

Humanization of autoantigen

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Transmissibility of characteristic lesions to experimental animals may help us understand the pathomechanism of human autoimmune disease. Here we show that human autoimmune disease can be reproduced using genetically engineered model mice. Bullous pemphigoid (BP) is the most common serious autoimmune blistering skin disease, with a considerable body of indirect evidence indicating that the underlying autoantigen is collagen XVII (COL17). Passive transfer of human BP autoantibodies into mice does not induce skin lesions, probably because of differences between humans and mice in the amino acid sequence of the COL17 pathogenic epitope. We injected human BP autoantibody into *Col17*-knockout mice rescued by the human ortholog. This resulted in BP-like skin lesions and a human disease phenotype. Humanization of autoantigens is a new approach to the study of human autoimmune diseases.

Evolution of a complex immune system in mammals can paradoxically lead to an increased likelihood of autoimmune disease, which affects approximately 5% of the general population¹. The immune system comprises two evolutionarily different responses: innate immunity and adaptive immunity. Vertebrates are capable of both adaptive and innate immunity, which is common to all metazoans, and are consequently exposed to autoimmune diseases—in which an aberrant, adaptive immune system recognizes a self-component as an autoantigen.

To understand the pathogenesis of these diseases and to develop new therapies, animal models corresponding to human autoimmune diseases are essential^{2,3}. A small number of experimental animal models of autoimmune diseases have been generated by the passive transfer of autoantibodies from individuals^{4,5}, but this approach has not been universally successful because of limited interspecies recognition of the autoantigen by the adaptive immune system. In reality, most animal models that reflect human autoimmune diseases have been identified from spontaneously arising diseases or have been generated by repeated immunization using host candidate autoantigens^{2,3}.

Although these animal models may develop certain aspects of human autoimmune diseases, there is as yet no reliable method to produce models that identically and faithfully reproduce human autoimmune disorders. Recently, transgenic mice expressing disease-associated human *HLA* alleles and T-cell receptors have been generated, which have the potential to provide further insight into the

pathogenesis of many diseases^{2,3,6,7}. Conversely, few advances have been made in humanizing autoantigens in animals.

Our study therefore focused on this approach in BP, the most common antibody-mediated autoimmune blistering skin disease. Although the sera of individuals with bullous pemphigoid (BP) are known to contain autoantibodies to the collagen XVII (COL17) autoantigen, the pathogenicity of COL17 autoantibodies has not been unequivocally proven as previous passive transfer experiments have failed^{8,9}. In the native COL17 epidermal protein, the pathogenic epitope is restricted to the noncollagenous 16th-A (NC16A) domain¹⁰, which shows distinct diversity among different species^{8,9}. Here, to assess the direct pathogenicity of human BP autoantibodies in a mouse model, we humanized the mouse BP autoantigen. Furthermore, we used this new mouse to demonstrate that COL17 decoy peptides block the pathogenic activity of BP-IgG *in vivo*.

RESULTS

Generation of COL17-humanized (*COL17^{m-h+}*) mice

We first generated *Col17*-knockout (*COL17^{m-/-}*) mice, in which the phenotypic features closely resembled the human disease non-Herlitz epidermolysis bullosa (OMIM: 226650) caused by null mutations in the *COL17A1* gene (Fig. 1 and refs. 11–13). When *COL17^{m-/-}* mice were born, blisters and erosions at sites of trauma were easily created by applying mild friction, and some pups exhibited spontaneous blister formation on their paws (Fig. 1c). Other characteristic findings seen in adult *COL17^{m-/-}* mice were genital erosions, hemorrhagic blisters around the digits, and diffuse, nonpigmented hair growth associated with hair loss (Fig. 1d,e). *COL17^{m-/-}* mice showed growth retardation compared with wild-type littermates, and most *COL17^{m-/-}* mice died within 2 weeks of birth. Mortality rates of the *COL17^{m-/-}* and wild-type littermates at 8 weeks were 80.1% and 4.0%, respectively (Supplementary Fig. 1 online). *COL17^{m-/-}* mice skin showed subepidermal blistering (Fig. 1f) associated with a lack of collagen XVII immunostaining (Fig. 1g), and ultrastructurally showed small and poorly formed hemidesmosomes (Fig. 1h) lacking prominent inner (IP) and outer (OP) plaques and keratin filament insertion compared to controls.

