

B cells are critical for autoimmune pathology in Scurfy mice

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Impaired regulatory T-cell function results in a severe chronic autoimmune disease affecting multiple organs in Scurfy mice and humans with the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. Previous studies have shown that T helper cells but not cytotoxic T cells are critical for the disease pathology. Whether this T-cell subset is responsible directly for tissue inflammation or rather indirectly via the interaction with B cells or myeloid cells is largely unknown. To study this and to identify potential therapeutic targets for this lethal disease we investigated the contribution of B cells to this complex autoimmune phenotype. We show that B cells and the production of autoantibodies plays a major role for skin, liver, lung, and kidney inflammation and therapeutic depletion of B cells resulted in reduced tissue pathology and in prolonged survival. In contrast, the absence of B cells did not impact systemic T-cell activation and hyperreactivity, indicating that autoantibody production by B cells may be a major factor for the autoimmune pathology in mice deficient for regulatory T cells.

Regulatory T cells (Treg) are critical for the maintenance of immunological tolerance (1–3). The transcription factor FoxP3 is critical for the development of functional Tregs and mutations affecting FoxP3 function result in a loss of immunological tolerance in mice and humans (4–7). The resulting chronic autoimmune phenotype in Scurfy mice and in human patients with the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is characterized by infiltrations of activated immune cells consisting of B cells, T cells, dendritic cells, monocytes, and eosinophils into several organs such as the skin, lung, kidney, and the liver, ultimately leading to organ failure and the premature death of affected individuals (3, 5, 8, 9). The only curative therapy for human IPEX patients so far is allogeneic stem cell transplantation, which in many cases is hampered by the bad overall health of affected patients (10). Thus, therapeutic strategies that can ameliorate systemic inflammation and organ damage would allow a window of time to be created for hematopoietic stem cell transplantation. In mice, this autoimmune phenotype can be recapitulated by the deletion of Tregs after birth (11, 12). The adoptive transfer of Tregs can rescue this phenotype and transfer of T cells depleted for the CD4/CD25^{high} Treg population into T-cell-deficient animals induces a Scurfy-like phenotype, providing strong evidence for the crucial role of Tregs for the maintenance of immunological tolerance (11, 13–16). Previous studies have shown that deletion of cytotoxic T cells has no effect on the disease phenotype, whereas removal of T helper cells and most forward the deletion of the costimulatory molecule CD28 leads to improved survival of the animals (17, 18). Further evidence suggesting that the interaction of CD28 or its inhibitory counterpart CTLA4 with the costimulatory molecules CD80 or CD86, which are expressed on activated antigen-presenting cells, are essential in maintaining immune homeostasis is provided by the Scurfy-like phenotype developing in cytotoxic T-lymphocyte

antigen 4 (CTLA4)-deficient mice (19, 20). Besides CD28, a variety of cytokine gene knockouts were bred to the Scurfy background indicating that especially IL2 may be critical for skin inflammation. In contrast, neither IL2, IL4, IL10, INF- γ , or signal transducer and activator of transcription (Stat6) signaling was required for liver inflammation (21). Besides professional antigen-presenting cells such as dendritic cells, activated B cells also express CD80 and CD86 and may be involved in the hyperactive T-cell phenotype and responsible for the elevated cytokine levels observed in Scurfy mice and human IPEX patients. Indeed, it was shown that B-cell tolerance is lost in Scurfy mice resulting in altered B-cell development, hyperimmunoglobulinemia, and autoantibody production, which may also contribute to tissue inflammation and recruitment of innate immune-effector cells (9, 22–25). More recently, a regulatory T-follicular helper cell subset was suggested to directly modulate germinal center reaction of B cells, which may explain at least in part the aberrant expansion of late B-cell developmental stages in Scurfy mice (26). To investigate which role B cells and the production of autoantibodies play in the Scurfy autoimmune phenotype, we generated B-cell-deficient Scurfy mice. We show that in the absence of B cells and autoantibodies the majority of autoimmune phenotypes are strongly reduced resulting in prolonged survival. Reconstitution of B-cell-deficient Scurfy mice with mature splenic B cells from wild-type mice reconstituted the disease phenotype and reduced survival. In contrast, the absence of B cells had no effect on T-cell activation and only minor effects on cytokine production, suggesting

Significance

The deficiency of regulatory T cells leads to a fatal systemic autoimmune disease in mice (Scurfy phenotype) and humans [IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome]. At present it is largely unclear to what degree the different deregulated parts of the immune system contribute to the disease pathology. To understand the role of B cells and the corresponding autoantibodies produced by these cells in this fatal disease we generated B-cell-deficient Scurfy mice and show that most of the systemic autoimmune phenotypes are greatly ameliorated. Consistent with this genetic approach, therapeutic depletion of B cells resulted in a similar increase in survival and reduction in multiorgan inflammation, suggesting that B cells may be a therapeutic target to ameliorate disease pathology.

