The American Journal of Pathology, Vol. 184, No. 8, August 2014

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The American Journal of
PATHOLOGY
ajp.amjpathol.org

ANIMAL MODELS

Fcγ Receptors III and IV Mediate Tissue Destruction in a Novel Adult Mouse Model of Bullous Pemphigoid

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Accepted for publication May 13, 2014.

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Bullous pemphigoid (BP) and epidermolysis bullosa acquisita are subepidermal autoimmune blistering diseases mediated by autoantibodies against type XVII collagen (Col17) and Col7, respectively. For blister formation, Fc-mediated events, such as infiltration of inflammatory cells in the skin, complement activation, and release of proteases at the dermal-epidermal junction, are essential. Although in the neonatal passive transfer mouse model of BP, tissue destruction is mediated by Fcγ receptors (FcγRs) I and III, the passive transfer model of epidermolysis bullosa acquisita completely depends on FcγRIV. To clarify this discrepancy, we developed a novel experimental model for BP using adult mice. Lesion formation was Fc mediated because γ-chain−deficient mice and mice treated with anti-Col17 IgG, depleted from its sugar moiety at the Fc portion, were resistant to disease induction. By the use of various FcγR-deficient mouse strains, tissue destruction was shown to be mediated by FcγRIV, FcγRIII, and FcγRIIB, whereas FcγRI was not essential. Furthermore, anti-inflammatory mediators in already clinically diseased mice can be explored in the novel BP model, because the pharmacological inhibition of FcγRIV and depletion of granulocytes abolished skin blisters. Herein, we extended our knowledge about the importance of FcγRs in experimental BP and established a novel BP mouse model suitable to study disease development over a longer time period and explore novel treatment strategies in a quasi-therapeutic setting. *(Am J Pathol 2014, 184: 2185−2196; http://dx.doi.org/10.1016/j.ajpath.2014.05.007)*

Bullous pemphigoid (BP) is by far the most frequent autoimmune bullous disease in Central Europe and Northern America.[1,2] Immunopathologically, BP is characterized by autoantibodies against type XVII collagen (Col17; alias BP180), a component of the dermal-epidermal junction (DEJ).[3,4] Most BP sera react with epitopes clustered within the 16th noncollagenous (NC16A) domain of Col17.[5] Several animal models for this disease have been developed during the past 20 years and fostered the extensive exploration of the sequence of events leading to separation of the DEJ.[6−12] Although we and others have shown that Fc receptor (FcR)−independent mechanisms may contribute to the pathogenesis,[13−15] FcR-mediated events, via anti-Col17 immunoglobulin−Col17 immune complexes, were shown to be pivotal. These events included the activation of complement and inflammatory cells, including mast cells, macrophages, and, most important, neutrophils.[16,17] Their release of reactive oxygen species and specific proteases finally leads to the separation of the DEJ.[18−21]

In contrast to the relatively well-understood pathogenesis of BP, little progress has been made in the treatment of the

Supported by Excellence Cluster Inflammation at Interfaces grant EXC306/1 (D.Z., R.L., and E.S.), the University of Lübeck (E.S.), Graduate College Modulation of Autoimmunity grant GRK1727/1-TP7 (E.S.), Swedish Research Council projects 2009-3759 and 2012-1875 (M.C.), Foundations of Åke Wiberg (M.C.), Alfred Österlund (M.C.), Gyllenstierna-Krapperup (M.C.), Royal Physiografic Society in Lund (M.C.), King Gustaf V's 80 years fund (M.C.), Swedish governmental funding for clinical research (M.C.), and Hansa Medical AB (M.C.).

Disclosures: Hansa Medical (HM) AB has filed patent applications for using EndoS as a treatment for antibody-mediated diseases. M.C. is listed as one of the inventors on these applications, has a royalty agreement with HM, and is a scientific consultant for HM. HM was not involved in any way in the design of the study, the writing of the manuscript, or the decision to publish.

http://dx.doi.org/10.1016/j.ajpath.2014.05.007

disease. Long-term use of superpotent topical or systemic corticosteroids is still the therapeutic backbone, frequently combined with further immunosuppressants/immunomodulants, such as dapsone, tetracycline, methotrexate, and azathioprine, for their potential, yet unproved, corticosteroid-sparing effect.[2,22] More specific therapeutic options are highly needed to reduce the adverse reactions associated with the current regimens that account for a high morbidity and mortality in the elderly patient population.[23,24]

In the present study, we focused on the role of FcγRs in the pathogenesis of experimental BP because of their high potential as future therapeutic targets in FcγR-mediated diseases.[25,26] Furthermore, we aimed at clarifying somewhat inconsistent results obtained in the neonatal mouse model of BP, in which a critical role of the FcγRIII, but not the FcγRI and FcγRIIB, was identified (no data about FcγRIV were available at that time), and experimental epidermolysis bullosa acquisita (EBA).[27,28] In the latter model, tissue destruction was completely dependent on FcγRIV with a clear protective effect of FcγRIIB, whereas no effect of FcγRI and FcγRIII was detected.[27] For this purpose, a novel experimental model of BP was established on the basis of the passive transfer of rabbit anti-murine BP180 15th noncollagenous domain (NC15A) IgG in adult mice. In this model, tissue destruction was completely dependent on the Fc portion of anti-Col17 IgG and mediated by FcγRIV and, to a somewhat lesser extent, by FcγRIII, whereas FcγRIIB was protective.

Materials and Methods

Mice

C57Bl/6J, BALB/c, *FcγR*$^{-/-}$ (B6;129P2-*Fcer1g*^{tm1Rav}/J), *FcγRIIB*$^{-/-}$, and *FcγRI*$^{-/-}$ (BALB/c background) were bred and housed in a 12-hour light-dark cycle at the experimental animal facility of the University of Lübeck (Lübeck, Germany). *FcγRIII*$^{-/-}$ mice (B6.129P2-*Fcgr3*^{tm1Sjv}/J) were obtained from The Jackson Laboratories (Bar Harbor, ME). *FcγRI*$^{-/-}$ (C57Bl/6 background) and *FcγRIV*$^{-/-}$ (C57Bl/6 background) mice were held at the University of Erlangen-Nuremberg (Erlangen, Germany). All experiments were performed on 8- to 12-week-old mice narcotized by i.p. injection of a mixture of 100 μg/g ketamine and 15 μg/g xylazine. Animal experiments were approved by the Animal Care and Use Committee of Schleswig-Holstein (21-2/11) and performed by F.S.S. and T.B.

Generation and Characterization of Rabbit Antibodies to mCol17

The extracellular portion of the NC15A of murine collagen type XVII (mCol17) covering the 76 amino acids directly adjacent to the transmembrane domain (amino acids 497 to 573 of murine BP180) was expressed as glutathione-*S*-transferase (GST) fusion protein and purified by affinity chromatography, as previously described.[10] Pathogenic anti-Col17 IgG was generated, as reported previously.[29] In brief, New Zealand white rabbits were immunized with recombinant forms of the GST-tagged NC15A domain of mCol17, the rabbit serum purified by affinity chromatography using protein G. Reactivity of IgG fractions was analyzed by indirect immunofluorescence (IF) microscopy on murine skin. The leukocyte-activating capacity of purified anti-Col17 IgG was evaluated *ex vivo* on cryosections of mouse skin, as reported.[30] Briefly, mouse skin was incubated with rabbit serum for 1 hour at 37°C. Human neutrophils were added and incubated for 3 hours at 37°C before washing with phosphate-buffered saline (PBS). Sections were stained with hematoxylin and eosin (H&E), and dermal-epidermal separation was evaluated microscopically. The *ex vivo* studies were approved by the local ethics committee (09-140). Normal rabbit serum was obtained from CCPro (Oberdorla, Germany).

