Autoantibodies to Multiple Epitopes on the Non-Collagenous-1 Domain of Type VII Collagen Induce Blisters

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Epidermolysis bullosa acquisita (EBA) is an autoimmune blistering disease of the skin and mucous membranes, characterized by autoantibodies against type VII collagen (COL7), a major component of anchoring fibrils. Different clinical EBA phenotypes are described, including mechanobullous and inflammatory variants. Most EBA patients' sera react with epitopes located within the non-collagenous 1 (NC1) domain of human COL7. However, it has remained unclear whether antibody binding to these different epitopes is pathogenically relevant. To address this issue, we generated recombinant proteins covering the entire NC1 domain. IgG reactivity with these proteins was analyzed in sera of 69 EBA patients. Most recognized clusters of epitopes throughout the NC1 domain. No correlation was detected between antibody specificity and clinical phenotype. To study the pathogenicity of antibodies specific to different NC1 subdomains, rabbit antibodies were generated. All these antibodies caused dermal–epidermal separation *ex vivo*. Antibodies against two of these subdomains were injected into mice carrying null mutations of mouse COL7 and the human COL7 transgene and induced subepidermal blisters. We here document that autoantibodies to COL7, independent of the targeted epitopes, induce blisters both *ex vivo* and *in vivo*. In addition, using COL7-humanized mice, we provide *in vivo* evidence of pathogenicity of autoantibodies binding to human COL7.

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INTRODUCTION

Autoimmune diseases are characterized by the presence of autoreactive T or B cells that mediate an uncontrolled inflammatory response and are responsible for clinical disease manifestation (Rose and Bona, 1993). Over the past decades, incidence of autoimmune diseases has constantly increased in developed countries (Bach, 2002). Epidermolysis bullosa acquisita (EBA) is a prototypical, organ-specific autoimmune disease. EBA is characterized by the presence of circulating and tissue-bound antibodies to type VII collagen (COL7), a major component of anchoring fibrils (Ludwig, 2013; Sakai *et al.*, 1986; Schmidt and Zillikens, 2013; Woodley *et al.*, 1984; Woodley *et al.*, 1986). Pathogenicity of anti-COL7 autoantibodies has been demonstrated in several experimental models (Ludwig *et al.*, 2013). Clinically, different EBA phenotypes are described: (i) classical mechanobullous (Roenigk *et al.*, 1971), and (ii) inflammatory type, including bullous pemphigoid- (Gammon *et al.*, 1984), Brunsting-Perry pemphigoid- (Kurzhals *et al.*, 1991), linear IgA dermatosis- (Zambruno *et al.*, 1994), and mucous membrane pemphigoid-like variants (Dahl, 1979). Whereas events leading to blister formation in the inflammatory EBA variant are relatively well characterized, it remains unclear which processes cause blistering in mechanobullous EBA—e.g., perturbation of interactions of COL7 and other components of the dermal–epidermal junction (Gupta *et al.*, 2012; Ishii *et al.*, 2010; Ludwig, 2012).

Detailed analysis of the autoantibody response in EBA patients showed that, in the majority of cases, IgG anti-COL7 autoantibodies are detected. Characterization of IgG isotypes demonstrated the prevalence of IgG1 and IgG4 subclasses (Bernard *et al.*, 1991; Oostingh *et al.*, 2005). In addition, IgA anti-COL7 reactivity, either exclusively or in combination with IgG autoantibodies, is observed in 50–60% of patients (Buijsrogge *et al.*, 2011; Gandhi *et al.*, 2000; Kim *et al.*, 2011). Yet, this understanding of the autoimmune response has not been useful to differentiate among the mechanobullous and

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the inflammatory EBA variants (Gandhi *et al.*, 2000). Epitope mapping of the COL7-specific B-cell immune response in EBA patients identified the NC1 domain of COL7 as the major antigenic site (Gammon *et al.*, 1993; Jones *et al.*, 1995; Tanaka *et al.*, 1994). Binding of patient autoantibodies to the collagenous or the NC2 domain is rarely observed (Ishii *et al.*, 2004; Saleh *et al.*, 2011). Further epitope mapping of EBA patients' sera identified 4 major antigenic epitopes within the NC1 domain (Chen *et al.*, 2007; Gammon *et al.*, 1993; Gandhi *et al.*, 2000; Ishii *et al.*, 2004; Lapiere *et al.*, 1993). However, it remained unclear whether the specificities of the anti-COL7 antibodies are associated with certain clinical EBA phenotypes.

The pathogenic relevance of patient's anti-COL7 IgG has been demonstrated by its ability to induce dermal–epidermal separation when incubated with cryosections of human skin in the presence of neutrophils (Sitaru *et al.*, 2002a) and by induction of subepidermal blisters in mice, which were injected with patient antibodies, affinity-purified against the NC1 domain (Woodley *et al.*, 2006). Experimental EBA can also be induced in mice by transfer of antibodies to epitopes located within the murine sixth–ninth fibronectin-3 (FNIII)-like repeat or by immunization with the same antigen (Sitaru *et al.*, 2006; Sitaru *et al.*, 2005). Furthermore, transfer of patient autoantibodies, affinity-purified against the cartilage matrix protein (CMP) domain, also induced EBA after transfer into mice (Chen *et al.*, 2007). Recently, pathogenicity of anti-von Willebrand factor 2 (anti-vWFA2) antibodies has been demonstrated, both by transfer of autoantibodies and by immunization of mice with vWFA2 (Iwata *et al.*, 2013). Collectively, sera from EBA patients bind to many epitopes located within the NC1 domain, but pathogenicity has only been demonstrated for few of these. It remains unclear whether the broad epitope recognition observed in EBA patients is an epiphenomenon resulting from intramolecular epitope spreading or whether this broad recognition pattern is required for blister induction.

