T Cells Are Required for the Production of Blister-Inducing Autoantibodies in Experimental Epidermolysis Bullosa Acquisita

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*J Immunol* 2010; 184:1596-1603; Prepublished online 28 December 2009;
doi: 10.4049/jimmunol.0901412

http://www.jimmunol.org/content/184/3/1596
T Cells Are Required for the Production of Blister-Inducing Autoantibodies in Experimental Epidermolysis Bullosa Acquisita

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Epidermolysis bullosa acquisita is a prototypical organ-specific autoimmune disease caused by autoantibodies against type VII collagen of the dermal-epidermal junction. Although mechanisms of autoantibody-induced blister formation were extensively characterized, the initiation of autoantibody production in autoimmune blistering diseases is still poorly defined. In the current study, we addressed the role of T cells for the production of blister-inducing autoantibodies in mice immunized with type VII collagen. To detect autoreactive type VII collagen-specific T cells, lymph node cells from immunized SJL mice were stimulated in vitro with recombinant Ag, and their proliferation was measured by radioactive thymidine incorporation and flow cytometry analysis of CFSE-labeled cells. Interestingly, using synthetic peptides of the immunogen, partly different T and B cell epitopes in mice immunized with type VII collagen were demonstrated. In contrast to wild-type mice, immunization with type VII collagen of SJL athymic nude mice lacking T cells did not induce an autoimmune response and blistering phenotype. Importantly, SJL nude mice repleted with T cells from immunized wild-type mice showed a robust and durable autoantibody production resulting in subepidermal blistering disease in the recipients. Our present results demonstrate that T cells are required for the initiation of autoimmunity against type VII collagen in experimental epidermolysis bullosa acquisita and provide a basis for developing T cell-directed immunomodulatory strategies for this and related autoimmune diseases.


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pidermolysis bullosa acquisita (EBA) is an organ-specific autoimmune disease characterized by subepidermal blisters and autoantibodies against type VII collagen, the main constituent of the anchoring fibrils at the dermal-epidermal junction (DEJ) (1, 2). The pathogenic relevance of (auto)antibodies against type VII collagen has been conclusively demonstrated ex vivo and in experimental animals (3–5).

Experimental EBA, which reproduces both the autoimmune response as well as immunopathological, histological, and clinical findings in patients with EBA, may be induced in mice by immunization with recombinant murine type VII collagen. This experimental model allows investigating the mechanisms of the production of blister-inducing autoantibodies (5).

Although the blister formation induced by Abs against type VII collagen has been extensively investigated in patients and experimental animals both ex vivo and in vitro, the afferent phase of the specific autoimmune response in EBA, in particular the mechanisms of pathogenic autoantibody production, is poorly characterized (2). Autoantibodies against type VII collagen were extensively characterized in patients with EBA. In contrast, autoreactive T cells have been demonstrated only in a few patients with EBA, and their pathogenic role is unknown (6). In experimental EBA, tissue damage, which represents the effector phase of the efferent autoimmune response, is independent of T cells, because blistering can be induced by the passive transfer of specific Abs into nude mice and ex vivo with purified granulocytes (3, 7, 8). These findings strongly suggest that T cells are not effector cells in EBA.

Studies using animal models of induced or spontaneous autoimmune diseases generally show that production of pathogenic autoantibodies is a T cell-dependent process (9–16). However, recent studies in models of lupus erythematosus demonstrated that the role of T cells is considerably more complex than previously believed and that, in certain experimental settings, B cells alone can drive systemic autoimmunity independently of T cells (17–19). The involvement of T cells for the generation of pathogenic autoantibodies in EBA has not yet been characterized. Therefore, in the current study, we set out to characterize the relevance of autoimmune T cell responses for the production of blister-inducing autoantibodies against type VII collagen in experimental EBA. Using recombinant forms of the autoantigen, we characterized the autoreactive T and B cell epitopes in SJL mice susceptible to experimental EBA. Although immunization of SJL nude mice with type VII collagen did not result in either autoantibody production or blistering disease, both autoantibodies and clinical disease were induced by the transfer of lymphocytes or purified T cells from immunized syngeneic SJL mice into nude mice. These findings demonstrate that T cells are required for the development of an autoimmune response in murine experimental EBA. The autoimmune response and the blistering disease could be adoptively transferred to nude mice using autoreactive T cells.

