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Characterization of pathogenic auto-antibodies directed against desmoglein 3 and desmocollin 3 in sera of pemphigus patients

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Abkürzungsverzeichnis

aa	amino acid
Ab	antibody
ABTS	2,2'-azino-di(3-ethylbenzthiazoline-6-sulphonate)
auto-Ab	auto-antibody
APC	antigen preenting cell
CD	cluster of differentiation
COOH-	carboxy-
Dsc	desmocollin
Dsg	desmoglein
Dsg3EC1-5	recombinant protein containing the entire ectodomain (aa 1-566) of desmoglein 3
Dsg3EC1	recombinant protein including aa 1–161 of desmoglein 3
Dsg3EC2	recombinant protein including aa 87–227 of desmoglein 3
Dsg3EC3	recombinant protein including aa 184–349 of desmoglein 3
Dsg3EC4	recombinant protein including aa 313–451of desmoglein 3
Dsg3EC5	recombinant protein including aa 424–566 of desmoglein 3
EC	extracellular subdomain
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immonosorbent assay
Fab	fragment antigen-binding
Fc	fragment crystallizable
His	histidin

HLA	human leukocyte antigen
IB	Immunoblot
IF	Immunofluorescence
Ig	immunoglobulin
IL	interleukin
M	molar
min	minute
ml	milliliter (10^{-3} liter)
mAb	monoclonal antibody
NH_2-	amino-
nm	nanometer (10^{-9} meter)
µl	microliter (10^{-6} liter)
µm	micrometer (10^{-6} meter)
OD	optical density
PBS	phosphate buffered saline
kDa	kilodalton
PF	pemphigus foliaceus
PNP	paraneoplastic pemphigus
PV	pemphigus vulgaris
r	recombinant
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Th	T helper cell
Treg	regulatory T cell

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Summary

Pemphigus vulgaris (PV) represents the most frequent clinical type of the pemphigus group of autoimmune bullous skin disorders. There is substantial evidence that blister formation in pemphigus patients is mediated by auto-antibodies (auto-Abs) targeted against certain desmosomal cadherins, namely desmoglein1 (Dsg1) and desmoglein3 (Dsg3). Several pathogenic epitopes of Dsg3 are located at the amino-(NH_2)-terminal end of the Dsg3 ectodomain, namely the extracellular domain 1 (EC1). On the other hand a great number of pemphigus patients exhibit auto-Abs directed against the more carboxy-(COOH)-terminal epitopes of Dsg3, e.g. within the EC4- and EC5-domain and these domains may play an essential role in maintaining desmosomal adhesion. Some pemphigus patients exhibit additional or solely auto-Abs against other desmosomal cadherins, especially desmocollin 3 (Dsc3). However, the pathogenic relevance of Dsc3-reactive immunoglobulin G (IgG) has not been directly shown. This study aimed to first establish a method to specifically isolate Dsg3-reactive IgG from PV sera and to further investigate their pathogenic capacity using a keratinocyte based *in vitro* assay. This method was further applied to sera of four Japanese patients suffering from atypical pemphigus all of them exhibiting a positive IgG reactivity against Dsc3. Sepharose based affinity chromatochraphy columns coated with recombinant baculovirus produced proteins of the extracellular domains of Dsg3 and Dsc3, respectively, were used to specifically isolate auto-Abs from pemphigus sera. Affinity purified IgG fractions were subsequently tested for antigen specificity using enzyme-linked immunosorbent assay (ELISA) and immunoblotting (IB). Reactivity with native Dsg3- and Dsc3-protein, respectively, was proven by immunofluorescence (IF) on cultured human keratinocytes, monkey esophagus and frozen sections of normal human skin. Finally, a keratinocyte based so-called dissociation assay served to investigate the *in vitro* pathogenicity of the affinity purified IgG fractions. Eight Dsg3-reactive PV patients showed Dsg3-domain-specific auto-antibodies by ELISA. Four of these patients were selected for further investigation based on their antibody profile, i.e. their epitope specificity. Two patients (#1 and #8) exhibited IgG directed against Dsg3EC1 and Dsg3EC4. Patient #2 exclusively expressed auto-Abs directed against Dsg3EC1 whereas patient #6 showed IgG reactivity against Dsg3EC4, only.

Serum IgG was then affinity purified using the respective recombinant Dsg3-subdomains. Antigen specificity of the eluted IgG-fractions was subsequently verified by IB and ELISA. Isolated IgG fractions showed a characteristic intercellular staining pattern by IF using cultured human keratinocytes indicating positive reactivity with native Dsg3-protein. Recognition of Dsg3EC1 but not of Dsg3EC4 by the isolated IgG fractions of patients #1 and #8 was Ca^{2+}-dependent. Finally Dsg3-, Dsg3EC1- and Dsg3EC4-specific IgG caused keratinocyte dissociation which was comparable to the positive control, a monoclonal antibody (AK23) directed against the NH_2-terminus of Dsg3. These techniques were then applied to the sera of four atypical, i.e. two pemphigus vegetans and two pemphigus herpetiformis patients, in order to isolate Dsc3-specific IgG. All but one of these patients, who showed additional Dsg1 reactivity, exhibited IgG reactivity exclusively against Dsc3 but no other desmosomal cadherin. From all sera IgG fractions were successfully isolated and antigen specificity to Dsc3 was verified. Dsc3-reacitve IgG showed a characteristic intercellular staining pattern by IF on cultured human keratinocytes, monkey esophagus and human skin. Finally all isolated IgG fractions were able to induce loss of keratinocyte adhesion *in vitro*.Taken together this data strongly suggests a significant acantholytic effect of IgG directed against COOH-terminal epitopes of Dsg3 in addition to the well known pathogenic epitopes at the NH_2-terminus of this auto-antigen. Furthermore recognition of COOH-terminal epitopes seems to be Ca^{2+} independent which goes in line with previous studies of our group. Moreover Dsc3-reactive IgG isolated from patients with atypical pemphigus variants proved to be pathogenic *in vitro*. For the first time these results directly show the acantholytic effect of Dsc3-reactive IgG and provides evidence for the pathogenic relevance of Dsc3-IgG in pemphigus patients lacking reactivity against other desmosomal cadherins. Further investigations are needed to elucidate the mechanisms by which auto-Abs directed against COOH-terminal epitopes of Dsg3 induce acantholysis. The pathogenic relevance of other epitopes of Dsg3, especially within the Dsg3EC5-domain, needs to be addressed. Finally screening of pemphigus patients' sera for Dsc3-reactive IgG should provide further knowledge about their correlation with atypical pemphigus variants.

Zusammenfassung

Pemphigus vulgaris (PV) repräsentiert die häufigste klinische Variante innerhalb der Pemphigus-Gruppe der autoimmunen bullösen Hautkrankheiten. Blasenbildung beim Pemphigus wird durch Auto-Antikörper (auto-Ak) gegen die desmosomalen Cadherine Desmoglein 1 (Dsg1) und Desmoglein 3 (Dsg3) ausgelöst. Einige pathogene Epitope des Dsg3 liegen im amino-(NH_2)-terminalen Bereich dieses Moleküls, genau genommen in der extrazellulären Domäne 1 (EC1). Auf der anderen Seite weist eine große Anzahl an Pemphigus Patienten auto-Ak gegen Carboxy-(COOH)-terminale Epitope des Dsg3, z.B. innerhalb der EC4- oder EC5-Domänen auf und möglicherweise spielen diese Domänen eine essentielle Rolle in der Aufrechterhaltung desmosomaler Zelladhäsion. Manche Pemphigus Patienten besitzen zusätzlich oder ausschließlich auto-Ak gegen andere desmosomale Cadherine, besonders Desmocollin 3 (Dsc3). Die pathogenetische Relevanz dieser Dsc3-reaktiven auto-Ak konnte bislang nicht direkt gezeigt werden. Zunächst wurde eine Methode etabliert, um Dsg3-reaktives Immunglobulin G (IgG) aus PV Seren spezifisch zu isolieren und die pathogenetische Wirkung mithilfe eines Zell-basierten *in vitro* Assays zu untersuchen. Diese Methode wurde anschließend auf Seren von vier japanischen Patienten mit atypischem Pemphigus, welche alle eine positive IgG-Reaktivität gegen Dsc3 aufwiesen, angewendet. Mithilfe von Affinitätschromatographie-Säulen, die rekombinante, im Baculovirus-System produzierte, Proteine der extrazellulären Domänen von Dsg3 bzw. Dsc3 enthielten, wurden auto-Ak aus den Seren von Pemphigus Patienten spezifisch isoliert. Die isolierten IgG-Fraktionen wurden anschließend bezüglich ihrer Antigenspezifität mittels enzyme-linked immunosorbent assay (ELISA) und Immunoblotting (IB) getestet. Immunfluoreszenz (IF) Untersuchungen auf kultivierten humanen Keratinozyten, Affenösophagus und Gefrierschnitten menschlicher Haut wiesen die Reaktivität mit nativem Dsg3 bzw. Dsc3 nach. Schließlich diente ein mit Keratinozyten durchgeführter, funktioneller, so genannter Dissoziations-Assay der Untersuchung der *in vitro*-Pathogenität der aufgereinigten IgG-Fraktionen. Acht PV Patienten zeigten im ELISA Dsg3-spezifische auto-Ak. Vier dieser Patienten wurden aufgrund ihres geeigneten Antikörperprofils, d.h. der Epitopspezifität, für die weiteren Untersuchungen ausgewählt. Zwei Patienten (#1 und #8) zeigten IgG-Reaktivität gegen

Dsg3EC1 und Dsg3EC4. Das Serum von Patient #2 beinhaltete ausschließlich auto-Ak gegen Dsg3EC1, wohingegen Patient #6 nur IgG-Reaktivität gegen Dsg3EC4 zeigte. Serum-IgG wurde anschließend mithilfe der jeweils entsprechenden rekombinanten Dsg3-Ektodomäne spezifisch aufgereinigt. Die Antigenspezifität der eluierten IgG-Fraktionen konnte anschließend mittels IB- und ELISA-Analysen verifiziert werden. Alle isolierten IgG-Fraktionen zeigten ein charakteristisches interzelluläres Färbemuster in der IF auf kultivierten humanen Keratinozyten. Durch diese Experimente wurde die positive Reaktivität mit rekombinantem und nativem Dsg3 nachgewiesen. IgG-Fraktionen der Patienten #1 und #8 zeigten eine Ca^{2+}-abhängige Reaktivität gegen Dsg3EC1, wohingegen die Reaktivität gegen Dsg3EC4 nicht Ca^{2+}-abhängig war. Dsg3-, Dsg3EC1- und Dsg3EC4-spezifisches IgG induzierte die Dissoziation von Keratinozyten in einem vergleichbaren Ausmaß wie die Positivkontrolle, ein monoklonaler Antikörper gegen den NH_2-Terminus von Dsg3 (AK23). Diese Technik wurde anschließend auf die Seren von vier atypischen, d.h. zwei Pemphigus Vegetans und zwei Pemphigus Herpetiformis Patienten, angewendet, um Dsc3-spezifisches IgG zu isolieren. Alle außer einem dieser Patienten, welcher zusätzlich Dsg1-reaktives IgG aufwies, zeigten ausschließlich Serum-Reaktivität gegen Dsc3 aber gegen kein anderes desmosomales Cadherin. Von allen vier Seren konnte Dsc3-reaktives IgG erfolgreich isoliert und die Antigenspezifität bestätigt werden. Die IgG-Fraktionen zeigten ein charakteristisches interzelluläres Färbemuster in der IF auf kultivierten humanen Keratinozyten, Affenösophagus und humaner Haut und induzierten den Adhäsionsverlust epidermaler Keratinozyten *in vitro*. Zusammengefasst legen diese Daten nahe, dass auch IgG gegen COOH-terminale Epitope des Dsg3, zusätzlich zu den bereits bekannten pathogenen NH_2-terminalen Epitopen, einen akantholytischen Effekt aufweist. Die Erkennung COOH-terminaler Epitope ist Ca^{2+}-unabhängig, was im Einklang mit Ergebnissen vorheriger Arbeiten aus unserer Gruppe steht. Dsc3-reaktives IgG, isoliert aus Seren von atypischen Pemphigus Patienten, ist pathogen *in vitro*. Diese Untersuchungen zeigen erstmalig den akantholytischen Effekt von Dsc3-reaktivem IgG und geben Hinweise auf die pathogene Relevanz von Dsc3-IgG in Seren von Pemphigus Patienten die keine Reaktivität gegen andere desmosomale Cadherine aufweisen. Weitere Untersuchungen sind nötig, um

die Mechanismen zu untersuchen, die dem akantholytischen Effekt von auto-Ak gegen COOH-terminale Dsg3-Epitope zugrunde liegen. Zudem sollte die pathogene Relevanz anderer Epitope des Dsg3, besonders innerhalb der Dsg3EC5-Domäne, näher untersucht werden. Schließlich erscheint das Screening von Pemphigus Patienten hinsichtlich Dsc3-reaktiven IgG sehr vielversprechend, um weitere Erkenntnisse über mögliche Korrelationen mit atypischen Varianten des Pemphigus zu erhalten.

1 Introduction

1.1 The skin

1.1.1 Structure and functions

The skin or cutis and its underlying layer consisting of connective and fatty tissue, the subcutis or tela subcutanea, together form the integumentum commune (Lüllmann-Rauch 2003). With a total surface area of 1.5 – 2m^2 and a weight of 3.5 – 10kg it is the largest organ of the human body (Moll 2010). The skin is composed of three layers: right on top there is a squamous epithelium, i.e. the epidermis, followed by a part mainly composed of connective tissue, called dermis. At the bottom lies the subcutis which is mainly formed by lobules of fatty tissue (Fritsch 2009). Integral parts of the skin are the skin appendages including cornifying products (hairs and nails) as well as the skin glands (sebaceous and sweat glands) (Lüllmann-Rauch 2003). The epidermis is a cornifying, multilayered squamous epithelium whose cells regenerate every four weeks (Welsch 2003). It is tightly interlocked with the dermis at the undulant running dermo-epidermal junction zone by cone shaped extensions of the dermis, called papillae, which are interjected by rete ridges of the epidermis. This fact and abundance of collagen and elastic fibers in the dermis contribute to the strong mechanical resistance of the cutis (Lüllmann-Rauch 2003). Whereas the dermis is very well vascularised via terminal blood vessels, the epidermis is completely vessel-free. The subcutis mainly consists of univacuolar fatty tissue. It builds the connection between the cutis and deeper structures like the fasciae of muscles or the periost of bones. Furthermore it functions as a cushioning layer and facilitates the flexibility of the integumentum (Lüllmann-Rauch 2003; Welsch 2003). The skin protects the body from mechanical trauma, high and low temperatures, dehydration, penetration of foreign animate and inanimate agents as well as ultraviolet radiation and finally builds the outermost part of the immune system (Fritsch 2009). Harbouring sensing organs the skin is able to perceive diverse stimuli from the environment and the complex vessel architecture together with the sweat glands makes it an important element of thermoregulation (Lüllmann-Rauch 2003). Finally the skin plays an essential role in the immune system of the body providing barrier functions like an acidic coverage as well as cellular and humoral immune

response mechanisms towards foreign organisms (Lüllmann-Rauch 2003; Welsch 2003).

1.1.2 The epidermis

The predominant cell type of this squamous epithelium is composed of keratinocytes, which form the different epidermal layers via their alternating phenotype (Lüllmann-Rauch 2003). In addition, there are pigment-producing melanocytes and neuroendocrine merkel cells, both immigrated from the neural crest. Finally bone marrow derived Langerhans cells play an outstanding role in the innate and adaptive immune system (Braun-Falco 2005).

The epidermis can be subdivided into 4 different layers:

- <u>Stratum basale</u>
  One layer of cubic cells directly connected to the basement membrane zone. It supports the epidermis with a permanent cell-supply deriving from clusters of stem cells that split frequently and migrate to higher levels. In healthy skin, mitosis takes place only within this area.
- <u>Stratum spinosum</u>
  2-5 cell layers of polygonal shaped cells which received their name from spinous ectensions that contact neighbouring cells via desmosomes. Cells are filled with a mesh-work of keratin filaments that insert into desmosomes exerting mechanical strength.
- <u>Stratum granulosum</u>
  About 3 layers of keratinocytes harbouring basophil keratohyalin granules that contain keratin filaments and Profillagrin important for the cornifying process.
- <u>Stratum corneum</u>
  Uppermost layers composed of completely cornified cells that have lost their nucleus and any other organelle. They are disk shaped with a diameter of about 30µm and form 25 to 100 cell layers depending on the site of the body

(Lippert 2003; Lüllmann-Rauch 2003; Welsch 2003).

The 3 lower layers are formed by living keratinocytes whereas the stratum corneum comprises dead corneocytes which are finally scaled off by succeeding cells from below. The complete turnover of the normal epidermis usually takes 28 days and from basal towards superficial layers keratinocytes undergo several differentiation steps (Lüllmann-Rauch 2003; Braun-Falco 2005). Reaching complete cornification keratinocytes form a nearly water tight diffusion barrier (Welsch 2003). Keratinocytes harbour certain cell surface structures which mediate cell-adhesion taking place between adjacent cells and cells anchored to the extracellular matrix (Lüllmann-Rauch 2003; Welsch 2003).

1.1.3 Cellular adhesion

Adhesion is mediated by so called cell adhesion molecules (CAM) which are mainly transmembrane proteins and belong to different subfamilies, i.e cadherins, selectins, connexins or integrins each of them delivering cellular adhesion to different structures (Welsch 2003). Sites of cell-cell or cell-extracellular matrix contact share a common feature: at the inner side of the involved plasma membrane there is a dense zone called plaque in which the cytoskeleton inserts (Franke 2009; Moll 2010). There are three major components:

1. Transmembrane proteins, that establish binding to neighbouring cells or the extracellular matrix mediated by their extracellular domain.
2. Plaque proteins, which mediate and stabilize cytoskeleton anchoring at the inner side of the plasma membrane.
3. Filaments of the cytoskeleton, which belong to either intermediate or actin filaments depending on the type of contact.

(Lüllmann-Rauch 2003; Franke 2009).

Cell-cell and cell-matrix contacts with mechanical functions are:

- Desmosomes / hemidesmosomes, insertion spot for intermediate filaments
- Adherens junctions / focal contacts, insertion spot for actin filaments

The transmembrane proteins of desomosomes and adherens junctions belong to the superfamily of calcium dependent adhesion molecules (cadherins) and bind to cadherins of adjacent cells (Lüllmann-Rauch 2003; Welsch 2003; Franke 2009; Moll 2010). Differences between these two contact types are

found within the structure, the types of cadherins (classical cadherins and desmosomal cadherins, respectively), the plaque proteins and the inserting filaments (Franke 2009). Important functions of cell adhesion include cell-cell identification in order to establish and maintain well regulated tissue structures, connection of the cytoskeleton to the cellular environment or temporary attachment of migrating cells. In addition many of the proteins involved are connected to intracellular signalling pathways via adaptor proteins and therefore are able to influence several functions such as cell proliferation and differentiation (Lüllmann-Rauch 2003). Keratinocytes are attached to each other by numerous desmosomes. Instead, the basal keratinocytes are anchored to the basement membrane zone via hemidesmosomes or focal contacts using integrins as CAMs (Welsch 2003). Both, desmosomes and hemidesmosomes are connected to the cytokeratin network (tonofilaments) which crosses the keratinocyte in various directions (Lüllmann-Rauch 2003).

1.1.4 Desmosomes

Desmosomes are dot-shaped, circumscribed intercellular junctions that are crucial to tissues which are subjected to mechanical stress like the stratified squamous epidermis, the myocardium, bladder, the mucous membranes or the arachnoidea mater (Delva et al. 2009; Franke 2009). These discoid junctions have a diameter of about 0,2-0,5µm and form an intercellular cleft of 24-30nm (Farquhar et al. 1963). Using electron microscopy there are three morphologically identifiable zones corresponding to overlapping/contacting sites of the different desmosomal proteins: An extracellular core region, an outer dense plaque and an inner dense plaque (both located intracellular) the latter being connected to the cytoskeleton network (Kelly 1966; Kelly et al. 1976) (figure 1.1). The architecture of a desmosome consists of three different types of proteins: desmosomal cadherins (trasmembrane proteins), as well as armadillo and plakin family members (plaque proteins) (Getsios et al. 2004; Garrod et al. 2008) (figure 1.1). Together they form a “half-desmosome” at the cell surface of one cell before finding its binding partner (another “half-desmosome”) on the adjacent cell and depending on a sufficiently high Ca^{2+} concentration resulting in the generation of a functional desmosome (Garrod 2010). The extracellular core region, sometimes called dense midline, is

supposed to be the site where cellular adhesion occurs mediated by desmosomal cadherins (Garrod et al. 2008). Desmosomal cadherins can be further subdivided into two separate subfamilies: desmogleins (Dsgs) and desmocollins (Dscs) (Nollet et al. 2000). The cytoplasmic tail of desmosomal cadherins interacts with members of the armadillo (plakoglobin and plakophilins) and plakin (desmoplakin) family of linker proteins thereby forming the outer dense plaque (Kowalczyk et al. 1994; Getsios et al. 2004). Plakoglobin directly binds to the cytoplasmic tails of both Dsgs and Dscs (Wahl et al. 1996; Witcher et al. 1996) and further interacts with desmoplakin (Cowin et al. 1996) which finally tethers the whole adhesion complex to the intermediate filament network of the cytoskeleton corresponding to the site of the inner dense plaque (Kowalczyk et al. 1994; Getsios et al. 2004; Garrod et al. 2008; Delva et al. 2009). A schematical illustration of the desmosomal architecture is shown in figure 1.1.

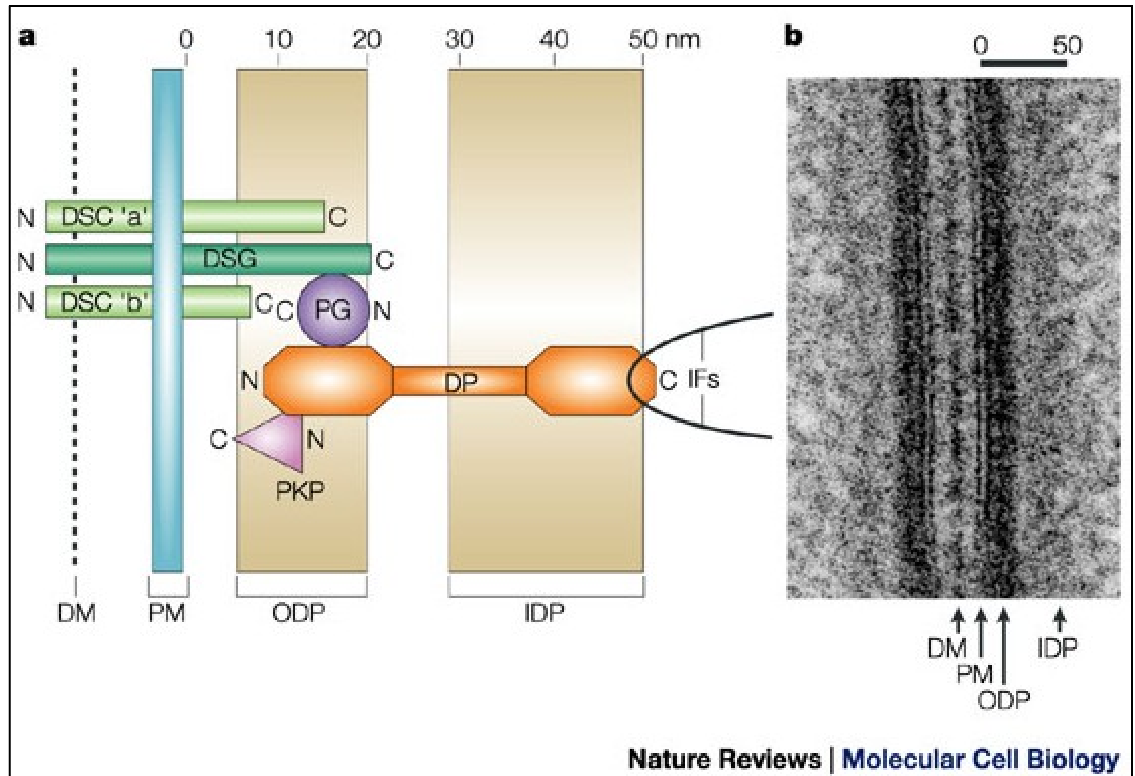


Figure 1.1: Molcular architecture of desmosomes. Schematic and electron microscopic picture of the molecular structure of a desmosome. Cadherin molecules span the intercellular space heading with their NH_2-terminus (N) forming the dense midline (DM) the site where cell adhesion takes place. Desmosomal cadherins comprise desmoglein (DSG) and desmocollin'a' and 'b' (DSC 'a' and 'b') isoforms. Further desmosomal proteins are plakoglobin (PG) which can interact with COOH-terminal (C) intracellular ends of DSG recognized by electron microscopy as the outer dense plaque (ODP), plakophilins (PKP) and the intermediate-filament (IF)-binding protein desmoplakin (DP) forming the inner dense plaque (IDP). Bar indicates relative distance (in nm) from the plasma membrane (PM) (Getsios et al. 2004).

1.1.4.1 Desmosomal cadherins

Desmosomal cadherins share multiple features with the classical cadherins found in adherens junctions. Both classical and desmosomal cadherins are type one integral membrane glycoproteins (Garrod et al. 2002). They contain 5 extracellular subdomains (ECs): four cadherin repeats (EC1-4) of about 110 amino acids, connected via calcium-sensitive flexible linkers, and a less related membrane proximal domain (EC5), sometimes called extracellular anchor domain (Pertz et al. 1999; Garrod et al. 2002; Dusek et al. 2007). A highly conserved, short amino acid sequence is present in the amino-(NH_2)-terminal EC1 subdomain of cadherins, the so called cell adhesion recognition (CAR) sequence which seems to be crucial for cell cohesion (Blaschuk et al. 1990).

Dsgs and Dscs share similarities in their cytoplasmic domains beginning with a juxtamembranuous anchor (IA) region that at least in the case of Dsc1 can interacts with desmoplakin (Troyanovsky et al. 1994b). Each Dsc-gene can be translated into two different forms by alternative splicing: An “a”-isoform with a cytoplasmic domain that is closely related to that of classical cadherins, and a shorter “b”-isoform lacking several carboxyterminal aminoacid-sequences (Collins et al. 1991) (figure 1.2). All Dsg- and Dsc”a”-isoforms contain a common intracellular cadherin typical sequence (ICS) which mediates binding to plakoglobin (Troyanovsky et al. 1994a). The Dsg isoforms have unique sequences further downstream of the cytoplasmic domain: an intracellular proline-rich linker (IPL) domain and a variable number of repeated-unit domains (RUD), followed by a glycine-rich desmoglein terminal domain (DTD) which forms the carboxyterminus (figure 1.2). The functions of these three domains however remain unclear (Getsios et al. 2004; Dusek et al. 2007). So far four desmoglein (Dsg1-4) and three desmocollin isoforms (Dsc1-3) are described and their genes are all clustered tightly on chromosome 18 (Cowley et al. 1997; Garrod et al. 2002; Whittock et al. 2003). Figure 1.2 gives a summary of the so far known desmosomal cadherins. The distribution pattern of the different isoforms varies according to the type of tissue. In the epidermis all seven desmosmal cadherins are present; however their genes are differentially expressed as keratinocytes undergo terminal differentiation (Delva et al. 2009). Dsg2 and Dsg3 can be found in the lower (basal and suprabasal) layers of the epidermis, whereas Dsg1 is present throughout the epidermis with much higher expression levels in the upper (subcorneal) layers (Kottke et al. 2006; Holthofer et al. 2007). Dsg4 is a major desmosomal component in the hair follicles and the granular layers of the epidermis (Kljuic et al. 2003). Dsc2 and Dsc3 like their desmoglein namesakes are expressed in the basal and spinous epidermal layers. And finally Dsc1 expression is most abundant in the granular layers (Kottke et al. 2006; Holthofer et al. 2007). However the expression pattern of desmosomal cadherins in non-cornifying stratified epithelium of the mucous membranes differs in respect to the epidermis. Here, Dsg3 is the predominant Dsg-isoform and can be found throughout all epithelial layers (Mahoney et al. 2006). These distribution patterns of desmsosomal cadherins are very important to keep in mind since the impaired or abolished function of only one Dsg or Dsc

molecule can lead to pathogenic effects that may be restricted to certain sites in the epidermis. This issue will be discussed later in more detail.

