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# B Cells, Dendritic Cells, and Macrophages Are Required To Induce an Autoreactive CD4 Helper T Cell Response in Experimental Epidermolysis Bullosa Acquisita

## Hiroaki Iwata,\* Katja Bieber,\* Benjamin Tiburzy,<sup>†</sup> Navina Chrobok,<sup>‡</sup> Kathrin Kalies,<sup>‡</sup> Atsushi Shimizu,<sup>§</sup> Sarah Leineweber,<sup>¶</sup> Akira Ishiko,<sup>§</sup> Artem Vorobyev,\* Detlef Zillikens,\* Jörg Köhl,<sup>†,||</sup> Jürgen Westermann,<sup>‡</sup> Karsten Seeger,<sup>¶</sup> Rudolf Manz,<sup>†</sup> and Ralf J. Ludwig\*

In autoimmune bullous dermatoses (AIBD), autoantibodies induce blisters on skin or mucous membranes, or both. Mechanisms of continued autoantibody production and blistering have been well characterized using AIBD animal models. Mechanisms leading to the initial autoantibody production, however, have not been investigated in detail. Epidermolysis bullosa acquisita (EBA) is an AIBD associated with autoantibodies to type VII collagen (COL7). The majority of EBA patients' sera recognize the noncollagenous domain 1, including the von Willebrand factor A–like domain 2 (vWFA2). In experimental EBA induced by immunization with GST-COL7, disease manifestation depended on the genetic background, a Th1 polarization, and the GST-tag. In this model, nude mice neither produced autoantibodies nor blisters. It has remained uncertain which APC and T cell subsets are required for EBA induction. We established a novel EBA model by immunization with vWFA2 fused to intein (lacking the GST-tag). All tested mouse strains developed autoantibodies, but blisters were exclusively observed in mice carrying H2s. In immunized mice, CD4 T cells specific for vWFA2 were detected, and their induction required presence of B cells, dendritic cells, and macrophages. Anti-vWFA2 autoantibodies located at the lamina densa bound to the dermal side of salt-split skin and induced blisters when transferred into healthy mice. Absence of CD8 T cells at time of immunization had no effect, whereas depletion of CD4 T cells during the same time period delayed autoantibody production and blisters. Collectively, we demonstrate the pathogenic relevance of Abs targeting the vWFA2 domain of COL7 and show the requirement of APC-induced CD4 T cells to induce experimental EBA. *The Journal of Immunology*, 2013, 191: 2978–2988.

utoimmunity is characterized by loss of self-tolerance and an induction of an immune response against self-Ags. Although the effector mechanisms of autoimmune diseases have been extensively investigated, the mechanisms of induction and maintenance of self-tolerance are less well characterized. Nevertheless, there are several reports that CD4 T cells are essential for disease induction in several autoimmune

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disorders (1–3). Autoimmune bullous dermatoses (AIBD) are paradigm organ-specific autoimmune diseases, characterized by autoantibodies to structural proteins of the skin, which directly or indirectly cause blister formation on skin and/or mucous membranes (4, 5).

Use of different animal models has greatly improved our understanding of the pathogenesis of AIBD (6). Especially, use of autoantibody transfer into mice has unraveled mechanisms leading to blister formation (7-13). Yet, insights into the loss of tolerance and autoantibody production cannot be provided by autoantibody transfer models. The latter can be in detail investigated by transfer of lymphocytes into immunodeficient mice; in these models, transferred lymphocytes are derived from mice lacking certain AIBD autoantigens or from animals immunized by skin grafting (14, 15). Regarding the cellular requirements of autoantibody production, these models clearly documented a dependency on both B cells and CD4 T cells (15-18). In principle, immunizationinduced models of AIBD (19-21) allow for investigating the cellular and molecular requirements that lead to loss of tolerance and subsequent autoantibody production. Yet, so far, detailed investigations on the cellular requirements in the early pathogenesis of these animal models had not been performed. The MHC association of immunization-induced AIBD (19, 22) pointed toward a contribution of T cells, which was confirmed by a resistance of T cell-deficient mice to develop epidermolysis bullosa acquisita (EBA) (23). It has so far remained unclear which T cell subsets and which APCs contribute to the loss of tolerance to structural proteins of the skin in AIBD.

To address the question which T cell subset(s) and APC(s) are important for early AIBD pathogenesis, namely, loss of tolerance

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Abbreviations used in this article: AIBD, autoimmune bullous dermatoses; AUC, area under the curve; B6, C57BL/6J; B6.s, B6.SJL-H2s C3c/1CyJ; COL7, type VII collagen; DC, dendritic cell; DEJ, dermal–epidermal junction; dLN, draining lymph node; DT, diphtheria toxin; EBA, epidermolysis bullosa acquisita; FNIII, fibronectin III–like domain; IF, immunofluorescence; IHC, immunohistochemistry; NC, noncollagenous domain; RT, room temperature; vWFA2, von Willebrand factor A–like domain 2.

and induction of autoantibodies, we made use of a novel mouse model for EBA, a prototypic organ-specific autoimmune disease. Clinically, EBA is characterized by chronic mucocutaneous subepidermal blistering (24). The autoimmune response is directed against a 290-kDa protein located at the dermal–epidermal junction (DEJ) (25), which has been identified as type VII collagen (COL7), a major component of anchoring fibrils (26). COL7 consists of a 145-kDa central collagenous triple-helical segment, which is flanked by a larger 145-kDa N-terminal noncollagenous domain 1 (NC1) and a smaller 34-kDa C-terminal NC2 (27, 28). The C-terminal subdomain of NC1 shows high homology with von Willebrand factor A and was therefore termed von Willebrand factor A–like domain 2 (vWFA2) (29). This domain has been shown to interact with type I collagen (30, 31) and was demonstrated to be targeted by a significant number of patients' sera (32).

