The Role of Proinflammatory Cytokines and the Inflammasome in Pemphigus

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Zusammenfassung

Pemphigus vulgaris (PV) ist eine blasenbildende Autoimmunerkrankung, die aufgrund schwerwiegender Komorbiditäten, welche durch aktuelle Behandlungsmethoden ausgelöst werden können, immer noch eine therapeutische Herausforderung darstellt. Hinzukommen ein oftmals refraktärer Verlauf und eine hohe Mortalität, falls die Erkrankung nicht behandelt wird. Die meisten Patienten weisen IgG Autoantikörper gegen die desmosomalen Adhesionsmoleküle Desmolglein 3 (Dsg3) und Desmoglein 1 (Dsg1) auf. Diese Autoantikörper führen zu einer Dissoziation der Keratinozyten, ein Vorgang der auch Akantholyse genannt wird und folglich einer Blasenbildung der Haut. Obwohl anti-Dsg IgG Antikörper in der Epidermis omnipräsent sind, findet die Blasenbildung nur in bestimmten Bereichen der Haut und der Schleimhaut statt. Daher ist es von großer Bedeutung, die Diskrepanz zwischen Bindung der pathogenen Antikörper und der fehlenden Blasenbildung aufzudecken, welche vermutlich durch einen lokalen Kofaktor verursacht wird, der die Manifestation der Erkrankung begünstigt.

Da Zytokine des angeborenem Immunsystems wie Interleukin-(IL-)1 und IL-6 in Blasenflüssigkeit und Serum von Pemphigus Patienten gefunden wurde, könnte eine Entzündung induziert durch das angeborene Immunsystem als Kofaktor dienen, der zur Blasenbildung führt. Somit könnte die regionale Aktivierung des angeborenem Immunsystems die Manifestation der Erkrankung an bestimmten Lokalisationen erklären.

Um die Rolle der innaten Zytokine und der Aktivierung des Inflammasoms näher zu untersuchen, wurden zunächst Stimuli identifiziert, die eine angeborene Immunantwort in Keratinozyten hervorrufen. Hierbei stellten die Bestrahlung mit UVA, mechanischer Stress und die Aktivierung von TLR3 durch Poly I:C vielversprechende Kandidaten dar.

Die Dissoziation des Zellrasen aufgrund von Beeinträchtigung der Desmosomen wurde mittels eines etabliertem Keratinozyten Fragmentation Assay gemessen, um Rückschlüsse auf die Pathogenität von Antikörper und Kofaktor zu ziehen. Hier wurde beobachtet, dass UVA und Poly I:C in Kombination mit anti-Dsg3 Antikörpern zu einer signifikanten Erhöhung der Fragmentzahl führte, verglichen mit einer alleinigen Behandlung mit anti-Dsg3. Dieser Effekt war abhängig von der Aktivität von Caspasen.

Um dies unter physiologischeren Bedingungen zu bestätigen, wurden Hautstanzen ex vivo mit UVA und anti-Dsg3 Antikörper behandelt. Hierbei wurde auch bereits nach alleiniger Behandlung mit anti-Dsg3 Antikörper eine suprabasale Blasenbildung beobachtet. Die eingesetzte Antikörperkonzentration wurde dann auf ein subpathogenes Level titriert, unter welchem keine Blasenbildung beobachtet werden konnte. Nach Bestrahlung der Stanzen mit einer nicht-toxischen Dosis UVA wurde auch in Kombination mit einer subpathogenen

Antikörper Konzentration eine Akantholyse festgestellt. UVA-Strahlen ohne weitere Behandlung führten im Gegensatz dazu zu keiner Blasenbildung.
Zusammenfassend scheint die Aktivierung des angeborenen Immunsystems als Kofaktor an der Entstehung der Blasenbildung in Pemphigus vulgaris beteiligt zu sein.

Abstract

Pemphigus vulgaris (PV) is an autoimmune blistering disease which is therapeutically still a challenge due to severe co-morbidities of current treatment options and, in part, a refractory course and a high mortality when untreated. Most patients have IgG autoantibodies directed against desmosomal adhesion proteins like Desmoglein 3 (Dsg3) or Desmoglein 1 (Dsg1). These antibodies lead to dissociation of keratinocytes also called acantholysis and subsequent blister formation of the skin. Although anti-Dsg IgG antibodies are present ubiquitously in the epidermis, blister sites occur at distinct areas of the skin and mucous membranes. Therefore, it is vital to elucidate the discrepancy between binding of pathogenic IgG autoantibodies but lack of blister formation, putatively by identification of local cofactors responsible for disease manifestation. Since cytokines of the innate immune system, like interleukin-1 (IL-1) and interleukin-6 (IL-6) were detected in blister fluids and sera of patients, The hypothesis arose that the induction of innate inflammation might serve as cofactor for blister formation. Thus, the regional activation of the innate immune system could explain the discontinuous disease manifestation observed in PV patients.

To dissect the role of innate cytokines and activation of the inflammasome in PV first stimuli were identified that induce an innate response in keratinocytes. Hereby, irradiation with UVA, mechanical stress and TLR3 activation through Poly I:C represent promising candidates.

Using an established keratinocyte fragmentation assay, the dissociation of the cell layer due to desmosome disruption was quantified to evaluate pathogenicity of antibody and cofactor. Here it was observed that UVA and Poly I:C in addition to treatment with anti-Dsg3 antibodies resulted in a caspase-dependent significantly higher number of fragments compared to treatment with anti-Dsg3 alone.

In a more physiological setting, ex vivo skin explants were treated with UVA and anti-Dsg3 antibody as well. In this setting the treatment with anti-Dsg3 IgG led to suprabasal acantholysis. The concentration of the antibody then was titrated to a subpathogenic concentration, meaning that acantholysis was not present. After irradiation with a non-toxic dose of UVA, acantholysis was observed histologically. Irradiation with UV alone did not lead to any blister formation. Taken together, the hypothesis was set up that innate immune activation acts as a possible cofactor in the disease manifestation of PV.

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Abbreviations

A2ML1	Alpha-2-Macroglobulin-Like Protein 1
AIM2	Absent in melanoma 2
AK23	Hybridoma derived anti-Dsg3 antibody constructed by Tsunoda et al.[1]
APC	Antigen-presenting cell
ASC	Apoptosis-Associated speck-Like protein containing a CARD
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ions
cAMP	Cyclic adenosine monophosphate
CARD	Caspase recruitment domains
CLR	C-type lectin receptor
DAMP	Damage-associated molecular patterns
DCs	Dendritic cells
DMEM	Dulbecco's modified Eagle's medium
Dsc1	Desmocollin-1
Dsc3	Desmocollin-3
Dsg1	Desmoglein-1
Dsg3	Desmoglein-3
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinases
FCS	Fetal bovine serum
H_2SO_4	Sulfuric acid
HaCaT	Spontaneously transformed aneuploid immortal keratinocyte cell line
HBSS	Hanks' Balanced Salt Solution,
HLA	Human leucocyte antigen
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunglobulin
IL	Interleukin
IL-1R1	Interleukin-1 receptor type 1
IL-1R2	Interleukin-1 receptor type 2
IL-1RA	Interleukin-1 receptor antagonist
IRAK	Interleukin-1 receptor associated kinase

JUNK	C-Jun N-terminal kinases
kDa	Kilodalton
LDL	Low-density lipoprotein
LPS	Lipopolysaccharides
MAL	Myelin and lymphocyte protein
MAPK	Mitogen-activated protein kinase
moDC	Monocyte-derived dendritic cell
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLR	NOD-like receptor
NLRC	NLR family CARD domain-containing protein
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PDMS	Polydimethylsiloxane
PF	Pemphigus foliaceaus
PKC	Protein kinase c
PMA	Phorbol-12-myristat-13-acetat
PMP22	Peripheral myelin protein 22
PNP	Paraneoplastic pemphigus
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern Recognition Receptors
PV	Pemphigus vulgaris
RhoA	Ras homolog family member A
RPMI	Growth medium for human lymphocytes
SARS	Severe acute respiratory syndrome
Sr	Scavenger receptor
Src	Sarcoma
TAP	Transporter associated processing gene
Tfh	Follicular B helper T cells
TGF	Transforming growth factor
TH1	T-helper cells type 1
TH17	T-helper cells type 17
TH2	T-helper cells type 2

TIR	Toll-Interleukin receptor
TLR	Toll-like receptor
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRP channel	Transient receptor potential channel
UV	Ultraviolet

1. Introduction

1.1 The innate immune system

The innate immune system is the first line of defense against invading pathogens. Additionally, it leads to an adaptive response by antigen presentation inducing a target-specific response and subsequently immunological memory [2]. Instead of a pathogen-specific reaction it clears threats by complement activation or phagocytosis. The innate immune system is rather primitive in evolutionary terms, mainly because it is entirely encoded within the genome in contrast to the adaptive immune system [3].

1.1.1 Inflammation

Upon detection of pathogens or other danger signals cytokines and chemokines are released by activated macrophages initiating a process called inflammation. This results in the recruitment of molecules and cells from the blood to the infected tissue to destroy these pathogens via phagocytosis and thereby control the danger. Some cells are able to process certain pathogenic proteins and present them as antigens to other cells. The increased flow of lymph carries microbes and antigen-presenting cells from infected tissue to lymphoid tissue. Here resident lymphocytes are activated and thereby initiate an adaptive immune response. A mechanism to clear a local infection is complement activation. The complement is a group of plasma proteins that can bind to antibodies covering the surface of pathogens. Binding initiates a cascade of proteolytic reactions resulting in coating of the microbes. This coat is recognized by complement receptors inducing phagocytosis.

Inflammation is described by the Latin words “calor, dolor, rubor and tumor” meaning “heat, pain, redness and swelling” all resulting from cytokines or other inflammatory mediators. Heat, redness and swelling are the clinical manifestations of an increased permeability of blood vessels which is necessary to bring cells and proteins faster to infected tissue. Pain is caused by the migration of cells into tissue and their local actions.

Several types of cells are involved in the formation of an inflammatory response. In the initial phase tissue-bound macrophages are normally first on site. Subsequently, neutrophils are recruited to the tissue in large numbers. These two cells types are also known as inflammatory cells [4]. Following the influx of neutrophils monocytes migrate into the tissue and differentiate into macrophages or monocyte derived dendritic cells (moDCs) [5]. This reinforces and sustains the innate immune response. Later eosinophils migrate into tissue and contribute to the destruction of invading microbes.

1.1.2 Pattern-recognition receptors (PRRs)

When it comes to recognition of self and non-self by the innate immune system Pattern-recognition receptors (PRRs) are extremely important. PRRs are highly conserved in evolution. They recognize discrete molecular components of invading pathogens called pathogen-associated molecular patterns (PAMPs) [2]. Upon activation of PRRs pro-inflammatory cytokines are released and phagocytosis and antigen presentation are initiated [6].

Key PRRs are Toll-like receptors (TLRs) that have 13 identified members in total of which 10 are described in humans [7]. TLR 1, 2, 4, 5 and 6 are located on the cell surface while TLR 3, 7, 8 and 9 are located intracellularly on the endosomes and endoplasmatic reticulum [8]. Toll-like receptors are transmembrane proteins with an ectodomain consisting of leucine-rich repeats, a transmembrane domain and an intracellular Toll/IL-1R (TIR) domain [9,10]. Upon activation specific adapter molecules are recruited to the intracellular domain. Depending on the type of TLR possible adaptor molecules are Myeloid differentiation factor 88 (MyD88), MyD88 adaptor like (MAL), TIR domain-containing adapter- inducing interferon β (TRIF) and REIF adaptor molecule (TRAM) [9,11]. There are two different pathways for downstream signaling of TLRs: The MyD88-dependent pathway and the MyD88-independent (TRIF) pathway. Only TLR 3 uses solely the TRIF pathway for signaling [12]. TLR4 can use both pathways and all other TLRs signal through the MyD88-dependent pathway [13]. Downstream of both pathways occurs the activation of nuclear transcription factors NFκB, JNK or MAPK [9,14]. This leads to the expression of pro-inflammatory cytokines and type 1 interferon gene induction [2]. Each receptor recognizes a distinct component of pathogens that are essential for their metabolism. This reassures that these components cannot be made undetectable by mutations [15]. TLR 1, 2 and 6 detect gram-positive bacterial cell wall components like lipoproteins, peptidoglycans and lipoteichoic acids [15]. TLR 4 recognizes lipopolysaccharide (LPS) which is part of the gram-negative bacterial cell wall [16]. Bacterial flagellin activates TLR 5, while double-stranded and single-stranded viral RNA is detected by TLR 3, 7 and 8 [15]. TLR 9 is triggered by non-methylated CpG dinucleotides that can be part of the bacterial DNA [17].

Other PRRs include scavenger receptors (SRs), c-type lectin receptors (CLRs), B2 integrins and NOD-like receptors (NLRs) [18]. NLRs detect intracellular danger and can be divided into three groups depending on whether they contain a nucleotide-binding oligomerization domain (NOD), an NLR-leucin-rich repeat and pyrin domain (NLRP) or an NLR-leucin-rich repeat and pyrin domain which also contains CARD (NLRC) [19].

PRRs can also get activated by damage associated molecular patterns (DAMPs), a range of endogenous ligands released as a result of tissue and cellular damage. DAMPs act as danger signals that initiate sterile inflammation [15]. Agonistic DAMPs for TLRs are for example the

extracellular matrix constituent hyaluronic acid, the nuclear protein HMGB1, heat shock proteins 60 and 70, oxidized LDL, fibrinogen and fibronectin [20].

1.1.3 Inflammasome

One of the key signaling platforms of the innate immune system are the inflammasomes. They are able to detect threats posed by pathogens and sterile stressors alike and take a major role in the maintenance of homeostatic tissue functions. An inflammasome consists of a group of multimeric protein complexes: The inflammasome sensor molecule, the adaptor protein ASC and caspase-1 [21]. Upon activation of the inflammasome sensor molecule the complex forms and subsequently activates caspase-1 which can proteolytically activate IL-1β and IL-18 [22]. Additionally, an active inflammasome can induce a rapid, proinflammatory form of cell death called pyroptosis [23]. Dysregulation of this complex was identified to be involved in various diseases such as atherosclerosis, type 2 diabetes, cancer and neurodegenerative diseases. This underlines that here balance is vital [21].

The adaptor protein ASC is encoded by PYCARD. It inhabits two death-fold domains: A pyrin domain and a Card-domain. The pyrin-domain interacts with the inflammasome sensor molecule which leads to an assembly of ASC into multimeric dimers that form so called ASC-specs [24]. The CARD-domain brings monomers of pro-caspase-1 into close reach resulting in the self-cleavage of caspase-1 into active heterotetrameric caspase-1 [25]. Caspase-1 is able to cleave the inactive pro-forms of IL-1β and IL-18 into active form [26,27]. These two IL-1-familiy proteins are then secreted together with IL-1α. There is a variety of inflammasome sensor molecules. The group of NLRs all contain a central NACHT nucleotide-binding domain that has ATPase activity and a domain of carboxy-terminal leucine-rich repeats (LRRs) that has regulatory functions for the complex [28]. On the amino-terminal end sits a death-fold domain which is the structure that can interact with ASC and/or caspase-1. Depending on the type of death-fold domain the inflammasome is labeled NLRP when it possesses a pyrin-domain or NLRC when it contains a CARD-domain. In addition to these structures human NLRP1 contains a function-to-find domain (FIIND) and a C-terminal CARD-domain [25]. Other inflammasomes that do not contain NLRs are the AIM2-inflammasome and the IFI16-inflammasome. The sensor molecule AIM2 consists of a pyrin domain to recruit ASC and a DNA-binding HIN domain [29]. IFI16 inhibits one pyrin domain and two HIN domains for DNA binding [21]. There are several activators, some specific to one sensor molecule: NLRC4 detects microbial proteinaceous ligands, NLRP1 muramyl dipeptides [30] and NLRP7 bacterial lipo-peptides [31]. NLRP3 is able to sense crystalline material, peptide aggregates and bacterial toxins [32]. Activators of NLRP6 and NLRP12 have not been identified yet [21].

1.1.4 Cutaneous immunology

Although it is discussed whether the skin really is largest organ of the human body [33], it is definitely one of the most vital and complex. Due to its barrier function to the environment, it is the first organ to encounter pathogens and other harmful environmental influences. Therefore, elements of the innate as well as the adaptive immune system reside within the different skin layers to protect the body from invaders.

1.1.4.1 Skin barrier

The first barrier for invading pathogens even before the activation of the immune system is the skin and mucosal epithelium. It acts as a physical as well as a chemical barrier. Mechanically the epithelium repels intruders through tight junctions between cells. Additionally, longitudinal flow of air and fluid in certain body parts clears skin from pathogens [4]. In the lung and oral cavity cilia ensure the removal of microbes while tears have the same function in the eyes and oral cavity. A chemical barrier is present on the skin made up of fatty acids, β-defensins, lamellar bodies and cathelicidin. Cathelicidins are antimicrobial peptides occurring in mammals and fish that can be incorporated into the bacterial cell wall and membrane where they lead to a loss of ions and small molecules and subsequently to a loss of cell integrity [34]. Furthermore, these peptides have the potential to modulate the immune response and even directly activate immune cells [35]. The gut additionally has a low pH and enzymes like pepsin, α-defensins and lecticidins to combat invading pathogens. Furthermore, the skin imposes a microbiological barrier by offering a habitat for commensal microbiota. These microorganisms compete with pathogens for nutrients and attachment sides on epithelial cells. Some also bear the benefit of producing antimicrobial substances or are able to stimulate epithelial cells to produce antimicrobial substances. These microorganisms live in delicate balance of growth and elimination by the immune system. Once the balance is destroyed an overgrowth of commensals can cause diseases as well as a considerable decrease of commensals can lead to replacement by pathogens [4].

1.1.4.2 Cellular components of cutaneous innate immunology

The importance of the skin barrier becomes even more apparent in wounds, burns or a general loss of barrier integrity. In those cases, pathogens are able to enter the body and cause infections which are still a major cause of morbidity and mortality today.

Several cell types of the innate immune system reside in the skin like dermal mast cells, macrophages and Langerhans cells, an epidermal dendritic cell type[36]. Additionally, some cells are readily recruited from the blood like neutrophils, macrophages, basophiles, eosinophils, NK cells and gamma-delta T cells [15]. All these cells express TLRs. Furthermore, TLRs are

expressed on non-immune cells of the epidermis and dermis. Keratinocytes for example can express TLR1 - 6 and TLR 9 and 10 [37]. Which TLR is expressed in the end depends on the position of the cell within the skin. This helps to keep tolerance to the healthy skin biome: While TLR5 is predominantly expressed in the basal layer, TLR9 is much more common in the upper epidermal layer [38]. Upon activation of TLR2, 3, 4, 5 and 9 keratinocytes release TNFα, IL-8, CCL2, a basophil chemokine, and CCL20 which attracts lymphocytes [39]. Furthermore TLR3 and TLR9 lead to the release of CXCL9 and CXCL10 both chemokines involved in T-memory cell activation and type 1 interferon production [39]. Fibroblasts even express the full set of human TLRs from 1 to 10 [40]. Activation of TLR2, 3, 4, 5 and 9 initiate the production of IFNγ, CXCL9, CXCL10 and CXCL11 thereby recruiting T and NK cells. TLR 4 results in release of IL-6, IL-8 and monocyte chemotactic protein [41]. Additionally, microvascular cells such as dermal endothelial cells initiate through the activation of TLR4 and to a lesser extent TLR2 activation of NFκB [42].

1.1.4.3 Important cytokines and chemokines in keratinocytes

The skin is a large resource of cytokines. Keratinocytes have the potential to synthesis and secrete many cytokines and on the other hand can also be influenced by a variety of cytokines [43].

Immunomodulatory cytokines produced in keratinocytes are IL-10, IL-12 and IL-18. IL-10 is able to shift the immune response to a Th2 pattern and thereby acts as an immune suppressor and an “antinflammatory” cytokine [44]. Contrary, IL-12 induces a Th1 response [45]. It furthermore plays a role in allergic contact dermatitis [46] and promotes the production of IFNγ which then again strengthens the Th1 pattern [47]. IL-18 is a structural homologue to IL-1 and similar to IL-1β its cleavage and secretion depends on inflammasome activation. The presence of IL-18 induces IFNγ as well [48,49].

The cytokines IL-7 and IL-15 can be labeled as cell-trophic. IL-7 is a growth factor for dendritic epidermal T-cells [50-52]. Its production is upregulated by IFNγ and downregulated by UVB [52,53]. IL-15 is usually present in inflammatory conditions and thereby plays a role in the migration of inflammatory cells such as eosinophils, neutrophils and $CD3^+$ T-cells [54]. Like IL-7 it is downregulated by UVB [55].

Nevertheless, the most important cytokines for this project are proinflammatory cytokines produced by keratinocytes. Some of them have already been well characterized and are long known like IL-1 that was thoroughly described in the 1980s [56,57]. Two important forms of the IL-1-family are IL-1α and IL-1β. They translate into a pro-form of 33 kDa for IL-1α and 31 kDa for IL-1β that reside in the cytoplasm. Although the two forms are only 23% homologue on amino acid level their tertiary structure is very similar. IL-1α is already active in its pro-form

nevertheless, it can be cleaved into a smaller 17 kDa form [58]. IL-1β is only biologically active in its cleaved 17 kDa form [59]. The receptors for IL-1, IL-1 receptor type 1 (IL-1R1) and type 2 (IL-1R2), can be found on the same cells that secret them allowing a feedback loop. Both IL-1 forms can bind to both receptor types. Activation of IL-1R1 leads to the recruitment of the adaptor molecule MyD88 and subsequently the activation of IRAK and transcription factor NF-κB. Relocation of latter stimulates an inflammatory response [60]. As inflammation mediated by IL-1 is therefore able to enhance itself through the feedback loop, it is necessary to have an antagonist for regulation [61]. The natural IL-1 receptor antagonist (IL-1RA) is thus used in a recombinant, slightly modified version as a therapy for rheumatoid arthritis under the name Anakinra [62].

IL-1 production can be triggered by various factors that induce a proinflammatory response. External triggers are, inter alia, wounding, burns [60], chemicals like sulfur mustard [63] and nickel [64], contact allergens [65] and mechanical stress [66]. Internally IL-1 is induced by cytokine released from leukocytes and as already mentioned through the IL-1R1 [60]. The production of IL-1 mediates the induction of keratin 6 expression and protects keratinocytes from apoptosis when exposed to TRAIL [43]. On top of that the E-cadherin expression of Langerhans cells can be downregulated. This leads to cell migration from the epidermis to the dermis and subsequently to the lymph nodes [67].

To be biologically active and secreted IL-1β needs to be cleaved by caspase-1 that is induced by inflammasome activation as described earlier. Additionally, its secretion is mediated by inflammasomes as well. IL-1α can be cleaved by calpains though this is not necessary for the activity of the cytokine [68]. Calpains are activated by small particles or Ca^{2+} influx. The secretion of IL-1α is either mediated through caspase-1 in an inflammasome-dependent way or independently of the inflammasome through enablers like small particles, C. difficile toxin B or activators of the TRP channels. All of those can also induce a strong Ca^{2+} influx hence IL-1α cleavage and secretion are highly entangled in the regulation of Ca^{2+} in the cell although the detailed mechanism is still unknown [69].

Another important proinflammatory cytokine is IL-6. It is expressed under a variety of conditions like UVB irradiation and treatment with TGFα [70-72]. Besides having many functions in the mediation of inflammation like inducing fever and an acute phase response it also plays a major role in keratinocyte proliferation which suggests its role in wound healing [73-75].

IL-8 is a chemokine which facilitates the migration of leukocytes from the blood vessel into inflamed skin. Like IL-1 it is expressed in an TLR-NF-κB-dependent manner [76]. Its production is induced by external stimuli like arsenic, contact sensitizers and irritants [77,78], but most importantly through pathogens [76]. IL-8 is known to be involved in autoimmune diseases like bullous pemphigoid and pemphigus herpetiformis [79,80].

Besides having receptors for cytokines which keratinocytes produce themselves, they also possess receptors for external cytokines. For example IL-4 can be sensed and induces proliferation of keratinocytes as well as IL-6 production [43]. IL-13 is known to induce IL-6 production as well [81]. Activation of the receptors for IL-17 modulates the effect of IL-4 or IFNγ on the activation of keratinocytes [82].

1.2 Autoimmunity

At the beginning of the 20th century the idea of autoimmunity was presented the first time by Paul Ehrlich. He termed it “horror autotoxicus” which is Latin for aversion of self-destruction as he did not believe the immune system could turn against its own host. In his view this would not be compatible with self-preservation of life. This was refuted in the 1950s by several scientific publications [83].

