

Regulatory T-cell deficiency leads to pathogenic bullous pemphigoid antigen 230 autoantibody and autoimmune bullous disease



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Background: Autoimmune bullous diseases/dermatoses (AIBDs) are severe autoantibody-mediated skin diseases. The pathogenic relevance of autoreactive CD4⁺ T cells for the induction of autoantibody production remains to be fully evaluated. Scurfy mice lack functional regulatory T (Treg) cells, experience spontaneous activation of autoreactive CD4⁺ T cells, and display severe erosive skin lesions suggestive of AIBDs.

Objective: We sought to determine whether AIBDs develop in Treg cell-deficient scurfy mice.

Methods: Histology, indirect immunofluorescence (IF) microscopy, direct IF, and ELISA were used to prove the presence of AIBDs in scurfy mice. Monoclonal autoantibodies from sera of scurfy mice were screened by using indirect IF on murine skin, and immunoprecipitation and mass spectrometry were used for target antigen identification, followed by confirmation in modified human embryonic kidney cells and murine keratinocytes. Pathogenicity was determined by injecting the autoantibody into neonatal mice and transferring scurfy CD4⁺ T cells into *nu/nu* mice.

Results: Autoantibodies against different known autoantigens of AIBDs spontaneously develop in scurfy mice. Histology reveals subepidermal blisters, and direct IF of skin of scurfy mice shows a predominant linear staining pattern. The mAb 20B12 shows a linear staining pattern in indirect IF, recognizes the murine hemidesmosomal protein bullous pemphigoid antigen 230 (BP230) as the target antigen, and cross-reacts with human BP230. Purified mAb 20B12 induces subepidermal blisters in neonatal mice. Transfer of scurfy CD4⁺ T cells is sufficient to induce antibodies with reactivity to AIBD autoantigens and

subepidermal blisters in the skin of recipient T cell-deficient *nu/nu* mice.

Conclusion: We show that the absence of Treg cells leads to AIBDs by pathogenic autoantibodies targeting BP230. (J Allergy Clin Immunol 2018;142:1831-42.)

Key words: Autoimmune bullous disease, bullous pemphigoid, bullous pemphigoid antigen 230, dystonin, regulatory T cells

Autoantibodies directed against structural proteins of the skin cause loss of cellular adhesion and induce autoimmune bullous diseases/dermatoses (AIBDs), such as pemphigus vulgaris (PV), bullous pemphigoid (BP), or epidermolysis bullosa acquisita (EBA).¹⁻⁴ For most AIBDs, the target antigens are known, such as desmoglein-3 for PV.⁵ In patients with BP, which is the most common AIBD with a linear basement membrane zone (BMZ) pattern in direct immunofluorescence (IF) microscopy, the autoantibodies target 2 components of the hemidesmosome: bullous pemphigoid antigen 180 (BP180) and bullous pemphigoid antigen 230 (BP230).^{6,7} BP180, also known as collagen XVII, is the major target antigen.^{8,9} However, in contrast to BP180, the pathogenic relevance of autoantibodies to BP230 in patients with BP is still a matter of debate. Even though the antigenic epitopes of the cytoplasmic protein BP230 have been identified and are distributed over the entire autoantigen,¹⁰⁻¹³ it has not yet been convincingly shown that autoantibodies to BP230 are capable of inducing blisters *in vivo*.

Identification of the autoantigens has allowed development of specific mouse models and studies on the pathogenesis of AIBDs.¹⁴⁻¹⁸ However, the exact mechanisms leading to B-cell activation and pathogenic high-affinity autoantibody production are not yet fully understood.¹⁹ Autoreactive CD4⁺ T cells specific for the respective autoantigens provide B-cell help for production of class-switched antibodies because the autoantibodies triggering AIBDs are mainly of the IgG type. Autoantigen-specific autoreactive CD4⁺ T cells for BP and PV have been identified in peripheral blood in both affected patients and corresponding mouse models.¹⁹⁻²² In addition, CD4⁺ T cells, but not CD8⁺ T cells, have been demonstrated to be crucial for the development of autoreactive B cells and disease development in a mouse model of EBA.²³

Forkhead box protein P3 (FoxP3)-positive regulatory T (Treg) cells are important for the maintenance of immune tolerance and prevention of autoimmunity.²⁴⁻²⁶ In the absence of functional Treg cells, as in FoxP3 mutant scurfy mice or patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, autoreactive T_H cells expand, leading to lethal multiorgan inflammation.^{27,28} Interestingly, in addition

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Abbreviations used

AIBD:	Autoimmune bullous disease/dermatosis
BMZ:	Basement membrane zone
BP:	Bullous pemphigoid
BP180:	Bullous pemphigoid antigen 180
BP180 NC15A:	Murine BP180 15th noncollagenous domain
BP230:	Bullous pemphigoid antigen 230
EBA:	Epidermolysis bullosa acquisita
FcγR:	Fcγ receptor
FoxP3:	Forkhead box protein P3
HEK:	Human embryonic kidney
HRP:	Horseradish peroxidase
IF:	Immunofluorescence
IPEX:	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
MALDI-TOF:	Matrix-assisted laser desorption ionization/time of flight
MS/MS:	Tandem mass spectrometry
PV:	Pemphigus vulgaris
TBS:	Tris-buffered saline
Treg:	Regulatory T
WT:	Wild-type

to T cell-mediated pathology, scurfy mice show increased humoral responses.^{29–31} B cell-mediated pathology has been shown to account for a relevant part of the scurfy phenotype because in B cell-deficient scurfy mice pathology is significantly alleviated and the lifespan is prolonged.³² Recent studies on a subtype of Treg cells, termed follicular Treg cells, have shown elegantly that these cells tightly control production of high-affinity autoantibodies during germinal center reactions.^{33–35}

We wanted to answer the question of whether AIBD development is controlled by Treg or follicular Treg cells, respectively, and whether the autoantibodies that develop in the absence of functional Treg cells are pathogenic. Therefore we investigated scurfy mice for the spontaneous development of AIBDs and detected autoantibodies against different known target antigens in sera of scurfy mice, as well as in skin of sick scurfy mice.

For analyses of pathogenicity and identification of the target antigen, we generated hybridomas of activated plasma cells of scurfy mice, enabling us to work with purified mAbs. One of these mAbs, termed 20B12, reveals a linear staining pattern in indirect IF microscopy, cross-reacts with human skin, and induces subepidermal blisters *in vivo* after transfer into neonatal mice. Interestingly, we identified murine BP230 as the target autoantigen of the mAb 20B12.

In summary, we show that Treg cells control humoral autoimmunity, leading to a subepidermal AIBD. Furthermore, we provide evidence that autoantibodies targeting BP230 are pathogenic *in vivo*.

METHODS**Mice**

Female heterozygous *B6.Cg-Foxp3^{sf}/J* mice were purchased from Jackson Laboratories (Bar Harbor, Me) and bred to *C57BL/6* wild-type (WT) male mice to generate hemizygous male *B6.Cg-Foxp3^{sf}/Y* (scurfy) offspring. WT male littermates were used as control mice. *B6.Cg-Foxn1nu/J* (B6/nude) and *B6.129S7-Rag1tm1Mom/J* (B6/RAG) mice were purchased from Jackson

Laboratories. All mice were held under specific pathogen-free conditions at the central animal facility of the Interfaculty Biomedical Faculty, University of Heidelberg, Heidelberg, Germany. Animal work was performed under the animal protocol (35-9185.81/G-195/11) approved by the animal care committee (Regierungspräsidium Karlsruhe).

Histologic analysis of skin inflammation and clinical skin scores

Skin tissue was fixed in 4% neutral buffered formalin and embedded in paraffin, and 5-μm sections were cut and stained with hematoxylin and eosin. Skin inflammation was scored in a graded fashion by using the following criteria: grade 0, no evident signs of skin disease; grade 1, mild thickening of the skin and mild scaling; grade 2, strong thickening of the skin and severe scaling; and grade 3, strong thickening of skin, severe scaling with crusts, and erosions.

Detection of autoantibodies by using IF microscopy

For indirect IF microscopy, freshly cut 5-μm cryosections of WT palates or normal human skin (human samples were provided by the tissue bank of the National Center for Tumor Diseases, Heidelberg, Germany, in accordance with the regulations of the tissue bank and the approval of the ethics committee of Heidelberg University [S-091/2011]) were fixed for 10 minutes in ice-cold acetone and blocked with 5% goat serum in Tris-buffered saline (TBS). Sera diluted 1:100 in TBS were used as the primary antibody, followed by goat anti-mouse IgG Alexa Fluor 488 (Invitrogen, Carlsbad, Calif) as the secondary antibody and fluorescence mounting media (DAKO, Agilent Technologies, Santa Clara, Calif).

For direct IF microscopy, 5-μm cryosections of scurfy or WT palates were used, and goat anti-mouse IgG Alexa Fluor 488, goat anti-mouse IgM Alexa Fluor 488 (SouthernBiotech, Birmingham, Ala), or goat anti-mouse C3 fluorescein isothiocyanate (MP Biomedicals, Eschwege, Germany) was used as a secondary antibody. For semiquantitative analyses, slides were scored (Axioskop 40; Zeiss, Göttingen, Germany), according to fluorescence intensity as follows: 0, no positive staining; 1, weakly positive staining; 2, intermediate positive staining; and 3, strongly positive staining. For indirect IF microscopy on salt-split skin, palatal tissue of WT mice was snap-frozen in liquid nitrogen, followed by incubation in 1 mol/L NaCl solution with a proteinase inhibitor (AEBSF; Sigma-Aldrich, Taufkirchen, Germany) for 6 hours, and 5-μm cryosections were then used for indirect IF.

Hybridoma generation

Five scurfy mice were chosen by using positive indirect IF before fusion. Total lymph node and splenic cells were pooled and fused with the murine myeloma cell line Sp2/0 (ATCC, Manassas, Va), according to the standard protocol.³⁶ Fused cells were selected by using HAT and HT medium (Sigma-Aldrich), and supernatants were screened for positive fluorescence on WT mouse palatal sections by using indirect IF. Clones yielding positive staining of epidermal structures, including the dermoepidermal junction, were chosen, and positive clones were grown in cDMEM20 and subcloned several times to reach monoclonality.

Passive transfer of mAbs in neonatal mice

Purified mAb 20B12 (0.5–3.5 mg total) was injected subcutaneously into WT neonatal mice at day 1 of life. Twenty-four to 48 hours after injection, mice were sacrificed, and whole skin, palate, and tail specimens were analyzed by using hematoxylin and eosin staining and direct IF microscopy.