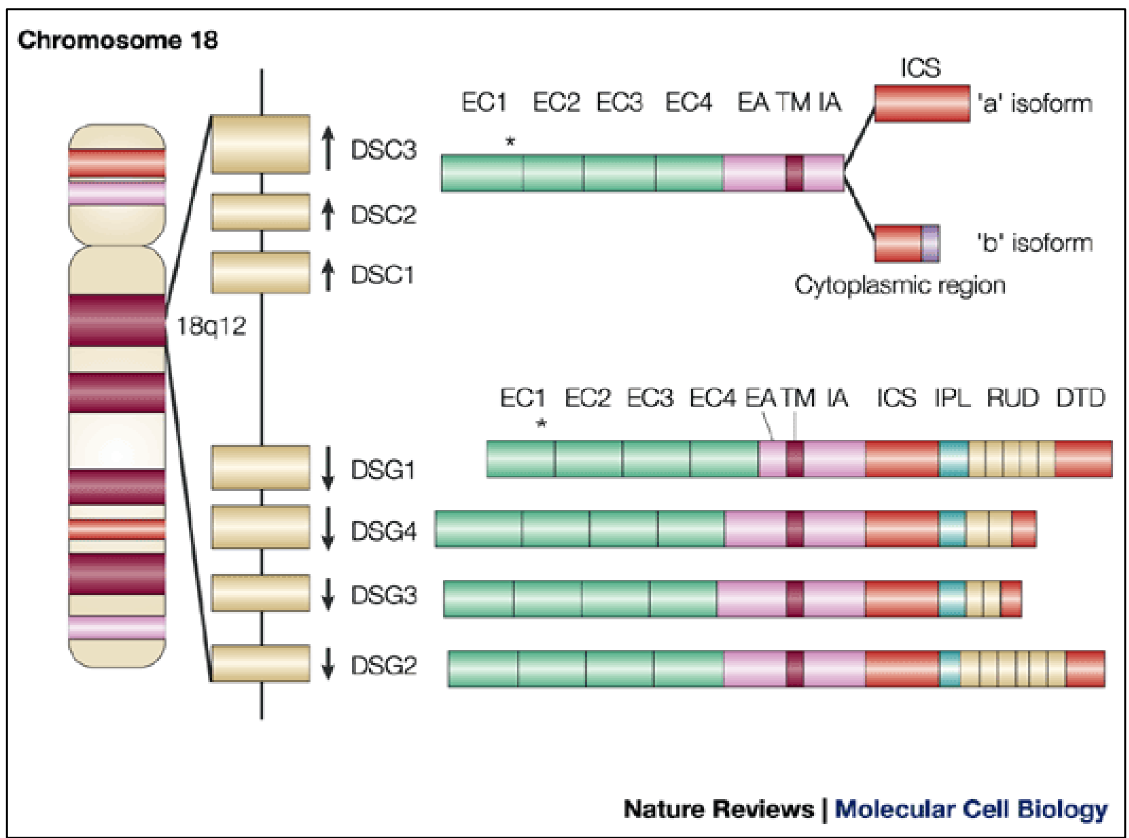


Figure 1.2: Structure of human desmosomal cadherins. The so far known human desmosomal cadherins comprise four desmoglein (Dsg1–4) and three desmocollin (Dsc1–3) genes which are found on the long arm of chromosome 18 (18q12). Desmosomal cadherins are type one integral membrane glycoproteins and harbor four highly conserved extracellular subdomains (EC1–4) and a more variable extracellular anchor (EA) also called EC5 domain. After a single transmembrane domain (TM) follows an intracellular anchor (IA) and depending on the type of cadherin additional cytoplasmic subdomains. The cell adhesion recognition (CAR) site is found within the EC1 subdomain (asterisks) mediating the adhesive function of the desmosomal cadherins. Dsc'a' and 'b' isoforms, as well as the Dsgs contain an intracellular cadherin-typical sequence (ICS). DSC isoforms however harbor a shorter cytoplasmic region that contains unique sequences (purple). Finally, desmoglein proteins possess an extended cytoplasmic region downstream of the ICS comprising an intracellular proline-rich linker domain (IPL), a variable number of repeated-unit domains (RUD) and a desmoglein-specific terminal domain (DTD). However, the function of these last three domains remains unclear at the moment (Getsios et al. 2004).

1.1.4.2 Desmosome assembly

Desmosomes represent very stable however not static structures. They underlie permanent changes of their cadherin repertoire also called cadherin trunover. Cadherins are constantly synthesized as propeptides and delivered to the plasma membrane through the classical secretory route (Green et al. 2010). Before reaching the cell surface they are processed via a furin-dependent mechanism, probably to prevent premature intracellular adhesion (Posthaus et al. 1998; Muller et al. 2004; Yokouchi et al. 2009). At the cell surface cadherins can be incorporated into existing desmosomes or may form new cell-cell contacts. In response to *de novo* cell contact or an elevation of extracellular calcium, cadherins cluster and become stabilized (Green et al. 2010). Desmosomal membrane and plaque proteins are delivered separately to the cell surface where they assemble to form an intact desmosome. In particular, desmoplakin is found at the cell membrane attached to intermediate filaments shortly after two adjacent cells get into contact. Groups of several cadherins then associate with desmoplakin allowing formation of cadherin transdimers and later incorporation into mature desmosomes (Green et al. 2007). Desmosomal and non-desmosomal cadherin transdimers are involved in cellular adhesion and may represent different targets in disease (Muller E.J. et al. 2008). *In vitro* studies on human keratinocytes suggest that in low-calcium-medium (LCM) desmosomal proteins form half desmosomes that are transported to the cell surface where they are unable to participate in adhesive binding and are therefore internalized and degraded leading to a short half-life (Penn et al. 1987; Demlehner et al. 1995). However when the extracellular calcium concentration is raised adhesion is triggered and desmosomes assemble at cell interfaces (Watt et al. 1984). A calcium concentration of 1mM is necessary to convert the extracellular cadherin domains from a clustured to a more extended/rigidified, adhesion competent configuration (Garrod 2010).

1.1.4.3 Mediating adhesion

Different ways of cadherin-cadherin interaction have been described. Intermolecular binding of two cadherin molecules on the same cell surface is called **cis interaction**, whereas between adjacent cells **trans interaction** (He et al. 2003; Getsios et al. 2004). Al-Amoudi and colleagues found two

predominant Dsg-interactions, which are presumably confined to the EC1 regions: A most favorable trans, W-shaped interaction between molecules emanating from opposing cell surfaces where a highly conserved amino-terminal tryptophan residue inserts into a hydrophibic pocket of the cell adhesion recognition-(CAR)-site of the corresponding cadherin forming an adhesive dimer of adjacent cells (Boggon et al. 2002; Al-Amoudi et al. 2007). The importance of this interaction has been shown by several studies where artificially synthesized peptides or antibodies targeting the CAR-site were able to block desmosomal adhesion (Tselepis et al. 1998; Runswick et al. 2001). The second EC1-mediated interaction has a cis, V-shaped appearance and occurs between molecules emanating from the same cell membrane leading to lateral dimerization which is considered a fundamental determinant of cell adhesion (He et al. 2003). Finally, densities observed at the positions close to the interdomain EC4-EC5 region suppose another cis interaction between proximal regions of neighboring molecules (Al-Amoudi et al. 2007). The EC1 subdomain of desmosomal cadherins seems to play an outstanding role in maintaining intercellular adhesion since targeting of the predicted binding motif of the EC1 domain of Dsg3 by a monoclonal antibody (AK23) is pathogenic *in vivo* (Tsunoda et al. 2003). In contrast to classical cadherins which exclusively undergo homophilic interactions, desmosomal cadherins are able to interact both homophilically and heterophilically. Homophilic binding of Dsg2, Dsc2 and Dsg3 was shown to occur in vitro as well as hetreophilc interactions between Dsg2 and Dsc1 or Dsc2 (Amagai et al. 1994b; Syed et al. 2002). However binding of Dsg1 to Dsg3 has been ruled out (Heupel et al. 2008). Using atomic force miscroscopy (AFM) and lazer tweezer experiments Spindler et al. found evidence which suggest that heterophilic binding of Dsgs and Dscs may induce an even stronger intercellular adhesion compared to homophilic interactions (Spindler et al. 2009). All these findings support an outstanding role of demsosomes in mediating cellular adhesion, especially in the epidermis.

1.2 From desmosome to disease

The first evidence for the involvement of disrupted desmosomal adhesion in the pathogenesis of a blistering skin disease evolved from findings in the early 1960s. Beutner and colleagues using immunofluorescence (IF) studies on

human skin detected circulating IgG auto-antibodies in sera of patients suffering from a blistering skin disease called pemphigus vulgaris (PV) which were directed against the cell surface of keratinocytes (Beutner et al. 1964; Beutner et al. 1965). Pemphigus derives from the Greek word “pemphix” meaning blister and stands for a group of chronic autoimmune blistering diseases of the skin and mucous membranes, characterized by intra-epidermal split formation (Lever 1953). The knowledge about the pathophysiologic backround of this relatively rare disease was very limited so far. According today’s knowledge, auto-antibodies of the IgG subclass are supposed to be the key players in the pathogenesis of PV by inducing loss of cell-cell adhesion, a process called acantholysis (Amagai 2010). This knowledge has been profoundly shaped by works from Schiltz and Anhalt in 1976 and 1982, respectively, which revealed the pathogenic effects of patients’ IgG *in vitro* and *in vivo* (Schiltz et al. 1976; Anhalt et al. 1982). More precisely, purified PV-auto-Abs were added to human skin cultures leading to a histological appearance indistinguishable from that of PV-lesions, i.e. extensive epidermal acantholysis of basal and suprabasal layers and IgG binding in an intercellular pattern at the site of blister formation (Schiltz et al. 1976). Furthermore, when injected intraperitoneally into neonatal mice, PV-IgG caused cutaneous blisters and erosions with ultrastructural and IF features resembling pemphigus in a dose dependent manner (Anhalt et al. 1982). Subsequently enormous effort was put into the identification of the auto-antigens detected by PV-IgG. Stanley et al. showed that sera from 5 out of 7 PV patients were able to specifically immunoprecipitate a 130kDa molecule from keratinocyte cell lysates which shared features of a membrane glycoprotein (Stanley et al. 1982). In addition, two years later the same group identified another molecule immunoprecipitated by sera from pemphigus foliaceus (PF) patients, a clinical subtype of pemphigus distinct from PV. This 160kDa protein could not be precipitated by PV sera indicating two different antigen specificities in line with the different clinical appearance of PV and PF (Stanley et al. 1984). Eyre et al. demonstrated biochemical similarities between the now called PV- and PF-antigen (PVA and PFA), i.e. PVA and PFA both seem to be cell surface glycoproteins, have the same isoelectric point and are capable of disulfide linkage to a 85kd polypeptide (later identified as plakoglobin (Cowin et al. 1986)). Immunoprecipitated PVA and PFA could be obtained from cultured

human keratinocytes and from human skin extracts, respectively (Eyre et al. 1988). Hashimoto et al. confirmed these results using immunoblot (IB) analysis. Nearly all PV sera but none of the PF sera were able to detect the 130kDa PVA, whereas sera from PF patients partially reacted with a 150kDa protein, most likely Dsg1, thereby introducing immunobloting as a method to routinely differentiate PV- and PF-Abs (Hashimoto et al. 1990). Jones and colleagues were able to demonstrate that PV and PF sera recognize desmosomes using IF and IB analysis of cell free desmosome preparations suggesting that PVA and PFA most likely play important roles in the adhesion of cells (Jones et al. 1986a; Jones et al. 1986b). Finally isolation of certain desmosomal proteins using monoclonal antibodies or PV/PF sera, respectively, and subsequent identification of their corresponding cDNA revealed that target antigens in pemhigus are desmogleins, namely Dsg1 and Dsg3 (Eyre et al. 1987; Koch et al. 1990; Amagai et al. 1991). The identification of Dsg3 as the target antigen in PV was a milestone in research on autoimmune bullous skin disorders since the presence of auto-Abs against desmosomal cadherins in pemhigus patients and the clinical appearance of mucocutaneous blisters could now be explained. These and other findings lead to the picture of pemphigus as a paradigm of an auto-ab mediated autoimmune disease. In order to get a better understanding of the clinical characteristics of this autoimmune disease the different pemphigus phenotypes will be subsequently described in more detail.

1.2.1 Clinical subtypes of pemphigus

Pemphigus can be subdivided according to the clinical and histological phenotype of the patients, i.e. the site of blister manifestation and the level of intraepithelial split formation (Mahoney et al. 1999a). The majority of pemphigus cases belong to three major subtypes, namely pemphigus vulgaris (PV), pemphigus foliaceus (PF) and paraneoplastic pemphigus (PNP) (Kneisel et al. 2011a). Furthermore there are rare and/or atypical pemphigus variants like IgA pemphigus, pemphigus herpetiformis, pemphigus erythematosus or pemphigus vegetans. They share certain antigenic targets as well as clinical and histological findings with the three major pemphigus variants (Hertl 2005) (see table 1.1).

1.2.1.1 Pemphigus vulgaris

Although being the most common pemphigus subtype PV is still a rare disease with an incidence of about 0.1 to 0.5 per 100,000 (Hertl et al. 2001b). It primarily affects individuals in the third to fifths decade without sex preference (Hertl 2005). The clinical picture of PV is characterized by painful erosions of the mucous membranes and the skin. Early Lesions can present as flaccid, fraglile walled blisters on erythematous ground which rupture easily leading to sharply outlined erosions that heal without scarring (Hertl 2005; Amagai 2010). Histologically, blister formation in PV occurs in the deep epidermis/mucosa caused by split formation between the basal and suprabasal keratinocyte layers (Mahoney et al. 1999a). Most of the PV patients exhibit involvement of the oral and pharyngeal mucosa, called mucosal dominant type, however other mucous membranes like the esophagus, conjunctiva, nasal mucosa, vagina, penis, anus and labia maybe involved as well (Amagai 2010). Chronic painful lesions of the mouth and pharynx can lead to reduced food or drink intake, hoarseness and difficulty in swallowing (Amagai 2010). On disease progression lesions can spread to the skin with a preferential involvement of the trunk resulting in a mucocutaneous PV type (Hertl 2005). According to the site of blister formation patients with exclusive mucous membrane involvement exhibit IgG autoantibodies against Dsg3 whereas appearance of mucocutaneous lesions correspond to a mixed anti-Dsg3/anti-Dsg1 antibody profile measured by ELISA using recombinant Dsg1/3 (Amagai et al. 1999b). Skin blisters can be induced by applying mechanical stress on healthy-appearing epidermis, a phenomenon called Nikolsky sign (Hertl et al. 2001b). Prior to the introduction of immunosuppressive therapies the prognosis of PV used to be fatal (75% died within a year) since the chronically disturbed barrier function of the skin and mucosa can lead to profound dehydration and bacterial superinfections, the latter still remains a serious clinical problem (Hertl 2005; Murphy et al. 2008; Amagai 2010).

1.2.1.2 Pemphigus foliaceus

In contrast to PV, PF predominantly affects the epidermis of the seborrheic areas, including the face, scalp and upper trunk. Lesions appear as scaly, crusted and well demarcated erosion often on an erythematous base (Amagai

2010). Another difference in relation to PV is the fact that splitting occurs between the upper granular layers of the epidermis (Mahoney et al. 1999a). PF-patients express IgG auto-antibodies against Dsg1 only (Amagai et al. 1999b), however a positive Nikolsky sign can be seen in PF as well. In very severe cases lesions can spread to nearly the whole integument resulting in erythrodermic exfoliative dermatitis (Hertl 2005; Waschke 2008; Amagai 2010). An endemic variant of PF is fogo selvagem which is most endemic in distinct regions of Brazil (Diaz et al. 1989). It is clinically indistinguishable from PF however the endemic appearance suggests that environmental factors initiate the autoantibody response in the host. Fogo selvagem seems to be an infectious disease, since it can be initiated by the bite of the black fly, which is thought to function as a vector for a so far unknown infectious organism or virus (Diaz et al. 1989). Recently however, Brazilian pemphigus foliaceus anti-desmoglein 1 autoantibodies have been shown to cross-react with a sand fly salivary antigen supporting the hypothesis of molecular mimicry (Qian et al. 2012).

1.2.1.3 Paraneoplastic pemphigus

PNP usually occurs in patients with an underlying neoplasm, mainly B-cell lymphoma (Hertl 2005). It is characterized by painful mucosal ulcerations and polymorphous skin lesions usually with progression to blistering eruptions on the trunk and extremities like erythematous macules, flaccid blisters and erosions, tense blisters, erythema multiforme like lesions, and lichenoid eruptions (Anhalt et al. 1990). The most constant clinical feature is a severe stomatitis that in the majority of cases presents as the first sign and characteristically extends to the vermillion of the lips. It presents with erosions and ulcerations and is very resistant to therapy (Amagai 2010). Contrary to PV and PF patients with PNP may present with involvement of lung epithelium leading to progressive respiratory failure (bronchiolitis obliterans) and is therefore the only pemphigus form affecting non-stratified squamous epithelium (Nousari et al. 1999; Amagai 2010). Lung involvement accounts for up to 30% of the terminal complications in PNP (Nousari et al. 1999). Like the clinical appearance the histologic findings are very heterogeneous. Keratinocyte necrosis and acantholysis may occur in various levels of the involved epithelia.

PNP-patients can develop IgG-auto-Abs against almost every desmosomal, hemidesmosomal as well as plaque-protein (Hertl 2005; Waschke 2008; Amagai 2010).

Table 1.1: Different clinical pemphigus variants.

Pemphigus variant (representative clinical picture)	**Target antigen**	**Clinical Phenotype**	**Histology/DIF**
Pemphigus vulgaris	**desmoglein 3** desmoglein 1	flaccid blisters, painful erosions, erythematous skin (skin + mucous membranes) a) mucosal dominant b) mucocutaneous	IgG deposits at basal/suprabasal levels of the epidermis, suprabasal split formation
Pemphigus foliaceus	**desmoglein 1**	scaly crusted erosions on erythematous ground, sebborheic distribution (only skin)	Subcorneal IgG deposits + subcorneal split formation
Paraneoplastic pemphigus	**desmoplakins I,II** **BP230** **desmoglein 1** desmoglein 3 **periplakin/envoplakin** **HD1/plektin**	Mucosal ulcers + polymorphic skin lesions (blisters, erosions, macules), severe stomatitis, involvement of lung epithelium ➔ bronchiolitis obliterans (skin and mucous membranes)	IgG deposits on entire epidermal cell surfaces +/- granular linear complement deposition along basement membrane zone; suprabasilar intraepithelial acantholysis, keratinocyte necrosis, vacuolar-interface change

Pemphigus variant (representative clinical picture)	**Target antigen**	**Clinical Phenotype**	**Histology/DIF**
Pemphigus vegetans	desmocollins cholinergic receptor pemphaxin α9 acetylcholine receptor	blisters + pustules, verruciform + papillomatous vegetations (skin ➔ intertriginous areas), PV-like oral lesions	Pustules and flaccid blisters; vegetations on erosive ground; hyperkeratotic plaques
IgA pemphigus - intraepidermal neutrophilic IgA dermatosis (IEN) type	desmoglein 1 **desmoglein 3**	Pruritic, flaccid vesicles and/or pustules in an annular pattern with central crusting, sometimes hypopyon (skin + rarely mucous membranes)	**- IEN type:** suprabasal pustules, neutrophil infiltration, scanty acantholysiss; IgA deposits in lower or entire epidermal cell surfaces
- subcorneal pustular dermatosis (SPD) type	desmocollin 1		**- SPD type:** subcorneal pustules, neutrophil infiltration, scanty acantholysis; IgA deposits on upper epidermal cell surfaces

Pemphigus variant	Target antigen	Clinical Phenotype	Histology/DIF
Pemphigus herpetiformis	**desmoglein 1 desmoglein 3**	pruritic, erythematous, vesicular, bullous, or papular lesions, herpetiform pattern (skin + occasionally mucous membranes)	Eosinophilic spongiosis +/- acantholysis, intaepidermal pustules filled with eosinophils or neutrophils; IgG deposits in upper or entire epidermal cell surfaces
Pemphigus erythematosus	**desmoglein 1**	Sharply demarcated erythematous plaques with scaling (Skin of face and upper trunk)	IgG and C3 deposits along the dermoepidermal junction zone similar to lupus erythematosus

Shown are clinical pictures and characteristics, known or suspected antigens, histopathological and immunofluorescence findings of the different clinical pemphigus variants. Adapted from (Robinson et al. 1999; Hertl 2005; Hertl et al. 2010; Kneisel et al. 2011a; Oktarina et al. 2012). Pictures (Kneisel et al. 2011a). DIF = direct immunofluorescence. C3 = complement component C3.

1.2.2 Auto-antibody profiles

Dsg1 and Dsg3 auto-antibody titers usually correlate with disease activity in pemphigus when measured by ELISA using recombinant proteins (Ishii et al. 1997). However, in some cases correlation between antibody titers and clinical phenotype was not found (Kwon et al. 2008). In PV pathogenic auto-Abs are polyclonal and initially belong to the IgG_4 subclass which is unable to activate the classical complement pathway. During disease progression patients can develop additional anti-Dsg3 IgG_1 titers with occasional appearance of IgA and IgE antibodies. Patients in remission mainly express low titers of Dsg3-reactive IgG_1, highlighting IgG_4 as the main pathogenic subclass in PV indicating a cell-independent directly pathogenic capacity (Bhol et al. 1995; Spaeth et al. 2001; Hertl et al. 2010). Finally a work examining the Th2-response in pemphigus patients has shown that acute onset PV is correlated with the occurrence of Dsg3-reactive IgG_4 and IgE, respectively (Nagel et al. 2010).

1.2.3 Knowledge from mice

To study the function of single desmosomal cadherins a very elegant approach is to use genetically engineered knockout (KO)-animal models. In a KO-mouse a certain gene has been turned off through targeted mutations, indicated as: $gene^{-/-}$, leading to the inability of these mice to express the gene product from the time point of fertilization. Subsequently the putative phenotypic effects of a targeted gene deletion can be studied macroscopically and microscopically. Conditional KO-models lead to the abrogated expression of a molecule restricted to a certain site of the organism or tissue, for example the skin. Several groups investigated the effects of targeted gene deletion of desmosomal cadherins in mice. Table 1.2 gives an overview of the established KO-mice. In line with the topic of this dissertation the gene deletions of Dsg3 and Dsc3 are of particular interest. Works by Koch and colleagues in 1997 and 1998 clearly demonstrated that $Dsg3^{-/-}$ -mice developed oropharyngeal inflammatory erosions by day 8-10 after birth as well as muscosal lesions exhibiting suprabasilar acantholysis with separation of desmosomes leading to a runted phenotype mainly due to reduced food intake (Koch et al. 1997). In adult mice lack of Dsg3 caused a patchy telogen hair loss and histological examination of the hair follicles revealed acantholysis between the cells

surrounding the telogen club and the basal layer of the outer root sheath epithelium (Koch et al. 1998). These data indicated an important role for Dsg3 in maintaining cellular adhesion at certain sites of the mucous membranes as well as providing the anchorage of telogen hair in the epidermis via desmosomal adhesion (Koch et al. 1997; Koch et al. 1998). In addition the phenotype of these mice was very similar to the mucosal lesions found in the human autoimmune blistering disease pemphigus vulgaris (Koch et al. 1997). Den et al. showed that complete deletion of Dsc3 leads to embryonic death in mice before implantation can take place (Den et al. 2006). Furthermore most mutants died before mature desmosomes were formed suggesting the importance of Dsc3 in early embryonic development (Den et al. 2006). A work by the same group in 2008 developed a conditional Dsc3 KO-mouse where Dsc3 gene inactivation is exclusively restricted to stratified epithelia using a K14-Cre transgene which is constitutively active in the basal layer of the epidermis beginning at embryonic day 14 (Chen et al. 2008). About 10% of these mice developed severe epidermal blistering within hours after birth and further lesion could be induced by mild mechanical stress. However these lesions were all restricted to the skin and microscopically revealed ancantholysis just above the basal cell layer and appearance of half desmosomes (Chen et al. 2008). Keratinocytes isolated from mutant mice showed significantly reduced adhesion properties in vitro. Finally mice that reached adulthood developed severe skin erosions and telogen hair loss (Chen et al. 2008). Taken together Dsg3 and Dsc3 seem to have profound influence on proper cellular adhesion in stratified epithelia and their Absence can independently lead to intraepidermal/-mucosal splitting of the basal and suprabasal epidermal keratinocytes.

Table 1.2: Desmoglein and desmocollin knock out mouse models.

KO-Gene	Author	Phenotype (mouse)
Dsc1	Chidgey et al. 2001	Epidermal flaking, defective barrier function, hyperproliferation, and granular layer acantholysis
Dsc3	Den et al. 2006	Leathal before implantation
Dsc3 (conditional)	Chen et al. 2008	Intraepidermal blistering in newborn; weak cell adhesion between mutant keratinocytes; skin erosions in adults; telogen hair loss,
Dsg2	Eshkind et al. 2002	Lethal shortly after implantation; decreased proliferation of embryonic stem cells
Dsg3	Koch et al. 1997, 1998	Runting, erosions of oral mucosa, supra-basilar epidermal acantholysis, patchy hair loss
Dsg4 (spontaneous)	Kljuic et al. 2003	Defective hair keratinization and premature differentiation of keratinocytes with hyperproliferation

Summarised are the established desmoglein (Dsg) / desmocollin (Dsc) knock out (KO) mouse models and their corresponding phenotypes. Adapted from (Green et al. 2007)

1.3 Pemhigus pathogenesis

The best characterised auto-antigens in pemphigus are Dsg1 and Dsg3 (Stanley et al. 1984; Eyre et al. 1988; Hashimoto et al. 1990; Karpati et al. 1993). Compelling evidence is available supporting the opinion that Dsg3-/Dsg1-specific auto-Abs alone are sufficient to induce acantholysis *in vitro* and *in vivo*, without help of the complement system or proinflammatory leukocytes (Anhalt et al. 1982; Takahashi et al. 1985; Amagai et al. 1992). Using the neonatal mouse model of pemphigus (established by Anhalt and colleagues 1982) affinity-purified human Dsg1- and Dsg3-specific auto-Abs have been proven to induce a blistering PV-like phenotype when injected intraperitoneally into newborn mice; injection of human IgG from healthy individuals had no pathologic effect at all (Anhalt et al. 1982). Furthermore preincubation of PV sera with recombinant Dsg1 and Dsg3, respectively, abolished their pathogenic ability in this passive transfer assay showing that the injection of antigen-specific pathogenic antibodies alone is sufficient to induce blisters in vivo

(Amagai et al. 1992; Amagai et al. 1994a; Amagai et al. 1995a). Studies by Amagai and colleagues in 2000 provided the first active mouse model for PV using the Dsg3-KO-mouse established by Peter Kochs' group (Koch et al. 1997; Amagai et al. 2000b). In detail: Dsg3-deficient mice were immunized by multiple intraperitoneal injections of recombinant Dsg3 leading to the production of anti-Dsg3 IgG titers. Splenocytes of these immunized mice were subsequently adoptively transferred into Dsg3 expressing recombination activating gene 2 (Rag-2)$^{-/-}$ recipient mice which lack the ability to produce an own T cell or B cell repertoire and therefore accept adoptive transfer of foreign immune cells. Anti-Dsg3 IgG was stably produced up to 6 months in the recipient mice without further boostering. Furthermore the Rag-2$^{-/-}$ mice developed lesions of their mucous membranes, like oral erosions, that strongly resembled PV lesions in humans and the Dsg3$^{-/-}$ phenotype (Amagai et al. 2000b). Further analysis of this disease model revealed that transplantation of both autoreactive T and B cells (which lost self-tolerance against Dsg3) from immunized Dsg3$^{-/-}$ animals are necessary to induce a PV-like phenotype in the recipient mice (Tsunoda et al. 2002). Even adoptive transfer of splenocytes from non-immunized Dsg3$^{-/-}$ mice induced a stable production of anti-Dsg3-IgG in Rag-2$^{-/-}$ recipient mice (Aoki-Ota et al. 2004). Together these findings show that impaired Dsg3-mediated adhesion either by mere Absence of Dsg3 in KO-mice or targeted via anti-Dsg3 IgG in an active mouse model can lead to a blistering phenotype in mice highly reminiscent of PV.

1.3.1 Cellular autoimmunity in pemphigus

Since the production of (auto-)antibodies by B cells requires the help of CD4$^+$ T helper lymphocytes (Th cells) the latter have been investigated according their pathogenic role in pemphigus. Hertl et al. proposed a crucial role for autoreactive CD4$^+$ T lymphocytes that recognize distinct epitopes of the Dsg3 ectodomain in initiating the autoimmune response. These T cells share a Th2 phenotype and secrete the interleukins (IL) IL-4, IL-5 and IL-13 upon Dsg3-stimulation in vitro probably leading to the production of anti-Dsg3 IgG$_4$ (Hertl et al. 2006) (figure 1.3). Especially IL-4 but not IL-10 seems to be the driving force in this process (Takahashi et al. 2008). However the appearance of autoreactive Th1 cells in PV patients has been reported as well (Hertl et al.