An autoimmune reaction resembles a normal response to pathogens but is directed against proteins of the host. Therefore, antigens are recognized as autoantigens targeted by autoreactive effector cells and autoantibodies [84]. Here, the discrimination of self from non-self is compromised. Approximately 5% of the population of Western countries is affected by such disorders with rising incident [4]. For the manifestation of an autoimmune disease a genetical predisposition is often required. The failure of intrinsic tolerance and/or environmental factors trigger the disease [85]. Autoimmune diseases can be classified in organ specific or systemic. Organ specific autoimmune disorders target only one or few organs like multiple sclerosis, psoriasis or Type 1 diabetes mellitus. In systemic diseases many organs are affected like in rheumatoid arthritis or primary Sjögren’s syndrome [4].

1.2.1 Pathogenic mechanisms of autoimmunity

To develop a chronic autoimmune disease positive feedback from inflammation and the inability to clear self-antigens are necessary, which results in a broadening of the disorder. It starts with an early activation phase with only few antigens. To induce an immune response to these antigens other components beside the adaptive immune system are necessary, like the innate immune system. Cytokines and chemokines released from inflamed tissue attract nonspecific effector cells, like macrophages and neutrophils. Tissue damage induced by inflammation induces more autoantigens which can be bound and presented by circulating B cells (see Fig. 1) [4]. The B cells then activate T cells specific for the self-peptides causing the B cells to differentiate into plasma cells that secrete large amounts of autoantibodies. Autoantibodies bind to autoantigens resulting in increased tissue damage. Therefore the reaction is maintained and even amplified by a constant supply of new autoantigens [4]. One

important step in the development of a chronic autoimmune disorder is the break of sequestration which in healthy humans imposes an important barrier by keeping self-antigens apart from the immune system [86]. Another important step upon the transition to a chronic stage is epitope spreading. Thereby new epitopes of already existing autoantigens or even new autoantigens are recognized by the immune system. This happens through the procession of internalized autoantigens by B cells that results in the presentation of previously hidden epitopes. A respond of T cells to these new epitopes leads to the recruitment of additional B cells and subsequently a greater variety of autoantibodies. It is also possible that B cells internalize other molecules closely associated with the target antigen causing presentation of epitopes from completely different proteins [4].

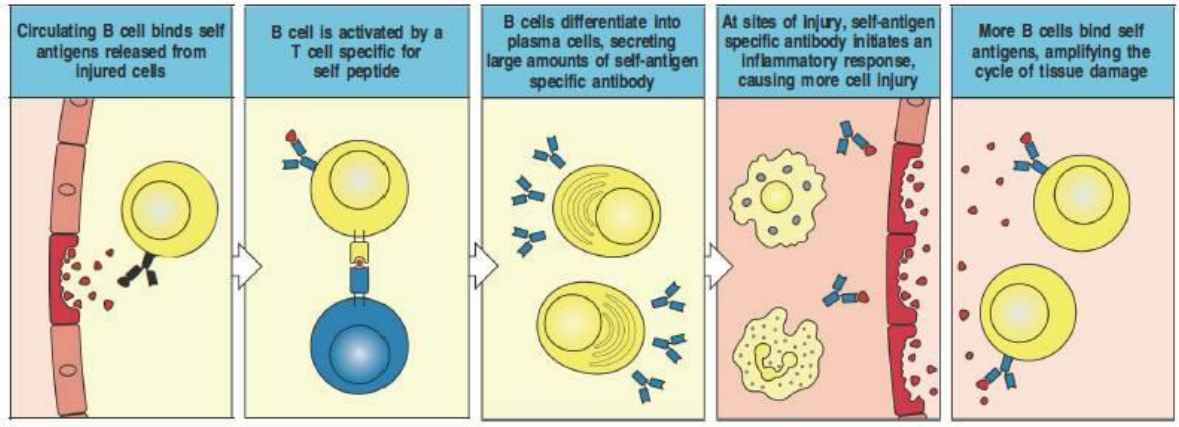

Figure 1: Cycle of tissue damage in autoantibody-mediated inflammation. Adapted from Murphy K, Travers P, Walport M, Janeway C. Janeway's immunobiology. New York: Garland Science; 2008. p. 628, Fig. 15.17

1.2.2 Genetic and environmental basis of autoimmunity

Although most autoimmune disorders require as already mentioned a genetic predisposition, an external event is usually necessary for the onset of the disease. This can be a geographic factor influencing the HLA-pattern as there is a heterogeneous distribution of many autoimmune diseases between continents and countries. The socioeconomic status, diet or exposure to environmental toxins [4]. or even an infection might create an environment that promotes lymphocyte activation through inflammation. The phenomenon known as molecular mimicry describes how antibodies originally targeted against pathogenic molecules can cross-react with self-antigens. Last but not least a pure chance encounter can be enough for the manifestation of an autoimmune disease: If in the peripheral lymphoid tissue a few autoreactive B and T cells are able to interact with each other and additionally pro-inflammatory signals from any infection are present, an autoimmune reaction can form [85].

1.3 Autoimmune bullous dermatoses

Autoimmune bullous dermatoses refer to a group of diseases with autoantibodies directed against adhesion molecules or structural proteins of the skin or mucosa involved. All types have in common that they clinically lead to blister formation. Although the symptoms are mostly confined to epithelial skin and the mucosa, quality of life can be severely impaired by superinfections, cachexia, scarring and chronification. The type of bullous dermatosis is defined by the protein targeted by the autoantibodies and the reaction in the skin [87].

Generally autoimmune bullous dermatoses are distinguished in three groups: Intraepidermal autoimmune blistering diseases known as pemphigus disorder, junctional autoimmune blistering diseases also called pemphigoid disorder and subepidermal autoimmune blistering diseases like epidermolysis bullosa acquisita and dermatitis herpetiformis [87]. While pemphigus carries antibodies against the epidermal adhesion complex also known as desmosome, antibodies in pemphigoid target hemidesmosomal proteins like BP180, BP230, α6β4 integrin or laminins [88]. In these entities a cleft formation within or below the basement membrane zone is observed. Epidermolysis bullosa acquisita is rather rare and shows an autoreactivity to dermal anchoring fibrils like type VII collagen [89]. Dermal blisters are present in dermatitis herpetiformis due to immune complexes of IgA and epidermal transglutaminase in the dermis [90].

With 40 million new cases per year in Europe the most common bullous autoimmune disorder is bullous pemphigoid [87]. Other disorders in the pemphigoid group include mucous membrane pemphigoid, pemphigoid gestationis, linear IgA bullous dermatosis, anti-laminin γ -1 pemphigoid and lichen planus pemphigoides [91].

1.3.1 Pemphigus

Pemphigus refers to a group of life-threatening autoimmune bullous diseases that are characterized by flaccid blisters and erosions [92]. The term “pemphigus” originates from the Greek word “Pemphix”, meaning blister [93].The prevalence is associated with two HLA-types: HLA-DRB1*04:02 and HLADQB1*05:03 [94-96]. The majority of affected patients have autoantibodies against the desmosomal cadherins desmoglein-1 (Dsg1) and/or desmoglein-3 (Dsg3) [97]. Hereby the presence of anti-Dsg1 and anti-Dsg3 relates to the clinical phenotype [98]. The concentration of these autoantibodies correlates in general to the clinical activity of the disease [99]. While pemphigus is therapeutically still a challenge due to severe co-morbidities of current treatment options [100,101] and high mortality when untreated [102], it offers a potent model to study antibody mediated immune disorders. The involved autoantigens are identified, and the tissue-specific expression pattern is characterized [103].

1.3.1.1 Pemphigus vulgaris

Pemphigus vulgaris (PV) is the most common clinical variant of the pemphigus group with 0.5-3.2 per 10^5 incidents per year[103,104]. There is no sex preference; affected are mostly adults between 30 and 60 years of age. Nevertheless, a rare juvenile form of pemphigus vulgaris exists, where young adults in their twenties are affected [92]. Untreated the disorder is fatal within five years with a 50% mortality after two years [100]. Death occurs mostly due to secondary problems like superinfections or malnutrition due to erosions in the mouth and throat that make food intake extremely painful [100].

As already mentioned, there is an HLA-associated prevalence which also correlates to specific ethnic groups, as here the specific HLA-types occur more often. HLA-DRB1*04:02 is very common amongst Jewish people and therefore leads to an increased incidence of PV in this group [105]. Among non-Jewish PV patients HLADQB1*05:03 is more frequent [106]. Here especially people from Iraq, Iran or India are affected. Besides the mentioned HLA-types, PV is also associated with HLA-G and the transporter associated processing (TAP) gene [107].

Clinical symptoms initially start with erosions of the oral mucosa in 70% or the genital mucosa in 20% of all cases. Very rarely the conjunctiva is also affected. Other sites of occurrence might be the lips, palate, cheeks, gingiva, larynx and pharynx resulting in impaired eating, swallowing, talking and hoarseness. Accompanying symptoms might be sialorrhoea, bloody salvia and fetor ex ore. Subsequently flaccid blisters develop on the trunk and transform into slowly healing, crusty erosions [108].

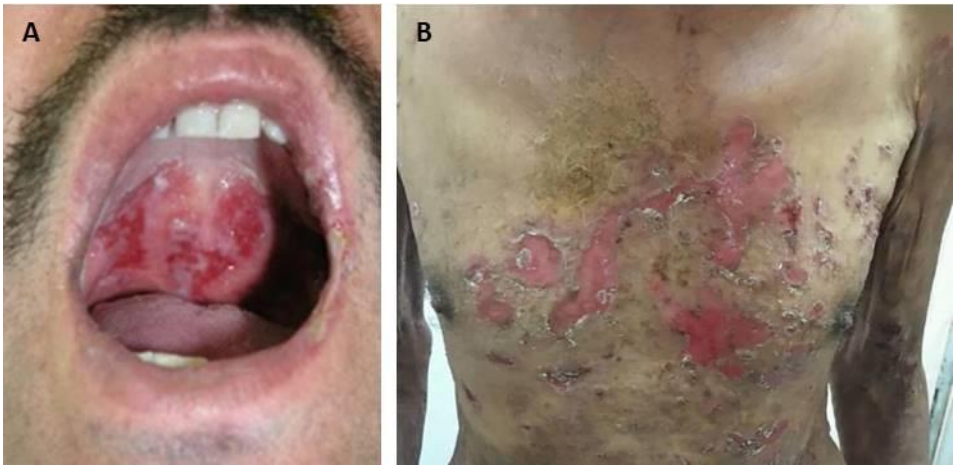

Figure 2: Mucosal (A)[109] and cutaneous (B)[110] involvement of pemphigus vulgaris.

1.3.1.2 Pemphigus foliaceus

The term pemphigus foliaceaus (PF) originates from the Latin word “Folium” meaning “leaf” and referring to the leafy scales many patients exhibit [111]. This type of pemphigus is much rarer and can be divided in a sporadic form and endemic forms occurring in Brazil (pemphigus

braziliensis) or in North Africa (fogo selvagem). Similar to PV there is no sex preference [103]. Children are even less affected than in PV [112].

In contrast to PV lesions only occur on keratinized skin, especially in the seborrheic area. Blisters rarely appear, the disease manifests with scaly crusts at the seborrheic body sites.

Brazilian pemphigus is more frequently in children and young adults [112]. In endemic areas a great prevalence of anti-Dsg1 autoantibodies were found even in healthy individuals. Therefore, it is very likely that there is a local rural factor causing this. The salvia of an endemic sand fly is discussed [113].

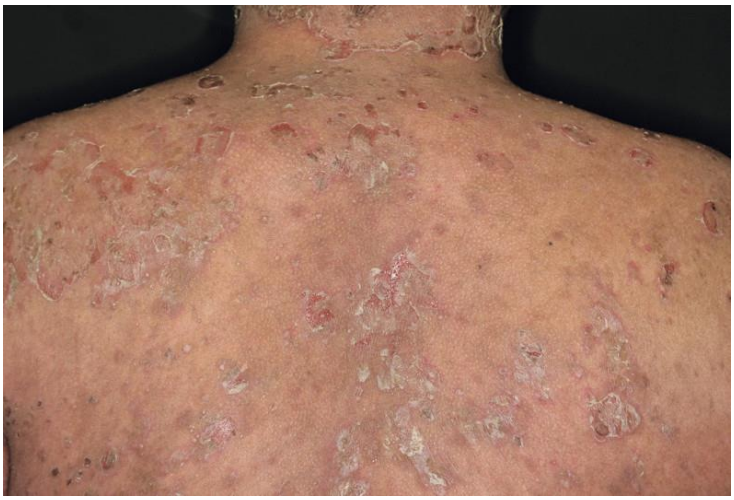

Figure 3: Characteristic erosions in pemphigus foliaceus[114].

1.3.1.3 Paraneoplastic pemphigus and rare types of pemphigus

A type of pemphigus with a very high mortality of approximately 90% is paraneoplastic pemphigus (PNP). This disorder is characterized by painful, extensive erosions of the mucosa together with lichenoid papules of the extremities. Additionally, it is associated with lymphoproliferative diseases like chronic lymphocytic leukemia, plasmocytoma, Castleman´s disease and B cell lymphoma [115]. In contrast to PV and PF PNP is not primarily associated with autoantibodies against desmogleins but against proteins of the plakin family. Targets are for example plaque proteins desmoplakin-I and –II, envoplakin and periplakin as well as the hemidesmosomal adhesion molecules plektin and BP230 [116,117]. The prognosis is very closely linked to the treatment of the underlying malignancy.

Another form of pemphigus is IgA pemphigus which as the name indicates is mediated mainly by IgA-autoantibodies. Clinically it is indicated by pustules instead of flaccid blisters and a mucosal involvement is rare [118]. By looking at the target proteins of the autoantibodies two forms of IgA pemphigus can be distinguished: An intraepidermal neutrophilic disease-type is associated with antibodies against Dsg1 or Dsg3 [119], while antibodies targeting desmocollin 1 (Dsc1) indicate a subcorneal pustular dermatosis [120,121].

A rare type of pemphigus with less than 5% is pemphigus vegetans which presents as hypertrophic vegetating plaques. It can be divided in the more aggressive Neumann type and the less aggressive Hallopeau type. Another rare form is pemphigus herpetiformis which is characterized by herpetiform, flaccide and cutaneous bullae [108,118].

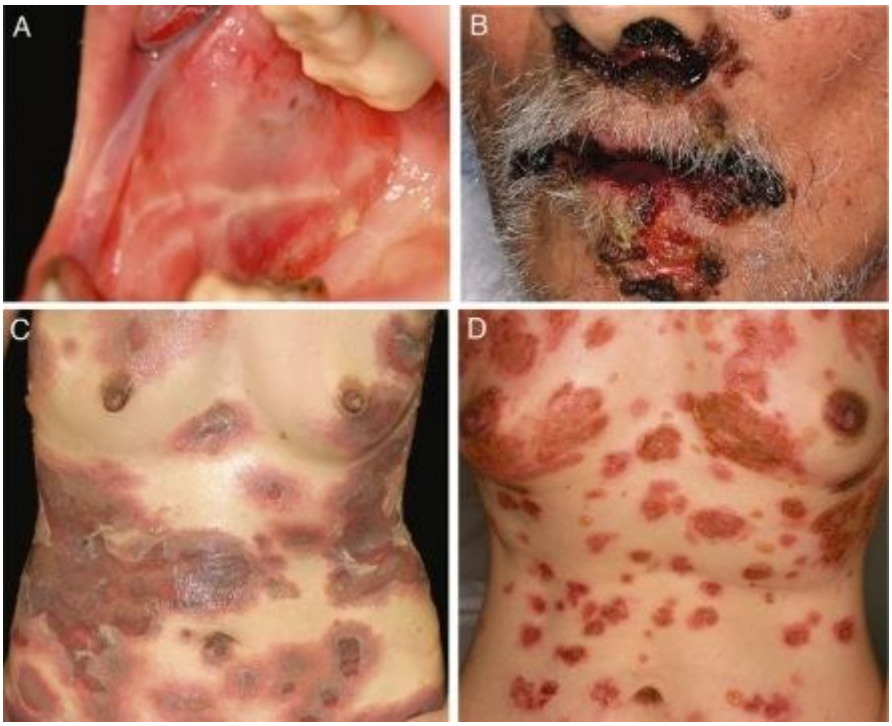

Figure 4: Clinical phenotype of paraneoplastic pemphigus[122].

1.3.2 Pathophysiology

Disease manifestation in pemphigus is connected to circulating autoantibodies that can bind to the desmosomes to induce a loss of cell-cell adhesion. However, the presence of autoantibodies alone does not lead to blister formation. In patients in remission autoantibody titers do not correlate to disease symptoms as blisters disappear long before antibody titers decrease [123]. Also, it is striking that, although autoantibodies are present ubiquitously, blistering only occurs in distinct areas of the body. Therefore, other cell-signaling events and antibody-independent factors have to be involved in disease manifestation of pemphigus.

1.3.2.1 Types of autoantibodies and mode of action

In general, there seems to be a difference between pathogenic and non-pathogenic antibodies although the overall mechanism that leads to disease manifestation only at defined parts of the body is still cryptic. One explanation is the existence of non-pathogenic antibodies that cannot induce acantholysis [124,125]. A theory is that the pathogenic potential is dependent on the recognition of distinct epitopes. NH2-terminal portion of Dsg3 has been suggested as a pathogenic target while non-pathogenic antibodies target epitopes of the membrane proximal

COOH-terminus of Dsg [1,126,127]. The major pathogenic isotype found is IgG4 [128,129]. A special case is Brazilian PF: Here non-pathogenic antibodies are of the IgG1 subtype while a class switch to IgG4 induces a clinical phenotype [130,131].

There are several effects induced by the binding of pemphigus autoantibodies: The binding to the NH2-terminal domain leads to sterical hinderance and therefore inhibits the trans-interaction of desmogleins [132,133]. There are also hints that binding of the antibody induces internalization and consecutive depletion of Dsg from the cell membrane [134-136]. This depletion then leads to acantholysis and seems to correlate with the pathogenicity [137]. It was shown that Dsg interacts with flotillins which are components of the lipid rafts. An antibody-induced interference might as well lead to an internalization of Dsg [138].

One major theory regarding antibodies in pemphigus is the Dsg1/Dsg3- compensation theory. The theory builds on the differential expression of autoantigens in PF and PV. Dsg1 is mostly located in the upper layer of the epidermis while Dsg3 occurs in the lower and suprabasal layers. Therefore, sera containing anti-Dsg3 antibodies alone are able to induce a loss of adhesion in the mucous membrane where Dsg1 is not expressed. In the skin where Dsg1 is present as well, anti-Dsg3 antibodies do not lead to suprabasal loss of adhesion. Nevertheless, whenever anti-Dsg3 and anti-Dsg1 antibodies can be found in sera, loss of adhesion can occur in skin as well.

In addition, in rare subtypes of pemphigus non-Dsg antibodies occur. PNP patients for example produce antibodies directed against Desmocollin-3 (Dsc3) [139,140]. Besides that, one major autoantibody in PNP is targeted against the protease inhibitor A2ML1 [141]. But even in more common types of Pemphigus autoantibodies against non-Dsg proteins were found. 85% of PV patients have IgGs targeting the keratinocyte α-acetylcholin receptor. There are hints that blocking of this receptor also leads to a desmosomal disassembly [142]. Other targets that have been observed are Dsc1 [143], desmoplakin 1 and 2 [144], Desmoglein 4 which is cross-reactive with Dsg1 [145], pemphaxin [146], E-cadherin [147] and PMP22/gas [148]. Their role is not completely understood yet. These autoantibodies might act synergistically with antibodies targeting Desmoglein or might even help to break tolerance.

1.3.2.2 Cell-signaling Events

At first glance pemphigus autoantibodies directed against Dsg1 and Dsg3 disturb the turnover of desmosomes and lead to a depletion of desmoglein. Other structures of the desmosomes are affected as well. For example, desmosomes are reduced in their size and number and keratin filaments are uncoupled. Still, pemphigus autoantibodies can impact various other intracellular signaling pathways although the exact purpose has yet to be discovered. Walter et al. investigated which pathways are activated in the mucosal, mucocutaneous and

cutaneous form of pemphigus [149]. The latter form is associated with PF in which only autoantibodies targeting Dsg1 are present. The mucosal and mucocutaneous forms occur in PV where antibodies directed against Dsg3 are mandatory and against Dsg1 optional [150]. They concluded that the p38MAPK pathway was active in mucosal and cutaneous pemphigus. The potential of PV-autoantibodies to modulate this pathway as well as the RhoA pathway were shown before [151,152]. Additionally, they suggested that Src and cAMP pathways are only activated in the mucosal type of pemphigus which is associated with anti-Dsg3 antibodies. Contrary Erk and Ca^{2+} pathways were activated in the cutaneous form of pemphigus connected to Dsg1 targeting antibodies. They conclude that these correlations between different autoantibodies and activation of different signaling pathways need to be further investigated, but still could be responsible for the different clinical phenotypes of pemphigus [149].

1.3.2.3 Autoantibody-independent factors

Shifts in expression and secretion of various cytokines that can be observed in PV patients have been identified as autoantibody-independent factors. On the one hand there is an increase in IL-6 and IL-10 as well as the TH2-cytokine IL-4 [153-155]. Th1-cytokines like IL-2 and IFNγ are on the other hand suppressed [155]. Furthermore, the TH17-cytokine IL-17a is increased [156] and levels of the Tfh-cytokines IL-21 and IL-27 are elevated [157]. Apart from that some studies also report an increase in IL-8 [158]. In blister fluid pro-inflammatory cytokines like IL-1α, IL-6 and TNF have been detected [159]. Furthermore IL-4 and IL-10 have been found [160] besides cytotoxic proteases [161] and complement activation [162]. Another important factor implicated in cell death is FasLigand (FasL) which was detected in pemphigus sera and induced apoptosis in keratinocytes via caspase 8 [163].

1.3.3 Diagnostic and current treatment options

There are four different options for diagnostics whenever a bullous autoimmune disease such as PV is suspected. Option one is a histopathology as in PV acantholysis is noted, which is the suprabasal loss of epidermal keratinocyte adhesion. In PF acantholysis is more superficial in the subcorneal stratum spinosum [164]. It is also possible to do a Tzanck test where single or grouped acantholytic keratinocytes can be retrieved by a smear from blisters or erosions [165].
Another option is a direct or indirect immunofluorescence staining. The goal is to detect tissue-bound or serum IgG or IgA autoantibodies. When using serum, monkey esophagus is most commonly used as a substrate for detecting anti-IgG serum autoantibodies. For the detection of plakin-specific IgG rat or monkey urinary bladder is used as a substrate [166].
A serological detection of IgG autoantibodies can also be done via ELISA. In PV with exclusive involvement of the mucous membrane IgG against Dsg3 are detected. In mucocutaneus PV

one would find antibodies against Dsg1 and Dsg3 and the sole presence of anti-Dsg1 IgG is typical for Pemphigus foliaceus. Furthermore, the concentration of serum IgG autoantibodies against desmoglein in many cases correlates with the clinical severity, therefore serving as a good serological marker of disease activity [167].

Further diagnostic options are the immunoblot and immune precipitation as more specific, but time consuming additional immune serological assays. This is mostly used for atypical pemphigus diseases like pemphigus vegetans, pemphigus herpetiformis or PNP where no commercial ELISA is available. Here anti-Dsc3 antibody is detected as the major autoantibody of interest in the immunoblot [139].

As an initial treatment a combination of high-dose systemic corticosteroids possibly in combination with steroid-sparing immunosuppressants is administered [100]. Another option is the application of high-dose intravenous immunoglobulins (IVIG therapy). This treatment often leads to a rapid reduction of the titers of serum autoantibodies followed by a clinical remission [168,169]. It is also possible to therapeutically remove circulating IgG by immunoadsorption which of course reduces the autoantibodies in the serum of pemphigus patients and often results in good clinical responses [170]. Nevertheless this is a rather expensive and for the patient very time-consuming treatment as it has to be repeated regularly [171].

A therapy option that arose quite recently is the treatment with the B-cell depleting monoclonal anti-CD20 antibody rituximab. In PV it seems to be especially effective for patients with refractory disease [172].

