Aus der Klinik für Dermatologie, Allergologie und Venerologie

der Universität zu Lübeck

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Untersuchungen zur Rolle von Komplement für die Blasenbildung der experimentellen Epidermolysis bullosa acquisita

Role of Complement Activation for Blister Formation in Experimental Epidermolysis Bullosa Acquisita

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Abbreviations

Bf – factor B

BP – bullous pemphigoid

BP180 – bullous pemphigoid antigen 2

BP230 – bullous pemphigoid antigen 1

DEJ – dermal-epidermal junction

EBA – epidermolysis bullosa acquisita

ELISA – enzyme-linked immunosorbent assay

GST– glutathione-S-transferase

IF – immunofluorescence

MASP – mannan binding lectin-associated protease

MBL – mannan binding lectin

mCOLVII – murine type VII collagen

MPO – myeloperoxidase

NC – non-collagenous

NRIgG – normal rabbit IgG

WT – wild type

Introduction

Autoimmune bullous skin diseases are chronic diseases of unknown origin characterized by blistering of the skin and associated with tissue-bound and circulating autoantibodies to structural components of the skin. Based on clinical and histopathological features, autoimmune bullous diseases may be divided into pemphigus-type and subepidermal autoimmune diseases. The first group of diseases includes blistering diseases characterized by intraepidermal blister formation due to the loss of adhesion of keratinocytes and is associated with autoantibodies to the intercellular junctions of keratinocytes. The second group of diseases is characterized by subepidermal blisters caused by the loss of attachment of basal keratinocytes to the underlying basement membrane and is associated with autoantibodies to the dermal-epidermal junction (DEJ).

Autoantibodies in patients with **pemphigus** were shown to specifically react with desmogleins (1-4) and desmocollins (5) (desmosomal transmembrane cell-adhesion molecules of cadherin type), with plakoglobin (an intracellular protein of the plakin family) (6), and with acethylcholine receptors (7, 8).

Over the past two decades, efforts have been made to identify antigens at the DEJ recognized by autoantibodies in patients with various forms of autoimmune **subepidermal blistering diseases (Fig. 1)**. Depending on the primary site of involvement, autoantibodies may bind to distinct DEJ molecules: the bullous pemphigoid (BP) antigens of 230kD (BP230) (9) or 180kD (BP180) (10, 11), plectin (12), and the α_3 (13, 14), $\beta_{3,}$ and γ_2 (15, 16) subunits of laminin 5; the α_6 (17) and β_4 (18-20) chains of $\alpha_6\beta_4$ integrin; a 168–170kD mucosal antigen (21); a 200 kDa dermal autoantigen (22),

type VII collagen (23), and the α_5 chain of type IV collagen (24). DEJ molecules that were identified as autoantigens in subepidermal blistering diseases are represented in **Figure 1**.

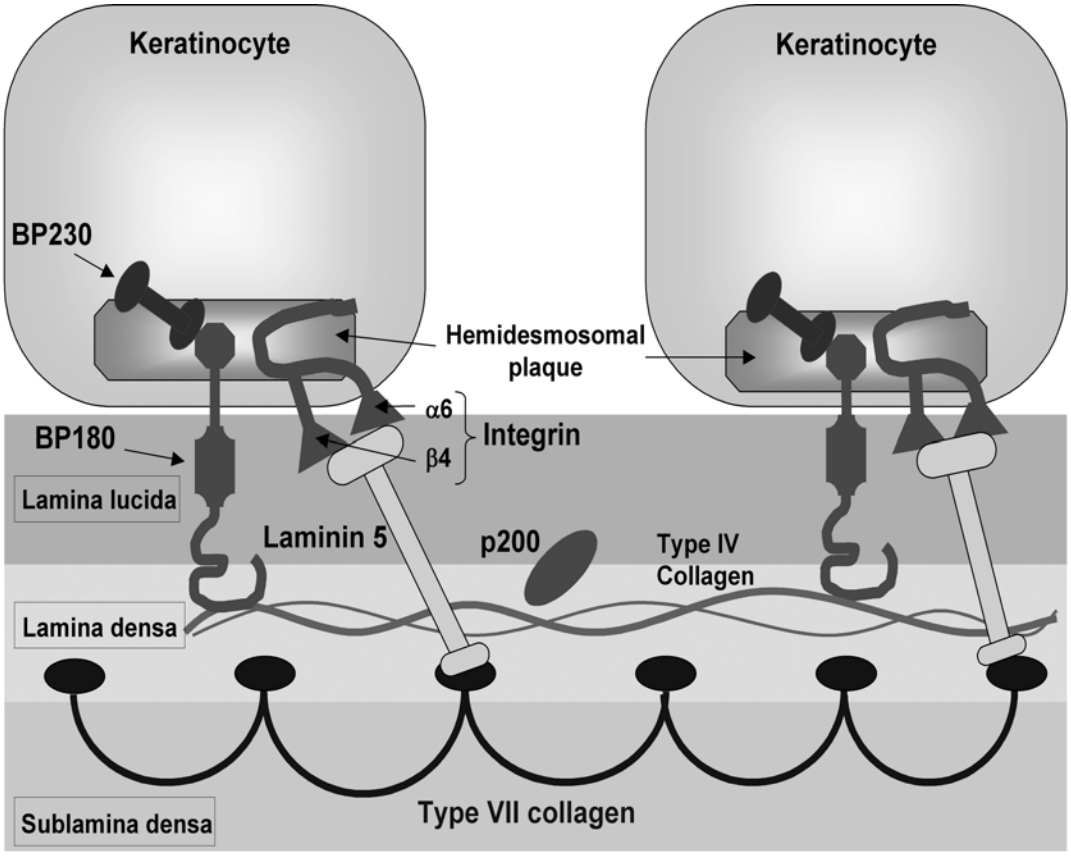

Figure 1. Schematic representation of autoantigens of the dermal-epidermal junction. Structural components of the skin basement membrane that may function as autoantigens in subepidermal blistering diseases and their relative position are shown. Plectin and BP230 are intracellular hemidesmosomal proteins. BP230 interacts with BP180 and $\alpha_6\beta_4$ integrin, which are transmembrane hemidesmosomal components. The α_6 chain of $\alpha_6\beta_4$ integrin interacts extracellularly with BP180. α_6 β_4 integrin is a known ligand for laminin 5 in the lower lamina lucida and lamina densa. P200 is a not yet fully characterized autoantigen localized to the lower lamina lucida. Beside other ubiquitous proteins like perlecan and

nidogen (not shown), laminin 5 and type IV collagen form a network in the lamina densa. Laminin 5 binds to type VII collagen, which represents the major component of the anchoring fibrils.

Epidermolysis bullosa acquisita (EBA) is a chronic blistering disease of skin and mucous membranes characterized by subepidermal blisters and tissue-bound and circulating autoantibodies to the dermal-epidermal junction (25). Type VII collagen, the main constituent of anchoring fibrils, was identified as the autoantigen of EBA (23, 26).

EBA is a clinically heterogeneous disease, which may present with an inflammatory or non-inflammatory phenotype. The first cases of a blistering disease with adult onset and features highly reminiscent of hereditary dystrophic epidermolysis bullosa were reported by Elliott more than 100 years ago (27). The mechanobullous, non-inflammatory form of EBA was defined in 1971 and is characterized by extreme skin fragility, trauma-induced blisters and erosions localized to the extensor skin surface, healing with scars and milia (28). In addition to the mechano-bullous variant, several inflammatory subtypes of EBA were described, clinically mimicking bullous pemphigoid, linear IgA disease, or mucos membrane pemphigoid (29-31). Certain EBA patients present with an inflammatory phenotype at the onset of the disease with overlapping or later evolving mechanobullous features (31, 32). EBA typically affects adults, but juvenile cases were also reported (33-39). EBA is a rare disease occurring in approximately 5% of unselected patients with basement membrane zone antibodies (40).

The disease is immunopathologically characterized by deposition of immunoreactants at the DEJ by direct immunofluorescence (IF) microscopy. The IgG and/or C3 deposits were shown to localize to the sublamina densa region of DEJ by direct immunoelectron microscopy (41). Indirect IF microscopy on 1M NaCl split-skin evidenced autoantibodies

to the dermal versant of the DEJ in serum of EBA patients (42), which label the sublamina densa zone by indirect immunoelectronmicroscopy (23). Although the site of immunoreactants deposition is always the lamina/sublamina densa region, in some EBA patients the cleavage plane localizes to the lamina lucida of the DEJ (43). Tissue bound and circulating antibodies in human EBA mainly belong to the IgG1 and IgG4 subclasses (44). EBA serum autoantibodies recognize the 290 kDa type VII collagen by immunoblotting with dermal extracts (23) and immunoprecipitation with keratinocyte and fibroblast extracts (45).

Type VII collagen, initially isolated from human chorioamniotic membranes, is an anti-parallel dimer recognized as the major constituent of anchoring fibrils (46-49). The gene COL7A1, encoding for the human type VII collagen, was mapped to the short arm of the chromosome 3 (50, 51). The COL7A1 gene consists of 118 exons, more than any previously described gene (52). Type VII collagen is composed of three identical α chains, each consisting of a 145-kDa central collagenous triple-helical portion, flanked by a large 145-kDa amino-terminal non-collagenous domain (NC1), and a smaller 34-KDa carboxy-terminal non-collagenous domain (NC2) (50). In the extracellular space, type VII collagen molecules form antiparallel tail-to-tail dimers stabilized by disulfide bonding through a small carboxy-terminal overlap (NC2), while a fragment of the NC2 domain is proteolitically removed (53, 54).

