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# Repetitive Immunization Breaks Tolerance to Type XVII Collagen and Leads to Bullous Pemphigoid in Mice

Misa Hirose,\* Andreas Recke,\* Tina Beckmann,\* Atsushi Shimizu,<sup>†</sup> Akira Ishiko,<sup>‡</sup> Katja Bieber,<sup>§</sup> Jürgen Westermann,<sup>§</sup> Detlef Zillikens,\* Enno Schmidt,\*<sup>¶</sup> and Ralf J. Ludwig\*

**Bullous pemphigoid (BP) is a subepidermal autoimmune blistering disease of the elderly associated with considerable morbidity and mortality. As unspecific immunosuppressants are still the mainstay of BP therapy, several animal models, based on the passive transfer of autoantibodies or immune cells, have been developed to obtain a better understanding of the pathogenesis of BP and evaluate novel therapeutic interventions. We describe in this study an experimental model inducing BP by immunization of immunocompetent mice with a recombinant form of the immunodominant 15th noncollagenous domain of murine BP180 (type XVII collagen). The homologous noncollagenous 16A domain of human BP180 has previously been identified as an immunodominant region in human BP. Immunization of female SJL/J mice with the murine peptide led to clinical disease within 14 wk in 56% of mice. In contrast, none of the other strains developed blisters despite the presence of autoantibodies. The clinical disease manifested for at least 8 wk without further manipulation. This novel immunization-induced model reflects key immunopathological characteristics of human BP, including binding of complement-fixing autoantibodies along the dermal–epidermal junction, elevated total IgE serum levels, and infiltration of skin lesions with eosinophilic granulocytes. The use of immunocompetent mice and the induction of sustained clinical disease not requiring additional interventions make this immunization-induced mouse model most suitable to further explore the pathogenesis of BP and novel therapeutic interventions for this and other autoantibody-mediated diseases. *The Journal of Immunology*, 2011, 187: 000–000.**

**B**ullous pemphigoid (BP) is a disease of the elderly characterized by tense blisters and severe pruritus (1–3). Autoantibodies in BP are directed against two hemidesmosomal proteins, BP230 and BP180 (type XVII collagen) (4–7). Although BP230 is an intracellular molecule of the hemidesmosomal plaque, BP180 is a transmembraneous protein with a long extracellular portion localizing to the lamina lucida of the dermal–epidermal junction (DEJ) (8). The majority of BP sera react with epitopes clustered within the 16th noncollagenous (NC16A) domain of BP180 (9). Evidence for the functional relevance of human anti-BP180 NC16A Abs was derived from the observations that serum levels paralleled disease activity in BP patients (10), and human BP180 NC16A-specific IgG induced subepidermal splits in cryosections of human skin (11). To obtain a better understanding of the pathogenesis and for evaluation of novel therapeutic strategies, autoantibody reactivity against

BP180 has been explored in various experimental animal models mimicking the human disease, mostly by transfer of anti-BP180 autoantibodies in neonatal rodents (12–20). However, no model has been available in which tolerance to BP180 has been lost and in which clinical disease was present in immunocompetent animals over a prolonged time period.

In this study, we describe a protocol that induces experimental BP in adult mice by immunization with a recombinant fragment of murine BP180. This immunization-induced BP model mirrors key features of the human disease, including: 1) skin lesions; 2) subepidermal blisters by histopathology; 3) skin infiltration by eosinophilic and neutrophilic granulocytes; 4) binding of IgG autoantibodies to BP180 at the DEJ by both direct and indirect immunofluorescence (IF) microscopy; and 5) complement activation at the DEJ.

## Materials and Methods

### Mice

SJL/J (H2s), BALB/c (H2d), B6.SJL-H2s (H2s), C57BL/6 (H2b), C57BL/10.Q (H2q), and DBA/1J (H2q) mice were purchased from The Jackson Laboratory. Mice were held at specific pathogen-free conditions and fed standard mouse chow and acidified drinking water ad libitum. All clinical examinations, biopsies, and bleedings were performed under anesthesia using i.p. administration of a mixture of ketamine (100 µg/g) and xylazine (15 µg/g). Animal experiments were approved by local authorities of the Animal Care and Use Committee (Kiel, Germany) and performed by certified personnel.

### Expression and purification of murine BP180 fragments

A recombinant fragment of murine BP180 (mCOL17A) was expressed as GST fusion protein (Fig. 1) using a prokaryotic expression system and purified by affinity chromatography as previously described (21). DNA sequence data from murine BP180 was retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) using accession number NC\_000085.5. The cDNA fragments were obtained by PCR on a cDNA pool generated by reverse transcription of total RNA from murine 212 keratinocytes. Restriction site for BamHI and SalI were introduced during PCR using

\*Department of Dermatology, University of Lübeck, Lübeck D-23538, Germany; <sup>†</sup>Department of Dermatology, Keio University School of Medicine, Tokyo 108-8345, Japan; <sup>‡</sup>First Department of Dermatology, School of Medicine, Faculty of Medicine, Toho University, Tokyo 143-8540, Japan; <sup>§</sup>Institute of Anatomy, University of Lübeck, Lübeck D-23538, Germany; and <sup>¶</sup>Comprehensive Center for Inflammation Medicine, University of Lübeck, Lübeck D-23538, Germany

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Address correspondence and reprint requests to Prof. Ralf J. Ludwig, Department of Dermatology, University of Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany. E-mail address: ralf.ludwig@uk-sh.de

Abbreviations used in this article: BP, bullous pemphigoid; DEJ, dermal–epidermal junction; EAE, experimental autoimmune encephalitis; EBA, epidermolysis bullosa acquisita; EM, electron microscopy; HPF, high-power field; IF, immunofluorescence; MBP, major basic protein; NC, noncollagenous; PBST, NaCl containing 0.05% Tween-20; RT, room temperature.

