Semin Immunopathol (2016) 38:57–74
DOI 10.1007/s00281-015-0541-1

REVIEW

Immune response in pemphigus and beyond: progresses and emerging concepts

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Received: 4 June 2015 / Accepted: 30 October 2015 / Published online: 23 November 2015

Abstract Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are two severe autoimmune bullous diseases of the mucosae and/or skin associated with autoantibodies directed against desmoglein (Dsg) 3 and/or Dsg1. These two desmosomal cadherins, typifying stratified epithelia, are components of cell adhesion complexes called desmosomes and represent extra-desmosomal adhesion receptors. We herein review the advances in our understanding of the immune response underlying pemphigus, including human leucocyte antigen (HLA) class II-associated genetic susceptibility, characteristics of pathogenic anti-Dsg antibodies, antigenic mapping studies as well as findings about Dsg-specific B and T cells. The pathogenicity of anti-Dsg autoantibodies has been convincingly demonstrated. Disease activity and clinical phenotype correlate with anti-Dsg antibody titers and profile while passive transfer of anti-Dsg IgG from pemphigus patients' results in pemphigus-like lesions in neonatal and adult mice. Finally, adoptive transfer of splenocytes from Dsg3-knockout mice immunized with murine Dsg3 into immunodeficient mice phenotypically recapitulates PV. Although the exact pathogenic mechanisms leading to blister formation have not been fully elucidated, intracellular signaling following antibody binding has been found to be necessary for inducing cell-cell dissociation, at least for PV. These new insights not only highlight the key role of Dsgs in maintenance of tissue homeostasis but are expected to progressively change pemphigus management, paving the way for novel targeted immunologic and pharmacologic therapies.

Keywords Autoantibody · Desmoglein · Pemphigus vulgaris · Pemphigus foliaceus · Fogo selvagem · Monoclonal antibody · Precursor Dsg · Mature desmoglein · Signaling pathways · Cadherins · Cell-cell adhesion · Desmosome · Pathogenesis · Acantholysis

This article is a contribution to the Special Issue on Immunodermatology - Guest Editors: Lars French and Alexander Navarini

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Abbreviations

AutoAb	Autoantibody
Dsg	Desmoglein
PV	Pemphigus vulgaris
PF	Pemphigus foliaceus
FS	Fogo selvagem
mAb	Monoclonal antibody
preDsg	Precursor Dsg
matDsg	Mature Dsg

Introduction

Pemphigus is a group of potentially fatal autoimmune blistering diseases of the skin and/or mucous membranes caused by IgG autoantibodies (autoAb) which predominantly target two transmembrane desmosomal cadherins: desmoglein (Dsg)1 and Dsg3 [1–3].

There are two major types of pemphigus: pemphigus vulgaris (PV) and pemphigus foliaceus (PF). PV patients characteristically develop oral lesions with buccal and/or gingival persisting erosions (mucosal dominant PV) which several weeks or months later may also spread to the epidermis (mucocutaneous PV). Clinically, there are localized or generalized flaccid bullae that quickly transform into post-bullous erosions and crusts. PF is characterized by widespread cutaneous fragile bullae which rapidly rupture, resulting in erosions, crusting, and scaling. In contrast to PV, the mucosa is usually not involved in PF. In pemphigus erythematosus, a localized variant of PF, lesions remain confined to the seborrheic areas of the face, scalp, back, and chest. The major clinical variants and respective antigenic targets of PF and PV are summarized in Table 1.

Histologically, the characteristic picture in pemphigus is loss of cohesion between keratinocytes, a phenomenon called acantholysis [2], due to shrinkage of desmosomes [4]. In PV, acantholysis is characteristically seen between the basal and suprabasal epithelial layer while in PF, blistering occurs between suprabasal and granular layers (Fig. 1).

According to the "desmoglein compensation" theory, the Dsg3/Dsg1 autoAb profile defines the clinical outcome depending on the specific expression pattern of Dsg3 and Dsg1 [5–7]. Consequently, mucosal PV patients typically exhibit Dsg3 without Dsg1 autoAbs and develop lesions restricted to the deep mucosa, where Dsg3 is expressed without compensating Dsg1. Mucocutaneous PV patients exhibit secondary Dsg1 autoAbs correlated with appearance of epidermal blisters where Dsg3 and Dsg1 are co-expressed. PF patients mainly harbor Dsg1 without Dsg3 autoAbs which affect the superficial epidermis, where Dsg1 is present without compensating Dsg3. Nevertheless, several cases of isolated oral mucosal blistering and isolated cutaneous lesions have been reported in patients with only anti-Dsg1 antibodies and anti-Dsg3 antibodies, respectively [8–10].

Clinically, direct immunofluorescence (DIF) microscopy studies are crucial for the diagnosis of PV and PF and characteristically demonstrate IgG deposits on the keratinocyte cell surfaces (Fig. 1) [1]. Pemphigus can further be confirmed by indirect IF studies using monkey and guinea pig esophagus as substrate. Nevertheless, nowadays, Dsg1 and Dsg3 ELISAs are predominantly used. These have a higher sensitivity and allow one to accurately define the autoAb profile [11].

In general, pemphigus remains a challenging disease to treat with prolonged periods of time needed to achieve remission. Likewise, there is a significant risk of relapse [12–15]. Pemphigus is further intrinsically associated with an increased risk of other autoimmune diseases, olfactory dysfunction [16, 17] as well as treatment related comorbidities including hyperglycemia, osteoporosis, and opportunistic infection [18–20]. Patients likewise demonstrate a decreased quality of life, particularly struggling with anxiety and depression [21] claiming the need to develop novel innovative therapies.

Table 1 Main clinical variants of pemphigus vulgaris and pemphigus foliaceus and their respective target antigens

Disease	Primary antibody	Clinical findings	
Pemphigus vulgaris	Dsg3 ± Dsg1	Mucosal	Oral lesions with buccal and/or gingival persisting erosions
		Mucocutaneous	Localized or generalized flaccid bullae that quickly transform into post-bullous erosions and crusts
Pemphigus vegetans	Dsg3 ± Dsg1, Dsc	Neumann type	Vesicles and bullae rupturing to form hypertrophic granulating erosions
		Hallopeau type	Pustular lesions that rupture and coalesce into vegetating erosions
Pemphigus foliaceus	Dsg1	Cutaneous fragile bullae which rapidly rupture, resulting in erosions, crusting, and scaling. No mucosal involvement	
Endemic *Pemphigus foliaceus*	Dsg1		
Fogo selvagem	Dsg1	Localized "forme fruste"	Superficial erosions, crusts, blisters, vesicles, yellow-brown keratotic plaques on seborrheic face and back areas
		Generalized	Widespread blistering, exfoliative erythroderma or disseminated keratotic plaques
Pemphigus erythematosus (*Pemphigus seborrhoicus*)	Dsg1	Fragile blisters and erosions, crusting, and scaling localized to the seborrheic areas of the face, scalp, back, and chest	