2006). Dsg3-reactive $CD4^+$ T-cells in peripheral blood of PV patients vary in their frequencies within the different stages of disease. Autoreactive Th2 cells are predominantly present in acute onset and remittent PV, whereas Dsg3-reactive $CD4^+$ T-cells expressing a Th1 phenotype exceed the number of Th2 cells in chronic active disease (Eming et al. 2000). These findings support a concept of auto-ab control in PV by Dsg3-reactive Th1 cells leading to the production of Dsg3-reactive IgG_1 and on the other hand by Th2 cells inducing secretion of Dsg3-reactive IgG_4 and IgE, which is preferentially seen in active stages of PV (Bhol et al. 1995; Spaeth et al. 2001; Nagel et al. 2010). PV is associated with certain human leukocyte antigen (HLA) class II alleles, namely HLA-DRB1*0402 and HLA-DQB1*0503 alleles (Hertl et al. 2006) (figure 1.3). However expressing these alleles does not implicate the onset of disease since carriers of these alleles which may harbour autoreactive Th clones do not necessarily develop a phenotype (Veldman et al. 2004b). Th1 and Th2 cell recognition of Dsg3 peptides seems to be restricted by PV-associated HLA-DRB1*0402 and/or HLA-DQB1*0503 alleles, since anti-HLA-DR and anti-HLA-DQ-antibodies blocked the proliferative response of autoreactive Th-cells (Veldman et al. 2003; Hertl et al. 2006). Furthermore, Dsg3 reactive Th1 and Th2 clones from PV-patients and HLA-matched healthy donors recognized, in association with the aforementioned HLA class II alleles, a limited set of immunodominant Dsg3 peptides indicating that loss of tolerance needs to take place on the T cell and B cell level (Veldman et al. 2004b). Nishifuji and colleagues discovered Dsg3-specific IgG producing B-cells in peripheral blood mononuclear cells of PV patients. IgG production of these cells seemed to be dependent on the presence of HLA class II-restricted autoreactive $CD4^+$ T cells (Nishifuji et al. 2000). Direct interaction of Dsg3-reactive $CD4^+$ T cells and B cells seems to be very important for the production of Dsg3-specific auto-Abs in vivo. Using the active PV mouse model, Dsg3-IgG production could be blocked by injection of anti-CD154 monoclonal antibody which interferes with interaction of activated $CD4^+$ Th cells and B cells (Yokoyama et al. 2010). Finally immunization of transgenic mice expressing human PV-associated HLA class II alleles and human CD4 receptor with recombinant human Dsg3 induced Dsg3-specific $CD4^+$ T cell responses and Dsg3-reactive antibody production (Hertl et al. 2006). Autoreactive T and B cells which escaped central tolerance

mechanisms in the thymus and bone marrow are thought to be controlled by certain peripheral T cell subsets with suppressive functions in healthy individuals, a process called peripheral tolerance (Murphy et al. 2008). These regulatory T cells (Treg) are composed of two major subsets: the forkhead box P3 *(*Foxp3) expressing, $CD4^{+}CD25^{+}$ naturally occurring regulatory T-cells (nTregs) and inducible, IL10 and transforming growth factor ß (TGF-ß) secreting Tr1 cells (Hertl et al. 2006; Murphy et al. 2008). The latter can be further subdivided into $Foxp3^{+}$ adoptive or inducible regulatory T cells (iTreg), IL-10-secreting T-inducible regulatory type 1 (Tr1) cells, and TGF-ß producing Th3 cells (Yokoyama et al. 2010). Veldman et al. showed that Dsg3-reactive, IL10- and TGF-ß-secreting T cells (Tr1 phenotype) were present in the majority of healthy carriers of PV-associated HLA class II alleles, but in less than 20% of the studied PV patients (Veldman et al. 2004a). These cells were capable of cell-independent suppression of Dsg3-specific autoreactive Th1 and Th2 effector cells in vitro by secretion of IL10 and TGF-ß indicating a protective function of these cells in healthy individuals (Veldman et al. 2004a). Furthermore there is some evidence that conversion from a Th into a Tr1 phenotype might be obtained via cell-cell contact with naturally occurring $CD4^{+}$ $CD25^{+}$ Tregs again underlining a possible protective role for these regulatory cells (Veldman et al. 2006a). In line with this Sugiyama demonstrated that the peripheral numbers of $CD4^{+}$ CD25(high) regulatory T cells are remarkably reduced in patients with PV (Sugiyama et al. 2007) and a possible imbalance between autoreactive Th2 cells and Dsg3-reactive Tr1 cells in PV patients has been proposed by Veldmann and colleagues (Veldman et al. 2004a). Taken together there seems to occur a dysregulation of Dsg3-specific Th1, Th2 and Treg responses in PV, with Th2 responses mainly leading to disease and protective Treg activity predominating in healthy individuals carrying PV-associated HLA class II alleles (Hertl et al. 2006). Figue 1.3 gives a synopsis of the proposed T and B cell involvement in the pathogenesis of pemphigus.

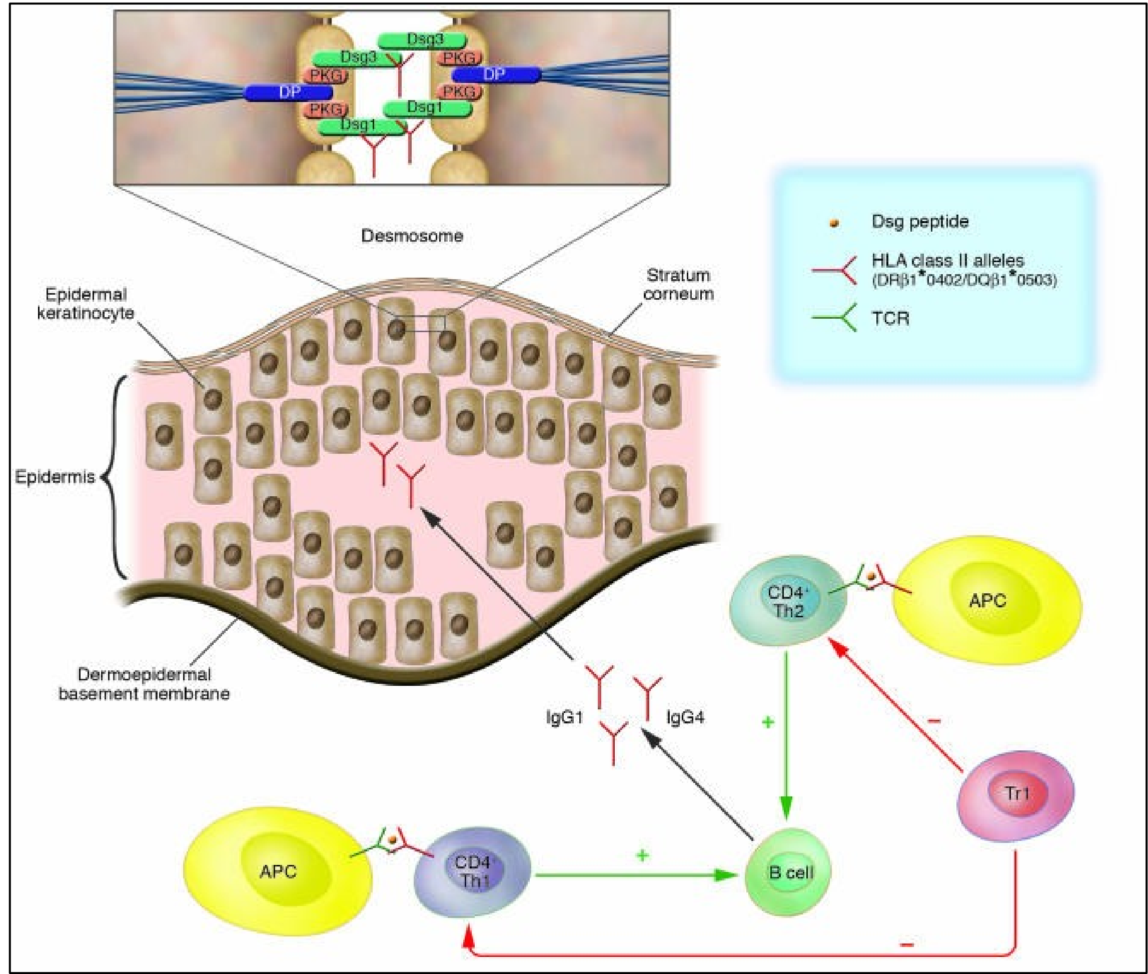


Figure 1.3: Role of T cells in the pathogenesis of PV. Impaired desmosomal adhesion leading to intraepidermal split formation primarily caused by auto-antibodies against Dsg3 and Dsg1 represents the hallmark in the pathogenesis of pemphigus vulgaris. Most auto-antibodies belong to the IgG_4 and IgG_1 subclasses. Autoreactive Th1 and Th2 cells which recognize epitopes of Dsg3 in association with the human leukocyte antigen (HLA) class II alleles HLA-DRβ1*0402 and HLA-DQβ1*0503 are presumably activated by antigen presenting cells (APC) leading to the production of auto-antibodies by autoreactive B cells. Interestingly, PV-associated HLA class II alleles are also found in healthy individuals who in addition harbour higher numbers of Dsg3-reactive type 1-regulatory (Tr1) cells thus suggesting a critical role of these cells in maintaining peripheral B cell tolerance to Dsg3 (Hertl et al. 2006). TCR = T cell receptor.

1.3.2 Disrupting adhesion of epidermal keratinocytes

Up to now, it remains unclear how auto-Abs induce loss of desmosomal adhesion. Three different pathophysiologic mechanisms have been proposed so far. Before the knowledge about pathogenic auto-Abs in pemphigus was available a favourable hypothesis was that proteolytic cleavage of cell adhesion molecules leads to skin blistering (Farb et al. 1978; Hashimoto et al. 1983; Amagai et al. 2000a; Cirillo et al. 2007; Santiago-Josefat et al. 2007). Later on

with the discovery of desmosomal cadherins as target antigens of PV and PF auto-antibodies several groups proposed a direct interference of pathogenic antibodies with the adhesive interface of Dsgs, a process called steric hindrance (Amagai et al. 1992; Futei et al. 2000; Tsunoda et al. 2003). Finally emerging evidence supports the idea that induction of intracellular signalling processes in response to auto-antibody binding may result in impaired cell-cell adhesion (Waschke et al. 2006; Muller E.J. et al. 2008; Grando 2012).

1.3.2.1 Proteolytic cleavage hypothesis

In 1978, Farb et al. added sera of pemphigus patients to cultured murine keratinocytes and observed a detachment of these cells from the culture plates (Farb et al. 1978). Furthermore cell detachment could be blocked by addition of two different proteinase inhibitors (soybean trypsin inhibitor and alpha2-macroglobulin) but did not interfere with cell surface binding of pemphigus IgG (Farb et al. 1978). Farb suggested that auto-antibody binding induces secretion or activation of an epithelial proteolytic enzyme which degrades proteins essential for cell adhesion (Farb et al. 1978). In recent years several groups suggested the involvement of different proteases in the pathogenesis of pemphigus either because of their increased expression by cultured keratinocytes incubated with pemphigus auto-Abs or by successful prevention of auto-antibody induced acantholysis by addition of specific protease inhibitors (Hashimoto et al. 1983; Feliciani et al. 2003). These proteases include plasminogen activator, urokinase-type plasminogen activator receptor (uPAR) system, matrix metalloproneinases (MMP) or the a disintegrin and metalloproteinase (ADAM) family members (Cirillo et al. 2007; Santiago-Josefat et al. 2007). However, no direct evidence for the induction of acantholysis by proteases subsequent to pemphigus IgG binding is available so far (Waschke 2008). Nevertheless Amagai et al. showed that the cause for epidermal blister formation in bullous impetigo and staphylococcal scalded skin syndrome is a serine protease secreted by the bacteria staphylococcus aureus (Amagai et al. 2000a). Even more striking was the fact that the specific cleavage site for this protease, also called exfoliative toxin A (ETA), is located within the extracellular portion of Dsg1 and that it’s enzymatic cleavage leads to a subcorneal split formation that is indistinguishable from histological findings in pemphigus

foliaceus (Amagai et al. 2000a). Taken together the proteolytic cleavage of desmosomal cadherins seems to be a less likely but still possible mechanism in initiating acantholysis in pemphigus.

1.3.2.2 Steric hindrance hypothesis

Since the discovery of demsosomal cadherins as antigens targeted by auto-antibodies in pemphigus a lot of effort was put on characterising the precise pathogenic epitopes within the extracellular domain of Dsg-proteins. Amagai et al. using ß-galactosidase fusion proteins of the different Dsg3 extracellular domains (EC1-5) performed immunoblotting experiments with 23 PV sera. Blot reactivities clearly revealed that the major immunogenic regions of Dsg3 are located within the EC1, EC2 and EC4 domain (Amagai et al. 1992). However affinity purification of PV IgG using fusion proteins representing the amino-terminus (EC1-2) and the carboxy-terminus (EC3-5) of Dsg3 showed that only autoantibodies directed against the amino-terminal epitopes of Dsg3 are pathogenic when injected into neonatal mice (Amagai et al. 1992). Futei et al. confirmed these findings using domain swapped recombinant Dsg3 molecules (Futei et al. 2000). Thus PV sera showed a clearly preferential reactivity with the amino-terminal residues 1-161 (EC1 domain) of Dsg3 using competition ELISA experiments in contrast to the carboxy-terminal domains (Futei et al. 2000). In addition IgG reactivity against the EC1 domain seem to coincide with active PV whereas the corresponding titers do not correlate with disease activity when analysed by ELISA using recombinant Dsg3 ectodomains expressed in an eukaryotic expression system (Muller et al. 2006). The same group however identified further EC2-, EC3- and EC4-reactive IgG in patients with active PV. Nearly all of these auto-antibodies reacted in a comformation-independent manner, i.e. Ca^{2+} -chelation or denaturarion of the domains EC1, EC2, EC4 and EC5 did not change the IgG reactivity measured by ELISA (Muller R. et al. 2008). Nevertheless another fact favours the major pathogenic role of EC1-reactive PV IgG. As already mentioned earlier the putative adhesive interface for trans-adhesion of desmosomal cadherins lies within the EC1 domain (Al-Amoudi et al. 2007; Heupel et al. 2009b). Thus it is a striking hypothesis that auto-antibodies in PV might directly interfere with desmosomal adhesion through binding to the involved adhesion sites, a process referred to as steric

hindrance (Waschke 2008). Finally using the active PV mouse model Tsunoda et al. identified several monoclonal mouse antibodies directed against Dsg3 (Tsunoda et al. 2003). However only the clone AK23 induced a blistering phenotype when its' hybridoma was injected into mice. Even more important epitope mapping revealed that AK23 recognizes a calcium-dependent conformational epitope within the EC1 domain, which consists of Dsg3-specific residues that form the adhesive interface between juxtaposed Dsgs (Tsunoda et al. 2003). When tested under cell free conditions AK23 as well as PV-IgG were able to interfere directly with homophilic Dsg3 binding again supporting their ability of steric hindrance (Heupel et al. 2008). On the contrary PF-sera containing Dsg1-specific antibodies did not abrogate homophilic Dsg1 binding in this assay but nevertheless were tested to be pathogenic in vitro using cultured keratinocytes. Thus PF-IgG may carry out their pathogenic effect through cell-dependent mechanisms, i.e. intracellular signalling (Heupel et al. 2008). Although antibodies against the middle or carboxy-terminal extracellular regions of Dsg3 did not show any pathogenic activity in the neonatal mouse model of PV, certain combinations of these antibodies clearly did indicating some kind of additive pathogenic effect (Kawasaki et al. 2006). A possible explanation for this finding could be allosteric effects, i.e. PV auto-antibodies directed against non-amino-terminal epitopes remote to the adhesive interface might induce conformational changes within the Dsg3 molecule leading to impaired desmoglein binding and subsequently to acantholysis (Waschke 2008). Using phage display it is possible to screen the antibody repertoire of a patient and additionally to isolate antigen-specific monoclonal antibodies together with their encoding cDNA. Payne and colleagues identified several pathogenic and non-pathogenic Dsg3-specific monoclonal auto-Abs in PV sera by phage display (Payne et al. 2005). As expected the epitopes of the pathogenic antibodies were located within the EC1 domain of Dsg3, however the effects of a combination of “non-pathogenic” antibodies has not been examined in this study (Payne et al. 2005). Very recently Di Zenzo and colleagues using proliferated Dsg3-reactive B cell clones isolated from the blood of two pemphigus patients identified a novel pathogenic epitope within the EC1 subdomain of Dsg3 which is crucial for the maintenance of a cis interaction between neighboring Dsg3-molecules (Di Zenzo et al. 2012). Taken together the contribution of specific

auto-antibodies directed against COOH-terminal epitopes of Dsg3 to the pathogenesis of PV needs to be further elucidated.

1.3.2.3 Signal transduction hypothesis

There is emerging evidence that initial events leading to acantholysis in pemphigus may be mediated by indirect mechanisms, i.e. intracellular signalling pathways, in contrast to direct inhibition (Seishima et al. 1995; Berkowitz et al. 2005). A convincing finding is that low temperature incubation of PV-IgG treated keratinocytes prevents cell dissociation but not antibody binding to the cell surfaces (Calkins et al. 2006). Furthermore, incubation of cultured keratinocytes with PV sera leads to a reduction of the Dsg3 half-life on the cell surface without changing its total amount (Cirillo et al. 2006). Interestingly PV-IgG seems to prevalently bind non-desmosomal Dsg3 thereby preventing its incorporation into mature desmosomes (Cirillo et al. 2006; Oktarina et al. 2011). In line with this other groups confirmed that auto-antibody binding and cell separation starts at inderdemosomal areas (Takahashi et al. 1985; Sato et al. 2000). Thus it's being speculated if this non-junctional Dsg3 fraction may be involved in an "outside-in signalling" and PV-IgG binding may interfere with this function (Muller E.J. et al. 2008). Finally, acantholysis subsequent to PV-IgG binding can be reduced or even abrogated by preincubation with several agents that modify intracellular signalling pathways. An overview of several candidate pathways proposed to be involved in the pathogenesis of PV is shown in table 1.3.

Protein kinase C

Ever since the discovery of a rapid and transient intracellular Ca^{2+} -increase in response to binding of PV-IgG in human keratinocytes (Seishima et al. 1995) the participation of several signal transduction pathways have been proposed. The intracellular Ca^{2+} (and inositol 1,4,5 triphosphate) increase for example was found to be phospholipase C (PLC) dependent and lead to the activation of both protein kinase C (PKC) and plasminogen activator (PA) (Kitajima et al. 1999; Seishima et al. 1999). Even more striking intracellular Ca^{2+} -chelation, and blocking of PKC or PA prevented PV-IgG induced acantholysis in vivo (Sanchez-Carpintero et al. 2004). However, since in PA-KO-mice still develop a

severe blistering phenotype after injection of PV-IgG, the PA-system is not believed to be crucial in this process (Mahoney et al. 1999b).

P38 mitogen-activated protein kinase (p38 MAPK)

Several studies have highlighted a possible role of the p38 mitogen-activated protein kinase (p38 MAPK) in the pathogenesis of pemphigus (Berkowitz et al. 2005; Berkowitz et al. 2006; Waschke et al. 2006; Woll et al. 2007; Berkowitz et al. 2008). This opinion derives from findings that p38 MAPK and one of its' downstream targets, heat shock protein (HSP) 25 (HSP27 = human homologue), get's phosphorylated in human keratinocyte cultures already 30 min after incubation with PV IgG (Berkowitz et al. 2005). Furthermore selective inhibiton of p38 MAPK prevented antibody-induced blister formation in vivo, blocked inactivation of the small GTPase RhoA, as well as cytokeratin retraction and reorganization of the actin cytoskeleton, both of the latter are known to precede acantholysis (Berkowitz et al. 2005; Berkowitz et al. 2006; Waschke et al. 2006; Chernyavsky et al. 2007). In addition high levels of phosphorylated p38 MAPK could be detected in skin lesions of PV patients (Berkowitz et al. 2008). However, other studies have qualified the role of p38 MAPK activation in PV pathogenesis (Lee et al. 2009; Mao et al. 2011). First there seems to be an even more important late peak in p38 MAPK phosphorylation subsequent to PV-IgG binding which may occur secondary to initial acantholysis (Lee et al. 2009). In line with this Mao et al. have shown that p38 MAPK activation is not necessary to induce initial Dsg3 endocytosis and blister formation but its' late activation leads to the endocytosis of Dsg3 and Dsc3 and may augment blister formation in PV (Mao et al. 2011). Grando proposed that p38 MAPK activation may even be secondary to apoptotic pathways since p38 MAPK phosphorylation could be abolished by inhibition of executioner caspases of the cell death programme (Grando 2012). Finally a phase 2 multicenter open-label uncontrolled pilot study using the allosteric p38 MAPK inhibitor KC706 had to be aborted due to severe adverse reactions (Grando 2012). A possible explanation of how p38 MAPK may induce or enhance blister formation maybe its influence on the keratinocyte cytoskeleton since serine phosphorylation of cytokeratin 8 has been shown to induce cytokeratin network disassembly which is a hallmark of acantholytic cells (Woll et al. 2007).

Several more signalling pathways involving epidermal growth factor receptor (EGFR), the tyrosine kinase Rous sarcoma oncogene (Src), the GTPase ras homolog family member A (RhoA), the desmosomal plaque protein plakoglobin (PG) and the cell cycle regulators myelocytomatosis viral oncogene homolog (c-Myc) and cyclin-dependent kinase 2 (CDK2) have been studied concerning their contribution to pemphigus pathogenesis. Table 1.3 below gives a summary of recent knowledge about these signalling molecules.

Table 1.3: Signalling pathways presumably involved in the pathogenesis of pemphigus.

Signalling molecule	Involvement in PV pathogenesis	references
ras homolog family member A GTPase (RhoA)	• PV-IgG induced keratinocyte dissociation is accompanied by p38 MAPK dependent inactivation of RhoA. • Specific inactivation of RhoA resulted in deep epidermal splitting, keratinocyte dissociation and actin reorganisation. • Specific activation of RhoA abrogated PV-IgG induced cytokeratin retraction, actin reorganization and reduction of Dsg3. ➔ RhoA activity may regulate desmoglein cytoskeletal anchorage.	(Izumi et al. 2004; Waschke et al. 2006; Spindler et al. 2007)
plakoglobin (PG)	• Plakoglobin deficienct mice show subcorneal acantholysis, loss of desmosomes, impaired cytoskeletal anchorage of desmogleins and c-Myc overexpression. • EGFR activation results in phosphorylation of plakoglobin, loss of desmoplakin from desmosomes and decreased adhesive strength. • PG deficient keratinocytes are resistant to PV-IgG induced dissociation, cytokeratin retraction and disruption of the desmosomal plaque. ➔ PG may help to transfer an “outside-in” signalling from auto-Ab bound Dsg3 which leads to disinhibition of c-Myc.	(Yin et al. 2005; Williamson et al. 2006; de Bruin et al. 2007; Muller E.J. et al. 2008)
epidermal growth factor receptor (EGFR)	• EGFR activity in cultured keratinocytes peaks 60min after PV-IgG treatment and finally leads to acantholysis and apoptosis. • EGFR induced acantholysis is mediated by plakoglobin phosphorylation. • EGFR activation seems to reduce protein levels of desmosomal cadherins. ➔ EGFR may act as a pro-proliferative counterpart to Dsg3/plakoglobin mediated signalling.	(Lorch et al. 2004; Yin et al. 2005; Frusic-Zlotkin et al. 2006; Chernyavsky et al. 2007)

Signalling molecule	Involvement in PV pathogenesis	references
sarcoma oncogene tyrosine kinase (Src)	• Src activity peaks 30min after PV-IgG treatment and contributes to EGFR and p38MAPK activation. • Src inhibition reduces PV-IgG induced acantholysis, cytokeratin retraction and apoptosis *in vitro*. • Non-desmosomal Dsg3 regulates activity of Src and its association with E-cadherin in adherens junction formation. ➔ Src is activated early after PV-IgG binding and may trigger EGFR and P38MAPK induced acatholysis.	(Sanchez-Carpintero et al. 2004; Chernyavsky et al. 2007; Tsang et al. 2012)
myelocytomatosis viral oncogene homolog (c-Myc) and cyclin-dependent kinase 2 (CDK2)	• PV-IgG induced PG depletion resulted in c-Myc overexpression (after 24h). • Inhibition of c-Myc and PG degradation abrogated PV-IgG induced skin blistering. • Pharmacological inhibition of cdk2 abolished PV-serum induced acantholysis in vivo. ➔ Regulators of cell cycle progression seem to be involved in loss of keratinocyte adhesion in PV.	(Williamson et al. 2006; Lanza et al. 2008; Muller E.J. et al. 2008)

Taken together it is not clear at present which of the above mentioned mechanisms is the initial trigger inducing acantholysis in PV. For a long time steric hindrance of junctional desmogleins was the favoured mechanism. However since keratinocyte detachment starts at interdesmosomal areas and incubation at low temperature can abolish acantholysis this hypothesis has to be revisited. On the other side several signalling mechanisms that are thought to be involved in PV pathogenesis are activated at late time points after PV-IgG treatment of cells when initial acantholysis has already been initiated (Lee et al. 2009; Mao et al. 2011). Thus the exact time kinetics of activated signalling molecules subsequent to antibody binding is needed to assess their relevance in initiating loss of cell adhesion. Finally PV-sera do not only contain antibodies to Dsg1 or Dsg3 (Nguyen et al. 2000c). Several studies have identified further keratinocyte surface molecules targeted by PV-IgG which in consequence may also lead to the alteration of intracellular signalling pathways that contribute to

acantholysis. Some of these non-Dsg targets will be discussed later in this chapter.

1.3.3 Epitope spreading

Findings in the active PV mouse model suggest the presence of Dsg3-reactive B-cells in normal individuals. These B cells may produce antibodies directed against non-pathogenic epitopes of the Dsg3 ectodomain (Ota et al. 2004) and in fact low levels of anti-Dsg3 antibodies were detected in healthy relatives of PV patients (Torzecka et al. 2007). However the mere presence of Dsg3-reactive antibodies is not sufficient to develop PV (Yokoyama et al. 2010). A possible explanation for this phenomenon is the occurrence of epitope spreading. Epitope spreading enables to the inclusion of new epitopes within the same or a different (auto-)antigen leading to the onset of an autoimmune disease or a phenotypical switch in an ongoing disease (Yokoyama et al. 2010). Intramolecular epitope spreading was found to take place in patients of the endemic PF form Fogo Selvagem (Li et al. 2003). Patients in preclinical stages presented with antibodies against the EC5 subdomain of Dsg1 and most interestingly with the onset of disease patients developed anti-Dsg1EC1 and/or anti-Dsg1EC2 auto-antibodies (Li et al. 2003). Salato et al. using IF and ELISA competition assays demonstrated that an intramolecular epitope spreading from COOH-terminal towards NH_2-terminal epitopes takes place within PV patients' sera just before the transition into a mucocutaneaous phenotype (Salato et al. 2005). Only antibodies directed against amino-terminal epitopes of Dsg3 were able to stain human skin in IIF analysis and their occurrence preceded an intermolecular epitope spreading from Dsg3 to Dsg1 (Salato et al. 2005). Additionally, shifts of the auto-ab profile in a group of 37 PV patients occurred with a frequency of 41% (Weitz et al. 2007). However no consistent relation between changes in anti-Dsg profile and changes in PV phenotype could be detected indicating the importance of other immunological mechanisms in the clinical manifestations of PV (Weitz et al. 2007). But how does epitope spreading occur on the cellular level? Takahashi et al. showed that a single Dsg3-reactive T cell clone is able to induce the production of anti-Dsg3 IgG by polyclonal naïve B cells independent of T cell epitopes on Dsg3 (Takahashi et al. 2009). That means a T cell clone auto-reactive against a peptide located

within the Dsg3 subdomain EC1 may induce IgG production by B cells specific for the EC4 domain of Dsg3. A possible explanation for this phenomenon is the fact that a B cell can act as an antigen presenting cell for T cells, i.e. a B cell may present various epitopes of a macromolecular complex and therefore might get help from a broad range of (auto-reactive) T cells (Yokoyama et al. 2010).

1.3.4 Desmoglein compensation theory

Patients with PV and PF not only present with different auto-antibody profiles, i.e. Dsg3- and/or Dsg1-specific IgG, they also exhibit different clinical sites of blister formation (Mahoney et al. 1999a). That is suprabasal blister formation of mucous membranes in PV and subcorneal epidermal splitting in PF, respectively. Furthermore patients suffering from mucocutaneous PV produce Dsg1 as well as Dsg3-sepecific IgG resulting in suprabasal acantholysis at epidermal and mucosal sites (Amagai et al. 1992; Ding et al. 1997; Amagai et al. 1999b; Hertl 2005). Using epidermal IF staining it has been demonstrated that PV and PF IgG show an inversely graded binding pattern. According to this PV IgG peferentially binds to the basal and suprabasal epidermal layers whereas PF IgG showed higher intensities in the upper epidermis. However both binding profiles seem to broadly overlap in the spinous layers (Shimizu et al. 1995; Amagai et al. 1996). These findings go in line with the expression patterns of Dsg1 and Dsg3 in human skin (Delva et al. 2009). Mahoney and colleagues could demonstrate that at least in mice Dsg1- and Dsg3-coexpressing epidermal layers were unsusceptible to pemphigus sera containing auto-antibodies against only one of the Dsg isoforms, i.e. Dsg1 or Dsg3, respectively (Mahoney et al. 1999a). On the other site human Dsg1- and Dsg3-specific IgG together induced blister formation in the superficial mouse epidermis. In the subcorneal layers where Dsg1 but no Dsg3 is expressed Dsg1-reactive PF-IgG alone was able to cause acantholysis. In this regard Dsg3-specific PV-IgG induced suprabasal split formation at layers of predominant Dsg3 expression (Mahoney et al. 1999a). These observations lead to the hypothesis that one Dsg isoform might functionally compensate for the loss of the other at sites of dual expression (Mahoney et al. 1999a). For instance Dsg1 compensates for loss Dsg3 function in PV patients harbouring Dsg3-reactive IgG and as a consequence no skin lesions occur. On the other

hand in layers where mainly one isoform is expressed no compensation can take place finally leading to acantholysis as happens in suprabasal layers of the mucous membranes where the low Dsg1 expression can not compensate for the loss of Dsg3 (Mahoney et al. 1999a). However the fact that PV patients developing additional IgG against Dsg1 exhibit suprabasal and no subcorneal epidermal split formation (like in PF patients) can not be explained by this hypothesis (Waschke 2008). Finally recent characterizations of the expression patterns of Dsg1 and Dsg3 in stratified epithelia revealed substantial differences between mice and humans, for example Dsg1 seems to be expressed to a much higher extend in the mucous membranes than primarily expected (Mahoney et al. 2006). Taken together although numerous studies support the idea of a specifically targeted reduction of desmogleins via IgG auto-Abs in stratified epithelia this concept is not able to sufficiently explain the clinical manifestation of blister formation (Nguyen et al. 2000c).

