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Role of p38MAPK in ultrastructural alterations of desmosomes in human *ex vivo* pemphigus model

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Summary

Desmosomes interconnect epithelial cells together and are abundant in tissues constantly challenged by shear forces. They are composed of different isoforms of intercellular adhesion proteins which include desmogleins (Dsg) and desmocollins (Dsc). These proteins of apposing cells interact in homophilic and heterophilic manner thereby conferring integrity to the tissue. When this crucial role of desmosomes is compromised, several desmosome-associated diseases such as pemphigus may occur. Pemphigus is a blistering disease of the skin and oral mucosa. It is caused by anti-Dg3 and anti-Dsg1 autoantibodies that bind to the extracellular domains of the desmogleins and perturb their interaction. There are different phenotypes of the disease depending on the autoantibody profiles. Pemphigus vulgaris (PV) is caused by autoantibodies (PV-IgG) targeting Dsg1 and Dsg3 whereas pemphigus foliaceus (PF) is associated with autoantibodies against Dsg1 only. Pemphigus vulgaris is recognized as two sub-types; i.e, the mucosal-dominant form (mdPV) caused by anti-Dsg3 autoantibodies and the mucocutaneus (mcPV) variant caused by both anti-Dsg1 and anti-Dsg3 autoantibodies.

Several lines of evidence demonstrated that pemphigus is caused by disruption of Dsg interaction when the autoantibodies are interposed between the interacting Dsg (steric hindrance) and signaling triggered by autoantibody binding. It has been widely accepted that different signaling pathways work in concert in the modulation of desmosome structure and dynamics. p38 mitogen activated protein kinase (p38MAPK) has been extensively studied and its phosphorylation was detected in cell cultures in response to PV-IgG binding as well as in skin lesions of pemphigus patients. Pharmacological inactivation of this pathway attenuated cell dissociation in cultures and blister formation in murine models. However, no data was available with respect to the role of p38MAPK in blister formation in human skin and mucosa.

Therefore, we tested the dependency of blister formation and desmosome ultrastructural alteration on p38MAPK signaling induced by PV-IgG in human skin and mucosa explant cultures. Accordingly, we adapted the existing *ex vivo* skin model and also established a novel *ex vivo* mucosa model, and employed histological, immune-histochemical as well as electron microscopy analyses to determine the role of p38MAPK signaling in PV pathogenesis. Human skin biopsies were treated with the mouse monoclonal Dsg3-specific antibodyAK23, in comparison to antibody fractions from patients with mucocutaneous PV (mcPV-IgG) or mucosal PV (mdPV-IgG). mcPV-IgG only were sufficient to induce blisters as well as alterations in desmosome ultrastructure. In contrast, in human labial mucosa explants both AK23 and mdPV-IgG were sufficient to induce blisters as well as alterations in desmosome ultrastructure. Moreover, inhibition of p38MAPK using the specific inhibitor SB202190 was effective to avert blister formation, rescue desmosome size and number as well as preserved keratin filament association with desmosomal plaques in human skin. However, in the newly established human *ex vivo* mucosa model, inhibition of p38MAPK with specific inhibitors SB202190 and SB203580 was not effective to prevent these alterations. Taken together, our data demonstrate that p38MAPK plays a key role in blister formation through modulation of desmosome ultrastructure in human skin. In contrast, blister formation and associated ultrastructural changes of desmosomes in mucosa may depend on steric hindrance and other signaling pathways independent of p38MAPK.

1 Introduction

1.1 Epidermis and oral mucosa

1.1.1 Epidermis

The epidermis is the outermost layer of the skin which comprises of a self- renewing stratified epithelium (Kanitakis, 2002) (Fig. 1). The predominant cell type of the epidermis is the keratinocyte (Wickett and Visscher 2006). These cells synthesize structural proteins called keratins which are the predominant structural protein of the epidermis (Rao et al., 2014). These are eventually assembled into intermediate keratin filaments (Fuchs, 1995) as the cells proceed towards terminal differentiation during their course across suprabasal layers (Blanpain and Fuchs, 2006, Wang et al., 2016). Keratinocytes are tethered to one another by various types of intercellular adhesion complexes (Simpson et al., 2011).

Keratinocytes differentiate and mature as they migrate through the suprabasal compartment, and as a result exhibit morphological variations ranging from columnar or cuboidal basal cells to the highly flattened cells of the stratum corneum (Blanpain and Fuchs, 2006; Arnette et al., 2016). Accordingly, there are different strata of the epidermis which exhibit variations in morphology and the type of keratins they express. The basal layer (stratum basale) is constituted mainly by a single layer of columnar or cuboidal cells anchored to the underlying basement membrane by hemidesmosomes. These represent the stem cells responsible for the regeneration of the epidermis (Watt, 2002). The spinous cell layer (stratum spinosum) is called so for its spiny appearance due to the presence of large number of desmosomes. It consists of several cell layers. The granular layer (stratum granulosum) consists of cells exhibiting characteristic dark-staining keratohyalin granules (Menon, 2002). The corneal layer (stratum corneum) is formed by flattened ‘terminally-differentiated’ cells called corneocytes,

devoid of nucleus and cytoplasmic organelles. These are dead cells which are eventually sloughed off from the skin surface (Blanpain and Fuchs, 2006). In thick skins such as those in the palm of hand and sole of foot, there is a subcorneal layer (stratum lucidum) in addition.

Keratinocytes offer various defensive functions by forming physical, functional, immunologic or microbiological barriers (Menon, 2002; Proksch et al., 2008; Barnard and Li, 2017). In addition, the skin plays a crucial role in sensory and body temperature regulation as well as in vitamin D synthesis (Menon, 2002). To this end, it harbors epidermal appendages such as sweat glands, sebaceous glands, and hair follicles (Meisel et al., 2018). When this epithelial barrier function is compromised secondary to weak intercellular junctions, patients will experience loss of water and electrolytes and could also be more susceptible to infections (Moens and Veldhoen, 2012).

1.1.2 Oral mucosa

Compared to the epidermis, the oral mucosa is generally more permeable, structurally intermediate between that of the epidermis and intestinal mucosa (Shakya et al., 2011). The oral mucosa compromises stratified squamous epithelia (Fig. 1) which exhibits a spatial structural and functional diversity (Collins and Dawes, 1987). It may regionally be identified as sublingual, gingival, buccal or labial and palatal (Shakya et al., 2011) as well as characterized morphologically as lining, masticatory or specialized mucosa (Squier, 1991). The lining mucosa is a non-keratinized stratified squamous epithelium separated from the underlying fat containing submucosa by a thin layer of loose connective tissue, the lamina propria (Bierbaumer et al., 2018). It forms the lining of about 60% of the oral cavity (Collins and Dawes, 1987) such as buccal, labial, and sublingual regions, whereas keratinized or parakeratinized epithelia constitute the masticatory mucosa of the gingiva and hard palate (Bierbaumer et al., 2018). The epithelial lining of the papillary region of the dorsum of tongue is recognized as a specialized mucosa for it entertains the characteristic features of

both (Squier, 1991). These epithelial linings play diverse roles such as barrier, secretory or absorptive functions. Bound to the apical cell surface of oral epithelium, is a thin film of mucus which forms a protective layer to the underlying cells (Shakya et al., 2011).The keratinized epithelia of the oral cavity share a lot of structural features with the epidermis especially in their pattern of maturation (Squier, 1991) including stratification, keratin expression as well as their terminal differentiation which results in cornified cell formation (Presland and Dale, 2000; Presland and Jurevic, 2002). However, they exhibit remarkable differences in histological features and in the type of differentiation markers they express (Gibbs and Ponec, 2000). The epithelia of the oral cavity are less differentiated owing to the rapid cellular turnover (Hashimoto et al., 1966) caused by mechanical stress imposed by external factors (Donetti et al., 2005). This high rate of proliferation can be evidenced by the presence of multiple viable cell layers and abundant Ki67 staining cells in the mucosa (Gibbs and Ponec, 2000).

1.2 Desmosomes

Cell–cell attachment is a characteristic feature of multicellular organisms. This is crucial for the integrity of normal epidermis (Garrod et al., 2002). It is mediated by a set of specialized membrane structures which form intercellular adhesion complexes (Dusek et al., 2007). Epithelial cells possess these junctional complexes which mainly consist of, from apical to basal, a tight junction, adherens junction, and desmosome (Oda and Takeichi, 2011). The desmosomes form the adhesive core of these intercellular junctions (Fig. 2). Gap junctions are located below these junctional complexes and are involved in cellular communications by regulating exchange of small molecules and ions (Herve and Derangeon, 2013). Adherens junctions and desmosomes both consist of Ca2+ dependent cadherin molecules but differ in the cytoskeletal components they are anchored to; namely, actins and keratins, respectively (Troyanovsky et al., 1993; Harris and Tepass, 2010). Adherens junctions form a complete ring

around a cell, hence the name zonula adherens, thereby defining epithelial cell polarity (Harris, 2012).

The term desmosome stems from the Greek words “desmo,” which means bond and “soma,” meaning body (Calkins and Setzer, 2007). Desmosomes form ‘‘spot welds,’’ referred to as maculae adherentes, which make a strong intercellular tethering. They form robust adhesive contacts between adjacent epithelial cells and confer tissue integrity when the epithelial sheet encounters shear forces (Garrod and Chidgey, 2008; Price et al., 2018). They are mainly built from three gene families: cadherins, armadillo proteins (plakoglobin, PG, and plakophilins, PKP), and plakins (desmoplakin, DP) (Nekrasova and Green, 2013). The desmosomal cadherins, so named for their dependency on Ca^{2+} for adhesion, mainly consist of desmogleins (Dsg) and desmocollins (Dsc) (Garrod and Chidgey, 2008). These are transmembrane proteins which mediate adhesion with adjacent cells through their extracellular domains, and interact with PG and PKP through their cytoplasmic tails (Fig. 2 b). DP plugs the entire complex to keratin intermediate filaments (Kowalczyk et al., 1999; Getsios et al., 2004) (Fig. 2a, b).

Different components of desmosomal proteins show a tissue-specific as well as a stratification-dependent pattern of distribution across the layers of stratified epithelia, and also dependent on differentiation status (Arnemann et al., 1993; Johnson et al., 2014). Humans express different types of desmosomal cadherin isoforms; four Dsgs (Dsg1–4) and three Dscs (Dsc1–3) as in the epidermis and oral cavity (Green and Simpson, 2007). Dsg2 and Dsc2 are present in all desmosome bearing tissues and are thus expressed in the heart and simple epithelia (Lowndes et al., 2014). In epidermis, Dsg3 and Dsc3 are expressed strongly in the deepest layers whose staining progressively fades out as the cells transit out of the proliferation zone, whereas Dsg2 is limited to the basal layer (Donetti et al., 2005; Mahoney et al., 2006). Dsg1 is first expressed in the interface between the basal and suprabasal cells

(Getsios et al., 2009), and together with Dsc1, shows an inverse distribution gradient with Dsg3 and Dsc3 as the cells differentiate and stratify (Arnemann et al., 1993; Elias et al., 2001). In mucosa, Dsg3 is the principal desmoglein present whereas Dsg1 is expressed at low level (Shirakata et al., 1998).

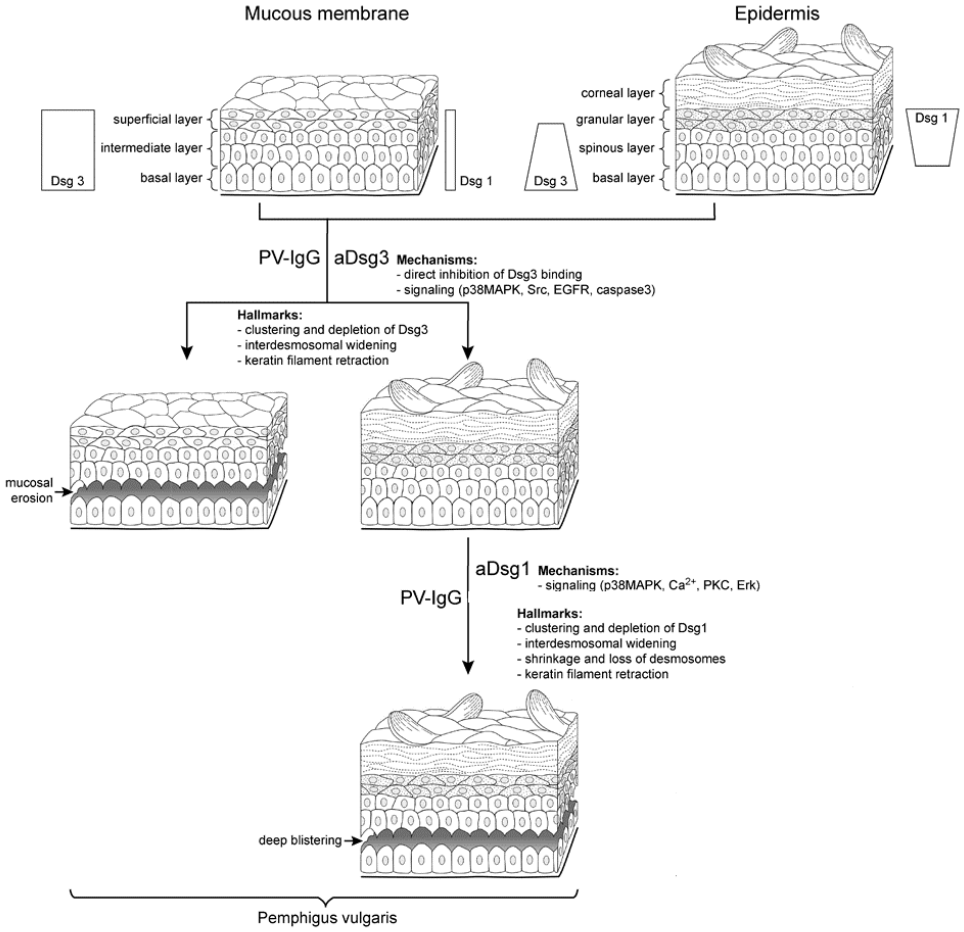

Figure 1. Schematic representing histological location of cleft formation in pemphigus vulgaris, and distribution of desmoglein isoforms in epidermis and mucosa. Dsg3 is strongly expressed in basal epidermis and across all layers of mucosa. Dsg1 is expressed in suprabasal and superficial layers in epidermis but its expression is minimal in mucosa. Anti-Dsg1 and 3 autoantibodies are the culprits in causing loss of adhesive contacts in pemphigus vulgaris. Modified from Spindler and Waschke 2018.