In a cohort of 69 EBA patients as well as in *ex vivo* and *in vivo* models of EBA, we therefore here addressed whether (i) the different clinical EBA phenotypes are associated with certain autoantibody specificities, and (ii) whether autoantibodies to certain domains of NC1 are capable to induce subepidermal blisters.

RESULTS

Expression of recombinant subdomains of COL7 NC1

Recombinant overlapping proteins for NC1 subdomains and NC2 domain were successfully produced in *E. coli* Rosetta DE3 (Supplementary Figure S1). CMP-FNIII1 and FNIII9-vWFA2 fragments were insoluble. After refolding, CMP-FNIII1 protein could be obtained in sufficient amounts and purity for rabbit immunization. FNIII9-vWFA2 protein purity did not allow using this protein for raising rabbit antibodies. Correct size of all proteins was confirmed by SDS-PAGE gel analysis (Supplementary Figure S1).

EBA patients' sera recognize multiple epitopes within NC1

For determination of epitope recognition patterns of patient autoantibodies, we analyzed IgG reactivity with recombinant proteins of NC1 domain. In preliminary studies, IgG1, IgG2, IgG3, and IgG4 secondary antibodies for detection of reactivity to the recombinant fragments were evaluated for specificity. Although the use of secondary antibodies specific for IgG1, IgG2, and IgG3 showed high reactivity with normal human sera, the use of IgG4 secondary antibodies did not or only rarely show this unspecific binding (not shown). This is in line with the routine diagnosis of EBA in our laboratory, where an anti-IgG4 antibody is used for the same reason (Schmidt and Zillikens, 2013). Furthermore, here (Supplementary Table S1 online) and elsewhere (Komorowski *et al.*, 2013) IgG4 reactivity correlated well with the COL7-specific binding of other IgG to this protein. Therefore, subsequent epitope mapping studies were carried out using anti-human IgG4 as a secondary antibody. In most cases, the IgG4 reactivity pattern in sera from EBA patients was not restricted to single epitopes, but rather showed clusters of reactivity, recognizing multiple subdomains of NC1 domain (Figure 1). Detailed analysis of the number of epitopes recognized by EBA sera showed that most sera (58%) had autoantibody reactivity to two stretches (defined as a continuous recognition of subsequent recombinant proteins). The mean length of such stretches consisted on an average of 3.5 ± 2.7 recombinant proteins. Sera of 16 patients recognized a single stretch with a length of 7.2 ± 2.7 recombinant proteins; autoantibodies from 13 patients were specific to three different stretches, mean length 2.0 ± 1.5. We also generated recombinant NC2. In 41 tested sera from our cohort of EBA patients, 9 reacted to this epitope.

Gender and age, but not the clinical EBA variant, correlate with autoantibody specificity

To test whether certain epitope recognition patterns are specific for the different clinical phenotypes of EBA, we performed homogeneity distribution analysis and Pearson's correlation coefficient analysis. Interestingly, no statistically significant correlation was found between the recognized epitopes within the NC1 domain and the clinical phenotype. However, IgG4 binding pattern was dependent on age and gender of the patients (Figure 2). Younger age was associated with recognition of C-terminally located recombinant proteins, as well as NC2. Male gender was associated with recognition of FNIII2-FNIII3 and FNIII3-FNIII4 fragments. Antibodies from female EBA patients bound more to FNIII8-FNIII9 (Figure 2).

Autoantibodies against specific human NC1 subdomains show intramolecular cross-reactivity and varying cross-reactivity with murine skin

For functional studies, polyclonal rabbit anti-COL7 antibodies were generated by immunizing rabbits with all recombinant proteins spanning the human NC1 domain with the exception of FNIII9-vWFA2. Subdomains of NC1 domain of COL7 consist of the flanking CMP and vWFA2 domains and 9 FNIII-like repeats; these subdomains share some homology (Parente *et al.*, 1991). We first evaluated the possibility of intramolecular cross-reactivity of autoantibodies directed to specific domains within NC1. For this, we performed western blotting analysis using affinity-purified anti-NC1 antibodies