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forward primer 5'-GSTCGGATCCCTGGCGGAGGAGGTAAGAAAG-3' and reverse primer 5'-GATCGTCTGACTCATCTGAGATTCCCGTTCTC CTG-3'. These primers were synthesized by MWG Biotech (Ebersberg, Germany). The reaction was run in thermal cycler GeneAmp PCR System 9700 (Applied Biosystems) for 35 cycles of 30 s denaturation at 96°C, 30 s annealing at 55°C, 29 s extension at 72°C, and finally 5 min extra extension for the last cycle. BP180 cDNA fragments were cloned into linearized pGEX-6P-1 (Amersham Biosciences, Heidelberg, Germany), resulting in the recombinant vector pGEX-mCOL17A. Correct ligation and in-frame insertion of the DNA fragments were confirmed by DNA sequence analysis. Recombinant fusion proteins were expressed in *Escherichia coli* Origami (Novagen) and purified by glutathione-agarose affinity chromatography (9). Protein concentrations were measured spectrophotometrically at 280 nm (BioPhotometer) and with SDS-PAGE.

#### *Immunization protocol, clinical evaluation of mice, and sample preparation*

Mice were immunized at the hind footpad with 60 µg murine BP180 (mCOL17A fused with GST) emulsified in the nonionic block copolymer adjuvant TiterMax (Alexis Biochemicals, Lörrach, Germany). Immunization was performed at week 0. Mice were either immunized once or received additional boosts 3 and 9 wk after initial immunization. Negative controls were generated by immunization with recombinant GST and TiterMax (Alexis Biochemicals). Subsequently, mice were evaluated at 2-wk intervals for presence of skin lesions until 14 wk after first immunization. Cutaneous lesions were counted, and the extent of skin disease was scored as involvement of the skin surface as reported previously (21). At the same time intervals, biopsies were obtained from nonlesional skin for determination of IgG and C3 deposits at the DEJ, and blood was obtained for analysis of circulating Abs. At week 14, lesional biopsies were taken for histopathological and electron microscopy (EM) analysis. For passive transfer studies, 50 µl (1 mg) IgG purified from diseased SJL/J or non-diseased BALB/c mice was injected intradermally into ears of C57BL/6 mice every second day. Forty-eight hours after the fourth injection, mice were killed, and ears were prepared for histopathological and immunopathological examination. For histopathological studies, tissue was stored in 4% buffered formalin. Tissues for EM analysis were prefixed with 2% glutaraldehyde containing 0.06% Na-cocodylate buffer, 0.6% paraformaldehyde, and 0.03% CaCl<sub>2</sub> and postfix with 1% osmium tetroxide.

#### *Histopathology*

Formalin-fixed skin samples were processed into paraffin blocks. Four-micrometer sections were stained with H&E according to standard protocols (22).

#### *Immunohistochemistry*

Six-micrometer cryosections of lesional skin obtained from murine BP180 or GST-immunized SJL/J mice were fixed in acetone at -20°C for 10 min. Air-dried sections were washed with PBS for 5 min three times and incubated with Dako Dual Endogenous Enzyme Block (DakoCytomation, Hamburg, Germany) for 30 min at room temperature (RT) for blocking endogenous peroxidase. To reduce nonspecific binding of secondary Ab, sections were incubated with 5% normal rabbit serum for 20 min at RT. After excess serum from sections was blotted, sections were incubated with rat anti-mouse major basic protein (MBP) Ab (1:1000 dilution in 2% normal rabbit serum/PBS; Lee Laboratory) for 1 h at RT (23). Slides were washed with PBS followed by incubation with rabbit anti-rat IgG (1:200 dilution in 2% normal rabbit serum/PBS; DakoCytomation) for 45 min at RT. After washing, sections were incubated with R.T.U. VECTASTAIN Elite ABC Reagent (Vector Laboratories) for 30 min at RT. To develop the peroxidase reaction, sections were incubated in HistoGreen (LINARIS, Wertheim-Bettingen, Germany) solution until appropriately color developed. Excess solution was washed in tap water, followed by counterstaining with hematoxylin, and sections were mounted. Eosinophilic granulocyte infiltrate was assessed on anti-MBP-stained sections using the following score: 0, no positive cell/high-power field (HPF); 1, 1–10 positive cells/HPF; 2, 10–20 positive cells; or 3, >20 positive cells/HPF.

#### *IF microscopy*

Methods for direct IF microscopy to detect tissue-bound IgG, complement deposits, and IgG subclasses—IgG1 (A85-1), IgG2b (R12-3), IgG2c (R19-15), and IgG3 (R40-82)—have been previously described (24). Staining intensity of immunoreactants at the DEJ of mice was quantified with ImageJ software (National Institutes of Health; <http://rsbweb.nih.gov/ij/>). For indirect IF microscopy, 1 M NaCl-split mouse skin was used as

a substrate. To generate salt-split skin, normal SJL/J mouse skin was incubated in a solution containing 1 M NaCl, 1 mM PMSF, and 5 mM EDTA at 4°C for 72 h. After washing, skin was embedded, and 6-µm sections were prepared. Sections were incubated with appropriately diluted mouse serum. Binding of autoantibodies to the skin was visualized using FITC-conjugated rabbit anti-mouse IgG (DakoCytomation). For comparison, sera from female SJL/J mice immunized with a fragment of murine type VII collagen (active epidermolysis bullosa acquisita [EBA] mouse) (25) were used.

#### *Transmission and immunogold EM*

Samples for transmission EM were taken from lesional skin of murine BP180-immunized mice and corresponding body parts of negative control mice. Fixed samples were processed for EM as described (26). Immunogold EM was performed to characterize the binding site of the serum obtained from immunized mice. Postembedding immuno-EM using normal mouse skin was performed as previously described (27). In detail, normal mouse skin was cryofixed with liquid propane cooled at -190°C, freeze-substituted with methanol, and embedded in Lowicryl K11M (Chemische Werke Lowi, Waldkraiburg, Germany). Ultrathin sections were incubated with either murine BP180-immunized mouse or negative control mouse serum for 4 h at RT followed by 15 nm colloidal gold-conjugated goat anti-mouse IgG (1:40; Amersham Biosciences) for 2 h at RT. Sections were stained with saturated uranyl acetate for 6 min followed by lead citrate for 1 min. Stained sections were examined by EM (Model JEOL JEM-1230; JEOL, Tokyo, Japan).