Next, we rescued *COL17^{m-/-}* mice by mating them with C57BL/6Ncr mice expressing human COL17 under the control of a human keratin 14 promoter (*COL17^{h+}*) (data not shown). After crossing heterozygote *COL17^{m-/+}* mice and human COL17 transgenic

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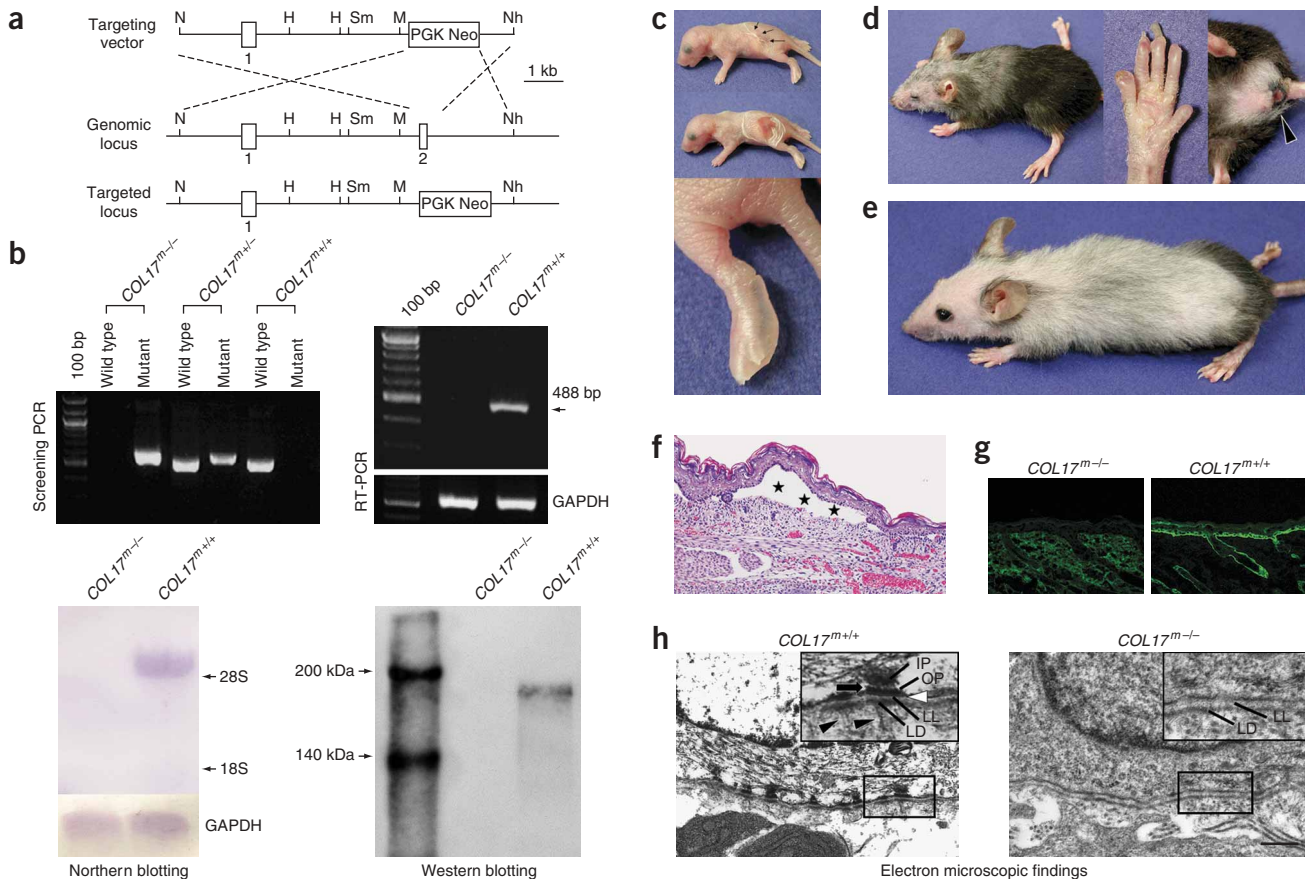


Figure 1 Generation of *COL17*^{m-/-} mice. **(a)** The targeting vector was constructed by disrupting exon 2. **(b)** PCR reaction amplified a 562-bp mutant band, and a 496-bp fragment corresponding to the wild-type genomic *Col17* DNA. RT and subsequent PCR reactions, northern and western blotting showed definite disruption of *COL17* mRNA and its transcript in *COL17*^{m-/-} mouse keratinocytes. These findings were characteristic of *COL17*^{m-/-} mice. **(c)** Epidermal detachment after gentle friction (arrows), and blister development on the paw. **(d)** Eight-week-old *COL17*^{m-/-} mouse. Note the white hair around the face and head, small hemorrhagic blisters and scar formation on the paw and digits, nail loss, and erosions within the genital area (arrowhead). **(e)** White hair growth over the entire body of a 12-week old *COL17*^{m-/-} mouse. **(f)** Light microscopy revealed blister formation beneath the epidermis of *COL17*^{m-/-} mouse skin (stars). **(g)** Immunofluorescence study of *COL17*^{m-/-} and wild-type mice skin. COL17 did not show any immunoreactivity (mNC16A antibody) along the dermal-epidermal junction in the *COL17*^{m-/-} mice. **(h)** Electron microscopy findings. In wild-type skin, hemidesmosomes were composed of prominent inner plaques (IP), outer plaques (OP) and sub-basal dense plates (arrow). Anchoring filaments (white arrowhead) spanned the lamina lucida (LL), and anchoring fibrils (black arrowheads) extended from lamina densa (LD) to the papillary dermis. In *COL17*^{m-/-} skin, there were a reduced number of hypoplastic (small) hemidesmosomal inner and outer attachment plaques with poor keratin filament attachment and less prominent anchoring filaments, whereas anchoring fibrils and the lamina densa (LD) were both well preserved. Scale bar, 300 nm.

