AUTOIMMUNE BLISTERING DISEASES

EDITED BY: Cezary Kowalewski, Takashi Hashimoto and Pascal Joly
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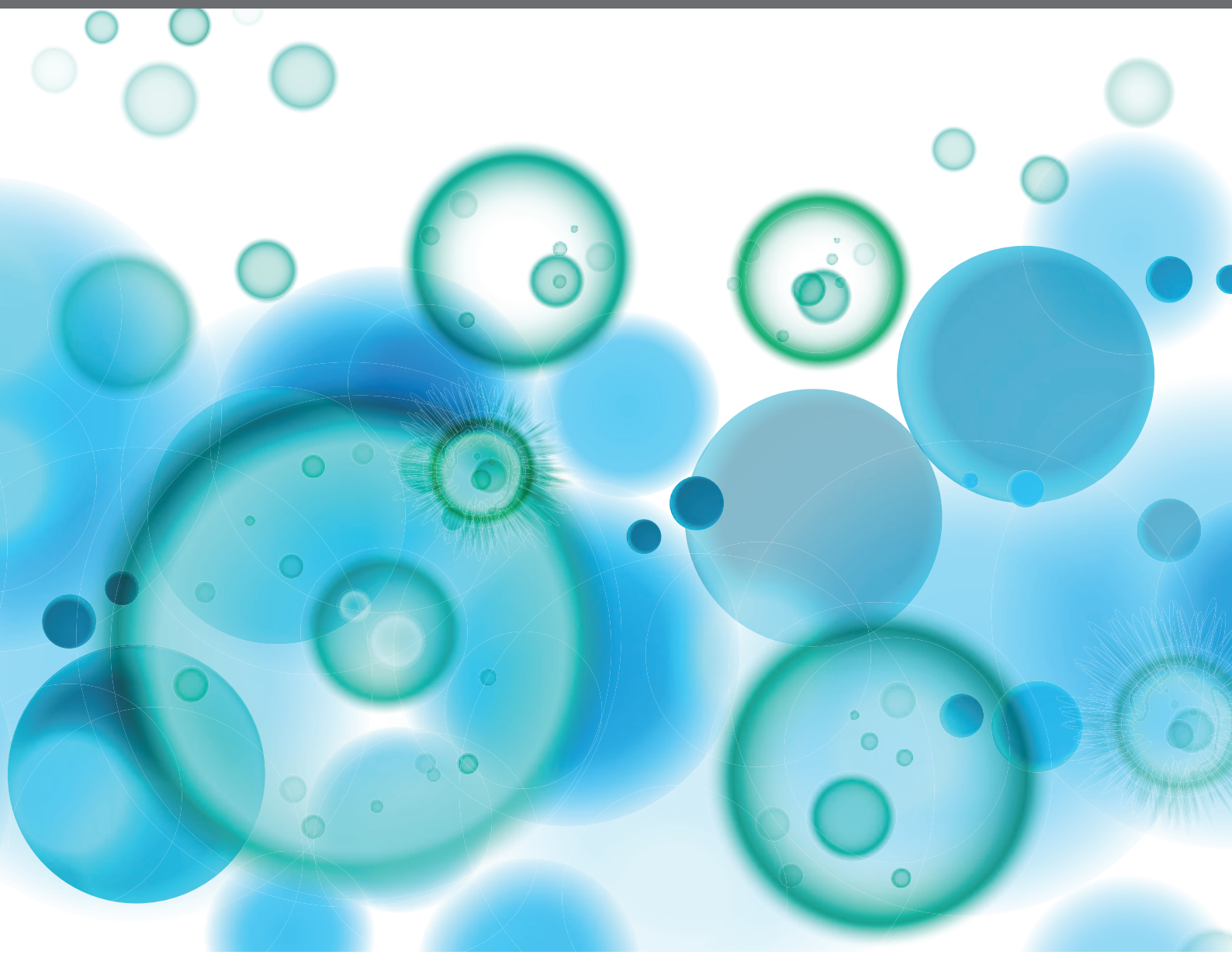

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AUTOIMMUNE BLISTERING DISEASES

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EDITORIAL
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Editorial: Autoimmune Blistering Diseases

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Editorial on the Research Topic

Autoimmune Blistering Diseases

Autoimmune blistering skin diseases are an extremely fascinating field of research. In recent years, most of the antigens recognized by autoantibodies in pemphigus and pemphigoid diseases have been characterized, and the pathomechanism of these diseases has been largely understood. However, several issues still need to be clarified. The content of this Research Topic consists of a series of original researches, review articles, and case reports, which expand our knowledge for the pathogenesis and new treatment strategies.

Di Lullo et al. identified a novel target antigen in pemphigus vulgaris (PV). Applying a method of the efficient immortalization of IgG+ memory B cells, the authors isolated human monoclonal antibody reactive with a non-Dsg antigen. Immunoprecipitation and immunoblotting studies on keratinocyte extracts identified α-catenin as the putative antigen, which was further confirmed by immunoblotting on the recombinant protein. The role of anti-α-catenin antibody in the mechanism of blister formation in pemphigus should be further analyzed.

In contrast to anti-α-catenin antibody, the pathogenic role of autoantibodies to desmogleins (Dsgs) and desmocollins (Dscs), desmosomal cadherins, are well-established. However, the precise mechanism of acantholysis is only partly understood. It is known that binding of pemphigus autoantibodies to Dsg3 leads to gross morphological changes in keratinocytes due to internalization of desmosomal proteins and keratin retraction. These structural alterations induced by pemphigus antibodies are mediated by intracellular signaling events via various molecules, such as p38 mitogen-activated protein kinase (p38MAPK) and extracellular-signal regulated kinase (ERK). Signaling mechanisms increase cytokine secretion and phosphorylation of structural proteins, which eventually lead to the loss of cell adhesion. It has been reported that single nucleotide polymorphism in the promoter region of the gene encoding suppression of tumorogenicity 18

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(ST18) causes ST18 up-regulation, increase in cytokine secretion and more prominent loss of keratinocyte cohesion induced by pemphigus antibodies. Radeva et al. studied the effects of pemphigus anti-Dsg3 antibodies on cytokine secretion and ERK activity in human keratinocytes transfected by ST18 construct. Without ST18 overexpression, both human and mouse pemphigus antibodies induced loss of keratinocyte cohesion and fragmentation of Dsg3 along cell borders, but the release of pro-inflammatory cytokines was not altered significantly. This result indicates that cytokine expression is not necessarily required for loss of keratinocyte cohesion in this disease model. Upon ST18 overexpression, fragmentation of cell monolayers increased significantly as well as production of IL-1α and IL-6. These results partially explain ethnic susceptibility and familial occurrence of PV described in the previous reports, and provide an evidence for a genetic predisposition to PV.

Oktarina et al. studied the fate of desmosomal proteins in the biopsies taken from perilesional skin of pemphigus foliaceus (PF) patients by double immunofluorescence staining method using battery of antibodies directed to different epitopes of the desmosomal proteins, Dsg1, Dsg3, Dsc1, Dsc3, plakoglobin (PG), desmoplakin, and plakophilin 3, as well as markers of lysosomes and endosomes. This study showed that staining for either cathepsin D or LAMP-1 did not overlap with Dsg1 or PG, suggesting that lysosomes have no role in the clearing process. The co-localization of Dgs1, plakoglobin and early endosomal antigen 1 suggested that endocytosis is part of the pathogenic process in PF.

French Bullous Disease Research Group for the first time showed that rituximab was more effective than a standard oral corticosteroid treatment in pemphigus, and that Dsg-specific-B cells (Dsg-positive B cells) were still detectable during the B cell recovery even in patients in clinical remission. In the article by Hébert et al. the authors characterized Dsg-positive B cells in patients in clinical remission after rituximab or corticosteroid treatment in comparison to those at baselines in the patients' active stages. They studied the expression profiles of 31 genes related to inflammatory cytokines, TNF receptors, and activation markers. At baseline, the patients' autoreactive B cells showed a significantly higher expression of genes encoding pro-inflammatory cytokines than non-autoreactive B cells. The gene expression profiles of Dsg-positive B cells collected from patients in clinical remission after rituximab treatment were similar to those of Dsg-positive B cells at baseline, except for lower expressions of the IL-1β and the CD27 memory marker genes. This study showed that the gene expression profiles in Dsg-positive autoreactive B cells are different from those in non-autoreactive B cells, and that rituximab and corticosteroids have different effects on the gene expression in autoreactive Dsg-positive B cells in pemphigus patients.

In addition to the above-mentioned original researches on the pathogenesis of pemphigus, there are two interesting case reports by Schauer et al. on radiation-associated pemphigus and by Solimani et al. on thymoma-associated paraneoplastic pemphigus.

In the review article by Didona et al., the authors presented current state of knowledge on etiopathogenesis, diagnostics, and therapeutic strategies in mild and severe form of pemphigus. Authors analyzed efficacy of corticosteroids, several immunosuppressants, intravenous immunoglobulin (IVIg), immunoadsorption and rituximab, and proposed algorithms for the induction and maintenance therapies, and therapy for relapse. In this review, the authors also presented future perspectives of pemphigus management, including the promising treatment approach using chimeric autoantibody receptor (CAAR)-T cell.

In the review by Izumi et al., the authors presented several promising ongoing clinical trials on pemphigus. These include rituximab, a novel defucosylated human IgG1 monoclonal antibody to BAFF receptor (VAY736) which depletes B cells, monoclonal antibody to neonatal Fc receptor which reduces circulating IgG antibody level, a Bruton's tyrosine kinase inhibitor (RN1008) which inhibits B cell receptor signaling and reduces B cell activation in autoimmunity, and polyclonal regulatory T cells (PolyTregs). The authors also summarized the ongoing clinical trials on bullous pemphigoid (BP) patients resistant to standard therapies, including IVIg, rituximab, a recombinant fully humanized monoclonal antibody targeting IL-17 (ixekizumab), a fully human monoclonal antibody targeting eotaxin-1 (bertilimumab) which impairs eosinophil infiltration into the skin.

The role of IL-17 and IL-23 for neutrophilic and eosinophilic infiltrations in the BP relapse was studied by Giusti et al..

A Japanese group previously reported that autoantibodies in BP induced by dipeptidyl peptidase-IV inhibitors (DPP4i), a widely used antihyperglycemic drugs, tend to target epitopes on non-NC16A regions of BP180. In the article by Mai et al. of the same group, the authors examined BP180 epitopes using various domain-specific recombinant proteins and plasmin-digested recombinant BP180 for 18 sera of DPP4i-induced BP targeting the non-NC16A domain. They showed that IgG1-class autoantibodies targeting epitopes on the processed extracellular domain of BP180, i.e., LABD97.

In the article by Ujiie et al. of the same group, the authors studied the humoral immune response to intra- and extracellular epitopes of BP180 using an active BP mouse model, in which BP is induced by the adoptive transfer of spleen cells from wild-type mice immunized with human BP180-expressing skin grafting to immunodeficient BP180-humanized (Rag-$2^{-/-}$, mouse $Col17^{-/-}$, human $COL17^{+}$) mice. Authors found that the immune response to the extracellular domain epitopes of human BP180, particularly the NC16*A* domain, triggered intramolecular epitope spreading to intercellular epitopes of human BP180 and intermolecular epitope spreading to murine BP230.

Tie et al. studied the mechanism of blister formation in BP using keratinocyte culture stimulated with IgG anti-BP180 antibodies in patient sera. Morphological and functional changes were evaluated by electron microscopy and a battery of laboratory methods. The authors found alterations in the

cell membrane structure and accumulation of intracellular vesicles. These morphological changes in the treated cells were accompanied by dysfunctional mitochondria, increased production of reactive oxygen species, increased motility, and cell detachment. These cellular alterations are reversed by pharmacological inhibitors of Rac1 or proteasome pathway molecule, suggesting that Rac1 and proteasome activation are involved in the effects of BP antibodies on cultured keratinocytes.

We hope that this Research Topic on autoimmune blistering skin diseases will be of interest to the scientific community. The articles published in this issue should expand our knowledge about the pathogenesis of these diseases, leading to development of new effective treatments.

AUTHOR CONTRIBUTIONS

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

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

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ORIGINAL RESEARCH
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Bullous Pemphigoid IgG Induces Cell Dysfunction and Enhances the Motility of Epidermal Keratinocytes via Rac1/Proteasome Activation

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Bullous pemphigoid (BP) is an autoimmune disease characterized by the formation of blisters, in which autoantibodies mainly target type XVII collagen (ColXVII) expressed in basal keratinocytes. BP IgG is known to induce the internalization of ColXVII from the plasma membrane of keratinocytes through macropinocytosis. However, the cellular dynamics following ColXVII internalization have not been completely elucidated. BP IgG exerts a precise effect on cultured keratinocytes, and the morphological/functional changes in BP IgG-stimulated cells lead to the subepidermal blistering associated with BP pathogenesis. Based on the electron microscopy examination, BP IgG-stimulated cells exhibit alterations in the cell membrane structure and the accumulation of intracellular vesicles. These morphological changes in the BP IgG-stimulated cells are accompanied by dysfunctional mitochondria, increased production of reactive oxygen species, increased motility, and detachment. BP IgG triggers the cascade leading to metabolic impairments and stimulates cell migration in the treated keratinocytes. These cellular alterations are reversed by pharmacological inhibitors of Rac1 or the proteasome pathway, suggesting that Rac1 and proteasome activation are involved in the effects of BP IgG on cultured keratinocytes. Our study highlights the role of keratinocyte kinetics in the direct functions of IgG in patients with BP.

Keywords: bullous pemphigoid, keratinocyte, IgG, cell adhesion, cell migration

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INTRODUCTION

Bullous pemphigoid (BP) is a skin-specific autoimmune disease characterized by subepidermal blisters (1–4). Systemic glucocorticoids and immunosuppressive agents are effective treatment options for BP (5, 6), but they increase the risk of lethal infections, particularly in elderly patients (7, 8). The autoimmune mechanisms involved in BP have been well-discussed based on clinical and experiential evidence (9, 10). IgG is the principal antibody involved, which recognizes the non-collagenous 16a domain (NC16a) of type XVII collagen (ColXVII, also known as BP180 or BPAg2) (9, 11–14).

ColXVII, a transmembrane protein, is located either at the core of the hemidesmosome in the skin (1) or in the hemidesmosome protein complexes of cultured keratinocytes (15, 16). ColXVII contains a long extracellular collagenous tail, which traverses the basement membrane zone (BMZ) (17), and serves as a signaling connector and/or a "mucilage" between the epidermis and the dermis (18–21). A genetic deficiency in ColXVII leads to junctional epidermolysis bullosa, resulting in the separation of the epidermis from the dermis (22, 23). Binding of the specific IgG to ColXVII is considered to contribute to intra-lamina lucida blistering in subjects with BP (24–26).

ColXVII-NC16a is a juxtamembrane region that plays a central role in the formation of the collagen-like-triple helix (27, 28). Moreover, IgG binding to ColXVII-NC16a is the most essential initial event in BP, and detection of the ColXVII-NC16a-specific IgG is important for diagnosing BP (10, 29, 30). The pathological progress of blistering after BP IgG binding to ColXVII-NC16a remains a debatable issue. Previously, complement activation was considered a major cause of blistering, because immunofluorescence studies of skin biopsies from patients with BP have shown that most patients with BP exhibit C3 deposition. Moreover, according to laboratory investigations, the binding of IgG to murine ColXVII triggers complement activation, mast cell degranulation, and neutrophil infiltration, suggesting that the formation of blistering lesions require both IgG and the recruitment and activation of complement (31–35). Recently, another BP pathomechanism has been advocated in which IgG exerts a direct effect on inducing blistering in patients with BP (36, 37). Evidence supporting these direct effects of BP IgG has been reported. Immunofluorescence studies have revealed that >10% BP cases are positive for BP IgG but negative for C3 deposition (31, 35). Based on the findings from some case reports, IgG staining shows an intercellular pattern in basal cells rather than a linear deposition along the BMZ (38). Clinical observations identified a non-inflammatory BP variant that is often associated with dipeptidyl peptidase-4 inhibitors application (39). BP IgG has been reported to directly induce blisters between the epidermis and the dermis without complement activation (40, 41), influence cell morphology (42), and "deplete" ColXVII in cell culture systems (37). The binding of BP IgG to ColXVII has also been reported to cause internalization of the immune complex by forming macropinosomes, resulting in decreased cell adhesion at the single cell level (43).

Abbreviations: BP, bullous pemphigoid; BMZ, basement membrane zone; BSA, bovine serum albumin; CLEIA, chemiluminescent enzyme immunoassay; ColXVII, type XVII collagen; DAPI, 4′, 6-diamidino-2-phenylindole; DCFHDA, 2′,7′-dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; DPBS, dulbecco's phosphate-buffered saline; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; GTPases, guanosine triphosphate phosphohydrolases; h, hour; IgG, immunoglobulin G; MG132, carbobenzoxy-Leu-Leu-leucinal; min, minutes; NC16a, noncollagenous 16a domain; NHEKs, normal human epidermal keratinocytes; NSC23766, n6-[2-(4-diethylamino-1-methyl-butylamino)-6-methyl-pyrimidin-4-yl]-2-methyl-quinoline-4,6-diamine trihydrochloride; Rac1, ras-related C3 botulinum toxin substrate 1; ROS, reactive oxygen species; RT, room temperature; SEM, scanning electron microscope; TBHP, tert-butyl hydroperoxide; TEM, transmission electron microscope; QY-1, n-butyl glycidyl ether; $\Delta\Psi$m, mitochondria membrane potential.

Although our understanding of the mechanism by which BP IgG induces blistering at the single-cell-level has improved, knowledge regarding macropinosome formation in the pathogenesis of BP is still lacking. A macropinosome is a large endocytic vacuole formed during macropinocytosis (44). Macropinosome formation acts as an entry site for intracellular pathogens (e.g., bacteria) that helps cells recognize the antigens/microbe-associated molecules (45, 46) and accumulate the metabolites required for proliferation (e.g., cancer cells) (47). However, hyperstimulation of macropinosomes leads to cell death (48, 49). Thus far, the changes in the morphology of keratinocytes during BP IgG-induced macropinosome formation are incompletely understood. We hypothesized that BP IgG contributes to the pathogenesis of BP by inducing keratinocyte dysfunction. The aim of this study was to clarify the morphological and functional changes in human keratinocytes incubated with BP IgG and the subsequent effects of inhibitors of macropinosome formation on these morphological and functional events.

MATERIALS AND METHODS

Cell Culture

Normal human epidermal keratinocytes (NHEKs) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in serum-free media (with calcium chloride at a final concentration of 0.09 mM, Defined Keratinocyte SFM supplemented with growth factors, Gibco, Invitrogen Corporation, Carlsbad, CA, USA), 1% penicillin-streptomycin (Wako Pure Chemical Industries, Japan), and 25 ng/ml amphotericin B (Wako Pure Chemical Industries) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were used after two to six passages in all experiments.

Antibody Purification

Plasma samples were obtained from three patients with BP (BP-1, BP-2, and BP-3), and eluents were obtained by a double filtration plasmapheresis treatment. BP IgGs fractions were isolated from plasma using a HiTrap Protein G HP column (GE Healthcare, Marlborough, MA, USA) with a fast protein liquid chromatography system (Amersham Biosciences, Marlborough, MA, USA), according to the manufacturer's protocol. The functions of BP IgGs were confirmed using indirect immunofluorescence staining of the salt-spilt human skin and a ColXVII-NC16a chemiluminescent enzyme immunoassay test (CLEIA, SRL Inc., Hachioji, Japan). Immunoblotting using the normal human epidermal extracts confirmed that the BP IgGs used in this study only reacted with ColXVII (180 kDa) (data not shown) and not with other antigens. The purified BP IgGs were concentrated through extensive washed with 0.9% NaCl and 50 K ultrafiltration (Millipore, Lexington, MA), and then filter-sterilized (pore size 0.22 μm; Millipore). The pooled IgGs from healthy people (normal IgG, Kenketsu Glovenin I, Nihon Pharmaceutical Co. Ltd., Tokyo, Japan) were dissolved in 0.9% NaCl to 50 mg/ml. Normal rabbit IgGs were purified using a Pierce classic IP kit (Thermo Fisher Scientific, MA, USA) with normal rabbit serum (ab7487, Abcam, Cambridge, UK). All IgGs

protein concentrations were measured spectrophotometrically at 280 nm and samples were stored at −20°C.

This study was approved by the Ethics Committee of Shimane University and the Dean of the Faculty of Medicine (approval nos. 1746 and 2679).

Reagents

Carbobenzoxy-Leu-Leu-leucinal (MG132), cytochalasin D, and n6-[2-(4-diethylamino-1-methyl-butylamino)-6-methyl-pyrimidin-4-yl]-2-methyl-quinoline-4,6-diamine trihydrochloride (NSC23766) were purchased from Sigma (St. Louis, MO, USA). MG132 and cytochalasin D were dissolved in DMSO to generate 10 and 2 mM stock solutions, respectively. NSC23766 was dissolved in distilled water to generate a 10 mM stock solution. All inhibitor stock solutions were frozen at −20°C. For the application of inhibitors to cell cultures, all reagents were diluted in warm culture medium at the indicated concentrations. NHEKs were pretreated with cytochalasin D for 30 min, NSC23766 for 1 h, or MG132 for 0 h, and then IgGs were added prior to the analysis.

Flow Cytometry Analyses

Upon reaching ∼60% confluence, NHEKs were treated with 2 mg/ml IgGs obtained from patients with BP (BP IgGs) or normal IgG. After 0, 0.25, 0.5, 1, 2, and 6 h incubations, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS). Non-trypsin detached cells were immediately fixed with 4% paraformaldehyde and resuspended in DPBS. Cells were first incubated with a monoclonal rabbit IgG specific for human ColXVII-COOH (ColXVII IgG, ab184996, Abcam) and then incubated with anti-rabbit IgG-Alexa488 (ab150077, Abcam) to analyze the cell surface ColXVII expression. Cells were directly incubated with anti-human IgG-FITC (Dako, Copenhagen, Denmark) and examined using flow cytometry to determine the amount of IgG bound to the cell surface.

Immunostaining Assay

NHEKs were seeded on sterile glass coverslips at a density of 0.2×10^6 cells/ml, and after a 16 h incubation, NHEKs were pretreated with or without cytochalasin D, NSC23766, or MG132, and incubated with BP IgGs (2 mg/ml), normal IgG (2 mg/ml), or ColXVII IgG (12 μg/ml) for 0, 0.5, or 2 h. Afterwards, the cells were washed with DPBS, fixed with cold 4% (w/v) paraformaldehyde for 10 min, permeabilized and then blocked with 0.3 M (w/v) glycine/1% (w/v) BSA/10% (v/v) normal rabbit serum/0.1% (v/v) Tween-20 in DPBS for 1 h at room temperature (RT). NHEKs that had been treated with IgGs for 0 h were further incubated with BP IgGs (2 mg/ml) or normal IgG (2 mg/ml) for 1 h at RT or incubated with ColXVII IgG (12 μg/ml) overnight at 4°C. Cells were then washed three times with DPBS. All cells treated with IgGs were subsequently incubated with Alexa488- or FITC-conjugated secondary antibodies and rabbit or human IgG for 1 h at RT. The nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific). Cells on the coverslips were washed, mounted in mounting medium (Vector Laboratories, Burlingame, CA, USA), and viewed under a confocal scanning laser microscope (FV-100V, Olympus, Tokyo, Japan).

For the 3D reconstruction assay, a series of z-planes captured at 0.38 μm intervals were imaged with an Olympus FV-100V confocal microscope after staining. The 2D z-stack images from each channel were projected onto one 3D plane using the Olympus FV-100V software 3D view function. A red box indicated projected z-stack images, a yellow boxed region indicated one frame of a z-stack image, and a cut-off XZ or XY-section was shown in a yellow slice to better display the staining.

Quantification of the fluorescence intensity was performed using ICY software (icy.bioimageanalysis.org).

Cell Counting Assay

NHEKs were cultured in 96-well culture plates to 40–60% confluence in the presence or absence of cytochalasin D, NSC23766, or MG132. Cells were labeled with Hoechst 33342 (Thermo Fisher Scientific) and treated with BP IgGs (2 mg/ml), normal IgG (2 mg/ml), ColXVII IgG (12 μg/ml), or normal rabbit IgG (2 mg/ml). Live cell imaging was performed with an In Cell analyzer 2000 (GE Biosciences, Piscataway, NJ, USA) equipped with a ×20 objective. After a 6 h incubation with IgGs, the numbers of adherent cells in a ×20 area (0.57 mm^2) were automatically counted every hour with the In Cell Analyzer 1000 Workstation software (GE Biosciences) using the cell count analysis module.

Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)

NHEKs were incubated with BP IgGs (2 mg/ml) or normal IgG (2 mg/ml). Cells grown on four-well culture slides to 60% confluence were observed with TEM and the cells grown on 15 mm round cover-slips to 60% confluence were observed with SEM. For TEM, after the incubation with the indicated IgGs, cells were washed and immediately fixed with 2.5% (v/v) glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (pH 7.4, Wako Pure Chemical Industries) for 2 h. Cells were then washed and postfixed with 1% (v/v) osmium tetroxide for 1 h and 1% (v/v) uranyl acetate for 1 h at 4°C in the dark. Dehydration was achieved in a graded series of ethanol solutions. Dehydrated cells were cleared in n-butyl glycidyl ether (QY-1), embedded in Epon (TAAB Epon 812 Resin, Berkshire, England), and cut into ultrathin sections at a thickness of 80 nm using an ultramicrotome. Sections were collected on a 300-mesh copper grid, and ultrathin sections were double stained with uranyl acetate and lead citrate. For SEM, after the incubation with the indicated IgGs, the debris were removed from the culture by gentle washes with sterile DPBS. Cells were fixed with 2.5% (v/v) glutaraldehyde for 2 h at 4°C and washed with a 0.1 M cacodylate buffer (pH 7.2) solution. Cells were postfixed with 1% (v/v) osmium tetroxide for 1 h at 4°C, stained with 1% (w/v) tannic acid for 1 h at 4°C in the dark, and then fixed with 1% (v/v) osmium tetroxide for 1 h at 4°C. Cells were subsequently dehydrated in an increasing gradient of ethanol solutions, critical point dried in liquid CO_2, mounted on aluminum stubs with the cell layer facing up, and then coated with gold. Cells were

observed with a JSM-6510 scanning electron microscope (JEOL Co., Ltd., Tokyo, Japan) at a 10 kV accelerating voltage.

LysoTracker® Green DND-26 Staining Assay

NHEKs seeded on a 35 mm dish (Ibidi GmbH, Martinsried, Germany) at ∼40% confluence were treated with BP IgGs (2 mg/ml) or normal IgG (2 mg/ml) for 2 h. Cells were stained with 50 nM Lysotracker® Green DND-26 (Thermo Fisher Scientific) for 15 min in the dark and the fluorescence of lysosomes was immediately detected using confocal microscopy (Olympus FV-1000). Images were quantified using CellProfiler image analysis software (cellprofiler.org) with the spot detection pipeline.

Analysis of Cell Morphology

NHEKs cultured in 96-well plate to 60% confluence were stained with 100 nM MitoTracker® Red CMXRos (Thermo Fisher Scientific) and 5 μg/ml Hoechst 33342. BP IgGs (2 mg/ml) or normal IgG (2 mg/ml) were then added to the culture, which was immediately imaged. Nuclear masks were generated from images of Hoechst 33342 staining at 0 and 2 h, and the found edge and overlay functions of ImageJ software were employed to identify enlarged cell nuclei. The cytoplasm was visualized by overlaying the images of MitoTracker® Red CMXRos staining with nuclear staining. The whole-cell area and the area of typical macropinosomes was determined in phase-contrast images, and the numbers of total observed cells and the cells that formed macropinosomes were counted using ImageJ software (imagej.nih.gov/ij/). For the cell size calculation, nuclear staining was used to mark each individual cell, and then phase-contrast images matched to nuclear channel images were employed to calculate the size of each cell using In Cell Analyzer 1000 Workstation software with the multitarget analysis module. The cell size was calculated from the sum of pixel areas for each cell based on the pixel sizes of the images (2.73 pixels/μm).

NHEKs were treated with BP-1 IgG (2 mg/ml) or normal IgG (2 mg/ml) and imaged to identify the rupture of the plasma membrane. Bright field images of cells were obtained at 10 min intervals up to 16 h. The found edge and overlay functions of ImageJ software were employed to analyze the images.

MitoTracker® Red CMXRos Staining Assay

NHEKs cultured in 96-well plates to 60% confluence were labeled with MitoTracker® Red CMXRos and Hoechst 33342 and treated with BP IgGs (2 mg/ml) or normal IgG (2 mg/ml) for 16 h to calculate the relative signal intensities and numbers of MitoTracker® Red CMXRos fluorescent dots per cell. Cells were fixed with cold 4% (w/v) paraformaldehyde for 10 min, and images of the cells were captured using an In Cell analyzer 2000 equipped with a ×20 objective (GE Biosciences). Images were quantified using CellProfiler image analysis software with the spot detection pipeline.

Cellular Reactive Oxygen Species (ROS) Assay

Intracellular ROS production in NHEKs was measured using the fluorescent dye 2′,7′-dichlorofluorescein diacetate (DCFHDA, Abcam) according to the manufacturer's instructions. Cells cultured in a 96-well plate to 60% confluence were incubated with DCFHDA for 45 min at 37°C in the dark, and fresh medium containing BP IgGs (2 mg/ml) or normal IgG (2 mg/ml) were added and cultured for 6 h at 37°C. The fluorescence intensity was determined using a fluorescence microplate reader (DTX880, Beckman Coulter, Brea, CA, USA). The relative ROS fluorescence intensity was calculated as follows: [T_{6h} (fluorescence intensity at 6 h)–T_{0h} (fluorescence intensity at 0 h)]/numbers of adherent cells measured by cell counting.

Mitochondrial Membrane Potential Assay

The mitochondrial membrane potential was measured using a JC-1 mitochondrial membrane potential assay kit (Mitosciences, Abcam) according to the manufacturer's instructions. NHEKs cultured in a 96-well culture plate to 60% confluence were stained with the JC-1 dye and incubated with BP IgGs (2 mg/ml) or normal IgG (2 mg/ml) for 20 h. The fluorescence intensity was analyzed using a fluorescence microplate reader (DTX880).

Analysis of C_{12}-Resazurin/SYTOX Green Staining

A C_{12}-resazurin/SYTOX green kit (Molecular Probes, Eugene, OR, USA) was used to visualize the cell viability according to the manufacturer's instructions. Briefly, NHEKs cultured to 60% confluence in T-25 flasks in the presence or absence of inhibitors were incubated with BP IgGs (2 mg/ml) or normal IgG (2 mg/ml) for 16 h. Cells were harvested with the TrypLE Express Enzyme (Gibco) and resuspended in 100 μl of DPBS containing 1 μM SYTOX green and 50 nM C_{12}-resazurin. After a 15 min incubation in the dark, 400 μL of DPBS were added. The cell suspension was mixed gently and then immediately subjected to flow cytometry analysis.

Cell Motility Assay

NHEKs were incubated in 96-well culture plates (Falcon) to ∼60% confluence. Cells cultured in the presence or absence of inhibitors were labeled with Hoechst 33342, and then treated with BP IgGs (2 mg/ml) or normal IgG (2 mg/ml). Images of the cells were captured at 10 min intervals up to 6 h using an In Cell analyzer 2000 equipped with a ×20 objective (GE Biosciences). The motility of ∼2,000 cells was analyzed using ImageJ software with WrMtrack plugins (www.phage.dk/plugins/wrmtrck.html). The Euclidean distance and velocity were calculated using the Chemotaxis and Migration Tool (Ibidi GmbH) and the mean square displacements were obtained using Motility Lab (www.motilitylab.net/).

Statistical Analysis

The results are presented either as the mean values ± standard errors of the means or mean values ± standard deviations (*SD*) from at least triplicate experiments. One-way or two-way ANOVA or Student's *t*-test was used to compare the means, and differences were deemed significant when the calculated *p*-value was < 0.05. All statistical analyses were performed with the R language (www.r-project.org).

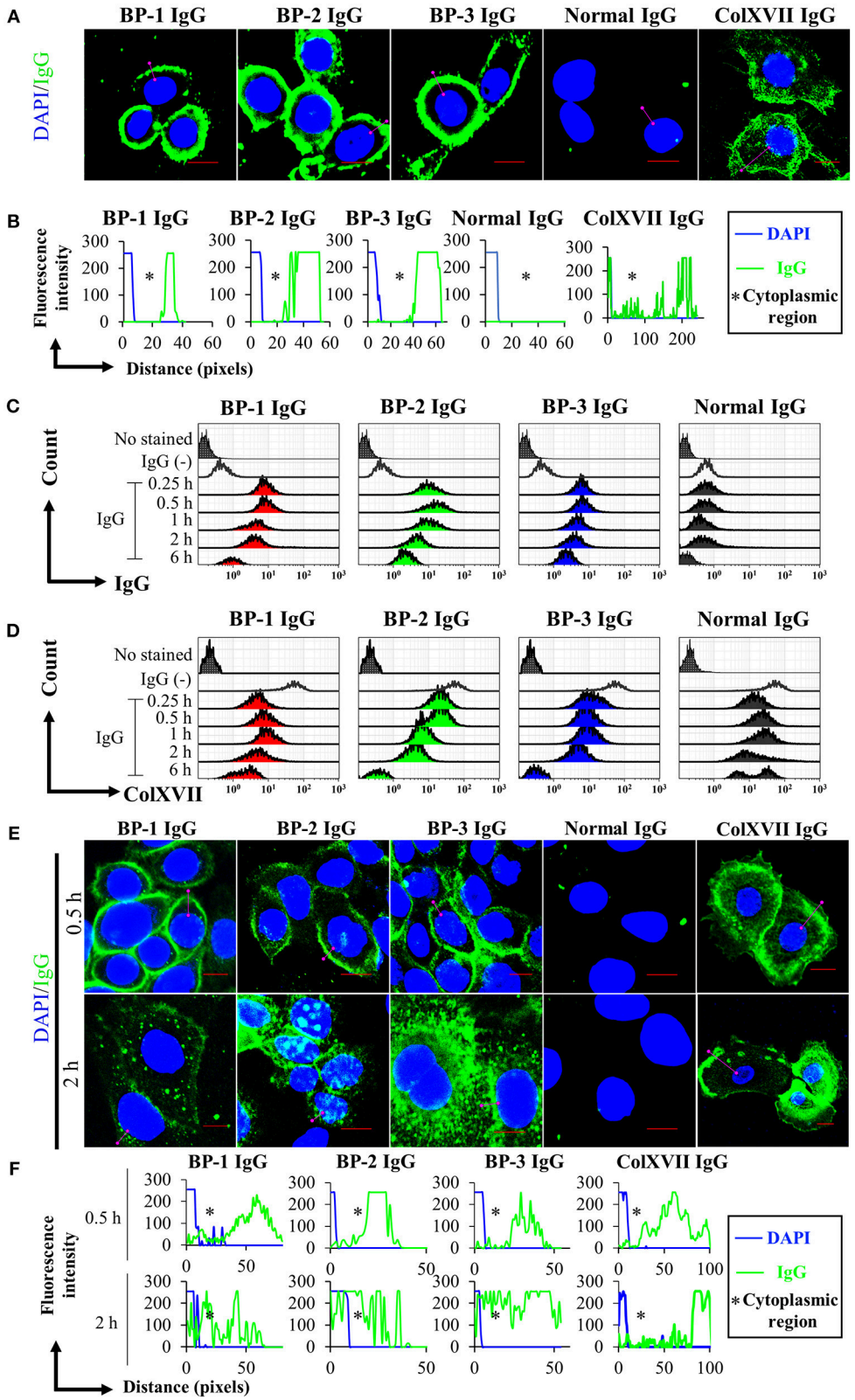

FIGURE 1 | Kinetics of the cell surface IgG binding and ColXVII expression. **(A)** Immunofluorescence staining for IgG bound to fixed NHEKs. NHEKs were fixed with paraformaldehyde and permeabilized. Cells were incubated with BP IgGs (BP-1, BP-2, or BP-3, 2 mg/ml), normal IgG (2 mg/ml), or rabbit anti-human

(Continued)

FIGURE 1 | ColXVII COOH IgG (ColXVII IgG, 12 μg/ml) and then subsequently stained with FITC- or Alexa Fluor 488-conjugated secondary antibodies (green). The nucleus was stained with DAPI (blue). Scale bar 10 μm. **(B)** Quantification of the fluorescence intensity of IgG bound to fixed NHEKs. Data were analyzed using ICY software. **(C)** Evaluation of the cell surface IgG binding using flow cytometry. NHEKs were cocultured with 2 mg/ml IgGs for the indicated times and then immediately fixed without permeabilization. The cells were directly incubated with anti-human IgG-FITC and were examined using flow cytometry. **(D)** Evaluation of the cell surface ColXVII expression using flow cytometry. NHEKs were cocultured with 2 mg/ml IgGs for the indicated times, and then immediately fixed without permeabilization. Then, cells were first incubated with an IgG specific for human ColXVII-COOH, and then incubated with Alexa Fluor 488-conjugated anti-IgG. Cells were examined using flow cytometry. **(E)** Fluorescence microscopy images of the binding of the indicated IgGs to NHEKs. NHEKs were incubated with BP IgGs (BP-1, BP-2, or BP-3, 2 mg/ml), normal IgG (2 mg/ml), or ColXVII IgG (12 μg/ml) for the indicated times. Then, the cells were fixed, permeabilized, subsequently stained with FITC- or Alexa Fluor 488-conjugated secondary antibodies (green). The nucleus was stained with DAPI (blue). Scale bar 10 μm. **(F)** Quantification of the fluorescence intensity of IgG bound to NHEKs. Data were analyzed using ICY software.

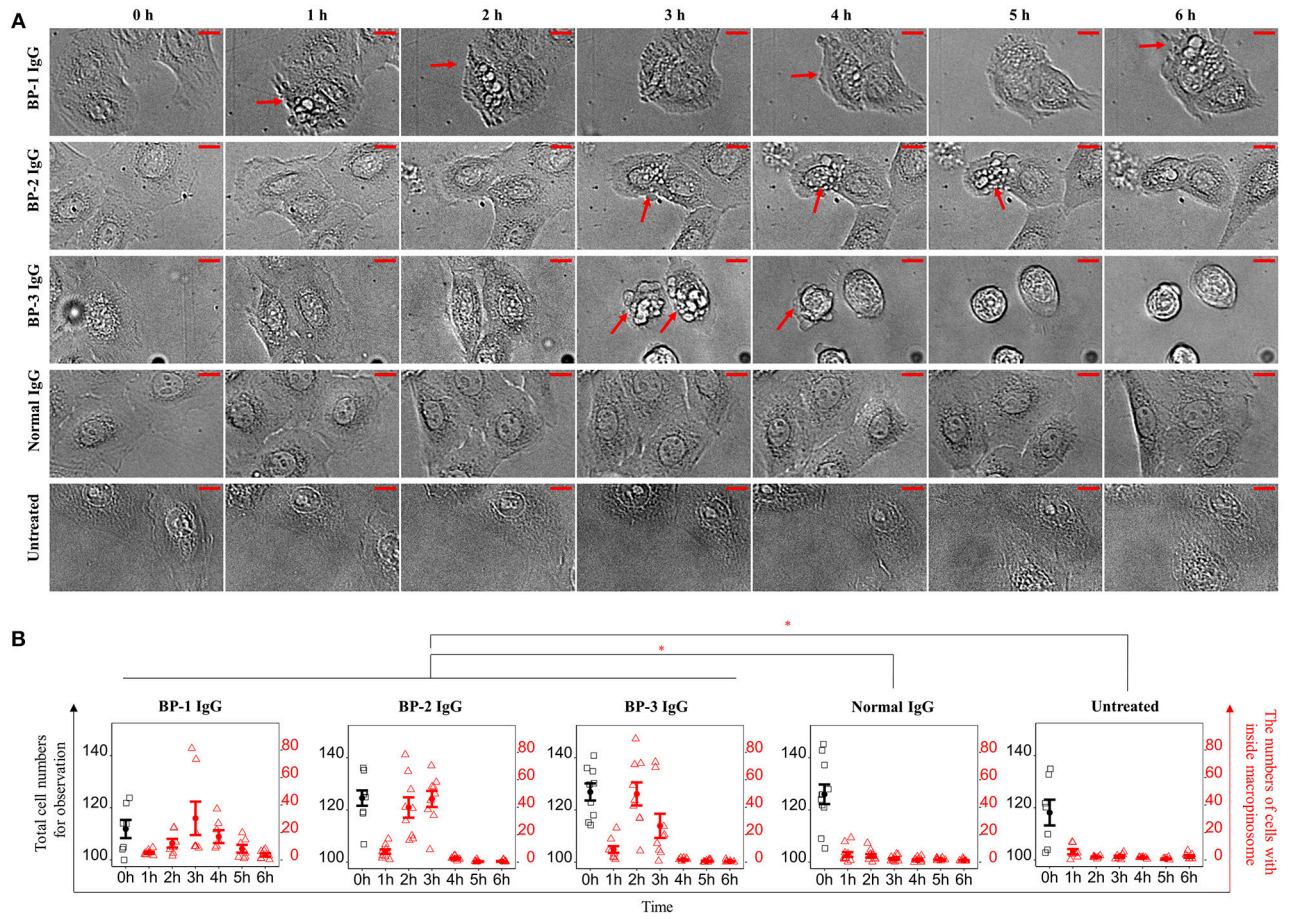

FIGURE 2 | Phase-contrast images of macropinosome formation in NHEKs treated with BP IgG. **(A)** Phase-contrast images were captured every 10 min after the addition of IgGs obtained from three patients with BP (BP-1, BP-2, and BP-3) or normal IgG to NHEKs and culture for up to 6 h. The figure shows images captured every 1 h. Cells with macropinosomes were indicated with arrows. Scale bar 10 μm. **(B)** The numbers of cells present during BP IgGs stimulation. Black: Total cell numbers observed at 0 h. Red: Numbers of cells containing macropinosomes at the indicated time points. Two-way ANOVA was used to analyze the significance of differences induced by BP IgGs. *p-value < 0.05.

RESULTS

Kinetics of the Cell Surface IgG Binding, ColXVII Expression, and the Numbers of Adherent NHEKs Treated With BP IgG

We performed immunostaining and analyzed the results with a confocal laser-scanning microscope and flow cytometry to obtain spatial and kinetic information about BP IgG binding and ColXVII expression in NHEKs treated with BP IgGs. BP IgGs binding to the NHEK surface were verified by positive immunostaining for FITC-labeled anti-human IgG, and ColXVII expression on the surface of NHEKs was verified using ColXVII IgG (**Figures 1A,B**). In the flow cytometry analysis, the cell surface IgG binding and ColXVII expression on the surface

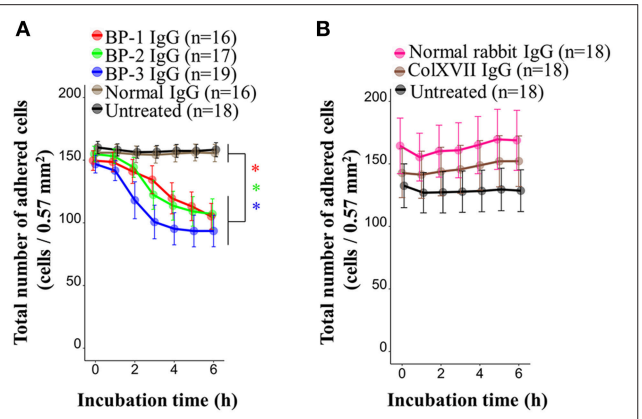

FIGURE 3 | Kinetics of the numbers of adherent NHEKs stimulated with BP IgG. Total numbers of adherent cells after stimulation with **(A)** BP IgGs, normal IgG, or **(B)** ColXVII IgG or normal rabbit IgG. The cell numbers were counted hourly in a ×20 area (0.57 mm^2) for up to 6 h. Two-way ANOVA was used to determine the significance of differences induced by BP IgGs. *p-value < 0.05.

of BP IgG-stimulated NHEKs decreased over time, whereas ColXVII expression and the binding of IgG to normal IgG-stimulated cells was not obviously altered (**Figures 1C,D**). Based on the confocal laser-scanning microscope observations, the cell surface localization of BP IgGs were visualized after a 0.5 h incubation, and most of the punctate IgGs staining were mainly localized in the cytoplasmic area at 2 h (**Figures 1E,F**). No significant IgG staining was observed when NHEKs were cocultured with normal IgG (**Figures 1A–E**). Immunostaining with ColXVII IgG did not show obvious internalization (**Figures 1A,B,E,F**). Phase-contrast images of NHEKs cocultured with BP IgGs displayed the BP IgG-induced formation of typical macropinosomes in NHEKs (**Figure 2A**), and compared to normal IgG- or untreated cells, BP IgG-stimulated cells exhibited a significant increase in the number of cells containing macropinosomes (**Figure 2B**).

BP IgG is known to cause the detachment of keratinocytes in 40% confluent cultures, although the adhesive function of ColXVII remains unclear (37). We performed a cell counting assay with living cells to determine the effect of BP IgGs on keratinocyte adhesion. BP IgGs significantly decreased the number of attached cells (reduced by ∼40%) compared to the cells treated with normal IgG or untreated cells, and the effects were evident beginning 2 h after the incubation (**Figure 3A**). We also assessed the effect of IgG on detaching the COOH-termini of ColXVII (ColXVII IgG) and found that the addition of ColXVII IgG to the NHEKs did not obviously affect adhesion (**Figure 3B**).

BP IgG Treatment Alters the Morphology of NHEKs

We hypothesized that internalization of BP IgG-ColXVII complexes alters the properties and morphology of NHEKs. First, we used SEM to characterize the status of the plasma membrane after BP IgGs were added to the culture. Compared to the cells incubated with normal IgG and/or the untreated cells, the BP IgG-stimulated cells exhibited alterations in the cell membrane structure (indicated with arrows in **Figure 4**).

The TEM examination revealed larger vesicles with villi-like structures inside the cytoplasmic space in BP IgG-treated NHEKs. BP IgG-treated NHEKs displayed vacuolar structures containing myelin-like structures. BP IgG-stimulated cells accumulated lysosomes and/or autophagosomes (**Figures 5A,B**), whereas cell nuclei showed neither damage nor chromatin condensation (**Figure 5A**). Since lysosomes have been suggested to play important roles in cell death (50), we further characterized the involvement of lysosomes in the BP IgG-induced cell death using a LysoTracker® Green DND-26 uptake assay. The live cell imaging revealed the accumulation of lysosomes 2 h after BP IgGs were added to the culture (**Figure 5B**), and the intracellular distribution of lysosomes was consistent with the TEM observations. Moreover, BP IgG-stimulated cells contained a greater number intracellular LysoTracker® Green DND-26-labeled dots with high fluorescent intensity (**Figures 5C,D**).

Morphological and Functional Changes in the Mitochondria of NHEKs Incubated With BP IgG

We used time-lapse microscopy to analyze cell morphology and calculated the sizes of individual cells to determine whether the increased number of intracellular vesicles increased the sizes of individual cells. The sizes of NHEKs increased 1 h after adding BP IgGs to the culture, followed by the rupture of the cell membrane (**Supplementary Figure S1A**). The larger cell sizes reverted to the average size of normal cells after 3 h (**Supplementary Figure S1B**). Following nuclear staining and the capture of phase-contrast images, the sizes of NHEKs that had been incubated with BP IgGs for 2 h increased (**Figures 6A,B**). The TEM observations of mitochondria revealed numerous swollen mitochondria and a loss of cristae in cells stimulated with BP IgGs (**Figure 6C**). The mitochondrial functions were further analyzed with MitoTracker® Red CMXRos staining to observe the effect of BP IgGs, and the mitochondrial numbers and staining intensity were quantified in each individual cell. After 16 h, the number of stained mitochondria per cell did not differ between BP IgG-treated cells and normal IgG-treated or untreated cells (data not shown), whereas the staining intensity of mitochondria in BP IgG-treated cells decreased (**Figure 7A**).

ROS were generated 6 h after BP IgGs were added to the culture, whereas normal IgG did not increase ROS levels compared to untreated cells (**Figure 7B**). By performing JC-1 staining to analyze the mitochondrial membrane potential ($\Delta\Psi m$), JC-1 aggregates, a sign of intact mitochondria, were more abundant in the normal IgG-stimulated cells, and untreated cells. On the other hand, dysfunctional mitochondria with a dissipation of $\Delta\Psi m$, as evidenced by the increased number of JC-1 monomers, were pronounced in BP IgG-treated NHEKs at 20 h (**Figure 7C**).

The two-color fluorescence assay that distinguishes metabolically active cells was performed using SYTOX

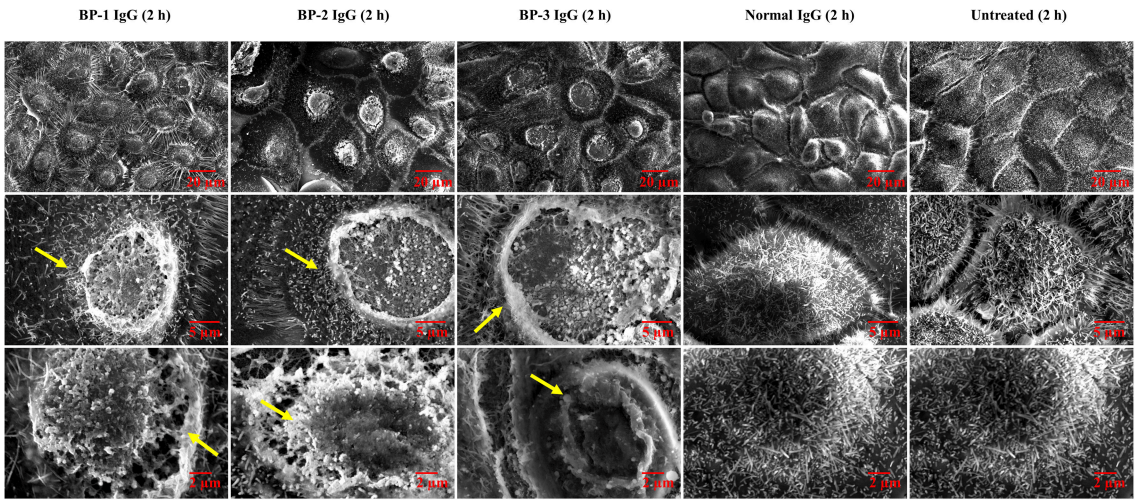

FIGURE 4 | SEM images of NHEKs stimulated with BP IgG. SEM images of NHEKs treated with BP IgGs (BP-1, BP-2, and BP-3) or normal IgG for 2 h. The lower magnification pictures show the connections of cells and filopodia. The higher magnification pictures show the cell surface and microvilli of individual cells. The alterations in the cell membrane structure are indicated with arrows.

green and C_{12}-resazurin dye. SYTOX green labels cells with compromised plasma membranes, and C_{12}-resazurin is used to assess mitochondrial metabolic activity. After a 2 h incubation with BP-1 IgG or normal IgG, the pattern of SYTOX green and C_{12}-resorufin staining did not differ between BP-1 IgG-, normal IgG-stimulated, or untreated NHEKs (data not shown). Therefore, we postulated that BP IgG-induced ColXVII internalization does not immediately induce a loss of metabolic activity in NHEKs. Since the dissipation of $\Delta\Psi$m is usually regarded as a sign of decreased cell viability (51) and $\Delta\Psi$m was not significantly decreased in NHEKs incubated with BP IgGs for up to 6 h (data not shown), we harvested the NHEKs that had been incubated with BP IgGs for 16 h and performed SYTOX green/C_{12}-resazurin staining. A greater percentage of BP IgG-stimulated cells was stained with SYTOX green and a relatively lower percentage was stained with C_{12}-resazurin than normal IgG-stimulated or untreated cells (**Figure 7D**), suggesting a decrease in the metabolic ability of BP IgG-treated cells. As DNA fragments were not found (data not shown) and chromatin condensation was not observed in our TEM images of BP IgG-stimulated cells (**Figure 5A**), we concluded that BP IgG did not induce apoptosis.

BP IgG Increased NHEK Motility

Next, we extended our observations to evaluate cell motility. According to the results of the electron microscopy analysis, BP-1 IgG-stimulated cells displayed longer lamellipodia (**Figure 4**), and the extension of lamellipodia is closely related to cell migration (52). Throughout the 6 h observation of live cells, BP IgG-treated cells showed increased cell motility compared with normal IgG-stimulated and untreated cells (**Figure 8**). Notably, both the migration distance (**Figures 8A,C**) and velocity (**Figure 8B**) increased.

Effects of cytochalasin D, NSC237766, and MG132 on the BP IgG-Induced Alterations in keratinocytes

The findings of BP IgG-induced cell dysfunction led us to investigate the pathway involved in the BP IgG-induced alterations. Three inhibitors that have been established as involved in the ColXVII internalization process were employed to further investigate the direct effect of BP IgGs on cell adhesion, metabolic activity, and motility.

Consistent with previous reports (43), application of cytochalasin D (inhibitor of the actin-cofilin interaction) inhibited the internalization of the BP IgG-ColXVII immune complex in BP IgG-stimulated NHEKs (**Figures 9A–C**). In the cell counting assay, the application of cytochalasin D did not exert a protective effect but instead increased the adhesion of NHEKs treated with BP IgGs (**Figure 10**). The cytochalasin D treatment did not completely relieve the BP IgG-induced loss of metabolic activity in NHEKs (**Figure 11**). In the motility assay, cytochalasin D significantly decreased the migration speed and distance of untreated NHEKs (**Figure 12A**), although it did not consistently exert the inhibitory effect on the NHEKs stimulated with BP IgGs (**Figures 12A,B**).

MG132 (inhibitor for proteasome) suppresses ColXVII depletion in both mouse and human keratinocytes stimulated with BP IgG (41). Thus, we hypothesized that the reduction in the adhesion of NHEKs treated with BP IgG is related to the depletion of ColXVII. MG132 did not prevent BP IgG-induced internalization (**Figures 9A–C**) or delay the BP IgG-induced decrease in cell number (**Figure 10**). However, MG132 protected NHEKs from the BP IgG-induced loss of

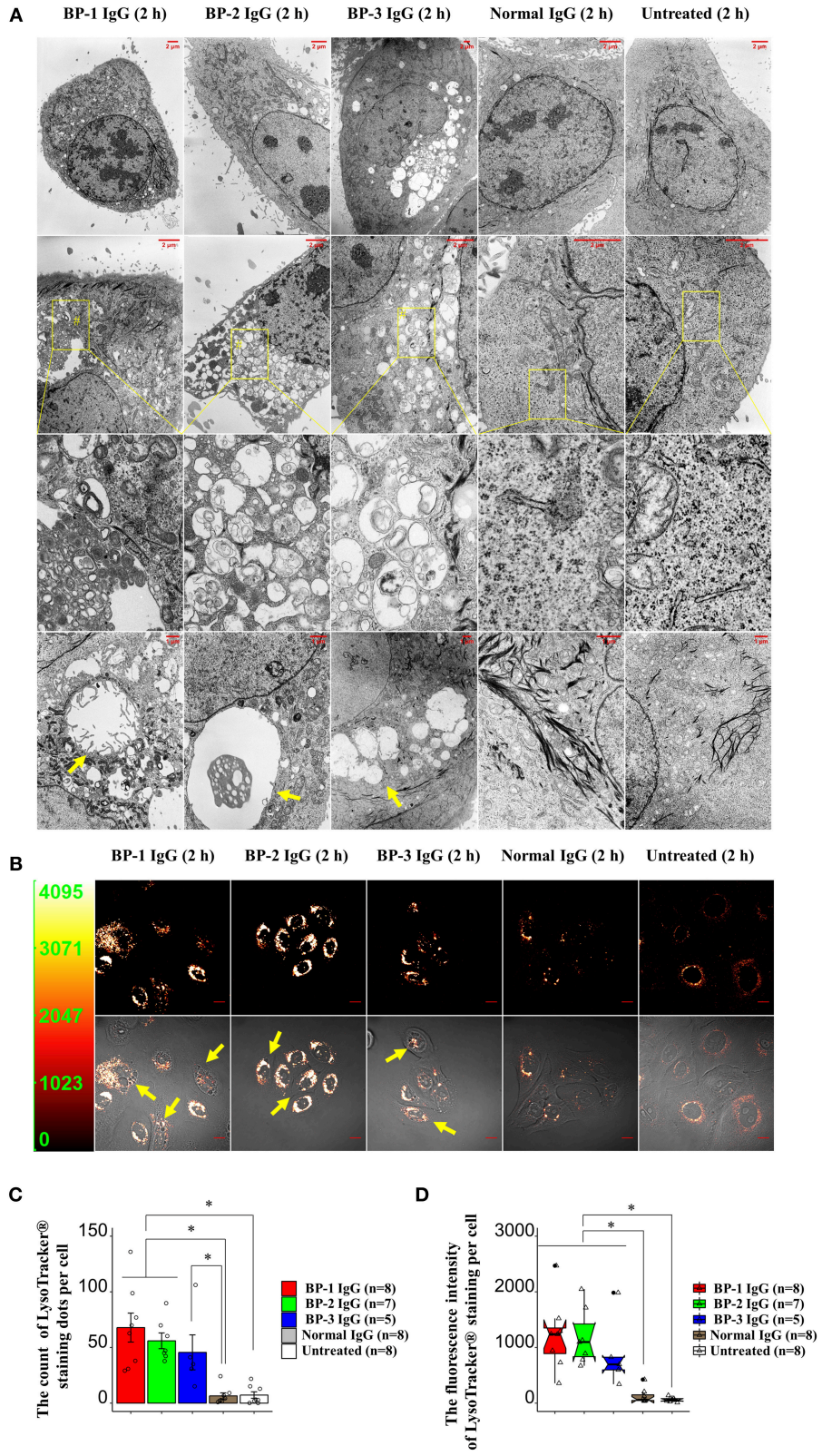

FIGURE 5 | Changes in the morphology of NHEKs stimulated with BP IgG. **(A)** TEM images of NHEKs stimulated with BP IgGs (BP-1, BP-2, and BP-3) or normal IgG for 2 h. Zoomed-out images labeled with the # symbol shows vacuolar structures containing myelin-like structures. Vacuolar structures are indicated with arrows.

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FIGURE 5 | (B) The intracellular distribution of lysosomes in NHEKs stimulated with BP IgGs (BP-1, BP-2, and BP-3) or normal IgG. After a 2 h incubation with IgGs, cells were incubated with 50 nM LysoTracker® Green DND-26 for 15 min in dark, and live cells were imaged using confocal microscopy. Arrows indicate vesicles surrounded by lysosomes. Scale bar 10 μm. Y-axis in the left panel: fluorescence intensity. **(C)** Quantification of the fluorescently stained dots in cells. The LysoTracker® Green DND-26-stained dots in the cytoplasmic region of individual cells were counted using the CellProfiler image analysis software. One-way ANOVA was used for the statistical analysis. *p-value < 0.05. **(D)** Measurement of the fluorescence intensity per cell. The fluorescence intensity of LysoTracker® Green DND-26-stained cells was measured using CellProfiler image analysis software. One-way ANOVA was used for the statistical analysis. *p-value < 0.05.

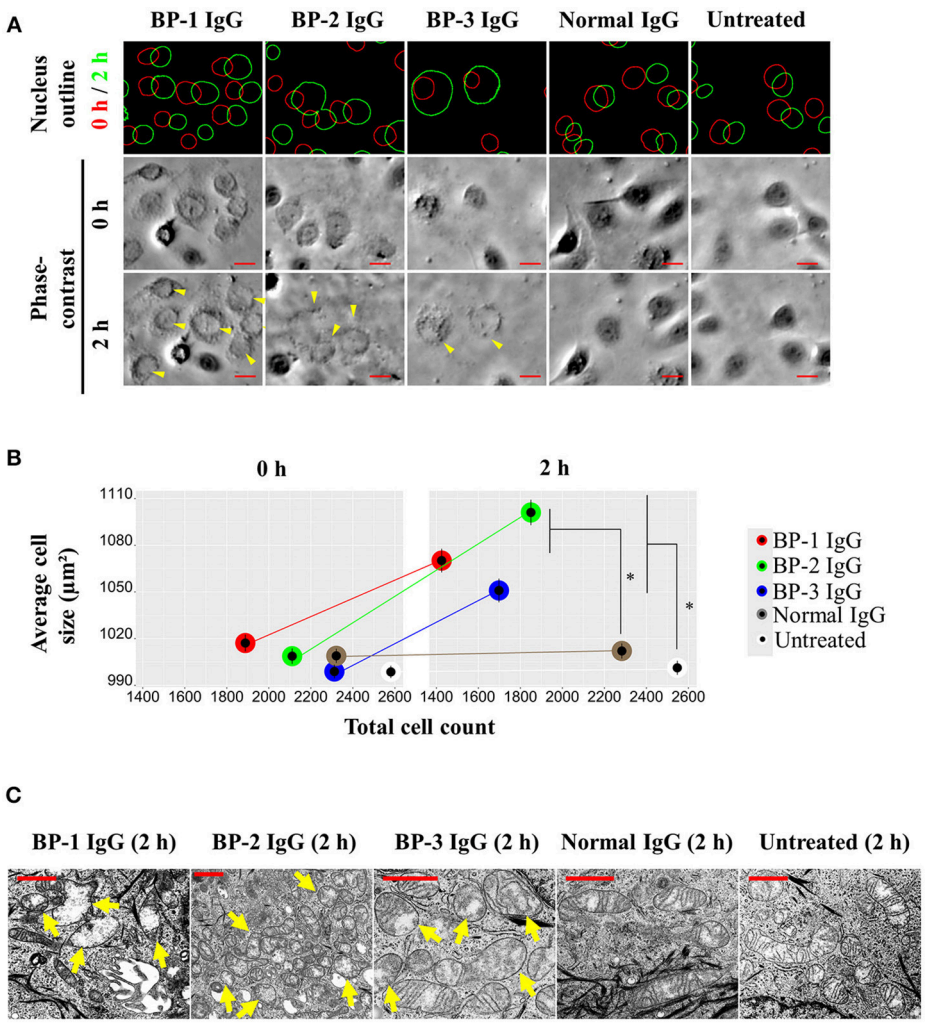

FIGURE 6 | Changes in the mitochondrial morphology in NHEKs treated with BP IgG. NHEKs were stained with Hoechst 33342 and then stimulated with BP IgGs (BP-1, BP-2, and BP-3) or normal IgG. **(A)** Nuclei were labeled at 0 h (red circle) and 2 h (green circle); the whole-cell area is shown with a phase-contrast image. Arrows indicate enlarged cells. Scale bar 20 μm. **(B)** Average cell sizes at 0 and 2 h were calculated from at least 1,500 individual cells per group. Two-way ANOVA was used for the statistical analysis. *p-value < 0.05. **(C)** TEM images of mitochondrial morphology in NHEKs that had been incubated with the indicated IgGs for 2 h. Damaged mitochondria are indicated with arrows. Scale bar 1 μm.

metabolic activity (**Figure 11**). These data support our hypothesis that the metabolic activity was associated with proteasome activation in NHEKs stimulated with BP IgGs. Additionally, MG132 slightly decreased the migration of BP IgG-treated cells (**Figures 12A,B**).

The formation of macropinosomes in epithelial cells as an early response to combat intracellular pathogens requires the activation of a Rho-GTPase such as Rac1 (46, 53). NSC23766 (a Rac1 inhibitor) suppresses the internalization of BP IgG-ColXVII immune complexes (54). In the immunostaining assay, we also confirmed that the inhibition of Rac1 activity prevented BP IgG-ColXVII internalization (**Figures 9A–C**). Surprisingly, the application of NSC23766 to the NHEKs treated with BP IgGs did not exert a protective effect on adhesion (**Figure 10**). As shown in **Figure 11**, the application of NSC23766 to the BP IgG-treated NHEKs prevented the loss of metabolic activity, but did not reduce the cell motility (**Figure 12**).

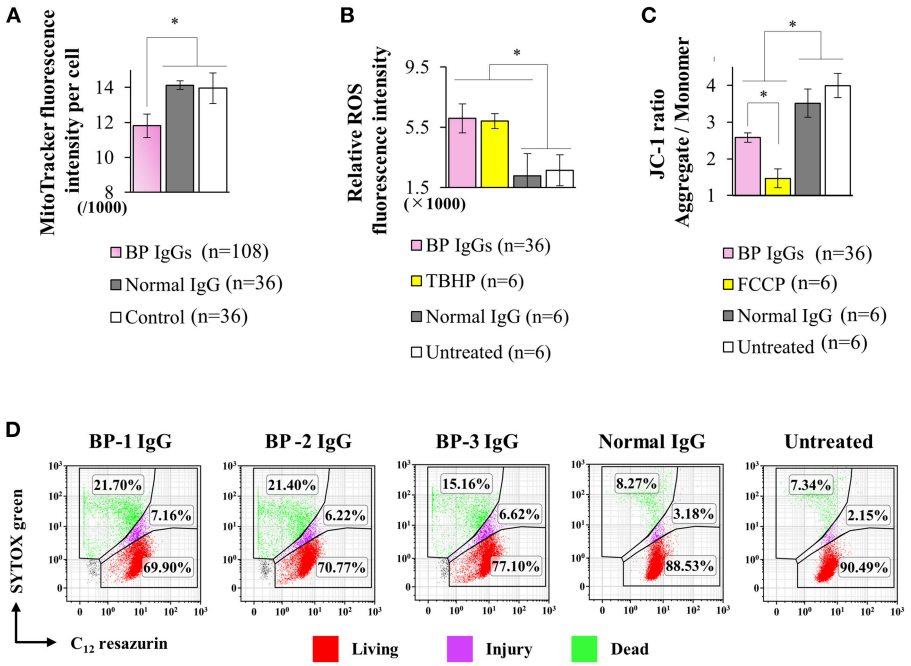

FIGURE 7 | Effects of BP IgG on mitochondrial function in NHEKs. **(A)** Mitochondrial staining intensities (MitoTracker® Red CMXRos) were evaluated in NHEKs incubated with a pool of BP IgGs (from BP-1, BP-2, and BP-3) or normal IgG for 16 h. One-way ANOVA was used for the statistical analysis. *p-value < 0.05. **(B)** ROS were detected in NHEKs stimulated with BP IgGs or normal IgG for 6 h. TBHP was used as a positive control. One-way ANOVA was used for the statistical analysis. *p-value < 0.05. **(C)** The mitochondrial membrane potential was assayed in NHEKs treated with BP IgGs or normal IgG for 20 h. FCCP was used as a positive control. One-way ANOVA was used for the statistical analysis. *p-value < 0.05. **(D)** NHEKs were collected after a 16 h incubation with the indicated IgGs, then stained with SYTOX green (stains dead cells) and C_{12}-resazurin (stains living cells), and subjected to flow cytometry.

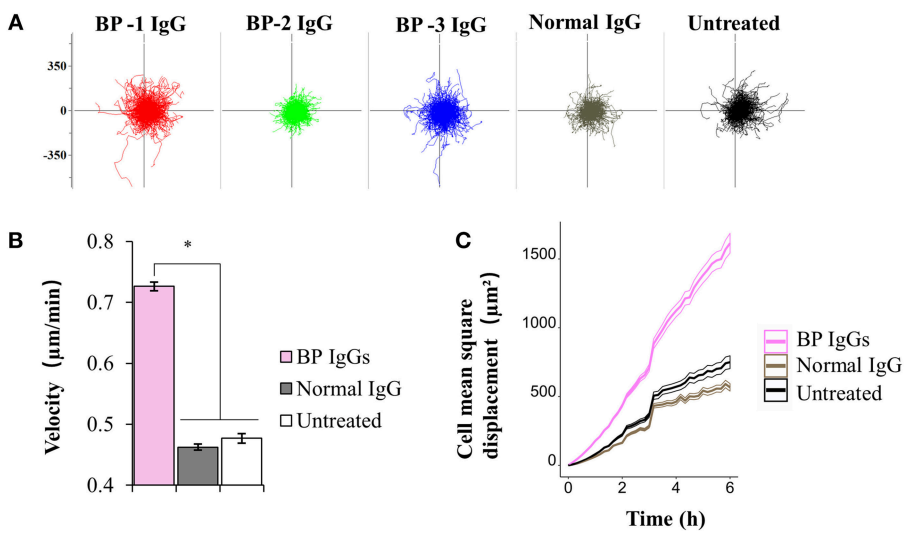

FIGURE 8 | Results of the cell motility assay using NHEKs stimulated with BP IgG. Hoechst 33342-labeled NHEKs were incubated with IgGs obtained from 3 patients with BP (BP-1, BP-2, and BP-3) or normal IgG, and imaged using time-lapse microscopy. Images were captured at 10 min intervals for up to 6 h. **(A)** Migration displacement maps were generated by centering the cell migration paths at a common starting point. Axes of vector diagrams = 500 μm. **(B)** Cell velocity of NHEKs treated with IgGs. One-way ANOVA was employed to determine the significance of differences induced by BP IgGs. *p-value < 0.05. **(C)** Mean square displacement of the NHEKs treated with IgGs.

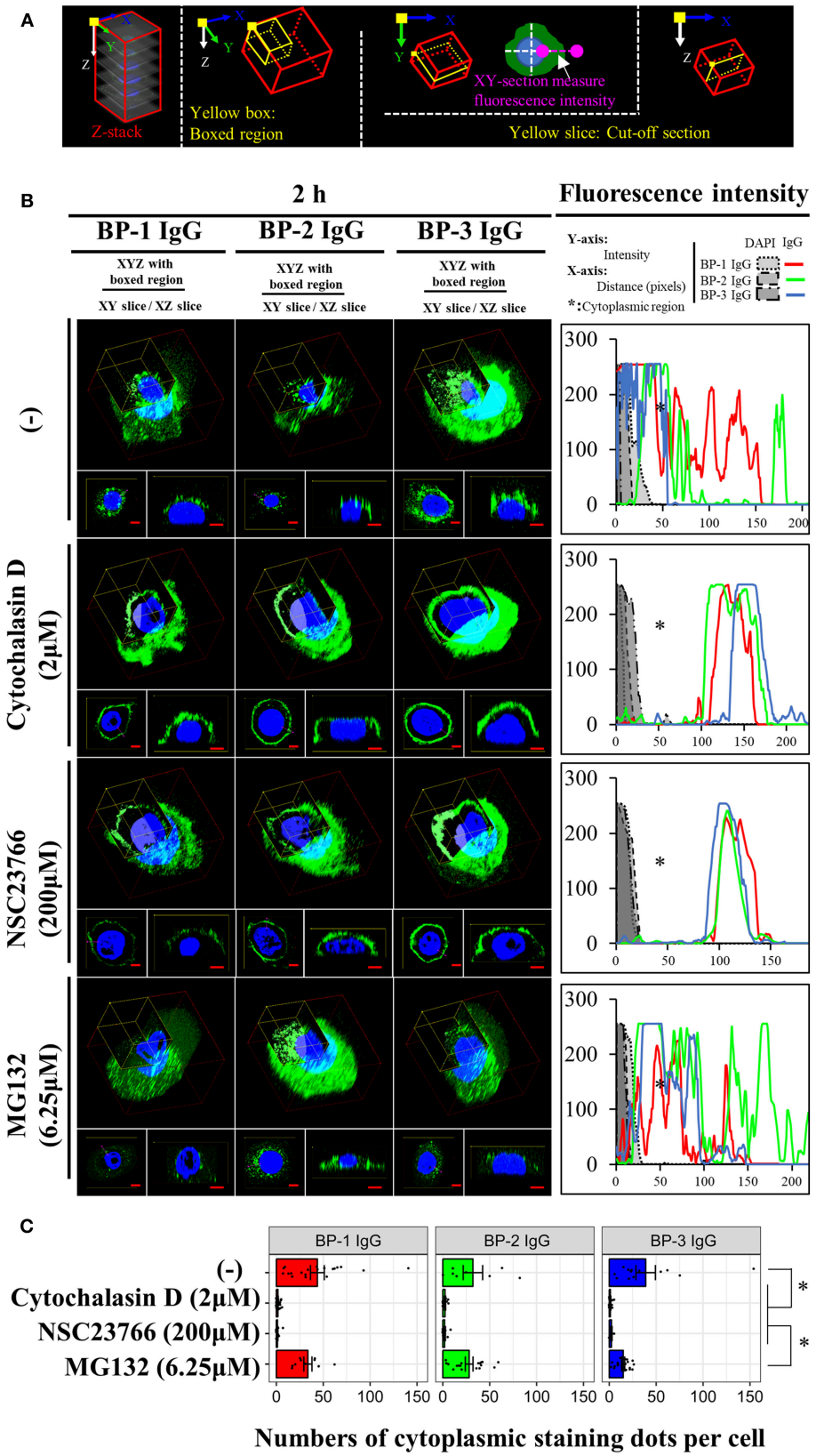

FIGURE 9 | Effects of cytochalasin D, NSC237766, and MG132 on the BP IgG-induced internalization. **(A)** Overview of the 3D reconstruction assay. The 3D reconstruction was performed as described in the methods. The boxed region indicates XYZ boxed area, XY slice indicates the horizontal slice and used to measure

(Continued)

FIGURE 9 | the fluorescence intensity, and XZ slice indicates the longitudinal slice. **(B)** Fluorescence microscopy images of the binding of the indicated IgGs to the NHEKs at 2 h. NHEKs were pretreated with cytochalasin D (2 μM) for 30 min, NSC23766 (200 μM) for 1 h, or MG132 (6.25 μM) for 0 h, and then 2 mg/ml BP IgGs (BP-1, BP-2, BP-3) or normal IgG were added to the cultured cells. The 3D reconstruction assay was applied to observe the changes. Scale bar 5 μm. **(C)** Quantification of the IgG-stained dots in cells. The anti-IgG-FITC-stained dots in the cytoplasmic region of individual cells were counted using the CellProfiler image analysis software. One-way ANOVA was used for the statistical analysis. *p-value < 0.05.

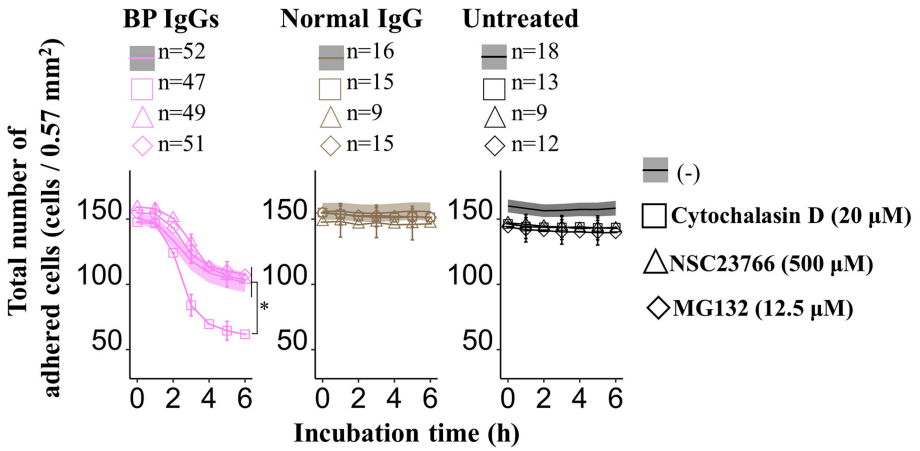

FIGURE 10 | Effects of cytochalasin D, NSC237766, and MG132 on the BP IgG-induced decrease in the number of adherent cells. NHEKs were pretreated with cytochalasin D (20 μM) for 30 min, NSC23766 (500 μM) for 1 h, or MG132 (12.5 μM) for 0 h, and then 2 mg/ml BP IgGs (BP-1, BP-2, and BP-3) or normal IgG were added to the cultured cells. Total numbers of adherent cells after the incubation with BP IgGs or normal IgG were counted every hour in a ×20 area (0.57 mm^2) for up to 6 h. One-way ANOVA was used for the statistical analysis. *p-value < 0.05.

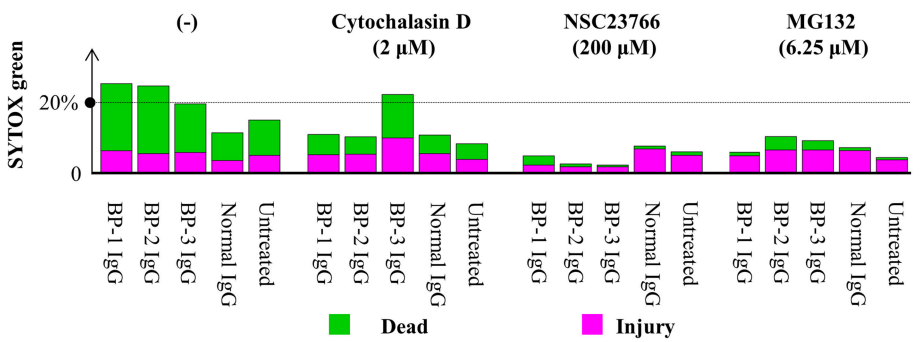

FIGURE 11 | Effects of cytochalasin D, NSC237766, and MG132 on the BP IgG-induced decrease in cellular metabolic activity. NHEKs were pretreated with cytochalasin D (2 μM) for 30 min, NSC23766 (200 μM) for 1 h, or MG132 (6.25 μM) for 0 h, and then 2 mg/ml BP IgGs (BP-1, BP-2, and BP-3) or normal IgG were added to the cultured cells for 16 h. Then, NHEKs were collected, stained with SYTOX green (stains dead cells) and C_{12}-resazurin (stains living cells), and analyzed using flow cytometry.

DISCUSSION

As shown in the present study, BP IgG directly induces NHEK dysfunction, as BP IgG stimulation increases SYTOX green staining, revealing structural disintegration of the plasma membrane, intercellular vesicle formation, and mitochondrial dysfunction, but not chromatin condensation. In addition, BP IgG induced cell dysfunction by activating Rac1 and the proteasome. Our findings support the hypothesis proposed by Ujiie et al. that BP IgG directly causes subepidermal bulla formation in patients with BP (41).

We considered two possible explanations for BP IgG-induced cell dysfunction: methuosis or oncosis. The well-known response of NHEKs to BP IgG stimulation is macropinosome formation (43, 54). In mammalian cells, hyperstimulation of macropinosomes is known to lead to methuosis, which is defined as the maturation of macropinosomes (48, 49). We first tested whether the BP IgG-induced accumulation of fluid-filled endocytic vesicles was catastrophic and ultimately merged with the lysosomes, as previously reported (55), to distinguish between these mechanisms. However, refuting our methuosis hypothesis, BP IgG-induced

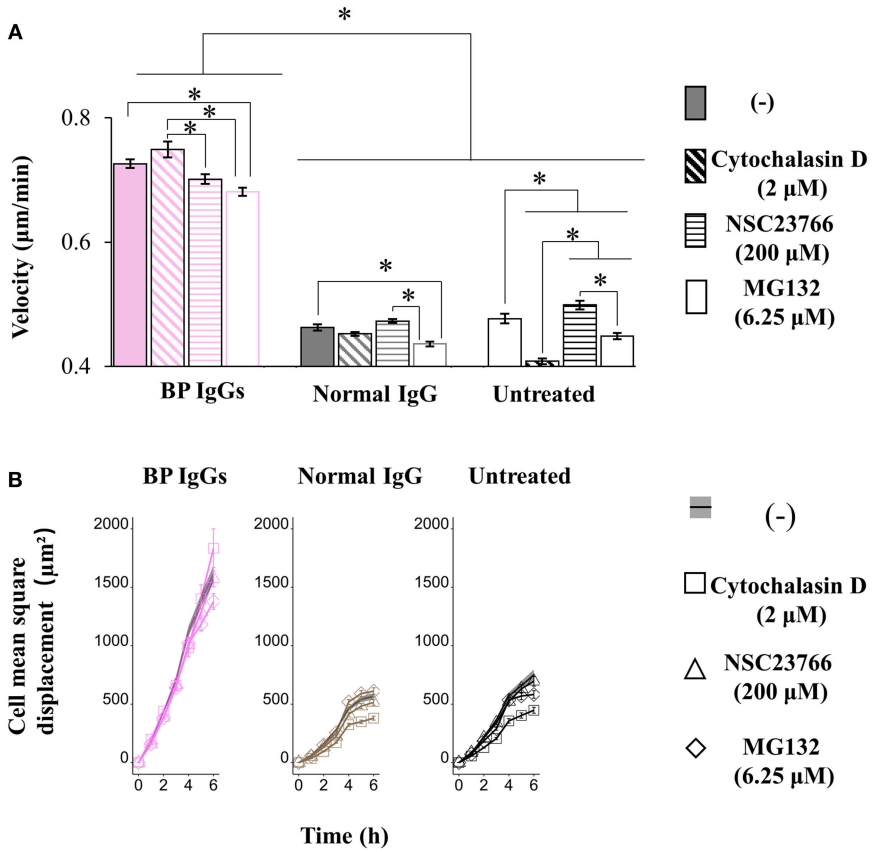

FIGURE 12 | Effects of cytochalasin D, NSC237766, and MG132 on the BP IgG-induced increase in cell motility. NHEKs were pretreated with cytochalasin D (2 μM) for 30 min, NSC23766 (200 μM) for 1 h, or MG132 (6.25 μM) for 0 h, and then 2 mg/ml BP IgGs (BP-1, BP-2, BP-3) or normal IgG were incubated with the cultured cells for 6 h. **(A)** Velocity of the NHEKs stimulated with IgGs. One-way ANOVA was used for the statistical analysis. *: p-value < 0.05. **(B)** Mean square displacement of the NHEKs stimulated with IgGs.

macropinosomes did not merge with lysosomes, excluding the possibility of methuosis. The alternative explanation is oncosis, a type of cell dysfunction caused by cell membrane damage characterized by cellular and/or organelle swelling (56). Our ultrastructural and morphological observations were consistent with these changes (**Figures 4**, **5** and **Supplementary Figure S1**).

Importantly, our data provided evidence supporting the relationship between BP IgG-induced cell dysfunction and lysosome accumulation and mitochondria dysfunction (**Figures 5**–**7**). Cell membrane injury directly results in acute cell dysfunction, and the dying cells release endogenous alarm signals, which trigger the innate immune system and modulate inflammation through the lysosome-induced increase in the levels and excessive formation of ROS (50, 57). In contrast, the lysosomes mediate plasma membrane repair and control cellular dysfunction (50, 58). Mitochondrial dysfunction usually leads to mammalian cell death and influences the immune system to perceive/react to the dying cells (51, 59). Lysosome-mediated cellular dysfunction is characterized by simultaneous membrane damage and the proteolysis of a wide range of proteins (60). Lysotracker® Green DND-26, a marker of the lysosome membrane potential (60), helped identify BP IgG-treated cells with a higher lysosome membrane potential. Conceivably, the proteolytic enzymes released from lysosomes trigger a forward loop promoting lysosome rupture after a 2 h treatment with BP IgG. Moreover, the proteasome inhibitor MG132 efficiently blocks lysosome rupture (61) and has attracted considerable attention as an anti-BP agent (41). Therefore, MG132 likely exerted its protective effects on BP IgG-induced cell dysfunction in our study through its antilysosome/proteasome effects. Not surprisingly, several skin diseases are reported to be associated with alterations in mitochondria-related metabolic pathways (59). Consistent with the results of our ROS assay, the BP disease process was recently reported to be characterized by ROS production (62). We provided direct evidence that cell membrane fragility follows ROS production induced by BP IgG stimulation, which ultimately significantly increases cell membrane fragility, although we have not identified the molecular events underlying ROS production. The cell membrane is the site of activation of numerous signaling cascades that induce

mitochondrial dysfunction. Interestingly, Rac1 is reported to be located both at the plasma membrane and mitochondrial membrane (63). The administration of NSC23766 decreases ROS production and promotes cell survival (64). Rac1 activation might serve as the initial step to "destroy" both the cell and mitochondrial membranes, and the pathways downstream of Rac1 also contribute to increasing ROS production to promote cellular dysfunction.

We conclude that the persistent stimulation with BP IgG induced the formation of macropinosomes in keratinocytes, resulting in a fragile plasma membrane, intercellular vesicle formation, lysosome accumulation, and ROS production, which ultimately contribute to mitochondrial dysfunction. The BP IgG-induced alterations in keratinocytes trigger the immune system to digest the basal keratinocytes, causing the formation of blisters on the skin along with BMZ.

Our study has limitations. For example, we had limited explanations for the association of BP IgG-induced keratinocyte alterations with blister formation. Similar to the results from a previous study of basal keratinocytes (*in vivo*) from patients with BP (65), intercellular vesicles filled with villi and swelled mitochondria were also observed in our TEM experiment. We first considered that the intercellular vesicles likely form in response to the maturation of macropinosomes, and the lysosome-mediated pathway contributes to vesicle expansion and exerts potential effects on skin blistering. However, the intracytoplasmic accumulation of lysosomes/autophagosomes are not observed in ultrastructural images *in vivo* (65–67). In addition, histopathology rarely reveals degenerated keratinocytes in patients with early-stage BP. We considered that the pattern of ColXVII expression differs between keratinocytes *in vivo* (hemidesmosome ColXVII) and *in vitro* (nonhemidesmosome ColXVII) (15, 16, 21); future studies should provide insights into the different changes in keratinocytes *in vitro* and *in vivo* during the BP IgG-induced blistering process.

We also describe the role of ColXVII in regulating cell adhesion and motility (**Figures 3**, **8**, **10**, **12**). The formation of the BP IgG-ColXVII complex has been shown to tear the weakened lamina lucida, leading to a specific split at the lamina lucida and induction of BMZ blistering (37). According to another report, ColXVII mediates the anchorage of basal keratinocytes by regulating cell motility (68). Thus, we speculate that the changes in the adhesion and motility of keratinocytes are involved in the pathogenesis of blistering in patients with BP. As shown in reports (69, 70), IgGs targeting proteins other than ColXVII-NC16a do not detach cells from culture dishes. Interestingly, an IgG targeting the C-terminus of ColXVII neither induced obvious IgG-ColXVII internalization nor had any significant effect on cell detachment. Together with the results of the *in vivo* study showing that IgGs targeting the ColXVII ectodomain fail to reproduce blistering in an animal model (71), the findings from previous studies and our data confirm the pathogenicity of the anti-ColXVII-NC16a antibodies in subjects with BP. Based on the existing literature, the reduction in the cell adhesion observed upon BP IgG stimulation can be accounted for by ColXVII internalization (43, 72). However, researchers have not clearly determined how ColXVII internalization might influence cell adhesion. In the present study, the BP IgG-induced cell detachment was not directly induced by macropinosome formation, because alterations in actin, the well-known and necessary molecule for macropinosome formation (73), did not completely prevent NHEK detachment. NHEKs disassembled their contacts with neighboring cells and detached from the culture dish following an incubation with BP IgG. Furthermore, epithelial cell "destabilization" has also been shown to require a step mediated by the proteasome (74). For this reason, we speculated and confirmed that the BP IgG-induced cell detachment was associated with proteasome activation, and the internalization of the IgG-ColXVII complex probably requires the initial event of proteasome activation. Another interesting aspect of this study was that the BP IgG treatment increased NHEK motility. Based on the BP IgG-induced cell detachment, we speculate that the BP IgG-induced alterations in cell motility are likely due to a decrease in the cell density. On the other hand, ColXVII has been shown to regulate keratinocyte motility, while changes in cell motility following the loss of ColXVII remain controversial (26). Studies using ColXVII-knockdown keratinocytes have reported that the loss of ColXVII reduces lamellipodial stability (75) and induces cell migration mediated by Rac1 (76, 77). Cell migration is associated with the remodeling of the actin cytoskeleton. However, cytochalasin D did not affect cell motility following the BP IgG treatment. This discrepancy might be explained by the binding of ColXVII to two different cytoskeleton systems in keratinocytes: actin-associated focal contacts and keratin-associated hemidesmosome compounds (15, 78, 79).

Our findings provide a better understanding of the direct effects of BP IgG on keratinocytes by increasing the fragility of the cell membrane, resulting in keratinocyte dysfunction, probably through oncosis. In addition, the BP IgG-induced cellular dysfunction was reversed by Rac1/proteasome inhibition. We believe that our identification of the Rac1/proteasome-mediated signaling pathway provides valuable new insights that have improved our understanding of the direct effects of BP IgG on keratinocytes.