1.4 Non-desmosomal antigen targets in pemphigus

Although the majority of pemphigus patients express auto-Abs against Dsg1 and Dsg3 the occurrence of auto-antibodies directed against several other desmosomal components and non-desmosomal keratinocyte surface proteins in the sera of these patients is remarkable (Nguyen et al. 2000c; Grando 2012). Even before the identification of Dsg3 as a target antigen in PV, Korman and colleagues demonstrated that pemphigus sera also immunoprecipitate the demosomal plaque protein plakoglobin (Korman et al. 1989). Injection of PV-IgG lacking auto-Abs against Dsg1 caused epidermal blistering in Dsg3-KO-mice indicating that antibodies against keratinocyte antigens other than desmogleins 1 and 3 can induce pemphigus vulgaris-like lesions (Nguyen et al. 2000c). In some pemphigus variants neither Dsg1- nor Dsg3-reactive IgG is present at all but intercellular IgG deposition in the epidermis can be clearly detected (Nakashima et al. 2010). Very promising targets in pemphigus seem to be cholinergic receptors and the annexin pemphaxin, since in up to 85% of PV and PF sera auto-antibodies against these antigens have been found (Grando 2006). In this context, a monoclonal antibody against the alpha9 acetylcholine receptor (AChR) of epidermal keratinocytes caused acantholysis in vitro and addition of the cholinergic agonist carbachol reversed these effects (Nguyen et

al. 2000a). Although antibodies against pemphaxin alone are not sufficient to induce blistering in vivo a synergistic action of antibodies to different keratinocyte self-antigens, including both acetylcholine receptors and desmosomal cadherins seems possible (Nguyen et al. 2000b). Finally, the second group of desmosomal cadherins the desmocollins (Dsc) may play a substantial role in the pathogenesis of pemphigus. Dsc1 has been previously identified as the target antigen in the subcorneal pustular dermatosis (SPD) type of IgA pemphigus (Hashimoto et al. 1997). Moreover, IgG auto-ab against Dsc1, Dsc2, or Dsc3 were detected in the sera of patients with paraneoplastic pemphigus and, occasionally, in patients with atypical pemphigus (Dmochowski et al. 1995; Hashimoto et al. 1995; Hisamatsu et al. 2004; Muller et al. 2009). However, the pathogenic relevance of Dsc-specific auto-Abs in these disorders remains unclear. The conditional Dsc3-KO-mouse provides a very useful tool to investigate the importance of Dsc3 in maintaining keratinocyte adhesion. These mice show a pronounced blistering phenotype with suprabasal loss of epidermal adhesion. Interestingly blistering affects in particular the epidermis and hair follicles and the phenotype seems more severe than in Dsg3-null mice (Chen et al. 2008). It has been shown that Dsc3, as Dsg3, is preferentially expressed in the basal and suprabasal layers of human epidermis the site where loss of adhesion occurs in PV (Yue et al. 1995; Delva et al. 2009). In desmosomes, Dsg1 and Dsg3 do not only interact via homophilic transinteraction but presumably also by heterophilic binding with Dsc isoforms, such as Dsc1 and Dsc3. Spindler and colleagues provided *in vitro* evidence that Dsc3 homo- and heterophilic trans-interaction is crucial for epidermal integrity and maybe impaired in pemphigus (Spindler et al. 2009). Taken together Dsc3 seems to be a promising antigenic target in pemphigus and further knowledge is required to address the pathologic relevance of Dsc3-specific auto-Abs in inducing a blistering phenotype.

1.5 Current therapeutic strategies in pemphigus

In PV, systemic corticicosteroids are the drugs of choice usually in combination with an adjuvant steroid sparing immunosuppressive drug (Hofmann et al. 2009; Martin et al. 2011). The aim of every therapeutic approach is to reach complete remission independent of the clinical phenotype. Thus initial high doses of

prednisolon equivalent are applied and subsequently tapered over months according to the clinical response (Hertl et al. 2010). Common immunosuppressive drugs comprise azathioprine, mycophenolate mofetil, cyclophosphamide and methotrexate (Hofmann et al. 2009; Martin et al. 2011). Most experience derives from the use of azathioprine as it has a well proven steroid sparing effect (Chams-Davatchi et al. 2007). However its immunosuppressive effect takes 8 to 12 weeks to begin. Mycophenolate mofetil has similar therapeutic and steroid sparing abilities but in contrast to azathioprine seems to be less hepatotoxic (Beissert et al. 2006; Beissert et al. 2010). For the treatment of refractory cases cyclophosphamide appears to be a possible alternative when used as a pulse therapy (Pasricha et al. 1988; Fleischli et al. 1999). Its potential is comparable to the one of azathioprine and mycophenolate mofetil however it harbours a higher risk for the development of toxic side effects like hemorrhagic cystitis, bone marrow depression, teratogenesis and an elevated risk for tumor diseases (Hertl et al. 2010). Systemic and topic application of cyclosporin A lead to a good response in some cases but a combination with prednisolon shows no significant advantage in relation to a monotherapy with steroids (Ioannides et al. 2000). Although methotrexate has been proven effective in PV treatment the necessity of high doses in some cases increased its toxic side effects immensely (Smith et al. 1999). The usage of high dose intravenous immune globulines (IVIG) for the treatment of problematic refractory cases of PV and PF has been successfully used as an adjuvant immune-modulatory therapy (Jolles 2001; Ahmed et al. 2006; Enk et al. 2009; Amagai et al. 2009b). IVIG therapies of 2-4 months induced significant disease regression and lead to a better response to a subsequent treatment with immune-suppressive drugs. Even in steroid-resistant cases IVIG treatment was able to achieve a better disease control (Ahmed et al. 2006; Enk et al. 2009; Amagai et al. 2009b). Further therapeutic strategies are based on the elimination of circulating IgG auto-antibodies using protein A columns or globaffin Absorber (Luftl et al. 2003; Schmidt et al. 2003; Eming et al. 2006b). These immunoadsorption techniques remove large amounts of IgG from the patients' sera including those auto-Abs responsible for blister induction (Eming et al. 2006a). A combination of adjuvant immunoadsorption with an immunosuppressive base line therapy is able to achieve an earlier disease

control in refractory pemphigus cases (Zillikens et al. 2007). Finally the B-cell depleting anti-CD20 monoclonal antibody rituximab has been successfully used to treat patients suffering from severe pemphigus. B-cell depletion lasts up to 6-9 months and correlates with reduced auto-ab titers and clinical improvement (Ahmed et al. 2006; Joly et al. 2007). Furthermore long term remission could be achieved in several cases (Espana et al. 2004). Since its profoundly positive effect on disease progression and its relatively mild side effects rituximab is likely to become a first line drug in the treatment of pemphigus (Hertl et al. 2008).

1.6 Aims of this study

Since cis-interactions between adjacent Dsg3 melocules at the EC4/EC5 extracellular site seem to be crucial for maintaining cellular adhesion (Al-Amoudi et al. 2007), it is prompting to further investigate the pathogenic relevance of pemphigus IgG directed against COOH-terminal epitopes of Dsg3. Furthermore, there is emerging evidence that desmocollins seem to play an essential role in maintaining desmosomal adhesion and may represent crucial antigenic targets for auto-Abs in pemphigus patients (Chen et al. 2008; Spindler et al. 2009). Since there is no real antigen specific therapy to treat pemphigus patients so far, it is really important to further elucidate potential antigenic targets within this disease. However, the knowledge about a certain antigen reactivity of Pemphigus IgG is not sufficient to allow a conclusion about its' pathogenic relevance. Therefore investigation of isolated highly antigen specific auto-Abs using pathogenicity assays are needed to answer this question. We therefore articulated the following aims of this study:

1) establishing a suitable technique to isolate domain-specific auto-Abs in PV patients

2) detection and isolation of auto-Abs in pemphigus sera specific for carboxy-terminal epitopes of Dsg3

3) application of this method to sera of four atypical pemphigus patients, in order to isolate Dsc3-specific auto-Abs

4) investigation of the pathogenic capacity of antigen-specific isolated auto-Abs in an in vitro assay using cultured human keratinocytes

2 Materials and Methods

2.1 Pemphigus patients and healthy controls

For the characterisation of Dsg3-subdomain-specific auto-Abs we included pemphigus vulgaris patients whose diagnosis was based on clinical, serological and histological charactersistics. Patients presented with mucous membrane lesions and/or flaccid blisters and erosions on erythematous skin, as well as high IgG-titers against the recombinant Dsg3-ectodomain. Biopsies from affected and perilesional skin revealed split formation within the basal and suprabasal epidermal layers and immunofluorescence detected IgG-deposits at the surface of epidermal keratinocytes. Finally indirect immunofluorescence on monkey esophagus showed positive intercellular staining for IgG of epithelial cells. Serological characteristics of all screened PV patients using ELISA are shown in figure 3.3. Figure 3.2 shows representative clinical, histopathological and immunofluorescence pictures of PV patient 1.

Four Japanese patients with atypical pemphigus (two pemphigus vegetans and two pemphigus herpetiformis) were investigated in cooperation with the laboratory of Dr. Takashi Hashimoto (Department of Dermatology, Kurume University School of Medicine, Fukuoka, Japan) who performed the clinical and serological analysis of the patients. Assessment of the clinical conditions revealed exophytic skin erosions of the fingers and oral erosions (patient 1), hypertrophic vegetative (verrucous) plaques with pustules and erosions on the foot, groin and scalp (patient 2), widespread erythema, pustules and erosions of the oral mucosa (patient 3) and multiple erythematous erosions on trunk and extremities (patient 4). The clinical diagnosis of pemphigus was confirmed by: histopathological evidence of intraepidermal loss of adhesion, IgG deposits on the surface of epidermal keratinocytes of perilesional skin by direct immunofluorescence, and cell surface IgG reactivity on Dsc3 transfected COS7-cells by indirect immunofluorescence. Clinical and immunoserological characteristics of the atypical pemphigus patients are shown in table 3.2. Except for patient 2 who also had Dsg1-reactive IgG, all the other patients showed predominantly IgG reactivity to Dsc3. Shown in figure 3.12 are representative clinical pictures of the studied patients. All patients gave written consent to participate in this investigation, which adhered to the Declaration of Helsinki Guidelines and was approved by the local ethics committee.

2.2 Antibodies and enzymes

Primary antibodies:

- monoclonal mouse anti-Dsc3 (clone: U114, 1µg/ml; Progen Biotechnik GmbH, Heidelberg, Germany)
- monoclonal mouse anti-E-Tag antibody (1:1000; Amersham Biosciences, Uppsala, Sweden)
- monoclonal mouse anti-Dsg3 (clone: AK23, 1µg/ml; kindly provided by Dr. Masayuki Amagai, department of Dermatology, Graduate School of Medicine, Keio University, Tokyo, Japan)

Secondary antibodies:

- horseradish peroxidase(HRP)-conjugated goat anti-human IgG (1:5000; Dako, Glostrup, Denmark)
- horseradish peroxidase(HRP)-conjugated rabbit anti-mouse IgG_1 (1:2000; Dako, Glostrup, Denmark)
- FITC-labeled goat anti-human IgG (1:200; dianova, Hamburg, Germany)
- FITC-labeled rabbit anti-mouse IgG_1 (1:500; dianova, Hamburg, Germany)

Enzmymes

- recombinant exfoliative toxin A (0,5µg/ml; Toxin Technology, Sarasota, FL)
- dispase I (1U/ml; Roche Applied Sciences, Mannheim, Germany)

2.3 Solutions, buffers, media and technical devices

Affinity chromatography column preparation

Buffer	composition
Swelling buffer	1mM HCl in Millipore-H_2O
Coupling buffer	0.1M $NaHCO_3$ + 0.5M NaCl in Millipore-H_2O, pH:8.3
Blocking buffer (a)	0.1M Tris-HCl in Millipore-H_2O, pH:8.0
Washing buffer 1	0.1M Acetat + 0.5M NaCl in Millipore-H_2O, pH:4.0
Washing buffer 2	0.1M Tris-HCl + 0.5M NaCl in Millipore-H_2O, pH:8.0
Washing buffer 3	0.5mM $CaCl_2$ in 1xPBS, pH:7.4
Storing buffer	0.01% NaN_3 + 0.5mM $CaCl_2$ in 1xPBS or 25% Ethanol + 0.5mM $CaCl_2$ in 1xPBS

Antigen specific Immunoadsorption

Buffer	composition
Washing buffer I	0.5mM $CaCl_2$ + 0.05% Tween in 1xPBS, pH:7.4
Washing buffer II	0.5mM $CaCl_2$ in 1xPBS, pH:7.4
Elution buffer	100mM Glycin/HCl in Millipore-H_2O, pH:2.7
Neutralising buffer	1M Tris/HCl in Millipore-H_2O, pH:9.0
Storing buffer	0.01% NaN_3 + 0.5mM $CaCl_2$ in 1xPBS or 25% Ethanol + 0.5mM $CaCl_2$ in 1xPBS
Regeneration buffer I	0.1M Tris-HCl + 0.5M NaCl, in Millipore-H_2O, pH:8.5
Regeneration buffer II	0.1M NaAcetate + 0.5M NaCl, in Millipore-H_2O, pH:4.5

ELISA analysis

Buffer	composition
Coating buffer	0.5 mM $CaCl_2$ in 1xPBS, pH:7.4
Washing buffer I	0.5 mM $CaCl_2$ + 0.05% Tween in 1xPBS, pH:7.4
Blocking buffer (b) / Reagent diluent	0.5 mM $CaCl_2$ + 0.05% Tween + 5% skimmed milk powder in 1xPBS, pH:7.4
Peroxidase substrate	ABTS: 2,2'-azino-di(3-ethylbenzthiazoline-6-sulphonate)

Immunoblot analysis

Buffer	composition
Deterging agent / probe buffer	sodium dodecyl sulphate (SDS)
Protein separation agent	polyacrylamide gel (10 and 12,5%, respectively)
Transfer buffer	25 mM Tris + 190 mM glycine in 20% Methanol
Blocking buffer (b) / Reagent Diluent	0.5 mM $CaCl_2$ + 0.05% Tween + 5% skimmed milk powder in 1xPBS, pH:7.4
Washing buffer I	0.5 mM $CaCl_2$ + 0.05% Tween in 1xPBS, pH:7.4
Peroxidase substrate	peroxide solution + luminol reagent (1/1) (Millipore)

Immunofluorescence studies

Buffer/medium	composition
Cultering medium	Dulbecco's modified Eagle's medium (DMEM high glucose, Invitrogen, Carlsbad, CA) + 10% fetal calf serum (PAA, Pasching, Austria) + 50 units/ml penicillin-G + 50 µg streptomycin and 2 mM L-Glutamine (Gibco, Karlsruhe, Germany)
Growing medium	Epidermal keratinocyte medium (CnT-57 medium; CELLnTEC Advanced Cell Systems, Bern, Switzerland)
Adhesion induction medium (high calcium medium)	keratinocyte medium (CnT-02 medium; CELLnTEC Advanced Cell Systems, Bern, Switzerland) + 1.2 mM $CaCl_2$
Washing buffer (a)	1.2 mM $CaCl_2$ + 1.0 mM $MgCl_2$ $6H_2O$ in 1xPBS
Fixative solution	1% paraformaldehyde in 1xPBS
Membrane solubilisation solution	0.5 % Triton X-100 in 1x PBS
Secondary antibody solution	1% bovine serum albumine (BSA) (dianova, Hamburg, Germany) + 1.2 mM $CaCl_2$ + 1.0 mM $MgCl_2$ $6H_2O$ in 1%PBS
Mounting medium	Dako, Glostrup, Denmark

Keratinocyte dissociation assay

Buffer/medium	composition
Growing medium	epidermal keratinocyte medium (CnT-57 medium; CELLnTEC Advanced Cell Systems, Bern, Switzerland)
Adhesion induction medium	keratinocyte medium (CnT-02 medium; CELLnTEC Advanced Cell Systems, Bern, Switzerland) + 1.2 mM $CaCl_2$
Washing buffer (b)	1xPBS, pH:7.4
Cell detachment solution	Dispase I, 1U/ml in 1xPBS (Roche Applied Sciences, Mannheim, Germany)
Fixative solution	10% formalin in Millipore-H_2O
Staining solution	Crystal violet (1:100) in Millipore-H_2O

Technical devices

Device	company
Analytical balance 770	Gottl. Kern & Sohn GmbH, Balingen-Frommern
Blotting chamber	Biometra, Göttingen
Clean bench HERAsafe®	Heraeus Kendro Laboratory Products GmbH, Langenselbold
CO_2-incubator HERAcell® 150	Heraeus Kendro Laboratory Products GmbH, Langenselbold
ELISA reader Multiskan Ex with Multiskan Ascent sowtware 2.6	Thermo Electron Corporation, Dreieich
Electropheresis chamber, Mini-Protean3 Dodeca™ Cell	Bio-Rad, München
Electropheresis chamber, Perfect Blue™ Gelsystem MaxiM	PeqLab, Erlangen
Fluorescence microscope	Nikon, Düsseldorf
Gel documentation system ChemiSmart 2000	PeqLab, Erlangen
Inverted microscope Invertoskop	Carl Zeiss MicroImaging GmbH,

ID03	Göttingen
Magnetic mixer IKAMAG® RET	IKA Labortechnik, Staufen
Multichannel pipette Discovery 20-200µl	ABIMED, Langenfeld
Multipipette plus	Eppendorf, Hamburg
Neubauer counting chamber	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen
pH-meter CyberScan pH 510	Eutech Instruments Europe bv, Nijkerk, Niederlande
Pipettor BD Falcon™ Express™	Becton Dickinson GmbH, Heidelberg
Pipettes Research® 10µl, 100µl, 200µl, 1000µl	Eppendorf, Hamburg
Reflected-light microscope Axiostar	Carl Zeiss MicroImaging GmbH, Göttingen
Refrigerated centrifuge Megafuge 1.0 R	Heraeus Kendro Laboratory Products GmbH, Langenselbold
Tumbler	Heidolph Instruments, Schwabach
Thermalcycler Touch Down	Thermo Electron Corporation, Dreieich
Vortex IKA® Genius 3	IKA Labortechnik, Staufen

2.4 Expression and purification of recombinant proteins

The recombinant Dsc and Dsg proteins, including the subdomains Dsg3EC1, Dsg3EC4 and Dsg3EC5 were expressed in insect cells (High Five; Invitrogen, Carlsbad, CA) by infection with recombinant baculoviruses, as previously described and were kindly provided by Dr. Ralf Müller (Muller et al. 2006). In short: Culture supernatants of infected High Five cells were collected after 3 days and recombinant His-tagged proteins were purified by affinity chromatography using nickel-nitrilotriacetic-linked agarose beads (Quiagen, Hilden, Germany) according to the manufacturer's instructions. Purified proteins were gradually dialysed against phosphate-buffered saline (PBS) supplemented with 0.5 mM $CaCl_2$ and stored at -20°C (Muller et al. 2006). By immunoblot analysis, the purified recombinant proteins were detected at the expected size and were specifically immunoreactive using a monoclonal mouse anti-Etag antibody (1:1000; Amersham Biosciences, Uppsala, Sweden) (Muller et al. 2006). In addition, serum of a patient with paraneoplastic pemphigus showed reactivity with recombinant Dsc1-3, Dsg1 and Dsg3 proteins (Figure 3.11 B, lower panel).

2.5 Purification of antigen-specific auto-antibodies from pemphigus sera

2.5.1 Affinity chromatography

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein and a specific ligand coupled to a chromatography matrix (Affinity Chromatograpy Handbook, GE Healthcare, Little Chalfont, United Kingdom). Biological interactions between ligand and target molecule can be a result of electrostatic or hydrophobic interactions, van der Waals' forces and/or hydrogen bonding. To elute the target molecule from the affinity medium the interaction can be reversed, either specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target molecules can be purified from complex biological mixtures, such as serum. A biospecific ligand is required that can be covalently attached to a chromatography matrix (Affinity Chromatograpy Handbook, GE Healthcare). The coupled ligand must retain its specific binding affinity for the target molecule and, after washing away unbound material, the binding between the

ligand and the target molecule must be reversible to allow the target molecule to be removed in an active form. Any component can be used as a ligand to purify its respective binding partner (Affinity Chromatograpy Handbook, GE Healthcare). A common method to isolate human antibodies from biological samples is purifying them via their fragment crystallizable (Fc)-region. In this case proteins with high affinity to the Fc-region, such as protein A or protein G, are used as antibody ligands which are bound covalently to a sepharose matrix. However this type of purification is unspecific since the Fc-region of an antibody is independent of its antigen-specificity. To achieve an antigen-specific isolation of human antibodies the antigen itself must be coupled onto the matrix as a ligand. By incubating this antigen-loaded column with a serum sample, antigen specific antibodies bind to the ligand via their fragment antigen-binding (Fab)-fragment and can be isolated specifically. A very suitable coupling method is to couple the ligand via its primary amine group to a pre-activated matrix, for example CNBr-activated sepharose (Amersham Biosciences, Uppsala, Sweden).

2.5.2 Preparation of affinity chromatography columns

In this study recombinant human Dsc- and Dsg-proteins as well as the Dsg3 extracellular subdomains Dsg3EC1 and Dsg3EC4 were used as ligands and were covalently attached to CNBr-activated sepharose 4B (Amersham Biosciences, Uppsala, Sweden) serving as the chromatography matrix. Sepharose 4B is a pre-activated medium for immobilization of ligands containing primary amines, like proteins, peptides and nucleic acids. The affinity columns were prepared according to the manufacturers instructions as following: The appropriate amount of sepharose 4B was diluted in 1mM HCl and let swell for 10 minutes. Washing with low pH preserves the activity of the reactive groups, which otherwise hydrolyse at high pH. 1g lyophilized CNBr-activated sepharose 4B yielded approximately 3,5ml final volume of sepharose medium and 5-10mg ligand/protein was being used for 1ml sepharose medium. Swollen medium was transferred into a 10ml-Polypropylen-column, which had been equipped with a frit to hold back the sepharose and let sediment completely. The sepharose was now being washed for 15 min with 1mM HCl and subsequently with two column volumes of 0,1M $NaHCO_3$ + 0,5M NaCl,

pH:8,3 (coupling buffer). Prior to coupling, the ligands (recombinant Dsc3, Dsg3, Dsg3EC1, Dsg3EC4) were dialysed against coupling buffer and incubated with the sepharose medium over night at 4°C on a tumbler. The next day, the coupled sepharose was subjected to 5 washes with coupling buffer followed by incubation with blocking buffer (0,1M Tris-HCl, pH: 8.0) for 2 hours at room temperature to remove any remaining covalent binding sites. The coupled sepharose was then washed 4 times with 0.1M acetate + 0.5M NaCl at pH 4.0 and 4 times with 0.1M Tris-HCl + 0.5M NaCl at pH 8.0 alternately. A final washing using PBS containing 0.5mM $CaCl_2$ at pH 7.4 was performed and then the columns were loaded with storing buffer and stored at 4°C in a fridge.

2.5.3 Auto-antibody purification

Pemphigus sera were diluted at 1:5 in PBS containing 0.5 mM $CaCl_2$ at pH 7.4. The respective column was incubated with the diluted sera overnight at 4°C on a tumbler. The next day, the preadsorbed sera were collected and the column was washed 3 times with PBS containing 0.5 mM $CaCl_2$ + 0.05% Tween 20 (PBST) at pH 7.4 and, subsequently, 3 times with PBS containing 0.5 mM $CaCl_2$ at pH 7.4. Bound Abs were eluted using 100 mM Glycin-HCl (pH 2.7) and 900 µl aliquots were collected in tubes containing 100 µl of 1 M Tris-HCl (pH 9.0). The collected aliquots were tested for eluted Abs by western blot analysis (see below). The aliquots containing Abs were combined and dialysed overnight against PBS with 0.5 mM $CaCl_2$ at pH 7.4. The purified IgG fractions were concentrated using Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA). The protein concentration of the eluted Abs was measured using a modified Lowry protein assay. Final protein concentrations ranged between 100 and 200 µg/mL.

2.5.4 Determination of protein concentrations

In order to quantitatively determine the protein concentrations of our eluted antibodies we used a modified Lowry assay (DC protein assay, Bio-Rad Laboratories, Hercules, CA). The principle of this assay is based on a chemically induced colour change which is dependent on the protein concentration of a certain sample and can be measured using photometric analysis. A serial dilution of bovine serum albumin (BSA, 1360µg/ml) has been

used as a standard. Dilution series as well as eluted Ab samples were pipetted in duplicates onto flat bottom microtiter plates (Corning Acton, USA) (5µl/ml). Subsequently 25µl of reagent A and 200µl of reagent B were added into each well. After 15min incubation time protein concentrations could be measured photometrically at 655nm.

2.6 Enzyme-linked immunosorbent assay (ELISA)

This assay is using an enzyme as a marker to determine antigen or antibody concentrations on the basis of the (enzyme) substrate turnover (Bratke 2009). There are different techniques of an ELISA assay in which either the antigen or the antibody is adsorbed to a solid phase (Bratke 2009). A competitive assay is suitable for measuring antigens as only one antigen-specific antibody is needed. However the antigen itself must harbour a marker to be detected. The sandwich assay instead uses a capturing and an enzyme linked detection antibody respectively to measure antigen concentrations. Finally to detect antibodies, for example within hybridoma supernatants or human sera, the direct assay is most suitable (Bratke 2009). Here the antigen is being immobilized on a solid phase and incubated with the antibody containing solution. After a certain incubation time in which the specific antibodies can bind to the antigen, unbound particles are washed away. Finally these specifically bound antibodies are detected using a secondary enzyme linked detection antibody which binds to the Fc-portion of the primary antigen bound antibody. The amount of substrate turnover correlates with the amount of the enzyme linked secondary antibody and thus as well with the antigen bound primary antibody (Bratke 2009). In this work the direct ELISA was being used as a qualitative method to detect Dsg- or Dsc-specific antibodies within human sera and antibody eluates respectively.

The recombinant proteins - Dsg3, Dsg3EC1, Dsg3EC2, Dsg3EC3, Dsg3EC4, Dsg3EC5, Dsc1, Dsc2, Dsc3 and collagen 7 were immobilized on 96-well polystyrene plates (Maxisorb Immunoplate; Nunc/Thermo Fisher Scientific, Wiesbaden, Germany) by coating each well with 0,5 µM of recombinant protein in PBS with 0.5mM $CaCl_2$ at 4°C over night. For conformation testing, the recombinant Dsg proteins and Dsg3 subdomains were coated onto microtitre plates in the presence of 3M urea or 10mM ethylenediaminetetraacetic acid

(EDTA). ELISA plates were then washed 6 times with PBST with 0.5mM $CaCl_2$ and blocked for 1 hour with 100µl of 5% skimmed milk powder in PBST with 0.5mM $CaCl_2$ (blocking buffer) at room temperature (for denaturation experiments Ca^{2+}-free washing and blocking buffers were used for the particular plates). Subsequently plates were incubated with pemphigus patients and control sera (1:50), as well as affinity-purified antibodies (1:2) or anti-E-tag monoclonal antibody (1:1000; Amersham Biosciences, Uppsala, Sweden) diluted in blocking buffer. After overnight incubation and 6 washing steps (as described above), plates were incubated with a horseradish peroxidase-labelled goat anti-human IgG (1:5000; Dako, Glostrup, Denmark) or rabbit anti-mouse IgG1 (1:2000; Dako, Glostrup, Denmark) respectively for one hour at room temperature. Plates were then subjected to a final washing (as described above) and IgG binding was visualized by adding the HRP-substrate ABTS (Calbiochem, Schwalbach, Germany). Plates were allowed to incubate for 30 minutes at room temperature before finally reactivity was measured at an optical density (OD) of 405nm (ELISA Reader Multiskan Ex with MultiskanAscent Software 2.6; Thermo Electron Corporation, Dreieich, Germany). Samples were run in duplicates and for evaluation we used mean values.