1.3.4 Involvement of interleukin-1 in the disease manifestation

The role of proinflammatory cytokines in pemphigus has been discussed in several publications. For examples high levels of IL-6 and TNF were found in sera and blister fluids of pemphigus patients and titers of those cytokines seem to correlate with disease activity [154]. Bhol et al. detected high levels of IL-1α and IL-1β in sera of untreated pemphigus patients. These levels decreased in clinical remission off therapy to levels similar to the healthy control. Additionally, they detected low levels of IL-1RA in sera of patients with active pemphigus disease and suggested that high levels of IL-1α and IL-1β and low levels of anti-inflammatory IL-1RA correlate with disease activity. IVIG therapy induced a decrease in IL-1α and IL-1β and an increase in IL-1RA *in vitro* during clinical remission in patients [173]. Feliciani et al. detected increased levels of IL-1α in lesional and perilesional skin of pemphigus patients with active disease. Furthermore, in vitro IgG from PV patients induced *IL1A* mRNA expression in the skin [159]. For the endemic form of PF Rocha-Rodrigues et al. claimed that PBMCs from these patients secreted significantly higher amounts of IL-1β after stimulation with LPS than PBMCs

from healthy individuals [174]. Nevertheless, it remains open how IL-1 is exactly involved in the mechanism of blister formation and to what extent it interacts with autoantibodies.

1.4 Aim of the project

The disease manifestation in PV is still cryptic as the sole presence of autoantibodies is not sufficient to provoke blisters or disease symptoms. Patients can carry detectable amounts of antibodies targeting Dsg1 and/or Dsg3 in the serum and the skin which are functional *in vitro* without showing any signs of PV. Thereby, functionality of the antibodies can be assessed *in vitro* by analyzing fragmentation in the dispase-based dissociation assay or by detecting binding-abilities to desmoglein via immunofluorescence staining. Another factor that is not completely understand today is the fact that PV manifests at very distinct regions of the body although autoantibodies are present and bind ubiquitously. Also, patients in remission usually lose symptoms long before antibody titers decrease [167]. Therefore, it is postulated that a yet unknown cofactor closes the gap from antibody binding to acantholysis within the skin and hence induce blister formation.

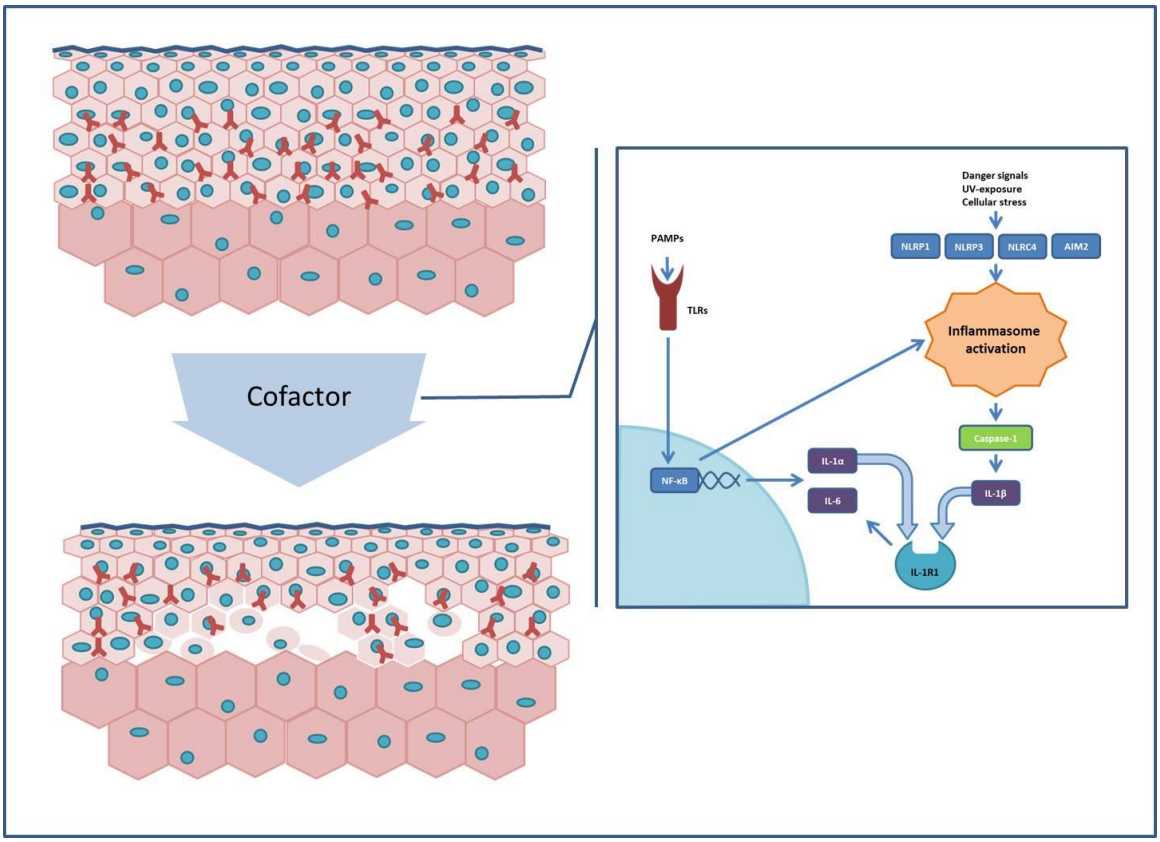

Figure 5: A yet unknown factor is needed to induce disease manifestation in pemphigus. Autoantibodies bind ubiquitously to desmosomes in the epidermis of pemphigus patients. Nevertheless, to achieve a loss of cell-cell adhesion resulting in acantholysis and subsequently blister formation a yet unknown factor is needed. Thus, the working hypothesis is that the innate immune system through TLR- and inflammasome activation, downstream signaling and the release of proinflammatory cytokines might be involved.

It has already been shown that the innate immune system can be involved in diseases of the adaptive immune system like for example psoriasis, a T-cell mediated disease. In blister fluid, blister tissue and sera of pemphigus patients proinflammatory cytokines like IL-1α, IL-6 and

TNF-α have been detected. Therefore, it might be possible that the involvement of the innate immune system induces disease manifestation in pemphigus diseases. The regional activation of the innate immune system could explain the discontinuous disease manifestation observed in PV patients. Thus, the overall aim of this project was to analyze whether and how the activation of the innate immune system is involved through proinflammatory in the disease manifestation of pemphigus.

2. Material and Methods

2.1 Material

Material	Manufacturer	Order number
ACK Lyse Puffer	Lonza	10-548E
Ac-YVAD-cmk	InvivoGen	inh-yvad
BSA-c	Aurion	900.022
Calciumchloriddihydrat	Roth	52392
CnT-07	CELLnTEC	cnt-07
Dispase II	Roche	4942078001
DMEM	Biochrom/ Merck	F 0435
DMSO	AppliChem	A3672
ELISA Auxiliary Kit	R&D Systems	DY008
Empty Lab Columns 2.5 ml	MoBiTec	S1012
Ethanol absolute p. A.	AppliChem	A3678
FCS	Sigma	F7524
Ficoll: Histopaque-1077 Hybrid-Max^{tm}	Sigma	H8889-500ML
Formaldehyd 37%	AppliChem	A0877
Gentamycin/Amphotericin	CELLnTEC	CnT-GAB10
HBSS (+Ca^{2+}, +Mg^{2+})	Thermo Scientific	14025050
Human IL-1 alpha/IL-1F1 DuoSet ELISA	R&D Systems	DY200
Human IL-1 beta/IL-1F2 DuoSet ELISA	R&D Systems	DY201
Human IL-6 DuoSet ELISA	R&D Systems	DY206
Human IL-8/CXCL8 DuoSet ELISA	R&D Systems	DY208
Nigericin	InvivoGen	tlrl-nig
Maxima First Strand cDNA Synthesis Kit	Thermo Scientific	K1642
MTT	Thermo Scientific	M6494
MTT	InvitroGen	M6494
PBS	Sigma	D8537-500ML
Penicillin/Streptomycin	Biochrom/Merck	A 2212
peqGOLD Total RNA Kit	Peqlab/VWR	732-2868
Pierce™ Protein A Columns, 1 mL	Thermo Scientific	20356
PMA	InvivoGen	tlrl-pma
Poly I:C	InvivoGen	tlrl-pic
Protein G Sepharose® 4 Fast Flow	GE Healthcare	17-0618-01
recombinant human IL-1α	Abcam	ab119165
recombinant human IL-1β	Abcam	ab9617
RNAlater™ Stabilization Solution	Thermo Scientific	AM7021
RPMI	Merck	F61215
Trypsin/EDTA-Lösung (0,05 %/0,02 %)	Biochrome/ Merck	L2143
Z-VAD-FMK	InvivoGen	tlrl-vad

2.2 Methods

2.2.1 RNA isolation

To evaluate the expression of proteins RNA was isolated from cell monolayers for real-time PCRs. This was done using the peqGOLD Total RNA Kit. Cells were lysed in the dish with 400 µl RNA Lysis Buffer T in case of 24- or 12-well plates or with 800 µl RNA Lysis Buffer T whenever lager dishes were used. The lysate was loaded onto DNA Removing Columns and centrifuged at 12000 x g for 1 minute. The flow-through was mixed thoroughly 1:1 with 70% ethanol. Next the suspension was loaded onto RNA binding columns and centrifuged at 10000 x g for 1 minute. The flow-through was discarded and the column was washed once with 500 µl wash buffer I and once with 600 µl wash buffer II. In-between buffers were removed by centrifuging the column at 10000 x g for 15 seconds. After the second washing step the columns were dried by centrifuging at 10000 x g for 2 minutes. The RNA was eluted with 50 µl RNase-free dH_2O through centrifugation at 5000 xg for 1 minute.
To calculate RNA concentration the absorption was measured via NanoDrop 1000 at 260 nm. The concentration was calculated as following:

$$RNA\ concentration\ \left(\frac{\mu g}{ml}\right) = absorption_{260} * 40$$

2.2.2 cDNA transcription

Before performing real-time PCR, it is necessary to transcribe RNA in cDNA. This was done using reverse transcriptase, an RNA-dependent DNA-polymerase. During this process RNA is spliced which means introns are cut out. Therefore, in contrast to genomic DNA cDNA only consists of exons.
For cDNA synthesis Maxima™ First Strand cDNA Synthesis Kit (ThermoFisher) was used. A typical set up would be the following:

5x reaction mix	4 µl
Maxima enzyme mix	2 µl
RNA	1 µg
DNAse-free H_2O	ad 20 µl

The reaction mix was incubated for 10 minutes at 25 °C and then for 30 minutes at 50° C. Afterwards the enzyme was inactivated at 85° C for 5 minutes. CDNA was stored at -20° C.

2.2.3 Real-time PCR

During real-time PCR a target section of the cDNA is amplified with a heat-stable DNA-polymerase similar to a classical polymerase chain reaction (PCR). In real-time PCR during the annealing stage a probe specific to the target section binds to the DNA. When the polymerase reaches the probe during amplification the polymerase separates a reporter dye on the probe from a quencher which leads to emission of fluorescence. The fluorescence is measured during the PCR and correlates directly to the amount of synthesized cDNA. Therefore, it is possible to draw conclusions regarding the inserted amount of target cDNA at the beginning of the reaction. Since target cDNA originates from isolated mRNA of the cell this correlates with the expression of the target protein.
Real-time PCR was performed using TaqMan assays from TIB MolBiol. A typical set-up consists of

0,5 µl	Primer fw
0,5 µl	Primer rev
0,5 µl	Probe
5 µl	Probemaster
2,5 µl	DNAse-free dH_2O
1 µl	cDNA

The PCR was performed for 50 cycles. As housekeeping gene β-actin was employed (referred as “control” below). For every sample triplicates were made. Values with a standard deviation higher than 0.5 were excluded. The data was analyzed using the comparative C_t method:

$$R = 2^{-[(\Delta Ct_{treated} - \Delta Ct_{control}) - (\Delta Ct_{untreated} - Ct_{control})]}$$

2.2.4 ELISA

ELISA stands for “Enzyme-linked immunosorbent assay”. In this assay the amount of a specific protein in a solution can be quantified through binding to antibodies. In the utilized sandwich ELISA plates are first coated with antibodies specific to the target protein. The protein solution of unknown concentration is then applied simultaneously with a standard of known concentration. After allowing some time for binding, protein solution and standard solution are withdrawn, and residuals are washed away. Bound protein is detected with a secondary antibody which is biotinylated to amplify the detection signal. In a next step streptavidin conjugated to horseradish peroxidase (HRP) is applied. The HRP catalyzes an enzymatic

reaction that leads to a blue colorization of the solution. By adding H_2SO_4 the reaction is stopped, marked by a color change to yellow. The absorbance of the samples can be measured in a photometer and correlate directly to the amount of antibody-bound protein.
For this thesis ELISA-Kits from R&D systems were used. The ELISA was executed according to the manufacturer´s protocol.

2.2.5 Isolation of primary keratinocytes

Primary keratinocytes were isolated from healthy, human foreskin. The procedure was approved by the medical ethical committee of the Eberhard Karls University Tübingen (ethical approval: 547/2011BO2). In a first step fat tissue and remaining blood vessels were removed. Then the skin tissue was cut in pieces 3-5 mm and incubated overnight at 4°C in CnT-07 medium containing 10 U/ml Dispase II to separate epidermis from dermis. The next day the epidermis was peeled from the skin tissue and transferred to pre-warmed Trypsin-EDTA. In the solution the tissue was cut in even smaller parts and afterwards incubated at 37°C for 10 – 15 minutes to dissolve the cell complex and free single cells. Subsequently, trypsinization was blocked by the addition of DMEM containing 10% FCS. The cell suspension was pipetted 20 times with a 10 ml-rod pipette to separate cells. Then the solution was filtered through a cell strainer (100 µm) and centrifuged at 1500 rpm for 5 min. The pellet was suspended in CnT-07 medium containing gentamycin and amphotericin and seeded in T75-flasks.
Cells were cultured at 37°C with 5% CO_2. Upon confluency freshly isolated cells were passaged once prior to being used for experiments or frozen at -80°C for storage.

2.2.6 Cell culture

All cells were cultured at 37° C with 5 % CO_2.
Primary keratinocytes were cultured in low-Ca^{2+} CnT-07 medium up until confluency was reached. For differentiation medium was replaced by high- Ca^{2+} medium for 24h.
HaCaT cells were generally cultured in DMEM with 10 % FCS. Before treatment, cells were exposed to low-calcium conditions to achieve an expression pattern of innate cytokines similar to primary keratinocytes. Therefore, cells were seeded and cultured in low-Ca^{2+} medium for at least 72 h. Upon confluency, cells were switched to high-Ca^{2+} medium for 24 h.

High-Ca^{2+} medium:

CnT-07 medium

5 % Penicillin/Streptomycin

1.8 mM Calcium

Low-Ca^{2+} medium:

CnT-07 medium

5 % Penicillin/Streptomycin

DMEM-medium:

DMEM

10 % FCS

5 % Penicillin/Streptomycin

2.2.7 Irradiation of cultured cells

For irradiation cells were cultured in 12-well or 6-well plates. Immediately before the UV-treatment medium was withdrawn and stored in reaction tubes. Irradiation was done via an UVA-lamp (medisun® Psori-Kamm, Schulze & Böhm, Germany) that was secured in 3 cm distance to the cell monolayer. The irradiation time that resulted in the best cytokine response while not affecting cell viability was titrated. As a result, cells were irradiated for 2:55 min resulting in an UVA-dose of 5 J/cm^2. In case of skin explants, specimens were irradiated for 19:00 min (15 J/cm^2). Afterwards medium was given back and cells were incubated for 4h.

2.2.8 Treatment of cells with activators and inhibitors

ZVad-FMK is a cell-permeable pan-caspase inhibitor that binds irreversibly to the catalytic site of caspase proteases[175]. It can inhibit apoptosis and is a potent inhibitor of caspase-1 activation. ZVad-FMK was added one hour prior any additional treatment to the cells in a concentration of 25 µg/ml. Similar to zVad-FMK, Ac-YVAD-cmk is also a cell-permeable caspase-inhibitor with a selective specificity to cysteine protease caspase-1 and some activity against caspase-4 and caspase-5 [176]. It was also applied one hour before any other treatment in a concentration of 25 µg/ml.

Phorbol-12-myristat-13-acetat (PMA) is a Protein kinase C (PKC) activator[177]. The best concentration and time point of stimulation for an optimal cytokine response was titrated. As a result, it was applied in a concentration of 5 µg/ml for 16 hours.

Polyinosinic:polycytidylic acid (Poly I:C) is a TLR3 activator that mimics viral infections[178]. It was used in a concentration of 10 µg/ml for 16 hours.

Recombinant human IL-1α and IL-1β were added in a concentration of 500 pg/ml. Afterwards cells were incubated for 4 hours.

Nigericin is a toxin produced by Streptomyces hygroscopicus bacteria. It induces the formation of the inflammasome and subsequently activates caspase-1 that can cleave IL-1β into its active form [179].

Nigericin was applied in a concentration of 2.5 mM for 40 or 15 minutes. Since Nigericin is dissolved in DMSO the negative control was treated with the corresponding volume of DMSO without Nigericin.

2.2.9 Isolation of PBMCs from whole blood

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from fresh blood diluted 1:1 with PBS. Diluted blood was carefully layered over 15 ml ficoll and centrifuged at 400 xg for 30 minutes without breaks. Afterwards, the buffy coat, a layer containing leucocytes and platelets, was gathered in a second reaction tube and washed with PBS. Next an erythrocyte lysis was performed by resuspending the cell pellet in 2 ml ACK lysis buffer and incubating for 2 minutes. Afterwards the reaction was stopped by adding 40 ml PBS and cells were pelletized by centrifugation. After one additional washing step with PBS cells were seeded in well plates with a density of $1*10^6$ cells per milliliter in FCS-free RPMI medium and incubated at 37° C with 5% CO_2. After one hour of incubation FCS-free medium was exchanged for RPMI containing 10% FCS.

2.2.10 Treatment of keratinocytes with PBMC supernatant

Freshly isolated PBMCs were incubated overnight before being used for experiments. Next day cells were treated with 100 ng/ml LPS for 2 hours to stimulate IL-1 expression. To cause a release of IL-1 into the supernatant cells were additionally stimulated with 5mM ATP for 30 minutes. Then supernatant was withdrawn and stored at -80° C. Cytokine levels in the supernatant were analyzed via ELISA. Afterwards supernatant was added on differentiated HaCaTs instead of DMEM medium. At the same time anti-Dsg3 antibody AK23 was added in a concentration of 20 µg/ml to the supernatant. After 4 hours incubation supernatant was stored and once again analyzed for cytokines by ELISA. Cell-cell adhesion strength of HaCaTs was determined by Dispase-based dissociation assay.

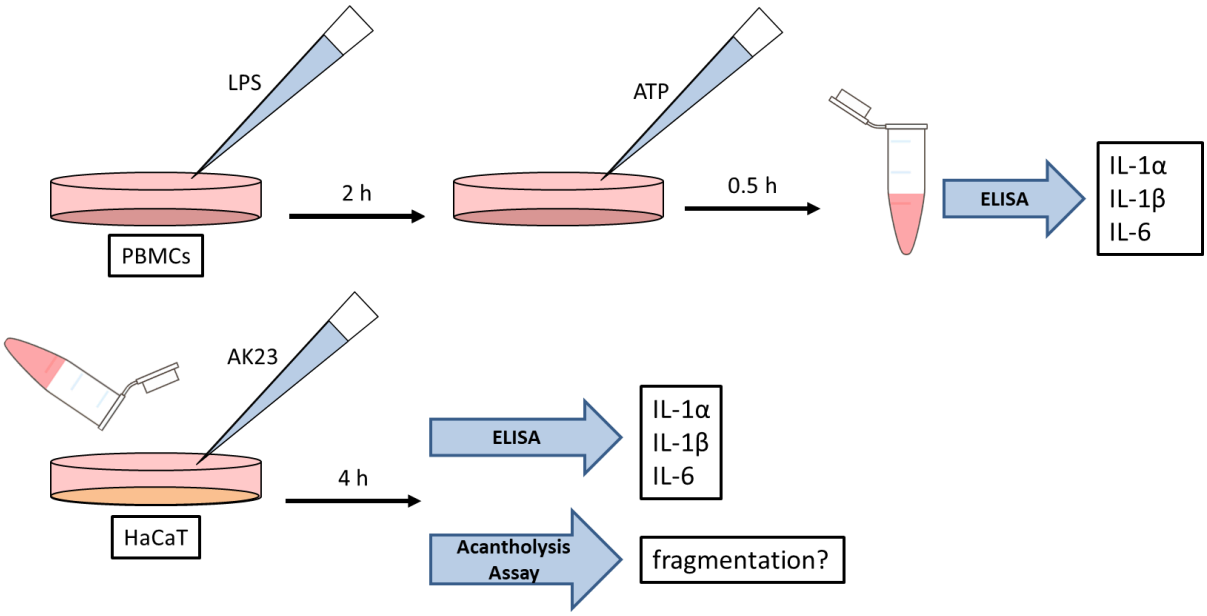

Figure 6: Experimental set-up of the treatment of HaCaT cells with PBMC supernatant.

2.2.11 Dispase-based dissociation assay

Cells were cultured till 100% confluency and then differentiated with high-Ca^{2+} medium for 24 hours. Afterwards IgGs purified from pemphigus patients were added in a concentration of 342 µg/ml and if required a cofactor was added for four to 24 hours, depending on the scientific question. After incubation supernatant was withdrawn and cells were washed twice with 0.5 ml HBSS. Then 0.5 ml Dispase II dissolved in HBSS with a total concentration of 2.5 U/ml was added to the cells to detach the monolayer from the plastic dish. Depending on the type of cells detaching took from 20 min for primary keratinocytes to 40 min for HaCaT-cells. Next Dispase-solution was carefully withdrawn and replaced by 0.5 ml fresh HBSS. To achieve a better visibility of the monolayer and to verify whether cells are still alive despite treatment with PV-IgG and cofactors, the monolayer was stained with MTT. MTT reagent was dissolved in HBSS to a final concentration of 200 µg/ml. Cells were then incubated 15 minutes to assess if they turn blue. Then shear stress was administered by pipetting the monolayer up and down with an electrical pipet. To make results comparable initially a positive control consisting of cells only treated with PV-IgG was shed for as many times as it took to just obtain fragmentation. Afterwards a negative control consisting of untreated cells was shed for the same number of repetitions. If in this case the monolayer stayed whole all other samples were pipetted for as many repetitions. Directly after shedding, 0.5 ml of an 8% formaldehyde-solution were added to the fragments to fix cells permanently. Then cell fragments in every well were counted and photographed.

2.2.12 Antibody purification from sera

IgG from Pemphigus patients were purified with Protein A columns using gravity flow according to the protocol provided by the manufacturer: Storing solution of the columns was discarded. Afterwards columns were washed with 5 ml PBS for equilibration. Serum samples were diluted 1:1 with PBS and centrifuged for 20 min at 10000 xg to lose any lipoprotein precipitation. The supernatant was then applied onto the column. Afterwards the column was washed with 15 ml PBS. The flow-through was saved for later analysis. Then bound protein was eluted from the column with a low pH elution buffer. This time flow-through was collected in 0.5 ml fractions and pH was adjusted to physiological conditions with 50 µl Neutralization buffer. To determine IgG-concentration in the fractions, absorbance was measured at 280 nm via NanoDrop. Fractions with measurable IgG-concentrations were stored while other fractions were discarded. The column was regenerated with 12 ml Elution buffer. For storage dH_2O containing 0.2% sodium azide was applied onto the column and let sink in.

Elution buffer:

dH_20

0.1 M glycine

pH adjusted to 2 - 3

Neutralization buffer:

dH_20

1M Tris

pH adjusted to 8.5 - 9

2.2.13 Hybridoma-derived antibody purification from cell supernatant

AK23 derived from Hybridomas was purified using protein G sepharose. Sepharose (1ml for ≈ 20 ml supernatant) was prepared in a column by washing with 20 ml PBS. Hybridoma supernatant was divided onto 50 ml tubes. Afterwards the column was closed with a bottom cap and the sepharose was resuspended with supernatant and transferred to a 50 ml tube. The mixture was then incubated over night at 4°C in a rotator.

The next day the sepharose-supernatant-mix was applied onto the column. The bottom cap was removed to allow supernatant to flow through. The sepharose was washed with 30 ml PBS. For elution 0.06 ml Neutralizing buffer was aliquoted in 1.5 ml reaction tubes. The antibody was then eluted with 6 ml elution buffer by letting 0.5 ml drop into each reaction tube.

Afterwards sepharose was restored by washing with 20 ml elution buffer and 20 ml PBS. For storage sepharose was kept in 0.02% sodium azide in H_20.
The antibody concentration was measured at 280 nm via NanoDrop.

