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The role of anti-laminin γ1 antibodies in diagnosis and pathogenicity of anti-p200 pemphigoid

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1 INTRODUCTION

1.1 Immune system

The immune system of mammalian species protects the integrity of the body against self and non-self pathogens. For this purpose, it comprises two modes of function, the innate and the adaptive immune system, which are both entangled in mounting a well balanced immune response. The innate immune system is considered as the first line of defence. It is represented by cells that provide the most basic functions for detection and removal of pathogens, as well as barriers like epithelial layers that shield the body against the environment. In addition, the innate immune system is responsible for support of the second line of defence, the adaptive immune system. The adaptive immune system has the ability to tailor a specific immune response against pathogens while minimizing collateral damage to the body. For this purpose, the adaptive immune system has evolved into two parts, the humoral and the cellular adaptive immune system represented by B and T lymphocytes, respectively. Both parts have the ability to recognize a nearly infinite number of pathogens by modification of the genome itself in an evolutionary process in single individual. The ability of the adaptive immune system to distinguish self from non-self or altered self is called tolerance. However, the mechanisms that control tolerance are highly advanced, but not free of defects. When tolerance mechanisms fail, the adaptive immune system starts to mount an immune response against the body it is meant to protect. The result is autoimmunity, which can manifest in a variety of diseases [1].

1.1.1 T cells, B cells and tolerance

T-cells are lymphocytes, which are developed in the thymus and characterized by an antigen recognition structure, the T cell receptor (TCR). The TCR recognizes only antigens presented by major histocompatibility complex (MHC) molecules and reacts with several other surface components. The most important is the CD4, which determines the interaction of the TCR with MHC class II molecules and the CD8, which determines the interaction of TCR with MHC class I molecules.

B-cells are lymphocytes developed in the bone marrow and characterized by the production of antigen recognition structures, the B cell receptor, and immunoglobulins. However the structure of the antigen recognition site is not terminally fixed when B cells leave the bone marrow. They go

through somatic mutation; thus the affinity of the antigen recognition site of immunoglobulins can mature during the immune response.

A very important function of the immune system is to distinguish self from non-self antigen. There are three important mechanisms to achieve this. The first mechanism is the phenomenon of central tolerance and is characterized by the deletion of potentially autoreactive lymphocytes. As soon as immature lymphocytes recognize a self antigen, by the expression of an antigen receptor, they are eliminated or inactivated. About 90% of self-reactive T and B cells are deleted in thymus and in processes such as apoptosis. However, 10% of these cells survive [2, 3]. The second mechanism implies on the concentration of an antigen. Self antigens are expressed in high concentrations in many tissues. This overexpression of antigens causes the tolerance of the lymphocytes to the specific self antigen, in contrast to a non self antigen which appears suddenly in the body and induces activation of the lymphocytes. The third mechanism corresponds to antigen in the periphery outside the thymus and bone marrow and therefore it is called peripheral tolerance. In this case, when naive lymphocytes recognize an antigen, they induce either an inhibitory signal or the development of regulatory lymphocytes to control harmful responses for a tissue.

The adaptive immune system can respond to millions of different antigens in a specific way. When B cell, naïve or memory B cell, encounters a pathogenic antigen, together with the collaboration of T cells, it proliferates and differentiates into activated B-cell and produce immunoglobulins [1, 4].

1.1.2 Immunoglobulins

There are five classes of immunoglobulins IgG, IgA, IgM, IgD, and IgE. IgG is the most abundant antibody isotype. In humans, IgGs are defined in 4 different subclasses IgG1, IgG2, IgG3 and IgG4. Similarly, in mice the subclasses which have been described are: IgG1, IgG2a/c, IgG2b and IgG3.

Immunoglobulins G are synthesized and secreted by plasma B cells, derived from B cells. Each IgG molecule has two antigen binding sites and is composed of four chains, two heavy chains and two light chains. The antigen binds to one part of the immunoglobulin (Ig) molecule, the antigen binding fragment (Fab), while the crystallizable region fragment (Fc) modulates immune cell activity by binding to specific Fc-receptor (FcR) or to complement peptides [1], which is discussed in the following chapter. FcR binds to the Fc part of Ig, which is attached to the antigen. This leads to antibody-mediated phagocytosis or antibody-dependent cell-mediated cytotoxicity. Fcγ receptors (FcγRs) are the most important FcR. FcγRs are expressed in several immune cells such as macrophages, monocytes, dendritic cells, neutrophils, B cells etc [5]. The FcγRs are divided in

activating (FcγRI, FcγRIIA FcγRIIIA, and FcγRIIC) and inhibitory (FcγRIIB) receptors[6] . In the mouse there are also different subclasses that have been identified as activating (FcγRI, FcγRIII, FcγRIV) and inhibitory receptors FcγRIIB. The ratio inhibitory/activating FcγRs influences the inflammatory response mediated by immune complexes [7]. There are many *in vivo* studies that demonstrate the significance of these receptors in autoimmune diseases [8]. Studies have shown the pathogenic potential of upregulated activating FcγRs and downeregulated FcγRsIIB in experimental rheumatoid arthritis, experimental arthritis, and immune-complex mediated nephritis. Recently genetic analysis and animal experimental models demonstrate the important role of activating FcγRIV and inhibitory FcγRIIB in pathogenesis of an autoimmune subepidermal blistering disease, epidermolysis bullosa acquisita [9, 10].

IgGs have also the ability to activate complement system by binding of Fc part to complement peptides. However, the affinity of each subclass depends of the complexity of the IgG. E.g. IgG4 does only weakly interact with components of the complement system.

1.1.3 Complement

Complement is an important component of the immune system. It is mainly part of the innate immune system but it can be activated by the adaptive immune system. It plays an important role in lysis of cells or viruses, phagocytosis, and secretion of molecules of immune system (e.g. cytokines). It also assists with the regulation of the innate and adaptive immune responses. Particularly, it prevents precipitation and aggregation of immune complexes (formed by binding of the antibodies to the antigen), while other functions are contribution in lipid metabolism and angiogenesis etc [11]. There are three pathways of complement activation: the classical, the alternative and the lectine pathway. Initially, immune complexes activate the classical pathway. Consequently C3 component is cleaved to C3a and C3b. C3b starts opsonization by binding to the surface of pathogens. Like C3a, C5a is also an anaphylatoxin and is released after cleavage of C5. The alternative pathway is independent of immune complexes but it is activated by the automatic activation of C3. Some components of the complement system like C5b form a membrane attack complex. This complex, together with antibodies, kills or damages pathogens or cells by osmotic lysis [12-14].

Complement is determined to destroy foreign microorganisms but it can also destroy body's own cells. It has been demonstrated that the presence of autoantibodies, the deficiency of complement components, and the increase of the levels of specific cytokines may lead to impairment of the complement function[15, 16]. Complement activation is present in many autoimmune diseases and is

also responsible for the development of several blistering diseases [13, 14, 17]. Deposition of components of the complement system at the site of inflammation is a typical finding in subepidermal blistering of the skin. Its contribution for disease severity is demonstrated by the findings that knock-out of complement components influences development of subepidermal autoimmune skin diseases in mice, which is discussed in chapter 1.6.2.

This thesis explores the pathophysiology of anti-p200 pemphigoid, a disease where antibodies become auto-reactive against structural components of the dermis of the skin, which manifests as an inflammatory blistering disease of the skin. Before talking about autoimmune blistering skin diseases, the following chapters provide information about the fundamental meanings of autoimmunity and skin.

1.2 Autoimmunity

The healthy human body is equipped with the powerful immune system for resisting the invasion of microorganisms. However this useful tool can attack the body itself. When an immune response, due to an excessive exposure to an antigen, results to an abnormal or pathological event, this phenomenon is characterized as hypersensitivity. Autoimmunity is a cause of hypersensitivity, which is in principle loss of immunological tolerance; as a result, the adaptive immune system is directed against self-antigens.

An autoimmune response is a physiological event during the development of an immune reaction and this event stops as soon as the triggered, harmful antigen, no longer exists. In autoimmune diseases the immune reaction can reach the autonomy so it carries on without the trigger antigen. In this case, there is no deference between harmful or not, self or non-self antigens. Then, the antigen presentation is more important than the deficiency of the immune response.

Several mechanisms have been proposed to play role in the pathogenesis of autoimmune diseases. Most of them focus on the breakdown of tolerance at both T-and B-cell levels. One of the mechanisms, hypothesized to be involved in the breakdown of tolerance, is the failure to dispose the autoreactive lymphocytes during ontogenesis. Molecular mimicry is also proposed as a pathogenetic mechanism for autoimmune disease and it is in fact the wrong detection of a self antigen which is alike to a microbial antigen [18]. Another interesting mechanism proposed is the epitope spreading. In this case, the development of an immune response is extended to new epitopes on the major autoantigen as well as to new autoantigen. The main self-antigen that harms tissues spreads the immune response to other epitopes. This leads to activation of lymphocytes

specific for other antigens and autoimmunity. Abnormal presentation of the self-antigen is also a cause of autoimmunity. This can be due to overproduction of autoantigens, binding of exogenous antigens to self antigens, disclosure of cryptic T-cell epitopes, and abnormal expression of MHC class II molecules.

A disease may be defined as autoimmune when it fulfils the following criteria:

1. the adaptive immune response is against a specific organ or tissue.
2. the specific organ or tissue is characterized by the presence of autoreactive T cells and/or autoantibodies
3. the transfer of the autoantibodies to healthy individuals or animals can induce disease
4. immunization of animals with the autoantigen can induce the disease
5. Improvement of clinical disease can be accomplished by suppression of autoimmune response [19].

There are more than 100 known autoimmune diseases affecting 3-5% of the general population [20]. The incidence rates are different among autoimmune diseases, and most of them can occur at any age. Autoimmunity is a main cause of death among young and middle-aged women in developed countries. The main causes of autoimmune disorders remain unknown. Autoimmune diseases usually affect more women than men. There are several theories for this, such as stronger inflammatory responses in women, hormonal differences between two genders, and genetic susceptibility [21]. Both genetic predisposition and environment play role in autoimmune and chronic inflammatory disorders [22-26]. However, few conventional case-control association studies investigated the genetic basis of autoimmune blistering diseases. There are quite a few genetic studies in systemic lupus erythematosus, rheumatoid arthritis, and other autoimmune diseases including pemphigus. Some of them focused on the association with the HLA (human leukocyte antigen) region but varied in different population groups. The MHC II and HLA was demonstrated to be associated with pemphigus [27], bullous pemphigoid [28, 29], epidermolysis bullosa acquisita [30], and dermatitis herpetiformis [31]. Non-MHC genes, FcγR polymorphisms and Interleukine-1beta gene, have also been associated with genetic predisposition but due to the small number of patients, the results are not conclusive [32-34]. Recently, in a case cohort study of Jewish, beside major histocompatibility complex locus, ST18 gene was found to be significantly associated with pemphigus vulgaris [35]. Autoimmune disease can affect any system of the body including the skin.

1.3 The skin

The skin is the largest and the heaviest organ of the human body and consists of three layers the epidermis, the dermis, and the hypodermis or subcutaneous fatty tissue. Skin has a variety of functions such as protection against injury, sun and radiation, thermoregulation, excretion of toxic substances with sweat, absorption, waterproofing and fluid conservation, mechanical support, as a sensory organ, and production of vitamin D. It also plays an important role as the first line of immunological defense [36].

The epidermis is the outer layer of the skin and is composed of at least four cell types: keratinocytes (95%), melanocytes, Merkel cells, and Langerhans cells. The epidermis has five well-defined layers: the basal (*stratum basale*), the squamous (*Stratum spinosum*), the granular (*Stratum granulosum*), the *stratum lucidum* and the horny layer (*Stratum corneum*). The dermis is located beneath the epidermis and contains the extracellular matrix. It consists of loose connective tissue with blood vessels, nerve processes and sensory nerves. The lower zone is the reticular dermis, is thicker and contains epidermal appendages, embedded nerves, blood vessels, fibroblasts, various inflammatory cells, mast cells, Langerhans cells, lymphocytes, and rarely, eosinophils. This component of the dermis reaches until the third layer or the skin, the hypodermis or subcutaneous fat. This is the deepest and thickest layer and is mainly composed of adipocytes, cells served as a storage site of fat. Apart from its function as an energy store, the hypodermis is also a thermal insulator and controls the production and metabolism of hormones [36].

1.3.1 Dermal-epidermal junction

The lower part of the epidermis and the top layer of the dermis (papillary dermis) are composed of complex network of proteins that are tightly connected through the cutaneous basement membrane zone, this is called dermal-epidermal junction. Ultrastructural examination shows two distinct layers. The upper layer is the lamina lucida and it is directly subjacent to the plasma membranes of basal keratinocytes (Figure 1). The lamina lucida contains heparin sulfate proteoglycans, fibronectin and laminin 332 (previously known as laminin 5). Laminin 332 is a major anchoring protein and connects BP180 (collagen type XVII) and integrin $\alpha 6\beta 4$ with collagen type VII. The lower layer is the lamina densa, which interacts with dermal collagens (type I, III, V) and elastic fibers of the sublamina densa [37]. Beside its major function, to provide the structural integrity of the skin, the dermal-epidermal junction controls the passage of the molecules between the epidermis and the dermis, depending on size and charge, regulates the permeability across the dermal-

epidermal interface, and interferes with keratinocyte migration, proliferation, and differentiation. Both fibroblasts and keratinocytes synthesize components of basement membrane like BP180, type VII collagen, type IV collagen etc [38, 39].

Within the dermal-epidermal junction anchoring complexes called hemidesmosomes are unique focal structural components. Hemidesmosomes consist of a) the cytoplasmatic hemidesmosomal inner plaque into which intermediate filaments are inserted, b) the outer plaque, and c) a sub-basal dense plate [37] (Figure 1). A schematic representation of the hemidesmosomes and the components of the dermal-epidermal junction recognized by autoantibodies are shown in Figure 1.

The dermal-epidermal junction is not a static region but changes continuously e.g. during wound healing. Alterations of this structure, due to genetic defects and inflammatory response following the binding of autoantibodies, result in well-defined disorders, epidermolysis bullosa hereditaria and autoimmune blistering skin diseases, respectively [40, 41].

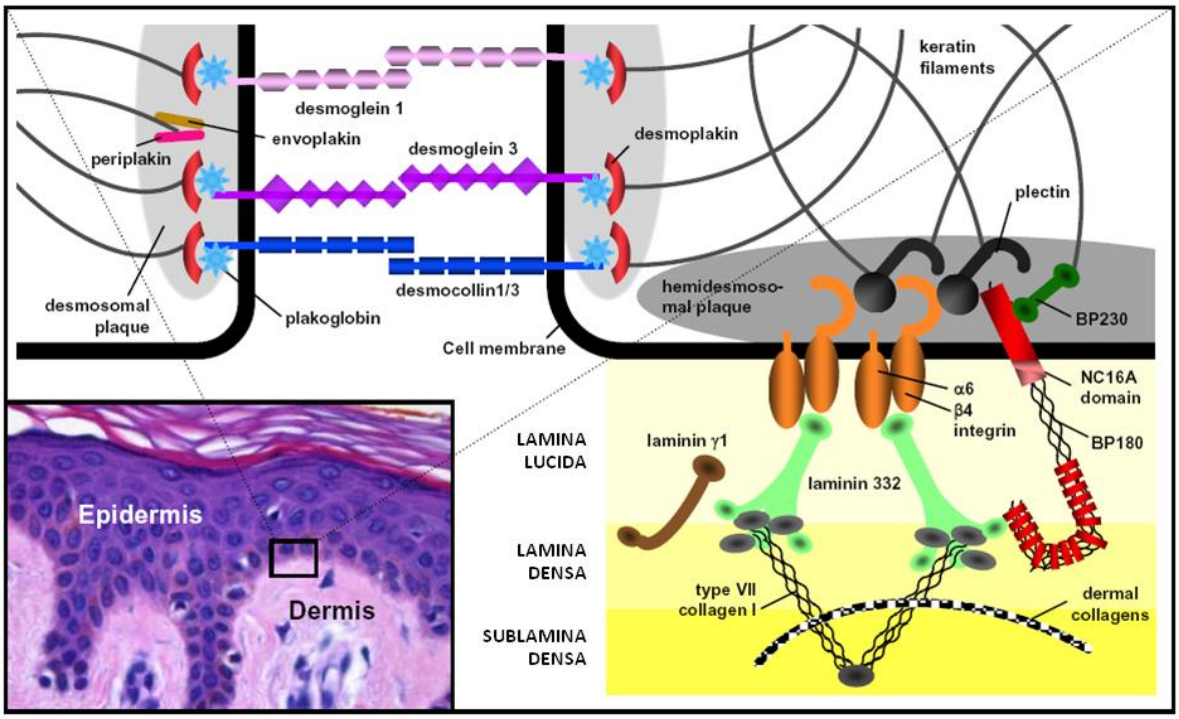

Figure 1. Schematic representation of the components of the dermal-epidermal junction. Many of the structural proteins of the skin basement membrane zone may function as autoantigens in subepidermal blistering diseases. Here, it is shown the relative position of these components and the connections between them. Bottom left: the epidermis, the dermis and the dermal-epidermal junction. Two neighboring basal keratinocytes are shown schematically, while on the left top in detail the extracellular portions mediate their

adhesion. Right side: Epidermis and dermis are attached via hemidesmosomal proteins, some of them are the target antigens in subepidermal autoimmune blistering diseases (adapted from[42]).

1.4 Autoimmune bullous diseases

Autoimmune bullous diseases are characterized by blisters of the skin and surface-close mucous membranes resulting in significant morbidity and mortality [41]. Autoimmune skin diseases are clinically diverse, have different prognoses, and differently respond to treatment[43]. Depending on the level of split formation (intra- or subepidermal) and the target structures of autoantibodies (desmosomal or hemidesmosomal protein) pemphigus and pemphigoid diseases can be differentiated [41, 44]. In addition, in dermatitis herpetiformis, subepidermal splitting occurs while no structural protein but gliadin and epidermal and tissue transglutaminase are targeted by autoantibodies [37].

1.4.1 Pemphigus group

Pemphigus diseases are characterized histologically by intraepidermal blisters due to acantholysis (break down of the intracellular connections between keratinocytes of the epidermis). Immunopathologically, these diseases are characterized by skin-bound and circulating autoantibodies against desmosomal proteins (e.g. desmoglein 1 and 3, Table 1), which mediate the cell-cell adhesion between neighbouring keratinocytes (Figure 1). In Europe pemphigus diseases are rare with an incidence of approximately 0.6-1.4/1,000,000 inhabitants/ year [45-48]. The predominant disease autoantibodies are of the IgG4 suclass. It is thought that IgG4 are pathogenic without the requirement of complement activation in order to induce blister [49].

1.4.2 Pemphigoid group

In Germany, 12,000 patients with pemphigoid diseases have been estimated with an annual incidence of 2,000 new cases[50]. Pemphigoid disorders are characterized clinically by the presence of cutaneous and mucosal blisters and erosions, histologically by subepidermal splitting, and immunopathologically by autoantibodies against hemidesmosomal proteins.

Pemphigoid diseases include bullous pemphigoid, pemphigoid gestationis, lichen planus pemphigoides, linear IgA disease, mucous membrane pemphigoid, anti-p200 pemphigoid, and epidermolysis bullosa acquisita [37, 41]. The different entities are characterized clinically and

immunopathologically. Table 1 summarizes target antigens and diagnostic methods of the different immunobullous diseases. The target antigens are also depicted in Figure 1.

Table 1. Classification of autoimmune bullous diseases, the respective autoantigen of each one and diagnostic method [42, 51]

Group	Immunobullous disease	Target antigen	Diagnostic method
Pemphigus	Pemphigus vulgaris	desmoglein 3, desmoglein 1	ELISA, IIF
	Pemphigus foliaceus	desmoglein 1	ELISA, IIF
	Paraneoplastic Pemphigus	envoplakin, periplakin, desmoglein 1 and 3, BP230, desmoplakin I/II, α2 macroglobulin-like 1	IB, ELISA
Pemphigoid	Bullous pemphigoid	BP180, BP230	ELISA, IIF
	Pemphigoid gestationis	BP180, BP230	ELISA, IIF
	Lichen planus pemphigoides	BP180, BP230	ELISA, IIF
	Mucous membrane pemphigoid	BP180, Laminin 332, BP230, $\alpha6\beta4$ integrin	IB, ELISA, IIF
	Linear IgA disease	LAD-1 (BP180 ectodomain), BP230	IB, IIF
	Anti-p200 pemphigoid (Anti-laminin γ1 pemphigoid)	**laminin γ1**	**IB, IIF**
	Epidermolysis bullosa acquisita	Type VII collagen	ELISA, IB, IIF

IIF, indirect immunofluorescence microscopy; IB, Immunoblotting; ELISA, Enzyme-linked immunosorbent assay.

1.4.3 Diagnosis of autoimmune bullous diseases

The current diagnostic gold standard of autoimmune bullous diseases is the detection of autoantibodies in skin or mucous membranes by direct immunofluorescence (IF) microscopy of a perilesional biopsy [50, 52]. Direct IF microscopy separates pemphigus from pemphigoid disorders but to further distinguish pemphigoid diseases from each other, serological analysis are necessary. Indirect IF microscopy, immunoblotting (IB), and Enzyme Linked Immunosorbent Assay (ELISA) are applied to detect serum autoantibodies [52]. For indirect IF microscopy 1 M NaCl-treated human skin

resulting in dermal-epidermal splitting is used as substrate for the screening of pemphigoid diseases. This method distinguishes between antigens localized on the epidermal side of the split such as BP180, BP230, and $\alpha 6\beta 4$ integrin and those that localize at the dermal side of the artificial split including laminin 332, p200 antigen, and type VII collagen. In bullous pemphigoid, 80-90% of patients' sera contain IgG autoantibodies reacting with the epidermal side of the salt-split skin by indirect IF microscopy [53]. In linear IgA dermatosis, IgA autoantibodies mostly bind to the epidermal side in case of lamina lucida type and to the dermal side in case of sublamina densa type. Autoantibodies bind to the floor of the artificial blister in anti-laminin 332 mucous membrane pemphigoid, anti-p200 pemphigoid, and EBA, thus can be distinguished from bullous pemphigoid, linear IgA dermatosis, pemphigoid gestationis, and anti-BP180 mucous membrane pemphigoid. However, additional methods like IB and ELISA using recombinant and cellular forms or fragments of the corresponding target antigens are then used for exact diagnosis [52].

1.5 Anti-p200 pemphigoid

Anti-p200 pemphigoid is an autoimmune subepidermal blistering disease first described in 1996 [54, 55]. It is a rare disease with less than 100 reported cases [56]. Due to the lack of available diagnostic methods, the disease is highly underdiagnosed/undereported. E.g. in the routine autoimmune laboratory of the Department of Dermatology in Lübeck, Germany, 26 and 28 new cases were serological diagnosed in 2011 and 2012, respectively. Anti-p200 pemphigoid is characterized by tissue-bound autoantibodies to a 200 kDa protein (p200) of the dermal-epidermal junction. By indirect immunogold electron microscopy, autoantibodies to p200 were localized to the lower portion of the lamina lucida [54, 55]. Clinically, the disease is characterized by tense blisters and urticarial eruptions resembling bullous pemphigoid (Figure 2a), the most frequent autoimmune blistering disease. In contrast to bullous pemphigoid, patients with anti-p200 pemphigoid tend to be younger (50-70 years old) and usually respond well to topical corticosteroids, prednisolone 0.5 mg/kg/d, and dapsone [50]. Histopathological examination of lesional skin demonstrates subepidermal blistering and superficial inflammatory infiltrate, mainly presence of neutrophils and rarely eosinophils at the dermal epidermal junction [57-59]. Histopathologically, anti-p200 pemphigoid can not be differentiated from other pemphigoid diseases such as bullous pemphigoid [59]. The main subclass of autoantibodies is IgG4 [60].

1.5.1 Current diagnosis of anti-p200 pemphigoid

Diagnosis of anti-p200 pemphigoid is mainly done by IF microscopy and IB. By direct IF microscopy of a perilesional skin biopsy IgG and C3 deposition along the dermal-epidermal junction are seen (Figure 2b). By indirect IF microscopy of salt-split skin IgG labels the dermal side of the artificial split (Figure 2c). The same labelling pattern is also seen in anti-laminin 332 mucous membrane pemphigoid and epidermolysis bullosa acquisita. For further differentiation, IB with extracts from human dermis that contains both the p200 antigen and collagen type VII, is employed (Figure 2d). Thus, diagnosis of anti-p200 pemphigoid is entirely depended on the quality of the dermal extract, and is available only in specialized laboratories. Recently, the C-terminus of laminin γ1 has been shown to be recognized by the majority of anti-p200 pemphigoid sera and may also provide a useful diagnostic target [61]. A precise and easy diagnostic method for the accurate diagnosis of anti-p200 pemphigoid was urgently needed.

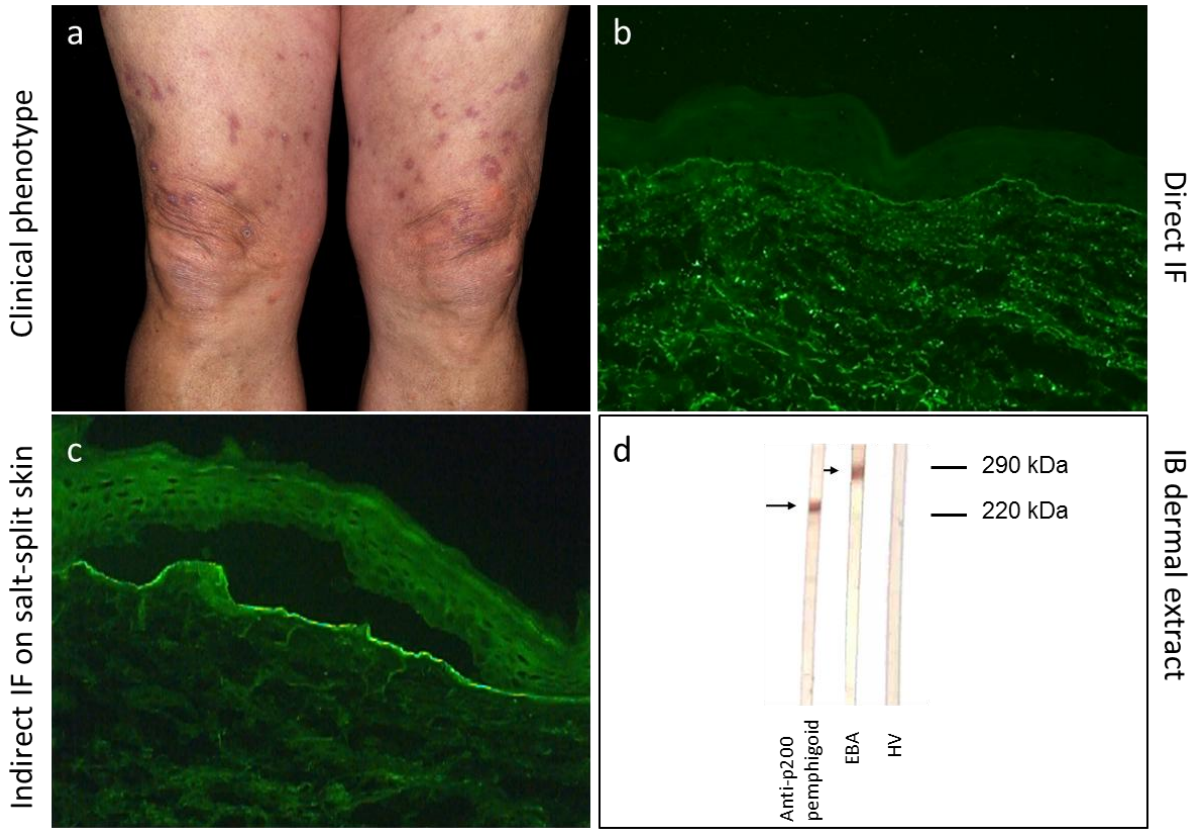

Figure 2. Anti-p200 pemphigoid, clinical picture, and diagnosis. Anti-p200 pemphigoid is clinically characterized by tense blisters and erosions (a), IgG deposition at the dermal-epidermal junction by direct IF microscopy (b), labelled the dermal side of the dermal-epidermal junction by indirect IF microscopy on

human salt-split skin (c), circulating IgG recognized a 200 kDa protein by immunoblotting with dermal extract (d). Photos a and b were kindly provided by the routine autoimmune laboratory, Department of Dermatology, Lübeck; photos c and d were taken by me. EBA, epidermolysis bullosa acquisita; HV, healthy volunteer; IF, immunofluorescence; IB, immunoblotting; kDa, kilodalton.

1.5.2 The target antigen of anti-p200 pemphigoid

For several years the identity of p200 was not known although several studies tried to further characterize the protein. The p200 protein was shown to be synthesized by keratinocytes and fibroblasts [62]. It is disulfide-bonded; forming highly insoluble S-S bonded oligomers or linked to other components of the extracellular matrix by disulfide bridges and takes part in calcium dependent molecular interactions [57]. Biochemical studies have shown that it is an acidic noncollagenous N-linked glycoprotein of the lower lamina lucida [54, 55, 63]. Several candidate molecules fulfilled some of the characteristics of the p200 protein including $\alpha6\beta4$-integrin, nidogen-2, laminin $\beta1$, and laminin $\gamma1$. $\alpha6\beta4$-integrin was excluded due to its epidermal localization. p200 was also distinct from nidogen-2, laminin 111, laminin 311, and fibronectin since anti-p200 pemphigoid sera showed no reactivity with these molecules [54, 58, 62]. The laminin $\beta2$ chain, as part of laminin 321/laminin 7 and laminin 521/laminin 11, is expressed in the upper lamina densa of the basement membrane zone and therefore was also excluded.

Initial studies on laminin $\gamma1$ showed that this molecule is not the autoantigen in anti-p200 pemphigoid due to lack of reactivity of sera from patients with the recombinant purified laminin 111 ($\alpha1\beta1\gamma1$) by IB study [54, 58]. Additionally, by indirect IF microscopy, the anti-laminin 111 antibody reacted with both the dermal basement membrane zone and blood vessels, whereas patients´ sera do not label the vessels by indirect IF microscopy. However, recently, the study of Dainichi *et al.* identified laminin $\gamma1$ as target antigen by 2D gel electrophoresis with dermal extracts followed by immunoblotting of patients' sera and subsequent mass spectrometry. About 90% of patients' sera showed reactivity with the recombinant trimer of laminin 111 (E8 fragment) and the term *anti-laminin $\gamma1$ pemphigoid* was introduced. Furthermore, the C-terminus of laminin $\gamma1$ was suggested as the immunodominant region of this protein [56, 61].

1.5.3 Laminin $\gamma1$

Laminins are a family of glycoproteins, which are major proteins of the dermal-epidermal junction. It has been shown that they play role in the structure of basement membrane but also cellular processes such as migration, differentiation and proliferation [64]. Laminins are large trimeric

proteins that contain 3 non-identical chains, an α-chain, a β-chain and a γ-chain. The three chains form a structure consisting of 3 short arms, each formed by a different chain, and a long arm composed of all three chains (Figure 3). Each chain has in its C-terminus a long α-helical domain (globular domain) that binds the other chains through disulfide bonds and mediates heterotrimer assembly. These three chains together form the coiled-coil domain (Figure 3). Laminins attach to other matrix macromolecules (integrins, collagen type IV, perlecans, nidogens, etc), which are mainly bound to the globular domain. These interactions are critical for cell differentiation, cell shape and movement, maintenance of tissue phenotype, and promotion of tissue survival [65, 66]. Laminin 111 is the most studied laminin and its expression is limited to epithelial basement membranes (Figure 3).

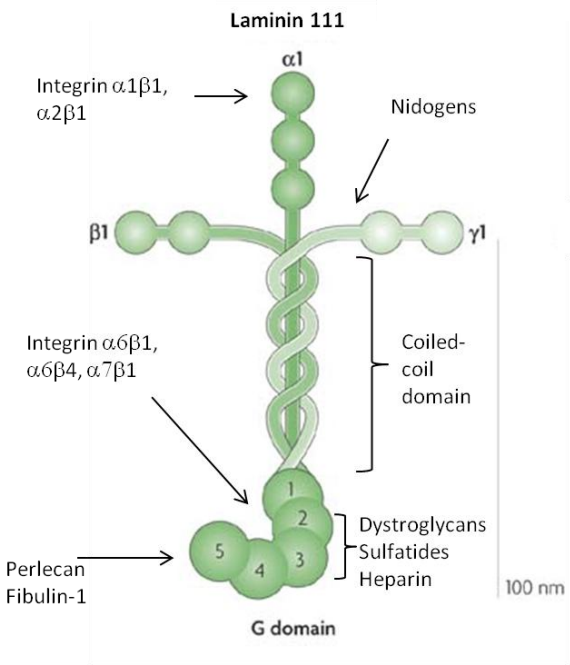

Figure 3. Schematic picture of laminin 111. Laminins are composed of three chains. The large globular domain (G domain) at the base of the long arm of the cross contains five repeating segments. It contains binding sites for various others integrins (adapted from [64, 67]).

The human laminin γ1 has 1,609 amino acids, and a size of 177.603 kDa with a pI 5.08. It is secreted and located in the extracellular matrix at the basement membrane outside hemidesmosomes. The

γ1-chains are responsible for laminin-integrin interaction, whereas nidogen is attached on the arm part. This interaction anchors laminin γ1 to type IV collagen in the basement membrane zone [68, 69].

Laminin γ1 is a component of various laminins, such as laminin 111, 311/32 (laminin 6/7) and 511 (laminin 10) [57]. The N-terminus interacts with nidogen while the C-terminus is the binding site for integrins in the basement membrane zone [70, 71]. As already mentioned, laminin γ1 has been identified as the target antigen in anti-p200 pemphigoid. Moreover, the C- terminus was suggested as the immunodominant region, more specifically, the C-terminal 246 amino acids [61]. The same study proposed as a possible pathogenic mechanism that as soon as autoantibodies against the C-terminus of laminin γ1 bind to the molecule, they disrupt the interaction between laminin and integrin, an event that has impact to the dermal-epidermal cohesion. The exact laminin, which is targeted by anti-laminin γ1 antibodies in anti-p200 pemphigoid, remains enigmatic. The list of laminins that contain α, β and γ chains is shown in Table 2 [72].

Table 2. The standard, abbreviated and previous names of all laminins

Standard name	Abbreviated	Previous name
Laminin α1β1γ1	111	1
Laminin α2β1γ1	211	2
Laminin α1β2γ1	121	3
Laminin α2β2γ1	221	4
Laminin α3β3γ2	332	5
Laminin α3β1γ1	311	6
Laminin α3β2γ1	321	7
Laminin α4β1γ1	411	8
Laminin α4β2γ1	421	9
Laminin α5β1γ1	511	10
Laminin α5β2γ1	521	11
Laminin α2β1γ3	213	12
Laminin α3β2γ3	323	13
Laminin α4β2γ3	423	14
Laminin α5β2γ3	523	15

1.6 Experimental models of autoimmune bullous disorders

To investigate the pathophysiological mechanisms of autoimmune bullous diseases and develop therapeutic strategies, *in vitro* studies, *ex vivo* and *in vivo* models have been established.

