A Novel Active Mouse Model for Bullous Pemphigoid Targeting Humanized Pathogenic Antigen

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A Novel Active Mouse Model for Bullous Pemphigoid Targeting Humanized Pathogenic Antigen

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Bullous pemphigoid (BP), the most common autoimmune blistering disease, is caused by autoantibodies against type XVII collagen (COL17). To establish an active stable BP animal model that demonstrates the persistent inflammatory skin lesions initiated by the anti-human COL17 Abs, we used COL17-humanized (COL17\(^{-/-}\),h\(^{+}\)) mice that we recently produced. First, we generated immunodeficient Rag-2\(^{-/-}\)/COL17–humanized mice by crossing Rag-2\(^{-/-}\) mice with COL17-humanized mice. Then, splenocytes from wild-type mice that had been immunized by grafting of human COL17-transgenic mouse skin were transferred into Rag-2\(^{-/-}\)/COL17–humanized mice. The recipient mice continuously produced anti-human COL17 IgG Abs in vivo and developed blisters and erosions corresponding to clinical, histological, and immunopathological features of BP, although eosinophil infiltration, one of the characteristic histological findings observed in BP patients, was not detected in the recipients. Although the depletion of CD8\(^{+}\) T cells from the immunized splenocytes was found to produce no effects in the recipients, the depletion of CD4\(^{+}\) T cells as well as CD45R\(^{+}\) B cells was found to inhibit the production of anti-human COL17 IgG Abs in the recipients, resulting in no apparent clinical phenotype. Furthermore, we demonstrated that cyclosporin A significantly suppressed the production of anti-human COL17 IgG Abs and prevented the development of the BP phenotype in the treated recipients. Although this model in an immunodeficient mouse does not exactly reproduce the induction mechanism of BP in human patients, this unique experimental system targeting humanized pathogenic Ag allows us to investigate ongoing autoimmune responses to human molecules in experimental animal models. The Journal of Immunology, 2010, 184: 2166–2174.

To investigate the pathogenic mechanisms of autoimmune diseases, the development of animal models is essential (1, 2). However, interspecies molecular differences in autoantigens make it difficult to develop autoimmune animal disease models in some cases. We recently overcame this issue by using the unique technique of humanization of autoantigens to generate an animal model for bullous pemphigoid (BP) (1).

BP is the most common autoimmune blistering disorder that is induced by autoantibodies against type XVII collagen (COL17, also called BP180 or BPAG2), a hemidesmosomal type II transmembrane protein that spans the lamina lucida and projects into the lamina densa of the epidermal basement membrane zone (3–7). The noncollagenous 16A domain located at the membrane-proximal region of COL17 is known as the major pathogenic epitope for BP (8, 9). Our group recently generated COL17-humanized mice (COL17\(^{-/-}\),h\(^{+}\)) that lack mouse COL17 but express human COL17 (hCOL17) (1). Autoantibodies from BP patients fail to recognize mouse COL17 due to differences in the amino acid sequence between human and mouse. In contrast, BP autoantibodies react to hCOL17 molecules expressed in COL17-humanized mice and induce BP-like skin lesions in the neonates. Thus, this passive-transfer BP mouse model directly demonstrated the pathogenicity of human BP autoantibodies (1). Our system makes it possible to investigate immune reactions mediated by Abs specific to human molecules even in animal models.

However, passive-transfer animal models demonstrate only transient disease activity. In this study, we have developed an active, stable BP model to further advance our knowledge of the BP pathogenic mechanisms for the dynamic process of developing chronic inflammatory skin lesions observed in BP patients. To develop such a model, we adoptively transferred the splenocytes immunized with hCOL17 into immunodeficient COL17-humanized recipients (10). This active, stable autoimmune disease model targeting humanized pathogenic Ag enables us to investigate ongoing autoimmune responses to human molecules in experimental animals.

Materials and Methods

Mice

C57BL/6J mice were purchased from Japan Clea (Hamamatsu, Japan). C57BL/6-background Rag-2\(^{-/-}\) mice were received as a gift from the Central Institute for Experimental Animals (Kawasaki, Japan). We crossed COL17\(^{-/-}\),h\(^{+}\) (COL17-humanized) mice that we had recently generated (1) with Rag-2\(^{-/-}\) mice. Mice that carried the heterozygous null mutations of both the Rag-2 and mouse COL17 genes and the transgene of human COL17 (Rag-2\(^{-/-}\)/COL17\(^{+/+}\),h\(^{+}\)) were bred to produce Rag-2\(^{-/-}\)/COL17\(^{-/-}\),h\(^{+}\) (Rag-2\(^{-/-}\)/COL17-humanized) mice. All of the animal procedures were conducted according to guidelines provided by the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.
Immunization of the mice by hCOL17-transgenic skin grafting

Immunization of the mice by hCOL17-transgenic (Tg) skin graft was performed according to the method reported by Olasz et al. (11). Briefly, full-thickness 1-cm² pieces of dorsal skin were removed from sacrificed hCOL17-Tg mice (COL17transg/+h-12) and grafted onto the backs of gender-matched 6-wk-old C57BL/6 wild-type (WT) mice. After topical application of antibiotic ointment, the grafted site was covered with gauze and an elastic bandage for 14 d. In selected experiments, WT mouse skin was grafted onto the backs of WT mice. Ab production was confirmed at 5 wk after skin grafting by indirect immunofluorescence (IF) analysis, as described below.

