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“The pathophysiological role of IL-17 in bullous pemphigoid”

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Abstract

IL-17A acts as a critical regulatory molecule in several autoimmune and chronic inflammatory diseases and is successfully targeted, e.g. in ankylosing spondylitis and psoriasis. Unlike most of the immunosuppressive treatments, anti-IL-17A therapy has a favourable safety profile. Bullous pemphigoid (BP) is the most frequent autoimmune blistering disease characterised by autoantibodies against a structural protein of the dermal-epidermal junction, BP180, also termed type XVII collagen (Col17). Treatment of BP is based on the long-term use of systemic or superpotent topical corticosteroids associated with a high rate of adverse effects and increased mortality. Thus, there is a high need for specific, effective, and safe treatment options in BP. To overcome the treatment limitations in this disease, it is essential to identify key pathogenic molecules in order to target them pharmaceutically. Here, I present conclusive data about the relevance of IL-17A in patients and several experimental models of BP. Elevated numbers of IL-17A$^+$ CD4$^+$ lymphocytes were found in the peripheral blood of BP patients, and CD3$^+$ cells were identified as a major source of IL-17A in early BP skin lesions. In addition, upregulation of *IL17A* and related genes in BP skin was revealed. Subsequently, we found several lines of evidence suggesting a functional role of IL-17A in the pathogenesis of BP. Specifically, an anti-IL-17A antibody reduced the reactive oxygen species (ROS) release from neutrophils in response to Col17-anti-Col17 IgG immune complexes. Anti-IL-17A treatment-induced inhibition of dermal-epidermal separation in cryosections of human skin incubated with anti-Col17 IgG and subsequently, with anti-IL-17A IgG-treated leukocytes. Furthermore, *IL17A*-deficient mice were shown to be protected against the otherwise pathogenic effect of anti-Col17 IgG, and pharmacological inhibition of IL-17A reduced the extent of skin lesions in the experimental mouse model of BP. Take together, these data indicated a pivotal role of IL-17A in the pathophysiology of BP and advocated IL-17A inhibition as a potential novel treatment for this disease.

Zusammenfassung

IL-17A wurde als wichtiges proinflammatorisches und regulatorisches Molekül bei verschiedenen Autoimmun- und chronischen Entzündungskrankheiten beschrieben, und eine IL-17A Inhibition wird erfolgreich z.B. bei der Spondylitis ankylosans und der Psoriasis eingesetzt. Im Gegensatz zu immunuppressiven Therapien hat die Anti-IL-17A Therapie ein günstiges Sicherheitsprofil. Das bullöse Pemphigoid (BP) ist die häufigste bullöse Autoimmunerkrankung und tritt fast ausschließlich im höheren Lebensalter auf. Das BP ist klinisch durch Blasen und Erosionen und immunologisch durch Autoantikörper gegen ein Strukturprotein der dermo-epidermalen Junktionszone, BP180 (auch als Kollagen Typ XVII, Col17, bezeichnet), charakterisiert. Die Behandlung des BP basiert auf der langfristigen Anwendung von systemischen oder superpotenten topischen Kortikosteroiden, die mit hohen Nebenwirkungen und erhöhter Mortalität verbunden ist. Daher besteht ein hoher Bedarf an spezifischeren, effektiveren und sichereren Behandlungsmöglichkeiten für diese Erkrankung.

Für die Entwicklung neuer Therapien ist es unerlässlich, pathogenetisch relevante Schlüsselmoleküle zu identifizieren, die pharmakologisch targetierbar sind. Die vorliegende Arbeit untersucht die Bedeutung von IL-17 und verwandter Mediatoren bei Patienten und in mehreren experimentellen Modellen des BP. Erhöhte Zahlen von IL-17A$^+$ CD4$^+$ Lymphozyten wurden im peripheren Blut von BP Patienten gefunden und CD3$^+$ Zellen als Hauptquelle von IL-17A in frühen Hautläsionen identifiziert. Darüber hinaus wurde die Hochregulation von *IL17A* und verwandten Genen in frühen Hautveränderungen von BP Patienten nachgewiesen. Zudem wurde in verschiedenen experimentellen Ansätzen Hinweise auf eine funktionelle Rolle von IL-17A in der Pathogenese des BP generiert. Anti-IL-17A Antikörper reduzierten die Freisetzung reaktiver Sauerstoffspezies aus Neutrophilen nach Stimulation mit Col17-Anti-Col17 IgG Immunkomplexen. Der Anti-IL-17A Antikörper inhibierte auch die subepidermale Spaltbildung in Kryoschnitten humaner Haut nach Inkubation mit Anti-Col17 IgG und anschließender Inkubation mit Leukozyten. Darüber hinaus waren IL17A-defiziente Mäuse gegen den ansonsten pathogenen Effekt von Anti-Col17-IgG geschützt, und die pharmakologische Hemmung von IL-17A reduzierte das Ausmaß der Hautveränderungen im Anti-Col17 IgG-induzierten Mausmodell des BP. Diese Daten beschreiben IL-17A als ein

Schlüsselmolekül in der Pathophysiologie des BP und weisen auf die IL-17 Inhibition als mögliche neue rationale Therapieoption für diese Erkrankung.

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Abbreviations

ABSA	Affected body surface area
AhR	Aryl hydrocarbon receptor
AIBDs	Autoimmune bullous diseases
APCs	Antigen-presenting cells
AUC	Area under the curve
Bcl	B cell lymphoma
BM	Bone marrow
BMZ	Basement zone
BP	Bullous pemphigoid
BPAG	Bullous pemphigoid antigen
BSA	Bovine serum albumin
C3	Complement component 3
CCL20	Chemokine C-C motif ligand 20
CCR6	Chemokine receptor 6
CD	Cluster of differentiation
CIA	Collagen-induced arthritis
COL	Collagenous
Col17	Type XVII collagen
CTLA-4	Cytotoxic T lymphocyte-associated antigen 8
CXCL	Chemokine C-X-C motif ligand
CXCR	C-X-C motif chemokine receptors
DAG	Diacylglycerol
DEJ	Dermal-epidermal junction
DEJ	Dermal-epidermal junction
DIF	Direct immunofluorescence microscopy
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal Bovine Serum.
FDA	Food and Drug Administration
FITS	Fluorescein isothiocyanate
Foxp3	Forkhead box P3

GCs	Germinal centres
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Growth macrophage colony stimulating factor
GST	Glutathione-S-transferase
H&E	Hematoxylin and eosin
HD	Hemidesmosome
i.p.	Intraperitoneal
IC	Immune-complex
ICOS	Inducible Costimulator
Ig	Immunoglobulin
IHC	Immunohistochemistry
IIF	Indirect immunofluorescence
IL	Interleukin
ILC3s	Group 3 innate lymphoid cells
INFγ	Interferon gamma
IRF4	Interferon regulatory factor 4
IP3	Inositol-triphosphate
JAK	Janus kinase
kDa	Kilodalton
KO	Knockout mice
Lcn-2	Lipocalin-2
LD	Lamina densa
LL	Lamina lucida
mAb	Monoclonal antibody
MCT	Mast cell tryptase
MHC	Major histocompatibility complex
MMP	Mucous membrane pemphigoid
MMPs	Matrix metalloproteinases
MPO	Myeloperoxidase
MUC	Mucin
NC	Non-collagenous
NK cells	Natural killer cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween

PD-1	Programmed Death-1
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PRRs	Pattern-recognition receptors
RA	Rheumatoid arthritis
RBCs	Red blood cells
ROR	Retinoic acid receptor-related orphan receptor
ROS	Reactive oxygen species
RPMI	Rosewell Park Memorial Institute
RT	Room temperature
s.c	Subcutaneous
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
T1D	Type-1 Diabetes
Tc	Cytotoxic T cells
T_{FH}	T-follicular helper cells
TGF-β	Transforming growth factor-beta
TLRs	Toll-like receptors
TMB	3,3′,5,5′-tetramethylbenzidine
TNFα	Tumor necrosis factor alfa
T_{regs}	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
TSR	T-cell receptor
Tyk2	Tyrosine kinase 2
WT	Wild-type

1. Introduction

1.1. Immune response

The immune system comprises an interactive network of cells, tissues, and organs. The functional abilities are to sense and foremost to protect an organism against foreign pathogens and signals. The first arm of defence is formed by cells of the innate immune system, including natural killer (NK) cell, mast cells, eosinophils, basophils, and phagocytic cells (neutrophils, macrophages and dendritic cells), as well as activation of the complement cascade. The innate immune system is the front arm of defence of the natural anatomical barriers (i.e. the skin, digestive tract and lungs) against danger signals. They recognise microbes via pattern-recognition receptors (PRRs) including the membrane-bound Toll-like receptors (TLRs) or cytosolic Nod proteins (Beutler and Hoffmann, 2004; Medzhitov, 2007). PRR signalling also primes the production of cytokines and chemokines to recruit and activate the cells of the adaptive immune system (Fearon and Locksley, 1996).
Vertebrates have developed a more specific, potent and systemic adaptive immune response. Adaptive immune responses, or the second arm of utmost importance, include humoral immunity, which is mediated by antibodies produced by B cells, and cellular immunity, mediated by T cells. B cells and T cells have an almost unlimited repertoire of receptors to ensure that any microbe can be recognised (Flajnik and Kasahara, 2010).

1.1.1. T cells

T cells are categorised by receptors and coreceptors expressed on the cell surface. They allow deciphering their specific antigen via the T cell receptor (TCR), which is a heterodimer of two chains, named TCR α and β chains (Garcia et al., 2010). A small subpopulation of T cells owns a TCR comprising a γ and a δ chain. The classical TCR is composed of a heterodimer of the variable α and β chains that together bind linear epitopes of the specific antigen. These linear epitopes/peptides are presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs). T cells to recognise the antigen and become active has to form a stable TCR:antigen: MHC complex (Garcia et al., 2010; Rudolph et al., 2006).

There are two main MHC molecules, MHC I and MHC II. MHC class I molecule will bind to the surface of protein expressed on CD8 T cells (cytotoxic T cells), while the MHC II molecule will recognise CD4 T cells (Gowans, 1996). The central role of CD4 T cells to the immune protection is present by their ability to recruit innate immune cells to the sites of infection and inflammation. This role is fulfilled through the production of massive soluble mediators like cytokines and chemokines, leading to the full parade of immune responses and helping B cells in class switching.

Initially, immunologists believed there were fundamentally two types of immune responses that require the action of CD4 T cells. One was antibody-mediated and involved B cells, while other was cell-mediated and involved T cells predominantly.

In 1986 Mossman and Coffman transformed the simple dichotomy into a complex array of a phenotype (Mosmann et al., 1986). The long-term CD4 T-cells are not a unitary set of cells but represent a series of distinct cell populations with different functions and subset-specific differentiation program. IFNγ producing-line called T helper cells type 1 (Th1) is critical for immunity to intracellular microorganisms. IL-4 line named as T helper cells type 2 (Th2) was described to be important for immunity against extracellular pathogens, including helminths (Mosmann et al., 2017; Taylor et al., 2012). During the past decade, the Th family expanded from Th1 and Th2 cells to follicular T cells (T_{fh}), Th9, Th22, Treg, and Th17 cells (Eyerich, 2014).

Different T helper cells differ between themselves by their pattern of differentiation, the genes they expressed, the sets of products they produce together with their functions, as well as the consequences and abnormalities they inflict. The classification concept comprises cytokines present in the microenvironment or produced directly by the APCs, the transcriptional regulators, specific migration properties and the strength of the stimulus determine the fate of naïve T cells.

Th1 cells are required to facilitate the clearance of pathogen-infected cells (Sacks and Noben-Trauth, 2002), activate macrophages by producing cytokines and induce delayed-type hypersensitivity responses. Generated Th1 cells secrete INFγ and TNFα (**Figure 1.1**). Their crucial roles in various autoimmune diseases including systemic lupus erythematosus, multiple sclerosis and rheumatoid arthritis (RA) have been previously shown (Burmester and Pope, 2017; Correale et al., 2017; Guimarães et al., 2017).

Th2 cells, characterized by production of IL-4, IL-5, IL-10 and IL-13 cytokines, (Eyerich and Zielinski, 2014; Sehra et al., 2008; Taylor et al., 2012; Tolieng et al., 2017), have

been linked to humoral immune responses and IgG1 and IgE class switching (Coffman and Carty, 1986). An over-exuberant Th2 response leads to pathogenic conditions, such as asthma and atopic airway hypersensitivity (Kay, 1991; Robinson et al., 1992). The long-known support of B cells responses by T helper cells is a well-established phenomenon. They support each other in the form of antibody class switching, affinity maturation and generation of B cell memory (Garside et al., 1998). A population of follicular $CD4^+$ T cells exhibit influence over B-cell responses. They present a substantial source of IL-21 (Hirota et al., 2013), but can also produce IL-4, IFNγ and IL-17 (Bauquet et al., 2009a; Morita et al., 2011; Reinhardt et al., 2009). Since class-switched autoantibodies characterise autoimmune diseases, the role of T_{fh} cells has been previously reported in many human autoimmune diseases and numerous animal models (Ohl et al., 2016; Simpson et al., 2010; Yang et al., 2005).

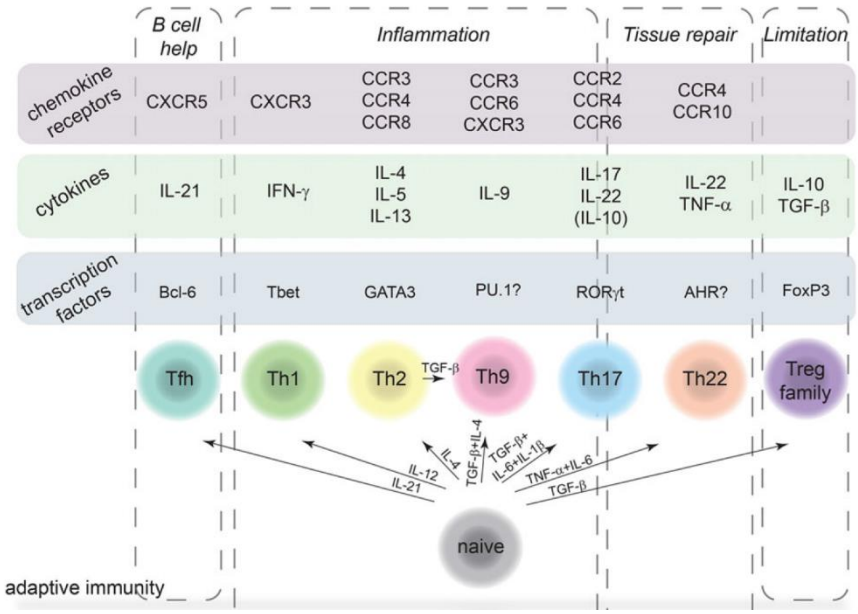

Figure 1.1: Overview of T helper cells subsets

T helper cells can be classified according to the cytokine production (green), expression of transcription factors (blue) or chemokine receptors (lavender). Given the plasticity between the different subsets, they can be further functionally divided into B-cell helper cells, inflammatory cells, tissue regeneration cells, and eliminatory cells (dashed line boxes); modified by (Eyerich and Zielinski, 2014)

Th9 cells are a recently identified subtype of Th cells. They produce high quantities of IL-9 and IL-10 (**Figure 1.1**). Although their function is not entirely clarified, there are indications they are involved in several inflammatory diseases (Chang et al., 2010; Ciccia et al., 2015; Kaplan, 2013).

In 2009, a new T cell population so-called Th22 extended the Th family. Th22 cells differentiation from naïve precursors cells is enhanced under polarisation with IL-6 and TNFa, (**Figure 1.1**). The impact of Th22 is still under debate with some studies showing their anti-inflammatory roles, while other report pro-inflammatory properties (Basu et al., 2012; Eyerich et al., 2009; Plank et al., 2017). So far, the role of Th22 was described to be important in various autoimmune allergic and oncological diseases such as thyroiditis, allergic rhinitis, hepatocellular carcinoma, rheumatoid arthritis, allergic asthma, and allergic rhinitis (Tamasauskiene and Sitkauskiene, 2017) as well as on keratinocytes in skin diseases, such as atopic dermatitis (Eyerich et al., 2009; Furue et al., 2017; Gittler et al., 2012; Jia and Wu, 2014).

While effector T cells promote inflammation, regulatory T cells control effector T cells, maintaining peripheral tolerance and immune homeostasis. Their characteristic is high suppressive capacity. There are two types of Tregs described in the thymus (naturally occurring Tregs (nTregs) or induced in the periphery-induced Tregs (iTregs) (Korn et al., 2009). Interestingly, iTreg and Th17 cells are antagonistically correlated; both produce high amounts of IL-10 and membrane-bound TGF-β and exert their suppressive effects by cell-cell contact (Nakamura et al., 2001). Their primer effect is preventing the induction of T effector responses against commensal bacterial antigens. Such induction could lead to the development of inflammatory diseases such as colitis and inflammatory bowel disease (IBD) (Atarashi et al., 2013). Wildin and colleagues showed a link between type-1 diabetes and the lack of Treg cells (Wildin and Freitas, 2005). Increasing evidence shows that deficiencies in the number and suppressive capacity of $CD4^{+}CD25^{high}$ Treg cells are crucial in the development of systemic lupus erythematosus (Miyara et al., 2005; Valencia et al., 2007). Recently, it was shown that Treg deficiency in mice leads to the production of pathogenic anti-BP230 autoantibodies in bullous pemphigoid (BP) (Haeberle et al., 2018).

1.1.2. Th17 cells discovery and polarisation

The discovery of IL-23, expand the simple dualism for Th1/Th2, and Th17 cells emerged on the scene, as a third main Th cell type. Th17 cells exhibit effector functions mainly by providing host defence against bacteria and fungi (Cua et al., 2003; Harrington et al., 2005; Langrish et al., 2005; Park et al., 2005). Transcription factors specific for Th17 are STAT3 and retinoic acid receptor-related orphan receptor-γτ (ROR

γτ). Th17 cells produce a different set of cytokines in human and mouse, but IL-17A, IL-17F, IL-21 and IL-22 are shared between both species (Korn et al., 2009).

IL-23 is a heterodimeric cytokine that is binding IL-12p40 and IL-23p19 subunits. The IL-12p40 subunit is shared with IL-12, while IL-23p19 subunit is unique for IL-23 (Cua et al., 2003; Duvallet et al., 2011). Previously, in graceful series of studies conducted in animal models of autoimmunity, specifically experimental autoimmune encephalomyelitis and collagen-induced arthritis, it was shown that mice deficient in IL-23p19 subunit were resistant to both diseases, collagen-induced arthritis and experimental autoimmune encephalomyelitis. In contrast, mice deficient in IL-12p35 subunit were susceptible to disease (Cua et al., 2003; Langrish et al., 2005; Murphy et al., 2003).

Initially, IL-23 thought to be a critical cytokine for polarization of Th17 cells. In 2006, three different groups showed the induction of Th17 cells *in vitro* without IL-23. Naive mouse $CD4^+$ T cells stimulated via their TCR in a combination of IL-6 and TGF-β can induce Th17 cells differentiation (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). Although IL-6 and TGF-β can stably induce mouse Th17 cells, the absolute requirement of TGF-β for Th17 differentiation in humans is still under debate. It seems that IL-1β replaced the function of TGF-β for Th17 differentiation in humans (Bettelli et al., 2006), while TGF-β is essential in mice (Korn et al., 2009) (**Figure 1.2)**. IL-6 signalling leads to phosphorylation of STAT3, the first transcription factor for appropriate Th17 differentiation (Muranski and Restifo, 2013; Yang et al., 2007). Thomas Korn and colleagues, using a mouse model of experimental autoimmune encephalomyelitis demonstrated that IL-6-deficient mice have a defect in Th17 cells generation. In these mice which were IL-6 deficient, $Foxp3^+$ Tregs response was dominating. Moreover, with depletion of Tregs, IL-6-deficient mice became susceptible to experimental autoimmune encephalomyelitis, suggesting a potential, the IL-6 independent mechanism driving Th17 cell differentiation (Korn et al., 2009; Miossec et al., 2009). More recently, in addition to IL-6 and TGF-β, it was shown that IL-21, a cytokine produced by Th17 cells themselves, provides autocrine amplificatory signal for stimulating Th17 cells (Korn et al., 2007; Liang et al., 2007; Murugaiyan and Saha, 2009; Nurieva et al., 2007; Ramirez-Carrozzi et al., 2011; Rickel et al., 2008; Toy et al., 2006; Yang et al., 2008a). It was suggested that this function of IL-21 is mediated via interferon regulatory factor 4 (Huber et al., 2008).

Nevertheless, none of these cytokines alone can induce or abrogate Th17 differentiation. It is preferably a “cytokine cocktail” in an individual disease setting that is required to intensify its potent-inflammatory response.

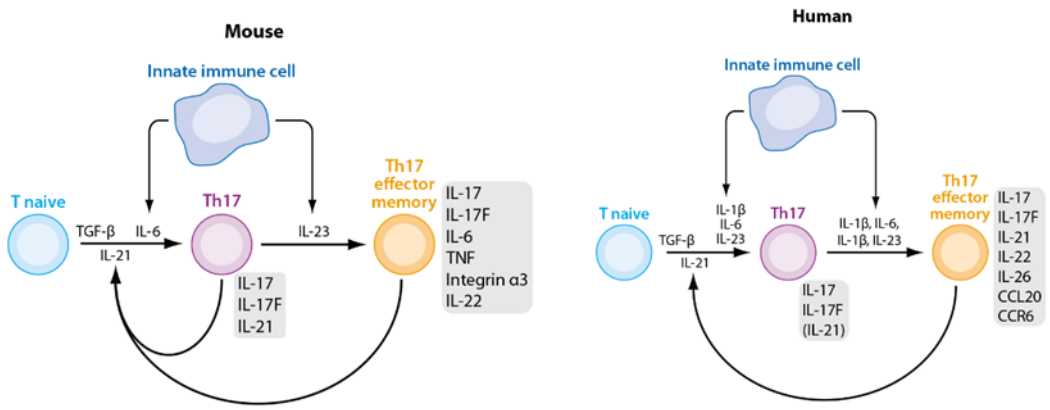

Figure 1.2: Difference in Th17 cell differentiation in mice and humans

Initiating factors (TGF-β, IL-6) help naïve cells to differentiate into Th17 cells, able to produce several cytokines (IL-17A, IL-17F, IL-21. IL-21 can act in an autocrine manner to self-stimulate Th17 cells for further differentiation. Additional stimulation of Th17 cells with mediators (IL-23) contribute to the differentiation of Th17 effector memory cells which can produce pathogenic cytokines and chemokines (IL-17A, IL17F, IL-6, TNF, Integrin α3, IL-22); adapted from (Korn et al., 2009)

Th17 cells have been recognised to play essential roles against bacterial and fungal infections at mucosal and epithelial surfaces (intestine, skin, lung and oral cavity) (Iwakura et al., 2008; Puel et al., 2011). Their requirement in mediating protective immunity relies on the induction of molecules that stimulate epithelial barrier function, like anti-microbial peptides (AMPs), including β-defensins, S100 proteins, and lipocalin-2 (Lcn2) (Yang et al., 1999).

Since their discovery, experiments using animal models of autoimmunity linked Th17 cells with inflammation and autoimmunity. Retinoic acid receptor-related orphan receptor-γτ (ROR γτ), a splice variant of RORγ and human counterpart RORC, is expressed as a second lineage-specific transcription factor (Ivanov et al., 2006, 2007; Manel et al., 2008). It is selectively expressed in *in-vitro*-differentiated T cells and IL-17^{+} T cells present in the lamina propria (Ivanov et al., 2006). Although being a master regulator, RORγτ is not sufficient to drive full Th17 differentiation, and instead acts as a combinatorial player with other transcription factors. Mice, which lack this transcription factor have reduced IL-17A levels. STAT3 preferentially activated by IL-6,

IL-21, and IL-23 (Ivanov et al., 2007; Mathur et al., 2007; Zhou et al., 2007), is required for induction of ROR γτ expression (Harris et al., 2007; Yang et al., 2007). Thus, its deficiency promotes the expression of T-bet and Foxp3 (Yang et al., 2007), while its overexpression increases IL-17A production, RORγt and RORα expressions (Harris et al., 2007; Yang et al., 2007, 2008b). RORα is another member of RORγ family believed to compensate for the absence of RORγτ and STAT3. RORγτ cooperates with several other transcription factors identified to be essential for Th17 differentiation and activation. RORγτ upregulation *in vivo* and upon *in vitro* polarisation with IL-6, TGF-β, require IRF4, with or without IL-1β and IL-23 (Heink et al., 2007; Huber et al., 2008). Mice, which lack IFN4 were resistant to experimental autoimmune encephalomyelitis, and T cells isolated from these mice failed to upregulate RORγτ under Th17 polarising conditions. Additionally, overexpression of RORγτ in IFN4 KO mice was unable to restore IL-17 induction (Brüstle et al., 2007). Studies looking into the contribution of c-Maf reported it as another transcription factor whose transactivation leads to increased IL-21 production (Bauquet et al., 2009b; Iwamoto et al., 2014; Qiu et al., 2017). However, more research needs to be done to support this notion further.

1.1.2.1. Cytokines of the IL-17 family

The production of IL-17 characterises Th17 cells as their signature cytokine. In 1993, interleukin IL-17A (IL-17A), was cloned and initially named as cytotoxic T lymphocyte-associated antigen 8 (CTLA-8) (Rouvier et al., 1993). The bioactivity of human IL-17A was sparked in 1996, alluding that it is responsible for IL-6 and IL-8 production in rheumatoid arthritis synoviocytes and that IL-17A exerts IL-6 function in inflammation and recruit neutrophils through IL-8 (Fossiez, 1996).

IL-17A is a disulfide-linked homodimer glycoprotein consisting of 155 amino acids (Yao et al., 1995a) and exerting part of its actions as a homodimer with a molecular weight of around 35 kDa (Yao et al., 1995b). A total of six proteins that share homology with IL-17A termed IL-17B, C, D, E, and F, have been identified. All members of the IL-17 family show conserved C-termini with five spatially conserved cysteine residues, accounting for a characteristic cysteine-knot structure of IL-17A and F (Hymowitz et al., 2001). Of these, IL-17F shares the highest degree of homology to IL-17A (55 %), and similar cell types produce it (Monin and Gaffen, 2017a). The encoding gene human IL-17F is adjacent to the IL-17A gene and transcribed in the opposite direction. Thus it

may share the same regulatory elements. IL-17E, also known as IL-25, displays the lowest degree of sequence conservation to IL-17A, with 16 % homology (Huang et al., 2015). IL-17A and IL-17F are secret as disulfide-linked homodimers, and heterodimeric IL-17A–IL-17F complexes (Gaffen, 2009). They are produced mainly by Th17 cells, but there are additional cell types that can produce IL-17A and IL-17F like type 3 innate lymphoid cells (ILC3s), natural killer (NK) cells, invariant NK T (iNKT) cells and neutrophils (Cua and Tato, 2010; Stockinger and Omenetti, 2017). Whereas the other IL-17 family members, IL-17B, IL-17C and IL-17D, are produced by non-T cellular sources.

The prevailing paradigm for most cytokines of the IL-17 family is that signalling occurs through heterodimeric receptors composed of a common IL-17RA chain and a second chain that determines ligand or signalling specificity: IL-17RC for IL-17A and IL-17F (Toy et al., 2006), IL-17RB for IL-17E (Rickel et al., 2008) and IL-17RE for IL-17C (Ramirez-Carrozzi et al., 2011). The receptor for IL-17D remains undefined yet (**Figure 1.3**). IL-17A and IL-17F can either exist as IL-17A homodimers and IL- 17F homodimers or as IL-17A-IL-17F heterodimers (Liang et al., 2007). Compared with IL-17A and IL-17F homodimers, the IL-17A and IL-17F heterodimer have moderate biological activity (Chang and Dong, 2007; Liang et al., 2007; Wright et al., 2007). IL-17RA has a 100-fold weaker affinity for IL-17F and even weaker affinity for IL-17B, C, D, and E. On the other hand, IL-17RC has a higher affinity for IL-17F than for IL-17A (Kuestner et al., 2007), which may explain the dominant role of IL-17A in driving autoimmunity (Zrioual et al., 2009). Receptor expression patterns also differ between the IL-17RA and IL-17RC. While the immune compartment, epithelial cells, fibroblasts and bone marrow stromal cells ubiquitously expressed IL-17RA (Gaffen, 2009; Monin and Gaffen, 2017a; Silva, 2003), IL-17RC has restricted expression to non- immune cells, predominantly epithelial cells, kidney and joints (Ishigame et al., 2009a; Kuestner et al., 2007). *Il17ra*-deficient mice are unable to control lung infection with *Klebsiella pneumoniae* (Ye et al., 2001) and exhibit increased susceptibility to cutaneous *S. aureus* infection (Boisson et al., 2013; Chan et al., 2015; Cho et al., 2010; Puel et al., 2011). Additionally, IL-17A is required to control *Candida albicans* infections (Puel et al., 2011; Whibley et al., 2016).

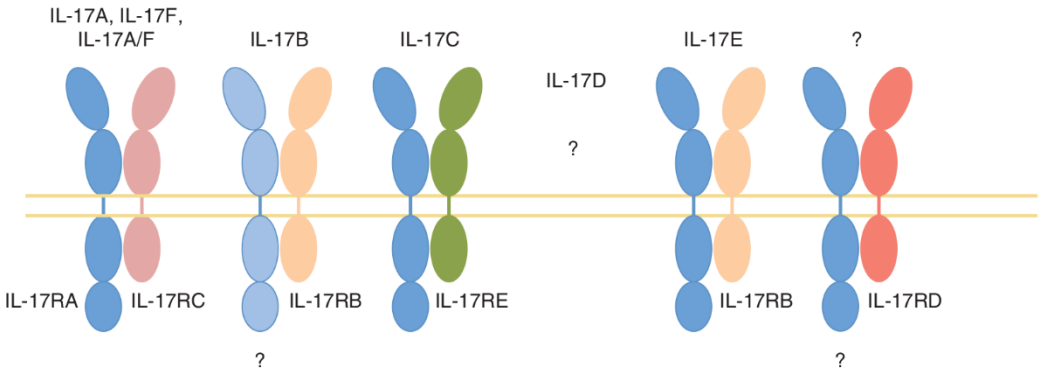

Figure 1.3: Cytokines of the IL-17 family and their receptors

Most IL-17 family cytokines signal via IL-17RA and a second chain that varies between the different member of the IL17A family. Also, the role of IL-17D and its ligands, as wells as the role of IL-17RD remain unknown; modified from Monin and Gaffen, 2017

Except for their role in protection against pathogens, IL17-family cytokines are associated with mediating inflammation in autoimmunity, allergy, and chronic inflammatory conditions. They contribute to the generation of proinflammatory milieu with an enhanced cytokine, chemokine, and matrix metalloproteinase (MMP) production including MMP-1, -2, -3, -8, -9 and -13 (Koenders et al., 2005).

IL-17A homodimers are very efficient in inducing chemokine production by epithelial cells and can expand and maintain populations of these cells at the site of infection. Indeed, chemokines such as CXCL1, CXCL5 and CCL2 stimulate neutrophil chemotaxis, whereas induction of granulocyte colony-stimulating factor (G-CSF) regulates neutrophil production. Also, IL-17A can induce the expression of acute-phase response mediators and other proinflammatory cytokines such as complement and IL-6 (Korn et al., 2009; Miossec et al., 2009). Similarly, CCL20, a key chemokine for recruiting IL-17-producing immune cells, that express the chemokine receptor 6 (CCR6) (Ogura et al., 2008). IL-17A can increase mRNA and protein for the mucins, MUC5AC and MUC5B, in primary human bronchial epithelial cells *in vitro* (Chen et al., 2003). Responses to IL-17A in different cell types vary based on the unique tissue distribution of IL-17 receptors in different organs and the specific cytokine milieu provided by immune cell sources.

More recently, it becomes clear that both IL-17A and IL-17F are produced by different immune cells, putting them between innate and adaptive immunity (Stockinger et al., 2007). Among these cellular sources are $CD4^+$ Th17 cells (Awasthi and Kuchroo, 2009;

Cosmi et al., 2008; Marks et al., 2009; Veldhoen, 2017) and innate tissue-resident cells that are rapidly activated on injury or pathogenic insult including γδ T cells (Cai et al., 2011; Cua and Tato, 2010; Dang et al., 2017; Takatori et al., 2009). In addition to Th17 cells, group 3 innate lymphoid cells (ILC3s), which predominate in the early stages of psoriasis disease (Cua, 2014; Villanova et al., 2014), $CD8^+$ cytotoxic T (Tc) cells (He et al., 2006; Huber et al., 2013),and natural killer T (NKT) cells (Kronenberg, 2005) are known to produce IL-17A and IL-17F. While the evidence for production of IL-17A from mast cells (Huizinga et al., 2010; Noordenbos et al., 2012, 2016) and neutrophils (Lin et al., 2011)(Fontao et al., 2012; Huppler et al., 2015; Katayama et al., 2013) are under debate.

Chronic overproduction of IL-17 contributes to inflammatory conditions and autoimmunity like psoriasis, multiple sclerosis, rheumatoid arthritis, allergic asthma, inflammatory bowel disease, type 1 diabetes and Sjogren syndrome (Awasthi and Kuchroo, 2009; Ciccia et al., 2015; Dong, 2014; Golden et al., 2013; Liu et al., 2011; Ouyang et al., 2012; Pan et al., 2013; Peters et al., 2011; Ye et al., 2013). IL-17A and IL-17F have an effect on a broad range of cells including chondrocytes, osteoblasts, endothelial cells, macrophages, fibroblasts and keratinocytes (Monin and Gaffen, 2017a). Moreover, locally IL-17A and IL-17F induce chemokine production of IL-8 and CCL20, resulting in neutrophil chemotaxis into the skin (Durand et al., 2002; Shahrara et al., 2009).