The epitopes recognized by EBA sera were mapped to the NC1 domain of type VII collagen and autoantibodies in a minority of sera were also shown to react with triple-helical or NC2 domains (26, 39, 54-57), while exclusive reactivity to the triple-helical domain was documented in only three children with EBA (58).

The biological significance of type VII collagen is further substantiated by the existence of a hereditary form of dystrophic epidermolysis bullosa. In the skin of these patients type VII collagen shows altered expression. Evidence accumulated that genetic defects in the correct synthesis, secretion, or in the molecular assembly of type VII collagen cause the different clinical forms of dystrophic epidermolysis bullosa (59-65). Interestingly, type VII collagen was found to have an elevated and topographically aberrant expression in the dermis of patients with systemic sclerosis under the regulatory action of transforming growth factor-beta (TGF-β). The presence of type VII collagen in the dermis may contribute to the tightly bound and indurated appearance of the affected skin in these patients (66).

The pathogenic relevance of antibodies against type VII collagen is supported by compelling evidence: 1) EBA autoantibodies were shown to recruit and activate leukocytes *ex vivo* resulting in dermal-epidermal separation in cryosections of human skin (67, 68). 2) Antibodies against type VII collagen induce subepidermal blisters when passively transferred into mice (69, 70). 3) Immunization with recombinant autologous type VII collagen induces an autoimmune response to this protein resulting in a blistering phenotype closely resembling human EBA (71).

Autoantibodies against type VII collagen are able to activate the complement system *in vivo* and *in vitro*. In the skin of EBA patients, deposition of different complement components, including C3, C5b, and membrane attack complex, are found with an incidence ranging from approximately 40% to 100% (72-74). In addition, deposition of C3 at the dermal-epidermal junction of murine skin is a constant feature in different passive transfer mouse models of EBA (69, 70) and in experimental EBA induced by

immunization of mice with autologous type VII collagen (71). These observations suggested that the complement system is involved in the autoimmune tissue injury in EBA. Indeed, this hypothesis was confirmed by recent studies showing that C5-deficient mice are resistant to blister induction by passive transfer of antibodies against type VII collagen (69).

Complement was first described in the 1980s as a heat-labile protein in serum that “complemented” heat-stable antibodies in the killing of bacteria. Now, the complement system, which consists of more than 30 plasma and and cell-surface proteins, is known to be a highly sophisticated host-defence system that is engaged both by innate immunity and as one of the main effector mechanisms of antibody-mediated immunity. Once the complement system is activated, a chain of reactions that involves proteolysis and assembly occurs, which results in cleavage of the third complement component (C3). The cascade that leads to the cleavage of C3 is called the activation pathway. It is followed by the lytic pathway, during which the membrane attack complex is formed (75, 76).

There are three different types of activation pathway: the classical, lectin, and alternative pathways **(Fig. 2)**. The classical pathway is activated primarily by antigen-antibody complexes binding to C1q (77). The lectin pathway is initiated by ficolins and mannan binding lectin (MBL) (78). The alternative pathway is continuously activated by factor B through a process called "tickover" of C3, and requires active control mechanisms to prevent autologous injury (79).

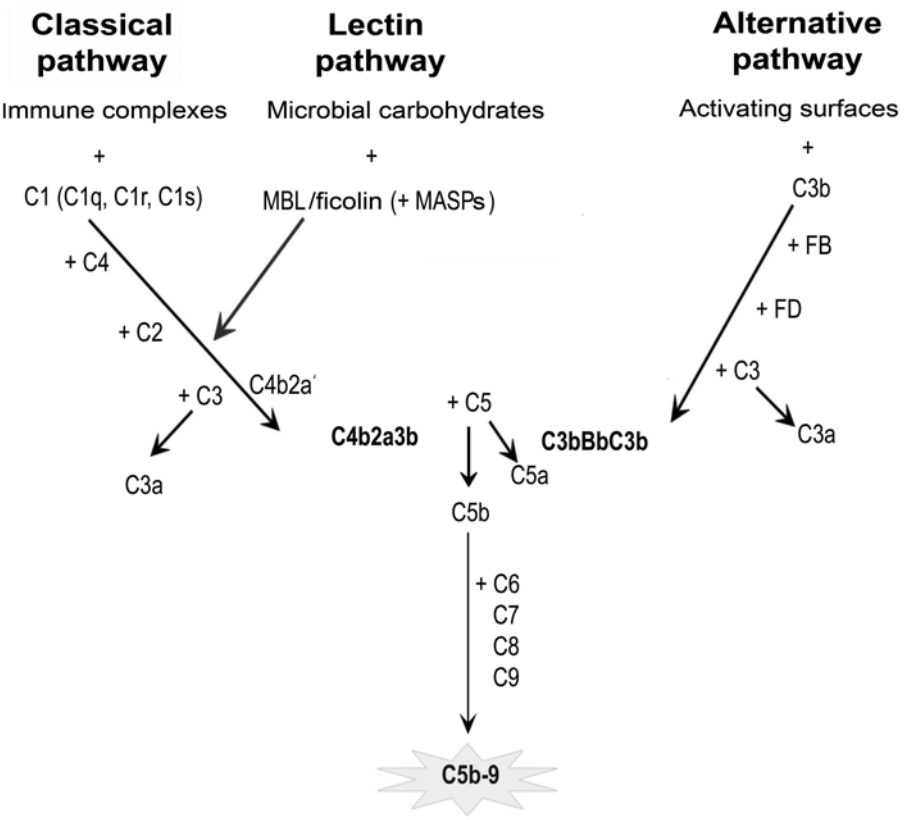

Figure 2. The three activation pathways of the complement system: the classical, lectin and alternative pathways. The classical pathway is initiated by the binding of C1 complex (consisting of C1q and two molecules each of C1r and C1s) to antibodies that are bound to antigens. The binding of the recognition subcomponent C1q to the Fc portion of immunoglobulins results in autoactivation of the serine protease C1r, which then cleaves and activates C1s. C1s first cleaves C4, and then cleaves C2, leading to the formation of a C4b2a enzyme complex, the C3 convertase of the classical pathway. The mannose-binding lectin pathway is initiated by the binding of either mannan-binding lectin (MBL) or ficolin-associated with MBL-associated serine proteases (MASPs): MASP1, MASP2, MASP3, and small MBL-associated protein (sMAP) - to arrays of mannose groups on the surface of a bacterial cell. Similar to C1s, MASP2 is responsible for the activation of C4 and C2, which leaves to the generation of the same C3 convertase (C4b2a) as in the classical pathway. MASP1 is able to cleave C3 directly. The alternative pathway is capable of autoactivation because of a process termed “tickover” of C3, which occurs spontaneously in the plasma, generating a conformationally altered C3, designated C3(H_2O), which is capable of binding factor B (FB). Once factor B associates with C3, factor B itself changes conformation

and can then be cleaved by the constitutively active serum protease factor D (FD), generating Ba and Bb. The Bb fragment remains associated with the complex and can then, through its own serine protease domain, cleave additional C3 molecules, generating a form designated C3b. Once C3b is generated, it associates with factor B to generate more alternative pathway C3-convertase (C3bBb). The alternative pathway can also be initiated as an “amplification loop” when fixed C3b that is generated by classical or lectin pathway activation binds factor B, again resulting in conformational changes in factor B that allow factor D to clave it similarly to the tickover process. All these pathways converge to C3 activation and formation of C5 convertase, which cleaves native C5 to produce soluble C5a, a potent chemoattractant for neutrophils, and C5b, which triggers the membrane attack complex assembly (C5b-9).

Activation of the complement system promotes three main biological activities: opsonization of pathogens; chemotaxis and activation of leucocytes; and direct killing of pathogens. Recently, accumulating evidence has shown that the complement system also acts as an adjuvant by enhancing and directing the adaptive immune response (75, 76). In addition, many studies have also elaborated on the pathogenic role of complement during ischemic, inflammatory, and autoimmune diseases (80).

The relevance of the different complement components and pathways of the complement cascade in experimental EBA, which could represent targets for treatment, have remained unknown.

Aim of the study

The aim of the present study was to examine the relative contribution of the different complement activation pathways for blister formation in experimental EBA. We have recently demonstrated that antibodies generated against murine type VII collagen induce a subepidermal blistering disease closely resembling human EBA when passively transferred into mice of different strains. When injected into C5-deficient mice, antibodies to type VII collagen failed to induce the disease, demonstrating that activation of terminal complement components is required for blister induction in experimental EBA (69). To further dissect the role of the complement system in this model, we injected mice unable to activate complement by the classical (C1q-deficient), the mannan binding lectin (MBL-null), and the alternative (factor B-deficient) pathways, and their control littermates (n=10/group) with rabbit antibodies against murine type VII collagen. Our findings provide the first direct evidence for the involvement of the alternative pathway in an autoantibody-induced blistering disease and a conceptual framework for developing rational therapeutic strategies for EBA and related diseases.