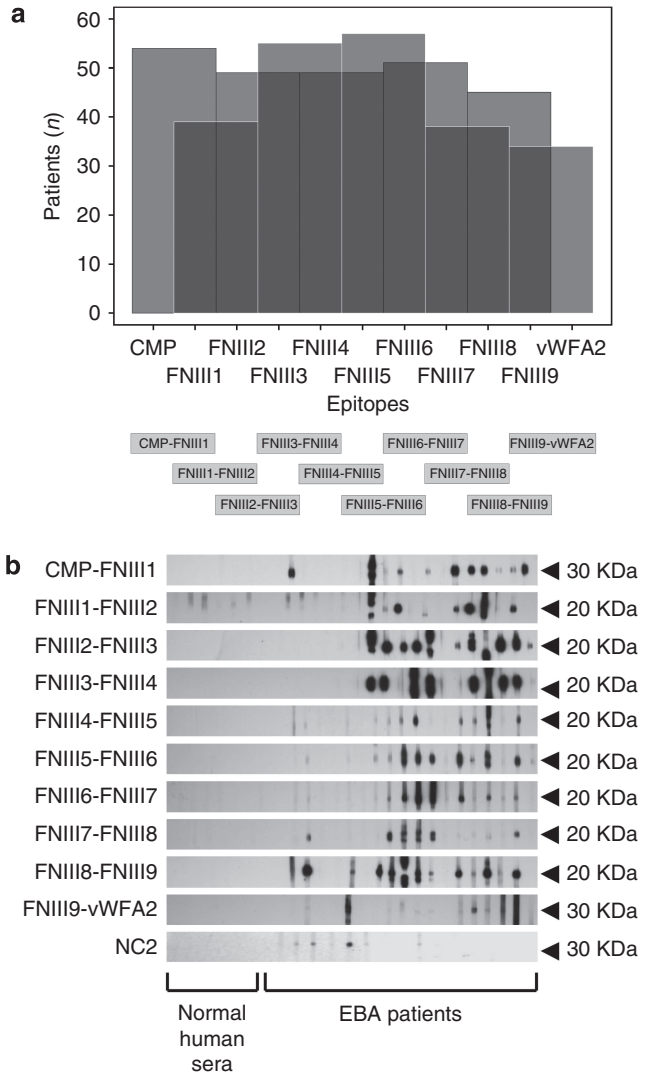

Figure 1. Epidermolysis bullosa acquisita (EBA) patients' sera recognize multiple epitopes within the non-collagenous 1 (NC1) domain. (**a**) Schematic representation of the epitope-specific reactivity of 69 EBA patients' sera tested by western blotting. The *y* axis corresponds to the number of EBA patient sera reacting with the epitopes specified on the *x* axis. (**b**) Representative images of western blotting analysis of selected EBA patients' sera. Bands show reactivity of 18 EBA sera with different recombinant proteins of human type VII collagen NC1. As negative controls, randomly selected normal human sera were used.

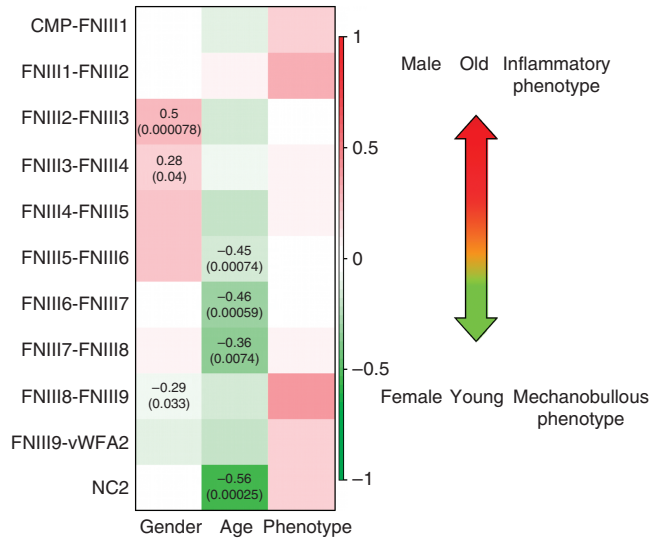

Figure 2. Correlation of gender, age, and clinical phenotype with autoantibody reactivity. Heatmap table showing Pearson's correlation coefficient of epitope-specific autoantibody reactivity with gender, age, and clinical phenotype. Nonsignificant values are shown as blank. Top line of the numbers in each box corresponds to Pearson's correlation coefficient and bottom line (in brackets) to *P*-value. In the left column, red and green colors correspond to male and female gender, in the middle column to older and younger age, and in the right column to inflammatory and mechanobullous phenotypes, respectively.

from rabbit sera and recombinant proteins. All antibodies showed reactivity to the respective antigen. Interestingly, for several antibodies, we observed binding to NC1 subdomains outside of the used immunogen. For example, FNIII1-FNIII2 was recognized not only by the corresponding anti-CMP-FNIII1, anti-FNIII1-FNIII2, and anti-FNIII2-FNIII3 antibodies but also by anti-FNIII4-FNIII5, anti-FNIII5-FNIII6, and anti-FNIII7-FNIII8 antibodies. (Figure 3a and b). Antibodies to NC2 were also generated. However, these did not bind to human salt-split skin (not shown). The presence of intermolecular cross-reactivity was also observed in the immunapheresis material of an EBA patient (Supplementary Figure S2A and B online).

Next, as NC1 portions of murine and human share some homology, we evaluated whether antibodies to human COL7 can also recognize murine COL7. All our polyclonal anti-COL7 antibodies bound to the dermal side of human salt-split skin. Endpoint titers ranged from 1:1,600 (anti-FNIII2-FNIII3) to 1:25,600 (anti-FNIII4-FNIII5 and anti-FNIII7-FNIII8) (Figure 3c). Using murine skin as a substrate for indirect immunofluorescence microscopy, we observed binding of most antibodies (Figure 3c). However, on murine skin, a similar endpoint titer was only observed for anti-CMP-FNIII1, whereas no binding to murine skin was observed for anti-FNIII3-FNIII4 and anti-FNIII7-FNIII8. All other antibodies showed a lower endpoint titer on murine, compared with human, skin.

Antibodies to different epitopes of COL7 induce dermal–epidermal separation *ex vivo*

We next determined the ability of anti-COL7 autoantibodies to induce dermal–epidermal separation *ex vivo* by incubating our anti-COL7 autoantibodies with cryosections of human skin in the presence of leukocytes. Interestingly, all anti-COL7 autoantibodies recruited leukocytes to the dermal–epidermal junction and caused dermal–epidermal separation, located in lamina lucida (Figure 4,Supplementary Figure S3 online). This is in line with previous observations of intra-lamina lucida blister formation in EBA patients (Fine *et al.*, 1989). For most immune sera tested, the extent of dermal–epidermal separation correlated with antibody concentration (Figure 4). Preimmune sera neither recruited leukocytes to the dermal–epidermal junction, nor induced dermal–epidermal separation.