(*COL17*^{m+/+,h+}) mice, we generated rescued COL17-humanized (*COL17*^{m-/-,h+}) mice (Fig. 2a–c). Notably, rescued COL17-humanized mice showed none of the abnormal manifestations seen in *COL17*^{m-/-} mice (Fig. 2a and Supplementary Fig. 1). The rescued mice were able to reproduce, in spite of the fact that the original *COL17*^{m-/-} mice were unable to multiply.

IgG transfer from BP patients to COL17-humanized mice

We then performed a passive transfer study using pathogenic IgG from individuals with BP. Preliminary studies in human COL17 transgenic (*COL17*^{m+/+,h+}) mice did not show skin detachment (data not shown); therefore we studied neonatal COL17-humanized mice instead.

The COL17-humanized mice received intraperitoneal injections of both total and affinity-purified IgG directed against COL17, and developed diffuse erythema and a positive Nikolsky sign (epidermal separation elicited by gentle skin friction) at 48 h after injection

(Fig. 2d and Table 1). Histopathologic studies of lesional skin revealed a separation between the epidermis and the dermis, and an inflammatory cell infiltrate including neutrophils and lymphocytes (Fig. 2e). Direct immunofluorescence studies revealed a linear deposition of human IgG along the dermal-epidermal junction (Fig. 2f). Electron microscopic findings showed dermal-epidermal separation in the lamina lucida between the plasma membrane of basal keratinocytes and the lamina densa (Fig. 2g). All these findings are consistent with, and precisely recapitulate, human BP skin lesions¹⁴, thus confirming that these mice are a good model for human BP disease. We demonstrate that this disease is initiated by an interaction between human BP autoantibodies and the human COL17 adhesion protein.

Recombinant peptide epitope decoy therapy

Finally, using this system, we evaluated the efficiency of a new approach to BP therapy. We synthesized six different short peptides

