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Complement is important but not essential in a new mouse model of bullous pemphigoid

Dissertation
for fulfilment of requirements
for the doctoral degree
of the University of Lübeck

-From the Department of Natural Sciences-

Submitted by

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Lübeck

2013

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Date of oral examination: 8 May 2014

Approved for printing. Lübeck, 9 May 2014

dedicated to my family

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1. Introduction

1.1 Autoimmune diseases

One of the main functions of the immune system is the protection of the organism from possibly harming invaders. It recognises structures that differ from "self" and reacts with different protection mechanisms. During development lymphocytes, which react to self-antigens, are eliminated. Autoimmune diseases are caused by failure in this self-guarding mechanism, the so called tolerance, when lymphocytes reacts to self-antigens in various tissues in a similar manner as they do to pathogens. This reaction can subsequently cause tissue damage. It is not known what causes the breakdown of central and/or peripheral tolerance to trigger autoreactive lymphocytes, but both environmental and genetic factors, such as the MHC genotype, are thought to be crucial [1].

Autoimmune diseases can be classified broadly as being antibody mediated or T-cell mediated as well as organ-specific or systemic. The systemic autoimmune disease rheumatoid arthritis, for example, is caused by antibody responses to synovial antigens and results in joint inflammation [2]. Systemic lupus erythematosous is characterised by autoantibody production by dysregulated B cells, target organ infiltration by inflammatory T cells and aberrant immune cell activation due to abnormal antigen presenting cell function [3]. Multiple sclerosis, a chronic inflammatory demyelinating disease affecting the central nervous system, is considered a T-cell-mediated autoimmune disease, although more recently a role for the humoral immune response has been attributed to a subgroup of multiple sclerosis patients [4,5]. Patients with the organ-specific type-1 diabetes

develop insulitis as a result of T-cell infiltration and destruction of the pancreatic islets [6].

In the present study I focus on an antibody mediated autoimmune disease, which is caused by antibodies against structural proteins of the skin.

1.2 The dermal-epidermal junction

The skin is composed of different layers. It can be divided in three parts; from the outside to the inside, the epidermis, the dermis and subcutaneous tissue. The epidermis and the dermis are connected via the dermal-epidermal junction (DEJ), also known as the dermal-epidermal basement membrane zone (BMZ). This DEJ consists of a number of extracellular proteins and molecules, such as collagens and polysaccharides. They are synthesised by basal keratinocytes and dermal fibroblasts, with both contributing to the development and repair of the DEJ. Using electron microscopy, two layers can be found within the DEJ: the upper, less electron dense lamina lucida and the lower, more electron dense lamina densa (Fig.1B). While the lamina lucida contains mainly hemidesmosomal proteins, the lamina densa mainly consists of fibronectins, heparan sulphate proteoglycan, type IV collagen and laminin 332 [7]. Hemidesmosomal proteins expanding into the lamina lucida are further connected to the upper dermis. Anchoring fibrils made of type VII collagen form semi-circular loop structures and firm connections around type I and III collagen fibres in the dermis, which links it to the lamina densa. This leads to a network which plays a critical role for adherence of the epidermis to the underlying dermis. Genetic alteration of or defects in these molecules or the DEJ as a target of autoimmunity result in fragility of the DEJ. This is clinically demonstrated by erosions and blisters following minor trauma [7,8].

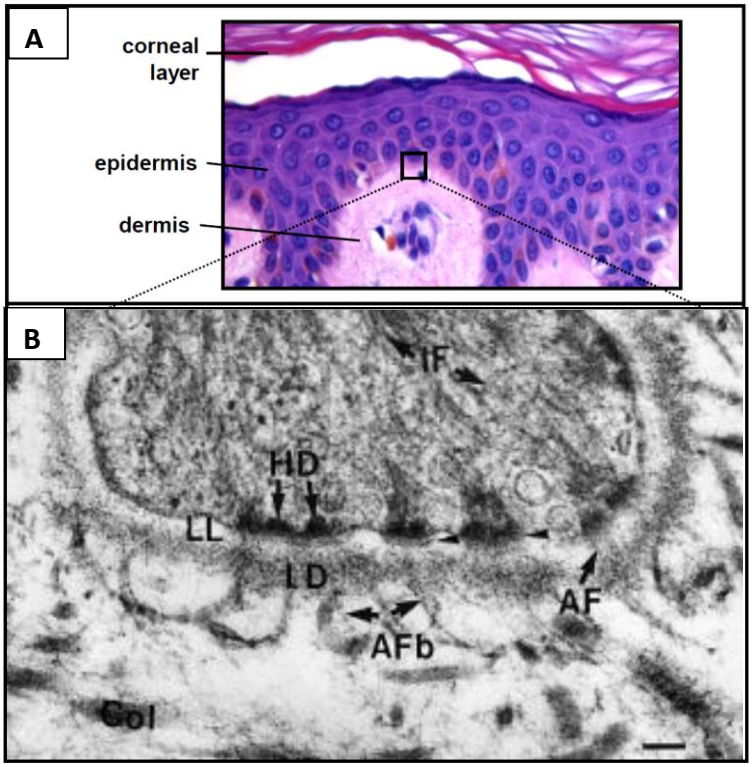

Figure 1. Structure of the skin and the DEJ in light and electron microscopy
(**A**) Light microscopy of the upper skin showing the corneal layer, the epidermis with the keratinocytes and the subjacent dermis [13]. Electronmicroscopic picture (**B**) shows the dermal epidermal junction with hemidesmosomes within the basal keratinocytes and underlying lamina lucida and lamina densa.
IF: keratin intermediate filament, HD: hemidesmosome, LL: lamina lucida, LD: lamina densa, AF: anchoring filament, AFb: anchoring fibrils, Col: collagen [10]

1.2.1 Hemidesmosome

The hemidesmosome is a specialised junction complex within the keratinocytes and is placed at the DEJ. Several different proteins form the hemidesmosome. Intracellularly, intermediate keratin filaments are connected to BP230 (BPAG1) and plectin. Hemidesmosome spanning integrin α6β4 is located mainly intracellularly with a small extracellular portion. The hemidesmosomal protein BP180 (type XVII collagen; BPAG2) is a transmembranous molecule with a type II membrane orientation. Its globular n-terminal portion lies intracellularly. 15 alternating collagenous repeats are located extracellularly and span the lamina

lucida forming a hook at the c-terminal end, which is anchored in the lamina densa, before kinking back into the lamina lucida. The 16th non-collagenous domain (NC16A; see below) is located directly outside of the cell membrane [9–11] (Fig.2).

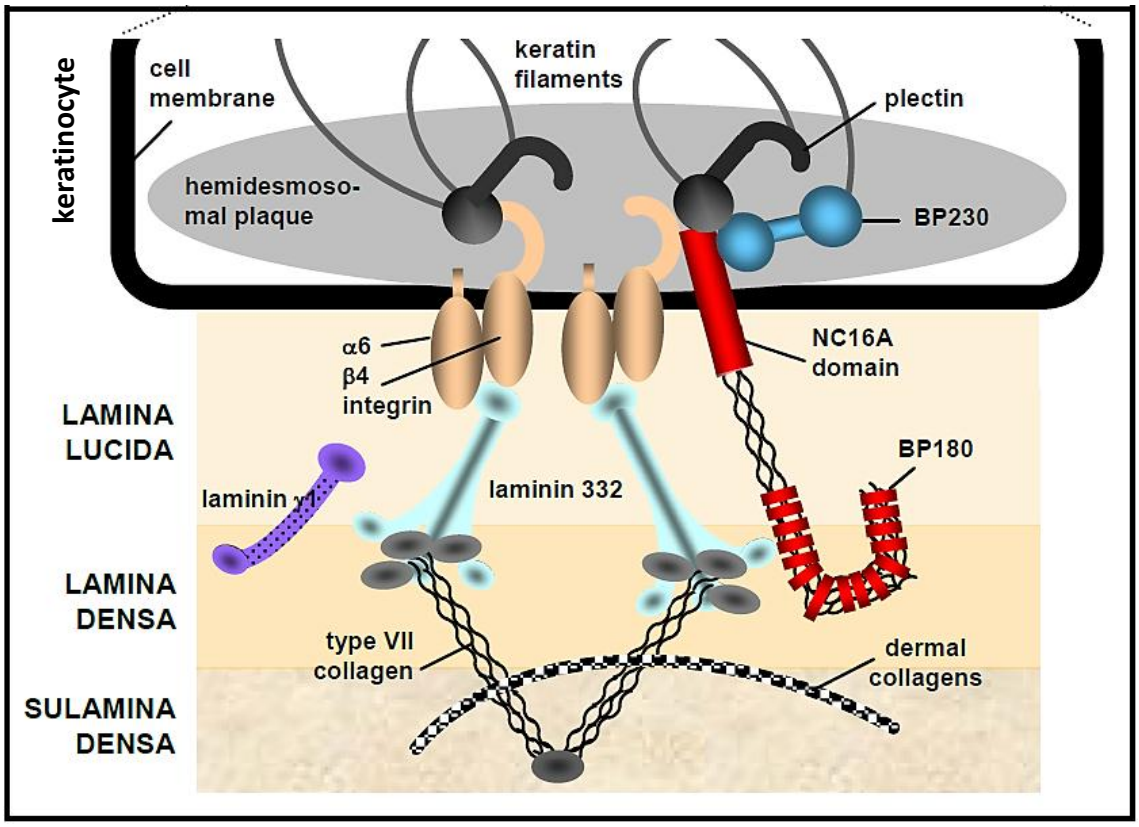

Figure 2. Schematic structure of the hemidesmosome
Schematic overview of the structure of the hemidesmosome, its proteins and the connection to the dermis via the lamina lucida and the lamina densa [13]. Within the hemidesmosomal plaque keratin filaments are connected to BP230 and plectin, which is further also connected to α6β4 integrin, BP230 and the N-terminal side of BP180. The extracellular part of α6β4 integrin is bound to laminin 332, which is connected to the dermal protein type VII collagen within the lamina densa. BP180 spans the lamina lucida, reaches the lamina densa before kinking back into the lamina lucida, and is anchored into the lamina densa with its C-terminal end.

1.3 Autoimmune blistering diseases

Autoimmune blistering dermatoses comprise a heterogeneous group of diseases that are characterised by autoantibodies to structural components of the skin and adjacent mucous membranes [12,13]. Clinically, they are characterised by blister

formation of the skin and/or surface-proximal mucosa [14]. The particular clinical, histological and serological manifestation of each disease varies with the target antigen. In 1953, Walter Lever already described histological differences between the two major disease groups, pemphigus and pemphigoid diseases, before serological diagnoses was available [15]. While pemphigus diseases show loss of adherence between the keratinocytes within the epidermal layer resulting in acantholysis, pemphigoid diseases show subepidermal blister formation. Final diagnosis is made by direct immunofluorescence (IF) microscopy of a perilesional biopsy and the detection of serum autoantibodies [14]. Respective target antigens have already been described for several autoimmune bullous diseases. In all pemphigus diseases direct IF microscopy reveals a net-like patterned binding of IgG/C3 around the keratinocytes. Pemphigus foliaceus is associated with antibodies against the desmosomal protein desmoglein (Dsg) 1 [16,17]; the main target antigen in pemphigus vulgaris is Dsg 3, with additional antibodies against Dsg 1 found in the mucocutanous subtype [18,19]. Dsg3 is also the target antigen in paraneoplastic pemphigus, together with proteins of the plakin family [20,21].

Pemphigoid diseases show a subepidermal staining of IgG/C3 in direct IF microscopy. The hemidesmosomal protein BP180 has been described as main target antigen in several pemphigoid diseases [12,13]. Besides the most common of the pemphigoid disorders, bullous pemphigoid [22] (see below), autoantibodies against BP180 are also associated with mucous membrane pemphigoid (formerly known as cicatricial pemphigoid) [23–26], linear IgA dermatosis [27,28], lichen planus pemphigoides [29] and pregnancy-associated pemphigoid gestationis (formerly termed herpes gestationis) [30,31]. Reactivity against BP230 can be found in all of

these diseases. In addition, autoantibodies in mucous membrane pemphigoid may also be directed against laminin 332 [23]. Another disease in the group of pemphigoid diseases is anti-laminin-γ1/p200 pemphigoid; its antigen has been determined as a 200kDa protein, that might be laminin-γ1 [32,33], though recent findings show that there may be another possible target antigen with the same size [34]. In epidermolysis bullosa acquisita, autoantibodies are directed against the dermal protein collagen type VII [35] .

Table 1: Classification of autoimmune bullous diseases, the respective autoantigens and diagnostic methods

	Disease	Target antigen	Diagnosis
Pempghigus	Pempghigus foliacieus	Desmoglein 1	ELISA, IIF
	Pemphigus vulgaris	Desmoglein 1 and 3	ELISA, IIF
	Paraneoplastic pemphigus	Envoplakin, Periplakin, Desmoglein 1 and 3, BP230, Desmoplakin I/II, α2 macroglobulin-like 1	ELISA, IB
Pemphigoid	Bullous pemphigoid	BP180; BP230	ELISA, IIF
	Mucus membrane pemphigoid	BP180, Laminin 332 BP230, α6β4 integrin	ELISA, IIF, IB
	Linear IgA dermatosis	BP180; BP230	IB, IIF
	Lichen planus pemphigoides	BP180; BP230	ELISA, IIF
	Pemphigoid gestationis	BP180; BP230	ELISA, IIF
	Anti-laminin-γ1/p200 pemphigoid	Laminin-γ1	IB, IIF
	Epidermolysis bullosa acquisita	Type VII collagen	ELISA, IIF, IB

IIF: indirect immunofluorescence; IB: immuno blot; ELISA, Enzyme-linked immunosorbent assay

As the main target antigens in autoimmune blistering diseases have been identified, serum antibodies can be detected by Western blotting and ELISA using

cell-derived or recombinant forms of the autoantigens. However, indirect IF microscopy on salt split skin, which is generated by treatment of normal human skin with 1M NaCl to obtain an artificial blister within the DEJ, still remains an important screening test for circulating autoantibodies in pemphigoid disorders. As some target antigens are located at the upper side of the split and some are at the bottom of the artificial blister, the screening reveals the location of the antigen in the DEJ and therefore can narrow down further diagnosis.

1.4 Bullous pemphigoid

Bullous pemphigoid (BP) is by far the most frequent autoimmune bullous disease in Central Europe and the US [36–43]. It occurs primarily in the elderly, is characterised by tense blisters and associated with severe pruritus [44]. Autoantibodies are directed against two hemidesmosomal proteins, the intracellular BP230 and the transmembranous glycoprotein BP180 [45–47].

BP is a geriatric disease with an onset between the age of 75 to 80 years [48], though it rarely also occurs in children [49]. Within the autoimmune blistering diseases, BP is the most common one in Central Europe and the United States with an incidence between 13 and 21 new cases per million citizens each year in Germany, Switzerland, Scotland, and France [38–41]. In the UK, an incidence of 42.8 per million each year has recently been reported [42]. In individuals older than 90 years of age, the incidence increases to 190 per million citizens each year [39]. One-year mortality rates of 28%, 23% and 38% were determined in large cohorts in Germany, United States, and France, respectively [41,43,50]. However, this may be more due to advanced age and associated medical conditions than to BP itself. Furthermore, a retrospective historical cohort in the UK demonstrated that the mortality of BP

patients is twice as high compared to the general population [42]. In addition, genetic studies have identified an association of BP with the MHC class II allele HLA-DQB1*0301 in the Caucasian population [51] and alleles HLA-DRB1*04 and/or HLA-DRB1*1101 in the Japanese population [52].

Clinically, BP is characterised by tense blisters on erythematous or normal skin, predominantly on the lower abdomen, the inner aspects of thighs and intertriginous areas accompanied by pruritus. In a premonitory stage, erythematous macules, eczematous lesions, papules and urticarial plaques may be present. BP is diagnosed by detecting the presence of autoantibodies to BP180 and/or BP230 in the skin and serum through the use of direct IF microscopy, indirect IF microscopy, ELISA and/or immunoblotting together with further clinical information.

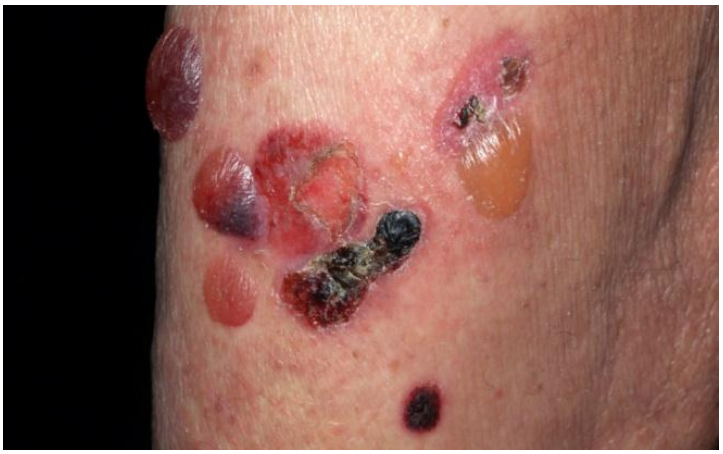

Figure 3. Clinical presentation of bullous pemphigoid
Skin lesions in bullous pemphigoid patients are characterised by tense and partly haemorrhagic blisters, erosions and crusts. [from Dept. of Dermatology, University of Lübeck]

BP is caused by antibodies against the hemidesmosomal proteins BP180 and BP230. As 50-90% of BP sera react against BP230, it was long believed that it was

the main target and immunodominant antigen in BP. Later, BP180, and more specifically, the extracellular portion of its 16th non-collagenous domain (NC16A) was identified as main target antigen [30,53,54]. BP180-NC16A specific autoantibodies predominantly belong to IgG subclasses IgG1 and IgG4 [55,56]. In 22% to 75% of the patients, additional IgE reactivity against BP180 NC16A can be found [55,57,58]. The majority of sera also react to additional antigenic sites outside the NC16A domain [59,60]. Furthermore, sera from patients with an advanced form of the disease contain antibodies that react to even more additional epitopes on either BP180 or on BP230 [61]. This seems to be due to inter- and intramolecular epitope spreading [62]. However, independent from the antibody class, disease severity correlates with the serum titre of the autoantibodies against BP180 [57,63].

Histopathology of patients' lesional skin reveals subepidermal separation located at the DEJ. In addition, prominent leukocyte infiltration, dominated by eosinophilic and neutrophilic granulocytes can be observed. Macrophages and mast cells can also be found in lower numbers [64,65] (Fig.4).

Diagnosis of BP is made by direct IF microscopy of a perilesional skin biopsy. IgG (mainly subclasses IgG1 and 4) or/and C3 deposition is observed along the DEJ (Fig. 5A). In addition, serum autoantibodies are detected by indirect IF microscopy on 1M NaCl-separated normal human skin as well as by ELISA or immunoblotting with the use of recombinant or cell-derived forms of BP180. By indirect IF microscopy IgG autoantibodies typically bind to the blister roof (Fig. 5B) [14]. In the BP180 NC16A-based ELISA, 80-90% of BP sera show reactivity [30].