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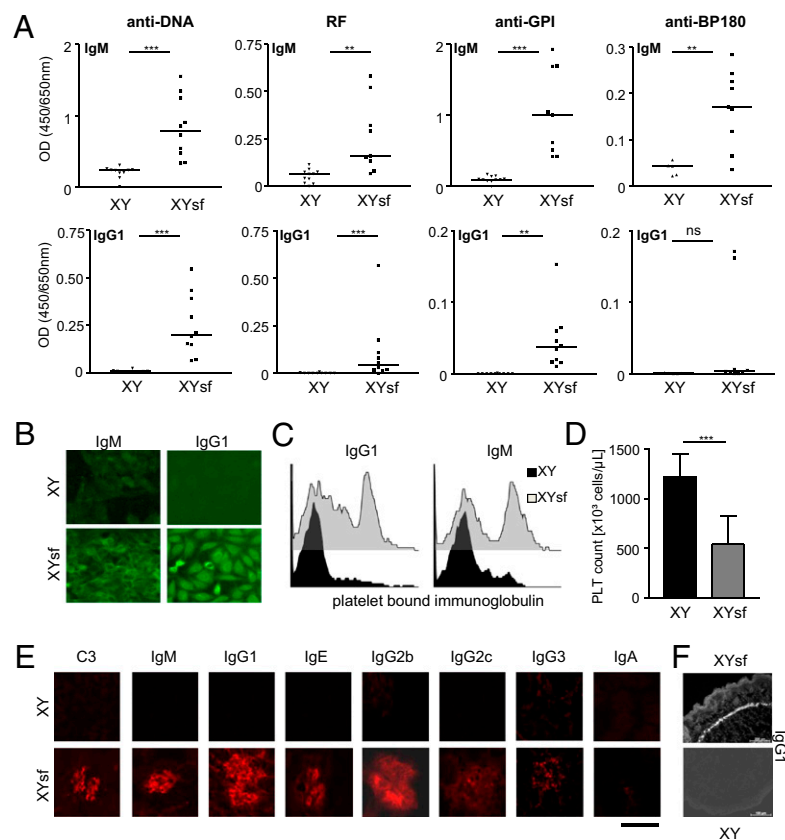


Fig. 1. Loss of humoral tolerance in Scurfy mice. (A) Detection of IgM and IgG1 autoantibodies directed against dsDNA (anti-DNA), rheumatoid factor, glucose-6-phosphate isomerase (anti-GPI) and BP180 (anti-BP180) in C57BL/6 wild-type (XY) and Scurfy (XYsf) animals by ELISA analysis. (B) Detection of IgM and IgG1 anti-nuclear antibodies in wild-type and Scurfy mice via immunofluorescence analysis on Hep2 cells. Detection of platelet-bound antibodies (C) and quantification of the PLT counts (D) in sera of C57BL/6 (XY) and Scurfy (XYsf) mice. (E) Detection of complement factor C3 (C3) and Ig deposits in the kidneys of C57BL/6 (XY) and Scurfy (XYsf) animals by immunofluorescence analysis; bar indicates 100 μ m. (F) Detection of skin-specific antibodies in the sera of C57BL/6 (XY) and Scurfy (XYsf) animals by incubation of serum with skin sections of newborn mice. In all experiments 4-wk-old male C57BL/6 wild-type (XY) and Scurfy (XYsf) animals were analyzed. Shown is one representative out of three independent experiments. Statistical analysis was performed with the Mann-Whitney test (nonparametric). * P < 0.05, ** P < 0.01, *** P < 0.001, ns: no significant difference.

that B cells may mainly contribute to tissue inflammation via the production of a wide array of autoantibodies.