IF analysis

Indirect IF using mouse sera was performed on the skin samples from human, COL17-humanized mice, or WT mice using standard protocols (1). In selected experiments, indirect IF was performed on 1 M NaCl-split normal human skin. We used FITC-conjugated Abs against mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA), mouse IgG1, IgG2a, IgG2b, and IgG3 (BD Pharmingen, San Diego, CA), and mouse IgG2c (Bethyl Laboratories, Montgomery, TX) as the secondary Abs.

ELISA

To determine the Ab titer against hCOL17 noncollagenous 16A domain (hNC16A) in the serum samples from the experimental mice, 96-well microtiter plates coated with recombinant hNC16A protein purchased from Medical & Biological Laboratories (Nagoya, Japan) were incubated with diluted mouse sera for 1 h at room temperature. After being washed, bound Abs were developed with a 40,000-fold–diluted, HRP-labeled Ab specific to mouse IgG (Jackson Immunoresearch Laboratories), and the OD was read at 450 nm using an ELISA plate reader (Mithras; Berthold Technologies, Bad Wildbad, Germany). The ELISA index value was defined by the following formula: index = (OD450 of tested serum – OD450 of negative control)/OD450 of positive control – OD450 of negative control) × 100 (10).

Immunoblotting

Immunoblotting was performed as described previously (1). Recombinant proteins were subjected to SDS-PAGE and electrotransferred onto nitrocellulose membrane (Trans-Blot; Bio-Rad, Hercules, CA). The membranes were blocked and incubated at room temperature for 1 h with diluted sera obtained from experimental mice. After being washed, the membranes were incubated with alkaline phosphatase-conjugated anti-mouse IgG (Zymed Laboratories, South San Francisco, CA). The bound Abs were detected with FITC-conjugated Abs specific to mouse C3 (Cappel; Valeant Medical & Biological Laboratories, CA) and observed with an automated freeze substitution system (Leica Microsystems; Cambridge, U.K.).

Complement fixation study

Complement activation induced by Abs obtained from the immunized WT mice against the COL17 in human skin samples was investigated by IF microscopy as previously described, with minor modifications (12). Cryosections of human skin were incubated with IgG (10 µg/ml) from immunized WT mice for 1 h at 37˚C. The membranes were treated with 0.02% Tween 20 as a complement source. One hour after incubation, in situ detection of mouse complement C3 was detected with FITC-conjugated Abs specific to mouse C3 (Cappel; Valeant Pharmaceuticals, Costa Mesa, CA).

Adoptive transfer of splenocytes

Splenocytes were isolated and pooled from several immunized WT mice at 35 d after the skin grafting and administered to Rag-2-/-/COL17–humanized mice or Rag-2-/- mice by i.v. injection into the tail vein with 2.0 × 10⁸ splenocytes in 500 µl PBS per mouse (10).

ELISPOT assay

ELISPOT assay was performed as previously described (10, 13) with some minor modifications. Polyvinylidene difluoride-bottomed 96-well multiwell-screen plates (Millipore, Bedford, MA) were coated with 30 µg/ml recombiant hNC16A protein. In some experiments, 30 µg/ml recombinant mouse noncollagenous 14A domain protein was coated as negative controls. Mononuclear cells isolated from the spleen, bone marrow, and lymph nodes of the Rag-2-/-/COL17–humanized recipients were incubated on the plate at 37˚C in a 5% CO₂ incubator for 4 h. IgG bound to the membrane was visualized as spots with alkaline phosphatase-conjugated anti-mouse IgG Abs. The number of spots was counted under a dissecting microscope (SMZ1500; Nikon, Tokyo, Japan), and the frequency of anti-hNC16A IgG-producing B cells was defined as the number of spots in 10⁵ mononuclear cells.

Evaluation of recipient mice

Weekly, the recipient mice were examined for their general condition and cutaneous lesions (i.e., erythema, hair loss, blisters, erosions, and crusts). Extent of skin disease was scored as follows: 0, no lesions; 1, lesions on <10% of the skin surface; 2, lesions on 10–20% of the skin surface; 3, lesions on 20–40% of the skin surface; 4, lesions on 40–60% of the skin surface; 5, lesions on >60% of the skin surface, as previously described (14) with minor modifications. Serum samples were also obtained from recipient mice weekly and assayed by indirect IF microscopy and hNC16A ELISA. Biopsies of lesional and perilesional skin were obtained between 2 and 5 wk after adoptive transfer for light microscopy (H&E), for toluidine blue staining to evaluate mast cell infiltration and degranulation, and for direct IF using FITC-conjugated Abs against mouse IgG, IgG1, IgG2a, IgG2b, IgG2c, IgG3, and C3.