Materials and Methods

Mice

Six- to 8-wk-old SJL/J (H-2b) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Nude, athymic SJL mice (SJL/nu-nu) (20) were bred at the Molecular and Immunological Pathology Department, University of Freiburg, Germany; and Molecular and Immunological Pathology, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden. Per Hultman, ‡ Werner Solbach,* and Cassian Sitaru †,§

Received for publication May 7, 2009. Accepted for publication November 25, 2009.

This work was supported by Grant E35-2008 from the Medical Faculty, University of Lübeck (to A.G.S.), Grant VII 507-31111.41-22.1 from the Innovationsfonds Schleswig-Holstein (subprojects B6 and A3; to C.S. and D.Z., respectively), and Grant 9453 from the Swedish Research Council, Branch of Medicine (to P.H.). A.S. received an Erasmus stipend.

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The online version of this paper contains supplemental material.

Abbreviations used in this paper: DEJ, dermal-epidermal junction; EBA, epidermolysis bullosa acquisita; IF, immunofluorescence; SJL/nu-nu, nude, athymic SJL mice; wt, wild-type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901412

The Journal of Immunology

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Linköping University, Linköping, Sweden. The nude mice were kept in a high-barrier unit under 12-h dark and 12-h light cycles and given sterilized food pellets and water ad libitum. The Institutional Animal Care and Use Committee, Schleswig-Holstein, Germany, has approved the experiments (reference number 4q/07), which were performed in accordance with the relevant guidelines and regulations.

**Autoantigen peptides**

Recombinant and synthetic forms of murine type VII collagen were prepared as previously described (4, 21, 22). Recombinant tagged fragments GST-mCVIIC and His-mCVIIC were produced using a prokaryotic expression system and purified by glutathione and metallochelate affinity chromatography, respectively (4, 22). Amino acid sequences of synthetic overlapping 20-mer peptides spanning mCVIIC are listed in Table I. All peptides were synthesized by JPT Peptide Technologies, Berlin, Germany. The purity was >90% by HPLC. The antigenic sites of these type VII collagen peptides were analyzed using the antigenic subroutine of the EMBOSS package and a software for the prediction of Ag epitopes (available at http://immanunax.dcfi.harvard.edu/Tools/antigenic.html; Dana-Farber Cancer Institute, Boston, MA) (23, 24).

**Phenotypic analysis of lymphocytes**

Flow cytometry analysis was performed as described with modifications (25–27). To stain cells, we used fluorochrome dye-labeled monoclonal rat Abs specific to CD3 (CT-CD3, IgG2a, R-Phycocerythrin; Caltag Laboratories, Burlingame, CA), CD4 (CT-CD4, IgG2a, FITC; BD Pharmingen, San Diego, CA), CD8 (CT-CD8, IgG2b, PE; BD Pharmingen), and CD45 (IgG2a, FITC; Caltag Laboratories). To block nonspecific binding of Abs, cells (1 × 10^6) were incubated with anti-CD16/32 mAbs (2.4G2; BD Pharmingen) for 10 min in ice-cold PBS with 0.2% BSA and 0.1% sodium azide. For staining, specific Abs at concentrations recommended by the manufacturer, or control isotype-matched irrelevant mAbs at the same concentrations, were added, and cells were further incubated for 15 min. After washing, flow cytometry was performed on 10^6 events using an FACSCalibur (BD Biosciences) and CellQuest software (BD Biosciences). Data were analyzed using WinMDI version 2.9 software (The Scripps Research Institute, La Jolla, CA; http://facs.scripps.edu/software.html).