1.1.2.2. IL-17-related cytokines

Besides, IL-17A and IL-17F, Th17 producing cells can also express IL-22 and IL-21 cytokines. These cytokines are predominantly produced by Th17 cell subset and act in an orchestrated manner mediate to tissue inflammation. IL-23 and aryl hydrocarbon receptor (AhR)-activating ligands influenced the expression of IL-22 (Stange and Veldhoen, 2013; Veldhoen et al., 2009).

IL-22 belongs to the IL-10 cytokine family. Its local overrepresentation in the skin mediates inflammation, promotes keratinocyte hyperplasia and is tissue sentinel against extracellular pathogens. These functions are showing the critical impact of IL-22 at the epithelia and its greatest importance to microbial burden (Dixon et al., 2016). IL-22 can induce the phosphorylation of tyrosine kinases Jak1 and Tyk2, which subsequently initiate the signalling cascade through the activation of STAT3

(Dumoutier et al., 2001; Lejeune et al., 2002). Based on the current knowledge, Th17 cells are the predominant source of IL-22, though Th1 cells and Th22 cells can also produce IL-22 (Eyerich et al., 2009; Liang et al., 2006). Other adaptive lymphocytes that express IL-22 are $CD8^+$ T cells and γδ T cells, as well as cells of the innate immune system like DCs and NK cells (Ouyang, 2010; Rutz et al., 2013; Zenewicz and Flavell, 2011).

IL-21 belong to the IL-2 cytokine family, induced by IL-6. IL-21 can act in an autocrine feedback loop, as self-renewing IL-21 and favour enhancing Th17 cells generation mediated via IRF4 (Nurieva et al., 2007; Zhou et al., 2007). The role of IL-21 is tightly linked with B cell differentiation and is dispensable for the formation of ectopic germinal centres (GCs) and class switching (Bryant et al., 2007). Tfh and Th17 cells, as well as NK cells, preferentially express IL-21.

1.2. Anatomy and organisation of human skin

The skin is the largest human organ of the integumentary system, with a total area of about 1.5-2 square meters. From bottom to top, the skin consists of 3 layers, i.e., epidermis, dermis and subcutis (Kanitakis, 2002).

The epidermis is the uppermost of the skin and acts as a physical barrier, preventing loss of water from the body and entry of substances and organisms into the body. The epidermis is mainly composed of regenerating keratinocytes. Dividing cells in the basal layer (stratum basale) give rise to the spinous layer (stratum spinosum). They move outwards and progressively differentiate to granular layer (stratum granulosum) and stratum corneum. Overall, the epidermis is composed of five layers: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum (**Figure 1.4**).

As the epidermis differentiates, the keratinocytes become flattened, a process in which filaggrins are involved. Migrating keratinocytes to the skin, go through a process of keratinisation and produce the so-called keratins. Keratins comprise the cellular cytoskeleton of keratinocytes, attached to intercellular adhesion complexes also known as the desmosomes (Kolarsick et al., 2011). The antigenic compartment of desmosomes comprises proteins from the Armadillo repeat protein family (e.g. plakoglobin) and the plakin family (e.g. plakophilin, periplakin, envoplakin, and desmoplakin). Altogether, these proteins create the desmosomal plaque and design

an elongated protein network interlinked with intermediate filaments (Garrod, 2010; Garrod and Chidgey, 2008) (**Figure 1.5**).

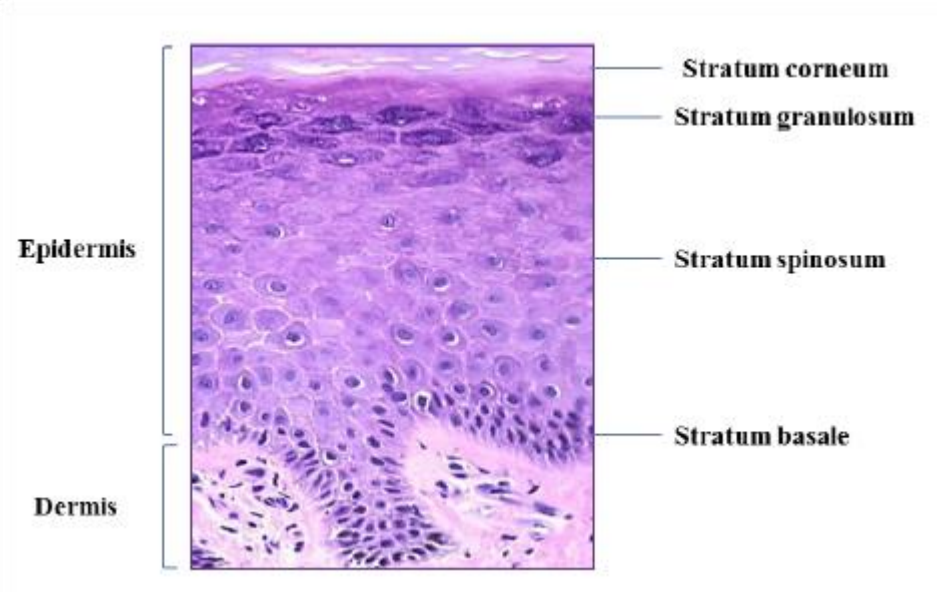

Figure 1.4: Structure of the skin

The epidermis includes stratum corneum, granular layer, spinous layer and basal layer. Basement membrane (dermal-epidermal junction) separate dermis and epidermis. The dermis is consisting of collagen, elastic fibres and many non-collagenous glycoproteins, including fibronectins, fibulins and integrins. In a normal situation, dermis includes mast cells, vascular smooth muscle cells, specialised muscle cells, fibroblast and immune cells like macrophages and lymphocytes

The interface between the lower epidermis and upper dermis includes the dermal-epidermal basement membrane zone (BMZ). Over 30 macromolecules many of which are glycoproteins, interact within the BMZ to form hemidesmosom (Bruckner-Tuderman and Has, 2014; Goletz et al., 2017; Mellerio, 1999). Based on electron microscopy, BMZ is composed of two layers, the basal lamina and underlying layer of reticular connective tissue. The basal lamina structurally contains two different layers, an upper layer called lamina lucida and a lower layer called lamina densa. Lamina lucida is made up of laminin, integrins, entactins and dystroglycans, while lamina densa is built up of a network of reticular collagen IV fibrils. The critical role of this network structure is vital in securing the adherence of the epidermis to the dermis, shown in various blistering diseases of the skin. Beside adhesion, the dermal-epidermal junction (DEJ) components also contribute to cell migration (collagens, laminins and integrins) and signalling events.

Early studies identified five major antigens of the hemidesmosome, divided into three different groups: cytoplasmic plaque proteins, transmembrane proteins, and basement membrane-associated proteins. The cytoplasmic plaque proteins, plectin and 230 kDa BP230, form the inner plaque of the hemidesmosome. The transmembrane proteins

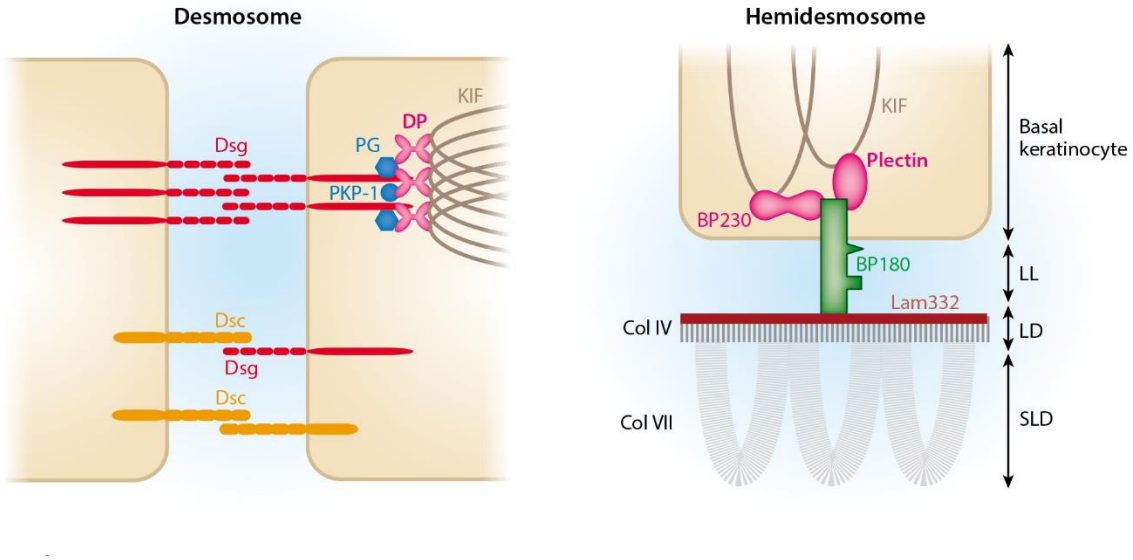

Figure 1.5: Schematics of the desmosome and hemidesmosome at the basement membrane

Desmogleins (Dsgs), the target antibodies for pemphigus and desmocollins (Dscs), belong to the cadherin-adhesion molecule supergene family and mediate cell-cell contact. Intracellular proteins of desmosomes (PG, PKP-1, and DP), link Dsgs and Dscs to KIFs. The hemidesmosome links basal keratinocytes to the epidermal basement membrane zone. BP180 and BP230 are the target molecules in BP patients BP230, and plectin link the hemidesmosome to KIFs. Abbreviations: BP, bullous pemphigoid; Col, collagen; DP, desmoplakin; Dsc, desmocollin; Dsg, desmoglein; KIF, keratin intermediate filament; Lam332, laminin 332; LD, lamina densa; LL, lamina lucida; PG, plakoglobin; PKP-1, plakophilin 1SLD, sublamina (Hammers and Stanley, 2016)

correspond to β4 and α6 integrin subunits and BP180 (type XVII collagen, Col17), a 180 kDa protein, form the outer plaque of hemidesmosomes (**Figure 1.6**).

Keratinocytes and filaggrins comprise 80-90 % of the epidermal cells. Other cells in the epidermis are melanocytes, Langerhans cells and Merkel cells. Melanocytes, located in the stratum basale, are associated with keratinocytes. The primary roles of melanocytes are the production and distribution of melanin pigment. Additionally, they are involved in regulation of the immune system through their ability to phagocytose and to present antigens to T-cells. Langerhans cells are antigen-presenting cells; they process antigens to local lymph nodes, playing a vital role in the adaptive immune

responses of the skin. Merkel cells are found in the basal layer and represent mechanosensory receptors (Rustin, 1990).

Fibrous connective tissue composed of proteins and glycoproteins attached the epidermis and dermis. There are two types of protein fibres: collagen and elastic tissue (elastin). Collagen is the major extracellular matrix protein. In total 29 proteins have been identified in the extracellular matrix (McGrath et al., 2010).

The subcutis, also called subcutaneous tissue, hypodermis or panniculus, is the lowermost layer of the integumentary system, below the dermis and beneath the epidermis. It consists of fatty tissue (adipocytes) and loose connective tissue and connects the skin with underlying structures. It is used mainly for fat storage. An additional type of cells found in subcutis are fibroblasts, macrophages, nerves, and blood vessels.

1.3. Autoimmunity and autoimmune diseases

Under homeostatic conditions in healthy individuals, the immune system generates T and B cells capable of reacting with self-antigens. Usually, these cells are either eliminated before becoming active within the immune system or killed by regulatory cells. Self-reactive immune cells that successfully escape tolerance mechanisms can pose a severe threat to health, as they can induce tissue inflammation and autoimmunity — escaping the self-tolerance results in the formation of autoreactive T and B cells. Autoantibody-producing B cells will mature and differentiate into antibody-secreting plasma cells. However, not necessarily, all autoantibodies are pathogenic. One disease to be defined as an autoimmune disease has to fulfil the criteria known as Witebsky's postulates (Rose and Bona, 1993). To provide direct evidence for transferring pathogenic antibody or T cells, indirect evidence that the disease is reproducible in experimental animals and circumstantial evidence from clinical clues for future research (Rose and Bona, 1993). Conferring National Institute of Environmental Health Sciences, up to date identified more than 80 autoimmune diseases. According to the expression profile of the antigen, self-reactive cells may induce organ-specific or systemic autoimmune diseases (Fairweather et al., 2008; Lis et al., 2012).

1.3.1. Bullous pemphigoid

The autoimmune bullous diseases (AIBDs) represent a group of disorders characterised by autoantibody-mediated responses against desmosomal and hemidesmosomal proteins of the skin and mucous membranes. According to the target antigens, they are characterised as pemphigoid group (hemidesmosomal proteins and proteins located in the BMZ), pemphigus group (desmosomes), and dermatitis herpetiformis (epidermal and tissue-type transglutaminase(Baum et al., 2014; Hammers and Stanley, 2016; Schmidt and Zillikens, 2013)(**Figure 1.6**).
Pemphigoid diseases include several well-distinguished entities indicated in **Table 1.1**

Table 1.1: Pemphigoid diseases and autoantibody specificities (adapted from (Schmidt and Zillikens, 2013))

Disease	Autoantibody targets
Bullous pemphigoid	BP180 NC16A BP230
Mucous membrane pemphigoid	BP180, laminin332 BP230, $\alpha6\beta4$ integrins, laminin 311
Linear IgA disease	LAD-1 BP230, (IgA reactivity)
Pemphigoid gestations	BP180 NC16A BP230
Anti-p200/laminin $\gamma1$ pemphigoid	p200 antigen, laminin $\gamma1$
Epidermolysis bullousa acquisita	Type VII collagen
Bullous systemic lupus erythematosus (SLE)	Type I: type VII collagen Type II: BP180, BP230, laminin 332
Lichen planus pemphigoides	BP180 NC16A BP230
Cicatricial pemphigoid	BP180, BP230 laminin 332

Bullous pemphigoid (BP) is the most frequent among the pemphigoid diseases and belongs to an organ-specific group of autoimmune diseases. Two target antigens have been identified in BP, BP180 and BP230, components of the hemidesmosomes, adhesion complexes promoting epithelial-stromal adhesion in stratified and other

complex epithelia (Baum et al., 2014; Goletz et al., 2017; Kershenovich et al., 2014; Schmidt and Zillikens, 2013; Vassileva et al., 2014).

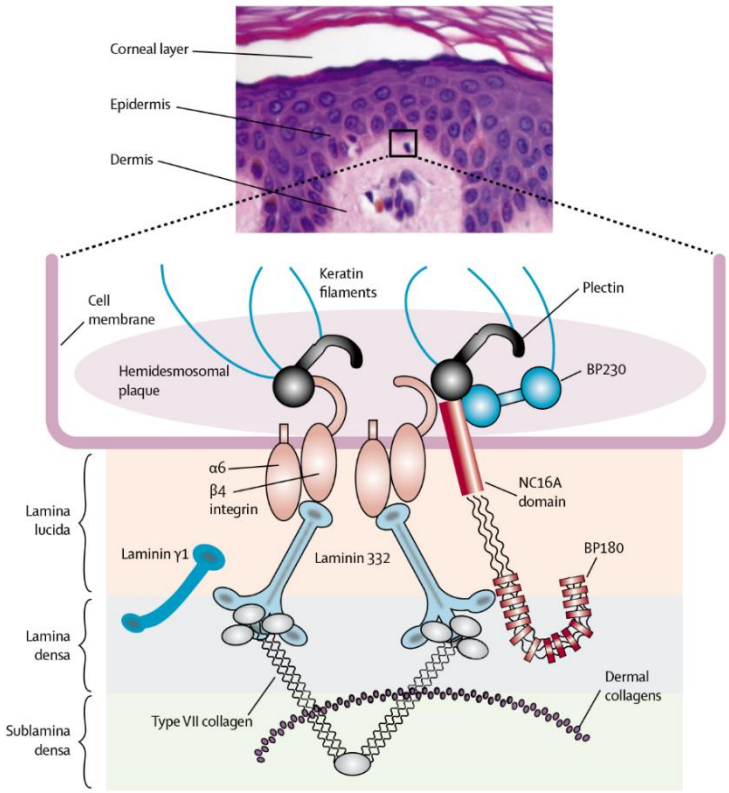

Figure 1.6: Target molecules in pemphigoid diseases

Plectin is a target protein in paraneoplastic pemphigus. BP230 and BP180 are the main proteins for bullous pemphigoid, α6β4 integrin and in laminin 332 are targeted in patients with mucous membrane pemphigoid. Laminin γ1 is described as the main antigen for anti-p200 pemphigoid and type VII collagen is the target in epidermolysis bullosa acquisita; adapted from (Schmidt and Zillikens, 2013).

Clinically, BP presents with tense blisters and erythema, frequently in conjugation with urticarial plaques. In almost all patients, severe pruritus is present (Schmidt and Zillikens, 2013). Trauma, burns, radiotherapy, ultraviolet radiation and vaccination-most frequently against influenza, old age, neurologic diseases (dementia, Parkinson's disease, cerebrovascular disease) are risk factors implicated in disease onset (Venning and Wojnarowska, 2006; Walmsley and Hampton, 2011). About 10-20 % of patients develop mild oral lesions, but other mucosal areas are rarely affected (Schmidt et al., 2012).

BP is a disease of the elderly, with onset usually in the late 70s. BP had the highest prevalence among all AIBDs with about 260 patients/million inhabitants (Hübner et al.,

2016). During the prodromal, non-bullous phase of BP, signs and symptoms are frequently non-specific. Mild to severe pruritus either alone or in association with eczematous, papular and urticarial cutaneous lesions may persist for several weeks or months (Bernard and Antonicelli, 2017; Schmidt and Zillikens, 2013).

Immunologically, BP is associated with a humoral and cellular immune response directed against two well-characterised antigens BP230 and BP180 (Col17). BP230 a member of the plakin family, was the first target antigen described in BP (Stanley et al., 1981). BP230 is placed in the hemidesmosomal inner plaque. Autoantibodies against BP230 are present in 50-60 % of all BP patients (Blöcker et al., 2012) and 25 % of mucous membrane pemphigoid (MMP) patients (Schmidt and Zillikens, 2013). The immunodominant epitopes are located in the globular C-terminal domain (Blöcker et al., 2012). Anti-BP230 IgG serum levels do not correlate with disease activity in patients with BP (Blöcker et al., 2012).

BP180 (BPAG2, Col17), was identified as the primary autoantigen in patients with BP (Diaz et al., 1990; Labib et al., 1986). BP180 is a homotrimeric type II transmembrane glycoprotein localised in the hemidesmosome. It is composed of three collagen α1 chains, each consisting of a globular intracellular N- terminal domain, a short transmembrane stretch and a large extracellular C- terminal domain comprising 15 collagen repeats separated by 16 non- collagenous (NC) subdomains (Goletz et al., 2017) (**Figure 1.7**). The extracellular portion of the 16th non-collagenous (NC16A) domain is the most immunogenic epitope in BP (Giudice et al., 1994; Kobayashi et al., 2002; Zillikens et al., 1997). It spans the lamina lucida, inserts into the lamina densa, than kinking back to the lamina lucida (Masunaga et al., 1997) (**Figure 1.7**).

The cytoplasmic domain of BP180 has been shown to contain multiple binding sites for other hemidesmosomal proteins such as plectin, BP230 and β4 integrin. The extracellular domain interacts with α6 integrin and laminin 332 (van den Bergh et al., 2011; Borradori, 1997; Koster, 2003). Serum levels of BP180 IgG are present in almost all BP patient and correlate with disease activity (van Beek et al., 2017).

Diagnosis of BP relay on a combination of clinical features, direct immunofluorescence microscopy (DIF) of a perilesional skin biopsy, which typically shows linear deposition of IgG and/or complement component 3 (C3) at the BMZ, and the detection of circulating IgG anti-BMZ autoantibodies by either indirect IF microscopy or by enzyme-linked immunosorbent assay (ELISA) via recombinant BP180 NC16A (Schmidt and Zillikens, 2013).

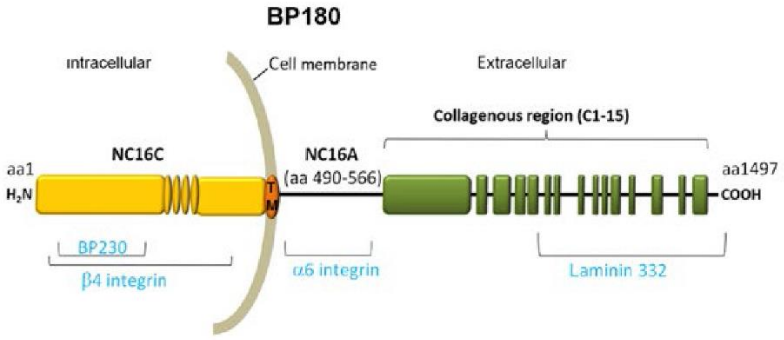

Figure 1.7: Structure of BP180

BP180 comprises of an intracellular N-terminal domain (NC16C), a short transmembrane domain (TM) termed NC16B and an extracellular domain with 15 collagenous repeats separated by 16 non-collagenous (NC) subdomain; adapted from (Goletz et al., 2017)

Pathology of BP can be subdivided between a prodromal nonbullous phase and a bullous phase (Amber et al., 2018; Bernard and Antonicelli, 2017; Schmidt and Zillikens, 2013). The two phases comprise autoimmunity, inflammation, and tissue destruction. Little is known about the autoimmune phase, why and how autoantibodies develop in humans and the events leading to the break of tolerance. Experts in the field believe that the manifestation pattern depends on the autoantibody titers, the predominant IgG subclass, the dominant immunoglobulin isotype (IgG, IgA, or IgE) of autoantibodies, and the recognised epitopes (Amber et al., 2018; Nishie et al., 2016). However, other modifying determinants like epigenetic, gene polymorphisms, defects in structural proteins, signalling molecules, environmental factors, will contribute to the final phenotype. Existing animal models of BP deepened the current understanding of the mechanism to a large extent. By animal work, two pathways are proposed to drive the pathogenesis of BP. The first pathway includes activation of the classical complement pathway at the DEJ after immune complexes have been formed. Rabbit anti-mouse NC14A antibody injected in neonatal mice, bound to the DEJ. Accompanied by complement activation, subsequently followed by mast cell degranulation, attraction and activation of neutrophils, degradation of BP180, and blister formation (Nelson et al., 2006). NC14A is the murine homolog of NC16A. DIF microscopy of BP patients is almost always positive for C3 as well (Schmidt and Zillikens, 2013; Witte et al., 2018). Besides, $C4^{-/-}$ mice were incapable of activating the classical complement pathway, $C5^{-/-}$ mice were also resistant to the pathogenic BP180 IgG (Heimbach et al., 2011; Kushner and Payne, 2018; Nelson et al., 2006; Sadik et

al., 2018). Mast cells degranulation attracts neutrophils to the BMZ and initiates the production of specific proteases such as neutrophil elastase and matrix metalloproteinase-9 (Heimbach et al., 2011; Le Jan et al., 2014; Lin et al., 2012a) In FcγRIII$^{-/-}$ mice, neutrophil activation is dependent on the binding of FcγRIII by the Fc domain of anti-Col17 (Zhao et al., 2006). Using several FcγR-deficient mouse strains, Schultze and colleagues showed that tissue destruction is mediated by FcγRIV, FcγRIII, and FcγRIIB, while FcγRI was not essential. In another study was shown that mAb against the C-terminus of Col17 in combination with Fc-binding proteins induced skin fragility in mice and BP180 depletion in keratinocytes (Iwata et al., 2018). Moreover, in a humanised mouse model of BP, anti- Col17 F(ab')2 fragments successfully blocked the binding of anti-Col17 IgG, preventing disease progression (Wang et al., 2010). Lastly, neutrophils elastase cleaves Col17, and fragments of Col17 were shown to act as a neutrophil chemoattractants (Lin et al., 2012b).

The second pathway was shown to be complement independent. In an elegant set of mouse studies were shown that non-complement dependent pathway lead to a depletion of Col17, facilitated by protein kinase C-regulated micropinocytosis (Karsten et al., 2018; Nelson et al., 2006; Ujiie et al., 2014). However, with or without complement dependency, effects of anti-Col17 IgG have been described in internalisation and depletion of BP180, as well as secretion of IL-6 and IL-8 from basal keratinocytes (Messingham et al., 2011; Nishie, 2014; Schmidt et al., 2000). Several studies have been performed and are still ongoing, to investigate the importance of different cell types and to identify more specific targets. Most of the research is focused on the effector phase of the disease and the role of neutrophils, mast cells, and eosinophils. These cells are orchestrating degradation of the anchoring complex, thus, disturbing the adhesion between dermis and epidermis eliciting subepidermal clefting, skin blisters and erosions (Hirose and Kasprick, 2017a; Le Jan et al., 2014; Samavedam et al., 2013, 2014a). Together with the activation of the complement system different chemokines and cytokines are produced, like TNFα, IL-1β, IL-17A and more (Chakievska et al., 2019; Le Jan et al., 2014; Ludwig and Schmidt, 2009; Plée et al., 2015; Schmidt and Zillikens, 2013; Zebrowska et al., 2013) The hypothetical pathomechanism leading to blister formation, include several well-orchestrated events (**Figure 1.8**).

As mentioned before, animal models are a helpful and indispensable tool for improving our understanding of the pathogenesis of each difficult-to-treat disease. Particularly in

BP, several mouse models have been described. Mouse models for BP constitute antibody-transfer to experimental animals, the adoptive transfer of autoantibody-producing B cells to immune-deficient mice and the construction of transgenic mice that produce autoantibodies. Antibody transfer-induced models of BP was first described in 1993 by Lui and colleagues. Injection of rabbit anti-mouse NC14A IgG into neonatal mice led to binding at DEJ accompanied by complement activation, mast cell degradation, and the recruitment of neutrophils and epidermal detachment (Liu et al., 1993). Later on, description of an adult mouse model of BP reflects a closer observation of the pathogenesis of BP, since this disease is characteristic for older adults (Chiriac et al., 2013; Oswald et al., 2012; Schulze et al., 2014). These models require frequent injections of rabbit anti-mCol17 s.c. or i.p. which results in the formation of skin erosions, crusts, erythema, and hair loss. Transgenic mouse models have been established to overcome the differences in the homology of the human NC16A and the mouse homolog. Col17-humanized mice, (Col17 $m^{-/-}$, h+) (Nishie et al., 2007) and partially Col17-humanized mice (Diaz et al., 2008) which showed dermal separation via mechanical friction. Immunisation-induced models of BPare based on immunisation with NC14A domain polypeptides. The difference between the antibody-transfer mouse model of BP is that circulating autoantibodies are persistently detected. A previous study showed that the immunisation of female SJL/J mice via mouse NC14A domain polypeptides (which correspond to the NC16A domain of hCOL17) could break the tolerance to mCOL17 in immunised mice, thereby showing similar immunopathological features like human BP (Hirose et al., 2011). Usage of the animal models, in addition to the understanding of the undergoing pathomechanisms, advances our possibilities to test more specific and safe therapeutic options for BP.

Treatment for BP relays on topical or systemic corticosteroids and immunosuppressive drugs including dapsone, doxycycline, methotrexate and azathioprine (Schmidt and Zillikens, 2013; Sticherling et al., 2017; Williams et al., 2017) Nearly 30 % of the patients with BP relapse during the first year of treatment (Fichel et al., 2014; Joly et al., 2002a, 2009; Kyriakis et al., 1999). Moreover, nearly 50% of the patients relapse after termination of the treatment (Bernard et al., 2009). The high age and the frequent adverse effects contribute to the quality of life and increased mortality in BP patients (Bernard et al., 2009). The high age and the frequent adverse effects contribute to the quality of life and increased mortality in BP patients. Only a few randomised controlled clinical trials have been performed in BP, but so far, no safe and efficient treatment has

been established. Therefore, this thesis will focus on skin inflammation after the binding of autoantibodies to the BMZ and the relevance of IL-17A and related cytokines in the pathogenesis of BP.

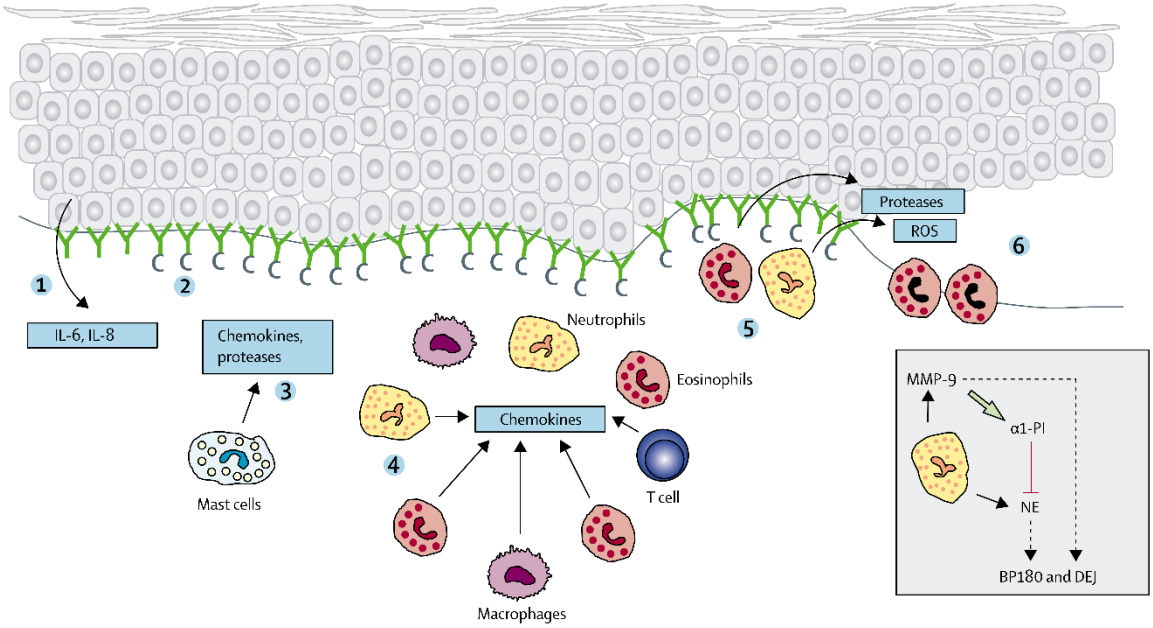

Figure 1.8: Proposed pathomechanism leading to blister formation in bullous pemphigoid

Binding of the immune complex initiates the release of interleukin 6 (IL-6) and IL-8 from basal keratinocytes (1). Complement is activated (2) at the dermal-epidermal junction (DEJ) (3).and inflammatory cells infiltrate into the upper dermis (4). Secretion of inflammatory mediators like proteases (insert) and reactive oxygen species (ROS) (5) induce dermal-epidermal splitting (6); adapted from (Schmidt and Zillikens, 2013).

A closer look at the pathogenic landscape of BP, show that proinflammatory cytokines and chemokines have a tremendous impact on disease development. So far, several potential therapeutic targets have been investigated in experimental animal models of pemphigoid diseases including TNFα, IL-1β, IL-6 and GM-CSF (Hirose and Kasprick, 2017b; Samavedam et al., 2013, 2014b), which showed little or no effect on the disease severity. Thus, there is an urgent requirement of further studies that identify critical molecules in the pathogenesis of BP, able to be pharmaceutically targeted.
Recent data highlighted the potential pathogenic role of IL-17A in BP (Arakawa et al., 2011; Le Jan et al., 2014; Plée et al., 2015). The authors showed elevated serum levels of IL-17A and its contribution to the overexpression of proteases. Also, high numbers of IL-17A-positive cells were observed in skin lesions of BP patients compared to the

skin of healthy volunteers (Arakawa et al., 2011). Recently, neutrophils have been shown to be a significant source of IL-17A in the lesional skin of BP patients (Le Jan et al., 2014). However, more data about the functional relevance of IL-17A in BP are needed. Investigation of the mechanisms leading to IL-17A production and its link with blister formation are still scarce in BP.

1.4. Aims of the study

Based on the available data, we hypothesised that IL-17A has a functional relevance in the pathogenesis of BP. The thesis aims to describe the role of IL-17A and IL-17-related cytokines in BP patients and explore the functional relevance of IL-17 *in vivo*.

Therefore, the following research questions will be addressed:

- Identify the cellular source of IL-17A in the peripheral blood of BP patients
- Unravel the leading producers of IL-17A in early BP skin lesions
- Analyse the expression of *IL17A* and related genes in early BP skin lesions
- Uncover the role of IL-17A in *in vitro* models of neutrophil activation
- Explore the functional relevance of IL-17A in the antibody transfer-induced mouse model of BP in adult mice by using IL-17A$^{-/-}$, and IL-17A/F$^{-/-}$ mice
- Elucidate pharmacological inhibition of IL-17A in both, a prophylactic and therapeutic setting in the antibody transfer-induced mouse model of BP

2. Materials and methods

2.1. Experiments with human material

In this study, 15 BP patients and 12 sex- and age-matched control patients were used for analysis of whole blood by flow cytometry, serum analysis by ELISA and multiplex assay. Patient material was collected between 2015-2017. 7 BP patients were men, and 8 were women, with a mean age of 73.5 years, ranging from 53 to 94 years.

The diagnosis of BP patients was made by histological subepidermal blisters, linear IgG deposition at the BMZ, and detection of circulating autoantibodies against BP180 or BP230 by ELISA (Euroimmun, Lübeck, Germany). The control group patients predominantly consist of non-inflammatory dermatoses and non-melanoma skin cancer. Samples (serum, whole blood and biopsies) were collected at the time of diagnosis before treatment was initiated. Whole blood samples were analysed immediately the same day, serum samples we stored within 30 min after collection at -80ºC, skin samples were frozen directly after collection and kept at -80ºC till analysed. Perilesional skin samples from seven BP patients and seven control patients with non-inflammatory dermatoses were used for quantitative PCR analysis. Additional 7 BP patients skin samples, without split formation by histopathology, were used for IF studies. Experiments with human material were performed by the Declaration of Helsinki and were approved by the ethics committee of the University of Lübeck (12-178, 15-051, 09-140). All study subjects were informed and gave written informed consent before inclusion in the study. The study was conducted in the Lübeck Institute of Experimental Dermatology (LIED), University of Lübeck.

