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Complement-Fixing Anti-Type VII Collagen Antibodies Are Induced in Th1-Polarized Lymph Nodes of Epidermolysis Bullosa Acquisita-Susceptible Mice

Christoph M. Hammers,*+ Katja Bieber,*+ Kathrin Kalies,*+ David Banchczyk,*+ Christoph T. Ellebrecht,*+ Saleh M. Ibrahim,*+ Detlef Zillikens,*+ Ralf J. Ludwig,*+1 and Jürgen Westermann*+1


E pidermolysis bullosa acquisita (EBA) is an organ-specific autoimmune bullous skin disease characterized by autoantibodies to a 290-kDa protein of the dermal–epidermal junction (DEJ) (1). Subsequently, these autoantibodies were shown to bind to the noncollagenous (NC)-1 domain of type VII collagen (COL7), a major constituent of the DEJ (2–4). The pathogenic relevance of anti-COL7 autoantibodies has been clearly demonstrated, as follows: 1) Sera from EBA patients and anti-NC1 domain-specific IgG, affinity purified from these patients, as well as recombinant anti-NC1 IgG1 and IgG3 induce neutrophil-dependent dermal-epidermal separation in cryosections of human skin (5,6). 2) Injection of either rabbit or human IgG to murine COL7 induces EBA in mice (7–10). 3) Immunization of mice with an immunodominant protein, located within the NC1 domain (termed mCOL7C), leads to loss of tolerance and production of anti-COL7 autoantibodies in most strains, whereas development of subepidermal blisters is restricted to certain strains (11,12).

Induction of this immunization-induced EBA mirrors the human inflammatory variant of the disease (13), requires T cells (14), and is associated with the H2s haplotype of the MHC (12). Furthermore, in vivo immunization-induced EBA upon in vitro restimulation with the autoantigen (14) requires T cells (14), and production of complement-fixing anti-COL7 Abs (11,12). Generation of complement-fixing autoantibodies is assumed to be related to Th1-type cytokines in several in vitro and in vivo models (15). We therefore hypothesized that clinical manifestation of experimental EBA is linked to a Th1-driven immune response leading to a predominance of complement-fixing autoantibodies, and thus to clinically overt disease. On the contrary, and following this hypothesis, resistance to experimental EBA induction should be linked to a Th2-type response with a predominant generation of nonpathogenic IgG1 Abs (16,17). This would be in line with the observed significant increase of IFN-γ, but not of IL-4, produced by lymphocytes from mice with immunization-induced EBA upon in vitro restimulation with the autoantigen (14). To challenge our hypothesis experimentally, mice of EBA-susceptible and -resistant strains were immunized with mCOL7C, and the clinical and immunological response to...
immunization was assessed. In detail, kinetics of the following were determined: 1) clinical disease manifestation; 2) circulating and tissue-bound anti-COL7 IgG isotypes; 3) complement deposition at the DEJ; and 4) cytokine gene expression at the site of disease induction (i.e., the draining lymph node [dLN]).

Materials and Methods

Mice

All mice (SJL/J, BALB/c, C57BL/6, and B6.SJL-H2s) were obtained from Charles River Laboratories (Sulzfeld, Germany). Animals, aged 8–10 wk, were fed acidified drinking water and standard chow ad libitum, and held under specific pathogen-free conditions on a 12-h light-dark cycle at the animal facility of the University of Lübeck. All protocols were approved by the Animal Rights Commission of the Ministry of Agriculture and Environment of Schleswig-Holstein and performed by certified personnel.

Recombinant peptides

The immunodominant mCOL7 epitope of the murine NC1 domain (aa 757–967) was produced as GST fusion protein using a prokaryotic expression system and purified by glutathione-affinity chromatography, as described (8). His-tagged mCOL7c was generated by subcloning the mCOL7c fragment into pQE40 (Qiagen, Hilden, Germany), using BamHI and HindIII restriction sites. His-tagged protein was expressed in Escherichia coli and purified using Talon-immobilized cobalt affinity chromatography (Clontech, Saint-Germain-en-Laye, France).

