diagnosis was also found (5). Yet, when following up the risk of developing hypertension was the same as the age and sex matched control population (HR = 1.0) and even significantly decreased (HR = 0.8) if the first year of observation was excluded thus not including patients who had died within the first observational year. This may be due to patients diagnosed with BP had more contacts to the health care system and would thus be treated for other diseases that could lead to hypertension. Alternatively, the results could indicate that BP may be the endstage of several systemic disease, or as it has been suggested; that certain drugs may increase the risk of BP (6).

The possible association between BP and cardiovascular diseases could be a result of inhibited fibrinolysis and coagulation activation in patients with BP. In an observational study of 20 BP patients compared to 20 age and sex matched healthy subjects levels of plasminogen activator inhibitor type 1 (PAI-1) antigen, tissue plasminogen activator (t-PA) antigen, fibrin fragment d-dimer and prothrombin fragment were compared (7). An increase in PAI-1 in patients with active BP but also an increase of t-PA, fibrin fragment d-dimer and prothrombin were found, indicating inhibited fibrinolysis and a coagulation activation in the patients with BP. Marzano et al. also found that during remission after treatment with corticosteroids levels of PAI-1 and fibrin fragment d-dimer were reduced, thus reducing the inhibition of fibrinolysis and decreasing the trombotic risk.

Another possible explanation of the association between BP and cardiovascular disease could be that the BP antigen (BP230) is expressed in cardiac muscle. Steiner-Champliaud showed that the BP230 is expressed in primate cardiac muscle cells (8). Also, Andrä et al. found disruption of the intercalated discs of the myocardium in BP230 deficient mice (9). Boyer et al. found signs of cardiac stress in BP230 deficient mice (10).

BULLOUS PEMPHIGOID AND NEUROLOGICAL DISEASE

The association between BP and neurodegenerative diseases is well-described (11, 12). There is evidence that having a neurodegenerative disease increases the risk for developing BP and that patients with neurodegenerative diseases have a higher standardized mortality rate. The molecular link between BP and neurodegenerative diseases is hypothesized to be autoantibodies against the antigens BP180 and BP230 expressed in neurons as well as the basement membrane of human skin. However, only few immune pathological studies have confirmed this theory. Yet, the question is: Which came first, the hen or the egg, hence the neurodegenerative disease or bullous pemphigoid?

Försti et al. studied 198 Finnish cases with BP and found that the most common comorbidities were cardiovascular diseases (76.3%) and neurodegenerative diseases (40.9%) (13). Teixeira et al. made a case control study with 77 cases and 176 controls (14). At least one neurological disease was present in 55.8% (43) of bullous pemphigoid cases before the diagnosis of BP compared with 20.5% (36) of controls (OR 5.36, 95% CI 2.97–9.66). Jeon et al. retrospectively evaluated 103 patients diagnosed with BP between 2006 and 2013 in Korea and found that among patients

TABLE 1 | Bullous pemphigoid and comorbidities.

	Study design	Cases (*N*)	Mean age (years)	Female: male ratio	Controls (*N*)	Confounders	Cardiovascular diseases* (%)	Neurological comorbidities (%)	Comparative measures
Kibsgaard et al. (1) (Denmark)	Case-series (retrospective)	98	78 (SD ± 10)	1.7	NA	Adjusted for age and sex	70	Stroke: 12.2, dementia: 17.3 PD: 4, MS: 1	NA
Banishahemi et al. (4) (Iran)	Retrospective descriptive study	122	69	NA	NA	NA	NA	Epilepsy: 0.8 PD: 1.6	NA
Casas-de-la-Asunción et al. (18) (Spain)	Observational, retrospective, case-control	54	80.8	1.5	108	Adjusted for age and sex	NA	NA	**OR** of dementia: 4.6 (2.1–10.1), PD: 5.9 (1.8–19.7), CVD: 3.1 (1.2–7.9)
Gambichler et al. (21) (Germany)	Retrospective database study AND experimental study	161	85/78 (±ND)	2.7 (+ND) 2.6 (–ND)	NA	NA	NA	Dementia: 48.2, Stroke: 17.6, PD: 14.1	NA
Gornowicz-Porowska et al. (19) (Poland)	Case-series (retrospective)	82	78.5/74 (±ND)	0.8 (+ND) 2.6 (–ND)	NA	NA	NA	24	Pearson X^2 test used to check for relationship between ND development and the BP180/360 anti IgG level
Kalinska-Bienias et al. (25) (Poland)	Case-series (retrospective)	205	76.2	0.6	NA	NA	38	Dementia: 22.4, stroke: 13.7 PD: 4.9, depression:4.4	NA
Khosravani et al.(15) (Iran/USA)	Cross-sectional case-control study	87	Cases: 64.2 Controls: 60.7	Cases: 1.1 Controls: 2.1	184	NA	NA	Cerebrovascular disease: 8 Dementia: 16.8	NA
Marzano et al. (7) (Italy)	Follow up study	20	76	1	20	NA	NA	NA	NA
Ren et al., 2017 (USA)	Cross sectional	2.105	75.9 (primary) 77.5 (secondary)	1.6	1.5	NA	CHF: 2.7, AFLI: 2	NA	**OR** Vascular dementia: 1.7, PD: 1.8
Tarazona et al. (16) (Brazil)	Cross sectional	25	73.9	2.6	NA	NA	NA	NA	NA
Brick et al. (17) (USA)	Case-control & matched cohort design	87	77.5	Cases: 1.4 Controls: 1.3	261	Adjustet for age (±2 years) and sex	NA	**Case group**: 17 **Control group**: 11	**OR** of dementia: 9.0 (2.4–33.2), PD: 7.2 (0.6-infinity), MS: 3.0 (0.19–48.0) & CVD: 1.8 (0.4–7.5) **HR** of dementia 1.3 (0.6–2.6), PD: 8.56 (1.6–47.3), CVD: 2.5 (0.9–7.1)
Försti et al. (13) (Finland)	Case-control & matched cohort design	4.524	NA	Cases: 1.5 Controls: 1.2	66.138	Adjusted for age and sex	NA	**Cases**: CNS: 41, Psyciatruc: 10.4 **Controls**: CNS: 16, Psyc: 7	**OR** of Alzheimers disease: 2.6 (2.3–3.0), PD: 2.4 (2.0–2.9), MS: 5.87 (3.93–8.53) **HR** of Alzheimers disease: 1.2 (1.1–1.4), PD: 1.1 (0.8–1.5), MS: 0.7 (0.1–5.0), schizophrenia 1.4 (0.6–3.4)
Kibsgaard et al. (5) (Denmark)	Case-control & matched cohort design	3.281	76.5 (SD± 12.6)	Cases: 1.3 Controls: 1.3	32.213	Adjusted for age and sex	Case group: 57.6 Control group: 45.6	**Case group**:34.5 **Control group**: 22.4	**OR** of Alzheimers: 2.6 (1.8–3.5), PD: 4.2 (3.1–5.8), MS: 9.7 (6.0–15.6) and hypertension: 2.0 (1.8–2.2) **HR** of Alzheimers: 0.83 (0.6–1.1), Parkinsonism: 0.7 (0.4–1.2) MS: 9.4 (4.9–18.0) and hypertension: 1.1 (1.0–1.2)

**Within the studied BP population.*

with BP the prevalence of dementia, and Parkinson's disease was higher than in the general population (3). Kibsgaard et al. found in a population-based cohort study of BP patients that the second most frequent comorbidity was neurologic disorders, comprising multiple sclerosis (5).

In a case control study a cerebrovascular accident (CVA) was the most common neurological disease which was seen in 7 patients (8.0%) in the case group and 4 patients (2.1%) in the control group ($p = 0.022$) (15), also dementia was significantly increased 16.8 vs. 1.0% ($p = 0.008$).

In a Brazilian cohort of patients with BP a significantly higher prevalence of neurological and/or psychiatric diseases was found (16). Especially cerebrovascular accident (CVA) and dementia were over-represented. Brick et al. found in their case control study an association of BP with neurologic disorders such as dementia and Parkinson disease (17). In a Spanish case-control study patients with BP were found to have a higher frequency of neurologic conditions (18).

The hypothetical pathogenetic association between BP and neurological disease has been questioned by Gornowicz-Porowska et al. finding insignificant differences in the autoantibody (BP180 and BP230) levels of patients with BP with and without neurological disease (19). Similarly, Ali et al. found no significant correlation between the transcripts of BPAG1a in the central nervous system and a diverse spectrum of neurological disorders related with BP (20).

Whether BP precedes neurological diseases or vice versa, fosters an ongoing discussion. Gambichler et al. found in their database of inpatients with BP a significantly increased frequency of neurological diseases in BP patients (21). They also studied brain tissue of mammalians treated with serum from nine patients with BP and elevated BP180 autoantibodies. These studies showed that raised BP180 titres and blood eosinophils were independent predictors for the presence of neurological disease in the patients with BP. However, the experimental data did not support previous results indicating that specific binding of BP180 antibodies in neuronal tissue plays a pathogenetic role in neurological disease. Taghipour et al. found that patients with BP and neurological disease exhibit an immune response to both BP180 and BP230, and thus hypothesize that both antigens may be exposed following a neurological insult followed by generation of an immune response in terms of BP (22).

In many patients with the co-existence of BP and neurological disease, the onset of neurological disease precedes the onset of BP with many years. We have considered this apparent paradox, and we hypothesize that the time lag between neurological disease and the onset of BP might be a result of the gradual exposure of autoantigens (BP180 and BP230) as the neurological disease progresses. The titres of anti-BP180/230 might increase as these antigens are exposed in the neurological tissues. Thereby increasing the risk of developing symptoms in other tissues containing BP180/230, for example skin and mucous membranes.

TREATMENT OF BULLOUS PEMPHIGOID

BP has traditionally been treated with topical steroids supplemented with systemic corticosteroid treatment if necessary. This is reflected in the British Association of Dermatologists' (BAD) 2012 guidelines for the management of BP (23). Superpotent topical corticosteroids are recommended as first-line treatment in localized and moderate disease, and in generalized BP the only validated systemic treatment is oral prednisolone. Yet, there is broad consensus among dermatologists to the use of other immunomodulating treatments in order to minimize the accumulated dose of systemic corticosteroids and the consequential detrimental side effects.

In the retrospective Finnish study by Försti et al., it was found that polypharmacy was very common in patients with BP, and the higher the number of drugs, the greater the mortality (24). Thus, the mortality for BP in Finland is 7.6-fold that of a reference population, due to malignancies and polypharmacy.

Kibsgaard et al. found in their retrospective consecutive case-series study of 98 BP patients a significant difference in admission time in favor of patients treated with low dose prednisolone (<45 mg/day) ($p = 0.02$) vs. patients treated with high dose prednisolone (>45 mg/day) (1). This may be due to the fact that patients treated with high doses of systemic glucocorticoid were initially suffering from more severe disease. However, this association could not be shown in the following sub-analysis. In contrast, rate of remission and relapse, median duration of systemic corticosteroid and immune modulating treatments, and total treatment duration were independent of the dichotomized initial doses of systemic glucocorticoids. There was no information on development of comorbidities in the two groups.

Similarly, Kalinska-Bienias et al. found that prednisone in moderate dose (0.5 mg kg−1) in monotherapy was an independent risk factor of fatal prognosis in the 1st year of follow-up, assessed by multivariate analysis (25). Patients treated with prednisone in monotherapy were in this study associated with almost a two-time increased risk of mortality. A weak correlation was found (in univariate analysis only) that the patients who received tetracycline plus nicotinamide showed decreased mortality within the 1st year of follow-up.

These results are in line with the co-morbidities of cardiovascular diseases and diabetes as described above, and since prednisolone may further accentuate these diseases and increase the mortality as can be seen in the studies by systemic corticosteroid may be responsible for the increased mortality during the first year after diagnosis.

In order to minimize the daily as well as the cumulative dose of systemic corticosteroid, so-called steroid sparing immune modulating drugs can be used to treat bullous pemphigoid. The immune modulating agents can be used in combination with topical steroids and/or systemic corticosteroids or alone.

A pragmatic, multicenter, randomized controlled trial by Williams et al. underpins that Doxycycline is non-inferior to standard treatment with oral prednisolone for short-term blister control in bullous pemphigoid and significantly safer in the

long-term (26). The role, dosing and duration of tetracycline (e.g., plus nicotinamide) treatment must, however, be further established in future randomized controlled trials.

Sticherlin et al. found that Dapsone appeared to have a moderately higher corticosteroid-sparing potential than azathioprine (27, 28). Yet, due to the lower than intended number of patients, the results of the primary and secondary endpoints were not or only barely significant. The combination regimen of either drug with oral methylprednisolone was associated with a relatively low 1-year mortality. This was a prospective, multicentre, randomized, non-blinded clinical trial comparing the efficacy and safety of two parallel groups of patients with BP treated with oral methylprednisolone in combination with either azathioprine or dapsone.

Azathioprine is also used as a steroid sparing agent against bullous pemphigoid. The effect and side effects of Azathioprine in the treatment of bullous pemphigoid was compared to Mycophenolate Mofetil, both in combination with oral prednisolone, in a prospective, multicenter, randomized, non-blinded clinical trial (29) showing equal efficacy.

Methotrexate is a commonly used as a steroid sparing agent for BP as found by Kibsgaard et al. considered equally effective as Azathioprine for the treatment of bullous pemphigoid. However, this has never been studied in a randomized controlled clinical trial.

Other prednisolone sparing immune modulating agents include Cyclosporine, Rituximab (28, 30, 31), intravenous immunoglobulin (32), Cyclophosphamide (33), and plasmapheresis (34, 35). Some of these treatments can be used in combination (36).

In our opinion oral prednisolone is an effective treatment with profound evidence of effectiveness in the treatment of severe BP. However, increased morbidity is strongly associated to especially long term use of oral prednisolone. Therefore, we recommend the immediate institution of steroid sparing treatment when the clinical diagnosis of BP has been pitched. Histological diagnosis should be awaited. For patients with diabetes and in the very old patients, we prefer to omit oral prednisolone and instead use highly potent topical steroids once or twice daily for some weeks and to treat the patients with oral Doxycycline (26, 37) or Dapsone (27, 38, 39). BP patients usually experience effect of Doxycycline or Dapsone, respectively, within 3–4 weeks.

A new publication by Quick et al. elucidates BP antigens as potential targets in future therapy of patients with BP (11).

CONCLUSION

BP is associated with increased risk for cardiovascular disease and neurological diseases. Whether the pathogenetic processes in BP predisposes to cardiovascular diseases and neurological diseases or vice versa has not been fully established yet.

The most well-established association is between multiple sclerosis and BP, which may also be explained biologically through the formation of antibodies against BP180 and BP230 which are expressed in the skin and in the neurological tissue. Thus biology and epidemiology is closely related.

In terms of cardiovascular disease the most prominent is hypertension which in many, but not all studies, seems to be associated with BP. As a consequence, there may also be an association with other cardiovascular diseases including valvular diseases.

Systemic corticosteroids are effective in the treatment of BP. However, the use of systemic corticosteroids prolongs admission time for the patients and increases the BP patients morbidity and mortality. Thus systemic corticosteroids have detrimental effects on hypertension and cardiovascular diseases and may as such bias the perception of the association between these diseases.

Although there are few studies on the use of non-corticosteroid immunemodulating treatment of BP, the findings of shorter admission time, and increased mortality within the first year of treatment and the fact that prednisolone monotherapy may double mortality leads us to conclude that non-corticsteroid treatment should be instituted as early as possible after the diagnosis of BP.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Growing Incidence of Bullous Pemphigoid: Overview and Potential Explanations

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Bullous pemphigoid (BP) is the most common type of subepidermal autoimmune bullous diseases. BP characteristically affects the elderly and is seen mainly in patients older than 70 years. While the annual incidence of BP has been estimated to be between 2.4 and 23 cases per million in the general population, it rises exponentially to 190–312 cases per million in individuals older than 80 years. In addition, a growing body of evidence reports a remarkable trend of increased incidence of BP, showing a 1.9- to 4.3-fold rise over the past two decades. This demonstrable increase warrants a higher awareness of the increased risk to develop BP. This review summarizes the current understanding of the epidemiological features of BP and sheds light on the putative explanations for its growing incidence.

Keywords: bullous pemphigoid, epidemiology, explanation, review of literature, incidence, DPP-4 inhibitors, longevity

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THE GENERAL EPIDEMIOLOGY OF BP

Bullous pemphigoid (BP) is the most common type of subepidermal autoimmune bullous diseases (1). The disease develops characteristically in the elderly, particularly in patients older than 70 years (2–5). The annual incidence of BP has been estimated to range between 2.4 and 21.7 new cases per million population in different populations worldwide (2, 3, 6–11). An even higher annual incidence of 42.8 cases per million population was reported in the United Kingdom (UK), although this report may be interpreted with caution as it is based on a computerized longitudinal general practice database (12). Also based on health insurance data, the prevalence of BP has recently been estimated at 259 per million population in Germany, i.e., ∼21,000 patients with BP lived in Germany as for 2014 (13). Despite this increase, BP is still considered as an orphan disease, i.e., <5 in 100,000 persons are affected by the disease (14).

BP is traditionally considered as a disease of the elderly population. The mean age of presentation ranges between 66 and 83 years in different cohorts across the globe (9, 15). The incidence rises exponentially with age, culminating at 190 to 312 cases per million per year in individuals older than 80 years of age (3, 11, 12, 16). Conversely, BP is rarely encountered in individuals younger than 50 years, with the reported incidence rates usually lower than 0.5 cases per million population in this age category (3, 8, 12, 17). This is atypical for an autoimmune disease, caused by an autoantibody response toward the hemidesmosomal proteins BP180 and BP230 (18–22), as autoimmune diseases usually arise during young adulthood (23–25).

An evident female preponderance was noted in the majority of studies, with a female-to-male ratio ranging between 1.04 and 5.1 (2, 4, 6, 7, 9, 12, 15, 26–28). Several studies found that the incidence rate seems to be higher in women until the age of 75, but thereafter the incidence is higher in men (3, 9, 17).

OVERVIEW OF THE INCREASING TREND IN DIFFERENT REGIONS IN THE LAST TWO DECADES

A growing incidence ranging from 1.9- to 4.3-fold in the past two decades has been reported in recent data from the UK, France, Germany, and Israel (**Table 1**) (2, 9, 12, 17, 28).

In a retrospective cohort study, the annual incidence of BP was estimated at 21.7 cases per million population throughout the years 2000–2005 in three French regions: Haute-Normandie, Limousin, and Champagne-Ardennes (9). This figure represents more than 3-fold increase relative to the annual incidence previously estimated in three adjacent French regions between the years 1986–1992: Limousin, Touraine, and Picardie, with very similar demographic characteristics (6.7 cases per million population) (2). This rise corresponds to an average increase of approximately one case per million per calendar year (9).

In accordance to the French study, very similar incidence rates had been concurrently calculated in two German regions during a similar period, namely Lower Franconia (6.1 cases per million population) and Northwestern Bavaria (6.6 cases per million population), between the years 1989–1997 and 1989–1994, respectively (28, 29). Bertram et al. (8) reported a 2.2-fold increase in annual incidence rate in Lower Franconia in 2001–2002 (13.4 cases per million population) as compared to the previous study.

A consistent rise of the annual incidence rates of BP has been reported in the UK during the last two decades; from a nadir of 10.0 cases per million population in 1985 (30), to 14.0 cases per million population during 1991–2001 (11), and up to 42.8 cases per million population during the 2001–2004 (12). However, the last study may be biased by overestimation as was previously discussed (12).

A recent population-based study investigating the epidemiology of BP in Northern Israel revealed a 1.9-fold increasing annual incidence: from 7.6 to 14.3 cases per million population in the calendar periods 2000–2005 and 2011–2015, respectively. This rise applied to both of the major ethnic populations residing in the region: Jews and Arabs (17).

PUTATIVE EXPLANATIONS FOR THIS SURGE

Increasing Life Expectancy of the Populations

Global mean life expectancy increased substantially by 5.5 years between 2000 and 2016, the most rapid increase observed since the 1960s (31). Over the past 15 years, a 2.9 year increase in life expectancy has occurred in the European Union countries, rising from 77.7 to 80.6 years; the rise was 2.4 years for women and 3.4 years for men (32). This rapid rise was attributed to several putative factors including the improved lifestyles, rising living standards, better education, and advanced healthcare and medicine (32). Given the increasing incidence rate of BP with age, the higher longevity account for part of the increased overall incidence of BP throughout the years.

In countries where rising incidence of BP was reported, a parallel rise in life expectancy was observed. In France and the UK between 1980 and 2000, life expectancy rose from 74.1 to 79.2 years and from 73.7 to 78.0 years, respectively (32, 33). In 2015, life expectancies in these two countries further increased to 82.4 and 81.0 years, respectively (32). In Germany, the life expectancy increased consistently, rising from 73.1 in 1980 to 78.3 in 2000 and reaching 80.7 in 2015 (32).When examining fluctuations in the life expectancy throughout the relevant period in Israel, a 3 years increase was identified; from 79.0 in 2000 to 82.1 in 2015 (33). Considering the aging trend of the European population, even more individuals are expected to develop BP in the coming decades.

Increasing Incidence of Disabling Neurological Conditions

A large body of evidence gathered within the past decade suggests a high association between neurological disorders and BP. The prevalence of neurological comorbidities among patients with BP ranges between 28 and 56% (34–37). In addition, neurological conditions were found to be an independent risk factor for the subsequent development of BP as revealed by several well-designed observational studies (16, 38–40). The presence of coexisting neurological disease at the onset of BP was found to be a bad prognostic factor (39, 41–43).

The burden of neurological disease has grown remarkably within the past few decades (44). The increasing number of individuals affected by these diseases has mainly been associated with the aging of the population and population growth (44). There was a large rise in the absolute numbers of prevalent cases of Alzheimer's disease and other dementias (44, 45). In addition, an increasing incidence of stroke (46), epilepsy (47), and intracranial malignancies in elderly people (48), as well as growing prevalence of multiple sclerosis (49, 50) have been reported in different regions. The increasing burden of these neurological conditions, found to be an established risk factor for BP, may account for part of the rising incidence of BP.

One of the widely accepted explanations for this association is the cross-reactivity between the neuronal and epithelial isoforms of BP Antigen-1 (BP230), which are both encoded by dystonin gene (*DST*) (51–55). Mice with mutations in this gene developed severe dystonia and sensory nerve degeneration, further supporting this concept (56). Additionally, a large amount of evidence suggests that BP180 may also be expressed in certain components of the central nervous system (54, 55).

Increasing Use of Certain Culprit Drugs

Dipeptidyl-Peptidase IV Inhibitors

Growing evidence suggests that dipeptidyl-peptidase IV inhibitors (DPP4i), new anti-hyperglycemic oral agents used to treat type 2 diabetes mellitus, may be implicated in the development of BP (57, 58). Sitagliptin was the first DPP4i agent to gain US Food and Drug Administration approval

TABLE 1 | Increase in bullous pemphigoid incidence across different populations.

Country	First incidence rate	Second incidence rate	Third incidence rate	Increase*	References
France	6.7 (1986–1992)	21.7 (2000–2005)		3.3-fold within 14 years	(2, 9)
Germany	6.1 (1989–1997)	6.6 (1989–1994)	13.4 (2001–2002)	2.2-fold within 8 years	(8, 28, 29)
United Kingdom	10.0 (1985)	14.0 (1991–2001)	42.8 (2001–2004)	4.3 within 17 years	(11, 12, 30)
Israel	7.6 (2000–2005)	12.6 (2006–2010)	14.3 (2011–2015)	1.9 increase within 10 years	(17)

*Incidence rates refer to cases per million population per year. Figures in brackets indicate the years when the incidence was determined. *The period in which the increase occurred was estimated by calculating the difference between the midpoint of the first and last follow-up periods.*

in 2006, followed by vildagliptin (2007), linagliptin (2011), and alogliptin (2013) (57). Since the approval of these agents, their administration has become more widespread as either monotherapy or in conjunction with insulin or other oral antihyperglycemic medications.

The role of DPP4i in triggering BP was grounded mainly on case reports (59–66) and national pharmacovigilance database analyses (67, 68) until well-designed controlled observational studies were recently conducted (57, 58). Benzaquen et al. (57) reported that DPP4i intake was associated with an increased risk for triggering BP (adjusted OR, 2.64), with vildagliptin being implicated with the highest risk (adjusted OR, 3.57). Varpuluoma et al. (58) found that DPP4i administration was associated with 2.2-fold increased risk for BP (adjusted OR, 2.19), particularly with vildagliptin (adjusted OR, 10.4). The aforementioned studies were underpowered to analyze the association between BP and linagliptin, which is most likely due to the limited number of cases under treatment with this new agent. Kridin and Bergman observed that overall DPP4i intake was associated with a 3-fold increased risk for BP (adjusted OR, 3.16). The adjusted ORs of vildagliptin and linagliptin were 10.67 and 6.65, respectively. Furthermore, a recent Greek retrospective cohort study observed a 38.4% increase in the prevalence of patients with comorbid type 2 diabetes mellitus in a cohort of 130 patients with BP. This rise was ascribed mainly to the growing prescription of DPP4i (69). Interestingly, DPP4i may in addition increase the risk for another pemphigoid disease, namely mucous membrane pemphigoid (MMP), where exposure to these agents accounted for 24 cases of a total of 313 MMP patients in a recent French cohort study (70).

The clinical and immunological characteristics of DPP4i-related BP were described only in few studies. Izumi et al. (71) reported that seven patients with DPP4i-associated BP tended to present with the non-inflammatory phenotype, characterized by reduced erythema and scant lesional eosinophilic infiltration. Autoantibody reactivity against the midportion of BP-180, but not against the NC-16A immunodominant domain of BP180, was detected in these patients. Recently, Chijiwa et al. (72) reported that the frequency of lesional eosinophilia and mucosal involvement was significantly lower in nine patients with DPP4i-related BP than in 21 patients with non-DPP4i-related BP. Garcia-Diez et al. (73) had recently reported that 4 (50%) out of their 8 patients with DPP4-related BP developed non-inflammatory phenotype, whereas 6 (75%) were tested positive for anti-NC-16A BP180 autoantibodies in ELISA. In their longitudinal follow-up across 9 years, Kawaguchi et al. (74) found that 8 patients developed BP while being managed with DPP4i, of whom 6 (75%) had a non-inflammatory phenotype, and 5 of the 6 (83.3%) had been tested negative for anti-NC-16A BP180 autoantibodies in ELISA. The susceptibility to the development of DPP4i-associated BP was found to associate with HLA-DQB1*03:01 allele (75).

The pathomechanism underlying the association between DPP4i and BP has yet to be fully elucidated. It is known that DPP4 is a cell-surface plasminogen receptor that activates plasminogen, which leads to the formation of plasmin (76). The latter is a major serine protease that cleaves BP180 within the immunodominant NC-16A domain and can be detected in lesional skin as well as in blister fluid of patients with BP (77). The inhibition of plasmin by DPP4i may alter the appropriate cleavage of BP180, which may affect its antigenicity and function (71). Additionally, DPP4 inhibition may substantiate the activity of eotaxin and other pro-inflammatory cytokines, leading to cutaneous eosinophil activation and blister formation (78).

Psychotropic Drugs

The intake of psychotropic medications, particularly phenothiazines with aliphatic side chains, was associated with an increased risk for the development of BP in two well-designed case-control studies (16, 79). The prescription of this group of medications has risen over the past few decades, according to studies across different countries (9, 80, 81). More alarming is the rise of polypharmacy of psychotropic medications within the last decade (80). Again, the mechanisms leading to psychotropic medication-induced BP remain unclear.

Checkpoint-Inhibitors

The use of checkpoint inhibitors, such as therapeutic monoclonal antibodies targeting cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed death ligand-1 (PD-L1) has greatly improved the survival of many cancer patients, especially with metastatic melanoma (82). Due to the inhibition of immune checkpoints, chronic inflammatory, and autoimmune diseases are among the most common adverse events of checkpoint inhibitor treatment in cancer (83). This also includes the induction of BP under checkpoint inhibitor treatment. So far, at least 22 cases of BP have been reported under checkpoint inhibitor treatment (84, 85). While this most likely does not greatly add to the rising incidence of BP, the induction of autoimmunity toward BP180 may be a useful indicator to predict checkpoint inhibitor treatment

outcomes (Poster LB1513 at the 2018 International Investigative Dermatology Meeting).

Increasing Awareness of the Atypical Variants of BP

Joly et al. (9) suggested that the improving awareness of the recently described clinical variants of BP, which were not recognized in the past, maybe another putative factor contributing to the increasing detection of BP. These BP variants that may appear in up to 20% of patients include the prurigo-like type (86), urticaria-like type (87), eczema-like type (88), dyshidrosiform type (89), erosive type (90), and erythema annulare centrifugatum-like type (30). In a recent systematic review of case reports and series that described BP patients lacking frank bullae, 132 patients with atypical non-bullous presentation had been identified. Urticarial plaques (52.3%) and papules/nodules (20.5%) were the most reported clinical features among these patients. Only 9.8% of patients developed bullae during the reported follow-up, thus leading to a substantial average delay of 22.6 months until a confirmatory diagnosis of BP (91).

Of interest, two previous studies reporting a growing incidence of BP revealed that a sizable portion of the patients presented with atypical clinical presentation. Joly et al. (9) found that 20.5% of their 502 BP patients had atypical clinical variants, of which the majority had prurigo-like and eczema-like variants. Kridin and Bergman (17) noted that 8.4% of their 287 patients had atypical clinical manifestation, with the urticaria-like and prurigo-like variants as the most common clinical picture seen among these patients.

This assumption is further supported by the recent survey conducted in the SSENIOR study (92). Herein the authors evaluated the prevalence of pruritus and pemphigoid in a high-risk population of nursing home residents. Almost half of the nursing home residents (48%) complained of pruritus. In addition. seven (6%) of 126 subjects had pemphigoid, of which four had chronic severe pruritus without blistering [non-bullous pemphigoid (91)], and three had known bullous pemphigoid. Hence, populations at risk, especially when pruritus is present, should be screened for the presence of BP using the appropriate measures. This further supports the assumption that the increased awareness has contributed to the increase in BP, but also highlights that it is likely that the incidence of BP will still increase, as approximately 4% of an at-risk population had undiagnosed BP.

Better Diagnostic Methods

In 1953, Dr. Lever distinguished BP from pemphigus based on distinct histologic features, which had been not noted before (93). This distinction based on histomorphologic changes was a landmark discovery, allowing differentiating between pemphigus and pemphigoid. Another breakthrough discovery, which contributed to an improved diagnosis of BP, was the discovery of IgG deposits by direct immunofluorescent (IF) microscopy in skin specimen from BP patients (94). By evaluating direct IF microscopy at high magnification, patterns, such as n- or u-serration become visible. This allows distinguishing between different pemphigoid diseases. However, the BP-associated, n-serrated pattern, is present in BP and other pemphigoid diseases (95). Lastly, the presence of circulating anti-basement membrane autoantibodies in BP patient sera by indirect IF microscopy on human salt-split skin as a substrate was described (96). Indeed, in addition to H&E stained lesional biopsies, direct and indirect IF microscopy are sufficient to diagnose BP if the disease is suspected. However, these techniques are mostly provided by tertiary centers. To overcome this limitation, methods allowing screening for BP (and other autoimmune skin blistering diseases) have been developed in the past. First, autoantibodies against BP180 and BP230 can be detected by specific ELISA systems (97–99). In addition, paper-based ELISA systems have also been described, but are, not commercially available so far (100).