In the immunization-induced EBA mouse model, we recently demonstrated that the GST-peptide tag coupled to the murine fibronectin III–like domains (FNIII) 7 and 8 of COL7 (termed mCOL7C) modulates the mCOL7C-specific plasma cell response and the induction of EBA (B. Tiburzy, M. Szyska, H. Iwata, N. Chrobok, U. Kulkarni, M. Hirose, R.J. Ludwig, K. Kalies, J. Westermann, D. Wong, R.A. Manz, submitted for publication). We therefore aimed at developing a novel immunization-induced EBA mouse model in which the disease develops independent of an immune response to proteins other than COL7. Because the vWFA2 domain had been demonstrated as an immunodominant site on COL7 (32) and represents the interaction site between type I collagen and COL7 (30, 31), we used in this article the murine vWFA2 protein for immunization of mice.

We identified mouse strains that generated only autoantibodies to the vWFA2 domain in the absence of clinical blistering, whereas others developed both autoantibodies and skin blistering. Interestingly, disease was induced independent of immune responses to any other protein than the autoantigen itself. In this study, we used this novel model to investigate whether CD4 and/or CD8 T cells contribute to loss of tolerance and initiation of autoantibody production in the early EBA pathogenesis. Furthermore, we investigated which APC cell types are required to induce the vWFA2-specific CD4 T cell responses.

#### **Materials and Methods**

#### Patient sera

For this study, serum samples from 21 EBA patients were used. All patients fulfilled the following inclusion criteria: 1) blisters/erosions on skin and/or mucous membranes, 2) binding of IgG autoantibodies to the dermal side of 1 M NaCl-split human skin as shown by indirect immunofluorescence (IF) microscopy, and 3) reactivity to NC1 and/or dermal extract (290 kDa) by immunoblot analysis. Sera from healthy volunteers served as negative controls. Written informed consent was obtained from all patients. The study was approved by the ethics committee of the University of Lübeck and was performed according to the Declaration of Helsinki.

# Expression of murine and human vWFA2 fragments and labeling with DyLight488

The immunodominant murine vWFA2 domain (Supplemental Fig. 1) of NC1 (aa 1048–1238 with 5 additional amino acids [GRAMG] at the N terminus) was expressed and purified as described previously (33). Before use, vWFA2 was dialyzed against PBS. For use in some experiments, recombinant murine vWFA2 was labeled with DyLight488 (Thermo Scientific, Rockford, IL) according to manufacturer's instructions. The DNA sequence of human NC1 domain of COL7 was obtained from the Uniprot database (http://www.uniprot.org). Primers for recombinant proteins' DNA cloning were obtained from VBC Biotech (VBC-Biotech Service, Vienna, Austria). The DNA sequence of human vWFA2 was amplified by PCR with Phusion polymerase (Fermentas, St. Leon-Rot, Germany) using cDNA from normal human skin as a template with appropriate primers (forward: 5'-ATCCATGGGCGATCTGGTGTTCCTACCACA-3' and re-

verse: 5'-GATGAATTCTCACAGACCACTGACTGCCTGGTC-3'). The amplified cDNA sequences were digested with NcoI and EcoRI Fast Digest restriction enzymes (Fermentas) and inserted into prokaryotic expression vector pTWIN1 (New England Biolabs, Ipswich, MA). Correct sequences of DNA fragments were confirmed by DNA sequence analysis of isolated plasmids (Eurofins MWG Operon, Ebersberg, Germany).

### Abs

IgG-FITC (DakoCytomation, Glostrup, Denmark), C3-FITC (Cappel, Solon, OH), human IgG-HRP (Sigma-Aldrich, Munich, Germany), IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA), IgG1-FITC (clone, A85-1), IgG2a/c-FITC (clone, 5.7), IgG2b-FITC (clone, R12-3), IgG3-FITC (clone, R40-82), IgG1-biotin (clone, A85-1), IgG2a/c-biotin (clone, 5.7), IgG2b-filt (clone, R12-3), IgG3-biotin (clone, R40-82), CD3e-FITC (clone, 145-2C11), CD3-PE (clone, 145-2C11), CD4-FITC (clone, GK1.5), CD4-Alexa Fluor 647 (clone, PC61.5), CD69-FITC (clone, H1.2F3), CD11b-PE (clone, M1/70), CD11c-PE (clone, HL3), CD19-allophycocyanin (clone, 1D3; BD Biosciences, San Jose, CA), CD8-PE (clone, 63-6.7), F4/80-PE (clone, BM8), Ly-6G-biotin (clone, PC61.5; eBiosciences, Frankfurt, Germany), CD154-PE (clone, MR1; Biolegend, Fell, Germany), CD19-PE (clone, 6D5) (Life Technologies, Darmstadt, Germany). Except Ab against human IgG-HRP, all other Abs recognize murine components.

#### Immunizations and evaluation of mice

C57BL/6J (B6), B6.SJL-H2s C3c/1CyJ (B6.s), BALB/c, SJL/J, and CD11c-DTR mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were fed acidified drinking water and standard chow ad libitum, and held on a 12-h light-dark cycle at the animal facility of the University of Lübeck. Mice aged 6-10 wk were used for the experiments. All clinical examinations, biopsies, and bleedings were performed under anesthesia with i.p. administration of a mixture of ketamine (100  $\mu$ g/g) and xylazine (15  $\mu$ g/ g). The experiments were approved by the Animal Care and Use Committee (Kiel, Germany) and performed by certified personnel. Mice were immunized at the hind footpad with 60  $\mu g$  recombinant murine COL7 vWFA2 domain emulsified in the nonionic block copolymer adjuvant TiterMax (ALEXIS Biochemicals, Norcross, GA). Control mice were immunized with PBS and TiterMax. Subsequently, mice were evaluated at 2-wk intervals for presence of skin lesions (i.e., erythema, blisters, erosions, crusts, and alopecia) until 12-36 wk after immunization. Disease severity was expressed as percentage of body surface area affected by skin lesions, and total clinical disease severity during observation period was calculated as area under the curve (AUC). Serum and tail skin samples were collected every fourth week. Serum, ear skin, and tail skin samples were obtained at the final day and prepared for examination by histopathology, IF microscopy, and electron microscopy.