Immunization of mice and observation protocol

Mice were immunized with mCOL7C, as previously described (18, 19). In brief, 60 µg mCOL7C, emulsified in the adjuvant TiterMax (Hiss, Freiburg, Germany), was i.v. injected into the hind footpads of mice. This protocol leads to the induction of experimental EBA, duplicating the findings of patients with the inflammatory variant of the disease. Controls were s.c. immunized with sterile PBS (Sigma-Aldrich, Munich, Germany). Extent of disease, determined on days 7, 14, 21, 28, 35, 42, and 49 after immunization, was expressed as percentage of body surface area covered by erythema, blisters, erosions, and crusts. At the end of the observation period, popliteal lymph nodes, peripheral blood, and ear skin were obtained. Peripheral blood cells (in lysis buffer) and serum were stored at −80°C until further analysis.

Evaluation of Gr−1+ cell infiltration and neutrophil-specific myeloperoxidase activity

Cryostat sections from ear skin samples were stained by H&E according to standard protocols. Staining for Gr−1+ cells was performed using rat anti-mouse Ly−6G and Ly−6C (BD Pharmingen, Heidelberg, Germany) as primary Ab, Alexa Fluor 555 goat anti-rat IgG (H+L) (Invitrogen, Darmstadt, Germany) as used as secondary Ab, and Hoechst 33342 (Invitrogen) was used to stain nuclei. Numbers of Gr−1+ cells were quantitatively analyzed and related to the measured area of the particular section using a Palm microbeam system (Zeiss, Jena, Germany). Activity of neutrophil-specific myeloperoxidase (MPO) was additionally assayed, as described elsewhere (20, 21).

Detection of circulating autoantibodies by ELISA

Serum levels of circulating anti-COL7 Ab subclasses were determined by ELISA using mouse IgG1, IgG2a, IgG2c, and IgG2b quantification sets (Bethyl, Montgomery, TX). In detail, each well was coated with 500 ng recombinant His-mCOL7c in 0.05 M PBS. After blocking, series of diluted samples were added and incubated for 60 min. Bound Abs were detected by HRP-conjugated goat anti-mouse Abs of the respective subclass (Bethyl) and tetramethylbenzidine (Invitrogen). The enzymatic color reaction was stopped with 2 M sulfuric acid (Carl Roth, Karlshruhe, Germany), and the change in OD was measured with a spectrophotometer (BioTek, Bad Friedrichshall, Germany) at 450 nm. Standard reference curves were established by using the provided mouse reference sera (Bethyl).

Detection of tissue-bound autoantibodies and C3 by direct immunofluorescence microscopy

Direct immunofluorescence (IF) microscopy was performed, as detailed (8). In brief, cryosections were prepared from ear skin biopsies and incubated with goat anti-mouse Abs reactive with murine IgG1 (Invitrogen), IgG2a (BD Pharmingen), IgG2b (BD Pharmingen), IgG2c (Bethyl), and C3 (Cappel MP Biomedicals, Solon, OH). Fluorescence intensity at the DEJ was determined using a confocal Zeiss Axiovert LSM 510 META laser-scanning microscope and corresponding software (Zeiss).

Immunohistochemical staining of proliferating cells

Cryostat sections of dLN were stained for B220 and the proliferation marker Ki-67, as described (22).

RNA isolation, cDNA synthesis, and quantitative real-time PCR

Analysis of cytokine gene expression was performed, as detailed elsewhere (23, 24). In brief, RNA was extracted from cryosections of whole lymph nodes or peripheral blood cells using the innuPREP RNA Mini Kit (Analytik Jena, Jena, Germany). After reverse transcription, cDNA was added to the qPCR MasterMix Plus (Eurogentec, Cologne, Germany) and amplified using a 7DS ABI 7000 or 7DS ABI 7900 system (Applied Biosystems, Darmstadt, Germany). The TaqMan probes, forward and reverse primers, were designed with CloneManager (SciEd, Cary, NC); the optimal primer concentrations used were 900 nM each for forward and reverse primers and 200 nM for the TaqMan probe. The primer sequences, amplicon sizes, and gene accession numbers are shown in Supplemental Table I. The same batch of cDNA (20 µl) was used to determine the cycles of threshold, and the amounts of the cytokine cDNA copies were normalized to the housekeeping gene metatlastic lymph node gene 51 (MLN51) and further normalized to the mean value of the controls using the 2−DDCT method. The cDNAs of FcγRI, FcγRIIb, FcγRIII, and FcγRV were analyzed using the qPCR MasterMix Plus for SYBR Green I (Eurogentec). The optimal primer concentrations found were to be 500 nM each.