Dsg1 desmoglein 1, *Dsg3* desmoglein 3, *Dsc* desmocollins

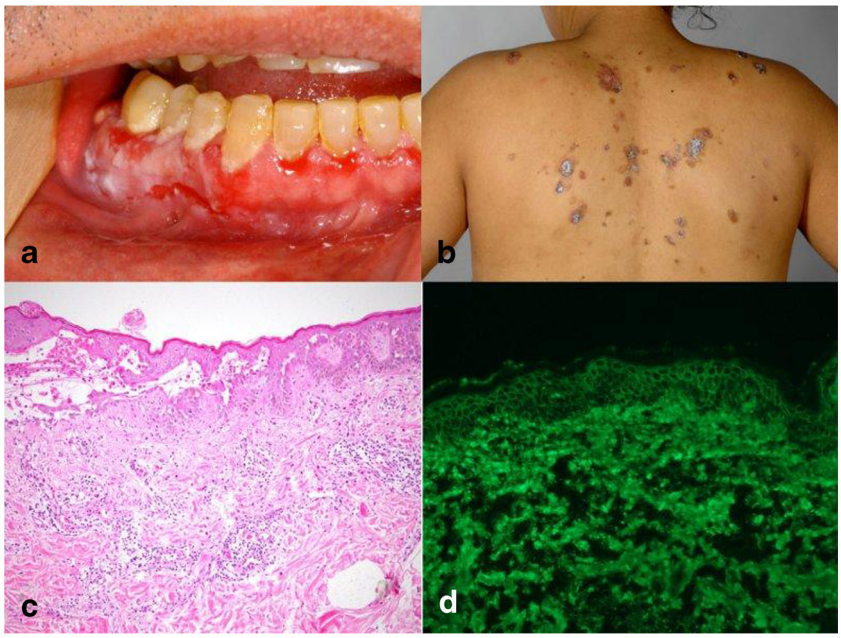

Fig. 1 Pemphigus vulgaris. **a** Oral mucosal involvement with gingival erosions. **b** Cutaneous involvement. Fluid-filled bullae arising on normal appearing skin and secondary lesions with erosions and crusting on the back. **c** Light microscopy studies. Formation of clefts and bullae in a predominant suprabasal location. Some acantholytic cells are round with a homogenous cytoplasm (H&E stains). **d** Direct immunofluorescence microscopy studies of a skin biopsy specimen detect presence of immune deposits of IgG on the cell surface of keratinocytes with a fishnet staining pattern

Epidemiology

Pemphigus is an orphan disease with estimated incidence of two to 10 cases per one million inhabitants in central Europe [22]. Higher incidences of pemphigus have been described in the Mediterranean population [23], Ashkenazi Jewish population [24, 25], and Macedonian Roma population [26]. Both genetic and environmental factors are involved in the development of pemphigus, with the former accounting for a higher predisposition in certain ethnic groups and the latter for endemic forms such as Brazilian pemphigus, called *Fogo Selvagem* (FS) [27], or endemic pemphigus in Tunisia [28] .

The genetic predisposition towards developing pemphigus is primarily due to polymorphisms in the human leucocyte antigen (HLA) class II alleles [29–33]. A meta-analysis of HLA polymorphisms demonstrated that the particular polymorphisms, DRB1*04, DRB1*08, and DRB1*14 significantly increase susceptibility to PV [34]. PF is similarly associated with certain predisposing HLA polymorphisms including HLA-DRB1*04 and HLA-DRB1*14 [31, 35–38]. However, many HLA polymorphisms are population dependent, appearing only among certain ethnic groups or geographic regions [30, 31, 34–36, 39–45]. HLA polymorphisms are thought to alter the conformation and electrical charges of HLA molecules, increasing the avidity of the HLA allele with the autoantigens Dsg1 and Dsg3, leading to autoimmunity [42]. A study comparing German to Egyptian PV patients noted HLA-DRB1*04:02 and HLA-DRB1*14:01 to be significantly associated with PV in both populations [46]. Studies of FS suggest an association between certain HLA types and a higher overall incidence of pemphigus. There is a 14-fold increased relative risk of FS in patients with predisposing polymorphisms, as well as an overall population incidence far greater than in non-endemic regions [47].

A paradigmatic example of an autoantibody mediated organ-specific disease

Pemphigus is a paradigmatic immunoglobulin-mediated organ-specific autoimmune disease of skin and or mucosae with (i) well-defined autoantigens, primarily Dsg1 and Dsg3 which by their own are sufficient to induce disease, (ii) available in vitro, ex vivo and in vivo models that recapitulate the major feature of the disease, and (iii) a remarkable amount of data that delineate a mechanism of blister formation with high complexity.

Dsg3 and Dsg1 are transmembrane desmosomal cadherin-type adhesion molecules [48]. Similar to classical cadherins, Dsgs comprise five extracellular subdomains of approximately equal size (EC1-EC5) (Fig. 2) Crystallographic and biochemical studies of classical cadherins have shown that residues in EC1 and EC2 domain mediate homophilic interaction in *trans* (between molecules emanating from opposing cell surfaces) and in *cis* (between molecules located on the same cell membrane) [49–54]. The crystallographic structure of Dsg1 and Dsg3 has not been established; however, the structural similarity between cadherin isoforms supports a conserved functional organization with regards to residues for *trans*- and *cis*-adhesion. PV autoAbs, which specifically target these domains in Dsg3, were found to disrupt *trans*- and potentially *cis*-adhesion between Dsg3 molecules [55, 56], a phenomenon which could so far not be confirmed for PF autoAbs binding to Dsg1 [57].

