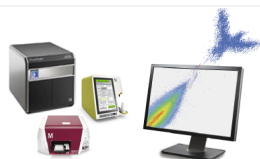




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Bullous Pemphigoid Autoantibodies Directly Induce Blister Formation without Complement Activation

Hideyuki Ujiie,* Tetsumasa Sasaoka,*[†] Kentaro Izumi,* Wataru Nishie,* Satoru Shinkuma,* Ken Natsuga,* Hideki Nakamura,* Akihiko Shibaki,* and Hiroshi Shimizu*

Complement activation and subsequent recruitment of inflammatory cells at the dermal/epidermal junction are thought to be essential for blister formation in bullous pemphigoid (BP), an autoimmune blistering disease induced by autoantibodies against type XVII collagen (COL17); however, this theory does not fully explain the pathological features of BP. Recently, the involvement of complement-independent pathways has been proposed. To directly address the question of the necessity of the complement activation in blister formation, we generated C3-deficient COL17-humanized mice. First, we show that passive transfer of autoantibodies from BP patients induced blister formation in neonatal C3-deficient COL17-humanized mice without complement activation. By using newly generated human and murine mAbs against the pathogenic noncollagenous 16A domain of COL17 with high (human IgG1, murine IgG2), low (murine IgG1), or no (human IgG4) complement activation abilities, we demonstrate that the deposition of Abs, and not complements, is relevant to the induction of blister formation in neonatal and adult mice. Notably, passive transfer of BP autoantibodies reduced the amount of COL17 in lesional mice skin, as observed in cultured normal human keratinocytes treated with the same Abs. Moreover, the COL17 depletion was associated with a ubiquitin/proteasome pathway. In conclusion, the COL17 depletion induced by BP autoantibodies, and not complement activation, is essential for the blister formation under our experimental system. *The Journal of Immunology*, 2014, 193: 4415–4428.

Bullous pemphigoid (BP), the most common autoimmune blistering disorder, is characterized by tense blisters with itchy urticarial erythema and plaques that develop on the entire body. Histologically, subepidermal blisters associated with inflammatory cell infiltration in the dermis are observed. BP is induced by autoantibodies against type XVII collagen (COL17, also called BP180 and BPAG2), a hemidesmosomal type II transmembrane protein that spans the lamina lucida and projects into the lamina densa of the dermal/epidermal junction (DEJ) (1–4). Studies to map the epitopes on COL17 have revealed that the juxtamembranous extracellular noncollagenous 16A domain (NC16A) is preferentially recognized by autoantibodies in the sera of BP patients (5, 6). The pathogenicity of IgG Ab from BP patients (BP-IgG) had been unclear because BP-IgG fails to recognize murine COL17 and to reproduce the skin detachment in

wild-type mice owing to differences in the amino acid sequence between humans and mice. Our recent studies using COL17-humanized (*COL17^{m-/-h+}*) mice, which lack murine COL17 but express human COL17, have directly demonstrated the pathogenicity of Abs against the NC16A of human COL17 (hNC16A), including BP-IgG, in vivo (7–13).

C3 is detected at the DEJ of the lesional/perilesional skin in most BP cases by direct immunofluorescence (IF) (14, 15). Findings from an IgG passive transfer neonatal mouse model have demonstrated that the binding of Abs to murine COL17 triggers immune reactions, including complement activation (16), mast cell degranulation (17), and neutrophil infiltration (18). The infiltrating neutrophils were shown to be activated via FcγRIII (19) and to release neutrophil elastase and matrix metalloproteinase-9, which are responsible for the dermal/epidermal separation (20). Thus, the binding of Abs to COL17 as well as subsequent immune reactions are thought to be essential for BP blister formation. However, this theory does not fully explain why Abs against a distinctive portion of NC16A are specifically pathogenic (13) or why separation occurs specifically within the lamina lucida (21). Recently, BP-IgG has been shown to be able to deplete the amount of COL17 in cultured normal human keratinocytes (NHKs) (22, 23) and to diminish their adhesive strength to the culture dish in vitro (22). More recently, we demonstrated that polyclonal F(ab')₂ Abs against NC16A induced dermal/epidermal separation in injected neonatal mice and reduced the amount of COL17 in cultured NHKs (13).

An accurate understanding of a disease's pathomechanism is essential for the development of disease-specific therapeutic strategies. To determine the necessity of the complement in BP blister formation clearly, we newly generated C3-deficient COL17-humanized mice and plural human and murine mAbs against hNC16A that have various degrees of complement activation ability. First, we demonstrate that BP-IgG induced blister

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Abbreviations used in this article: BP, bullous pemphigoid; BP-IgG, IgG Ab from BP patient; COL17, type XVII collagen; DEJ, dermal/epidermal junction; hIgG, human IgG; hNC16A, noncollagenous 16A domain of human COL17; IF, immunofluorescence; mC3, murine C3; mIgG, murine IgG; NC16A, noncollagenous 16A domain; NHK, normal human keratinocyte; NHS, normal human skin; rhIgG, recombinant human IgG; V_H, V region H chain; V_LC_L, V region and C region L chain.

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formation in neonatal C3-deficient COL17-humanized mice. Next, we used mAbs to show that the deposition of Abs at NC16A was more relevant to the induction of blister formation than was complement activation in neonatal and adult mice. BP-IgG reduced the amount of COL17 in vivo and in vitro, and the COL17 depletion was mediated by a ubiquitin/proteasome pathway. These findings provide new insights into the blister formation and approaches to the treatment of BP.

Materials and Methods

Mice

The COL17-humanized mice were generated as previously described (7). Briefly, human COL17-transgenic mice (C57BL/6 background) expressing the squamous epithelium-specific K14 promoter and human COL17A1 cDNA (*COL17^{tm1.1/+}*) (24) were crossed with heterozygous murine *Col17^{+/-}* mice (the F₁ mouse had a 129/SvEv × C57BL/6 background, backcrossed with C57BL/6 for 10 generations) to produce COL17-humanized mice (*COL17^{tm1.1/+}*). *C3^{-/-}*/COL17-humanized mice were generated by crossing COL17-humanized mice with B6;129S4-*C3^{tm1.Crr/J}* mice (C3-deficient strain, C57BL/6 background) purchased from The Jackson Laboratory. *Rag-2^{-/-}*/COL17-humanized mice were generated by crossing COL17-humanized mice with C57BL/6 background *Rag-2^{-/-}* mice (Central Institute for Experimental Animals, Kawasaki, Japan) as reported previously (9). All animal procedures were conducted according to guidelines of the Hokkaido University Institutional Animal Care and Use Committee.

Preparation and purification of BP-IgG

Sera from four BP patients were used. These patients met three criteria: 1) subepidermal blistering lesions present clinically on skin, 2) in vivo deposition of IgG at the DEJ in skin samples from patients, and 3) circulating anti-DEJ IgG Abs that react with the epidermal side of 1 M NaCl-split normal human skin (NHS). Total IgG was purified from the patients' sera by protein G-Sepharose column chromatography (GE Healthcare, Uppsala, Sweden) and concentrated by Amicon ultrafiltration 50K (Millipore, Lexington, MA). The purity of BP-IgGs was confirmed by SDS-PAGE. The collection of samples was approved by the local ethics committee and the Institutional Review Board of Hokkaido University. Full, written informed consent was obtained from all individuals. This study was performed according to the Declaration of Helsinki.

Passive transfer studies

We performed passive transfer of Abs into mice as described previously (7, 9). Briefly, each neonatal COL17-humanized mouse or *C3^{-/-}*/COL17-humanized mouse (1 d old) received a single i.p. injection of BP-IgG1 and BP-IgG2 (1000 µg/mouse), BP-IgG3 and BP-IgG4 (1500 µg/mouse), normal human IgG (hIgG; Nihon Pharmaceutical, Tokyo, Japan) (1500 µg/mouse), recombinant hIgG1 (rhIgG1; 4.5–30 µg/mouse), control hIgG1 (Sigma-Aldrich, St. Louis, MO) (30 µg/mouse), rhIgG4 (7.5–50 µg/mouse), control hIgG4 (Sigma-Aldrich) (50 µg/mouse), monoclonal murine IgG1 (mIgG1; TS39-3, TS21-1, and TI66-1) (25–100 µg/mouse), monoclonal mIgG2c (TS4-2) (50–500 µg/mouse), or control mIgG (100–500 µg/mouse) without marking. To inhibit FcγR in vivo, neonatal COL17-humanized mice were injected i.p. with 20 µg rat anti-mouse mAb 2.4G (specific for FcγRII and FcγRIII; BD Biosciences) or control rat IgG2b (BD Biosciences) and 2 h later were injected i.p. with 50 µg rhIgG4 according to the reported method (19). To obtain a sufficient effect, higher doses of 2.4G were administered than in a previous study (19). For proteasome inhibition, neonatal mice were injected i.p. with 1.5 µg proteasome inhibitor MG-132 (Calbiochem, San Diego, CA) and 3 h later were injected i.p. with 50 µg rhIgG4. The same dose of MG-132 was also injected at 24 h after the injection of rhIgG4. The dose of MG-132 was determined based on previous studies (25, 26). At 48 h after Ab injection, back skins of the injected mice were evaluated for detachment with gentle rubbing performed up to four times. In some experiments, skin fragility was evaluated at 6 and 12 h after Ab injection. For histological investigations, back or ear skin was obtained from the mice after the evaluation of skin detachment and was processed for light microscopy (H&E) and for direct IF using FITC-conjugated Abs against hIgG, mIgG, and mC3.

Construction of rhIgG1 and rhIgG4 expression vector

rhIgG1 and rhIgG4 mAbs to hNC16A were generated using the previously described Fab-B4 fragment (10). Conversion of Fab-B4 to rhIgG1 or

rhIgG4 was performed as previously described (27), with some modifications. The V region and C region L chain (V_LC_L) and the V region H chain (V_H) of the Fab-B4 fragment were amplified by PCR with primers containing a restriction site as follows: V_LC_L, forward primer, 5'-CTACTCTGGCTC-CGCGGTGCCAGACAGTCTGTGTTGACGCAGCGG-3', reverse primer, 5'-TCGACTGGCGCGCCCTATGAACATTCTGTAGGGGCCACTGTCTC-3'; V_H, forward primer, 5'-TTCCTCTACTAGTGGCAGCTCCCA-GATGGGTCTCTGTCAGGTGACGT GGTGCAGTCTGG-3', reverse primer, 5'-GGTGGAGGCACTCGAGACGGTGACCGTGGTCC-3'. After digestion with SacII and AscI for the V_LC_L region and with SpeI and XhoI for the V_H region, the PCR products were subcloned into an hIgG1 construction vector (Medical and Biological Laboratories, Nagoya, Japan). To generate the rhIgG4 construction vector, C region H chains of hIgG1 were converted to those of hIgG4. The constructed hIgG1 and hIgG4 cassettes were further cloned into the pCMV-Script expression vector (Stratagene, Santa Clara, CA) (see Fig. 2A).