2.2. Experiments with animals

C57Bl/6J WT mice were purchased from The Jackson Laboratory (Bar Harbour, USA). IL17A$^{-/-}$ mice (Il17a^{tm1Yiw}) (Nakae et al., 2002) were kindly provided by Prof. Yoichiro Iwakura (Tokyo University of Science, Tokyo, Japan) and bred at the experimental animal facility of the Christian-Albrechts-University Kiel. IL-17A/F$^{-/-}$ mice (C57BL/6-Il17AF$^{tmPrinz /J}$) (Haas et al., 2012; Ishigame et al., 2009a), were bred in an animal facility in Lübeck. IL-17A/F$^{-/-}$ were generously provided by Dr. rer. nat. Christoph

Hölscher, Research Center Borstel, Germany. IL-17A$^{-/-}$ mice and IL-17A/F$^{-/-}$ were kept on C57Bl/6 background. Animals used in the experiments were 6-12 weeks old. Mice were maintained on a 12-hour light-dark cycle under specific, pathogenic free conditions and fed acidified drinking water and standard chow ad libidum. All protocols were approved by local authorities of the Animal Care and Use Committee of Schleswig-Holstein, ("Pathogenesis and therapeutic modulation of experimental bullous pemphigoid (BP) in the passive transfer model of adult mice", Tierversuchsvorhaben-Nummer: 21-2/11, 40-3/15) and performed by certified personnel.

IL17A$^{-/-}$ mice were previously generated by replacing exon 1 and 2 with a cassette containing an EGFP gene followed by a floxed neomycin resistance gene, **Figure 2.1** (Nakae et al., 2002). IL-17A$^{-/-}$ mice did not show any obvious phenotypic abnormalities.

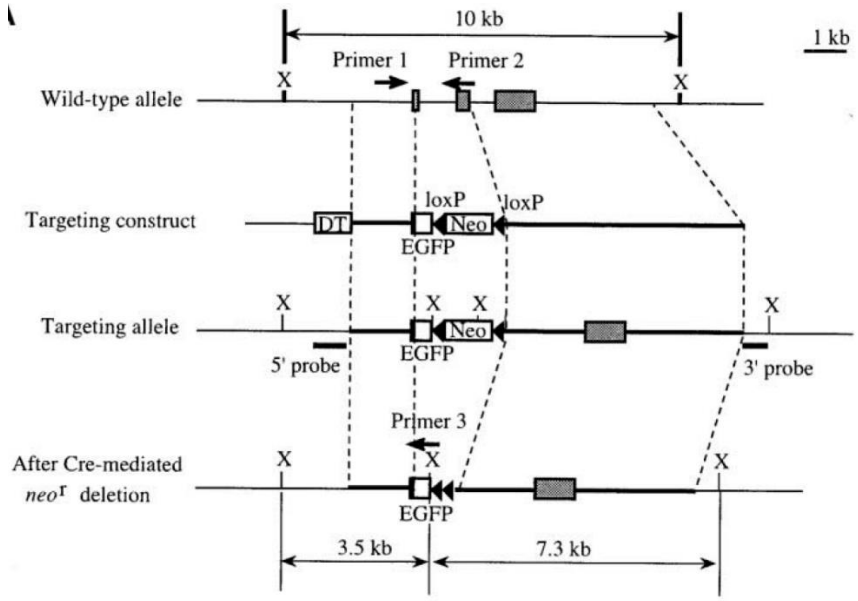

Figure 2.1: Generation of IL-17$^{-/-}$ mice

Structure of the mouse *il-17* locus (Wildtype allele), the IL-17 targeting construct, targeting allele and after neomycin resistance gene (neo^r) deletion. Exon 1 and 2 of the il-17 gene were replaced with the EGFP gene and the neo^r gene flanked by lox P sequences; adapted from (Nakae et al., 2002, Haas et al., 2012).

IL-17A/F$^{-/-}$ mice had been previously generated by substituting exons 2 and 3 of the *Il17f* gene with the hygromycin B phosphotransferase (*hph*) resistance gene using *Il17a*$^{+/-}$ embryonic stem cells, **Figure 2.2** (Ishigame et al., 2009a).

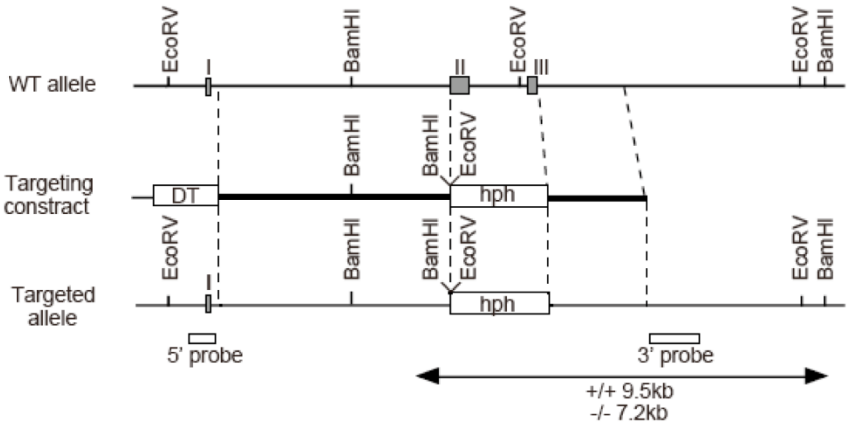

Figure 2.2: Generation of *Il17a*$^{-/-}$*Il17f*$^{-/-}$ (Il17a/f$^{-/-}$) mice

Il17f$^{-/-}$ mice and *Il17af*$^{-/-}$ mice were generated by replacing exons 2 and 3 of the Il17f gene. *Il17f* locus structure of the mouse (WT allele), the IL-17F-targeting construct targeted allele. Substitution of exons 2 and 3 of the *Il17f* gene with the *hph* resistance gene; adapted from (Ishigame et al., 2009)

2.1. Antibody transfer-induced mouse model of bullous pemphigoid

Induction of the experimental antibody transfer-induced mouse model of BP was performed according to a previously described protocol (Schulze et al., 2014). Briefly, mice received six subcutaneous injections (s.c.) of 10mg affinity-purified rabbit anti-mCol17-IgG IgG every second day over 12 days unless indicated otherwise. Clinical examination of mice was performed under anaesthesia with intraperitoneal (i.p.) injection of ketamine (70-80mg/kg, Sigma-Aldrich, Hamburg) and xylazine (7-10mg/kg, Sigma-Aldrich, Hamburg).

2.1.1. Evaluation of disease severity in the antibody transfer-induced bullous pemphigoid mouse model

The extent of cutaneous disease was presented as a percentage of body surface area by the lesions on the skin surface as described before for the antibody transfer mouse model of EBA (Sitaru et al., 2005). The affected body surface area was calculated by allotting individual percentile fraction to each part of the body based on the relative size of the body (**Table 2.1**). The area under the curve (AUC) was calculated using scores of different time points during the experiment. AUC denotes

the disease severity through the experiment. For calculation of AUC, the following formula was used:

$$: AUC = \frac{1}{2} * \left(\frac{\%\ ABSA;t_1}{t_v}\right) + \left(\frac{\%\ ABSA;t_2}{t_v}\right) + \cdots \left(\frac{\%\ ABSA;t_{v\text{-}1}}{t_v}\right) + \frac{1}{2} * \left(\frac{\%\ ABSA;t_v}{t_v}\right)$$

(ABST= affected body surface area, t=time, t_1=time point 1, and t_v=end point time).

Time-course disease progression index was calculated by the graph slope (disease score vs time) using the following formula:

$$Slope = \frac{\%\ ABSA;t_v - \%\ ABSA;t_{v\text{-}1}}{t_v - t_{v\text{-}1}}$$

Table 2.1: Scoring table used to calculate the affected body surface area

Body parts	Ear (left)	Ear (right)	Eye (right)	Eye (left)	Snout	oral mucosa	head&neck	front legs	rear legs	tail	trunk	total
%	2.5	2.5	0.5	0.5	2.5	2.5	9	5	10	10	40	100

2.1.2. Generation and characterisation of rabbit antibodies to mCol17

2.1.2.1. Expression and purification of mCol17

The extracellular portion of the NC15A of murine collagen type XVII (mCol17) covers 76 amino acids (amino acids 497 to 573 of murine BP180, **Figure 2.3**). This recombinant fragment was expressed as glutathione-S-transferase (GST) fusion protein and purified by affinity chromatography, as previously described (Hirose et al., 2011). NC15A is a murine homolog of the NC16A domain in humans. Expression and purification of the protein were performed as already described (Hirose et al., 2011; Sitaru et al., 2005). Briefly, a colony with the vector pGEX-mCol17A transformed *Escherichia coli* (Rozzeta DE3), was cultured in 1L LB medium containing 100 mg carbenicillin. The culture was incubated at 37ºC in a shaking incubator (220 revolutions/minute). Expression was induced by adding 100mM IPTG. After 3h incubation, bacteria were cooled down on the ice and centrifuged at 4,000xg at 4ºC for 30 min. A sample of 1mL was taken before and after induction with IPTG and stored at -20º C for validation of expression by SDS-PAGE. The obtained pellet was

resuspended on ice in 13 mL solution buffer, **Table 2.2**) and sonicated on ice three times (50 % power, level 4-5, 30 pulses). In this step, the protein was released from included in inclusion bodies.

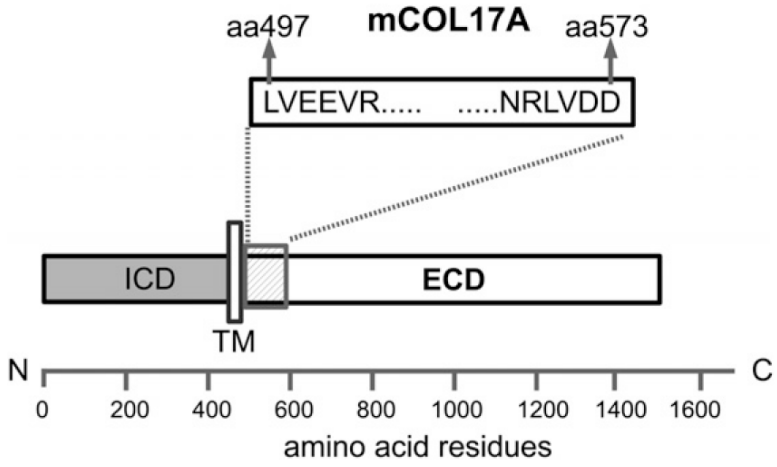

Figure 2.3: Schematic organisation of murine BP180 constructs generated for expressing recombinant peptides of the NC15 domain

BP180 (mCOL17A) cDNA has been cloned into pGEX-6P-1 and expressed in E. coli. ICD, intracellular domain; ECD, extracellular domain; adapted from (Hirose et al., 2011)

After sonication, 100 μL lysozyme, 10 µL benzoase, and 50 µL $MgCl_2$ were added and mixed well. Then 12.4mL of lysis buffer was added, mixed and incubated for 30-60 min at RT on a shaker. In this step, the cell wall was enzymatically broken down to improve protein extraction efficiency. After 350 μL NaEDTA was added, the tube was immersed in liquid nitrogen until no more bubbles were produced. The frozen lysate was next incubated for 30 min at 37ºC. Later, 200 μL $MgCl_2$ was added, and the sample was incubated for 30-60 min until viscosity decreased. Subsequently, 350 µL of NaEDTA were added, the sample was centrifuged 20 min 11,000xg at 4ºC. Supernatant #1 was taken and frozen at -20ºC. The outstanding pellet was resuspended in 10 mL washing buffer with Triton and sonicated on ice. The sample was centrifuged 20 min 11,000xg at 4ºC, and the supernatant was stored as a #2.at -20ºC The pellet was resuspended one more time in washing buffer without Triton, sonicated, and centrifuged 20 min 11,000xg at 4ºC. Supernatant #3 was frozen, and the pellet was further dissolved in 9 mL 8 M guanidine buffer, pH 8, containing 4 mM DTT. The pellet was frozen at -80ºC.

Table 2.2: Buffers for expression and purification of mCol17

Buffer	Composition
Solution buffer, pH 8.0	50mM Tris-HCl, 25% saccharose, 1mM NaEDTA, 0.1% NaN3, 10mM DTT
Lysis buffer, pH 8.0	50mM Tris-HCl, 1% Triton X-100, 1% Na-deoxycholate, 100mM NaCl, 0,1 NaN3, 10mM DTT
Washing buffer with Triton X-100, pH 8.0	50mM Tris-HCl, 0.5% Triton X-100, 100mM NaCl, 1mM NaEDTA, 0.1% NaN3, 1mM DTT
Washing buffer Triton X-100, pH 8.0	50mM Tris-HCl, 100mM NaCl, 1mM NaEDTA, 0.1% NaN3, 1mM DTT
Lysozym	50mg/mL in aqua dest
MgCl2-solution	0.5M MgCl2 in aqua dest
NaEDTA-buffer, pH 8.0	0.5M NaEDTA in 50mM Tris-HCl
Guanidium + DTT, pH 8.0	8M Guanidium, 4mM DTT in aqua dest

2.1.2.2. Refolding of mCol17 and affinity purification with glutathione-agarose column

After purification, the protein is in an unfolded form, therefore needs to be refolded to its three-dimensional structure. For this purpose, 1mL of the dissolved pellet in 8M guanidium buffer was added dropwise in refolding buffer three times (**Table 2.3**). The protein containing extract was stirred slowly on 4º C for 8 hours. The buffer was later changed to PBS through dialysis, three times for 12 hours against 5 µL 1x PBS.

Table 2.3: Buffers for refolding and affinity purification of mCol17

Buffer	Composition
Refolding buffer	100mM Tris-HCl, 400mM L-Arginine, 2mM NaEDTA, 0.5mM oxidized glutathione, 5mM reduced glutathione, 50µL protease inhibitor cocktail
Elution buffer, pH 8.0	924mg reduced glutathione in 150mL PBS
Boratbuffer, pH 8.5	0.1M Borat acid, 0.5M NaCl in aqua dest
Acetic acid buffer, pH 4.5	0.1M Acetic acid, 0.5M NaCl in aqua dest
PBS, pH 7.2	9g NaCl, 1.74g Na2HPO4 *2H2O, 0.18g NaH2PO4 *H2O in 1L aqua dest

In the final step of preparation, the dialysed protein extract was affinity purified using a glutathione-agarose (GST) column. The column was in the first step equilibrate with PBS, and afterwards, the sample was added. Flow through was collected, and load again on the column. Next, the column was cleaned with PBS and elution step was performed with 100mL of elution buffer. The column was further washed with elution

buffer and subsequently with 50mL borate buffer containing NaCl, PBS, acetic acid buffer with NaCl, and one more time with PBS. The column was stored in PBS with 1mM NaN_3 to avoid contamination.

2.1.2.3. Validation of mCol17 by SDS-PAGE

The purity and the amount of purified protein were confirmed by SDS-PAGE electrophoresis was performed. 20 μL of samples were mixed with 5x loading buffer and cooked for 5 min at 95º C for denaturation of the protein. Samples were applied on 15 % separating gel (**Table 2.4**). In addition to the samples, BSA in known concentrations (2 mg/ml, 1,5 mg/ml, 1 mg/ml, and 0,5 mg/ml) was also added in order to estimate the concentration of our protein. SDS-PAGE was run at 120 V, 25 mA until the end of the gel. As marker Spectra Multicolour Broad Range Protein Ladder (eBioscience, Santa Clara, California, USA.) was used. The gel later was stained with Coomassie blue solution (**Table 2.5**).

Table 2.4: Separating and stacking gel

Separating gel (15%)	Stacking gel (4%)
3mL aqua dest.	2.35mL aqua dest.
1.25mL 0,5M Tris-buffer, pH 6.8	2.5mL 1.5M Tris-buffer, pH8.8
670µL 30% Acrylamide	5mL 30% Acrylamide
50µL 10% SDS	100µL 10% SDS
12µL TEMED	12µL TEMED
70µL 10% APS	80µL 10% APS

Table 2.5: Solutions and buffers for SDS-PAGE electrophoresis

Solution/Buffer	Composition
Tris-buffer, pH 8.8	1.5M Tris-base in aqua dest
Tris-buffer, pH 6.8	0.5M Tris-base in aqua dest
Loading buffer	15g Tris-base, 72g glycin, 5g SDS in 1L aqua dest
5x SDS loading buffer	25mL 1.5M Tris buffer, 12g SDS, 60mL glycerol, 13mL aqua dest, 2mL 0.2% bromophenol blue solution, 37,3mL β-mercaptoethanol
Sensitive Coomassie-staining solution	50g Aluminiumsulfat(14-18)-hydrat, 100mL ethanol, 0.2g coomassie brilliant blue G250, 20mL phosphoric acid in 1L aqua dest
10% SDS	10% SDS in aqua dest
10% APS	10% Ammoniumpersulfat in aqua dest

The recombinant GST-tagged NC15A domain of mCol17 was injected in New Zealand white rabbits. Sera from the rabbits was received in regular intervals and further processed for affinity purification of total IgG. Immunisation and bleeding were performed by Eurogentec (Seraing, Belgium).

2.1.3. Affinity column purification and titration of anti-mColX17 IgG

Affinity chromatography is a well-known method for separating biochemical mixtures based on a highly specific interaction between antigen and antibody, protein and ligand, coupled to a chromatography matrix. The coupled target protein is later recovered by changing the pH, ionic strength or polarity. For purification of anti-mCol17 total IgG, protein G agarose was used. First, the column was equilibrated with binding buffer 2x followed by glycine buffer 1x, NaCl 1x and 2x binding buffer. Then, the rabbit serum was incubated for 1 hour at 4º C on a shaker. The flow-through was discarded, and the column was washed with binding buffer until concertation of 0.05 absorption. Elution was performed with 0.1M glycine buffer. 5-7 mL of neutralising Tris buffer was added in the elution tube to avoid destruction of the IgG. Eluates were collected until the OD was less than 0.1. Collected IgG was further concentrated, and the buffer was changed to PBS. The column was washed with 1x glycine buffer, 1x NaCl and 2x binding buffer. For preventing contamination, the column was stored with 20 % ethanol in binding buffer (**Table 2.6**). The binding of the anti-mCol17 IgG was tested by immunofluorescence and ELISA.

Table 2.6: Buffers for IgG purification by protein G affinity chromatography

Buffer	Composition
Binding buffer, pH7.0	20mM Na_2HPO_4, 150mM NaCl in aqua dest
Glycine buffer, pH 2.8	0.1 M Glycine in aqua dest
NaCl	1M NaCl in aqua dest
Tris-Bbse-buffer, pH 9.0	1M Tris-base in aqua dest

2.3. Skin microscopy

2.3.1. Indirect Immunofluorescence microscopy (IIF)

IIF microscopy was used to determine the titers of anti-BMZ reactivity in sera of rabbits immunised with mCol17. Sections of tail from healthy mouse were incubated with different dilution of rabbit sera (1:100; 1:5, 000; 1:10, 000; 1:20,000; 1:40,000; 1:80,000; 1:160,000; 1:320,000, and 1:640,000) for 1h at RT. Samples were washed with 1x PBS 2 x 10 min and subsequently incubated with polyclonal donkey anti-rabbit-FITC conjugated antibody (Jackson ImmunoResearch, Cambridgeshire, UK) at a dilution of 1:100 for 1h RT. Slides were washed 3 x 5 min with 1x PBS, dried and mounted with mowiol® 4-88 (Carl Roth, Karlsruhe, Germany). Slides were finally read using a EUROStat Microscope (Euroimmun).

2.3.2. Direct immunofluorescence microscopy (DIF)

DIF was used to evaluate the deposition of IgG and C3 in mice injected with pathogenic anti-mCol17 IgG. Mouse-ear and perilesional skin embedded in Tissue-Tek® Cryomold (Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherland) were cut with Cryostat, Leica CM 3050S (Leica Mikrosystem, Wetzlar, Germany) into 6 μm sections and stained with donkey anti-mouse IgG-FITC (Dianova, Hamburg, Germany), and goat IgG fraction to mouse complement C3 (MPI, Solon, Ohio, USA) respectively. Sections were incubated for 1h at RT, washed 3x 5 min with 1x PBS and covered with mowiol® 4-88 (Carl Roth). For visualisation, the Light/Fluorescence Microscope BZ-9000 (Keyence, Neu-Isenburg, Germany) was employed.

2.3.3. Immunopathology (H&E staining)

Tissue sections embedded in paraffin were stained with haematoxylin and eosin according to a standard protocol. Briefly, sections were fixed in formalin for 5 min. Formalin was removed from the sections by washing with tap H_2O. Then sections were rinsed in Haematoxylin for 4 min and washed again with tap H_2O. The sections were subsequently rinsed for 20 seconds in acetic acid (1%) followed by washing with tap H_2O. Next, sections were cleaned for 10 seconds in ammonium water (0.2%) and then

washed with dH_2O. In the next step, sections were incubated for 1 min in eosin and without washing the sections were dehydrated in 95% ethanol, 100% ethanol and 2 changes of xylene, 5 minutes each. Slides were covered with coverslip slides using Micromount xylene-based mounting medium (Leica Mikrosystem). Finally, slides were analysed by Light/Fluorescence Microscope BZ-9000 (Keyence).

2.3.4. Immunofluorescence microscopy (IF)

IF for infiltrating cells was applied on paraffin-embedded 6 µm sections of perilesional skin from BP patients. Sections were deparaffinated at 56°C for 20 min, slowly cooled down and incubated 2x 10 min in fresh xylene. Sections were then rehydrated using an ethanol gradient starting from absolute ethanol, 96 %, 70 % and 50 % each incubated for 5 min and at the end for 1 min in dH_2O. Sections were then washed 3x 5 min in TBS-T. Heat-induced retrieval of the epitopes was performed to demask the epitopes them. By target retrieval buffer pH6 (Dako), the steamer was pre-heated in until 60°C, and slides were added. When the buffer reached 95°C, sections were incubated for 30 min. Slides were cooled down slowly, washed 3 x 5 min in TBS-T and then processed for further staining. Sections were subsequently blocked using 3% BSA/TBS-T (**Table 2.7**) for 1h, then simultaneous staining was performed with monoclonal mouse anti-human mast cells tryptase (0.34 mg/L), polyclonal rabbit anti-human myeloperoxidase (22 mg/L), monoclonal mouse anti-human CD3 antibodies (2.76 mg/L), monoclonal mouse anti-human CD68 (0.3 mg/L, all Dako), rat monoclonal anti-CD4 antibody Daylight 488 (0.025 mg/L, Novus Bio, Colorado, USA) diluted in blocking buffer and incubated overnight at 4°C. IgG1 kappa (1:200; Biolegend, San Diego, USA) served as isotype control. Details about the antibodies used in IF staining are given in **Appendix (Table 7.3)**. As secondary antibodies, polyclonal donkey anti-mouse IgG (1:200, Abcam, Cambridge, UK) and polyclonal chicken anti-rabbit IgG (1:200, Invitrogen, Carlsbad, California, USA) were incubated for 1h at RT and afterwards, sections were stained with polyclonal goat anti-human IL-17A (1:400, R&D Systems, Minneapolis, Minnesota, USA) or mouse monoclonal anti-human IL-17A antibody-FITC conjugated (0.25 mg/mL, eBioecience, Santa Clara, California) overnight at 4°C. Next day, polyclonal goat anti-human IL-17 was additionally conjugated with AlexaFluor488 AffinityiPure F(ab)'2 fragment donkey anti-goat IgG (H+L) and incubated for 1h at RT. Sections were washed, covered with

Fluoromount-G® (SouthernBiotech, Birmingham, Alabama, USA) and analysed. Staining was controlled with isotype controls, and additionally by omitting the secondary antibody control. Slides were analysed by Light/Fluorescence Microscope BZ-9000 (Keyence) and Olympus BX40 (Olympus, Tokyo, Japan), confocal microscope.

Table 2.7: Buffers used in immunofluorescence microscopy analysis

Buffer	Composition
TBS-T	100 mL of TBS 10X, 900 mL of distilled water, 1 mL tween 20
3% BSA/TBS-T	3g in 100mL TBS-T
mowiol	6.0g Glycerol, 2.4g mowiol 4-88, 6.0ml aqua dest, 12ml 0.2M Tris-HCl-solution

2.3.5. Quantification of immunostained cells

Quantification of immunostained cells was performed manually using a counting grid as described by Bologna-Molina and colleagues (Bologna-Molina et al., 2011) in combination with ImageJ. Briefly, pictures of 400 x magnification were captured using the Olympus® BX40 microscope system. Five photomicrographs were taken for each sample. Each file was opened using Microsoft Office PowerPointTM (Microsoft Corporation, USA) and also with ImageJ. The 6 x 6 grid was placed over the entire image. Counting procedure is presented in **Figure 2.4**. Finally, the average number of cells per slide was taken. The same procedure was applied for all biopsies from the 7 BP patients. The counting was validated in parallel by ImageJ using the same procedure.

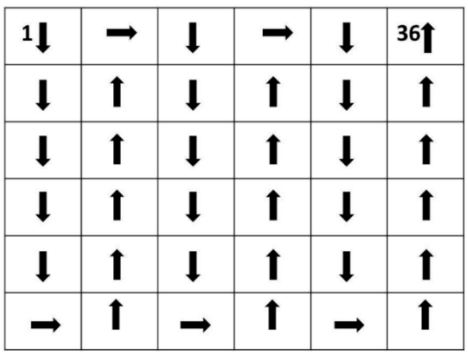

Figure 2.4: Schematic figure for counting immunostained cells

Cell counting started in the top left corner (1) and finished in the top right corner (36)

2.4. Enzyme-linked Immunosorbent Assay (ELISA)

ELISA measured purified anti-mCol17 IgG. The general principle was applied. Shortly, Nunc-Immuno® MaxiSorp® 96-well plates (Nunc, Roskilde, Denmark) were coated with mCol17 antigen at a concentration of 20 μg/mL in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (coating buffer) and incubated overnight at 4ºC. Plates were washed with PBS-T and blocked for 90 min at RT with 1% BSA/PBS-T (**Table 2.8**). After washing with PBS-T plates were incubated with 100 μL mCol17 IgG or sera from mice in different dilutions (1:2-1:1,000) at RT for 1 hour. Plates were washed with PBS-T and appropriate horseradish peroxidase (HRP)-conjugated antibody was applied, at a dilution of 1:1,000 for 1h at RT. Development was performed by adding 100 μL 1-Step Turbo TMB-ELISA solution (Thermo Scientific, Waltham, MA, USA). By adding 100 μL stop solution (0.9 M H_2SO_4), the reaction was terminated, and samples were measured by GlowMax® Discover System microplate reader (Promega, USA). All samples were tested in duplicates. From the mean OD value for each sample, the mean OD value of the blank was subtracted.

Table 2.8: Buffers used in ELISA

Buffer	Composition
Coating buffer	3.7g $NaHCO_3$, 0.64g Na_2CO_3, 1L aqua dest.
PBS-T	100 mL of PBS 10X, 900 mL of distilled water, 1 mL Tween 20
1% BSA/PBS-T	1g in 100mL TBS-T
Stop solution 0.9 M H_2SO_4	53.3 mL sulfuric acid in 1L aqua dest.

2.5. RNA extraction and quantitative reverse-transcription-polymerase chain reaction-based gene expression analysis of skin biopsies

6 mm punch biopsies from perilesional skin of BP patients, and perilesional skin of mice were taken. Total RNA was extracted from frozen skin biopsies using RNAse Mini Kit (Qiagen, Hilde, Germany) according to the manufacturer's protocol. Briefly, 15 mg skin was disrupted and homogenised in 600 µL of lysis RLT buffer supplied with β-mercaptoethanol (β-ME). 600 µL of 70 % ethanol was used to wash out the salts and "soak-out" the water from the nucleic acid. Biomolecules were washed with 700 µL RW1 buffer and traces of salts was further washed with 500 µL RPE buffer twice. RNA

was eluted in 30 µL of RNase-free water. The concentration was determined by NanoPhotometer® NP180 (Implen, München, Germany) under 260/280nm absorbance. Total RNA was stored at -80°C until used.

500 ng of total RNA was reverse-transcribed into complementary DNA using First Strand cDNA Synthesis Kit (Thermo Fisher) following the manufacturer's protocol. Shortly, 500 ng of template RNA and random hexamer primers were used for cDNA conversion in 20 µL reaction. The synthesis was performed by incubation for 5 min at 25°C followed by 60 min at 37°C. The reaction was completed by heating at 70°C for 5 min. The product of the first strand PCR was stored at -80°C until the next step.

RT-PCR was performed using SYBR Green-gene expression assay (Bio-Rad) and custom plate where the primers were dried on the plate (Bio-Rad). 25 ng of cDNA was used for expression analysis in 20 µL reaction. The following program of the thermal cycling protocol was: activation for 30 sec at 95ºC for one cycle; denaturation for 5 sec at 95ºC for 40 cycles; annealing for 30 sec at 60-82ºC with a temperature gradient in different columns for 40 cycles; melting curve at 72ºC for one cycle. Results were calculated using the $2^{\Delta\Delta}$Ct method. Primers used to detect *CCL2, CCL20, CCR6, CD4, CD8a, CSF2, CSF3, CXCL1, CXCL2, ELANE, ICAM1, IFNg, IL10, IL12B, IL12RB1, IL17A, IL17F, IL17RA, IL17RC, IL2, IL21, IL22, IL23A, IL23R, IL27, IL4, IL6, IRF4, KLRB1, RORc, SOCS3,* and *TNF* are detailed in **Appendix (Table 7.1** and **Table 7.2)**.

PCR products were analysed by 1 % gel electrophoresis. The gel was prepared by mixing 1 % agarose (w/v) (Biozym Biotech, Hessisch Oldendorf, Germany) and 0.5 µg/mL ethidium bromide to 0.5 x Tris-Borate-EDTA buffer (Carl Roth). This prepared solution was heated gently before pouring into a pre-made cast. The ladder loading was prepared by adding 1 µL of 180 base pair DNA Ladder (New England Biolabs, Ipswich, USA) with 1 µL 6 x Blue Loading Dye (New England Biolabs) and 4 µL of distilled water. The samples and ladder were loaded onto the agarose gel and ran at 95 volts for 45 min. Images of electrophoresis gels were captured using the transilluminator Imaging System (Vilber Lormat, Collegien, France).

2.6. Cell isolation from skin

In order to isolate the inflammatory cells from mouse skin, lesional skin was taken at the last day of the experiment (day 12), cut into small pieces, and put into liberase medium (2.5 mg/mL, Sigma-Aldrich, Darmstadt, Germany). Samples were

incubated for 1.5 h at 37ºC on a shaker. 1 mL of RPMI 1640 (Lonza Cologne GmbH, Köln, Germany) was added to inactivate the liberase and samples were passed on a 70 μm cell strainer. Samples were centrifuged by 500 x g for 5 min at 4ºC and washed with FACS buffer. Cells were then counted with the Neubauer chamber, and 250,000 cells per panel were subjected to further FACS staining.

2.7. Fluorescence-activated cell sorting (FACS) analysis

FACS analysis is a sophisticated analytical method that use fluorescence light to count and profile cells in a biological fluid. A panel with 13 colours was used to determine the phenotype and frequency of immune cells and cytokines in the blood and cells isolated from the skin in BP patients and controls.

1 x 10^6 cells from heparin-NH_4-treated patient/control blood were resuspended in 100 µL RPMI 1640 medium and stimulated with 250 ng/ml phorbol myristate acetate (PMA) plus 5 µg/ml ionomycin in the presence of brefeldin A (Biolegend), a protein transport inhibitor, for 6 hours at 37°C with 5% CO_2. PMA is a small organic compound which diffuses through the cell membrane into the cytoplasm, mimics the endogenous signalling molecule diacylglycerol (DAG), and therefore, directly activates protein kinase C (PKC), omitting the 'need' of surface receptor stimulation. Because its action as a calcium ionophore, ionomycin is used to trigger the intracellular concentration of Ca^{++} and thus enhances the hydrolysis of membrane phosphoinositides into inositol-triphosphate (IP3) and DAG. The emerging second messenger IP3 mobilises Ca^{++} from intracellular and extracellular stores, and DAG further activates PKC. As a monovalent cationic ionophore, brefeldin A inhibits transport processes of proteins from the endoplasmic reticulum to the Golgi apparatus and therefore, leads to accumulation of intracellular synthesised cytokines. After stimulation cells were stained for surface markers, except CD4, because its signal was affected by the permeabilisation step. All antibodies used for staining are summarised in **Appendix (Table 7.3)**. Samples were incubated 30 min at RT in the dark. Afterwards, life/dead staining was applied in a final concentration of 1:1,000 in 200 µL and incubated for 20 min in the dark. Cells were then lysed with 1x RBC Lysis/Fixation Solution (Biolegend) for 10 min and washed with 1 mL cold PBS. In the next step, cells were permeabilised by using fixation/permeabilisation buffer from True Nuclear transcription Kit (Biolgened) for 75 min at RT, then washed 2x with permeabilisation buffer and resuspend in 100 µL of

permeabilisation buffer. Then, the intracellular staining and CD4 staining were performed. Samples were further incubated for 30 min at RT in the dark and afterwards washed two times with permeabilisation buffer. Cells were fixed in fixation/permeabilisation buffer for 20 min, washed, and resuspended in 200 µL FACS buffer (1.5% BSA/PBS). Samples were measured by Benchtop analyzer LSRII (BD Biosciences, San Jose, CA, USA) and analysed by FlowJo.