Materials and methods

Mice. C1qa$^{-/-}$ and Bf$^{-/-}$ mice, backcrossed to BALB/c mice (for 10 and 7 generations, respectively), and MBL-null mice backcrossed to C57BL/6J mice (for 7 generations), were previously described (81-83). Age- and sex-matched BALB/c and C57BL/6J mice were obtained from Charles River (Sulzfeld, Germany). All injections and bleedings were performed on mice narcotized by inhalation of isoflurane or intraperitoneal administration of a mixture of ketamine (100 µg/g) and xylazine (15 µg/g). The experiments were approved by the local authorities of the Animal Care and Use Committee (6/g/04) and performed by certified personnel.

Affinity-purification of antibodies. Rabbits were immunized with recombinant forms of murine type VII collagen as described (69). IgG from immune and preimmune rabbit sera was purified by affinity chromatography using Protein G affinity, as previously reported (69). Reactivity of IgG fractions was analyzed by IF microscopy on murine skin.

Induction of blistering in vivo and phenotype assessment. Passive transfer studies followed published protocols with minor modifications (69). Briefly, mice received 6 injections of 7.5 mg rabbit IgG. Blisters or erosions were counted and the extent of skin disease was scored as follows: 0, no lesions; 1, < 10 lesions or < 1% of the skin surface; 2, > 10 lesions or 1-5% of the skin surface; 3, 5-10%; 4, 10-20%; and 5, >20% involvement of the skin surface. Biopsies of lesional and perilesional skin were obtained

2 days after the last injection of IgG and prepared for examination by histopathology and IF microscopy as described (69, 71). Tissue bound murine C5 was detected by incubation of the frozen sections prepared from tissue biopsies with a monoclonal antibody specific to murine C5 (BB5.1) (84) and, finally, with a FITC-labeled antibody specific to mouse IgG (DAKO, Glostrup, Denmark). The staining intensity of immunoreactants in the skin of immunized mice was assessed semiquantitatively using a score comprising 0, for no staining; 1, faint staining; 2, medium; and 3, intense staining (71).

Neutrophil infiltration of murine skin was assayed as described (85), with minor modifications. Briefly, in both clinically diseased and not diseased mice, left ear was removed after killing the mice and skin samples (approximately 10 x 5 mm in size) were extracted by homogenization in a buffer containing 0.1 M Tris-Cl, pH 7.6, 0.15 M NaCl, 0.5% hexadecyl trimethylammoniumbromide (Sigma). Myleoperoxidaze (MPO) activity in the supernatant fraction was measured by the change in optical density at 460 nm resulting from decomposition of H_2O_2 in the presence of *o*-dianisidine (Sigma). A standard reference curve was established using known concentrations of purified MPO (Sigma). MPO content was expressed as units of MPO activity per mg of protein. Protein concentrations were determined by the Bradford dye-binding assay (Bio-Rad Laboratories, Richmond, CA).

For the *in vivo* reconstitution with leukocytes, murine granulocytes were isolated from peripheral blood and bone marrow of donor BALB/c mice by 3% dextran sedimentation followed by density gradient centrifugation using Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and hypotonic lysis in 0.2% NaCl. Only cell preparations with viability above 95% as assessed by trypan-blue exclusion were used.

These consisted of >90% granulocytes as revealed by Giemsa staining and by flow cytometry as described (68). Granulocytes were defined as Gr-1^{hi} $CD11b^{hi}$ using mAb specific to murine Gr-1 (RB6-8C5) and to CD11b (M1/70) (both from BD Biosciences). Mice were injected with 5 x 10^6 cells in 50µl medium intradermally in the ears. The animals were examined clinically after 12 and 24 h, subsequently killed and samples prepared and analysed as described above.

Detection of antibody levels by ELISA. ELISA using recombinant murine type VII collagen was performed at room temperature on 96-well microtiter plates as previously reported (71) with minor modifications. Briefly, each well was coated with 500 ng of the recombinant protein glutathione-S-transferase (GST)-mCOL7C or with an equimolar amount of GST in 0.1 M bicarbonate buffer, pH 9.6 and incubated with 200-fold dilutions of mouse serum for 60 min. Bound antibodies were detected using a 10,000-fold dilution of an horseradish peroxidase-labelled goat anti rabbit IgG antibody (DAKO) and ortho-phenylene diamine (Sigma). The colour reaction was read at 490 nm using a multilabel counter (Victor 3; Perkin Elmer, Wellesley, MA). To evaluate reactivity against the epidermal basement membrane, for each serum, the mean optical density reading obtained with GST was subtracted from the mean reading with GST-mCOL7C.

Statistical analysis. We used OpenStat2 free software for Linux (http://www.agrivisser.com/cgibin/English/OpenStat2.htm). Differences in disease severity and in MPO activity were calculated using the Chi-square and Student's *t* test, respectively. The Mann-Whitney *U* test was used to compare values for semiquantitative

scoring of immunohistochemistry. Means are presented ± S.E.M.; $p < 0.05$ was considered statistically significant.

Results

C1q-deficient mice are susceptible to skin blistering induced by antibodies against type VII collagen

To examine the role of the C1q complement component for subepidermal blister formation in experimental EBA, we injected C1qa$^{-/-}$ (n=10) and wild type (n=10) mice with rabbit IgG against murine type VII collagen. All mice injected with antibodies against type VII collagen developed single blisters 4 days after the first injection. Widespread lesions, including blisters, erosions and crusts, occurred 6 days after the first injection **(Fig. 3, a and b)**.

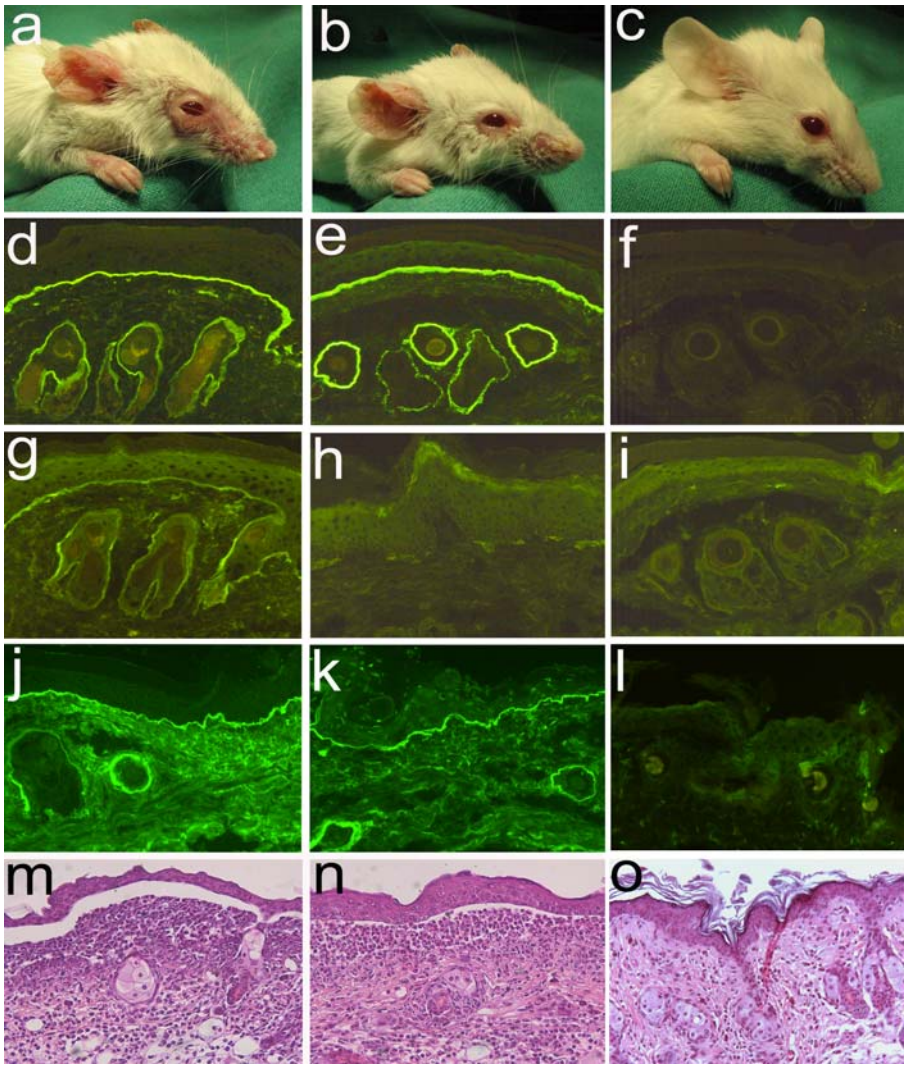

Figure 3. C1q-deficient mice are susceptible to the induction of subepidermal blisters by antibodies specific to type VII collagen. Skin lesions, including blisters and erosions covered by crusts on the ear and front leg, and alopecia of the snout and around eyes, developed in both **(a)** wild type and **(b)** C1qa$^{-/-}$ mice, which received a total dose of 45 mg of rabbit IgG against type VII collagen (day 12). A C1qa$^{-/-}$ mouse receiving the same amount of control rabbit IgG **(c)** did not develop skin lesions. Immunofluorescence microscopy analysis of perilesional skin revealed deposition of rabbit IgG at the dermal epidermal junction of both **(d)** wild type and **(e)** C1qa$^{-/-}$ mice injected with antibodies against type VII collagen, **(f)** but not in the C1qa$^{-/-}$ mouse treated with control rabbit IgG. Deposits of mouse C3 along the dermal epidermal junction were strong in the skin of **(g)** wild type and weak in the skin of **(h)** C1qa$^{-/-}$ mice injected with antibodies against type VII collagen, and **(i)** absent in the C1qa$^{-/-}$ mouse treated with control rabbit IgG. Staining for murine C5 revealed similar deposits in the skin of the **(j)** wild type and of the **(k)** C1qa$^{-/-}$ mice treated with antibodies against type VII collagen, but (**l**) was absent in the C1qa$^{-/-}$ mouse injected with control rabbit IgG. Histological analysis of lesional skin revealed extensive dermal-epidermal separation and a neutrophil-rich inflammatory infiltrate in both **(m)** wild type and **(n)** C1qa$^{-/-}$ mice injected with antibodies against type VII collagen. No histological appearance **(o)** in the skin of the C1qa$^{-/-}$ mouse injected with control rabbit IgG.