Immunoelectron microscopy

Postembedding immunoelectron microscopy of cryofixed and cryosubstituted skin samples taken from the Rag-2-/-/COL17–humanized mice at 5 wk after the adoptive transfer of immunized splenocytes was performed as previously described (15, 16) with minor modifications. Small pieces of fresh skin were cryofixed by plunging them into liquid propane at −190˚C using a freeze–plunge apparatus (Leica Microsystems, Cambridge, U.K.). Skin samples were then cryosubstituted with methanol at −80˚C using an automated freeze substitution system (Leica Microsystems) and embedded in Lowicryl K11M (Ladd Research Industries, Williston, VT) at −60˚C. Ultrathin sections were incubated with 5-nm gold-labeled goat anti-mouse IgG (Biocell Laboratories, Rancho Dominguez, CA) and observed with a transmission electron microscope (H-7100; Hitachi High-Technologies, Tokyo, Japan).

Preparation of IgG fractions from mice and passive-transfer studies

Sera were obtained from Rag-2-/-/COL17–humanized mice at 8 d after the adoptive transfer of immunized splenocytes. Total IgG was prepared from the sera by affinity chromatography using a HiTrap Protein G HP (GE Healthcare Biosciences, Uppsala, Sweden). We performed passive transfer of IgG into mice as previously described (1). A 60-µl dose of sterile IgG in PBS was administered to neonatal COL17–humanized mice by i.p. injection (0.1, 0.5, or 1.0 mg/g body weight). As a control, we prepared the total IgG fractions from WT mice and i.p. injected them into neonatal COL17–humanized mice (1.0 mg/g body weight). We judged skin phenotype at 48 h after the injection. The animals were then sacrificed, and skin sections were taken for histological examination.

Depletion of CD4⁺ or CD8⁺ T cells or CD45R⁺ B cells from immunized splenocytes

For adoptive transfer of immunized splenocytes without CD4⁺ or CD8⁺ T cells or CD45R⁺ B cells, we depleted each fraction from splenocytes of the immunized WT mice by using microbeads conjugated to monoclonal anti-mouse CD4 (L3T4), anti-mouse CD8a (Ly-2), or anti-mouse CD45R (B220) Abs (Miltenyi Biotec, Auburn, CA). The depletions of CD4⁺ or CD8⁺ T cells or CD45R⁺ B cells were confirmed by flow cytometric analysis on a FACS Aria (BD Pharmingen) as described below. Approximately 1.0 to 2.0 × 10⁶ splenocytes depleted with CD4⁺ or CD8⁺ T cells or CD45R⁺ B cells were adoptively transferred to Rag-2-/-/COL17–humanized mice.

Administration of cyclosporin A to the BP model mice

Cyclosporin A (CsA) (Novartis Pharma, Basel, Switzerland) dissolved in olive oil was given i.p. at the dose of 35 mg/kg (100 µl) from 2 d after the adoptive transfer and continued daily for 14 d. The dose was chosen based on a previous study (17) and our preliminary data. As a control, the same volume of olive oil was injected into the BP model mice. The treated BP model mice were observed for 5 wk to evaluate the efficacy of the treatments.

Flow cytometry

The following mAbs were purchased from BD Pharmingen: 145-2C11–FITC (anti-CD3ε), H129.19–FITC (anti-CD4), 53-6.7–PE (anti-CD8), and RA3-6B2–PE (anti-CD45R/B220). One million cells were stained and subjected to analysis using a FACS Aria.

Statistical analysis

To compare ELISA index values of Abs, the weights of mice, and the numbers of splenocytes, Student t tests were applied. We determined the statistical differences between groups of indirect IF titer and disease severity using the
Mann-Whitney U test or ANOVA with the Scheffe F test. Data were expressed as mean ± SE. We considered p values of <0.05 as significant.

Results

High titer of anti-hCOL17 IgG is induced in WT mice by hCOL17-Tg skin graft immunization

To induce anti-hCOL17 IgG Abs, WT mice were immunized by skin grafting from hCOL17-Tg mice, which express hCOL17 in the epidermis under the control of the human keratin 14 promoter (1, 11, 18). As reported by Olasz et al. (11), a high titer of IgG Abs specific to hNC16A was produced within 5 wk after the skin grafting. Levels of IgG Abs specific to hNC16A were measured by ELISA. The ELISA index values of sera from WT mice immunized by hCOL17-Tg skin grafting showed significantly higher reactivity than those values of sera from control WT skin-grafted WT mice (74.9 ± 13.5 versus 1.5 ± 1.0, p < 0.01) (Fig. 1A). Immune serum was analyzed by indirect IF, which revealed the deposition of IgG Abs at the dermal–epidermal junction (DEJ) of COL17-humanized mouse skin (Fig. 1B). There was no reactivity against WT mouse skin (Fig. 1C). We prepared the IgG fractions of sera from WT mice immunized by hCOL17-Tg skin grafting by affinity chromatography and determined the complement-fixing activity of purified IgG by indirect IF analysis. We found that 10 µg purified IgG could fix mouse C3 in freshly prepared mouse serum to the DEJ of normal human skin, whereas mouse C3 without purified IgG could not bind to the DEJ (Fig. 1D, 1E). The IgG subclass of anti-hCOL17 Abs present in the serum of each WT mouse immunized by hCOL17-Tg skin grafting was assessed by indirect IF analysis (n = 10). The deposition of IgG1, IgG2a, IgG2b, IgG2c, or IgG3 at the DEJ of normal human skin was observed in 100, 20, 10, 70, or 0% of the analyzed sera, respectively. Thus, immunized WT mice produce high titers of IgG1 and IgG2c anti-hCOL17 Abs that have complement-fixing activity.