Elution buffer:

dH_20

0.2 M Trisodium citrate

pH adjusted to 2.4

Neutralization buffer:

dH_20

2M sodium carbonate

pH adjusted to 9

2.2.14 Cell stretching

To simulate mechanical stress cell monolayers were cyclic stretched and relaxed over a set period of time. This was done in cooperation with the group of Prof. Dr. Kemkemer, Department of Applied Chemistry, Hochschule Reutlingen. For the experiment, cells were cultured on the PMS-membranes in until 100% confluency and then differentiated with high-Ca^{2+} medium for 24 hours. Depending on the scientific question other cofactors, PV-IgG or inhibitors were added in concentration and timely manners as previously tested. Then membranes were clamped in a cyclic stretching machine and stretched at 1 Hz with 7% expansion. Since the stretching device is portable and could therefore be set up in an incubator, normal cultivating conditions of keratinocytes such as 37° C, 5% CO_2 and high humidity could be sustained throughout the procedure. After stretching supernatant was withdrawn and stored for ELISA testing. The cells were scraped in 0.5 ml PBS and the suspension was mixed with 0.8 ml RNAlater.

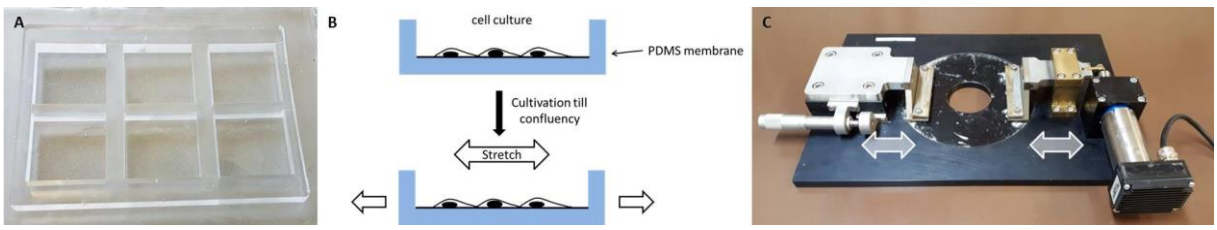

Figure 7: Set-up of cell-stretching. Keratinocytes were seeded into a flexible PDMS membrane (A) and cultivated till confluency. Then the PDMS membrane was clamped into a cyclic stretching machine (C) and stretched two-ways in a cyclic manner (B).

2.2.15 Human skin explants *(ex-vivo*-model)

For the skin explant model left-over skin tissue from the safety-margins of skin surgeries, so called “Dog ears” are utilized. The procedure was approved by the medical ethical committee of the Eberhard Karls University Tübingen (ethical approval: 662/2019BO2). The tissue is washed twice in PBS and then cut into small pieces of about 0.3 - 0.4 mm in diameter. Then tissue pieces are put on hanging cell culture inserts with a pore size of 5.0 µm and cultured overnight with 500 µl medium in 24-well cell culture dishes. Cell culture medium was kindly provided by Prof. Dr. Jens Baron, Department of Dermatology, RWTH Aachen University [180]. Overnight skin explants were cultured in medium containing amphotericin and gentamycin to clear existing colonization with bacteria or fungi. Next day skin explants were transferred to antibiotic-free medium and treated with the monoclonal anti-Dsg3 antibody AK23 and cofactors. After 48 h of incubation the tissue is prepared for histological staining and kept overnight in 4.5% formaldehyde. The histological staining was performed by the department for dermatology, histology units of the universities in Tübingen and Aachen.

3. Results

3.1 IL-1 inducing cofactors

Primary aim was to find possible cofactors that might play a role in pemphigus and are able to activate the innate immune system. As a surrogate parameter for innate immune activation the expression and secretion of proinflammatory cytokines was measured. Especially IL-1 which is secreted as a result of inflammasome activation played a crucial role.

3.1.1 UVA

The involvement of irradiation of the skin with UVA is discussed in many skin disorders because of its mutagen and non-specific damaging properties [181]. Furthermore, due to its environmental abundance nearly every human is subjected to UVA. Therefore, UVA might serve as a possible cofactor in the clinical manifestation of pemphigus.
HaCaT cells and primary human keratinocytes were irradiated with 5 J/cm². The incubation time for optimal induction of innate cytokine expression and secretion was adapted from Stefanie Reinknecht's thesis "The ATF3/IL-6-axis as determinator between innate and adaptive immunity". After 4 hours, cells were lysed and subjected to Real-time PCR to determine cytokine expression and cell supernatant was analyzed via ELISA for cytokine secretion.

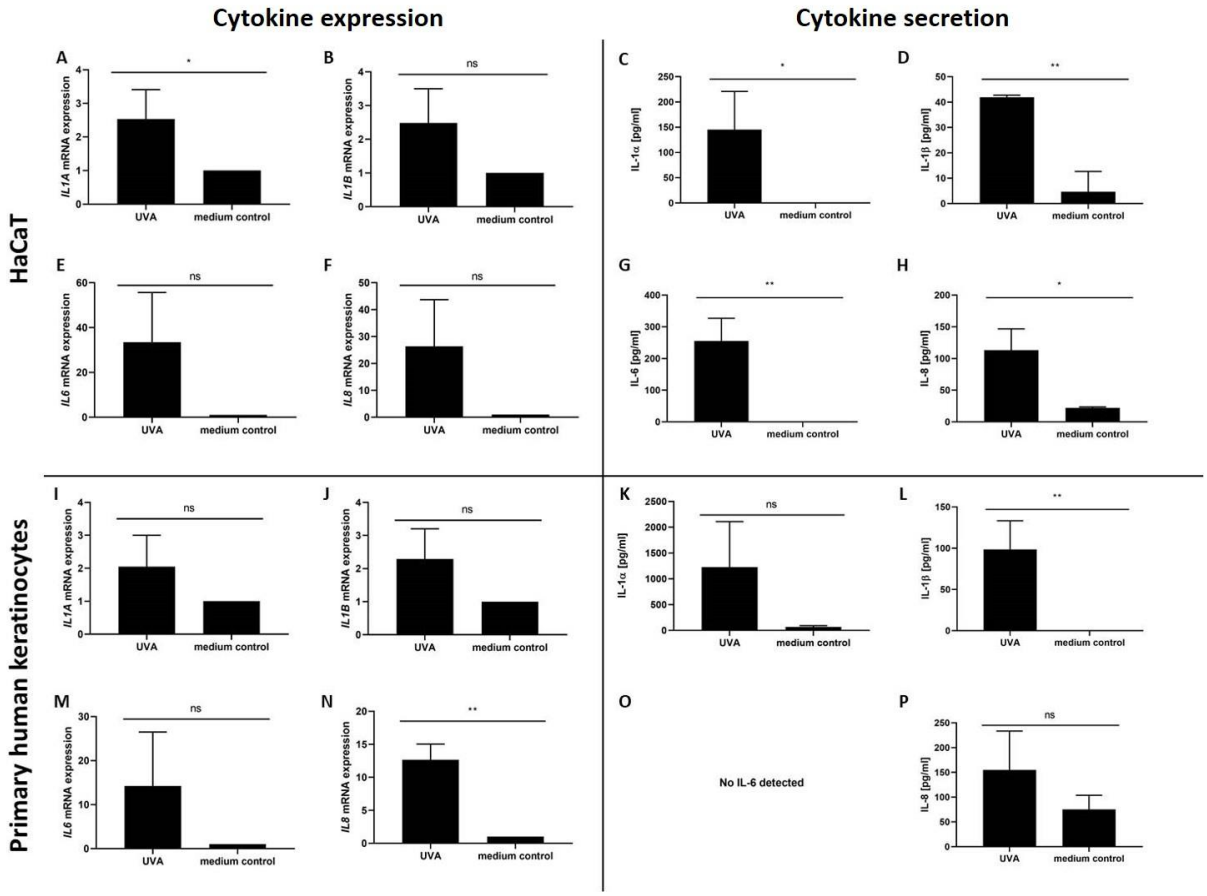

Figure 8: Activation of cytokine response through UVA: HaCaT cells (A – H) and primary human keratinocytes (I – P) were irradiated with UVA and expression of *IL1A*, *IL1B*, *IL6* and *CXCL8* were determined via real-time PCR (A, B, E, F, I, J, M, N). Protein secretion in the supernatant was measured by ELISA (C, D, G, H, K, L, O, P).

In HaCaT cells the expression of *IL1A, IL1B, IL6* and *CXCL8* mRNA was increased after irradiation with UVA. For *IL1A* the increase of 2.5-fold was significant. *IL1B* was increased slightly, while expression of *IL6* and *CXCL8* was increased multiple times. The secretion of those cytokines was significantly elevated after UVA irradiation. IL-1α secretion outnumbered the secretion of IL-1β after UVA. As non-IL-1 cytokines, which are conventionally secreted, IL-6 and IL-8 were expelled after UVA irradiation.

Similarly, upon irradiation with UVA cytokine expression was increased in primary human keratinocytes. Expression of *IL1A* and *IL1B* was double the amount compared to medium control. The mRNA expression of *IL6* was increased 14-fold and the expression of *CXCL8* 13 fold. Upon irradiation large amounts of IL-1α and less IL-1β were detected in the medium. In opposition to the cell lines, human primary keratinocytes did not secrete IL-6.

3.1.2 Mechanical stress

Mechanical stress plays an important role in blister formation of pemphigus. The Nikolsky's sign describes a phenomenon where a blister can be induced or moved by pushing and squeezing of the skin [182]. To evaluate whether innate immunity is involved in this process, the

expression and secretion of the innate cytokines IL-1 and IL-6 was measured as a response to mechanical stress. Differentiated primary human keratinocytes were seeded on a flexible membrane that could be stretched two-way in a cyclic manner imitating continuous mechanical stress exerted on the stratum corneum.

To set-up optimal conditions for innate immune activation cytokine expression and secretion was determined after three and six hours of stretching. Additionally, cells stretched for six hours were incubated for another 18 hours to assess whether an activated state of the immune system is kept up. All stretched samples were compared to a sample containing cells that were grown on a similar, flexible membrane and stored during the stretch in the same incubator.

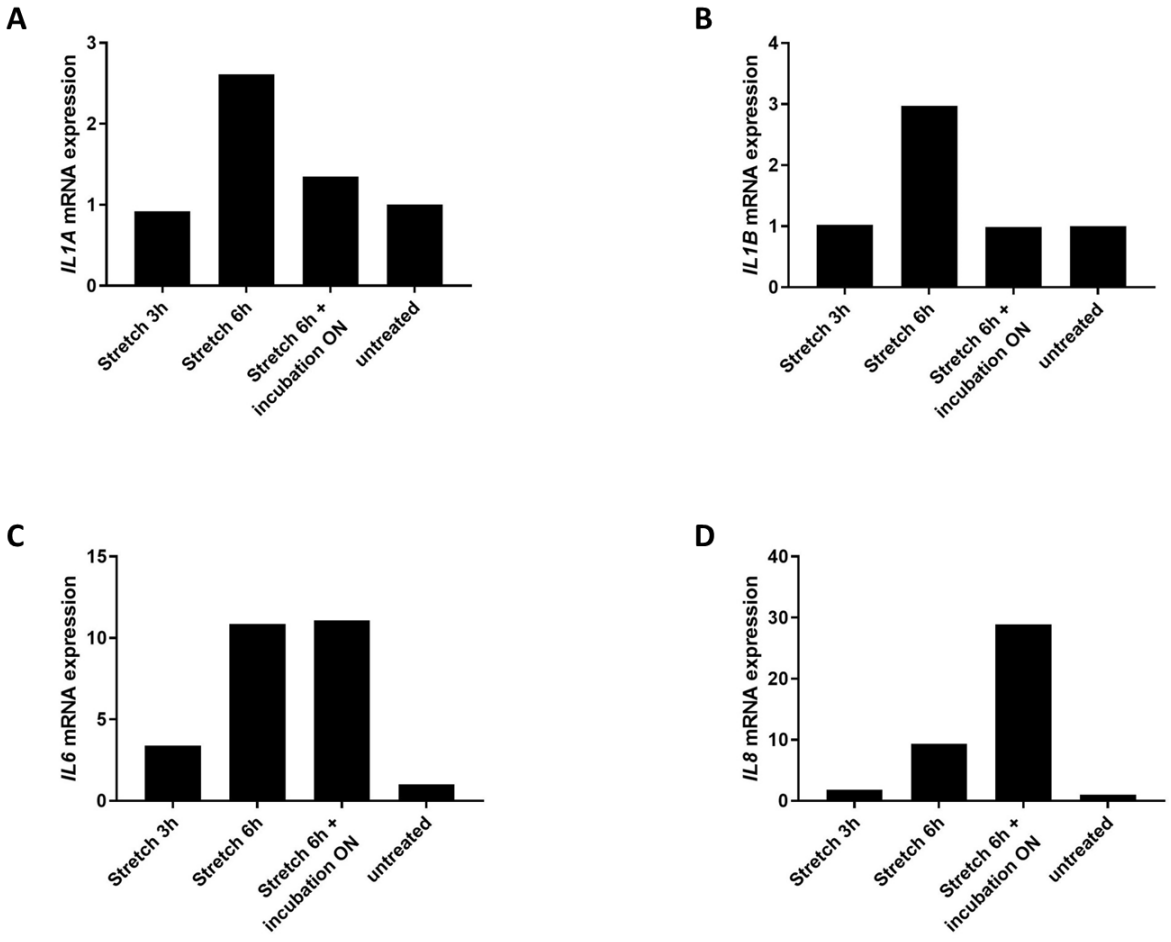

Figure 9: Cytokine expression through mechanical stress: Primary human keratinocytes were exposed to mechanical stress for 3 h or 6 h. Directly afterwards mRNA levels of *IL1A* (A), *IL1B* (B), *IL6* (C) and *CXCL8* (D) were determined by Real-time PCR. Additionally, samples exposed for 6 h were incubated further overnight without mechanical stress before cytokine expression was assessed**.**

After three hours of stretching the expression of *IL1A* and *IL1B* was not elevated compared to the control. MRNA expression of both *IL6* and *CXCL8* was slightly increased. After six hours of stretch a slight induction of mRNA expression of *IL1A* and *IL1B* compared to untreated cells could be observed. Both the expression of *IL6* and *CXCL8* were increased notably. After an

additional 18 hours of incubation without stretch, the mRNA expression of *IL1A* and *IL1B* decreased to a level similar to the negative control. The expression of *IL6* stayed at a similar level as observed immediately after 6 hours of stretch. MRNA expression of *CXCL8* increased about three times more than directly after termination of the cyclic stretch.

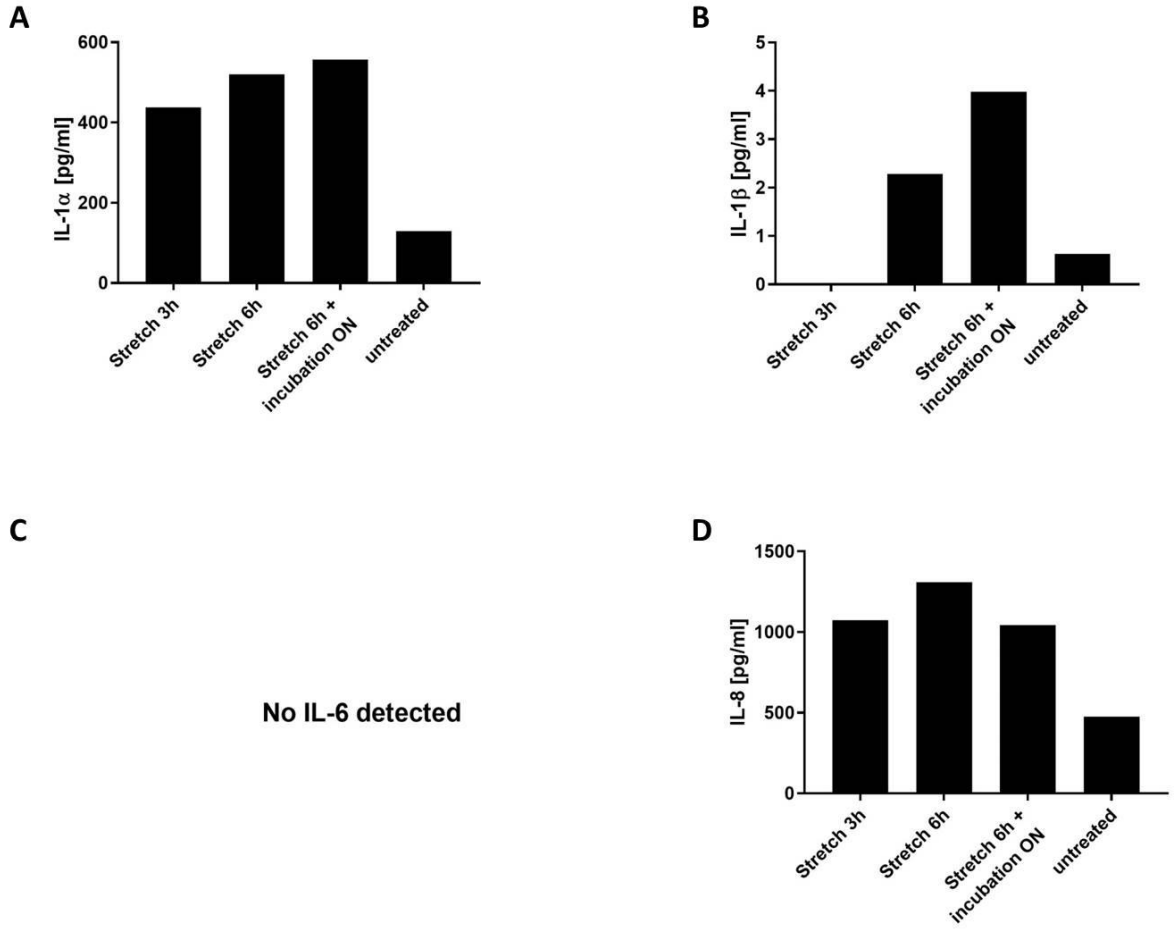

Figure 10: Activation of cytokine secretion through mechanical stress: Primary human keratinocytes were exposed to mechanical stress for 3 h or 6 h. Directly afterwards the amount of IL-1α (A), IL-1β (B), IL-6 (C) and IL-8 (D) secreted into the supernatant was determined via ELISA. Additionally, samples exposed for 6 h were incubated further overnight without mechanical stress before cytokine levels were tested**.**

Interestingly, a major amount of IL-1α was secreted into the supernatant after 3 hours of stretch compared to the negative control, hinting towards an impact of inflammasome activation. At the same time point no IL-1β was detectable. The secretion of IL-8 into the supernatant after 3 hours of stretch was double the amount of the negative control. Nevertheless IL-8 was found in a considerable amount in the control, pointing to a strong baseline induction of IL-8. IL-6 secretion was not detected in any condition.

After 6 hours of stretching the secretion of IL-1α had further increased by around 100 pg/ml. Secretion rose even further after incubation overnight without stretch. For IL-1β no protein could be detected in the supernatant after 6 hours of stretching. After additional incubation overnight no IL-1β was measured as well. Looking at IL-8, an already major amount of cytokine

could be found in the supernatant after 6 hours stretched. Here the concentration decreased after the additional incubation time to a similar cytokine level that was detected after 3 hours of stretch.

In a next step 100% confluent cells were pretreated with PV-IgG prior to mechanical stress. Since 6 hours of 7% stretch at 1 Hz led to sufficient immune activation and to elevated levels of IL-1 expression and IL-1α secretion, these settings were applied for all further stretch experiments. Additionally, the pan-caspase-inhibitor zVad was applied 1 hour prior to stretch.

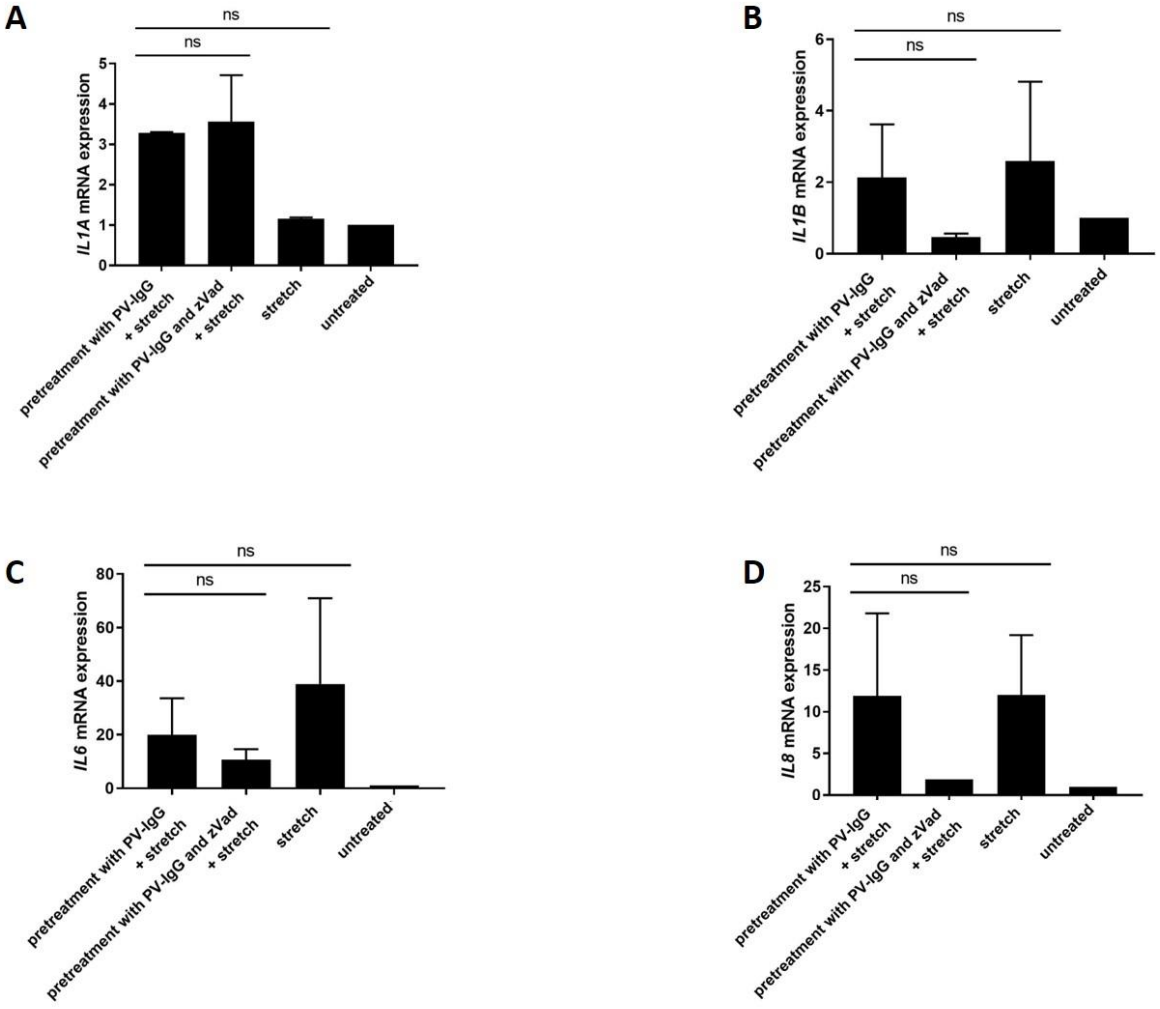

Figure 11: Effect of PV-IgG and mechanical stress on cytokine expression of keratinocytes: Right before being exposed to 6 h of mechanical stress human primary keratinocytes were treated with IgG isolated from patient serum (PV-IgG) for 24 h. Afterwards cells were stretched for 6h before mRNA levels of *IL1A* (A), *IL1B* (B), *IL6* (C) and *CXCL8* (D) were analyzed. To block caspase activity the pan-caspase inhibitor zVad was applied 1 h prior stretching.

Regarding expression levels, *IL1A* was slightly raised after 6 hours of stretch compared to the untreated control. With pretreatment with IgG the expression increased about 3-fold. Expression levels were similar with an additional pretreatment with zVad. Looking at *IL1B* the expression was increased 2-fold after stretch regardless of pretreatment with PV IgG.

Pretreatment with zVad was able to decrease expression of *IL1B* even below control levels. Expression of *IL6* was already increased multiple times with stretch alone. Additional pretreatment with PV-IgG reduced expression compared to stretch only but still yielded an increase compared to the control. Nevertheless, standard deviations are high, making it difficult to make any valid statement. After pretreatment with zVad *IL6* mRNA expression was reduced by half. The expression levels of *CXCL8* after stretch and stretch plus pretreatment with PV-IgG were both increased in a similar amount. With the addition of zVad expression levels decreased notably to control levels.