#### *ELISA*

*Detection of circulating mCOL17A-specific IgG.* Ninety-six-well microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) were coated with murine BP180 or GST at a concentration of 5 µg/ml (250 ng/well) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Plates were washed three times with 0.05 M phosphate (pH 7.2), 150 mM NaCl containing 0.05% Tween-20 (PBST). Nonspecific binding was reduced by blocking plates with 1% BSA in PBST at RT for 90 min. Subsequently, plates were incubated with mouse sera at RT for 90 min. Serum dilutions ranged from 1:5,000 to 1:125,000 for detection of mCOL17A reactivity and 1:1,000 to 1:25,000 for detection of GST reactivity. After washing with PBST, wells were incubated with rabbit anti-mouse IgG conjugated with HRP (BD Pharmingen, Heidelberg, Germany; dilution 1:10,000 with 1% BSA containing PBST) for 1 h at RT. One-step Turbo TM-ELISA (Thermo Scientific, Rockford, IL) substrate was added for visualization of Ab binding. OD was measured by absorbance at 450 nm (Wallac 1420 VICTOR; PerkinElmer). OD values were adjusted based on those of negative control mouse sera in both murine BP180-coated and GST-coated plates.

*Detection of circulating mCOL17A-specific IgG isotype.* Coating of ELISA plates with murine BP180 or GST peptide was performed as described above. After washing plates three times with PBST, nonspecific binding was reduced by blocking plates with 1% biotin-free BSA (Roth, Karlsruhe, Germany) in PBST at RT for 90 min. Subsequently, plates were incubated with mouse sera at RT for 90 min. Dilutions of mouse sera ranged from 1:5,000 to 1:125,000 for detection of mCOL17A reactivity and 1:1,000 to 1:25,000 for detection of GST reactivity. After washing with PBST, wells were incubated with either rat anti-mouse IgG1, IgG2b, or IgG2c conjugated with biotin (BD Pharmingen; dilution 1:1,000 for IgG1 or 1:500 for IgG2b and IgG2c with 1% biotin-free BSA containing PBST) for 1 h at RT. Alkaline phosphatase-conjugated streptavidin (1:1,000; Jackson ImmunoResearch Laboratory, Suffolk, U.K.) was applied as a detection substrate and the reaction was developed by 1-step Turbo TM-ELISA (Thermo Scientific) substrate for visualization of Ab binding. OD was measured by absorbance at 450 nm (Wallac 1420 VICTOR; PerkinElmer). OD values were adjusted based on readings for control mouse serum in both murine BP180-coated and GST-coated plates.

*Detection of circulating total IgE and mCOL17A-specific IgE.* Total IgE was detected by ELISA (Mouse IgE ELISA MAX Deluxe Set; BioLegend, San Diego, CA) following the instructions provided. Levels of mCOL17A-specific IgE were measured by a slightly modified sandwich ELISA. In brief, rat anti-mouse IgE Ab (BD Pharmingen) was coated with 2 µg/ml as capture Ab. Biotinylated mCOL17A was used for detection. mCOL17A was labeled with Sulfo-NHS-LC-Biotin (Thermo Scientific) following the manufacturer's instructions. Alkaline phosphatase-conjugated streptavidin (1:1,000; Jackson ImmunoResearch Laboratory) was applied as a detection substrate, and the reaction was developed by 4-methylumbelliferylphosphatase-free acid (Calbiochem, Darmstadt, Germany) for 30 min at 37°C.

### Statistics

Sigma plot 11.0 (Systat Software, Chicago, IL) and R (<http://www.r-project.org/>) were used to perform statistical analysis. A *p* value <0.05 was considered statistically significant. Applied tests are indicated in the respective table and figure legends.

## Results

### Immunization against BP180 induces autoantibody production in mice

As SJL/J mice have been shown to be susceptible to EBA induction (24), this mouse strain was selected for our initial attempt to induce BP in mice. In a first set of experiments, we immunized 12 female SJL/J mice against murine BP180 (Fig. 1), followed by boosts after 3 and 9 wk. One mouse died 4 wk after the initial injection. Three SJL/J female mice immunized with GST alone did not develop disease (data not shown). Subsequently, six additional male SJL/J mice were immunized using the same protocol. None of the six mice developed skin lesions. In addition, 12 female SJL/J mice receiving a single immunization did not show any skin lesion (data not shown). Levels of circulating IgG against a recombinant fragment of mouse BP180 (mCOL17A) were measured by ELISA. None of the preimmune mouse sera showed IgG reactivity to mCOL17A. All immunized SJL/J female mice (11 out of 11) developed Ag-specific IgG and peaked at 4 wk after the initial immunization, though no correlation with disease severity was observed (data not shown). By indirect IF microscopy using 1 M NaCl-split mouse skin, circulating IgG from mice immunized against murine BP180 bound to the epidermal side of the split (Fig. 2A). In contrast, serum from mice immunized with murine type VII collagen bound to the dermal side (Fig. 2B). Immunogold EM using murine BP180-immunized mice sera showed binding of autoantibodies mainly to the plasma membrane of the hemidesmosomal complex of nonimmunized control mice (Fig. 2C), whereas sera from GST-immunized mouse sera showed no staining (Fig. 2D).

### Skin lesions of immunization-induced BP reflect histological characteristics of human BP

Six out of 11 female SJL/J mice immunized with murine BP180 developed skin lesions, including erythema, erosions, and crust (Fig. 3A–C) between 6 and 14 wk after the initial immunization. Clinical disease scores increased with longer observation periods, and most diseased mice reached a plateau of disease severity at

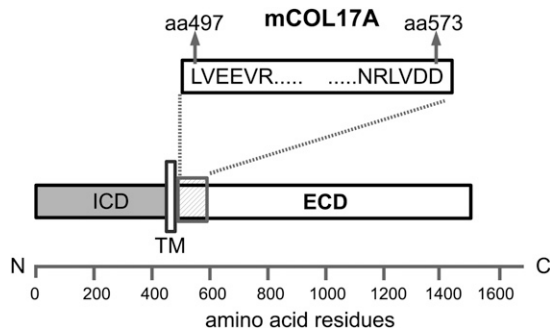


**FIGURE 1.** Schematic organization of murine BP180 constructs generated for this study for expressing recombinant peptides of the NC15 domain. BP180 is composed of an intracellular domain (ICD), a transmembrane domain (TM), and an extracellular domain (ECD). A recombinant fragment of murine BP180 (mCOL17A) cDNA was cloned into pGEX-6P-1 and expressed in *E. coli*. The N- and C-terminal amino acid sequence parts of the fragment are depicted; location of the fragment within the complete murine BP180 aa sequence (UniProt, <http://www.uniprot.org/>; UniProt ID Q07563, isoform 1) is indicated above.