Detection of COL7-specific plasma cells in dLN

Immunohistochemical double staining of mCOL7C-specific CD138+ cells was performed, as described (19). Prior to further analysis, immunohistochemical double staining of mCOL7C-specific CD138+ cells was confirmed by IF stainings. Therefore, cryostat sections were incubated with biotinylated recombinant His-mCOL7C and stained with Alexa Fluor 488 streptavidin-conjugate (Invitrogen) after fixation. These sections were then incubated with rat anti-mouse CD138 (BD Pharmingen) and visualized using Alexa Fluor 555 goat anti-rat IgG (H+L) conjugate (Invitrogen). mCOL7C-specific CD138+ cells were counted in representative immunohistochemically stained cryosections of each dLN, and numbers were related to the measured area of the respective section using PALM MicroBeam system (Zeiss). Direct IF imaging was carried out on a confocal Zeiss Axiovert LSM 510 META laser-scanning microscope with corresponding software (Zeiss). The same procedures were applied on spleen cryosections.

Statistical analysis

Statistical calculations were performed using SigmaStat (Systat Software, San Jose, CA) and GraphPad Prism (GraphPad Software, La Jolla, CA). A p value <0.05 was considered statistically significant (**p < 0.001, ***p < 0.01, *p < 0.05).

Results

BALB/c mice are resistant to immunization-induced EBA

The immunological and clinical response to immunization with a recombinant fragment of COL7 was monitored in SJL/J and BALB/c mice. In SJL/J mice, starting 21 d after immunization, erythema, erosions, and crusts, predominantly on the ears, were observed. Disease severity progressed during the observation period, showing clinical lesions also in other regions, including nose, temples, periocular skin, neck, back, legs, and tail. In contrast, BALB/c mice were completely resistant to induction of skin lesions (Figs. 1, 2A). Accordingly, microscopic blisters analyzed by H&E staining were only seen in SJL/J mice (data not shown).

Complement fixation at the site of autoantibody binding is characteristic in many autoimmune conditions (25) and has also been shown to be associated with clinical disease manifestation in experimental EBA (8, 11, 14, 26). Fig. 2B shows analysis of the kinetics of C3 deposition at the DEJ from mCOL7C-immunized mice by direct IF microscopy. In contrast to BALB/c mice, C3
Regarding kinetics of DEJ-bound autoantibody isotypes were specific autoantibodies, marked differences between both strains mouse strains (Fig. 3). A similar (IgG2b + IgG2a/c)/IgG1 ratio was obtained in both serum concentrations, but at a lower level (Fig. 3A). Consequently, IgG deposition in EBA-resistant BALB/c mice was characterized by an early response of noncomplement-fixing IgG1 on day 7, which continued to be the major deposited IgG isotype. In BALB/c mice, deposition of IgG2b and IgG2a isotypes was only observed at later time points and at a low degree. Calculation of the (IgG2b + IgG2c/a)/IgG1 ratio showed a significant difference between susceptible SJL/J and resistant BALB/c mice, with a predominant IgG2 response in SJL/J mice (Fig. 3D, 3E).

Association of polarization toward Th1-type cytokine expression with EBA susceptibility

As production of complement-fixing autoantibodies (IgG2b and IgG2a/c) has been shown to be related to Th1 cytokine expression (15), we next investigated cellular responses in lymph nodes draining the area of immunization. Proliferation of Ki-67+ T cells was determined immunohistochemically in paracortical zones of dLN. A higher degree of proliferating cells on days 7–21 after immunization was detected in SJL/J mice compared with BALB/c mice (Fig. 4A, 4B).