Splenocytes transferred from the immunized mice produce a high titer of pathogenic anti-hCOL17 IgG Abs in the Rag-2−/−/COL17-humanized recipients

To develop an active disease model for BP targeting humanized hNC16A and the DEJ of COL17-humanized mouse skin, we generated immunodeficient COL17-humanized (Rag-2−/−/COL17−−/−) mice. First, we crossed COL17-humanized (COL17−−/−/−) mice with immunodeficient Rag-2−/− mice to generate Rag-2−/−/COL17−−/− mice. Next, those Rag-2−/−/COL17−−/− mice were crossed with each other, and the genotypes of the offspring were carefully screened. After four to five repeated crossings, we finally obtained the Rag-2−/−/COL17−−/− mice. As a next step, splenocytes from some of the immunized WT mice were pooled after isolation and then adoptively transferred into the Rag-2−/−/COL17−−/− mice that expressed hCOL17 protein in vivo (n = 10). Because these Rag-2−/−/COL17−−/− mice had no mature T or B cells, they were able to accept the transferred splenocytes. All of the Rag-2−/−/COL17−−/− mice that received immunized splenocytes produced IgG Abs against hCOL17 (Fig. 2C, 2D, Table I). Indirect IF examination revealed that IgG Abs produced in the recipients bound to the DEJ of normal human skin and COL17-humanized mouse skin but not to WT mouse skin. IF analysis using 1 M NaCl-split normal human skin as a substrate showed linear deposition of IgG on the epidermal side (Fig. 2A). Immunoblot analysis revealed that the recipients’ sera reacted with both recombinant hCOL17 and hNC16A (Fig. 2B). Time-course analysis revealed that anti-DEJ IgG, which reflects the presence of anti-hCOL17 IgG, became detectable in recipients’ sera within 1 wk after the transfer and that the titer peaked around day 9 after the transfer. Although the titer gradually decreased after the peak, it remained high (>5120×) for >10 wk without boosting (Fig. 2C). ELISA analysis revealed that anti-hNC16A IgG Abs appeared in the recipients’ sera as early as 1 wk after the transfer. The Abs level rapidly increased, peaking around day 9 after the transfer. Although the titer gradually decreased, falling to a stable level at 6 wk after the transfer, anti-hNC16A IgG Abs were detectable for >10 wk without boosting (Fig. 2D). These results demonstrate that splenocytes from immunized WT mice can survive in the Rag-2−/−/COL17−−/− mice and produce a high titer of anti-hCOL17 Abs containing IgG against hNC16A. ELISPOT assay revealed that anti-hNC16A IgG-producing B cells in the Rag-2−/−/COL17−−/− mice existed mainly in the spleen and lymph nodes but not in bone marrow at days 9 and 10. Although the number of anti-hNC16A IgG-producing B cells decreased at day 52, it still remained detectable (Table II).

As a control, immunized splenocytes were also transferred into Rag-2−/− mice (n = 6). Interestingly, neither anti-DEJ nor anti-hNC16A IgG Abs were detected in control Rag-2−/− recipients (Fig. 2C, 2D, Table I). To exclude the possibility that transferred splenocytes cannot survive in the Rag-2−/− recipients, we grafted hCOL17-Tg skin onto the Rag-2−/− recipients 5 wk after the adoptive transfer of immunized splenocytes (n = 3). A high titer of anti-hCOL17 IgG Abs was detected within 14 d after the skin grafting in the Rag-2−/− recipients (data not shown). These results demonstrate that splenocytes transferred from the immunized WT mice can produce a high titer of anti-hCOL17 IgG Abs in the Rag-2−/−/COL17−−/− mice but not in the Rag-2−/− recipients. Rag-2−/−/COL17−−/− mice given immunized splenocytes develop the BP phenotype

The phenotypes observed in the recipient mice are summarized in Table I. Around day 7 after the adoptive transfer, the Rag-2−/−/COL17−−/− recipients began to scratch their snouts, muzzles, ears, and chests. Patchy hair loss associated with erythema began to develop on the chest between 9 and 14 d after the transfer, which is accompanied by a high titer of anti-hCOL17 IgG Abs detected by ELISA. The phenotype in the recipient mice was similar to that previously reported (11).
adoptive transfer in most of the recipients. Then, blisters and erosions spontaneously developed in the dehaired areas on the trunk (Fig. 3A). Genital erosions and ears swelling with crusts were also observed (Fig. 3B, 3C). The dehaired patches gradually enlarged and spread to other regions on the trunk over the next 2–4 wk, resulting in large areas of alopecia (Fig. 3D, 3E). The epidermis on the trunk and tail easily detached from the dermis by gentle friction (Fig. 3F, 3G). Disease severity, scored by the percentage of skin surface with the BP phenotype (14), plateaued 5 wk after the transfer (Fig. 4). Conversely, none of the control Rag-2^{-/-} recipients developed any skin lesions (Fig. 4). Also, splenocytes from untreated WT mice produced very low levels of anti-hCOL17 and anti-hNC16A IgG Abs and failed to induce any phenotypic changes in the Rag-2^{-/-}/COL17–humanized recipients (data not shown). In addition, 3 out of 10 Rag-2^{-/-}/COL17–humanized recipients showed changes on 20% of the skin surface (Table I). In one of those three recipients, the index value of the hNC16A ELISA at day 9 was far lower than the average (85.3 versus 907.8), whereas the anti-hCOL17 IgG titer at day 9 checked by indirect IF analysis was similar to the average. The other two recipients with changes of 20% of the surface showed no obvious difference in the IgG titers compared with the average. Long-term follow-up of

