Generation of Antibodies of Distinct Subclasses and Specificity Is Linked to H2s in an Active Mouse Model of Epidermolysis Bullosa Acquisita

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Epidermolysis bullosa acquisita (EBA) is an autoimmune blistering disease, characterized by antibodies to type VII collagen (COL7). EBA can be induced in mice by immunization with a fragment of the non-collagenous 1 domain of murine COL7. Contrary to other autoimmune diseases, e.g., rheumatoid arthritis, little is known about the genetic susceptibility for EBA. We therefore used the EBA mouse model to address the hypothesis that disease induction depends on the major histocompatibility complex (MHC) haplotype. Mice from different inbred strains were immunized with recombinant murine COL7. Five distinct responses were observed: induction of (i) severe disease in SJL/J (H2s) and female MRL/MpJ (H2k), (ii) mild and transient disease in C57Bl/10.s (H2s), (iii) microscopic blistering in DBA/1J (H2q), (iv) only presence of non-pathogenic autoantibodies in C57Bl/6J (H2b), NZM2410/J (H2z), BXD2 (H2b), and male MRL/MpJ, and (v) complete resistance to EBA in NOD/ShiLtJ (H2g7) and C57Bl/10.q (H2q) mice. Overall, susceptibility to EBA was strongly associated with H2s. In addition, the diseased phenotype was associated with autoantibodies to specific regions of COL7. Our findings show that induction of antibodies with a distinct specificity is linked to the MHC haplotype in experimental EBA. Furthermore, our data are the basis for future studies with the goal of identifying non-MHC EBA susceptibility genes.

Journal of Investigative Dermatology (2011) **131,** 167–176; doi:10.1038/jid.2010.248; published online 19 August 2010

INTRODUCTION

The murine major histocompatibility complex (MHC) locus, *H2*, was identified and termed for its role in histocompatibility (Snell, 1964). The human MHC, or human leukocyte antigen (HLA) region, was named according to its original demonstration on the surface of white blood cells (Dausset, 1958). In 1981, the central importance of these antigens in immune response was recognized (Benacerraf, 1981). The MHC is one of the most extensively studied regions in the genome, because of the contribution of multiple variants at this locus in inflammatory diseases, including autoimmune disorders (Satsangi *et al.*, 1996; Harbo *et al.*, 2004; Fernando *et al.*, 2008). In contrast to most autoimmune diseases, little is known about susceptibility genes in autoimmune bullous skin diseases (ABSDs). This heterogeneous group of disorders is characterized by circulating and tissue-bound antibodies to structural components of the skin and mucous membranes (Zillikens, 2009). ABSDs are prototypes of organ-specific autoimmune disorders. Autoantibodies in ABSDs are directed to distinct antigens, and pathogenic relevance of these autoantibodies has been demonstrated in several of the diseases (Sitaru and Zillikens, 2005; Bieber *et al.*, 2009). Epidermolysis bullosa acquisita (EBA) is an ABSD characterized by autoantibodies to type VII collagen (COL7), the major component of anchoring fibrils (Woodley *et al.*, 1984, 1988). Epitopes recognized by the majority of EBA sera were mapped to the non-collagenous 1 domain of type VII collagen (Woodley *et al.*, 1988; Remington *et al.*, 2008).

Studies on the genetic basis of ABSDs in patients were inconsistent, varied considerably between different populations, and were based on small patient numbers. Pemphigus vulgaris was found to be associated with HLA-DRW4 in a Jewish cohort (Park *et al.*, 1979), HLA-DQw1 and -DQw3 polymorphisms in Ashkenazi Jews (Szafer *et al.*, 1987), HLA-DRB1*04 and DRB1*1401 in Iranian patients (Shams *et al.*, 2009), HLA-DRB1*0402 haplotypes in Venezuelan patients (Saenz-Cantele *et al.*, 2007), HLA-DQB1*0503 or DRB1*1405 in Japanese patients, and HLA-B38(35) and

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Abbreviations: ABSD, autoimmune bullous skin disease; DEJ, dermal–epidermal junction; EBA, epidermolysis bullosa acquisita; HLA, human leukocyte antigen; IF, immunofluorescence; MHC, major histocompatibility complex

Received 9 November 2009; revised 10 June 2010; accepted 1 July 2010; published online 19 August 2010

HLA-Bw55 in an American cohort. For bullous pemphigoid, MHC association studies have also not been conclusive (Delgado *et al.*, 1996; Okazaki *et al.*, 2000). In 29 patients with EBA, an association with HLA-DR2 has been described (Gammon *et al.*, 1988). Interestingly, 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).

Studies on genetic susceptibility using animal models for ABSDs are also rare, as most models use the passive transfer of autoantibodies into mice (Bieber *et al.*, 2009). 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 DQ8 and the NOD/ShiLtJ background are required for blister formation (Marietta *et al.*, 2004).

To further clarify the relevance of MHC association in ABSDs, in this study we used a recently established active mouse model for EBA, which reproduces the inflammatory form of human EBA. This model appeared to be particularly suitable to address this question, as repetitive immunizations with a recombinant fragment of the NC1 domain of COL7 lead to loss of tolerance in 82% of SJL/J, 56% of BALB/c mice, and 45% of FcγRIIB-deficient mice on the C57Bl/6J genetic background, but not in outbred SKH-1 mice. In this model, induction of complement-fixing autoantibodies against COL7 was associated with clinical disease (Sitaru *et al.*, 2006). In addition, we have previously shown that a single immunization is sufficient to trigger the disease in over 95% of SJL/J mice (Ludwig *et al.*, 2008), but fails to induce disease in BALB/c mice (unpublished data).