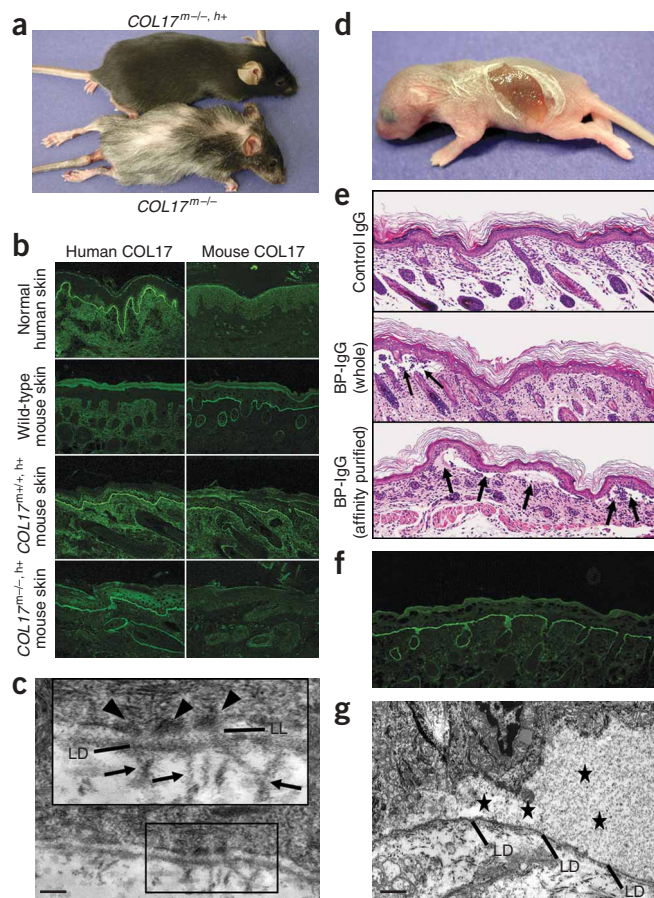


Figure 2 Phenotype, immunohistochemistry and histology of the humanized model mice. (a) *COL17*^{-/-} mouse and rescued *COL17*-humanized mouse (*COL17*^{-/-}h+). Compared to the *COL17*^{-/-} mouse, the *COL17*-humanized mouse showed no abnormal clinical manifestations. (b) Immunofluorescence studies using antibody to human COL17 and mouse COL17 showed that in human skin, only human COL17 was positively stained, whereas mouse COL17 did not stain. By contrast, mouse COL17 was positively stained only in mouse skin. In human COL17 transgenic (*COL17*^{+/+,h+}) mice skin, both human COL17 and mouse COL17 were positively stained. Note that in the rescued *COL17*-humanized mice skin, mouse COL17 was absent whereas human COL17 was present. (c) Electron microscopy findings from the rescued *COL17*-humanized mice skin. Note the distinct, normal-looking hemidesmosomes with clear keratin filament attachment (arrowheads) adjacent to the lamina lucida (LL), lamina densa (LD) and anchoring fibrils (arrows). Scale bar, 200 nm. (d) *COL17*-humanized mouse injected with pathogenic IgG showed diffuse erythema and the epidermis was frequently detached from the dermis. (e) Histological findings in IgG-injected mice skin. There were no abnormal findings in control mice skin, whereas mice skin injected with the whole IgG fraction showed subepidermal blisters. Furthermore, mice skin injected with *COL17* affinity-purified IgG showed a greater degree of subepidermal blistering with inflammatory cells. (f) Direct immunofluorescence microscopy showed linear IgG deposition along the dermal-epidermal junction. (g) Electron microscopy of rescued mice skin injected with BP-IgG revealed skin separation as well as putative blisters (stars) developing within the lamina lucida overlying the lamina densa (LD). Scale bar, 1 μ m.

0.01). We observed no adverse reactions, such as anaphylaxis or dermatitis, to the peptide treatments.

DISCUSSION

Here we succeeded in generating a *COL17*^{-/-} mouse, which represents a human disease model of non-Herlitz junctional epidermolysis bullosa^{11–13}. We also generated *COL17*-humanized (*COL17*^{-/-}h+) mice with a normal appearance, clearly showing that the transgenic epidermal *COL17* product that originated from the transfected *COL17* cDNA is able to abolish the clinical features of the *COL17*^{-/-} mouse. This supports the previously proposed notion that genetic diseases with defective epidermally expressed genes, such as those in the epidermolysis bullosa group of diseases, can be treated by gene therapy using an appropriate cDNA, even one from another species.

The potential pathogenic role of *COL17* has been supported in an experimental mouse passive transfer model using rabbit IgG directed against the murine homolog of the immunodominant epitope of human *COL17* (ref. 9). Indeed, many previous attempts have been made to try to directly confirm the pathogenicity of human *COL17* BP autoantibodies. Of note, a new model was developed by administering IgG fractions from individuals with BP into human skin grafted onto adult severe combined immunodeficient (SCID) mice. No blister formation was observed, however, although human IgG and mouse C3 were both deposited at the epidermal basement membrane zone of the grafted human skin¹⁵.

Here we were able to generate BP lesions in experimental mice using autoantibodies from human BP patients. Several factors have contributed to the success of this model system. First, rescued *COL17*-humanized mice possess a functional, humanized *COL17* protein that is faithfully recognized by IgG autoantibodies from individuals with BP. Second, we performed this passive transfer study using neonatal mice, which might have resulted in a higher index value of circulating IgG in the host mice (because of smaller blood and body volumes). Third, we observed no epidermal detachment in human *COL17* transgenic (*COL17*^{+/+,h+}) mice that had been given pathogenic BP-IgG. The transgenic mice possessed both human and mouse *COL17*, and the inflammatory process induced by BP-IgG in this model might

overlapping a number of antigenic *COL17* NC16A microdomains (Fig. 3a). *In vitro* immune-adsorption studies showed that a recombinant peptide composed of 77 amino acids (R1) most efficiently suppressed the BP antibody index (defined as follows: index = (optical density (OD) of tested serum – OD of negative control)/(OD of positive control – OD of negative control) $\times$ 100; all index values against BP antibodies were measured by ELISA using *COL17* NC16A domain peptide; see Methods) from BP sera (Fig. 3b and Supplementary Methods online). Furthermore, we found that the suppression efficacy of R1 was increased after fusion of glutathione S-transferase (GST) (Fig. 3b). This R1-GST peptide reduced index values of BP-IgG in a dose-dependent manner in an *in vitro* binding assay (Fig. 3c).

For *in vivo* studies, after intraperitoneal injection of 1 mg/g (body weight) total IgG from individuals with BP, we treated *COL17*-humanized mice with R1-GST peptide (total dose of 600 μ g, injected subcutaneously). This resulted in a marked reduction in blister formation (specifically, no blisters or skin fragility in 16 out of 17 mice), in contrast to controls treated with GST alone (extensive blisters in 12 of 16 mice) (Fig. 3d and Table 2). Histopathologically, mouse skin treated with R1-GST showed no subepidermal blister formation, whereas distinctive blister formation developed in control mouse skin (Fig. 3d). Direct immunofluorescence studies showed substantially reduced human IgG staining along the dermal-epidermal junction in the group treated with recombinant protein compared to the control group, indicating that the R1-GST peptide can efficiently neutralize pathogenic IgG *in vivo* (Fig. 3d). BP antibody index value was also significantly decreased in the treated group (Fig. 3e, $n = 15$, $P <$

Table 1 Characteristics of serum and IgG from BP patients showing the extent of disease in neonatal mice