AUTHOR CONTRIBUTIONS

DT designed the study and wrote the initial draft of the manuscript. XD contributed to data collection and interpretation, and critically reviewed the manuscript. KN contributed to data interpretation and critically reviewed the manuscript. NY and OY contributed to the electron microscopy experiments and data interpretation, and OY critically reviewed the manuscript. EM supervised the entire study, provided critical intellectual input, and approved the final version of the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work and

ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00200/full#supplementary-material

Supplementary Figure S1 | Morphometric features and cell sizes. **(A)** NHEKs were cocultured with BP-1 IgG or normal IgG. Outlines of single cells at the indicated time points are shown. Arrows indicate the rupture of the plasma membrane. Scale bar 20 μm. **(B)** Analysis of the sizes of NHEKs incubated with IgG obtained from three patients with BP (BP-1, BP-2, and BP-3) or normal IgG. Images of nuclear staining were used to clearly segment the cells, and phase-contrast images were used to calculate cell sizes. Yellow dots indicate the average cell size. Approximately 1,500 cells from each group were analyzed. Student's *t*-test was used for the statistical analysis. $*p$-value < 0.05.

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NET Formation in Bullous Pemphigoid Patients With Relapse Is Modulated by IL-17 and IL-23 Interplay

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Background: DNA extracellular traps (ETs), released by neutrophils (NETs), or eosinophils (EETs), play a pathogenic role in several autoimmune disorders. However, to date, NETs have never been investigated in bullous pemphigoid (BP) with respect to clinical and immunological activities, both at baseline and at time of relapse which have been characterized with specific IL-17 and IL-23 patterns.

Objective: We sought to assess whether ETs were associated with BP as well as the relative contribution of IL-17 axis cytokines to NET induction.

Methods: Skin biopsy specimens were obtained from 11 patients with BP. Immuno-detection of neutrophils and eosinophils combined to DNA staining allowed us to investigate the *in-situ* presence of NETs and EETs using confocal scanning microscopy. NETs release was evaluated *ex vivo* by stimulating polymorphonuclear cells from BP patients with BP biological fluids in presence of IL-17A and IL-23 or of glucocorticoids.

Results: At baseline, ETs were observed in BP lesions at the site of dermal-epidermal cleavage. Despite an important infiltrate of eosinophils, ETs were essentially associated with neutrophils *in situ* and were not related to BP clinical activity at diagnosis. *In situ* observation of NETs was associated in 6 among 8 patients with serum capacity of NET induction. Notably both blister fluid and sera from BP patients at diagnosis and at time of relapse could induce NET formation *ex vivo*. In contrast, a longitudinal investigation showed a decrease of NET formation with time of treatment in patients undergoing remission. Mimicking relapse, complementation of sera from BP patients with ongoing remission with either IL-17A or IL-23 increased NET formation. Conversely, IL-17A inhibited NET formation induced by serum from BP patients with relapse supplemented or not with IL-23. Finally, glucocorticoids also inhibited NET formation *ex vivo* in BP.

Conclusion: NET formation is an associated phenomenon with BP. Furthermore, we showed that IL-23 favored NET formation, whereas the effects of IL-17A are environment dependent. Indeed, IL-17A displayed a protective effect on NET formation when associated with IL-23, showing for the first-time differential effects of these two cytokines in BP.

Keywords: neutrophil extracellular traps, eosinophil extracellular traps, cytokine, autoimmunity, inflammation, bullous pemphigoid

INTRODUCTION

Bullous pemphigoid (BP) is an invalidating autoimmune subepidermal blistering disease affecting preferentially the elderly. Eosinophils and neutrophils are the most represented cells in the skin inflammatory infiltrate of BP patients (1). Both cells have the capacity to form DNA traps (2–5) which play a pathogenic role in several autoimmune diseases (6, 7). The participation of eosinophil DNA extracellular traps (EET)s to blister formation has recently been reported in BP (7, 8). In contrast, neutrophil extracellular trap (NET) presence has not been demonstrated yet. Additionally, DNA trap formation has never been investigated in BP with respect to the clinical features of the disease i.e., activity and outcome. Also, the relationship between NET formation and the autoimmune or inflammatory responses, which characterize BP development, needs to be clarified.

Neutrophils and eosinophils are both involved in BP pathological process (8–13). Neutrophils play a crucial role in most BP experimental models to induce dermal-epidermal separation (10, 14–16). The cooperation with monocytic cell-line and the stimulation by CXCL-10, IL-17, or IL-23, lead neutrophils to release metalloproteinase MMP-9 and neutrophil elastase, which are responsible for dermal-epidermal junction (DEJ) disruption and BP180 cleavage (17–22). Besides, eosinophils are the predominant cells in the human BP inflammatory infiltrate (1). The activated eosinophils release a potent army of cytokines (9, 11) and granule proteins such as Eosinophil Cationic Protein (ECP), which serum concentrations parallel BP activity (23). The actions of both granulocytes converge to MMP-9 production, which suppresses the neutrophil elastase natural inhibitor, and thus contributes to dermal-epidermal splitting (24).

Stimuli inducing NETs release are numerous and highly variable. For instances auto-antibodies (AAb)s, as well as cytokines, appear to be able to induce this phenomenon (5, 6, 25). Indeed, AAbs from patients with rheumatoid arthritis and IL-17 were recently shown to induce NET formation in an *ex vivo* model of rheumatoid arthritis (25). In BP, the pathogenic role of anti-BP180 antibodies has been illustrated by both *in vitro* and *in vivo* studies, and their serum concentrations at diagnosis have been correlated with disease activity (14, 26–30). Cytokines also play a key role in BP pathogeny (17–19, 31–34). In previous studies, we showed that IL-17 levels were elevated in blister fluids, linked to a local production by neutrophils and mastocytes (17), and a relationship between IL-17 axis cytokines and BP outcome (18). More precisely, we evidenced an increased serum level of IL-23 or a high sustained serum level of IL-17 despite treatment in BP patient who later relapsed (18). Moreover, these inflammatory mediators are involved in BP pathophysiological process, as they enhance MMP-9 production by innate immune cells from patients (17, 18).

In the present study, we investigated DNA extracellular traps in BP with respect to clinical and immunological characteristics of the disease. Therefore, the aim of this study was to determine whether NETs or EETs or even both were associated with BP at tissue level, and to investigate IL-17 and IL-23 influence on NET formation *ex vivo*.

MATERIALS AND METHODS

Patients

This prospective, observational, and translational study was conducted in our tertiary Referral Center for Autoimmune Bullous Diseases at the Reims University Hospital. Consecutive patients with newly diagnosed BP were included in this prospective study. Diagnosis of BP was made when the following criteria were met: presence of at least 3 out of 4 established clinical criteria by Vaillant et al in combination with positive direct immunofluorescence findings (30). Routine skin biopsy specimens of 11 BP patients were provided by the Pathology Department of the Reims University Hospital to perform *in situ* analysis. *Ex-vivo* assays were performed with sera from these 11 patients collected at time of diagnosis (at the same time as the biopsy) and with biological samples [sera and polymorphonuclear cells (PMN)] collected at time of diagnosis and around 150 and 360 days after, from 17 other consecutive BP patients. Sera were also collected at time of relapse in patients who underwent relapse despite treatment. PMNs used for *ex vivo* experiments were freshly isolated PMNs from patients with BP collected at any time point (between D1 and D360 after diagnosis) throughout the course of the study. Seven sera and PMN from healthy controls were provided by French Blood Agency and volunteers (mean age 66.4 years).

In situ Analysis of NETs/EETs

Immunofluorescence and confocal analysis of NETs and EETs were performed on paraformaldehyde-fixed and paraffin-embedded skin biopsy specimens from 11 BP patients. DNA staining along with neutrophils and eosinophils immunostaining was performed as follow on tissues. Four consecutive deparaffanized sections were used by patient, using a distinct eosinophil marker on each. After 15 min heat

retrieval in sodium citrate buffer pH6, the sections were then blocked with PBS-BSA 3% for 30 min at room temperature. Then, simultaneous staining was performed for 30 min at room temperature with mouse anti-human myeloperoxidase (1:150; *R&D Systems, Minneapolis, USA)* for neutrophil staining and another primary antibody (Ab) for eosinophil staining: either rabbit anti-human IL-5Rα (1:150; *Proteintech*, *Chicago, USA*), or rabbit anti-human MBP, EDN, or ECP (1:150; *Novus Biologicals, Littleton, USA*). This was followed by 30 min of incubation at room temperature with matched secondary Abs: chicken anti-rabbit IgG Alexa Fluor 488 and chicken anti-mouse IgG Alexa Fluor 594 (Invitrogen). Sytox Orange Nucleic Acid Stain (5 μM) (*Molecular Probes, Invitrogen, Carlsbad, USA)* was finally used to stain DNA prior to mounting slides with Dako® fluorescent mounting medium. The presence of NETs *in situ* was confirmed by an immunostaining performed with a rabbit polyclonal Ab to Histone H3 (1:100 overnight; *Abcam*, *Cambridge, UK)* revealed by a secondary chicken anti-rabbit Ab Alexa Fluor 594 (1:200. *Invitrogen, Carlsbad, USA*) and a mouse monoclonal Ab to human myeloperoxydase coupled to a FITC fluorochrome *(1:100; Abcam, Cambridge, UK)*.

Extracellular DNA filaments associated to both eosinophils and neutrophils were investigated by 2 independent investigators using 3-dimensional imaging by confocal scanning microscope LSM 710 NLO Zeiss (Zeiss SAS, Germany). Image analysis was performed by using ZEISS ZEN software (Zeiss SAS, Germany) and IMARIS Software (Bitplane, Switzerland). ETs were identified as shapes of DNA extruding from cell and stained by nucleic acid probe SYTOX orange. NETs were defined as extracellular DNA filaments stained by Sytox Orange extruding from cells labeled by MPO Ab, while cell MBP, EDN, ECP or IL-5R labeling characterized EETs.

To qualify eosinophil and neutrophil cell infiltrate, hematoxylin-eosin coloration of serial sections was performed for these 11 biopsy specimens. A rating from 0 to + + + was assigned by two operators to eosinophil and neutrophil infiltrates in the papillary dermis and in the blister cavity to allow a comparative semi-quantification of these cells.

Ex vivo Analysis of NET Formation

Peripheral PMNs cells were obtained by density-gradient centrifugation from 25 mL heparinized-treated whole blood (Granulosep, Eurobio-Abcys). NET generation was performed following the protocol proposed by Brinkmann et al. (35) with the difference that SYTOX Orange was used to stain extracellular DNA. 2.10^5 PMNs were incubated with either 5% blister fluids or 5% sera from BP patients, following preliminary optimization assays (**Supplementary Figure S1**). To investigate their potential influence on NET formation, recombinant IL-17A and IL-23 were added to cell culture medium at concentrations close to those determined in the sera of BP patients (1.2 ng/mL) (18). To assess the effects of treatment on NET induction by BP sera, methylprednisolone MP (10 μM), and Compound A (10 μM) were added to the cell culture medium in presence of BP sera. Each condition was tested with biological fluids from at least 3 different subjects (patients or controls).

NETs were visualized using an inverted epifluorescence microscope (AxioObserver Z1: Zeiss, Germany; camera Cool Snap HQ2: Roper Scientific, France). For each slide, a mosaic of 4 × 4 consecutive images was taken (scale image: 0.625 μm/pixel, driven by Metamorph Software (Roper scientific, Evry, France), which represents an area of around 2 mm^2 per coverslips. Fluorescence signal was collected with bandpass 545/25 excitation filter and bandpass 605/70 emission filter. For each acquisition, fluorescence, and transmitted images were taken.

To assess NETs formation, the area occupied by the DNA filaments was determined using a dedicated home-made macro based on "skeletonize function" and the "analyse particle" tool running under IMAGE J® software (ImageJ, U. S. National Institutes of Health, Bethesda, USA.). Briefly, we skeletonized the fluorescent images, setting 40 pixels as the minimal resolution, and transformed these images into drawings of DNA filaments, which were measured and processed by the "Analyze particles" tool (**Supplementary Figure S1**). Area of each filament was measured, and the mean was calculated, making the conversion from pixels to μm (1pixel = 0.625 μm). The total number of cells was counted using the counter cell tool of Image J® software on the transmitted image acquired simultaneously with fluorescent image. Thus, this method allowed measuring the total number of PMNs, and the total area of NETs. Results were expressed in mean area of NETs per PMN (as proposed by Rebernick et al. (36). As variations were observed when using PMNs from different subjects, results were also expressed in ratio between mean area of NETs per cell for condition X and mean area of NETs per cell for the reference condition with the same PMNs.

Statistical Analysis

As the distribution of the variables could not be assumed to be normal, comparisons between two groups were performed using the Wilcoxon matched pairs signed rank test for paired data and the Mann-Whitney test for unpaired data. When more of two matched groups were compared, Friedman test was performed. Ratio paired *t*-test was used to compare NETs induction by sera with and without treatment (by cytokines, methylprednisolone or compound A). Statistical difference was considered significant if *p*-value was 0.05 or less.

RESULTS

DNA Extracellular Traps Mainly Originated From Neutrophils *in situ*

To investigate the presence of either NETs or EETs or even both in BP at site of lesion, immune-detection of neutrophils and eosinophils along with DNA staining was performed on paraffin-embedded skin biopsy specimens from 11 BP patients. The mean age of the 11 newly diagnosed BP patients (6 female (54.5%) and 5 males (45.5%), sex ratio F/M: 1.2) was of 78.9 years. At baseline, the clinical BP activity was assessed by the Bullous Pemphigoid Disease Assessment Index (BPDAI) score and by the number of new daily blisters. The BPDAI median value was of 53 [23–90], and 9 (81.8%) BP patients presented with a severe disease

characterized by more than 10 blisters a day. Finally, 5 (45.5%) BP patients experienced a relapse despite treatment during the first year of follow up (**Table 1**).

Extracellular DNA filaments were identified *in situ* in 8 out of the 11 (73%) BP patients (**Figures 1A–C**). NETs were observed on lesional skin biopsy specimen, in the papillary dermis at the edge of the dermal-epidermal separation (**Figures 1A–G**). A 3-dimensional reconstruction picture corresponding to **Figure 1E** allowed defining NETs as shapes of DNA extruding from neutrophils stained by MPO (**Supplementary Video S1**; **Figure 1F**). EETs were only observed in one patient despite a higher number of eosinophils than neutrophils in BP lesional skin, corroborated by both HES staining and immunofluorescence using specific eosinophils markers including MBP, EDN, ECP, or IL-5Rα (**Figure 1H**; **Table 2**). DNA extracellular trap was observed neither in blister cavity nor in perilesional dermis, despite the presence of numerous inflammatory cells. Besides, in those 8 BP patients, NETs were absent from skin biopsy specimens performed at distance of the lesions (data not shown). Also, no DNA traps were evidenced in 4 plastic surgical normal skin specimens (mean age of controls 66.5 years) (data not shown).

In situ occurrence of NETs appeared to be independent of the disease activity at time of diagnosis. Indeed, patients in whom NETs were seen, showed miscellaneous BPDAI scores with values ranged from 23 to 90 and a median of 50.5 (**Tables 1**, **3**). Furthermore, these 8 BP patients were evenly distributed according to the BPDAI score, as 3 patients displayed a BPDAI score superior to 56, 2 with a BPDAI close to mean value between 48 and 53, and 3 with a BPDAI inferior to 36. Also, NETs were observed in both patients with a multibullous BP (displaying more than 10 new blisters per day and up to 120 new daily blisters), and in those with a limited disease (down to only one blister per day). Likewise, the presence of NETs was not associated with BP relapse, as among the 8 BP patients displaying NETs at baseline, 4 later underwent a relapse and 4 remained on remission over the one-year of clinical follow-up (**Table 1**).

Both Neutrophils and Biological Fluids From Patients With BP Were Required for NET Formation

To further investigate the factors involved in NET formation associated with BP, we established an *ex vivo* NET generation model with peripheral blood PMNs from patients with BP stimulated with blister fluid collected at time of diagnosis (**Figure 2A**). Similar NET formation level was observed when PMNs from BP patients were stimulated with BP serum (**Figures 2B–D**, $p = 0.25$, Wilcoxon matched pairs test). Besides, immunostaining of myeloperoxydase and citrullinated histones allowed to confirm that neutrophils were the source of the observed NETs in this *ex vivo* model (**Figure 2C**). The induction of NETs by blister fluids was independent from BPDAI score at diagnosis and from BP 180 Ab levels in blister fluids (**Figures 2E,F**). We then investigated the capacity of the sera from the 11 BP patients for whom *in situ* exploration of NETs was performed, to induce NET formation. Among the 8 patients previously identified with NETs *in situ*, 6 showed capacity of NET induction compared with 1 patient among the 3 for whom *in-situ* assessment was negative (**Table 1**).

In contrast, the capability of sera from healthy donors to induce NET formation by PMN from BP patients was significantly lower than sera from BP patients (0.15 vs. 0.74 μm^2/cell, $p = 0.0006$ Mann-Whitney test) (**Figures 2G,H**). To investigate whether circulating PMNs from BP patients were primed to release NETs, we assessed the ability of PMNs isolated from healthy donor peripheral blood to generate NETs upon BP serum stimulation. As showed in **Figure 2I**, NET quantification remained low in those conditions. Also, when PMNs from healthy donors were stimulated with heterologous sera from healthy donors, NET area remained low (**Figure 2J**).

BP Serum Capacity to Induce NET Formation Decreased in BP Patients With Ongoing Remission

Then, we evaluated the ability of BP sera collected during patient's follow up to induce NET release. Compared with baseline values, NET formation was gradually reduced with time of treatment as illustrated by *ex vivo* experiments conducted with sera collected at day150 and day 360 (0.18, 0.13, and 0.06 μm^2/cell, $p = 0.0001$Friedman test) (**Figures 3A–C,E**). Conversely, NET levels remained as elevated as with BP serum at diagnosis when NET generation was performed with the BP sera collected at time of relapse from the same patients (0.13 at baseline vs. 0.15 μm^2/cell at time of relapse, $p = 0.68$ Wilcoxon matched pairs test) (**Figures 3D,F**).

IL-17 and IL-23 Display Differential Effects on NET Formation

As we previously reported that IL-17 and IL-23 remained elevated or even increased in the sera of patients at risk of relapse (18), we further investigated whether these cytokines participated to NET induction in BP. In this line, we complemented BP sera collected after 150 days from patients with ongoing remission with either IL-17A or IL-23 or even both. Both IL-17A and IL-23 independently enhanced NET formation with an even greater effect under IL-23 stimulation (1.9-fold increase with IL-17, $p = 0.029$, and 10-fold increase with IL-23 with respect to the stimulation with D150 sera alone, $p = 0.04$ ratio paired *t*-test, **Figures 4A–D,F,G**). We then assessed the combined effects of both cytokines, IL-17A and IL-23 on NET generation. The high NET formation level induced by addition of IL-23 was reduced when IL-17A was added to the cell culture medium (**Figures 4E,H**).

To further investigate the potential lowering effects of IL-17A on IL-23-induced NETs release, we added IL-17A to the potent NET inducer sera collected at the time of relapse. Supporting the above-mentioned observation, IL-17A addition (alone or combined with IL-23) significantly decreased the area occupied by NETs with respect to the area obtained with the serum of BP patients collected at time of relapse (0.09, and 0.17 vs. 0.45

TABLE 1 | Individual characteristics of BP patients.

Patient	NETs		Baseline						Follow-up							
			Age at diagnosis	Sex	Nb blisters/ day	BPDAI	Serum antibodies		Relapse	Day 150			Day 360			
	Presence *in situ*	*Ex vivo* NET induction (mean area of NET μm^2/cell)					Anti-BP180 (U/mL)	Anti-BP230 (U/mL)		BPDAI	Anti-BP180 (U/mL)	Anti-BP230 (U/mL)	BPDAI	Anti-BP180 (U/mL)	Anti-BP230 (U/mL)	
1	+	**1.141**	84	F	120	**90***	**150**	**94**	+	34	**59**	**30**	7	**52**	**71**	
2	+	**2.02**	95	F	30	**65***	**64**	**83**	+	**59***	**47**	7	10	**81**	**32**	
3	+	0.285	81	F	73	**65***	**81**	**64**	–	7	**22**	**14**	7	**10**	**9**	
4	+	0.130	58	F	26	53	**56**	1	+	32	**74**	5	D	D	D	
5	+	**0.471**	75	M	10	48	**116**	5	–	2	9	5	0	0	0	
6	+	**0.501**	87	M	15	36	1	1	–	2	7	5	0	7	7	
7	+	**0.798**	78	M	10	23	1	2	+	0	2	4	4	0	0	
8	+	**0.503**	81	M	1#	23	9	**20**	–	1	7	**10**	1	7	20	
9	–	0.382	79	M	20	**73***	**68**	**35**	+	31	**41**	**21**	6	8	5	
10	–	0.433	90	F	10	**56***	**79**	3	–	5	**38**	3	10	**21**	5	
11	–	**0.636**	60	F	5#	30	**26**	1	–	6	**16**	0	23	**11**	5	

**Severe disease according to BPDAI ≥56;*

#patients with moderate disease defined by a number of new daily blisters <10; Relapse was defined as the reappearance of at least 3 new daily blisters in between one-year follow-up. Bold characters: out of normal range values i.e., NET area per PMN >0.445 μm^2/PMN, which is the mean value obtained by stimulation of the same PMNs with healthy sera + 2SD; Anti-BP180 Ab >9 U/ml, Anti-BP230 Ab >7 U/ml. BPDAI, bullous pemphigoid disease area index, Nb number.

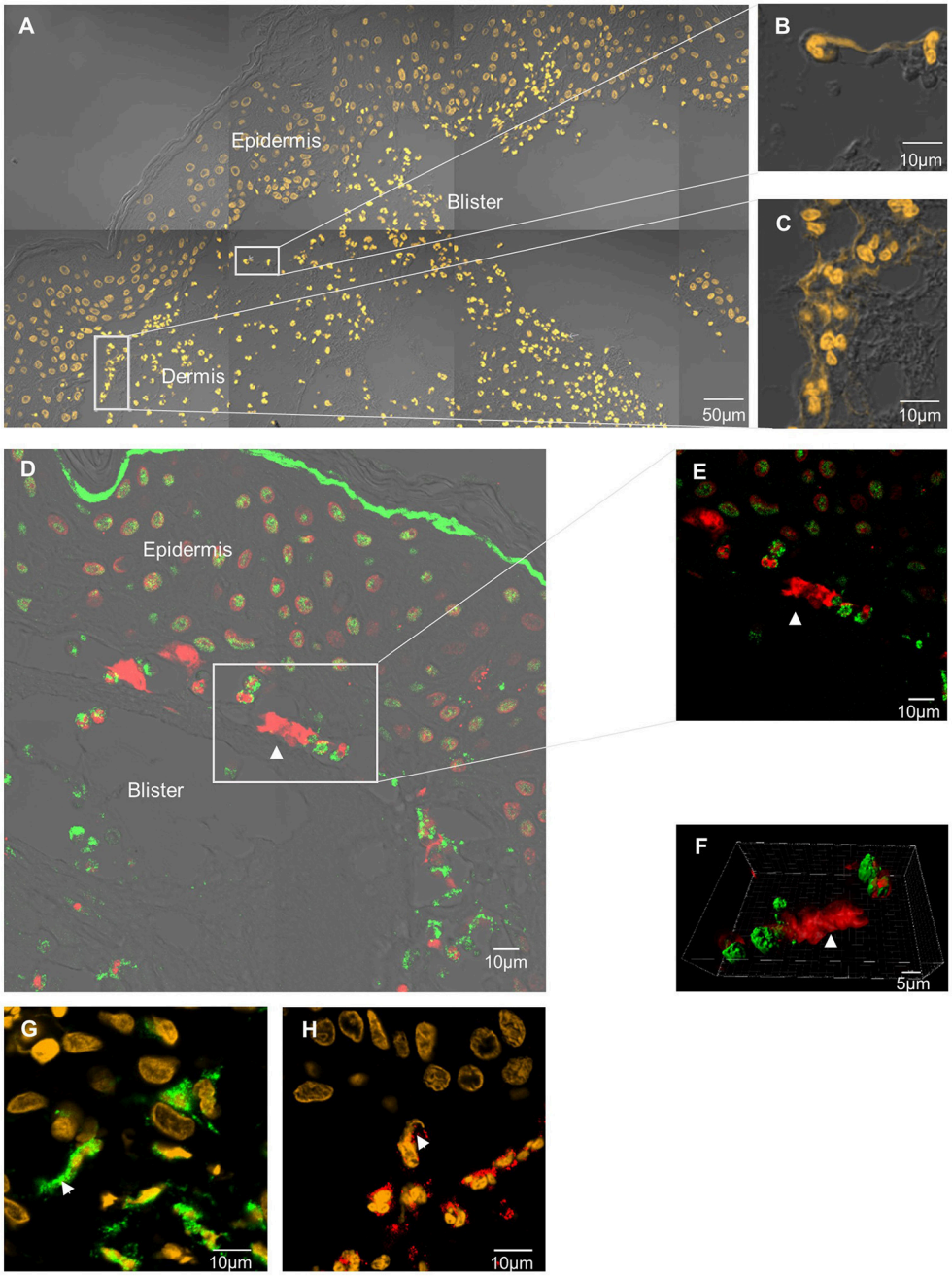

FIGURE 1 | *In situ* exploration of DNA traps in BP. DNA extracellular traps are located at the edge of the DEJ splitting, in the papillary dermis of BP patient skin. Skin biopsy specimen stained by SYTOX Orange nucleic acid probe **(A–C)**. Neutrophil extracellular trap stained by anti-histone H3 Ab *(red)* and anti-myeloperoxidase Ab *(green)* **(D–F)**. 3-dimensional reconstruction imaging by confocal microscopy and IMARIS® software of this NET **(F)**. Magnification 80x, numerical aperture of 1, z-stack with 0.67µm step-size. Neutrophil extracellular trap stained by Sytox orange and anti-myeloperoxidase *(green)* **(G)**. Eosinophil extracellular trap stained by Sytox orange and anti-eosinophil-derived-neurotoxin Ab *(red)* **(H)**.

µm²/cell, $p = 0.05$ and $p = 0.02$, respectively, ratio paired t-test) (**Figures 5A–C,E,F**). In contrast, supplementation of these sera collected at time of relapse with IL-23, did not affect their capacity to induce NET formation (0.48 vs. 0.45 µm²/cell, $p = 0.89$ ratio paired t-test) (**Figures 5D,G**).

NET Formation Is Inhibited by Methylprednisolone and by Compound A

Having demonstrated that the capacity of BP sera to induce NET release progressively decreased in BP patients with ongoing remission upon treatment, we then wondered whether

TABLE 2 | Inflammatory cell infiltrate in BP skin according to the presence of NETs forming cells *in situ*.

Patient	Blister cavity		Papillary dermis		NETS
	Eosinophils	Neutrophils	Eosinophils	Neutrophils	
1	+	0/+	0/+	0/+	+
2	+++	+	++/+++	+/++	+
3	++	+++	0/+	0/+	+
4	++/+++	++	++/+++	++	+
5	+++	++	++	+	+
6	+++	0/+	+++	+	+
7	+++	++	++/+++	+	+
8	+	+	0/+	0/+	+
9	+++	+/++	+++	+	–
10	+++	+/++	++	0/+	–
11	++	+++	+	+	–

Quality of cell infiltrate assessed independently by two operators on Hematoxylin-eosin stained sections with respect to the presence of NETs determined by immunofluorescence on a serial section of the same skin biopsy. A rating from 0 to +++ was assigned to eosinophil and neutrophil infiltrates in the papillary dermis and in the blister cavity to allow a comparative semi-quantification of these cells. (n = 11).

TABLE 3 | Clinical and biological characteristics of BP patients according to the *in-situ* observation of NETs.

	"NETs positive" patients	"NETs negative" patients
CLINICAL CHARACTERISTICS		
Number of patients, *n*	8	3
Age (y); sex ratio F/M	79.9y; 1	76.3y; 2
BPDAI total score, mean ± SD	50.3 ± 23	53 ± 21.6
Total skin activity, mean ± SD	49.9 ± 23	50.6 ± 23.6
Blisters/Erosions, mean ± SD	32.2 ± 16.7	41.7 ± 13.8
Erythema/urticaria, mean ± SD	17.6 ± 9.6	9 ± 14.7
Patients with severe disease according BPDAI,[a] *n*	3/8	2/3
Patients with at least 10 daily new blisters, *n*	7/8	2/3
Relapse[b]	4/8	1/3
BIOLOGICAL CHARACTERISTICS		
Positive serum anti-BP180 NC16A IgG, *n*	5/8	3/3
Mean anti BP180 NC16A IgG, U/ml	93.4	57.7
Positive serum anti-BP230 IgG, *n*	4/8	1/3

[a] *Severe disease was defined by BPDAI ≥56.*

[b] *Relapse was defined as the reappearance of at least 3 new daily blisters in between one-year follow-up. No statistically significant difference was observed using Mann Whitney test. n, effective, y, years. BPDAI, bullous pemphigoid disease area index.*

corticosteroid therapy also reduced the capability of BP sera collected at baseline to induce NET formation. NET quantification revealed that both methylprednisolone (10 μM) and compound A (also named 2-(4-acetoxyphenyl)-2-chloro-N-ethyl ammonium chloride, a natural glucocorticoid receptor ligand (37), 10 μM) significantly decreased the capacity of BP serum to induce NET formation by BP PMNs (0.05 and 0.04 vs. 0.14 μm^2/cell, $p = 0.04$ and $p = 0.01$, ratio paired *t*-test) (**Figure 6**).

DISCUSSION

NET formation has recently been reported in several autoimmune diseases as a phenomenon potentially associated to tissue damage and/or loss of tolerance mechanisms (6). In this study, we brought *in situ* evidence of the presence of NETs in BP at the extremity of the blister where dermis separates from epidermis. Using an *ex vivo* model, we also showed that both BP neutrophils and biological fluids (blister fluid, serum) from BP patients were required for NET formation. Analysis of NET presence at tissue level with respect to BP clinical and to immunological data revealed that NET formation was not associated with specific characteristics of the disease at baseline. However, a longitudinal analysis of NET formation using our *ex vivo* model discriminated sera from BP patients with ongoing remission under treatment from those collected at time of relapse. Based on our previous studies (17, 18), we identified IL-23 as a potent NETs enhancer in BP. In contrast, we evidenced in BP a protective role of IL-17A despite the presence of potent inducers such as IL-23 or relapse sera, demonstrating for the first time in BP antagonist functions for these 2 cytokines.

To the best of our knowledge, we here evidenced for the first time the presence *in situ* of NETs in BP. Actually, NETs were observed in 8 out of 11 patients with BP at baseline. In contrast, EETs were present only in 1 case despite the use of 4 different markers on 4 sequential sections for eosinophil immunostaining. The absence of EETs could not be explained by the quality of the inflammatory infiltrate, as eosinophils were the most represented immune cells where NETs were detected. It is possible that EETs formation rather occurs in the skin of BP patients with tissue eosinophilia as previously described (7). So far, in our study NETs were observed in the skin biopsy from BP patients whatever disease activity, although the quantity of NETs observed remained low in all cases. Thus, our observations suggest that NET formation is a common but quantitatively rare process associated with BP.

Noticeably, NETs localized precisely at the very edge of dermal-epidermal separation. NET formation could be dictated by skin microenvironment. Furthermore, most patients for whom *in situ* exploration was positive, displayed at the same time capacity to induce NET formation *ex vivo* when BP PMNs were cultured in presence of their sera. Actually, we showed that both body fluid and PMNs from BP patients were required for NET formation. Such specific NET localization at the extremity of blister where the epidermis separates from the dermis could be related to a role of NETs in blister formation as it was demonstrated using an *ex vivo* model that degradation of DNA filaments inhibited DEJ splitting (8).

Although immunoglobulins have been associated with NET formation (38–42), such process could not be foreseen in BP. Indeed, no correlation could be drawn between NETs' presence *in situ* or NET formation *ex vivo* and serum or blister fluids levels of anti-BP180 AAb. Besides, the fact that NETs were observed in most skin biopsy specimen independently of disease

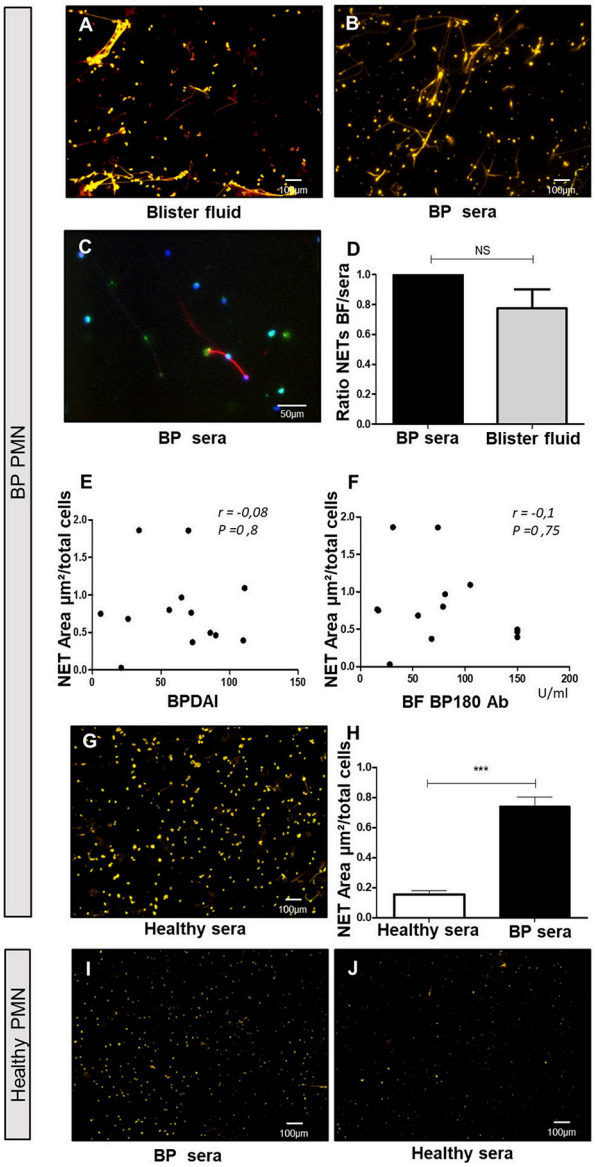

FIGURE 2 | Blister fluids and BP sera induce NET formation by BP PMN but not by control PMN. *Ex vivo* stimulation of BP PMNs with BP blister fluids (BF) **(A)**, and BP sera **(B,C)** stained by Sytox orange **(A,B)** or anti-Histone H3 and anti-MPO Ab **(C)**. NET area induced by BP sera vs. BF from the same patients on heterologous BP PMNs **(D)** ($n = 3$); results expressed in ratio of NET area induced by BF to NET area induced by serum of the same patient, results represent mean ± SEM, NS, non-significant. Correlation of NET area induced by heterologous BF with BPDAI **(E)** and BF concentration of BP180 Ab **(F)**. Stimulation of BP PMN with healthy control sera **(G)** and comparison with NET area induced by BP sera **(H)** ($n = 7$), $^{***}p < 0.001$, results represent mean ± SEM. Stimulation of PMNs from healthy controls by BP sera **(I)**, or heterologous healthy sera **(J)**. Magnification 20x. BPDAI, Bullous Pemphigoid Disease Area Index; BF BP180 Ab, concentrations of anti-BP180 antibodies in blister fluids.

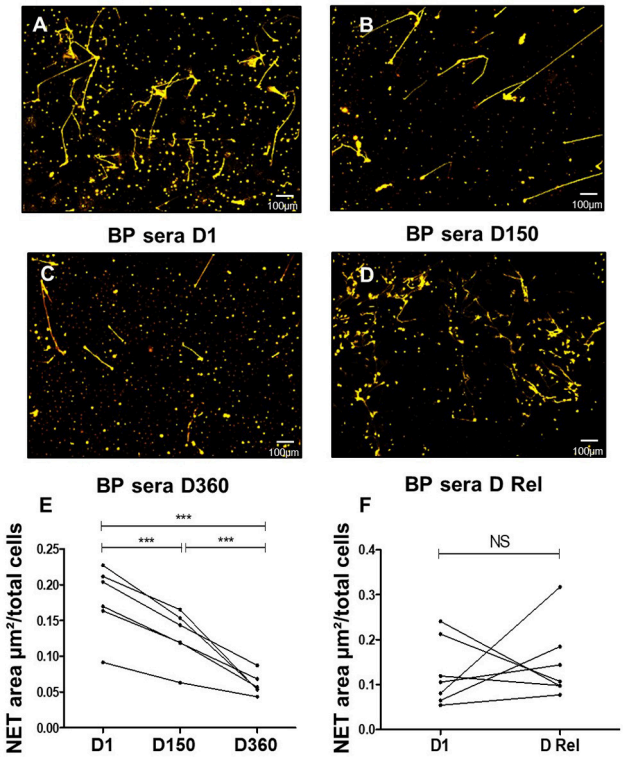

FIGURE 3 | NETs are induced more efficiently by BP sera at diagnosis and at time of relapse. *Ex vivo* stimulation of BP PMNs with heterologous BP sera collected: at baseline **(A)**, around 150 days after diagnosis in patients with controlled disease **(B)**, around 360 days after diagnosis in patients undergoing remission **(C)**, or at time of relapse in patients undergoing relapse **(D)**. Magnification 20x. NET area measured for each condition: stimulation of BP PMNs with BP sera collected Baseline (D1) vs matched sera collected at Day 150 and Day 360 **(E)** ($n = 6$), $^{***}p < 0.0001$. NET area measured after stimulation of BP PMNs with BP sera collected at baseline (D1) vs. matched sera collected at time of relapse (D Rel) from the same patients **(F)** ($n = 7$), NS: non-significant.

activity, suggests that NET inducers may be present in all body fluids from BP patients at baseline. Indeed, sera from BP patients in clinical remission were less efficient to induce NET formation. Altogether, this suggests that NET induction is rather associated with inflammatory mediators than with the autoimmune response in BP. In this setting, it was shown that inflammatory molecules such as cytokines and complement components were potent inducers of DNA traps (5, 6, 43–45).

Neutrophils' priming is a required step for NET formation in BP. In BP skin environment, the presence of cytokines able to activate innate cells such as neutrophils, eosinophils and monocytes has already been largely described (17–19, 31). Such inflammatory response may be responsible for neutrophils' priming and explain why NET formation level remained low in neutrophils from healthy donors even when stimulated with BP serum. Accordingly, it has been shown that neutrophils from normal individuals released NETs upon IL-17A stimulation only after having been primed by TNF-α (25). In BP, the importance of neutrophils was also highlighted by the capacity of both classical corticoids and Compound A to inhibit NET generation induced by serum collected at baseline. However, further studies are required to determine the cytokines involved in neutrophils sensitization to NET formation in BP.

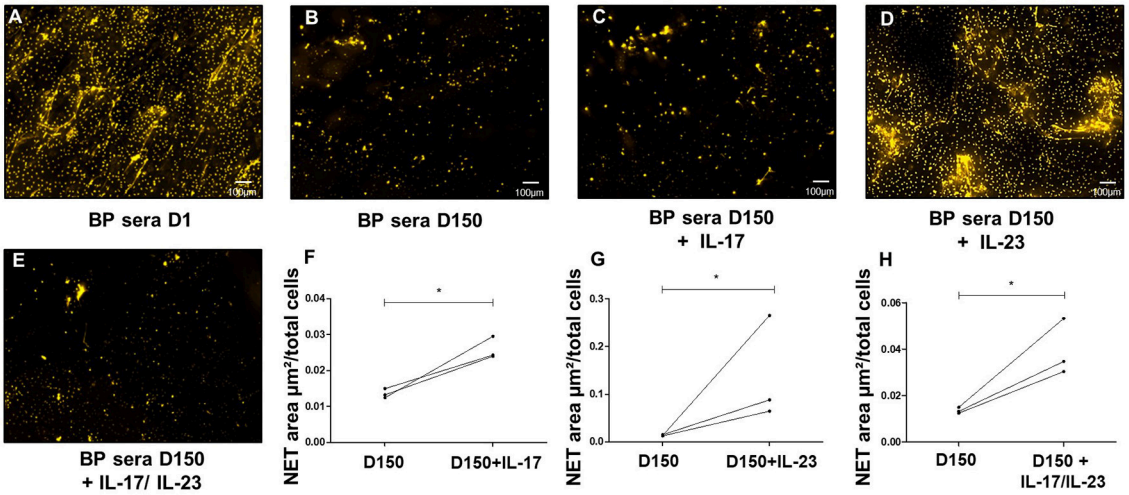

FIGURE 4 | IL23 is critical to NETs formation. *Ex-vivo* stimulation of BP PMNs with BP sera collected at baseline **(A)**, around 150 days after diagnosis in patients with controlled disease **(B)**, and day 150 sera added with either IL-17A **(C)**, IL-23 **(D)**, or both IL-17A and IL-23 **(E)** (1.2 ng/mL for both cytokines). Comparison of NET area measured when cytokines IL-17A, IL-23, or both were added to day 150-serum with NET area induced by day 150- serum alone **(F–H)** ($n = 3$), * $p<0.05$.

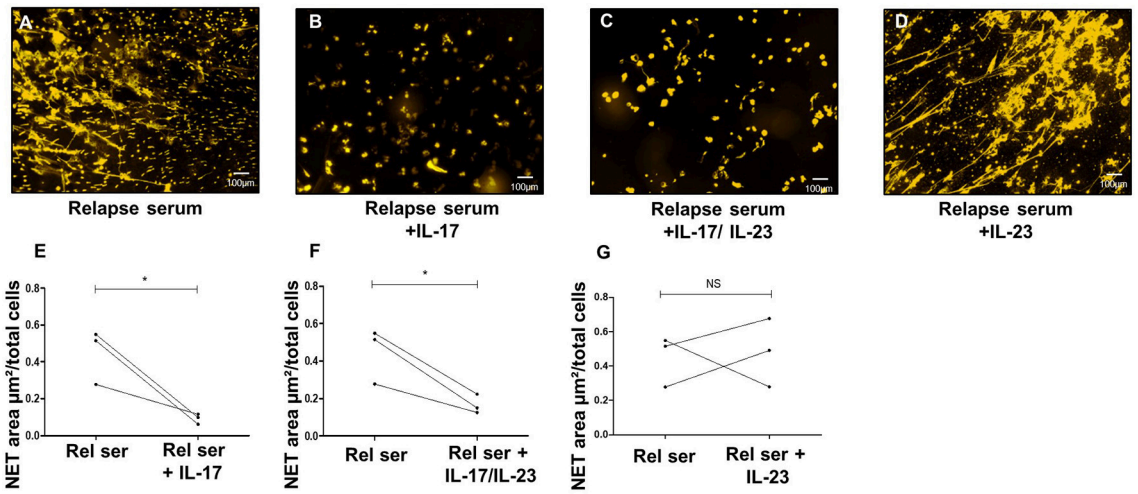

FIGURE 5 | IL-17A inhibits NET formation by BP sera at time of relapse. *Ex vivo* stimulation of BP PMNs with BP sera collected at time of relapse alone **(A)**, or in presence of either IL-17 **(B)**, or both IL-17 and IL-23 **(C)**, or IL-23 **(D)**. Comparison of NET area measured when cytokines were added to serum collected at time of relapse with NET level generated in presence of this relapse serum (Rel Ser) alone **(E–G)** ($n = 3$), *$p<0.05$.

Serum from BP patients with relapse holds on high level of NET formation. This suggests that NETs' triggers remained present in the serum of those patients even under therapy. In this line, we previously reported that the concentration of IL-17 and IL-23 remained elevated or even increased in the sera of BP patients at risk of relapse (18). Noteworthy, using our *ex vivo* model, we demonstrated that IL-23, and to a lesser extent IL-17A, could enhance NET formation in BP neutrophils. While IL-17 is known to potently recruit neutrophils, a role and the mechanisms associated with this cytokine in the induction of NET have still not been fully elucidated. Activation of p38-Mitogen-activated protein kinase (p38 MAPK) and Nuclear Factor Kappa B (NF-κB) pathways by IL-17A (46–49) may explain this property, as both are involved in NET formation (50–52), and p38 MAPK pathway mediates IL-17 induced ROS production (46). To our knowledge, the role of IL-23 in NET generation had not been evaluated yet. Neutrophils constitutively express low amount of IL-23R, which are up-regulated upon activation (53).Three activation cascades may be activated following IL-23 transduction signal among which the above-mentioned p38 MAPK and NF-κB pathways but also the mammalian target of rapamycin (mTOR) pathway which play a pivotal role in NET formation and may explain the higher capacity of IL-23 to induce NET formation in BP (50, 51, 53, 54). In this line, no correlation could be drawn between

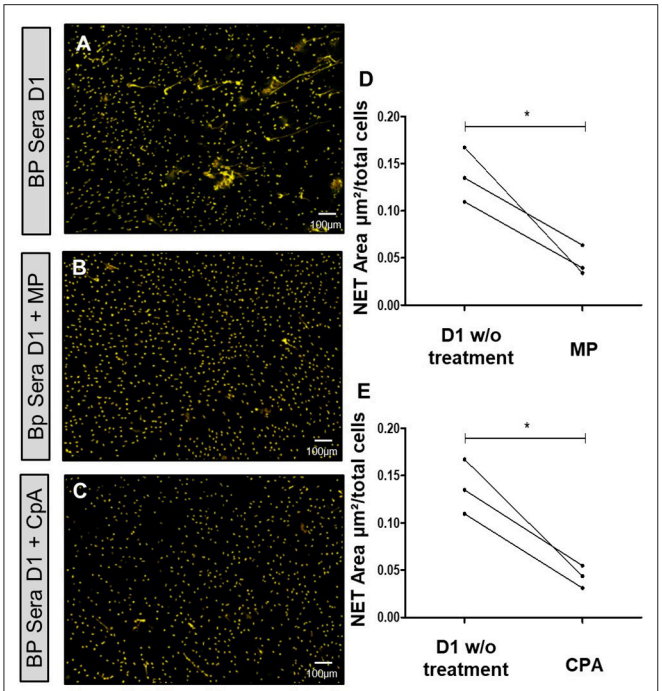

FIGURE 6 | NET formation is inhibited by methylprednisolone or Compound A. *Ex vivo* stimulation of BP PMNs with BP sera collected at diagnosis (D1) alone **(A)**, or in presence of either methylprednisolone **(B)**, or compound A **(C)**. Comparison of NET area measured when treatments were added to baseline serum with NET formation by this baseline serum without treatment **(D,E)** ($n = 3$). *$p < 0.05$, mean ± SEM are represented. MP, Methylprednisolone; CpA, Compound A; w/o, without.

IL-17 or IL-23 blister fluids concentrations and NET formation ($r = -0.5$, $p = 0.14$, and $r = -0.71$, $p = 0.06$ respectively), suggesting that IL-17A and IL-23 were not the only triggers of NET formation in BP. Observed effects of these cytokines may also result from synergic actions of several cytokines and further studies are needed to understand mechanisms of NET induction in BP.

This study is the first to our knowledge to demonstrate tendentious functions of IL-17A with respect to IL-23 presence. Indeed, we observed a protective effect of IL-17A supplementation on NET formation induced either by IL-23 itself or by sera from BP patients with relapse, highlighting the role of disease specific microenvironment in cytokine's function. In the same line, blocking IL-17 signaling in inflammatory bowel diseases resulted in an increased expression of pro-inflammatory chemokines and cytokines (55, 56). Some molecules have recently been described in such regulation mechanisms. Suppressor of cytokine signaling 3 (SOCS3) is therefore known to inhibit Janus kinase 2 activity, thereby decreasing IL-23 induced effects (57–59). Yet, SOCS3 expression has recently been correlated with severity of inflammation, expression of proinflammatory cytokines, and activation of p38 MAPK pathway (60). SOCS3 expression is also induced by IL-17 family cytokines (61, 62). Then, although this has to be proven, we hypothesized that in BP IL-17A attenuates IL-23 induced NET formation by inducing SOCS3 expression as previously demonstrated in the airway epithelium (62). Such effects could explain the limited quantity of NETs observed in the skin of BP patients. Furthermore, our present results demonstrate that, in the future, patients with bullous pemphigoid could be further divided into subgroups according to the biomarkers expressed to identify an adequate candidate for biotherapy maintenance in relay of corticosteroid in patients at risk of relapse.

NETs are involved in loss of tolerance mechanisms in several autoimmune diseases (6). In BP, NETs release could participate to both autoimmune and inflammatory responses. Auto-antigens may be present at the surface of NETs, which prolongs their exposition, subsequently favoring an autoimmune response (63, 64). In this line, NET formation is associated with production of reactive oxygen species, and granule enzymes cover the DNA filaments (2, 6, 65). Several studies showed the involvement of NETs in tissue damage (66, 67). Brinkmann et al. reported the association of NETs with various proteases such as neutrophil elastase (5). Yet, neutrophil elastase has largely been implicated in BP pathological processes, as well as matrix metalloproteinase MMP-9 (14, 68). Thus, although NET formation does not directly correlate with BP activity, we showed in this study that NET formation depends on the presence of inflammatory cytokines, and therefore that the inflammatory response associated with BP may participate to BP antigen immunogenicity or to the perpetuation of the disease. Subsequently, our results showing NETs at the dermal-epidermal splitting area, along with the serum capacity to induce NETs at the same time, really advocate for a role of neutrophil in extracellular DNA release, although NET implication in BP still needs to be further demonstrated. Based on the literature, we hypothesize that NET-associated mechanisms may be involved in BP180 antigen immunogenicity, and further in epitope spreading in BP.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

The investigation was conducted under the approval of the Ethic Committee of the University Hospital of Reims (CNIL authorization DR-2013-320), and all of the subjects gave their informed and written consent before participating in the study in accordance with the Helsinki Declaration.

AUTHOR CONTRIBUTIONS

PB, FA, and BP conceived the study. DG, EB, SL, GG, PB, FA, and BN contributed to the study design and data analysis. AD, CM, PB, and FA contributed to the clinical and

histological metadata collection. DG, EB, CT, KD, SL, SN, CM, and BP contributed to the sample processing. CT conceived the macro allowing *ex vivo* NET quantification. DG, EB, and SL performed the statistical analyses. DG, PB, FA, and BP wrote the manuscript and all authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00701/full#supplementary-material

Supplementary Figure S1 | Optimal culture conditions to induce NETosis with BP biological fluids. Representative microphotographs of NETs and their respective drawings generated by *ex vivo* stimulation of BP PMNs with either healthy sera **(A, A', B, B')**, BP sera **(C, C', D, D')** or BP blister fluids **(E, E', F, F')** collected at baseline. PMNs were incubated with 5% biological fluids during either 1 h **(A, A', C, C', E, E')** or 3 h **(B, B', D, D', F, F')**. NETs area generated by BP PMNs after 1 or 3 h stimulation by healthy sera, BP sera and BP BF **(G)**. BF: blister fluids.

Supplementary Video S1 | 3-dimensional-reconstruction of the NET corresponding to **Figures 1E,F** 3D-reconstruction of a netting neutrophil *in situ* by IMARIS® (Bitplane® software, Belfast, UK). Red, Histone; Green, myeloperoxidase. Magnification 80x, numerical aperture of 1, z-stack with 0.67 μm step-size.

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Role of Src and Cortactin in Pemphigus Skin Blistering

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Autoantibodies against desmoglein (Dsg) 1 and Dsg3 primarily cause blister formation in the autoimmune disease pemphigus vulgaris (PV). Src was proposed to contribute to loss of keratinocyte cohesion. However, the role and underlying mechanisms are unclear and were studied here. In keratinocytes, cell cohesion in response to autoantibodies was reduced in Src-dependent manner by two patient-derived PV-IgG fractions as well as by AK23 but not by a third PV-IgG fraction, although Src was activated by all autoantibodies. Loss of cell cohesion was progredient in a timeframe of 24 h and AK23, similar to PV-IgG, interfered with reconstitution of cell cohesion after Ca^{2+}-switch, indicating that the autoantibodies also interfered with desmosome assembly. Dsg3 co-localized along cell contacts and interacted with the Src substrate cortactin. In keratinocytes isolated from cortactin-deficient mice, cell adhesion was impaired and Src-mediated inhibition of AK23-induced loss of cell cohesion for 24 h was significantly reduced compared to wild-type (wt) cells. Similarly, AK23 impaired reconstitution of cell adhesion was Src-dependent only in the presence of cortactin. Likewise, Src inhibition significantly reduced AK23-induced skin blistering in wt but not cortactin-deficient mice. These data suggest that the Src-mediated long-term effects of AK23 on loss of cell cohesion and skin blistering are dependent on cortactin-mediated desmosome assembly. However, in human epidermis PV-IgG-induced skin blistering and ultrastructural alterations of desmosomes were not affected by Src inhibition, indicating that Src may not be critical for skin blistering in intact human skin, at least when high levels of autoantibodies targeting Dsg1 are present.

Keywords: src, cortactin, adhesion, skin blistering diseases, pemphigus vulgaris (PV)

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INTRODUCTION

Pemphigus vulgaris (PV) is a severe autoimmune skin blistering disease. Patients suffer from mucocutaneous erosions and blisters caused by autoantibody-induced acantholysis (1, 2). It is accepted that loss of keratinocyte cohesion is primarily caused by autoantibodies directed against the desmosomal cadherins desmoglein 3 (Dsg3) and Dsg1 (3). Autoantibodies were proposed to

directly interfere with trans-interaction of Dsg3 and to require cellular signaling mechanisms to induce loss of cell adhesion (2). Pemphigus autoantibodies modulate the activity of signaling pathways such as Ca^{2+} -influx, protein kinase C (PKC), p38 mitogen-activated protein kinase (p38MAPK) and sarcoma-associated kinase (Src), and interfere with the turnover of desmosomal components (2). It was shown that Src can be activated by PV-IgG containing antibodies against Dsg3 as well as by AK23, which constitutes a Dsg3-specific monoclonal autoantibody from a pemphigus mouse model (4, 5). However, the role of Src in the loss of keratinocyte cohesion and the underlying mechanisms are not fully understood. Src is a signal-transducing non-receptor protein kinase which is involved in several signaling pathways (6). It is enriched in various cancer diseases and plays an important role in metastasis, cell migration and motility as well as cell survival and proliferation (6–9). Furthermore, Src is located at adherens junctions in various cell types (10), and the inhibition of Src-kinase activity is proposed to stabilize cadherin dependent cell-cell contacts (11). In previous studies we showed that PV-IgG-mediated loss of keratinocyte cohesion can be abrogated by Src inhibition (4), and that the activity of Src in combination with E-cadherin is necessary for the cytoskeletal anchorage of Dsg3 (12). Moreover, Dsg3 forms a complex with Src and regulates its activity (12, 13). All these findings led to the hypothesis that Dsg3 organizes cell contacts coordinating cell adhesion with signaling responses required for cellular behavior (14). Thus, we speculated that Src could be a key regulator of desmosomal adhesion in PV pathogenesis. In this context, it was demonstrated that autoantibodies from pemphigus patients caused Src-dependent phosphorylation of Pkp3 paralleled by Dsg3 translocation to the cytoplasm and destabilization of cell adhesion (15). Since we observed that Src is required for desmosome assembly, which is regulated by actin-binding proteins such as adducin (12, 16), we focused on the actin binding protein cortactin, which was identified as a major substrate for Src (17). Among many other functions, phosphorylation of cortactin is crucial for cadherin-mediated intercellular adhesion strength (18). In the work presented here, we show for the first time that cortactin regulates reconstitution of cell adhesion in pemphigus and provide new insights into the role and function of Src in PV.

MATERIALS AND METHODS

Cell Culture

The immortalized human keratinocyte cell line HaCaT was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA) supplemented with 10% FCS (Biochrom, Berlin, Germany), 50 U/ml penicillin and 50 g/ml streptomycin (both AppliChem, Darmstadt, Germany) in a humidified atmosphere of 5% CO_2 at 37°C.

Preparation of Mouse Keratinocytes

Murine keratinocytes from the epidermis of newborn cortactin-deficient ($CTTN^{-/-}$) and cortactin wildtype ($CTTN^{+/+}$) mice were isolated and immortalized according to the literature for preparation of mouse keratinocytes (19–21). In brief, the skin was taken and incubated for 16 h in 2.4 U/ml dispase II in PBS supplemented with Gentamicin/AmphotericinB (CELLnTEC, Bern, Switzerland) at 4°C. After separating the dermis and epidermis, the epidermis was incubated for 20 min with accutase (CELLnTEC) at room temperature, in order to dissociate the cells. Mouse keratinocytes were resuspended and then grown in complete FAD medium (0.05 mM CaCl) on collagen I-coated culture dishes (rat tail; BD Bioscience, New Jersey, USA). The cells were cultivated in a humidified atmosphere containing 5% CO_2 at 35°C. After reaching confluence, cells were switched to 1.2 mM Ca^{2+} and used for experiments after 48 h.

TABLE 1 | Antibody profiles of pemphigus vulgaris patients' IgG fractions as determined by ELISA for Dsg1 or Dsg3, respectively, and clinical phenotype.

ELISA	Dsg1 (U/ml)	Dsg3 (U/ml)	
PV1-IgG	375	11.55	Mucocutaneous PV
PV2-IgG	212.27	181.44	Mucocutaneous PV
PV3-IgG	101.18	106.72	Mucocutaneous PV
PV4-IgG	5542	711	Mucocutaneous PV

Test Reagents, Antibodies, and Purification of PV-IgG Fractions

The Src-inhibitor PP2 (Calbiochem, Darmstadt, Germany) was used at 10 µM for the respective time periods. For short time-incubations PP2 was preincubated for 2 h. The following commercial primary antibodies were used: anti- cortactin (clone 4F-11; Milipore), anti-phospho-cortactin tyr421 (Milipore), anti-alpha-tubulin (Abcam), anti-desmoplakin (DP) (Abcam), anti-desmoglein 3 (Dsg3) (Clone H154, Santa Cruz), anti-Src (Clone 32G6, Cell Signaling), anti-phospho-Src Tyr 416 (Cell Signaling), anti-plakophilin 3 (Pkp3) (Progen), anti-phospho-plakophilin Tyr 195 (kindly provided by Ansgar Schmidt, Marburg, Germany). Filamentous actin (F-actin) was visualized using an Alexa Fluor™ 488 phalloidin dye (Life Technologies). The corresponding secondary antibodies for immunofluorescence analysis were purchased from Dianova (Hamburg). AK23, a monoclonal pathogenic antibody, derived from a pemphigus mouse model, was purchased from Biozol (Eching, Germany) and used at 75 µg/ml. PV-IgG (all including autoantibodies against both Dsg1 and Dsg3) and IgG fractions pooled from three healthy donors (c-IgG) were purified as described previously (22). The autoantibody profiles were determined using enzyme-linked immunosorbent assay (ELISA), (Euroimmun, Luebeck, Germany). ELISA scores of antibodies against Dsg1 and Dsg3 were determinded before purification (cut-off value: 20 U/ml). The scores are shown in **Table 1**. All patients had mucocutaneous involvement. Patients and donors gave written consent for research use. A positive vote of the Ethics Committee from the Medical Faculty of the University of Marburg was given.

Western Blotting

Cells were washed with PBS, lysed with SDS-lysis buffer (25 mmol/l HEPES, 25 mmol/l NaF and 1% SDS, pH 7.4) and sonicated on ice. Protein amounts were determined

using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, USA). Cell lysates were mixed with laemmli buffer containing 50 mM dithiothreitol. Electrophoresis and western blotting were performed according to standard procedures. Membranes were incubated at 4°C overnight with respective primary autoantibody in tris-buffered-saline containing 0.05% tween (TBS-T), and supplemented with 5% bovine serum albumin (BSA).

Triton X-100 Protein Fractionation

Cells were washed with ice-cold PBS and incubated in Triton buffer (0.5% Triton X-100, 50 mM MES, 25 mM EGTA, 5 mM $MgCl_2$) supplemented with 1 mM PMSF (Roth, Germany), Aprotinin, Pepstatin A (both Applichem, Germany), and Leupeptin (VWR, Germany) for 20 min on ice under continuous shaking. Thereafter, cell lysates were centrifuged at 13.000 rpm for 5 min, which leads to separation of the soluble cytosolic and insoluble cytoskeleton bound fraction. Subsequently, the pellets (insoluble fractions) were resuspended in SDS lysis buffer for Western blotting or with RIPA buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.1% SDS, 1% Nonidet P-40, 0.1 mM EDTA) for immunoprecipitation and followed by sonification. Protein concentrations were calculated as described above and equivalent amounts used for Western blotting or immunoprecipitation analyses.

Immunoprecipitation

Following Triton X-100 mediated solubilization, cell lysates (protein amount of 1,000 μg) were precleared with 25 μl Protein A/G Agarose Beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 4°C, thereafter centrifuged at 10,300 rpm for 5 min at 4°C. The supernatant (IP-lysat) was incubated with 1 μg of anti-Dsg3 antibody (Santa Cruz) or IgG control, for 3 h at 4°C with gentle rotation. The lysate was then added to 40 μl of beads and incubated overnight at 4°C under rotation. IP lysates were washed with RIPA buffer and subjected to Western blot analyses.

In situ Proximity Ligation (PLA) Assay

Spatial proximities of Dsg3 and cortactin were investigated using the Duolink *in situ* kit (Olink, Bioscience) as described previously (23).

Histology and Immunostaining

Samples were embedded in Tissue Tec (Leica Biosystems, Nussloch, Germany) and thereafter serial-sectioned at 7 μm thickness using a cryostat microtome (Cyrosstar NX70, Thermo Fisher). Hematoxylin and esoin (H.E.) staining was performed according to standard protocols (24), and mounted in DEPX (Sigma-Aldrich, St. Louis, MO, U.S.A). Images were captured using a Leica DMi8 microscope with a HC PL APO 40x/0.85 dry objective. For immunostaining, cells were seeded on coverslips and grown to confluence. After respective treatment, cell monolayers were washed with PBS and fixed with 2% paraformaldehyde in PBS for 10 min (HaCaT) or fixed with 4% paraformaldehyde in PBS for 20 min ($CTTN^{-/-}$ and $CTTN^{+/+}$ keratinocytes). Next, samples were rinsed several times with PBS, permeabilized with 0,1% Triton X-100 for 5 min and after final washing with PBS, blocked with 3% bovine serum albumin and 1% normal goat serum for 60 min. The primary antibodies were incubated overnight at 4°C. After washing with PBS, respective secondary antibodies were applied for 60 min at room temperature. Subsequently, coverslips were washed and mounted with 1.5% n-propyl gallate in glycerol. Images were taken with a Leica SP5 confocal microscope using a 63x/1.40 PL APO oil objective (Leica, Mannheim, Germany).

Ca^{2+} Switch Assay

Cells were grown to confluence and, after respective treatment, incubated with 2.5 mM EGTA for 30 min (Ca^{2+}-depletion), which leads to a Ca^{2+}-dependent disruption of cell-cell junctions. Reformation of junctions was induced by medium change with corresponding growth medium containing 1.8 mM Ca^{2+} for 8 h (Ca^{2+} repletion).

Dispase-Based Dissociation Assay

After incubation with test reagents, confluent cell monolayers were washed with Hank's buffered saline solution (HBSS; Sigma Aldrich) and subjected to 2.4 U/ml dispase II (Sigma- Aldrich) in HBSS for 20 min at 37°C and 5% CO_2. After detachment of the monolayer the reaction was stopped by replacing the dispase II solution with HBSS. Defined shear stress was applied with an electrical pipette. Resulting fragments were counted using a binocular microscope (Leica, Mannheim, Germany). All independent experiments were performed in duplicates.

Neonatal Mouse Model

The model was used as described before (25). Newborn cortactin-deficient ($CTTN^{-/-}$) and cortactin wt ($CTTN^{+/+}$) mice were injected intra-dermally into the back skin with a total volume of 50 μl containing 2 mg/ml AK23 without or in combination with 10 μM PP2. The area injected was marked. Twenty hours after incubation the injection site was exposed to defined mechanical stress. Skin was explanted, embedded into cryo freezing medium (Leica, Mannheim, Germany), frozen on dry ice, followed by preparation for cryo-cutting. The experimental protocol was approved by the institutional animal care and use committee of Cinvestav (IACUC), Mexico-City.

Ex vivo Human Skin Model

Biopsies of healthy human skin were acquired from cadavers from the human body donor program from the institute of Anatomy and Cell Biology, Ludwig-Maximilians-Universität München, Germany. Written informed consent was given from body donors for the use of research samples. Biopsies were taken only if death occurred <24 h before arrival at the institute. From each body donor, a skin piece of approximately 5 × 5 cm size was removed from the shoulder, gently stripped off fat including excessive connective tissue. The skin was cut into 1 cm^2 pieces and injected intra-dermally with 50 μl of the respective IgG-fraction (PV-IgG and c-IgG) with or without PP2 (10 μM), using a 30G syringe. Samples were incubated floating on DMEM at 37°C and 5% CO_2 for 24 h. After incubation sheer stress was applied using a rubber head with equal frequency and magnitude. Treated samples were cut into two parts and processed for hematoxylin and eosin (HE) stainings and electron microscopy

analyses. Blister score of the human samples was measured as described below.

Scoring of Blister Size

Each section was evaluated and sorted into the following score system as published previously (23): absence of intraepidermal separation, score 0; cleft size covering 1–25% of total section length, score 1; cleft size between 26 and 50% of section length, score 2; cleft size between 51 and 75% section length, score 3; cleft size between 76 and 100%, score 4.

Electron Microscopy

The injected and incubated human skin explants were cut into small pieces of approximately 2 mm in diameter and fixed with 2–5% glutaraldehyde (Sigma Aldrich). After washing in PBS, tissue samples were post-fixed with 2% osmium tetroxide (Merk Millipore), and dehydrated through a graded ethanol series followed by clearance in propylene oxide, embedding into EPON 812 (Serva Eletrophoresis GmbH, Heidelberg, Germany) and curing at 80°C for 24 h. Resulting blocks were trimmed and sectioned at 60 nm thick slices with a Reichert-Jung Ultracut E ultra-microtome using a diamond knife (DiATOME Electron Microscopy Sciences, Hatfield, PA, USA). Silver-appearing sections were placed onto a 150 mesh copper/rhodium grid (Plano GmbH, Wetzlar, Germany). Sections were then contrasted with alcoholic uranyl-acetate and lead citrate. For imaging a Libra 120 transmission electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) equipped with a SSCCD camera system (TRS, Olympus, Tokyo, Japan) was used.

Statistical Analysis

Data were analyzed in Exel (Microsoft, Redmond, WA) and compared using one-way ANOVA followed by Bonferroni *post-hoc* test (for Gaussian-distributed samples) using Graphpad Prism (Graphpad Software, LaJolla, CA). Error bars represent SEM. Significance was assumed with $p \leq 0.05$. Data are shown as mean ± SEM. Each n represent one independent experiment.

RESULTS

Role of Src for Autoantibody-Induced Loss of Keratinocyte Cohesion

To investigate the role of Src for loss of cell adhesion in pemphigus, human keratinocytes (HaCaT) were incubated with PV-IgG in combination with the Src family kinase inhibitor PP2 and intercellular cohesion was measured by dispase-based dissociation assay (26). Incubation with PV-IgG from pemphigus vulgaris patient 1 (PV1-IgG) significantly increased the number of fragments in comparison to incubation with control IgG (c-IgG) for all time periods (15 min, 60 min and 24 h). Src inhibition using PP2 abrogated loss of intercellular adhesion after incubation with PV1-IgG for 15 and 60 min. In contrast, after 24 h incubation of PP2 together with PV1, no protective effect was observed (**Figure 1A**). After incubation of PV1-IgG for 15, 30 and 120 min, Western blot analysis showed a phosphorylation of Src at Tyr 416, which is one of the major phosphorylation sites and leads to autophosphorylation (9). In contrast, no activation of Src was detectable after 24 h of incubation, indicating that inhibiton of Src was only protective at time points when Src was activated (**Figure 1B**). To get more insights into the importance of Src in PV, two more IgG fractions from different pemphigus patients (PV2-IgG and PV3-IgG) as well as a monoclonal autoantibody against Dsg3 from a pemphigus mouse model (AK23) (27), were included for respective time points. In dissociation assays, PV2- and PV3-IgG as well as AK23 caused keratinocyte monolayer fragmentation after 15 min (**Figure 1C**), 1 h (**Figure 1D**), 2 h (**Figure 1E**), and 24 h (**Figure 1F**) compared to incubation with c-IgG. PV3-IgG and AK23-induced loss of cell cohesion was significantly reduced by PP2 at all time-points whereas inhibition of Src was not effective to modulate loss of adhesion caused by PV2-IgG (**Figures 1C–F**). Western blot analysis showed that all autoantibody fractions were effective to activate Src (**Figure 1G**).

Cadherin-based adhesion is Ca^{2+}-dependent. *In vitro*, the assembly of intercellular junctions in keratinocytes can be induced by a switch from low to high Ca^{2+} medium (28, 29). Vice versa, reduction of the extracellular Ca^{2+} concentration leads to translocation of cadherins from cell-cell borders to the cytosol, followed by disassembly of adherens junctions and desmosomes (28, 30). Since inhibition of Src was protective for adhesion of kerationcytes after treatment with AK23 and PV3-IgG, we investigated the role of Src for desmosomal reassembly. Ca^{2+}-depleted HaCaTs were re-exposed to Ca^{2+} for 8 h in combination with c-IgG, AK23, and PV3-IgG with or without PP2 and then subjected to a dispase-based dissociation assay. Ca^{2+} repletion in combination with autoantibodies did not restore cell adhesion indicating that autoantibodies interfered with desmosome re-assembly, which likely contributes to loss of cell cohesion when autoantibodies are applied for longer periods such as shown in **Figure 1**. However, PP2 reduced this negative effect of both AK23 and PV3-IgG on reconstitution of cell cohesion (**Figure 2A**).

The Src Target Cortactin Colocalizes and Interacts With Dsg3

The actin binding protein cortactin was identified as a major substrate of Src (17), and is proposed to be activated by Src-mediated tyrosine phosphorylation (31). Therefore, we were interested if cortactin plays a role in Src-mediated effects on desmosomal adhesion. First, we investigated the correlation of cortactin and Dsg3 in desmosomal assembly by immunofluorescence analysis under Ca^{2+} switch conditions. Under control conditions, both proteins are located at cell borders and cortactin was phosphorylated on the Src-dependent phosphorylation site Tyr 421. Depletion of Ca^{2+} resulted in Dsg3 dissociation form the cell borders, accompanied by disruption of cortactin and phospho-cortactin immunostaining. Re-administration of Ca^{2+} led to restored Dsg3, cortactin, and phospho-cortactin staining at cell-cell borders (**Figure 2B**). Moreover, immunostaining showed that cortactin and Dsg3 partially co-localized at the cell membrane (**Figure 2C**) which was validated by a proximity ligation assay (PLA), also revealing a close association with the cortical actin cytoskeleton (**Figure 2D**, upper images) at the cell periphery

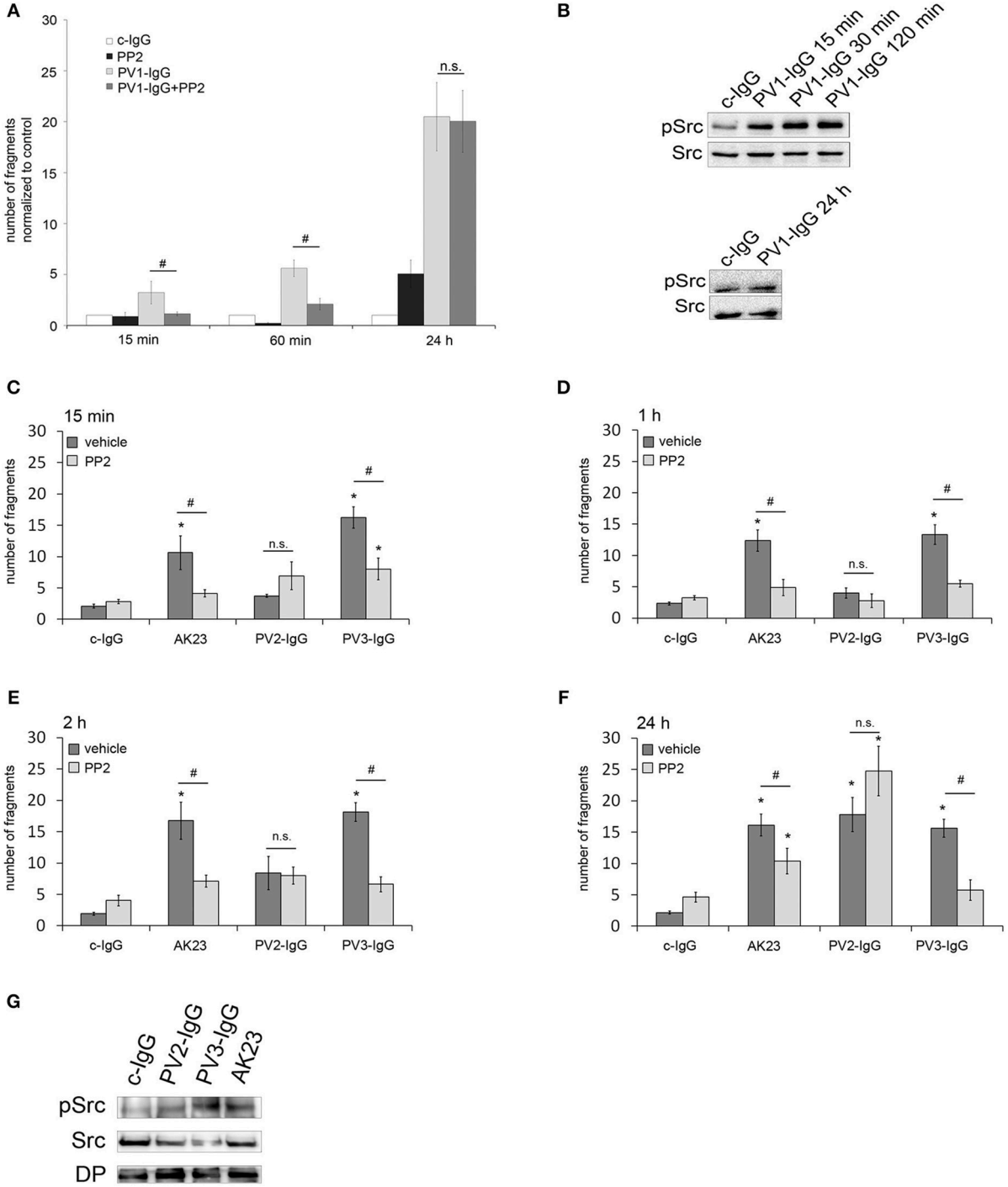

FIGURE 1 | Protective effect of Src inhibition against autoantibody-induced loss of cell cohesion is variable. **(A)** HaCaT cells were incubated with PV1-IgG or with control IgG (c-IgG) and subjected to dispase-based dissociation assays. Inhibition of Src by PP2 prevented fragmentation of cell monolayers after incubation with PV1 for 15 min and 60 min but not for 24 h ($n = 5$; $^{\#}p < 0.05$; $^{*}p < 0.05$ vs. c-IgG). **(B)** Western blot analysis revealed that Src was phosphorylated after 15, 30, and 120 min but not after 24 h of incubation with PV1 ($n = 3$). **(C–F)** PV2- and PV3-IgG as well as AK23 were applied for several time points: 15 min **(C)**, 1 h **(D)**, 2 h **(E)**, and 24 h **(F)**, with keratinocytes being subsequently subjected to dissociation assays. Co-incubation with PP2 led to significantly reduced fragment numbers in PV3-IgG- and AK23- but not PV2-IgG-treated cells ($n > 7$; $^{\#}p < 0.05$; $^{*}p < 0.05$ vs. c-IgG). **(G)** Corresponding Western blot analysis for 2 h revealed that all autoantibody fractions were effective to activate Src ($n = 3$).

(**Figure 2D**, XZ presentation). Next, immunoprecipitation analysis verified that cortactin co-precipitated with Dsg3 in the triton-soluble protein pool but not in the triton-insoluble fraction (**Figure 2E**). This implied that cortactin may play a role in Src-mediated regulation of desmosome assembly. In contrast, phosphorylation of Pkp3 after incubation with AK23 was not detectable (**Supplemental Figure 1**).

Cortactin Is Important for Src-Mediated Reconstitution of Cell Cohesion *in vitro* and in a Pemphigus Mouse Model *in vivo*

To study the role of cortactin in more detail, we isolated primary keratinocytes form newborn cortactin wild type (wt) and cortactin-deficient mice (32). Cortactin-deficient ($CTTN^{-/-}$) mouse keratinocyte mono-layers showed significantly more fragments in a dispase-based dissociation assay, indicating that cortactin is required for cell cohesion (**Figure 3A**). The knockout of cortactin was verified by Western blot analysis (**Figure 3B**), which also showed no changes of desmosomal proteins such as desmoplakin (DP) and Dsg3 in $CTTN^{-/-}$ cells. Next, we subjected cells to a dissociation assay after application of PP2 in combination with AK23 for 2 h (**Figure 3C**) or 24 h (**Figure 3D**). In wt mouse keratinocytes, cell adhesion was rescued by Src inhibition when AK23 was applied for 2 h or for 24 h. In contrast, in $CTTN^{-/-}$ cells, PP2 blocked AK23-induced loss of adhesion only when AK23 was incubated for 2 h but not for 24 h. This suggested that short-term effects auf AK23 were Src- but not cortactin-dependent, whereas long-term effects at least in part required both Src and cortactin. To examine the importance of cortactin for restoring of cell adhesion, Ca^{2+}-switch assay was performed under the same conditions (**Figure 3E**). Again, dissociation assay experiments showed that Src inhibition by PP2 was not effective to restore cell cohesion in $CTTN^{-/-}$ cells in presence of AK23, indicating that reconstitution of cell adhesion is both Src- and cortactin-dependent.

To verify these findings *in vivo*, we used the neonatal passive immuno-transfer mouse model as described previously using AK23 (23). Therefore, we injected AK23 alone and in combination with the Src-inhibitor PP2 into the skin of newborn $CTTN^{+/+}$ and $CTTN^{-/-}$ mice. After incubation for 24 h, defined shear stress was applied. H.E. staining of serial sections showed AK23-induced gross blistering at injection sites of both wt and cortactin-deficient pubs (**Figures 4A–C**). However, in wt animals co-incubation with the Src inhibitor abrogated blister formation almost completely whereas in cortactin-deficient mice, blisters were still observed. These results indicate that AK23-induced skin blistering *in vivo* is Src- and cortactin-dependent, at least, in mice.

Inhibition of Src Was Not Protective Against PV-IgG-Induced Blistering in Human Skin *ex vivo*

Finally, to study the role of Src for skin blistering in intact human skin, we used a human *ex vivo* skin model as reported previously using PV-IgG (22, 33). After injection and incubation of human skin with PV4-IgG alone or in combination with the Src inhibitor PP2 for 24 h, HE-stained serial sections revealed blister formation after treatment with PV-IgG alone as well as in combination with PP2 (**Figures 5A,B**). Analysis of the ultrastructure showed no differences after PV-IgG incubation in conjunction with vehicle or PP2 treatment (**Figure 5A**, lower panel, **Figures 5C–E**). Under both experimental conditions, the number of desmosomes (**Figure 5C**) and desmosome length (**Figure 5D**) were reduced in comparison to control conditions and inter-desmosomal widening was observed (**Figure 5E**). This data show that PP2 was in the long term not protective against PV-IgG-induced skin blistering in human skin.

DISCUSSION

The study provides further insights into the role and mechanisms of Src-mediated epidermal blistering in pemphigus. Inhibition of Src was protective against AK23-induced skin blistering in an *in vivo* mouse model but not against PV-IgG-induced skin blistering in human epidermis. Moreover, the role of Src for loss of cell cohesion appeared to be patient-dependent and to negatively correlate at least in part with autoantibody scores against Dsg1. Src-mediated loss of keratinocyte cohesion engaged mechanisms which were both cortactin-dependent and -independent, the former of which were involved in reconstitution of keratinocyte adhesion.

Role of Src in Pemphigus Autoantibody-Induced Skin Blistering

In accordance with the literature, PV-IgG fractions as well as AK23 led to activation of Src (4, 5, 34, 35). In this context, it was shown that PV-IgG induce Src activation early after application of autoantibodies and thus before other signaling molecules such as p38MAPK or EGFR were activated (5). The first question is by which autoantibodies Src signaling is triggered. It was shown that activation of Src was detectable only when PV-IgG fractions contained antibodies against Dsg3 but not when pemphigus foliaceus (PF) autoantibodies against Dsg1 were applied (4). Since AK23, which is specifically directed against Dsg3, was effective to activate Src it can be concluded that Dsg3 is sufficient to modulate Src signaling (4). Nevertheless, experiments using siRNA-mediated depletion of Dsg1 and Dsg3 suggest that antibodies against other targets may also be capable of activating Src (5).

Consistent with these previous findings, our data confirmed activation of Src after PV-IgG and AK23 treatment for 15 min and up to 2 h. Furthermore, loss of cell adhesion caused by AK23 was completely abolished by PP2 in cell culture *in vitro* as well as in the neonatal mouse model *in vivo*, demonstrating that Src plays an important role in loss of cell cohesion caused by autoantibodies directed against Dsg3. This supports previous findings where either broad-spectrum tyrosin kinase inhibitors or PP2 were efficient to inhibit PV-IgG-induced skin blistering in mice (35, 36).

However, inhibition of Src was not effective to consistently protect against PV-IgG-induced loss of adhesion when autoantibody fractions from different donors were applied.

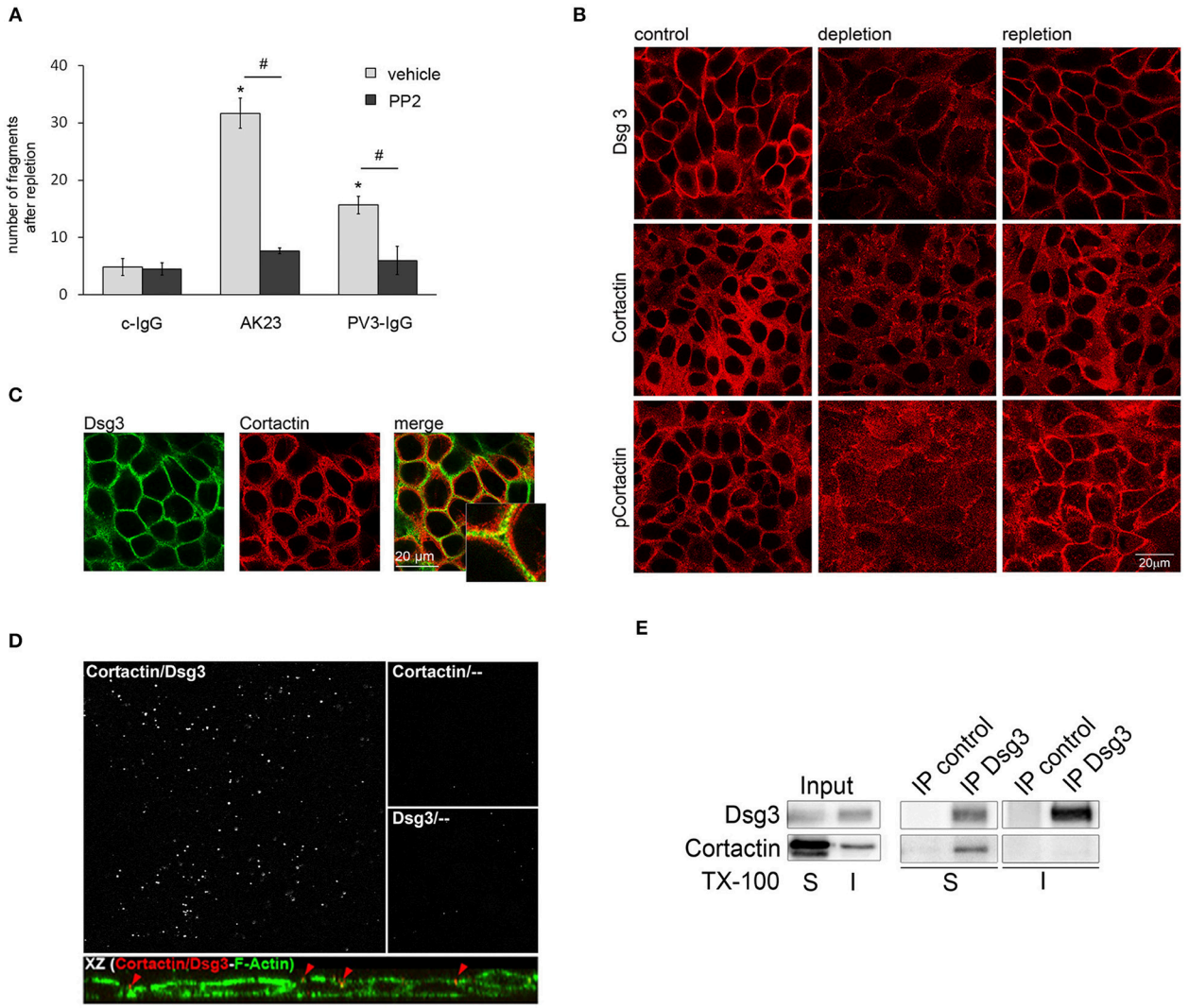

FIGURE 2 | Cortactin colocalizes and interacts with Dsg3. **(A)** Under conditions of desmosome re-assembly (Ca^{2+}-repletion) co-incubation with PP2 significantly reduced PV3-IgG- and AK23-induced monolayer fragmentation ($n > 7$; $^{\#}p < 0.05$; $^{*}p < 0.05$ vs. c-IgG). **(B)** Immunostaining revealed that under control conditions Dsg3 and cortactin as well as phosophorylated cortactin were in part localized at cell borders, which was reduced by Ca^{2+}-depletion. Following Ca^{2+}-repletion for 8 h, all proteins relocated along the cell-membrane (scale bar 20 µm; $n = 4$). **(C)** Under basal conditions, Dsg3 and cortactin partly co-localized at cell borders (scale bar 20 µm; insets represent 3.2x magnifications of indicated areas; $n = 3$) **(D)** Proximity ligation assay revealed co-localization of Dsg3 and cortactin close to the cell periphery. Cells were illuminated with F-actin to localize cell-structures. Incubation with cortactin or Dsg3 only served as negative control ($n = 3$). **(E)** Immunoprecipitation (IP) of Dsg3 documented a complex of cortactin within the Triton-soluble but not the -insoluble fraction ($n = 3$).

For one fraction, Src inhibition abrogated loss of cell adhesion only when autoantibodies were incubated short-time. Using a second fraction, no protection against loss of cell cohesion was detectable at all (27). Since all autoantibody fractions were equally effective to induce loss of cell cohesion *in vitro*, this suggests that different mechanisms maybe involved in different patients. This is supported by the observation that PP2 was not effective to inhibit PV-IgG-induced skin blistering in human skin *ex vivo*. The data presented here do not allow the conclusion that Src is not involved in human skin at all. Nevertheless, the data show that PP2 under the conditions where it is effective to block AK23-mediated blistering in mice and to prevent loss of keratinocyte cohesion in response to some patients' IgG fractions *in vitro*, is not consistently protective in intact human skin.

