Identification of Quantitative Trait Loci in Experimental Epidermolysis Bullosa Acquisita

Ralf J. Ludwig[1,3], Susen Müller[1,3], Andreia d.C. Marques[1], Andreas Recke[1], Enno Schmidt[1,2], Detlef Zillikens[1], Steffen Möller[1] and Saleh M. Ibrahim[1]

Epidermolysis bullosa acquisita (EBA) is a chronic mucocutaneous autoimmune skin blistering disease. Several lines of evidence underscore the contribution of autoantibodies against type VII collagen (COL7) to the pathogenesis of EBA. Furthermore, EBA susceptibility is associated with the MHC haplotype in patients (HLA-DR2) and in immunization-induced EBA in mice (H2s). The latter study indicated an additional contribution of non-MHC genes to disease susceptibility. To identify non-MHC genes controlling EBA susceptibility, we intercrossed EBA-susceptible MRL/MpJ with EBA-resistant NZM2410/J and BXD2/TyJ as well as Cast mice. Mice of the fourth generation of this four-way autoimmune-prone advanced intercross line were immunized with a fragment of murine COL7 to induce EBA. Anti-COL7 autoantibodies were detected in 84% of mice, whereas deposition of complement at the dermal–epidermal junction (DEJ) was observed in 50% of the animals; 33% of immunized mice presented with overt clinical EBA. Onset of clinical disease was associated with several quantitative trait loci (QTLs) located on chromosomes 9, 12, 14, and 19, whereas maximum disease severity was linked to QTLs on chromosomes 1, 15, and 19. This more detailed insight into the pathogenesis of EBA may eventually lead to new treatment strategies for EBA and other autoantibody-mediated diseases.

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INTRODUCTION

Both genes and environment determine susceptibility to complex diseases, such as autoimmune and chronic inflammatory disorders (Naldi *et al.*, 2005; Han *et al.*, 2009; Bang *et al.*, 2010; Franke *et al.*, 2010). In contrast to most autoimmune diseases (Fernando *et al.*, 2008), little is known about the genetic basis of autoimmune skin blistering diseases (ASBD). ASBD are clinically characterized by cutaneous or mucocutaneous blistering, directly or indirectly caused by binding of autoantibodies to structural components of the skin (Amagai, 2008; Borradori and Bernard, 2008).

Most studies on the genetic basis of ASBD in patients focused on associations with the HLA region, but varied considerably between different populations. This may be due to a relatively small number of patients recruited for these association studies. In more detail, pemphigus vulgaris, an ASBD with Dsg 3 as the target antigen (Amagai *et al.*, 1991), was found to be associated with HLA-DRW4 in a Jewish cohort, with HLA-DQw1 and -DQw3 polymorphisms in Ashkenazi Jews, HLA-DRB1*04 and DRB1*1401 in Iranian patients, HLA-DRB1*0402 haplotypes in Venezuelan patients, HLA-DQB1*0503, -B*1507 or -DRB1*1405 in Japanese patients, as well as HLA-B38 and HLA-Bw55 in an American cohort (Park *et al.*, 1979; Szafer *et al.*, 1987; Miyagawa *et al.*, 1999, 2002; Lee *et al.*, 2006; Saenz-Cantele *et al.*, 2007; Shams *et al.*, 2009). For bullous pemphigoid (BP), an ASBD with antibodies directed against hemidesmosomal proteins termed BP180 (type XVII collagen, COL17) and BP230 (Stanley *et al.*, 1981, 1988; Diaz *et al.*, 1990), major histocompatibility complex (MHC) association studies have also not been conclusive (Delgado *et al.*, 1996; Okazaki *et al.*, 2000). In 29 patients with epidermolysis bullosa acquisita (EBA) and 5 patients with bullous lupus erythematodes, an association with HLA-DR2 has been described (Gammon *et al.*, 1988). In dermatitis herpetiformis, a disorder associated with gluten-sensitive enteropathy and IgA antibodies to epidermal and tissue transglutaminase (Sardy *et al.*, 2002), a strong association with HLA-DQ2 and HLA-DQ8 has been reported (Spurkland *et al.*, 1997).