#### Histological and electron microscopical studies

Samples of skin were fixed in 4% buffered formalin. Four-micrometer-thick sections from paraffin-embedded tissues were stained with H&E. Transmission electron microscopy was performed as described previously (21). To characterize the binding site of the immune serum, postembedding immunogold electron microscopy using normal murine skin (B6) was performed as reported previously (21).

# IF microscopy, immunohistochemistry, immunoblot, and ELISA analysis

Tissue-bound Abs and complement were detected by direct IF microscopy on 6-µm frozen skin sections prepared from tissue biopsies using 100-fold diluted FITC-labeled Abs specific to murine IgG or IgG subclasses, including IgG1, IgG2a/c, IgG2b, and IgG3, as well as murine C3. A total of 1 M NaCl-split murine skin was prepared as described previously (21). To investigate which APCs may contribute to the formation of immunogenspecific CD4 T cells, we obtained draining lymph nodes (dLNs) from SJL/ J 3 wk after immunization with fluorescent-vWFA2. Frozen dLN sections were stained with Abs against CD11b-PE, CD11c-PE, or CD19-PE; subsequently, nuclei were stained with DAPI (Roche Diagnostics, Mannheim, Germany). Sections were observed by fluorescence microscopy (Keyence, Osaka, Japan). Neutrophil infiltration in the skin was detected by immunohistochemistry (IHC) as described earlier with modifications (20). Sixmicrometer frozen skin sections were incubated with biotin-conjugated Ly-6G (1:200 dilution) Ab at 37°C for 1 h. Subsequently, sections were incubated with Ultra-Sensitive ABC Peroxidase Staining Kit (Thermo Scientific Pierce Protein Research Products) at room temperature (RT) for 0.5 h and were developed by 3,3'-diaminobenzidine (Merck, Darmstadt, Germany). Dermal extracts of murine skin were prepared as described (34).

Dermal extract and recombinant murine and human vWFA2 protein were fractionated by 6 and 12% SDS-PAGE, respectively, transferred to nitrocellulose, and analyzed by immunoblotting (35). For epitope mapping, patients' sera (1:100 dilution) were incubated at 4°C overnight and were incubated Ab against human IgG-HRP (dilution 1:1000) at RT for 1 h. Circulating mouse vWFA2-specific Abs were detected by ELISA. In brief, 96-well microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) were coated with 250 ng murine vWFA2. Nonspecific binding was reduced by blocking plates with 1% BSA in PBS-T at RT for 1 h. Subsequently, plates were incubated with mouse sera at RT for 1 h. Serum dilutions ranged from 1:5,000 to 1:20,000. Plates were incubated with HRP-conjugated antimurine IgG (dilution 1:20,000) and biotin-conjugated anti-murine IgG subclasses, including IgG1, IgG2a/c, IgG2b, and IgG3 (dilution 1:20,000), at RT for 1 h. Plates were incubated with ultrasensitive ABC peroxidase staining kit (Thermo Scientific) for IgG subclasses at RT for 0.5 h. Onestep Turbo TM-ELISA (Thermo Scientific) substrate was added for visualization. Cutoff was determined by average of negative controls +3 SDs of negative controls.

#### Immunoadsorption test

To test for a possible epitope spreading, we obtained sera from SJL/J mice 36 wk after immunization. Sera (dilution 1:10) were incubated with vWFA2 protein (2  $\mu$ g) at 4°C overnight. Subsequently, these preadsorbed sera were analyzed by indirect IF microscopy as mentioned earlier.

# Ab purification from immunized mice and passive transfer studies

To test for the pathogenicity of Abs from immunized mice, we performed passive transfer studies as described previously with minor modification (21). Sera were obtained from immunized SJL/J, B6.s, B6, and BALB/c mice at the end of the experiment (weeks 12 [B6.s] or 20 for all other mouse strains). Total IgG was prepared from the sera by affinity chromatography using a HiTrap Protein G HP (GE Healthcare Biosciences, Uppsala, Sweden). A total of 1 mg total IgGs (20 mg/ml) was injected intradermally into the ears of healthy B6 mice. Injections were done every second day for a total of four times. Starting after the first IgG injection, ears were examined for evidence of skin lesions (i.e., erythema, blisters, erosions, and crusts) every day, until 2 d after last injection. On the final day of the experiment, ears were obtained for histological and immunopathological examination. When mice experienced development of skin lesions before the fourth injection, IgG injections were discontinued.

#### In vivo cell depletion

To investigate which T cell subset(s) are required for the early pathogenesis of EBA, we depleted CD4 or CD8 T cells before immunization and maintained cell depletion for at least 2 wk thereafter. Anti-CD4 and anti-CD8 mAbs were obtained from the hybridoma cell lines, GK1.5 or 53-6.72, respectively. Hybridoma cell lines were grown in RPMI 1640 medium (Life Technologies, Darmstadt, Germany) supplemented with 10% heat-inactivated IgG free FCS (PAA Laboratories, Pasching, Austria), 100 U/ml PenStrep (PAA Laboratories), 2 mM L-glutamine, and 50 µM 2-ME (Sigma-Aldrich). Supernatants were purified over a protein G column (GE Healthcare Biosciences), dialyzed in PBS, and concentrated in polyethylene glycol 20,000 (Merck, Darmstadt, Germany). In a pilot experiment, the ability to deplete the respective cell population in SJL/J mice was confirmed by flow cytometry (FACS) analysis of peripheral blood cells from treated mice (data not shown). One day before immunization, mice were injected i.p. with anti-CD4 mAb 400 µg/body (concentration, 2 mg/ml) and anti-CD8 mAb 5 mg/kg (concentration, 1 mg/ml), respectively (36, 37). According to the results from the pilot depletion study, anti-CD4 or anti-CD8 mAbs were injected every second or fifth day, respectively.