2.7 Western blot analysis

Western blotting in combination with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a useful technique to separate and detect different proteins according to their molecular weight (western blotting guide, abcam plc, Cambridge, United Kingdom). Prior to separation proteins are incubated with a deterging agent, normally the negatively charged sodium dodecyl sulphate (SDS). SDS denatures proteins by “wrapping around” the polypeptide backbone. It confers a negative charge to the polypeptide in proportion to its length. Therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight (western blotting guide, abcam plc, Cambridge, United Kingdom). Samples are subsequently subjected to polyacrylamide gel-electrophoresis (PAGE) which separates the denatured proteins according to size. The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The pore

size of a gel is determined by two factors: the total amount of acrylamide present and the amount of cross-linker. When the denatured proteins have been transferred into the gel, the latter is subjected to an electric field causing the negatively-charged proteins to migrate across the gel towards the positive electrode (anode). Depending on their size, each protein will move differently through the gel matrix: short proteins will more easily fit through the pores in the gel, while larger ones will have more difficulty. The proteins are then transferred to a nitrocellulose membrane and detected using antibodies specific to the target protein (western blotting guide, abcam plc, Cambridge, United Kingdom).
The recombinant Dsg- and Dsc-proteins were run in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis system (10% and 12,5% gel, respectively) and blotted onto nitrocellulose membranes. Membranes were blocked with 5% milk powder in PBS, 0.05% Tween-20 (PBS-T) with 0.5mmol/L $CaCl_2$. Pemphigus sera (1:100), purified antibodies (1:20) and the monoclonal mouse anti-E-Tag antibody (1:1000; Amersham Biosciences, Uppsala, Sweden) were diluted in PBS-T with 0.5mmol/L $CaCl_2$ and 5% milk powder and were incubated with the blotted membranes overnight at 4°C. After three washes with PBS-T supplemented with 0.5mmol/L $CaCl_2$, the nitrocellulose membranes were incubated with horseradish peroxidase(HRP)-conjugated anti-human IgG (1:5000; Dako, Glostrup, Denmark) or HRP-conjugated anti-mouse IgG_1 (1:2000; Dako, Glostrup, Denmark) respectively for 1 hour at room temperature. Specific immunoreactivity was then visualized using a commercial HRP subtrate (Immobilon Western Chemiluminescent HRP substrate; Millipore, Billerica, MA).

2.8 Immunofluorescence studies

Since recombinant proteins, even when expressed in an eucaryotic system, cannot fully imitate the native expression and conformation within living cells additional assays are needed to confirm the binding of the eluted Abs to native antigens, i.e. Dsg3 and Dsc3. Immunoflurescence studies using living cells or tissues sections are suitable to detect antibody binding to native intra and/or extracellular antigens. Substrate cells are fixed on a slide and incubated with a blocking solution to avoid unspecific binding. Subsequently cell supernatants or sera are incubated on the blocked substrates for a certain amount of time. Finally after several washing steps bound antibodies are detected using a

secondary antibody coupled with a fluorescent dye. This signal is captured by a fluorescence microscope which harbours a certain wavelength filter. The type of the fluorescence pattern in correspondence to a positive control antibody gives information about the occurrence of antigen specific antibodies within the probes.

The immortalized keratinocyte cell line HaCaT was cultured in Dulbecco's modified Eagle's medium (DMEM high glucose, Invitrogen) supplemented with 10% fetal calf serum (PAA), 50 units/ml penicillin-G, 50 µg streptomycin and 2 mM L-Glutamine (Gibco). For immunofluorescence staining, HaCaT cells were grown subconfluently in chamber slides (Nunc/Thermo Fisher Scientific) using epidermal keratinocyte medium (CnT-57 medium; CELLnTEC Advanced Cell Systems). Subconfluent keratinocyte cultures were switched to defined keratinocyte medium (CnT-02 medium; CELLnTEC Advanced Cell Systems) supplemented with 1.2 mM $CaCl_2$ 24h prior to adding the IgG fractions. Keratinocytes were then incubated for 2 hours at 4°C with purified antibodies diluted at 1:2, pooled IgG from healthy donors 1:2 diluted or anti-Dsc3 monoclonal ab (clone U114, 1µg/mL; Progen) and anti-Dsg3 monoclanal ab (AK23, 1µg/ml; Dr. Amagai, Keio University Tokyo, Japan), respectively. After 3 washes with PBS containing 1.2 mM $CaCl_2$ and 1.0 mM $MgCl_2$ $6H_2O$, keratinocytes were fixed in 1% paraformaldehyde-PBS for 20 minutes at room temperature. After 3 washes, cells were incubated with PBS containing 0.5 % Triton X-100 for 10 minutes at room temperature and were finally incubated with FITC-labelled anti-human IgG (1:200, dianova) in 1% BSA-PBS. Frozen sections of normal human skin were blocked for 30 minutes with PBS supplemented with 10% normal goat serum. Samples were incubated for 1 hour at room temperature with purified antibodies (diluted in PBS- with 2% goat serum at 1:2), IgG pooled from healthy donors at the same dilution or anti-Dsc3 monoclonal IgG (U114, 1µg/mL; Progen) as positive control for Dsc3 reactivity. After 3 washes with PBS, the skin sections were incubated with FITC-labelled rabbit anti-human IgG (1:200; dianova) for 30 min at room temperature. Indirect immunofluorescence on monkey esophagus was performed following a standardized protocol according to the manufacturer's instructions (The Binding Site, Birmingham, UK).

2.9 Dispase-based keratinocyte dissociation assay

There are several cell-free and cell-based *in vitro* approaches to study the pathogenicity of Dsg- or Dsc-specific auto-Abs. Ishii et al. presented the "In vitro keratinocte dissociation assay for evaluation of the pathogenicity of anti-Desmoglein 3 IgG autoantibodies in Pemphigus Vulgaris" (Ishii et al. 2005). In this assay primary human keratinocytes expressing high levels of Dsg1 and Dsg3 but low levels of Dsg2 are grown in 12 well plates to form a confluent monolayer. After incubation with total serum or isolated PV-IgG, the staphylococcus aureus enzyme exfoliative toxin A (ETA) which specifically cleaves Dsg1 is added so that the remaining binding forces are mostly related to Dsg3. Cell sheets are then subjected to mechanical stress and cell dissociation is achieved. The number of keratinocyte particles correlates well with the pathogenic strength of added PV-IgG since sera from patients with active disease yield much higher fragmentation as sera from patients in remission or normal human sera. To exclude interassay variability Ishii et al. also established a dissociation score based on the positive control AK23 using the following formula: dissociation score = ((number of particles with serum – number of particles without serum) / (number of particles with serum – number of particles with AK23 or U114)) x 100 (Ishii et al. 2005).

Primary human epidermal keratinocytes were seeded in 12-well plates and grown to confluence in CnT-57 medium (CELLnTEC Advanced Cell Systems). The day before the assay, cells were cultured in CnT-02 medium (CELLnTEC Advanced Cell Systems) containing 1.2 mM $CaCl_2$ and incubated with purified antibodies (at 20 µg/mL), pooled control IgG (20 µg/mL), anti-Dsg3 monoclonal ab (AK23, 1µg/ml, Dr. Amagai, Keio University Tokyo, Japan) or anti-Dsc3 monoclonal ab (U114, 1 µg/mL; Progen), overnight at 37°C. Recombinant exfoliative toxin A (Toxin Technology) was added at 0.5 µg/mL to cleave Dsg1 for the last two hours of the assay. After 2 washes with PBS, the adherent keratinocyte monolayer was incubated at 37°C for 20 minutes with dispase I (Roche Applied Sciences) resulting in a non-adherent monolayer. The monolayers were carefully washed twice with PBS and subjected to mechanical stress by pipetting ten times with a 1 mL pipet. Fragments were fixed in 1 mL of a 10% formalin solution and stained with crystal violet. Images were captured using a digital camera and fragments were counted by five different blinded

observers. Relative dissociation scores were calculated using the number of keratinocyte fragments in relation to the maximal number of cell fragments obtained by the positive control (anti-Dsg3 monoclonal ab (AK23) or anti-Dsc3 monoclonal ab (clone U114) respectively).

3 Results

3.1 Purification and characterisation of desmoglein 3 extracellular subdomain-specific auto-antibodies from pemphigus vulgaris patients' sera

First we sought to establish a setting of experiments to investigate the pathogenicity of Dsg3-specific auto-Abs, *in vitro*. To achieve this aim we isolated epitope-specific Abs from PV patients who exhibited auto-Ab reactivity against certain subdomains of the extracellular portion of Dsg3. Eight PV patients were screened for their IgG reactivity against COOH-terminal epitopes of the Dsg3 extracellular domain. Four of these sera underwent affinity purification assays to isolate and to further characterise their antigen specific IgG *in vitro*. Affinity purification was performed using recombinant Dsg3 proteins produced in a baculovirus expression system (Muller et al. 2006). Figure 3.1 shows a scheme of the recombinant Dsg and DsgEC proteins used in this study.

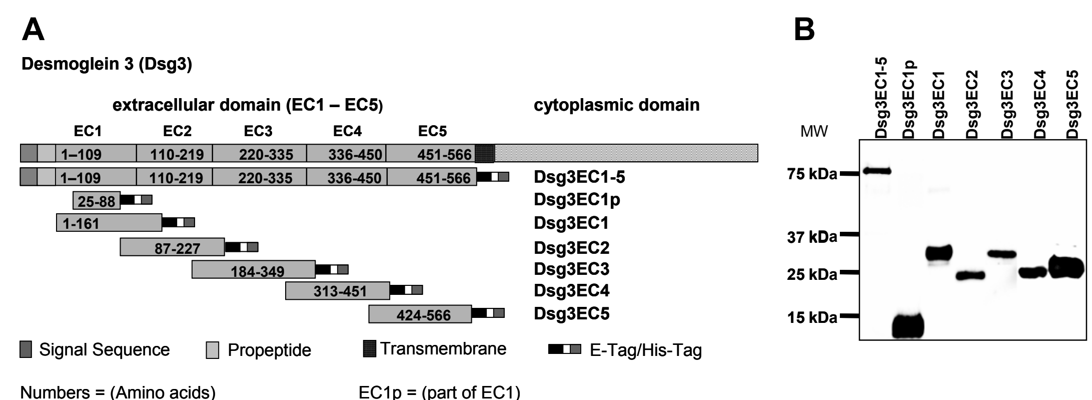


Figure 3.1: Recombinant desmoglein 3 and desmoglein 3 extracellular subdomain proteins used in this study. (A) Scheme of the recombinant Dsg3 and Dsg3EC proteins applied in this study, i.e. the entire ectodomains of Dsg3EC1-5 and the 5 different extracellular domains of Dsg3, linked to E- and 6xHis-Tag. (B) Detection of the recombinant proteins by immunoblot analysis using an anti-E-tag monoclonal antibody (Muller R. et al. 2008). Molecular weight (MW) shown in kilo Dalton (kDa).

3.1.1 Clinical and serological characteristics of pemphigus vulgaris patients

For this study we investigated the sera of eight PV patients who demonstrated high IgG titers against Dsg3. All PV patients presented with a clinical phenotype characteristic for pemphigus vulgaris, i.e. flaccid blisters and erosions on erythematous ground on the trunk and/or the mucous membranes as shown in figure 3.2. Histopathology of affected skin revealed suprabasal blister formation and intercellular IgG deposition by IF. Patients presented with active or chronic active stages of PV.

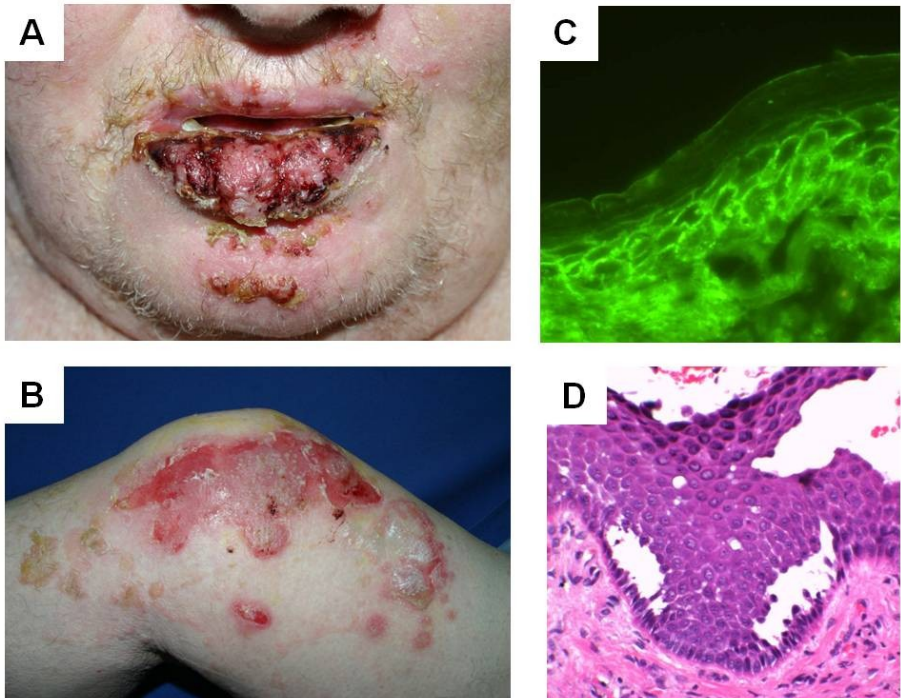


Figure 3.2: Clinical features, immunoflurescence and histopathology of selected pemphigus patients. Shown are representative clinical pictures of patient 1 with erosions on mucous membranes and the skin (A and B). Direct immunofluorescence shows IgG deposition and an intercellular staining pattern of the epidermis (C). Histology of affected skin revealed suprabasal split formation (D). Pictures are courtesy of the Department of Dermatology and Allergy, Philipps Universität Marburg, and adapted from (Kneisel et al. 2011a).

The sera were screened for the presence of auto-Ab reactiv against the Dsg3 extracellular subdomains by ELISA. As shown in figure 3.3 all patients reacted with recombinant Dsg3 whereas no unspecific protein reactivity, as shown by a negative reactivity against type 7 collagen, could be detected. Furthermore in the sera of patients 1, 2, 4 and 8 IgG reactivity against the NH_2-terminal

subdomain, Dsg3EC1, was detected. A positive reaction against the carboxy-terminal Dsg3 subdomain EC4 was seen in patient 1, 6 and 8. The optical density-(OD)-values of patient 7 were not sufficient to determine a clearly positive reactivity against any of the EC-constructs. Patients 2 and 3 showed additional IgG reactivity against Dsg1. These results lead to the selection of patients 1, 2, 6 and 8 for the following experiments since all but one (patient 2) expressed IgG-auto-Abs against the carboxy-terminal Dsg3-epitope EC4. Patient 2 was used later on as a reference for patients with exclusive reaction against NH_2-terminal epitopes of Dsg3.

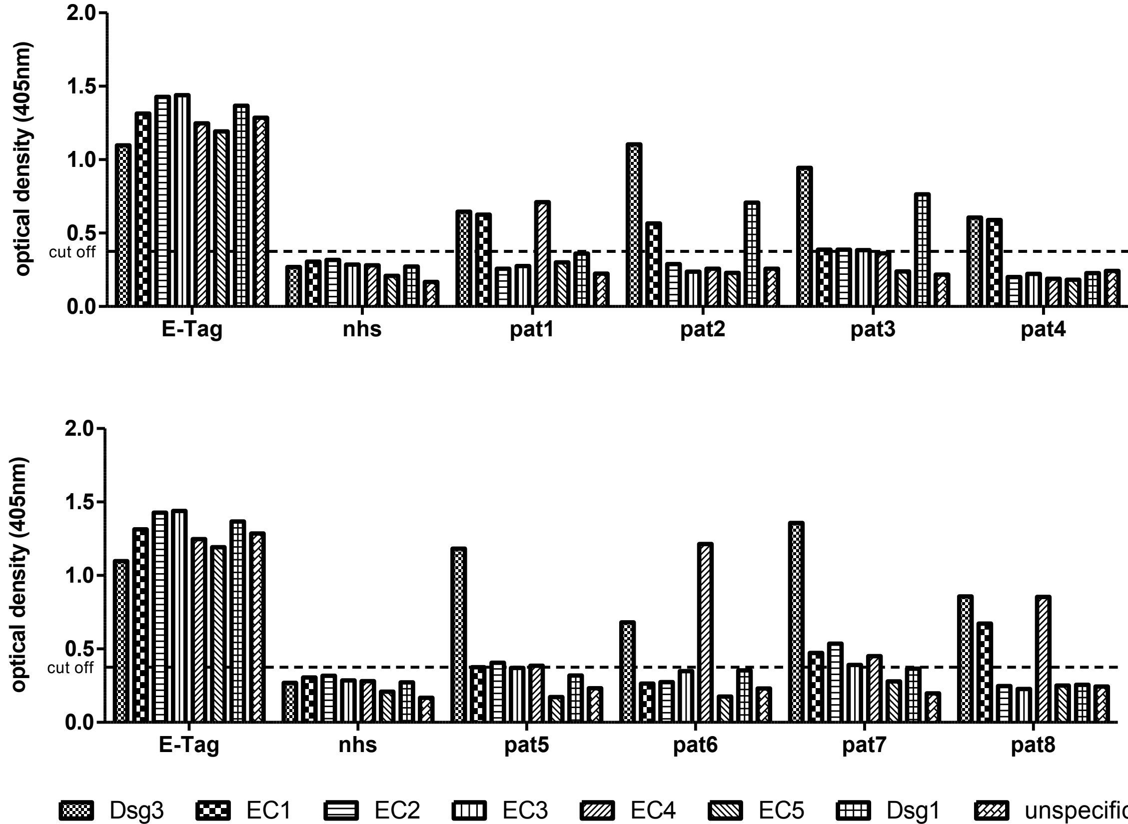


Figure 3.3: PV sera reactivity to recombinant desmoglein 3 and desmoglein 3 extracellular subdomain proteins. ELISA showing serum reactivity of screened pemphigus patients against Dsg3 and Dsg3EC-proteins. A monoclonal anti-E-Tag-ab serves as loading control. All patients show positive reactivity against recombinant Dsg3EC1-5 as expected. Patient 1, 6 and 8 show additional reactivity against the EC4 domain of Dsg3. Positive EC1 reactivity can be detected in the sera of patients 1, 2, 4 and 8. Normal human serum (nhs) serves as negative control. No unspecific protein reactivity against type 7 collagen (unspecific) could be detected. Cut off value is determined at an optical densitiy of 0.376 (405nm wavelength).

To reconfirm serum reactivity against the recombinant EC-proteins of Dsg3 in a different system we performed immunoblotting analysis for the selected PV-patients' sera. Recombinant proteins were separated by SDS-page and blotted onto nitrocellulose membranes. Pemphigus sera were incubated on the membranes overnight and IgG-reactivity was detected using a chemiluminescence system. As shown in figure 3.4 A, all patients' sera contained auto-Abs against the whole Dsg3- extracellular domain. Furthermore patient 1 and 8 exhibited reactivity against the EC1 and EC4 subdomains of Dsg3, whereas patient 2 and 6 had additional auto-Abs directed against Dsg3EC1 or Dsg3EC4, respectively. A monoclonal mouse anti-E-tag antibody indicated the molecular size of the Dsg3-constructs and served as a positive control. Normal human serum of healthy individuals showed no reactivity to any of the recombinant Dsg3-proteins. Indirect immunofluorescence studies on cultured primary human keratinocytes were performed with the patient's sera to confirm a positive reactivity with native Dsg3 as well. As seen in figure 3.4 B, all patients' sera revealed a clear intercellular staining pattern as compared to the positive control, a mAb against Dsg3 (AK23), indicating the recognition of native human Dsg3 by IgG auto-Abs. Table 3.1 shows the definitive serological profile of the PV-patients' sera.

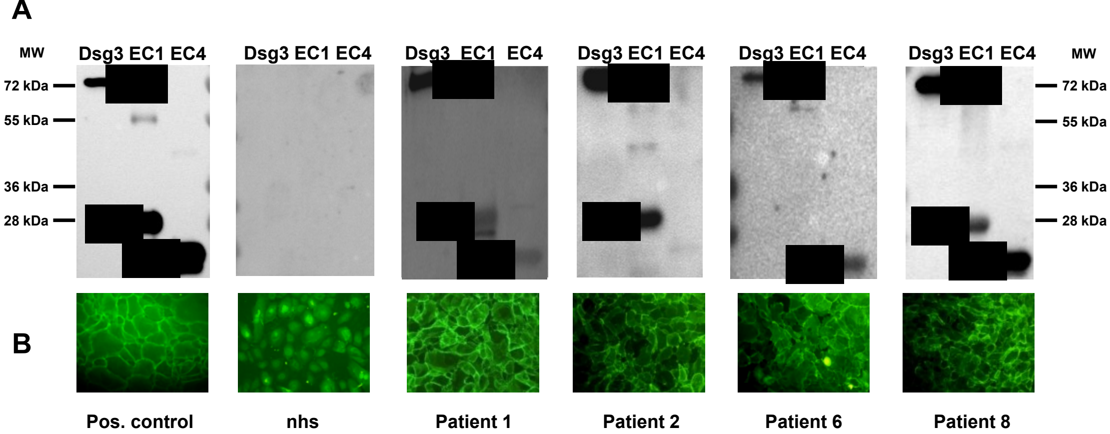


Figure 3.4: PV sera react with recombinant and native desmoglein 3. (A) Immunoblot confirming the antigen reactivity of the selected PV sera, i.e. patients 1, 2, 6 and 8 recognize the whole ectodomain of Dsg3, patients 1, 2 and 8 additionally recognize the Dsg3EC1 subdomain and finally Dsg3EC4 reactivity is detected for patients 1, 6 and 8. (B). All serum samples show a positive intercellular staining pattern using immunofluorescence studies on human primary keratinocytes indicating recognition of native Dsg3. Molecular weight (MW) in kilo Dalton (kDa). Recombinant proteins running at their expected size (arrows).

Table 3.1: Serological characteristics of selected pemphigus patients.

Patient	Dsg3-IgG ELISA/WB	Dsg3EC1-IgG ELISA/WB	Dsg3EC4-IgG ELISA/WB	Dsg3EC5-IgG ELISA/WB	IF
1	+/+	+/+	+/+	-	+
2	+/+	+/+	-/-	-	+
6	+/+	-/-	+/+	-	+
8	+/+	+/+	+/+	-	+

Summary of the auto-antibody-profile of the selected patients showing enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF) and western blot (WB) analysis of the patients' sera.

Given these findings the four PV patients suited well to isolate and compare their domain-specific Abs since the NH_2-terminal epitope Dsg3EC1 and the COOH-terminal epitope Dsg3EC4 were targeted by the selected patients' auto-Abs.

3.1.2 Purification of antigen-specific auto-antibodies via affinity chromatography

According to the serum reactivity of the patients' sera three different antigen-coupled-CNBr sepharose columns were established, namely a Dsg3-, an EC1- and an EC4-column, in order to isolate the corresponding auto-Abs. As schematically shown in figure 3.5 A, patients' sera were subjected to antibody purification according to their previously defined autoantibody profile. Dsg3-affinity purified antibodies were isolated from all the sera, whereas EC1- and EC4 affinity-purified antibodies were obtained from patients 1, 2 and 8 and 1, 6 and 8, respectively. The amount of the purified antibodies was determined using a modified Lowry protein assay and the final concentration ranged between 100 and 200 µg/mL. In addition whole IgG fractions from four healthy donors were purified using a protein A affinity chromatography column. This normal human IgG (nh-IgG) served as a negative control in the following analysis. Finally all Abs were subjected to western blot and ELISA as previuously done for the patients' sera. This step was necessary to proof the successful antigen-specific isolation of the auto-Abs and to exclude a possible unspecific reactivity against other antigens.

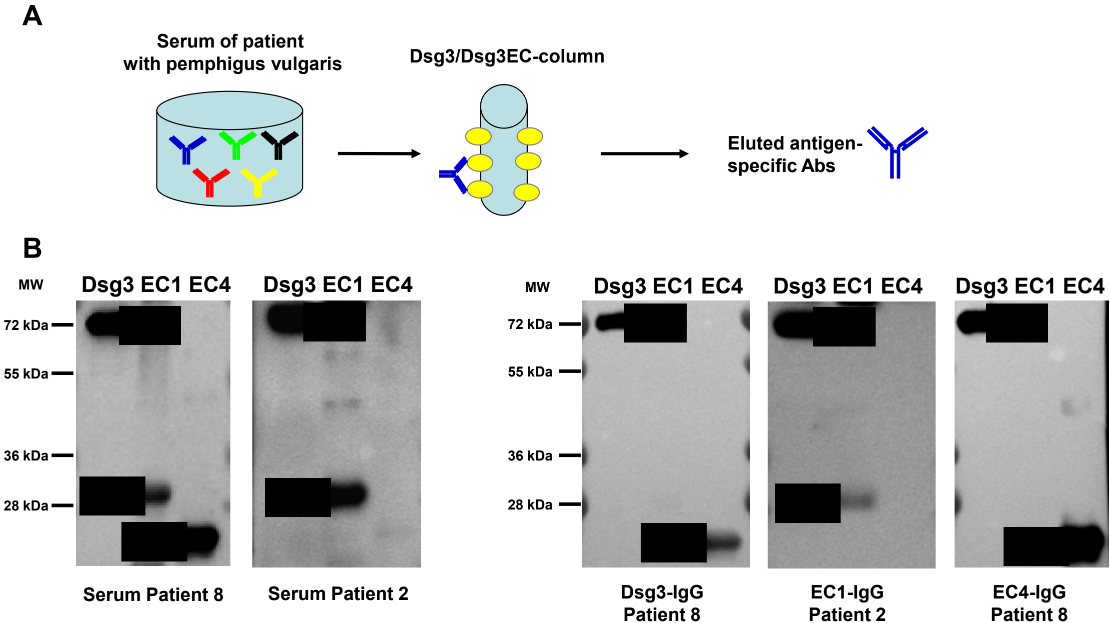


Figure 3.5: Affinity chromatography of pemphigus IgG. (A) Scheme of Dsg3-specific affinity chromatography using antigen coupled sepharose B columns. (B) Western blot reactivity of two representative pemphigus sera (patient 2 + 8) against recombinant Dsg3 proteins prior and after antigen specific purification. Purified IgG shows specific reactivity against the recombinant proteins that were used to purify them, i.e. IgG from patient 2 affinity-purified by an EC1-column shows positive reaction with EC1, Dsg3 but not EC4. Furthermore IgG from patient 8 affinity-purified by an EC4-column is specific for EC4, Dsg3 but not EC1. Molecular weight (MW) in kilo Dalton (kDa). Recombinant proteins running at their expected size (arrows).

3.1.3 Purified pemphigus vulgaris immunoglobulin G is antigen-specific for recombinant extracellular subdomains of desmoglein 3

Since all of the EC-constructs represent subdomains of the extracellular part of human Dsg3, the affinity purified Abs should always recognize the whole recombinant protein Dsg3 as well as the EC-construct which has been used for purification. Whereas Abs purified via the whole ectodomain should react with recombinant Dsg3 and one or more of the subdomains. Conformational epitope reactivity cannot be represented by this kind of western blot since the single proteins are blotted onto the membrane under denaturing conditions. Figure 3.5 B shows representative western blots of patients 2 and 8 before and after affinity purification. Serum blots on the left side demonstrate that both patients' sera have a positive IgG reactivity for the entire ectodomain of Dsg3. Patient 8 shows additional reactivity with the EC1 and EC4 subdomains whereas the serum of patient 2 contains EC1-specific IgG. As expected after purification all

isolated auto-Abs showed a strong reaction with the whole Dsg3 ectodomain. However Abs of patient 2 affinity-purified via the EC1 domain expressed additional reactivity only against EC1. The same could be demonstrated for EC4-affinity-purified Abs of patient 8. None of these subdomain-specific auto-Abs reacted with any other Dsg3 subdomain (data not shown). Successful antigen-specific purification was further reconfirmed by ELISA. All IgG eluates were tested for reactivity with Dsg3, Dsg3EC1, Dsg3EC4 and Dsg3EC5 respectively as shown in figure 3.6. It can be clearly seen that EC1- and EC4-affinity purified Abs express a strong IgG reactivity against their corresponding subdomain and the whole Dsg3 ectodomain while lacking reactivity for other subdomains, like EC5 (fig. 3.6). On the other side nh-IgG fractions did not show any positive reaction with the constructs used in this assay. Taken together these data confirmed the antigen specificity of our affinity purified Abs making it possible to subsequently investigate the pathogenic role of this highly specific IgG.