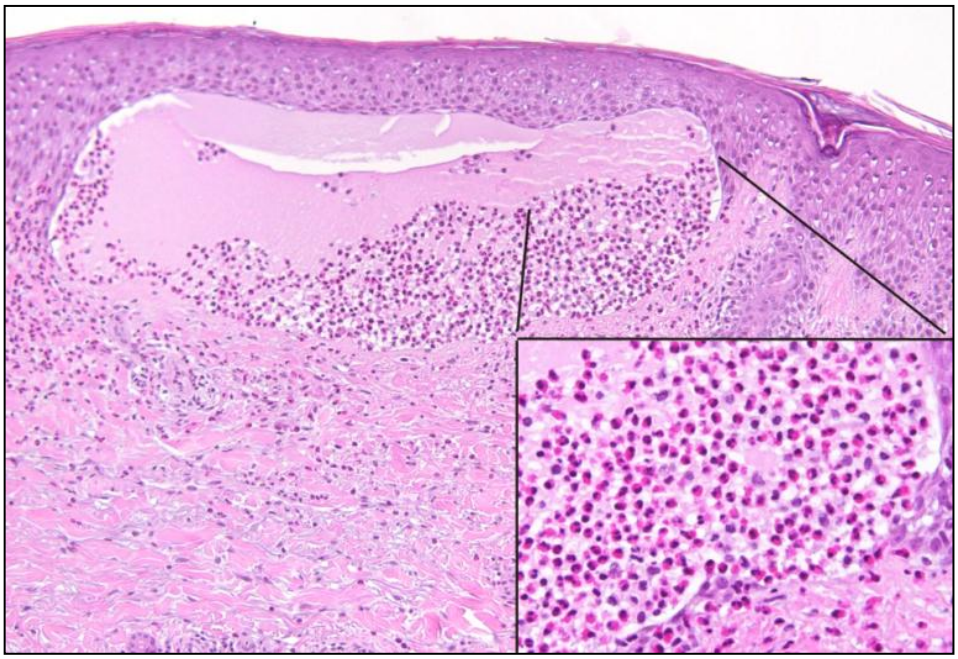

Figure 4. Histopathology of lesional skin from a bullous pemphigoid patient
Histopathologically, lesions are characterised by subepidermal blister and a mixed inflammatory infiltrate containing numerous eosinophil granulocytes. [from Dept. of Dermatology, University of Luebeck]

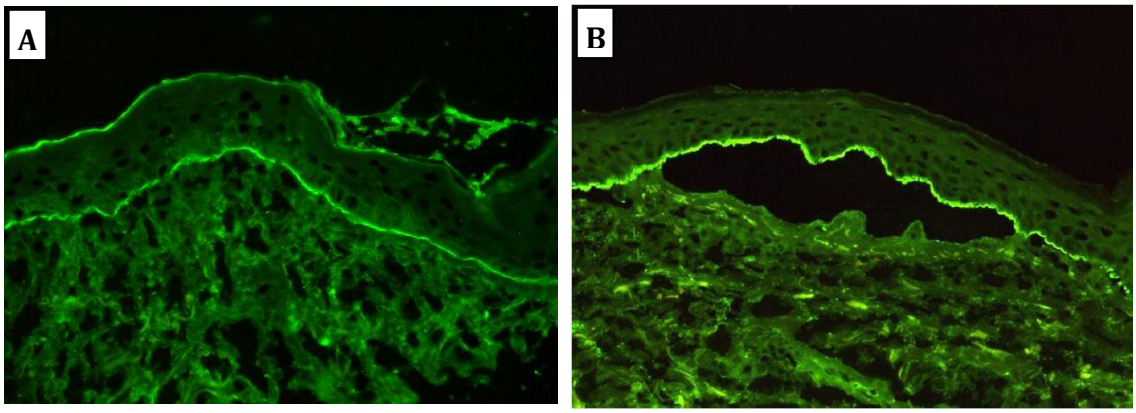

Figure 5. Immunofluorescence microscopy of bullous pemphigoid
(**A**) Direct immunofluorescence microscopy of perilesional skin in bullous pemphigoid. Linear deposition of complement C3 at the DEJ.[12] (**B**) By indirect immunofluorescence microscopy on 1M NaCl separated human skin, IgG serum autoantibodies bind to the epidermal side of the artificial split.[13]

Unspecific immunosuppressive therapy is still the mainstay of BP therapy. BP is usually treated by topical high-potent or systemic corticosteroids (e.g. tapering doses of prednisolone 0.5mg per kg of body weight per day). Most physicians add further immunomodulants/immunosuppressants such as daposone, tetracyclines,

methotrexate and azathioprine to reduce the cumulative corticosteroid use. In patients with refractory disease, high-dose intravenous immunoglobulins, immunoadsorption and rituximab can be applied respectively [12,13]. However, treatment of BP is associated with relatively high morbidity in the elderly patient population and a higher dose of systemic corticosteroids has been linked to higher mortality. Therefore, novel, more specific therapeutic options are urgently needed. A further understanding of key pathogenetic events during BP development will be helpful for this approach. In addition, suitable animal models are required to study novel treatment approaches.

1.4.1 Pathogenesis

The binding of autoantibodies to BP180 leads to a series of events that ultimately results in detachment of the dermis from the epidermis and subsequent subepidermal blister formation. How these events are connected to each other and what exactly causes this detachment is still unknown. A complex inflammatory reaction precedes the blister formation and includes activation of complement, infiltration of inflammatory cells into the skin and release of proteases [13].

Data from BP patients, *in vitro*, *ex vivo* and also *in vivo* can help to predict what is happening during disease development.

In BP patients, cells found in the inflammatory milieu include eosinophilic granulocytes as the predominant cell type, but also neutrophilic granulocytes, lymphocytes, macrophages and mast cells [64,65]. These cells can be activated by a number of inflammatory mediators, which are additionally found in this milieu. These molecules include granular proteins derived from degranulated leukocytes,

for example, eosinophilic major basic protein (MBP), neutrophil derived myeloperoxidase (MPO) [66] or chemoattractants and cytokines such as C5a, histamine and several interleukins such as IL-6 or IL-8 [67–70]. Other components found in blister fluids of BP patients, which are likely to be involved, are proteolytic enzymes including collagenase, neutrophil elastase and gelatinases A (MMP-2) and B (MMP-9) [71–73].

In vitro experiments demonstrated that binding of autoantibodies to BP180 triggers cultured keratinocytes to release IL-6 and IL-8 [74,75], which might act as strong attractants to immune cells in the first instance. In addition, with the cryosection assay the leukocyte recruiting capability of patient's serum was demonstrated. In this assay, normal human skin cryosections were incubated with BP patient serum. Autoantibodies in these serum samples bound to their antigens. The subsequent incubation with human leukocytes from healthy volunteers led to an accumulation of cells at the DEJ and finally to dermal-epidermal sparation [76]. Furthermore, studies on human skin cryosections demonstrated that elastase and gelatinase B (MMP-9) are essential for granulocyte-mediated proteolysis and further blister formation[77] .

Initial attempts to induce BP in animals through passive transfer of patients' sera into rhesus monkeys or mice were unsuccessful [78,79]. This is due to the fact that the amino acid sequence of BP180 shows only a 86% homology between human and mice [80]. Anhalt et al. were successful in inducing lesions in the cornea of New Zealand rabbits through passive transfer of patients' sera, but the disease was limited locally [81]. Following this rabbit model, further models were developed with separately produced antibodies in different species. The first model mimicking

human BP in mice was developed by Liu et al. in 1993 [82]. Neonatal Balb/c mice were injected with rabbit IgG against the murine BP180 domain NC15A, which is the murine homologue to human NC16A. The mice developed a BP-like phenotype with skin fragility and blister formation after mechanical friction, IgG and complement deposition at the DEJ was detected with direct IF microscopy and subepidermal blisters in histopathology [82]. The sequence of events, including relevant cell types and enzymes that ultimately lead to blister formation, was dissected in this model (see below).

In 2002, Yamamoto et al. induced an experimental BP in neonatal Syrian hamsters through passive transfer of rabbit anti-hamster IgG [83]. Nishie et al. produced a mouse that carries the whole human BP180 instead of the murine one [84]. Passive transfer of patients' sera into these neonatal transgenic humanised mice caused BP like disease with characteristics of human BP. Liu et al. generated a similar humanised mouse, in which only the sequence of the immunodominant murine NC15A domain was replaced with the sequence for the human NC16A [85]. Passive transfer of patients' sera as well as rabbit-anti human NC16A IgG into these neonatal mice induced a BP-like disease. Based on the humanised mouse model different studies were done with skin grafts and crossbreeding or transplants to further study different effector phase aspects of BP [84,86,87]. To study the relevance of BP230, Kiss et al. injected rabbit anti BP230 IgG into neonatal isogenic CBA/Ca. However, this only resulted in IgG and complement deposition and no clinical signs [88].

Autoantibodies of BP patients are mainly of the IgG isotype, but also serum levels of IgE anti-BP180 NC16A antibodies were reported to correlate with disease

activity and to play an important role in BP development. Two models are available, in which IgE was passively transferred into adult mice with human skin grafts. While Zone et al. transferred IgE against BP180 generated from hybridoma cells into C.B/lcrSCID mice and could detect microscopic blistering accompanied by eosinophilic infiltration [89], injection of patients' IgE against BP180NC16A into nu/nu mice by Fairley et al. resulted in eosinophilic infiltration but no blistering [90].

Besides the models with passive transfer of antibodies, models were developed in which loss of tolerance was induced by injection of recombinant murine NC15A into adult mice. Xu et al. injected human NC16A and/or murine NC15A in combination with complete Freud's adjuvant into Balb/c mice, which resulted in loss of tolerance but no clinical signs of disease [91]. Hirose et al. have recently developed an active mouse model for BP, which is based on the immunisation of SJL mice with recombinant NC15A [92]. In this model, the major immunopathological and clinical features of the human disease are reflected. Another active model with human BP180 has been developed by Ujiie et al., who induced the disease by transfer of splenocytes from C57BL/6Ncr mice that were transplanted with humanised BP180 mouse skin, into immunodeficient Rag2 knockout mice which also express human BP180 [87]. The recipient mice developed BP lesions which showed clinical similarity to human BP.

Summarising all available data mainly derived from the neonatal mouse models, the following sequence of events was devised. After anti-BP180 antibodies bind to their antigen on basal keratinocytes and form immune complexes (ICs), mast cells seem to be the first cells which appear at the sites of the ICs and react with degranulation [93]. This leads to recruitment of neutrophil granulocytes and

macrophages, which are essential for blister development [82,94]. Interestingly, tissue destruction seems to be independent of the infiltration of B- and T-lymphocytes in skin lesions [79]. Once neutrophils and macrophages have reached the inflammation site, their Fc-receptors can bind to the Fc-portions of the autoantibodies. It was shown in mouse models that the Fc part of the antibody plays a critical role in blister formation [85,95]. The injection of autoantibodies lacking the Fc part did not result in any disease development in mice. Binding to the Fc receptor leads to activation of the effector cells and the subsequent release of more modulating molecules such as cytokines and chemokines [96]. Besides binding to Fc receptors, ICs can activate the complement system, which results in the generation of further cell attractants [97]. In the neonatal BP model, complement was found to be a prerequisite [95,98] for blister formation. C1q-, C4- and C5-deficient neonatal mice were protected from blister formation and factor B deficient mice show delayed onset of disease when injected with anti BP180 antibodies [95,98]. As a probable function of the complement system the generation of C5a for the activation of mast cells was suggested [93]. Activated immune cells do not only release chemoattractants for further cell recruitment, but also release a number of proteases like elastase or metalloproteases and produce reactive oxygen species (ROS). These molecules can finally induce weakening of the dermal-epidermal bond and, ultimately, its detachment [13].

1.5 Fc and the Fc-receptor

In mice, class IgG antibodies can be divided into four different subclasses: IgG1, IgG2a, IgG2b and IgG3. The 150kDa IgG glycoprotein is composed of two identical light chains and two identical heavy chains. The heavy chain consists of one

variable domain (VH) and three constant domains (CH), while the light chain contains one variable domain (VL) and only one constant domain (CL). The CH1 and CH2 domains are separated by a protease sensitive hinge region. Papain can cleave this region, which results in the antigen-binding Fab fragments and the Fc fragment. The Fc region interacts with effector function promoting molecules, including complement factor C1q and Fc receptors on effector cells [99]. Within the CH2 domains, there is a conserved N-glycosylation site at Asn297. The functional relevance of this glycosylation has been investigated in several studies. For instance, murine IgG lacking the oligosaccharide does not activate complement, bind to Fc-receptors on macrophages nor induce antibody-dependent cellular cytotoxicity in vivo [100]. When an IgG antibody is bound to its Fcγ receptor (FcγR), it forms a horseshoe-like structure, which seems to be crucial for efficient binding. This conformational change is mediated by the sugar moieties within the CH2 domain of the Fc fragment [101]. In addition to subclass variations, the composition of the sugar moiety therefore can influence the binding affinity of IgG molecules to their different FcγRs and even compromise the clearance of antibody-antigen complexes from the circulation [100]. Importantly, the Fc N-glycans are highly dynamic and accessible to glycan-modifying enzymes or glycan recognition domains [102]. It is suggested that modification of the Fc N-glycan composition may affect the biological properties of the IgG molecules. For instance, EndoS, an endoglycosidase that specifically hydrolyses the N-linked glycan [103,104], reduces binding to activating FcγR and increases binding to the inhibitory FcγRIIB [105].

FcγRs belong to the large immunoglobulin superfamily [101]. Functionally, they act as a bridge between the innate and the adaptive immune system. They can bind

immunoglobulins via their Fc part, which leads to activation of several types of innate immune cells. The prerequisite for efficient cell activation is the specific binding of immunoglobulins to their target antigen which results in the formation of ICs. This leads to accumulation of the FcRs on the cell surface. Binding of immune complexes to FcγRs induces phagocytosis, antibody-dependent cellular cytotoxicity, the transcription of cytokine genes and the release of inflammatory mediators [106]. FcγRs can be divided into high or low affinity FcγRs [101] as well as into activating or inhibitory FcγRs [107–109]. In mice, four different subclasses of FcγRs have been identified: FcγRI, FcγRIIB, FcγRIII and FcγRIV [110]. These are expressed on the surface of natural killer (NK), NKT and on B cells [111–114], but not on T cells [1]. The receptors are all co-expressed on immune cells, except on NK cells, which exclusively express FcγRIII, and on B cells, which only express FcγRIIB. The different IgG subclasses bind with different affinities and specificities to the different FcγRs [106,115–118]. In mice, FcγRI, FcγRIII and FcγRIV belong to the group of activating FcγRs. FcγRIIB is the only inhibitory FcγR. All activating FcγRs in mice are composed of an IgG binding α-chain and a signal transducing adaptor molecule that contains an immunoreceptor tyrosine-based activation motif in its cytoplasmic domain, which is the common γ-chain. In addition to its signalling function, the γ-chain promotes the accumulation and cell surface transport of the different activating FcγR γ-chains. Therefore, mice deficient in the γ-chain are functional FcγRs knockouts [119]. In contrast, the inhibitory FcγR, FcγRIIB, contains an immunoreceptor tyrosine-based inhibitory motif in its cytoplasmic domain, which decreases the activation of molecules involved in further cell signaling.

1.6 Complement system

In the neonatal mouse model of BP, the classical pathway of the complement system appeared to be pivotal [95,98], while the alternative pathway was found to play a minor role in disease development [98].

The complement system is a part of the humoral immune system. It consists of around 30 plasmaproteins, which can be classified into nine types from C1 to C9. They are generally synthesised by the liver and normally circulate as inactive precursors. They work together in a cascade manner and several methods of activating the complement system are currently known. Depending on the trigger, different initiation and regulatory mechanisms act together and ultimately result in cleavage of C3 and C5 [97].

Two pathways lead to the generation of the C3 convertase C2bC4b. The classical pathway is often referred to as antibody-dependent because it is strongly triggered by IgM or IgG clusters. However, C1q activates complement by directly recognising distinct structures on microbial and apoptotic cells or through endogenous pattern recognition molecules such as immunoglobulins. Binding of C1q leads to activation of the proteases C1r and C1s, which are also part of the C1 complex $C1qr_1s_2$ [120,121]. C1s subsequently cleaves C4 into C4a and C4b, which leads to covalent deposition of C4b on surfaces in the surrounding area of the activation sites. In addition, C1s cleaves C2 into C2a and C2b. This results in the generation of the classical pathway C3 convertase C2b4b.

The same C3 convertase is generated by activation of the lectin pathway. Here, mannose-binding lectin (MBL) and ficolins predominantly recognise carbohydrate

patterns. MBL or ficolin assembles with MBL-associated serine proteases (MASPs) which share structural similarity with C1r and C1s. While MASP-2 generates the same C3 convertase as in the classical pathway by cleaving both C4 and C2, MASP-1 cleaves C2 but not C4. Therefore, MASP-1 can support the lectin pathway response once initiated [122] and increases the efficiency of convertase formation [123,124].

In contrast to the classical and the lectin pathway, the alternative pathway represents three different but partially overlapping processes. The central molecule of the alternative pathway is C3. In its native form, C3 has only few ligands and is relatively inactive. However, a small fraction of the C3 molecules are hydrolysed to $C3H_2O$, exposing new binding sites. The factor B (fB) protease binds $C3H_2O$ and is cleaved by factor D (fD). This leads to generation of an initial C3 convertase $C3H_2OBb$, which activates complement by cleaving C3 into its fragments, C3a and C3b. Subsequently, C3b can covalently bind to amine and carbohydrate groups on target surfaces. This tagging is quickly amplified on foreign cells. In addition, binding to specific carbohydrates may lead to preferential opsonisation of foreign particles and represent a basic pattern recognition mechanism [125,126]. Furthermore, the alternative pathway also includes an initiation mechanism, which is similar to those found in both the lectin and classical pathways. Properdin (factor P) recognises several pathogen- or damage-associated molecular patterns on foreign and apoptotic cells. Once bound, it initiates and amplifies the complement response by attracting C3b to recognised surfaces [127], which results in more convertase formation by stabilising the C3 convertase complexes (C3bBbP) [128].

Eventually, cleavage of C3 by all surface-bound C3 convertases, independent of its origin, can induce the alternative pathway. The generated C3b forms the main alternative pathway C3 convertase (C3bBb) in the presence of fB and fD. This results in an efficient cycle of C3 cleavage and convertase production that amplifies the response. Therefore, the alternative pathway might account for up to 80–90% of total complement activation, even when initially triggered by the classical pathway or lectin pathway [129]. Amplification by the alternative pathway increases the density of C3b and leads to formation of C5 convertases by adding one more C3b molecule to the C3 convertases. C4b2b3b and C3bBb3b subsequently cleave C5 into the anaphylatoxin C5a and the fragment C5b. When C5b associates with C6 and C7, the formed complex is inserted into cell membranes and interacts with C8. Subsequent binding of several units of C9 then induces formation of a lytic pore, the membrane attack complex (MAC) [130].

Proinflammatory signalling and phagocytosis are essential for complement-mediated defence against most foreign cells. The active complement fragments C3a and C5a trigger proinflammatory signalling through their corresponding G-protein–coupled receptors, C3a receptor (C3aR) and C5a receptor (C5aR; also called CD88). A third, G-protein–independent C5a receptor, C5L2 (GPR77), has been discovered recently [131]. Theories of its role range from decoy to regulatory or even proinflammatory functions [132,133], but its exact functions have not been completely determined yet [134]. While C3aR binds only C3a, C5aR recognises both C5a and its degradation product $C5a^{desArg}$, the latter one with less affinity. C5L2 interacts with $C5a^{desArg}$ and C5a and, interestingly, has comparable affinities for both [135]. Although there is evidence for a functional link between C3a or $C3a^{desArg}$

and C5L2, proof of a direct interaction remains controversial. Anaphylatoxins C3a and especially C5a are highly potent effectors and have a number of crucial roles in immune response and inflammation [136,137]. The biological effects include increased vascular permeability, chemotaxis of inflammatory cells, respiratory burst, cytokine and chemokine release and promotion of phagocytosis through the interaction of opsonins with complement receptors [137–139]. The reacting effector cells are neutrophils, macrophages and monocytes, mast cells and also endothelial cells, T lymphocytes and cardiomyocytes [139,140]. As powerful chemoattractants, anaphylatoxins guide neutrophils, monocytes and macrophages toward sites of complement activation. The binding of C5aR to T-cells leads to their activation as well as the inhibition of the IL-12 family regulation, which is an inhibitory pathway, after binding to C5aR on antigen presenting cells [134].

Additional methods of complement activation to the three described above have emerged in recent years. Although it has been already known that proteases such as plasmin, thrombin, elastase and plasma kallikrein, can cleave and activate C3 [141–143], the extrinsic protease pathway has gained more attention as it might be involved in potential crosstalk between complement and coagulation pathways [144]. Thrombin can induce the generation of C5a in the absence of C3 [142], which indicates that the extrinsic protease pathway also includes C5. In addition, target-bound MBL can activate C3 independent from MASP-2, C2 and C4 *in vitro* [145], but the physiological consequences of this bypass require further investigation [146].

Aim of the study

Currently, there are several mouse models available for BP. A major disadvantage of the preferably studied neonatal mouse models is the use of neonates for a disease of the elderly. In addition, in the neonatal mouse model of BP, experiments run for only 48 hours, a setting that does not allow modulating the tissue destruction in clinically diseased animals. Furthermore, not all immunopathological characteristics of the human disease e.g. the infiltration of eosinophils are recapitulated in the available animal models.

The first aim of my study was to establish a new mouse model for BP with the use of adult mice. This would allow a longer time of disease than in the neonatal mouse models, and would therefore mimic the patients' situation more closely. Furthermore, this would allow the exploration of new, more specific anti-inflammatory modalities. Injection of antibodies against collagen type XVII should induce disease in mice, which reflects the main features of human BP. Clinically, diseased mice were expected to show tense blisters accompanied by erythema, crusts and erosions. The blisters should be located subepidermally within the lamina lucida and an inflammatory infiltrate dominated by eosinophils and neutrophils should be present in the dermis. Direct IF microscopy is expected to reveal linear deposition of antibodies and complement along the DEJ. Similar to BP patients, disease activity should correlate with serum levels of anti-BP180 antibodies. In previous studies with the available experimental BP models it was shown that binding of the Fc-fragment to its respective receptors was crucial for the development of pemphigoid diseases. I claimed the same key role of the Fc-fragment for the development of an experimental BP in my new model and verified

this hypothesis with the use of knockout mice and modified antibodies. Furthermore, the complement system was previously shown to be pivotal for experimental BP. Besides confirming this notion in the new mouse model, I aimed at dissecting the importance of the main complement components, C3 and C5 as well as their receptors. Finally, I wanted to exemplify the usefulness of the novel model to investigate therapeutic substances by inhibiting of the tissue destruction phase in both a prophylactic and a therapeutic setting i.e. in already clinically diseased animals.