Only at the end of the observation period, C1qa$^{-/-}$ mice demonstrated significantly less extensive skin disease compared with control mice **(Fig. 4a)**. The levels of rabbit antibodies against type VII collagen in serum of mice of both groups were similar **(Fig. 4b).**

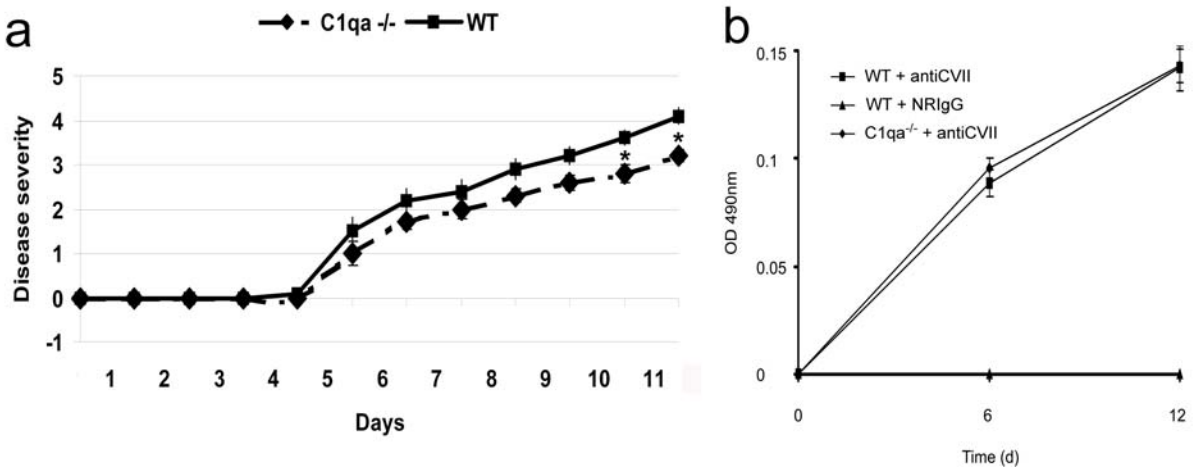

Figure 4. Time course and disease severity in C1q-deficient and control mice. (a) C1qa$^{-/-}$and wild type (WT) mice (n=10 per group) were injected with 7.5 mg of purified rabbit IgG to murine type VII collagen every second day, over a period of 12 days, and evaluated for skin lesions, as described in Methods. *represents significant difference of disease activity ($p < 0.05$) between the 2 groups. **(b)** Serum levels of antibodies to type VII collagen in C1qa$^{-/-}$ and wild type (WT) mice (n=10 per group) injected with rabbit antibodies against type VII collagen (antiCVII) or normal rabbit IgG (NR IgG) as detected by ELISA using recombinant antigen. All values are mean ± S.E.M.

No clinical lesions were observed in C1qa$^{-/-}$ (n=2) and wild type mice (n=2) that received normal rabbit IgG at any time during the observation period (**Fig. 3c**). IF microscopy analysis of perilesional mouse skin revealed linear deposition of rabbit IgG at the dermal-epidermal junction in all mice that received IgG specific to murine type VII collagen **(Fig. 3, d and e)**. No IgG deposits were observed in the skin of mice injected with normal rabbit IgG (**Fig. 3f**). Staining for murine complement C3 was bright in the skin of wild type mice **(Fig. 3g)** and significantly less intense or absent in the skin of C1qa$^{-/-}$ mice **(Fig. 3h)** (C1qa$^{-/-}$ versus wild type mice: 1.3 ± 0.15 versus 2.0 ± 0.24; $p < 0.05$) injected with antibodies against type VII collagen. No C3b deposits were observed

in the skin of mice injected with normal rabbit IgG **(Fig. 3i).** However, staining for murine complement C5 was similar in the skin of wild type **(Fig. 3j)** and C1qa$^{-/-}$ mice **(Fig. 3k)** injected with antibodies against type VII collagen, as shown by grading intensity of C5 staining in perilesional skin biopsies obtained from the two groups of mice (C1qa$^{-/-}$ versus wild type mice: 2.6 ± 0.22 versus 2.8 ± 0.26; $p > 0.05$). No C5 deposits were observed in the skin of mice injected with normal rabbit IgG **(Fig. 3l).** Histological examination of lesional skin biopsies from diseased wild type **(Fig. 3m)** and C1qa$^{-/-}$ **(Fig. 3n)** mice injected with IgG against type VII collagen revealed extensive dermal-epidermal separation accompanied by dense inflammatory infiltrates that were dominated by neutrophils. No histological alterations were observed in mice injected with normal rabbit IgG **(Fig. 3o).**

MBL-null mice develop subepidermal blisters when injected with antibodies against type VII collagen

In further experiments, we analyzed the contribution of MBL for complement activation and blister formation in experimental EBA. MBL-null (n=10) and wild type (n=10) mice were treated with antibodies against murine type VII collagen. Both MBL-null and control mice developed widespread blistering disease **(Fig. 5, a and b)**.

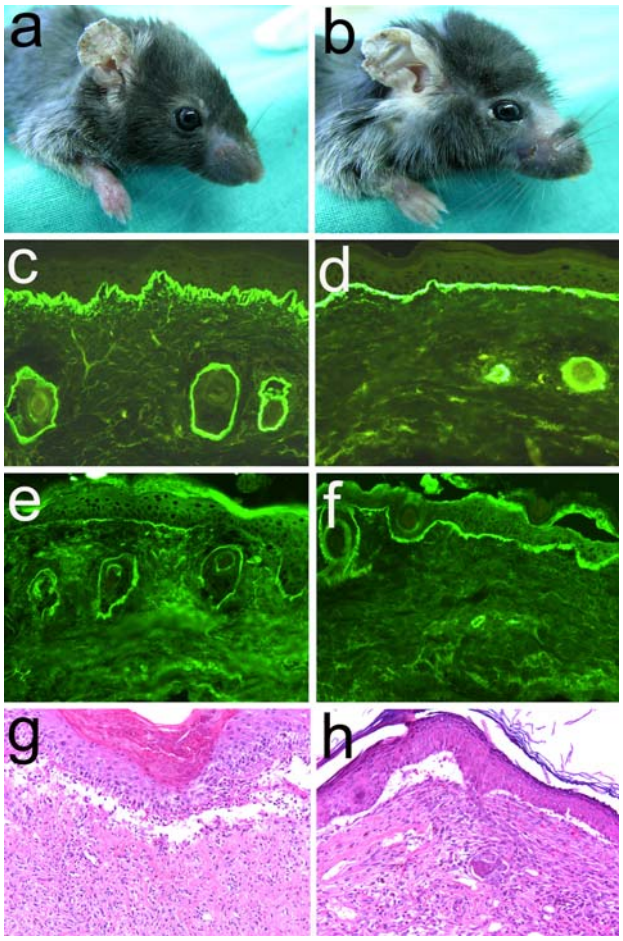

Figure 5. Antibodies against type VII collagen induce subepidermal blisters in MBL-null mice. Skin lesions, including blisters and erosions covered by crusts on the ear and front leg and alopecia of the snout and around eyes developed in both **(a)** MBL-sufficient and **(b)** MBL-null mice, receiving a total dose of 45 mg of rabbit IgG against murine type VII collagen (day 12). IF analysis of perilesional skin revealed deposition of **(c, d)** rabbit IgG and **(e, f)** murine C3 at the dermal epidermal junction of both MBL-sufficient and MBL-null mice. Histological analysis of lesional skin revealed extensive dermal-epidermal separation and a neutrophil-rich inflammatory infiltrate in both **(g)** MBL-sufficient and **(h)** MBL-null mice.

Levels of circulating antibodies against type VII collagen and disease severity were similar in MBL-null and control mice at any time during the observation period **(Fig. 6, a and b)**.