1.6.1 Experimental models of pemphigus

There are many studies that demonstrate the significant role of IgG autoantibodies against desmoglein 1 and desmoglein 3 in the pathogenesis of pemphigus. In pemphigus patients, disease activity correlates with antibody titers [73-75]. Studies using cultured keratinocytes have shown that serum and pathogenic antibodies of pemphigus patients targeting desmoglein 3 disrupt desmoglein 3 transinteraction and cause a loss of cell cohesion [76-80]. Moreover, transfer of autoantibodies from pemphigus patients into neonatal mice induces lesions similar to human disease[81]. Additionally, signaling pathways, involving p38 MAPK, which induces internalization and depletion of desmoglein 3 in pemphigus vulgaris, RhoA, plakoglobin etc, have been shown to play a role in disruption of skin cohesion and blister formation [82-90]. Consequently, autoantibodies bind to desmoglein 3, cause internalization, which leads to depletion of desmosomal desmoglein 3, loss of cohesion, and blistering. In contrast to bullous pemphigoid, complement is not required for blister formation [91, 92]. Also, an active mouse model was generated using desmoglein knock out mice. Desmoglein 3-depleted lymphocytes were transferred to immunodeficient mice that express desmoglein 3. Mice produced antibodies and developed clinical phenotype [93].

1.6.2 Experimental model of pemphigoid diseases

Bullous pemphigoid (BP)

When normal human keratinocytes are incubated with purified IgG from BP sera or IgG from rabbits immunized with a recombinant form of human BP180 a signal transduction event was triggered. That led to expression and secretion of the proinflammatory cytokines interleukine-6 and interleukine-8 in a dose and time-dependent manner [94]. These cytokines are known to be elevated in the blister fluids and sera of BP patients [95, 96]. In the same experimental design, Messingham *et al*. demonstrated that the interleukin-8 release can also be stimulated by BP IgE[97]. Moreover, FcR-independent effects of anti-BP180 antibodies were also observed by the internalization of BP180 upon stimulation of cultured human keratinocytes [94, 96, 98, 99]. This resulted in the impairment of hemidesmosome formation, decrease of adhesive strength, and blistering.

Several studies show the association of disease activity with circulating levels of IgG antibodies against the non-collagenous NC16a domain and other epitopes on the BP180 ectodomain[100-104]. The so-called cryosection model is an *ex vivo* model for the investigation of the pathogenicity of patient, rabbit, or mouse antibodies. As a first step, cryosections of normal human or mouse skin are incubated with autoantibodies followed by incubation of leukocytes from healthy volunteers. After 2-3 hours of incubation, dermis and epidermis are detached and split formation occurs[105]. This subepidermal splitting is mediated by reactive oxygen species and proteases such as neutrophil elastase, gelatinase G, and matrix metallopeptidase 9 [106, 107].

Animal models for autoimmune bullous diseases can be mainly classified in two categories, passive transfer models and "active" models[108]. In passive transfer models, autoantibodies isolated from rabbits immunized with murine BP180 were injected into neonatal mice. Consequently, histological, ultrastractural, and immunologic features similar to human disease are observed within hours. Using this passive transfer model several pathological mechanisms have been studied [109]. *In vivo* studies have shown the importance of the proteases for blister formation, as mentioned before, including neutrophil elastase, gelatinase, matrix metalloproteinase 9, plasmin, alpha 1 proteinase inhibitor and mast cell proteinase 4 [108, 110, 111].

Complement-deficient mice did not develop blisters after injection of pathogenic rabbit anti-murine-BP180 antibodies. This demonstrates the significance of complement, in blister formation [112, 113]. Mast-cell genetically deficient mice or mast cell-sufficient mice pretreated with an inhibitor of mast cell degranulation failed to develop bullous pemphigoid. Moreover wild-type mice injected intradermally with pathogenic anti-mBP180 IgG exhibited extensive mast cell degranulation in the skin, which lead to blister formation [114, 115]. Many inflammatory cells are observed in the biopsies of patients with bullous pemphigoid. These inflammatory cells are mainly neutrophils, eosinophils, lymphocytes, and macrophages [116, 117]. BALB/c mice treated with neutrophil-specific antibodies and consequently depletion of neutrophils were not susceptible to anti-mBP180 IgG. These findings demonstrate the significant role of neutrophils and other inflammatory cells in blister formation [118].

In the "active" models either lymphocytes are transferred or mice are immunized using recombinant fragments of the target antigen (Table 3). An active model has also been developed to investigate autoimmune responses. Splenocytes, from wild-mice that have been immunized by grafting of humanized-BP transgenic mouse skin, were tranferred into immunodeficient BP180-humanized mice. The recepient mice although developed disease, they did not have main characteristic findings observed in bullous pemphigoid patients such as eosinophil infiltration [119]. A

recent study using humanized mice demonstrated the significance of NC16a-reactive CD4 T cells [120]. In another study, human skin was also transplanted onto Severe Combined Immunodeficiency (SCID) mice. However after injection of BP-IgG into the transplant, no blister formation was observed [121]. Recently, studies using humanized model showed that transgenic grafts on wild-type mice developed neutrophil-rich infiltrates and subepidermal blisters [122].

In contrast to BP180, the pathogenic relevance of IgG against BP230, the second antigenic target of bullous pemphigoid, remains unclear. Two animal models attempted to investigate the pathogenicity of BP230 antibodies, but blisters were not observed [123-125].

Mucous Membrane Pemphigoid (MMP)

The pathogenic relevance of autoantibodies in mucous membrane pemphigoid has been demonstrated both *in vitro* and *in vivo*. Antibodies to α6β4 integrin induce separation along the dermal–epidermal junction in organ cultures [126, 127]. Additionally, studies in cultured conjunctival fibroblasts showed that several profibrotic factors such as heat shock protein 47, interleukine 4 and 5 play role in scarring [128, 129].

Anti-laminin 332 antibodies induced noninflammatory subepidermal blisters when injected into mice or human skin grafted onto immunocompromised mice [130, 131]. Similarly, subepidermal blisters were also observed in mice injected with antigen binding fragments against laminin 332. Interestingly, in contrast to bullous pemphigoid, mice lacking complement, mast cells or T cells demonstrate clinical manifestations suggesting that blistering can be induced by antibodies without inflammatory factors [130, 132].

Epidermolysis Bullosa Acquisita (EBA)

Pathogenicity in epidermolysis bullosa acquisita has been studied using various *ex vivo* and *in vivo* models. Studies using the *ex vivo* cryosection model have shown that binding of autoantibodies to type VII collagen, following release of cytokines and reactive oxygen species, activation of Fcγ receptors and metalloproteinases lead to split formation [133].

In vivo animal models have also been developed to study the pathogenesis of epidermolysis bullosa acquisita. Antibodies against type VII collagen are injected into mice and clinical disease is developed. Also, immunization of mice with a recombinant fragment of murine collagen VII induces blisters and clinical phenotype in mice resembling human disease. It was further demonstrated that the binding of autoantibodies to antigen causes recruitment and activation of neutrophils via

FcγRIV, a very important step for blister formation [10]. Noteworthy, glycosylation of autoantibodies against collagen type VII modulates the expression of activating and inhibitory FcγRs [9].

However, so far, no experimental evidence for the pathogenic role of autoantibodies in anti-p200 pemphigoid has been provided. Table 3 presents the experimental animal models that have been developed to study pemphigoid skin diseases.

Table 3. Available animal models for pemphigoid diseases.

	Animal models	**Pemphigoid skin disease**
Passive transfer model	autoantibodies in the rabbit cornea	Bullous pemphigoid[134]
	autoantibodies into neonatal mice	Bullous pemphigoid[109, 112, 115, 118, 123, 124, 135]
		Mucous membrane pemphigoid[130]
	autoantibodies into adult mice	Epidermolysis bullosa acquisita[106, 136-140]
		Mucous membrane pemphigoid[131]
	autoantibodies in humanized mice	Bullous pemphigoid[141, 142]
		Mucous membrane pemphigoid[131]
Active model	Transfer of autoreactive lymphocytes	Bullous pemphigoid[119]
	Transgenic mice	Bullous pemphigoid[122]
	Forced immunization models	Bullous pemphigoid[125, 143]
		Epidermolysis bullosa acquisita[9, 144]

2 Aim of the study

This study focused on anti-p200 pemphigoid and consists of two experimental parts. In the first part, a simple diagnostic method to facilitate the diagnosis of anti-p200 pemphigoid was developed. In the second part, the pathogenicity of antibodies in anti-p200 pemphigoid was explored by the use of several *ex vivo* and *in vivo* models.

Establishment of a novel diagnostic method for anti-p200 pemphigoid

Clinically, anti-p200 pemphigoid can not be distinguished from bullous pemphigoid. However, anti-p200 pemphigoid responds better to treatment. Therefore, a simple diagnostic test that can be easily used would help to differentiate anti-p200 pemphigoid from bullous pemphigoid and other autoimmune subepidermal blistering diseases. So far, the detection of antibodies against the p200 protein in the extract of human dermis by immunoblotting was required for the diagnosis of anti-p200 pemphigoid. Preparation of dermal extract requires specialized clinical laboratories and cannot be easily standardized. ELISA systems are simple in application and also, more easily certified in quality management systems. As the C-terminal part of laminin γ1 has been previously described as a target antigen in anti-p200 pemphigoid [61], the first aim of this thesis was to develop a detection system, i.e. an ELISA, based on the recombinant C-terminal of laminin γ1 that would facilitate the diagnosis of the disease and would allow to monitor serum autoantibodies during the course of the disease. To this line, several tasks had to be addressed:

1. Generation of high amounts of recombinant C-terminus of laminin γ1 using a suitable expression system.

2. Examining the optimal coating and incubation conditions to apply the C-terminus of laminin γ1 by ELISA

3. To determine the sensitivity, specificity and reproducibility of the novel ELISA.

A large collection of well-characterized sera from patients with anti-p200 pemphigoid had already been established by the routine autoimmune laboratory of the Department of Dermatology, Lübeck University in cooperation with Prof Takashi Hashimoto, Department of Dermatology, Kurume University, Fukuoka, Japan.

Pathogenicity of anti-p200 pemphigoid

So far, nothing was known about the pathogenic relevance of antibodies in anti-p200 pemphigoid. The second and major aim of this thesis was to investigate the pathogenic relevance of antibodies against laminin γ1.

Therefore the following 6 research questions were set up.

1. Are serum antibodies in anti-p200 pemphigoid patients pathogenic?

Previously, an *ex vivo* model has been developed, in which incubation of IgG from patients with bullous pemphigoid and epidermolysis bullosa acquisita induced leukocyte-dependent dermal epidermal separation in cryosections of human skin [105, 133]. Therefore, sera from anti-p200 pemphigoid were employed in this *ex vivo* model.

2. Is the pathogenicity of patients' autoantibodies restricted to the C-terminus of laminin γ1?

To answer this question, preadsorption studies will reveal two fractions, one containing specific antibodies against the C-terminus of laminin γ1 (hLAMC1-cterm) and the other fraction will be depleted of the reactivity of these antibodies. The pathogenic potential of the anti-LAMC1-cterm antibodies will be subsequently tested by use of the *ex vivo* cryosection model.

3. Is the C-terminus of laminin γ1 the immunodominant epitope in anti-p200 pemphigoid?

To address this, epitope mapping studies of laminin γ1 are required. Immunoblotting of anti-p200 pemphigoid sera to the different fragments of laminin γ1 would demonstrate the presence of other potential epitopes outside C-terminus.

4. Are rabbit autoantibodies against the C-terminal fragment of murine laminin γ1 pathogenic using the *ex vivo* model?

Rabbit IgG generated against the murine C-terminal fragment of laminin γ1 will be tested using the *ex vivo* cryosection model.

5. Are *rabbit* autoantibodies against the C-terminal fragment of murine laminin γ1 pathogenic *in vivo*?

Previous studies have shown that the injection of anti-murine type VII collagen antibodies into mice led to blistering phenotypes in adult mice closely mimicking epidermolysis bullosa acquisita and bullous pemphigoid, respectively [109, 136]. The rabbit antibodies against the C-terminal fragment of murine laminin γ1 should be further passively transferred into mice. Consequently, clinical, histological and immunological evaluations will be assessed in all mice.

6. Are *murine* autoantibodies against the C-terminal fragment, raised by immunization of mice with the murine recombinant laminin γ1 pathogenic *in vivo*?

Previous studies have shown that immunization of susceptible mouse strains with recombinant murine type VII and type XVII collagen led to blistering phenotypes in adult mice reproducing the human epidermolysis bullosa acquisita and bullous pemphigoid disease, respectively [143, 144]. Based on that, mice will be immunized with the recombinant C-terminal fragment of murine laminin γ1. Consequently, clinical, histological and immunological evaluations will be assessed in all mice.

3 MATERIALS AND METHODS

3.1 Materials

The lists of materials used in this study including laboratory equipment, consumables, chemicals, antibodies, bacteria, plasmids, vectors, cell lines, kits, enzymes, and buffers are shown in the paragraphs 3.1.1 - 3.1.10.

3.1.1 Laboratory equipment

Air incubator containing 5% CO_2	Memmert, Schwabach, Germany
Animal facility	University of Lübeck
Automatic microplate washer	Columbus Pro, Tecan, Crailsheim, Germany
Biophotometer	Eppendorf AG, Hamburg, Germany
Centrifuge BIOFUGE fresco	Heraeus Instruments GmbH, Hanau, Germany
Centrifuge Varifuge 3.0 R	Heraeus Instruments GmbH, Hanau, Germany
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 sterilizer	Binder GmbH, Tuttlingen, Germany
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 pippet	EPPENDORF AG, Hamburg, Germany
Freezer (-20°C)	Bulle, Switzerland
Microscopy Olympus BX40	Olympus Deutschland GmbH, Hamburg, Germany
PCR Cycler, Thermal cycler GeneAmo PCR System 9700	Applied Biosystems Darmstadt, Germany
pH-meter ph526	MultiCal WTW, Weilheim, Germany
Refrigerator (4°C)	Liebherr International AG
Sonicator	Bandelin, Berlin, Germany
UV Transilluminator	Life Technologies, Darmstadt, Germany
Voltex	Scientific Industries, Bohemia, NY, USA

3.1.2 Consumable materials

Amicon Ultra-15 filters	Millipore, Bradford, MA, USA

Cover glasses	Gerhard Menzel, Glasbearbeitungswerk GmbH&Co. KG, Braunschweig, Germany
Cover slide glasses	Gerhard Menzel, Glasbearbeitungswerk GmbH&Co. KG, Braunschweig, Germany
Culture slide chambers	BD, Ontario, Canada
Dark chamber	Werner Hassa GMbH, Heidelberg, Germany
Dialysis tubing visking, cellulose	Roth, Karlsruhe, Germany
Disposable cuvets	BRAND GmbH, Wertheim, Germany
Disposable needle	Becton Dickinson GmbH, Heidelberg, Germany
Disposable syringe	Becton Dickinson GmbH, Heidelberg, Germany
Embedding compound Tissue-Tek® O.C.T. CompoundTM	Sakura Finetek Europe B.V. Alphen aan den Rijn, Netherlands
Embedding mold Tissue-Tek® CryomoldTM	Sakura Finetek Europe B.V. Alphen aan den Rijn, Netherlands
Gel electrophoresis apparatus	Bio-Rad, Munich, Germany
Gravity-flow column	Qiagen, Hilden, Germany
MaxiSorb™ plates	Nunc, Roskilde, Denmark
Microlon™200 plates	Greiner, Frickenhausen, Germany
Nitrocellulose membrane	Whatman, GmbH, Dassel, Germany
Slide glasses	Gerhard Menzel, Glasbearbeitungswerk GmbH&Co. KG, Braunchweig, Germany
Steril filter	Sarstedt AG&Co., Nuembrecht, Germany
Tubes 1.5 ml/2.0 ml	Sarstedt AG&Co., Nuembrecht, Germany

3.1.3 Chemical reagents

Acetic acid	Merck, Darmstadt, Germany
Affi-Gel 15	Bio-Rad, Munich, Germany
Acrylamide	
Affi-Gel agarose beads	Millipore, Temecula, USA
APS	Sigma, Steinheim, Germany
Aquatex	Merck, Darmstadt, Germany
BSA	Roth, Karlsruhe, Germany
BSA biotin-free	Sigma-Aldrich, Munich, Germany
Carbenicillin	Life Technologies, Darmstadt, Germany
Dextran 500	Nycomed, Oslo, Norway
Diaminobenzidine	Merck, Darmstadt, Germany
DMEM	Invitrogen, Karlsruhe, Germany
DMSO	Sigma, Steinheim, Germany
DNA loading dye 6x	Thermo Scientific, Fermentas, Rockford, USA
dNTPs	Thermo Scientific, Fermentas, Rockford, USA
EDTA	Roth, Karlsruhe, Germany
Eosin	Merck, Darmstadt, Germany
Ethanol	Roth, Karlsrue, Germany
ethanolamine	Applichem, Darmstadt, Germany

Fast Digest Buffer 10x	Thermo Scientific, Fermentas, Rockford, USA
Formaldehyde	Roth, Karlsrue, Germany
Gel stain GelStar Nucleic Acid	Lonza, Rockland, USA
Glycine	Roth, Karlsrue, Germany
Glycerol	Roth, Karlsrue, Germany
Guanidin-HCl	Roth, Karlsrue, Germany
Hematoxyline	Merck KGaA, Darmstadt, Germany
HEPES	Roth, Karlsrue, Germany
H2O Millipore	Ampuwa, Bad Homburg, Germany
Imidazole	Alfa Aesar, Karlsruhe, Germany
Isopropyl-β-D- thiogalactopyranosid	Life Technologies, Darmstadt, Germany
Ketamine	Sigma-Aldrich, Hamburg, Germany
LB medium	PM Biomedicals, Illkirch, France
Nitro blue tetrazolium	Roth, Karlsruhe, Germany
Penicillin/Streptomycin	Life Technologies, Darmstadt, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Roth, Karlsruhe, Germany
phosphate acid	Merck, Darmstadt, Germany
Phusion HF buffer 5x	Thermo Scientific, Fermentas, Rockford, USA
primers	VBC-Biotech, Vienna, Austria
Protein G Sepharose Fast Flow affinity Column chromatography	GE Healthcare, Munich, Germany
Proteinase inhibitor cocktail	Calbiochem/Merck, Darmstadt, Germany
SDS	Roth, Karlsrue, Germany
Skimmed milk powder	Roth, Karlsruhe, Germany
Sodium chloride	Roth, Karlsrue, Germany
Sodium dihydrogen phosphate	Roth, Karlsrue, Germany
Talon affinity resin/matrix	Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France
TALON superflow	Clontech, Palo Alto, CA, USA
TiterMax®	Alexis, Lörrach, Germany
TMB-ELISA solution 1-Step Turbo	Fisher Scientific, Schwerte, Germany
Tris-base	SERVA, Heidelberg, Germany
Tris-HCl	Roth, Karlsruhe, Germany
Trypan blue	Sigma, Steinheim, Germany
Tween 20	Sigma, Saint Louis Missouri, USA
Trypsin	Roche, Cölbe, Germany
Urea	Roth, Karlsruhe, Germany
Xylazine	Sigma, Hamburg, Germany
β-mercaptoethanol	Sigma, Steinheim, Germany

3.1.4 Antibodies

Antibody	*Company*	*Dilution*
Monoclonal mouse anti-penta-His antibody	Qiagen, Hilden, Germany	50pg-2ng/ml

Cy™3-conjugated AffiniPure Goat Anti-mouse IgG	Jackson ImmunoResearch Laboratories, PA, USA	1:100
PE-mouse anti-human IgG4 clone HP6025	SouthernBiotech, Birmingham, Alabama	2 μg/ml
FITC-goat IgG fraction to mouse complement C3	Cappel Organon-Teknika, Durham, NC	1:50
FITC-monoclonal anti-human IgG4 clone HP6025	Sigma Aldrich, Munich, Germany	10 μg/ml
FITC-polyclonal rabbit anti-mouse IgG	DAKO, Hamburg, Germany	1:100
FITC-rat anti-mouse IgG1	BD Pharmingen, Heidelberg, Germany	1:50
FITC-rat anti-mouse IgG2a	BD Pharmingen, Heidelberg, Germany	1:50
FITC-rat anti-mouse IgG2b	BD Pharmingen, Heidelberg, Germany	1:50
FITC-rat anti-mouse IgG3	BD Pharmingen, Heidelberg, Germany	1:50
HRP mouse anti-human IgG4-antibody clone HP6023	Southern Biotech, Birmingham, Alabama, USA	0.1 μg/ml
HRP polyclonal goat anti-rabbit antibody	DAKO, Hamburg, Germany	1:2,000
HRP polyclonal rabbit anti-mouse IgG antibody	DAKO, Hamburg, Germany	1:1,000
Mouse monoclonal anti-LAMC1 clone B4 (sc-13144)	Santa Cruz Biotechnology, CA, USA	IB, 1-4μg/ml; IF, 20-100μg/ml
FITC- Polyclonal swine anti-rabbit IgG	DAKO, Hamburg, Germany	1:1000
Rabbit polyclonal anti-mLAMC1 clone H-190 (sc-5584)	Santa Cruz Biotechnology, CA, USA	1:200 1:10
Streptavidin-HRP antibody	Becton, Dickinson, Heidelberg, Germany	1:2,000

3.1.5 Bacteria

E.coli Nova blue Genotype:endA1 hsdR17 (r_{K12}^{-} m_{K12}^{+}) supE44 thi-1 recA1 gyrA96 relA1 lac F′[$proA^{+}B^{+}$ $lacI^{q}ZΔM15$::Tn10] (Tet^{R})	Novagen⁄Merck, Darmstadt, Germany
E.coli strain Rosetta DE3 Genotype: F^{-} ompT $hsdS_B$(r_B^{-} m_B^{-}) gal dcm (DE3) pRARE (Cam^{R})	Novagen⁄Merck, Darmstadt, Germany
Z-Competent™ *E.coli*	ZymoResearch, Irvine, USA

3.1.6 Plasmids and vectors

hLAMC1-cterm-pMA obtained vector	Mr. Gene, Regensburg, Germany
mLAMC1-cterm-pMA obtained vector	Mr. Gene, Regensburg, Germany
pEE14.4 expression vector	Lonza, Cologne, Germany
pJET cloning vector	Thermo Scientific, Rockford, USA
pQE-40 expression vector	Qiagen, Hilden, Germany
pTriEx-LAMC1-FL obtained vector	Euroimmun, Lübeck, Germany

3.1.7 Media for cells

Keratinocyte Growth medium 2	PromoCell, Heidelberg, Germany
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
RPMI 1640 Medium	Life Technologies, Karlsruhe, Germany
DMEM	Life Technologies, Karlsruhe, Germany

3.1.8 Kits

BCA Assay	Thermo Scientific, Rockford, USA
DNA Clean and ConcentratorTM-5	Zymo Research, Irvine, CA, USA
ECL plus	GE Healthcare, Amersham, UK
FuGENE Transfection	Roche, Mannheim, Germany
High pure PCR product purification kit	Roche, Mannheim, Germany
MicroLink Protein Coupling Kit	Thermo Scientific, Rockford, USA
PCR reaction	VBC-Biotech, Vienna, Austria
Phusion High-Fidelity PCR Kit	Thermo Scientific, Rockford, USA
peqGOLD plasmid mini-prep kit	peqlab, Erlangen, Germany
RNeasy kit	Qiagen, Hilden, Germany
Omniscript Reverse Transcription Kit	Qiagen, Hilden, Germany
pJET Kit	Thermo Scientific, Rockford, USA

3.1.9 Enzymes

DNA Polymerase	Thermo Scientific, Rockford, USA
T4 DNA Ligase	Invitrogen, Carlsband, USA
HindIII restriction enzyme	Thermo Scientific, Fermentas, Rockford, USA
BamHI restriction enzyme	Thermo Scientific, Fermentas, Rockford, USA
KpnI restriction enzyme	Thermo Scientific, Fermentas, Rockford, USA

3.1.10 Buffers

PBS	8 g/L sodium chloride, 0.2 g/L KCl, 1.44 g/L $Na_2HPO_4K*2H_2O$, 0.27g/L K_2HPO_4 in distilled water, pH at 7.2 with phosphoric acid
PBST	8 g/L sodium chloride, 0.2 g/L KCL, 1.44 g/L $Na_2HPO_4K*2H_2O$, 0.27g/L K_2HPO_4 in distilled water, 0.05 ml Tween 20 in 1 L PBS, pH at 7.2
Preparation of dermal extract	
Buffer A	PBS, pH 7.2, 1 M sodium chloride, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM EDTA
Buffer B	12.5 mM Tris-HCl pH 6.8, 4 M urea, 5 mM EDTA, 1 mM PMSF
extraction buffer	12.5 mM Tris-HCl, 9 M urea, 2% w/v SDS, 10% β-mercaptoethanol, 5 mM EDTA, and 1 mM PMSF
protein purification	
washing buffer	50 mM sodium phosphate, 300 mM sodium chloride, pH 7.0, proteinase inhibitor cocktail
elution buffer	50 mM sodium phosphate, 300 mM sodium chloride, 150 mM

	imidazole, pH 7.0
protein purification denature conditions	
washing buffer	50 mM sodium phosphate, 300 mM sodium chloride, 6 M guanidine-HCl, pH 7.0
elution buffer	50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole, 6 M guanidine-HCl, pH 7.0
Gel agarose electrophoresis	
Tris-Acetate-EDTA (TAE buffer)	242 g Tris Base, 57 ml acetic acid, 0.05 M EDTA, pH 8 in 1 L distilled water
2% gel agarose	50ml TAE, 1 g agarose, 5 µl gel stain
SDS-PAGE electrophoresis	
Separation gel 10%	Distilled water 4.08 ml, 2.5 ml 1.5 M Tris pH 8.8, 3.3 ml 30% Acrylamide, 100 µl 10% SDS, 12 µl TEMED, 35 µl 10% APS
Separation gel 6%	Distilled water 2.65 ml, 1.35 ml 1.5 M Tris pH 8.8, 2 ml 30% Acrylamide, 100% glycerol 2.5 ml, 1 ml 1 M glycine, 400 µl 10% SDS, 12 µl TEMED, 150 µl 10% APS
Collection gel 4%	Distilled water 3 ml, 1.25 ml 1.5 M Tris pH 6.8, 0.67 ml 30% Acrylamide, 50 µl 10% SDS, 12 µl TEMED, 70 µl 10% APS or Distilled water 2.1 ml, 0.7 ml 1.5 M Tris pH 6.8, 0.65 ml 30% Acrylamide, 100% glycerol 1.25 ml, 40 µl 0.5 M EDTA pH 7, 200 µl 10% SDS, 12 µl TEMED, 100 µl 10% APS
SDS buffer 5x	15 Tris pH, SDS 5 g, 72 g glycine in 1L distilled water
Tris glycine buffer 10x	1.92 M glycine, Tris Base 0.25 M in 1 L distilled water
Transfer buffer	Tris-glycine buffer x10 100 ml, methanol 200 ml in total 1 L distilled water
coomassie staining	50 g Aluminiumsulfate hydroxide, 100 ml ethanol, 0.2 g coomassie brilliant blue, 20 ml phoshoric acid in 1 L distilled water
Immunoblotting	
BST	Tris-buffered saline Tween-20, 10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20, pH 7.2
blocking buffer	1% TBST, 5% skimmed milk powder
incubation buffer	1% TBST, 5% skimmed milk powder plus 1% bovine serum albumin
IgG purification	
glycine buffer	0,1 glycine pH 2.8
Tris buffer	1 M Tris-Base in distilled water
ELISA	
Blocking buffer	PBST with 5% skimmed milk
Incubation buffer	PBST with 5% skimmed milk and 1 % PBS

3.2 Human sera

Serum samples from patients with anti-p200 pemphigoid (n=35) were obtained from the autoimmune laboratory of Department of Dermatology, University of Lübeck. Some sera were kindly provided by Prof Takashi Hashimoto, Department of Dermatology, Kurume University, Fukuoka, Japan. All patients had active disease and were characterized by (i) blisters on the skin, (ii) linear deposits of IgG and/or C3 at the DEJ by direct IF microscopy, (iii) binding of IgG at the dermal side of 1 M NaCl-split normal human skin by indirect IF microscopy, and (iv) serum IgG antibodies against a 200-kDa protein by immunoblotting with extract of human dermis. Twenty one of these sera also contained IgG reactivity against hLAMC1-cterm by immunoblotting. Seven of these sera were also used for studies on the pathogenicity of anti-p200 pemphigoid. In addition, sera from patients with typical clinical and immunopathological features of bullous pemphigoid (n=101), anti-laminin 332 mucous membrane pemphigoid (n=14), epidermolysis bullosa acquisita (n=10) and pemphigus vulgaris (n=51) were used. Sera from healthy volunteers (n=131) were included as negative controls. All sera were stored at -20°C until used. The study was performed following the Declaration of Helsinki and was approved by the ethics committee of the University of Lübeck (11-143).

3.3 Rabbits

Rabbits SA6539 and SA6794 were generated against the recombinant C terminal fragment of murine laminin γ1 (mLAMC1-cterm). The rabbits were immunized and boosted twice subcutaneously with 250 µg mLAMC1-cterm suspended in Freund´s complete adjuvant (Eurogentec, Cologne, Germany). Immune sera were collected at regular intervals and were analyzed by immunoblotting using mLAMC1-cterm and dermal extracts, and IF microscopy on cryosections of murine skin. Rabbit IgG against murine BP180 NC15, and preimmune rabbit serum were used as controls. All analyses of rabbit sera were performed by me.

3.4 Mice

Six-to eight-week-old C57BL/6, BALB/c, and SJL female mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and neonatal C57BL/6 mice were bred and kept in cages in the University of Lübeck under standard laboratory conditions.

All injections and bleedings were performed on mice anesthetized by intraperitoneal administration of a mixture of ketamine (100 µg/g) and xylazine (15 µg/g). Experiments were approved by the Animal Rights Commission of the Ministry of Agriculture and Environment, Schleswig-Holstein (V312-72241.122-5(79-6/09) and V312-72241.122-5(80-6/09)). All mouse experiments were performed by me.

3.5 Cell cultures

Human embryonic kidney cell line (HEK293T cells) were cultured in Dulbecco´s modified Eagle medium (DMEM, Life Technologies, Karlsruhe, Germany) high glucose medium supplemented with fetal calf serum (FCS, Biochrom, Berlin, Germany) 10% and Penicilin/Streptomycin to full confluence and passaged every 2 days using Trypsine (Roche, Cölbe, Germany).

The human keratinocyte cell line (HaCaT cells, Human Adult Low Calcium High Temperature Keratinocytes) was cultured in Keratinocyte Growth Medium 2 (PromoCell, Heidelberg, Germany) to full confluence and passaged every 3 days using trypsine. HaCaT cells were seeded into 4-well culture slide (BD, Ontario, Canada) in the Keratinocyte Growth Medium 2 and were maintained in a humidified air incubator containing 5% CO_2 (Memmert, Schwabach, Germany). Cultivation and cell seeding were performed by me and a technical assistant with the supervision of Dr. Stephanie Groth.

3.6 Preparation of dermal extracts

Human and murine dermal extract for IB analysis was prepared according to the protocol established for human dermis with minor modifications[54]. Human skin or freshly shaved mouse skin was cut in small pieces (4cm^2) and kept in buffer A (PBS, pH 7.2, 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM EDTA) at 4°C for 2 (for mouse skin) or 4 days (for human skin). After scraping off the epidermis, the skin pieces were placed in the chamber, which was consisted of 2 plates. The skin pieces were placed in such way so that they were exposed under the holes of the covered plate. This epidermal-exposed surface of the dermis was incubated with buffer B (12.5 mM Tris-HCl pH 6.8, 4 M urea, 5 mM EDTA, 1 mM PMSF) at room temperature. After 10 minutes, buffer B was discarded and the epidermal surface was incubated with extraction buffer (12.5 mM Tris-HCl, 9 M urea, 2% SDS, 10% β-mercaptoethanol, 5 mM EDTA, and 1 mM PMSF) for 1 hour at room temperature for the extraction of the proteins of the surface of the dermis. After 1

hour, the extraction buffer, which contained the proteins of the dermis, was collected and stored at -80°C. Preparation of dermal extract was done by me.

3.7 Molecular biology techniques

In this study, several recombinant proteins have been produced. In paragraph 2.7.1 the different recombinant proteins used in this study are introduced, while in paragraph 2.7.2-2.7.12 the different molecular biology techniques, applied in this study are described.

3.7.1 Recombinant fragments

hLAMC1-ctrerm (prokaryotic, eukaryotic)

The C-terminal fragment of human laminin γ1 (hLAMC1-cterm; mRNA: 4349–5086 bp, 738 bp, accession number NM_002293) was provided in the obtained vector hLAMC1-cterm-pMA by Mr Gene (Mr. Gene, Regensburg, Germany). It was designed, synthesized, and optimized for expression as His-tagged (6xHistidines in N-termini) fusion protein in *E. coli* (Novagen/Merck, Darmstadt, Germany). hLAMC1-cterm was also expressed in the human cell line HEK293T. The production of hLAMC1-cterm was performed by Dr. Stephanie Groth in collaboration with me.

mLAMC1-cterm

The C-terminal fragment of murine laminin γ1 (mLAMC1-cterm; mRNA: 4330-5069 bp, 740 bp, accession number NM- 010683) was provided in the obtained vector mLAMC1-cterm-pMA by Mr Gene. It was designed, synthesized, and optimized for expression as His-tagged fusion protein in *E. coli*. The production of mLAMC1-cterm was performed by me.

E8 fragment

The recombinant E8 fragment of laminin 111, a heterotrimer of the truncated C-terminal portions of α1, β1, and γ1 chains was kindly provided by Dr. Sekiguchi (Institute for Protein Research, Osaka University, Japan)[61].

Laminin γ1

The full length protein of laminin γ1 (hLAMC1-FL) in the plasmid pTriEx-1 was kindly provided by the Euroimmun AG (Lübeck, Germany).

Five fragments covering the laminin γ1

The 5 overlapping fragments of hLAMC1, comprising the portion not covered by hLAMC1-cterm, were synthesized using RNA isolated from human skin with molecular biology techniques. DNA sequence data for human LAMC1 was retrieved from GenBank (accession number: NM-002293). Furthermore, the fragments were expressed as His-tagged fusion proteins in *E. coli*. The design of the different fragments covering laminin γ1 was done by Dr. Andreas Recke. The generation of the 5 recombinant fragments covering the laminin γ1 was performed by me.