To investigate which APC(s) are important for induction of Ag-specific CD4 T cells, we depleted APCs for 7 d. To deplete dendritic cells (DCs), we injected CD11c-DTR and B6 control mice i.p. with 8 ng diphtheria toxin (DT; Sigma-Aldrich, Munich, Germany) 1 d before immunization. DT was injected every third day for a total of three injections according to the results from the pilot study (data not shown). To deplete macrophages in dLNs, we injected SJL/J mice s.c. into both footpads with 100 µl clodronate liposomes (Clodronateliposomes, Haarlem, the Netherlands) 5 d before immunization according to previously published protocols (38). Control SJL/J mice were injected the same amount of PBS liposomes (Clodronateliposomes). To deplete B cells, we injected SJL/J mice i.v. into tail vein with 100 mg/kg anti-CD20 mAb (kindly provided by Genentech, San Francisco, CA) 1 d before immunization. Depletions of DCs, macrophages, and B cells were confirmed by FACS analyses using Abs against CD11c-PE, F4/80-PE, and CD19-allophycocyanin, respectively (Fig. 7A, 7D, 7G).

### Detection of vWFA2-specific CD4 T cells in vitro

To analyze Ag-specific CD4 T cells, we described protocols in detail elsewhere with some modifications (B. Tiburzy et al., submitted for publication). In brief, single-cell suspensions of dLNs from SJL/J, CD11c-DTR, and SJL/J mice injected with anti-CD20 mAb or clodronate liposomes were prepared. Cells were stimulated with 100  $\mu$ g/ml vWFA2 fused to intein (GRAMG) in RPMI 1640 medium (Life Technologies, Darmstadt, Germany) in 48- or 96-well flat-bottom plates. As a negative control, 100  $\mu$ g/ml OVA or recombinant mCOL7C, which contains the GRAMG sequence of vWFA2, produced by same procedure as vWFA2 or PBS was used for restimulation. Positive control was achieved by stimulation with PMA (Sigma-Aldrich) and Ionomycin (Merck, Darmstadt, Germany). The expression of CD154, CD69, or CD25 on CD4 T cells was analyzed 5 h, 20 h or 2 d after, respectively. To detect CD154, we stimulated cells in the presence of monensin (eBiosciences).

#### Flow cytometry analysis

In T cell depletion studies, blood samples were obtained at 0, 1, 2, 4, 8, and 12 wk after immunization. A total of 50  $\mu$ l blood was taken from tail vein with 25  $\mu$ l of 20 mM EDTA. Cells were stained using CD3-FITC, CD4-FITC, CD8-PE, and CD19-allophycocyanin. Samples were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences), and the data were evaluated using Cell Quest Pro (BD Biosciences). Cells were analyzed on a gate set on lymphocyte-sized cells. Cell suspensions from dLNs were stained with CD3, CD4, CD154, CD69, and CD25 after stimulation with several different Ags to analyze Ag-specific CD4 T cells. For CD154 staining, cells were fixed before intracellular staining using Cytofix/Cytoperm (BD Biosciences). Samples were analyzed on a BD Biosciences LSRII flow cytometer, and the resulting data were evaluated using FlowJo software (Tree Star, Ashland, OR).

### Real time RT-PCR

To determine IL-4 and IFN- $\gamma$  mRNA expression in dLNs, we performed PCR in dLNs obtained from mice treated with either anti-CD4, anti-CD8, or isotype control mAbs as described earlier. Primers and PCR protocols were described in detail elsewhere (39).

#### Statistical analysis

Statistical calculations were performed using SigmaPlot (Version 12.0; Systat Software, Chicago, IL). To compare the parameters, we used the independent samples' Student *t* test. Differences in Ag-specific CD4 T cell responses and Th1/Th2 cytokine balances were calculated using one-way ANOVA test. A *p* value <0.05 was considered statistically significant.

### Results

# Almost 50% of sera from human EBA patients recognizes the vWFA2 domain

Recombinant human vWFA2 protein was separated with 12% SDS-PAGE and transferred onto nitrocellulose membrane. For epitope mapping in human EBA, 21 patients and 10 normal human sera were tested for reactivity with vWFA2 by immunoblotting. In total, 10 of 21 patients' sera reacted against human vWFA2, whereas none of the control sera showed reactivity to vWFA2. A representative blot is shown in Supplemental Fig. 2.

# Immunization with the vWFA2 domain of COL7 induces specific B and T cell responses in mice

SJL/J (n = 16), B6.s (n = 11), BALB/c (n = 16), and B6 (n = 17) mice were immunized with 60 µg vWFA2 emulsified in TiterMax. Control SJL/J (n = 4), B6.s (n = 2), BALB/c (n = 6), and B6 (n = 4) mice were immunized with PBS and TiterMax. All SJL/J, B6.s, and B6 mice, immunized with vWFA2, produced specific IgG 8 wk after immunization. However, only 11 of 15 BALB/c mice developed specific IgG (Table I). Detailed results on circulating autoantibodies, including isotypes, are summarized in Table I. IgG from SJL/J mice bound to the dermal side of 1 M NaCl-split murine skin by indirect IF microscopy (Fig. 1a) and targeted a 290-kDa protein of murine dermal extract (Fig. 1b). By indirect immunogold electron microscopy using murine skin as a substrate, serum from SJL/J mice bound to the lamina densa (Fig. 1c).

Table I. Extent of skin disease and immunopathological findings in different mouse strains immunized with vWFA2

							Serum ELISA			Tissue Direct IF			
Strain	MHC	Sex	n	Clinical Disease (%)	Onset (wk)	AUC <sup>a</sup>	IgG	IgG1	IgG2a	IgG2b	IgG	C3	IgG Transfer
SJL/J	H2s	М	8	100 (8/8)	4.3	50.5 ± 14.9	16/16	16/16	16/16	16/16	16/16	16/16	3/3
		F	8	100 (8/8)	5.5	$54.1 \pm 23.8$							
B6.s	H2s	Μ	5	100 (5/5)	4	$47.8 \pm 29.6$	11/11	11/11	8/11	10/11	11/11	11/11	3/3
		F	6	83.3 (5/6)	4.4	$37.0 \pm 33.2$							
B6	H2b	Μ	9	0 (0/9)		0	16/16	8/16	14/16	16/16	13/16	0/16	0/3
		F	7	0 (0/7)	_	0							
BALB/c	H2d	Μ	8	0 (0/8)		0	$11/15^{b}$	7/11	0/11	11/11	$3/15^{b}$	$0/15^{b}$	0/3
		F	8	0 (0/8)		0							

All mouse strains produced autoantibodies 8 wk after immunization, but only SJL/J and B6.s mice developed skin lesions 4–5 wk after immunization. Overall clinical disease severity, expressed as AUC, was not significantly different in male and female mice. IgG from diseased SJL/J or B6.s mice induced skin lesions when transferred into healthy B6 mice.