2. Methods

2.1 Mice

C57BL/6J, Balb/c, FcRγ$^{-/-}$ mice (B6;129P2-*Fcer1g*^{tm1Rav}/J), C5aR$^{-/-}$ (on C57BL/6 background), and C5L2$^{-/-}$ mice (on Balb/c background) were bred and housed in a 12 hour light-dark cycle at the experimental animal facility at the University of Lübeck. C5$^{-/-}$ mice (B10.D2-*Hc*^{0} *H2*^{d} *H2-T18*^{c}/oSnJ), the respective C5-sufficient controls (B10.D2-*Hc*^{1} *H2*^{d} *H2-T18*^{c}/nSnJ), and C3$^{-/-}$ mice (B6;129S4-C3tm1Crr/J) were obtained from The Jackson Laboratories (Bar Harbor, ME, USA). All injections and bleedings were performed on eight to twelve week old mice narcotised by intraperitoneal (i.p.) injection of a mixture of ketamine (100µg/g) and xylazine (15µg/g). All animal experiments were approved by the Animal Care and Use Committee of Schleswig-Holstein (Kiel, Germany; 21-2/11) and performed by me as certified personnel.

2.2 Expression and purification of murine type XVII collagen fragments

The extracellular portion of the 15th non-collagenous domain (NC15A) of mCOL17 (aa497-573 of murine BP180) was expressed as glutathione-*S*-transferase (GST) fusion protein and purified by affinity chromatography as previously described [92]. DNA sequence data from mCOL17A was retrieved from GenBank using the access number NC_000085.5. The cDNA fragments were obtained from murine 212 keratinocytes. mCOL17A cDNA fragments with inserted restriction sites for *BamHI* and *SalI* (Fermentas Life Sciences, St. Leon-Rot, Germany) were cloned into

linearised pGEX-6P-1 (Amersham Biosciences, Heidelberg, Germany) resulting in the recombinant vector pGEX-mCOL17A. Correct ligation and in-frame insertion of the DNA fragments were confirmed by DNA sequence analysis.

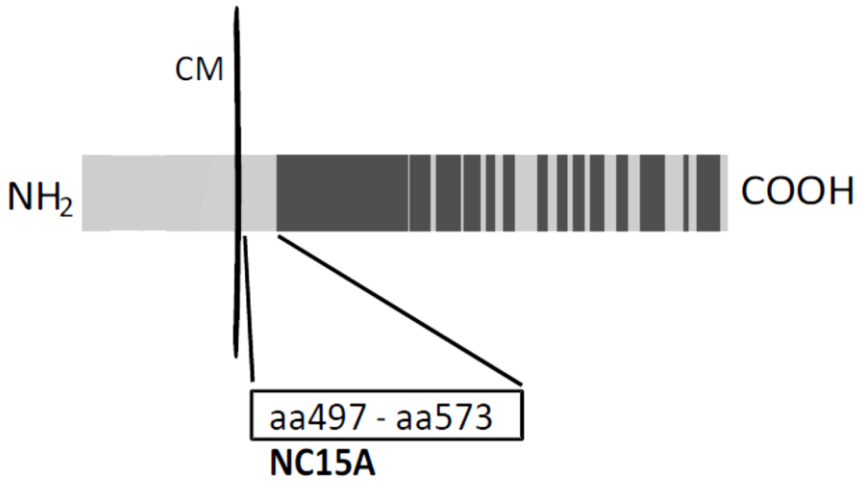

Figure 6. Schematic organisation of the murine BP180 protein constructs generated for this study for expressing recombinant peptides of the NC15 domain
BP180 is composed of an intracellular domain at the NH2 site, a transmembrane domain going through the cell membrane (CM) and an extracellular domain. A recombinant fragment of murine BP180 (NC15A) cDNA was cloned into pGEX-6P-1 and expressed in *E.coli*. The N- and C-terminal amino acid sequence parts of the fragment are depicted; the location of the fragment within the complete murine BP180 amino acid sequence (UniProt ID Q07563, isoform 1) is indicated above.

Previously generated recombinant fusion proteins were then expressed and purified by me. Expression was conducted in *E.coli* Origami (Novagen, WI, USA). 5ml overnight cultures in LB-medium (MP Biomedicals, Illkirch, France) were transferred into 500ml LB-medium. Growing was monitored by measuring the OD_{600} and protein expression was induced in the log-phase (OD_{600} ~0.6-0.8) by adding IPTG (0.1mg/ml). After 3 hours the bacteria were harvested by centrifugation of the cultures. The pellets were resuspended in 13ml buffer (50mM Tris-Cl, 25% sucrose, 1mM NaEDTA, 0.1% NaN_3, 10mM DTT; pH 8.0) and sonicated three times (50%, level 4-5, 30 pulses) on ice. After adding 100µl lysozyme, 10µl

benzoase and 50µl $MgCl_2$ the suspension was mixed thoroughly and 12.5ml lysis buffer (50mM Tris-Cl, 1% Triton X-100, 1% Na-deoxycholate, 100mM NaCl, 0.1% NaN_3, 10mM DTT; pH 8.0) were added. After incubation for 45minutes at room temperature 350µl of a NaEDTA solution (0.5M NaEDTA in 50mM Tris-Cl; pH 8.0) were added and the suspension was frozen with N_2 and thawed at 37°C. After adding 200µl of 5M NaCl, another 30minutes incubation at room temperature and addition of 350µl NaEDTA solution (0.5M NaEDTA in 50mM Tris-Cl; pH 8.0) the suspension was centrifuged at 11,000g for 20 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 10ml buffer (50mM Tris-Cl, 0.5% Triton X-100, 100mM NaCl, 1mM NaEDTA, 0.1% NaN_3, 1mM DTT; pH 8.0) and sonicated three times (50%, level 4-5, 30 pulses) on ice. The suspension was again centrifuged (11,000g, 20min, 4°C), resuspended in 10ml buffer (50mM Tris-Cl, 100mM NaCl, 1mM NaEDTA, 0.1% NaN_3, 1mM DTT; pH 8.0) and sonicated (50%, level 4-5, 30 pulses) on ice. After centrifugation (11,000g, 20min, 4°C) the supernatant was discarded and the pellet was resuspended in 9ml 8M guanidinium (pH8.0, including 4mM DTT). 1ml fractions were stored at -80°C.

Refolding of the protein, dialysis and protein purification were done at 4°C. Purification of the protein was performed with the use of glutathione-agarose affinity chromatography [22]. For protein refolding 3 times 1ml (1ml every 8 hours) of the frozen guanidinium fractions were diluted in 200ml continuously stirring refolding buffer (100mM Tris-Cl, 400mM L-arginine, 2mM NaEDTA, 0,5mM ox. glutathione, 5mM rd. glutathione, 50µl protease inhibitors [Protease Inhibitor Set II, Calbiochem/Merck, Darmstadt, Germany]). After 24 hours the protein containing buffer was dialysed against PBS three times. The solution was loaded

onto a PBS-equilibrated glutathione-agarose column (Sigma, Munich, Germany). The glutathione-agarose was washed with 100ml PBS. The protein was eluted with 100ml fresh elution buffer (924mg glutathione red. in 150ml PBS; pH 8.0). The agarose was washed with 50ml elution buffer to clean it from all remaining protein, followed by further washing steps (150ml 0.1 M Borate Buffer containing 0.5M NaCl, pH 8,5; 150ml PBS, 150ml 0.1M Acetic Acid containing 0.5M NaCl, pH 4,5, 150ml PBS), and finally stored in 2M NaCl + 1mM NaN_3. The protein eluate was concentrated and washed with PBS with the use of Amicon Ultra filter tubes (Millipore, Bradford, MA, USA). Protein concentrations were measured spectrophotometrically at 280 nm (BioPhotometer; Eppendorf, Hamburg, Germany) and with the use of SDS-PAGE.

2.3 Generation and characterisation of rabbit antibodies to murine collagen type XVII (mCOL17)

Pathogenic anti-mCol17 IgG was generated as reported previously [147]. New Zealand white rabbits were immunised with recombinant forms of the GST-tagged NC15A domain of mCOL17 at EUROGENTEC (Seraing, Belgium). All further investigations with the rabbit serum were conducted by me. The rabbit serum was purified by affinity chromatography with the use of protein G following the manufacturer's manual. Reactivity of IgG fractions was analysed by indirect IFM on murine skin. The leukocyte-activating capacity of purified rabbit IgG was evaluated *ex vivo* on cryosections of mouse skin as reported [76]. Mouse skin was incubated with rabbit serum for one hour at 37°C. Human neutrophils were added and incubated for 3 hours at 37°C before washing with PBS. Sections were stained with hematoxylin and eosin and dermal-epidermal separation was evaluated

microscopically. The *ex vivo* studies were approved by the local ethics committee (09-140). Normal rabbit serum was obtained from CCPro (Oberdorla, Germany).

2.4 EndoS preparation and IgG hydrolysis *in vitro*

Pretreatment of rabbit IgG was performed as previously described (at the laboratory of Prof. Mattias Collin [Lund University, Lund, Sweden]) [103,148]. 1mg of rabbit anti-mCOL17 IgG was incubated with 5µg recombinant GST-EndoS in PBS for 16 hours at 37°C followed by affinity removal of GST-EndoS by serial passages over Glutathione-Sepharose 4B columns (GE Healthcare, Uppsala, Sweden). IgG hydrolysis was verified by SDS-PAGE and lectin blot analyses as previously described [149]. Glycosylated IgG was detected by using 5µg/ml biotinylated *Lens culinaris agglutinin* -lectin (Vector Laboratories, Burlingame, CA, USA) and 1µg/ml streptavidin-horseradish peroxidase (Vector Laboratories) and Super Signal West Pico peroxidase substrate (Pierce, Rockford, IL, USA).

2.5 Passive transfer mouse model

IgG injection into mice followed a previously established model for anti-mCol VII [150] . Affinity-purified rabbit anti-mCol17 IgG or normal rabbit IgG was injected subcutaneously into the neck of the mice every second day over a period of 12 days. At the time of IgG injections, the mice were weighed and examined for their general condition and evidence of cutaneous lesions (i.e., erythema, blisters, erosions, and crusts). Cutaneous lesions were counted, and the extent of skin disease was scored as involvement of the skin surface. On day 12, blood was taken and both lesional and perilesional biopsies were collected for histopathological analysis (stored in 4% buffered formalin) and direct IF microscopy (stored at -80°C) respectively. C5a receptor antagonist PMX53 was provided by Dr. Trent

Woodruff (University of Queensland, Brisbane, Australia). 20µg of PMX53 per mouse were injected i.p. daily either from day 0 to 11 or from day 4 to 11.

2.6 Immunoblot analysis

As described previously [28], serum from immunised and from preimmune rabbits was preadsorbed with GST produced by *E.coli* (dilution 1:100,000) for 24h at 4°C. Recombinant GST-tagged mCol17 (BP180 NC15A) was separated by SDS-PAGE (15% separation gel, 4% collecting gel) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% milk powder in TBS-T. Preabsorbed serum or mouse anti-GST antibody (1:1,000; Abcam, Cambridge, UK) was incubated on the membrane overnight at 4°C. After washing with PBS, HRP-tagged goat anti-rabbit IgG (1:2,000, Dako, Hamburg, Germany) and HRP-tagged rabbit anti mouse IgG (1:2,000, Dako) were incubated for 1 hour at room temperature. After washing with TBS-T bound antibodies were visualised with 10ml 3,3'-Diaminobenzidine (DAB) solution (Merck, Darmstadt, Germany) containing 20µl H_2O_2.

2.7 Human leukocyte preparation

Human leukocytes were prepared by me. Peripheral blood leukocytes from healthy volunteers were isolated by a sedimentation gradient containing sodium diatrizoate and dextran 500 following the instructions of the manufacturer (Nycomed, Oslo, Norway). Cells were harvested, washed twice in RPMI 1640 (Life Technologies, Karlsruhe, Germany) and resuspended in the same medium at a density of $6*10^7$cells per ml. The cell suspension was kept on ice and cell viability was tested using trypan blue; only preparations with viability greater than 95% were used.

2.8 Cryosection assay

Following a previously described protocol [76], I performed a series of cryosection assays. 6µm cryostat sections from normal mouse back skin were washed with PBS for 5min to remove embedding medium before incubation with 30µl of diluted rabbit sera preparations (1:3 in PBS) for 90min at room temperature. After washing the sections with PBS twice, chambers were prepared as described elsewhere [151] with minor modifications. Briefly, tissue containing slides were covered with a second slide to which transparent adhesive tape (Beiersdorf AG, Hamburg, Germany) had been placed around each end. The tape prevented the skin sections from contacting the covering slide, thus creating a chamber of approximately 0.3mm thickness and 0.5ml volume. Both slides were taped together and the leukocyte suspension was injected into the chambers. Chambers were incubated in a humidified air incubator containing 5% CO_2 (Memmert, Schwabach, Germany) for 3 h at 37°C. Subsequently, chambers were disassembled and sections were washed in PBS for 10min to remove excess serum and non-adherent cells, air dried for 10min, fixed in formalin, and finally stained with hematoxylin and eosin.

2.9 Immunofluorescence microscopy

IF microscopy was performed to characterise the rabbit anti-mCol 17 antibody and detection of tissue-bound autoantibodies and complement deposits in the skin of mice. Rebecca Cames, Lübeck, helped preparing the cryosections.

6µm sections of healthy C57BL/6 mouse skin and healthy mouse skin in which dermal-epidermal splitting was induced by incubation with 1M NaCl ('salt-split skin') were prepared for indirect IF microscopy to evaluate the tissue-binding

ability of rabbit anti-mCol17 IgG. Sections were incubated with appropriately diluted rabbit serum and evaluated using a fluorescence microscope (EUROstar, EUROIMMUN AG, Groß Grönau, Germany).

The tissue-bound autoantibodies and complement deposits were detected by direct IF microscopy of frozen sections with the use of FITC-conjugated rabbit anti-mouse IgG (1:100; Dako), FITC-conjugated murine anti-mouse C3 (1:50; Cappel, MP Biomedicals, Solon, OH, USA), and murine anti-mouse C5 antibody (1:100; Cell Sciences, Canton, MA, USA) detected by FITC-labelled rabbit anti-mouse IgG (1:100; Dako). The staining intensity of immunoreactants at the DEJ was quantified with ImageJ (http://rsbweb.nih.gov/ij/) software.

2.10 Histopathology

Histopathology staining was performed by Rebecca Cames according to the following protocol. Formalin-fixed skin samples were processed into paraffin blocks. 6µm sections were stained with hematoxylin and eosin according to standard protocols.

Immunohistochemistry analysis was performed by me. For the visualisation of eosinophils 6µm cryosections of lesional mouse skin (cut by Ms. Cames) were fixed in acetone for 10 minutes. Air dried sections were washed 3 times with PBS before incubation with Dako Dual Endogenous Enzyme Block (Dako) for the blocking of endogenous peroxidase. After subsequent washing the sections were incubated with rat anti-mouse MBP antibody (1:1,000; Lee Laboratory, Mayo clinic Arizona, Scottsdale, AZ, USA [152]) and subsequently, with HRP-coupled rabbit anti-rat IgG (1:500; Dako). Detection was done with HistoGreen (Linaris, Wertheim-Bettingen, Germany), counterstaining was done by Haemalaun staining.

2.11 Electron microscopy

Electron microscopy was performed by Prof. Akira Ishiko at the Department of Dermatology at the Keio University (Tokyo, Japan). Samples for transmission electron microscopy were taken from lesional skin of anti-mCol17 IgG-injected mice and corresponding body parts of negative control mice. Fixed samples were processed as described and stained sections examined with an electron microscope (Model JEOL JEM-1230, Tokyo, Japan) [153].

2.12 Neutrophil preparation

Mouse neutrophils were purified as previously described [154]. Bone marrow cells from hind femurs and tibiae were flushed, red blood cells lysed with hypotonic NaCl and cells were separated by 62.5% Percoll™ (GE Healthcare, Uppsala, Sweden) gradient. For higher purity of neutrophils, T- and B-cells were depleted by MACS separation with PE- labelled anti-CD3ε and anti-CD19-antibodies (each 10µl/10^9 cells; Miltenyi, Bergisch-Gladbach, Germany) and anti-PE-microbeads (equal volume to primary antibodies; Miltenyi). Purity of neutrophils was consistently >90% as determined by cell sorting analysis. Cell samples before and after MACS were sorted by size and granularity in a FACS separator. The population of neutrophils was clearly distinguishable from T- and B-cells and also from potentially remaining macrophages so could therefore be quantified as percentage of the total cell amount.

2.13 ROS release assay

For the ROS-release and the neutrophil migration assay serum was used as a source of complement. When serum is heated for a certain time, the complement

components are destroyed. For the ROS release and migration assay, I split the required volume for total volume of heat-inactivated and normal serum and kept one half at 4°C and incubated the other half for 30minutes at 56°C ('heat-inactivated').

The ROS release assay was performed as described previously [155]. 96-well plates were coated with 10µg/ml of mCol17 (NC15A) overnight. After blocking with PBS containing 1%BSA and 0.05% Tween, 1 mg/ml rabbit anti-mCOL17 IgG was incubated for one hour at 37°C. Generation of reactive oxygen species by phagocytes was determined in a microplate luminometer (Wallac 1420 VICTOR TM, Perkin Elmer, CA, USA) by measurement of chemiluminescence in the presence of 60µg/ml luminol (5-amino-2,3-dihydro-1,4-phthalazindione; Roche Diagnostics, Mannheim, Germany). Luminol, cells (10^5) in chemiluminescence medium (RPMI 1640 without phenol red with stabilised L-glutamine [Biochrom, Berlin, Germany], 25mM HEPES, 1%FCS [Biochrom]) together with 5% serum, heat-inactivated (30' at 56°C) serum or only chemiluminescence medium were added to the wells with a final volume of 200µl. Chemiluminescence was recorded for 60 min and data were expressed as relative light units (RLU).

2.14 Neutrophil migration assay

The assay was performed as reported previously with the following modifications [156]. 24-well plates were coated with 10µg/ml of mCol17 (NC15A) overnight. After blocking with PBS containing 1%BSA and 0.05% Tween, 1mg/ml rabbit anti-mCol17 IgG was incubated for one hour at 37°C. After washing with PBS transwells (6.5mm, 3µm pore, Corning, New York, NY, USA) $1.0*10^6$ neutrophils in 100µl of

migration medium (RPMI 1640 [Life Technologies, Karlsruhe, Germany]), 1M HEPES, 0.5% FCS, and 1% penicillin/streptavidin [Biochrom]) were placed into the 24 well-plates filled with 600µl of migration medium containing 5% mouse serum or heat-inactivated (30' at 56°C) serum and incubated for 1.5 hours at 37°C. Transwells were discarded and the plates were centrifuged for 5min at 260g. Cells were washed 3 times with PBS and resolved in 350µl migration medium. Cells were then lysed with 75µl 1% triton X-100 and incubated with p-nitrophenyl-β-glucuronide in 0.1M acetate buffer (pH4) overnight at 37°C. The reaction was stopped with 0.4M glycine buffer (pH10.3). The number of migrated cells was approximated by glucuronidase levels measured through photospectometry (405nm; Wallac 1420 VICTOR TM). Uncoated wells and migration medium with and without IL-8 (50ng/ml; Calbiochem, San Diego, CA, USA) were used as positive and negative controls, respectively.

2.15 Clinical score, cumulative score and statistical analysis

To compare the disease activity in mice, the clinical score (CS) was determined from each mouse. Erythema, crusts and erosions were calculated as affected body surface area in per cent [%]. To compare the overall disease activity, the clinical scores from each sampling day were cumulated ('cumulative disease activity'). For this purpose the area under the curve (AUC) was calculated for each mouse: AUC = $(4*CS_{Day4})/2+(4*CS_{Day4}+(CS_{Day8}-CS_{Day4})/2)+4*CS_{Day8}+(CS_{Day12}-CS_{Day8})/2)$. The data are represented as mean values; the differences within the groups are shown as standard error of the mean values. The AUC for the ROS-release was taken as the summary of each value from each measured minute.

To perform statistical analysis, the software programs Sigma plot 11.0 (Systat Software Inc., Chicago, IL, USA) and R (http://www.r-project.org/) were used.

Differences in the clinical scores between groups were determined by t-test or Rank Sum test respectively, if the data were not normally distributed. To calculate differences between the cumulative scores, a t-test or a One Way ANOVA was performed. For the comparison of the immunofluorescence values (2.9), One Way ANOVA was used for statistical analysis. A p-value of <0.05 was considered statistically significant.