Production and purification of rhIgG1 and rhIgG4

Expression of rhIgG1 and rhIgG4 was performed by transfection of the expression vectors into CHO-K1 cells. Stably expressing cells were selected under 700 µg/ml Geneticin (Life Technologies). For large-scale production of rhIgGs, CHO-K1 cells were stationary-cultured in CHO-S-SFM II (Invitrogen, Carlsbad, CA) for 3 d at 37°C in 5% CO₂, and the culture supernatant was harvested. Purification of rhIgGs was performed using protein A-Sepharose (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. The purified rhIgGs were dialyzed against PBS and concentrated by Amicon ultrafiltration 30K (Millipore) and were then characterized by SDS-PAGE, hNC16A ELISA, and indirect IF on NHS obtained from healthy volunteers.

Establishment of HEK293 human COL17 cells and human COL17ΔNC16A cells

The HEK293 human COL17 cells used in this study were previously established (11). Briefly, the constructed plasmid pcDNA5/FRT (Invitrogen) that had been inserted with human COL17A1 cDNA (a gift from Dr. K.B. Yancey, University of Texas Southwestern Medical Center, Dallas, TX) and pOG44 was cotransfected into FlpIn 293 cells (Invitrogen) and was then cultured in selective medium (DMEM, 100 µg/ml hygromycin B [Invitrogen], and 10% FBS). The construct of human COL17ΔNC16A cells was generated according to the reported method (28). The constructed plasmid of human COL17ΔNC16A cells was transfected into FlpIn 293 cells in the same way as for generating the HEK293 human COL17 cells.

IF studies

Direct IF was performed on the skin samples from neonatal mice that were passively transferred with IgG using standard protocols (7). Briefly, mouse skins were mounted and snap frozen into optimal cutting temperature compound, and 5-µm cryosections were prepared. The sections were blocked with 10% BSA for 1 h at 37°C and incubated with Abs, including FITC-conjugated Abs against hIgG (Jackson ImmunoResearch Laboratories), mIgG (Jackson ImmunoResearch Laboratories), or murine C3 (mC3; MP Biomedicals, Solon, OH) in a 1:100 dilution. Indirect IF study was performed on skin samples from human or COL17-humanized mice prepared as above. The sections were blocked with BSA and incubated with rhIgGs or monoclonal mIgGs for 1 h at 37°C and detected by secondary Abs, including FITC-conjugated Abs against hIgG (Jackson ImmunoResearch Laboratories), hIgG1, hIgG4 (Beckman Coulter, Brea, CA), mIgG (Jackson ImmunoResearch Laboratories), mIgG1, mIgG2a, mIgG2b, and mIgG3 (BD Biosciences) in a 1:100 dilution, or mIgG2c (Bethyl Laboratories, Montgomery, TX) in a 1:500 dilution.

HEK293 human COL17, HEK293 human COL17ΔNC16A, and HEK293 cells cultured in a collagen I-coated eight-well chambers (BD Biosciences) were fixed with 100% methanol for 5 min at -20°C. Chambers were washed once with PBS and incubated with primary Abs for 30 min at 37°C. After two washings with PBS, they were incubated with FITC-conjugated Abs to hIgG and propidium iodide (Dojindo Laboratories, Kumamoto, Japan) in a 1:100 dilution for 30 min at 37°C.

ELISA assay

Serum mC3 levels were examined by ELISA (Kamiya Biomedical Company, Seattle, WA) according to the manufacturer's instructions. The titers of BP-IgGs and rhIgGs against hNC16A were determined by using a BP180 ELISA kit (Medical and Biological Laboratories) according to the manufacturer's instructions. To measure the titers of mIgGs against hNC16A in hybridoma-transferred mice, serum diluted to 1:300 was used

as the primary Ab, and HRP-conjugated anti-mIgG Ab (Jackson ImmunoResearch Laboratories) diluted to 1:40,000 was used as the secondary Ab. Bound Abs were read at the OD of 450 nm using an ELISA plate reader (Mithras, Berthold Technologies). The ELISA index value was defined by the following formula: index = $[(OD_{450} \text{ of tested serum} - OD_{450} \text{ of negative control}) / (OD_{450} \text{ of positive control} - OD_{450} \text{ of negative control})] \times 100$. Titers of mIgGs were simply shown by the OD_{450} value.

Dot blot analysis

To detect the epitope of rhIgG1 and rhIgG4 in hNC16A (Glu⁴⁹⁰ to Arg⁵⁶⁶), dot blotting was performed by using four chemically synthesized peptides conjugated with BSA: R4 (Glu⁴⁹⁰ to Ile⁵⁰⁹), R5 (Arg⁵⁰⁶ to Gln⁵²⁵), R7 (Asp⁵²² to Gln⁵⁴⁵), and R8 (Ser⁵⁴² to Arg⁵⁶⁶) (Greiner Bio-One) (13). The amino acid numbering system is based on the human COL17 sequence (NP_00485.3). Synthesized peptides were diluted to 0.1 $\mu\text{g}/\mu\text{l}$ in distilled water, and 10 μl of this was spotted on nitrocellulose membrane. The membrane was blocked for 30 min at room temperature with 2% skimmed milk in TBS and then incubated with rhIgGs, Fab-B4, normal hIgG, monoclonal mIgGs, or normal mIgG. Bound Abs were visualized enzymatically using HRP-conjugated anti-hIgG Ab (DakoCytomation, Glostrup, Denmark), anti-human λ L chain Ab (Bethyl Laboratories), or anti-mIgG Ab (Jackson ImmunoResearch Laboratories). The blots were detected using the ECL Prime detection kit (GE Healthcare, Fairfield, CT).

Complement fixation study

Complement activation induced by rhIgG1, rhIgG4, or Fab-B4 in human skin samples was investigated by IF study as previously described, with minor modifications (9, 10). Cryosections of human skin were incubated with Abs in a 1:100 dilution for 1 h at 37°C. BP-IgG1 in a 1:100 dilution was used as a positive control. The sections were washed three times with PBS and incubated with mouse serum complement (Innovative Research, Novi, MI) in a 1:50 dilution for 1 h at 37°C. Then, in situ deposition of mC3 was detected with FITC-conjugated Ab specific to mC3 in a 1:100 dilution.

C1q-binding assay

The human C1q-binding activity of rhIgGs and Fab-B4 was determined by C1q-binding assay by ELISA as previously described, with minor modifications (11). The rhIgGs and Fab-B4 with 2-fold serial dilution (starting concentration, 20 $\mu\text{g}/\text{ml}$) in 0.1 M sodium carbonate buffer (pH 9.5) were coated in a Microtest 96-well ELISA plate (BD Biosciences) and left overnight at 4°C. The plate was washed three times with PBS containing 0.05% Tween 20 and blocked for 1 h at room temperature with ELISA assay diluent (BD Biosciences), then incubated for 2 h with 100 μl 4 $\mu\text{g}/\text{ml}$ human complement component C1q (Sigma-Aldrich) in assay diluent. The plate was washed four times and incubated with 100 μl of a 1:400 dilution of sheep polyclonal Ab to human C1q (HRP) (Abcam, Japan) for 1 h at room temperature. After five washings, the plate was displayed in 100 μl of a 1:1 mixture of substrate reagent A containing hydrogen peroxide and substrate reagent B containing tetramethylbenzidine (BD Biosciences) and then stopped by 100 μl 1 M phosphoric acid (BD Biosciences). The OD was determined at 450 nm. To correct for background, the OD at 620 nm was subtracted from the OD at 450 nm. Binding activity was calculated by the following formula: index value = $100 \times [(OD_{\text{test}} - OD_{\text{blank}}) / (OD_{\text{St.}} - OD_{\text{blank}})]$, where St. indicates standard hIgG1 (Sigma-Aldrich) and blank indicates the assay diluent. In the mouse C1q-binding assay with monoclonal mIgGs, the starting concentration of monoclonal mIgGs was 20 $\mu\text{g}/\text{ml}$. As a source of mC1q, a 1:50 dilution of mouse complement serum (Innovative) was used. As a secondary Ab, we used a 1:5000 dilution of mouse polyclonal Ab to mC1q (Abcam, Cambridge, U.K.) that was conjugated with peroxidase by peroxidase labeling kit-NH2 (Cosmo Bio, Tokyo, Japan). As a standard sample, mIgG2a (Sigma-Aldrich) was used.

Development of hybridomas that produce monoclonal mIgG Abs against hNC16A

Wild-type mice (C57BL/6) were immunized by grafting skin from human COL17-expressing mice (COL17^{m+/+,h+/+}) to their backs (9, 24). Five weeks after the skin graft, spleen cells were removed from the immunized mice and were fused with P3U1 myeloma cells by a polyethylene glycol 400 procedure (29, 30). Hybridoma supernatants were screened on 96-well plates coated with recombinant hNC16A protein (7). After the hybridomas were cloned, the monoclonal mIgGs were purified from the culture supernatant by protein G-Sepharose column chromatography (GE Healthcare, Uppsala, Sweden) and concentrated by Amicon ultrafiltration 50K (Millipore). IgG subclasses were confirmed by indirect IF and IsoStrip (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Transfer of hybridomas

For each mouse, 8×10^6 hybridoma cells were washed twice, suspended in 0.2 ml PBS, and then injected s.c. into the lower back of the Rag-2^{-/-}/COL17-humanized mice. Tumors were visible at the injection site after ~7 d. Tumor size for all of the mice selected in the study ranged from 2.5 to 3.5 cm in diameter when the evaluation was terminated on day 28. Some of the mice were observed until day 21 because they died before day 28. At day 28, tail skins were evaluated for detachment with gentle rubbing performed up to five times. Biopsies of lesional or perilesional skin were obtained between 21 and 28 d after the transfer for light microscopy (H&E) and for direct IF using FITC-conjugated Abs against mIgG and mC3.