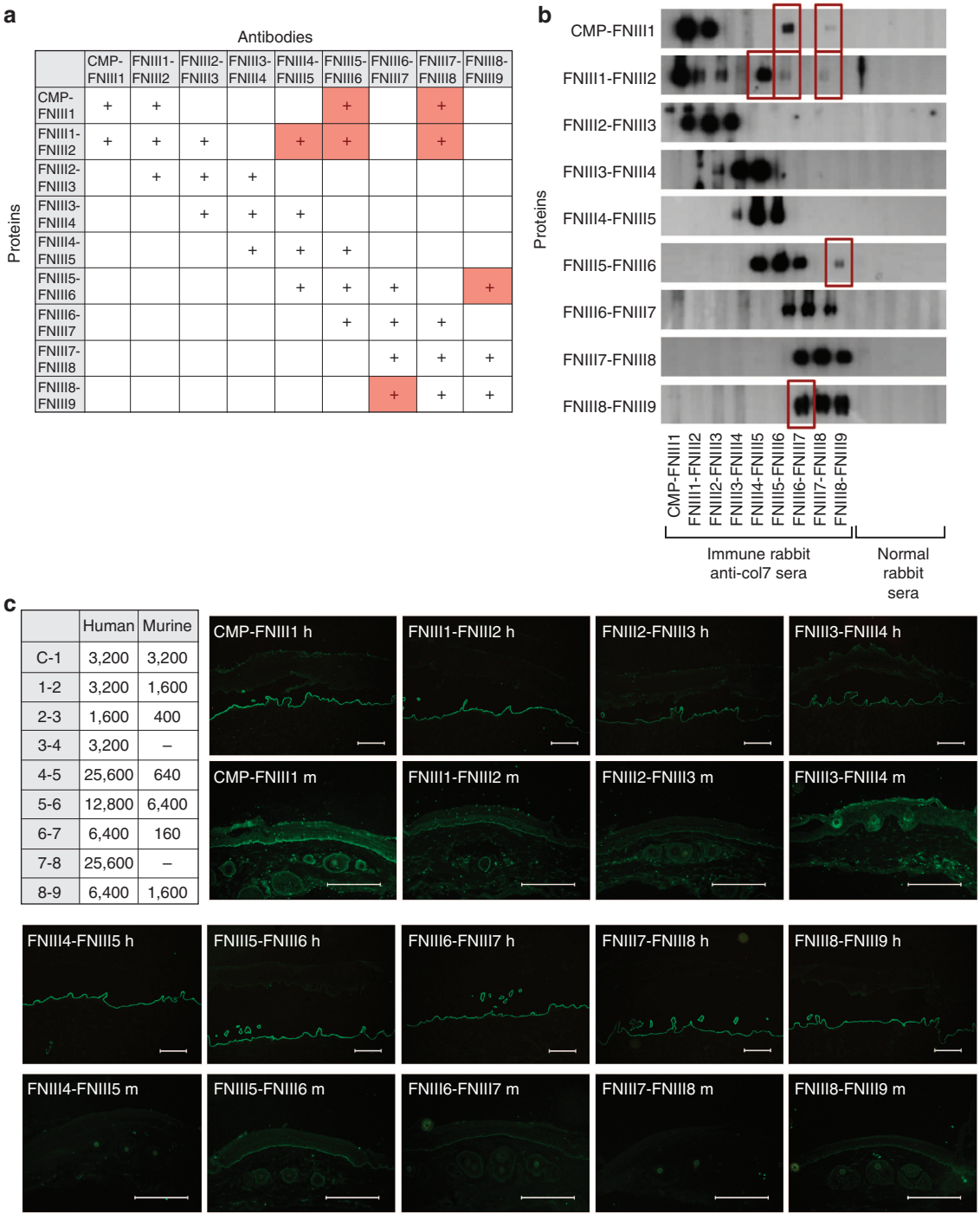

Figure 3. The presence of intramolecular cross-reactivity of antibodies to type VII collagen (COL7) non-collagenous 1 (NC1) and cross-reactivity of antibodies between human and murine skin. (**a**) Summary and (**b**) corresponding images of western blotting analyses, showing cross-reactivity of immune sera with recombinant fragments of human COL7. Marked in red is reactivity outside the antigen used for immunization. (**c**) Summary and corresponding images of end point titers of the generated rabbit immune sera, specific to different subdomains of COL7 NC1, on human 1 M salt-split skin (marked with h) and murine skin (marked with m) by indirect immunofluorescence microscopy. Bar = 100 μm. Serum dilutions used for the shown images were adjusted to a 100-fold lower dilution compared with the end point titer of each epitope-specific serum determined on human skin as substrate. For immunofluorescence images on human and murine skin, the same serum dilutions were used. The summary table on the left side of top panel shows end point titers of the subdomain-specific sera on human salt-split skin and murine skin. Abbreviations: C-1, CMP-FNIII1, 1-2, FNIII1-FNIII2, all other abbreviations correspond to this format.