Table I. Summary of phenotypes observed in recipient mice

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>n</th>
<th>Serum Abs</th>
<th>Subepidermal Separation in H&amp;E Staining</th>
<th>Mast Cell Degranulation in Toluidine Blue Staining</th>
<th>Skin Immunopathology</th>
<th>Skin Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 immunized by Tg^ skin grafting</td>
<td>Rag-2^{-/-}/COL17^-/-h^*</td>
<td>10</td>
<td>10/10</td>
<td>10/10</td>
<td>8/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>C57BL/6 immunized by Tg^ skin grafting</td>
<td>Rag-2^{-/-}/COL17^-/-h^*</td>
<td>6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

^A Linear deposition of IgG at the DEJ of the skin was detected by indirect IF microscopy on normal human skin cryosections using 40-fold–diluted recipient mouse serum obtained 2 wk after the adoptive transfer of splenocytes.

^B Circulating IgG was tested with ELISA against recombinant hNC16A protein using 300-fold–diluted mouse serum obtained 2 wk after the adoptive transfer. The cutoff index value was set at 20.

^C In vivo IgG and complement C3 deposition at the DEJ of the skin was determined by direct IF of perilesional skin biopsies. Medium or intense staining was regarded as positive.

^D Skin changes including erythema, hair loss, bullae, and erosions exceeding 20% of the skin surface were considered significant.

^E Tg: hCOL17-transgenic. The recipients in this table are those shown in Figs. 2C, 2D, and 4.
Immunoelectron microscopy demonstrates that mouse IgG Abs deposit at the DEJ close to the plasma membranes of basal cells in the skin of the lesional skin biopsy reveals linear deposition of mouse IgG (arrows) (Fig. 3J, original magnification ×400). Histologic examination of diseased mice reveals separation between dermis and epidermis with mild inflammatory cell infiltration in H&E staining (original magnification ×200). Mast cell degranulation in the dermis is observed in toluidine blue staining (Fig. 5D, original magnification ×200). Direct IF examination revealed linear deposition of mouse IgG and C3 at the DEJ (Fig. 5D, 5E). In contrast, the recipient mice that received 1.0 mg/g body weight of IgG purified from WT mice showed no skin detachment, nor any histologic or immunopathologic changes (n = 5) (Fig. 5F–J). These findings show that IgG Abs purified from the Rag-2−/−/COL17–humanized recipients are capable of inducing subepidermal blistering in COL17–humanized neonatal mice, which is associated with the binding of IgG Abs to the DEJ, followed by in situ activation of mouse complement and mast cell degranulation.

CD4+ T cells as well as CD45R+ B cells are essential for the stable production of anti-hCOL17 IgG Abs in the Rag-2−/−/COL17–humanized recipients

To determine the pathogenic roles of T cells and B cells in the Rag-2−/−/COL17–humanized recipients, we depleted CD4+ or CD8+ T cells or CD45R+ B cells from splenocytes of the immunized WT

#### Table II. ELISPOT assay of the anti-hNC16A IgG-producing B cells

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Daya</th>
<th>Spleen</th>
<th>Lymph Node</th>
<th>Bone Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>#488</td>
<td>9</td>
<td>135 ± 16.2</td>
<td>43.5 ± 4.0</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>#109</td>
<td>10</td>
<td>70.5 ± 5.4</td>
<td>84.0 ± 10.5</td>
<td>3.8 ± 0.6</td>
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<td>20</td>
<td>17.0 ± 2.7</td>
<td>10.5 ± 2.3</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>

*Rag-2−/−/COL17m−/−* mice were transferred with splenocytes of the immunized WT mice. The number of the anti-hNC16A IgG-producing B cells is displayed per 10⁶ cells in spleen, lymph nodes, and bone marrow.

### Notes

- Number of days after the transfer of immunized splenocytes.

BP model mice demonstrated a trend in which the disease severity started to decrease around 12 wk after the transfer; however, there was variation among the individual mice.

Histopathologic analysis of the lesional skin demonstrated the dermal–epidermal separation associated with mild inflammatory cell infiltration (Fig. 3H). Mast cell degranulation was observed in the dermis in toluidine blue staining (Fig. 3J). In the control Rag-2−/− recipients, no significant histopathologic changes were detected (Table I). Direct IF analysis of perilesional skin biopsies revealed linear deposition of IgG (Fig. 3J) and C3 (Fig. 3K) at the DEJ in all of the Rag-2−/−/COL17–humanized recipients, whereas no IgG deposition was detected in the control Rag-2−/− recipients (Table I). Time-course analysis of the in situ deposition of IgG Abs in the Rag-2−/−/COL17–humanized recipients (n = 3) by direct IF at days 4, 9, 14, and 21 demonstrated intense deposition of anti-hCOL17 IgG Abs at the DEJ as early as day 9 after the adoptive transfer, and the same levels of deposition were observed at days 14 and 21.