Tissue-bound, but not circulating, complement-fixing COL7-specific autoantibodies are associated with clinical disease activity

In disease-prone SJL/J mice, circulating anti-COL7-specific IgG2b were first detected 7 d after immunization, and exceeded IgG1 and IgG2c concentrations starting on day 14, establishing a plateau over the entire observation period. In this strain, significant correlations were found between the kinetics of C3 deposition and IgG2c (r = 0.77, p = 0.0005, Spearman rank order correlation) and IgG2b (r = 0.55, p = 0.0273, Spearman rank order correlation). In contrast, IgG deposition in EBA-resistant BALB/c mice was characterized by an early response of noncomplement-fixing IgG1 on day 7, which continued to be the major deposited IgG isotype. In BALB/c mice, deposition of IgG2b and IgG2a isotypes was only observed at later time points and at a low degree. Calculation of the (IgG2b + IgG2c/a)/IgG1 ratio showed a significant difference between susceptible SJL/J and resistant BALB/c mice, with a predominant IgG2 response in SJL/J mice (Fig. 3D, 3E).

FIGURE 1. SJL/J, but not BALB/c, mice are susceptible to immunization-induced EBA. Representative clinical presentation, complement factor C3 deposition (arrowheads, scale bar, 100 μm), and infiltration with Gr-1+ cells (red, DAPI counterstain in blue, white dotted line represents DEJ, scale bar, 50 μm) of SJL/J and BALB/c ear skin 49 d after single immunization with mCOL7C.

FIGURE 2. Kinetics of clinical, histological, and immunological observations in SJL/J and BALB/c mice after immunization with mCOL7C. A, Development of clinical disease in SJL/J and BALB/c mice until 49 d after immunization. B, Deposition of complement factor C3 at the DEJ. C, Infiltration with Gr-1+ cells. Data shown in A–C correspond to two mice per time point and strain.

FIGURE 2. Kinetics of clinical, histological, and immunological observations in SJL/J and BALB/c mice after immunization with mCOL7C. A, Development of clinical disease in SJL/J and BALB/c mice until 49 d after immunization. B, Deposition of complement factor C3 at the DEJ. C, Infiltration with Gr-1+ cells. Data shown in A–C correspond to two mice per time point and strain.
mCOL7C-specific plasma cells (data not shown), indicating an almost exclusive immune response in dLN after immunization.

Apparently, the adaptive immune responses in the dLN clearly differ between susceptible SJL/J and resistant BALB/c mice. However, disease development in susceptible SJL/J mice strongly depends on the activation of neutrophils. Thus, we asked whether the innate immune response in the dLN would also differ between SJL/J and BALB/c mice, by analyzing the gene expression of the neutrophil-attracting chemokine CXCL1. No difference in the expression of CXCL1 was found (0.082 copies CXCL1/copy MLN51 ± 0.014 in SJL/J versus 0.065 copies CXCL1/copy MLN51 ± 0.011 in BALB/c mice, mean ± SEM, n = 10).

**Restoration of disease susceptibility and Th1-type cytokine expression in C57BL/6 mice by introduction of MHC haplotype H2s**