To validate these findings using human sera, we affinity-purified IgG antibodies direct against the different epitopes within the NC1 domain from immunoapheresis material from one EBA patient. Here, we could isolate IgG antibodies against all NC1 fragments used within the study. The obtained material was not sufficient to be tested in the above *ex vivo* dermal–epidermal separation assay. To test for a possible neutrophil activating capability of these autoantibodies, we

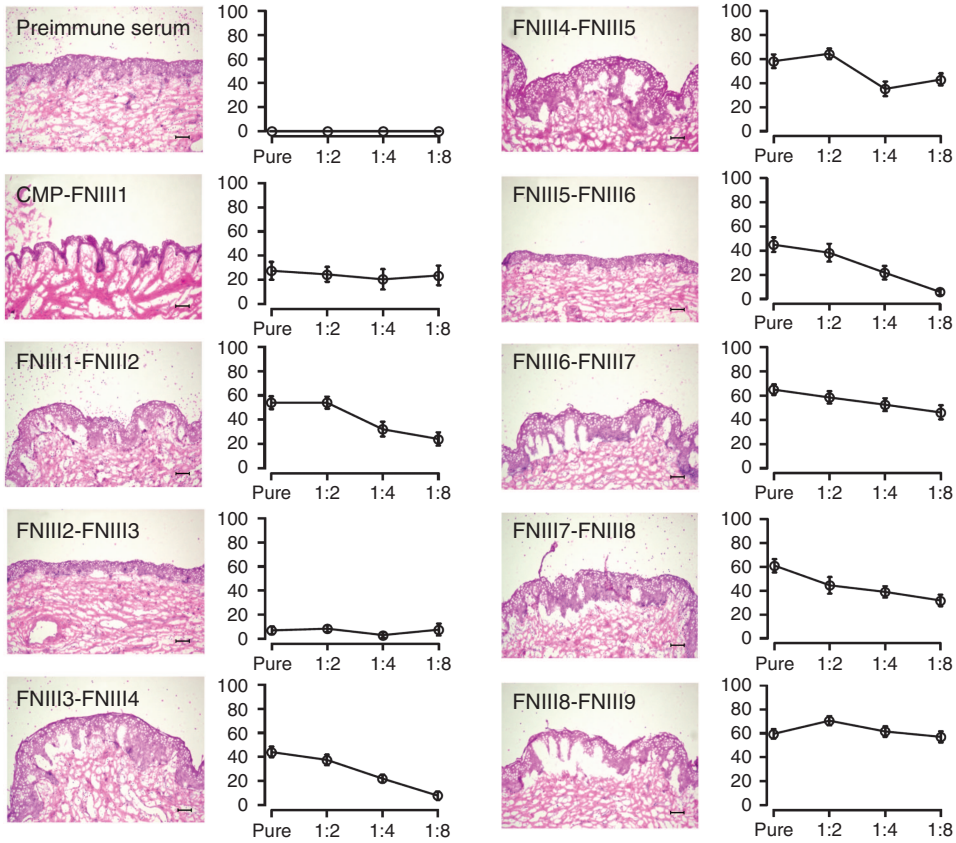

Figure 4. Anti-type VII collagen (anti-COL7) non-collagenous 1 (NC1) antibodies induces dermal–epidermal separation *ex vivo* independent of the targeted epitope. Cryosections of human skin were incubated with epitope-specific rabbit anti-human COL7 NC1 antibodies for 1 hour and subsequently with leukocytes from healthy volunteers for 3 hours. All sera were used as pure 1:2, 1:4, and 1:8 dilutions. On the *y* axis, the percentage of dermal–epidermal separation is shown. Each experiment was performed five times with leukocytes from five volunteers. SEM is depicted. Representative results for each experiment are shown (bar = 100 μm).

generated immune complexes using the different affinity purified antibodies and recombinant NC1 protein. In parallel to above findings using rabbit immune sera, immune complexes of NC1 and either one of the affinity-purified IgG were able to activate neutrophils (Supplementary Figure S2C online).

Antibodies to different epitopes of COL7 cause blister formation when injected into COL7$^{m-/-,h}$ mice

To validate findings from the *ex vivo* assay *in vivo*, affinity-purified antibodies against FNIII4-FNIII5 and FNIII7-FNIII8 subdomains were injected into COL7$^{m-/-,h+}$ (COL7-humanized) mice. Injection of either one of these antibodies into COL7-humanized mice induced clinical and histological changes resembling human EBA (Figure 5). In detail, skin lesions were apparent already on the fourth day after initial injection of antibodies. To assess whether the effect of these anti-COL7 antibodies was dose dependent, lower doses were injected. In fact, these experiments showed a dose-dependent effect of the antibodies in the skin of COL7-humanized mice. To further characterize this EBA mouse model, F(ab)2 fragments of these anti-COL7 IgG were generated. When injected at an equimolar concentration, mixture of anti-FNIII4-FNIII5 and anti-FNIII7-FNIII8 induced subepidermal blisters, the corresponding F(ab)2 fragments did not induce any clinical or microscopic phenotype (Supplementary Figure S4 online). Investigation of the infiltrating cells showed a predominance of neutrophils in the skin lesions (Figure 5, Supplementary Figure S4 online).

DISCUSSION

We here performed a detailed epitope mapping of reactivity to COL7 using 69 sera from EBA patients. By immunoblotting, we demonstrate a broad spectrum of epitopes located within the NC1 domain that are targeted by the autoantibodies. We next demonstrated that autoantibodies to different epitopes induce blisters *ex vivo*. This finding was confirmed by injection of two different anti-COL7 antibodies into mice carrying null mutations of mouse COL7 and the human COL7 transgene (COL7$^{m-/-,h+}$), which induced subepidermal blisters resembling EBA. Although previous studies clearly demonstrated *in vivo* pathogenicity of autoantibodies to mouse COL7 (Chen *et al.*, 2007; Sitaru *et al.*, 2005; Woodley *et al.*, 2005; Woodley *et al.*, 2006), we here provide *in vivo* evidence of the pathogenicity of autoantibodies directed to human COL7.