Results and Discussion

Loss of Humoral Tolerance in Scurfy Mice. To verify the loss of humoral tolerance in Scurfy mice we first analyzed the level of serum immunoglobulins and the presence of autoantibodies in hemizygous males affected by the disease. Indeed, we noted a pronounced hyperglobulinemia in IgM, IgG1, IgG3, IgA, and IgE subclasses and a higher level of Ig-secreting cells in the spleen, lymph nodes, and bone marrow (Fig. S1 A–D). More importantly, autoantibodies specific for double-stranded DNA, glucose-6-phosphate isomerase, and skin antigens were present in these animals, and Ig and complement deposits were detectable in the kidneys of Scurfy mice (Fig. 1 A–F). Furthermore, increased levels of IgM and IgG antibodies could be detected on platelets of Scurfy mice, which may either represent platelet-specific antibodies or immune complexes deposited on the platelet surface (Fig. 1C). Consistent with the presence of these antibodies, Scurfy mice showed a pronounced immunothrombocytopenia (ITP) (Fig. 1D). We could further detect Ig-producing B cells and lymphoid like structures in the liver, suggesting that at least some of these Ig deposits may be caused by B cells present in these organs (Fig. S2).

Absence of B Cells Ameliorates Chronic Inflammation and Prolongs Survival. To investigate to what extent B cells and the respective autoantibodies are responsible for the different inflammatory phenotypes and the reduced survival, we generated B-cell-deficient Scurfy mice by crossing Scurfy mice to μ MT animals. The resulting mice were deficient in peripheral B cells and did not produce immunoglobulins (Fig. S3 A and B). Of note, B-cell-deficient Scurfy mice had an increased body weight compared with their B-cell-sufficient littermates and did not show the splenomegaly typically observed in Treg-deficient animals (Figs. 2A and B). As Scurfy mice deficient in CD4 T cells still had

a splenomegaly these results indicate that B cells, although present at reduced numbers in Scurfy mice, are a major cause for the increased spleen size (17). Moreover, kidney function was restored and the severe immunothrombocytopenia was absent in B-cell-deficient Scurfy mice (Fig. 2 C–E). In a similar manner, the inflammatory infiltrate in the liver consisting of activated innate and adaptive immune cells was strongly reduced and contained lower amounts of activated cells (Fig. 2 F and G). Despite this reduction, lymphoid like structures consisting of dendritic cells and T cells were still detectable in the liver (Fig. S2C). Another characteristic feature of Scurfy mice is a severe skin and lung inflammation. B-cell-deficient Scurfy animals showed a strong amelioration in skin and lung inflammation evident by a reduced skin thickness and an absent inflammatory infiltrate in both organs, suggesting that autoantibodies directed for example against skin antigens, such as keratin-14 or BP180, which have been identified in Scurfy mice and human IPEX patients, are indeed a major driver of inflammation (Figs. 1F and 2 H–J) (22). Consistent with this reduction in systemic inflammation and organ damage, B-cell-deficient Scurfy mice showed an approximately threefold increased average survival with individual mice living up to 200 d (Fig. 2K).

B-Cell Transfer Into B-Cell-Deficient Scurfy Mice Restores Autoimmune Disease. To provide direct evidence that B cells are responsible for this reduction in inflammation and enhanced survival we adoptively transferred splenic B cells from the CD45 congenic B6.SJL- $Ptpr^a$ Pepe^b/BoyJ mice into B-cell-deficient wild-type and Scurfy animals. A strong increase in the development of CD138-positive plasma cells or plasma blasts was evident on the Scurfy background and coincided with the production of autoantibodies and the presence of IgG1, IgM, and IgE deposits in the kidneys (Fig. 3 A–C). In a similar manner, a strong trend toward an increase in liver inflammation was evident and the reconstitution with B cells resulted in a significant reduction in survival of B-cell-deficient Scurfy mice (Fig. 3 D–F). This may indicate that B cells and