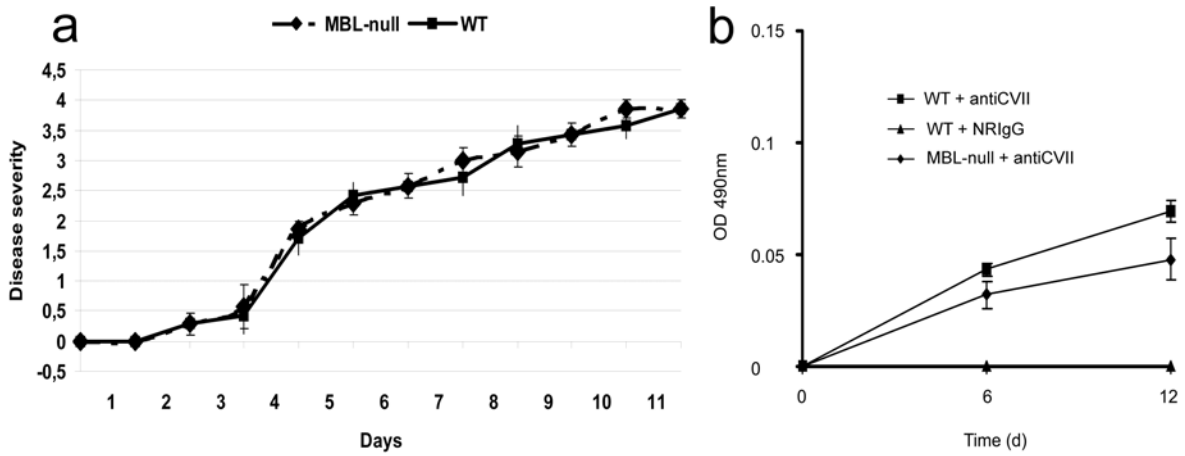

Figure 6. Time course and disease severity in MBL-null and control mice. (a) MBL-null and control mice (WT) (n=10 per group) were injected with 7.5 mg of purified rabbit IgG to murine type VII collagen every second day, over a period of 12 days, and evaluated for skin lesions, as described in Methods. **(b)** Serum levels of antibodies to type VII collagen in MBL-null and wild type (WT) mice (n=10 per group) injected with rabbit antibodies against type VII collagen (antiCVII) or normal rabbit IgG (NR IgG) as detected by ELISA using recombinant antigen. All values are mean ± S.E.M..

MBL-null and control mice (n=2 per group) treated with normal rabbit IgG did not develop skin disease (data not shown). IF microscopy revealed linear deposition of rabbit IgG **(Fig. 5, c and d)** and murine C3 **(Fig. 5, e and f)** at the dermal-epidermal junction of mice from both groups. Histological analysis of lesional skin showed

subepidermal cleavage and a neutrophil-rich inflammatory infiltrate in all mice injected with rabbit IgG to murine type VII collagen **(Fig. 5, g and h)**.

Antibodies against type VII collagen induce a delayed and milder blistering in factor B-deficient mice

To assess the contribution of the alternative pathway, we injected antibodies against murine type VII collagen in mice lacking factor B. Wild type mice (n=10) developed initial blisters 4 days after the first injection of rabbit IgG to type VII collagen, while widespread disease was observed 6 days after the first injection **(Fig. 7a)**. Importantly, $Bf^{-/-}$ mice (n=10) treated with antibodies against type VII collagen developed a delayed and significantly less severe blistering phenotype compared with control mice **(Fig. 7b, c and 8)**.

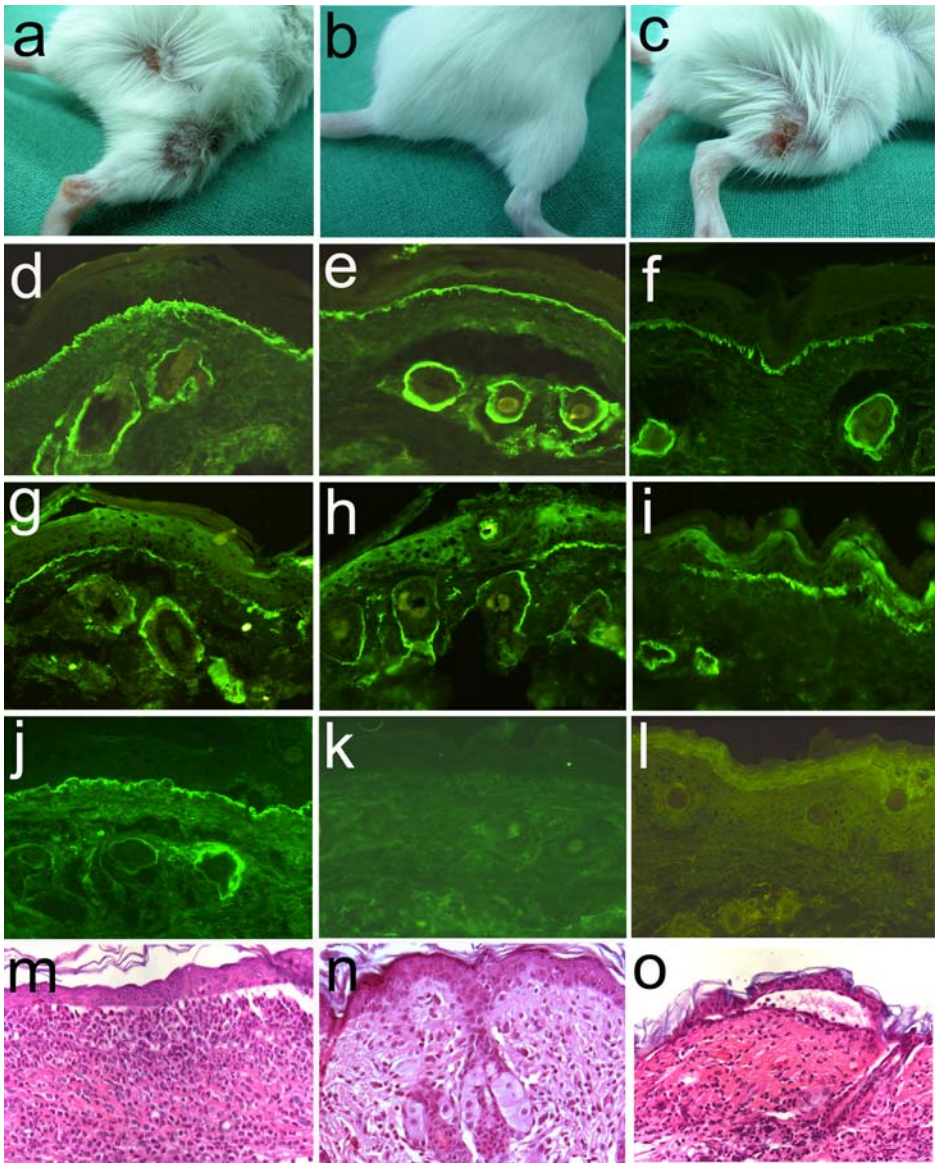

Figure 7. Factor B-deficient mice are relatively resistant to the induction of subepidermal blisters by antibodies against type VII collagen. (a) Erosions covered by crusts on the back and leg of a wild type mouse, which received a total dose of 22.5 mg rabbit IgG against murine type VII collagen, but not in a **(b)** $Bf^{-/-}$ mouse challenged with the same dose of pathogenic IgG (day 7). **(c)** crusted erosions on the hind leg of a $Bf^{-/-}$ mouse injected with 45 mg of pathogenic IgG. Immunofluorescence microscopy analysis of mouse skin revealed deposition of rabbit IgG in **(d)** wild type and $Bf^{-/-}$ mice on days 7 **(e)** and **(f)** 12 of the experiment. Staining for murine C3 revealed similar deposits in the skin of both **(g)** wild type and $Bf^{-/-}$ mice on days 7 **(h)** and **(i)** 12 of the experiment. Deposits of mouse C5 along the dermal epidermal junction were strong in the skin of a **(j)** wild type mouse and absent in the skin of $Bf^{-/-}$ mice on days 7 **(k)**

and **(l)** 12 of the experiment. Histological analysis of murine skin revealed extensive dermal-epidermal separation and a rich inflammatory infiltrate, consisting mainly of neutrophils in **(m)** wild type, but not in **(n)** $Bf^{-/-}$ mice on day 7. **(o)** In contrast, $Bf^{-/-}$ mice on day 12 demonstrated subepidermal cleavage and dense dermal granulocytic infiltration.

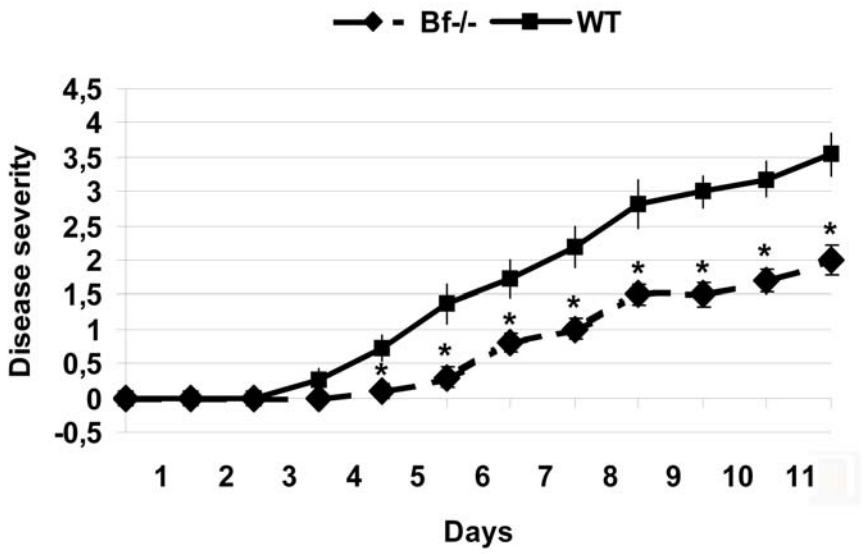

Figure 8. Factor B-deficient mice develop a delayed and significantly less severe blistering disease compared with control mice. Wild type (WT) and $Bf^{-/-}$ mice were injected with 7.5 mg of purified rabbit IgG to murine type VII collagen every second day, over a period of 12 days, and evaluated for skin lesions, as described in Methods. Disease activity is represented as mean ± S.E.M. in 10 wild type and $Bf^{-/-}$ mice mice. *represents significant difference of disease activity between the 2 groups ($p < 0.05$).