Regarding the contribution of non-MHC genes to the susceptibility to ASBD, conflicting results on Fc gamma receptor polymorphisms have been reported in patients with BP (Weisenseel *et al.*, 2007; Guilabert *et al.*, 2010). In addition, polymorphisms in the *IL-1β* gene have been

[1]*Department of Dermatology, University of Lübeck, Lübeck, Germany and* [2]*Comprehensive Center for Inflammation Medicine, University of Lübeck, Lübeck, Germany*

[3]*These authors contributed equally to this work.*

Correspondence: Ralf J. Ludwig, Department of Dermatology, University of Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany. E-mail: ralf.ludwig@uk-sh.de

Abbreviations: ASBD, autoimmune skin blistering diseases; BP, bullous pemphigoid; COL7, type VII collagen; DEJ, dermal–epidermal junction; EBA, epidermolysis bullosa acquisita; IF, immunofluorescence; MHC, major histocompatibility complex; QTLs, quantitative trait loci

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reported in female patients with BP (Chang *et al.*, 2006). However, these studies included relatively low numbers of patients.

Studies on genetic susceptibility using animal models for ASBD are also rare, as most models employ the passive transfer of autoantibodies into mice (Bieber *et al.*, 2010). On the basis of a strong association of dermatitis herpetiformis with HLA-DQ2 and HLA-DQ8 (Spurkland *et al.*, 1997), autoimmune-prone NOD/ShiLtJ mice were crossed with DQ8+ mice, lacking endogenous mouse MHC II, to generate congenic NOD/ShiLtJ DQ8+ mice. Development of gluten-dependent blistering, associated with IgA deposition along the dermal–epidermal junction (DEJ), was observed in 16% of mice, immunized with crude gluten. No blistering or IgA deposition was seen in NOD/ShiLtJ or DQ8 congenic mice after gluten sensitization, indicating that both the DQ8 and the NOD/ShiLtJ background are required for blister formation (Marietta *et al.*, 2004).

To further clarify the relevance of MHC association in ASBD, we used an established immunization-induced mouse model for EBA, which reproduces the inflammatory form of human EBA (Sitaru *et al.*, 2006; Ishii *et al.*, 2011). EBA is an autoimmune blistering disease, which is characterized by antibodies to type VII collagen (COL7; Woodley *et al.*, 1988). Immunization of several inbred mouse strains identified a significant association of EBA development with the MHC haplotype H2s. However, this data also pointed towards a contribution of non-MHC genes to EBA susceptibility. More specifically: (i) MRL/MpJ mice (H2k) developed experimental EBA; (ii) C57Bl/10.s mice developed a milder (compared with SJL/J and MRL/MpJ) and mostly transient disease phenotype (Ludwig *et al.*, 2011); and (iii) C57Bl/6J mice were susceptible to EBA induction when lacking expression of the inhibitory Fc gamma RIIB receptor (Sitaru *et al.*, 2006).

In order to investigate the contribution of non-MHC genes to EBA susceptibility, in the present study, we generated an outbred four-way autoimmune-prone intercross mouse line, originating from MRL/MpJ, BXD2/TyJ, NZM2410/J, and Cast mice. Using a classical quantitative trait mapping approach in this outbred family, we identified six loci controlling susceptibility to murine EBA.

RESULTS

EBA susceptibility of inbred mouse strains

Immunization of MRL/MpJ, NZM2410/J, and BXD2/TyJ mice with mCOL7C confirmed the previously reported EBA susceptibility of MRL/MpJ mice (Ludwig *et al.*, 2011). In contrast, NZM2410/J, BXD2/TyJ, and Cast mice were resistant to EBA induction (data not shown). Hematoxylin and eosin studies were in line with the clinical observations, i.e., subepidermal blistering accompanied by a dermal leukocyte infiltration was only observed in MRL/MpJ mice. All strains showed circulating and tissue-bound anti-COL7 IgG (data not shown), supporting the notion that induction of complement activating antibodies is associated with the development of clinical EBA (Sitaru *et al.*, 2006; Ludwig *et al.*, 2011).