According to clinical presentation, mechanobullous and inflammatory EBA is distinguished. In addition, clinical presentation of an individual EBA patient may change during the course of the disease or may show two different pre-

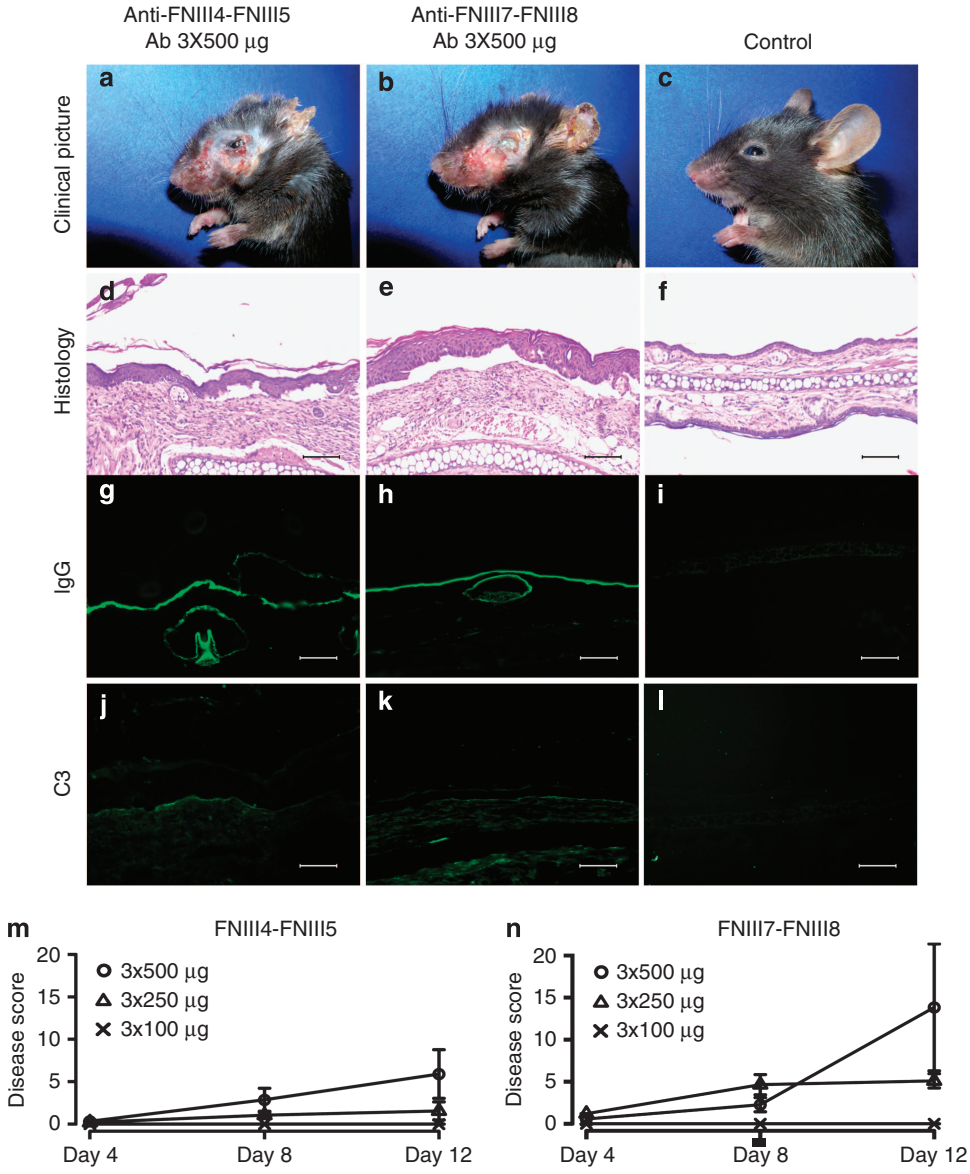

Figure 5. Transfer of anti-FNIII4-FNIII5 or anti-FNIII7-FNIII8 antibodies induces blisters in type VII collagen (COL7)-humanized mice. COL7-humanized mice, injected with 3 × 500 µg of (**a**, **d**) FNIII4-FNIII5- or (**b**, **e**) FNIII7-FNIII8-specific antibodies, developed blisters and erosions clinically and subepidermal splits histologically, whereas (**c**, **f**) control mice remained healthy (bar = 100 µm). By direct immunofluorescence microscopy, COL7-humanized mice injected with FNIII4-FNIII5- or FNIII7-FNIII8-specific antibodies showed (**g**, **h**) strong IgG and (**j**, **k**) weak C3 deposits, respectively, at the dermal–epidermal junction, whereas no immunoreactants were found in (**i**, **l**) control mice (bar = 25 µm). Graphs show disease scores corresponding to body surface area affected by skin lesions after injecting COL7-humanized mice with 3 × 500 µg, 3 × 250 µg, or 3 × 100 µg of (**m**) anti-FNIII4-FNIII5, or (**n**) anti-FNIII7-FNIII8 affinity-purified antibodies. SDs are indicated.