Ultrastructurally, desmosomes show three morphologically distinct regions: the extracellular core region (EC), the outer dense plaque (ODP), and the inner dense plaque (IDP) (North et al., 1999) (Fig. 2 b). The ODP is composed of cytoplasmic tails of the Dsg and Dsc, as well as the armadillo proteins, PG and PKP whereas the IDP mainly comprises DP (Delva et al., 2009). A dense midline bisects the desmosomes interconnecting neighboring cells corresponding to sites of trans-interactions between the extracellular adhesive domains of opposing cadherins (Al-Amoudi et al., 2007) and this represents a Ca^{2+} independent hyperadhesive desmosomes characteristic of normal epidermis (Garrod et al., 2005).

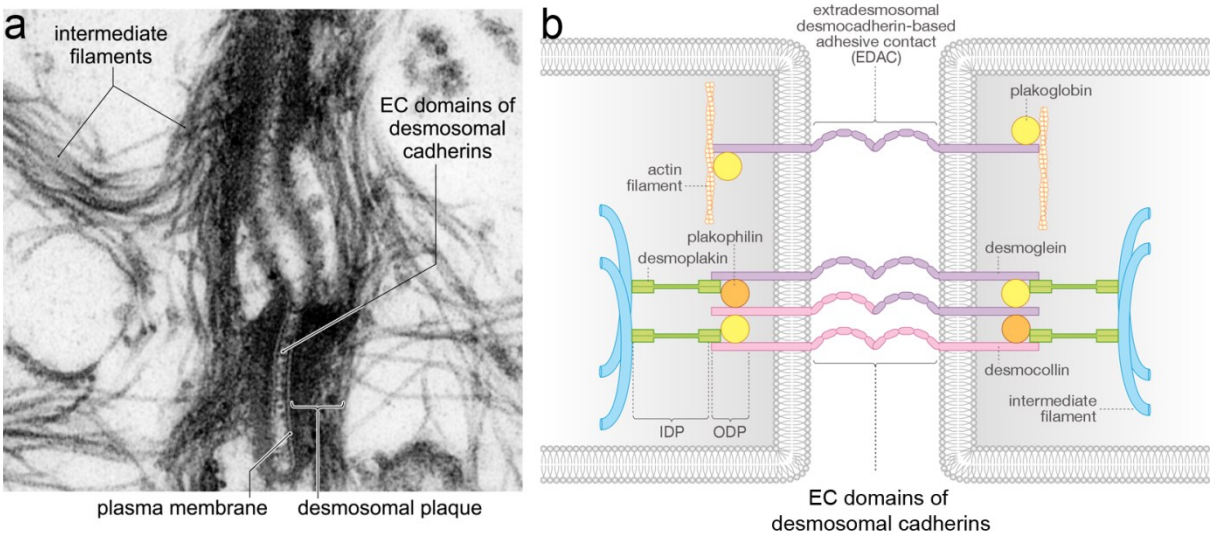

Figure 2. Desmosome ultrastructure. a) Electromicrograph of desmosomes showing desmosomal plaques and associated keratin intermediate filaments; (b) schematic representation of the various components of desmosomes with interacting extracellular domains, ODP (outer dense plaque), IDP (inner dense plaque). Modified from (a) Waschke 2008 (b) Waschke and Spindler 2014.

Dsg and Dsc span the membrane and have an extracellular N-terminal domain (head) which make *cis* and *trans* interactions with desmosomal cadherins of same or adjacent cells, respectively (Al-Amoudi et al., 2007). Different homophilic (between similar) and

heterophilic (between different) interactions among Dsg and Dsc have been reported in the literature (Chitaev and Troyanovsky, 1997, Syed et al., 2002, Vielmuth et al., 2018a). The C terminus resides within the ODP where it binds the armadillo proteins. Dsg and Dsc exhibit a remarkable similarity, however differ mainly in their tail structure. Both contain four extracellular cadherin repeats (EC 1-4) and an extracellular anchor (Garrod et al., 2002). The latter is attached to a single transmembrane segment which in turn binds to the intercellular anchor on the inner side. Dsc occurs in two isoforms, designated as Dsc-a and Dsc-b representing long and short forms, respectively (Garrod and Chidgey, 2008). The latter lacks the PG-binding intracellular segment region (Troyanovsky et al., 1993). Dsg possesses extended cytoplasmic domains with subdomains. Inwards to the intercellular segment successively, are a proline rich linker, a repeating unit domain whose repeats differ among the isoforms, the glycine-rich desmoglein terminal domain being the deepest (Berika and Garrod, 2014)).

1.3 Desmosome-associated diseases

Several diseases are implicated when desmosomal functions are compromised producing diverse disease phenotypes (Dusek et al., 2007; Waschke, 2008). Desmosomes are targets of genetic, infectious and autoimmune diseases which result in a largely weakened cellular cohesion leading to impaired functions (Broussard et al., 2015). These diseases result from dysfunction or altered expression of some of the desmosomal protein components (Waschke, 2008). Mutations associated with obligate desmosomal proteins such as Dsg, Dsc or DP (Getsios et al., 2004; Stahley and Kowalczyk, 2015) bring about debilitating conditions in the respective tissues (Al-Jassar et al., 2013). For example, mutation of Dsg2 and Dsc2 of the intercalated discs of the heart muscles causes arrhythmogenic right ventricular cardiomyopathy (Bhuiyan et al., 2009). Similarly, mutations in Dsg1 and DP have been shown to be associated with inherited inflammatory skin diseases such as severe allergies,

multiple allergies and metabolic wasting syndrome (McAleer et al., 2015, Samuelov et al., 2013).

Overexpression or down regulation of desmosomal proteins in epithelial cell carcinomas has been reported by various researchers. But the exact role is not well known (Waschke, 2008; Al-Jassaret al., 2013). On the other hand, infectious agents such as staphylococcus aureus are known to produce exfoliative toxin which cleaves Dsg1 thereby inducing blisters as in bullus impetigo and staphylococcal scalded skin disease locally or away from the infection site, respectively (Amagai, 2010). The most extensively studied human autoimmune disease, which is associated with desmosome dysfunction, is pemphigus (Kasperkiewicz et al., 2017).

1.4 Pemphigus

In the epidermis and oral mucosa, autoantibodies that target desmosomal proteins cause pemphigus, a disease manifested by mucosal and skin lesions (Kasperkiewicz et al., 2017; Schmidt et al., 2019). All lesions are histochemically characterized by binding of PV-IgG autoantibodies to keratinocyte cell surface proteins (Amagai, 2010). Different pemphigus phenotypes occur based on autoantibody profile, tissue specificity and histological location of cleft formation (Amagai et al., 1999; Amagai and Stanley, 2012). The main types are pemphigus vulgaris (PV) and Pemphigus foliaceus (PF) (Fig. 3).

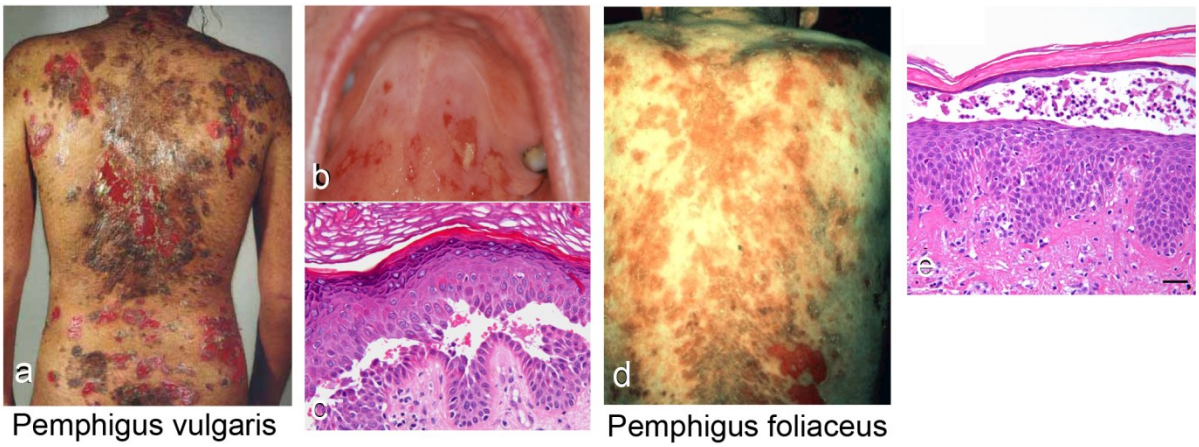

Figure 3. The major clinical phenotypes of pemphigus. A patient suffering from pemphigus vulgaris (a) with mucosal involvement (b), and hematoxylene eosin staining showing suprabasal cleft formation (c), a pemphigus foliaceus patient (d) with subcorneal histological location of the lesion (e). Modified from Waschke 2008

1.4.1 Pemphigus vulgaris

Pemphigus vulgaris is caused by circulating autoantibodies which specifically engage the extracellular adhesive domains of Dsg3 and Dsg1 in desmosomes (Pollmann et al., 2018) (Fig. 1). It initially appears as erosions of oral mucosa with subsequent involvement of the skin (Fig. 3 a, b), hence called mucocutaneous PV (mcPV), and as suprabasal clefting in histological sections (Payne et al., 2004) (Fig. 3c). The mucosal subtype (mucosal dominant PV, mdPV) involves mucous membranes only and is triggered by anti-Dsg3 autoantibodies (Amagai et al., 1999) (Fig. 3b). Pemphigus foliaceus causes blistering in the superficial epidermis where Dsg1 is highly expressed and Dsg3 is lacking (Fig. 3 d, e). Thus, autoantibodies targeting Dsg1 presumably are responsible for the clinical phenotype (Kitajima, 2013). Histological locations of clefting as well as tissue-specific manifestation of the disease can be explained by what is widely known as ‘desmoglein compensation hypothesis’ (Mahoney et al., 1999). In mucosa, since Dsg3 is the predominant desmosomal protein and Dsg1 is present at a lower level in the suprabasal layers (Shirakata et al., 1998), anti-Dsg3-autoantibodies are able to disrupt Dsg3 interaction where compensatory adhesion by Dsg1 is lacking resulting in suprabasal blisters. This same mechanism has been proposed to apply to the suprabasal blistering of the epidermis as well (Amagai et al., 1996). In PF, anti-Dsg1-autoantibodies cause subcorneal blistering because Dsg3 is absent to compensate for adhesive function at the superficial layers in the epidermis (Ding et al., 1997; Shirakata et al., 1998) (Fig. 3e). The effect of PV-IgG on cell adhesion has been well characterized under controlled conditions. Many laboratory studies have shown that PV-IgG causes Dsg3

depletion from membranes in cultures and animal models as well as in human skin explants (Aoyama and Kitajima, 1999; Shu et al., 2007; Yamamoto et al., 2007; Spindler et al., 2011; Egu et al., 2017) indicating that blister formation and Dsg3 internalization are two interconnected events leading to loss of cell adhesion (Jolly et al., 2010).

1.4.2 Pathomechanisms of pemphigus

The exact pathomechanism underlying loss of cell contacts of keratinocytes owing to pemphigus autoantibodies is not fully unravelled. Among several hypotheses, however, it has been widely accepted that steric hindrance and signaling as the most plausible mechanisms that drive loss of adhesive contacts in keratinocytes resulting in acantholysis (Spindler et al., 2018; Spindler and Waschke, 2018).

1.4.2.1 Role of steric hindrance

It is well known that epidermal blistering in PV patients is caused by autoantibodies directed against the amino-terminal adhesive interface of Dsg3 exodomain (Amagai et al., 1992; Tsunoda et al., 2003). IgGs from PV patients was sufficient to induce intraepidermal blisters when injected to a mouse (Anhalt et al., 1982; Schulze et al., 2012) or into an *ex vivo* human skin (Hu et al., 1978; Egu et al., 2017). To be more precise, the amino-terminal of the first two cadherin repeats of the Dsg3 extracellular domain (EC1 and EC2) are the main targets to which PV-IgG preferentially bind (Amagai et al., 1992; Ding et al., 1999; Sekiguchi et al., 2001; Tsunoda et al., 2003; Di Zenzo et al., 2012). These cadherin residues, crucial for adhesive interactions, predominantly contain those epitopes recognized by pemphigus autoantibodies (Chan et al., 2010; Di Zenzo et al., 2012; Ohyama et al., 2012). However, other studies have shown that a significant amount of PV-IgG bind to the Dsg membrane proximal domains (Sekiguchi et al., 2001) which implies that these domains are also, at least in part, involved in the disease (Amagai et al., 1992). Hence, a complete adsorption of the entire extracellular domain from a pemphigus patient serum was required to abolish blister

formation (Amagai et al., 1992; Langenhan et al., 2014). Direct inhibition of desmoglein interaction is, hence, caused by the interference of the autoantibodies through binding to the extracellular domains of desmogleins (Stanley and Amagai, 2006)). Interestingly, direct inhibition of Dsg interaction has been found for Dsg3 but not for Dsg1, and was detectable on the surface of living keratinocytes (Waschke et al., 2005; Heupel et al., 2008; Vielmuth et al., 2015).