<sup>a</sup>AUC was calculated at 12 or 20 wk in B6.s or SJL/J mice, respectively.

<sup>b</sup>One BALB/c mouse died before.

Direct IF microscopy of perilesional tail skin revealed linear deposits of murine IgG at the DEJ in all SJL/J and B6.s mice (Fig. 1d). IgG deposits of SJL/J mice were detected as early as 2 wk after immunization. In case of positive IgG deposits, IgG subclasses (IgG1, IgG2a/c, IgG2b, and IgG3) were determined by direct IF microscopy. In SJL/J and B6.s mice, IgG1, IgG2a/c, and IgG2b, but not IgG3, were detected (Fig. 1e–h). Tissue-bound IgG was found in 13 of 16 B6 mice and 3 of 15 BALB/c mice. All control mice showed no IgG deposits at the DEJ (Fig. 1i). In contrast, SJL/J (n = 16) mice immunized with 60 µg vWFA2, emulsified in Alum, did not produce autoantibodies as detected by ELISA and direct IF microscopy (data not shown).

To study vWFA2-immunized mice for specific T cell responses, we first harvested dLNs 3 wk after immunization to analyze for Agspecific CD4 T cells by CD154. Stimulation of single-cell suspensions from dLNs with vWFA2 induced 1.6% CD154 cells, whereas OVA induced 0.2% (Fig. 2A, 2B; p < 0.01). Because vWFA2 protein contains the amino acids GRAMG due to cloning into the pTWIN1 vector, FNIII7-8 (mCOL7C) fused to intein was used as an additional negative control. Next, T cell activation markers of CD69 and CD25 on CD4 cells were analyzed 7 d after immunization. vWFA2 induced 11.2% CD69 cells (Fig. 2C, 2D) and 19.9% CD25 cells (Fig. 2E, 2F) in CD4 cells. Stimulation with OVA or mCOL7C showed no specific T cell responses (Fig. 2).



**FIGURE 1.** Immunization with the vWFA2 domain of COL7 induces pathogenic autoantibodies in mice. (a) IgG from diseased SJL/J mice reacted against the dermal side of 1 M NaCl-split murine skin by indirect IF microscopy. (b) IgG from diseased SJL/J mice targeted a 290-kDa protein of dermal extract of murine skin (*lane 1*, Before immunization, *lane 2*, Immunized with PBS, *lane 3*, Immunized with vWFA2, *lane 4*, Immunized with mCOL7C-GST) (18). (c) By indirect immunogold electron microscopy using murine skin as substrate, serum from diseased SJL/J mice bound to the lamina densa (arrowheads). By direct IF microscopy of diseased SJL/J mice, deposits of (d) total IgG, (e) IgG1, (f) IgG2a/c, and (g) IgG2b, but (h) not of IgG3 at the DEJ. (i) Control SJL/J mice, immunized with PBS, did not show IgG deposits at the DEJ. (j) Transfer of 1 mg of total IgG from diseased SJL/J mice induced skin lesions in healthy B6 mice (n = 3). (k) Four milligrams total IgG ( $4 \times 1$  mg) from diseased B6.s mice induced skin lesions in healthy B6 mice (n = 3). (m) In mice that developed skin lesions after IgG transfer, subepidermal blistering accompanied by inflammatory cells was observed histologically, and (n) total IgG and (o) murine C3 were deposited at the DEJ by direct IF microscopy. D, Dermis; E, epidermis. Original magnification ×400 (M), ×200 (a, d–i, n, and o). Indirect IF staining (a); direct IF staining (d–i, n, o); H&E staining (m).



**FIGURE 2.** Detection of vWFA2-specific T cells in dLNs of immunized mice. (**A**) dLN cells were harvested 3 wk after immunization and restimulated with OVA, PBS (no Ag), vWFA2, or PMA + Ionomycin, as indicated. Stimulation with vWFA2 fused to intein showed significantly higher CD154 cells than stimulation with OVA or PBS (\*p < 0.01). (**B**) Representative FACS analyses of cells stimulated under the four different conditions. (**C**) dLN cells were harvested 7 d after immunization. vWFA2 stimulation induced ~11.2% CD69 cells, whereas mCOL7C fused to intein or PBS induced 1.8% CD69 cells (\*p < 0.01). (**D**) Representative FACS analyses of cells stimulated under the four different conditions. (**E**) vWFA2 stimulation induced ~9% CD25 cells (\*p < 0.01). (**F**) Representative FACS analyses of cells stimulated under the four different conditions. Data are based on a total of five mice per group.

#### Immunization with vWFA2 induces clinical skin lesions in mice

Overall disease incidence of each mouse strain, immunized with vWFA2 emulsified in TiterMax, is summarized in Table I. In detail, all SJL/J and 10 of 11 B6.s immunized mice (91%) developed skin blistering. SJL/J mice developed first lesions on ears and/or eyelids within 6 wk after immunization (Fig. 3b). Subsequently, skin blistering spread to tail (Fig. 3c) and trunk (Fig. 3d). The average disease severity, determined by the affected body surface area, reached a plateau at 8 or 12 wk after immunization in male or female mice, respectively (Fig. 3a, left panel). In B6.s mice, disease severity increased continuously until the experiment had to be terminated for reasons of animal welfare (Fig. 3a, right panel). Similarly to SJL/J, in B6.s mice, initial lesions were observed on ears, and subsequently on eyelid, head, neck, limbs, and trunk (Fig. 3e). In both SJL/J and B6.s mice, overall disease severity expressed as AUC showed no sex difference (Table I). Control SJL/J and B6.s mice, immunized with PBS and TiterMax, as well as BALB/c and B6 mice, immunized with vWFA2 with TiterMax, did not show any skin blistering (Fig. 3f).