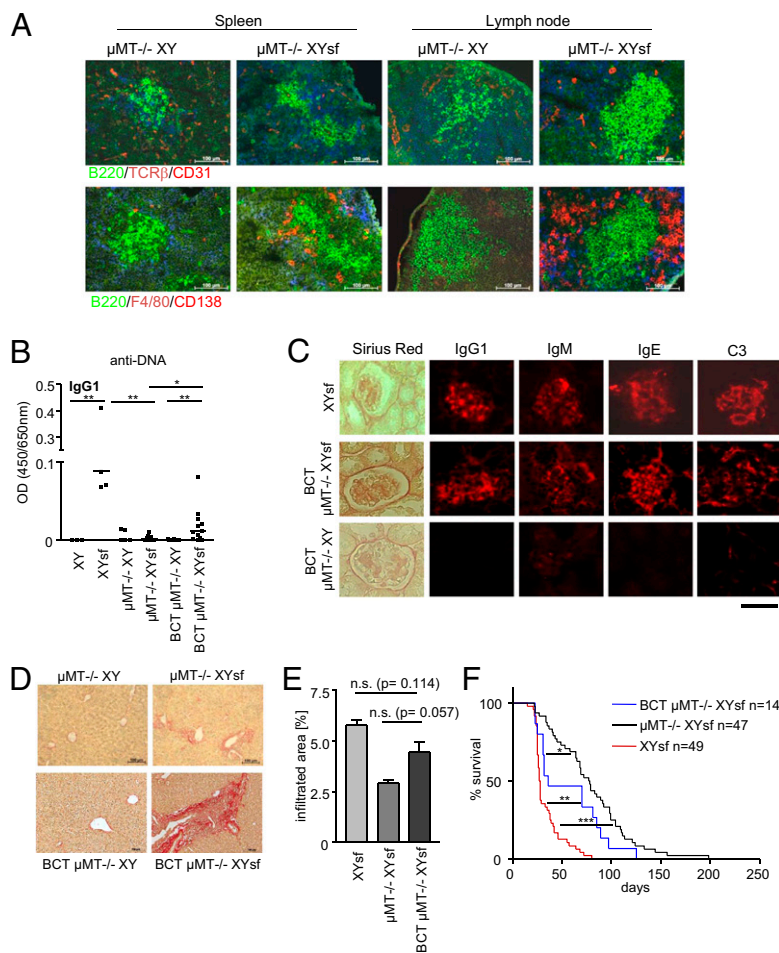


Fig. 3. Reconstitution of B-cell-deficient Scurfy mice with mature B cells. (A) Immunofluorescence analysis of B cells (B220), T cells (TCR- β), endothelial cells (CD31), macrophages (F4/80), and plasma cells (CD138) in the spleen and lymph node of B-cell-deficient male wild-type ($\mu\text{MT}^{-/-}$ XY) and Scurfy mice ($\mu\text{MT}^{-/-}$ XYsf) two weeks after B-cell transfer into the indicated mouse strains. (B) Presence of IgG1 anti-DNA antibodies in the serum of the indicated mouse strains as detected by ELISA. (C) Detection of fibrosis via Sirius red staining and Ig and complement deposits in the kidney of the indicated mouse strains 2 wk after the adoptive transfer of B cells (BCT). Age-matched Scurfy mice (XYsf) served as controls and the bar represents 100 μM . Histopathological detection of liver fibrosis (D) and quantification of the inflammatory infiltrates in the liver of the indicated mouse strains (E). (F) Survival of Scurfy (XYsf) mice, B-cell-deficient Scurfy mice ($\mu\text{MT}^{-/-}$ XYsf) and $\mu\text{MT}^{-/-}$ XYsf mice adoptively transferred with splenic B cells from B6.SJL-Ptprc^{pep}c/BoyJ mice (BCT). Shown is one representative out of three independent experiments for A–C; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

escaped central tolerance induction, or by silencing autoreactive B cells generated de novo during germinal center reactions. Indeed, previous studies have demonstrated a loss of humoral

tolerance in Scurfy mice and could show that regulatory T cells did not prevent the entry of autoreactive B cells into the germinal center but rather limited the following maturation of the