1.4.2.2 Role of signaling

A large body of experimental data has shown that pharmacologic inhibition of signaling pathways ameliorated intraepidermal cleft formation *in vivo* and cell dissociation *in vitro* (Berkowitz et al., 2006; Waschke et al., 2006; Delva et al., 2008). It is believed that both mechanisms are relevant but not necessarily independent events (Stahley and Kowalczyk, 2015), although signaling cascades may be activated downstream of antibody binding (Getsios et al., 2010; Spindler and Waschke, 2018) or following loss of cell adhesion (Mao et al., 2011). Some investigators assert that other factors such as clustering and endocytosis of cell surface Dsg3 (Stahley et al., 2016) as well as autoantibody mediated perturbation of desmosome assembly (Nekrasova and Green, 2013) in addition may synergistically orchestrate blister formation (Kasperkiewicz et al., 2017).

Several cellular responses to autoantibody binding in pemphigus have been attributed to different signaling pathways. These include, among others, groups of mammalian mitogen-activated protein kinases (MAPKs) such as p38MAPK and extracellular signal-regulated kinases (ERK) as well as Rho GTPase, epidermal growth factor receptor (EGFR), Rous sarcoma-related kinase (Src) and protein kinase C (PKC) (Esaki et al., 1995; Osada et al., 1997; Sanchez-Carpintero et al., 2004; Berkowitz et al., 2005; Frusic-Zlotkin et al., 2006; Waschke et al., 2006; Getsios et al., 2009; Jolly et al., 2010; Spindler et al., 2011; Espana et

al., 2013; Harmon et al., 2013; Walter et al., 2017; Kugelmann et al., 2019; Walter et al., 2019,).

p38MAPK is one of the most extensively studied signaling molecule in pemphigus pathology. It exists in different isoforms (α, β, γ, δ) displaying a species-specific expression pattern (Jiang et al., 1996; Jiang et al., 1997; Cuenda and Rousseau, 2007). The α and β isoforms are ubiquitously expressed across cell lines and tissues, the α subtype being the most common isoform in adult tissues (Jiang et al., 1997; Cargnello and Roux, 2011). p38MAPK mediates cellular responses triggered by inflammation or other environmental responses such as chemicals, UV, and oxidative stress (Kim et al., 2008). Deletion of p38MAPK α has been shown to reduce proinflammatory gene expression in epithelial cells which underscores its role in inflammatory reactions (Kim et al., 2008). Interestingly, p38MAPK has been shown to be activated secondary to PV-IgG binding (Berkowitz et al., 2005) and its phosphorylation was detected in lesioned skin of PV patients (Berkowitz et al., 2008) and in keratinocyte cell cultures treated with PV patient serum (Berkowitz et al., 2005, Kawasaki et al., 2006). Furthermore, there is abundant evidence that PV-IgG-induced activation of p38MAPK resulted in internalization of Dsg 3 (Jolly et al., 2010), and its subsequent depletion from endosomes (Stahley et al., 2016), retraction of keratin intermediate filaments (Berkowitz et al., 2005; Spindler et al., 2013; Vielmuth et al., 2018b) thereby perturbing the dynamics of desmosome assembly (Mao et al., 2011). Interestingly, a drug-induced inhibition of this molecule was sufficient to prevent all these PV-IgG-induced hallmark features of the disease. As a result, p38MAKP has become the main focus of interest in pemphigus research among the plethora of signaling molecules implicated in PV pathogenesis.

There are strata of protein kinase cascades functionally subordinate to p38MAPK whose phosphorylation mediates the dynamics of their target gene expression (Kim et al., 2008). PV-IgG-induced activation of p38MAPK has been shown to augment the phosphorylation of its

downstream targets such as mitogen-activated protein kinase 2 (MK2) in a dose-dependent manner (Mao et al., 2014). MK2 regulates several cellular activities such as actin remodeling (Kotlyarov et al., 2002), a process which is relevant to PV pathogenesis. The latter study reported a significant reduction of p38MAPK levels in MK2-deficient mice asserting that MK2 functions to maintain baseline p38MAPK protein levels. Specific inhibition of p38MAPK was sufficient to prevent MK2 phosphorylation, its major downstream substrate (Mao et al., 2014). Moreover, inhibition of MK2 has been shown to block PV-IgG-mediated spontaneous blister formation in mice as well as loss of cell surface Dsg3 in human keratinocytes (Mao et al., 2014).

In this study, we demonstrated that pharmacological inhibition of p38MAPK was sufficient to avert intra-epithelial blister formation, attenuate reduction in the number and size of desmosomes, and preserve keratin filament association with desmosomal plaque in human skin (Egu et al., 2017). As a result, we speculate that blistering in PV is mediated by p38MAPK through modulation of desmosome ultrastructure in human skin but not in mucosa.

1.5 Aim of the study

The epidermis has been best characterized among stratified keratinizing epithelia for its availability, ease to study and its versatility to be used in the investigation of various skin related diseases (Presland and Dale, 2000). Most investigations involving the oral mucosa are mainly focused on permeability studies primarily related to oral transmucosal delivery of drugs (Squier, 1991; Shakya et al., 2011). Although many diseases which affect the skin such as pemphigus vulgaris also involve the mucosa, few data are available to date related to mucosal studies and only embrace animal models (Schulze et al., 2012). Therefore, *ex vivo* mucosa model will be a useful approach because, similarly to skin studies, such experiments utilize large samples, which is the major drawback in studies involving patient biopsies.

The importance of desmosomes in conferring integrity to tissues is best illustrated by the various diseases that occur when this essential function is compromised which primarily affects the barrier function resulting is loss of electrolytes and water as well as increased susceptibility to infections. Desmosomes are targets for autoimmune diseases such as pemphigus.

Studies investigating pemphigus pathogenesis have been going on for many decades and the precise pathomechanism is not yet fully unraveled. The present study was aimed at adapting the existing *ex vivo* skin model and establishing a novel *ex vivo* mucosa model to assess the role of p38MAPK signaling in the acantholytic changes resulting from PV-IgG in humans. This is required to understand the pathomechanism and shed light on possible signaling targeted treatment options in the future. To this end, we employed electron microscope studies to characterize ultrastructural changes in the context of p38MAPK signaling

2 Results

2.1 Inhibition of p38MAPK signalling prevents epidermal blistering and alterations of desmosome structure induced by pemphigus autoantibodies in human epidermis.

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Inhibition of p38MAPK signalling prevents epidermal blistering and alterations of desmosome structure induced by pemphigus autoantibodies in human epidermis

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Summary

Background Pemphigus vulgaris (PV) is a skin blistering disease caused by autoantibodies targeting the desmosomal adhesion proteins desmoglein (Dsg) 3 and 1. The mechanisms underlying pemphigus skin blistering are not fully elucidated but p38 mitogen-activated protein kinase (p38MAPK) activation is one of the signalling events necessary for full loss of cell cohesion. However, it is unclear whether ultrastructural hallmarks of desmosome morphology as observed in patients' lesions are mediated by p38MAPK signalling.

Objectives In this study, we tested the relevance of p38MAPK for blister formation and the ultrastructural changes induced by PV autoantibodies in human skin.

Methods Human skin samples were injected with IgG fractions of one patient suffering from mucocutaneous PV (mcPV-IgG), one from mucosal-dominant PV (mdPV-IgG) or AK23, a pathogenic monoclonal Dsg3 antibody derived from a pemphigus mouse model. Samples were processed for histological and electron microscopy analyses.

Results mcPV-IgG and AK23 but not mdPV-IgG reduced desmosome size, caused interdesmosomal widening and formation of split desmosomes, and altered keratin filament insertion. In contrast, full epidermal blister formation and lower desmosome number were evident in tissue samples exposed to mcPV-IgG only. Pharmacological inhibition of p38MAPK blunted the reduction of desmosome number and size, ameliorated interdesmosomal widening and loss of keratin insertion and prevented mcPV-IgG-induced blister formation.

Conclusions Our data demonstrate that blistering can be prevented by inhibition of p38MAPK in the human epidermis. Moreover, typical morphological alterations induced by mcPV-IgG such as interdesmosomal widening and the reduction of desmosome size at least in part require p38MAPK signalling.

What's already known about this topic?

- p38 mitogen-activated protein kinase (p38MAPK) inhibition blocks loss of cell cohesion in keratinocyte cultures and blister formation in mouse models induced by pemphigus vulgaris autoantibodies.

What does this study add?

- Inhibition of p38MAPK signalling is sufficient to abrogate skin blistering in human and ultrastructural data suggests that p38MAPK contributes to blister formation via reduction of the number and size of desmosomes and modulation of the keratin filament cytoskeleton.

What is the translational message?

- This study demonstrates the relevance of p38MAPK signalling for epidermal blistering in human skin and links this pathway to the alterations of desmosome morphology found in patients with pemphigus.

Desmosomes are intercellular adhesion complexes that tether adjacent cells and are important for maintaining tissue integrity.[1] They consist of the transmembrane adhesion molecules desmoglein (Dsg) 1–4 and desmocollin 1–3 as well as intracellular plaque proteins (plakoglobin, desmoplakin and plakophilins), providing the link to the keratin filament cytoskeleton.[2,3] Desmosomes are targets in pemphigus vulgaris (PV), an autoimmune disease causing weakening of cell–cell adhesion. PV is characterized clinically by blister formation and histologically by the appearance of clefts in the lower epidermis and within mucous membranes of the oral cavity.[4] The mucosal-dominant PV variant is caused by IgG antibodies which target Dsg3 (mdPV-IgG) whereas mucocutaneous PV (mcPV-IgG) affecting both mucous membranes and epidermis is a result of antibodies against Dsg3 and Dsg1.[5] It is well established that both steric hindrance of the extracellular domain of Dsg3 and intracellular signalling events contribute to loss of cell adhesion.[6,7] In addition to steric hindrance, various signalling pathways have been implicated in PV pathogenesis. Activation of p38 mitogen-activated protein kinase (p38MAPK) is one of the best-characterized signalling events associated with the acantholytic changes induced by PV-IgG. Specific inhibition of this molecule using SB202190 was effective to prevent loss of adhesive contacts in cell cultures and mouse models.[8,9] It is not completely understood whether and how p38MAPK contributes to blister formation in human skin. Moreover, no data are available on whether the morphological alterations of desmosomes, which have been characterized recently on the ultrastructural level in lesions of patients with pemphigus,[10,11] are induced by p38MAPK.

Material and methods

Tissue culture

Biopsies of human skin were acquired from cadavers of the human body donor programme without history of skin diseases from the Institute of Anatomy and Cell Biology, Ludwig-Maximilians-Universität München, Germany. Written informed consent was obtained from body donors for the use of research samples. Biopsies were acquired only if death occurred less than 24 h before arrival at the institute. Viability of the skin biopsy was assured with MTT assay [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] before and after incubation. From each body donor, a skin piece of approximately 5 × 5 cm size was removed from the shoulder and gently stripped of fat and excessive connective tissue. The biopsy was further divided into 1 × 1 cm pieces for injection of pemphigus autoantibodies and the p38MAPK inhibitor used in the study. The skin pieces were intradermally injected using a 30G syringe with either 50 μL of the pathogenic monoclonal Dsg3 pemphigus autoantibody AK23 (200 μg per specimen), 50 μL of mcPV-IgG or mdPV-IgG (50 μg per specimen). mcPV-IgG contained antibodies against Dsg3 and Dsg1 [enzyme-linked immunosorbent assay (ELISA) score: 5542 U mL^{-1} and 711 U mL^{-1}, respectively], mdPV-IgG was positive for anti-Dsg3 only (ELISA score: 163 U mL^{-1}). Controls received IgG from a healthy volunteer. In order to assess the effect of p38MAPK on desmosome morphology, some samples were pretreated with 50 μL of 30 μmol L^{-1} p38MAPK inhibitor SB202190 [Merck, Darmstadt, Germany, 1 : 100 in phosphate-buffered saline (PBS)] for 1 h followed by injection with autoantibodies. All other samples received 50 μL of a 1 : 100 dilution of dimethyl sulphoxide in PBS as solvent control. Samples were incubated floating on Dulbecco modified Eagle medium (DMEM) at 37 °C and 5% CO_2 for an additional 24 h with the epidermis facing upwards without any additional support. Each condition was evaluated in biopsies from three to four different body donors. Shear stress was applied gently on the skin samples using a rubber head with equal frequency and magnitude. Finally, samples were cut into two parts and processed for haematoxylin and eosin (HE) and immunostaining or electron microscopy analyses.

Histology and immunostaining

Samples were embedded in TissueTec (Leica Biosystems, Nussloch, Germany) and were serially sectioned at 7-μm thickness using a cryostat microtome (HM 500 OM, Microm International GmbH, Walldorf, Germany). HE staining was performed according to standard procedures and mounted in DEPX (Sigma-Aldrich, St Louis, MO, U.S.A.).

For immunofluorescence, sections were drawn randomly from the pool of serial sections. Sections were heated, fixed with 2% paraformaldehyde in PBS (20 min), permeabilized with 0·1% Triton X-100 (45 min) and blocked with 3% bovine serum albumin and 1% normal goat serum for 60 min. A primary monoclonal anti-Dsg3 antibody directed against the ectodomain of Dsg3 (Invitrogen, Carlsbad, CA, U.S.A.) was incubated overnight at 4 °C in the dark. Cy3-conjugated goat-antimouse and goat-antihuman secondary antibodies were applied for 1 h at room temperature (Dianova, Hamburg, Germany). Some slides were stained with secondary

antibody only as a primary antibody control. Finally, slides were mounted with 1·5% n-propyl gallate in glycerol and images were captured using a Leica SP5 confocal microscope with a X63 NA 1·4 PL APO objective (both Leica, Mannheim, Germany).