In a separate experiment, 14 SJL/J mice were immunized with vWFA2 and selected mice were observed until 36 wk after immunization. All mice developed skin disease 4 wk after immunization, which reached its maximum on week 8 (Supplemental Fig. 3A, black line). In these mice, serum titer of circulating autoantibodies to the DEJ increased rapidly and reached a plateau around 4 wk after immunization (Supplemental Fig. 3A, red line). Circulating anti-vWFA2 IgG was detected throughout the observation period. Skin blistering on ear and snout persisted with a similar severity for as long as 36 wk after immunization (Sup-

plemental Fig. 3B). To test for a possible epitope spreading, we performed immunoadsorption test using serum from mice 36 wk after immunization. After adsorption with vWFA2, diseased SJL/J mice serum did not bind to the DEJ by indirect IF microscopy (Supplemental Fig. 3C, 3D).

### Blisters are located beneath the lamina densa

Light microscopic analysis of skin lesions revealed extensive subepidermal blisters accompanied by mononuclear cells (Fig. 3g). Early blisters showed many neutrophils in the upper dermis by IHC using Ly-6G Ab (Fig. 3h). Transmission electron microscopy of lesional skin in SJL/J mice demonstrated split formation in the upper dermis (Fig. 3i), and the split formation was located beneath the lamina densa (Fig. 3j). Control, all BALB/c, and all B6 mice demonstrated no histological changes in the skin (data not shown). Murine complement C3 deposits at the DEJ were observed in all 16 SJL/J mice and in 9 of 11 B6.s mice (Table I); 1 diseased and 1 nondiseased B6.s mouse did not show C3 deposits.

# Transfer of IgG from diseased mice into healthy mice induces clinical skin blistering

To confirm the pathogenicity of IgG from diseased mice, total IgG from immunized mice was injected into the ears of healthy B6 mice (Table I). As early as 2 d after first injection of 1 mg total IgG from diseased SJL/J mice, erythema and erosion were observed on the skin of recipient mice (Fig. 1j). Injection of 4 mg IgG from diseased B6.s mice also induced skin blistering in B6 mice (Fig. 1k). IgG from nondiseased B6 or BALB/c mice did not induce any skin blistering in B6 mice (Fig. 1l). Histologically, subepidermal blistering was associated with neutrophil infiltrates (Fig. 1m) and

FIGURE 3. Incidence of clinical disease is associated with the H2s locus. (a) Clinical disease severity of SJL/J (left panel) and B6.s (right panel) male and female mice after single immunization with vWFA2 or PBS, both emulsified in adjuvant TiterMax. SJL/J mice immunized with vWFA2 demonstrated skin lesions on (**b**) ear, (**c**) tail, and (**d**) trunk, whereas (e) B6.s mice showed a more severe clinical phenotype. (f) No disease in mice immunized with PBS and TiterMax. In diseased SJL/J, histologically, (g) subepidermal blistering was accompanied by inflammatory cells and fibrosis in the dermis. (  $\boldsymbol{h})$  IHC using Ly6-G Ab showed many neutrophils in the upper dermis. (i) Transmission electron microscopy of lesional skin in SJL/J mice demonstrated split formation in the upper dermis, (j) and split formation was located beneath the lamina densa (arrowheads indicate lamina densa, asterisk indicates vacular change). Data are based on a total of eight male and eight female SJL/J mice for vWFA2 immunization, five male and six female B6.s mice for vWFA2 immunization, and six SJL/J mice and two B6.s mice for PBS control, respectively. Original magnification  $\times 200$  (g),  $\times 400$ (h). H&E staining (q); IHC staining using Gr-1 Ab (h).



linear murine IgG (Fig. 1n) and C3 (Fig. 1o) deposits at the DEJ by direct IF microscopy.

# CD4, but not CD8, T cells are required for autoantibody production

To investigate which T cell subset(s) are required for the early pathogenesis of EBA, we injected mice with either anti-CD4 (n = 9) or anti-CD8 (n = 10) mAbs to deplete the corresponding lymphocyte subpopulation. Depletion was maintained for the first 2 wk after immunization (Fig. 4A, 5A). Isotype-injected animals served as controls. All isotype-injected animals (n = 5 for CD4 and CD8, respectively) produced autoantibodies 2 wk after immunization (Fig. 4B, 5B). On the other hand, production of autoantibodies in CD4 T cell-depleted mice was delayed for 4 wk compared with isotype-injected mice (Fig. 4B). However, serum titer level and tissue-bound IgG were not different at the final day of the experiment (Fig. 4B, 4C). The mice injected with anti-CD8 mAb started to produce autoantibodies at similar time points as isotype-injected mice (Fig. 5B), and no differences in tissue-bound IgG were observed at the final day (Fig. 5C).

In line with these serological observations, isotype-injected animals developed skin blistering 3–5 wk after immunization (Fig. 4D, 5D). In contrast, mice injected with anti-CD4 mAb did not develop any skin blistering during follow-up of 6–9 wk after immunization (Fig. 4D). Overall disease severity scored as AUC was significantly less in CD4 T cell–depleted mice compared with isotype-injected animals (p = 0.03; Fig. 4D). Although mice injected with anti-CD4 showed significantly less disease than isotype-injected mice between 4 and 10 wk after immunization (Fig. 4E), no histological differences were observed at the final

day of the experiment (Fig. 4F). In contrast, mice injected with anti-CD8 developed disease at similar time points as control animals. Anti-CD8 treatment did not affect disease severity (Fig. 5D, 5E) and histological features at the final day (Fig. 5F).

### CD4 T cells are required for a Th1 polarization in dLNs

Because the expression of IFN- $\gamma$  and IL-4 in dLNs has recently been demonstrated to be critical for skin blistering in experimental EBA (39), we investigated IFN- $\gamma$  and IL-4 mRNA expression in mice injected with either anti-CD4, anti-CD8, or isotype control mAbs. Compared with isotype-injected animals, depletion of CD8<sup>+</sup> cells had no impact on the ratio of IFN- $\gamma$ /IL-4 in dLNs. In contrast, CD4 T cell–depleted mice showed a decrease of this ratio by 60% (p = 0.011) and by ~90% compared with anti-CD8 mAbinjected animals (p = 0.016; Fig. 6).