Immunization-induced EBA-resistant C57BL/6 mice (H2b) respond with a Th1- or Th2-like polarization in different models of infection and allergy (28–33). To elucidate a possible association of Th1-type cytokine gene expression with susceptibility-defining H2s in experimental EBA, we therefore evaluated the clinical and immunological response in C57BL/6 and congenic C57BL/6 mice carrying the H2s of SJL/J mice (B6.SJL-H2s). In line with previous observations, C57BL/6 mice were resistant to EBA induction (Fig. 5A). Furthermore, predominance of IgG1 anti-COL7 Abs, lack of complement fixation, and trend to a Th2-type polarization were observed (Fig. 5B, 5D, 5E). In contrast, congenic B6.SJL-H2s developed skin blisters, as well as deposition of both complement-fixing Abs of the IgG2 isotypes and C3 at the DEJ (Fig. 5A, 5B). Cytokine expression analysis in dLN of congenic mice revealed a mixed, but Th1-type IFN-γ–dominated Th1/Th2 response (Fig. 5D, 5E). Despite similar paracortical T cell proliferation and a comparable binding pattern of autoantibody subclasses in both susceptible strains (Fig. 5B, 5C), SJL/J and B6. SJL-H2s mice differed in their disease progression (Fig. 5A; means of 0.5% versus 0.2% of affected total body skin on day 21, FIGURE 3. Kinetics of autoantibody levels in the serum and deposition at the DEJ. A, Quantitative analysis of circulating serum autoantibodies. B, Pooling of (IgG2 + IgG2c/a)/IgG1 ratios of serum samples obtained between day 7 and 49 did not show a significant difference in between SJL/J and BALB/c mice, using Mann–Whitney U test. C, Quantitative analysis of kinetics of IgG binding to the DEJ showed strong deposition of IgG in SJL/J mice starting on day 14 (IgG2c > IgG2b ≥ IgG1). In BALB/c mice, early IgG1 binding exceeded that of IgG2a and IgG2b throughout the observation period. D, Pooling of (IgG2b + IgG2c/a)/IgG1 ratios of skin samples obtained between day 7 and 49 revealed a significant difference between SJL/J and BALB/c mice, using Mann–Whitney U test. E, Representative images of IgG binding to the DEJ (arrowheads) on day 49. Scale bar, 100 μm. A–D, Data have been obtained from two mice per time point and strain.
4.4% versus 1.5% on day 49 in SJL/J or B6.SJL-H2s mice, respectively). To investigate possible differences of the cytokine milieu in dLN of susceptible SJL/J and B6.SJL-H2s mice, expressions of other pro- and anti-inflammatory cytokines were determined (Supplemental Fig. 2). Among those, a divergent expression pattern was noted for IL-6 and IL-21 only. More specifically, SJL/J mice exhibited a significant and exclusive increase of expression of IL-6, whereas resistant C57BL/6 and susceptible, but less affected, B6.SJL-H2s mice displayed a significant increase in IL-21 expression, which could not be found in SJL/J mice.

Increased FcγRIV expression on circulating leukocytes in EBA-susceptible mice

To investigate regulation of autoantibody-induced tissue injury by differential FcγR expression, we assessed expression of FcγRI, IIb, III, and IV on peripheral leukocytes in EBA-susceptible and -resistant mice after immunization with mCOL7C. Compared with C57BL/6 mice, SJL/J and B6.SJL-H2s mice showed higher expression of FcγRIV. In addition, and in contrast to the two B6 strains (C57BL/6, B6.SJL-H2s), an increased FcγRI expression was noted in SJL/J mice (Fig. 6). No significant differences between the three strains were seen for expression of FcγRIIb and FcγRIII.