Electron microscopy

Tissue samples from the injected skin explants were dissected into small pieces of approximately 2 mm in diameter. They were fixed in 2·5% glutaraldehyde and washed in PBS. They were then post-fixed with 2% osmium tetroxide and dehydrated through a graded ethanol series. The samples were subsequently cleared in propylene oxide, embedded in EPON 812 (Serva Electrophoresis GmBH, Heidelberg, Germany) and finally cured at 80 °C for 24 h. The resulting blocks were trimmed and sectioned at 60-nm thick slices with a Reichert-Jung Ultracut E ultramicrotome using a diamond knife (DiATOME Electron Microscopy Sciences, Hatfield, PA, U.S.A.). Silver-appearing sections were placed on a 150 mesh copper/rhodium grid (Plano GmbH, Wetzlar, Germany). Samples were then contrasted using alcholic uranylacetate and lead citrate. The sections were imaged using a Libra 120 transmission electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) equipped with a SSCCD camera system (TRS, Olympus, Tokyo, Japan).

Quantification of autoantibody effects

Serial sections stained with HE were evaluated for blister formation and extent as described.[12] Cleft lengths were scored in whole numbers ranging from 0 to 4 based on the following principle: 0 (no blister), 1 (cleft covering 1%–25% of section length), 2 (26%–50% coverage), 3 (51%–75% coverage) and 4 (76%–100% coverage).

For the ultrastructural analyses, about 10–20 micrographs from each body donor of basal and suprabasal epidermal layers at 4000 × magnification were selected randomly and in total 200–300 desmosomes per condition were analysed with ImageJ (Wayne Rasband; https://imagej.nih.gov/ij/). To assess the number of desmosomes, a freehand line was drawn along cell membranes and all desmosomes intersected by the line were counted for each photomicrograph. To account for variability in the number of cells present in a micrograph, the number of desmosomes was expressed per μm of membrane length.[13] For measuring desmosome length, the distance between the two ends of the desmosome was measured and expressed in μm. For quantification of interdesmosomal widening, the freehand selection tool was used to draw a line between successive desmosomes along the cell boundaries of adjacent cells until the free ends of the line met. The resulting area was expressed in $\mu m.^2$ For desmosome–keratin association, those desmosomes without or with reduced keratin association were counted and expressed as a percentage of altered keratin insertion from the total number of desmosomes for each condition.

Dispase-based dissociation assays

HaCaT human keratinocytes were cultured at 37 °C and 5% CO_2 in DMEM containing 10% fetal calf serum, 50 U mL^{-1} penicillin and 50 g mL^{-1} streptomycin. Dispase-based dissociation assays were carried out on confluent HaCaT monolayers as previously described.[14] Briefly, after 24 h of IgG fraction incubation, cell monolayers were washed with Hank's balanced salt solution (HBSS) (Sigma-Aldrich) and detached from the well bottom by 20-min incubation of Dispase-II (Sigma-Aldrich) at 37 °C. Eventually, the Dispase solution was substituted by 350 μL HBSS and mechanical stress was applied by shearing the monolayer 10 times with an electrical 1-mL pipette (Finnpipette, Thermo Fisher Scientific, Waltham, MA, U.S.A.). Ten μL thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich) was added after shearing to increase the visibility of the cell fragments, whose number correlate to loss of cell cohesion. Images of the entire well were acquired using a stereomicroscope (Stemi 508, Carl Zeiss Microimaging, Jena, Germany) and evaluated using ImageJ.

Statistical analysis

A mean value per body donor was calculated from the aquired data points and used for statistics. Error bars represent SEM. Data were compared using one-way ANOVA followed by Bonferroni post-test (for Gaussian-distributed samples) and Kruskal–Wallis analysis with Dunn's post-correction (for non-Gaussian distribution) using Prism (GraphPad Software, La Jolla, CA, U.S.A.). Statistical significance was assumed when $P < 0·05$.

Results

Biopsies of human skin were acquired from cadavers from the Anatomy body donor programme within 24 h after decease, followed by explant culture and immediately injected and incubated for 24 h with mcPV-IgG, mdPV-IgG or the pathogenic monoclonal Dsg3 pemphigus autoantibody AK23, similar to earlier studies.[15] AK23 was included to determine the strict anti-Dsg3 dependency of the observed effects in comparison with PV-IgG fractions containing a multitude of different antibodies. To address the role of p38MAPK, some samples were injected with the p38MAPK inhibitor SB202190 1 h prior to autoantibody injection. Serial sections of the biopsies were stained with HE and evaluated for blister formation and extent. Blisters were evident in all samples injected with mcPV-IgG, but ameliorated in samples treated with mcPV-IgG in combination with SB202190 (Fig. 1a, b). In contrast, AK23 induced minor but not significant epidermal splitting, although some intercellular widening was observed. mdPV-IgG had no effect, although the autoantibody fraction was capable of disrupting cell–cell adhesion of cultured HaCaT keratinocytes as detected by dispase-based dissociation assays (Fig. S1a; see Supporting Information). Secondary antibody controls revealed that all autoantibody fractions bound to

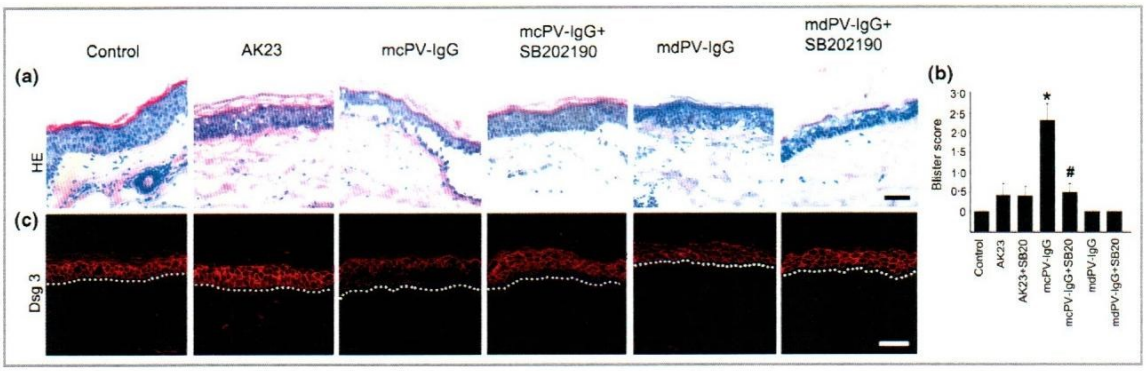


Fig 1. Histological alterations of intact human skin incubated with pemphigus autoantibodies *ex vivo*. AK23, mcPV-IgG or mdPV-IgG were applied in the absence or presence of the p38MAPK inhibitor SB202190. (a) Haematoxylin and eosin staining. (b) Blister score. (c) Dsg3 immunostaining (n = 3–4 body donors for each condition). Scale bar = 50 μm. *$P < 0{\cdot}05$ vs. control, #$P < 0{\cdot}05$ vs. AK23 or mcPV-IgG, respectively. AK23, pathogenic monoclonal Dsg3 antibody derived from a pemphigus mouse model; Dsg, desmoglein; mcPV, mucocutaneous pemphigus vulgaris; mdPV, mucosal-dominant PV; p38MAPK, p38 mitogen-activated protein kinase.

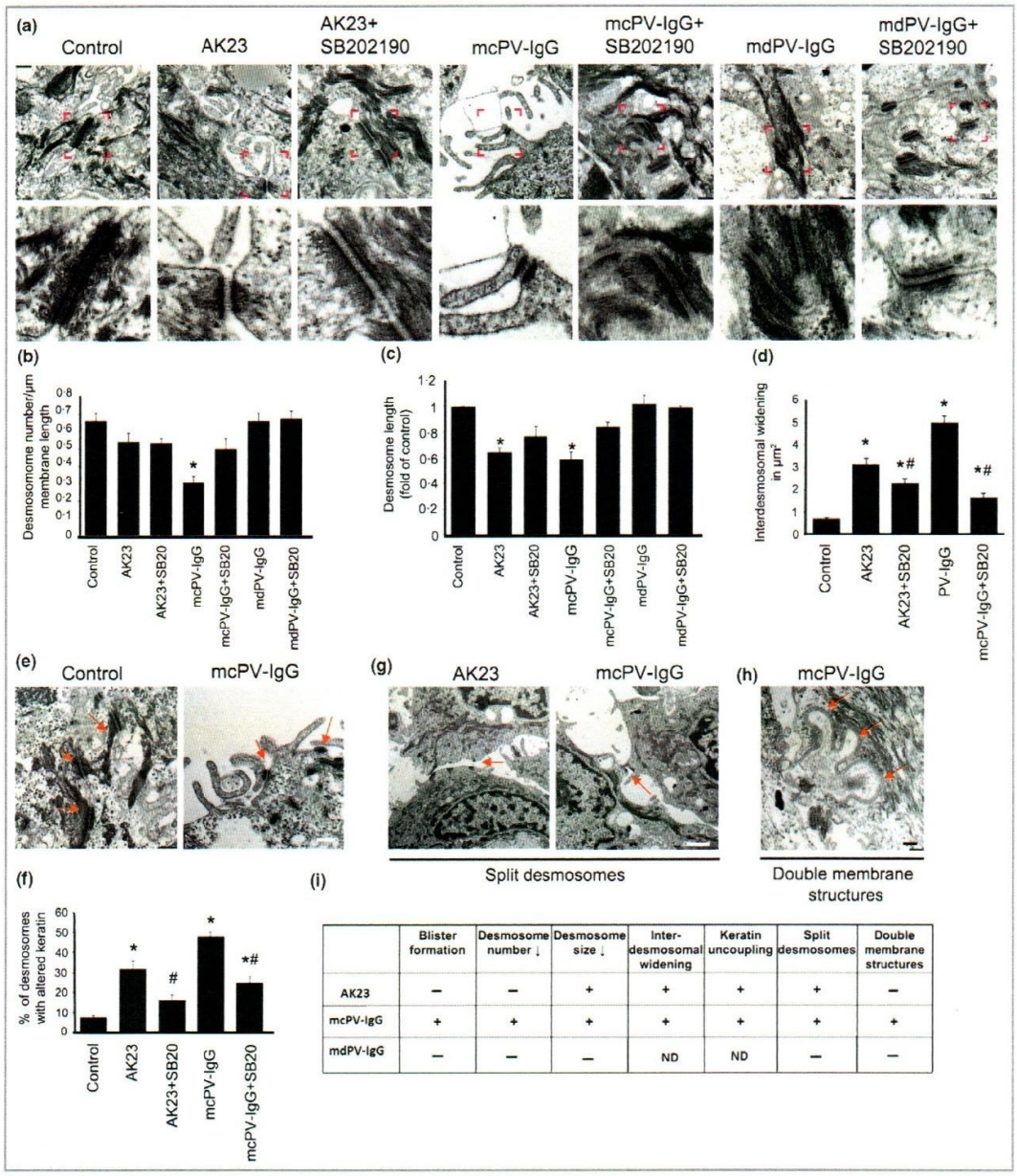
keratinocytes in the epidermis in the absence or presence of SB202190 (Fig. S1b). Examination of Dsg3 distribution by immunostaining showed fragmented Dsg3 staining, mostly in the basal and suprabasal areas in mcPV-IgG-treated samples, which was blocked by p38MAPK inhibition (Fig. 1c).

Ultrastructural studies involving skin of patients with PV and mouse models have demonstrated a reduction in number and size of desmosomes, and further showed that the two desmosomal halves can be partially or fully separated ('split').[11,16,17] In our human skin model, the number of desmosomes was significantly reduced in the basal areas in mcPV-IgG-injected biopsies (Fig. 2a, b). The desmosome size varied considerably between the different body donors, ranging from 294 nm to 556 nm. Thus, desmosome size was normalized to the respective control injection of each body donor. We detected a reduction of desmosome size in skin samples injected with mcPV-IgG, whereas the injection of mdPV-IgG had no effect (Fig. 2a, c). p38MAPK inhibition prevented both the reduction of desmosome number and size in response to mcPV-IgG treatment.

Interestingly, AK23 induced a significant reduction in desmosome size, which was ameliorated by SB202190, whereas the number of desmosomes remained unaffected. Thus, histologically evident blister formation correlated with reduction of desmosome number but not size. Although the increase in extracellular space at areas between desmosomes may not be a prerequisite for acantholysis,[10] it is considered an early event after autoantibody binding as a result of extradesmosomal Dsg loss. We observed this increased interdesmosomal widening in AK23-injected samples and, to a higher extent, in mcPV-IgG injected biopsies (Fig. 2a, d). Interestingly, p38MAPK inhibition only slightly ameliorated AK23-induced interdesmosomal widening and blunted the effect of mcPV-IgG to the level of AK23 injection. Next, we assessed the association of desmosomes with keratin filaments as the uncoupling of keratin filaments is an immunohistological hallmark of PV.[18] In both AK23- and mcPV-IgG-treated samples, increased numbers of desmosomes with lost or altered keratin insertion were detectable, which was ameliorated in samples pretreated with the p38MAPK inhibitor (Fig. 2e, f). We observed split desmosomes in all mcPV-IgG-treated and to lesser extents in all AK23-injected samples (Fig. 2g), but not when SB202190 was pre-applied (not shown). Furthermore, no split desmosomes were observed following mdPV-IgG injection. We also noticed double-membrane structures in 60% of the samples treated with mcPV-IgG (Fig. 2h) that were absent in biopsies incubated with AK23 or mdPV-IgG as well as in samples pretreated with the p38MAPK inhibitor (not shown). The double-membrane structures show a juxtaposition of the plasma membranes of adjacent cells in various shapes including linear or circular structures and are believed to reflect the ultrastructural correlate of an endocytic process.[10]

Discussion

Our data demonstrate that inhibition of p38MAPK is protective against PV-IgG-induced blistering in human skin, similar to a previous study in which an IgG fraction containing only Dsg3 antibodies together with exfoliative toxin A was used.[19] Interestingly, blistering in AK23-injected human skin was absent, which is in contrast to our study showing shear–stress-induced blistering in neonatal Balb/c mice induced by AK23.[12] Possibly, species-specific differences in Dsg expression may account for this observation, which would underscore human skin as the best model for investigation of antibody-dependent mechanisms. Therefore, one goal of the study was to define whether ultrastructural alterations known from the lesions of patients with pemphigus were detectable in the *ex vivo* human skin model. This model would allow investigating the mechanisms known to be involved in pemphigus pathogenesis and to conclude which ultrastructural hallmarks can be allocated to specific autoantibodies and/or signalling pathways, respectively.