Applied tests are also indicated in the figure legends.

3. Results

3.1 Development of a novel mouse model

3.1.1. Rabbit IgG against murine Col17 (BP180 NC15A) induced dermal-epidermal splitting *ex vivo*

New Zealand white rabbits (n=10) were immunised with a recombinant murine homologue of the human NC16A domain of Col17 (BP180 NC15A). In a Western Blot serum of the immunised rabbits, but not serum from before immunisation, specifically bound to NC15A-GST that was absorbed with GST before it was blotted to the membrane (Fig.7A). In addition, indirect IF microscopy with incubated protein G purified IgG from immunised rabbit serum on murine 1M NaCl-split skin revealed binding of the antibody to the epidermal side of the artificial blister (Fig. 7B). Purified IgG from normal rabbit serum did not show any binding (data not shown).

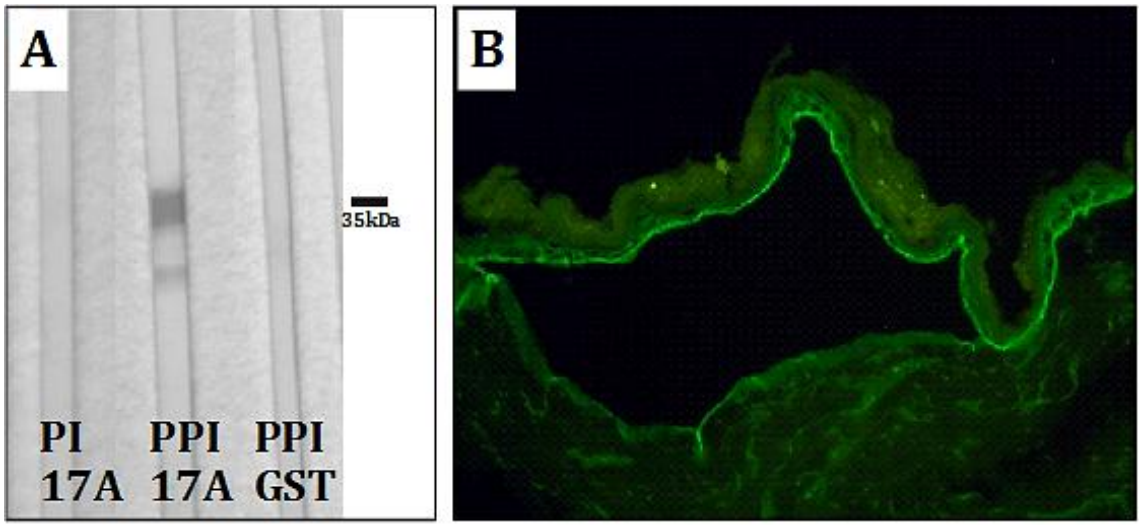

Figure 7. *In vitro* characterisation of rabbit anti mCol17 IgG
(**A**) In Western blot, serum from rabbits immunised with GST tagged mCol17 (NC15A) (=PPI), but not from preimmune rabbits (=PI), specifically bound to GST-preabsorbed NC15A-GST (17A), but not to GST (GST). (**B**) Protein G purified IgG from rabbits immunised with GST tagged mCol17 labelled the epidermal side of the artificial splitting by indirect immunofluorescence microscopy on murine 1M NaCl-split skin. Magnification: 1:200

The leukocyte-activating and dermal-epidermal split-inducing capacity of rabbit anti-Col17 IgG was demonstrated with the use of a cryosection assay. It was shown before that antibodies against proteins of the DEJ can initiate dermal-epidermal separation in the presence of human leukocytes [76,157]. Therefore, murine skin was incubated with serum of mCol17-immunised and preimmune rabbits respectively. Following incubation with human leukocytes, separation of the epidermis from the dermis was detected by H&E staining. Only incubation with serum from immunised rabbits resulted in split formation (Fig.8A), while incubation with serum from preimmune rabbits showed no separation (Fig. 8B).

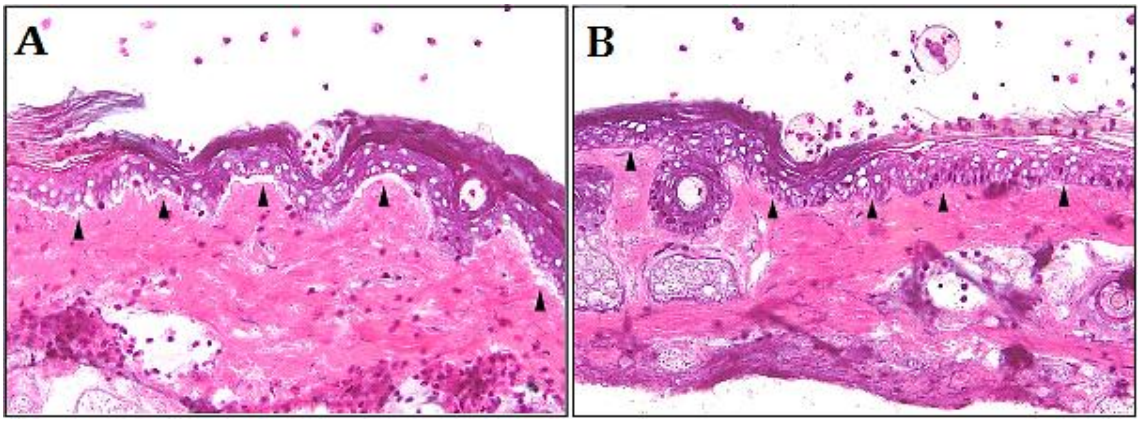

Figure 8. Split inducing capacity of anti-mCol17 IgG
Cryosections of normal murine skin was incubated with serum from immunised or preimmune rabbits and washed prior to incubation with human leukocytes. Sections were stained with H&E. Serum from immunised (**A**) but not from preimmune rabbits (**B**) induced dermal-epidermal separation in the presence of human leukocytes. Arrow heads show localisation of separation (A) and unaltered DEJ (B). Magnification: 1:200

3.1.2 Passive transfer of anti-mCol17 IgG in adult mice reproduced major clinical and immunopathological characteristics of the human disease

8- to 12-week-old C57Bl/6 mice were injected subcutaneously with anti mCol17-specific rabbit IgG every second day over a period of 12 days with each injection

containing 5mg IgG. On day 12 perilesional skin biopsies were taken for IF microscopy, histology, immunohistochemistry and electron microscopy.

Direct IF microscopy of the biopsies revealed linear deposits of rabbit IgG at the DEJ. Furthermore, linear deposition of murine complement components C3 and C5 was also detected along the DEJ (Fig. 9). Mice injected with the same amount of normal rabbit IgG showed no deposits of IgG, C3 and C5 at the DEJ in direct IF microscopy (data not shown).

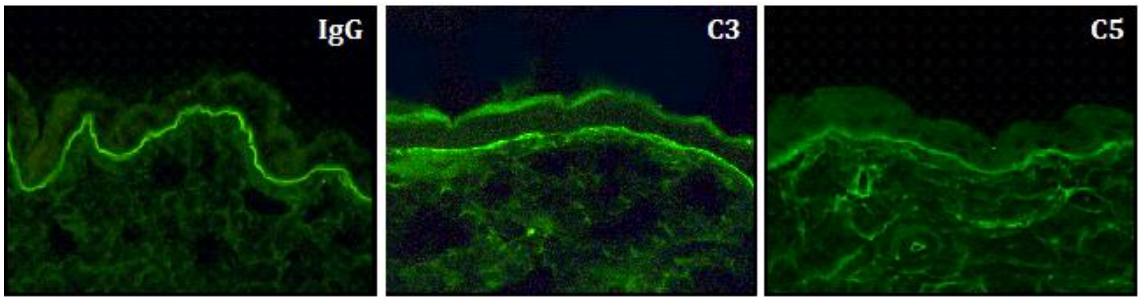

Figure 9. Rabbit IgG against murine type XVII collagen (anti-mCol17 IgG) binds at the dermal-epidermal junction (DEJ) in vivo and activates complement
C57BL/6 mice were injected s.c. with anti-mCol17 IgG over a period of 12 days. Rabbit IgG bound along the dermal-epidermal junction of injected mice (**A**) and activated murine C3 (**B**) and C5 (**C**) as shown by direct immunofluorescence microscopy on skin biopsies from day 12. Magnification: 1:200

Histopathology with the use of H&E staining showed split formation between the dermis and the epidermis. The epidermis showed thickening and an inflammatory infiltrate was detected in the upper dermis. This infiltrate was dominated by neutrophils (Fig. 10). In biopsies of mice that were injected with the same amount of normal rabbit IgG, no thickening of the dermis or microscopic cutaneous lesions were found (data not shown).

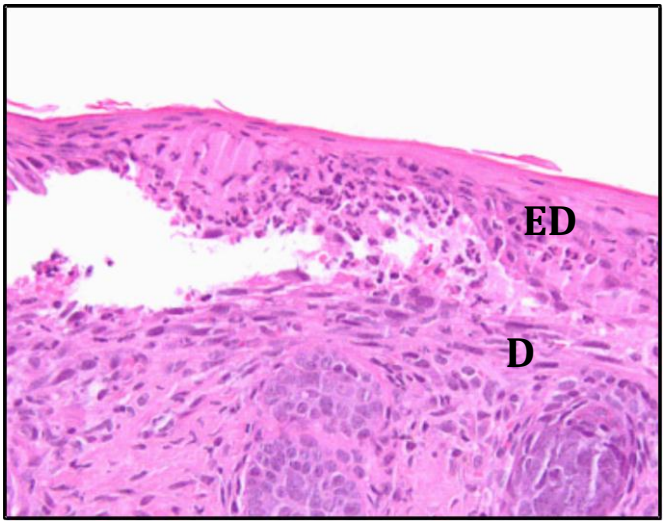

Figure 10. Rabbit IgG against murine type XVII collagen (anti-mCol17 IgG) induced subepidermal split formation
Histopathology (H&E) of skin of mice injected with anti-mCol17 IgG over a period of 12 days showed dermal-epidermal separation and inflammatory infiltrate dominated by neutrophils. ED: Epidermis; D: Dermis; Magnification: 1:200

In human BP, the inflammatory infiltrate is dominated by eosinophils. To determine the presence of eosinophils in the inflammatory infiltrate, immunohistochemistry was performed with the use of antibodies against murine MBP, a surface protein of eosinophils. Staining showed eosinophil presence in the inflammatory infiltrate in the dermis of perilesional skin (Fig.11).

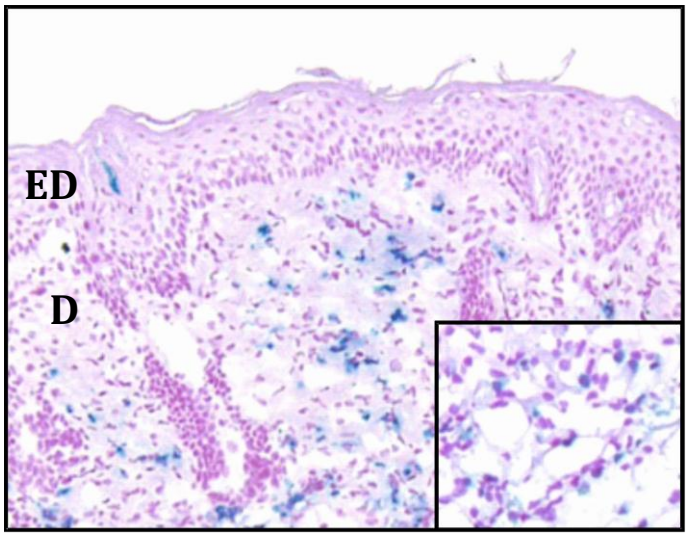

Figure 11. Inflammatory infiltrate of perilesional skin contained eosinophils
Immunohistochemistry of perilesional skin from mice injected with anti-mCol17 IgG over a period of 12 days. Staining against murine major basic protein showed the presence of eosinophils within the inflammatory infiltrate in the dermis. ED: Epidermis; D: Dermis; Magnification: 1:200, Detail: 1:400

To determine the exact location of the dermal-epidermal split, transmission electron microscopy of lesional skin biopsies from day 12 was performed by Prof. Ishiko at the Keio University in Tokyo, Japan. As expected, the split was detected directly below the keratinocytes and above the dermis, therefore within the region of the lamina lucida (Fig. 12).

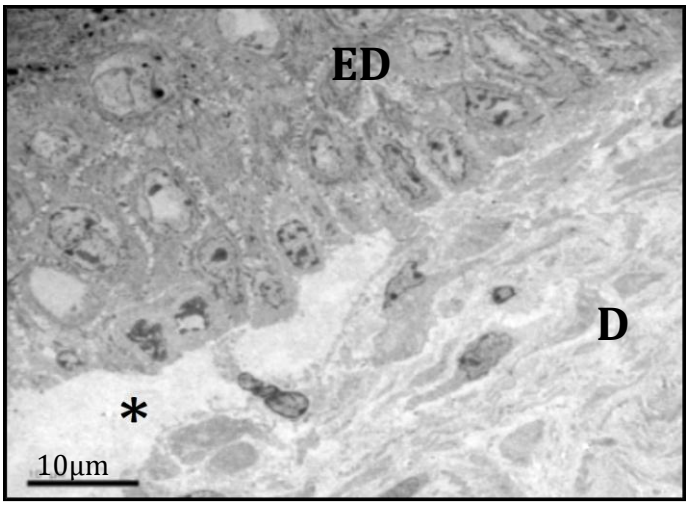

Figure 12 Rabbit IgG against murine type XVII collagen (anti-mCol17 IgG) induced split formation located within the lamina lucida
By transmission electron microscopy of a lesional skin biopsy a blister (*) was shown at the lamina lucida level. D: dermis; ED: epidermis

Clinically, mice showed erythema on the ears and neck on day 4. Erythema was also found on the back and front legs, on the heads, around the eyes and around the snouts of the mice. Furthermore, developing crusts and erosions were observed at the sites where the erythema had developed. On day 12 erosions and crusts were found around the injection site on the neck and ears and at more distant sites such as around the eyes and snout (Fig.13). In contrast, mice injected with the same amount of normal rabbit IgG showed no clinical signs of disease like erythema, crusts or erosions (data not shown).

Figure 13. Rabbit IgG against murine type XVII collagen (anti-mCol17 IgG) induced cutaneous lesions in adult mice
C57BL/6 mice were injected s.c. with anti-mCol17 IgG over a period of 12 days. Representative clinical presentation on day 12; lesions were characterised by erythema, erosions, and crusts predominantly involving the ears.

To determine the relevance of the amount of injected antibody during disease development, I applied different amounts of pathogenic anti-mCol17 antibodies. Adult C57Bl/6 mice were injected once (n=5; on day 0; in total 15 mg IgG), twice (n=5; on days 0 and 2; in total 30mg IgG), and three times (n=5; on days 0, 2 and 4; in total 45mg IgG) respectively, with 15mg of pathogenic IgG per injection. A fourth group of mice received six injections with 5mg IgG (n=5; on days 0, 2, 4, 6, 8, and 10; in total 30 mg IgG) (Fig.15). Mice injected once with 15mg of pathogenic IgG showed significantly less total disease activity compared to mice injected twice (AUC; p=0.005) or three times (AUC; p=0.002) with 15mg pathogenic IgG (Fig.14A). Interestingly, mice injected with a total of 30mg either with two injections of 15mg or with six injections of 5mg pathogenic IgG showed no difference in the extent of skin lesions on day 12. However, a different disease development was observed between the two groups resulting in statistically different disease activities measured over the entire experimental period of 12 days (AUC p=0.002). Cutaneous lesions developed more rapidly in mice which received two doses of 15mg compared to mice that received six doses of 5mg of pathogenic IgG (Fig.14B). A somehow similar effect was observed between the group of mice that received one dose of 15mg and six doses of 5mg. At day six they

showed the same disease score. In the group of mice that received one dose of 15mg on day 0, lesions developed more rapidly than in the group of mice that received three doses of 5mg of IgG until that day (Fig.14B). In summary, a clear dose-dependent effect of the amount of anti-mCol17 antibodies was observed. The dose dependency was later corroborated using C5-deficient mice and their respective controls (Fig. 19).

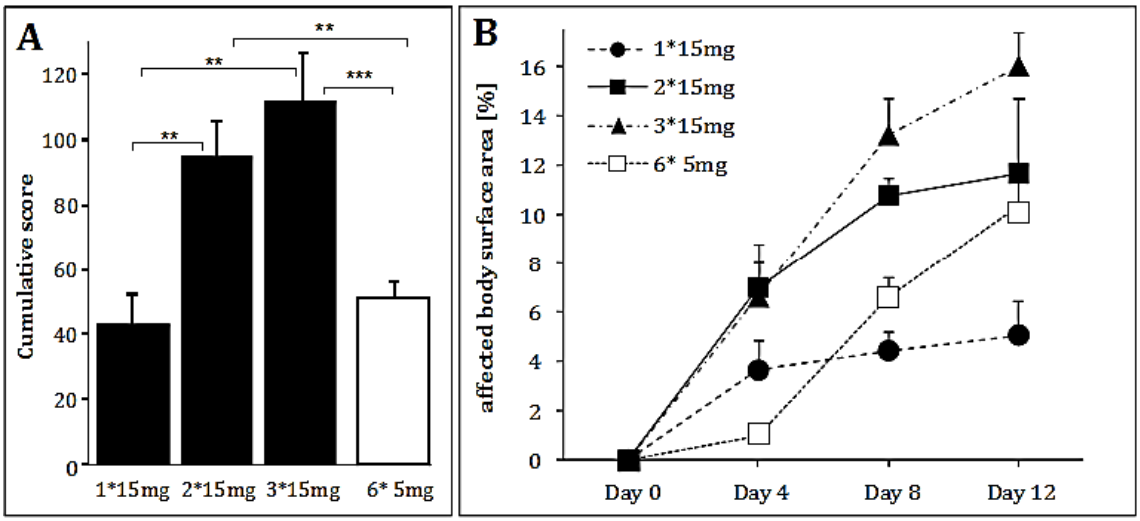

Figure 14. Disease activity was related to the amount of injected anti-mCol17 IgG.
C57Bl/6 mice were injected subcutaneously with anti-Col 17IgG. (**A**) Cumulative disease score; three injections of 15mg anti-mCol17 IgG (on days 0, 2, and 4) resulted in significantly more extensive disease compared to 2 injections on days 0 and 2, a single injection on day 0, and 6 injections of 5 mg anti-mCol17 on day 0, 2, 4, 6, 8, and 10. Two injections of 15mg anti-mCol17 IgG in the beginning (on day 0 and 2) resulted in a significantly more extensive disease than 6 injections of 5mg. (**B**) The extent of skin lesions was measured as percentage of the body surface area. Means of individual clinical scores (n=5) are shown before the first injection (day 0) and on days 4, 8, and 12. *** $p<0.001$, ** $p<0.01$, performed statistical test: One Way ANOVA

3.2 Experimental bullous pemphigoid in adult mice was FcR-dependent

To determine the impact of the Fc fragment in disease development, adult C57Bl/6 mice (n=5) and mice lacking the activating γ-chain of the Fc receptor (FcRγ$^{-/-}$; n=5) were injected three times with 15mg of pathogenic rabbit anti-mCol17 IgG. While wild-type animals developed skin lesions as described above and a final score of

around 12% affected body surface area, FcRγ$^{-/-}$ mice were completely protected from disease (Fig.15). Significant differences were already seen at day 4 where control mice already reached a score of 2, while FcRγ$^{-/-}$ mice stayed healthy (p=0,007). During the course of the experiment wildtype mice developed more extensive disease, while the FcRγ$^{-/-}$ mice did not show any indication of disease until the end of the experiment (day 8 p<0.001; day 12 p<0.001). As a result of the differences on the sampling days, the overall disease activity was significantly different (AUC p<0.001).