3.7.2 Isolation of RNA from human tissue.

The isolation of RNA from human tissue was performed with the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's description. The isolation of RNA was performed by me in collaboration with a technical assistant.

3.7.3 cDNA synthesis

For cDNA synthesis, the Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany) was used. A master mix was prepared according to the Omniscript Reverse Transcription handbook, using approximately 0.1 ng-1 μg of RNA. The master mix contained the following components:

component	volume	
5x Buffer RT	12 μl	MasterMix
dNTP Mix (5 mL each dNTP)	2 μl	
Oligo-dT primer (10 mM)	2 μl	
RNAase inhibitor (10 units/ml)	1 μl	
Reverse transcriptase	1 μl	
template RNA	0.1 ng-1 μg	
RNAse -free water	up to 20 μl	

First RNA was incubated in RNAase-free water for 5 minutes at 65°C, placed on ice and then was mixed with the master mix. All the components were mixed and incubated for 60 minutes at 37°C. The cDNA synthesis was performed by me in collaboration with Dr. Stephanie Groth.

3.7.4 Polymerase chain reaction (PCR)

The cDNA fragments of LAMC1 were obtained by PCR with the Phusion High-Fidelity PCR Kit (Thermo Scientific, Rockford, USA). A master Mix was prepared as indicated:

component	volume	
5X Phusion HF Buffer	4 µl	Master Mix
dNTPs	0.4 µl	
Forward primer	0.5 µM	
Reverse primer	0.5 µM	
Phusion DNA polymerase	0.2 µl	
Nuclease-free water	up to 20 µl	
DMSO	0.6 µl	
Template DNA	~1 µl (<250 ng)	

The reactions were run in a thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, Darmstadt, Germany) following 2-step protocol with a repetition (denaturation-extension) of 35 cycles:

Cycle step	Temperature	Time	
Initial denaturation	98°C	30 seconds	
Denaturation	98°C	10 seconds	35 cycles
Annealing	-	-	
Extension	72°C	30 seconds	
Final extension	72°C	7 minutes	

The different primer pairs used to amplify the different fragments of laminin γ1 are mentioned in table 4. The PCR was performed by me in collaboration with Dr. Stephanie Groth.

3.7.5 Enzymatic digestion of DNA and preparation of expression plasmids

The prokaryotic and eukaryotic hLAMC1-cterm was subcloned from the obtained vector hLAMC1-cterm-pMA into the expression vector pQE-40 and pEE14.4 respectively (Qiagen, Hilden, Germany) using a BamHI /HindIII cutting site to produce a His-tagged protein[145].

The prokaryotic murine LAMC1-cterm was subcloned from the obtained vector mLAMC1-cterm-pMA into the expression vector pQE-40 (Qiagen) using a BamHI /HindIII cutting site to produce a His-tagged protein.

For amplification of the fragments (1-5) covering the laminin γ1 outside the C-terminal fragment, cloning procedures were performed. The amplification products were subcloned into pJET cloning vector (Thermo Scientific, Rockford, USA) or pQE40 expression vector, using a *BamHI/KpnI* cutting site to produce His-tagged proteins. Restriction sites for *BamHI* and *KpnI* were introduced during PCR using primer pairs (Table 4). All restriction enzymes and buffers were purchased from Fermentas, ThermoScientific, Rockford, USA. A typical reaction for DNA with the restriction enzymes was according to the manufacturer's instructions:

component	volume
10x FastDigest buffer	2 µl
FastDigest enzyme 1	1 µl
FastDigest enzyme 2	1 µl
Nuclease-free water	Up to 20 µl
Template DNA	Up to 1 µg (~2 µl)

The components were mixed gently and incubated for 5 minutes at 37°C (water bath).

Table 4. Primer sequence for PCR amplification of cDNA fragments of human laminin γ1 chain

Fragment	Size (bp)	Primer sequence (5'-3')
1	740	F: AT**G-GATCC**CGGCCGCAGCGCTGCA
		R: GC**GGTAC-C**TCATCTGCCACCTACAGCAAAATCAGAGA
2	670	F: AT**G-GATCC**ACTGTAGGTGGCAGATGTAAATGTAATG
		R: AT**GGTAC-C**TCAGCAGAAGCAGGGTGTGCAACC
3	706	F: AT**G-GATCC**TGCTTCTGCTTTGGGCATTCT
		R: AT**GGTAC-C**TCAAAGCACACATGGACTGTATGGTC
4	958	F: AT**G-GATCC**AGTGTGCTTTGCGCCTGCAATG
		R: AT**GGTAC-C**TCAGGCTGGACATTCCTGGCAGCCAG
5	1021	F:AT**G-GATCC**GAATGTCCAGCTTGTTACCGG
		R: AT**GGTAC-C**TCAAGCTTCTTGTAAGGTATCCCG

F, forward primer; R, reverse primer; bold letters, restriction site sequence.

The samples were further applied onto an agarose gel for analysis. After agarose gel electrophoresis, the bands of interest were excised, purified, cleaned, concentrated and prepared for ligation and transformation. The enzymatic digestion and preparation of the plasmids were performed by me in collaboration with Dr. Stephanie Groth.

3.7.6 Agarose gel electrophoresis

After PCR reaction, each sample was applied for gel electrophoresis onto a 2% agarose gel in Tris-acetate-EDTA (TAE: 242 g Tris Base, 57 ml acetic acid, 0.05 M EDTA, pH 8 in 1 L distilled water) buffer with a GelStar Nucleic Acid Gel stain 0.01% (LONZA, Rockland, USA). After adding the Gel stain onto the chamber (Bio-Rad Laboratories, Munich, Germany), the gel was left to polymerize for 25 minutes. The DNA samples were mixed with a 6xDNA loading dye (Thermo Scientific, Fermentas, Rockford, USA) and the samples were loaded onto the gel. Gels were run at 80 Volt for 50-60 minutes depending on the size of each fragment. The electrophoresis was performed by me.

3.7.7 Gel extraction

The bands of interest were cut under UV light (UV Transilluminator, Life Technologies, Darmstadt, Germany). Then, purification, cleansing and concentration of the PCR products were performed using High pure PCR product purification Kit (Roche, Mannheim, Germany) and DNA clean and concentrator kits Zymo research (Zymo research, Irnvine, CA, US) according to manufacturer's instructions. The gel extraction was performed by me.

3.7.8 Ligation

Ligation between vector and each insert was performed using T4 DNA Ligase (Invitrogen, Carlsband, USA). For cloning purposes, the pJet cloning vector was used, and for expression purposes, pQE40 was used. The ligation for pJET was performed following manufacturer’s description (ThermoScientific, Rockford, USA), while for pQE40 the ligation reaction was performed as follows:

component	volume
vector DNA	y µl (50ng)
insert	x µl
T4 DNA ligase	2 µl
T4 DNA ligase buffer 5x	4 µl
ddH2O	up to 20 µl

The amount x of the insert for ligation mix was calculated with the following formula:

amount of vector (50 ng)/bases of vector (for pQE40: 3420bp)*bases of insert*3-5

The samples were mixed briefly, centrifuged for 3-5 seconds, and incubated overnight at room temperature. The ligation was performed by me.

3.7.9 Transformation

After ligation, the reaction mix was used for transformation to *E. coli* strain Nova blue for cloning and *Rosetta DE3* for expression purposes. LB-ampicilin plates are pre-warmed at 37 °C. For transformation, 50 µl of Z- competent™ bacteria (ZymoResearch, Irvine, USA) were picked from -80 °C and left on ice to thaw. The ligation mixture, 3-5 µl, was transferred in a new clean tube and mixed with the bacteria. The mix was plated immediately on the LB-ampicilin agar plate and kept in the incubator overnight at 37°C.

For validation of the correct ligation and in-frame insertion of the DNA fragments, plasmids were sent for DNA sequence analysis. For plasmid purification, peqGOLD plasmid miniprep kit (peqlab, Erlangen, Germany) was used following the manufacture's description. The transformation was performed by me.

3.7.10 Protein expression of prokaryotic proteins

All prokaryotic recombinant fusion proteins were expressed in *E. coli* strain *Rosetta DE3* (Novagen ⁄Merck, Darmstadt, Germany)[145]. For this approach, a fresh bacterial colony was used to inoculate 10 ml LB medium supplemented with 50 µg/ml carbenicillin (Life Technologies, Darmstadt, Germany). The culture was incubated at 37°C over night in a bacterial shaker. The primary culture was used to inoculate 1 l LB medium with carbenicillin and incubated with shaking for 2-3 hours in 37°C, until the bacterial suspension reached an absorbance OD_{600nm} (optical density) between 0.6

and 0.8. Protein expression was induced with 1 mM *isopropyl*-β-D- thiogalactopyranosid (Life Technologies, Darmstadt, Germany) for 3 hours with shaking at 37°C. Cells were then centrifuged for 20 minute at 6000xg at 4°C. The supernatant was discarded and the cell pellets were stored at -20°C until used. From each step 1 ml sample was collected, centrifuged at 13000xrpm for 15 minutes and kept for electrophoretic analysis. The protein expression of prokaryotic proteins was performed by me.

3.7.11 Protein purification of prokaryotic proteins

For protein purification of the prokaryotically expressed proteins, the pellets were resuspended in 16 ml washing buffer (50 mM sodium phosphate, 300 mM sodium chloride, pH 7.0, proteinase inhibitor cocktail (Calbiochem/Merck)).

After sonication (3 times, 20 seconds, 7 cycles; Bandelin, Berlin, Germany) and centrifugation (34000 g, 30 minutes, 4°C) the supernatant was carefully transferred to a gravity-flow column (Qiagen, Hilden, Germany) and each His-tagged protein was purified by immobilized metal affinity chromatography using Talon affinity resin/matrix (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) following the manufacturer's instructions. In detail, the resin TALON resin (6 ml), before use, was washed with washing buffer following centrifugation (1200xrpm, 2 minutes, 19°C). The sample containing the expressed protein was mixed with the resin and incubated for 20 minutes at room temperature to allow the polyhistidine-tagged protein to bind the resin. Then, the mix was centrifuged again and the supernatant was discarded. The resin was washed 3-4 times with the washing buffer. The polyhistidine tagged protein was eluted by adding elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole, pH 7.0). The elutes were collected in 2 ml tubes (in total 16 ml). From each step a sample was kept for electrophoretic analysis and determination of the fragments that contained the polyhistidine-tagged protein. The elutes containing the protein were pooled together and the elution buffer was replaced with PBS by dialysis with a cellulose (Roth, Carlsruhe, Germany). The protein concentration was determined by Bradford assay (BioRad, Munich, Germany) and BCA assay (Thermo Scientific, Rockford, USA) following the manufacturer’s instractions.

Some fragments were expressed in inclusion bodies (insoluble). For this reason denaturing conditions (washing buffer: 50 mM sodium phosphate, 300 mM sodium chloride, 6 M guanidine-HCl, pH 7.0) were used for purification following the manufacturer’s instructions. The elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole, M guanidine-HCl, pH 7.0)

was replaced with 8 M urea, pH 7.5, by dialysis and the protein concentration determined by BCA assay. For analysis of expression and purification of the recombinant proteins, SDS-PAGE followed by Coomassie staining (50 g Aluminiumsulfate hydrate, 100 ml ethanol, 0.2 g coomassie brilliant blue, 20 ml phoshoric acid in 1 l distilled water) and IB was used. The purification of the recombinant proteins were performed by me.

3.7.12 Transfection, expression and purification of eukaryotic proteins

The eukaryotic hLAMC1-cterm was expressed in the human cell line HEK293T. The sequence included a signal peptide from Ig kappa, for secretion into the culture medium. For transfection, HEK293T cells were seeded and grown until 70-80% confluence, and transfected with FuGENE transfection reagent, a proprietary mixture of lipids and other components, (63 µl, Roche, Mannheim, Germany) and eukaryotic expression plasmid pEE14.4 (18 mg, Lonza, Cologne, Germany) following the manufacturer's instructions (Roche, Mannheim, Germany). After 48 hours, the supernatant was collected and purified by immobilized metal affinity chromatography on TALON superflow (Clontech). Transfection of HEK293T cells of hLAMC1-cterm-FL and further purification of the protein were performed similarly. For analysis of expression and purification of the recombinant proteins, SDS-PAGE followed by Coomassie staining and IB was used. The transfection of the eukaryotically expressed hLAMC1-cterm and hLAMC1-FL into HEK293T cells were performed by Dr. Stephanie Groth. The purification of the proteins was performed by me.

3.8 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated according to their electrophoretic mobility by SDS-PAGE electrophoresis. This system was consisted of 2 gels. All the recombinant proteins were run in 10% separating gel and 4% stacking gel. The dermal extracts were fractionated by 6% and 4% separating and stacking gel relatively. The lower gel was applied onto the chambers and was let for 10-20 minutes. After polymerization the stacking gel was applied together with the comb. The recombinant proteins were run at 50 mA for 1 to 1.5 hours. The dermal extracts were run at 20 mA for 15 minutes and 50 mA for 1 to 1.5 hours. The visualization of the proteins was done by staining with Coomassie Brilliant Blue. All SDS-PAGE analyses were performed by me.

3.9 Immunoblotting

For immunoblotting, recombinant proteins and dermal extract were transferred to nitrocellulose membrane (Whatman, GmbH, Dassel, Germany) in transfer buffer at 120 Volt on ice for 1 hour.

First, membranes or stripes of membrane of 1 millimeter were blocked with 5% milk powder in 1% TBST (tris-buffered saline Tween-20, 10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20) with incubation at room temperature for 1 hour. Then membrane or stripes were incubated with the first antibody or sera overnight at 4°C. In detail: human (1:50), mouse (1:50), and rabbit sera (1:1,000), polyclonal rabbit anti-hLAMC1 crossreacting with mLAMC1 (clone H-190, 1:2000), and mouse monoclonal anti-hLAMC1 (clone B4; 1μg/ml, Santa Cruz Biotechnology, CA, USA) were diluted in TBST containing 5% skimmed milk powder plus 1% bovine serum albumin (BSA, Roth, Carlsruhe, Germany), and biotin-conjugated anti-penta-His antibody (Qiagen, Hilden, Germany; 1:2,000) was diluted in 5% biotin-free BSA (Sigma-Aldrich, Munich, Germany) in TBST. After overnight incubation in 4°C the membrane or strips were washed 3 times for 10 minutes with TBST. As secondary antibody, horseradish peroxidase (HRP)-conjugated monoclonal mouse anti-human IgG4 antibody (1:2,000, 0.1 μg/ml Southern Biotech, Birmingham, Alabama, USA), polyclonal rabbit anti-mouse IgG antibody (1:1,000, DAKO, Hamburg, Germany), polyclonal goat anti-rabbit antibody (1:2,000, DAKO) and streptavidin-horseradish peroxidase (1:2,000, Becton, Dickinson, Heidelberg, Germany) were used, respectively. The secondary antibodies were diluted in TBST containing 5% skimmed milk powder plus 1% bovine serum albumin and membranes and stripes were incubated for 1 hour in room temperature. After washing 3 times for 10 minutes with TBST the proteins were visualized by diaminobenzidine (Merck, Darmstadt, Germany) or by ECL plus kit (GE Healthcare, Amersham, UK). Immunoblot studies were performed by me in collaboration with Dr. Stephanie Groth.

3.10 Purification of antibodies

3.10.1 Coupling

Coupling of human and murine LAMC1-cterm

The recombinant proteins, hLAMC1-cterm and mLAMC1-cterm, were covalently coupled to Affi-Gel 15 (Bio-Rad, Munich, Germany). For coupling of the recombinant proteins onto the agarose beads, the storage buffer was exchanged against HEPES buffer (100 mM HEPES, pH 7.5) by dialysis. The

protein concentration was in the range of 0.5-1 mg/ml in a total volume of 7 ml. Protein was incubated with the Affi-Gel 15 overnight at 4°C with gentle end-over-end mixing into a column. After coupling, the gel was washed with HEPES buffer at least with 20 times the volume of the gel until the OD_{280} was less than 0.01. The flow through was kept for electrophoretic analysis and evaluating the coupling efficiency. Then the gel was incubated with 0.5 ml 1 M ethanolamine and 7 ml Millipore H_2O for 1 hour at 4°C with a gentle end-over-end mixing. The column was washed with PBS at least 20 times the volume of the gel and stored in PBS with 0.1 % sodium acid. The coupling of the human and murine LAMC1-cterm was performed by me.

Coupling of E8 fragment, LAMC1-FL, and eukaryotic hLAMC1-cterm

For the purification of antibodies against eukaryotic expressed hLAMC1-cterm, E8 fragment and LAMC1-FL, proteins were coupled to AminoLink Plus Coupling Resin (MicroLink™ Protein Coupling Kit, Thermo Fisher Scientific p/a Pierce Biotechnology, Rockford, USA), following the manufacturer´s instructions. In detail, the resin was mixed with the purified protein (0.5-1 mg/ml) dissolved in coupling buffer (0.1 M sodium phosphate, 0.15 M NaCl pH 7.2) and sodium cyanoborohydride (5 M, 2 µl) overnight in 4°C with gentle end-over-end mixing. After centrifugation (1000xg, 1 minute) the flow through was kept for evaluation of the coupling efficiency and the resin was washed twice with the coupling buffer, and three times with the Quenching buffer (1 M Tris-HCl, 0.05% sodium acide, pH 7.4). Then, resin was incubated with Quenching buffer and sodium cyanoborohydride (5 M, 2 µl) at room temperature for 30 minutes mixed every 15 minutes. After several centrifugations and wash steps with coupling buffer and NaCl (0.5 M), the column was ready to be used for affinity purification of specific IgG from anti-p200 pemphigoid patients' sera. The coupling of E8 fragment, LAMC1-FL, and eukaryotic hLAMC1-cterm was performed by me.

3.10.2 Purification of antibodies

Affinity purification of specific IgG against h- and m- LAMC1-cterm

Human LAMC1-cterm and murine LAMC1-cterm-loaded matrix were incubated with patients' sera and purified rabbit IgG for 1 hour at 4°C respectively. After extensive washes with PBS (OD< 0.005) antibodies were eluted with 0.1 M glycine buffer (pH 2.8), neutralized with Tris-HCL (pH 9), and concentrated under extensive washing with PBS (pH 7.2) using Amicon Ultra-15 filters (Millipore, Bradford, MA, USA). Concentrations of specific IgG were determined using BCA assay. Reactivity and

purity of specific antibodies against hLAMC1-cterm and mLAMC1-cterm were analyzed by indirect IF microscopy on human and mouse skin, by IB with extracts of human and mouse dermis, and recombinant h-and mLAMC1-cterm, respectively. The affinity purification of specific IgG against h- and m-LAMC1-cterm was performed by me.

Affinity purification of the specific antibodies against eukaryotic expressed proteins

Antibodies to hLAMC1-cterm (eukaryotic expressed forms), E8 fragment of laminin 111, as well as antibodies to hLAMC1-FL were affinity purified from sera of anti-p200 pemphigoid patients using the columns, which contain the respective coupled protein. In brief, the serum was diluted equally with PBS and was incubated with the resin end-over-end rotation for 2 hours at room temperature. The following flow through and washes were kept to evaluate the purification efficiency. Elution of the specific antibodies was done by incubating the resin with elution buffer (glycine pH 2.8) for 10 minutes at room temperature. Elution fractions were directly neutralized by adding Tris 1 M pH 9 and pooled. The elution buffer was exchanged to PBS by centrifugation with amicon filters. Autoantibodies to hLAMC1-FL were generated using the sera affinity-purified against eukaryotic expressed hLAMC1-cterm protein followed by incubation with the immobilized hLAMC1-FL protein. After both steps of affinity-purification, eluted antibodies were pooled and used for the experiments. Concentrations of IgG were determined using BCA assay. Flow through and eluted fractions containing the specific autoantibodies were analyzed by indirect IF microscopy on human foreskin and by IB with human dermal extract and eukaryotic expressed hLAMC1-cterm, hLAMC1-FL and hLAMC1-E8 protein. Affinity purification of the specific antibodies against eukaryotic expressed proteins was performed by me.

Purification of rabbit IgG

IgG from rabbit sera were isolated using Protein G Sepharose Fast Flow affinity column chromatography (GE Healthcare, Munich, Germany) as described previously[94]. Antibodies were eluted with 0.1 M glycine buffer (pH 2.8), neutralized with Tris-HCL (pH 9), and concentrated under extensive washing with PBS (pH 7.2) using Amicon Ultra-15 filters (Millipore, Bradford, MA, USA). Concentrations of IgG were determined using BCA assay. Reactivity of antibodies was analyzed by indirect IF microscopy on mouse tail, by IB with extracts of mouse dermis, and mLAMC1-cterm, respectively. Purification of the rabbit IgG was performed by me.

3.11 Cryosection Assay

Blister-inducing capacity of patients' autoantibodies and rabbit IgG was evaluated using the cryosection assay, an *ex vivo* model of autoantibody-induced dermal-epidermal separation originally described by Gammon *et al*[146] and modified by Sitaru *et al*[105].

Preparation of cryosections

Neonatal human foreskin obtained from routine circumcision, and murine skin were washed in cold PBS, cut in pieces embedded in optimum cutting temperature compound (Sakura Finetek Europe BV, Zoeterwonde, The Netherlands), and stored at -80°C. Skin cryosections of 6 µm thickness were placed on a Superfrost Plus microscope slide (Menzel Gläser, Braunscweig, Germany)[146]. Human and murine skin was prepared by me, placement of the skin onto the slides was performed by a technical assistant.

Isolation of peripheral blood leukocytes

Human leukocytes were isolated from 80 ml fresh peripheral blood of healthy donors by 3% dextran (80 ml; Nycomed, Oslo, Norway) sedimentation. After 20-30 minutes incubation at room temperature the upper layer contained the leukocytes was transferred into new tubes and cells were centrifuged (1200 rpm, 12 minutes, 19°C). After discarding the supernatant, cells were washed with 30 ml RPMI medium (Life Technologies, Karlsruhe, Germany). Further, the remaining erythrocytes were lysed with 0.2% (20 ml) and 1.6% NaCl (20 ml) and centrifuged (1200 rpm, 12 minutes, 4°C). After the last wash with RPMI (30 ml; 1200 rpm, 7 minutes, 4°C), cells were resuspended in the same medium at a density of 6 x 10^7 cells per ml and kept on ice. The cell viability was assessed by trypan blue (Sigma, Steinheim, Germany). Preparation of the leukocyte suspension was performed by me.

Treatment of cryostat sections

Cryosections of neonatal human foreskin and mouse tail were incubated for 1 hour at 37°C with 50 µl of patients and rabbit sera (dilution 1:3 in PBS). In addition, hLAMC1-cterm-specific IgG and sera depleted from anti-hLAMC1-cterm reactivity were used after adjusting to serum IgG concentrations. Flow through from affinity purification was diluted properly, so that the final dilution was equal to the dilution of the serum. After washing the sections with PBS, chambers

were prepared[146]. Tissue containing slides were covered with a second slide. Both slides were taped together and the 0.5 ml of leukocyte suspension was injected into the chambers. Chambers were incubated in a humidified air incubator containing 5% CO_2 for 3 hours at 37°C. Subsequently, chambers were disassembled and sections were washed in PBS for 10 minutes to remove excess serum and detached cells, air dried for 10 minutes, fixed in formalin for 5 minutes, and then stained for 4 minutes in papanicolaou stain. After washing with tap water, slides were left in ethanol for 20 seconds and subsequently in ammonia for 10 seconds. After washing with water, slides were stained with eosin (one minute) following ascending concentrations of eosin and alcohol. Finally the slides were mounted into a glycerol solution and covered with coverlids. Analogous experiments were performed using the eukaryotic expressed hLAMC1-cterm, the E8 fragment of laminin 111, and the hLAMC1-FL. Sections were examined by two blinded independent examiners at x200 magnification. Treatment of the cryosections was performed by me.

Activation of neutrophils

To determine the activation of neutrophils, the cell suspension was treated with 0.05% nitro blue tetrazolium-chloride (Roth, Karlsruhe, Germany) before incubation with the cryosections. On each slide 500 µl of this mixed cell suspension was applied and slides were incubated for 1.5 hour at 37°C. Then, slides were washed in PBS and mounted into Aquatex® (Merck, Darmstadt, Germany). The presence of dark-blue formazan was observed by light microscopy. Determination of the activation of neutrophils was perfomed by me.

3.12 Immunofluorescence microscopy

3.12.1 Direct immunofluorescence microscopy

Antibodies bound on the dermal-epidermal junction were detected by IF microscopy on 6 µm frozen sections prepared from tissue biopsies using FITC-labelled polyclonal rabbit anti-mouse IgG (1:100, DAKO), FITC-labelled polyclonal swine anti-rabbit IgG (dilution 1:100, DAKO), FITC-labelled monoclonal anti-human IgG4 (20 µg, Sigma Aldrich, Hamburg, Germany) and FITC-goat IgG fraction to mouse C3 complement (1:50, Cappel Organon-Teknika, Durham, NC). For detection of different subclasses of IgG FITC-rat anti-mouse IgG1, FITC-rat anti-mouse IgG2a, FITC-rat anti-mouse IgG2b, and FITC-rat anti-mouse IgG3 were used. All the antibodies were used in dilution 1:50 and purchased from BD Pharmingen, Heidelberg, Germany. After 45 minutes incubation in room

temperature, slides were washed with PBS and mounted in buffered glycerol (1:1 diluted in PBS). IgG distribution was examined in fluorescent microscopy. Direct immunofluorescence microscopy studies were performed by me.

3.12.2 Indirect immunofluorescence microscopy

Detection of serum autoantibodies in mice followed published protocols with minor modifications[136]. In brief, 6 μm sections of human and mouse skin were incubated for 45 minutes with human, mouse, rabbit sera, monoclonal anti-LAMC1 antibody (clone B4, 20-100 μg/ml, Santa Cruz Biotechnology, CA, USA) or polyclonal anti-murine LAMC1 antibody (H-190, Santa Cruz Biotechnology, CA, USA) diluted appropriately in PBS. Then, slides were washed 3 times for 5 minutes with PBS. For detection of human antibodies, monoclonal anti-human IgG4 (clone HP6025 20 μg/ml 1:50) was used. For detection of mouse antibodies, FITC-labelled polyclonal rabbit anti-mouse IgG (1:100, DAKO) was used. For detection of rabbit antibodies, polyclonal swine anti-rabbit IgG (dilution 1:100, DAKO) was used. After 45 minutes incubation in room temperature, slides were washed 3 times for 5 minutes with PBS and mounted in buffered glycerol (1:1 diluted in PBS). IgG deposition was examined by fluorescent microscopy. Indirect immunofluorescence microscopy studies were performed by me.

2.12.3 Indirect immunofluorescence microscopy of HaCaT cells

For IF analysis of LAMC1 expression in keratinocytes, HaCaT cells that reached 75% confluency, were fixed with formaldehyde 2% (Roth Karlsruhe, Germany). Then, the cells were incubated with anti-p200 pemphigoid sera, anti-p200 pemphigoid sera depleted of anti-hLAMC1-cterm antibodies, monoclonal anti-laminin γ1 antibody (clone B4, 20-100 μg), and sera from healthy volunteers (all samples in dilution of 1:50) at room temperature. After 45 minutes the cells were washed 3 times with 500 μl PBS. Mouse anti-human IgG4-PE (1:50, 2 μg/ml, clone HP6025n SouthernBiotech, Birmingham, USA) and Cy^{TM}3-conjugated AffiniPure goat anti-mouse IgG (1:100; Jackson ImmunoResearch Laboratories, PA, USA), were used as secondary antibodies for the detection of the human antibodies and monoclonal antibodies. After 45 minutes incubation at room temperature, slides were washed with PBS (3 times with 500 μl PBS) and mounted in buffered glycerol and examined by fluorescent microscopy. Immunofluorescence microscopy of HaCaT cells was performed by me.

3.13 Enzyme-linked immunosorbent assay (ELISA) LAMC1-cterm

To perform an enzyme-linked immunosorbent assay, the antigen is immobilized to the surface of polysterene microplate wells. Then, the antigen specific antibodies bind to the antigen and as a further step the secondary antibody (conjugated to a fluorofor) binds to the antigen-antibody complex. Finally, the detection of the signal, which is proposional to the amount of the antibodies in the sample, is measured using an ELISA photometer reader at 450 nm. Here, hLAMC1-cterm ELISA for the detection of anti-hLAMC1-cterm antibodies in anti-p200 pemphigoid and control sera and mLAMC1-cterm ELISA for the detection of anti-mLAMC1-cterm antibodies in mice sera were developed.

3.13.1 hLAMC1-cterm ELISA

To define the optimal working conditions of the ELISA, different parameters including the amount of coated protein (native and denatured), material of microtiter plates, blocking buffers, and concentration of secondary antibody were analyzed.

For the hLAMC1-cterm ELISA the following conditions were evaluated: (i) coating of native hLAMC1-cterm and protein denatured by 8 M urea, respectively, (ii) amount of coated protein (between 5,000 ng, 1,650 ng, 550 ng, 180 ng, 62 ng, 21 ng, 7 µg, 2.3 ng), (iii) plastic material (MaxiSorb™ plates and Microlon™200 plates, high and medium binding), and (iv) blocking solutions (1% BSA; 5% BSA; 1% skimmed milk; 5% skimmed milk in TBST). In addition, the secondary antibody was employed in various dilutions (1:1,000-1:4,000). Serum dilution (1:50) and incubation times of sera and secondary antibody were kept constant. Selected anti-p200 sera (n=3) and sera from healthy volunteers (n=5) were analyzed used under the different conditions.

Finally, MaxiSorb™ plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with 2 µg/ml hLAMC1-cterm per well diluted in 8 M urea in PBS. After overnight incubation at 4°C, plates were washed five times with PBST (PBS with 0.05% Tween 20, pH 7.2,) using an automatic microplate washer (Columbus Pro, Tecan, Crailsheim, Germany), and blocked with PBST containing 5% skimmed milk for 1 hour. After another washing step, wells were incubated for 1 hour with patient diluted 50-fold in blocking buffer. Followed by washing, bound antibodies were detected using HRP-conjugated mouse anti-human IgG4-antibody (Invitrogen) diluted 1:1,000, followed by addition of 1-Step Turbo TMB-ELISA solution (Fisher Scientific, Schwerte, Germany) for 15 minutes. The OD_{450nm} was measured using a VICTOR3 Wallac 1420 microplate reader (Perkin-Elmer LAS, Rodgau, Germany). All

steps were carried out at room temperature. All sera were tested in duplicates. From the mean OD value for each serum sample, the mean OD value of the blank (PBST) was subtracted. The development of the hLAMC1-cterm ELISA was performed by Dr. Stephanie Groth in collaboration with me.

3.13.2 mLAMC1-cterm ELISA

For the detection of anti-mLAMC1-cterm antibodies in mice sera, the coated antigen was the recombinant mLAMC1-cterm.

ELISA was performed using MaxiSorb^{TM} plates. The wells were coated with 4 µg/ml mLAMC1-cterm diluted in PBS. Plates were incubated overnight at 4°C and then washed five times with PBST (PBS with 0.05% Tween 20, pH 7.2,) using an automatic microplate washer and blocked with PBST containing 5% skimmed milk for 1 hour. After another washing step, wells were incubated for 1 hour with mouse sera diluted in blocking buffer. After several washing steps, HRP-conjugated polyclonal rabbit anti-mouse IgG antibody and polyclonal goat anti-rabbit antibody diluted 1:2,000 in blocking buffer were added and incubated for 1 hour. Development was performed by 1-Step Turbo TMB-ELISA solution The OD_{450nm} was measured using a VICTOR3 Wallac 1420 microplate reader. All steps were carried out at room temperature. All sera were tested in duplicates. From the mean OD value for each serum sample, the mean OD value of the blank (PBST) was subtracted. The development of the mLAMC1-cterm ELISA was performed by me.

3.14 Mouse experiments

3.14.1 Passive transfer models

Adult mice

In the passive transfer model, purified rabbit IgG generated against mLAMC1-cterm (15 mg) and normal rabbit IgG (15 mg) were injected into adult mice (C57CL/6, BALB/c) subcutaneously in abdominal skin every second day over a period of 12 days. Similarly, adult mice were also injected with specific IgG against mLAMC1-cterm (1 mg/ml) following the same protocol. Every second day mice were examined for any evidence of lesions (i.e., erythema, blisters, erosions, and crusts). To evaluate the autoantibody levels, sera were obtained from mice. Also, biopsies of the tails was obtained for detection of IgG deposition. On day 12, mice were sacrificed. Ears and tails were

subjected and prepared for examination by histopathology and snap-frozen for direct IF microscopy. For histopathology, biopsies of tail and ear were fixed in 4% buffered formalin. Blood samples were collected and after centrifugation, (13,000xg, 15 minutes, 4°C), sera were stored at -20°C (Figure 4).

Neonatal mice

Total IgG purified from rabbit immunized with mLAMC1-cterm (10 mg/g body weight) was injected into neonatal mice (C57CL/6) subcutaneously in the head-neck area every second day over a period of 12 days. During this period neonates were kept with their mothers. Specific IgG against the mLAMC1-cterm was injected into neonatal mice at a concentration of 1 mg/ml body weight following the same protocol. Rabbit antibodies were concentrated up to 10-50 mg/ml, to avoid loss of IgG during the injection. Every day mice were examined for evidence of clinical lesions by softly scratching the epidermis with a tweezer. On day 12, mice were sacrificed. Blood samples were taken for evaluation of the antibody levels. For histopathology, biopsies of tail and ear were fixed in 4% buffered formalin. Blood samples were collected and after centrifugation (13.000xg, 15 minutes, 4°C) sera were stored at -20°C (Figure 4).

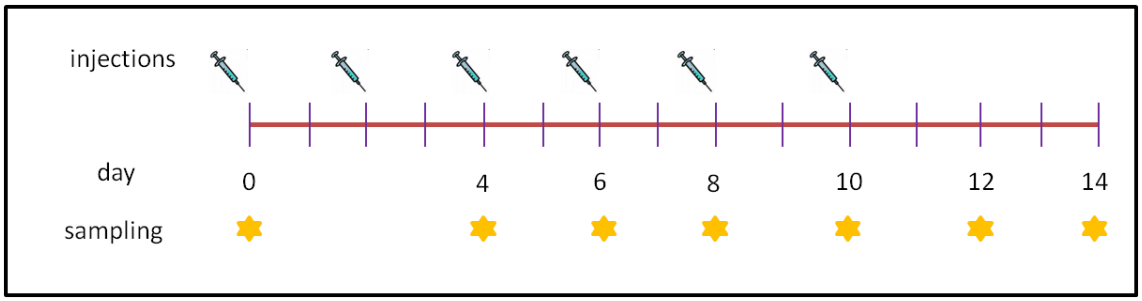

Figure 4. Experimental design of the passive transfer model. Polyclonal antibodies against mLAMC1-cterm were generated in rabbits by immunization with recombinant mLAMC1-cterm. The IgG fraction from immune rabbit sera was purified by protein G chromatography and, subsequently, injected into mice (adult or neonatal) every second day for a period of 12 days. At day 14 the mice were sacrificed. The yellow stars indicate the sampling or scoring days and syrings represent the injection days.