Wild type (n=2) and $Bf^{-/-}$ (n=2) mice treated with normal rabbit IgG did not develop skin disease (data not shown). By histopathology, subepidermal blisters and neutrophil infiltration were observed in biopsies of lesional skin obtained from wild type mice **(Fig. 7m)**, while no or reduced dermal-epidermal separation and neutrophil influx were detected in the skin of $Bf^{-/-}$ mice **(Fig. 7, n and o)**. IF microscopy of skin biopsies showed

linear deposition of rabbit IgG at the dermal-epidermal junction of both wild type and $Bf^{-/-}$ mice **(Fig. 7, d, e and f)**. Staining for murine complement C3 was similar in the skin of $Bf^{-/-}$ **(Fig. 7, h and i)**, compared with wild type mice **(Fig. 7g)**, as shown by grading intensity of C3 staining in the perilesional skin biopsies obtained from the two groups of mice ($Bf^{-/-}$ versus wild type mice: 1.11 ± 0.11 versus 1.22 ± 0.15; $p > 0.05$). In contrast, staining for murine complement C5 was bright in the skin of wild type mice **(Fig. 7j)** and significantly less intense or absent in the skin of $Bf^{-/-}$ mice **(Fig. 7, k and l)** ($Bf^{-/-}$ versus wild type mice: 0.33 ± 0.24 versus 1.33 ± 0.33; $p < 0.05$).

The levels of rabbit antibodies against type VII collagen were comparable in serum of $Bf^{-/-}$ and wild type mice **(Fig. 9a)**. Interestingly, granulocytes were significantly less recruited into the dermis of the $Bf^{-/-}$ mice injected with IgG against type VII collagen compared with the wild type mice as demonstrated by histological analysis and measurements of the MPO activity in skin biopsies **(Fig. 9b)**.

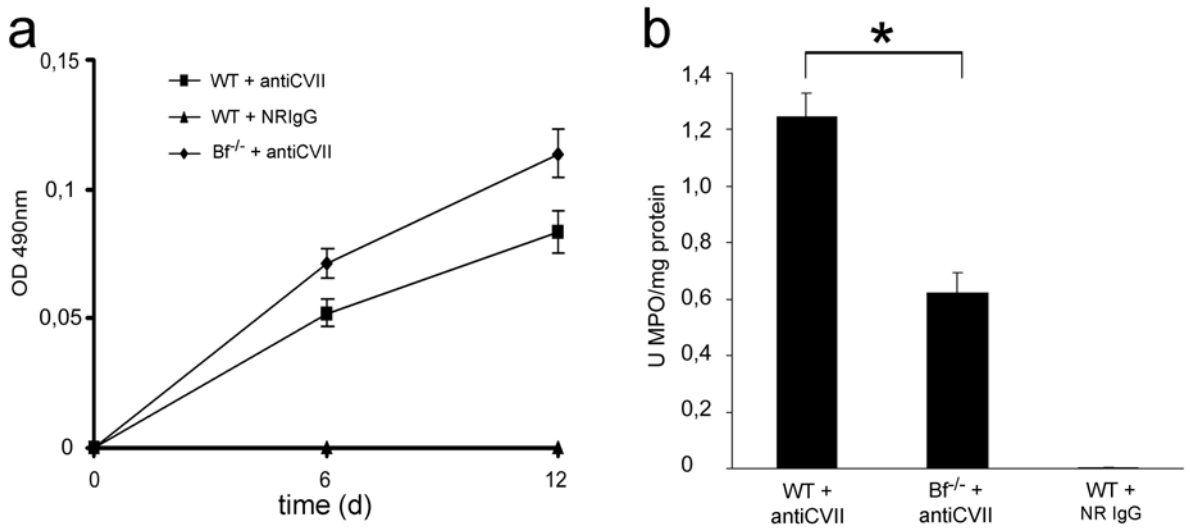

Figure 9. Injection of antibodies against type VII collagen results in less neutrophil infiltration in factor B-deficient compared with wild type mice. (a) Serum levels of antibodies to type VII collagen in

$Bf^{-/-}$ and wild type (WT) mice (n=10 per group) treated with 7.5 mg per injection of rabbit antibodies against type VII collagen (antiCVII) or normal rabbit IgG (NR IgG) as detected by ELISA using recombinant antigen. **(b)** Neutrophil infiltrates in the skin of wild type (WT) and $Bf^{-/-}$ mice treated with the antibodies against type VII collagen or control antibody as assessed by measuring myeloperoxidase (MPO) activity in skin biopsies as described in Methods (*significant difference, $p < 0.01$). All values are means ± S.E.M.

Reconstitution with granulocytes renders factor B-deficient mice readily susceptible to blistering by antibodies against type VII collagen

To verify whether a lower recruitment of granulocytes is responsible for the inhibition of blistering, $Bf^{-/-}$ mice were treated with antibodies against murine type VII collagen and locally injected on day 6 with granulocytes purified from wild type and $Bf^{-/-}$ mice (intradermal injection). Both wild type mice (n=4) treated with IgG against type VII collagen alone **(Fig. 10a)** and $Bf^{-/-}$mice (n=4) injected with antibodies against murine type VII collagen and locally reconstituted with wild type **(Fig. 10b)** and $Bf^{-/-}$ (data not shown) granulocytes (n=4 per group) developed blisters and erosions on their ears. In contrast, $Bf^{-/-}$ mice (n=2) treated with control rabbit IgG and reconstituted with wild type granulocytes did not show any skin alterations **(Fig. 10c)**. Histological analysis revealed an infiltrate of neutrophils in the dermis of all groups of mice **(Fig. 10, d, e, and f)**. Recruitment of granulocytes to the dermal-epidermal junction and subepidermal cleavage was found in the skin of control mice injected with antibodies against murine type VII collagen **(Fig. 10d)** and of $Bf^{-/-}$ mice injected with antibodies to murine type VII collagen and locally reconstituted with wild type **(Fig. 10e)** and $Bf^{-/-}$ (data not shown) granulocytes, but not in the skin of $Bf^{-/-}$ mice treated with control rabbit IgG and reconstituted with murine granulocytes **(Fig. 10f)**.

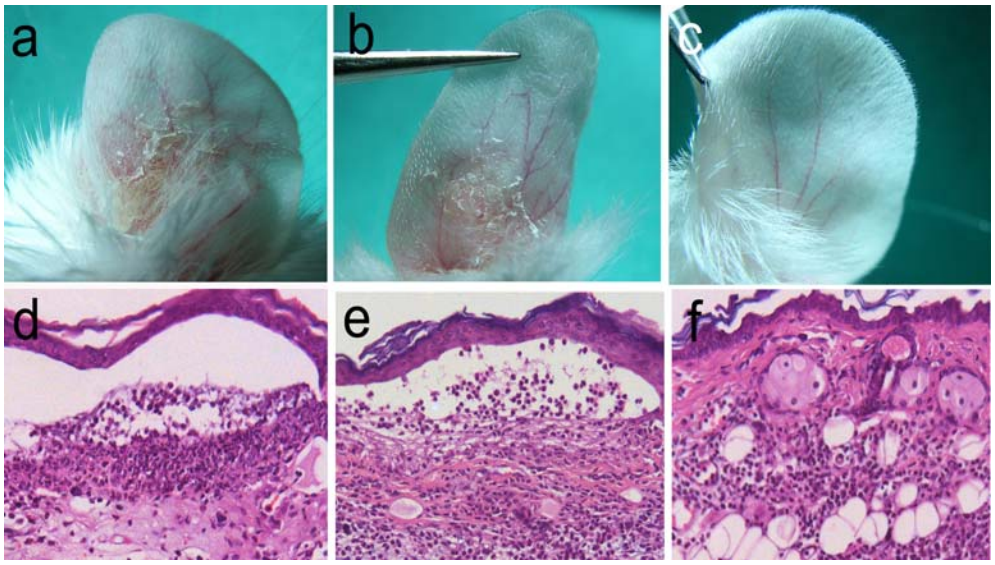

Figure 10. Local reconstitution with granulocytes renders factor B-deficient mice susceptible to the antibody-induced skin blistering. Mice were treated with a total dose of 22.5 mg rabbit IgG subcutaneously into the back and, subsequently, ears were injected with 5 x 10^6 murine granulocytes in 50 µl medium intradermally. Both a **(a)** wild type mouse injected with rabbit antibodies to murine type VII collagen and a **(b)** $Bf^{-/-}$ mouse injected with pathogenic rabbit IgG and locally reconstituted with granulocytes developed blisters and erosions on their ears. **(c)** A $Bf^{-/-}$ mouse treated with control rabbit IgG and reconstituted with granulocytes did not show skin alterations. **(d, e, f)** Histological analysis revealed an infiltrate of neutrophils in the dermis of all mice. Dermal-epidermal separation was observed in the skin of both the **(d)** wild type mouse injected with rabbit antibodies to murine type VII collagen and the **(e)** $Bf^{-/-}$ mouse injected with pathogenic rabbit IgG and locally reconstituted with granulocytes, but not in the skin of the **(f)** $Bf^{-/-}$ mouse treated with control rabbit IgG and reconstituted with granulocytes.