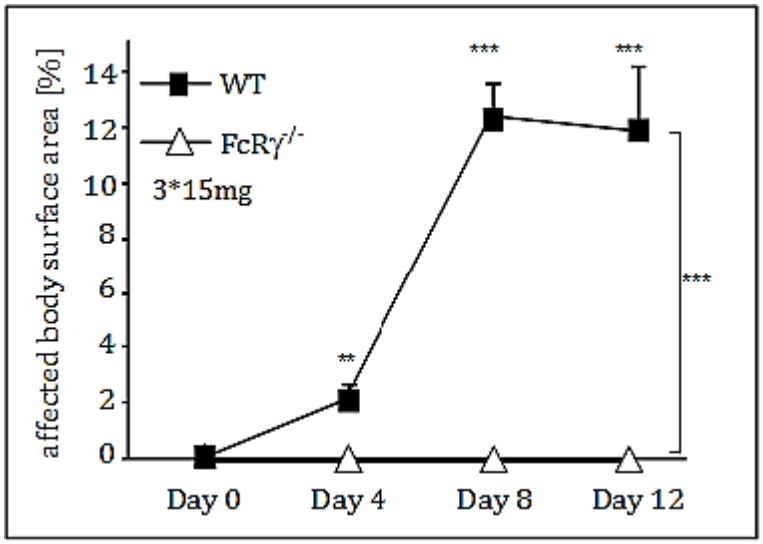

Figure 15. In FcRγ-chain-deficient (FcRγ$^{-/-}$) mice clinical disease was abolished
FcRγ$^{-/-}$ (n=5) and wild type (WT) mice (n=5) were injected subcutaneously with anti-mCol17 IgG (15mg on days 0, 2, and 4). While the wild type animals developed skin lesions, FcRγ$^{-/-}$ mice remained without clinical disease. ** p<0.01; *** p<0.001, performed statistical tests: t-test

The impact of the FcγR-dependent effects in the novel experimental BP model was further addressed with the use of EndoS-treated mCol17-specific IgG. EndoS, an endoglycosidase that specifically hydrolyses the N-linked glycan on IgG heavy chains [103,104], reduces binding to activating FcγR and increases binding to the inhibiting FcγRIIB [105]. Mice injected with untreated pathogenic IgG (n=5; six times

5mg IgG) developed a phenotype as described above, while injection of in adult C57/Bl6 mice (n=8; six times 5mg IgG) resulted in only few BP lesions as only erythema in the neck was found. Significant differences were found in the disease activity already on day 4 (p=0.003), and in the further course of the disease on scoring day 8 (p=0.023) and on day 12 (p=0.045). Therefore, a significantly higher overall disease activity in the wildtype mice compared to the mice injected with EndoS-pretreated anti-Col17 IgG was detected (AUC p=0.002; Fig. 16).

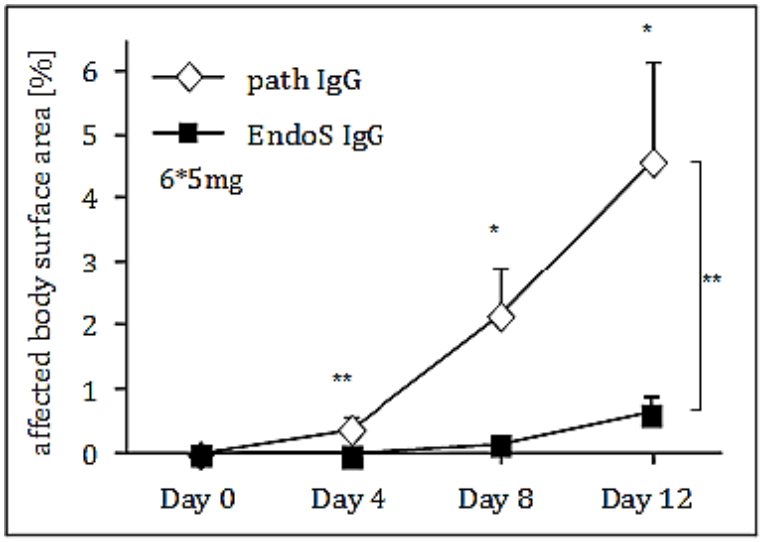

Figure 16. In mice injected with EndoS-treated anti-mCol17 IgG clinical disease is nearly abolished
Mice were injected with EndoS-treated (n=5) and untreated rabbit anti-mCol17 IgG (n=8) (5 mg on days 0, 2, 4, 6, 8 and 10). Mice injected with EndoS-pretreated anti-Col17 IgG showed significantly lower clinical scores compared to mice that had received untreated anti-Col IgG. Cumulative disease score is shown on the right side with a bracket around the two curves * $p<0.05$; ** $p<0.01$; performed statistical tests: clinical score: t-test, Rank Sum test, AUC: t-test

By direct IF microscopy of perilesional skin biopsies taken on day 12, all mice showed binding of rabbit IgG at the DEJ (Fig.17A,B,C). As expected, skin samples from wildtype mice also showed deposition of the complement components C3 and C5 at the DEJ (Fig 17D,G). FcR$\gamma^{-/-}$ mice and C57Bl/6 mice injected with EndoS-treated Col17-specific IgG were protected from developing disease and showed no

skin lesions. Interestingly, direct IF microscopy of skin biopsies taken on day 12 revealed deposition of complement factors C3 and C5 at the DEJ (Fig 17 E,H and F,I).

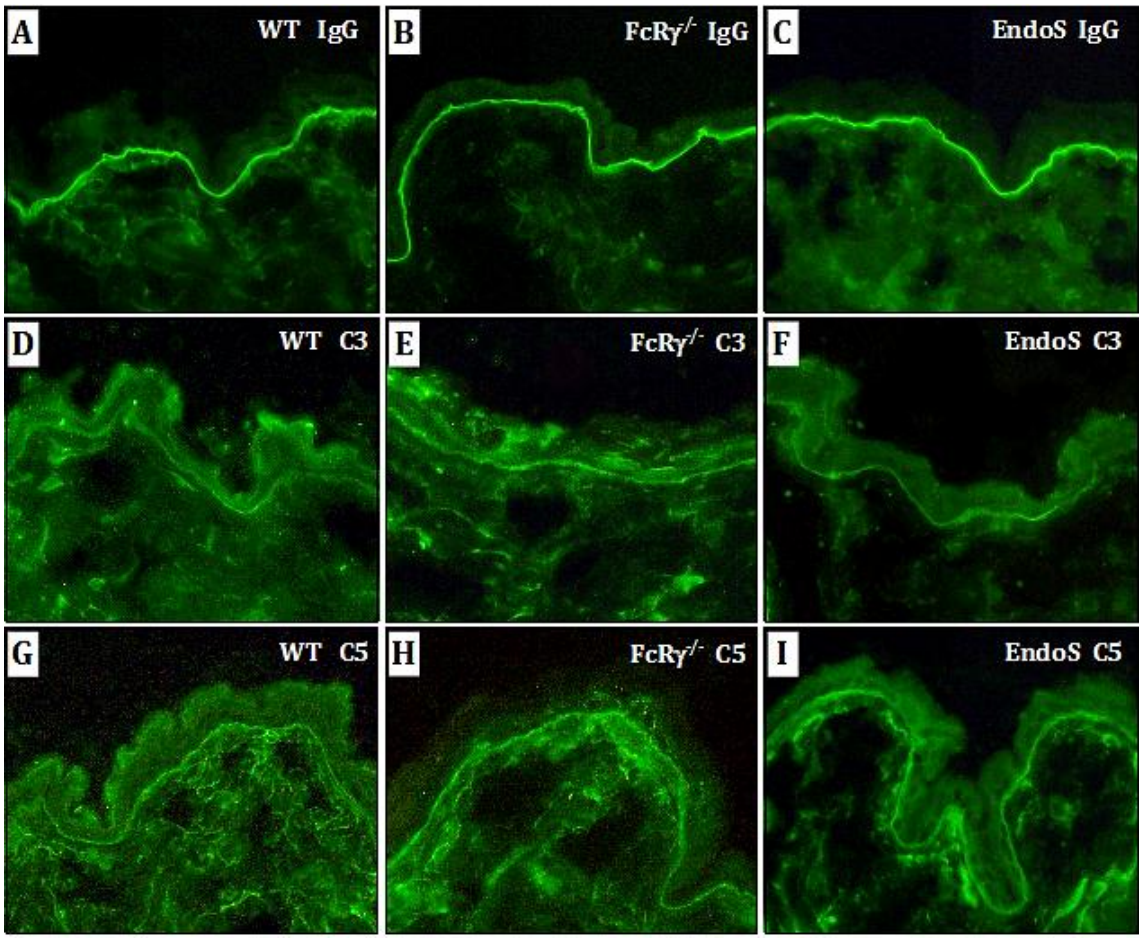

Figure 17. *In vivo* deposition of IgG and complement in FcRγ$^{-/-}$ mice and mice injected with EndoS-treated anti-mCol17 IgG
Wildtype (WT) mice and FcRγ$^{-/-}$ were injected with anti-mCol17 IgG (3x15 mg IgG), further wild type mice were injected with EndoS-pretreated anti-mCol17 IgG (EndoS) (6x5 mg IgG). By direct immunofluorescence microscopy of non- and perilesional mouse skin obtained on day 12 from wild type mice (**A, D, G**),FcRγ$^{-/-}$ (**B, E, H**) and EndoS-pretreated (**C, F, I**) *in vivo* deposits of IgG (**A-C**), C3 (**D-F**), and C5 (**G-I**) at the dermal-epidermal junction were visualised. Magnification: 1:200

The intensity of the deposits at the DEJ was then quantified by the use of the software ImageJ. No differences were observed in the intensity of bound IgG between FcRγ$^{-/-}$ mice and mice injected with EndoS-pretreated anti-mCol17 IgG compared to the respective control mice (Fig.18A). In addition, no differences in

the deposition of C3 (Fig. 18B) and C5 (Fig.18C) along the DEJ were observed between the experimental groups.

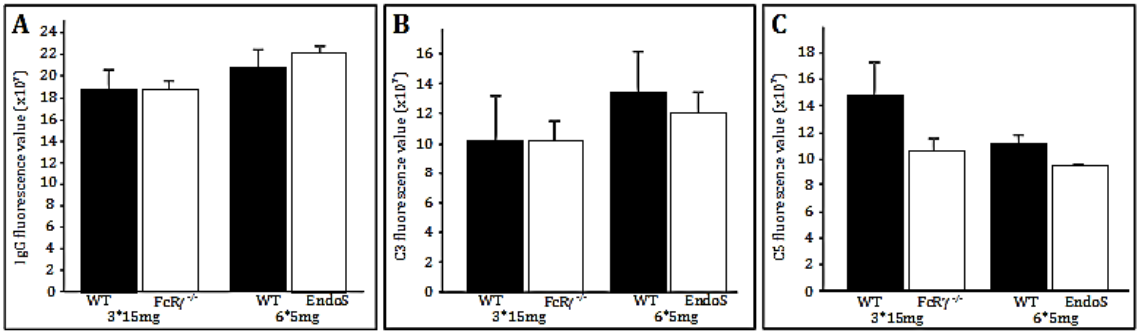

Figure 18. *In vivo* deposition of IgG and complement were unaltered in FcRγ$^{-/-}$ mice and mice injected with EndoS-treated anti-mCol17 IgG
Wildtype (WT) and FcRγ$^{-/-}$ mice were injected with anti-mCol17 IgG (3x15 mg IgG), further wild type mice were injected with EndoS-pretreated anti-mCol17 IgG (EndoS) (6x5 mg IgG). By direct immunofluorescence microscopy of non- and perilesional mouse skin obtained on day 12 from wild type mice, FcRγ$^{-/-}$ and EndoS-pretreated, in vivo deposits of IgG, C3, and C5 at the dermal-epidermal junction were visualised. When the intensity of fluorescence was quantified using ImageJ software, no significant difference was observed between the groups. Performed statistical tests: One Wav ANOVA

3.3 Role of complement in the new mouse model

3.3.1 In experimental bullous pemphigoid in adult mice, complement activation was important but not a prerequisite for disease development

Mice without clinical signs of experimental BP showed deposition of complement components C3 and C5 at the DEJ to the same extent as their respective controls (Fig. 17 and 18). As complement was expected to play a critical role in disease development, my next step was the investigation of the role of complement in the new mouse model for BP. I injected mice that were deficient for the complement components C5 (C5$^{-/-}$) and C3 (C3$^{-/-}$) respectively, to induce experimental disease.

Direct IF microscopy of perilesional skin samples taken on day 12 was performed to detect complement deposition at the DEJ.

$C5^{-/-}$ and their corresponding wildtype mice were injected six times with 5, 10 and 15mg of pathogenic anti-mCol17 IgG respectively (each group: n=5). Depending on the injected dose, $C5^{-/-}$ mice developed cutaneous BP lesions with a final disease score from 4.5 and 7.5%, while wildtype mice reached a score between 10 and 16% (Fig 19A). Within each setting with the same amount of injected antibody, overall disease activity in $C5^{-/-}$ mice was significantly reduced to between 40 and 50% compared with the corresponding wild type animals (Fig.19B). The largest difference between wildtype and knockout mice was found within the group of mice which received six injections of 15mg IgG: the overall disease activity in the wildtype mice was nearly 100, while knockout mice reached only 46 ($p=<0.001$; Fig.19B). Injection of lower doses (six times 5 and 10mg, respectively) resulted also in a significantly higher total disease score in wildtype compared to knockout mice: a total score of 81 was found in wildtype mice compared to 52 in knockout mice after six injections of 10mg IgG ($p=0.032$; Fig.19B). Six injections of 5mg IgG resulted in a total score of 67 in wildtype mice compared to 32 in knockout mice ($p=0.012$; Fig.19B). In addition, as already shown before in C57BL/6 mice (Fig. 14), a dose-dependency was observed in the wildtype mice. Mice injected with a total of 90mg (six injections with 15mg) anti-mCol17 IgG developed significantly more severe disease than mice that had received 30mg IgG (six injections with 5mg) ($p=0.017$; Fig.19B). In contrast, $C5^{-/-}$ mice did not show any significant differences in disease activity between the different groups.

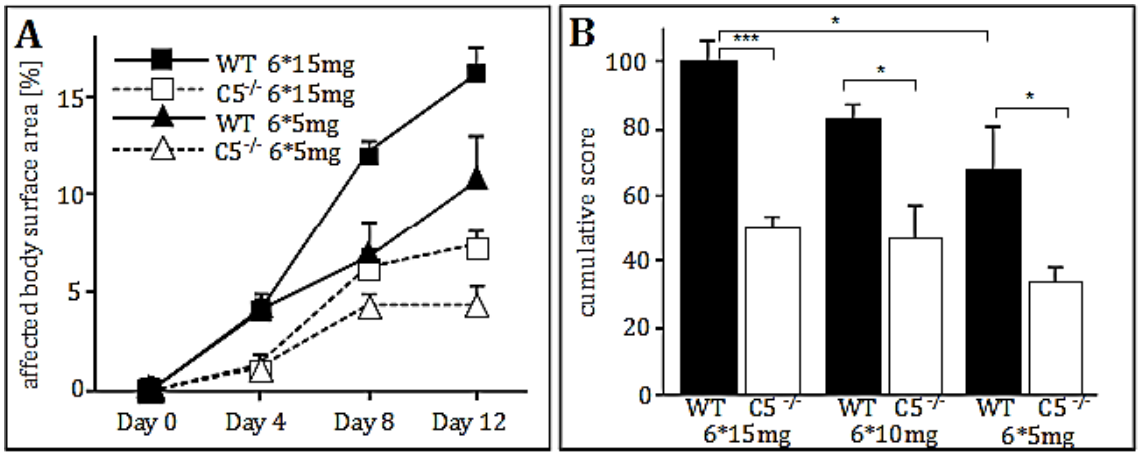

Figure 19. Disease activity is reduced in C5$^{-/-}$ mice and is related to the amount of injected anti-mCol17 IgG.
C5$^{-/-}$ and corresponding wildtype (WT) mice were injected with different amounts of anti-mCol17 IgG over a period of 12 days. (**A**) The extent of skin lesions was measured as percentage of the body surface area. Means of individual clinical scores (n=5) are shown before the first injection (day 0) and on days 4, 8, and 12. (**B**) The extent of the disease of each mouse was cumulated to compare the overall disease activity. Six injections of 15mg anti-mCol17 IgG resulted in significantly more extensive disease compared to six injections of 5mg anti-mCol17). Disease activity of knockout mice was reduced 40-50%. * $p<0.05$, *** $p<0.001$; performed statistical test: One Way ANOVA

By direct IF microscopy, linear deposition of murine complement component C3 was detected at the DEJ in the wildtype as well as in the C5$^{-/-}$ mice (Fig. 20). As a control, staining against murine C5 was performed and, as expected, deposition of C5 was detectable in the wildtype, but not in the C5$^{-/-}$ mice (data not shown).

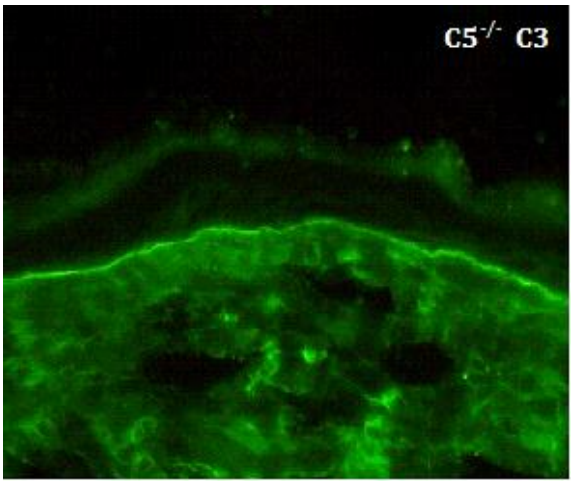

Figure 20. *In vivo* deposition of C3 at the DEJ of C5$^{-/-}$ mice after injection with anti-mCol17 IgG
Direct immunofluorescence microscopy of skin biopsies taken on day 12 from C5$^{-/-}$ mice that were injected with anti-mCol17 IgG revealed deposition of C3 at the dermal-epidermal junction. Magnification: 1:200

To investigate the role of C3 during disease development, I injected $C3^{-/-}$ mice (n=6) and wildtype controls (n=6) with pathogenic rabbit anti-mCol17 IgG. All mice developed erythema, crusts and erosions around the ears, neck and head as described above. Interestingly, C57Bl/6 control and $C3^{-/-}$ mice developed skin lesions to almost the same extent with a final disease score of 7% in the wildtype and 8% in the $C3^{-/-}$ group (p=0.582). During the development of the disease there was no difference on day 4 (p=0.329) and day 8 (p=0.072). Although the $C3^{-/-}$ mice tended to show a slightly more extensive disease on day 8 (p=0.072; Fig. 21B), overall disease activity of the wildtype (AUC=32) was not significantly different from the $C3^{-/-}$ knockout animals (AUC=44) (p=0.256) (Fig. 21B).

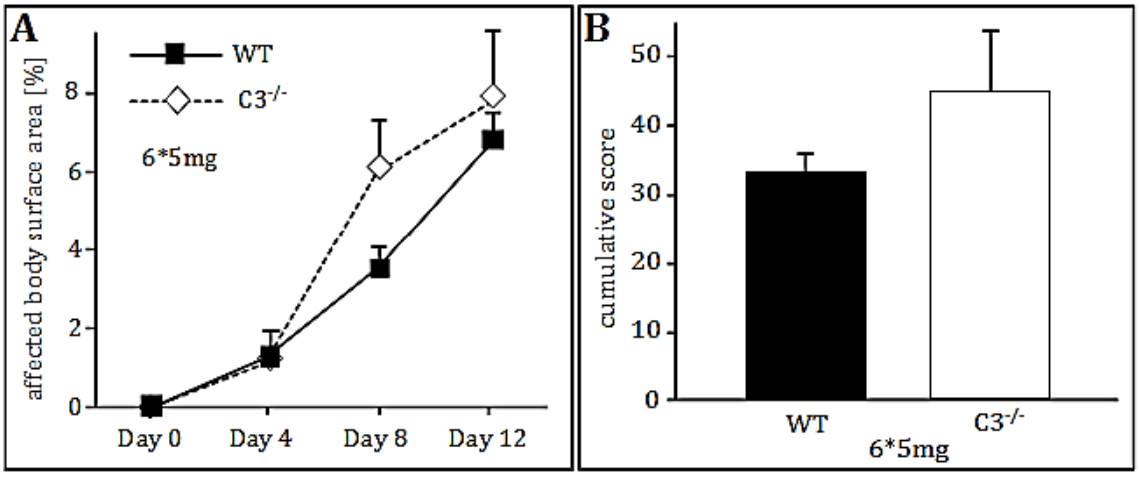

Figure 21. Deficiency in complement component C3 did not reduce disease activity of experimental BP

$C3^{-/-}$ and corresponding wild type (WT) mice were injected with anti-mCol17 IgG. (**A**) The extent of skin lesions was measured as percentage of the body surface area. Means of individual clinical scores (n=6) are shown before the first injection (day 0) and on days 4, 8, and 12. (**B**) $C3^{-/-}$ mice developed the same overall disease activity compared to the wildtype controls after injection of anti-mCol17 IgG. Performed statistical tests: t-test

As expected, direct IF microscopy revealed linear deposits of C3 in perilesional skin samples from wildtype mice, but not in samples from $C3^{-/-}$ mice. Interestingly, although complement component C5 is acting downstream of C3, linear C5 deposition was found at the DEJ of all samples from $C3^{-/-}$ mice (Fig. 22).