Second, if substrates for IF microscopy or ELISA are not available, biochips spotted with primate salt-split skin, recombinant BP180 and BP230 expressing cells can be purchased and used for indirect IF microscopy for the detection of circulating autoantibodies directed against BP180 or BP230 (101). This may be even automated, for high-throughput analysis (102, 103).

Taken together, direct IF microscopy of a perilesinal skin biopsy for the detection of tissue bound IgG/A and/or C3, and indirect IF microscopy on human salt-split skin for the detection of circulating anti-basement membrane autoantibodies are the minimal diagnostic criteria for BP. If these techniques are not available, specific ELISA systems and/or biochips may be used for BP diagnosis. Hence, a broad spectrum of diagnostic tests has become available, and thus certainly contributed to the increased incidence of BP.

CONCLUSION

While BP is still a rare disease, its incidence rises exponentially in the elderly and is associated with remarkable burden. The growing incidence of BP warrants an expanded awareness of the disease, especially on its atypical non-bullous presentations. Alongside practicing dermatologists, other physicians caring for the elderly patients, including general practitioners and geriatricians, should be attentive of the increasing incidence of BP and to refer patients with a suggestive clinical picture to experienced centers. Caution and careful evaluation should be exercised with regard to the use of DPP4i, particularly in high-risk patients like those with disabling neurological conditions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Genetics of Pemphigus Vulgaris

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Pemphigus vulgaris (PV) is a severe autoimmune blistering disease caused by auto-antibodies (auto-Abs) directed against epithelial desmosomal components and leading to disruption of cell-cell adhesion. The exact mechanisms underlying the disease pathogenesis remain unknown and treatment is still based on immunosuppressive drugs, such as corticosteroids, which are associated with potentially significant side effects. Ethnic susceptibility, familial occurrence, and autoimmune comorbidity, suggest a genetic component to the pathogenesis of the disease, which, if discovered, could advance our understanding of PV pathogenesis and thereby point to novel therapeutic targets for this life-threatening disorder. In this article, we review the evidence for a genetic basis of PV, summarize the different approaches used to investigate susceptibility traits for the disease and describe past and recent discoveries regarding genes associated with PV, most of which belong to the human leukocyte antigen (HLA) locus with limited data regarding association of non-HLA genes with the disease.

Keywords: pemphigus, genetics, autoimmunity, blistering disorders, HLA

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INTRODUCTION

Pemphigus is a group of rare, chronic, autoimmune blistering diseases, which affect the skin and mucosal membranes (1). The annual incidence of pemphigus varies among different populations and is estimated to range between 0.75 and 5 new cases per million (1). PV, the most common subtype of the disease, is characterized by ulcerations or flaccid blisters on mucous membranes and on the skin that easily rupture to cause painful, large erosions, which do not easily heal and which, if not properly treated, can lead to serious life-threatening infections and metabolic disturbances. The use of immunosuppressive drugs, which are the mainstay of treatment, have reduced the mortality from the disease to around 10%, though the adverse effects of corticosteroid treatment still cause considerable morbidity (1). PV is traditionally considered to result from the deleterious action of circulating auto-Abs, which are directed against desmosomal components, primarily desmoglein (Dsg) 3 and Dsg1, and lead to loss of keratinocytes cell-cell adhesion within the epidermis, a phenomenon known as acantholysis (2). In recent years, in addition to desmosome destabilization, blister formation in PV was suggested to result from other pathomechanisms that may be involved in PV pathogenesis and include increased secretion of pro-inflammatory mediators, abnormalities in intercellular signaling, activation of apoptosis and activation of specific muscarinic receptors expressed by keratinocytes (3–7).

Numerous studies provide support for a genetic contribution to the pathogenesis of PV as evident from the ethnic clustering of PV, the familial aggregation of the disease and the higher prevalence of additional autoimmune conditions in both PV patients and their family members (8–13). Unfortunately, although multiple attempts to identify susceptibility traits were set forth, our knowledge regarding the genetic basis of PV is far from complete. Discoveries concerning the

genetics of PV will improve the understanding of the mechanisms underlying this severe disease, which in turn may point to novel potential therapeutic targets. In this article, we review our current understanding of the genetics of PV. We describe previous attempts at identifying disease susceptibility loci and review the evidence for the contribution of human leukocyte antigen (HLA) and non-HLA genes to PV pathogenesis.

EVIDENCE FOR A GENETIC BASIS OF PV

Evidence for genetic susceptibility to contract a given disease often stems from observational studies showing varying disease prevalence in different populations, familial aggregation, and higher concordance among monozygotic vs. dizygotic twins. Over the years, many such studies have firmly established that susceptibility to PV is to a large extent genetically determined.

First, PV prevalence and incidence are low but differ significantly among diverse ethnic populations as evident by the wide range of the worldwide annual incidence of the disease, estimated to be between 0.75 and 5 new cases per million (1). For example, the disease is between 4- and 10-fold more common in the Jewish population in comparison with other Caucasian populations, with an annual incidence ranging from 15 to 30 cases per million (8). Differences in PV incidence between ethnic groups living in the same environment have also been shown and highlight the contribution of genetic, rather than environmental, factors to PV. An epidemiological study performed in Hartford County, USA, showed a 7.5-fold increase in PV incidence in Jewish adults as compared to the overall adult population (14) and a higher incidence of PV was observed in Turks and Italians living in Germany as compared to native Germans (15). A north-south gradient in PV incidence has also been observed, with PV being more common in lower latitudes, such as in Italy or Tunisia (3 and 6.7 cases per million, respectively) than Northern countries such as France or Finland (1.7 and 0.76 cases per million, respectively). This distribution could be influenced by environmental factors but could also be due to specific genetic factors (8).

In addition to population studies, familial aggregation offers yet another clue for a genetic contribution to PV. Though not common, familial cases of PV have been documented, usually involving a first-degree relative (16–19). Moreover, circulating PV-IgG Abs have been found more frequently in first-degree unaffected relatives of PV patients as compared with healthy controls (9). The prevalence of autoimmune conditions in family members of PV patients was also shown to be significantly increased, supporting an inherited susceptibility for autoimmunity, influenced by genetic factors. Compared to controls, family members of PV patients, most commonly first-degree relatives, exhibited a higher prevalence of type 1 diabetes mellitus (T1DM), autoimmune thyroid disease (AITD), and juvenile rheumatoid arthritis (10, 20).

Supporting a genetic background common to PV and other autoimmune conditions, a cross-sectional study of almost 800 PV patients revealed a significant increase in the prevalence of AITD, rheumatoid arthritis (RA), and T1DM in PV patients, in comparison with the general population (13). Comparable results were obtained in a study of 295 PV patients, showing a higher incidence of hypothyroidism, inflammatory bowel disease, and T1DM (21). In contrast, a study of 1998 PV patients, conducted in Taiwan, did not find an association between PV and RA or AITD but discovered a higher incidence of systemic lupus erythematosus (SLE) in PV patients and demonstrated that female PV patients are more likely to suffer from Sjögren's syndrome as well as from alopecia areata (22).

STRATEGIES FOR THE IDENTIFICATION OF GENETIC FACTORS IN PV

PV is a multifactorial disease, in which the risk of an individual to be affected with the disease is dependent on a combination of multiple genetic as well as environmental factors. For polygenic conditions, risk alleles are more probabilistic than deterministic, as a person carrying a high-risk trait can be only mildly prone to develop the disease. Nevertheless, identifying these susceptibility genes is crucial for a better understanding of a disease pathogenesis. One common method to identify genetic variants determining the propensity to develop a complex disease is known as association studies (23), in which the frequencies of genetic variations are compared between individuals with the disease and unaffected controls. When an allele shows a higher frequency in the affected individuals, it is considered to be in association with an increased risk to develop the disease. This association points to a region in which the causative variant lies in close proximity to the disease-associated allele (23). Association studies can be conducted in two ways. First, by using a candidate-gene driven approach, in which the regions chosen to be inspected and genotyped are ones carrying genes that, based on previous knowledge, may possibly be involved in the disease pathogenesis. Second, they can be performed without any prior hypothesis over the entire genome and are then known as genome wide association studies (GWAS).

The use of the candidate-gene approach in PV is mainly focused on genes encoding proteins of relevance to immune dysregulation and autoimmunity, such as target antigens, antigen-processing or presenting molecules, proteins related to lymphocytes function, or structure and cytokines. Over the years, most of the candidate gene-driven case-control studies, aimed at identifying genetic factors in PV, have shown associations between several HLA alleles and PV in specific ethnic groups of PV, as described below. In addition, PV was found to be associated with non-HLA genes at the HLA locus, such as *TNF-α*, *IL-6, IL-10,* and *TAP2* (24–28) as well as with genes encoding pemphigus autoantigens (29, 30). However, these data have not been reproduced in similarly designed studies and in additional populations and conflicting results have been published regarding a possible association between single nucleotide polymorphisms (SNPs) in genes coding for cytokines and PV (24, 26, 31). In recent years, new approaches to identify candidate genes are emerging. Among these, is the use of gene expression analysis to identify genes which are differentially expressed between diseased and healthy individuals and that

can be further investigated. Pathway analysis of these selected genes can lead to the identification of additional genetic factors which might be involved in the disease pathogenesis. Using this approach, Dey-Rao et al. have discovered subsets of disease-promoting and disease-preventing genes in a study of 21 PV patients (32) while Sezin et al. identified shared gene signatures between PV and SLE and discovered a possible involvement of the gene *GP9* in PV, which encodes a glycoprotein related to platelet adhesion (33).

In contrast to the candidate-gene strategy, a genome wide approach offers a hypothesis-free study and reveals associations with genes with an unknown relevance to the pathogenesis of the disease (34). The aim of genomic-wide scans is to identify co-segregation of genetic markers, previously consisting of fragment length polymorphisms and tandem repeats and more recently of SNPs, in order to define chromosomal regions containing susceptibility loci. However, in order for the results to reach statistical significance, a genome wide approach requires a large study cohort and until lately, the low prevalence of PV has represented a significant obstacle to GWAS in this disease. Sarig et al. reasoned that performing a GWAS in a genetically homogenous study group, with a relatively high prevalence of PV, such as the Jewish population (8) could help to filter out false positive association signals while uncovering significant associations, even from a relatively low number of participants (35). They performed the first GWAS in PV in a Jewish population and discovered several PV-associated markers, as described below.

While both of these methods have been used over the years in order to identify genetic factors involved in PV, novel study designs, combining the two approaches and utilizing new technologies are emerging. The advantage of utilizing gene expression studies in order to prioritize target candidate genes for SNPs in a GWAS has already been demonstrated (36) and was also implemented in PV, leading to the identification of a number of transcriptional hot spots, harboring several genes with possible involvement in PV (32). The use of next generation sequencing (NGS) in PV, including whole genome sequencing and exome sequencing may also increase the chance to identify novel genetic variants as was performed in other polygenic skin disorders (37) and could also assist in identifying causal variants underlying genome-wide associations (38).

ASSOCIATION OF HLA GENES WITH PV

The majority of studies regarding genetic predisposition to PV have been focused on the association between the disease and genes in the major histocompatibility complex, which is termed HLA in humans. This approximately 4 Mb-long region on chromosome 6p21.3, encodes above 200 genes and has the highest gene density in the human genome (39). The HLA locus is divided into three main regions: the class I region encodes the polymorphic HLA-A, HLA-B, and HLA-C genes, which assist in the presentation of antigenic peptides to cytotoxic T-cells and are expressed ubiquitously; the class II region contains polymorphic HLA-DQ, HLA-DR, and HLA-DP genes that are expressed on antigen-presenting cells (APCs) and assist in peptides display to helper T-cells; and the class III region which consists of multiple important immune system-related genes (for example, *TNF-α, C2,* and *C4*) (39). A large number of autoimmune disorders were found to be associated with the HLA region, making HLA associations a hallmark of autoimmunity (40).

So far, the association between PV and HLA class II genes remains the strongest and the most reported. As seen in **Figure 1**, a vast number of studies have been able to show an association between PV and several class II HLA alleles in specific ethnic groups of PV (8, 41–57). While some of the HLA types are more population specific, there are also ones associated with PV in numerous ethnic groups. The two most common PV-associated alleles are DQB1*0503 and DRB1*0402, both of which were found to associated with the disease in the Spanish, French, Italian, Slovak, North American and Brazilian populations (42–48) (**Figure 1**). In the Jewish population, an association was found between PV and several HLA alleles, such as HLA-DRB1*0402, and DQB1*0302 (8) while HLA-DQB1*0503 was found in association with PV in non-Jewish populations (58). A meta-analysis of the correlation between PV and HLA-DRB1 concluded that HLA-DRB1*04 and HLA-DRB1*14 are indeed statistically significant susceptibility factors for PV along with an additional HLA allele, DRB1*08 (59). Interestingly, while HLA-DRB1*0402 confers susceptibility to PV, it was found to encode a sequence motif that exerts a protective effect against RA (60).

Over the years, several studies provided evidence regarding association between PV and certain HLA class I alleles. These include HLA-A3, -A26, and -B60 in Han Chinese population (56), HLA-B38, -C12, -B57, and -C15 in the Brazilian population (47, 61), HLA-A10 and -B15 in the Japanese population (62, 63), HLA-B35 and -B44 in the Turkish population (64), HLA-B38 in the Jewish (50) and in the Spanish (45) populations, and HLA-B4402, -C0401, and -C1502 in the Iranian population (65). However, it remains unclear how do HLA class I alleles contribute to PV susceptibility. In addition, several studies have shown an association between PV and non-classic HLA class Ib alleles (HLA-E, -F, and -G). HLA-G polymorphism was found in a significant association with Jewish PV patients (66) while HLA-E, previously demonstrated to play a role in multiple autoimmune conditions (67, 68), was found in association with Caucasian and Ashkenazi Jewish patients and was suggested to be involved in the disruption of immune tolerance in PV (69).

ASSOCIATION OF NON-HLA GENES WITH PV

To date, only a limited number of studies have been able to show an association between non-HLA genes and PV (**Table 1**). Most of these studies have used a candidate gene approach to investigate a possible association between PV and autoimmune-related genes, mainly autoantigens, cytokines, and immunoglobulins known to play a role in PV pathogenesis, such as TNF-α, IL-6, and IL-10 (1, 5, 72, 73). However, the results of these studies have often been conflicting, limited to specific ethnic groups and could not be replicated in similarly

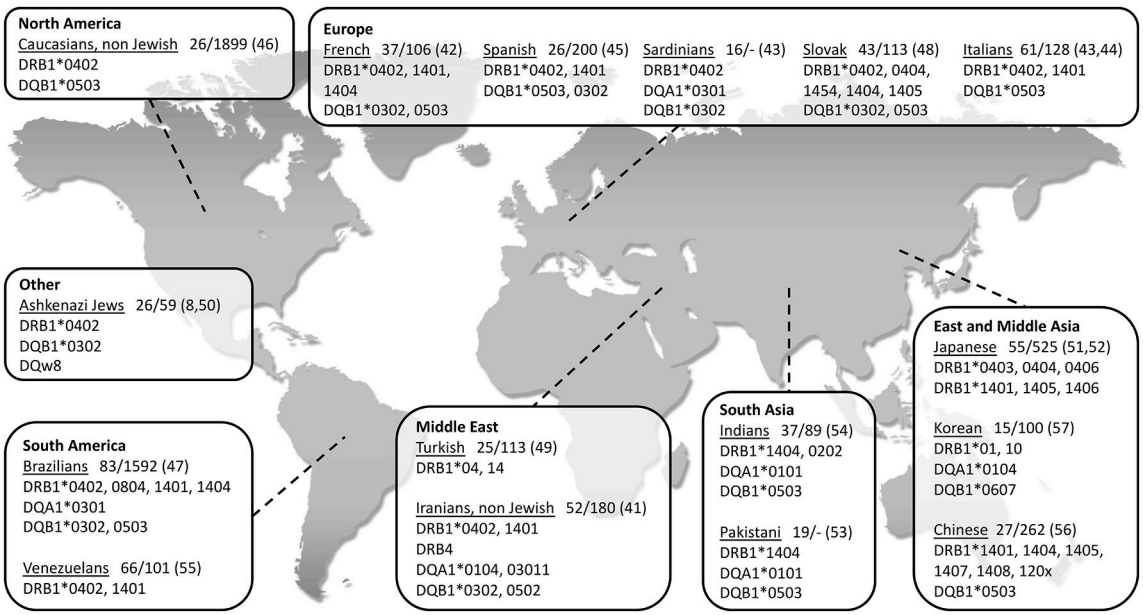

FIGURE 1 | World map showing association of type II HLA genes with PV in different populations, grouped according to geographical regions. The name of each investigated population is followed by: 1. Patients/control (*n*); 2. Reference number in brackets.

TABLE 1 | Association of non-HLA genes with PV.

Gene	Role of protein	Population	Patients/Control (*n*)	References
DSG3	Autoantigen in PV	United Kingdom	62/154	(29)
		India	28/98	(29)
VH3	Part of the variable region of the immunoglobulin heavy chain	French, Italian, and Jewish	12/–	(70)
TNF-α	Inflammatory cytokine	Slovak	34/140	(25)
		Egyptian	51/203	(27)
TAP2	Assembly and transport of peptides to HLA class I antigens	Jewish	37/37	(28)
IL-6	Pro-inflammatory interleukin	Egyptian	51/203	(27)
IL-10	Pro-inflammatory interleukin	Slovak	34/140	(25)
		Argentinian	17/24	(24)
ST18	Transcription factor with roles in the regulation of inflammation and apoptosis	Jewish	100/400 59/285	(35)
		Egyptian	126/246	(35)
CD86	A type I membrane protein which is expressed on antigen presenting cells and provides costimulatory signals necessary for T-cell activation and survival	Serbian	48/486	(71)

designed studies and/or other populations. *TNF-α* was found to be weakly associated to PV in the Slovak population (25) but not in Polish (26) or Argentinian (24) patients while in the Egyptian population only one genotype within the *TNF-α* and *IL-6* genes were found in association with PV (27). A genetic variant within the *IL-10* gene was found in association with PV in Argentinian patients (24) but not in the Slovak population, where only a haplotype inside *IL-10* showed an association to the disease (25). Slomov et al. have shown an association between PV in the Jewish population and the *TAP2* gene, encoding for a protein involved in peptides assembly and transport to HLA class I antigens (28) but this was not reproduced in the Japanese population (74). As for autoantigens, two different haplotypes within the *DSG3* gene were found in association with PV in British and Northern Indian patients, respectively. Interestingly, patients carrying any one of these two risk haplotypes were always found to carry PV-associated HLA class II alleles, suggesting possible additive effects for these two loci (29). A study of 12 PV patients discovered an association between PV and a SNP within the *VH3* gene, encoding part of the variable region of the immunoglobulin heavy chain. However, a sampling error is possible due to the small size of the patient group. Of note, no association was found between PV and the genes encoding for the constant regions of the kappa light chain or heavy chain of the immunoglobulin

(70, 75). Tanasilovic et al. reported an association between PV and a SNP within the *CD86* gene (71), encoding for a protein expressed on APCs, which has a role in T-cell activation and IgG4 production by B cells (76). This SNP has the potential to alter CD86 signaling, suggesting that it may have an effect on the production of Dsg3-specific IgG4 Abs, shown to be implicated in PV (77). Using a GWAS in the Jewish population, Sarig et al. has managed to show an association of several genetic variants with PV, including SNPs within the *ST18* gene (35), encoding for a transcription factor (TF) shown to be involved in inflammatory and apoptotic processes (78). Although not showing association with PV in German or Chinese patients, *ST18* was also found to be associated with the disease in the Egyptian population and to be overexpressed in the non-lesional skin of Jewish PV patients (35, 79). A subsequent study showed that the promoter region of *ST18* harbors a PV-associated SNP, which was demonstrated to increase gene transcription. ST18 was additionally shown to stimulate PV serum-induced acantholysis and secretion of key inflammatory molecules, supporting a direct role for ST18 in PV pathogenesis (80). In the Tunisian population, SNPs within the *FOXP3* gene were found in association with the susceptibility and clinical course of Pemphigus Foliaceus (PF), a different subtype of the disease (81). *FOXP3* encodes for a TF with a central role in the development and function of regulatory T (Treg) cells, suggesting it may be involved in the disruption of immune self-tolerance in the disease. Of interest, a significantly reduced number of Treg cells was discovered in the peripheral blood of both PV and PF patients in comparison to controls (82).

CONCLUSIONS

The important role of genetic factors in determining the propensity to develop PV is evident from the numerous epidemiological and association studies reviewed in this article. To date, the majority of studies aimed at identifying susceptibility genes for this complex disease, mainly focused on the HLA locus, showing various associations between PV and HLA alleles. In recent years, new methods, including a genome wide approach, gene expression analysis and NGS, are utilized and new discoveries regarding non-HLA genes involvement in PV are emerging. As the search for the genetic basis of PV continues, our understanding of PV pathomechanism is improving, paving the way for innovative and possibly safer therapeutic approaches.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Involvement of Nail Apparatus in Pemphigus Vulgaris in Ethnic Poles Is Infrequent

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Pemphigus vulgaris lesions have a tendency to localize around natural body orifices. The aim here was to analyze the involvement of nail apparatus in pemphigus vulgaris. Sixty seven ethnic Poles suffering from pemphigus vulgaris on photographic files archiving initial presentation were retrospectively evaluated. Pemphigus vulgaris was diagnosed using combination of clinical data, H+E histology, direct immunofluorescence of plucked scalp hair and/or perilesional tissue also for IgG1 and IgG4 deposits evaluation, indirect immunofluorescence on mosaic substrate and/or monkey esophagus, mono-analyte ELISA with desmoglein 1/3 or multi-analyte ELISA. The nail apparatus involvement was found in 9 of 67 patients (13.4%; 3 females and 6 males). Periungual fingernail lesions were found in 6 patients (2 females, 4 males), whereas periungual toenail lesions in just 3 patients (1 female, 2 males). Our patients nail apparatus changes included, by order of frequency, paronychia, nail discoloration, onychorrhexis, Beau lines, periungual hemorrhages, onychomadesis, cross-ridging, onycholysis, and trachyonychia. The average time between the onset, as recalled by patients, and the diagnosis of pemphigus vulgaris with direct immunofluorescence was not statistically different in PV patients with and without nail apparatus lesions. In this article the molecular and immunological rationale for of periungual involvement is discussed. Our single-center study suggests that nail apparatus involvement is infrequent in pemphigus vulgaris in ethnic Poles. Due to the fact that nail apparatus lesions in pemphigus vulgaris may clinically resemble onychomycosis, giving the proper diagnosis can be difficult particularly when other lesions are overlooked or misinterpreted.

Keywords: pemphigus vulgaris, nails, desmoglein 3, desmoglein 1, immunofluorescence, paronychia, onychomycosis, orifices

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INTRODUCTION

Pemphigus vulgaris (PV) is the most common constitute of pemphigus group of autoimmune blistering dermatoses (ABD). Although relatively rare, it is a severe, potentially life-threatening condition of a 3-fold increased risk of death than normal population (1). Scant literature data indicate yearly PV incidence as ranging between 0.076 and 1.6 per 100.000 persons (2, 3), yet it seems to be varied geographically, ethnically, and sex-dependent (with woman predominance noted in some studies) (3–7). While considered a disease characteristic for quintagenarians, it may occur at any age including childhood (7, 8).

There are two main forms of clinical manifestation of PV. In mucocutaneous PV (mcPV), pathological autoimmunity targets desmoglein 3 (DSG3; abundantly expressed in basal

and parabasal layers of the epidermis and the mucosa) and desmoglein 1 (DSG1; abundantly expressed in upper layers of the epidermis but scantly expressed in the mucosa), whereas in mucosal dominant PV (mdPV)—DSG3 is classically targeted (9). Painful flaccid intraepidermal/suprabasal blisters in PV, lead to oozing, crusted, usually superinfected erosions. Nevertheless, in some patients no link between autoantibody profile and non-stereotypical clinical manifestation was noted, that led to distinction of, the so called, atypical variant of PV (e.g., cutaneous PV[cPV] with anti-DSG3 or anti-DSG1/3 IgG autoantibodies) (10, 11).

Nowadays, immunopathological studies (direct and indirect immunofluorescence; DIF, IIF) and enzyme-linked immunosorbent assay (ELISA) are regarded indispensable diagnostic tools in ABD diagnostics (12, 13). There are commercially available serological assays designed for precise target antigen identification, including novel biochip mosaic-based IIF and bioplex-based techniques (14–17). Moreover, some kits can be modified for specific IgG4 autoantibody detection and the identification of an active, Th-2 mediated stage of the disease (18, 19). IIF study on monkey esophagus in PV reveals circulating IgG/IgG4 class of pemphigus-type autoantibodies against desmosomal proteins of keratinocytes. Classic DIF of perilesional skin/mucosa in PV shows "fishing-net"/"honeycomb"/"basket weave"/"chicken wire" pattern (intercellular IgG+/-C3 deposits) (20) and "dew drops on spider web" appearance (punctate/granular intercellular IgG4 deposits) (21). For low-invasive diagnosis—perilesional skin can be substituted with plucked hair (21).

Although oral mucosa seems to be primarily affected in 50–70% of cases (20) due to increased density of DSG3 containing desmosomes, PV mucosal blisters and erosions also typically have a tendency to involve certain areas characterized by transitive epithelia—e.g. nasopharynx, external ear canal, conjunctivae, tear canals, lids, vermilion, armpits, groins, areolae, esophagus, scalp (hair follicles as a natural body orifices), navel, nails, anus, genitourinary mucosa of vagina/labia, urethra and penis/preputium. Transitional epithelium may be a focus of autoimmune/autoinflammatory process regardless of whether the orifices are natural or not (scars, fistulas) (22–24). Thus, if these lesions are isolated, they may mimic many diseases (mostly infectious or neoplastic) and pose a significant diagnostic challenge for a non-dermatologist (25). Although PV patients occasionally present nail apparatus involvement, there are scant studies thoroughly investigating this topic and even fewer utilizing statistical methods. The aim of this single-center, retrospective, observational study was to analyze the PV nail apparatus involvement in ethnic Poles.

MATERIALS AND METHODS

This work was a part of studies approved by the local Ethical Committee of the Poznan University of Medical Sciences in Poland and informed written consent was obtained from each individual.

We analyzed retrospectively photographic archives (showing initial patients' fingernail/toenail involvement before the treatment) and medical charts of 67 ethnic Slavs, i.e., Poles (29 males, 38 females) suffering from PV recorded at the Department of Dermatology, Poznan University of Medical Sciences (Poznan, Poland) in the years 2002-2017. The diagnoses were based on the combination of clinical data, H+E histology, and DIF of plucked scalp hair and/or perilesional tissue also for IgG1 and IgG4 deposits evaluation, gradually introduced/changing diagnostic tools within assessed period: IIF on mosaic substrate and/or monkey esophagus (Euroimmun, Germany or MBL, Japan), mono-analyte ELISA with DSG1/3 (Euroimmun, Germany or MBL, Japan) or multi-analyte ELISA with envoplakin, type VII collagen, DSG1, DSG3, BP180, BP230 (Euroimmun, Germany). Paraneoplastic pemphigus patients were excluded from the study based on the laboratory/clinical/imaging findings and immunofluorescence studies. Bacteriological and fungal cultures as well as direct mycological KOH tests were performed to exclude infections in every case of nail involvement. Due to cost-effectiveness no nail clipping or PAS staining was performed. Nail apparatus involvement was statistically assessed concerning sex (n=67), sites (n=9)(fingernails/toenails) differences and PV subtype (n=44) with Fisher exact test (CI 0.95). In 51 cases of PV (9 with nail apparatus involvement, 42 without nail involvement) we compared the time between the onset of the disease and the diagnosis made with DIF (weeks till diagnosis, wtD) with Mann-Whitney U test with correction for continuity (CI 0.95). The comparison of the wtD in mcPV and mdPV subtypes was assessed with Mann-Whitney U test with correction for continuity (CI 0.95), whereas the comparison of wtD in mcPV, mdPV, cPV subtypes was evaluated with Kruskal-Wallis test (CI 0.95). All statistical tests were performed using Statistica 12.0, (Tibco Software Inc., US).

RESULTS

The nail apparatus involvement was found in 9 of 67 patients (13.4%; 3 females and 5 males with mcPV, 1 mdPV male) (**Tables 1**, **2** and **Figure 1**). Periungual fingernail lesions were found in 6 patients (2 females, 4 males), whereas periungual toenail lesions in just 3 patients (1 female, 2 males). None of the patients had concomitant fingernail and toenail lesions recorded in the archive. There were no significant differences in nail apparatus involvement neither between sexes (p=0.2460), sites (p=1.000) or PV subtypes (p=0.3891). There was no significant difference in wtD between PV patients with and without nail apparatus involvement (p=0.3126). No significant difference in wtD was observed either in mdPV and mcPV subtypes (p=0.3802) or between all the PV subtypes (p=0.3464).

DISCUSSION

The fingernails in PV are usually more affected than toenails (26, 27). Nail apparatus involvement may herald the recurrence and exacerbation of PV, while its intensity

Abbreviations: PV, pemphigus vulgaris; mcPV, mucocutaneous PV; mdPV, mucosal dominant PV; cPV, cutaneous PV; DIF, direct immunofluorescence study.

TABLE 1 | Nail apparatus involvement type and lesion location in ethnic Poles with pemphigus vulgaris (2002–2017).

Nail apparatus involvement type	Number of patients with certain lesions ($n = 9$)	Fingernail involvement (males[m], females[f])	Toenail involvement (males[m], females[f])	Certain nail apparatus involvement differences regarding sites (fingernail vs. toenail; Fisher's exact test)	Certain nail apparatus involvement differences regarding sex (m vs. f; Fisher's exact test)
Paronychia	9 (100%)	6 (66.67%) (5m, 2f)	3 (33.33%) (1m, 1f)	$p = 1.000$	$p = 0.278$
Nail discoloration	7 (77.78%)	4 (44.44%) (2m, 2f)	3 (33.33%) (2m, 1f)	$p = 1.000$	$p = 0.456$
Beau lines	5 (55.56%)	5 (55.56%) (3m, 2f)	0	$p = 1.000$	$p = 0.645$
Periungual hemorrhages	5 (55.56%)	3 (33.33%) (1m, 2f)	2 (22.22%) (2m)	$p = 0.400$	$p = 1.00$
Onychorrhexis	5 (55.56%)	2 (22.22%) (2m)	3(33.33%) (2m, 1f)	$p = 0.400$	$p = 0.158$
Onychomadesis	4 (44.44%)	4 (44.44%) (2m, 2f)	0	$p = 1.000$	$p = 1.000$
Cross-ridging	2 (22.22%)	2 (22.22%) (2m)	0	$p = 1.000$	$p = 0.184$
Onycholysis	1 (11.11%)	1 (11.11%) (1m)	0	$p = 1.000$	$p = 0.433$
Trachyonychia	1 (11.11%)	1 (11.11%) (1m)	0	$p = 1.000$	$p = 0.433$

TABLE 2 | Average time between the PV onset and the diagnosis with DIF (wtD).