Passive transfer of CD4⁺ T cells into *Rag*^{−/−} or *nu/nu* mice

After serologic screening for autoantibodies with reactivity for skin antigens, scurfy mice were chosen for T-cell isolation. CD4⁺ T cells from lymph nodes and spleens of scurfy or WT mice were isolated by using CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4⁺ T cells

(2×10^6 ; purity $\geq 95\%$) in 100 μ L of PBS were transferred into 4- to 6-week-old B6/Rag^{-/-} or B6/nude mice through tail vein injection. Four to 8 weeks after injection, depending on disease severity, mice were sacrificed and sera were analyzed for autoantibody production by means of ELISA and indirect IF microscopy.

Immunoprecipitation

Palates of WT mice were homogenized in lysis buffer containing 1% NP-40 (pH 8) with protease inhibitors by using a gentleMACS dissociator (Miltenyi Biotec). The tissue lysate was precleared with 50 μ L of protein G beads (Thermo Fisher Scientific, Waltham, Mass) and then incubated with 5 μ g of purified mAb 20B12 or isotype control overnight. Fifty microliters of prewashed protein G beads were added for 3 hours. The beads were washed, boiled in 1 $\times$ reducing loading buffer, separated on a 12% SDS-PAGE gel, and analyzed by using matrix-assisted laser desorption/ionization/time-of-flight (MALDI-TOF) mass spectrometry at the protein core facility of the DKFZ (Heidelberg, Germany; see the [Methods](#) section in this article's Online Repository at www.jacionline.org).

Statistical analysis

Data are expressed as means $\pm$ SDs, if not otherwise indicated. Significance was analyzed by using Prism and InStat software (GraphPad Software, La Jolla, Calif), and *P* values of less than .05 were considered significant. Significance in ELISA experiments with 2 groups was determined by using the 2-tailed unpaired *t* test with Welch correction; for more than 2 groups, 1-way ANOVA with the Bonferroni posttest was used.

RESULTS

Severe erosive skin disease with spontaneous blister formation in scurfy mice

We and others have previously reported that scurfy mice have spontaneous severe skin disease.^{26,37} Scurfy mice have scales and erosions on their trunk and especially on their ears, tail, and mucosal areas, including the eyes and anus ([Fig 1, A](#)). During their limited 4-week lifespan, scurfy mice experience skin disease, which first becomes clinically visible around day 5 to 7 after birth and worsens over time (see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org). At 3 weeks of age, the majority of scurfy mice show signs of severe skin disease ([Fig 1, B](#)). Lesional histopathology reveals a dense dermal infiltrate of lymphocytes and mononuclear cells, as well as acanthosis and hyperkeratosis ([Fig 1, C](#)). Although the presence of erosions on the skin might indicate blistering disease, it is difficult to identify skin blistering in adult mice because of the presence of fur. Therefore we looked carefully for intact blisters using histology and identified subepidermal blisters in less hairy areas of the skin, such as the legs ([Fig 1, C](#)).

Identification of autoantibodies to skin in sera from scurfy mice

Palatal tissue from WT mice, which is hairless and contains several layers of keratinocytes, was used to screen sera for autoantibodies to skin antigens. The majority of scurfy sera had positive test results in indirect IF microscopy on WT palatal tissue. Interestingly, different fluorescence patterns were detected ([Fig 1, D and E](#)). Some sera contained predominantly autoantibodies, which show a predominantly linear positive staining of the BMZ, whereas others show positive staining in the epidermis. The positive epidermal staining pattern was

summarized as a “reticular” pattern because the method does not allow clear differentiation between staining of cytoplasmic or intercellular target antigens, especially if mice, such as scurfy mice, display a polyclonal antibody response with potentially overlapping patterns. However, the majority of the sera from scurfy mice tested showed several staining patterns simultaneously, including linear, reticular, cytoplasmic, and nuclear staining patterns, as suspected for a polyclonal humoral response (data not shown).

To directly determine whether autoantibodies were present and bound to the skin *in situ* in sick scurfy mice, we tested palatal tissue from scurfy mice using direct IF microscopy for the presence of tissue-bound IgG antibodies ([Fig 1, F](#)). The majority of scurfy palates examined had positive results with direct IF, whereas no IgG autoantibodies could be detected on palatal tissues of WT mice ([Fig 1, G](#)). Interestingly, we found a strong predominance of linear staining patterns for IgG, but only a minority of mice had a reticular staining pattern ([Fig 1, H](#)). Results of direct IF microscopy were also positive in scurfy mice if other regions of the skin were tested, such as the ear or tail (see [Fig E1, B and C](#)). In addition to IgG, we also detected tissue-bound IgM in the majority of palatal tissue from scurfy mice but no C3 deposition (see [Fig E2](#) in this article's Online Repository at www.jacionline.org).

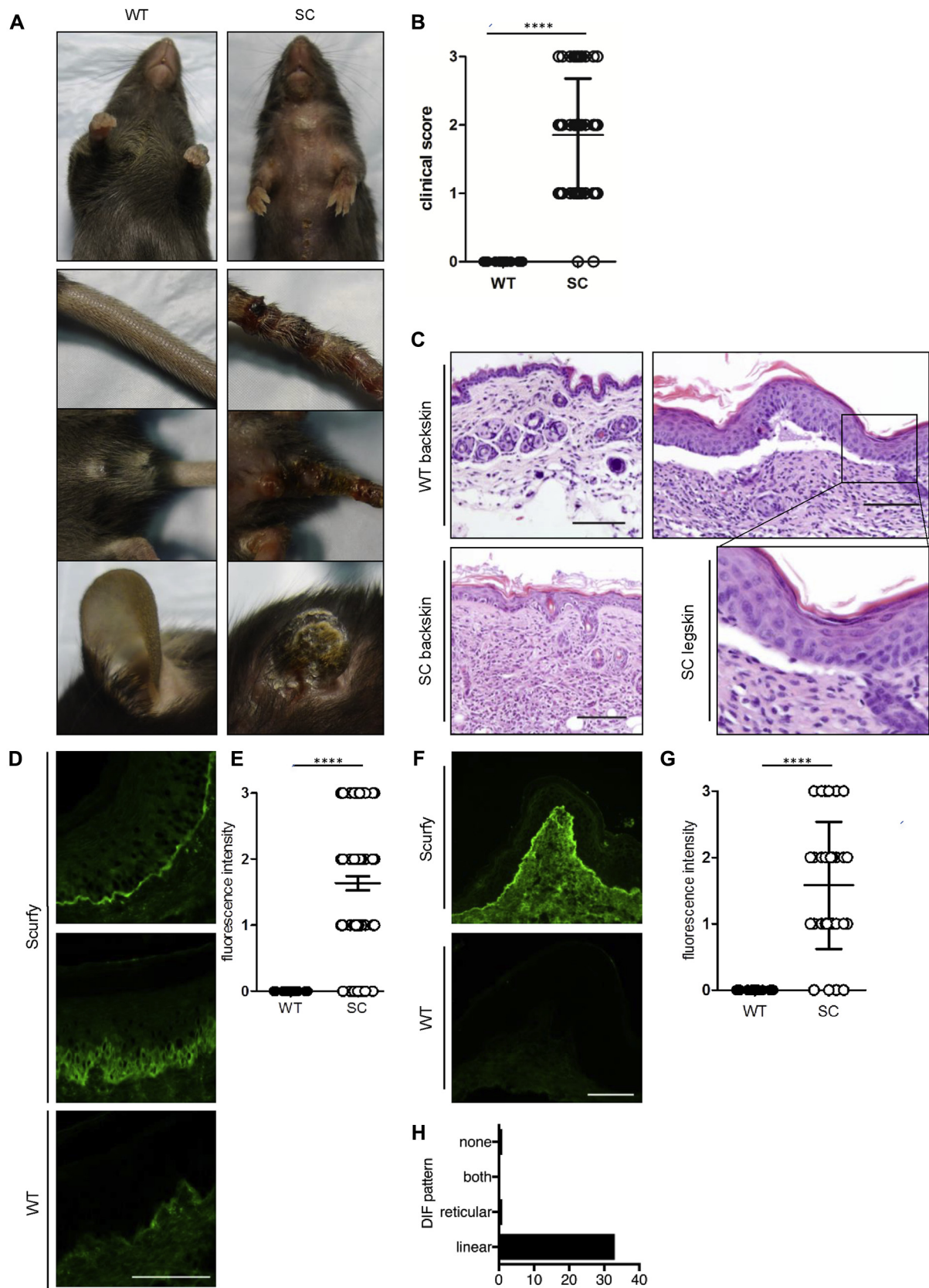
Scurfy sera contain autoantibodies with reactivity against antigens of the dermal-epidermal junction

Because direct IF analyses revealed tissue-bound autoantibodies predominantly at the basal membrane zone, we performed ELISA with sera from scurfy mice to detect autoantibodies specific for known target antigens of AIBDs located within the BMZ.³⁸⁻⁴² We found reactivity of some sera to the immunodominant murine BP180 15th noncollagenous domain (BP180 NC15A), as well as to the intracytoplasmic domain and the C-terminal fragment ([Fig 2, A](#)). WT sera were not reactive to any of these autoantigens. We further found reactivity of scurfy sera against the C-terminus of laminin γ 1, which is the target antigen of anti-p200 pemphigoid ([Fig 2, B](#)),^{41,43} different parts of laminin α 3, the main target antigens in anti-laminin 332 mucous membrane pemphigoid,² and murine type VII collagen, the autoantigen of EBA ([Fig 2, C](#)).⁴⁴ We confirmed the results of ELISA experiments using Western blot analyses (see [Fig E3](#) in this article's Online Repository at www.jacionline.org).

To further verify that scurfy sera contain autoantibodies against different proteins of the BMZ, we performed indirect IF microscopy with scurfy sera on salt-split WT palatal tissue. We found staining on the floor (see [Fig E4, A](#), right panel, in this article's Online Repository at www.jacionline.org) and on the roof (see [Fig E4, A](#), left panel) of the artificial blister; some scurfy sera revealed staining of both the blister floor and blister roof (see [Fig E4, C](#)), whereas WT sera did not show positive staining (see [Fig E4, B](#)). Thus activation of the immune response in the absence of Treg cells results in the development of autoantibodies against known target antigens of AIBDs.