A limitation of the study is that the conditions used in the different experimental models do not necessarily reflect the situation in patients. Regarding the inhibition of Dsg3 with the monoclonal autoantibody AK23 derived from a pempighus mouse model (27), it should be considered that the concentration used was supraphysiologic compared to patients' autoantibody levels. This could lead to an excessive direct inhibition of Dsg3 binding and activation of signaling by Src and p38MAPK (37), both of which may not be typical in PV patients. However, in contrast to the neonatal mouse model used here, AK23 in a

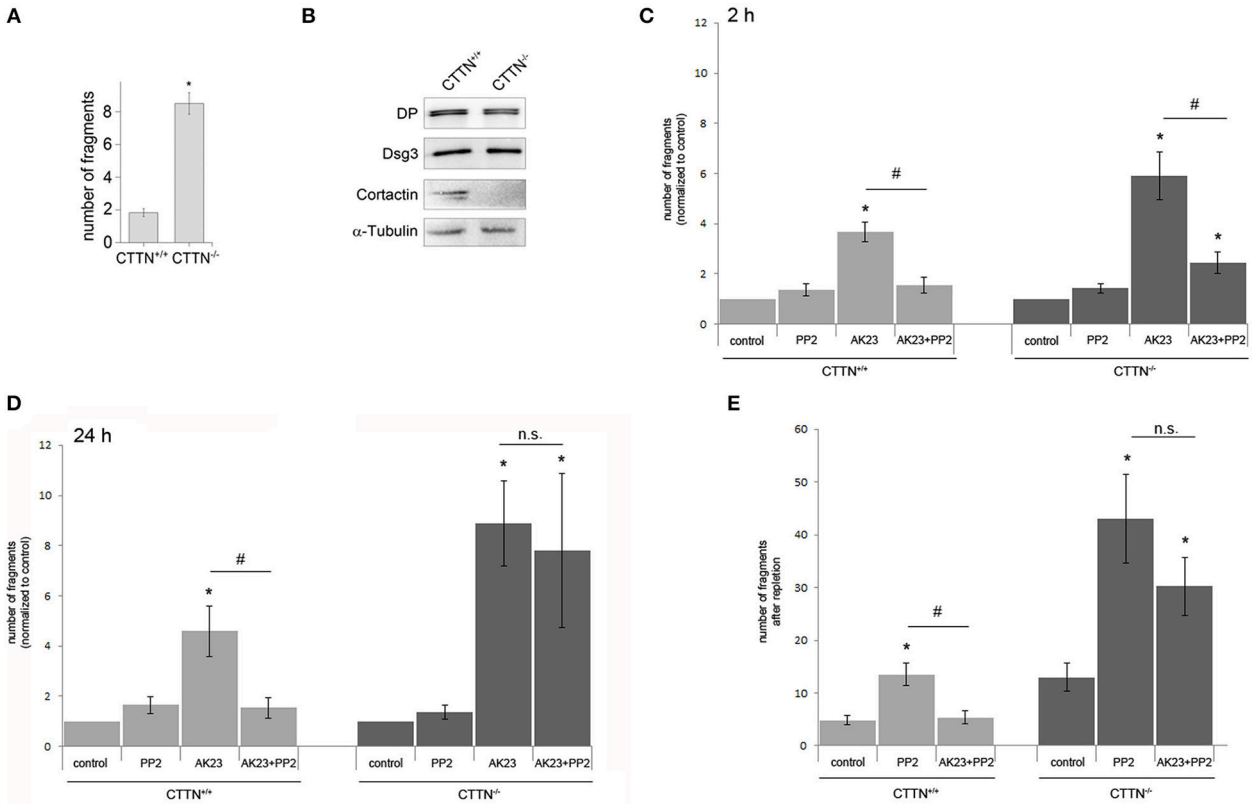

FIGURE 3 | Role of cortactin in Src-mediated modulation of AK23-induced loss of cell cohesion. **(A)** Cortactin is important for cell cohesion, indicated by enhanced fragmentation of cortactin-deficient (CTTN$^{-/-}$) cell monolayers in dispase-based dissociation assay ($n = 5$; $^{*}p < 0.05$ vs. control). **(B)** Confirmation of cortactin knockout by Western blot, α-Tubulin served as loading control ($n = 3$). **(C,D)** CTTN$^{-/-}$ and wt mouse keratinocytes exposed to a dissociation assay following either short time (2 h) or long time (24 h) incubation with AK23 ($n > 7$, $^{\#}p < 0.05$; $^{*}p < 0.05$ vs. control). **(E)** Reconstitution of cell adhesion measured by dissociation assay after Ca^{2+} switch in CTTN$^{-/-}$ and wt keratinocytes in absence or presence of Src inhibitor PP2 ($n > 7$, $^{\#}p < 0.05$; $^{*}p < 0.05$ vs. control).

human skin model was not effective to induce blister formation and reduce desmosome numbers indicating that mechanisms triggered by autoantibodies against Dsg1 may be required for blister formation as well (33). Similarly, the autoantibody profiles of the PV-IgG fractions and the abundance of aDsg1 autoantibodies used in this study may at least in part explain the different roles of Src for loss of keratinocyte adhesion. PV3-IgG, the effects of which were consistently ameliorated by Src-inhibition, contained comparable amounts of autoantibodies against Dsg1 and Dsg3, whereas in all other PV-IgG fractions the relative amount of Dsg1 autoantibodies was higher. Especially, PV4-IgG used for the *ex vivo* human skin model contained excessive levels of autoantibodies against Dsg1. However, for PV2-IgG this explanation falls short because Src inhibition was not protective at all despite comparable levels of antibodies against Dsg1 and Dsg3. Since we have no information about antibodies against other antigens including desmocollin isoforms or others, we cannot rule out that such antibodies may trigger mechanisms others than Src in loss of keratinocyte adhesion.

Nevertheless, the results are in line with the hypothesis that the different clinical phenotypes of pemphigus may at least in part be determined by the mechanisms which are involved in loss of cell cohesion and engaged by antibodies targeting Dsg3 or Dsg1 (4). Moreover, the data indicate that a therapeutic paradigm to modulate Src activity alone unlikely would be effective to treat the majority of patients with epidermal blistering as this usually is associated with antibodies against Dsg1 (1). This may be related to the different mechanisms induced by autoantibodies against Dsg1 in pemphigus pathogenesis.

Mechanisms by Which Src Regulates Cell Cohesion In Pemphigus

For some of the PV-IgG fractions such as PV1-IgG and PV2-IgG, loss of cell cohesion was clearly progressive from 15 min up to 24 h as studied in dispase assays. For other autoantibody fractions such as PV3-IgG and AK23, this effect was less pronounced which is in line with previous observations (37). This may indicate that at early time-points the underlying mechanisms may be different from later stages of adhesion loss. This is supported by the observation that short time co-incubation of AK23 with PP2 in cortactin-deficient cells can abrogate loss of cell cohesion whereas long-term incubation is not affected by Src inhibition. We conclude that short time effects of autoantibodies are in part Src- but not cortactin-dependent whereas cortactin at later stages of cell cohesion loss becomes more relevant.

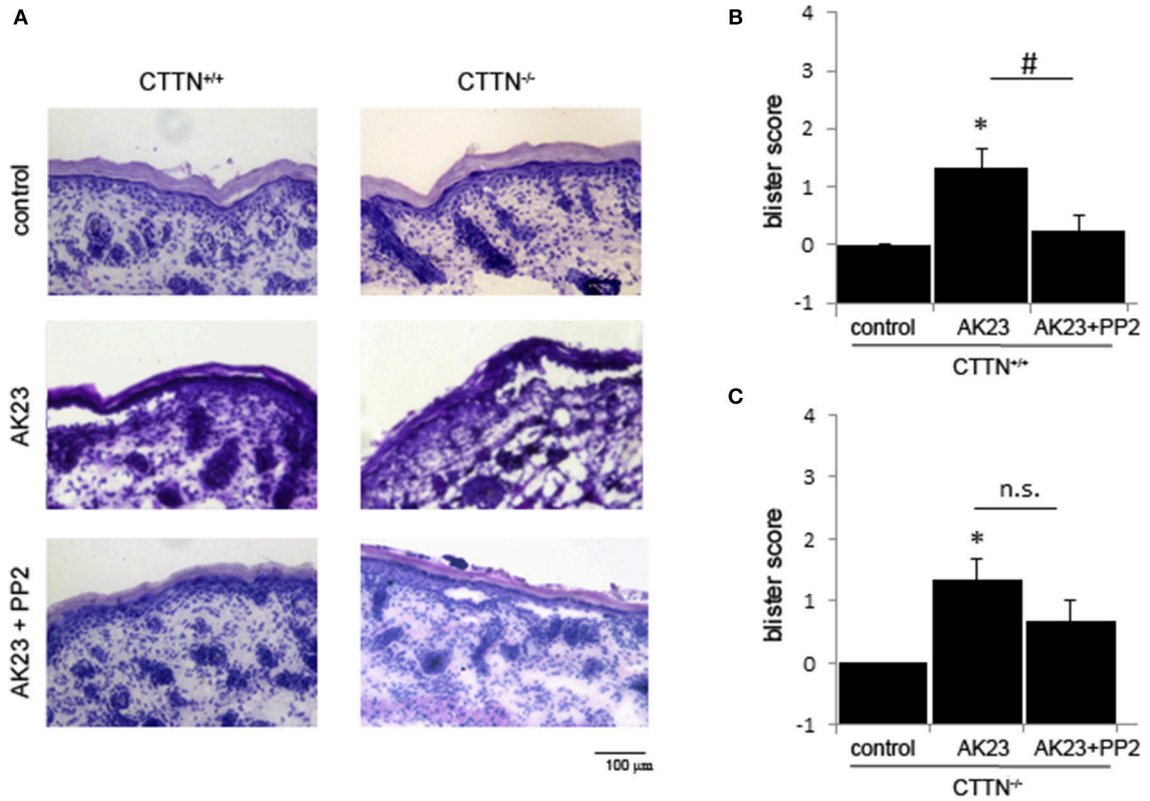

FIGURE 4 | AK23-induced blister formation *in vivo* is Src- and cortactin-dependent. **(A)** H.E.-stained serial sections showed that acantholysis induced by AK23 is almost completely blocked by inhibition of Src in wt but not CTTN$^{-/-}$ mice. Scale bar = 100 µm. **(B,C)** Blister scores for experiments described above ($n = 3$, $^{\#}p < 0.05$; $^{*}p < 0.05$ vs. control).

We observed that autoantibodies indeed interfered with reconstitution of cell adhesion in a manner dependent on Src. Moreover, because phosphorylation of the Src substrate cortactin at cell junctions correlated with junction integrity and cortactin binds to extra-desmosomal Dsg3, we reasoned that cortactin may be involved in desmosome assembly. The interaction of cortactin with desmosomal cadherins recently has been shown for Dsg1 also (38). More detailed studies are required to elucidate how cortactin is important in this context. Here, we observed that short-time effects of autoantibodies cause loss of cell cohesion by mechanisms which do not impair formation of new desmosomes whereas at later stages, impaired reformation of junctions may contribute to adhesion loss. This can be concluded from Ca^{2+}-switch assays in cortactin-deficient monolayers where Src inhibition was no longer protective against autoantibody-induced adhesion reconstitution. These data are in line with the observation that Dsg3 and Src form a complex together with E-cadherin, which appears to be involved in formation of new desmosomes (12). Since both in intact human skin *ex vivo* as well as in neonatal mouse skin *in vivo* autoantibodies were applied for 24 h, it is possible that cell cohesion in neonatal skin is more sensitive to mechanisms impairing formation of new cell contacts.

Since the pathogenesis of pemphigus appears to be complex and to include direct inhibition of Dsg3 binding as well as signaling events (2), the question arises about the role of Src and cortactin for loss of cell cohesion when compared to other mechanisms. Here we show that the short-time effects by which autoantibodies interfere with keratinocyte adhesion are at least in part dependent on Src but are independent of cortactin. Therefore, it is possible that phosphorylation of plakophilin (Pkp) 3 and its dissociation from desmosomes may be involved as has been suggested for experiments with PV-IgG (15). However, for AK23 we did not observe Pkp3 phosphorylation (**Supplemental Figure 1**) indicating that phosphorylation of Pkp3 can be independent of Dsg3. Aside from Src, other signaling molecules likely contribute to loss of cell cohesion in pemphigus. Electron microscopy in *ex vivo* human skin revealed that ultrastructural alterations such as loss of desmosomes, shortening of desmosomes and inter-desmososomal widening, all of which are observed in patients' lesions as well (39), were independent of Src. In contrast, these effects were shown previously to be mediated by p38MAPK (33). This is in line with the observation that pemphigus autoantibody fractions including autoantibodies targeting Dsg3 activate both Src and p38MAPK (4).

Finally, it is well-established that autoantibody titers correlate with the clinical phenotype of pemphigus (1, 3). For pemphigus autoantibodies from patients with muco-cutaneous PV or with PF, influx of Ca^{2+} and activation of ERK were also detected

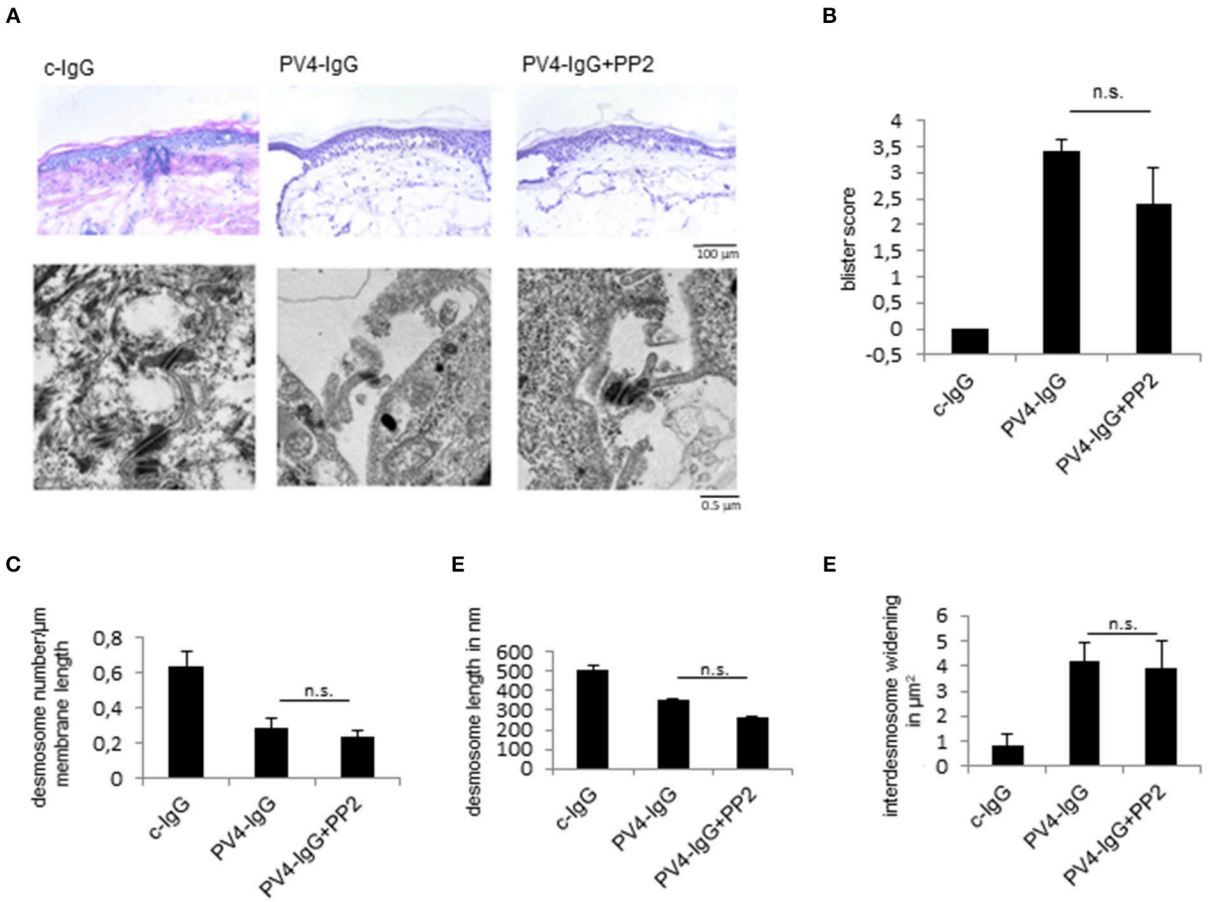

FIGURE 5 | Src inhibition does not prevent PV-IgG-induced skin blistering and desmosome alterations in human skin *ex vivo*. **(A)** upper images, **(B)** H.E.-stained serial sections revealed no change in blister formation after incubation with PV4-IgG in presence of Src-inhibitor PP2 (scale bar = 100 µm). (**A**, lower images) Ultrastructural alterations of desmosomes were evaluated by transmission electron microscopy in absence or presence of PP2 (scale bar = 0.5 µm). **(C–E)** Quantification of number of the desmosomes **(C)**, desmosome length **(D)** and inter-desmosomal widening **(E)** for the conditions described above ($n = 5$).

(4). Since Dsg1 was shown to suppress EGFR/ERK signaling by interacting with the ErbB2 binding protein Erbin (40), it is possible that autoantibodies targeting Dsg1 interfere with ERK signaling via this pathway. Taken together, we show that Src contributes to loss of cell adhesion primarily downstream of antibodies against Dsg3 in pemphigus by mechanisms which are both cortactin-dependent and –independent. The notion that actin-binding proteins such as cortactin and adducin become increasingly recognized to control desmosome function and cell behavior (12, 38), opens a new field for research on desmosome regulation.

AUTHOR CONTRIBUTIONS

DK, VR, EW, DE, MF, and HV-R performed experiments. DK, VR, EW, DE, and MF analyzed data. DK, EW, FV, MS, MH, RE, KR, AS, VS, and JW discussed data and interpreted results. DK and JW designed the study and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00626/full#supplementary-material

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ST18 Enhances PV-IgG-Induced Loss of Keratinocyte Cohesion in Parallel to Increased ERK Activation

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Pemphigus is an autoimmune blistering disease targeting the desmosomal proteins desmoglein (Dsg) 1 and Dsg3. Recently, a genetic variant of the Suppression of tumorigenicity 18 (ST18) promoter was reported to cause ST18 up-regulation, associated with pemphigus vulgaris (PV)-IgG-mediated increase in cytokine secretion and more prominent loss of keratinocyte cohesion. Here we tested the effects of PV-IgG and the pathogenic pemphigus mouse anti-Dsg3 antibody AK23 on cytokine secretion and ERK activity in human keratinocytes dependent on ST18 expression. Without ST18 overexpression, both PV-IgG and AK23 induced loss of keratinocyte cohesion which was accompanied by prominent fragmentation of Dsg3 immunostaining along cell borders. In contrast, release of pro-inflammatory cytokines such as IL-1α, IL-6, TNFα, and IFN-γ was not altered significantly in both HaCaT and primary NHEK cells. These experiments indicate that cytokine expression is not strictly required for loss of keratinocyte cohesion. Upon ST18 overexpression, fragmentation of cell monolayers increased significantly in response to autoantibody incubation. Furthermore, production of IL-1α and IL-6 was enhanced in some experiments but not in others whereas release of TNF-α dropped significantly upon PV-IgG application in both EV- and ST18-transfected HaCaT cells. Additionally, in NHEK, application of PV-IgG but not of AK23 significantly increased ERK activity. In contrast, ST18 overexpression in HaCaT cells augmented ERK activation in response to both c-IgG and AK23 but not PV-IgG. Because inhibition of ERK by U0126 abolished PV-IgG- and AK23-induced loss of cell cohesion in ST18-expressing cells, we conclude that autoantibody-induced ERK activation was relevant in this scenario. In summary, similar to the situation in PV patients carrying ST18 polymorphism, overexpression of ST18 enhanced keratinocyte susceptibility to autoantibody-induced loss of cell adhesion, which may be caused in part by enhanced ERK signaling.

Keywords: pemphigus, desmosome, desmoglein, ST18, ERK, cytokines

INTRODUCTION

Pemphigus vulgaris (PV) is a potentially fatal autoimmune disease of the skin and the mucous membranes. The skin disorder is manifested by mucocutaneous blister formation caused by loss of keratinocytes intercellular cell adhesion, which is primarily promoted by IgG autoantibodies directed against desmosomal adhesion molecules such as desmoglein (Dsg) 1 and Dsg 3 (1). However, autoantibodies targeting desmocollin (Dsc) 3, which are rarely present in cases when antibodies against Dsg3 are missing, were also shown to be pathogenic (2–4). These desmosomal cadherins are transmembrane proteins forming the adhesive core of desmosomes, a special intercellular junction maintaining the mechanical integrity of tissues and bearing tension primarily if exposed to high levels of mechanical stress such as in the epidermis (5). With their cytoplasmic tail, via Plakoglobin (PG), and Desmoplakin (DP), the cadherins are linked to the intermediate filament skeleton, another structural unit providing mechanical strength to cells (6, 7). Besides their mechanical functions, desmosomes serve as signaling hubs coordinating tissue-specific functions with intercellular adhesion, which is required for instance during wound healing (8).

In response to binding of pemphigus autoantibodies to the keratinocyte surface, Dsg3-mediated homophilic interaction is impaired and desmosomal proteins are depleted from the membrane leading to keratin retraction and Dsg internalization (9–12). These structural alterations are induced by various PV-mediated outside-in intracellular signaling events such as p38 mitogen-activated protein kinase (p38MAPK) (13–15) and extracellular-signal regulated kinase (ERK) (16, 17). Signaling mechanisms have been shown to be important for loss of cell adhesion (17) and the importance of several signaling mechanisms is highlighted by the protective effects of pharmacological inhibitors *in vivo*, *in vitro,* or *ex vivo* (18–20).

Despite all efforts, the etiology of the disease is still not completely understood. Interestingly studies reporting ethnic susceptibility and familial occurrence of the PV- skin disorder provided evidence for a genetic predisposition to PV (21–25). In this respect, most of the reports provided data for PV associated genes belonging to a human leukocyte antigen (HLA) locus. Few studies, however, described PV genetic association of non-HLA genes (25). Amongst the latter is suppression of tumorigenicity 18 (ST18), the product of which functions as a transcription factor and thereby controls the mRNA levels of numerous proappoptotic and pro-inflammatory genes (26), participating in regulation of processes with potential relevance for loss of cell adhesion in PV (27, 28). Recently, a genetic variant located within the ST18 promoter was reported to cause ST18 up-regulation, associated with PV-IgG-mediated increase in cytokine secretion and more prominent loss of keratinocytes cohesion (29). In line with this, single nucleotide polymorphisms (SNP) identified in the ST18 gene were proposed to predispose to PV in a population-specific manner. SNPs promoted augmentation in ST18 expression was shown to be associated with more severe disease manifestation, indicative for the direct role of ST18 in PV pathogenesis (30, 31).

In the present study we investigated in both HaCaT and normal human epidermal keratinocytes (NHEK) the release of key pro-inflammatory molecules such as IL-1α, IL-6, TNF-α, and IFN-γ upon PV-IgG treatment. Additionally, the effect of ST18 overexpression on cytokine release but also on the modulation of pemphigus-associated ERK signaling was evaluated since both events would render keratinocytes more susceptible to PV-IgG-induced loss of keratinocyte adhesion.

MATERIALS AND METHODS

Cell Culture, Isolation of Human Primary Keratinocytes

For all experiments, primary normal human epithelial keratinocytes (NHEK) and HaCaT, a spontaneously immortalized human skin keratinocyte cell line were used. HaCaT cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), (Life Technologies; Carlsbad; CA; USA) supplemented with 10% FCS (Biochrom, Berlin, Germany), 50 U/ml penicillin and 50 U/ml streptomycin, both antibiotics were purchased from AppliChem, Darmstadt, Germany.

NHEK were generated at Universitäts-Hautklinik Tübingen. The procedure was approved by the medical ethical committee of the Eberhard Karls University Tübingen (ethical approval: 547/2011BO2). Briefly, the cells were isolated from juvenile foreskin derived from patients, who have given written informed consent. The skin was divided into epidermis and dermis by using 50 mg/ml Dispase II (Roche; Basel, Switzerland). Epidermal cells were then separated from each other by using a trypsin-EDTA solution (Merck, Darmstadt, Germany) and afterwards cultivated in CnT-07 medium (CELLnTEC Advanced Cell Systems AG, Switzerland) containing 10 μg/ml gentamycin and 0.25 μg/ml amphotericin B. The cells were cultured in low calcium conditions (0.06 mM Ca^{2+}). 24 h prior treatment, cell differentiation was induced by increasing the Ca^{2+} concentration to 1.8 mM. All experiments were conducted on NHEK cells between passages 3 to 8.

Both cell lines were grown in a humidified atmosphere containing 5% CO_2 at 37°C. After cells reached confluency the medium was changed and 24 h later cell monolayers were treated accordingly.

Constructs and Transfection

The human ST18 construct (pCMV6-ST18-Myc/DDK) was generated by inserting the translated region of a human ST18 cDNA into the pCMV6-Entry target vector (Origene Technologies Company, Rockville, MD, USA). The latter was used also for a control transfection.

ST18 overexpression was accomplished by transient transfection with a plasmid DNA into HaCaT cells at 80% confluence using Turbofect transfection reagent (Fermentas, ThermoFisher Scientific). 24 h after transfection, medium was changed and cell monolayers were further exposed to treatment with various IgG fractions.

RNA Isolation, Complementary DNA (cDNA) Preparation, and PCR Performance

To confirm sufficient transfection efficiency with ST18 construct, PCR was performed with cDNA transcribed from an equal amount of total RNA. The latter was isolated by using RNeasy Plus mini kit (Qiagen, Venlo, Netherlands). cDNA was synthesized through the usage of SuperScript II reverse transcriptase kit (Invitrogen, ThermoFisher Scientific). All preparations were carried out according to manufacturer's instructions.

The transfection efficiency of each experiment requesting ST18 overexpression was tested by PCR using following primer pair:

hST18-164bp-Fw CACTAATCCAGGAGCTCAGTGTTG
hST18-164bp-Rev CCTGTCACTGCGGTCTTCTTG

To validate the PCR assay, positive (pCMV-ST18-Myc/DDK vector) and negative (instead of cDNA, H_2O was used as a template) controls were used. An empty negative control reflects the lack of contaminations, whereas manifestation of amplicon with correct size in the positive control guarantees accurate PCR conditions.

PCR was carried out as follows: 95°C for 3 min, followed by 22 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, the final extension was at 72°C for 5 min. Then, the PCR products were visualized by gel electrophoresis.

Dispase-Based Keratinocytes Dissociation Assay

Keratinocytes were seeded in 24-well plates and grown to confluency. NHEK cells were subsequently differentiated for 24 h by increasing the Ca^{2+}-concentration to 1.8 mM. HaCaT monolayers were transiently transfected either with control or pCMV-ST18-Myc/DDK vector 24 h prior to experimental procedure. Monolayers treated with PBS (Vehicle) or control IgG (c-IgG) for 24 h were considered as controls. Experimental conditions were treated with AK23 or different PV-IgG in a similar fashion.

The loss of cell cohesion was tested by dispase-based dissociation assay. The latter was performed as described elsewhere (32, 33). Briefly, after treatment, cell monolayers were washed with pre-warmed Hank's Balanced Salt Solution (HBSS) and subsequently released from the well-bottom by incubation with 2.4 U/ml Dispase II (Sigma-Aldrich) dissolved in HBSS for 20 min at 37°C. Next, the protease solution was substituted by HBSS and each condition was treated additionally for 10 min with thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich) at a final concentration of 10 μM to better visualize the monolayer sheets. The integrity of a single intact monolayer was compromised by applying controlled shear stress through pipetting the monolayers with an electrical 1 ml pipette. Resulting fragments were counted under a binocular microscope (Leica, Mannheim, Germany) as their number is an inverse measure of intercellular adhesion. Higher number of fragments correspond to stronger loss of intercellular adhesion. A Canon EOS 750D camera was used to record the dissociation experiments. Each experiment was repeated 6 to 10 times.

Analysis of the Cytokine Secretion

Simultaneous quantitative cytokine measurements were accomplished by high-throughput bead-based multiplex assays. A Milliplex kit (HCYTOMAG-60K, Merck KGaA, Darmstadt, Germany), precustomized for cytokines of interest, i.e., IL-1α, IL-6, IFN-γ, TNF-α, was used. Along with the serial standards, each sample was analyzed in duplicate or triplicate following the manufacturer's instructions. Collection of the data was achieved by using Luminex LX100/200 system (Luminex Corporation, Austin, Texas). Data analysis was performed using MILLIPLEX® Analyst 5.1.Software (Merck KGaA, Darmstadt, Germany). To estimate the cytokine concentration (expressed in pg/ml), standard curves derived from known reference concentrations supplied by the manufacturing company were utilized.

Immunostaining

Human keratinocytes (HaCaT and NHEK) were grown to confluence on glass coverslips. Twenty four hours prior treatment, the medium was changed and in the case of NHEK cells, the Ca^{2+} concentration was increased to 1.8 mM in order to induce cell differentiation. After 24 h of incubation at 37°C with different IgG fractions the culture medium was removed, cell monolayers were washed with PBS and subsequently fixed (with 2% paraformaldehyde (PFA) for 10 min) and permeabilized (with 0,1% Triton-X-100 for 5 min). To prevent unspecific binding the intact monolayers were blocked with a mix of 1% normal goat serum and 3% bovine serum albumin solved in PBS for at least 30 min at room temperature (RT). Consequent overnight incubation at 4°C with primary rabbit anti-Dsg3 antibody (Biozol Diagnostica Vertrieb GmbH, Germany) was performed. After washing with PBS, the cell monolayers were incubated with Cy3- conjugated secondary goat-anti-rabbit antibody (Dianova, Hamburg, Germany) for an hour at RT. After rinsing the monolayers with PBS and H_2O the cells were mounted on a glass slide with N-propyl gallate, an anti-fade reagent, which is used to reduce photobleaching of the fluorescent probes. Images were collected with 63xNA1.4 PL APO objective using a Leica SP5 confocal microscope.

PV Sera and Purification

Patient blood sera were obtained from the Department of Dermatology and Allergology, Philipps-Universität Marburg, Marburg, Germany. Prior serum collection, all patients gave their written informed consent. For disease determination, clinical and histological studies were carried out and the antibody scores for aDsg1 and 3 were obtained during disease diagnosis. Control serum was donated from healthy volunteers. Before experimental usage, patient sera were purified by column affinity chromatography as described elsewhere (34). Briefly, Protein-A Agarose (ThermoFisher, Waltham, USA) was washed with PBS. Subsequently, beads were incubated with patient sera for 2 h at room temperature on a rotator, which enables IgG binding to the beads. After washing the beads with PBS, IgGs were detached from the Agarose by using sodium citrate (20 mM, pH 2.4) followed up by neutralization with sodium carbonate. Next, a filter unit (Amicon Ultra−4, 100 k; Merck Millipore, Darmstadt, Germany) was used to wash the resulting salts out of the IgG

fractions by centrifugation at 19,000 g for 20 min. As a final step the IgGs were resuspended in PBS. Protein amount of purified IgG fractions was determined by Bicinchoninic acid kit (BCA) (Thermo Fisher Scientific, Schwerte, Germany) and used for further experiments at a dilution of 1:50.

The monoclonal antibody AK23 reacting to Dsg3 was purchased from Biozol (Eching, Germany).

Triton X-100 Protein Fractionation, Electrophoresis, Western Blotting, and Tested Antibodies

Signaling pathway modulation was examined in either non-transfected or transfected keratinocytes exposed to different IgGs or AK23 for a time period of 30 min. After the incubation was accomplished, cell monolayers were washed with ice cold PBS. As next, extraction buffer (0.5% Triton X-100, 50 mmol/L MES, 25 mmol/L EGTA, 5 mmol/L $MgCl_2$) containing 0.1% of Leupeptin, Pepstatin and Aprotinin as well as 1% PMSF was applied for 15 min, on ice, under moderate shaking. Lysates were collected by scraping. In order to achieve successful fractionation of the cytoskeletal bound insoluble from the soluble fraction, a centrifugation step at 19,000 g for 10 min at 4°C was performed. Resulting supernatants defined the cytosolic pools. The obtained pellet, representing the cytoskeletal bound fraction, was further washed with Triton extraction buffer, centrifuged for 10 min and consequently re-suspended in SDS-buffer (25 mmol/l HEPES, 2 mmol/l EDTA, 25 mmol/l NaF, and 1% SDS, pH 7,4, complete Protease Inhibitor Cocktail). After sonication, protein concentration of both fractions was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Schwerte, Germany), following the manufacturer's instructions. Equal amounts of samples were mixed with Laemmli buffer and consequently subjected to Western blotting as described elsewhere (17).

Following primary antibodies were incubated overnight at 4°C in 5% bovine serum albumin (BSA) in Tris-buffered-saline (TBS) with 0.05% tween (TSB-T): p-ERK (Cell Signaling Technologies, Cambridge, United Kingdom), p44/42 MAPK pAB (Erk1/2) (Cell Signaling Technologies), GAPDH mAB (Santa Cruz), Desmoplakin I/II pAB (H-300) (Santa Cruz). Following peroxidase coupled secondary antibodies were used for 1 h at room temperature in TBS-T: HRP-coupled goat anti-mouse, a HRP-coupled goat anti-rabbit (Dianova, Hamburg, Germany). Afterwards antibodies were visualized by chemiluminescence with ECL reaction (GE Healthcare, Munich, Germany).

Data Analysis and Statistic

One-way ANOVA followed by Bonferroni correction (GraphPad Software, La Jolla, CA) was used to assess the differences among three or more treatments in dispase-based assay. To determine the cell response to different treatments in cells transfected with EV and ST18- vector either two-way ANOVA followed by Sidak's multiple comparisons test or multiple *t*-tests corrected by Holm-Sidak's method were applied as indicated. Each data point represents one single experiment. Data are presented as mean ± SEM. *P*-values less or equal to 0.05 assume significant differences between analyzed groups.

RESULTS

Pathogenicity of Pemphigus Autoantibodies on Human Keratinocytes

The pathogenic effect of IgG fractions derived either from an active pemphigus mouse model (AK23, a monoclonal autoantibody targeting Dsg3, (35)) or from pemphigus patients (PV-IgG) was evaluated by dispase-based dissociation assay in cultured immortalized HaCaT and primary normal human epidermal keratinocytes (NHEK) (**Figure 1A**). In contrast to control conditions, in which confluent monolayer exposed to PBS (vehicle) or control IgG (c-IgG) obtained from healthy volunteers remained intact, application of both AK23 and PV-IgG (PV-1 and PV-2) for 24 h induced loss of cell adhesion.

In HaCaT cell monolayers, the effect of pathogenic IgG-fractions on desmosomes was visualized by Dsg3 immunostaining. In all controls (vehicle and c-IgG), linearly organized Dsg3 staining at cell borders was observed. In contrast, 24 h incubation with autoantibodies induced fragmentation of the Dsg3 staining pattern along the borders (**Figure 1B**). Under the same conditions, NHEKs exhibited changes similar to those observed in HaCaT cells (**Supplementary Figure 1**).

ST18 Overexpression Promotes PV-IgG-Mediated Loss of Keratinocyte Cohesion

Recently, it has been reported that a PV-associated SNP leads to a significant increase in the expression of ST18 gene. As a consequence, in NHEK cells PV-IgG caused loss of keratinocyte adhesion as well as the release of key inflammatory molecules (29). In order to validate the effect of ST18 overexpression in human HaCaT keratinocytes, cells were transiently transfected with either empty vector (EV) or with ST18 expression vector. Consequently, the intact monolayers were exposed to control or pemphigus autoantibodies. Twenty four hours later, dispase-based dissociation assay was performed. Upon ST18 overexpression, the fragmentation of cell monolayers increased significantly in response to PV-IgG and AK23 incubation (**Figures 2A,B**). For each single experiment, sufficient transfection efficiency was confirmed by PCR analysis (**Figure 2C**).

Effect of Pemphigus Autoantibodies and ST18 Overexpression on Cytokine Release

Besides preserving the structure of the skin by forming strong intercellular junctions and thus functioning as a physical barrier, keratinocytes also mediate inflammation by synthesizing and secreting various cytokines in response to physical or chemical damage, inflammatory signals or UV radiation (36–38). Therefore, in the present study, the pattern of cytokines expressed by non-transfected or transiently transfected human keratinocytes exposed to either c-IgG or pathogenic IgG was further analyzed.

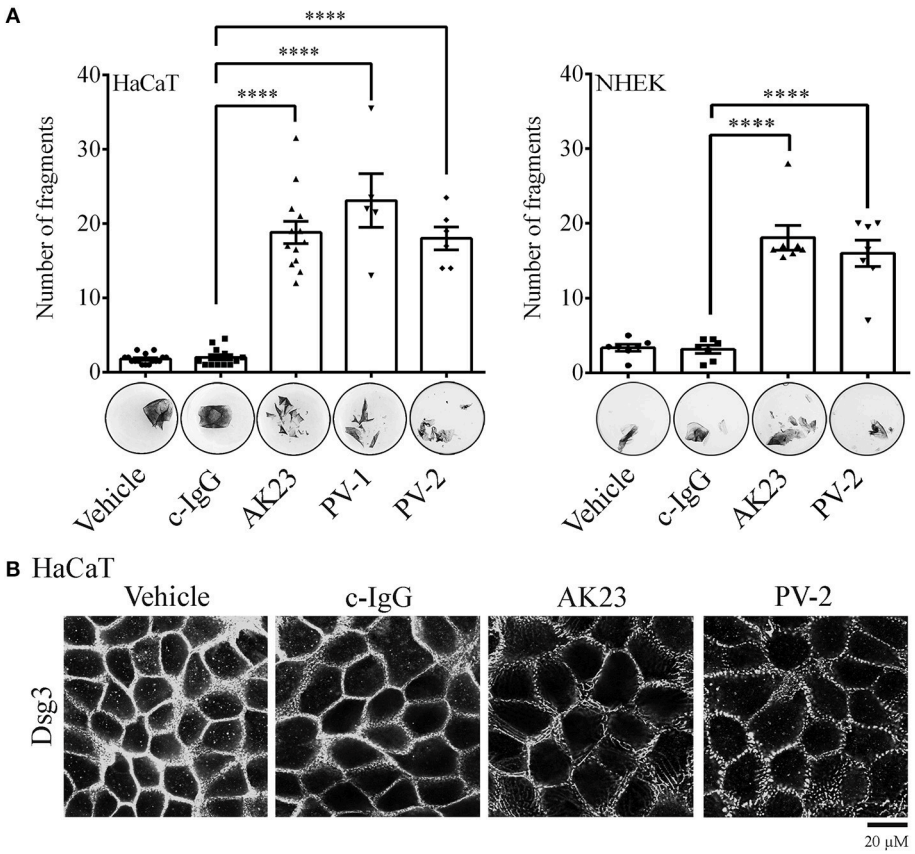

FIGURE 1 | Pemphigus autoantibody application induces loss of keratinocyte cohesion and fragmentation of Dsg3 immunostaining. **(A)** Dispase-based dissociation assay in human HaCaT and NHEK keratinocytes after treatment with pemphigus autoantibodies. Besides PBS-treated monolayers (Vehicle), cells exposed to c-IgG were also used as a control. Representative images of cell sheets pre-stained with MTT and subjected to defined mechanical stress ($n \geq 6$, One-way ANOVA, ****$p \leq 0.0001$ vs. c-IgG). **(B)** Dsg3 immunostaining in response to pathogenic autoantibodies (PV-IgG and monoclonal autoantibody AK23, targeting Dsg3). Representative images of $n \geq 4$ (Scale bar: 20 μm).

Twenty four hours after reaching confluency HaCaT and NHEK cells were exposed to pathogenic IgG for another 24 h. Respective controls (PBS or c-IgG treated) were run in parallel. After the incubation was completed, supernatants were collected and simultaneously assessed for multiple cytokines by Luminex assay. Cytokine secretion of IL-1α, IL-6, TNF-α, and IFN-γ in both HaCaT and primary NHEK cells was not affected significantly (**Figures 3A,B**). In case of IFN-γ, independent on the treatment, cytokine release in HaCaT was either not altered, similar as in NHEK cells, or often under the detection limit (**Supplementary Figures 2A,B**). The latter observation was in line with a recent study in PV-patients, where the serum levels of IFN-γ were undetectable (39). Taken together, these data demonstrate that at least *in vitro*, cytokine release is not strictly required for loss of keratinocyte cohesion.

It was reported that ST18 overexpression in NHEKs results in a PV-IgG-mediated release of key inflammatory cytokines (29). In order to validate the effect of ST18 overexpression in human HaCaT keratinocytes, cells were transiently transfected with either EV or ST18- expression vector. For the recent study, experiments were carried out in HaCaT keratinocytes since the transfection efficiency in NHEK was not consistent. The intact monolayers were consequently exposed to IgG fractions. Supernatants were collected and secretion profiles of IL-1α, IL-6, TNF-α, and IFN-γ were analyzed by Luminex assay. ST18 overexpression induced no significant increase in the secretion of any cytokine analyzed (**Figure 3C**). However, IL-1α and IL-6 levels were augmented in some experiments, but not in others. When compared to c-IgG treatment, the release of TNF-α was even reduced significantly upon PV-IgG application in both EV and ST18 transfected monolayers. Secretion of IFN-γ was either undetectable or not affected by ST18 overexpression (**Supplementary Figure 2C**).

Modulation of ERK Signaling Upon Treatment With PV-IgG Fractions and ST18 Overexpression

Several signaling pathways, including ERK signaling, have been implicated in the pathogenesis of pemphigus (16, 17). In order to evaluate ERK activity upon pemphigus autoantibody treatment, 24 h after differentiation with 1.8 mM Ca^{2+}, confluent NHEK

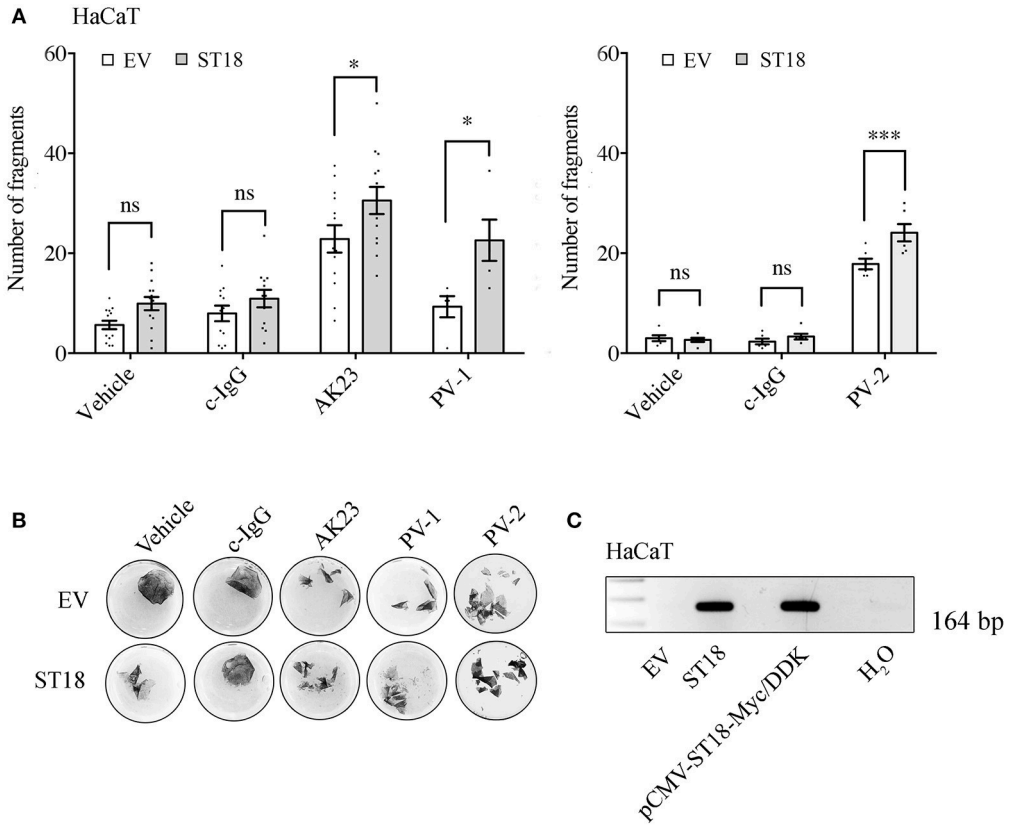

FIGURE 2 | ST18 enhances AK23- and PV-IgG-mediated loss of cohesion in cultured keratinocyte monolayers. **(A)** Dispase-based dissociation assay in HaCaT cells transiently transfected with a control empty (EV) or a ST18-expression vector and exposed to either c-IgG, AK23, or PV-IgG fractions ($n \geq 0.05$, two-way ANOVA, $^{*}p \leq 0.05$ and $^{***}p \leq 0.001$ vs. respective monolayers transfected with EV). **(B)** Representative images of keratinocyte monolayers pre-stained with 10 μM MTT for better visualization of the fragments. **(C)** PCR analysis validating sufficient transfection efficiency was performed for each single experiment. Positive (pCMV-ST18-Myc/DDK vector) and negative (H_2O) controls were run in parallel.

monolayers were incubated with different control or pathogenic IgGs for 30 min. ERK phosphorylation was investigated by triton fractionation and subsequent western blot analysis. The bands of phosphorylated and total ERK were densitometrically quantified and ERK activity was expressed as a ratio of phosphorylated-ERK to non-phosphorylated total ERK. Analysis revealed that compared to c-IgG, treatment with PV-2 IgG but not with AK23 induced significant activation of ERK (**Figure 4A**).

To evaluate the effect of ST18 overexpression on ERK signaling, 24 h after transfection HaCaT cells were incubated for 30 min with different IgGs (**Figure 4B**). Overexpression of ST18 resulted in significantly increased activity of ERK after c-IgG and AK23 treatment. Prominent but not significant elevation of ERK activity was observed in response to PV-2 application. Thus, we hypothesize that activation of ERK signaling is required to induce significant loss of cell cohesion as a result of ST18 overexpression. In order to test this hypothesis, inhibition of ERK activation by U0126, a widely used MEK inhibitor (40, 41), was performed in dispase assays using cell monolayers transiently transfected with either EV or ST18. Twenty four hours after transfection HaCaT cells were treated with 5 μM U0126 for an hour followed by incubation with either c-IgG, PV-IgG, or AK23 for another 2 h. Inhibition of ERK activation completely abolished autoantibody-induced loss of cell adhesion in ST18 transfected monolayers (**Figure 4C**).

Next, to test whether overexpression of ST18 modulates baseline adhesion via ERK, dispase-based dissociation assays were performed in vehicle or U0126-treated HaCaT keratinocytes transiently transfected with either EV or ST18 expression vector. Analysis revealed that neither in EV- nor in ST18- transfected cells adhesion was affected by ERK inhibition (**Figure 4D**). This indicates that an additional factor such as PV-IgG is required to facilitate ST18/ERK-mediated loss of adhesion.

DISCUSSIONS

Autoantibodies targeting Dsg1 and Dsg3 are pathogenic and cause blister formation by inducing structural desmosomal changes in the skin of PV patients (42). However, the mechanisms underlying disease development and the factors enhancing its manifestation have not yet been fully elucidated. Secondary

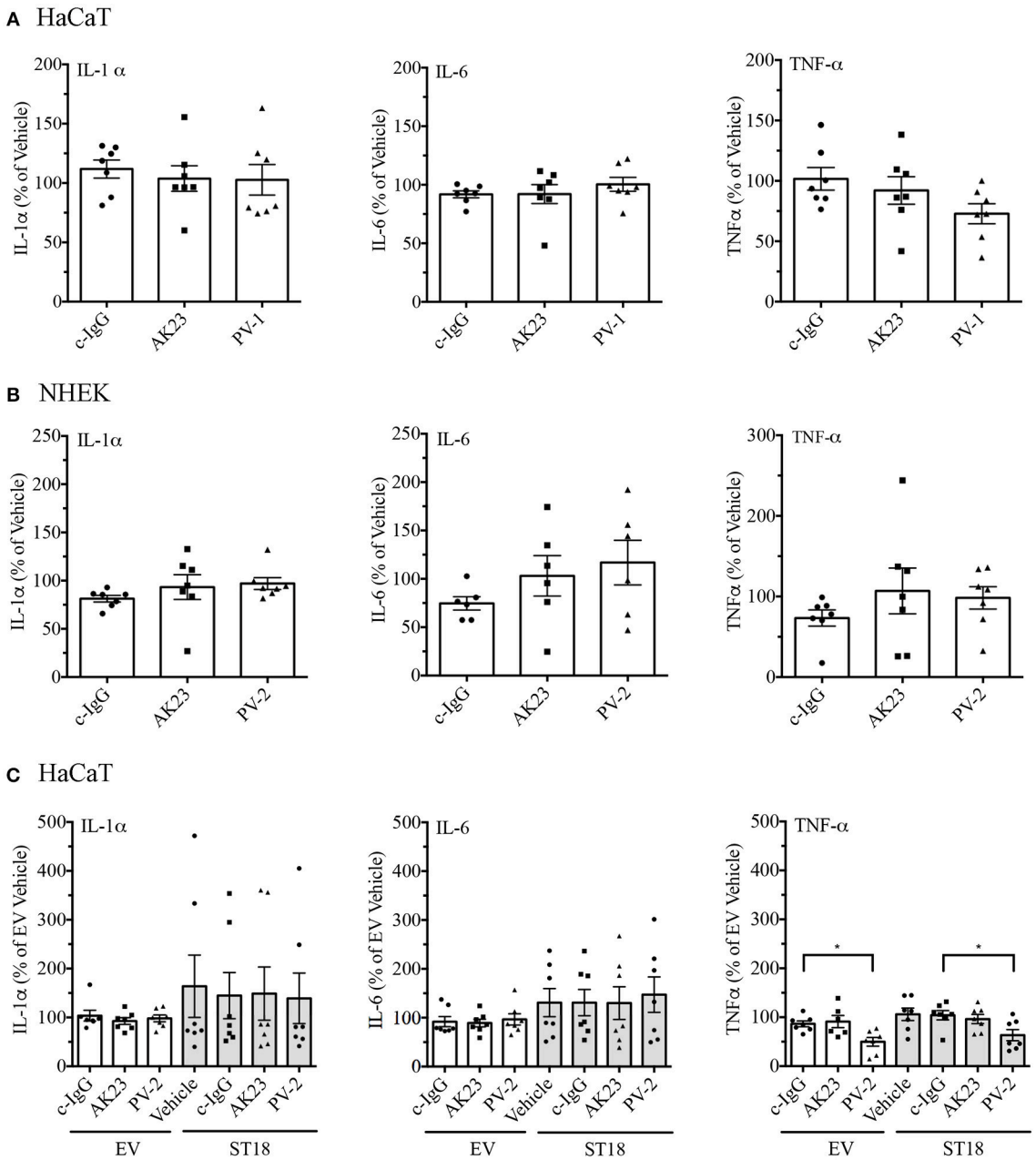

FIGURE 3 | Release of pro-inflammatory cytokines in response to pemphigus autoantibodies and ST18 overexpression. The content of cytokines in supernatants collected from cell monolayers, which were either treated with controls (Vehicle, c-IgG), AK23 or PV-IgGs, was evaluated in both HaCaT **(A)** and NHEK **(B)** cells. **(C)** Cytokine release by HaCaT cells transiently transfected with either EV or ST18 expression vectors and exposed to PV-IgG or AK23 was determined. All data are presented as percent of Vehicle ($n \geq 6$, two-way ANOVA; $^*p \leq 0.05$ vs. respective c-IgG).

factors promoting severity of the disease include non-Dsg antibodies (43) as well as genetic alterations such as recently reported ST18 SNPs (29–31) and may also entail keratinocyte-derived cytokine release.

Here, we investigate the effect of ST18 overexpression and cytokine secretion on PV-IgG mediated loss of adhesion. We observed that in both HaCaT and NHEK the release of key pro-inflammatory molecules such as IL-1α, IL-6, TNF-α, and IFN-γ is not strictly required for PV-IgG-induced loss of cell cohesion. However, ST18 overexpression did not only modulate cytokine release in some of the experiments but also altered ERK activity.

Pemphigus, as an autoimmune disease, depends highly on the activity of T-cells, the differentiation of which is regulated by autocrine cytokine signaling and is modulated by paracrine cytokine secretion from other cell type such as keratinocytes (44, 45). In skin, the production and secretion of cytokines has been

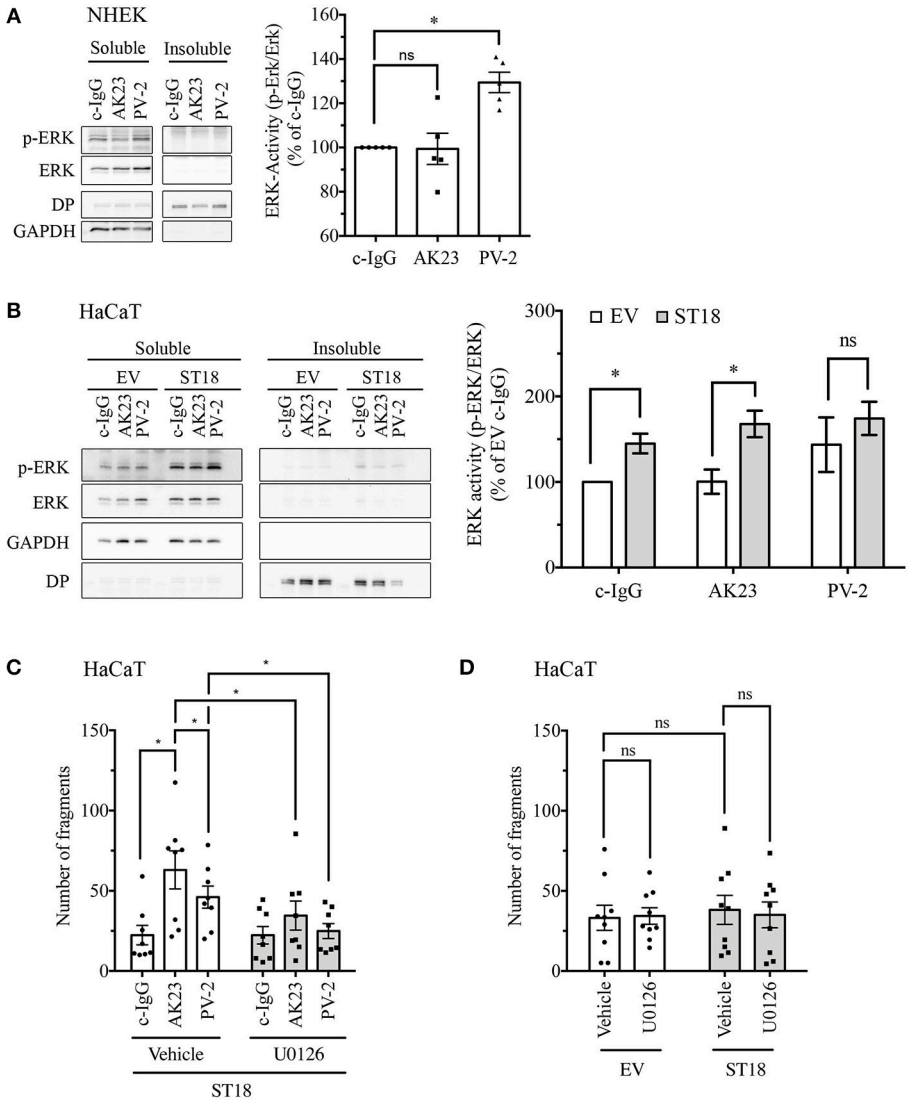

FIGURE 4 | Modulation of ERK signaling after incubation with pemphigus autoantibodies and ST18 overexpression. Twenty four hours after differentiation, NHEK cells were exposed for 30 min to different IgG fractions. Triton X-100-soluble (cytosolic) and Triton X-100-insoluble (representing cytoskeletal- bound) fractions were generated and further analyzed by Western blot. **(A)** Representative blot of ERK phosphorylation in response to IgG for each Triton X-100 fraction produced. GAPDH was used as a cytosolic marker, while Desmoplakin (DP) served as a cytoskeletal-bound marker. Densitometric quantification of ERK activity ($n = 5$, One-Way ANOVA, $^*p < 0.05$ vs. c-IgG). **(B)** Triton X-100 fractionation of HaCaT cells transfected with EV or ST18 vector and treated with IgG fractions for 30 min. Representative western blots showing total and phosphorylated ERK with respective markers for protein fractionation. Densitometric analysis of ERK activity within the soluble fraction using ImageJ software ($n = 7$, multiple *T*-tests, $^*p < 0.05$). **(C)** In HaCaT cells, 24 h after ST18 transfection an U0126 was applied for an hour followed by 2 h incubation with different IgG fractions, as indicated. Dispase-based assay evaluating the effect of ST18 mediated ERK activation in cell monolayers treated with autoantibody fractions. **(D)** Impact of U0126-mediated ERK inhibition on baseline cell adhesion in EV- and ST18-transfected monolayers assessed by dispase-based dissociation assays ($n \geq 8$, two-way ANOVA, $^*p < 0.05$).

recognized long ago and therefore intensely studied (46). These pro-inflammatory cytokines can also modulate keratinocyte behavior and could therefore be relevant for blister formation in pemphigus (47, 48).

Most studies on PV and cytokine release, however, are conflicting and report patient serum cytokine levels, which are most likely derived from T-cell signaling (49–52). Only few studies include cytokine assessment of blister fluid and only single reports evaluate production and secretion of cytokines *in vitro* (53). In the latter, polyclonal IgG fractions containing a plethora of antibodies, which could also include non-Dsg antibodies, were used (53). Therefore, in the present study, in addition to patient-derived PV-IgG the ability of a specific aDsg3 antibody, i.e., AK23, to induce cytokine secretion in keratinocytes was investigated. Both, AK23 and PV-IgG did not provoke significant cytokine secretion in HaCaT and NHEK cells (**Figures 3A,B**) but were efficient to impair keratinocyte adhesion. Therefore, secretion of keratinocyte-derived cytokines

seems not to be primarily important for induction of loss of cell adhesion. Other antibodies such as mitochondrial antibodies or soluble FAS-ligand might change cytokine secretion profiles and thereby augment the disease as a secondary factor (54, 55).

Studies available on the transcription factor ST18 are limited. However, one of the first reports validated ST18 as a suppressor of tumor growth in breast cancer cell lines (56). Additionally, in fibroblasts, ST18 was reported to promote the expression of pro-inflammatory and pro-apoptotic genes, including cytokines (26). Furthermore, a relationship between a ST18 polymorphism and PV has now been shown to be relevant for Israelian, Egyptian (30) as well as Iranian (31) but not German (30) or Chinese (57) populations. In this respect, it was reported that increased loss of adhesion was associated with cytokine secretion in primary NHEK cells after introduction of ST18 expression vector and incubation with PV-IgG (29). Similarly, in HaCaT cells, we also detected decrease of cell adhesion upon simultaneous ST18 overexpression and pathogenic autoantibody treatment. However, we found elevated cytokine secretion in some but not all experiments following ST18 overexpression independently of IgG treatment. The discrepancy between both studies could possibly be based on the usage of different cell lines since in the study of Vodo et al. (29) NHEK cells were used whereas our experiments were performed with HaCaT cells. Nevertheless, these results indicate that mechanisms independent of cytokine secretion may also contribute to ST18-induced enhancement of adhesion loss.

Activation of several signaling pathways regulates desmosome stability and is involved in pemphigus skin blistering (58, 59). We focused on ERK because it was observed previously that this pathway is activated only when antibodies against Dsg1, which are known to be primarily important for epidermal blistering in pemphigus, were present in autoantibody fractions (1, 16, 17, 60). Similarly to ST18, ERK modulation also was shown to be associated with cytokine signaling (61–65) and could thereby link desmosome destabilization and ST18 expression. Here we show, that expression of ST18 induced significant ERK activation after c-IgG and AK23 treatment, which led to the hypothesis that ST18 promoted increase in disease severity is mediated by ERK activation independent of IgG treatment. In order to validate this hypothesis, ERK activation was inhibited by U0126. The mediator abolished loss of cell cohesion in monolayers transfected with ST18 and incubated with pemphigus antibodies indicating that ST18-mediated loss of adhesion is at least in part induced by ERK signaling pathway modulation.

Mechanisms underlying loss of cell adhesion in response to AK23 or PV-IgG may differ as the latter contain antibodies against Dsg1 and possibly against various other antigens (43). Moreover, it has been shown that in contrast to antibodies targeting Dsg3 such as AK23, which are capable to prevent homophilic interactions between Dsg3 molecules (66), antibodies targeting Dsg1 were not observed to inhibit Dsg1 interactions (34).

We previously reported that ERK was not activated by AK23 or mucosal-dominant PV-IgG both of which are targeting Dsg3, but rather by IgG fractions containing aDsg1 antibodies (17). Here we confirmed these results as we detected increased ERK phosphorylation after incubation with mucocutaneous PV-IgG but not AK23 in NHEK cells. However, the underlying ERK-dependent mechanisms relevant for pemphigus skin blistering are not yet completely understood. Interestingly, upon ST18 overexpression in HaCaT monolayers, ERK was activated under AK23 as well as c-IgG treatment, suggesting that ST18 may generally modulate ERK signaling. Nevertheless, further analysis revealed that baseline adhesion in ST18-transfected cells was unaffected by ERK inhibition (**Figure 4D**) indicating that PV-IgG is required as an additional factor to facilitate ERK-mediated loss of adhesion in response to ST18 overexpression.

Taken together, our study adds new insight on the mechanisms by which ST18 may contribute to pemphigus pathogenesis and indicates that altered signaling mechanisms regulating desmosomal adhesion may render keratinocytes more susceptible to autoantibody-induced loss of cell adhesion.

ETHICS STATEMENT

Human sera were collected and used in accordance with recommendations of the ethics committee of the Philipps-Universität Marburg, Marburg, Germany. Name of the indicated project: Phänotypische und funktionelle Analyse von Immunzellen des peripheren Bluts beim Pemphigus vulgaris.

AUTHOR CONTRIBUTIONS

MR and EW performed the experiments, participated in study design, interpreted results, and wrote the manuscript. RS isolated the primary NHEK cells. JW designed the experiments, interpreted results, revised the manuscript and provided the funding. AY, NS, OS, and ES revised the manuscript and actively participate in the scientific discussions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00770/full#supplementary-material

Supplementary Figure 1 | NHEK monolayers treated with c-IgG, AK23 and PV-IgG were immunostained for Dsg3. Representative immunofluorescence images from n ≥ 4. Bar scale is 20 μm.

Supplementary Figure 2 | Release of IFN-γ is unaffected by pemphigus autoantibody application or ST18 overexpression. Confluent keratinocyte monolayers were exposed to PV-IgG or AK23 for 24 h. Respective controls were run in parallel. IFN-γ concentration was assessed in HaCaT **(A)** and NHEK **(B)** cells. **(C)** HaCaT cells transiently transfected with control (EV) or ST18 expression vectors were treated as indicated. 24 h later, the release of IFN-γ was evaluated. Secretion of IFN-γ is presented as raw data (pg/ml), as each data point represents the mean of a single experiment. A dotted red line depicts the detection limit of the cytokine assay; $n \geq 6$.

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Current Clinical Trials in Pemphigus and Pemphigoid

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Autoimmune bullous dermatoses (AIBDs) are a group of rare chronic inflammatory skin diseases, which clinically manifest as blisters and erosions of the skin and/or mucosa. Immunologically, AIBDs are characterized and caused by autoantibodies targeting adhesion molecules in the skin and mucosa. According to the histological location of the blistering, AIBDs are classified into the following two main subtypes: pemphigus (intraepidermal blistering) and pemphigoid (subepidermal blistering). Most AIBDs were potentially life-threatening diseases before the advent of immunosuppressive drugs, especially systemic steroid therapies, which suppress pathogenic immunological activity. Although there have been recent advancements in the understanding of the pathogenesis of AIBDs, glucocorticosteroids and/or adjuvant immunosuppressive drugs are still needed to control disease activity. However, the long-term use of systemic immunosuppression is associated with major adverse events, including death. Based on the growing understanding of AIBD pathogenesis, novel treatment targets have emerged, some of which are currently being evaluated in clinical trials. Within this article, we review the current clinical trials involving pemphigus and pemphigoid and discuss the rationale that lead to these trials. Overall, we aim to foster insights into translational research in AIBDs to improve patient care.

Keywords: autoimmune blistering skin disease, pemphigus, pemphigoid, clinical trial, treatment

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INTRODUCTION

Autoimmune bullous dermatoses (AIBDs) are a heterogeneous group of skin diseases that are characterized and caused by autoantibodies targeting adhesion molecules in the skin and/or mucous membranes. Depending on the targeted adhesion molecules and the location of the blistering, AIBDs are classified into the following two major types: pemphigus diseases with autoantibodies targeting desmosomal proteins (1) and pemphigoid diseases with autoantibodies targeting the structural proteins of dermal-epidermal junction (2). In pemphigus diseases, including pemphigus vulgaris (PV) and pemphigus foliaceus (PF), autoantibody binding leads to the disruption of epidermal adhesion, resulting in the clinical finding of flaccid blisters and the histological finding of intraepidermal blistering (1). In pemphigoid diseases, the linear deposition of autoantibodies along the dermal-epidermal junction causes subepidermal blistering, resulting in tense blisters. Based on the target molecules of the autoantibodies and the clinical manifestations, pemphigoid diseases are classified as bullous pemphigoid (BP), mucous membrane pemphigoid (MMP), epidermolysis bullosa acquisita (EBA), anti-laminin-γ1/p200 pemphigoid (p200), pemphigoid gestationis (PG), lichen planus pemphigoides (LPP), linear IgA bullous dermatosis (LAD), and dermatitis herpetiformis (DH), which is associated with gluten-sensitive enteropathy and characteristic granular IgA deposits in the upper dermis (3–5).

Although the advent of systemic steroid therapy significantly improved the prognosis of AIBDs, these groups of diseases are still potentially life-threatening, now mainly due to adverse events resulting from corticosteroid treatment (6). Due to the chronicity of AIBDs, the prolonged administration of systemic steroids is often needed to induce and maintain clinical remission, leading to various adverse effects such as cytopenia, diabetes mellitus, osteoporosis, hypertension, gastrointestinal ulcers, and infections due to immunosuppression (7). Furthermore, severe infection induced by an immunocompromised state is one of the most important causes of death during AIBD treatment (8, 9). Thus, the development of alternative treatment modalities that have fewer adverse events is urgently needed for the treatment of AIBD patients.

Based on the growing understanding of AIBD pathogenesis (1, 10, 11), novel therapeutic targets and/or treatment modalities have been identified (12–15). Some of those new treatments are currently being evaluated in clinical trials. To foster translational AIBD research, in this article, we summarize the current clinical trials involving pemphigus and pemphigoid diseases. For this purpose, we searched clinicaltrials.gov[1] and the EU clinical trials register[2] through December 2018 and selected clinical trials on pemphigus or pemphigoid disease with the status "recruiting (the study is currently recruiting participants)," "active, not recruiting (the study is ongoing, and participants are receiving an intervention or being examined, but not currently being recruited or enrolled)," and "completed (the study has ended normally, and participants are no longer being examined or treated)," and an initiation in 2013 or later (**Tables 1**, **2**, **Figure 1**). To provide a more comprehensive overview, we list the trials before this time frame in **Supplementary Tables 1, 2**. In addition, we included one clinical trial that is registered with the Australia and New Zealand Clinical Trials Registry, which was recently presented at the 5th International Pemphigus and Pemphigoid Foundation Scientific Conference in Orlando (16).

PEMPHIGUS

Anti-CD20 in Pemphigus (Rituximab or Ofatumumab)

Rituximab is a human chimeric IgG1 monoclonal antibody targeting CD20, which is a cell surface marker expressed by B cells (17). Rituximab exerts its treatment effects via the depletion of B-cells following its binding to CD20. Regarding the treatment of pemphigus with rituximab, case reports have suggested the efficacy of rituximab as a second- or third-line therapy (18–20). There are two main rituximab regimens for pemphigus; one regimen, which is based on the lymphoma protocol, is composed of a total of 4 doses of 375 mg/m^2 weekly infusions (21), while the other regimen, which is based on the rheumatoid arthritis protocol, consists of a total of two doses of 1,000 mg (or 500 mg) biweekly intravenous infusions (22). Prior to evaluating the efficacy of a single cycle regimen of rituximab in refractory pemphigus cases, a multicenter, single arm, phase 2/3 clinical trial was conducted in France (23). At 3 months after rituximab treatment, 18 (86%) of 21 cases achieved complete remission (CR). Furthermore, 18 (86%) of 21 cases maintained CR after a median follow-up of 34 months. In addition, 8 of these 18 cases did not receive systemic corticosteroid therapy. To evaluate the safety and efficacy of rituximab in a controlled clinical trial, a prospective, parallel-group, open-label, randomized, phase 3 clinical trial of rituximab as a first-line treatment for moderate to severe cases of pemphigus (**NCT00784589**) was initiated (**Table 1**). Patients with PV or PF were randomized to receive either oral prednisolone at 1.0 to 1.5 mg/kg/day that was tapered over 12–18 months or oral prednisolone at 0.5–1.0 mg/kg/day that was tapered over 3–6 months plus rituximab 1 g on days 0 and 14 and 0.5 g at months 12 and 18. The primary endpoint was the proportion of patients who achieved CR off-therapy at month 24. The results of this clinical trial were recently reported (24). Regarding the primary endpoint, 34% of patients in the prednisolone arm achieved CR at month 24. In the rituximab plus prednisolone arm, CR was reached by 89% of the patients; the difference was significant. Furthermore, fewer grade 3–4 adverse events were observed in the rituximab plus prednisone group than in the prednisone alone group (27 events in 16 out of 46 patients; mean 0.59 [SD 1.15] vs. 53 events in 29 out of 44 patients; mean 1.20 [1.25]). Overall, this trial demonstrated that the first-line treatment of pemphigus with rituximab and lower doses of prednisolone is more effective and safer compared to high-dose prednisolone treatment. Based on the results of this clinical trial, the Food and Drug Administration approved the expansion of health insurance coverage of rituximab for pemphigus vulgaris in the United States.

In addition to rituximab, ofatumumab, a fully human anti-CD20 monoclonal antibody, has been demonstrated to be safe and effective for the treatment of autoimmune disorders other than pemphigus or pemphigoid (25). Preclinical studies suggested that ofatumumab shows a high affinity for CD20 and activates complement-dependent cytotoxicity (26). A double-blind, randomized, placebo-controlled, phase 3 clinical trial evaluating the efficacy of ofatumumab in pemphigus was completed in January 2018 (**NCT01920477**). This clinical trial enrolled moderate

[1]https://clinicaltrials.gov/

[2]https://www.clinicaltrialsregister.eu/

Abbreviations: AIBDs, autoimmune bullous dermatoses; PV, pemphigus vulgaris; PF, pemphigus foliaceus; BP, bullous pemphigoid; EBA, epidermolysis acquisita; p200, anti-laminin-γ1/p200 pemphigoid; PG, pemphigoid gestationis; LPP, lichen planus pemphigoides; LAD, linear IgA bullous dermatosis; DH, dermatitis herpetiformis; RA, rheumatoid arthritis; MS, multiple sclerosis; CR, complete remission; MMF, mycophenolate mofetil; BAFF, B cell activating factor of the tumor necrosis factor family; BAFF-R, B cell activating factor of the tumor necrosis factor family receptor; BCR, B cell receptor; PDAI, Pemphigus Disease Area Index; FcRn, neonatal Fc receptor; IVIg, intravenous immunoglobulin; BTK, Bruton's tyrosine kinase; Tregs, regulatory T cells; GVHD, graft vs. host disease; Dsg, desmoglein; PRP, Platelet-rich plasma; CAR, chimeric antigen receptor; Dsg3-CAART, Dsg3 chimeric autoantibody receptor T cell; IL, interleukin; EAE, experimental autoimmune encephalomyelitis; DAS, disease activity score; NLRP3, nucleotide-binding domain, leucine-rich repeat family, pyrin domain-containing 3; BPDAI, bullous pemphigoid disease area index; ECP, eosinophil cationic protein; SYK, spleen tyrosine kinase; FcγR, Fc gamma receptor; LTB4, leukotriene B4.

TABLE 1 | Current clinical trials in pemphigus.

NCT number	Disease	Interventions	Target	Allocation	Masking	Phase	Status
NCT01930175	PV	VAY736	BAFF-R	Randomized	Double blind	2	Active, not recruiting
NCT01920477	PV	Ofatumumab	CD20	Randomized	Double blind	3	Completed
NCT03334058	PV	ARGX-113	FcRn	Single group	None	2	Recruiting
NCT02704429	PV or PF	PRN1008	BTK	Single group	None	2	Recruiting
NCT03762265	PV or PF	PRN1008	BTK	Randomized	Quadruple blind	3	Recruiting
NCT02383589	PV	Rituximab MMF	CD20 IMPDH	Randomized	Double blind	3	Active, not recruiting
NCT03239470	PV or PF	Poly Tregs	Immune tolerance	Non- randomized	None	1	Recruiting
NCT02828163	PV	PRP	Wound healing	Randomized	Double blind	3	Recruiting
NCT00784589	PV or PF	Rituximab	CD20	Randomized	None	3	Completed
NCT03075904	PV or PF	SYNT001	FcRn	Non-randomized	None	1/2	Completed

PV, pemphigus vulgaris; PF, pemphigus foliaceus; BAFF-R, B cell activating factor of the tumor necrosis factor family receptor; FcRn, neonatal Fc receptor; BTK, Bruton's tyrosine kinase; MMF, mycophenolate mofetil; IMPDH, inosine 5′-monophosphate dehydrogenase; Tregs, regulatory T cells; PRP, Platelet rich plasma.

TABLE 2 | Current clinical trials in bullous pemphigoid.

NCT number	Interventions	Target	Allocation	Masking	Phase	Status
NCT03099538	Ixekizumab	IL-17	Single group	None	2	Recruiting
NCT01408550	NPB-1	FcRn	Randomized	Double blind	3	Completed
NCT03286582	AC-203	Inflammasome	Randomized	None	2	Recruiting
NCT02226146	Bertilimumab	eotaxin	Single group	None	2	Completed
NCT00525616	Rituximab	CD20	Single group	None	3	Completed

No clinical trials are being performed for any other pemphigoid diseases. FcRn, neonatal Fc receptor.

to severe PV patients with a history of at least failure of tapered steroid therapy. Participants received a stable dose of prednisone/prednisolone from a minimum of 20 mg/day up to a maximum of 120 mg/day or 1.5 mg/kg/day for 2 weeks prior to randomization. Thirty-five patients with PV were randomized to receive either ofatumumab or a placebo. Ofatumumab at 40 mg was subcutaneously injected at week 0 and week 4. From week 8, subjects were subcutaneously administered ofatumumab 20 mg every 4 weeks through week 56. The primary endpoints were the time to sustained CR on minimal steroid therapy (prednisone/prednisolone dose to <10 mg/day) and the duration of CR on minimal steroid therapy. The results of this study have thus far not been reported.

Similar to these two trials, another double-blind, randomized, phase 3 clinical trial of adjuvant rituximab vs. mycophenolate mofetil (MMF) as a therapy for pemphigus is ongoing (**NCT02383589**). In that study, 135 PV patients who are receiving standard systemic steroid treatment (oral prednisone 60–120 mg/day or equivalent) will randomly receive either (i) MMF and a rituximab-matched placebo or (ii) an MMF-matched placebo and rituximab. MMF is orally administered twice daily from day 1 to week 56. The initial dose of MMF is 500 mg, and the dose will be titrated to achieve a goal of 1,000 mg. Rituximab is administered at a dose of 1,000 mg intravenously on days 1, 15, 168, and 182. The primary endpoint is the proportion of patients who achieve sustained CR.

VAY736 in Pemphigus

B cell activating factor of the tumor necrosis factor family (BAFF) is a crucial cytokine for regulating B cell development in mice and humans (27, 28). BAFF functions by binding to the BAFF receptor (BAFF-R), B cell maturation antigen, and tumor necrosis factor receptor superfamily member (29). Although at physiological concentrations BAFF cannot rescue B cell apoptosis due to a strong B cell death signal, which is transduced via the B cell receptor (BCR) stimulated by autoantigens, at higher concentrations, BAFF causes the survival of autoreactive B cells, which contributes to the pathogenesis of autoimmune diseases (27, 30, 31). For example, elevated BAFF serum levels have been detected in various autoimmune diseases such as RA, systemic lupus erythematosus, Sjögren's syndrome, and systemic sclerosis (32–34). Taken together, BAFF is a likely therapeutic target for the treatment of autoimmune diseases. Regarding clinical translation, the anti-BAFF antibody belimumab was licensed for the treatment of systemic lupus erythematosus in 2011 (35). However, clinical trials of belimumab have not been conducted in pemphigus thus far. Alternatively, with regard to the inhibition of BAFF, its receptor can be blocked to achieve similar results. BAFF-R signaling drives B cell differentiation, proliferation and survival (36). VAY736 is a novel, defucosylated, human IgG1 monoclonal antibody targeting BAFF-R, providing both enhanced antibody-dependent cellular cytotoxicity-mediated depletion of B cells and the blockade of BAFF. To investigate the safety, tolerability and efficacy of VAY736 in PV, a randomized, placebo-controlled, double-blind,

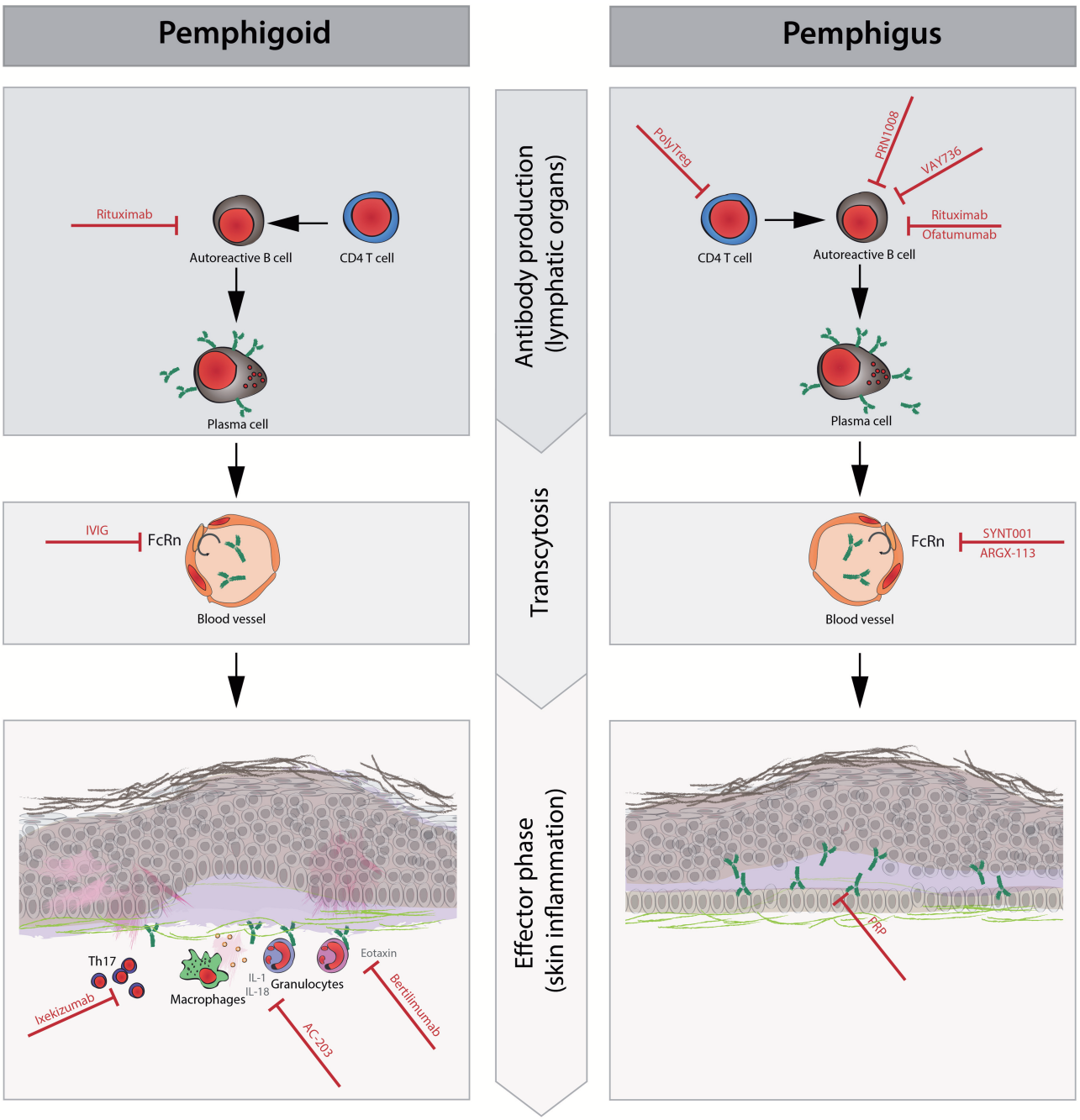

FIGURE 1 | Schematic representation of the pathophysiology and new therapeutic targets of pemphigus and pemphigoid diseases. The pathophysiology of pemphigus and pemphigoid diseases consists of the following three phases: (1) CD4+T cells promote autoreactive B cell activation, proliferation, and differentiation to plasma cells that produce pathogenic autoantibodies. (2) Circulating pathogenic antibodies are transferred to the dermal epidermal junction or intracellular space of the epidermis. Neonatal Fc receptor (FcRn) plays a role in prolonging the half-life of IgG antibodies during this phase. (3) After the binding of pathogenic autoantibodies to target molecules, pro-inflammatory cells such as granulocytes and macrophages are recruited to the immune complex in lesional skin by chemokines (e.g., eotaxin). Then, granulocytes elicit reactive oxygen species (ROS), elastases, and proteases, resulting in tissue damage such as blisters and/or erythema, which are clinical symptoms in pemphigoid diseases but not in pemphigus diseases. Cytokines [e.g., interleukin (IL)-1beta and IL-18] and Th17 polarization are thought to enhance local inflammation. During the antibody production phase, rituximab and ofatumumab deplete autoreactive B cells to prevent their differentiation to plasma cells. PolyTregs act on CD4+ T cells, and VAY736 and PRN1008 act on autoreactive B cells, resulting in less activation of autoreactive B cells. In the transcytosis phase, intravenous immunoglobulin (IVIg), SYNT001, and ARGX-113 saturate FcRn, contributing to the shortened half-life of pathogenic autoantibodies. In the effector phase, ixekizumab restores Th17 polarization and suppresses inflammatory augmentation. AC-203 modulates cytokines such as IL-1beta and IL-18, contributing to decreased inflammation. The inhibition of eotaxin with bertilimumab ameliorates the recruitment of eosinophils to local inflammation sites in pemphigoid disease, especially bullous pemphigoid. Platelet-rich plasma (PRP) is thought to promote wound healing in erosions.

phase 2 clinical trial is currently ongoing (**NCT01930175**). In this trial, 16 mild-moderate PV patients will randomly receive either intravenous VAY736 or a placebo once. The primary endpoint is the efficacy of single cycle VAY736 administration in reducing Pemphigus Disease Area Index (PDAI) scores at week 12 compared to at the baseline. No results of this trial are currently available.

Anti-neonatal Fc Receptor (FcRn) in Pemphigus (SYNT001, ARGX-113)

FcRn plays an essential role in regulating host circulating IgG levels (37). FcRn protects IgG from intracellular digestion, leading to the prolongation of its half-life. This "IgG recycling system" is essential for host defense. However, it also maintains the concentration of circulating pathogenic IgG in various autoimmune diseases, including AIBD. Specifically, FcRn-deficient mice do not (or to a lesser extent) develop experimental AIBD after the injection of AIBD-inducing antibodies (10). Interestingly, saturation of the FcRn by the administration of high-dose human IgG (IVIg) reduces the pathogenic effects in antibody-transfer AIBD models. Thus, FcRn inhibition is one possible mode of action of IVIg therapy in AIBD (38, 39). Based on these observations, anti-FcRn targeting treatments have been developed. Currently, the following 2 clinical trials targeting FcRn are being conducted in pemphigus: SYNT001 (**NCT03075904**) and ARGX-113 (**NCT03334058**). SYNT001 is a humanized, deimmunized IgG4 monoclonal antibody that blocks the binding of FcRn to the Fc portion of IgG (40). ARGX-113 is a human IgG1-derived Fc-modified fragment with increased affinity for FcRn that reduces the circulating IgG concentration (41). In addition, both SYNT001 and ARGX-113 do not alter serum levels of albumin. As both trials are ongoing, no results have been published so far. Regarding **NCT03075904**, 16 PV or PF patients will be sequentially assigned to receive three different doses of intravenous SYNT001 weekly for either 5 or 14 weeks, and the primary endpoint is the count and percentage of adverse events (time frame; days 0–112 or days 0–175). At the 2018 pre-International Investigative Dermatology meeting in Orlando, the first results were reported for SYNT001. The infusion of SYNT001 in human subjects resulted in a rapid lowering of the circulating levels of IgG (mean total IgG reduction of 56% by day 30) with good safety and tolerability. Furthermore, 5 of the 7 subjects showed a reduction in disease activity by day 42 (42). Regarding **NCT03334058**, 12 newly diagnosed or relapsed PV patients will receive ARGX-113 intravenously. The primary endpoints are safety and tolerability up to 17 weeks.

PRN1008 in Pemphigus

B cell receptor signaling is a key player in B cell development and function. Bruton's tyrosine kinase (BTK) belongs to the Tec family of nonreceptor tyrosine kinases, and it is a vital component of B cell receptor signaling (43). BTK is predominantly expressed by B-lymphocytes from the pre-B cell stage to the mature B cell stage (44, 45). Based on the crucial role of BTK in B cell function, it has been identified as a potential target for the treatment of autoimmune disorders. Several studies have shown that ibrutinib, which is one of the BTK inhibitors under development, binds to BTK with high affinity, leading to the inhibition of B cell receptor signaling and resulting in the reduction of B cell activation involved in autoimmunity (46). In pemphigus treatment, a previous case report showed that ibrutinib improved clinical cutaneous lesions of paraneoplastic pemphigus complicated with chronic lymphocytic leukemia (47). However, there have been no case reports or series investigating the effects of this BTK inhibitor in PNP, PV and/or PF. PRN1008 is another BTK inhibitor that was evaluated in a phase 1 clinical trial that enrolled 80 healthy volunteers (Australian New Zealand Clinical Trials Registry No. **ACTRN12614000359639**). In that study, PRN1008 was considered safe. Regarding the pharmacokinetics and pharmacodynamics, BTK occupancy of more than 90% was observed within 4 h after dosing in both the single and multiple dose regimens and was closely correlated with the maximum plasma concentration (48). Based on the promising results from the phase 1 trial, an open-label, single-armed, phase 2 clinical trial of PRN1008 for PV treatment has been conducted (**NCT02704429**). In this trial, 27 pemphigus patients (including PV and PF) received PRN1008 orally for 12 weeks with a 12-week follow-up period. The primary efficacy endpoint was the initial control of disease activity during the first 4 weeks of therapy, during which new lesions cease to form and existing lesions begin to heal, without the need for prednisone-equivalent corticosteroid doses >0.5 mg/kg/day. More than 50% of patients have achieved control of disease activity within 4 weeks of starting PRN1008 thus far[3]. Furthermore, Principia has recently initiated a global, randomized, double-blind, placebo-controlled, pivotal phase 3 clinical trial (**NCT03762265**) in approximately 120 PV or PF patients to evaluate PRN1008 vs. a placebo. The primary endpoint is the proportion of participants who are in CR from week ≤ 29 to 37 with a prednisone dose of ≤5 mg/day.

Polyclonal Regulatory T Cells (PolyTregs) in Pemphigus

Among the T cell subtypes, regulatory T cells (Tregs) play an important role in regulating the immune system and preventing autoimmune disease development. Based on findings in animal models, including AIBD (49, 50), it is hypothesized that naturally occurring Tregs may be utilized for the treatment of autoimmune diseases and potentially replace the use of chronic immunosuppressive therapies that are associated with multiple adverse effects. A clinical trial of Treg adoptive therapy was started by treating graft vs. host diseases (GVHD) with expanded allogeneic Tregs (51). There has been a small study demonstrating the safe administration of autologous Tregs with decreased disease activity in patients with insulin-dependent diabetes (52). Subsequently, Brunstein et al. reported that HLA-matched umbilical cord blood-derived Tregs decreased the incidence of GVHD after double umbilical cord blood transplantation (53), indicating the potential efficacy and safety profile of the passive transfer of autologous Tregs in humans. The application of Tregs for lupus, cancer and organ transplantation

[3]https://ir.principiabio.com/news-releases/news-release-details/principia-biopharma-reports-positive-prn1008-phase-2-top-line

has been addressed (54). The suppressive effects of Tregs have been reported by studies using an active PV mouse model (55). A recent study indicated that Treg induction via the anti-CD28 antibody reduces pathogenic IgG directing desmoglein (Dsg) 3 in the HLA-DRB1*04:02- transgenic PV mouse model (56). Although Tregs are expected to improve PV symptoms, there are no case reports or case series of autologous Treg injections in patients with PV to date. To evaluate the effects of Tregs on the manifestation of PV, a nonrandomized, open-label, phase 1 clinical trial is ongoing (**NCT03239470**). In this trial, 12 PV or PF patients will receive one infusion of autologous expanded Tregs (CD4+CD127lo/negCD25+) at one of the following doses: either 2.5 x 10^8 poly Tregs or 10 $\times$ 10^8 poly Tregs. The primary endpoint is the number of significant grade 3 or higher adverse events by week 52. The results from this study have thus far not been reported.