sentations simultaneously. The clinical distinction between mechanobullous and inflammatory EBA has so far, however, not been reflected by laboratory findings—e.g., autoantibody subclasses (Gupta *et al.*, 2012). As binding of autoantibodies to the vWFA2-domain of NC1 leads to disruption of COL7/COL1 interactions, as shown by nuclear magnetic resonance (Leineweber *et al.*, 2011), and as interactions of COL7 with both COL4 and laminin-332 have been described (Brittingham *et al.*, 2006; Chen *et al.*, 1997; Rousselle *et al.*, 1997), we hypothesized that autoantibody binding to certain epitopes within the NC1 domain of COL7 is associated with a distinct EBA phenotype. However, in our cohort of 69 EBA patients, we found no correlation of clinical phenotype with the fine specificity of anti-COL7 IgG. We do not think that reactivity to the collagenous domain of COL7 can explain these findings, as only very few patients had been described to have reactivity to epitopes located within the collagenous domain and/or the hinge region of COL7 (Ishii *et al.*, 2009). Hence, other factors are likely to cause the different clinical phenotypes. In an antibody-transfer model of the disease, inbred mouse lines showed different susceptibilities to blister induction; e.g., in C57BL6/J, the extent of clinical disease was higher compared with BALB/C mice (Kasperkiewicz *et al.*, 2012). As the immune system differs among inbred mouse lines, mechanobullous EBA may develop in patients who do not mount a strong inflammatory reaction in response to

binding of autoantibodies to COL7, if the autoantibody targets a structurally relevant epitope. Careful clinical observations together with stringent immunophenotyping—e.g., analyzing the patients' neutrophils for the ability to mount an oxidative burst upon stimulation with autoimmune complexes—will allow testing this assumption. In line with this assumption, a major interindividual variation of immune complex-induced neutrophil activation has been described (Recke *et al.*, 2010).

After demonstrating a broad epitope recognition of EBA patient IgG, we next evaluated the potential of antibodies targeting different epitopes of NC1 to induce subepidermal splits. When these antibodies were tested for reactivity with the proteins used for immunization, binding to epitopes outside these proteins was observed for anti-FNIII3-FNIII4, anti-FNIII5-FNIII6, anti-FNIII6-FNIII7, anti-FNIII7-FNIII8, and anti-FNIII8-FNIII9 antibodies. This phenomenon may be explained by the high homology among the FNIII-like repeat domains. Hence, in EBA patients, an autoantibody may cross-react with different epitopes on COL7. In addition to inter- and intramolecular epitope spreading (Di Zenzo *et al.*, 2011), this binding of an individual antibody to different epitopes underscores the complexity of the autoimmune response in acquired bullous dermatoses. Although the epitope mapping study used IgG4 as a surrogate marker for the total IgG reactivity to COL7 for sensitivity and specificity purposes, we here either used whole patient IgG or used whole immune IgG from rabbits.

We further showed that all antibodies generated here, which were directed to different epitopes of human COL7 NC1, led to dermal–epidermal separation *ex vivo*, as reported previously using other fragments of COL7 (Csorba *et al.*, 2010). Split formation was dependent on the presence of neutrophils, whereas incubation of human skin with antibodies alone did not cause pathology. This is in line with previous observations demonstrating that patients' antibodies, purified against the entire human NC1 domain, depend on the presence of neutrophils (Sitaru *et al.*, 2002a), more specifically, on generation of reactive oxygen species (Chiriac *et al.*, 2007) and proteases (Shimanovich *et al.*, 2004). To validate the blister-inducing potential of the generated antibodies *in vivo*, rabbit anti-FNIII4-FNIII5 or anti-FNIII7-FNIII8 was injected into COL7-humanized mice. Both these antibody preparations bound to the dermal–epidermal junction, led to complement deposition, and induced subepidermal blisters in a dose-dependent manner, resembling the inflammatory variant of human EBA. This blistering phenotype underscores the pathogenic relevance of the used antibodies, as mere binding of autoantibodies to the dermal–epidermal junction, i.e. formation of immune complexes, does not inevitably lead to blister formation. For example, this is reflected by the lack of blister formation, despite of IgG deposition at the dermal–epidermal junction in mouse models of EBA (Ludwig *et al.*, 2012; Sitaru *et al.*, 2006) and bullous pemphigoid (Liu *et al.*, 1995). This EBA animal model also provides direct evidence that binding of antibodies to human COL7 induces blistering *in vivo*. Previous mouse models of EBA demonstrated pathogenicity of antibodies, directed to mouse or human COL7, that bound murine COL7 *in vivo* (Chen *et al.*, 2007; Sitaru *et al.*, 2006; Sitaru *et al.*, 2005; Woodley *et al.*, 2005). The pathogenicity of different IgG subclasses, as well as IgA directed against COL7, still remains a controversial issue: although some data suggest a potential of IgG4 to induce subepidermal splits *ex vivo* (Mihai *et al.*, 2007), others have demonstrated opposite findings (Recke *et al.*, 2010). Furthermore, IgA has recently been demonstrated to lead to subepidermal splits *ex vivo* (Recke *et al.*, 2014; van der Steen *et al.*, 2012). On the basis of the developed animal model here, these findings can now be addressed *in vivo* to investigate the potential pathogenic contribution of IgA, as well as IgG subclasses in EBA.