**Assessment of autoantibody response**

Tissue-bound autoantibodies were detected by immunofluorescence (IF) microscopy on frozen sections prepared from tissue biopsies using 100-fold diluted FITC-labeled Abs specific to mouse IgG (DakoCytomation, Carpinteria, CA), IgG1, IgG2a, IgG2b, IgG3 (all obtained from BD Pharmingen), and murine C3 (Cappel-Organon Teknika, Scarborough, Ontario, Canada) as previously described (5). Detection of serum autoantibodies in mice was performed by IF microscopy on frozen sections prepared from tissue biopsies using 100-fold dilution of mouse sera for 60 min. Bound Abs were detected using 3,3′-diaminobenzidine tetrahydrochloride. Peroxidase skin biopsies were assessed by IF microscopy. Serum autoantibodies were detected by IF microscopy and/or ELISA. T cell responses to type VII collagen were investigated by measuring [3H]thymidine incorporation after stimulation of lymphocytes with autoantigenic peptides and cytokine production by ELISA.

**Cytokine analysis**

IL-2, IFN-γ, IL-4, IL-10, and IL-17 were measured in the supernatants of lymphocyte cultures by sandwich ELISA following the instructions of the manufacturer (R&D Systems, Minneapolis, MN). The detection limits of the assays performed were: 3 pg/ml for IL-2, 2 pg/ml for IL-4, 2 pg/ml for IFN-γ, 5 pg/ml for IL-17, and 4 pg/ml for IL-10.

**Induction of EBA and phenotype analysis**

Experimental EBA was induced in mice by active immunization as described (5), with minor modifications. Briefly, mice were injected s.c. in the hind footpads with 25 μl of emulsion containing 100 μg of GST-mCVIIC in TiterMax (Alexis Biochemicals, Lausen, Switzerland). Mice were examined every second day for their general condition and for evidence of cutaneous lesions (i.e., erythema, blisters, erosions, and crusts) (5). Biopsies of lesional skin were fixed in 4% buffered formalin. Sections from paraffin-embedded tissues were stained with H&E. Perilesional skin biopsies were assessed by IF microscopy. Serum autoantibodies were detected by IF microscopy and/or ELISA. T cell responses to type VII collagen were investigated by measuring [3H]thymidine incorporation after stimulation of lymphocytes with autoantigenic peptides and cytokine production by ELISA.

**Adaptive transfer of lymphocytes**

For cell transfer experiments, lymphocyte suspensions were obtained from draining lymph nodes of immunized SJL mice. Figure 1: a) An immunohistochemistry staining of the anti-NF-kB p65 monoclonal Ab in the biopsies of the skin lesions is shown. b) Flow cytometry analysis of lymphocytes using an FACSCalibur and CellQuest software (BD Biosciences). Data were analyzed using WinMDI version 2.9 software (The Scripps Research Institute, La Jolla, CA; http://facs.scripps.edu/software.html).

**FIGURE 1.** Dose- and time-dependent proliferation of T cells from SJL mice immunized with type VII collagen after in vitro restimulation with the Ag. SJL mice were immunized with 100 μg GST-mCVIIC in adjuvant. Fourteen days after immunization, lymphocytes were collected from the draining lymph nodes and stimulated in vitro with recombinant His-mCVII as described in Materials and Methods. Cell proliferation was expressed as a stimulation index representing the ratio of [3H]thymidine uptake in cultures with Ag and without Ag. a, Cell proliferation by stimulation with type VII collagen in different concentrations ranging from 100–0.1 μg/ml (n = 7 mice), b, Cell proliferation after incubation with irradiated spleen cells and 50 μg/ml Ag for different times (n = 3 mice). Data is given as mean ± SD. *p < 0.05.
Bergisch Gladbach, Germany). A total of $10^7$ lymphocytes were injected into the tail vein of naive syngeneic SJL nu/nu mice. Observation of mice, collection, and preparation of biological samples for histopathology and immunopathology analysis were performed as described previously (5).

**Statistical analysis**

All analyses were performed using gnumeric (v.1.9.5; http://projects.gnome.org/gnumeric/). Continuous variables were compared using the Student t test.