We therefore hypothesized that the MHC haplotype controls tolerance to COL7. To challenge this assumption, experimental EBA was induced in several inbred mouse strains. Clinical phenotype, histological characteristics, and immunological response were evaluated subsequently. As MHC governs the presentation of antigen (Miller *et al.*, 1976), we also hypothesized that development of clinical EBA is associated with autoantibodies recognizing specific epitopes within the murine COL7.

RESULTS

H2s haplotype confers susceptibility to clinical EBA development

We hypothesized that the MHC haplotype controls tolerance to COL7. To address this assumption, experimental EBA was induced in different strains of inbred mice by immunization with a recombinant fragment of murine COL7. Overall, we observed three distinct clinical responses: (i) induction of severe disease in SJL/J mice of both sexes (H2s) and in female MRL/MpJ mice (H2k), (ii) induction of a mild and mostly transient disease in C57Bl/10.s mice (H2s), and (iii) resistance to EBA induction in all other strains of mice (Figure 1, Table 1). Overall, 11 out of 11 SJL/J mice (H2s) developed EBA during the 10-week observation period. Clinical EBA lesions were observed at 4 weeks after immunization, and disease severity increased during the observation period. In C57Bl/10.s mice, disease severity was significantly lower compared with that in SJL/J mice, and was transient in most mice ($P=0.001$ and 0.015, for weeks 4 and 6, respectively; rank-sum test—no significant difference was observed at weeks 8 and 10, but disease incidence was too low in C57Bl/10.s mice to allow final conclusions). Immunized female MRL/MpJ mice were also susceptible to EBA induction. In contrast to SJL/J and C57Bl/10.s mice, disease developed 8 weeks after immunization. Clinical scores in female MRL/MpJ mice were comparable to the ones observed in SJL/J mice. All other tested strains were resistant to EBA induction. The association of H2s with EBA was statistically significant ($P<0.001$, χ^2). In summary, 75% of mice carrying the H2s haplotype developed clinical EBA. In contrast, only 5% of mice with other haplotypes developed disease (Figure 1a, Tables 1 and 2).

In accordance with the clinical observation, microscopic blistering, determined by hematoxylin and eosin staining of ear specimen obtained at the end of the observation period, was evident in 8 of 11 SJL/J, 5 of 11 C57Bl/10.s, and 4 of 4 female MRL/MpJ mice (Figure 1b). In addition, 3 of 10 DBA/1J mice revealed microscopic but not macroscopic blistering. All other strains showed no evidence of microscopic blistering and leukocyte infiltration (Table 2). By direct immunofluorescence (IF) microscopy, deposition of IgG was noted in all strains, with the exception of C57Bl/10.q and NOD/ShiLtJ mice. The rate of complement deposition at the DEJ was highest in SJL/J, female MRL/MpJ, DBA/1J, and C57Bl/10.s mice (100%, 100%, 75%, and 71%, respectively; Table 2).

Levels of complement-fixing autoantibodies at the DEJ correlate with subepidermal blister formation

On the basis of clinical, histopathological, and immunopathological findings, five distinct responses to immunization with mCOL7C can be distinguished: (i) induction of severe EBA, (ii) induction of a mild and mostly transient disease, (iii) induction of microscopic blistering, (iv) induction of non-pathogenic autoantibodies, and (v) resistance to produce anti-mCOL7C antibodies. In line with previous findings (Sitaru *et al.*, 2006), subepidermal blistering was associated with a predominant deposition of complement-fixing IgG2a and IgG2b antibodies at the DEJ in mice with C3 deposition at the DEJ, whereas IgG1 was preferentially detected in mice without C3 deposition (Figure 2, Table 2). Interestingly, disease-prone female MRL/MpJ mice showed a predominant IgG1 response. However, a strong C3 deposition was observed at the DEJ.

Autoantibodies from mice with clinical disease react with distinct peptides within the NC1 domain of murine COL7

As the MHC haplotype greatly controls susceptibility to EBA development, we hypothesized that autoantibody response is directed to separate epitopes in different strains of mice. To