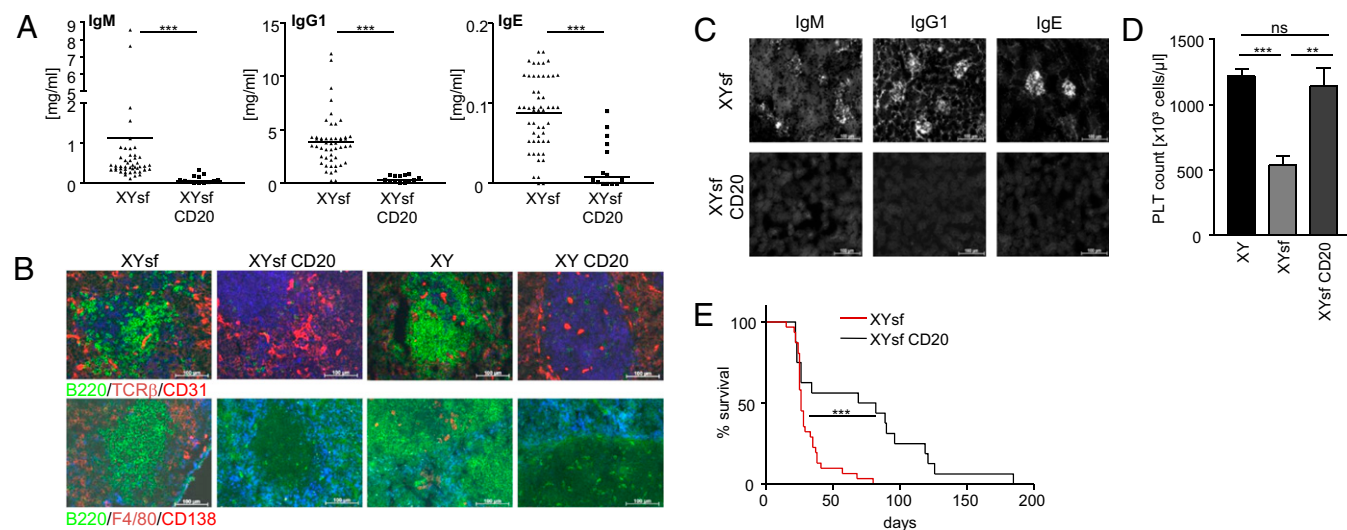


Fig. 4. Therapeutic B-cell depletion in Scurfy mice. (A) Quantification of IgM, IgG1, and IgE in the serum of Scurfy mice either injected with PBS (XYsf) or injected weekly with a CD20-specific B-cell depleting antibody (XYsf CD20). (B) Detection of B cells (B220), T cells (TCR- β), endothelial cells (CD31), macrophages (F4/80), and plasma cells (CD138) in the spleen of male wild-type (XY) or Scurfy mice (XYsf) either left untreated or depleted of B cells by immunofluorescence analysis. (C) Detection of Ig deposits in the kidneys (C) and of the platelet count in the blood depicted as the mean $\pm$ SEM (D) of Scurfy mice (XYsf) either left untreated or B-cell depleted (XYsf CD20). (E) Survival of Scurfy mice (XYsf) either left untreated or depleted of B cells (XYsf CD20). Shown is one out of three independent experiments for A–D; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