**Results**

**Presence of type VII collagen-specific T cells in experimental EBA**

SJL ($n = 10$) mice were immunized using a GST fusion protein containing a sequence of the type VII collagen NC1 domain (GST-mCVII; aa 757–957). Two weeks later, T lymphocytes were purified from lymph nodes and spleens of immunized mice. We first examined the proliferative ability of the T cells by culturing lymph node cells with irradiated syngeneic spleen cells and a His-tagged recombinant type VII collagen (His-mCVII). Lymph node cells from SJL mice immunized with type VII collagen showed a robust dose- and time-dependent proliferation after in vitro stimulation with the Ag (Fig. 1a, 1b). These results were confirmed using CFSE labeling. Lymph node cells from immunized mice were labeled with CFSE and cultured in the presence of recombinant His-mCVII. Cell division was analyzed by flow cytometry several time points during the culture (data not shown). Cells from immunized mice showed very few cell divisions when cultured in medium alone (2.38%), but strongly proliferated after stimulation with the autoantigen (28.30%) or with anti-CD3 and anti-CD28 Abs (75.97%).

**T cells and autoantibodies recognize several different epitopes**

The proliferation of T cells purified from lymph nodes of mice ($n = 10$) immunized with GST-mCVII was investigated by thymidine incorporation after stimulation with synthetic peptides covering the stretch of type VII collagen used for immunization (aa 757–957; Table I). As shown in Fig. 2a, stimulation with P811–830, P820–839, and P859–902, similar to the recombinant mCVII, induced a strong proliferation of in vivo-primed T cells. Epitopes targeted by serum autoantibodies were characterized by ELISA using the synthetic peptides (Fig. 2b). Autoantibodies recognized epitopes scattered over the type VII collagen sequence used for immunization, including peptides P793–812, P865–884, and P938–967. Analysis of the predicted antigenic sites of the type VII collagen sequence used for immunization by specialized bioinformatic software revealed several epitopes with high scores contained by peptides P725–776, P793–812, P829–848, P838–857, P847–866, P910–939, P929–949, P938–893, P938–967, P983–902, and P982–911. The peptides recognized by autoreactive T and B cells only partly matched the ones containing the predicted antigenic sites (e.g., peptide P811–830 was recognized by T cells, but not autoantibodies and the antigenic score of this sequence was low).

**Cytokine secretion profile of T cells stimulated with type VII collagen**

Previous results showing that autoantibodies against type VII collagen belong to both IgG2a/b and IgG1 subclasses suggested a mixed Th1/Th2 cytokine secretion profile of the autoreactive T cells (5). To investigate the cytokine secretion of autoreactive T cells, supernatants from lymph node cell cultures of SJL mice immunized with type VII collagen were analyzed for the presence of IL-2, IL-4, IL-10, IL-17, and IFN-γ, respectively, by ELISA. As shown in Fig. 3, Ag-stimulated cells secreted dose-dependently large amounts of IL-10 (up to 120 pg/ml culture supernatant), IL-17 (up to 2000 pg/ml culture supernatant), and IFN-γ (up to 4500 pg/ml culture supernatant).

### Table I. Synthetic peptides used in this study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>P1 (757–776)</td>
<td>RVAGVDGAPASVVVRVTAKP</td>
</tr>
<tr>
<td>P2 (766–785)</td>
<td>SVVVRTATPEPVSVVSKLIQIL</td>
</tr>
<tr>
<td>P3 (775–794)</td>
<td>VGSVSKLQILNAGSSDVLRVT</td>
</tr>
<tr>
<td>P4 (784–803)</td>
<td>NAGSSDVLRVTVWGVGATSTY</td>
</tr>
<tr>
<td>P5 (793–812)</td>
<td>WGVGATSYKLAMGRSEQG</td>
</tr>
<tr>
<td>P6 (802–821)</td>
<td>KALAMGRSEQFFMKHRILPGN</td>
</tr>
<tr>
<td>P7 (811–830)</td>
<td>PMKRILPGNKESAERIDLE</td>
</tr>
<tr>
<td>P8 (820–839)</td>
<td>KESAERIDLEBGGVSYSVVRKT</td>
</tr>
<tr>
<td>P9 (838–857)</td>
<td>GGVSTYVRSTETLAVGDREGAP</td>
</tr>
<tr>
<td>P10 (838–857)</td>
<td>VSIVITTPPATTALLETQLQV</td>
</tr>
<tr>
<td>P11 (847–866)</td>
<td>TPALLETLQVVQGSEHSLR</td>
</tr>
<tr>
<td>P12 (856–875)</td>
<td>VQGSEHSLRLRWEVPVPAGP</td>
</tr>
<tr>
<td>P13 (865–884)</td>
<td>RWEVPVPGAPFRHALHQPPEGG</td>
</tr>
<tr>
<td>P14 (874–893)</td>
<td>FRHALHQPQEGQCSSLTGALPE</td>
</tr>
<tr>
<td>P15 (883–902)</td>
<td>QCSSLTGALPEGSNLYNGVLE</td>
</tr>
<tr>
<td>P16 (892–911)</td>
<td>SNSYLVGCLEPATTYQVWLT</td>
</tr>
<tr>
<td>P17 (901–920)</td>
<td>PATKYQVLHVTLvLQCTGEGPP</td>
</tr>
<tr>
<td>P18 (910–939)</td>
<td>VLQGVTQEGPQPKVTAYTEPS</td>
</tr>
<tr>
<td>P19 (929–948)</td>
<td>RKVTAYTEPSHIFSTELVRVD</td>
</tr>
<tr>
<td>P20 (938–967)</td>
<td>RKVTAYTEPSHIFSTELVRVD</td>
</tr>
</tbody>
</table>