3.14.2 Immunization of mice

In the immunization-induced model, mice (C57CL/6, BALB/c and SJL) were immunized subcutaneously into footpads 4 times (with 3 weeks interval) with 60 µg of purified recombinant protein (mLAMC1-cterm) emulsified in adjuvant (TiterMax®, Alexis, Lörrach, Germany). Mice were examined every second week for evidence of cutaneous lesions (i.e., erythema, blisters, erosions, or crusts). Control mice were immunized with PBS and TiterMax® (same amount as the protein). All mice were observed for at least 16 weeks. From every mouse, serum and tissues samples were obtained at weeks 2, 4, 6, 8, and 16. Serum samples were assayed by IF microscopy and for autoantibody level by ELISA and immunoblotting using recombinant protein or murine dermal extract. Biopsies of tail and ear were collected at the end of the observation period and prepared for examination by histopathology, and direct IF microscopy. Biopsies were fixed in 4% buffered formalin. Sections from paraffin-embedded tissues were stained with hematoxylin and eosin (Figure 5). All mice experiments were performed by me.

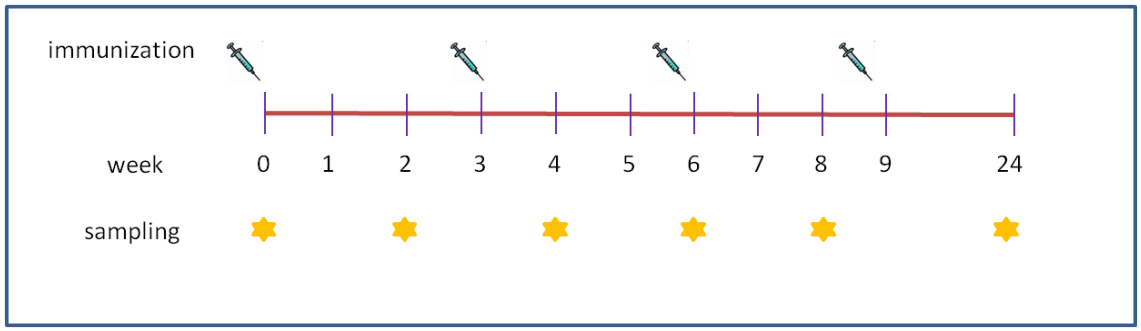

Figure 5. Experimental design of the immunization protocol. Mice were immunized with the recombinant protein (60 µg) every third week, in total 4 times. The yellow stars indicate the scoring sampling time points and syrings represent the immunization time points. At week 24 the mice were sacrificed.

3.15 Statistical analysis

For all statistical analyses, Gnu R open access software, version 2.12 (R Development Core Team 2009; R Foundation for Statistical Computing, Vienna, Austria) and SigmaPlot were used. Generally, for hypothesis testing, p values below 0.05 were considered significant.

To analyze and evaluate the discriminatory capability of the ELISA method, receiver-operator characteristics (ROC) analysis was performed, employing the package 'DiagnosisMed' for Gnu R. The following characteristics of the ELISA method were determined by ROC analysis.

Value	***Calculation and meaning***
Area under curve (AUC)	Fractional area below the ROC curve
Sensitivity	true positive / (true positive + false negative)
Specificity	1 - false positive / (true negative + false positive)
Accuracy	(true negative + true positive) / whole number of samples
Cut-off value	Threshold to determine positive reactivity of the ELISA test

The cut-off value of the hLAMC1-cterm ELISA was optimized to maximize the accuracy of the ELISA method. Accuracy, sensitivity and specificity were estimated when a certain cut-off value was given.

For calculation of confidence intervals for specificity, sensitivity and accuracy, Wilson confidence intervals were calculated analytically. For calculation of the expected value und confidence intervals for sensitivity at a given specificity, bootstrapping was used (package 'boot'). In short, in each bootstrap resampling step *k*, data points x_i from healthy controls were given weights *wi* (re-weighting strategy as provided by package 'boot'), which were the used to calculate the weighted quantiles (function 'wtd.quantile' from package 'Hmisc'). The cut-off at the quantile specified by specificity was then used to calculate the sensitivity for this resampling step:

$$\text{Sensitivity} = {\sum_{x_i > cutoff} x_i w_i} \Big/ {\sum_{i=1}^{N} w_i}$$ for all data points xi from the patient group.

The mean and the 2.5%-97.5% quantiles for the resulting bootstrapping distribution of the sensitivity were given as expected value and confidence interval.

To determine the reproducibility of the ELISA test, the interest correlation coefficient (ICC) was calculated by use of the 'psych' package.

Furthermore, the following robust method for outlier detection was used (after: F.R. Hampel, "The breakdown points of the mean combined with some rejection rules." In: Technometrics 27 (1985), S. 95-107):

$$|\tilde{x} - x_a| > 5.2\widetilde{D} \implies x_a \text{ is an outlier,}$$

with $\widetilde{D}$ as the median absolute derivation and $\tilde{x}$ as the median of a given set of measured values x.

All data from mice were exported and stored in Excel (Microsoft, USA). Data was subsequently analyzed for statistical significance with SigmaPlot. *was considered significant (P≤0.05). The statistical analysis for the ELISA was performed by me in collaboration with Dr. Andreas Recke.

4 RESULTS

4.1 Diagnosis of anti-p200 pemphigoid using a hLAMC1-cterm ELISA

Diagnosis of anti-p200 pemphigoid is very limited due to difficult preparations of dermal extracts, which is only available in specialized laboratories. Recently, laminin γ1 (LAMC1) and specifically the C-terminus (hLAMC1-cterm) of this protein, was suggested to be the immunodominant epitope. The first part of the thesis examined whether specific antibodies against LAMC1-cterm can be used as a diagnostic marker in anti-p200 pemphigoid sera and therefore, to develop an easy and simple enzyme-linked immunosorbent assay (ELISA) for this disease.

4.1.1 Generation of a recombinant monomeric C-terminal fragment of human laminin γ1 (hLAMC1-cterm).

The recombinant C-terminal fragment of human laminin γ1 (hLAMC1-cterm; amino acid residues 1,364 to 1,609, Figure 6a) was subcloned from obtained vector hLAMC1-cterm-pMA into the expression vector pQE40 including a His-tag. Then, the fragment was purified by immobilized metal affinity chromatography using Talon affinity resin. After SDS-PAGE, the migration position of hLAMC1-cterm was visualized by Coomassie blue staining and immunoblotting with an anti-His-tag antibody (Figure 6b).

4.1.2 Only anti-p200 pemphigoid sera reacted with the recombinant hLAMC1-cterm by immunoblotting

In a pilot experiment, sera from patients with anti-p200 pemphigoid and other autoimmune skin diseases were analyzed for reactivity of hLAMC1-cterm by immunoblotting on nitrocellulose membranes. Sera from patients with anti-p200 pemphigoid (n=10), bullous pemphigoid (n=10), epidermolysis bullosa acquisita (n=5), anti-laminin-332 mucous membrane pemphigoid (n=5), and pemphigus vulgaris (n=10), as well as healthy volunteers (n=10) were tested for reactivity to hLAMC1-cterm by IB. Eight of the 10 anti-p200 pemphigoid sera reacted with hLAMC1-cterm while none of the control sera showed reactivity (Figure 7).

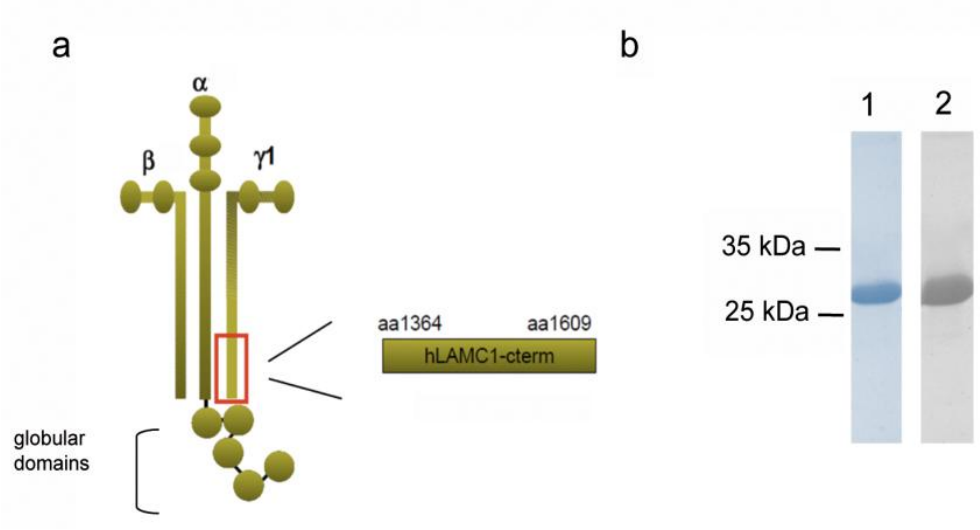

Figure 6. Generation of the recombinant monomeric C-terminal fragment of human laminin γ1 (hLAMC1-cterm). (a) Schematic diagram of the laminin structure and the hLAMC1-cterm localized in the coiled-coil domain. (b) hLAMC1-cterm was migrated in 28 kDa size visualized by Coomassie staining (lane 1) and IB with an anti-his antibody (lane 2) (adapted from Groth *et al.*[145]).

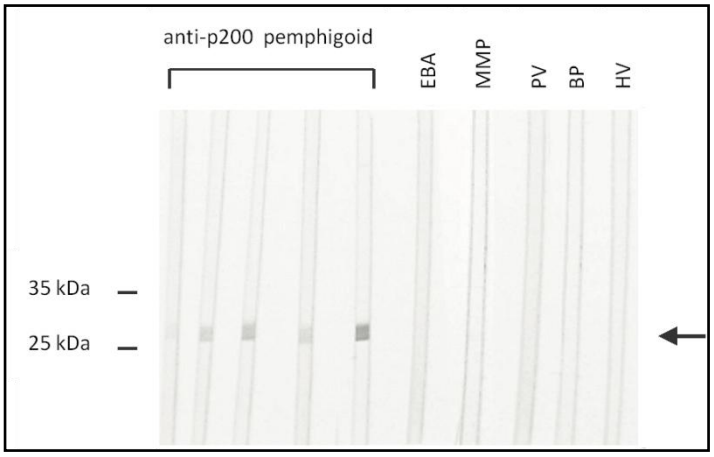

Figure 7. Immunoblot reactivities of anti-p200 pemphigoid and control sera with a monomeric C-terminal fragment of human laminin γ1. Here, representative blotting of five anti-p200 pemphigoid sera and control sera. As controls, sera from healthy volunteers, as well as sera from other autoimmune blistering diseases were used. Only the anti-p200 pemphigoid sera showed reactivity with recombinant hLAMC1-cterm. The arrow indicates the migration position of hLAMC-cterm. EBA, epidermolysis bullosa acquisita; MMP, anti-laminin-332 mucous membrane pemphigoid; PV, pemphigus vulgaris; BP, bullous pemphigoid; HV, healthy volunteers, (adapted from Groth *et al*.[145]).

4.1.3 Development of a sensitive and specific ELISA for detection of antibodies against hLAMC1-cterm in anti-p200 pemphigoid sera.

For optimisation of the novel ELISA, different conditions were evaluated including (i) plastic material (MaxiSorb™ plates and Microlon™200 plates, high and medium binding), (ii) concentration of coated hLAMC1-cterm (between 5,000 ng, 1,650 ng, 550 ng, 180 ng, 62 ng, 21 ng, 7 µg, 2.3 ng), (iii) blocking buffer (1% BSA; 5% BSA; 1% skimmed milk; 5% skimmed milk in TBST), and (iv) concentration of the secondary antibody (1:1,000-1:4,000). The best discrimination between anti-p200 pemphigoid and control sera was seen by using MaxiSorb™ plastic plates, coating of 100 ng of hLAMC1-cterm, 5% skimmed milk as blocking buffer, and secondary antibody at a dilution of 1:1,000.

Subsequently, a large number of sera was tested including patients with anti-p200 pemphigoid (n=35), bullous pemphigoid (n=101), epidermolysis bullosa acquisita (n=10), anti-laminin 332 mucous membrane pemphigoid (n=14), and pemphigus vulgaris (n=51), as well as sera from healthy volunteers (n=131). The analysis of sensitivity and specificity of the hLAMC1-cterm ELISA at different cut-offs is presented in Figure 8. To differentiate between positive and negative sera in this test, a cut-off was used. The properties of the test procedure were analyzed by receiver-operator characteristic (ROC) curves, with parameters sensitivity, specificity and accuracy. Sensitivity measures the proportion of people with disease who were correctly identified as having the disease. This function indicates the ability of a test to identify true positive sera. Specificity measures the proportion of healthy people who are correctly identified as healthy. Accuracy of a test procedure is the proportion of correctly identified sera overall. Accuracy was selected as a parameter to be optimized by choosing the best cut-off value for the ELISA. The overall test performance was expressed by the area under curve (AUC), which reaches a value of 1 for perfect discrimination and 0.5 for no discrimination between patients' and healthy control sera.

Twenty four out of 35 (69%) anti-p200 pemphigoid patients sera showed IgG reactivity to hLAMC1-cterm, whereas none of the epidermolysis bullosa acquisita and pemphigus vulgaris patients, 2 of 101 (2%) bullous pemphigoid patients, 2 of the 14 anti-laminin 332 mucous membrane pemphigoid patients (14%), and 0 of 131 healthy volunteers were reactive based on the cut-off defined by ROC analysis (Figure 9). One of the 2 reactive bullous pemphigoid and 1 reactive anti-laminin 332 mucous membrane pemphigoid sera, respectively, also recognized hLAMC1-cterm by IB.

Based on a cut off optimized for accuracy, a sensitivity of 69% (95% Wilson confidence interval 52% - 81.5%, 24 of 35 anti-p200 pemphigoid patients) and a specificity of 98.7% (95% Wilson confidence

interval 92.9 - 99.5%, 2 false positives from 307 controls) were calculated for the novel ELISA (Table 5).

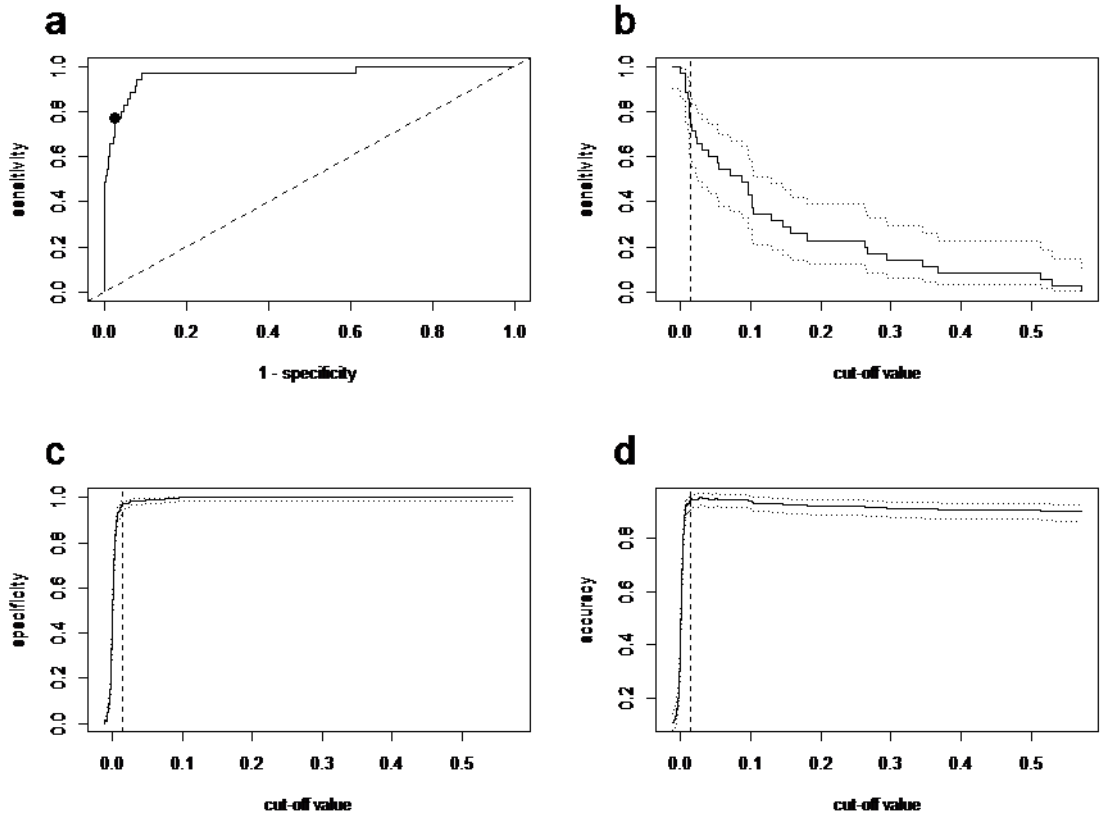

Figure 8. Receiver operating characteristic (ROC) curve analysis for the hLAMC1-cterm ELISA. To identify the point of the greatest efficiency (a, black point), the sensitivity and 1 – specificity at different cut-offs were plotted. Relationship between cut-off values and sensitivity (b), and specificity (c, based on sera from healthy volunteers and patients with PV), and accuracy (d) for the hLAMC1-cterm ELISA were presented. The cut-off value was set as the point at which the difference in the sensitivity and specificity was minimal (represented as dotted line). The cut-off with the highest accuracy (95.6%), for identifying anti-p200 pemphigoid sera from other autoimmune skin diseases, was 0.027 (d). Dashed line, reference line.

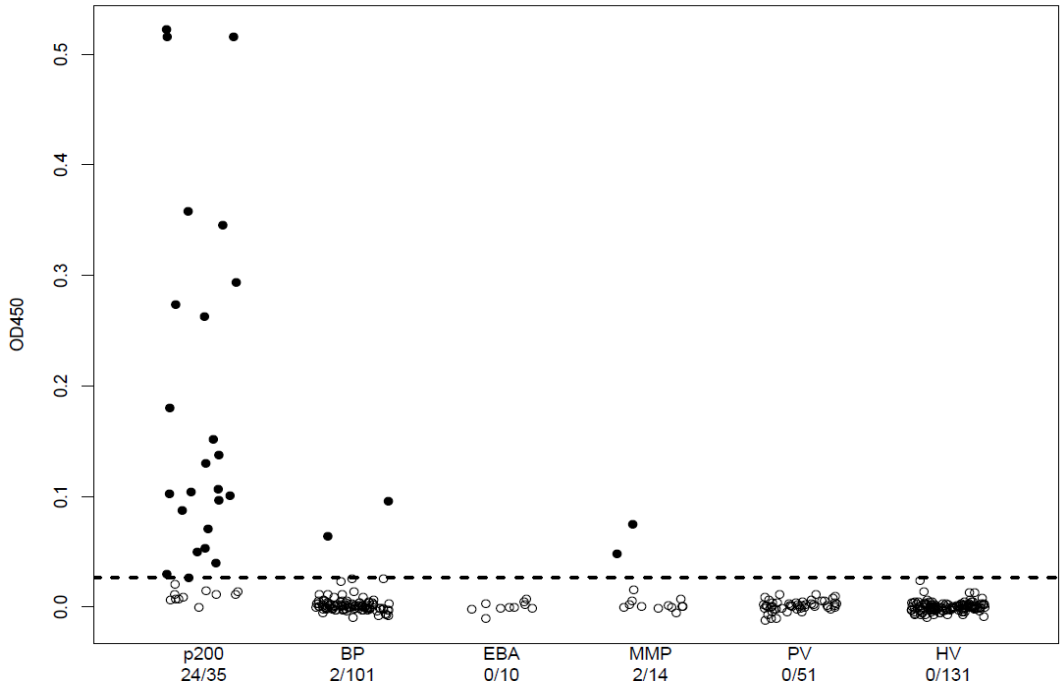

Figure 9. ELISA reactivities of anti-p200 pemphigoid and control sera with a hLAMC1-cterm. Sera of patients with anti-p200 pemphigoid and control sera from patients with BP, EBA, anti-laminin 332 MMP, PV, and HV were quantitatively analyzed by the novel ELISA system based on the hLAMC1-cterm. Each serum was tested in duplicates and plotted data points represent optical density (OD) at 450 nm. The dotted line represents the cut-off value of this assay (0.027 at OD_{450nm}). Numbers show positive and total numbers of tested sera, respectively. p200, anti-p200 pemphigoid; BP, bullous pemphigoid; EBA, epidermolysis bullosa acquisita; MMP, anti-laminin 332 mucous membrane pemphigoid; PV, pemphigus vulgaris; HV, healthy volunteers (adapted from Groth *et al.*[145]).

Since dual reactivity of anti-p200 pemphigoid sera with other antigens of the dermal-epidermal junction, such as BP180, BP230, laminin 332, and type VII collagen has previously been described[51, 58, 147, 148], specificity was based on sera from healthy volunteers and patients with pemphigus vulgaris. The test parameters are shown in Table 5.

To calculate the intra-class correlation coefficient (ICC for random raters) ELISA values of the same sample on different plates and different days were compared. Forty-two sera, including anti-p200 pemphigoid (n=12) and control sera (n=30), were studied at four different days. The results showed a high inter-rate reliability with a calculated ICC of 0.97, which indicates a low variance of ELISA values between separate experiments (Figure 10).

Table 5. Characteristics of hLAMC1-cterm ELISA (adapted from Groth *et al*.[145])

Parameters	hLAMC1-cterm	95% CI
Area under curve	0.96	0.93 - 1.00
Sensitivity	68.6%	52% - 81.5%
Specificity	98.7%	92.9 - 99.5%
Cut-off [1] (OD_{450nm})	0.027	
Accuracy	95.6%	92.9% - 97.3%
Maximal sum of sensitivity and specificity	188.4%	180.6 - 193.2%
Cut-off (OD_{450nm})	0.007	
Sensitivity at fixed specificity 99%	61.5%	40.0 - 81.3%
Cut-off [2] (OD_{450nm})	0.047	
Sensitivity at fixed specificity 98%	69.7%	50.0 - 86.7%
Cut-off [2] (OD_{450nm})	0.024	

[1]The optimal cut-off was determined by maximal accuracy. Specificity was based on sera from healthy controls (n=131) and patients with pemphigus vulgaris (n=51).

[2]Expected values and 95% confidence intervals (95% CI) of sensitivity at a fixed specificity were calculated by numeric simulation. OD_{450nm}, optical density at 450 nm.

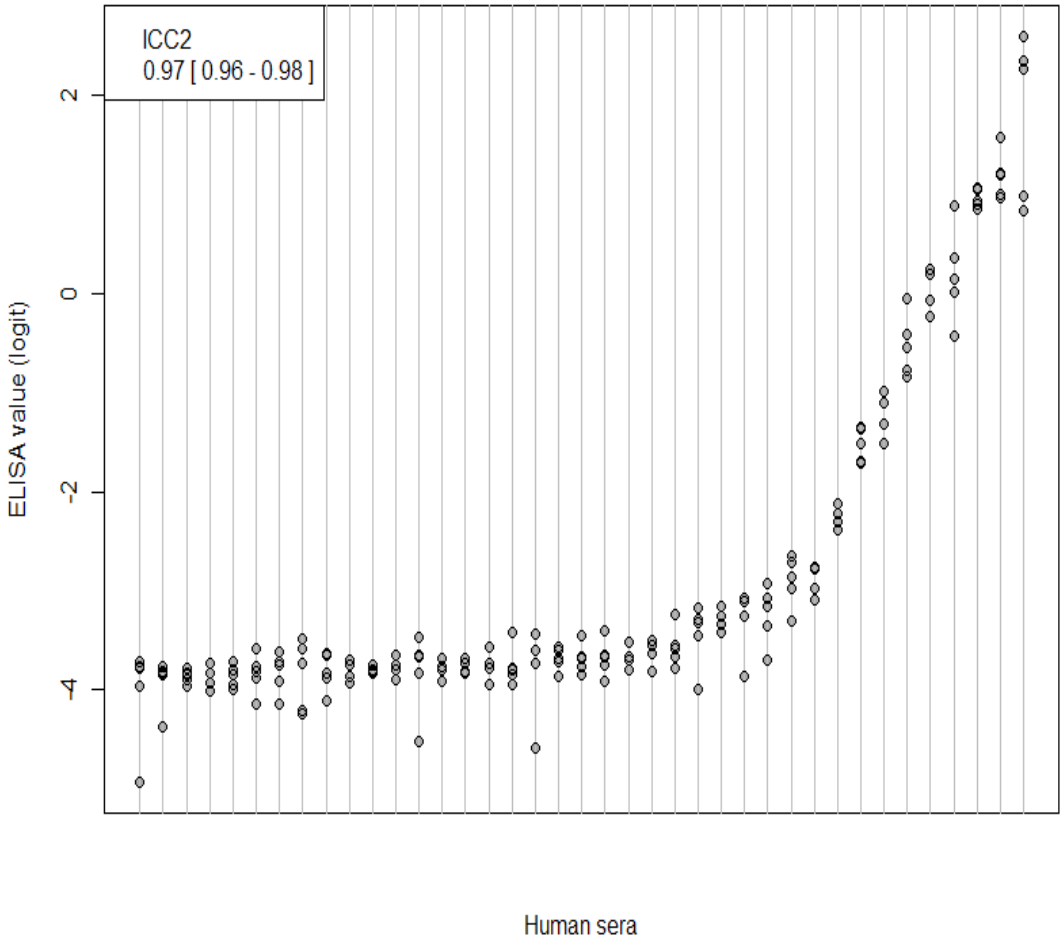

Figure 10. Reproducibility of ELISA measurements. ELISA values of 42 different sera (columns) covering the entire measurement range of the novel ELISA were determined on 4 different days (each dot represents one measurement of separate experiments). Data were normalized by logit transformation for calculation of the intra-class correlation coefficient (ICC2). The high ICC2 value of 0.97 (95% confidence interval 0.96-0.98) demonstrates a high correlation between repeated ELISA experiments, indicating a good reproducibility of this method. To improve visual evaluation of data, columns representing individual sera are sorted increasingly by ELISA values (adapted from Groth *et al.*[145]).

As a summary of this part, a simple ELISA for the detection of antibodies in anti-p200 pemphigoid patients against the C-terminal fragment of laminin γ1 was developed. This ELISA has a sensitivity of 69% and a specificity of 99% and can thus be used as a diagnostic method in routine laboratories. These findings also show the significance of anti-hLAMC1-cterm antibodies as a diagnostic marker of anti-p200 pemphigoid.

4.2 Pathogenicity of autoantibodies in anti-p200 pemphigoid.

The second part of the thesis explored the role of anti-laminin γ1 antibodies in blister formation by the use of *ex vivo* and *in vivo* experiments.

4.2.1 Sera from anti-p200 pemphigoid patients recruited neutrophils to the dermal-epidermal junction and induced subepidermal splitting in cryosections of human skin

To investigate the ability of antibodies in anti-p200 pemphigoid sera to induce split formation, sera from 7 patients were subjected to the cryosection assay (an autoantibody-mediated leukocyte-dependent neutrophil activation model). Serum samples from a patient with bullous pemphigoid and from a healthy volunteer were used as positive and negative controls. To study the time course of leukocytes recruitment and the time point when dermal-epidermal separation (DES) appeared, sera-pre-treated-cryosections were incubated for 1, 2, and 3 hours with leukocyte from healthy donors. After 1 hour incubation, leukocytes were attached to the epidermal basement membrane in sections incubated with the bullous pemphigoid (Figure 11a) and anti-p200 pemphigoid serum (Figure 11b), respectively, while after 3 hours of incubation DES occurred (Figure 11 d and e). Sera from healthy volunteers (HV) neither induced neutrophil recruitment nor DES (Figure 11 c and f).

4.2.2 Epitope mapping of laminin γ1

A previous study suggested that the C-terminus of laminin γ1 is the immunodominant region of this protein. By IB, 90% of anti-p200 pemphigoid sera recognized the recombinant laminin γ1 C-terminal E8 fragment (which is the recombinant trimer of laminin 111) and the IB reactivity of anti-p200 pemphigoid sera with E8 was inhibited by adding a monoclonal anti-laminin γ1 antibody against the C-terminus[61]. This study aimed to confirm this finding and to exclude the presence of other major antigenic sites on laminin γ1 outside hLAMC1-cterm. Thus, 5 overlapping recombinant fragments covering the entire laminin γ1 molecule outside hLAMC1-cterm were designed, synthesized, cloned and expressed in *E. coli* as His-tagged fusion proteins (Figure 12). These fragments were further purified by immobilized metal affinity chromatography using Talon affinity resin and run in SDS-PAGE electrophoresis. The migration position of each fragment was visualized by Coomassie blue staining and IB with an anti-His-tag antibody (Figure 13).

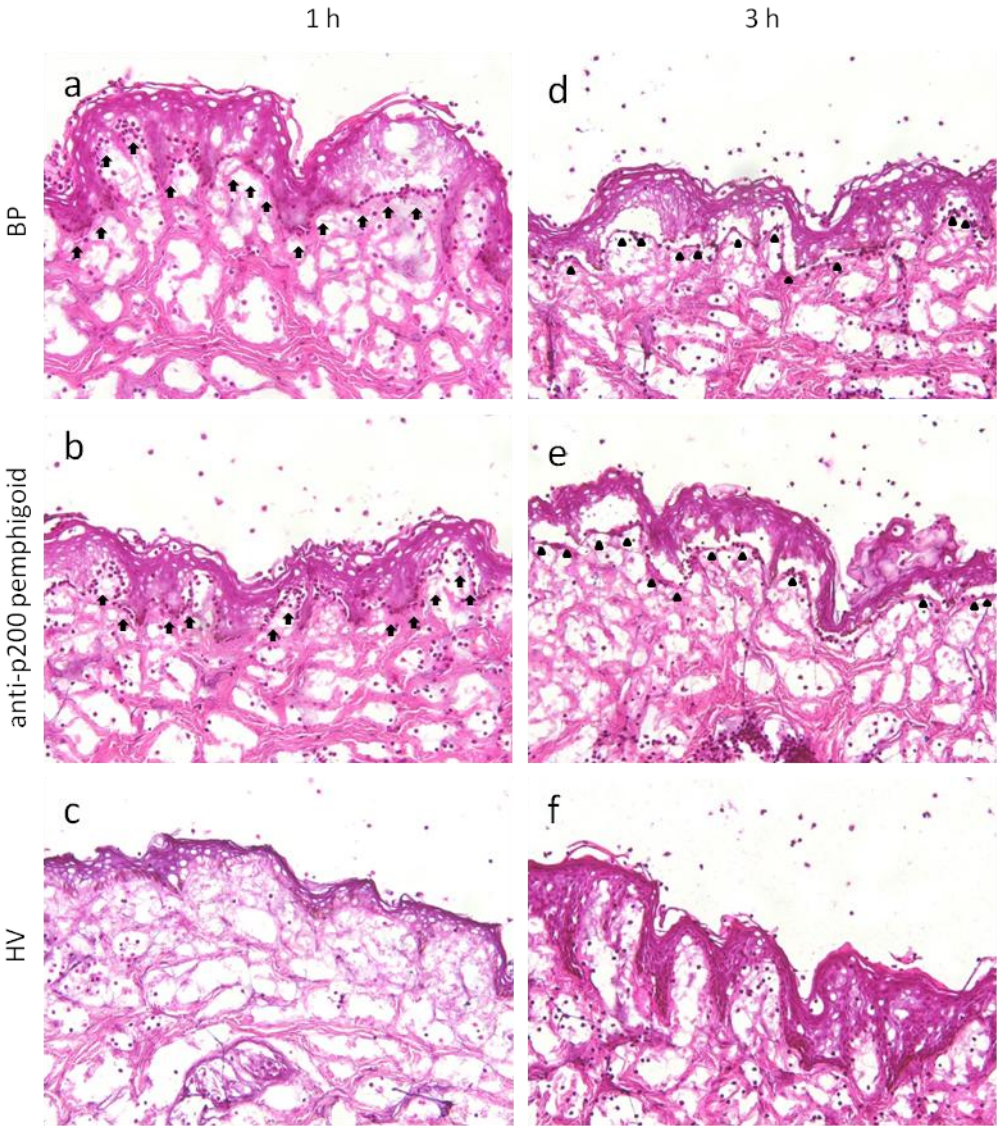

Figure 11. Autoantibodies from anti-p200 pemphigoid patients recruit neutrophils to the dermal-epidermal junction (DEJ) and induce dermal-epidermal separation (DES) in cryosections of human skin. Sera from patients with anti-p200 pemphigoid (b, e), similarly as a serum from a BP patient, which served as positive control, (a, d) recruited leukocytes to the DEJ after 1 hour of incubation (a, b) and induced DES after 3 hours (d, e). In contrast, serum from a HV did not recruit leukocytes to the DEJ and no DES was observed after 3 hours of incubation (c, f). Base of the split is marked by black triangles. All sections were stained with hematoxylin and eosin. BP, bullous pemphigoid; HV, healthy volunteer Magnification, x200 (adapted from Vafia *et al*.[149]).

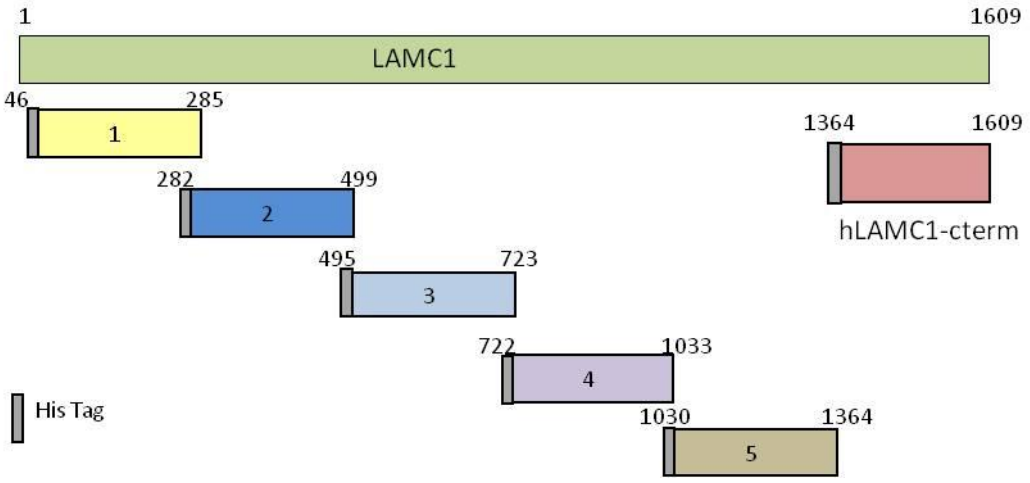

Figure 12. Schematic diagram of the 6 recombinant fragments of human laminin γ1 (hLAMC1). For epitope mapping, 5 overlapping recombinant fragmants 1 to 5 were generated. All the fragments including the recombinant C-terminus (hLAMC1-cterm) were fused with an N-terminal His-tag. Amino acid numbers are shown next to the fragments. LAMC1, laminin γ1; hLAMC1-cterm, C-terminus of laminin g1; His Tag, 6 histidines tagged to the N-terminus of each recombinant protein.