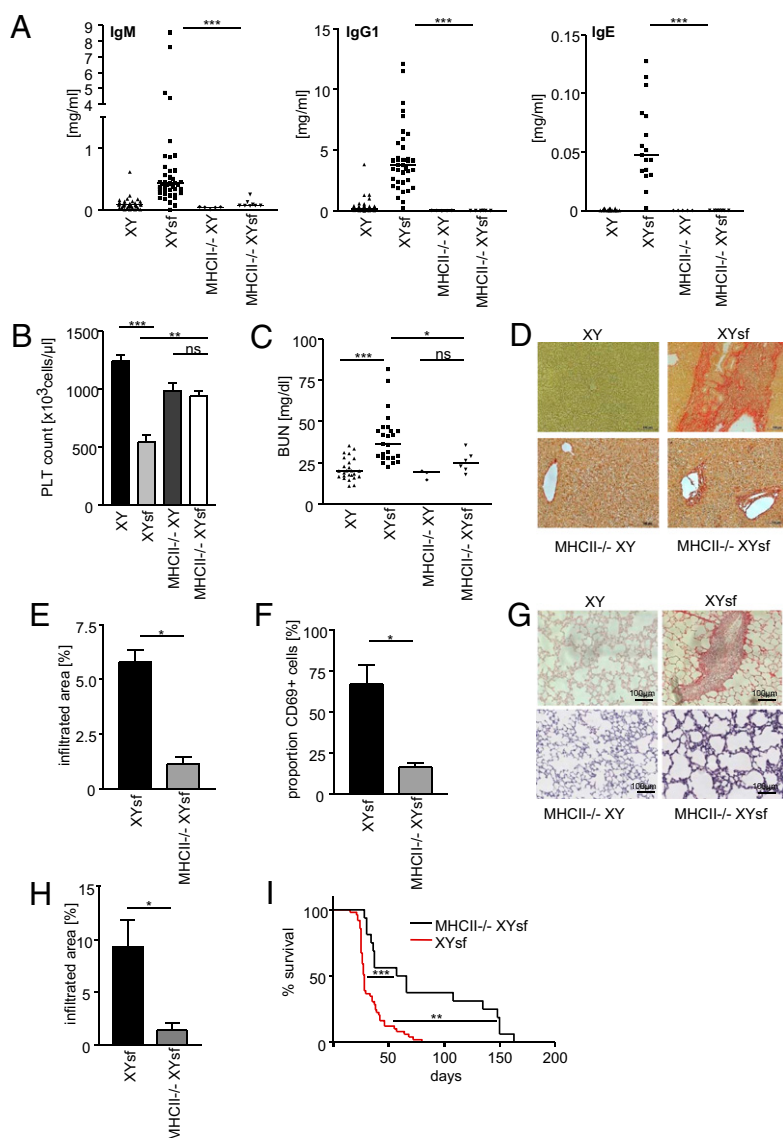


Fig. 5. Impact of MHC class II deficiency on the autoimmune phenotype of Scurfy mice. Shown are the levels of serum IgM, IgG1, and IgE (A), the platelet count (B), the BUN level (C) in male C57BL/6 (XY), Scurfy (XYsf), MHC class II deficient (MHCII^{-/-}XY) and Scurfy MHC class II deficient mice (MHCII^{-/-}XYsf) at 4 wk of age. (D) Representative histopathology of liver sections from the indicated mouse strains stained with Sirius red to detect liver fibrosis. Quantification of the size of the inflammatory infiltrates (E) and the amount of activated (CD69 positive) cells (F) in the liver of 4-wk-old male mice of the indicated strains. (G) Representative histopathology of lung sections of the indicated mouse strains stained with hematoxylin/eosin and quantification of the size of the inflammatory infiltrate in the lung (H). (I) Kaplan-Meier-survival curve of Scurfy mice (XYsf) and major histocompatibility complex 2 deficient Scurfy (MHCII^{-/-}XYsf) mice. Statistical analysis was performed with the Mann-Whitney test (nonparametric). Error bars represent the mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001.

autoantibody response (23–25, 28). A potential clinical relevance for our observations is highlighted by the fact that depletion of B cells in affected Scurfy mice resulted in a reduction of inflammation and a prolonged survival. To our knowledge, there is only one case study in human IPEX patients so far, showing that the skin phenotype was ameliorated under B-cell depletion therapy in one treated IPEX patient (29). Although this is not direct proof that B-cell depletion may have a therapeutic benefit in human IPEX patients, it at least suggests that autoantibody production by B cells also contributes to the disease phenotype in humans. Despite this general improvement of the autoimmune phenotype it should be noted that even B-cell-deficient Scurfy mice eventually died, suggesting that other antibody independent pathways are critical for the full-blown autoimmune phenotype. This is in line with studies showing that the transfer of T helper cells depleted for Tregs into B- and T-cell-deficient recombination activating gene (Rag)^{-/-} mice can induce a Scurfy-like autoimmune pathology (30). Apart from B cells, T cells, and T-cell-derived cytokines are also critical to control the activation of innate immune effector cells, such as monocytes and macrophages, which might be responsible for the delayed inflammation and the death of affected animals (21). Further studies designed to analyze the mechanism of B-cell independent

induction of autoimmune pathology will be necessary to understand this in more detail.

Experimental Procedures

Animals. B6.SJL-Ptprc^aPepr^b/BoyJ, Scurfy, μ MT, and MHC class II knockout mice were purchased from the Jackson Laboratories. Scurfy mice were bred with μ MT and MHC class II knockout mice to generate B-cell and MHC class II deficient Scurfy mice (μ MT^{-/-} and MHCII^{-/-} Scurfy mice, respectively). All mice were held under specific pathogen-free conditions and in isolated ventilated cages in the animal facilities of the University of Erlangen, according to state and institutional guidelines and the rules and regulations of the German animal welfare laws.

ELISpot Analysis. ELISpot plates (Millipore) were activated with 70% ethanol for 1 min. After washing, membranes were coated with the 2 μ g/mL goat anti-mouse IgM/G/E antibodies (Jackson Immuno Research) in Tris/EDTA (TE) buffer at 4 °C overnight. Membranes were washed with PBS and blocked with PBS/2% FCS for 2 h at room temperature; 20,000 cells from spleen, lymph node, or bone marrow were seeded in four replicates in RPMI/10% FCS/1% Penicillin/Streptomycin/1% nonessential amino acids/1% sodium pyruvate/1% glutamate and incubated for 2 hrs at 37 °C and 5% CO₂. Ig-Spots were detected by incubation with peroxidase conjugated goat anti-mouse IgM/G/E antibodies (Bethyl) followed by the addition of 3,3',5,5'-tetramethylbenzidine-peroxidase substrate (KPL). Membranes were measured with the AID ELISpot Reader ELR 03 and analyzed with ELISpot 5.0 software.