Autoantibody 20B12 targets the BMZ at the blister roof and is pathogenic *in vivo*

To overcome the experimental restrictions caused by the limited amounts of scurfy sera available and to be able to further



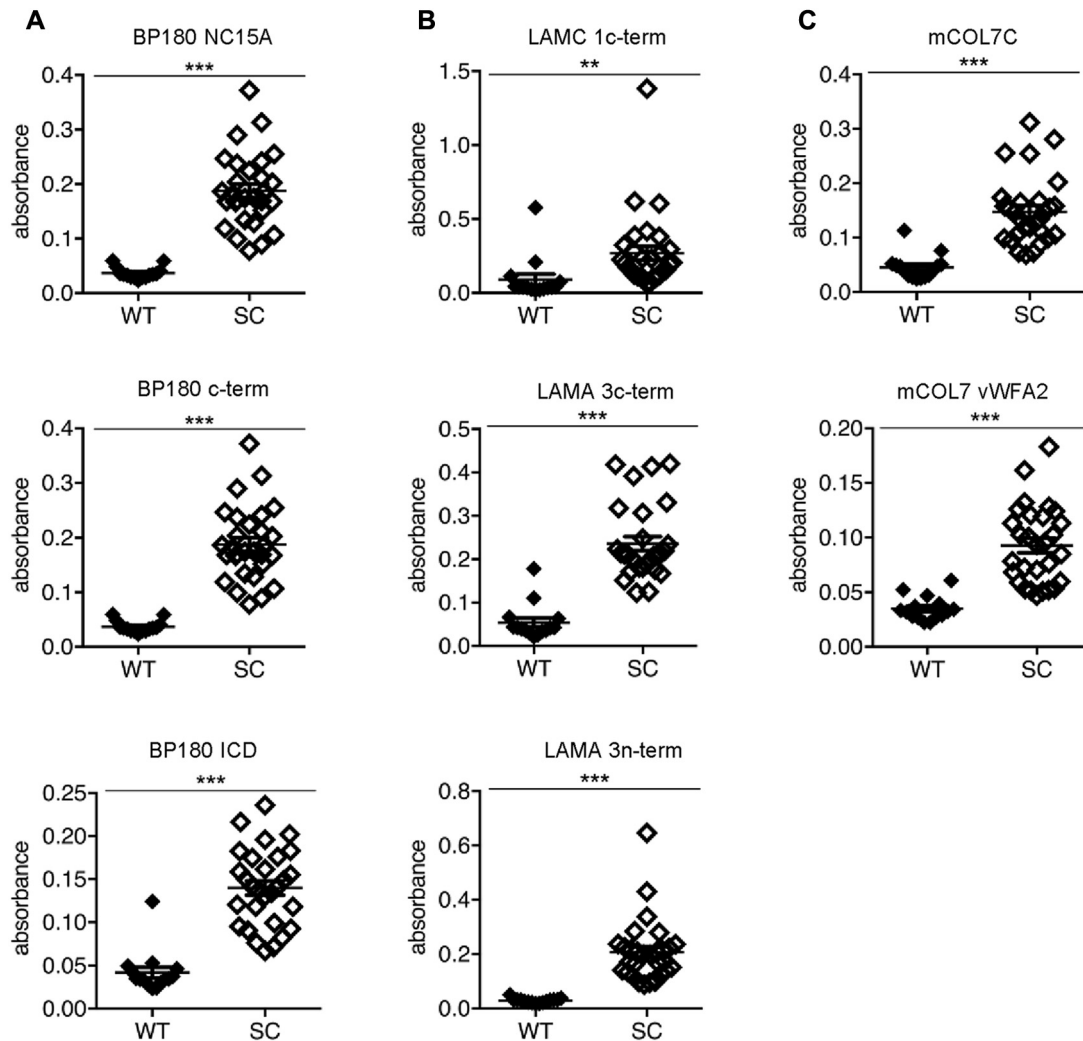


FIG 2. Reactivity of autoantibodies in scurfy (SC) sera to different skin autoantigens of the dermal-epidermal junction. ELISA of sera from scurfy ($n = 30$) and WT ($n = 16$) shows reactivity to different autoantigens located at the dermal-epidermal junction: **A**, BP180 intracellular domain (BP180 ICD), BP180 C-terminal domain (BP180 c-term), and BP180 NC15A; **B**, laminin $\gamma 1$ C-terminal domain (LAMC 1c-term), laminin 332 $\alpha 3$ chain C-terminal domain (LAMA 3c-term), and laminin 332 $\alpha 3$ chain N-terminal domain (LAMA 3n-term); and **C**, murine collagen VII C-terminal domain (mCOL7C) and murine collagen VII von Willebrand factor A-like domain 2 (mCOL7 vWFA2). The 2-tailed unpaired t test with the Welch correction was used. ** $P < .01$ and *** $P < .001$.

dissect the fine specificities of the polyclonal humoral response, we generated hybridomas of activated plasma cells from sick scurfy mice. We chose 5 scurfy mice after prescreening with indirect IF microscopy for the presence of autoantibodies and used their *in vivo*-activated pooled lymph node and splenic cells for fusion to the mouse myeloma cell line Sp2/0. Supernatants of

the resulting hybridomas (>500) were screened by using indirect IF microscopy on WT palatal tissue, and in addition to several supernatants with positive nuclear staining, we identified several hybridomas with a positive reticular epidermal staining pattern and a positive linear staining pattern, all of which were of the IgG₁ subclass (data not shown). One mAb, termed 20B12, shows

FIG 1. Scurfy mice have AIBDs. **A**, Representative macroscopic picture of the skin of scurfy (SC) and WT mice. **B**, Summary of skin disease in scurfy ($n = 49$) and WT ($n = 15$) mice. Bar indicates mean $\pm$ SD. **** $P < .0001$, Mann-Whitney U test. **C**, Left panel shows representative histology of scurfy and WT back skin (hematoxylin and eosin [H&E] staining, $\times 100$ original magnification). Scale bars = 100 μ m. Right panel shows subepidermal blister in scurfy leg skin with close-up of blister split (H&E staining, $\times 200$ original magnification). Scale bar = 100 μ m. **D**, Representative indirect IF microscopy with scurfy and WT sera on WT palatal sections. Scale bar = 50 μ m. **E**, Summary of indirect IF staining in scurfy ($n = 26$) and WT ($n = 26$) sera. Bar indicates means $\pm$ SDs. **** $P < .0001$, Mann-Whitney U test. **F**, Representative direct IF microscopy (IgG) of scurfy and WT palatal sections. Scale bar = 100 μ m. **G**, Summary of direct IF microscopy (IgG) in scurfy ($n = 24$) and WT ($n = 24$) palatal sections. Bar indicates means $\pm$ SDs. **** $P < .0001$, Mann-Whitney U test. **H**, Pattern analysis of direct IF microscopy (IgG) in scurfy palatal tissue ($n = 33$).

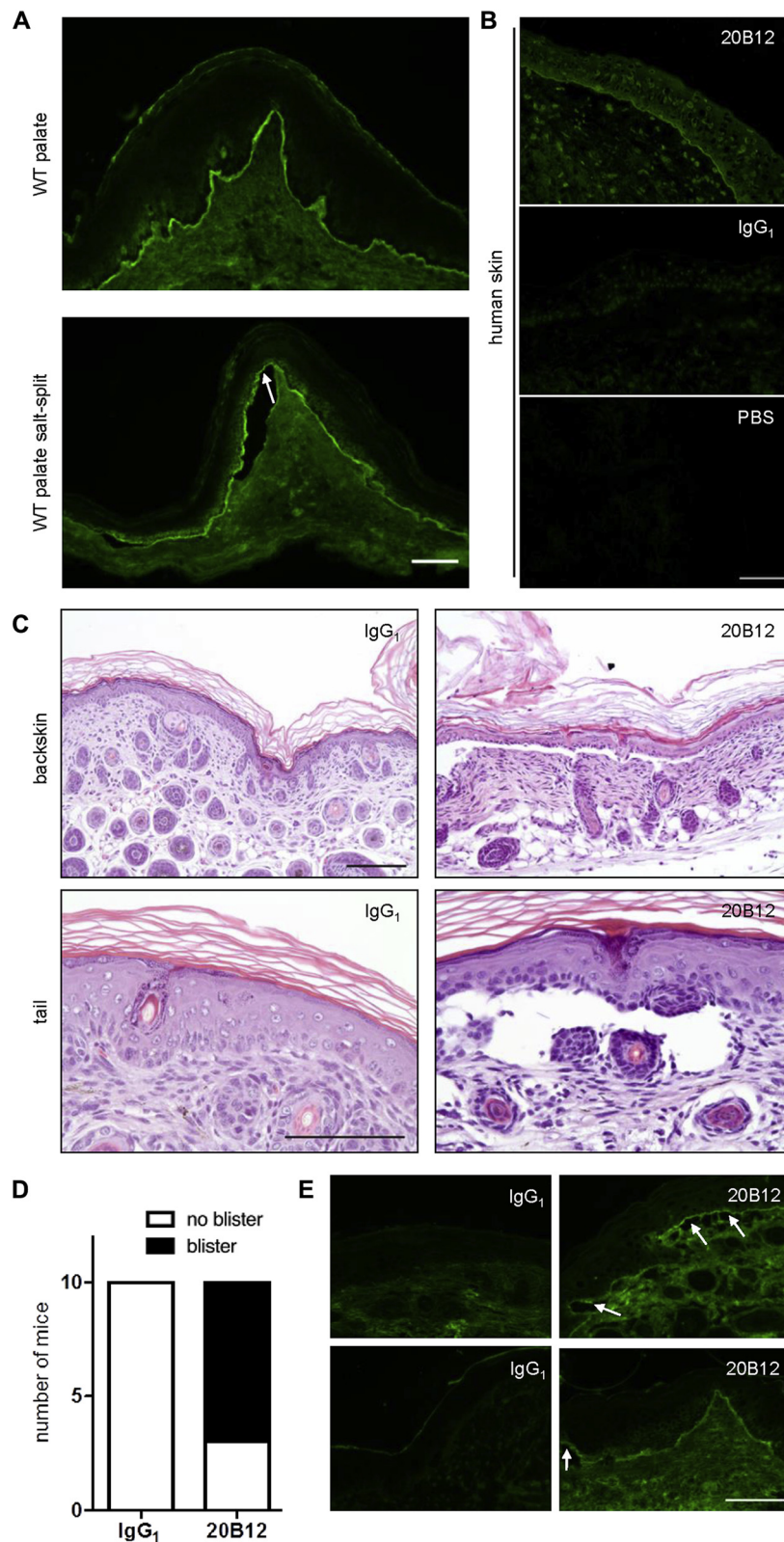


FIG 3. Autoantibody 20B12 recognizes its antigen in the dermal-epidermal junction, cross-reacts with human protein, and is pathogenic *in vivo*. **A**, mAb 20B12 shows a linear staining pattern in indirect IF microscopy on WT palate (*upper panel*) and staining of the blister roof on salt-split WT palate (*lower panel*). Scale bar = 100 μ m. **B**, Indirect IF microscopy on healthy human skin with the mAb 20B12, control IgG₁, or PBS. Scale bar = 100 μ m. **C**, Histology of skin of neonatal mice after injection of purified mAb 20B12 or murine IgG₁ as a control (hematoxylin and eosin staining, $\times 200$ [*upper panel*] and $\times 400$ [*lower panel*] original magnification). Scale bars = 100 μ m. **D**, Summary of injection experiments of neonatal mice with 20B12. **E**, Direct IF microscopy of skin of neonatal mice after injection of purified mAb 20B12. White arrows indicate initial blister split. Scale bar = 100 μ m.