The subclasses of IgG produced in the Rag-2−/−/COL17–humanized recipients were also analyzed by direct IF (n = 10). All of the Rag-2−/−/COL17–humanized recipients showed a positive reaction with IgG1, IgG2a, IgG2b, and IgG2c Abs at the DEJ.
mice and adoptively transferred them into the \( \text{Rag}^{-2-/-} / \text{COL17}-\)humanized mice. All of the recipient mice given immunized splenocytes after the depletion of CD8\(^+\) T cells produced a high titer of anti-hCOL17 IgG Abs and developed severe BP lesions associated with histopathologic and immunopathologic changes indistinguishable from those of the \( \text{Rag}^{-2-/-} / \text{COL17}-\)humanized mice given whole immunized splenocytes (\( n = 4 \)) (Fig. 6). In contrast, the depletion of CD4\(^+\) T cells or CD45R\(^+\) B cells inhibited the production of anti-hCOL17 IgG Abs and the development of the BP phenotype (\( n = 4 \), respectively) (Fig. 6). These findings indicate that CD4\(^+\), but not CD8\(^+\), T cells and CD45R\(^+\) B cells are crucial for the production of anti-hCOL17 IgG Abs and for the development of the BP phenotype.

To further investigate the pathogenic role of CD4\(^+\) T cells in the \( \text{Rag}^{-2-/-} / \text{COL17}-\)humanized recipients, we examined the efficacy of CsA (19–22). Approximately 35 mg/kg of CsA dissolved in olive oil (\( n = 5 \)) or a control vehicle (\( n = 5 \)) was i.p. injected into the \( \text{Rag}^{-2-/-} / \text{COL17}-\)humanized recipients from 2 d after the adoptive transfer of whole immunized splenocytes, once daily for 14 d. When the numbers of splenocytes at day 9 after the transfer were compared, the mean number of splenocytes in both groups was not significantly different (8.3 \( \times 10^7 \) cells in the CsA-treated mice versus 11.0 \( \times 10^7 \) cells in the control mice, \( p > 0.05 \)). Although the mean percentage of CD3\(^+\) T cells was significantly lower in the CsA-treated mice than that in the control mice (14.1\% in the CsA-treated mice versus 24.5\% in the control mice, \( p < 0.05 \)), the mean percentages of CD45R\(^+\) B cells were similar in both groups (28.8\% in the CsA-treated mice versus 26.5\% in the control mice, \( p > 0.05 \)). Disease severity and the titers of circulating anti-hNC16A IgG Abs were significantly lower in the treated mice than those in the controls (Fig. 7). This result further suggests that CD4\(^+\) T cells play a pivotal role in the pathogenesis of this BP model.

**Discussion**

This is the first active BP model that stably produces pathogenic anti-hCOL17 Abs and spontaneously develops blisters and erosions on the skin. Because amino acid sequences of COL17, especially those of the noncollagenous 16A domain region, are different between human and mouse, an animal model using COL17-humanized mice that express hCOL17 is suitable for analyzing pathogenic mechanisms of human BP. Therefore, we developed an active BP model in which the targeted pathogenic Ag is hCOL17 but not mouse COL17. Immunized splenocytes transferred into immunodeficient \( \text{Rag}^{-2-/-} / \text{COL17}-\)humanized recipients survived and continuously produced a high titer of anti-hCOL17 Abs in vivo for >10 wk after the adoptive transfer. Those Abs bound to the hCOL17 molecules that were expressed in the recipients’ skin, which initiated subsequent immune reactions including complement activation and mast cell degranulation, resulting in dermal–epidermal separation. This array of immune responses was consistent with the pathogenic mechanisms of BP previously demonstrated in passive-transfer neonatal mouse models (1, 12, 23–25). Furthermore, the \( \text{Rag}^{-2-/-} / \text{COL17}-\)humanized recipients developed blisters and erosions on erythematous skin areas that lasted >10 wk. The pathogenicity of anti-hCOL17 IgG Abs was confirmed by passive-transfer experiments that revealed that IgG Abs obtained from the \( \text{Rag}^{-2-/-} / \text{COL17}-\)humanized recipients could induce the BP phenotype in COL17-humanized neonatal mice. Thus, pathogenic anti-hCOL17 IgG Abs produced in the \( \text{Rag}^{-2-/-} / \text{COL17}-\)humanized recipient binds to the target Ag in vivo and induces the BP phenotype. By using COL17-humanized mice, we can observe the dynamic immune reactions induced by pathogenic Abs against hCOL17 molecules. These strategies for the production of active autoimmune disease models targeting humanized pathogenic Ag can also be applied to other autoimmune diseases.

In BP, complement activation is considered to be critical for blister formation (26). The first evidence suggesting the pathogenic role of complements in BP is the demonstration of C3

**FIGURE 4.** \( \text{Rag}^{-2-/-} / \text{COL17}-\)humanized mice given immunized splenocytes develop the BP disease phenotype. Disease severities of the \( \text{Rag}^{-2-/-} / \text{COL17}-\)humanized recipients gradually increase, plateauing 5 wk after the transfer. None of the control \( \text{Rag}^{-2-/-} \) recipients develop any skin lesions (\( n = 10 \), controls, \( n = 6 \)). Disease severity is scored as described in Materials and Methods.