	PV with nail apparatus involvement ($n = 9$)	PV without nail apparatus involvement ($n = 52$)	Mucocutaneous PV ($n = 29$)	Mucosal dominant PV ($n = 13$)	Cutaneous PV ($n = 2$)
Average wtD (weeks)	27.78	23.60	22.86	23.85	56

seems to be associated with the severity of the disease and area affected, with poor prognosis correlating with the presence of periungual/subungual/intraungual hemorrhages (26, 28–35). Periungual PV manifestations include paronychia, onychomadesis, onycholysis, Beau's lines, trachyonychia (rough nails), onychorrhexis (brittle nails), subungual hyperkeratosis, pterygium, nail dystrophy, nail discoloration, cross ridging, hemorrhagic nails, and periungual vegetating and verrucous lesions (31, 36–47). Our patients nail apparatus changes included, by order of frequency, paronychia, nail discoloration, onychorrhexis, Beau lines, periungual hemorrhages, onychomadesis, cross-ridging, onycholysis and trachyonychia. We found nail apparatus involvement in 13.4% of our PV patients. This is in contrast to the findings in previous studies from India (80%) (48), Iran (31.6%) (30), and USA (47%) (41) that showed higher prevalence. It is possible that ethnic and genetic differences between those populations and Polish one (e.g., haplogroups, HLA class II alleles) may be responsible for these odds. This hypothesis should be verified by further comparative studies in other European and Slavic populations, as this report seems to be the first in this ethnic group. Possibly, due to relatively low numbers of patients with PV nail apparatus involvement in our study, statistical analysis displayed no significant differences between sexes, sites and subtypes (**Table 1**). Although not statistically significant, in our study nail apparatus involvement was more frequent in males than in females in both sites (fingernails/toenails). Physical work pursued more likely by males due to sociocultural reasons may lead to higher tendency for traumatization, a known factor for developing PV lesions (42, 43). Unfortunately, having incomplete retrospective data about patients' occupation, we were not able to verify this presumption. The influence of male/female hormone balance modulating the inflammatory process at this specific site, may be another explanation of higher prevalence of PV nail involvement in man.

The most common symptoms of nail apparatus PV are paronychia (60%) and onychomadesis (30%) (31), what partially was also confirmed in our study (100, 44.44%, respectively). The most common causes of acute inflammation of the periungual folds include bacterial (*Streptococci, S. aureus*), viral (herpetic withlow) and fungal infections (*Fusarium, Candida, Neoscytalidum*), while drugs (including chemotherapy and targeted therapy) (49) and PV were reported to be the joint 4th most common cause (5% of all cases) (37, 50). Paronychia or paronychia mimics may also be triggered by several various pathologies: trauma (ingrown nail, nail biting, nail-sucking), parasitic infections, psoriasis (proximal nailfold psoriasis, acrodermatitis continua Hallopeau), neoplasms, and benign tumors. Onychomadesis is a state when the nail plate separates from the nail matrix, yet remains attached to the nail bed, that finally leads to nail plate shedding. Although the most common causes of onychomadesis are infections, severe illnesses, and drugs. The proper diagnosis may demand meticulous collection of medical history, examination and selection of accessory tests. Nail abnormalities in PV may not only succeed skin lesions and develop concomitantly with mucosal/skin lesions, but may also precede them (46, 51, 52). In such circumstances it may be a valuable hint easing the diagnostic process but also leading it astray and delaying the proper treatment. However, that process may be two-way.

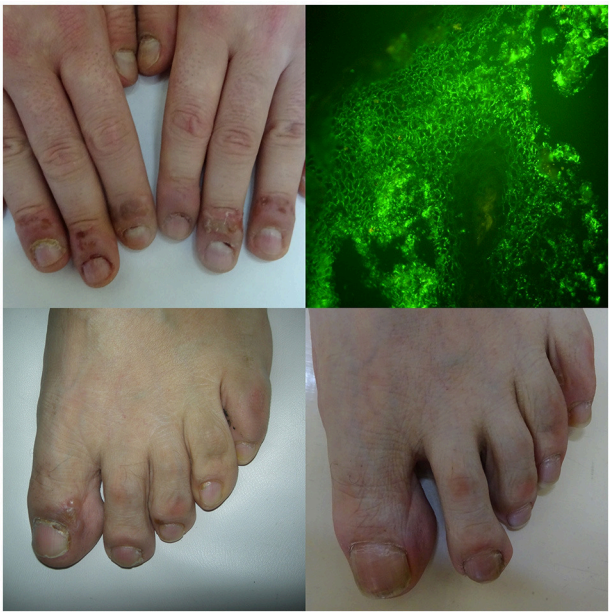

FIGURE 1 | A young male, manual laborer, with mucocutaneous pemphigus vulgaris affecting also fingernails apparatus (featuring paronychia, onychomadesis, periungual hemorrhages, trachyonychia, Beau lines, and onychorrhexis), also suffering from onychotillomania and taking escitalopram for depression (**A**, upper panel left). Direct immunofluorescence of perilesional skin showed pemphigus intercellular IgG4 (++) deposits in both follicular and perifollicular epithelium having dew drops on spider web appearance (**B**, upper panel right) and multi-analyte ELISA revealed elevated levels of serum anti-DSG1 (value 1.8) and anti-DSG3 (value 4.4) IgG antibodies (cut-off values below 1). A middle-aged male with mucocutaneous pemphigus vulgaris having also toenails apparatus lesions (featuring paronychia, nail discoloration, onychorrhexis, and periungual hemorrhages)—before (**C**, lower panel left) and after immunosuppressive treatment (**D**, lower panel right). Direct immunofluorescence of perilesional skin showed pemphigus IgG1(+) and IgG4 (+ + +) intercellular deposits and mono-analyte ELISA revealed elevated levels of serum anti-DSG1 (>200 RU/ml and anti-DSG3 166.796 RU/ml) IgG antibodies (cut-off levels 20 RU/ml).

The diagnosis of PV should not discourage the physician from further investigation. Interestingly, 12% of already treated PV patients in Thai study, were found to have concomitant clinical onychomycosis (although only 5% were confirmed by mycological culture) (53). Similarly, an Egyptian study on PV female patients on immunosuppressive treatment also indicated 24% prevalence of onychomycosis, yet also 24% prevalence of bacterial periungual infection (54). What is worth noticing, fungal, and bacterial infection may mimic PV nail apparatus lesions and pose a threat to immunocompromised patients if left untreated, especially considering additional immunosuppressive treatment. Humidity of the periungual area (erosions, crusts, subungual hemorrhages) could be the predisposing factor for development of these infections in PV patients. Thus the hot climate of Egypt and Thailand may lead to relatively high incidence of infectious paronychia and onychomycosis, whereas the mild climate in Poland might be beneficial in that respect. Topical therapy was reported to be insufficient in nail manifestations of PV. Thus, systemic treatment is required to achieve nail recovery, usually with no subsequent nail deformation (31). All nail apparatus lesions in our PV patients responded to first-line immunosuppressive treatment schemes recommended for this disease with no subsequent nail involvement relapses, while oral lesions were commonly stubborn.

The precise mechanism leading to the initiation of pathological nail apparatus involvement in PV has not been elucidated. Hence, autoimmune process taking place in nail bed, matrix, and periungual nail fold could be explained by plausible complex associated with different expression of desmosomal cadherins in nail structures epithelia in comparison with skin and mucous membranes. Transitive stratified squamous epithelium could be regarded a target of immunization similarly as at other affected sites, what corresponds with acantholysis and PV patterns of IgG, IgG1, and IgG4 in DIF (21). Relative rarity of nail apparatus involvement is considered by some a consequence of partial sequestration of target antigens in proximal nail matrix (PNM) and reduced number of antigen presenting cells as well as depression of their functions in comparison to mucosal and skin immune system, but resembling the one of hair follicle (31, 55, 56). Moreover, apart from locally increasing levels of potent immunosuppressive cytokines, i.e., transforming growth factor-β1 (TGF-β1) and α-melanocyte stimulating hormone (α-MSH), PNM keratinocytes downregulate MHC class I, Langerhans cells downregulate MHC class II and CD209 expression, while numbers and functions of NK lymphocytes and mast cells in periungual area are also reduced giving the "immune privilege" conditions against autoimmunity (55, 56). Still, hairy skin seems to be preferentially affected by PV lesions compared to nail apparatus, which may suggest that the sheer surface size, and not "immune privilege," is the critical factor determining appendageal sites of predilection. Nonetheless, compensation theory explains the involvement of these sites as areas supposedly more prone to anti-DSG3-directed autoimmunity and subsequent keratinocyte detachment without any relation to anatomical/structural morphology of the epithelium where low DSG1 expression is accompanied by compensatory higher expression of DSG3. Interestingly, this is not the case in nails, where both DSG1 and DSG3 are expressed at low level due to the absence of granular layer in nail matrix and thickness (2–3 cell layers) of nail bed epithelium (57). Thus, only high titers of anti-DSG1/anti-DSG3 or anti-DSG3 autoantibodies alone cause the collapsing of "immune privilege" and PV nail apparatus involvement (57, 58).

As nail apparatus involvement was not the sole presentation in any of our PV cases, we suspect that it is just a sign of a severe and protracting disease. We assumed that nail involvement in PV may encourage the patients to seek help earlier. Interestingly, wtD in patients with nail involvement was greater than in patients without nail involvement (**Table 2**), although the differences were not shown to be statistically significant. This ca. 1-month delay in periungual PV diagnosis may suggest that these symptoms might have been treated as a fungal/bacterial/viral infection by the physician or were regarded a minor/non-related issue either by the patient or a physician. On the other hand, it is possible that the periungual PV patients, driven by the embarrassment

and lowered self-esteem were reluctant to seek professional help until full-blown PV developed. Further studies should be performed in this group to evaluate the seriousness of this burden concerning the social impact of PV. No subtype was diagnosed significantly faster than other (**Table 2**), however the assessment of vast range of symptoms and locations involved may be a limitation. Hence, this relation should also cover the aspect of severity of the disease subtype measured with uniform scoring system in the future studies. Relation between nail apparatus involvement in PV and IIF, ELISA, DIF was not assessed in this study due to incomplete database for all the patients, as in 2002–2017 diagnostic algorithms changed and new diagnostic tools were implemented (in our department multi-analyte ELISA superseded mono-analyte ones and we ceased using subjective imaging IIF), suppliers changed (different titration units) and some became redundant or were used occasionally as supplemental procedures. Further studies should provide more information on the association between nail involvement and severity of the disease.

It is concluded that nail apparatus involvement is infrequent in pemphigus vulgaris in ethnic Poles. Thus, nail apparatus lesions can be misleading when practicing dermatologists examine just periungual body areas that bother patients most, overlook, and/or misinterpret other lesions. Conversely, nail apparatus involvement, although embarrassing for the patient, can be an invaluable hint as to PV diagnosis when meticulously analyzed in the clinical context, such as symptoms evolution, medical histories including family history, concomitant malignancies (59), culprit medications (60), radiation (61), trauma (42) and airborne and topical chemical compounds exposure (61–63). Proper treatment, including cessation of triggering factors, can facilitate good control, and faster recovery, reduce the burden of aggressive treatment and prevent aggravation of PV, possibly the lethal disease.

AUTHOR CONTRIBUTIONS

MD and PP contributed conception and design of the study. PP organized the database and performed the statistical analysis. PP and MD wrote the first draft of the manuscript. PP, MB-D, MD, and JG-P wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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The Evolving Story of Autoantibodies in Pemphigus Vulgaris: Development of the "Super Compensation Hypothesis"

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Emerging data and innovative technologies are re-shaping our understanding of the scope and specificity of the autoimmune response in Pemphigus vulgaris (PV), a prototypical humorally mediated autoimmune skin blistering disorder. Seminal studies identified the desmosomal proteins Desmoglein 3 and 1 (Dsg3 and Dsg1), cadherin family proteins which function to maintain cell adhesion, as the primary targets of pathogenic autoAbs. Consequently, pathogenesis in PV has primarily considered to be the result of anti-Dsg autoAbs alone. However, accumulating data suggesting that anti-Dsg autoAbs by themselves cannot adequately explain the loss of cell-cell adhesion seen in PV, nor account for the disease heterogeneity exhibited across PV patients has spurred the notion that additional autoAb specificities may contribute to disease. To investigate the role of non-Dsg autoAbs in PV, an increasing number of studies have attempted to characterize additional targets of PV autoAbs. The recent advent of protein microarray technology, which allows for the rapid, highly sensitive, and multiplexed assessment of autoAb specificity has facilitated the comprehensive classification of the scope and specificity of the autoAb response in PV. Such detailed deconstruction of the autoimmune response in PV, beyond simply tracking anti-Dsg autoAbs, has provided invaluable new insights concerning disease mechanisms and enhanced disease classification which could directly translate into superior tools for prognostics and clinical management, as well as the development of novel, disease specific treatments.

Keywords: autoantibodies, desmoglein, thyroid peroxidase, acetylcholine receptor, mitochondria, desmocollin, plakophilin, protein array technology

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INTRODUCTION

Pemphigus vulgaris (PV) is an autoimmune skin disease that results from the production of autoAbs that target keratinocyte proteins. Binding of these autoAbs results in the loss of keratinocyte cell-cell adhesion (termed acantholysis) just superior to the basal cell layer in the epidermis resulting in the development of painful, flaccid bullae on the skin and/or mucosal membranes that easily rupture. The discovery that autoantibodies (Abs) targeting desmoglein (Dsg) 1 and Dsg3 cause blister formation has been potentially the most critical event in understanding disease pathogenesis in PV to date. Numerous studies have been dedicated to characterizing the isotype and fine epitope specificity of anti-desmoglein autoAbs and investigations aimed at

uncovering the mechanisms underlying autoAb-induced blister formation focused primarily on studying the effects downstream of anti-desmoglein Ab binding. Accordingly, the majority of currently proposed disease models are desmoglein-centric. These models, however, fail to explain a number of disease phenomena such as patients that present in active disease without detectable anti-desmoglein autoAbs and the lack of tight correlation between anti-desmoglein autoAb titers and disease activity. Additionally, these models cannot adequately account for the degree of disease heterogeneity exhibited by PV patients. Here, we focus on: (i) the seminal studies that led investigators to identify Dsg3 and 1 as targets of pathogenic autoAbs, and how these studies shaped our understanding of disease, and (ii) the identification of non-Dsg autoAbs, with a particular focus on the contribution of comprehensive autoAb profiling facilitated by protein microarray technology, as well as the potential role of these autoAbs in disease, and how these findings may re-shape/direct how we ultimately view the pathogenesis of PV.

EARLY STUDIES

Several observations have suggested a role for autoAbs in the pathogenesis of PV (**Figure 1**). Neonates born to mothers with PV were observed to experience transient disease at birth (1), and the addition of the IgG fraction alone from patient sera (PVIgG), without the presence of complement or other immune cells, could recapitulate disease in a skin organ culture model as well as disturb cell-cell adhesion in a keratinocyte monolayer (2, 3). Patient sera was shown to be capable of inducing disease when passively transferred to mice (4). Furthermore, PVIgG stained epidermal tissue in a "fishnet pattern." Early efforts to determine the target of these autoAb revealed that PV sera recognized a number of then unknown proteins, with molecular weights of 20, 22, 33, 50, 66, 68, 80, 105, 130, 140, 160, 210, and 220 kDa (5–14). The effect of PV sera on cell adhesion led researchers to hypothesize, and eventually prove, that PV sera recognized a desmosomal protein (15, 16). In 1991, using PV sera to screen a phage display library created from cDNA cloned from normal human epidermal keratinocytes, Amagai et al. (6), demonstrated that the antigen recognized by PV autoAbs was a novel 130 kDa cadherin protein that shared a high degree of homology with desmoglein 1, a previously discovered desmosomal cadherin. Eventually this novel cadherin was named desmoglein 3. However, in order to identify this clone, only PV autoAbs purified from the 130 kDa band were used to screen the phage display library, because initial screening of the library with PV sera identified over 200 clones, and none of those 200 clones were capable of being recognized by all sera samples (6).

After the discovery of Dsg3 as a major antigenic target of PV autoAbs, a number of studies focused on establishing the pathogenicity of anti-Dsg3 autoAbs. In an early experiment, PVIgG was exposed to fusion proteins consisting of various Dsg3 extracellular domains (ECs) conjugated to beta galactosidase. PVIgG from 17 of 23 patients recognized at least one of the fusion proteins, but 6 patients did not possess autoAbs reactive to any of the constructs. Two different fusion proteins, expressing EC1-2 and EC3-5 respectively, were then used to deplete anti-Dsg3 autoAb from PVIgG. When autoAbs affinity purified by the EC1-2 construct were passively transferred to mice, they were capable of eliciting blister formation. However, instead of the gross blister formation seen when using PVIgG, these purified autoAbs only produced microscopic blisters. Additionally, PVIgG depleted using the EC1-2 construct was still capable of eliciting blister formation upon passive transfer. AutoAbs purified using the EC3-5 construct failed to induce blister formation upon passive transfer. From these experiments, the authors concluded that anti-Dsg3 autoAb are in fact disease causing, and the failure of the Dsg3 constructs to be recognized by all patient sera, as well inhibit the pathogenicity of PVIgG, was due to improper conformation of the Dsg3 constructs (17).

A different Dsg3 construct, consisting of the extracellular domains of Dsg3 combined with the constant region of IgG1 (named PVIg), was generated to create a protein that would more accurately reflect the conformation of native Dsg3. Preabsorption of PVIgG with PVIg reduced the indirect immunofluorescent titers of 6/17 patients to zero, significantly lowered the indirect immunofluorescent titers of all but one patient's IgG, which remain unaffected. Preabsorption of PVIgG with this construct was also capable of preventing the formation of gross blisters when transferred to mice, although microscopic suprabasilar acantholysis was still detected in some areas (18). It should be noted that the specificity of the autoAbs purified using this construct were not assessed in this experiment.

A number of mouse models also seemed to support the notion that anti-Dsg3 could be sufficient to cause disease in PV. Splenocytes from $Dsg3^{-/-}$ mice immunized with Dsg3 were adoptively transferred to $rag2^{-/-}$ mice which subsequently developed blisters and suprabasilar acantholysis (19–21). Histological examination of the epidermis from mice with a targeted deletion of Dsg3 displayed suprabasilar acantholysis as well as the characteristic "tombstoning" of basal keratinocytes as seen in PV, but still lacked any gross signs of spontaneous blister formation (22). In another experiment, mice expressing a truncated Dsg3 displayed swelled paws, flaky skin, widened intercellular space between keratinocytes and a number of other epidermal abnormalities. Again, blister formation and suprabasilar acantholysis were absent in these mice (23).

THE DESMOGLEIN COMPENSATION HYPOTHESIS

The identification of Dsg3 as a major antigenic target represented a critical moment in the process of understanding PV and, after its discovery, the prevailing view of disease immediately narrowed. For the next decade, the design and interpretation of almost all experiments were informed by an underlying assumption that anti-Dsg autoAb were the sole drivers of disease in pemphigus, ignoring the potential role of other, non-Dsg autoAbs. Although the importance of anti-Dsg3 autoAb is clear, this limited view may have slowed the progression of

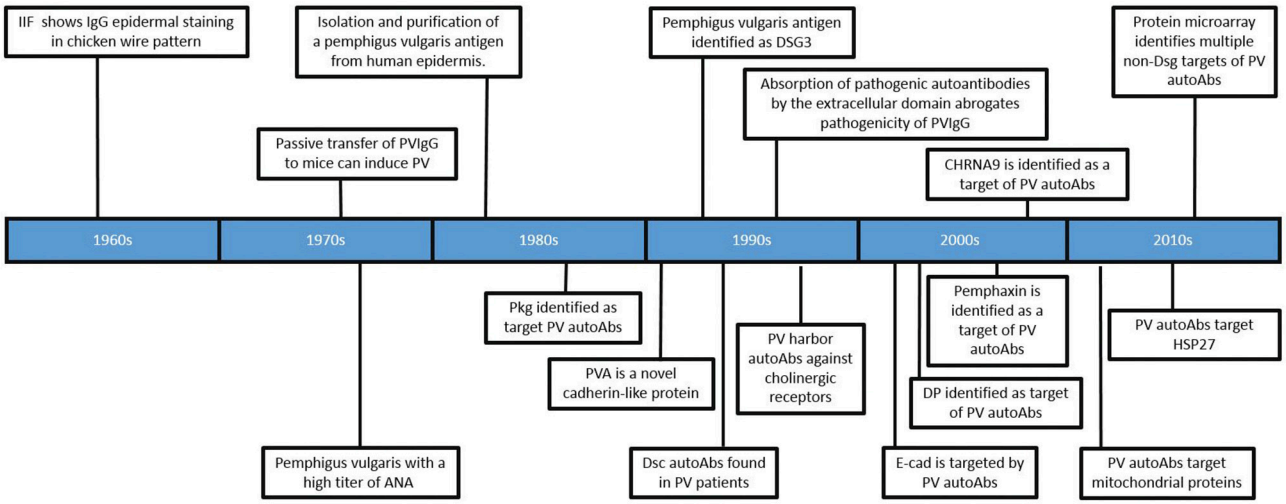

FIGURE 1 | Timeline of significant findings regarding autoantibodies in Pemphigus vulgaris. Important developments in the field are depicted in chronological order (see text for detailed description).

understanding the true complexity of disease. The impact of how this desmoglein-centric view fundamentally influenced the way researchers understood PV is epitomized by the development of the *desmoglein compensation hypothesis*. This elegant hypothesis asserts that anti-Dsg3 and anti-Dsg1 autoAb profiles can predict which epithelial surface(s) will be affected, as well at what level the loss of cell-cell adhesion will occur in the epidermis (24). The foundation of this hypothesis are the differential expression patterns of Dsg3 and Dsg1 between mucosal and cutaneous epidermis, and the idea that Dsg3 or Dsg1 alone can sustain cell-cell adhesion. In a series of experiments Mahoney et al. demonstrated that: (1) murine mucosal tissue expresses Dsg3 throughout the entire epidermis, with strongest expression in the superficial layers, while Dsg1 expression is highest in the superficial layers and very low in the deeper layers, and (2) murine cutaneous epidermal tissue expresses Dsg3 most highly in the basal layer with lower expression seen in the more superficial layers, whereas Dsg1 expression is high in superficial epidermis and decreased in the deeper layers. The tissue specific expression patterns of Dsg3 and 1 in mice are similar to that of human epidermis, with the exception that Dsg1 expression in human mucosal epidermis is very low (25–27).

Next, a series of passive transfer experiments using PVIgG injected into wildtype C57BL/6J mice or Dsg3 null mice demonstrated that anti-Dsg1 autoAbs induce blister formation when transferred to Dsg3 null mice and both anti-Dsg3 and anti-Dsg1 autoAb are required to promote blister formation in parts of the epidermis that express both Dsg3 and 1. It should be noted that the Dsg3 null mice used in this experiment were shown to spontaneously develop inflammatory erosions along with a loss of cell-cell adhesion in the superficial layers of the epidermis (22). Still, from these results, the authors proposed that blister formation in PV occurs initially within the mucosa at the suprabasilar level where Dsg3, but not Dsg1, is expressed. Cutaneous lesions in PV patients only occur when patients develop additional autoAbs, directed against Dsg1, later in disease. This hypothesis also attempts to explain blister formation in Pemphigus foliaceus (PF), a related autoimmune blistering disease where autoAbs directed against Dsg1 cause cutaneous blister formation in the superficial layers of the epidermis.

Aside from the assertion that the epidermal architecture of the Dsg3 null mice used in these experiments may not have been an optimal model, there exists a plethora of clinical and experimental evidence that does not align with the desmoglein compensation hypothesis. If true, PV patients exhibiting both anti-Dsg3 and Dsg1 autoAbs might be expected to demonstrate a loss of cell-cell adhesion throughout the entire epidermis, instead of just at the suprabasilar level. Moreover, assessment of Dsg3 and 1 titers in PV patients have demonstrated the existence of cutaneous only patients (with no history of mucosal lesions) with no detectable anti-Dsg1, mucosal only patients with no detectable anti-Dsg3, as well patients that lack detectable titers of anti-Dsg3 or 1 autoAbs (28–43), all of which cannot be explained by the desmoglein compensation hypothesis (**Table 1**).

NON-DESMOGLEIN TARGETS OF AUTOANTIBODIES IN PV

The desmoglein compensation hypothesis cannot adequately account for disease presentation in PV, and newer models are needed to more precisely understand disease mechanisms. The idea that unique profiles of autoAbs may underlie differences in disease expression represents the beginning of a more sophisticated understanding of immune dysregulation in PV. The failure of anti-Dsg autoAbs alone to fully explain disease spurred the notion that additional autoAb specificities may be relevant in PV, and subsequent experiments have resulted in a growing pool of evidence that suggests autoAbs directed at *non-Dsg targets* may play a role in PV.

TABLE 1 | Postulates and limitations of the desmoglein compensation hypothesis (DHC).

Postulates of the DCH	Limitation of the DCH
- Patients expressing only anti-Dsg3 autoAbs exhibit suprabasal acantholysis in mucosal epidermis only.	- Patients expressing only anti-Dsg3 autoAbs can exhibit cutaneous acantholysis either alone or in combination with mucosal acantholysis.
- Patients expressing both anti-Dsg3 and anti-Dsg1 autoAbs will exhibit only suprabasal acantholysis in both mucosal and cutaneous epidermis.	- Patients with no detectable levels of anti-Dsg3 or anti-Dsg1 autoAbs can exhibit cutaneous acantholysis either alone or in combination with mucosal acantholysis.
- Patients expressing only anti-Dsg1 autoAbs will exhibit acantholysis in the superficial cutaneous epidermis only (PF).	- Patients expressing both anti-Dsg3 and anti-Dsg1 autoAbs can exhibit cutaneous lesions only, rather than cutaneous and mucosal lesions.
	- Patients expressing both anti-Dsg3 and anti-Dsg1 autoAbs exhibit only suprabasal acantholysis in both cutaneous and mucosal lesions.

Initial evidence that non-Dsg autoAb may be relevant to disease came from experiments demonstrating the formation of blisters in Dsg3 null mice upon the passive transfer of PVIgG that did not contain any anti-Dsg1 autoAbs (44). Although this observation seemed to contradict previous studies that demonstrated the ability of a Dsg3 fusion protein to absorb out pathogenic antibodies in PVIgG, it was soon shown that autoAbs eluted from this protein bound to a number of distinct protein bands when exposed to the lysate of keratinocytes lacking expression of Dsg3 (45). Potentially, the ability of this construct to absorb out non-specific IgG is attributable to Fc-Fc interactions (46–48). Additional evidence that non-Dsg autoAbs may be relevant to disease came from studies that demonstrated a lack of correlation between anti-Dsg autoAb titers and disease activity in a subset of patients (29, 41, 42, 49, 50). These studies emphasized the importance of identifying other targets of autoAbs in PV, and soon more than 50 non-Dsg antigens were reported to be recognized by PV patient autoAbs (**Table 2**).

Some of the first non-Dsg targets of autoAbs to be discovered were those directed against acetylcholine receptors. Using PVIgG to immunoprecipitate keratinocytes whose cholinergic receptors were first radiolabeled using [^{3}H]propylbenzilylcholine mustard, it was shown that 34/40 PV patients precipitated cholinergic receptors (44). In an attempt to identify which cholinergic receptor may be recognized by autoAbs, it was shown that pre-incubation of monkey esophagus with PVIgG blocked the binding of antibodies directed at alpha9 acetylcholine receptor. Using antibodies derived from rabbits this group was able to show that these Abs had similar effects on the cell morphology of oral keratinocytes as PVIgG, but passive transfer of such antibodies was unable to induce blister formation (51).

PV autoAbs have also been shown to target mitochondrial proteins. PVIgG can penetrate keratinocytes and bind targets on the mitochondrial membrane. In one study 6/6 PV sera contained autoAbs that recognized mitochondrial preparations purified from keratinocytes, although the molecular weights of reactive proteins varied from sample to sample. Removal of these mitochondrial autoAbs by pre-incubation with mitochondrial preparations abolished the ability of PVIgG to cause acantholysis in a keratinocyte monolayer and lessened the severity of suprabasilar blister formation in a passive transfer model (75). In a separate experiment, PVIgG was also shown to precipitate various mitochondrial nicotinic cholinergic receptor subtypes. The mitochondrial nicotinic subtype α3 was precipitated by 3/5 patients, α5 by 2/5, α10 by 2/5, β2 by 1/5, and β4 by 1/5 (67).

Other studies have shown that some PV sera bind desmocollins (Dsc) 1−3. An immunoblot of bovine desmosomal preparation identified 4/16 PV sera recognizing Dsc 1/2 (68), while another study also performing immunoblot analysis identified Dsc 1/2 autoAbs in 8/39 PV patients. Constructs consisting of the extracellular domains of each Dsc isoform, however, were not recognized by these sera (69). Yet another study demonstrated that 8/39 PV samples immunoprecipitated Dsc3, and that preabsorption of sera with recombinant Dsc3 prevented the ability of this PVIgG to cause acantholysis in a cell monolayer (53). Recently, another study using ELISAs made with Dsc proteins expressed in mammalian cells found that in a group of 22 PV patients, 2/22, 3/22, and 1/22 patients were positive for autoAbs against Dsc1, 2, and 3 respectively (54).

Another keratinocyte antigen found to be detected by PVIgG was an annexin-like protein, now known as pemphaxin. To identify this protein, PVIgG was purified using the PVIg construct and eluted autoAbs that recognized a 75 kDa band were used to screen a library of keratinocyte proteins. Preabsorption of PVIgG using a recombinant version of pemphaxin eliminated the ability of PVIgG to cause blister formation when passively transferred to mice. However, autoAb eluted from this column, while able to restore acantholytic ability to previously pre-absorbed PVIgG, was not sufficient to induce blister formation in mice (52).