Discussion

The mechanisms of autoantibody-induced blistering in EBA are not fully understood. Several possible mechanisms were proposed that include: 1) complement activation, recruitment and activation of inflammatory cells by autoantibodies bound to anchoring fibrils, 2) autoantibodies specific to type VII collagen may directly interfere with the antiparallel dimer formation and its proper incorporation into the anchoring fibrils, and 3) disruption of the interaction between type VII collagen and its ligands. While *in vivo* experimental evidence supporting a direct, Fc-independent, non-inflammatory mechanisms of blister induction in EBA is still lacking, a growing body of clinical and experimental evidence favors the inflammatory hypothesis (86). Blistering induced by binding of antibodies to type VII collagen seems to be dependent on triggering down-stream inflammatory pathways, including complement (69) and leukocytes (87). However, the relevance of the different complement components and pathways of the complement cascade in experimental EBA, which could represent targets for treatment, have remained unknown. Therefore, in the present study, we injected mice unable to activate complement by the classical (C1q-deficient), the mannan binding lectin (MBL-null), and the alternative (factor B-deficient) pathways with rabbit antibodies against murine type VII collagen.

We made the unanticipated observation that C1q-deficiency and blockade of complement activation by the classical pathway does not inhibit tissue injury induced by antibodies against type VII collagen in mice. Instead, we found the activation of the alternative complement pathway to play a pivotal role for the antibody-induced blistering. The passive transfer of antibodies against type VII collagen into mice provokes within

days a subepidermal blistering disease reproducing the clinical, histopathological, immunopathological and electron microscopical findings in patients with EBA. A likely scenario is that, after binding to their target at the dermal-epidermal junction, antibodies against type VII collagen trigger a cascade of events that includes the activation of the complement network and/or engagement of FcRs on leukocytes recruited into the skin. Potent inflammatory mediators, including cytokines, reactive oxygen species, and proteases released by granulocytes most probably amplify local inflammation and are instrumental for tissue destruction (88).

This scenario bears similarities to the effector phase of other autoantibody-induced inflammatory diseases, including arthritis (89), vitiligo (90), cryoglobulinemia (91, 92), bullous pemphigoid (93) and anti-phospholipid syndrome (94). In contrast, the pathogenic effects of autoantibodies in other autoimmune diseases such as pemphigus (95) are mediated strictly by binding to their target antigen and do not involve the complement network. An overall remark with regard to the role of complement in tissue injury in autoimmune diseases is that its contribution varies depending on the particular tissue involved and the genetic background. Examining complement activation pathways in different autoimmune diseases in patients and experimental animals is therefore mandatory to identify key molecular effectors of inflammatory tissue injury for therapeutic interventions.

It is textbook knowledge that complement-fixing (auto)antibodies bind to the C1q and trigger the complement cascade by the classical pathway. It has been therefore proposed that antibody-induced tissue injury essentially depends on complement activation by the classical pathway. In the present study, we found the complement component C1q to be dispensable for the induction of blisters by antibodies against type

VII collagen. In line with the present observations, C1q-deficiency does not protect from autoantibody-induced tissue injury in cryoglobulin-induced immune complex glomerulonephritis (91). In addition, the blockade of the classical pathway in $C4^{-/-}$ mice does not abolish the pathogenic effects of K/BxN-derived anti-GPI serum or of anti-collagen II antibodies in a passive transfer model of rheumatoid arthritis (89, 96). Fetal loss and growth restriction triggered by anti-cardiolipin antibodies in mice, main features of the anti-phospholipid syndrome, and blistering induced by antibodies to BP180 in experimental bullous pemhigoid, also depend on complement activation (93, 97, 98). However, in these experimental models, the resistance of $C4^{-/-}$ mice to tissue injury demonstrates an important role of an intact classical complement activation pathway (94, 98). The differences with regard to the classical pathway contribution to pathology in these experimental disease models have not yet been explained. One may only speculate that differences of targeted antigens and binding of antibodies as well as different isotypes and glycosylation of antibodies may influence the interaction of antigen-antibody complexes with various components of the complement network.

To evaluate the contribution of factor B for antibody-induced blistering, we analyzed disease expression in $Bf^{-/-}$ mice (82). Induction of blistering was delayed and the extent of cutaneous disease was significantly lower in $Bf^{-/-}$ compared with wild type mice. This interesting finding raised the question of what activates the alternative pathway. A classical explanation is that the alternative pathway is initiated as "an amplification loop" by fixed C3b generated by classical or lectin pathways. However, blockade of complement activation by the classical pathway in C1q-deficient mice or by the lectin pathway in MBL-null mice did not alter the antibody-induced blistering. These findings suggest that initial complement activation occurs by both classical and lectin pathways,

which can thus compensate for each other. An additional possibility is that the activation and amplification of the alternative pathway is due to a breakdown of the active control of the alternative pathway in the skin caused by binding of antibodies, which may act as activating surfaces. Although it has not been reported that ficolins bind immunoglobulins, our study cannot rule out a possible involvement of ficolins that are also able to initiate the lectin pathway (99, 100).

Granulocyte recruitment to the dermal-epidermal junction by antibodies is a prerequisite for blister induction in the animal model used in this study (87) and in the *ex vivo* cryosection model of EBA (67). Our present findings show that the absence of factor B impairs the recruitment of granulocytes into the skin of mice injected with antibodies against type VII collagen. In line with these results, inhibition of neutrophil recruitment into the joints has been observed in factor B-deficient mice passively transferred with K/BxN-derived anti-GPI serum (89). The relevance of granulocyte recruitment into the skin is further supported by the fact that local reconstitution of factor B-deficient mice with granulocytes following injection of antibodies against type VII collagen resulted in blister formation.

It has been suggested that neutrophils promote complement deposition by causing damage that triggers complement activation and by secreting C3 and/or properdin at sites of inflammation to amplify complement activation via the alternative pathway. Properdin functions by stabilizing the interaction of factor B with spontaneously generated C3(H_2O) and the formation of C3 convertase C3bBb (101, 102)**.** As depletion of neutrophils abolishes blister induction in the animal model used in this study (87), an alternative explanation for the importance of the alternative pathway in our model could be that infiltrating cells, such as neutrophils, bring in C3 and properdin that increase

activation specifically at that site by providing additional substrate for the alternative pathway. Linkage of the alternative pathway activation with neutrophil infiltration has been suggested to contribute to the resistance of mice deficient in factor B to joint damage after treatment with arthriogenic antibodies (89), and to fetal loss and growth restriction after injection of anti-cardiolipin antibodies (97) in passive transfer models that parallel our model. However, the finding that factor B-deficient granulocytes, similar to wild type cells, also mediate blister formation in our model strongly suggests that factor B produced by granulocytes is not essentially required for antibody-induced blistering in experimental EBA.

Deposition of the complement protein C3 at sites of autoantibody binding in tissues is a hallmark of autoimmune diseases in humans (103). Our present results show that C3 deposition in the skin is not imperiously associated with antibody-induced tissue injury. On one hand, a reduced C3 deposition was found in C1q-deficient mice susceptible to experimental EBA. On the other hand, C3 deposits were found in factor B-deficient mice resistant to blister induction by antibodies against type VII collagen. Interestingly, a markedly reduced C5 deposition was found in factor B-deficient mice compared with controls. Indeed, it has been recently shown that generation of C5a does not absolutely require C3 (104). Proteases released by leukocytes recruited at the dermal-epidermal junction could also contribute to generation of C5a in our model (105, 106). Why complement activation at the dermal-epidermal junction in C1q-deficient mice is curtailed beyond the C3 activation step is unclear, but reminiscent of the targeted and restricted complement activation that may occur on modified self tissues (107). Taken together, these findings suggest that generation of C5a in our model is required for disease expression, but partly independent of the classical C5 convertase (C4b2a3b) availability.

Among the complement activation products, C5a is one of the most potent inflammatory peptides, with a broad spectrum of functions: it attracts and activates neutrophils, monocytes and mast cells, and stimulates the release of inflammatory mediators, including reactive oxygen species, proteolytic enzymes, chemokynes and cytokines, as well as complement components (108). Neutrophil-dependent animal models similar to our animal model have identified the proinflammatory sequelae of C5a: C5a receptor interactions as being a critical intermediate linking pathogenic antibodies to tissue damage (89, 94). In previous studies, we have found C5-deficient mice to be resistant to the induction of blisters by antibodies to type VII collagen, demonstrating that activation of terminal complement components is required for blister formation in experimental EBA (69). However, C5 deficiency does not only prevents the downstream activation of terminal complement components with subsequent formation of the membrane attack complex, but also eliminates the production of C5a. In this context, we can only speculate on the key effector role played by the C5a molecule in the granulocyte-dependent tissue injury in our model. However, the effect of C5a inhibition on the outcome of experimental EBA still needs to be elucidated.