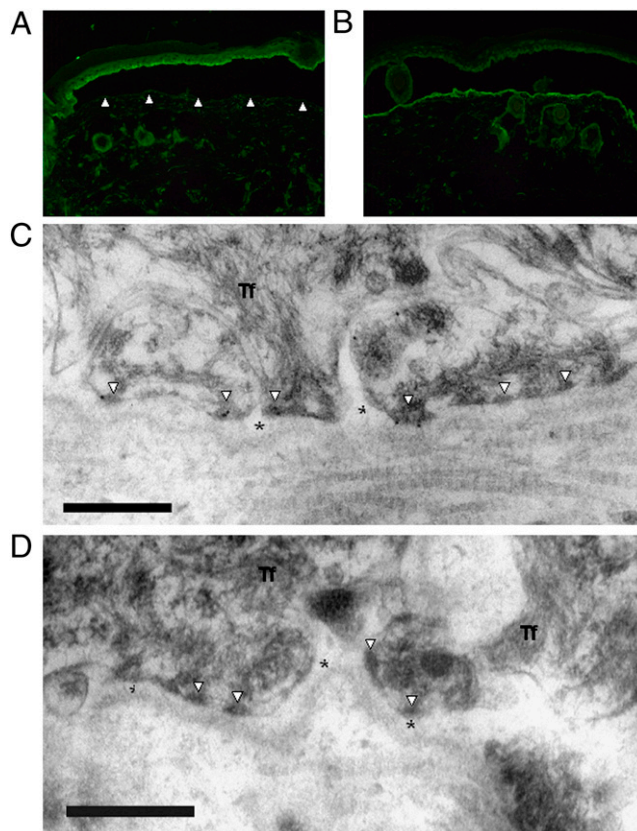


**FIGURE 2.** Immunization of female SJL/J mice against murine BP180 induces specific autoantibody production. *A*, By IF microscopy using 1 M NaCl-split mouse skin, serum obtained from immunized female SJL/J mice binds to the epidermal side of the split. White triangle ( $\Delta$ ) demonstrates the upper edge of the dermis. *B*, Sera from mice immunized with murine type VII collagen bind to the dermal side of the salt-split skin. Original magnification  $\times 400$  (*A*, *B*). *C* and *D*, Immunogold EM using serum obtained from an immunization-induced BP mouse and healthy mouse skin substrate shows the autoantibodies bound to the plasma membrane of hemidesmosome complex, indicating the presence of Ab recognizing near the transmembrane domain of BP180. In addition, there may be Abs that recognize intracellular domain of BP180 (*C*), whereas IgG from GST-immunized control does not reveal any binding (*D*). Serum dilution 1:120; secondary Ab, 15-nm gold-labeled goat anti-mouse IgG. White triangle ( $\nabla$ ) indicates hemidesmosome; asterisk (\*) marks lamina lucida. Scale bars, 500 nm. Tf, tonofilament.

week 12 (Fig. 3D). Histopathologically, in all diseased female SJL/J mice, lesional skin biopsies showed subepidermal blisters accompanied by a dense inflammatory infiltrate in the underlying dermis (Fig. 3E, Table I). Furthermore, eosinophilic granulocytes were scattered in the upper dermis of lesional skin confirmed by immunohistochemistry using anti-murine MBP Abs specific for eosinophilic granulocytes (Fig. 3F). By quantitative evaluation of immunohistochemistry, eosinophilic infiltration was significantly higher in immunization-induced BP compared with negative controls (Fig. 3G). Transmission EM showed the split localizing to the lamina lucida with the lamina densa at the blister floor (Fig. 3H) and hemidesmosomes at the blister roof (Fig. 3I).

### Disease phenotype observed in female SJL/J mice is associated with local deposits of IgG and complement C3 at the DEJ

Tissue-bound IgG was examined by direct IF microscopy. Mouse IgG deposits at the DEJ peaked at week 6 (Fig. 3J), and declined until week 14 (Fig. 3K), whereas C3 deposits at the DEJ were weaker at week 6 (Fig. 3L) compared with week 14 (Fig. 3M). At