Detection of COL17 in mice skin

To detect the COL17 in mice skin, we newly generated polyclonal rabbit Ab (09040IgG) against the C-terminal domain of COL17. A recombinant protein covering C-terminal domain of COL17 (Val¹¹⁹² to Pro¹⁴⁹⁷) was generated as a GST fusion protein using expression vector pGEX6P-1 (GE Healthcare) and bacteria BL21 (GE Healthcare), and purified using GSTrap HP (GE Healthcare). Rabbits were immunized with the recombinant protein. Rabbit IgG (09040IgG) was purified from the rabbit antisera using HiTrap protein G HP (GE Healthcare). Skin samples from BP-IgG (BP-IgG1 and BP-IgG2, 1000 $\mu\text{g}/\text{mouse}$; BP-IgG3 and BP-IgG4, 1500 $\mu\text{g}/\text{mouse}$) injected or untreated C3^{-/-}/COL17-humanized mice were separated into epidermis and dermis by 2 mM EDTA with protease inhibitor mixture (Sigma-Aldrich) at 4°C for 24 h. The epidermis was rapidly frozen in liquid nitrogen, smashed using the Multi-Beads Shocker (Yasui Kikai, Osaka, Japan), and then lysed in RIPA buffer (Thermo Scientific, Waltham, MA) with protease inhibitor mixture (Sigma-Aldrich). The lysates were centrifuged at 11,000 rpm for 30 min. After the protein concentration was determined by Bradford protein assay (Bio-Rad), the lysates were combined with Laemmli sample buffer. Immunoblotting was performed as described previously, with modifications (13). The samples were heated at 95°C for 5 min, applied to 4–12% gradient Bis-Tris gels (Invitrogen), and transferred to a polyvinylidene difluoride membrane. The membrane was blocked for 1 h at room temperature in 5% skimmed milk dissolved in TBS with 0.05% Tween 20. Blots were incubated with 1:1000 diluted 09040IgG for 1 h at room temperature, followed by incubation with 1:2000 diluted HRP-conjugated anti-rabbit IgG for 30 min at room temperature. To detect β -tubulin, membranes were incubated with 1:2000 diluted anti- β -tubulin Ab (Abcam, Cambridge, U.K.) for 1 h at room temperature followed by incubation with 1:2000 diluted HRP-conjugated anti-rabbit IgG Ab for 30 min at room temperature. Bound Abs were detected using the ECL Prime detection kit.

Depletion of COL17 in cultured NHKs by Abs

Ab stimulation of NHKs was performed as previously described (13, 22). NHKs were purchased from Life Technologies (human epidermal keratinocytes, neonatal). The NHKs were cultured in serum-free keratinocyte growth medium (Clonetics, San Diego, CA) containing 0.03 mM calcium, epidermal growth factor (0.1 mg/ml), bovine pituitary extract (0.1 mg/ml), insulin (0.5 mg/ml), and hydrocortisone (0.5 mg/ml) at 37°C under 5% CO₂. The NHKs used in this study had been cultivated for no more than four passages. Abs (150 $\mu\text{g}/\text{ml}$ BP-IgG1, 300 $\mu\text{g}/\text{ml}$ BP-IgG2, 690 $\mu\text{g}/\text{ml}$ BP-IgG3, 900 $\mu\text{g}/\text{ml}$ of BP-IgG4, 40 $\mu\text{g}/\text{ml}$ rhIgG1 or control hIgG1, 60 $\mu\text{g}/\text{ml}$ rhIgG4 or control hIgG4, 150 $\mu\text{g}/\text{ml}$ mIgGs) were added to the culture medium of the NHKs. Doses of BP-IgGs and rhIgGs were determined by the results of hNC16A ELISA. After 6 h of incubation, the cells were lysed in Laemmli sample buffer. To evaluate the effect of proteasome inhibitor on the COL17 depletion in cultured NHKs, several doses of MG-132 were added with rhIgG1 (40 $\mu\text{g}/\text{ml}$), rhIgG4 (60 $\mu\text{g}/\text{ml}$) or BP-IgG1 (150 $\mu\text{g}/\text{ml}$). Immunoblotting of cell lysates was performed as described above. To detect COL17, blots were incubated with 1:2000 diluted murine mAb NC16A-3 (31) for 1 h at room temperature, followed by incubation with 1:5000 diluted HRP-conjugated anti-mouse IgG Ab for 1 h at room temperature.

Detection of ubiquitinated COL17

To detect the ubiquitinated COL17, immunoprecipitation was done with mAb TS39-3. Cell lysates from NHKs, which were treated with rhIgG4 (60 $\mu\text{g}/\text{ml}$) and/or MG-132 (6.25 μM), were incubated with TS39-3 conjugated with Dynabeads protein G (Invitrogen) overnight at 4°C, or directly incubated with Dynabeads protein G for 2 h at 4°C. The beads were denatured and applied to 4–12% gradient Bis-Tris gels. Blots were incubated with 1:2000 diluted mAb NC16A-3 or 1:500 diluted anti-ubiquitin mAb P4D1 (Santa Cruz Biotechnology, Heidelberg, Germany)

for 1 h at room temperature, followed by incubation with 1:5000 diluted HRP-conjugated anti-mouse IgG for 1 h at room temperature. Bound Abs were detected using the ECL Prime detection kit.

Statistical analysis

Data are expressed as means $\pm$ SEM. A Dunnett multiple comparison test was used to identify differences between the COL17/ β -tubulin ratios from mice skin treated with versus without BP-IgGs, those from NHKs treated with versus without BP-IgGs, or those from NHKs treated with versus without monoclonal mIgGs. A Tukey all-pairwise comparison test was applied to identify differences between the COL17/ β -tubulin ratios from NHKs treated with rhIgGs versus control hIgGs, those from NHKs treated with versus without MG-132, or to identify differences between the affected body surface areas of hybridoma-transferred mice. The p values < 0.05 were considered significant.

Results

BP-IgG induces blister formation in complement-deficient COL17-humanized mice

To evaluate the pathogenicity of Abs against human COL17 in the absence of complements, we established in this study C3-deficient COL17-humanized ($C3^{-/-}$ /COL17-humanized) mice. Serum mC3 levels examined by ELISA were below the detection limit in neonatal $C3^{-/-}$ /COL17-humanized neonatal mice and were 380 ± 35.2 μ g/ml in neonatal COL17-humanized mice ($n = 7$ for both). We prepared BP-IgG from four patients and measured the titer of IgG specific to hNC16A by ELISA (index values: BP-IgG1, 5794.9; BP-IgG2, 2734.5; BP-IgG3, 1192.4; BP-IgG4, 842.6). The high purity of the BP-IgGs was confirmed by Coomassie blue staining (Fig. 1A). We i.p. injected these Abs (BP-IgG1 and BP-IgG2, 1000 μ g/mouse; BP-IgG3 and BP-IgG4, 1500 μ g/mouse) or normal hIgG (1500 μ g/mouse) into neonatal COL17-humanized mice and $C3^{-/-}$ /COL17-humanized mice. As previously reported (7), BP-IgGs induced dermal/epidermal separation in COL17-humanized mice (Fig. 1B, 1C). Direct IF revealed linear deposition of hIgG and mC3 at the DEJ of the injected mice skin (Fig. 1D, 1E). Interestingly, BP-IgGs also produced blisters in the $C3^{-/-}$ /COL17-humanized mice (Fig. 1F). Subepidermal separation and slight inflammatory cell infiltration were observed in the skin samples of the IgG-injected $C3^{-/-}$ /COL17-humanized mice (Fig. 1G). Direct IF revealed linear deposition of hIgG but not mC3 at the DEJ (Fig. 1H, 1I). Normal hIgG did not produce skin detachment. Neither hIgG nor mC3 was detected in skin samples from the control hIgG-injected mice (data not shown). The skin

detachment results are summarized in Table I. Next, we evaluated the BP-IgG1 transferred mice at 6 and 12 h after transfer. As shown in Table II, there is no significant difference in time span between COL17-humanized mice and $C3^{-/-}$ /COL17-humanized mice, suggesting that the presence of complement does not affect the time span of blister induction in neonatal mice. Thus, BP-IgGs were proven to induce blister formation in neonatal mice in a complement-independent manner.

Monoclonal hIgG Abs against hNC16A induce blister formation in mice in complement- and Fc γ RII/III-independent manners

Because the BP-IgGs were polyclonal, each might recognize different epitopes and have different pathogenicity. Therefore, we sought to compare the pathogenicity of Abs that recognize an identical epitope in hNC16A and have different abilities to produce complement activation. For this, we generated rhIgG1 and rhIgG4 mAbs against hNC16A by combining an Fab-B4 fragment that reacts to hNC16A (10) with Fc portions of hIgG1 or hIgG4 (Fig. 2A). It is acknowledged that the IgG1 subclass can activate all types of Fc receptors and the complement C1 component, whereas the IgG4 subclass has restricted Fc receptor-activating abilities and does not activate C1q (32–34). High purity of the rhIgGs was confirmed by Coomassie blue staining (Fig. 2B). The reactivity of the rhIgGs against hNC16A was confirmed by IF study using skin samples and human COL17-expressing mammalian cells (HEK293) (Fig. 2C, 2D) and ELISA. The ELISA index values for 0.6 μ g/ μ l purified rhIgG1 and rhIgG4 were 3013 and 1794, respectively. Epitope mapping of the rhIgGs and Fab-B4 by using four small synthesized peptides of hNC16A (13) revealed both rhIgGs bound to the R5 (Arg⁵⁰⁶ to Gln⁵²⁵) peptide that was also recognized by Fab-B4 (Fig. 2E). Complement fixation analysis by indirect IF demonstrated that rhIgG1 as well as BP-IgG1 were able to fix mC3 to the DEJ of NHS, whereas rhIgG4 and Fab-B4 were not (Fig. 3A). C1q-binding assay by ELISA showed that rhIgG4 and Fab-B4 demonstrated much lower binding activity to C1q than that of rhIgG1 (Fig. 3B).

To examine the pathogenicity of the rhIgGs in vivo, Abs (30 μ g rhIgG1 or control-hIgG1, 50 μ g rhIgG4 or control-hIgG4) were i.p. injected into neonatal COL17-humanized mice. Doses were determined by the results of hNC16A ELISA. As expected, rhIgG1 produced dermal/epidermal separation (Fig. 3C, 3D), al-

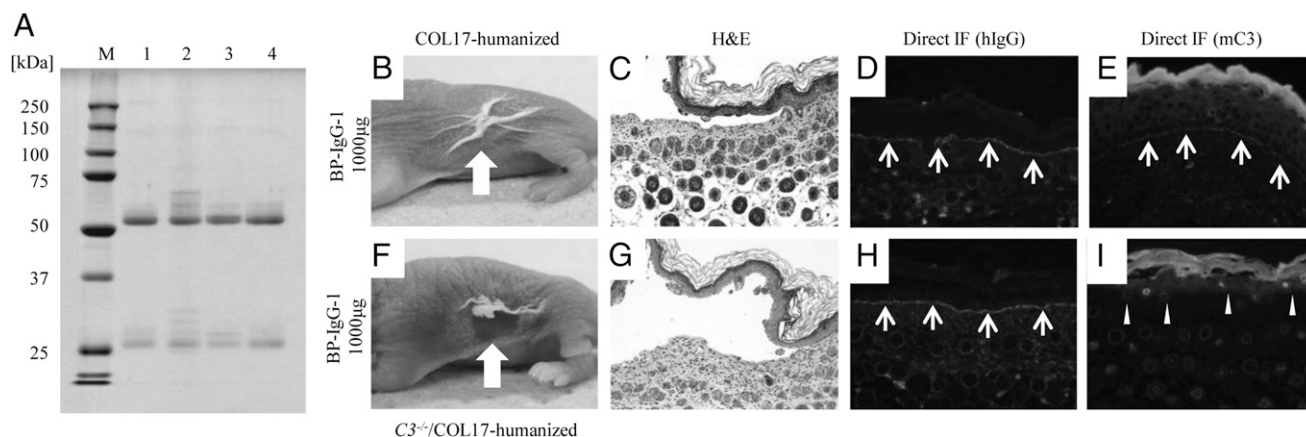


FIGURE 1. BP-IgGs induce blister formation in C3-deficient COL17-humanized mice. (A) Coomassie blue staining of affinity-purified BP-IgGs separated by SDS-PAGE on 12.5% polyacrylamide gel. M, marker. Lane 1, 1 μ g BP-IgG1; lane 2, 1 μ g BP-IgG2; lane 3, 1 μ g BP-IgG3; lane 4, 1 μ g BP-IgG4. (B–I) Representative clinical presentations, H&E staining, and hIgG and mC3 deposition 48 h after the passive transfer of BP-IgG1 into COL17-humanized mice (B–E) and into C3-deficient COL17-humanized mice (F–I). Skin detachment is indicated by an arrow. Linear deposition of hIgG and mC3 at the DEJ in direct IF is indicated by arrows. Negative results are indicated by arrowheads. H&E, original magnification $\times 20$; direct IF, original magnification $\times 20$.