New mechanistic insights into the role of complement activation in the pathogenesis of autoimmune inflammatory diseases facilitate targeting complement pathways for therapeutic drug development. In addition to many reports of a beneficial effect of complement blockade in animal models, anti-C5 therapy is well tolerated and effective in patients with paroxysmal nocturnal hemoglobinuria (109, 110). Our present study identifies factor B as an additional target of therapy in inflammatory autoimmune blistering diseases. Selective inhibition of this pathway may not result in side effects seen with inhibitors of C3 and C5 convertases. Such an inhibitory monoclonal antibody

against factor B prevents antiphospholipid antibody-induced pregnancy loss in mice (79). To avoid a global inhibition of complement activation, more specific approaches were explored to target inhibitors of complement activation to the site of inflammation by linking inhibitors to the complement receptor (CR) 2 (111, 112). C3 breakdown products deposited at sites of complement activation are natural ligands for CR2. Thus blockers of the alternative pathway could be directed to the dermal-epidermal junction by designing fusion proteins of CR2 and single-chain variable fragments (scFv) of mAb or inhibitory peptides.

In conclusion, our results demonstrate that an intact alternative complement activation pathway is required for blistering induced by antibodies against type VII collagen. Thus selectively blocking this pathway may offer therapeutic benefit in patients with EBA and related autoantibody-mediated diseases.

Summary

Epidermolysis bullosa acquisita is a subepidermal blistering disease associated with tissue-bound and circulating autoantibodies against type VII collagen, a major constituent of the dermal-epidermal junction. The passive transfer of antibodies against type VII collagen into mice induces a subepidermal blistering disease dependent upon activation of terminal complement components. To further dissect the role of the different complement activation pathways in this model, we injected C1q-deficient, mannan binding lectin-deficient (MBL-null), and factor B-deficient mice with rabbit antibodies against murine type VII collagen. The development and evolution of blistering had a similar pattern in MBL-null and control mice, and was initially only marginally less extensive in C1q-deficient mice compared with controls. Importantly, factor B-deficient mice developed a delayed and significantly less severe blistering disease compared with factor B-sufficient mice. A significantly lower neutrophilic infiltration was observed in factor B-deficient mice compared with controls, and local reconstitution with granulocytes restored the blistering disease in factor B-deficient mice. Our study provides the first direct evidence for the involvement of the alternative pathway in an autoantibody-induced blistering disease and should facilitate the development of new therapeutic strategies for epidermolysis bullosa acquisita and related autoimmune diseases.

Zusammenfassung

Die Epidermolysis bullosa acquisita ist eine subepidermal blasenbildende Autoimmunerkrankung, die mit Autoantikörpern gegen Typ VII Kollagen einhergeht. Durch passiven Transfer von Autoantikörpern gegen Typ VII Kollagen in Mäuse konnten wir erstmals eine subepidermale blasenbildende Erkrankung hervorrufen, die mit der Aktivierung von Komplement assoziiert war. Um die Bedeutung des Komplementsystems für die Pathogenese der EBA näher zu charakterisieren, injizierten wir genetisch veränderten Mäusen mit Defizienzen von C1q (klassischer Komplement-aktivierungsweg), von Faktor B (alternativer Komplementaktivierungsweg) und von Mannan bindendem Lektin (Lektinweg der Komplementaktivierung) pathogene Antikörper. Die Entwicklung der Blasen war bei MBL-null und Kontrollmäusen ähnlich und bei C1q-defizienten Mäusen gegen Ende der Experimente etwas geringer als bei den Kontrollmäusen. Demgegenüber entwickelten die Faktor B-defizienten Mäuse deutlich verspätet und weniger Blasen als die Faktor B-suffizienten Mäuse. Im Vergleich zu den Kontrolltieren wurde bei Faktor B-defizienten Mäusen auch eine signifikant geringere Infiltration von Neutrophilen beobachtet, während die lokale Rekonstitution mit Granulozyten die Blasenbildung in Faktor B-defizienten Mäusen wiederherstellte. Die vorliegende Arbeit identifiziert erstmals den alternativen Komplementweg als einen Effektormechanismus für die durch Autoantikörper induzierte Blasenbildung und soll die Grundlage für spezifischere Therapieansätze für die entzündliche Gewebeschädigung durch Autoantikörper darstellen.

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List of own original articles generated during the thesis work

1. **Mihai S**, Chiriac MT, Takahashi K, Thurman JM, Holers VM, Zillikens D, Botto M, Sitaru C. The alternative pathway of complement activation is critical for blister induction in experimental epidermolysis bullosa acquisita. ***J Immunol***, in press (IF 6.38).
This work constitutes the main body of the doctoral thesis.

2. Sitaru C, **Mihai S**, Otto C, Chiriac MT, Haußer I, Dotterweich B, Saito H, Rose C, Ishiko A, Zillikens D. Induction of dermal-epidermal separation in mice by passive transfer of antibodies to type VII collagen. ***J Clin Invest*** 2005, 115: 870-878 (IF 15,05).
The work presented here describes the first passive transfer model for the autoimmune blistering skin disease epidermolysis bullosa acquisita. With some modifications, most of the protocols published in this study, including the purification of antibodies, the injection protocols and the scoring for the immunoreactants deposited in situ were successfully applied throughout the in vivo experiments performed in the doctoral thesis.

3. Sitaru C, Chiriac MT, **Mihai S**, Büning J, Gebert A, Ishiko A, Zillikens D. Induction of complement fixing IgG4 autoantibodies against type VII collagen results in subepidermal blistering in mice. ***J Immunol*** 2006 177(5): 3461-8 (IF 6.38).
Some methods and protocols published in this paper, including ELISA with recombinant protein and evaluation of the disease activity in vivo, were adapted and used in the doctoral thesis.

4. Shimanovich I, **Mihai S**, Oostingh GJ, Ilenchuk T, Bröcker E-B, Opdenakker G, Zillikens D**,** Sitaru C. Granulocyte-derived elastase and gelatinase B are required for dermal-epidermal separation induced by autoantibodies from patients with epidermolysis bullosa acquisita and bullous pemphigoid. ***J Pathol*** 2004, 204: 519-527 (IF 6.21).

5. Olaru F, **Mihai S**, Petrescu I, Zillikens, D, Sitaru C. Generation and characterization of monoclonal antibodies against the intracellular domain of human type XVII collagen. ***Hybridoma*** 2006, 25(3): 158-62 (IF 0.4).

6**. Mihai S**, Chiriac MT Herrero-Gonzales JE, Goodall M, Jefferis R, Savage CO, Zillikens, D, Sitaru C. Non-complement-fixing IgG4 autoantibodies activate leukocytes and induce dermal-epidermal separation. Submitted.

List of review articles generated during the thesis work

1. Sitaru C, **Mihai S**, Zillikens D. The relevance of IgG subclass of autoantibodies for blister induction in autoimmune bullous skin diseases. ***Arch Dermatol Res*** *2007*, in press (IF 1.21).

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List of abstracts published during the thesis work

1. Sitaru C, **Mihai S**, Otto C, Chiriac MT, Hausser I, Dotterweich B, Saito H, Rose C, Ishiko A, Zillikens D. Induction of dermal-epidermal separation in mice by passive transfer of antibodies to type VII collagen. 34th Annual Meeting of the European Society for Dermatological Research (ESDR), 9-12 September 2004, Vienna, Austria. J Invest Dermatol 2004, 123: A 13.

2. Sitaru C, **Mihai S**, Otto C, Chiriac MT, Hausser I, Dotterweich B, Saito H, Rose C, Ishiko A, Zillikens D. Induction of dermal-epidermal separation in mice by passive transfer of antibodies to type VII collagen. Joint Annual Meeting of the German and Dutch Societies for Immunology (JAMI), 20-23 October, Maastricht, The Netherlands. Immunobiology 2004; 209: 302.

3. **Mihai S**, Zillikens D, Sitaru C. Non-complement-fixing IgG4 autoantibodies from bullous pemphigoid patients induce dermal-epidermal separation in cryosections of human skin. 32nd Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), 03-05 March 2005, Innsbruck, Austria. Arch Dermatol Res 2005; 296: 389**.**

3. Sitaru C, Chiriac MT, **Mihai S**, Ishiko A, Zillikens D. Development of an active disease model for epidermolysis bullosa acquisita in mice. 32nd Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), 03-05 March 2005, Innsbruck, Austria. Arch Dermatol Res 2005; 296: 389.

4. Sitaru C, Chiriac MT, **Mihai S**, Ishiko A, Zillikens D. Development of an active disease model for epidermolysis bullosa acquisita in mice. 10th European Meeting on

Complement in Human Disease, 09-13 September 2005, Heidelberg, Germany. Mol Immunol 2006; 43: 158.

5. **Mihai S,** Sitaru C, Otto C, Chiriac MT, Hausser I, Dotterweich B, Saito H, Rose C, Ishiko A, Zillikens D. Entwicklung eines tiermodells für die Epidermolysis bullosa acquisita. 7th Meeting of the German-Japanese Society of Dermatology (GJSD), 03-05 June 2005, Dresden. Hautarzt, 2005; 56: 506-516.

6. **Mihai S**, Herrero-Gonzalez JE, Goodall M, Jefferis R, Savage CO, Zillikens D, Sitaru C. Non-complement-fixing IgG4 autoantibodies from bullous pemphigoid patients activate leukocytes and induce blisters in cryosections of human skin. 35th Annual Meeting of the European Society for Dermatological Research (ESDR), 22-24 September 2005, Tübingen, Germany. J Invest Dermatol 2005, 125 (3): A 45

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