**FIGURE 5.** COL17-humanized neonatal mice injected with IgG purified from the \( \text{Rag}^{-2-/-} / \text{COL17}-\)humanized recipients at 8 d after the adoptive transfer show skin fragility and histologic and immunopathologic changes similar to BP. A. The recipient mice develop epidermal detachment by gentle friction at 48 h after injection of the 1.0 mg/g IgG purified from the \( \text{Rag}^{-2-/-} / \text{COL17}-\)humanized recipients (\( n = 5 \)). B. Histologic examination reveals subepidermal separation associated with mild inflammatory cell infiltrates in H&E staining (original magnification ×200). C. Mast cell degranulation in the dermis (arrow heads) is observed in toluidine blue staining (original magnification ×400). D and E. Direct IF studies show linear deposition of mouse IgG (arrows) (D) and C3 (arrows) (E) at the DEJ (original magnification ×200). F–J, No phenotypic or histological findings are observed in the mice injected with 1.0 mg/g IgG purified from sera of WT mice grafted with WT skin (\( n = 5 \)).
deposition at the basement membrane zone of the lesional and perilesional skin by direct IF (27). By means of the passive-transfer experiments using C5-deficient mice, Liu et al. (25) further showed that complement activation is a pivotal step in subepidermal blister formation triggered by rabbit anti-mouse COL17 IgG Abs in their BP animal model. Consistent with these previous studies, linear deposition of complement C3 was observed at the DEJ in all of the diseased Rag-2\(^{-/-}\)/COL17–humanized recipients. We also demonstrated that sera from both the immunized WT mice and the Rag-2\(^{-/-}\)/COL17–humanized recipients contained complement-fixing Abs of the IgG2 subclass and could fix complements at the DEJ of the normal human skin and the COL17-humanized skin. Analysis of the subclass distribution of IgG autoantibodies in human BP revealed that complement-fixing IgG1 was present as the predominant subclass of autoantibodies (28). These findings suggest that complement activation mediated by Abs of the IgG2 subclass against hCOL17 may induce blister formation in the present BP model.

It is unclear why the anti-hCOL17 IgG titer decreases in a short period. To examine the possible compartmentalization of anti-hCOL17 IgG response to the skin, we checked in situ deposition of anti-hCOL17 IgG in the skin of BP model mice by direct IF analysis sequentially at days 4, 9, 14, and 21. Intense deposition of anti-hCOL17 IgG Abs was detected at the DEJ as early as day 9 of the adoptive transfer, and the same levels of deposition were observed at days 14 and 21 (\(n=3\)). This indicates that the compartmentalization of the anti-hCOL17 IgG response to the skin is not the main reason for the spontaneous reduction of the anti-hCOL17 IgG titer in this BP model. Alternatively, some regulatory mechanism against hCOL17-specific T cells, B cells, or both may be induced in this BP model. In experimental autoimmune myasthenia gravis, an autoimmune neuromuscular disease model induced by anti-acetylcholine receptor Abs, regulatory T cells (Tregs) generated ex vivo or expanded in vivo suppress pathogenic T cell and Ab responses (29, 30). In experimental autoimmune encephalomyelitis, a myelin-reactive T cell-dependent multiple

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** The production of anti-hCOL17 IgG Abs requires CD4\(^+\) T cells and CD45R\(^+\) B cells but not CD8\(^+\) T cells. A. All of the recipients given CD8\(^+\) T cell-depleted splenocytes (\(n=4\)) develop severe BP lesions similar to those of the recipients given whole splenocytes, whereas the recipients given CD4\(^+\) T cell-depleted splenocytes (\(n=4\)) or CD45R\(^+\) B cell-depleted splenocytes (\(n=4\)) demonstrate no erosive lesions. B. The depletions of CD4\(^+\) T cells or CD45R\(^+\) B cells significantly inhibit the production of anti-hNC16A IgG Abs (\(p<0.01\) at day 9). C. The recipients given CD4\(^+\) T cell-depleted or CD45R\(^+\) B cell-depleted splenocytes show significantly lower disease severities than those in other groups (\(p<0.05\) at days 14 and 35).

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** Results of CsA treatment in the Rag-2\(^{-/-}\)/COL17–humanized recipients. Approximately 35 mg/kg CsA was administered daily from 2 d after the adoptive transfer for 14 d (CsA, \(n=5\); control vehicle, \(n=5\)). A. Skin lesions of the Rag-2\(^{-/-}\)/COL17–humanized recipients treated with CsA are markedly diminished with CsA treatment (day 28). B. CsA significantly suppresses the production of anti-hNC16A IgG (\(p<0.01\) at days 8, 15, and 21). C. The treated mice show significantly lower disease severity than that of the controls (\(p<0.01\) at days 8, 15, 21, 28, and 35).
sclerosis model, natural resolution correlates with the accumulation of myelin-reactive Tregs expanded during the course of experimental autoimmune encephalomyelitis in the inflamed CNS (31, 32). Similar to these autoimmune disease models, Tregs may contribute to the spontaneous decline of the anti-hCOL17 IgG titer in this BP model. Further studies examining Treg function in this BP model may provide clues for controlling the autoimmune reaction in BP patients.