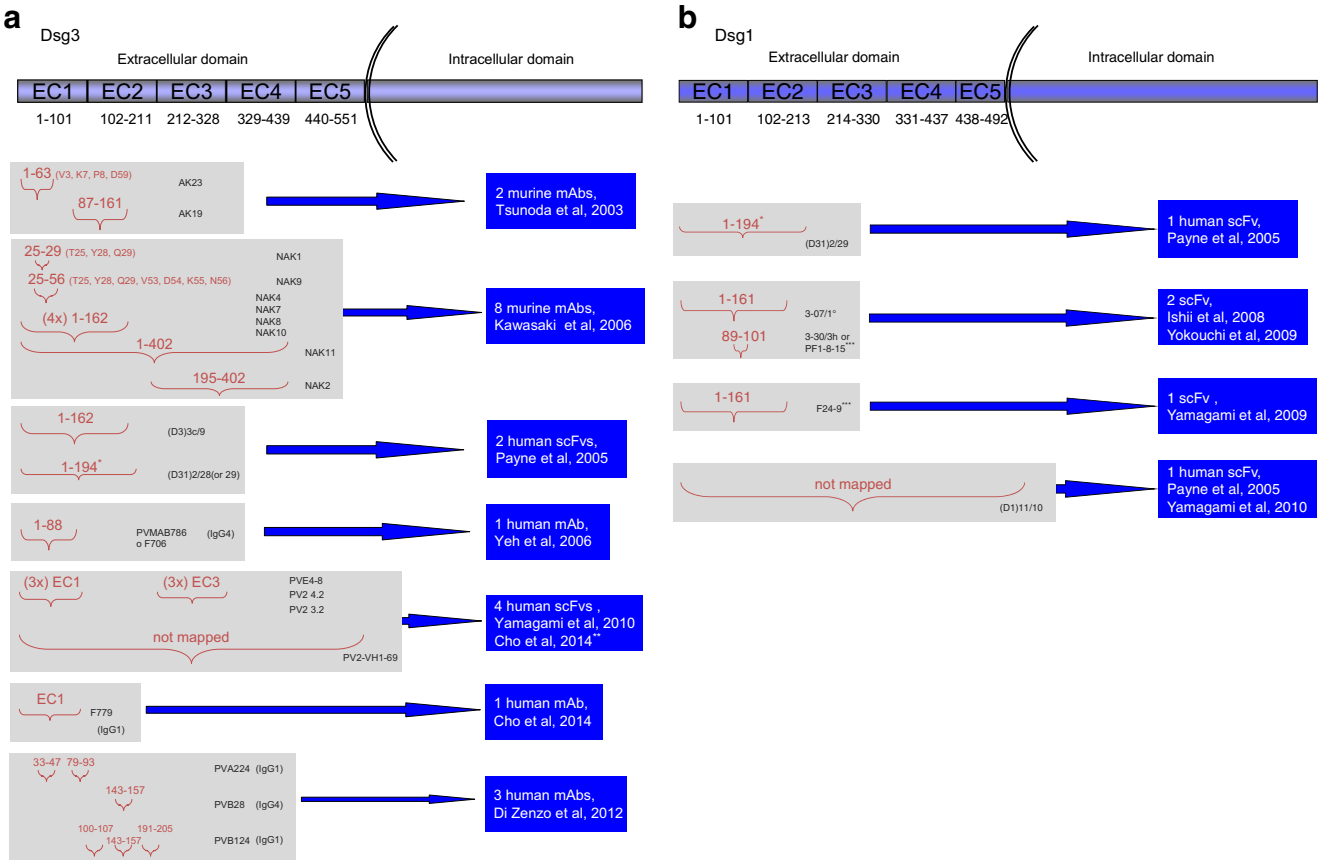

Fig. 2 Regions on the extracellular domains of Dsg targeted by human pathogenic monoclonal antibodies. Survey and schematic representation of the antigenic regions recognized by human pathogenic mAbs specific for Dsg3 (**a**) and Dsg1 (**b**) isolated from PV and PF patients. Reported amino acid stretches of Dsg1 and Dsg3 subdomains correspond to the EC subdomains identified in previous epitope mapping studies of mAbs [214]. *Epitope mapping performed using murine Dsg1. (**) Four mAbs isolated and mapped by Yamagami and coworkers and by Cho and coworkers, respectively. (***) In epitope mapping and cross-competition studies, these mAbs bind the same epitope (AA 89–101) [114]

The critical role of autoAb binding to Dsg in pemphigus pathogenesis is corroborated by the observation that (1) the activity of PV and PF generally correlates with anti-Dsg3 and Dsg1 IgG autoAb titers [58, 59]; (2) newborns of mothers with active pemphigus contemporarily exhibit blisters due to the diaplacentar transfer of maternal anti-Dsg autoAbs [60, 61]; (3) pemphigus-like lesions are induced in neonatal and adult mice by passive transfer of purified anti-Dsg IgG from pemphigus patients [62–67]; (4) transfer of splenocytes from Dsg3-immunized Dsg3−/− mice into immunodeficient Rag2 −/− animals expressing Dsg3 results in anti-Dsg3 autoAb production and PV-like lesions in mucous membranes [68], and (5) Dsg autoAbs from pemphigus patients are sufficient to dissociate a monolayer of cultured keratinocytes [69].

Role of autoantibody isotype

Pemphigus autoAbs display pathogenic activity independently of their Fc portions, hence without involving Fcγ receptor activation. In fact, bivalent (F(ab')2), monovalent (Fab), or recombinant single-chain variable (scFv) autoAb fragments are sufficient to cause acantholysis of keratinocytes in culture [70–72] or when passively transferred into neonatal mice [62]. Accordingly, complement activation is dispensable for pemphigus acantholysis as was further confirmed by passive transfer of autoAbs into C5-deficient mice [73]. In line with these findings, several studies have demonstrated that pathogenic autoAbs in pemphigus patients belong, with some exceptions, to the IgG4 subclass which has poor complement- and leucocyte-activating properties [55, 74–79].

A switch from IgG1, which is non-pathogenic to pathogenic IgG4, is exemplified in FS, the endemic PF subtype in subtropical areas of Brazil. Up to 55 % of healthy individuals have IgG1 Dsg1 autoAbs, and onset of disease is associated with antibody subclass as well as epitope switching from IgG1 to IgG4 and EC5-Dsg1 to EC1/2-Dsg1, respectively [47, 80, 81]. The model for FS pathogenesis predicts that a non-pathogenic IgG1 antibody response to the EC5 subdomain of Dsg1 is mounted by antigenic mimicry to an environmental antigen [82, 83]. Overt disease subsequently develops in individuals with certain HLA susceptibility genes [47, 84] and is

associated with the production of pathogenic anti-EC1/2-Dsg1 IgG4 antibodies, presumably through intramolecular epitope spreading [80, 81]. Despite the prevalence of pathogenic IgG4 in pemphigus [85], pathogenic IgG1 anti-Dsg3 monoclonal antibodies (mAbs) have recently been isolated from PV patients indicating, however, that at least for PV, the IgG subclass might not be the major determinant of pathogenicity [55, 79].

Pathogenic and non-pathogenic anti-Dsgs autoAbs

The existence of both pathogenic and non-pathogenic anti-Dsg autoAbs has been broadly documented. For example, pemphigus patients in clinical remission harbor anti-Dsg autoAbs targeting linear epitopes in the C-terminal ectodomain of Dsg3. These are considered non-pathogenic as they are unable to dissociate a keratinocyte monolayer [86–88]. Non-pathogenic anti-Dsg autoAbs also occur in healthy first-degree relatives of pemphigus patients who have a predisposition but do not develop pemphigus [89, 90]. Finally, patients with rheumatoid arthritis taking thiol compounds have autoAbs targeting non-conformational epitopes of either Dsg1 or Dsg3 without developing skin lesions [91].

The generation of both pathogenic and non-pathogenic autoAbs has recently been underscored by isolation of individual human mAbs from PV and PF patients [55, 71, 72, 78, 79, 92–94], suggesting that the pathogenic potential of anti-Dsg autoAbs primarily depends on the targeted epitope.

Epitopes targeted by pemphigus sera and mAbs

In previous seminal works, Amagai and collaborators and subsequently other investigators have demonstrated that recombinant eukaryotic Dsg1 and Dsg3 proteins are able to adsorb pathogenic, conformation (but not glycosylation) dependent autoAbs from PF and PV patient sera [95, 96]. In addition, PV IgG affinity purified on the EC1/2 subdomains of Dsg3, but not those purified on the EC3-5 subdomains, caused acantholysis in neonatal mice [64]. In line with these findings, PV and PF sera in active disease mainly target the N-terminus of Dsgs [97, 98]. Specifically, most of the critical epitopes mapped to residues 26–87 of EC1-Dsg1 and 25–88 of EC1-Dsg3 [99]. Although these findings indicate that the autoAbs pathogenicity depends on the reactivity with conformation-dependent epitopes in the N-terminal EC1/EC2 region of Dsg 1 and Dsg3, autoAbs targeting non-conformational epitopes or epitopes in the Dsg3 C-terminal ectodomain can also contribute to loss of cell adhesion [100–102].