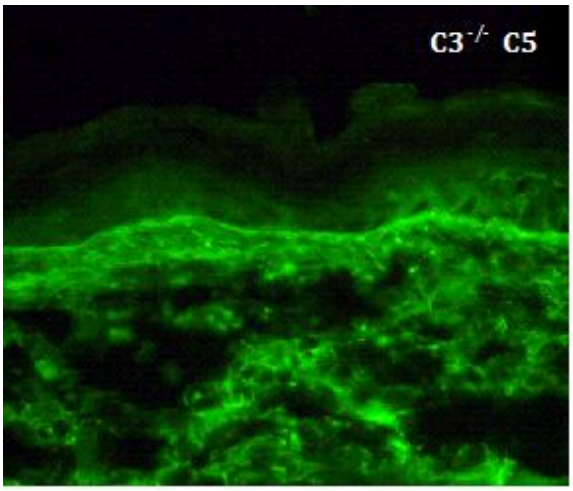

Figure 22. *In vivo* deposition of C5 at the DEJ of $C3^{-/-}$ mice after injection with anti-mCol17 IgG
Direct immunofluorescence microscopy of skin biopsies taken on day 12 from $C3^{-/-}$ mice that were injected with anti-Col17IgG revealed deposition of C5 at the DEJ. Magnification: 1:200

3.3.2 C5aR mediated the pathogenic effect of anti-mCol17 IgG induced C5 while C5L2 was protective

I further investigated the role of the complement component C5 and, more precisely, the active cleavage product and anaphylatoxin C5a. Mice that were deficient in the corresponding C5a receptor C5aR (CD88) or C5L2 (GRP77) were injected six times with 5mg of pathogenic anti-mCol17 IgG. In a further approach, wildtype mice were treated with an antagonist for C5aR (PMX53).

After six injections of 5mg pathogenic anti-mCol17 IgG $C5aR^{-/-}$ mice (n=10) developed significantly less disease compared to corresponding wildtype animals

(n=10) (day 12; p=0.007). In addition, the disease score was already significantly lower in C5aR$^{-/-}$ compared to wildtype mice after receiving 10mg of anti-mCol17 IgG (day 4; p<0.001). Overall disease activity was significantly higher in the wildtype mice at the end of the experiment (p=0.035) (Fig. 23).

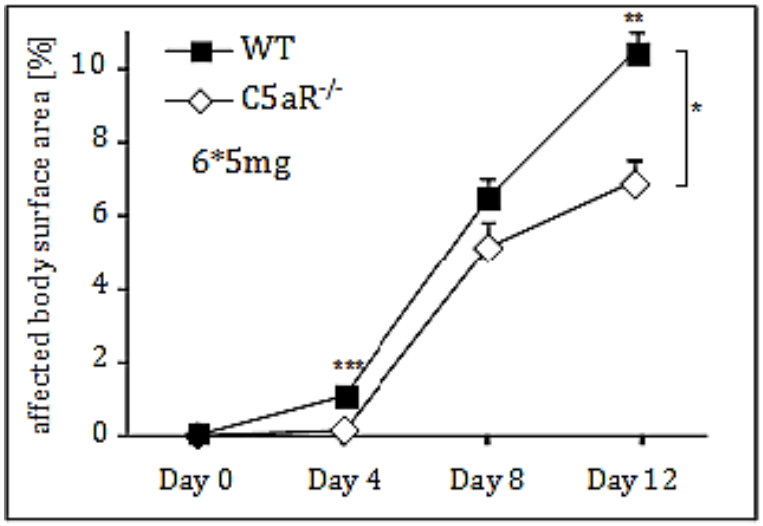

Figure 23. C5a receptor C5aR mediates tissue destruction by anti-mCol17 IgG
C5aR-deficient (C5aR$^{-/-}$) (n=10) and corresponding wild type (WT) mice (n=10) were injected with 6*5mg anti-mCol17 IgG over a period of 12 days. Injection of pathogenic anti-mCol17 IgG in C5aR$^{-/-}$ mice resulted in significantly less bullous pemphigoid lesions compared to WT mice. A significant difference in the cumulative disease score is shown on the right side with a bracket around the two curves * p<0.05; ** p<0.01;*** p=<0,001; performed statistical tests: clinical score: t-test, rank sum test, AUC: t-test

In contrast to C5aR$^{-/-}$ mice, C5L2$^{-/-}$ mice (n=15) developed significantly more skin lesions during disease development compared to wildtype animals (n=18) after receiving pathogenic anti-Col17 IgG. After injection of a total of 10mg and 20mg on day 4 and day 8 respectively, C5L2$^{-/-}$ mice showed a significantly higher disease score than their controls (day 4: p=0.018; day 8: p=0.033). At the end of the experiment, the overall disease activity was significantly elevated in the knockout animals (p=0.021) (Fig. 24).

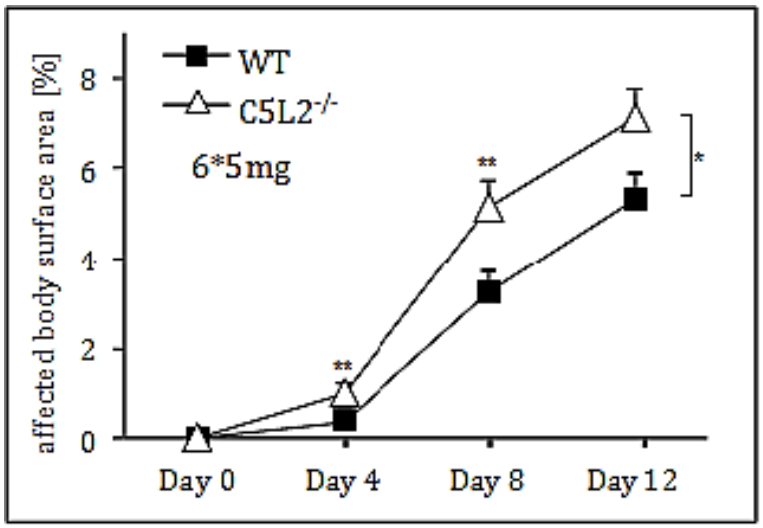

Figure 24. C5a receptor C5L2 is protective during development of experimental BP
C5L2-deficient ($C5L2^{-/-}$) (n=15) and corresponding wild type (WT) mice (n=18) were injected with 6*5mg anti-mCol17 IgG over a period of 12 days Injection of pathogenic anti-mCol17 IgG in $C5L2^{-/-}$ mice resulted in significantly more bullous pemphigoid lesions compared to wild type mice. A significant difference in the cumulative disease score is shown on the right side with a bracket around the two curves *, $p<0.05$; performed statistical tests: clinical score: t-test, rank sum test, AUC: t-test

To corroborate the importance of the C5aR in the novel model and its potential for therapeutic interventions, C57Bl/6 mice were injected six times with 5mg of pathogenic anti-mCol17 IgG. Additionally, they received daily injections of 200µg of the C5aR antagonist PMX53 and PBS as control respectively. Two different approaches were chosen. In a preventive approach, the antagonist was given from day 0 until day 11 (treatment and control; each n=15). In a second, more therapeutic approach, the antagonist was given from day 4, when skin lesions had already started to develop, until day 11 (treatment and control; each n=5). In the preventive approach, skin lesions were reduced in the PMX53-injected mice compared to control mice (p=0,082; Fig. 25A). This reduction was, however, not statistically significant. In the therapeutic approach, disease activity did not differ between PMX53-injected and control mice (Fig. 25B).

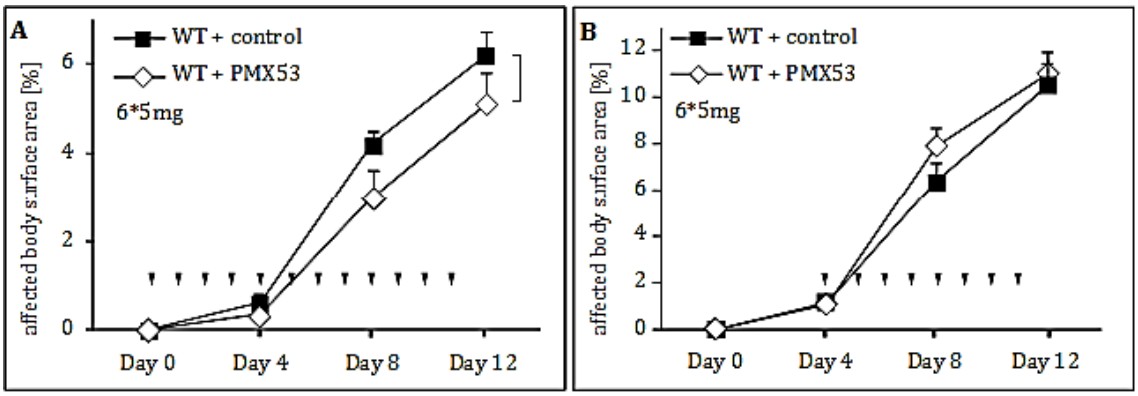

Figure 25. PMX53 is protective when applied at the beginning but is not effective when applied after disease development
Wildtype mice (WT) were injected six times with pathogenic anti-mCol17 IgG and with either PBS (control) or the C5aR inhibitor PMX53 (PMX53). (**A**) When mice were injected daily with PMX53 (n=15) and PBS (n=15), respectively, fewer BP lesions were found in the PMX53-treated mice compared to the control mice. (p=0,082) (**B**) In contrast, when injection of PMX53 and PBS was started on day 4, no difference between the PMX53-injected (n=5) and the control mice (n=5) was observed. Arrow heads indicate injections of PMX53 and PBS respectively. Performed statistical tests: rank sum test

3.3.3 C5aR did not enhance the immune complex-mediated ROS release from neutrophils

Release of ROS from neutrophils can lead to weakening of the dermal-epidermal junction targeted by autoantibodies as shown for autoimmunity to type VII collagen (epidermolysis bullosa acquisita) [158]. The role of C5aR in the ROS release from neutrophils in response to immune complexes (ICs) of recombinant mCol-17 NC15A and anti-mCol17 IgG was explored by the use of a previously described *ex vivo* assay [155]. Immune complexes were coated into plates. Neutrophils of FcR$\gamma^{-/-}$ mice, C5aR$^{-/-}$ mice, C5L2$^{-/-}$ mice and corresponding wildtype mice were added together with either normal mouse serum as source of complement, heat inactivated mouse serum, which should no longer contain any active complement components, or medium only. Addition of neutrophils resulted in different activation of ROS release. Almost no ROS was released from FcR$\gamma^{-/-}$ neutrophils in

any of the setups (Fig. 26A). Neutrophils from C5aR$^{-/-}$ and C5L5$^{-/-}$ mice showed ROS release in all setups which included the ICs. Interestingly, no difference in the ROS release was observed neither between wildtype, C5aR$^{-/-}$ and C5L2$^{-/-}$ neutrophils when stimulated with only ICs or together with untreated or heat-inactivated serum (IC: p=0.167; IC+serum: p=0.933; IC+heat-inactivated serum: p=0.270) nor between the settings within the strains (control: p=0.134; C5aR$^{-/-}$: p=0.589; C5L2$^{-/-}$: p=0.095) (Fig. 26B, representative data from one strain). Furthermore, stimulation with serum in the absence of the ICs did not result in any ROS release from neutrophils of wildtype, C5aR$^{-/-}$ and C5L2$^{-/-}$ mice (Fig.26B).

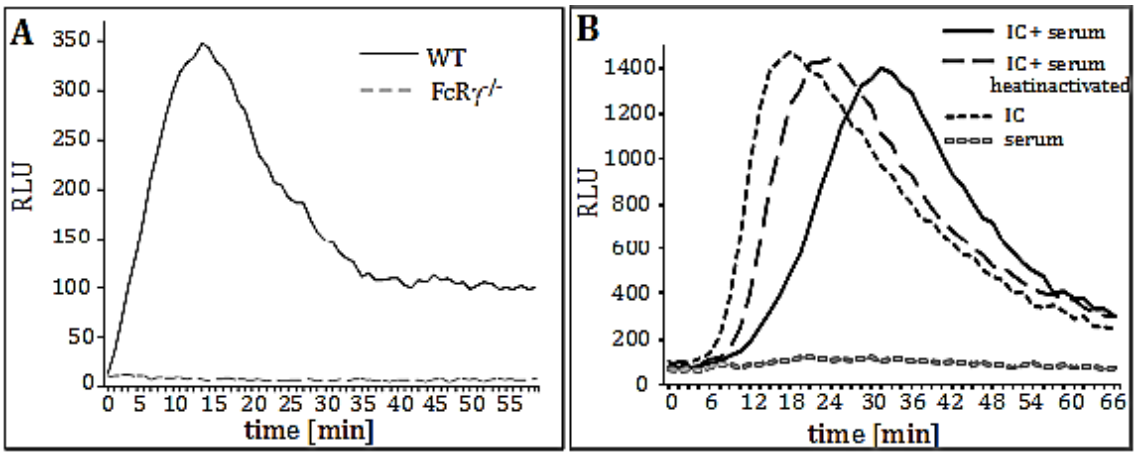

Figure 26. ROS release assay after stimulation in the presence or absence of serum
Immune complexes (IC) were coated into 96 well-plates. Murine neutrophils together with medium, mouse serum as source of complement or heat-inactivated serum, which did not contain active complement, were added and ROS release was measured immediately. (**A**) Neutrophils from FcRγ-chain $^{-/-}$ (FcRγ$^{-/-}$) mice showed no ROS release after stimulation with IC while C57Bl/6 (B6) mice did. (**B**) Representative ROS release of neutrophils from wildtype (WT), C5aR$^{-/-}$ and C5L2$^{-/-}$ mice: Neutrophils stimulated with either only IC, IC together with serum or heat inactivated serum showed no differences in ROS release. Stimulation with only serum did not result in any ROS release. RLU: relative light units; performed statistical test: One Way ANOVA of AUCs

C5a is known to be important for chemotaxis during immune reactions. Therefore, I used a migration assay with neutrophils from C5aR$^{-/-}$ and C5L2$^{-/-}$ mice to investigate the effect of complement system on migration towards immune

complexes. Wells were coated with ICs (recombinant mCol-17 NC15A and anti-mCol17 IgG) and filled with medium, serum as a source of complement or heat-inactivated serum without active complement. Neutrophils were added into transwells, which were placed into the coated wells. The neutrophils were separated from the serum or medium only by a permeable membrane. Although, most likely also as a result of the low number of n=4 in each group, statistical tests showed no differences between any of the settings, slight tendencies were observed. Neutrophils derived from wild type mice showed more migration towards the ICs in the presence of untreated serum compared to heat-inactivated serum. This was also observed with neutrophils from C5L2$^{-/-}$ mice. Interestingly, neutrophils from C5aR$^{-/-}$ mice showed the opposite phenomenon; neutrophils migrated more towards the ICs in the presence of heat-inactivated serum than with normal serum. These data were compatible with the *in vivo* experiments pointing to a role of complement activation in the early stage of the disease e.g. the chemotaxis of neutrophils but not at later stager e.g. the tissue destruction phase represented by release of ROS.

The presence of only C5aR in C5L2$^{-/-}$ mice mediated the enhanced migration as seen with C5L2$^{-/-}$ neutrophils. C5aR also seemed to be the relevant C5 receptor in wildtype mice. The presence of C5L2 in C5aR$^{-/-}$ mice seemed to have an inhibiting effect on migration of neutrophils when complement was available (Fig.27).

Data were derived from two independent experiments, each with neutrophils from two different mice in each group.

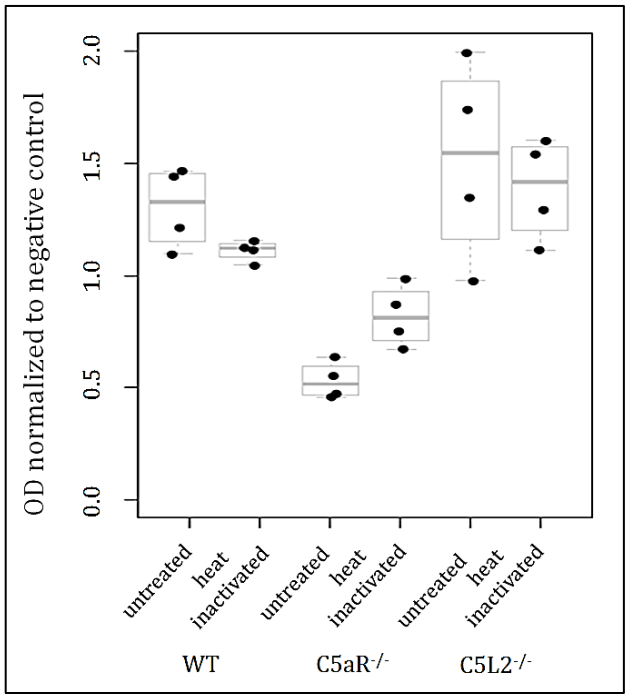

Figure 27. Migration of neutrophils towards immune complexes in the presence of serum
Neutrophils showed tendency to different migration potential when immune complexes composed of recombinant mCol17 NC15A and anti-mCol17 IgG were presented together with serum or heat-inactivated serum. Heat inactivation led to the destruction of complement factors. Wildtype (WT) and C5L2$^{-/-}$ neutrophils seemed to migrate less when the serum was heat-inactivated while C5aR$^{-/-}$ migrate more when the serum was heat-inactivated compared to untreated serum. Each group: n=4 Low numbers of individuals did not show significant difference in performed statistical test (ANOVA)

4. Discussion

Bullous pemphigoid (BP) is by far the most common autoimmune blistering disease [36–43]. With the use of direct immunofluorescence (IF) microscopy of a perilesional skin biopsy and various serological assays, diagnosis is usually rapidly confirmed. In contrast, treatment relies on unspecific immunosuppression by the long-term use of high-potent topical and/or systemic corticosteroids associated with considerable morbidity and mortality [13]. Several mouse models of BP have previously been developed for a better understanding of the pathophysiology [78,159,160]. In the present study, I established a new passive transfer model in adult mice. It reflects main clinical and immunopathological features of the human disease and overcame several shortcomings of the currently used models. This novel model may be useful in further dissecting the mechanisms of autoantibody-mediated tissue destruction and, ultimately, may help to study novel, more specific therapeutic approaches in autoantibody-mediated diseases.

Development of a new mouse model for bullous pemphigoid

Several studies have revealed the 16th non collagenous (NC16A) domain of BP180 as the major pathogenic epitope of human BP [53,54]. A study on epitope spreading in an active mouse model by di Zenzo et al. also detected more epitopes occurring at later stages of the disease but all sera showed activity against murine BP180 NC15A, which corresponds to the human NC16A [161]. For the new passive transfer model rabbit antibodies against the murine epitope NC15A (mCol17) were generated. As expected, these antibodies bound to the recombinant mCol17 protein by Western blotting and binding of the rabbit antibodies was seen at the

blister roof by indirect IF microscopy on murine salt-split skin. Furthermore, using the previously developed *ex vivo* cryosection assay [76] I was able to confirm the pathogenic relevance of the generated rabbit anti-NC15A IgG. Human neutrophils induced split formation at the DEJ after incubation of the generated rabbit IgG followed by addition of human leucocytes on murine skin sections.

Following the protocol for the previously established passive transfer mouse model for epidermolysis bullosa acquisita [150] mice were injected with pathogenic rabbit anti-mCol17 IgG over a period of 12 days. Perilesional skin sections of mice injected with the pathogenic IgG showed binding of rabbit antibodies at the DEJ as well as deposition of complement factors C3 and C5. These findings are in line with other available animal models for BP including the neonatal mouse model, the models with humanised mice and also the model, in which patient's IgE is applied [82,84,87,90]. In all existing models, even if no clinical blistering was detected, linear binding of IgG and, often, complement deposition along the DEJ was found in direct IF microscopy. Moreover, BP patients are diagnosed by direct IF microscopy based on the deposition of IgG and/or C3 along the DEJ [12]. Similar as in the patients skin biopsies [12], all diseased animals showed dense infiltrates of immune cells, dominated by neutrophils in the upper dermis. Until now, cellular infiltrate has been always dominated by neutrophils in mouse models for BP, in which rabbit IgG, patients' sera or purified patients' IgG was used [159,160]. In contrast, in the new model, immunohistochemistry of perilesional skin biopsies also revealed eosinophils in the inflammatory infiltrate, even though they were less frequent than neutrophils. Interestingly, in two mouse models of BP, in which mice were injected with anti-BP180 IgE, cellular infiltration was dominated by eosinophils

[89,90]. In skin lesions of the recently developed immunisation-induced mouse model of BP [78], eosinophils were also found in the cellular infiltrate. In this model, mice were immunised with recombinant mCol17 and developed BP-like disease with elevated IgG and IgE levels, as well as neutrophil and eosinophil infiltration at the erosion site. In BP patients, eosinophils are frequently the dominant inflammatory cell type in skin lesions [12] and pathogenic antibodies have been identified belonging to the subclasses IgG1 and IgG4 as well as to the IgE isotype [55,56]. As hypothesised, mice injected with rabbit anti-mCol17 IgG developed a dermatological disease and showed IgG and complement binding at the DEJ. In addition, I was able to induce infiltration not only of neutrophils but also of eosinophils, which reflects the patients' situation more closely than other models do.