**FIGURE 2.** Epitope recognition by type VII collagen-specific T and B cells. SJL mice ($n = 6$) were immunized with 100 μg GST-mCVII in adjuvant. a, Fourteen days after immunization, lymphocytes were collected from the draining lymph nodes and stimulated in vitro with synthetic overlapping peptides and their proliferation measured as described in Materials and Methods. b, Blood samples of immunized SJL were obtained 14 d after immunization. Levels of IgG autoantibodies in serum were measured by an ELISA using synthetic overlapping peptides of mCVII, as described in Materials and Methods. OD readings are represented as means ± SD. *p < 0.05.
pg/ml culture supernatant), but no dose-dependent response and only low levels were found for IL-4 (up to 20 pg/ml culture supernatant). The dose-dependent IL-2 production (up to 60 pg/ml culture supernatant) closely matched the proliferative responses of the Ag-stimulated cells.

**Immunization of SJLnu/nu mice with type VII collagen does not result in autoimmune skin disease**

SJLnu/nu (n = 6) and wild-type (wt) SJL (n = 6) mice were immunized with the GST-mCVIIC three times at 3-wk intervals. Preimmune sera from the mice did not show IgG reactivity to the DEJ by IF microscopy (data not shown). None of the immunized SJLnu/nu mice developed skin lesions during the observation period (Fig. 4a). As expected, blisters, erosions, alopecia, and scarring developed in immunized wt SJL mice (Fig. 4b). Histopathologically, the skin of SJLnu/nu mice showed a normal appearance (Fig. 4c), whereas lesional biopsies from wt mice demonstrated dermal-epidermal separation and an inflammatory cell infiltrate consisting mainly of neutrophils (Fig. 4d). In contrast to nude mice (Fig. 4e), in all wt mice (Fig. 4f) after immunization with type VII collagen, linear deposition of mouse IgG was detected at the epithelial basement membrane of skin. A robust dose-dependent proliferation of lymph node cells after in vitro stimulation with His-mCVIIC was observed in diseased wt, but not in SJLnu/nu mice (Fig. 5a). In contrast to wt mice, SJLnu/nu mice did not develop serum IgG autoantibodies against the DEJ as detected by IF microscopy using murine skin as a substrate (data not shown) or by ELISA using recombinant Ag (Fig. 5b).

**FIGURE 3.** In vitro restimulation with type VII collagen induces a dose-dependent secretion of IL-10, IL-17, and IFN-γ in lymph node cells of immunized SJL mice. SJL mice (n = 6) were immunized with 100 μg GST-mCVIIC in adjuvant. Fifteen days after immunization, lymphocytes were collected from the draining lymph nodes and stimulated with different amounts of recombinant His-mCVIIC. After 4 d of culture, supernatants were collected. The amounts of IL-2 (a), IL-4 (b), IL-10 (c), IL-17 (d), and IFN-γ (e) were measured by ELISA as described in Materials and Methods. The measurements were performed in duplicates, and data are given as mean ± SD. Dotted lines represent the values obtained from cells incubated with medium alone. *p < 0.05.