Patient	Serum from BP patients				Passive transfer of IgG into COL17-humanized mice	
	IIF titer human skin	IIF titer mouse skin	BP180 ELISA index value	BP230 ELISA index value	Dose of IgG or COL17-affinity IgG (mg per g body weight)	Skin detachment
1	×320	Neg	171.4	Neg	2	5/6
					1	7/7
					0.5	3/6
					0.25	0/7
					<u>0.05</u>	5/5
2	×320	Neg	162.5	27.06	2	7/7
					1	7/7
					0.5	3/5
					0.25	2/6
					<u>0.05</u>	5/5
3	×320	Neg	142.4	Neg	2	5/5
					1	5/5
					0.5	3/6
					0.25	2/6
					<u>0.05</u>	5/5
4	×320	Neg	184.4	Neg	2	5/6
					1	6/8
					0.5	5/7
					0.25	4/5
					<u>0.05</u>	ND
5	×40	Neg	125.5	Neg	2	5/5
					1	5/5
					0.5	5/6
					0.25	2/5
					<u>0.05</u>	ND
Healthy control (<i>n</i> = 5)	Neg	Neg	Neg	Neg	2	0/5
					1	0/5
					0.5	0/5
					0.25	0/5
					0.05	0/5

The COL17-humanized mice that were injected intraperitoneally with whole IgG fractions and with COL17 affinity-purified (underlined) IgG fraction developed distinct skin fragility (positive Nikolsky sign) at 48 h. In contrast, control IgG from healthy volunteers resulted in no detectable abnormal skin lesions (*n* = 5, all mice were injected with total IgG because COL17 affinity IgG was not obtainable). Neg, negative; ND, not done.

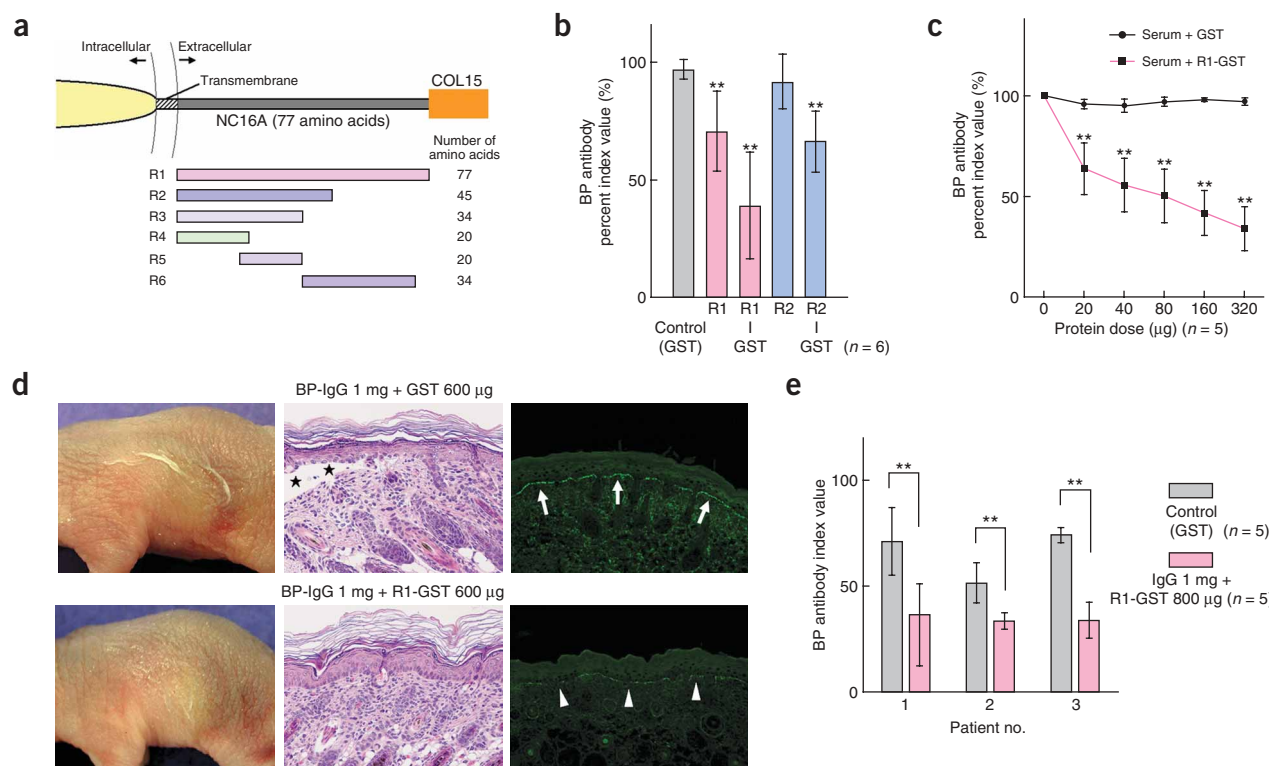
have been insufficient for blistering or disrupting mouse COL17 dermal-epidermal adhesion. In fact, the BP-IgG-treated COL17-humanized mice developed the blistering disease because they expressed only the human COL17 and not the mouse antigen. Last, our study showed that COL17-humanized mice have a normal mouse immune system, in contrast to previous findings in SCID mice. We showed a direct induction of human BP lesions in COL17-humanized mice, which provides strong and irrefutable evidence that human BP autoantibodies directly cause subepidermal blisters. Humanization of autoantigens in model animals therefore represents a powerful technical approach capable of improving our understanding of this and other autoimmune diseases.