EndoS Preparation and IgG Hydrolysis *in Vitro*

Pretreatment of rabbit IgG was performed as previously described.[31,32] Rabbit anti-Col17 IgG (1 mg) was incubated with 5 μg recombinant GST-EndoS in PBS for 16 hours at 37°C, followed by affinity removal of GST-EndoS by serial passages over glutathione-Sepharose 4B columns (GE Healthcare, Uppsala, Sweden). IgG hydrolysis was verified by SDS-PAGE and lectin blot analyses, as previously described.[32,33] Glycosylated IgG was detected by using 5 μg/mL biotinylated *Lens culinaris agglutinin*—lectin (Vector Laboratories, Burlingame, CA), 1 μg/mL streptavidin—horseradish peroxidase (Vector Laboratories), and Super Signal West Pico peroxidase substrate (Pierce, Rockford, IL).

Passive Transfer Mouse Model

Affinity-purified rabbit anti-Col17 IgG or normal rabbit IgG was injected s.c. into the neck of mice every second day over 12 days at doses of 5, 10, and 15 mg, respectively. The IgG dose for each experiment is specified in *Results*. At the time of IgG injections, mice were weighed and examined for their general condition and evidence of cutaneous lesions (ie, erythema, blisters, erosions, and crusts). Cutaneous lesions were counted, and the extent of skin disease was scored as involvement of the skin surface, as previously described for a passive transfer model of EBA.[29] In brief, each body part of the mouse was assigned with a certain percentage of body surface (ears, 5%; eyes, 1%; snout, 2.5%; forelegs, 10%; hind legs, 20%; tail, 10%; trunk, 40%; oral mucosa, 2.5%; and head and neck, 9%). The percentage of body surface affected by skin lesions (erythema, crusts, and erosions) was then calculated, resulting in an overall clinical score. The cumulative disease score was calculated as the area under the curve (AUC) determined by individual clinical scores obtained on days 4, 8, and 12, unless stated otherwise. At day 12, all mice were sacrificed, blood was taken, and both lesional and perilesional biopsy specimens were obtained for histopathological analysis (stored in 4% buffered formalin) and direct IF microscopy (stored at −80°C),

respectively. FcγRIV-blocking monoclonal antibody 9E9 (provided by F.N.) or the isotype control antibody (clone A19-3; BD Biosciences, Heidelberg, Germany) was injected i.p. into mice at a dose of 200 μg on days 0, 4, and 8 or 4, 6, and 8, respectively. Anti-granulocyte differentiation antigen-1 (Gr-1) antibody RB6-8C5 (BioXcell, West Lebanon, NH) and isotype control antibody (clone LTF-2; BioXcell) were injected i.p. at a total dose of 1 mg (200 μg on days -2, 2, 4, and 6; and 100 μg on days 0 and 8), respectively, for the prophylactic approach; for the quasi-therapeutic setting, 250 μg on days 4, 6, 8, and 10 (total dose, 1 mg) was administered.

IF Microscopy

Tissue-bound anti-Col17 IgG and complement deposits were detected by direct IF microscopy of frozen sections using fluorescein isothiocyanate (FITC)–conjugated swine anti-rabbit IgG (1:100; Dako, Hamburg, Germany), FITC-conjugated murine anti-mouse C3 (1:50; Cappel, MP Biomedicals, Solon, OH), and murine anti-mouse C5 antibody (1:100; Cell Sciences, Canton, MA) detected by FITC-labeled rabbit anti-mouse IgG (1:100; Dako). The staining intensity of immunoreactants at the DEJ was quantified with ImageJ version 1.45 (NIH, Bethesda, MD). Sections (6 μm thick) of healthy C57BL/6 mouse skin and healthy mouse skin in which dermal-epidermal splitting was induced by incubation with 1 mol/L NaCl (salt-split skin) were prepared for indirect IF microscopy to evaluate the tissue-binding ability of rabbit anti-Col17 IgG. Sections were incubated with appropriately diluted rabbit serum and evaluated using a Keyence BZ-9000 microscope (Keyence, Neu-Isenburg, Germany).

Histopathological Data

Formalin-fixed skin samples were processed into paraffin blocks. Sections (4 μm thick) were stained with H&E, according to standard protocols. For the visualization of eosinophils, cryosections (6 μm thick) of lesional mouse skin were fixed in acetone for 10 minutes. Air-dried sections were washed three times with PBS before incubation with Dako Dual Endogenous Enzyme Block (Dako) for blocking of endogenous peroxidase. After subsequent washing, sections were incubated with rat anti-mouse major basic protein antibody (1:1000; Lee Laboratory, Mayo Clinic Arizona, Scottsdale, AZ)[34] and, subsequently, with horseradish peroxidase–coupled rabbit anti-rat IgG (1:500; Dako). Counterstaining was done with HistoGreen (Linaris, Wertheim-Bettingen, Germany).

Electron Microscopy

Samples for transmission electron microscopy were taken from lesional skin of anti-Col17 IgG-injected mice and corresponding body parts of negative control mice. Fixed samples were processed as described, and stained sections were examined with an electron microscope (model JEM-1230; JEOL Ltd, Tokyo, Japan).[35]

Neutrophil-Specific MPO Activity

Myeloperoxidase (MPO) activity, corresponding to the total granulocyte infiltration, was assessed in homogenized ear specimens, as described in previous protocols.[36] MPO content was expressed as units of MPO activity per milligram of protein. Protein concentrations were determined by a dye-binding assay (Thermo Scientific, Rockford, IL) using bovine serum albumin as a standard.

Flow Cytometry

Peripheral blood was taken before injection of anti–Gr-1 antibody and every second day during the whole observation period. After lysis of erythrocytes, cells were stained with a phosphatidylethanolamine-conjugated Gr-1–specific antibody RB6-8C5 (BD Biosciences) and analyzed by FACSCalibur (BD Biosciences). The percentage of Gr-1–positive cells was determined.

Statistical Analysis

Sigma plot version 11.0 (Systat Software Inc., Chicago, IL) and R version 2.10.1 (*http://www.r-project.org*) were used to perform statistical analysis. The P value was determined by t-test.