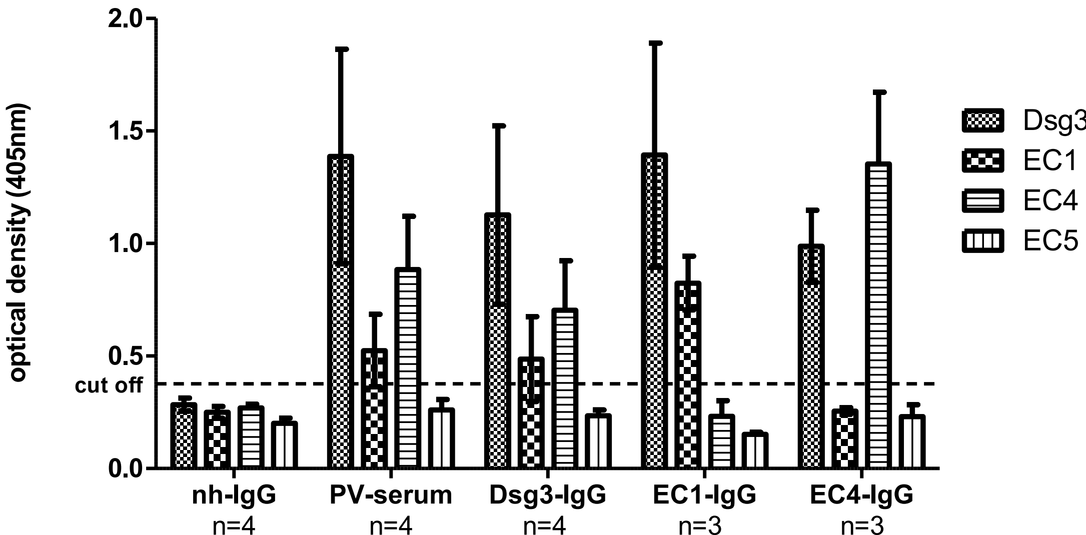


Figure 3.6: Antigen specificity of purified IgG shown by ELISA. Patients' sera and affinity purified IgG reactivity against recombinant Dsg3 proteins. Antibodies purified via EC1 (EC1-IgG) show exclusive binding to desmoglein 3 and the EC1 subdomain. Similarly, EC4 affinity purified IgG (EC4-IgG) shows positive reactivity with desmoglein 3 and EC4, respectively. Normal human IgG (nh-IgG) served as negative control and does not show any Dsg3 reactivity. Cut off value is determined at an optical densitiy of 0.376 (405nm wavelength). Optical density (OD) values are shown as mean values of the three or four affinity puried patients' IgG fractions, respectively.

3.1.4 Extracellular subdomain 4-specific auto-antibodies belong to the immunoglobulin G_1 and immunoglobulin G_4 isotype classes

Having proven the antigen specificity of the affinity purified antibodies, we now were interested in the IgG isotype profile present in the eluates. Therefore the purified antibodies of patient 1 were investigated on ELISA plates coated with the whole recombinant ectodomain of human Dsg3 (figure 3.7). Monoclonal mouse anti-human IgG Abs served to target the different human IgG isotypes, i.e. IgG_1, IgG_2, IgG_3 and IgG_4, as well as IgE and IgA. Total patients' serum contained Abs of the IgG_1, IgG_2 and IgG_4 isotypes. These isotypes were distributed within the purified Ab fractions as following: Purified Abs specific for the whole ectodomain of Dsg3 showed IgG_1 and IgG_4 reactivity. The same distribution could be obtained from Abs affinity purified via the EC4-subdomain. In contrast EC1-specific Abs belonged to the isotypes IgG_1, IgG_2 and IgG_4. IgE or IgA Abs were not present at all. The presence of IgG_1 and IgG_4 isotypes goes in line with the clinical phenotype of (chronic) active PV (Bhol et al. 1995). However the presence of EC1-specific IgG_2 Abs was surprising. Unfortunately this assay could not be performed for the purified Abs of the remaining patients due to shortage of eluted Ab material.

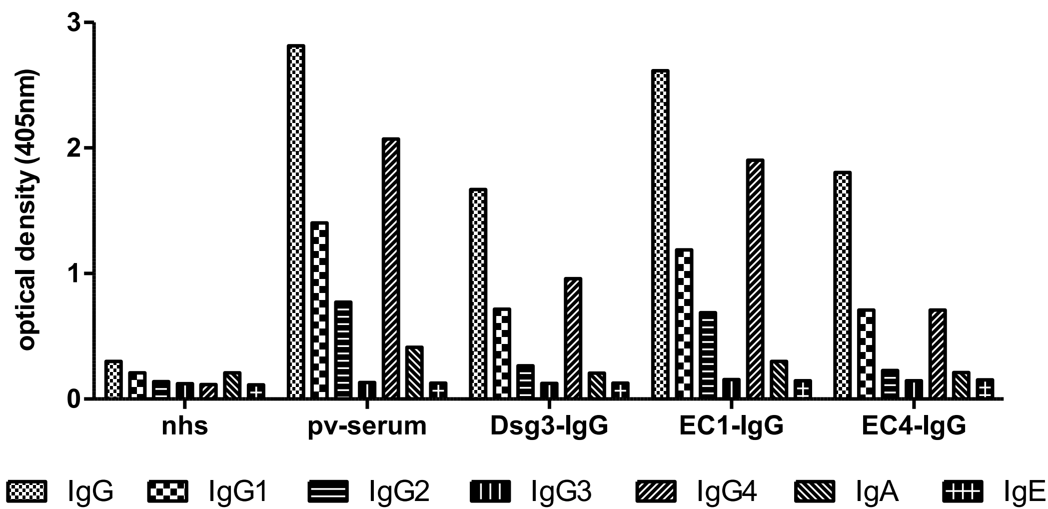


Figure 3.7: IgG isotype distribution of affinity purified pemphigus IgG. ELISA showing reactivity of affinity purified, antigen specific (Dsg3-, EC1- and EC4-IgG), total IgG and IgG isotypes from patient 1 against recombinant Dsg3. EC1-IgG contains IgG_1, IgG_2 and IgG_4 isotypes respectively whereas EC4-IgG only harbors IgG_1 and IgG_4 subclasses. IgA or IgE reactivity could not be detected. Optical densitiy measured at 405nm wavelength.

3.1.5 Recognition of desmoglein 3 extracellular subdomain 4 is calcium independent, whereas desmoglein 3 extracellular subdomain 1-reactive immunoglobulin G binds to conformational epitopes

It has been previously shown that in a cohort of 25 PV patients the pre-treatment of the whole ectodomain of Dsg3 and the Dsg3 subdomain EC3 with denaturing agents (Urea, EDTA) leads to a significantly decreased IgG-reactivity when tested by ELISA (Muller R. et al. 2008). These results demonstrated clearly the presence of Abs recognizing conformational epitopes. However the IgG-reactivity in the sera did not differ when denaturing the extracellular Dsg3 subdomains EC1, EC2, EC4 and EC5 indicating IgG auto-antibodies which recognize non-conformational epitopes. These findings prompted us to investigate the existence of conformation dependent recognition of Dsg3 in our purified Ab fractions. Therefore we coated the whole ectodomain of Dsg3 onto an ELISA plate under normal and denaturing conditions and incubated it with antigen specific Abs of patient 1 and 8 over night (figure 3.8). As suspected the serum IgG reactivity of the two patients against the whole ectodomain decreased dramatically but nevertheless did not fall under the cut of value of 0,376. A similar result could be obtained with the Dsg3-specific purified Abs indicating conformation dependent and independent epitope recognition within the Dsg3-specific Ab fraction. The most profound change in the IgG-reactivity however could be observed in the EC1-specific Ab eluate. Denaturing of Dsg3 led to almost a complete loss of epitope recognition by EC1-specific IgG which goes in line with conformation dependent epitope reactivity. Interestingly antibodies affinity purified via the EC4 subdomain did not differ in their IgG-reactivity against Dsg3 highlighting the presence of non-conformational epitope recognition within this auto-Ab subset.

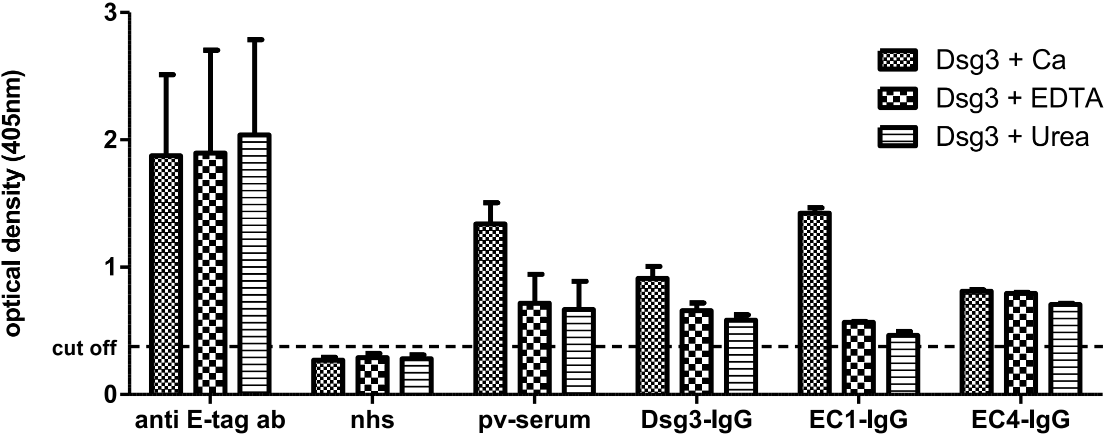


Figure 3.8: Recognition of conformational desmoglein 3 epitopes. Reactivity of sera and purified antigen specific IgG from patients 1 and 8 against Dsg3 under normal (Dsg3+Ca), Ca^{2+}-depleted (Dsg3+EDTA) and denaturing conditions (Dsg3+Urea). PV sera and Dsg3-reactive IgG show a decreased but not negative reactivity under denaturing conditions indicating the presence of conformation dependent and independent epitope recognition. Recognition of the EC1 epitope of Dsg3 seems to be conformation dependent as seen by the reduced reactivity under denaturizing and Ca^{2+}-depleted conditions. In contrast, EC4 specific IgG reactivity is not being altered by Dsg3 denaturation indicating conformation independent epitope recognition. Cut off value is determined at an optical densitiy of 0.376 (405nm wavelength). Optical density (OD) values are shown as mean values of the two affinity puried patients' IgG fractions.

3.1.6 Desmoglein 3 subdomain-specific imunoglobulin G recognizes native desmoglein 3

Since the specificity of the isolated auto-Abs for their corresponding recombinant protein has been shown in previous experiments, we now sought to find out if these Abs exhibit the ability to recognize native Dsg3 as well. For this purpose indirect immunofluorescence studies were performed on cultured human primary epidermal keratinocytes (HPEK). To proof the expression of Dsg3 and the expected staining pattern on these cells we used the mAb AK23 which is directed against the NH_2-terminal region of Dsg3. As shown in figure 3.9 AK23 stained the cell surfaces in a clear intercellular pattern, which is absolutely missing when looking at the negative control (nh-IgG). Affinity purified auto-Abs of PV patients expressed a similar staining pattern on human keratinocytes when compared with the positive control (figure 3.9). Taken together these data clearly demonstrate that Abs purified by recombinant Dsg3-

constructs are able to recognize the native Dsg3 protein suggestive of a possible effect on desmoglein mediated keratinocyte adhesion.

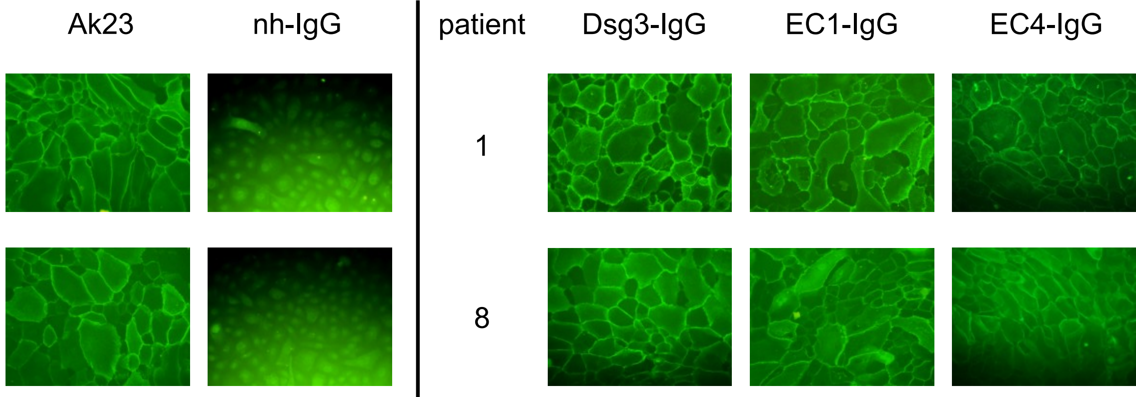


Figure 3.9: Affinity purified IgG recognizes native desmoglein 3. Immunofluorescence of human primary keratinocytes. The Dsg3-reactive monoclonal antibody AK23 serves as positive control and shows a clear intercellular staining pattern (left). Similarly antigen specific purified IgG of patients 1 and 8 shows an intercellular staining comparable with the positive control indicating recognition of native Dsg3 (right). Normal human IgG (nh-IgG) does not show any specific staining (left).

3.1.7 Desmoglein 3 extracellular subdomain 4-specific immunoglobulin G induces loss of keratinocyte adhesion *in vitro*

The positive Dsg3-staining pattern of the AK23 on HPEK made these cells suitable for an assay which can be used to demonstrate the pathogenic capacity of Dsg3-reactive IgG independent of Dsg1-driven adhesion. Ishii et al established the dispased based keratinocyte dissociation assay to investigate the pathogenic effect of patients' sera or total IgG on keratinocyte adhesion *in vitro* (Ishii et al. 2005). We therefore used this functional assay to investigate a possible acantholytic effect of our Dsg3-purified Ab fractions. Patients' sera and purified IgG were incubated with keratinocyte monolayers over night and the number of cell sheet fragments was measured on the next day after application of mechanical stress on the monolayer offering a semi-quantitive value for the *in vitro* pathogenic strength of the added Abs. Shown in figure 3.10 are the dissociation scores which reduce interassay variability and stand for a representative value. We performed the assay with the purified Abs in order to compare the pathogenic ability of EC1- and EC4-specific Abs in PV patients (figure 3.10). The mAb AK23 served as a positive control, since its acantholytic

effect has been proven to be exclusively mediated by Dsg3-driven intercellular cohesion (Tsunoda et al. 2003). As expected the AK23 showed a profound dissociation of the keratinocyte monolayer at a concentration of 1µg/ml whereas neither normal human sera nor normal human IgG lead to a significant fragmentation (figure 3.10). In contrast all patients' sera as well as all of the Dsg3- and Dsg3-subdomain specific IgG fractions induced strong keratinocyte dissociation comparable with the positive control antibody (AK23). These *in vitro* findings demonstrate that PV auto-Abs directed against the COOH-terminal subdomains of Dsg3, especially EC4, clearly impair keratinocyte adhesion to a similar extend like EC1-specific IgG.

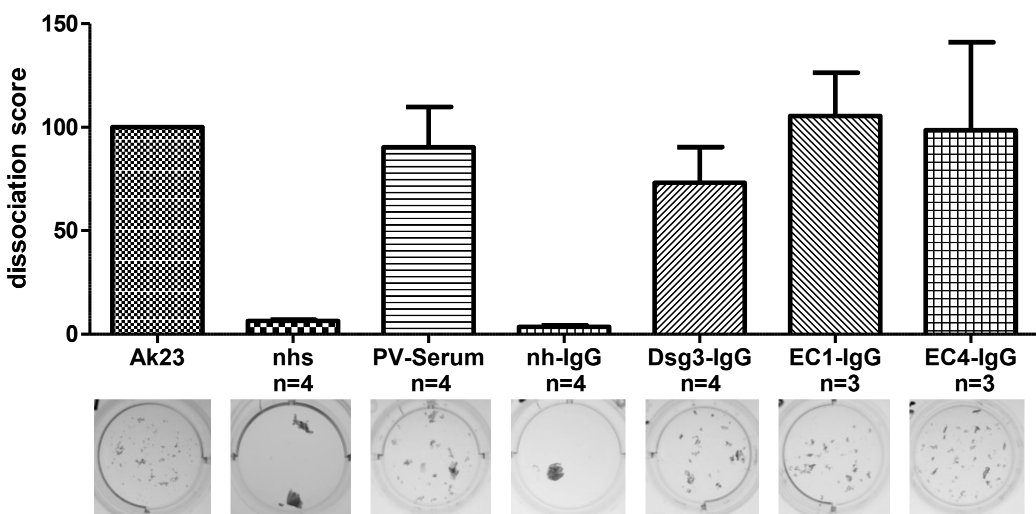


Figure 3.10: Affinity purified IgG induces loss of keratinocyte adhesion *in vitro*. PV-patients' sera as well as all affinity-purified antigen specific IgG, i.e. Dsg3-IgG, EC1-IgG and EC4-IgG, respectively, induce fragmentation of keratinocyte monolayers as illustrated by representative photographs of dissociation assays (lower panel). Pathogenicity of antigen specific IgG is quantified using the well established dissociation score (Ishii et al. 2005) which reduces interassay variability (upper panel). Results are shown as mean values of scores of three or four PV patients' sera/IgG, normal human serum (nhs) or normal human IgG (nh-IgG), respectively. EC1 and EC4 specific IgG reach similar dissociation scores indicating strong pathogenic capacity for both auto-Abs *in vitro*. The Dsg3 specific monoclonal antibody AK23 served as positive control. Neither normal human serum nor normal human IgG induce relevant acantholysis in this assay. Dissociation score = ((number of particles with serum – number of particles without serum) / (number of particles with serum – number of particles with AK23)) x 100 (Ishii et al. 2005).

3.2 Purification and characterisation of desmocollin 3-specific auto-antibodies in atypical pemphigus patients

Patients suffering from atypical pemphigus show IgG reactivity against a variety of auto-antigens including the second main group of desmosomal cadherins, the desmocollins (Dscs). Thus we were interested in characterising the pathogenic capacity of affinity purified Dsc-specific IgG using our previously established assays. When the methods to successfully affinity purify antigen specific auto-Abs from patients' sera and subsequent *in vitro* pathogenicity testing were established, we applied this set of experiments on the sera of four atypical pemphigus patients, which were a kindly provided by Dr. Takeishi Hashimoto, Departement of Dermatology, Kurume University, Japan. To identify the desmosomal target proteins recognized by the IgG auto-Abs of these patients, the sera were incubated with defined baculovirus-derived proteins including Dsg1, Dsg3, and Dsc1, Dsc2, Dsc3, respectively. Figure 3.11 shows a schematic illustration and western blot-detection of these proteins by anti-E-Tag mAb and serum of a PNP patient.

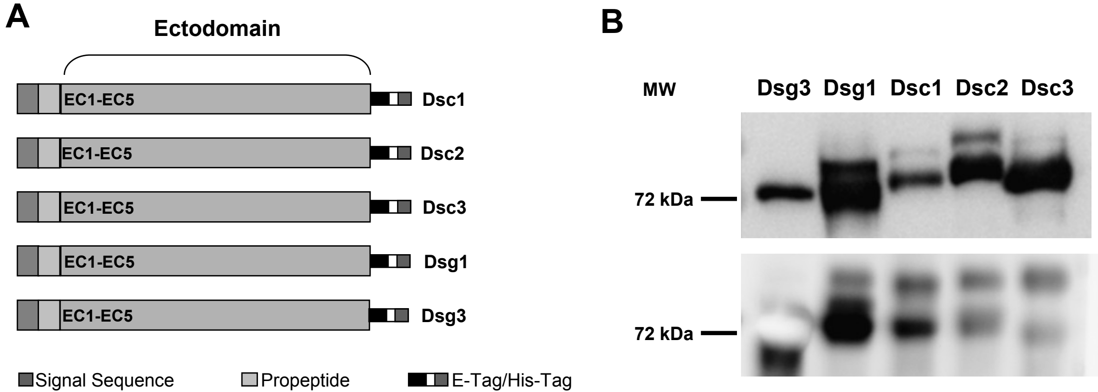
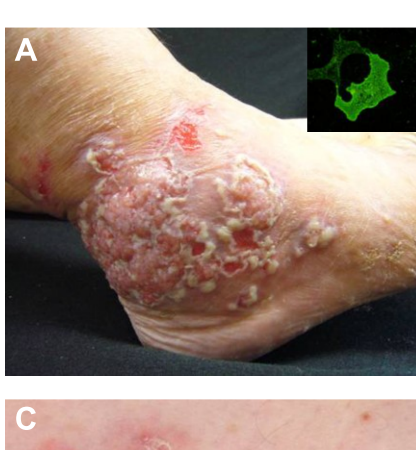
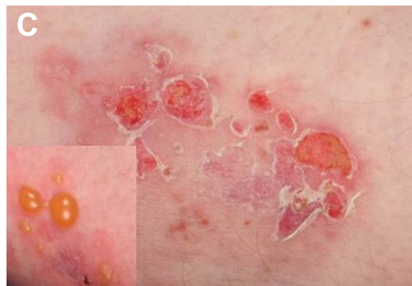


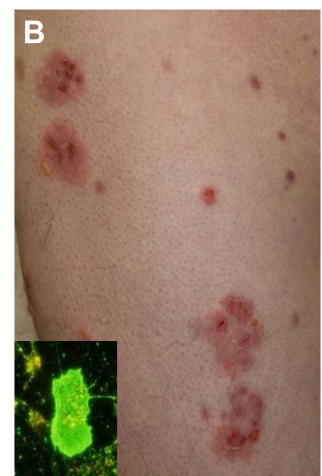
Figure 3.11: Recombinant desmoglein and desmocollin proteins used in this study. (A) Scheme of the recombinant proteins applied in this study, i.e. the entire ectodomains of Dsc1, Dsc2, Dsc3, Dsg1 and Dsg3, linked to E- and 6xHis-Tag (Muller et al. 2006; Muller et al. 2009). (B) Detection of the recombinant proteins by immunoblot analysis using an anti-E-tag monoclonal antibody (upper panel) or serum of a patient with paraneoplastic pemphigus (PNP) (lower panel) (Rafei et al. 2011). Molecular weight (MW) shown in kilo Dalton (kDa). Recombinant proteins running at their expected size at 72 kDa.

3.2.1 Clinical and serological characteristics of atypical pemphigus patients

Sera of two patients with pemphigus herpetiformis and two patients with pemphigus vegetans were included in this study (table 3.2 and figure 3.12). Patients presented with multiple erythematous herpetiform erosions and with hypertrophic verrucous plaques with pustules and erosions, respectively, as illustrated in Figure 3.12. Histopathology analysis of lesional skin showed intraepidermal loss of keratinocyte adhesion at the suprabasilar layer. Furthermore, direct immunofluorescence revealed an intercellular staining pattern, i.e. positive IgG deposits on the surface of epidermal keratinocytes (data from Dr. Takeishi Hashimoto, Departement of Dermatology, Kurume University, Japan).







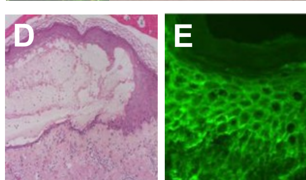


Figure 3.12: Clinical phenotype of representative patients with atypical pemphigus. Clinical pictures of two representative patients with atypical pemphigus (pemphigus vegetans and pemphigus herpetiformis) showing hypertrophic verrucous plaques with pustules and erosions of the foot (A) and multiple erythematous herpetiform erosions of the trunk and extremities (B, C). Insets of A and B show IgG reactivity of the patients' sera with human desmocollin 3-transfected COS-7 cells. Histopathology of lesional skin reveals intra-epidermal loss of keratinocyte adhesion at the suprabasilar level (D). Direct immunofluorescence shows IgG deposits on the surface of epidermal keratinocytes (E) (Rafei et al. 2011).

Table 3.2: Synopsis of clinical and serological characteristics of atypical pemphigus patients.

Patient	Autoab isotype	Diagnosis	Clinical characteristics	Desmosomal target antigens				
				Dsc1	Dsc2	Dsc3	Dsg1	Dsg3
#1	IgG	Pemphigus vegetans	Exophytic skin erosions and oral erosions	-	-	+	-	-
#2	IgG	Pemphigus vegetans	Hypertrophic verrucous plaques with pustules and erosions on the foot, groin and scalp	-	-	+	+	-
#3	IgG	Pemphigus herpetiformis	Multiple pustules, erythematous herpetiform erosions, oral erosions	-	-	+	-	-
#4	IgG	Pemphigus herpetiformis	Erythematous cutaneous erosions	-	-	+	-	-

(Rafei et al. 2011)

3.2.2 Desmocollin 3-reactive auto-antibodies in the sera of four patients with atypical pemphigus

To evaluate the desmosomal target proteins recognized by the IgG auto-Abs of these patients, the sera were incubated with defined baculovirus-derived proteins including Dsg1, Dsg3, Dsc1, Dsc2 and Dsc3. Immunoblot analysis revealed the presence of IgG against Dsc3 in all the studied patients (figure 3.13 upper pannel). In addition, the serum of patient 2 showed IgG reactivity against Dsg1. However no IgG-reactivity against Dsg3 could be detected at all (figure 3.13 upper pannel). As already mentioned the pathogenic role of Dsc3-specific IgG is not fully understood. Whether they represent an epiphenomenon or have capacities to induce mucocutaneous lesions remains unclear at the moment. Therefore it seemed suitable to isolate the Dsc3-specific auto-Abs from these sera and to investigate their pathogenic effect in the keratinocyte dissociation assay. Unfortunately due to the small amounts of patients' serum, specificity experiments were limited to western blot analysis and no ELISA was performed.

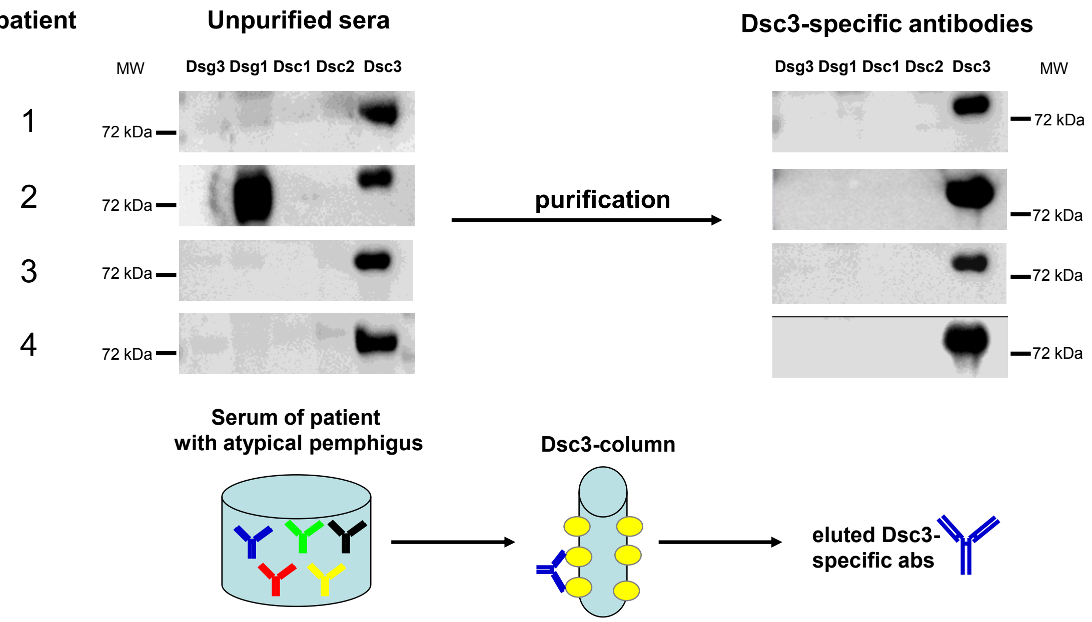


Figure 3.13: Affinity purification of desmocollin 3-reactive IgG auto-antibodies from four patients with atypical pemphigus. Immunoblot analysis showing IgG reactivity of the four atypical pemphigus sera against the recombinant proteins before and after affinity purification using Dsc3 columns (upper panel). Serum of patient 2 shows additional IgG-reactivity with Dsg1 prior to Dsc3-column-purification. Affinity purification technique using Dsc3-coupled CNBr columns is schematically shown in the lower panel (Rafei et al. 2011).

3.2.3 Desmocollin 3-specific immunoglobulin G recognizes both recombinant and native desmocollin 3

Serum samples of 4 atypical pemphigus patients were affinity purified using a Dsc3-coupled-CNBr sepharose column in order to obtain and further analyze the Dsc3-reactive IgG-fraction (figure 3.13 lower pannel). After purification, the IgG fractions of the four studied patients reacted exclusively with recombinant Dsc3 as shown by immunoblot analysis and were therefore thought to be highly Dsc3-specific (figure 3.13 upper pannel). To determine the reactivity of the Dsc3-purified IgG also with native Dsc3, immunofluorescence studies of cultured human keratinocytes (HaCat) and human skin samples was performed as well (figure 3.14). The anti-Dsc3 monoclonal ab U114 was used as a positive control and showed intercellular staining of both cultured keratinocytes and human skin, whereas pooled IgG from healthy control sera did not show any specific staining. The Dsc3-purified IgG fractions of patients 1-4 demonstrated intercellular reactivity with cultured human keratinocytes and human epidermis in a similar intensity as the positive control. In addition, indirect

immunofluorescence was performed on monkey esophagus to confirm the previous results. The anti-Dsc3 monoclonal ab, U114, elicited an intercellular staining pattern predominantly at the expected basal and suprabasal levels of the mucosal epithelium. Very similar results were obtained subjecting the Dsc3-purified IgG fractions of patients 1-4 to immunofluorescent staining on monkey esophagus, demonstrating the binding of these Dsc3-purified auto-Abs to native Dsc3. Taken together the purified IgG-fraction of the four atypical PV patients were highly specific for recombinant Dsc3 and were able to stain native Dsc3 on three different substrates as well.