Using an active mouse model of PV, Tsunoda and coworkers cloned several murine anti-Dsg3 mAbs [103, 104] (Fig. 2a). These studies confirmed that the majority of mAbs are directed against EC1/EC2-Dsg3 and that a combination of weakly pathogenic mAbs targeting different epitopes on Dsg3 can augment the pathogenic effect [104]. However, one mAb termed AK23 exhibited maximal pathogenic activity on its own in the active mouse model and in passive transfer studies. The binding site of AK23 was mapped to the putative *trans*-adhesion domain of Dsg3 (based on the predicted crystal structure). It consists of a calcium-dependent conformational epitope in the EC1 domain of Dsg3, composed of the V3, K7, P8, and D59 residues [52, 103] (Fig. 2a). In another study using atomic force microscopy, evidence was provided that AK23 binding alone is sufficient to disrupt *trans*-adhesion between interacting Dsg3 molecules [105], similarly than do IgG autoAbs from PV patients.

Isolation of pathogenic antibodies from pemphigus patients confirmed the findings on disease-relevant epitopes in murine models. Using hetero-hybridomas from B cells immortalized by Epstein-Barr virus and fused with mouse myeloma cells, two human pathogenic anti-Dsg3 mAb were generated targeting EC1-Dsg3 (AA 1–88; and not defined) [78, 79] (Fig. 2a). In addition, in other studies using antibody phage display libraries, anti-Dsg3 pathogenic mAbs and anti-Dsg1 pathogenic mAbs were isolated from both PV and PF patients targeting both, the EC1 and EC2 subdomains [71, 72, 94] (Fig. 2a, b). Furthermore, Di Zenzo and coworkers isolated three pathogenic anti-Dsg3 mAbs from PV patients which specifically bind to epitopes in the EC1 and EC2 subdomains thought to be involved in *cis*-adhesive interactions [55]. In this context, it should be noted that a recent study investigating the higher ordered structure of Dsg2 has provided evidence that the rows of desmosomal cadherins are spaced too far apart to undergo *cis*-interaction [106]. It is therefore likely that these pathogenic antibodies specifically bind to the *cis*-adhesive interface to disrupt *trans*-adhesion of Dsg3 molecules belonging to the extra-desmosomal pool, that is, those not associated with fully assembled desmosomes (see below).

In conclusion, the vast majority of isolated pathogenic mAbs target conformation-dependent epitopes in the EC1/EC2 subdomains of Dsg1 and 3 (Fig. 2). These pathogenic autoAbs are able to disrupt keratinocyte adhesion by interfering most likely with either putative *trans*- or *cis*-adhesive interfaces of Dsg molecules. Such binding leads to loss of *trans*-adhesion triggering an intracellular signaling response, which ultimately leads to cell-cell disadhesion (see below) [107–112].

Mature and premature forms of Dsgs and autoAbs pathogenicity

Cadherins, including Dsgs, were shown to be synthesized as inactive precursor proteins (preDsg) in the endoplasmic reticulum bearing a N-terminal propeptide [113, 114]. The

propeptide is subsequently cleaved by a Golgi proprotein convertase to yield mature adhesive Dsgs (matDsg). These are transported to the plasma membrane where they are stabilized through *trans*-adhesion before assembling into desmosomes [115]. Desmosome assembly and disassembly are continuously ongoing dynamic processes in which matDsg3 transit through biochemically distinct extra-desmosomal cadherin pools (see below) [110, 116].

Two studies classified several mAbs cloned from two PF patients into three groups based on their pathogenic potential and ability to recognize preDsg1 and matDsg1: pathogenic anti-matDsg1 mAbs, non-pathogenic anti-matDsg1 mAbs, and non-pathogenic anti-preDsg1 mAbs [72, 93]. These findings suggest that pathogenic autoAbs target N-terminal epitopes in the EC1 domain that are unmasked by proteolytic processing into the mature form of Dsg1. Competition ELISAs further showed that the majority of PF sera target the same or nearby epitopes of the putative *trans*-adhesion domain in EC1 defined by one pathogenic anti-Dsg1 mAb [114]. In analogy, pathogenic anti-Dsg3 mAbs also appear to specifically bind to the matDsg3, whereas non-pathogenic ones react with both mature and precursor forms of Dsg3 [117]. In line with these data, disease activity in PF patients correlated with reactivity against matDsg [114]. Together, these findings indicate that epitopes masked by the prosequence are very important in pemphigus pathogenesis, although not all pathogenic autoAbs target these masked epitopes. In fact, pathogenic mAbs targeting both precursor and/or mature form of Dsg1 (3-07/1e in Fig. 2b) and Dsg3 (AK19, PVMAB786, PVA224, PVB28, PVB124 in Fig. 2a) have also been isolated [55, 72, 78, 103]. Interestingly, the Dsg3 binding of a mAb that targets both precursor and mature form (PVA224) is inhibited by virtually all PV sera tested, while the binding of AK23 that binds the mature form of Dsg3 is inhibited only by a portion of PV sera [55]. This seems to indicate, as already insinuated by studies described above [103, 104], that PV autoAbs can target different epitopes with similar pathogenic outcome.

Genetic analysis of autoAbs and idiotypes

Like for other autoimmune diseases [118, 119], the immunologic properties of both anti-Dsg1 and anti-Dsg3 mAbs mainly correlate with heavy chain (VH) gene usage [55, 71, 72, 93]. Currently, several anti-Dsg3 and anti-Dsg1 pathogenic mAbs have been isolated from either PV or PF patients (Fig. 3). The VH gene usage was found to be relatively restricted, whereas light-chain (VL) gene usage was more promiscuous [71, 72, 94]. However, Di Zenzo et al. described a polyclonal anti-Dsg3 IgG antibody response in two PV patients, in which all autoAb used different VH and VL genes [55]. The most frequently identified genes belong to VH1 and VH3 families for PF and VH1, VH3, and VH4 families for PV [55, 71, 72, 93, 120, 121]. However, while pathogenic autoAbs obtained from different patients do not necessarily use the same VH gene (Fig. 3), both pathogenic and non-pathogenic mAbs can share the same gene suggesting that pathogenic autoAbs evolve from non-pathogenic ones through affinity maturation [55, 93, 94]. PV and PF autoAbs carry mutations in their complementarity determining regions (CDRs, binding to Dsgs) at higher frequencies then in the framework regions (FWRs). This is indeed consistent with an affinity maturation process related most likely to a chronic stimulation by Dsgs [55, 71, 72, 93]. Furthermore, a frequent usage of the VH1-46 gene has been reported for PV but not for PF autoAbs, indicating common humoral immune responses among PV patients [79].

CDR3s on VHs (H-CDR3) are thought to provide the most important variable sequences for antibody specificity and binding [122]. Since pathogenicity and binding of pemphigus autoAbs mainly correlate with VH gene usage, comparative analyses of the H-CDR3 of the cloned pathogenic mAbs were conducted and revealed a putative consensus amino acid motif (D/E-X-X-X-W) [94] (Fig. 3). However, it was found that this is not required for pathogenicity [94]. Nevertheless, at least for VH1-46 pathogenic mAbs, specific acidic amino acid residues in the CDRs appear necessary and sufficient to confer binding to Dsg3 [79]. Finally, as illustrated in Fig. 3, there may be other not yet characterized recurrent motifs (in orange, green, and blue) in the pathogenic anti-Dsg3 mAbs. However, as two different pathogenic anti-Dsg1 mAbs (F24-9, PF1-8-15) bearing a different H-CDR can bind to the same epitope (AA 89–101), the search for H-CDR consensus motifs might be called into question.