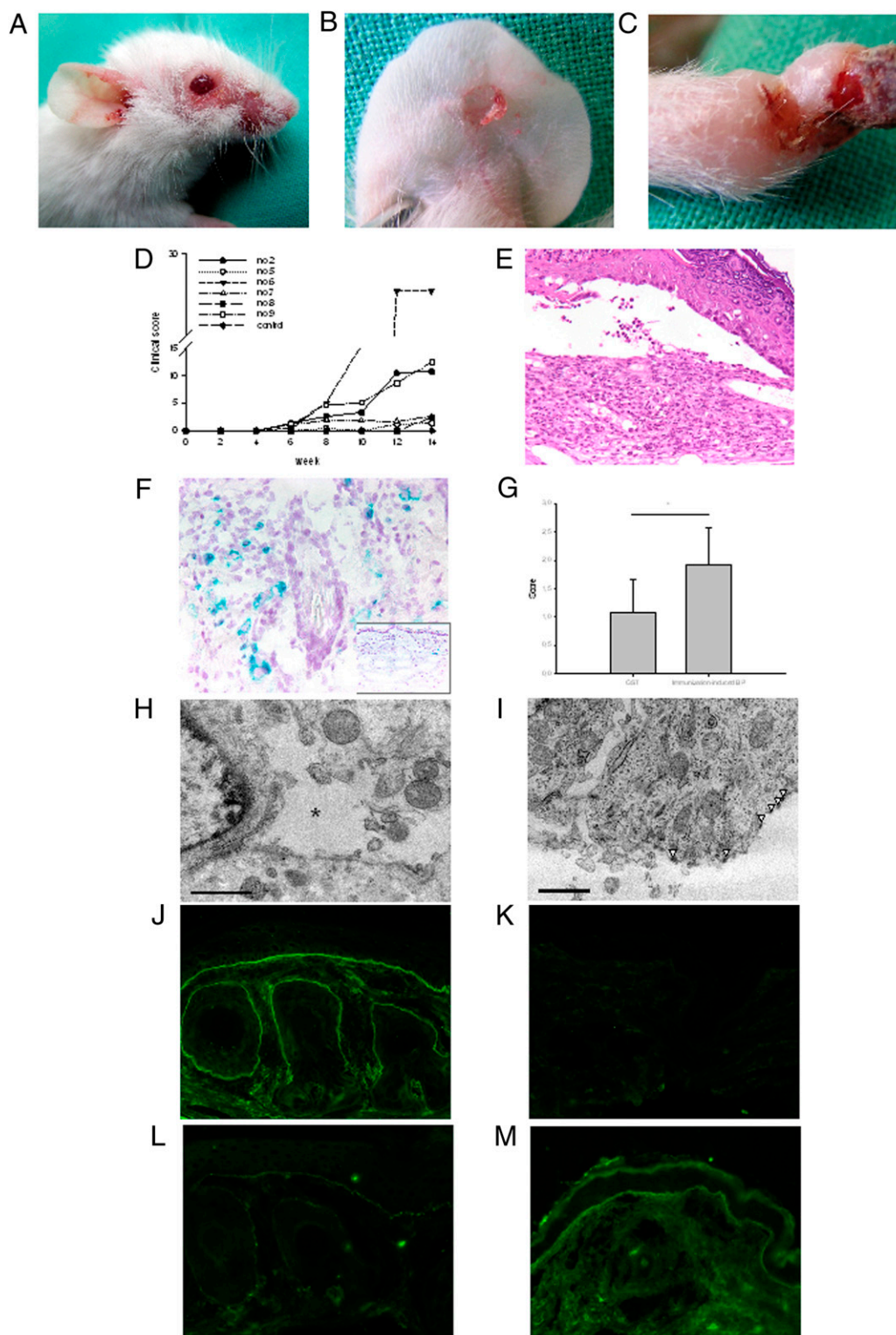


**FIGURE 3.** Lesions in immunization-induced BP duplicate findings in patients at clinical, histological, electron microscopical, and immunopathological levels. A–C, Representative clinical presentation of diseased murine BP180-immunized female SJL/J mice at week 14. Lesions are characterized by erythema, erosions, and crusts around eyes and snout (A) as well as on ear (B) and tail (C). D, Clinical disease activity expressed as a percentage of body surface area affected by skin lesions during the observation period of 14 wk in diseased immunized mice and a control mouse. E, Histological examination of lesional skin biopsies from a diseased SJL/J mouse at week 14 reveals extensive subepidermal cleavage accompanied by a dense inflammatory dermal infiltrate consisting of granulocytes and lymphohistiocytes (original magnification  $\times 400$ ). F, Immunohistochemistry staining with anti-mouse MBP Abs (original magnification  $\times 400$ ); blue color indicates eosinophilic granulocytes. *Inset* shows anti-mouse MBP staining of skin from a control mouse (original magnification  $\times 200$ ). G, Eosinophilic granulocyte infiltrate was assessed using the following score: 0, no positive cell/HPF; 1, 1–10 positive cells/HPF; 2, 11–20 positive cells/HPF; and 3,  $>20$  positive cells/HPF. H and I, Transmission EM examination of a lesional skin biopsy from a diseased mouse at week 14. Blister (\*) is formed at the lamina lucida level (H). Scale bar, 1  $\mu$ m. Note that basal lamina remains on the floor of the blister. Some hemidesmosomes (arrowheads) remain on the basal keratinocyte in blister roof (I). Scale bar, 1  $\mu$ m. Direct IF microscopy of skin biopsies from immunized mice: IgG deposits at the DEJ at weeks 6 (J) and 14 (K); C3 deposits at the DEJ at weeks 6 (L) and 14 (M). Original magnification  $\times 400$  (J–M).  $*p = 0.04$  (*t* test).

Table I. Different mouse strains show distinct susceptibility to the induction of skin lesion after immunization with murine BP180

| Strain     | MHC Haplotype | Gender | No. of Mice | Serum Autoantibodies     |                    | Tissue Deposits <sup>c</sup> |      | Skin Blisters |
|------------|---------------|--------|-------------|--------------------------|--------------------|------------------------------|------|---------------|
|            |               |        |             | Indirect IF <sup>a</sup> | ELISA <sup>b</sup> | IgG                          | C3   |               |
| SJL/J      | H2s           | Female | 11          | 11/11                    | 11/11              | 10/11                        | 7/11 | 6/11          |
| SJL/J      | H2s           | Male   | 6           | 6/6                      | 6/6                | 4/6                          | 2/6  | 0/6           |
| B6.SJL-H2s | H2s           | Female | 6           | 6/6                      | 6/6                | 6/6                          | 0/6  | 0/6           |
| B6.SJL-H2s | H2s           | Male   | 6           | 6/6                      | 6/6                | 5/6                          | 0/6  | 0/6           |
| BALB/c     | H2d           | Female | 6           | 6/6                      | 6/6                | 3/6                          | 1/6  | 0/6           |
| C57BL/6    | H2b           | Female | 6           | 6/6                      | 6/6                | 5/6                          | 0/5  | 0/6           |
| C57BL/10.Q | H2q           | Female | 6           | 6/6                      | 6/6                | 0/6                          | 0/6  | 0/6           |
| DBA/1J     | H2q           | Female | 5           | 5/5                      | 5/5                | 3/5                          | 0/5  | 0/5           |

Time points were selected according to the maximal reactivity/incidence in SJL/J mice.  
<sup>a</sup>Reactivity of IgG autoantibodies was determined by indirect IF microscopy on murine skin sections using 10-fold diluted mouse serum obtained 4 wk after immunization against BP180.  
<sup>b</sup>Serum autoantibodies to BP180 were detected by ELISA using recombinant murine Ag as described in *Materials and Methods*; serum samples were obtained 4 wk after immunization.  
<sup>c</sup>Skin biopsies obtained at week 6 were analyzed for IgG and those obtained at week 14 were analyzed for complement C3 deposits at the DEJ by direct IF microscopy.