Comparison of Injections of Steroids to Autologous Platelet-Rich Plasma (PRP) in Oral Erosions in PV

Oral erosive lesions are a major hallmark of PV. These oral lesions cause severe pain, resulting in problems with eating and drinking (57). To improve oral lesions in PV, adjuvant topical or intralesional steroids are used, and based on evidence from case reports, the treatment is effective (58, 59). To date, however, no controlled clinical trial of the treatment of oral lesions in PV has been performed. PRP, which is concentrated plasma derived from autologous whole blood, is believed to promote wound healing (60). In a case report series, El-Komy et al. reported that six of seven PV patients showed improvement of their oral PDAI scores after PRP intralesional injection (61). To evaluate the effects of PRP in PV, an open-label, dose-escalation, multicenter phase 1 trial using autologous PRP has been conducted in adults with active PV (**NCT02828163**). This clinical trial was designed to compare the effects of PRP to those of intralesional steroid injection. Eleven PV patients received a 10 mg/mL triamcinolone injection on one side of the oral mucosa and a 1-mL PRP injection on the other side every 2 weeks for 3 months. The primary endpoint was the improvement of oral PV lesions in 3 months. Nine out of 11 participants completed the protocol, and 7 (78%) of those 9 patients showed improvement in oral PDAI and/or pain scores at the PRP injection sites. Although PRP resulted in clinical improvement, as in the previous study, there were no significant differences between PRP and intralesional steroid injections (62). Thus, autologous PRP might be used for the treatment of resistant oral erosions in pemphigus patients when intralesional steroid injection is contraindicated.

Future Potential Clinical Trials in Pemphigus

B cell depletion therapy using an anti-CD20 monoclonal antibody leads to short-term remission in the majority of pemphigus patients; however, we often observe relapsing disease after treatment. Although disease remission is related to the depletion of circulating Dsg3-specific B cells, the expansion of the same pathogenic B cell clone is observed during relapsing disease (63). Therefore, to maintain complete remission in PV, targeted removal of anti-Dsg3 memory B cells is essential. Recently, chimeric antigen receptor (CAR) technology was developed and resulted in novel treatments that led to the prolonged remission of refractory B cell leukemia and lymphoma. Adapted from that strategy, Dsg3 chimeric autoantibody receptor T cell (Dsg3-CAART) therapy has been reported to result in serological and histological improvements in experimental pemphigus mice without detectable off-target toxicity (15). A phase 1 clinical trial of Dsg3-CAART in PV patients is planned to investigate its safety and therapeutic potential.

PEMPHIGOID

Ixekizumab in BP

Th17 cells were first identified by their production of interleukin (IL)-17 (64, 65). A previous study indicated that the transfer of IL-17-producing Th17 cells into healthy SJL/J mice induced experimental autoimmune encephalomyelitis an animal model of MS (66). The prominent role of the Th17 polarization of T cells, as well as that of IL-17, in inflammation and autoimmunity was recognized after this observation. In addition to EAE, the Th17-IL-17 axis is also important in the pathogenesis of RA (67–69). Notably, neutralizing anti-IL-17 antibodies significantly decreased joint inflammation, cartilage destruction, and bone erosion in a collagen-immunized arthritis mouse model. Several recent studies suggest that Th17 cells and their cytokines also play roles in the pathogenesis of AIBD, including BP (70). More specifically, Le Jan et al. reported that IL-17A expression was significantly higher in both serum and lesional skin in BP patients than in normal healthy individuals (71). Furthermore, Chakievska et al. reported that IL-17A -/- mice were protected from the development of experimental BP induced by the transfer of anti-COL17 IgG (72). Taken together, these results indicate that IL-17A is a potential target for the treatment of BP. Ixekizumab is a recombinant fully humanized monoclonal antibody targeting IL-17 and is used for the treatment of psoriasis (73). However, there are neither case reports nor clinical studies reporting the effect of ixekizumab or other IL-17-targeting compounds on BP. To determine the efficacy of ixekizumab for the treatment of BP, an open-label, single-group phase 2 clinical trial is currently ongoing (**NCT03099538**). In this trial, twelve BP patients will receive subcutaneous injections of 160 mg ixekizumab on day 0, and then they will receive 80 mg of ixekizumab every 2 weeks until week 12. The primary endpoint is the median time from the start of treatment to the cessation of blister formation during the 12 weeks of therapy. There is no available report of the results of this trial to date.

NPB-01 in BP Unresponsive to Corticosteroids

With regard to pemphigus, the efficacy and safety of IVIg in refractory BP patients has been suggested by case reports and case report series (74–76). Based on these observations, several guidelines and consensus statements recommend the use of IVIg for refractory BP (77–79). Recently, Sasaoka

et al. reported that IVIg reduced serum IL-6 concentrations, circulating pathogenic IgG levels, and skin inflammation disease activity in an active BP mouse model (80). Kamaguchi et al. reported that an anti-idiotypic antibody in IVIg significantly reduced the BP180 depletion of cultured keratinocytes stimulated with BP pathogenic IgG (81). To evaluate the effectiveness of IVIg in BP, a multicenter, randomized, double-blind, placebo-controlled, phase 3 clinical trial was conducted (**NCT01408550**). In this trial, 56 BP patients were parallelly assigned to receive either IVIg or a placebo. The IVIg group received an intravenous drip infusion of human IgG at 400 mg/kg/day for 5 consecutive days. The primary endpoint was the disease activity score (DAS) of skin lesions on day 15. The results of that study have recently been published (82). The DAS on day 15 was evaluated as the primary endpoint. Although the DAS on day 15 in the IVIg group (19.8 ± 22.2) was 12.5 points lower than that in the placebo group (32.3 ± 31.5), the difference between the groups was not significant ($p = 0.089$). However, a *post hoc* analysis of covariance using the DAS on day 1 as a covariate showed a significant difference between the IVIg and placebo groups ($p = 0.041$). Regarding the safety of IVIg in BP, the incidence of adverse drug reactions was 37.9% ($n = 11/29$) in the IVIg group vs. 18.5% ($n = 5/27$) in the placebo group. No significant difference in the incidence of adverse drug reactions was observed between the IVIg and placebo groups ($p = 0.143$). No patients in either group experienced any severe adverse drug reactions. Therefore, this report suggested that IVIg is a beneficial treatment modality for refractory BP cases treated with a moderate dose of systemic steroids.

Topical AC-203 in BP

The nucleotide-binding domain, leucine-rich repeat family, pyrin domain-containing 3 (NLRP3) inflammasome regulates the activation of caspase-1 and the release of pro-inflammatory cytokines such as IL-1β and IL-18 in macrophages (83–87). It is an essential innate immune sensor that is activated in response to various damage-associated molecular patterns (83, 84). Previous reports have suggested that dysregulation of the inflammasome attenuates several chronic diseases, including autoimmune diseases such as EAE (88) and systemic lupus erythematosus (89, 90). In addition, polymorphisms in both NLRP3 and caspase-1 recruitment domain-8 genes led to increased IL-1β production and are related to disease susceptibility and severity in RA (91). A recent study demonstrated that the expression levels of NLRP3 and inflammasome components are significantly higher in peripheral blood mononuclear cells from BP patients than in those from healthy donors (92). Furthermore, higher NLRP3 levels were positively correlated with BP disease activity. Although the precise mechanism by which NLRP3 contributes to pathogenesis in BP is still unclear, pharmacological modulation of the inflammasome pathway could be a novel therapeutic strategy in BP. However, in a mouse model of inflammatory EBA, caspase-1/11-deficient mice developed clinical disease manifestations identical to those of the wild-type controls after the injection of autoantibodies against type VII collagen (93).

To evaluate the therapeutic efficacy of caspase-1 inhibition in BP, AC-203, which is a topical ointment formulation of a modulator of the inflammasome and IL-1β pathways, was developed. To investigate the effects of AC-203 on BP, a randomized, open-label phase 2 clinical trial is currently conducted (**NCT03286582**). Forty BP patients received either topical AC-203 or clobetasol 0.05% topical ointment twice a day (time frame is 10 weeks). The primary endpoint is the incidence of adverse effects during the treatment period. No results of this trial are currently available.

Bertilimumab in BP

While the presence of eosinophils in the dermal infiltrate is one of the histological hallmarks of BP, their contribution to the pathogenesis of BP remains to be determined (94–97). Recent data, however, support the notion that eosinophils promote subepidermal blistering in BP (98). In line with the presence of eosinophils in the dermal infiltrate in BP, elevated expression levels of IL-5, eotaxin, and eosinophil colony-stimulating factor were detected in BP blister fluids (99). Furthermore, matrix metallopeptidase 9 is secreted by eosinophils in BP lesional skin (100), eosinophil cationic protein (ECP) is detected at the dermal-epidermal junction of lesional skin, and serum concentrations of ECP correlate with BP disease activity (101, 102). Based on these findings, eotaxin, which is released upon eosinophil activation (103), has emerged as a therapeutic target for the treatment of BP.

Bertilimumab is a fully human monoclonal antibody targeting eotaxin-1 (104). Therefore, the administration of bertilimumab is expected to impair eosinophil infiltration into the skin in BP. This hypothesis has been recently tested in an open-label, single arm, phase 2 clinical trial (**NCT02226146**). Eleven moderate to severe BP patients received 10 mg/kg of bertilimumab intravenously on days 0, 14, and 28 with approximately 13 weeks of follow-up. The primary endpoint was safety, including the incidence of adverse effects. The results of the study were presented at the 2018 American Academy of Dermatology annual meeting. Of the 11 subjects enrolled, 2 patients withdrew consent and 9 received bertilimumab. Bertilimumab was well tolerated in all 9 subjects, and no drug-associated serious adverse events were reported[4] Preliminary analyses indicated that the subjects had an 81% decline in their Bullous Pemphigoid Disease Area Index (BPDAI) scores. Based on these results, bertilimumab has been granted fast track designation for the treatment of BP[5].

Rituximab in BP

Rituximab has a well-documented efficacy in treating pemphigus (24). However, controlled clinical trials in pemphigoid diseases, including BP, are missing. Several case reports and case report series reported good outcomes of rituximab treatment for BP. Although the efficiency of rituximab for the treatment of BP varied among the studies, the overall CR rates were

[4]https://www.immunepharma.com/what-we-do/bertilimumab

[5]https://ir.immunepharma.com/press-releases/detail/168/bertilimumab-granted-fast-track-designation-for-the

60–70% and the relapse rates were approximately 20% in BP patients receiving rituximab therapy (105, 106). To evaluate the efficacy and safety of rituximab for the treatment of BP, an open-label, prospective, phase 3 clinical trial was conducted (**NCT00525616**). Eighteen BP patients received two intravenous injections of 1,000 mg of rituximab at 15-day intervals. The primary endpoint was clinical and biological disease control for up to 2 years. To date, no results of this trial are available.

Future Potential Clinical Trials in Pemphigoid

Spleen tyrosine kinase (SYK) is a nonreceptor cytoplasmic enzyme that is mainly expressed in hematopoietic cells and plays a pivotal role in regulating cellular responses to extracellular pathogens or antigen-immunoglobulin complexes (107). Because SYK acts downstream of activating Fc gamma receptor (FcγR), it has been considered a candidate drug target for antibody-induced diseases, such as pemphigoid diseases. In fact, a previous study has shown that SYK inhibition led to less activation of neutrophils stimulated with immune complexes and prevented the development of skin lesions in a preclinical EBA mouse model (108). In lesional skin in pemphigoid diseases, both eicosanoid leukotriene B4 (LTB4) and complement factor C5, the precursor of anaphylatoxin C5a, are found; however, their significance in the pathogenesis of pemphigoid diseases still needs to be elucidated. A previous preclinical mouse model of pemphigoid disease indicated a critical role of LTB4 in the recruitment of polymorph nuclear cells to the dermal epidermal junction (109). It is suggested that LTB4 may closely interact with C5a in the regulation of skin inflammation. Thus, inhibiting these two factors individually or in parallel might be effective for the treatment of pemphigoid diseases. These newly identified therapeutic targets should be addressed in future clinical trials.

CONCLUSIONS

The clinical trials discussed here, which include several trials investigating novel therapeutic targets, demonstrate that translational research in pemphigus and pemphigoid is a fast-growing field. We thus expect that several novel treatments will be shortly available for the treatment of pemphigus and pemphigoid patients. Given the high, and thus far unmet, medical need in this field (110), this is highly encouraging and will hopefully improve the quality of life of the affected patients. In addition to the compounds and targets described here, several new targets have been recently identified in preclinical model systems, such as PDE4 or PI3Kδ inhibitors (111, 112). Hence, the preclinical pipeline is well developed and will contribute to the growing number of clinical trials in pemphigus and pemphigoid.

AUTHOR CONTRIBUTIONS

KI and RL wrote the manuscript. KB prepared the figure. All authors read, commented on, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00978/full#supplementary-material

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Preferential Reactivity of Dipeptidyl Peptidase-IV Inhibitor-Associated Bullous Pemphigoid Autoantibodies to the Processed Extracellular Domains of BP180

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Bullous pemphigoid (BP) is a common autoimmune blistering disease in which autoantibodies target the hemidesmosomal components BP180 and/or BP230 in basal keratinocytes. In BP, 80 to 90% of autoantibodies target the juxtamembranous extracellular non-collagenous 16th A (NC16A) domain of BP180. Recently, the administration of dipeptidyl peptidase-IV inhibitors (DPP4i), which are widely used as antihyperglycemic drugs, has been recognized to be a causative factor for BP. DPP4i-associated BP (DPP4i-BP) autoantibodies tend to target epitopes on non-NC16A regions of BP180, and the pathomechanism for the development of the unique autoantibodies remains unknown. To address the characteristics of DPP4i-BP autoantibodies in detail, we performed epitope analysis of 18 DPP4i-BP autoantibodies targeting the non-NC16A domains of BP180 using various domain-specific as well as plasmin-digested polypeptides derived from recombinant BP180. Firstly, Western blotting showed that only one DPP4i-BP serum reacted with the epitopes on the intracellular domain of BP180, and no sera reacted with the C-terminal domain of the molecule. In addition, only 2 DPP4i-BP sera reacted with BP230 as determined by enzyme-linked immunosorbent assay. Thus, DPP4i-BP autoantibodies were found to mainly target the non-NC16A mid-portion of the extracellular domain of BP. Interestingly, Western blotting using plasmin-digested BP180 as a substrate revealed that all of the DPP4i-BP sera reacted more intensively with the 97-kDa processed extracellular domain of BP180, which is known as the LABD97 autoantigen, than full-length BP180 did. All of the DPP4i-BP autoantibodies targeting the LABD97 autoantigen were IgG1, and IgG4 was observed to react with the molecule in only 7 cases (38.9%). In summary, the present study suggests that IgG1-class autoantibodies targeting epitopes on the processed extracellular domain of BP180, i.e., LABD97, are the major autoantibodies in DPP4i-BP.

Keywords: bullous pemphigoid, dipeptidyl peptidase-IV inhibitor, dipeptidyl peptidase-IV inhibitor-associated bullous pemphigoid, autoantibodies, IgG subclass, BP180, BP230, autoimmune disease

INTRODUCTION

Bullous pemphigoid (BP) is a common autoimmune blistering disease in which autoantibodies target the hemidesmosomal components BP180 and/or BP230 in basal keratinocytes (1). In BP, almost 90% of autoantibodies target the juxtamembranous extracellular non-collagenous 16th A (NC16A) domain of BP180 (2). Recently, the administration of dipeptidyl peptidase-IV inhibitors (DPP4i), which are widely used as antihyperglycemic drugs, is recognized to be a causative factor for BP (3). In 2016, a strong relationship between the onset of BP under DPP4i exposure was reported from the France Pharmacovigilance Database (4). Thereafter, retrospective case-control studies from Finland, (5) Switzerland, (6) France, (6) Israel (7), and Japan (the Japanese Adverse Drug Event Report Database) (8) also confirmed that the administration of DPP4i, especially vildagliptin, was associated with increased risk of BP development. In addition, based on the National Healthcare Insurance Agency of France, the observed frequencies of DPP4i intake in the BP population are higher than those in the general population (9). Although the increased risk of BP development after the use of DPP4i has been intensively studied, the mechanisms whereby DPP4i administration causes BP development remains to be elucidated.

We and other groups have recently reported that Japanese patients with DPP4i-associated BP (DPP4i-BP) may show unique clinical and immunological characteristics (10–14). Clinically, DPP4i-BP patients tend to show a less severe erythematous phenotype than typical non-DPP4i BP patients show. In terms of the Bullous Pemphigoid Disease Area Index (BPDAI) (15) a commonly used disease severity score for BP, patients with typical BP do not differ significantly from patients with DPP4i-BP in terms of erosion/blister scores, whereas patients with DPP4i-BP have significantly less severe erythema/urticaria than patients with typical BP have. Immunologically, DPP4i-BP autoantibodies preferentially target the mid-portion of the extracellular region of BP180 without reactivity toward the NC16A domain (10). Interestingly anti-BP180 NC16A autoantibodies may be produced during the clinical course of DPP4i-BP as a result of epitope spreading (13, 14). After the production of anti-BP180 NC16A autoantibodies, clinical manifestations of DPP4i-BP may resemble those of typical non-DPP4i-BP (13, 14). Thus, anti-BP180 NC16A autoantibodies may be observed also in DPP4i-BP.

In this study, we collected 18 cases of "pure" DPP4i-BP in which autoantibodies did not target the NC16A domain of BP180. As a result, it has been revealed that DPP4i-BP autoantibodies are IgG1-class autoantibodies which target epitopes on the non-NC16A processed extracellular domains of BP180.

Abbreviations: BP, bullous pemphigoid; NC16A, the 16th non-collagenous domain; DPP4i, dipeptidyl peptidase-IV inhibitor(s); DPP4i-BP, dipeptidyl peptidase-IV inhibitor-associated bullous pemphigoid; ELISA, enzyme-linked immunosorbent assay; BPDAI, bullous pemphigoid disease area index.

MATERIALS AND METHODS

Patient Characteristics

BP was diagnosed based on clinical, histopathological, and immunological findings (1). The clinical and immunological characteristics of the DPP4i-BP cases in this study are listed in **Table 1** and summarized in **Table 2**. Direct immunofluorescence study showed positive results for IgG and/or C3 deposition at the basement membrane zone in all cases. The mean age was 78.8 and ranged from 57 to 93. The female/male ratio was 6:12. The mean index value of full-length BP180 ELISA was 108.2 and ranged from 60.9 to 171.7 (cutoff < 4.64). None of our cases was positive for BP180 NC16A by chemiluminescent enzyme immunoassay. BPDAI scores for blister/erosion, urticaria/erythema, and total mucosa are summarized in **Table 1**. Vildagliptin was the most common DPP4i (38.9%), followed by teneligliptin (33.3%), and Sitagliptin (27.8%) (**Table 3**).

Preparation of Recombinant Proteins

Full-length human BP180 recombinant protein (Met^{1} – Pro^{1497}) and polypeptides corresponding to the intracellular domain (Met^{1} to Trp^{467}) and the C-terminus region (Leu^{1281} – Pro^{1497}) of BP180 were produced using the Flp-In 293 system (Invitrogen, CA) as previously reported (10). Processed BP180 extracellular fragments of 120-kDa and 97-kDa forms, which are known as LAD-1 and LABD97, respectively, were generated by limited plasmin digestion of the full-length recombinant BP180 protein (10). Schematics of the recombinant proteins and the plasmin-digested proteins are given in **Figure 1A**. Mixture substrate samples of full-length BP180, LAD-1, and LABD97 were used for Western blotting, of which even doses were confirmed by Coomassie Blue staining (**Figure 1B**).

Immunofluorescence Study

For indirect immunofluorescence study using 1 M NaCl-split skin, normal human skin was incubated in 1 M NaCl solution for 48 h at 4°C. Thereafter, the skin was mounted and snap-frozen in OCT compound (Thermo Fisher Scientific, MA), and 5-μm cryosections were prepared. The sections were then incubated with patients' sera (dilution 1:10–20) for 40 min at 37°C. After washing with PBS 3 times, the sections were incubated with FITC-conjugated antibodies to human IgG (dilution 1:100) (Dako Cytomation, Denmark) for 30 min at 37°C.

Western Blotting

Protein samples were separated by SDS-PAGE electrophoresis using 7 or 10% SDS-polyacrylamide gel. The gels were transferred to nitrocellulose membranes (Bio Rad, CA). The membranes were blocked for 30 min at room temperature with 2% skimmed milk in TBS. After incubation with 1:200 diluted patient serum with 2% skimmed milk in TBS for 1 h at room temperature, horseradish peroxidase-conjugated secondary anti-human IgG (1:1,000) (Dako Cytomation, Denmark), IgG1 (1:500) (Thermo Fisher Scientific, MA), or IgG4 (1:500) (Thermo Fisher Scientific, MA) antibodies in the same buffer were reacted at room temperature for 1 h. Signals were visualized with Clarity Western ECL Substrate (Bio Rad, CA). Each protein band was quantified using Fiji (16). Relative intensities were calculated for each band

TABLE 1 | List of DPP4i-BP cases.

No	Age	Sex	Full-length BP180	BP180 NC16A	BP230	DPP4i	DIF	ssIIF	Delay after BP onset	BPDAI
1	55–60	Male	94.4	(–)	2.3	Teneligliptin	IgG, C3	roof side	–	–
2	75–80	Male	86.6	(–)	1.5	Vildagliptin, Teneligliptin, Sitagliptin	IgG, C3	roof side	7 months	–
3	90–95	Male	150.6	(–)	2.3	Anagliptin	IgG, IgA, IgM, C3	roof side	7 months	52–7–0
4	90–95	Female	130.6	(–)	3.4	Vildagliptin	IgG, C3	roof side	2 months	36–NA–5
5	70–75	Male	103.8	(–)	1.7	Teneligliptin, Vildagliptin	IgG, C3	roof side	6 months	–
6	80–85	Male	102.3	(–)	4.7	Vildagliptin	IgG, C3	roof side	2 months	–
7	65–70	Male	60.93	(–)	0.4	Sitagliptin	IgG, C3	roof side	3 months	–
8	85–90	Female	109.9	(–)	1.7	Teneligliptin	IgG, IgM, C3	roof side	2 months	total 30
9	80–85	Female	171.7	(–)	1.1	Omarigliptin	C3	roof side	2 months	–
10	90–95	Female	98.9	(–)	20.3	Alogliptin, Sitagliptin	IgG, C3	roof side	2 months	–
11	85–90	Male	113.2	(–)	1.9	Teneligliptin	IgG, C3	roof side	1 month	7–6–0
12	55–60	Female	139	(–)	2.6	Vildagliptin	IgG, C3	roof side	2 months	9–7–15
13	70–75	Male	81.7	(–)	4.1	Vildagliptin, Sitagliptin	IgG, IgA, C3	roof side	–	13–0–0
14	85–90	Male	111.2	(–)	3.3	Alogliptin, Linagliptin	IgG, IgA, IgM, C3	roof side	2 months	38–0–0
15	70–74	Male	103.7	(–)	14.3	Vildagliptin	IgG, C3	roof side	3 months	46–3–0
16	75–80	Male	84.5	(–)	2.5	Teneligliptin	IgG C3	roof side	2 weeks	–
17	50–60	Female	79.5	(–)	2.3	Anagliptin, Linagliptin	IgG, C3	roof side	8 months	–
18	90–95	Male	125.4	(–)	3.7	Sitagliptin, Linagliptin	IgG, C3	roof side	2 weeks	–

Full-length BP180 ELISA: Index values, cutoff<4.64. BP230ELISA: Index values, cutoff < 9.0. NA, means not available.

TABLE 2 | Summary of patient attributes.

Age, mean (range), years	78.8 (57–93)
Female/male, *n*; sex ratio	6/12
Full-length BP180 ELISA, mean (range), index values	108.2 (60.9–171)
BP180 NC16a, positive rate, *n* (%)	0 (0%)
BP230 ELISA, mean (range), index values	4.2 (1.1–20.3)
BP230 ELISA, positive rate, *n* (%)	2 (11.1%)

TABLE 3 | DPP4i use.

Vildagliptin	7 (38.9%)
Teneligliptin	6 (33.3%)
Sitagliptin	5 (27.8%)
Linagliptin	3 (16.7%)
Alogliptin	2 (11.1%)
Anagliptin	2 (111%)
Omarigliptin	1 (5.6%)

based on the intensity of the 180-kDa bands. Case No.11 was excluded since the intensity of the 180-kDa band was faint.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA using full-length BP180 recombinant proteins was performed as previously described, with a minor modification (10). Briefly, 96-well plates (Thermo Fisher Scientific, MA) were coated with 1 μg/well of the recombinant proteins in 50 mM carbonate buffer pH 9.5 and then blocked with Blocking Reagent for ELISA (Roche, Swiss) for 2 h at room temperature. Patient sera were diluted to 1:100 and incubated for 1 h at room temperature. After washing, the plates were incubated with 1:500 diluted horseradish peroxidase-conjugated anti-human IgG antibody for 1 h at room temperature. Subsequently, the plates were reacted with a substrate solution, 3, 3′, 5, 5′tetramthylbenzidine dihydrochloride single solution (Thermo Fisher Scientific, MA). After color development, the reaction was stopped with the addition of 0.12 N hydrochloric acid, and absorbance was measured at 450 nm by a microplate reader with the correlation wavelength set at 620 nm (Tecan Austria GmbH, Austria). To detect anti-BP230 autoantibodies, BP230 ELISA (MESACUP BP230 ELISA kit, MBL, Japan) was performed according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, USA). Data were analyzed using two-way analysis of variance, followed by Tukey's multiple comparison test. p-values are indicated as $^{*}0.01 < p < 0.05$, $^{**}0.001 < p < 0.01$, and $^{****}p < 0.0001$.

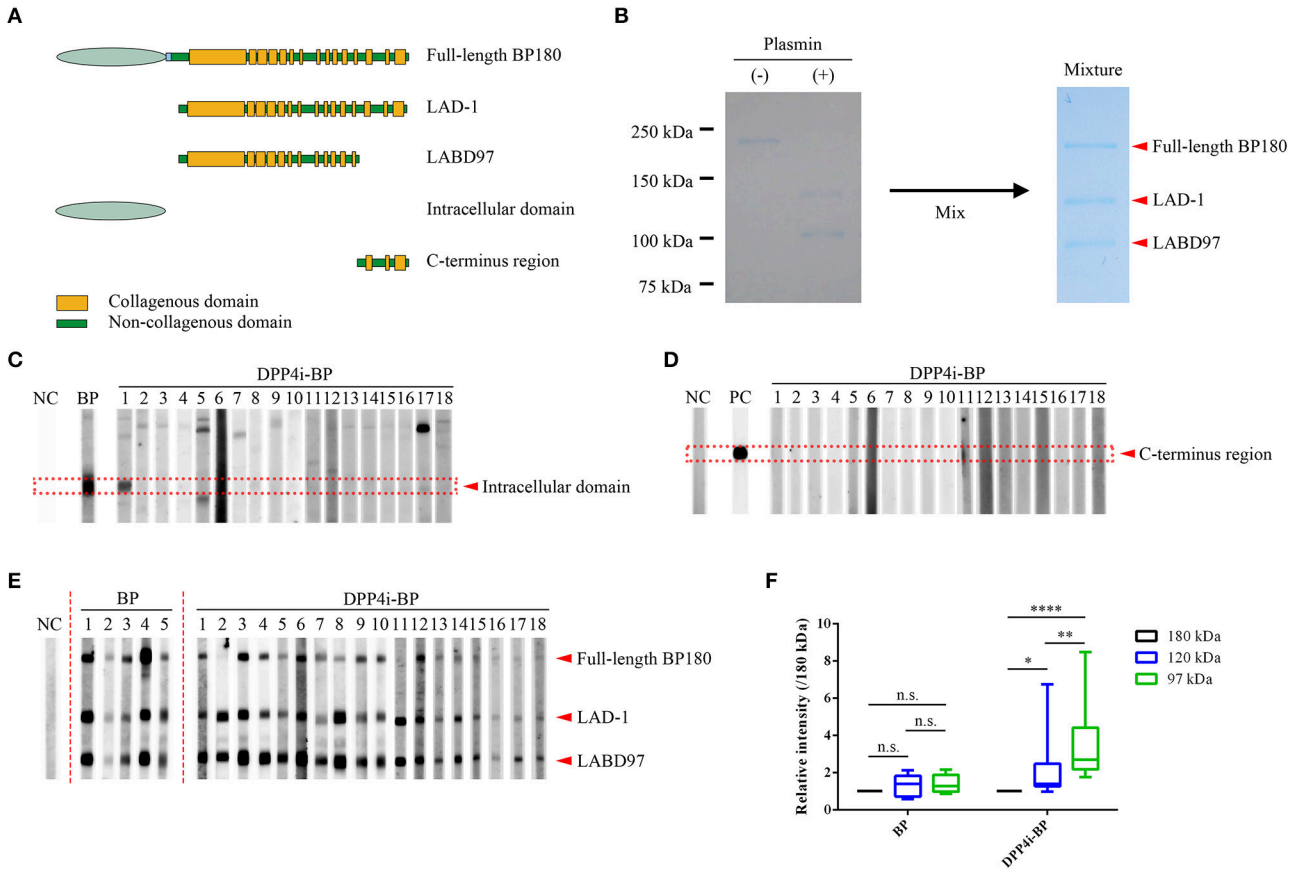

FIGURE 1 | Epitope mapping of DPP4i-BP autoantibodies. **(A)** Schematic of recombinant proteins. **(B)** Coomassie Blue staining using a mixture of substrates including the full-length recombinant BP180 and plasmin-digested BP180 proteins. **(C–E)** Western blots using the intracellular domain of BP180 **(C)**, C-terminal regions of BP180 **(D)**, and a mixture of full-length recombinant BP180 and plasmin-digested BP180 proteins **(E)**. Relative intensities of 180-kDa, 120-kDa, and 97-kDa bands in BP and DPP4i-BP **(F)**. Data were analyzed using two-way analysis of variance, followed by Tukey's multiple comparison test. *p*-values are indicated as *$0.01 < p < 0.05$, **$0.001 < p < 0.01$, and ****$p < 0.0001$. n.s., Not significant; NC, Negative control using healthy individual sera; PC, Positive control using anti-FLAG antibody.

RESULTS

Preferential Reactivity of DPP4i-BP Autoantibodies to the Processed Extracellular Domain of BP180

Firstly, we performed Western blotting using the recombinant intracellular and C-terminal proteins of BP180 (**Figures 1C,D**). Only one case had autoantibodies targeting the intracellular region of BP180, and no cases had autoantibodies targeting both fragments (**Figures 1C,D**). In addition, autoantibodies targeting BP230 were only detected in two cases (11.1%) by BP230 ELISA (cutoff < 9.0) (**Tables 1**, **2**). We also confirmed that there were no IgG autoantibodies targeting the dermal side of the artificial blisters in 1 M NaCl-split skin indirect immunofluorescence (**Supplemental Figure 1**).

To identify the epitopes of IgG autoantibodies in DPP4i-BP, we performed Western blotting using mixture samples of full-length BP180 and plasmin-digested 120-kDa (LAD-1) and 97-kDa (LABD97) fragments (**Figure 1E**). IgG autoantibodies of all cases except for case No.11 reacted to the full-length BP180 (**Figure 1E**), which is mostly consistent with the result of full-length BP180 ELISA (**Table 1**). Interestingly, IgG autoantibodies showed more intense reactivity to LABD97 than to LAD-1 or full-length BP180 (**Figures 1E,F**). In contrast, the autoantibodies in typical non-DPP4i BP reacted almost equally to each band (**Figures 1E,F**). Since none of the cases reacted with the NC16A domain of BP180, all of the DPP4i-BP autoantibodies tested in this study showed preferential reactivities toward the processed extracellular domain of BP180.

IgG1 Autoantibodies to LABD97 Are Major Autoantibodies in DPP4I-BP

It is known that both IgG1 and IgG4 are the predominant autoantibodies in BP (17). Therefore, we evaluated IgG1 and IgG4 autoantibody reactivities to the full-length BP180 and the plasmin-digested LAD-1 and LABD97 fragments of BP180. Although all cases had IgG1 autoantibodies targeting each form of BP180, it should be noted that significant reactivities to LABD97 were observed (**Figure 2**). In contrast, 7 of 18 cases (38.9%) had IgG4 autoantibodies targeting

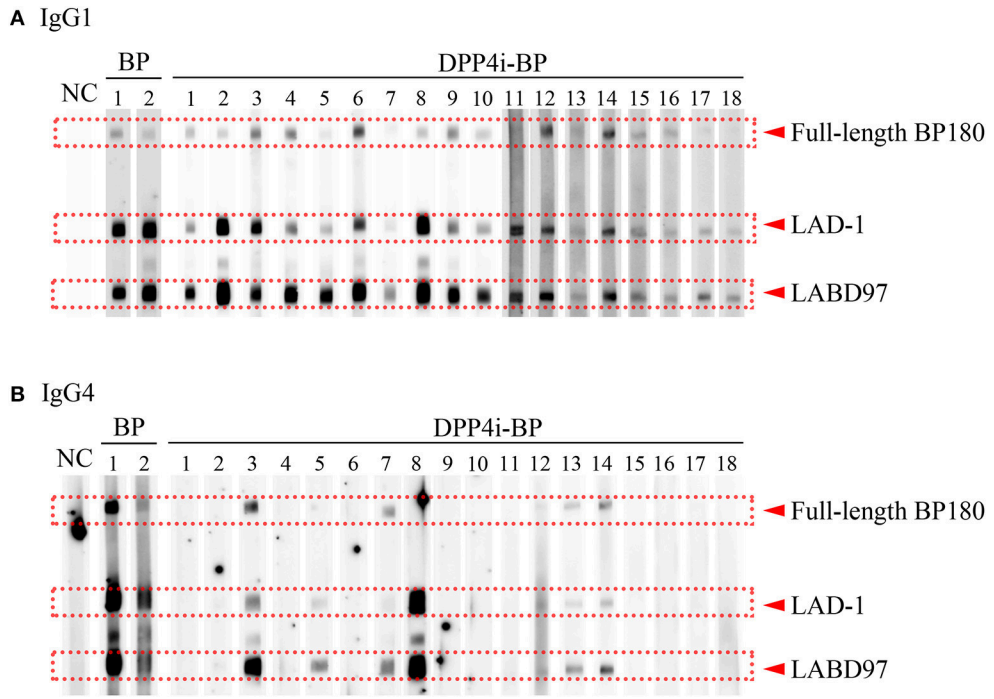

FIGURE 2 | IgG subclass analysis of DPP4i-BP. Detection of IgG1 **(A)** and IgG4 **(B)** autoantibodies by Western blot using a mixture of full-length recombinant BP180 and plasmin-digested BP180 proteins.

various forms of BP180 (**Figure 2**). These findings suggest that IgG1 autoantibodies to LABD97 are major autoantibodies in DPP4i-BP.

DISCUSSION

In this study, we analyzed autoantibodies of 18 DPP4i-BP cases without reactivity to the NC16A domain of BP180. Consistent with our previous study using small numbers of DPP4i-BP cases, (10) all of the autoantibodies showed positive reactivity to the processed extracellular domain of BP180. Interestingly, all of the DPP4i-BP autoantibodies tested in this study showed preferential reactivity to LABD97. A similar characteristic of autoantibodies can be found in another autoimmune blistering skin disease, linear IgA bullous dermatosis. In linear IgA bullous dermatosis, autoantibodies preferentially react with neoepitopes that selectively develop on LAD-1 and LABD97, and they do not react with full-length BP180 (18, 19). BP180 can be processed by several proteases under physiological or pathological conditions, Franzke et al. (20), Hofmann et al. (21) and the N-terminal deletion resulting from cleavage within the NC16A domain of BP180 results in the production of LAD-1 (20). In addition, further C-terminal deletion produces LABD97 (21). This processing of BP180 may induce conformational changes on the molecule, which also may induce new epitopes distinct from those on the native full-length form of BP180 (22–25). Several studies have shown that antibodies targeting the 15th collagenous domain or its neighboring regions in BP180 preferentially react with LAD-1 and LABD97 (22, 23). In addition, our previous study revealed that the C-terminus processing of BP180 induces neoepitopes on the 15th collagenous domain of BP180 (24). Thus, not only does cleavage within the NC16A domain cause neoepitopes to develop on the 15th collagenous domain of BP180, but so does C-terminal processing (25). Although fine epitope mapping of DPP4i-BP autoantibodies will be necessary in future studies, the preferential reactivity of DPP4i-BP autoantibodies to LABD97 may suggest the possibility that putative epitopes may exit on the 15th collagenous domain or in its neighboring regions.

The present study also revealed that all of the DPP4i-BP autoantibodies which preferentially targeted the LABD97 fragment of BP180 were IgG1. In contrast, IgG4-subclass autoantibodies were only observed in 38.9% of our cases. These results indicate that IgG1 autoantibodies targeting epitopes on LABD97 are the main autoantibodies in DPP4i-BP. This finding was unexpected, because it is well-known that IgG1 BP autoantibodies are able to activate the complement pathway associated with the development of inflammation, (26) whereas DPP4i-BP tends to show a non-inflammatory phenotype associated with scant urticarial erythema and fewer infiltrating eosinophils than those of non-DPP4i BP, despite activating the complement at dermal-epidermal junctions (10–14). Thus, the predominance of IgG1 autoantibodies in DPP4i-BP stands in contrast to the previous concept of BP pathogenesis (26). Although further studies are necessary to resolve this issue, the present study indicates that complement activation is insufficient to induce inflammation in DPP4i-BP.

The pathogenicity of IgG4 is controversial. An experimental mouse model showed that IgG4 plays a protective role against the development of BP (27). In contrast, it is experimentally shown that IgG4 BP autoantibodies have the capacity to induce leucocyte-dependent dermal-epidermal separation (28). In addition, BP cases with only IgG4 autoantibodies have been reported (29). As this IgG4-type BP shows a non-inflammatory phenotype, IgG4 autoantibodies are assumed to play complement-independent roles in BP development, such as a role in BP180 depletion in keratinocytes, the Fc-dependent activation of neutrophils, or leucocyte-dependent dermal-epidermal separation (26, 28, 30). In our subclass analysis of DPP4i-BP, IgG4 autoantibodies preferentially reacted to LABD97, similar to IgG1 autoantibodies. However, the positive rates for IgG4 autoantibodies to LABD97 and full-length BP180 were lower than those of IgG1 autoantibodies, especially to full-length BP180. This discrepancy may suggest that the development of IgG1 autoantibodies might precede IgG4 autoantibody development in DPP4i-BP, although other possibilities cannot be ruled out.

CONCLUDING REMARKS

In conclusion, major epitopes of DPP4i-BP were located in the mid-portion of the extracellular domain, and these epitopes can be presented on LABD97. All such autoantibodies were in the IgG1 class. The unique immunological characteristics of DPP4i-BP autoantibodies may help us understand the pathological mechanism behind the development of the disease.

ETHICS STATEMENT

This report on a single patient complies with the Declaration of Helsinki. The patient gave written informed consent for publication. This study was approved by the institutional review board of Hokkaido University (institutional review board number: 016-0061).

AUTHOR CONTRIBUTIONS

YM, WN, and KI drafted the paper and collected the clinical data. HS supervised the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01224/full#supplementary-material

Supplemental Figure 1 | Indirect Immunofluorescence Study Using 1 M NaCl-split Skin. NC, Negative control using healthy individual sera.

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REVIEW
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Dermatitis Herpetiformis: Novel Perspectives

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Dermatitis herpetiformis (DH) is an inflammatory disease of the skin, considered the specific cutaneous manifestation of celiac disease (CD). Both DH and CD occur in gluten-sensitive individuals, share the same Human Leukocyte Antigen (HLA) haplotypes (DQ2 and DQ8), and improve following the administration of a gluten-free diet. Moreover, almost all DH patients show typical CD alterations at the small bowel biopsy, ranging from villous atrophy to augmented presence of intraepithelial lymphocytes, as well as the generation of circulating autoantibodies against tissue transglutaminase (tTG). Clinically, DH presents with polymorphic lesions, including papules, vesicles, and small blisters, symmetrically distributed in typical anatomical sites including the extensor aspects of the limbs, the elbows, the sacral regions, and the buttocks. Intense pruritus is almost the rule. However, many atypical presentations of DH have also been reported. Moreover, recent evidence suggested that DH is changing. Firstly, some studies reported a reduced incidence of DH, probably due to early recognition of CD, so that there is not enough time for DH to develop. Moreover, data from Japanese literature highlighted the absence of intestinal involvement as well as of the typical serological markers of CD (i.e., anti-tTG antibodies) in Japanese patients with DH. Similar cases may also occur in Caucasian patients, complicating DH diagnosis. The latter relies on the combination of clinical, histopathologic, and immunopathologic findings. Detecting granular IgA deposits at the dermal-epidermal junction by direct immunofluorescence (DIF) from perilesional skin represents the most specific diagnostic tool. Further, assessing serum titers of autoantibodies against epidermal transglutaminase (eTG), the supposed autoantigen of DH, may also serve as a clue for the diagnosis. However, a study from our group has recently demonstrated that granular IgA deposits may also occur in celiac patients with non-DH inflammatory skin diseases, raising questions about the effective role of eTG IgA autoantibodies in DH and suggesting the need of revising diagnostic criteria, conceivably emphasizing clinical aspects of the disease along with DIF. DH usually responds to the gluten-free diet. Topical clobetasol ointment or dapsone may be also applied to favor rapid disease control. Our review will focus on novel pathogenic insights, controversies, and management aspects of DH.

Keywords: dermatitis herpetiformis, epidermal transglutaminase, coeliac disease, non-coeliac gluten sensitivity, direct immunofluorescence

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INTRODUCTION

About 135 years ago, the American dermatologist Luis Duhring reported for the first time an itching and multiform skin eruption with erythema, papules, bullae, pustules, and above all, grouped vesicles localized at typical areas (1). At that time, he had seen 15 cases of such a disease, and he decided to name it "dermatitis herpetiformis," due to its clinical similarity to herpes virus infection (1). After the identification of dermatitis herpetiformis, several years passed before the second important step in its early story until 1950, when dapsone was accidentally found to be effective for the treatment of the disease (2). Then, 50 years ago, Van der Meer described the typical IgA deposits at the papillary tips, which represent the immunological hallmark of DH as well as the clue for the diagnosis of the disease (3).

However, it was only in the 60s and, above all, in the 70s that DH was clearly related to gluten intolerance, allowing to consider the disease as the specific cutaneous manifestation of celiac disease (CD). In fact, patients with DH were shown to have in most cases intestinal changes similar to those found in CD (4) and to share the same genetic background of CD (5), and gluten-free diet (GFD) was demonstrated to improve skin symptoms (6), although more slowly than the gastrointestinal ones.

Since then, several issues about the pathogenesis and the management of DH have been pointed out (7). Among them, the identification of reliable serologic markers of the disease (8) and the discovery of epidermal transglutaminase (eTG) as the autoantigen of DH were some of the most remarkable steps (9).

DH can be defined as a disease presenting with: (i) symmetrical polymorphic lesions involving typical areas such as the extensor aspects of the limbs and sacral region, (ii) a predominant neutrophilic infiltrate at the dermal papillae at histopathology, (iii) granular IgA deposits along the dermal-epidermal junction, (iv) an invariable association with CD, and (v) a response to a lifelong GFD (7).

However, despite the growing knowledge about the disease, several issues have to be clarified yet, and some of the cornerstones of DH need further discussion. For example, the specificity of IgA deposits in the perilesional skin of patients with DH could be questioned and the diagnostic algorithm should be revised (10), taking also into account the identification of non-coeliac gluten sensitivity (NCGS) as a new entity among the spectrum of gluten-related disorders (11); moreover, the pathogenic role of IgA anti-eTG antibodies needs further demonstration, since they may also occur in patients without DH (12). Finally, even the therapeutic role of GFD is discussed, since some authors suggest that it can be interrupted in the (rare) case of DH remission (13).

The current review will try to address or at least to focus on these and other issues that are still under debate in the context of DH. The picture that hopefully will come out would be that of a lively disease that represents both a pathogenic model of autoimmunity and a paradigmatic example of how the skin can be the mirror of an internal condition such as CD.

THE EPIDEMIOLOGY OF DERMATITIS HERPETIFORMIS: A (RARE) DISEASE OF THE WESTERN COUNTRIES

DH is a rare disease that occurs prevalently in Caucasian individuals. In Europe and USA, the prevalence of DH ranges from 11.2 to 75.3 per 100.000, with the highest reported in Finland; whereas, the incidence ranges from 0.4 to 2.6 per 100.000 people per year (14–16). DH is extremely rare among African and Asian populations. Reasons explaining the low prevalence of DH among such populations include the absence of DH predisposing human leukocyte haplotypes (HLA) DQ2 and DQ8, which are always found in Caucasian DH patients, and the low wheat consumption in these geographic areas (17). However, in the Asians, including Chinese and Japanese populations, the association between DH and CD seems to be weaker than in Western countries, despite DH presents with similar clinical and immunopathological features. The extreme rarity of CD in these populations may have led, indeed, to overlook the diagnosis of DH and possibly underestimate the prevalence of the disease (17, 18).

During the last decades, the overall incidence of DH is significantly decreased, although the incidence of CD is increasing (19–22). One plausible explanation for the opposite trends in the epidemiology of CD and DH could be the increased awareness of CD among physicians and patients and the wide prescription of CD screening tests even in patients without typical gastrointestinal manifestations, leading to identify early patients with latent or potential CD.

DH can occur at any age, but typically occurs during adulthood, and mostly between the third and the fourth decade of life (14). Interestingly, in one Finnish study including 477 patients diagnosed with DH over a 40 year period, the patients' age at DH diagnosis was shown to significantly increase over time. This is presumably related to a parallel decrease of the annual consumption of wheat during the same period, leading to a lower lifetime gluten load (16).

DH has been also reported to occur in pediatric patients, but the exact incidence of DH during childhood is unknown. In one study by our group in 2013 including 159 DH patients, about 36% were diagnosed below the age of 20 (23). Other authors have suggested a possible underestimation of pediatric DH due to clinical overlapping features with atopic dermatitis, which still accounts for the most prevalent dermatologic disease among children (24). Unlike CD, DH seems to occur most commonly in man, although the male/female ratio has reportedly ranging from 2:1 to 1:1 according to different studies (16, 19, 24). Interestingly, a Finnish study found that in females there is a longer diagnostic delay compared to male (25).

Associated Diseases

Like CD, DH has been found to be associated with several autoimmune disorders, including type I diabetes mellitus, autoimmune thyroid diseases, and connective tissue diseases, such as Sjögren syndrome (24). Of interest is also the reported association between DH and bullous pemphigoid (BP), a

subepidermal autoimmune blistering disease characterized by autoimmunity against the hemidesmosomal antigens BP180 and BP230 (26, 27). Accordingly, in a retrospective case-control study, diagnosis of DH was found to increase of 22 folds the risk of BP, compared to only a 2-fold higher risk of developing BP amongst coeliac patients. The mean time between the diagnosis of DH and BP development was about 3 years (27).

Since virtually all patients with DH have CD, DH patients also carry an increased risk of non-Hodgkin lymphomas and gastrointestinal malignancies (28). However, unlike CD, mortality of DH patients is not increased (29–31). Accordingly, in a large population-based study including 476 patients with DH, a reduced mortality for all the causes of death compared to the general population and a significantly reduced mortality related to cerebrovascular diseases were shown. Mortality due to non-Hodgkin lymphomas was increased only during the first 5 years following the diagnosis but not thereafter. DH patients were also found to have less hypercholesterolemia, and there were fewer smoker compared to the control population (32). Ali and Lear speculated that smoking might have a protective effect against DH, since it suppresses natural killer (NK) lymphocytes and reduces intestinal IgA secretion (33–36). Finally, an association between DH and higher social class have been reported, and may contribute to the observed reduced mortality reflecting the "many facets of better living" of a high socioeconomic status (33).

Clinical Manifestations

The polymorphism and the symmetrical distribution of the lesions represent the major clinical hallmarks of DH (37, 38).

The disease usually presents with grouped erythematous papules and urticarial plaques with overwhelming vesicles. The latter may then coalesce into small tense blisters with sero-hemorrhagic content, which are characterized by a centrifugal growth pattern. Erosions, excoriations, and crusts are likely to occur because of blisters rupture and because of the scratching secondary to the associated pruritus (15, 38, 39). Lesions eventually heal leaving post-inflammatory hypo- and hyper-pigmentation (**Figures 1**, **2**).

Typically, DH lesions symmetrically localized at the extensor surfaces of upper and lower limbs, mostly at the elbows and knees, buttocks, and the sacral region; the abdomen, upper back, shoulders, nuchal area, and the scalp may also be involved, whereas the face and groin are rarely affected.

Likewise, mucosal involvement has sporadically been reported. Oral manifestations of DH mainly consist of erosions affecting both the oral mucosa and the tongue; associated symptoms include pain and burning sensation (15, 40). However, it is not clear whether oral involvement has to be considered as a specific manifestation of DH or, rather, a sign of the underlying CD; accordingly, oral aphthosis, erosions, and/or ulcerations are also frequently found in CD (40–44).

Pruritus is the leading symptom of DH and its absence is a strong argument against the diagnosis (45). In one study by our group including a cohort of 159 patients, almost all complained of severe pruritus, which had a significantly negative impact on the patient's quality of life. Moreover, in many cases, pruritus along with a stinging and burning sensation of the skin was shown to be the presenting sign of DH, preceding of 12–24 h the appearance of the cutaneous lesions (46). Notably, pruritus has reportedly occurred even months before the onset of skin lesions (47, 48).

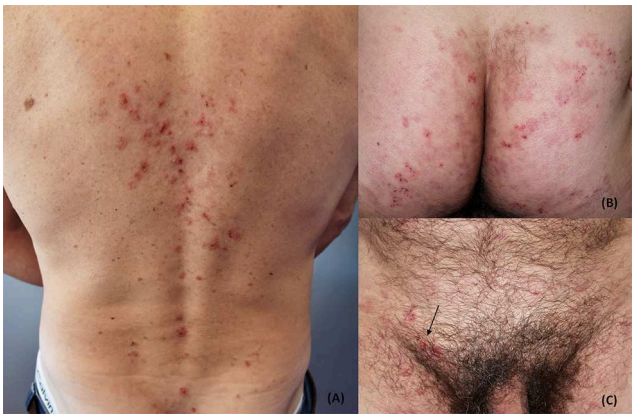

FIGURE 1 | Clinical presentation of dermatitis herpetiformis (DH): erythematous grouped papules and vesicles associated with excoriations and crusts at the back **(A)**, sacral region and buttocks **(B)**. Rarely, DH may also affect the groin and pubis (arrow) **(C)**. The patient gave written informed consent for the publication of these pictures.

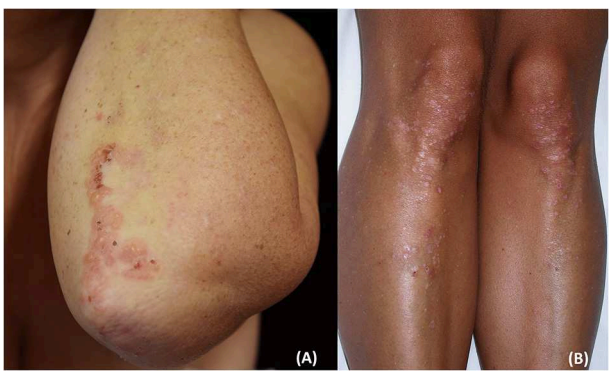

FIGURE 2 | Clinical presentation of dermatitis herpetiformis: grouped papules and vesicles associated with excoriations and crusts at the elbows **(A)** and lower limbs **(B)**. Post-inflammatory pigmentary changes such as hypo-pigmentation could be also appreciated **(B)**. The patient gave written informed consent for the publication of these pictures.

Atypical Cases of Dermatitis Herpetiformis: Are They Always Dermatitis Herpetiformis?

Several atypical cases of DH have been reported in the literature. Asymptomatic palmoplantar petechiae, occurring either alone or in association with characteristic DH clinical findings, have been reported in some cases of pediatric DH. Petechiae were found to occur prevalently at the dominant hand or foot, suggesting repeated microtraumatism as a possible trigger (44, 49–55).

Moreover, Naylor et al. reported a case of DH presenting with a diffuse petechial rush and microscopic changes consistent with both DH and vasculitis (56). Kern et al. reported a patient with DH presenting as pseudovasculitis, characterized

by a diffuse petechial rush and a large ulcer at his extensor forearm, and histopathologic and direct immunofluorescence (DIF) findings consistent with DH without additional signs of small-vessel inflammation (57). A possible hypothesis of the association between DH and vascular damage might be the presence of perivascular IgA immune complexes leading to small vessel inflammation.

Finally, cases of DH presenting as palmoplantar keratosis (58), purpuric lesions with chronic urticaria (59), and prurigo pigmentosa-like lesions (60) have been also reported.

PATHOGENESIS

DH represents a paradigmatic model of autoimmune disease, owing that it can be switched on or off by a known external trigger: the gluten. The pathogenesis of DH, which relies on a complex inflammatory network along the gut-skin axis, remains at present only partly understood. Over the past 30 years, major efforts have led to the identification of eTG as the main autoantigen of DH and to well-characterize the inflammatory microenviroment underlying skin lesions development. However, controversies still persist about the mechanisms by which (i) anti-eTG autoantibodies develop, (ii) they form the typical granular aggregates at the dermal papillary tips, and (iii) eventually induce the appearance of the skin lesions. Herein, we summarized the current knowledge of DH pathogenesis, highlighting the major controversial issues that may represent the starting point for future researches (**Figure 3**).

HLA DQ2/DQ8 and Gluten Are Required for the Occurrence of Dermatitis Herpetiformis

About 5–10% of DH patients have a first-degree relative affected by either DH or CD (61–63), suggesting that genetic factors play a major pathogenic role in the disease. Previous studies have found an association between DH and both HLA class I and II molecules, including HLA A1, B8, DR3, and DPB1 (64). However, the closest association occurs with HLA-DQ2 (combination of the DQA1*0501 and DQB1*02 alleles) and DQ8 (combination of the DQA1*03 and DQB1*0302 alleles), which can be found in roughly 85 and 15% of the patients, respectively (65). Other studies have tried to investigate the role of more than 40 other non-HLA gene polymorphisms in the pathogenesis of both CD and DH, but none has yielded convincing results (66–69).

Both HLA-DQ2 and DQ8, whose genes map on the chromosome 6, are crucially involved in processing the gluten antigen gliadin (70), corresponding to the same predisposing haplotypes found in coeliac patients (71, 72). In CD, tissue TG (tTG) catalyze deamidation of gliadin, creating epitopes that increase gluten-peptide binding affinity to HLA-DQ2 and DQ8 expressed on the surface of antigen presenting cells, leading to an adaptive immune reaction against both tTG and gliadin. In parallel, activation of innate immunity leads to characteristic small bowel alterations, including reversible villous atrophy in the upper part of the jejunum, crypt hyperplasia on small bowel mucosal samples and prominent intraepithelial lymphocytosis (73–75). Virtually all DH patients show evidence of a potential or manifest, usually mild, CD, suggesting that DH represents a specific cutaneous manifestation of CD (76). The pathogenic relevance of HLA-DQ and gluten intake in DH has been further demonstrated experimentally by Marietta and coworkers. Firstly, they observed that, unlike other autoimmune blistering dermatoses, passive transfer of DH serum into athymic mice with human skin engrafts did not induce DH lesions. However, DH lesions developed in about 17% of HLA-DQ8 positive autoimmune prone NOD mice when they were exposed to gluten through periodic intraperitoneal injections. This suggests that DH develops only in the presence of predisposing HLA-DQ antigens together with gluten exposure (77, 78).

An exception to what aforementioned is DH occurring in Japanese populations. To date, only two studies have delineated the clinical and immunological features of Japanese DH, including a total of 116 patients, suggesting the extreme rarity of DH in Japan. Although these studies were biased by the fact that the results of some analyzed parameters, including the presence of villous atrophy, circulating antibodies, and response to a GFD, were available only for a few patients, some peculiar characteristics of Japanese DH have emerged, including (i) the absence of HLA DQ2/DQ8 haplotypes, (ii) the absence of an underlying CD, (iii) a higher clinical involvement of non-predilection sites such as the extremities and trunk, (iv) fibrillar rather than granular IgA deposits in the papillary dermis of a substantial proportion of patients, and (v) a lower incidence of CD-associated autoimmune diseases and non-Hodgkin lymphomas (**Table 1**) (18, 79). Even in some Chinese DH patients, the disease was shown to occur outside the setting of CD, which is also very rare in China (80). Altogether, these observations raise speculation on the existence of a subgroup of DH that may be not elicited by gluten, although showing some clinic-pathologic features like in gluten-related DH.

Triggers Other Than Gluten Possibly Involved in the Pathogenesis of Dermatitis Herpetiformis

There is evidence that external triggers other than gluten may induce or worsen DH. For example, potassium iodide, a common compound of expectorants, has reportedly been a trigger of DH both after oral ingestion and when applied topically (81). Similarly, Snider et al. described two cases of DH elicited by a cleaning solution (82).

Kovaleski et al. reported on two cases of DH following a gastric stapling procedure and a gastrectomy with partial pancreatectomy and colectomy, respectively (83). Similarly, a non-coeliac patient developing DH after a mini-gastric bypass surgery was reported (84). In these cases, the authors hypothesized that the surgery-induced enteral inflammation promoted a cross-reaction between cutaneous and intestinal anti-TG antibodies, that seems to be in accordance with the recent demonstration of the intestinal origin of anti-eTG IgA autoantibodies (85).

Hormonal factors, and specifically the hypothalamic-pituitary-gonadal axis, may play a role in DH. Accordingly,

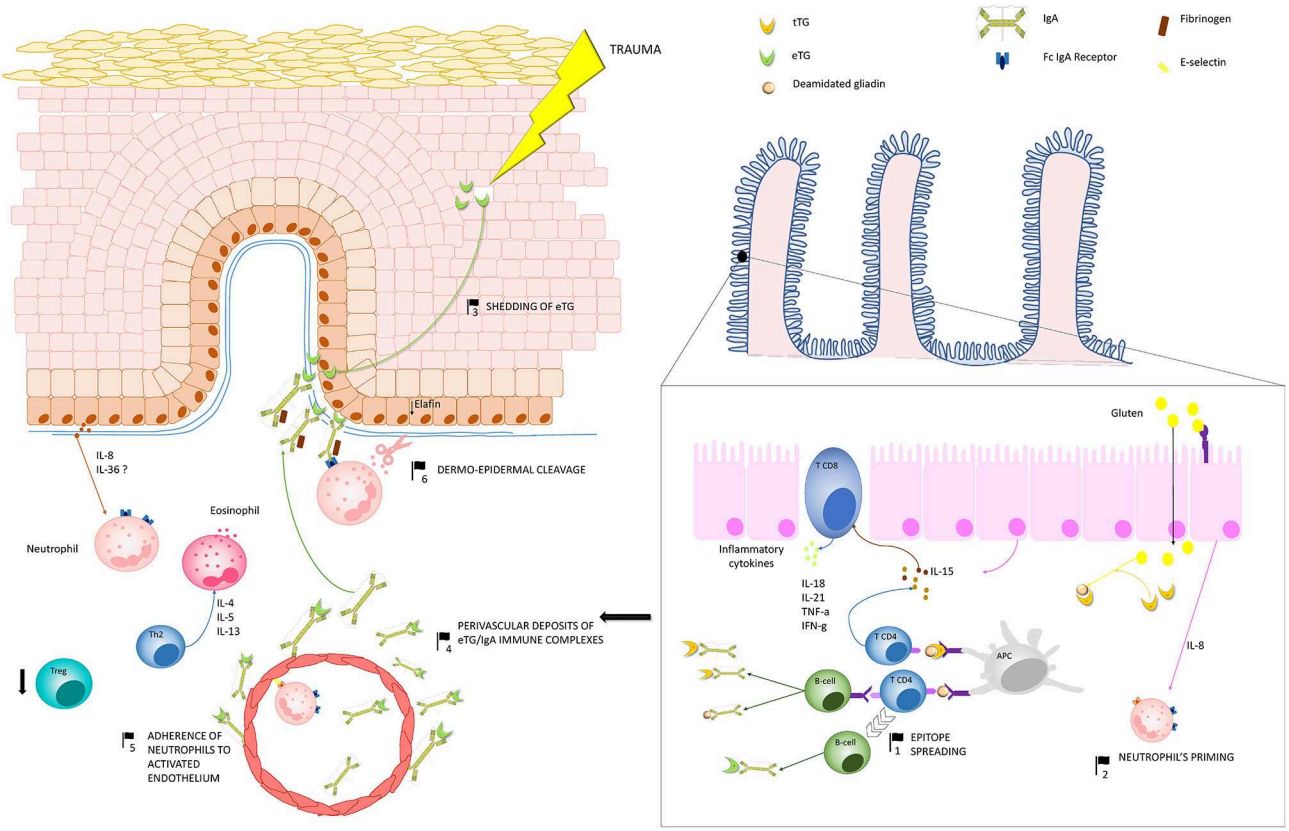

FIGURE 3 | Pathogenesis of dermatitis herpetiformis (DH): Production of IgA autoantibodies against eTG occurs in the gut, probably as a result of an epitope spreading phenomenon (1), due to the high sequence homology between tissue TG, which is a major autoantigen in coeliac disease, and eTG. Activation of innate immunity in the gut leads to increased release of IL-8, which is thought to be responsible for the initial priming of neutrophils (2). One theory suggests that, in region of trauma, keratinocyte damage leads to shedding of eTG to the dermal-epidermal junction (3), where it binds to anti-eTG IgA. An alternative hypothesis suggests that eTG/IgA complexes exists as circulating immune complexes, which can deposit both at the dermal-epidermal junction and around dermal vessels (4). A complex interplay between inflammatory cytokines and the activation of fibrinogen stimulate neutrophil adherence to the activated endothelium (5) and migration to the dermal papillae. Herein neutrophils, which probably bind to IgA aggregates through the Fc IgA receptor, release proteases, which finally induce the cleavage of the dermal-epidermal junction (6). In parallel, hyper-activation of Th2 cells activate eosinophils, which also co-operate with neutrophils to the cleavage of the dermal-epidermal junction. Down-regulation of Treg cells do also occur, favoring the maintenance of a pro-inflammatory microenvironment in DH skin. Abbreviations in the figure: eTG, epidermal transglutaminase; tTG, tissue transglutaminase; IL, interleukin; Th, T helper cell; Treg, regulatory T cell; APC, antigen presenting cell.

hormone replacement therapy for panhypopituitarism has been reported to cure DH (86). One study reported DH following progesterone contraception (87). Other two studies reported DH occurrence after a therapy with leuprolide acetate, an analog of the gonadotropin-releasing hormone (GnRH). In both cases, lesions resolved upon drug discontinuation. However, in one case, skin lesions recurred once the patient was started on biculatamide, another GnRH analog (88, 89).

Intriguingly, drugs may be also a potential trigger of DH. Marakli et al. described a patient who developed DH while on therapy with the Tumor Necrosis Factor-alfa inhibitor infliximab for an ankylosing spondylitis (90); whereas, Mochel et al. recently illustrated a patient who developed DH after several weeks of ipilimumab treatment for a metastatic melanoma. Interestingly, the patient had a positive result of anti tTG IgA antibodies prior to the diagnosis of the metastatic cancer but neither a confirmed diagnosis of coeliac disease nor active skin rush before taking ipilimumab (91). The latter is a monoclonal antibody targeting CTLA-4 and is thought to exert its anti-tumor activity by promoting T-cell activation and down-regulation of Treg function (92). It is arguable that the reported patient had a latent or silent gluten-sensitive enteropathy with an immunological reaction against tTG and eTG, and that inhibition of Tregs precipitated the manifestation of DH, in agreement with the critical role of Treg in the disease pathogenesis (93).

Finally, DH has reportedly been occurred close to a diagnosis of an adenocarcinoma of the lung and an autoimmune pancreatitis in two Japanese patients, who had neither serum IgA antibodies against eTG nor signs of an underlying CD (94).

Interestingly, a gastrointestinal infection appears to be an essential factor for the induction of CD. Several pathogenes have been suggested to trigger CD, including rotavirus, Epstein Bar Virus, Cytomegalovirus, HCV, HBV, *Bacteroides* species, *C. jejuni, Pneumococcus, M. tuberculosis*, and *H. pylori* (95).

TABLE 1 | Different characteristics between Caucasian and Japanese patients with dermatitis herpetiformis.

Characteristics	Caucasian DH	Japanese DH
HLA	HLA-DQ2 (DQB1*02:01)−85% HLA-DQ8 (DQB1*03:02)−15%	HLA-DQ2 (DQB1*02:01)−0% HLA-DQ8 (DQB1*03:02)−37%*
Sites of involvement	Elbow, buttock, knee, face, ear, neck, scalp, groin	Elbow, buttock, knee, face, ear, neck, scalp, groin Non-predilection sites, including the extremities and trunk. Whole body
Villous atrophy	Most of the patients	Not known**
Circulating anti-tTG IgA	50–95%	38%
Circulating anti-eTG IgA	50–95%	43%
DIF	Granular IgA deposits	Granular and fibrillar IgA deposits
Response to the GFD	Most of the patients	Lack of consistent data
Association with autoimmune diseases	Frequent	Rare

HLA, Human Leucocyte Antigen; DIF, Direct Immunofluorescent; GFD, gluten-free diet.
**The frequency of HLA-DQ8 refers to the study by Ohata et al. (18), where the allele was found in six (37%) out of 16 Japanese patients with DH (19).*
***Found in three out of six patients in the study by Ohata et al. (79), including a total of 91 patients in 2012.*

A recent study demonstrated a link between Reovirus, an avirulent pathogen that elicit protective immunity, and the loss of peripheral tolerance against dietary antigens, resulting in a Th1-type immunity to dietary antigens. Moreover, the study found an increased titer of antibodies against Reovirus in patients with active CD and elevated serum anti-tTG autoantibodies, suggesting a direct link between the pathogen and the induction of CD (96). Whether there might be an infectious trigger also for DH is far less clear (97).

To conclude, complex endocrine and immunologic factors seem to play a role in modulating the inflammatory response in DH, suggesting that its pathogenesis is much more complex than a simple interaction between HLA-DQ antigens and gluten.

Epidermal Transglutaminase Is the Main Autoantigen of Dermatitis Herpetiformis

Epidermal transglutaminase (eTG) belongs to a nine-member Ca^{2+}-dependent enzyme family that promotes the formation of covalent cross-links between proteins (98). eTG is physiologically expressed in the spinous layer of the epidermis, and contribute to epidermal terminal differentiation, formation of the cornified cell envelop, and protection of keratinocytes against UVB-induced apoptosis (99–102).

While tTG was shown to be a major autoantigen of CD, Sardy et al. identified eTG as the main autoantigen of DH (9). Specifically, they observed that CD and DH patients had autoantibodies targeting both tTG and eTG; however, IgA autoantibodies binding selectively and with high avidity to eTG were found only in DH patients. Moreover, eTG, but not tTG, was found to co-localize with IgA in the granular deposits at the papillary tips of DH skin (9). The mechanism by which both CD and DH patients develop an autoimmune response against eTG remains still obscure. One suggested hypothesis is related to epitope spreading (99). The phenomenon of epitope spreading involves the development over time of a humoral or cell-mediated immune response from an initial dominant epitope to a secondary one, belonging to the same (intramolecular) or a distinct (intermolecular) antigen (103). Evidence supporting the theory of epitope spreading in DH include: (i) the high sequence homology between tTG and eTG (9); (ii) the presence of an autoimmunity also against neuronal TG (or TG6), which is also highly similar to tTG and eTG, in both CD and DH (99); (iii) the lower prevalence of anti-eTG IgA autoantibodies in pediatric compared to adult CD patients, which (iv) parallels the decreased, albeit not abolished, incidence of DH during childhood (23, 104).

One recent study demonstrated that patients with active DH secreted considerably high amounts of anti-eTG IgA in the organ culture medium of small bowel mucosal biopsies, and had eTG-binding IgA-positive cells in the lamina propria, thereby suggesting that autoimmunity against eTG possibly develops in the gut (85). Interestingly, small bowel secretion of eTG-targeting IgA did not occur in CD patients, despite they showed elevated levels of such autoantibodies in the serum (85).

An unmet issue concerns the mechanism underlying the formation of eTG/IgA aggregates in the skin. One theory suggests that, in regions of trauma, where DH lesions classically occurs, epidermal damage leads to eTG shedding from the spinous layer to the upper dermis, where it binds to circulating anti-eTG IgA. A study by Zone et al. supports this hypothesis, showing that passive transfer of goat anti-eTG IgG or DH serum into mice with human skin grafts reproduced DH-like deposits only in the engrafted skin, the only source of human eTG (105). An alternative hypothesis is that eTG/IgA aggregates exists as circulating immune complexes. Accordingly, DH patients may show asymptomatic IgA immune complex deposition in the kidney (106); Preisz et al. described deposits of eTG/IgA1 complexes in both upper and deep dermal vessels in roughly 64% of DH patients (107); rare clinical manifestations of DH include digital purpura and ecchymosis, that show evidence of small vessels vasculitis on microscopic examination (49, 108); circulating eTG/IgA immune complexes can be found in patients with DH and their concentrations decrease under the GFD (109).

TABLE 2 | Evidence which seem to support or point against the pathogenic relevance of eTG/IgA deposits which are typically found in the perilesional skin of patients with DH.

Evidence supporting the pathogenic role of eTG/IgA aggregates in DH
1) Circulating eTG IgA correlate with disease activity and disappear after a GFD
2) eTG/IgA complexes are enzimatically active, and activate fibrinogen at the tips of the papillary dermis
3) Circulating and skin resident neutrophils express Fc IgA receptor (CD89), suggesting a direct interaction between neutrophils and IgA.
Evidence against the pathogenic role of eTG/IgA aggregates in DH
1) eTG/IgA complexes can be found in the healthy skin of patients with DH
2) eTG/IgA complexes can be detected in the skin of coealic patients without DH
3) eTG/IgA complexes disappears even years after the introduction of the GFD and the resolution of the skin rash
4) Passive transfer of goat anti-eTG IgG or human DH sera in mice with human skin grafts reproduces DH-like granular deposits in the engrafted skin, but not DH lesions

DH, dermatitis herpetiformis; eTG, epidermal transglutaminase; IgA, immunoglobulin A; GFD, gluten free diet.

Pathogenic Relevance of IgA Autoantibodies Against Epidermal Transglutaminase

A major controversial issue concerns the role of eTG/IgA aggregates in DH, since some studies clearly support their pathogenic relevance, whilst others did not (**Table 2**). For example, serum anti-eTG IgA autoantibodies has proven to correlate positively with disease activity (110): along with anti-tTG IgA and anti-endomysium autoantibodies (EmA), they significantly decrease in patients achieving clinical remission owing to the GFD, while increasing in patients who suffer a relapse (111). However, passive transfer of goat anti-eTG autoantibodies or DH human serum into SCID mice with human skin grafts fails to induce DH lesions, despite the formation of the typical granular eTG/IgA deposits into the engrafted skin (105, 112).

In the skin, eTG/IgA aggregates have been shown to activate fibrinogen, which can be found at the tips of the papillary dermis in a pattern similar to that of eTG/IgA aggregates (113). Fibrinolysis directly contributes to the blister formation in DH, and it is also believed to function as a chemoattractant for neutrophis, T-cells and macrophages, which are major components of the inflammatory infiltrate of DH (114). Both circulating and skin resident neutrophils in DH have been shown to highly express Fc IgA receptors, suggesting activation dependent on the interaction with the eTG/IgA complexes (115, 116). However, Donaldson et al. observed that granular IgA deposits could be also detected in the non-affected skin of DH patients (114). More intriguingly, Cannistraci et al. documented eTG/IgA co-localization in the papillary dermis, at the dermal-epidermal junction and in the vessel walls of coeliac patients without skin manifestations both before and during a GFD (10, 117, 118). It is also worth of mention that disappearance of eTG/IgA deposits from the skin occurs only in a subset of patients despite a long and strict GFD and no active skin rash, and takes much longer than serum autoantibodies or immune complexes (76, 119).

Recently, Taylor and Zone found that Potassium Iodide, a known precipitating factor of DH, increases the capacity of eTG/IgA complexes to bind the substrate cadaverin in normal skin cryosections from DH patients on dapsone or on a GFD. Thus, one could speculate that, during disease remission, eTG/IgA complexes preserve a baseline enzymatic activity, allowing tight binding with anchoring fibrils of the BMZ but not the activation of fibrinogen (76, 120).

Cytokine Network in Dermatitis Herpetiformis and Mechanisms of Blister Formation

The mechanisms leading to tissue damage in DH are only partly understood. The microscopic finding of neutrophil accumulation at the papillary dermis and the responsiveness of skin lesions to dapsone support the key role of neutrophils in DH inflammation. Circulating neutrophils in DH show an increased expression of CD11b, decreased cell surface L-selectin, and increased Fc IgA receptor function, suggesting that they have already been primed before migrating into the skin (115). Indeed, neutrophils priming is likely to occur in the gut under the influx of gut-derived IL-8 (121). Accordingly, IL-8 mRNA was shown to be significantly increased in the small bowel of DH patients on a normal diet compared to that on a GFD. Circulating IL-8 decreases following the GFD, while persists elevated in DH patients who take a normal diet. A positive correlation between serum IL-8 and anti-tTG IgA antibodies has been also demonstrated, suggesting that the cytokine levels parallel the ongoing mucosal inflammation in the gut and depend directly on gluten ingestion (121). Likewise, the underlying mucosal inflammation leads to enhanced expression of the adhesion molecule E-selectin in endothelial cells from both lesional and non-lesional skin (122). Local production of cytokines and chemokines, including IL-8 and GM-CSF, in the presence of eTG/IgA deposits eventually allow migration of adhered neutrophils to the papillary dermis (121, 123). A recent study demonstrated a down-regulation of elafin, a serine protease inhibitor that inhibits a neutrophil mediated inflammatory response, in keratinocytes from DH skin. A similar finding could be also found in the epithelial cells from the small bowel of patients with active CD (124).

Activated neutrophils release neutrophil elastase and granzyme B, which induce subepidermal split by cleaving adhesion molecules of the BMZ, such as collagen VII (125). Accordingly, immunomapping studies have shown that dermal-epidermal detachment in DH mainly occurs within the lamina lucida, between collagen VII and laminin 332, and probably involves destruction of laminin 332 (126, 127). In addition, basal keratinocytes over-expresses collagenase, stromelysin-1, and urokinase-type plasminogen activator, which contribute to degrading basement membrane zone proteins (128).

The activation of the coagulation cascade is thought to be an additional pathomechanism of DH. Accordingly, Bognar

et al. found a high prevalence of criofibrinogenaemia in untreated DH patients (129). Another study demonstrated an impaired fibrinogen/fibrin turnover in the disease. More in detail, untreated DH patients showed significantly prolonged clot lysis time and thicker fibrin fibers, which could be normalized by the *in vitro* adjunct of dapsone (130). Conversely, unlike BP, no studies demonstrated significant skin expression of tissue factor as well as elevation of serum D-dimer in patients with DH compared to healthy individuals (131, 132).

Previous studies have also suggested that other inflammatory cells participate to the pathogenesis of DH (133). In particular, T-lymphocytes, mononuclear phagocytes and B-cells were shown to accumulate around dermal vessels during the early phases of DH lesion formation (134). Other studies found increased Th2-related cytokines in the skin but not in the serum of DH patients (133, 135). By comparison, Makino et al. recently reported a significant elevation of IL-4, IL-5, IL-13, and eotaxin in fibrillary-type DH, whereas Th1-related cytokines, including IL-12 and IFN-γ, did not show significant differences compared to healthy controls (136).

In another study, our group demonstrated that, in DH skin, Tregs and IL-10 were significantly reduced compared to the skin of healthy subjects, whereas both coeliac and DH patients had a similar number of Tregs in duodenal biopsies, suggesting that the down-regulation of Tregs in the skin may be critical for the development of DH lesions (93).

The Pathogenesis of Pruritus in Dermatitis Herpetiformis

While over the recent years different studies have gradually shed light on the major pathogenic mechanisms of DH, the pathogenesis of pruritus is far less clear. Probably, different pathways are involved, including neurogenic inflammation, mechanical itch dysesthesias, and release of inflammatory cytokines. Accordingly, Cynkier et al. demonstrated an over-expression of neuropeptides, including corticotropin releasing factor and the receptor for endotelin B in DH lesional skin, which may be released by activated keratinocytes (137). In DH, pruritus may be evoked or worsened by mechanical stimuli (allokinesis), such as clothing. Moreover, the intensity of the perceived pruritus is also enhanced (hyperkinesis).

Among inflammatory cytokines involved in pruritogenesis, IL-31 has gained a major interest. IL-31 belongs to the cytokine family of IL-6. IL-31 interacts with a heterodimeric receptor, which comprises the IL-31 receptor A (IL-31RA) and the oncostatin M receptor (138), and is expressed on various immune cells including T cells, keratinocytes, dendritic cells, eosinophils, basophils, macrophages, and dorsal root ganglia (139–145). In mice, over-expression of IL-31 was shown to evoke pruritus and induce inflammatory cells accumulation with increased number of mast cells. Injection of IL-31 in the dog also triggers a scratching behavior. Several studies have demonstrated an over-expression of IL-31 in the skin of different pruritic dermatoses, including atopic dermatitis (146), psoriasis (147), cutaneous T-cell lymphomas (148), nephrogenic pruritus (149), and mastocytosis (145).

IL-31 seems to be particularly implicated in pruritic dermatoses related to a prevalent Th2 type inflammation (150). A recent study demonstrated that eosinophils are the major source of IL-31 in BP, a prototype of highly pruritic Th2-mediated autoimmune blistering dermatoses (151). We have previously shown that Th2 type cytokines are also elevated in the skin of DH patients, thereby allowing speculation about a possible role of IL-31 in the associated pruritus. Interestingly, while a previous paper did not show significant elevation of serum IL-31 concentration in DH patients (152), a recent study by our group demonstrated that IL-31 was not only elevated in DH serum, but also significantly over-expressed in the skin, where it co-localized with IL-31RA (153).

Intriguingly, a monoclonal antibody targeting IL-31 is currently under clinical investigation in atopic dermatitis (154). If the role of IL-31 in DH pruritus were to be confirmed, IL-31 monoclonal antibodies will open interesting therapeutic perspectives, potentially allowing a faster control of DH pruritus, which is typically refractory to either topical or systemic treatments and improve only after months following a GFD.

GLUTEN AND THE SKIN: NOT ALWAYS DERMATITIS HERPETIFORMIS

Although DH is the specific cutaneous manifestation of CD, several other skin diseases secondary to gluten ingestion are increasingly reported, especially in the last years, when the focus on gluten intolerance has grown steadily. In fact, despite the exclusion of gluten from the diet is even becoming a fashion, data from the literature demonstrate a higher incidence of skin diseases in patients with gluten-related disorders (14).

In 2012, Sapone et al. reviewed the spectrum of gluten-related disorders, including CD, DH, wheat allergy, gluten ataxia, and non-celiac gluten sensitivity (NCGS) (155). In their review, the authors highlighted that gluten ingestion could cause the involvement of different organs (such as bowel, nervous system, and skin), via the activation of different pathogenic mechanisms.

Accordingly, in recent years, several studies focused on skin manifestations different from DH that occurred in patients with both CD and NCGS.

The Skin in Celiac Patients

According to the classification proposed by Humbert et al. (156), our group recently reviewed in detail the cutaneous and mucosal manifestations associated with CD (43). As a result, different groups of CD-related skin diseases were identified; however, the main distinction was between those that improve after a GFD and those that are just occasionally associated with CD.

In general, common dermatological diseases such as psoriasis, atopic dermatitis, urticaria, aphtous stomatitis, and rosacea are more frequently diagnosed in celiac patients than in the general population (157). Notably, their diagnosis is often difficult because of atypical clinical presentation, and their course is sometimes characterized by the resistance to standard therapies and the response (or at least the improvement) after the introduction of a GFD.

In this regard, the relationship between psoriasis and CD has been studied in depth, and patients with psoriasis seem to have a 3-fold increased risk of CD (158). Moreover, in a recent meta-analysis, patients with psoriasis were demonstrated to have an increased risk of positivity for serologic markers of CD and GFD was suggested to be of potential benefit for celiac antibody-positive patients with psoriasis (159).

Despite the association between the aforementioned skin diseases, testing patients having psoriasis, atopic dermatitis, or other dermatologic diseases for CD is not advisable, since the relative risk is low. There are only few conditions in which the screening is recommended, such as type 1 diabetes mellitus, autoimmune thyroiditis, autoimmune liver disease, juvenile chronic arthritis, Down syndrome, Turner syndrome, Williams syndrome, IgA deficiency, as well as patients having first-degree relatives with CD (160); among them, no skin disorders have been included yet.

Non-celiac Gluten Sensitivity: The Skin as a Major Target

NCGS is a new entity within the group of gluten-related disorders that is being increasingly reported in the medical literature. Despite the lack of specific biomarkers, that makes the diagnosis of NCGS one of exclusion, the reported high frequency of the disease and its potential impact on the quality of life make NCGS a health challenge.

NCGS mainly affects the bowel; however, extra-intestinal manifestations are common, being the skin one of the major target of the disease.

To date, only two studies have investigated the cutaneous manifestations of NCGS, including one by our group. In general, skin lesions were found to be predominantly located on the extensor surfaces of the upper and lower limbs, and consisted of erythema, papules, crusts, and sometimes, vesicles, resembling subacute eczema, or even DH. Some patients had hyperkeratotic scales overlying mild erythematous plaques similar to psoriasis, while others showed urticarial plaques or wheals (161, 162). Besides the prevalent clinical phenotype, all the patients suffered from intense pruritus, that in about 10% of the cases represented the only symptom.

Interestingly, in both studies, patients were shown to respond well to a GFD, with some patients achieving disease remission within only 1 month following the GFD (161). It is worth mentioning that about 80% of the patient included in our study showed at DIF the presence of DH-like granular C3 deposits at the dermal-epidermal junction, but none showed the presence of IgA.

This immunopathologic profile, together with itching and the resolution of the skin lesions after the introduction of a GFD were considered as the main features of this new entity, referred to as "cutaneous gluten sensitivity."

Despite the low number of enrolled patients, suggesting that further studies are required to confirm these data, the introduction of the concept of specific skin manifestations related to NCGS may be helpful for the management of the patients, as is for DH and CD.

In fact, the main issue of NCGS is represented by the diagnosis, that relies on a procedure that is impractical to be implemented in the clinical setting (i.e., double blind placebo controlled challenge with duodenal biopsy) (163). Accordingly, the identification of specific skin features such as cutaneous gluten sensitivity might allow the diagnosis of NCGS without the need of invasive investigations.

DIAGNOSIS OF DERMATITIS HERPETIFORMIS

DH is a difficult disease to be diagnosed. Accordingly, the delay from the occurrence of the first symptoms or clinical signs to the diagnosis is usually of several months or even years, although it is reported to be decreasing in the last decades, probably due to a major awareness both of DH itself and of coeliac disease (25).

This diagnostic delay is caused by the rarity of DH and the polymorphic cutaneous manifestations, which can be misdiagnosed as other chronic pruritic dermatoses including autoimmune blistering diseases such as BP or Linear IgA bullous dermatosis but also atopic dermatitis, eczema, prurigo, urticaria, or scabies (7). Moreover, in the last years skin manifestations of NCGS have emerged as a novel diagnostic challenge in patients with gluten intolerance, since they can clinically resemble DH and can share similar intestinal involvement (161, 162).

The Role of Skin Biopsy: Are IgA Deposits Still Pathognomonic of Dermatitis Herpetiformis?

After taking patient's history and clinical examination, the first step to make a diagnosis of DH is to perform a skin biopsy. The biopsy specimen can be investigated for histopathological examination, which may show typical features such as subepidermal vesicles and blisters associated with accumulation of neutrophils at the papillary tips (7). However, in a third of the cases (164), histopathology is non-specific; atypical findings have been also reported, including acantholysis, leading to a possible histopathologic overlap with pemphigus (165).

Therefore, although histopathological examination may be helpful, DIF from the perilesional skin is still considered the gold standard for the diagnosis. The main finding is the presence of granular IgA deposits at the dermal papillae and/or at the dermal-epidermal junction (**Figure 4**) (7); in some cases, and in up to 50% of Japanese patients (79), a fibrillar deposition of IgA could be found.

Besides IgA, other immunoreactants can be found at the dermal-epidermal junction or at the dermal papillae in the perilesional skin. Among them, IgG granular deposits are infrequently detected, while IgM and C3 granular deposits are present in more cases.

In some cases, patients may show only granular deposition of C3 in the absence of IgA, IgG, or IgM. Recently, a case series of 20 patients showing these findings at DIF has been reported (166). Half of them had clinical features mimicking DH and very few of them showed low titres of anti-eTG, anti-tTG, anti-gliadin, and anti-BP180 antibodies. The authors proposed the

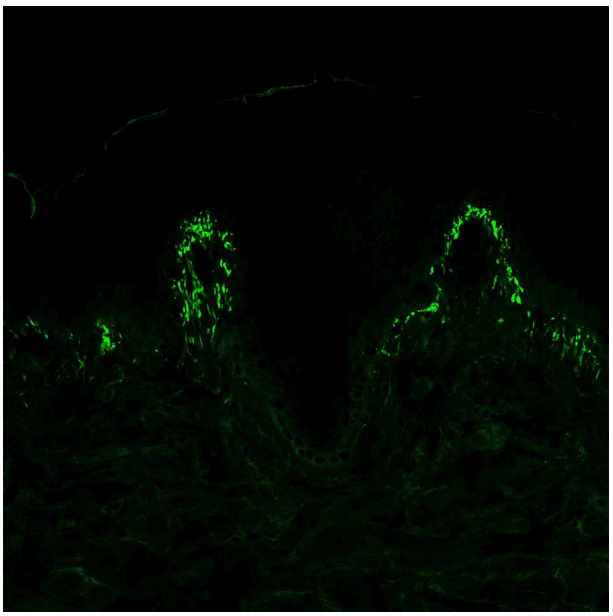

FIGURE 4 | Direct immunofluorescence of perilesional skin specimens from patients with dermatitis herpetiformis (DH). Granular IgA deposits at the dermal papillary tips are considered a pathognomonic finding of DH (magnification 400×).

term granular C3 dermatosis to describe this condition (166), that might be considered as an umbrella concept including different diseases (DH, cutaneous gluten sensitivity, non-DH dermatoses occurring in patients with CD) rather than a distinct clinical entity.

Although its prominent role in the diagnostic algorithm for DH, due to its high sensitivity and specificity, DIF can have some limitations. As an example, several papers reported patients diagnosed with DH according to compatible clinical features, histopathology, concomitant presence of CD, the response to the GFD or dapsone, and the recurrence of cutaneous lesions after a gluten challenge, but showing negative DIF results (167, 168).

In some instances, DIF may result negative because of the site of the biopsy; in fact, when the skin specimen is taken in lesional skin or too far from the skin lesions, it may lack the presence of IgA deposits (7). However, in some confirmed DH cases, even repeated biopsies performed at different times gave negative DIF results, suggesting that false-negative patients are possible (169, 170).

Another pitfall can be related to the interpretation of DIF. On this point, a recent paper from our group reported a case-series of six celiac patients presenting with skin diseases different from DH, such as contact eczema, dermatophytosis, granuloma annulare, pytiriasis rosea, lichenoid dermatitis, and psoriasis. Notably, all these patients showed a granular deposit of IgA at the dermal-epidermal junction and/or at the papillary tips (10). As previously mentioned, Cannistraci et al. observed granular IgA deposits in the healthy skin of nine celiac patients without any cutaneous manifestation (117). Both these studies raise the hypothesis that granular IgA deposits may occur also as a cutaneous marker of CD. Interestingly, while in study by Cannistraci et al. coeliac patients showed IgA/eTG co-localization in the granular deposits, no IgA/eTG co-localization was documented in any case from our study.