Together, the clonal analysis of human autoAbs has provided new insights into the immune response in pemphigus patients. Anti-Dsg pathogenic autoAbs do not appear to share a common CDR (idiotype), but pathogenicity may rather be due to the recognition of different epitopes. Future cross-competition and fine epitope mapping studies of pathogenic autoAbs will expand our knowledge on different pathogenic epitopes and of the corresponding pathogenic idiotypes.

Somatic mutation processes in the anti-Dsg immune response

Recently, efforts have been made to revert isolated mAbs to their germline version. In one study, three pathogenic and one non-pathogenic autoAbs isolated from two PV patients were retromutated [55]. Noteworthy, all the retromutated germline antibodies failed to bind to Dsg3 [55]. Based on these results, the investigators suggested that autoreactivity is related to somatic mutations which occur in response to an antigen unrelated to Dsg3; thus, Dsg3 does not seem to represent the

a anti-Dsg3 mAbs

Patient	Clone	VH chain	VH CDR3	Recognized region	Ref.
PV1	(D3)3c/9	VH3-07	YYCAS GGVVDFDH WGQ	AA 1-162	71
PV1	(D31) 2/29 (or 28)	VH1-69	YYCAR GGDYSGWYNFDY WGQ	AA 1-194	71
PV1	PVE4-8*	VH1-46	YYCAR DRQGFDLDV WGQ	EC1/EC3	94
PV2	PV2 VH1-69	VH1-69	YYCAR DRWRFQESEGFDY WGQ	NK	94
PV3	PV2 4.2*	VH1-46	YYCAR DQSLGMDV WGQ	EC1/EC3	94
PV3	PV2 3.2*	VH1-46	YYCAR DLGGFDFDY WGQ	EC1/EC3	94
PV4	F779	VH1-46	AR SIESISGRTLGY	EC1	79
PV5	PVMAB786 or F706*	VH1-46	AR GVGTLDH	AA 1-88	78
PVA	PVA224	VH3-48	AR ESRSYYYYFGMDV WGQ	AA 33-47; AA 79-93	55
PVB	PVB28	VH4-39	AR DGAAGLYLEK WGQ	AA 143-157	55
PVB	PVB124	VH4-39	AR SDGDYVEGWYFDI WGR	AA 100-107 AA 143-157 AA 191-205	55

b anti-Dsg1 mAbs

Patient	Clone	VH chain	VH CDR3	Recognized region	Ref.
PF1	3-07/1e	VH3-07	Not reported	AA 1-161	72
PF1	3-30/3h or PF1-8-15	VH3-30	YYCAR DRVEGYVWGGTFDH WGQ	AA 89-101**	72, 114
PF2	F24-9	VH3-53	YYCVR GPAYYDIDY WGQ	AA 1-161**	93
PV1	(D1) 11/10	VH4-b	YYCAR TTTAYWYFDL WGR	NK	71, 93

Fig. 3 Survey of human pathogenic monoclonal antibodies isolated from pemphigus patients with characterization of VH genes. Clone name, VH usage, H-CDR3 amino acid sequence, and recognized epitope of all human pathogenic anti-Dsg3 (**a**) and anti-Dsg1 (**b**) mAbs isolated from PV and PF patients are depicted. YYCAR/S (in *red*) indicates end of VH FWR3, while WGQ the beginning of FWR4. (*) 4 mAbs isolated and mapped by Yamagami and coworkers and by Cho and coworkers, respectively. (**) In epitope mapping and cross-competition studies, these mAbs bind the same epitope (AA 89–101) [114]. The *blue*, *orange*, and *green colors* highlight some recurrent amino acid motifs among different autoAb CDR sequences. Amino acids FDY/H/I/L are at the end of several CDR3 sequences because present in recurrent joining segments

primary trigger of the pathogenic B cell clones [55]. In line with this idea, it has been found that two anti-DNA autoAbs isolated from systemic lupus erythematosus patients were derived from non-autoreactive B cells, an observation suggesting that they developed as a byproduct of a normal immune response [123].

Another recent study showed that pathogenic anti-Dsg3 mAbs bearing the VH1-46 gene had relatively few replacement mutations in the CDRs and require little to no mutations to bind Dsg3 [79]. However, these mAbs exhibited an almost log-fold lower affinity for Dsg3 when compared with non-VH1-46 mAbs. Furthermore, three of five VH1-46 autoAbs maintained the ability to bind to Dsg3 following reversion to germline. In contrast the highly mutated non-VH1-46 mAbs lost their ability to bind Dsg3 upon reversion to the germline versionas described above [55]. Site-directed mutagenesis has demonstrated that acidic amino acid residues introduced by somatic mutation or heavy chain VDJ recombination are necessary and sufficient for Dsg3 binding. Since VH1-46 autoAbs bind Dsg3 even with no or only a few amino acid mutations, this feature may favor their early selection in the autoimmune response [79]. It has thus been proposed that Dsg3-autoreactive B cells bearing VH1-46 gene occur naturally, that is without previous antigen exposure. These Dsg3-autoreactive B cells may survive and escape deletion or anergy, as Dsg3 is not expressed centrally in the bone marrow. In contrast, Dsg3-autoreactive T cells are deleted centrally in the thymus where Dsg3 is expressed. However, in carriers of HLA alleles associated with PV susceptibility, a low number of Dsg3-autoreactive T cells may escape into the periphery [124]. In these subjects, the presence of appropriate co-stimulatory signals may then result in an overt autoimmune response. To support this idea, it will be necessary to confirm the presence of VH1-46 mAbs in more PV patients as well as in normal healthy individuals. However, other mechanisms may also trigger the immune response. For example, germlined mAbs may recognize an antigen, through which somatic mutated pathogenic anti-Dsg3 autoAbs may then develop.

In PF, the presence of anti-preDsg1 autoAbs seems to be important for the development of the disease. The majority of

mAbs isolated from two PF patients were non-pathogenic and reacted against preDsg1 [72, 93]. PreDsg1 mAbs showed a lower replacement to silent mutation ratio in their CDRs when compared to the anti-matDsg1 mAbs. This is probably due to the intracellular localization of the Dsg1 precursor, making stimulation of B cells difficult [93]. In normal individuals, anti-preDsg1 (but not anti-matDsg1) mAbs have been isolated. Noteworthy, the rate of somatic mutations in the anti-preDsg1 mAbs was lower when compared to those in PF patients. In the latter, exposure to preDsg1 may occur upon its release from damaged epidermis [93]. Furthermore, almost all non-pathogenic anti-preDsg1 mAbs isolated so far from both PF and non-PF individuals have used the same variable H chain gene (VH3-09). In contrast, the pathogenic and non-pathogenic anti-matDsg1 mAbs isolated from PF patients possess different VH genes, an observation indicating that in PF, anti-matDsg1 B cells are not derived through somatic mutation from the anti-preDsg1 B cells. Together, these findings suggest that the intracellular preDsg1, which under normal conditions is not exposed to the immune system, can allow B cells to escape tolerance and may be involved in the initiation of autoimmunity. Yamagami et al. have speculated that in the context of tissue destruction, anti-preDsg1 B cells present peptides of the matDsg1 derived from the processing of preDsg1 to T cells that have lost tolerance to matDsg1. Thereafter, these T cells could provide help not only to the original anti-preDsg1 B cells but also to any B cells that have escaped from tolerance to matDsg1 [93]