By using the same protocol, whole blood with heparin and cells isolated from the skin of mice were stained for 6 different cell types including Th17 cells, CD8 cells, γδ T cells, neutrophils, monocytes/macrophages, and mast cells. At the same time production of IL-17A, IL-17F, IL-21 and IL-22 were analysed. The full list of applied antibodies is shown in the **Appendix (Table 7.3)**. Samples were measured by MACSQuant analyzer 10.

2.8. Purification of neutrophils with Polymorphprep

To analyse the reactive oxygen species (ROS) activity of polymorphonuclear leukocytes, PMN were isolated from healthy volunteers using Polymorphprep. In a 15 mL tube, 5 mL of Polymorphprep™ (Axis-Shield, Heidelberg, Germany), was added, overlayed with 5 mL of whole blood and then centrifuged for 35 min at 500g at RT without a break. The upper cell band containing PBMC and the plasma were removed. The second band (granulocytes) was collected in a 50 mL tube. 5 mL of half medium (RPMI 1640 diluted 1:1 with LPS free ddH_2O) was added and filled with RPMI 1640 till 50 mL. Samples were centrifuged for 10 min at 400 x g at RT. The supernatant was discarded, cells were lysed with 3 mL lysis buffer (D-PBS diluted 1:5 with LPS free ddH_2O) within 30 sec, and subsequently, the tube was filled with RPMI 1640 up to 50 mL. Samples were centrifuged for 10 min at 400 x g at 4ºC. The supernatant was aspirated, 20 mL of CL medium (RPMI 1640 without phenol red stabilised with L-glutamine, 25 mM HEPES and 1% FCS) was added, and the sample was centrifuged for another 10 min at 400 x g at 4ºC. The pellet was resuspended in 5 mL CL medium and cells were counted with Neubauer counting chamber.

2.9. Reactive oxygen species release assay

The amount of ROS produced by neutrophils and other cells was used as an indicator for the degree of Fc-receptor-mediated cell activation. A fluorescence-based assay which uses luminol-enhanced chemiluminescence was applied to measure ROS production. To study whether neutralising anti-IL-17A antibody can prevent the ROS production from immune complex activated cells or neutrophils activated with recombinant IL-17A, the ROS release assay was performed. The experiment followed an already established protocol (Chou et al., 2010).In brief, immune complexes were generated using human BP 180 antigen and serum from BP patients. High-binding 96-well plates were incubated with 20 µg/ml human BP180 antigen in 50 mM carbonate/bicarbonate buffer (pH 9.6) overnight at 4ºC. Subsequently, the plate was washed with 0.01 M PBS pH 7.2 and then blocked for 1 hour with 10% FCS in 0.01 M PBS pH 7.2. The plate was washed again and therefore incubated with sera from BP patients diluted in 1:2 in PBS for 3 hours. Antigen coated wells incubated with PBS only served as negative control. In parallel, PMN from healthy volunteers were isolated. Cells concentration was adjusted to $1x10^6$ cells/ml in RPMI w/o phenol red supplemented with 1 gr of glucose and 25mM HEPES. Afterwards, 0.2 mM luminol was added. Following an extensive wash of the wells, 200,000 cells were added to each well. The chemiluminescence reaction was monitored for 2 hours by GlowMax reader at 37˚C.

Table 2.9: Buffers used for purification of neutrophils with Polymorphprep

Buffer	Composition
Coating buffer	0.05 M Na2CO3, 0.05 M NaHCO3 in aqua dest
Blocking buffer pH.7.2	1 % biotin free BSA, 0.05 % Tween-20 in PBS
Washing buffer, pH 7.2 (PBS-T)	0.05 % Tween-20 in PBS
Chemiluminescence (CL-Medium)	Modified RPMI 1640 without Phenol red, L-glutamine, 25 mM HEPES, 1% FCS
Luminol solution	2 mg/mL 5-Amino-2,3-dihydro-1,4-phthalazinedione (Luminol) in aqua dest

2.10. Cryosection assay

Separation of the dermal-epidermal junction in cryosections of healthy human skin was evaluated using an *ex vivo* model as previously described (Gammon et al.,

1981; Heppe et al., 2017; Sitaru et al., 2002; Vafia et al., 2012). In brief, 4 x 20 mL heparin-blood from healthy donors was used to isolate granulocytes, which were applied on the skin section pre-incubated with serum from BP patients or mice injected with mCol17 for 1h at 37°C. A sedimentation gradient purified leukocytes with dextran 500 (Carl Roth) were incubated 30 min at RT. The supernatant was transferred in new 50 mL tubes and centrifuged for 12 min at 1200rpm at RT. The supernatant was removed, and cells were washed with 10 mL RPMI 1640. After centrifugation, cells were lysed with 20 mL 0.2 % NaCl within 20 seconds and immediately after that 20 mL of 1.6 % of NaCl was added. Tubes were filled with RPMI 1640 until 50 mL After centrifugation. Samples were centrifuged 12 min 1,200rpm 4°C. Cells were counted by using a Neubauer counting chamber. About $1x10^6$ cells were added together with 100 µg, 200 µg, and 300 µg neutralising anti-IL-17A antibody and isotype control (Novartis), respectively, and incubated for 3 hours at 37°C before intense washing with PBS. Skin sections were proceeded for staining with haematoxylin and eosin (H&E), and dermal-epidermal separation was evaluated microscopically (Keyence).

Table 2.10: Buffers used for cryosection assay

Buffer	Composition
Dextran-/NaCl solution	3% Dextran 500, 0.9% NaCl in aqua dest
0,2% NaCl-solution	0.2% NaCl in aqua dest
1,6% NaCl-solution	1.6% NaCl in aqua dest

2.11. Statistical analysis

Data are presented as the median or mean ± standard deviation. Statistical calculations were performed using GraphPad Prism version 6 (GraphPad Software Inc, San Diego, USA). For a comparison of more than two groups, Two-way ANOVA with Sidak's multiple comparisons test was used. When data were nonparametric Mann-Whitney-U test or Kruskal-Wallis test were applied followed by a Dunn postdoc or Bonferroni t-test for multiple comparisons. Spearman product-moment correlation was used to test for correlations. In all tests, a p-value of 0.05 was considered to be statistically significant.

3. Results

3.1. Human studies

3.1.1. CD4-positive lymphocytes are the major source of IL-17A in the peripheral blood of patients with bullous pemphigoid

In order to evaluate the frequency and phenotype of circulating IL-17A, peripheral blood cells from newly diagnosed BP patients (n=15) and sex- and age-matched controls with other non-inflammatory dermatoses (n=12) were stained for cytokine expression on $CD4^+$ cells, $CD8^+$ cells, monocytes, and neutrophils (gating strategy is presented in **Appendix (Figure 7.1)**. Furthermore, we addressed the question of whether $CD4^+$ cells in the peripheral blood of BP patients, in addition to IL-17A, produced IL-17A-related cytokines. After *in vitro* stimulation with PMA and ionomycin, cells were stained for IL-17A, IL-17F, IL-21, and IL-22.

Activated and functionally differentiated $CD4^+$ cells expressed significantly higher levels of IL-17A compared to control patients, but not $CD8^+$ cells, neutrophils, and monocytes (**Figure 3.1**). Interestingly, significantly enhanced IL-22 production was seen only in $CD4^+$ cells of BP patients compared to controls (**Figure 3.2**). In contrast,

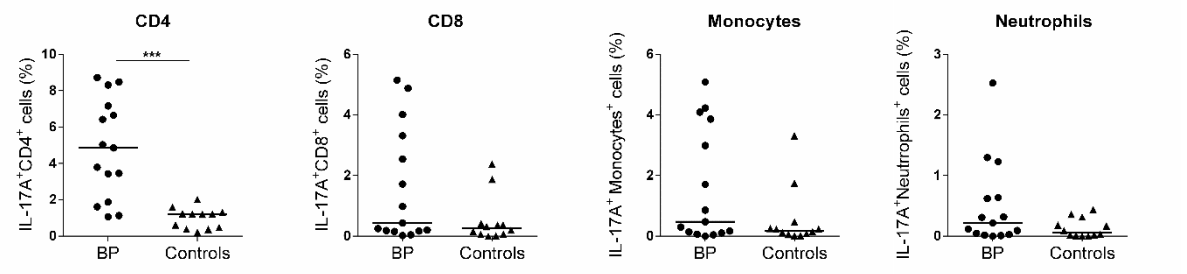

Figure 3.1: $CD4^+$ cells are the main producers of IL-17A in the peripheral blood of bullous pemphigoid patients compared to control patients

When $CD4^+$ cells, CD8+ cells, neutrophils, and monocytes of the peripheral blood from treatment naïve BP patients and age-and sex-matched controls were analysed by flow cytometry for production of IL-17A, significantly more $IL\text{-}17A^+CD4^+$ cells were found in BP patients compared to controls. No difference in the number of IL-17A producing cells between BP patients and controls was seen in $CD8^+$ cells, neutrophils, and monocytes. Data are presented as a median. Statistical analysis was carried out using the Mann Whitney test. $p \leq 0.05$ was considered significant. ***, $p \leq 0.001$

no difference in the production of IL-17F and IL-21 were found on $CD4^+$ cells, $CD8^+$ cells, neutrophils, and monocytes in BP patients compared to control patients.

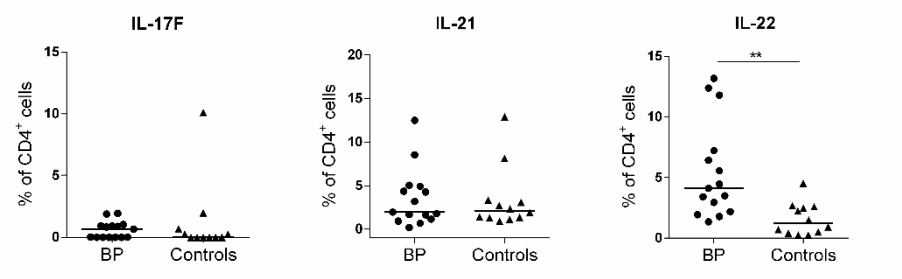

Figure 3.2: Increased number of IL-22 producing $CD4^+$ cells in the peripheral blood of patients with bullous pemphigoid compared to control patients

$CD4^+$ cells in the peripheral blood from treatment naïve BP patients and age-and sex-matched controls were analysed by flow cytometry for their potential to produce IL-17F, IL-21, and IL-22. Significantly more IL-22-producing cells were detected in BP patients compared to controls. Data are presented as a median. Statistical analysis was carried out using the Mann Whitney test. $p \leq 0.05$ was considered as a significant. **, $p \leq 0.01$

3.1.2. Expression of IL-17A and related mediators in early skin lesions of patients with bullous pemphigoid

Aside from specific effector cytokines, the exclusive pathogenic properties of Th17 cells in the skin, possible depend on Th17 specific molecules differently regulated on mRNA level. Quantitative RT-PCR was applied to identify potential candidates related to IL-17A in perilesional skin biopsies of BP patients and patients with non-inflammatory dermatoses. The expression of 32 genes related to the IL-17 regulatory network was studied. The array contained primers for cytokines, chemokines, their receptors, cell signalling molecules, and transcriptional factors. GAPDH was used as an endogenous control for normalisation.

Eighteen out of twenty-four genes were significantly ≥ 2-fold up-regulated in BP patients skin compared to control skin. Among eighteen significantly up-regulated genes were cytokines as *IL17A, IL6,* and *TNFα;* receptors like *IL-17RC, CCR6,* and *IL23R*; as well as chemokines such as *CXCL1* and *CXCL2*. Three out of twenty-four genes were found to be ≥ 2-fold down-regulated. Only one gene, *CD8*, was found to be significantly less expressed in perilesional skin of BP patients (n=7) in comparison

to control patients with other non-inflammatory dermatoses (n=7) (**Figure 3.3, Appendix-Table 7.8**).

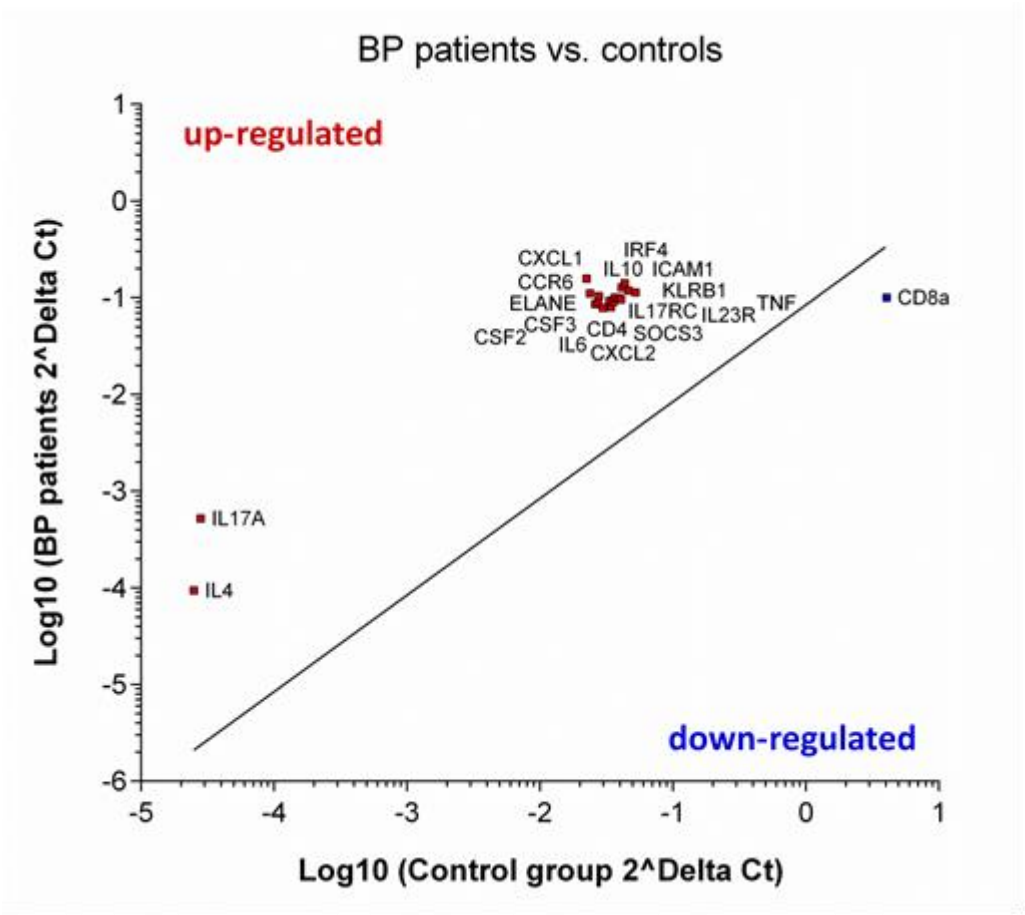

Figure 3.3: mRNA levels of IL-17A and related mediators were upregulated in perilesional skin of bullous pemphigoid patients

In perilesional skin of BP patients, 18 of 32 analysed IL-17-related genes were significantly upregulated (red), while CD8 was significantly down-regulated (blue). BP patients (n=7) versus control patients (n=7). Results represent the relative expression ($2^{-\Delta CT}$) normalised to GAPDH. The p values are calculated based on Student's t-test of the replicate for each gene in the control group and BP group. p values less than 0.05 were considered to be significant.

3.1.3. $CD3^+CD4^+$ T lymphocytes are the main source of IL-17A in early skin lesions of patients with bullous pemphigoid

To gain insight into the local production of IL-17A in early BP skin lesions, perilesional skin of BP patients (n=7), reflecting the early phase of the disease, was evaluated by immunofluorescence staining of the cell populations known to produce IL-17A, i.e. $CD3^+$ lymphocytes, $CD4^+$ lymphocytes, mast cells, neutrophils, and

macrophages. Neutrophils were detected by polyclonal rabbit anti-human myeloperoxidase (MPO) and mast cells by monoclonal mouse anti-human mast cells tryptase (MCT), respectively. Our initial analysis revealed that 70 % of $CD3^+$ cells, 70 % of neutrophils and 69 % of mast cells were IL-$17A^+$ (**Figure 3.4**). Due to the relatively high percentage of double positive cells, we speculated that this anti-IL-17A antibody revealed unspecific binding (Tamarozzi et al., 2014; Velden et al., 2012). Subsequently, we applied another antibody, a mouse monoclonal antibody against IL-17A (eBioscience). With this antibody 40.5 % of $CD3^+$ cells were reactive, 45 % of MCT^+ cells, and 35 % MPO^+IL-$17A^+$ (**Figure 3.5**). Therefore, in the following experiments, only the mouse monoclonal anti-human IL-17A was used. When stained for $CD4^+$ lymphocytes and $CD68^+$ macrophages, 61 % of $CD4^+$ cells 45 % of macrophages were IL-$17A^+$, respectively **(Figure 3.6)**.

Confocal microscopy was then used to determine if the IL-17A expression was intracellular or if the cytokine may have bound to a receptor expressed on the cell surface. IL-17A-producing cells of a certain cell population were analysed concerning the total number of IL-17A-producing cells. By this method, 41 % of total $CD3^+$ cells expressed IL-17A (**Figure 3.6**). 61 % of $CD4^+$ cell, 45 % of $CD68^+$ cells and 45 % of mast cells. In addition, 35 % of neutrophils were found to express IL-17A (**Figure 3.6**). In summary, $CD3^+$ cells appeared to be the main source of IL-17A representing 60% of the IL-17A producers followed by $CD68^+$ macrophages (17 %), neutrophils (17 %), whereas mast cells present only 6 % of whole IL-17A positive cells (**Figure 3.6**).

To validate the findings obtained by IF microscopy, staining for CD4, CD8, and CD68 was repeated by routine immunohistochemistry in perilesional skin of BP patients. Additionally, samples were stained for CD20 as a marker for B cells. Results revealed that $CD4^+$ cells are the most abundant cell type in perilesional skin of BP patients (**Figure 3.7**), corresponding well to the findings by IF staining.

3.1.4. The potential role of IL-17A in blister formation of bullous pemphigoid

So far, it becomes clear that patients with BP reveal an elevated number of IL-17A-expressing $CD4^+$ cells in the peripheral blood. Also, early skin lesions of BP, IL-17A and related genes were significantly up-regulated, and $CD3^+$ cells appeared to be the leading producers of IL-17A. Thus, the potential role of IL-17A in blister formation

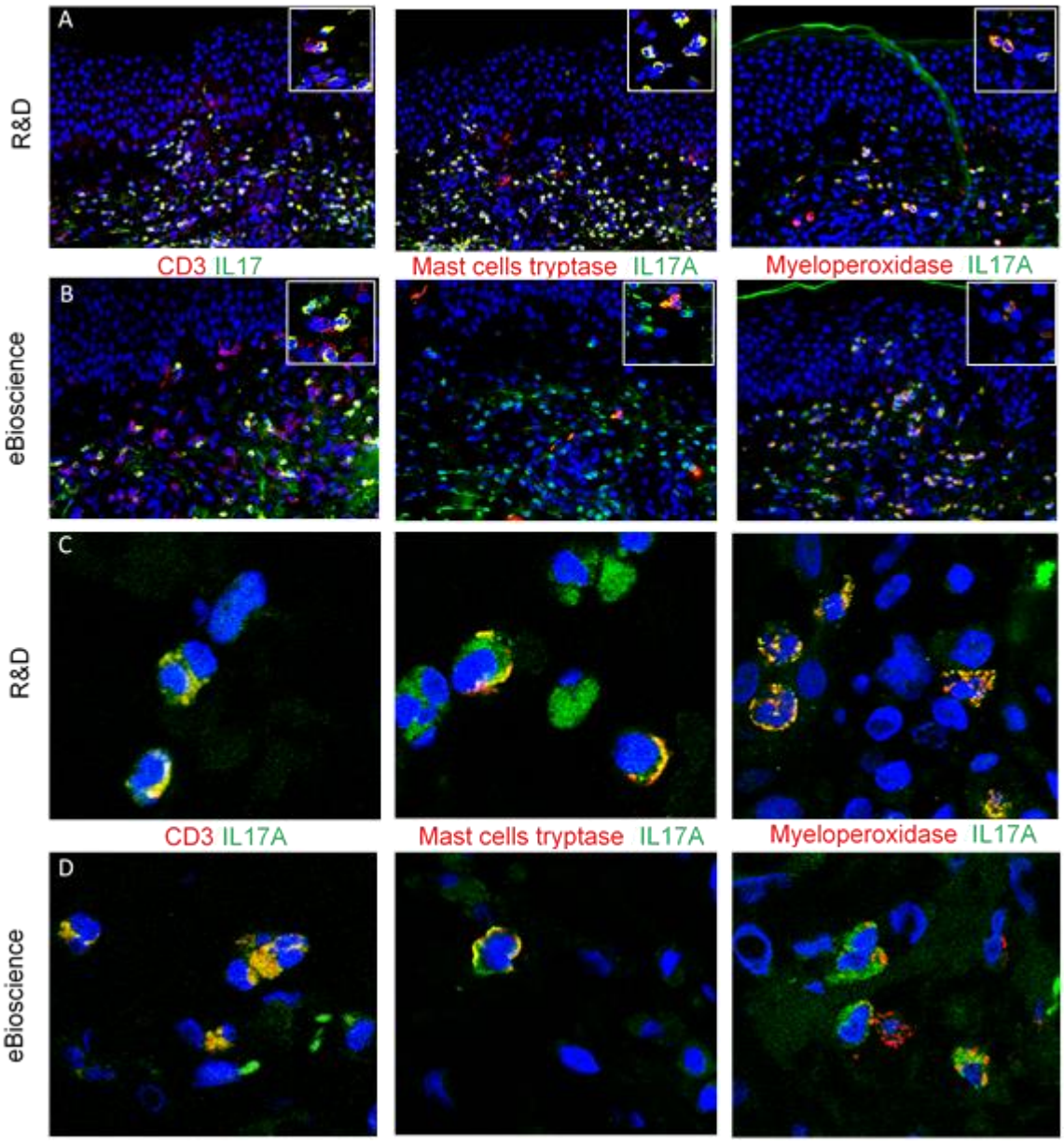

Figure 3.4: Comparison between polyclonal goat anti-human IL-17A antibody (R&D) and mouse monoclonal anti-human IL-17A (eBioscience)

A, B; x200 magnification, 100μm scale, Light/Fluorescence Microscope BZ-9000 (Keyence); C, D; x600 magnification, 5μm scale, confocal microscope Olympus BX40 (Olympus); A, C; 3 different cell types stained with polyclonal goat anti-human IL-17A antibody (R&D Systems). B, D; staining with mouse monoclonal anti-human IL-17A (eBioscience). $CD3^+$ cells, mast cell tryptase, myeloperoxidase (red); IL-17A (green); DAPI (blue).

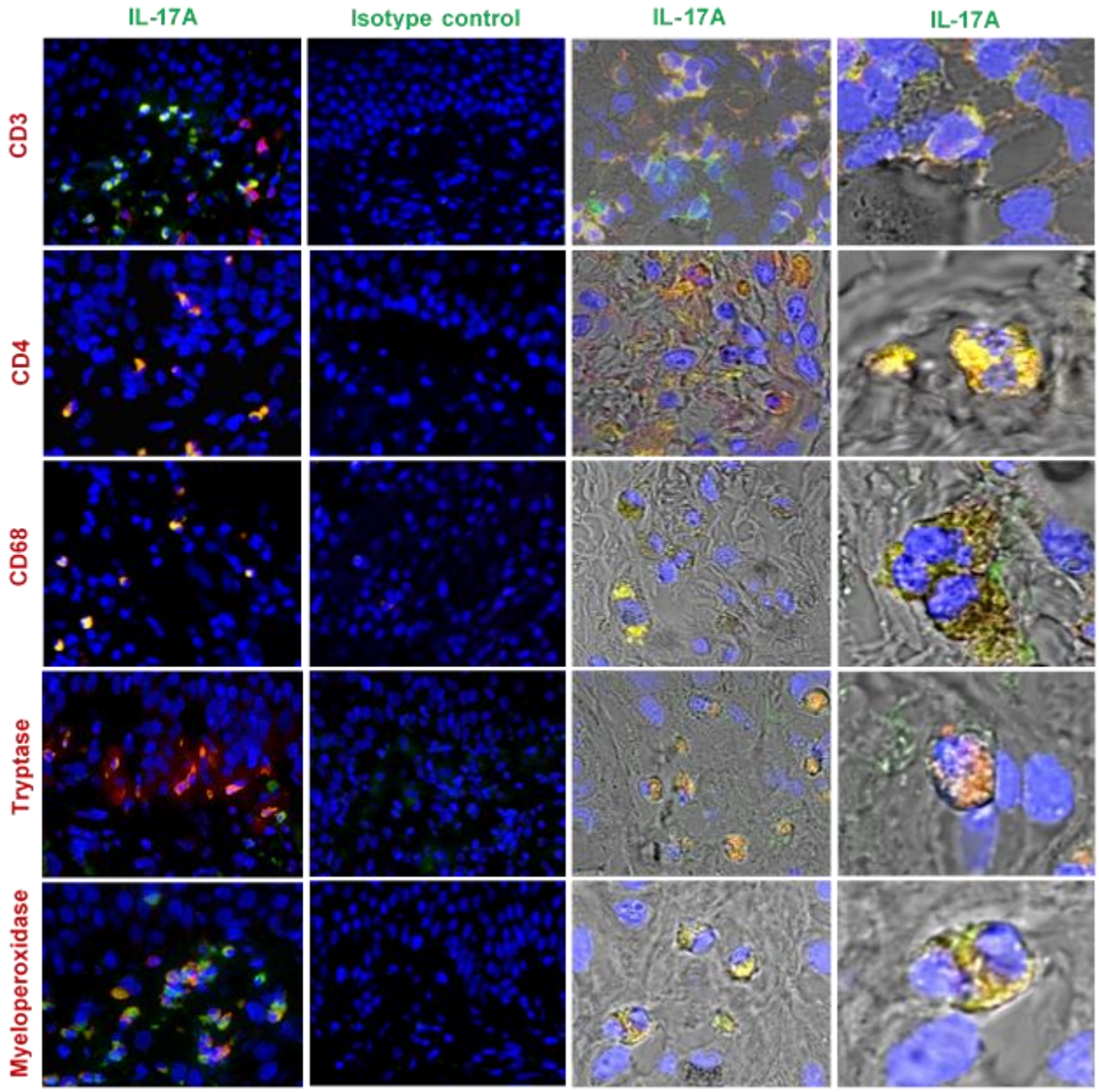

Figure 3.5: CD3$^+$ lymphocytes are the main source of IL-17A in perilesional skin of bullous pemphigoid patients

Paraffin sections of perilesional skin were subjected to double staining with antibodies against IL-17A (green) and CD3, CD4, CD68, tryptase, and myeloperoxidase, respectively (red; line 1, 3 and 4). Staining with isotype antibodies was used as controls (lane 2). Confocal microscopic pictures show IL-17A staining both within the cell and on the cell surface of some cells (lanes 3 and 4). Counterstaining of nuclei was done with DAPI (blue), merged (yellow). x200 magnification, 100μm scale; x600 magnification, 5μm scale. Confocal microscope Olympus BX40 (Olympus).

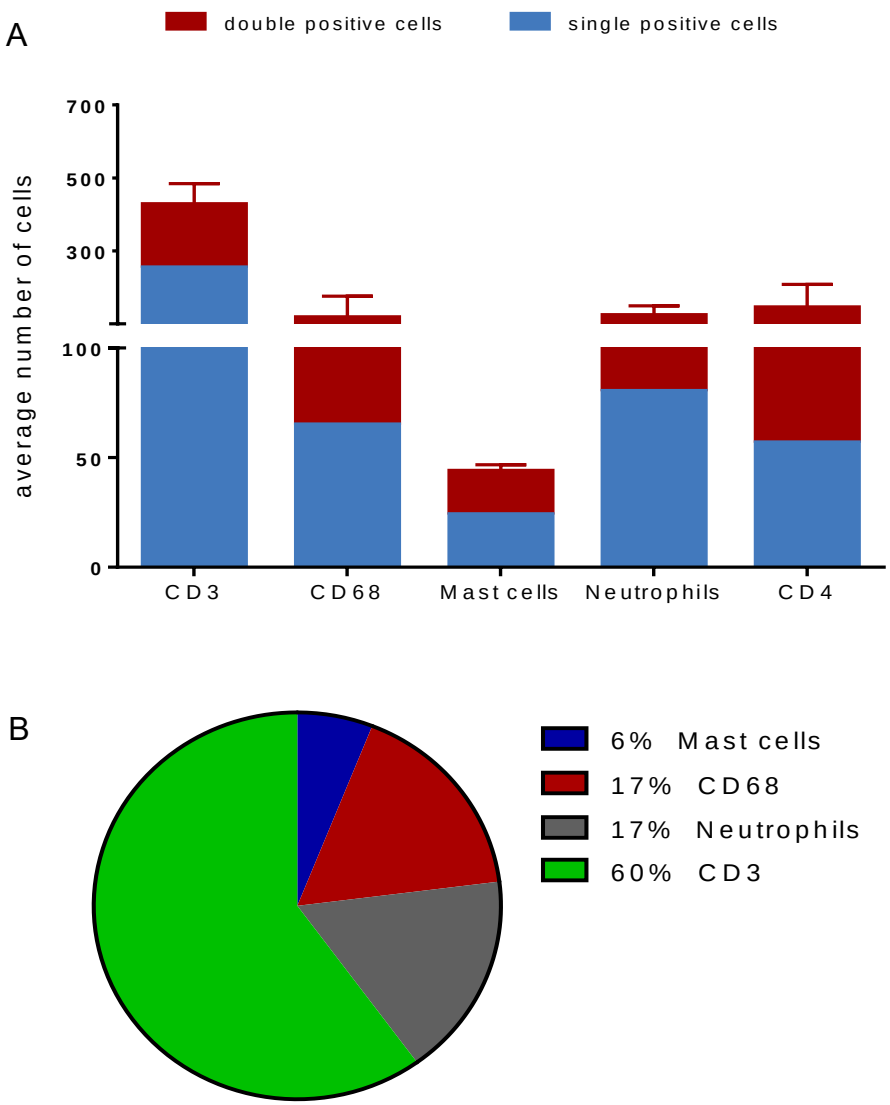

Figure 3.6: Quantification of IL-17A$^+$ cells in early skin lesions of patients with bullous pemphigoid

A, Statistical analyses of double-stained cells with intracellular fluorescence. Five representative pictures of 2 sections were analysed. Bars show mean +SD. B, Diagram of the percentage of IL-17A-producing cells of a certain cell type in relation to the total number of IL-17A-producing cells. B; Summary in % for IL-17A-producing cells in BP patients

was investigated in a series of in vitro assays. In the following actions cryosection assay, ROS release assay, and neutrophils stimulation experiments were employed.

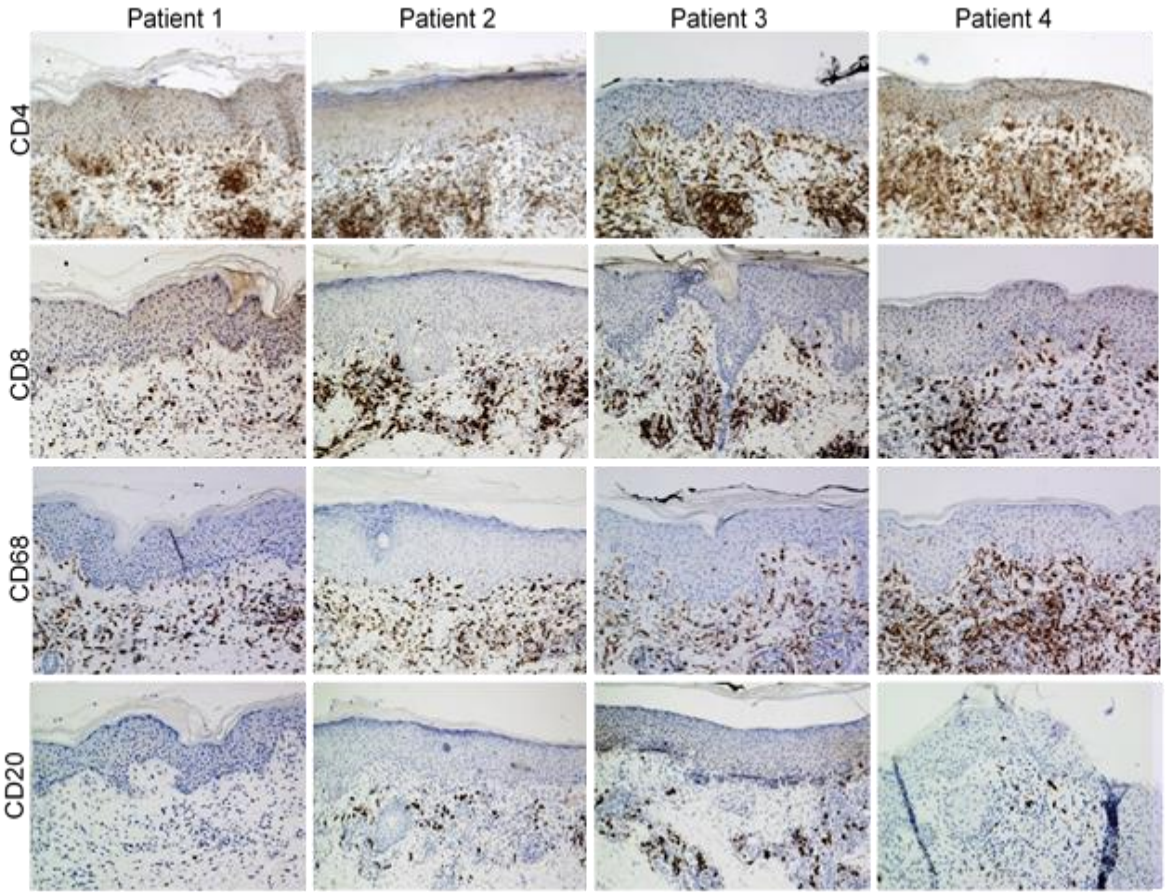

Figure 3.7: Immunohistochemistry of different cell types in early skin lesions of bullous pemphigoid patients

Perilesional skin from 4 BP patients has been stained for reactivity with CD4, CD8, CD68, and CD20, respectively.