In addition, we showed that a therapeutic challenge with a recombinant peptide sequence containing the BP pathogenic epitope as a decoy markedly inhibited the formation of BP skin lesions and decreased disease severity. One or more epitopes for BP autoantibodies are clustered within a limited area of the NC16A domain¹⁰. This domain resides within the COL17 ectodomain, in a region that is closest to the transmembrane domain and where circulating autoantibodies have easy access¹⁶. We used this known characteristic of BP antibodies to design an effective 'trap' for the BP autoantibodies. We did this by synthesizing a short recombinant peptide harboring the

pathogenic BP epitope sequence, which is capable of acting as a decoy. Moreover, we found that the capacity of the R1 antigenic peptide to bind antibodies to NC16A BP-IgG was increased after coupling with GST. This is consistent with a previous report showing that COL17 NC16A domain peptides obtain a more beneficial conformation, including α -helix and β -turn, after linking with GST, in terms of a higher ligand affinity for the substrate (as determined by ELISA; ref. 17). Here we showed that this epitope decoy strategy has potential for the treatment of antibody-mediated autoimmune diseases in clinical practice. Overall, we believe that the new mouse model and the technical approach we have followed provide a strong basis for future studies that can provide new insight into the pathomechanisms and therapy of human autoimmune diseases.

METHODS

Generation of COL17^{m/-} mouse. We cloned a 14.7-kb mouse genomic DNA Col17 fragment from the mouse 129Sv/Ev genomic library (Stratagene). We subcloned a 11.5-kb *NheI* to *NotI* fragment to make the targeting vector. We inserted the PGK/Neo cassette between 6 bp upstream of the ATG start codon in exon 2 and 1.2 kb downstream in intron 2. We transfected the targeting vector by electroporation into 129 Sv/Ev embryonic stem cells, then micro-injected the correctly targeted embryonic stem cell line into blastocysts



obtained from C57BL/6J mice (Jackson Laboratories) to generate chimeric mice, which we then mated with C57BL/6J females. We crossed F1 heterozygotes with C57BL/6J over more than four generations, and then intercrossed them to generate *COL17*^{tm-/-} mice.

Screening of *COL17*^{tm-/-} mice by PCR, RT-PCR, northern and western blotting, histology, electron microscopic findings and immunofluorescence study. Details of these procedures are in the **Supplementary Methods**.

Generation of rescued *COL17*^{tm-/-h+} COL17-humanized mice. We crossed transgenic mice (C57BL/6 background) expressing the squamous epithelium-specific K14 promoter and a human *COL17* cDNA (*COL17*^{tm+/+,h+}) with heterozygous *COL17*^{tm+/-} mice. Mice that carried both the heterozygous null mutation of *COL17* and the transgene of human *COL17* (*COL17*^{tm+/-h+}) were bred to produce rescued *COL17*^{tm-/-h+} COL17-humanized mice.

Table 2 Epitope decoy therapy with COL17 antigenic peptide

	Skin detachment
IgG 1 mg	15/16
IgG 1 mg + GST 800 µg	12/16
IgG 1 mg + R1-GST 600 µg	1/17

The recombinant peptide R1-GST carrying COL17 antigenic sequences markedly suppressed skin detachment in experimental mice injected with BP autoantibodies *in vivo*.

Preparation and characterization of IgG fractions from individuals with BP.

We obtained sera from five individuals with BP during the early, active phase of the disease. We performed indirect immunofluorescence (IIF) using normal human skin as a substrate obtained from surgical operations. We defined the IIF titer as the highest dilution of the sera that showed positive fluorescence along the dermal-epidermal junction. We measured the ratio of autoantibody index values in the individuals' sera recognizing COL17 (measured by ELISA using the COL17 NC16A domain peptide) and BP230 using ELISA kits as per the manufacturer's instructions (MBL). We defined the ELISA index value by the following formula: index = (optical density (OD) of tested serum - OD of negative control)/(OD of positive control - OD of negative control) × 100. We prepared the total IgG fractions from these serum samples by affinity chromatography using HiTrap Protein G HP (Amersham Biosciences), and we followed a selection of sera ($n = 3$) by affinity purification directed against entire region of COL17 NC16A domain peptide (R1, amino acids 490-566; see below) using HiTrap NHS-activated HP column (Amersham) as per the manufacturer's instructions. We dialyzed the IgG fractions against PBS and concentrated by ultrafiltration (Millipore).

Passive transfer studies. We performed passive transfer of IgG into mice as described previously with minor modifications^{4,5,9}. Briefly, each mouse received a single intraperitoneal injection of IgG (dose: 0.05-2 mg/kg body weight). At 48 h after the injection, we judged whether a distinct Nikolsky sign was observed in the skin or not. We then killed the mice and studied the skin samples by light microscopy and direct immunofluorescence microscopy.

using FITC-conjugated rat antibody to human IgG (1:100; Jackson ImmunoResearch Laboratories).