Table I. Passive transfer of BP-IgG into neonatal mice

Injected Neonatal Mice	Abs	Ratio of Mice with Skin Detachment ^a
COL17-humanized	BP-IgG1 (1000 μ g)	5/5
	BP-IgG2 (1000 μ g)	4/5
	BP-IgG3 (1500 μ g)	5/5
	BP-IgG4 (1500 μ g)	5/5
	Normal hIgG (1500 μ g)	0/5
$C3^{-/-}$ /COL17-humanized	BP-IgG1 (1000 μ g)	5/5
	BP-IgG2 (1000 μ g)	5/5
	BP-IgG3 (1500 μ g)	4/5
	BP-IgG4 (1500 μ g)	4/5
	Normal hIgG (1500 μ g)	0/5

^aGentle skin rubbing test performed at 48 h in neonatal mice.

though control-hIgG1 or control-hIgG4 did not induce blister formation (Fig. 3G, 3H, 3O, 3P). Direct IF revealed linear deposition of hIgG and mC3 at the DEJ of the rhIgG1-injected mice skin (Fig. 3E, 3F). Neither hIgG nor mC3 was detected in skin samples from the control hIgG-injected mice (Fig. 3I, 3J, 3Q, 3R). Notably, all the mice injected with rhIgG4 developed dermal/epidermal separation, which is associated with linear deposition of hIgG but is not associated with a deposition of mC3 (Fig. 3K–N). Little inflammatory cell infiltration was observed in the skin samples of the rhIgG1- or rhIgG4-injected mice (Fig. 3D, 3L). At lower doses of rhIgGs, blister formation was more frequently induced by rhIgG1 (18 μ g, 87.5%; 9 μ g, 75%) than by rhIgG4 (30 μ g, 50%; 15 μ g, 62.5%) (Table III). The lowest doses of rhIgGs (4.5 μ g rhIgG1, 7.5 μ g rhIgG4) induced no skin detachment. Next, we injected 50 μ g rhIgG4 into human and murine COL17-expressing ($COL17^{m+/+,h+}$) mice. Despite the deposition of hIgG4 at the DEJ, no blister formation was induced (Fig. 2S). This suggests that IgG binding to hNC16A does not affect the function of murine COL17 in vivo. We also transferred rhIgGs into neonatal $C3^{-/-}$ /COL17-humanized mice. Both rhIgG1 (30 μ g) and rhIgG4 (50 μ g) induced blister formation in all $C3^{-/-}$ /COL17-humanized mice associated with linear deposition of hIgG but not mC3 at the DEJ of the skin (Table III). These results demonstrate that monoclonal hIgGs against hNC16A induce blister formation in mice regardless of complement activation.

We considered the possibility that rhIgGs may activate cellular immune responses not through complement activation but via the molecular interaction between the Fc portion of rhIgGs and the Fc γ R on the inflammatory cells. Therefore, we compared the rhIgG4-injected mice with versus without the administration of an Fc γ R blocker that inhibits both Fc γ RII and Fc γ RIII. Six of seven neonatal COL17-humanized mice pretreated with the Fc γ R blocker developed blisters (Table III). The same results were observed in mice pretreated with the equivalent dose of control rat IgG. Little inflammatory cell infiltration was observed in either group. Direct IF revealed linear deposition of hIgG but not mC3 at the DEJ in the injected mice in both groups. This suggested that rhIgG4 cause blister formation in neonatal mice in an Fc γ RII/III-independent manner. This is in line with the results that little in-

flammatory cell infiltration had been observed in the dermis of the rhIgG-injected mice.

The deposition of monoclonal murine IgG Abs at hNC16A was more relevant to the induction of blister formation than was in situ complement activation

We considered that the pathogenicity of mouse-derived Abs should also be examined, because the in vivo experiments in this study used mice. Moreover, we wanted to investigate the pathogenicity of Abs that react to the different epitope of hNC16A from that recognized by rhIgGs. We therefore generated hybridomas that produce murine mAbs against hNC16A. Using splenocytes from wild-type mice (C57BL/6) immunized by grafting their backs with human COL17-expressing mouse skin (9, 24), we obtained three mIgG1 clones (TS39-3, TS21-1, and TI66-1) and one mIgG2c clone (TS4-2) against hNC16A that strongly reacted to NHS (Fig. 4A). The hybridomas were derived from C57BL/6 mice; therefore, TS4-2 should be regarded as IgG2c instead of as IgG2a (35). The reactivity of TS4-2 to COL17-humanized mouse skin was quite weak (Fig. 4B). Epitope mapping of those monoclonal mIgGs using the four small synthesized peptides of hNC16A revealed that all the Abs bound to R7 (Asp⁵²² to Gln⁵⁴⁵) peptide (Fig. 5A). It is commonly known that the mIgG2 and mIgG3 subclasses are potent activators of complements, whereas mIgG1 is a weak activator (36–38). As we expected, C1q-binding activities were high in IgG2c (TS4-2) and low in IgG1 (TS39-3, TS21-1, and TI66-1) (Fig. 5B). We i.p. injected the Abs into neonatal COL17-humanized mice. As shown in Fig. 5C, a low dose of monoclonal IgG1 Abs (TS39-3, TS21-1, and TI66-1, 25 μ g) induced blisters in all the injected mice that were associated with weak deposition of mC3 at the DEJ (three of three), whereas a high dose of IgG2c (TS4-2, 500 μ g) caused weak deposition of IgG and distinct deposition of mC3 at the DEJ but showed a low potential to induce blister formation in the injected mice (positive: two of six) (Table IV). Low doses of monoclonal mIgG1 Abs (TS39-3, TS21-1, and TI66-1, 25 μ g) induced blisters in all of the neonatal $C3^{-/-}$ /COL17-humanized mice (Table IV), showing that monoclonal mIgG against the R7 region of hNC16A also induces blister formation in mice in a complement-independent

Table II. Time course of passive transfer of BP-IgG1 into neonatal mice

Abs (1000 μ g Each)	COL17-Humanized Mice with Skin Detachment		$C3^{-/-}$ /COL17-Humanized Mice with Skin Detachment	
	6 h	12 h	6 h	12 h
BP-IgG1	3/4	4/4	6/7	4/4
Normal hIgG	0/4	ND	0/3	ND

ND, not done.

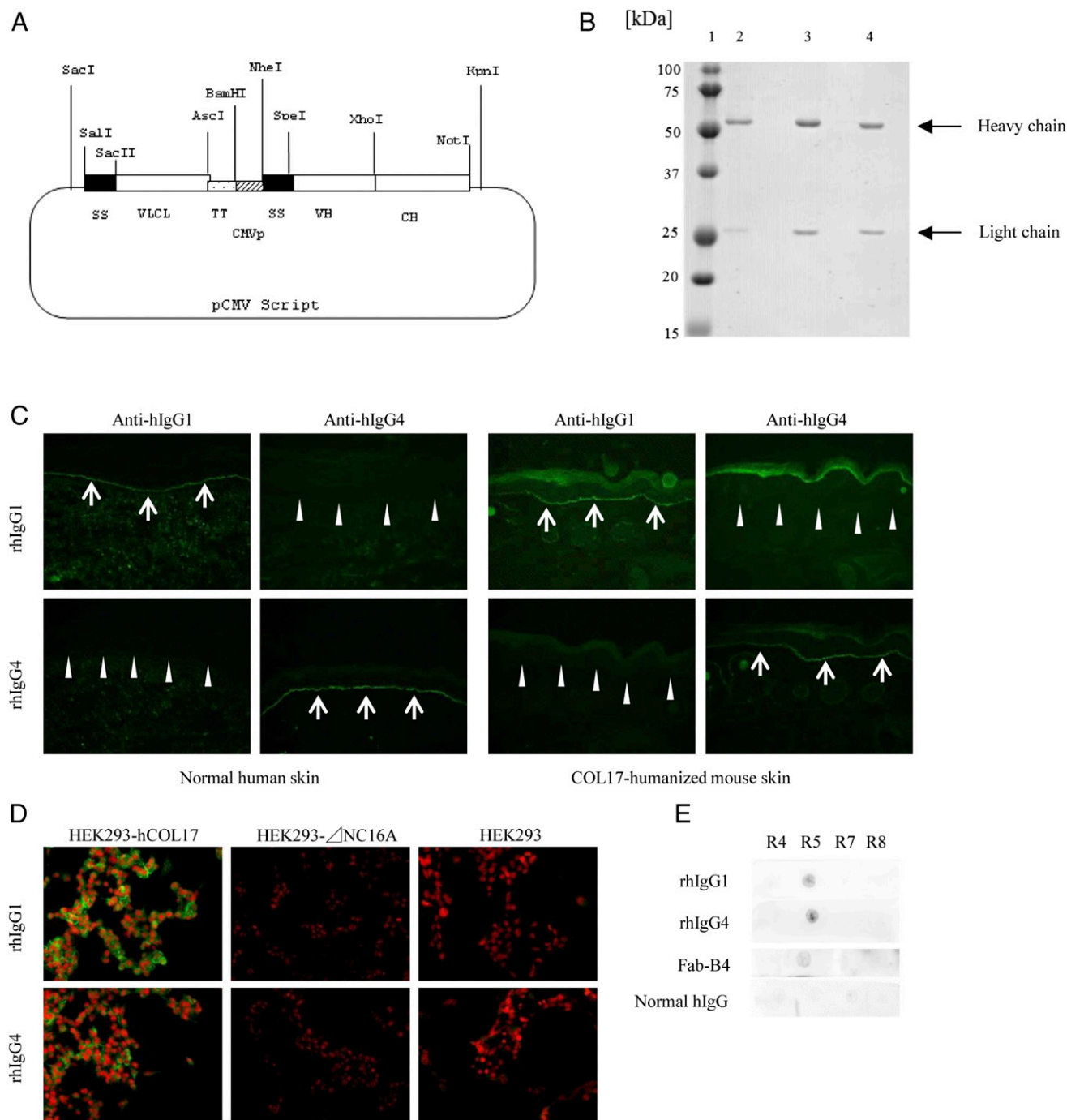


FIGURE 2. Monoclonal rhIgG1 and rhIgG4 Abs react to hNC16A with different ability to fix complement to the DEJ. **(A)** Scheme for expression vectors of rhIgGs. CH, C region H chain; SS, signal sequence; TT, terminator. **(B)** Coomassie blue staining of affinity-purified rhIgGs separated by SDS-PAGE on 12.5% polyacrylamide gel. Lane 1, marker; lane 2, 1 µg rhIgG1; lane 3, 1 µg rhIgG4; lane 4, 1 µg control IgG1. **(C and D)** The reactivity of the rhIgGs against hNC16A was examined by indirect IF using NHS and COL17-humanized mouse skin (C) or using HEK293 cells expressing full-length human COL17 (HEK293-COL17), HEK293 cells expressing human COL17 other than the NC16A domain (HEK293-ΔNC16A), or HEK293 cells (D). Linear deposition of hIgG at the DEJ is indicated by arrows, and negative results are indicated by arrowheads. **(E)** Epitopes of rhIgGs were examined by dot blot using four small synthesized peptides of hNC16A. (C and E) Original magnification $\times 20$. (D) Original Magnification $\times 40$.

manner. These results further indicate that the induction of BP blister formation is more directly related to the deposition of Ab against hNC16A than to that of complement.

