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

Ana Gabriela Sitaru, Alina Sesarman, Sidonia Mihai, Mircea T. Chiriac, Detlef Zillikens, Per Hultman, Werner Solbach and Cassian Sitaru

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Epidermolysis 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 effenter 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 adaptively 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 Lu¨beck, Lu¨beck; and the Department of Dermatology, University of Freiburg, Germany; and the Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden.

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Address correspondence and reprint requests to Dr. Cassian Sitaram, Department of Dermatology, University of Freiburg, Hauptstrasse 7, 79104 Freiburg, Germany; E-mail address: csitaru@fastmail.fm

*Institute for Medical Microbiology and Hygiene and †Department of Dermatology, University of Lübeck, Lübeck; ‡Department of Dermatology, University of Freiburg, Freiburg, Germany; and †Molecular and Immunological Pathology, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

The online version of this paper contains supplemental material.

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

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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 gluthatione 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 subroute of the EMBLSS package and a software for the prediction of Ag epitopes (available at http://immunex.dfci.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-Phycocerythin; Caltag Laboratories, Burlingame, CA), CD4 (CT-CD4, IgG2a, FITC; BD Pharmingen, San Diego, CA), CD8 (CT-CD8, IgG2a, R-Phycocerythin; BD Pharmingen), and CD45 (IgG2a, FITC; Caltag Laboratories). To block nonspecific binding of Abs, cells (1 × 10^5) 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 FACS Calibur (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 of normal murine skin or ELISA using recombinant and/or synthetic type VII collagen peptides as described with minor modifications (5). Briefly, after incubating with diluted mouse serum, the frozen sections of murine skin were treated with 10^6 cells were further incubated for 15 min. After washing, flow cytometry was performed on 10^6 events using an FACS Calibur (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 T cell proliferative response

To investigate the autoreactive T cells ex vivo, T cell proliferation assays were performed as described with minor modification (28, 29). Briefly, single-cell suspensions were obtained from draining lymph nodes and spleens of mice and cultured in RPMI 1640 culture medium supplemented with 1 mM sodium pyruvate, 2 mM glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, nonessential amino acids, and 10% FCS. A total of 5 × 10^5 cells were cultured with different amounts of autoantigen (His-mCVIIC or synthetic peptides) in the presence of 5 × 10^5 irradiated spleen cells from syngeneic mice as APCs in 96-well plates. Control cells were cultured in the presence of tissue culture plate-bound anti-CD3 mAbs (2 µg/ml, Caltag) and soluble anti-CD28 mAbs (1 µg/ml, Caltag). After 72 h, 100 µl of culture supernatant was carefully removed for measurement of cytokines and the remaining cells were pulsed with 1 µCi [3H]thymidine for a further 20 h. Cells were then harvested by standard liquid scintillation counting. Experiments were set up in quadruplicates and results expressed in counts per minute.

Labeling of lymphocytes with CFSE was performed according to the manufacturer’s instruction. Briefly, 1 × 10^7 cells were incubated in 2.5 µM CFSE (Molecular Probes Europe, Leiden, The Netherlands) in PBS in dark, at room temperature for 8 min, and then three times washed with prewarmed RPMI 1640 culture medium.

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 TierMax (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 autoantigen peptides and cytokine production by ELISA.

Adoptive transfer of lymphocytes

For cell transfer experiments, lymphocyte suspensions were obtained from draining lymph nodes of immunized SJL mice 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.

**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 SJLnu/nu mice. Observation of mice, col- 

mumopathology analysis were performed as described previously (5).

were immunized with 100

FIGURE 2.

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-mCVIIC; 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-mCVIIC). 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. 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-mCVIIC. 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-mCVIIC was investigated by thymidine incor- 

poration 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, P838–902, and P902–911, 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 P892–911. 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 P757–776, P793–812, P829–848, P838–857, P847–866, P910–939, P929–948, P938–967, P947–967, and P992–911. 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 auto-