To conclude, the diagnosis of DH should be the result of an overall assessment, including clinical, histological, and immunopathological findings and could not rely only on DIF findings. Whether assessing the co-localization between IgA and eTG deposits in the perilesional skin might be helpful to confirm the diagnosis of DH in doubtful cases warrants further investigation.

Beyond Direct Immunofluorescence: Serology and Other Investigations

Among other examinations that can help in the diagnosis of DH, serology plays a primary role, mainly as a screening tool. In fact, as happens for CD, patients with DH test positive in the majority of cases for anti-tTG, anti-EMA and anti-deamidated synthetic gliadin-derived peptides, that overall showed a sensitivity ranging from 50 to 95%, and a specificity higher than 90% (171). Moreover, in the last years, after the demonstration of eTG as the main autoantigen for DH, anti-eTG antibodies have been shown to be a promising tool for the diagnosis of DH (12, 104, 111). Unfortunately, although their sensitivity and specificity for DH are close to those of anti-tTG antibodies, anti-eTG antibodies can be found in about 30 to 50% of the patients with CD (12, 172), and therefore, are not helpful to differentiate between DH and other dermatoses occurring in celiac patients.

Other antibodies have been tested in patients with DH showing promising results, such as anti-neo epitope tissue TG (173) and anti-GAF3X antibodies (174); however they were no further tested and their high sensitivity and specificity values should be confirmed.

A comprehensive assessment of patients with DH can include other investigations that may be helpful in doubtful cases. Duodenal biopsy is usually not required but, since it is the gold standard for the diagnosis of CD, it should be performed if the clinical picture or other findings are highly evocative for the diagnosis of DH but DIF results negative (7). In that case, the presence of villous atrophy at histopathology is important to confirm (or to exclude) the diagnosis of CD and to support the introduction of a GFD, at least in the Caucasians. Accordingly, it is worth noting that duodenal biopsy might be not helpful in Japanese DH patients, because of the weaker association of DH with CD in that population and because diseases other than CD represent a more frequent cause of villous atrophy in Asia, including tropical sprue, parasitic infections, immunoproliferative small intestinal diseases and combined variable immunodeficiency disease (175).

Since virtually all the patients with CD and DH, at least in western countries, have HLA-DQ2 or DQ8 aplotypes, HLA testing may be helpful for its high negative predictive value in order to exclude a diagnosis of DH and to avoid repetition of unnecessary investigations (171).

Finally, in the last years, other potential diagnostic tools have been proposed for DH. As an example, dermoscopy was found

to be helpful in the differential diagnosis of autoimmune bullous diseases involving the scalp. In particular, patients with DH usually show the absence of yellow scales, that are more typical of pemphigus patients, but display extravasation and clustered dotted vessels (176), that seem to be a specific finding of the disease and are found even in the petechial lesions of palms and soles (177).

Other more complex investigations, such as the analysis of microbiota and of metabolic signature of the patients, may provide some information on the patients with DH. However, although they were investigated in detail in patients with CD, paving the way for their potential use in the clinical setting (178, 179), very scarce data are available for DH (180), and further studies are needed to address the questions that are still open in this field.

TREATMENT

The Central Role of Gluten: What's Beyond Gluten-Free Diet?

Since DH is the specific cutaneous manifestation of CD, at least in the Caucasians, all the patients require a GFD. Although some authors suggested that a subgroup of the patients with DH can go into remission and, therefore, gluten-free diet may be stopped (13), other studies demonstrated that DH is associated with an increased risk of associated diseases, such as non-Hodgkin lymphomas (28). Moreover, although this risk is higher in the first 5 years after the diagnosis, it overlaps that of the general population thereafter (32). As a result, since GFD in DH is a way to prevent complications rather just a symptomatic treatment for skin lesions, it should be maintained for all the life (181).

Gluten-free diet requires strict monitoring of all ingested foods, it is time-consuming, and socially restricting (182), and patient compliance depends on different individual and environmental factors, with a self-reported diet adherence in adult patients with CD ranging from 36 to 96%(183). By contrast, patients with DH seem to have a better compliance to gluten-free diet, with an overall adherence of 98% (percentage that decreases to 72% for strict adherence) (32). However, since gluten contamination is possible even in supposed gluten-free food, nutritional monitoring, and participation in support groups are recommended for both patients with CD and DH (184). Moreover, some patients that adhere to gluten-free diet are refractory even several years after the introduction of the diet (185).

According to the limitations of gluten-free diet reported above, novel approaches to treat patients with CD and DH are currently under investigation. Such new therapies rely on blocking at different steps the pathogenic process occurring in gluten-sensitive disorders.

For example, the reduction of gluten immunogenicity via the production of genetically modified grains may lead to a decrease of the number of patients that are able to develop autoimmunity to gliadin-tissue TG complexes (186). RNA interference is another way that could be applied in order to reduce gluten toxicity (187). Moreover, gluten exposure could be reduced using some binders that allow its sequestration in the bowel lumen, or by hydrolysis of gluten peptides that are resistant to proteolysis in a physiological setting using orally administered glutenases (188). Finally, the exposure to immunogenic peptides can be prevented by altering gut permeability; in this regard, the blockade of zonulin, a tight junction protein that regulates the epithelial transit of molecules, with inhibitors such as larazotide has proven to ameliorate symptoms of patients with CD (189).

Other steps that can be targeted in patients with CD are related to the activation of the immune response. tTG activity inhibition, HLA-DQ2 blocking with gluten peptide analogs or immune tolerance induction using specific gluten epitopes are under study or are being tested in patients with CD (190, 191).

Pharmacologic Treatment of DH: When Gluten-Free Diet Is Not Enough

All the approaches reported above are being developed for patients with CD but, since they are oriented to prevent gluten sensitization or to restore gluten tolerance, they might work even for DH. The latter, however, has some specific therapeutic issues that differ from those of CD. In fact, while intestinal symptoms usually resolve in few weeks, cutaneous manifestations may last months or even years after the introduction of a GFD. Therefore, in most cases, pharmacologic treatment is required in order to control itching as well as the skin rash (7).

Despite the lack of randomized controlled trials, dapsone has been considered the first line treatment in patients with DH for over 70 years. Dosages of about 50 to 100 mg per day are usually sufficient to clear the skin rash. At these dosages, side effects are usually rare. However, hemolytic anemia and methemoglobinemia, that are dose-dependent side effects, can be seen in some patients. As a result, all the patients taking the drug should be followed up with frequent testing of hemoglobin levels and reticulocytes. In patients with glucose-6-posphate dehydrogenase deficiency dapsone treatment is contraindicated (7).

In two case reports, topical dapsone 5% gel was also shown to be effective as an adjuvant treatment for DH, which is not adequately controlled by GFD or oral dapsone (192, 193). Compared to the oral counterpart, topical dapsone shows a lower incidence of side effects and can be administered safely in patients with glucose-6-posphate dehydrogenase deficiency (192).

Possible alternatives to dapsone are the so-called sulfonamides, including sulfasalazine, sulfapyridine, and sulfamethoxypyridazine, that may potentially cause hemolytic anemia and gastrointestinal upset, but require a less strict monitoring than dapsone (7). Furthermore, a recent report suggested that combination therapy with dapsone and sulfasalazine may be effective in patients who do not tolerate increasing doses of dapsone monotherapy (194).

Several other pharmacologic treatments have been proposed for DH. Case reports do exist showing that cyclosporin A, azathioprine, colchicine, heparin, tetracyclines, nicotinamide, and mycophenolate may control the acute rash in patients with DH (171). Among them, some authors suggested that colchicine may be used as a second choice after dapsone, due

to its antineutrophilic and antithrombotic activity (195), since DH was reported to be associated with a decreased fibrinolytic potential (130).

Besides these "old" drugs, biologics may become the next step in DH pharmacologic treatment. Among them, rituximab has been proven to be effective in a patient resistant to GFD, dapsone, sulfasalazine, and conventional immunosuppressive agents such as azathioprine, and was suggested as a viable treatment option for recalcitrant DH (196).

Celiac Disease and Dermatitis Herpetiformis: Is Prevention Possible?

In the last years, some authors focused on the risk factors associated with the development of CD, aiming at the prevention of the disease in genetically at-risk infants or children. Randomized controlled trials investigated the possible role of the age at which gluten is introduced, showing that neither ingestion of small gluten amounts between weeks 16–24, nor delayed introduction of gluten (at 6 or at 12 months) modified the incidence of CD in the studied groups (197, 198).

Besides gluten, microbiota has been suggested to act as a trigger in several autoimmune diseases, including CD. In the latter, both genetic and environmental factors, such as rotavirus infection, may modified the gut microbiota of at-risk patients that, in turn, can affect bowel immunity and permeability (199). In particular, altered gut microbiota increases the production of pro-inflammatory cytokines, impairs the mucosal barrier, and produces microbial TG (199), that is a target of antibodies found in celiac patients (200). Therefore, targeting the gut microbiota via probiotics may be a reasonable approach in order to prevent CD in at-risk individuals, and trials are currently underway (199).

Notably, all these data are available for CD. However, due to the close correlation with CD, it could be assumed that such strategies may work even for the prevention of DH, and studies on these topics would be advisable.

FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

DH is an autoimmune bullous disease associated with a chronic, usually asymptomatic, autoimmune enteropathy, that arises in genetically susceptible individuals, in the presence of gluten proteins consumed in common grain products.

One of the main concerns of DH is still represented by misdiagnosis, due to its rarity, the growing report of atypical clinical presentations and the possible occurrence of IgA deposits in non-DH skin diseases. In the near future, the diagnostic challenge is expected even to increase, due to the falling incidence of DH (16). This is reflected by the quite long diagnostic delay, found even in high prevalence areas (25).

In the last years, NCGS has emerged as a new entity within the spectrum of gluten-related disorders. Similarities in both clinical and immunopathological findings may enhance the diagnostic challenge and have important therapeutic implications. In fact, although GFD is the first line treatment for both DH and NCGS, patients with DH should be followed up closely for dietary adherence, nutritional deficiencies, and potential complications.

Recent advances in the pathogenesis of DH have paved the way for the development of new treatments to be used in the time window between the beginning of the GFD and the complete resolution of skin lesions. As an example, the recent finding of elevated serum levels of IL-17 and IL-36 in DH patients supported their possible role in the activation of neutrophils and NK cells, making them as possible targets for new therapeutic strategies (201). In addition, the involvement of IL-31 pathway in DH suggests a connection among the immune system, the nervous system and itch, and its targeting holds promise for the treatment of the patients (153).

AUTHOR CONTRIBUTIONS

EA, RM, and MC designed the study and drafted the manuscript. LQ, AV, DB, and VB drafted the manuscript. EA, RM, and MC revised the whole manuscript. All the authors approved the final content of the manuscript.

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Immune Reaction to Type XVII Collagen Induces Intramolecular and Intermolecular Epitope Spreading in Experimental Bullous Pemphigoid Models

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Bullous pemphigoid (BP), the most common autoimmune blistering disease, is induced by autoantibodies to type XVII collagen (COL17). Previous studies demonstrated that COL17 harbors several epitopes targeted by autoreactive T and B cells and that the target epitopes change sequentially during the disease course. To elucidate the details of the humoral immune response to COL17, we used an active BP mouse model in which BP is induced by the adoptive transfer of spleen cells from wild-type mice immunized with human COL17-expressing skin grafting to immunodeficient COL17-humanized (Rag-2$^{-/-}$, mouse Col17$^{-/-}$, human COL17^{+}) mice. By immunoblot analysis, antibodies to the NC16A domain and other extracellular domains (ECDs) of COL17 were detected earlier than antibodies to intracellular domains (ICDs) in the active BP model. Time course analysis by enzyme-linked immunosorbent assay demonstrated a delayed peak of antibodies to ICD epitopes in active BP model. The blockade of CD40–CD40 ligand interaction soon after the adoptive transfer suppressed the production of antibodies to the non-collagenous 16A (NC16A) domain but not to an ICD epitope, suggesting the sequential activation from T and B cells against the ECD epitopes including the NC16A domain to those against ICD epitopes *in vivo*. Both wild-type mice immunized with a fragment of the NC16A domain and the recipients of those spleen cells produced IgG antibodies to ICD and ECD epitopes, showing intramolecular epitope spreading from the NC16A domain to other epitopes of COL17. Furthermore, we found that a portion of the active BP model mice show intermolecular epitope spreading from human COL17 to murine BP230. The appearance of antibodies to ICD epitopes of COL17 or of antibodies to murine BP230 did not correlate with the skin changes in the mice, suggesting that those antibodies have low pathogenicity. These results suggest that the immune response to the ECD epitopes of COL17, especially to the NC16*A* domain, triggers intramolecular, and intermolecular epitope spreading to ICD epitopes of COL17 and to murine BP230. These novel findings provide insight into the mechanism of epitope spreading in organ-specific, antibody-mediated autoimmune disorders.

Keywords: BP180, COL17, BP230, active mouse model, CD40 ligand, autoimmunity, autoantibody, NC16A domain

INTRODUCTION

Bullous pemphigoid (BP) is the most common autoimmune blistering disorder and is characterized by tense blisters with itchy urticarial plaques and erythema on the entire body. BP characteristically affects the elderly, and recent studies reported a trend of increased incidence of BP (1). BP is reported to be associated with increased risk for cardiovascular disease and neurological disease (2). Autoantibodies in BP react with two structural components of the dermal-epidermal junction (DEJ): type XVII collagen (COL17, also called BP180, or BPAG2) and BP230 (also called dystonin or BPAG1). The autoantibodies to COL17 are considered to trigger the inflammatory and non-inflammatory processes, resulting in the disruption of dermal-epidermal connection. COL17 is a hemidesmosomal transmembrane protein that spans the lamina lucida and projects into the lamina densa of the DEJ (3–10). The extracellular portion of COL17 contains 15 collagenous domains separated from one another by non-collagenous domains (4). The juxtamembranous extracellular non-collagenous 16A (NC16A) domain, located at the membrane-proximal region of COL17, is preferentially recognized by autoantibodies in BP patients (11, 12). Several studies have demonstrated that the serum levels of autoantibodies to the NC16A domain of COL17 are related to the disease activity of BP (13, 14). The passive transfer of IgG antibodies to the NC16A domain of human COL17 or its murine counterpart into neonatal mice directly demonstrates the *in vivo* pathogenicity of those antibodies (15, 16). Thus, the NC16A domain of COL17 contains the major pathogenic epitope for BP. In addition, it is well-known that the intracellular domain (ICD) and the extracellular domain (ECD) of COL17 are also targeted by autoantibodies of BP (17, 18). A previous study demonstrated that 47% of BP sera reacted to the C-terminal region of COL17 (19). Autoantibodies to the C-terminal region of COL17 are thought to be involved in mucous membrane pemphigoid (7). Furthermore, a recent study demonstrated that autoantibodies in BP patients which react to the full-length recombinant COL17 protein but not to the NC16A domain preferentially react to epitopes within the mid-portion of the ECD of COL17 (20).

BP230 is another autoantigen of BP and was originally identified as the major antigen of BP (21, 22). BP230 is a cytoplasmic component of hemidesmosomes that belongs to the plakin family; it promotes the linkage of keratin intermediate filaments to hemidesmosomes (23). More than 80% of BP sera show reactivity to BP230 (24, 25). It remains uncertain whether anti-BP230 autoantibodies directly contribute to blister formation or whether they are just by-products of epitope spreading associated with disease extension, although several studies have pointed to the pathogenicity of autoantibodies to BP230 (26–28).

Abbreviations: BP, bullous pemphigoid; COL17, type XVII collagen; NC16A, non-collagenous 16A; ICD, intracellular domain; ECD, extracellular domain; CD40L, CD40 ligand; IF, immunofluorescence; ELISA, enzyme-linked immunosorbent assay; NHS, normal human skin; skin-grafted wild-type mice, wild-type mice grafted with human COL17-expressing skin

Epitope spreading is a phenomenon in which the targets of T- and/or B-cell responses can extend from the initial dominant epitope to other epitopes on the same protein (intramolecular epitope spreading) or to other proteins in the same tissue (intermolecular epitope spreading) over time (29, 30). Intramolecular epitope spreading has been reported in several autoimmune disorders, such as multiple sclerosis (31) and myasthenia gravis (32). It is well-known that epitope spreading frequently occurs in BP. *In vivo* experiments using a human COL17-expressing skin-grafted BP mouse model showed that IgG antibodies to human COL17 initially react to the ECD epitopes and that, subsequently, the humoral immune responses target additional ECD and ICD epitopes (33). A prospective multicenter study demonstrated that 49% of 35 BP patients showed epitope spreading that preferentially occurred at an early stage of the disease and was associated with disease severity (34). Thus, epitope spreading has been shown in both experimental murine BP and human BP. However, many questions remain, such as whether the T- and B-cell interactions for different epitopes of COL17 occur at different times and whether an immune response to the NC16A domain of COL17 actually triggers intramolecular epitope spreading to other epitopes of COL17 and/or intermolecular epitope spreading to other hemidesmosomal antigens.

To address these issues, we utilized an active disease model for BP that we previously established (35). It is generated by the adoptive transfer of human COL17-immunized spleen cells into adult immunodeficient *Rag-2*$^{-/-}$/COL17-humanized (*COL17*$^{m-/-,h+}$) mice. This model continuously produces IgG antibodies to human COL17 in a CD4^{+} T-cell-dependent manner and reproduces the BP disease phenotype. By using this active BP model, the current study demonstrates that the production of antibodies to the ECD epitopes of COL17 precedes that to the ICD epitopes, especially to the inner portion of the ICD. The interference of T- and B-cell interaction by monoclonal antibody to the CD40 ligand (CD40L) shows that T- and B-cell interactions for ECD epitopes precede those to ICD epitopes of COL17 in an active BP model. Wild-type mice that were immunized with a fragment of the NC16A domain produced antibodies to ICD epitopes of COL17. Furthermore, the active BP model generates antibodies to murine BP230 as a result of intermolecular epitope spreading. These findings clarify the details of epitope spreading in BP.

MATERIALS AND METHODS

Mice

C57BL/6-background *Rag-2*$^{-/-}$ mice were received as a gift from the Central Institute for Experimental Animals (Kawasaki, Japan). We crossed *COL17*$^{m-/-,h+}$ (COL17-humanized) mice that we had previously generated (16) with *Rag-2*$^{-/-}$ mice to produce *Rag-2*$^{-/-}$/*COL17*$^{m-/-,h+}$ (Rag-2$^{-/-}$/COL17-humanized) mice.

Generation of Recombinant Proteins and Synthesized Peptides

Recombinant proteins covering all parts of human COL17 were generated in a previous study (36). Briefly, human ICD-1 (amino acids Met^{1} to Ser^{204}), ICD-2 (Thr^{197} to Lys^{466}), NC16A (Glu^{490} to Arg^{566}), ECD-1 (Gly^{567} to Gly^{860}), ECD-2 (Leu^{853} to Gly^{1218}), and ECD-3 (Ser^{1211} to Pro^{1497}) were generated as GST-fusion proteins using the expression vector pGEX6P-1 (GE Healthcare) and the bacteria BL21 (GE Healthcare). These GST-fusion proteins were purified using GSTrap HP (GE Healthcare) according to the manufacturer's instructions. NC16A-R7 (Asp^{522} to Gln^{545}), 2 ICD peptides (ICD-149: Ala^{149} to Ser^{172}, ICD-320: Thr^{320} to Lys^{343}) and 3 ECD peptides (ECD-917: Lys^{917} to Ser^{940}, ECD-1084: Ser^{1084} to Pro^{1107}, ECD-1330: Ala^{1330} to Gly^{1353}) of human COL17 were chemically synthesized (Greiner Bio-One, Kremsmünster, Austria). The amino acid numbering system is based on the human COL17 sequence (NP_000494.3). Recombinant proteins covering all parts of murine BP230 were generated as previously reported (37). Briefly, total RNA of murine BP230 was extracted from murine keratinocytes, and cDNAs were synthesized by reverse transcription polymerase chain reaction. Plasmid vectors of 3 fragments of murine BP230 with His-tag at the C-terminus were designed. The 3 cDNA fragments were named BP230-1 (Met^{1}-Gly^{3264}), containing the N-terminal (plakin) domain; BP230-2 (Glu^{3223}-Pro^{5562}), containing the (coiled-coil) rod domain; and BP230-3 (Asp^{5533}-Gln^{7833}), containing the C-terminal (intermediate filament-binding) domain, according to GenBank (AF396877.1) and a previous report (38). These cDNAs were inserted into the pSeq Tag2/Hygro B vector (Thermo Fisher Scientific). Vectors were transfected into HEK293 cells with Lipofectamine 2000 (Thermo Fisher Scientific) for transient expression of BP230. Supernatants were collected and centrifuged for purification by using Amicon Ultra Centrifugal Filters (Merck Millipore, Darmstadt, Germany).

Generation of the Active BP Model

To make an active BP model, we first immunized wild-type mice with human COL17-expressing mouse skin graft according to the reported method (39). Briefly, full-thickness 1-cm^2 pieces of dorsal skin were removed from sacrificed COL17-humanized mice and grafted onto the backs of gender-matched, 6- to 8-week-old C57BL/6 wild-type mice. After the topical application of antibiotic ointment, the grafted site was covered with gauze and an elastic bandage for 14 days. Antibody production was confirmed at 5 weeks after skin grafting by immunofluorescence (IF) analysis, as described below. Spleen cells were isolated 5 weeks after the skin graft and pooled from several immunized wild-type mice, and 2.0×10^8 cells/mouse were adoptively transferred into *Rag-2*$^{-/-}$/COL17-humanized mice through a tail vain in 500 μL of PBS as previously reported (35). In some experiments, the wild-type mice were immunized at the hind footpad with 50 μg NC16A-R7 (fused with KLH) peptides emulsified in the adjuvant TiterMax Gold (TiterMax USA, Norcross, GA). The mice received an additional boost at the tail base 1 week after initial immunization. Negative controls were generated by immunization with phosphate-buffered saline (PBS) and TiterMax Gold. Spleen cells were isolated 5 weeks after immunization and pooled from several immunized wild-type mice, and 1.0×10^8 cells/mouse were transferred into *Rag-2*$^{-/-}$/COL17-humanized mice by retroorbital injection in 100 μL of PBS as previously described (40).

In vivo Anti-CD40L Antibody Treatment

Rag-2$^{-/-}$/COL17-humanized recipients that were adoptively transferred with immunized spleen cells were intraperitoneally injected with 1,000 μg hamster monoclonal antibody MR1 specific to mouse CD40L (Taconic Farms, Hudson, NY) at day 0 soon after the adoptive transfer of immunized splenocytes as previously reported (41). All of the treated mice were carefully observed for at least 10 weeks after adoptive transfer.

Evaluation of Recipient Mice

Weekly, the recipient mice were examined for general condition and for percentage of body surface area affected by cutaneous lesions (i.e., erythema, hair loss, blisters, erosions, and crusts). Serum samples were also obtained from recipient mice weekly and assayed by indirect IF microscopy and enzyme-linked immunosorbent assay (ELISA).

Indirect IF Study

Indirect IF using mice sera was performed on normal human skin (NHS) and wild-type mouse skin using standard protocols. We used 1:20 diluted mouse sera as the primary antibodies and 1:100 diluted FITC-conjugated antibodies to murine IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) as the secondary antibodies.

Immunoblot Analysis

Immunoblotting of recombinant proteins covering human COL17 was performed as described previously (36). Briefly, each sample was solubilized in Laemmli's sample buffer and applied to SDS-polyacrylamide gels and then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature in 3% skimmed milk in TBS and then incubated with 1:20 diluted mouse serum samples overnight at 4°C. Bound antibodies were visualized using 1:500 diluted HRP-conjugated goat anti-rat IgG (Jackson ImmunoResearch). Color was developed with 4-chloro-1-naphthol in the presence of H_2O_2. For the detection of antibodies to murine BP230, recombinant murine BP230 proteins were used as a substrate. The membranes were incubated with 1:20 diluted mouse serum samples overnight at 4°C. Bound antibodies were visualized using 1:5,000 diluted HRP-conjugated goat anti-rat IgG (Jackson ImmunoResearch). As a positive control, 1:5,000 HRP-conjugated mouse anti-His-tag mAb-HRP-DirecT (MBL, Nagoya, Japan) was used. The blots were detected using the ECL Plus Detection Kit (GE Healthcare, Fairfield, CT).

ELISA

To determine the titer of antibodies to the NC16A domain of human COL17 in serum samples from the experimental mice, 96-well microtiter plates coated with recombinant NC16A protein purchased from MBL were incubated with diluted

mouse sera for 1 h at room temperature. After being washed, bound antibodies were developed with 1:40,000-diluted, HRP-conjugated antibodies to mouse IgG (Jackson ImmunoResearch Laboratories), and the OD was read at 450 nm. The ELISA index value was defined by the following formula: index = (OD_{450} of tested serum—OD_{450} of negative control)/(OD_{450} of positive control—OD_{450} of negative control) ×100 (35). To examine the titers of antibodies to various regions of human COL17, synthesized peptides in 0.1 M sodium carbonate buffer (pH 9.5) were coated on F96 Maxisorp Nunc-Immuno plates (Thermo Scientific, Roskilde, Denmark) at 5 μg/ml and left overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20 and were blocked for 1 h at room temperature with ELISA assay diluent (BD Biosciences). They were incubated for 2 h at room temperature with 1:100-diluted mouse sera for NC16A-R7 or 1:25 for other peptides in assay diluent. The plates were washed four times and were incubated for 30 min at room temperature with 1:20,000-diluted HRP-conjugated antibodies to mouse IgG for NC16A-R7 or 1:10,000 for other peptides in an assay diluent. After four washings, the plates were displayed in a 1:1 mixture of substrate reagent A containing hydrogen peroxide and substrate reagent B containing tetramethylbenzidine (BD Biosciences) and were then stopped with 1 M phosphoric acid (BD Biosciences). The results were shown by OD at 450 nm. In some experiments, binding activities were calculated by the following formula: index value = (OD_{450} of tested serum—OD_{450} of negative control)/(OD_{450} of positive control—OD_{450} of negative control) ×100.

Statistical Analyses

Data were expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, California, USA). The unpaired *t*-test with Welch's correction was used for comparisons of ELISA index in wild-type mice grafted with human COL17-expressing skin (skin-grafted wild-type mice). *P*-values of <0.05 were considered significant compared with the control.

RESULTS

Antibodies to the NC16A Domain of COL17 Decreased Earlier Than Those to the DEJ in the Active BP Model

First, we prepared the active BP mouse model by the adoptive transfer of spleen cells from a wild-type mouse that was immunized with human COL17-expressing skin grafting onto adult immunodeficient $Rag^{-/-}$/COL17-humanized mice ($n = 4$) (**Figure 1A**). Because human COL17-expressing skin contains full-length human COL17, the skin grafting theoretically induces polyclonal IgG antibodies to various regions of human COL17 in the skin-grafted wild-type mice as well as $Rag^{-/-}$/COL17-humanized recipient mice. As we previously reported (35), around day 7 after the adoptive transfer, the $Rag^{-/-}$/COL17-humanized recipients began to scratch their snouts, ears, and chests. Patchy hair loss with erythema started to develop on the neck and chest around 14 days after the adoptive transfer in the recipients. Ear swelling with crusts was also observed (**Figure 1B**). The dehaired patches gradually enlarged and spread to other regions on the trunk, face and extremities over the next 2–4 weeks, resulting in large areas of alopecia (**Figure 1B**). Dermal-epidermal separation with mild inflammatory cell infiltration was also seen in some of the ear samples at 5 weeks (**Figure 1B**). The affected body surface area plateaued 6 weeks after the transfer and remained high until 10 weeks after the transfer (**Figure 1C**).

Indirect IF examination using sera from the active BP model revealed that IgG bound to the DEJ of normal human skin. Time-course analysis revealed that IgG antibodies to the DEJ, which reflects the presence of antibodies to human COL17, became detectable in recipients' sera 1 week after the transfer. Although the titer gradually decreased after the peak, it remained high ($> \times 2{,}560$) for 10 weeks (**Figure 1D**). We next examined the titer of antibodies to the NC16A domain of human COL17 by ELISA (MBL, Nagoya Japan). ELISA analysis revealed that antibodies to the NC16A domain of COL17 appeared in the recipients' sera as early as 1 week after the transfer. The antibody level peaked around day 9 after the transfer and then rapidly decreased, falling to a low level (<10% of the peak) at 6 weeks after the transfer (**Figure 1E**). These results demonstrate that the titers of total antibodies to human COL17 remained elevated for longer than did those to the NC16A domain, suggesting the presence of additional antibodies which are elevated in the late phase of the disease.

Antibodies to ECD and ICD Epitopes of COL17 Become Detectable at Different Times in the Active BP Model

To examine the presence of antibodies to various regions of human COL17 in the active BP model, we utilized 6 fragments of COL17 which were previously reported (**Figure 2A**) (36). By immunoblotting using those fragments from 4 active BP model mice, we analyzed the sera at days 8 and 56. All sera at day 8 strongly reacted to the NC16A domain and ECD fragments, but only one serum clearly reacted to ICD-2 and no serum bound to ICD-1 (**Figure 2B**). Interestingly, the reactivity of sera to NC16A, ECD-1, and ECD2 was apparently decreased at day 56, while the reactivity to ICD-2 was increased and ICD-1 was recognized by all sera (**Figure 2B**). The relative intensity of the reactivity is summarized in **Figure 2C**. To confirm these findings, we additionally analyzed sera from 4 active BP model mice at days 8, 35, and 56 after the adoptive transfer, and they showed a similar tendency (**Supplementary Figure 1**) Thus, antibodies to ECD epitopes of human COL17 are detectable from the early phase of the disease, whereas antibodies to ICD epitopes, especially to the inner portion of the ICD, were elevated in the late phase in the active BP model.

The Peak of Antibodies to an ICD Epitope of COL17 Lags That to the NC16A Domain and ECD Epitopes in the Active BP Model

To investigate the time course of antibodies to various epitopes of human COL17 in the active BP model, we newly generated

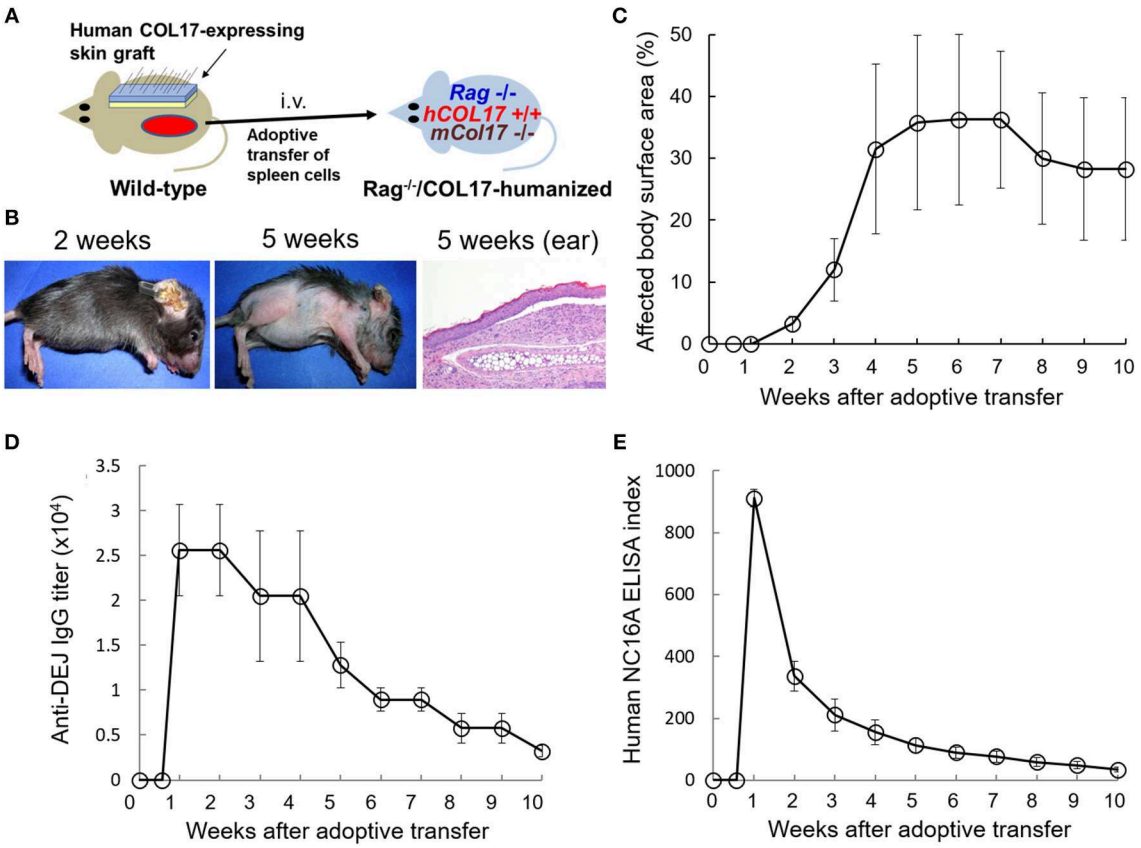

FIGURE 1 | Antibodies to the NC16A domain of COL17 decrease more rapidly than those to the dermal-epidermal junction (DEJ) in an active BP mouse model. **(A)** Schematic of the generation of an active BP mouse model. **(B)** Representative clinical presentations of the active BP model at 2 and 5 weeks after the adoptive transfer of immunized spleen cells and hematoxylin and eosin staining of the ear at 5 weeks (original magnification ×20). **(C)** Time course of disease severities for the $Rag^{-/-}$/COL17-humanized recipients ($n = 4$). **(D)** Time course of titers of circulating IgG antibodies to the dermal-epidermal junction (DEJ) of the skin as determined by indirect IF using sera of an active BP model and normal human skin ($n = 4$). **(E)** Time course of the titers of circulating IgG antibodies to the NC16A domain as determined by ELISA ($n = 4$). Results are shown as mean ± SEM.

an ELISA using synthesized peptides of human COL17. As a fragment of the NC16A domain, we utilized the NC16A-R7 (Asp^{522} to Gln^{545}) peptide because we previously reported that IgG antibodies to R7, but not to other portions of the NC16A domain, are pathogenic *in vivo* (36). We also synthesized 2 ICD peptides (ICD-149: Ala^{149} to Ser^{172}, ICD-320: Thr^{320} to Lys^{343}) and 3 ECD peptides (ECD-917: Lys^{917} to Ser^{940}, ECD-1084: Ser^{1084} to Pro^{1107}, ECD-1330: Ala^{1330} to Gly^{1353}) with reference to previous studies (18, 33, 42) (**Figure 3A**). First, we measured the titers of antibodies in skin-grafted wild-type mice ($n = 16$) at 5 weeks after the skin grafting and compared them to those in untreated wild-type mice ($n = 8$) by ELISA. Although we expected the skin grafting to induce antibodies to every epitope of human COL17, it preferentially induced antibodies to NC16A-R7 but not to ICD-149 nor to ECD-1330 (**Figure 3B**). Next, we examined the titers of antibodies in the active BP model ($n = 6$). As expected, the titer of antibodies to NC16A-R7 peaked at day 9 and decreased rapidly thereafter (**Figure 3C**). The titers of antibodies to ECD-917, −1084 and −1330 also peaked at day 9 and then rapidly decreased, similar to the antibodies to NC16A-R7 (**Figure 3D**). The titer of antibodies to ICD-320 also peaked at day 9 and remained stable after the peak. Notably, the titer of antibodies to ICD-149 slowly increased and peaked at day 21 and then gradually decreased (**Figure 3D**). It should be noted that the titers of antibodies to NC16A-R7 were far higher than those to other epitopes of COL17, because 1:100 and 1:25 diluted sera were used to check the reactivity to NC16A-R7 and to other peptides, respectively. These results suggest that humoral immune response occurs preferentially to the NC16A domain in skin-grafted wild-type mice and then spreads to other epitopes of COL17 in the active BP model in which the response to ECD epitopes precedes that to ICD epitopes.

Blockade of CD40–CD40 Ligand Interaction at Day 0 Preferentially Suppresses the Production of Antibodies to the NC16A Domain but not to Other Epitopes of COL17

To further investigate the timing of T- and B-cell responses to various epitopes of COL17 in an active BP model, we interfered with the interaction between T and B cells by administrating

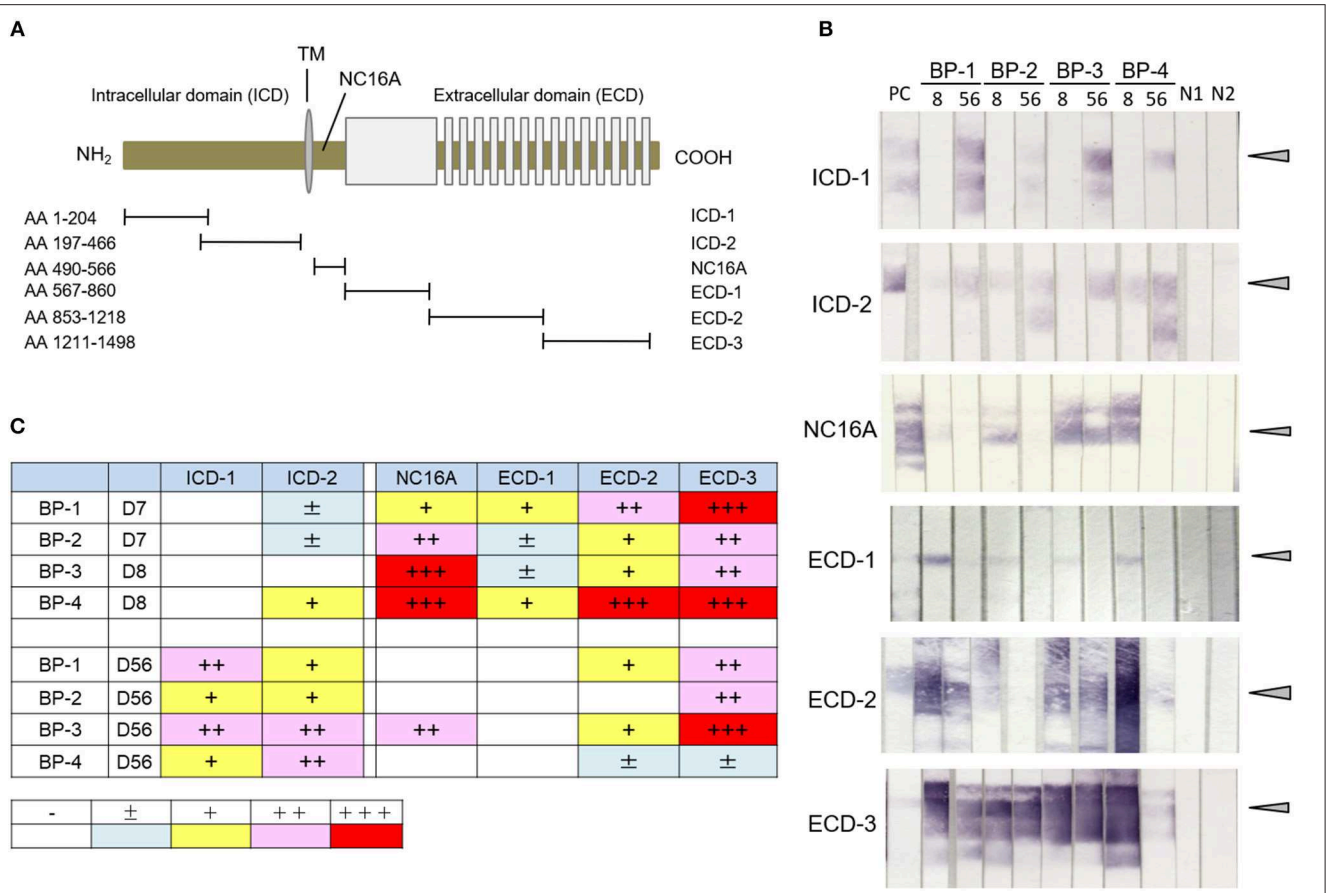

FIGURE 2 | Antibodies to ICD epitopes of COL17 become detectable later than those to ECD epitopes by immunoblotting in an active BP model. **(A)** Schematic of human COL17 and recombinant proteins. TM, transmembrane domain; NH_2, N-terminus; COOH, C-terminus; AA, amino acid. **(B)** Reactivity of sera from active BP model mice ($n = 4$, days 8 and 56) to fragments of COL17 as measured by immunoblotting. PC; positive control (BP patient serum), 8, day 8; 56, day 56; BP-1-4, active BP model mice #1-4, respectively; N1, negative control-1 (wild-type serum); N2, negative control-2 (normal human serum). Arrowheads indicate positive levels. **(C)** Relative intensities of reactivity as measured by immunoblotting. –, negative; ±, faint; +, weakly positive; ++, positive; +++, strongly positive.

monoclonal antibodies to CD40 ligand (CD40L) at day 0 of the adoptive transfer ($n = 4$) and compared the titers of antibodies in untreated active BP model ($n = 9$) (**Figure 4A**). As we previously reported (41), a single dose of antibodies to CD40L at day 0 strongly suppressed the production of antibodies to the NC16A domain (**Figure 4B**). Antibodies to ECD epitopes of COL17 (ECD-917 and 1330) were not suppressed by the treatment (**Figures 4D,E**). Interestingly, the peak of antibodies to ICD-149 was increased and prolonged by the treatment compared to the untreated active BP model (**Figure 4C**). These results suggest that T- and B-cell interaction for the ECD epitopes occurs very soon after adoptive transfer, whereas the interaction regarding ICD epitopes occurs late, so it is unaffected by the early treatment of antibodies to CD40L.

Immunization With NC16A-R7 Induces Antibodies to ICD and ECD Epitopes of COL17 *in vivo*

Because COL17-humanized skin contains full-length human COL17, skin grafting can activate lymphocytes to multiple epitopes of COL17 in wild-type mice. To simply analyze epitope spreading from the NC16A domain to other domains of COL17, we immunized wild-type mice with NC16A-R7 peptides fused with KLH twice at days 0 and 7 (**Figure 5A**). The immunized mice started to produce antibodies to NC16A-R7 within 1 week. However, interestingly, the mice also produced antibodies to ICD-149, which were detected from 2 weeks after the immunization and which gradually increased (**Figure 5B**). Meanwhile, the titers of antibodies to ECD-1330 were very low. Indirect IF study demonstrated that NC16A-R7-immunized wild-type sera reacted to the DEJ of normal human skin (NHS) (positive/total = 5/5). Additionally, they reacted to the surface of basal keratinocytes in wild-type mouse skin (positive/total = 4/5) (**Figure 5C**), suggesting the presence of antibodies that recognize murine COL17 and/or other keratinocyte antigens. We transferred the spleen cells to *Rag-2*$^{-/-}$/COL17-humanized mice at 5 weeks after immunization ($n = 8$) (**Figure 5A**). As a control, spleen cells obtained from untreated wild-type mice were transferred to *Rag-2*$^{-/-}$/COL17-humanized recipients ($n = 8$). As expected, the recipients receiving NC16A-R7-immunized

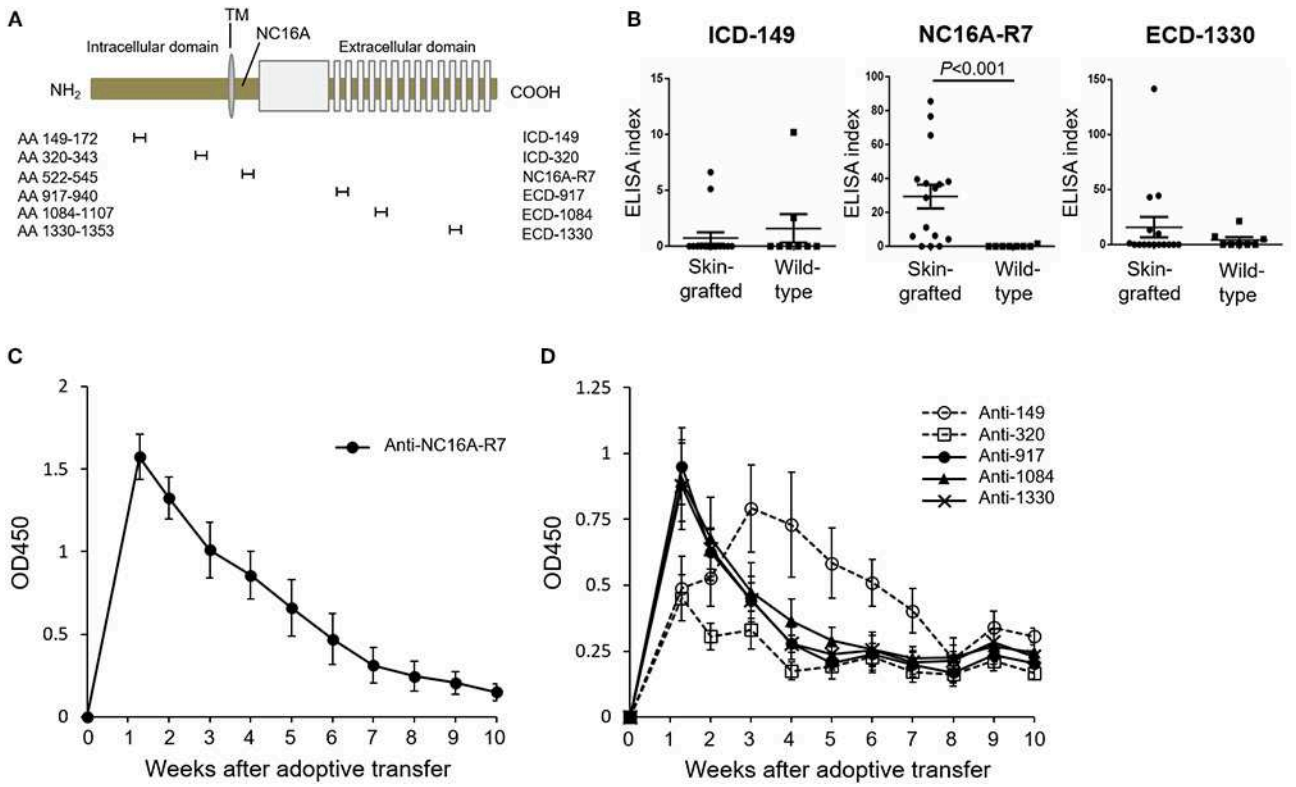

FIGURE 3 | The peak of antibodies to ICD-149 lags that of antibodies to the NC16A domain and ECD epitopes by ELISA in an active BP model. **(A)** Schematic of human COL17 and synthesized peptides. TM, transmembrane domain; NH_2, N-terminus; COOH, C-terminus; AA, amino acid. **(B)** ELISA index of skin-grafted wild-type sera (day 35, $n = 16$) and untreated wild-type sera ($n = 8$) measured by ELISA. Sera were diluted to 1:100 for NC16A-R7 and 1:25 for ICD-149 and ECD-1330. **(C)** Time course of titers of circulating IgG antibodies to NC16A-R7 as determined by ELISA ($n = 6$). **(D)** Time course of titers of circulating IgG antibodies to ICD-149, ICD-320, ECD-917, ECD-1084 and ECD-1330 as determined by ELISA ($n = 6$, respectively). Results are shown as mean ± SEM.

spleen cells developed antibodies to NC16A-R7 shortly after adoptive transfer, and then the titer gradually decreased (**Figure 5D**). Additionally, antibodies to ICD-149 and ECD-1330 became detectable 2 weeks after transfer and then slowly increased. These results indicate that the re-activation of T and B cells to ICD-149 occurs later than that to NC16A-R7 in the recipients. All the recipients developed faint or no skin changes that accounted for <2% of skin surface area, suggesting that the immune response in the recipients is unrelated to the inflammation of the skin. Notably, the recipients of spleen cells from the untreated wild-type mice produced low titers of antibodies to ICD and ECD epitopes but not to the NC16A domain at a late phase (**Figure 5E**), suggesting that a small number of naïve T and B cells from untreated wild-type mice can weakly react to ICD and ECD epitopes of human COL17 in the recipients.

A Portion of the Active BP Model Mice Produce a Low Titer of Antibodies to Murine BP230

Next, we examined whether intermolecular epitope spreading occurs in an active BP model. First, we performed indirect IF analysis using sera from an active BP model and NHS containing human COL17, and COL17-deficient mouse skin lacking both murine and human COL17. As expected, sera from the active BP model strongly reacted to the DEJ of NHS; furthermore, they showed weak reactivity around the basal keratinocytes in COL17-deficient mouse skin (**Figure 6A**), suggesting the presence of antibodies to the antigens on basal keratinocytes other than COL17. Then, we focused on BP230, another major autoantigen of BP, and examined the presence of antibodies to murine BP230 by using recombinant proteins. Recombinant murine BP230 proteins were prepared as 3 fragments; the N-terminus domain (referred to as BP230-1), the rod domain (BP230-2) and the C-terminus domain (BP230-3) (37) (**Figure 6B**). The sizes were confirmed by Western blotting using anti-His-tag antibody (**Figure 6C**). We examined sera from skin-grafted wild-type mice at day 35 ($n = 8$), and they showed no reactivity (data not shown). We then examined sera from the active BP model at days 8, 21 and 84 ($n = 8$). No reactivity was observed in sera at day 8 (data not shown) nor at day 84 (**Figure 6D**), but interestingly, 4 sera at day 21 weakly reacted to BP230-1 (**Figure 6D**). No reactivity was observed on BP230-2 or BP230-3 fragments. There was no correlation between the appearance of antibodies to BP230-1 and disease severity (data not shown). Thus, the humoral immune response that started from COL17 extended to BP230 *in vivo*.

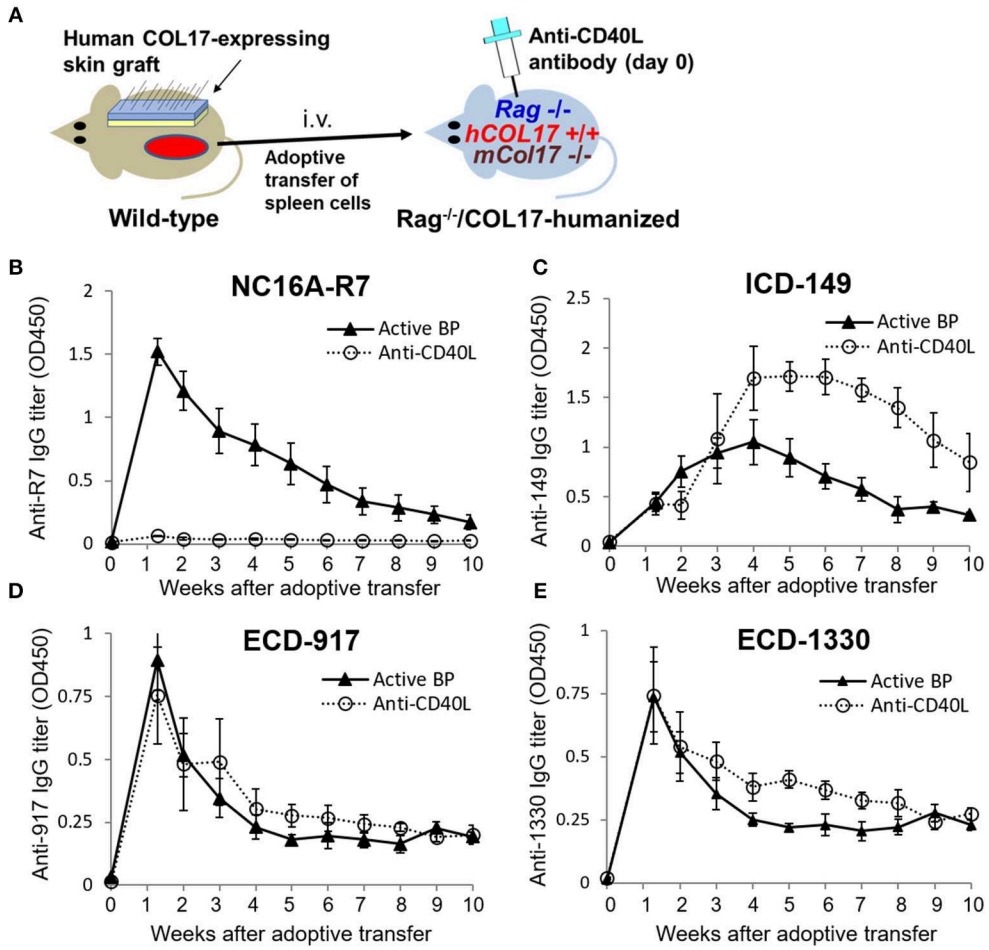

FIGURE 4 | Blockade of CD40–CD40 ligand interaction preferentially decreases antibodies to the NC16A domain but not to other epitopes of COL17. **(A)** Schematic of treatment of the active BP model with antibodies to murine CD40 ligand (CD40L). **(B–E)** Time course of titers of circulating IgG antibodies to NC16A-R7 **(B)**, ICD-149 **(C)**, ECD-917 **(D)**, and ECD-1330 **(E)** in an anti-CD40L antibody-treated active BP model ($n = 4$) and in an untreated active BP model ($n = 9$) as determined by ELISA. Results are shown as mean ± SEM.

DISCUSSION

This study has shown that intramolecular epitope spreading within COL17 and intermolecular epitope spreading from COL17 to BP230 occurs in experimental BP mouse models (**Figure 7**). Although the titer of antibodies to the NC16A domain drops rapidly (**Figure 1E**), the active BP model maintains a similar clinical disease severity for a long period (**Figure 1C**) in association with the clear deposition of IgG at the DEJ of lesional skin, even 35 days after the adoptive transfer of spleen cells as we previously reported (43). We had also noticed the discrepancy in time-course between the titer of antibodies to the DEJ as measured by indirect IF and that to the NC16A domain as measured by ELISA. In this study, we showed that this discrepancy is due to the delayed increase of antibodies to ICD epitopes of COL17. This is consistent with a previous study using a human COL17-expressing skin-grafted mouse model (33). In that study, antibodies to ICD epitopes emerged mostly later than 40 days after skin grafting, which is also consistent with our results in which antibodies to ICD-149 were not detected at day 35 in skin-grafted wild-type mice (**Figure 3B**). As reasons for the delayed increase of antibodies to ICD epitopes in the active BP model, we consider two possibilities. First, intramolecular epitope spreading from the NC16A domain to ICD epitopes occurs shortly after adoptive transfer. Second, ICD epitope-reactive T and B cells were already activated in skin-grafted wild-type mice as a result of intramolecular epitope spreading, and when these cells were transferred into $Rag^{-/-}$/COL17-humanized recipients, they encountered ICD antigens probably a little bit later than ECD antigens *in vivo*, resulting in the delayed peak of antibodies to ICD epitopes.

Unexpectedly, wild-type mice immunized with NC16A-R7 produced antibodies to ICD-149 (**Figure 5B**). We speculate that NC16A-R7-reactive T and B cells also recognized a corresponding site of murine COL17, which induced

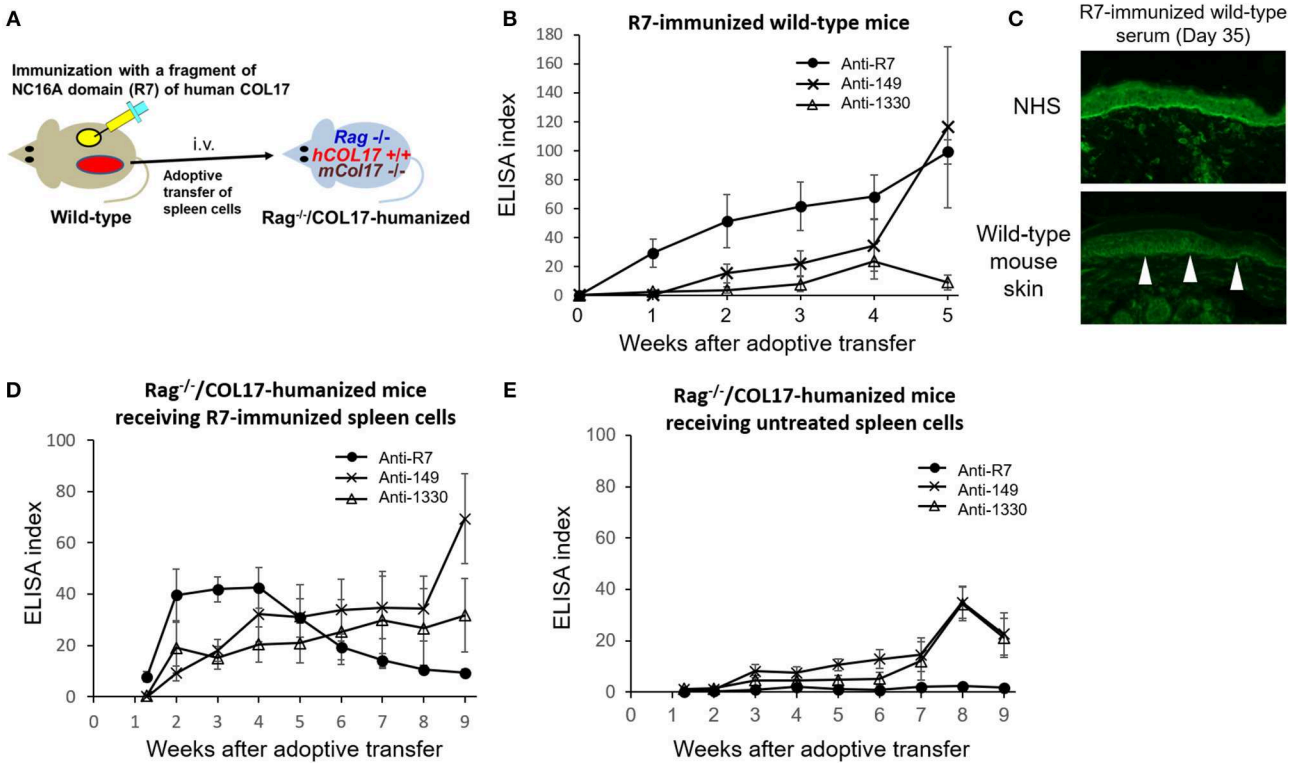

FIGURE 5 | The immune response to the NC16A domain spreads to ICD and ECD epitopes of COL17 *in vivo*. **(A)** Schematic of the immunization of wild-type mice with NC16A-R7 and the adoptive transfer of spleen cells to make the active BP model. **(B)** Time course of titers of circulating IgG antibodies to NC16A-R7, ICD-149, and ECD-1330 in NC16A-R7-immunized wild-type mice ($n = 5$) as determined by ELISA. **(C)** Representative images of IgG deposition in indirect IF study using normal human skin (NHS) and wild-type mouse skin as substrates and 1:20 diluted sera from NC16A-R7-immunized wild-type mice at day 35 as primary antibodies. Arrowheads indicate IgG deposition around the basal keratinocytes. **(D,E)** Time course of titers of circulating IgG antibodies to NC16A-R7, ICD-149, and ECD-1330 in $Rag^{-/-}$/COL17-humanized mice that received spleen cells from NC16A-R7-immunized mice ($n = 8$) **(D)** and in those that received spleen cells from untreated wild-type mice ($n = 8$) **(E)** as determined by ELISA. Results are shown as mean ± SEM.

intramolecular epitope spreading to other epitopes of murine COL17 in wild-type mice. Although the homology of the amino acid sequence of NC17A-R7 (human COL17) and the corresponding site of murine COL17 is 54.1%, that of ICD-149 (human COL17) and the corresponding site of murine COL17 is 95.8%. The positive reactivity of NC16A-R7-immunized sera at day 35 to mouse skin shown in **Figure 5C** supports our hypothesis. Thus, intramolecular epitope spreading from the NC16A domain to ICD epitopes occurs in NC16A-immunized wild-type mice.

Two major mechanisms are considered to be behind the epitope spreading phenomenon: an "independent" or "dependent" of a physical association of antigens (30, 44). The former involves the development of secondary epitopes due to the release of antigens or the disclosure of parts of antigens during a chronic inflammatory or autoimmune response. The latter is independent of inflammatory processes. T cells specific for one epitope of an antigen can activate B cells that are specific for other antigens of the same multi-antigen complex, resulting in the generation of autoantibodies to antigens that are not initially targeted by the immune response (45). In this study, we demonstrated epitope spreading from the NC16A domain to other epitopes of COL17 in NC16A-R7-immunized wild-type mice, even though the mice developed no skin changes. This suggests that intramolecular epitope spreading within COL17 can occur under a mechanism that is "dependent" on a physical association of antigens.

We can estimate the timing of COL17-reactive T-cell activation more easily in the active BP mouse model than in the skin-grafted BP model, because in the former model, it starts soon after the adoptive transfer of spleen cells. By utilizing this advantage, we previously demonstrated that the activation of anti-human COL17 NC16A IgG-producing B cells via CD40–CD40L interaction is completed within 5 days after the transfer of spleen cells (41). The study also showed that antibodies to the DEJ were restored long after the single administration of antibodies to CD40L at day 0 and that the restored antibodies do not react to the NC16A domain. The results in **Figure 4** suggest that the restored antibodies are mainly specific for ICD epitopes of COL17 and that the interaction between T and B cells regarding ICD epitopes may arise slightly bit later than that to the NC16A domain after the adoptive transfer of spleen cells. We previously reported that the blockade of CD40L at

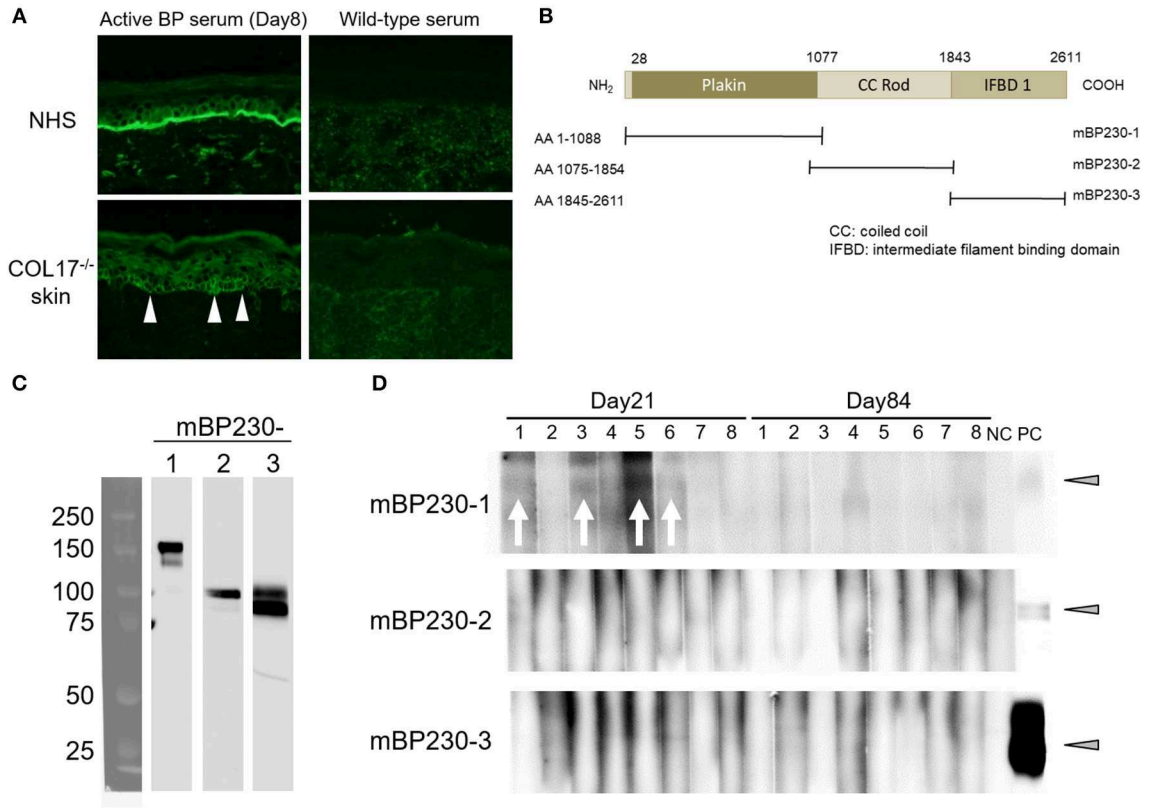

FIGURE 6 | Antibodies to murine BP230 are detected in a portion of the active BP model mice. **(A)** Representative images of IgG deposition in indirect IF study using normal human skin (NHS) and murine COL17$^{-/-}$ mouse skin as substrates and 1:20 diluted sera from an active BP model at day 35 and from wild-type mice as primary antibodies. Arrowheads indicate IgG deposition around the basal keratinocytes. **(B)** Schematic of murine BP230 and recombinant proteins. NH_2, N-terminus; COOH, C-terminus; AA, amino acid; mBP230, murine BP230. **(C)** The sizes of recombinant proteins were confirmed by immunoblotting using anti-His-tag antibody. **(D)** Reactivity of sera from the active BP model ($n = 8$, days 21 and 84) to fragments of murine BP230 was measured by immunoblotting. NC, negative control (wild-type serum); PC, positive control (anti-His-tag antibody). Arrowheads indicate a positive level. Arrows indicate a positive band.

day 13, 16, and 19 after the adoptive transfer did not suppress antibodies to the NC16A domain (examined by ELISA), nor did it suppress antibodies to the DEJ (examined by indirect IF) (41). This strongly suggests that the delayed blockade of CD40-CD40L interaction does not suppress the production of antibodies to ICD epitopes and that the CD40-CD40L interaction between T and B cells regarding ICD epitopes was completed within 13 days after the adoptive transfer. The reason for the discrepancy between the results for NC16A-R7 and those for other ECDs in **Figure 4** is unclear. As mentioned above, the reactivity of antibodies to NC16A-R7 is far stronger than those to ECD-917 and ECD-1330 in the untreated active BP model. This would be due to the high antigenicity of the NC16A domain of COL17 as reported previously (36, 46). It is commonly known that CD40L is transiently expressed on the surface of activated CD4$^+$ T cells (47). Given the above, we consider that a treatment of CD40L-blocking antibody preferentially affects the highly activated T cells reacting to the NC16A domain rather than those reacting to the other ECD epitopes. Further investigations are required to confirm this.

This study also showed intermolecular epitope spreading from COL17 to BP230 in an active BP model. As mentioned above, intermolecular epitope spreading commonly occurs among different antigens of a single molecular complex or among antigens that colocalize to the same site (30). Both COL17 and BP230 are hemidesmosomal proteins, and they directly interact with each other at their N-termini (48). Di Zenzo et al. examined IgG reactivity to COL17 and BP230 during the course of the disease in 35 BP cases and detected epitope spreading in 17 cases (49%). Notably, 3 of those 17 cases showed intermolecular epitope spreading from COL17 to BP230, but the reactivity to BP230 never preceded that to COL17 (34). We demonstrated the reactivity of active BP mouse sera to murine BP230, but it was very weak, as shown in **Figure 6D**. This weak reactivity may be due to the strong self-tolerance in the wild-type mouse, a source of transferred spleen cells in the active BP model, because murine BP230 is originally expressed in wild-type mice. This hypothesis is supported by our recent finding that regulatory T cells, a main player in peripheral tolerance, play a crucial role in maintaining self-tolerance to murine BP230 in mice (37).

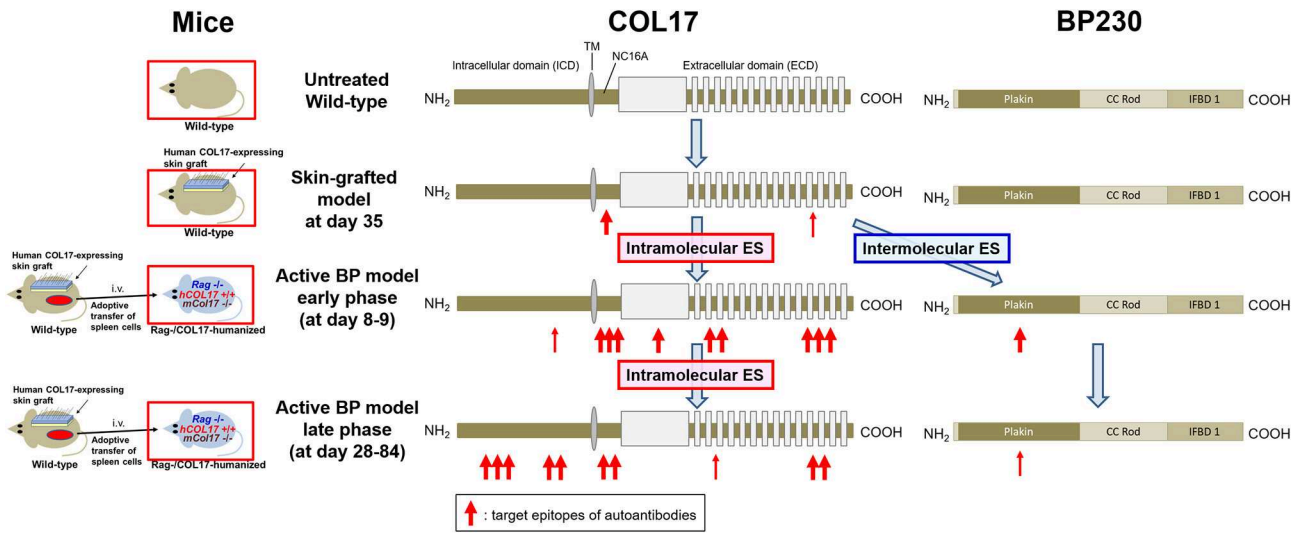

FIGURE 7 | Epitope spreading in the skin-grafted mouse model and in the active BP model. A schematic of COL17 and BP230. In the skin-grafted mouse model, antibodies mainly react to the NC16A domain and weakly react to ECD epitopes of COL17. After the adoptive transfer of immunized spleen cells, intramolecular epitope spreading to ECD epitopes and to the outer portion of the ICD as well as intermolecular epitope spreading to BP230 occur, followed by intramolecular epitope spreading to the inner portion of the ICD. ES: epitope spreading.

There are several limitations to this study. First, the synthesized peptides that were used for the ELISA account for only a small portion of human COL17. The titers of antibodies to ICD-1 (recombinant protein of 204 amino acids) were higher at day 56 than at day 8, as shown in **Figure 2C**, whereas the titers of antibodies to ICD-149 (synthesized peptides of 24 amino acids) were higher at day 8 than at day 56, as shown in **Figure 3D**. These results suggest that active BP mouse sera contain antibodies to ICD-1 epitopes (Met^{1} to Ser^{204}) other than ICD-149 (Ala^{149} to Ser^{172}). Second, a correlation between disease severity and epitope spreading remains unclear. As mentioned above, previous studies demonstrated that the disease severities of BP are associated with the appearance of antibodies to ICD epitopes in both mouse models and human patients (33, 34). Meanwhile the appearance of antibodies to BP230-1 or BP230 was not related to the disease severity of the active BP model in this study, which is probably due to the low titers of those antibodies. Third, we have not examined intramolecular epitope spreading that starts from ICD or ECD epitopes other than the NC16A domain. We recently reported 3 cases of dipeptidyl peptidase-4 inhibitor-associated BP in which autoantibodies to the NC16A domain were initially undetectable but became positive during the course of BP (49). To investigate this phenomenon, we immunized wild-type mice with recombinant proteins of ICD or ECD shown in **Figure 2A** and adoptively transferred the spleen cells to $Rag^{-/-}$/COL17-humanized mice. However, the titers of antibodies to COL17 were very low in all the immunized mice and the recipient mice (data not shown). This is probably due to the lower antigenicity of those proteins compared to that of the NC16A domain. Fourth, we have not examined intermolecular epitope spreading to hemidesmosomal proteins such as plectin, integrin α6, integrin β4, or laminin 332, which were reported to interact directly with COL17 (50–54), although those molecules become targets of autoantibodies less frequently than COL17 and BP230 do. We should also confirm whether the interaction between human COL17 and murine hemidesmosomal proteins in COL17-humanized mice is normal. To overcome these limitations, further investigations are necessary.

In conclusion, the immune reaction to human COL17 that starts from ECD epitopes, especially those of the NC16A domain, was found to spread over time to ICD epitopes and to murine BP230 in an experimental BP model. The timing of the interaction between COL17-reactive T and B cells differs *in vivo*, and it is dependent on the target epitope. These novel findings elucidate certain details of epitope spreading in BP and give us a hint for new therapeutic strategies for BP that involve the regulation of this phenomenon.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the local ethics committee and the Institutional Review Board of Hokkaido University, with written informed consent obtained from all subjects in accordance with the Declaration of Helsinki. All animal procedures were conducted according to guidelines provided by the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.

AUTHOR CONTRIBUTIONS

HU and NY performed the experiments. KN, KM, HI, and WN provided materials. HU and HS designed the experiments. HU wrote the manuscript, and all the coauthors reviewed the manuscript and gave final approval of the submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01410/full#supplementary-material

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Identification of a Novel Non-desmoglein Autoantigen in Pemphigus Vulgaris

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Pemphigus vulgaris (PV) is an autoimmune bullous disease of the skin and mucous membranes characterized by the presence of circulating and tissue-bound autoantibodies against keratinocyte cell surface antigens, specifically desmoglein (Dsg) 1 and 3. The pathogenic role of anti-Dsg antibodies is well-established, while the mechanism of blister formation is only partly defined. We have applied a previously developed method for the efficient immortalization of IgG+ memory B cells to identify novel target antigens in PV. A human monoclonal antibody reactive with a hitherto unreported non-Dsg antigen was isolated. Immunoprecipitation and immunoblotting studies with keratinocyte extracts indicated α-catenin as the putative antigen, then confirmed by immunoblotting on the recombinant protein. Four of ten PV sera reacted with recombinant α-catenin. Although the isolated human monoclonal antibody was *per se* unable to dissociate keratinocyte monolayers and also to synergize with a pathogenic antibody *in vitro,* further studies are warranted to assess its possible *in vivo* contribution in the multifactorial pathogenesis and heterogeneous manifestations of PV disease.

Keywords: pemphigus vulgaris, non-desmoglein autoantigens, autoantibodies, memory B cells, α-catenin, skin, mucous membranes

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INTRODUCTION

Pemphigus vulgaris (PV) is a rare but highly disabling and, if untreated, almost always fatal immunobullous disease of the skin and mucous membranes. PV is characterized histologically by loss of cell-cell adhesion between suprabasal keratinocytes, leading to acantholysis, and immunopathologically by the presence of circulating and tissue-bound autoantibodies (autoAbs) against keratinocyte cell surface antigens, specifically desmoglein (Dsg) 1 and 3. PV is considered as a paradigmatic organ-specific autoimmune disease in view of (i) present knowledge of disease autoantigens and pathogenesis and (ii) reproducibility of major clinical and pathogenic features in animal models (1). The existence of both pathogenic and non-pathogenic anti-Dsg autoAbs has recently been underscored by isolation of human monoclonal antibodies (hMabs) from pemphigus patients. Anti-Dsg hMabs characterization has shown that their pathogenic potential primarily depends on the targeted epitopes (1). We have been interested in characterizing the repertoire of naturally occurring autoreactive epithelium-specific memory B cells in pemphigus vulgaris patients. In a first work, we focused on autoantibodies targeting Dsg3 (2). However, (i) the lack of tight correlation between anti-Dsg autoAb titers and disease activity in some patients and (ii) the significant degree of disease heterogeneity point at the importance

of non-Dsg autoAbs, that have been progressively, even though not exhaustively, described (3, 4). In fact, besides Dsg3 and Dsg1, other non-desmoglein autoAbs, either pathogenic or non-pathogenic, have been identified in pemphigus patients. AutoAbs endowed with an acantholytic potential target desmocollin 3, α-acetylcholine receptor, pemphaxin, and keratinocyte mitochondria (5–8). On the other hand, the pathogenic role of autoAbs recognizing other autoantigens, such as ATP2C1, desmocollin 1, BP230, periplakin, E-cadherin, desmoglein 4, desmoplakin 1, and desmoplakin 2, remains to be demonstrated (9). In line with this interest, our current work aimed to identify autoAbs targeting non-Dsg membrane-bound or membrane-associated intracellular antigens.

In the present study, we report on the characterization of a hMab isolated from a PV patient and directed to a novel non-Dsg antigen. The hMab reacts with α-catenin that is recognized by almost half of PV sera analyzed.

MATERIALS AND METHODS

Patients, Sera and Isolation of hMabs From a PV Patient

Peripheral blood was obtained from 2 patients (PVC and PVF) suffering from active mucocutaneous PV. The patients showed typical clinical, histological, and immunopathological features and had high-titer anti-Dsg circulating autoantibodies (PVC: Dsg3, 127 U/ml, Dsg1, 90 U/ml; PVF: Dsg3, 191 U/ml, Dsg1, 170 U/ml), as assessed by ELISA kits based on ectodomain of Dsg1 and Dsg3 (MBL, Nagoya, Japan). hMabs were isolated by a highly-efficient protocol for the immortalization of IgG+ memory B cell with Epstein Barr virus (EBV) in the presence of a Toll-like receptor agonist, as previously described (2). In detail, IgG+ memory B cells were isolated from cryopreserved peripheral blood mononuclear cells using anti-CD22 microbeads (Miltenyi Biotec, Bo, Italy) followed by depletion of cells carrying IgM, IgD, and IgA by cell sorting. Multiple replicate microcultures of 10–30 IgG+ memory B cells/well (for a total of 2 to 8×10^4 purified cells) were infected with EBV and CpG as previously described (10). Culture supernatants were tested for binding to Dsg1- and Dsg3-coated ELISA plates and for binding to HaCaT keratinocyte cell line monolayers (both on live cells and on fixed and permeabilized cells) by immunofluorescence (IF) assay using an automated fluorescence microscope (Pathway 855, BD). The specificity of positive polyclonal cultures was further assessed by IF on primary human keratinocytes. Positive reactivities were confirmed by the propagation of oligoclonal cultures. Positive cultures were cloned by limiting dilution and expanded; antibodies were purified using protein G columns. Serum samples were collected from 10 PV and 16 bullous pemphigoid (BP) patients and 10 healthy donors. This study was carried out in conformity with the Helsinki guidelines and with approval of the IDI-IRCCS Ethics Committee. All the biological samples were obtained after patient's informed consent.

Immunofluorescence Analyses

IF studies were performed according to the procedure described in Di Zenzo et al. with minor modifications (2). Briefly, supernatants from immortalized human memory B cells were screened on monolayers of live and fixed/permeabilized HaCaT cells. After washing with phosphate-buffered saline, cells were stained with Alexa Fluor 488–conjugated goat anti-human IgG (Invitrogen, Carlsbad, CA, USA). The isolated monoclonal antibodies were further tested on permeabilized HaCaT cells and on primary keratinocytes. Non-keratinocyte cell lines, i.e., MRC9, Hela, and SKMEL cells, were used as controls for IF analyses on cell monolayers. Human antibodies of irrelevant specificity were used as negative controls. Serial images of stained keratinocyte monolayers were acquired by the BD Pathway 855 automated fluorescence microscope. Staining was also performed on cryo sections of normal human skin, guinea pig and monkey esophagus and revealed with fluorescein isothiocyanate-conjugated anti-human IgG antibody (Agilent DAKO, Santa Clara, CA, USA).

Immunoprecipitation and Immunoblotting Analyses

Immunoprecipitation (IP) of ^{35}S-labeled keratinocyte extracts by hMab PVF144 was carried out as previously described (11). The precipitated proteins, separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 6% gels under reducing conditions, were detected by autoradiography. Immunoblotting (IB) experiments on keratinocyte extracts were performed as previously reported using both horseradish peroxidase (HRP)-conjugated and alkaline phosphatase-conjugated secondary antibodies (12). Bands were quantified using ImageJ Software (National Institutes of Health, Bethesda, MD, USA) and their intensities were normalized respect to the positive control signal obtained by anti-α-catenin antibody. The cut-off value was set as the medium value +2 standard deviations obtained measuring signals obtained with healthy donors. Commercial primary antibodies were purchased from Progen Biotechnik GmbH (Heidelberg, Germany) (anti-desmocollin 2), BD Biosciences (San Jose, CA, USA; anti-α-catenin), and Santa Cruz Biotechnology, Inc (Dallas, TX, USA; anti-γ-catenin, anti-β-catenin, anti-p120, and anti-E-cadherin). Recombinant tagged human α1-catenin was purchased from Abcam (Cambridge, UK). To rule out that PV sera were mainly reacting against the fused glutathione S-transferase (GST) moiety of the α-catenin recombinant protein, IB experiments by using commercial tagged protein and equimolar GST were performed (data not shown).

Enzyme Linked ImmunoSorbent Assay (ELISA)

Briefly, recombinant Dsg1 and Dsg3 ectodomains were produced in baculovirus and used for coating of ELISA plates. The plates were, then, blocked with 1% bovine serum albumin and incubated with antibodies followed by HRP-conjugated anti-human IgG (Jackson ImmunoResearch, Baltimore, PA, USA) (2).

Keratinocyte Dissociation Assay

The assay was performed as previously reported (13). Briefly, primary human keratinocyte cells were seeded onto 12-well plates and 24 h post-confluence treated with monoclonal antibodies.

After adding exfoliative toxin A to cleave Dsg1 protein, the cell monolayers were detached with dispase I (Merck, KGaA, Darmstadt, Germany) and subjected to mechanical stress by pipetting. The monolayer fragments, fixed by adding a 3% formaldehyde solution, were subsequently stained using crystal violet. To investigate a possible synergistic effect PVF144 was applied to the monolayer together with a pathogenic antibody (PVB28) (2) at optimal (4 μg/ml) and suboptimal concentrations (1 μg/ml and 0.25 μg/ml; **Figure 4**) and a non-pathogenic antibody (2, PVB28 in a germline version that is not able to dissociate the keratinocyte monolayer; data not shown).

RESULTS

Isolation of hMabs Specific for Non-Dsg Epithelial Antigens in Pemphigus Patients

In order to identify hMabs targeting non-Dsg membrane-bound or membrane-associated intracellular antigens, we took advantage of the same strategy by which we had previously isolated and finely characterized several Dsg-reactive PV patient-derived monoclonal autoantibodies (2). In detail, peripheral blood samples were collected from 2 patients with mucocutaneous PV: one with long-lasting steroid-resistant disease (PVC) and the other prior to treatment initiation (PVF). IgG+ memory B cells were isolated by magnetic and fluorescence-activated cell sorting, seeded in 96-well microplates, and immortalized with EBV in the presence of irradiated mononuclear cells and oligodeoxynucleotides containing CpG motifs, as previously described (10). The reactivity of antibodies secreted in the supernatants of growing polyclonal cultures was screened by IF staining both on live and on fixed and permeabilized cells from the human keratinocyte HaCaT cell line. The polyclonal antibodies produced by the vast majority of isolated cultures bound to surface antigens on the keratinocyte membrane and were reactive with Dsg1 and/or Dsg3 ectodomains by ELISA, as previously reported (2, and data not shown). Besides such prevalent pattern of reactivity, supernatants of rare cultures from both patients showed a distinctive membrane-associated fishnet reactivity, detected only on permeabilized keratinocytes, and were negative in ELISA on Dsg1 and Dsg3 ectodomains. The human Mabs PVF144 (IgG1-isotype), PVC6 (IgG1-isotype), and PVC33 (isotype IgG3-isotype) were cloned by limiting dilution and showed the same IF pattern as the original polyclonal cultures also on permeabilized HaCaT keratinocytes (**Figure 1A**), suggesting their specificity for a membrane-associated intracellular (i.e., non-exposed) autoantigen. In addition, they showed an intercellular staining pattern by IF on human skin, guinea pig, and monkey esophagus (**Figures 1B–D**), very similar to IF staining pattern of anti-Dsg3 antibodies (2, and data not shown). Supernatants from the selected clones were tested on non-keratinocyte cell lines, i.e., MRC-9 (fibroblast), SKMEL (melanoma), and Hela (epithelial) cells: they failed to stain both live and permeabilized cells, apart from a reactivity on permeabilized Hela cells (data not shown), hinting at a specificity for membrane-associated intracellular antigens expressed on keratinocytes and other epithelial cells.

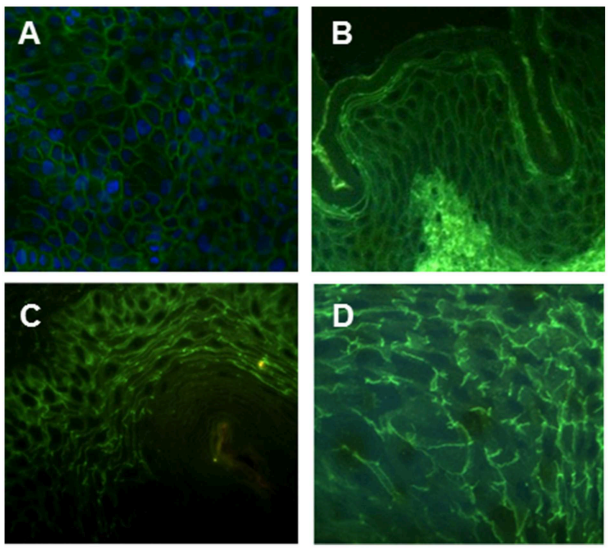

FIGURE 1 | Intercellular staining pattern of hMab PVF144. PVF144 binds a membrane associated epithelial antigen showing a typical intercellular staining pattern on permeabilized HaCaT keratinocytes (nuclear counterstain is obtained with DAPI) (20X) **(A)**, human skin (40X) **(B)** guinea pig (40X) **(C),** and monkey esophagus (40X) **(D)**.

Identification of the Intracellular Epithelial Target Antigen of hMab PVF144

IP studies with the 3 selected clones on radiolabelled keratinocyte extracts to identify the target antigen showed reactivity to a 100 kDa antigen for PVF144 (**Figure 2A**) and to 190–210 kDa antigens for PVC6 and PVC33, respectively (data not shown). As the IP results from the latter two clones pointed at members of the plakin-family as candidate target antigens and autoreactivity of PV sera to plakin family members has been already documented (14, 15), we chose to further characterize PVF144 with the aim to identify a possible novel membrane-associated PV autoantigen. Subsequent IB experiments using commercial antibodies reacting with keratinocyte membrane-associated proteins with a molecular weight of ∼100 kDa (desmocollin 2–115 kDa; α-catenin-102 kDa; E-cadherin-120 kDa; β-catenin-92 kDa; γ-catenin-83 kDa; P120-catenin 80–100 kDa) indicated α-catenin as the putative antigen (**Figure 2B**). Sequential IP and IB experiments further supported α-catenin as the target antigen of PVF144. Specifically, a protein immunoprecipitated from keratinocyte extracts with PVF144 was recognized by a commercial anti-α-catenin antibody, and an anti-α-catenin antibody was in turn able to immunoprecipitate a protein recognized by PVF144 in IB (**Figure 2C**). Finally, IB experiments with the recombinant tagged protein unequivocally confirmed the specific binding of PVF144 to α-catenin (**Figure 2D**).