	Blister formation	Desmosome number ↓	Desmosome size ↓	Inter-desmosomal widening	Keratin uncoupling	Split desmosomes	Double membrane structures
AK23	–	–	+	+	+	+	–
mcPV-IgG	+	+	+	+	+	+	+
mdPV-IgG	–	–	–	ND	ND	–	–

Fig 2. Ultrastructural alterations revealed by transmission electron microscopy. (a) Shows qualitative changes that were quantified for desmosome number in (b) (n = 3–4 body donors); desmosome length in (c) (n = 3–4 body donors) and interdesmosomal widening in (d) (n = 3 body donors). Bar = 0·5 µm for upper panel and 0·25 µm for lower panel. Keratin insertion (arrows) as shown in (e) was evaluated in (f) (n = 3 body donors). Bar = 0·25 µm. (g) Depicts formation of desmosome splits (arrows, bar = 1 µm) and (h) of double-membrane structures (arrows) for samples incubated with mcPV-IgG (bar = 0·25 µm). (i) Table summarizes morphological alterations (*P < 0·05 vs. control, #P < 0·05 vs. AK23 or mcPV-IgG, respectively). AK23, pathogenic monoclonal Dsg3 antibody derived from a pemphigus mouse model; mcPV, mucocutaneous pemphigus vulgaris; mdPV, mucosal-dominant PV.

This is important because a plethora of signalling pathways is associated with pemphigus skin blistering but the mechanisms are only partially understood. The elucidation of possible connections of these pathways and the integration into known mechanisms of pemphigus pathogenesis such as steric hindrance of Dsg3 interaction are tasks for future

pemphigus research and human skin injections may serve as a highly relevant model here.[20]

We observed the hallmarks of PV by injection of mcPV-IgG in healthy human skin explants which were ameliorated through p38MAPK inhibition (Fig. 2i). Some of the ultrastructural hallmarks of skin of patients with PV such as reduction of desmosome size, interdesmosomal widening, split desmosome formation and keratin uncoupling were to some extent also present in AK23-injected samples. In another study in human skin explant injections,[19] AK23 did not induce ultrastructural changes of desmosomes using a lower antibody amount compared with our study, indicating that these effects may be induced in a dose-dependent manner. In addition, here we applied defined shear stress to the skin samples to better mimic the patient situation, in which the skin is constantly challenged by tension. As it was also shown that shear–stress-induced (i.e. Nikolsky-positive) blistering may be associated with p38MAPK signalling[21] and the effects of AK23 were ameliorated by p38MAPK inhibition, at least some of the ultrastructural changes in response to AK23 injection such as desmosome splitting may be attributed to the application of shear stress. Nevertheless, in contrast to mcPV-IgG, blister formation and reduction of desmosome number but also formation of double-membrane structures were absent following AK23 injections. In line with ultrastructural studies from patients suffering from mucosal PV,[17] mdPV-IgG injection did not induce ultrastructural changes and led to desmosome numbers comparable with controls. These data indicate that at least some ultrastructural changes in the epidermis can be induced by high amounts of a pathogenic monoclonal anti-Dsg3 antibody, whereas others changes such as the reduction in desmosome number require the presence of anti-Dsg1 autoantibodies.

This variation in degree of pathogenicity may also be attributed to specific responses to the monoclonal antibody AK23 vs. polyclonal PV-IgG, the latter of which may potentiate the effect of Dsg-specific autoantibodies.[22] Our ultrastructural analysis showed that inhibition of p38MAPK ameliorates the reduction of desmosome number and size as well as uncoupling of keratin filaments. Therefore, we conclude that inhibition of p38MAPK signalling is sufficient to abrogate skin blistering, which suggests that p38MAPK contributes to blister formation via reduction of the number and size of desmosomes and modulation of the keratin filament cytoskeleton. Because p38MAPK inhibition only slightly ameliorated AK23- and PV-IgG-induced interdesmosomal widening, this may indicate that other mechanisms such as steric hindrance of Dsg3 interactions independent from signalling contribute to this phenomenon.

Quantitative electron microscopy is very extensive and thus evaluation of a higher number of autoantibodies from patients' sera is not feasible using this approach, especially when every repetition is done in skin from a different body donor. Nevertheless, it is possible that autoantibody fractions from other patients may induce different alterations of desmosomal ultrastructure that have not been observed here or were described in the recent study using nanotomy of patients' skin.[10] Together, our study is a first important step showing that a central pathway in pemphigus pathogenesis such as p38MAPK is associated with alterations of desmosome morphology in human skin.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig S1. Dissociation assays and secondary antibody controls: (a) Mucosal-dominant pemphigus vulgaris-IgG and AK23 incubation on HaCaT keratinocyte monolayers demonstrated the efficacy of these autoantibody fractions ($n = 3$). (b) Binding of the autoantibody fractions to the epidermis was ensured by staining with goat-antimouse (gam-Cy3) and goat-antihuman (gah-Cy3) antibodies. Bar = 50 μm; $n = 3–4$ body donors.

2.2 A new ex vivo human oral mucosa model reveals that p38MAPK inhibition is not effective in preventing autoantibody-induced mucosal blistering in pemphigus.

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A new *ex vivo* human oral mucosa model reveals that p38MAPK inhibition is not effective in preventing autoantibody-induced mucosal blistering in pemphigus

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Summary

Background Pemphigus vulgaris (PV) is an autoimmune disease characterized by blister formation in the epidermis and oral mucosa due to loss of keratinocyte cohesion. Autoantibodies present in patients with PV (PV-IgG) are known to primarily target desmoglein (Dsg)1 and Dsg3 in desmosomes. The mucosal-dominant subtype of PV (mdPV) is caused by PV-IgG autoantibodies against the cadherin-type adhesion molecule Dsg3. p38 mitogen-activated protein kinase (p38MAPK) signalling has been characterized as an important pathway downstream of PV-IgG binding and its inhibition is protective in *ex vivo* human skin. However, the role of p38MAPK signalling in mdPV is unknown as no experimental model has been available.

Objectives To establish a human *ex vivo* oral mucosa culture, and evaluate the p38MAPK dependency of blister formation and of ultrastructural alterations of desmosomes induced by mdPV-IgG.

Methods Human labial mucosa was injected with mdPV-IgG as well as AK23, a pathogenic mouse monoclonal Dsg3 antibody, in the presence or absence of p38MAPK inhibitors. Viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and apoptosis by terminal deoxynucleotidyl transferase dUTP nick-end labelling assay. Blister score was determined following haematoxylin and eosin staining and Dsg3 distribution by immunostaining. Samples were processed for transmission electron microscopy to analyse desmosome ultrastructure.

Results Both AK23 and mdPV-IgG induced blisters and caused reduction in desmosome size and number in labial mucosa. Inhibition of p38MAPK was not effective in preventing these alterations.

Conclusions In contrast with human epidermis, PV-IgG and AK23 induce blisters and desmosome ultrastructural changes in labial mucosa via a mechanism not dependent on p38MAPK.

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Conflicts of interest
None to declare.

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What's already known about this topic?

- Pemphigus vulgaris IgG (PV-IgG) induces blistering as well as a reduction in desmosome number and size mediated by p38 mitogen-activated protein kinase (p38MAPK) signalling in *ex vivo* human skin.

What does this study add?

- This study establishes a new human *ex vivo* mucosa model to test pathomechanisms mediated by PV-IgG.

- The study demonstrates that both AK23 and mucosal-dominant PV induce blisters and associated ultrastructural changes in labial mucosa via a mechanism not dependent on p38MAPK signalling.

What is the translational message?

- This study highlights the respective tissue-specific responses of oral mucosa and skin related to PV pathogenesis, similar to the patient situation.

Pemphigus vulgaris (PV) represents an autoimmune disease characterized by suprabasal blister formation due to loss of keratinocyte cohesion in stratified epithelia such as skin and oral mucosa that are subjected to substantial shear forces.[1–3] In line with this, it was demonstrated that desmosomes experience tension when keratinocytes are exposed to mechanical strain.[4] Autoantibodies present in patients with PV (PV-IgG) are known to primarily target desmoglein (Dsg)1 and Dsg3, the cadherin-type adhesion molecules in desmosomes.[5] The mucosal-dominant subtype of PV (mdPV) is believed to be caused primarily by anti-Dsg3 antibodies, whereas the mucocutaneous type (mcPV) is caused by autoantibodies targeting both Dsg1 and 3.[2,6]

The most relevant mechanisms by which pemphigus autoantibodies induce cell dissociation have been attributed to direct inhibition of Dsg interaction and altered signalling leading to disturbed desmosome turnover, and uncoupling from the keratin filament cytoskeleton.[7,8] p38 mitogen-activated protein kinase (p38MAPK) signalling was characterized as one of the important pathways downstream of PV-IgG antibody binding; pharmacological inhibition of this molecule has been shown to prevent cell detachment *in vitro* and blistering in animal models.[9,10] Recently, it was reported that p38MAPK inhibition was effective in attenuating blister formation, as well as in reducing desmosome size and number in human epidermis after PV-IgG injection into cultured skin explants.[11] These data indicate that desmosomes serve as signalling hubs to integrate cell adhesion with cellular signalling pathways.[12]

However, it is unclear at present whether signalling pathway modulation is a treatment option in pemphigus, in particular whether inhibition of p38MAPK is feasible in patients; thus, data from experimental models on this approach are of clinical relevance and remain to be determined. A clinical study using the p38MAPK inhibitor KC706 in an open-label trial with 15 patients with PV was not successful.[13]

Although mucosal biopsies from patients with mdPV have been characterized ultrastructurally,[14] there are no data available to date characterizing the mechanisms by which pathogenic pemphigus autoantibodies targeting Dsg3 cause erosions in the lining epithelium of the oral cavity. This is at least in part because studies in mucosa have been possible so far only in animal models.[15] Moreover, although a variety of cell culture models of mucosal barriers exist, there is no real standard so far.[16] Ultrastructural analysis of a human oral mucosa model would be desirable, especially as the relevance of Dsg3 for desmosomal integrity was proposed to be different between mice and men.[17]

Hence, in this study we present a novel *ex vivo* model in which we characterized blister formation and ultrastructural alterations of desmosome morphology in the inner lining of the labial mucosa to test the relevance of p38MAPK after injection of IgG from a patient with mdPV, in comparison with the monoclonal anti-Dsg3 antibody AK23, which was derived from a pemphigus mouse model.[18]

Materials and methods

Tissue culture

Mucosa biopsies from the inner lining of the lips were harvested from body donors with no history of oral lesions at the Institute of Anatomy and Cell Biology, Ludwig-Maximilian-Universität, München, Germany. Written informed consent was obtained from body donors for use of tissue samples in research. Only those bodies arriving within 12 h after decease were considered for the study.

Patient biopsies were obtained from volunteers not suffering from pemphigus by punch biopsy during clinical diagnosis. Ethical approval for use of patient biopsies in research was given by the ethics committee of the University of Lübeck, Germany (file reference, 12-178).

A strip of mucosa, approximately 2 × 6 cm, was gently excised from the lower as well as upper lip, and fat was subsequently stripped off. Eventually, the underlying muscle tissue was sliced off until only a thin sheet remained to provide space for intramucosal injections. The tissue sample was divided into 2 × 2cm pieces for injection of pemphigus autoantibodies and the p38MAPK inhibitor used in the study. A larger piece of the specimen was required as the tissue is very delicate, and hence areas grasped with tweezers were carefully removed to avoid any damage incurred from handling (Fig. S1a; see Supporting Information).

A 30G syringe was allowed to pass through the mucosa sample along its long axis (Fig S1b). Then, either 50 µL of the pathogenic monoclonal Dsg3 pemphigus autoantibody AK23 (200 µg per specimen) or 50 µL of mdPV-IgG was injected into the sample. mdPV-IgG contained antibodies against Dsg3 only (enzyme-linked immunosorbent assay score: 163 U mL^{-1}). Controls were injected with IgG from a healthy volunteer. In case of p38MAPK inhibition, samples were treated with 50 µL

of 30, 60 and 120 μmol L^{-1} SB202190 (Merck, Darmstadt, Germany), dissolved in dimethyl sulfoxide (DMSO) 1 : 100, 2 : 100 and 4 : 100 in phosphate-buffered saline (PBS); or 30 μmol L^{-1} SB203580 1 h prior to antibody incubation. Additionally, all other samples received 50 μmol L^{-1} of a respective DMSO in PBS vehicle control corresponding to the concentration in inhibitor samples. Injection areas were marked (Fig. S1c) and samples were allowed to float on Dulbecco's modified Eagle medium with the mucosa facing upwards without any additional support, then incubated at 37 °C and 5% CO_2 for indicated time periods (Fig. S1d). Viability of the mucosal tissue sample was confirmed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for all conditions before and after incubation. Negative control samples were heat inactivated at 65 °C for 30 min (Fig. S1a). Each condition was evaluated in biopsies from at least three different body donors. The number of independent body donors is indicated in the figures of each evaluation. No shear stress was applied unless resulting from sample handling. Finally, specimens were cut into two parts and processed either for haematoxylin and eosin (H&E) and immunostaining or electron microscopy analyses.