Generation of a four-way autoimmune-prone intercross mouse line

In order to generate a genetically diverse mouse line, EBA-susceptible (MRL/MpJ) and -resistant mice, and the offspring of each generation were intercrossed for several generations. Genetic diversity of this so termed four-way autoimmune-prone intercross mouse line is reflected by the different morphological traits, including weight, tail length, or fur color. Figure 1 reflects these differences in morphology; all mice shown are from one litter, and all mice present an individual fur color.

Mice from the four-way autoimmune-prone intercross line are susceptible to COL7-induced EBA

We next immunized 250 mice from G4 of the four-way autoimmune-prone intercross mouse line with GST-mCOL7C emulsified in the adjuvant TiterMax (HiSS Diagnostics GmbH, Freibrug, Germany). After immunization, mice were clinically monitored for a period of 12 weeks for the presence of skin lesions. For analysis, 233 mice were included; the remaining 17 mice died during the observation period. The overall incidence of clinically evident EBA was 33% (Figure 2). On average, onset of disease was observed 7.9 ± 2.8 weeks after immunization. We also observed a (not statistically significant) trend towards a higher disease incidence in male (40%) as opposed to female mice (28%). Most diseased mice presented with mild clinical disease. However, in 22% of diseased mice, 10% or more of the body surface area was affected by skin lesions (Figure 2). Disease extent, expressed as body surface area covered by skin

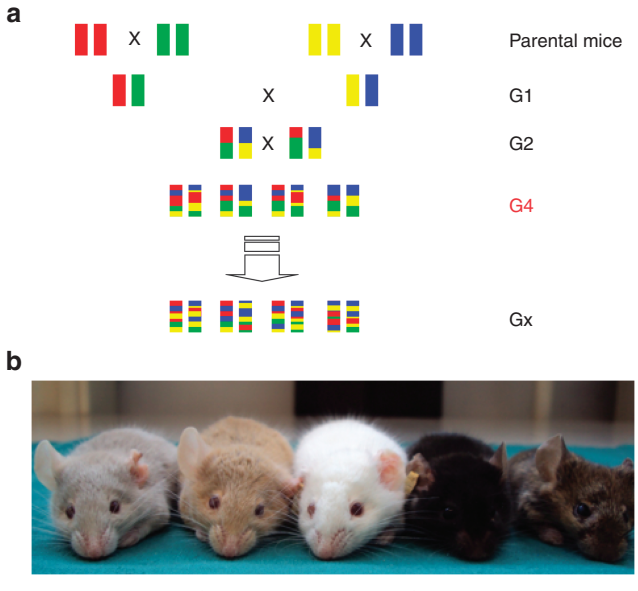

Figure 1. Generation of genetic diversity in the four-way autoimmune-prone intercross line. (**a**) Schematic diagram of mouse breeding in order to generate the four-way autoimmune-prone intercross line. Each color represents the genetic background of the parental inbred mouse lines. By intercrossing each generation (G) of mice, genomic diversity was obtained. For this study, mice of the fourth offspring generation (G4) were used, as marked in red. (**b**) Female offspring mice from one litter of G6 mice, each showing a different fur color, thus representing the genetic diversity of this mouse line.