Contribution of non-classical autoantibodies to tissue damage

Besides Dsg3 and Dsg1, several other non-desmoglein autoAbs have been linked to the tissue damage and acantholysis observed in pemphigus. For example, autoAbs directed against desmocollin 3, which are detectable in some PV patients, are able to induce loss of cell-cell adhesion similar to that seen with other PV autoAbs [125–127]. Antibodies against an α-acetylcholine receptor that regulates keratinocyte adhesion have also been implicated in the pathogenesis [128]. Similarly, autoantibodies to pemphaxin, an annexin-like molecule acting as cholinergic receptor, have been described. These cholinergic receptors do not possess acantholytic potential on their own but may act synergistically with anti-Dsg3 antibodies to increase acantholysis [129]. It has also been claimed that antibodies targeting keratinocyte mitochondria contribute to the process of acantholysis [130]. AutoAbs directed against a calcium pump encoded by ATP2C1, desmocollin 1, BP230, periplakin, E-cadherin, desmoglein 4, desmoplakin 1, and desmoplakin 2 as well as the intracellular domains of Dsg1 and Dsg3 have been detected in pemphigus, but so far, there is little proof for their pathogenic roles [126, 131–137]. In line with this idea, by exploiting the Epstein-Barr virus immortalization of human memory B cells, we have been able to isolate different human non-desmoglein mAbs labeling the keratinocyte cell surface or cytoplasm. However, these had no effect when tested by a keratinocyte dissociation assay (unpublished data). Table 2 depicts a survey of non-Dsg1 and 3 autoAbs for which acantholytic activity has been demonstrated.

Finally, several non-organ-specific antibodies have been detected in pemphigus patients. Anti-cardiolipin (IgG and IgM), anti-gliadin (IgG), and anti-reticulin levels may be significantly increased in PV, while in PF anti-thyroglobulin, autoAbs have been found [138–140].

Role of T cells in pemphigus development

T cells play a crucial role not only in the pathogenesis of pemphigus but also in the maintenance of tolerance in healthy individuals carrying distinct pemphigus associated HLA class II alleles conferring disease susceptibility [141]. The interaction of T and B cells critically modulates the development of pemphigus. Dsg3-activated type 2 helper T (Th2) cells are capable of activating unprimed B cells [142], whereas reduction of Dsg3-specific T cells leads to a decrease in autoantibody generation [143]. Conversely, depletion of peripheral B cells is associated with a marked reduction in the frequency of Dsg3-specific autoreactive T cells [144]. This reduction only occurs in Dsg3-specific autoreactive T cells. In fact, T helper cells specific to recall antigens are not affected by B cell depletion [145].

Early seminal experiments in murine models of pemphigus have convincingly shown that pathogenic anti-Dsg3 antibodies are generated in immunodeficient Rag2−/− mice only when both T cells and B cells from Dsg3-primed mice are transferred. Thus, both B and T cells with autoreactivity towards Dsg3 are required for the development of pemphigus [146]. Through the use of an anti-CD154 mAb, Aoki-Ota et al. were able to block the CD154/CD40 interaction of T and B cells, leading to suppression of anti-Dsg3 IgG production [147]. Thus, by preventing the cross-talk between activated T cells and B cells, autoimmunity is mitigated. Furthermore, when splenocytes from anti-CD154 treated mice and splenocytes from Dsg3 −/− mice are both transferred into syngeneic Dsg3 +/+ mice, the recipient mice demonstrate significantly reduced levels of anti-Dsg3 IgG [147]. These findings are probably directly due to the anti-CD54 treatment of mice which is expected to increase the T regulatory (Treg) cells, which in turn suppress the immune response against Dsg3 [148].

Activation of autoreactive CD4+ T cells is critically affected by autoantigen presentation by HLA class II molecules [33]. Dsg3-specific T cells can be detected through the use

Table 2 Survey of selected non-Dsg1/Dsg3 autoantibodies with potential pathogenic role in pemphigus

Family	Pathogenic antibodies	Function
Annexin	Pemphaxin [129]	Ca^{2+}-dependent phospholipid-binding cholinergic receptor
Cadherin	Desmocollin 3 [125, 127]	Transmembrane epithelial Ca^{2+}-dependent glycoprotein mediating intercellular adhesion
Nicotinic acetylcholine receptor	α9-acetylcholine receptor [128]	Ca^{2+}-permeable acetylcholine-gated ion channel with dual muscarinic and nicotinic properties

of HLA-DRB1*0402 tetramers loaded with immunogenic portions of Dsg3 [149]. They are present in both PV patients and healthy carriers with PV-associated HLA polymorphisms. In fact, healthy carriers of the DRB1*04:02 and DQB1*05:03 polymorphisms demonstrate the same Dsg3 specific CD4+ T cell response to Dsg3 [124, 150]. Thus, a loss of T cell tolerance is not enough on its own to initiate disease in the genetically predisposed individuals.

T cell tolerance is regulated both centrally and peripherally. Central tolerance occurs during T cell development within the thymus. The transcription for autoimmune regulator (AIRE) allows for the expression of a large repertoire of self-antigens, including Dsg3 [151]. In normal circumstances, negative selection results in deletion of the auto-Dsg3 reactive T cell within the thymus [152]. Thus, the increased risk of developing pemphigus in patients with thymoma likely represents a defect in the regulation of tolerance [153].

The inactivation of autoreactive T cells that have escaped from the thymus is termed peripheral tolerance. This is primarily carried out by CD4+CD25+ regulatory T regulatory cells (Treg) that express the transcription factor Forkhead box P3 (FoxP3) [154]. In vivo removal of these FoxP3+Treg cells results in a proliferation of autoreactive T cells with the development of clinically overt autoimmunity [155]. FoxP3 is constitutively expressed in Dsg3-specific type 1 T regulatory cells (Tr1) [156]. These Tr1 cells are found at a higher frequency in healthy individuals with the PV-associated HLA polymorphisms compared to PV patients [156]. Treg cells transferred from a Dsg3−/− mouse into a mouse with autoimmunity to Dsg3 were able to suppress anti-Dsg3 production [157]. Conversely, inactivation of FoxP3 in these Tr1 cells leads to a loss of their immunosuppressive abilities, resulting in a proliferative response to Dsg3 and a Th2 cytokine profile [158]. These findings confirm the regulatory role of these T cells. Clinically, PV patients show a high ratio of Th2/Tr1 cells, whereas healthy carriers of the PV-associated HLA polymorphisms exhibit a low Th2/Tr1 ratio [156].

Dsg3 reactive Th2 lymphocytes are present at all stages of the disease [159, 160]. Elevated levels of Dsg3-reactive Th1 cells are primarily found in patients with chronic active PV but also in healthy individuals with PV-associated HLA polymorphisms. During the acute phase of disease, both Th1 and Th2 cells circulate with similar frequencies and chemokines from each of these T helper cells are needed for disease development. Furthermore, the Th1/Th2 ratio may change during the different phases of the disease process [161, 162]. Finally, Th1 cells may also directly contribute to the tissue damage, since Dsg3-reactive Th1 cells can cause an interface dermatitis in mice [163].