TABLE I. Blister formation after subcutaneous injection of mAb 20B12 or control antibody

Mice per experiment	mAb 20B12 (mg/mouse)	Macroscopic blister	Microscopic blister
3	1.0-2.0	0/3	3/3
4	1.5-2.0	0/4	2/4
1	0.5	0/1	1/1
2	0.5-1.0	0/2	1/2
Mice per experiment	IgG ₁ (mg/mouse)	Macroscopic blister	Microscopic blister
3	1.0-2.0	0/3	0/3
3	1.5-3.0	0/3	0/3
1	1.0	0/1	0/1
3	1.0-2.5	0/3	0/3

positive linear staining at the dermoepidermal junction on indirect IF (Fig 3, A, upper panel), as well as linear staining at the blister roof (Fig 3, A, lower panel) by using salt-split skin for indirect IF as a common technique to differentiate between reactivity to autoantigens above or below the lamina lucida. Next, we tested binding of the murine autoantibody 20B12 on human healthy skin and found cross-reactivity with human skin (Fig 3, B).

To test whether the autoantibody 20B12 contributes to skin pathology in scurfy mice, we injected the purified antibody into neonatal mice as the gold standard method to test for blister formation.⁴⁵ None of the injected neonates showed macroscopic blisters, but the majority of mice had microscopic subepidermal blisters in different parts of the skin, such as the back, head, and tail (Fig 3, C, and Table I). The IgG₁ control did not induce blisters in neonatal mice (summary in Fig 3, D). As expected, mAb 20B12 was present in the tissue, and direct IF analysis revealed the previously observed linear pattern and some initial subepidermal blisters (Fig 3, E). In the majority of mice, mAb 20B12 induced subepidermal blisters *in vivo* (summarized in Table I), clearly indicating that this autoantibody is pathogenic.

mAb 20B12 targets BP230

To identify the target antigen, we used 20B12 for immunoprecipitation with murine palatal tissue lysate, followed by MALDI-TOF analysis. In 2 separate runs the murine protein BP230 (dystonin or bullous pemphigoid antigen 1 epidermal) was identified (see Fig E5, A, in this article's Online Repository at www.jacionline.org). To confirm these results, we first performed Western blot analyses with purified 20B12 antibody. Because human and murine BP230 show 84% homology, lysate of human embryonic kidney (HEK) cells transfected with human BP230 and rabbit anti-human BP230 serum was used as a positive control (Fig 4, A). 20B12 showed a positive band at the same molecular weight as the positive control when applied to lysate of the murine keratinocyte cell line, as well as murine palatal tissue lysate and HEK cells transfected with human BP230 (Fig 4, A). The same results were obtained with lysate of primary murine keratinocytes (Fig 4, B). As expected from positive direct IF of 20B12 on human skin, 20B12 shows cytoplasmic staining in primary murine keratinocytes, as well as primary human keratinocytes, and did not stain fibroblasts (Fig 4, C). Next, HEK cells transfected with

human BP230 were used for indirect IF staining and compared with cells transfected with human BP180 as a control for unspecific binding. 20B12 stained the BP230⁺ cells, but not the control cells, transfected with human BP180 (Fig 4, D).

To further characterize the antigenic epitope of mAb 20B12, we used a chip-based approach with overlapping peptides covering the whole BP230 protein. After incubation with mAb 20B12, fluorescence-based chip analysis yielded 5 potential antigenic epitopes of 7 to 10 amino acids in length (data not shown). We then synthesized the 5 potential antigenic epitopes as biotinylated peptides, performed ELISA, and found reactivity of mAb 20B12 to one particular peptide epitope (see Fig E5, B). We next screened scurfy and WT sera using ELISA for reactivity to the newly identified target peptide of 20B12, and the majority of scurfy but not WT sera recognized the peptide epitope (Fig 4, E). Thus the pathogenic autoantibody 20B12 recognizes BP230.

Transfer of autoreactive scurfy CD4⁺ T cells into T cell-deficient *nu/nu* mice induces skin-reactive autoantibodies and subepidermal blisters in skin of recipient mice

To prove that activated autoreactive CD4⁺ T cells are sufficient to initiate autoantibody production, finally leading to AIBDs, we transferred isolated scurfy and WT CD4⁺ T cells into nude mice. Nude mice display a normal B-cell repertoire but are devoid of T cells. Recipient nude mice have an inflammatory skin phenotype resembling that of scurfy mice within 3 to 4 weeks after transfer of scurfy CD4⁺ T cells, whereas nude mice, which received WT CD4⁺ T cells, were unaffected (Fig 5, A, upper panel). On histologic examination, we found subepidermal blisters in the majority of mice after scurfy CD4⁺ T-cell transfer (Fig 5, A, lower panel, and Fig 5, B). Analysis of monoclonal autoantibodies from hybridoma supernatants derived from splenocytes of recipient nude mice after transfer of scurfy CD4⁺ T cells revealed a linear staining pattern on indirect IF microscopy (Fig 5, C). Sera of recipient mice were analyzed for the presence of autoantibodies targeting known autoantigens of AIBDs. Only sera of nude mice after transfer of scurfy CD4⁺ T cells were positive for autoantibodies against different autoantigens of AIBDs and displayed a similarly diverse humoral response as scurfy mice (Fig 5, D-F). Nude mice transferred with WT CD4⁺ T cells did not have autoantibodies.

To exclude that a contamination of scurfy CD4⁺ T cells with autoantibody-producing plasma cells was responsible for the autoantibody production in recipient nude mice, we used T and B cell-deficient *Rag*^{-/-} mice transferred with scurfy CD4⁺ T cells as a control, and as expected, these mice did not have autoantibodies after transfer (Fig 5, D-F). We then screened the sera of the transferred mice using ELISA for reactivity to the newly identified target peptide of 20B12 and found reactivity in the majority of mice transferred with scurfy, but not WT, CD4⁺ T cells (Fig 5, G).

DISCUSSION

We report here that AIBDs develop in the absence of functional Treg cells. Within the polyclonal humoral immune response in scurfy mice, we identified the murine IgG₁ autoantibody 20B12, which had spontaneously evolved in a

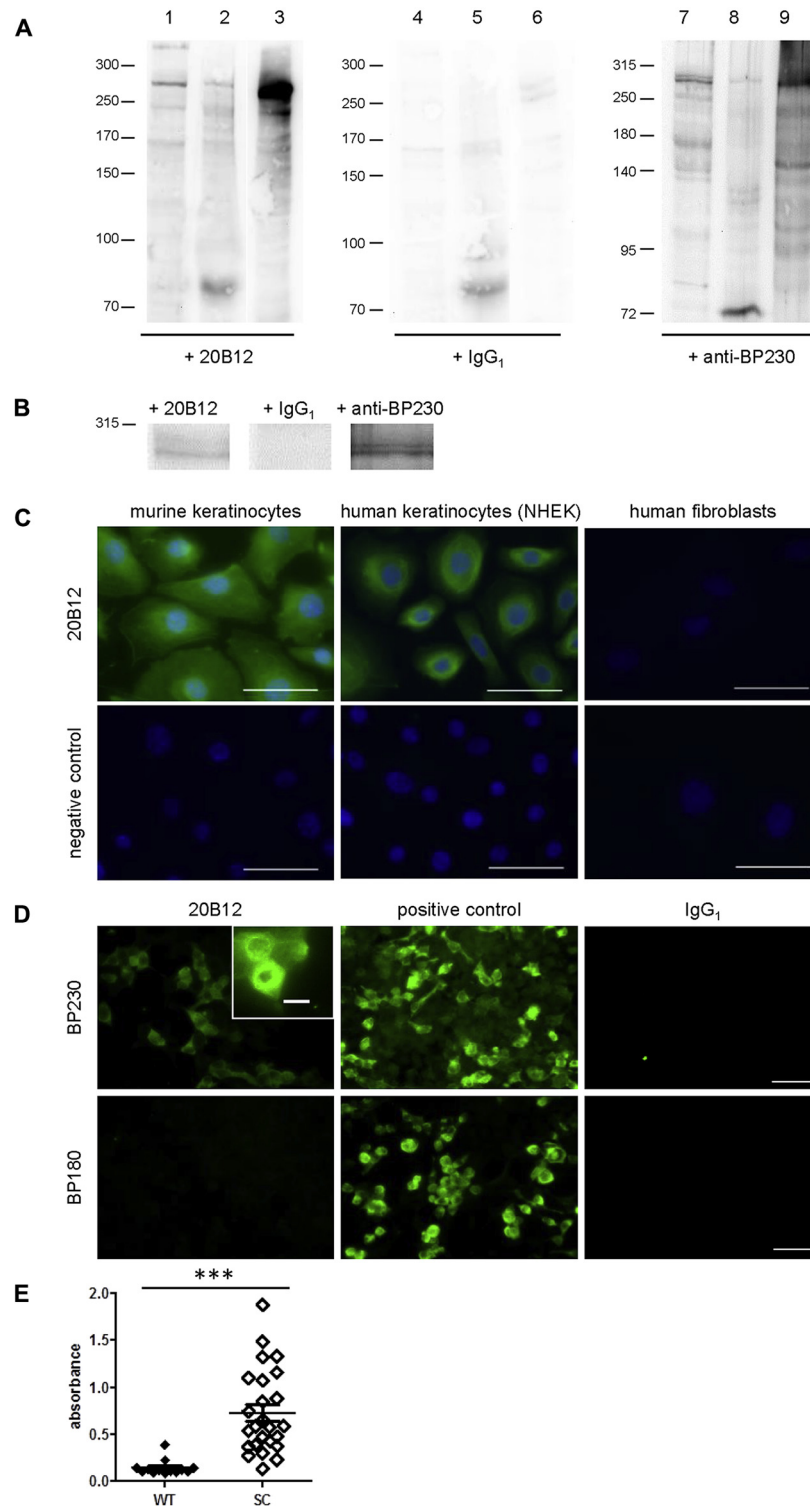


FIG 4. Autoantibody 20B12 recognizes murine and human BP230. **A**, Western blot with purified 20B12 (lines 1-3), isotype control (lines 4-6), or rabbit anti-BP230 serum on murine and human cell/tissue lysates (lines 1, 4, and 7: murine keratinocyte cell line MCA-3D; lines 2, 5, and 8: WT skin lysate; lines 3, 6, and 9: lysate of HEK cells overexpressing human BP230). **B**, Western blot with purified 20B12, isotype control, and rabbit anti-BP230 serum on murine primary keratinocytes. **C**, Primary murine keratinocytes (left panel), human keratinocytes (NHEK; middle panel), and human fibroblasts (right panel) stained with 20B12 ($\times 400$ magnification). Scale bars = 50 μ m. **D**, HEK cells overexpressing human BP230 (upper panel) and human BP180 as a control (lower panel) stained with purified 20B12, positive control (rabbit anti-BP180 IgG and rabbit anti-BP230 IgG), or isotype control ($\times 400$ magnification). Scale bars = 50 μ m. Inset = $\times 630$ original magnification, scale bar = 10 μ m. **E**, ELISA of scurfy (SC; $n = 25$) and WT ($n = 13$) sera shows reactivity to antigenic peptide epitope of mAb 20B12 (2-tailed unpaired t test with the Welch correction). *** $P < .001$.