Histology and immunostaining

Samples for histological analysis were embedded in Leica Tissue Freezing Medium (Leica Biosystems, Nußloch, Germany), and serial sections of 7 μm thickness were made using a cryostat microtome (Microtome Cryostat HM 500 OM; MICROM International GmbH, Walldorf, Germany) until the entire sample was processed. Sections were subjected to H&E staining according to standard procedures and mounted in DPX Mountant for histology (Sigma-Aldrich, St Louis, MO, U.S.A.). For phenotypic analysis, every 10th section was considered. Images were taken on a Leica DMi8 Microscope (Leica Microsystems, Wetzlar, Germany).

Details on performance of immunostainings can be found in the online materials and methods (File S1; see Supporting Information).

Electron microscopy

Electron microscopy was performed as described previously.[11] A detailed description can be found in the online materials and methods (File S1).

Terminal deoxynucleotidyl transferase dUTP nick-end labelling assay

A DeadEnd™ Fluorometric TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling) System (cat. no. G3250; Promega, Madison, WI, U.S.A.) was purchased and assays performed as instructed by the manual. A detailed description can be found in the online materials and methods (File S1). Skin explants were used as a biological control, whereas DNase I-treated sections served as technical positive controls. Signal in the TUNEL panel colocalizing with 4′,6-diamidino-2-phenylindole staining indicated apoptosis.

Tissue lysis, gel electrophoresis and Western blot analysis

Injected mucosa samples were subjected to cell lysis and Western blotting to evaluate signalling pathway activation. A detailed description of the experimental procedure can be found in the online materials and methods (File S1). Dsg3 served as a loading control to ensure similar amounts of keratinocytes were present in all samples.

Quantification of autoantibody effects

H&E-stained serial sections were evaluated for blister formation and extent, similarly to previous studies.[19] Cleft lengths were scored in whole numbers ranging from 0 to 4 in accordance with the following criteria: 0 (no blister), and cleft covering 1–25%, 26–50%, 51–75%, 76–100% of section length as 1, 2, 3 or 4, respectively.

Micrographs were taken from basal and suprabasal mucosal layers; 15–25 micrographs were randomly selected from each body donor and analysed at 4000 × magnification. In total, 200–300 desmosomes for each condition were analysed using *ImageJ* software (https://imagej.nih.gov/). Desmosomes in the basolateral and suprabasal areas were counted as previously discussed[11] and the result was expressed as number of desmosomes per μm of membrane length. This helps to account for the variability in the number of cells present in each micrograph. Desmosome size was measured as the distance between the two ends of a desmosome and expressed in μm.

Statistical analysis

From the data points acquired, a mean value per body donor was considered for statistical analysis; error bars represent SEM. Comparison of data was done using one-way ANOVA followed by Bonferroni post-hoc test (for Gaussian-distributed samples) using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, U.S.A.). Statistical significance was assumed when $P < 0{\cdot}05$.

Results

Validation of a new human *ex vivo* mucosa model

In principle, the labial mucosa model is similar to the original human skin model[20] we used to study the role of p38MAPK in PV-IgG induced skin blistering.[11] Firstly, we evaluated the distribution of Dsg and desmocollin (Dsc) isoforms in mucosa controls by immunostaining (Fig. 1a). Staining was strongest for Dsg3, which was homogeneously present throughout the oral mucosa. Dsg1 immunostaining was less pronounced but additionally present in all mucosal layers except the basal layer. In contrast, Dsg2 staining was very faint and detectable in the basal layer only where Dsc3 was also expressed most strongly. However, Dsc1 staining was absent.

Next, we tested mucosa for viability and apoptosis. For the TUNEL assay (Fig. 1b) we used *ex vivo* skin cultured for 24 h in comparison and included a positive control from the TUNEL kit. In both skin and mucosa, no signs for apoptosis were observed. Viability of mucosa tissue was controlled by MTT following incubation with autoantibodies in the presence or absence of p38MAPK inhibitor SB202190 (Fig. 1c). As revealed by the heat-inactivated negative control, mucosa samples were viable under all conditions after 24 h of incubation. Antibody deposition was controlled by direct immunostaining using cy3–goat anti-human conjugates (data not shown).

Effect of p38 mitogen-activated protein kinase inhibition on pemphigus vulgaris IgG- and AK23-induced blister formation in human oral mucosa

Next, we performed H&E staining on cryosections of samples for all experimental conditions (Fig. 2a). In controls, mucosa was largely intact and closely resembled patient biopsies directly frozen after explantation, indicating hole-like structures to be cutting artefacts (Fig. S2a; see Supporting Information). We assessed slices for the presence or absence of blisters and quantified the extent of blister formation using a scoring scale ranging from 0 to 4. In all samples, blister formation was evident on injection with AK23 (Fig. 2b, d) as well as with mdPV-IgG (Fig. 2c, d), whereas control mucosa explants showed no blistering (Fig. 2a, d). Similarly to the previous study in *ex vivo* human epidermis,[11] we tested the efficacy of p38MAPK inhibition in human mucosa. However, mucosa samples pretreated with the specific p38MAPK inhibitor SB202190 showed the same extent of blister formation as those treated with AK23 or mdPV-IgG alone (Fig. 2b–d). This was confirmed for mdPV-IgG by using higher concentrations of SB202190 (60 μmol L^{-1} and 120 μmol L^{-1}, respectively) (Fig. S2b, c), as well as by application of another p38MAPK inhibitor, SB203580, at a concentration of 30 μmol L^{-1} (Fig. S2c, d), the latter of which was effective in blocking loss of keratinocyte cohesion previously *in vitro*.[21]

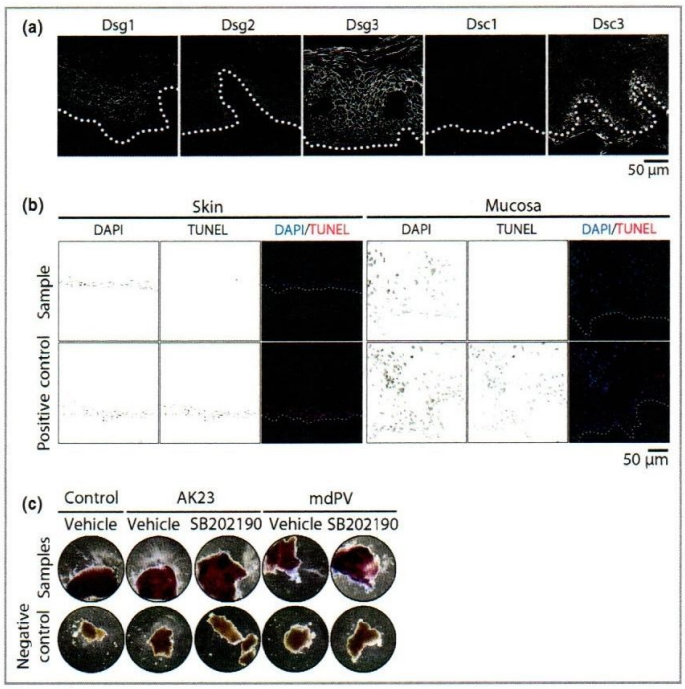

Fig 1. Validation of *ex vivo* model by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and characterization. (a) Immunostaining of desmosomal proteins to characterize mucosal explants (n = 3). Desmoglein (Dsg)3 shows a higher intensity staining across all layers, whereas Dsg1 shows a lower intensity staining that was missing in basal and suprabasal keratinocytes. In contrast, Dsg2 and desmocollin (Dsc)1 were almost absent, whereas Dsc3 was detectable by immunostaining in the basal and suprabasal layer only (n = 3). (b) Representative immunostaining of terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay showing skin and mucosa samples treated with and without DNase I as positive control. Apoptosis was not detectable in samples indicated by negative TUNEL staining (n = 3). Dotted lines represent basement membrane. (c) A positive test for viability was observed as viable tissues induced a colour change from yellow to blue detecting mitochondrial activity; boiled negative control samples did not yield any colour change. DAPI, 4′,6-diamidino-2-phenylindole; mdPV, mucosal-dominant pemphigus vulgaris.

Additionally, Western blot analysis was carried out after mucosa was incubated in the presence or absence of AK23 for 30 min, to evaluate phosphorylation of MAPK-activated protein kinase 2 (MK2), a downstream target of p38MAPK shown to be relevant for blister formation in pemphigus (Fig. 2e).[22] Controls exhibited a low baseline activity of MK2, whereas incubation with AK23 resulted in pronounced MK2 phosphorylation. Incubation of SB202190 (30 μmol L^{-1}) for 1 h before application of AK23 reduced MK2 activity to control levels, confirming that this concentration is effective in blocking p38MAPK-dependent signalling events.

To evaluate the effects of autoantibody fractions on desmosome integrity under the different conditions, we used immunostaining against Dsg3 as a first step. Immunostaining revealed fragmentation of Dsg3 staining in response to autoantibody treatment predominantly at the blister floor, which was not rescued by SB202190 (Fig. 2f).

Ultrastructural analysis of autoantibody-treated mucosa

Finally, we employed transmission electron microscopy to evaluate ultrastructural alterations in mucosal desmosomes in

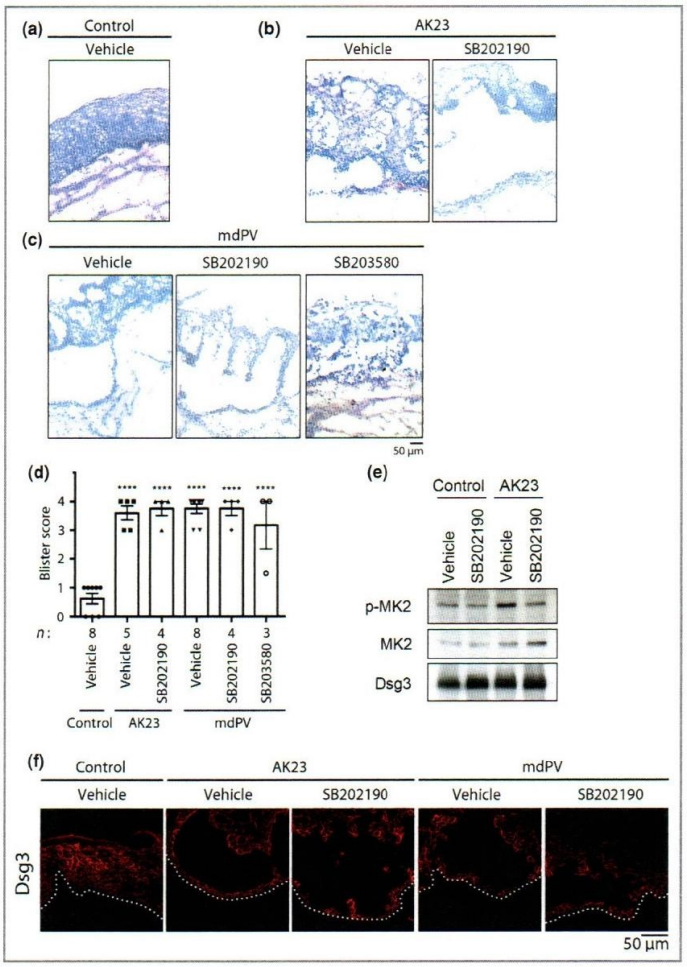

Fig 2. Histological changes in healthy human labial mucosa incubated with pemphigus vulgaris (PV) autoantibodies *ex vivo*. AK23 antibodies or mucosal-dominant PV (mdPV)-IgG were applied for 24 h in the absence or presence of p38 mitogen-activated protein kinase inhibitors SB202190 or SB203580, preincubated for 1 h. (a–c) Representative haematoxylin and eosin stainings of cryosections from aforementioned samples. (d) Corresponding blister scores (n = 4–6 of independent body donors). ****$P \leq 0{\cdot}0001$ vs. control. (e) Mucosa samples injected and incubated for 30 min with AK23 in presence or absence of SB202190 to evaluate phosphorylation of MAPK-activated protein kinase 2 (p-MK2 vs. MK2) in Western blot analysis (representative image, n = 3). Desmoglein (Dsg)3 served as loading control. (f) Corresponding immunostaining for Dsg3 of samples treated with SB202190 (n = 4). Dotted lines indicate basement membrane.

the basal layer. Interestingly, desmosomes in control tissue lacked a dense midline. Moreover, in all samples treated with AK23 or mdPV-IgG with or without SB202190, the desmosome number was significantly reduced by about 60% as compared with the control (Fig. 3a, b). In controls, mean (± SD) desmosome length was 289·92 ± 20·03 nm. Following incubation with AK23 or mdPV-IgG, there was a significant reduction in desmosome length by about 22% relative to controls (Fig. 3c). The p38MAPK-specific inhibitor SB202190 was not effective in modulating reduction of desmosome size.

Discussion

Here, we successfully established a new *ex vivo* model to study the mechanisms involved in mucosal pemphigus pathogenesis in human tissue. Histological analysis showed that the model reproduces the situation in patients, with mdPV-IgG and AK23 resulting in suprabasal blisters with reduced desmosome number and size. Additionally, AK23 is able to activate the p38MAPK signalling pathway in human oral mucosa; nevertheless, in contrast with human epidermis, blister formation and ultrastructural alterations of desmosomes are independent of p38MAPK in oral mucosa.