Results

Rabbit IgG against Murine BP180 NC15A Induces Dermal-Epidermal Splitting *ex Vivo*

New Zealand white rabbits ($n = 10$) were immunized with a recombinant murine homologue of the human NC16A

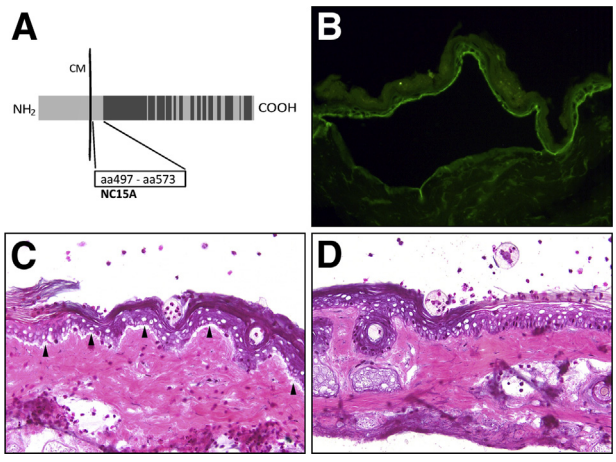

Figure 1 Schematic diagram of type XVII collagen and *in vitro* effect of rabbit anti-Col17 IgG. **A:** The extracellular portion of NC15A was expressed in *Escherichia coli*. Amino acid (aa) residue numbers are shown. **B:** Rabbit IgG generated against the murine NC15A domain labels the epidermal side of mouse NaCl-split skin by indirect IF microscopy. Anti-Col17 IgG (**C**), but not preimmune rabbit IgG (**D**), induces dermal-epidermal separation (**arrowheads**), when incubated with cryosections of murine skin in the presence of human leukocytes. CM, cell membrane.

domain of Col17 (Col17 NC15A) (Figure 1A). IgG affinity purified from these rabbits, but not from normal rabbits, bound to the epidermal side of the artificial split, as visualized by indirect IF microscopy on murine 1 mol/L NaCl-split skin (Figure 1B). The leukocyte-activating and dermal-epidermal split–inducing capacity of rabbit anti-Col17 IgG was demonstrated by the use of cryosections of murine skin incubated with human leukocytes (Figure 1C). In contrast, incubation with preimmune rabbit IgG did not result in split formation (Figure 1D).

Passive Transfer of Anti-Col17 IgG in Adult Mice Reproduces Major Clinical and Immunopathological Characteristics of the Human Disease

C57Bl/6 mice aged 8 to 12 weeks were injected with rabbit anti-Col17 IgG every second day over 12 days. Perilesional skin biopsy specimens obtained at day 12 revealed linear deposits of IgG, C3, and C5 at the DEJ by direct IF microscopy (Figure 2, A–C). Histopathological analysis of a lesional biopsy specimen obtained at day 12 showed dermal-epidermal separation and dense inflammatory infiltrates dominated by neutrophils in the upper dermis (Figure 2D). By immunohistochemistry (IHC), infiltration of eosinophils was also demonstrated (Figure 2E). Transmission electron microscopy of lesional skin biopsy specimens (day 12) localized the splitting within the lamina lucida (Figure 2F). Clinically, mice developed erythema on the ears (on day 4) and, subsequently, erosions and crusts on the ears, the neck, and around the eyes and the snout (Figure 2, G–I). Mice injected with the same amount of normal rabbit IgG showed no macroscopic and microscopic cutaneous lesions, and no deposits of IgG, C3, and C5 were seen at the DEJ by direct IF microscopy (data not shown).

Next, we applied different amounts of pathogenic anti-Col17 IgG. Adult C57Bl/6 mice were injected once ($n = 5$ on day 0; total, 15 mg IgG), twice ($n = 5$ on days 0 and 2; total, 30 mg IgG), and three times ($n = 5$ on days 0, 2, and 4; total, 45 mg IgG), respectively, with 15 mg of pathogenic IgG. A fourth group of mice received six injections with 5 mg IgG ($n = 5$ on days 0, 2, 4, 6, 8, and 10; total, 30 mg IgG) (Figure 3). Mice injected once with 15 mg of pathogenic IgG showed significantly less disease compared with mice injected two times (AUC, $P = 0.005$) and three times ($P = 0.002$) with 15 mg pathogenic IgG, respectively. At day 12, no difference in the extent of skin lesions was observed in animals that received 15 mg twice or 5 mg six

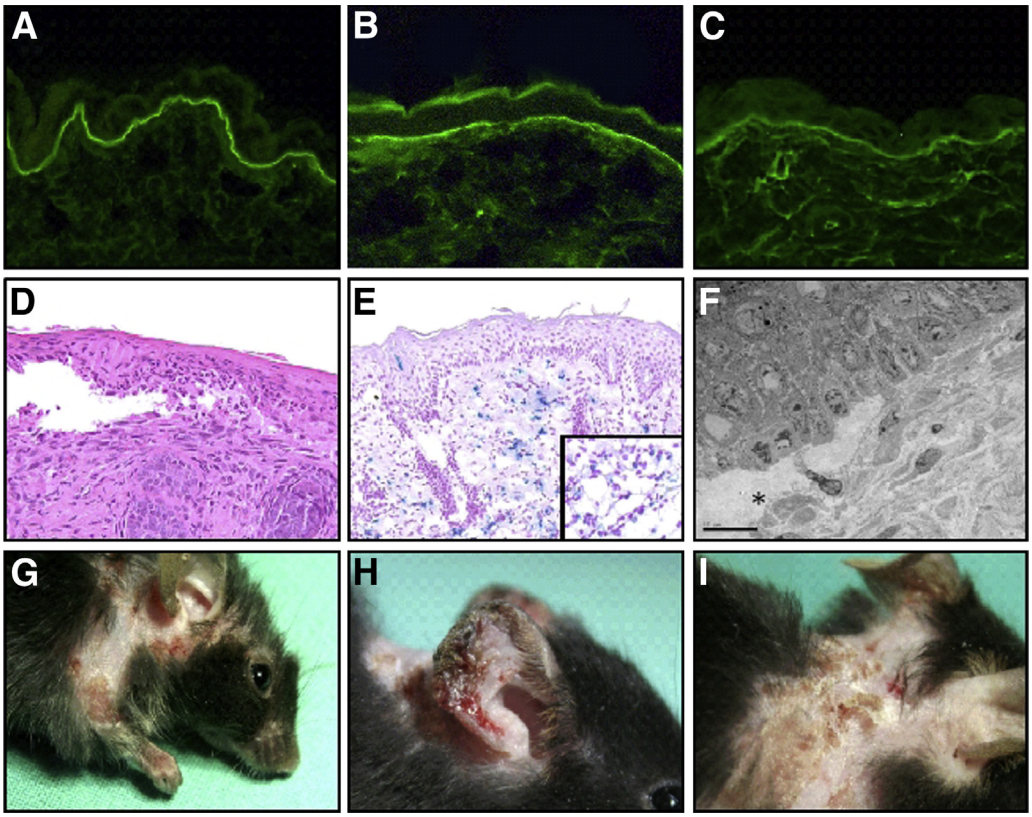

Figure 2 Rabbit IgG against murine type XVII collagen (anti-Col17 IgG) binds at the dermal-epidermal junction (DEJ) *in vivo*, activates complement, and induces subepidermal splitting and cutaneous lesions in adult mice. C57Bl/6 mice were injected s.c. into the neck with anti-Col17 IgG over a period of 12 days with six injections, each containing 5 mg IgG every second day. Rabbit anti-Col17 IgG binds along the DEJ of injected mice (**A**) and activates murine C3 (**B**) and C5 (**C**), as shown by direct IF microscopy. **D:** Histopathological analysis of a skin lesion reveals subepidermal cleavage and a dense inflammatory infiltrate dominated by neutrophils and eosinophils (H&E staining). **E:** IHC with anti-mouse major basic protein antibody. **F:** Transmission electron microscopy of a lesional skin biopsy specimen reveals the formation of a blister (**asterisk**) at the lamina lucida level. **G–I:** Representative clinical presentation at day 12. Lesions are characterized by erythema, erosions, and crusts, particular by involving the ears.