Cell-biologic mechanisms of blister formation: role of cell signaling

In recent years, much progress has been made in unraveling molecular mechanisms of blister formation in pemphigus, particularly in PV. PV antibody cloning as described above, and hence the use of monospecific rather than polyclonal IgG coupled with modern imaging techniques and biochemical fractionation, have revealed both extra- and intra-desmosomal functions of Dsg3. This has led to a progressive shift in our view of the pathomechanism, enabling us today to propose novel therapies in PV.

In a nutshell, PV antibodies are now widely documented to primarily bind to and exert their adhesion disrupting activity via extra-desmosomal Dsg3 receptors, which are present at the plasma membrane outside of desmosomes [164, 165] (Fig. 4). Antibody binding, likely followed by loss of *trans*-adhesion between these Dsg3 receptors, evokes a rapid intracellular signaling response which ultimately leads to shrinkage of desmosomes and acantholysis [107–112]. Current advances in the field suggest that extra-desmosomal Dsg3 also exhibit mechano-transducing activities which feed into the biochemical signal response, reminiscent of classical or type I cadherins to which a wealth of recent literature attests mechano-sensing activities in surveillance of tissue damage [166–168].

Extra-desmosomal cadherin functions are still discussed with some skepticism as they have not been extensively studied outside of PV. However, prior to identifying Dsg3 as a major antigenic target in PV, previous studies had already pointed towards the possibility that PV antibodies mainly bind between desmosomes [169–171]. Consistent with this observation, microblisters were reported as the earliest pathogenic

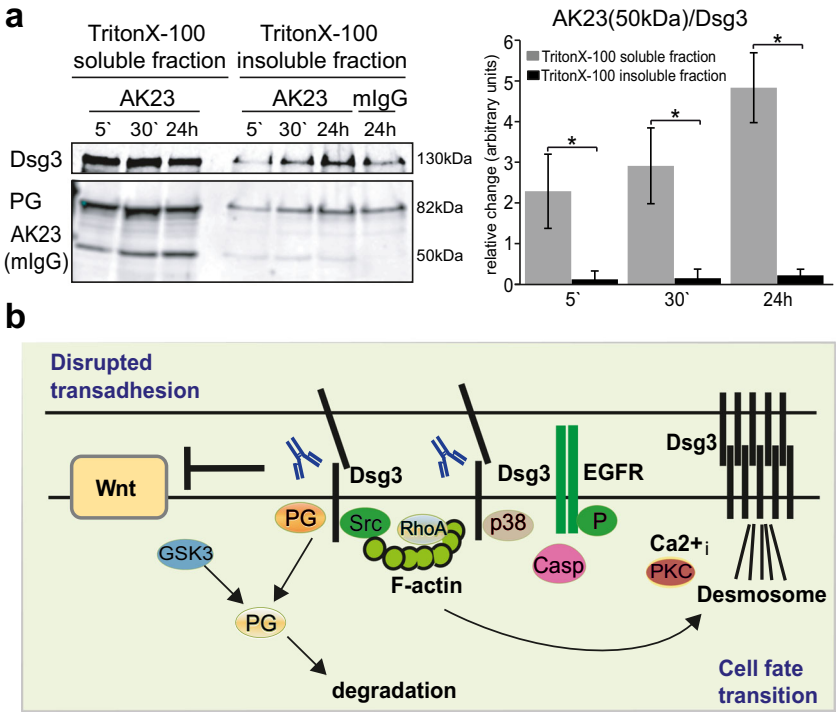

Fig. 4 Pathogenic events in pemphigus vulgaris (PV). **a** AK23, the pathogenic anti-Dsg3 antibody disrupting *trans*-adhesion [103] mainly binds to TritonX-100 soluble, extra-desmosomal Dsg3 receptors. Soluble and insoluble protein fractions were obtained from mouse keratinocytes switched to 1.2 mM calcium for 2 h and incubated on ice with AK23 or control mouse IgG (in vivo labeling) for the indicated time points. One representative blot with corresponding fractions is shown on the left and graph with quantified signals on the right. $n=3$/group. The mean±SEM of quantified and normalized signals of AK23 over Dsg3 is reported, $^*p<0.05$. Note that similar results were obtained using chemical cross-linking or with PV IgG. **b** Synthesis of major signaling pathways involved in PV pathogenesis; they comprise (from *left to right*) inhibition of Wnt-like signaling implicating GSK3 and plakoglobin [69, 70, 115] (and unpublished results), modulation of RhoA that can cross-talk to Src kinase, the actin cytoskeleton, and E-cadherin [178, 190], pathways involved in cellular stress responses such as EGFR, p38 MAP kinase, hsp70, and non-apoptotic caspase 3 activation [178–182] as well as pathways affecting desmosome-mediated adhesive strength via PKCα [185, 186, 188]

sign in presence of intact desmosomes in mouse models for PV using passive antibody transfer [62, 172]. About a decade later, desmosomal cadherins were found to exist in two distinguishable biochemical pools in cultured keratinocytes [173]: a first extra-desmosomal, low ionic soluble fraction containing desmosomal cadherins which are associated with plakoglobin but not desmoplakin or intermediate keratin filaments and a second pool which typically resists solubilization by low anionic detergents and contains fully assembled, keratin-anchored desmosomes.

As of today, modern imaging techniques have confirmed extra-desmosomal Dsg3 by atomic force microscopy in HaCat keratinocytes [164]. Consistently, these cadherins are routinely extracted by biochemical fractionation from mouse skin [67, 174] and are particularly evidenced by "in vivo labeling" of intact cells incubated with PV antibodies at 4 °C followed by biochemical fractionation (Fig. 4a) or IgG pull down [115]. Furthermore, extra-desmosomal cadherins partition to membrane rafts [165, 175, 176] which are free-floating lipid platforms responsible for a variety of cellular processes including signaling and endocytosis [177]. Noticeably, if lipid rafts are chemically disrupted, cells become irresponsive to PV antibodies in terms of Dsg3 endocytosis and shrinkage of desmosomes [165]. This supports the notion that disturbance of these regulatory entities by PV antibody binding affects desmosomes by enhancing endocytosis involving intracellular signaling mechanisms. Monospecific or polyclonal PV autoAb binding to extra-desmosomal Dsg3 indeed evokes a wide range of signals. Their involvement in pathogenicity has been functionally confirmed by using either pharmacological inhibitors and/or genetically manipulated mice which reduced loss of intercellular adhesion and blister formation. To honor the vast literature on signal transduction, the readers are referred to some excellent reviews in the field [107–112]. Briefly, as mostly inferred from mouse models, PV antibody-induced loss of *trans*-adhesion interferes with effectors attributable to the RhoA-Src kinase-plakoglobin axis and a stress response involving epidermal growth factor receptor (EGFR), MAP kinases, and non-apoptotic caspase-3 while inhibiting Wnt-like signaling resulting in glycogen synthase kinase (GSK) 3 activation (Fig. 4b) [69, 70, 115, 178–184]. Furthermore, the increase in intracellular calcium, reported in PV many years ago, may support protein kinase C activation

[185, 186], known to result in alterations of desmosomal morphology and function [107–112] (Fig. 4).