For further characterisation of the new model, it was mandatory to determine the exact level of splitting of the induced blisters. As H&E staining showed subepidermal blistering and indirect IF microscopy of the generated rabbit antibody on murine salt-split skin showed binding to the blister roof, I expected the split at the DEJ; more precisely, in the lamina lucida. Direct electron microscopy confirmed my hypothesis. This is in line with all other existing mouse models of BP, irrespective of the use of IgG or IgE antibodies against BP180 [159,160], where split formation was also detected at the lamina lucida.

Clinically, patients with BP show tense blisters and suffer from pruritus [12]. In models with disease induction by IgE antibodies, itching was already described [89,90]. In the novel mouse model, due to the mechanical irritation by scratching, macroscopic blisters were not seen, but lesions like erosions and crusts

surrounded by erythema, which are also typically seen in BP patients, developed spontaneously. They were mainly found around the injection site, but also at more distinct areas like the snout. In contrast to the neonatal mouse models of BP [82,84,98], in the novel model lesions arise spontaneously and do not need to be induced by mechanical friction. The method used in the neonatal models is highly dependent on how friction is applied and only poorly reflects blister formation in patients.

Compared to the other existing mouse models, there are additional advantages of the new model. The first efficient model for BP was the neonatal mouse model by Liu et al. [82,84]. A significant difference of all neonatal models is the short experiment time of just 24 to 48h. Furthermore, due to the low body weight of the mice, only low amounts of IgG are required. However, BP is a disease of the elderly and not of newborns; the immune system of newborns differs from that of adults [114]. Immunological events found in the neonatal mice might therefore not reflect the situation of the elderly patients. Two previously established models in adult mice have overcome this shortcoming [86,87]. In these models, organ transplants from different mice are used to induce disease. In one model, wildtype mice were transplanted with skin from humanised mice [86]. For the other model, two transplantations were performed: splenocytes from wildtype mice, which were grafted with skin from mCol17-humanised mice, were transferred into immunodeficient Rag2$^{-/-}$ mCol17-humanised mice [87]. Although the recipient mice in both models developed BP-like disease, these methods of disease induction require a lot of effort. Furthermore, transplantation of cells and skin into immunocompromised mice might provoke unwanted immunomodulating events. In addition, the use of knockout strains to further explore the pathogenicity is

difficult. Extensive crossbreeding of the human molecule would have to be repeated for each knockout mouse strain which is used.

In contrast to my new model, direct application of patients' sera or IgG is possible in the available mouse models, in which the whole murine BP180 molecule or the NC15A domain have been replaced by the human homologue [84,71]. In these mice, no antibodies against the murine protein need to be produced. The main disadvantage of these models is that again only neonatal mice are used for the studies. This is likely due to the limited amount of patients' sera. Besides the earlier mentioned disadvantage of a different immune system in neonatal compared to adult mice, the short time of the experiments gives only opportunity to explore some aspects of the pathophysiology in preventive, but not in therapeutic settings. Research on therapeutic strategies is rarely possible with the use of neonatal mice. Ultimately, although pathogenic human IgG from BP patients can bind to the human antigen in the humanised mice, the final immune reaction still remains murine.

In human BP, disease activity correlates with serum levels of anti-BP180 NC16A antibodies [44,115]. I have shown a similar effect by detection of a dose dependency in the new model. Injection of low amounts of pathogenic antibody resulted in a low disease activity, while a high amount of pathogenic antibody led to more extensive disease activity. The highest dose of the pathogenic antibody resulted in the highest clinical score at the end of the experiment (on day 12) while the lowest dose, which was only a third of the highest antibody amount, resulted in the lowest disease activity. In addition, injection of a defined dose of antibody results in a more rapid development of lesions when injected only once compared to continuous injection over a longer period.

Taken together, I developed a new passive transfer mouse model of BP which reflects major clinical and immunopathological features of human BP. Mice developed crusts, erosion and erythema. Unfortunately, no macroscopic blisters were detected, which seemed to be due to the scratching activity of the mice. Immunopathological analysis of lesional skin revealed deposition of antibodies and complement along the DEJ and subepidermal blistering, which was defined at the lamina lucida, together with an inflammatory infiltrate. Unlike the situation in BP patients, the infiltrate was not dominated by eosinophils and neutrophils, but by neutrophils with a minor concentration of eosinophils. In the new model, disease severity also depended on the amount of injected antibodies, reflecting the situation in BP patients.

Role of Fc and the Fc receptor

Antibodies can react on immune cells via their Fc portion. Fc dependency in antibody-induced autoimmune diseases has been already described in several models [119,150,163–170]. Mice that are deficient for the activating common γ-chain (FcRγ$^{-/-}$) of the receptor are unable to phagocytose IgG-opsonised particles or to mediate antibody dependent cellular cytotoxicity by NK cells and they respond poorly to IgE-mediated mast-cell activation [119]. FcRγ$^{-/-}$ mice are resistant to the induction or spontaneous onset of various autoimmune diseases such as vasculitis, glomerulonephritis and experimental autoimmune encephalomyelitis [164–167,170,171]. Tsuboi et al. showed importance of FcγRs, in particular FcγRIIA, on neutrophils during the development of rheumatoid arthritis, when the susceptibility could be restored by the expression of FcγRIIA in γ-chain deficient mice [172,173]. Furthermore, FcγRIIB on macrophages play an important role in protection from experimental

arthritis [174]. γ-chain-deficient mice lacking FcγRI and FcγRIII are resistant to the development of experimental immune hemolytic anemia, which is primarily a consequence of ineffective erythrophagocytosis, resulting from the lack of FcγRs on mononuclear phagocytes [163]. Zhao et al showed in experimental BP that neonatal FcγRIII$^{-/-}$ mice were protected from disease. In addition, studies in different mouse models for BP [168,169] and the passive transfer model for EBA [150] showed, that induction of clinical disease with only $F(ab)_2$ fragments against BP180 or collagen type VII was not possible, although binding of the $F(ab)_2$ fragments along the DEJ was detected.

To show the same Fc dependency in the new model, I injected FcRγ$^{-/-}$ mice with the pathogenic anti-mCol17 antibody. As expected, the mice did not develop disease although linear deposition of IgG at the DEJ was detected in direct IF microscopy. In addition, the level of bound antibody was the same compared to wildtype control mice. To exclude an undesired reaction of the receptor cells caused by the absence of the γ-chain, in a further experiment, pathogenic anti-BP180 IgG was treated with EndoS before injection into the mice. EndoS is an endoglycosidase derived from *Streptococcus pyogenes* that specifically hydrolyses the N-linked glycan of native IgG. It has previously been shown to modulate the interaction between the Fc portion of autoantibody and Fcγ receptors on leukocytes [104]. In the novel model, wildtype mice injected with EndoS-treated antibodies developed significantly less disease than control mice, although binding of the same level of antibodies as in the wildtype controls was observed. Therefore, I could clearly demonstrate that the new animal model is dependent on the binding of pathogenic antibodies to the Fcγ-receptors of inflammatory cells. Cells that carry Fc-receptors

include a number of immune cells such as B-cells, macrophages, granulocytes, and mast cells [1]. The relevance of several immune cells to the development of experimental neonatal BP has been previously demonstrated. Mice deficient for mast cells [93] or neutrophils [175] were resistant to disease induction. In addition, it was also suggested, that mast cells trigger macrophage dependent neutrophil infiltration [93].

In cultured human keratinocytes, another Fab-mediated effect of anti-BP180 antibodies was described. IgE and IgG against BP180 triggered a signal transducing event in cultured keratinocytes which led to the release of interleukin- (IL-) 6 and IL-8 [75]. Elevated levels of these cytokines had also been detected in BP patients' sera [176]. In addition, anti-BP180 IgG-treated keratinocytes also showed a decrease in the number of hemidesmosomes at the basal side of the cell, which may weaken the cell attachment [75]. Similarly, IgG from BP patients depleted cultured keratinocytes of the BP180 and weakened cell attachment [177]. In the present work, these FcR-independent effects have not been directly addressed. Interestingly, Natsuga et al. demonstrated an additional mechanism of disease induction. Injection of only $F(ab')_2$ fragments of anti-human Col17 antibodies into neonatal humanised mice resulted in skin fragility without any complement involvement. This finding was explained by their *in vitro* experiments with human keratinocytes. Incubation with anti-humanCol17 antibodies or $F(ab')_2$ fragments resulted in a decreased expression of the Col17 in the cultured keratinocytes, which may induce instability within the DEJ [178]. However, since $FcR\gamma^{-/-}$ mice were completely protected from the effect of anti-mCol17 IgG, FcR-mediated effects of autoantibody

have the proposed crucial relevance for disease development in my novel mouse model.

My experiments on the Fc-FcR-interaction also brought up an unexpected finding. Besides linear binding of the rabbit IgG, I also found deposition of the complement factors C3 and C5 along the DEJ in all mice. Interestingly, there was no difference in the amount of deposits between wild-type and FcR$\gamma^{-/-}$ animals as well as mice that had received EndoS-pretreated IgG. Therefore, absence of FcRγ or altering the Fc portion does not prevent complement activation at the DEJ. Similarly, in the passive model of EBA, application of EndoS-treated autoantibody resulted in substantially reduced disease activity, while complement was deposited at the DEJ to the same extent as mice injected with untreated pathogenic autoantibody [154].

Role of complement

The main function of the complement has long been thought to be restricted to the clearance of invading microorganisms. Original opsonisation and lysis of foreign particles and cells as well as generation of proinflammatory peptides like the anaphylatoxins give the potential to damage host tissue when the complement system is activated inappropriately. This dysfunction can appear due to genetic disorders or inaccurate functions of the immune system, as present in autoimmune diseases. These dysfunctions were explored in association with diseases like rheumatoid arthritis, glomerulonephritis and systemic autoimmune diseases like cryoglobulinemic and anti-neutrophil cytoplasmic antibody associated vasculitis or systemic lupus erythematosus [179,180]. Studies performed on systemic autoimmune disease mouse models have confirmed that an absence of the classical pathway is associated with the development of autoimmunity [181]. One generally

accepted hypothesis is that the high prevalence of autoimmune reactions in complement-deficient patients and animals is associated with an impairment of the clearance of dying cells and immune complexes due to missing complement proteins. An alternative hypothesis is that complement determines the thresholds for B- and T-cell activation, and the absence of complement proteins disturbs the tolerance for self-antigens [179,181]. In addition to inflammatory diseases, the development of neurodegenerative diseases like Alzheimer's disease, Parkinson's disease [182], multiple sclerosis [183] and schizophrenia [184] was found to be related to the complement system.

The pathogenic relevance of complement activation has previously been studied in both neonatal mouse models of BP [82,98] and in adult mice in the passive transfer model of EBA [150,185]. Through the use of knockout mice in the EBA model it has been shown that the alternative pathway is related to disease development [185]. Mice deficient in component factor B were resistant to disease induction, while MBL $^{-/-}$ and C1q$^{-/-}$ mice developed experimental EBA. Furthermore, C5$^{-/-}$ mice were protected from disease in this model [150]. Neonatal C5$^{-/-}$ mice injected with IgG against BP180 also failed to develop experimental BP [82]. In contrast to EBA, the classical pathway was shown to be the more important pathway in the neonatal mouse model for BP, as C4$^{-/-}$ and C1q$^{-/-}$ mice were resistant to disease induction, while factor B$^{-/-}$ mice showed only a delayed onset and less intense disease [98]. This indicates a crucial role for complement in development of BP.

Interestingly, in my new experimental BP, model C5$^{-/-}$ mice were protected from disease development by only about 50%. Even more surprisingly, mice deficient in complement component C3, which acts upstream of C5, developed experimental

BP with the same extent of disease activity compared to their wildtype controls. This indicates I) that, in contrast to my initial hypothesis, the complement system is not as important for disease development as expected from the data obtained in the neonatal mouse model of BP and II) there has to be a fourth, C3 independent way of cleaving C5.

Liu et al. have proven, using a neonatal mouse model, a crucial effect of the complement system [95]. Neonatal mice were used in Liu's model, so the reaction may have been different in neonatal compared to adult mice. In addition, there may have been some dose effect; the amount of IgG Liu et al. applied showed a complete complement dependency in the neonatal mouse model of BP. However, it may be speculated that injection of higher amounts of pathogenic IgG would have also elicited complement-independent effects. To exclude a dose dependency of complement activation in the novel BP mouse model, three different amounts of anti-mCol17 IgG were applied. In all three approaches, $C5^{-/-}$ mice developed disease with a total score of 50% of wildtype mice, pointing to a minor importance of the complement during disease development. However, it cannot be excluded that by the use of even lower amounts of anti-mCol17 IgG a complement-dependency might be detectable, i.e. $C5^{-/-}$ mice may be more protected from the effect of anti-mCol17 IgG. In line with this, two further observations have questioned the complete complement dependency of experimental BP. In an active model for BP, in which IgG from the mother was transferred to her neonates, complement deposition was found but no blistering could be detected [160]. In addition and in concordance with my data, as mentioned above, Natsuga et al.

recently showed a complement-independent way of producing instability of the DEJ in the BP180-humanised mouse of BP [178].

Several molecules that might be possible candidates for cleaving C5 during disease development have previously been identified, e.g. thrombin [142], plasmin [186] and neutrophil elastase [143]. Neutrophil elastase may be a possible candidate in the development of the new experimental BP. Shown by histopathology of perilesional skin biopsies from diseases mice, there is a massive infiltrate of neutrophils, a source of neutrophil elastase. This protease was previously described to be essential in the neonatal BP model [187]. Neutrophil elastase-deficient mice were resistant to disease induction but became susceptible after reconstitution with wildtype neutrophils. A further role for the enzyme has been examined by Lin et al, who described neutrophil elastase in their mouse model as an important enzyme for the development of blisters in experimental BP [188]. Injection of neutrophil elastase into neonatal mice resulted in recruitment of neutrophils into the skin. In addition, they demonstrated a degradation of BP180 in murine and human skin at the immunodominant extracellular domain, which would lead to skin fragility. Further *in vitro* and *in vivo* experiments are required to determine the role of neutrophil elastase in the model and to show if this is the responsible enzyme or if another molecule might be relevant. *Ex vivo* and *in vivo* studies with deficient mice for the candidate molecule, for example neutrophil elastase knockout mice might reveal the relevant role of the respective molecule. Cryosection assays with neutrophils of these mice could give a clue for the split-inducing capacity. In addition, similar to Liu et al. [187], induction of disease in the deficient mice and, in

case of no disease development, restoring this with wildtype neutrophils, could be a possible setting.

The mechanism of cleaving C5 notwithstanding, I further focussed on the role of the active cleaving product C5a in development of my experimental BP. The function of C5aR has been analysed so far in different diseases. Activation of cells of myeloid origin and activated mast cells undergo chemotaxis, degranulation and secretion [139]. In a mouse model for sepsis, activation of neutrophils by C5aR results in the paralysis of cell signalling and the inability to assemble NADPH-oxidase, which leads to the loss of innate immune functions like chemotaxis and phagocytosic. The binding to C5aR to cardiomyocytes results in defective contractility, the consequence of which is cardiac shock and, ultimately, cell death [189,190].

In an asthma model, different effects for C5aR have recently been found. C5-deficient mice were more susceptible to the development of airway hyperresponsiveness and pulmonary inflammation in response to allergen exposure than wild-type mice [191,192]. Köhl et al. suggested a dual role for C5a/C5aR signalling in allergic asthma, which is protective during allergen sensitisation but proallergic in an established inflammatory environment [193]. In further studies, it was found that C5a protects from the development of maladaptive Th2 immunity during allergen sensitisation. During the course of the disease, the C5aR was downregulated on pulmonary dendritic cells [194].

For BP, an interesting function of the C5aR on mast cells has recently been found in the neonatal mouse model of BP. Mice deficient in mast cells or C5aR were protected from disease development and showed a decrease in phosphorylation of

p38 mitogen activated protein kinase (p38MAPK), a key signalling molecule involved in translating extracellular environmental conditions into cellular responses. Reconstitution with mast cells only from wildtype but not from C5aR deficient mice resulted in subepidermal blister formation and restored p38MAPK levels. In addition, local injection of recombinant C5a induced phosphorylation in wildtype but not mast cell deficient mice and also cultured mast cells showed an increase in phosphorylation of p38MAPK and degranulation after treatment with recombinant C5a [195].

C5L2 is expressed on most of the same cell types as C5aR but it lacks the G-coupled activation pathway. The function of C5L2 is not yet fully understood. Some experimental data suggest that C5L2 functions as a non-signalling decoy receptor. Knockout or blockage of C5L2 was found to exacerbate the inflammatory response in mice [196,197]. In addition, it was shown that C5L2 is required to facilitate C5a signalling in neutrophils, macrophages and fibroblasts *in vitro*, while lack of C5L2 *in vivo* resulted in reduced ovalbumin-induced airway hyperresponsiveness, inflammation and mildly delayed haematopoietic cell regeneration after γ-irradiation [198]. Studies on C5L2 in experimental allergic asthma showed evidence for critical roles in the development of the disease through direct effects on myeloid dendritic cells as well as on other pulmonary cells [199]. In immune complex induced lung injury, C5L2 has a protective effect, as lack of the receptor results in enhanced injury [197]. In contrast, C5L2 was shown to have contrasting functions in sepsis. In 'mid-grade' sepsis, which means a survival rate in patients of 30-40%, blockage or absence of one of the receptors led to improvement, while in high grade sepsis only blockage of both receptors led to greater survival rate [132].

In the new BP mouse model, C5aR$^{-/-}$ mice were largely protected from disease, while C5L2$^{-/-}$ mice showed significantly more clinical disease compared to the corresponding wildtype mice. Therefore, while C5aR shows an enhancing effect, C5L2 seems to play a protective role in the development of experimental BP. Consequently, the 50% reduced disease activity in C5$^{-/-}$ mice seems to be a result of two different facts: First, C5$^{-/-}$ mice cannot produce C5a. Therefore the enhancing C5aR, which only binds C5a and C5a^{desArg}, cannot be activated. This is a similar situation as in mice lacking the receptor C5aR, where I showed the same experimental outcome in my experiments. Secondly, C5L2 has a protective effect in experimental BP. As C5L2 also interacts with C3 and C3^{desArg}, which are produced upstream of C5, this possibility of reduction of disease activity is also available in C5$^{-/-}$ mice.

Besides the previously described activating functions of the effector cells, C5aR can also have an influence on Fc receptors. C5aR signalling lowers the threshold for Fcγ receptor activation by upregulating the expression of activating Fcγ receptors (FcγRI and III) and downregulating the expression of the inhibitory FcγRIIB [200,201]. Conversely, FcγR activation enhances the synthesis of C5, which subsequently can enhance C5a generation [200]. This C5a-FcγR crosstalk is important in infection, where it promotes the clearance of microbial intruders by combining phagocytosis with the specificity of IgG antibodies. In autoantibody-mediated autoimmune diseases like BP, complement-FcγR crosstalk might therefore exacerbate autoimmune pathology.

To examine possible functions of the C5a and its receptors during the development of the novel experimental BP *in vivo* and its potential as a therapeutic target,

specific inhibition of C5aR at different time points was performed. For these purposes, I treated mice with the C5aR antagonist PMX53. Application from day 0 together with the pathogenic antibody resulted in a reduced overall disease activity. In contrast, application of PMX53, when disease already was clinically detectable (at day 4), no difference in the clinical score was observed compared to control mice. Therefore, blocking C5aR did not appear to be useful in established BP. Nevertheless, with this experiment I was able to demonstrate the usability of the new model for the treatment of diseased animals. With the new model it is now possible to perform studies on novel, more specific drugs.

Besides this, the results from the experiments with PMX53 indicate an early function of C5a during the process of blister formation. One of the functions of C5a might be its chemotactic activity. I explored the cell attracting potential of C5a through the use of bone marrow-derived mouse neutrophils from different C5aR-deficient strains. I could detect enhanced chemotactic activity of C5aR and an inhibiting effect of C5L2 compatible with my *in vivo* observations. However, my *ex vivo* studies only covered functions of C5aRs on neutrophils and not on other cell types, for example mast cells, which had previously been suggested to be important in the pathogenesis of BP [195]. In addition, only a limited number of repetitions of the experiment were conducted, so further repetitions of the migration studies would be necessary to confirm the data. Experiments with mast cells and, potentially, other cell types would also be necessary to make a reasonable conclusion on the chemotactic role of C5a in BP development. In another ex vivo approach, I found no influence of complement in ROS release of neutrophils, which is compatible with my hypothesis that C5a is important in the

early phase of blister formation. There was no difference in ROS release when neutrophils containing only one of the C5aRs were stimulated with ICs formed by anti-mCol17 IgG and recombinant mCol17 in presence or absence of complement. Therefore, ROS release from neutrophils may be a complement-independent event. Of note, ROS-release itself, however, was previously demonstrated to be crucial for blister formation in the passive transfer mouse model of EBA [160] and may thus also be relevant in my BP model.