A number of other experiments, where identification of PV autoantigens was not the primary goal, have still provided information concerning the reactivity of autoAbs in PV. Immunoblotting PVIgG identified 3/44 pemphigus sera containing autoAbs that recognized full length collagen XVII (71). Sera from two PV patients was shown to react with a recombinant Dsg4 protein (76). In a case review, a patient with PV was shown by immunoblot to have antibodies against desmoplakin (72). Another experiment which coupled immunoprecipitation with immunoblotting identified anti-E-cadherin autoAbs in 33/40 PV patients. However, indirect immunofluorescence of A431DE cells, which express E-cadherin but not Dsg1, was negative. These results indicate that E-cadherin positivity in PV patients may be a result of cross reactivity of Dsg1 autoAbs with E-cadherin (73). Plakophilin 3 (Pkp3) reactivity was observed in 1/4 PV patients when immunoblotting against the lysate of HEK293 cells transfected with a gene encoding for Pkp3 (70). Using an ELISA specific for FcER1, it was determined that 12/28 PV patients had autoAbs directed against FcER1 (74). Several additional studies have assessed anti-thyroid peroxidase (TPO) autoAb levels and found that between 14 and 40% of PV patients have autoAbs directed against TPO (58–62).

TABLE 2 | Ranking evidence for non-desmoglein antigens.

Level of evidence	Symbol	Name	IB/IP/ELISA	Protein microarray	*In vitro*	*In vivo*	References
3	CHRNA9	Cholinergic Receptor Nicotinic Alpha 9			x	x	(51)
3	ANXA9	Annexin A9, Pemphaxin	x		x	x	(52)
3	DSC3	Desmocollin 3	x	x	x		(53–57)
3	TPO	Thyroid Peroxidase	x	x	x		(58–63)
2	CD2	T-cell surface antigen T11/Leu-5, LFA-2, LFA-3 receptor	x	x			(64)
2	CD33	Sialic acid binding Ig-like lectin 3	x	x			(55, 64)
2	CD36	Thrombospondin receptor	x	x			(64)
2	CD37	Cluster of Differentiation 37 Molecule, Leukocyte antigen 37	x	x			(64)
2	CD40	Cluster of Differentiation 40 Molecule	x	x			(64)
2	CD84	Cluster of Differentiation 84 Molecule	x	x			(64)
2	CEACAM6	Carcinoembryonic Antigen Related Cell Adhesion Molecule 6	x	x			(64)
2	CHRM1	Cholinergic Receptor Muscarinic 1	x	x			(64)
2	CHRM3	Cholinergic Receptor Muscarinic 3	x	x			(63, 65, 66)
2	CHRNA5	Cholinergic Receptor Muscarinic 5	x	x			(67)
2	CHRNA10	Cholinergic Receptor Nicotinic Alpha 10 Subunit	x	x			(67)
2	CHRNB4	Cholinergic Receptor Nicotinic Beta 4 Subunit	x	x			(67)
2	DSC1	Desmocollin 1	x	x			(54, 68, 69)
2	DSC2	Desmocollin 2	x	x			(54, 68, 69)
2	HBE1	Hemoglobin Subunit Epsilon 1	x	x			(64)
2	ICAM1	Intercellular Adhesion Molecule 1	x	x			(64)
2	IGHG2	Immunoglobulin Heavy Constant Gamma 2	x	x			(64)
2	IL1RAPL2	Interleukin 1 Receptor Accessory Protein Like 2	x	x			(64)
2	IRF8	Interferon Regulatory Factor 8	x	x			(64)
2	NMNAT2	Nicotinamide Nucleotide Adenylyltransferase 2	x	x			(64)
2	PECAM1	Platelet And Endothelial Cell Adhesion Molecule 1	x	x			(64)
2	PKP3	Plakophillin 3	x	x			(70)
2	PMP22	Peripheral Myelin Protein 22	x	x			(55, 64)
1	ATP2C1	ATPase Secretory Pathway Ca2+ Transporting 1		x			(55)
1	ANXA8L1	Annexin A8 Like 1		x			(55)
1	CD1B	Cluster of Differentiation 1B molecule; Integrin beta 2		x			(55)
1	CD32	Cluster of Differentiation 32 molecule, Fc-fragment of IgG		x			(55)
1	CD88	CD88 molecule, complement component 5a receptor 1		x			(55)
1	CDH8	Cadherin 8		x			(55)
1	CDH9	Cadherin 9		x			(55)
1	CHRM4	Cholinergic Receptor Muscarinic 4		x			(63)
1	CHRNA3, –A5, A7, –A9, A10, –B2, and –B4	Cholinergic Receptor Nicotinic Subunits Alpha 3, –Alpha 5, Alpha 7, Alpha 9, Alpha 10, Beta 2 and Beta 6	x				(55)
1	CHRND	Cholinergic Receptor Nicotinic Delta Subunit		x			(55)
1	CHRNE	Cholinergic Receptor Nicotinic Epsilon Subunit		x			(55)
1	COL21A1	Collagen Type XXI Alpha 1 Chain		x			(55)
1	COLXVII	Collagen Type XVII Alpha 1 Chain	x				(71)
1	CYB5B	Cytochrome B5 Type B		x			(55)

(Continued)

TABLE 2 | Continued

Level of evidence	Symbol	Name	IB/IP/ELISA	Protein microarray	*In vitro*	*In vivo*	References
1	DSP	Desmoplakin	x				(72)
1	ECAD	E-Cadherin	x				(73)
1	FCER1	Fc Fragment of IgE receptor 1	x				(74)
1	FH	Fumarate Hydratase		x			(55)
1	GBP1A	Glycoprotein Ibα					(55)
1	HLA-DRA	Major Histocompatibility Complex, Class II, DR Alpha		x			(55)
1	HLA-E	Major Histocompatibility Complex, Class I, E		x			(55)
1	NDUFS1	NADH:Ubiquinone Oxidoreductase Core Subunit S1		x			(55)
1	PDHA1	Pyruvate Dehydrogenase E1 Alpha 1 Subunit		x			(55)
1	SCL36A4	Solute Carrier Family 36 Member 4		x			(55)
1	SOD2	Superoxide Dismutase 2		x			(55)

Black (level of evidence: 1) indicates that PV-relevant antigens were found by one study and/or one methodology. Green (level of evidence: 2) indicates that PV-relevant antigens were found by one or more studies and two independent methods. Red (level of evidence: 3) indicates that PV-relevant antigens were found by one or more studies and three independent methods and/or confirmed in vivo.

PROTEIN ARRAY TECHNOLOGY

Protein microarrays are powerful tools that allow for the assessment of protein interactions in a high-throughput manner. Compared to previous techniques such as ELISA, protein microarrays are more sensitive, require less sample volume, and can probe for multiple protein-protein interactions simultaneously, making them an especially powerful tool for assessing the autoAb response in autoimmune diseases. The use of protein arrays has facilitated the identification of novel antigenic targets in multiple autoimmune diseases, including the identification of biomarkers in RA which predate disease by months to years and specific autoAb profiles that predict disease phenotype and prognosis in polyomyositis (77).

Recently, protein array technology has been used to characterize the scope of antigens targeted by autoAbs in PV (**Figure 2**). Kalantari-Dehaghi et al. (64) probed autoAb reactivity of seven PV patients and five healthy controls using a protein microarray consisting of 785 keratinocyte antigens (expressed using a cell-free expression system). These authors detected 16 antigens with significantly higher reactivity in PV sera compared to healthy sera: thrombospondin receptor (CD36), immunoglobulin heavy chain constant region gamma 2 (IGHG2), TNF receptor superfamily member 5 (CD40), CD37, nicotinamide/nicotinic acid mononucleotide adenylyltransferase 2(NMNAT2), CD84, peripheral myelin protein 22 (PMP22), hemoglobin epsilon 1 (HBE1), interferon regulatory factor 8 (IRF8), CD2, carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), platelet/endothelial cell adhesion molecule (PECAM1), cholinergic receptor, muscarinic 1 (CHRM1), CD33, interleukin 1 receptor accessory protein-like 2 (IL1RAPL2), intercellular adhesion molecule 1 (ICAM1). These findings were then confirmed by immunoblot (64). This experiment indicated the autoAb response in PV is more complicated than initially thought, but the power of analysis was limited due to the small number of patients.

The same group later ran a similar experiment comparing the IgG autoAb reactivity of 264 PV patients with158 healthy controls. This analysis identified a large number of proteins that were targeted at least 10 fold greater by autoAbs in PV sera vs. that of controls: sialic acid-binding immunoglobulin-like lectin 3 (CD33; ratio = 27.7) and glycoprotein Iba (GP1BA; 27.7), d subunit of nicotinic AChR (CHRND; 17.6), proton-coupled amino acid transporter 4 (SLC36A4; 17.3), the antigen-presenting protein CD1B (13.1), Fc-fragment of IgG (CD32; 12.5), cadherin 8 (CDH8; 11.3) and 9 (CDH9; 11.5), peripheral myelin protein 22 (PMP22; 11.0), the MHC class I molecule E (HLA-E; 10.8) and the mitochondrial proteins NADH-ubiquinone oxidoreductase (NDUFS1; 16.2), cytochrome b5 outer mitochondrial membrane isoform precursor (CYB5B; 13.1), superoxide dismutase (SOD2) a subunit of pyruvate dehydrogenase E1 component (PDHA1; 10.3) and fumarate hydratase (FH; 10.1). The antigens that were recognized by the majority of PV patients were DR α chain of the class II major histocompatibility complex (MHC) encoded by the human leukocyte antigen (HLA)-DRA gene (45% PV patients), Dsc1 and Dsc3, respectively; 44% each), ATPase, Ca++ transporting, type 2C, member 1 (ATP2C1; 43%), plakophilin 3 (PKP3; 43%), M3 subtype of muscarinic acetylcholine receptor (AChR) (CHRM3; 42%), collagen a1, type XXI, (COL21A1; 42%), annexin A8-like 1 molecule (ANXA8L1; 42%), complement component 5a receptor 1 (CD88; 42%) and e subunit of nicotinic AChR (CHRNE; 41%) (55).

Concurrently, our group also sought to characterize autoAb reactivity (both Dsg as well as non-Dsg) in PV patients using protein microarray technology (**Figure 2**). In contrast to previous studies, we designed a focused, disease-specific custom protein array that included (in addition to relevant biological and technical controls): Dsg1-4, Dsc 2 and 3, CHRM1 and 3-5, Pkg, E-cad, TPO, FCER1, and ANXA9, all identified as potential targets of disease relevant autoAbs by a thorough review of the literature at the time of experimentation. Since

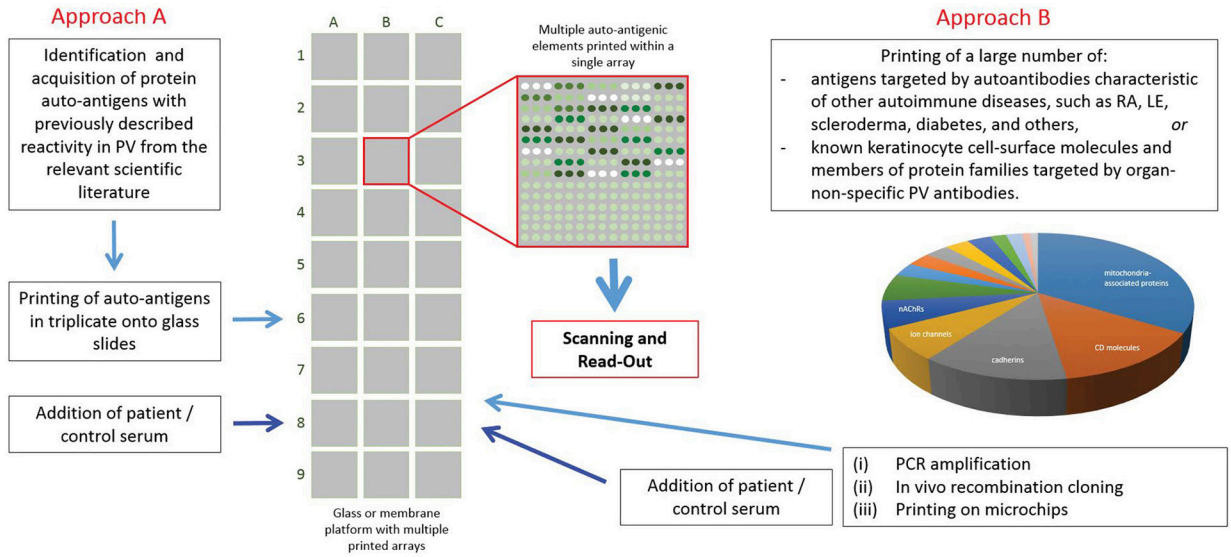

FIGURE 2 | Use of protein array technology in Pemphigus vulgaris. To date, protein array technology has been used by 2 groups with differing sets of antigens printed, patient and control populations used, and varying approaches to analysis. Both groups found some overlap in the autoantibody response in PV for both anti-Dsg and non-Dsg targets.

post-translational modifications are known to affect the reactivity of autoAbs in PV (17, 78, 79), printed antigens were produced in cell-based expression systems to more closely mimic typical posttranslational modifications. Analysis of autoAb using sera from 40 active PV patients and 20 healthy controls revealed significantly increased IgG reactivity toward Dsg3, CHRM 3,4,5, and TPO in PV patients (63). Interestingly, PV patients also exhibited a significant *decrease* in IgM reactivity to the same 5 antigens compared to healthy controls, while healthy controls with no history of autoimmune disease, who were first or second degree relatives of PV patients, had *increased* IgG autoAb reactivity to these same antigens. Further investigation suggested that this IgG reactivity in healthy related controls was partially linked to the expression of specific HLA alleles (DQB01*0503 and DRB01*0402), which are known to be strongly associated with PV (80, 81). This highlights the unique ability of protein microarrays to examine how genetic elements can impact the immune response.

In a subsequent study, we utilized an expanded protein microarray encompassing a wider range of putative PV autoantigens to better characterize the autoAb response in PV and identify patterns of autoAb reactivity that might underlie disease heterogeneity (82). Using this next generation array, we performed the largest known analysis assessing IgG autoAb reactivity in PV (466 patient and 216 control samples) and identified significantly increased reactivity toward 35 Ags, including all four non-Dsg autoAgs identified in our previous array. Again, the PV associated HLA risk alleles described above were shown to impact the atoAb profiles in patients and HLA-matched healthy controls. In addition, we also identified significantly increased reactivity toward 19 Ags in patient samples obtained from patients in the active phase of disease when compared to samples obtained from patients who were in disease remission as defined by consensus definitions (83). Furthermore, by comparing autoAb reactivities from samples obtained from patients who exhibited distinct disease morphologies at the time of sample collection [either mucosal (M), cutaneous (C), or mucocutaneous, (MC)], we were able to identify distinct profiles of autoAb reactivity that correlated to disease morphology.

Additional longitudinal analyses of samples obtained from patients across various time points and in different phases of disease activity demonstrated that changes in autoAb profiles were associated with variance in disease activity in all cases. However, the precise sets of antigens recognized was unique to individual patients. Finally, using specific patterns of autoAb reactivity identified in the previous analysis, and accounting for the known history of disease morphology, we were able to accurately predict the disease activity and expression in de-identified patient samples, indicating the potential for identifying serum biomarkers with clinical utility.

Together, these data strongly support the idea that non-Dsg autoAbs underlie disease complexity in PV and, furthermore, demonstrates the utility of comprehensive autoAb profiling to accurately classify, monitor and predict disease activity.

FUNCTIONAL ROLE OF NON-DESMOGLEIN AUTOANTIBODIES IN PV

Currently, a direct and definitive functional role in PV has yet to be established for any non-Dsg autoAb. However, there is evidence suggesting a potential role in the disease process for a number of these autoAbs. Here, we assess both the methods utilized in the detection of the non-Dsg autoAbs, as well as

data with implications to disease function in order to better understand how non-Dsg autoAb may be relevant to disease pathology.

Cholinergic Receptors

Epidermal keratinocytes express both nicotinic and muscarinic acetylcholine receptors, and both receptor subtypes function together in order to maintain cell-cell adhesion (84). The importance of cholinergic signaling in cell adhesion, as well as its relevance to PV, is emphasized by the ability of both: (1) muscarinic and nicotinic agonists to abolish PVIgG induced acantholysis *in vitro* and *in vivo*, and (2) muscarinic and nicotinic antagonists to induce cell separation in cultured keratinocytes (85–87). It should also be noted that these cholinergic receptors exhibit differential expression throughout the layers of the epidermis as well as between cutaneous and mucosal tissue (88), targeting of which could potentially contribute to the various disease phenotypes and characteristic level of acantholysis seen in PV.

Alteration of cholinergic signaling is also relevant in the clinical treatment of pemphigus. One study showing that PV patients who smoked cigarettes had better response to therapy and that smokers are less likely to develop PV (89–91) may implicate imbalances in nicotinic cholinergic signaling in disease pathogenesis. However, the therapeutic effects of cigarette smoking may very well be a function of the ability of nicotine to increase endogenous glucocorticoid production (92) or suppress B cell proliferation (92), rather than action on keratinocyte receptors. In another study, Mestinon, a compound which interferes with the breakdown of acetylcholine, was used to treat 6 PV patients as well as 1 patient with paraneoplastic pemphigus (PNP) and another with PF. Three of the 6 PV patients treated with this compound exhibited significant improvement (93). However, the therapeutic effects of these compounds may arise simply due to their pro-adhesive effects, and even though they may represent a therapeutic target, the observed effects alone do not indicate a clear role for anti-cholinergic receptor autoAbs in disease.

Although a direct pathologic role has yet to be established, the presence of anti-cholinergic receptor autoAbs in PV and the known functional association of cholinergic receptors to cell-cell adhesion establish their candidacy as disease relevant autoAbs. Of the cholinergic receptors identified as targets of autoAbs, investigation of the functional effects of anti-CHRM3 and anti-annexin (ANXA)9 autoAbs appear to be of highest relevance, as autoAbs targeting each were identified by two distinct approaches (55, 64, 85–87). Furthermore, the relatively large study groups used in the protein array studies (55, 64) indicate that these autoAbs are prevalent in the PV population.

Mitochondrial Proteins

Anti-mitochondrial autoAbs are found in several other autoimmune disorders in addition to PV, such as primary biliary sclerosis and systemic scleroderma (67, 75, 94–96). Despite a lack of specificity to PV, a preponderance of evidence exists that links anti-mitochondrial autoAbs to pathogenesis in PV. Characteristically, PV patients have been shown to exhibit increased levels of oxidative stress and reactive oxygen species along with abnormalities in lipid peroxidation and mitochondrial enzyme activity, all changes associated with mitochondrial dysfunction (97–102). Additionally, anti-mitochondrial autoAbs in PV have been shown to disrupt mitochondrial oxygen respiration, membrane potential across the mitochondrial membrane, and increase cellular reactive oxygen species (103). These changes are sufficient to induce apoptotic mechanisms [reviewed in (104)], which, despite some controversy, have been shown by some groups to play a role in cell-cell detachment in PV (105). A role for anti-mitochondrial autoAb induced mitochondrial dysfunction is further supported by the reduction of blister formation in mice treated with mitochondrial protective drugs: Cyclosporin A, minocycline, and nicotinamide (103). However, it is also possible that the protective effects of these compounds, particularly minocycline and cyclosporine, may be due to their well-documented immunosuppressive effects.

Studies demonstrating that anti-mitochondrial autoAbs can penetrate keratinocytes may help to explain how autoAbs targeting intracellular proteins could contribute to disease processes (75). Recently, it was discovered that the internalization of anti-mitochondrial autoAbs (and others) in keratinocytes is dependent upon the neonatal Fc receptor (FcRn). FcRn binds IgG in a) endosomes after pinocytosis of IgG, or B) in it's membrane bound form, followed by internalization of the IgG–FcRn complex. The mechanisms by which receptor bound IgG avoid degradation is not currently understood, although one explanation may be that these endosomes are trafficked directly to the mitochondria, the site of their antigenic targets (106, 107). Blocking of the FcRn was shown to ameliorate PVIgG induced acantholysis *in vitro* (108), demonstrating the potential functional significance of this pathway. Interestingly, these experiments also found non-anti-mitochondrial autoAbs internalized through the same pathway. Given that FcRn is predominantly expressed by basal keratinocytes in the epidermis (109), this unique expression pattern may shed light on the characteristic suprabasilar site of acantholysis seen in PV.

Identifying the predominant target (or targets) of anti-mitochondrial autoAbs in PV is critical. Although functional studies have demonstrated how mitochondrial disruption could contribute to blister formation, the exact antigenic targets have not been elucidated. The increased reactivity toward a number of mitochondrial proteins as determined from protein microarray data (55) is promising, but further validation is required. Similarly, although the immunoprecipitation of mitochondrial nicotinic AChRs confirmed the presence of autoAb reactivity against 4 proteins identified by protein microarray (67), the relatively small sample size tempers the conclusions that can be drawn concerning the prevalence of such autoAbs across PV patients as a group.

Non-Dsg Adhesion Proteins

AutoAb in PV are also known to target a number of non-desmoglein adhesion proteins. Of those recognized, the Dsc proteins are the most similar to the Dsg proteins. Dsc1-3 represent desmosomal cadherins (in addition to the desmogleins)

that are involved in cell-cell adhesion (110–114). Similar to the desmogleins, there exists a differential expression of Dsc isoforms through the layers of the epidermis, with Dsc1 having highest expression in the most superficial layers and Dsc3 expressed primarily in the suprabasilar/deep epidermis (115–119). In addition to the adhesive functions of their extracellular domains, the cytoplasmic tails of these proteins are also known to play a role in formation of the desmosomal plaque and attachment of desmosomes to the intermediate filament network (120–124). The high degree of structural and functional similarity between these two groups of proteins reflects the potential functional relevance of autoAbs directed at these targets.

Dsc3 in particular represents a good target candidate for potentially disease relevant autoAbs. Dsc3 knockout mice develop a PV-like phenotype with spontaneous suprabasilar blister formation (56). Additionally, anti-Dsc Abs can cause acantholysis in both keratinocyte monolayers as well as in a model of human epidermis (57). PVIgG pre-absorbed to remove anti-Dsc3 autoAbs is no longer pathogenic (53). The observation that greater than 40% of patients harbor anti-Dsc3 autoAbs (55) further supports the notion that Dsc3 may represent a target of disease relevant autoAbs in PV.

Plakophillins, in conjunction with plakoglobin, facilitate the attachment of the desmosomal cadherins to desmoplakin and the keratin intermediate filament network (125–128). Plakophillins have also been shown to play a key role in the assembly of desmosomes (129, 130), and mutations of plakophilins are known to cause ectodermal dysplasia-skin fragility syndrome, a disease similar to PV that is characterized by mechanical stress-induced blister formation (131). Given their role in cell adhesion, it is possible that the binding of autoAb to these targets may lead to dysfunction, resulting in impaired cell-cell adhesion. However, it has not yet been shown that PVIgG interacts with intracellular plakophillins *in vivo*.

Although autoAbs targeting other non-desmoglein adhesion associated proteins have been identified in PV patients, little functional data exists to suggest a role for these autoAbs in disease. However, evidence describing the relationship between E-cadherin and desmosomes may suggest a role for autoAbs targeting this protein in PV. E-cadherin, like the desmogleins, is a member of the cadherin family of proteins. However, unlike desmogleins, E-cadherin is known to associate with adherens junctions as opposed to desmosomes (132). While not directly involved in desmosomal adhesion, E-cadherin has been shown to play a role in the recruitment of both Dsg3 and Pkp3, suggesting a role for E-cadherin in the early stages of desmosomal development (133, 134). Given this relationship, anti-E-cadherin autoAbs identified in PV patients (73) may interfere with the normal functioning of E-cadherin, resulting in impaired desmosomal formation.

Additional Targets

Autoantibodies to a number of additional targets have been found in PV, as detailed above. Their potential functional significance in PV is explored below.

Thyroid Peroxidase (TPO)

TPO, an enzyme that functions in the organification of iodine, is a major target of autoAbs in autoimmune thyroid disease (135). The increased risk of autoimmune thyroid disease in both PV patients and first degree relatives highlight the association of autoimmune thyroid disease and PV (58–60, 136, 137). Recently, our lab has found an increased prevalence of anti-TPO autoAbs in PV patients vs. controls that is linked to the absence of both PV-typical HLA alleles and of anti-Dsg Abs (62). In a separate study, we also show that anti-TPO Abs can induce cell fragmentation in keratinocyte dissociation assays, and affect intracellular Ca levels along with p38MAPK activation in a manner similar to anti-Dsg3 autoAbs (82). Establishing a direct pathogenic role for these autoAbs is a continuing effort. Although, TPO mRNA has been shown to be expressed by qPCR analysis of human skin biopsies (138), protein expression in keratinocytes has yet to be demonstrated. If TPO is expressed by keratinocytes, it is possible that anti-TPO autoAbs may function in a similar manner as they do in Hashimoto's thyroiditis, inflicting cell damage via compliment fixation and/or antibody dependent cell-mediated cytotoxicity (ADCC) (139–145), though the paucity of immune cell infiltrate characteristic of PV may exclude ADCC as a major contribution to disease pathogenesis.

Another possibility is that anti-TPO Abs cross-react with other, yet to be identified non-TPO keratinocyte protein. For example, anti-TPO autoAbs may exert their pathogenic effect due to cross reactivity of these autoAbs with heat shock protein 60 (Hsp60), a mitochondria chaperone (146). Anti-Hsp60 autoAbs have been associated with a multitude of autoimmune diseases (147–151). Furthermore, these autoAbs have also been shown to reduce mitochondrial activity and activate caspase 3 (152). Additional cross reactivity observed between anti-Hsp60 Abs and acetylcholine receptors (153, 154) may also suggest that anti-TPO autoAbs could interfere with cholinergic signaling in the skin. Additionally, the selectivity of HLA-DR expressing APCs to activate T cells through Hsp60 presentation may offer an intriguing insight into the mechanisms underlying the genetic susceptibility seen in PV patients expressing the HLA DRB*0402 allele (155). Although it is clear that the precise mechanisms need to be worked out, the efficacy of Hsp60 tolerization in treating autoimmune conditions in both mice and humans (156–158) may represent a novel therapeutic approach to PV treatment.

Peripheral Myelin Protein 22 (PMP22)

Autoreactivity to both peripheral myelin protein (PMP)22 and CD33 was noted by Kalantari-Dehaghi et al. to be expressed at levels 10x or greater in active patients vs. controls (55). CD33 represents a transmembrane sialic acid receptor expressed on both myeloid and lymphoid cell, with no clear relationship to PV. PMP22, on the other hand, is an integral membrane protein structurally related to Perp (also seen by protein microarray to be recognized by 31% autoAbs of PV patients and only 5% of healthy controls) (55). Perp is associated with desmosomes and is integral to cell-cell adhesion (159). Deletion of Perp in mice leads to the disruption of desmosomes and spontaneous blister formation (160), and is also known to activate the extrinsic apoptotic

pathway via caspase 8 activation (161). Although both Perp and PMP22 are in the same protein family, little is known about PMP22. Mutations in PMP22 are associated with Charcot Marie Tooth disease (162). PMP22 mRNA is expressed highly in all ectodermal tissues, including the skin, and staining of the MDCK cell line reveals that PMP22 localizes to areas of cell-cell contact in epithelial monolayers (163, 164). While there is no mention of epidermal alterations in any of the mouse models lacking PMP22 (165), recent studies have shown that PMP22 may play a role in anchoring the actin cytoskeleton to the plasma membrane (166). More studies ascertaining the function of PMP22 in the epidermis are needed before we can speculate on a potential role in PV.

Human Leukocyte Antigen (HLA) Proteins

Expression of certain HLA-DR and HLA-E alleles is associated with susceptibility to PV (80, 167). Interestingly, antibodies to both HLA-DR and anti-HLA-E antibodies may play a role in PV pathogenesis as well. HLA-DR is expressed in low levels on basal keratinocytes, and studies have shown that expression of HLA-DR is elevated in both lesional and non-lesional skin in PV (168, 169). HLA-E expression has not been previously associated with PV skin, but keratinocytes near blisters in Stevens Johnson's Syndrome have been shown to increase expression of HLA-E, which enhances the chances of cell death by NK T cells, who require the atypical class I HLA-E molecule to be primed (170). Our group has additionally shown increased HLA-E expression in Dsg specific T-cells in the peripheral blood of patients (unpublished data). Finally, anti-HLA autoAbs have been shown to be pathogenic in pemphigoid gestations, another autoimmune skin blistering disease (171).

Calcium Transporting ATPase Type 2C (ATP2C1)

Calcium transporting ATPase type 2C (ATP2C1) encodes for a calcium pump typically located in the Golgi apparatus. This calcium ATPase represents a particularly interesting putative target for PV autoAbs because genetic mutations in this pump are known to cause Hailey-Hailey disease (172), which manifests as a loss of epidermal adhesion at the same level of the epidermis as PV. Additionally, alterations in intracellular calcium, which underlie pathogenesis in Hailey- Hailey disease, are also implicated in the pathogenesis of PV (173).

EVOLVING CONCEPTS IN PV: DEVELOPMENT OF THE "SUPER-COMPENSATION" HYPOTHESIS

Just as the discovery of anti-Dsg autoAbs guided the formation of the monopathic view of PV pathogenesis, the elucidation of additional autoantigenic targets has spurred the metamorphosis of understanding toward a more comprehensive and complex model that is better equipped to explain the more subtle nuances seen in PV. This shift in how PV pathogenesis is viewed is epitomized by the development of the "Multiple Hit Hypothesis" (174). According to this theory, blister formation in PV occurs from a synergistic effect of autoAbs targeting multiple keratinocyte antigens. In the past, the relative lack of data pertaining to the scope and specificity of autoAbs in the population of PV patients and tools which could quickly and efficiently determine autoAb targets limited the ability to test this hypothesis. However, the advent of protein array technology and a greater understanding of relevant antigenic targets in PV has facilitated the dissection of the complex relationship between autoAb expression and disease phenotype.

Expanding the current view of disease pathology in PV also has considerable implications concerning the framework for assessing the underlying disease mechanisms. Alterations in numerous signaling pathways have been associated with the binding of PVIgG to keratinocyte antigens, including: PLC, PKC, Cdk2, p38MAPK, EGFR, Src, JNK, MMP-9, c-myc, GSKbeta, Fas/FasL, p53, BAX, and caspases 1,3, and 8 (75, 173, 175–186). Compared to the monopathic view, incorporation of multiple disease relevant autoAbs could allow for a more precise integration of these pathways, where specific autoAbs may alter specific pathways.

In consideration of the data reviewed in this manuscript, we propose a "*super-compensation hypothesis*" that purports that the binding of specific autoAbs in combination with the unique epidermal expression of the various autoantigens results in the characteristic alteration of signaling pathways and the development of acantholysis only if the *sum* of these effects exceeds a set threshold (**Figure 3).** Weakly pathogenic autoAbs alone, or in combination do not trigger these effects. However, highly pathogenic autoantibodies alone, or multiple combinations of pathogenic or subpathogenic autoAbs could potentially exceed this threshold (**Figure 3**). Furthermore, specific autoAb expression profiles may underlie variations in disease expression to better explain clinical heterogeneity across phenotypic subgroups. The role of multiple autoAbs in PV has been previously postulated (174). Here, we extend this line of thought based on the accumulating evidence from the literature and our lab presented above to formulate a novel hypothesis underlying autoAb-mediated acantholysis. This model of PV has the potential to integrate autoAb profiles, disease variability and the mechanistic effect of autoAbs in a way that was impossible to achieve when viewing PV as the result of strictly anti-Dsg autoAbs. Consequently, each of the autoantibodies potentially involved in PV pathogenesis would lead to activation of specific downstream signaling pathways that either result in pathway amplification and/or in additive/combinatorial effects relevant to acantholysis [see (186) for a comprehensive review of autoantibody signaling in PV].