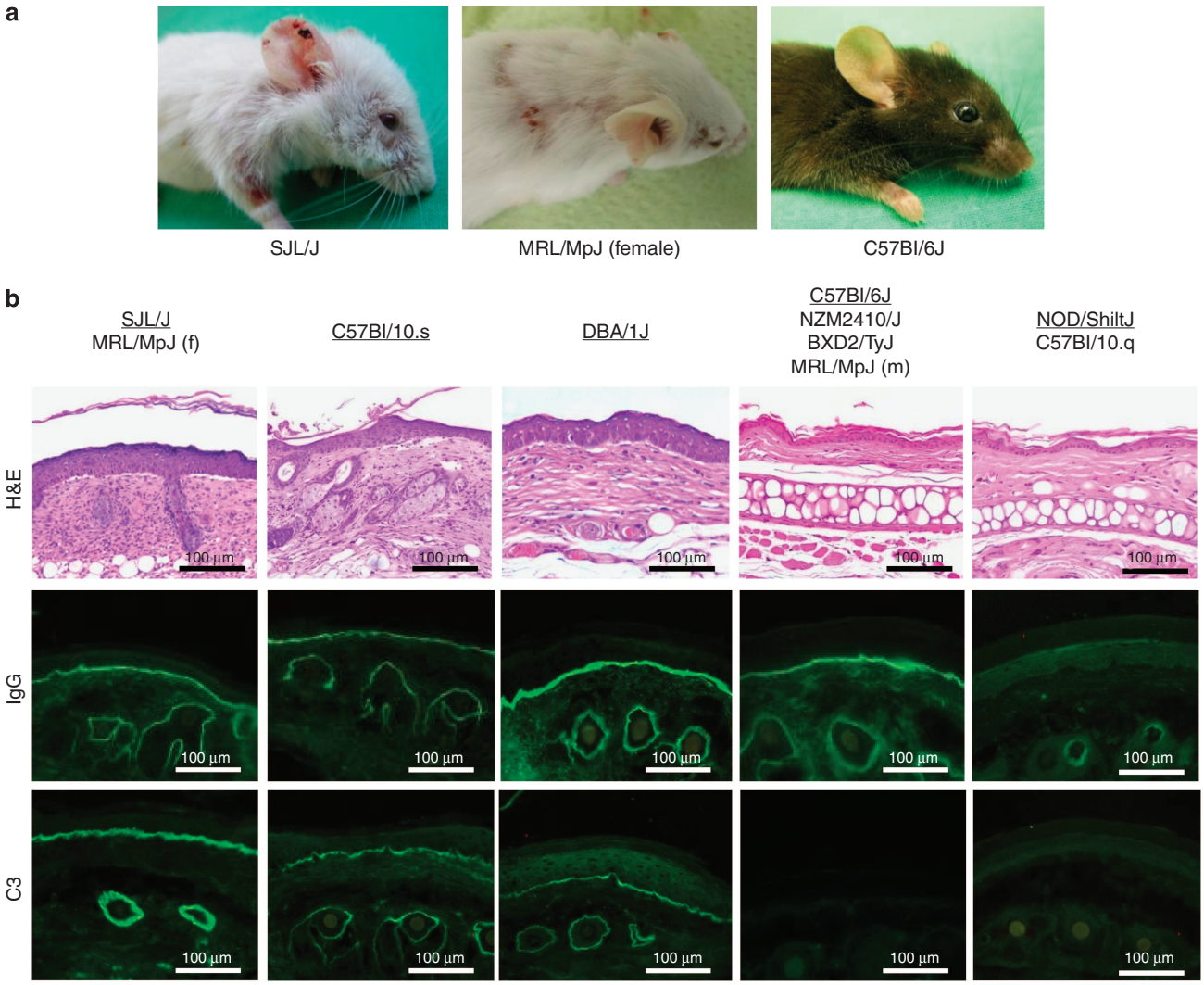

Figure 1. Immunization with mCOL7C leads to different clinical phenotypes and immunological responses in various strains of mice. (**a**) Representative clinical phenotypes from the three different strains are shown. (**b**) Hematoxylin and eosin (H&E)-stained sections showed subepidermal blistering accompanied by leukocyte infiltration only in those strains that developed clinical lesions. In contrast, all other strains, with the exception of DBA/1J mice, did not develop microscopic blistering. Clinical or microscopic evidence of epidermolysis bullosa acquisita (EBA) was associated with complement activation along the dermal–epidermal junction. Mice without microscopic or clinical EBA either developed non-complement-binding antibodies (C57Bl/6, NZM2410/J, BXBD/TyJ, and male MRL/MpJ mice), or were completely protected from the development of an autoimmune response (NOD/ShiLtJ and C57Bl/10.q mice). Shown are representative images corresponding to the underlined mouse strain. Bar = 100 µm.

address this, IgG reactivity to 20 overlapping peptides spanning the entire region of murine NC1 amino acids 757–967 (mCOL7C) from mice immunized with mCOL7C was assessed using enzyme-linked immunosorbent assays. The cutoff for each peptide was defined as the mean OD450 using serum from healthy SJL/J mice ($n = 8$). With the exception of peptide 4 (0.07), peptide 17 (0.09), and peptide 18 (0.15), the cutoff was <0.06. Using analysis of variance (ANOVA), the reactivity of serum from immunized mice to each peptide was compared with that of unimmunized control mice. We observed a broad spectrum of epitopes recognized by autoantibodies from mice that had developed anti-COL7-specific antibodies (Figure 3). However, autoantibody specificity in mice prone to develop clinical disease had a distinct profile. Only disease-prone SJL/J and C57Bl/10.s mice developed antibodies to peptides 1, 7, and 8. With the exception of DBA/1J and male MRL/MpJ mice, none of the other tested strains developed antibodies with this specificity. In disease-prone female MRL/MpJ mice, antibody response was restricted to peptides 1 and 20. This response toward peptide 1 in female MRL/MpJ mice was significantly higher compared with that of male mice of the same strain. In contrast to SJL/J and C57Bl/10.s mice, DBA/1J mice, prone to microscopic blistering, had developed reactivity to peptides 7 and 8, but not to peptide 1.

No evidence of intramolecular and intermolecular epitope spreading in diseased mice

Intramolecular and intermolecular epitope spreading has previously been observed in several human autoimmune blistering skin diseases, and its importance in pathogenicity has been documented for endemic pemphigus foliaceus

Table 1. Differences in disease severity in EBA-susceptible strains

		Incidence of EBA and clinical score							
		Week 4		Week 6		Week 8		Week 10	
Strain	MHC	Incidence	Score	Incidence	Score	Incidence	Score	Incidence	Score
SJL/J	H2s	11/11	3.3 ± 0.6	11/11	3.3 ± 0.9	11/11	3.7 ± 1.0	11/11	4.0 ± 1.2
C57Bl/10.s	H2s	6/11	1.8 ± 0.4	5/11	2.0 ± 0.7	3/11	2.7 ± 1.5	2/11	4.0 ± 1.4
MRL/MpJ (f)	H2k	0/4	NA	0/4	NA	3/4	2.7 ± 0.6	3/4	3.0 ± 1.0