Pemphigus pathogenesis is more complex than assumed initially and several mechanisms, including direct inhibition of Dsg3 binding and signalling pathway modulation, are known to be important.[17,23] Recently, it was proposed that different signalling pathways may correlate with antibody profiles and thus may contribute to different clinical phenotypes in pemphigus.[24] To test this hypothesis further, establishment of a human mucosa model was required. The model reported here appears feasible, as cultured oral mucosa proved to be viable without signs of apoptosis. In contrast with their effects on mucosa, neither AK23 nor mdPV-IgG were effective in inducing blisters in human epidermis,[11] indicating that these two human *ex vivo* models for epidermis and mucosa reflect the situation in patients.[1,2] Additionally, the composition of desmosomal cadherins in explanted mucosa showed results comparable with previous reports in the literature where mucosa *in situ* was studied by immunostaining as well as by quantitative polymerase chain

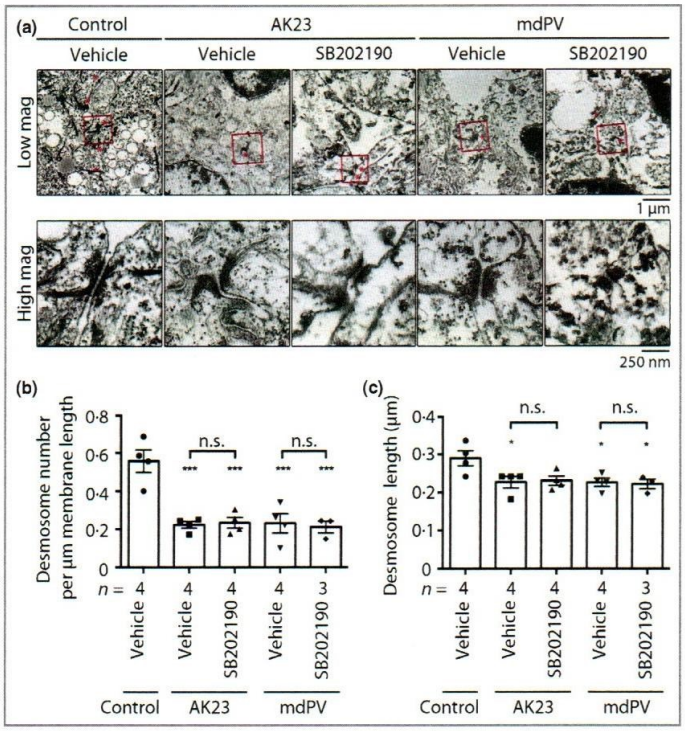

Fig 3. Changes in desmosome ultrastructure as revealed by transmission electron microscope. (a) Representative electron micrographs showing desmosomal ultrastructure in *ex vivo* mucosa after 24 h of IgG treatment. Asterisks mark desmosomes in upper panel, and lower panel magnifies desmosomes for size comparisons (n = 3–4). (b) Quantitative analysis for changes in desmosome number (n = 3–4 independent body donors). (c) Quantification of desmosome length (n = 3–4 independent body donors). For each body donor at least 20 images were analysed for desmosome number and every desmosome size was evaluated. mdPV, mucosal-dominant pemphigus vulgaris; mag, magnification. ***$P \leq 0{\cdot}001$ vs. control; *$P \leq 0{\cdot}05$ vs. control; n.s., not significant.

reaction.[25,26] Our data support Dsg3 as the predominant isoform of the desmosomal cadherins throughout all layers in oral mucosa whereas Dsg1 staining intensity was lower and absent in the basal layer.

Beyond establishment of a new experimental model, our data provide a first insight into the role of signalling mechanisms in mucosal PV. Firstly, the data underscore the importance of autoantibodies targeting Dsg3 for mucosal erosion formation because the monoclonal anti-Dsg3 antibody AK23 was similarly effective in inducing blisters to mdPV-IgG containing a variety of autoantibodies beside anti-Dsg3. In contrast, with the same antibodies, no blistering was induced in human epidermis, compatible with the notion that autoantibodies against Dsg1 are required for epidermal blistering as well.[8,11,27] However, in contrast with human epidermis, the novel data indicate that p38MAPK is not required for loss of cell cohesion in oral mucosa because inhibition of p38MAPK did not prevent autoantibody-induced blister formation. Importantly, under the conditions used, the p38MAPK downstream target MK2 was activated after incubation with AK23 and this phenomenon was blocked by SB202190. Together with the observations that Dsg3 is the predominant desmosomal cadherin in the basal and suprabasal layer of oral mucosa, it is possible that loss of Dsg3 interaction through autoantibody-induced direct inhibition alone might be sufficient to induce blisters in human oral epithelia.[28,29] For AK23, this has been proposed for epidermis as well.[30] In this context, it may be relevant that we observed mucosal desmosomes to be smaller and lacking a dense midline, when compared with epidermis. Previous ultrastructural studies of normal human buccal epithelia also revealed reduced keratin formation in the basal mucosa.[31] Other studies in mice skin showed a positive correlation between loss of keratins and a largely reduced size and number of desmosomes,[32] which underscores the role of keratins in maintaining intercellular adhesion and desmosomal integrity.[33] All of these observations suggest that compared with epidermis, mucosal desmosomes may be less mature and more fragile, and therefore mucosa may be more prone to blistering caused by pathogenic mechanisms such as direct inhibition. However, it cannot be ruled out that signalling pathways other than p38MAPK are critical for autoantibody-induced loss of keratinocyte cohesion in human oral mucosa as well. This requires more detailed analyses, which are clearly beyond the scope of this study.

Finally, we hope that the new mucosa *ex vivo* model may be helpful for further investigations on pemphigus pathogenesis and highlight differences between mucosal and epidermal tissue, as well as the function of different desmosomal proteins.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig S1. *Ex vivo* human mucosa model.
Fig S2. Validation of inhibitor concentrations.
File S1. Supplementary Material and Methods.

2 Discussion

2.1 Mucocutaneous PV-IgG but not mucosal-dominant PV-IgG were effective to induce blisters in *ex vivo* human skin

p38MAPK has been central in researches involving pemphigus pathogenesis. A large body of experimental data has been generated regarding the role of p38MAPK signaling in mediating blister formation and other hallmarks of pemphigus (Spindler et al., 2018, Spindler and Waschke, 2018). Especially, pharmacological inhibition of this molecule was shown to avert cell dissociation *in vitro* and blister formation in mouse models (Berkowitz et al., 2005; 2006). Skin biopsies from pemphigus patients have been studied mainly pertaining to layer and tissue-specific binding of IgG from various PV phenotypes (Shimizu et al., 1995; Amagai et al., 1996). Similarly PV-IgG-triggered ultrastructural changes in desmosomes have been characterized using patient samples (Sokol et al., 2015; Stahley et al., 2016). However, no sufficient data were available to explain the mechanisms orchestrating these ultrastructural changes in human skin. To this end, we used human skin biopsies obtained from body donors to evaluate the p38MAPK dependency of blister formation and other hallmarks after treatment of healthy skin samples with IgG purified from pemphigus patient serum.

From histological analysis, only those skin samples treated with mcPV-IgG developed blisters but not those treated with either mdPV-IgG or AK23 (Egu et al., 2017). This is expected because only IgG containing both anti-Dsg3 and anti-Dsg1 autoantibodies are sufficient to induce suprabasal blisters which recapitulated the situation in PV patients. Although AK23 was potent enough to induce blisters in mouse skin (Spindler et al., 2013), it necessitated the application of exfoliative toxin together with AK23 to effect blister in human skin explants (Saito et al., 2012).

On the other hand, immunostaining of PV-IgG-treated samples showed a fragmented staining pattern of Dsg3 along the blister floor and roof compared to the smooth pattern in controls

which indicates that Dsg3 in desmosomes were depleted owing to PV-IgG binding (Aoyama et al., 1999; Saito et al., 2012).

2.2 Inhibition of p38MAPK prevented blister formation in epidermis

In our study, inhibition of p38MAPK using SB202190 was sufficient to avert PV-IgG-induced blister formation in skin explants similar to other studies in murine models (Berkowitz et al., 2008; Saito et al., 2012) and rescued Dsg3 depletion from membranes in *ex vivo* human skin cultures (Egu et al., 2017). This highlights the essential role of p38MAPK as a key regulator in bister formation in human skin.

2.3 p38MAPK inhibition preserved desmosome ultrastructure in skin

Our ultrastructural analysis shows that alterations in desmosomal structure which are ultrastructural hallmarks characteristic of pemphigus, including shortening and splitting up of desmosomes as well as alterations in keratin association with desmosomal plaques, have been reproduced in our model. Accordingly both desmosome number and size have been significantly reduced in PV-IgG-treated samples, and this was sufficiently prevented in those pretreated with the inhibitor SB202190. Although AK23 was able to cause a significant reduction in desmosome size but not number, no blisters developed in the respective samples. It means that blister formation which was evident in histological sections in samples treated with mcPV-IgG corresponded with reduction in desmosome number but not desmosome size in ultrastructural evaluation (Egu et al., 2017). This is in agreement with a study in which ultrastructural analysis of lesioned pemphigus patients' skin asserted that blister formation was correlated to loss of desmosomes (van der Wier et al., 2012; Sokol et al., 2015). Moreover, we observed a progressive reduction in size, splitting up and absence of desmosomes in the vicinity of blisters, which implies that decrease in desmosome size may be among the initial episodes leading to loss of desmosomes and eventually resulting in acantholysis (van der Wier et al., 2012). Although split desmosomes, intercellular widening

and keratin filament dis-association from desmosomes were evident in both mcPV-IgG- and AK23-injected skin samples, it occurred to a lower extent in the latter. Tissue and cell specific response to autoantibodies present in pemphigus patient serum was demonstrated by a study in which mdPV-IgG, when injected to a neonatal mouse, showed no pathogenicity whereas mcPV-IgG was pathogenic (Ding et al., 1997). Therefore, the absence of blister in skin samples injected with AK23 may be understood in terms of species as well as tissue specificity in autoimmune response (Ding et al., 1997) or compensatory role of Dsg1 counteracting the dys-cohesive effects impacted by anti-Dsg3 autoantibodies (Mahoney et al., 1999) or varying cellular responses to monoclonal vs polyclonal antibodies (Saito et al., 2012).

After incubation, we applied mechanical shear force (Nikolsky positive) to the samples to reproduce similar shear stress encountered by the patients' skin because this was correlated with activation of p38MAPK signaling events (Mao et al., 2011). Interestingly, specific inhibition of p38MAPK blunted reduction in desmosome number and size as well as preserved keratin association with desmosomal plaque in both PV-IgG- and AK23-treated samples (Egu et al., 2017). We also observed intercellular widening associated with reduced and/or split desmosomes in the interface of adjacent basal cells as well as in that of basal and suprabasal cells, marking the initial pathological events leading to acantholysis as suggested occurring in PF (van der Wier et al., 2012) and PV (Takahashi et al., 1985; Diercks et al., 2009) although intercellular widening has been suggested not been a pre-requisite for acantholysis (Sokol et al., 2015). Split desmosomes were recognized at the edge of blisters (Shimizu et al., 2002; 2004; Wang et al., 2009; van der Wier et al., 2012; Sokol et al., 2015; Stahley et al., 2016) using electron microscopy and SIM. However, there are divergent opinions on the mechanism of their occurrence. Some postulate that splitting of desmosomes occurs when PV-IgG autoantibodies are interposed between interacting desmogleins thereby

weakening adhesion (steric hindrance), followed by shear forces (Shimizu et al., 2002; 2004; Wang et al., 2009; Stahley et al., 2016) or outside-in-signaling (Diercks et al., 2009). In *in vivo* experiments in neonatal mouse, it has been shown that desmosomes split up before they are eventually internalized (Takahashi et al., 1985). This has been suggested to be an initial step leading to acantholysis (Takahashi et al., 1985; Wang et al., 2009) although this argument has been challenged (Aoyama et al., 2010). In our study, however, split desmosomes were significantly reduced in size which may indicate that desmosomal components were depleted in this process, which highlights the involvement of signaling mechanisms (Spindler and Waschke, 2018) that may alter the dynamics of desmosome assembly and disassembly (Stahley et al., 2016).

2.4 p38MAPK inhibition ameliorated PV-IgG-dependent keratin filament dissociation from desmosomal plaques

In our study, a gradient of plaque density was evident in the vicinity of blisters depending on the degree of acantholysis. In addition, retraction of keratins from the plaques was observed similarly to other studies (Takahashi et al., 1985; Wang et al., 2009). The relevance of keratin filaments in cell adhesion was demonstrated by the studies in which absence of keratins has been linked to impaired adhesive function associated with altered desmosome morphology (Kroger et al., 2013; Bar et al., 2014). Recent works with keratin-deficient cells pinpointed that keratins are crucial in regulating intercellular adhesion through stabilizing desmosomes fine tuned by signaling (Loschke et al., 2015; Vielmuth et al., 2018b). Earlier studies revealed that keratins unplug from desmosomes in response to PV-IgG binding in a mechanism dependent on p38MAPK activity (Berkowitz et al., 2005). Moreover, inhibition of p38MAPK abrogated keratin granule formation and dissolution, which represents the disassembly of the keratin filament network (Woll et al., 2007). In light of this, in our *ex vivo* model, preservation of keratin association via inactivation of p38MAPK pathway underscores the crucial role of

keratin filaments in p38MAPK-dependent stabilization of desmosomal contacts (Egu et al., 2017).