Generation of recombinant COL17 NC16A peptide. We synthesized 77 amino acids spanning the COL17 NC16A domain (R1, amino acids 490–566) and the 45 amino acids in its N terminus half (R2, amino acids 490–534) as GST-fusion proteins using the expression vector pGEX2-T (Amersham Biosciences) and bacteria BL21 (Amersham Biosciences), as reported previously¹⁰. In this study, we used both native GST-fusion forms, R1-GST and R2-GST, and GST-cleaved forms (R1 and R2). Other amino acids R3 (amino acids 490–525), R4 (amino acids 490–509), R5 (amino acids 506–525) and R6 (amino acids 526–559) peptides were chemically synthesized (R3 and R6, Greiner Bio-one; R4 and R5, Nippi).

Therapeutic study of COL17 NC16A domain antigenic peptide. Preliminary *in vitro* immune-adsorption studies showed that a 77-amino acid recombinant peptide (R1) most efficiently suppressed the BP antibody index value from BP sera (see Fig. 3b and Supplementary Methods). We selected the R1-GST peptide for use in the *in vivo* decoy therapy. For the *in vivo* study, each mouse received a subcutaneous injection of 300 µg of R1-GST fusion protein desalted in 20 mM Tris-HCl, pH 7.5, to the dorsal skin soon after and 24 h after a 1 mg intraperitoneal injection of total IgG from three individuals with BP ($n = 17$). For controls, the same dose of GST was used ($n = 16$).

All mouse procedures were approved by the Institutional Animal Care and Use Committee of Hokkaido University, and full, written informed consent was obtained from all individuals for the use of their human materials.

Statistical analysis and ethical considerations. We examined growth differences between groups for statistical significance using analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) test. For therapeutic analysis of the effects of synthetic peptide treatments, we determined statistical significance using the Student's *t*-test. We considered *P* values less than 0.05 as significant.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

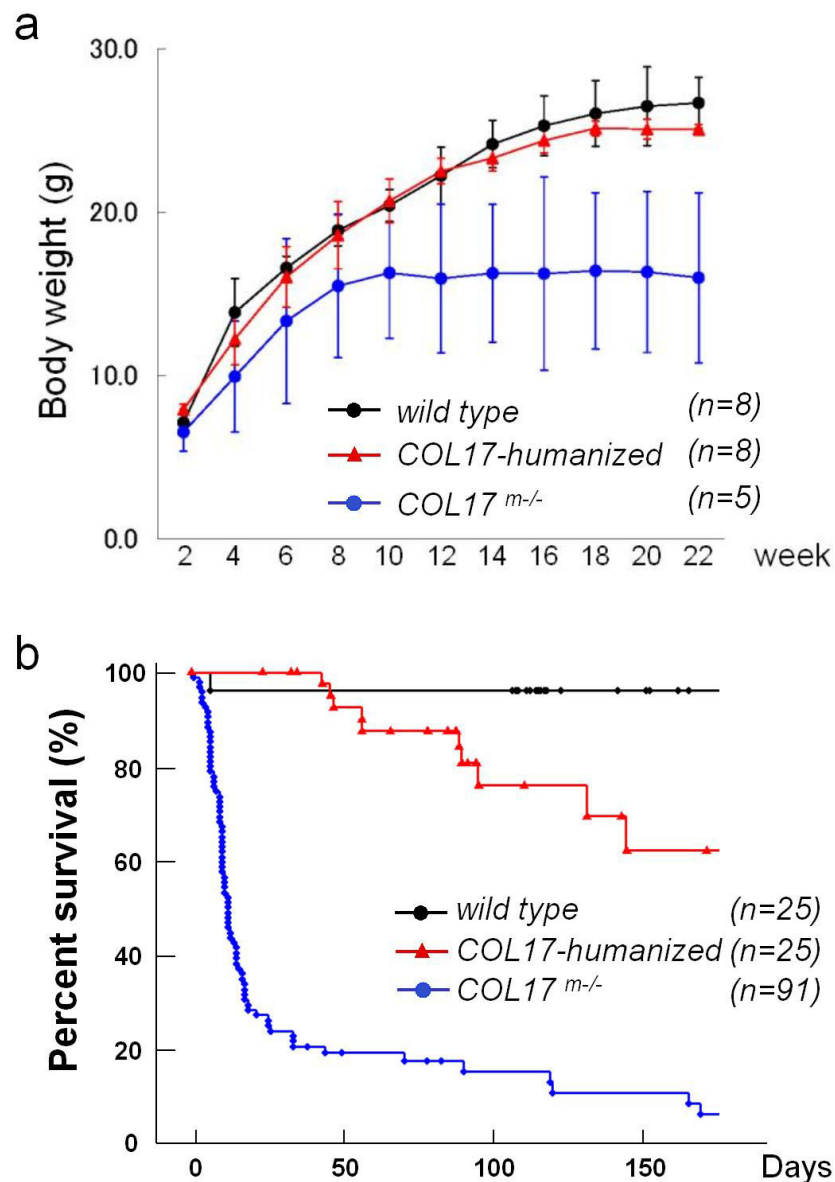
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Supplementary Fig. 1. Growth and Kaplan-Meier survival curves of wild type, *COL17*^{m/-}, and rescued *COL17*-humanized mice. **(a)** *COL17*^{m/-} mice showed growth retardation compared with wild-type littermates, and **(b)** most *COL17*^{m/-} mice died within 2 weeks of birth. Mortality rates of the *COL17*^{m/-} and wild-type littermates at 8 weeks were 80.1% and 4.0%, respectively. Notably, the rescued *COL17*-humanized mice showed normal growth and had lost the high neonatal pup mortality rate seen in *COL17*^{m/-} mice.