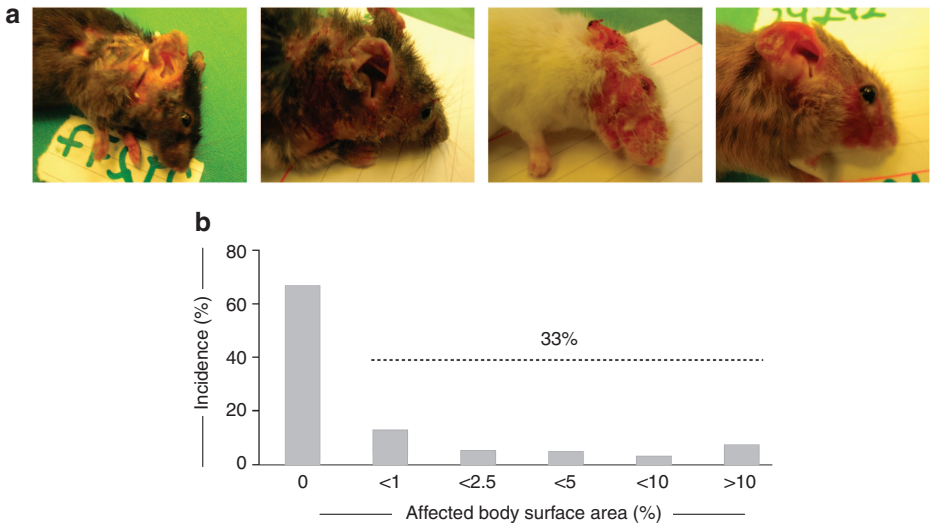

Figure 2. Mice of the four-way autoimmune-prone intercross line are susceptible to epidermolysis bullosa acquisita (EBA) induction. (**a**) Representative clinical presentations of mice with skin lesions 12 weeks after immunization. (**b**) Distribution of disease scores (expressed as body surface area covered by skin lesions) in 233 mice of the four-way autoimmune-prone intercross line 12 weeks after immunization.

Table 1. Induction of complement-fixing antibodies is associated with skin lesions in experimental EBA

Phenotype	IgG (total, %)	IgG1 (%)	IgG2a/c (%)	IgG2b (%)	IgG3 (%)	C3 (%)
EBA	88	86	91	79	46	61
Healthy	83	88	63	77	43	47

Abbreviation: EBA, epidermolysis bullosa acquisita.
Numbers correspond to the percentage of positive IgG/C3 deposition at the dermal–epidermal junction from all 233 mice. Regarding IgG subclasses, IgG2a/c is binding differently between healthy and affected individuals ($P<0.0001$, Fisher's exact test).

lesions, ranged from 0.1 to 51.5% with a median of 2%. Regarding sex, median disease severity was similar in female and male mice (median 2.6%, range 0.1–51.5%, median 1.3%, range 0.1–28.5%, respectively; $P=0.09$, rank sum test). We observed no significant influence of weight or fur color on EBA susceptibility or severity (data not shown). Immunization of mice from the same intercross line (G10) with GST control peptide emulsified in TiterMax did not lead to the development of skin lesions.

Hematoxylin and eosin–stained sections from a selection of diseased and clinically healthy mice was in accordance with the clinical observation, i.e., we detected subepidermal blisters associated with an inflammatory cell infiltrate in clinically diseased mice, whereas no histopathological changes were observed in clinically unaffected animals (data not shown). Direct immunofluorescence (IF) microscopy demonstrated IgG deposition in 83% of non-diseased mice, and in 88% of mice with skin lesions. Deposits of C3 were detected in 47% of healthy mice, as opposed to 61% in diseased animals ($P=0.037$, χ^2, Table 1). Detailed analysis of IgG subclasses (Table 1) showed a predominant IgG2a/c response in diseased versus healthy mice ($P<0.001$, Fisher's exact test). Furthermore, within diseased mice, analysis of variance identified decreased IgG1 binding to be significantly associated with the severity of the disease ($P=0.019$). No association with severity could be found for IgG2a/c, IgG2b, or IgG3. In line with the clinical observation, no IgG or C3 deposits were observed in mice immunized with control peptide and adjuvant.

Identification of quantitative trait loci in experimental EBA

Susceptibility to immunization-induced EBA was then shown to be associated with several quantitative trait loci (QTLs). We analyzed the data regarding the onset of disease (Figure 3a, Table 2) as well as maximum disease score (Figure 3b, Table 3). In detail, EBA onset is associated with four very distinct QTLs on chromosomes 9, 12, 14, and 19. Chromosome 9 has its peak on rs4135590 (log*P* 3.8, bp 42796186) and is flanked by the immediate steep drop to rs13480150 (bp 39531947) and rs13480173 (bp 46336655). This log*P* score was not reached by any of the 1,000 permutations ($P<0.001$). The region of only 7 Mbp covers 147 genes. Chromosome 12 has a significant peak at rs3716095 (log*P* 3.2, exceeded by 2 of 1,000 permutations, bp 87934638) at the end of the q arm. It sharply drops towards the centromere at rs13481561 (bp 83267516) and towards the terminus at rs6390948 (bp 109917259). This region covers for 300 genes. Chromosome 14 harbors just a