Discussion

Using immunization-induced EBA, we demonstrate that production of autoantibodies of specific Ig subclasses is associated with the cytokine milieu of the dLN. More specifically, we show the following: 1) a Th1-like cytokine profile in dLN is associated with skin blistering, whereas a Th2-dominated Th1/Th2-like response determines resistance to EBA induction; 2) in addition to Th1 and Th2 cytokines, IL-6 and IL-21 are involved in mediating the loss of tolerance to COL7; and 3) the FcγR repertoire is shaped differently in EBA-susceptible versus -resistant mice. Characterization of the kinetics of immunization-induced EBA demonstrated that first signs of clinical disease started at day 21 and were accompanied by both C3 fixation and Gr-1⁺ cell infiltration at the DEJ. As Gr-1 is expressed by monocytes, neutrophils, and...
eosinophils in the peripheral blood (34), we confirmed skin infiltration by neutrophils with a specific MPO assay (20, 21). C3 fixation and neutrophil infiltration were preceded by IgG2b and IgG2a/c autoantibody binding to the DEJ at day 14, in line with the previously shown association of complement fixation with binding of IgG2 subclasses to the DEJ (11, 12). Detailed investigation of COL7-specific IgG isotype Abs in EBA-susceptible and -resistant mice showed a significant difference of the calculated (IgG2b + IgG2c/a)/IgG1 ratio for tissue-bound, but not circulating, IgG. These observations suggest that analysis of IgG binding to the DEJ may be a more sensitive approach to investigate disease kinetics, possibly because of better reflecting binding avidity to the actual skin target compared with the use of a recombinant form of the autoantigen. Resistance to EBA in BALB/c mice was found to be related to early deposition of noncomplement-fixing IgG1 starting day 7 after immunization, which continued to be the predominantly deposited isotype of IgG. Although serum IgG2b peaked on day 21 in these mice, DEJ-bound IgG1 dominated continuously, suggesting resistance to EBA by established saturation of the DEJ with IgG1. This assumption is supported by previous findings of reduced clearance rates of DEJ-bound IgG compared with circulating IgG in immunization-induced EBA (18) and also points to an advantage of analyzing tissue-bound compared with circulating IgG. These findings are in line with observations using recombinant anti-human COL7 Abs. Whereas anti-human COL7 IgG1 and IgG3 induced complement activation and dermal-epidermal separation ex vivo, IgG2 and IgG4 neither activated complement, nor led to a significant dermal-epidermal separation (6). Overall, autoantibody-induced tissue injury in experimental EBA most likely is a stepwise process, which mirrors the inflammatory variant of the human disease and does not represent the full clinical spectrum of EBA, including the mechnano-bullos variant of the disease: autoantibody-induced tissue injury in experimental EBA-susceptible and -resistant mice. Disease-prone SJL/J and B6-SJL-H2s mice showed comparable increases in T cell proliferation in dLN on day 21 after immunization. Significances are indicated for comparison between the three different strains, immunized with mCOL7C, and for comparison with the control group of the same strain (8–10 mice/group; means ± SEM). D, Cytokine gene expression in dLN on day 21 after immunization. Obtained data were normalized to the mean value of controls for each cytokine gene and mouse strain. Significances are indicated for comparison with the three different strains, immunized with mCOL7C, and for comparison with the control group of the same strain (8–10 mice/group; means ± SEM). E, In dLN, SJL/J and B6-SJL-H2s mice showed a Th1-type polarization of cytokine expression, significantly different from C57BL/6 mice with a Th2-polarized immune response. Significances are indicated for comparison with the three different strains, immunized with mCOL7C (8–10 mice/group; means ± SEM). Significances in A–E were calculated with the Mann–Whitney U or the Kruskal–Wallis test with Dunn’s posttest, when appropriate.

FIGURE 5. Restoration of disease susceptibility and a Th1-type cytokine gene expression pattern in C57BL/6 mice by introduction of the MHC haplotype H2s. A, Clinical disease severity in SJL/J, C57BL/6, and B6.SJL-H2s mice. Data for day 21 are based on 8–10 mice/group; data for day 49 obtained from 5 mice/group. Significances refer to 5–10 controls/group. B, (IgG2b + IgG2c/a)/IgG1 ratios obtained from skin evaluation showed dermal-epidermal binding predominantly of IgG2bc in EBA-susceptible SJL/J and B6.SJL-H2s mice. In contrast, EBA-resistant C57BL/6 mice showed binding predominantly of IgG1. C3 deposition was also significantly increased in SJL/J and B6.SJL-H2s mice, compared with C57BL/6 (8–10 mice/group). C, Disease-prone SJL/J and B6-SJL-H2s mice showed comparable increases in T cell proliferation in dLN on day 21 after immunization. Significances are indicated for comparison between the three different strains, immunized with mCOL7C, and for comparison with the control group of the same strain (8–10 mice/group; means ± SEM). D, Cytokine gene expression in dLN on day 21 after immunization. Obtained data were normalized to the mean value of controls for each cytokine gene and mouse strain. Significances are indicated for comparison with the three different strains, immunized with mCOL7C, and for comparison with the control group of the same strain (8–10 mice/group; means ± SEM). E, In dLN, SJL/J and B6-SJL-H2s mice showed a Th1-type polarization of cytokine expression, significantly different from C57BL/6 mice with a Th2-polarized immune response. Significances are indicated for comparison with the three different strains, immunized with mCOL7C (8–10 mice/group; means ± SEM). Significances in A–E were calculated with the Mann–Whitney U or the Kruskal–Wallis test with Dunn’s posttest, when appropriate.