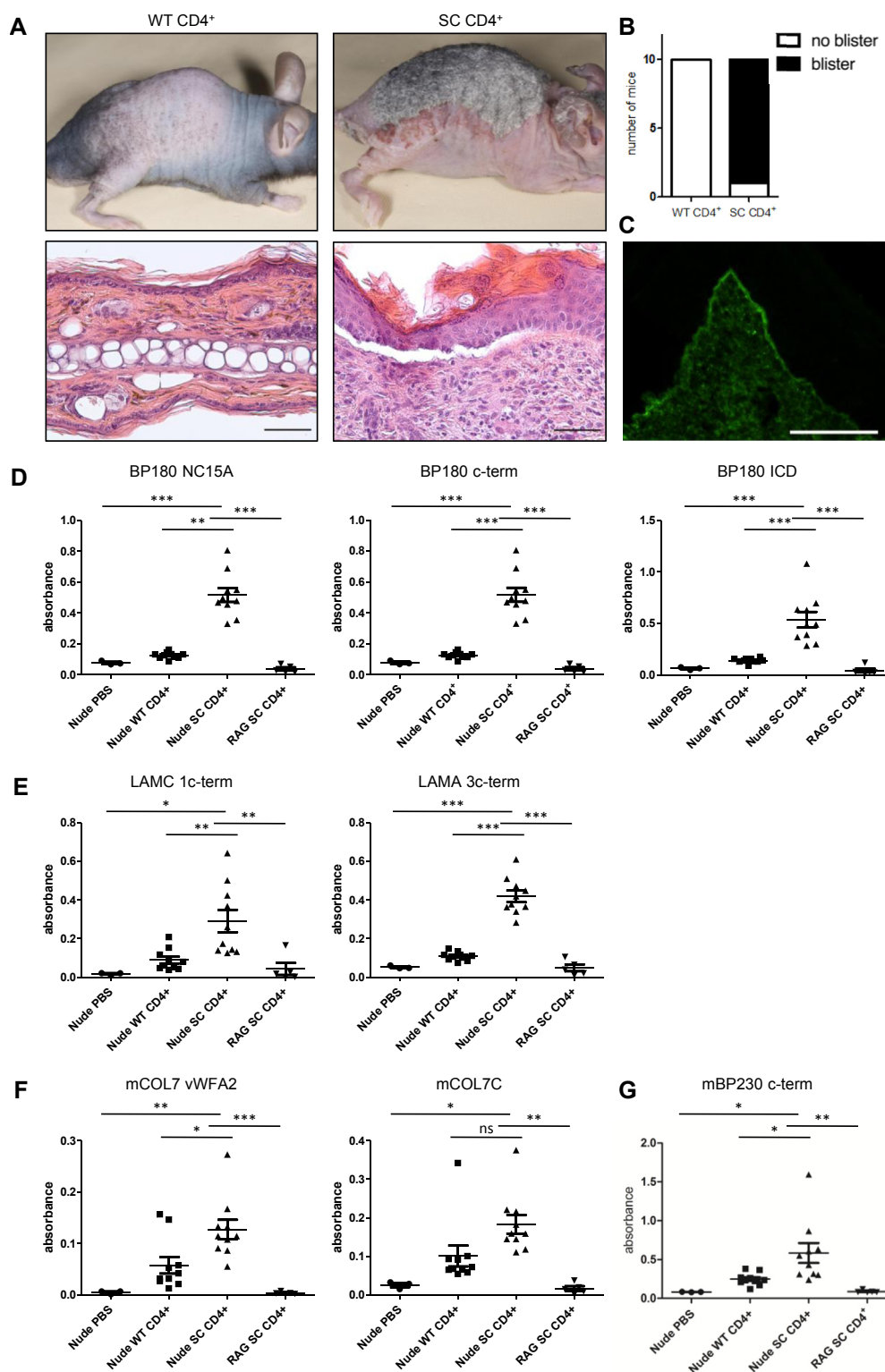


FIG 5. Transfer of autoreactive CD4⁺ T cells into T cell-deficient nude mice induces pathogenic autoantibodies, leading to AIBDs. **A**, Representative macroscopic picture (*upper panel*) and histology (*lower panel*) of nude mice 4 to 8 weeks after transfer of 2×10^6 scurfy (SC) or WT CD4⁺ T cells. Representative data of 2 independent experiments are shown (original magnification $\times 400$). Scale bars = 50 μ m. **B**, Summary of histologic analyses for subepidermal blister formation ($n = 7$ in both groups). **C**, Representative indirect IF staining of monoclonal autoantibody derived from nude mice transferred with scurfy CD4⁺ T cells ($\times 200$ original magnification). Scale bar = 100 μ m. **D-F**, ELISA with sera from nude mice transferred with either PBS ($n = 3$), WT CD4⁺ T cells ($n = 10$), or scurfy CD4⁺ T cells ($n = 10$) and Rag^{-/-} mice transferred with scurfy CD4⁺ T cells ($n = 5$). Pooled data of 3 independent transfer experiments are shown (1-way ANOVA with the Bonferroni posttest). In Fig 5, **F**, ELISA with sera from transferred nude mice shows reactivity to antigenic peptide epitope of mAb 20B12 (1-way ANOVA with the Bonferroni posttest). * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, Not significant.

sick scurfy mouse and is pathogenic *in vivo*. 20B12 binds to its target antigen BP230 and induces subepidermal blisters in healthy skin.

BP230 is an intracellular constituent of the hemidesmosomal plaque and is known as one antigenic target of human BP.¹⁰⁻¹³ However, in contrast to BP180, little is known about the pathogenic relevance of autoantibodies to BP230 in patients with BP. The vast majority of patients with BP display autoantibodies to BP180, especially to the BP180 extracellular 16th noncollagenous domain,⁸ and autoantibodies to BP230 have been shown to evolve after epitope spreading.⁴⁶ However, a minority of patients with clinically confirmed BP have only autoantibodies to BP230 and no humoral response to BP180. Recently, this cohort of patients with BP was clinically analyzed and revealed a less severe phenotype with a smaller affected skin area, as well as a better therapeutic response to steroids, compared with patients with only BP180 autoantibodies.⁴⁷ Thus the authors proposed to classify these patients as a novel subtype of BP, termed anti-BP230 BP. The fact that this group of patients has skin disease suggests that BP230 autoantibodies are pathogenic but that data from different animal studies are controversial: immunization of rabbits with peptides derived from human BP230 yielded circulating antibodies but no blisters,⁴⁸ and injection of polyclonal rabbit anti-murine BP230 antibody did not lead to blister formation.⁴⁹ However, the significance of the results in the latter study is limited because the antibodies were directed only against C-terminal epitopes of murine BP230, so that antibodies directed against N-terminal epitopes or the central rod domain could still be pathogenic,⁴⁹ even more so because it is known that the antigenic epitopes in patients with BP are distributed over the entire BP230 protein.¹⁰⁻¹³ In contrast, another animal study showed that injection of neonatal mice with rabbit antibodies against conserved sequences of BP230 induced blisters in the majority of animals.⁵⁰ Our results now demonstrate that autoantibodies against BP230 are pathogenic *in vivo*. The fact that 3 of 10 mice did not have blisters might be due to antibody doses in a suboptimal range for blister induction in these mice. Because our antibody cross-reacts with human skin, the antigenic epitope is likely to be located in the conserved regions of BP230.

Because BP230 is an intracellular constituent of the hemidesmosomal plaque, the pathogenic autoantibodies likely enter the cell to cause loss of adhesion at the dermal-epidermal junction. Cell-penetrating antibodies have been shown to be pathogenic in patients with different autoimmune diseases, including lupus erythematosus.⁵¹⁻⁵³ After cell penetration, pathogenic autoantibodies likely interfere with intermediate filaments by binding to the intracellular BP230 protein and causing instability of the hemidesmosomal plaque, resulting in loss of cell adhesion. This hypothesis is supported by the fact that BP230 physically interacts with keratin filaments in the basal keratinocytes.⁵⁴ Furthermore, in BP230 knockout mice, the inner plate of the hemidesmosome is missing, and association with keratin filaments is lost.⁵⁴ Interestingly, BP230 knockout mice show subepidermal blistering predominantly on the tail and oral mucosa, resembling the pathology of scurfy mice. Furthermore, we previously identified keratin 14, which is predominantly expressed in the basal layers of stratified epithelia and known to interact with BP230, as a target of the humoral and cellular immune response in scurfy mice and patients with IPEX syndrome.²⁹

Many studies, mostly in mouse models of BP, have elucidated the pathogenic importance of humoral and cellular autoimmunity against BP180 and further delineated the Fc/Fc γ receptor (Fc γ R)-dependent and Fc/Fc γ R-independent mechanisms of blister formation.⁵⁵⁻⁵⁸ After transfer of our anti-BP230 mAb, we detected the autoantibody in palatal tissue, but we could not identify colocalized staining for C3 (see Fig E2, C and D), suggesting that the antibody might cause blisters in a Fc/Fc γ R-independent fashion. In line with our results, a study in C3-deficient COL17-humanized mice, in which injection of BP autoantibodies induced subepidermal blisters, proved that C3 is dispensable for blister formation.⁵⁷

First mouse models of BP were hampered by the fact that human autoantibodies against BP180 did not induce skin pathology in mice, and it was not until the generation of active mouse models of BP that the importance of autoreactive T cells for initiation of AIBDs was analyzed.^{15,42} Immunization of WT mice with recombinant murine NC15A induces a BP phenotype in mice. In another experimental system *Rag2*^{-/-} Col17-humanized mice transferred with splenocytes of WT mice, which had been previously immunized with human type XVII collagen, had autoantibodies and reproduced a clinical BP phenotype.¹⁵ Further studies in this mouse model showed the importance of NC15A-specific CD4⁺ T cells for the development of disease.²² These data were in line with previous reports analyzing specific CD4⁺ T-cell responses in patients with BP.⁵⁹

We and others have shown that scurfy mice and patients with IPEX syndrome have a predominantly T_H2 phenotype of autoreactive CD4⁺ T cells in the skin and a mixed T_H1 and T_H2 response in skin-draining lymph nodes and peripheral blood.^{37,60} Interestingly, patients with BP and a certain HLA susceptibility locus were found to have a mixed T_H1 and T_H2 response in their T-cell compartments, whereas control subjects had a T_H1 response.^{59,61} These studies suggested a role for autoreactive CD4⁺ T cells in initiation of blistering disease. Our study now provides compelling evidence that autoreactive CD4⁺ T cells, which are activated in the absence of Treg cells, are crucial for the initiation of blistering diseases. It was already proposed that the autoantibodies in scurfy mice are not only an epiphenomenon but contribute substantially to skin pathology because scurfy mice crossed to B-cell knockout mice have a longer lifespan.³² Our data prove the pathogenicity of the anti-BP230 antibody, which had developed because of the missing Treg cell control, and strongly support this finding.