Abbreviations: EBA, epidermolysis bullosa acquisita; MHC, major histocompatibility complex; NA, not applicable.
EBA induction and clinical evaluation was performed as described in Materials and Methods. After immunization, a severe EBA was induced in SJL/J mice. In addition, disease severity increased during the observation period. In contrast, compared with SJL/J, C57Bl/10.s mice showed significantly lower disease activity, and the induced EBA was transient. Female MRL/MpJ mice were also susceptible to EBA induction, however, in contrast to the other two strains, clinical lesions started to develop with a 4-week delay. All other strains, including DBA/1J (H2q, *n*=10), NZM2410/J (H2z, *n*=7), male MRL/MpJ (H2k, *n*=5), NOD/ShiLtJ (H2g7, *n*=10), C57Bl/10.q (H2q, *n*=10), BXBD/TyJ (H2b, *n*=8), and C57Bl/6J (H2b, *n*=10) mice, were resistant to EBA induction. The indicated score is calculated from diseased mice only.

Table 2. H2s confers susceptibility to induction of experimental EBA

Strain	H2	Sex	Clinical disease	Microscopic blistering	Infiltration	MPO ($\mu g\,\mu l^{-1}$)	DIF C3	DIF IgG	Ratio DIF (IgG2a+IgG2b)/IgG1
SJL/J	s	Both	11/11	8/11	2.6 ± 0.2	5.9 ± 4.6	5/5	5/5	10.1 ± 2.9
C57Bl/10.s	s	Both	7/11	5/11	1.7 ± 0.3	3.8 ± 4.7	5/7	7/7	12.0 ± 5.3
MRL/MpJ	k	Female	3/4	4/4	2.0 ± 0.4	ND	4/4	4/4	1.7 ± 1.0
	k	Male	0/5	0/5	0.2 ± 0.2	0.0 ± 0.0	0/5	5/5	2.1 ± 1.0
DBA/1J	q	Both	0/10	3/10	1.5 ± 0.2	1.5 ± 0.6	4/5	5/5	4.5 ± 2.1
C57Bl/6	b	Both	0/10	0/10	0.0 ± 0.0	0.0 ± 0.1	0/5	5/5	1.1 ± 0.6
NZM2410/J	z	Both	0/7	0/7	0.5 ± 0.3	ND	0/3	3/3	2.4 ± 1.1
BXD2	b	Both	0/8	0/8	0.4 ± 0.3	0.0 ± 0.3	0/4	4/4	2.8 ± 0.8
NOD/ShiLtJ	g7	Both	0/10	0/10	0.3 ± 0.2	0.0 ± 0.1	0/5	0/5	DIF negative
C57Bl/10.q	q	Both	0/10	0/10	0.5 ± 0.3	ND	0/3	0/3	DIF negative

Abbreviations: EBA, epidermolysis bullosa acquisita; H&E, hematoxylin and eosin; IF, immunofluorescence; MPO, myeloperoxidase; ND, not done.
Clinical disease scoring was based on the entire body surface area, whereas histopathology evaluation and direct IF microscopy were performed using individual ears. In contrast to all other strains, which were resistant to EBA induction, 75% of mice carrying the H2s haplotype and all female MRL/MpJ (H2k) mice developed skin lesions. Degree of neutrophil infiltration (week 10) was assessed by scoring H&E stained sections (Ludwig *et al.*, 2005) and by determining MPO reactivity using the enzymatic assay described in Materials and Methods. Degree of neutrophils infiltration correlated with MPO levels (r=0.7, $P<0.001$, Spearman's rank order correlation). In the last column, the ratio of IgG2a+IgG2b to IgG1 staining at the dermal–epidermal junction is displayed. High values point to a predominant IgG2a and IgG2b binding.

(Li *et al.*, 2003). To explore the relevance of intramolecular epitope spreading in experimental EBA, sera from diseased SJL/J mice (10 weeks after the initial immunization) were preadsorbed using mCOL7C-GST, followed by indirect IF microscopy on normal mouse skin. In all tested samples, ability to bind to the skin was lost after preadsorption with mCOL7C-GST (Figure 4a). Similar results were obtained using less-diluted serum samples (1:100, data not shown). To confirm the results from indirect IF microscopy, we conducted similar experiments using mouse dermal extract as a substrate. In line, preadsorbtion of sera from diseased SJL mice completely inhibited binding at 290kDa (Figure 4b). Overall, this indicates that epitope spreading to regions outside the mCOL7C does not occur in experimental EBA within the observed time period. In transiently diseased C57Bl/10.s mice, fine epitope mapping was performed at weeks 4, 6, and 8, in addition to week 10, to investigate a possible intramolecular epitope spreading. Yet, we observed no epitope spreading outside the mCOL7C peptide, and no loss of reactivity with epitopes during the observation period (data not shown).

DISCUSSION

Recently, two animal models for EBA have been developed, recapitulating the inflammatory form of the human disease. Experimental EBA can be induced by repeated injection of rabbit or human antimouse COL7 IgG into adult mice (Sitaru *et al.*, 2005; Woodley *et al.*, 2005, 2006). These antibody-transferring (''passive'') mouse models of EBA allow the investigation of mechanisms involved in autoantibody-induced