antibodies 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

Table I. Synthetic peptides used in this study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>P1 (757–776)</td>
<td>HVAGVDGAPSVVVRTAPEP</td>
</tr>
<tr>
<td>P2 (766–785)</td>
<td>SVVVRTAPEPVSVSXKLQIL</td>
</tr>
<tr>
<td>P3 (775–794)</td>
<td>VSVSKLQILNASVSDVRVT</td>
</tr>
<tr>
<td>P4 (784–803)</td>
<td>NASSDLYRTWTVGPASTY</td>
</tr>
<tr>
<td>P5 (793–812)</td>
<td>WVGGPGATSYKLAWRSSEG</td>
</tr>
<tr>
<td>P6 (802–821)</td>
<td>KFLGWRSEGPGWKHRILPS</td>
</tr>
<tr>
<td>P7 (811–830)</td>
<td>PMKRHLIPGKESEARIDLE</td>
</tr>
<tr>
<td>P8 (820–839)</td>
<td>KESAEIRDELGVSYSVVRT</td>
</tr>
<tr>
<td>P9 (829–848)</td>
<td>GGSYSVRVTALVGREGAP</td>
</tr>
<tr>
<td>P10 (838–857)</td>
<td>ALVGREGAPVSIVITTPPA</td>
</tr>
<tr>
<td>P11 (847–866)</td>
<td>VSIVITTPPATPALLELTVQ</td>
</tr>
<tr>
<td>P12 (856–875)</td>
<td>TPALLETLQVVQSGEHSLRL</td>
</tr>
<tr>
<td>P13 (865–884)</td>
<td>VQSGEHSLRLVWEPVPAG</td>
</tr>
<tr>
<td>P14 (874–893)</td>
<td>RWEPVPAEPFRLHNPQEG</td>
</tr>
<tr>
<td>P15 (883–902)</td>
<td>FRLHNPQEGGQESLTLGPE</td>
</tr>
<tr>
<td>P16 (892–911)</td>
<td>QEQLTLGPEXNSYNLNGLE</td>
</tr>
<tr>
<td>P17 (901–920)</td>
<td>SNSYNLNGLEPATKLYQVWL</td>
</tr>
<tr>
<td>P18 (910–939)</td>
<td>PATKLYQVWLTVQGEGFP</td>
</tr>
<tr>
<td>P19 (929–948)</td>
<td>VQLGCTGGPSEPVRKTVAY</td>
</tr>
<tr>
<td>P20 (938–967)</td>
<td>RKVTAYTEGRHSTELRVD</td>
</tr>
</tbody>
</table>

Peptide Sequence

P1 (757–776) HVAGVDGAPSVVVRTAPEP
P2 (766–785) SVVVRTAPEPVSVSXKLQIL
P3 (775–794) VSVSKLQILNASVSDVRVT
P4 (784–803) NASSDLYRTWTVGPASTY
P5 (793–812) WVGGPGATSYKLAWRSSEG
P6 (802–821) KFLGWRSEGPGWKHRILPS
P7 (811–830) PMKRHLIPGKESEARIDLE
P8 (820–839) KESAEIRDELGVSYSVVRT
P9 (829–848) GGSYSVRVTALVGREGAP
P10 (838–857) ALVGREGAPVSIVITTPPA
P11 (847–866) VSIVITTPPATPALLELTVQ
P12 (856–875) TPALLETLQVVQSGEHSLRL
P13 (865–884) VQSGEHSLRLVWEPVPAG
P14 (874–893) RWEPVPAEPFRLHNPQEG
P15 (883–902) FRLHNPQEGGQESLTLGPE
P16 (892–911) QEQLTLGPEXNSYNLNGLE
P17 (901–920) SNSYNLNGLEPATKLYQVWL
P18 (910–939) PATKLYQVWLTVQGEGFP
P19 (929–948) VQLGCTGGPSEPVRKTVAY
P20 (938–967) RKVTAYTEGRHSTELRVD

Statistical analysis

All analyses were performed using gnupl(t) (v.1.9.5; http://projects.gnome. org/gnupl(t)). 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-mCVIIC; 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-mCVIIC). Lymph node cells from SJL mice immunized with type VII collagen showed a robust dose- and time-dependent proliferation after in vitro stimulation with

FIGURE 2. Epitope recognition by type VII collagen-specific T and B cells. SJL mice (n = 6) were immunized with 100 μg GST-mCVIIC 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 mCVIIC, 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 SJL\textsuperscript{nu/nu} mice with type VII collagen does not result in autoimmune skin disease**

SJL\textsuperscript{nu/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 SJL\textsuperscript{nu/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 SJL\textsuperscript{nu/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 SJL\textsuperscript{nu/nu} mice (Fig. 5a). In contrast to wt mice, SJL\textsuperscript{nu/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-\(\gamma\) in lymph node cells of immunized SJL mice. SJL mice (n = 6) were immunized with 100 \(\mu\)g 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-\(\gamma\) (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 represents the values obtained from cells incubated with medium alone. *\(p<0.05\).