α-Catenin Is Recognized by PV Patient Sera

To determine whether autoAbs of the same specificity as PVF144 were present in the sera of PV patients, we performed IB experiments with 10 PV sera on recombinant tagged α-catenin

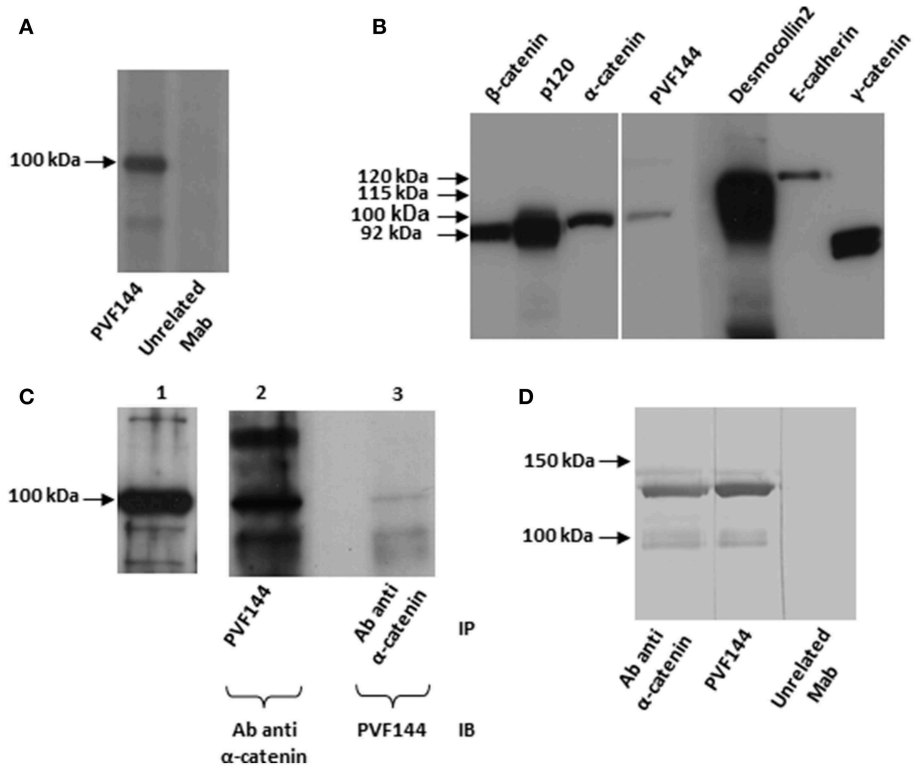

FIGURE 2 | PVF144 binds a membrane-associated epithelial antigen: α-catenin. PVF144 immunoprecipitates (IP) an unknown antigen of 100 kDa from radiolabeled normal human keratinocyte extracts **(A)**. Immunoblotting (IB) experiments on keratinocyte extracts by using PVF144 and commercial antibodies suggest that α-catenin could be the putative antigen of 100 kDa: PVF144 and a monoclonal murine anti-α-catenin antibody (Ab) react with a keratinocyte antigen of similar molecular weight (100 kDa) **(B)**. Anti-α-catenin commercial antibody reacts to α-catenin from keratinocyte extracts by IB (lane 1); PVF144 immunoprecipitates α-catenin recognized by the commercial anti-α-catenin antibody by IB (lane 2) and, viceversa, anti-α-catenin antibody immunoprecipitates α-catenin recognized by PVF144 by IB (lane 3). The faint reactivity observed in lane 3 could be related to the epitope recognized **(C)**. IB performed with a recombinant GST-tagged human α-catenin (120 kDa) confirms that the target of PVF144 is α-catenin. The lower bands are likely degradation products **(D)**.

(**Figure 3**). Four out of 10 PV patients (40%) showed the same reactivity of PVF144 to α-catenin, whereas the other PV sera and 10 normal human control sera showed only a faint signal, indicating that this autoreactivity was well-represented in PV patients, even though not shared by all patients. In order to evaluate whether this reactivity is disease-specific, 16 BP patient sera were analyzed on recombinant α-catenin by IB. Only one of 16 BP (7 representative BP sera shown and other 9 not shown) reacted to α-catenin (**Supplementary Figure 1**) underlining the specificity of this autoantigen. The weak signals found in the other remaining PV, PB, and healthy donor sera might be ascribed to reactivity to the tag protein (GST).

PVF144 Is Not Able to Dissociate a Keratinocyte Monolayer and to Synergize With a Human Pathogenic Antibody

The presence of anti-α-catenin autoAbs in several PV sera raised the question as to their pathogenic potential. Previous studies showed the ability of intact autoAbs to enter the cytosol or nucleus of living cells (16, 17). More recently, Marchenko et al. reported that PV autoAbs could penetrate keratinocytes and react with intracellular mitochondrial proteins (8). These findings suggested that a hMab to an intracellular antigen could exert its pathogenic ability on live keratinocytes. Thus, an *in vitro* dissociation assay was used to evaluate the pathogenic activity of the hMab PVF144. This approach allows to measure the ability of a specific antibody or a mixture of antibodies, such as a serum, to fragment a monolayer of primary human keratinocytes seeded to confluence. The keratinocyte monolayers, incubated for 24 h with PVF144, remained intact, similarly to monolayers incubated with an unrelated hMab used as negative control (**Figure 4**). As expected, human (PVB28) and murine (AK23) pathogenic anti-Dsg3 antibodies were able to dissociate the monolayers (2, 18) (**Figure 4**). In addition, PVF144 failed to synergize with a pathogenic antibody (**Figure 4**). These findings exclude a primary pathogenic function of anti-α-catenin autoAbs in PV, nevertheless a potential secondary role in the immunobiology of the disease cannot be excluded and warrants future studies.

DISCUSSION

In the present work we went forward in the characterization of the diverse targets of epithelium-specific autoreactive B cells from PV patients. To this purpose, we took advantage of the

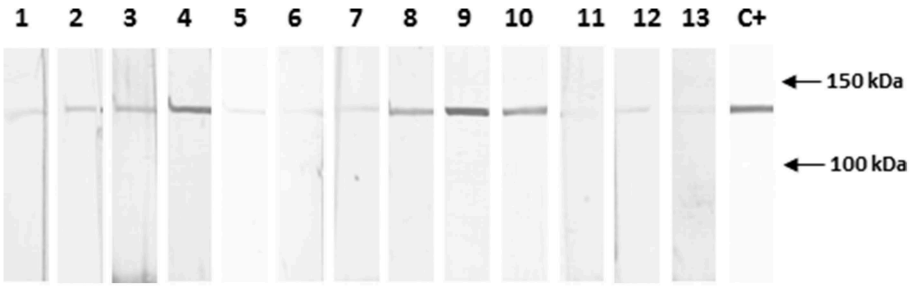

FIGURE 3 | Almost half of PV sera specifically react with recombinant α-catenin. Immunoblotting with sera obtained from 10 pemphigus vulgaris (PV) patients shows that 4 of 10 sera (4, 8, 9, 10) react with the novel epithelial antigen, while 3 healthy donor sera (11, 12, 13), representative of 10 sera analyzed, show only a background signal. The positive control (C+) is the commercial anti-α-catenin antibody. The background signal, could be also due to reactivity to the tag protein (GST). Quantification and normalization of bands using ImageJ analysis (data not shown) have confirmed the reported results (see Materials and Methods section).

high-efficiency immortalization protocol of IgG+ memory B cells we had previously developed (10) and applied to isolate and molecularly characterize a number of anti-Dsg3 hMabs (2). Our present focus was to detect plasma-membrane-associated antigens, either expressed on the cell surface or intracellularly, therefore we chose to screen the isolated polyclonal cultures on both live and permeabilized keratinocytes. Our choice was based on the hypothesis that antigens associated to the cell membrane, even those with intracellular localization, could have a higher chance to contribute to pemphigus pathogenesis, which is due to the loss of cell-cell adhesion.

As expected, the vast majority of cultures resulted specific for Dsg1 and/or Dsg3, further confirming the major role of these antigens in pemphigus pathogenesis. Among polyclonal cultures that reacted only with permeabilized keratinocytes, we selected and cloned those giving a membrane-associated fishnet-like staining pattern on stratified epithelia. Among the cloned cultures, we further characterized clone PVF144 and demonstrated by IF, IP and IB studies that α-catenin is its target. α-catenin is a component of adherens junctions (AJs), i.e., cell-cell anchoring structures that, together with desmosomes, allow keratinocytes to adhere to one another and maintain epithelial integrity. α-catenin binds to β-catenin in AJs and is required for their formation and maintenance (19, 20). In addition, α-catenin was reported to be necessary for the organization of desmosomes in epithelial cells (21).

A previous study reported a reactivity of pemphigus sera to another AJ component, i.e., E-cadherin (22). Likewise, we showed that anti-α-catenin autoreactivity was: (i) well-represented in PV patient sera, as α-catenin was recognized by almost half of the PV sera tested; (ii) more frequently associated with pemphigus than with other autoimmune bullous diseases, as α-catenin was very rarely recognized by the BP sera analyzed.

Then, considering that previous studies demonstrated the ability of intact autoAbs to enter living cells (8, 16, 17), we addressed the potential pathogenicity of α-catenin-specific Mab PVF144 by evaluating its acantholytic activity in an *in vitro* keratinocyte dissociation assay. In this regard, several observations suggest that AJs, and in particular E-cadherin, may be involved in pemphigus pathogenesis (23, 24). Of note, with the exception of anti-desmocollin 3 autoAbs, all known non-Dsg-reactive autoAbs with reported pathogenicity do not possess acantholytic potential on their own but may act synergistically with anti-Dsg antibodies (1, 25, 26). Accordingly, Marchenko et al. described a pathogenic role for intracellular anti-mitochondrial autoAbs, even though not on their own (8). In our hands, the anti-α-catenin Mab PVF144 was not able to dissociate the keratinocyte monolayer either alone or in combination with a suboptimal dose of a pathogenic anti-Dsg3 antibody. Nevertheless, we cannot exclude that regions of α-catenin different from that recognized by PVF144 could be recognized by PV sera and contribute to acantholysis. In addition, a possible role of anti-α-catenin hMabs as co-factors in disease initiation or maintenance could not be exhaustively addressed by our approach. In previous studies, several antibodies against intracellular antigens have been considered as triggers for autoimmunity. Mabs specific for the cytoplasmic precursor form of Dsg1 (preDsg1) have been cloned from pemphigus patients and from healthy individuals (25, 26). Yamagami et al. postulated that the presence of anti-preDsg1 B cells is involved in the initiation of the autoimmune response in pemphigus patients. In particular, in the context of tissue damage they could present peptides which are part of mature Dsg1 (i.e., the extracellular autoantigen recognized by most pathogenetic autoAbs) derived from the processing of preDsg1. This intramolecular epitope spreading phenomenon could lead to the production of pathogenic autoAbs targeting mature Dsg1 and to the initiation of disease pathogenesis (27, 28). Moreover, natural autoAbs (NAAs), i.e., antibodies to intracellular autoantigens that naturally occur in the healthy population and are *per se* unable to cause immune phatology, have been theorized as cofactors in the onset of autoimmunity, possibly by participating in the mechanisms of chronic tissue injury at the basis of intermolecular epitope spreading (29–31).

In conclusion, while our previous study (2) demonstrated that pathogenic PV antibodies primarily target the Dsg-3 cis-interface, thus leading to desmosome disruption, our current results reveal novel antibodies targeting intracellular keratinocyte proteins. We are tempted to speculate that the cellular damage

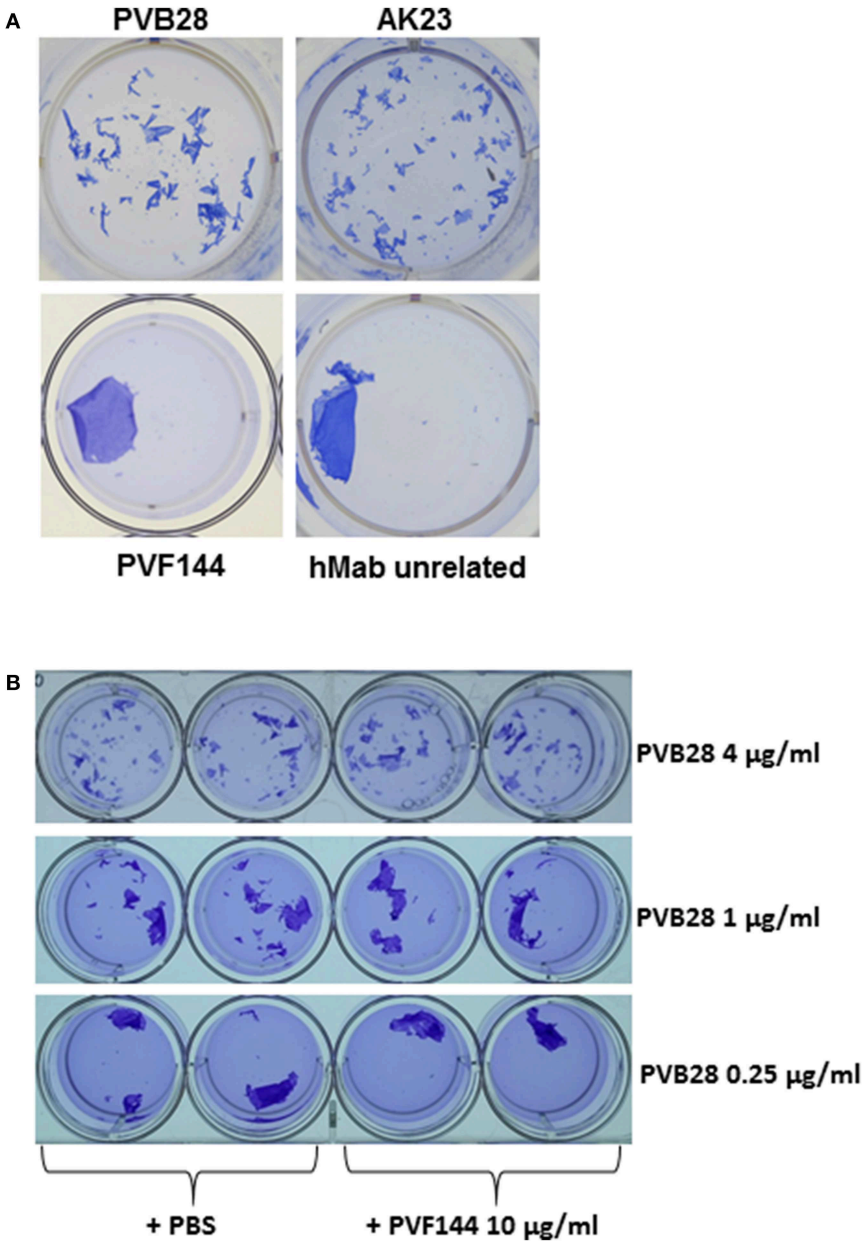

FIGURE 4 | PVF144 is not able to dissociate a keratinocyte monolayer even in the presence of suboptimal concentrations of PVB28. A representative keratinocyte dissociation experiment. Primary human keratinocytes, seeded to confluence, were incubated with PVF144, an unrelated human Mab (negative control) and, as positive controls, the human pathogenic Mab PVB28 (7) and the murine pathogenic Mab AK23 (14), both Dsg3-specific **(A)**. To investigate synergistic potential of cloned antibody we have employed PVF144 (10 µg/ml) together with optimal (4 µg/ml) and suboptimal concentrations (1 µg/ml and 0.25 µg/ml) of the pathogenic anti-Dsg3 antibody PVB28 without obtaining any difference in ability of PVB28, with or without PVF144, to dissociate the keratinocyte monolayer **(B)**.

induced by pathogenic anti-Dsg antibodies may trigger an intermolecular epitope-spreading phenomenon resulting in an antibody response against intracellular antigens, among which α-catenin. Further studies are needed: (i) to evaluate whether PVF144 may act synergistically with anti-Dsg antibodies using more informative approaches, such as *in vitro* organ culture or *in vivo* models; (ii) to assess the possible role of anti-α-catenin autoAbs in pemphigus initiation and evolution *in vivo*; and (iii) to characterize this novel antigen as a possible target of epitope spreading phenomena in PV.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Helsinki guidelines, IDI-IRCCS Ethics Committee. The protocol was approved by the IDI-IRCCS Ethics Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

GDZ and GDL have written the manuscript. GDZ, GZ, and AL have designed the study. GDZ, GDL, VC, and FM performed the experiments. All the authors have revised the manuscript and given the final approval for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01391/full#supplementary-material

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CASE REPORT
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Thymoma-Associated Paraneoplastic Autoimmune Multiorgan Syndrome—From Pemphigus to Lichenoid Dermatitis

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Introduction: Paraneoplastic autoimmune multi-organ syndrome (PAMS) is a rare clinical condition characterized by variable and heterogeneous clinical phenotypes in the presence of neoplasias which largely depend on the activation of humoral and cellular immune responses. Clinically, these patients present with a spectrum of antibody-driven pemphigus-like lesions to graft-vs.-host-disease-like exanthemas with a lichenoid inflammatory infiltrate in the skin. PAMS is occasionally associated with thymoma, in which altered immune surveillance eventually leads to multiorgan autoimmunity which often includes variable cutaneous symptoms. This disorder is associated with a profound disturbance of peripheral immune tolerance against human autoantigens.

Objectives: We here present a patient with relapsing thymoma who developed PAMS with several cutaneous and extracutaneous autoimmune disorders.

Materials: Peripheral blood mononuclear cells (PBMC), sera, and lesional skin biopsies were obtained at different clinical disease stages. Peripheral T cell subsets were characterized phenotypically and the cytokine profile of the peripheral blood T cellular response against distinct epidermal and dermal autoantigens of the skin was analyzed by ELISpot assay. Serological screening was performed by ELISA and immunoblot analysis. Skin biopsies were subjected to immunohistochemical analysis of distinct T cell subsets. Thymoma tissue was analyzed for the presence of T regulatory cells and compared with adult thymus and indolent thymoma.

Results and Conclusions: In the present case, thymoma was the cause of the observed multi-organ autoimmune syndromes as its recurrence and surgical removal was associated with the relapse and regression of the cutaneous symptoms, respectively. Initially, the patient presented with two autoimmune disorders with Th2/Th1 imbalance, myasthenia gravis (MG) and pemphigus foliaceus (PF), which regressed upon immunosuppressive treatment. Months later, the patient developed a lichenoid exanthema with a Th1-dominated skin infiltrate. Further clinical evaluation revealed the recurrence of the thymoma and the lichenoid exanthema gradually regressed

upon thymectomy. Our contention that T cell recognition against distinct cutaneous autoantigens, such as desmoglein 1 (Dsg1), shifted from a Th2 to a Th1-dominated immune response could not be fully substantiated as the patient was on a stringent immunosuppressive treatment regimen. We could only observe a decrease of the initially present serum IgG autoantibodies against Dsg1. Phenotypic analysis of the associated thymoma showed a lower number of T regulatory cells compared to adult thymus and indolent thymoma, suggesting that impaired thymus-derived immune surveillance had a direct impact on the outcome of the observed cutaneous autoimmune disorders.

Keywords: *Pemphigus foliaceous*, autoimmunity, thymoma auto-immunity, PAMS, myasthenia (myasthenia gravis—MG), GVHD-like disease

BACKGROUND

PAMS is an extremely rare clinical syndrome which arises in patients with lymphoproliferative or solid tumors, like thymomas (1, 2). It is increasingly debated whether PAMS and paraneoplastic pemphigus (PNP) should be considered as one entity, since, in both cases, production of IgG autoantibodies against desmosomal adhesions molecules, such as Dsg1 and/or Dsg3, are related to an altered immune surveillance induced by the underlying neoplasia (3–5). In contrast to pemphigus vulgaris (PV) or PF, two well-characterized autoimmune bullous disorders of the skin associated with IgG autoantibodies against Dsg3 and Dsg1, respectively, PAMS is characterized by a wide clinical heterogeneity (6), ranging from classic pemphigus-like muco-cutaneous lesions with erosions, and blisters to lichenoid, graft vs. host disease (GVHD)-like, bullous pemphigoid (BP)-like, and erythema multiforme like-skin lesions (1). As in PNP, mortality of PAMS is high and largely dependent on the underlying malignancy or opportunistic infections (6, 7). As previously reported, PAMS may be associated with thymoma, a neoplasm which arises from epithelial cells of the thymus and accounts for ∼50% of all mediastinal tumors (8–15).

Thymomas are often asymptomatic and are sometimes detected by chance by routine radiographic examinations (16). Nonetheless, given the central role of the thymus in adaptive immune regulation, ensuring T-cell tolerance against self-antigens and preventing the maturation of self-reactive T-cells, it is not surprising that thymomas can frequently lead to autoimmune syndromes (7, 17). Approximately 50% of the patients with thymomas experience associated autoimmune diseases, including MG, pure red cell aplasia, systemic lupus erythematosus, and Goodpasture's syndrome (17, 18). Cutaneous disorders associated with thymomas are widely heterogeneous and include pemphigus, BP, lichen planus (LP), vitiligo, alopecia areata, and lupus erythematosus (17).

CASE PRESENTATION

A 51 year old Caucasian woman presented with a diffuse skin rash associated with abdominal pain and diarrhea with a 2 weeks duration. Her past medical history was remarkable for a B2-type thymoma which was diagnosed about 7 years earlier. The patient underwent complete surgical resection of the tumor and then received adjuvant radiotherapy (50.4 Gy). Few months after surgical removal of the thymoma, the patient developed diffuse muscle weakness and was diagnosed with MG. She was started on azathioprine and pyridostigmine resulting in a good clinical control of her symptoms. Physical examination revealed erythematous plaques with shallow erosions and overwhelming yellow-to-brown crusts, involving the trunk, mainly back, upper and, to lesser extent, lower limbs, dorsal aspect of hands, face, and scalp (**Figure 1A**). There was no mucosal involvement. The results of the routine laboratory investigations were unremarkable except for elevated serum concentrations of the transaminases, GOT and GPT.

Initially, after surgical removal of the thymoma, direct immunofluorescence (DIF) from perilesional biopsy of the scaly erythematous skin rash revealed deposits of both IgG and C3 on the surface of epidermal keratinocytes (**Figures 2A,C**). Another DIF which was taken later at the time of GVHD-like dermatitis revealed instead linear deposits of IgG and C3 along the dermal-epidermal basement membrane zone (BMZ) (**Figures 2B,C**). Indirect immunofluorescence (IIF) on normal human skin, 1M NaCl-split human skin and monkey esophagus showed neither IgG autoantibodies to either the surface of epithelial cells nor on the dermal-epidermal BMZ (not shown). In addition, IIF on rat bladder was negative on transitional epithelia (not shown). Of note, the patient had IgG autoantibodies against Dsg1 (719 relative units (RU)/ml, cutoff < 20 RE/ml) and desmocollin 1 (Dsc1) (0.448 OD, cutoff < 0.200 OD) by ELISA and IgG autoantibodies against laminin 332 by immunoblot analysis (**Table 1**).

Based on clinical, histologic, and immunologic findings, the diagnosis of PF with additional anti-BMZ IgG reactivity was established. As there was neither evidence for IgG antibodies against desmosomal plaque proteins nor the 170 kDa alpha2-macroglobulin-like protein-1, the diagnosis of PNP was abandoned.

The patient initially received a cycle of intravenous immunoglobulins (IVIg) at 2 g/kg/cycle, followed by infusions of rituximab 2 × 1 g two weeks apart (19, 20). The patient's skin erosions improved significantly and fully regressed eventually, in association with the decrease of anti-Dsg1 serum IgG antibodies.

Several months later, she developed diffuse erythroderma (**Figure 1B**). Erythematous targetoid plaques, resembling

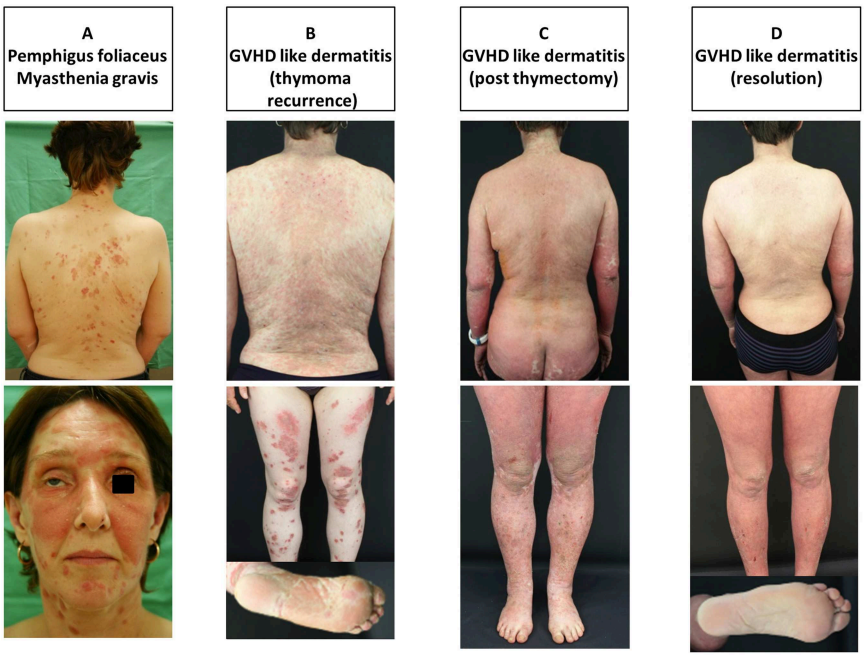

FIGURE 1 | (A–D) Clinical manifestations of the patient during clinical observational period. **(A)** Initially pemphigus foliaceus with involvement of the seborrheic skin areas and ptosis in myasthenia gravis, **(B)** lichenoid eruption at the time of thymoma recurrence, **(C)** erythroderma with lichenoid eruption right after thymectomy, and **(D)** resolution of the lichenoid eruption.

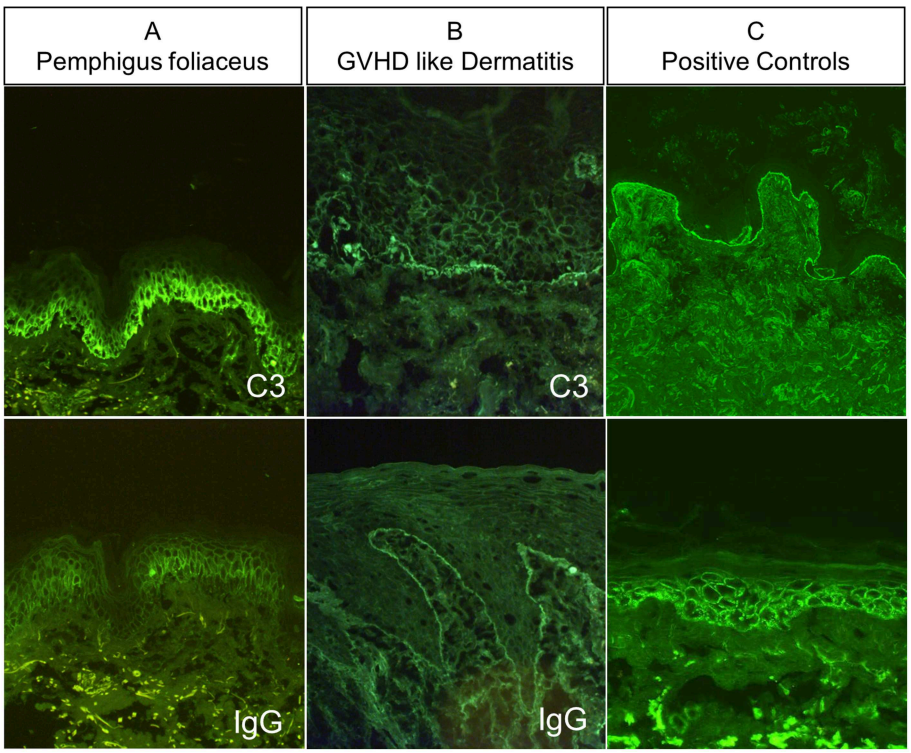

FIGURE 2 | Immune serological characteristics of the patient, **(A)** direct immunofluorescence (DIF) at time of pemphigus foliaceus, which showed IgG and C3 deposits at the surface of epidermal keratinocytes and **(B)** DIF at time of GVHD-like dermatitis which showed IgG C3 deposits along the BMZ. **(C)** positive DIF controls of perilesional BP skin, showing linear deposition of IgG along the BMZ (above), and positive DIF from perilesional PV skin presenting deposits at the surface of epidermal keratinocytes.

TABLE 1 | Autoantibody profile of the studied patient with thymoma-associated paraneoplastic autoimmune multiorgan syndrome.

Antigens	pemphigus foliaceus + myasthenia gravis	Thymoma reoccurrence	After thymectomy	Immunoassay
Dsg1	719 RE/ml (Cutoff < 20 RU/ml)	71 RU/ml	6 RU/ml	ELISA
Dsg3	0 (Cutoff < 20 RU/ml)	0	0	ELISA
Desmocollin 1	0.448 OD (Cutoff < 0.200 OD)	nd	nd	ELISA
Plakins	–	–	–	Immunoblot
BP180	0 (Cutoff < 20 RU/ml)	0	0	ELISA
BP230	0 (Cutoff < 20 RU/ml)	0	0	ELISA
Laminin γ-1	–	–	–	Immunoblot
Laminin 332	+	+	+	Immunoblot
Human type VII collagen	0 (Cutoff < 6 RU/ml)	0	0	ELISA
Skeletal muscle (titin)	++	+	–	BIOCHIP MOSAIC
Parotid gland	–	nd	nd	BIOCHIP MOSAIC
Stomach and bowels	–	nd	nd	BIOCHIP MOSAIC
Granulocytes, eosinophils, platelets, lymphocytes	–	nd	nd	BIOCHIP MOSAIC
Spinal cord, cerebrum, nerves, and cerebellum	–	nd	nd	BIOCHIP MOSAIC
HEP-2 cells	–	nd	nd	BIOCHIP MOSAIC
Liver, kidney, heart	–	nd	nd	BIOCHIP MOSAIC

nd, not determined; OD, optical densitiy; RU, relative units.

erythema multiforme (EM), and hyperkeratotic plaques appeared at her lower limbs and soles, respectively (**Figure 1B**). A skin biopsy from the erythrodermic skin revealed liquefactive degeneration and apoptotic keratinocytes and a band-like lymphocytic infiltrate along the BMZ (**Figure 3A**). These cutaneous symptoms were associated with persistent diarrhea and elevated liver enzymes and were thus considered as thymoma-associated GVHD-like disease. A chest X-ray and magnetic resonance imaging revealed a large mass in the left anterior mediastinum which was diagnosed by histopathology as a recurrent type-B2 thymoma. Prior to surgical removal, the patient received a cycle of intravenous cyclophosphamide (1,300 mg total dose) and IVIg (2 g/kg given on three consecutive days). After tumor resection, erythroderma with multiform lesions gradually regressed (**Figure 1C**) and eventually disappeared (**Figure 1D**). Of note, MG significantly improved and anti-Dsg1 serum IgG antibodies were no longer detectable (**Table 1**). A third skin biopsy revealed findings consistent with GVHD-like erythroderma (**Figure 3A**). Despite the clinical response, the patient eventually died because of an opportunistic bacterial infection leading to fulminant sepsis.

LABORATORY INVESTIGATIONS

Autoantibody Profile in the Patient With Thymoma-Associated Autoimmune Syndrome

The patient's serum IgG antibodies were reactive with Dsg1 and Dsc1 by ELISA and laminin 332 by immunoblot analysis (**Table 1**). Immunoblot analysis with epidermal extracts failed to identify IgG against plakins including periplakin and envoplakin (**Table 1**). Autoantibody profiling against various tissues showed the presence of IgG antibodies against muscle tissue (anti-titin IgG antibodies) which is characteristic for MG (**Table 1**) (21). More detailed information regarding the IgG autoantibody profile is given in the **Supplementary Material**. For information regarding detection of autoantibodies against laminin-γ1 please see (22).

Immunohistochemical Analysis of the T Cell Infiltrate in Skin Lesions and Thymoma Tissue

Skin samples taken from PF lesions, pre- and post-thymectomy GVHD-like skin lesions were further analyzed

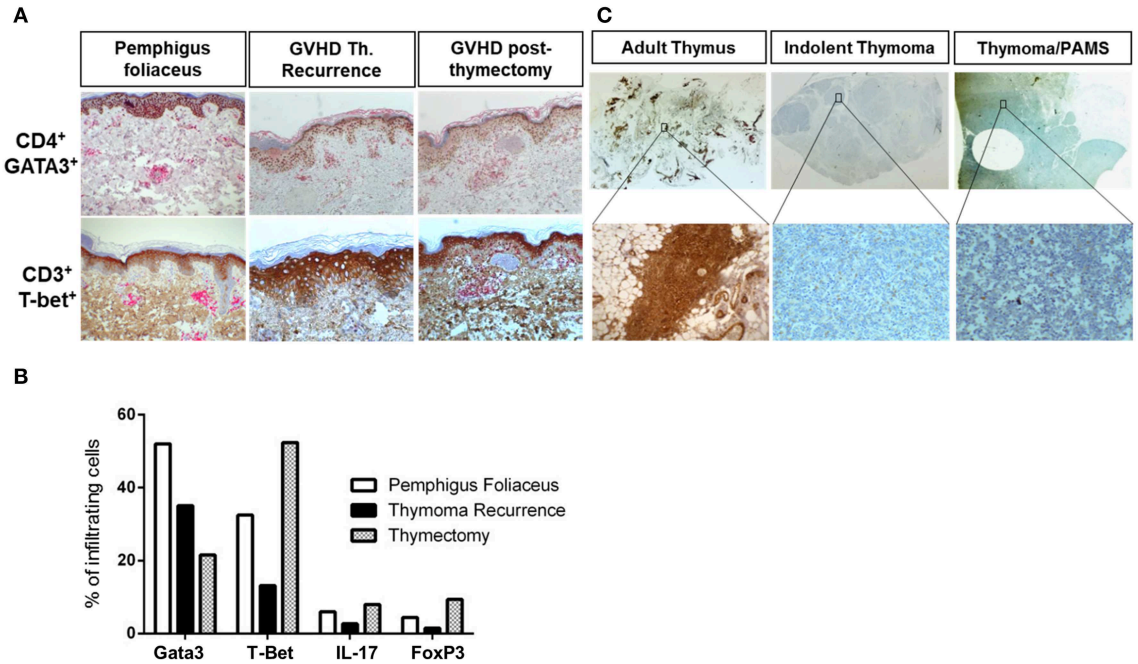

FIGURE 3 | (A) Immunohistochemical analysis of T cell subsets in pemphigus foliaceus (PF) and lichenoid skin lesions during the observational period. **(A)** $CD3^+$/$Tbet^+$ and $CD4^+$/$GATA3^+$ T cell skin infiltrate in PF, the lichenoid eruption at thymoma recurrence, and after thymectomy. **(B)** Percentage of $CD4^+$/$GATA3^+$, T-Bet+, $IL17A^+$, and $FoxP3^+$ T cells in PF skin lesions, at thymoma recurrence and after thymectomy, **(C)** expression pattern of FoxP3+ T cells in adult thymus, indolent thymoma, and in PAMS-associated thymoma (present case).

by immunohistochemistry as recently described (23) (more detailed information is given in the **Supplementary Materials**). PF skin lesions showed more $CD4^+GATA\text{-}3^+$ Th2 cells than $CD3^+T\text{-}Bet^+$ compared to the Th1-dominated GVHD-like skin lesions which inversely showed a stronger presence of $CD3^+T\text{-}Bet^+$ Th1 cells than $CD4^+GATA\text{-}3^+$ Th2 cells (**Figures 3A,B**). The numbers of $IL\text{-}17^+$ T cells and of $FoxP3^+$ Treg cells were not different between PF and GVHD-like skin lesions (**Figure 3B**). Moreover, the number of $FoxP3^+$ Treg cells in the patient's thymoma at the time of recurrence was significantly lower compared to adult persistent thymus and indolent thymoma without associated autoimmune syndromes (**Figure 3C**).

Peripheral Blood T Cell Response Against Cutaneous Autoantigen

By ELISpot assay, the peripheral blood T cell response of the patient against different cutaneous autoantigens, i.e., Dsg1, Dsg3, BP180, and BP230, was studied at different time points as recently described (21) (more detailed information is given in the **Supplementary Materials**).

Tetanus toxoid served as a control protein. Due to the strong immunosuppressive therapy, it was impossible to systematically study peripheral blood T cell responses against distinct cutaneous autoantigens (**Supplementary Figure 1**). Except for the initial treatment phase of PF with rituximab, Th1 and Th2 responses against the recall antigen, i.e., tetanus toxoid, were visible. After thymectomy, distinct Th1 and Th2 responses against Dsg3 and BP180 became apparent.

DISCUSSION

The association of thymoma, MG and PF is uncommon and is only rarely described in the literature (12). PF, MG, and thymoma can manifest as part of multiple autoimmune syndrome type 1, which consists of at least three autoimmune diseases including polymyositis, autoimmune thyroid disease, and giant cell myocarditis (18, 24). There have been sporadic cases of co-existing MG and PF which were not linked to thymoma (7, 8, 25). The etiopathogenesis of the simultaneous occurrence of MG, PF, and thymoma is only poorly understood. It has been proposed that a defective negative selection of autoreactive T-cells in neoplastic thymus leads to a loss of tolerance associated with autoantibody production against epithelial cells in Hassall's corpuscles and myeloid cells. These autoantibodies may then cross-react with homologous antigens of the epidermis and striate muscle, respectively (12). This contention is supported by the finding that the number of $FoxP3^+$ Treg cells in thymomas is inversely correlated with the occurrence of associated autoimmune disorders, i.e., impaired Treg cell function favors the onset of autoimmune disorders (26, 27).

Tsuchisaka et al. reported on a patient with PF associated with thymoma which expressed higher levels of Dsg3, Dsc2, Dsc3, and autoimmune regulator (AIRE) but not Dsg1 compared to normal adult thymus, indolent thymomas, and thymic carcinoma. These findings suggest that lack of AIRE-induced expression of Dsg1 lead to an autoimmune response against this epidermal adhesion protein (28).

Our patient was diagnosed with PF based on the characteristic skin lesions and presence of anti-Dsg1 IgG antibodies. In addition, serum IgG against Dsc1 was also initially detected, and was likely linked to impaired desmosomal function (29). The patient did not fulfill all the criteria for PNP based on; (i) a lack of mucosal involvement, (ii) negative IIF on urinary bladder, and (iii) the absence of serum IgG antibodies against plakins, which are major autoantigens of PNP (6, 30).

Anti-laminin 332 antibodies are pathogenic autoantibodies in anti-laminin 332 mucous membrane pemphigoid, a distinct subepidermal autoimmune bullous disorder showing predominant mucosal lesions. However, the pathogenic role of anti-laminin 332 antibodies detected in the present case is unclear, as the patient neither presented subepithelial blisters on the skin nor on the mucous membranes. Due to the profound impaired immunological tolerance status, the presence of IgG autoantibodies against laminin 332 is likely the consequence of epitope spreading (31).

Although considered as a separate entity based on its characteristic spectrum of pathology, there is not a clear-cut distinction between thymoma associated multiorgan autoimmunity (TAMA) and PAMS. Accordingly, (i) thymomas are the second most frequent neoplasms associated with PAMS, following Castleman's disease (2); (ii) among the various cutaneous presentations, which also include pemphigus-like, BP-like or multiforme lesions, several cases of PAMS resembling cutaneous GVHD have been reported (2); (iii) involvement of liver and colon in PAMS has been also reported (2). As for thymoma, both PAMS and TAMA are thought to occur as a result of incomplete deletion of self-reactive T cells in the tumors (32). A decreased number of Treg cells in neoplastic thymus is believed to be also involved (33). In line with previous studies, we also observed a reduction of Treg cells in our patient's thymoma compared to adult thymus and indolent thymoma. The observed clinical spectrum, ranging from pemphigus-like to lichenoid lesions, likely depends on the T-cellular profile of the cutaneous immune response, either humoral (Th2), and later T cell-mediated (Th1).

IgG antibodies against envoplakin and periplakin are characteristic serological markers of PAMS, while anti-Dsg1 and anti-Dsg3 IgG antibodies are rarely seen (34–36). Conversely, anti-Dsc1-3 IgG antibodies are seen in almost 60% of PAMS cases, as the presence of anti-Dsc1 IgG in the present case. Although unusual, the detection of initial epidermal cell surface staining and, at a later time point, linear staining at the BMZ is a classic feature of PAMS. The presence of both patterns has been considered as a criteria for the diagnosis of PAMS (2, 37), while systematic studies on the prevalence of IgG antibodies against epidermal or BMZ antigens in TAMA are lacking. In addition, one case of cutaneous thymoma associated GVHD-like erythroderma was reported to show IgG deposits on the surface of epidermal keratinocytes by DIF (38).

In our case, even though IIF failed to show the presence of IgG autoantibodies against epidermal keratinocytes, DIF taken from perilesional skin at two different clinical stages showed initially the presence of IgG and C3 deposits on the surface of epidermal keratinocytes (**Figure 2A**) and, later on, the presence of linear IgG and C3 deposits at the BMZ (**Figure 2B**), which was supported by the detection of anti-Dsg1, anti-Dsc1, and anti-laminin 332 IgG antibodies. It could be argued that Dsg1, Dsc1, and/or laminin 332 function as antigenic targets and triggers for the activation of autoreactive T cells driving GVHD-like erythroderma (39). This contention is supported by a recent study of our group which suggested that lichenoid dermatoses such as LP harbor a T-cellular response against epidermal (Dsg3) or BMZ (BP180) autoantigens commonly associated with autoimmune blistering diseases (23). As recently suggested by Amber, it should be discussed if PNP should be classified as a subtype of PV or if PNP is just a variant of PAMS, a large group of clinically variable disorders which are all linked the occurrence of various neoplasias. We also tend to believe that PNP, as TAMA, should be considered as a variant of PAMS, but this requires a broader consensus (3). In conclusion, the present study proposes an immunologic conversion from an initial Th2-driven to a Th1-type dominated cutaneous immune response triggered by the recurrence of the thymoma (**Figures 1**, **3A**). This shift to a Th1 response was paralleled by the complete resolution of the initial Th2 driven autoimmune disorders, PF and MG (**Figure 1**), and by the disappearance of serum anti-Dsg1/Dsc1 IgG antibodies (**Table 1**).

Of note, a study by Fujisao and Tsuda (40) described a patient with thymoma-associated pure red aplasia in which thymectomy resulted in an increased ratio of peripheral blood Th1/Th2 cells leading to significant amelioration of anemia (40). In 2015, Garrwan et al. reported on a patient with thymoma and associated proteinuria due to minimal change glomerulonephritis, in which antitumoral therapy with belinostat, cisplatin, doxorubicin, and cyclophosphamide resulted in a complete resolution of the tumor, reduction of proteinuria and increase in the Th1/Th2 ratio (41). Further accumulation of cases is required to better understand how thymectomy modulates thymoma-associated autoimmune syndromes via specific T-cell signatures and impacts on the course of these associated paraneoplastic syndromes.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

In accordance with the declaration of Helsinki, written consent for the publication of this case report, and the accompanying images was obtained from the patient.

AUTHOR CONTRIBUTIONS

FS, RM, RP, and TS wrote the manuscript, performed the experiments, analyzed data, and designed figures. RE and MH designed and supervised the study. TH and NI provided help for extensive serological diagnostic. BT, AK,

DD, and JP helped during clinical follow-ups and helped during manuscripts writing. RP, TS, RE, MH, and TH revised the study. All authors revised and finally approved the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01413/full#supplementary-material

Supplementary Figure 1 | Analysis of peripheral blood T cell subset responses against cutaneous autoantigens during the disease course. ELISpot analysis of IL-10^+, IFN-γ^+, IL-$17A^+$, and IL-5^+ peripheral blood T cells against tetanus toxoid (recall antigen), bullous pemphigoid (BP) 180, BP230, desmoglein (Dsg) 1, and Dsg3. Numbers of autoreactive T cells were determined at the initial stage (pemphigus foliaceus), at thymoma recurrence, after thymectomy and at resolution of the lichenoid eruption.

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REVIEW
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Pemphigus: Current and Future Therapeutic Strategies

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Pemphigus encompasses a heterogeneous group of autoimmune blistering diseases, which affect both mucous membranes and the skin. The disease usually runs a chronic-relapsing course, with a potentially devastating impact on the patients' quality of life. Pemphigus pathogenesis is related to IgG autoantibodies targeting various adhesion molecules in the epidermis, including desmoglein (Dsg) 1 and 3, major components of desmosomes. The pathogenic relevance of such autoantibodies has been largely demonstrated experimentally. IgG autoantibody binding to Dsg results in loss of epidermal keratinocyte adhesion, a phenomenon referred to as acantholysis. This in turn causes intra-epidermal blistering and the clinical appearance of flaccid blisters and erosions at involved sites. Since the advent of glucocorticoids, the overall prognosis of pemphigus has largely improved. However, mortality persists elevated, since long-term use of high dose corticosteroids and adjuvant steroid-sparing immunosuppressants portend a high risk of serious adverse events, especially infections. Recently, rituximab, a chimeric anti CD20 monoclonal antibody which induces B-cell depletion, has been shown to improve patients' survival, as early rituximab use results in higher disease remission rates, long term clinical response and faster prednisone tapering compared to conventional immunosuppressive therapies, leading to its approval as a first line therapy in pemphigus. Other anti B-cell therapies targeting B-cell receptor or downstream molecules are currently tried in clinical studies. More intriguingly, a preliminary study in a preclinical mouse model of pemphigus has shown promise regarding future therapeutic application of Chimeric Autoantibody Receptor T-cells engineered using Dsg domains to selectively target autoreactive B-cells. Conversely, previous studies from our group have demonstrated that B-cell depletion in pemphigus resulted in secondary impairment of T-cell function; this may account for the observed long-term remission following B-cell recovery in rituximab treated patients. Likewise, our data support the critical role of Dsg-specific T-cell clones in orchestrating the inflammatory response and B-cell activation in pemphigus. Monitoring autoreactive T-cells in patients may indeed provide further information on the role of these cells, and would be the starting point for designating therapies aimed at restoring the lost immune tolerance against Dsg. The present review focuses on current advances, unmet challenges and future perspectives of pemphigus management.

Keywords: pemphigus, CAAR T-cell, rituximab, anti-CD 20 antibodies, BTK inhibitors, neonatal Fc receptor (FcRn)

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INTRODUCTION

Definition

Pemphigus encompasses a heterogeneous group of autoimmune chronic blistering skin diseases, which affect both mucous membranes and the skin. Pemphigus group diseases are characterized by IgG autoantibodies directed against epidermal adhesion complexes (desmosomes) of keratinocytes, leading to loss of cell–cell adhesion, a phenomenon called acantholysis (1, 2).

Pemphigus can be divided into three major forms: pemphigus vulgaris (PV), pemphigus foliaceus (PF), and paraneoplastic pemphigus (PNP). Autoantibodies directed against Dsg3 and Dsg1 are mainly identified in PV; anti-Dsg1 autoantibodies are the serological hallmark of PF (3). In addition, autoantibodies targeting non-Dsg antigens have been reported in PV patients (4), such as IgG against alpha9 acetylcholine receptor (5), various mitochondrial nicotinic cholinergic receptor subtypes (4) and desmocollins 1-3 (4).

A variety of IgG autoantibodies have been described in PNP patients, including IgG against adhesion proteins of the plakin family, plakophilin 3, desmocollins 1 and 3, Dsg1, and Dsg3 and a 170 kD protein which has been recently identified as the protease inhibitor, alpha-2 macroglobulin-like 1 (A2ML1) (6).

Alike PV, PF and PNP, IgA pemphigus is an extremely rare variant of pemphigus, in which IgA but not IgG autoantibodies against epidermal antigens can be identified (3, 7, 8).

Epidemiology

Epidemiology of Pemphigus Vulgaris

PV is the most common clinical pemphigus variant. The annual incidence rate has been reported between 0.76 and 16.1 per million population, depending on the geographical area and the ethnicity (9, 10), with the highest incidence reported in Ashkenazi Jews (10, 11). This observation has been related to the more frequent occurrence of particular Human Leukocyte Antigen (HLA) class II genes in PV patients of Jewish origin, particularly HLA-DRB1*04:02; while HLA-DQB1*05:03 is more common in non-Jewish PV patients (12) and was also shown to have the strongest association with PV in a Chinese study using next generation sequence analysis (13).

The exact prevalence of pemphigus is unknown. A German analysis reported a point prevalence of 0.009% (14), while a Danish analysis estimated the pemphigus prevalence at 0.006% (15, 16). In addition, in a recent analysis on the US population, an overall standardized point prevalence of 5.2 cases per 100,000 adults has been reported (17). The age at initial PV presentation varies from 36.5 and 72.4 years (12). The mean age of PV onset is 50–60 years, although several cases of PV in children have been described (12). A female predominance has been globally reported, with an estimated female to male ratio of 5.0 in the American PV population (11).

Epidemiology of Pemphigus Foliacues

The annual incidence of sporadic PF in the Caucasian population is ∼0.04 per 100.000 inhabitants (10, 12). Sporadic PF corresponding to ∼20% of pemphigus cases (10, 12). People in the fifth decade are mainly affected, without sex preference (10, 12). HLA-DRB1*04:01, HLA-DRB1*04:06, HLA-DRB1*14, DRB1*01:01, have been associated with a higher risk of PF (13, 18). No ethnic predisposition has been reported (10, 12).

Endemic PF (*fogo selvagem*) has been reported in some areas of Brazil, Colombia, and Tunisia (19). Most of the patients are young rural workers, who live in forest areas adjacent to rivers and streams (19). In these areas, some insects including black fly (simulium species), are though to trigger the disease, leading to an immune reaction against Dsg1 via molecular mimicry (20, 21). This hypothesis is supported by high positivity rates of anti-Dsg1 IgG autoantibodies in the sera of healthy individuals living in endemic regions of *fogo selvagem* (21). In Brazilian population HLA-DRB1 alleles *04:04, *14:02, *14:06, and *01:02 have been reported as risk factors for *fogo selvagem* (22).

Epidemiology of Paraneoplastic Pemphigus

PNP is considered a rare disease, with about 500 cases reported in the literature (6, 23). Patients between 45 and 70 years of age are usually affected (6, 23). PNP accounts for 3–5% of all pemphigus cases (6, 23). Furthermore, PNP can affect also children and adolescents, particularly in association with Castleman's disease (6, 23). In this sub-group of patients, a predisposition in patients with Hispanic roots was described (24). An association with HLA class II DRB1*03 and HLA Cw*14, respectively, was reported in Caucasian and in Han Chinese patients (25, 26).

Major Clinical Variants

Pemphigus Vulgaris

More than half of the patients develop flaccid cutaneous blisters (3, 8, 27) (**Figure 1A**), which evolve into oozing erosions on erythematous skin. The entire skin may be affected, although lesions mostly occurs in areas exposed to increased mechanical stress (e.g., intertriginous areas) (3, 8, 27) and seborrheic areas (3, 8, 27). Bacterial or viral superinfections of cutaneous and mucosal lesions are fairly common. Cutaneous blisters and erosions usually transform into crusts followed by re-epithelisation without scars. Post-inflammatory hypo and/or hyperpigmentation are common.

In most instances, PV initially manifests with extremely painful erosions of the oral mucosa, particularly the buccal mucosa, the gingiva, the tongue, and the hard and soft palate (3, 8, 27) (**Figure 1B**). These lesions lead impaired food uptake which results in progressive weight loss. Hoarseness of the voice may be indicative of laryngeal involvement. In the early stages, oral lesions may be misinterpreted as recurrent aphthae, herpetic gingivostomatitis, or erosive lichen planus (3, 8, 27). Other mucous membranes might be less frequently involved, such as laryngeal, esophageal, conjunctival, nasal, anal, and genital mucosa (28).

PV may also involve the nail apparatus. In one study, nail involvement occurred in circa 13% of PV patients. Nail alterations included paronychia, nail discoloration, onychorrhexis, periungual hemorrhages, and onycholysis (29).

Erosions of the intertriginous areas, the scalp and face might evolve into papillomatous or vegetative lesions characterized by abnormal growth of keratinocytes (30) (**Figure 2**). This

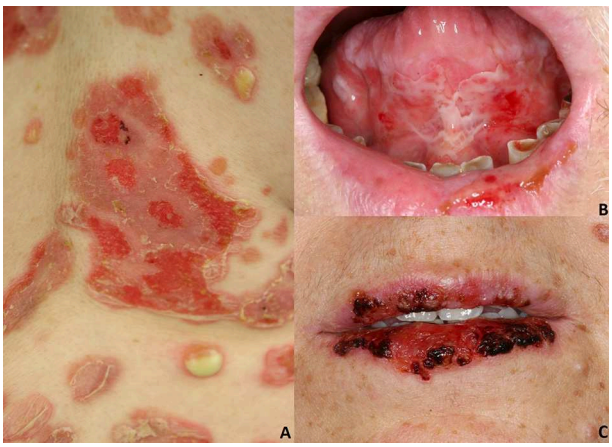

FIGURE 1 | Pemphigus vulgaris: **(A)** Flaccid cutaneous blisters associated with erosions; **(B)** Multiple erosions of the tongue and of the lips; Paraneoplastic pemphigus: **(C)** haemorrhagic crusts and erosion of the lips. All the patients gave written informed consent for the publication of the pictures.

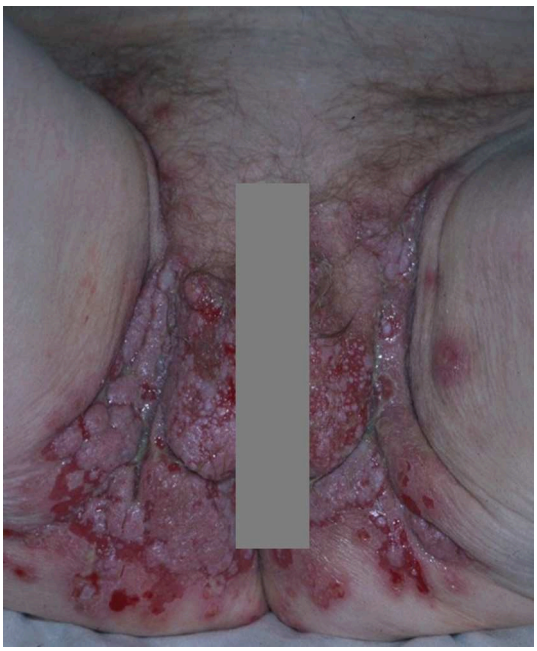

FIGURE 2 | Pemphigus vegetans: vegetative lesions and erosions of the groin and genitals.

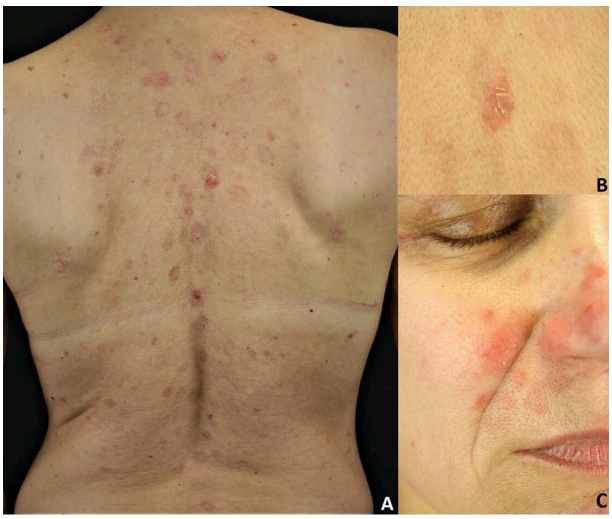

FIGURE 3 | Pemphigus foliaceus: **(A)** Scaly and crusted erythematous plaques on the seborrheic areas; **(B)** Leafy and crusted circumscribed erosion on the back; **(C)** Scaly erythematous plaques on the seborrheic areas. All the patients gave written informed consent for the publication of the pictures.

phenomenon represents the clinical hallmark of pemphigus vegetans (PVe) (30), which accounts for <5% of pemphigus cases (30).

A substantial number of PV patients shows a transition from a mucosal dominant to a mucocutaneous phenotype with skin lesions characteristic of PF as a result of epitope spreading, a process of diversification of B and/or T-cell responses from the initial dominant epitope (i.e., Dsg3) to a secondary one (i.e., Dsg1) (31). Based on the involved area, PV can be clinically divided in mucosal dominant, mucocutaneous, and, less frequently, cutaneous dominant (3, 8, 27).

Pemphigus Foliaceus

Sporadic PF is characterized by the absence of mucosal involvement (3, 8, 27). It presents with leafy, scaly and crusted circumscribed erosions on erythematous skin (3, 8, 27) (**Figure 3**). Seborrheic areas, including the upper trunk and the face, are mainly involved. Flaccid, fragile blisters are rarely seen because of their fragility. Skin lesions can dramatically progress leading to exfoliative erythroderma. PF onset is often subtle, with a few scattered crusted lesions that resembling impetigo. Furthermore, the scaly erythema on the scalp may be misdiagnosed for seborrheic dermatitis. The endemic variant of PF *(fogo selvagem)* is clinically and pathologically indistinguishable from the sporadic one (3, 8, 27). Pemphigus erythematosus (Senear-Usher syndrome) is a rare clinical variant of PF (3, 8, 27), characterized by malar erythemato-squamous plaques and vesicles involving the face in a butterfly-like distribution pattern, the trunk and sun-exposed areas resembling lupus erythematosus (32). In addition, a diagnosis of psoriasis should be also ruled out. Pemphigus seborrhoicus is a very superficial variant of PF with extensive superficial, crusty erosions and erythematous plaques affecting seborrheic areas, particularly the face (3, 27).

Paraneoplastic Pemphigus

PNP is a rare pemphigus variant which is always associated with underlying neoplasms, both malignant and benign. Up to 84% of all PNP cases are secondary to hematologic malignancies (33), including non-Hodgkin lymphomas (38.6%), chronic lymphocytic leukemia (18.4%), Castleman's disease (18.4%), thymoma (5.5%), Waldenstrom's macroglobulinemia (1.2%),

Hodgkin lymphoma (0.6%), and monoclonal gammopathy (0.6%) (6, 33). Less frequently, epithelial carcinomas (8.6%), sarcomas (6.2%) and gastric cancers have been described in association with PNP (6, 33–35). Some cases of PNP have been reported to be triggered by anti-neoplastic drugs, including fludarabin and bendamustine (36) or radiotherapy (37). PNP has a polymorphic clinical appearance, probably related to the variable presence of different IgG autoantibodies in addition to anti Dsg3/Dsg1 IgG (38). PNP typically presents with a painful stomatitis, and with extensive erosions of the oral cavity and oropharynx (**Figure 1C**). Usually, the vermillion border of the lips is involved (6). Differential diagnosis includes erythema multiforme (EM), toxic epidermal necrolysis (TEN) and Stevens-Johnson's syndrome; in pediatric cases, oral involvement may be mistaken for a herpetic stomatitis (6).

The nasopharynx, anogenital region, and esophagus may be also affected. Ocular involvement occurs in about 70% of cases (39). Usually, skin lesions, including diffuse erythema, vesicles, blisters, papules, scaly plaques, exfoliative erythroderma, erosions or ulcerations, appear after the onset of the mucosal lesions (38). Moreover, erythema may appear as macular, urticarial, targetoid or polymorphous lesions and a single patient may present different types of lesions, that could evolve from one type to another (40). Lichenoid lesions are also common and occur more frequently in children (24).

The peculiar clinical features of PNP can be explained by both, antibody-driven and cell-mediated pathogenetic mechanisms (41). The first usually determine a PV-like clinical phenotype, while the second features lead to a lichenoid phenotype. More than 90% of PNP cases show an involvement of the respiratory epithelium with dyspnea, obstructive lung disease, and bronchiolitis obliterans, which is one of the main causes of death in PNP (6, 42, 43). Recently, a correlation between bronchiolitis obliterans and anti-epiplakin Ig Abs was found in Japanese PNP patients (42).

IgA Pemphigus

IgA pemphigus is characterized by intraepidermal pustules or vesicles with neutrophilic infiltration (3, 7, 8). Acantholysis is usually absent. Depending on the level of pustule formation, IgA pemphigus is divided into two major subtypes, namely subcorneal pustular dermatosis type (IgA-SPD), characterized by subcorneal pustules in the upper epidermis, and intraepidermal neutrophilic type (IgA-IEN), characterized by suprabasilar pustules located at the lower or entire epidermis (3, 7, 8).

Pathogenesis

Dsg1/Dsg3 Compensation Theory

Because of the different expression of the pemphigus autoantigens (Dsg1 and Dsg3) in the cornified and non-cornified epithelium, skin and mucosae are differentially affected by anti-Dsg IgG autoantibodies. PF patients show only anti-Dsg1 IgG autoantibodies; whilst, patients affected by mucosal-dominant PV have only anti-Dsg3 IgG autoantibodies. Furthermore, in patients with mucocutaneous PV both anti-Dsg3 and anti-Dsg1 IgG autoantibodies are detected (44). In the skin and mucosae, the expression of Dsg1 and Dsg3 is different: cutaneous Dsg1 is expressed in the entire epidermis, but more strongly in the superficial layers; cutaneous Dsg3 is expressed in the lower epidermis, mainly in the basal and parabasal layers. On the contrary, mucosal Dsg1 and Dsg3 are expressed in the entire squamous layer, but the expression of Dsg3 is much higher.

Therefore, sera with only anti-Dsg1 IgG lead to superficial blisters and only in the skin, as in PF, because Dsg3 compensates for the loss of Dsg1. In contrast, anti-Dsg3 IgG lead to impairment of mucosal epidermal adhesion because of the low expression of Dsg1, that is not adequate to compensate the loss of Dsg3 adhesion. When anti-Dsg1 and anti-Dsg3 IgG is present, skin and mucous membranes are affected (45).

Blister Formation and Acantholysis: Auto-Antibody Dependent Factors

Ig autoantibodies directed against Dsg antigens lead to epithelial acantholysis presumably through several synergistic mechanisms. A model in which acantholysis is produced by interference through antibodies in desmosome adhesion and/or assembly has been proposed. Furthermore, an altered outside-in-signaling caused by antibodies has been thought to cooperate in damaging the desmosomal integrity (46).

The pivotal role of antibodies in pemphigus has been extensively reported (47). Furthermore, it has been highlighted that the sole monovalent antibody fragments can lead to skin lesions (48). In addition, IgG4 antibodies have been mainly reported in pemphigus, which do not involve the complement cascade (49).

The most important targets for Ig antibodies in pemphigus are extracellular domains of Dsg. Dsg show five extracellular cadherin repeats domains (EC1-EC5); the amino-terminal EC1 and EC2 domains, which play a pivotal role in adhesive interactions, are usually targeted by pemphigus antibodies. Indeed, anti-Dsg3 autoantibodies form PV patients and model mice bind directly to residues involved in trans-adhesion (50) and cis-adhesion (51). Thus, antibodies to the NH2-terminal cadherin domains likely compete with or block cellular cohesion. Di Zenzo et al. (51) propose that human anti-Dsg3 autoantibodies bind to the cis-adhesive Dsg3 interface inducing acantholysis. Furthermore, in contrast to IgG autoantibodies directed against other epitopes of Dsg1 and Dsg3, the serum concentrations of these IgG Abs correlate with disease activity (52, 53).

Depletion of Dsg results from several steps: desmosomes lose adhesive properties, probably through a direct interference of trans-interaction of Dsg; further, different signaling pathways cause Dsg endocytosis and depletion, leading to loss of demosomal integrity and adhesion (45). Moreover, the depletion of extradesmosomal Dsg located in association with lipid raft components may affect the *ex novo* expression of desmosomes (54). Furthermore, it has been reported that polyclonal IgG antibodies from PV patients can directly inhibit homophilic Dsg3 trans-interactions. These evidences provide support for the steric hindrance model of pemphigus pathogenesis (52, 53).

Further mechanisms have been thought to be involved in pemphigus acantholysis. Dsg endocytosis and desmosome disassembly have been reported as triggered by both

IgG autoantibodies from PV patients and recombinant monovalent human anti-Dsg3 autoantibodies (55, 56). In addition, intercellular widening at non-acantholytic cell layers induced by pathogenic pemphigus antibodies have been detected by both immunofluorescence and electron microscopy findings (57, 58).

Autoantibody-triggered cellular signaling pathways have been also reported as pathogenetic co-mechanisms in pemphigus. Specifically, it has been shown that polyclonal IgG antibodies from serum of PF patients can lead to dissociation of Dsg1 junctions without blocking homophilic Dsg1 trans-interactions (59). In addition, several molecules and signaling pathways have been reported as playing a role in pemphigus acantholysis, including p38 mitogen-activated protein kinase (MAPK) and MAPK-activated protein kinase 2 (MK2) (60, 61). p38MAPK directly modulates intermediate filament formation and the maintenance of the desmosomal structure. It was recently reported that p38 MAPK signaling and Dsg3 internalization play a pivotal role in pemphigus acantholysis (62); however, it has been also highlighted that blisters induced by monoclonal autoantibodies from PV patients are not affected by p38 or MK2 inhibition, indicating that this mechanism of blisters formation might be mainly related to steric hindrance (60, 61, 63).

Furthermore, Saito et al. (63) demonstrated that monoclonal and polyclonal autoantibodies are both complementary involved in acantholysis; indeed, an inducible Dsg clustering has been reported with polyclonal serum IgG, but not with monoclonal antibodies. Furthermore, several other signaling molecules and pathways have been reported as altered by anti-Dsg autoantibodies in pemphigus acantholysis, such as EGFR, caspases (64), and MYC (65). However, none of these events can solely induce acantholysis.

TABLE 1 | Diagnostic algorithm in pemphigus [adapted from Witte et al. (76)].

HISTOPATHOLOGY
Suprabasal acantholysis (IgA-IEN, PNP, PV)
Subcorneal acantholysis (IgA-SPD, PF)
Interface dermatitis with vacuolization of the basal cells and lichenoid infiltrate at the DEJ (PNP)
DIF
Reticular binding of IgG and/or C3 to the surface of epidermal keratinocytes (PF, PV)
Reticular binding of IgA and/or C3 to the surface of epidermal keratinocytes (IgA-IEN, IgA-SPD)
Linear deposits of IgG and/or C3 at the BMZ (PNP)
Net-like IgG and/or C3 deposits on the surface of epidermal keratinocytes and along the DEJ (PNP)
IIF
Reticular pattern of cell surface reactivity of IgG antibodies on the epithelium of monkey esophagus (PF, PNP, PV)
Reticular pattern of cell surface reactivity of IgA antibodies on the epithelium of monkey esophagus (IgA-IEN, IgA-SPD)
Net-like pattern of cell surface reactivity of IgG antibodies on monkey esophagus epithelia, normal human skin, and plakin-rich urinary bladder (PNP)
ELISA
Alpha-2-macroglobulin-like-1 (PNP)
BP230 (PNP)
Desmocollin 1 (IgA-SPD)
Desmocollin 3 (IgA-SPD, PNP)
Desmoglein 1 (IgA-IEN, PF, PNP, PV)
Desmoglein 3 (IgA-IEN, PV, PNP)
Periplakin/Envoplakin (PNP)

BMZ, Basal membrane zone; DEJ, dermo-epidermal junction; DIF, direct immunofluorescence; ELISA, enzyme-linked immunosorbent assay; IgA-IEN, intraepidermal neutrophilic type of IgA pemphigus; IgA-SPD, subcorneal pustular dermatosis type of IgA pemphigus; IIF, indirect immunofluorescence; PF, pemphigus foliaceus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris.

Blister Formation and Acantholysis: IgG-Independent Factors

Beside of the Abs role in acantholysis, several autoantibody-independent factors have been thought to be involved in acantholysis. However, the distinct role of these factors is not completely understood.

An increment of Th2 cytokines, such as interleukin (IL)-4, IL-6, and IL-10, has been extensively reported in sera of PV patients, while a reduction of Th1 cytokines, such as IL-2 and Interferon-gamma (IFN-γ) has been also reported (66). In addition, an increase of IL-17a, produced by Th17-cells, and IL-21 and IL-27, synthesized by T follicular helper cell, has been detected (67). Of note, IL-17a was also shown in PV blisters (67). Furthermore, complement activation, cytotoxic proteases and high levels of IL-4 and IL-10 were observed (66, 68). In addition, Tumor Necrosis Factor-alpha (TNF-α) RNA is widely expressed in PV skin lesions and TNF-α serum concentrations correlate with disease activity and IgG autoantibody titers (69, 70).

The importance of apoptosis of epidermal keratinocytes in acantholysis is still under debate. Indeed, some groups considered this process a downstream event after loss of cell–cell adhesion (71), while others suggested it as an upstream event (72). Caspase 8 activation induced by Fas ligand (FasL) detected in PV sera was described to induce apoptosis in keratinocytes. It has been reported *in vitro* and *in vivo* that hindrance of FasL protein causes an inhibition of PV IgG-induced apoptosis of epidermal keratinocytes, suggesting a pivotal role of that FasL in PV pathogenesis (73, 74). Moreover, Lotti et al. (73) highlighted that apoptosis precedes acantholysis, as Fas overexpression, caspase activation before cell detachment *in vivo*.

Furthermore, it was reported that the secretion of cytokines from keratinocytes could be stimulated by PV-IgG (75). Indeed, the expression of the transcription factor ST18 in keratinocytes was reported in response to PV-IgG, leading to both secretion of cytokines and loss of keratinocyte cohesion; therefore, it has been concluded that that cytokines contribute to blistering downstream of autoantibodies (75).

Diagnostics

The proper diagnosis of pemphigus is based on four criteria, namely clinics, histopathology of the lesional skin, direct immunofluorescence microscopy (DIF) of perilesional skin, and detection of serum autoantibodies by indirect immunofluorescence microscopy (IIF), enzyme-linked immunosorbent assay (ELISA) and/or additional techniques such as Biochip, immunoblot analysis or immunoprecipitation (7, 76) (**Table 1**).

Histopathologically, PV is characterized by intraepidermal acantholysis (7) (**Figure 4A**), with basal keratinocytes still attached to the basement membrane zone assuming a characteristic tombstone-like morphology. In contrast to PV, PF lesions show a more superficial, subcorneal acantholysis (**Figure 4B**). In PNP, the histopathological features are polymorphic. Bullous lesions show suprabasal acantholysis with dyskeratosis and a scattered inflammatory infiltrate (6). In maculopapular lesions, a lichenoid interface dermatitis is more frequently observed (6). Clinically mixed maculopapular and bullous lesions show both acantholysis and lichenoid interface dermatitis (23). IgA pemphigus is characterized by intraepidermal pustules or vesicles with neutrophilic infiltration whereas acantholysis is usually absent (7).

In all pemphigus variants, DIF of perilesional skin shows reticular binding of IgG and/or C3 to the surface of epidermal keratinocytes (7, 77) (**Figure 4C**). In IgA pemphigus, DIF detects IgA instead of IgG (7, 77). In PNP, net-like IgG and/or C3 deposits on the surface of epidermal keratinocytes and along the dermo-epidermal junction can be detected in <50% of cases (6). In contrast to PV and PF, PNP lesions show linear deposits of IgG and/or C3 at the basal membrane zone by DIF (6).

In IIF routine diagnostics, monkey esophagus is used as the major tissue substrate. A reticular pattern of cell surface reactivity of IgG antibodies with epithelial cells is characteristic (7, 77). In IgA pemphigus, intercellular deposits of IgA directed against Dsg 1 and Dsg 3 (IgA-IEN), as well as against Dsc 1 and Dsc 3 (IgA-SPD) are detected by IIF (7, 77). IgA autoantibodies in IgA-SPD may be detected by IIF on Dsc1-transfected COS-7 cells (78).

In PNP, IgG antibodies directed against plakins can be detected; among those, IgG against envoplakin and periplakin are the most common (23). In PNP, IIF also shows a net-like staining pattern with normal human skin and plakin-rich urinary bladder, the latter being the substrate of choice since it shows for the detection of plakin-reactive IgG autoantibodies (83%) (6, 23, 76).

In PV, IgG autoantibodies against Dsg 1 and Dsg 3 can be detected by ELISA. Patients affected by dominant cutaneous PV show only or preferentially anti-Dsg 1 autoantibodies, while patients with mucosal dominant PV show only or preferably anti-Dsg 3 IgG autoantibodies. In muco-cutaneous PV, both anti-Dsg 1 and anti-Dsg 3 autoantibodies can be detected. In contrast to PV, patients with PF show only IgG against Dsg 1 in the vast majority of cases (7, 77). In general, anti-Dsg 1 and anti-Dsg 3 serum antibody concentrations correlate with disease activity (7, 77). However, in case of atypical pemphigus, autoantibodies against Dscs are detected, while reactivity against Dsg 3 and/or Dsg 1 lacks. In atypical pemphigus, both IgA and IgG against different Dscs are detected (79). However, routine evaluation of serum IgG and IgA against Dscs does not play a significant role in making the diagnosis of PV and PF (79).

Depending on the subtype, different IgA autoantibodies are detected in IgA pemphigus by ELISA, including IgA against Dsc 1, Dsg 1, and Dsg 3.

The spectrum of IgG autoantibodies is more diverse in PNP, including IgG autoantibodies against Dsg 1, Dsg 3, desmoplakin 1, desmoplakin 2, Dsc 1, Dsc 3, envoplakin, periplakin, plectin, BP180, BP230, and the protease inhibitor, alpha-2-macroglobulin-like-1 (6, 80). However, ELISA lacks sensitivity in PNP patients due to the wide range of autoantigens targeted by Ig autoantibodies (81).

Immunoblotting and immunoprecipitation are considered useful techniques for diagnosing PNP and can show IgG antibodies against several antigens, including plakins, periplakin, desmoplakin, BP180, BP230, and alpha-2-macroglobulin-like-1 (6, 80). Indeed, IgG autoantibodies against envoplakin and periplakin and/or alpha-2 macroglobulin-like-1 confirm the diagnosis of PNP. Therefore, in PNP patients two of three serological techniques (IIF on rat bladder, immunoblot and immunoprecipitation) should be performed to establish the correct diagnosis (76).

Immunoblot analysis is performed with recombinant proteins or extracts of dermis, epidermis, bovine gingiva, amnion membrane or cultured keratinocytes (76). They can be used for the detection of several autoantibodies, such as anti-envoplakin, anti-periplakin, anti-desmoplakin, anti-BP180, and anti-BP230 Ig (76).

Recently, a novel lateral flow immunoassay (LFIA) was developed (82). It detects anti-Dsg 3 IgG in human sera. In contrast to other diagnostic procedures, the assay is simpler and faster. LFIA was validated on a collection of 200 sera and showed a sensitivity and specificity of 78.1 and 97.1%, respectively (82).

TREATMENT

Since the advent of targeted therapies, the management of pemphigus has gradually changed. Until now, systemic corticosteroids (CS) and immunosuppressants have been the mainstay of pemphigus therapy. Among conventional adjuvant immunosuppressants, both EADV and BAD guidelines suggest azathioprine (AZA) and mycophenolate mofetil (MMF) as a first line steroid-sparing agent (83, 84). However, different variables, including patients' comorbidities, single institutional experience and costs have to be taken into account, and other drugs, such as methotrexate and cyclophosphamide, also demonstrate efficacy. Notably, these drugs have mainly a CS-sparing rather than a morbostatic effect (85–87). Accordingly, they do not lead to an improvement in achieving remission, but reduce the risk of relapse by 29% in comparison to CS alone (85). A recent prospective multicentre study by Joly et al. (88), now supports using RTX as a first line adjuvant therapy for pemphigus, showing superior efficacy compared to CS alone and reduced incidence of CS-related serious adverse events and overall mortality. The administration of intravenous immunoglobulin (IVIg) or immunoadsorption (IA) is a therapeutic option in patients with severe/refractory PV. Proposed algorithms for the induction and maintenance therapy as well as therapy of relapse are summarized in **Figures 5**–**7**.

Corticosteroids and Immunosuppressants

Corticosteroids

In pemphigus, prednisolone is recommended as a first-line therapy in combination with an immunosuppressive agent, such as azathioprine (AZA) and mycophenolate mofetil (MMF), or RTX (83, 84). In addition, prednisolone alone at a dose of

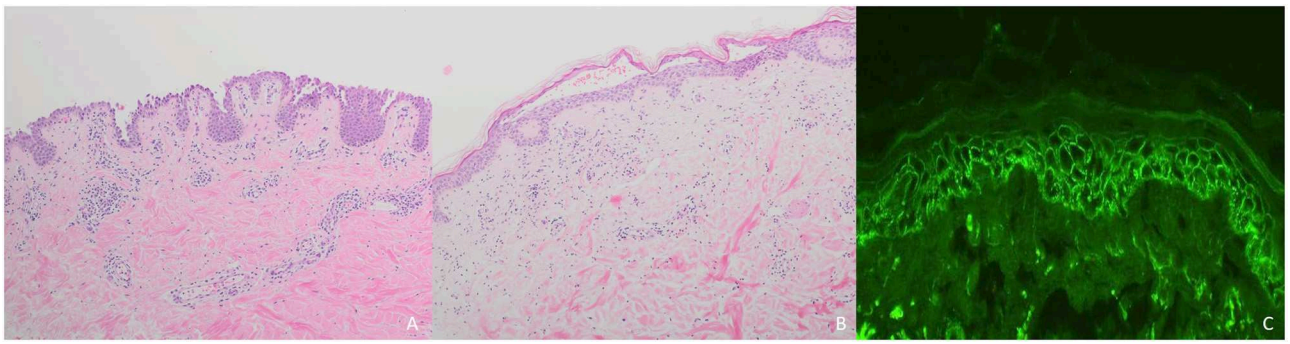

FIGURE 4 | Diagnostic of pemphigus: **(A)** Intraepidermal acantholysis in pemphigus vulgaris; **(B)** Subcorneal loss of adhesion in pemphigus foliaceus; **(C)** Reticular binding of IgG in pemphigus vulgaris.

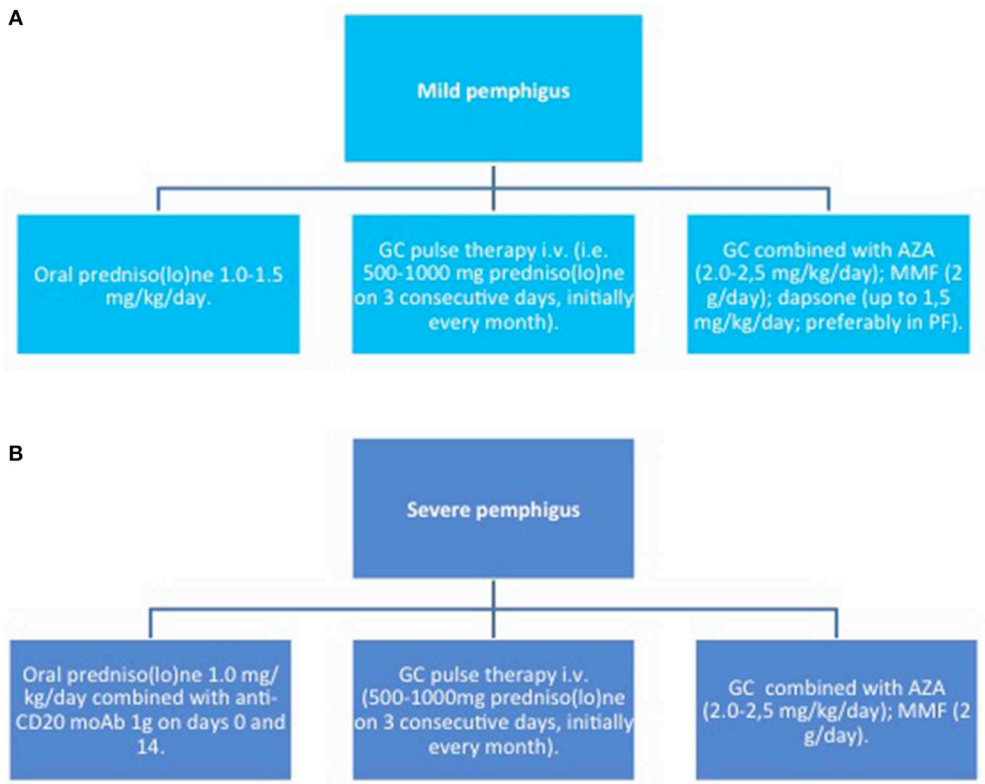

FIGURE 5 | Induction therapy in pemphigus. **(A)** mild pemphigus; **(B)** severe pemphigus. AZA, azathioprine; GC, glucocorticoids; MMF, mycophenolate mofetil; moAb, monoclonal antibody.

1–1.5 mg/kg/day is still recommended as first line therapy in patients who are not eligible for treatment with RTX or other immunosuppressive adjuvants.

Higher CS doses (up to 1.5 mg/kg) may be administered, if disease control is not achieved within 3 weeks. As soon as disease control is reached, the doses should be tapered by a 25% reduction every other week. If lesions reappear, CS should be increased until two steps back at the previous dose to lead to disease control (84). However, the optimal dose has not been validated by randomized clinical trials RCT.

No significant differences regarding the duration of remissions and relapse rates have been reported in PV patients receiving low-dose oral prednisolone (45–60 mg/day) or high-dose oral prednisolone (120–180 mg/day) (89). In the maintenance period of PV, no advantages in terms of remission, death, relapse or withdrawal rates have been reported in patients on a pulsed CS therapy in comparison to conventional oral CS therapy. Indeed, the two RCTs about this topic reported opposite results (90, 91).

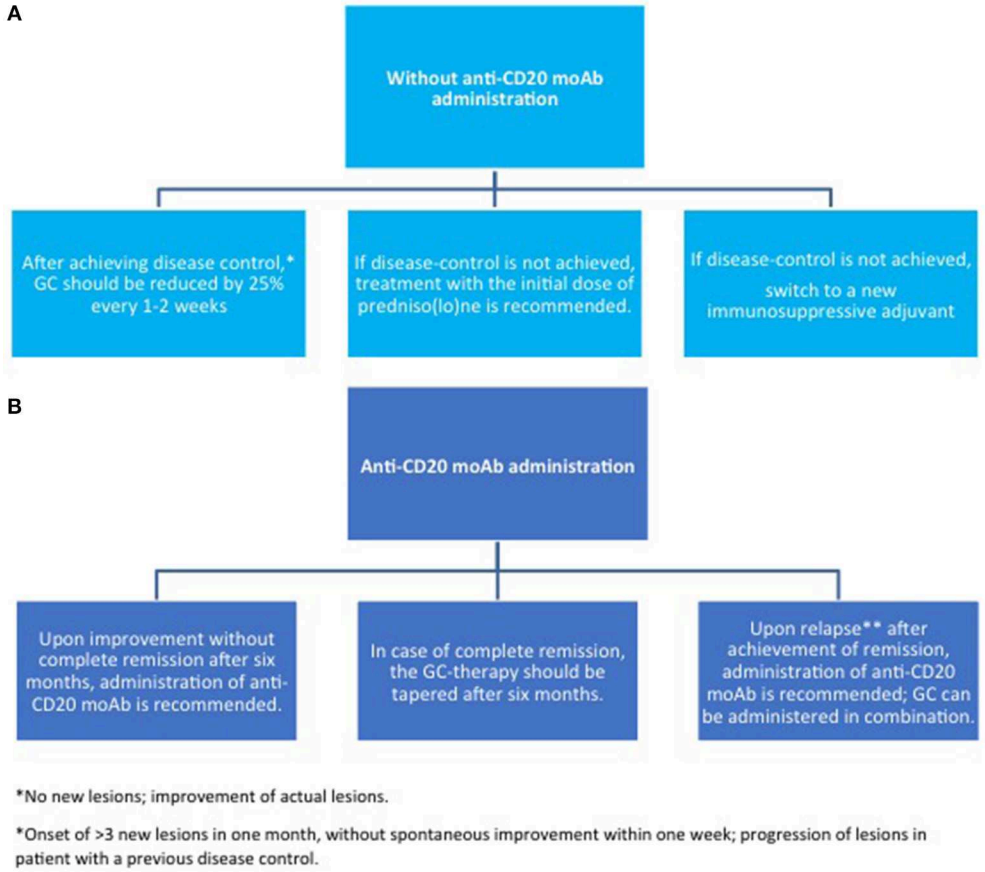

FIGURE 6 | Maintenance therapy in pemphigus. **(A)** without anti-CD20 moAb; **(B)** with anti-CD20 moAb. GC, glucocorticoids; moAb, monoclonal antibody.

If the required CS dose is higher than 100 mg/day, a pulse therapy should be considered, in order to reduce the risk of adverse effects (92). Still, the advantage of combined oral CS pulses and immunosuppressive adjuvants is under debate (90, 93).

CS increase the expression of anti-inflammatory proteins and inhibit the production of pro-inflammatory proteins interacting with the cytoplasmic corticosteroid receptor (94). Indeed, the corticosteroid receptor reduces the expression of transcription factors as well as their co-activator molecules, such as NF-κB and activator protein 1 (94). In addition, CS induce the downregulation of IL-2, leading to a reduction in both B-cell clone expansion and autoantibodies synthesis (94). Furthermore, the reduction IL-2 expression inhibits the cell-mediated immunity and reduces T-cell proliferation (95). Therefore, CS lead to multiple signal transduction pathways producing anti-inflammatory, immunosuppressive, antiproliferative, and vasoconstrictive effects.

Several adverse effects have been described in patients undergoing long-time CS therapy, including increased overall susceptibility to infections and infestations, secondary adrenal insufficiency, osteoporosis, transient hyperglycaemia, hypertension, and posterior subcapsular cataract (96). In addition, cutaneous adverse effects have been described, including purpura, telangiectasias, atrophy, striae rubrae, acneiform or rosacea-like eruptions, infections, stern obesity and facial oedema.

Immunosuppressive Adjuvants

Azathioprine

AZA is a prodrug that converts to 6-mercaptopurine after oral administration. AZA down-regulates purine metabolism leading to a block of DNA, RNA and proteins synthesis. Furthermore, AZA inhibits mitosis and leads to immunosuppression in several ways (97). AZA reduces the number of monocytes and Langerhans cells, decreases γ-globulin production, and lower T-cell as well as suppressor B cell activity. Furthermore, it blocks T-helper-cell dependent responses of B cells (97, 98). 6-mercaptopurine can be inactivated to 6-methyl-mercaptopurine by thiopurine methyltransferase (TPMT) enzyme (96).

AZA is a safe CS-sparing agent, recommended as a first-line adjuvant immunosuppressant (83). The dosage of AZA is adapted to the TPMT activity and measurement of TPMT activity should be performed before AZA administration (99). Usually, 2.0 mg AZA kg/day are recommended by normal TPMT activity, while 1 mg AZA kg/day is recommended for patients

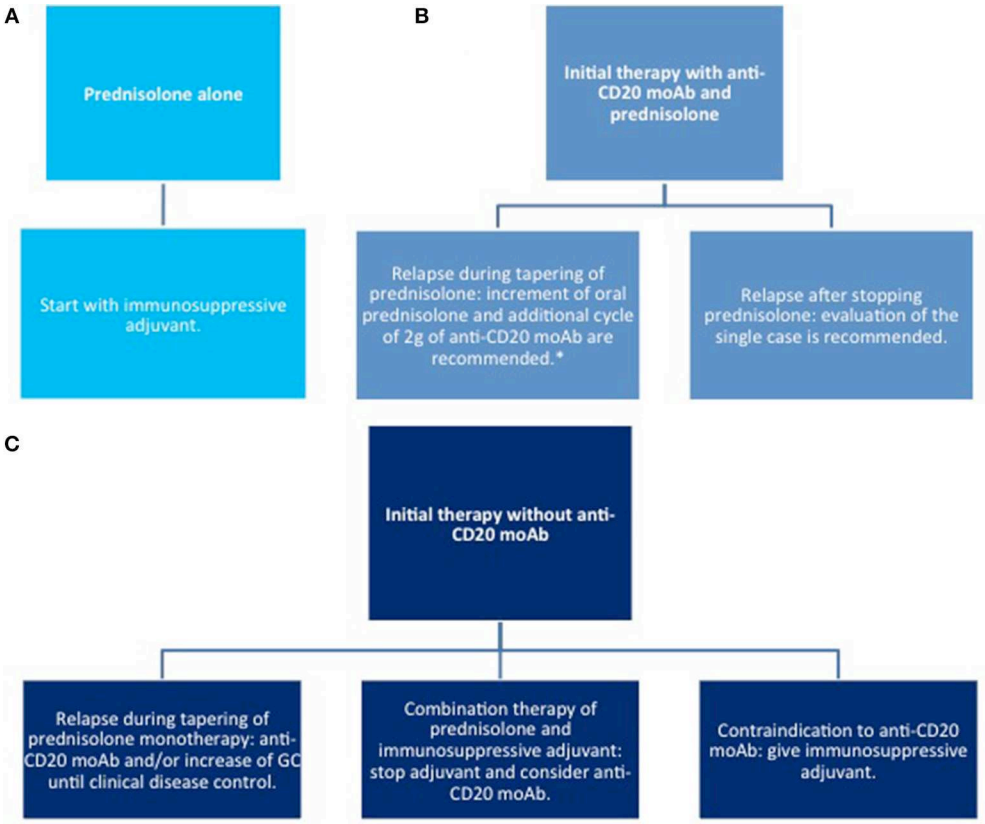

FIGURE 7 | Therapy of relapse. **(A)** with corticosteroids only; **(B)** systemic corticosteroids combined with anti CD20 moAb; **(C)** systemic corticosteroids combined with other immunosuppressive agents. moAb, monoclonal antibody.

with TPMT enzyme mutations (84). A dose of 50 mg AZA per day is recommended as initial therapy; the dose can be increased to the optimal dose based on TPMT activity (84). Adverse effects have been reported in 15–30% of patients. Severe adverse effects include myelosuppression and pancytopenia, and hepatotoxicity (99). However, myelosuppression may occur despite normal TPMT. Therefore, despite normal TPMT activity, a routine complete blood count including liver enzymes throughout the treatment period should be performed (99). In addition, long-term immunosuppression raises the risk of infections and cancer (100). Indeed, AZA shows a mutagenic potential that might provoke hematologic malignancies (100). Therefore, AZA is not recommended in pregnancy and breastfeeding (101). Other adverse effects include nausea, pancreatitis, diarrhea, aphthous stomatitis, maculopapular rashes, and anaphylaxis (99).