# *B* cells, DCs, and macrophages are required for induction of an *Ag-specific CD4 T cell response*

To investigate which APCs are required for the induction of Agspecific CD4 T cell responses in EBA, we fluorescently labeled vWFA2 and immunized mice with this protein. Subsequently, dLNs were obtained from immunized mice 3 wk after immunization, when an autoantibody production was detected in all immunized mice (data not shown), and sections were stained for B cells, DCs, and monocytes/macrophages. In dLNs of immunized mice, most of green fluorescence, correlating to vWFA2, was located in the subcapsular or cortical sinus. Colocalization of vWFA2 was also detected with CD11b<sup>+</sup>, CD11c<sup>+</sup>, or CD19<sup>+</sup> cells (Supplemental Fig. 4), indicating the presence of vWFA2 in several APCs. Next, Ag-specific CD4 T cell responses were analyzed in mice that were

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FIGURE 4. CD4 T cells are required for autoantibody production. (A) CD4 T cells were depleted (<1.55% of total lymphocytes) between 0 and 2 wk after immunization and recovered to  $\sim 40\%$  at 4 wk and to 83% at 8 wk after immunization, respectively. CD4 T cells percentages were significantly different during the entire time period of the first 8 wk after immunization as indicated by asterisks. Upper graph demonstrates average percentage of CD4 T cells; lower graph shows representative results from FACS analyses at 1 wk before immunization, week of immunization, and 4, 8, and 12 wk after immunization. (B) Levels of circulating anti-vWFA2 IgG were determined by ELISA in mice with the indicated treatments. Anti-vWFA2 IgG production in CD4 T cell-depleted mice was delayed for 4 wk. Anti-vWFA2 IgG production in CD4 T celldepleted mice was significantly lower than control mice during 2–4 wk after immunization (p =0.03 and p < 0.01 at weeks 2 and 4, respectively). (C) Direct IF microscopy of tissue-bound IgG at termination of experiment (week 12) showed no significant difference between both treatment groups. Original magnification ×200; direct IF staining. (D) Mice of the indicated treatment groups were scored every second week for presence of skin lesions, expressed as the percentage of body surface area covered by skin lesions. Disease onset in CD4 T cell-depleted mice was delayed for 4 wk (left panel), and overall disease severity, expressed as AUC, was significantly reduced (p < 0.01, right panel). (E) Representative clinical pictures at 10 wk after immunization in a CD4 T cell-depleted mouse (left panel) and an isotype-injected animal (right panel). (F) Histologically, no significant differences were observed between anti-CD4 mAb- (left panel) and isotype-injected mice (right panel) at termination of experiment (week 12). Original magnification ×200; H&E staining. Data are based on a total of nine mice for anti-CD4 mAb injections and five mice treated with isotype control.



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depleted of DCs, macrophages, or B cells (Fig. 7A, 7D, 7G). In B cell-depleted mice, an Ag-specific CD4 T cell response was completely absent and comparable with that of control cells (Fig. 7B, 7C). Depletion of DCs induced vWFA2-specific CD4 T cells, but also led to a significant reduction of the vWFA2-specific CD4 T cell response (Fig. 7E, 7F). A similar partial reduction of Agspecific CD4 T cells was observed in macrophage-depleted mice (Fig. 7H, 7I).

### Discussion

In this article, we established a novel mouse model of EBA induced by immunization with recombinant vWFA2 of murine COL7, reproducing the clinical, histological, electron microscopical, and immunopathological findings of human EBA. Detailed characterization of this model showed that it predominantly reflects the inflammatory variant of the human disease (40). Compared with the previous model of immunization-induced EBA (20), immunization with vWFA2 fused to intein leads to both autoantibody production and skin blistering independent of the GST tag. In the

previous model, disease was induced by an immunodominant protein covering epitopes from the end of the sixth to the beginning of the ninth FNIII (mCOL7C) fused with GST (20). Therefore, in this model, the immune response was directed against both mCOL7C and GST. Because neither His-mCOL7C nor mCOL7C, which contain five additional amino acids (GRAMG), induced skin blistering (B. Tiburzy et al., submitted for publication), we recently investigated the immune response in the previous model in detail. The vast majority of the T cell response was directed against GST, whereas mCOL7C-specific T cell responses were hardly detected. Collectively, these data suggested that the mCOL7C-specific plasma cell response is driven by T cells specific for GST (B. Tiburzy et al., submitted for publication). To focus on an immune response directed only to the autoantigen itself, we established the present model based on immunization with immunodominant domain of COL7 fused intein. Using timerestricted depletion of certain cell subsets, this new model was applied to investigate the cellular requirements for the early stages of EBA pathogenesis.

FIGURE 5. CD8 T cells are not required for autoantibody production. (A) CD8 T cells were depleted (<5.04% of total lymphocytes) between 0 and 2 wk after immunization and recovered to 48% at 4 wk and to 71% at 8 wk after immunization, respectively. CD8 T cells percentages were significantly different during the entire period of the first 8 wk after immunization as indicated by asterisks. Upper graph demonstrates average percentage of CD8 T cells; lower graph shows representative results from FACS analyses at 1 wk before immunization, week of immunization, and 4, 8, and 12 wk after immunization. (B) Levels of circulating antivWFA2 IgG were determined by ELISA in mice with the indicated treatments. Anti-vWFA2 IgG production was similar between both groups throughout the entire observation period. (C) Direct IF microscopy of tissue-bound IgG at termination of experiment (week 12) showed no significant difference between both treatment groups. (D) Mice of the indicated treatment groups were scored every second week for presence of skin lesions, expressed as the percentage of body surface area covered by skin lesions. The disease onset and severity were not significantly different between anti-CD8 mAb- and isotypeinjected mice during the whole observation period (left panel), and overall disease severity, expressed as AUC, was also identical (right panel). (E) Representative clinical pictures of mice immunized with vWFA2: a CD8 T cell-depleted mouse (left panel) and an isotype-injected animal (right panel) 10 wk after immunization. (F) Histologically, no significant differences were observed between anti-CD8 mAb- (left) and isotype-injected mice (right) at termination of experiment (week 12). Original magnification ×200; H&E staining. Data are based on a total of 10 mice for anti-CD8 mAb injections and 5 mice treated with isotype control.