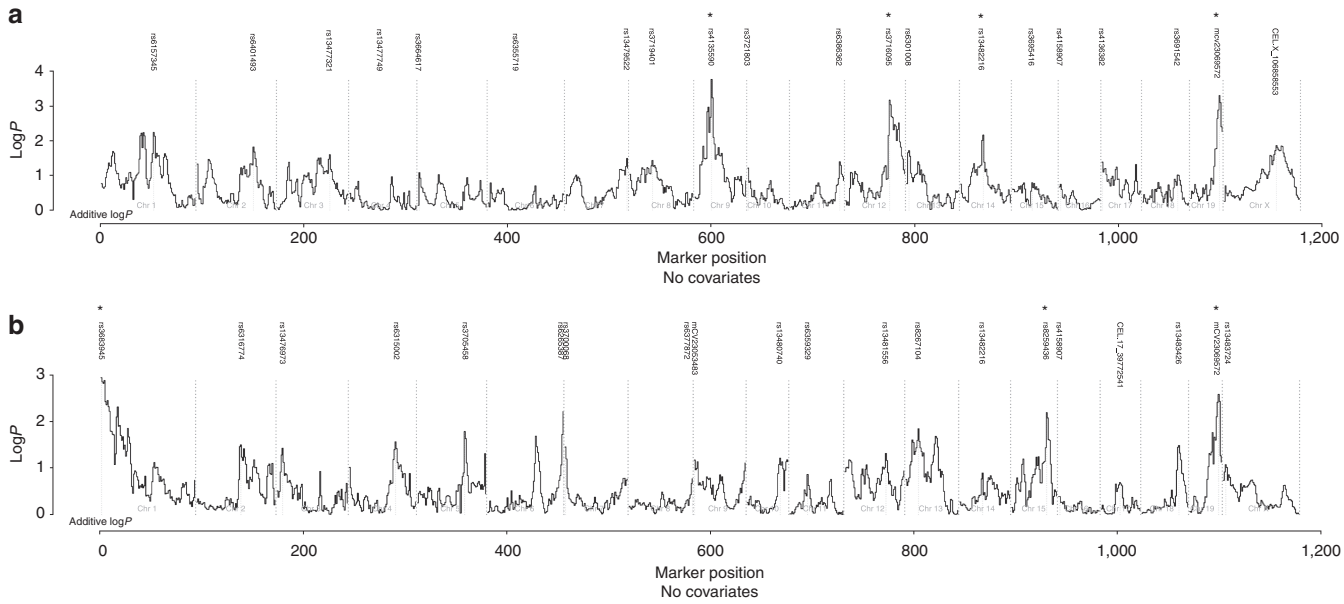

Figure 3. Identification of quantitative trait loci (QTL) for anti-COL7 IgG production and immunization-induced epidermolysis bullosa acquisita (EBA). QTL for (**a**) onset and (**b**) maximum disease score of immunization-induced EBA. On *y* axis the log *P*-value is indicated, while the *x* axis corresponds to the mouse genome. *Indicates an association with either onset or maximum score of EBA. Chr, chromosome.

Table 2. QTLs for onset of immunization-induced EBA

Chromosome	Flanking–peak–flanking QTL	bp Range
9	rs13480150–rs4135590–rs13480173	39531947–46336655
12	rs13481561–rs3716095–rs6390948	83267516–109917259
14	rs13482170–rs13482216–rs3672425	49147300–68964185
19	mCV23390953–mCV23069572–end of chr.19	46090165–end of chr.19

Abbreviations: chr., chromosome; EBA, epidermolysis bullosa acquisita; QTLs, quantitative trait loci.