Recent large-scale electron microscopy studies of tissues obtained from pemphigus patients underscore that biochemical alterations at a single cell level, alluded to above, have a wide ranging impact eventually involving the entire tissue from basal keratinocytes to the stratum corneum [4]. Lesions in PV and PF, as mentioned in the introduction, are restricted to basal and granular layer keratinocytes, respectively. However, widening of intercellular spaces is observed away from lesions in the stratified epithelium. This recalls pioneering work which revealed that altered pressure, tension, or shear stress exerted onto the tissue is mechanically perceived at long range without need of protein synthesis [187, 188]. Studies done two decades ago evoked that intercellular adhesion molecules bridge the cytoskeleton from one cell to another to form a supracellular "entangled net" of interconnected microtubules, actin, and intermediate filaments [189]. Hence, changes in mechanical tension of single cells appear to be transmitted to the surrounding tissue through adhesive structures pulling onto their cytoskeleton anchor. Most recent findings seem to attribute mechano-transducing abilities also to Dsg3 which might explain some of the morphological tissue changes in PV as described above [107–112].

Extra-desmosomal Dsg3 which are typically devoid of intermediate filament anchorage have been reported to associate with and organize F-actin via RhoA [190]. Consistently, modulation of RhoA activity has been associated with PV pathogenesis [178]. RhoA is the principal driver of actomyosin (F-actin and its myosin motor) contractility and cellular tension. It is typically activated by classical cadherins transmitting mechanical outside-in signals for actin remodeling [191]. Might it be farfetched to envisage alterations in mechano-transducing properties to the actin cytoskeleton in PV pathogenesis? In our view, the response is no, also taking in consideration a previous study which revealed that myosin IIA is implicated in loss of intercellular adhesion in PV [192]. The authors further reported that the distribution of adherens junction components, which anchor to the actin cytoskeleton, remained unaffected in response to AK23 mAb treatment, consistent with previous findings using PV patient's antibodies [193, 194]. However, the study further revealed that these junctions exhibited properties of enhanced tension [192]. A potential implication of altered mechanical tension in disassembly of desmosomes exerted via the actin cytoskeleton is compatible with the recent observation that cellular stiffness rapidly decreases and, after 1 h, increases in response to pathogenic Dsg3-specific antibodies [195]. The increase in cellular stiffness is a phenomenon typically observed in cells out of force equilibrium due to heightened tension on the actin cytoskeleton [191]. Although little is known about the mechano-sensing abilities of extra-desmosomal and desmosomal cadherins, a potential conclusion drawn from these observations is that the initial disruption of extra-desmosomal Dsg3 *trans*-adhesion might intermittently relax tension on the actin cytoskeleton (and reduce RhoA), which is then built up to counter cell shape changes implicating, as a consequence, shrinkage of desmosomes. Incidentally, PV signals feature typical properties of classical cadherin-mediated mechano-transduction; they involve modulation of RhoA [178], MAPK [196], and GSK3 [115], followed by cytoskeletal reorganization [69, 194, 197] as well as altered cell fate featuring increased proliferation [115, 198] (Fig. 1b).

In conclusion, mechanisms of blister formation in PV are still incompletely understood. Recent results now indicate, however, that PV pathophysiology may not only involve biochemical but also biomechanical mechanisms.

From the bench to the bedside: new and future approaches to the treatment of pemphigus

Management of PV and PF is currently mainly based on the use of systemic corticosteroids and immunosuppressants. Although the latter have allowed to drastically reduce the mortality associated with pemphigus and are often effective in controlling the mucous and cutaneous disease, continuous therapy results in substantial side effects and comorbidities [199]. In this context, patients would greatly benefit from novel, more pathogenesis-focused treatment options potentially exhibiting fewer side effects.

In recent years, removal of pathogenic IgG autoAb by immunoadsorption has shown some promise in reducing the clinical activity of pemphigus [199]. Nevertheless, immunoadsorption techniques are potentially associated with severe procedure-related complications, which question their use and indication. In contrast, B cell depletion therapy using the anti-CD20 mAb rituximab has shown impressive rapid and long-term responses in a significant portion of patients with both refractory chronic disease as well as in newly diagnosed disease with a good safety profile. Therapeutic response to rituximab is associated with a rapid and persistent B cell depletion [86, 200] and a reduction of autoaggressive Th2 cells. The reduction of autoaggressive Dsg-3-specific Th2 cells has been related to their requirement of B cells as antigen-presenting cells [145, 201–204]. Furthermore, the induction of IL-10-producing B regulatory cells may be important for maintenance of long-term clinical remission in rituximab-treated pemphigus patients. These B regulatory cells may contribute to the down-regulation of autoaggressive T and B cells [201, 205, 206]. Furthermore, during the remission phase, there is a default of the B cell repertoire with disappearance of pathogenic B cells [201, 204].

Rituximab therapy results in a rapid decrease of pathogenic IgG autoAb, an observation suggesting that Dsg-specific plasma cells are presumably short-lived [144, 207]. Furthermore, in

a recent longitudinal study of PV patients, it has been shown that patients with active disease exhibit persistence of the same sets of anti-Dsg3 B cell clones over time, whereas rituximab-treated patients, who are in remission and off therapy lack detectable anti-Dsg3 B cell clones [208]. These findings strongly indicate targeted therapies that are able to eliminate specific anti-Dsg3 clones should cure the disease. It should be also addressed whether treatments with either mAb or peptides specifically interfering with the reactivity of the pathogenic antibodies with the EC1/EC2 subdomains of Dsgs may also be therapeutically promising in both PV and PF.

Besides therapies focusing on the humoral response, the therapeutic potential of antigen-specific T cell tolerance induction represent another potential approach for treatment of pemphigus, based on the promising results observed in experimental autoimmune encephalomyelitis [33, 209, 210] as well as in a clinical phase trial in multiple sclerosis [211]. Induction of either anergy of Dsg3-specific autoaggressive T cells, of protective Dsg-specific T helper cells, or, finally, of T regulatory cells is a potential novel immunological approach to treat pemphigus [33]. The characterization of autoaggressive Th2 cells from PV patients [199] and of immunodominant Dsg3 epitopes provides the basis and requisite for the further development of this area of investigation.

Finally, the dissection of signaling pathways downstream from Dsg3 receptors that are critically implicated in mediating cell-cell dissociation and thus skin blistering provides for new pharmacologic and more targeted therapies, at least in PV [212]. As inferred from various mouse models of the diseases, EGFR, GSK3, and MAPK effectors such as MK2 are potential adjunctive targets for therapy of PV using specific inhibitors [115, 179, 184, 213]. Nevertheless, it is important to obtain a more precise understanding of the signaling networks surveyed by Dsg3/1 and of titrating carefully the various activators and inhibitors used in the in vitro and in vivo models of PV. These points appear critical prerequisites to ensure the successful introduction of inhibitors in clinical trials for pemphigus [179, 212].

Acknowledgments The present work has been supported by the following bodies: the Italian Ministry of Health (Ricerca Finalizzata grant n. RF2309790 and Ricerca Corrente 2015, to G.D.), the Framework Programme FP7 (Coordination theme I, Health-F2, 2008–2013, to E.M and L.B), the Swiss National Foundation for Scientific Research (31003A-135689 to E.M.; 3100A0-121966 to L.B.) and the Martha Foundation Zürich to E.M.. We are greatly indebted to many investigators and friends who supported our studies over the years by sharing ideas and providing support and working tools.

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