Not all scurfy mice display manifest blistering disease. One reason might be the polyclonal nature of the humoral response. We have identified autoantibodies specific for several known antigenic targets of blistering disease, such as BP180, laminin γ 1, laminin α 3, and type VII collagen, but not all of these antibodies might be pathogenic. Based on the present data, for the first time, we show that autoantibodies with the capacity to induce an AIBD develop as a consequence of a missing Treg cell control. However, autoantibody-mediated pathology takes more time to evolve than T cell-mediated pathology, and because scurfy mice die early in life of severe multiorgan autoimmune inflammation, the AIBDs might not have fully evolved.

The importance of our data concerning the pathogenic relevance of a Treg cell defect for generation of pathogenic

autoantibodies and initiation of AIBDs in human subjects is supported by a report describing a case of BP in a 7-year-old boy with IPEX syndrome.⁶² This is a very unusual age of disease manifestation because BP typically affects elderly patients.² However, missing Treg cell control leads to the development of pathogenic autoantibodies and disease manifestation at this early age.

In summary, we show that a pathogenic autoantibody targeting BP230 develops in Treg cell-deficient scurfy mice, indicating that Treg cells control autoreactive T cells and thereby prevent the development of AIBDs.

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Key messages

- Scurfy mice lacking functional Treg cells have a spontaneous autoantibody-mediated AIBD.
- Autoreactive scurfy CD4⁺ T cells induce autoantibodies with reactivity to AIBD autoantigens and subepidermal blisters in skin after transfer to T cell-deficient *nu/nu* mice.
- Autoantibodies targeting the hemidesmosomal protein dystonin, also known as BP230, induce blisters *in vivo*.

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METHODS

Detection of BP230 on overexpressing HEK cells

Slides with fixed and permeabilized HEK cells, which were transiently transfected with human recombinant full-length BP180 and BP230, were kindly provided by Euroimmun (Lübeck, Germany). Slides were blocked with 5% goat serum in TBS followed by the primary antibody (1 mg/mL) or positive control (rabbit anti-BP180 IgG [1:500] or rabbit anti-BP230 IgG [1:10]) for 30 minutes. After washing with PBS-Tween, slides were incubated with the secondary antibody (for 20B12 and isotype, goat anti-mouse IgG Alexa Fluor 488 [1:500]; for positive controls, goat anti-rabbit IgG Alexa Fluor 488 [1:500]; Invitrogen).

Western blot analysis with mAb 20B12

Primary murine keratinocytes or cells of a murine keratinocyte cell line (MCA-3D; kindly provided by Professor Y. Samstag, Heidelberg, Germany) were lysed with RIPA buffer. Skin samples were homogenized with RIPA buffer by using a gentleMACS dissociator (Miltenyi Biotec). Three hundred micrograms of lysate was boiled with reducing SDS loading buffer, run on an 8% SDS-PAGE gel, and transferred by means of wet transfer on a nitrocellulose membrane (Immobilon; Millipore, Darmstadt, Germany). After blocking with 5% skim milk, the primary antibody (mAb 20B12 or isotype [0.1 mg/mL diluted in 5% BSA in TBS-Tween]) was added for 1 hour, followed by the horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse IgG-Fc-HRP, 1:5,000; Jackson ImmunoResearch, West Grove, Pa). As a positive control, sera of rabbits immunized with human BP230 (1:3,000 in 5% BSA in TBS-Tween) were used as a primary antibody detected by using a goat anti-rabbit IgG-Fc-HRP secondary antibody (1:20,000; Dianova, Hamburg, Germany). Signals were visualized with ECL solution (Amersham, GE Healthcare, Little Chalfont, United Kingdom) and documented with a gel imaging system (Bio-Rad, Munich, Germany).

Western blotting with murine recombinant protein fragments

For Western blot analysis, 15 μ g of recombinant proteins (BP180 NC15A,^{E1} murine collagen VII von Willebrand factor A-like domain 2,^{E2} murine collagen VII C-terminal domain,^{E3} laminin 332 α 3 chain N-terminal domain,^{E4} and laminin γ 1 C-terminal domain^{E4}) were loaded with reducing SDS buffer, separated on a 12% polyacrylamide gel, and transferred by means of semidry transfer on a nitrocellulose membrane (Immobilon; Millipore). After blocking with 5% skim milk, scurfy or WT sera (1:20 prediluted in 5% BSA in PBS-Tween) were incubated for 1 hour at room temperature. After washing with PBS-Tween, goat anti-mouse IgG-Fc-HRP (1:10,000, Jackson ImmunoResearch) was applied as a secondary antibody for 1 hour at room temperature. Signals were visualized with ECL solution (Amersham, GE Healthcare) and documented with a gel imaging system (Bio-Rad).

ELISA with murine recombinant protein fragments

Ninety-six-well plates (NUNC MaxiSorb; eBioscience, Frankfurt, Germany) were coated with 250 ng per well of different murine recombinant protein fragments (BP180 intracellular domain, murine BP180 C-terminal domain, BP180 NC15A,^{E1} murine collagen VII von Willebrand factor A-like domain 2,^{E2} murine collagen VII C-terminal domain,^{E3} murine laminin 332 α 3 chain C-terminal domain and N-terminal domain,^{E4} and murine laminin γ 1 C-terminal domain^{E5}) in 0.05 mol/L carbonate/bicarbonate buffer (pH 9.6) overnight at 4°C. After blocking with 1% BSA (Sigma-Aldrich), wells were incubated with 50 μ L of scurfy or WT sera (1:20 prediluted in 1% BSA PBS-Tween) and PBS-Tween, followed by incubation with 50 μ L of goat-anti mouse IgG-Fc-HRP (1:10,000; Bethyl, Montgomery, Tex). After washing, plates were developed with 50 μ L of 3,3',5,5'-Tetramethylbenzidine. All samples were run in duplicates, and absorbance was measured at a wavelength of 450 nm.

ELISA with biotinylated peptides

Streptavidin-coated 96-well plates (Pierce Streptavidin High Binding Capacity, Thermo Fisher Scientific) were used and incubated with the 5 different C-terminal biotinylated peptides, which were previously identified by using epitope mapping with mAb 20B12 (HIQENSAYFEFFN-biotin, RDSYHPLDDWIQ-biotin, KEHEHQLLRLQCEI-biotin, LNQACSRDEE FQF-biotin, and VDCQCFLLPFGE-biotin). The peptide-biotin incubation was performed for 4 hours at room temperature, followed by washing 5 times with washing buffer (PBS containing 0.1% BSA and 0.05% Tween). Purified mAb 20B12 (0.3 μ g/mL), purified isotype (0.3 μ g/mL), or sera (1:20 prediluted in washing buffer) were used as primary antibodies and incubated for 30 minutes at room temperature. After washing, the secondary antibody goat-anti mouse IgG-Fc-HRP (1:10,000, Bethyl) was applied for 30 minutes at room temperature. The plates were developed with 70 μ L of TMB. All samples were run in duplicates, and absorbance was measured at a wavelength of 450 nm.

Epitope mapping

The sequence of BP antigen 1 epidermal (or BP230) was translated into 7- and 13-amino-acid peptides with peptide-peptide overlaps of 6 and 12 amino acids, respectively. After peptide synthesis, all peptides were cyclized through a thioether linkage between a C-terminal cysteine and an appropriately modified N-terminus. The resulting conformational BP antigen 1 epidermal peptide microarrays contained 5,232 different peptides printed in duplicates (10,464 peptide spots) and were framed by additional HA (YPYDVPDYAG, 228 spots) control peptides. The microarray was blocked with blocking buffer for 30 minutes and incubated with 20B12 hybridoma supernatant for 16 hours at 4°C. After washing 2 times with PBS-Tween, the microarray was incubated with goat anti-mouse IgG(Fc) DyLight680 (1:5,000; Rockland, BioMol, Hamburg, Germany) for 45 minutes at room temperature. Staining anti-HA DyLight800 (1:2,000, clone: 12CA5 produced by Dr Gerd Moldenhauer, DKFZ, Heidelberg, Germany) was used as a control. Fluorescence was detected by using a LI-COR Odyssey Imaging System (LI-COR Biosciences, Lincoln, Neb). Epitope mapping was performed by PEPperPRINT GmbH (Heidelberg, Germany).

MALDI-TOF

Sample preparation, gel electrophoresis, and tryptic digestion. Beads were incubated with 30 μ L of LDS buffer containing reducing agent (Invitrogen) for 10 minutes at 70°C to elute the proteins. Subsequently, proteins were separated by using 1-dimensional gel electrophoresis on a NuPAGE 4-12% Bis-Tris gradient gel (Invitrogen) with a MOPS buffer system. The gel was stained with colloidal Coomassie Blue, and each lane was cut into 9 pieces. Each gel piece was washed once with 100 μ L of H₂O and 100 μ L of H₂O/acetonitrile 50/50 (vol/vol) and incubated for 5 minutes at 37°C. After removing the solution from the gel plugs, proteins were alkylated with 100 μ L of 55 mmol/L iodoacetamide in 40 mmol/L NH₄HCO₃ for 30 minutes at 25°C in the dark, followed by 3 alternating washing steps, each with 150 μ L of water and H₂O/acetonitrile 50/50 (vol/vol) for 10 minutes at 37°C. Gel pieces were dehydrated with 100 μ L of neat acetonitrile for 1 minute at room temperature, dried for 15 minutes, and subsequently rehydrated with porcine trypsin (sequencing grade; Promega, Mannheim, Germany) with a minimal volume sufficient to cover the gel pieces after rehydration (100 ng of trypsin in 40 mmol/L NH₄HCO₃). After overnight digestion, the supernatant was collected in PCR tubes, and gel pieces were subjected to 4 further extraction steps (acetonitrile/0.1% TFA 50/50 [vol/vol]). The combined solutions were evaporated to dryness, redissolved in 0.1% TFA/2.5% hexafluoroisopropanol, and subsequently analyzed by using nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (MS/MS).