Next, we sought to examine the necessity of complements in the induction of skin lesions in adult mice, because BP patients are adult humans and not newborns. For this, we s.c. transferred mIgG1 hybridoma or mIgG2c hybridoma into the backs of *Rag-2^{-/-}*/COL17-humanized mice that we previously generated (9). Four weeks after the transfer, each hybridoma formed a large mass on

the back. Mild skin changes develop on the ear and snout in the mIgG2c (TS4-2) hybridoma-transferred mice, which are associated with distinct mC3 deposition and with weak mIgG deposition (Fig. 6A, 6E). Meanwhile, significantly severer skin changes, that is, erythema, erosions with crusts, and hair loss, were observed on the snout and ears and around the eyes, legs, and tail in the mIgG1 (TS39-3, TS21-1, or TI66-1) hybridoma-transferred mice, which are associated with strong deposition of mIgG but not mC3 (Fig. 6B–E). Blister formation was not apparent. Ear samples from

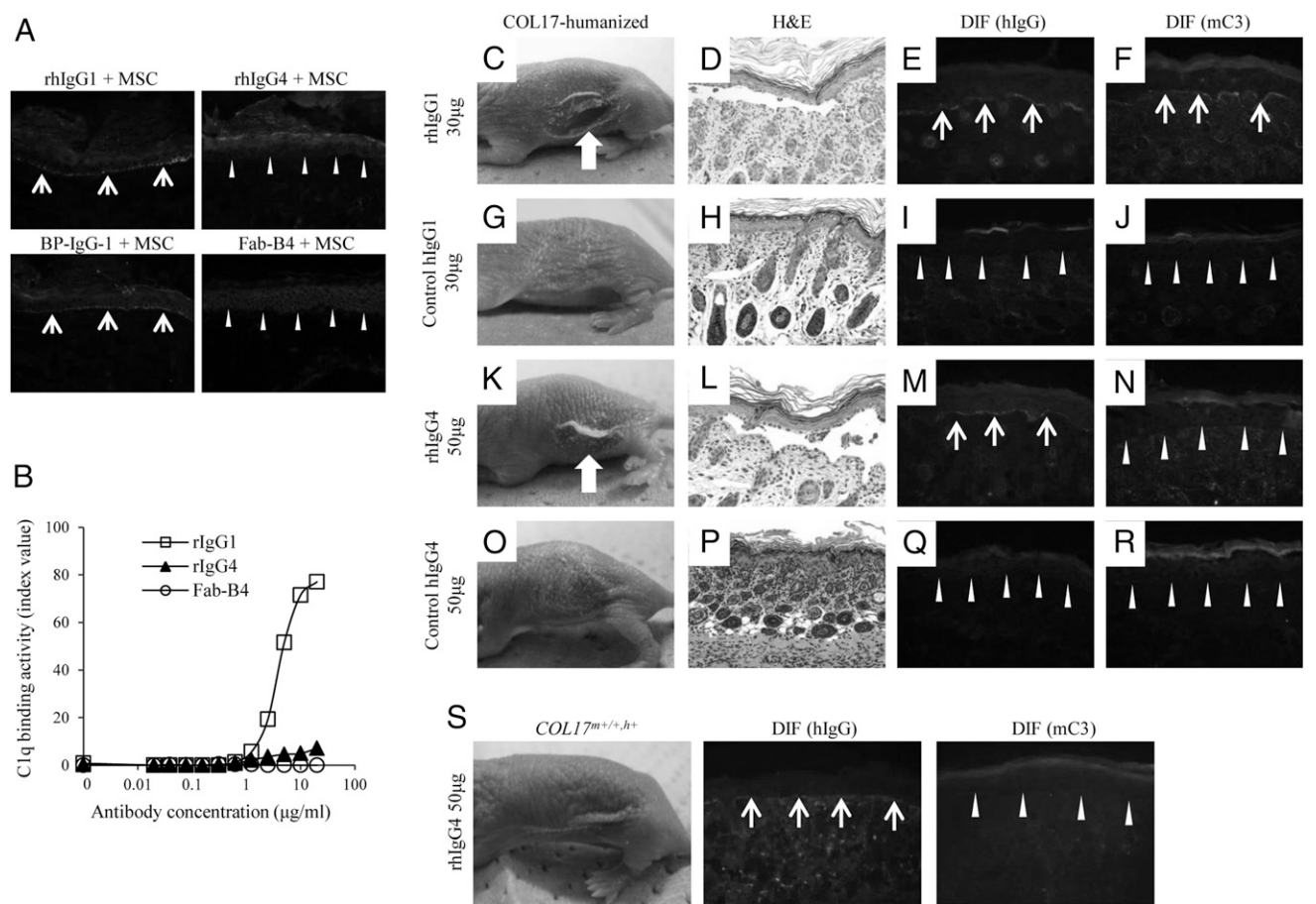


FIGURE 3. Monoclonal rhIgG Abs against hNC16A induce blister formation in mice regardless of complement activation. **(A)** The complement-fixing activity of the rhIgGs was evaluated by indirect IF using NHS and mouse serum complement (MSC). Linear deposition of mC3 at the DEJ is indicated by arrows, and negative results are indicated by arrowheads. **(B)** The C1q-binding activity of the rhIgGs was determined by human C1q-binding assay by ELISA. One representative result out of three is shown. **(C–R)** Representative clinical presentations, H&E staining, and hIgG and mC3 deposition 48 h after the passive transfer of rhIgG1, control hIgG1, rhIgG4, and control hIgG4 into neonatal COL17-humanized mice. Doses of rhIgGs were determined by the results of hNC16A ELISA. **(S)** Representative clinical presentations and hIgG and mC3 deposition in neonatal human COL17-transgenic (*COL17^{+/+,h+}*) mice at 48 h after passive transfer of rhIgG4. Linear deposition of hIgG at the DEJ in direct IF is indicated by arrows. Negative results are indicated by arrowheads. H&E, original magnification $\times 20$; direct IF, original magnification $\times 20$.

each group showed similar extents of inflammatory cell infiltration of neutrophils, lymphocytes, and histiocytes (Fig. 6A–D). Dermal/epidermal separation was also seen in some of the ear samples (positive/total: TS4-2, one of five; TS39-3, two of four; TS21-1, one of four; TI66-1, two of five; untreated, none of five). At day 28, most of the mice that were transferred with the mIgG1

Table III. Passive transfer of rhIgG1 and rhIgG4 against human COL17 NC16A into neonatal mice

Injected Neonatal Mice	Abs	Ratio of Mice with Skin Detachment ^a
COL17-humanized	rhIgG1 (4.5 μg)	0/4
	rhIgG1 (9 μg)	6/8
	rhIgG1 (18 μg)	7/8
	rhIgG1 (30 μg)	5/5
	Control hIgG1 (30 μg)	0/4
	rhIgG4 (7.5 μg)	0/4
	rhIgG4 (15 μg)	5/8
	rhIgG4 (30 μg)	4/8
	rhIgG4 (50 μg)	6/6
	Control hIgG4 (50 μg)	0/4
<i>C3^{-/-}</i> /COL17-humanized	FcγR blocker (20 μg) plus rhIgG4 (50 μg)	6/7
	FcγR blocker control (20 μg) plus rhIgG4 (50 μg)	6/7
	rhIgG4 (50 μg) plus MG-132	4/5
	rhIgG1 (30 μg)	4/4
	Control hIgG1 (30 μg)	0/4
<i>COL17^{+/+,h+}</i>	rhIgG4 (50 μg)	6/6
	Control hIgG4 (50 μg)	0/4
	rhIgG4 (50 μg)	0/6

^aGentle skin rubbing test performed at 48 h in neonatal mice.