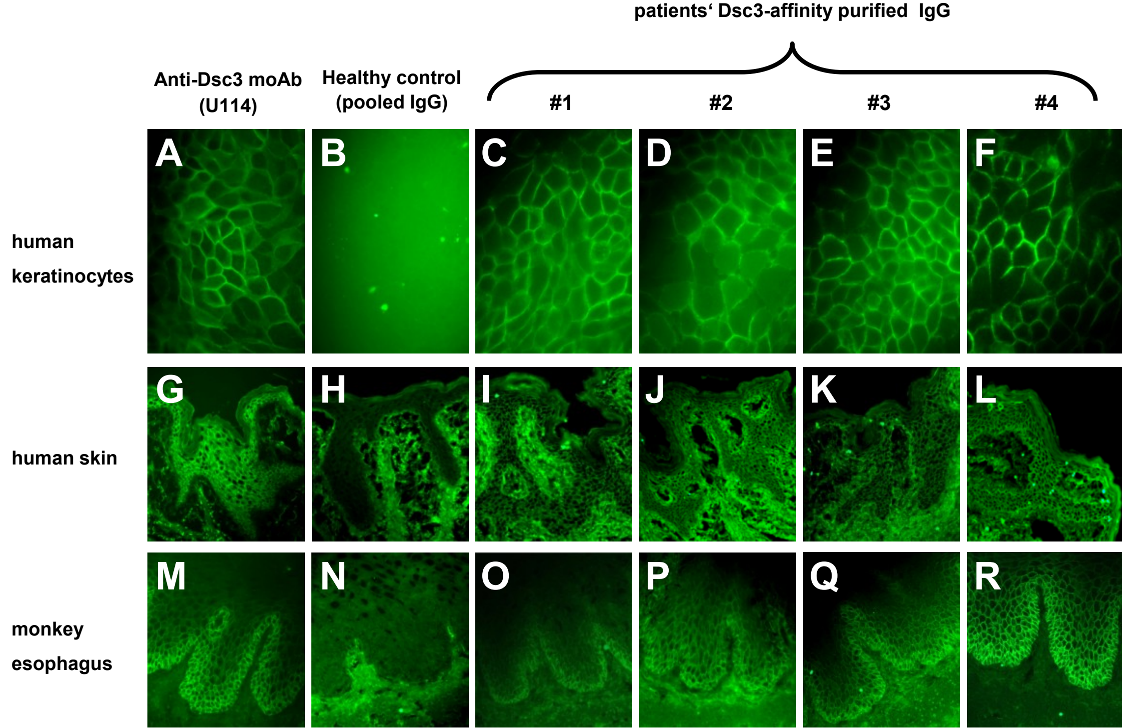
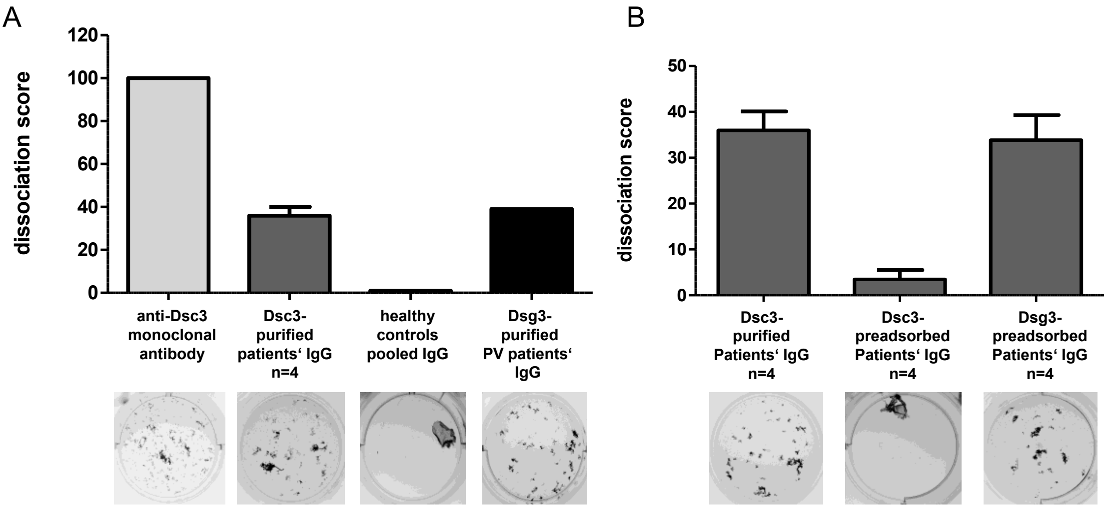


Figure 3.14: Reactivity of the purified desmocollin 3-reactive IgG auto-antibodies with cultured human keratinocytes, human epidermis and monkey esophagus. Dsc3 affinity-purified IgG from four patients with atypical pemphigus shows intercellular reactivity with cultured human keratinocytes (HaCaT cells) (patients 1-4 shown as C-F), human epidermis (I-L) and monkey esophagus (O-R). Monoclonal anti-Dsc3 antibody (clone U114) (A, G and M) and pooled IgG of healthy control sera (B, H and N) served as positive and negative controls, respectively (Rafei et al. 2011).

3.2.4 Desmocollin 3-specific immunoglobulin G induces loss of keratinocyte adhesion *in vitro*

After confirming the reactivity of Dsc3-purified IgG auto-Abs with recombinant and native Dsc3, their pathogenic capacity was investigated *in vitro*. Interference with keratinocyte adhesion is considered the pathogenic hallmark of auto-Abs in pemphigus. Thus, we applied a well established *in vitro* system for evaluating the capability of Dsc3-purified IgG auto-Abs to induce loss of keratinocyte adhesion. Dsc3-purified IgG of pemphigus patients 1-4 was applied to a dispase-based keratinocyte dissociation assay using primary human keratinocytes. Confluently grown primary keratinocytes were incubated with Dsc3-purified IgG fractions of patients 1-4, and afterwards Dsg1 was degraded by incubation with exfoliatoxin A (ETA). Next, mechanical stress was applied to the keratinocyte monolayer in order to obtain cellular fragments illustrating the loss of keratinocyte adhesion in these keratinocyte cultures and thus, the pathogenicity of the Dsc3 auto-Abs studied. Figure 3.15 A shows that the anti-Dsc3 monoclonal ab, U114, induces a dramatic weakening of keratinocyte adhesion, leading to a high number of keratinocyte fragments, while control IgG from pooled healthy individuals does not affect keratinocyte adhesion. Keratinocyte cell sheets incubated with Dsc3-purified IgG were dissociated into numerous smaller fragments, indicating that Dsc3-reactive IgG of patients' sera is capable of inducing loss of keratinocyte adhesion (figure 3.15 A). In comparison Dsg3-specific purified IgG from a patient with classical pemphigus vulgaris induced cell dissociation to a very similar extend indicating comparable pathogenic capacities of Dsg3- and Dsc3-specific Abs. Furthermore, it was crucial to investigate whether this effect on keratinocyte adhesion was specific for the Dsc3-reactive auto-Abs and related to the presence of Dsc3 in desmosomes. Preadsorption of the Dsc3-purified IgG fractions with recombinant Dsc3 almost completely blocked the loss of keratinocyte adhesion in the dissociation assay. In contrast preadsorption with recombinant Dsg3 did not alter the pathogenic effect of the Dsc3-purified IgG fractions in this assay (figure 3.15 B). To exclude non-specific binding of IgG auto-Abs to recombinant Dsc3, we also applied Dsg3-purified IgG from PV patients to the keratinocyte dissociation assay. As previously described, Dsg3-reactive auto-Abs of a pemphigus vulgaris patient induces loss of keratinocyte adhesion in this assay.

However, preincubation of anti-Dsg3 IgG with recombinant Dsc3 protein did not abrogate this effect (figure 3.15 C). Thus, these results strongly suggest that the Dsc3-purified IgG fraction in the atypical pemphigus patients studied here is pathogenic and induces loss of keratinocyte adhesion by specifically binding and/or blocking Dsc3 and such effect can be blocked by preincubation with the recombinant Dsc3-protein but not with Dsg3.



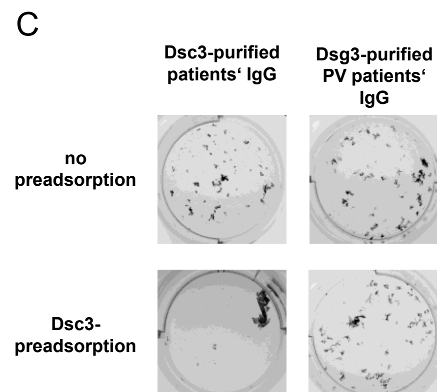


Figure 3.15: Desmocollin 3-affinity purified IgG from four patients with atypical pemphigus induces loss of keratinocyte adhesion. Shown are results of the dispase-based keratinocyte dissociation assay using primary human epidermal keratinocytes. Keratinocyte monolayers were incubated either with the monoclonal anti-Dsc3 IgG antibody (clone U114), Dsc3-affinity purified IgG from the four atypical pemphigus patients, pooled IgG from healthy donors or desmoglein 3 (Dsg3)-affinity purified IgG from a pemphigus vulgaris patient. (A) Quantification of the keratinocyte fragments shown as dissociation scores. Representative images out of three independent experiments are shown below the diagram. (B) Number of keratinocyte fragments after incubation with Dsc3-affinity purified IgG from four atypical pemphigus patients (B, left column). The induction of keratinocyte dissociation is Dsc3-specific, since preadsorption of the Dsc3-affinity purified IgG fraction with recombinant Dsc3 (B, middle column and C, left panel) but not with human Dsg3 (B, right column) blocks keratinocyte dissociation. To exclude non-specific binding of IgG to recombinant Dsc3 protein, Dsg3-purified IgG from a pemphigus vulgaris patient was pre-adsorbed with Dsc3 protein (C, right panel). Dsg3 affinity purified IgG induces loss of keratinocyte adhesion (A and C) which is not inhibited by preadsorption with human Dsc3 protein (C, right panel) (Rafei et al. 2011). Dissociation score = ((number of particles with serum – number of particles without serum) / (number of particles with serum – number of particles with U114)) x 100 (Ishii et al. 2005).

4 Discussion

4.1 Role of anti-desmoglein 3 auto-antibodies recognizing carboxy-terminal epitopes in the pathogenesis of pemphigus

The results from the first part of this study clearly demonstrate that auto-Abs directed against non-amino-terminal epitopes of Dsg3, like the Dsg3EC4-subdomain, react with native Dsg3 and are pathogenic *in vitro*. These findings challenge the dogma of Dsg3EC1-reactive IgG antibodies as the major pathogenic Abs to some extent, since auto-Abs directed against carboxy-terminal epitopes may at least contribute to the induction of acantholysis in patients suffering from pemphigus (Amagai et al. 1992; Futei et al. 2000; Tsunoda et al. 2003; Muller R. et al. 2008). Shortly after identification of Dsg3 as the major pathogenic antigen in PV, Amagai et al. using β-galactosidase fusion proteins (FP) produced by E.coli showed that in a cohort of 23 PV patients most were able to react with the EC1, EC2 and EC4 domains of human Dsg3 (Amagai et al. 1992). Antigen specific immunoadsorption of IgG out of these sera using Dsg3EC1-2 and Dsg3EC3-5 FPs and subsequent intraperitoneal injection into neonatal mice resulted in suprabasal split formation accounting only for the Dsg3EC1-2-affinity purified fraction. However, the pass through IgG of the Dsg3EC1-2 FP column still exhibited acantholitic capacity when injected intraperitoneally indicating the presence of pathogenic Dsg3-epitopes other than EC1 and EC2 (Amagai et al. 1992). Data from Bhol et al. in 1995 already suggested that EC2-specific IgG_4 is probably the main acantholytic autoantibody, while EC1- specific IgG_4 may act as a facilitator or enhancer of the pathogenic process (Bhol et al. 1995). Furthermore our group has previously shown that active stages of PV were associated with the presence of IgG directed against the NH_2-terminus and Dsg3 subdomains EC2-4 (Muller R. et al. 2008). Even more convincing, the same PV patients did not exhibit auto-Abs against these epitopes anymore while in remission. Finally Dsg3EC1-4 specific auto-Abs seemed to predominantly occur in patients with a mucosal dominant phenotype and in one case titers decreased, together with clinical improvement, upon treatment (Muller R. et al. 2008).

4.1.1 Epitope spreading and synergistic effects of “non-pathogenic” pemphigus vulgaris auto-antibodies

A possible explanation for the above mentioned observations provides a study by Salato et al. using baculovirus-produced domain-swapped Dsg1 and Dsg3 molecules, respectively (Salato et al. 2005). This study could show that in patients with mucosal dominant PV an intramolecular epitope spreading from carboxy- to NH_2-terminal epitopes of Dsg3 preceded an intermolecular epitope spreading from Dsg3 to Dsg1 and the transition to a mucocutaneous phenotype. Patients with mucosal lesion had antibodies only reactive with COOH-terminal epitopes (Salato et al. 2005). However, our lab previously detected auto-Abs directed against the COOH-terminal epitope Dsg3EC5 in patients with mucocutaneous PV as well (Muller R. et al. 2008). Furthermore PV patients in remission still presented with high titers against the whole Dsg3-ectodomain indicating the presence of pathogenic and non-pathogenic auto-Abs (Muller R. et al. 2008). This goes in line with findings from Payne et al. using phage display techniques to isolate a repertoire of monoclonal Dsg3-reactive antibodies as single-chain variable-region fragments (scFvs) from PV sera (Payne et al. 2005). Only two among several isolated scFvs showed pathogenic effects when injected in neonatal mice or tested in the keratinocyte dissociation assay (Payne et al. 2005). However, several of the “non-pathogenic” scFvs were not injected together to investigate a possible synergistic effect. But exactly this synergistic effect may provide an explanation of how primarily non-pathogenic Dsg3 auto-Abs may contribute to the pathogenesis of PV. Kawasaki et al. demonstrated an additive pathogenic effect of monoclonal mouse Abs directed against middle- or carboxy-terminal extracellular regions of the Dsg3-ectodomain when pooled in certain combinations. These pools were able to induce a PV like phenotype when injected into neonatal mice (Kawasaki et al. 2006). Further evidence that intramolecular epitope spreading may modulate clinical activity in pemphigus comes from the endemic PF variant Fogo Selvagem (FS). Patients in preclinical stages presented with Abs against the EC5 subdomain of Dsg1 and with the onset of disease developed anti-Dsg1EC1 and/or anti-Dsg1EC2 auto-Abs (Li et al. 2003). Finally sera from FS patients in remission showed reactivity restricted to Dsg3EC5 (Li et al. 2003).

The study presented here shows for the first time that specifically isolated antibodies directed against a COOH-terminal extracellular subdomain of Dsg3, i.e. Dsg3EC4, are able to induce keratinocyte dissociation *in vitro* and therefore may contribute to acantholysis in pemphigus. Three out of four patients in this study presented with EC1-specifc IgG and two of these had additional EC4-reactive antibodies. Since pathogenicity *in vitro* could be demonstrated for both Dsg3EC1- and Dsg3EC4-affinity purified IgG respectively, it is not possible to determine the major pathogenic auto-Ab entity leading to the blistering phenotype. Interestingly patient 6 exhibited only Dsg3EC4-specific IgG but lacked auto-Abs directed against other Dsg3 subdomains and any other desmoglein or desmocollin, respectively. Thus it is very likely that EC4-specific IgG may induce acantholysis in this patient. Unfortunately it wasn't possible to follow up serological changes in patient 6 which may have shown the appearance of auto-Abs reactive with NH_2-terminal epitopes supporting the role of intramolecular epitope spreading in disease modulation. Therefore future studies should address the phenomenon of Dsg3-epitope spreading during the course of PV to further elucidate its relevance for disease progression. Ohyama et al. recently addressed this question in a large-scale longitudinal study using Dsg2-based domain-swapped molecules (Ohyama et al. 2012). Dsg1 and Dsg3EC-domain reactivity of 212 sera collected from 53 PV patients at multiple time points during the course of the disease was detected by immunoprecipitation-immunoblotting (Ohyama et al. 2012). Sera were incubated with Dsg2 molecules, previously swapped with one of the extracellular domains EC1-5 of Dsg1 and Dsg3, respectively, to determine epitope specific reactivity of the Dsg1-/Dsg3-reactive IgG. Since PV sera do not react with any Dsg2EC domain, positive reactivity in this assay can be directly related to the swapped Dsg1EC/Dsg3EC domains (Ohyama et al. 2012). Ohyama could show that epitope spreading occurred only in very few PV cases during the disease course and that most patients harboured a constantly high IgG reactivity against the NH_2-terminal region of Dsg1 and Dsg3 going in line with several previous studies (Amagai et al. 1992; Futei et al. 2000; Payne et al. 2005; Ohyama et al. 2012). Interesting, however 18.9% of all sera contained Abs against the Dsg3EC4 domain and around 17% of those antibodies were found during active and moderate disease (Ohyama et al. 2012). Even more

astonishing 12 out of 14 patients suffering from praneoplastic pemphigus which were also included into this study revealed a positive IgG reactivity against the Dsg3EC4 epitope indicating a high abundance of these auto-Abs among PNP patients (Ohyama et al. 2012). Furthermore, this study revealed high prevalence for other COOH-terminal epitope specific auto-Abs like Dsg3EC3-IgG (50.5%) and Dsg3EC5-IgG (12.3%), respectively, in PV sera, similar to previous findings by our group (Muller R. et al. 2008; Ohyama et al. 2012). Taken together with the fact that around 61% of all sera showed a positive IgG reactivity against at least 3 or more Dsg3EC domains, a possible synergistic effect of these auto-Abs even if they recognize non-NH_2-terminal epitopes can not be excluded. As already mentioned above, Kawasaki et al. were able to show that in mice primarily non pathogenic Dsg3-reactive auto-Abs can exert a pathogenic effect when pooled together (Kawasaki et al. 2006). Interestingly non-pathogenic mAbs directed against middle or COOH-terminal epitopes induced blister formation when injected into neonatal mice together with other non-pathogenic mAbs reactive with NH_2-terminal epitopes indicating that IgG recognizing the middle to COOH-terminal extracellular domains of Dsg3 can also take part in the pathogenic process of blister formation (Kawasaki et al. 2006). Finally, although epitope spreading in the auto-Ab profile of PV patients seems to be a rare event the presence of serum IgG reactive with several different Dsg3 epitopes including primarily non-pathogenic ones (specific for middle or COOH-terminal epitopes) together with our findings of pathogenic EC4-specific IgG *in vitro* clearly emphasize a possible synergistic effect of these auto-Abs in PV pathogenesis.

4.1.2 Possible pathogenic molecular mechanisms of desmoglein 3 extracellular subdomain 4-specific immunoglobulin G

The molecular pathology of PV IgG reactive with middle or carboxy-terminal epitopes of Dsg3 is not known so far. Steric hindrance by direct inhibition of the adhesion interface seems unlikely since these Abs do not react with the NH_2-terminal part of the Dsg3-ectodomain (Heupel et al. 2008). However Dsg3EC4-reactive Abs may induce allosteric hindrance through a conformational change of the extracellular portion of Dsg3 leading to impaired desmosomal adhesion, a mechanism that may act synergistically with direct inhibition at the adhesive

interface by EC1-specific IgG (Waschke 2008). Heupel et al. nicely demonstrated that Dsg1-reactive IgG from PF patients which was not able to block Dsg1 homophilic interactions under cell free conditions, induced dissociation of keratinocyte cell sheets and reduced binding of Dsg1 coated beads to the surface of cultured keratinocytes (Heupel et al. 2008). These findings indicate that PF IgG presumably excerts its pathologic effects rather via intracellular changes in the signalling cascades of the keratinocyte (Heupel et al. 2008). The authors also suggest that Dsg3-reactive PV IgG may harbour similar capacities to induce keratinocyte dissociation by activation of intracellular signalling in addition to direct inhibition (Heupel et al. 2008). Finally they mention the pathogenic effect of a mAb directed against the EC2 domain of Dsg1, suggesting allosteric conformational changes of Dsg1 (Heupel et al. 2008). Although this effect could not be reproduced using mAbs specific for non-NH_2-terminal epitopes of Dsg3, Heupel et al. did not examine a possible synergistic effect of these Abs when pooled together. Therefore the further investigation of our isolated Dsg3EC4-specific IgG in cell-dependent and independent pathogenicity assays are necessary to elucidate the molecular mechanisms underlying their pathogenic capacity in inducing acantholysis.
Another hypothesis for the pathogenic effect emerging from PV-IgG binding to the keratinocyte surface is the induction of intracellular signalling pathways which may result in the depletion of membrane bound Dsg3 finally leading to impaired cell adhesion (Muller E.J. et al. 2008; Thomason et al. 2010). As already mentioned earlier several studies have investigated the effect of IgG binding to junctional/desmosomal and transadhering non-junctional/non-desmosomal Dsg3 (Sato et al. 2000; Oktarina et al. 2011; Tsang et al. 2012). Targeting of this non-junctional, non-keratin-anchored Dsg3 by PV-IgG seems to induce two major mechanisms. On the one hand it leads to Dsg3 internalisation and lysosomal degradation within 30-60 min after PV-IgG treatment thereby preventing its’ attachment to keratin filaments and its’ integration into mature desmosomes as demonstrated by time-lapsed labeling of cultured keratinocytes incubated with PV IgG (Sato et al. 2000). This goes in line with the finding that split formation between keratinocytes starts in the interdesmosomal areas since intraperitoneal PV-IgG injection into BALB/c mice resulted in early epidermal cell detachment after 1 h visible as widening of the

epidermal intercellular spaces (Takahashi et al. 1985). Six hours after PV IgG injection interdesmosomal areas of the cell membrane had detached completely. Desmosomal junctions separated at last, occurring at 12-18 hours upon injection (Takahashi et al. 1985). On the other hand binding of Dsg3-specific IgG may induce an "outside-in" signal that alters protein phosphorylation and transcription finally leading to acantholysis promoting events (Muller E.J. et al. 2008). One of those signals may be the degradation of desmosomal Dsg3 but since changes in the desmosomal pool of Dsg3 do not seem to occur before 18-24 hours after PV-IgG treatment other intracellular mechanisms are speculated to be involved (Aoyama et al. 1999a; Aoyama et al. 1999b; Yamamoto et al. 2007).

Most of the observations on keratinocyte cell signalling have been performed *in vitro* using cultured keratinocytes mimicking the transitional phase, which is classified as the time point where the epidermal keratinocyte exits the stem cell compartment and becomes a transit amplifying cell until it is growth arrested and has committed to terminal differentiation (Muller E.J. et al. 2008). This applies for keratinocytes residing in the suprabasal layers of the epidermis, the site where blister formation occurs in PV (Amagai et al. 1996; Mahoney et al. 1999a). The fact that at the beginning of the transitional phase Dsg3 and Dsc3 are the main demsosomal cadherins displayed at the cell surface of keratinocytes renders these types of experiments a suitable setting to study the effects of PV-IgG and for example Dsc3- or Dsg3EC- affinity purified IgG binding (Green et al. 2000; Dusek et al. 2007).

A sound explanation of how anti-Dsg3-IgG is involved in inducing acantholysis derives from the finding that disturbed cadherin transinteraction between opposing cells results in a hyperproliferative signal in correlation with an upregulation of c-Myc and disrupted desmosomal organization (Williamson et al. 2006; de Bruin et al. 2007). Under physiological conditions Dsg3 homophilic interaction leads to the Src-family-kinase dependent phosphorylation of Dsg3-associated plakoglobin (PG) which then associates with phosphatidylinositol triphosphate kinase (PI3K) (Calautti et al. 1998). This complex induces increased activity of the serine-threonine protein kinase AKT finally resulting in the reduced expression of the proto-oncogene c-myc which renders the cell into cell cycle arrest (Muller E.J. et al. 2008). There is emerging evidence that anti-

Dsg3 Abs oppose Dsg3-mediated signalling by disrupting transadhesion between non-junctional Dsg3 for example by steric and/or allosteric hindrance (Oktarina et al. 2011; Schulze et al. 2012; Tsang et al. 2012). This binding may provide a signal of “no cell contact”, epidermal growth factor receptor- (EGFR-)signalling fails to be inhibited, keratinocytes in the basal layer of the epidermis continue to proliferate due to disinhibited c-myc expression, Dsg3 is being depleted from desmosomes leading to weekened adhesiveness and finally suprabasal blisters occur (Schulze et al. 2012). Further evidence derives from findings that PV-IgG treatment of cultured keratinocytes leads to increased EGFR activity (Chernyavsky et al. 2007) and c-myc overexpression is found in the epidermis of PV-patients (Williamson et al. 2007). Taken together Dsg3 mediated antiproliferative events seem to physiologically act as a counterpart of EGFR-mediated proliferative signalling and thus PV-IgG binding may interfere with Dsg3 signalling leading to disinhibited EGFR activation and cell proliferation (Muller E.J. et al. 2008).

Another proposed role for non-junctional “Dsg3-signalling” is its’ influence on the assembly and disassembly of adherens junctions. In this context the tyrosine kinase Src seems to play an essential role. Very recently is has been shown that non junctional human desmoglein 3 acts as an upstream regulator of Src in E-cadherin adhesion, a pathway possibly involved in the pathogenesis of pemphigus vulgaris (Tsang et al. 2012). Src phosphorylation in response to PV-IgG binding preceeds EGFR- and P38MAPK activation in keratinocytes (Chernyavsky et al. 2007) and therefore may act as an early intracellular branch point that triggers further downstream pathways (Chernyavsky et al. 2007). The importance of P38MAPK in the pathogenesis of pemphigus has been weakened to some extend at least in early blister formation since *in vitro* studies have shown that p38 MAPK phosphorylation occurred after initial Dsg3 endocytosis (Mao et al. 2011). Nevertheless p38 MAPK seems to augment late Dsg3/Dsc3 endocytosis and blister formation (Mao et al. 2011). In summary, activation of intracellular signalling pathways in response to PV-IgG binding to non-junctional cadherins provides a possible explanation of how initial blister formation is triggered in pemphigus.

4.1.3 Immunoglobulin G isotype distribution patterns of auto-antibodies ractive with amino- and carboxy-terminal epitopes of desmoglein 3

In this study we showed that auto-Abs of a PV patient directed against the whole ectodomain of Dsg3 and its subdomains EC1 and EC4 mainly belonged to the IgG_1 and IgG_4 isotypes matching to a patient in a chronic active stage of the disease (Hertl et al. 2010). While EC4-specific IgG exclusively belonged to the IgG_1 and IgG_4 subclass the EC1-specific antibody pool further contained IgG_2 auto-Abs which similar to IgG_4 do not or only to a limited extend activate the classical complement pathway (Murphy et al. 2008). Furthermore IgG_2 can not bind to Fc receptors on phagocytes highlighting a possible directly pathogenic role of this subtype. Interesting information about the distribution of Dsg3 specific IgG_2 comes from a large study on Dsg3 reactivity in PV patients and healthy relatives using immunoblotting analysis (Torzecka et al. 2007). In this study circulating Dsg3 specific IgG_2 isotype was observed in 60% of a cohort of relatives of PV patients and IgG_4 was detected in 23.3% of them. On the other side in active PV patients IgG_4 and IgG_1 were the dominant isotypes (96 and 76% relatively) while in clinical remission Abs predominantly belonged to the IgG_2 (75%) and IgG_4 (37.5%) isotype (Torzecka et al. 2007). The frequency of IgG_1 and IgG_4 isotypes was significantly higher in acute disease compared to patients in clinical remission or seropositive healthy relatives (Torzecka et al. 2007). These results confirmed the dependence of the different IgG isotypes in pemphigus on certain stages of the disease (Bhol et al. 1995; Torzecka et al. 2007). Together with our findings these data indicate that EC4 specific IgG_1 and IgG_4 belong to the major pathogenic IgG isotypes which are present in active PV. On the other hand, EC1 specific IgG_2 may play a minor role in pemphigus pathogenesis since IgG_2 reactive with Dsg3 is frequently found in healthy relatives of PV patients or patients in remission.