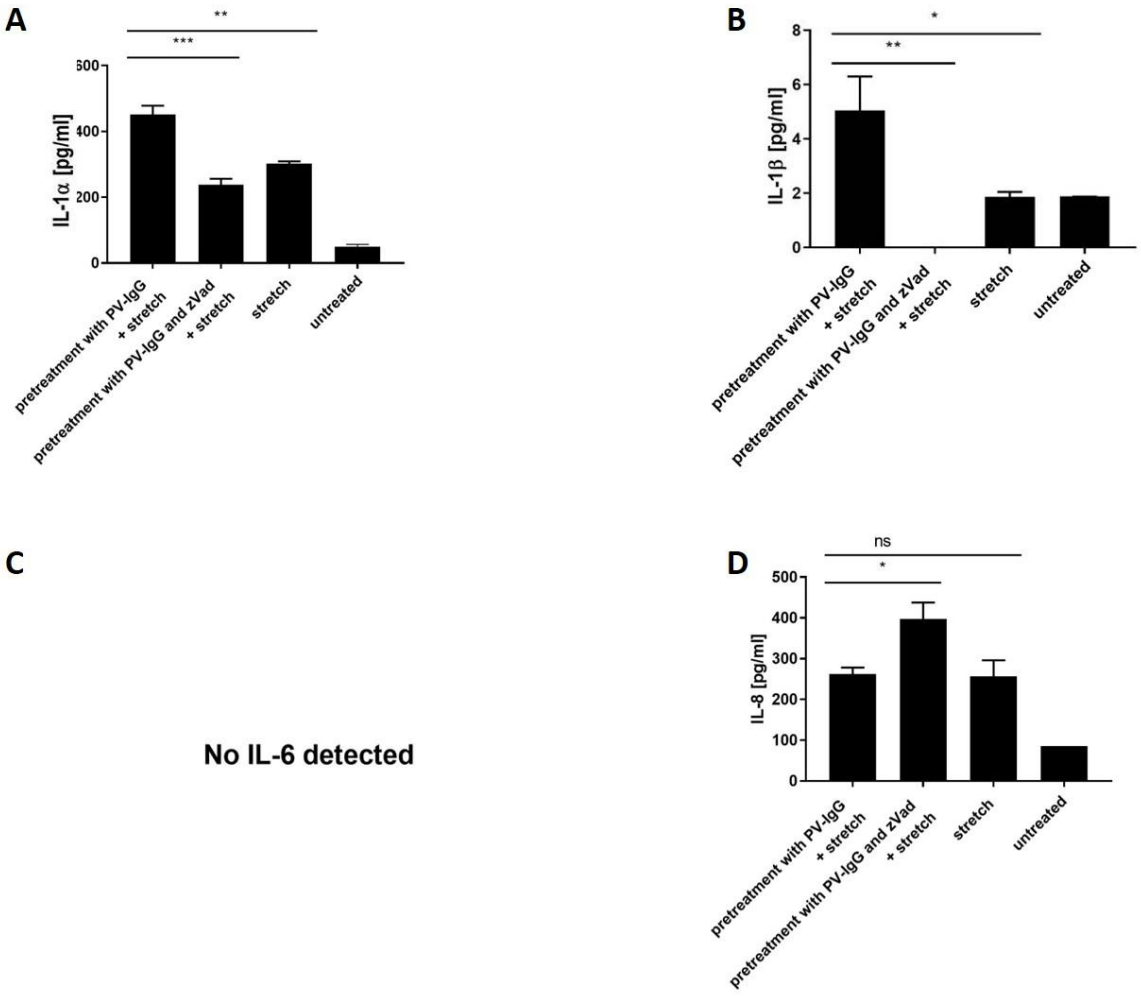

Figure 12: Effect of PV-IgG and mechanical stress on cytokine secretion of keratinocytes: Right before being exposed to mechanical stress human primary keratinocytes were treated with PV-IgG for 24 h. Afterwards cells were stretched for 6 h before levels of IL-1α (A), IL-1β (B), IL-6 (C) and IL-8 (D) protein secreted into the supernatant were analyzed. zVad was applied 1 h prior stretching.

Stretch alone induced in this experiment a strong secretion of IL-1α. Pretreatment with PV-IgG further increased this secretion, while additional pretreatment with zVad reversed the effect and resulted in a cytokine concentration similar to stretch only. Both findings were significant. Looking at IL-1β stretch alone evoked no considerable secretion of IL-1β in any condition. Similar to previous findings IL-6 secretion was never observed. For IL-8 a notable background

was detected in the control. Nevertheless, after stretch an apparent increase in IL-8 secretion could be detected. Pretreatment with PV-IgG led to no significant increase of secretion and additional pretreatment with zVad even resulted in a further enhanced cytokine secretion.

3.1.1 Phorbol-12-myristat-13-acetat

Phorbol-12-myristat-13-acetat (PMA) is small molecule drug that activates protein kinase C (PKC) and subsequently the MAPK/ERK pathway [177]. HaCaTs were treated with PMA with and without PV-IgG for 16 hours. Afterwards the expression and secretion of IL-1, IL-6 and IL-8 were determined.

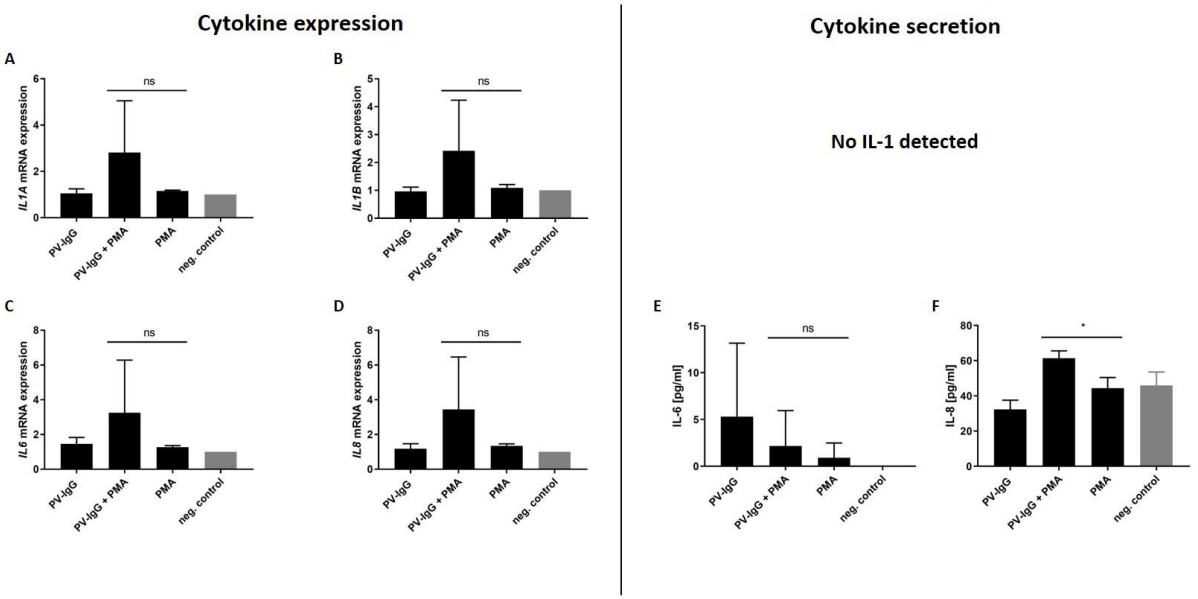

Figure 13: Effect of PMA on cytokine induction in HaCaTs: PMA was applied together with PV-IgG for 16 hours. The expression of *IL1A* (A), *IL1B* (B), *IL6* (C) and *CXCL8* (D) was determined as well as the secretion of IL-6 (E) and IL-8 (F).

Treatment with PMA alone did not change the expression of these cytokines. Addition of PV-IgG resulted in a 3-fold increase of *IL1A*, *IL6* and *CXCL8* expression. *IL1B* expression was increased by 2-fold. Nevertheless, standard deviations were high and none of these effects were significant. Neither IL-1 nor IL-6 were released into the supernatant. The secretion of IL-8 after treatment with PMA was not augmented either. Addition of PV-IgG led to a significant increase in IL-8 secretion (60 pg/ml).

3.1.2 Polyinosinic:polycytidylic acid (Poly I:C)

Polyinosinic:polycytidylic acid (Poly I:C) is an analog to double-stranded RNA and binds to the endosomal TLR3 [178]. Therefore, it can be used to simulate the effect of a viral infection. To cover viral infections as a possible cofactor HaCaT cells and human primary keratinocytes were treated with Poly I:C alone and in combination with the anti-Dsg3 antibody AK23. To

determine whether any occurring effects are caspase-dependent, samples were incubated with zVad before being treated with Poly I:C and anti-Dsg3.

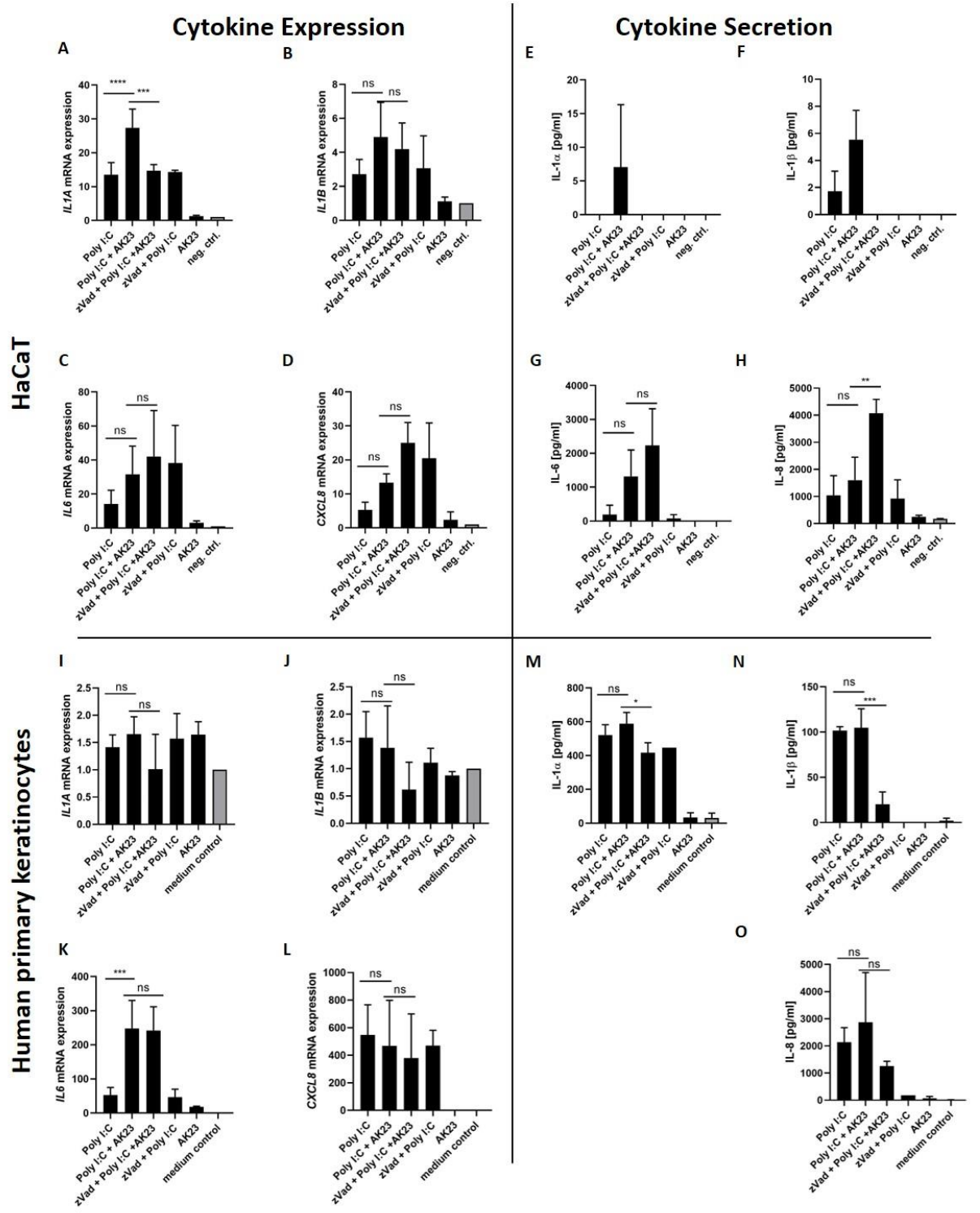

Figure 14: Effect of Poly I:C on cytokine induction in HaCaT cells (A – H) and primary human keratinocytes (I – O): Poly I:C was applied together with anti-Dsg3 antibody AK23 for 16 hours. Afterwards mRNA expression (A-D, I - L) and secretion (E – H, M - O) of IL-1α, IL-1β, IL6 and IL-8 was determined.

Compared to an untreated medium control the expression of *IL1A* in HaCaT cells was increased after treatment with Poly I:C and addition of AK23 led to an even stronger increase.

This was however not significant. Inhibition of caspases with zVad or specifically caspase-1 with yVad significantly reduced the increase in expression. For *IL1B* the expression was double the amount of the control after treatment with Poly I:C. Anti-Dsg3 increased mRNA expression further. Here treatment with zVad could only slightly decrease expression. Treatment with Poly I:C led to a notable increase in *IL6* expression, which was doubled by addition of anti-Dsg3 antibody. This could not be reversed by caspase inhibition. A similar picture occurred for *CXCL8*: Treatment with Poly I:C alone led to a slight increase in expression. Addition of anti-Dsg3 increased expression considerably while treatment with zVad and yVad did not impair this effect.

Looking at cytokine secretion the levels measured for IL-1α and IL-1β were below detection limit. IL-6 was secreted after Poly I:C-treatment in a mediocre amount. Nevertheless, secretion levels were strongly elevated after addition of anti-Dsg3 antibody. Treatment with Poly I:C activates interferons. Therefore, IL-6 as an interferon signal is secreted upon activation. Both the expression and the secretion of IL-6 were not affected by zVad or yVad as expected. Treatment with Poly I:C induced robust IL-8 secretion, which was further increased by anti-Dsg3 antibody. Pretreatment with zVad interestingly augmented the secretion of IL-8.

In human primary keratinocytes expression of *IL1A* or *IL1B* was not affected by treatment with Poly I:C. Additional treatment with anti-Dsg3 antibody did also not influence the expression. As expected, expression of *IL6* was massively increased by Poly I:C, which could be further enhanced significantly by anti-Dsg3. *CXCL8* mRNA was increased 5-fold after treatment with Poly I:C. Addition of anti-Dsg3 antibody or pretreatment was not relevant for *CXCL8* mRNA induction.

IL-1α or IL-1b were not secreted after treatment with Poly I:C or Poly I:C and anti-Dsg3 antibody. As seen before in primary human keratinocytes, IL-6 was not secreted. IL-8 was secreted in a robust level for all conditions and therefore not altered by any treatment applied.

3.1.5 Nigericin

Nigericin is a bacterial toxin that besides its antibiotic properties against gram-positive bacteria is known to activate the NLRP3 inflammasome in myeloid cells [179]. Nevertheless, there is strong evidence that in keratinocytes Nigericin does not activate NLRP3 but NLRP1, which is suggested to be the more relevant inflammasome in these cells [183].

Nigericin is a potent inflammasome activator that can induce the transcription and production of IL-1. Hence, HaCaT cells as well as primary human keratinocytes were treated with Nigericin with or without additional anti-Dsg3 antibody AK23. To inhibit caspases zVad or yVad for a specific inhibition of caspase-1 were used. To assess cytokine induction mRNA expression of

IL-1α, IL-1β, IL-6 and IL-8 were detected. Additionally, the expression of inflammasome components *NLRP1* and *NLRP3* was measured.

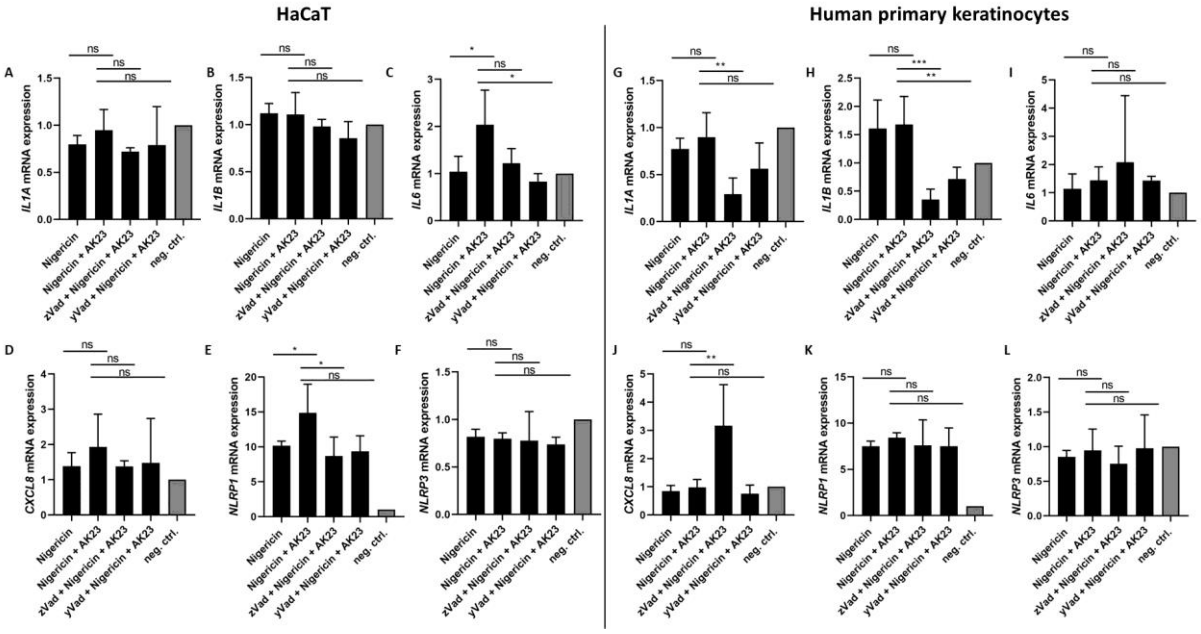

Figure 15: Effect of Nigericin on cytokine induction in HaCaT cells (A – F) and primary human keratinocytes (G – L): Anti-Dsg3 antibody AK23 was applied for 3 hours 45 minutes. Afterwards Nigericin was added and incubated for another 15 minutes. Afterwards mRNA expression of *IL1A* (A, G), *IL1B* (B, H), *IL6* (C, I), *CXCL8* (D, J), *NLRP1* (E, K) and *NLRP3* (F, L) was determined.

In HaCaT cells treatment with Nigericin alone did not increase the expression of *IL1*, *IL6, CXCL8* or *NLRP3*. Nevertheless, the combination of both Nigericin and anti-Dsg3 antibody led to a slight increase in *IL1B, CXCL8* and *NLRP3*. This could be decreased by pretreatment with zVad or yVad. In contrast, the expression of *NLRP1* was elevated noticeably after treatment with Nigericin only which could be further enhanced by addition of anti-Dsg3 antibody. Pretreatment with zVad or yVad here reduced expression to levels similar as observed with Nigericin only.

In human primary keratinocytes neither the analyzed cytokines nor *NLRP3* showed an increased expression after treatment with Nigericin or the combination of Nigericin and anti-Dsg3 antibody. However, expression of *CXCL8* was increased slightly after treatment with zVad in addition to Nigericin and anti-Dsg3 antibody. The mRNA expression of *NLRP1* was increased significantly after treatment with Nigericin only and even more after treatment with Nigericin and anti-Dsg3 antibody. Pretreatment with zVad or yVad reduced the increase observed after addition of antibody to a similar level as observed by treatment with Nigericin only.

3.2 Effect of IL-1 inducing cofactors on acantholysis *in vitro*

After having identified proinflammatory cytokine and especially IL-1 inducing agents the next step was to test their effect on keratinocyte adhesion. In PV cell-cell adhesion is compromised due to antibodies targeting desmoglein. Nevertheless, the sole binding of the antibody is usually not sufficient to induce the loss of adhesion termed acantholysis. Therefore, further signals are necessary to induce blister formation. To validate the hypothesis that the innate immune system serves as a cofactor, agents that were shown to induce innate cytokines were tested in a Dispase-based dissociation assay. In this assay a keratinocyte monolayer is treated with anti-Dsg3 antibodies in combination with possible cofactors. Then mechanical stress is applied to the monolayer to test the adhesion strength of the cells. Untreated keratinocyte monolayers usually only disrupt into few fragments. The number of fragments that are attained by treating cells with anti-Dsg3 antibody act as a positive control and at the same time set a baseline. Number of fragments that occur in other samples are always compared to the positive control and therefore either enhance or reduce acantholysis. So, whenever the addition of a cofactor changes the number of fragments compared to the baseline, one could draw conclusion regarding the cofactor`s potential to increase or inhibit acantholysis. This then can indicate whether a cofactor also might promote disease manifestation in patients.

3.2.1 Effect of UVA on acantholysis

As UVA irradiation induced IL-1 and IL-6 in keratinocytes its effect on acantholysis of keratinocyte monolayers was tested. Therefore, cells were treated with UVA only or in combination with the anti-Dsg3 antibody AK23. To determine whether any effect was caspase-dependent samples were treated with zVad, a pan-caspase inhibitor, and yVad which specifically blocks caspase-1 [184].

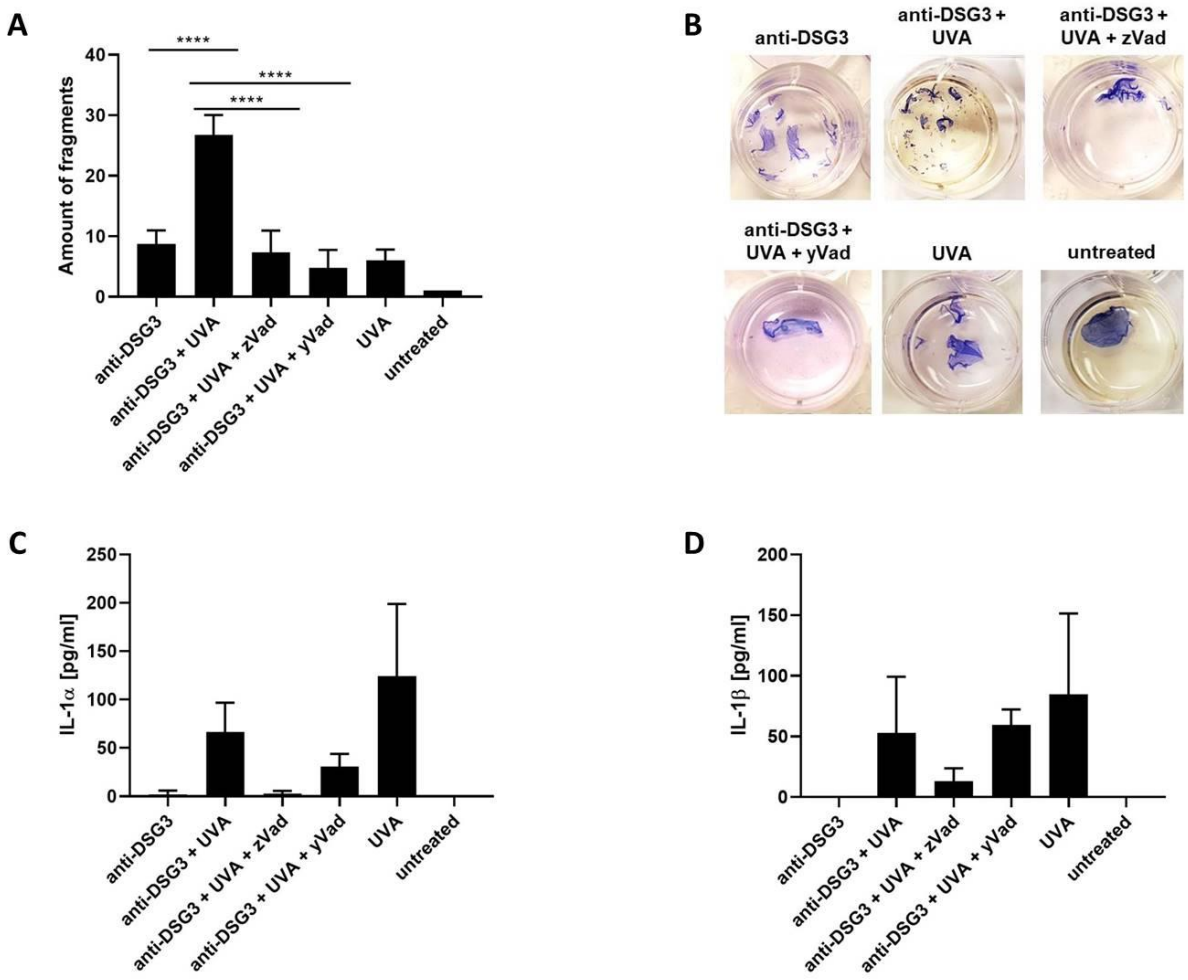

Figure 16: The effect of UVA on acantholysis in HaCaTs: Cells were irradiated with UVA. Directly afterwards anti-Dsg3 antibody was added. Caspase-inhibition was achieved by treating cells with zVad or yVad for 1 hour prior to irradiation. After 4 hours of incubation supernatant was removed and analyzed for IL-1α (C) and IL-1β (D) secretion. Cell-cell adhesion strength within the monolayer was tested by Dispase-based dissociation assay (A, B).