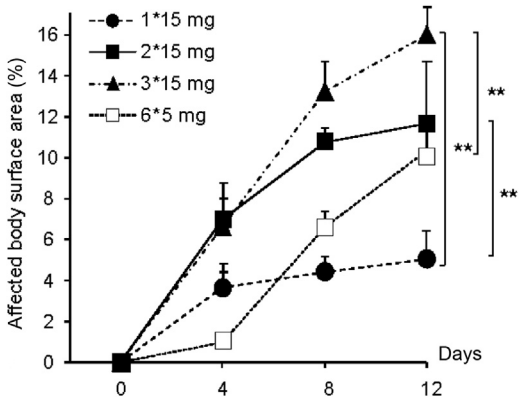

Figure 3 Disease activity is related to the amount of injected anti-Col17 IgG. The extent of skin lesions was determined as percentage of the body surface area. Means of individual clinical scores ($n = 5$) are shown before the first injection (day 0) and on days 4, 8, and 12. Three injections of 15 mg anti-Col17 IgG (on days 0, 2, and 4) result in significantly more extensive disease compared with two injections (on days 0 and 2), a single injection (on day 0), and six injections of 5 mg anti-Col17 (on days 0, 2, 4, 6, 8, and 10). $^{**}P < 0.01$.

times of pathogenic IgG (AUC, $P = 0.694$). As expected, cutaneous lesions developed more rapidly in mice injected two times with 15 mg compared with mice injected six times with 5 mg pathogenic IgG (Figure 3). These results showed a clear dose-dependent effect of anti-Col17 IgG.

Experimental BP in Adult Mice Is FcR Dependent

Adult C57Bl/6 mice ($n = 5$) and mice lacking the activating γ-chain of the Fc receptor ($FcR\gamma^{-/-}$; $n = 5$) were injected three times with 15 mg of rabbit anti-Col17 IgG. Although wild-type (WT) animals developed skin lesions, $FcR\gamma^{-/-}$ mice were completely protected (Figure 4, A, C, D, F, and G). The impact of FcγR-dependent effects in the novel experimental BP model was further addressed by the use of EndoS-treated anti-Col17 IgG. EndoS, an endoglycosidase that specifically hydrolyzes the N-linked glycan on IgG heavy chains, reduces the binding to activating FcγR and increases the binding to the inhibitory FcγRIIB.[31,37] Injection of EndoS-pretreated anti-Col17 IgG in adult C57Bl/6 mice ($n = 5$; 5 mg IgG given six times) resulted in only a few BP lesions compared with injection of untreated anti-Col17 IgG ($n = 8$; 5 mg IgG given six times; AUC, $P = 0.002$) (Figure 4, B, C, E, F, and H). When the intensity of bound IgG at the DEJ, as detected by direct IF microscopy of perilesional skin biopsy specimens obtained at day 12, was quantified, no differences were seen between $FcR\gamma^{-/-}$ mice and mice injected with EndoS-pretreated anti-Col17 IgG compared with the control mice ($P = 0.667$ and $P = 0.195$) (Figure 4, I–K).

FcγRI Has No Role in the Effector Phase of Experimental BP in Adult Mice

Adult $Fc\gamma RI^{-/-}$ mice (C57Bl/6 background; $n = 5$), $Fc\gamma RI^{-/-}$ mice (BALB/c background; $n = 8$), and the corresponding WT mice ($n = 6$ and $n = 8$, respectively) were injected s.c. six times with 5 mg anti-Col17 IgG every second day (day 0 to day 10). As in the C57Bl/6 mice (Figure 5, A–C), BALB/c developed erythema and erosion on the ears, the snout, and the neck, subepidermal split formation, and a neutrophil-rich inflammatory infiltrate in the upper dermis by histopathological analysis, and they revealed binding of IgG and C3 along the DEJ by direct IF microscopy (Figure 5, D–I). No differences in the severity of the clinical disease between $Fc\gamma RI^{-/-}$ and control animals were observed in both mouse strains (C57Bl/6, AUC, $P = 0.100$; BALB/c, AUC, $P = 0.855$) (Figure 5). Also, the intensity of bound IgG at the DEJ, as seen by direct IF microscopy of perilesional skin biopsy specimens obtained at day 12, was not different between $Fc\gamma RI^{-/-}$ mice and WT animals (data not shown).

Tissue Destruction Is Mediated by FcγRIV and FcγRIII, whereas FcγRIIB Is Protective, in Experimental BP in Adult Mice

To analyze the pathogenic importance of $Fc\gamma RIII^{-/-}$ mice ($n = 8$) and WT mice ($n = 5$), mice were injected with anti-Col17 IgG (5 mg given six times). After the observation period of 12 days, $Fc\gamma RIII^{-/-}$ mice showed a 48% reduced disease score compared with control mice (AUC, $P = 0.045$) (Figure 6, A, D, and M). When $Fc\gamma RIV^{-/-}$ ($n = 5$) and WT ($n = 6$) mice were injected with anti-Col17 IgG (5 mg given six times), clinical disease in the $Fc\gamma RIV^{-/-}$ animals was reduced by 77% (AUC, $P < 0.001$) (Figure 6, B, G, and M). Such as in the $Fc\gamma RIII^{-/-}$ animals, no dermal-epidermal separation and only a few inflammatory cells in the upper dermis were observed in $Fc\gamma RIV^{-/-}$ mice (Figure 6, E and H).

FcγRIIB is the only known inhibitory FcγR. Injection of anti-Col17 IgG (5 mg given six times) in $Fc\gamma RIIB^{-/-}$ ($n = 5$) and WT ($n = 5$) mice led to significantly more clinical disease in $Fc\gamma RIIB^{-/-}$ mice (AUC, $P < 0.001$) (Figure 6, C, J, and M). This difference could be observed beginning from day 4 throughout the whole observation period. In all mouse strains, IgG deposition at the DEJ appeared not to be different (Figure 6, F, I, L, and O). Histological analysis of lesional skin in both control and $Fc\gamma RIIB^{-/-}$ mice showed split formation at the DEJ and a more pronounced inflammatory infiltrate in the upper dermis compared with $Fc\gamma RIII^{-/-}$ and $Fc\gamma RIV^{-/-}$ mice (Figure 6, E, H, K, and L). Consistently, MPO activity in the lesional skin of $Fc\gamma RIII^{-/-}$ and $Fc\gamma RIV^{-/-}$ mice was significantly lower compared with WT animals. In contrast, $Fc\gamma RIIB^{-/-}$ mice showed 10 times higher MPO activity compared with the WT mice (Supplemental Figure S1).

Pharmacological Inhibition of FcγRIV Inhibits Disease Induction and Prevents Disease Progression in Already Clinically Diseased Mice

Consistent with previous experiments, C57Bl/6 mice were injected with anti-Col17 IgG on days 0, 2, 4, 6, 8, and 10.