Electrospray ionization-MS/MS. Tryptic peptide mixtures were separated with a nano-Acquity Ultra Performance Liquid Chromatography system (Waters GmbH, Eschborn, Germany). Peptides were trapped on a nano-Acquity C18 column (180 μ m $\times$ 20 mm; particle

size, 5 μm ; Waters GmbH). The liquid chromatography separation was performed on a C18 column (BEH 130 C18, 100 $\mu\text{m} \times 100 \text{ mm}$; particle size, 1.7 μm ; Waters GmbH) with a flow rate of 400 nL/min. Chromatography was carried out with a 1-hour gradient of solvent A (98.9% water, 1% acetonitrile, and 0.1% formic acid) and solvent B (99.9% acetonitrile and 0.1% formic acid) in the following sequence: from 0% to 4% solvent B in 1 minute, from 4% to 40% solvent B in 40 minutes, from 40% to 60% solvent B in 5 minutes, from 60% to 85% solvent B in 0.1 minute, 6 minutes at 85% solvent B, from 85% to 0% solvent B in 0.1 minute, and 9 minutes at 0% solvent B. The nano-ultraperformance liquid chromatography system was coupled online to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). The mass spectrometer was operated in data-dependent mode to automatically measure MS1 and MS2. The following parameters were set: electrospray ionization voltage, 2400 V; capillary temperature, 200°C; and normalized collision energy, 35 V. Data were acquired by using scan cycles of 1 Fourier transform mass spectrometric scan with a resolution of 60,000 at 400 m/z and a range from 300 to 2000 m/z in parallel with 6 MS/MS scans in the ion trap of the most abundant precursor ions.

Database searches

The mgf files generated by using Xcalibur software (Thermo Scientific) were used for database searches with the MASCOT search engine (version 2.4; Matrix Science, London, United Kingdom) against the UniProt database (version Uniprot_Swissprot_Trembl_2015_08) with taxonomy set to *Mus musculus*. Each slice was analyzed separately by using MS/MS, and

MS/MS data of each slice were merged into a single file before protein database search. Peptide mass tolerance for database searches was set to 5 ppm, and fragment mass tolerance was set to 0.4 Da. Carbamidomethylation of C was set as fixed modification. Variable modifications included oxidation of M and deamidation of N and Q. One missed cleavage site in case of incomplete trypsin hydrolysis was allowed. Furthermore, proteins were considered identified if at least 2 unique peptides had an individual ion score exceeding the MASCOT identity threshold. Identified proteins were summarized in an Excel table.

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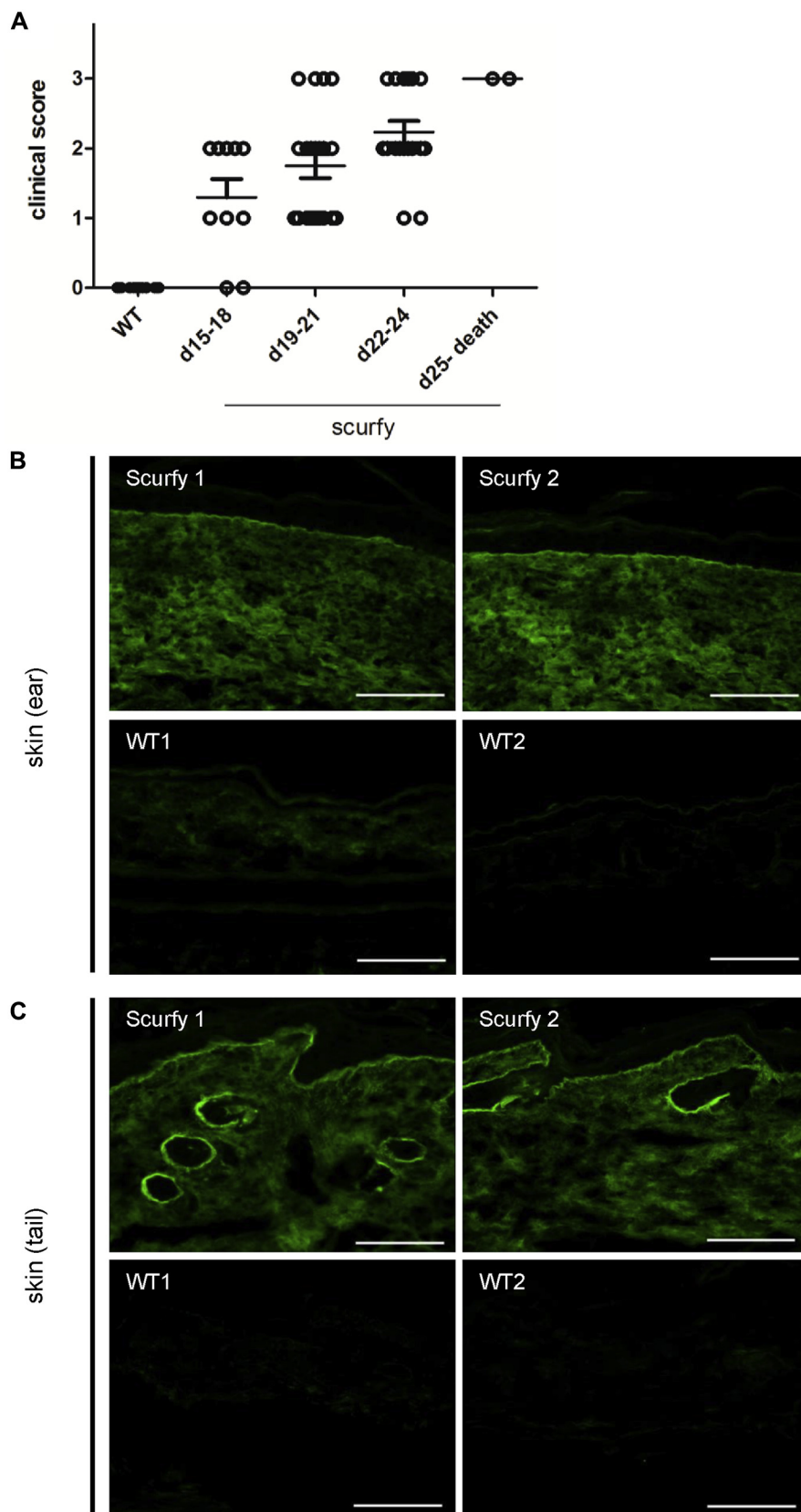


FIG E1. Skin disease in scurfy mice over time and direct IF microscopy on scurfy and WT ear and tail skin. **A**, Skin disease in scurfy and WT mice at different ages. *Bar* indicates mean $\pm$ SD. **B**, Representative direct IF microscopy of 2 scurfy and 2 WT ear skin sections. *Scale bars* = 100 μ m. **C**, Representative direct IF microscopy of 2 scurfy and 2 WT tail skin sections. *Scale bars* = 100 μ m.

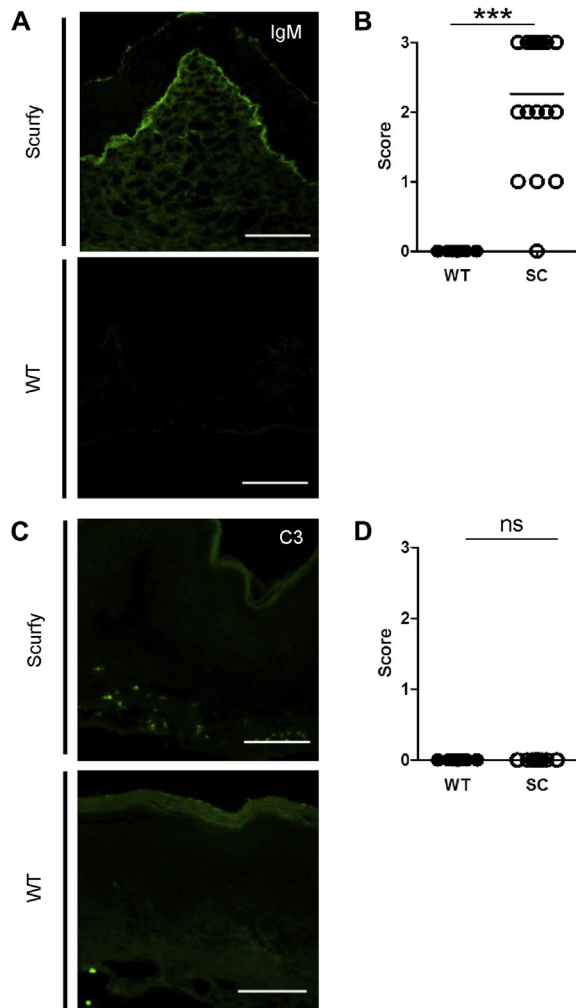


FIG E2. Positive linear IgM staining but no C3 deposition in palatal tissue of scurfy (SC) mice. **A**, Representative direct IF microscopy (IgM) in scurfy and WT palatal sections. Scale bar = 100 μ m. **B**, Summary of direct IF microscopy (IgM) on scurfy (n = 20) and WT (n = 20) palatal sections. Bar indicates mean. *** P < .001, Mann-Whitney U test. **C**, Representative direct IF microscopy (C3) of scurfy and WT palatal sections. Scale bars = 100 μ m. **D**, Summary of direct IF microscopy (C3) in scurfy (n = 20) and WT (n = 20) palatal sections. Bar indicates mean. P = not significant (Mann-Whitney U test).