Twenty five anti-p200 pemphigoid sera, including 21 sera that recognized hLAMC1-cterm, were probed for reactivity with the 5 laminin γ1 fragments by IB. Only weak reactivity outside hLAMC1-cterm was detected in together 32% of patients' sera. The highest reactivity was found with fragment 5 in 4 of 25 sera, 16% (Table 6). These results confirm previous observations that the C-terminus of LAMC1 is the most immunodominant region of this protein in anti-p200 pemphigoid. This fragment was further used for affinity purification of IgG from anti-p200 pemphigoid sera.

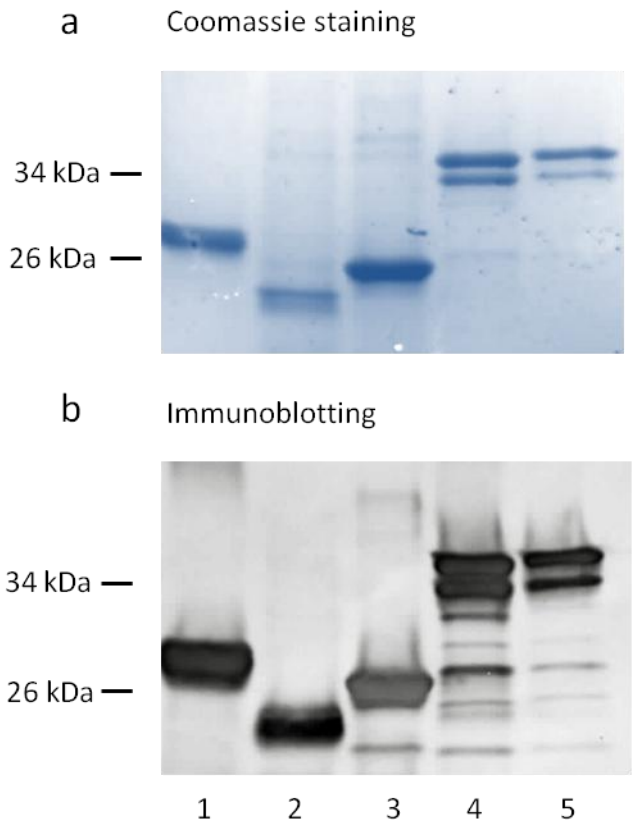

Figure 13. Coomassie blue staining and immunoblotting of the 5 recombinant His-tagged fragments (1-5) of laminin γ1. Bacterially expressed fragments of laminin γ1 were purified from *E. coli* strain Rosetta DE3 using immobilized metal affinity chromatography, visualized on a 10% SDS-PAGE and stained with sensitive Coomassie blue (a). The His-tagged recombinant fragments (1-5) were transferred onto nitrocellulose and analyzed with an anti-His antibody by immunoblotting (b). Fragments 4 and 5 were partly fermented but due to high concentration they were considered enough for identification. The fragments migrated according to their size (25-38 kDa).

Table 6. Serum autoantibody reactivity in anti-p200 pemphigoid patients with overlapping fragments of laminin γ1 covering the entire molecule (adapted from Vafia *et al*[149]).

	fragments outside hLAMC1-cterm					
	1	2	3	4	5	hLAMC1-cterm
positive sera	1 of 25	2 of 25	3 of 25 8 of 25	1 of 25	4 of 25	21 of 25

4.2.3 Anti-hLAMC1-cterm-specific IgG from patients with anti-p200 pemphigoid did not induce split formation in cryosections of human skin

To explore the pathogenic effect of anti-hLAMC1-cterm antibodies, IgG from anti-p200 pemphigoid patients (n=5) was affinity-purified using recombinant prokaryotic expressed hLAMC1-cterm (chapter 2.10.2). Anti-hLAMC1-cterm-specific IgG and sera depleted from anti-hLAMC1-cterm reactivity fractions were tested by IB of dermal extracts and recombinant hLAMC1-cterm. Monoclonal anti-LAMC1-cterm, unfractionated sera and sera from healthy volunteers were used as positive and negative controls, respectively. In all 5 sera, hLAMC1-cterm-specific IgG, as well as the monoclonal anti-LAMC1 antibody (clone B4, 1 μg/ml), recognized both recombinant hLAMC1-cterm and the p200 antigen by IB. The fraction depleted of anti-hLAMC1-cterm antibodies did not recognize the recombinant protein but, interestingly, labelled the p200 antigen by IB (Figure 14).

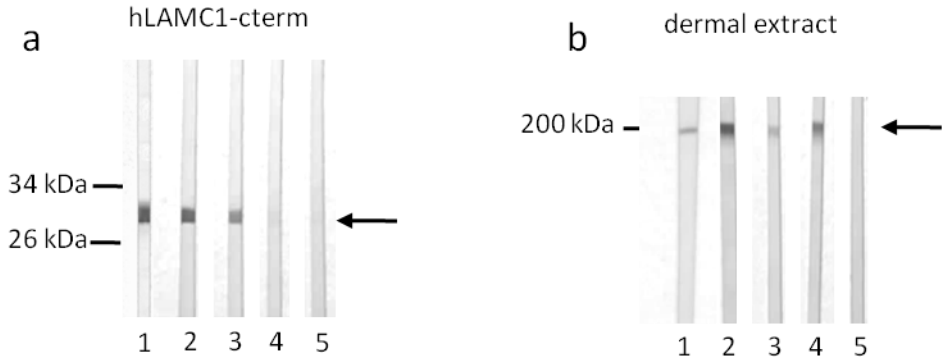

Figure 14. Detection of IgG from anti-p200 pemphigoid serum against the procaryotic expressed form of hLAMC1-cterm by immunoblotting. Using recombinant hLAMC1-cterm, IgG specific for hLAMC1-cterm (a, b; lane 3) and serum depleted from anti-hLAMC1-cterm reactivity (a, b, lane 4) were generated by afinity purification from anti-p200 pemphigoid sera (a, b; lane 2; dilution 1:50) as shown by immunoblotting with recombinant hLAMC1-cterm (a) and extract of human dermis (b). Interestingly, serum depleted from anti-hLAMC1-cterm reactivity (a, lane 4) still labeled the p200 protein in dermal extract (b, lane 4). Monoclonal antibody against LAMC1 (a, b; lane 1, dilution 1:200, 1 μg/ml) and serum from a healthy volunteer (a, b; lane 5) were used as controls. Arrows indicate the positions of the proteins; hLAMC1-cterm, C-terminal fragment of human laminin γ1 (adapted from Vafia *et al.*[149]).

Then, the above fractions (anti-hLAMC1-cterm and sera depleted from anti-hLAMC1-cterm reactivity) were examined by indirect IF microscopy for binding of IgG to the dermal-epidermal junction. All 5 sera, hLAMC1-cterm-specific IgG, as well as the monoclonal anti-LAMC1 antibody (clone B4, 20 μg/ml), labelled the DEJ by indirect IF microscopy on human skin (Figure 15 a-c). In addition, all 5 sera depleted from anti-hLAMC1-cterm reactivity labelled the dermal-epidermal junction by indirect IF microscopy of human skin (Figure 15 d). Interestingly, a discontinuous staining pattern of the basal keratinocytes was noticed with both the monoclonal anti-LAMC1 antibody (Figure 15 a, insert) and patient hLAMC1-cterm-specific IgG (Figure 15 c, insert). In contrast, patient sera and sera depleted from anti-hLAMC1-cterm antibodies revealed a linear staining throughout the specimen (Figure 15 b and d, inserts). Serum from a healthy volunteer, served as negative control, did not label the dermal-epidermal junction.

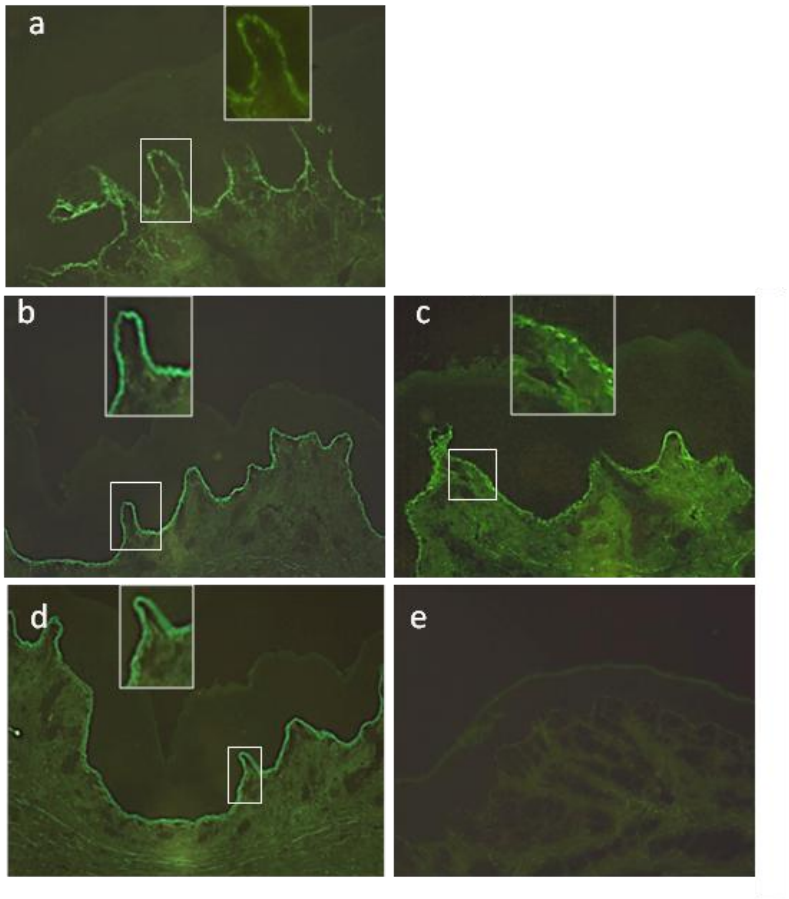

Figure 15. Binding of anti-hLAMC1-cterm IgG on human skin by indirect IF microscopy. hLAMC1-cterm-specific patients' IgG (c) and the monoclonal anti-LAMC1 antibody (a, 20 μg/ml) labeled the dermal-epidermal junction (DEJ) by indirect immunofluorescence (IF) microscopy. As well, serum depleted from reactivity with

hLAMC1-cterm (d) and unfractionated patient's serum labeled the DEJ in a similar way (b). Interestingly, while both anti-p200 serum (b) and serum depleted from anti-hLAMC1-cterm reactivity (d) stained the DEJ of human skin in a linear pattern (b, d inserts), the monoclonal anti-hLAMC1-antibody (a) and hLAMC1-cterm-specific patient's IgG (c) showed an additional cell surface staining of the basal portion of basal keratinocytes (a, c, inserts). Serum from a healthy volunteer was used as negative control (e). Magnification: x200 (adapted from Vafia *et al*.[149])

Next, the pathogenic effect of each fraction was explored in the cryosection model. hLAMC1-cterm-specific IgG from all 5 sera, as well as the monoclonal anti-LAMC1 antibody (clone B4, 20 μg/ml) did not induce splitting (Figure 16 c and a). In contrast, all 5 sera, as well as sera deleted from hLAMC1-cterm reactivity induced DES in the cryosection model (Figure 16 b and d).

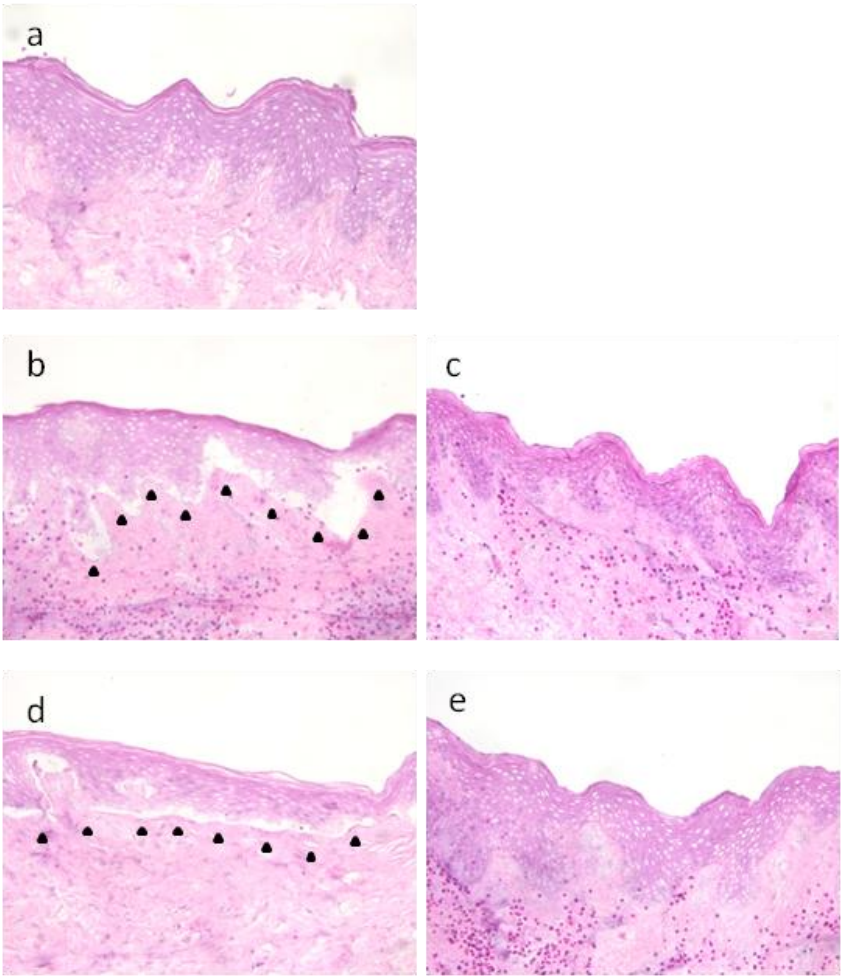

Figure 16. Anti-hLAMC1-cterm antibodies from anti-p200 pemphigoid sera were not pathogenic in cryosections of human skin. hLAMC1-cterm-specific patients IgG (c) and the monoclonal anti-LAMC1

antibody (a) did not induce dermal-epidermal separation (DES) in cryosection of human skin after incubation with leukocytes. In contrast, serum depleted from reactivity with hLAMC1-cterm (d) and unfractionated patient's serum (b) resulted in DES (black triangles mark base of the split). Magnification: x200 (adapted from Vafia *et al*.[149]).

To exclude suboptimal dosing, hLAMC1-cterm-specific IgG was applied at a 5-fold higher concentration and high titer (tested by IB) compared to the serum. The concentrated hLAMC1-cterm-specific IgG also failed to induce DES.

The activation of leukocytes attached to the dermal-epidermal junction was determined by the reduction of nitro-blue tetrazolium. The cryosections already treated with sera from anti-p200 pemphigoid patients, anti-hLAMC1-cterm IgG, sera depleted from anti-hLAMC1-cterm IgG reactivity and sera from healthy volunteers were incubated with leukocytes together with nitro blue tetrazolium. After 1 hour of incubation, nitro blue tetrazolium was reduced and dark-blue precipitates (formazan) appeared along the DEJ in the sections treated with serum and serum depleted from anti-hLAMC1-cterm reactivity, but not in sections treated with anti-hLAMC1-cterm specific antibodies. As expected, in sera of healthy volunteers, no blue precipitates appeared along the dermal-epidermal junction (Figure 17).

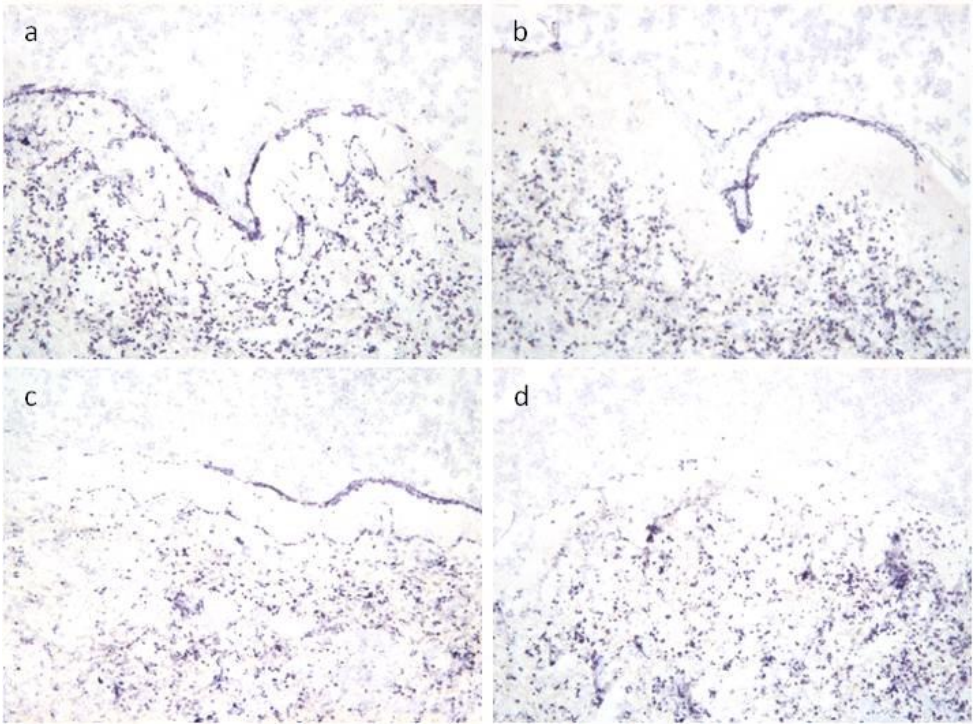

Figure 17. Antibodies against hLAMC1-cterm did not activate leukocytes *ex vivo*. Activated leukocytes reduced nitro blue tetrazolium to formazan (dark precipitates along the dermal-epidermal junction). Neutrophils were induced when cryosections of normal human skin were incubated with anti-p200 pemphigoid sera (a) and sera depleted from anti-hLAMC1-cterm IgG reactivity (c). However, no precipitates were observed in cryosections incubated with anti-hLAMC1-cterm IgG (b) and sera from healthy volunteers (d). Magnification: x200.

4.2.4 Antibodies against the eukaryotic expressed hLAMC1-cterm from patients with anti-p200 pemphigoid did not induce split formation in cryosections of human skin

Laminin γ1 is known to be N-glycosylated[60]. To examine whether modifications like glycosylations are involved in determining its antigenicity, the C-terminal fragment was expressed in eukaryotic cells. The sequence of hLAMC1-cterm, including a secretion of a signal peptide, was subcloned in the eukaryotic expression plasmid pEE14.4, which was used for transfection of HEK293T cells. The purified eukaryotic protein was used to affinity-purify IgG from anti-p200 pemphigoid sera (n=3). In all 3 sera, the obtained hLAMC1-cterm-specific IgG recognized both recombinant hLAMC1-cterm and the p200 antigen by IB (Figure 18 a, b; lane 2). The fraction depleted of anti hLAMC1-cterm antibodies recognized weakly the recombinant protein (Figure 18 a; lane 3) but interestingly labelled the p200 antigen by IB (Figure 18 b; lane 3). As negative control, serum from healthy volunteer was used (Figure 18 a, b; lane 4).

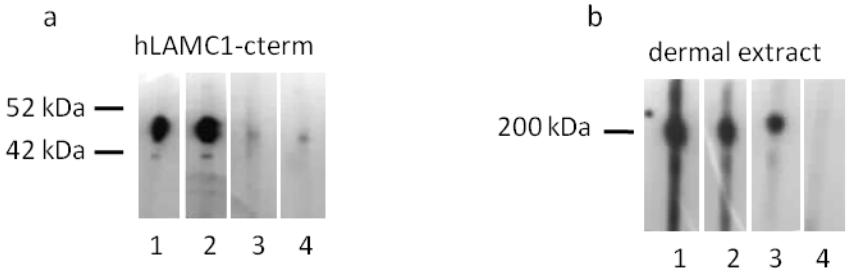

Figure 18. Detection of IgG from anti-p200 pemphigoid serum against the eukaryotic expressed form of hLAMC1-cterm by immunoblotting. Autoantibodies to LAMC1 from patient's serum were purified using a recombinant form of the C-terminus of LAMC1 chain expressed in eukaryotic cells covalently coupled to an agarose matrix. By IB, both eluted specific IgG antibodies (a, b; lane 2) and a serum sample from the same patient (a, b; lane 1) recognized the recombinant protein (a) and the 200 kDa protein of human dermal extract (b). The sera depleted from anti-hLAMC1-cterm reactivity (a, lane 3) showed the same low reactivity as the serum from healthy volunteer (a, lane 4) with the recombinant protein (a). However it showed strong

reactivity with the 200 kDa protein band of the dermal extract (b, lane 3) in contrast to sera from healthy volunteers which did not show any reactivity (b, lane 4), hLAMC1-cterm, C-terminal fragment of human laminin γ1.

Then, the above fractions were examined by indirect IF microscopy for binding of IgG to the dermal-epidermal junction (Figure 19). The affinity purified hLAMC1-cterm-specific IgG, did not label the DEJ by indirect IF microscopy on human skin (Figure 19 b). In contrast, sera and sera depleted from anti-hLAMC1-cterm reactivity labelled the DEJ (Figure 19 a, c). Serum from a healthy volunteer did not label the DEJ and served as negative control (Figure 19d).

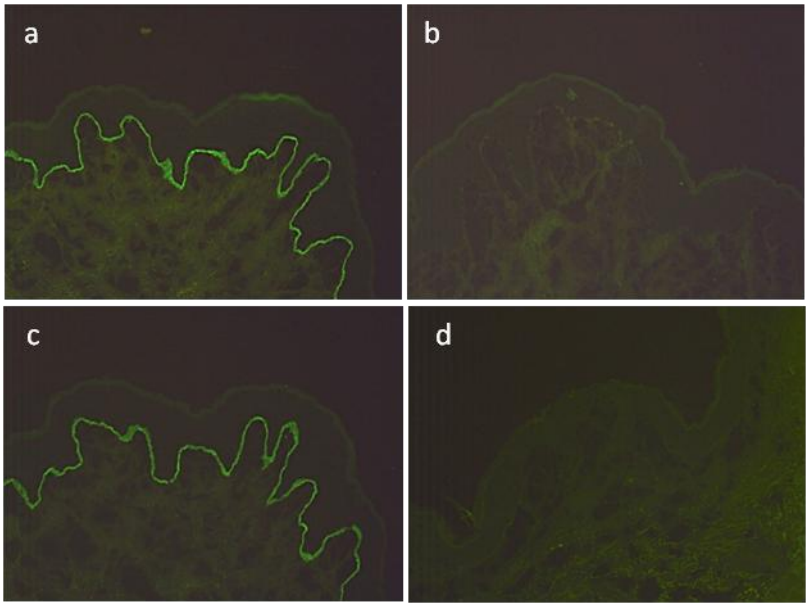

Figure 19. Binding of IgG against the eukaryotic expressed hLAMC1-cterm to human skin by indirect IF microscopy. By indirect immunofluorescence microscopy on foreskin, eluted hLAMC1-cterm-specific IgG (b) showed weak binding to the dermal-epidermal junction (DEJ), whereas serum (a) and sera depleted from anti-hLAMC1-cterm reactivity (c) showed strong IF reactivity. The control serum did not show any reactivity with DEJ (d). Magnification: x200.

The same fractions were also analyzed in the cryosection model. Cryosections of human skin were incubated with sera, purified hLAMC1-cterm-specific IgG, sera depleted from these IgG and sera from healthy volunteers, and consequently with leukocytes suspension for 3 hours. The hLAMC1-

cterm-specific IgG fraction did not induce DES in cryosections of human skin (Figure 20 b). In contrast, anti-p200 pemphigoid sera and sera depleted from IgG against the eukaryotic hLAMC1-cterm reactivity resulted in DES (Figure 20 a, c). As negative control, serum from a healthy volunteer was used (Figure 20 d).

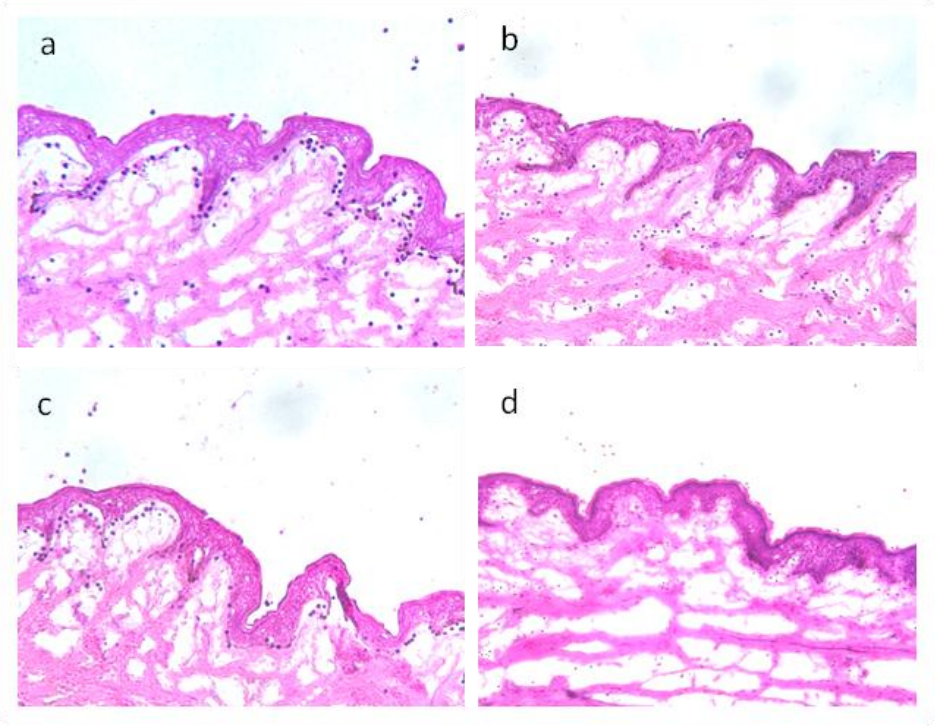

Figure 20. Antibodies against the eukaryotically expressed form of hLAMC1-cterm are not pathogenic in cryosections of human skin. When cryosections of human skin were incubated with hLAMC1-cterm-specific IgG, as well as serum from healthy volunteer, did not show blister-inducing potential (b and d), in contrast to patients' serum (a) and sera depleted from anti-hLAMC1-cterm reactivity (c) which induced dermal-epidermal separation. Magnification: x200.

4.2.5 Antibodies against the heterotrimeric E8 fragment of laminin 111 did not induce split formation in cryosections of human skin

In the study of Dainichi *et al*. the recombinant E8 fragment of laminin 111, a heterotrimer of the truncated C-terminal portions of α1, β1 and γ1 chains, was used. By IB they found that 90% of anti-p200 pemphigoid patients' sera recognized this recombinant E8 fragment of laminin γ1[61]. In the next set of experiments, the recombinant E8 fragment was employed to affinity purify IgG from sera of anti-p200 pemphigoid patients (n=3). Again, IB analysis, indirect IF microscopy, and cryosection model, were performed to test the ability of anti-E8 IgG to induce DES. Similarly to the previous

approaches (prokaryotically and eukaryotically expressed hLAMC1-cterm) the E8-specific IgG reacted with the recombinant protein and the p200 protein in dermal extract by IB, stained weakly the DEJ by indirect IF microscopy but failed to induce DES in the *ex vivo* cryosection assay. In contrast, serum depleted from anti-E8 reactivity reacted with a 200 kDa protein in dermal extract, did not react with the recombinant E8 protein, labelled the DEJ by indirect IF, and induced blisters similar to the unfractionated sera in the *ex vivo* model.

4.2.6 Antibodies against the whole LAMC1 did not induce split formation in cryosections of human skin

In the previous experiments, several C-terminal forms of laminin γ1 were used; however, no pathogenic relevance of anti-hLAMC1-cterm-specific IgG was demonstrated. Subsequently IgG against the whole protein chain of LAMC1 was analysed. In this set of experiments, the entire protein hLAMC1-FL was employed to affinity purify patients' sera (n=3). Again, the affinity-purified IgG reacted with the p200 protein in dermal extract (Figure 21 b, lane 2) stained the dermal-epidermal junction by indirect IF microscopy (Figure 22 b), but did not induce dermal-epidermal separation in cryosection assay (Figure 23 b). In contrast, serum depleted from hLAMC1-FL reactivity did not recognized the hLAMC1-FL (Figure 21 a, lane 3), but reacted with a 200 kDa protein in dermal extract (Figure 21 b lane 3), labelled the dermal-epidermal junction by indirect IF microscopy (Figure 22 c) and induced dermal-epidermal separation similar to the patient's sera in the *ex vivo* model (Figure 23 c).

As a summary of the above sets of experiments, human antibodies to laminin γ1 labelled weakly the dermal-epidermal junction but did not mediate dermal-epidermal separation in the cryosection model.

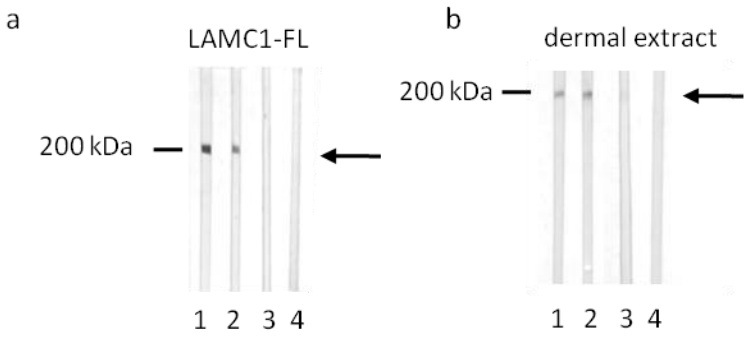

Figure 21. Detection of IgG from anti-p200 pemphigoid serum against laminin γ1 (LAMC1-FL) by immunoblotting. Using the recombinant form of the full length laminin γ1 (LAMC1-FL), IgG specific for

LAMC1-FL (a, b; lane 2) was generated from anti-p200 pemphigoid serum (a, b; lane 1), as well as serum depleted from LAMC1-FL reactivity (a, b; lane 3) respectively, as shown by immunoblotting with recombinant LAMC1-FL (a), and extract of human dermis (b). The serum depleted from LAMC1-FL reactivity (a, b; lane 3) still labeled the p200 protein in dermal extract (b, lane 3). Serum from healthy volunteer did not show any reactivity (a, b; lane 4).

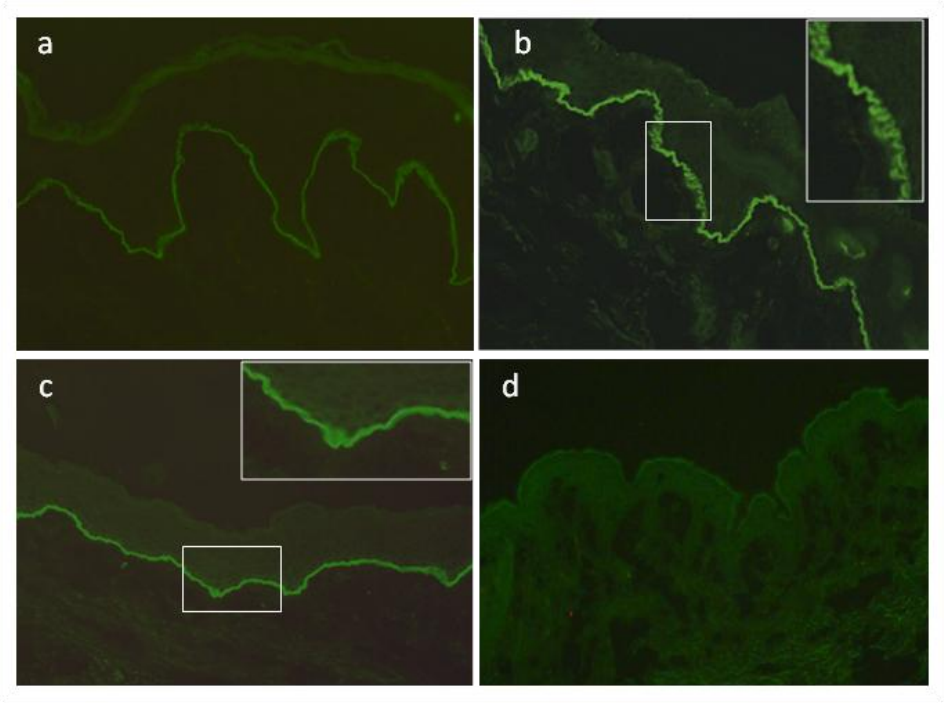

Figure 22. Binding of patient's IgG against laminin γ1 (LAMC1-FL) to human skin by indirect immunofluorescence (IF) microscopy. LAMC1-FL-specific patients IgG labeled the dermal-epidermal junction (DEJ) by indirect IF microscopy (b). Serum depleted from reactivity against LAMC1-FL (c), and patient's serum (a) stained the DEJ of human skin in a linear pattern in comparison to specific anti-hLAMC1-FL IgG (b, c inserts). Serum from a healthy volunteer was used as control (D). Magnification: x200, (adapted from Vafia *et al*.[149]).