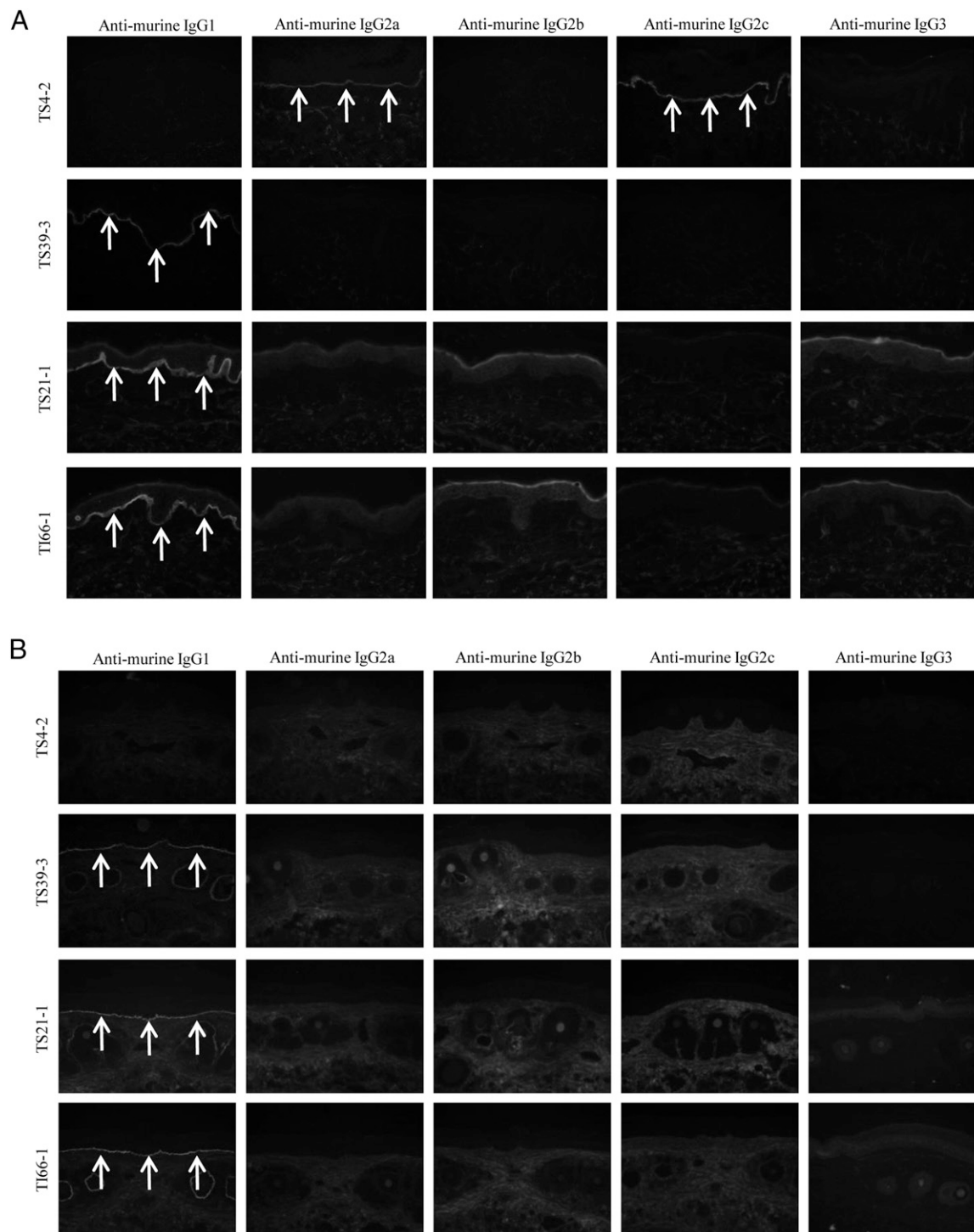


FIGURE 4. Indirect IF study of monoclonal mIgGs on NHS (**A**) and on COL17-humanized mouse skin (**B**). Anti-mIgG1, mIgG2a, mIgG2b, mIgG2c, and mIgG3 Abs were used as secondary Abs. Linear deposition of mIgG at the DEJ in direct IF is indicated by arrows. Original magnification $\times 20$.

hybridoma developed skin fragility on the tail (positive/total: TS39-3, four of four; TS21-1, four of four; TI66-1, four of five), whereas only one of the mice that were transferred with the mIgG2c hybridoma showed skin detachment (positive/total: TS4-2, one of five). We also examined the levels of circulating IgG against hNC16A in the sera of hybridoma-transferred mice by ELISA. The mean OD values in each group are as follows: TS4-2, 0.465 ± 0.033 ; TS39-3, 0.404 ± 0.013 ; TS21-1, 0.410 ± 0.017 ; TI66-1, 0.417 ± 0.028 ; untreated, 0.023 ± 0.001 (mean $\pm$ SD). Thus, each hybridoma produced roughly the same level of Abs in the host mice. As shown in Fig. 6F, disease severity in individual mice did

not relate to the level of circulating anti-hNC16A IgG. Altogether, blister formation in adult mice seems to correlate more directly with the amount of hNC16A-binding IgG Abs than with the extent of complement deposition or inflammatory cell infiltration.

Treatments of Abs against COL17 reduce the amount of COL17 both in vivo and in vitro

The results above strongly suggested that complement- and Fc γ R-independent effects would induce blister formation in vivo. Recent studies showed that Abs against COL17 deplete the amount of COL17 in cultured NHKs in a complement-independent manner

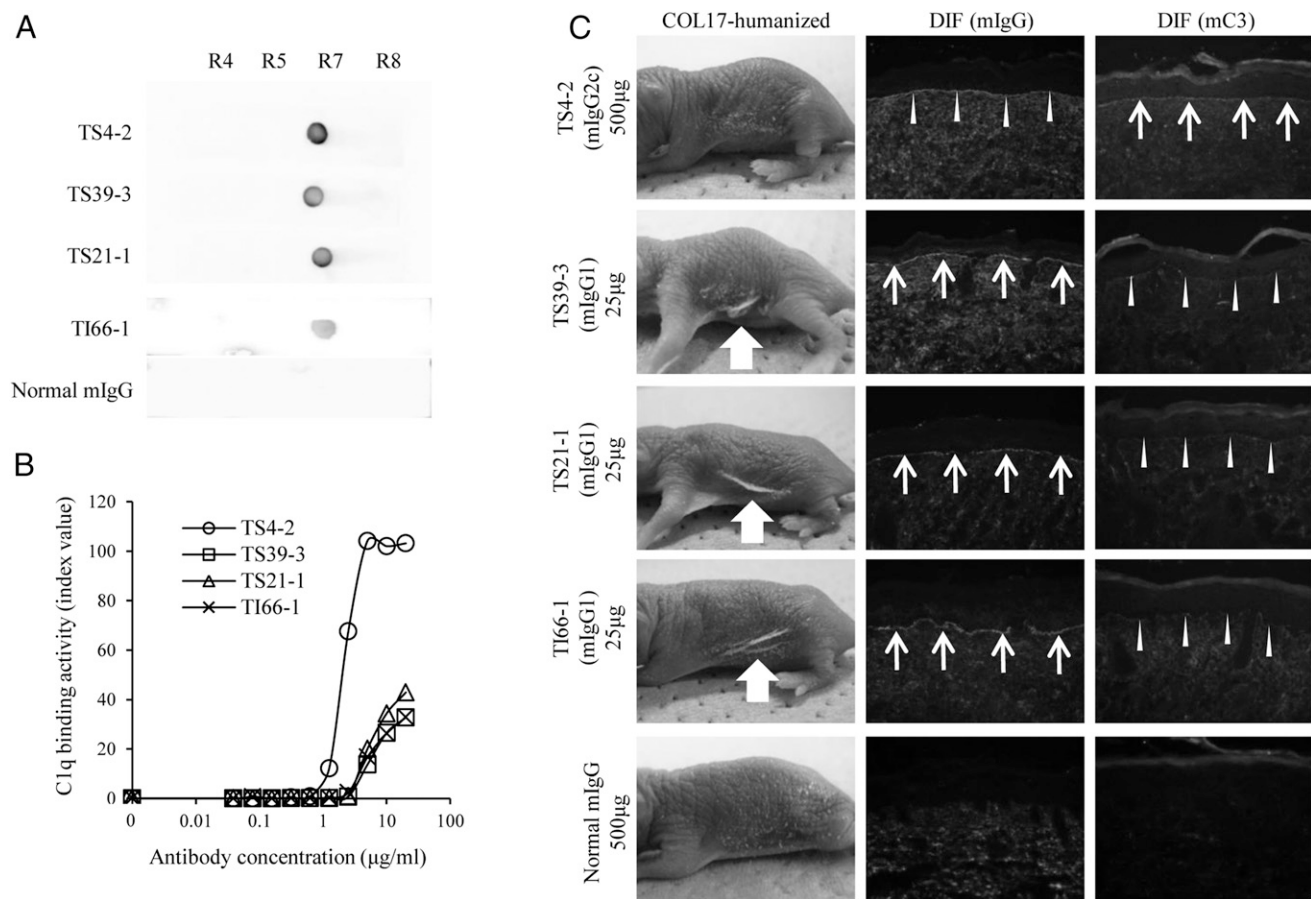


FIGURE 5. The deposition of monoclonal mIgG Abs at hNC16A is more relevant to the induction of blister formation than is complement activation. **(A)** Epitopes of monoclonal mIgGs from hybridomas were examined by dot blot using four small synthesized peptides of hNC16A. **(B)** The C1q-binding activity of the monoclonal mIgGs was determined by murine C1q-binding assay by ELISA. One representative result out of three is shown. **(C)** Representative clinical presentations and mIgG and mC3 deposition 48 h after the passive transfer of monoclonal mIgG2c, mIgG1s, or normal mIgG into neonatal COL17-humanized mice. Linear deposition of mIgG or mC3 at the DEJ is indicated by arrows (strongly positive) or arrowheads (weakly positive). Direct IF, original magnification $\times 20$.

(13, 22). To investigate this mechanism in the present experimental system, we first examined the amount of COL17 in the skin of BP-IgG-treated $C3^{-/-}$ /COL17-humanized mice at 48 h after injection. When compared with untreated mice as normalized to β -tubulin, the amount of COL17 was found to be reduced by ~ 15 – 25% by BP-IgG treatment, although the difference was not statistically significant (Fig. 7A). We then performed *in vitro* experiments. When compared with the results of control hIgG treatments, $\sim 50\%$ ($p < 0.01$) of the COL17 treated with BP-IgG1, BP-IgG3, and BP-IgG4, or 40% ($p < 0.05$) of the COL17 treated with BP-IgG2 was depleted from the cells at 6 h (Fig. 7B). Additionally, rhIgG1, rhIgG4, and mIgG1s, but not mIgG2c, significantly depleted COL17 in NHKs when compared with the results

of control-IgG-treated cells (Fig. 7C, 7D). Combined with the results of passive transfer experiments, these findings suggest that the induction of blister formation correlates with the affinity of Abs to hNC16A and with COL17 depletion.

Ab-bound COL17 is ubiquitinated and degraded by proteasome

We attempted to further investigate the mechanism whereby COL17 is depleted by pathogenic Ab binding. The ubiquitin/proteasome pathway is well known as a major system for protein degradation (39). To assess whether the pathway is involved in COL17 depletion, the ubiquitination of COL17 in rhIgG4-treated NHKs was examined. Before that, we confirmed that up to $12.5\ \mu\text{M}$ MG-132, a very potent proteasome inhibitor, did not

Table IV. Passive transfer of murine IgG1 and IgG2c against human COL17 NC16A into neonatal mice

Abs (Subclass)	COL17-Humanized Mice with Skin Detachment ^a				$C3^{-/-}$ /COL17-Humanized Mice with Skin Detachment ^a	
	500 μg	100 μg	50 μg	25 μg	500 μg	25 μg
TS4-2 (IgG2c)	2/6	0/3	0/3	ND	2/3	0/3
TS39-3 (IgG1)	ND	3/3	3/3	3/3	ND	3/3
TS21-1 (IgG1)	ND	3/3	3/3	3/3	ND	3/3
TI66-1 (IgG1)	ND	3/3	3/3	3/3	ND	3/3
Normal mIgG	0/4	0/4	ND	ND	0/3	ND

^aGentle skin rubbing test performed at 48 h in neonatal mice.
ND, not done.