3.1.4.1. Inhibition of dermal-epidermal separation by anti-IL-17A antibody

In the cryosection model, dermal-epidermal separation can be modulated *ex vivo*, by incubation of normal human skin sections with anti-Col17 IgG and subsequently, with leucocytes from healthy volunteers (Gammon et al., 1981; Heppe et al., 2017; Sitaru et al., 2002). While anti-Col17 IgG-treated skin sections reveal dermal-epidermal separation, incubation with IgG from healthy volunteers does not. Here, was tested whether the splitting is prevented by inhibition of IL-17A. Leukocytes were pre-treated with neutralising anti-IL-17A antibody or isotype control (IgG2), respectively, in different concentrations (100 μg, 200 μg and 300 μg) before applied to the skin sections. As a negative control, normal human serum was used. When

leukocytes were pre-treated with 100 µg of anti-IL-17A antibody, dermal-epidermal split formation occurred (**Figure 3.8**). In contrast pre-treatment with 200 µg and 300 µg of anti-IL-17A antibody prevented dermal-epidermal splitting. In sections incubated with normal human serum, no split formation was detected (**Figure 3.8**).

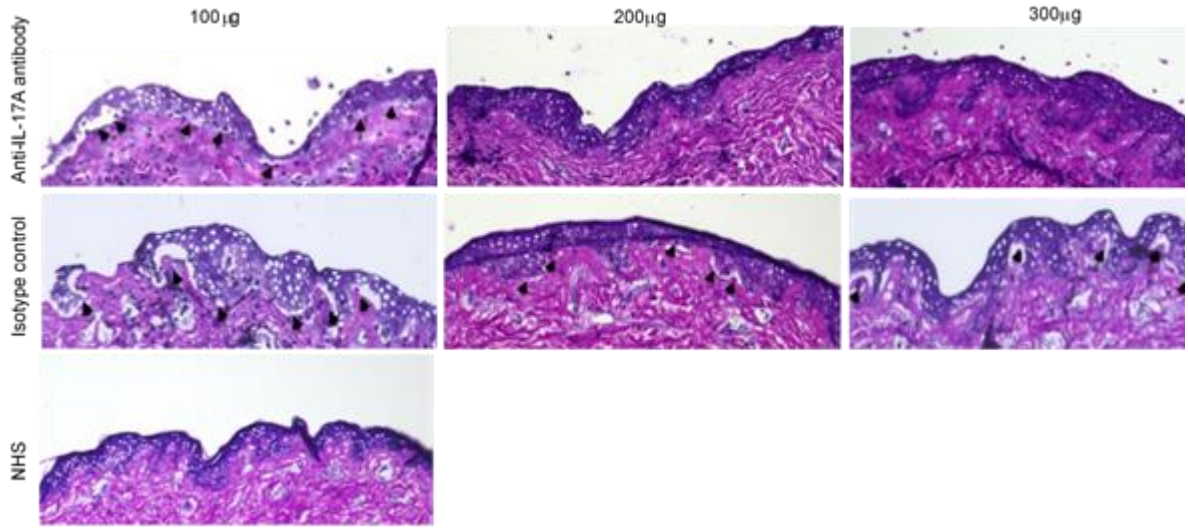

Figure 3.8: Anti-Col17 IgG-mediated dermal-epidermal splitting is prevented by inhibition of IL-17A

Treatment of cryosections of normal human skin with BP serum and subsequently, with leukocytes from healthy volunteers plus an isotype control (100 µg, 200 µg and 300 µg) resulted in dermal-epidermal separation (black triangles). Incubation of leukocytes with 200 µg (B) and 300 µg (C), but not with 100 µg anti-IL-17A IgG (A) prevented splitting. Representative pictures of 2 independent experiments with 3 blood donors are shown. Normal human serum (NHS) was incubated alone with neutrophils (x200 magnification).

3.1.4.2. The Col17-anti-Col17 IgG-mediated release of reactive oxygen species from normal human neutrophils is reduced by inhibiting IL-17A

Reactive oxygen species (ROS) generated during various metabolic and biochemical reactions were monitored *in vitro*. These sets of experiments aimed to investigate whether the inhibition of IL-17A can prevent ROS formation. Neutrophils purified from peripheral blood of healthy individuals were activated by immune complexes composed of Col17 NC16A and anti-Col17 NC16A IgG.

ROS release from neutrophils was significantly reduced in the samples pre-treated with neutralising anti-IL-17A antibody in a dose-dependent manner. Samples pre-treated

with 200 μg and 300 μg (p=0.32, p=0.26) of neutralising antibody showed lower ROS release compared to samples treated with isotype control antibody (**Figure 3.9**). In line, neutrophils pre-treated with recombinant IL-17A (rIL-17A) induced an increased ROS release compared to untreated neutrophils. As a negative control, neutrophils incubated with normal human serum were used. ROS production was calculated every 30 min throughout 90 minutes (**Figure 3.9**). Another interesting result from this experiment was that the immune complex together with rIL-17A resulted in much stronger ROS production compared to incubation with the immune complexes alone, reaching the response of ROS release obtained with PMA pre-treated neutrophils.

3.1.4.3. Activation of normal human neutrophils with recombinant IL-17A

From the previous experiments, we concluded that the anti-IL-17A antibody had an impact on neutrophils by preventing split formation and ROS production. In the next set of experiments, we set out to delineate how this antibody may affect neutrophil function. Therefore, neutrophil activation was quantified after 1 h stimulation with 100 ng recombinant IL-17A. Neutrophils stimulated with PMA were used as positive control, while neutrophils alone served as negative control. Live cells were gated from $CD45^{+}CD16^{+}$, $CD14^{-}CD193^{-}$. CD62L (L-selectin) marker was used as an activation marker together with CD66b. Consequently, $CD66b^{+}CD62^{-}$ cells were considered as active neutrophils and $C66b^{+}CD62L^{+}$ cells as non-activated (**Figure 3.10**).
Untreated neutrophils showed only 10 % activation, while stimulation with rIL-17A had a compatible impact on the activity of neutrophils as PMA (**Figure 3.10**). The gating strategy is presented in the Appendix **(Figure 7.3)**.

3.2. Mouse studies

An antibody transfer induced a model of BP was used to investigate the functional relevance of IL-17A *in vivo* (Schulze et al., 2014). For disease induction, 10 mg of affinity purified total rabbit IgG against mCol17 IgG was injected s.c. into C57/Bl6 mice. Animals received a total of 6 injections every second day starting from day 0 (**Figure 3.11**). The model closely reflects the human situation by clinical phenotype (Bieber et al., 2010). Usually, the first lesions start to develop around day 4 appearing

on the ears and neck. The peak of the disease is between day 8 and day 12. Every 4th day, mice were scored for the affected body surface area (Schulze et al., 2014).

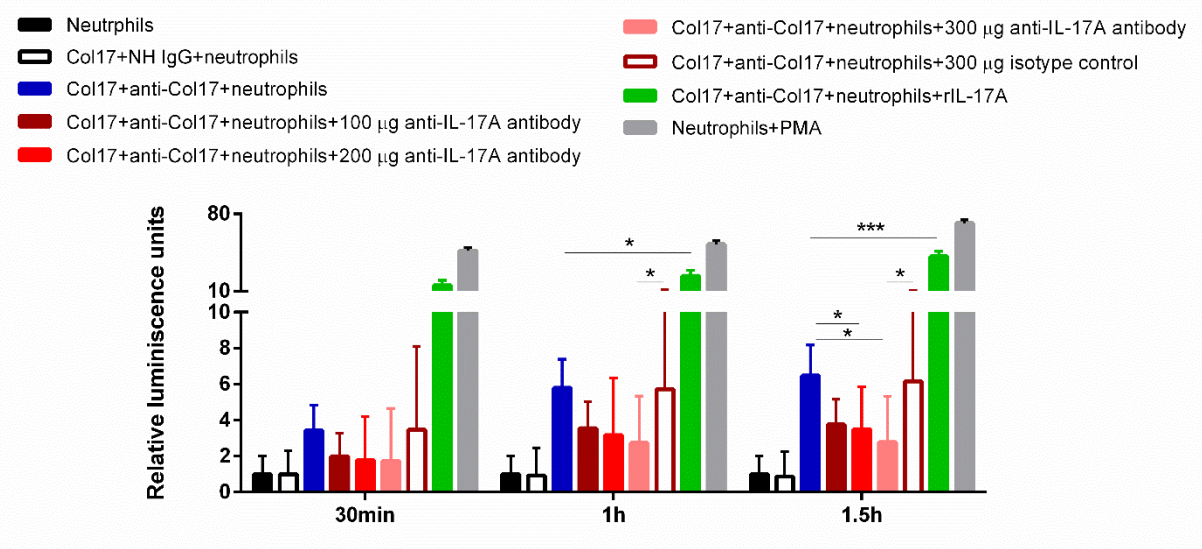

Figure 3.9: Anti-IL-17A prevents ROS production in vitro

The release of reactive oxygen species from neutrophils was measured throughout 1.5h. Incubation of Col17-anti-Col17 IgG immune complexes with normal neutrophils (blue column); neutrophils exposed to Col17 incubated with normal human (NH) IgG (white column); co-incubation of neutrophils with recombinant IL-17A (green column); neutrophils co-incubated with different doses of anti-IL-17A antibody, 100 µg/ml, 200 µg/ml, 300µg/ml (red columns); neutrophils co-incubated with an isotype control (300 µg/ml; red framed column); treatment of neutrophils with phorbol myristate acetate (PMA) served as positive control (grey column).Results are shown as mean ± SD. Statistical analysis was performed using Kruskal-Wallis test, multiple comparisons (GraphPad Prism 6.0). Significance levels were set at *, $p<0.05$; ***, $p<0.001$. Results from 3 independent experiments with 8 replicates are shown.

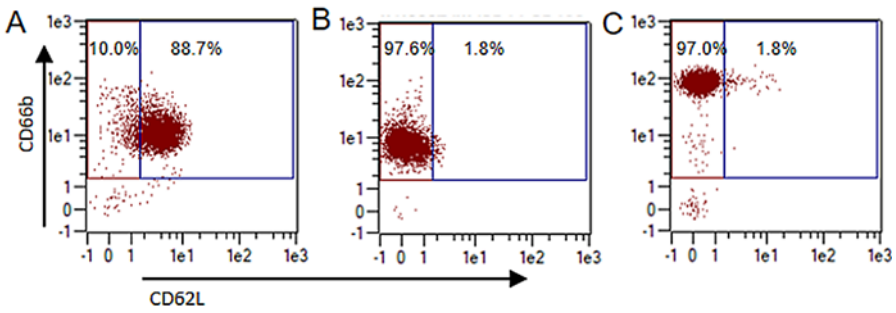

Figure 3.10: IL-17A activates normal human neutrophils

Human neutrophils purified from a healthy volunteer revealed lower expression of CD62L, indicating a higher activation level, after incubation with recombinant IL-17A (B) compared to unstimulated cells (A). Phorbol myristate acetate (PMA) was used as a positive control (C).

3.2.1. IL-17A$^{-/-}$ mice are protected from the pathogenic effect of anti-Col17 IgG

To determine the role and the contribution of IL-17A in the pathogenesis of BP, knock-out mice for IL-17A were used. Experimental mouse model of BP was performed as previously described (Schulze et al., 2014). Schematic overview of the experimental protocol is presented in **Figure 3.11**.

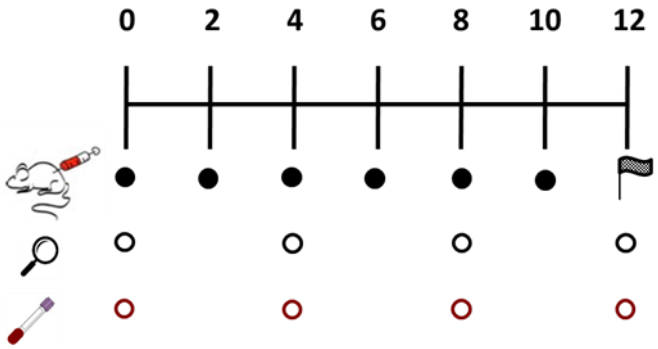

Figure 3.11: Schematic diagram of the experimental plan in the antibody transferred-induced mouse model of bullous pemphigoid

The experiment lasted 12 days. mCol17 IgG was injected 6 times after day (10 mg, s.c) (mouse); the extent of the skin lesions was scored every 4th day (magnifier), and blood was taken every 4th day (blood tube).

IL-17A$^{-/-}$ mice were almost completely protected from the pathogenic effect of anti-mCol17 IgG. Compared to wild-type (WT) mice, IL-17A$^{-/-}$ mice showed significantly less erythema and erosion over the body surface (day 4, p=0.007; day 8, p=0.0003; day

12, p=< 0.0001). The cumulative disease score at day 12 was significantly lower in IL-17A$^{-/-}$ mice compared to WT mice (p= 0.0001) (**Figure 3.12**). Deposition of IgG at the dermal-epidermal junction was present in the skin of both, IL-17A$^{-/-}$ mice and WT mice, while split formation was visible only in the WT group (**Figure 3.13**). Interestingly, perilesional skin of IL-17A$^{-/-}$ mice revealed a similar dense inflammatory cell infiltrate as WT mice, but showed no dermal separation.

3.2.2. IL-17A/F$^{-/-}$ mice are partially protected from anti-Col17 IgG

Since IL-17A and IL-17F share the same signalling receptors IL-17RA/IL-17RC, the effect of IL-17F in experimental BP was also investigated. Therefore, mice deficient of both IL-17A and IL-17F, IL-17A/F$^{-/-}$ mice, were used in the same experiment and compared with IL-17A$^{-/-}$ and WT mice. At day 12, IL-17A/F$^{-/-}$ mice showed an intermediate phenotype, but significantly fewer skin lesions compared to WT mice (p= 0.013,), but significantly more than IL-17A$^{-/-}$ mice (p=0.04) (**Figure 3.12**). The cumulative disease score of IL-17A/F$^{-/-}$ mice was statistically significantly lower than WT and IL-17A$^{-/-}$ mice (p= 0.013, p=0.017) (**Figure 3.12**). No difference in the IgG deposition was detected between IL-17A/F$^{-/-}$, IL-17A$^{-/-}$ and WT mice. Histology of the perilesional skin biopsies revealed dermal-epidermal separation in IL-17A/F$^{-/-}$ mice and WT mice only (**Figure 3.13**).

3.2.2.1. Difference between cell infiltration in skin and blood

To characterise the inflammatory infiltrate in the skin and blood WT mice, IL-17A$^{-/-}$ mice and IL-17A/F$^{-/-}$ mice, were subjected to FACS analysis. Whole blood and lesional skin were taken. At day 12, and mice were tested for five different cell populations including Th17 cells, $\gamma\delta$ T cells, neutrophils, monocytes/macrophages, and mast cells. In the lesional skin, the most present cell type was macrophages, followed by Th17 cells and neutrophils. Interestingly, among the three groups, IL-17A$^{-/-}$ mice had the highest number of Th17cells, significantly more compared with WT mice

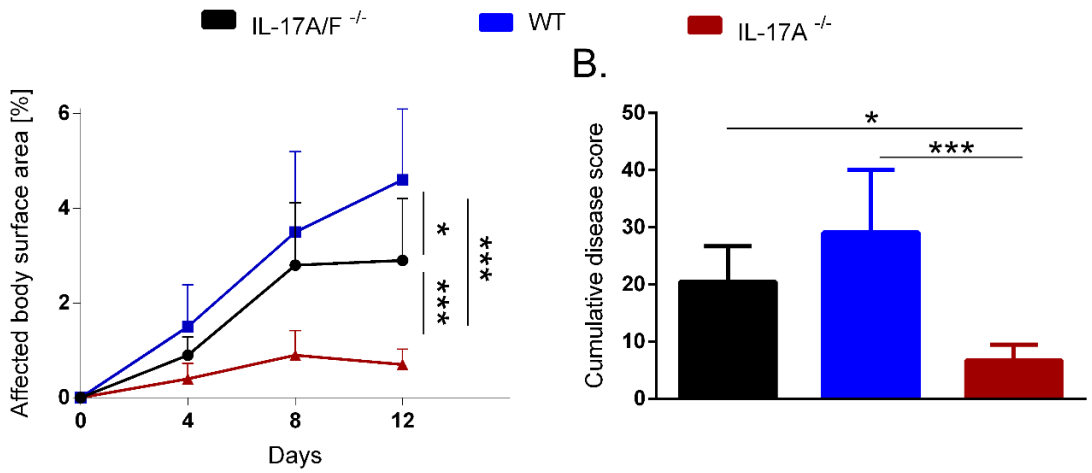

Figure 3.12: Affected body surface area and cumulative disease score of IL-17A$^{-/-}$, IL-17A/F$^{-/-}$ and wild-type mice

Affected body surface area during the course of the disease (left graph) and cumulative disease score between the three groups. IL-17A$^{-/-}$ mice (red); IL-17A/F$^{-/-}$ mice (black) and WT mice (blue). Statistical analysis was performed using Kruskal-Wallis test, multiple comparisons (GraphPad Prism 6.0); significance levels were set at *, p<0.05; **; ***, p<0.001.

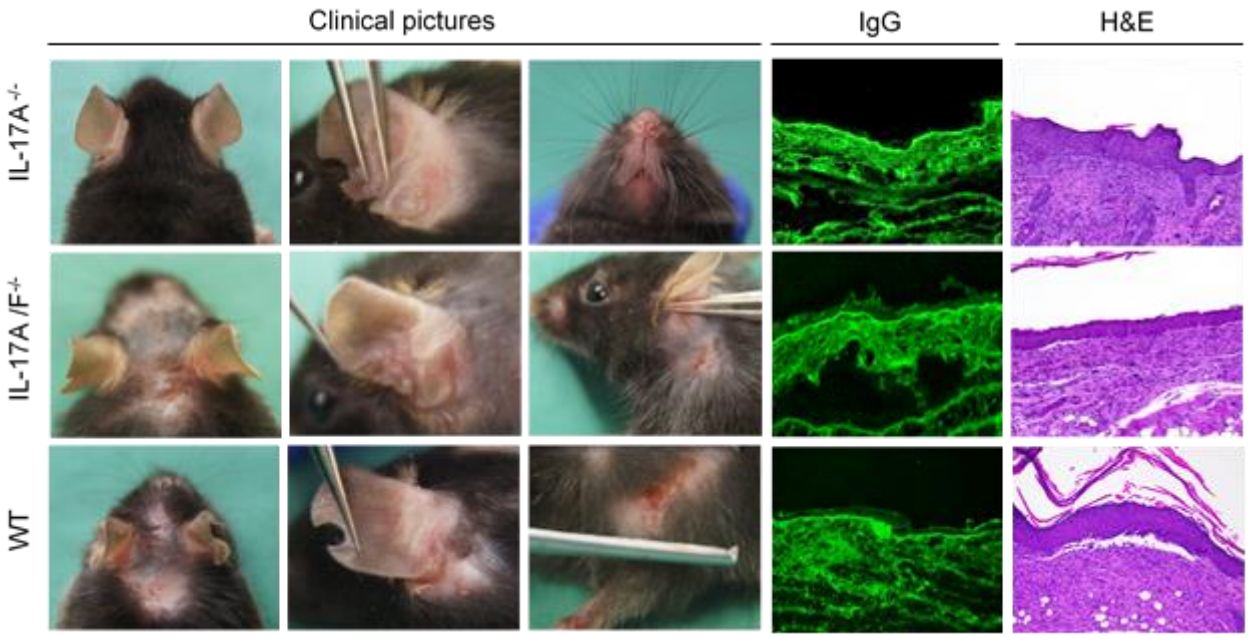

Figure 3.13: Representative clinical pictures, IgG deposition and lesional histopathology in IL-17A$^{-/-}$ IL-17A/F$^{-/-}$, and wild-type mice

Clinical pictures from IL-17A$^{-/-}$ mice, IL-17A/F$^{-/-}$ and WT mice; linear deposition of IgG at the dermal-epidermal junction of perilesional skin biopsies; histology revealed subepidermal split formation in IL-17A/F$^{-/-}$ and more pronounced in WT mice.

(p= 0.0191) and no difference with IL-17A/F$^{-/-}$ mice (p= 0.6308). Neutrophils were significantly more frequent in lesional skin of IL-17A$^{-/-}$ mice compared to WT mice (p= 0.0446), while no difference with IL-17A/F$^{-/-}$ mice was observed (p= 0.4397). Macrophages were another cell type that was significantly increased in IL-17A$^{-/-}$ mice compared to IL-17A/F$^{-/-}$ mice (p= 0.0442). γδ T cells and mast cells were less abundant with about 2% and less than 1 % of total cell number in the skin, respectively (**Figure 3.14**). When the same cell types were analysed in the peripheral blood, only the number of neutrophils appeared to be significantly different between the 3 groups. In the peripheral blood, neutrophils in IL-17A$^{-/-}$ mice were significantly less abundant compared to IL-17A/F$^{-/-}$ (p= 0.0001) and WT mice (p= < 0.0001), respectively. IL-17A$^{-/-}$ mice showed the lowest number of neutrophils in the peripheral blood. A significant difference was detected between IL-17A/F$^{-/-}$ mice and WT mice (p=<0.0001). Monocytes were present with up to 5 % of total cell number, and no difference among the different strains was observed. Th17 cells, were present in about 3 % of the total cell number, γδ T cells approximately in 1.5 %, while mast cells, as expected were not detectable in the peripheral blood of same mouse strains (**Figure 3.14**). The gating strategy for all cell types with cellular markers is presented in **Appendix (Figure 7.2)**.

3.2.2.2. Cytokine production in the lesional skin of IL-17A$^{-/-}$, IL-17A/F$^{-/-}$ and wild-type mice after injection of anti-Col17 IgG

The expression of IL-17A, IL-17F, IL-21 and IL-22 in lesional skin of IL-17A$^{-/-}$, IL-17-A/F$^{-/-}$ and WT mice was measured by FACS analysis. IL-17A, IL-17F, IL-21, and IL-22 were evaluated on different cell types including Th17 cells, γδ T cells, neutrophils, macrophages, and mast cells (**Figure 3.15, Figure 3.16, Figure 3.17, Figure 3.18**).

IL-17A

The expression of IL-17A in the lesional skin was detected only in WT mice and not in IL-17A$^{-/-}$ and IL-17A/F$^{-/-}$ mice (**Figure 3.15**). Compared with IL-17A$^{-/-}$ and IL-17A/F$^{-/-}$ mice, WT mice showed significantly higher number of IL-17A^{+}CCR6^{+} T cells (Th17 cells) (p=0.004, p=0.0006) and neutrophils (p= 0.0002, p=0.0005). While γδ T cells, macrophages, and mast cells did not show expression of IL-17A.

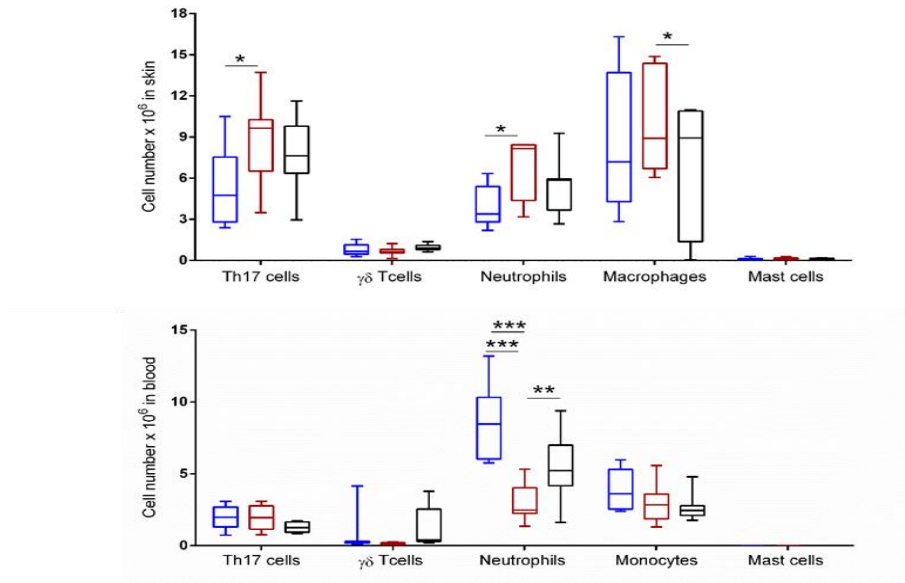

Figure 3.14: Frequencies of different cell types in peripheral blood and lesional skin of IL-17A$^{-/-}$ mice, IL-17A/F$^{-/-}$ and wild-type mice after injection of anti-Col17 IgG

Comparison among the three groups of mice, IL-17A$^{-/-}$ mice (red); IL-17A/F$^{-/-}$ mice (black) and WT mice (blue). Peripheral blood and lesional skin were taken at day 12 after injection of anti-Col17 IgG. Results are shown as a mean ± SD. Statistical analysis was performed using Tukey's multiple comparisons test, (GraphPad Prism 6.0). Significance levels were set at *, p<0.05; **, p<0.01; ***, p<0.001.

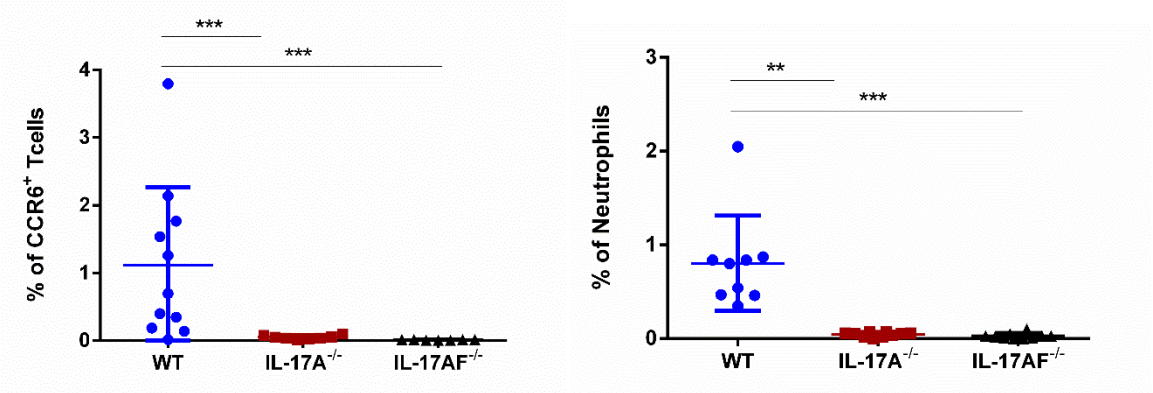

Figure 3.15: Expression of IL-17A in lesional skin of wild-type mice, IL-17A$^{-/-}$ and IL-17A/F$^{-/-}$ mice

CCR6^{+} T cells (Th17) and neutrophils after injection of anti-Col17 IgG. Comparison between the three groups of mice, WT mice (blue), IL-17A$^{-/-}$ mice (red); IL-17A/F$^{-/-}$ mice (black). Results are shown as mean ± SD. Statistical analysis was performed using Kruskal-Wallis multiple comparisons test, (GraphPad Prism 6.0). Significance levels were set at **, p<0.01; ***, p<0.001.

IL-17F

The expression of IL-17F in the lesional skin was found in WT mice and IL-17A$^{-/-}$ mice. Compared with IL-17A$^{-/-}$ and IL-17A/F$^{-/-}$ mice, WT mice showed significantly more IL-17A on CCR6^{+} T cells (Th17 cells) (p=0.004, p=0.0006) and neutrophils (p= 0.0002, p=0.0005). In contrast, γδ T cells, macrophages, and mast cells did not show any expression of IL-17A (**Figure 3.16**).

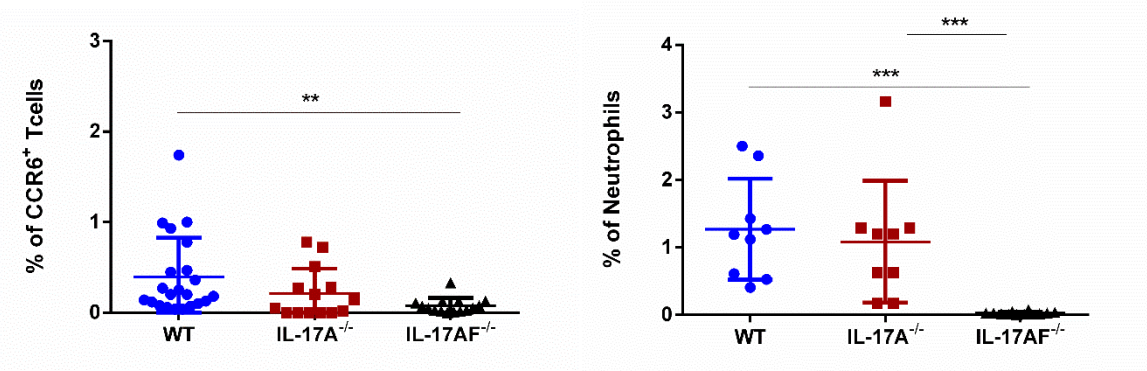

Figure 3.16: Expression of IL-17F in lesional skin of wild-type mice, IL-17A$^{-/-}$ and IL-17A/F$^{-/-}$ mice

CCR6^{+} T cells (Th17) and neutrophils after injection of anti-Col17 IgG. Comparison between the three groups of mice, WT mice (blue), IL-17A$^{-/-}$ mice (red); IL-17A/F$^{-/-}$ mice (black). Results are shown as mean ± SD. Statistical analysis was performed using Kruskal-Wallis multiple comparisons test, (GraphPad Prism 6.0). Significance levels were set at **, p<0.01; ***, p<0.001.

IL-21

The production of IL-21 was detected mainly detected in WT mice and low levels in IL-17A$^{-/-}$ and IL-17A/F$^{-/-}$ mice. IL-21 was predominantly produced by CCR6^{+} T cells (Th17 cells), and γδ T cells (**Figure 3.17**) In IL-17A$^{-/-}$ and IL-17A/F$^{-/-}$ mice, CCR6^{+} T cells (Th17 cells) were found to produce significantly lower IL-21 levels compared to WT mice (p= 0.0458, p=0.0350).In contrast, IL-21 expression on γδ T cells was significantly different in IL-17A$^{-/-}$ mice compared to both WT and IL-17A/F$^{-/-}$ mice (p=0.0122, p=0.0035).

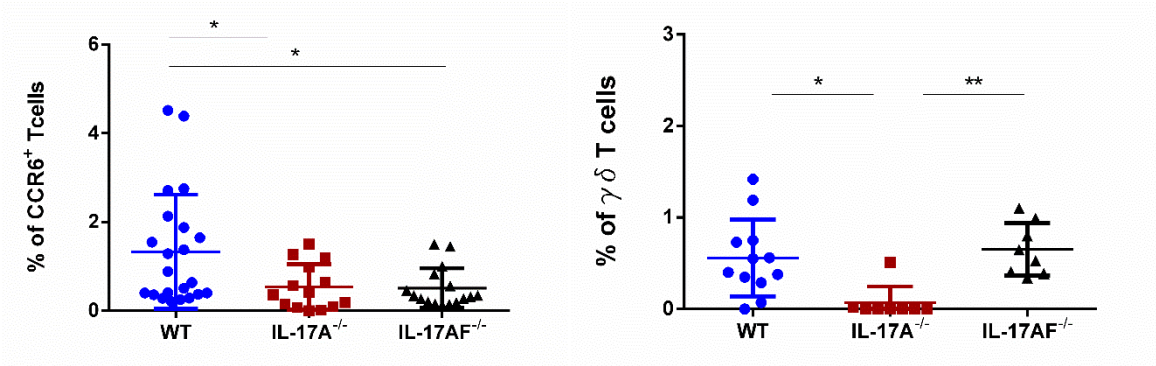

Figure 3.17: Expression of IL-21 in lesional skin of wild-type mice, IL-17A$^{-/-}$ and IL-17A/F$^{-/-}$ mice

CCR6+ T cells and γδ T cells after injection of anti-Col17 IgG. Comparison between the three groups of mice, WT mice (blue), IL-17A$^{-/-}$ mice (red); IL-17A/F$^{-/-}$ mice (black). Results are shown as mean ± SD. Statistical analysis was performed using Kruskal-Wallis multiple comparisons test, (GraphPad Prism 6.0). Significance levels were set at *, p<0.05 **, p<0.01; ***.

IL-22

Expression of IL-22 in lesional skin of mice was detected only on CCR6^{+} T cells (Th17 cells). WT mice showed the highest production of IL-22. A significant difference in IL-22 expression of CCR6^{+} T cells (Th17 cells) was detected between WT mice and IL-17A/F$^{-/-}$ (p=0.0299), but not between WT and L-17A$^{-/-}$ and IL-17A/F$^{-/-}$ mice (**Figure 3.18**). No IL-22 expression was detected on γδ T cells, neutrophils, macrophages, and mast cells.