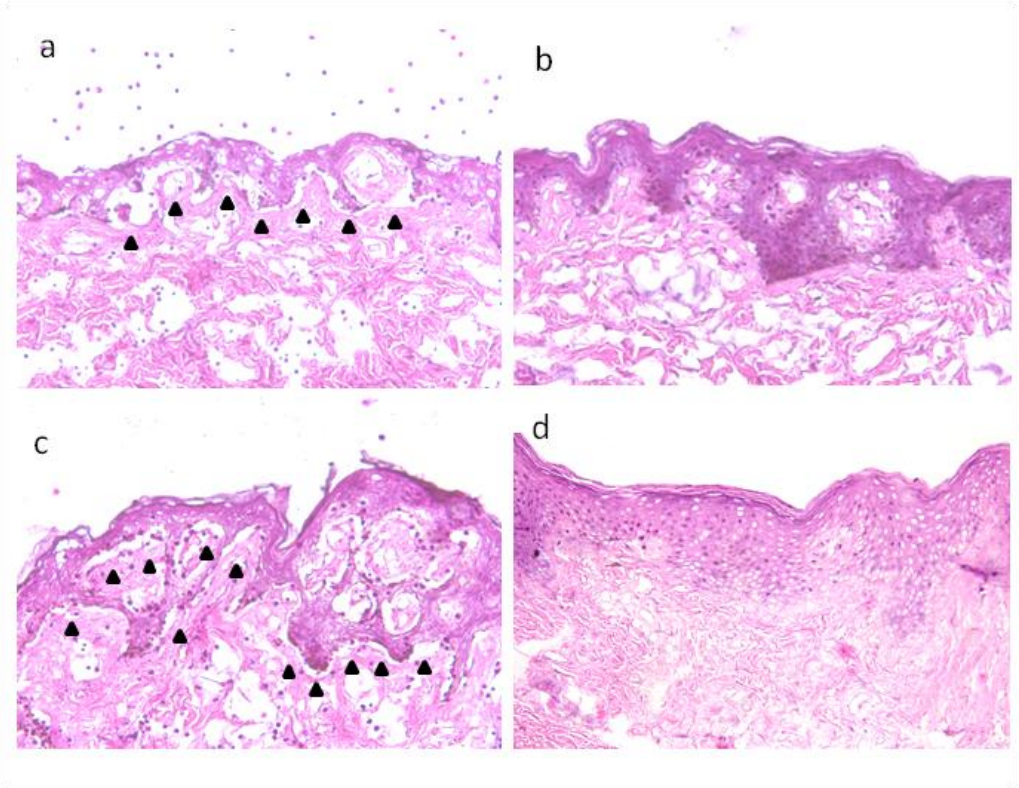

Figure 23. Autoantibodies against laminin γ1 (LAMC1-FL) in anti-p200 pemphigoid sera were not pathogenic in cryosections of human skin. LAMC1-FL-specific patients IgG (b) did not induce dermal epidermal separation (DES). In contrast, serum depleted from reactivity against LAMC1-FL (c), as well as patient's serum (a) resulted in DES (black triangles mark base of the split). Serum from a healthy volunteer was used as control (d). Magnification: x200 (adapted from Vafia *et al*.[149]).

4.2.7 HaCaT keratinocytes showed different staining pattern when incubated with anti-p200 pemphigoid and anti-LAMC1 antibodies

Indirect IF studies in human skin showed different staining patterns when skin was incubated with anti-hLAMC1-cterm IgG and anti-p200 pemphigoid sera (chapter 3.2.3). To further investigate this observation, sera and purified specific IgG were assessed by indirect IF using the human keratinocyte cell line HaCaT. Hofmann *et al*. have shown that the p200 protein is synthesized by cultured keratinocytes[62]. Therefore, HaCaT cells, in 70% confluency, were seeded in cultured slide and fixed with 2% formaldehyde. Cells were incubated with the monoclonal antibody against LAMC1 (clone B4, 20 μg/ml), anti-p200 pemphigoid sera, sera depleted from anti-LAMC1 antibodies, and serum from a healthy volunteer. Two different staining patterns were observed by indirect IF microscopy (Figure 24). In detail, B4 monoclonal antibody showed an intense,

intracellular, dotted, discontinuous, patchy staining pattern (Figure 24 a), in comparison to patient's sera which demonstrated a more homogenous and diffuse staining throughout the cytoplasm (Figure 24 b). A similar pattern was observed when cells were incubated with sera depleted from anti-laminin γ1 reactivity (Figure 24 c). In contrast, no staining was observed with sera from a healthy volunteer, which served as negative control. This observation points to different target antigen, and consequently laminin γ1 and the p200 protein, may not be identical.

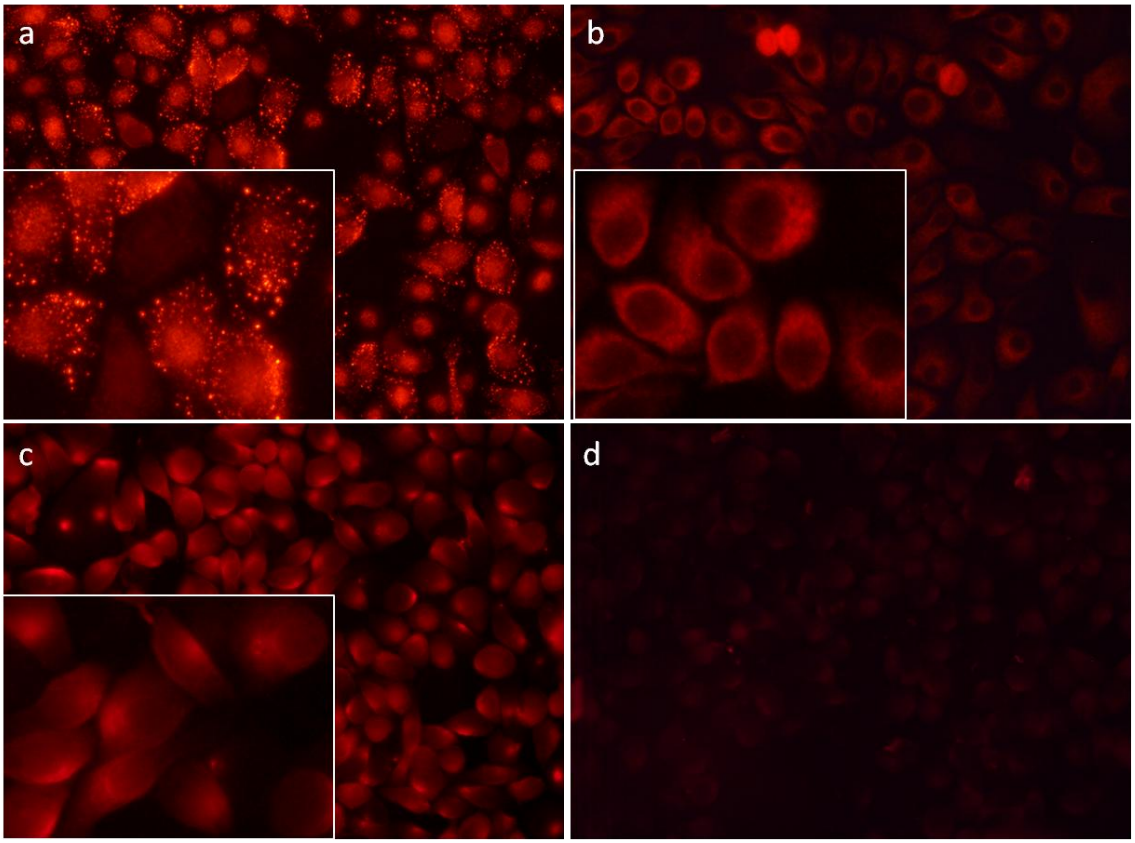

Figure 24. Binding pattern of monoclonal anti-LAMC1-cterm and anti-p200 pemphigoid IgG to cultured keratinocytes. Binding of monoclonal antibody against LAMC1-cterm (a) results in an intense intracellular dotted pattern in comparison to the diffuse staining around the cell membrane in cells treated with anti-p200 pemphigoid sera (b) and anti-p200 pemphigoid sera depleted from hLAMC1-cterm reactivity (c). Serum from a healthy volunteer served as negative control; hLAMC1-cterm, the C-terminal fragment of laminin γ1 (d). Magnification x400.

4.2.8 IgG against the recombinant murine LAMC1-cterm (mLAMC1-cterm) did not induce dermal-epidermal separation

Human and murine laminin γ1 have 89.4% homology (http://embnet.vitalit.ch/software/LALIGN_form.html)). To test the pathogenic effect of antibodies against LAMC1-cterm *in vivo*, murine LAMC1-cterm was generated. Murine LAMC1-cterm was subcloned from obtained vector hLAMC1-cterm-pMA into the vector pQE40 and expressed in the Rosetta 2 *E.coli* strain DE3 as a His-tagged protein. Then, the fragment was purified by immobilized metal affinity chromatography using Talon affinity resin. Each fraction was run in SDS-PAGE and visualized with coomassie blue staining (Figure 25).

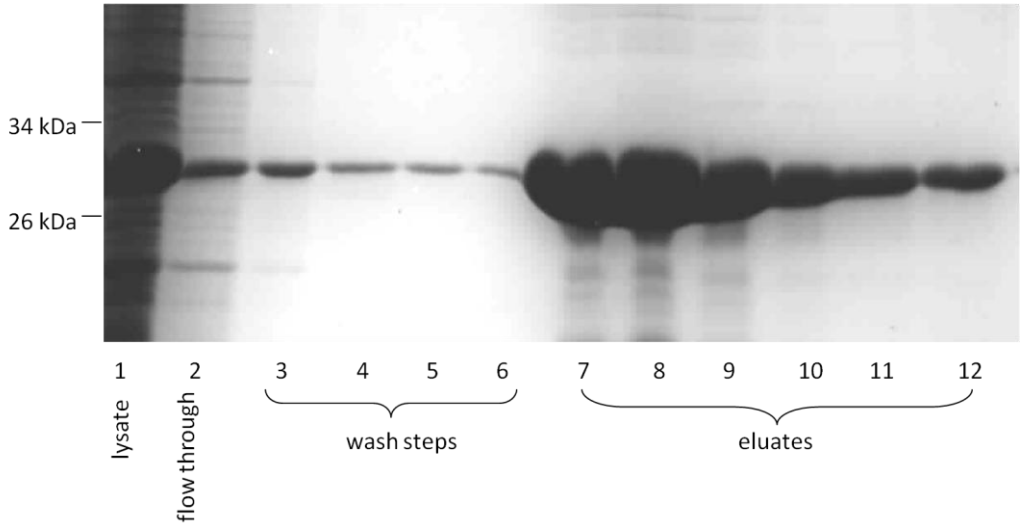

Figure 25. SDS-PAGE of the steps of the His-purification of the recombinant mLAMC1-cterm by immobilized metal affinity chromatography. Lane 1: unpurified protein (lysate). Lane 2: flow through of mLAMC1-cterm lysate after binding of the protein to the TALON resin. Lanes 3-6: wash steps with washing buffer. Lanes 7-12: collected fractions with the purified recombinant His-mLAMC1-cterm protein.

The identity of mLAMC1-cterm was confirmed by immunoblotting using an antibody specific for histidine residues, and a rabbit polyclonal antibody against laminin γ1 (clone H-190, Santa Cruz). Subsequently, rabbit IgG against mLAMC1-cterm was generated, by immunization of rabbits with the recombinant protein, and reacted with a 200 kDa protein in the extract of murine dermis by IB (Figure 26 lane 2). The same band was labelled by the commercial polyclonal anti-murine LAMC1 antibody clone H-190 (Figure 26 lane 1, 1 μg). Both rabbit IgG against mLAMC1-cterm and

polyclonal anti-murine LAMC1 antibody H-190 also recognized the recombinant mLAMC1-cterm, used for the immunization of the rabbits.

Furthermore, the ability of rabbit anti-mLAMC1-cterm antibodies to bind to the DEJ was tested by indirect IF microscopy. Rabbit anti-mLAMC1-cterm IgG stained the DEJ of mouse skin at a titre of 1:100. The staining of the basal keratinocytes (Figure 27 b) was similar to the extracellular staining pattern seen with patient hLAMC1-cterm specific IgG on human skin (Figure 15 c, insert). Faint binding was also observed with the polyclonal anti-LAMC1 antibody H-190 by indirect IF microscopy on murine skin (Figure 27 a).

The pathogenicity of rabbit anti-mLAMC1-cterm antibodies was tested by the *ex vivo* cryosection model on mouse skin. Rabbit anti-mLAMC1-cterm IgG did not recruit neutrophils at the DEJ and did not induce DES (Figure 28 c), although the titres tested by immunoblotting with murine dermal extract were very high (>1:10^5). Same findings were obtained using the polyclonal LAMC1 antibody H190 (Figure 28 b) and preimmune rabbit IgG (Figure 28 d). In contrast, as shown previously[136], rabbit anti-mBP180 NC15A IgG (reactive against the immunodominant region of BP180) resulted in DES (Figure 28 a).

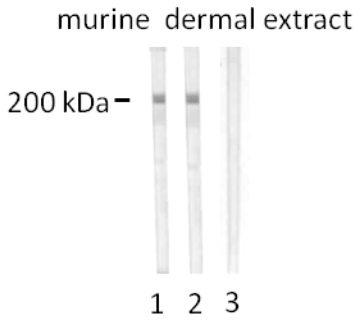

Figure 26. Immunoblotting of the rabbit IgG generated against the murine laminin γ1 C-terminus (mLAMC1-cterm). Rabbit anti-mLAMC1-cterm (lane 2) and commercial rabbit antibody H-190 against mLAMC1 (lane 1) recognized the p200 protein by immunoblotting with extract of murine dermis. In contrast, the preimmune rabbit IgG, which was used as negative control, did not show reactivity (lane 3), (adapted from Vafia *et al*.[149]).

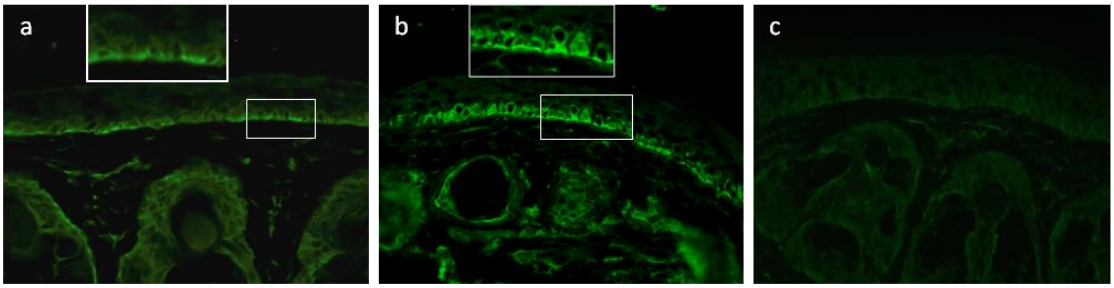

Figure 27. Rabbit IgG generated against the mLAMC1-cterm labeled the mouse skin by indirect IF microscopy. Commercial rabbit antibody H-190 against mLAMC1 and rabbit anti-mLAMC1-cterm labeled the DEJ (a, b). In addition to linear binding at the DEJ, this rabbit IgG stained the cell surface of the basal portion of basal keratinocytes by indirect immunofluorescence microscopy on murine skin (a, b, inserts) similar to that seen with monoclonal anti-hLAMC1-antibody and hLAMC1-cterm-specific patient IgG (Figure 15 a, c). Preimmune rabbit IgG (c) was used as negative control. Magnification: x400, (adapted from Vafia *et al*. [149]).

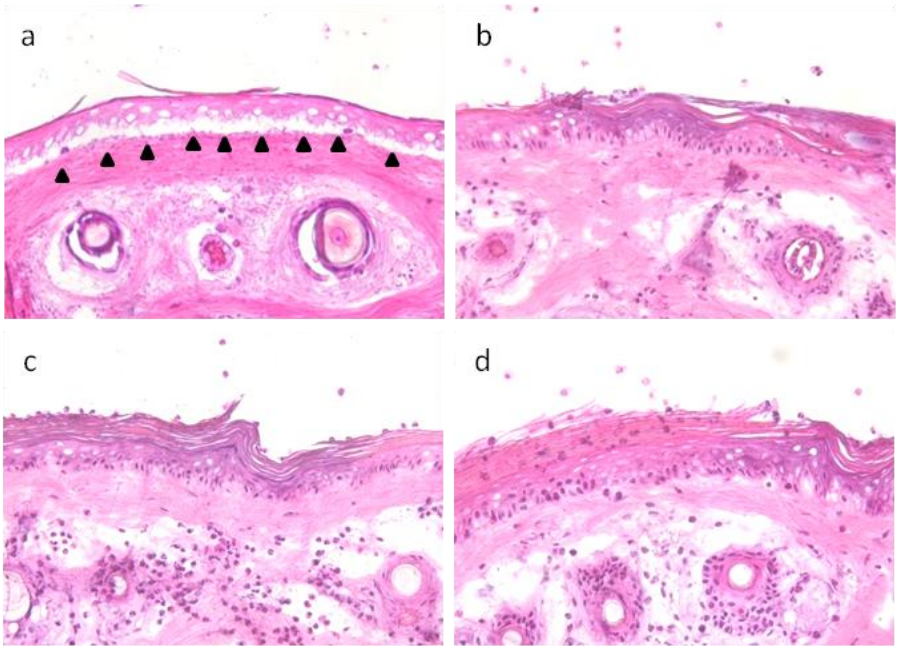

Figure 28. Rabbit IgG generated against the mLAMC1-cterm did not induce DES in cryosections of mouse skin. Commercial rabbit antibody H-190 against mLAMC1 and rabbit anti-mLAMC1-cterm did not induce dermal-epidermal separation (DES) in cryosections of mouse skin (b, c). In contrast to the rabbit IgG against murine BP180 NC15A (a), used as positive control, induced splits in cryosections of mouse skin. Black triangles mark base of the split. Also, preimmune rabbit IgG, served as negative control, did not induced DES (d). Magnification: x400 (adapted from Vafia *et al*. [149]).

4.2.9 Passive transfer of IgG into neonatal mice

Previously, the injection of rabbit IgG against the murine homologue of immunodominant human BP180 into neonatal mice reproduced important clinical and immunopathological features of human BP[109]. Antibodies against human laminin γ1 did not cross-react with murine skin[62]. Therefore, rabbit anti-mLAMC1-cterm antibodies purified from rabbits immunized with the murine LAMC1-cterm were used for passive transfer studies in mice. First, 8 C57BL/6 neonatal mice were injected with rabbit anti-mLAMC1-cterm IgG and 2 C57BL/6 with preimmune rabbit IgG (normal IgG), served as control, respectively, at a concentration of 10 mg/g body weight. In none of the mice clinical disease was observed (Figure 29) and histopathological examinations of back skin did not reveal dermal inflammation or subepidermal blisters (Figure 30 a). Direct IF microscopy of back skin showed linear deposits of rabbit IgG in 2 out of 8 mice injected with anti-mLAMC1-cterm IgG, while in none of the mice, deposits of murine complement C3 were detected at the DEJ (Figure 30 b and c). All sera, from mice injected with mLAMC1-cterm IgG, taken on day 12 showed IgG staining of the DEJ by indirect IF microscopy of normal mouse skin at titres between 1:30 and 1:40 (Figure 30 d). Staining was observed on the basal layer of keratinocytes at the DEJ (Figure 30 d, insert). In contrast no IgG deposition was observed in control mice. In all mice injected with anti-mLAMC1-cterm IgG, strong reactivity with the recombinant protein was also detected by ELISA (Figure 31) and IB, as well as mice sera labelled the 200 kDa protein in extract of murine dermis (Figure 32 a and b). In contrast, sera from control mice did not react with the mLAMC1-cterm by ELISA (Figure 31), they did not label the recombinant protein by immunoblotting neither the 200 kDa protein by immunoblotting in extract of murine dermis.

Figure 29. Rabbit IgG against the murine laminin γ1 C-terminus (mLAMC1-cterm) was not pathogenic when passively transferred into neonatal C57BL/6 mice. Representative 12-day old C57BL/6 mouse did not develop any blister after injection of rabbit anti-mLAMC1-cterm IgG at a concentration of 10 mg/g body weight every second day for 10 days.

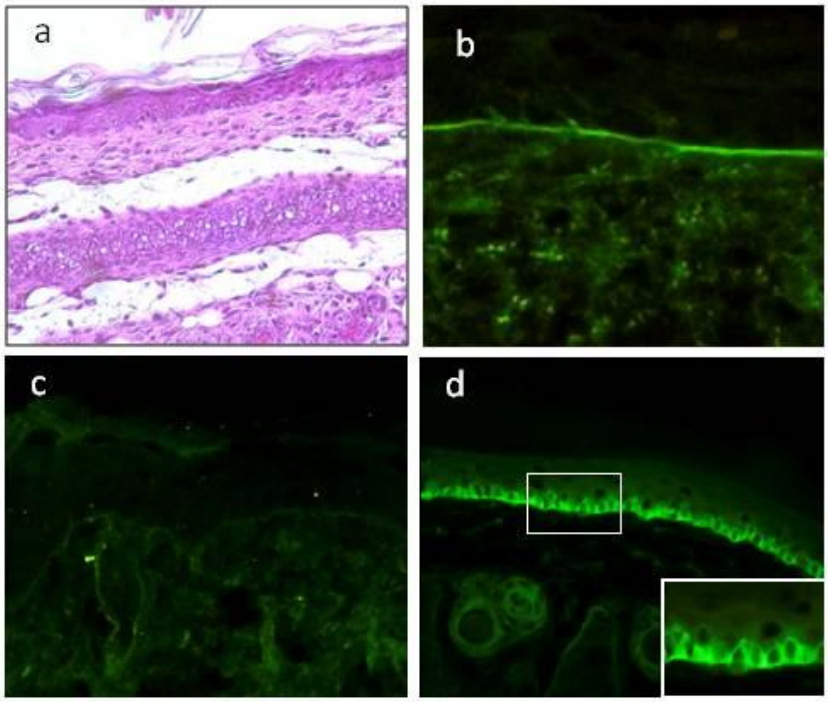

Figure 30. Histological and serological examination of neonatal C57BL/6 mice injected with rabbit IgG against the murine laminin γ1 C-terminus (mLAMC1-cterm). Injection of rabbit anti-mLAMC1-cterm IgG at a concentration of 10 mg/g body weight every second day for 10 days did not result in histopathological (a) lesions on day 12. Linear deposition of rabbit IgG at the dermal-epidermal junction was only observed in 2 of 8 mice (b), while staining of murine C3 was always negative (c) by direct IF microscopy. In sera of all mice, rabbit IgG stained the DEJ of normal mouse skin by indirect IF microscopy (d) (adapted from Vafia *et al*.[149]).

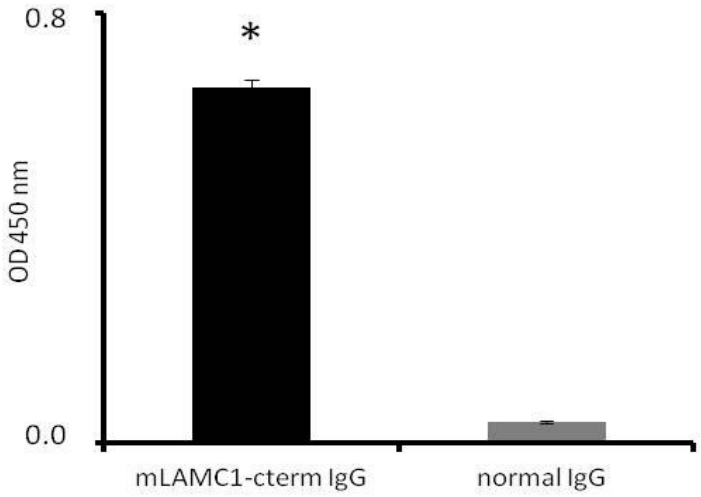

Figure 31. Mice sera tested for mLAMC1-cterm reactivity by ELISA. Sera from neonatal mice (n=8), injected with rabbit anti-mLAMC1-cterm antibodies, reacted with recombinant mLAMC1-cterm by ELISA giving high values of OD_{450nm} (mLAMC1-cterm IgG). In contrast mice injected with normal rabbit IgG (purified from preimmune rabbit IgG) did not show any reactivity (normal IgG) *p<0.01 by two sided Student's t Test (adapted from Vafia *et al*.[149]).

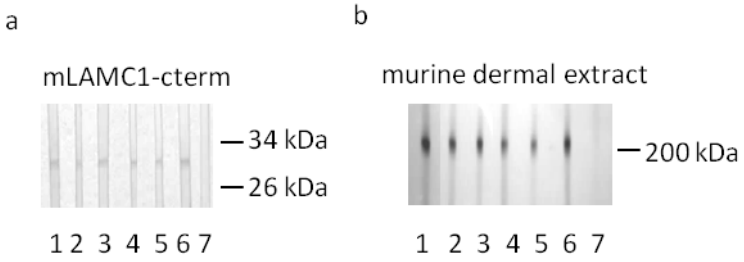

Figure 32. Immunoblotting of sera from neonatal mice injected with rabbit mLAMC1-cterm IgG and normal IgG. Representative blot of 6 mice sera, at day 12, in which rabbit IgG labeled the recombinant mLAMC1-cterm (a, lanes 2-6) and a 200 kDa protein in extract of murine dermis by IB (b, lanes 2-6). Polyclonal rabbit antibody H-190 against mLAMC1 (a, b; lane 1) and normal mouse serum (a, b; lane 7) were used as controls; mLAMC1-cterm, C-terminus of murine laminin γ1 (adapted from Vafia *et al*.[149]).

4.2.10 Passive transfer of specific mLAMC1-cterm IgG into neonatal mice

To exclude the possibility of insufficient amounts of total rabbit IgG, anti-mLAMC1-cterm IgG was further affinity-purified with mLAMC1-cterm. This IgG fraction was injected into neonatal mice

(n=3) at a concentration of 1 mg/g of body weight. Control mice were injected with IgG purified from preimmune rabbit sera (n=2). The results were similar to the experiments of the transfer of the total IgG to neonatal mice (chapter 4.2.9). None of the mice developed clinical disease. Only 1 out of 3 neonatal mice injected with the specific anti-mLAMC1-cterm IgG showed IgG deposition along the dermal-epidermal junction by direct IF microscopy. However, high levels of circulating antibodies were detected by immunoblotting of mLAMC1-cterm and mouse dermal extract labelling a 200 kDa protein. Circulting antibodies were also observed by indirect IF microscopy in normal mouse skin (Figure 33). Control mice did not show any clinical disease, sera did not demonstrate any IgG deposition in dermal-epidermal junction by indirect IF microscopy and did not label the recombinant protein or the 200 kDa protein by immunoblotting.

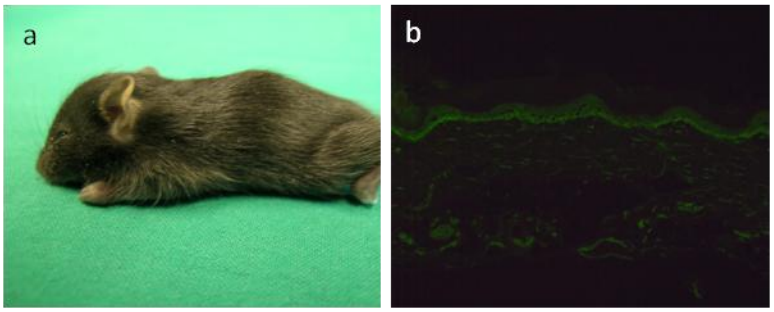

Figure 33. Passive transfer of specific mLAMC1-cterm IgG into neonatal mice did not result in clinical disease. Representative 12-day old C57BL/6 mouse did not develop any blister after injection of rabbit anti-mLAMC1-cterm specific IgG at a concentration of 1 mg/g body weight every second day for 10 days (a). However, mouse serum demonstrated IgG deposition along the DEJ on mouse skin by inirect immunofluorescence microscopy (b). mLAMC1-cterm, C-terminus of murine laminin γ1.

4.2.11 Passive transfer of IgG into adult mice

Then, rabbit anti-mLAMC1-cterm IgG was injected in adult mice following a previously established protocol for experimental epidermolysis bullosa acquisita[136]. C57BL/6 (n=5) and BALB/c (n=5) adult mice were injected, every second day, for 12 days, with 15 mg of purified IgG from rabbits immunized with mLAMC1-cterm. For control, one mouse per strain was injected with preimmune or normal (not immunized) rabbit IgG. None of the mice treated with IgG to mLAMC1-cterm or normal IgG showed clinical signs (Figure 34) or histopathological skin lesions (Figure 35). Direct IF

microscopy of perilesional mouse skin revealed linear, although weak deposits of rabbit IgG in 2 out of 5 C56BL/6 and 1 BALB/c mice injected with mLAMC1-cterm IgG (Figure 36), while murine complement C3 at the DEJ was negative in all cases (Figure 37). In addition, all mice injected with antibodies from immunized rabbits demonstrated depositions of IgG at the DEJ of skin sections (Figure 38). The indirect IF titres of circulating rabbit IgG in the mice ranged from 30 to 40. In contrast, there were no deposits of rabbit IgG at the DEJ in mice injected with control IgG.

Circulating autoantibodies to mLAMC1-cterm were measured at day 4, 6, 8, 10, and 12 by ELISA using recombinant mLAMC1-cterm. From the day 4 the levels of IgG, in mice injected with anti-mLAMC1-cterm IgG, were high and remained at high values until the end of the experiment (Figure 39). By immunoblot analysis, IgG targeted both the recombinant form of mLAMC1-cterm (Figure 40 a) and the 200 kDa protein at murine dermal extracts as a substrate (Figure 40 b). In contrast, sera from control mice did not react with the mLAMC1-cterm by ELISA (Figure 39), they did not label the recombinant protein by immunoblotting neither the 200 kDa protein by immunoblotting in extract of murine dermis.

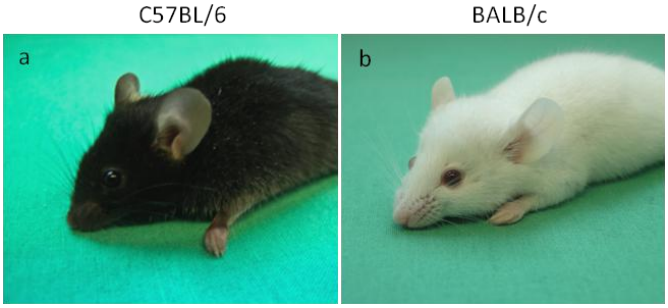

Figure 34. Rabbit IgG against the mLAMC1-cterm was not pathogenic when passively transferred into adult C57BL/6 and BALB/c mice. Here, representative C57BL/6 (a) and BALB/c (b) mice at day 12, after 6 injections of 15 mg rabbit anti-mLAMC1-cterm IgG every second day, did not show any clinical manifestations. mLAMC1-cterm, C-terminus of murine laminin γ1.

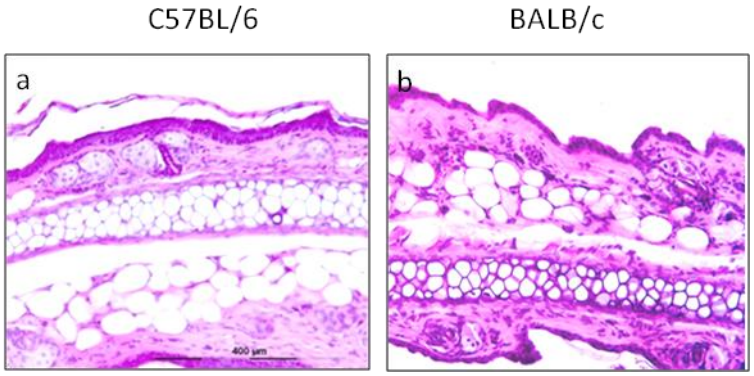

Figure 35. Histological examination of mice injected with rabbit IgG against the mLAMC1-cterm. Injection of 15 mg rabbit anti-mLAMC1-cterm IgG every second day for 10 days did not result in histopathological lesions on day 12 in C57BL/6 (a) and BALB/c (b) mice. Here are shown representative examples of a C57BL/6 and a BALB/c mouse. mLAMC1-cterm, C-terminus of murine laminin γ1 (adapted from Vafia *et al*.[149]).

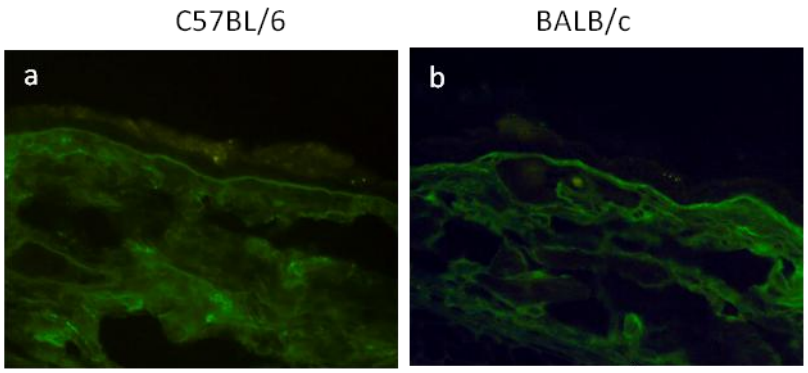

Figure 36. Direct immunofluorescence microscopy of mice injected with rabbit IgG against the mLAMC1-cterm. Linear deposition of rabbit IgG at the dermal-epidermal junction was observed in 2 of 5 C57BL/6 (a) and 1 of 5 BALB/c mice (b). Here are shown representative examples of a C57BL/6 and a BALB/c mouse with a positive direct IF staining at day 12. mLAMC1-cterm, C-terminus of murine laminin γ1 (adapted from Vafia *et al*.[149]).

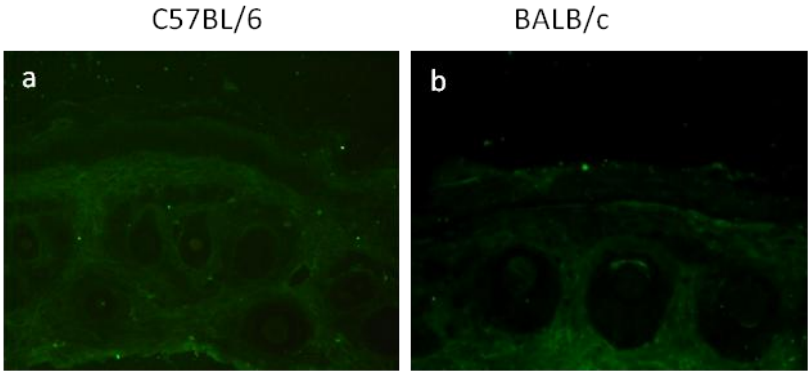

Figure 37. Direct immunofluorescence microscopy showed no C3 deposition in mice injected with rabbit IgG against the mLAMC1-cterm. Staining of murine C3 was negative in all C57BL/6 (a) and BALB/c mice (b) at day 12 after 6 injections of 15 mg rabbit anti-mLAMC1-cterm IgG every second day for 10 days. Here are shown reperesentative examples of a C57BL/6 and a BALB/c mouse. mLAMC1-cterm, C-terminus of murine laminin γ1 (adapted from Vafia *et al*.[149]).