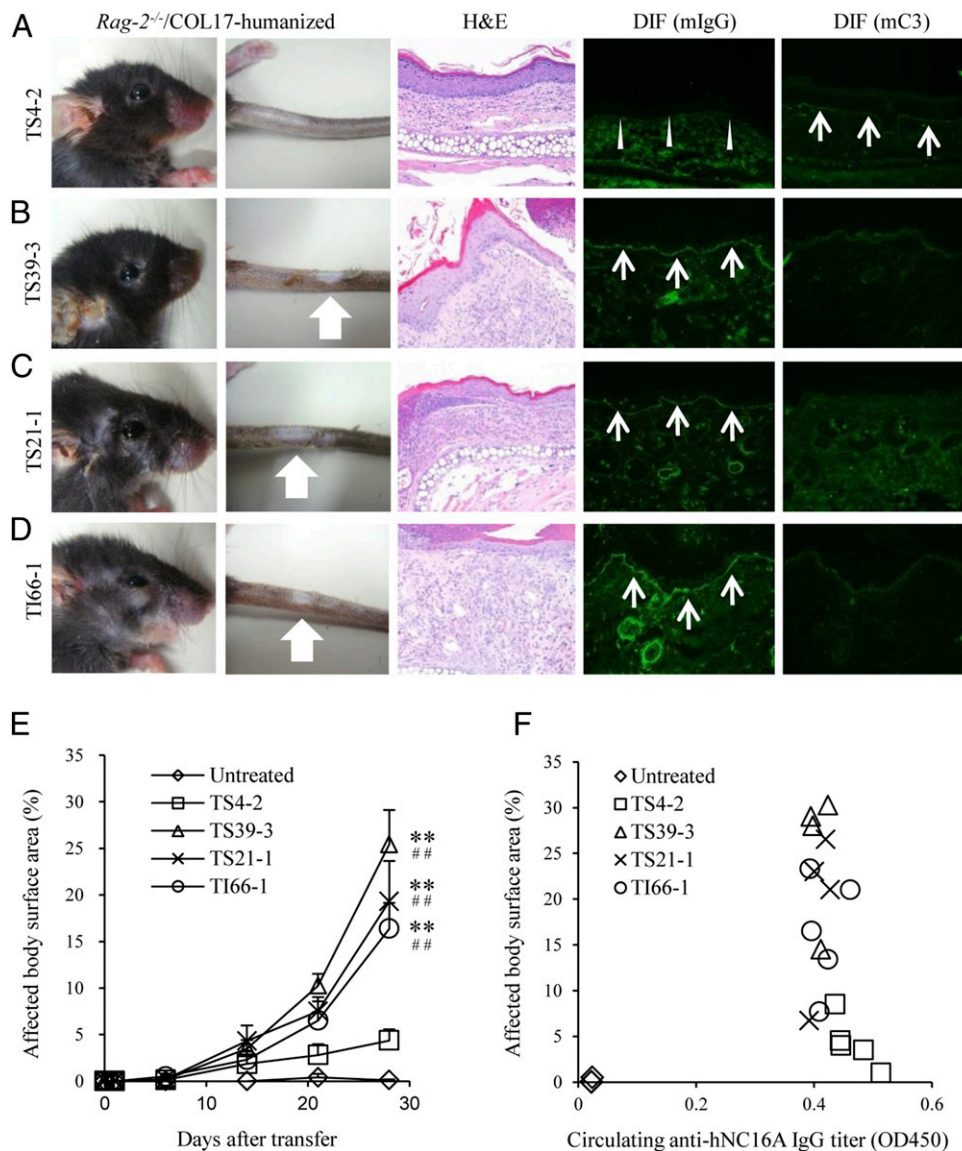


FIGURE 6. Mice receiving hybridomas that produce monoclonal mIgG Abs against hNC16A develop skin lesions in a complement-independent manner. (A–D) Representative clinical presentations, H&E staining, and mIgG and mC3 deposition 28 d after the adoptive transfer of mIgG2c hybridomas or mIgG1 hybridomas on the back of *Rag-2*^{-/-}/COL17-humanized mice. Linear deposition of mIgG or mC3 at the DEJ in direct IF is indicated by arrows. Weak deposition of mIgG is indicated by arrowheads. H&E, original magnification $\times 14$; direct IF, original magnification $\times 20$. (E) The affected body surface area of the transferred mice. Results are expressed as means $\pm$ SEM. Days 0–21: $n = 5$ in each group; day 28: $n = 4$ in TS39-3 and TS21-1, and $n = 5$ in untreated, TS4-2, and TI66-1. ** $p < 0.01$ versus untreated, *** $p < 0.01$ versus TS4-2. (F) Summary of the affected body surface area and circulating anti-hNC16A IgG titers of the transferred mice at day 28.

affect the viability in cultured NHKs (data not shown). COL17 was purified from NHKs after the treatment of rhIgG4 and/or MG-132 by immunoprecipitation using IgG Ab against hNC16A (TS39-3), and ubiquitination was checked by immunoblotting. Ubiquitination of COL17 in NHKs treated with rhIgG4 was more clear than that in untreated NHKs, indicating that rhIgG4 treatment promotes the ubiquitination of COL17 (Fig. 7E). Ubiquitination of rhIgG4-bound COL17 was more obvious when the COL17/rhIgG4 complex was purified from NHKs by immunoprecipitation using protein G beads (Fig. 7F). Next, we added different concentrations of MG-132 to NHKs with or without rhIgG1, rhIgG4, or BP-IgG1 and examined the expression of COL17. MG-132 was found to significantly inhibit the COL17 depletion in a dose-dependent manner (Fig. 7G). These results indicate that the ubiquitin/proteasome pathway might be involved in the degradation of IgG-bound COL17. We further evaluated the

effect of proteasome inhibitor in vivo. Intraperitoneal injection of MG-132 prior to the injection of rhIgG4 did not prevent the induction of blister formation in the treated mice (Table III), suggesting that several steps might exist between the blister formation by IgG binding and the COL17 depletion.

Discussion

To our knowledge, this study is the first to demonstrate the pathogenicity of Abs against COL17 in complement-deficient mice. BP-IgG caused blisters in neonatal C3-deficient COL17-humanized mice, of course, without complement activation. We recently reported that differences in Ab epitopes may considerably influence blister formation in neonatal COL17-humanized mice (13). To exclude the influence of the difference in epitopes, we generated novel rhIgG1 and rhIgG4 mAbs that strongly bind to an identical epitope on hNC16A. It was not only rhIgG1, which has

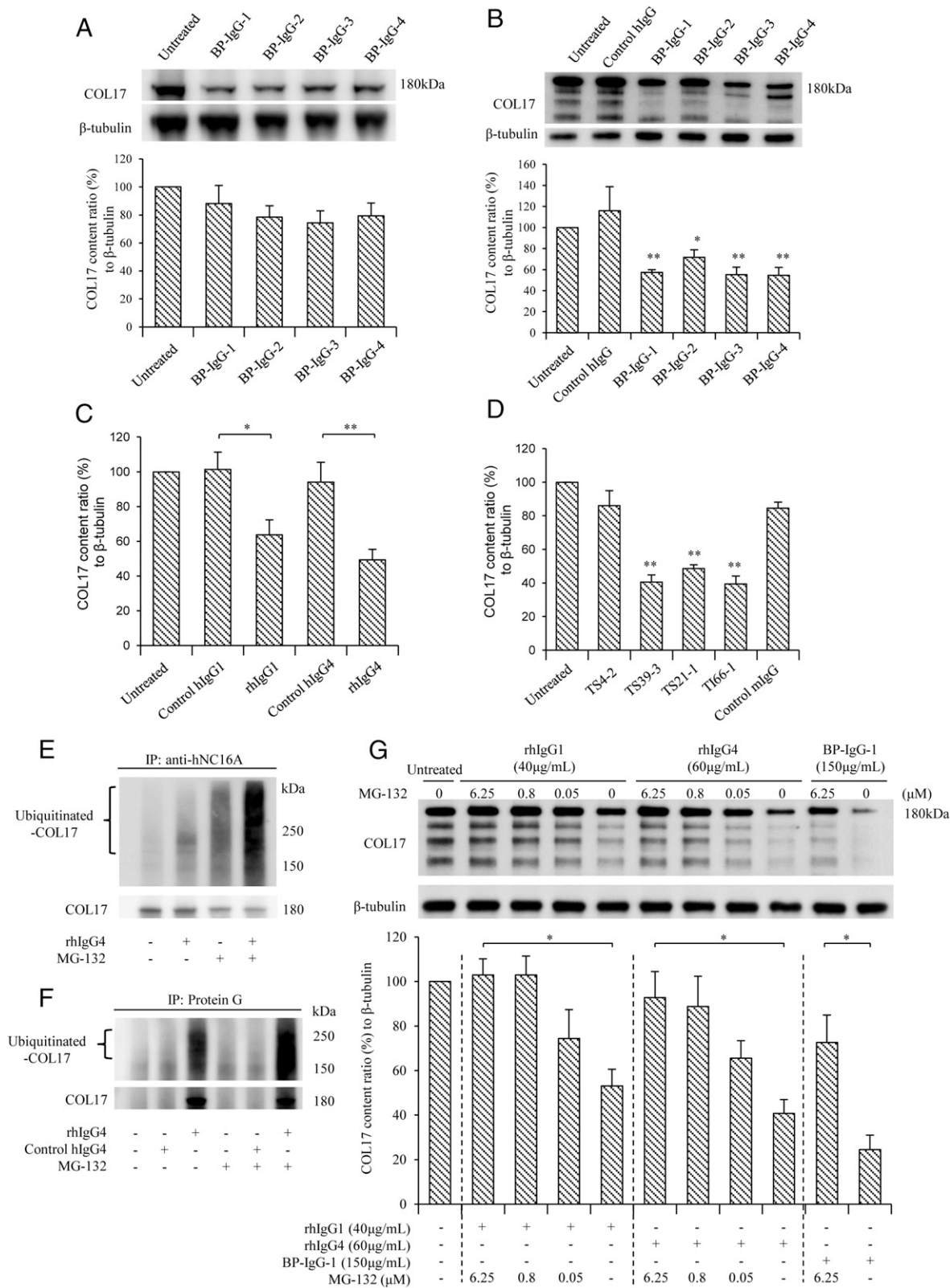


FIGURE 7. IgG binding to COL17 induces COL17 depletion via the ubiquitin/proteasome pathway. **(A)** Neonatal C3^{-/-}/COL17-humanized mice were injected with BP-IgG. The amount of COL17 (180 kDa) in the skin was examined by immunoblotting. COL17 content was normalized to β -tubulin. The COL17/ β -tubulin ratio of untreated mice skin was calculated at 100%. Results are expressed as means $\pm$ SEM ($n = 5$). **(B)** Cultured NHKs were incubated with BP-IgGs or control hlgG. The amount of COL17 (180 kDa) in the cell lysates was detected by immunoblotting. Results are expressed as means $\pm$ SEM ($n = 6$). * $p < 0.05$, ** $p < 0.01$ versus control hlgG. **(C)** Cultured NHKs were incubated with rhlgG1, control hlgG1, rhlgG4, or control hlgG4 for 6 h. The amount of COL17 (180 kDa) in the cell lysates was detected by immunoblotting. Results are expressed as means $\pm$ SEM ($n = 6$). * $p < 0.05$ versus control hlgG1, ** $p < 0.01$ versus control hlgG4. **(D)** Cultured NHKs were incubated with mlgGs from hybridomas or control normal mlgG for 6 h. The amount of COL17 (180 kDa) in the cell lysates was detected by immunoblotting. Results are expressed as means $\pm$ SEM ($n = 5$). ** $p < 0.01$ versus control mlgG. **(E)** NHKs were untreated or treated with rhlgG4 and/or MG-132, and lysates were subjected to immunoprecipitation with (Figure legend continues)

high complement activation ability, that led to blister formation in neonatal COL17-humanized mice. rhIgG4, which lacks complement activation ability, also led to such formation. Additionally, we showed that monoclonal mIgGs against the different epitope of hNC16A from those recognized by rhIgGs also cause blister formation in a complement-independent manner both in neonatal and adult mice. Thus, we confirmed the complement-independent pathogenicity of IgG Abs against COL17 in three distinct ways.