Recent work by our group assessing the functional capacity of anti-TPO autoAbs present in patient IgG provides support for the idea that multiple autoAb specificities may function together to contribute to disease. By depleting PVIgG of anti-TPO autoAbs, we were able to demonstrate that anti-TPO autoAbs contributed to PVIgG induced loss of cell adhesion, as well as PVIgG induced activation of p38MAPK and increases in intracellular calcium *in vitro* (187). These results demonstrate that additional, non-Dsg autoAbs contribute to PVIgG induced pathogenesis. However, these experiments also provide key insights concerning how multiple autoAb specificities may be working together in unique ways to drive blister formation in PV. Specifically, the effects

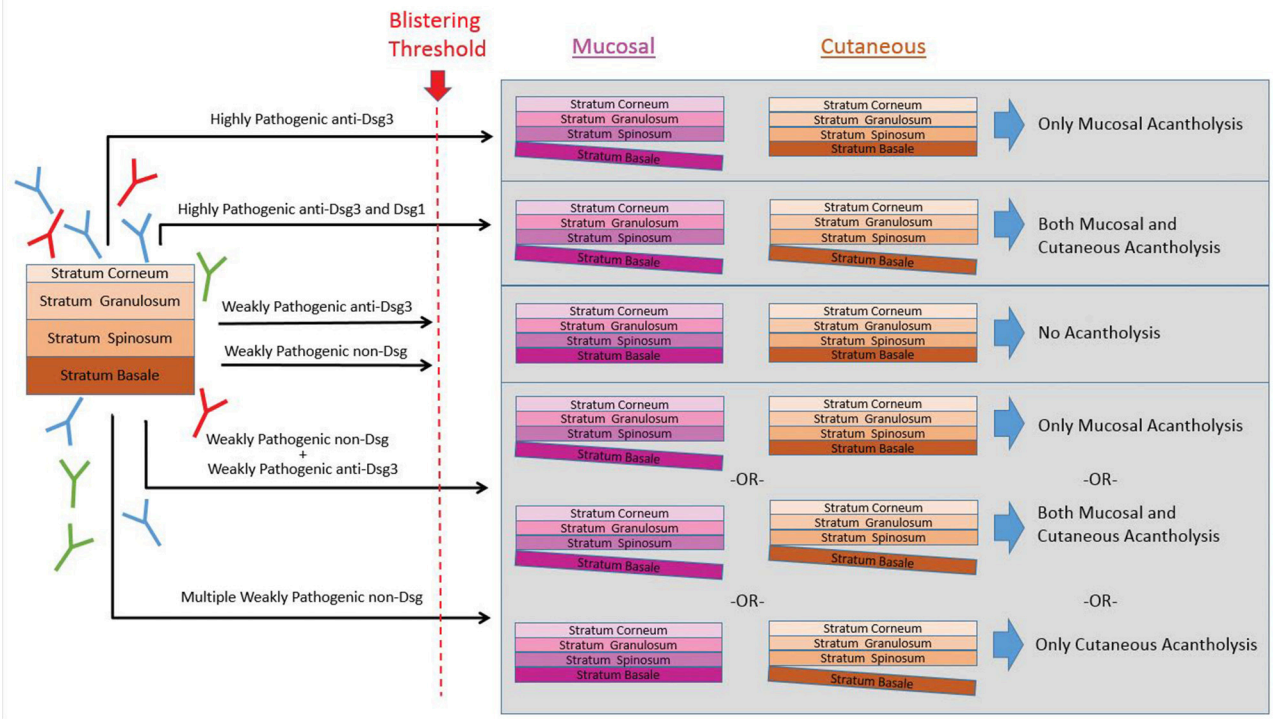

FIGURE 3 | The super-compensation hypothesis. We hypothesize the binding of specific autoAbs in combination with the unique epidermal expression of the various autoantigens results in the characteristic alteration of signaling pathways and the development of acantholysis only if the *sum* of these effects exceeds a set threshold. In this theory, highly pathogenic antibodies to either anti-Dsg3 alone, or anti-Dsg3 and−1 together can exceed the blistering threshold. Similarly, multiple combinations of subpathogenic anti-Dsg3/1 autoAbs together with non-Dsg autoAbs could potentially exceed this threshold. However, weakly pathogenic anti-Dsg or non-Dsg autoAbs alone, or sometimes even in combination, do not breach the threshold for triggering acantholysis.

of anti-TPO depletion were most significant when anti-Dsg3 autoAbs were not present. This could potentially help to explain why some patients who are negative for both anti-Dsg3 and anti-Dsg1 still exhibit disease activity. Interestingly, in support of this notion, we find the highest prevalence of anti-TPO Abs in the subgroup of patients that lack anti-Dsg Abs (62). Additional experiments investigating the precise effects of multiple autoAb specificities are required to more fully characterize how different autoAbs function together to elicit blister formation in PV.

Additionally, researchers should consider that the role of autoAbs is not always restricted to driving pathogenesis. Natural autoAbs of the IgM subgroup have been shown to play a number of beneficial roles, with subsets of these autoAbs modulating disease severity, and even protecting against the development of in autoimmune disease (188–190). It is entirely possible that some of the autoantibodies found in PV are protective against disease, similar to the role of certain g-protein coupled receptors, such as CXCR4, in experimental autoimmune encephalomyelitis (191).

FUTURE DIRECTIONS

Ultimately, the primary objective of investigation into PV is to identify avenues of intervention to improve patient quality of life. With our current understanding of disease, the best available treatments remain the administration of glucocorticoids or other broadly immunosuppressive agents, which by themselves pose a significant risk to patient health. The lack of actionable biomarkers to monitor disease progression, response to therapy, or help predict prognosis makes it almost impossible for physicians to maximize treatment efficacy while minimizing harmful side effects.

Recent characterization of autoAb specificity represents a significant step toward achieving a broader understanding of PV. However, these results must first be validated and the autoAb repertoire of even larger patient cohorts must be assessed in order to have an accurate estimation of auto antigenic targets across all PV patients. Given the well documented importance of conformation and post-translational modifications on the immunogenicity of proteins, subsequent experiments should also be conducted using antigens produced in cell systems that will parallel those of human keratinocytes. Once the full repertoire of autoAb specificity is clear, the effects of these autoAbs on keratinocyte adhesion and any effects on cellular signaling must be ascertained.

The foundation for the significance of this proposed work lies on the identification of autoAb signatures capable of distinguishing the phenotypic variations seen in PV. To this end, our group has taken the approach to define highly specific

disease subgroups stratified by both variable characteristics (disease activity, morphology, treatment, and disease duration) and static characteristics (age of onset, sex, HLA type). Establishing specific immunoprofiles for these groups will significantly impact the clinical treatment of PV. We expect that a more in depth understanding of disease relevant autoAbs will: (1) facilitate the identification of actionable biomarkers, allowing for a more precise classification of disease while simultaneously enabling physicians to predict disease progression and response to therapy, (2) provide new insights into the mechanistic pathways responsible for acantholysis, facilitating the identification of novel therapeutic targets, and (3) allow for a higher degree of personalized medicine where autoAb profiles dictate treatments individualized toward a specific patient.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Interaction of Psoriasis and Bullous Diseases

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Patients with psoriasis are frequently complicated with autoimmune bullous diseases, especially, pemphigoid diseases. It has been known that one-third cases of anti-laminin gamma1 pemphigoid, formerly anti-p200 pemphigoid, are associated with psoriasis whereas bullous pemphigoid is the most frequently associated bullous disease in psoriasis cases regardless of the lack of detectable levels of the accompanying anti-laminin gamma1 autoantibodies. Despite several suggestions, however, the definitive reason of the striking association of psoriasis and these autoimmune bullous diseases remains elusive. In this review, we look over the epidemiological evidence of the association of psoriasis and autoimmune bullous diseases and the information of genetic susceptibilities of each disease, and discuss the possible mechanisms of their complication with reference to the recent understandings of each pathogenesis.

Keywords: autoimmunity, Th2, Th17, psoriasis, pemphigoid, laminin, MMP, senescence

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INTRODUCTION

Autoimmune bullous diseases, as well as psoriasis, are skin disorders affecting the epidermis. In both diseases, immune reactions target the epidermis, and induce the development of the skin lesions following the failures in epithelial cell contacts or the defects in epithelial cell proliferation and differentiation. There is remarkable progress in the understandings of their pathogenesis in these decades, respectively. Nevertheless, (1) what triggers the pathogenic immune reactions, (2) which cells by which molecules respond to the internal or external changes and direct the subsequent immune reactions, and (3) which step is critical for the decision of the immune type, have not yet been fully elucidated.

Physicians and dermatologists have long time been aware that psoriasis patients are frequently complicated with autoimmune bullous diseases. Indeed, epidemiological evidence indicates that the incidence of some pemphigoid diseases in psoriasis patients is significantly higher than that in the control individuals without psoriasis. Moreover, recent investigations have suggested that there are in part similarities and shared players in their pathogenesis.

In this review, first we look over the epidemiological evidence of the association of psoriasis and autoimmune bullous diseases. Second, we compare their genetic susceptibilities. And third, we discuss the possible mechanisms of their association with reference to the current understandings on each pathogenesis.

EPIDEMIOLOGICAL EVIDENCE

Psoriasis and Pemphigus

Most reported cases of pemphigus developed in psoriasis patients were pemphigus foliaceus including pemphigus erythematosus. A case series of 145 patients with concomitant psoriasis and autoimmune blistering diseases from Japan reported that all four (2.8%) pemphigus cases with psoriasis were pemphigus foliaceus (124). The first case-control study of 51,800 psoriasis patients from Taiwan demonstrated the significantly higher prevalence rate of pemphigus in the patients than that in the control subjects (odds ratio (OR), 41.8; 95% confidence interval (CI), 12.4–140.9; $P < 0.0001$) (125).

There is another study evaluating their association in an inverse direction: a case-control study of 1985 pemphigus patients from Israel demonstrated that the prevalence rate of psoriasis in pemphigus patients was also higher than that in the controls (OR, 2.84; 95% CI, 2.09–3.85, $P < 0.001$) (126).

Psoriasis and Pemphigoid Diseases Including Epidermolysis Bullosa Acquisita

Complication of psoriasis cases with pemphigoid diseases are much more commonly experienced than those with pemphigus whereas the number of the report of the psoriasis cases with pemphigoid is only about three times as many as those with pemphigus (**Figure 1**). Indeed, in the case series of 145 patients with psoriasis and autoimmune blistering diseases from Japan, almost all the cases are complicated with bullous pemphigoid (63%), anti-laminin γ1 pemphigoid (formerly anti-p200 pemphigoid) (37%), or their combination (8%) (**Figure 2**). Psoriasis including pustular psoriasis precedes the development of pemphigoid in most cases. Of note, 111 (78.7%) cases had no history of any phototherapies in this case series (124).

The case-control study of 51,800 psoriasis patients from Taiwan also demonstrated the higher prevalence rate of pemphigoid in the patients than that in the control subjects (OR, 14.8; 95% CI, 5.00–43.50, $P < 0.0001$) (125).

Inversely, early case-controlled study has shown that 7 out of 62 (11%) pemphigoid cases are complicated with psoriasis and the prevalence was significantly higher than expected in the controls ($P < 0.01$) (40). Following studies also confirmed that psoriasis cases are significantly associated with bullous pemphigoid: A study of 3,485 bullous pemphigoid cases from Taiwan (OR 2.02; 95% CI 1.54–2.66, $P < 0.003$) (127), and another of 287 bullous pemphigoid cases from Israel (OR 4.39; 95% CI 2.17–8.92, $P < 0.0001$) (128), respectively.

Anti-laminin γ1 pemphigoid is originally reported as pemphigoid developed in psoriasis patients with circulating autoantibodies against unknown autoantigen. Around one-third of the following cases have also been associated with psoriasis (129).

There are only a few reported cases of psoriasis associated with other pemphigoid diseases. The case series of 145 patients with psoriasis and autoimmune blistering diseases from Japan included three cases with linear IgA bullous dermatosis and two cases with epidermolysis bullosa acquisita (124). There are few independent reports of a case with epidermolysis bullosa acquisita (51, 112), or with anti-laminin 332 mucous membrane pemphigoid (109).

Psoriasis and Other Blistering Diseases

Intriguingly, as far as we looked up, there is no reported case of psoriasis in any type of epidermolysis bullosa: simplex, junctional, or dystrophic type, except for one case report of the dystrophic type without confirmation by DNA sequencing analysis (116). There are seven reports of a case with psoriasis in Hailey-Hailey disease since the first reported case (117).

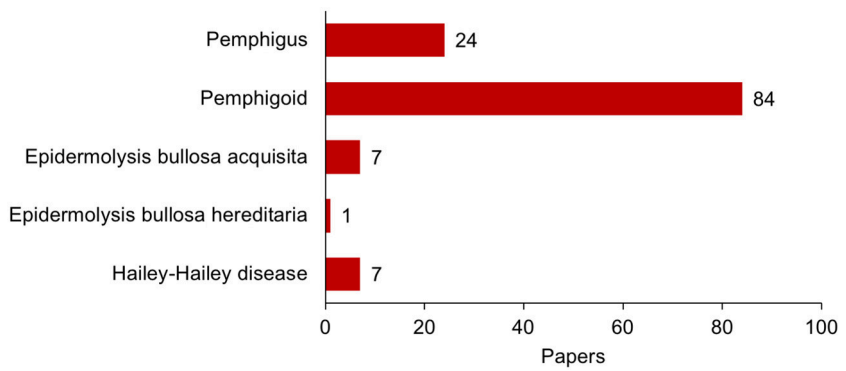

FIGURE 1 | Publication of the report of cases with association of psoriasis and bullous diseases until the end of 2017. All the publications were searched in PubMed database, and case reports and case series were selected manually with exclusion of redundancy. Cases with coexistence of two or more autoimmune blistering diseases were counted in each category: (psoriasis[tiab] AND pemphigoid[tiab]) for pemphigoid diseases; (psoriasis[tiab] AND pemphigus[tiab] NOT pemphigoid[tiab]) for pemphigus; psoriasis[tiab] AND (epidermolysis bullosa acquisita[tiab]) for epidermolysis bullosa acquisita; psoriasis AND (epidermolysis bullosa hereditaria OR epidermolysis bullosa simplex OR junctional epidermolysis bullosa OR dystrophic epidermolysis bullosa OR kindler's syndrome OR kindler syndrome) for epidermolysis bullosa hereditaria; psoriasis[tiab] AND (hailey-hailey OR familial pemphigus OR familial benign chronic pemphigus) for Hailey-Hailey disease. References are as follows. Pemphigus (24): 1990 or earlier (5) (1–5); 1991–2000 (5) (6–10); 2001–2010 (6) (11–16); 2011 or later (8) (17–25). Pemphigoid (84): 1980 or earlier (7) (26–32); 1981–1990 (16) (3, 33–47); 1991–2000 (10) (48–57); 2001–2010 (21) (58–78); 2011 or later (30) (79–109). Epidermolysis bullosa acquisita (7) (51, 110–115). Epidermolysis bullosa hereditaria (1) (116). Hailey-Hailey disease (7) (117–123).

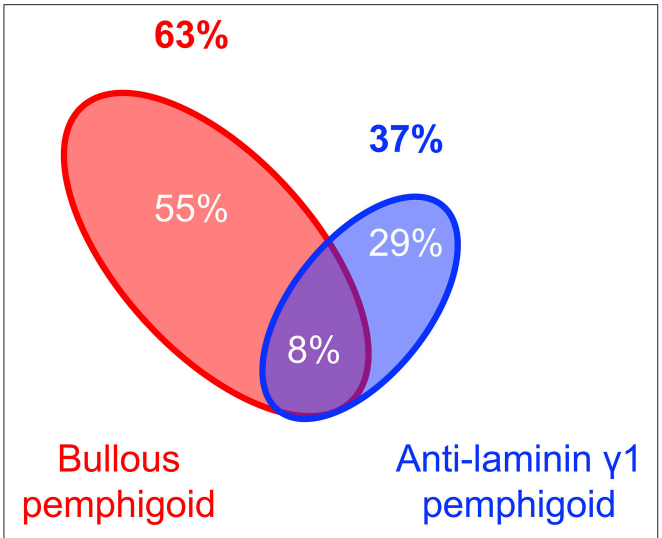

FIGURE 2 | Component of pemphigoid diseases associated with psoriasis (124).

SUSCEPTIBILITIES OF PSORIASIS AND BULLOUS DISEASES

HLA

No shared susceptibility human leukocyte antigen (HLA) alleles have been reported between psoriasis and bullous diseases that can be associated with psoriasis: HLA-Cw*0602 allele has been identified in psoriasis susceptibility 1 (*PSORS1*), a major psoriasis susceptibility locus (130). On the other hand, HLA-DRB1 alleles, such as DRB1*1401, DRB1*0402, and DRB1*08 alleles are associated with pemphigus vulgaris (131). HLA-DQB1*0301 allele has been identified as a susceptibility gene for bullous pemphigoid. Epidermolysis bullosa acquisita is associated with DRB1*15:03 allele (132).

Other Susceptibility Genes

Studies for single nucleotide polymorphisms have been defined several psoriasis susceptibility genes (130, 133) (**Table 1**) whereas it has been challenging to identify the susceptibility genes of pemphigus or pemphigoid diseases and there is much less information about their susceptibility genes. As for two major bullous diseases that can be associated with psoriasis, following genes are suggested to be associated with the disease susceptibility:, *IL1B* (135), *CD16* (136), *ATP8* (137), and *CYP2D6* (138) in bullous pemphigoid; and *CD40L*, *CD40*, *BLYS* (139), *CTLA4* (140), and *CD59* (141) in pemphigus foliaceus. However, they are not included in the major psoriasis susceptibility genes except for the risk loci at *IL1B* in late onset psoriasis (142). Susceptible SNPs in mucous membrane pemphigoid were recently reported (143) whereas mucous membrane pemphigoid rarely accompanied with psoriasis.

Transcriptomic Studies

Whereas transcriptomic analyses are preferentially demonstrated to investigate the pathogenesis of psoriasis, it is not in the case of autoimmune bullous diseases. The increased expression levels of *CD1D* (4.0) and *LILRB2* (4.7) were reported in pemphigus foliaceus (144), neither of them were included in the upregulated genes in psoriasis lesions (134) (**Table 2**).

Consequently, these results suggest that the complication of psoriasis with bullous pemphigoid or pemphigus foliaceus are not attributed to the shared susceptibility. Therefore, it would be more reasonable to consider that the epigenetic events in psoriasis lesions give rise to the increased rate of the complication with autoimmune bullous diseases.

POTENTIAL MECHANISMS OF THE ASSOCIATION OF PSORIASIS AND PEMPHIGOID DISEASES

Local Inflammation

Psoriasis plaques are the frequently affected sites for the blister formation of associated autoimmune bullous diseases, such as bullous pemphigoid (58), anti-laminin γ1 pemphigoid (49), and pemphigus foliaceus (7). It would be reasonable to consider that epigenetic changes altered by psoriasis lesion may trigger or accelerate autoreactive response to specific antigens resulting in autoantibody production, blistering formation, and further positive loop of organ-specific autoimmunity (145). Whereas detailed speculations in this context are described below, it is of not that local inflammation exacerbates cutaneous manifestations in a murine autoimmune pemphigus model (146), suggesting effective recruitment of autoantibodies into psoriasis lesions and further autoimmune loop.

Th17

There are much more psoriasis cases complicated with bullous pemphigoid than those with pemphigus. We have demonstrated that the percentages of interleukin (IL)-17+ cells in CD4+ cells in the lesional skin from bullous pemphigoid are significantly higher than those in the lesional skin from pemphigus foliaceus, and that the serum levels of IL-17 in patients with bullous pemphigoid is higher than those in healthy controls (147). Although IL-17 from T helper type 17 (Th17) cells have an essential role in pathogenesis of psoriasis, it does not explain the common order of the disease development: bullous pemphigoid following psoriasis despite the existence of a rare, inverse case: psoriasis following bullous pemphigoid (77). However, one may speculate that pathological events around the epidermis shared between psoriasis and bullous pemphigoid is related to the activation of Th17 in these diseases, and incidental switch of the immune response from Th1 to Th2 induce the production of the IgG autoantibodies resulting the complication of psoriasis with bullous pemphigoid (77) (**Figure 3**). Because, animal studies have demonstrated that single helper T cell clone specific for desmoglein 3 is sufficient to recapitulate autoimmune blister formation whereas the Th17-deviated T cell clone specific for desmoglein 3 induces

TABLE 1 | SNPs in psoriasis and the related bullous diseases (130, 133).

Disease	Category	Symbols
Psoriasis	HLA	*HLA-C*12:03, HLA-B, HLA-A, HLA-DQA1*
	MHC class-I processing	*ERAP1*
	NF-κB signaling	*REL, TNIP1, NFKBIA, CARD14*
	IFN signaling	*IL28RA, TYK2*
	T-cell regulation	*RUNX3, IL13, TAGAP, ETS1, MBD2, PTPN22*
	Antiviral signaling	*IFIH1, DDX58, RNF114*
	IL-23/IL-17 axis	*TNFAIP3, IL23R, IL12B, TRAF3IP2, IL23A, STAT3*
	Th2	*IL4, IL13*
	Late cornified envelope	*LCE3B, LCE3C, LCE3D*
	Ubiquitin pathway	*ZNF313*
	Unknown	*CDKAL1*
Bullous pemphigoid		*IL1B, CD16, ATP8, CYP2D6*
multicolumn1lPemphigus foliaceus		*CD40L, CD40, BLYS, CTLA4, CD59*

TABLE 2 | Top 25 upregulated genes in the psoriasis lesions relative to the non-lesional skin (134).

#	Symbol	Description	Fold change
1	*SERPINB4*	serpin peptidase inhibitor, clade B (ovalbumin), member 4	661
2	*S100A12*	S100 calcium binding protein A12	328
3	*TCN1*	transcobalamin I (vitamin B12 binding protein, R binder family)	309
4	*S100A7A*	S100 calcium binding protein A7A	260
5	*SPRR2C*	small proline-rich protein 2C (pseudogene)	167
6	*DEFB4A*	defensin, beta 4A	138
7	*AKR1B10*	aldo-keto reductase family 1, member B10 (aldose reductase)	89
8	*PI3*	peptidase inhibitor 3, skin-derived	80
9	*IL8*	interleukin 8	66
10	*TMPRSS11D*	transmembrane protease, serine 11D	63
11	*SERPINB3*	serpin peptidase inhibitor, clade B (ovalbumin), member 3	62
12	*S100A9*	S100 calcium binding protein A9	60
13	*OASL*	29-59-oligoadenylate synthetase-like	56
14	*ATP12A*	ATPase, H+/K+ transporting, nongastric, alpha polypeptide	54
15	*LCN2*	lipocalin 2	53
16	*RHCG*	Rh family, C glycoprotein	52
17	*IGFL1*	IGF-like family member 1	48
18	*KYNU*	kynureninase (L-kynurenine hydrolase)	48
19	*IL1F9*	interleukin 1 family, member 9	43
20	*KLK6*	kallikrein-related peptidase 6	43
21	*LTF*	lactotransferrin	36
22	*CCL20*	chemokine (C-C motif) ligand 20	35
23	*C10orf99*	chromosome 10 open reading frame 99	34
24	*HPSE*	heparanase	33
25	*ADAMDEC1*	ADAM-like, decysin 1	33

psoriasiform dermatitis (148, 149). Occasional production of autoantibodies against BP180 and desmogleins in lichen planus cases has been reported regardless of accompanying blister formation, probably because of the consequence of interface dermatitis, suggesting Th1/Th2 dichotomy among lichen planus vs. pemphigus or pemphigoid diseases (150). In psoriasis, however, production of neither autoantibodies against BP180 nor desmogleins, but α6 integrin (151), in psoriasis has been reported without complication with blistering diseases. It is therefore unlikely that psoriasis and bullous pemphigoid or pemphigus diseases are sharing their primary effector memory T cells.

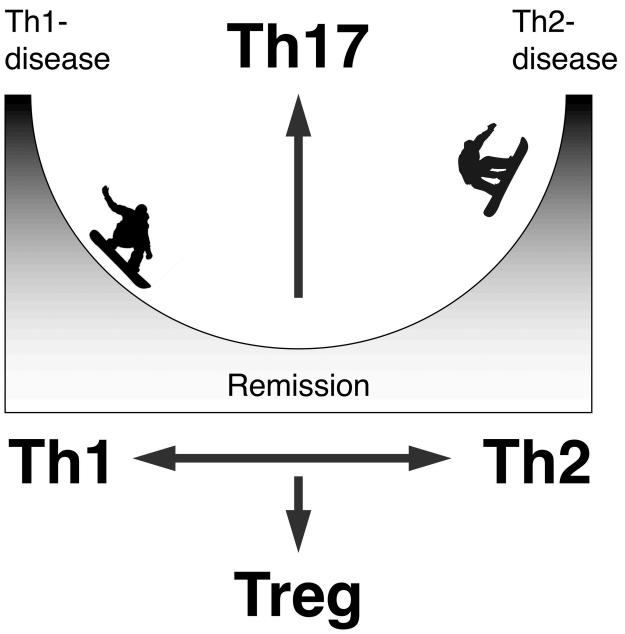

FIGURE 3 | A mechanistic model to produce autoantibodies in psoriasis. Can switch from the Th1/Th17-dominant to Th2/Th17-dominant state be involved?.

Neutrophils and MMP

Keratinocytes in both psoriasis and bullous pemphigoid produce neutrophil chemoattractants, such as IL-8, and infiltration of neutrophil is a common histologic feature in these diseases (130, 131). Consequently, neutrophils release a series of metalloproteases, and it might be related to the substantial degradation of matrix proteins and the subsequent exposure of the antigenic epitopes from matrix autoantigens composing the dermal-epidermal junction. Specifically, a disintegrin and metalloprotease (ADAM) 9, ADAM10, and ADAM17/ tumor necrosis factor-alpha converting enzyme (TACE) degrade BP180/type XVII collagen (152), which is a major autoantigen in bullous pemphigoid while matrix metalloprotease (MMP) 2, 7, 8, 12, 14, 15, and 19 degrades laminins (153), of which trimers are targeted in anti-laminin γ1 pemphigoid (154) and anti-laminin 332 mucous membrane pemphigoid (155).

Laminins

One may be tempted by the following idea: very high prevalence of psoriasis in anti-laminin γ1 pemphigoid can be explained by a positive loop of laminin degradation in psoriasis (129). In psoriasis, as well as in trauma or staphylococcal infections, degradation of laminin is accelerated through the increased expression levels of α5β1 integrin, fibronectin, and plasminogen activators (156). The laminin degradation is also stimulated by MMP9 released from neutrophils. Furthermore, laminin fragments stimulate the MMP9 expression. This laminin degradation loop may be contributed to decrease the threshold of spontaneous production of autoantibodies against laminin γ1 in the development of anti-laminin γ1 pemphigoid in psoriasis patients.

Senescence

The median age of the development of bullous pemphigoid is around 80 years of age. Cell cycle and turnover of the epidermal keratinocytes are extremely accelerated in psoriasis whereas keratinocytes in psoriasis are not immortalized like carcinoma cells. Therefore, it is a plausible idea that the extracellular matrix in psoriatic skin simulates the senescent extracellular matrix and contribute to the development of bullous pemphigoid if the development of bullous pemphigoid is triggered by the senescence of the extracellular matrix produced by senescent keratinocytes. The shortened telomere lengths in psoriasis have not yet determined in keratinocytes or dermal fibroblasts, but in lymphocytes (157). In terms of senescence, type XVII collagen (BP180) changes its distribution (158) and the protein amount due to proteolysis (159) by aging. Despite several suggestions, however, the definitive reason of the predilection of bullous pemphigoid in an extremely old age remains to be elucidated.

CONCLUDING REMARKS

Epidemiological studies have confirmed that psoriasis is highly complicated by the subsequent development of autoimmune bullous diseases. The order of the disease development and the lack of shared susceptibility genes ask whether epigenetic events and molecular circumstances in psoriasis lesions raise the susceptibility to the organ-specific autoimmunity in the skin. The high prevalence of bullous pemphigoid and anti-laminin γ1 pemphigoid in patients with psoriasis promotes following investigations on the pathogenesis of each disease, especially about their unique types of immune responses, as well as the involvement of the degradation and senescence of extracellular proteins around the dermal-epidermal junctions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Role of Eosinophils in Bullous Pemphigoid: A Developing Model of Eosinophil Pathogenicity in Mucocutaneous Disease

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Bullous pemphigoid (BP) is an autoimmune blistering disease which carries a significant mortality and morbidity. While historically BP has been characterized as an IgG driven disease mediated by anti-BP180 and BP230 IgG autoantibodies, developments in recent years have further elucidated the role of eosinophils and IgE autoantibodies. In fact, eosinophil infiltration and eosinophilic spongiosis are prominent features in BP. Several observations support a pathogenic role of eosinophils in BP: IL-5, eotaxin, and eosinophil-colony stimulating factor are present in blister fluid; eosinophils line the dermo-epidermal junction (DEJ) in the presence of BP serum, metalloprotease-9 is released by eosinophils at the site of blisters; eosinophil degranulation proteins are found on the affected basement membrane zone as well as in serum corresponding with clinical disease; eosinophil extracellular DNA traps directed against the basement membrane zone are present, IL-5 activated eosinophils cause separation of the DEJ in the presence of BP serum; and eosinophils are the necessary cell required to drive anti-BP180 IgE mediated skin blistering. Still, it is likely that eosinophils contribute to the pathogenesis of BP in numerous other ways that have yet to be explored based on the known biology of eosinophils. We herein will review the role of eosinophils in BP and provide a framework for understanding eosinophil pathogenic mechanisms in mucocutaneous disease.

Keywords: bullous pemphigoid, eosinophils, eosinophilia, major basic protein, eosinophil cationic protein, pruritus, cytokines

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INTRODUCTION TO BULLOUS PEMPHIGOID

Clinical Presentation of Bullous Pemphigoid

Bullous pemphigoid (BP) is the most common autoimmune blistering disease with an estimated annual incidence between 2 and 22 new cases per million people (1–8). BP mainly affects the elderly with an age of onset in the late 70s (8–10). Association with neurological disorders such as dementia, Parkinson's disease and cerebrovascular disease is seen in between 28–56% of BP patients (8, 11).