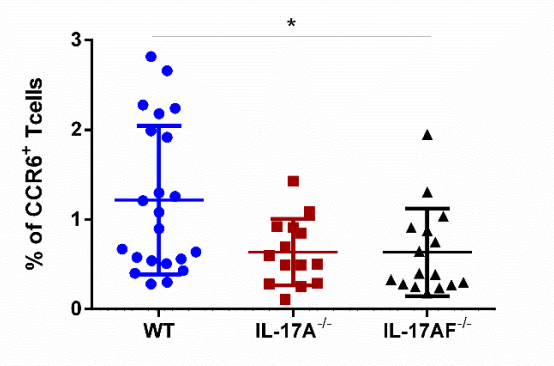

Figure 3.18: Expression of IL-22 in lesional skin of wild-type mice, IL-17A$^{-/-}$ and IL-17A/F$^{-/-}$ mice on CCR6^{+} T cells

Comparison between the three groups of mice, WT mice (blue), IL-17A$^{-/-}$ mice (red); IL-17A/F$^{-/-}$ mice (black). Results are shown as mean ± SD. Statistical analysis was performed using Kruskal-Wallis multiple comparisons test, (GraphPad Prism 6.0). The significance level was set at *, p<0.05.

3.1.1. Down-regulation of IL-17A and related genes in perilesional skin of IL-17A$^{-/-}$ mice compared to wild-type mice

To characterise the regulatory network in the antibody transfer-induced mouse model of BP the expression of 28 genes connected to Th17 cells were analysed for their expression in IL-17A$^{-/-}$ and WT mice at day 12 after the injection of anti-Col17 IgG, (**Appendix, Table 7.2, Table 7.9**). When the gene expression in the skin was compared between IL-17A$^{-/-}$ and WT mice, 15 of 28 genes were found to be down-regulated in IL-17A$^{-/-}$ animals including *il17f, il17rc, il17ra,* and *il22,* as a part of Th17 lineage. *il6* and *Ly6g,* which were shown to be directly involved in the pathogenesis of BP (Schmidt and Zillikens, 2013), as well as *cd4, cd69, cd244, cd8a, Itgam, Icam1, Casc3* and *il-1β* were also down-regulated in IL-17A$^{-/-}$ mice (**Figure 3.19**). There was no significant difference between the down-regulated genes from IL-17A$^{-/-}$ and WT mice (**Appendix, Table 7.9**). No changes in the expression of *Foxp3, Klrb1c, Ccl20, Il-21, Ccr6, Il12b, Il23a, Stat3, Cd3e, Il23r* and *Il27* was detected between the investigated groups (IL-17A$^{-/-}$ and WT mice). As expected, *Il17a* was not detectable in IL-17A$^{-/-}$ mice, and the average threshold cycle in WT mice was relatively high (>30), meaning that its relative expression level was too low. A full description of the gene expression is presented in **Appendix (Table 7.9)**.

3.1.2. Anti-IL-17A antibody as a therapeutic option for bullous pemphigoid

In subsequent experiments, the pharmacological inhibition of IL-17A was analysed with the functional relevance of IL-17A in *ex vivo* experiments, and the effect of the drug *in vivo* in an experimental mouse model of BP. First, the anti-IL-17A antibody was tested in a prophylactic approach and afterwards in a therapeutic setting.

3.1.2.1. Neutralising anti-IL-17A antibody prevents split formation in the cryosection assay

The functional relevance of monoclonal mouse anti-IL-17A antibody was analysed in the cryosection model. Skin from healthy mice was incubated with serum of mice treated with mCol17 IgG for 1h RT. Neutrophils derived from the bone marrow

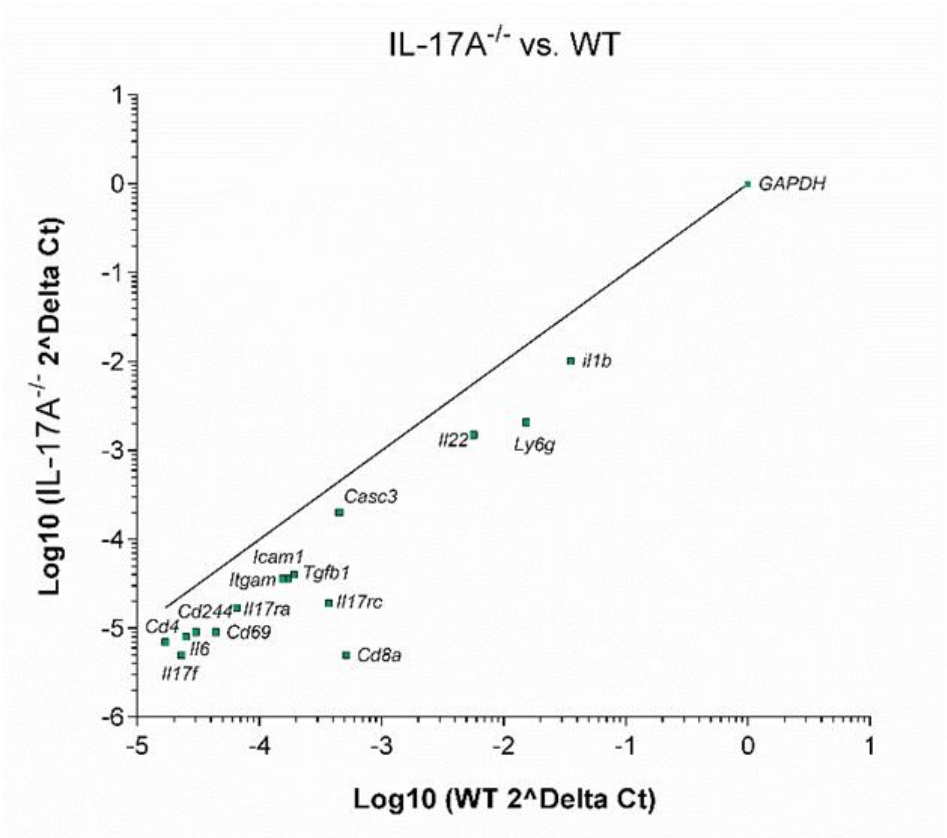

Figure 3.19: Down-regulated genes in the skin of IL-17A$^{-/-}$ mice and wild-type mice at day 12 after injection of anti-Col17 IgG

Results represent the relative expression ($2^{-\Delta CT}$) normalised to GAPDH. The p values are calculated based on a Student's t-test of the replicate for each gene in the control group (WT mice) and IL-17A$^{-/-}$ mice, and p values less than 0.05 were considered to be significant.

of WT mice were pre-incubated with the anti-IL-17A antibody or corresponding isotype control, IgG2a, in a concentration of 200 µg per section. In the WT mice and IL-17A/F$^{-/-}$ mice, 200 µg of the agent was able to block the dermal-epidermal separation, while in the isotype control split formation was detectable (**Figure 3.20**). When in subsequent experiments neutrophils isolated from the bone marrow of IL-17A$^{-/-}$ mice and IL-17A/F$^{-/-}$ mice were applied, the dermal-epidermal separation was also abrogated (**Figure 3.20**).

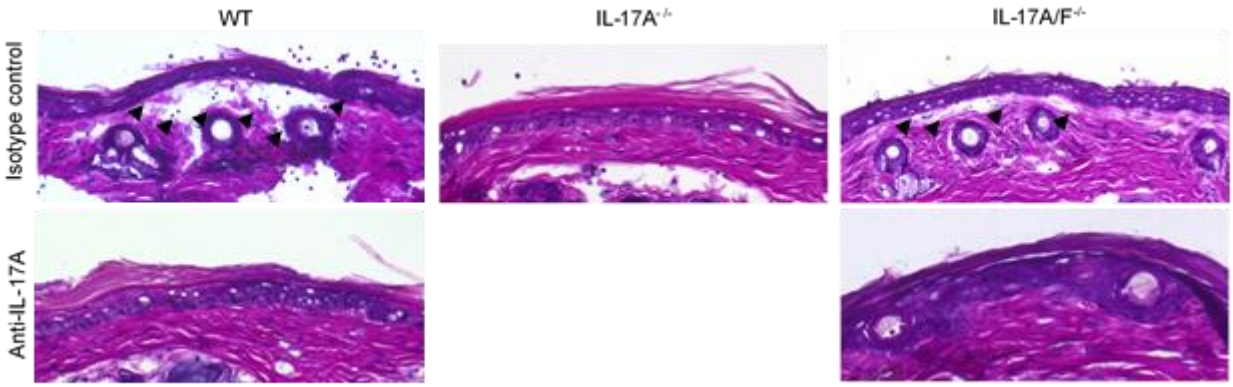

Figure 3.20: Anti-IL-17A antibody successfully prevents dermal-epidermal separation in the cryosection model

By the use of bone marrow-derived neutrophils from WT and IL-17A/F$^{-/-}$ mice treated with 200 μg anti-IL-17A antibody or neutrophils from IL-17A$^{-/-}$ mice no split formation was. In contrast, employing isotype control, (IgG2a) in WT mice used in 200μg concentration showed split formation. Representative pictures from three independent experiments are shown.

3.1.2.2. Prophylactic use of anti-IL-17A antibody significantly reduce disease activity in the antibody transfer-induced mouse model of bullous pemphigoid

The anti-IL-17A antibody was applied 2 days before induction of the disease, by injection of anti-Col17 at day0. Animals were treated with 200 μg of anti-IL-17A antibody (n=8) or isotype control (n=8) every other day. This dose was considered effective from our *ex vivo* experiments. From day 0, mice received 6 injections of 10 mg of mCol17 IgG, every second day (**Figure 3.21**).

Until day 8 mice from both groups, treatment group and isotype control group, developed clinical disease in parallel. The first visible difference started from day 8, and at the end of the experiment at day 14, a significant difference (p= 0.0104) was seen in the extent of skin lesions between the anti-IL-17A antibody- and the isotype control-injected mice. The cumulative disease score, however, was not significant between both groups (**Figure 3.22**). Clinical picture and representative histology picture at day 14 are presented in **Figure 3.25.**

The prophylactic approach was then repeated, with a longer duration of 18 days to account for the apparent period of about 8 days before anti-IL-17A inhibition become effective (**Figure 3.23**). Comparable to the first experiment, the two experimental

groups started differentiating in their disease severity after day 8. Disease scores on day 14 (p=0.04), day 16 (p=0.004¸) and day 18 (p=0.0002) were significantly different

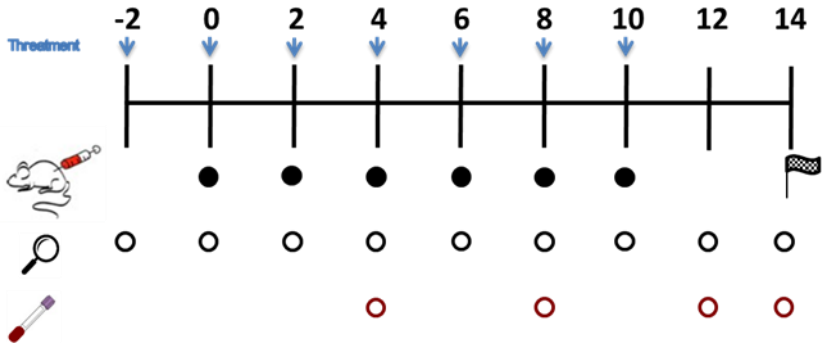

Figure 3.21: Experimental design of preventive approach with blocking anti-IL-17A antibody

Anti-IL-17A antibody or isotype control antibody was injected at the dose of 200 μg/ mouse every another day until day 10 (blue arrows). Anti-Col17 IgG was injected from day 0 to day 12 every second day at a dose of 10mg.

No significant difference in the cumulative disease score between days 0-18 was observed (n=8). By the previous experiment (**Figure 3.22**), where the effect of IL-17A inhibition become apparent after day 8, the cumulative disease score was calculated between day 8-18, and a significant difference was seen between the two experimental groups (p=0.02). Lesional histopathology showed that the isotype control treated mice revealed split formation at the dermal-epidermal junction and a dense inflammatory infiltrate in the upper dermis, repeating the clinical efficiency (**Figure 3.25**).

3.1.2.3. Use of the inhibiting anti-IL-17A antibody in a quasi-therapeutic setting reduces disease activity in antibody transfer-induced mouse model of bullous pemphigoid

After successful treatment with anti-IL-17A antibody in the preventive approach, the next experiment explored the effect of the drug in a quasi-therapeutic setting. For this purpose, the disease was induced by injecting 10 mg of anti-mCol17A IgG s.c. every other day. When the mice reached a score of 2 % of affected body surface area,

they were randomised to the experimental groups receiving i.p. treatment either with 200 μg of anti-IL-17A antibody or with 200 μg isotype control IgG2a (**Figure 3.26**).

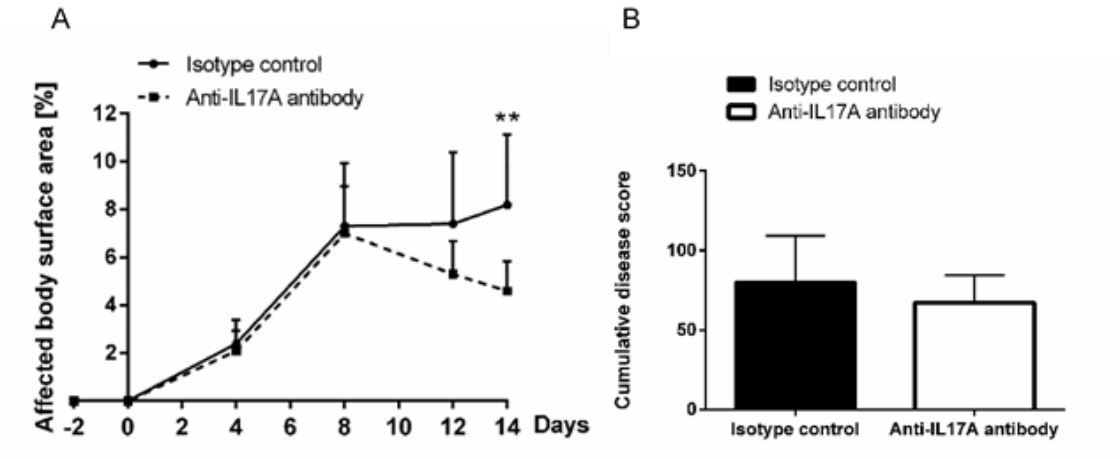

Figure 3.22: Prophylactic use of anti-IL-17A antibody in the antibody transfer-induced mouse model of bullous pemphigoid

Experimental BP was induced by injection of anti-mCol17A IgG every second day. Treatment with anti-IL-17 antibody or isotype control antibody was started on day -2; Results are shown as a mean + SD; Statistical analysis was performed using Mann Whitney test (GraphPad Prism 6.0); Significance level was set at **, $p<0.01$.

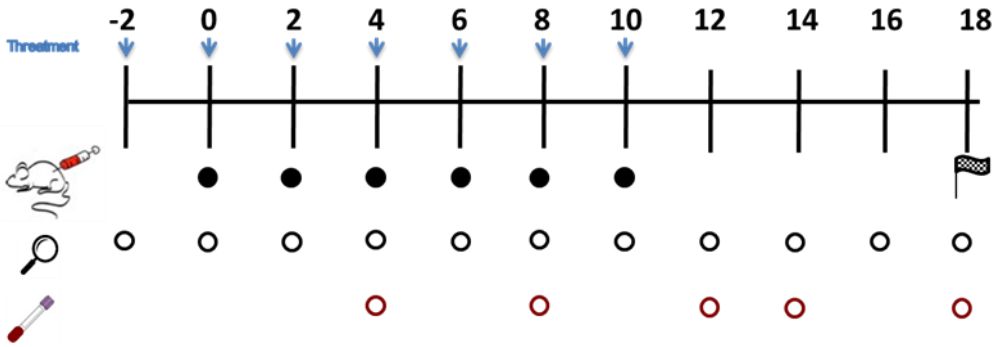

Figure 3.23: Experimental design of preventive approach with neutralising anti-IL-17A antibody

Anti-IL-17A antibody or isotype control antibody was injected at the dose of 200 μg/ mouse very another day until day 10 (blue arrows). Anti-Col17 IgG was injected from day 0 to day 12 every second day at a dose of 10mg.

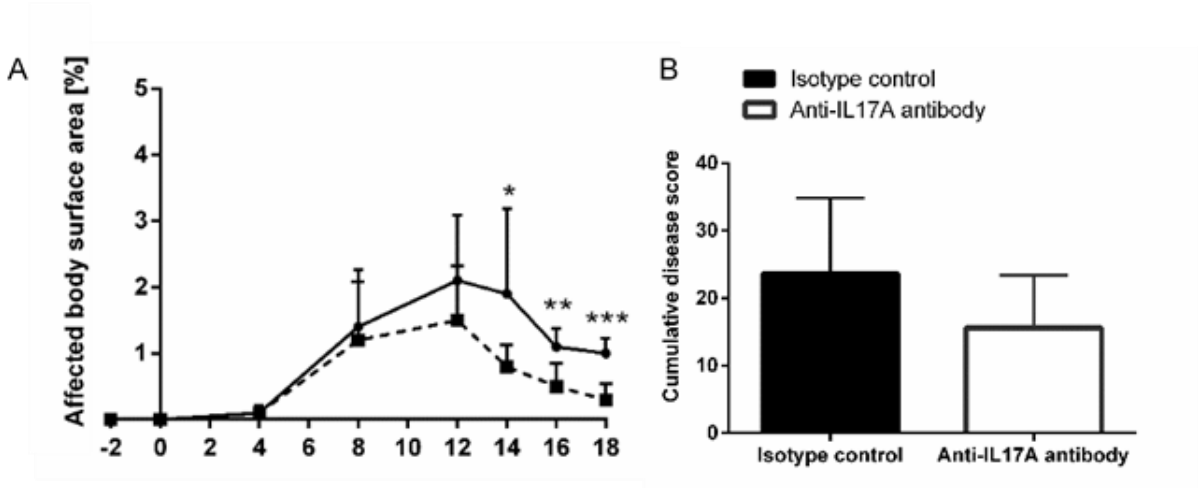

Figure 3.24: Prophylactic use of anti-IL-17A antibody in an experimental mouse model of bullous pemphigoid

The experiment was induced by injection of anti-mCol17A IgG every second day. Treatment with anti-IL-17 antibody was started on day -2. A. Affected body surface area; B. Cumulative disease score. Results are shown as a mean + SD; Statistical analysis was performed using Mann Whitney test (GraphPad Prism 6.0); Significance level was set at *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

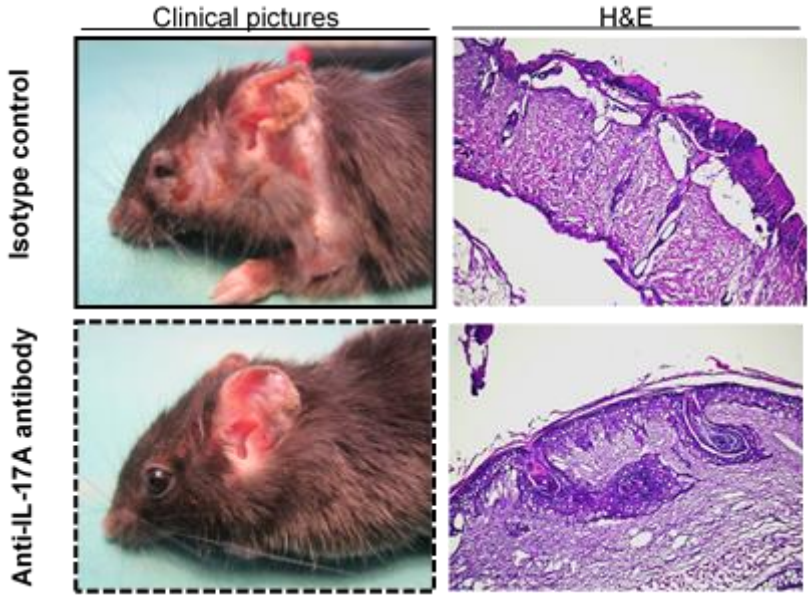

Figure 3.25: Representative pictures from prophylactic approach treated animals

Representative clinical pictures and lesional histopathology of perilesional skin biopsies are shown. The subepidermal split formation was only present in mice that had received the isotype control antibody.

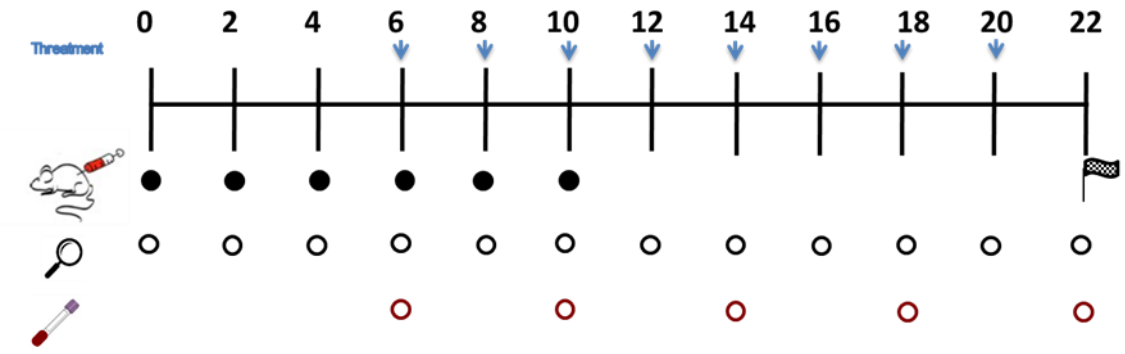

Figure 3.26: Experimental design of therapeutic approach with neutralising anti-IL-17A antibody

Anti-Col17 IgG was injected from day 0 to day 10 every second day at a dose of 10mg. Anti-IL-17A antibody or isotype control antibody was injected at the dose of 200 μg/ mouse every other day after the mice reached 2 % affected body surface area (blue arrows).

After the total experimental period of 22 days, a significant difference in the extent of the skin lesions between the two groups was seen on day 16 (p=0.028) and day 18 (p=0.012) (**Figure 3.27**). The cumulative disease scores calculated for the entire treatment period and the period starting 8 days after the initial anti-IL-17A / isotype control IgG injection and day 22 were not significantly different between mice injected with anti-IL-17A n=antibody and the isotype control, respectively (p=0.802, p=0.2) (**Figure 3.27**).

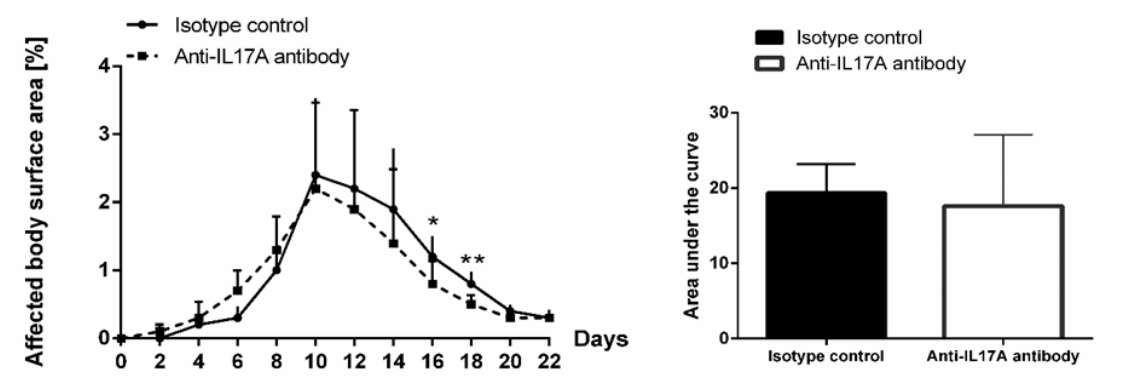

Figure 3.27: Therapeutic approach of anti-IL-17A antibody in experimental bullous pemphigoid

The experiment was induced by injection of anti-mCol17A IgG every second day. Treatment with anti-IL-17 antibody was started after the mice reached 2 % affected body surface area. A. Affected body surface area; B. Cumulative disease score. Results are shown as a mean + SD; Statistical analysis was performed using Mann Whitney test (GraphPad Prism 6.0); Significance level was set at *, p<0.05; **, p<0.01.

4. Discussion

Bullous pemphigoid (BP) is the most frequent autoimmune blistering disease in Central Europe and North America with a prevalence of about 21.000 patients in Germany (Hübner et al., 2016; Joly et al., 2012; Kridin and Ludwig, 2018; Marazza et al., 2009; Wanke et al., 2018). The only demonstrably effective therapies for BP are systemic or superpotent topical corticosteroids that are, however, associated with significant adverse events (Bech et al., 2018; Joly et al., 2002b). The number of severe adverse events and the increased mortality of BP patients are, at least in part, due to the corticosteroid treatment (Rzany et al., 2002; Schmidt and Zillikens, 2013). While recent randomised controlled clinical trials suggested some effect of doxycycline and dapsone (Sticherling et al., 2017; Williams et al., 2017), there is an utmost need of more specific, effective, and safe treatment options for BP.

Tissue destruction in BP is caused by autoantibodies against Col17, more specifically against the extracellular part of the 16th non-collagenous domain of the protein (NC16A) (Goletz et al., 2017; Schmidt and Zillikens, 2013). The reported *in vitro* and *in vivo* studies in BP have proposed two main pathways that are driving the pathogenesis in BP. The first pathway is complement-dependent, where immune complexes of autoantibodies against Col17 activate the complement system in the upper dermis (Karsten et al., 2018; Schmidt and Zillikens, 2013). The secondly proposed pathway is complement-independent and leads to depletion of Col17 from the cell surface (Iwata et al., 2018; Ujiie et al., 2014) The pathogenesis of BP is characterised by excessive inflammatory infiltration in the upper dermis. It is not clear which pathway is driving the inflammation in BP. Various chemokines and cytokines coordinate the inflammatory stage, e.g. (IL-8, and IL-6), essential for migration and activation of the different cell types, like neutrophils. Finally, neutrophils and macrophages activated by cytokines and chemokines start to secrete specific proteases and reactive oxygen species, that are ultimately responsible for blister formation (Li et al., 2018; Shimanovich et al., 2004). In addition to the critical role of cytokines and chemokines that they play in protection against pathogens and tissue maintaining homeostasis, it is of crucial importance their activity to remains controlled. Presence of anti-Col17 autoantibodies binding to Col17 has been demonstrated to trigger the inflammatory cascade response, including increased expression of pro-

inflammatory cytokines like IL-1β, IL-6, IL-8, TNF−α, GM-SCF (Nakashima et al., 2007; Samavedam et al., 2013, 2014b).

IL-17A is a pleiotropic cytokine with particular significance at epithelial barrier sites including the skin and oral cavity. It can induce the expression of proinflammatory cytokines and chemokines in various stromal and innate immune cells (Krueger and Brunner, 2018; Kuwabara et al., 2017; Veldhoen, 2017). IL-17A is of particular importance for the initiation of the immune response by mediating the influx of neutrophils and monocytes and keeping these cells at the site of inflammation (Dubin and Kolls, 2008; Veldhoen, 2017). In addition to its role in host defence against microorganisms the importance of IL-17A in autoimmunity and chronic inflammatory disorders such as inflammatory bowel disease and psoriasis has been previously highlighted (Boehncke and Schön, 2015; Kleinewietfeld et al., 2013; Ramesh et al., 2014; Veldhoen, 2017). The biologic agents targeting the inflammatory mediators revolutionised the treatment of autoimmune and inflammatory diseases. Clinical studies performed with neutralising antibodies against IL-17A have shown promising results in psoriasis and rheumatoid arthritis, reducing the disease severity to 90% (Brembilla et al., 2018; Cui et al., 2018; Kaufman and Alexis, 2018; Kurschus and Moos, 2017).

Herein, was explored the cellular sources and functional relevance of IL-17A and related inflammatory mediators in the pathophysiology of BP and probed IL-17A inhibition in the treatment of experimental murine BP.

4.1. Source of IL-17A in patients with bullous pemphigoid

These data showed that genes of the classical Th17 cell pathway are differentially expressed in the tissue of BP patients. In accordance, the expression of 18 Th17 cell-related regulatory molecules was significantly elevated. All of them are known to be involved in the development of pathogenic Th17 cells, leading to the production of the IL-17-related cytokines such us IL-17A, IL-21 and IL-22. IL-17-related cytokines expression has been already shown to be differently regulated in other inflammatory dermatoses, mucosal immunity and autoimmune diseases, including rheumatoid arthritis, inflammatory bowel diseases, and psoriasis (Kellner, 2013; Li et al., 2014; McInnes et al., 2014; Qu et al., 2013; Song et al., 2016). Thus, in agreement with published findings, these data suggest a possible pathogenic role of IL-17A in BP.

The observation that Th17 cell-associated cytokines are dysregulated at the site of blister formation in BP is indicated by up-regulated expression of *IL17RA, IL-17RC, CCR6, CD4* and *IL-23R*. These receptors are all critical for Th17 cell activation and IL-17A production (Isailovic et al., 2015; Song et al., 2016). Additionally, the growth factors *CSF2* and *CSF3*, which are involved in Th17 cell development, were found to be significantly up-regulated in the perilesional skin of BP patients. IL-17A is a potent inducer of *CXCL1* and *CXCL2* expression (Kuwabara et al., 2017), which are powerful chemoattractants for neutrophils. Up-regulated *ELANE* expression in the skin, encoding for neutrophil elastase, confirm previous data concerning the importance of neutrophils and neutrophil elastase in the pathogenesis of BP (Le Jan et al., 2014; Kuwabara et al., 2017; Liu et al., 2000; Shimanovich et al., 2004).

In addition to these findings on mRNA level, data presented in this thesis showed that IL-17A has a different expression locally and systemically. In the peripheral blood of BP and control patients, we were able to detect IL-17A and IL-22 on $CD4^+$ cells, whereas $CD8^+$ cells, neutrophils, and macrophages did not show a significant contribution to IL-17A production in BP patients.

When looking into a local expression using immunofluorescence analysis, IL-17A was detectable on the site of early skin lesion, when analysing perilesional skin of BP patients. It is possible that IL-17A is locally produced by cellular sources that are migrating to the skin in the late stage of the disease. By published data by Le Jan and colleagues, blister fluid of BP patients contain significantly higher levels of IL-17A compared to serum (Le Jan et al., 2014). The notion that IL-17A is locally produced is further supported by the fact that IL-17A and IL-17F play critical roles in defence against pathogens in the epithelial surfaces and mucous tissue (Crome et al., 2010; Heather et al., 2018; Wanke et al., 2018). In such a response, they elicit the expression of antimicrobial peptides (Yang et al., 1999). Also, they act on epithelial cells to promote the generation of the proinflammatory milieu with the production of chemokines, cytokines and matrix metalloproteinase. All these factors lead to activation and recruitment of immune cells to the site of infection.

Using peripheral blood from BP patients, also IL-22 was found to be significantly elevated analysed to sex- and age-matched patients with non-inflammatory dermatoses. Increased IL-22 levels presented in this study are in line with already published data, which support the notion of a synergistic effect of several Th17 cell cytokines (Li et al., 2014; Patel and Kuchroo, 2015). It has been shown that IL-22 is a

potent pathogenic player in psoriasis-like skin inflammation. (Ma et al., 2008; Res et al., 2010; Wolk et al., 2009a) Have been shown that IL-22 can accumulate in lesional psoriatic skin and that transgenic mice overexpressing IL-22 have aberrant skin phenotypes that mimic psoriasis (Cochez et al., 2016; Eyerich et al., 2017; Wolk et al., 2009b). In addition to psoriasis, IL-22 has been elevated in plasma and synovial fluid in patients with ankylosing spondylitis and rheumatoid arthritis (Kim et al., 2012; Zhang et al., 2012).

In contrast, IL-21 levels did not differ between BP patients and controls. IL-21 exerts broad pleiotropic functions in immune cell differentiation, proliferation and function. The primary effect of IL-21 is in the promotion of B cell activation and plasma cell differentiation (Ding et al., 2013; Moens and Tangye, 2014) and negatively regulate the balance between Th17 cells and Tregs (Fantini et al., 2007). In several studies has been shown its potential in promoting autoimmune diseases and inflammatory disorders (Liu et al., 2012; Nakou et al., 2013; Pallone et al., 2014). IL-17F showed significantly higher levels in BP patients sera and an up-regulated expression in the perilesional skin of BP patients. By so far published data, IL-17F play distinct roles in host defence against pathogens (Aujla et al., 2008; Ishigame et al., 2009b).

Regarding the pathogenic roles in autoimmunity like collagen-induced arthritis and experimental autoimmune encephalitis are still under debate (Haak et al., 2009; Sarkar et al., 2014). Whereas IL-17A/F$^{-/-}$ mice developed an intermediate phenotype compared to IL-17A$^{-/-}$ mice which were largely protected from the disease pathogenesis in experimental BP, we hypothesise that IL-17F may have an anti-inflammatory role. IL-17F$^{-/-}$ mice in experimental BP mouse model will give us a clear answer about the role of IL-17F. In this experiment, I would expect that IL-17F$^{-/-}$ mice will not be protected from the pathogenic effect of anti-Col17 IgG. My expectations simply could be explained by the fact that BP is characterised with extensive skin lesions, resulting in the disturbed epithelial barrier, which in turn leads to higher bacterial diversity.

To identify the cellular source of IL-17A immunofluorescence analyse was applied. Perilesional skin of BP patients was used in order to investigate which cell types produce IL-17A. Since in this tissue, subepidermal splitting has not yet occurred, but it is close to a macroscopic blister, we assume that the biopsy site is representative for the early stage of the disease. For this experiment, the polyclonal goat anti-human IL-17A antibody (clone Ile20Ala155), which is extensively used by other researchers in

the field, was initially applied (Burgler et al., 2009; Le Jan et al., 2014). Importantly, Velden et al., and Tamarozzi et al., showed that this polyclonal goat anti-human IL-17A antibody revealed several bands by western blotting, but none of the detected bands corresponds to IL-17A (Tamarozzi et al., 2014; Velden et al., 2012). When we found out that this detection antibody was contaminated with myeloperoxidase, a mouse monoclonal anti-human IL-17, (clone eBio64DEC17) was used. Comparing the two anti-IL-17A detection antibodies, we found less positive IL-17A cells with mouse monoclonal anti-human IL-17 than polyclonal goat anti-human IL-17A. By noticed differences and the published data between the anti-IL-17A antibodies, further analyses were performed with the mouse monoclonal anti-human IL-17A.