**FIGURE 4.** Immunization of SJLnu/nu mice with type VII collagen does not result in bullous skin disease. Mice (n = 6) were immunized with 100 μg GST-mCVIIC in adjuvant three times at 3-wk intervals and observed for 80 d. In contrast to an SJLnu/nu mouse (a), a wt SJL mouse (b) shows blisters, erosions, and crusts on the right ear and trunk 70 d after immunization. Histopathology analysis shows normal findings in an SJLnu/nu (c), but dermal-epidermal separation (d) and an inflammatory infiltrate dominated by neutrophils in a wt SJL mouse. By IF microscopy, the skin of a SJLnu/nu mouse (e) lacks, whereas the biopsy from a wt SJL mouse (f) does show deposition of murine IgG at the DEJ.
The transfer of lymphocytes from immunized syngeneic mice results in autoimmune blistering disease in SJLnu/nu mice

SJLnu/nu mice (n = 6) were given i.v. 1 x 10^7 lymphocytes isolated from the draining lymph nodes of SJL mice immunized with murine type VII collagen. The recipient SJLnu/nu mice were further immunized s.c. two times with type VII collagen at 3-wk intervals. Ten weeks after the transfer of lymphocytes two of six transferred SJLnu/nu mice developed blisters and erosions on their ears as well as ventral and dorsal area of the trunk (Fig. 6a). Reduced adhesion of the epidermis to dermis was observed in the diseased mice (Fig. 6b). Skin biopsies were obtained for histopathological examination at the end of the observation period. In all mice that showed skin lesions at the time of tissue collection, light microscopic analysis of skin biopsies revealed extensive dermal-epidermal separation associated with low dense inflammatory infiltrates (Fig. 6c). Circulating IgG autoantibodies binding to the DEJ of murine skin were detected in all SJLnu/nu mice injected with lymphocytes (Fig. 6d). In perilesional skin biopsies obtained from SJLnu/nu mice treated with lymphocytes, deposition of IgG (Fig. 6e) and, less intensely, of C3 (Fig. 6f) was demonstrated at the DEJ by IF microscopy. All SJLnu/nu mice reconstituted with lymphocytes showed a dose-dependent proliferation of lymph node cells after in vitro restimulation with the autoantigen (Fig. 7a). Importantly, these mice also developed autoantibodies against type VII collagen as measured by ELISA using recombinant Ag. The levels of these autoantibodies were comparable with those of immunized wt SJL mice, which developed skin disease (Fig. 7b). The clinical, histopathological, and immunopathological data of this experiment are summarized in Supplemental Table I. In two of six mice, blisters and erosions were demonstrated clinically and histopathologically (Supplemental Fig. 1A). In the skin of all SJLnu/nu mice repleted with T cells, deposits of IgG and less intense deposits of C3 were detected at the DEJ by IF microscopy analysis (Supplemental Fig. 1B, 1C). To demonstrate that transferred T cells persist in the SJLnu/nu mice, CFSE-labeled T cells from wt SJL mice were transferred to SJLnu/nu mice (n = 2). Flow cytometry analysis of cells isolated from spleen and lymph nodes of these SJLnu/nu mice 2 wk after the transfer revealed the presence of CFSE-positive and CD3^hi lymphocytes (data not shown).

Autoantibodies induced in SJLnu/nu mice by the transfer of autoreactive T cells belong to both IgG1 and IgG2a/b subclasses