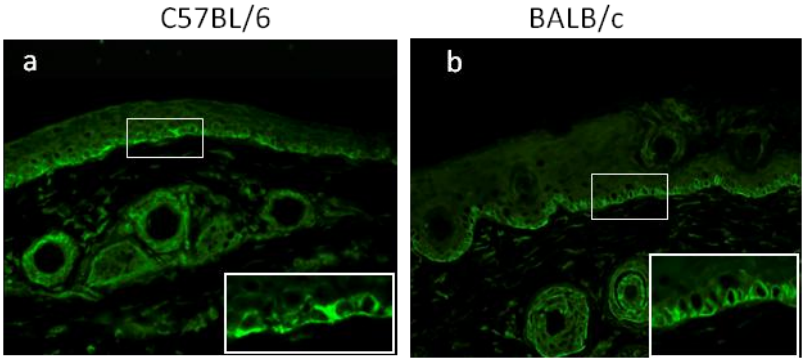

Figure 38. Indirect immunofluorescence (IF) microscopy examination in mice injected with rabbit IgG against the mLAMC1-cterm. At day 12, and after 6 injections of 15 mg rabbit anti-mLAMC1-cterm IgG every second day, in sera of all C57BL/6 (a) and BALB/c (b) mice, rabbit IgG labeled the dermal-epidermal junction of normal mouse skin by indirect IF microscopy. Here are shown representative examples of a C57BL/6 and a BALB/c mouse. mLAMC1-cterm, C-terminus of murine laminin γ1 (adapted from Vafia *et al*.[149]).

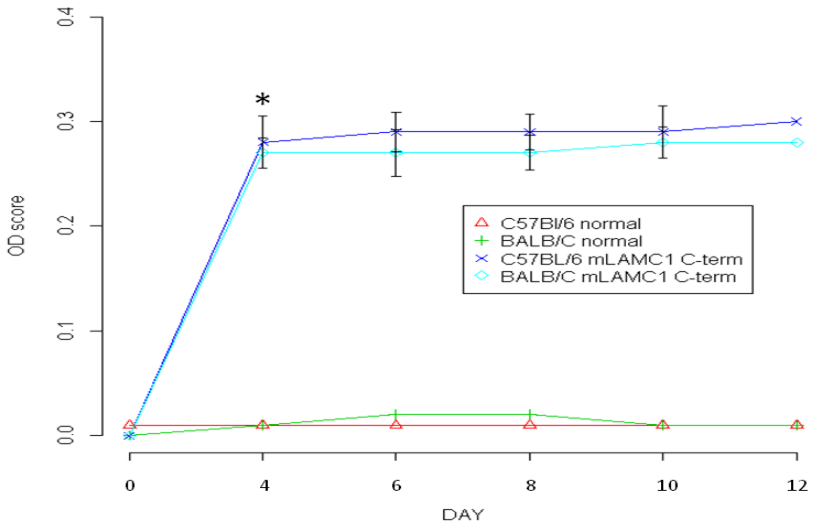

Figure 39. Mice sera tested for mLAMC1-cterm reactivity at different time points by ELISA. Sera from C57BL/6 (n=5) and BALB/c mice (n=5) injected with rabbit anti-mLAMC1-cterm antibodies reacted with the recombinant mLAMC1-cterm by ELISA giving high values of OD_{450nm} from day 4 (C57BL/6 mLAMC1 C-term and BALB/c mLAMC1 C-term). In contrast, mice injected with normal rabbit IgG (purified from preimmune rabbit IgG did not show any reactivity (C57BL/6 normal and BALB/c normal) *p<0.01 by two sided Student's t Test, mLAMC1-cterm, C-terminus of murine laminin γ1; OD, optical density; ELISA, enzyme-linked immunosorbent assay; C57BL/6 normal, C57BL/6 injected with preimmune rabbit IgG; BALB/c normal, BALB/c mice injected with preimmune rabbit IgG; C57BL/6 mLAMC1 C-term, C57BL/6 mice injected with the recombinant C-terminal fragment of laminin γ1; BALB/c mLAMC1 C-term, BALB/c mice injected with the recombinant C-terminal fragment of laminin γ1, OD, optical density.

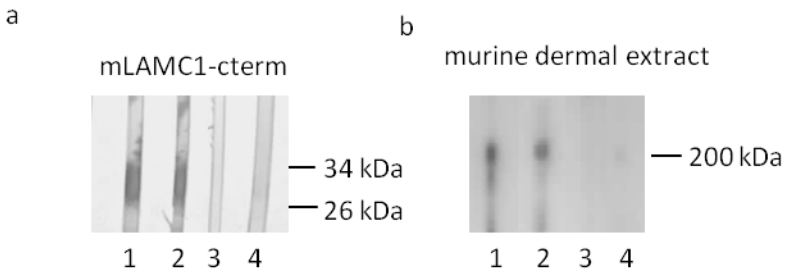

Figure 40. Immunoblotting of sera from mice injected with rabbit mLAMC1-cterm IgG and normal IgG. At day 12, sera from all mice were tested for mLAMC1-cterm reactivity by IB. Here representative blots from C57BL/6 (a, b; lane 1) and BALB/c (a, b; lane 2) mice rabbit IgG labeled the recombinant mLAMC1-cterm (a)

and the 200 kDa protein in extract of murine dermis by IB (b). Mice C57BL/6 (a, b; lane 3) and BALB/c (a, b; lane 4) injected with normal rabbit IgG were used as controls; mLAMC1-cterm, C-terminal fragment of murine laminin γ1 (adapted from Vafia *et al*.[149]).

4.2.12 Passive transfer of specific mLAMC1-cterm IgG into adult mice

Similar to neonatal mice mLAMC1-cterm-specific IgG was purified from total rabbit IgG obtained from rabbits immunized with mLAMC1-cterm. One mg of anti-mLAMC1-cterm specific IgG was injected into C57BL/6 adult mice (n=3) on day 0 and every second day for in total 6 injections. The results were similar to the experiments of the transfer of the specific IgG to neonatal mice (chapter 4.2.10). Likewise, no clinical signs were observed, while IgG deposition along the DEJ was seen in 2 mice by direct IF microscopy. In contrast, high levels of circulating anti-murine p200 (by IB of murine dermis), and anti-mLAMC1-cterm (by IB of mLAMC1-cterm) were found in the sera of all mice injected with specific mLAMC1-cterm IgG. Control mice did not show any clinical disease, sera did not demonstrate any IgG deposition in dermal-epidermal junction by indirect IF microscopy and did not label the 200 kDa protein by immunoblotting or the recombinant protein by immunoblotting or ELISA.

4.2.13 Immunization with recombinant mLAMC1-cterm induced autoantibody production in mice of different strains but no clinical lesions

Following previously established protocols for the induction of experimental epidermolysis bullosa acquisita and bullous pemphigoid[143, 144, 150], C57BL/6 (n=5), BALB/c (n=5), and SJL (n=5) mice were immunized 4 times with 60 μg of recombinant mLAMC1-cterm and equal amount of TiterMax®. Control C57BL/6 (n=1), BALB/c (n=1), and SJL (n=1) were immunized with an emulsion containing PBS and TiterMax®. After 16 weeks, no clinical (Figure 41) or histopathological changes were detected in mLAMC1-cterm-immunized mice of the 3 strains (Figure 42). By direct IF microscopy of perilesional skin, 2 C57BL/6 mice immunized with the recombinant protein showed faint deposits at the dermal-epidermal junction (Figure 43). Murine C3 deposition at the dermal-epidermal junction was observed in none of the mice (Figure 44). Moreover, indirect IF microscopy on murine skin demonstrated IgG deposition along the DEJ (at a titre of 1:30; Figure 45). Also, all mice sera contained antibodies against recombinant mLAMC1-cterm as it was shown by IB (titre $1:10^8$, Figure 46 a), and ELISA (Figure 47), and they recognized the p200 protein in extract of murine dermis (titre 1:5,000, Figure 46 b). In contrast, sera from mice immunized with PBS and TiterMax® did not react

with the mLAMC1-cterm by ELISA (Figure 47), they did not label the recombinant protein by immunoblotting neither the 200 kDa protein by immunoblotting in extract of murine dermis (Figure 46).

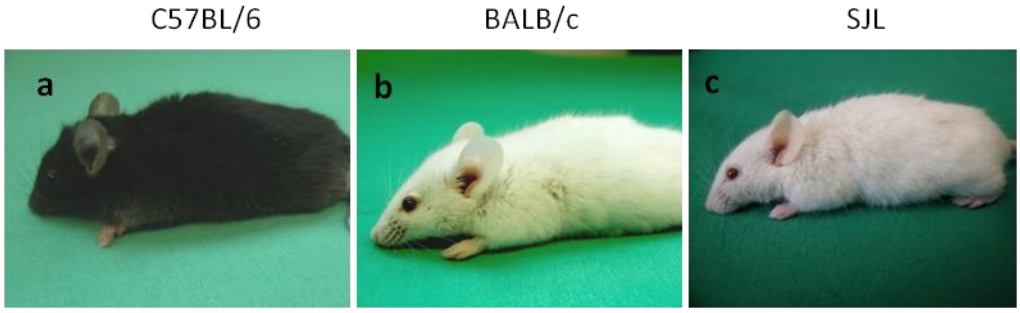

Figure 41. Immunization of different mouse strains with the recombinant mLAMC1-cterm did not induce clinical disease. Three different mouse strains, C57BL/6, BALB/c, and SJL; (n=5/strain) were immunized 4 times with 60 µg of recombinant mLAMC1-cterm in conjunction with TiterMax®. Here, representative photos of a C57BL/6 (a), a BALB/c (b) and an SJL (c) mouse show no clinical manifestations 16 weeks after the first immunization. mLAMC1-cterm, C-terminal fragment of murine laminin γ1.

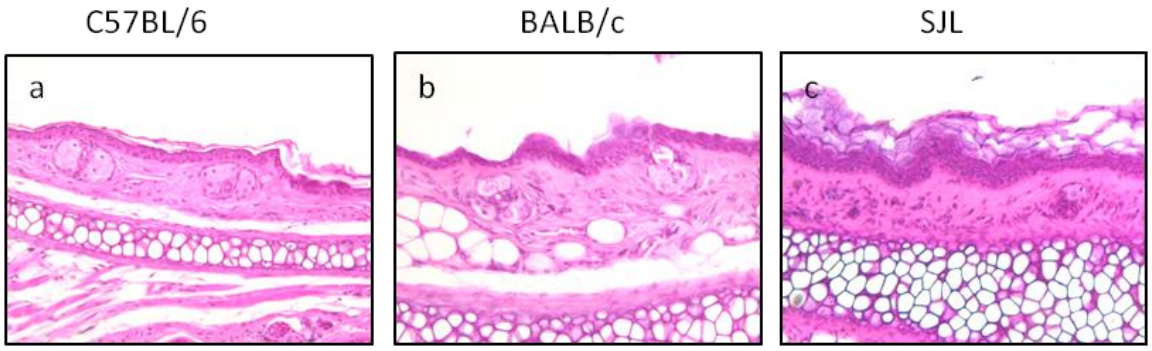

Figure 42. Histological examination of mice immunized with the recombinant mLAMC1-cterm. Sixteen weeks after the first immunization, no histopathological changes were seen in any of the mice. Here, representative histological photos from a C57BL/6 (a), a BALB/c (b), and an SJL (c) mouse are shown, mLAMC1-cterm, C-terminal fragment of murine laminin γ1 (adapted from Vafia *et al.*[149]).

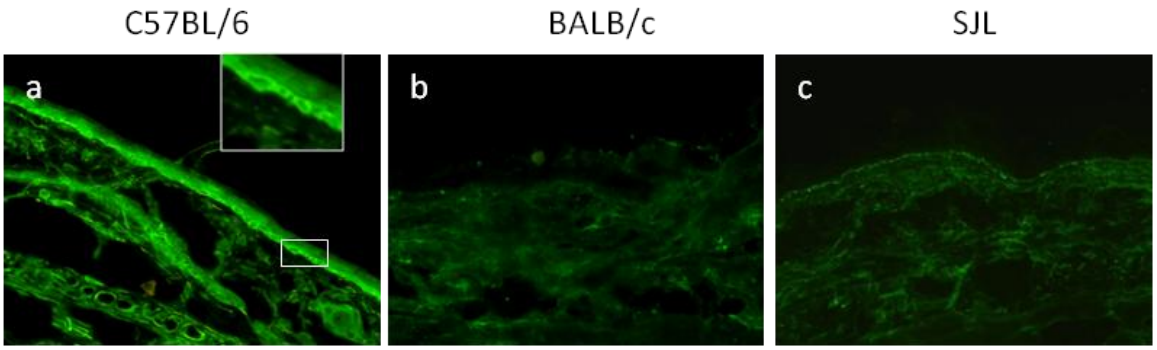

Figure 43. Direct immunofluorescence microscopy of mice immunized with the recombinant mLAMC1-cterm. Deposition of mouse IgG at the dermal-epidermal junction (DEJ) was found in only 2 C56BL/6 mice (a). In all other mice, neither BALB/c (b), nor SJL (c) demonstrated IgG deposition, 16 weeks after the first immunization. Here are shown representative photos of each strain; mLAMC1-cterm, C-terminal fragment of murine laminin γ1 (adapted from Vafia *et al*.[149]).

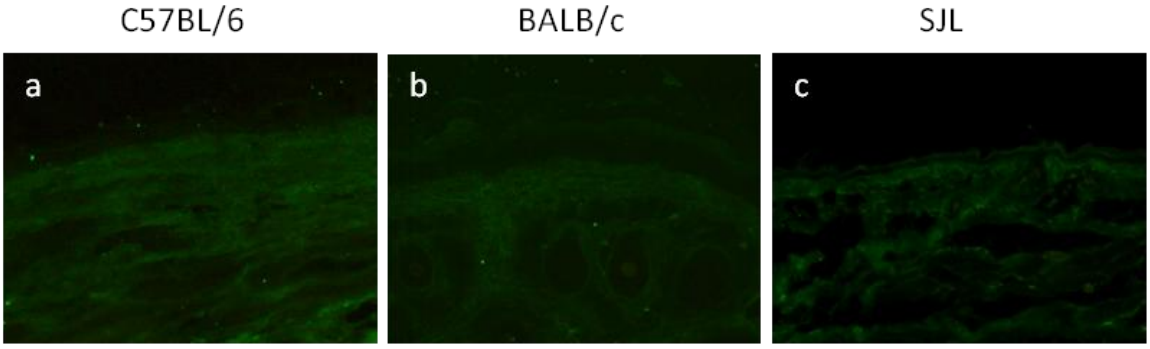

Figure 44. Direct immunofluorescence microscopy showed no C3 deposition in mice immunized with the recombinant mLAMC1-cterm. Staining of murine C3 was negative in all C57BL/6 (a), BALB/c (b), and SJL (c) mice after 16 weeks and 3 times immunization of mLAMC1-cterm. Here, representative photos of each strain are shown; mLAMC1-cterm, C-terminal fragment of murine laminin γ1 (adapted from Vafia *et al*.[149]).

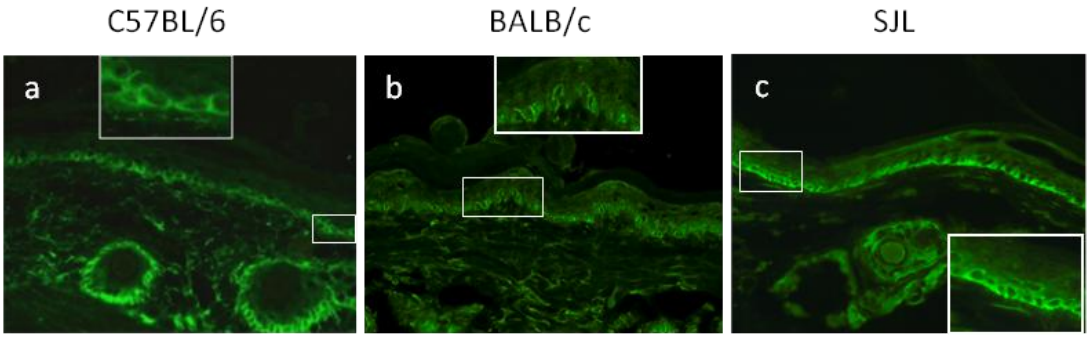

Figure 45. Immunization of different mouse strains with the recombinant mLAMC1-cterm induced high levels of anti-mLAMC1-cterm antibodies. Serum autoantibodies from all mice immunized with the mLAMC1-cterm labeled the DEJ of normal murine skin. Here representative photos of indirect IF microscopy of a C57BL/6 (a), a BALB/c (b), and an SJL (c) mouse 16 weeks after the first immunization, mLAMC1-cterm, C-terminal fragment of murine laminin γ1 (adapted from Vafia *et al*.[149]).

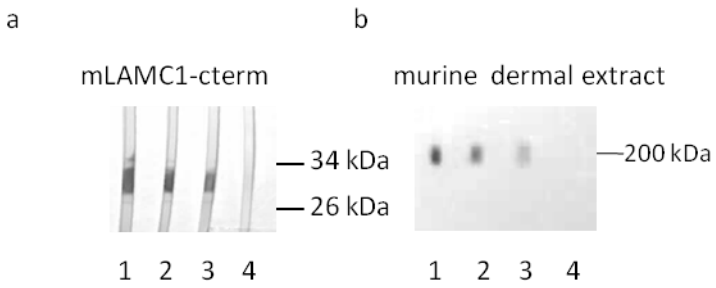

Figure 46. Immunoblotting of sera from mice immunized with mLAMC1-cterm and PBS. Representative blots of sera from C57BL/6, BALB/c mice and SJL mice immunized with mLAMC1-cterm reacted with the recombinant mLAMC1-cterm (a; C57BL/6, lane 1; BALB/c, lane 2; SJL, lane 3) and a 200 kDa protein in dermal extracts (b; C57BL/6, lane 1; BALB/c, lane 2; SJL, lane 3) by IB. In contrast mice immunized with PBS and TiterMax® did not show any reactivity in any of the membranes (a and b; lane 4); mLAMC1-cterm, C-terminal fragment of murine laminin γ1 (adapted from Vafia *et al*.[149]).

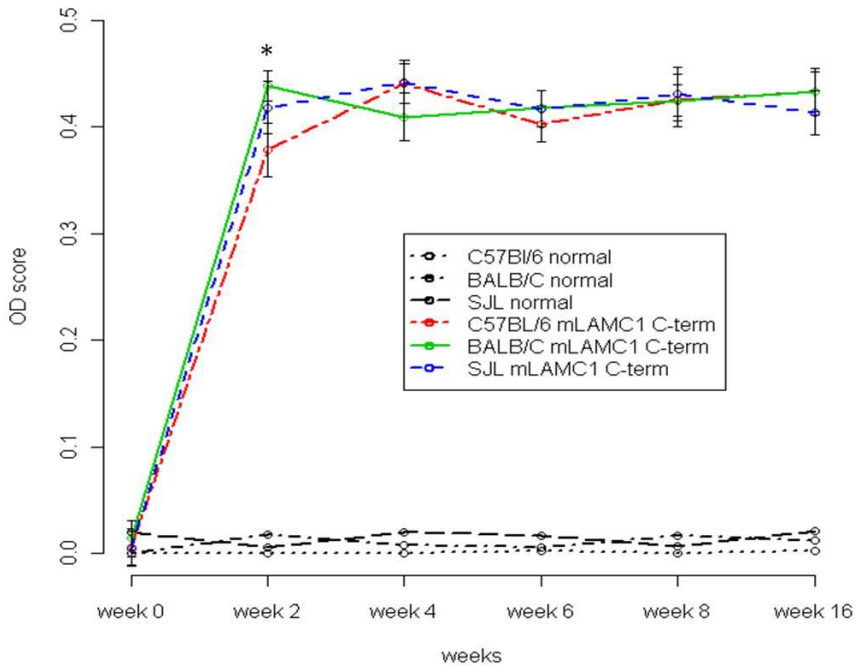

Figure 47. Mice sera tested for mLAMC1-cterm reactivity at different time points by ELISA. Sera from C57BL/6 (n=5), BALB/c mice (n=5) and SJL (n=5) mice immunized with mLAMC1-cterm reacted with the recombinant mLAMC1-cterm by ELISA giving high values of OD_{450nm} from week 4 (C57BL/6 mLAMC1 C-term, BALB/c mLAMC1 C-term, SJL mLAMC1 C-term). In contrast, mice immunized PBS and TiterMax® emulsified with PBS (C57BL/6 normal, BALB/c normal, SJL normal) did not show any reactivity *$p<0.01$, by two sided Student's t Test; C57BL/6 mLAMC1 C-term, C57BL/6 mice immunized with the C-terminus of laminin γ1; BALB/c mLAMC1 C-term, BALB/c mice immunized with the C-terminus of laminin γ1; SJL mLAMC1 C-term; SJL mice immunized with the C-terminus of laminin γ1; C57BL/6 normal, C57BL/6 mice immunized with PBS; BALB/c normal, BALB/c mice immunized with PBS; SJL normal, SJL mice immunized with PBS

5 DISCUSSION

5.1 Anti-p200 pemphigoid patients can be diagnosed by a novel ELISA using a recombinant fragment of laminin γ1

Autoimmune skin diseases share many common characteristics; however each disease is unique in clinical presentation, histopathology, and immunofluorescence patterns which demand specific diagnosis and efficient treatment. Accurate diagnosis allows the patients to start an appropriate and disease-specific treatment, which in many cases is complex and requires knowledge of pathogenesis.

Anti-p200 pemphigoid is a rare autoimmune blistering disease described in 1996[54]. The gold standard for the diagnosis of this group of diseases is the detection of tissue-bound autoantibodies or C3 component of the complement system in perilesional patient's skin or mucous membranes. For the diagnosis of anti-p200 pemphigoid further serological examinations are needed. By indirect IF microscopy on salt-split normal human skin, sera from anti-p200 pemphigoid patients label the dermal side of the artificial split. A similar binding pattern is seen with sera from patients with epidermolysis bullosa acquisita and anti-laminin 332 mucous membrane pemphigoid. In order to distinguish anti-p200 pemphigoid from these autoimmune blistering diseases, detection of serum antibodies against a 200 kDa protein in the extract of human dermis by immunoblotting is required [54]. This assay is highly depended on the extraction method and only available in a few specialized laboratories [60, 61]. This makes the diagnosis of this disease insufficient, and patients are probably misdiagnosed as epidermolysis bullosa acquisita or bullous pemphigoid. Therefore, a simple, sensitive and specific diagnostic assay should be advantageous. The diagnostic pathway in anti-p200 pemphigoid is shown in Figure 48. Exact diagnosis of the different subepidermal immunobullous disorders is important since prognoses differ and more specific treatment modalities have increasingly been adapted for these disorders. E.g., anti-p200 pemphigoid appears to have a more benign course compared to other entities and potent topical corticosteroids, in some patients combined with dapsone, have been found sufficient to achieve clinical remission in the majority of patients [57].

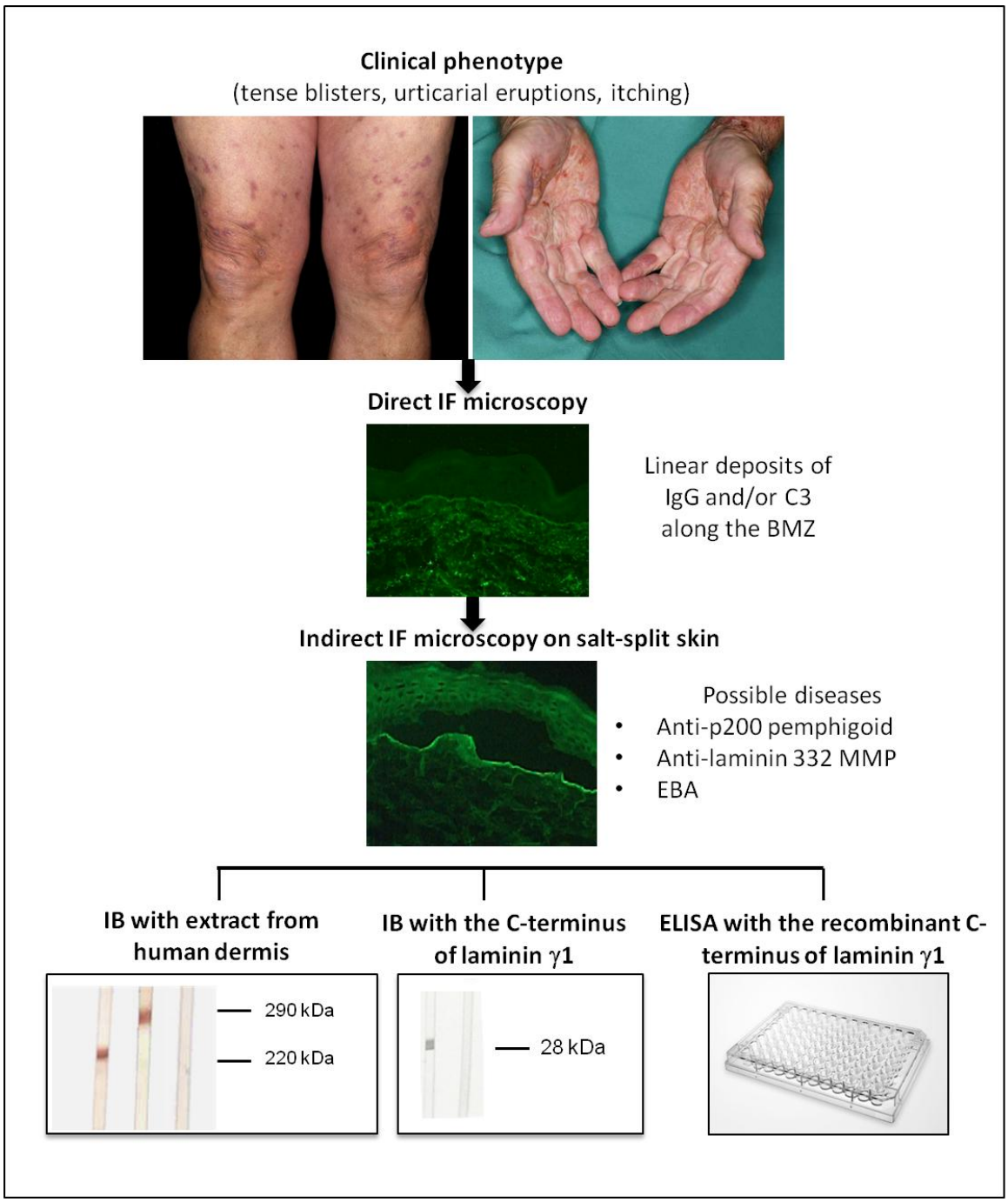

Figure 48. **Diagnostic pathway in anti-p200 pemphigoid.** Anti-p200 pemphigoid patients develop tense blisters and urticarial eruptions, sometimes accompanied by itching. Direct IF microscopy demonstrates IgG and C3 deposition along the BMZ. Autoantibodies bind to the dermal side of the salt-split human skin by indirect IF microscopy. To differentiate anti-p200 pemphigoid from other diseases further serological examinations are required. Autoantibodies react with the 200 kDa protein in human dermal extract. Alternatively, laminin γ1 specific antibodies are detected using the recombinant C-terminus of laminin γ1 by IB or hLAMC1-cterm ELISA. The photos were kindly provided from the routine autoimmune laboratory,

Department of Dermatology, Lübeck. IF, Immunofluorescence; IB, immunoblotting; EBA, epidermolysis bullosa acquisita; MMP, mucous membrane pemphigoid; C3, complement component C3; BMZ, basement membrane zone; ELISA, enzyme-linked immunosorbent assay.

In a previous study by Dainichi *et al*. a heterotrimer of the C-termini of laminin α1, β1, and γ1 (E8 fragment) was used as source of laminin γ1 [61]. In the present study, a monomeric recombinant form of laminin γ1 (hLAMC1-cterm) was cloned, expressed in *E.coli*, and purified by immobilized metal affinity chromatography in high amounts. By IB with the recombinant hLAMC1-cterm, sera from patients with anti-p200 pemphigoid but no sera from other autoimmune blistering disorders were reactive. Subsequently, an ELISA was developed based on monomeric hLAMC1-cterm. To optimize this system several conditions have been tested; different plates, a range of antigen and secondary antibodies concentrations, and different blocking buffers were evaluated for the best discrimination between anti-p200 pemphigoid, and control sera including other autoimmune skin diseases. Thirty five sera from patients with anti-p200 pemphigoid and 176 sera from different other autoimmune blistering diseases, including bullous pemphigoid, epidemolysis bullosa acquisita, anti-laminin 332 mucous membrane pemphigoid, and pemphigus vulgaris, as well as healthy volunteers (n=131) were tested by this ELISA. The results indicated a sensitivity of 69% and a specificity of 98.7%.

There are several sensitive and specific ELISA systems for the detection of autoantibodies in autoimmune bullous diseases; some of them are already commercialized. For the detection of serum autoantibodies against BP180 ELISAs were based on various recombinant forms of BP180 including the immunodominant NC16a domain [103, 151-153]. In these systems, sensitivity and specificity reached 90 and 100% respectively or even 95% using the anti-tetrameric NC16a ELISA [154]. Self-assembling radiolabeled tetramers discriminate antibodies against folded or denatured protein. Multimerization of self-antigen has been shown to increase the antigen recognition by autoantibodies that have a low binding affinity or are present at low concentrations [101, 155-158].

Ninety percent of pemphigoid gestationis serum samples contain IgG autoantibodies against NC16a domain of BP180. Thus, NC16a-ELISA is sensitive tool for the detection and monitoring of autoantibodies against BP180 in patients with pemphigoid gestationis and highly specific in differentiating the latter from a pruritic urticarial papules and plaques of pregnancy, one of the most important differential diagnoses of pemphigoid gestationis [159, 160].

Several ELISAs for the detection of autoantibodies to BP230 in patient's sera have also been developed but they appeared less sensitive. These ELISA assays used either six non overlapping synthetic peptides representing sequences in the carboxyl domain of the 230-kDa BP antigen[161] or bacterial fusion proteins [162] covering only small portions of the molecule or a fragment of murine BP230 [163, 164], respectively. Of note, it was demonstrated that circulating autoantibodies in patients with bullous pemphigoid recognize multiple epitopes on the 230-kDa BP antigen. Although the high diagnostic specificity of these tests (90%), the sensitivity was not more than 60% for anti-BP230 [165-167]. Two more ELISA systems for the detection of autoantibodies in patients' sera are commercially available applying recombinant fragments of BP230. The ELISA was generated using five overlapping cDNA fragments covering the entire length of BP230 and expressed in baculovirus-infected Sf21 insect cells (derived from ovaries of Spodoptera frugiperda). The second ELISA was developed using the bacterial recombinant proteins of N-terminal and C-terminal domains. These systems revealed sensitivities of 63% and 72.4% and specificities of 93% and 99.5%, respectively. Most of these studies suggest the combined use of BP180 and BP230 ELISA as the most appropriate diagnostic methods for the detection of all bullous pemphigoid sera [168]. These test characteristics are comparable with the hLAMC1-cterm ELISA of the present study. Of note, although BP230 is one of the two target antigens in bullous pemphigoid, various studies failed to univocally demonstrate the pathogenic relevance of anti-BP230 antibodies [101, 157, 166, 167, 169].

For the detection of autoantibodies against type VII collagen in epidermolysis bullosa acquisita patients, 2 highly sensitive and specific ELISAs were established. It was reported that major epitopes are located within the NC1 domain and minor epitopes within NC2 domain of collagen type VII. The ELISAs based on the recombinant NC1 domain expressed in human eukaryotic HEK293 cells, initially by Chen *et al*. and further optimized by Komorowski *et al*. reaching sensitivity of 98.7% and specificity of 98.7% [170, 171]. Interestingly, none of the anti-p200 pemphigoid sera, used as controls showed reactivity in this ELISA. An ELISA coated with a mixture of recombinant NC1 and NC2 domains, revealed 93.8% sensitivity and 98.1% specificity and ELISA scores vary in parallel with the disease activity of the patients [172]. Additionally, the results of an ELISA, using baculovirus-derived Col VII-NC1 recombinant proteins, confirm the high significance of NC1 domain for the serological diagnosis of epidermolysis bullosa acquisita [173].

In pemphigus vulgaris and pemphigus foliaceus, the main target antigens are the keratinocyte proteins desmoglein 1 and desmoglein 3. Cloning and generation of the recombinant proteins

facilitated the development of a sensitive and specific ELISAs [73]. Subsequently, several ELISA systems have been developed using extracts of bovine skin and recombinant fragments of desmoglein 3 and 1 expressed in *E. coli* or Sf21 insect cells [73, 163, 174, 175]. In another study, the recombinant ectodomains of the two proteins were expressed in the human cell line HEK293, to include protein folding and post-translational modification, and applied in two ELISA systems. These ELISAs revealed a sensitivity and specificity of 100% and 99.6% respectively, for anti-desmoglein 3 antibodies, as well as 96% and 99.1% respectively, for anti- desmoglein 1 antibodies. These ELISA systems were shown to be most suitable for both the serological diagnosis of pemphigus vulgaris and pemphigus foliaceous, as well as monitoring disease activity during the course of the disease [176-179]. An ELISA system has also been developed for detection of autoantibodies against envoplakin and periplakin in paraneoplastic pemphigus. This system, which uses the recombinant N-termini of the proteins, has also high diagnostic accuracy of around 80% and can help to differentiate paraneoplastic pemphigus from pemphigus vulgaris[180].

Here, in 11 of the 35 anti-p200 pemphigoid sera, no anti-hLAMC1-cterm reactivity was detected. This finding could be explained by reactivity with epitopes on laminin γ1 outside of hLAMC1-cterm or by reactivity with another 200 kDa dermal protein. This hypothesis was excluded after epitope mapping studies (chapter 4.2.2). The possibility that the amount of coated recombinant hLAMC1-cterm was too little to bind low serum autoantibody concentrations was excluded because the coated protein was up to 50-fold higher amounts and that yielded no higher sensitivity but a lower specificity.

There were also sera from other bullous diseases that showed ELISA reactivity with hLAMC1-cterm. In detail, 2 bullous pemphigoid and 2 anti-laminin 332 mucous membrane pemphigoid sera were positive in the novel ELISA. Of these 4 sera, one bullous pemphigoid and one anti-laminin 332 mucous membrane pemphigoid sera also reacted by IB with this protein. None of the 2 bullous pemphigoid sera, however, stained the dermal side of the artificial split when re-investigated by indirect IF microscopy on human split-skin. This negative IF finding may still be compatible with anti-hLAMC1-cterm reactivity and may be explained by the lower sensitivity of human salt-split skin for anti-laminin γ1 antibodies.

In autoimmunity, autoantibodies against more than one epitope and more than one protein have frequently been described and are termed intra- and intermolecular epitope spreading phenomenon. Tissue damage from a primary inflammatory process may cause the release and

exposure of immunological “hidden” antigens to the immune system. This leads to a secondary autoimmune response against this previously hidden epitope [181-183].

Reactivity against multiple antigens of the DEJ is common in autoimmune subepidermal bullous diseases and is likely due to this phenomenon [184]. In fact, a substantial number of anti-p200 pemphigoid sera have previously been described to contain antibodies against other target antigens of the DEJ, including BP180 [58, 147, 148, 185], BP230 [55, 58], the α3 chain of laminin 332 [186], and type VII collagen [187, 188]. Most recently, a patient with anti-p200 pemphigoid developed additional anti-BP180 NC16a autoantibodies during the course of his disease [185]. Alternatively, concomitant autoantibody binding against 2 different antigens may result from cross-reactivity between antigens, which share a high degree of homology. The high homology between the laminin γ1 and laminin γ2 chains (34% identity and 27% similarity) may be responsible for the reactivity of the 2 anti-laminin 332 mucous membrane pemphigoid sera by the hLAMC1-cterm ELISA. Indeed, all 3 laminin γ1-reactive mucous membrane pemphigoid sera showed reactivity against the laminin γ2 chain.