2.5 Mucosal-dominant PV-IgG and AK23 were effective in inducing blisters in *ex vivo* human oral mucosa

It has been recently hypothesized that different autoantibody profiles determine the specific clinical phenotype in pemphigus consequently triggering specific signaling pathways (Walter et al., 2017). Besides, since mcPV also involves the mucous membranes mainly associated with the oral cavity, we tested the role of p38MAPK in *ex vivo* mucosa model which was in principle similar to the skin model we used to investigate a similar role in human epidermal explant culture (Egu et al., 2017). As a result, it necessitated establishment of an *ex vivo* human oral mucosa model. Viability tests using MTT and TUNEL assays yielded positive results qualifying the tissues for the intended study. In addition, expression of desmosomal proteins in human oral mucosa matched previous reports in the literature using immunostaining and quantitative PCR (Shirakata et al., 1998; Teh et al., 2011). Accordingly, our data confirmed Dsg3 to be the predominant isoform of the desmosomal cadherins expressed across the entire epithelial layers in oral mucosa, whereas Dsg1 expression was minimal and absent in the basal layer (Egu et al., 2020). Histological examination revealed that samples injected with mdPV-IgG or AK23 developed suprabasal blisters characteristics of mdPV patients (Kasperkiewicz et al., 2017; Pollmann et al., 2018). In contrast, in our skin model mdPV-IgG and AK23 were not sufficient to cause blistering signifying the pathogenic role of anti-Dsg1 autoantibody in deep epidermal and mucosal layer blister formation (Mahoney et al., 1999).

2.6 Inhibition of p38MAPK was not effective to block blister formation in oral mucosa.

In contrast to the epidermis, inhibition of p38MAPK was not effective to blunt the acantholytic effect of both mdPV-IgG and AK23, in which anti-Dsg3 but not anti-Dsg1

autoantibodies were present. Then we tested whether MK2, a major downstream effector of p38MAPK, was activated in response to AK23 activity and whether this activation could be inhibited as well. This yielded a positive result similarly to previous reports in which activation of MK2 was detected in lesional skin of pemphigus patients (Mao et al., 2014). In our present study, however, this inactivation of p38MAPK was not paralleled by abrogation of blistering induced by AK23 which is in agreement with a previous finding in which AK23 was used in combination with exfoliative toxin (Saito et al., 2012). This data demonstrate that the p38MAPK inhibitor used in this study was effective to inhibit autoantibody-induced activation of p38MAPK in mucosa. However, inhibition of this signaling pathway was not sufficient to blunt blistering in mucosa indicating that its relevance for blistering is different for mucosa compared to epidermis (Egu et al., 2020).

2.7 Inhibition of p38MAPK did not preserve desmosome ultrastructure in oral mucosa

In our ultrastructural analyses of mucosa samples, we observed a significant reduction in size and number of desmosomes similarly to other ultrastructural studies in oral mucosa of pemphigus patients (Sokol et al., 2015; Stahley et al., 2016). Because inhibition of p38MAPK was sufficient to block blistering and rescue desmosome ultrastructure in human skin explants, we assumed p38MAPK would play a similar role in desmosome regulation in mucosa. However, unlike in the epidermis, p38MAPK inactivation, which was confirmed by Western blot analysis, didn't avert decrease in desmosome number and size in human labial mucosa explants (Egu et al., 2020).

It has been proposed that p38MAPK is activated in response to disruption of cell contacts caused by PV-IgG in Nikolsky positive skin, and thereby resulting in depletion of desmosomal proteins (Mao et al., 2011). Thus, it can be understood why inhibition of p38MAPK did only attenuate the increased skin fragility but didn't block blister formation

(Mao et al., 2011) which reflects the situation in our mucosa model. Besides, inactivation of p38MAPK did not prevent blistering induced by AK23 as well similar to other studies (Saito et al., 2012). On the other hand, the oral cavity is exposed to an incessant environmental stress including moisture, chemicals, heat and abrasion (Presland and Jurevic, 2002) which necessitates a rapid healing process (Hashimoto et al., 1966). As a result, oral epithelial cells have a faster turnover (Donetti et al., 2005) leading to the formation of less differentiated organelles (Hashimoto et al., 1966). In line of this, we observed small desmosomes lacking a dense mid-line, characteristic of mature desmosomes, in the basal and suprabasal layers of labial mucosa which might have contributed to increased tissue fragility. Furthermore, a previous ultrastructural study of healthy human buccal mucosa showed less number of keratin filaments along with reduced desmosome size in the basal keratinocytes as compared to the skin (Hashimoto et al., 1966). Reports from studies of mouse skin also correlated loss of keratin filaments with a largely reduced size and number of desmosomes (Bar et al., 2014), which highlights the essential role of keratins in maintaining intercellular adhesion and desmosome integrity (Vielmuth et al., 2018b). Moreover, Dsg1 is expressed at a lower degree in oral mucosa whereas it is the major desmoglein isoform in the superficial epidermis. In the latter, Dsg1 was detectable in desmosomes tethering the basal and suprabasal cells where the cells enter into a differentiation phase which highlights the essential role of Dsg1 in promoting keratinocyte differentiation (Getsios et al., 2009). In another study, ectopic expression of desmoglein in keratinocyte cell cultures rescued defective differentiation caused by UVB (Johnson et al., 2014). Therefore, absence of Dsg1 in mucosal keratinocytes may limit the potential of the cells to differentiate and may impair the recovery of the cells after PV-IgG-induced desmosome dysfunction. Taken together, the unique nature of the composition of desmosomal proteins in mucosa, the tissue and layer specific properties of signal modulation, and the high turnover rate of the cells might have contributed to the failure

of the mucosa cells to withstand the PV-IgG-induced loss of adhesion after p38MAPK inhibition unlike in epidermis (Egu et al., 2020).

We can hereby speculate that PV-IgG autoantibody binding to desmogleins by direct inhibition of Dsg3 interaction perturbs the interconnection between desmosomes of adjacent cells eventually causing cell dissociation (Fig. 4). Autoantibody binding triggers p38MAPK activation which augments the pathogenesis by further activating downstream cascades thereby causing disturbance of desmosome assembly and disassembly dynamics, ultimately

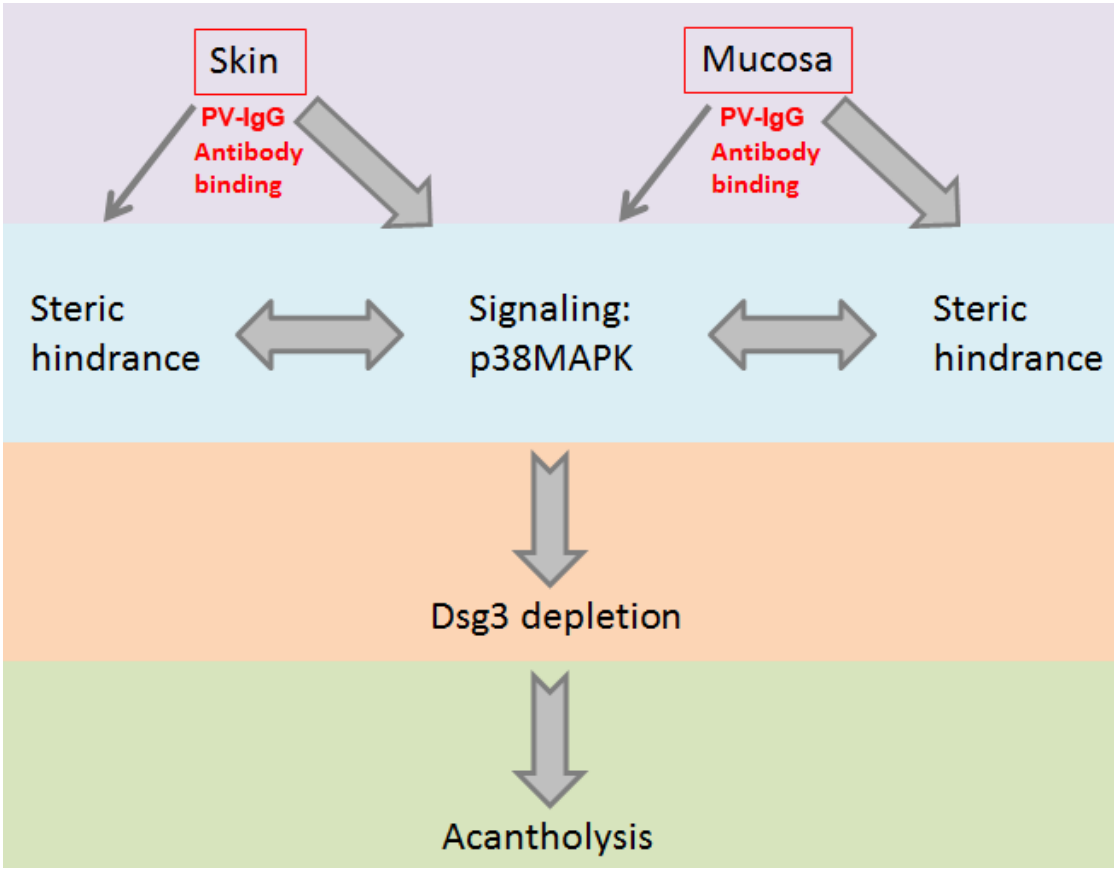

Figure 4. Schematic showing possible pathomechanisms in pemphigus. The underlying pathomechanism appears to be different for epidermis and mucosa. While p38MAPK signaling predominates in epidermis (designated by a thick arrow), steric hindrance (represented by a thin arrow) and other downstream signaling events independent of p38MAPK may orchestrate the processes leading to mucosal acantholysis.

resulting in Dsg3 depletion and endocytosis (Fig. 4). Inhibition of p38MAPK effectively rescues loss of cell cohesion in skin but this is not effective in mucosa due to tissue fragility caused by high cell turnover as well as absence of Dsg1 to promote cell adhesion.

In summary, the use of *ex vivo* models is of paramount importance since it allows assessing the role of various signaling molecules under controlled conditions. Besides, larger specimens could be utilized in these models which would solve the problem related to harvesting a sizable sample from patient biopsies. However, *ex vivo* studies in mucosa are mainly limited to permeability studies regarding drug absorption aimed at identifying most efficient route of drug administration (Caon and Simoes, 2011, Squier, 1991). To this end, we successfully established a novel mucosa *ex vivo* model which will be helpful for further investigation not only of pemphigus pathogenensis but also other mucosa related-diseases.

In conclusion, since cellular responses to the various signaling molecules differ among tissues and between species, it appears to be highly invaluable to evaluate the different signaling pathways and test the potency of the respective inhibitors, characterized in cell cultures and mouse models, in human skin and mucosa as a subsequent step in the development of an innovative and effective therapeutics to pemphigus. In view of this, p38MAPK, being a key regulator in pemphigus pathogenesis, could be one potential therapeutic signaling target as evidenced by its specific inhibition which rescued desmosomes and preserved the desmososome-keratin filament association in our *ex vivo* skin.

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4 Annex

4.1 Abbreviations

Ca2+	calcium ion
Dsc	desmocollin
Dsg	desmoglein
DP	desmoplakin
DMEM	Dulbecco's modified eagle medium
EGFR	epidermal growth factor receptor
ELISA	Enzyme-linked Immunosorbent Assay
EC	extracellular
h	hour
IF	immunofluorescence
Cy3	Indocarbocyanin 3
IgG	immunoglobulin
µl	microliter
µm	micrometer
µg	microgram
µM	micromolar
mm	millimeter
MAPK	mitogen-activated protein kinase
M	Molar [g/mol]
mcPV	mucocutaneous pemphigus vulgaris
mdPV	mucosal dominant pemphigus vulgaris
min	minutes
nm	nanometer
PV	pemphigus vulgaris
PF	pemphigus foliaceus
Pg	plakoglobin
Pkp	plakophilin
Pkc	protein kinase C
SEM	standard error of the mean
Src	rous sarcoma kinase
TEM	transmission electron microscope
U	unit

4.2 Author contribution

Inhibition of p38MAPK signalling prevents epidermal blistering and alterations of desmosome structure induced by pemphigus autoantibodies in human epidermis. Br J Dermatol. 2017 Dec;177(6):1612-1618. doi: 10.1111/bjd.15721. Epub 2017 Nov 14.

This study was planned and designed by Jens Waschke, Volker Spindler and me. I performed all the experiments and data acquisition, interpreted the results except the dispase assay done by Elias Walter. I arranged the figures, and wrote the manuscript. Jens Waschke and Volker Spindler edited the manuscript.

A new ex vivo human oral mucosa model reveals that p38MAPK inhibition is not effective to prevent autoantibody-induced mucosal blistering in pemphigus. Br J Dermatol. 2020;182(4):987-994. doi:10.1111/bjd.18237

This study was planned and designed by Jens Waschke and me. I performed all experimets and data acquisition, interpreted the results except the Western blot analysis and HE and immunostaining of patients' mucosal biopsies done and resulting data interpreted by Anna Sigmund. Elias Walter partly participated in planning and designing the experiment, performed TUNEL assay, arranged the figures and did the statistics. I wrote the manuscript which was edited by Elias Walter and Jens Waschke.

4.3 Affidavit (Eidesstattliche Versicherung)

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Affidavit

Egu, Desalegn Tadesse

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I hereby declare, that the submitted thesis entitled

Role of p38MAPK in ultrastructural alterations of desmosomes in human ex vivo pemphigus model

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

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4.4 Confirmation of congruency between printed and electronic version of the doctoral thesis

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is congruent with the printed version both in content and format.

Munich, 21.10.2019

Place, date

Desalegn Tadesse Egu

Signature doctoral candidate

Congruency of submitted versions

July 2019

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