In a RCT involving 120 PV patients, a combined therapy of CS and adjuvant AZA (2.5 mg/kg daily) showed a higher CS-sparing effect than CS alone and a combination therapy with MMF (102). Furthermore, in a previous RCT study, adjuvant AZA (2.5 mg/kg daily) was compared to CS alone (103). During 1-year follow-up, a significant CS-sparing effect has been shown only in the last 3 months. Furthermore, disease activity was also significantly lower in the AZA group only in the last 3 months in comparison to the CS only. In addition, in a non-randomized study on PV patients, high-dose oral prednisone daily (1.5 mg/kg/day) vs. low-dose oral prednisone (40 mg every other day) plus AZA (100 mg/day) have been compared. It was shown a shorter main time to remission in high-dose oral prednisone monotherapy group, although the rate of adverse effects was higher (104). In summary, there is good evidence for a higher CS-sparing effect of AZA than CS monotherapy and MMF (83).

Mycophenolate mofetil

MMF is a prodrug that converts to mycophenolic acid (MPA) upon oral administration. MPA downregulates the immune system by selective impairment of inosine monophosphate dehydrogenase, leading to a blockade of the *de novo* pathway of purine synthesis in T and B cells, affecting both cellular and humoral immunity. Because lymphocytes are mainly dependent on the *de novo* pathway for purine biosynthesis, lymphocytes are the primary target of MPA. Because this target profile, MMF shows a safer profile in comparison to other less selective immunosuppressants, such as AZA (105).

As AZA, MMF is also recommended as a first-line adjuvant immunosuppressant (83, 84). The recommended dose of 2 g/day divided in two doses. In patients with a reduced renal function a reduced dosage should be administered (106). Initially, a dose of 500 mg MMF/day should be administered and an increase by

500 mg may be possible. A final dose of 2 g/day has been proposed in order to reach a better gastrointestinal tolerance (97).

Severe adverse effects have been rarely reported. Mild gastrointestinal symptoms, such as nausea, vomiting, and diarrhea are commonly seen. Rare are opportunistic infections, hematologic abnormalities, esophagitis, and gastritis (107). Studies on transplant recipients in therapy with MMF have demonstrated an increased risk of developing lymphomas and skin cancer (86). MMF is not recommended in pregnancy and breastfeeding, because of an increased risk of spontaneous abortion and congenital malformations (101).

Beissert et al. (108) compared CS plus MMF with CS plus placebo in PV patients. It has been highlighted that the MMF group showed a faster response to therapy, a longer disease-free interval, and a statistically significant CS-sparing. However, adjuvant MMF was not superior to CS in inducing disease control (108). Furthermore, no significant differences in cumulative CS dose, efficacy, or adverse effects were reported between AZA plus oral methylprednisolone and MMF plus oral methylprednisolone (109). In addition, Chams-Davatchi et al. (102) showed no significant difference in efficacy or safety between a combination therapy of prednisolone plus MMF (2 g/day) and prednisolone plus AZA. Regarding the CS-sparing effect, MMF was superior to prednisolone alone, but inferior to AZA, while compared to Cyclophosphamide (CYP), non-conclusive data were reported (102). The optimal MMF dose in PV has not yet been found. In a multicentric randomized controlled trial (RCT), no clear conclusions regarding the use of standard MMF dose (2 g daily) vs. a high one (3 g daily) were reported (108).

Cyclophosphamide

CYP is an alkylating prodrug with antineoplastic and immunosuppressive properties. CYP is converted in the liver into two active metabolites, phosphoramide mustard and aldophosphamide, which downregulate DNA replication and induce cell death. CYP shows also a blocking activity on proliferation, cytokine production, and lymphocyte-induced inflammation (85, 92).

The recommended oral dose is 2 mg/kg/day (97). Because of its rather unfavorable safety profile, CYP is not recommended as a first-line CS-sparing agent but rather as a rescue drug.

Several frequent adverse effects have been reported, including nausea, vomiting, diarrhea, hyperpigmentation of the skin/nails, and alopecia. Leukopenia, anemia, and thrombocytopenia may also occur. A severe complication is haemorrhagic cystitis, which may be prevented by adequate fluid intake and sodium 2-mercaptoethane sulfonate.

CYP shows a carcinogenic and teratogenic activity (86). As a result CYP administration is not allowed in pregnancy and breastfeeding (101). Moreover, temporary or permanent gonadal dysfunction has been described.

Three RCTs evaluated the CS-sparing effect of CYP. Chrysomallis et al. (110) compared oral CS monotherapy with a combined therapy of oral CYP and CS as well as a combined therapy of cyclosporine and CS. No difference in efficacy between these treatments were observed, but adverse events were higher in patients on combination treatment. An RCT comparing intravenous CYP pulse therapy (15 mg/kg monthly) plus CS vs. CS alone showed no conclusive difference in remission and relapse rates, cumulative steroid doses, and adverse events (111). Moreover, RCT regarding CYP pulse therapy (1 g monthly for 6 months, then 1 g every 2 months) plus CS in comparison to prednisolone alone and in combination with adjuvant AZA or MMF showed inconclusive results regarding efficacy and CS-sparing effect (102). Finally, oral methylprednisolone (2 mg/kg/day) combined with AZA (2–2.5 mg/kg/day) and a pulse CYP treatment protocol (500 mg intravenous CYP in combination with 100 mg intravenous dexamethasone for 3 consecutive days) were evaluated in a multicentric prospective RCT and did not show significant differences (112).

Different dexamethasone–CYP pulse therapy regimens were evaluated in two RCTs. In the first study, a combination of dexamethasone i.v. (100 mg on three consecutive days per months), CYP i.v. (500 mg once a month), and oral CYP (50 mg/day) was compared with CYP pulse therapy (15 mg/kg monthly) and prednisolone. In the first group a cutaneous, but not mucosal, response was faster achieved, while in the second group, the remission was seen earlier, but more severe CS-related adverse effects were reported (113). In the second study, patients under oral CYP alone (50 mg daily) and under a combination of dexamethasone i.v. (100 mg on three consecutive days monthly) with CYP i.v. (500 mg monthly) or oral CYP (50 mg daily on days between the pulses) were evaluated. No significant differences were reported regarding relapse rate, anti-Dsg1 and anti-Dsg3 autoantibodies titres, and the presence of tissue-binding autoantibodies by DIF (114).

Dapsone

Dapsone is used alone or in combination with topical clobetasol as first-line therapy in mild PF. Evaluation of serum glucose-6-phosphate dehydrogenase (G6PD) activity is mandatory before administration. The role of DA in the maintenance phase of PV has been evaluated only in one RCT, showing no statistical significance (115).

Methotrexate

Methotrexate (MTX) (10–20 mg/week) is considered a third-line CS-sparing drug in PV (83). In a retrospective single-center study, it has been reported that >80% of PV patients were able to reduce CS after 6 months on adjuvant MTX (15 mg/week) (116). Furthermore, Tran et al. (117) reported in a retrospective single-center study that 70% of PV patients stopped completely CS, mainly after 18 months. These findings support the concept that MTX has a CS-sparing effect in PV.

Cyclosporine

There are only limited data regarding the adjuvant use of cyclosporine in PV. Chrysomallis et al. (110) reported an inconclusive effect of adjuvant cyclosporine and a higher incidence of toxicities in combination treatment with prednisolone. Ioannides et al. (118) showed no advantage of this adjuvant drug over treatment with CS alone. Based on this data, CS is not recommended as adjuvant therapy in PV by the EADV or BAD guidelines (83, 84).

Rituximab

Rituximab—Mode of Action

The putative mode of action of RTX in pemphigus is shown in **Figure 8**. RTX is a chimeric type I monoclonal anti CD20 antibody, consisting of a human Fc portion and a murine variable region which serve as CD20 binding site (119). RTX was first licensed for use in B-cell malignancies (120), however, it is currently used in several autoimmune disorders, and has been recently licensed as first-line treatment in pemphigus (121–124). RTX target, CD20, is a transmembrane receptor, that is expressed across various developmental stages of the B-cell, from the pre-B cell to the mature; while, early precursor pro-B cells and antibody-producing plasma cells do not express it (125). Probably, it functions as a Ca^{2+} channel, that regulates intracellular Ca^{2+} influx through interaction between the intracytoplasmic domain and the activated B-cell receptor (119, 126). Noteworthy, $CD20^{-/-}$ mice display normal B-cell development and function, and no enhanced susceptibility to infections (127). RTX binds near the large extracellular loop of CD20 (128). RTX binding to CD20 induces B-cell depletion by, at least, four different mechanisms: (i) direct induction of programmed cell death, which is dependent on activation of caspases and involves intracellular molecules, including Src kinases, p38 MAPK and NFkB (129–131); (ii) complement-dependent cytotoxicity, that happens when C1s binds to RTX opsonized cells and triggers complement activation and formation of the membrane attack complex (MAC), which eventually induces cell lysis (132); (iii) antibody-dependent cytotoxicity, which consists of activation of NK cells through binding the human Fc portion of RTX to the FcRIII receptor: this activates NK cells to release cytotoxic mediators, including perforins and granzyme B, which induces caspases-dependent cell death in the target lymphocyte (133); (iv) antibody-dependent phagocytosis, in which neutrophils, monocytes and macrophages bind RTX opsonized B-cells through the Fcγ Receptor (132). Recently, a new mechanism, referred to as trogocytosis, or shaving, has been characterized. In trogocytosis, macrophages remove RTX-CD20 complexes by transferring plasma membrane; this triggers cell death through a yet-to-be identified mechanism (134).

RTX is a highly effective therapy in pemphigus (1). By depleting B-cells, RTX leads to marked decrease of circulating anti-Dsg autoantibodies, and, since the pathogenic role of such autoantibodies, significant amelioration of the lesions (135). Multiple lines of evidence, however, suggest that RTX exerts a deep modulation of both humoral and acquired immune function in pemphigus, explaining the fact that, in many cases, disease amelioration lasts longer than B-cell re-appearance in the peripheral blood of the patients (136). First, it should be noted that RTX, in parallel with a drastic decrease of pathogenic autoantibodies, induces drop of total serum IgM, but does not modify total serum IgG, thus suggesting that long-lived plasmablasts accounting for antibody production against microbes are not affected (137). Previously, we showed that in PV patients, RTX induced elevation of B-cell activating factor (BAFF) in parallel with decreasing pathogenic antibody levels and increased IgG titer against Varicella Zoster virus and Epstein Barr virus: thus elevation of BAFF may at least partly exerts a stimulatory role on long-lived plasma blasts (124). Likewise, one study recently showed that while autoimmune blistering disease patients receiving RTX showed reduced circulating memory B-cells against the influenza virus compared to healthy people, they showed comparable recall response to vaccination, suggesting the existence of a memory B-cell compartment, probably resident in lymphoid tissues, which is not depleted by RTX (138). Interestingly, when B-cells re-appear following RTX treatment, there is a substantial increase in the proportion of naive and transitional B-cells and an increased naive/memory B-cell ratio (137). Patients in complete clinical response also display increased number of IL-10-producing regulatory B-cells and absence of Dsg3-IgG^+ B-cells (136). Altogether, these findings suggest that RTX induces a complete reset of the B-cell repertoire in pemphigus, favoring early appearance of immature B-cells and anti-inflammatory regulatory B (Breg) cells, and delayed reappearance of Dsg-specific memory B-cells, which eventually account for disease relapse (136, 139).

Our group demonstrated that, besides pleiotropic effects on B-cells, RTX inhibits auto-reactive Th1 and Th2 cells, by interfering with the T-B cell cross-talk, in which Dsg-specific B-cells probably serve as antigen presenting cells. Decreasing Th1 and Th2 functions occurred early following RTX and lasted around 6 and 12 months, respectively. Conversely, we did not observe inhibition of Dsg-specific regulatory T (Treg)-cells, which account for maintaining peripheral tolerance against Dsg antigens. Finally, we demonstrated that T-cells specific for the tetanus toxoid as well as the total count of $CD3^+CD4^+$ T cells were not decreased by RTX (140). Similar results were found in a subsequent study by Leshem et al. (141), confirming that RTX impairs autoreactive, rather than global T-cell function in pemphigus.

RTX side effects mostly include infections and infusion-related adverse events. In fact, while single RTX infusions may not impair significantly memory responses against previously encountered pathogens (138), patients mounts a defective immune reactions against newly encountered pathogens and serious and life-threatening infections, including sepsis, following RTX treatment have been variously reported (120, 142–147). Opportunistic infections can also occur, including cytomegalovirus and *Pneumocytic jiroveci* infections (148–153). In this regard, it is not known yet whether pemphigus patients receiving RTX may benefit from prophylaxis against *Pneumocystis jiroveci* infection (154). A theoretical risk of reactivation of hepatitis B and C viruses as well as tuberculosis should be also taken into account (138, 155–157). Infusion-related adverse events mostly occur during the time of infusion and include type I hypersensitivity reaction and anaphylaxis, and cytokine release syndrome (158–162), although the latter has never been reported in pemphigus patients (142, 163). Delayed reactions include serum sickness, vasculitis and Steven Johnson syndrome (164–167). Interestingly, there have been some cases of paradoxical pemphigus flares following RTX treatment. In one case, paradoxical pemphigus flare

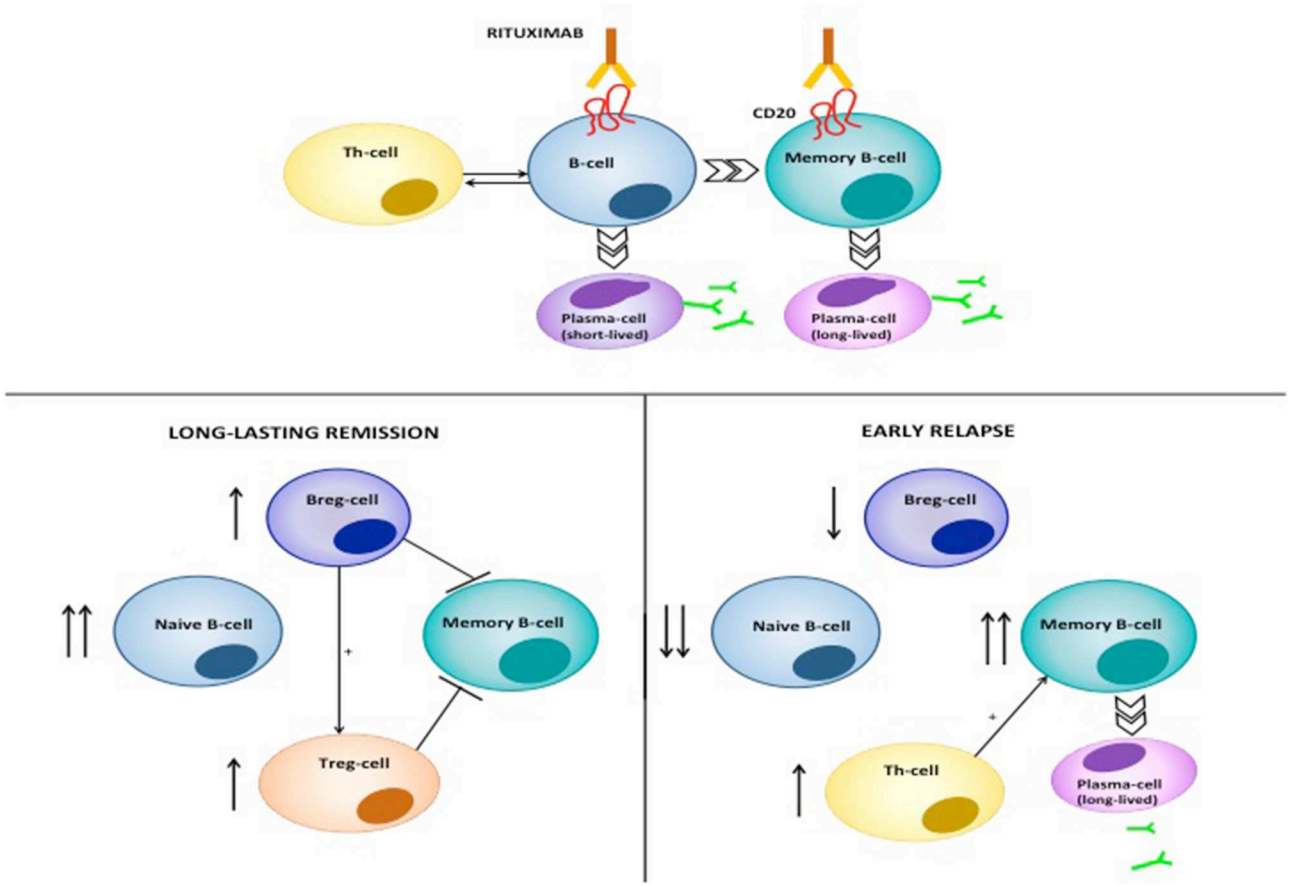

FIGURE 8 | Mode of action of RTX in pemphigus. RTX induces depletion of B-cells and lymphoid resident memory B-cells by different mechanisms, including direct cell apoptosis, complement-dependent cytotoxicity and antibody-dependent cytotoxicity. The latter consists of the phagocytosis of opsonized B-cells by neutrophils, monocytes and macrophages, which express the Fcγ Receptor. Moreover, RTX significantly decreases T-cell function, by depleting antigen presenting B cells. Patients achieving durable responses have an increased naïve/memory B-cell ratio. Regulatory B-cells (B-regs) and regulatory T-cells (T-regs) are also increased, and are inhibitory on Dsg3-specific memory B-cells. On the contrary, patients with early relapses following B-cell repopulation have a decreased naïve/memory B-cell ratio. Reappearance of auto-reactive, Dsg3-specific T-cells contributes to activation of autoreactive B-cells and subsequent anti-Dsg IgG production.

was accompanied by increased serum concentrations of anti-Dsg3 IgG autoantibodies (168). In any case, disease flare predicted treatment failure. Suggested underlying mechanisms include increased cytokine release from apoptotic B-cells, immediate depletion of regulatory B-cells or a transient lymphocyte activation following RTX-CD20 interaction (141, 163, 168–171).

There is much controversy about the optimal RTX dose in pemphigus. Two main protocols are used: the rheumatoid arthritis protocol, which consists of two 1,000 mg infusions 2 weeks apart, and the lymphoma protocol, which consists of four 500 mg infusions once a week (135). There are not yet randomized trials assessing which protocol is better in terms of efficacy and safety. On the other hand, high dose regimens should be preferred instead of low-dose regimens, due to longer disease response (163). Nowadays, one study recently reported on a pemphigus patient achieving successful disease remission with an ultra-low dose of RTX (200 mg in a single infusion), with persistent B-cell depletion after 6-month follow-up (172, 173). This highlights the need of further investigating individual factors that may influence RTX efficacy in an effort to personalize treatment schedules and optimize the safety.

Rituximab in Pemphigus Vulgaris and Pemphigus Foliaceus

RTX was initially shown to be effective for pemphigus patients resistant to standard immunosuppressive therapies. In one study in 2006, Ahmed et al. (174) reported complete clinical remission, allowing successful tapering of immunosuppressive therapies, in 9 out of 11 patients with refractory pemphigus following a protocol combining IVIg and 10 RTX infusions over a 6 months period (**Table 1**). After 10-year follow-up most patients were shown to maintain clinical remission (175). In 2007, in a larger clinical series, Joly et al. (176) reported durable clinical response with significant corticosteroid sparing effects in 86% of 21 patients with refractory pemphigus following a single cycle of RTX. Similar results were reported in a series of 42 patients by Cianchini et al. (177), they also observed that a recall infusion

of 500 mg was effective for patients who relapsed following initial disease control. One study including 136 patients with refractory pemphigus from four different European countries reported a 95% overall response rate, with two third of patients achieving complete remission (178). Likewise, review articles and meta-analysis estimated complete clinical remission occurring in 76–90% of patients within a median time of ∼6 months (146, 163). Mean remission duration ranged from 15 to 17 months, although only a small percentage of patients maintained remission off therapy (146, 163). In two studies, RTX use early in the disease course resulted in significantly higher and longer clinical response (136, 179). The same finding emerged also in a study by Amber and Hertl, reviewing clinical outcomes of 155 pemphigus patients treated with a single cycle of RTX (179, 180). These preliminary observations have lead different research groups to investigate the potential benefit of RTX applied as a first line therapy in pemphigus. Indeed, first-line therapy with RTX, combined either with high potency topical corticosteroids or IVIg, was shown to be effective in pemphigus patients with contraindication to systemic steroids (181, 182). One retrospective study found significantly higher rate of complete remission off immunosuppressive therapy in patients who were administered RTX as a first-line steroid sparing agent compared to patients who received RTX after failing other immunosuppressants (183). In 2017 a large prospective randomized trial, comparing RTX combined with a short course of prednisone vs. prednisone alone in patients with newly diagnosed pemphigus, demonstrated significantly higher complete remission rate off therapy in patients receiving RTX, resulting in a dramatic decrease of the cumulative steroid dose and significantly fewer adverse events. Furthermore, re-treatment with a single RTX dose of 500 mg after 12 and 18 months was highly effective and well-tolerated in achieving long-term clinical remissions (88). Interestingly, also patients with PF, in whom the rate of remission with RTX was estimated around 50% in the refractory setting, were shown to respond well to RTX when applied as a first line therapy (88, 184–186). In summary, while RTX was initially recommended as a third line therapy in patients without adequate disease control with standard immunosuppressants, several studies have definitively demonstrated that patients may benefit from early RTX treatment, in terms of both clinical efficacy and safety, leading current guidelines to recommend it as the gold standard for new onset pemphigus (187, 188).

Rituximab in Paraneoplastic Pemphigus

PNP usually occurs secondary to B-cell neoplasms, hence RTX appears to be a reasonable treatment, targeting both autoreactive and malignant B-cells (189–191). Several cases in the literature have shown remarkable responses in B-cell malignancies-associated PNP using RTX either alone or in combination with immunosuppressants or chemotherapy (192–195). However, the overall efficacy of RTX in PNP is much less consistent than PV and PF (196); mucosal lesions were shown to be particularly resistant to RTX treatment (197). Moreover, some authors pointed out that RTX treatment of the underlying malignancy may paradoxically trigger PNP (198).

PNP combines clinical and histologic features of PV and lichenoid/interface dermatitis, reflecting a mixed B- and T-cell response against epidermal autoantigens. Indeed, while declining B-cells autoreactivity, RTX may be ineffective against clinical manifestations secondary to auto-reactive T-cells activation (199). Indeed, RTX use to treat the underlying malignancy may lead to overlook the diagnosis of PNP in patients presenting with lichenoid dermatitis or toxic epidermal necrolysis-like lesions without detectable circulating autoantibodies and negative DIF. This was recently hypothesized in an interesting study by Kwatra et al. where the authors observed a significant reduction in the cases of B-cell lymphoma-associated PNP from 2011 to 2017 compared to the period from 2003 to 2010. All the patients diagnosed with PNP during or after 2011 had already received RTX; whereas, most of the cases before 2011 did not (199).

Is There Evidence for a Maintenance Therapy With Rituximab in Pemphigus?

Relapse following RTX occurs in about 40–80% of the patients (163), within a mean time ranging from 6 to 24 months (153, 200). Additional cycles of RTX were shown to be effective in relapsed patients, suggesting that patients may benefit from maintenance with RTX (88, 176, 177). However, the exact timing of RTX re-treatment to prevent relapse is uncertain (187). Re-treatment at 6 months has been adopted empirically, but it is not currently validated in the setting of randomized trials (201). Noteworthy, there is a subset of patients who achieves long-lasting remission even with a single cycle of RTX (136, 176, 202). In these patients, additional prophylactic cycles of RTX not only result in unneeded costs, but also substantially increase the risk of adverse events. Developing biomarkers that could identify patients at higher risk of relapse following RTX is therefore an urgent need.

Relapse following RTX can be attributed to persistence of autoreactive B-cells, because of incomplete B-cell depletion, or re-appearance of Dsg-specific B-cell clones during B-cell repopulation. Thus, monitoring the B-cell repertoire appears to be a suitable tool to predict the risk of relapse following RTX. In one study, shorter time to relapse was found in patients receiving adjuvant immunosuppressants during and following RTX treatment, suggesting a possible effect of prolonged immunosuppression on immunosurveillance, favoring an early re-appearance of autoreactive B-cells (203). In 2008, a consensus of German experts recommended checking the number of $CD19^+$ B-cells in the blood at baseline and after RTX treatment (204). In one retrospective study by Albers et al. (200) including 62 pemphigus patients treated with a total of 99 RTX cycles, the number of $CD19^+$ B-cells were shown to be a useful predictor of relapse. A time to B-cell repopulation lower than 12 months also correlated with the risk of relapse. However, relapse has reportedly occurred even before B-cell re-population (205). In these cases, it is conceivable that lymphoid tissues served as a reservoir for autoreactive B-cells and protected them from RTX.

Longitudinal analysis of the naïve/memory B-cells ratio and the number of B-regs may also provide useful information (206). Interestingly, Albers et al. (200) found an inverted correlation

between $CD4^+$ T-cell counts following RTX and the risk of relapse. Autoreactive T-cells are essential in pemphigus to orchestrate the B-cell responses and autoantibody production against Dsgs. RTX also is effective in decreasing peripheral T-cell response against Dsgs (140). Thus, high $CD4^+$ T-cells count would be expected to predict relapse. However, Albers et al. (200) speculated that the protective role of $CD4^+$ T-cells could be attributed to Treg cells, which prevented further expansion of autoreactive B-cell clones. Levels of circulating anti-Dsg autoantibodies were also shown to be involved in the relapse of patients with pemphigus (207). In Albers et al. (200), levels of anti-Dsg3 IgG were predictive of relapse in patients with mucocutaneous and mucosal disease, whereas anti-Dsg1 IgG were predictive for the subset of mucosal PV patients with cutaneous involvement. However, in other studies, elevation of anti-Dsg3 IgG was also noted in patients maintaining a clinical remission, suggesting that in some cases anti-Dsg3 IgG may target non-pathogenic epitopes of the Dsg3 ectodomain (208). Mouquet et al. (137) found that elevation of Dsg1 autoantibodies was associated with early relapse following RTX.

In a prospective study, a high baseline index of anti-Dsg1 IgG was found in early relapsing patients compared to late relapsing patients following RTX. Baseline anti-Dsg1 IgG, but not Dsg3-IgG, indeed showed a significant positive correlation with a risk of relapse within 12 months after RTX treatment, thus suggesting that patients with high anti-Dsg1 IgG before treatment deserve a close monitoring during the 12 months following treatment, or at least may benefit from a prophylactic RTX dose during the first 12 months. Also in this study, later B-cell repopulation was found in patients experiencing a late relapse compared to patients experiencing an early relapse (209). By contrast, in a retrospective study including 40 pemphigus patients treated with RTX, mucosal involvement was found to be associated with a poor clinical outcome and relapse (210).

Intravenous Immunoglobulin

IVIg consist of human plasma-derived IgG, sugars, salts and solvents. IVIg derived from large plasma pools. Albeit not immunosuppressive, they exerts various anti-inflammatory effects, including Fc receptor blockade, stimulation of antibodies production against different subclasses of T lymphocytes, inhibition of different T-cell functions, complement hindrance via inactivating C3 precursors, dendritic cell downregulation, B-lymphocyte apoptosis, inhibition of phagocytosis, and increment of response to steroids (211). However, the main mode of action is an increased catabolism of immunoglobulins via binding to the neonatal Fc receptor (FcRn) (211).

A dose of 2 g per kg body weight per treatment cycle is recommended. High-dose IVIg was shown to independently increase disease control in pemphigus (85). IVIg is mostly used as an adjuvant therapy to CS and immunosuppressive drugs in recalcitrant PV. Indeed, Amagai et al. (212) reported that high dose IVIg (0.4 g/kg per day) over 5 days resulted in significantly reduced disease activity and autoantibody titres in 51 patients with CS-resistant PV. Furthermore, Svecova et al. (213) reported a significant improvement in Pemphigus Disease Area Index (PDAI) and a reduction of 90% of the CS dose in a cohort of 10 CS-resistant PV. Combination of IVIg and RTX also demonstrated efficacy (**Table 2**).

Adverse effects have been reported in $< 5\%$ of patients and occur more often in patients who are IVIg-naive or at risk of bacterial infections (217, 218). Immediate adverse effects (occurring within the first hour of infusion) include headache, nausea, fever, tachycardia, malaise, arthralgia, and dyspnoea. Delayed reactions include headache, acute renal failure, thromboembolic events, and pseudohyponatremia (218). Myocardial infarction, thrombosis, pulmonary embolus and Stevens–Johnson syndrome, have been also described. Thrombosis can be provoked by hypercoagulability due to increased blood viscosity, augmented fibrinogen production, and raised platelet activity (219). Therefore, high-risk patients, such as elderly or people affected by hypertension or coronary heart disease, should be screened appropriately and prophylactic anticoagulation need to be considered. In a series of 54 patients on IVIg treatment, an incidence of aseptic meningitis of 11% has been reported. Risk factors were a previous history of migraine and high-dose IVIg regimen (220). Finally transient acute kidney injury has been also described (221).

Immunoadsorption

IA consists of the passive removal of IgG from the patient's systemic circulation. In IA, the blood is passed through adsorber columns, in which molecules with high affinity for IgG, i.g. protein A (Immunosorba®) or the synthetic peptide PGAM146 (Globaffin®), function as a ligand (135, 222). Basic principles of IA are, indeed, similar to plasmapheresis, but, compared to the latter, IA does not remove plasma proteins, such as albumin and clotting factors. The use of plasmapheresis in pemphigus has been largely abandoned due to significant incidence of serious adverse events, such as sepsis (223). The fact that IA does not require replacement of fresh frozen plasma and albumins allows processing higher plasma volume per treatment session, resulting in a lower, albeit not abolished, risk of adverse events. Nevertheless, infections are still the most frequently encountered complication and can occur either secondary to the IA procedure, i.g. cathether-associated infections, or secondary to decreased serum concentrations of protective antibodies (224–226).

IA is an ideal treatment for pemphigus patients with severe and extensive disease at baseline. Combining IA with immunosuppressive therapies provides faster clinical responses compared to the immunosuppressive therapy alone, since IA allows immediate removal of pathogenic antibodies, whose serum concentration reflects both disease activity and severity. Once circulating antibodies are removed, a positive gradient between the skin and blood leads skin-bound autoantibodies to move into the systemic circulation. To avoid a rebound increase of the autoantibody titer, IA is therefore performed on 3 or 4 consecutive days, and then repeated on a monthly base based on the disease response, autoantibody serum concentrations and treatment tolerability (227).

Current guidelines indicate IA as a reliable first-line treatment in pemphigus patients, in whom lesions cover (1) $> 30\%$ of the body surface or (2) $> 25\%$ of oral or genital mucous membranes or involve (3) the conjunctiva or (4) the esophagus; it can

TABLE 2 | Combination therapy of rituximab with intravenous immunoglobulins (IVIg) or immunoadsorption (IA).

Study	Study design	Synopsis	Adverse events
RTX PLUS IVIg			
Ahmed et al. (174)	Prospective, including 11 refractory PV patients, treated with 2 cycles of RTX 375 mg/Kg/m^2 once a week for 3 weeks followed by a cycle of IVIg 2 g/Kg in the fourth week. Maintenance with RTX and IVIg infusions once a month for 4 months	9 out of 11 patients achieved a complete remission in parallel with a rapid decreasing of the serum concentrations of anti-Dsg autoantibodies, which allowed successful discontinuation of steroids and adjuvant immunosuppressants. Clinical responses lasted 22–37 months (follow-up after discontinuation of RTX: 15–37 months). In 2 patients experiencing a relapse, retreatment with RTX was effective	No relevant side effects reported, including infections or infusion-related reactions
Ahmed et al. (175)	10-year follow-up study of Ahmed et al. (174)	All the 10 patients previously treated with RTX and IVIg retained clinical remission after 10 years from the last RTX infusion	Not observed
Feldman et al. (205)	Retrospective, including 19 patients with refractory pemphigus. RTX was given at week 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 20, 24. IVIg were given at week 0, 4, 8, 12, 16, 20, 24	11 patients achieved long-term remission, allowing discontinuation of corticosteroids and other immunosuppressants. 8 suffered at total of 15 relapses. Re-treatment with RTX and IVIg was effective in achieving a long-term remission. Relapse was associated with incomplete B-cell depletion, B-cell repopulation and raise of serum anti-Dsg autoantibodies	Not reported
RTX PLUS IA			
Behzad et al. (208)	Retrospective, including 10 patients with refractory PV. IA was administered at 4-week intervals, followed by RTX according to either the lymphoma protocol or the RA protocol	8 out of 10 patients obtained a complete remission on therapy at 6 months following the first IA treatment. In 6 of them complete remission on therapy persisted at 12 months. Treatment with IA and RTX leads to a successful tapering of oral prednisone	No serious adverse events reported
Kasperkiewicz et al. (214)	Clinical series including 23 consecutive patients. IA was given on 3 consecutive days and repeated at initially 3 and then 4 weeks until lesions clearance of 90%. RTX 1,000 mg was infused at weeks 1 and 3. Patients also received intravenous dexamethasone pulses and oral azathioprine or mycophenolic acid	19 patients achieved long-term complete remission. Over a period of a mean of 29-month follow-up, 6 patients suffered a relapse	A *Staphylococcus aureus sepsis* associated with an infected central intravenous line and an episode of extensive herpes simplex infection
Kolesnik et al. (215)	Retrospective, including 4 patients with PV and 2 with PF. The treatment protocol included a combination of protein A IA and RTX (375 mg/m^2 once a week the day after each IA session) (Magdeburg treatment protocol). Patients with sub-epidermal blistering dermatoses were also included	Complete or partial remission was observed in 88 and 12% of patients, respectively, within an average follow-up of 22 months. Relapse occurred in one patient with PF. Treatment was associated with a substantial decrease of serum autoantibody concentrations	Erysipelas at the lower leg of one patient due to trauma
RTX PLUS IVIg PLUS IA			
Shimanovich et al. (216)	Clinical series, including 5 patients with PV and 2 with PF. Treatment included a combination of protein A IA and RTX (375 mg/m^2 once a week per 4 consecutive weeks). All patients received adjuvant immunosuppressive therapies. IVIg were given in non-responder patients	Long-term remission was achieved by 3 patients. Partial remission was induced in 1. Three refractory patients achieved long-term disease control following IVIg therapy	*Staphylococcus aureus* bacteremia, deep venous thrombosis and *P. carinii* pneumonia

RTX, rituximab; IA, immunoadsorption; IVIG, intravenous immunoglobulins; PV, pemphigus vulgaris; PF, pemphigus foliaceus; RA, rheumatoid arthritis.

be also recommended in refractory patients with more than 3 months of active disease despite at least two immunosuppressive therapies (84, 187, 188).

Possibly, IA may also exert immunomodulating properties, which account for its synergistic effect in combination with RTX (**Table 2**). Accordingly, in a study by Amber and Hertl, IA was the only adjuvant treatment resulting in a lower risk of relapse following RTX (180). Indeed two studies, including one by our group, demonstrated rapid and durable clinical response combining RTX and IA with or without oral immunosuppressants (208, 214). Moreover, a yet unpublished German multicentric prospective randomized trial comparing IA plus the best medical therapy vs. the best medical therapy alone found that the adjunct of IA resulted in faster withdrawing

prednisone and reduced cumulative steroid dose to achieve pemphigus remission (DRKS 00000566).

Langenhan et al. (228) developed specific adsorbers using Dsg1 and Dsg3 ectodomains as a ligand which were shown to efficiently remove pathogenic autoantibodies by 25 and 21%, respectively, without significant variation of anti-EBNA 1 IgG. The same group demonstrated that Dsg3/Dsg1 specific IA eliminated the capacity of PV sera to induce Dsg3 internalization *in vitro* and blistering in neonatal mice (229). Hopefully, future clinical application of these absorbers would lead to increase the safety of IA, reducing the incidence of infections secondary to hypogammaglobulinemia.

Double filtration plasmapheresis (DFPP) is a relatively new procedure that, similar to IA, removes selectively immunoglobulins, while minimizing the loss of albumin. In small case series and one retrospective study DFPP also demonstrated efficacy in drug resistant pemphigus (230–232).

EMERGING THERAPIES

B-Cell Therapies Other Than Rituximab

Although RTX has dramatically improved the overall prognosis of pemphigus, treatment failure or early relapse may be observed. RTX is a chimeric monoclonal antibody, whose murine component is thought to be responsible for the observed allergic reactions during the infusion. However, it also accounts for the appearance of human anti-chimeric antibodies (HACAT), which may potentially limit the efficacy of the drug (233). Over the recent years, different monoclonal antibodies targeting CD20 have been developed. Second generation anti-CD20 mAb differ from RTX in that they are humanized or fully human, taking advantage of being less immunogenic (234) (**Figure 9**). Amongst second-generation anti-CD20 monoclonal antibodies, ofatumumab has been the first to be approved. It is a type I anti-CD20 monoclonal antibodies that targets the extracellular portion of CD20 close to the B-cell membrane, resulting in a more potent complement-dependent cytotoxicity compared to RTX (235). Ofatumumab demonstrated efficacy in a patient with pemphigus, in whom RTX loses efficacy presumably because of the appearance of HACAT (236). Unfortunately, a randomized controlled trial of ofatumumab in pemphigus has been prematurely terminated due to financial restrictions (186). Similarly, Ellebrecht et al. (237) described successful treatment with subcutaneous veltuzmab in a pemphigus patient who only achieved a partial remission with RTX. Veltuzumab is a type I humanized anti CD20 monoclonal antibody that has similar complementary-determining regions of RTX, but a 2.7-fold greater binding avidity and effect on complement-dependent cytotoxicity than RTX. It can be also administered subcutaneously, resulting in lower side effects than intravenous RTX (234).

Obinutuzumab is a third generation glycoengineered type II humanized anti-CD20 monoclonal antibody. Differently from type I monoclonal antibodies, obinutuzumab shows enhanced capacity to induce direct apoptosis (via a caspase-independent factor) and antibody-dependent cytotoxicity, whereas it does not induce complement-dependent cytotoxicity (238–240). Obinutuzumab has shown to induce superior B-cell depletion compared to RTX in blood samples from patients with rheumatoid arthritis and systemic lupus erythematosus. More intriguingly, it has been shown to induce significant cytotoxicicty also in naive and class-switched memory B-cells, a high number of which may be implicated in early relapse following RTX treatment in pemphigus (241). Other therapies of interest include belimumab and atacicept, a monoclonal human IgG1 antibody and a fully human recombinant fusion protein, which respectively, target BAFF and a proliferating-induced ligand (APRIL), which are involved in B-cell differentiation in antibody producing plasma cells (242, 243). It is worth mentioning that a monoclonal anti-BAFF-receptor antibody (VAY736) is being investigated in a randomized, partial-blind, placebo- controlled multicentre trial (NCT01930175) (125).

Bruton kinase (BTK) inhibitors are also a promising B-cell targeting therapy in pemphigus. BTK is a member of the Tec family of kinases, which is mainly expressed on B-cells, excluding antibody-producing plasma cells. Activation of BTK following antigen-recognition by the BCR activates different downstream molecules including p38MAPK, MEK/ERK, and NFkB, whose related signals are key regulator of B-cells survival, proliferation, maturation and antigen-presentation (244, 245). BTK inhibitors such as ibrutinib have shown impressive clinical responses in patients with B-cell malignancies, but also hold promise for the treatment of autoimmune disorders. In particular, over-activation of BTK have been shown to drive autoimmunity by enhancing autoantibody production and class switching, promoting B-T cell cross talk and peripheral B-cell loss of tolerance (244). Furthermore, enhanced expression of BTK in B-cells induces differentiation of T follicular helper cells, which have been shown to be involved in the pathogenesis of pemphigus (67, 246). Interestingly, ibrutinib has been successfully used in two cases of PNP associated with B-cell malignancies (247, 248). The efficacy of PRN1008, an oral inhibitor of BTK is currently being evaluated in a clinical trial (NCT02704429) (125, 249).

Monoclonal antibodies targeting $CD19^+$ (a B-cell surface molecule which is also expressed on antibody producing plasma cells) such as inebilizumab, would be an effective strategy in pemphigus, since persistence of long-lived plasmablasts producing anti-Dsg IgG autoantibodies is presumably amongst the mechanisms of disease resistance to RTX treatment (125). **Figure 9** summarizes how emerging anti-B-cell therapies works in pemphigus.

Chimeric Autoantibody Receptor (CAAR)-T Cell: A Most Promising Treatment Approach in Pemphigus

Chimeric antigen receptor (CAR)-T-cell therapy has shown remarkable efficacy in otherwise untreatable hematologic malignancies (250). Currently, two CD19-directed CAR-T-cell therapy, tisagenlecleucel and axicabtagene ciloleucel have been approved for treatment of heavily refractory/relapsed acute lymphoblastic leukemia and B-cell aggressive lymphomas (251–254). The noteworthy antitumor activity of CAR-T cells in hematologic malignancies has recently led to

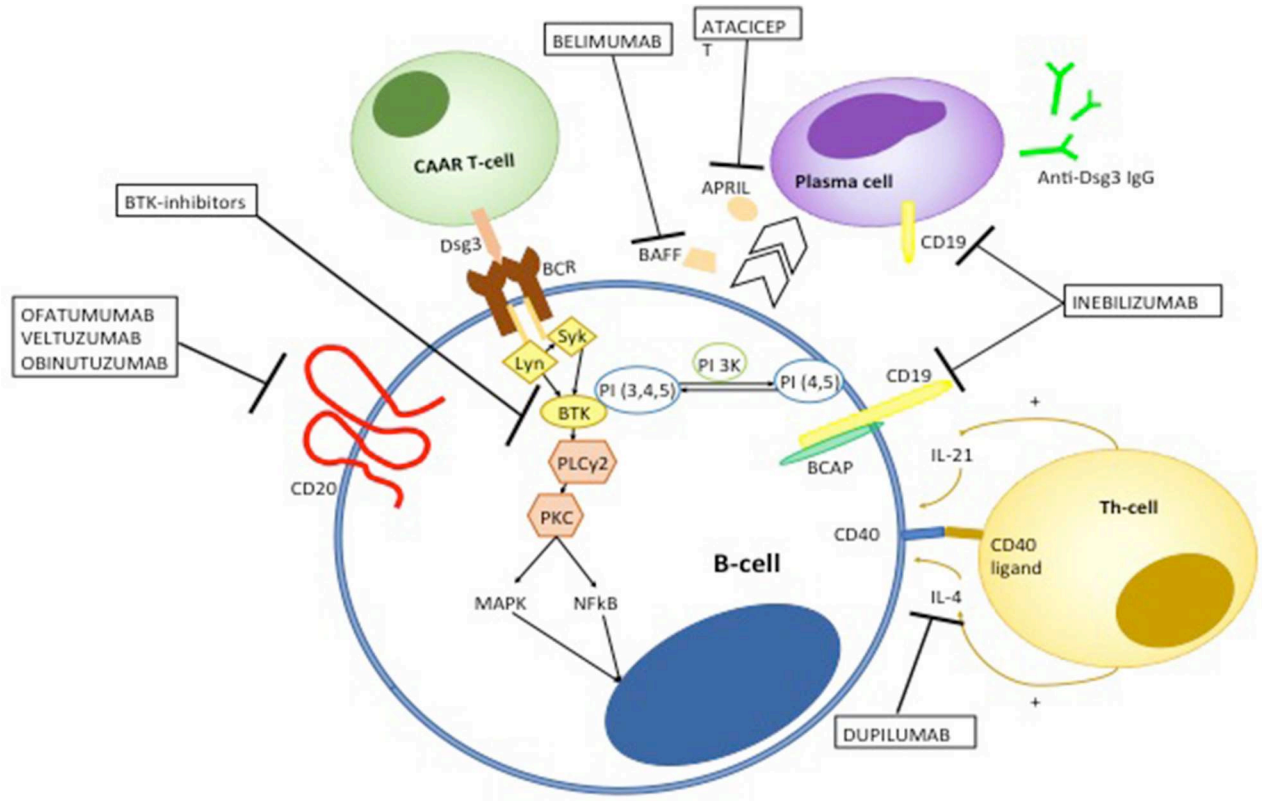

FIGURE 9 | Emerging therapies targeting auto-reactive B and T-cells in pemphigus. Ofatumumab, veltuzumab, and obinutuzumab are fully human or humanized monoclonal antibodies targeting CD20. Ofatumumab and veltuzumab are class I anti-CD20 monoclonal antibody, with a higher capacity of binding CD20 and inducing complement-dependent cytotoxicity compared to RTX. Obinutuzumab is a class II anti-CD20 monoclonal antibody, that has an increased affinity to the FcγIII receptor, resulting in a more potent antibody-dependent cytotoxicity. Bruton kinase (BTK) inhibitors interfere with B cell activation. BCR signaling induces migration of BTK from the cytosol to the cell membrane, though the interaction with phosphatidylinositol 3,4,5-triphosfate generated by phosphoinositide 3-kinase (PI3K). BTK is activated by Lyn and Syk and then actives downstream molecules including phospholipase C gamma 2 (PLCγ2) and Protein kinase C. The latter in turns activate different pro-inflammatory pathways including mitogen associated protein kinases (MAPK) and Nuclear Factor k B (NFkB). Chimeric autoantibody receptor (CAAR)-T-cells are engineered T-lymphocytes which express Dsg3 ectodomain, which allows recognition and subsequent killing of B-cells targeting Dsg3. Belimumab and atacicept target B-cell derived B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), respectively, which promote differentiation toward autoantibody-producing plasma cells. Inebilizumab is a monoclonal antibody targeting CD19 which is not only expressed on B cells but also plasma cells. Dupilumamb is a monoclonal antibody targeting interleukin (IL-4), which is one the main cytokine produced by T helper 2 cells and T follicular helper cell which induces autoantibody production by autoreactive B-cells.

investigate potential clinical application of such a therapy in solid tumors (255–257); an intriguing scenario has been also opened with regard to autoimmune diseases (258). CAR-T cell therapy represents an example of adoptive cell transfer therapy: patient's derived T-cells are modified *ex-vivo* to express a CAR, which allows selective recognition of the antigen of interest and consequent killing, via an MHC-unrestricted manner, of the antigen-bearing cells (259–261).

CARs are fusion proteins whose structure comprises three domains: (i) the extracellular domain, which consists of a single chain variable fragment and serves as antigen recognition domain; (ii) the transmembrane domain; and (iii) the intracellular domain, which consists of the zeta (ζ) chain of the CD3, a component of the endogenous T-cell receptor (262). In second and third-generation CARs, the intracellular domain is linked to co-stimulatory molecules, specifically 4-IBB and CD28, which promote survival and proliferation of CAR-T cells following antigen recognition, resulting in higher clinical efficacy (263). Production of CAR-T-cells requires different steps. Briefly, the CAR protein is cloned into lentiviral or retroviral plasmids. Viral vectors are then transfected to packaging cell lines, such as HEK293 cells, in order to obtain large amounts of the CAR-bearing plasmids. Patient's T-cells, which are obtained by leukapheresis, are incubated with the viral vector, which enters into the cells and introduces the CAR encoding-RNA. The latter is reverse-transcribed in DNA and stably integrates into the T-cell genome. The CAR protein can be then transcribed and translated and eventually expressed on the T-cell surface. CAR T-cells are finally expanded, concentrated and cryopreserved to be then re-infused into the patient (263, 264).

Recently, Ellebrecht et al. (265) created a "chimeric autoantibody receptor" (CAAR), whose extracellular domain consisted of Dsg3 fragments. T-cells engineered to express Dsg3 CAAR were shown to selectively target human anti-Dsg3 B-cells *in vitro*. Anti-Dsg3 antibodies derived from patients' sera did not abolish CAAR-T cells activity. In a PV murine model, CAAR-T cells reduced pathogenic IgG antibodies and ameliorated disease severity. Interestingly CAAR-T-cells were shown to target B-cells bearing antibodies against different Dsg3 epitopes, providing support for their efficacy in a disease typically characterized by oligo-clonality (265, 266). Apart from the possibility that Dsg3-CAAR T-cells could potentially target keratinocytes expressing desmocollins and desmogleins, which physiologically binds to Dsg3, the authors did not observe any significant toxicity (the so-called off-target toxicity) against keratinocytes. Finally, the authors reported similar activity against anti-Dsg3 B-cells between CAAR-T-cells and $CD19^+$ CAR T-cells, hence suggesting that selective target of self-reactive B-lymphocytes does not result in reduced treatment efficacy (265).

A major advantage of CAAR-T cell therapy in PV involves the possibility to target memory B cells, which still accounts for the observed relapse following anti CD20 therapies (136, 267). Moreover, since part of CAAR-T-cells differentiates toward memory cells (267), this may prevent future formation and expansion of Dsg 3-reactive B-cells, conferring such a kind of "immunity against autoimmunity." A second advantage of CAAR-T-cell therapy is reduced immunosuppression, since normal B-cells are not expected to be killed (265).

Since auto-reactive B-cells in pemphigus account for only a minor subset of total B-cells, it is unlikely that CAAR-T-cell therapy may lead to serious adverse events as has been observed in the onco-hematologic setting (265, 268).

One potential limitation of Dsg3 CAAR-T-cell therapy in pemphigus IgG-driven autoimmunity not only against Dsg3 but also against Dsg1. Moreover, patients with Dsg3 mucosal dominant-PV were shown to possibly relapse as PF (or vice versa), probably via intermolecular epitope spreading (269, 270). These findings highlight the importance of targeting simultaneously both Dsg1 and Dsg3 reactive B-cells. A major challenge of CAR-T cell therapy in cancer is the ability of neoplastic cells to escape by down-regulating the expression of the target antigen. A novel strategy to overcome tumor antigen loss is modifying individual T cells with two distinct CAR molecules (dual-signaling CAR) or with one CAR molecule containing two different binding domains, referred to as Tandem CAR (271, 272). A similar strategy could be adopted to develop CAAR-T-cells targeting at the same time Dsg1 and Dsg3 reactive B-cells.

Also, another intriguing therapeutic perspective would be developing CAR-Treg cells to down-regulate ongoing immune reaction against Dsg (273, 274). In this regard, of interest is a study by Fransson et al. involving a murine model of autoimmune encephalitis, in which mouse $CD4^+$ T cells were modified to express a CAR targeting myelin oligodendrocyte glycoprotein (MOG) in trans with the murine FoxP3 gene promoting Treg differentiation. Myelin targeting Treg cells successfully suppressed inflammation and improved symptoms in the treated mice (275).

Another potential limitation of CAAR-T-cell therapy in pemphigus includes prohibitive costs. For example, one single infusion of the CD19 CAR T-cell products costs around $ 373,000–475,000 (276). However, taking into account the economic impact of a virtually life-long immunosuppressive therapy, if the aim of pemphigus cure with front-line CAAR T-cell therapy were to be realized, reductions in cumulative spending may conceivably occur. **Figure 9** represents how CAAR-T cells works in pemphigus.

Targeting the Neonatal Fc Receptor in Pemphigus

FcRn is a heterodimer including the MHC class I-like H chain and the β_2-microglobulin L chain (277). This receptor is involved in the transport of IgG from mother to fetus; different studies however have shown that FcRn also plays a critical role in regulating IgG homeostasis; accordingly, when IgG enter into FcRn-expressing cells, the IgG-FcRn binding in acidified endosomes avoids degradation of IgG and allows subsequent recycle and release of IgG in the extracellular space at a near-neutral pH. IgM and IgA do not bind to FcRn and consequently have a reduced half-life compared to IgG (278, 279). Blocking the interaction between FcRn and pathogenic autoantibodies in autoimmune diseases may thereby accelerate pathogenic autoantibody catabolism (280). Interestingly, keratinocytes have been shown to express FcRn (281). Indeed, different studies have demonstrated a critical role of FcRn in pemphigus. Knock-out mouse lacking FcRn do not develop acantholysis by passive transfer of anti-Dsg antibodies (282). Therapeutic efficacy of IVIg can be at least in part attributed to saturation of FcRn, allowing faster degradation of anti-Dsg autoantibodies (282). *In vitro*, an excess of normal IgG accordingly protects cultured keratinocytes from anti-Dsg autoantibodies-induced apoptolysis (283). One recent study demonstrated that FcRn induces enter of anti-mitochondrial antibodies, which are also found in pemphigus sera and contribute to keratinocyte shrinkage due to mithocondial damage. In this study, blocking of FcRn abolished the capacity of PV sera to cause detachment of keratinocytes *in vitro* (284). In another study, Recke et al. (285) found that an allelic variant harboring an amino acid replacement of His435 to Arg favoring high affinity of IgG3 to FcRn was associated with an increase risk of PV. Altogether these studies raise evidence for a potential benefit of targeting FcRn in pemphigus. In 2018, the results of a randomized double-blind placebo-controlled first in-human study on efgartigimod, an antagonist of FcRN, including 62 healthy volunteers showed that the drug was effective in reducing IgG levels by about 75% on after multiple dosing (286). The treatment was well-tolerated and no serious adverse events were recorded (286). A phase II study evaluating the safety and efficacy of ARGX-113, a human IgG1-derived Fc fragments binding

to FcRn in patients with PV and PF is currently ongoing (NCT03334058) (287).

Targeting T-Cells and the T-B Cell Cross Talk in Pemphigus

Autoreactive T-cells are critically involved in the pathogenesis of pemphigus (288). Both Th1 and Th2 cells reacting against Dsg were detected in patients with pemphigus at different stages of the disease. However, while Dsg3-reactive Th1 cells can be found in healthy individuals carrying PV predisposing HLA class II alleles, Dsg3-reactive Th2 cells are restricted to pemphigus patients (289, 290). In a recent study by our group, we demonstrated that patients with PV developed a predominant Th2-type response against Dsg3, in contrast to patients with lichen planus, who developed a Th1-type response against the identical autoantigen (291). Indeed, high amount of serum IL-4 sustaining autoantibody production by autoreactive B-cells have been found in pemphigus patients (292). Our group demonstrated the presence of IgE targeting Dsg3 in patients with pemphigus, which further supports the critical role of Th2 cells in orchestrating the inflammatory response and autoantibody production in pemphigus (293). Interestingly, dupilumab, a monoclonal antibody targeting IL-4 has been recently shown to be effective in bullous pemphigoid, a disease in which Th2 cells targeting BP180 and both IgG and IgE against BP180 have also a prominent pathogenic role (294, 295). As far as we are aware, no studies have yet investigated the efficacy of dupilumab in pemphigus. However, pathogenic evidence suggests that the drug may be of potential benefit (**Figure 6**). In parallel to activation of Th2 cells, in pemphigus there is a marked down-regulation of Dsg-specific Foxp3-expressing Treg cells (289). In mouse experiments, Treg cells created in Dsg3$^{-/-}$ mice and transferred into mice with PV were able to reduce autoantibody production (296). Thus, enhancing Treg functions in pemphigus appears to be a promising strategy to restore the lost immune tolerance against Dsg. Infusion of autologous polyclonal regulatory T cells is currently being studied in a phase 1 open-label multicenter trial in patients with active pemphigus vulgaris and pemphigus foliaceus (NCT03239470) (135, 296).

Current work of our group aims at restoring immune tolerance to Dsg3 via targeting of autoreactive T cells. Specifically, we observed in a preclinical mouse model of pemphigus that injections of immunodominant HLA-DRß1*04:02-binding T cell epitopes of Dsg3 conjugated either to antigen-presenting cells lacking a second co-stimulatory signal or to nanoparticles prevented the production of pathogenic anti-Dsg3 IgG.

As previously mentioned, RTX has an inhibitory effect on the co-stimulation of autoreactive B and T-cells in pemphigus by depletion B cells which act as antigen presenting cells (140). In this process, the interaction between CD40 expressed on the surface of B-cells and CD40 L expressed on the surface of T-cells is thought to play an essential role (297). Interestingly, targeting CD40 L demonstrated efficacy in an active mouse PV model. Specifically, Rag2-deficient mice expressing Dsg3 and treated with a monoclonal antibody targeting CD40L did not develop PV after having received splenocytes from Dsg3 deficient mice. However, this preventive effect did not occur when the monoclonal antibody was given after the adoptive cell transfer suggesting that targeting T-B-cell cross-talk may be an effective tool to prevent disease recurrences in patients who have already achieved disease remission (**Figure 9**) (297–299).

Is There a Possibility for a Local Targeted Therapy in Pemphigus?

Vinaj et al. (300) reported on 3 patients with oral PV lesions refractory to immunosuppressants, including intravenous RTX, in whom intralesional RTX injections led to meaningful clinical improvement, suggesting a local immunomodulating effect of the drug. A landmark paper by Yuan et al. (301) demonstrated an accumulation of Dsg-3 and Dsg-1 specific B-cells in the skin of pemphigus lesions. Additionally they showed that the skin in pemphigus serves as a tertiary lymphoid organ in which a close interaction between IL-21- and IL17A-producing CD4^{+} T cells leads to in loco production of anti-Dsg autoantibodies by auto-reactive B-cells. It is possible that the formation of this tertiary lymphoid organ in the skin may contribute to the resistance of pemphigus lesions to immunosuppressive treatments, including RTX. The discovery by Yuan et al. (301) may also provide a plausible explanation for the successful use of intralesional RTX in oral pemphigus lesions observed by Vinaj et al. (300). It is interesting to note that secretion of IL-21 is associated with activation of JAK1 and JAK3. Tofacinib, an inhibitor of both JAK1 and JAK3, was suggested as a possible therapeutic in pemphigus (302). Interestingly, tofacinib has been successfully applied in different autoimmune disorders (303, 304). Moreover, a topical preparation of tofacinib was shown to be effective in alopecia areata (305). Based on these findings, it would be intriguing to evaluate the potential benefit of topical JAK inhibitors as a local adjuvant therapy in pemphigus.

We have previously shown that mechanisms of acantholysis in pemphigus require activation of different intracellular signaling, including p38MAPK. *In vitro* studies showed that inhibition of p38MAPK eliminated PV IgG-induced blister formation in human skin biopsies (306). Oral p38MAPK inhibitors have been tried in clinical trials in rheumatoid arthritis, Crohn's disease and psoriasis, as well as in pemphigus, but had resulted in severe adverse events (1). Nevertheless, topical p38MAPK inhibitors have been shown to exert potent anti-inflammatory effects in the skin of mice following burn injury (307). Indeed, topical p38MAPK inhibitors may represent a therapeutic strategy in pemphigus to overcome the significant toxicity observed with the systemic counterpart. Finally in a study by Mao et al. (308), inhibition of STAT3 by hydrocortisone, rapamicin, an inhibitor of mTOR, or Stat3 inhibitor XVIII prevented blister formation in a passive transfer PV mouse model by up-regulating the expression of Dsg3. Interestingly, sirolimus, a systemic inhibitor of mTOR, was shown to be effective in PV, whereas topical rapamicin did not (309, 310). Notably, intratumoral injection of Stat3

oligonucletide decoy demonstrated mild efficacy in a trial including patients with head and neck tumors (311). Due to its pivotal implication in the mechanisms of acantholysis in pemphigus, Pharmacologic inhibition of Stat3 through topical drugs may also hold promise in the field of targeted therapy in pemphigus.

DEDICATION

This article is dedicated to Stephen I. Katz, MD, PhD, who enormously contributed to a better understanding of autoimmune bullous skin diseases and immunological skin disorders in general. It was his institute which identified desmoglein 3 as the autoantigen of pemphigus vulgaris and firstly described the central role of autoreactive T cells in this disorder. Steve Katz trained world leaders in immunodermatology from all over the world and will be unforgotten as a superb mentor and wonderful person.

AUTHOR CONTRIBUTIONS

RM and MH designed the manuscript. DD, RM, RE, and MH drafted the manuscript and approved the final version of the manuscript. MH revised critically the final version of the manuscript.

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Modifications of the Transcriptomic Profile of Autoreactive B Cells From Pemphigus Patients After Treatment With Rituximab or a Standard Corticosteroid Regimen

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Pemphigus Vulgaris is an autoimmune disease of the skin and mucous membranes, which is due to the production of pathogenic autoantibodies targeting desmoglein (DSG) 1 and 3, which are adhesion proteins of the keratinocytes. Rituximab is an anti-CD20 mAb which induces a prolonged depletion of blood B cells. We recently showed that rituximab was more effective than a standard oral corticosteroid (CS) treatment, allowing 90% of patients to achieve complete remission (CR). Additionally, we showed that DSG-specific-B (DSG positive) cells were still detectable during the B cell recovery which follows the initial rituximab-induced B cell depletion, even in patients in CR. In order to characterize DSG positive B cells in patients in CR after rituximab or CS treatment relative to those detectable at baseline in patients with an active pemphigus, we studied the expression profile of 31 genes of interest related to inflammatory cytokines, TNF receptors and activation markers. Using quantitative Polymerase Chain Reaction performed on one cell with a microfluidic technique, we found that patients' autoreactive B cells collected at baseline had a significantly higher expression of genes encoding for IL-1β, IL-23p19, and IL-12p35 pro-inflammatory cytokines and the IRF5 transcription factor, than non-autoreactive B cells. Surprisingly, the gene expression profile of DSG positive B cells collected after rituximab treatment in patients in CR was close to that of DSG positive B cells at baseline in patients with active pemphigus, except for the IL-1β and the CD27 memory marker genes, which were under-expressed after rituximab compared to baseline. Conversely, we observed a decreased expression of genes encoding for IL-1β and IL-23p19 in patients treated with CS relative to baseline. This study showed that: (i) DSG positive autoreactive B cells have a different gene expression profile than non-autoreactive B cells; (ii) rituximab and CS have different effects on the genes' expression in autoreactive DSG positive B cells from pemphigus patients.

Keywords: pemphigus, rituximab, corticosteriods, autoreactive B cell, transcriptomic analysis

INTRODUCTION

Pemphigus is a rare life-threatening autoimmune blistering disease involving the skin and mucosa, leading to erosions and major weight loss, which severely impair patients' quality of life (1–5). It is characterized by the production of pathogenic autoantibodies directed against two desmosomal proteins involved in keratinocyte adhesion: desmoglein 1 (DSG1) and desmoglein 3 (DSG3) (1, 6). DSG3 is mainly found in deep skin epidermal layers and mucosa, while DSG1 is present throughout the epidermis. Anti-DSG3 antibodies (Abs) are observed in pemphigus vulgaris (PV) with mucosal involvement, and anti-DSG1 Abs are observed in patients with pemphigus foliaceus (PF) and those with PV and skin involvement. Binding of anti-DSG Abs to their target antigen results in a loss of keratinocyte adhesion, leading to the so called "acantholysis" phenomenon, which results in the formation of skin and mucosal blisters (7–9).

Until recently, high-dose of oral corticosteroids (CS) sometimes associated with immunosuppressive drugs (azathioprine, mycophenolate mofetil) were the mainstay of pemphigus treatment (10). A recent large randomized controlled study (RCT) from our group demonstrated the interest of the first line use of rituximab in pemphigus (11). A large difference was evidenced in the primary outcome: 89% of patients in the rituximab + short term CS arm were in complete remission off therapy after 2 years, compared with 34% of patients treated with prednisone alone ($p < 0001$).

Rituximab is a chimeric murine-human monoclonal antibody that binds to the CD20 antigen of B-lymphocytes (anti-CD20 mAb), which is expressed by pre-B-cell and pre-plasma cells. A stop in the renewal of the plasma cell pool is thought to be one of the main mechanisms of the short-term action of rituximab (12, 13). However, the long-term clinical course of pemphigus patients treated with rituximab has not been extensively assessed yet. In particular, apart from a reversal of the naïve/memory B-cell *ratio*, the long-term evolution of reappearing DSG1– and DSG3–specific B cells after rituximab treatment remains largely unknown (14). Furthermore, the effects of oral CS on DSG specific-B cells in pemphigus patients has not been studied yet.

In addition to producing antibodies, B cells secrete multiple cytokines with pro- or anti-inflammatory functions, which can either enhance or downregulate the immune response. Since understanding the long-term modifications of the DSG-specific autoimmune B cell response after rituximab treatment might improve the way we use this drug, we longitudinally analyzed the transcriptomic and phenotypic profiles of one-cell sorted autoreactive DSG-specific B cells from patients with a newly diagnosed pemphigus (during the active phase of disease) and after treatment with rituximab or a standard CS regimen, and compared these results with those observed from non-autoreactive B cells.

RESULTS

Baseline Analysis

Using quantitative polymerase chain reaction (qPCR), we analyzed the single cell mRNA expression of 33 genes encoding for membrane markers, cytokine receptors or transcription factors (**Table SI**) which are known to be expressed in B cells from 20 patients. Among them, nine pemphigus patients were treated by rituximab and assessed at baseline and at Month 36 (18 months after the last rituximab infusion); all of them were in complete remission at M36. Eleven pemphigus patients were treated by oral corticosteroids and assessed at baseline and Month 12, after tapering of CS doses; five of these 11 patients were in clinical complete remission at Month 12. The 20 patients had a pemphigus vulgaris with either isolated mucosal involvement or skin and mucosal involvement. Clinical, biological, and immunologic characteristics of patients are presented in **Tables 1**, **2**. Finally, we performed a transcriptomic analysis in seven out of 14 healthy donors (HD) who were studied with phenotypic analyses. Indeed, DSG1-positive (**Figure 1A**) and DSG3-positive (**Figure 1B**) B cells were detectable in HD, although at a lower frequency than in the 20 patients with active pemphigus (DSG1 positive 0.11 vs. 0.20%, $p < 0.001$; DSG3 positive 0.10% vs. 0.20%, $p < 0.001$).

We first compared the baseline gene expression between non-autoreactive DSG-negative B cells (201 single cells) and autoreactive DSG-positive B cells (191 single cells) from pemphigus patients. We observed that the mRNA of 11 genes (IL-2, IL-5, IL-9, IL-12p40, IL-13, IL-17F, IL-21, IL-27p28, IFNγ, TGF β2, and APRIL) was not detected at baseline neither in DSG-positive, nor in DSG negative B cells from pemphigus patients, whereas all housekeeping genes were detected. Therefore, these latter genes were not further analyzed. The 22 remaining genes detected were used to realize a heat map representing the ΔCT expression of these genes between autoreactive and non-autoreactive B cells at baseline (**Figure 1C**).

At baseline, autoreactive DSG-positive and non-autoreactive DSG-negative sorted B cells showed distinct mRNA expression profiles for the three pro-inflammatory cytokine genes IL-1β, IL-12p35, IL-23p19, and for the transcription factor IRF5 gene which were found to be overexpressed by the DSG-positive relative to the DSG-negative B cell populations (**Figure 1D**), whereas no difference of expression of these four genes was evidenced between DSG-positive (107 single cells) and DSG-negative (69 single cells) B cell populations from HD (data not shown).

Longitudinal Analyses in Patients Treated With Rituximab

We performed a longitudinal analysis by flow cytometry in the nine rituximab-treated pemphigus patients previously analyzed by qPCR. Frequency of DSG1-positive and DSG3-positive autoreactive B cells did not vary significantly between baseline and Month 36 when patients were in complete remission (DSG1 positive: 0.20% vs. 0.22%, $p = 0.67$; DSG3 positive 0.23% vs. 0.25, $p = 0.61$; **Figures 2A,B**).

We then analyzed the expression of selected genes in 306 single DSG-positive B cells from the nine rituximab-treated pemphigus patients, including 191 sorted-cells from blood samples collected at baseline and 115 sorted-cells from samples collected at Month 36 after RTX treatment. One hundred

TABLE 1 | Clinical and biological characteristics of patients from the rituximab group.

Age	Treatment	Type of pemphigus	Severity	ELISA anti-DSG1 Ab value at D0	ELISA anti-DSG3 Ab value at D0	Clinical status at M36	ELISA anti-DSG1 Ab value at M36	ELISA anti-DSG3 Ab value at M36
51	RTX	Mucosal	Moderate	30	750	Complete remission	<20	80
45	RTX	Mucosal	Severe	<20	360	Complete remission	<20	<20
63	RTX	Mucosal	Severe	<20	2,050	Complete remission	<20	111
28	RTX	PV skin + mucosa	Severe	330	120	Complete remission	<20	555
76	RTX	Mucosal	Severe	<20	530	Complete remission	<20	157
80	RTX	PV skin + mucosa	Severe	410	1,805	Complete remission	<20	<20
52	RTX	PV skin + mucosa	Severe	260	4,000	Complete remission	<20	<20
64	RTX	PV skin + mucosa	Severe	200	131	Complete remission	<20	<20
48	RTX	PV skin + mucosa	Severe	356	176	Complete remission	<20	<20

TABLE 2 | Clinical and biological characteristics of patients from the standard corticosteroids (CS) group.

Age	Type of pemphigus	Severity	ELISA anti-DSG1 Ab value at D0	ELISA anti-DSG3 Ab value at D0	Clinical status at M12	ELISA anti-DSG1 Ab value at M12	ELISA anti-DSG3 Ab value at M12
76	PV skin + mucosal	Severe	107	2,400	Complete remission	<20	<20
54	PV skin + mucosal	Severe	1,960	179	Complete remission	<20	<20
66	PV skin + Mucosal	Severe	27	3,350	Complete remission	<20	126
48	PV skin + mucosa	Severe	176	760	Complete remission	<20	<20
33	PV skin + mucosa	Severe	60	143	complete remission	<20	<20
79	PV skin + mucosa	Severe	1,110	553	Incomplete remission	<20	<20
45	PV skin + mucosa	Severe	156	5,217	Incomplete remission	30	1040
61	PV skin + mucosa	Severe	170	1,750	Incomplete remission	<20	550
71	PV skin + mucosa	Severe	665	225	Incomplete remission	77	<20
58	PV skin + mucosa	Severe	1,070	1,065	Incomplete remission	<20	<20
58	PV skin + mucosa	Severe	214	1,979	Incomplete remission	<20	128

and seven single DSG-positive B cells from the seven healthy individuals were used as controls (**Figure 3**).

Interestingly, the expression of the IL-1β gene in DSG-positive B cells, which was higher at baseline in pemphigus patients than in healthy individuals (12 vs. 6%, $p = 0.048$), returned to normal values after rituximab treatment (12 vs. 4%, $p = 0.039$). We also observed that, whereas the frequency of expression of the CD27 memory marker gene in desmoglein-positive B cells collected from pemphigus patients at baseline was close to that observed in healthy individuals (23 vs. 17%, $p = 0.24$), the CD27 gene expression decreased from baseline to Month 36 in pemphigus patients treated with rituximab (23 vs. 10%, $p = 0.03$).

Surprisingly, we did not observe any statistically significant variation in the frequency of the other genes studied between samples collected from pemphigus patients at baseline and after rituximab treatment.

In non-autoreactive desmoglein-negative B cells, rituximab treatment did not induce modification of gene expression for the pro-inflammatory cytokine IL-1β (2 vs. 4.1%, $p = 0.73$) whereas it induced a decreased frequency of expression of the CD27 memory marker gene (15.1 vs. 6.9%, $p = 0.04$; **Figure 4**).

Longitudinal Analyses in Patients Treated With Corticosteroids Alone

We performed a longitudinal flow cytometry analysis in the same 11 corticosteroids-treated pemphigus patients previously analyzed by qPCR. In these 11 CS-treated patients, 311 single DSG-positive cells were isolated including 191 sorted-cells from samples collected at baseline and 120 sorted-cells from samples collected at Month 12. This Month 12 evaluation was chosen because patients still received low doses of prednisone at this time point.

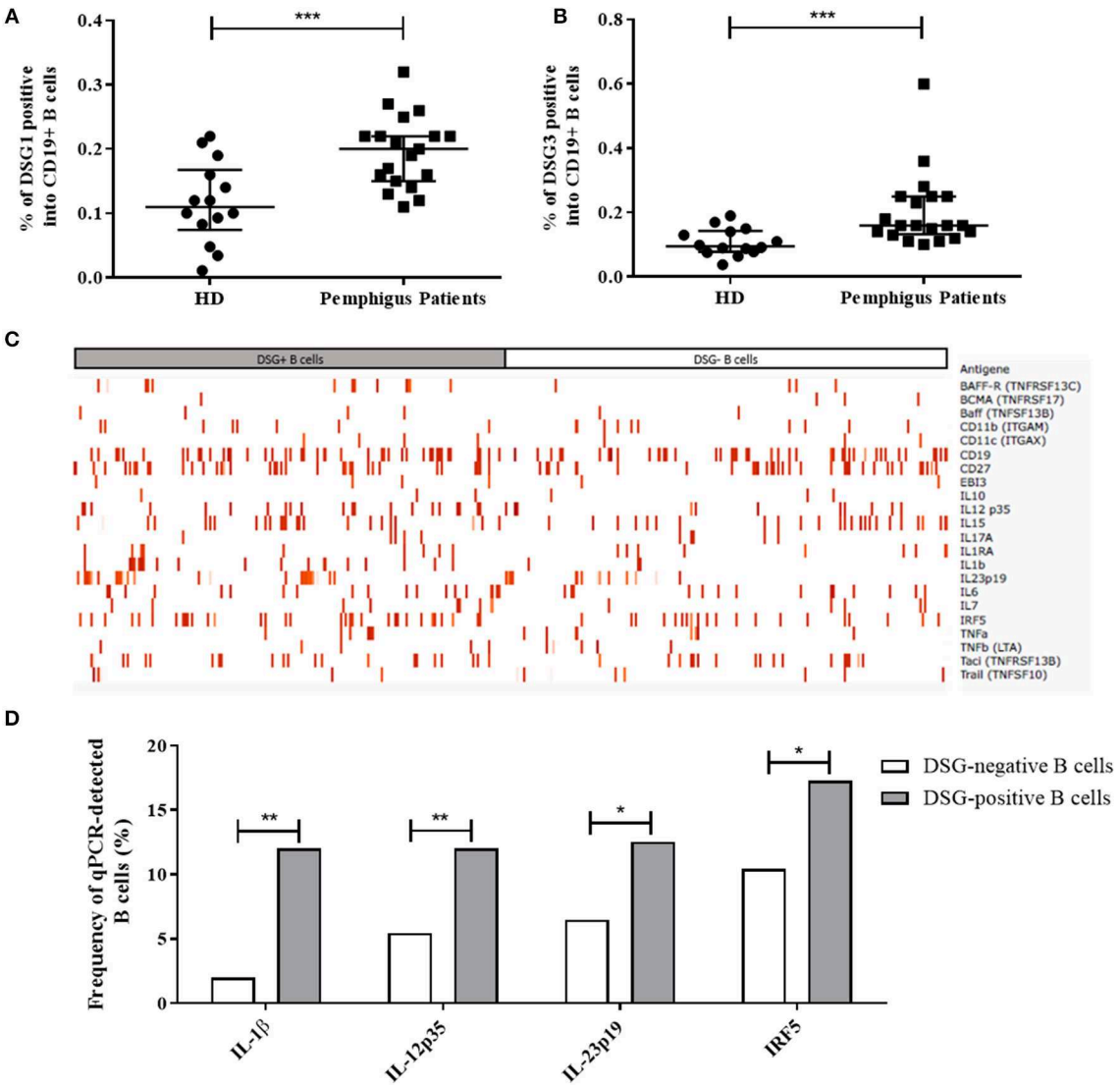

FIGURE 1 | (A,B) Percentage of autoreactive DSG1-positive (CD19+DSG1+) B cells **(A)** and DSG3-positive B cells (CD19+DSG3+) **(B)** among total B cells (CD19+) collected in 14 healthy donors and 20 pemphigus patients at baseline. Median with interquartile range were compared using the Mann-Whitney *t*-test. **(C)** Intensity of gene expression in one-cell sorted DSG-positive B cells (in gray $n = 191$ cells) and non-autoreactive DSG-negative B cells (in white, $n = 201$ cells) from pemphigus patients. The color intensity in each sorted cell depended on the ΔCT value relative to GAPDH house-keeping gene expression. Non-detected genes in one-cell qPCR were excluded from the map. **(D)** Comparison of the baseline frequencies of cells expressing the IL-1β, IL-12p35, IL-23p19 genes, and the transcription factor IRF5 gene between DSG-positive autoreactive B cells (gray columns, $n = 191$ cells) and non-autoreactive DSG-negative B cells (white columns, $n = 201$ cells) from pemphigus patients. Frequencies were compared using the Fisher's exact test.

After treatment with systemic corticosteroids (**Figure 5**), IL-1β gene was downregulated (12–1.7%, $p = 0.003$) allowing to return to values close to those observed in HD. Although the baseline gene expression of the pro-inflammatory cytokine IL-23p19 in desmoglein-positive B cells from patients was close to that detected in healthy individuals (12.5 vs. 15%, $p = 0.59$), we observed a downregulation of the gene after corticosteroids treatment (12.5 vs. 1.2%, $p < 0.005$). We did not observe significant variation in the expression of the other genes studied, including the CD27 memory marker gene (baseline: 23.5% vs. after CS treatment 26.6%, $p = 0.48$).

In non-autoreactive desmoglein-negative B cells (**Figure 6**), corticosteroids treatment induced non-significant variations of the IL-23p19, IL-1β, and CD27 genes, as other genes from the panel studied.

Assessment of the CD27 Memory Marker in Autoreactive B Cells After Rituximab

To extend the results of our transciptomic analysis, we studied the evolution of the CD27 expression on the cell surface of DSG-positive and DSG-negative B cells during treatment with rituximab or corticosteroids. According to the findings from

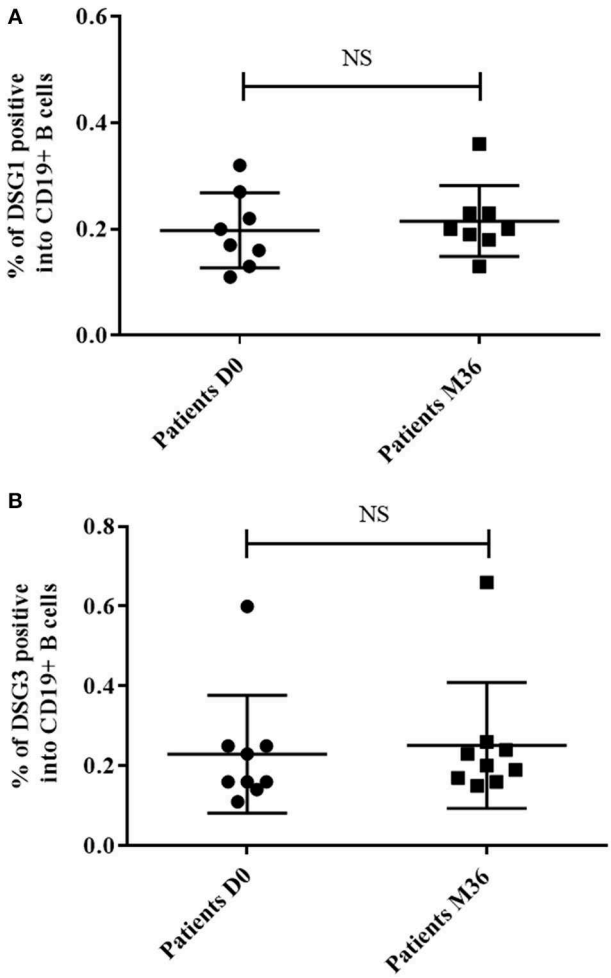

FIGURE 2 | Percentage of autoreactive DSG1-positive B cells (CD19+DSG1+) **(A)** and DSG3-positive B cells (CD19+DSG3+) **(B)** among total B cells (CD19+) collected in pemphigus patients at baseline and at Month 36 after rituximab treatment. Means ± standard deviations were compared using the Wilcoxon paired test.

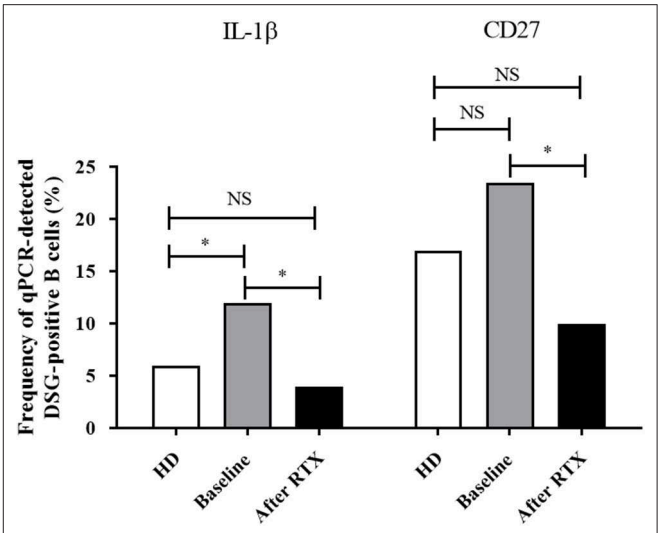

FIGURE 3 | Frequency of cells with detectable mRNA encoding for IL-1β and CD27 in one-cell sorted desmoglein-positive autoreactive B cells from healthy donors (white columns $n = 107$ cells) compared to pemphigus patients before treatment (gray columns, $n = 191$ cells) or after rituximab treatment (black columns, $n = 115$ cells). Frequencies were compared using the Fisher's exact test.

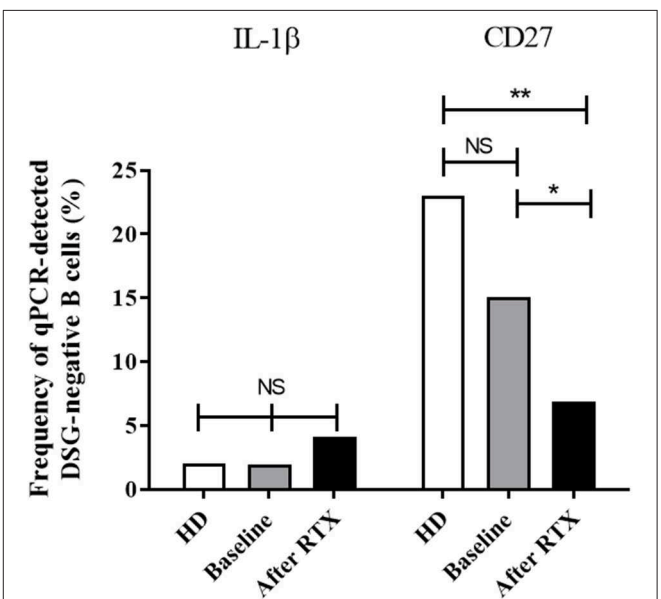

FIGURE 4 | Frequency of cells with detectable mRNA encoding for the IL-1β and CD27 genes in one-cell sorted desmoglein-negative B cells from healthy donors (white columns, $n = 69$ cells) and pemphigus patients at baseline (gray columns, $n = 201$ cells) and after rituximab treatment (black columns, $n = 106$ cells). Frequencies were compared using the Fisher's exact test.

transcriptomic analyses, we observed a significant decrease in the frequency of memory CD19+CD27+ autoreactive DSG-positive and DSG-negative B cells after rituximab treatment (**Figure 7A**), that were not observed in patients treated with CS alone without rituximab (**Figure 7B**).

ELISA Assays

In order to evaluate the consequences of the decreased IL-1β gene expression in DSG-positive B cells from pemphigus patients whether they were treated with corticosteroids or rituximab, we performed longitudinal dosages of serum IL-1β using ELISA assay.

We observed a non-significant decrease of serum IL-1β ELISA values in pemphigus patients during the first 3 months of treatment, that was secondarily followed by a re increase of serum levels to baseline values when corticosteroids doses were tapered during patients' follow-up (**Figure 8**).

Finally, the IL-23p19 cytokine, whose gene expression was downregulated after CS treatment in autoreactive B cells, was also analyzed by ELISA. No significant variation in the IL-23p19 serum level was detected in pemphigus patients whether they were treated with rituximab or CS alone (data not shown).

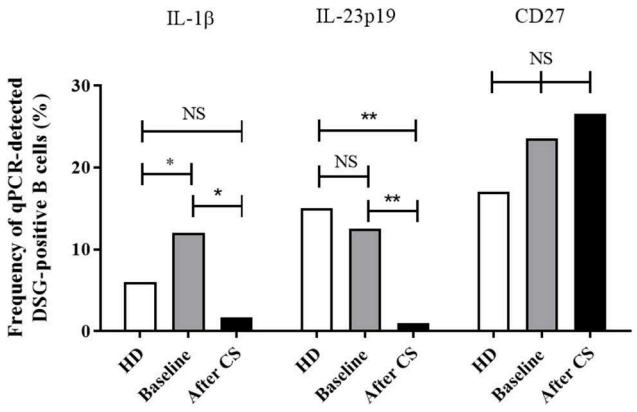

FIGURE 5 | Frequency of cells with detectable mRNA encoding for IL-1β, IL-23p19, and CD27 in one-cell sorted desmoglein-positive from healthy donors (white columns, $n = 107$ cells) compared to pemphigus patients before treatment before (gray columns, $n = 191$ cells) and after CS treatment (black columns, $n = 120$ cells). Frequencies were compared using the Fisher's exact test.

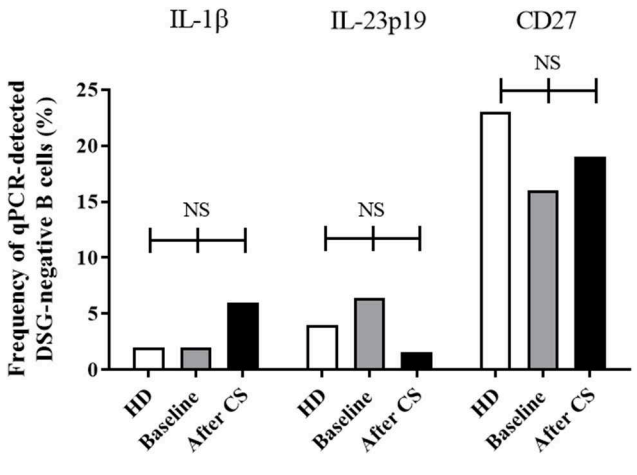

FIGURE 6 | Frequency of cells with detectable mRNA encoding for IL-1β, IL-23p19, and CD27 in one-cell sorted desmoglein-negative B cells from healthy donors (white columns, $n = 69$ cells), and pemphigus patients at baseline before treatment (gray columns, $n = 201$ cells) or after corticosteroids treatment (black columns, $n = 123$ cells). Frequencies were compared using the Fisher's exact test.

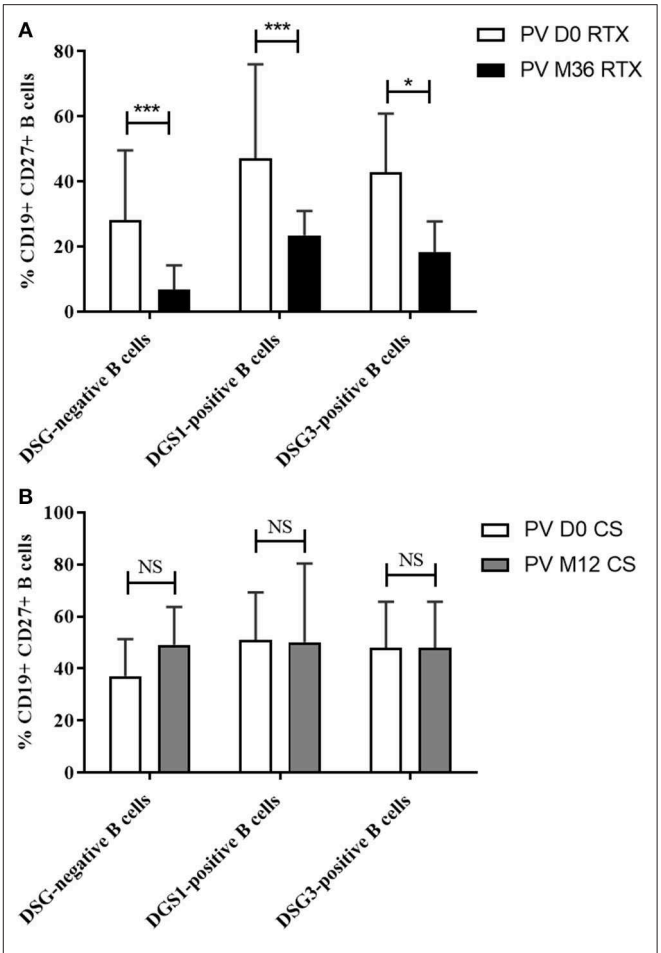

FIGURE 7 | (A) Percentage of memory B cells among non-desmoglein specific, desmoglein 1-specific (DSG1+), and desmogelin three-specific (DSG3+) B cells before (white columns) and after treatment with rituximab (black columns). **(B)** Percentage of memory B cells among non-desmoglein specific, desmoglein 1-specific (DSG1+), and desmogelin 3-specific (DSG3+) B cells before (white columns) and after treatment with corticosteroids (gray columns). Means ± standard deviations were compared using the Wilcoxon paired test.