Table 3. QTLs for maximum disease score of immunization-induced EBA

Chromosome	Flanking–peak–flanking QTL	bp Range
1	rs3683945–rs3707642–rs3677683	3187481–27321156
15	rs3683495–rs8259436–rs13482687	61034058–85776069
19	rs3687275–mCV23069572–end of chr.19	43144678–end of chr.19

Abbreviations: chr., chromosome; EBA, epidermolysis bullosa acquisita; QTLs, quantitative trait loci.

few neighboring markers contributing to the QTL, i.e., steep drops to either side. The maximum is reached at rs13482216 (log*P* 2.2, bp 62510281). Its log*P* value is 2.2 and exceeded by 1.2% of the permutations, but is still considerably stronger than peaks located on other chromosomes. With a demanded drop of 1.5, the region is flanked by markers rs4230248 (bp 31996605) and rs13482265 (79420389), spanning a 38-Mbp region. Chromosome 19 harbors a highly significant narrow peak at mCV23069572, flanked by mCV23390953 and the end of the chromosome (log*P* 3.3, not exceeded by any permutation, bp 52876222).

Maximum disease activity (EBA max. score) as a trait confirmed the identified locus on chromosome 19. This region also has the strongest log*P* value of 2.5 for maximum disease activity, exceeded by only 0.2% of the permutations. The region is slightly wider than determined for EBA onset, with its peak again at mCV23069572 (bp 52876222), flanked by rs3687275 (bp 43144678) and the end of the chromosome. A second strong effect was observed on chromosome 1 at the very beginning of the p arm from rs3683945 (log*P* 2.92, only 4 of 1,000 permutations where stronger, bp 3187481) to rs3677683 (bp 27321156). In addition, chromosome 15 harbors a fairly strong

peak on rs8259436 (log*P* 2.15, exceeded by 8 of 1,000 permutations, bp 74680379) that is flanked by rs3683495 (bp 61034058) and rs13482687 (bp 85776069).

Generation of anti-COL7 antibodies is genetically controlled, but distinct from EBA QTLs

In addition, we also identified QTLs controlling the generation of anti-COL7-specific IgG, including all murine IgG subclasses (Table 4). Interestingly, analysis of a possible common genetic control of specific IgG production and clinical EBA manifestation did not show an overlap of the respective QTLs (Table 4).

DISCUSSION

In this study, we identified several QTLs controlling the susceptibility to experimentally induced EBA. With the exception of an *IL-1β* polymorphism in female BP patients (Chang *et al.*, 2006) and conflicting data on Fc gamma receptor polymorphisms in BP (Weisenseel *et al.*, 2007; Guilabert *et al.*, 2010), the contribution on non-MHC genes has not been reported in patients with ASBD or animal models for these diseases. The identified QTLs for EBA onset were found in chromosomes 9, 12, 15, and 19, while QTLs for the maximal disease score were linked to chromosomes 1, 6, and 19. Interestingly, QTLs for both the traits showed an overlap on chromosome 19.

From the association data described here, we have identified a set of possible EBA-associated genes, providing chromosome assignment. At the moment the data provided does not allow to link EBA to distinct genes. However, several genes associated with inflammation and autoimmunity, such as (i) caspase-7 (Garcia-Lozano *et al.*, 2007; Teixeira *et al.*, 2008; Akhter *et al.*, 2009), (ii) programmed cell death 4 (Hilliard *et al.*, 2006), (iii) C1q, which initiates complement activation through the classical pathway (Lachmann and Hughes-Jones, 1984) and partially contributes to autoantibody-induced tissue injury in experimental EBA (Mihai *et al.*, 2007), and (iv) alpha-mannosidase, which is linked to chronic activation of the innate immune system (Green *et al.*, 2007). An activation of innate immune cells is also required for blister formation in experimental EBA (Chiriac *et al.*, 2007). However, despite being located within the identified QTL for EBA, all of the above genes need to be validated by further intercrossing and tested for functional relevance; e.g., by use of respective knockout mice. Also of note, the genetic control of anti-COL7 IgG production did not overlap with the QTL of clinical EBA manifestation. This may indicate that the identified QTL predominantly control the autoantibody-induced tissue injury, rather than the loss of tolerance.

Interestingly, full-blown disease was detected in 33% of mice. By intercrossing one EBA-susceptible and three EBA-resistant mouse strains, we expected a disease incidence of ~25% following a Mendelian distribution of susceptibility genes from the MRL/MpJ strain. The observed higher incidence suggests that EBA susceptibility genes are also located in EBA-resistant mice (BXD2, Cast, and NZM2410).