Based on the assumption that reactivity of bullous pemphigoid and anti-laminin 332 mucous membrane pemphigoid sera is due to epitope spreading and cross-reacting antibodies, respectively, a robust method for the identification of outlier values was applied which resulted in a sensitivity of 77% and a specificity of 99.3% for the novel ELISA. Interestingly, no ELISA reactivity with hLAMC1-cterm in epidermolysis bullosa acquisita sera was observed. Higher numbers of epidermolysis bullosa acquisita sera may, however, be necessary to confirm this observation.

For routine diagnosis of blistering disorders, the hLAMC1-cterm ELISA may be recommended when indirect IF microscopy on salt-split skin revealed a staining along the dermal side of the artificial blister (Figure 48). In laboratories that use indirect IF microscopy on monkey oesophagus or BP180 NC16a ELISA as initial screening assays, an OD just above the cut-off in the hLAMC1-cterm ELISA, but below OD 0.1, may require subsequent indirect IF microscopy on salt-split skin to exclude bullous pemphigoid.

The high ICC (0.97) of the novel ELISA reflected the low variance of ELISA values between separate experiments. Because of anti-p200 pemphigoid sera with ELISA reactivity just above the cut-off, the ELISA may still be sensitive to additional confounding factors when performed in other laboratories. In cases with slightly elevated hLAMC1-cterm ELISA reactivity, MMP can

usually clinically be differentiated from anti-p200 pemphigoid since predominant mucosal lesions have not been described in these patients [57].

The first aim of this thesis was to develop an ELISA for the diagnosis of anti-p200 pemphigoid. Therefore, the recombinant C-terminal fragment of laminin γ1 was generated and purified by immobilized metal affinity chromatography in high amounts. This protein was further applied with optimal conditions for the development of an ELISA and sera were collected and tested for reactivity of anti-hLAMC1-cterm antibodies. This ELISA system revealed sensitivity and specificity of 77% and 99.3% respectively as well as very high reproducibility. In conclusion, the hLAMC1-cterm antibodies can be used as diagnostic marker and the ELISA based on the monomeric recombinant hLAMC1-cterm was shown to be a sensitive and specific tool for the serological diagnosis of anti-p200 pemphigoid.

5.2 Pathogenicity of anti-laminin γ1 antibodies

Autoantibodies were shown to be pathogenic in various autoimmune bullous diseases such as pemphigus, bullous pemphigoid, epidermolysis bullosa acquisita, and laminin 332 mucous membrane pemphigoid. However, pathophysiology was shown to differ between those disorders. In bullous pemphigoid, the most common and best-studied autoimmune blistering disease, antibody-target interaction followed by inflammatory cascade leads to blister formation. Specifically, antibody binding to the target antigen results in the release of interleukin 6 and interleukin 8, complement activation at the dermal epidermal junction, and mast cell degranulation. These events induce infiltration of inflammatory cells, release of proteases and reactive oxygen species which finally lead to blister formation [41]. Similarly, in experimental epidermolysis bullosa acquisita, anti-type VII collagen autoantibodies induce activation of the complement system, release of cytokines, neutrophil recruitment in the dermal-epidermal junction, activation of Fcγ receptors and proteinases. All these events result to blister formation. In contrast, in pemphigus, autoantibodies are sufficient to induce clinical disease and complement is not required. As well as in the experimental model of anti-laminin 332 mucous membrane pemphigoid, mice demonstrated clinical disease without inflammatory factors including complement, mast cells and T cells. Anti-p200 pemphigoid is an autoimmune subepidermal blistering disease and shares many clinical and immunological characteristics with bullous pemphigoid. But so far, no studies on the pathogenesis of this disease are available.

Antibodies in anti-p200 pemphigoid are pathogenic

In the second part of the thesis, the pathogenic relevance of antibodies in anti-p200 pemphigoid and specifically antibodies against the laminin γ1 was addressed. To answer the first research question of the second aim of the thesis, an *ex vivo* model of autoantibody-mediated leukocyte-dependent neutrophil activation and dermal-epidermal separation (DES) was applied [146]. This *ex vivo* model, originally described by Gammon *et al.*, involving cryosections of human skin incubated with sera or IgG preparations and leukocytes from healthy donors [146]. This model has been already used to demonstrate that autoantibodies of patients with other subepidermal blistering diseases, like epidermolysis bullosa acquisita [133] and bullous pemphigoid [105], induce DES in cryosections of human skin. All 7 anti-p200 pemphigoid sera subjected to this *ex vivo* model induced DES, indicating their pathogenic potential. Moreover, similar to the previous studies in bullous pemphigoid and epidermolysis bullosa acquisita [105, 133], DES was preceded by leukocyte attachment along the DEJ. When the time-dependent-leukocyte attachment and the induction of split formation were studied, it was observed that after 1 hour of incubation, leukocytes are localized to the DEJ, while split formation started thereafter. The greatest extent of DES was visible after 3 hours in skin incubated with anti-p200 pemphigoid sera, in contrast to sections incubated with serum of healthy donors, where neither splits nor neutrophil attachment at the DEJ were detected. These data also point to the importance of leukocytes, mainly neutrophils, in the induction of DES.

Antibodies against LAMC1-cterm did not mediate pathogenicity ex vivo.

Since the C-terminus of laminin γ1 has previously been described as immunodominant region in anti-p200 pemphigoid [61] this study aimed to dissect the impact of autoantibody reactivity against this stretch in the *ex vivo* model. In bullous pemphigoid sera, the split inducing capacity was mapped to the NC16a domain using recombinant NC16a for preadsorption and depletion approaches similar as the present studies using recombinant hLAMC1-cterm [105]. To answer the second research question, whether antibodies against C-terminus of laminin γ1 (hLAMC1-cterm) are pathogenic, IgG affinity-purified with the hLAMC1-cterm and serum depleted from hLAMC1-cterm-reactivity were prepared from anti-p200 pemphigoid sera. While latter serum fractions, recruited leukocytes at the DEJ and induced DES, hLAMC1-cterm-specific IgG was not pathogenic. The same results were obtained using IgG specific to recombinant eukaryotic

hLAMC1-cterm, IgG specific to eukaryotic E8 fragment, a C-terminal portion of laminin 111, and IgG specific to full-length laminin γ1 (Figure 49).

All these approaches did not result in DES while total serum and serum depleted from anti-laminin γ1 reactivity did clearly point to antibody-mediated tissue damage in anti-p200 pemphigoid. These results together with the strong labeling of serum depleted of antibodies against laminin γ1 to a 200 kDa protein in dermal extract turns to reconsider the true identity of the autoantigen in anti-p200 pemphigoid (Figure 14).

The following hypotheses to explain inefficacy in the *ex vivo* model were compiled: i) the concentration of anti-LAMC1-cterm-IgG was too low, ii) glycosylation-dependent epitopes mediate pathogenicity, iii) the elution process of hLAMC1-cterm-specific IgG during the affinity purification procedure impaired pathogenicity, iv) antibodies against a heterotrimeric form of laminin γ1 are pathogenic.

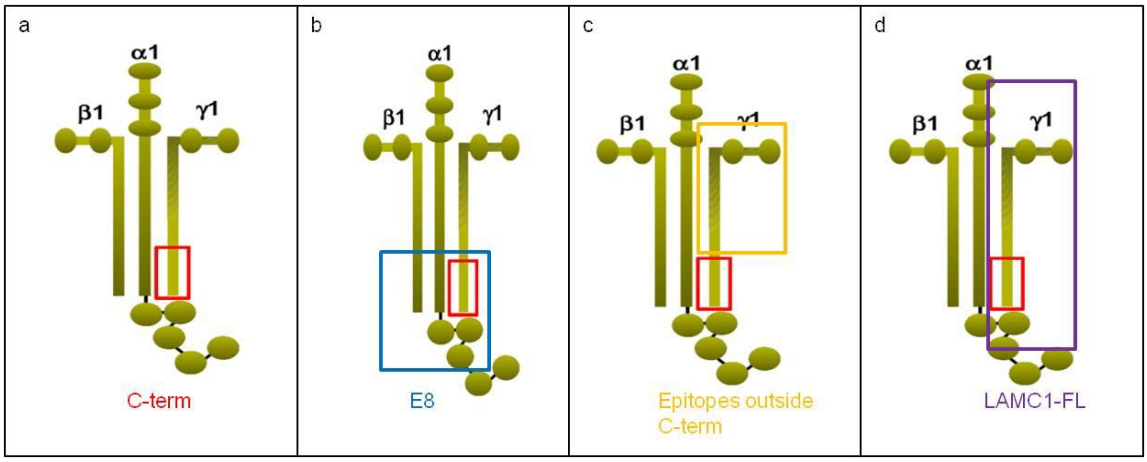

Figure 49. Different parts of laminin γ1 tested in this study for reactivity and pathogenicity in anti-p200 pemphigoid. Red box: the C-terminal fragment of laminin γ1 (a-d); blue box: the E8 fragment, a truncated version of laminin 111 consisting of the C-terminus and the 3 laminin G-like modules (b); yellow box: the fragment of laminin γ1 outside C-terminal fragment (c); purple box: the entire laminin γ1 molecule (d).

To address the first hypothesis, a 5-fold higher IgG concentration compared to the corresponding patient sera as well as high concentrations of both rabbit IgG generated against murine LAMC1-cterm and commercially available anti-laminin γ1 IgG were used in the cryosection model, but no DES was observed (chapters 4.2.3, 4.2.8).

Since both the p200 protein and laminin γ1 were shown to be N-glycosylated [60, 61], then, it was further investigated whether glycosylation-dependent epitopes mediate DES in this *ex vivo* model. When patient IgG, affinity purified against eukaryotic expressed hLAMC1-cterm was applied, also no effect was observed (chapters 4.2.4).

The third hypothesis, claiming a potential damage of IgG following the elution process, was excluded, since hLAMC1-cterm-specific IgG showed strong reactivity with recombinant hLAMC1-cterm (Figure 14 a line 3), the p200 protein in human dermis by immunoblotting Figure 14 b line 3), and the human DEJ by indirect IF microscopy (Figure 15c), respectively. Besides, when purified IgG from anti-p200 pemphigoid patient's sera applied to cryosection assay, it induced splits similarly to unfractionated sera. In addition, both rabbit serum and concentrated rabbit IgG generated against mLAMC1-cterm, as well as a monoclonal anti-laminin γ1 antibody (not subjected to affinity purification) were ineffective in the *ex vivo* model, although the strong reactivity with murine dermal extract by IB and normal murine skin by indirect IF microscopy (chapter 4.2.8).

To test the fourth hypothesis a recombinant trimer of laminin 111 (E8 fragment) was used. This E8 fragment was used by Dainichi *et al.* for the detection of anti-laminin γ1 reactivity in anti-p200 pemphigoid sera [61]. They demonstrated that 90% of anti-p200 pemphigoid sera recognized this fragment. Similar to experiments with hLAMC1-cterm, E8 fragment-specific IgG was ineffective in the cryosection assay and serum depleted from anti-E8 antibodies still reacted with the p200 protein in dermal extract and induced DES in the *ex vivo* model.

The C-terminal fragment of laminin γ1 is the immunodominant epitope

To answer the third research question discrete domains were further examined within the laminin γ1 chain for the ability to be recognized by the antibodies in 25 sera of patients with anti-p200 pemphigoid by immunoblotting. Five anti-His fused proteins outside the C-terminal fragment were cloned expressed in *E.coli* and purified by immobilized metal affinity chromatography. Sera from anti-p200-pemphigoid patients were further tested of reactivity with the other fragments outside the C-terminus. However epitope mapping experiments revealed the binding of only a minority of anti-p200 pemphigoid sera outside LAMC1-cterm. In total only 8 out of 25 sera reacted with one or more epitopes outside the C-termini. Thus, major pathogenic sites on laminin γ1 outside the LAMC1-cterm domain were excluded. These results

confirm previous suggestions that the epitope targeted by patients' antibodies is located within the C-terminal 246-amino acid residues of the laminin γ1 chain [61] (chapter 4.2.2).

Additionally, since the recombinant fragments used in the epitope mapping studies were overlapping by only 0 to 5 amino acids and some B-cell epitopes could thus have been missed, using the entire laminin γ1 chain employed in the cryosection model this possibility was excluded. This also excludes the hypothesis whether modifications-dependent epitopes of the whole laminin γ1 are important to mediate pathogenicity. Therefore the entire human laminin γ1 chain (LAMC1-FL) was generated, expressed in HEK293T cells and purified. Antibodies affinity-purified from three anti-p200 pemphigoid patients' sera against LAMC1-FL did not result in dermal-epidermal splitting in cryosections of human skin. In contrast, patients' sera depleted from anti-LAMC1-FL antibodies retained their pathogenic ability in this model (chapter 4.2.6). Once again the blisters were not mediated by the specific antibodies, while serum depleted from anti-full length laminin γ1 retained its DES-inducing capacity.

Rabbit antibodies against mLAMC1-cterm are not pathogenic ex vivo

To address the fourth research question, the C-terminal fragment of murine laminin γ1 was cloned, expressed in *E.coli*, and purified by immobilized metal affinity chromatography in high amounts. Rabbits were immunized with this fragment and rabbit autoantibodies against the mLAMC1-cterm were purified and applied in the *ex vivo* model. A similar approach was previously chosen in experimental model in bullous pemphigoid[105] and epidermolysis bullosa acquisita [9]. However here, rabbit anti-mLAMC1-cterm autoantibodies, in spite of their ability to bind to mouse skin and recognize both the mLAMC1-cterm protein and the 200 kDa protein in dermal extract, did not induce neutrophil recruitment nor dermal-epidermal separation in cryosections of human skin.

Antibodies against mLAMC1-cterm are not pathogenic in vivo

To address the fifth research question, rabbit anti-mLAMC1-cterm antibodies were passively transferred into neonatal and adult mice. In 1982, Anhalt *et al*. developed an animal model for pemphigus vulgaris by injecting IgG from a patient with pemphigus vulgaris into neonatal BALB/c mice at a dose of 1.5 to 16 mg/g of body weight. Eighteen hours after injection clinical, histological, ultrastructural, and immunological features of the human disease were observed [81]. Similar results were obtained by the injection of serum or IgG from a patient with pemphigus

foliaceus with IgG at an even higher dose of 10 mg/g body weight per day for four injections [81, 189]. Later, the pathogenic potential of pemphigus IgG was attributed to anti-desmoglein 3 and 1 antibodies, respectively [190-192]. Applying IgG from a patient with paraneoplastic pemphigus, anti-desmoglein 3 were identified to mediate blister formation [193].

IgG from anti-p200 pemphigoid sera only weakly reacted with mouse skin by indirect IF microscopy and IB with murine dermis demonstrating the lack of cross-reactivity between human anti-p200 IgG with murine skin [62]. Therefore, in the present study, anti-p200 pemphigoid patient's IgG was not injected in mice, but mLAMC1-cterm was generated and rabbits were immunized with this fragment. Consequently, rabbit antibodies against the murine C-terminus of laminin γ1 was employed to mice, following the strategy that has been successfully used in several studies [109, 130, 136].

To explore the ability of these antibodies to induce anti-p200 pemphigoid *in vivo*, high doses of rabbit IgG and IgG specific to mLAMC1-cterm were injected into adult and neonatal mice, respectively. Despite sustained injections over longer times and an increased total amount of IgG as compared to the previous reported models, in which antibodies were pathogenic, no disease was observed. In principle, circulating anti-mLAMC1-cterm antibodies were present in high levels in all animals, and bound weakly at the DEJ, however they did not attract C3 at the DEJ, and did not result in clinical or histopathological lesions (chapters 4.2.9 – 4.2.12).

In contrast, previously, rabbit IgG generated against type VII collagen and type XVII collagen (BP180) recapitulated the human diseases epidermolysis bullosa acquisita and bullous pemphigoid, respectively, when IgG was injected in adult (epidermolysis bullosa acquisita) and neonatal mice (bullous pemphigoid) at doses of 0.3-0.9 mg/ml of body weight and 0.03-27 mg/ml of body weight, respectively [109, 136, 140, 142]. Furthermore, complement, mast cells, and neutrophils were shown to be essential in the development of experimental BP [109, 112, 115, 118]. Of note, when the same approach was used with rabbit IgG against human BP230 only one out of 7 mice developed blisters [124]. Animal models using neonatal mice were not always successful. Purified human autoantibodies from epidermolysis bullosa acquisita patients did not induce blisters in neonatal mice [194].

Recently, clinical disease mimicking the human disorders bullous pemphigoid (BP) and epidermolysis bullosa acquisita (EBA) was induced by immunizing susceptible mice strains with the immunodominant regions of type XVII collagen and type VII collagen, respectively, following a similar protocol as applied in the present study [143, 144]. In case of epidermolysis bullosa

acquisita, one week after immunization with the recombinant NC1 domain of type VII collagen mice developed autoantibodies and blisters were induced as early as the second week after immunization [143, 144]. However, not all mice strains were susceptible [144]. Studies on the genetic basis of epidermolysis bullosa acquisita demonstrated a dependence of the major histocompatibility complex (MHC) haplotype to the disease induction and susceptibility to epidermolysis bullosa acquisita. Furthermore the onset of clinical disease as well as the maximum disease severity was associated with several quantitative trait loci (QTLs) located on different chromosomes [150, 195]. To answer the sixth research question, mice from 3 different strains were immunized 4 times. All of them showed high levels of circulating IgG but no clinical disease was observed (chapter 4.2.13).

Autoimmunity against laminin 332, the target antigen in a subgroup of patients with mucous membrane pemphigoid was induced in mice by the passive transfer of human and rabbit anti-laminin 332 IgG [130, 131]. These data show that autoimmunity against target antigens including collagens and laminins could be mounted by both passive transfer of autoantibodies and immunization-induced autoantibodies mimicking the clinical disease of the corresponding subepidermal blistering diseases.

It may be speculated that the failure to induce clinical disease in our mouse models, although high levels of serum anti-mLAMC1-cterm IgG were present, can be attributed to the weak binding of autoantibodies at the DEJ. The relatively low affinity of autoantibodies against mLAMC1-cterm speaks against a major pathogenic role in anti-p200 pemphigoid. This notion is fuelled by the peculiar binding pattern of anti-LAMC1-cterm IgG at the basal keratinocytes of the DEJ that differs from the exclusively linear binding observed in patients' skin and patients' serum autoantibodies labeling human skin. Furthermore, the unique reactivity of patient's autoantibodies with the DEJ, but not with any other tissues, which express the laminin γ1 chain, remains enigmatic. Several studies could not detect any reactivity of sera from anti-p200 pemphigoid to placenta laminin [58], purified laminin γ1 [54, 58], or laminin 311 [58] by IB.

The current findings are in line with the results of a parallel recent published study of Koga *et al*. Their attempt to develop an animal model by injecting rabbit IgG against a shorter (107 aa) recombinant protein of the C-terminal of murine laminin γ1 or the immunization of mice with this protein did not demonstrate any clinical disease [196].

Laminin γ1 as the target antigen in anti-p200 pemphigoid

The autoantigen in anti-p200 pemphigoid had been unknown for many years, until laminin γ1 was suggested. 2D gel electrophoresis of dermal extracts and mass spectrometry analysis recognized a unique protein band as the laminin γ1 protein. By immunoblotting, 90% of sera from patients with anti-p200 pemphigoid recognized the recombinant laminin 111 [61]. The previous candidates of p200 kDa protein were several components of the extracellular matrix, such as α6β4-integrin, nidogen-2, laminin β1. All of them were finally excluded due to epidermal localization and lack of reactivity with patients' sera. Laminin γ1 was also a candidate, but several past studies could not detect any reactivity of sera from anti-p200 pemphigoid to placenta laminin, purified laminin, and laminin 311 [54, 58]. Furthermore, the unique reactivity of patient's autoantibodies with the DEJ, but not with any other tissues that express the laminin γ1 chain remains enigmatic.

Laminins have been described in a variety of biological and pathologic processes. Antibodies against laminin have been found in autoimmune diseases. Mucous membrane pemphigoid, as has previously been mentioned, is characterized by autoantibodies against laminin 332, and mutations of any of the chains of laminin 5 results to junctional epidermolysis bullosa type Herlitz, while antibodies against laminin 111 have been diagnosed in patients with systemic lupus erythematosus. Animal studies showed that knocking out laminin γ1 in the cerebral cortex leads to defects in neurite outgrowth and neuronal migration [197]; however there are not studies that relate laminin γ1 with the pathogenicity of other autoimmune or skin diseases.

Autoantibodies from anti-p200 pemphigoid patients are associated with pathogenicity only to the skin and not to other organs. Except for one case, patients' sera did not show reactivity with blood vessel walls [57, 58, 61]. Nevertheless, laminin γ1 is widely expressed in different basement membrane zones. This has been explained due to posttranslational modifications, such as glycosylations, occurred in these areas and therefore the organ specificity of the disease, however, when using the eukaryotic form of this protein the number of reactivity of patient's sera did not increased by immunoblotting. It is also suggested that circulating anti-laminin γ1 antibodies in the patients may inhibit the interaction between laminin and integrin in the skin and this interaction is more essential to the skin than other organs [56].

In vitro studies with keratinocytes come to support the previous observations. Previous studies have shown that fibroblasts and keratinocytes synthesize several components of the cutaneous

basement membrane zone such as collagen IV, collagen VII, laminin 111, and p200 [62, 198, 199]. This was also confirmed in this study when sera from anti-p200 pemphigoid stained HEK293T cells. Interestingly, cells incubated with sera depleted of anti-hLAMC1-cterm IgG reactivity were also stained in diffused pattern similar to cells incubated with sera. In contrast, anti-hLAMC1-cterm-specific IgG and the monoclonal anti-laminin γ1 antibody, B4, showed a different discontinuous staining pattern (chapter 4.2.7).

Based on these data, it was concluded that while the C-terminus of laminin γ1 is a major target of autoantibodies in anti-p200 pemphigoid, there was no demonstration of the pathogenic potential of anti-laminin γ1 antibodies using different experimental approaches.

A somehow similar constellation is known in bullous pemphigoid. Autoantibodies in bullous pemphigoid recognize two antigens, BP180 and BP230. Initially, BP230 has been described as the major target antigen recognized by the majority of bullous pemphigoid patients [123, 200]. However, the pathogenic relevance of BP230 antibodies has not yet been unequivocally demonstrated [124, 125]. Subsequently, antibodies against BP180 were described [201, 202] and numerous evidences for their pathogenic relevance has been gathered [94, 104, 105, 109, 142, 203].

5.3 Conclusion

To summarize the results shown in this study, a hLAMC1-cterm enzyme linked immunosorbent assay (ELISA) for the detection of antibodies against the C-terminal fragment of laminin γ1 was designed, developed and validated. This assay is shown to be reliable, sensitive and specific and will therefore be valuable for serological studies. The routine use of this ELISA in conjunction with the salt-split skin for the screening of autoimmune blistering diseases would provide an effective and efficient method for the detection of anti-LAMC1-cterm antibodies in anti-p200 pemphigoid.

It is further confirmed that laminin γ1 is a major antigen of autoantibodies in anti-p200 pemphigoid and the C-terminal fragment is the immunodominant epitope. Although antibodies against hLAMC1-cterm may be of potential prognostic value; however they were particularly not associated with blistering and clinical phenotype using *ex vivo* and *in vivo* approaches. Taken these data together, it has been shown that antibodies against laminin γ1 are not pathogenically

relevant, and therefore, it is suggested that laminin γ1 is not the major target antigen in anti-p200 pemphigoid.

Future lines of research will focus on the identification of an antigen recognized by the pathogenic antibodies. This would shed more light on the characteristics and pathogenicity of this disease, which could finally contribute to prognosis and treatment.

6 ABSTRACT

Anti-p200 pemphigoid is a rare subepidermal blistering skin disease characterized by autoantibodies against a 200-kDa protein (p200) of the dermal-epidermal junction (DEJ). Diagnosis of anti-p200 pemphigoid requires detection of serum IgG at the dermal side of salt-split skin by indirect immunofluorescence microscopy and labeling of a 200-kDa protein by immunoblotting with extract of human dermis. These techniques are only available in few laboratories. In addition, the detection of anti-p200 antigen antibodies is highly dependent on the quality of the dermal extract. Therefore anti-p200 pemphigoid is not recognized in many cases although this entity differs from other autoimmune bullous diseases with respect to treatment response and diagnosis. Establishing an easy and accurate diagnostic method is necessary. The autoantigen of anti-p200 pemphigoid had been unknown for many years, until laminin γ1 was recently identified as the target antigen and its C-terminus described as the immunodominant region. In addition, no data are available on the pathogenic relevance of autoantibodies in anti-p200 pemphigoid. Thus, this study aimed 1) to develop a simple, sensitive and specific enzyme linked immunosorbent assay (ELISA) to facilitate the serological diagnosis of anti-p200 pemphigoid and, in its major part 2) to improve our knowledge on the pathogenesis of this disease by exploring the pathogenic potential of antibodies against laminin γ1. Initially, sera from patients with anti-p200 pemphigoid, other autoimmune bullous diseases and healthy volunteers were tested by a novel ELISA that employed a recombinant C-terminal fragment of human laminin γ1 (hLAMC1-cterm). The novel ELISA revealed a sensitivity and specificity of 69% and 99% respectively. To investigate the pathogenic relevance of anti-laminin γ1 antibodies, *ex vivo* and *in vivo* studies were performed. It was shown that anti-p200 pemphigoid sera (n=7) attracted neutrophils and induced dermal-epidermal separation (DES) in a time-depended manner in an autoantibody mediated leukocyte-depended *ex vivo* model. Also, epitope mapping studies followed by immunoblot analysis of anti-p200 pemphigoid sera with the 5 different fragments, covering the whole laminin γ1 molecule, confirmed that the C-terminal fragment of laminin γ1 is the immunodominant epitope. Further, different forms of C-terminal fragment of laminin γ1 (prokaryotically and eukaryotically expressed hLAMC1-cterm, truncated trimer of laminin 111, full length of LAMC1 and murine LAMC1-cterm) were generated. Antibodies specific to these different forms of laminin γ1 did not develop any split formation in the cryosection *ex vivo* model indicating the lack of pathogenic ability. Interestingly, in all cases patient's sera depleted from anti-laminin γ1 antibodies induced DES similarly to patients' sera. Moreover,

repeated injections of rabbit anti-murine LAMC1-cterm IgG into both neonatal and adult mice, as well as repetitive immunization of various mouse strains with murine LAMC1-cterm failed to induce clinical disease, although high levels of circulating antibodies were present in all mice. In conclusion, the novel ELISA based on recombinant hLAMC1-cterm will facilitate the diagnosis of anti-p200 pemphigoid. However, different *ex vivo* and *in vivo* approaches could not demonstrate the pathogenic potential of antibodies to laminin γ1. The notion that laminin γ1 is the main autoantigen in anti-p200 pemphigoid should therefore be reconsidered. Alternatively, a yet unknown splice variant of laminin γ1 in human skin may be targeted.

7 ZUSAMMENFASUNG

Das Anti-p200 Pemphigoid ist eine seltene subepidermale Autoimmundermatose der Haut, die durch Autoantikörper gegen ein 200-kDa schweres Protein (p200) der dermo-epidermalen Junktionszone (DEJ) charakterisiert ist. Die Diagnose des Anti-p200-Pemphigoid erfordert den Nachweis der Bindung von Serum-IgG an der dermalen Seite des artifiziellen Spaltes in der indirekten Immunfluoreszenz mit NaCl-gespaltener Haut sowie des 200 kDa schweren Proteins im Immunoblot unter Verwendung von Extrakten humaner Dermis. Bis jetzt ist die Qualität des dermalen Extraktes ausschlaggebend für die Diagnose der Erkrankung. Zudem werden diese Techniken nur in wenigen spezialisierten Laboratorien eingesetzt. Die Qualität des dermalen Extraktes, welches nur ein wenigen Laboren hergestellt wird, ist ausschlaggebend für die Diagnose der Erkrankung und somit der limitierende Faktor. Die Laminin γ1-Kette wurde kürzlich als Zielantigen des Anti-p200 Pemphigoid identifiziert und der C-Terminus als immundominante Region beschrieben. Bisher gibt es keine Untersuchungen zur pathogenetischen Relevanz der Autoantikörper beim Anti-p200 Pemphigoid. Ziel dieser Studie war 1) die Entwicklung eines sensitiven und spezifischen ELISA (enzyme linked immunosorbent assay) zur Detektion von Anti-Laminin γ1/Anti-p200 Pemphigoid Autoantikörpern und 2) der Nachweis des pathogenetischen Potential der Anti-Laminin γ1 Antikörper. Zunächst wurden Seren von Anti-p200 Pemphigoid Patienten, Patienten mit anderen bullösen Autoimmundermatosen und gesunden Blutspendern mit Hilfe des neuen ELISA untersucht, in dem ein rekombinantes C-terminales Fragment von Laminin γ1 (hLAMC1-cterm) zum Einsatz kommt. Dieses neue Testsystem weist eine Sensitivität und Spezifität von 69% und 99% auf. Um der Frage nachzugehen, ob Anti-Laminin γ1 Antikörper pathogen sind, wurden verschiedenen *ex vivo* und *in vivo* Studien durchgeführt. Zunächst konnte gezeigt werden, dass Anti-p200-Pemphigoid Seren (n=7), eine zeitabhängige dermo-epidermalen Spaltbildung (DES) im Autoantikörper-vermittelten Leukozyten-abhängigen ex-vivo Modell induzieren. Epitopkartierung und Immunoblot verschiedener Anti-p200 Pemphigoid-Seren, mit den 5 verschiedenen Fragmenten, die das gesamte Laminin γ1-Molekül abdecken, bestätigten, dass es sich beim C-terminale Laminin γ1-Fragment um das immunodominante Epitope Handelt. Darüberhinaus wurden verschiedene Formen des C-terminalen Fragments von Laminin γ1 (prokaryotisch und eukaryotisch exprimierter hLAMC1-cterm, verkürztes Trimer von Laminin 111, Volllängen-LAMC1 und murines LAMC1-cterm) generiert. Spezifische Antikörper gegen diese Formen von Laminin γ1 induzierten keine DES im Gefrierschnitt *ex vivo* Modell, was auf eine

fehlende Pathogenität des Antikörpers hinweist. Interessanterweise behielt allerdings das von Laminin γ1-Reaktivität depletierte Serum sein DES-induzierendes Potenzial. Darüberhinausführte, die wiederholte Injektion von Kaninchen anti-murinem LAMC1-cterm IgG in neonatale und adulte Mäuse, sowie die wiederholte Immunisierung von verschiedenen Maus-Stämmen mit murinem LAMC1-cterm nicht zu der Induktion einer Erkrankung. Zusammenfassend lässt sich daher sagen, dass der neue hLAMC1-cterm-ELISA die Diagnose des Anti-p200-Pemphigoids erleichtern wird Allerdings konnte durch den Einsatz verschiedener *ex vivo* und *in vivo* Modelle kein pathogenetisches Potenzial der Laminin γ1 Antikörper nachgewiesen werden. Die Annahme, dass es sich bei Laminin γ1 um das Hauptautoantigen des Anti-p200 Pemphigoids handelt, sollte daher überdacht werden. Es ist daher zu diskutieren, ob Laminin γ1 wirklich der Haupt Autoantigen der Anti-p200-Pemphigoid darstellt. Allerdings kann nicht ausgeschlossen werden, dass eine bis jetzt unbekannte Splicevariante von Laminin γ1 in der Haut existiert, die pathogenetisch relevante Epitope enthält.

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9 APPENDIX

9.1 List of tables

9.2 List of figures

9.3 List of abbreviations

2D	2 dimentional
aa	amino acid
APS	Ammonium persulfate
B4	monoclonal anti-LAMC1 antibody
BMZ	basement membrane zone
bp	base pair
BP	bullous pemphigoid
BP180	Bullous pemphigoid antigen 2 or collagen XVII
BP230	Bullous pemphigoid antigen 1
BSA	Bovine Serum Albumin
C3	complement component 3
cDNA	complementary DANN
CI	confidence interval
cm	Centimeter
Col VII-NC1	non-collagenous domain 1 of type VII collagen
DEJ	dermal-epidermal-junction
DES	dermal-epidermal separation
DIF	direct immunofluorescence
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dT	Deoxy-thymine
E.coli	Escherichia coli
E8	recombinant trimer of laminin 111
EBA	epidermolysis bullosa acquisita
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
F	Forward primer
Fc	Fragment crystallizable
FcR	Fc receptor
FcγR	Fc gamma receptor
FCS	Fetal Calf Serum
FITC	fluorescein isothiocyanate
g	Gram
G	Centrifugal force
HaCaT	human keratinocyte line
HCl	hydrogen chloride
HEK293T	Human embryonic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	High fidelity
His-tag	6xHistidines tagged to a recombinant protein

HLA	Human Leukocyte Antigen
hLAMC1	human laminin γ1
hLAMC1-cterm	recombinant C-terminus of laminin γ1
hLAMC1-FL	recombinant laminin γ1 full length
HRP	horseradish peroxidase
HV	healthy volunteers
IB	Immunoblotting
ICC	intra-class correlation coefficient
IF	Immunofluorescence
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgG1	immunoglobulin G1
IgG2a	immunoglobulin G2a
IgG2b	immunoglobulin G2b
IgG3	immunoglobulin G3
IgG4	immunoglobulin G4
kDa	kilo dalton
l	Liter
LAD	linear IgA dermatosis
LAMC1	laminin γ1
LB	Lysogeny Broth
µ	micro (10^{-6})
M	Mole
m	milli (10^{-3})
mA	Milliampere (10^{-3})
MAPK	Mitogen-activated protein kinase
MHC	major histocompatibility complex
mLAMC1-cterm	murine laminin γ1 C-terminus
MMP	mucous membrane pemphigoid
NaCl	sodium chloride
NC16a	non-collagenous 16a domain of BP180
ng	Nanogram
nm	Nanometer
OD	optical density
p200	200 kDa protein
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
PCR	polymerase chain reaction
PMSF	Phenylmethanesulfonylfluoride
PV	pemphigus vulgaris

QTL	Quantitative trait locus
R	Reverse primer
RNA	Ribonucleic acid
ROC	receiver-operating characteristics
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Reverse Transcription
SDS	sodium dodecyl sulfate
SLE	Systemic lupus erythematosus
TAE	Tris Acetate EDTA
TEMED	N, N, N´, N´-Tetramethylendiamin
TBST	Tris-Buffered Saline with Tween 20
TMB	3,3',5,5'-Tetramethylbenzidine

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Declarations

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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