DISCUSSION

In this study, we assessed the transcriptomic profiles of 33 genes of potential interest in pemphigus on one-cell sorted autoreactive B cells collected from pemphigus patients at different stages of their disease, in particular during the acute phase of the disease, and after two treatments (systemic corticosteroids alone or associated with rituximab) which were compared in a randomized controlled trial (11).

This methodology has several major interests. First, it allowed to study *ex vivo* autoreactive B-lymphocytes, since pemphigus is one of the rare autoimmune diseases in humans in which autoantibodies directed against DSG are pathogenic (15). Second, auto-reactive DSG positive B cells are the pre-stage of antibody-secreting plasma cells, which cannot be easily studied *ex vivo* in humans. Third, the one-cell sorting assays are likely relevant since a very low number of circulating autoreactive B cells in the peripheral blood are enough to trigger the disease, as we showed in the baseline analyses. To the best of our knowledge, this single-cell transcriptome analysis of autoreactive cells is the first one performed in patients with an auto immune disorder.

We first evidenced that the transcriptomic profiles of autoreactive and non-autoreactive B cells collected during the active phase of disease were different. Four out of the 31 genes studied (IL-1β, IL-12p35, IL-23p19, IRF5) were overexpressed in autoreactive relative to non-autoreactive B cells. The most surprising finding in this study was the observation that in addition to the fact that DSG-positive B cells are still detectable after rituximab treatment, arguing against a simple deletion mechanism, the gene expression profile of DSG-positive autoreactive B cells collected after rituximab treatment in patients

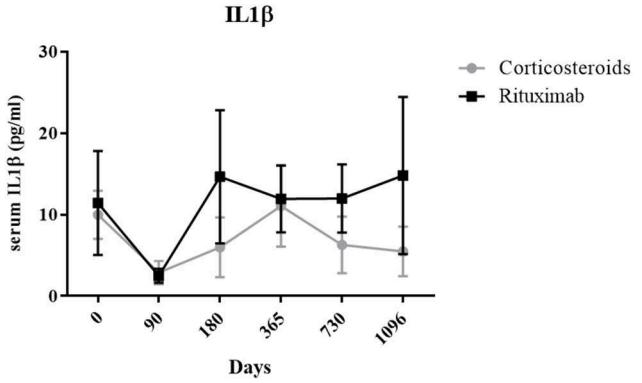

FIGURE 8 | Longitudinal analysis of mean serum IL-1β level in rituximab (black) and corticosteroids-treated (gray) patients. Means ± standard error of the mean were compared with baseline level in each group (RTX and CS) using one-way analysis of variance followed by Bonferroni *post-hoc* test.

in complete remission was actually pretty close to that of DSG-positive B cells collected at baseline in patients with active pemphigus, except for the IL-1β and the CD27 genes, which were downregulated after rituximab compared to baseline.

The downregulation of the CD27 memory marker gene observed in transcriptomic analysis was confirmed by flow cytometry, which showed a lower expression of the CD27 protein at the cell surface of autoreactive B cells after rituximab as well as in non-autoreactive B cells. Thus, the decreased expression of the CD27 gene after rituximab was not specific for DSG-positive B cells. However, the decreased expression of the CD27 marker reflects the blockage of B cell maturation, leading to a prolonged repopulation with naïve B cells, and a delayed reappearance of memory B cells. It has been suggested that autoantibodies are produced by a transitory subpopulation of autoreactive plasmablasts which are continuously refund from autoreactive switched memory B cells (CD20+IgG+CD27+) (13, 16). Thus, changes in CD27 expression which correlates with the disappearance of circulating DSG-positive IgG(+) B lymphocytes are likely responsible for the decreased production of anti-DSG autoantibodies, that contributes to the long lasting clinical remission of the disease after rituximab treatment (14). Another argument which supports this hypothesis is the fact that by using an ELISPOT assay, we showed that DSG3+CD27+ B cells but not DSG3+CD27− B cells are able to differentiate into antibody secreting cells after 72 h of stimulation by IL-2 and R848, a TLR7 and eight ligands (still unpublished data). This mechanism was specifically related to rituximab, since it was not observed in patients treated with CS alone, whether they were in complete or incomplete remission.

Conversely, changes in CD27 do not influence the production of alloreactive antibodies which are produced by long-lived plasma cells which do not express the CD20 marker, explaining the absence of modification of total serum IgG levels in after rituximab in most patients.

We also observed that the expression of the IL-1β gene in DSG specific B cells, which was higher in baseline samples from pemphigus patients than in healthy individuals, and returned to normal values after rituximab or oral CS treatment, whereas no modification was observed in non-autoreactive desmoglein-negative B cells. This observation might suggest the potential role of IL-1β in the pathogenicity of pemphigus, since the downregulation observed in autoreactive B cells paralleled disease remission, whether patients were treated with rituximab or oral CS. Indeed, IL-1β is a pro-inflammatory cytokine mainly produced by hematopoietic cells such as blood monocytes, tissue macrophages and skin dendritic cells in response to TLR ligand, activated complement components or other cytokines such as TNF-α and IL-1β itself (17, 18). In our work, we observed a slight decrease of serum IL-1β during the first 3 months of treatment in both CS- and RTX-treated groups, which was likely related to the effect of high doses of CS. It was then followed by a re-increase of serum levels to baseline values when corticosteroids doses were tapered. The role of IL-1β has previously been suggested by Feliciani et al. who demonstrated that an IL-1β KO mouse model was less susceptible to passive transfer of IgG isolated from the serum of a PV patient. Moreover, Increased concentrations of IL-1α and IL-1β were detected in the serum of untreated PV patients with active disease relative to healthy controls. Additionally, the *in vivo* and *in vitro* production of IL-1α and IL-1β decreased in patients in clinical remission after treatment with IVIG. Finally, IL-1β promotes Th17 polarization, which correlates with disease activity (19–24).

Since the frequency of autoreactive B cells in pemphigus patients was surprisingly only doubled compared to healthy donors, it is likely that the different gene expression profiles of IL-1β and CD27 play a major role in the onset of pemphigus by promoting the emergence/appearance of DSG + CD27+ IgG+ B memory cells, which were only detected in pemphigus patients but not in healthy individuals.

Despite the fact that we observed a difference in the baseline expression of the IL-12p35 between DSG-positive and DSG-negative B cells, we did not observe significant modification in RNA expression in patients in complete remission after rituximab, nor variation in the serum concentration of IL-12p35 during patients' follow-up, which does not support a major role for IL-12p35 in the healing of RTX-treated patients.

We did not analyse the serum concentration of cytokines relevant for B cell activation such as BAFF, APRIL, or IL-6, because we did not observe any modification of the gene expression of these cytokines by autoreactive DSG-positive B cells in our transcriptomic analyses.

Overall, this study showed that self-reactive and non-self-reactive B cell populations from pemphigus patients do not express the same genes during the acute phase of the disease, in particular genes encoding for IL-1β, IL-12p35, IL-23p19, and IRF5. Additionally, DSG-positive autoreactive B cells can still be observed in patients in complete remission, even in those treated with rituximab, when blood B-lymphocytes reappeared after the initial rituximab-induced depletion. Surprisingly, the gene expression of autoreactive DSG-positive B cells in remitted patients was close to that observed at baseline, except for the CD27 gene which was downregulated in patients treated with rituximab, and the IL-1β gene, which was down regulated in both treatment groups, likely related to the effect of oral CS.

METHODS

Clinical Study

In the randomized controlled trial, 90 newly-diagnosed pemphigus patients were randomly assigned to receive a standard regimen of CS vs. rituximab associated with a short-term CS regimen. Patients in the rituximab group were treated with the autoimmune regimen (two infusions of 1,000 mg of rituximab at day 0 and day 14) and a maintenance treatment corresponding to two infusions of 500 mg at Month 12 and Month 18. They also received an initial medium dose of prednisone, 0.5–1 mg/kg/day, depending on initial pemphigus severity (moderate vs. severe), which was rapidly tapered over 3–6 months. Patients assigned in the standard oral CS group were given a higher initial dose of prednisone, 1–1.5 mg/kg/day, with a progressive tapering over 12–18 months, depending on initial pemphigus severity. Patients who participated in the Ritux 3 clinical trial were included in the present study (ClinicalTrials.gov number, NCT00784589).

Patients

Blood samples from rituximab-treated patients were analyzed at baseline before treatment (Day 0) and after 36 months of follow-up, since we had to wait for the recovery of blood B-lymphocytes after the repeated infusions of rituximab at Day 1 and Month 12 and Month 18. Blood samples from CS-treated patients were analyzed before treatment and at Month 12 at which time patients in the standard CS group were still treated with oral CS.

Serum Auto-Antibody Titers and Cytokine Levels

Titers of IgG antibodies against DSG1 and three were measured by a DSG enzyme-linked immunosorbent assay test (ELISA) with 1:100 diluted serums (EUROIMMUN, Germany, Lübeck). Serum levels of IL-1β and Il-23p19 were quantified by ELISA (RD and ebioscience, respectively), according to the manufacturer's protocol.

Phenotyping of Desmoglein-Specific B Cells

In order to analyse DSG1 and DSG3 specific B cells, B cells were isolated using Dynabeads Untouched Human B-cells kit (Life Technologies) according to the manufacturer's instruction. Then, purified B cells were incubated for 30 min at 4°C with histidine-tagged recombinant DSG1 or DSG3 (30 ng/μl). After washing, B cells were stained with anti-human IgG antibodies (BD Biosciences). Cells were then incubated with Fc Block (eBioscience), LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies), anti-human antibodies directed against CD19, CD27, IgM (BD Biosciences). Anti-histidine coupled with phycoerythrin (R&D Systems) was used to identify desmoglein-specific B cells.

One-Cell Sorting and Pre-amplification

DSG-positive single B cells were sorted using FACS ARIA III into 96-well plates containing 10μL Platinum Taq polymerase and SuperScript III reverse transcriptase (Invitrogen), a mixture of Taqman primer-probes at 0.2× concentration specific for the transcripts of interest (**Table SI**) and CellsDirect qRT-PCR buffer (Invitrogen). Immediately after cell sorting, samples were centrifuged, incubated at 55°C for 10 min, and subjected to 30 cycles of PCR (50°C 15 min then 95°C for 15 s for the reverse transcription, followed by 30 cycles of 95°C 15 s, and 60°C 4 min for amplification). Subsequent pre-amplified single-cell cDNA was stored at −20°C until analysis.

Real-Time qPCR

After ¼ dilution in TE buffer, each cDNA sample was then separated into 48 separate reactions for further qPCR using the BioMark 48.48 dynamic array nanofluidic chip (Fluidigm, Inc.). Briefly, following hydraulic chip priming, 48 pre-amplified cDNA samples were mixed with a mild detergent loading solution to allow capillary flow. Samples were then added to a 48.48 nanofluidic chip (Fluidigm, Inc.) along with 38 individual Taqman primer-probe mixtures listed in **Table SI** (Applied Biosystems) specific for individual transcripts of interest, allowing a combination of each sample to mix with each probe in every possible combination (a total of 2,304 reactions). The chip was then thermocycled through 40 cycles and fluorescence in the FAM channel was detected using a CCD camera placed above the chip, normalized by ROX (6-carboxy-X-rhodamine) intensity. A well containing 100 CD19+ cells and a well without cell were used as positive and negative controls, respectively. To limit potentially biased measurement, cells with <2 expressed genes among the five control genes (HPRT1, B2M, GUSB, TUBB, and GAPDH) were excluded from the analysis. Data were analyzed using Real Time PCR Analysis software with or without normalization of the Ct value for each gene using GAPDH as calibrator gene. We considered that sorted cells expressed a gene if the Ct (threshold of detection) value was < 40 and if the detection curve was a sigmoid. Eleven genes were found to be unexpressed by single B cells including IL-2, IL-5, IL-9, IL-12p40, IL-13, IL-17F, IL-21, IL-27p28, IFNγ, TGFβ2, and APRIL. This absence of detection was due to a too low RNA amount since positive control wells containing 100 CD19+ cells showed detectable expression levels of all tested cytokine genes. We compared the frequency of cytokine gene expressing B cells using qPCR between treatment groups.

ΔCt Analysis

The ΔCt analysis allows to determine the intensity of the gene of interest expression in cells by comparing its threshold of detection (Ct) with the Ct of the housekeeping gene.

Statistical Analysis

Statistical comparison of the frequency of gene expressing cells was performed with Fisher's exact test. Statistical comparison of percentages of autoreactive B cells in flow cytometry were calculated using the Mann-Whitney *t*-test to compare healthy donors and pemphigus patients; and using the Wilcoxon *t*-test to compare patients before and after treatment. All analyses were performed using the GraphPad Prism Software. A p-value ≤ 0.05

was considered as significant. The ∗, ∗∗, ∗∗∗ refer to statistical values explained in the legends.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

VH: transcriptomic, cytometric acquisitions, analyses, ELISA assays, and writer. MP: transcriptomic acquisitions. MM-V: transcriptomic, cytometric acquisitions, and ELISA assays. M-LG: cytometric acquisitions. GR: cytometric analyses. CD: transcriptomic analyses. OB: drafting and revision. SC and PJ: transcriptomic analyses, cytometric analyses, drafting, and revision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01794/full#supplementary-material

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Endocytosis of IgG, Desmoglein 1, and Plakoglobin in Pemphigus Foliaceus Patient Skin

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Pemphigus foliaceus (PF) is one of the two main forms of pemphigus and is characterized by circulating IgG to the desmosomal cadherin desmoglein 1 (DSG1) and by subcorneal blistering of the skin. The pathomechanism of blister formation in PF is unknown. Previously we have shown that PF IgG induces aggregation of DSG1, plakoglobin (PG), and IgG outside of desmosomes, what in immunofluorescence of PF patient skin visualizes as a granular IgG deposition pattern with a limited number of coarse IgG aggregates between cells. Here we have investigated the fate of these aggregates in skin and found that these are cleared by endocytosis. We performed double immunofluorescence staining on snap-frozen skin biopsies of six PF patients for the following molecules: IgG, the desmosomal proteins DSG1 and DSG3, desmocollins 1 and 3, PG, desmoplakin and plakophilin 3, and for the endosomal marker early endosomal antigen 1 and the lysosomal markers cathepsin D and lysosomal-associated membrane protein 1. Endosomes were present in all cells but did not make contact with the aggregates in the basal and suprabasal layers. In the higher layers they moored to the aggregates, often symmetrically from two adjacent cells, and IgG, DSG1, and PG were taken up. Finally these endosomes became localized perinuclear. Endocytosis was only observed in perilesional or lesional skin but not in non-lesional skin. Older immunoelectron microscopic studies have suggested that in PF skin endocytosis of detached desmosomes takes place but we found no other desmosomal proteins to be present in these endosomes. Double staining with cathepsin D and LAMP-1 revealed no overlap with IgG, DSG1, or PG suggesting that lysosomes have no role in the clearing process. Collectively, our results show that endocytosis is part of the pathogenic process in PF but that no detached desmosomes are taken up but instead the deposited IgG is taken up together with DSG1 and PG.

Keywords: pemphigus foliaceus, desmoglein 1, desmosomes, acantholysis, endocytosis

INTRODUCTION

Pemphigus is an autoimmune blistering disease of the skin and mucous membranes, which is characterized by a loss of cell-cell adhesion, known as acantholysis. Pemphigus foliaceus (PF) is one of the two major forms of pemphigus and it is characterized by blistering of the skin only and immunologically by circulating autoantibodies against desmoglein 1 (DSG1) (1, 2). DSG1 is a glycoprotein of desmosomes and is expressed through all the epidermal layers. Desmosomes are intercellular adhesion structures and are build-up of transmembrane desmosomal cadherins desmogleins and desmocollins (DSC), that by heterophilic interaction directly interconnect two opposite half-desmosomes, and intracellular desmosomal plaque proteins including plakoglobin (PG), desmoplakin (DP), and plakophilins (PKP) (3–6). The intracellular proteins connect the cytoplasmic tails of the desmosomal cadherins to the intermediate keratin filaments thereby providing strength to the tissue. The pathomechanism of the loss of cell-cell adhesion in PF is not exactly known and compared to PV only few studies have addressed it. It is clear that the anti-DSG1 IgG is the pathogenic factor that induces acantholysis as injection of PF IgG causes skin blistering in mice and in an *ex vivo* living human skin model (7–9). Injecting of PF IgG in mice also results in activation of p38MAPK and an inhibitor to p38MAPK abolishes this blistering (7). Waschke et al. showed that PF IgG reduces Rho A activity and in an *ex vivo* human skin model PF induced blistering could be abrogated by Rho A activation (10). Recent research by Walter et al. found that anti-DSG3 IgG and anti-DSG1 IgG led to activation of different signaling pathways. While both activated p38MAPK, anti-DSG3 activated Src while in contrast anti-DSG1 activated ERK, indicating that the pathomechanisms between PV and PF might differ (11). Steric hindrance i.e., obstruction of DSG transinteraction is also considered as a possible pathomechanism. Based on single molecule atomic force measurements and by laser trapping of surface- bound DSG1-coated microbeads Waschke et al. however found no evidence for this (12). We have shown that in the anti-DSG1 IgG induces a shift in distribution of DSG1 and PG in PF patient skin (8). In healthy human skin staining for DSG1 shows an evenly distributed signal over the cell membranes in line with a desmosomal distribution while instead in PF skin, especially in the lower epidermal layers, DSG1 is present in coarse clusters that also contain PG and IgG but no other desmosomal proteins. These clusters can be induced by bivalent PF IgG in the *ex vivo* living human skin model, but not by monovalent Fab fragments of this same IgG which suggest that crosslinking of DSG molecules underlies cluster formation (8, 9). Recently further evidence was provided that the polyvalence of bivalent PF IgG is responsible for clustering. A mixture of non-pathogenic PF monoclonal antibody (mAb) and pathogenic PF mAb is needed to induce clusters, but only pathogenic PF mAb is able to induce the loss of cell-cell adhesion (13). The clusters in PF skin contain DSG1 and PG, but no other desmosomal components which suggests that desmosomes

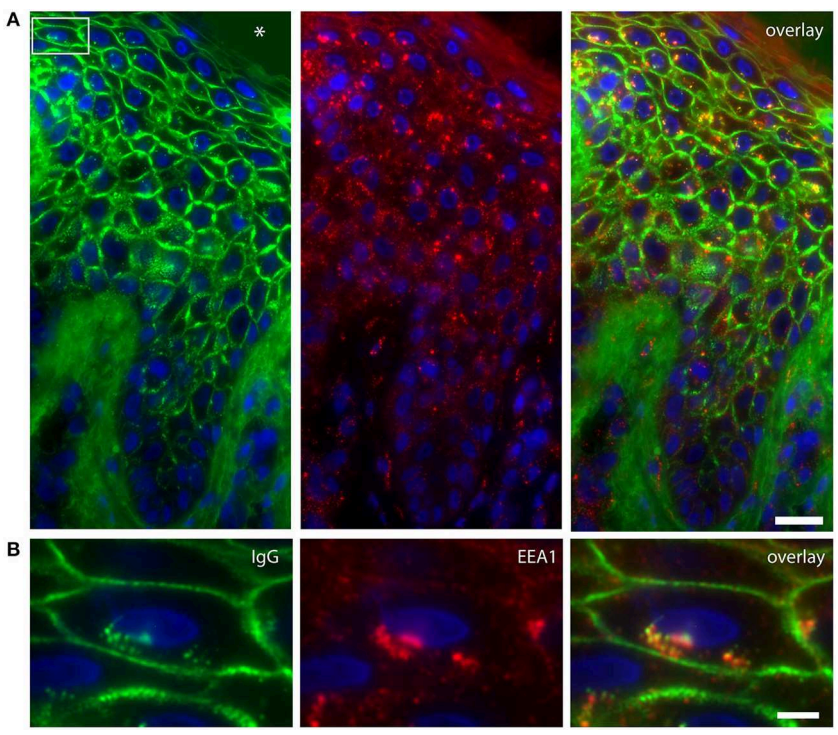

FIGURE 1 | Colocalization of IgG and endosomes in lesional PF patient skin. **(A)** IgG (green) and EEA1 (red) colocalize in the higher skin layers (right panel). The white box depicts the cell shown in **(B)**. The blister cavity is indicated by an asterix. White bar: 25 μm. **(B)** Detail showing the perinuclear localization of IgG. White bar: 5 μm.

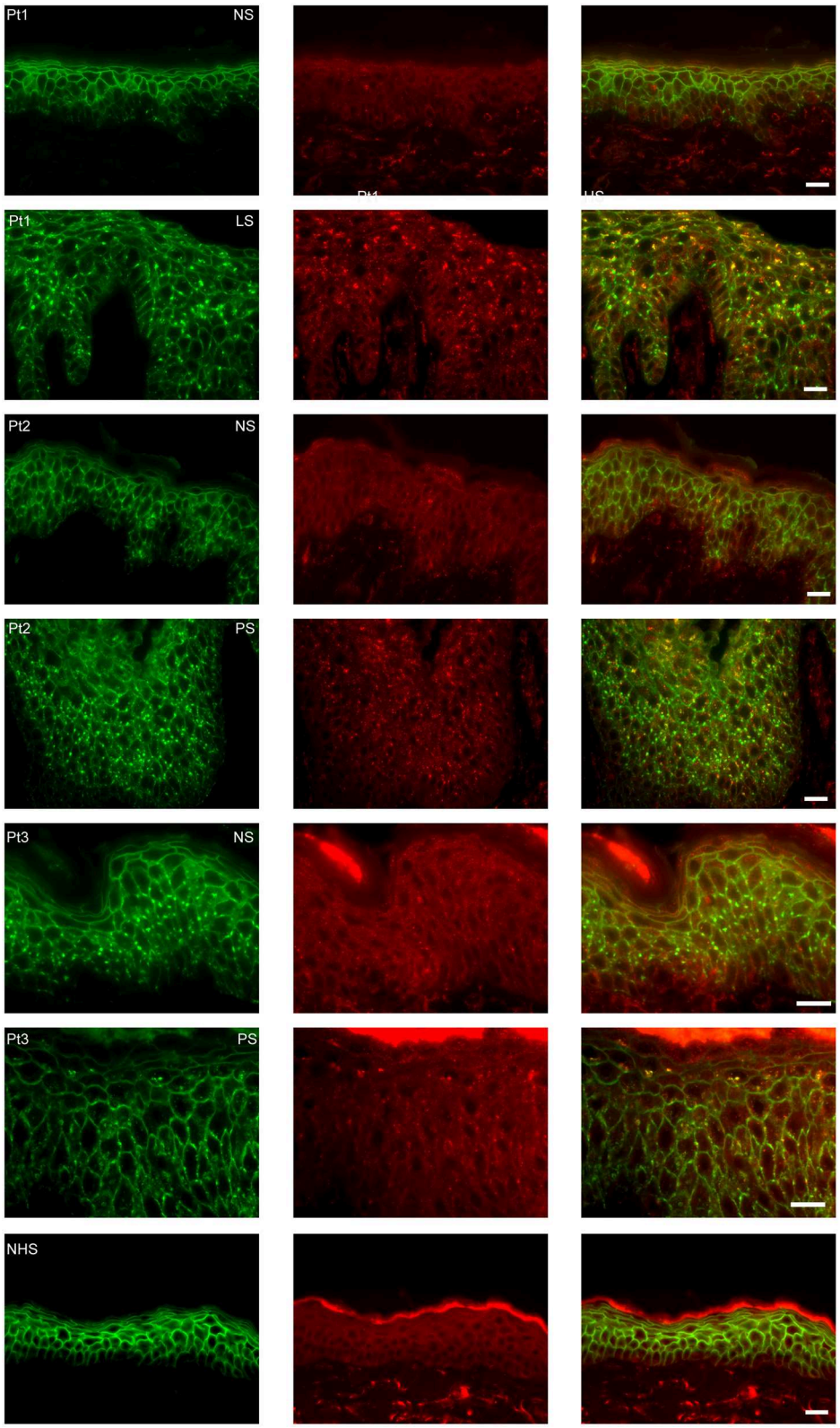

FIGURE 2 | Endocytosis from clusters is not present in non-lesional skin. The images are from three patients of whom we had biopsies of non-lesional skin next to biopsies of perilesional or lesional skin. As control healthy normal human skin is used. In green plakoglobin and in red early endosomal antigen. The panels to the right are overlays. Pt, patient; NS, non-lesional skin; PS, perilesional skin; LS, lesional skin; NHS, normal human skin. White bar is 20 μm.

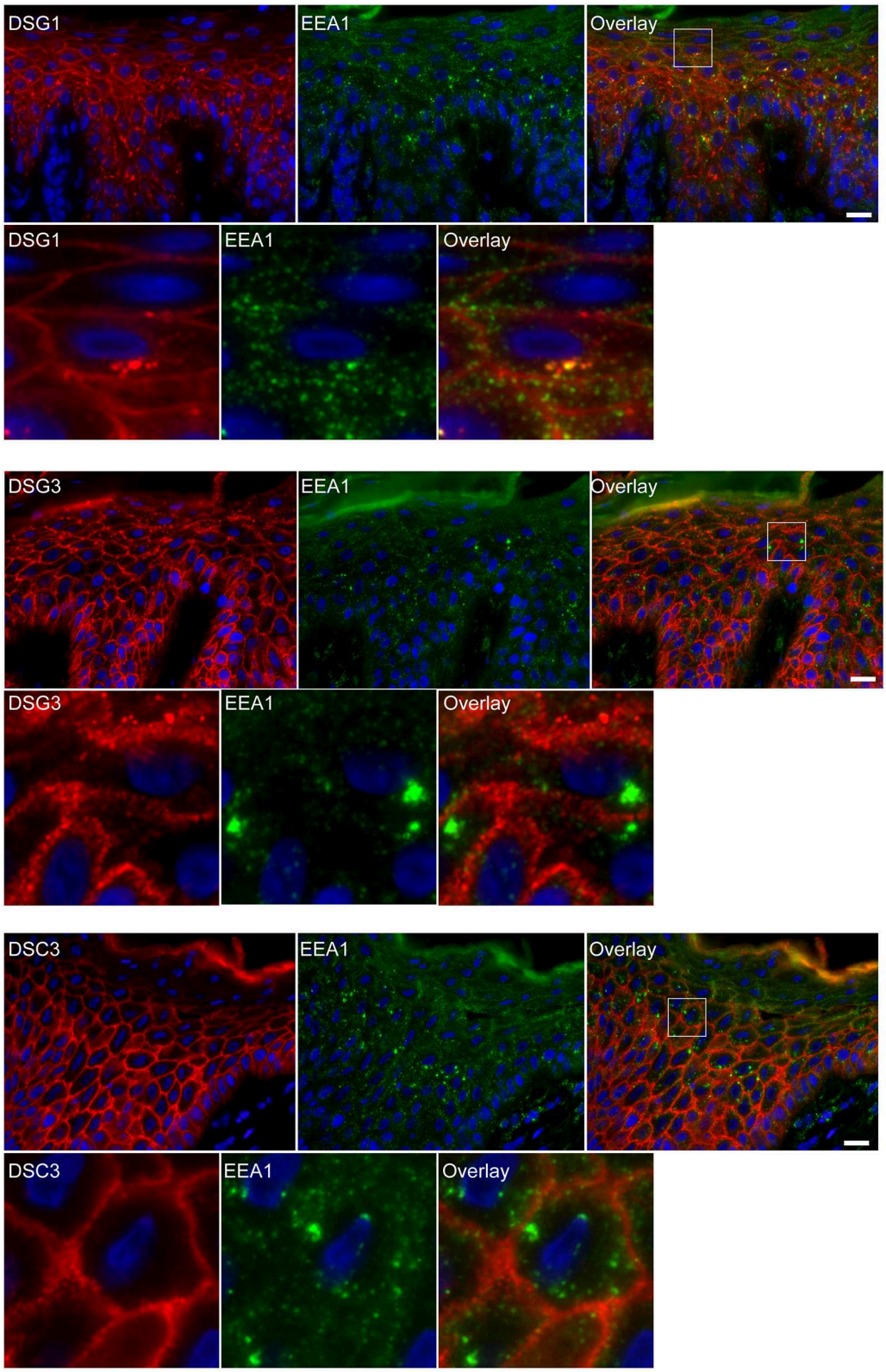

FIGURE 3 | Double staining shows colocalization of EEA1 with desmoglein 1 but not with desmoglein 3 and desmocollin 3. The upper panels show the overviews, the lower panels shows the details from the white boxes in the upper panels. White bar is 20 μm.

become depleted of DSG1 (8). In skin where IgG has induced clustering of DSG1 desmosomes become reduced in size and number (14). The reduction in size of the desmosomes can be induced in *ex vivo* living human skin by both pathogenic or non-pathogenic PF mAb, while their mixture enhances this effect (13).

Tada and Hashimoto (15) studied patient skin by immunoelectron microscopy and found what they called curvicircular cytoplasmic bodies in PF but not in PV skin. These were present in the higher, but not in the lower layers of the epidermis. These structures labeled positive for DSG1, PG, IgG, and connexin 43 (CNX43), and did not contain attachment plaques or inserted tonofilaments. These were hypothesized to be internalized IgG-bound desmosome-gap-junction complexes that transformed into curvicircular structures. In 1999, Iwatsuki et al. described cytoplasmic vesicles in acantholytic keratinocytes that labeled positive for DSG1 and seemed to contain detached desmosomes. We recently described the curvicircular structures to be double membrane structures, that may look intracellular but in fact seem the result of closely sealed (40 nm width) neighboring membranes (www.nanotomy.org). In perilesional skin they are present in the lower epidermal layers and they spread upwards to higher layers in lesional skin (16). We have speculated that these double membrane structures likely are the DSG1-PG-IgG clusters seen by light microscopy.

In order to further unravel the pathomechanism of PF we address in the present the faith of DSG1-PG-IgG clusters and investigate if endocytosis of DSG1 takes place in PF patient skin.

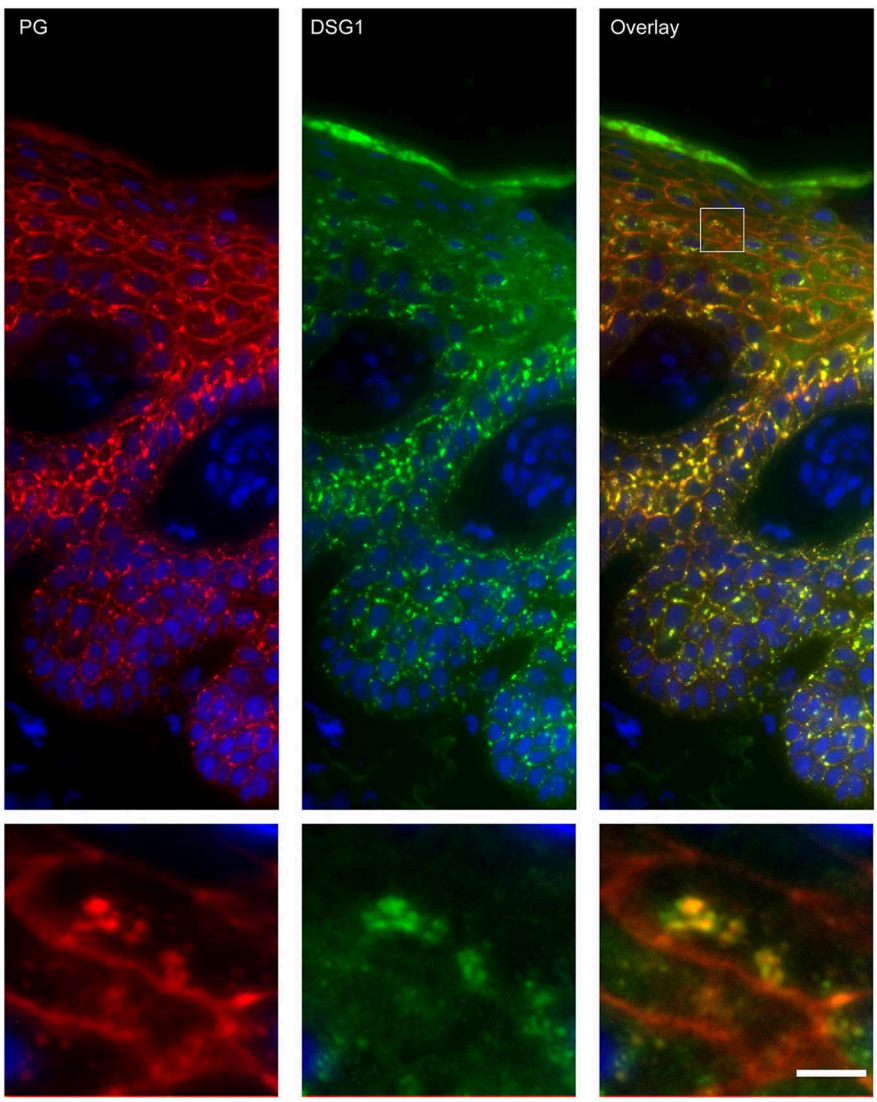

FIGURE 4 | Double staining of desmoglein 1 and plakoglobin 1 shows that these are both present in the endosomes. The upper panel shows the overview, the lower panel shows the detail from the white box in the upper panel. White bar is 5 μm.

MATERIALS AND METHODS

Patient Biopsies

We included 6 PF patients; from these 5 had a biopsy from lesional skin, 1 from perilesional skin, and from three patients we also had a concomitant biopsy from healthy skin.

The diagnosis in all six patients had been established on clinical criteria and laboratory investigation, including histology, positive immunofluorescence of skin (DIF), positive binding of serum IgG (1/40 dilution) to monkey esophagus in an epithelial cell surface (ECS) pattern, and serum anti-DSG1 antibodies by MESACUP-2 ELISA test for anti-DSG1 (MBL, Japan). The skin specimens had been immediately frozen in liquid nitrogen and stored at −80°C. Skin obtained from breast reduction was used as healthy control skin. According to national regulations this type of retrospective non-interventional study on leftover tissue from diagnostics does not need approval from the local medical ethical committee.

Immunofluorescence Microscopy

The procedure for immunofluorescence staining and image collection has been described in detail before (8). For visualization of adhesion molecules we used the following monoclonals: DSG1-P23 and DSG1-P124 (DSG1 ectodomain), 27B2, DG3.10, 18D4 and B-11 (DSG1 endodomain), DSG-G194 (DSG3), U100 (DSC1), U114 (DSC3), 15F11 (PG), DP2.15 (DP), and PKP3-270.6.2 (PKP3). Early endosomal antigen (EEA1) was stained with 14/EEA1, cathepsin D (CTS D) was stained with CTD-19, LAMP-1 with H4A3, and connexin 43 with 4E6.2. Double staining of IgG and adhesion molecules IgG was performed with fluoresceinthiocyanate (FITC)-conjugated Fcγ-specific goat F(ab')2 anti-human IgG (Protos Immunoresearch, Burlingame, CA, U.S.A) and Alexa 568-conjugated goat anti-mouse IgG (Molecular Probes Eugene, OR, U.S.A) as secondary steps. For double staining with two different mouse monoclonals we used

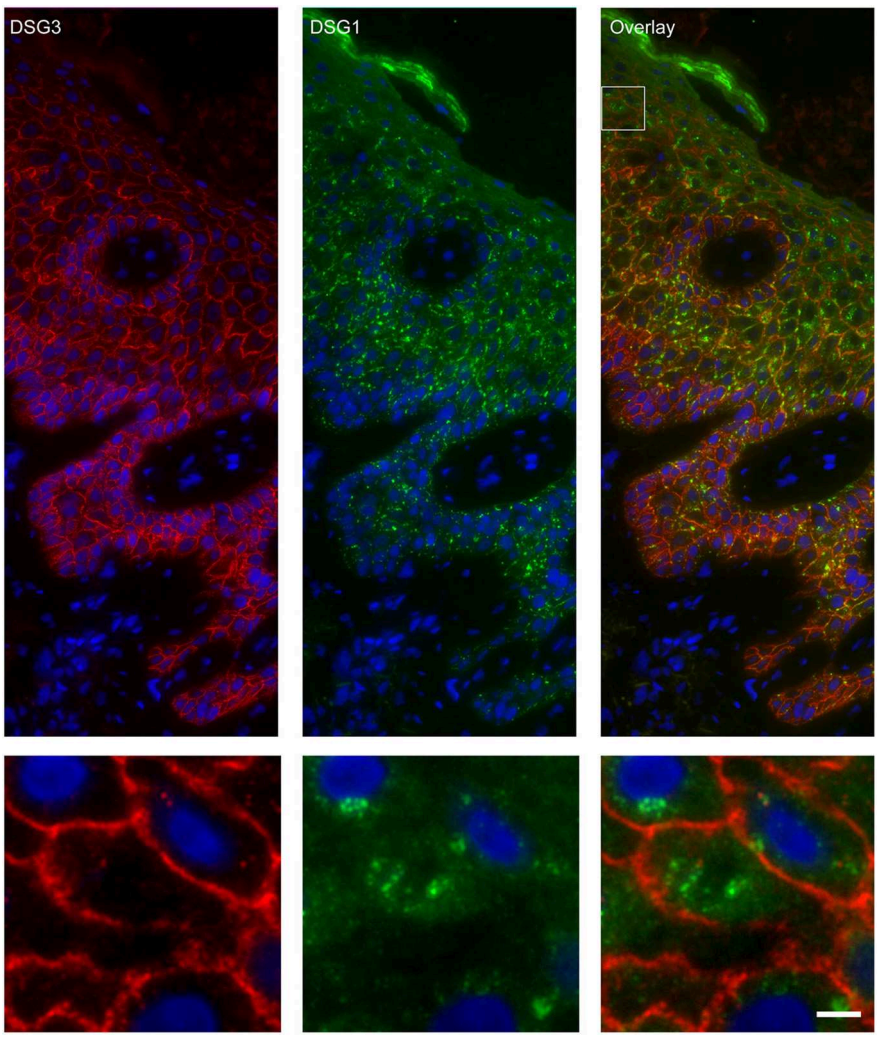

FIGURE 5 | Double staining of desmoglein 1 and desmoglein 3 shows no overlap in endosomes. The upper panel shows the overview, the lower panel shows the detail from the white box in the upper panel. White bar is 5 μm.

Zenon® Mouse IgG Labeling Kits Alexa Fluor®488 and Alexa Fluor®568 (Molecular Probes, Invitrogen, USA) by following technical protocols. **Supplementary Table 1** contains details on antibodies used.

RESULTS

Endosomal Uptake Takes Place in the Upper Layers of Lesional Pemphigus Foliaceus Skin

In order to examine the fate of DSG1, PG, and IgG clusters, we double stained the perilesional and lesional biopsies for EEA1 and IgG. In 4 of the 6 biopsies EEA1 colocalized with IgG demonstrating that IgG is taken up in endosomes. This is most prominent in the higher epidermal layers, next to and beneath the blister (**Figure 1A**). In more differentiated cells these endosomes are largely localized perinuclear (**Figure 1B**). In the 3 biopsies of non-lesional skin of our PF patients no endocytosis was observed. **Figure 2** compares endocytosis of PG in non-lesional skin with perilesional or lesional skin of the same patients. In normal healthy human skin no overlap of EEA1 and PG was observed (**Figure 2**).

Endosomes Contain Desmoglein 1 and Plakoglobin but No Other Desmosomal Proteins

Apart from IgG and PG also DSG1 is present in the same endosomes. In order to examine if other desmosomal components are also endocytosed we examined the localization of other desmosmal proteins in relation to the EEA1/DSG1/PG clusters. No overlap was found. **Figure 3** shows colocalization of EEA1 with DSG1 but not with DSG3 and DSC3. Where DSG1 and PG (**Figure 4**) colocalized in the intercellular

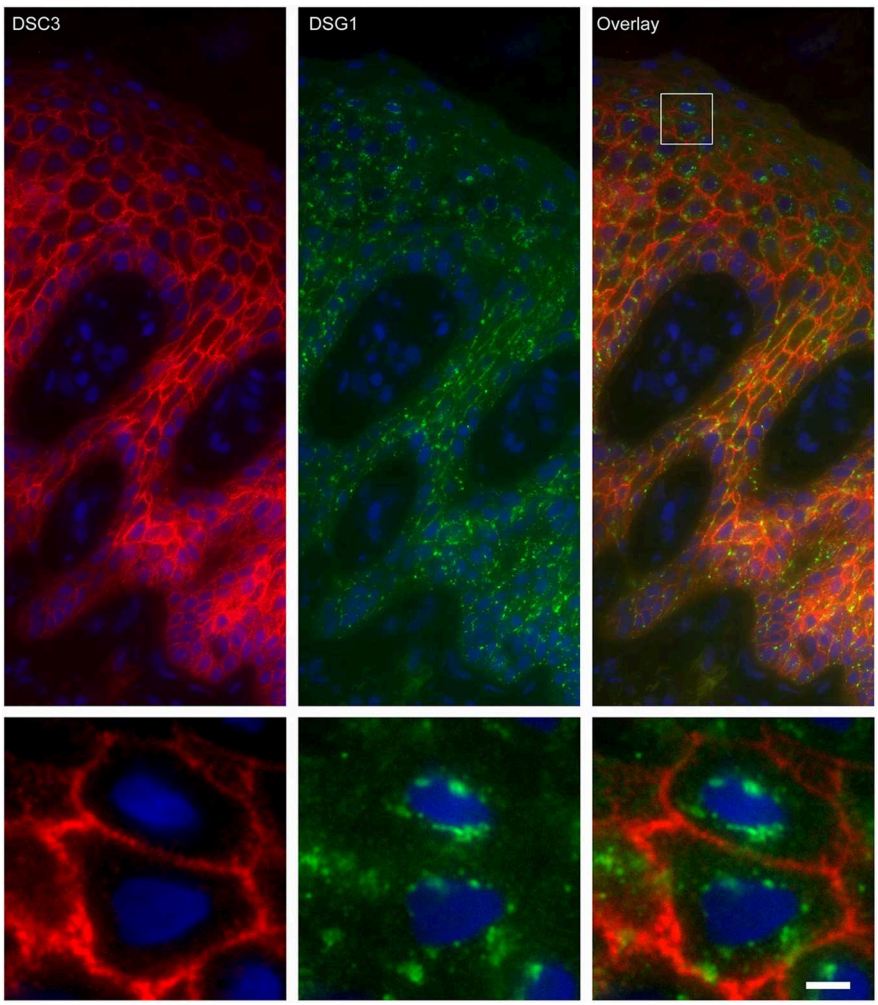

FIGURE 6 | Double staining of desmoglein 1 and desmocollin 3 shows no overlap in endosomes. The upper panel shows the overview, the lower panel shows the detail from the white box in the upper panel. White bar is 5 μm.

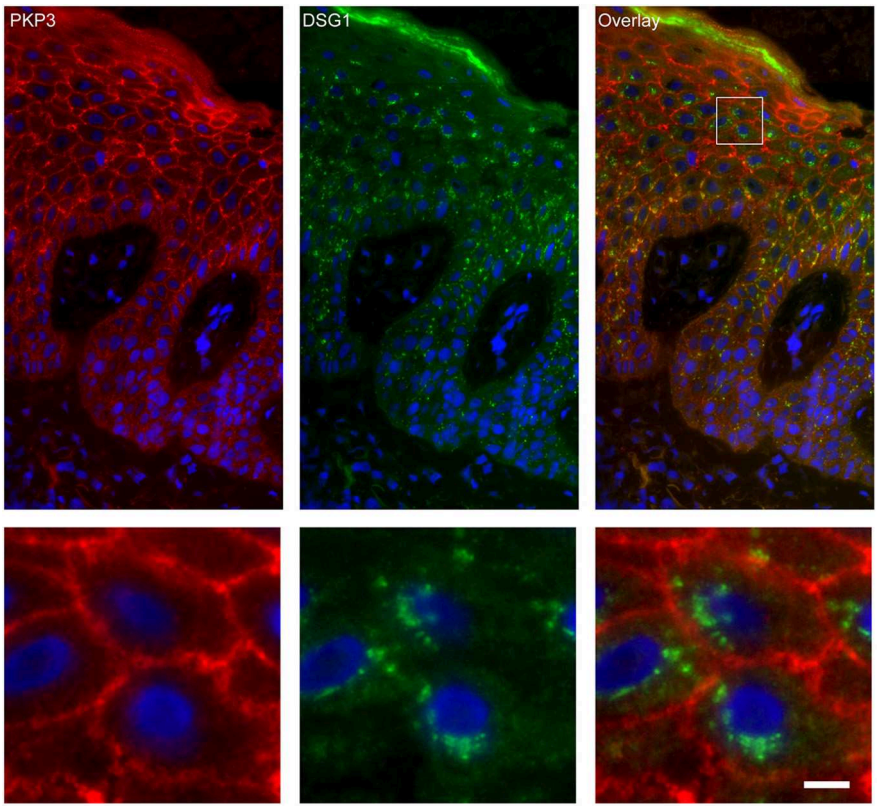

FIGURE 7 | Double staining of desmoglein 1 and plakophilin 3 shows no overlap in endosomes. The upper panel shows the overview, the lower panel shows the detail from the white box in the upper panel. White bar is 5 μm.

vesicles no co-localization of DSG1 with other desmosomal proteins was observed (**Figures 5–8**). The absence of desmosomal proteins as for instance DP in the clusters suggests that extra-desmosomal rather than desmosomal DSG1 is endocytosed.

Endosomal Uptake Starts From the IgG Clusters

In the basal layer endosomes do not contact the IgG clusters (**Figure 9C**). Upwards in the spinal layer endosomes moor on to the clusters and internalization starts to take place (**Figure 9B**). Then finally they become localized perinuclear (**Figure 9A**). Confocal microscopy shows that the endocytosis from the cluster takes place symmetrically in both cells that share the cluster (**Figure 9D**, white arrows).

Endosomes Do Not Contain Connexin 43 and Do Not Fuse With Lysosomes

Double staining of DSG1 and CNX43 showed that both were present in intracellular vesicles but these did not colocalize, implying they originate from different mechanisms (**Figure 10**). Surprisingly CTS D and LAMP-1 did also not colocalize with DSG1 indicating that lysosomes are not a destiny of the IgG-DSG1-PG clusters (**Figure 10**).

DISCUSSION

In this study we demonstrate that the clustered deposits of IgG in PF patient skin are cleared by endocytosis together with the co-clustered components DSG1 and PG (8). Although clusters are present in the basal epidermal layers active endocytosis starts in the spinous layer. Endocytosis is symmetrical in the way that in takes simultaneously place in both cells that share the cluster and progresses in more differentiated keratinocytes toward the cell nucleus.

Tada and Hashimoto suggested that binding of PF autoantibodies induce internalization of intact desmosomes and gap junctions in form of curvicircular structures that contained IgG, DSG1, PG, and CNX43 (15). Our double labeling results however show that CNX43 is present in separate organelles that are likely internalized annular gap junctions (17). Furthermore we did not find internalizations that contained other desmosomal components meaning that endocytosis of intact or half-desmosomes does not take place in PF skin as suggested before (18). Cirillo et al. demonstrated internalization of DSG1 upon incubation with PF patient sera in a monolayer of cultured cells (19). Cultured cells have characteristics of cells from the basal epidermal layer and no internalization of DSG1 was seen in the basal cells in any of PF biopsies. Therefore,

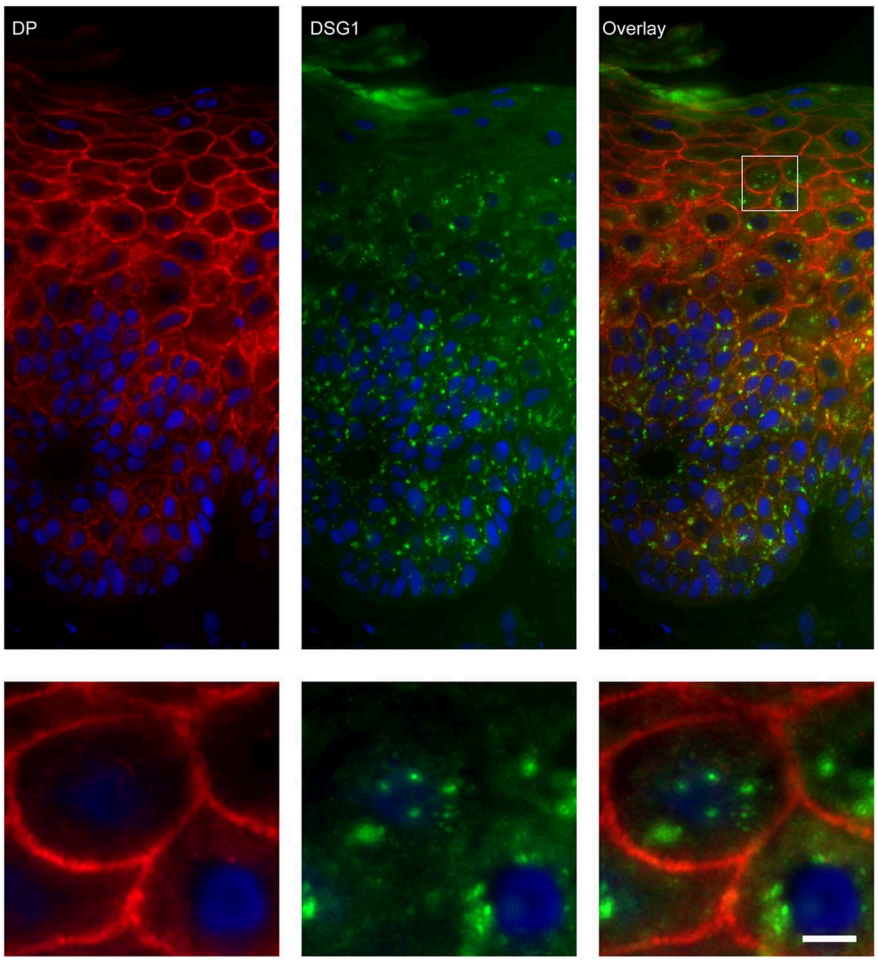

FIGURE 8 | Double staining of desmoglein 1 and desmoplakin shows no overlap in endosomes. The upper panel shows the overview, the lower panel shows the detail from the white box in the upper panel. White bar is 5 μm.

it seems that conclusions reached with cell models have to be interpreted with care.

In contrast with PF, PV was extensively studied in cultured cells and it is shown that PV IgG upon incubation is taken up by endocytosis together with DSG3 and PG (20–23). After 3 h the IgG-DSG3-PG complex colocalized with lysosomes which suggests that cultured cells degrade internalized IgG. However, in PF patient skin we could not find evidence for such a degradation pathway. This suggests that internalized PF-IgG containing endosomes remain in the cell and are finally lost by desquamation. Furthermore in cell monolayer experiments it was shown that upon incubation PV IgG rapidly depletes soluble DSG3 from the membrane followed later by disappearance of desmosomal DSG3 (20, 21). Jennings et al. showed that prior to its internalization insoluble DSG3 is reorganized together with other desmosomal proteins into structures that are perpendicular to the cell membranes that were named linear arrays (23). Stahley et al. by super resolution microscopy observed structures in mucocutaneous PV skin they believed to be the linear arrays from which endocytosis of insoluble DSG3 takes place (24). Although we used simple immunofluorescence microscopy our data do not in any way suggest that such arrays are present in PF skin. Therefore, PV and PF pathogenesis might differ in the way IgG and DSG1 are endocytosed. An indication for this is that in PV skin colocalization of IgG and EEA1 was present in multiple small puncta near the cell membranes while we observed in PF skin that colocalization of IgG and EEA1 is in very large endosomes and only in the upper epidermal layers (24). Furthermore unlike in PV skin endocytosis in PF skin is restricted to the clusters that are induced by the anti-DSG1 IgG only.

Taken together we propose the following sequence of the events in PF patient skin. PF IgG binding to non-desmosomal DSG1 induces clustering and reorganization of non-desmosomal DSG1 together with PG which is bound to the cytoplasmic tail of DSG1. As other molecules as DP are absent desmosomal DSG1 is likely not included in these clusters. This causes lack of DSG1 in desmosomal turnover resulting in depletion of DSG1 from desmosomes and reduction of their size. Reorganized DSG1-PG-IgG are trapped into clusters which are likely the double membrane structures seen by electron

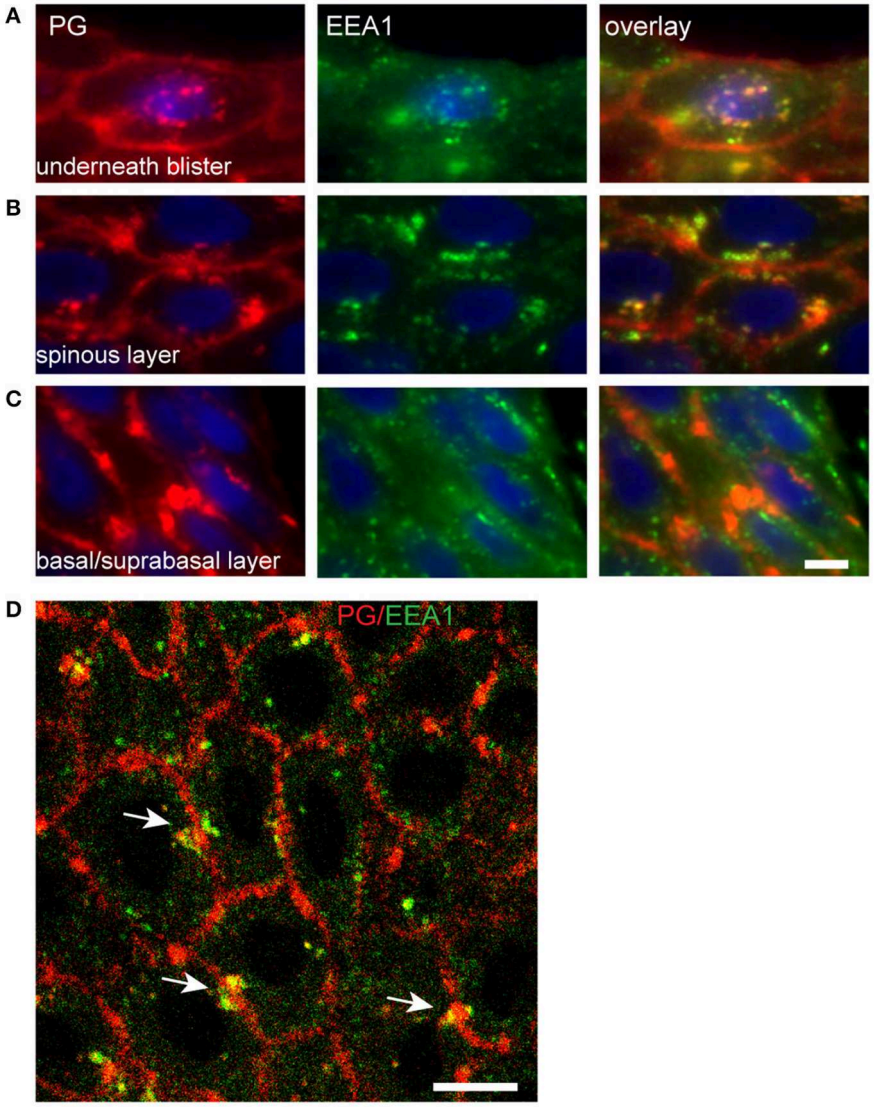

FIGURE 9 | Endocytosis of plakoglobin starts in the spinous layer. Lesional PF skin stained for PG (red) and EEA1 (green). **(A)** Below the blister cavity; **(B)** spinous layer; **(C)** basal/suprabasal layer. Note the colocalization of PG and EEA1 in the spinous layer and below the blister cavity. White bar: 5 μm. **(D)** Confocal microscopy of lesional PF skin stained for PG (red) and EEA1 (green). White arrows indicate symmetrical endocytosis into neighboring keratinocytes. White bar: 10 μm.

microscopy (16). Proteins from these clusters are taken up in endosomal compartments in the upper epidermal layers. This endosomes remain perinuclear until removed with desquamation of keratinocytes. Due to depletion of desmosomes from DSG1 and absence of compensatory DSG3 in the subcorneal layer desmosomes will weaken and finally "melt" away here what leads to blistering. Future research has to address how previous observations on effects of PF serum on signal transduction and the activation of signal transduction molecules are connected to the ultrastructural findings. What does emerge from this and previous studies is that many factors have to be taken into consideration, which complicates such research. For instance why acantholysis only occurs when DSG3 is absent, meaning that whatever is the primary cause of acantholysis, steric hindrance, signal transduction or DSG1 depletion, neither one of them is able to induce acantholysis when DSG3 is still present. It is possible that clustering is a main cause of DSG1 depletion of desmosomes, but PF blisters can also be induced in organ culture in the absence of clustering (8, 10). Then for endocytosis of DSG1 it is unclear why it is absent in the basal layer, although endosomes are present here. Clustering starts in the basal layer as this is the first layer that encounters the pathogenic PF IgG, so something must be missing to start endocytosis.

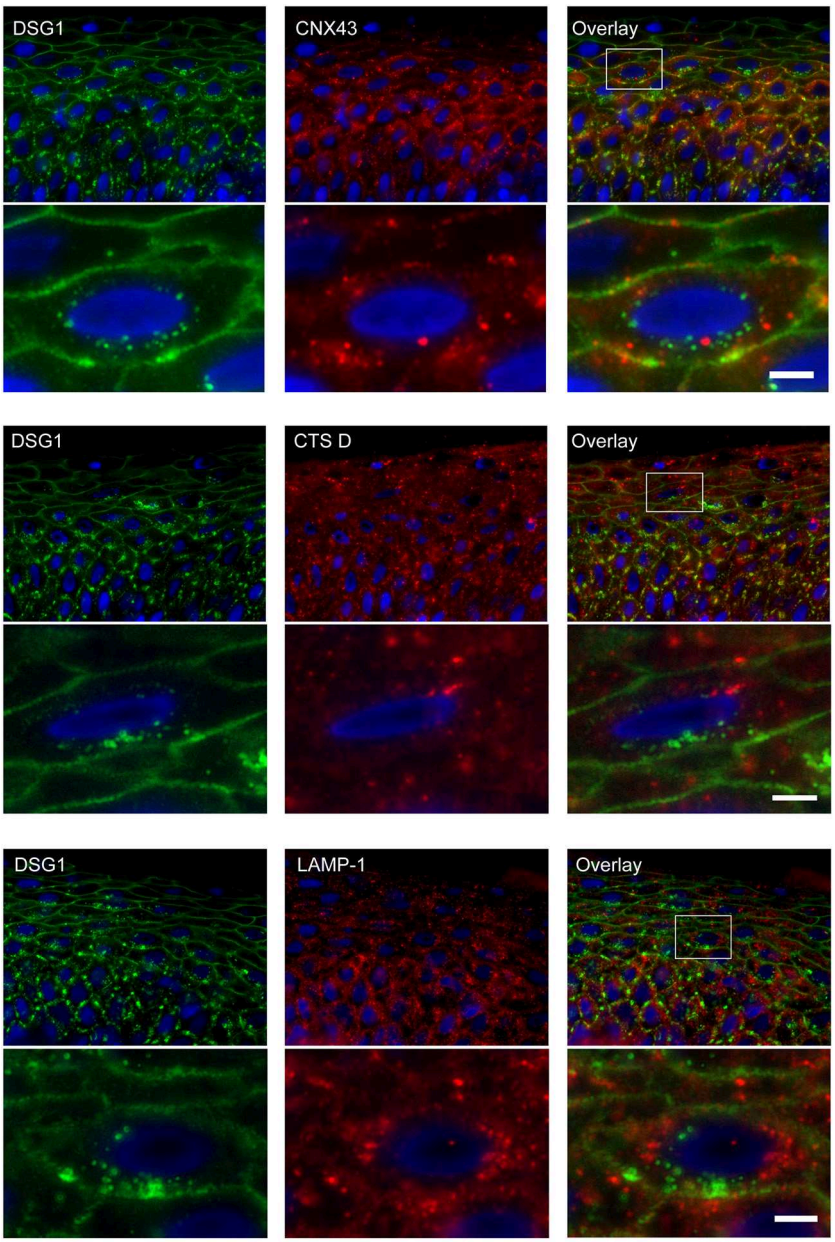

FIGURE 10 | No colocalization of endocytosed desmoglein 1 with connexin 43 and lysosomes in PF skin. Lesional PF patient skin stained for DSG1 and CNX43, upper panel overview with detail underneath, DSG1 and CTS D, middle panel overview with detail underneath, and DSG1 and LAMP-1, lower panel overview with detail underneath. The location of the detailed area is indicated by a white box in the overview. No colocalization is observed. White bar: 5 μm.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DO, HP, and MJ contributed to the design of the study. DO, DK, and ES performed the experiments. DO and ES wrote the manuscript. HP and MJ revised the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02635/full#supplementary-material

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CASE REPORT
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Radiation-Associated Pemphigus Vulgaris in a Patient With Preceding Malignancy: Treatment With Rituximab as a Valuable Option

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Pemphigus is a chronic autoimmune blistering disorder, characterized by (muco-)cutaneous erosions due to autoantibodies against desmoglein 3 and/or 1. Pemphigus induction might be associated with drugs, malignancy or radiation therapy (RT); the latter being only rarely described. A rigorous literature review revealed around 30 cases of RT-associated pemphigus, which had been primarily treated with topical and/or systemic steroids, in some cases also dapsone or few other immunosuppressive agents were given. The most common underlying cancer type was breast cancer. We here present a 63-year-old male patient, who was pre-treated with adjuvant RT for larynx carcinoma 3 months before admission. He developed extensive cutaneous, ocular, and oral erosions. Despite the clinical picture comparable to a paraneoplastic pemphigus, the diagnosis of pemphigus vulgaris of mucocutaneous type was established based on the direct immunofluorescence, showing positive cell surface IgG and discrete C3 deposits, with matching cell surface IgG pattern on monkey esophagus. Serum autoantibodies to desmoglein 1 and 3 were highly positive. No further autoantibodies were found, thus paraneoplastic pemphigus was excluded. The patient was treated with high dose prednisolone, partially given intravenously up to 2 mg/kg per day, as well as topical disinfectants and class IV steroid cream. To stabilize the disease rituximab 2 × 1,000 mg was given, leading to clinical and serological remission for up to 2 years now. We show that rituximab represents a good treatment option for the frequently treatment-refractory RT-associated pemphigus, a clinically and immunologically specific RT-induced skin disorder, resulting in long-term clinical, and serological remission.

Keywords: autoimmune blistering disorder, desmoglein, desmosome, desmosomal adhesion, radiation therapy

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INTRODUCTION

The incidence of pemphigus has been estimated to be between 0.5 and 34 cases/million inhabitants/year (1), thus the disease is considered to be rare. As factors related to the induction of pemphigus, specific drugs and malignancy, but also radiation therapy (RT) have been proposed. Malignancy-associated pemphigus has an incidence of 5–11%, while malignancy-associated bullous

pemphigoid has a reported incidence of 5.8–10.2% in retrospective studies (2, 3). As the underlying malignancy, lung cancer was most common in pemphigus, and gastric cancer in bullous pemphigoid (3). Cases of RT-associated pemphigus have only rarely been described in the literature (**Table 1**). In this study, we report the development of pemphigus vulgaris (PV) in a patient who received RT for cancer treatment and review the literature on RT-associated pemphigus. Search terms included irradiation, radiation, radiotherapy, and cobalt therapy. The data provided include age, gender, underlying malignancy, dosage of RT, time intervals between RT and the onset of pemphigus, immunofluorescence data, and the treatment regime. In our patient we show that treatment with rituximab induces long-term clinical and serological remission for over two and a half years.

METHODS

Immunofluorescence (IF) Studies

Direct immunofluorescence (DIF) and indirect immunofluorescence (IIF) were performed in the Department of Dermatology, Medical Center—University of Freiburg. The FITC-labeled antibodies were anti-human IgG, IgA, IgM, and C3c (Dako, Hamburg, Germany) at a dilution of 1:200, 1:50, 1:50 and 1:500, respectively. For IIF on monkey esophagus, patients' sera were diluted 1:10; secondary antibodies used were FITC-labeled anti-human IgG (Dako, Hamburg, Germany) at a dilution of 1:100.

Immunoblotting Studies

Immunoblotting of normal human epidermal extracts (31, 32), the BP180 NC16a domain recombinant protein (33), the BP180 C-terminal domain recombinant protein (34), the concentrated culture supernatant of HaCaT cells (35), normal human dermal extract (36), and purified human laminin-332 for both IgG and/or IgA antibodies (37) were performed at the Kurume University and Osaka City University as previously described.

Enzyme-Linked Immunosorbent Assays (ELISA) Studies

Commercially available IgG ELISAs of Dsg1 and Dsg3 (MESACUP, MBL, Nagoya, Japan) were conducted according to the manufacturer's instructions and measured by ELISA reader.

RESULTS

We present a 63-year-old male patient, who was referred to our department due to extensive cutaneous (**Figures 1A–C**) and mucosal erosions (eyes, nose, lips, mouth, and esophagus). No paronychia-like or lichen planus-like lesions were observed. Until 3 months prior to admission, the patient had received adjuvant RT, following surgical reconstruction and bilateral neck lymph node dissection to treat larynx carcinoma. At that time point, the patient had received 54 Gy (single dose 1.8 Gy each) for his cervical right (level II-IV) and left region (III-IV), as well as a single boost at the tumor bed and additional 63.9 Gy (single dose 2.13 Gy each) for the left cervical lymph nodes (level II). The tumor appeared to be in remission. Histopathology of a skin specimen showed an intraepidermal split with the characteristic tombstone pattern of the basal keratinocytes (not shown). DIF of a skin specimen from the thigh revealed cell surface deposition of IgG (**Figure 2A**) and C3 in the entire epidermis. IIF with normal human skin and monkey esophagus (**Figure 2B**) revealed circulating cell surface IgG autoantibodies at a titer of 1:160. No reaction to the basement membrane zone or transitional epithelium of rat bladder was observed.

Immunoblotting of normal human epidermal extracts demonstrated IgG autoantibodies against the 130-kDa Dsg3 (**Figure 2C**, lane 4) (32), but no reactivity with Dsg1, envoplakin, periplakin, BP230, BP180, LAD-1 antigen, collagen VII, or laminin-332. Other immunoblotting methods did not detect any other autoantigens, including BP180, LAD-1 antigen, collagen VII, laminin gamma-1, and laminin-332. ELISA results were positive for both anti-Dsg1 (191 units; positive index $\geqq$ 20), and anti-Dsg3 antibodies (144 units; positive index $\geqq$ 20). ELISAs for recombinant eukaryotic desmocollin 1-3 proteins were negative (38). The discrepancy between immunoblotting and ELISA for anti-Dsg1 antibodies can be explained by that the PV sera tend to react with non-conformational epitopes, which are destroyed during the procedure of immunoblot, but not ELISA. The higher sensitivity in ELISA diagnostics has been well-established by previous studies (39). In fact it has been shown that in immunoblot with normal epidermal extracts most PV sera react with Dsg3, while only about two-thirds or PF sera react with Dsg1 (32). PV with underlying malignancy and PNP are sometimes difficult to distinguish. However, although our case had severe oral and ocular mucosal paraneoplastic pemphigus-like lesions, immunoblotting showed reactivity neither with envoplakin nor periplakin, excluding the diagnosis of PNP.

Initially, the patient was treated with high dose prednisolone, first given intravenously up to 2 mg/kg per day, topical disinfectants, and class IV steroid creams, combined with meticulous wound care for several weeks. Administration of rituximab 2 × 1,000 mg led to quick stabilization of the disease and to clinical and serological remission. Currently, the follow-up-time is two and a half years (**Figure 1D**), showing that rituximab is a valuable treatment for this rare pemphigus subtype; it improved the patient's quality of life tremendously.

A rigorous literature search for RT-associated pemphigus revealed 29 reported cases (**Table 1**). Of these, 23 represented PV and eight pemphigus foliaceus (PF). The age of the patients ranged from 37 to 92 years, with a median of 62 years. In 11 cases, the disease onset was within <1 month, in 12 cases skin lesions developed within the first 3 months after therapy initiation. Twenty-seven of 30 (90%) cases initially presented with lesions at the irradiated side with the majority of these patients (24/30 cases, corresponding to 80%) having a progression to the non-irradiated skin (11/24 cases, corresponding to 46%) or even to a generalized distribution (13/24 cases, corresponding to 54%).

Abbreviations: DIF, direct immunofluorescence; HMGB1, High mobility group box 1; IIF, indirect immunofluorescence; ELISA, Enzyme-linked immunosorbent assay; PF, pemphigus foliaceus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris; RAGE, receptor for advanced glycation endproduct; RT, radiation therapy.

TABLE 1 | Patients with radiation-associated pemphigus described in the literature.

No	Age	Diagnosis	Time from radiation to start of eruption	DIF (cell surface)	IIF (cell surface)	Neoplasm	Radiation characteristics	Localization (irradiated area, non-irradiated area, generalized)	Treatment	References
1	63	PV	1 month	IgG	IgG	Hypopharynx carcinoma	LN cervical R II-IV, L III-IV 54 Gy, tumor bed, and LN cervical II 63,9 Gy	Irradiated area with generalized progression	Prednisolone 2 mg/kg/day, topical clobetasol propionate 0.05% ointment (body), followed by rituximab 2 × 1,000 mg	Current case
2	48	PV	3 months	+	na	Medullary breast cancer	100 Gy	Irradiated area with progression to non-irradiated area	Prednisone 150 mg/day	(4)
3	56	PV	1 year	na	na	Epidermoid bladder carcinoma	65 Gy	Generalized mucocutaneous type	Prednisone 60 mg/day	(5)
4	65	PV	<1 month	na	na	Breast cancer	n.a	Irradiated area with generalized progression	Prednisone 80 mg/day, later 120 mg/day, methotrexate 25 mg, then azathioprine 100 mg/day	(5)
5	70	PV	14 days	IgG, C3	+	Gastric lymphosarcoma	40 Gy	Irradiated area with generalized progression	Prednisone 120 mg/day	(6)
6	70	PV	4 months	+	+	Solar keratosis on the forehead	48 Gy	Irradiated area to non- irradiated area	Prednisone 100 mg/day	(7)
7	52	PV	3 weeks	na	na	Bronchial squamous cell carcinoma	40 Gy	Irradiated area with generalized progression	Methylprednisolone intravenously 1,250 mg 6 days, then 1 mg/kg BW and tapering 45 days	(8)
8	73	PV	3 months	+	+	Breast cancer	55 Gy	Irradiated area with generalized progression	Prednisone 50 mg/day	(9)
9	70	PF	1 month	+	+	Laryngeal squamous cell carcinoma	60 Gy	Irradiated area with progression to non-irradiated area	Prednisone 2 mg/kg/day, azathioprine 50 mg/day	(9)
10	54	PV	3 weeks	+	IgG	Lymphoma	38 Gy	Irradiated area with generalized progression	Prednisone 2 mg/kg/ day	(10)
11	77	PV	na	+	+	Basal cell carcinoma	60 Gy	Irradiated area with progression to non-irradiated	Prednisone 100 mg/day, dapsone 100 mg/day	(11)
12	45	PV	1 week	+	na	Breast cancer	68 Gy	Irradiated area	Prednisone 80 mg/day	(12)
13	61	PV	2 months	+	na	SCC of the lower lip	70 Gy	Irradiated area with progression to non-irradiated	Prednisone 1 mg/kg/day	(13)
14	54	PV	1 month	IgG, C3	IgG	SCC of the lung	59.4 Gy	Irradiated area with progression to non-irradiated	Methylprednisolone intravenously, then oral prednisone	(14)
15	73	PV	3 weeks	+	na	Epidermoid carcinoma	66 Gy	Irradiated area with progression to non-irradiated	Prednisone 1.5 mg/day, followed by rituximab 6 x 375 mg/m^2	(15)
16	49	PV	4 weeks	+	na	Breast cancer	50 Gy	Non-irradiated area (mouth and esophagus)	Prednisone 60 mg/day, methotrexate 15 mg weekly	(16)

(Continued)

TABLE 1 | Continued

No	Age	Diagnosis	Time from radiation to start of eruption	DIF (cell surface)	IIF (cell surface)	Neoplasm	Radiation characteristics	Localization (irradiated area, non-irradiated area, generalized)	Treatment AIBD	References
17	48	PV	6 months	na	+	Breast cancer (ductal carcinoma *in situ*)	Megavoltage radiation therapy−50 Gy	Irradiated area	Prednisone 100 mg/day, topical steroids	(17)
18	61	PV	1 month	IgG, C3	IgG (rabbit tongue)	Epidermoid carcinoma of the piriform sinus	70 Gy tumor bed, 54 Gy cercical area	Irradiated area with generalized progression	Methylprednisolone 1 g, followed by oral prednisolone 1.5 mg/kg/day	(18)
19	47	PV	Within days (face), 6 months body	C4	IgG	Acinic cell carcinoma of the parotid gland	39, 6 Gy (cheek and chin)	Irradiated area with generalized progression	Prednisolone 1 mg/kg/day, azathioprine 150 mg/day and rituximab 2 × 375 mg/m^2	(19)
20	58	PV	21 days	IgG, C3	+	Low grade infiltrating ductal carcinoma	Tumor area 65 Gy, subclavicular area (46 Gy), right internal mammary node chain (50 Gy)	Irradiated area with progression to non-irradiated area	Prednisone 2 mg/kg/day, MMF 2 g/day	(20)
21	84	PV	8 months	IgG	IgG	Breast cancer	60 Gy	Non-irradiated area (mouth)	Topical clobetasol propionate ointment, oral prednisolone 1 mg/kg/day, azathioprine 2.5 mg/kg/day	(21)
22	58	PV	14 months	IgG	+	Breast cancer (*in situ* ductal)	na	Irradiated area with progression to non-irradiated	Prednisone 60 mg/day, azathioprine 50 mg/day	(22)
23	68	PV	< 1 month	+	na	Breast cancer	40 Gy	Irradiated area with generalized progression	Prednisolone, MMF	(23)
24	37	PF	<1 month	+	+	Malignant thymoma	30 Gy	Irradiated area with generalized progression (after sun exposure)	Methylprednisolone 12 mg, azathioprine 50 mg/day, dapsone 100 mg/day	(24)
25	92	PF	3 months	+	na	Breast cancer (*in situ* intraductal)	50,4 Gy	Irradiated area with generalized progression	na	(25)
26	70	PF	12 months	IgG, C3	IgG	Breast cancer	60 Gy	Irradiated area with progression to non-irradiated	Dapsone 100 mg/ day, topical clobetasol propionate ointment	(26)
27	59	PF	1 month	IgG, C3	na	Extramammary Paget disease	52.5 Gy	Irradiated area with progression to non-irradiated	Prednisolone 0.5 mg/kg/day	(27)
28	65	PF	2 months	IgG, C3	na	Breast cancer	50 Gy	Irradiated area	Prednisolone 30 mg/day	(28)
39	44	PF	< 2 months	IgG and C3, focal staining C3d, and C4d	na	Breast cancer	na	Irradiated area with generalized progression	Oral prednisolone 1 mg/kg/day, topical hydrocortisone 2.5% ointment (face), clobetasol propionate 0.05% ointment (body), oral dapsone	(29)
30	66	PF	1 month	IgG, C3	na	Breast cancer (ductal carcinoma *in situ*)	50 Gy	Irradiated area with generalized progression	Oral triamcinolone 40 mg/day	(30)

BW, body weight; C3, complement 3; C4, complement 4; CS, cell surface; PF, pemphigus foliaceus; PV, pemphigus vulgaris; MMF, mycophenolate mofetil; na, not available; +, positive (fluorescence pattern not described).

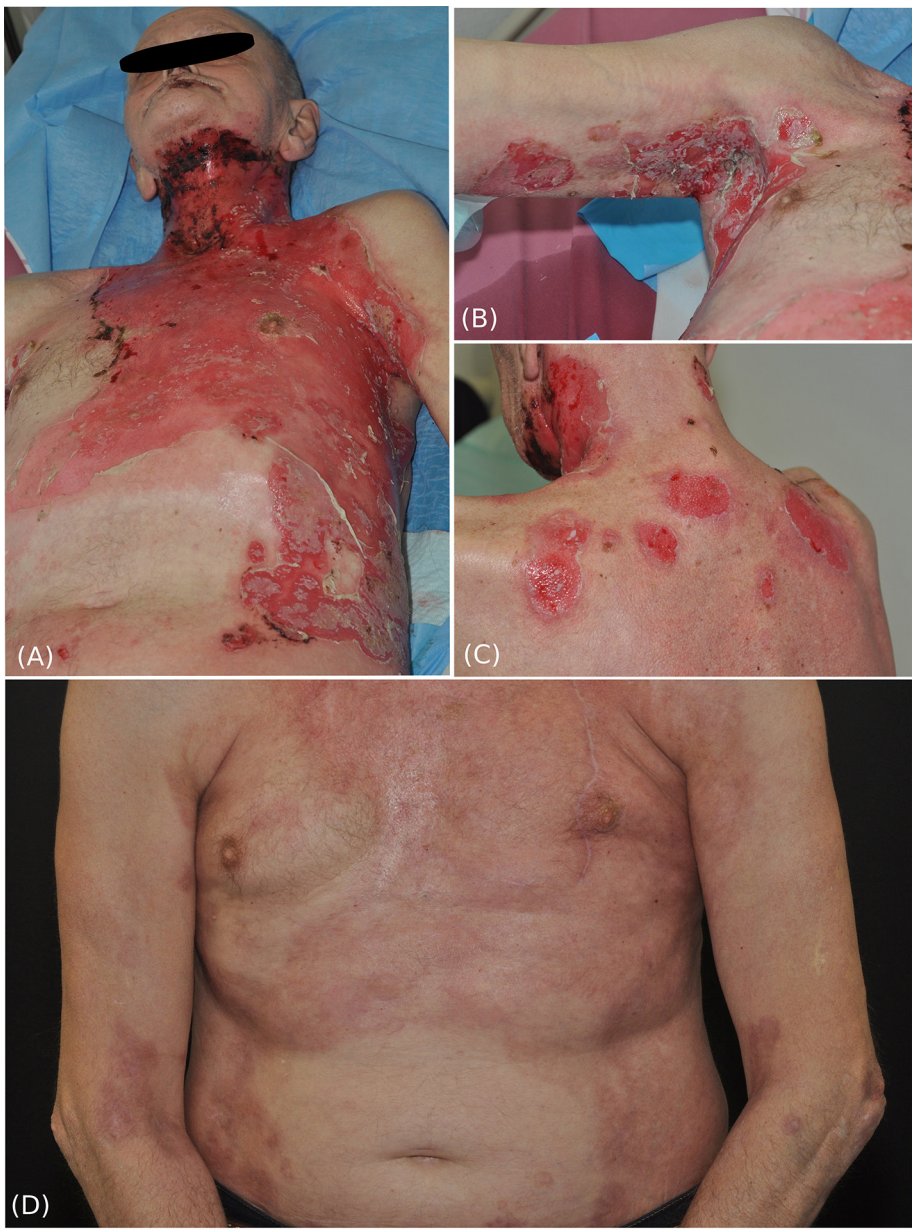

FIGURE 1 | Clinical pictures of the patient at initial presentation and after treatment. Extensive erosive skin detachment of the frontal trunk and neck **(A)**, right axillary fold **(B),** and shoulders and neck **(C)**. Three months after systemic treatment with rituximab and ongoing low dose glucocorticosteroids, intact skin with postinflammatory hyperpigmentations was observed **(D)**.

The skin lesions stayed at the irradiation side in only three patients (3/30 cases, corresponding 10%). Two patients developed erosions in the non-irradiated area at their first presentation, while one patient showed a generalized distribution pattern of PV. At least three cases (including ours) clinically imitated PNP. The pemphigus diagnosis was made in 20 cases by DIF, and in 14 of these cases additionally by IIF. ELISA was performed in only four cases. Fifteen patients ($n = 9$ PV, $n = 6$ PF) were treated for breast cancer. Others received radiotherapy for miscellaneous forms of cancer, for example lymphoma, gastric lymphosarcoma, squamous cell carcinoma of the skin or the lung, and bladder carcinoma. The RT dose varied from 38 to 100 Gy, conducted in fractional application. All published patients, but one, have received oral, or intravenous glucocorticosteroids (prednisolone or methylprednisolone) in a medium to high dose. Ten individuals, representing one third of the reported, needed additional agents like dapsone, azathioprine or mycophenolate mofetil, at least temporarily. In two previously published cases

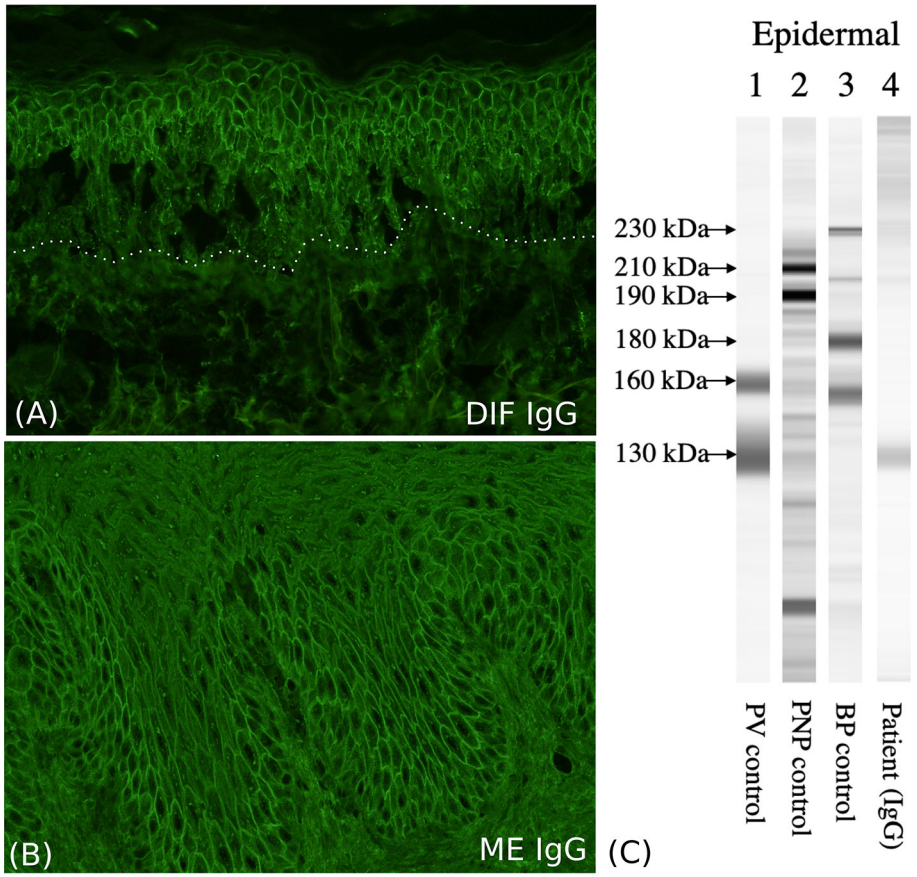

FIGURE 2 | Immunological characterization of the patient. **(A)** The direct immunofluorescence (DIF) shows cell surface IgG deposition on the keratinocytes of the entire epidermis. **(B)** Indirect immunofluorescence (IIF) with monkey esophagus (ME) shows circulating cell surface IgG autoantibodies. **(C)** Immunoblotting with normal human epidermal extracts shows IgG antibodies reactive with 130-kDa Dsg3 (lane 4).

rituximab had been given in different dosages (2 × 375 and 6 × 375 mg/m^2) and resulted in remission with a follow up of up to 6 months (15, 19). Three patients had a pre-existing PF (27–29) that aggravated during radiotherapy; suggesting that in patients with a history of an autoimmune blistering skin disorder, RT should be cautiously considered. Nevertheless, it is not an absolute contraindication, since patients with PV have tolerated radiation without exacerbation of the disease (40).

DISCUSSION

Here we report a severely affected patient with PV in whom RT for larynx cancer triggered the onset of the disease. So far, 29 cases RT-associated pemphigus have been reported in the literature with the majority of cases having lesions first at the RT site, soon followed by generalized erosions (**Table 1**). This clinical sign facilitates differentiation from a classical RT-induced dermatitis. In our case, high dose prednisolone and B-cell depletion by rituximab was required to induce clinical and serological remission for more than two and a half years, without need for further immunosuppression. Since this is the third case of RT-associated PV treated with rituximab, showing a quick response and resulting in clear remission in all three cases, we propose that rituximab should be considered early, when treating such patients. Rituximab selectively binds to CD20 and leads to B-cell depletion due to antibody- and complement-dependent cytotoxicity, as well as induction of apoptosis. The use of immunosuppression in patients with malignancies should be avoided, whenever possible. However, in around one third of the already reported RT-associated pemphigus cases such drugs had to be given to control disease (**Table 1**). Thus, especially for the most severely affected patients, requiring additional immunosuppression than steroids, rituximab given at the already published regimen for PV (41) (1,000 mg rituximab on days 1 and 15) would be probably the most advantageous treatment option.

Another autoimmune skin blistering disorder that is pathogenetically often connected to malignancy or RT is bullous pemphigoid (BP). The so far published cases comprise similarly to RT-associated pemphigus more than 30 (42), including a stage IV melanoma patient with dual exposure to PD-1

checkpoint inhibition and RT therapy (43). Nguyen et al. have summarized 29 patients with BP following radiation for malignancy treatment. BP was localized on the irradiated area in 25 of them, in two it was localized in non-irradiated sites and in another two it was generalized (44). All cases had a rather benign course, with corticosteroids being sufficient to control the disease. RT-associated pemphigus appears to have a more severe disease course, since it tends to spread to non-irradiated skin and in one third of cases further treatment with other immunosuppressants was required to control disease.

Several hypotheses have been proposed about the role of RT in induction of autoimmune skin blistering disorders. The efficacy and role of RT in anti-tumor therapy is believed to be due to the RT-induced DNA damage to malignant cells, but recent evidence demonstrates that RT also activates the innate immune system, promoting specific danger signals like complement, calreticulin, and high mobility group box 1 protein (HMGB1), as well as a variety of cytokines and chemokines (45). Beside a few patients who may have circulating autoantibodies before RT, the substantial role of complement in activation of BP is well-established, while the role of complement in pemphigus has a controversial standing (46). HMGB1, released by damaged cells can stimulate macrophages and dendritic cells, thus resulting in an activation of T cells. Increased serological concentration, paired with plentiful cytoplasmic overexpression of HMGB1 and its receptor RAGE has been observed in the epidermis of pemphigus patients (47). This results in tissue destruction and unmasking of epidermal structures, thus promoting auto-reactivity in the irritated, immunocompromised area (48, 49). Furthermore, there are no sufficient data about the effects of RT damage and recovery of the thymus, which is in anatomical proximity to the tumor region in our patient. Post-RT effects in total body RT mouse model comprised reduced thymocyte numbers and long-term suppression of thymopoesis (50), supporting a hypothesis of impaired T-cell education and disturbed selection processes inducing autoimmunity. Finally, RT-associated pemphigus appears to have a similar incidence as pemphigoid. This contrasts the situation in the non-radiated population, where BP is more common. That is another argument for the direct induction of autoantibodies by the tumor in the RT-associated pemphigus. Additional studies to characterize the pathogenesis of RT-associated pemphigus are required to gain a better understanding of the disease.

Taken together, we show that rituximab represents a good treatment option for the frequently treatment-refractory RT-associated pemphigus, resulting in long-term clinical, and serological remission.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The study has been approved by the Ethics committee in Freiburg (Number 235/15). All analyzes were performed with written informed consent of the patient, after ethics approval and in accordance to the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

FS and DK contributed conception and design of the study, they also wrote the initial manuscript draft. NI and TH performed part of the diagnostics and wrote sections of the manuscript. FS, MM, and DK cared for the patient, performed part of the diagnostics, and interpreted the results. LB-T interpreted the data and revised the manuscript critically for important intellectual content. All authors contributed to manuscript revision, read and approved the submitted version.

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