The IgG subclass of autoantibodies bound at the DEJ in mice was assessed by IF microscopy of perilesional skin biopsies from SJLnu/nu mice (n = 6) that received CD3^hi positive cells i.v. Representative results of this analysis are depicted in Fig. 8. In addition to IgG1 deposits (Fig. 8a), strong deposition of IgG2a (Fig. 8b) and IgG2b (Fig. 8c) were documented. In contrast, no deposits of IgG3 (Fig. 8d) were observed. Grading the intensity of IgG staining at the epidermal basement membrane showed lower, but not significantly different, scores for IgG (SJLnu/nu versus SJL wt: 3.6 ± 0.54 versus 3.8 ± 0.44; p > 0.5), IgG1 (SJLnu/nu versus SJL wt: 3 ± 0.7 versus 3.8 ± 0.44; p > 0.5), and IgG2a (SJLnu/nu versus SJL wt: 2.2 ± 1.3 versus 3.2 ± 0.44; p > 0.5) in treated nude and immunized wt mice. Significantly less deposition of IgG2b (SJLnu/nu versus SJL wt: 2 ± 1.2 versus 3.8 ± 0.44; p < 0.5) and C3 (SJLnu/nu versus SJL wt: 1.2 ± 0.44 versus 2.8 ± 0.44; p < 0.5) in treated nude compared with immunized wt mice.
SJLnu/nu mouse reveals extensive subepidermal cleavage (original magnification ×400). Indirect IF microscopy using serum of a SJLnu/nu mouse immunized with 100 μg GST-mCVIIC in adjuvant twice at 3-wk intervals. SJLnu/nu mice were observed for at least 80 d after the cell transfer. a, Vesicles, erosions, and crusts developed on the left ear of a SJLnu/nu mouse 70 d after the transfer of lymphocytes. b, Upon tangential pressure, epidermal detachment could be induced, and the epidermis could easily be lifted up from the dermis. c, Histological examination of a skin biopsy from a diseased SJLnu/nu mouse reveals extensive subepidermal cleavage (original magnification ×400). d, Indirect IF microscopy using serum of a SJLnu/nu mouse treated with lymphocytes reveals binding of IgG autoantibodies at the epidermal basement membrane in frozen sections of normal murine skin (original magnification ×400). Skin biopsies from treated SJLnu/nu mice were assessed by IF microscopy for the presence of IgG and C3 deposits. Strong IgG (e) and less intense C3 (f) deposits were found at the epidermal basement membrane (original magnification ×400).

**Discussion**

In the present work, we provided an initial characterization of the T cell response against type VII collagen in experimental EBA and addressed its relevance for blister induction. We used a previously established mouse model of EBA induced by immunization with type VII collagen (5) and a newly developed lymphocyte transfer model.

In initial experiments, we investigated the T cell autoreactivity in EBA-susceptible SJL mice immunized against type VII collagen. As expected, the presence of autoantigen-specific T cells after peptide immunization could be demonstrated by both thymidine incorporation and CFSE labeling assays. In line with our findings, type VII collagen-specific T cells have been recently reported in patients with EBA (6). Although the major epitopes of human EBA are located in the NC1 domain of collagen VII with an m.w. of ~145 kDa, they are distributed over its entire length without preferential clustering within this region (30, 31). In mouse models reproducing the subepidermal blistering by the passive transfer of specific Abs, the epitopes targeted by the pathogenic Abs are also localized within the NC1 domain of murine collagen VII (4, 5, 32). Although several epitopes seem to be pathogenically relevant, we have used for our present study a fragment containing the sequence aa 757–957, which has been shown to induce an autoimmune response and active blistering disease by immunization (4, 5, 33). In addition, the passive transfer of Abs specific to this fragment results in subepidermal blistering disease in mice (4, 5).

To characterize the type VII collagen epitopes recognized by T and B cells in further experiments, using synthetic overlapping peptides of the autoantigen, we performed proliferation assays and ELISA, respectively. Interestingly, in contrast to data from one patient with EBA suggesting similar T and B cell epitopes (6), we found T and B cells to exhibit a partly different profile of peptide specificity. However, in the previous study, recombinant fragments of ~600 aa, which may contain numerous epitopes, have been used (6). In addition, due to the use of short peptides in our study, it is likely that only autoantibodies recognizing linear epitopes on murine type VII collagen were measured, which could explain the low reactivity observed by ELISA. Nevertheless, although conformational B cell epitopes may have been missed, some of the peptides recognized by autoantibodies did not stimulate T cells to proliferate. In addition, the predicted antigenic sites were only partly contained by the peptides recognized by T cells and autoantibodies. Although the significance of this interesting finding has not been followed in the current study, dissimilarities between T and B cell epitopes have been reported also in other autoimmunity models in SJL mice (34).