Although complement has previously been found to play a critical role in the neonatal mouse model of BP, I have clearly shown that complement activation is important for lesion formation but not a prerequisite since C5 deficient mice were still susceptible to the pathogenic effect of anti-mCol17 IgG, however to a lesser extent. These data were very recently supported by Natsuga et al., who found a complement-independent way of BP development by direct destabilisation of the DEJ by the bound antibodies [178]. In further experiments I have proven that the novel model is suitable to analyse the therapeutic efficacy of novel therapies in already clinically diseased mice by application of the C5aR inhibitor PMX53 in a therapeutic setting (treatment started on day 4). Since disease severity could not be reduced in this approach but was effective when applied is a prophylactic setting (treatment started on day 0) inhibition of the C5a-C5aR axis did not appear as a potential therapeutic target in patients with BP.

Conclusion

I have developed a new passive transfer mouse model of BP, which reflects major clinical and immunopathological features of human BP, including eosinophil infiltration in lesional skin. Importantly, in contrast to two other mouse models of BP, adult animals are used, which allows the study of disease development over a longer time period. Furthermore, in the novel model, blistering is spontaneous and does not have to be induced by friction. Finally, the new model can be employed in exploring new, more specific therapeutic approaches in already clinically diseased mice. I have also proven for the first time that complement activation is not a prerequisite for lesion formation in BP. My data also indicate that therapeutic targeting of complement activation may not be effective when clinically disease is already established.

5. Abstract

Bullous pemphigoid (BP) is by far the most common autoimmune blistering diseases. It is associated with considerable morbidity and mortality within the elderly patient population. Treatment is based on nonspecific immunomodulation and immunosuppression and leads to severe adverse effects in a high number of patients. Thus, more specific treatment approaches are necessary. Two structural proteins of the dermal-epidermal junction, BP180 (type XVII collagen) and BP230 were identified as target antigens in BP. In this study, I have developed a new mouse model based on the repetitive injection of rabbit anti-murine BP180NC15A IgG ("mCol17"; the corresponding murine epitope to the human immunodominant NC16A domain) into adult mice. These mice developed BP-like disease reflecting major clinical and immunopathological features of the human disease. Disease induction in Fc receptor γ-chain knockout (FcRγ$^{-/-}$) mice failed as well as injection of antibodies with disabled binding properties to the Fc-receptor in wildtype mice. Mice lacking the complement component C3 developed disease to the same extent as their controls, while mice lacking serum C5 exhibited only half of the disease activity compared to wildtype controls. Subsequently, I identified the C5a receptor (C5aR) as pathogenetically relevant, while mice deficient of the second C5a receptor, C5L2, developed significantly more disease. Pharmacological inhibition of C5aR was protective when applied as a preventive strategy together with the injection of anti-mCol17 IgG but was not effective when applied after clinical disease had already been established. *Ex vivo* migration and ROS-release studies with bone marrow-derived neutrophils showed that complement enhanced neutrophil migration towards mCol17-containing immune complexes, whereas

complement did not increase the ROS release from neutrophils stimulated with these immune complexes. These data suggest that the complement system is important at the beginning of the disease, e.g. for mediating migration of inflammatory cells towards the dermal-epidermal junction. In summary, the novel mouse model shed new light on the role of complement in BP and may be a useful tool for exploration of both the inflammatory events leading to blister formation and novel, more specific treatment modalities in this disease.

5. Zusammenfassung

Das bullöse Pemphigoid (BP) ist bei Weitem die häufigste Blasen bildende Autoimmunerkrankung. Das BP ist mit einer hohen Morbidität und Mortalität assoziiert. Die Behandlung basiert auf einer unspezifischen Immunmodulation und langfristigen Immunsuppression und führt bei vielen Patienten zu schweren Nebenwirkungen, was spezifischere Behandlungsmöglichkeiten notwendig macht. Zwei Strukturproteine der dermo-epidermalen Junktionszone, BP180 (Typ XVII Kollagen) und BP230 wurden als Zielantigene des BP identifiziert. In der vorliegen Arbeit habe ich ein neues Mausmodell entwickelt, in dem die wiederholte Injektion von Kaninchen IgG, das gegen die murine BP180NC15A Domäne („mCol17"; das murine Pendant zur humanen immundominanten NC16A-Domäne) in adulte Mäuse zu einem der humanen Erkrankung ähnlichem Krankheitsbild führte. Die Erkrankung konnte in Mäusen mit fehlender γ-Kette des Fc-Rezeptors oder durch Injektion von Anti-mCol17 IgG, bei dem die Zuckerkette am Fc Teil abgespalten war, nicht induziert werden. Bei beiden Versuchen zeigten sich jedoch deutliche Ablagerungen von C3 und C5 an der dermo-epidermalen Junktionszone. Interessanterweise war die Erkrankung in C3-defizienten Mäusen ebenso stark ausgeprägt wie in Wildtypmäusen, während sie in C5-defizienten Tieren um die Hälfte reduziert war. Anschließend konnte ich den C5a Rezeptor (C5aR) als relevanten Rezeptor für die Pathogenese identifizieren. Mäuse, denen der zweite C5a Rezeptor, C5L2, fehlte, zeigen hingegen eine signifikant stärkere Ausprägung der Krankheit. Pharmakologische Blockade des C5aR führte in einem präventiven Ansatz zu einer reduzierten Krankheitsaktivität, nicht aber im therapeutischen Ansatz, nachdem sich bereits erste Hautveränderungen ausgebildet hatten. *Ex vivo*

Migrations- und ROS-Freisetzungs-Studien mit Knochenmarksneutrophilen zeigten eine verstärkte Migration bei Anwesenheit von Komplement, während Komplement keinen Effekt auf die ROS-Freisetzung hatte. Meine Daten deuten darauf hin, dass Komplementaktivierung an der dermo-epidermalen Junktionszone zu Beginn der Krankheit wichtig ist, z.B. bei der Einwanderung von Entzündungszellen, jedoch, anders als im neonatalen Mausmodell des BP beschrieben, keine zwingende Voraussetzung darstellt. Das neue Mausmodell sollte erlauben, neue Erkenntnisse zur Pathogenese der Erkrankung zu gewinnen und spezifischere Therapiestrategien zu entwickeln.

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7. Appendix

I. Materials

Laboratory equipment

Air incubator containing 5% CO_2	Memmert, Schwabach, Germany
Animal facility	University of Lübeck
Automatic microtiterplate washer	Columbus Pro, Tecan, Crailsheim, Germany
Biophotometer	Eppendorf AG, Hamburg, Germany
Camera for IF microscopy/histology ProgRes C10 plus	Jenoptik, LaserOpticsystems, 07739 Jena
Camera for Mice, Eos 350D	Canon Deutschland, Krefeld, Germany
Centrifuge BIOFUGE fresco	Haereus Instruments GmbH, Hanau, Germany
Centrifuge Varifuge 3.0 R	Haereus Instruments GmbH, Hanau, Germany
Centrifuge Avanti J-E	Beckman Coulter Inc., Brea, CA, USA
Cold room	Viessmann GmbH&Co. KG, Allendorf, Germany
Cryostat Leica CM 3050S	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany
Deep freezer (-80°C) C660	New Brunswick Scientific, England
Dry heat steriliser	Binder GmbH, Tittlingen, Germany
Electron microscope	Model JEOL JEM-1230, Tokyo, Japan
Electrophoresis system Mini-Protean®3, Electrophoresis Cell	Bio-Rad Laboratories, Munich, Germany
ELISA microplate reader, VICTOR3 Wallac 1420	Perkin-Elmer LAS, Rodgau, Germany
ELISA PLATE washer	TECAN, Maennedorf, Switzerland
Eppendorf pipette	EPPENDORF AG, Hamburg, Germany
FACS Calibur	Becton Dickinson GmbH, Heidelberg, Germany
Fluorescence microscope	EUROstar, EUROIMMUN AG, Groß

	Grönau, Germany
Freezer -20C/Refrigerator +4°C	Liebherr International AG, Bulle, Switzerland
PCR cycler, GeneAmp PCR System 9700	Applied Biosystems Deutschland GmbH, Darmstadt, Germany
pH meter, ph526	MultiCal WTW, Weilheim, Germany
Shaker/incubator for bacterial cultures Certomat IS	B.Braun, Melsungen, Germany
Sonicator; Sonoplus GM/UW 2070	BANDELIN electronic, Berlin, Germany
Vortex, Genie2	Scientific Industries, Bohemia, New York, USA

Consumable materials

1,5ml/2,0ml tubes	Sarstedt AG&Co., Nuernberg, Germany
15/50ml tubes	Sarstedt AG&Co., Nuernberg, Germany
Amicon Ultra-15 filters	Millipore, Bradford, MA, USA
Cover glasses	Gerhard Menzel, Glasbearbeitungswerk GmbH&Co. KG, Braunschweig, Germany
Dark chamber	Werner Hassa GmbH, Heidelberg, Germany
Disposable cuvettes	BRAND GmbH, Wertheim, Germany
Disposable needle	Becton Dickinson GmbH, Heidelberg, Germany
Disposable syringe	Becton Dickinson GmbH, Heidelberg, Germany
Embedding compound TissueTek® O.C.T. CompoundTM	Sakura Finetek Europe B.V. Alphen aan den Rijn, Netherlands
Embedding mold TissueTek® CryomoldTM	Sakura Finetek Europe B.V. Alphen aan den Rijn, Netherlands
Non-transparent 96 well microtiterplate	Nunc, Roskilde, Denmark
Nitrocellulose membrane	Whatman, GmbH, Dassel, Germany
Transwell plates 6.5mm, 3µm pore	Corning, New York, NY, USA
Slide glasses	Gerhard Menzel, Glasbearbeitungswerk GmbH&Co. KG, Braunschweig, Germany

Sterile filter	Sarstedt AG&Co., Nuernberg, Germany

Chemical and biological reagents

Actic Acid	Roth, Karlsruhe, Germany
L-Arginine	Roth, Karlsruhe, Germany
Benzoase	Novagen /Merck, Darmstadt, Germany
Borate	Roth, Karlsruhe, Germany
BSA	Roth, Karlsruhe, Germany
BSA biotin-free	Sigma-Aldrich, Munich, Germany
Carbenicillin	Roth, Karlsruhe, Germany
DDT	Roth, Karlsruhe, Germany
Dextran 500	Nycomed, Oslo, Norway
Diaminobenzidine	Merck KgaA, Darmstadt, Germany
Dual Endogenous Enzyme Block	Dako, Hamburg, Germany
E.coli strain Origami	Novagen /Merck, Darmstadt, Germany
EDTA	Roth, Karlsruhe, Germany
Eosin	Merck KgaA, Darmstadt, Germany
Formaldehyde	Roth, Karlsruhe, Germany
Glutathione-Agarose	Sigma-Aldrich, Munich, Germany
Glutathione oxidised	Roth, Karlsruhe, Germany
Glutathione reduced	Sigma-Aldrich, Munich, Germany
Glycine	Roth, Karlsruhe, Germany
Glycerol	Roth, Karlsruhe, Germany
Hematoxyline	Merck KgaA, Darmstadt, Germany
HEPES	Roth, Karlsruhe, Germany
HistoGreen	Linaris, Wertheim-Bettingen, Germany
IL-8	50ng/ml; Calbiochem, San Diego, CA, USA
IPTG	Roth, Karlsruhe, Germany
Ketamine	Sigma-Aldrich, Munich, Germany
LB medium	MP Biomedicals, Illkirch, France
Lens culinaris agglutinin -lectin	Vector Laboratories, Burlingame, CA, USA

Luminol	Roche Diagnostics, Mannheim, Germany
Lysozyme	Fluka/ Sigma-Aldrich, Munich, Germany
MgCl	Merck KgaA, Darmstadt, Germany
NaCl	Roth, Karlsruhe, Germany
Na dodecyoxycholate	Sigma-Aldrich, Munich, Germany
NaEDTA	Roth, Karlsruhe, Germany
NaN_3	Merck KgaA, Darmstadt, Germany
Normal rabbit serum	CCPro, Oberdorla, Germany
Penicillin	Biochrom, Berlin, Germany
Percoll™	GE Healthcare, Uppsala, Sweden
PMX53	University of Queensland, Brisbane, Australia
Primers *Bam*HI and *Sal*III	MWG-Biotech, Ebersberg, Germany
Protein G Sepharose Fast Flow Column chromatography	Millipore/Merck, Darmstadt, Germany
Proteinase inhibitor cocktail	Calbiochem/Merck, Darmstadt, Germany
RPMI 1640	Life Technologies, Karlsruhe, Germany
Skimmed milk powder	Roth, Karlsruhe, Germany
Streptavidin	Biochrom, Berlin, Germany
Streptavidin-HRP	Vector Laboratories, Burlingame, CA, USA
Sucrose	
Super Signal West Pico peroxidase substrate	Pierce, Rockford, IL, USA
Transparent adhesive tape	Beiersdorf AG, Hamburg, Germany
Tris-HCl	Roth, Karlsruhe, Germany
Triton X-100	Sigma-Aldrich, Munich, Germany
Trypan blue	Sigma-Aldrich, Munich, Germany
Tween 20	Sigma-Aldrich, Munich, Germany
Vector pGex 6P-1	Amersham Biosciences, Heidelberg, Germany
Xylazine	Sigma-Aldrich, Munich, Germany

Antibodies

Antibodies	Clone #	Company	Dilution	Concentration Vial
FITC labelled polyclonal goat anti-mouse C3	-	Cappel Organon-Teknika, Durham, NC	1:50	4mg/ml
FITC labelled polyclonal rabbit anti-mouse IgG	-	DAKO, Hamburg, Germany	1:100	1.9mg/ml
FITC labelled polyclonal swine anti-rabbit IgG	-	DAKO, Hamburg, Germany	1:100	0.5mg/ml
HRP labelled polyclonal goat anti-rabbit IgG	-	DAKO, Hamburg, Germany	1:2,000	0.25mg/ml
HRP labelled polyclonal rabbit anti-mouse IgG	-	DAKO, Hamburg, Germany	1:2,000	1.3mg/ml
HRP labelled-polyclonal rabbit anti-rat IgG	-	DAKO, Hamburg, Germany	1:500	1.3mg/ml
Mouse IgG1 anti-PE conjugated microbeads	n/a	Miltenyi, Bergisch-Gladbach, Germany	Total volume of primary ab	n/a ; Order# 130-048-801
Monoclonal mouse anti-mouse C5 antibody	BB5.1	Cell Sciences, Canton, MA, USA	1:100	1mg/ml
PE- labelled hamster IgG1 anti-CD3ε	145-2C11	Miltenyi, Bergisch-Gladbach, Germany	10µl per $10*10^9$ cells	55µg/ml
PE- labelled monoclonal rat IgG2a anti-CD19	6D5	Miltenyi, Bergisch-Gladbach, Germany	10µl per $10*10^9$ cells	33µg/ml
Monoclonal mouse anti-mouse GST antibody	BDI340	Abcam, Cambridge, UK	1:1,000	1mg/ml
Rat anti-mouse major basic protein antibody	MT 14.7	Lee Laboratory, Mayo clinic Arizona, Scottsdale, AZ, USA	1:1,000	1mg/ml

II. List of figures and tables

III. List of abbreviations

µg	microgram
AF	anchoring filament
Afb	anchoring fibril
APC	antigen presenting cell
BMZ	basement membrane zone
BP	bullous pemphigoid
BPAG	bullous pemphigoid antigen
BSA	bovine serum albumin
C1q	complement component 1q
C4	complement component 4
C3	complement component 3
C5	complement component 5
C6	complement component 6
C9	complement component 9
CD	cluster of differentiation
CL	chemiluminescence
Col	collagen
CP	cicatricial pemphigoid
DAB	3,3'-diaminobenzidine
DDT	1,1,1-trichlor-2,2-bis(4-chlorphenyl)ethan
DEJ	dermal-epidermal-junction
DH	dermatitis herpetiformis
Dsg	desmoglein
EBA	epidermolysis bullosa acquisita
ELISA	enzyme-linked immunosorbent assay
fB	factor B
fD	Factor D
FACS	fluorescence activated cell sorting
FcRγ	common γ-chain of the Fc receptor
FCS	fetal calf serum
FcγR	Fc receptor γ
FITC	fluorescein isothiocyanate
GST	glutathione-*S*-transferase
H&E	hematoxilin and eosin staining
HD	hemidesmosome
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horse raddish peroxidase
i.p.	intraperitoneal
IC	immune complex
IF	immunofluorescence
IgE	immunoglobulin E
IgG	immunoglobulin G
IL	interleukin

IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilo dalton
LB	lysogeny broth
LD	lamina densa
LL	lamina lucida
MAC	membrane attack complex
MBL	mannan-binding lectin
mg	milligram
MHC	major histocompatibility complex
ml	milliliter
mm	millimeter
MMP	mucous membrane pemphigoid
MPO	myeloperoxidase
NaCl	sodium chloride
NaEDTA	sodium 2,2',2'',2'''-(ethane-1,2-diyldinitrilo)tetraacetic acid
NaN_3	sodium azide
NC	non-collagenous
NE	neutrophil elastase
NK	natural killer
nm	nanometer
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	preimmunised
PPI	immunised
RA	rheumatiod arthirtis
RLU	relative light units
ROS	reactive oxygen species
s.c.	subcutaneous
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	single nucleotid polymorphism
TBS-T	tris-buffered saline with Tween 20
Tris-HCl	tris(hydroxymethyl)amino methane

IV. Published articles

Hirose, M, Recke, A, **Beckmann, T**, Shimizu, A, Ishiko, A, Bieber, K, Westermann, J, Zillikens, D, Schmidt, E, Ludwig, RJ. Repetitive immunization breaks tolerance to type XVII collagen and leads to bullous pemphigoid in mice. *Journal of Immunology* 187, 1176–83 (2011).

Vafia, K, Groth, S, **Beckmann, T**, Hirose, M, Dworschak, J, Recke, A, Ludwig, RJ, Hashimoto, T, Zillikens, D, Schmidt, E. Pathogenicity of Autoantibodies in Anti-p200 Pemphigoid. *PloS one* 7, e41769 (2012).

Hellberg, L, Samavedam UKSRL, Holdorf, K, Hänsel, M, Recke, A, **Beckmann, T**, Hardt, K, Boehncke, WH, Kirchner, T, Möckel, N, Solbach, W, Zillikens, D, Schmidt, E, Ludwig, RJ, Laskay, T. Methylprednisolone blocks autoantibody-induced tissue damage through inhibition of neutrophil activation. *Journal of Investigative Dermatology.* 133(10),2390-9 (2013)

V. Acknowledgement

I would like to express my utmost gratitude to Prof. Dr. Detlef Zillikens, Director of the Department of Dermatology, Allergology and Venereology, University of Lübeck for providing me this opportunity to start my scientific career in the immunodermatology field, and for his supervision and guidance.

I gratefully acknowledge my supervisor, Prof. Dr. Dr. Enno Schmidt. Throughout this research project, I have very gratefully received numerous thoughtful and helpful suggestions and guidance.

I am also heartily thankful to Prof. Jürgen Westermann, Director of the Institute of Anatomy for providing critical comments and thoughtful suggestions as my secondary mentor and for being chairman. Thanks are also due to Prof. Ulrich Schaible, Head of Department of Cellular Microbiology of the Research Center Borstel for being my secondary doctoral referee.

I very sincerely appreciate the valuable assistance and advice I have been given by my peers and supervisors and colleagues in the completion of this project. I have been truly fortunate to work in such a marvelous environment. In particular, I greatly thank Franziska Sophie Schulze, who supported the purification of pathogenic IgG and sample collection of mice and Dr. Andreas Recke who helped me with statistical analysis. My histology samples were greatly prepared by Rebecca Cames.

I also greatly acknowledge the supportive collaborators as there are Prof. Jörg Köhl, Director of the Institute of Systemic Inflammation Research, who greatly supported me with knockout mouse strains and thoughtful suggestions on the complement system, Prof. Akira Ishiko, Director of Department of Dermatology of Toho University in Japan, who greatly supported the electron microscopic analysis, Prof. Mattias Collin from the Division of Infection Medicine at the Department of Clinical Sciences of Lund University, Sweden, who supported the EndoS treatment, and Dr. Trent Woodruff from the University of Queensland, Brisbane, Australia, who kindly provided me with the PMX53.

VII. Declarations/ Copy right statement

Declaration

No part of the work referred to in this dissertation has been submitted in support of an application for any degree or qualification of The University of Lübeck or any other University or Institute of learning.

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