In summary, our mapping studies with NC1 and NC2 domains of COL7 show that autoantibodies in EBA patients recognize a broad spectrum of epitopes within COL7 NC1. No correlation was detected between antibody specificity and clinical phenotype. Both *ex vivo* and using COL7 humanized mouse *in vivo,* these autoantibodies induce subepidermal blisters, independent of their specificity. Furthermore, we demonstrate intramolecular cross-reactivity of autoantibodies directed to a specific epitope within the NC1 domain.

MATERIALS AND METHODS

A more detailed description of the materials and methods used is provided in the Supplementary Materials and Methods online.

Patient sera and human skin

Serum samples from 69 EBA patients for detection of circulating anti-COL7 antibodies were used in this study. As the presence of circulating anti-COL7 antibodies was required for epitope mapping studies, EBA patients diagnosed by detection of an u-serrated pattern in the absence of circulating autoantibodies (Buijsrogge *et al.*, 2011) were not included. Sera from healthy volunteers served as controls. Neonatal human foreskin, obtained from routine circumcision, was snap, cut in 6 μm sections, and mounted on SuperFrost slides (Thermo Scientific, Braunschweig, Germany). Prior to all procedures, written informed consent was obtained from all patients. The study was approved by the ethics committee of the University of Lübeck and was performed according to the Declaration of Helsinki.

Mice

$COL7^{m-/-,h+}$ (COL7-humanized) mice that carry the homozygous null mutations of mouse Col7a1 genes and the transgene of human COL7A1 were generated as described previously (Ito *et al.*, 2009). COL7-humanized mice aged 6–8 weeks were intraperitoneally injected with 100–500 μg in a total of rabbit antibody specific to NC1 domain of human COL7 or phosphate-buffered saline (PBS) at days 0, 2, and 4. The extent of blistering was evaluated at days 4, 8, and 12 after the initial IgG injection. Skin samples for hematoxylin and eosin staining and direct immunofluorescence microscopy and serum samples were obtained at day 12. All animal procedures were conducted according to guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.

Cloning of cDNA clones for human NC1 and NC2 domains of COL7 recombinant proteins

DNA sequence of the NC1 and NC2 domains was obtained from Uniprot database (www.uniprot.org).

Expression and purification of recombinant proteins

E. coli Rosetta DE3 cells were transfected with plasmids. Recombinant proteins were purified by affinity chromatography using chitin beads (New England Biolabs, Ipswich, MA). As proteins CMP-FNIII1 and FNIII9-vWFA2 were purified from inclusion bodies as described (Hirose *et al.*, 2011), they were refolded using oxidized-reduced glutathione buffer, dialyzed against protein elution buffer, and purified by affinity chromatography over chitin beads.

Western blotting with patient sera

Please see Supplementary Materials and Methods online.

Rabbit polyclonal antibody production

Rabbit polyclonal antibodies against different fragments of COL7 were produced as previously described (Sitaru *et al.*, 2005). IgG was purified from preimmune and postimmune serum using protein G-agarose as described (Sitaru *et al.*, 2005). Corresponding F (ab)2 fragments were generated using the F(ab)2 preparation kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol.

Indirect immunofluorescence microscopy

Indirect immunofluorescence studies were performed on murine and 1 M NaCl-split human skin. Cryosections of mouse skin and human salt-split skin of 6-μm thickness were incubated with serial dilutions of immune rabbit serum for 1 hour at room temperature, washed with PBS, and then treated with 1:100 diluted FITC-conjugated polyclonal swine-anti rabbit Ig (Dako, Hamburg, Germany).

Affinity purification of antibodies from rabbit serum

Recombinant proteins were dialyzed in 0.1 M MOPS, pH 7.5, and coupled to Affi-Gel 10 or 15 (according to isoelectric point of proteins) following the manufacturer's protocol (Bio-Rad, Munich, Germany). IgG previously purified from postimmune serum was affinity-purified using corresponding Affi-Gel coupled recombinant proteins, then dialyzed against PBS, and concentrated by Amicon Ultra (30 KDa, Millipore, Billerica, MA).

Affinity purification of antibodies from EBA patient serum

Plasmapheresis material from EBA patient was affinity-purified using corresponding Affi-Gel coupled recombinant proteins, then dialyzed against PBS, and concentrated by Amicon Ultra (30 KDa, Millipore, Billerica, MA).

Purification of normal human leukocytes

Leukocytes were isolated from heparin-anticoagulated blood of five different healthy volunteers following a published protocol (Sitaru *et al.*, 2002b). Leukocytes used in this study (determined by trypan blue) had a viability of $\geqslant$90%.

Immune-complex activation of human leukocytes *in vitro*

Please see Supplementary Materials and Methods online.

Determination of dermal–epidermal separation *ex vivo*

Cryosections of 6-μm thickness human foreskin were incubated with different dilutions of rabbit serum (pure, 1:2, 1:4, 1:8, 1:16) for 1 hour at 37 °C, washed with PBS, and subsequently incubated with normal human leukocytes from five different healthy donors at 37 °C for 3 hours. As a negative control, preimmune rabbit serum was used. Subsequently, sections were washed and stained with hematoxylin and eosin. Percentage of dermal–epidermal separation was evaluated by two investigators unaware of the section treatment.

Statistical analysis

R was used for statistical analyses (R Foundation for Statistical Computing, Vienna, Austria). A P-value <0.05 was considered significant. Correlation of epitopes with gender, age, and clinical phenotype was assessed by homogeneity distribution analysis and Pearson's correlation. For Pearson's correlation, the coefficient analysis WGCNA package was used. Homogeneity distribution analysis was performed using homals package.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary Material is linked to the online version of the paper at http://www.nature.com/jid

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