Determination of the Platelet Count. Platelet counts were determined in blood diluted in PBS and 5% BSA (1:2 to 1:10 dilution) and analyzed in an Advia 120 hematology system (Siemens) as described (27).

ELISA. Sera of 4-wk-old mice were collected and stored at -80°C until further use. For quantification of the serum concentration of different Ig isotypes the corresponding Bethyl Mouse ELISA Quantification Kit (Biomol) was used according to the manufacturer's instructions. The optical density (OD) was measured with a VersaMax tunable microplate reader (Molecular Devices) at 450 and 650 nm. For the detection of anti-DNA antibodies, ELISA plates were coated with 10 $\mu\text{g}/\text{mL}$ methylated BSA (Sigma) in PBS for 2 h at room temperature. After washing, the plates were coated with 50 $\mu\text{g}/\text{mL}$ calf thymus DNA (Sigma) in PBS at 4°C overnight. Blocking of unspecific binding was performed with PBS/0.1% Gelatin/3% BSA/1 mM EDTA. Sera were diluted 1:100 in the blocking solution and incubated for 1 h at room temperature. As a detection antibody, the HRP-conjugated IgM and IgG1 antibodies (Bethyl) were used at a dilution of 1:20,000 in blocking solution. For detection 3,3',5,5'-tetramethylbenzidine-peroxidase substrate (TMB Solution, Sigma) was added and the reaction was stopped with 6% orthophosphoric acid. To detect GPI-specific antibodies, ELISA plates were coated with 1 $\mu\text{g}/\text{mL}$ GPI in PBS at 4°C overnight. Blocking was performed with PBS/3% BSA for 1 h at room temperature. Sera were diluted 1:100 in PBS/3% BSA and incubated 1 h at room temperature. Detection antibody was diluted 1:20,000 in PBS/3% BSA for 1 h at room temperature. Detection of bound mouse antibodies was performed as described for the anti-dsDNA ELISA. Rheumatoid factor was detected by coating 1 ng/mL mouse IgG2a on ELISA plates overnight at 4°C . Unspecific binding was blocked with PBS/3% BSA for 1 h at room temperature. Sera were diluted 1:100 in PBS/3% BSA and incubated for 1 h at room temperature and detection of bound antibodies was carried out as described before. Anti-BP180 specific antibodies were identified by coating 5 $\mu\text{g}/\text{mL}$ murine collagen 17 protein (kindly provided by Prof. Detlef Zillikens, University of Lübeck) on ELISA plates overnight at 4°C . Nonspecific binding was blocked with PBS/1% BSA for 1 h at room temperature. Sera were diluted 1:100 in PBS/1% BSA and incubated for 1 h at room temperature and detection of bound antibodies was carried out as described before.

Detection of Anti-Nuclear Antibodies. Autoantibodies specific for nuclear antigens were detected on Hep-2 slides (Viro-Immun). Experiments were carried out using the manufacturer's recommended protocol using serum diluted in PBS at 1:100 followed by detection with an FITC-conjugated anti-mouse IgM (clone AF6-78; Biolegend) and anti-mouse IgG1 (clone A85-1; BD Pharmingen) secondary antibody. Slides were mounted using Fluoromount

imaging medium (Sigma) and analyzed on a Zeiss AxioVert system with the Axiovision 4.8 software. Images were taken using a 100x oil immersion lens.

Determination of the Blood Urea Nitrogen Level. For quantification of the total serum concentration of blood urea nitrogen (BUN) the corresponding Urea Nitrogen Enzymatic Test Kit (Stanbio Laboratory) was used according to the manufacturer's instructions. OD was measured with VersaMax tunable microplate reader (Molecular Devices) at 600 nm.

Adoptive B-Cell Transfer. Resting naïve B cells were isolated from B6.SJL-Ptprca^bPepcb/BoyJ (Ly5.1) splenocytes using mouse anti-CD43 and mouse anti-CD11c magnetic cell sorting (Miltenyi Biotech). After confirmation of B-cell purity by flow cytometry, 1×10^7 naïve B cells were i.v. injected into 2-wk-old $\mu\text{MT}^{-/-}$ and $\mu\text{MT}^{-/-}$ Scurfy mice.

CD20-Antibody Production and B-Cell Depletion. CD20-IgG2a was produced by transient calcium phosphate transfection of 293 T cells followed by purification of the secreted antibody from the culture supernatant using protein G as described (27). B-cell depletion was achieved by weekly injections of 100 μg of the antibody starting from the day of birth.