week 6, IgG binding was observed in 10 out of 11 female SJL/J mice immunized against murine BP180, whereas complement C3 deposits to the DEJ were found in 7 out of 11 female mice and 2 out of 6 male mice at week 14 (Table I). All female SJL/J mice with clinical skin lesions showed IgG deposits at week 6 and 14, and C3 deposits at the DEJ at week 14 (six out of six), whereas

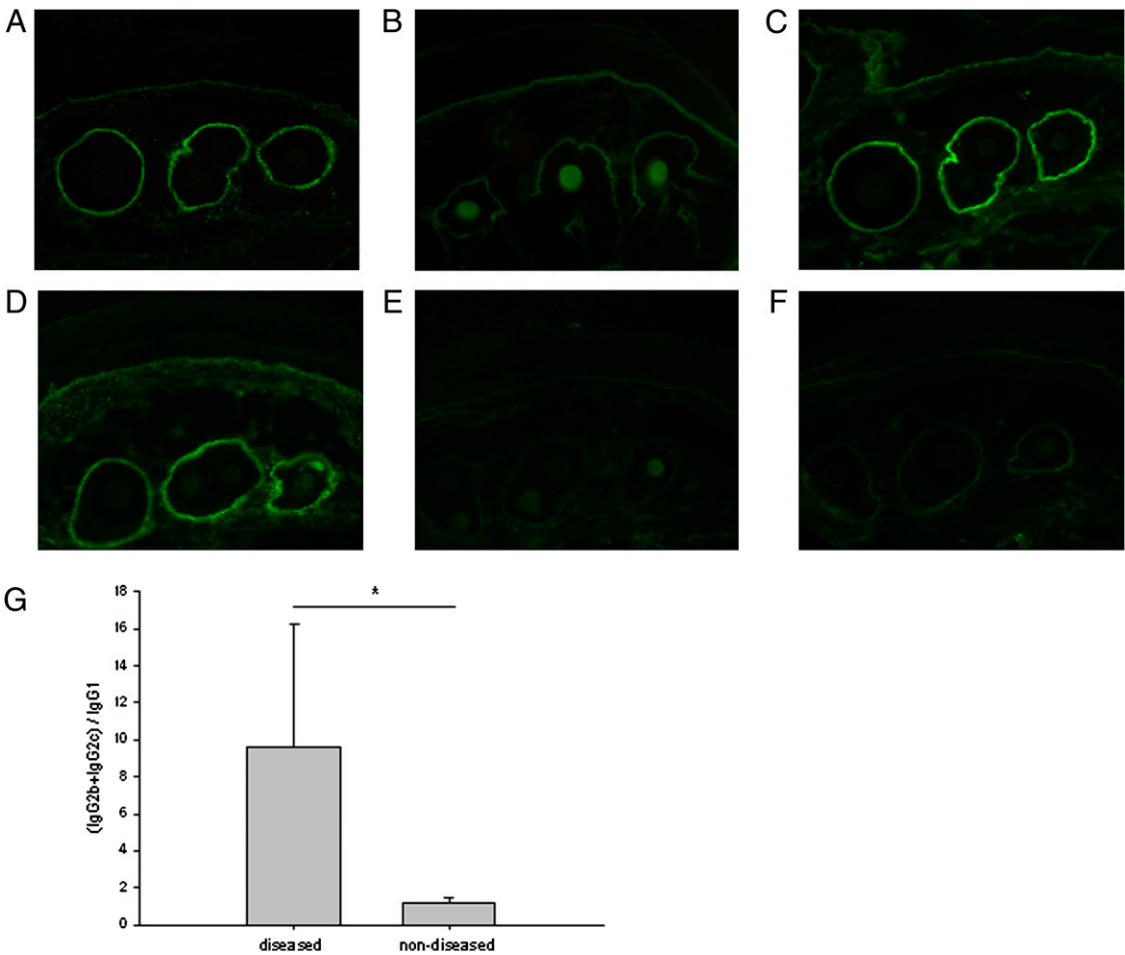


**FIGURE 4.** Clinical phenotype is associated with IgG2b, IgG2c Abs bound to the DEJ. Representative direct IF microscopy for IgG subclasses of perilesional skin biopsies from diseased ( $n = 6$ ; A–C) and nondiseased murine BP180-immunized female SJL/J mice ( $n = 5$ ; D–F) at week 6. Strong deposits of IgG1 were observed at the DEJ in diseased (A) and nondiseased (D) mice. IgG2b and IgG2c deposits were found in the diseased (B, C) and to a lesser extent also in nondiseased (E, F) mice. Original magnification  $\times 400$  (A–F). G, Extent of IgG1, IgG2b, and IgG2c staining was quantified using ImageJ (National Institutes of Health) software. The ratio of tissue-bound complement fixing and noncomplement-fixing IgG at the DEJ in diseased mice was calculated in each individual subject. This demonstrates the ratio is significantly higher in diseased compared with nondiseased mice.  $*p = 0.03$  (Mann–Whitney rank sum test).

one out of the five nondiseased mice showed C3 deposition. None of the skin biopsies from preimmune SJL/J mice demonstrated IgG or C3 deposits at the DEJ (data not shown).

*Immunization-induced BP is associated with complement-fixing Abs at the DEJ*

Based on the observed difference in C3 deposits at the DEJ between diseased ( $n = 6$ ) and nondiseased ( $n = 5$ ) murine BP180-immunized mice (Table I), IgG subclasses were further analyzed using skin biopsies of these mice. Staining of perilesional skin biopsies from diseased (Fig. 4A–C) and nondiseased mice (Fig. 4D–F) for IgG1, IgG2b, and IgG2c demonstrated linear deposits of these Ab subclasses at the DEJ by direct IF microscopy. Stainings for IgG3 were negative in both groups (data not shown). Extent of IgG1, IgG2b, and IgG2c deposits was quantified using ImageJ (National Institutes of Health). Compared to nondiseased SJL/J mice (Fig. 4D), deposits of noncomplement-fixing Abs (IgG1) (Fig. 4A) were less intensive, whereas staining for complement-fixing Abs (IgG2b and IgG2c; Fig. 4B, 4C) was increased in diseased (Fig. 4B, 4C) compared with nondiseased mice (Fig. 4E, 4F). Furthermore, the ratio of tissue-bound complement-fixing

and noncomplement-fixing Abs was calculated in each individual subject. The calculated ratio was significantly higher in diseased compared with nondiseased mice ( $p = 0.03$ ; Fig. 4G). By ELISA, IgG1, IgG2b, and IgG2c Abs to mCOL17A were detected in sera from all immunized SJL/J mice (11 out of 11), whereas levels were not significantly different between diseased and nondiseased immunized mice (data not shown). Hence, clinical disease was dependent on complement fixation at the DEJ.