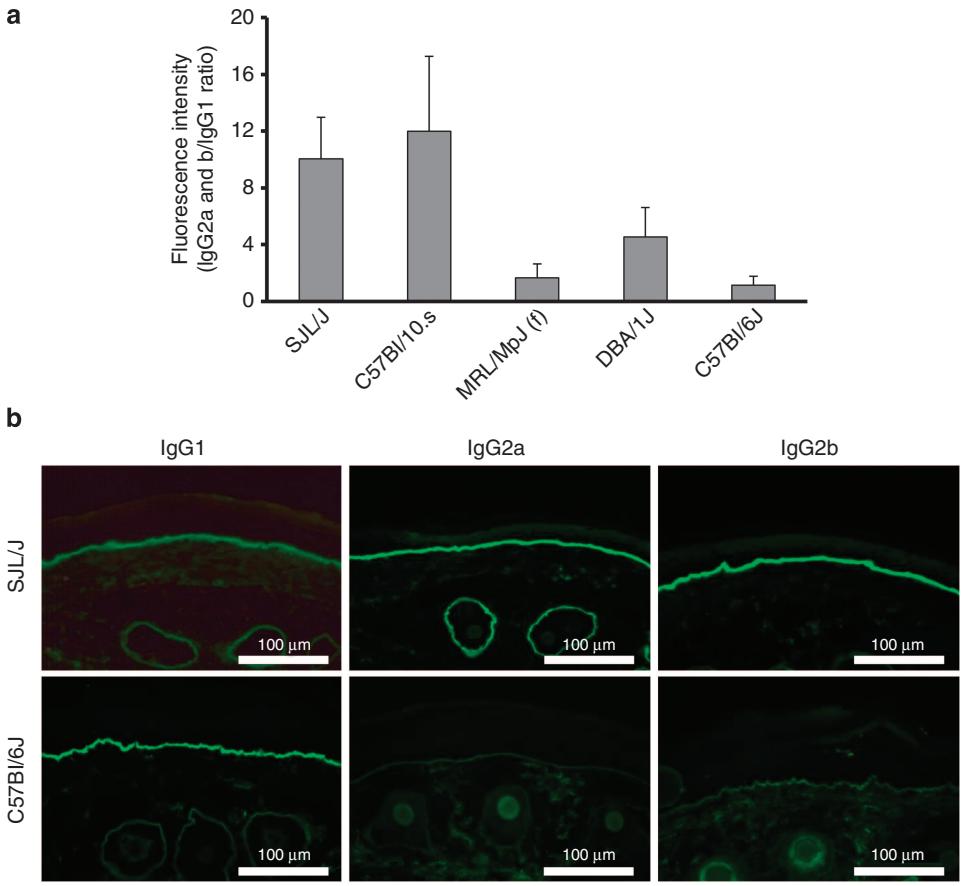

Figure 2. Staining intensity of complement-fixing autoantibodies at the dermal–epidermal junction correlates with subepidermal blister formation. To further validate the association of complement-fixing antibodies with development of clinical epidermolysis bullosa acquisita (EBA), we assessed deposition of complement-fixing IgG2 antibodies, and non-complement-fixing IgG1 antibodies at the dermal–epidermal junction by direct immunofluorescence microscopy. Staining intensity was determined by ImageJ. (**a**) With the exception of female MRL/MpJ mice, the ratio of IgG2a and IgG2b to IgG1 binding shows a bias to IgG2a and IgG2b in mice prone to the development of clinical EBA (SJL/J versus C57Bl/6: $P=0.002$; C57Bl/10.s versus C57Bl/6: $P<0.001$; C57Bl/10.s versus DBA/1J: $P=0.014$; analysis of variance (ANOVA), multiple comparisons calculated using Bonferroni). The difference between DBA/1J and C57Bl/6J mice was not statistically significant. (**b**) Representative DIF stainings from one SJL/J and C57Bl/6J mouse are shown. Bar = 100 µm.

tissue damage; e.g., we have shown that complement activation by the alternative pathway (Mihai *et al.*, 2007; Sesarman *et al.*, 2008a), neutrophils (Chiriac *et al.*, 2007), and reactive oxygen species (Chiriac *et al.*, 2007) are required to elicit disease. Alternatively, experimental EBA can be induced by immunization with an immunogenic peptide from murine COL7 (Sitaru *et al.*, 2006; Ludwig *et al.*, 2008). In this ''active'' EBA model, clinical disease was accompanied by deposition of complement-activating antibodies at the DEJ (Sitaru *et al.*, 2006). We hereby confirm and extend this finding in several strains of inbred mice. The observation of an association of clinical disease with complement-activating antibodies has also been noted in other autoimmune and chronic inflammatory diseases, such as collagen-induced arthritis, experimental myasthenia gravis, experimental autoimmune encephalomyelitis, anti-red blood cell-induced hemolytic anemia, and vasculitis (Staines *et al.*, 1990; Ichikawa *et al.*, 1999; Karachunski *et al.*, 2000; Azeredo da *et al.*, 2002; Baiu *et al.*, 2005).

In addition, we provide evidence that induction of disease is strongly associated with the MHC haplotype H2s. More specifically, 75% of mice carrying H2s developed EBA, whereas this was observed only in 5% of mice with haplotypes other than H2s. Furthermore, C57Bl/10.s mice developed EBA, whereas the genetically identical (with the exception of the MHC) C57Bl/10.q mice were completely resistant to EBA induction. Our data also point toward genes outside the MHC that control susceptibility to EBA development: (i) Immunization of SJL/J mice led to a severe and robust disease induction, whereas C57Bl/10.s mice with the same MHC haplotype developed a mild and transient disease. (ii) Female, but not male, MRL/MpJ mice were susceptible to EBA. (iii) Mice lacking the inhibitory Fcγ-recptor IIB on the otherwise EBA-resistant C56Bl/6 (H2b) genetic background develop blisters (Sitaru *et al.*, 2006). These observations encourage further investigations to study the genetic basis of ABSDs in humans. The present data on genetic susceptibility to autoimmune blistering in human populations are contradictory and based on relatively small cohorts (Gammon *et al.*, 1988; Delgado *et al.*, 1996; Banfield *et al.*, 1998; Okazaki *et al.*, 2000). The observed association of EBA susceptibility with H2s in experimental EBA strongly