Based on the findings presented in this article and elsewhere, we propose the following model of the early EBA pathogenesis: Both human (41, 42) and experimental EBA (22) are strongly associated with certain MHC alleles. In addition, genes outside the MHC locus have recently been shown to influence susceptibility to experimental EBA (43). Therefore, EBA manifests in the context of a certain, yet to be more defined, genetic context. Furthermore, failure to induce autoantibodies and blistering in SJL nude mice by immunization with COL7 (23) indicated that T cells were required either for the induction of the autoimmune response and/or for the maintenance of autoantibody production. To determine whether CD4 and/or CD8 T cells are required for the induction of an immune response to the vWFA2 domain, we depleted both subsets in separate experiments. In accordance with other models of autoimmune diseases (1-3), lack of CD4 T cells delayed both autoantibody production and onset of clinical disease. The results from Ag-specific T cell detection in vitro and CD4 T cell depletion in vivo suggested that vWFA2 fused to intein induced vWFA2specific CD4 T cells, which supported autoantibody production in

this model. In some autoimmune diseases, CD8 T cells were shown to modulate autoantibody production (44–46). However, in both pemphigoid and pemphigus, CD8 T cells were not required for autoantibody production after cell transfer into immunodeficient mice (14, 15). In the development of an antiviral immune response, CD8 T cells were associated with a predominant Th1 polarization (47). Such a Th1 response is also associated with skin blistering in experimental EBA (39). Our results, however, document that CD8 T cells are not required for production of Abs. These are similar to previous reports on the generation of Ab to external Ags (48).

Next, we investigated which APCs induce Ag-specific CD4 T cells. Based on cell-depletion results, induction of Ag-specific CD4T cells was completely B cell dependent in this model of immunization-induced EBA. Although B cells are well-known as professional APCs (49), we expected they might play a partial role in Ag presenting. However, our results clearly indicate that B cells are crucial for loss of tolerance in our experimental model. In addition to B cells, CD11c<sup>+</sup> cells, which are predominantly DCs,

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**FIGURE 6.** CD4 T cells are required for a Th1 polarization in dLNs. The ratio of IFN- $\gamma$ /IL-4 in dLNs was determined by RT-PCR in mice injected with either anti-CD4, anti-CD8, or isotype control mAbs. Data are based on five mice in each group. The ratio of IFN- $\gamma$ /IL-4 in CD4 T cell–depleted mice was reduced to ~60% compared with isotype-injected mice (\*\*p = 0.011) and reduced to ~90% compared with mice that received anti-CD8 mAb (\*p = 0.016).

and macrophages lead to a significant, but not complete, reduction of Ag-specific CD4 T cells, despite their incomplete reduction. Overall, this indicates that signals from several APCs are required to lead to the formation of vWFA2-reactive CD4 T cells. Previous studies showed that different APCs were required in the Agpresenting process (50–52). Particularly in the collagen-induced arthritis model in rats, similar results like in our model were observed (50). In CD4-depleted mice, autoantibody production and disease onset delayed for 4 wk. This indicates that Ag presenting may be lasting in vivo. To address this issue, we immunized mice with DyLight488-vWFA2, and dLNs were analyzed. At the time of 3 wk after immunization, some fluorescence colocalized with APCs in dLNs. This result suggested that Ag presenting remained and induced Ag-specific T cell responses even 4 wk after immunization in CD4-depleted mice.

In addition, our data provide insights into pathogenically relevant epitopes on COL7. Whereas in bullous pemphigoid the majority of sera from patients reacts with the relatively small NC16A portion of BP180 (53), autoantibodies from EBA patients bind to numerous epitopes, predominantly located within the NC1 domain (32, 54-58). Reactivity to the NC2 domain has also been noted in a few patients (57, 58). So far it has been unclear whether this broad epitope recognition is of pathogenic relevance or is due to epitope spreading to nonpathogenic epitopes. In vivo evidence for pathogenicity-relevant epitopes has been obtained by Ab transfer studies and immunization of mice. Transferring Abs directed to either the cartilage matrix protein domain (12) or multiple epitopes located within the FNIII domains (10) into mice induced skin blisters. Similarly, immunization with a protein covering epitopes from the end of the sixth to the beginning of the ninth FNIII (mCOL7C) induced skin lesions (20). In this article, we demonstrate that autoantibodies targeting the vWFA2 domain are also

FIGURE 7. Different APCs are involved in induction of vWFA2specific CD4 T cells. (A) Representative FACS analyses of depletion efficacy shows that anti-CD20 mAb treatment induced 96% reduction of CD19 cells. (B) B cell-depleted mice neither induced CD69 expression (\*p < 0.01) (**C**) nor CD25 expression (p < 0.01) on CD4 T cells after in vitro stimulation with vWFA2. Data are based on five mice in each group. (D) DT-injected CD11c-DTR mice showed a reduction of CD11c DCs by 79%. (E) In DC-depleted mice, percentage of CD69 cells was reduced to 67%. \*p < 0.01. (**F**) Regarding CD25 cells, a reduction to 43% was observed. Data are based on four mice in each group. \*p < 0.01. (**G**) Clodronate liposome injections induced a 79% depletion of F4/80 cells. (H) In clodronate liposome-injected mice, 41% of CD69 cells and (I) 31% of CD25 cells were decreased. Data are based on five mice in each group. (H) \*p < 0.01.



capable of inducing EBA, underlining that COL7 harbors multiple pathogenically relevant epitopes.

In conclusion, we established a novel mouse model of EBA induced by immunization with the vWFA2 domain of murine COL7. Autoantibodies to this domain induce skin blistering. By celldepleting studies, we provide novel insights into the early steps of EBA pathogenesis and identify APC-induced CD4 T cells as a key cellular requirement to induce autoantibodies and clinical disease.

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### Disclosures

The authors have no financial conflicts of interest.

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