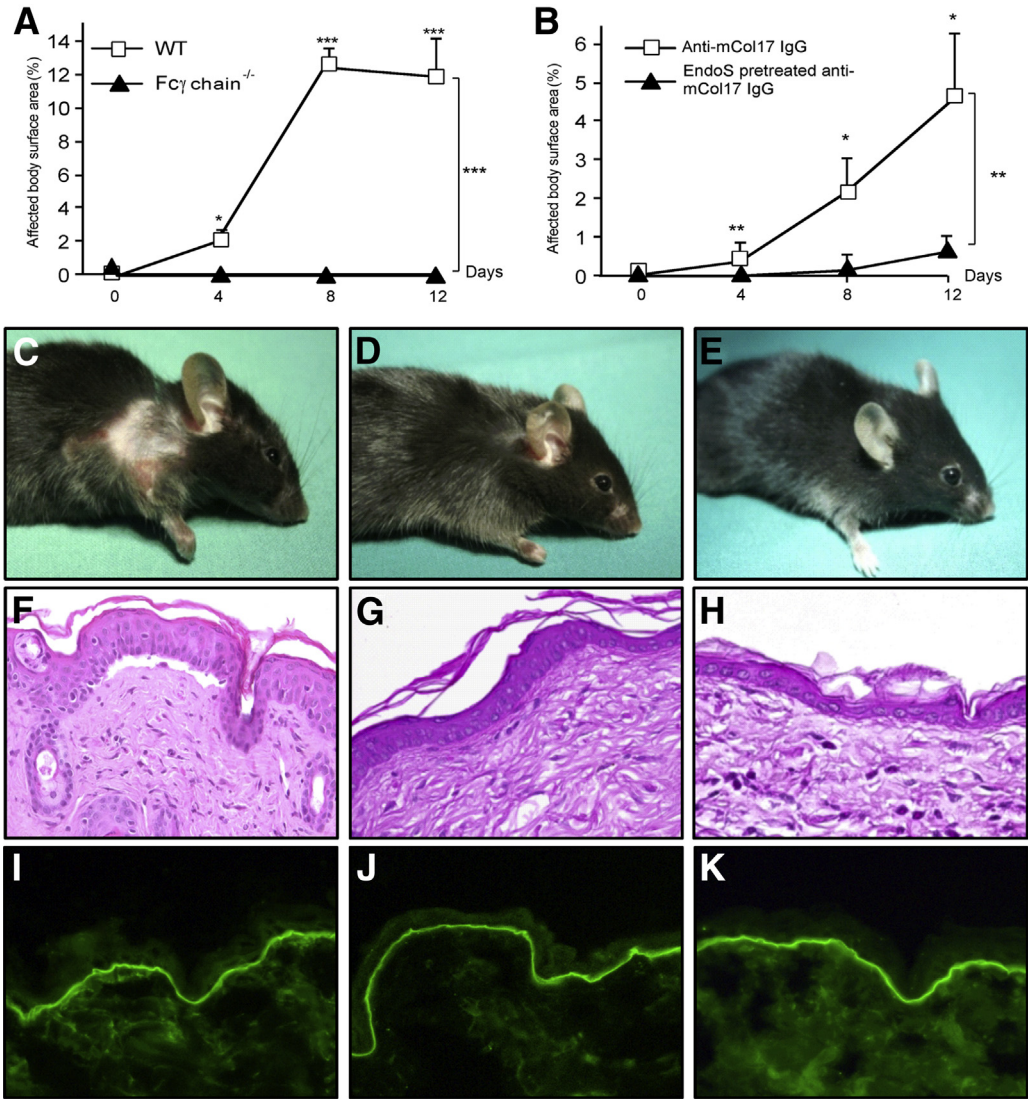

Figure 4 In Fcγ chain-deficient ($FcR\gamma^{-/-}$) mice and mice injected with EndoS-pretreated anti-Col17 IgG, clinical disease is abolished, whereas *in vivo* deposition of IgG is unaltered. $FcR\gamma^{-/-}$ ($n = 5$; **D**, **G**, and **J**) and WT ($n = 5$; **C**, **F**, and **I**) mice were injected s.c. with anti-Col17 IgG (15 mg on days 0, 2, and 4). **A:** Although WT animals develop skin lesions, $FcR\gamma^{-/-}$ mice remain without clinical disease (AUC, $P < 0.001$). **B:** Mice injected with EndoS-pretreated anti-Col17 IgG ($n = 5$; **E**, **H**, and **K**) demonstrate a significantly lower clinical score compared with mice that had received untreated anti-Col17 IgG (5 mg on days 0, 2, 4, 6, 8 and 10; AUC, $P < 0.01$). In histopathological specimens (**F**–**H**) taken from ears, subepidermal splitting is only present in WT mice injected with untreated anti-Col17 IgG (**F**). By direct IF microscopy of nonlesional mouse skin obtained from WT mice (15 mg IgG three times; **I**), $FcR\gamma^{-/-}$ mice injected with anti-Col17 IgG (15 mg IgG three times; **J**), and WT mice injected with EndoS-pretreated anti-Col17 IgG (5 mg IgG six times; **K**), *in vivo* deposits of IgG at the DEJ were not different between the three groups. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. DEJ, dermal-epidermal junction.

The administration of FcγRIV-blocking antibody 9E9 or an isotype control on days 0, 4, and 8 resulted in significantly less clinical disease in the 9E9-treated animals (AUC, $P = 0.034$) (Figure 7, A–E). When the injection of FcγRIV-blocking antibody 9E9 was started on day 4 (when the first clinical lesions have already appeared), followed by further administration on days 6 and 8, the extent of skin lesions, MPO activity determined in the right ear, and density of the inflammatory infiltrate in the ear section were not significantly lower compared with mice that received the isotype control (Figure 7, F–H). Only disease progression between days 6 and 10 was significantly reduced ($P = 0.035$).

Granulocytes Are Essential for Lesion Formation

To investigate the role of granulocytes in this model, the anti–Gr-1 antibody RB6-8C5 that depletes Gr-1–expressing cells, such as mature granulocytes and monocytes at certain differentiation stages, was used. RB6-8C5 and isotype control were applied on days -2, 0, 2, 4, 6, and 8 (C57Bl/6 mice, $n = 8$; prophylactic approach) and on days 4, 6, 8, and 10 ($n = 8$; therapeutic approach), respectively, whereas anti-Col17 IgG (10 mg given six times) was injected as usual on days 0, 2, 4, 6, 8, and 10. Disease activity was significantly reduced for both the prophylactic (AUC on days 0 to 12, $P = 0.028$; AUC on days 0 to 10, $P = 0.015$) (Figure 8A) and the