For HaCaT cells treatment with anti-Dsg3 antibody AK23 led to a baseline of 9 fragments. Irradiation with UVA increased the number of fragments significantly. Both pretreatment with zVad and yVad reduced the number of fragments below baseline level. These findings were again significant. UVA irradiation alone did only induce minor fragmentation. To correlate findings to IL-1 production of the cells and therefore a possible activation of the innate immune system secreted IL-1 was measured by ELISA. Here a strong induction of IL-1α and IL-1β secretion could be observed after irradiation with UVA only. Addition of anti-Dsg3 did also induce IL-1 secretion although the amount was less than UVA only. When zVad was added IL-1α secretion could be blocked and IL-1β secretion strongly diminished. YVad diminished IL-1α secretion as well while IL-1β secretion was not affected.

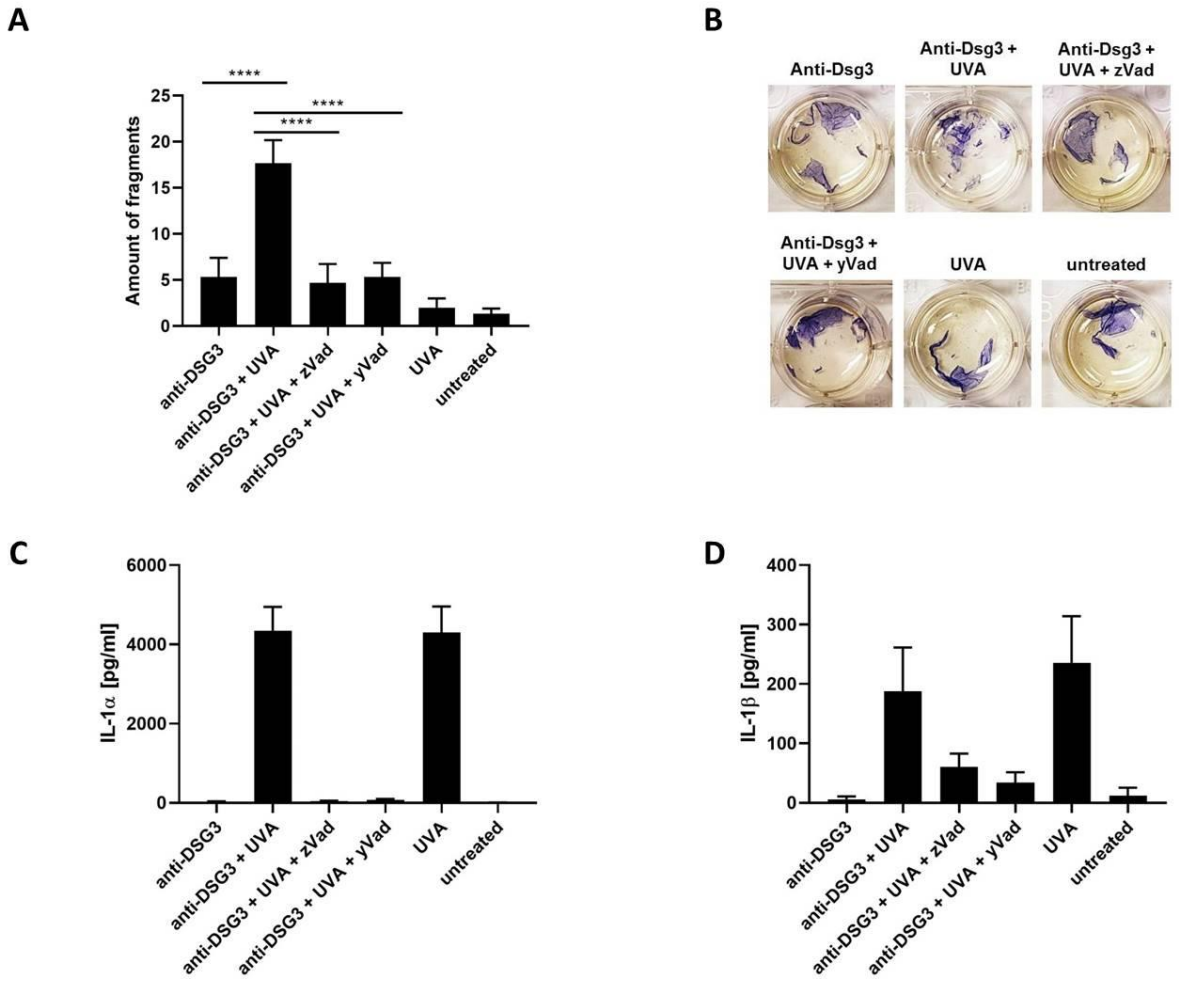

Figure 17: The effect of UVA on acantholysis in primary human keratinocytes: Cells were irradiated with UVA. Directly afterwards anti-Dsg3 antibody was added. Caspase-inhibition was achieved by treating cells with zVad or yVad for 1 hour prior to irradiation. After 4 hours of incubation supernatant was removed and analyzed for IL-1α (C) and IL-1β (D) secretion. Cell-cell adhesion strength within the monolayer was tested by Dispase-based dissociation assay (A, B).

Treatment of primary human keratinocytes with anti-Dsg3 antibody AK23 led to dissociation of the monolayer into three fragments opposing the baseline for this experiment. Additional treatment with UVA light increased the number of fragments significantly. This event could be reversed by pretreatment with pan-caspase inhibitor zVad or caspase-1 inhibitor yVad: Here fragmentation occurred at baseline level. UVA light alone did not induce fragmentation. Treatment with UVA led to a heavy secretion of IL-1α and IL-1β. Addition of anti-Dsg3 induced similar IL-1 secretion levels as UVA only. The secretion of IL-1α could be blocked by pretreatment with zVad or yVad. In case of IL-1β both inhibitors were able to reduce secretion but could not completely block it.

3.2.2 Effect of PMA on acantholysis

After having shown that PMA induces the secretion of innate cytokines, the effect of PMA on cell-cell adhesion in HaCaT cells was tested. Hence, cells were treated with PMA with and without IgG isolated from serum of PV patients and then subjected to a Dispase-based dissociation assay.

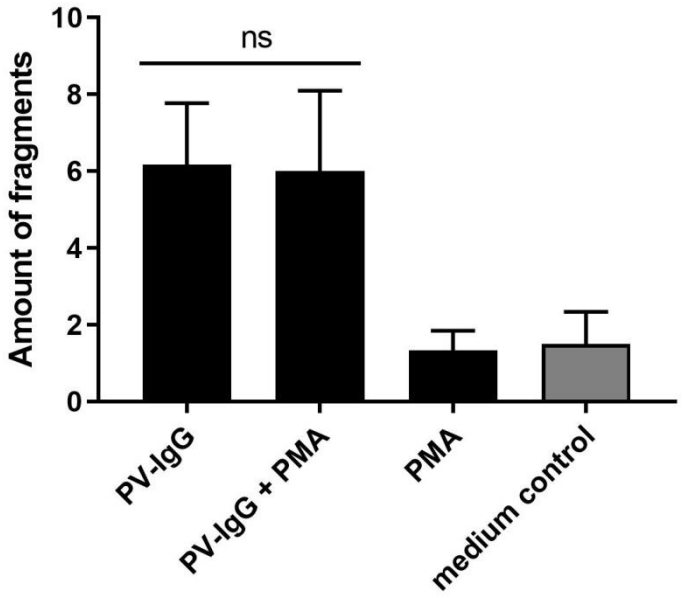

Figure 18: The effect of PMA on acantholysis in HaCaT cells: Cells were treated with PMA, IgG isolated from pemphigus patient sera or a combination of both. After 16 hours of incubation cell-cell adhesion strength was tested by Dispase-based dissociation assay.

Cells treated with PMA only as well as the medium control dissociated in 1.3 and 1.5 fragments. Treatment with IgG from PV patients led to an average of 6 fragments. Addition of PMA did not affect the number of fragments.

3.2.3 Effect of Poly I:C on acantholysis

To test the effect of Poly I:C on cell-cell adhesion strength HaCaT cells were treated similarly to the experiment described in chapter 3.1.4.

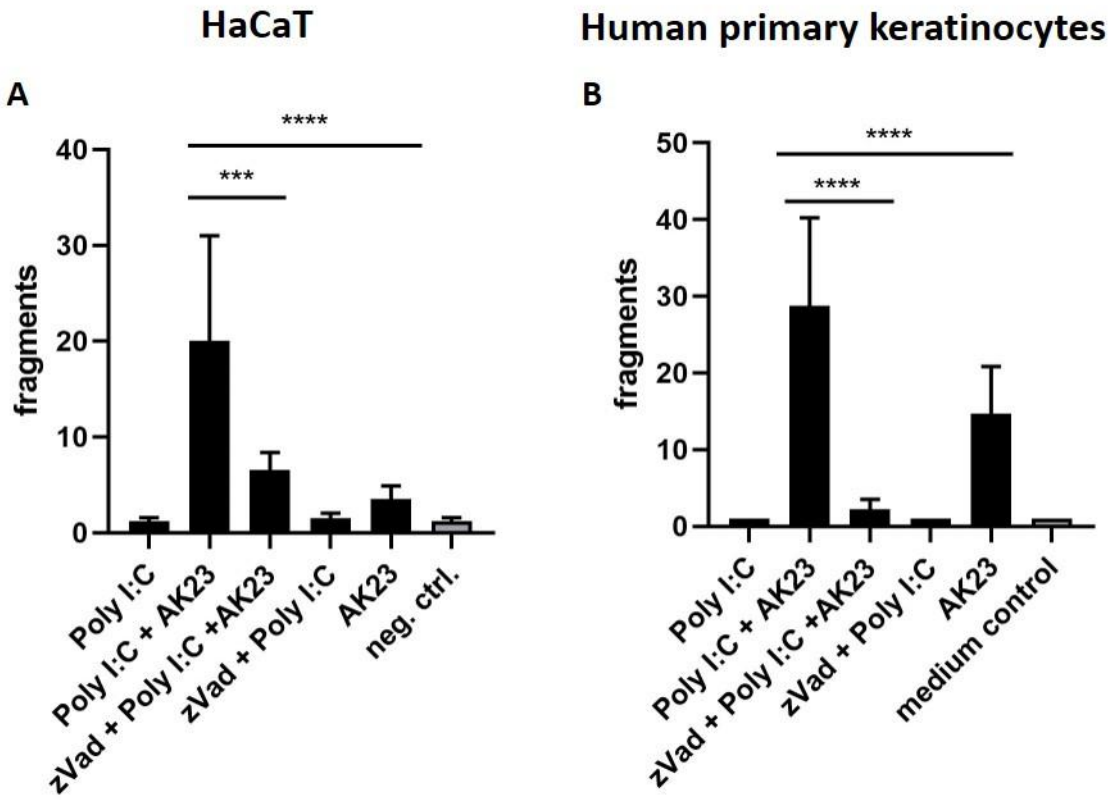

Figure 19: The effect of Poly I:C on acantholysis in HaCaT cells (A) and primary human keratinocytes (B): Cells were treated with Poly I:C, anti-Dsg3 antibody or a combination of both. Caspase-inhibition was achieved by treating cells with zVad for 1 hour prior to stimulation with Poly I:C and antibody. After 16 hours of incubation cell-cell adhesion strength was tested by Dispase-based dissociation assay.

Treatment of HaCaT cells with anti-Dsg3 antibody AK23 let to 3.5 fragments. Addition of Poly I:C and AK23 resulted in a significant increase while pretreatment with zVad reversed that effect.

A similar picture unfolded with primary human keratinocytes: The baseline was at 14 fragments. Addition of Poly I:C increased acantholysis significantly. When pretreated with zVad the dissociation of keratinocytes treated with anti-Dsg3 antibody and Poly I:C was significantly decreased.

3.2.4 Influence of recombinant IL-1 on acantholysis

To test whether elevated levels of IL-1 are responsible for increased fragmentation in the Dispase-based dissociation assay HaCaT cells were treated with recombinant IL-1 and anti-Dsg3 antibody. Therefore 500 pg/ml recombinant IL-1 was administered, which is round about the same concentration that prevails in cell culture after treatment with UVA. At the same time anti-Dsg3 antibody AK23 was added, and cells were incubated for 4 hours. Then IL-1 and IL-6 were measured in the supernatant and cell-cell adhesion was tested in a Dispase-based dissociation assay.

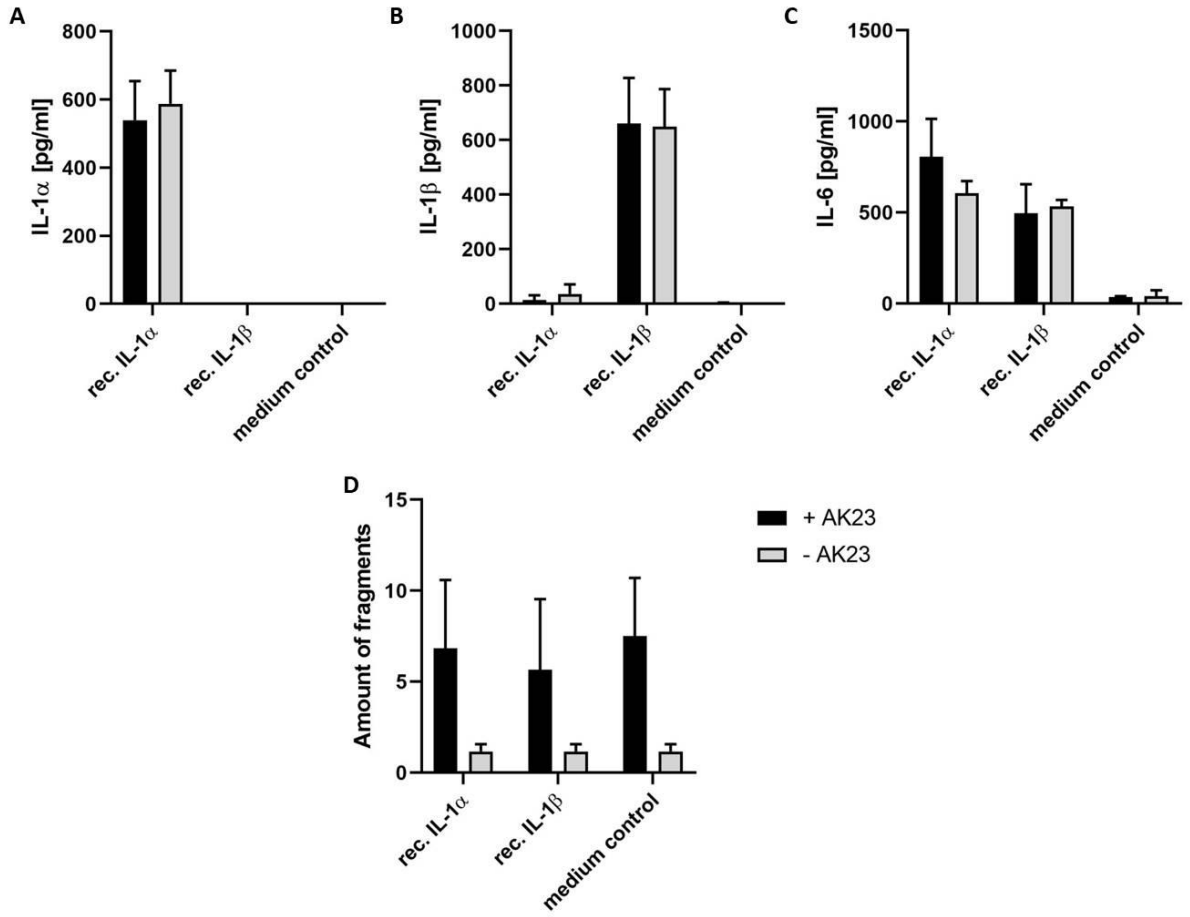

Figure 20: Influence of IL-1 on acantholysis: HaCaT cells were treated with recombinant IL-1α or IL-1β in combination with anti-Dsg3 antibody AK23 for 4 hours. Afterwards the secretion of IL-1α(A), IL-1β (B) and IL-6 (C) was determined. Cell-cell adhesion strength was measured by Dispase-based dissociation assay (D).

After analyzing the secretion of IL-1α upon treatment with recombinant IL-1α, cytokine levels similar to the applied dose were detected. Therefore, quit possibly no additional IL-1α was produced. Addition of anti-Dsg3 antibody did not affect the secretion. Treatment with recombinant IL-1β did also not induce secretion of IL-α. A similar picture unrolled for IL-1β: Only the amount applied was detected after incubation, indicating that no IL-1β was produced by the cells. Again, this was not affected by addition of anti-Dsg3 antibody. The amount of IL-1α that was measured was below the detection limit. In contrast to that IL-6 was secreted robustly after treatment with recombinant IL-1α or IL-1β. However, addition of anti-Dsg3 antibody did not affect the secretion in a significant way.

In the Dispase-based dissociation assay treatment with anti-Dsg3 alone led to 7.5 fragments. Addition of recombinant IL-1α or IL-1β did not influence fragmentation. Samples without anti-Dsg3 antibody all exhibited a mean of 1.2 fragments regardless of whether recombinant IL-1 was added or not.

3.2.5 Effect of nigericin on acantholysis

To test whether the activation of the NLRP1-inflammasome has an influence on acantholysis, cells were treated with Nigericin analog to the experiment described in chapter 3.1.5. Afterwards cells were subjected to a Dispase-based dissociation assay to test cell-cell adhesion strength.

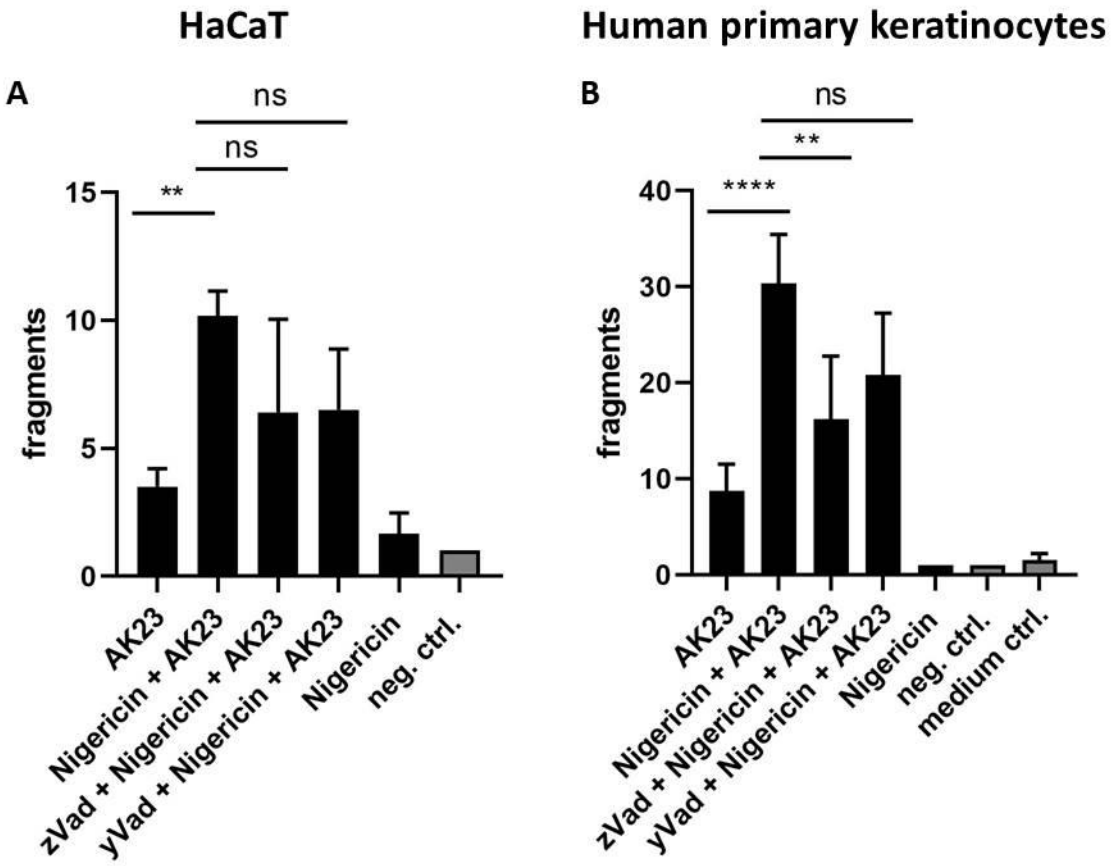

Figure 21: The effect of Nigericin on acantholysis in HaCaT cells (A) and primary human keratinocytes (B): Cells were treated with Nigericin for 15 minutes, anti-Dsg3 antibody AK23 for 4 hours or a combination of both. Inhibition of all caspases was achieved by treating cells with zVad and selective inhibition of caspase-1 by treating cells with yVad. Both substances were applied 1 hour prior to addition of anti-Dsg3 antibody. After incubation cell-cell adhesion strength was tested by Dispase-based dissociation assay

In HaCaT cells treatment with anti-Dsg3 antibody led to a baseline of 3.5 fragments. Addition of Nigericin significantly increased the number of fragments about 3-fold. This could be decreased through pretreatment with zVad or yVad. Treatment with Nigercin only resulted in no fragmentation.

Primary human keratinocytes dissociated into 8.75 fragments upon treatment with anti-Dsg3. This could be significantly increased by around 3-fold through addition of Nigericin. Pretreatment with zVad significantly decreased the number of fragments. Addition of yVad decreased fragmentation as well. However, this was not significant. In primary keratinocytes Nigericin on its own did not induce any fragmentation.

3.2.6 Caspase activation after treatment with nigericin

As no IL-1 secretion or expression could be detected after treatment of keratinocytes with Nigericin, although NLRP1 was activated, the question arose whether downstream caspases had been activated. Therefore, a caspase staining of HaCaT cells treated with Nigericin was conducted. As Nigericin should induce activation of caspase-1 a noticeable staining of caspases with green FAM-FLICA dye was expected. Furthermore, Nigericin was applied for 15 minutes or 40 minutes to see how the activation changes upon treatment in a timely manner. Dead cells were stained using PI and appear red.