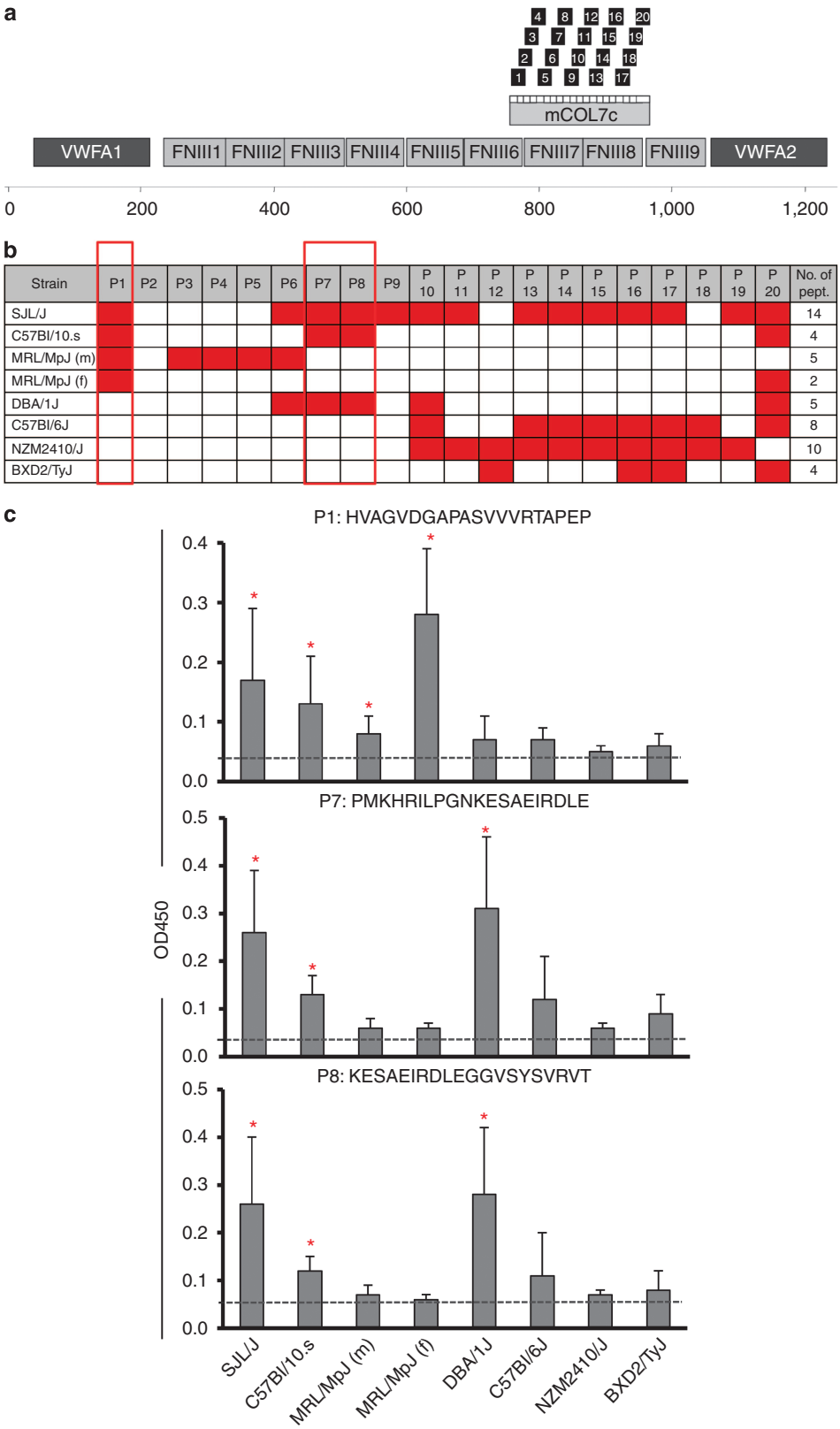

Figure 3. Distinct specificities of autoantibody responses in epidermolysis bullosa acquisita (EBA)-susceptible and -resistant mice. (**a**) Schematic diagram of the murine NC1 domain, its subdomains, and the overlapping fragments used for mapping. (**b**) Reactivity of sera with the 20 overlapping fragments. Displayed in red are sera that had a statistically significant (analysis of variance (ANOVA)) higher reactivity than did the control serum (normal SJL/J mice). A distinct reactivity pattern was observed in mice with clinical EBA lesions, which reacted with peptides 1, 7, and 8. (**c**) Mean ± SD reactivity to peptides 1, 7, and 8. Dotted line indicates the mean reactivity of control serum. *Indicates statistical significance compared with normal SJL/J mice (ANOVA).