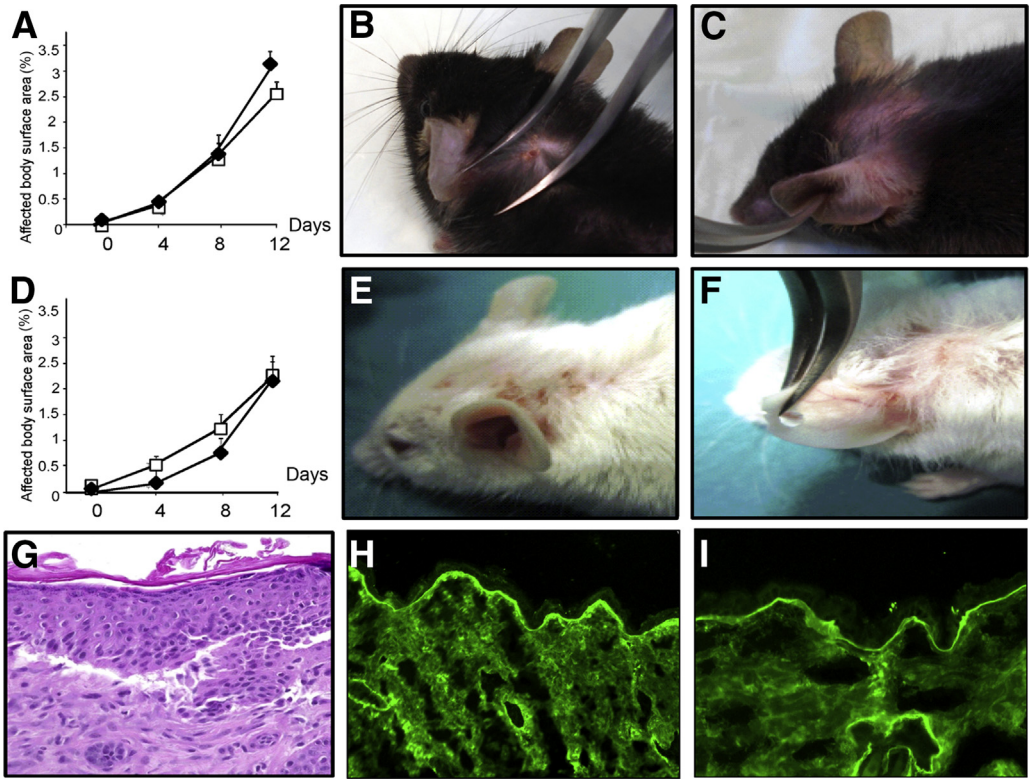

Figure 5 FcγRI has no effect on disease development in experimental BP. *FcγRI*$^{-/-}$ mice (black diamonds) (C57Bl/6, **B**; BALB/c, **E**) and corresponding WT (white squares) (**C** and **F**) controls were injected six times with 5 mg of anti-Col17 IgG. **A** and **D:** *FcγRI*$^{-/-}$ of both strains develop the same extent of clinical disease as WT controls. BALB/c WT mice exhibit skin lesions, subepidermal blister formation (**G**), and IgG (**H**) and C3 (**I**) deposition at the DEJ, similar to C57Bl/6 WT mice. BP, bullous pemphigoid; DEJ, dermal-epidermal junction.

therapeutic (AUC on days 0 to 12, $P = 0.001$; AUC on days 4 to 12, $P = 0.001$) approaches (Figure 8B). By flow cytometric analysis of peripheral blood taken during the experimental period, the percentage of Gr-1−expressing cells was, as expected, largely depleted 2 days after the first injection with RB6-8C5 (Figure 8, C and D).

Discussion

Several animal models have previously been developed that greatly increased our knowledge about the pathophysiological characteristics of BP.[6−12,38] Most of the data were obtained by the group of Liu and Diaz[17] in the passive transfer neonatal mouse model of BP. This model and the passive transfer humanized mouse model have several disadvantages: i) disease is induced in neonatal mice,[6,11] whereas BP is a disease of elderly persons; ii) skin lesions are induced within 48 hours by mechanical friction,[6,11] whereas in patients, lesions develop spontaneously over a longer time period, with a prodromal nonbullous phase characterized by pruritic erythematous or eczematous lesions; iii) lesional eosinophils have not been reported but represent the predominant cell type in the lesional skin of patients[6−9,11]; and, most important, iv) the exploration of anti-inflammatory agents in animals, in which skin lesions have already been developed, is not possible.[6−8,11] In addition, in the humanized mouse models, mice deficient in a molecule with potentially pathophysiological relevance require backcrossing,[9] and in the IgE anti-Col17 models, the clinical phenotype does not reflect the human disease.[12,38] Although the novel mouse model lacks these shortcomings, it only reflects the effector phase of the disease, allowing the investigation of the tissue destruction by anti-Col17 antibodies. The initial disease phase, including the break of tolerance against Col17, is not covered. The latter important issue may be addressed in the active humanized[9] and the immunization-induced BP models.[10]

Our aim was to clarify the role of FcγRs in tissue destruction of experimental BP. To overcome some of the disadvantages of the available BP animal models, an experimental design was chosen that reproduced major clinical and immunopathological characteristics of the human disease, including the following: i) erythema and erosions that developed over some days without the application of friction, ii) subepidermal blister formation that occurred within the lamina lucida, iii) deposition of complement-fixing IgG along the DEJ, and iv) lesional infiltration of neutrophils and, to a much lesser extent, eosinophils. Although eosinophils have not been reported in the lesional infiltrate of the established mouse models of BP,[6,9,11] they are the predominant inflammatory cell in skin lesions of most patients with BP[2] and in human skin grafted on the back of mice with severe