Most commonly, BP presents with chronic and recurrent blisters, usually arising on urticarial or eczematized skin, favoring the abdomen, and flexural aspects of the extremities. Blisters turn into erosions by mechanical friction with subsequent crust formation and healing. Prior to the development of the blisters, a prodrome of pruritus with or without urticarial lesions commonly

occurs (12). Oral involvement is seen in 10–20% of BP patients (13, 14). Pruritus alone may be the only symptom of BP in some of the cases, though it is controversial whether these patients represent falsely seropositive elderly patients with other causes of pruritus, or are in fact pre-clinical cases of BP (15, 16).

Numerous clinical variants exist, with atypical clinical variants accounting for approximately 20% of cases (8, 10, 13, 16). Likewise, medications can also induce bullous pemphigoid, (8, 17–20) with a more atypical clinical and immunologic phenotype seen particularly in patients with dipeptidyl-4 inhibitor induced BP, who demonstrated a decrease in peripheral eosinophil infiltration (21–24).

Diagnosis of Bullous Pemphigoid

Histological sections of BP typically show a subepidermal blister with variable degree of inflammatory infiltrate composed of lymphocytes, neutrophils, and characteristically eosinophils. Histological presentation may vary depending on the clinical presentation. Urticarial lesions may present with spongiosis and eosinophils infiltrating the epidermis, also termed eosinophilic spongiosis, with an absence of subepidermal clefting (**Figure 1**) (25, 26).Peripheral eosinophilia is present in around 50% of treated patients (27–29).

The diagnosis of BP requires further immunology workup in the form, of immunofluorescence and serologic studies. Direct immunofluorescence reveals linear deposition of IgG and complement component 3 (C3) at the dermal-epidermal junction (DEJ); linear IgA or IgE positivity is sometimes appreciated (8, 30, 31). Indirect immunofluorescence (IIF) shows linear deposition of IgG at the basement membrane on monkey esophagus or the epidermal side of salt-split human skin (32). Circulating antibodies against the proteins BP180 and BP230 can be detected in serum samples by ELISA, with sensitivities ranging from 66 to 100% (33–36). Sensitivity of the BP180 non-collagenous 16A (NC16A) domain ELISA is comparable with that of IIF with salt-split skin (37).

Pathogenesis of Bullous Pemphigoid

Evidence points to formation of autoantibodies against the hemidesmosomal proteins BP180 and BP230 as the leading events in blister formation in BP both clinically and experimentally (38, 39). BP180 (type XVII collagen) is a transmembrane glycoprotein with an extracellular C-terminus that mediates adhesion between the epidermis and the basement membrane (28). Association of disease activity has been demonstrated to clinically correlate with serum concentration of IgG antibodies against NC16A which is considered to contain the main pathogenic epitope of BP (40). Experiments with cultured human keratinocytes have shown cell detachment with reduced BP180 expression after tissue was incubated with antibodies against BP180 protein (41); moreover, anti-BP180 IgG and IgE induce signal transduction events with upregulation of interleukin-6 and interleukin-8 confirmed at the protein and mRNA levels (42–44). These results have been reproduced by transgenic mice expressing human BP180 (45).

Utilizing this humanized BP180 model, investigators demonstrated the role of T-cells as helpers of B-cells to differentiate into plasma cells and produce autoreactive IgG. Particularly they showed that NC16A-reactive $CD4^+$ T cells in these mouse model could activate B-cells to produce anti-NC16A IgG via CD40-CD40L interaction (46). In humans, there is an association between BP and the HLA-DQB1*03:01 allele, as stimulation with BP180 to both healthy and BP patients with this allele can exert a T-cell response. Patients with BP, however, demonstrate a Th2 response while healthy HLA matched controls demonstrate a Th1 response (47–49).

BP230 is an intracellular plakin-like protein of the hemidesmosomal plaque (28). The pathogenic role of anti-BP230 antibody has not been found to be as conclusive as anti-BP180 antibody because blisters have not been observed

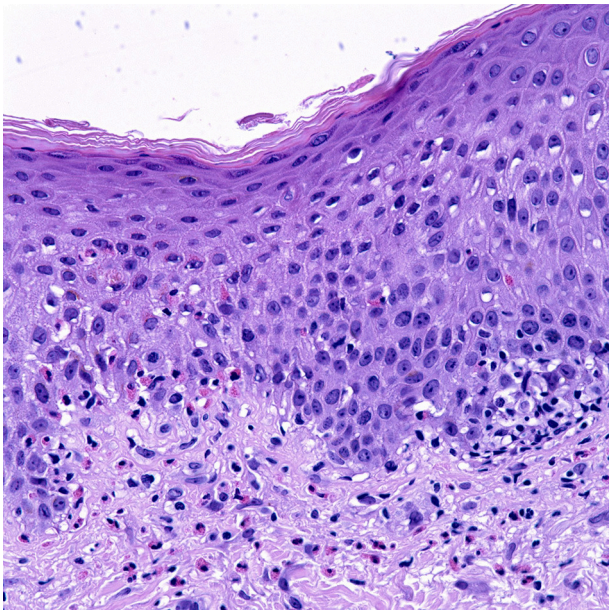

FIGURE 1 | Uriticarial bullous pemphigoid. Histological section shows dermal inflammatory cells, predominantly composed of eosinophils, which line up at the dermoepidermal junction. Notice the spongiosis and exocytosis of eosinophils through the basement membrane into the spinous layer. H&E × 4000. (Courtesy of Dr. Philip LeBoit and the Dermatopathology Service at UCSF).

Abbreviations: AP-1, activator protein-1; APC, antigen presenting cell; APRIL, activation and proliferation-induced ligand; BAFF, B cell-activating factor; BMZ, basement membrane zone; BNP, brain natriuretic peptide; BP, bullous pemphigoid; C3, Complement 3; CCL5, RANTES (regulated on activation, normal T-cell expressed and secreted); CCL11, Eotaxin-1; CCL24, Eotaxin 2; CCL26, Eotaxin 3; ChAT, choline acetyltransferase; CRTH2, prostaglandin DP2 receptor; DEJ, Dermal-epidermal junction; ECP, eosinophil cationic protein; EDN, eosinophil derived neurotoxin; EET, eosinophil extracellular traps; EPX, eosinophil peroxidase; FcεRI, human high-affinity IgE receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IIF, indirect immunofluorescence; ICAM-1, intercellular adhesion molecule-1; IP-10, IFN- γ- inducible protein 10; MBP, major basic protein; MCP, monocyte chemoattractant protein; MIG, monokine induced by IFN- γ; MMP-9, Metalloprotease 9, Gelatinase B; NC16a, non-collagenous 16A domain of BP180; NE, neutrophil elastase; NGF, nerve growth factor; NK1R, neurokinin-1 receptor; NK2R, neurokinin-2 receptor; PAF, platelet activating factor; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; VAChT, vesicular acetylcholine transporter; VCAM-1, vascular cell adhesion molecule-1.

consistently in animal models in which antibodies to BP230 are present. Furthermore, relationship between serum anti-BP230 autoantibody and disease activity has not been clearly established (12). In clinical practice, the use of ELISA for antibody to BP230 protein increases the sensitivity only 5–10% when combined with that for BP180 protein (50–52). Still, anti-BP230-type BP has been described a distinct entity, thus suggesting a pathogenic role of these autoantibodies as well as a potential for synergy with other pathogenic anti-basement membrane zone (BMZ) autoantibodies (53).

Despite the clear pathogenic role of anti-BP180 IgG autoantibody, many clinical manifestations and pathways are not easily explained by this alone. Effector cells of cell-mediated autoimmunity must be considered as significant contributors to the pathogenesis of BP. Eosinophils, have several known pathogenic roles in BP. Likewise, several known functions of eosinophils that have yet to be described in BP may play an additional role in the pathogenesis and symptoms of BP. We will review both known contributions of eosinophils to the pathogenesis of BP, as well as known mechanisms of pathogenic action of eosinophils that have yet to be evaluated specifically in BP.

INTRODUCTION TO EOSINOPHILS

Eosinophils are effector cells found in various organs including the skin. Their impact on biological processes is likely mediated primarily by their cytoplasmic granules. These granules are classified as primary, secondary, small granules, and lipid bodies (54). Secondary granules contain four toxic basic proteins: the major basic protein (MBP), eosinophil peroxidase (EPX), eosinophil derived neurotoxin (EDN) and eosinophil cationic protein (ECP). The crystalloid core of secondary granules is constituted by highly cationic MBP and is covered by the 2 ribonucleases: ECP and EDN (55). ECP is used extensively as a marker to assess activity in various inflammatory diseases (56). Granules are secreted when eosinophils become activated. These granules are highly toxic to microbes, parasites, and tumor cells (57). For instance, ECP is a cytotoxic ribonuclease with the ability to exterminate parasites, bacteria and virus *in vitro* (56). Moreover, ECP forms pores or transmembrane channels, which ultimately results in cellular damage and death (58). ECP can also lead to epithelial and neuronal apoptosis (59–61). MBP toxicity is mediated by affecting the charge of cellular surface membranes resulting in disruption and altered permeability leading to cellular injury (54, 62). Eosinophils are also implicated in the production of various cytokines, chemokines, lipid mediators, and superoxide. Complex immunomodulatory functions have been attributed to eosinophils which can also act as antigen-presenting cells (APCs) (57).

Peripheral eosinophilia is the result of the secretion of several factors such as IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-3 (63). Migration of eosinophils from circulation into the skin is mediated at least in part by very late activation antigen-4 (VLA4) which is expressed in eosinophils and binds to vascular cell adhesion molecule 1 (VCAM-1) on vascular endothelium. Other chemoattractants include eotaxin-1 (CCL11), eotaxin-2 (CCL24), eotaxin-3 (CCL26), RANTES (CCL5), and monocyte chemoattractant proteins which can bind to eosinophils and lead them to lesional sites (57, 64, 65).

Known Mechanisms by Which Eosinophils Can Contribute to the Pathogenesis of Bullous Pemphigoid

There are several lines of evidence suggesting the role of eosinophils in the pathogenesis of BP. Peripheral blood eosinophilia is present in ∼50% of affected patients (27–29). Furthermore, elevated serum concentrations of secretory granules, such as ECP, are significantly elevated in patients with BP, with levels paralleling disease severity (55, 66–71). A similar relationship has been documented to occur with IL-5 levels which runs parallel not only to disease severity, but also to ECP levels (68, 72–80). This is consistent with an increase in eosinophil activation, as confirmed by expression CD69, in peripheral blood and lesional skin of BP patients (81). While an increase in blood and tissue eosinophils has long been known (82), the actual role of eosinophils in the pathogenesis of BP is becoming more readily understood.

Production of Metalloproteases

Proteases including gelatinase B (92-kD gelatinase, matrix metalloproteinase [MMP]-9) and neutrophil elastase (NE) play a significant role in degrading BP180 and cleaving the DEJ (83). MMP-9 is released as a zymogen and is subsequently activated by a series of proteases including MMP-2, 3, 7, 10, and 13, as well as cathepsin G, plasmin, and trypsin. MMP-9 intervenes in tissue remodeling and facilitates cellular migration, extracellular matrix degradation and tissue destruction (84). Studies in isolated human eosinophils have documented that tumor necrosis factor-α (TNF-α) is a potent stimulator for a rapid release of pro-MMP-9 (85). Experiments in peripheral blood of allergic volunteers demonstrated that IL-3 in combination with TNF-α induces significant MMP-9 synthesis by eosinophils (84).

MMP-9 can cleave the extracellular collagenous domain of recombinant 180-kD BP antigen (86). Eosinophils appear to be the principal culprit in MMP-9 secretion. Strong signal for gelatinase mRNA has been detected in eosinophils but not in neutrophils at site of blister formation. *In vitro* studies conducted in Matrigel have likewise demonstrated eosinophils' ability to degrade the BMZ, identifying MMP-9 as the key protease. Notably, release of MMP-9 was increased only in the presence of both IL-5 and platelet activating factor (PAF) (87).

The direct role of MMP-9 in cleaving BP180 has been challenged, based on its ability to regulate neutrophil elastase (NE). In mouse models, MMP-9 regulates NE activity by inactivating α1-proteinase inhibitor, thus contributing to further degradation of BP180 and DEJ separation (88). Studies by Verraes et al showed that despite the presence of the proform of MMP-9 in human lesional skin, BP180 degradation could be inhibited by a specific elastase inhibitor, but not by a wide spectrum of matrix metalloproteinase inhibitor, suggesting the

importance of the regulatory role of MMP-9 on NE in blister formation (83).

Production of Eosinophil Degranulation Proteins

Eosinophils and neutrophil granule proteins can be detected in the blister fluid and serum of BP patients (55, 66, 67, 89). Moreover, studies have found peroxidase positive eosinophil granules along lamina lucida of the BMZ in BP patients (90). Scanning electron microscopy studies have revealed granule release into basal cells (91). Eosinophil granule protein deposition has been demonstrated not only in fully developed blisters, but also at the earliest stages of blister development and urticarial lesions of BP (73, 92). Eosinophil degranulation has also been observed in pemphigoid gestationis, a gestational variant of BP, whereby MBP is deposited extracellularly in the dermis (93).

More recent *in vivo* observations have demonstrated the presence of ECP, EDN, EPX, and MBP in skin. In addition, *in vivo* experiments in guinea pig skin have demonstrated the presence of these granules for weeks after intradermal injection at concentrations seen in human disease. During these experiments, Davis et al demonstrated increased cutaneous vascular permeability as an effect of the degranulation; however, basement membrane and epidermal alterations such as spongiosis were not examined (94).

In vitro experiments have shown that eosinophils may be activated through augmenting cell-surface receptors and receptor-linked oxidative metabolism (95). Upon activation, eosinophils degranulate (55, 66, 67, 89). Tsuda et al. demonstrated that degranulated eosinophils adhered to basal keratinocytes suggesting that eosinophil granules may directly damage basal keratinocytes leading to DEJ separation (89). We have demonstrated that MBP has a concentration dependent cytotoxic effect on cultured keratinocytes (96).

As eosinophils also release tissue factor (TF), an initiator of blood coagulation, Marzano et al, hypothesized a role for local activation of the coagulation cascade in BP. This was confirmed to be the case (97), with subsequent correlation between ECP levels and prothrombotic markers (70). Cases with higher coagulation, like ECP, are associated with more severe disease (97). Whether this contributes to blister development is, however, unclear.

Production of Eosinophil Extracellular Traps

Relevance of extracellular traps produced by eosinophils (EET) have increased, at least in part due to the interesting findings obtained from studies on neutrophils. Extracellular traps consist of network-like structures containing DNA, granule proteins and nuclear proteins. These traps can expand up to 15 times the size of the cell, thereby increasing the effective targeting area (98). Experiments performed on skin biopsies from human participants have shown that EET are present in infectious skin diseases, allergic diseases and autoimmune diseases including BP. The number of eosinophils releasing DNA appear to be around 10%, though this phenomenon was most commonly observed in Well's syndrome, whereby trap formation was seen in up 30% (99). *Ex vivo* experiments utilizing human skin and isolated human eosinophils have shown that EET may contribute to DEJ splitting, after the observation that DNase significantly reduced DEJ separation (99). Still, the mechanism by which EET contribute to DEJ separation is unclear.

Link Between Anti-BP180 IgE Autoantibodies and Dermo-Epidermal Junction Separation

Evidence supporting the pathogenic role of IgE autoantibodies in BP as well as its relationship with eosinophils has increased in recent years. Passive transfer of anti-BMZ IgE autoantibodies results in erythema, pruritis, eosinophil infiltration, and histologic blistering (100). This study did, however, use the LABD97 portion of the BP180 protein rather than the NC16A portion. Lin et al. created a transgenic mouse model expressing human hNC16A and human high-affinity IgE receptor (FcεRI), showing that anti-NC16A IgE from BP patients induced subepidermal split as well as eosinophil infiltration and IgE deposition at the DEJ. Particularly, they found that eosinophils are essential in order to induce BMZ separation in the presence of anti-NC16A IgE *in vivo*. This step appears to be key in DEJ separation, thus supporting the pathogenic role of IgE autoantibodies against the NC16A region of the BP180. Likewise, eosinophils in this animal model expressed FcεRI, thus providing a further link between IgE autoantibodies, eosinophils and blister formation, occurring independently of neutrophils (101).

The expression of FcεRI in monocytes, mast cells, basophils, eosinophils, dendritic cells, and platelets has been highlighted recently as a link between the biology of these cells in the presence of IgE autoantibodies (102). FcεRI, the high affinity IgE receptor is typically minimally expressed on eosinophils, but it is highly expressed on eosinophils in BP and other diseases characterized by high IgE and eosinophilia (103). FcεRI consist of an α-chain, which controls IgE binding, and β- and γ-chains, which intervene in signal transduction. These chains have been found in either tetrameric ($\alpha\beta\gamma_2$) or trimeric ($\alpha\gamma_2$) form in humans (103). Interestingly, investigators have found mRNA for α-,β-, and γ-chains in eosinophils of BP patients. These FceRI eosinophils appear to predominate in the dermis rather than epidermis (104). The capability of eosinophils to bind IgE may thus influence their subsequent degranulation (103).

Elevated serum IgE in patients with BP was first described in 1974 (105). Since then, several studies have demonstrated elevated IgE levels in up to 40–50% of patients, with the notable discovery of anti-basement-membrane IgE autoantibodies (29, 106–108). Advancements in technology led scientists in the 1990s to identify IgE autoantibodies to different BP molecules such as BP230 (109, 110). Later in the 2000s, IgE autoantibodies targeting NC16A, the principle epitope of BP180 to which IgG4 antibodies preferentially react, were described (111). IgE reactivity with other epitopes, particularly the intracellular domain of BP180 was subsequently described (112). This intracellular domain is critical for incorporating proteins into hemidesmosomes, raising the possibility that autoantibodies against it could

impair the interaction of BP180 with other constituents of the hemidesmosome (112). Experiments conducted by Freire et al provided evidence that IgE and BP180 form immune complexes in BP skin. Furthermore, *in vitro* experiments found that IgE-BP180 complexes have the potential to cross-link FcεRI in basophils leading to activation and degranulation (113). Approximately half of patients demonstrate IgE autoantibodies against BP180 and BP230 (114). Ultimately, the use of an eosinophil deficient mouse demonstrated that eosinophils are the essential link between anti-BP180 IgE antibodies and BP (101).

Despite the prevalence and clear causal role of anti-BP180 IgE in inducing BP, the clinical significance of these autoantibodies remains unclear (114). A systematic review of studies associating disease phenotype with the presence of anti-BMZ IgE found no association with phenotype, though the presence of anti-BMZ IgE was associated with greater disease severity (115). Treatment results with omalizumab, a monoclonal antibody blocking soluble IgE from binding to its receptors, have been mixed with moderate efficacy, showing only a limited role as a monotherapy (116–118). As anti-BP180 IgE ELISA is not routinely available, selecting BP patients for this treatment regimen remains a challenge in the routine clinical practice.

Production of Key Cytokines and Chemokines

Evidence of both inflammatory and anti-inflammatory cytokines have been described to be present in BP. Elevated levels of proinflammatory (IL-6, TNF-alpha, IL-8) and anti-inflammatory cytokines (IL-4, IL-10) have been reported (119). Moreover, Giomi et al has suggested that cytokine milieu varies according to the chronicity of BP. An initial Th0/Th2-like response would be seen in early stage of BP with IL-4, IL-5, and low levels of IFN- γ. Whereas in chronic phases, a Th1 response would follow with significant expression of IFN- γ (120).

A complex network of chemokines also contributes to the development of BP. Both Th1 and Th2 chemokine profiles are exhibited in BP as follows: macrophage inflammatory protein-1 β (MIP-1β) and IFN- γ- inducible protein 10 (IP-10) for Th1; and eotaxin, monocyte chemoattractant protein (MCP)-4 for Th2. Overall, there is predominance of Th2 chemotactic activity compared to Th1 cells (79). Other studies have shown significantly levels of MCP-1, and IP-10, monokine induced by IFN- γ (MIG) for Th1 (121), and, eotaxin, CCR3, and MCP-4 for Th2 (77, 79, 122). Experiments conducted by Gounni-Abdelilah et al found that eotaxin and MCP-4 were present in eosinophil granules in the bullae of patients with BP, as well as in the epidermis and infiltrating eosinophils in skin of BP patients and these were secreted by eosinophils when stimulated by IgG, IgA, or IgE immunocomplexes (79). This autocrine pathway may thus perpetuate the immune response in BP, leading to chronicity of lesions (79). Cytokines and chemokines known to be produced by eosinophils are summarized in **Table 1**.

TABLE 1 | Cytokines and chemokines capable of being secreted by eosinophils (65).

Cytokines	Chemokines
A proliferation-inducing ligand (APRIL)	CCL3/macrophage inflammatory protein-1α (MIP-1α)
Granulocyte/macrophage colony-stimulating factor (GM-CSF)	CCL5/RANTES
Interleukin-1α	CCL11/eotaxin
Interleukin-1β	CCL13/monocyte chemoattractant protein-4 (MCP-4)
Interleukin-2	CCL17/thymus activation regulated chemokine (TARC)
Interleukin-3	CCL22/macrophage-derived chemokine (MDC)
Interleukin-4	CCL23/myeloid progenitor inhibitory factor 1 (MPIF-1)
Interleukin-5	CXCL1/Groα
Interleukin-6	CXCL5/epithelial-derived neutrophil-activating peptide 78 (ENA-78)
Interleukin-10	CXCL8/interleukin-8
Interleukin-11	CXCL9/monokine induced by gamma interferon (MIG)
Interleukin-12	CXCL10/interferon γ induced protein 10 (IP-10)
Interleukin-13	CXCL11/interferon-inducible T cell alpha chemoattractant (I-TAC)
Interleukin-16	
Interleukin-17	
Interleukin-25	
Interferon-γ (IFNγ)	
Tumor necrosis factor-α (TNF)	

KNOWN MECHANISM OF EOSINOPHILS WITH POTENTIAL ROLES IN BP (HYPOTHESIS)

Learning From Other Eosinophil-Mediated Conditions

Numerous conditions involve a predominance of eosinophils such as allergic reactions, parasitic infections, and certain malignancies. From these, a large amount of information regarding the function of eosinophils has been discovered. However, it is important to note that not all eosinophils are the same, even within the same disease and organ system. Eosinophils differ in their molecular pattern as observed by Lingblom et al. when investigating differences in children and adults with eosinophilic esophagitis (123). Moreover, investigations in healthy individuals revealed age-dependent differences in levels of eosinophil markers. For instance, levels of CD44 increased with age, while levels of CD54, prostaglandin DP2 receptor (CRTH2), and galectin-10 decreased with age. In addition, they demonstrated that young healthy children express highest levels of galectin-10, CRTH2, and CD54 and that these diminish with age (123). Similarly, the gastrointestinal system

hosts substantial number of eosinophils exhibiting differences to eosinophils in the lungs or blood. For example, intestinal eosinophils rarely degranulate, and their lifespan is far longer than of those found in inflammatory sites (124, 125).

Despite these limitations, many functions of eosinophils appear to be retained across allergic diseases. To what degree they contribute specifically to BP, however, remains to be determined. We thus review these known mechanisms, which have a scientific rationale for contributing to the pathogenesis and symptomatology of BP.

The Role of Eosinophils in BP Related Pruritis

Pruritis is a hallmark of BP. In certain cases, it can be the presenting symptom, even when a rash is not present (16). While the depletion of BP180 in itself can generate itch as seen in BP180 knockout mice (126), several other pathways potentially contribute to this cardinal symptom of BP.

Interleukin-31

IL-31 belongs to the IL-6 family of cytokines produced in part by activated Th2 cells (127). It has a significant role in itch, by activating endothelin-1 responsive neurons and by increasing the release of brain natriuretic peptide (BNP), a central mediator of itch (128, 129). IL-31 additionally induces cutaneous nerve growth and branching (130).

In BP, elevated levels of IL-31 have been demonstrated in serum and in lesional skin of patients (131) and has been significantly associated with both eosinophilia and elevated anti BP-180-IgE (132). Eosinophils are capable of producing IL-31 (132, 133). In fact, eosinophils were recently shown to be the primary source of IL-31 in BP (134).

Substance P

Substance P is a major pruritogen and vasodilator released from peripheral nerves. The presence of substance P in BP has, however, varied between studies (135, 136). Substance P can have significant interactions with eosinophils. In mouse models of atopic dermatitis, degranulated eosinophils have been found surrounding an increased number of substance P-positive nerve fibers in lesional skin (137). Also, nasal provocation with substance P in patients with allergic rhinitis leads to an increased number of eosinophils (138, 139). Substance P acts on cells via binding to neurokinin-1 receptor (NK1R) and neurokinin-2 receptor (NK2R). Interestingly, effects of substance P on eosinophils include inhibition of apoptosis in a comparable manner to IL-3, a known apoptosis inhibitor, which contribute to extend eosinophil survival and may perpetuate its biological effects in disease (139).

Substance P can also induce the release of nerve growth factor (NGF) and IL-31 from eosinophils, in addition to mast cells. NGF may play a significant role mediating pruritus due to its ability to sensitize primary itching sensing neurons (140). NGF released from eosinophils may then stimulate neighbor nerves to further release substance P. Other roles of substance P on eosinophils include chemotaxis, activation and survival, thus potentially perpetuating the itch cycle. Mast cell-eosinophil crosstalk can also develop a neuro-immune communication axis and subsequently induce distinctive substance P itch (140).

Direct Interaction With Peripheral Nerves

Eosinophils interact with nerve cells leading to enhanced growth and branching resulting in enhanced innervation of the skin, as documented in cultured dorsal root ganglion neurons (141, 142). Eosinophils also coordinate changes in neurotransmitter release, and protection from cytokine-induced apoptosis. In part, these interactions occur as a result of activation of neural NFκB, activated by adhesion of eosinophils to neural intercellular adhesion molecule-1 (ICAM-1) (143).

The close relationship of eosinophils and nerves has been demonstrated in human skin samples of atopic dermatitis patients where investigators observed increased nerve density near eosinophil granule proteins. These findings were reproduced in mice whereby histological samples of murine skin showed that IL-5-stimulated eosinophils were present in the same epidermal foci of increased nerves. *In vitro* experiments with cultures of eosinophils have shown a dramatic increase in branching of sensory neurons. Collectively, these findings are in favor of an important role for eosinophils in cutaneous nerve growth (141).

Eosinophil granules may mediate a crosstalk between nerves and eosinophils. In histological samples of prurigo nodularis patients, for example, ECP- and EDN/EPX-containing eosinophils were primarily distributed in the upper dermis where nerves were also increased in number. Some of these nerves were even in direct contact with eosinophils (144).

Interaction With the Autonomic Nervous System

Eosinophils and the autonomic nerve system demonstrate a two-way cross talk. *In vitro* experiments demonstrate that the adherence of eosinophils to cholinergic nerves triggers a series of molecular events including activation of NFκB and activator protein (AP)-1 in the nerve cells, ultimately promoting nerve growth (145). In guinea pigs, adhesion of eosinophils to parasympathetic nerves results in release of reactive oxygen species (ROS) via neuronal NADPH oxidase, as well as activation of p38 MAP kinase (146). Eosinophils have also been implicated in the remodeling of neurites of the cholinergic nerve cell line (146).

Individual eosinophil derived granule proteins have been shown to affect cholinergic nerves. At non-cytotoxic concentrations, eosinophil cationic proteins have been shown to induce nerve cell signaling pathways by phosphorylation of the MAP kinases ERK 1/2, p38, and AKT and subsequent activation of the nuclear transcription factor NFκB (147). EPX has been shown to upregulate choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) gene expression while MBP upregulated VAChT alone. These enzymes coordinate the production of acetylcholine whereby ChAT catalyzes the production of acetylcholine from choline and acetyl-CoA, and VAChT regulates packaging into vesicles for synaptic release (148).

MBP and NGF have also been implicated in upregulating muscarinic M2 receptor expression *in vitro;* observed changes

were associated with a reduction of intracellular neural acetylcholine and an increase in choline content (149, 150). MBP also protects nerve cells from apoptosis by upregulation of adhesion-dependent activation of ERK1/2, inducing expression of the antiapoptotic gene bfl-1 and bfl-2 (147, 149, 151). Thus, MBP released from eosinophils at inflammatory sites may regulate peripheral nerve plasticity by inhibiting apoptosis (147). In animal models, eosinophil MBP is associated with the hyperreactivity of cholinergic nerves (148, 152–155).

Cholingeric nerves additionally can influence eosinophils. Eosinophil degranulation has been demonstrated in tissue taken from patients with inflammatory bowel disease and asthma in which eosinophils adhered to cholinergic nerves (156, 157). Interestingly, nicotinic agonists decrease eosinophil infiltration in lungs and airways of mice (158).

Eosinophils Act as Antigen Presenting Cells

While eosinophils had traditionally been considered an effector cell, recent advances have elucidated the multifaceted nature of eosinophils which can affect tissue homeostasis, metabolism, and immune regulation in both disease and the steady state (159). Eosinophils can effectively process antigen, express co-stimulatory molecules, traffic to the lymph node and induce a T-cell response (160–163). This can be stimulated by GM-CSF. Studies in wild-type mice have demonstrated that eosinophils in the lamina propria of the intestine express surface markers such as MHC II and CD80 suggesting that eosinophils in this location are capable of functioning as APCs. Moreover, investigators identified intraepithelial eosinophils exhibiting dendrites with extensive reaches. Further experiments with antigen sensitized mice revealed that despite the presence of two distinct populations, both populations of eosinophils acquired intestinal antigen *in vivo* (159). Double stain-immunohistochemistry has likewise been used to demonstrate T-cell activation and tissue eosinophils expressing MHC-II in specimens of eosinophilic esophagitis (164). Still, the efficiency of eosinophils as APCs, their function in the skin, and their ability to process key antigens in BP remains unknown.

Direct Pathogenic Actions of Degranulation Proteins and Reactive Oxygen Species

Eosinophil degranulation proteins are capable of inducing cytotoxicity through several mechanisms. ECP induces pore formation in the cell membranes contributing to inflict cellular damage (58), while MBP increases smooth muscle reactivity due to selective allosteric antagonism of vagal muscarinic M2 receptors (165) and triggers degranulation of mast cells and basophils (166, 167). While we demonstrated a cytotoxic effect of degranulation proteins on keratinocytes at physiologic doses seen in BP (96), it is unclear the extent of damage *in vivo* as necrosis is not a histologic feature of BP.

Eosinophil peroxidase generates hydrogen peroxidase as well as superoxide, causing additional damage (168). Interestingly, blockade of ROS was capable of inhibiting blister formation in an *ex vivo* model of BP (169). Limitations to the cryosection model however may overstate the role of ROS in DEJ separation. A similar study in neutrophil mediated BP evaluated luteolin, a plant-derived flavonoid with potent anti-oxidative and anti-inflammatory properties effects in *ex vivo* cryosection model of BP, resulting in a significant reduction of autoantibody-induced DEJ separation. However *in vivo* mouse experiments did not yield comparable results (170). Thus, further *in vivo* studies are needed to determine the role of antioxidants in inhibiting eosinophil induced ROS in BP.

Can Eosinophils Sustain Local Immune Response?