**FIGURE 4.** Immunization of SJL\textsuperscript{nu/nu} mice with type VII collagen does not result in bullous skin disease. Mice (n = 6) were immunized with 100 \(\mu\)g GST-mCVIIC in adjuvant three times at 3-wk intervals and observed for 80 d. In contrast to an SJL\textsuperscript{nu/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 a SJL\textsuperscript{nu/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 SJL\textsuperscript{nu/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 SJL<sup>nu/nu</sup> mice

SJL<sup>nu/nu</sup> mice (n = 6) were given i.v. 1 × 10<sup>7</sup> lymphocytes isolated from the draining lymph nodes of SJL mice immunized with murine type VII collagen. The recipient SJL<sup>nu/nu</sup> 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 SJL<sup>nu/nu</sup> mouse 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 SJL<sup>nu/nu</sup> mice injected with lymphocytes (Fig. 6d). In perilesional skin biopsies obtained from SJL<sup>nu/nu</sup> mice treated with lymphocytes, deposition of IgG (Fig. 6e) and, less intensely, of C3 (Fig. 6f) were demonstrated at the DEJ by IF microscopy. All SJL<sup>nu/nu</sup> 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. To demonstrate that transferred T cells persist in the SJL<sup>nu/nu</sup> mice, CFSE-labeled T cells from wt SJL mice were transferred to SJL<sup>nu/nu</sup> mice (n = 2). Flow cytometry analysis of cells isolated from spleen and lymph nodes of these SJL<sup>nu/nu</sup> mice 2 wk after the transfer revealed the presence of CFSE-positive and CD3<sup>hi</sup> lymphocytes (data not shown).

Autoantibodies induced in SJL<sup>nu/nu</sup> 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 determined by both IF microscopy on murine skin and ELISA using recombinant autoantigen (data not shown). 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 SJL<sup>nu/nu</sup> 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 SJL<sup>nu/nu</sup> mice, CFSE-labeled T cells from wt SJL mice were transferred to SJL<sup>nu/nu</sup> mice (n = 2). Flow cytometry analysis of cells isolated from spleen and lymph nodes of these SJL<sup>nu/nu</sup> mice 2 wk after the transfer revealed the presence of CFSE-positive and CD3<sup>hi</sup> lymphocytes (data not shown).

T cells are required for the autoimmune blistering disease in SJL<sup>nu/nu</sup> mice

To further delineate the pathogenic relevance of T cells for the adoptively transferred EBA, T lymphocytes were isolated from lymph nodes of immunized SJL mice (n = 16). A total of 1 × 10<sup>7</sup> purified CD3<sup>hi</sup> cells were injected i.v. in SJL<sup>nu/nu</sup> mice (n = 6). The treated SJL<sup>nu/nu</sup> mice were further immunized two times s.c. with type VII collagen at 3-wk intervals. In all of these SJL<sup>nu/nu</sup> mice, serum autoantibodies against type VII collagen were demonstrated by both IF microscopy on murine skin and ELISA with recombinant autoantigen (data not shown). 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 SJL<sup>nu/nu</sup> 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 SJL<sup>nu/nu</sup> mice, CFSE-labeled T cells from wt SJL mice were transferred to SJL<sup>nu/nu</sup> mice (n = 2). Flow cytometry analysis of cells isolated from spleen and lymph nodes of these SJL<sup>nu/nu</sup> mice 2 wk after the transfer revealed the presence of CFSE-positive and CD3<sup>hi</sup> lymphocytes (data not shown).
SJLnu/nu mouse reveals extensive subepidermal cleavage (original magnification ×400). Skin biopsies from treated SJLnu/nu mice were assessed by IF microscopy for the presence of IgG and C3 deposits. F, Upon tangential pressure, epidermal detachment could be induced, and the epidermis could easily be lifted up from the dermis. 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 (ε) and less intense C3 (f) deposits were found at the epidermal basement membrane (original magnification ×400).

FIGURE 6. Transfer of lymph node cells from wt SJL mice immunized with type VII collagen into SJLnu/nu mice induces a subepidermal blistering disease. Wild-type SJL mice (n = 6) were immunized with 100 μg GST-mCVIC in adjuvant. On day 14 after the immunization, the mice were killed to obtain cells from draining lymph nodes. A total of 10^7 cells were injected into the tail vein of SJLnu/nu (n = 6), and these mice were subsequently immunized with 100 μg GST-mCVIC 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 (ε) 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 autoimmune 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), pemphigus 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 IgG2a as well as significantly less deposits of IgG2b 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.

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Disclosures
The authors have no financial conflicts of interest.

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