FIGURE 6. Determination of FcγR gene expression in peripheral blood leukocytes in EBA-susceptible and -resistant mice. Compared with C57BL/6 and B6.SJL-H2s, only SJL/J mice showed a significant increase of both FcγRI and FcγRIV expression in peripheral blood leukocytes upon immunization with mCOL7C. In susceptible B6.SJL-H2s mice, a significant, but less pronounced increase of FcγRIV expression was observed, when compared with SJL/J. No significant differences between the three strains were seen for expression of FcγRIIa and FcγRIII. Significances are indicated for comparison with the three different strains, immunized with mCOL7C, and for comparison with the control group of the same strain (8–10 mice/group, means ± SEM; Mann–Whitney U or Kruskal–Wallis test with Dunn’s posttest, when appropriate).
EBA is initiated by deposition of autoantibodies at the DEJ. Subsequently, a proinflammatory milieu may be generated in the skin, including complement activation (26). Lack of complement activation may be compensated by other mechanisms, such as mast cell activation. The proinflammatory milieu promotes recruitment of neutrophils (35), leading to FcR-dependent binding of neutrophils to autoantibodies. Upon activation of neutrophils, reactive oxygen species and proteolytic enzymes mediate blister formation (35).

Addressing the T cell response, we observed a strong and lasting paracortical T cell proliferation along with a clear IFN-γ cytokine gene expression and sustained increase in numbers of mCOL7C-specific CD138+ plasma cells in dLN of SJL/J mice. Th1-type polarization of cytokine gene expression correlated with dominance of complement-fixing IgG subclasses at the DEJ (IgG2b/c > IgG1). In contrast, EBA-resistant BALB/c mice had a mixed, but IL-4-dominated, cytokine expression profile in dLN, which was associated with a IgG1-dominated immune response to COL7. These findings clearly indicate that clinical manifestation of immunization-induced EBA is associated with a Th1-like milieu in dLN, whereas a Th2-type polarization protects from clinical disease manifestation. In line with these findings, EBA-resistant C57BL/6 mice showed a trend to a Th2-type polarization upon immunization with mCOL7C. Resistance to EBA induction on the genetic background of C57BL/6 mice was overcome by immunizing H2s-congenic B6.SJL-H2s mice. Differences in polarization of cytokine gene expression are thus most likely dependent on the MHC and may be explained by divergent MHC-encoded mechanisms of Ag presentation. Strength of interaction between TCRs and Ag-loaded MHC class II molecules of APCs, the ratio of APCs with high and low ligand densities on the cell surface, and the Ag dose have been shown to influence T cell proliferation, Th1/Th2 differentiation, and IgG subclass distribution of Abs with and without complement-fixing properties in other models (16, 36–40). These mechanisms may also be responsible for shaping the Th1/Th2 cytokine ratio and the relative distribution of pathogenic/nonpathogenic autoantibodies to COL7 in our experimental model of EBA. Based on these and other findings (12), immunization-induced EBA strongly depends on the MHC haplotype.