*IgG from immunization-induced BP mice is pathogenic after passive transfer into healthy recipients*

To study the blister-inducing potential of autoantibodies from diseased and nondiseased mice, IgG purified from six diseased SJL/J or six nondiseased BALB/c mice was injected into the ears of healthy C57BL/6 mice. Macroscopically, erythema, erosion, and crust were observed in mice injected with IgG purified from SJL/J (Fig. 5A), but not with IgG from BALB/c mice (Fig. 5B). In line, histopathological analysis demonstrated subepidermal blisters associated with a dense leukocyte infiltration in biopsies from mice injected with IgG purified from SJL/J (Fig. 5C) but not BALB/c (Fig. 5D). In addition, direct IF microscopy of perilesional skin revealed linear deposits of C3 at the DEJ in mice injected with IgG purified from SJL/J (Fig. 5E) but not BALB/c mice (Fig. 5F). Deposition of IgG1 at the DEJ was found in mice injected with IgG from both SJL/J and BALB/c mice (Fig. 5G, 5H). However, deposits of IgG2b and IgG2a/c were only observed in mice injected with IgG purified from SJL/J (Fig. 5I) but not from BALB/c mice (Fig. 5J).

*SJL/J mice immunized against murine BP180 show elevated serum levels of total IgE*

All immunized female SJL/J mice showed increased serum levels of total IgE that peaked 4 wk after first immunization (Table II). Serum IgE levels were not significantly different between diseased and nondiseased immunized mice (Table III). In none of the sera from immunized mice was mCOL17A-specific IgE detected (data not shown). No IgE deposits were found at the DEJ of perilesional skin in immunized SJL/J mice.

*Induction of skin disease is observed only in SJL/J mice*

To evaluate a possible genetic association of experimental murine BP with a certain MHC gene, in addition to SJL/J mice, five other strains (BALB/c, B6.SJL-H2s, C57BL/6, C57BL/10.Q, and DBA/1J) were immunized against murine BP180. In none of these additional strains, even in B6.SJL-H2s, which have the same H2s haplotype as SJL/J mice, development of skin lesions was observed even though most mice developed circulating autoantibodies (Table I). By direct IF microscopy of skin biopsies obtained at week 6, binding of IgG was observed in five out of six male, six out of six female B6.SJL-H2s, C57BL/6 (five out of six), and BALB/c mice (three out of six), whereas none was detected in C57BL/10.Q mice (Table I). Deposits of C3 at the DEJ at week 14 were found in only one of six BALB/c mice, whereas female/male B6.SJL-H2s, C57BL/6, C57BL/10.Q, and DBA/1J demonstrated no C3 deposits (Table I).

## Discussion

We established in this study an immunization protocol leading to the loss of tolerance to BP180 and a subepidermal blistering disease mimicking human BP. Transmission EM revealed split formation within the lamina lucida. Inflammatory cell infiltration was dominated by neutrophilic granulocytes. Eosinophilic granulocytes were also present, and the number of infiltrating eosinophilic granulocytes in diseased mice was increased compared with mice

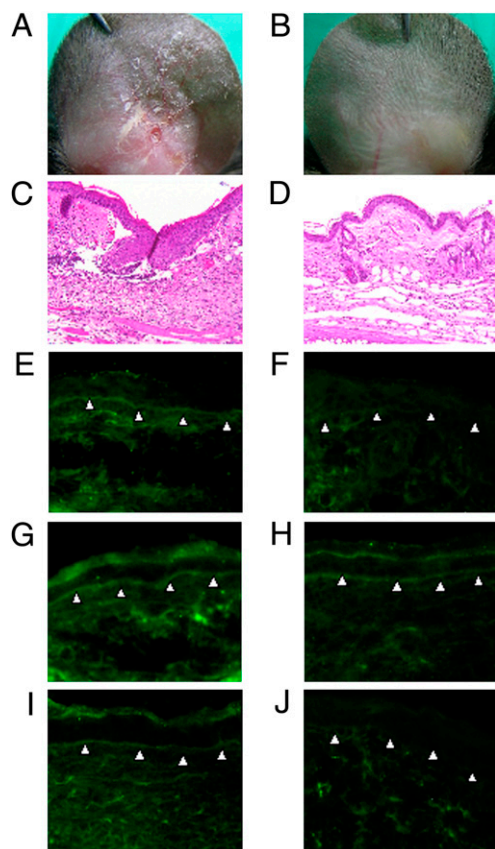


**FIGURE 5.** IgG purified from diseased mice induces dermal-epidermal separation when passively transferred into mice. A and B, A total of 50  $\mu$ l (1 mg) IgG purified from six diseased SJL/J (A) or nondiseased BALB/c (B) mice was injected intradermally into the ears of C57BL/6 mice every other day ( $n = 4$ ). After four injections, histopathological analysis shows subepidermal blisters in mice injected with IgG purified from SJL/J (C), but not from BALB/c (D) mice. IF microscopy of perilesional skin reveals linear deposits of C3 at the DEJ in mice injected with IgG purified from SJL/J (E), but not BALB/c (F) mice. Deposition of IgG1 was observed in mice injected with IgG from both SJL/J (G) and BALB/c (H) mice, whereas deposits of IgG2b were found only in mice injected with IgG purified from SJL/J (I), but not from BALB/c (J) mice. White triangle ( $\triangle$ ) indicates the DEJ. Original magnification  $\times 200$  (C, D),  $\times 400$  (E–J).

Table II. Elevated total IgE serum levels in murine BP180-immunized female SJL/J mice

|                                  | Week         |              |                |               |               |               |               |
|----------------------------------|--------------|--------------|----------------|---------------|---------------|---------------|---------------|
|                                  | 0            | 2            | 4              | 8             | 10            | 12            | 14            |
| Total serum IgE (ng/ml) $\pm$ SD | 12 $\pm$ 3.2 | 13 $\pm$ 5.1 | 21 $\pm$ 13.4* | 35 $\pm$ 66.5 | 30 $\pm$ 37.2 | 19 $\pm$ 12.6 | 21 $\pm$ 17.5 |

Total serum IgE levels were detected by ELISA.