Previous studies in immunization-induced EBA demonstrated deposits of C3 in all diseased mice (Sitaru *et al.*, 2006; Ludwig *et al.*, 2011). Interestingly, in our four-way autoimmune-prone intercross line, we found disease also in mice lacking C3 deposition at the DEJ by direct IF microscopy. In addition, we observed deposition of complement also in mice with no EBA skin lesions. This suggests that autoantibody-induced tissue injury in experimental EBA may be a stepwise process, which is initiated by deposition of autoantibodies at the DEJ (Figure 4). Subsequently, a

Table 4. QTLs for generation of anti-COL7 IgG isotypes in immunization-induced EBA

IgG isotype	QTL (flanking–peak–flanking QTL)
Anti-COL7 IgG1	rs13476472–rs3674936–gnf02.076.311
	rs13479071–rs13479014–rs3658783
	rs3699409–rs3669563–end of chr. 10
Anti-COL7 IgG2a/c	gnf01.036.770–rs4137502–rs13475851
	rs3684076–rs13481009–rs13481061
	rs3655061–gnf13.079.671–rs13481992
Anti-COL7 IgG2b	rs3671709–rs3718776–rs6330932
	rs13479807–rs3672639–rs4227253
	rs13479956–CZECH.8_78291790–rs4227398
	rs13482135–rs4230248–rs13482231
Anti-COL7 IgG3	rs13480421–rs6385971–end of chr. 9
	rs6309315–rs3686467–gnf19.035.019

Abbreviations: chr., chromosome; EBA, epidermolysis bullosa acquisita; QTLs, quantitative trait loci.

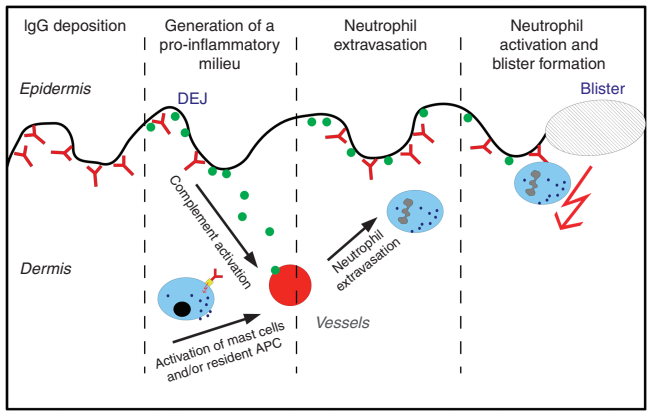

Figure 4. Proposed model of autoantibody-induced tissue injury in experimental epidermolysis bullosa acquisita (EBA). After deposition of binding of anti-COL7 autoantibodies to the dermal–epidermal junction (DEJ), Fc-dependent mechanisms lead to the generation of a pro-inflammatory milieu. This includes the activation of the complement system. On the basis of the observed induction of EBA in mice without the deposition of C3 at the DEJ, other mechanisms, e.g., activation of mast cells of resident antigen-presenting cells (APC) seem to contribute to the generation of the pro-inflammatory milieu. This will lead to the CD18-dependent (Chiriac *et al.*, 2007) extravasation of neutrophils. Activation of neutrophils, including reactive oxygen species (ROS) release (Chiriac *et al.*, 2007) and proteolytic enzymes (Shimanovich *et al.*, 2004), ultimately leads to blister formation.