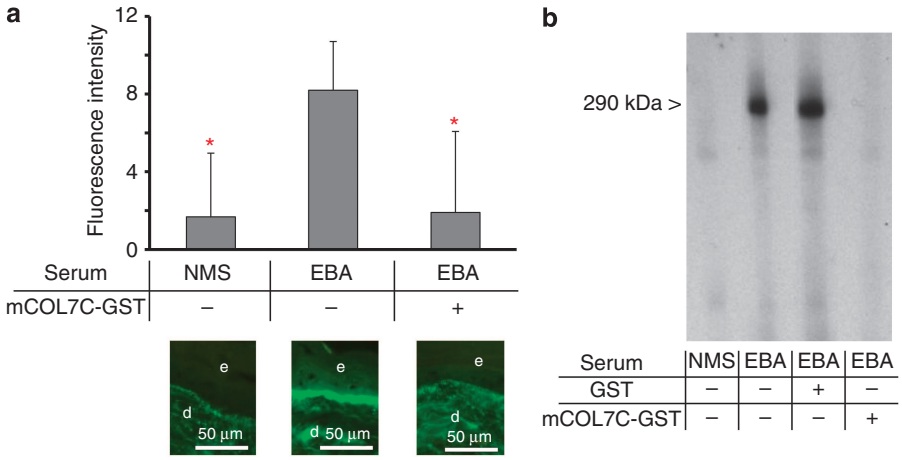

Figure 4. No evidence of molecular epitope spreading in diseased mice. (**a**) To study inter- and/or intramolecular epitope spreading, sera from diseased SJL/J mice were preadsorbed using mCOL7C-GST, and tested for reactivity with skin and dermal extract. Fluorescence intensity at the dermal–epidermal junction was analyzed using ImageJ (mean ± SD). *Indicates statistical significance. Representative stainings are shown below. In all samples, ability to bind to skin was lost after preadsorption with mCOL7C-GST. This indicates that inter- and intramolecular epitope spreading did not occur in experimental epidermolysis bullosa acquisita (EBA) within the observed time period. Bar = 50 µm. (**b**) Results from immunofluorescence microscopy inhibition studies were confirmed using western blotting, with mouse dermal extract as a substrate. d, dermis; e, epidermis; NMS, normal mouse serum.

supports the notion that the human disease is also linked with MHC haplotypes. Likewise, such an association is also observed in many models of autoimmune or chronic inflammatory diseases, e.g., in multiple sclerosis/experimental autoimmune encephalitis, type 1 diabetes, systemic lupus erythematosus, and rheumatoid arthritis (Gunther *et al.*, 1978; Woodley *et al.*, 1981; Kotzin and Palmer, 1987; Wicker *et al.*, 1987; Happ *et al.*, 1988; Fernando *et al.*, 2008). As the observed gender difference in MRL/MpJ mice regarding the susceptibility toward EBA has not been noted in other mouse strains and in humans, interpretation of this observation can only be preliminary. It is most likely restricted to this particular mouse strain, as female MRL/MpJ mice are also more prone to develop spontaneous autoimmune pancreatitis (Kanno *et al.*, 1992).

As the presentation of antigen by MHC is influenced by the haplotype, and susceptibility to induction of experimental EBA is associated with the H2s haplotype, we hypothesized that antibodies to certain epitopes within the NC1 domain of COL7 preferentially develop in mice prone to disease. The results of our B-cell epitope mapping using 20 mer peptides covering the mCOL7C fragment confirmed this assumption. With the exception of female MRL/MpJ mice (reactivity to peptides 1 and 20), EBA-susceptible mice recognized peptides P1, P7, and P8, whereas this pattern of reactivity was not observed in the other strains. Future studies will address whether these epitopes are indeed pathogenically relevant. However, epitopes from peptides 7 and 8 are also recognized by autoreactive T cells in this model (Sitaru *et al.*, 2010). So far, experimental EBA has been induced using polyclonal antibodies (Woodley *et al.*, 2005, 2006; Sitaru *et al.*, 2005; Chen *et al.*, 2007). Characterizing the specificity of blister-inducing autoantibodies in these mouse models demonstrated that disease can be induced by polyclonal antibodies directed against the entire NC1 domain (Woodley *et al.*, 2005, 2006), as well as more defined regions within NC1, including the cartilage matrix protein domain and fibronectin-III-like domains 3, 4, 7, and 8 (Sitaru *et al.*, 2005). In addition, disease was induced by affinity-purified polyclonal antibodies to the cartilage matrix protein domain (Chen *et al.*, 2007). Using an *ex vivo* model of EBA, recombinant human autoantibodies binding within fibronectin-III-like domains 4–6 induced dermal epidermal separation (Recke *et al.*, 2009b). This seeming discrepancy between human EBA, with pathogenic relevant epitopes to the cartilage matrix protein domain (Chen *et al.*, 2007) and to fibronectin-III-like domains 4–6 (Recke *et al.*, 2009b), and immunization-induced EBA, with pathogenic antibodies within the fibronectin-III-like domains 7–9, may be explained by the fact that none of the other epitopes outside those regions, which are observed at a high frequency in patients (Lapiere *et al.*, 1993), as well as to autoantibodies to the NC2 or triple-helical domains (Delgado *et al.*, 1996; Ishii *et al.*, 2004, 2009), have been tested for pathogenic relevance.

In addition to the isotype and specificity of autoantibodies, intramolecular and intermolecular epitope spreading has been observed in patients with ABSDs (Chan *et al.*, 1998; Fairley *et al.*, 2004; Recke *et al.*, 2009a). This has been best demonstrated for endemic pemphigus foliaceus (Li *et al.*, 2003). Our data suggest that epitope spreading to regions outside mCOL7C is not required to induce disease in the active EBA mouse model, as binding of sera to the skin was completely abolished by preadsorption with mCOL7C.

In summary, in this study we show that (i) susceptibility to the inflammatory-type EBA is strongly associated with the H2s haplotype, (ii) genes outside the MHC are likely to contribute to the disease, (iii) induction of EBA is accompanied with specific isotypes and autoantibody specificities, (iv) and epitope spreading does not contribute to EBA development. Furthermore, our data are the basis for future studies identifying non-MHC EBA susceptibility genes.