Eosinophils express a series of cytokines involved in plasma cell survival such as activation and proliferation-induced ligand (APRIL), IL-6, IL-4, IL-5, IL-13, IL-10, and TNF (171, 172). Therefore, they could in theory provide a local stimulus sustaining Ig producing plasma cells in the dermis. Eosinophil IL-16 a key cytokine in T-cell recruitment, as well as IL-4, IL-5, and IL-13 can stimulate Th2 immunotype. Thus, eosinophils could also in theory perpetuate T-cell stimulation and a Th2 milieu.

Effect of Eosinophils on B-Cells

B-cell responses are regulated by a series of signals including IL-2, IL-4, IL-7, IL-15, and members of the TNF family such as CD40 ligand (173). In the past two decades, two TNF family molecules: B cell-activating factor of the TNF family (BAFF) and APRIL have been recognized as key regulators of normal B cell functions and autoimmune B cell induction, both of which are expressed by eosinophils (174).

APRIL binds to receptors such as transmembrane activator and calcium modulator ligand interactor (TACI) and B cell maturation antigen (BCMA) (175, 176). Normal functions of APRIL include: increasing B-cell antigen presentation, stimulation of antigen-activated B-cells, enabling isotype switching in B cells, and augmenting plasma cell survival (174, 177, 178). BAFF acts as a potent B-cell growth factor as well as stimulus for immunoglobulin production (173, 179).

BAFF levels are significantly elevated in patients with BP. Interestingly, BAFF levels increased before the anti-BP180 antibody level and quickly decreased in response to treatment, making it a useful marker for early disease (180). Levels of APRIL are likewise elevated in BP, and closely correlate with BAFF. Levels of APRIL similarly occur extremely early on in the development of disease, and thus appear to be a key mediator prior to the development of detectable levels of autoantibodies (181). Whether the APRIL and BAFF in BP primarily comes from eosinophils or other immune cells remains unknown.

Effect of Eosinophils on T-Cell Recruitment

CCL5 (RANTES) is a chemoattractant for $CD4^+$ memory T cells, monocytes, and eosinophils (182, 183). *In vitro* studies have shown that RANTES activates T lymphocytes in an antigen-independent manner (184). RANTES may also activate eosinophils, upregulating their expression of adhesion molecules and enhance transendothelial migration (182, 183, 185, 186). IL-16 is likewise a strong attractant of CD4+ T-cells. Eosinophils

release both IL-16 and RANTES. Even at very low concentrations, RANTES and IL-16 induce migration of T-lymphocytes. Thus, eosinophils can potentially amplify the immune response by recruitment of CD4+ lymphocytes as well as additional eosinophils (187). These interactions have yet to be elucidated in BP.

Effect of Eosinophils on Th2 Polarization

Th2 polarization may be driven by eosinophil production of IL-4, IL-5, and IL-13 (188). In addition, eosinophil expression of indoleamine 2,3-dioxygenase (IDO) which catalyzes the conversion of tryptophan to kynurenines, can regulate T cell subset selection toward Th2 (189). Thus, eosinophil products can serve as Th2 adjuvants via dendritic cell regulation (190, 191).

Eotaxin and MCP-4 are two chemokines that play a significant role in the selective recruitment of not only Th2 effector cells, but also eosinophils to the inflammatory site of BP, both of which are present at elevated levels in tissue and blister fluid (79, 122, 192). In a series of experiments investigators have found that eotaxin and MCP-4 mRNA were expressed in all biopsies of BP patients, present in the epidermis, and were also expressed in eosinophils. Immunohistochemical studies confirmed that these chemokines were localized to the granules of eosinophils (79). Overall, the levels of Th2 associated chemokines (eotaxin and MCP-4) in blister fluid are significantly greater than Th1 associated chemokines (MIP-1B and IP-10). Whether eosinophils or keratinocytes are the primary source of these chemokines is unclear (193).

Keratinocytes Exposed to Anti-BP180 Antibodies Express Key Cytokines and Chemokines Involved in Eosinophil Chemotaxis

In BP, eosinophils are classically aligned along the basement membrane, with several eosinophils often traveling into the epidermis via exocytosis. While eosinophil binding to the BMZ is known to require IgG and complement (not IgE), the ability of eosinophils to exocytose into the epidermis likewise cannot sufficiently be explained by this. Thus, keratinocyte signaling is likely to have a pivotal role, as keratinocytes can express key chemokines such as IL-8 and eotaxins.

Interleukin-8

IL-8 is produced by keratinocytes when exposed to BP autoantibodies (42, 44, 194–197). IL-8 is a known chemoattractant for neutrophils (198, 199). Studies investigating relationship of neutrophils, eosinophils and IL-8 have shown that IL-8 stimulates neutrophils to induce trans-basement

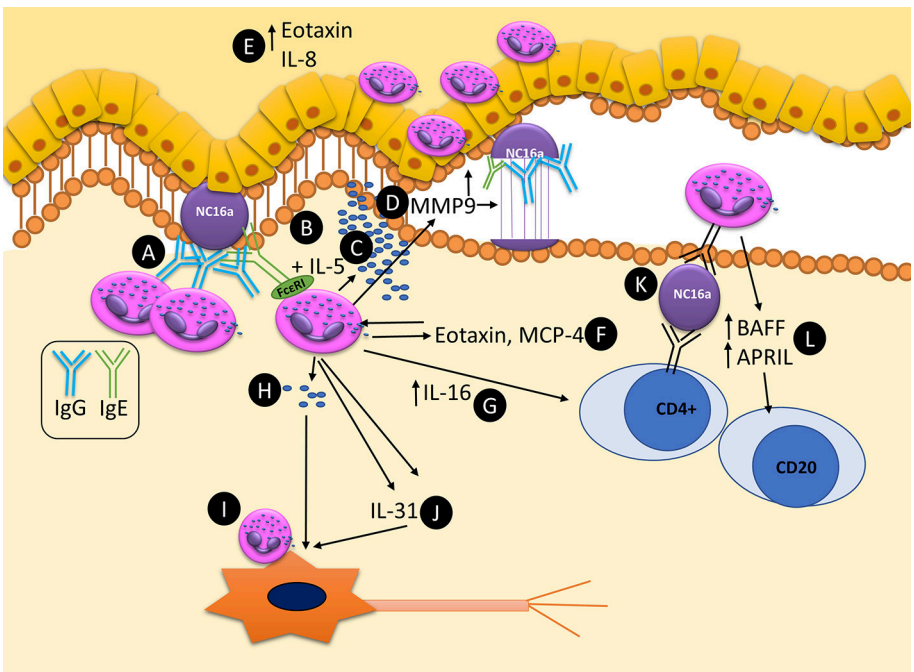

FIGURE 2 | Schematic representation of known and potential pathways by which eosinophils can contribute to the pathogenesis and maintenance of autoimmunity in bullous pemphigoid. **(A)** Eosinophils bind anti-BP180 IgG, aligning along the BMZ. **(B)** Dermal eosinophils express FcεRI which can bind to anti-BP180 IgE leading to DEJ separation. **(C)** Upon activation with IL-5 eosinophils can lead to DEJ separation and degranulation. **(D)** MMP9 is secreted from eosinophils and is capable of cleaving BP180. **(E)** Eotaxin and IL-8 are expressed in the epidermis, acting as eosinophil chemotactic chemokines, attracting further tissue eosinophilia. **(F)** Eotaxin and MCP-4 are released from eosinophil granules, further driving tissue eosinophilia and Th2 polarization. **(G)** IL-16 is released from eosinophils and is capable of stimulating T-cell response. **(H)** Eosinophils can directly degranulate on and **(I)** directly bind to neurons leading to increase branching and potentially pruritis. **(J)** Eosinophils secrete IL-31, a major pruritogen which can stimulate nerves. **(K)** Eosinophils are capable of acting as antigen presenting cells, potentially leading to T-cell responses by binding bound antigen via MHC-II to T-cell receptors. **(L)** Eosinophils express BAFF and APRIL, potentially stimulating local autoimmune B-cells.

membrane migration of eosinophils in the airways of asthmatic patients (200). Upon lipopolysaccharide (LPS) and IL-8 stimulation, neutrophils produce several chemoattractants for eosinophils such as leukotriene B4 and PAF that can recruit eosinophils and induce trans-BMZ migration (201, 202). Interestingly, IL-8 does not stimulate eosinophils alone to migrate through artificial BMZ (Matrigel) (203). Therefore IL-8 seems to play a key role to stimulate neutrophils resulting in subsequent trans-basement membrane migration of eosinophils (197).

Autoantibodies to BP180 mediate the release of IL-8 from human keratinocytes in a dose and time dependent manner (42, 195). In fact, this cytokine is known to be elevated in sera and blister of BP patients with significantly higher levels of IL-8 in blister fluid as compared to serum (42, 194, 196).

Eotaxins

Aside from stimulating Th2 polarization, eotaxins have a key role in the attraction of eosinophils into their target tissues. Eotaxins consists of three chemokines CCL11, CCL24, and CCL26 (204). The main eotaxin receptor is CCR3 which is expressed on all eosinophils in peripheral circulation (205).

The presence of elevated levels of eotaxin, IL-5 and CCR3 has been demonstrated in blister fluid, lesional and perilesional skin in BP (77, 122). Significant correlation with these markers and the number of dermal infiltrating eosinophils has also been demonstrated (77). In addition, studies investigating specific ligands have shown that CCL11 and CCL26 are significantly associated with activated eosinophils (204). Epidermal expression of eotaxin appears to be a consistent feature among all eosinophilic dermatoses (193).

CONCLUSION

Eosinophils are complex cells with numerous functions. They generally make up the predominant inflammatory cell-type seen in BP. In recent years, the overall understanding of eosinophils has significantly improved, leading to new avenues to pursue in the pathogenesis of BP. Several eosinophil pathways have well-defined roles in the pathogenesis of BP that demonstrate not only a correlative role in disease and severity, but rather a causative.

- MMP9 is secreted from eosinophils and is capable of cleaving BP180 and activating NE
- Eosinophil degranulation proteins are deposited on basal keratinocytes
- Eosinophil extracellular traps can contribute to DEJ separation. This can be abrogated with DNAse treatment
- BP180 IgE autoantibodies need eosinophils in order to mediate DEJ separation *in vivo*. This requires FcεRI which while not typically expressed on eosinophils, is significantly overexpressed in BP.
- Eotaxin and MCP-4 are seen in eosinophil granules in BP patients, thus perpetuating tissue eosinophilia.

Aside from these known direct roles in the pathogenesis of BP, several known functions of eosinophils have a scientific rationale to contribute to symptomatology and the pathogenesis of BP. Limitations to drawing further conclusions are summarized below:

- Eosinophils are the key producer of IL-31, a known pruritogen, in BP. Whether this is the primary pruritogen in BP is not known.
- Eosinophils can directly attach or degranulate onto peripheral and autonomic nerves, inducing branching and nerve growth which can lead to pruritis. While known to occur in other skin disease, this has not been studied in BP.
- Eosinophils can act as functional APCs. It is not known whether eosinophils in the skin can function as APCs, and whether they can effectively process the BP180 antigen
- Eosinophil degranulation proteins are known to be cytotoxic and have been shown to be cytotoxic to keratinocytes. Whether this cytotoxicity has *in vivo* contributions to the pathogenesis of BP has not been studied.
- Eosinophils cause generation of ROS which when blocked in *ex vivo* models, can prevent DEJ separation. Whether eosinophil induced ROS is sufficient to lead to disease *in vivo* has not been studied. In neutrophils, this is not sufficient to prevent blister formation.
- Eosinophils are known to produce BAFF and APRIL, two key regulators of autoimmune B-cells. Whether they produce BAFF and APRIL in BP, and whether this is indispensable in promoting B-cell responses in BP is not known.
- Eosinophils are known to secrete IL-16 and RANTES, two key T-cell recruiting molecules. Whether this occurs in BP, and whether this is indispensable for T-cell involvement in BP is not known.
- Eosinophils are known to secrete IL-4, IL-5, and IL-13 which can promote Th2 polarization. Whether they are the primary source of IL-4, IL-5, and IL-13 in BP, as well as whether they are indispensable in promoting Th2 polarization in BP is not known.
- Keratinocytes express Eotaxin and IL-8, both strong attractants for eosinophil migration. Whether this has pathologic significance is not known.

The principle mechanism by which eosinophils can potentially contribute to the pathogenesis of BP are summarized as a schematic in **Figure 2**. Future studies addressing these uncertainties will provide a more thorough understanding of the roles of eosinophils in BP, as well as eosinophils in the skin.

AUTHOR CONTRIBUTIONS

KA: Substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data for the work; KA, MV, KK, SG: Drafting the work

or revising it critically for important intellectual content, Final approval of the version to be published, Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Autoimmune Thyroid Diseases and Thyroid Cancer in Pemphigus: A Big Data Analysis

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There is a little consensus regarding the association of pemphigus with autoimmune thyroid diseases. While this association had been confirmed by some observational studies, others had refuted it. We aimed to study the association between pemphigus and Hashimoto's thyroiditis, Grave's disease, and thyroid cancer using a large-scale real-life computerized database. A cross-sectional study was performed to compare pemphigus patients with age-, sex-, and ethnicity-matched control subjects regarding the prevalence of overt thyroid diseases. Chi-square and *t*-tests were used for univariate analysis, and a logistic regression model was used for multivariate analysis. The study was performed using the computerized database of Clalit Healthcare Services ensuring 4.5 million individuals. A total of 1,985 pemphigus patients and 9,874 controls were included in the study. The prevalence of Hashimoto's thyroiditis (12.9 vs. 11.9%; $P = 0.228$), Graves's disease (0.7 vs. 0.7%; $P = 0.986$), and thyroid cancer (0.7 vs. 0.5%; $P = 0.305$) were comparable among patients with pemphigus and control subjects. In sex-stratified analysis, pemphigus associated significantly with Hashimoto's thyroiditis among male patients (OR, 1.36; 95% CI, 1.04–1.79). In multivariate analysis adjusting for potential confounding factors, no independent associations between the conditions were revealed. Study findings were robust to sensitivity analysis that included only patients under pemphigus-specific treatments. In conclusion, Hashimoto's thyroiditis was found to be associated with pemphigus only among male patients, but not among all patients. Physicians treating patients with pemphigus might be aware of this possible association. This study does not provide a clue for an association of pemphigus with Grave's disease or thyroid cancer.

Keywords: pemphigus, thyroid gland, autoimmunity, comorbid conditions, comorbidity, association, cancer

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INTRODUCTION

Pemphigus is a rare group of organ-specific autoimmune diseases affecting the skin and the mucous membranes. This intraepidermal bullous diseases manifest with vesicles and erosions on the epithelium of mucous membranes and skin, and is often accompanied with increased morbidity and mortality (1, 2). The pathogenesis of pemphigus is characterized by the production

of autoantibodies against different proteins of the desmosomes. The binding of these autoantibodies to desmosomal components disrupts intra-epidermal adhesion, leading to acantholysis and intraepithelial blister formation (3, 4). Patients with pemphigus were observed to experience an increased prevalence of several autoimmune diseases (5).

Hashimoto's thyroiditis and Grave's disease are the most widespread autoimmune thyroid disease (AITD). These diseases represent a prototypical organ-specific autoimmune disorder, in which extrinsic factors trigger the development of an immune response directed against thyroid antigens in genetically susceptible individuals (6). AITDs were found to coexist with other comorbid autoimmune disorders, and the concept of an autoimmune diathesis is widely accepted in these diseases (7).

The association between pemphigus and autoimmune thyroid diseases (AITD) is controversial. While several controlled studies revealed a higher prevalence of overt autoimmune thyroid diseases (8–10) and greater positivity of anti-thyroid peroxidase (TPO) among pemphigus patients (11, 12), other studies did not demonstrate a significant association between pemphigus and clinical AITD (11–15).

The objective of the current study is to investigate the association of pemphigus with Hashimoto's thyroiditis, Graves's disease, and thyroid cancer using a large-scale real-life cross-sectional study.

METHODS

Study Design and Dataset

This study was designed as a cross-sectional retrospective study utilizing the database of Clalit Healthcare Services (CHS)- the largest managed care organization in Israel, serving a population of approximately 4,500,000 enrollers in 2016. CHS has a database with incessant real-time input from medical, administrative, and pharmaceutical computerized operating systems. The validity of diagnoses in this registry, which are grounded on hospital and primary care physicians and specialists reports, has been found to be of high reliability (16, 17). This database undergoes a regular validation procedures by logistic checks (such as comparing the diagnoses from different sources), as well as by direct validation of the diagnoses by the managing physicians.

The current study was approved by the institutional ethical board of Ben-Gurion University and CHS.

Study Population

Patients were defined as having pemphigus and each one of the thyroid diseases when there was a documented diagnosis of these entities at least twice in the medical records registered by a physician in the community, or when they have been registered in the diagnoses of discharge letters from hospitals. To raise their validity, the definition of Hashimoto's thyroiditis and Grave's diseases were based on the relevant ICD-9 codes and prescription of thyroid hormone replacements and anti-thyroid preparations, respectively, for more than 6 months.

A control group of up to 5 controls per each case were selected, matched randomly by age, sex, and ethnicity. The age matching was grounded on the exact year of birth (1-year strata).

Covariates and Outcome Measures

Outcome measures were controlled for comorbid conditions as determined using Charlson comorbidity index (18). Outcome measures were also controlled for overutilization of health services, in order to ensure that observed associations were not merely due to ascertainment bias. Healthcare utilization was determined by the number of total visits per individual in the year preceding the diagnosis of pemphigus in cases and the enrollment of control subjects.

Sensitivity analysis was undertaken by repeating all calculations following the inclusion of only cases prescribed "pemphigus-related medications": systemic corticosteroids or adjuvant immunosuppressive agents (excluding methotrexate) for more than 6 months; or cases prescribed one or more cycles of rituximab.

Statistical Analysis

The distribution of sociodemographic and clinical factors was compared between cases and control subjects using Chi-square test for sex, socioeconomic status, and *t*-test for age. Logistic regression was then used to calculate ORs, and 95% CIs, to compare cases and controls with respect to the specified malignancies. Homogeneity of ORs across strata was tested using Breslow–Day and Tarone's tests. The exact age matching permitted the use of unconditional logistic regression (19). All statistical analysis was performed using SPSS software, version 23 (SPSS, Chicago, IL, USA).

RESULTS

Our study population comprised 1,985 patients with pemphigus and 9,874 age-, sex-, and ethnicity-matched control subjects (**Table 1**). The mean (±SD) age at the onset of pemphigus was 72.1 ± 18.5, which is identical to the age of control subjects at their enrollment date. In all, 797 (40.2%) of pemphigus patients were male and similar proportion was observed in controls. No significant differences in ethnic background and socioeconomic status were noted between the two groups. Comorbidity rates, measured by the Charlson index, were higher in cases, with 1,059 (53.4%) patients having severe comorbidity compared with 4,055 (41.1%) in controls (**Table 1**).

Table 2 demonstrates the proportions of cases and controls with thyroid diseases stratified by sex and age category. The prevalence of Hashimoto's thyroiditis (12.9 vs. 11.9%) and thyroid cancer (0.7 vs. 0.5%) was slightly higher in patients with pemphigus than in controls, although without reaching the level of statistical significance ($P = 0.228$ and $P = 0.305$, respectively). The prevalence of Grave's disease was comparable among cases and controls (0.7 vs. 0.7%; $P = 0.986$).

Table 3 presents the results of univariate and logistic regression models and summarizes ORs for AITD and thyroid cancer among patients with pemphigus. In univariate analysis, no statistically significant association was established between pemphigus and Hashimoto's thyroiditis (OR, 1.09; 95%CI, 0.95–1.22), Grave's disease (OR, 0.99; 95% CI, 0.55–1.81), or thyroid cancer (OR, 1.38; 95% CI, 0.74–2.55). However, in sex-stratified

analysis, Hashimoto's thyroiditis was significantly associated with pemphigus among men (OR, 1.36; 95% CI, 1.04–1.79).

We further performed a sensitivity analysis, including only pemphigus patients who were prescribed one of the following "pemphigus-related treatments": systemic corticosteroids or adjuvant immunosuppressive agents (azathioprine, mycophenolate mofetil, cyclophosphamide); or cases prescribed one or more cycles of rituximab. The association of pemphigus with the three aforementioned thyroid diseases lacked statistical significance also in this analysis (Hashimoto's thyroiditis: OR, 1.07; 95% CI, 0.91–1.22; Grave's disease: OR, 1.01; 95% CI, 0.57–1.84; thyroid cancer: OR, 1.34; 95% CI, 0.68–2.66; **Table 3**).

In a multivariate logistic regression model, no association was observed between pemphigus and Hashimoto's thyroiditis, Grave's disease, and thyroid cancer after adjusting for comorbidities and over-utilization of healthcare services (**Table 3**). The multivariate analysis was performed despite the lack of a significant outcome in univariate analysis in order to control for putative negative confounders that may interfere with the association between the entities by suppression effect (20).

TABLE 1 | Descriptive characteristics of the study population.

Characteristic	Patients with pemphigus (*N* = 1985)	Controls (*N* = 9874)	*P*-value
AGE, YEARS			
Mean ± SD	72.1 ± 18.5	72.1 ± 18.5	1.000
Median (range)	77.4 (0–103.0)	77.4 (0–103.1)	
Male sex, *N* (%)	797 (40.2%)	3,962 (40.1%)	0.934
ETHNICITY, *N* (%)			
Jews	1,805 (90.9%)	8,866 (89.8%)	0.136
Arabs	180 (9.1%)	1,008 (10.2%)	
BMI, kg/m^2 (Mean ± SD)	27.7 ± 6.6	27.9 ± 6.6	0.355
Smoking, *N* (%)	510 (25.7%)	2,758 (27.9%)	0.045
SES, *N* (%)			
Low	634 (31.9%)	3,249 (32.9%)	0.386
Intermediate	830 (41.8%)	4,263 (43.2%)	0.250
High	423 (21.3%)	2,217 (22.5%)	0.241
CHARLSON COMORBIDITY SCORE, *n* (%)			
None (0)	344 (17.3%)	2,636 (26.7%)	<0.001
Moderate (1–2)	582 (29.3%)	3,183 (32.2%)	0.011
Severe (≥3)	1,059 (53.4%)	4,055 (41.1%)	<0.001
HEALTHCARE UTILIZATION, *n* (%)			
0 visits	286 (14.4%)	770 (7.8%)	<0.001
1–12 visits	411 (20.7%)	2,094 (21.2%)	0.248
≥13 visits	1,288 (64.9%)	7,010 (71.0%)	<0.001

N, Number; SD, standard deviation; BMI, body mass index; SES, socioeconomic status.

DISCUSSION

This large-scale study is the first population-based study aiming to investigate the associations between pemphigus and autoimmune thyroid diseases and thyroid cancer. Our findings revealed that Hashimoto's thyroiditis was found to be associated with pemphigus among male patients, whereas no association was observed between pemphigus and Grave's disease and thyroid cancer.

"Autoimmune diathesis" is a concept alleging that individuals affected by an autoimmune disease are more susceptible to develop other autoimmune disease (21–23). In accordance with the findings of controlled observational studies, pemphigus was found to associate with other autoimmune diseases, including rheumatoid arthritis (9, 10), type I diabetes mellitus (10), Sjögren's syndrome (15), systemic lupus erythematosus (15), alopecia areata (15), ulcerative colitis (24), and myasthenia gravis (2). In addition, both Hashimoto's thyroiditis and Grave's disease are known to cluster with a wide range of autoimmune disorders (6, 25).

With regard to the link between pemphigus and AITD, there is a little consensus, as results from the fact that different studies are inconsistent. Leshem et al. (9) reported that the prevalence of AITD was greater among 110 patients with pemphigus as compared to 969 of their first-degree relatives. The diagnosis of AITD, in this study, was determined differentially using serological analyses in cases and using questionnaires in controls. Parameswaran et al. (10) found that the prevalence of AITD was 6-fold greater among pemphigus patients as compared to

TABLE 2 | Demographics of cases and controls with overt thyroid diseases.

Characteristic	Hashimoto's thyroiditis (*n* = 1,434), *n* (%)		Grave's disease (*n* = 78), *n* (%)		Thyroid cancer (*n* = 60), *n* (%)	
	Pemphigus (*n* = 1,985)	Control (*n* = 9,874)	Pemphigus (*n* = 1,985)	Control (*n* = 9,874)	Pemphigus (*n* = 1,985)	Control (*n* = 9,874)
All (*n* = 11,859)	256 (12.9)	1,178 (11.9)	13 (0.7)	65 (0.7)	13 (0.7)	47 (0.5)
Male (*n* = 4,759)	73 (9.2)	273 (6.9)	2 (0.3)	12 (0.3)	3 (0.4)	8 (0.2)
Female (*n* = 7,100)	183 (15.4)	905 (15.3)	11 (0.9)	53 (0.9)	10 (0.8)	39 (0.7)
AGE CATEGORY (YEARS)						
<40 (*n* = 872)	5 (3.5)	12 (1.6)	0 (0.0)	1 (0.1)	0 (0.0)	3 (0.4)
40–59 (*n* = 1,768)	26 (8.8)	87 (5.9)	2 (0.7)	5 (0.3)	0 (0.0)	8 (0.5)
60–79 (*n* = 4,121)	102 (14.8)	404 (11.8)	5 (0.7)	17 (0.5)	7 (1.0)	24 (0.7)
≥80 (*n* = 5,098)	123 (14.3)	675 (15.9)	6 (0.7)	42 (1.0)	6 (0.7)	12 (0.3)

n, Number. The figures inside the brackets represent the percentage of the positive cases of all individuals in the same strata.

TABLE 3 | The association between pemphigus and thyroid diseases.

Disease	Pemphigus (n = 1,985), n (%)	Controls (n = 9,874), n (%)	OR (95% CI)	Univariate P-value	Male-specific OR (95%CI)	Female-specific OR (95%CI)	Sensitivity analysis OR (95%CI)	Adjusted OR (95%CI)[a]	Adjusted OR (95%CI)[b]
Hashimoto's thyroiditis	256 (12.9)	1,178 (11.9)	1.09 (0.95–1.26)	0.228	**1.36 (1.04–1.79)**	1.01 (0.85–1.20)	1.07 (0.91–1.22)	1.00 (0.86–1.16)	1.01 (0.87–1.17)
Grave's disease	13 (0.7)	65 (0.7)	0.99 (0.55–1.81)	0.986	0.83 (0.19–3.71)	1.03 (0.54–1.98)	1.01 (0.57–1.84)	0.93 (0.51–1.70)	0.88 (0.48–1.62)
Thyroid cancer	13 (0.7)	47 (0.5)	1.38 (0.74–2.55)	0.305	1.87 (0.49–7.05)	1.28 (0.64–2.57)	1.34 (0.68–2.66)	1.17 (0.63–2.17)	1.23 (0.66–2.30)

n, Number; OR, odds ratio; CI, confidence interval. [a]Adjusted for Charlson score. [b]Adjusted for healthcare utilization. Bold indicates significant values.

the general population. Similarly, the definition of outcome measure was differential; the prevalence of AITD was determined according to a questionnaire in cases and obtained from the published literature and the Centers for Disease Control and Prevention for the general population. In a Turkish study including 80 patients and 80 control subjects, the frequency of anti-TPO antibodies and the prevalence of Hashimoto's thyroiditis were significantly higher among cases (8).

Conversely, two small case-control studies including 15 and 22 patients with pemphigus demonstrated higher detection rate of anti-TPO antibodies in the sera of patients with pemphigus relative to controls. However, no differences were noted between cases and controls in terms of clinical thyroid diseases including AITDs (11, 12). Another Iranian case-control study comprising 75 patients and 65 controls found no significant differences between cases and control subjects whether in serum positivity to anti-TPO and anti-thyroglobulin antibodies or in the prevalence of Hashimoto's thyroiditis (13).

Apparently, the conclusiveness of the aforementioned studies is severely hampered by the small size of the cohorts utilized. By using one of the largest pemphigus cohorts reported so far, we overcame one of the main drawbacks of previous studies, which hinder a better understanding of pemphigus associations and comorbidities. Lack of association with Hashimoto's thyroiditis among female patients and with Grave's disease across the entire cohort align with the findings of another population-based Taiwanese study aiming to evaluate the prevalence of comorbid autoimmune disease in pemphigus patients (15). In this large-scale study including 1,998 patients, AITDs were not found to associate with pemphigus (15).

Strengths and Limitations

The large sample size provides sufficient power to exclude chance as the basis for the findings, and enables a precise estimation of the association between uncommon conditions which have otherwise been unavailable. The population-based setting minimizes the probability of selection bias. The study limitations include the lack of data concerning the immunopathological subtype, clinical features, and severity of pemphigus. The utilization of routinely collected data interferes with a direct validation of diagnoses; however, it is improbable that significant misclassification will have meaningfully affected our findings. The diagnoses of both pemphigus and thyroid diseases in our study are very reliable, because pemphigus in Israel is diagnosed in secondary and tertiary care facilities, relying on skin biopsies, direct and indirect immunofluorescence (26), and because the chronic diseases registry of CHS undergoes continuous validation process including verifying diagnoses according to laboratory analyses, making the diagnosis of AITD more precise. To further refute this misclassification, we performed a sensitivity analysis to validate the diagnosis of pemphigus among cases, and we relied on the prescription of relevant treatments to define the diagnosis of AITD.

In conclusion, the present study demonstrates that pemphigus may be associated with Hashimoto's thyroiditis only among male patients. Physicians treating patients with pemphigus might be aware of this possible association. This study does not provide a clue for an association of pemphigus with Grave's disease or thyroid cancer.

AUTHOR CONTRIBUTIONS

KK, AC contributed to study concept and design. KK drafted the manuscript. KK, EB, and DC contributed to the acquisition, analysis, and interpretation of data. AC, MK supervised the study. All authors take responsibility for the integrity of the data and the accuracy of the data analysis.

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BRIEF RESEARCH REPORT
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High Index Values of Enzyme-Linked Immunosorbent Assay for BP180 at Baseline Predict Relapse in Patients With Bullous Pemphigoid

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Bullous pemphigoid (BP) presenting with erythema plaques and tense blisters is the most frequent autoimmune bullous disease. Immunologically, BP is characterized by the presence of circulating anti-epidermal basement membrane zone (BMZ) antibodies. The autoantigens in BMZs targeted by patient's antibodies are mainly BP180 (type XVII collagen) and BP230. Previous reports have indicated that IgG to the immunodominant region of BP180 in BP, 16th non-collagenous domain (NC16A), and anti-BP180NC16A IgE are related to disease activity. In the cytokine profile, serum levels of IL-6, TNF-α, IL-15, and CCL18 were associated with the severity or activity of the disease. Blood eosinophilia is seen frequently, especially in severe cases. These biomarkers are helpful to evaluate efficacy of treatment and disease severity. Due to the high frequency of disease relapse, prediction of relapse at initiation of treatment (baseline) must be beneficial for clinicians. Therefore, we evaluated biomarkers anti-BP180 IgG (BP180 ELISA), anti-BP230 IgG (BP230 ELISA), peripheral eosinophils, and serum IgE at baseline between BP patients with ($n = 16$) and without ($n = 31$) relapse. We found significantly higher index values of BP180 ELISA in the relapse group, whereas no significant difference was found in BP230 ELISA, peripheral eosinophils, and serum IgE. This study indicated that a high index value of BP180 ELISA (cutoff value, 53.09 U/mL; sensitivity, 81.3%; specificity, 48.4%) at baseline may predict relapse in patients with BP. This may help clinicians treating BP patients in decision-making regarding duration and intensity of treatment.