The tissue was stained for cell populations known to express IL-17A, i.e. neutrophils, macrophages, $CD3^+$ lymphocytes, and mast cells (Le Jan et al., 2014; Zebrowska et al., 2014). IL-17A expression was found to be mainly co-expressed with $CD3^+CD4^+$ and to a lesser extent on mast cells and neutrophils. In details, $CD3^+$ cells present 60 % of infiltrated cells. 40.5 % of them were IL-$17A^+$. $CD4^+$ cells present 33.8 % of total cell number, or 83 % of $CD3^+$ cells. 60.6 % of $CD4^+$ cells were found to be double positive for IL-17A. 18 % of the total cell number belongs to macrophages marked as $CD68^+$ cells, while 44.5 % of them were detected as IL-$17A^+$ cells. Neutrophils present 15 % of the cell infiltrate and 35 % of them were double positive for IL-17A. Mast cells were the less frequent population detected in the early lesions from BP patients, presented with 7 %, but beside the frequency, almost half of all mast cells or 45 % of them were found to be $IL17^+$ cells. From obtained results, $CD3^+CD4^+$ cells represent the major source of IL-17A in the tissue of BP patients. Taking into account the small per cent of the presence of mast cells and the high positive IL-17 cells expressed on the surface, they represent a significant source of this cytokine. Followed by macrophages and the last cell type which expresses IL-17A are neutrophils. In the next step, confocal microscopy was applied to differentiate between cells that showed IL-17A staining on the cell surface and cells that showed intracellular IL-17A signals. Assuming that in cells IL-17A reacting on the cell surface, was bound to the ubiquitously expressed IL- 17RA/IL-17RC, e.g. on different T cell subsets and neutrophils or they phagocyte, store and release bioactive IL-17A (Gaffen, 2009; Lin et al., 2011; Lombard et al., 2016; Monin and Gaffen, 2017a; Noordenbos et al., 2016). In the next set of experiments, we aimed at confirming the data stained by immunofluorescence by measuring mRNA levels of *IL17A* in perilesional BP skin

compared to the skin of patients with other non-inflammatory dermatoses. The expression of IL-17A was confirmed on mRNA level where *IL17A* was found to be significantly upregulated in the perilesional skin. In addition to IL-17A, we found *CCR6*, a receptor expressed on the surface of Th17 cells and *IL17RC*, receptor critical for IL-17A signalling, to be also significantly expressed. More important for BP was that *ELANE*, a gene representative for neutrophil elastase, and *CSF2*, *CSF3* and *IL6* were also found to be significantly upregulated. We know that these genes and their expressed cytokines are critical in the pathogenesis of BP in the infiltration of different inflammatory cells and the production of ROS, leading to blister formation Taking together studies on mRNA level of IL-17A confirmed the findings of elevated IL-17A expression by immunofluorescence. The data from the experiment with immunofluorescence are in part contradicting to previously published data from Le Jan *et al.*, (Le Jan et al., 2014), that showed that neutrophils to be the primary source of IL-17A in lesional skin of BP. There are two explanations for these deceptive results 1) the use of different tissue for staining. Whereas Le Jan et al. used lesional skin from BP patients, in this study, perilesional skin was taken. 2) Le Jan and co-workers used the polyclonal goat anti-human IL-17A, revealed unspecific staining in two other studies (Tamarozzi et al., 2014; Velden et al., 2012), whereas in the present study the mouse monoclonal anti-human IL-17A antibody was employed. The literature for possible sources of IL-17A is contradictory. Most of the studies showed that IL-17A is mainly produced by Th17 cells and tissue resident cells like $\gamma\tau$ T cells, while phagocytic cells only store and transport the cytokine, but they do not synthesize it (Huppler et al., 2015; Noordenbos et al., 2016; Quesniaux et al., 2013; R et al., 2012; Schulz et al., 2008; Vazquez-Tello et al., 2012). Therefore, further experiments are required to authorise the primary source of IL-17A in BP, e.g. playing single-cell mRNA sequencing.

The presence of $CD3^+$ cells in BP patient skin obtained with immunofluorescence analysis, was additionally, validate with routine immunohistochemistry for $CD4^+$ and $CD8^+$. This staining validated the findings obtained by immunofluorescence microscopy. We could show that the majority of infiltrating cells in the perilesional BP skin are $CD4^+$ cells with 65 % of the counted cells, followed by $CD8^+$ cells with 21 %. CD68 was used as a marker for macrophages, which are also present in an enviable number, accounted to be present with 12 %. Only B cells, identified by CD20 expression were not detected in high amount, 2 %, in the perilesional skin BP patients.

Detection of B cells is not unexpected since the major production of anti-Col17 antibodies and consequently, of Col-17 specific B cells is thought to be present. Interestingly, however, autoantibodies specific B cells have recently been found in the skin of patients with pemphigus, another blistering autoimmune disease (Kasperkiewicz et al., 2017).

4.2. Functional relevance of IL-17A in experimental bullous pemphigoid

The role of numerous individual cytokines in the disease development of bullous pemphigoid is still debated. In a set of studies in an experimental model of bullous-like disease, EBA, was shown that IL-6 is essential in the tissue destruction. Treatment in mice with anti-IL-6 antibody showed reduced disease score (Samavedam et al., 2013). The protective effect of IL-6 was thought to be mediated throw IL-1ra. Additionally, this was proved with prophylactic administration of IL-1ra in mice injected with anti-Col7 IgG (Samavedam et al., 2013). Another study from the same group demonstrated that patient with EBA end mice injected with anti-Col7 IgG has a significant increase in the expression of IL-1β. Inline, mice treated with neutralising IL-1b antibody, as well as IL-1r antagonist, were less diseased compared to wild-type mice (Sadeghi et al., 2015). The third study reported elevated levels of TNF in mice injected with anti-Col7 IgG. When soluble TNF receptor fusion protein etanercept (Enbrel®) was used or a monoclonal antibody to murine TNF, mice showed significantly less disease (Hirose and Kasprick, 2017a).

IL-17A was shown to be important in the pathogenesis of autoimmune diseases and inflammatory diseases such as psoriasis, rheumatoid arthritis, and inflammatory bowel disease (Boehncke and Schön, 2015; Hueber et al., 2010; Kellner, 2013; Ma, 2012; Miossec, 2009). Psoriasis patient treated with anti-IL-17A antibody respond successfully with a 90 % reduction. In psoriasis, IL-17A stimulates the production of chemokines, cytokines, and other proinflammatory mediators from keratinocytes thereby enabling IL-17A to bridge the connecting the innate and adaptive immune systems to sustain chronic inflammation (Lynde et al., 2014). In rheumatoid arthritis, it is believed that IL-17A up-regulates the expression of critical inflammatory genes in target cells such as keratinocytes and fibroblasts. This result in increased production of chemokines, cytokines including IL-17A, antimicrobial peptides and more mediators contributing to clinical disease features (Lubberts, 2015). The effect of IL-17A has also

been described in inflammatory bowel disease. Its mild effect it is not clear yet how it is regulated on the molecular level (Neurath, 2014). Several previous studies have shown that IL-17A may act as a chemoattractant for neutrophils (Muranski and Restifo, 2013; 2015).

The role of IL-17A in blister formation of BP was investigated in several *in vitro* experiments. These *in vitro* experiments indicated that IL-17A is relevant for blister formation in BP. The split formation was successfully prevented by reabsorption of leukocytes with 200 μg and 300 μg of anti-IL-17A antibody. In the cryosection model of both human and mouse skin. Gammon and colleagues and later Sitaru *et al*., described the importance of the leukocytes and autoantibodies in the dermal-epidermal separation in BP (Gammon et al., 1982; Sitaru et al., 2002). Additionally, that IL-17A has a substantial impact was also demonstrated by blocking the reactive oxygen species (ROS), formation induced by immune complexes of Col17 and anti-Col17 IgG, with an anti-IL-17A antibody. ROS is one critical factor leading to dermal-epidermal separation (Liu et al., 1997). Kasparkiewicz and colleagues also showed the vital role of FcγRIV and FcγRIIB receptors in mediating the skin inflammation in mice. Another group demonstrated that glycosylated IgG1 immune complexes bind to the inhibitory FcγRIIB and dectin-1 complex, and inhibit C5aR-mediated effects in neutrophils in experimental bullous like disease-Epidermolysis bullosa acquisita (Karsten et al., 2012; Kasperkiewicz et al., 2012) In experimental BP lesion formation depended on FcgRIV and FcgRIII, whereas FcgRIIB is protective. (Schulze et al., 2014). Immune complexes alone showed less ROS production compared to immune complex-activated neutrophils preincubated with recombinant IL-17A antibody or neutrophils with recombinant IL-17A only.

Th17 cells have been linked to various autoimmune diseases including multiple sclerosis, psoriasis, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, and asthma. (Cho, 2013; Harden et al., 2015; Monin and Gaffen, 2017a; Niu et al., 2012; Peters et al., 2011; Wen et al., 2013). Our paucity knowledge about the contribution of Th17 cells in the pathogenesis of BP, the role of IL-17A and IL-17F was investigated in the antibody transfer-induced mouse model of BP. We used WT mice as a control for IL-17A$^{-/-}$ mice and IL-17A/F$^{-/-}$ mice. While IL-17A$^{-/-}$ mice were largely protected from the disease, IL-17A/F$^{-/-}$ mice showed an intermediate phenotype. IL-17A/F$^{-/-}$ mice were significantly less diseased compared to WT mice and significantly more skin lesions compared to IL-17A$^{-/-}$ mice. The results may be

explained due to IL-17F obliteration. It is possible that the deletion of IL-17A leads to overexpression of IL-17F in the skin. Therefore, we assume that IL-17F has an anti-inflammatory function. In our setting when mice lack both, IL-17A and IL-17F, there is no compensation effect of IL-17F and BP can be induced earlier in the mice.

Further experiments are required to confirm the protective effect of IL-17F, e.g. by the injection of anti-Col17 IgG in IL-17F$^{-/-}$ mice. We hypothesise that IL-17F$^{-/-}$ mice will develop more skin lesions compared to WT mice after injection of Col17 specific IgG. To get further insights into the regulatory effect of IL-17A, we analysed the production of IL-17A and related cytokines in the skin of IL-17A$^{-/-}$, IL-17A/F$^{-/-}$, and WT mice. Interestingly, increased numbers of IL-17F^{+} neutrophils were detected in the skin of IL-17A$^{-/-}$ mice. Of note, Th17 cells were present in plethora in the skin of IL-17A$^{-/-}$ mice, and also significantly more neutrophils were found in the skin lesions of IL-17A$^{-/-}$- mice compared to both, IL-17A/F$^{-/-}$ mice and WT mice. These results mean that neutrophils are attracted to the site of inflammation, but they are unable to be activated. This conclusion is further supported by our *in vitro* finding that neutrophils can be activated by recombinant IL-17A, able to release ROS and proteases that finally lead to dermal-epidermal splitting. It may be speculated that IL-17A is not essential for attracting neutrophils to the skin, but may be critical for their activation. In fact, strong neutrophil attractants such as IL-8 and C5a have been previously described to be present in BP skin and have a functional role in blister formation of BP (Karsten et al., 2014; Liu et al., 1993; Sadik et al., 2018; Schmidt et al., 2001). Additional experiments are required to confirm this hypothesis.

Another exciting point from this experiment was the histopathological finding from the three groups of mice (IL-17A$^{-/-}$, IL-17A/F$^{-/-}$, and WT) that showed that WT mice had severe split formation, IL-17A/F$^{-/-}$ mice also revealed dermal-epidermal separation to the same extent, while IL-17A$^{-/-}$ mice did not show any separation but intriguing, did not differ in the cellular infiltration. IL-17A$^{-/-}$ mice had as many inflammatory cells as WT mice and IL-17A/F$^{-/-}$ mice. The fact that IL-17A$^{-/-}$ mice are protected from disease but not IL-17A/F$^{-/-}$ mice once again is going in line with the previous hypothesis of the protective effect of IL-17F in this disease. It is known that IL-17A has a 100 fold stronger affinity to bind to the IL-17RA/IL-17RC receptor complex compared to IL-17F (Awasthi and Kuchroo, 2009; Hirota et al., 2012; Monin and Gaffen, 2017a, 2017b). They share the same binding receptor complex IL-17RA, IL-17RC as homodimers or IL-17RA/RC

heterodimer. Additional experiments for the presence of IL-17F in the tissue in IL-17A$^{-/-}$ mice and immunofluorescence analysis will help to confirm our hypothesis.
Concerning the production of IL-17 related cytokines in the skin of mice, we found that IL-21 and IL-22 are mainly detected in CCR6^{+} cells, a receptor which is mainly expressed on Th17 cells. Only minor or no production of IL-21 and IL-22 was detected on the remaining cell types in the skin. These findings are in line with published data of IL-22 in BP (Le Jan et al., 2014). Le Jan *et al.,* found significantly more IL-22 in the blister fluid of BP patient. Additionally, it is in line with other inflammatory dermatoses demonstrating the role of IL-21 and IL-22, and their contribution to disease severity. In psoriasis Cho *et al.,* reported that IL-22 and IL-17A enhanced skin inflammation through stimulation of IL-1β production and ROS release (Cho et al., 2011), while Wolk *et al.,* reported the potential of IL-22 to induce the secretion of IL-20 in psoriasis (Wolk et al., 2009b). It was shown that IL-21 contribute to IL-17A production from Th17 cells (Korn et al., 2007). Additionally, IL-21 has been shown to act as an amplifier of Th17 cells. Therefore it enhances and maintains their presence at the site of skin inflammation (Wei et al., 2007; Zenewicz et al., 2007).
Our findings of elevated expression of IL-17A and related cytokines in early skin lesions of BP patients were validated with a similar approach was chosen in mice. A panel of 28 genes related to IL-17A was selected to check their expression, including cytokines, receptor, regulatory molecules and chemokines. The expression was detected in the skin from IL-17A$^{-/-}$ mice injected with mCol17 IgG and compared with WT mice injected with anti-Col17 IgG. Fifteen genes were found to be down-regulated in IL-17A$^{-/-}$ mice, among those 15 genes are cytokines, chemokines like *Il22, Il6, Il17f, Il1β.* Receptors for IL-17A, *Il17ra* and *Il17rc*, *as* well as *Ly6g*, a molecule for neutrophils. Mentioned cytokines result in the inflammatory response in BP. IL-22 and IL-6 act on keratinocytes; they continue to synthesise signals for attracting neutrophils and other cells. Neutrophils than migrate to the upper dermis and produce reactive oxygen species, responsible for the blister formation, a hallmark in BP patients. Released IL-17A binds to the IL-17RA, and IL-17RC expressed on the cell surface and further support the inflammation, e.g. with activation of the neutrophils. Several clusters of differentiation molecules like *Cd4* for T cells*, Cd8a* for cytotoxic T cells*, Cd68* for macrophages*, Cd244* for dendritic cells were significantly downregulated. Most of the down-regulated genes in the IL-17A$^{-/-}$ mice, with exception to *CD8a*, were up-regulated in the skin of BP patients. Of note, the great similarity between our findings in the skin

of BP patients and WT mice injected with anti-Col17 IgG corroborate the assumption that indeed, the antibody transfer-induced mouse model of BP reflects primary immunopathological characteristics of human BP. Moreover, these results indicate that IL-17A has a significant impact on the inflammatory process in the antibody transfer-induced mouse model of BP. Levels of IL-17A were not detectable in the skin of IL-17A$^{-/-}$ mice. Low expression of IL-17A was detected in the skin of WT mice.

The therapeutic potential of IL-17A inhibition became famous following a successful clinical trial in psoriasis. The successful treatment of psoriasis patients with 300mg secukinumab in PASI 75 was 81.6% showed a response to the treatment within 12 weeks (Gisondi et al., 2014; Langley et al., 2014; McInnes et al., 2014; Patel et al., 2013). The available anti-IL-17A antibodies include secukinumab and ixekizumab, as well as anti-IL-17RA inhibitor Brodalumab. In January 2015, Secukinumab, an IL-17 inhibiting monoclonal antibody was approved by the FDA approved in 2015, for the treatment of moderate to severe plaque psoriasis and psoriatic arthritis. Brodalumab is approved for psoriasis. Also, Cosentyx has been approved in Japan for use in treating psoriatic arthritis. In this study, we found that IL-17A has a functional relevance in the pathogenesis of BP in several *in vitro* studies. We also showed that IL-17A$^{-/-}$ mice were largely protected from the pathogenic effect of anti-Col17 IgG. Thus, in the next set of experiments, we tested the effect of an anti-IL-17A analogue in the antibody transfer- induced mouse model of BP in a preventive setting, using the effective dose determined in our *in vitro* experiments. With this approach, the effect of the anti-IL-17A antibody was seen after six injections of 200 μg, which at the end always yielded in a significant reduction in disease progression in comparison to isotype treated group. Of note, the effect of anti-IL-17A antibody was only seen after 12 days. Several explanations may be discussed why no complete prevention of disease development was noted: 1) the applied dose of 200 μg/injection was not sufficient to completely deplete IL-17A function in the skin; 2) the endogenous levels of IL-17A may have been higher in WT mice compared to IL-17A$^{-/-}$ mice; 3) the anti-IL-17A antibody distribution was not adequate; 4) other pathogenic factors different from IL-17A could compensate for IL-17A. Another reasonable explanation for our results could be the mechanism of action of the anti-IL-17A antibody itself. We could demonstrate that the anti-IL-17A antibody successfully prevented immune-complex mediated blister formation in cryosection of human skin and ROS release from human leukocytes isolated healthy

volunteers. We hypothesise that the anti-IL-17A prevents the binding of IL-17A to the receptor, and at the same time blocks IL-17F.

The anti-IL-17A antibody was also tested in a quasi-therapeutic setting and showed a similar effect. After six injections of 200 μg/injection of anti-IL-17A antibody, the first visible differences in the extent of the skin lesions were observed between the anti-IL-17A antibody treated group and isotype-treated group. These *in vivo* experiments suggested IL-17A inhibition as a promising treatment model in BP. Further studies in experimental murine BP will aim at identifying the primary target cell of IL-17A in the skin lesions to further explore how IL-17A exerts its effect in BP.

Cytokines have multiple faces, depending on the surrounded mediators. Their crucial role in innate and adaptive immune response has been verified many times in the past. Such events include induction of higher expression of pattern recognition receptors, endorse chemotaxis of phagocytes (macrophages and polymorphonuclear leucocytes), as well as activation and proliferation of T cell population and antibody-producing B cells and tissue repair (Hattori et al., 2015). Disbalance between pro-inflammatory and anti-inflammatory cytokines initiates an appropriate immune response. Cytokines control numerous inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease and many other (Dinarello, 2000; Neurath, 2014; Zhang and An, 2007). Autoimmune bullous diseases are also modulated by cytokines and chemokines (D'Auria et al., 1999; Ludwig and Schmidt, 2009; Schmidt and Zillikens, 2013). IL-6, GM-CSF, IL-1β and TNFα, are some of the cytokines, which have already been tested for their therapeutic potential in bullous diseases. Inhibition of these cytokines only showed minor or no effect in different mouse models (Kasperkiewicz et al., 2016; Samavedam et al., 2013, 2014b) The current therapy for BP patients relies on topical or systemic corticosteroids. Thus, the identification of key inflammatory molecules that can be targeted pharmacologically is highly needed in BP.

5. Conclusions

Based on the present study, we conclude that in this study IL-17A as a cytokine with a potential pathogenic role in BP and as a promising new therapeutic target. We demonstrated that IL-17A is mainly present at the site of lesions, primarily produced by T cells and that it is silencing successfully prevented blister formation and ROS release production from neutrophils *in vitro*. We also showed that specific deletion of IL-17A in mice protected them from the pathogenic effect of anti-Col17 IgG. Furthermore, treatment of mice in experimental BP with an anti-IL-17A antibody in prophylactic and therapeutic settings could ameliorate the disease activity. Taken all together, the results of this study encourage investigating the therapeutic potential of IL-17A inhibition in clinical trials in patients with BP.

Outlook

Future studies can more precisely determine the crucial role of IL-17A in bullous pemphigoid. Having in mind that the animal model of bullous pemphigoid is of great help for exploring the pathogenesis of these diseases, the same can be used for more mechanical experiments.

1. A kinetic experiment will show at which time point IL-17A is produced in bullous pemphigoid patients.
2. Experiment with IL-17RA$^{-/-}$ mice is essential to confirm the signalling of IL-17A, which subunit is dominant in IL-17A response, and further help for better understanding and contribution of IL-17F in bullous pemphigoid
3. IL-17A-GFP reporter mice will precisely show which cell type is essential in the production of IL-17A during bullous pemphigoid and further help for silencing the cytokine specifically on that cell type
4. Mice lacking IL-17RA receptor on the specific cell types will uncover which cell type is required for IL-17A signalling at first place and give a direction for further experiments.

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7.3. Materials and methods

Table 7.1: Description of human Th17 cells pathway-related genes

Unique Assay ID	Symbol	Description
qHsaCID0011608	*CCL2*	Chemokine (C–C motif) ligand 2
qHsaCID0011773	*CCL20*	Chemokine (C–C motif) ligand 20
qHsaCED0034607	*CCR6*	Chemokine (C-C motif) receptor 6
qHsaCED0005613	*CD4*	CD4 molecule
qHsaCED0034773	*CD8a*	CD8a molecule
qHsaCED0002766	*CSF2*	Colony stimulating factor 2 (granulocyte–macrophage)
qHsaCED0033948	*CSF3*	Colony stimulating factor 3 (granulocyte)
qHsaCED0046130	*CXCL1*	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
qHsaCED0002502	*CXCL2*	Chemokine (C-X-C motif) ligand 2
qHsaCED0048210	*ELANE*	Elastase, neutrophil expressed
qHsaCED0004281	*ICAM1*	Intercellular adhesion molecule 1
qHsaCID0017614	*IFNg*	Interferon, gamma
qHsaCED0003369	*IL10*	Interleukin 10
qHsaCID0016458	*IL12B*	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
qHsaCEP0057499	*IL12RB1*	Interleukin 12 receptor, beta 1
qHsaCID0015941	*IL17A*	Interleukin 17A
qHsaCID0011295	*IL17F*	Interleukin 17F
qHsaCID0014875	*IL17RA*	Interleukin 17 receptor A
qHsaCED0001213	*IL17RC*	Interleukin 17 receptor C
qHsaCID0015409	*IL2*	Interleukin 2
qHsaCID0008071	*IL21*	Interleukin 21
qHsaCID0022987	*IL22*	Interleukin 22
qHsaCJD0034582	*IL23A*	Interleukin 23, alpha subunit p19
qHsaCED0045995	*IL23R*	Interleukin 23 receptor
qHsaCID0014412	*IL27*	Interleukin 27
qHsaCID0013645	*IL4*	Interleukin 4
qHsaCED0044677	*IL6*	Interleukin 6 (interferon, beta 2)
qHsaCED0044756	*IRF4*	Interferon regulatory factor 4
qHsaCED0056899	*KLRB1*	Killer cell lectin-like receptor subfamily B, member 1
qHsaCID0008528	*RORc*	RAR-related orphan receptor C
qHsaCED0003543	*SOCS3*	Suppressor of cytokine signalling 3
qHsaCED0037461	*TNF*	Tumour necrosis factor

Table 7.2: Description of mouse Th17 cells pathway-related genes

Unique Assay ID	Symbol	Description
qMmuCED0039691	*Ccl20*	Chemokine (C–C motif) ligand 20
qMmuCED0039839	*Ccr6*	Chemokine (C-C motif) receptor 6
qMmuCID0022320	*Cd4*	CD4 molecule
qMmuCID0016523	*Cd8a*	CD8a molecule
qMmuCED0046689	*Ly6g*	Lymphocyte antigen 6 complex, locus G
qMmuCID0005575	*Icam1*	Intercellular adhesion molecule 1
qMmuCID0022424	*Il12b*	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
qMmuCID0026592	*Il17a*	Interleukin 17A
qMmuCID0006376	*Il17f*	Interleukin 17F
qMmuCID0005263	*Il17ra*	Interleukin 17 receptor A
qMmuCID0026621	*Il17rc*	Interleukin 17 receptor C
qMmuCED0045758	*Il21*	Interleukin 21
qMmuCED0052207	*Il22*	Interleukin 22
qMmuCED0045759	*Il23a*	Interleukin 23, alpha subunit p19
qMmuCID0016373	*Il23r*	Interleukin 23 receptor
qMmuCID0007264	*Il27*	Interleukin 27
qMmuCID0005613	*Il6*	Interleukin 6 (interferon, beta 2)
qMmuCID0025602	*Klrb1c*	Killer cell lectin-like receptor subfamily B, member 1
qMmuCID0006484	*Rorc*	RAR-related orphan receptor C
qMmuCED0044698	*Stat3*	Signal transducer and activator of transcription 3
qMmuCID0017320	*Tgfb1*	Transforming growth factor, beta 1
qMmuCID0026016	*Cd244*	CD244 natural killer cell receptor 2B4
qMmuCID0027036	*Cd3e*	CD3 antigen, epsilon polypeptide
qMmuCID0013811	*Cd69*	CD69 antigen
qMmuCED0045755	*Il1b*	Interleukin 1 beta
qMmuCID0022414	*Foxp3*	Forkhead box P3

Table 7.3: Antibodies used for flow cytometry (human samples)

Antibody	Clon	Cat.no	company
Brilliant Violet 510™ anti-human CD3 Antibody	UCHT1	300447	Biolegend, San Diego, CA USA
PerCP/Cy5.5 anti-human CD4	RPA-T4	300529	Biolegend, San Diego, CA USA
Alexa Fluor® 700 anti-human CD8a	RPA-T8	301027	Biolegend, San Diego, CA USA
Brilliant Violet 711™ anti-human CD14	M5E2	301837	Biolegend, San Diego, CA USA
Brilliant Violet 421™ anti-human CD25	M-A251	356113	Biolegend, San Diego, CA USA
Brilliant Violet 785™ anti-human CD45	HI30	304047	Biolegend, San Diego, CA USA
Brilliant Violet 605™ anti-human CD127(IL-7Rα)	A019D5	351333	Biolegend, San Diego, CA USA
Zombie NIR™ Fixable Viability Kit		423105	Biolegend, San Diego, CA USA
Intracellular antibodies for FACS			
PE/Dazzle™ 594 anti-human IL-17A	BL168	512335	Biolegend, San Diego, CA USA
Alexa Fluor® 647 anti-human IL-21	3A3-N2	513005	Biolegend, San Diego, CA USA
PE anti-human IL-22	BG/IL2	515303	Biolegend, San Diego, CA USA
Brilliant Violet 650™ anti-human IFN-γ	4S.B3	502537	Biolegend, San Diego, CA USA
Alexa Fluor® 488 anti-human FOXP3	259D	320212	Biolegend, San Diego, CA USA
Anti-Human IL-17F PerCP-eFluor® 710	SHLR17	46-7169	eBioscience Santa Clara, CA, USA
Activation status of neutrophils			
Brilliant Violet 510™ anti-human CD45 Antibody		304035	Biolegend, San Diego, CA USA
Pacific Blue™ anti-human CD16 Antibody (FcγRIII)	HI30	302032	Biolegend, San Diego, CA USA
APC anti-human CD66b Antibody	3G8	305118	Biolegend, San Diego, CA USA
PerCP/Cy5.5 anti-human CD62L Antibody	G10F5	304824	Biolegend, San Diego, CA USA
PE/Cy7 anti-human CD14 Antibody	DREG-56	325618	Biolegend, San Diego, CA USA
PE anti-human CD193 (CCR3) Antibody	HCD14	310706	Biolegend, San Diego, CA USA
FITC Annexin V	5E8	640906	Biolegend, San Diego, CA USA
Antibodies for immunofluorescence microscopy			
Monoclonal Mouse Anti-human Mast cells Tryptase		M7052	Dako, Glostrup, Denmark
Polyclonal Rabbit Anti-human Myeloperoxidase		A0398	Dako, Glostrup, Denmark
Monoclonal Mouse Anti-human CD3		M7254	Dako, Glostrup, Denmark
Polyclonal Donkey Anti-Mouse IgG-AlexaFluor 594	AA1	ab150112	Abcam, Cambridge, UK
Polyclonal Chicken anti-Rabbit IgG-AlexaFluor 594	F7.2.38	A-21442	Invitrogen, Carlsbad, CA, USA
Rat Monoclonal Anti-CD4 Antibody DyLight 488	YNB46.1.8	NB100-64884G	Novusbio, Centennial, CO, USA

Table 7.4: Antibodies used for flow cytometry (mouse samples)

Antibody	Clone	Cat.no	company
Brilliant Violet 510™ anti-mouse CD45 Antibody	30-F11	103138	Biolegend, San Diego, CA USA
APC/Cy7 anti-mouse CD90.2 Antibody	30-H12	105327	Biolegend, San Diego, CA USA
APC/Cy7 anti-mouse Ly-6G/Ly-6C (Gr-1) Antibody	RB6-8C5	108423	Biolegend, San Diego, CA USA
APC/Fire™ 750 anti-mouse CD3ε Antibody	500A2	152307	Biolegend, San Diego, CA USA
APC/Fire™ 750 anti-mouse Ly-6G Antibody	1A8	127651	Biolegend, San Diego, CA USA
APC/Fire™ 750 anti-mouse/human CD45R/B220 Antibody	RA3-6B2	103259	Biolegend, San Diego, CA USA
Biotin anti-mouse CD183 (CXCR3) Antibody	CXCR3-	126503	Biolegend, San Diego, CA USA
APC/Cy7 Streptavidin	173	405208	Biolegend, San Diego, CA USA
FITC anti-mouse CD11c Antibody	N418	117305	Biolegend, San Diego, CA USA
FITC anti-mouse FcεRIα Antibody	MAR-1	134305	Biolegend, San Diego, CA USA
FITC anti-mouse TCR β chain Antibody	H57-597	109205	Biolegend, San Diego, CA USA
PE anti-mouse F4/80 Antibody	BM8	123109	Biolegend, San Diego, CA USA
PE/Cy7 anti-mouse CD117 (c-kit) Antibody	ACK2	135111	Biolegend, San Diego, CA USA
PE/Cy7 anti-mouse CD196 (CCR6) Antibody	29-2L17	129815	Biolegend, San Diego, CA USA
PE/Cy7 anti-mouse CD8a Antibody	53-6.7	100721	Biolegend, San Diego, CA USA
PE/Cy7 anti-mouse TCR γ/δ Antibody	GL3	118123	Biolegend, San Diego, CA USA
PE/Cy7 anti-mouse/human CD11b Antibody	M1/70	101215	Biolegend, San Diego, CA USA
Anti-mouse CD4 FITC	GK1.5	130-102-541	Miltheniy, Bergisch Gladbach Germany
Zombie NIR™ Fixable Viability Kit		423105	Biolegend, San Diego, CA USA
Intracellular antibodies for FACS			
	BL25168		
Alexa Fluor® 647 anti-mouse IL-21	TC11-	516803	Biolegend, San Diego, CA USA
Brilliant Violet 421™ anti-mouse IL-17A Antibody	18H10.1	506925	Biolegend, San Diego, CA USA
PE anti-mouse IL-17F Antibody	9D3.1C8	517008	Biolegend, San Diego, CA USA
Anti-Mouse IL-22 PerCP-eFluor® 710	1H8PWSR	46-7221	Biolegend, San Diego, CA USA
Antibodies used for iIF and DIF			
Donkey F(ab')2 anti-Rabbit IgG (H+L)-FITC		711-095-152	Dianova, Hamburg, Germay
Donkey F(ab')2 anti-Mouse IgG (H+L)-FITC		715-096-150	Dianova, Hamburg, Germay

Table 7.5: Laboratory devices

Name	Company

Autoclave	Webeco, Selmsdorf, Germany
Avanti® J-E centrifuge	Beckman Coulter, Brea, CA, USA
Bio-photometer 8,5 mm	Eppendorf, Hamburg, Germany
Cell incubator containing 5% CO2	Memmert, Schwabach, Germany
Centrifuge 5415 C	Eppendorf, Hamburg, Germany
Centrifuge 5810 R	Eppendorf, Hamburg, Germany
Centrifuge, BIOFUGE Fresco	Haereus Instruments, Hanau, Germany
CERTOMAT® IS heating/circulation	B. Braun Biotech International, Melsungen, Germany
CFI Plan Apo λ 100x lense	Keyence Deutschland, Neu-Isenburg, Germany
CFI Plan Apo λ 10x lense	Keyence Deutschland, Neu-Isenburg, Germany
CFI Plan Apo λ 20x lense	Keyence Deutschland, Neu-Isenburg, Germany
CFI Plan Apo λ 40x lense	Keyence Deutschland, Neu-Isenburg, Germany
Cold room (4°C)	Viessmann, Allendorf, Germany
Cryostat, Leica CM 3050S	Leica Mikrosysteme Vertrieb, Wetzlar, Germany
Drying and hitting chamber	Binder, Tuttlingen, Germany
Electrophoresis Cell	Bio-Rad Laboratories, Hercules, CA, USA
ELISA plate washer	Tecan Group., Maennedorf, Switzerland
Eppendorf Repeater® M4	Eppendorf, Hamburg, Germany
EUROStat Microscope	Euroimmun, Lübeck, Germany
GloMax® Discover System	Promega, Madison, WI, USA
Inverse confocal microscope system FV 1000	Olympus Corporation, Tokyo, Japan
Laminair HB2448 flow hood, Biowizard	Kojair Tech Oy, Vilppula, Finnland
Light/Fluorescence Microscope BZ-9000	Keyence Deutschland, Neu-Isenburg, Germany
LSR II Flow Cytometer	BD Bioscience, San Jose, CA, USA
MACSQuant®Analyzer 10	Miltenyi Biotec, Gladbach, Germany
Mastercycler ep realplex	Eppendorf, Hamburg, Germany
Micro centrifuge Micro Star 17R	VWR International, Darmstadt, Germany
Microfuge Sigma 1-14	Sigma-Centrifuges, Shropshire, UK
Microtome	Leica Mikrosysteme Vertrieb, Wetzlar, Germany
Mini centrifuge C-1202	Labnet International, Edison, NJ, USA
Mini protean tetra system	Bio-Rad Laboratories, Hercules, CA, USA
NanoPhotometer® NP180	Implen, München Germany
Neubauer chamber	Paul Marienfeld, Lauda Königshofen, Germany
pH-meter, ph526	WTW, Weilheim, Germany
pipetus®	Hirschmann Laborgeräte, Eberstadt, Germany
Plate reader, VICTOR3 Wallac 1420	Perkin Elmer, Waltham, MA, USA
Power Pac 200	Bio-Rad Laboratories, Hercules, CA, USA