4.1.4 Conformation dependence of amino- and carboxy-terminal epitopes of desmoglein 3

Denaturing of the whole extracellular domain of Dsg3 by calcium depletion using EDTA or treatment upon urea lead to a reduced binding of Dsg3- and Dsg3EC1-affinity purified Abs indicating the recognition of conformational epitopes by these auto-Abs. These data perfectly match with the findings of

Tsunoda et al. who identified the pathogenic monoclonal antibody AK23 recognizing a calcium-dependent conformational epitope within the EC1 subdomain of Dsg3 (Tsunoda et al. 2003). However our study shows that Dsg3 epitope recognition by EC4-affinity purified IgG does not differ among denaturation or calcium depletion indicating the presence of non-conformational epitopes within the EC4 subdomain of Dsg3. These findings were recently confirmed by Ohyama et al. using domain swapped Dsg2/Dsg3 and Dsg2/Dsg1 molecules (Ohyama et al. 2012). According to their findings anti-Dsg1 and anti-Dsg3 IgG Abs recognize calcium-dependent epitopes of the EC1-3 domains of Dsg1 and Dsg3, respectively, whereas recognition of epitopes on the EC4-5 domains is calcium independent (Ohyama et al. 2012). Furthermore in a large cohort of pemphigus patients our group has previously shown by ELISA analysis that IgG-reactivity to the recombinant extracellular domain EC4 was not being diminished by denaturising the protein or by reducing the Ca^{2+}-level (Muller R. et al. 2008). Taken together these data indicate that EC4-recognition is conformation independent and thus EC4-affinity purification using recombinant proteins seems to be a suitable tool to isolate and investigate EC4-specific IgG in PV patients' sera.

4.1.5 Role of carboxy-terminal subdomains of desmoglein 3 in maintaining keratinocyte adhesion

Most knowledge about how desmosomal cadherins interact on the molecular level derives from studies on classical cadherins, like E- or C-Cadherin. Nevertheless these findings are thought to be widely applicable on desmosomal cadherins as well since they share approximately 30% amino acid identity with classical cadherins and harbour the same basic structure in their extracelllular domains (Garrod et al. 2002). There are two different hypotheses how cadherins first interact when arriving on the cell surface before assembling into mature desmosomes. One is the formation of **cis** dimers between two neighbouring cadherins on the same cell which then come together on adjacent cells to interact in **trans**, followed by a zippering of the adhesive interface (Green et al. 2010). A possible site for a cis interaction represent the EC1 domain and interestingly a region close to the interdomain EC4-EC5 as shown by cryo-electron tomography of vitreous sections of human epidermis (Al-

Amoudi et al. 2007). The second hypothesis favours the initial formation of weakly bound trans dimers between two molecules protruding from opposing cells which is subsequently strengthened by lateral cadherin clustering and cytoskeleton attachment (Green et al. 2010). Although the above mentioned trans interaction seems to be largely dependent on two EC1 domains forming adhesive complexes between adjacent cells, the strength of this interaction is rather low (Green et al. 2010). To enlarge trans binding forces another possibility is the involvement of other EC domains into this kind of interaction. The importance of the involvement of more than only one Dsg-EC-domain into trans interaction has been elucidated by Chappuis-Flament et al. using bead aggregation and cell attachment-based adhesion assays (Chappuis-Flament et al. 2001). Sivasankar et al. proposed that rather than binding in a single unique orientation, cadherins adhere in three distinct alignments. The strongest adhesion was observed when opposing extracellular fragments were completely interdigitated including the involvement of the carboxy-terminal regions EC4 and/or EC5 (Sivasankar et al. 2001). Taken together both initial cis or trans interactions between adjacent or opposing cadherins seem to include middle or COOH-terminal EC domains as well highlighting those regions as important players in maintaining cellular adhesion and giving rise to the possibility that auto-Abs in pemphigus sera directed against the EC4 or EC5 domain of Dsg3 may interfere with desmosomal adhesion as well.

4.2 Role of desmocollin 3-specific auto-antibodies in the pathogenesis of pemphigus

4.2.1 Evidence for pathogenic desmocollin 3-specific auto-antibodies

This study shows for the first time that affinity-purified Dsc3-reactive auto-Abs from patients with atypical pemphigus induce loss of epidermal keratinocyte adhesion *in vitro*. These results extend recent findings of Spindler et al who identified Dsc3 as a crucial component of desmosomal adhesion especially in the suprabasal layers of the epidermis (Spindler et al. 2009). First, incubation of human skin biopsies with a mAb against Dsc3 (U114) induced suprabasal split formation, the site where acantholysis usually starts in PV patients (Spindler et al. 2009). Using adhesion force measurement experiments Spindler et al. investigated single molecule homophilic and heterophilic interactions between

different desmosomal cadherins (Spindler et al. 2009). They were able to show that Dsc3 does not only interact homophilically but also heteophilically with Dsg1. Heterophililic interaction was based on an additional hydrogen bond and hydrophobic interactions and seemed to be even slightly stronger (Spindler et al. 2009). Although hetreophilic Dsc3/Dsg1 interaction could be blocked by incubation with mAbs against Dsc3 and Dsg1, respectively, the addition of PF-IgG exclusively reactive with Dsg1 did not reduce the heterophililic binding activity in this cell free system. Interestingly heterophilic binding of Dsc3 to Dsg3 could not be detected indicating that heterophilic cadherin interaction does not seem to be a general rule (Spindler et al. 2009). Furthermore Spindler et al. tested the influence of impaired Dsc3 function in the keratinocyte dissociation assay, which has been used in our work as well. Dsc3 gene silencing using small hairpin RNA (shRNA) and incubation with a mAb against human Dsc3 (U114) increased fragmentation of the cell monolayer significantly (Spindler et al. 2009). Finally, Dsc3-coated bead binding to keratinocyte monolayers was impaired by mAbs against Dsc3 and Dsg1 as well as Dsg1 reactive human IgG (Spindler et al. 2009). Strikingly preincubation of keratinocyte monolayers with the P38 MAPK inhibitor SB202190 abolished the effect of Dsg1-reactive IgG indicating the involvement of cellular signalling mechanisms subsequently to Ab binding leading to impaired Dsc3 bead binding (Spindler et al. 2009). Nevertheless, the PV and PF sera used in this study were not tested for their reaction against non-desmosomal antigens like cholinergic receptors. Thus the acantholytic effects seen here may not be exclusively related to Dsg1- and/or Dsg3- reactive IgG (Grando 2012).

The best evidence that Dsc3 is critically involved in desmosomal adhesion of epidermal keratinocytes comes from a recently established conditional Dsc3^{-} knock out mouse model (Chen et al. 2008). Mice with a targeted Dsc3 deficiency in the skin and hair follicles show an impressive phenotype with extensive intra-epidermal blistering and telogen hair loss. This phenotype was even more profound than the one found in mice deficient of Dsg3, the auto-antigen of PV, where mostly mucosal lesions evolved (Koch et al. 1997; Chen et al. 2008). In addition, cultured keratinocytes from the Dsc3 conditional knock out mice showed impaired cellular adhesion when tested in the keratinocyte dissociation assay similar to the findings of Spindler et al. as mentioned above

(Chen et al. 2008; Spindler et al. 2009). Knowledge about impaired Dsc3 function in humans is rarely found in the literature. Since total knock out of Dsc3 in mice resulted in a pre-implantation lethal phenotype suggesting an essential role of this cadherin in early embryonic development (Den et al. 2006), hereditary defects of Dsc3 in humans may also result in prenatal abortion. However, Ayub et al. were able to identify a homozygous nonsense mutation in the human Dsc3 gene that affected four members of a large afghan family. All individuals were affected with hereditary hypotrichosis and the appearance of recurrent skin vesicle formation (Ayub et al. 2009). Thus, in addition to Dsg3 and Dsg1, Dsc3 must be considered as a relevant component for desmosomal adhesion of epidermal keratinocytes.

Very recently Mao et al. identified 6 out of 38 PV sera that were able to immunoprecipitate recombinant human Dsc3-protein (Mao et al. 2010). One of these sera belonged to a patient with mucosal dominant PV and exclusively reacted with Dsc3. Isolated IgG of this patient was able to induce acantholysis in the keratinocyte dissociation assay and preincubation of anti-Dsc3-IgG with recombinant Dsc3 abolished this effect indicating a specific disruption of Dsc3-mediated adhesion (Mao et al. 2010). But again, the PV sera used in this study were not investigated for additional reactivity against non-desmosomal antigens. Therfore isolated IgG fractions from these patients may still harbour pathogenic Abs against non-desmosomal pathogenic antigens. In our approach we specifically isolated PV IgG using recombinant human Dsc3. Isolated IgG fractions showed no cross-reactivity with other desmosomal cadherins and were able to induce acantholysis *in vitro*. Since acantholysis could only be blocked by preincubation with recombinant Dsc3, these effects can be directly referred to the action of Dsc3 reactive IgG. Finally these findings provide the first evidence that specific targeting of Dsc3 by auto-Abs in pemphigus patients induces loss of keratinocyte adhesion *in vitro* and may contribute to the blistering phenotype.

4.2.2 Presence of desmocollin-reactive immunoglobulin G in clinical pemphigus variants

IgG auto-Abs against Dsc3 are not the only auto-Ab-specificity detected in atypical pemphigus since our group and others have also identified IgG reactivity against Dsg1 and Dsg3 in single cases of pemphigus herpetiformis

(Kozlowska et al. 2003; Lebeau et al. 2010). Furthermore, Mao et al. identified 6 patients suffering from classical pemphigus demonstrating Dsc3-reactive auto-Abs (Mao et al. 2010). Until now, there was only circumstantial evidence for a pathogenic role of IgG auto-Abs against distinct Dsc isoforms. Dsc1 has been previously identified as a target antigen of IgA auto-Abs in the subcorneal pustular dermatosis type of IgA pemphigus (Hashimoto et al. 1997; Heng et al. 2006). Except for the findings of a single *in vitro* study, there is currently no clear evidence for the pathogenicity of IgA auto-Abs on desmosomal cell-cell adhesion (Supapannachart et al. 1993). IgG- and IgA-auto-Abs against Dsc have been occasionally identified in patients with atypical pemphigus (Dmochowski et al. 1995; Hashimoto et al. 1995; Kozlowska et al. 2003; Hisamatsu et al. 2004; Muller et al. 2009) and paraneoplastic pemphigus (Chorzelski et al. 1994; Hashimoto 2001; Muller et al. 2009). It is currently a matter of debate whether IgG or IgA auto-Abs against Dsc are a rare epiphenomen in pemphigus or whether their detection is limited by the currently available diagnostic tools. Hisamatsu et al. did not detect IgG or IgA auto-Abs against Dsc1-3 in 45 sera from classical pemphigus patients by ELISA with recombinant proteins (Hisamatsu et al. 2004). The sensitivity of the Dsc-ELISA was limited since all the sera from eight patients with IgA pemphigus showed IgA reactivity with Dsc1 expressed on COS cells but only one serum was IgA positive by ELISA (Hisamatsu et al. 2004). Utilizing baculovirus-derived, eukaryotic recombinants of Dsc1, Dsc2, and Dsc3, Müller et al. were not able to detect Dsc-specific IgG in the sera of a cohort of 74 European patients with PV by ELISA and immunoblot, respectively (Muller et al. 2009). However, a considerable number of sera from patients with atypical forms of pemphigus, paraneoplastic pemphigus and IgA pemphigus showed either IgG or IgA reactivity with Dsc1, Dsc2, or Dsc3 (Muller et al. 2009). This observation is also reflected by the findings of the present study that four Dsc3-reactive sera were collected from patients with atypical pemphigus (pemphigus vegetans and pemphigus herpetiformis) and only one patient demonstrated IgG-reactivity against Dsg1 (Rafei et al. 2011). Thus, the low detection rate of Dsc-reactive IgG auto-Abs in PV and PF is presumably not due to technical limitations of the respective assays but rather due to the low prevalence of Dsc-reactive auto-Abs in classical PV or PF (Rafei et al. 2011). In summary, the present findings

demonstrate that IgG auto-Abs against Dsc3 induce loss of keratinocyte adhesion *in vitro* strongly suggesting their pathogenic relevance in pemphigus. Since Dsc3-reactive IgG is rarely found in patients with classical PV or PF, the observed *in vitro* pathogenicity of anti-Dsc3 IgG auto-Abs provides a sound explanation why Dsc3-reactive patients with atypical pemphigus lacking IgG against Dsg3 or Dsg1 develop skin blistering (Rafei et al. 2011).

4.3 Potential role of antigen-specific immunoadsorption in the therapy of pemphigus

As mentioned before one additional second line treatment strategy in pemphigus is to remove large amounts of circulating IgG from the patients' serum by immunoadsorption including the pathogenic auto-Abs (Eming et al. 2006a). More precisely blood is withdrawn from the patient using peripheral or central venous catheters and cellular components are seperated from plasma by centrifugation or filtration. The plasma is passed through an adsorber with high affinity to human immunoglobulin, such as protein A, a major cell wall component of staphylococcus aureus. Protein A harbours high affinity to human IgG, especially IgG_1, IgG_2 and IgG_4 and to a lesser extend IgG_3 (Eming et al. 2006a).

This immunoadsorption technique eliminates Abs via their Fc-portion including IgG specific for auto-antigens like Dsg3 as well as protective pathogen-specific Abs such as measles- or CMV/EBV-reactive IgG. Nevertheless, immunoadsorption has already been successfully applied and well tolerated in the treatment of difficult to treat pemphigus patients (Luftl et al. 2003; Schmidt et al. 2003; Eming et al. 2006b). Furthermore, immunoadsorption in combination with the B-cell depleting mAb rituximab induced fast and prolonged remissions in difficult to treat pemphigus (Behzad et al. 2011). The first antigen specific approach is the "Coraffin adsorber" which has been developed based on peptide epitopes recognized by anti-β1 adrenergic receptor antibodies in patients suffering from idiopathic dilatative cardiomyopathy, thus removing predominantly antigen specific auto-Abs from patients' plasma (Eming et al. 2006a). There have been many pieces of evidence that antigen-specific immunoadsorption of IgG from pemphigus sera can successfully ameliorate their pathogenicity. Amagai and collegues nicely showed that recombinant

proteins produced in a baculovirus expression system can be used to remove Dsg1- and/or Dsg3-specific IgG from PV sera. Those preadsorbed sera were not able to induce blister formation upon injection into neonatal mice anymore (Amagai et al. 1994a; Amagai et al. 1995a). These studies already suggested antigen-specific immunoadsorption as a possible therapeutic strategy in the treatment of patients suffering from pemphigus. Since subsequent studies using a phage display technique have demonstrated that Dsg3 harbours pathogenic and non-pathogenic epitopes (Payne et al. 2005) it is obvious to think about a more epitope specific elimination of circulating pathogenic auto-Abs in PV patients. This work for the first time identifies EC4 as a potential additional pathogenic epitope of Dsg3 since EC4-specific isolated IgG showed an ancantholytic effect *in vitro*. Thus epitope-based plasmapheresis may represent a more targeted therapy in pemphigus treatment in the future. On the other hand desmocollins, and in particular Dsc3, have emerged as relevant non-desmoglein auto-antigens in the pathogenesis of pemphigus (Muller et al. 2009; Spindler et al. 2009). This study has shown that purified Dsc3-reactive IgG is pathogenic in vitro and that preadsorption of Dsc3-IgG using recombinant proteins can significantly reduce their pathogenicity (Rafei et al. 2011). Taken together antigen-specific immunoadsorption represents a promising method to reduce circulating pathogenic antibodies from the blood of pemphigus patients which might lead to a far more specific treatment option with less side effects. However, since additive pathogenic functions of auto-Abs directed against primarily non-pathogenic desmosomal and/or non-desmosomal epitopes can’t be ruled out, it may be still necessary to eliminate all epitope-specific IgG from the blood to avoid these additive effects (Grando 2012). Figure 4.1 gives a summarised picture of the potential pathogenic mechanisms in pemphigus.

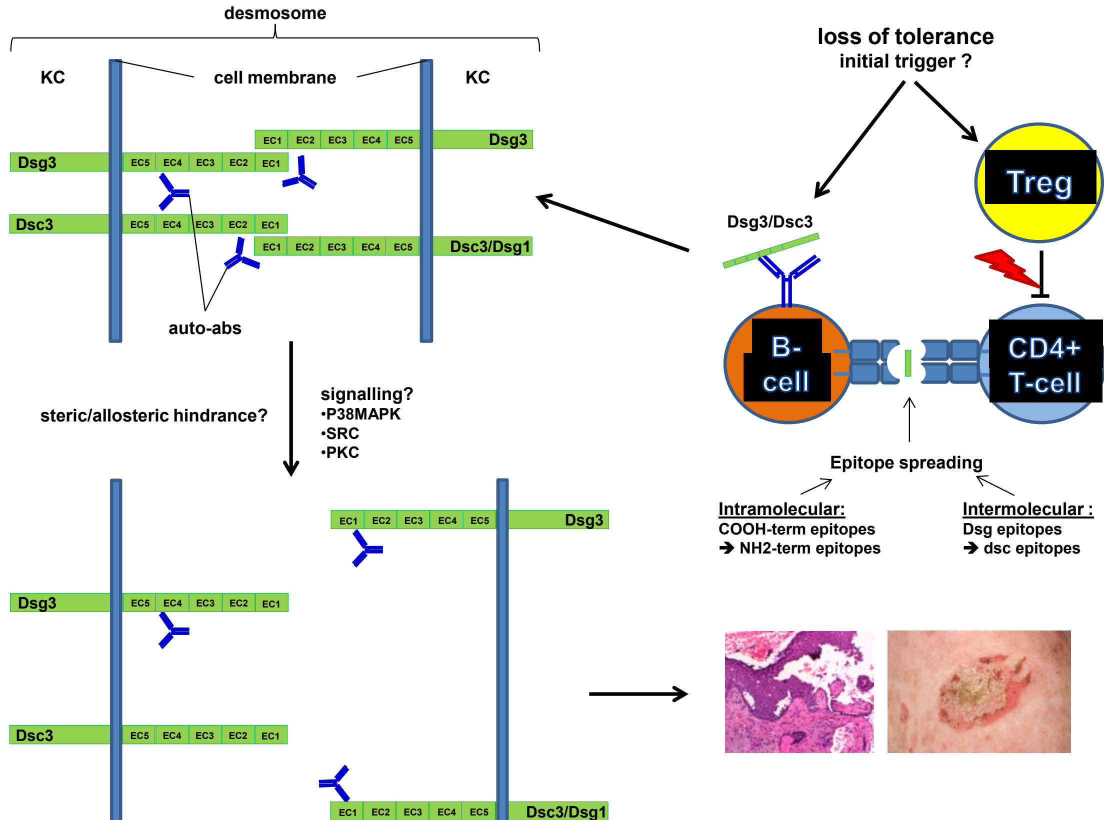


Figure 4.1: Synopsis of potential pathogenic mechanisms in pemphigus. The initial trigger for the loss of tolerance in pemphigus pathogenesis might be the liberation of antigen, i.e. desmoglein 3 or desmocollin 3, through keratinocyte lysis and subsequent protein uptake by B-cells and antigen presentation to auto-reactive CD4+T-cells. Intra- and/or inter-molecular epitope spreading may take place at this point of B- and T-cell cross-talk. Reduced control of these T-cells by impaired functioning of regulatory T-cells (Treg) may facilitate this process. B-cells then transform into plasma-cells producing auto-Abs directed against different epitopes of desmogleins and/or desmocollins. Auto-Abs may excert their pathogenic capacity by direct inhibition of desmosomal adhesion through binding at NH2-terminal and/or COOH-terminal epitopes or induction of intracellular signalling cascades finally leading to loss of keratinocyte adhesion and blister formation. In addition, auto-antibodies directed against different epitopes of desmoglein 3 may act synergistically to induce acantholysis probably by mechanisms of allosteric hindrance. Presence of desmocollin 3-reactive IgG may contribute to the pathogenesis in pemphigus as well. Finally desmoglein 3- as well as desmocollin 3-specific IgG may not only reduce homophilic desmoglein 3- and desmocollin 3-binding, respectively, but may also interfere with heterophilic binding between desmoglein 1 and desmocollin 3. Clinical pictures are courtesy of the Department of Dermatology and Allergy at the Philipps-Universität Marburg. P38 MAPK = P38 mitogen-activated protein kinase, SRC = sarcoma oncogene tyrosine kinase, PKC = protein kinas C, Treg = regulatory T cell, KC = keratinocyte.

5 Conclusions

- There is compelling evidence that cis interactions between neighbouring Dsg3 molecules at COOH-terminal regions play an important role in maintaining cellular adhesion of epidermal keratinocytes.
- IgG against COOH-terminal epitopes of Dsg3 are found in a great number of pemphigus patients including atypical variants, such as pemphigus herpetiformis and pemphigus vegetans.
- Although single monoclonal-Abs against COOH-terminal epitopes were not found to be pathogenic *in vivo*, they may act synergistically to facilitate blister formation in pemphigus.
- Dsg3EC4-specific IgG causes acantholysis *in vitro* and is likely to contribute to the blistering phenotype in pemphigus.
- Dsg3EC4-specific IgG recognizes conformation independent epitopes and belongs to the IgG_1 and IgG_4 isotypes.
- The exact mechanism how Dsg3EC4-specific IgG disrupts desmosomal adhesion remains unknown. Allosteric hindrance and the induction of intracellular signalling pathways are very promising hypotheses and need to be further investigated.
- IgG auto-Abs against Dscs are found in classical as well as in atypical pemphigus variants some of them exclusively react to Dsc3.
- Dsc3-specific IgG causes keratinocyte dissociation *in vitro* and may therefore provide an explanation why Dsg-negative pemphigus patients develop blisters.

6 Outlook

- Further *in vitro* and *in vivo* studies are needed to investigate the importance and the mode of action of auto-Abs directed against COOH-terminal epitopes of Dsg3 and against Dsc3, respectively.
- The pathogenic relevance of other epitopes of Dsg3, especially Dsg3EC5, needs to be addressed and if these pathogenic effects are exerted separately or in an additive manner.
- Screening of the presence of Dsc3-reactive IgG in the sera of pemphigus patients should provide further knowledge about the pathogenic role of these auto-Abs in pemphigus pathogenesis and their possible correlation with atypical pemphigus variants.
- Antigen-specific immunoadsorption is a promising targeted therapy option in auto-immune diseases. Therefore the investigation of the relevant repertoire of pathogenic epitopes in pemhigus should help to develop ways to specifically eliminate acantholytic auto-Abs.

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8 ELISA-Messdaten und dissociation scores

PV sera reactivity to recombinant Dsg3 and Dsg3EC-constructs (figure 3.3).

Values = optical density at 405nm wavelength. Unspecific = type 7 collagen.

PV-patient Serum	Dsg3	Dsg3EC1	Dsg3EC2	Dsg3EC3	Dsg3EC4	Dsg3EC5	Dsg1	unspecific
1	0,6465	0,6265	0,2575	0,2750	0,7115	0,3010	0,3600	0,2245
2	1,1045	0,5665	0,2900	0,2380	0,2570	0,2295	0,7080	0,2575
3	0,9440	0,3880	0,3875	0,3850	0,3625	0,2390	0,7635	0,2180
4	0,6065	0,5895	0,2010	0,2225	0,1900	0,1830	0,2270	0,2420
5	1,1830	0,3755	0,4060	0,3710	0,3855	0,1730	0,3195	0,2320
6	0,6810	0,2635	0,2735	0,3485	1,2140	0,1765	0,3540	0,2300
7	1,3575	0,4725	0,5350	0,3905	0,4515	0,2795	0,3660	0,1980
8	0,8570	0,6720	0,2470	0,2270	0,8545	0,2510	0,2555	0,2445

Confirmation of antigen specificity of purified IgG to recombinant Dsg3 and Dsg3EC-constructs (figure 3.6).

Values = optical density at 405nm wavelength.

	Dsg3				Dsg3EC1				Dsg3EC4				Dsg3EC5			
Antibodies/Sera	Pat. 1	Pat. 2	Pat. 6	Pat. 8	Pat. 1	Pat. 2	Pat. 6	Pat. 8	Pat. 1	Pat. 2	Pat. 6	Pat. 8	Pat. 1	Pat. 2	Pat. 6	Pat. 8
PV-Serum	1,412	2,0465	0,9675	1,1205	0,6375	0,921	0,199	0,3385	0,5115	0,4515	1,203	1,373	0,3785	0,1915	0,182	0,2915
Dsg3-IgG	0,9665	1,6945	0,7835	1,0635	0,1690	1,0300	0,4220	0,3240	0,2965	0,6020	0,5850	1,3280	0,1700	0,2845	0,2625	0,2230
Dsg3EC1-IgG	1,1260	1,9680	n/a	1,0845	0,8578	1,0125	n/a	0,6020	0,1860	0,3700	n/a	0,1380	0,1400	0,1715	n/a	0,1425
Dsg3EC4-IgG	1,1650	n/a	0,9465	0,8515	0,2385	n/a	0,2435	0,2845	0,7290	n/a	1,5470	1,7825	0,1705	n/a	0,1830	0,3370

IgG subclass distribution of affinity purified pemphigus IgG of patient 1 (fig. 3.7).

Values = optical density at 405nm wavelength.

Antibodies/serum of patient 1	IgG	IgG_1	IgG_2	IgG_3	IgG_4	IgA	IgE
PV-Serum	2,8135	1,4025	0,7730	0,1325	2,0715	0,4130	0,1275
Dsg3-IgG	1,6690	0,7165	0,2645	0,1250	0,9595	0,2075	0,1275
Dsg3EC1-IgG	2,6145	1,1875	0,6875	0,1545	1,9020	0,3010	0,1465
Dsg3EC4-IgG	1,8060	0,7100	0,2270	0,1465	0,7080	0,2110	0,1525

Conformation dependent recognition of Dsg3 epitopes by affinity purified IgG of patient 1 and 8 (figure 3.8). Values = optical density at 405nm wavelength.

	Dsg3 + Ca^{2+}		Dsg3 + EDTA		Dsg3 + Urea	
Antibodies/Sera	patient 1	patient 8	patient 1	patient 8	patient 1	patient 8
PV-Serum	1,1730	1,5045	0,4915	0,9445	0,4420	0,8900
Dsg3-IgG	1,0045	0,8195	0,7190	0,5965	0,6250	0,5440
Dsg3EC1-IgG	1,4665	1,3850	0,5735	0,5615	0,4360	0,4940
Dsg3EC4-IgG	0,8215	0,7995	0,8015	0,7860	0,7170	0,6960

Dissociation-Scores of Dsg3 and Dsg3-subdomain-specific IgG (figure 3.10).

Dissociation score = ((number of particles with serum – number of particles without serum) / (number of particles with serum – number of particles with AK23)) x 100.

patient	Serum	Dsg3-IgG	Dsg3EC1-IgG	Dsg3EC4-IgG
1	49,13044	33,47826	93,47826	82,17391
2	85,66038	112,8302	146,0377	n/a
6	143,0303	87,27273	n/a	34,54546
8	83,39484	59,4096	76,75277	178,9668

Dissociation-Scores of Dsc3-specific IgG (figure 3.15).

Dissociation score = ((number of particles with serum – number of particles without serum) / (number of particles with serum – number of particles with U114)) x 100.

patient	Dsc3-IgG	Dsc3-preadsorbed patients' IgG	Dsg3-preadsorbed patients' IgG
1	41,95	1,46	28,38
2	32,17	5,53	39,31
3	43,59	2,07	32,15
4	26,24	4,18	40,36

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Diese Seite enthält persönliche Daten und ist nicht Bestandteil der Online-Ausgabe.

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10 Verzeichnis der akademischen Lehrer

Meine akademischen LehrerInnen an der Philipps-Universität Marburg waren die Damen und Herren:

Adamkiewicz, Alter, Aumüller, Barth, Bartsch, Basler, Bauer, Baum, Becker K., Becker S., Benes, Czubayko, Daut, del Rey, Donner-Banzhoff, Eggert, Eilers, Eming, Fendrich, Feuser, Fuchs-Winkelmann, Görg, Gress, Grosse, Grundmann, Grzeschik, Hamer, Hassan, Hellmeyer, Hertl, Heverhagen, Höffken, Hofmann, Höglinger, Hoyer, Jansen, Kaltenborn, Kann, Kircher, Klose, Knake, König, Koolman, Kühnert, Leonhardt, Lill, Löffler, Lohoff, Maier, Maisch, Mandrek, Mennel, Michl, Moll, Moosdorf, Mueller, Mutters, Neubauer, Neumüller, Nimsky, Oertel, Opitz, Pagenstecher, Plant, Printz, Renz, Richter, Riße, Röhm, Röper, Rothmund, Ruchholtz, Schäfer H., Schäfer J., Schmidt, Schneider, Schofer, Schrader, Sekundo, Tackenberg, Vogelmeier, Wagner, Waldegger, Weihe, Werner, Westermann und Wulf.

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12 Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel

„Characterization of pathogenic auto-antibodies directed against desmoglein 3 and desmocollin 3 in sera of pemphigus patients“

in der Klinik für Dermatologie und Allergologie unter Leitung von PD Dr. med. R. Eming ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.
Teile der vorliegenden Arbeit wurden wie folgt veröffentlicht:

1. Rafei, D., Muller, R., Ishii, N., Llamazares, M., Hashimoto, T., Hertl, M. and Eming, R. (2011). IgG autoantibodies against desmocollin 3 in pemphigus sera induce loss of keratinocyte adhesion. Am J Pathol **178**(2): 718-23.

2. Rafei, D., Llamazares, M., Hertl, M. and Eming, R. Dsg3 specific IgG directed against C-terminal epitopes in pemphigus vulgaris induces loss of keratinocyte adhesion in vitro. 2012; J Invest Dermatol, 132: S14-S35 (Abstract 089) – 42nd Annual European Society for Dermatological Research (ESDR) Meeting 2012, Venedig.

Freiburg,

David Ali Rafei-Shamsabadi