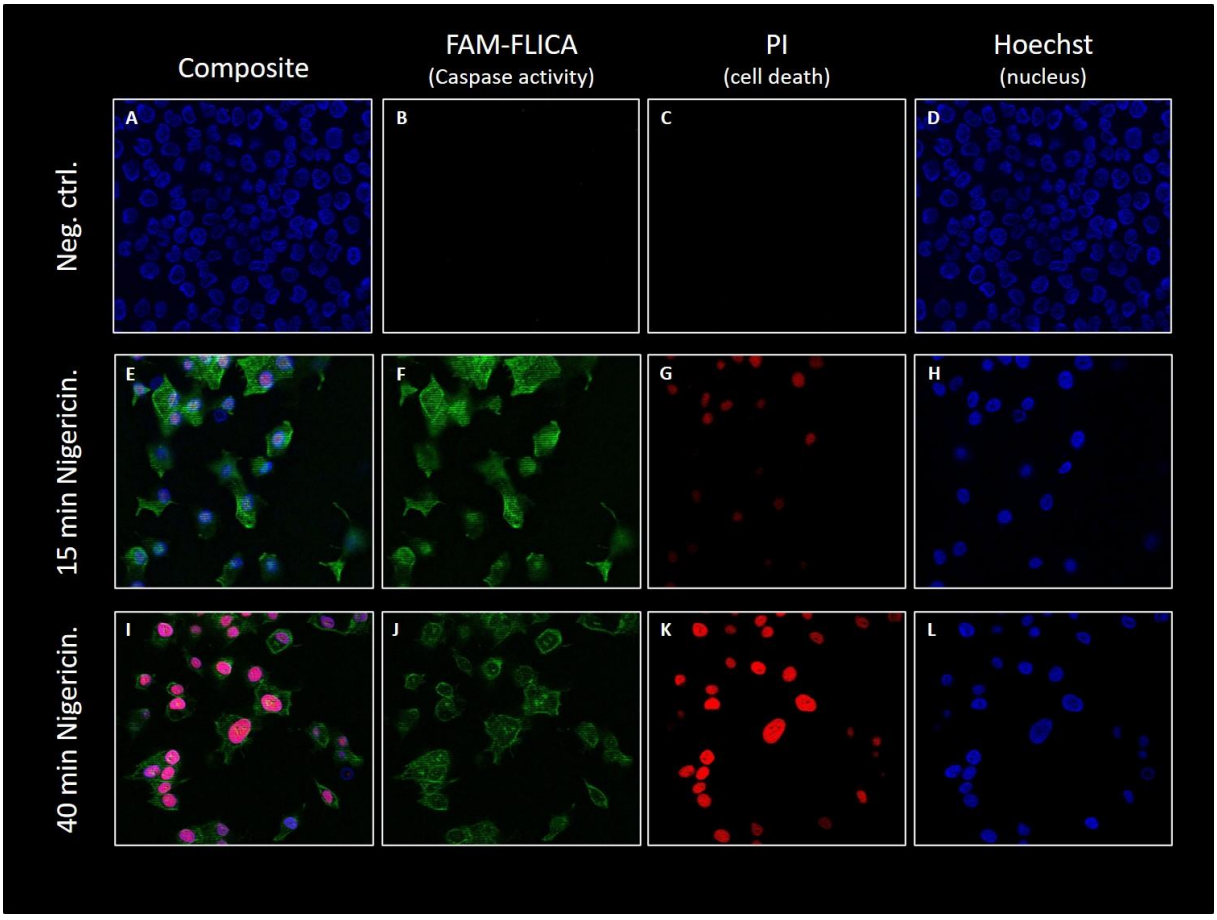

Figure 22: Caspase activation in HaCaT cells after treatment with Nigericin. Nigericin was applied either for 40 minutes (I – L) or 15 minutes (E – H) and compared to a negative control (A – D). Afterwards activated caspases were stained using FAM-FLICA which appears green (B, F, J). Apoptotic cells were detected by PI staining visualized in red (C, G, K). Cell nuclei were stained in blue using Hoechst 33342 (D, H, L).

In the negative control no fluorescence signal correlating to the FAM-FLICA dye or PI could be detected. After 15 minutes treatment with Nigericin a green fluorescent signal could be detected in multiple cells. The signal was thereby present throughout the cytosol of the cells. Additionally, a faint red fluorescent signal could be detected at the nucleus of some cells. After 40 minutes treatment with Nigericin the green fluorescent signal was much weaker than after 15 minutes, and signal was mostly located at the cell membrane or at the nucleus of the cells. In contrast more cells exhibited a red fluorescent signal at the nucleus and additionally the

signal had increased notably in intensity. Also, it should be mentioned that Nigericin did reduce the number of cells that could be stained in total even after 15 minutes of incubation. Staining of nuclei by Hoechst 33342 indicated a notable reduction in cell number on the slide.

3.2.7 Effect of cytokines secreted by PBMCs on acantholysis

In the skin both, keratinocytes and invading inflammatory cells, can provide cytokines. To mimic the influence of the infiltrate, PBMCs were stimulated with LPS or LPS plus ATP to induce inflammasome activation and the release of innate cytokines like IL-1 and IL-6 into the supernatant. The supernatant was collected, and cytokine levels were determined via ELISA. In a next step PBMC supernatant was applied with and without anti-Dsg3 antibody onto HaCaT cells. After 4 hours of incubation levels of IL-1 and IL-6 were determined again and cell-cell adhesion of the HaCaT cells was tested by Dispase-based dissociation assay.

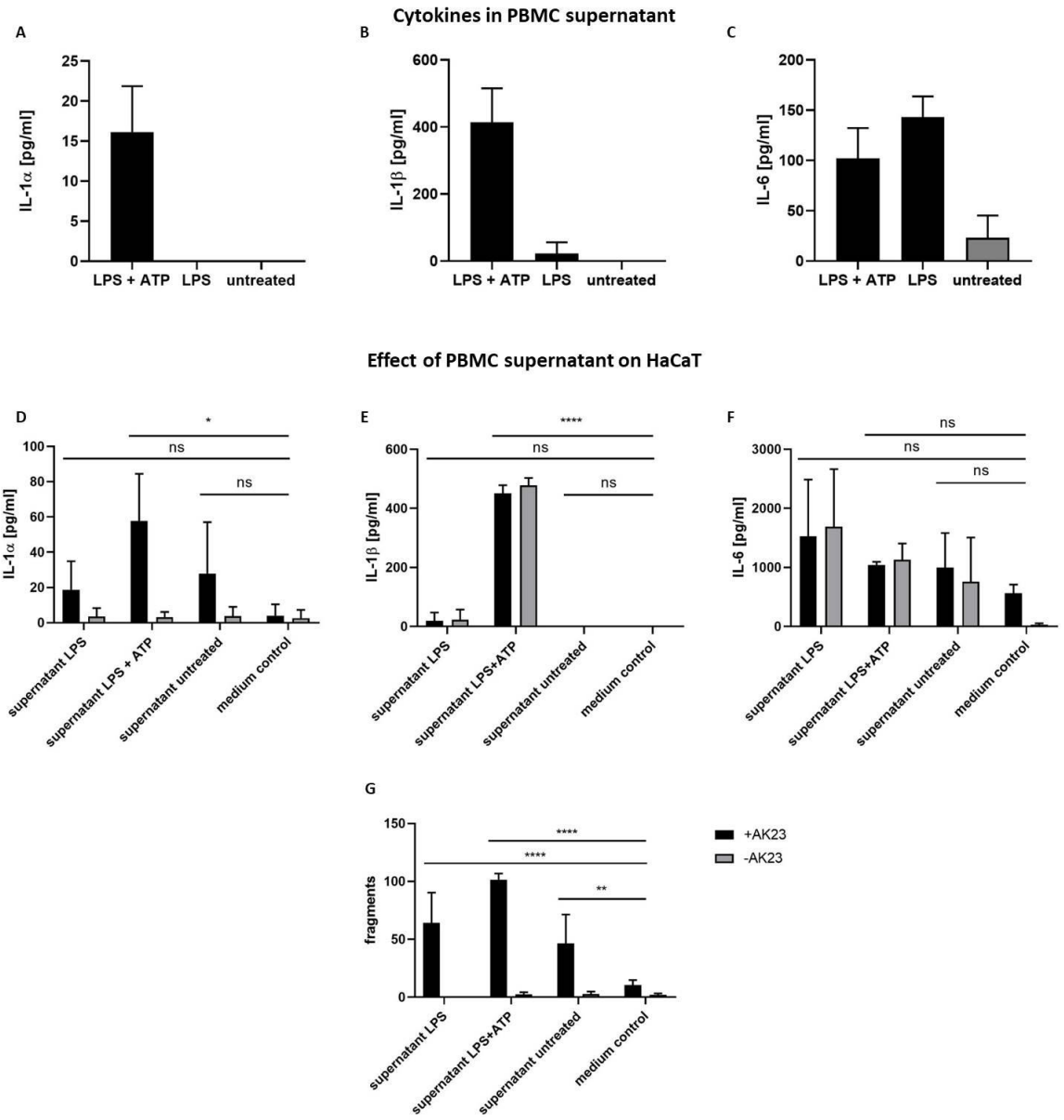

Figure 23: Effect of cytokines secreted by PBMCs on acantholysis: PBMCs were stimulated with LPS or LPS + ATP. Afterwards supernatants were collected and the amount of secreted IL-1α (A), IL-1β (B) and IL-6 (C) was determined. PBMC supernatants were then applied in combination with anti-Dsg3 antibody AK23 onto HaCaT cells. After 4 hours of incubation secretion of IL-1α (D), IL-1β and IL-6 (F) was measured by ELISA. Cell-cell adhesion strength of treated HaCaTs was tested by Dispase-based dissociation assay (G).

Supernatant of PBMCs stimulated with LPS and ATP contained a small amount IL-1α which is very close to the undermost detection limit. Stimulation with LPS alone did not induce secretion of IL-1α. Regarding the secretion of IL-1β the combination of LPS and ATP led to a notable secretion. LPS without ATP resulted in an amount of IL-1β that lies below the detection limit. Stimulation with LPS or LPS and ATP amounted both to a mediocre amount of IL-6 in the supernatant. Also, untreated cells secreted a minor amount of IL-6.

HACAT cells treated with one of the PBMC supernatants and anti-Dsg3 antibody secreted minor amounts of IL-1α. Without the added antibody no IL-1α was secreted. For IL-1β a high

secretion was detected upon treatment with LPS + ATP supernatant which was significant. This was however not influenced by the addition of anti-Dsg3 antibody.
HaCaTs treated with PBMCs supernatant all secreted very high amounts of IL-6 regardless of the stimulation of the PBMCs and whether anti-Dsg3 antibody was applied. Also, treatment with anti-Dsg3 antibody only already induced a strong secretion of IL-6.
In the Dispase-based dissociation assay HaCaTs treated with anti-Dsg3 antibody only dissociated into 11 fragments. This could be massively increased by addition of any PBMC supernatant although the supernatant of PBMCs treated with LPS and ATP showed the strongest increase. Without anti-Dsg3 antibody no fragmentation could be observed regardless of whether PBMC supernatant was applied or not.

3.3 Effect of IL-1 on acantholysis *ex vivo*

The epidermis is not only a layer of keratinocytes, but rather multilayer construct with different stages of differentiation and keratinization. Therefore, we used a skin explant model next, to work in more physiological setting.

3.3.1 Human skin explant model

The skin explants were taken from left-over skin tissue from surgeries. After preparation, the tissue was irradiated with 15 J/cm² UVA and subsequently treated with anti-Dsg3 antibody AK23. After 12 hours of incubation samples were fixated in formaldehyde for histological staining. Additionally, the content of innate cytokines within the culture medium of each sample was determined via ELISA. Tissue samples were HE-stained to visualize.

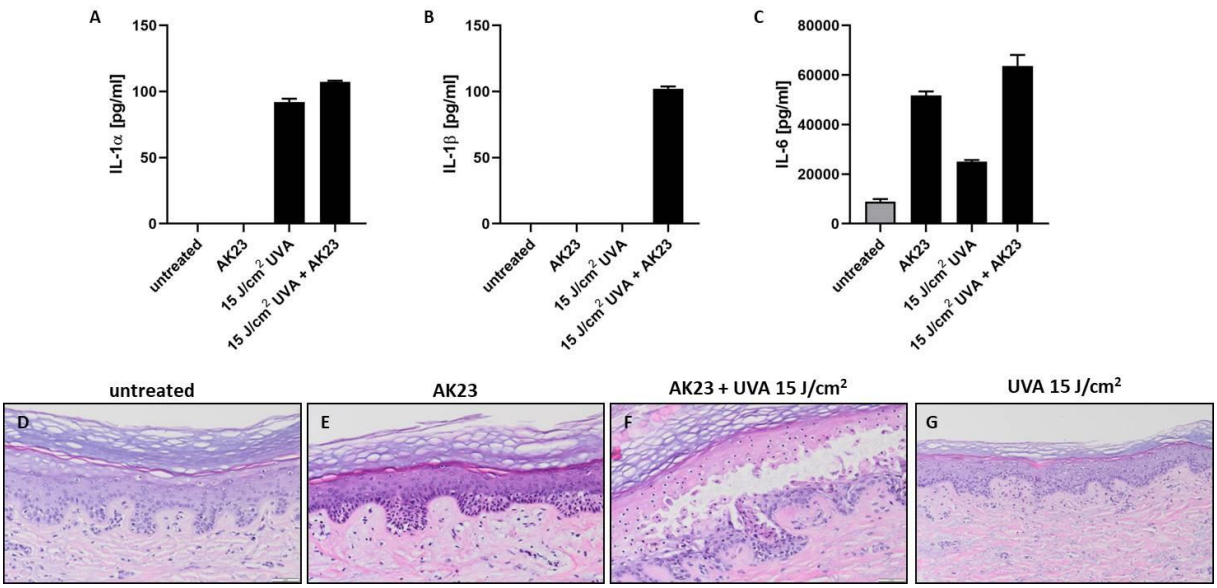

Figure 24: The effect of UVA on acantholysis *ex vivo*: Human ex vivo skin explants were irradiated with 15 J/cm^2 UVA and treated with 15 µg/ml anti-Dsg3 antibody AK23. After 4 hours of incubation secreted IL-1α (A), IL-1β (B) and IL-6 (C) was determined by ELISA. Skin explants were sliced, and HE stained to detect acantholysis within the skin (D – G).

Samples treated with AK23 only did not display acantholysis (Fig. 18, E). Similarly, UVA irradiation alone did not affect the integrity of the cell layers (Fig. 18, G). Nevertheless, a combination of both treatments induced major loss of cell-cell adhesion at the suprabasal layer (Fig. 18, F). When looking at the secretion of IL-1 by tissue samples, the treatment with both UVA and AK23 led to the release of a robust amount of IL-1α and IL-1β. The treatment with UVA alone led to a secretion of IL-1α but no IL-1β, while anti-Dsg3 antibody AK23 did not induce any IL-1 secretion. In addition, the secretion of IL-6 was measured: Here even without additional stimulation extremely high IL-6 levels within nanogram range were measured. Compared to the medium control, samples treated with UVA secreted three times more IL-6. Addition of anti-Dsg3 antibody significantly increased the secretion further. Nevertheless, treatment with anti-Dsg3 antibody only led to a strong increase of IL-6 in comparison to the medium control.

As a next step, tissue samples were treated with zVad to evaluate whether the loss of cell-cell adhesion is caspase dependent. Here, tissue samples were incubated with zVad for 1 hour before UVA irradiation and anti-Dsg3 treatment were applied. Again, tissue samples were HE-stained after incubation and cytokine content in the medium was determined by ELISA.

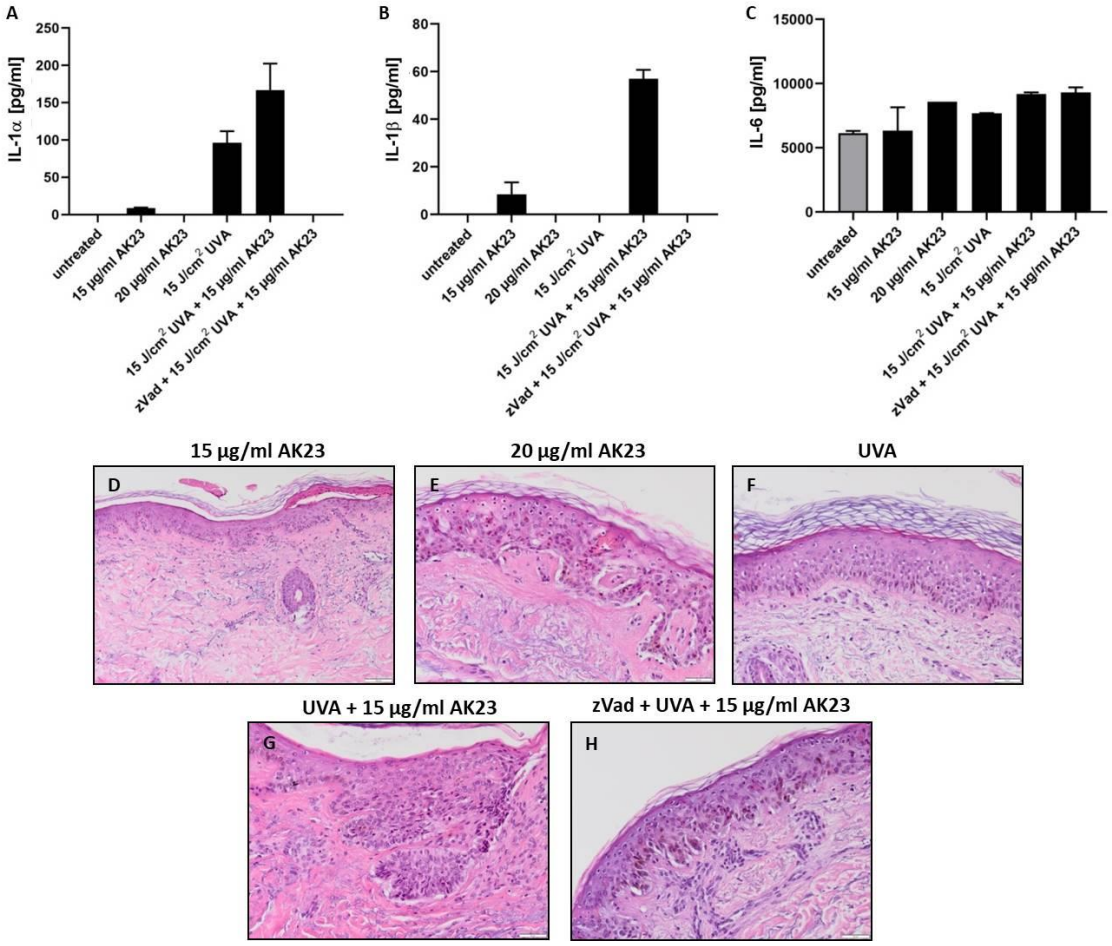

Figure 25: The effect of UVA and caspase inhibition on acantholysis *ex vivo*: Human ex vivo skin explants were irradiated with 15 J/cm^2 UVA and treated with a subpathogenic concentration of 15 µg/ml or a pathogenic concentration of 20 µg/ml anti-Dsg3 antibody AK23. To inhibit caspases samples were treated with zVad for 1 hour prior to irradiation. After 4 hours of incubation secreted IL-1α (A), IL-1β (B) and IL-6 (C) was determined by ELISA. Skin explants were sliced, and HE stained to detect acantholysis within the skin (D – H).

Again, UVA irradiation alone did not induce acantholysis. The effect of anti-Dsg3 antibody AK23 was concentration-dependent: While 15 µg/ml did not lead to a loss of cell-cell adhesion this effect was visible after treatment with 20 µg/ml AK23. However, a combination of UVA irradiation and 15 µg/ml AK23 induced acantholysis in the *ex vivo* tissue samples resulting in visible clefts at the suprabasal skin layer. The pretreatment with zVad was not able to completely block that effect, but reduced size and frequency of clefts.

Again IL-1α and IL-1β were secreted upon treatment with UVA and anti-Dsg3 in a stable, mediocre amount. This secretion could be completely inhibited by addition of zVad. UVA only induced IL-1α, but no IL-1β secretion. Detected amounts of IL-1 after treatment with anti-Dsg3 antibody only were below detection limit. IL-6 was again heavily secreted in all samples. Compared to the already high secretion in the medium control, treatment with UVA increased secretion of IL-6. Addition of anti-Dsg3 antibody led to a further increase of secretion. As expected zVad had no influence on IL-6 secretion.

4. Discussion

The innate immune system as the first line of defense against pathogens takes a major role in the barrier function of the skin. Besides its immunoprotective function it can also cause a variety of inflammatory skin diseases. Pemphigus as an antibody mediated autoimmune disease is considered a disease of the adaptive immune system. Nevertheless, it has been suggested that the innate immune system is involved through T-cell modulation in various autoimmune diseases like for example psoriasis [185]. Proinflammatory cytokines like IL-1 and IL-6 were found in blister fluid and sera of pemphigus patients [159]. As the disease manifestation of pemphigus requires additional factors the involvement of the innate immune system might be an explanation. The discontinuous disease manifestation could hereby be explained through a regional activation of the innate immune system. Hence, the aim of this project was to determine if and how the innate immune system can mediate blister formation.

4.1 Influence of potential cofactors on acantholysis

At first, the aim was to find stimuli that are able to induce an innate immune response and might be of clinical relevance in PV patients. UVA for example is known as a potent inductor of inflammation and is suspected to be involved in the other autoimmune diseases like lupus erythematosus [186]. Almost everybody is regularly exposed to UV light. Still, differences in quantity of exposure due to living conditions in areas with high UV index or regular visits of a solarium could explain, why not every individual with a genetic predisposition develops the disease. On the other hand, mucosa which can also be affected by pemphigus and often is the site where first symptoms occur, is practically never exposed to UV light. However, at least in pemphigus foliaceus where the mucosa is not involved case reports suggest UV light as a trigger for the disease [187,188]. Another property of UV light is the induction of reactive oxygen species (ROS). ROS are highly reactive molecules that are formed upon cellular stress like nutrient deficiency, toxicity, and defense of pathogens. When ROS accumulate severe damage occurs to the cell which is called oxidative stress [189]. Unsurprisingly, many inflammatory and autoimmune diseases are linked to extensive ROS production like arthritis, lupus, Hashimoto thyroiditis, inflammatory bowel disease and multiple sclerosis [190,191].

Irradiation of HaCaT cells with UVA induced the expression as well as secretion of IL-1α, IL-1β, IL-6 and IL-8. Similarly, in primary human keratinocytes the expression of those cytokines was increased as well as the secretion of IL-1 and IL-8. Secretion of proinflammatory cytokines upon UV irradiation has already been described [192]. Secretion of IL-6 was not detected in any experiment with primary keratinocytes. Therefore, it can be assumed that *IL6* is expressed but

to be secreted a second signal is needed that was absent in the experiments. However, in HaCaTs a notable IL-6 secretion was detectable. Irradiation with UV can lead to cell damage which subsequently induces the secretion of DAMPs. These molecules will then be sensed by TLRs of neighboring cells and lead to the secretion of type I-interferons like IL-10, IL-1β, TNF and IL-6 [193,194]. Furthermore, IL-8 secretion was increased under all conditions even in the medium control. UVA can induce IL-8 in keratinocytes through oxidative stress and the activation of the transcription factor AP-1 [195]. IL-8 secretion in the control could be due to cellular stress caused through technical procedures during the experiment. For example, withdrawing and changing of medium induces low shear stress that was shown to induce IL-8 secretion in endothelial cells [196].

After having identified UVA as a potent stimulus of innate cytokines, its potential to influence blister formation was investigated. Blisters in pemphigus patients occur through the loss of adhesion of keratinocytes at the desmosomes, a process called acantholysis [197]. Two vital components of the desmosome, Dsg1 and Dsg3, are targeted by antibodies, which leads to impairment of their function. Nevertheless, blister formation cannot be observed in monolayer cell culture. Therefore, acantholysis induced by antibodies targeting Dsg is the decisive process. This can be analyzed with a Dispase-based dissociation assay.

In HaCaT cells irradiated with UVA in addition to anti-Dsg3 antibody the number of fragments significantly increased compared to the baseline. This correlated with the secretion of IL-1 into the supernatant. At the same time no fragmentation was observed when cells were irradiated without the presence of anti-Dsg3 antibody. Therefore, fragmentation is probably not increased due to the accumulation of dead cells through UV damage. Inhibition of caspases and also the specific inhibition of caspase-1 diminishes the increase, resulting in fragmentation at baseline level. Hence, UVA most likely triggers a cellular process that is caspase-dependent and induces an enhancement in acantholysis but is not sufficient to induce acantholysis on its own. As that process is caspase-1 dependent and leads to the secretion of IL-1β the inflammasome might be involved. Nevertheless, IL-1β secretion in the experiment is not widely blocked by caspase-1 inhibition. It has been shown in neutrophils that other proteases like hymase or cathepsin G can compensate for caspase-1 in pro-IL-1β cleavage [198]. If similar mechanisms are active in keratinocytes this could explain the findings.

A dispase-based dissociation assay with primary human keratinocytes irradiated with UVA leads to similar results to the one obtained with HaCaTs. Taken together UVA serves as s a promising cofactor that induces an innate, proinflammatory immune response and might be involved in disease manifestation in pemphigus as it is able to increase acantholysis *in vitro*.

Another potential cofactor that was further investigated for its potential to induce innate cytokine release was mechanical stress. In pemphigus it is possible to induce blisters or move

blisters to a different location by pushing and squeezing of the skin. This phenomenon is called Nikolsky`s sign [182]. Mechanical stress also plays an important role in the onset of other diseases like epidermolysis bullosa [199]. Here a genetic defect affecting keratins leads to blister formation and rupture of the epidermis. However, especially in milder cases mechanical stress is needed to induce those lesions [200]. Also, in arthritis mechanical stress seemingly induces the side-specific inflammation that leads to disease manifestation [201]. This disease therefore has similarities to pemphigus in terms of disease manifestation: Although an underlying systemic autoimmunity is present, only regional inflammation at the joints can be observed. A study from Cambré et al suggests that chemokines induced by mechanical stress are the cofactor that leads to disease manifestation in arthritis [201].