Supplementary Methods

Nishie et al “Humanization of autoantigen”

Screening of recombinant clones and genotyping of *COL17*^{m-/} mice by PCR.

Primer, YA1 (5'-CATACCAGGGCCAACTTTGA-3') was designed downstream outside the region used to generate the targeting construct. The primer N1 (5'-TTGTGTAGCG CCAAGTGCCA-3') and WT1 (5'-CCTTATATCCCTTGACTGCC-3') was designed at the 5' end of the Neo cassette and within the region used to generate the targeting construct, respectively.

RT-PCR. For RT-PCR, total RNA from tissues was extracted using ISOGEN, according to manufacturer's recommendations (Nippon Gene, Tokyo, Japan). Three micrograms of total RNA was used for cDNA synthesis in SuperScript III reverse transcriptase according to manufacturer's instructions (Invitrogen, Carisbad, CA). The following primers specific for mouse BP180 chain sequences (NM: 007732) were used for RT-PCR: 5'-AGAAGAAAA GCATCCGAGGG-3' (RT-F); and 5'- TGGTTGAAGAAGAGGCGAGT-3' (RT-B). As a control, we used the primers for mouse GAPDH (NM:001001303): 5'-TTAGCCCCC CTGGCCAAGG-3' (mGAPDH-F) and 5'-CTTACTCCTTGGAGGCCATG-3' (mGAPDH-B), which amplified a 541-bp fragment.

Northern blotting. For northern analysis, denatured RNA gels using 13 µg of total RNA per lane were transfer to Zetaprobe membrane (Bio-Rad, Hercules, CA) and Northern blotting was carried out as described elsewhere^{1,2}. Membranes were hybridized with 5' labeled probes (residues 351–690) amplification products of the mouse COL17 cDNA by use of a digoxigenin DNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany).

Western blotting. Western blotting was performed as previously described³. Tissue extracts were subjected to SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The membranes were blocked with 1% non-fat dry milk in PBS, probed with rat monoclonal antibodies against mouse COL17 NC16A domain (mNC16A mAb, 1:250,000; a gift from T Tanaka, Shiga Medical University, Japan), and then allowed to react with goat anti-rat IgG antibody coupled with horseradish peroxidase.

Histology and electron microscopy. The tissue samples were fixed in 10% buffered formalin followed by paraffin embedding. Five-µm sections were stained with hematoxylin-eosin. Transmission electron microscopy was performed as previously described⁴, using a transmission electron microscope (Hitachi, H-7100).

Immunofluorescence. Five - μ m cryosections of OCT-embedded skin were cut and placed onto microscope slides and subjected to indirect immunofluorescence. The following primary antibodies and dilutions were used: mNC16A mAb (1:5000) and mouse monoclonal antibodies against human COL17 (D20, 1:160; a gift from K Owaribe, Nagoya University, Japan). The following secondary antibodies were used, FITC-conjugated donkey anti-rat IgG, FITC-conjugated goat anti-mouse IgG (Jackson, West Grove, PA).

***In vitro* immune-adsorption study.** To assess the therapeutic potential of recombinant peptides carrying COL17 antigenic sequences, preliminary *in vitro* immunoadsorption studies were performed. Briefly, 20 μ g of each of the recombinant peptides was incubated with 10 μ l of serum from BP patients in 37°C for 30 minutes, followed by testing using a BP180 ELISA kit according to manufacturer's instructions (MBL, Japan). Preliminary immunoadsorption study of the suppression rate (%) compared with control (GST) showed; R1: 29.02 $\pm$ 17.18 (p=0.009), R1-GST: 60.18 $\pm$ 22.35 (p=0.004), R2: 7.6 $\pm$ 11.54 (p=0.16), R2-GST: 33.89 $\pm$ 13.1 (p=0.001), R3: 10.38 $\pm$ 5.47 (p=0.01), R4: -0.44 $\pm$ 3.87 (p=0.791), R5: 12.03 $\pm$ 5.05 (p=0.002), R6: 0.2 $\pm$ 3.91 (p=0.907). From these data, we selected to use the R1-GST peptide for the *in vivo* decoy therapy studies.

References

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