Previous studies using a polyclonal rabbit IgG passive transfer mouse model suggested that complement activation has primary importance in the pathogenesis of BP (16, 40). Our previous study showed that the administration of Fab fragments to hNC16A into neonatal COL17-humanized mice inhibits the complement pathway and ameliorates skin detachment (10). We also reported that hIgG1 against hNC16A with mutations at the C1q-binding Fc portion has lower pathogenicity in neonatal COL17-humanized mice than has nonmutated hIgG1 (11). More recently, we demonstrated that passive transfer of F(ab')₂ fragments of BP-IgG or rabbit IgG against hNC16A induces skin detachment in neonatal COL17-humanized mice (13). These contradictory results can be explained by the differences in the target epitopes on COL17, the applied dose of Abs, the affinity of Abs to the target molecules, or the clonality of Abs in each experimental system. Our present data demonstrate that, at low doses, rhIgG4 has less potential to induce blister formation than does rhIgG1 (Table III). Thus, complement activation may make some contribution to blister formation in neonatal mice, although it is dispensable under our experimental system.

We also demonstrated that blockade of FcγRII/III does not inhibit skin detachment in rhIgG-injected mice. Little inflammatory cell infiltration was observed in skin samples from the rhIgG-injected neonatal mice. Taken together, cellular immune responses mediated by the Fc portions of rhIgG may not be involved in the pathogenesis of our experimental system. A recent study showing FcR-independent effects of BP-IgG and BP-IgE on cultured NHKs and human skin organ cultures (23) is consistent with our results.

Although the IgG4 subclass accounts for only ~5% of total IgG in normal human sera, IgG4 autoantibodies have been reported to predominate in BP (41, 42). The capacity of the predominant IgG4 to induce tissue damage has been demonstrated by cryosection assay (34). In that study, IgG4 autoantibodies were found to have no capacity to fix complements but did show lower pathogenicity than did IgG1 with inducing the activation of leukocytes in an FcR-dependent manner in vitro. Our present data demonstrate that rhIgG4 induces blister formation in neonatal mice in complement- and FcγR-independent ways. These findings strongly suggest that not only do IgG1 autoantibodies contribute to the development of BP blister formation, but also do IgG4 autoantibodies.

COL17 depletion has been proposed as an alternative pathomechanism of BP. BP-IgG has been demonstrated to reduce the amount of COL17 in cultured NHKs (22). Additionally, we recently reported that the pathogenic IgG autoantibodies against hNC16A depletes COL17 in cultured NHKs (13). The present study shows that the administration of IgG against hNC16A reduces the amount of COL17 in mice skin and in cultured NHKs. We also demonstrated that the administration of rhIgG4 to human

COL17-transgenic (*COL17^{m+/+.h+}*) mice, which express both human and murine COL17, induces no blister formation, suggesting that the rhIgG which binds to hNC16A does not affect the function of murine COL17 in vivo. These findings show that IgGs against hNC16A directly deplete human COL17, which results in the impairment of hemidesmosomal formation and the decrease of adhesive strength. Additionally, monoclonal mIgG2c (TS4-2), which has a low affinity to hNC16A, a low ability to deplete COL17, and a high ability to activate complements, did not induce skin detachment in most treated mice. This strongly suggests that IgG binding to COL17 and subsequent COL17 depletion more directly correlate with the induction of blister formation than does complement activation. As a possible mechanism of COL17 depletion by IgG binding, the internalization of COL17 has been shown (43, 44). It was recently reported that BP-IgG induces COL17 internalization via a macropinocytic pathway and that intracellular and extracellular domains of COL17 are internalized simultaneously, suggesting that ectodomain shedding of COL17 does not necessarily occur in COL17 internalization and would not be essential to BP disease onset (45). Intracellular proteins are degraded to amino acids and replaced by newly synthesized proteins. Most proteins destined for degradation are labeled by ubiquitin and then digested to small peptides by a proteasome (39). We demonstrated that COL17 is ubiquitinated by the treatment of rhIgG and that a proteasome inhibitor inhibits the COL17 depletion in a dose-dependent manner. These indicate the contribution of the ubiquitin/proteasome pathway to COL17 depletion. Administration of a proteasome inhibitor to the rhIgG4-injected mice did not prevent the induction of blister formation, suggesting that additional processes such as the internalization of COL17 cause the reduction of the adhesive strength before degradation by proteasomes. A recent study showed that the administration of mAbs against anaplastic lymphoma kinase induces the internalization of cell surface anaplastic lymphoma kinase, resulting in degradation by proteasomes (46). Further investigations of the mechanism of the internalization of COL17 caused by IgG binding may provide a new insight into the pathomechanisms of BP.

In interpreting the results, the limitations of each experimental system should be taken into consideration. In this study, we evaluated the pathogenicities of BP-IgG and human and murine mAbs mainly based on the blister formation of neonatal mice subjected to artificial rubbing. From the passive transfer model, IgG binding to COL17 seems to have enough of a direct effect to significantly weaken the adhesive strength of the neonatal skin. The COL17 depletion must induce blister formation in humans, because mutations in COL17 seen in junctional epidermolysis bullosa patients cause subepidermal blistering without neutrophil infiltration (47). Nevertheless, we cannot exclude the possibility that subsequent inflammatory immune reactions are necessary for spontaneous blister formation in human BP patients, because BP patients generally develop itchy edematous erythema as well as tense blisters. Similarly, an active adult BP mouse model that may more closely reproduce human BP than the neonatal model shows itchy erythema and blisters associated with strong deposition of mIgG and mC3 at the DEJ (9). Also note that the mechanically

mAb TS39-3 against hNC16A. Ubiquitin and COL17 were detected by immunoblotting with Abs against ubiquitin (*top*) or against hNC16A (*bottom*), respectively. One representative result out of two is shown. (F) NHKs were untreated or treated with rhIgG4, control hIgG4, and/or MG-132, and lysates were subjected to immunoprecipitation with protein G. Ubiquitin and COL17 were detected by immunoblotting with Abs against ubiquitin (*top*) or against hNC16A (*bottom*), respectively. One representative result out of two is shown. (G) NHKs were incubated with rhIgG1, rhIgG4, or BP-IgG1 with several doses of MG-132. The amount of COL17 (180 kDa) in the cell lysates was examined by immunoblotting. Results are expressed as means ± SEM (*n* = 5).

**p* < 0.05.

induced blister formation in the passive transfer model does not exactly reproduce the spontaneous blistering that characterizes the human disease. Additionally, the transient disease activity does not fully allow us to examine the pathogenic mechanisms of the chronic disease. To address these limitations of the passive transfer model, we also performed a hybridoma transfer study using adult mice. Remarkably, to our knowledge, that study is the first demonstration that adult mice develop skin lesions such as erythema, erosions, and hair loss by the binding of IgG Ab against COL17 without complement deposition. However, spontaneous blister formation was not observed in the hybridoma transfer model, and its skin changes were less severe than those seen in the active adult BP mouse model mentioned above, suggesting a possible contribution of complement activation in the development of skin lesions in adult mice. Previous *in vivo* and *in vitro* studies have demonstrated that blister induction in BP depends on the presence of neutrophils (18, 48). As previously mentioned (22), a combination of COL17 depletion and nonspecific inflammatory enzymatic activity at the DEJ induced by Abs against COL17 may be necessary for the development of the severe BP disease phenotype observed in BP patients and in the active adult BP mouse model. Interestingly, the mIgG1 hybridoma-transferred mice showed inflammatory cell infiltration including neutrophils despite a lack of mC3 deposition. It has been reported that Abs against COL17 directly promote the production of IL-6 and IL-8, proinflammatory cytokines that are reported to be elevated in the sera and tissues of BP patients, by keratinocytes (23, 49). This pathway would enable Abs against COL17 to recruit neutrophils to the DEJ regardless of complement activation.

We should also consider species-related differences between human IgGs (BP-IgGs and rhIgGs) and murine innate immune components, for example, complement and neutrophils, in our experimental systems. Human autoantibodies against COL17 have been reported to have a lower capacity to fix murine complement and a reduced ability to activate murine leukocytes when compared with human complement and cells, respectively (50). In this study, complement activation was observed in the skin of neonatal COL17-humanized mice that were injected with BP-IgGs or rhIgG1, although this experimental system may not reflect the *in vivo* situation in BP patients in terms of species difference. To overcome this issue, we further performed experiments using mIgGs and reached the same conclusion.

Although humanization of COL17 enables us to evaluate the pathogenicity of BP-IgGs against hCOL17 *in vivo*, it raises the disadvantage of cross-species interaction between hCOL17 and other murine hemidesmosomal components. Hemidesmosomes are multimolecular complexes that promote the stable adhesion of epithelial cells to the underlying extracellular matrix. COL17 is known to interact with BP230, plectin, $\alpha_6\beta_4$ integrin, laminin 332, and collagen IV (51–53). There is a possibility that COL17-humanized mice have less skin stability than do wild-type mice owing to the insufficient interaction between hCOL17 and other murine components, which may lead to skin fragility in COL17-humanized mice in a complement-independent manner, although we have shown that without the transfer of pathogenic Abs, neonatal COL17-humanized mice skin is intact (Tables I–IV). To further address this issue, experiments using pathogenic mAbs against murine COL17 and $C3^{-/-}$ mice are needed.

In conclusion, this study revealed that BP autoantibodies induce blister formation in neonatal and adult mice in a complement-independent manner. The blister formation mainly depends on the COL17 depletion caused by IgG binding to hNC16A that is presumably followed by the internalization, ubiquitination, and degradation of COL17 molecules by proteasomes in the cytoplasm.

Our results indicate that the blocking of autoantibody binding to COL17, but not the prevention of complement activation, should be considered as the foremost therapeutic strategy for BP.

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Disclosures

The authors have no financial conflicts of interest.

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