Keywords: bullous pemphigoid, BP180 ELISA, BP230 ELISA, eosinophil, IgE, predictive marker, relapse

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INTRODUCTION

Bullous pemphigoid (BP) presents with erythema plaques and tense blisters, and is one of the pemphigoid diseases occurring most frequently among autoimmune bullous diseases, with an especially high prevalence in the elderly. Immunologically, BP is characterized by presence of circulating anti-epidermal basement membrane zone (BMZ) antibodies, which are detected by indirect immunofluorescence (IF), and antibodies and/or complement deposition on BMZ, which

Abbreviations: BMZ, basement membrane zone; BP, bullous pemphigoid; ELISA, enzyme-linked immunosorbent assay; IF, immunofluorescence; IVIG, high-dose intravenous immunoglobulins; NC16A, non-collagenous 16A.

is detected by direct IF. The autoantigens in BMZ targeted by patient's antibodies are mainly BP180 (type XVII collagen) and BP230 (1). The immunodominant region of BP180 in BP is the 16th non-collagenous domain (NC16A) and anti-BP180NC16A IgG is related to disease activity (2), whereas anti-BP230 is not (3). Total serum IgE level is elevated in BP and anti-BP180NC16A IgE recently was reported to be associated with disease activity (4, 5). In the cytokine profile, serum levels of interleukin (IL)-5, IL-6, and IL-8 were elevated in BP and serum levels of IL-6, tumor necrosis factor (TNF)-α, IL-15 and chemokine (C-C motif) ligand 18 (CCL18) were associated with severity or disease activity (6–9). Blood eosinophilia is seen frequently, especially in severe cases (10). These biomarkers are helpful to evaluate efficacy of treatment and disease severity.

In terms of treatment, dapsone, immunosuppressants, such as azathioprine, cyclosporine, and methotrexate, and emerging therapies, including high-dose intravenous immunoglobulins (IVIG), immunoadsorption, rituximab, and omalizumab, are available, although systemic corticosteroid remains in main strategy for treatment (11). However, previous studies reported disease relapse frequently ranging from 29.2 to 39% during the first year (12, 13). To date, several studies have reported predictive factors of relapse, including BP180 enzyme-linked immunosorbent assay (ELISA) titer > 23 U/mL 150 days later at baseline (13) and a positive finding on direct IF and BP180 ELISA titer > 27 U/mL at cessation of therapy (14).

Prediction of relapse at initiation of treatment (baseline) must be more beneficial for clinicians. Therefore, extensive disease activity, defined as the occurrence of at least 10 new blisters daily, and association with dementia were found to be risk factors for relapse in multivariable analysis (14). However, prediction of relapse by biomarker at baseline in BP has not yet been fully analyzed. We evaluated biomarkers at baseline previously reported as being associated with disease activity in BP patients with and without relapse.

MATERIALS AND METHODS

Patients

Patients with BP who visited the Kurume University Hospital between October 2005 and May 2016 and were followed at our hospital for more than 1 year were enrolled in this study. To avoid treatment bias, patients treated with systemic corticosteroid and/or immunosuppressants at first visit to our hospital were excluded. A total of 47 patients were analyzed. Patients with BP were diagnosed based on the combination of clinical, histopathological, and immunological findings of circulating IgG reaction on the roof of 1 M NaCl-split-normal human skin, and/or positive finding on BP180 ELISA. Relapse was defined in this study when the dose of prednisolone was increased 1.5-fold (former dose >10 mg/day) or over 10 mg/day (former dose <10 mg/day) after the consolidation phase defined by Murrell et al. (15). Information on treatment and the number of peripheral eosinophils was obtained from the medical record. Regarding treatments, the present cases were primarily treated with systemic and topical corticosteroids. The intractable cases were treated with an additional immunosuppressant, pulse corticosteroid therapy with methylprednisolone, and double-filtration plasmapheresis. Only one case in non-relapse group was treated with IVIG. No patient was treated with either rituximab or omalizumab.

Enzyme-Linked Immunosorbent Assay (ELISA)

Circulating IgG to BP180NC16A and BP230 were detected using commercial kits (MESACUP BP180 and BP230 ELISA kits; MBL Co., Nagoya, Japan) and were used according to the manufacturer's instructions. Further dilution to 1:1,600 and/or 1:16,000 was performed for samples with an index >100 with 1:101 dilutions to obtain reliable index values (16–18). For total serum IgE, a human IgE ELISA quantification set (Bethyl Laboratories, Montgomery, TX, USA) was used according to the manufacturer's instructions.

Immunofluorescence (IF)

Direct IF was performed using perilesional skin specimens from each patient as described previously (19). Indirect IF using 1 M NaCl-split-normal human skin was performed as described previously (20, 21).

Receiver Operating Characteristic (ROC) Analysis

ROC analysis was performed on indexes of BP180 ELISA to assess predictive accuracy for relapse (ROC-AUC 0.68). The highest Youden Index (0.40) set the cutoff value at 90.07 U/mL, with 75.0% sensitivity and 64.5% specificity.

Statistical Analysis

Data were presented as mean ± SD. Statistical analysis of age, BP180 ELISA index, BP230 ELISA index, peripheral eosinophils, and serum IgE at baseline in the two groups was performed using a two-tailed Mann-Whitney *U*-test, and sex in the two groups was analyzed using the Fisher's exact test using GraphPad Prism (Version 6.05; GraphPad Software, San Diego, CA). A *P*-value of 0.05 was considered statistically significant.

RESULTS

In our study, 34% of the patients experienced relapse. The latency to relapse was 367 ± 285 days; 10 were in the first year, 5 were in the second year, and 1 was in the fourth year. BP180 ELISA indexes at baseline were 1138 ± 2161 U/mL, 459 ± 334 U/mL, and 375.2 U/mL, respectively. In the 16 patients with relapse, 4 were on "complete remission off therapy," 10 were on "Minimal therapy," and 2 were on "treatment with more than 0.1 mg/kg/day of prednisolone" before relapse.

We measured BP180 and BP230 ELISA indexes, and total serum IgE levels using patients' sera at baseline and compared the results in the two groups of patients with or without relapse

TABLE 1 | Characteristics of BP patients at baseline.

	Total ($n = 47$)	Non-relapse ($n = 31$)	Relapse ($n = 16$)	p-value
Sex ratio (female/male)	1.35	1.21	1.67	0.758
Age (years)	74.68 ± 13.00	73.55 ± 10.83	76.88 ± 16.59	0.157
BP180 ELISA index (U/mL)	550.3 ± 1273	381.2 ± 959.9	877.8 ± 1718	0.041
BP230 ELISA index (U/mL)	144.9 ± 283.3	145.7 ± 321.3	143.4 ± 199.2	0.303
Peripheral eosinophils (/μL)	1002 ± 1010	848.0 ± 872.9	1320 ± 1218	0.135
Serum IgE (ng/mL)	8907 ± 14483	6614 ± 9355	13350 ± 20887	0.176
POSITIVITIES To BP180/BP230 ELISA				
BP180 alone (n)	13 (27.7%)	8 (25.8%)	5 (31.3%)	
BP230 alone (n)	3 (6.4%)	2 (6.5%)	1 (6.3%)	
Double positive (n)	28 (59.6%)	18 (58.1%)	10 (62.5%)	
Double negative (n)	3 (6.4%)	3 (9.7%)	0 (0.0%)	

(**Table 1**). Of note, BP180 ELISA index was significantly higher in the relapse group (877.8 ± 1718 U/mL) than in the non-relapse group (381.2 ± 959.9 U/mL). On the other hand, sex ratio, age, BP230 ELISA index, peripheral eosinophils, and total serum IgE were not significantly different between the two groups, although serum IgE level was higher in the relapse group (13,350 ± 20,887 ng/mL) than in the non-relapse group (6614 ± 9355 ng/mL) ($p = 0.176$). To determine the cutoff value for prediction of relapse, a receiver operating characteristic (ROC) curve was obtained from BP180 ELISA indexes at baseline (ROC-AUC 0.68). The cutoff value set by the Youden Index was 90.07 U/mL, with 75.0% sensitivity and 64.5% specificity. To reduce false-negatives, we set the cutoff value at 53.09 U/ml with 81.3% sensitivity and 48.4% specificity (**Figure 1**). We also checked positive reaction to BP180 and BP230 by ELISA in our cases. The positive rates to BP180, BP230, and both combined were similar between the two groups (**Table 1**). However, of note, all three cases with negative reactivity in both ELISAs were from the non-relapse group, and none from the relapse group.

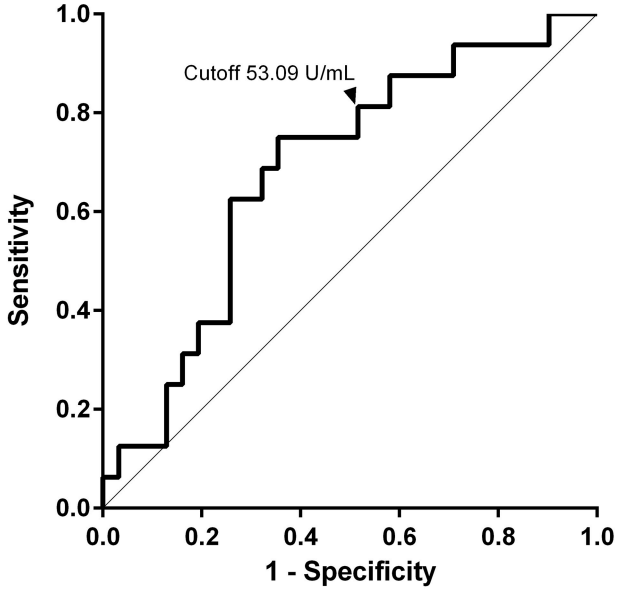

FIGURE 1 | Values of anti-BP180NC16A IgG at baseline for prediction of relapse. The ROC curve was used to evaluate the ability of BP180 ELISA index at baseline to predict relapse. The cutoff value was set at 53.09 U/ml with 81.3% sensitivity and 48.4% specificity.

DISCUSSION

We showed that the concentration of anti-BP180NC16A IgG antibodies in serum at baseline measured by BP180 ELISA was significantly higher in the relapse group than in the non-relapse group. This result suggested that a high BP180 ELISA index before starting treatment is a predictive marker for clinical relapse in later stages of BP. Fichel et al. (13) did not reveal significant differences at baseline in the BP180 ELISA indexes between patients with and without relapse in their retrospective analysis. The significance difference of BP180 ELISA index found in our study might result from proper dilution of serum for ELISA. ELISA must be performed using appropriate serum dilutions to demonstrate linear correlations between antibody concentration and index value. To be precise, with regard to the ELISA supplied from MBL Co., serum samples whose index is >100 must be tested with further dilutions (18). We performed further dilution in cases whose ELISA index exceeded 100. This is in accordance with previous reports showing that a high index of BP180 ELISA during the disease course or at cessation of therapy was predictive for relapse (13, 14). Our result suggested that an index value (53.09 U/ml) even at baseline was useful for prediction of clinical relapse. However, we also noted that some patients without relapse had a high index of BP180 ELISA at baseline and vice versa. It should be noted that a patient with a low BP180 ELISA index at baseline may possibly have relapse.

Bernard et al. (22). reported that a positive anti-BP180 detected by Western blotting was associated with poor prognosis. Although a high index of BP180 ELISA at baseline possibly is

related to disease outcome, we did not analyze prognosis in our study.

Our results showed relatively higher serum IgE levels in the relapse group, which may indicate a risk for relapse, although there was no significance in this study. A previous report also showed no statistically significant correlation between serum IgE levels and extent of the disease (23). Interestingly, all three patients with negative reaction to both BP180 and BP230 ELISA at baseline were in the non-relapse group. Negative reaction to BP180 and BP230 ELISA might be a predictive sign of non-relapse. Overall, our study contained a small number of samples, although the frequency of relapse (34%) in our study was similar to that reported previously in Europe (12, 13) and Asia (24). Therefore, further study with larger sample sizes will be required.

CONCLUSION

In this study, we reported a serological predictive marker at baseline for relapse in patients with BP. Our results indicated that higher index of BP180 ELISA (>53.09 U/mL) is a predictive marker for relapse at baseline. This may help clinicians treating BP patients in decision-making regarding duration and intensity of treatment.

ETHICS STATEMENT

This retrospective study was approved by the ethics committee of the Kurume University and was performed in accordance with the Declaration of Helsinki guidelines.

AUTHOR CONTRIBUTIONS

HK and KT: contributed to study concept and design; HK: wrote the manuscript; HK, NI, and CO: contributed to the acquisition, analysis, and interpretation of data; TN: supervised the study. All authors had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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ORIGINAL RESEARCH
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Direct Immunofluorescence Using Non-Lesional Buccal Mucosa in Mucous Membrane Pemphigoid

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Mucous membrane pemphigoid (MMP) is a rare organ-specific autoimmune subepithelial blistering disease with predominantly mucosal erosions, most frequently affecting the gingiva. Erosions in the oral cavity usually result in markedly decreased quality of life. The major autoantigens are BP180 and laminin332, which are components of basement membrane proteins in the skin and mucosa. Diagnosis is usually difficult due to histological destruction of the tissue and low autoantibody titers. In this study, we evaluated the diagnostic value of direct immunofluorescence (DIF) using non-lesional buccal mucosa in seven cases of MMP. In all seven patients, gingival lesions were clinically observed, and in one of the seven patients, buccal lesions were also clinically observed. First, we performed DIF to detect tissue-bound autoantibodies and complement. DIF from non-lesional buccal mucosa revealed linear deposits of IgG and C3 at the basement membrane zone in all cases. To detect autoantibodies, indirect immunofluorescence (IIF), BP180-NC16A ELISA and immunoblotting were performed. Surprisingly, circulating autoantibodies were unable to be detected in any of the cases by ELISA, IIF, or immunoblotting. Furthermore, histological separation was observed in one patient. In conclusion, DIF using non-lesional buccal mucosa was found to be superior to histological and serological tests for diagnosing mucous membrane pemphigoid. The procedure is technically easy and has high diagnostic value.

Keywords: autoimmune disease, direct immunofluorescence, mucous membrane pemphigoid, oral mucosa, autoantibody

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INTRODUCTION

The prevalence and incidence of autoimmune disorders are increasing, with many people suffering from such disorders. Autoimmune subepidermal blistering diseases, e.g., bullous pemphigoid, mucous membrane pemphigoid (MMP) and epidermolysis bullosa acquisita, are organ-specific autoimmune disorders that are characterized by autoantibodies to components of the skin basement membrane zone (BMZ) (1–4). Clinically, MMP shows predominant mucosal involvement, most frequently affecting the oral cavity, followed by the conjunctiva,

Abbreviations: MMP, mucous membrane pemphigoid; BMZ, basement membrane zone; IIF, indirect immunofluorescence; DIF, direct immunofluorescence.

the nasal cavity, and the esophagus (4). In the oral cavity, the gingiva is most commonly affected (70% of cases), followed by the buccal mucosa (60%), the palate (27%), and the tongue and lips (13%) (5). Histological analysis shows junctional separation at the BMZ (4, 6). In immunofluorescence microscopy, linear deposits of IgG and/or complement, and sometimes IgA at the BMZ, are characteristic (4, 7). Several autoantigens are involved in MMP, including BP180 (also called type XVII collagen), laminin332, integrin α6/β4 and type VII collagen, although BP180 and laminin332 are the major autoantigens (4, 7).

The diagnosis of MMP is confirmed based on the combination of clinical findings, histological analysis, and immunological findings. Immunological tests reveal tissue-bound autoantibodies by direct immunofluorescence and circulating autoantibodies by indirect immunofluorescence (IIF), ELISA, or immunoblotting (8). Circulating autoantibodies are frequently difficult to detect; several studies reported that the autoantibodies are detected in approximately 40% of cases (5, 9). By contrast, autoantibodies are detected in more than 80% of cases in bullous pemphigoid, which is an autoimmune subepidermal blistering disease in which BP180 is targeted (10, 11). This difference tends to be due to the low titers of the autoantibodies in MMP (4). Recently, we reported the usefulness of mucosal substrates to detect autoantibodies in MMP (12). Furthermore, histological study fails to show junctional separation because of tissue destruction in the fragile oral mucosa. For these reasons, it frequently takes time to make diagnose MMP and start treatment.

In cases that are difficult to diagnose, direct immunofluorescence (DIF) using the patient's tissue is a valuable test for diagnosing MMP. Although histological analyses generally should be performed on the affected lesions, DIF samples can be taken from perilesional areas in autoimmune blistering diseases (13). Therefore, we can get specimens in which the structure is maintained, so that we can evaluate the tissue-bound autoantibodies. We, here, report the usefulness of DIF on non-lesional buccal mucosa for diagnosing MMP.

MATERIALS AND METHODS

Patients

All the patients were referred to the dermatology department or to the oral medicine and diagnosis department of Hokkaido University Hospital. The patients demonstrated multiple erosions around the gingiva. DIF tests were performed on non-lesional buccal mucosa.

Patients were selected according to the following criteria: (1) clinically, MMP was suspected and (2) DIF was performed on non-lesional buccal mucosa.

The diagnostic criteria for MMP are as follows: (1) clinical findings of blisters and/or erosions, (2) linear deposits of IgG and/or C3 at the BMZ by DIF., and/or (3) circulating autoantibodies detected by IIF using normal human skin as a substrate, BP180-NC16A ELISA or immunoblotting using normal human epidermal extract.

Hematoxylin and Eosin (H&E) Staining

Hematoxylin and eosin staining was performed using paraffin embedded sections. After the sections were deparaffinized, specimens were stained with Mayer's hematoxylin (Muto, Tokyo, Japan) for 3 min. After being rinsed in distilled water, the specimens were stained with 1% eosin Y (Wako, Osaka, Japan) for 1 min, followed by dehydration with 99.5% ethanol.

Direct Immunofluorescence

The specimens were taken from non-lesional buccal mucosa using a 4-mm punch biopsy tool under local anesthesia (**Figures 1A,B**). The tissues were frozen on the dry ice, and 5-μm-thick sections were prepared by cryostat (Leicabiosystems, Tokyo, Japan). The sections were stained with FITC-conjugated goat anti-human IgG, IgA, IgM, and C3 (1:100, DakoCytomation, Glostrup, Denmark) for 45 min at 37°C.

Serological Tests to Detect Autoantibodies

Indirect immunofluorescence was performed on normal human skin. The sections were incubated with sera from dilutions of 1:10 to 1:320 for 45 min at 37°C, followed by incubation with 1:100 diluted FITC-conjugated anti-human IgG. Immunoblotting was performed to identify the autoantigens. Normal human epidermal extract was derived as described previously (14). The extracts were applied to 6% SDS-polyacrylamide gel and were then transferred to nitrocellulose membrane. The membrane was blocked for 1 h at room temperature. After incubation with 1:200 diluted sera overnight at

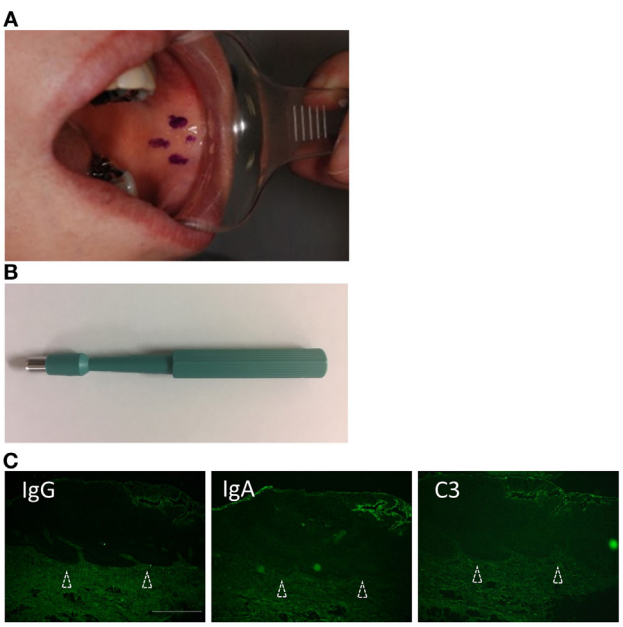

FIGURE 1 | Biopsy from non-lesional buccal mucosa. **(A)** Non-lesional buccal mucosa marked by crystal violet was biopsied under the local anesthesia. **(B)** The samples were taken using a 4-mm punch biopsy tool. No closing sutures were needed. **(C)** A healthy individual shows no evidence of IgG, C3, or IgA. Scale bar = 100 μm.

4°C, HRP-conjugated goat anti-human IgG (1:5,000 dilution, Life Technologies, Carlsbad, CA, USA) was reacted for 1 h at room temperature. The BP180-NC16A ELISA was performed using 1:100 diluted sera according to the manufacturer's instruction (MBL, Nagoya, Japan).

Statistical Analysis

The sensitivities of the diagnostic procedures were determined including 95% confidence interval (CI). Graph Pad PRISM software Version 7.0 was used to analyze the data.

RESULTS

Findings of Tissue-Bound IgG Taken from Normal Buccal Mucosa

We evaluated seven patients. The clinical findings are shown in **Figure 2** and **Table 1**. All of the patients had gingival lesions, and one patient (case 1) also showed erosions on the buccal mucosa. The mean duration between the initial symptoms and diagnosis was 2.3 years. Four patients received histological analysis, and one patient demonstrated dermal–epidermal separation (#3). In two

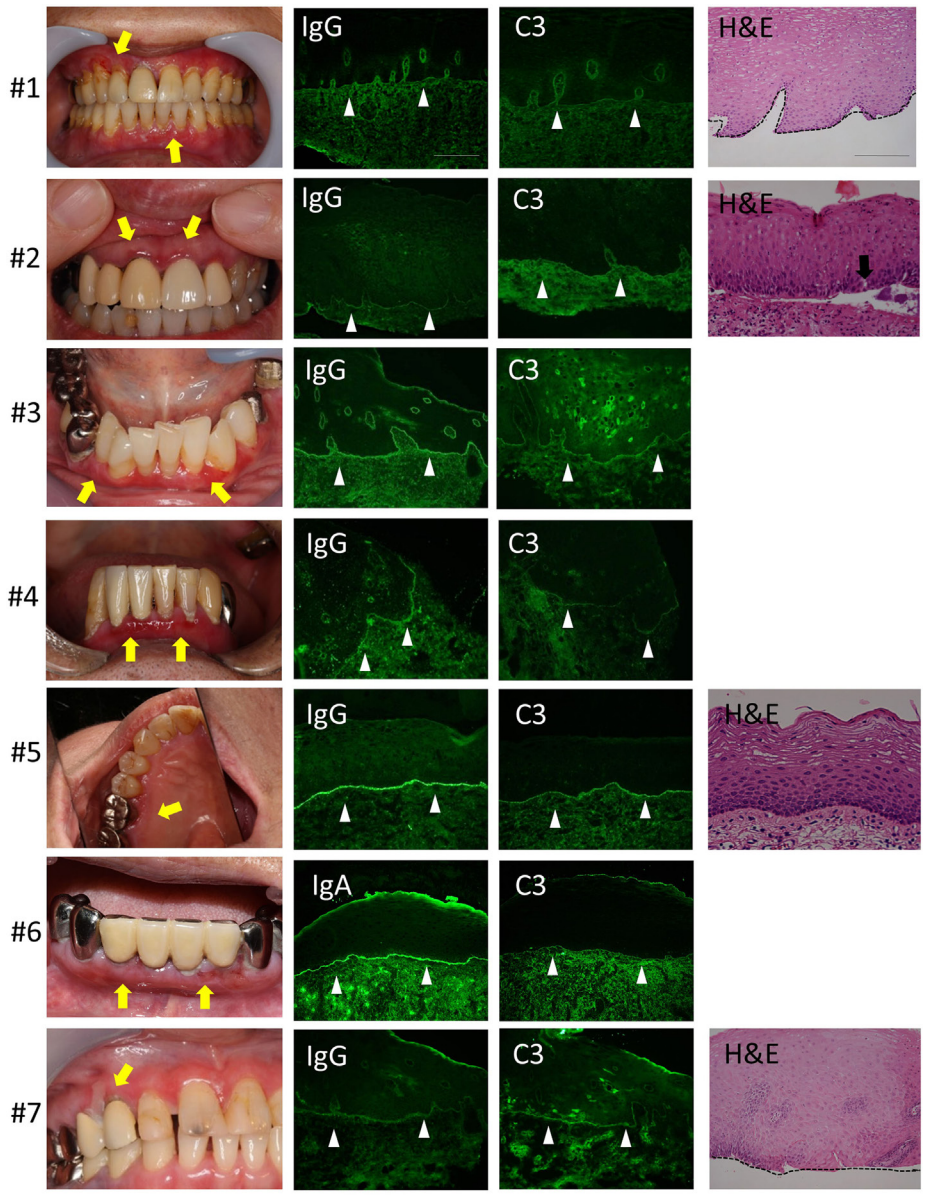

FIGURE 2 | Clinical, histological, and direct immunofluorescence (DIF) findings. All the patients have gingival lesions (yellow arrows). The biopsy samples for DIF were taken from non-lesional buccal mucosa, and all samples shows the linear deposition of IgG or IgA, and C3 at the basement membrane zone (BMZ) in all cases (white arrow heads). Histological analysis shows the junctional separation at the BMZ (#3) (black arrow). The epidermal and dermal tissue are completely separated because of tissue destruction (#1,7). There is no evident separation at the BMZ (#5). Scale bar = 100 μm.

TABLE 1 | Summary of clinical information.

Case	Age	Disease duration (years)	Skin	Oral cavity	H&E	DIF	IIF	BP180-NC16A ELISA	IB epidermal[a]
1	71–75	3	–	Gingiva, bucal, phalangeal	–	G, C3, A	–	–	–
2	51–55	0.75	–	Gingiva	ND	G, C3	–	–	–
3	66–70	3	–	Gingiva, soft plate	+	G, C3, A	–	–	–
4	76–80	2	–	Gingiva	ND	G, C3	–	–	–
5	71–75	5	–	Gingiva	–	G, C3, A	–	–	–
6	81–85	1	–	Gingiva	ND	G, C3, A	–	–	ND
7	61–65	1.5	–	Gingiva	–	G, C3	–	–	ND
Mean	70	2.3							

[a]*Immunoblotting using normal human epidermal extract.*

H&E, hematoxylin and eosin stain; DIF, direct immunofluorescence; IIF, indirect immunofluorescence; G, IgG; A, IgA; ND, No data.

patients, epidermal and dermal tissues are completely separated because of tissue destruction (#1,7). In the other one patient, there is no evident separation at the BMZ (#5) (sensitivity of H&E: 14.286%, 95% CI: 0.361–56.872). Neither IIF nor BP180-NC16A ELISA was able to detect any circulating autoantibodies (sensitivity of IIF or ELISA: 0%, 95% CI: 0–40.962). We took the DIF samples from the buccal mucosa, and these showed linear deposits of IgG or IgA, and C3 in all cases (sensitivity of DIF: 100%, 95% CI: 59.038–100) (**Figure 2**). By contrast, the healthy individual showed no evidence of IgG, C3, or IgA (**Figure 1C**).

DISCUSSION

Direct immunofluorescence showing tissue-bound antibodies is the strongest evidence for antibody-induced autoimmune diseases. Such immunofluorescence reveals deposits of immunoglobulins, complement components, and other protein substances in the patient's tissue by the use of fluorescence-labeled antibodies. DIF is performed when various autoimmune diseases as suspected, such as rheumatoid arthritis, lupus nephritis, autoimmune blistering diseases, and thyroid diseases (15, 16). Although the samples are usually taken from affected lesions, it is possible to detect deposits of autoantibodies and complements in perilesional areas in cases of autoimmune blistering diseases (13). This has the major advantage of maintaining the structure. MMP frequently involves the gingiva and only rarely involves the buccal mucosa. Recently, one study showed the usefulness of DIF using unaffected oral mucosa in MMP (17). However, they did not describe the biopsy regions on the oral mucosa. We selected non-lesional buccal mucosa due to easy access. Biopsying the gingiva is technically more difficult than biopsying the buccal mucosa. In all seven cases, tissue-bound autoantibodies and complements were clearly detected by DIF using non-lesional mucosa (sensitivity: 100%, 95% CI: 59.038–100).

Serological tests are technically easier to perform than histological examinations and DIF, which involve surgical procedures. ELISA has particularly high sensitivity and quickly determines the autoantigens and titers of autoantibodies. Therefore, it can be widely used for most autoimmune diseases. However, the biggest problem with ELISA is the limitation of autoantigens: Recombinant targeting proteins are required for ELISA tests. MMP has multiple autoantigens, and ELISA tests are available for only a few of them. For example, BP180 is one of targeted autoantigen by MMP autoantibodies. However, previous reports demonstrate that 30–42% of MMP autoantibodies are detected by BP180-NC16A ELISA (5, 12, 18). To overcome this problem, Izumi et al. reported the usefulness of recombinant full-length BP180 ELISA (18). The full-length BP180 ELISA detects autoantibodies not only to the NC16A domain but also to parts of BP180 outside of the NC16A domain. The sensitivity is increased from 42% in the BP180-NC16A ELISA into 75% in the full-length BP180 ELISA. Immunoblotting using normal human skin extract or IIF using normal human skin cover greater ranges of antigens. However, these tests are less sensitive than ELISA, and many of the autoantibodies are rarely detected. Indeed, none of the autoantibodies were detected by IIF or immunoblotting in this study (sensitivity: 0%, 95% CI: 0–40.962). Because of these diagnostic difficulties, it frequently takes time to diagnose and start treatments for most MMP patients. Moreover, the diagnosis delay was reported to be site specific. The oral mucosal lesions are thought to be more difficult to detect than the skin lesions such as those on limbs or trunk (19). The average time between initial symptom and diagnosis was more than 2 years in our study.

In conclusion, to confirm the diagnosis of MMP, we highly recommend DIF using non-lesional buccal mucosa. The procedure is technically easy and has high diagnostic value.

ETHICS STATEMENT

All studies conformed to the guidelines of the medical ethics committee of Hokkaido University and the Declaration of Helsinki Principles. Written informed consent was obtained before any samples were collected. A full review and approval by an ethics committee of Hokkaido University were not required, according to local guidelines.

AUTHOR CONTRIBUTIONS

MK and JS participated in data generation and analysis, and MK and HI wrote the paper. MK, HI, HU, IU, and YK contributed samples and clinical data. HI, HU, YK, and HS supervised the study. All the authors had final approval of the submission.

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