To investigate whether mechanical stress might be involved in blister formation in PV a monolayer of primary human keratinocytes was subjected to cyclic stretch. In a first experiment the aim was to identify a time point with the optimal induction of innate cytokines. Here after 6 hours of stretch elevated expression of proinflammatory cytokines *IL1A, IL1B* and *IL6* were detected as well as notable secretion of IL-1α. The activation of proinflammatory cytokines like IL-1β and IL-6 upon mechanical stress has been shown in human dental pulp cells as well as an IL-6 induction in macrophages [202,203]. However, in this experiments IL-1β was not secreted which might be because IL-1β needs to be cleaved from pro-IL-1β into its active form. This happens upon a second stimulus that activates the inflammasome which then again induces the cleavage. So, it is possible that in keratinocytes the stretch only serves as a first signal that induces the expression and production of pro-IL-1β but is not sufficient to induce the secretion. Also, IL-8 was strongly expressed and secreted after cyclic stretching. IL8 is a chemotactic factor that is able to draw leucocytes, especially neutrophils, into inflammatory tissue [204]. Neutrophils have been found in plaques of some rare types of pemphigus and IgA pemphigus as well as bullous pemphigoid [205]. In their study which elucidates on the mechanisms of onset in arthritis Cambré et al suggested that chemokines produced upon mechanical stress were a crucial mediator [201]. By drawing in inflammatory monocytes to a specific region, joint inflammation was promoted or even induced. It might be that IL-8 induced by mechanical stress of the skin draws in inflammatory cells from the vascular system in pemphigus and hence mediate the side-specific inflammation.

In a next step PV antibody was added to a keratinocyte monolayer before the stretch starts to mimic the situation in a patient’s body where antibodies are present and tearing and squeezing of the skin induces a blister. The addition of antibodies from pemphigus patients increased the secretion of both IL-1 cytokines. This has been observed in the previous experiment with UVA as a cofactor and here increase in IL-1 correlated to an increase in acantholysis. Hence, mechanical stress might also be a potential cofactor in pemphigus. Again, the IL-1 increase

could be blocked by caspase inhibition, indicating that the inflammasome upstream had been activated. IL-6 and IL-8 induction were not affected by the addition of PV antibody.
In epidermolysis bullosa where mechanical stress plays a vital role for disease manifestation the clinical activity has been shown to correlate with IL-6 levels in the serum of patients [206]. Additionally, elevated expression of chemokines like IL-8 has been observed [207]. Wally et al. showed that IL-1β signaling was extremely active in this disease resulting in the activation of the JNK-stress pathway and subsequently overexpression of IL-1β in a feedback loop [208]. This indicates that mechanical stress can be a potent activator of innate, proinflammatory cytokines and might be involved in other blistering diseases as well. Although, the above-mentioned studies do not enlarge upon whether the inflammatory cytokines mediate or are even the reason for disease manifestation or are induced as a reaction to impaired skin.

Another possible cofactor that was tested was PMA. PMA resembles diacylglycerol, the natural activator of the signaling molecule PKC [177]. After activation PKC signals downstream through the MAPK-ERK pathway and thereby impacts for example regulation of cell proliferation, contractility and permeability of blood vessels, cytokine production and activation of T- and B-cells [209,210]. Nevertheless, expression and secretion of IL-1 and IL-6 was not increased in keratinocytes after treatment with PMA compared to the medium control. This was also not significantly affected by addition of IgG from PV patients. IL-8 was induced by PMA and the secretion significantly increased after addition of IgG from pemphigus patients.
In the dispase-based dissociation assay addition of PMA did not lead to an increase of fragmentation above baseline. Therefore, PMA and its pathways are not a suitable factor in PV manifestation. IL-8 release can occur unspecifically to various factors that induce cellular stress. Due to the point that IL-1 secretion is not initiated by PMA it is very likely that the acantholysis-increasing mechanism is linked to IL-1 secretion.

Poly I:C is a potent TLR3 activator that can mimic a viral infection as viral components can bind to the same receptor. RNA can be found in many viruses that are classified as RNA-viruses like influenza, rota, hepatitis, measles or SARS [211]. Viral infections have been described to induce or modulate several autoimmune diseases. Underlying mechanisms for that are molecular mimicry, epitope spreading or bystander T-cell activation [212]. In molecular mimicry a virus disguises itself as a component of the host by carrying antigens that are structurally similar. This can lead to a cross reactive response by the host immune system against self and non-self-antigens [213]. Another risk during viral infection is that persistent inflammation and non-specific immune responses in the area lead to the release of self-antigens from damaged tissue. These antigens are then processed and presented by APCs causing priming of autoreactive T-cells. During epitope spreading recognition of this one self-

antigen leads to an attack of the immune system against the corresponding host protein which subsequently induces the presentation of different epitopes of that protein and therefore more self-antigens [214]. This process enhances the autoimmunity. During bystander T-cell activation signals which are released due to a non-specific immune response activate autoreactive T-cells that are close by [215]. Additionally, it is discussed that EBV (Epstein-Barr virus) is able to immortalize infected autoreactive B-cells [216,217]. Such a non-specific immune response needed for bystander T-cell activation can be induced amongst others by TLR3 activation. TLR3 signals to the adaptor protein TRIF. Downstream either IFNβ is produced through the activation of TRAF3 or NF-κB is activated through TRAF6 [218]. NF-κB then induces transcription of proinflammatory cytokines.

For PV a connection between an infection with Herpes simplex and an onset of the disease has been discussed [219]. However, Herpes simplex is a dsDNA-virus. Still, others reported a correlation between an infection with coxsackievirus and the manifestation of pemphigus [220]. This is an RNA-virus that usually leads to cold-like symptoms but can be more severe by inducing myocarditis or meningitis [221]. It can also affect the skin by inducing hand-foot-and-mouth disease that leads to skin lesions. Additionally, an infection with this virus has been connected to triggering autoimmune diseases like Sjögren's syndrome, diabetes type I and chronic autoimmune myocarditis [222-224].

The expression of *IL1*, *IL6* and *CXCL8* was increased in HaCaTs as a response to treatment with Poly I:C. Additional treatment with anti-Dsg3 antibody AK23 increased the expression of all four cytokines mentioned above. Blockage of caspases with zVad led to a decrease in expression of *IL1A* and *IL1B*. In primary human keratinocytes only the transcription of *IL6* and *CXCL8* were increased while anti-Dsg3 antibody could only affect *IL6* expression positively. As already mentioned, Poly I:C activates TLR3 which can lead to the production of proinflammatory cytokines as well as inflammasome and subsequently caspase-1 activation. Therefore, increased expression of *IL1*, *IL6* and *CXCL8* would be expected after treatment of cells with Poly I:C. Anti-Dsg3 antibody affects this signaling pathway as expression of those proinflammatory cytokines is increased when antibody is present. Since the antibody alone does not influence the expression, combination of both seems to be needed. Blocking caspases does not affect the transcription of proinflammatory cytokines directly as TLR3 signals independently of caspases. But by preventing the secretion of IL-1β, that can bind to IL-1R1 which then again activates NF-κB, it is possible that inhibition of caspases can influence the transcription of *IL1* as seen in the experiment. As expected *IL6* and *CXCL8* expression is not decreased by caspase inhibition. Looking at the secretion Poly I:C only does not lead to IL-1α secretion in HaCaT cells but notably in human primary keratinocytes. Anti-Dsg3 antibody did not affect IL-1 secretion. IL-6 and IL-8 are both secreted massively as a reaction to Poly I:C by HaCaTs and IL-8 also by human primary keratinocytes. However, it was not possible to

increase the secretion significantly by addition of anti-Dsg3 antibody. Secretion of IL-6 and IL-8 upon stimulation with Poly I:C has already been reported in corneal fibroblasts as well as endothelial cells [225,226]. IL-6 secretion has also been observed in primary keratinocytes while IL-8 secretion was studied by Takada et al. in HaCaT cells [227,228].

Regarding acantholysis, the addition of Poly I:C significantly increased fragmentation compared to baseline. As UVA has similar properties regarding induction of proinflammatory cytokines and acantholysis both cofactors probably trigger the same mechanism within the cell. UVA is known to activate and translocate NF-κB through the generation of oxidative stress [229]. Poly I:C is also able to activate NF-κB through TLR-activation and downstream signaling. Hence, NF-κB might be the common feature that is responsible for the increase in acantholysis. NF-κB is a transcription factor that can influence the transcription of a variety of genes involved in cell proliferation, cell death and inflammation processes. As mentioned above, it is also able to induce transcription of proinflammatory cytokines, like IL-1 and IL-6 which are expressed as well in the experiments involving Poly I:C and UVA. Again, the increase in acantholysis could be diminished by caspase inhibition, indicating that the mechanism initiated by Poly I:C is also caspase-dependent.

So, taken together Poly I:C as a mimic of viral RNA induces a proinflammatory immune response. This could certainly lead to a manifestation of an autoimmune response through bystander T-cell activation or epitope spreading. Enforced by the non-specific, proinflammatory immune response Dsg1 and Dsg3 that were released from inflamed and damaged tissue would be falsely recognized by the immune system as hostile antigens. However, this would then lead to a systemic autoimmunity and could therefore only explain the sudden onset of Pemphigus symptoms in patients that were not affected previously. The discontinuous disease manifestation in terms of regional blister formation would hereby not be explained. Nevertheless, the increased fragmentation observed in the dispase-based dissociation indicates that there has to be an effect on keratinocytes that occurs when Poly I:C and anti-Dsg3 antibody are encountered together. This suggests that viral RNA could also locally induce acantholysis and subsequently blister formation.

4.2 The role of the inflammasome and IL-1 in acantholysis

So far, all tested cofactors that lead to an increase of fragmentation also induced a secretion of proinflammatory cytokines. Especially the correlation between acantholysis and IL-1 secretion was striking. Members of the IL-1 family and IL-1β in particular have been discussed to be involved or even causative in many autoinflammatory diseases [230]. For IL-1α excessive production in rheumatoid arthritis and increased circulating levels in Alzheimer's disease have been described [231,232]. IL-1β is excessively produced in rheumatoid arthritis as well and highly

expressed in psoriasis. Also increased circulating levels have been found in Alzheimer's disease and multiple sclerosis [233-237]. In pemphigus elevated IL-1α and IL-1β serum levels have been described in untreated patients with active disease. With clinical remission serum levels would then decrease while the amount of detectable IL-1RA would enhance [173]. Stimulation of PBMCs of patients with endemic PF resulted in a higher IL-1β secretion compared to a healthy control group [174]. Additionally, Feliciani et al. showed that injection of PV-patient serum into IL-1-knockout mice inhibited blister formation [159].

Thus, the question occurs whether secreted IL-1 itself is responsible for the increase in acantholysis. To investigate that, cells were treated with recombinant IL-1α or IL-1β that was added to the medium with and without anti-Dsg3 antibody. Neither form of IL-1 was able to induce fragmentation on its own or increase it in combination with anti-Dsg3 antibody. Also treatment with recombinant IL-1 did not lead to an increased IL-1 secretion which would theoretically be possible through a feedback loop where IL-1 would bind to IL-1R1 [238]. Contrary, IL-6 secretion was strongly induced by IL-1α as well as IL-1β. Binding of IL-1 to its receptor IL-1R1 induces a strong IL-6 response as confirmed here [239]. So, all in all IL-1 is probably not the factor that is responsible for the increase in acantholysis after UVA or Poly I:C treatment. The added IL-1 was functional as seen by the strong induction of IL-6 secretion. However, neither additional IL-1 secretion nor fragmentation was affected. Thus, the mechanism responsible for the increase in acantholysis must be an intracellular mechanism that is induced by identified cofactors and ultimately leads to IL-1 production. This makes IL-1 a by-product and not the actual activator of acantholysis.

Since IL-1 is most likely not the factor responsible for increased acantholysis it has to be a component upstream of IL-1 induction. Therefore, the effect of inflammasome activation was investigated by stimulating cells with Nigericin. The inflammasome activates caspase-1 which is vital for the cleavage and subsequently the release of IL-1β. Nigericin is a potent activator of NLRP3-inflammasome in lymphocytes [179,240]. However, it has been suggested that in keratinocytes the main inflammasome is of NLRP1-type which can also be stimulated by Nigericin [241]. In bullous pemphigoid an increased expression of NLRP3 and elevated expression levels of NLRP1 in PV have been observed [242,243].

In the experiment no upregulation of cytokine expression could be observed but a notable increase in NLRP1 expression was detected. NLRP3 was not upregulated which underlines the suggestion of Burian et al. that the NLRP1-inflammasome is key inflammasome in keratinocytes. In addition, no cytokine secretion was detected. Others have found increased IL-1 secretion in myeloid cells or IL-6 and IL-8 induction in HeLa cells upon inflammasome activation [69,244]. However, it is possible that the time point chosen specifically for the dispase-based dissociation assay was too short for cytokine secretion. The dispase-based dissociation

assay resulted in a significant increase in fragmentation when Nigericin was added together with anti-Dsg3 antibody. Hence, Nigericin possibly through inflammasome activation induces a mechanism that is able to increase acantholysis. The role of caspase-activation through the inflammasome is not totally clear as only in HaCaT cells, but not in primary human keratinocytes a decreased fragmentation through caspase inhibition occurred.
Hence, caspase-activation upon treatment with Nigericin was visualized via staining with FAM-FLICA. Here it was visible that initially caspases were activated and present ubiquitously in keratinocytes. However, with prolonged incubation of Nigericin caspase-activity declined and at the same time the number of dead cells detected by PI increased. Nigericin induces a form of inflammatory, programmed cell death called pryoptosis via inflammasome- and caspase-activation. Staining with PI is not able to differentiate between those types of cell death as it stains cellular DNA whenever the cell membrane is impaired. Besides the staining the declining number of cells that were visible after treatment with Nigericin regardless of the incubation time indicates that Nigericin leads to a very fast induction of cell death.

Patients` blisters and lesions are often infiltrated by lymphocytes[245,246]. Therefore, to get closer to an *in vivo* situation, keratinocytes were treated with the supernatant of stimulated PBMCs. Supernatant was added on top of a monolayer of HaCaT cells with and without the addition of anti-Dsg3 to test the effect on acantholysis. PBMCs stimulated with LPS and ATP secreted both IL-1α and IL-1β. This induction is due to LPS binding to TLR4 that subsequently leads to expression and production of IL-1 [247]. For the secretion of IL-1β a second signal is needed that activates the inflammasome and subsequently caspase-1 to cleave IL-1β in its active form [69]. Here ATP serves as a second signal. The minor amounts of IL-1β secreted after treatment with LPS only must be due to other danger signals that lead to a minor activation of caspase-1. IL-6 was secreted as a reaction to both LPS and LPS + ATP. Here the activation of NF-κB through downstream signaling of TLR4 is sufficient to produce and release IL-6. Therefore, three different PBMC supernatants were applied onto HaCaT cells: One containing IL-1, especially high amounts of IL-1β and IL-6, one containing predominantly IL-6 and one of untreated PBMCs, containing none of those proinflammatory cytokines.
Here mainly IL-1α not IL-1β was secreted in response to all three supernatants in combination with anti-Dsg3 antibody. IL-6 was also secreted extensively upon treatment with PBMC supernatants regardless of whether anti-Dsg3 antibody was added. In addition, fragmentation was heavily increased by combining anti-Dsg3 antibody and PBMC supernatant treatment. The largest increase could here be observed with the supernatant of PBMCs treated with LPS + ATP. Thus, a substance that is always secreted by PBMCs in culture but even more by stimulated PBMCs must induce in combination with anti-Dsg3 antibody an intracellular mechanism in keratinocytes that increases acantholysis and at the same time induces IL-1

secretion. The results again underline that it cannot be the IL-1 itself as supernatant from unstimulated PBMCs did not contain any IL-1 while the supernatant had a similar effect as the other two. Unpublished data obtained by our group showed that the addition of Fas-L in combination with anti-Dsg3 antibody would increase acantholysis. Fas-L is transmembrane protein of the TNF family that plays a vital role in the induction of apoptosis. However, the enhancement in acantholysis seems to happen prior to cell death. In the light of this it could be possible that Fas-L secretion by PBMCs induced the major increase in acantholysis. Fas-L has been described to be secreted by monocytes and macrophages upon cellular stress like cultivation in general and stimulation with LPS in particular [248,249]. Fas-L downstream activates caspases like caspase-3 and caspase-8. As caspase inhibition via zVad impaired acantholysis this would underline possible link between anti-Dsg3 antibody and Fas-L. Also, the stress-induced transcription factor AP-1 is able to activate Fas-L [250]. As mentioned above AP-1 can be induced for example by UV via ROS and trigger IL-8 secretion which could explain why increased fragmentation sometimes goes along with increased IL-8 secretion.

4.3 Acantholysis in *ex vivo* skin model

To investigate whether these findings are also transferable to the situation in patients, cofactors were tested ex vivo in a human skin explant model. Thereby skin explants are taken into culture and treated with anti-Dsg3 antibody with and without additional cofactors. Acantholysis can be assessed afterwards in histological HE staining of the explants.

The goal of a first experiment here was to simply induce acantholysis through combined treatment with antibody and UVA as it has been observed in vitro. Results of the HE staining show that anti-Dsg3 antibody only induces small blisters in the stratum basale right above the dermis. In accordance with in vitro findings in the Dispase-based dissociation assay UVA only did not induce blistering. The combination of UVA and anti-Dsg3 antibody led to major clefts in the suprabasal epidermis. As sign of inflammasome activation UVA induced the secretion of IL-1α and the addition of anti-Dsg3 antibody increased this even further. IL-1β was only secreted after treatment with both UVA and anti-Dsg3 antibody. This could be due to general stress of preparation and culturing of the explants. Still, irradiation with UVA increased the secretion and the highest secretion was measured after treatment with the combination. However, contrary to in vitro findings the antibody led to an increase in IL-6 secretion as well. As IL-6 is also observed in patients' sera 158 it might be possible that in a setting closer to the situation in patients the antibody is already sufficient to induce this cytokine. This can either be due to the multilayer epidermis compared to a monolayer or due its differentiation or the presence of fibroblasts. As clefts in the ex vivo models histologically resemble the situation in

PV patients the human skin explant model serves as a valid model to study acantholysis ex vivo. The utilized concentration of anti-Dsg3 antibody in this experiment is subpathogenic as no major clefts could be induced with it. UVA irradiation is here able to increase acantholysis which underlines its possible role as a cofactor. The secretion of IL-1 in samples with and without acantholysis looks similar to the monolayer in vitro situation suggesting that the same intracellular mechanism is responsible here.

In a next experiment, samples were treated to inhibit caspases analog to experiments in vitro. Here the same concentration of anti-Dsg3 antibody that was used in the previous experiment again proves to be subpathogenic as no suprabasal clefting could be observed. Small clefts occurring in stratum spinosum are probably due to shear stress of the preparation process of the slices. Additionally, a higher concentration of anti-Dsg3 antibody was used in this experiment that shows that ex vivo the antibody on its own is sufficient to induce clefts: Small gaps in the stratum basale were visible that indicate a suprabasal acantholysis. This concentration of anti-Dsg3 antibody is therefore pathogenic. UVA irradiation on its own again did not induce any gaps and cells within the skin layers look normal which confirms the results of the first ex vivo experiment. The combination of UVA irradiation and anti-Dsg3 antibody in a subpathogenic concentration led to clefting similar to that achieved with the pathogenic concentration of anti-Dsg3. The clefts occur in the stratum basale above the dermis as well. Hence, UVA is able to increase acantholysis ex vivo and the question arises whether it is also responsible to break tolerance and induce disease manifestation in patients. When caspases were inhibited, clefts were still visible in the stratum basale right above the dermis. Nevertheless, size and frequency of the gaps was notably reduced indicating that the inhibition of caspases is able to decrease the effect but not completely diminish it. Still, this result confirms that the underlying mechanism is connected to caspases ex vivo as well. Regarding cytokine secretion UVA only led to the induction of IL-1α and IL-1β secretion that could be increased in both cases with the addition of anti-Dsg3 antibody. After inhibition of caspases the secretion of both cytokines was diminished. This confirms that the inhibition worked. As seen in the previous experiment IL-6 was secreted majorly in all samples. The concentration in the supernatant was increased in some treated samples but overall, there is no distinct pattern visible. This leads to the conclusion that IL-6 is mostly secreted as a reaction to the preparation of the explants and not due to specific stimuli. All in all, these results indicate that findings in vitro could be replicated ex vivo. UVA light is still a hot contender as a cofactor that is able to induce acantholysis whenever anti-Dsg3 antibody is only present in a subpathogenic concentration. As acantholysis observed in experiments can be seen as an indicator for the potential to induce blister formation in patients, UVA could possibly induce a clinical disease manifestation. Once more it was underlined that the underlying cellular mechanism is caspase dependent and leads to the production and secretion of IL-1.

5. Summary

5.1 Conclusion

Aim of this project was to analyze how the innate immune system through proinflammatory cytokines or the inflammasome are involved in disease manifestation and blister formation in pemphigus. First it was shown that UVA, mechanical stress and Poly I:C are able to induce an innate immune response in keratinocytes marked by the induction of proinflammatory cytokines IL-1, IL-6 and IL-8. UVA and Poly I:C are also able to increase acantholysis *in vitro* when applied in combination with anti-Dsg3 antibody. These results could be confirmed in an ex vivo skin model where acantholysis took the form of suprabasal clefts. Hence, both stimuli present possible cofactors that might promote disease manifestation in pemphigus. Together with increased acantholysis an induction of IL-1 secretion was detected in most cases. Nevertheless, treatment with recombinant IL-1 showed that not the cytokine itself is responsible for the induction of acantholysis. Therefore, a component upstream of IL-1 has to be involved. The activation of the inflammasome through Nigericin was also able to induce an increase in acantholysis and at the same time as expected cell death was initiated. Additionally, treatment of keratinocytes with supernatant of stimulated and unstimulated PBMCs increased acantholysis in combination with anti-Dsg3 antibody and led to an induction of IL-1 as well. Thus, a component secreted by PBMCs and even more so by stimulated PBMCs must be involved in the induction of acantholysis. Therefore, theory arose that the increase in acantholysis is due to an activation of the apoptotic pathway through Fas-L and downstream caspases. Fas-L could be secreted by stimulated PBMCs as well as keratinocytes as a reaction to cellular stress. Stress could be induced through oxidative stress via ROS and UVA irradiation as well as Poly I:C as an analog to viral RNA. This is supported by the fact that the induction of acantholysis was shown to be caspase-dependent. As the apoptotic pathway relies on the activation of various caspases like caspase-3 and caspase-8, blocking those could also inhibit the increase in acantholysis. In this scenario proinflammatory cytokines like IL-1 would be a byproduct as Fas-L is able to activate NF-κB.

5.2 Outlook

The next steps in this project would be to verify whether the theory that connects increased acantholysis to the apoptotic pathway is valid. Therefore, it should be evaluated if components of the apoptotic pathway are present after stimulation with identified cofactors like UVA or Poly I:C. Possible targets could be Fas-L and caspase-3 or -8. Additionally, to investigate further whether in vitro findings with keratinocyte monolayers are also applicable to the situation in patients, experiments could be repeated using a human 3D-skin model. This in vitro model consists of all layers of the skin created from scratch by growing the associated cell types above each other. Therefore, in contrary to human ex vivo skin explant these models are highly standardized [251-254]. If it would be possible to induce an acantholysis in this model the effect of cofactors and possibly the apoptotic pathway could be studied in a situation much closer to actual human skin. It is possible to co-cultivate those models with PBMCs or even get lymphocytes to infiltrate the skin equivalents [252]. This would offer the possibility to investigate further which component secreted by PBMCs is ultimately responsible for the increase in acantholysis. Also, by using knockout cells to build those skin equivalents the effect of components of the apoptotic pathway could be studied further: If a knockout of Fas-L or one of the caspases inhibits the induction of acantholysis this would greatly strengthen the case that this is the missing link to disease manifestation.

Mucous skin acts very differently in pemphigus as first lesions in pemphigus vulgaris often occur here while it is not involved in Pemphigus foliaceus. Since the group around Prof. Baron has also established and published a 3D-skin model resembling mucosa it might be interesting to study acantholysis and the effect of potential cofactors here [255].

Appendix A: Resources

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