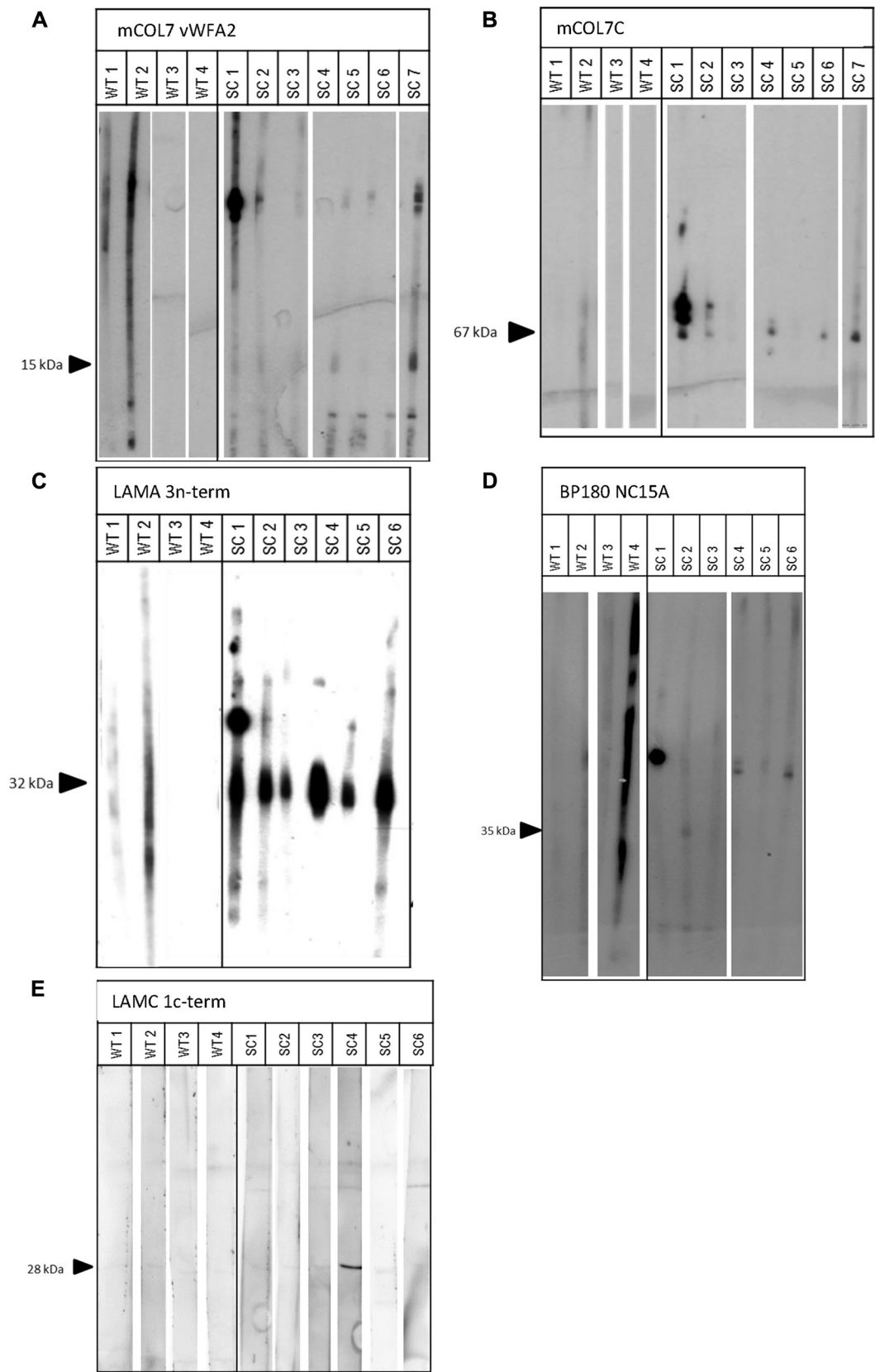


FIG E3. Scurvy and WT sera show reactivity against different proteins of the BMZ by using Western blotting. mCOL7 vWFA2 (**A**), murine collagen VII C-terminal domain (*mCOL7C*; **B**), laminin 332 α 3 chain N-terminal domain (*LAMA 3n-term*; **C**), BP180 NC15A (**D**), and laminin γ 1 C-terminal domain (*LAMC 1c-term*; **E**) were used for Western blot analyses with 6 different scurvy and 4 different WT sera.

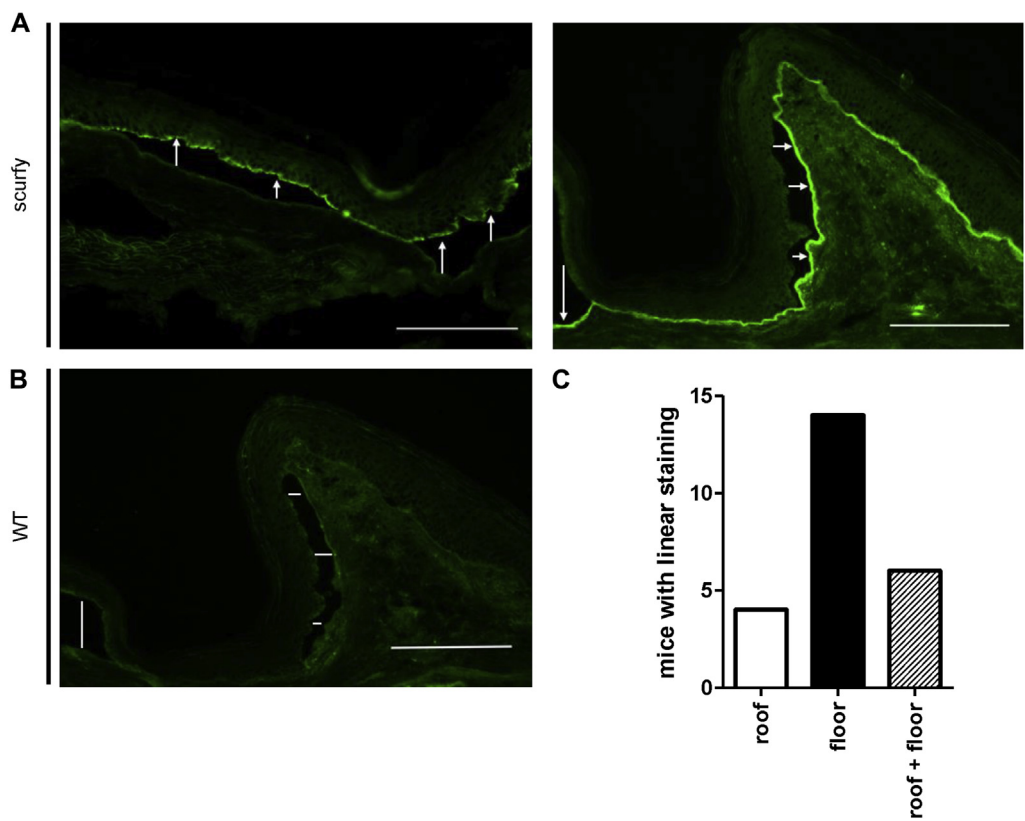


FIG E4. Positive staining of blister floor and roof by using indirect IF microscopy of scurfy sera on salt-split WT palatal tissue. **A** and **B**, Representative indirect IF microscopy of 2 different scurfy (Fig E4, A) and WT (Fig E4, B) sera on salt-split WT palatal tissue sections. *Scale bars* = 100 μ m. **C**, Summary of the location of positive staining on salt-split WT palatal tissue of positively tested scurfy sera (n = 24).

A

Hit No.	Accession No.	Protein Description	Score	Mass [Da]	sign Prot Sequences	Coverage [%]
1	A0A087WPR7	Dystonin (Fragment) OS=Mus musculus GN=Dst PE=1 SV=1	1207	334308	18	6,9
2	P50446	Keratin, type II cytoskeletal 6A OS=Mus musculus GN=Krt6a PE=1 SV=3	1014	59641	14	28,9
3	Q542G9	Annexin OS=Mus musculus GN=Anxa2 PE=2 SV=1	1008	38937	14	44
4	A0A075B5P4	Ig gamma-1 chain C region secreted form (Fragment) OS=Mus musculus GN=Ighg1 PE=1 SV=1	974	36292	13	60,8
5	Q61781	Keratin, type I cytoskeletal 14 OS=Mus musculus GN=Krt14 PE=1 SV=2	945	53176	12	29,3
6	Q32P04	Keratin 5 OS=Mus musculus GN=Krt5 PE=2 SV=2	918	61957	14	19,1
7	A0A075B5Q4	Protein Ighv5-12 (Fragment) OS=Mus musculus GN=Ighv5-12 PE=4 SV=1	819	13238	8	70,9
8	Q9QWL7	Keratin, type I cytoskeletal 17 OS=Mus musculus GN=Krt17 PE=1 SV=3	769	48417	11	24,5
9	Q3TEK2	Putative uncharacterized protein OS=Mus musculus GN=Hspa8 PE=2 SV=1	739	71041	9	25,9
10	Q6IFX2	Keratin, type I cytoskeletal 42 OS=Mus musculus GN=Krt42 PE=1 SV=1	724	50444	11	23,9

Hit No.	Accession No.	Protein Description	Score	Mass [Da]	sign Prot Sequences	Coverage [%]
1	Q3UV17	Keratin, type II cytoskeletal 2 oral OS=Mus musculus GN=Krt76 PE=2 SV=1	2262	63319	31	49,3
2	P07744	Keratin, type II cytoskeletal 4 OS=Mus musculus GN=Krt4 PE=1 SV=2	1806	56590	25	45,7
3	P50446	Keratin, type II cytoskeletal 6A OS=Mus musculus GN=Krt6a PE=1 SV=3	1797	59641	23	44,8
4	Q0VDR7	Krt6b protein OS=Mus musculus GN=Krt6b PE=2 SV=1	1665	60578	21	41,3
5	Q61781	Keratin, type I cytoskeletal 14 OS=Mus musculus GN=Krt14 PE=1 SV=2	1554	53176	20	57,2
6	P08730	Keratin, type I cytoskeletal 13 OS=Mus musculus GN=Krt13 PE=1 SV=2	1428	48066	24	46,5
7	Q3SYP5	Keratin 16 OS=Mus musculus GN=Krt16 PE=2 SV=1	1283	51973	17	48
8	Q32P04	Keratin 5 OS=Mus musculus GN=Krt5 PE=2 SV=2	1239	61957	17	32,2
9	A2A513	Keratin, type I cytoskeletal 10 OS=Mus musculus GN=Krt10 PE=1 SV=1	1238	57178	17	30,8
10	A0A087WPR7	Dystonin (Fragment) OS=Mus musculus GN=Dst PE=1 SV=1	1005	334308	14	6,4

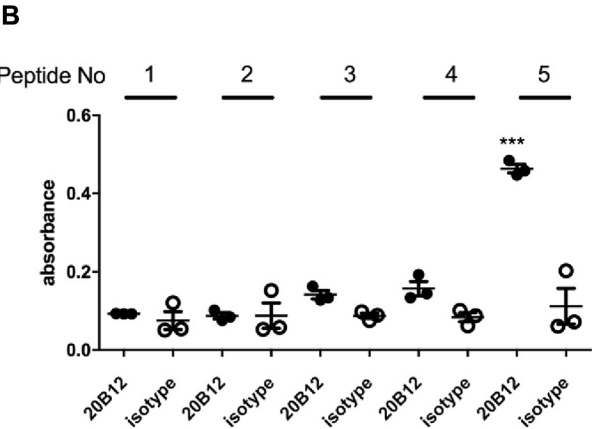


FIG E5. Identification of BP230 as an antigenic target of mAb 20B12. **A**, Results of 2 separate MALDI-TOF analyses of immunoprecipitation with purified mAb 20B12 in pooled WT palatal lysate. The first 10 hits with the highest scores of both experiments are displayed. **B**, ELISA with mAb 20B12 and IgG control antibody on the 5 potential antigenic peptides yielded by the peptide-based epitope mapping. ELISA was performed with 3 batches of purified antibody. *** $P < .001$, 1-way ANOVA.