In addition to experimental EBA, a MHC association is also observed in two other models of autoimmune skin-blistering diseases. Because dermatitis herpetiformis (DH) in patients has been found to be strongly associated with HLA-DQ8, DQ8-transgenic mice were backcrossed in NOD mice, which are prone to autoimmune disease (The Jackson Laboratory; http://jaxmice.jax.org/strain/001976.html). A single immunization of DQ8-transgenic Mice with crude gluten along with CFA as adjuvant and pretreatment with pertussis toxin led to development of a blistering skin phenotype. Immunization was accompanied by deposition of IgA at the DEJ and neutrophil infiltration. Interestingly, complete reversal of symptoms was observed after introducing a gluten-free diet. Neither NOD mice nor DQ8-transgenic mice developed cutaneous lesions after immunization with gluten, indicating that both the transgene and the NOD background are required to induce DH. In contrast to patients with DH, small bowel pathology was not observed in this model (41). More recently, we have established an immunization protocol that leads to experimental bullous pemphigoid (BP) in mice (42). In this model, disease could only be induced in SJL/J mice, whereas all other strains, including B6.SJL-H2s, were resistant to BP induction. We therefore concluded that the MHC haplotype may contribute to induction of experimental BP. However, as disease could only be induced in SJL/J mice, the immunological uniqueness of this strain, rather than the MHC haplotype, may lead to skin lesions in this experimental model of BP.

Different disease progression in SJL/J and B6.SJL-H2s may be explained by differences in cytokine gene expression in dLN (Th1/Th2 ratio, IL-6, IL-21), and/or in expression of activating FcγR on peripheral blood leukocytes. Binding to and signaling through different FcγR take considerable effect on immunopathology in the development of several autoimmune diseases (25). According to our results, initiation of EBA-specific immunopathology via the Fc portion of IgG2b and IgG2c Abs is restricted to the intermediate-affinity FcγRIV in B6.SJL-H2s mice, whereas the same IgG subclasses can interact with high-affinity FcγRI and intermediate-affinity FcγRIV in SJL/J mice. In experimental EBA induced by passive transfer of anti-COL7 IgG, recent findings showed a dependency of autoantibody-induced tissue injury on FcγRIV expressed on neutrophils (43) and Gr-1+ cells (44). Therefore, differences in disease severity in SJL/J and B6.SJL-H2s mice may be due to the differences in FcγRIV expression found in the current study.

In conclusion, our data indicate the following: 1) skin lesions in experimental EBA are associated with a shift to complement-fixing autoantibodies; 2) induction of clinical disease is only observed in mice featuring a predominant expression of Th1-type cytokines in dLN; and 3) severity of disease is associated with expression levels of activating FcγRs on peripheral blood leukocytes. These data provide new insights into mechanisms associated with loss of tolerance to murine COL7 and subsequent autoantibody-induced tissue injury. Modulation of these processes may be a promising therapeutic approach for EBA and other autoantibody-mediated diseases.

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Disclosures
The authors have no financial conflicts of interest.

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Supplement Figure 1. Kinetics of IFN-γ and IL-4 gene expression in draining lymph nodes (dLN) of SJL/J and BALB/c mice.

Day 21 after immunization with mCOL7C provided best discrimination in dLN cytokine gene expression analysis by real-time PCR (2 mice per point of time and strain). Lines demonstrate mean of data (continuous for SJL/J, dashed for BALB/c).

Supplement Figure 2. Gene expression of various pro- and anti-inflammatory cytokines in draining lymph nodes of SJL/J, C57Bl/6 and B6.SJL-H2s mice.

On day 21 after immunization, IL-6 was almost exclusively expressed by SJL/J mice, whereas IL-21 was only up-regulated in mouse strains carrying a B6 genetic background (C57Bl/6, B6.SJL-H2s). Both susceptible SJL/J and B6.SJL-H2s mice showed significant increase in IL-10 gene expression. Significances are indicated for comparison with the 3 different strains immunized and for comparison with the control group of the same strain (8-10 mice/group, means±SEM; Mann-Whitney U or Kruskal-Wallis test with Dunn’s post test, when appropriate).
(Suppl. Fig. 1)

![Graph showing the x-fold change in cytokine gene expression over time for IFN-γ and IL-4. The graph includes data for SJLU and BALB/c strains.](image-url)
### Table I

<table>
<thead>
<tr>
<th>Primer name</th>
<th>5’ Nucleotide sequence</th>
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1 Primer sequences, amplicon sizes, and NCBI gene accession numbers of the analyzed genes (http://www.ncbi.nlm.nih.gov/nuccore/).