\* $p = 0.036$  (paired  $t$  test; compared with levels at week 0).

immunized with GST only. These observations closely resemble the histopathological findings in human BP (28). Of note, eosinophilic infiltration in lesional skin, a hallmark of human BP, is not present in other IgG-mediated experimental models of BP (12–15, 18–20). In contrast, eosinophilic infiltration has been observed in IgE-mediated mouse models of BP (16, 17). More specifically, the model developed by Fairley et al. (17) showed presence of eosinophils in human skin transplanted onto the back of immunodeficient nu/nu mice after injection of IgE derived from BP patients into the graft. Zone et al. (16) demonstrated eosinophilic infiltration in lesional skin after intradermal injection of IgE anti-human BP180-producing hybridoma cells into normal human skin grafted onto immunodeficient C.B-17/lcr-SCID mice. Both IgE-mediated models lacked IgG and C3 deposits at the DEJ.

In our model, IgG/C3 deposits were present at the DEJ, and circulating mCOL17A-specific IgG was observed in all immunized SJL/J mice. In contrast, none of other strains mice, with the exception of one BALB/c mouse, demonstrated C3 deposition at the DEJ. Time-course studies showed that IgG deposits decreased over time, whereas C3 deposition was strongest at week 14 (Fig. 3). As demonstrated by immunogold EM, autoreactive Abs bound to both intracellular and extracellular parts of the hemidesmosomal complex, where BP180 is located. Because immunization was performed with mCOL17A, the murine homolog to the NC16A domain of human BP180, reactivity against intracellular hemidesmosomal portions may be due to intramolecular epitope spreading, a phenomenon well recognized in human BP (29, 30). Correlating disease manifestation with IgG subclasses, we found that skin lesions were associated with tissue-bound complement-fixing IgG subclasses (Fig. 4). This corresponds to findings in immunization-induced EBA (22, 24) and with the detection of complement deposits at the DEJ in BP patients (31).

Another observation frequently observed in human BP (32), which was reflected in our experimental model, was increased serum levels of total IgE. SJL/J mice produce less IgE compared with other strains, including BALB/c mice (33, 34). Hence, we expected low serum levels of total IgE in immunized SJL/J mice, but in fact, increased levels of total IgE were observed. In contrast to the IgE-dependent BP models (16, 17), we did not observe tissue-bound IgE at the DEJ or circulating BP180-specific IgE. This is in line with the low frequency of IgE deposits at the DEJ in BP patients (35, 36).

After immunization, induction of skin lesions was restricted to SJL/J mice carrying the H2s haplotype even though mouse strains other than SJL/J developed circulating anti-mCOL17A Abs, and some of these mice demonstrated tissue-bound IgG (Table I). This observation parallels the finding of an MHC association observed in Japanese BP patients (37) and immunization-induced EBA mice (22). However, as B6-SJL.H2s mice also carrying H2s did not develop BP, it is likely that genes outside of the MHC locus contribute to disease susceptibility, which has also been noted in humans (e.g., association of BP with low-affinity Fc $\gamma$ RIIIa gene in white patients) (38, 39). However, these changes may be also due to specific alterations of the SJL/J mouse strain. More specifically, in addition to its higher incidence of Hodgkin's disease, SJL/J mice are commonly used for induction of experimental autoimmune encephalitis (EAE), supporting the immunological uniqueness of this strain. In SJL/J mice, incidence of immunization-induced EAE is significantly higher in female mice. This observation is paralleled by elevated levels of circulating CD4<sup>+</sup> T cells in female, as compared with male SJL/J mice (40). Interestingly, castration of SJL/J male mice leads to an increase in EAE severity. In contrast, castration of C57BL/6 males showed no change in disease severity (41). Another study demonstrated androgen treatment reduced EAE disease severity in female SJL/J mice (42). Taken together, even though disease induction was exclusively observed in mice with the H2s haplotype, interpretation of a possible genetic association in immunization-induced BP has to be carefully considered because the SJL/J mouse strain demonstrates unique features as described above.

The majority of previous animal models of BP used the passive transfer of BP180-specific IgG into neonatal mice (12, 14, 18, 19). In these models, rabbit or human IgG was administered that may influence the inflammatory cascade, leading to blister formation. In addition, in these passive transfer models, the loss of tolerance to BP180 cannot be studied. Recently, an elaborate murine BP model has been reported by Ujiie et al. (20), in which disease is induced by transferring splenocytes of C57BL/6Ncr mice, which were transplanted with humanized BP180 mouse skin, into immunodeficient Rag2 knockout mice expressing human BP180. Recipient mice developed BP lesions clinically; similar to human BP. In this experimental model, loss of tolerance is induced by contact of a foreign (human) Ag, which may not be a true autoantigen. Furthermore, recipient mice are immunodeficient, which may influence the full exploration of loss of tolerance and evaluation of novel therapeutics and may also affect maintenance of the autoimmune response.

We describe in this study an immunization-induced mouse model of BP that mirrors major aspects of the human disease: 1) erosions and subepidermal blistering; 2) in vivo binding of complement-fixing IgG to the DEJ; 3) infiltration of eosinophilic and neutrophilic granulocytes into the skin; and 4) detection of circulating complement-fixing IgG directed to BP180. This novel experimental model will be helpful to further dissect the pathogenesis of BP, including mechanisms leading to loss of tolerance,

Table III. AUC of serum total IgE levels (quantified by ELISA) in diseased and nondiseased female SJL/J mice immunized with murine BP180

| Clinical Status              | AUC $\pm$ SD  |
|------------------------------|---------------|
| Diseased mice ( $n = 6$ )    | 381 $\pm$ 376 |
| No diseased mice ( $n = 5$ ) | 235 $\pm$ 193 |

AUC, area under the curve.

and will facilitate the development of novel treatment options for this and other Ab-mediated autoimmune diseases.

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## Disclosures

The authors have no financial conflicts of interest.

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