MATERIALS AND METHODS

Mice

Mice aged 6–8 weeks were obtained from Charles River (Sulzfeld, Germany). Animals were maintained on a 12-hour light–dark cycle at the animal facility of the University of Lübeck. Mice were held under SPF conditions and fed acidified drinking water and standard chow *ad libidum*. Protocols were approved by the Animal Rights Commission of the Ministry of Agriculture and Environment, Schleswig-Holstein (70-7/08).

Recombinant and synthetic peptides

The immunodominant mCOL7C epitope of the murine NC1 domain (amino acids 757–967) was produced as glutathionyl-s-transferase fusion protein using a prokaryotic expression system and purified by glutathione-affinity chromatography as described (Sitaru *et al.*, 2005, 2006; Sesarman *et al.*, 2008b). For determination of COL7-specific antibodies by enzyme-linked immunosorbent assays, mCOL7C was expressed in a similar manner and purified using polyhistidine-tag (his-mCOL7C). A total of 20 overlapping 20-mer peptides with N-terminal biotinylation, covering mCOL7C, were synthesized by JPT Peptide Technologies GmbH (Berlin, Germany; Supplementary Table S1 online).

Immunization of mice and observation protocol

For induction of an autoimmune response toward murine COL7, mice were immunized according to a slightly modified protocol (Sitaru *et al.*, 2006; Bieber *et al.*, 2009). In brief, 60 μg GST-mCOL7C emulsified in 60 μl adjuvant (TiterMax, Alexis, Lörrach, Germany) was injected subcutaneously into footpads. For each mouse strain and sex, a minimum of four mice were immunized. Additional experiments using adjuvants other than TiterMax, including complete Freund's adjuvant/incomplete Freund's adjuvant and AbISCO-100, have also been conducted. Induction of experimental EBA is, however, only possible using TiterMax (Supplementary Table S2 online). The extent of disease, determined by the percentage of body surface area covered by lesions, was evaluated 4, 6, 8, and 10 weeks after immunization. As described (Sitaru *et al.*, 2006), mice were assigned a score ranging from 0 to 5, corresponding to 0%, $<1\%$, $\geqslant 1\%$ to $<5\%$, $\geqslant 5\%$ to $<10\%$, $\geqslant 10\%$ to $<20\%$, and $\geqslant 20\%$, respectively. From every mouse, serum and skin samples were obtained at the end of the 10-week observation period. Serum was stored at $-20^{\circ}C$; skin samples were fixed in 4% buffered formalin and snap-frozen.

Detection of circulating autoantibodies

Circulating autoantibodies were detected by incubating diluted serum samples on his-mCOL7C-coated plates. To measure serum levels of antibodies against COL7 or to peptides, enzyme-linked immunosorbent assays using synthetic or recombinant peptides of COL7 as target antigens were performed as described, with minor modifications (Sitaru *et al.*, 2006).

Detection of tissue-bound autoantibodies by direct IF microscopy

Direct IF microscopy was performed as detailed (Sitaru *et al.*, 2005). In brief, frozen sections were prepared from tissue biopsy samples and incubated with goat antimouse antibodies reactive with total IgG, IgG1, IgG2, IgG3, and murine C3 (Dako, Hamburg, Germany).

Evaluation of neutrophil infiltration

Histopathology: sections from paraffin-embedded tissues were stained with hematoxylin and eosin. Sections were then semiquantitatively analyzed by two blinded observers. Infiltration of neutrophils was scored ranging from 0 to 3, corresponding to no, mild, moderate, or severe infiltration (Ludwig *et al.*, 2005). Myeloperoxidase assay: neutrophil infiltration of murine skin was assayed as described (Bradley *et al.*, 1982; Ludwig *et al.*, 2005). Briefly, the left ear was removed. Myeloperoxidase was extracted by homogenization in a buffer containing 0.1 M Tris-Cl, pH 7.6, 0.15 M NaCl, and 0.5% hexadecyl trimethylammoniumbromide (Sigma, Munich, Germany). Myeloperoxidase activity in the supernatant fraction was measured by the change in optical density at 460. A standard reference curve was established using known concentrations of purified myeloperoxidase (Sigma).

Preparation of dermal extract

Murine dermal extracts were prepared as described for human skin (Zillikens *et al.*, 1996).

Indirect IF microscopy and inhibition studies

Sera of three different diseased SJL/J mice were analyzed by indirect IF microscopy on cryosections of murine tail skin at a dilution of 1:1,000. Antibodies bound to the DEJ were detected by fluorescein isothiocyanate-conjugated goat antimouse IgG antibodies (Dako). Serum was either directly added to sections or preincubated with GST-mCOL7C at a concentration of $1\,\mu g\,ml^{-1}$ for 1 hour at room temperature. Fluorescence intensity at the DEJ was determined by ImageJ (http://rsbweb.nih.gov/ij/), using dermal fluorescence for background subtraction. In addition, western blots were performed with the same sera using mouse dermal extract as a substrate: mouse sera were diluted 1:50 with 1% bovine serum albumin/0.05% Tween 20/phosphate-buffered saline. For detection, enhanced chemiluminescence on X-ray films, together with peroxidase-coupled polyclonal rabbit antimouse pan-IgG antibody (Dako), was used. For preadsorption experiments, diluted mouse sera were preincubated for 30 minutes at room temperature with $100\,\mu g\,ml^{-1}$ mCOL7c:GST and GST, before being applied on immunoblot membranes.

Statistical analysis

Statistical calculations were performed using SigmaStat (Systat Software, Chicago, IL). Applied tests are indicated in the respective table and figure legends. A *P*-value <0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

Grant support: Excellence Cluster *Inflammation at Interfaces* (EXC 306/1) and the Deutsche Forschungsgemeinschaft (DFG LU 877/5-1).

SUPPLEMENTARY MATERIAL

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

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