To directly address the pathogenic relevance of T cells for the induction of the autoimmune response and skin blistering in experimental EBA, we immunized SJLnu/nu mice with type VII collagen. In contrast to wt mice, nude mice did not develop measurable cellular and humoral autoimmune responses or skin blistering. Our findings are in agreement with results from other experimental autoimmune diseases, including myasthenia gravis (11, 16), autoimmune encephalomyelitis (35), pneumocystis vulgaris (14, 15), and Goodpasture’s syndrome (12), showing that T cells are required for the development of autoimmune conditions induced by immunization with peptide/protein. It is interesting to note in this context that low but measurable Ab responses were obtained in nude mice immunized with cardiolipin (13). Although the exact cause of these divergent findings is not clear, it appears likely that a T cell-independent mechanism might contribute to the production of Abs against lipids.

In further experiments, we reproduced the blistering phenotype by transferring lymphocytes from wt mice immunized with the autoantigen into nude mice. Importantly, EBA was also reproduced by the transfer of purified T cells, further confirming that T lymphocytes are required for inducing pathology in this experimental model. Although temporarily autoantibody production reached levels similar to those measured in wt mice, toward the end of the observation period, significantly lower autoantibody levels have been measured in treated nude mice. Overall, less deposition of IgG, IgG1, and IgG2b as well as significantly less deposits of IgG2a and complement C3 were seen in the skin of nude mice repleted with lymphocytes. These findings may explain the lower incidence of skin blistering, which occurred in 33% of the treated nude mice compared with ~80% in immunized wt mice and should help improving the lymphocyte transfer model of EBA.

The autoantibodies in SJLnu/nu mice, which received autoantigen-specific T cells, belonged to IgG1, IgG2a, and IgG2b subclasses. These findings confirm previous observations in wt SJL mice immunized with type VII collagen (5) and suggest that, in light of the Th1/Th2 paradigm, the autoreactive T cells show a mixed cytokine secretion profile in experimental EBA. Autoantibodies in patients with EBA also belong to both complement-fixing and noncomplement-fixing IgG subclasses, mainly IgG1.
and IgG4 (36, 37), which adds further support to the notion that the autoimmune response in EBA is associated with a mixed cytokine secretion pattern. This hypothesis is directly supported by the present cytokine secretion analysis showing that T cells from wt mice immunized with type VII collagen secrete cytokines characteristic of Th1, Th2, and Th17 cells. Although we clearly show that conventional T cells are required for the production of blister-inducing autoantibodies against type VII collagen, the functional significance of the Th1, Th2, and Th17 subsets for disease pathogenesis still needs to be addressed and should make the object of future investigations. To validate our present results, these initial observations should be followed by more extensive descriptive investigation of the T cell autoimmune response in patients with EBA. Ideally, our findings should be confirmed using spontaneous models of EBA and other subepidermal autoimmune blistering diseases in animals (38–43). Further characterizing the collagen-specific T cells in EBA should help developing therapeutic interventions aimed at re-establishing T cell tolerance and curbing the aberrant autoimmune response. These include depletion of autoreactive T cells and modulation of their function (e.g., by induction of regulatory T cells, oral tolerance, and raising the threshold for T cell activation).

In conclusion, our results demonstrate that T cells are required for the development of the autoimmune disease in murine experimental EBA induced by immunization with the autoantigen or by transfer of syngeneic lymphocytes into immunodeficient mice. In addition, the new disease model reproducing EBA in immunodeficient mice by transferring syngeneic Ag-experienced lymphocytes clearly demonstrates that both T and B cells are required for disease induction and will greatly facilitate dissecting the role of the different lymphocytes subpopulations for EBA pathogenesis.

Acknowledgments
We thank Thomas Bollinger, Lübeck, Germany, for helpful advice. The experiments of this work were performed at the University of Lübeck. After Dr. Cassian Sitaru left the University of Lübeck, he contributed to drafting this manuscript at his new affiliation.

Disclosures
The authors have no financial conflicts of interest.

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