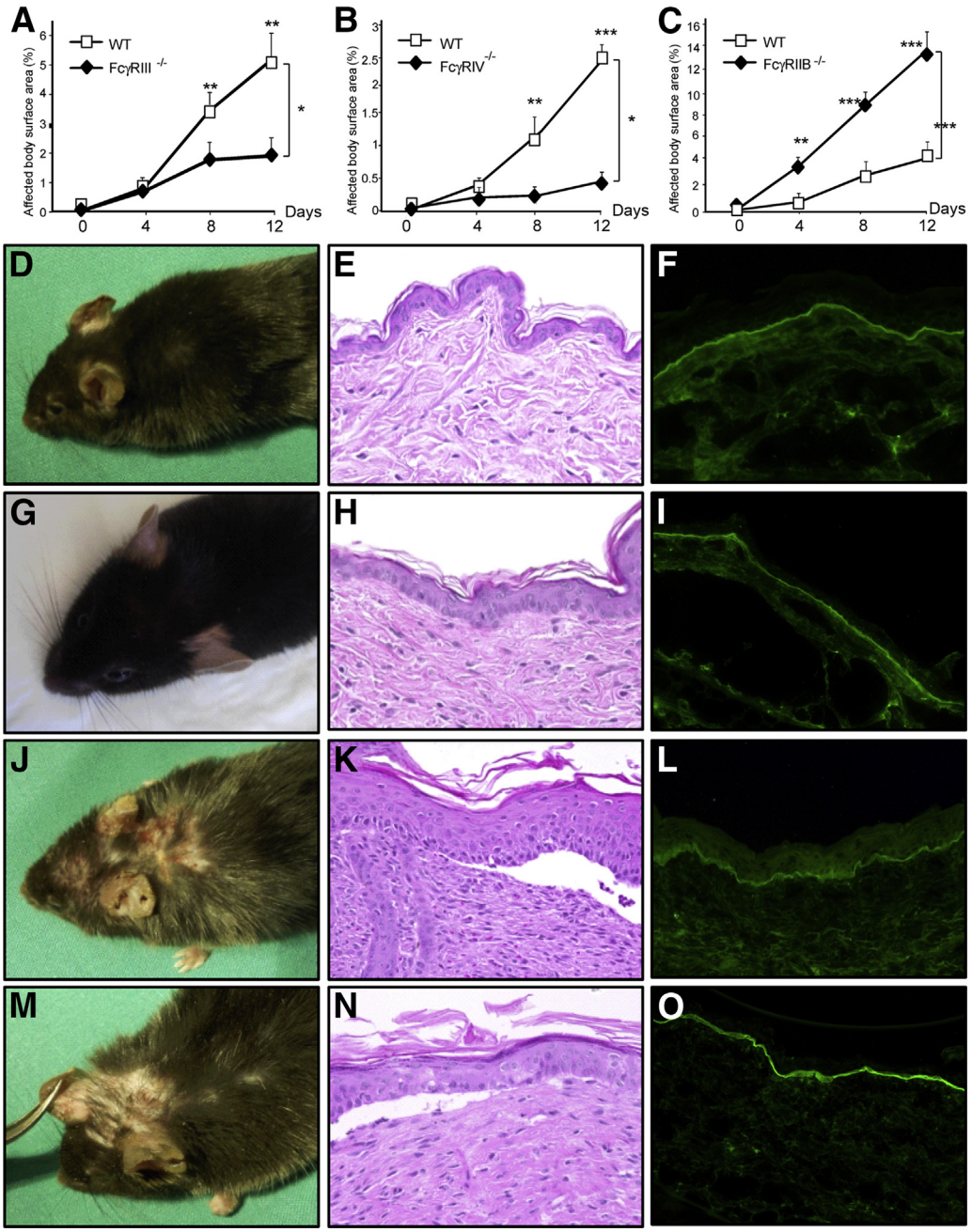

Figure 6 FcγRIIB is protective, whereas FcγRIII and FcγRIV mediate tissue destruction in experimental BP. *FcγRIII*$^{-/-}$ (**D**–**F**) and *FcγRIV*$^{-/-}$ (**G**–**I**) mice develop less disease compared to WT mice (**M**–**O**) after six injections of 5 mg anti-Col17 IgG every second day (AUC, $P < 0.045$ and $P < 0.001$; **A** and **B**). Accordingly, *FcγRIII*$^{-/-}$ and *FcγRIV*$^{-/-}$ mice show no dermal-epidermal separation and a less dense inflammatory infiltrate in the upper dermis by histopathological analysis of the right ear (**E**, **H**, and **N**). In contrast, *FcγRIIB*$^{-/-}$ mice (**J**–**L**) reveal a dermal-epidermal separation and a dense inflammatory dermal infiltrate, as well as significantly more disease, compared with WT mice after injection of anti-Col17 IgG (AUC, $P < 0.001$; **C**). In all mouse strains, no difference in the extent of IgG (**F**, **I**, **L**, and **O**) deposition at the DEJ was seen. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. BP, bullous pemphigoid; DEJ, dermal-epidermal junction.

combined immunodeficiency that were injected with anti-hCol17 IgE-secreting hybridoma cells.[38] We, therefore, speculate that IgE anti-Col17 antibodies may promote eosinophil infiltration in the skin.

Consistent with the present approach, injection of anti-murine Col7 IgG in adult mice has previously resulted in a skin disease with major clinical and immunopathological findings of the human disease EBA, leading to new insights in the pathogenesis of this disease.[27,29,32] In the novel model for BP, the pivotal role of the Fc portion of anti-Col17 antibodies was shown. First, mice lacking the FcRγ were completely protected from cutaneous disease. This observation is consistent with findings in other autoantibody-mediated diseases, such as anti-glomerular basement disease, EBA, and the neonatal mouse model of BP.[29,39,40] In a separate experiment, in mice injected with anti-Col17

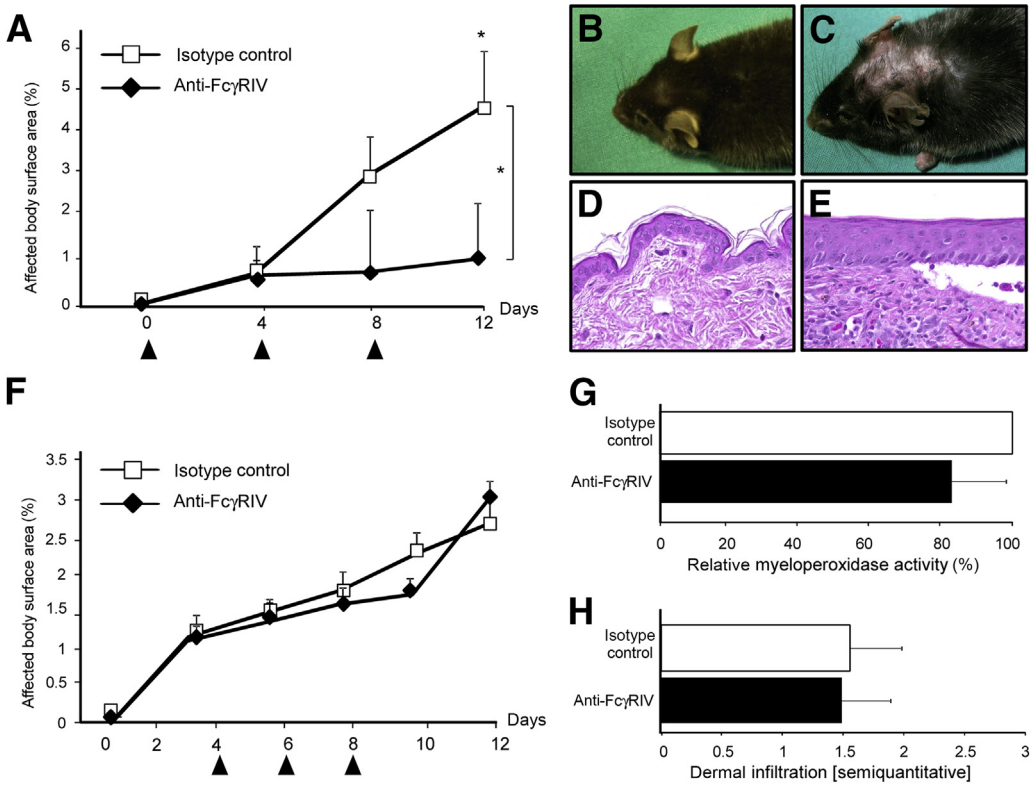

Figure 7 Pharmacological inhibition of FcγRIV function reduces disease progression in a prophylactic approach. C57Bl/6 mice were injected with anti-Col17 IgG on days 0, 2, 4, 6, 8, and 10. FcγRIV-blocking antibody 9E9 and isotype control antibody (**arrowheads** on *x* axes, **A** and **F**) were given on days 0, 4, and 8 in the prophylactic approach (**A**—**E**) and on days 4, 6, and 8 in the therapeutic setting (**F**—**H**), respectively. **A**—**E:** In the prophylactic approach, significantly less macroscopic and microscopic disease is observed in the 9E9-treated compared with isotype-injected animals. In the quasi-therapeutic setting (when clinical disease was already visible at the first injection of 9E9 or control antibody), disease progression between days 6 and 10 is significantly reduced in the 9E9-treated mice ($P = 0.035$). Overall disease activity during days 4 and 12 ($P = 0.428$; **F**) and myeloperoxidase activity reflecting the extent of neutrophil infiltration in the skin ($P = 0.812$; **G**) and the amount of inflammatory cells in skin lesions determined by histopathological analysis ($P = 0.232$; **H**) do not differ between 9E9-treated and control mice. $^{*}P < 0.05$.