Power Pac basic	Bio-Rad Laboratories, Hercules, CA, USA
Precision scale, Analytical Plus	Ohaus, Parsippany, NJ, USA
Refrigerator (4°C)/freezer (-20°C)	Liebherr International, Bulle, Switzerland
Serological pipettes	Eppendorf, Hamburg, Germany
Shaker, Duomax 1030	Heidolph Instruments, Schwabach
Sonopuls UW 2070	Bandeln electronic, Berlin, Germany
Stuart Magnetic Hotplate Stirrer CB162	Biocote Limited, Coventry, UK
Thermo Electron's Px2 Thermal Cycler	Thermo Electron Corporation, Waltham, MA, USA
Tissue-Homogenizer-Omni-TH	Omni International, Kennesaw, GA, USA
Transferpette S (10 µl, 100 µl, 1000 µl)	BRAND, Wertheim, Germany
Ultra-Low-Temperature Freezer, -80°C	Edison, NJ, USA
Vilber Lormat Serial N°	Vilber Lormat, Collégien , France
Vortex	Scientific Industries, Inc., Bohemia, NYC, USA
Water baths Aqualine AL 5	Lauda Dr. R. Wobser, Lauda-Königshofen, Germany
Wide Mini-Sub Cell GT System	Bio-Rad Laboratories, Munich, Germany
Feather disposable scalpel 10	FEATHER Safety Razor., Osaka, Japan
Scotsman AF 20 Ice Flaker	Scotsman® Ice systems, Vernon Hills, IL, USA

Table 7.6: Chemicals and reagents

Name	Company
1-Step™ Turbo TMB-ELISA solution	Thermo Scientific, Waltham, MA, USA
0.1M EDTA disodium salt solution	Carl Roth, Karlsruhe, Germany
32% Hydrochloric acid (HCl)	Merck, Darmstadt, Germany
4′,6-Diamidino-2-phenylindole dihydrochloride	Sigma-Aldrich Chemie, St. Louis, MO, USA
5-Amino-2,3-dihydro-1,4-phthalazindion (Luminol)	Sigma-Aldrich Chemie, St. Louis, MO, USA
ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)	Sigma-Aldrich Chemie, St. Louis, MO, USA
Acetic acid	Carl Roth, Karlsruhe, Germany
Aceton	Carl Roth, Karlsruhe, Germany
Acrylamide-Lösung 30%	Bio-Rad Laboratories, Hercules, CA, USA
Ammonium persulfate (APS)	Sigma-Aldrich Chemie, St. Louis, MO, USA
Ampuwa dH2O	Fresenius Kabi, Germany
Antibody diluent	Dako, Glostrup, Denmark
Bovine serum albumin (BSA)	Carl Roth, Karlsruhe, Germany
Bovine serum albumin (BSA) biotin-free	Sigma-Aldrich Chemie, St. Louis, MO, USA
Carbenicillin disodium salt	Carl Roth, Karlsruhe, Germany
Coomassie Brilliant Blue G250	Merck, Darmstadt, Germany
Coomassie brilliant blue R-250	Bio-Rad Laboratories, Hercules, CA, USA
Dextran 500	Nycomed, Oslo, Norway
D-Glucose solution	Sigma-Aldrich Chemie, St. Louis, MO, USA
DL-Dithiothreitol (DTT)	Sigma-Aldrich Chemie, St. Louis, MO, USA
EDTA	Carl Roth, Karlsruhe, Germany
Eosin	Merck, Darmstadt, Germany
Escherichia coli-BL21	Novagen/Merck, Darmstadt, Germany
Ethanol 70%	Carl Roth, Karlsruhe, Germany
Ethanol 96%	Carl Roth, Karlsruhe, Germany
FBS superior	Merck, Darmstadt, Germany
Fetal calf serum (FBS Superior)	Biochrom, Berlin, Germany
Ficoll-Paque	GE Healthcare Chicago, IL, USA
Fluoromount-G®	SouthernBiotech, Birmingham, AL, USA
Formaldehyde	Carl Roth, Karlsruhe, Germany
Glutathione-Sepharose	GE Healthcare Chicago, IL, USA
Glutathione	Sigma-Aldrich Chemie, St. Louis, MO, USA
Glycerol	Carl Roth, Karlsruhe, Germany
Glycin	Carl Roth, Karlsruhe, Germany
Guanidium	Merck, Darmstadt, Germany

Hank’s balanced salt solution (HBSS)	Gibco/Thermo Fisher Scientific, Waltham, MA, USA
Hematoxyline	Merck, Darmstadt, Germany
Heparin-Natrium 25000	Ratiopharm, Ulm, Germany
HEPES	Carl Roth, Karlsruhe, Germany
HEPES-Buffer	Biochrom, Berlin, Germany
Hydrochloric acid (2N)	Carl Roth, Karlsruhe, Germany
Hydrochloric acid 32%	Merck, Darmstadt, Germany
Immobilon-P polyvinylidene difluoride (PVDF) membrane	Millipore, Burlington, MA, USA
Isopropyl-b-D-thiogalactopyranoside (IPTG)	Carl Roth, Karlsruhe, Germany
Ketamine hydrochloride	Sigma-Aldrich Chemie, St. Louis, MO, USA
LB (lysogene broth)-Medium	MP Biomedicals, Illkirch, France
LB medium	PM Biomedicals, Illkirch, France
L-Glutamine	Biochrom, Berlin, Germany
Liberase™ Reasearch Grade	Sigma-Aldrich Chemie, St. Louis, MO, USA
Luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione)	Sigma-Aldrich Chemie, St. Louis, MO, USA
Lysozyme	Merck, Darmstadt, Germany
Magnesiumchlorid (MgCl2)	Merck, Darmstadt, Germany
Mowiol 4-88	Carl Roth, Karlsruhe, Germany
Mowiol-488 mounting medium	Carl Roth, Karlsruhe, Germany
N,N,N´,N´-Tetramethyl ethylenediamin (TEMED)	Sigma-Aldrich Chemie, St. Louis, MO, USA
Natriumazid (NaN3)	Merck, Darmstadt, Germany
ortho-Phosphoric acid (H_3PO_4) 85%	Merck, Darmstadt, Germany
Percoll	GE Healthcare Chicago, IL, USA
Polymorphprep™	Axis-Shield, Heidelberg, Germany
Proteaseinhibitorcocktail Set III, Animal Free	Calbiochem/Merck, Darmstadt, Germany
Protein G Sepharose Fast Flow affinity	GE Healthcare Chicago, IL, USA
Proteinase inhibitor cocktail	Calbiochem/Merck, Darmstadt, Germany
Protein-G-Resin Protein-G-Agarose Fast Flow	GenScript, Leiden, Netherland
Roti®-Histofix	Carl Roth, Karlsruhe, Germany
RPMI 1640 with L-glutamine	Lonza Basel, Switzerland
RPMI 1640 with L-glutamine w/o glucose w/o phenol red	Genaxxon bioscience, Ulm, Germany
SDS	Carl Roth, Karlsruhe, Germany
Sodium carbonate (Na2CO3)	Merck, Darmstad, Germanyt
Sodium chloride	Carl Roth, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth, Karlsruhe, Germany
Sodium citrate dihydrate	Sigma-Aldrich Chemie, St. Louis, MO, USA

Sodium deoxycholate	Sigma-Aldrich Chemie, St. Louis, MO, USA
Sodium dihydrogen phosphate monohydrate Na_2HPO_4	Carl Roth, Karlsruhe, Germany
Sodium ethyleneditetraacetic acid (NaEDTA)	Carl Roth, Karlsruhe, Germany
Sodium hydrogen phosphate	Carl Roth, Karlsruhe, Germany
Sodium hydrogencarbonate (NaHCO3)	Merck, Darmstadt, Germany
Sodium hydroxide	Merck, Darmstadt, Germany
Sodium phosphate	Carl Roth, Karlsruhe, Germany
Sulfuric acid (H2SO4)	Millipore, Burlington, MA, USA
TALON metal affinity resin	Clontech, Saint-Germain-en-Laye, France
TALON superflow	Clontech, Saint-Germain-en-Laye, France
Tris-hydroxymethyl aminomethane ($C_4H_{11}NO_3$)	Serva Electrophoresis, Heidelberg, Germany
Tris-base	Serva Electrophoresis, Heidelberg, Germany
Tris-hydrochloride	Carl Roth, Karlsruhe, Germany
Triton X-100	Carl Roth, Karlsruhe, Germany
Tween 20	Sigma-Aldrich Chemie, St. Louis, MO, USA
Xylazin	Carl Roth, Karlsruhe, Germany
Xylene	Sigma-Aldrich Chemie, St. Louis, MO, USA
β-mercaptoethanol	Sigma-Aldrich Chemie, St. Louis, MO, USA

Table 7.7: Materials

Name	Company
Centrifugal Filters Amicon® Ultra-15	Millipore, Burlington, MA, USA
Centrifuge tube 30ml for Ultracentrifuge	Beckmann Coulter, Brea, CA, USA
Centrifuge tube 500ml for Ultracentrifuge	Beckmann Coulter, Brea, CA, USA
Cover glass slides (24x32)	Paul Marienfeld, Lauda-Königshofen, Germany
Cover glasses (24x60mm)	Th. Geyer, Renningen, Germany
Dako-pen	Dako, Glostrup, Denmark
dark chamber	Hassa, Lübeck, Germany
Disposable filter Ministart 0.2 µm	Sartorius Stedim Biotech, Göttingen, Germany
Disposable OD-cuvettes	Brand, Wertheim, Germany
Disposable scalpel No.10	Feather Safety Razor, Osaka, Japan
Disposable UV-Cuvette micro 70µm	Brand GmbH, Wertheim, Germany
EDTA-syringes	Sarstedt, Nuembrecht, Germany
ELISA plate Maxisorp®	Nunc/Thermo Fisher Scientific, Waltham, MA, USA
ELISA plate seal	Sarstedt, Nuembrecht, Germany
epT.I.P.S.® Standard 200µl ,300µl	Eppendorf, Hamburg, Germany
Falcon tubes (15 ml)	Sarstedt., Nuembrecht, Germany
Falcon tubes (15 ml)	Sarstedt., Nuembrecht, Germany
Falcon® 70µm cell strainer	Corning Inc, Corning, USA
Filtropur S 0.45 µm	Sarstedt, Nuembrecht, Germany
Folded Filter Papers 110mm Whatman®	GE Healthcare, Uppsala, Schweden
Glutathione-Sepharose	GE Healthcare Chicago, IL, USA
High binding Micropalte F-bottom	Greiner bio-one, Kremsmünster, Austria
Homogenisator Omni Th	Omni International, Kennesaw, GA, USA
Membrane-Adapter	Sarstedt, Nuembrecht, Germany
Microtubes, light protection	A. Hartenstein, Würzburg, Germany
Niddles BD Microlance 3 (26Gx1/2“)	Becton Dickinson, Franklin Lakes, NY, USA
Non-binding Micropale F-bottom	Greiner bio-one, Kremsmünster, Austria
Parafilm®M	Th. Geyer Renningen, Germany
Pasteur pipettes ISO 7712	Karl Hecht "Assistent" , Altnau Schweiz
Pipette tips 10µl, 200µl, 1000µl	Sarstedt, Nuembrecht, Germany
Pipettes	Eppendorf, Hamburg, Germany
Protein-G-Resin Protein-G-Agarose Fast Flow	Millipore, Burlington, MA, USA
Reaction tubes (1.5 ml; 2 ml)	Sarstedt, Nuembrecht, Germany
SafeSeal tube o.5ml	Sarstedt, Nuembrecht, Germany
Serological pipettes (5 ml; 10 ml; 25 ml)	Sarstedt, Nuembrecht, Germany
Serum S-Monovette® 7.5ml Z	Sarstedt, Nuembrecht, Germany

S-Monovette® 9ml AH NH4 Heparin	Sarstedt, Nuembrecht, Germany
SuperFrost/Plus-slide glasses	Gerhard Menzel, Braunschweig, Germany
Syringe (20 ml) BD Discardit II	Becton Dickinson, Heidelberg, Germany
Syringe (5 ml) BD Discardit II	Becton Dickinson, Heidelberg, Germany
Syringe (5 ml; 20 ml)	Becton Dickinson, Heidelberg, Germany
Syringe 1 ml *Omnican F (30Gx1/2")*	B.Braun, Melsungen, Germany
Tissue-Tek® Cryomold	Sakura Finetek, Alphen aan den Rijn, Netherland
Tissue-Tek® O.C.T. Compound	Sakura Finetek., Alphen aan den Rijn, Netherland
Transfer pipette 1ml	Sarstedt, Nuembrecht, Germany
Transfer pipette 3.5ml	Sarstedt, Nuembrecht, Germany

7.4. Supplementary results

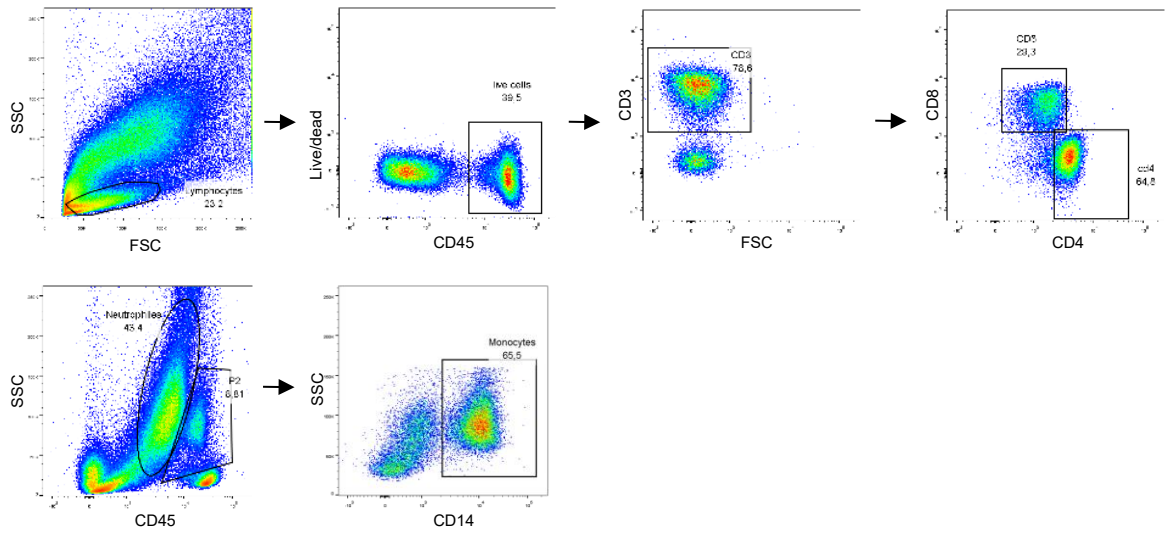

Figure 7.1: Gating strategy for different cell types in human peripheral blood

Representative plots from stimulated cells with PMA and Ionomycin gated for CD4, CD8, neutrophils and monocytes. Lymphocytes were identified based on FSC and SSC profile, neutrophils were identified based on the size.

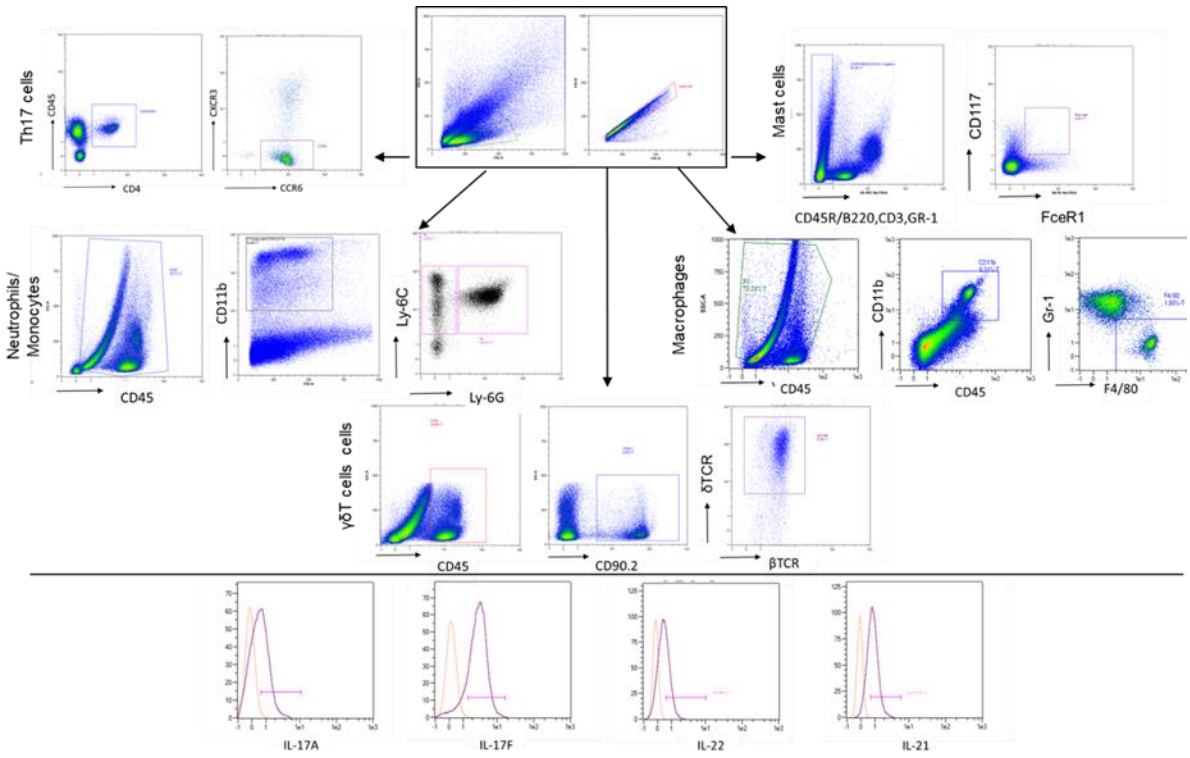

Figure 7.2: Gating strategy for cell types in mice from two different compartments, blood and skin

From FCS and SSC, all singlets were gated, and after that, cells were gated for Th17 cells, γδ T cells, neutrophils, monocytes, macrophages and mast cells. From these cell types additionally were analysed IL-17A, L-21, IL-22 and IL-17F, showed in the histograms plots

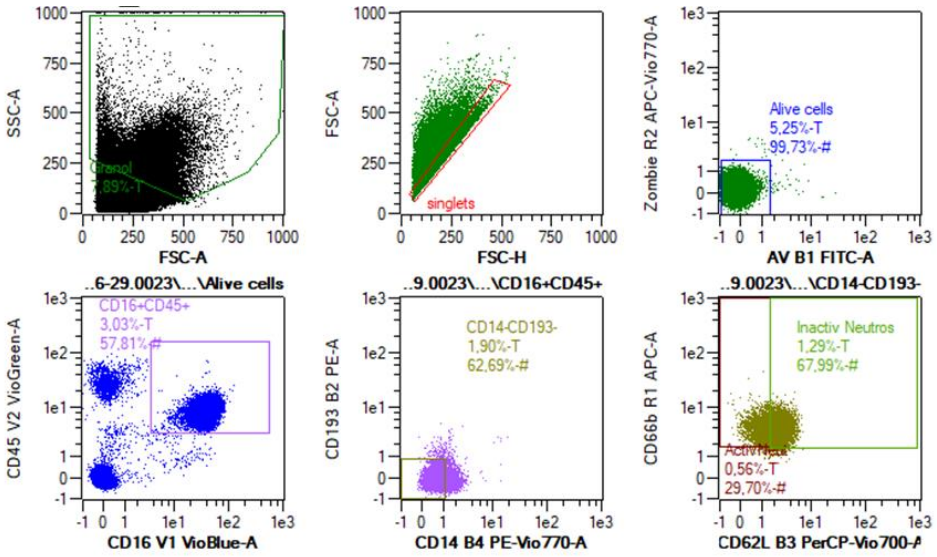

Figure 7.3: Gating strategy used for determination of active status of the neutrophils

Live neutrophils were gated from granulocytes from CD45+CD16+, CD14-CD193-.CD62L-CD66b+.Results are presented in % from the parent population

Table 7.8: mRNA expression of Th17 cells-related genes in the skin of bullous pemphigoid patients and controls

Gene[1]	$2^{-DD}Ct$		Fold change	P value
	BP patients	Controls		
IL4	9.40E-05	2.50E-05	3.75 ↑	0.001
IL17RC	9.07E-02	3.56E-02	2.55 ↑	0.001
CXCL1	1.57E-01	2.25E-02	7.01 ↑	0.003
CSF3	8.89E-02	2.73E-02	3.26 ↑	0.003
IRF4	1.41E-01	4.35E-02	3.24 ↑	0.004
ELANE	1.04E-01	2.77E-02	3.76 ↑	0.005
IL10	1.28E-01	4.12E-02	3.11 ↑	0.008
CD4	9.34E-02	3.32E-02	2.82 ↑	0.009
ICAM1	1.19E-01	4.61E-02	2.58 ↑	0.013
SOCS3	1.00E-01	3.91E-02	2.57 ↑	0.014
IL6	7.85E-02	2.97E-02	2.64 ↑	0.015
CCR6	1.11E-01	2.38E-02	4.67 ↑	0.020
CSF2	8.58E-02	2.60E-02	3.30 ↑	0.021
KLRB1	1.14E-01	5.24E-02	2.17 ↑	0.024
IL23R	1.00E-01	3.64E-02	2.76 ↑	0.026
TNF	9.61E-02	4.07E-02	2.36 ↑	0.034
CXCL2	8.01E-02	3.42E-02	2.34 ↑	0.034
IL17A	5.22E-04	2.80E-05	18.96 ↑	0.046
CD8a	1.01E-01	4.05E+00	-40.22 ↓	0.048
RORc	4.41E-02	1.75E-02	2.53 ↑	0.061
IL2	1.28E-04	3.40E-05	3.71 ↑	0.064
IL17RA	2.21E-02	4.92E-03	4.49 ↑	0.177
IL12RB1	2.78E-04	7.20E-05	3.86 ↑	0.208
IL23A	2.56E-04	1.03E-04	2.47 ↑	0.219
IL27	2.03E-03	5.80E-05	35.30 ↑	0.275
CCL2	5.69E-03	2.05E-03	-2.78 ↓	0.297
IL22	3.12E-04	4.70E-05	6.63 ↑	0.344
IL21	8.30E-05	3.30E-05	2.52 ↑	0.396
CCL20	1.43E-04	4.40E-05	3.25 ↑	0.515
IL17F	5.69E-03	1.86E-03	3.05 ↑	0.673
IL12B	1.11E-04	4.00E-05	2.78 ↑	0.761
IFNg	1.14E-04	4.10E-05	2.74 ↑	0.766

Detail description in **Table 7.8**

↑ up-regulated

↓ down-regulated

Table 7.9: mRNA expression of Th17 cells-related genes in the skin of experimental bullous pemphigoid mice and wild-type mice

Gene[1]	$2^{-\Delta\Delta}$Ct		Fold change	P value
	IL-17A$^{-/-}$	WT mice		
Ccr6	0.006381	0.00554	1.15	0.94
Icam1	0.000036	0.00017	0.21 ↓	0.30
Il22	0.001493	0.005664	0.26 ↓	0.86
Rorc	0.000106	0.000105	1.02	0.40
Cd244	0.000009	0.00003	0.31 ↓	0.34
Il12b	0.000005	0.000009	0.52	0.26
Il23a	0.008502	0.005	1.70	0.68
Stat3	0.006708	0.006408	1.05	0.86
Cd3e	0.000015	0.000021	0.71	0.52
Il17a	0.000005	0.000007	0.71	0.14
Il23r	0.000006	0.000011	0.52	0.34
Tgfb1	0.00004	0.000192	0.21 ↓	0.29
Cd4	0.000007	0.000017	0.38 ↓	0.35
Il17f	0.000005	0.000023	0.21 ↓	0.35
Il27	0.000006	0.000011	0.57	0.34
Cd69	0.000009	0.000044	0.20 ↓	0.32
Il17ra	0.000017	0.000065	0.26 ↓	0.32
Il6	0.000008	0.000025	0.31 ↓	0.35
Cd8a	0.000005	0.000516	0.01 ↓	0.34
Il17rc	0.000019	0.000371	0.05 ↓	0.35
Itgam	0.000036	0.000154	0.24 ↓	0.34
Casc3	0.000202	0.000451	0.45 ↓	0.35
Foxp3	0.000006	0.000007	0.80	0.51
il1b	0.010027	0.035452	0.28 ↓	0.30
Klrb1c	0.000005	0.000007	0.71	0.14
ccl20	0.006096	0.006579	0.93	0.51
il21	0.007219	0.005555	1.30	0.67
Ly6g	0.00207	4	0.13 ↓	0.10

[1] Detail description in Appendix Table 7.9

↓ down-regulated

7.5. Curriculum vitae

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2014-2019	**Dr. rer. nat.**
	University Hospital Schleswig-Holstein, University of Lübeck, Lübeck Institute of Experimental Dermatology, Lübeck Germany
	Thesis title: "*The pathophysiological role of IL-17 cells in bullous pemphigoid*"
2011-2013	**Master of science**
	Sofia University "St. Kliment Ohridski", Sofia, Bulgaria Faculty of Biology
	Thesis title: "*Cloning, expression and purification of the monomeric form of recombinant cystatin.*"
2006-2010	**Bachelor Degree**
	University St. Kliment Ohridski", Bitola, Macedonia Higher Medical School, Bitola, Macedonia
	Thesis title:" *Urinary IL-18 as a marker for acute tubular necrosis in humans."*

Publications

1. **Sezin, T., Krajewski, M., Wutkowski, A., Mousavi, S., Chakievska, L., Bieber, K., Ludwig, R.J., Dahlke, M., Rades, D., Schulze, F.S., et al. (2017). The Leukotriene B4and its Receptor BLT1 Act as Critical Drivers of Neutrophil Recruitment in Murine Bullous Pemphigoid-Like Epidermolysis Bullosa Acquisita. J. Invest. Dermatol. 137, 1104–1113.**

2. **Chakievska, L., Holtsche, M.M., Künstner, A., Goletz, S., Petersen, B.S., Thaci, D., Ibrahim, S.M., Ludwig, R.J., Franke, A., Sadik, C.D., et al. (2019). IL-17A is functionally relevant and a potential therapeutic target in bullous pemphigoid. J. Autoimmun. 96, 104–112.**

Achievement	
ORAL PRESENTATIONS:	1. **L. Chakievska**, M. M. Holtsche, C. M. Hammers, A. Künstner, M. Hofrichter, S. Goletz, B. Petersen, D. Thaci, S. M. Ibrahim, R. J. Ludwig, A. Franke, C. D. Sadik, D. Zillikens, C. Hölscher, H. Busch, E. Schmidt ***IL-17A is functionally relevant and a potential therapeutic target in bullous pemphigoid*** 27th European Academy of Dermatology and Venereology Congress, Paris, France, September 2018 2. **L. Chakievska**, M. M. Holtsche, C. M. Hammers, S. Goletz, D. Zillikens, F. S. Schultze, C. Hölscher, E. Schmidt ***A rationale for anti-IL-17A treatment in bullous pemphigoid*** 45th Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), Zurich, Switzerland, March 2018 3. **L. Chakievska**, S. Goletz, D. Zillikens, F. S. Schultze, C. Hölscher, E. Schmidt ***Elevated levels of IL-17A in the blood and skin of patients with bullous pemphigoid*** Autoimmune Blistering Skin Diseases Meeting, Göttingen, Germany, March 2017 4. **L. Chakievska**, S. Roy, S. Goletz, C. Hölscher, D. Zillikens, E. Schmidt, F. S. Schulze ***The pathophysiological role of Th17 cells in Bullous pemphigoid*** 3th Round Table, Arbeitsgemeinschaft Dermatologische Forschung, Bad Segeberg, October 2016 5. **L. Chakievska**, S. Roy, S. Goletz, C. Hölscher, D. Zillikens, E. Schmidt, F. S. Schulze ***IL-17A governs tissue destruction in bullous pemphigoid*** 10th International Congress on Autoimmunity, Leipzig, Germany, April 2016

POSTER PRESENTATIONS:		1. **L. Chakievska**, M. M. Holtsche, C. M. Hammers, S. Goletz, D. Zillikens, F. S. Schultze, C. Hölscher, E. Schmidt ***A rationale for anti-IL-17A treatment in bullous pemphigoid*** 45th Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), Zurich, Switzerland, March 2018 2. **L. Chakievska**, F. S. Schulze, S. Goletz, S. Roy, C. Hölscher, D. Zillikens, E. Schmidt ***Rationale for anti-IL-17A treatment in bullous pemphigoid*** 2nd Interdisciplinary Congress on IL-17, München, Germany, November 2017 3. **L. Chakievska**, F. S. Schulze, S. Goletz, S. Roy, C. Hölscher, D. Zillikens, E. Schmidt ***IL-17A is a key regulator in BP*** 47th Annual Meeting of the European Society for Dermatological Research (ESDR), Salzburg, Austria, September 2017 4. **L. Chakievska**, F. S. Schulze, S. Roy, S. Goletz, C Hölscher, D. Zillikens, E. Schmidt ***The pathophysiological role of Th17 cells in experimental bullous pemphigoid*** Meeting of the International Pemphigus and Pemphigoid Foundation (IPPF), Lübeck, Germany, June 2017 5. F. S. Schulze, **L. Chakievska**, S. Goletz, S. Roy, C. Hölscher, D. Zillikens, E. Schmidt ***Elevated levels of IL-17A in the blood and skin of patients with bullous pemphigoid*** Meeting of the International Pemphigus and Pemphigoid Foundation (IPPF), Lübeck, Germany, June 2017 6. **L. Chakievska**, S. Goletz, S. Roy, C. Hölscher, D. Zillikens, E. Schmidt, F. S. Schulze ***Elevated levels of IL-17A in the blood and skin of patients with bullous pemphigoid*** 44th Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), Göttingen, Germany, March 2017 7. **L. Chakievska**, S. Goletz, F. S. Schulze, D. Zillikens, C. Hölscher, E. Schmidt ***The pathophysiological role of IL-17 in bullous pemphigoid*** 6th ADF Winter School, Zugspitze, Germany, January 2017 8. **L. Chakievska**, S. Roy, S. Goletz, C. Hölscher, D. Zillikens, E. Schmidt, F. S. Schulze ***Role of IL-17A in bullous pemphigoid*** 1st Interdisciplinary Congress on IL-17, München, Germany, December 2016 9. **L. Chakievska**, S. Roy, S. Tofern, S. Goletz, D. Zillikens, C. Hölscher, E. Schmidt, F S. Schulze ***IL-17A governs tissue destruction in bullous pemphigoid*** 39th Symposium of the North German Immunologists (NDI), Borstel, Germany, October 2016 10. **L. Chakievska**, S. Roy, S. Goletz, C. Hölscher, D. Zillikens, E. Schmidt, F. S. Schulze ***The pathophysiological role of Th17 cells in Bullous pemphigoid*** 3th Round Table, Arbeitsgemeinschaft Dermatologische Forschung, Bad Segeberg, October 2016

		11. **L. Chakievska**, F. S. Schulze, S. Goletz, C. Hölscher, D. Zillikens, E. Schmidt ***New mechanic for your skin bubbles!*** Uni im Dialog, Luebeck, Germay, June 2016
AWARDS:		1. **Best publication** ***IL-17A is functionally relevant and a potential therapeutic target in bullous pemphigoid*** RTG1727-Autumn retreat, Crivitz, Germany, November 2018 2. **Travel award** ***IL-17A is functionally relevant and a potential therapeutic target in bullous pemphigoid*** 27th European Academy of Dermatology and Venereology Congress, Paris, France, September 2018 3. **Translational Research Award** ***A rationale for anti-IL-17A treatment in bullous pemphigoid*** 45th Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), Zurich, Switzerland, March 2018 4. **The best presentation and best abstract** ***A rationale for anti-IL-17A treatment in bullous pemphigoid*** 45th Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), Zurich, Switzerland, March 2018 5. **Travel award** ***A rationale for anti-IL-17A treatment in bullous pemphigoid*** 2nd Interdisciplinary Congress on IL-17, München, Germany, November 2017 6. **Travel award** ***Role of IL-17A in bullous pemphigoid*** 1st Interdisciplinary Congress on IL-17, München, Germany, December 2016

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7.7. Declaration and copyright statements

Declarations

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