Interestingly, none of the control Rag-2−/− recipients given im- munized splenocytes produced anti-hCOL17 IgG Abs or developed the BP phenotype despite the presence of living splenocytes in vivo. We further demonstrated that the grafting of hCOL17-Tg skin onto Rag-2−/− mice 5 wk after the adoptive transfer of immunized splenocytes could induce a high titer of anti-hCOL17 IgG Abs. These results indicate that transferred splenocytes need endogenous hCOL17 molecules to produce anti-hCOL17 IgG Abs. In addition, the depletion of CD4+ T cells from the immunized WT splenocytes suppressed the production of anti-hCOL17 IgG Abs, whereas the depletion of CD8+ T cells showed no effects. This clearly suggests that CD4+ T cells, and not CD8+ T cells, are essential for the production of Abs against hCOL17 in this BP model.

Generally, the production of Abs by B cells requires the help of CD4+ T cells. In experimental autoimmune myasthenia gravis, both MHC class II gene-disrupted mice and CD4 gene knockout mice have been proven to be resistant to induction of clinical experimental autoimmune myasthenia gravis (33, 34). In experimental pemphigus vulgaris, an autoimmune blistering disease caused by anti-desmoglein 3 Abs, the production of autoantibodies required both CD4+ T cells and B cells from naïve desmoglein 3 knockout mice (35). To further investigate the pathogenic role of CD4+ T cells, we administered CsA, an immunosuppressant that inhibits T cell function, to the Rag-2−/−/COL17−humanized recipients after the adoptive transfer of immunized splenocytes. Because active disease models possess more persistent disease activity than passive-transfer neonatal disease models (10, 36, 37), we can easily analyze the time-course changes of disease activity altered by such an intervention. CsA significantly suppressed the production of anti-hNC16A IgG Abs and diminished the disease severity. These results strongly suggest that CD4+ T cells play a pivotal role in the production of the autoantibodies through the presentation of the endogenous autoantigen. In human BP, the presence of autoreactive CD4+ T cells has been reported, indicating the contribution of CD4+ T cells to the pathogenesis of human BP (38–40). In addition, particular MHC class II alleles are more frequent in BP patients (41). These results further indicate that the autoreactive CD4+ T cells may be activated through an interaction with the specific MHC class II molecule in BP. The pathogenic function of CD4+ T cells shown in this BP model may provide a new insight into the pathogenic mechanism of BP and the development of a novel therapeutic strategy that targets T cell-mediated immune reactions.

Although this BP model is a useful tool for investigating the pathophysiology of BP, limitations are still present in our experimental system. First, the induction phase of the autoimmune response, such as the breakdown of self-tolerance, cannot be investigated in this BP model because the immune response to hCOL17 is induced by adoptive transfer of immunized WT splenocytes. To investigate the induction of autoimmunity in BP, Xu et al. (42) have aimed to induce autoimmune responses to mouse COL17 by using the immunocompetent BALB/c mice. Multiple immunizations of BALB/c mice with peptides of the hNC16A domain, its mouse equivalent, or both successfully induced anti-mouse COL17 IgG Abs, although no overt skin changes were observed. Similar experiments have been performed to establish an animal model for epidermolysis bullosa acquisita, a subepidermal blistering disorder induced by Abs against type VII collagen, another hemidesmosomal protein present at the basement membrane zone (43). Anti-type VII collagen Abs and subepidermal blisters were successfully induced in the mice by repeated immunizations with recombinant mouse type VII collagen protein mixed with adjuvant, although the development of the disease phenotype depended on the strain of mice. These results indicate that repeated exposure of the self Ag in conjunction with inflammatory stimulation, such as by bacterial components, may break down peripheral tolerance and induce autoantibody production in patients with a specific genetic background. This concept is further supported by the clinical findings that BP develops preferentially in elderly people and that particular HLA class II alleles correlate with BP patients (41). Second, this BP model demonstrates immune responses against a humanized Ag of the skin; however, the response still occurs in a murine milieu. The lack of eosinophilic infiltration, a characteristic trait of human BP, in this model could be related to the difference of the effector cell function between human and mouse immune systems. Furthermore, because the MHCs in mice are different from those in humans, MHC-dependent presentation of the pathogenic Ag to the T and B cells cannot be simulated in this current BP mouse model. To overcome these issues, not only the pathogenic Ag but also the immune system should be humanized in experimental animals. Recently, quasi-human immune systems have been stably reconstituted in supra-immunodeficient NOG mice using human CD34+ stem cells from various sources including bone marrow, umbilical cord blood, and peripheral blood (44, 45). This system has become a common tool for studying human immunity and diseases relating to it (46, 47). However, even in that system the development of human B cells was partially blocked, and the human T cells lost their function in the periphery (48). Further technical advances would be required to establish more accurate and reliable humanized animal models that could be used toward better understanding human diseases that involve autoimmunity.

In summary, using immunodeficient COL17-humanized mice, we have successfully developed a novel active disease model for BP that continuously produces pathogenic anti-hCOL17 IgG Abs and reproduces the BP phenotype. This study indicates that a humanized animal model is quite valuable not only for analyzing biological function of human molecules but also for investigating pathogenic mechanisms of autoimmune diseases against human proteins. This new BP model can be used for the investigation of underlying mechanisms in the development and progression of BP. Furthermore, it should facilitate the development of novel therapeutic strategies for BP.

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Disclosures
The authors have no financial conflicts of interest.

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