pro-inflammatory milieu is generated in the skin, including complement activation (Mihai *et al.*, 2007). The pro-inflammatory milieu promotes recruitment of neutrophils (Chiriac *et al.*, 2007), leading to Fc receptor–dependent binding of neutrophils to autoantibodies (Sitaru *et al.*, 2002, 2005; Sesarman *et al.*, 2008). After the activation of neutrophils, reactive oxygen species (Chiriac *et al.*, 2007) and proteolytic enzymes (Shimanovich *et al.*, 2004) mediate blister formation. It is therefore tempting to speculate that genetic variations in one of these steps, such as defects in neutrophil activation caused by mutations of caspase-7, C1q, or α-mannosidase, prevents blister formation. However, this model, would also allow for a compensation of one missing factor by others; e.g., the lack of C3 deposition may be compensated by other pro-inflammatory stimuli induced by autoantibody binding to the DEJ. This hypothetical model of EBA pathogenesis could explain the absence of EBA skin lesions despite deposition of complement at the DEJ, and the induction of skin lesions in mice lacking C3 deposition at the DEJ. The 12% of mice with clinical EBA, but negative staining in direct IF microscopy, we assume, are false negative either due to the kinetics of IgG deposition at the DEJ (Kasperkiewicz *et al.*, 2010) or due to false negative DIF readings, which has a sensitivity of ∼90–95% in humans (Chan *et al.*, 2003; Pohla-Gubo *et al.*, 2011).

As the identified QTLs for EBA onset and maximum disease severity harbor several genes, fine mapping of these risk alleles is currently pursued by further intercrossing of the four-way autoimmune-prone mouse line. These studies may eventually lead to identification of new therapeutic targets for experimental EBA, which may also have implications for the human disease and other autoantibody-mediated disorders.

MATERIALS AND METHODS

Animal experiments

Generation of an outbred mouse line. Parental mouse stains (MRL/MpJ, NZM2410/J, BXD2/TyJ, and Cast) were intercrossed at an equal strain and sex distribution. Parental origin of generation 1 (G1) offspring mice was considered when setting up mating for the generation of G2 mice in order to maintain an equal distribution of the original strains. This procedure was also followed for intercrossing G2 mice. At least 50 breeding pairs were used as parentals for the next generation of mice. Animals were held under specific pathogen conditions at a 12-hour light/dark cycle with food and water ad libitum. All animal experiments were approved by the governmental administration of the State of Schleswig-Holstein.

Induction of experimental EBA

Induction of EBA has been performed by immunization with an immunodominant peptide (GST-mCOL7C) within the murine NC1 domain as described (Kasperkiewicz *et al.*, 2010, 2011; Ludwig *et al.*, 2011). Immunization with GST emulsified in the same adjuvant was used as control. Every fourth week after immunization, extent of clinical disease was assessed by evaluating the percentage of body surface area covered by skin lesions for a total period of 12 weeks. At the end of the experiment, biopsies from lesional and non-lesional skin, and serum samples were obtained. In total, 6–8 mice, aged 8–10 weeks, from the four parental strains and 250 mice from the outbred mouse line were immunized with GST-mCOL7C (G4), and 24 mice (G10) with control protein (GST affinity tag alone expressed by the use of pGEX-6P-1, GE Healthcare, Frankfurt/Main, Germany). Parental strains were purchased from Charles River Laboratories (Sulzfeld, Germany).

Histology and direct IF microscopy

Hematoxylin and eosin staining of skin biopsies (ears) and direct IF microscopy were performed as described (Kasperkiewicz *et al.*, 2010). All specimen were obtained at the end of the 12-week observation period.

ELISA

Detection of circulating COL7 autoantibodies was performed as described (Kasperkiewicz *et al.*, 2010). In brief, 96-well plates were coated with His-mCOL7C, followed by incubation with serum from mice. Bound IgG antibodies were detected using a horseradish peroxidase-labelled goat anti-mouse IgG (Bethyl Laboratories, Montgomery, TX) and 3,3′,5,5′-tetram-ethyl benzidine substrate solution (Thermo Scientific, Illkirch, France). The color reaction was read at 450 nm using a multilabel counter (Victor 3, Perkin Elmer, Waltham, MA).

Statistical analysis

In total 233 individuals were analyzed, each genotyped by the same set of 1400 single-nucleotide polymorphisms. The analysis was performed with the R version of HAPPY (Mott *et al.*, 2000; Durrant and Mott, 2010) in version 2.3 beta on Debian Linux (Möller *et al.*, 2010). The plotting across multiple chromosomes was added locally and contributed back to the authors of the package. The flanking regions of QTLs were determined manually by a drop of 1.5 to both sides to the peak. All QTLs where found significant ($P<0.001$) by a point-wise permutation test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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