IgG that had been treated with EndoS, an endoglycosidase that specifically hydrolyzes the N-linked glycan on IgG heavy chains,[31,37] only a few skin lesions occurred. Hydrolysis of IgG glycan has previously been shown to reduce the binding to activating FcγRs and, thus, the proinflammatory effect of autoantibodies.[32,33,41] Interestingly, binding of IgG at the DEJ was not reduced in both $FcR\gamma^{-/-}$ mice and mice injected with EndoS-treated anti-Col17 IgG. Similarly, EndoS-treated anti-collagen IgG2b lost its capacity to induce arthritis in B10.RIII mice, whereas deposition of anti-collagen IgG2b remained unaltered.[41]

To further dissect the role of the different FcγRs, the corresponding knockout mice and an inhibitor of FcγRIV were applied in the passive transfer model of BP in adult mice. We found that FcγRIII-deficient mice and mice deficient for FcγRIV were widely protected from the pathogenic effect of anti-Col17 IgG, whereas FcγRIIB-deficient mice developed significantly more disease. Four classes of murine

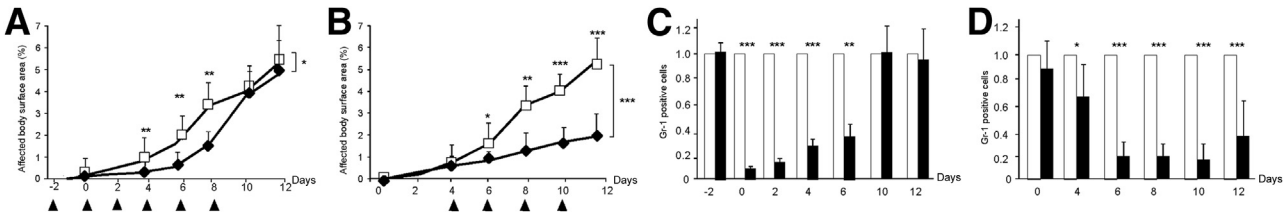

Figure 8 Granulocytes are pivotal, and their depletion in already clinically diseased mice delays disease progression. C57Bl/6 mice ($n = 8$ per group) were injected with anti-Col17 IgG on days 0, 2, 4, 6, 8, and 10. Anti—Gr-1 (black diamonds) and isotype control antibody (white squares) were injected on days -2, 0, 2, 4, 6, and 8 (prophylactic approach; **A** and **C**) and on days 4, 6, 8, and 10 (therapeutic approach; **B** and **D**). Disease activity was significantly reduced in anti—Gr-1—injected mice (AUC on days 0 to 12; $P = 0.028$ and $P = 0.001$, respectively). **C** and **D:** After injection of anti—Gr-1 antibody, the percentage of Gr-1—positive cells expressed in relation to cell numbers in isotype control-injected mice was significantly lower. **Arrowheads** on *x* axes (**A** and **B**) indicate time points of anti—Gr-1 antibody and isotype control injection, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

FcγRs have been described, composed of the activating FcγRI, FcγRIII, and FcγRIV and the inhibitory FcγRIIB.[42] With the exception of FcγRI, FcγRs display low affinity for IgG and are generally triggered in the context of IgG-containing immune complexes, rather than monomeric soluble IgG.[43] Because in BP, the effector phase is driven by anti-Col17 IgG-Col17 immune complexes, the low-affinity FcγR may be relevant. Via FcγR, various cellular responses are initiated, including antigen presentation and release of inflammatory mediators, which can then trigger and enhance inflammatory and autoimmune processes. Most immune cells, with the exception of T and B lymphocytes and natural killer (NK) cells, co-express activating and inhibitory FcγR.[43] Thus, the level of activating and inhibitory signals determines the activation status of the cell.

FcγRIII is expressed on neutrophils, monocytes, macrophages, dendritic cells, basophils, and mast cells.[44] Neutrophils, macrophages, and mast cells were shown to exert important effector functions in the neonatal mouse model of BP.[17,45,46] In the same model, FcγRIII has been identified as the major FcγR for lesion formation.[28] This finding is compatible with our results. In contrast, in experimental EBA, FcγRIII has previously been shown to be irrelevant for tissue destruction.[27]

Consistent with the latter model,[27] FcγRIV was crucial for lesion formation in the novel mouse model of BP. FcγRIV is highly expressed on neutrophils, macrophages, and dendritic cells, whereas it is undetectable on mast cells, NK cells, and T and B lymphocytes.[43] The lack of FcγRIV expression on mast cells may point to a rather minor role of these cells in autoimmunity to Col7, whereas mast cells have repeatedly been shown to be pivotal in the neonatal mouse model of BP.[18,46,47] These data support the notion that, as in humans, autoimmunity to Col17 and Col7 may differ with respect to disease expression in mice. Further studies will aim at dissecting further differences in the effector phase of experimental BP and EBA.

In both models of BP and EBA in adult mice, and in experimental anti-glomerular basement membrane glomerulonephritis, immunothrombocytopenia, hemolytic anemia, collagen-induced arthritis, and autoantibody-mediated vasculitis, lack of FcγRIIB expression resulted in significantly higher disease activity.[27,48] In contrast, FcγRIIB, which is expressed on all cells of the immune system with the exception of T and NK cells,[43,44] had no role in lesion formation in the neonatal mouse model of BP.[28] This discrepancy may be due to differences in the neonatal and adult immune systems, longer follow-up periods in the novel model, or the specificity of the rabbit anti-Col17 IgG.

In humans, six different FcγRs are known: FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA, and FcγRIIIB. On the basis of their structure and type of signals triggered by FcR, cross-linking murine and human FcγRs can be, to some extent, matched. Such as in the mouse, FcγRIIB is the only inhibitory FcγR. Although the human FcγRIIA and FcγRIIIA correspond with the murine FcγRIII and FcγRIV, respectively, FcγRIIC and FcγRIIIB are unique to humans.[44] Consistent with the present results, previous *in vitro* studies using human neutrophils (that do not express FcγRIIIA) have attributed the pathogenic effect of anti-human Col17 IgG to FcγRIIA and FcγRIIIB.[49]

To investigate the potential use of the novel model in the evaluation of anti-inflammatory agents in a quasi-therapeutic setting (ie, in mice that had already developed skin lesions), injections of FcγRIV-blocking and granulocyte-depleting anti−Gr-1 antibodies were initiated on day 4. In this approach, the FcγRIV-blocking antibody was only marginally effective, whereas the anti−Gr-1 antibody resulted in significantly reduced disease activity. These data indicate that, although granulocytes are pivotal for lesion formation in BP, the impact of FcγRIV appears to be mainly restricted to the initial phase of the disease.

Herein, we showed that lesion formation in experimental BP depended on two FcγRs, FcγRIV and FcγRIII, whereas FcγRIIB was protective. Furthermore, our data indicate that the novel mouse model of BP should be suitable to explore both mechanisms of autoantibody-mediated tissue destruction and novel exploratory treatment options in a mouse model with already existing skin lesions.

Acknowledgment

We thank Rebecca Cames for excellent technical assistance.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2014.05.007*.

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