Detection of Platelet-Specific Antibodies. Anti-platelet (PLT) antibodies in the sera of Scurfy mice were detected using flow cytometry analysis. PLT were enriched from C57BL/6 whole-blood samples by centrifugation (300 g for 5 min). Supernatant was mixed with PBS and centrifuged for an additional 15 min at 1,500 g. Pellet was washed (15 min; 1,500 g). Erythrocytes were removed by centrifugation (10 min, 100 g). PLT in the supernatant were incubated on ice with sera from Scurfy or wild-type mice for 15 min, followed by washing and further incubation with combinations of the following antibodies: anti-CD61 (clone 2C9.G2; Biolegend), anti-IgM (clone AF6-78; Biolegend), or anti-IgG1 (clone A85-1; BD Pharmingen) and analyzed on a FACS Canto II (BD Bioscience).

Statistical Analysis. Statistical differences between experimental groups were calculated with the Mann-Whitney *u* test. Differences in survival were analyzed with Kaplan-Meier estimates, and groups were compared with the log rank test; $P < 0.05$ was considered significant.

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Supporting Information

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SI Experimental Procedures

Flow Cytometry. Flow cytometry analysis was done on a FACS Canto II (BD Bioscience) with single-cell suspensions of peripheral blood. Blood was obtained from the retroorbital plexus and erythrocytes were lysed. To reduce unspecific binding to Fc receptors cells were incubated on ice for at least 10 min with Fc block (clone 2.4G2, 0.5 μ g per sample). Cells were washed and incubated on ice for at least 20 min with combinations of the following antibodies: anti-B220/CD45R (clone RA3-6B2; BD Pharmingen) and anti-IgM (clone AF6-78; Biolegend). Analysis was restricted to viable cells, which were identified by exclusion of the nucleic acid-binding dye DAPI. Data acquisition and analysis was performed using FACS Diva software (BD Bioscience).

Immunofluorescence. Here, 4 μ m frozen kidney, liver, lung, spleen, and lymph node sections were fixed in acetone. Kidneys were stained with phycoerythrin (PE)-conjugated anti-mouse IgM (clone AF6-78; Biolegend), IgG1 (clone A85-1; BD Pharmingen), IgG2b (clone ATTO488; Caltag), IgG2c (clone $\times$ 5563; Caltag), IgG3 (Biozol), IgA (clone 11-44-2; Southern Biotech) or FITC-conjugated anti-mouse IgE (clone R35-72; BD Pharmingen) and complement component C3 (clone CL7503F; Cedarlane) antibodies. Spleen and lymph node sections were either stained with a combination of Alexa488-conjugated B220/CD45R (clone RA3-6B2; BD Pharmingen), allophycocyanin (APC)-conjugated T cell receptor (TCR)- β (clone H57; BD Pharmingen) and PE-conjugated CD31 (clone 390; Biolegend) or PE-conjugated CD138 (clone Syndecan-1; BD Pharmingen), Alexa647-conjugated F4/80 (clone MCA497; AbD Serotec) and Alexa488-conjugated B220/CD45R (clone RA3-6B2; BD Pharmingen) antibodies. Liver sections were either stained with PE-conjugated CD69 (clone

H1.2F3; Biolegend) and APC-conjugated TCR- β (clone H57; BD Pharmingen) or with Alexa488-conjugated B220/CD45R (clone RA3-6B2; BD Pharmingen), Alexa647-conjugated F4/80 (clone MCA497; AbD Serotec), APC-conjugated CD11b (clone M1/70; BD Pharmingen), PE-conjugated SiglecF (clone E50-2440; Biolegend), and APC-conjugated CD11c (clone N418; BD Pharmingen) antibodies.

Histological Analysis. Kidneys, lungs, and livers were removed, fixed in 10% buffered formalin, and embedded in paraffin. The 4- μ m paraffin sections were stained using Sirius red to detect collagen depositions and evaluated by light microscopy. To stain activated cells in the liver, paraffin-embedded sections were incubated with the biotinylated anti-mouse CD69 (clone H1.2F3; Biolegend) antibody after endogenous biotin blocking using an Avidin-Biotin blocking kit (Invitrogen) according to manufacturer's instructions. A streptavidin-conjugated alkaline phosphatase (AP)-coupled anti-biotin antibody (BD Pharmingen) was used as secondary antibody followed by development with the Alkaline Phosphatase Substrate Kit III (Vector Laboratories) and staining with Sirius red. Analysis of ear thickness was performed on H&E stained sections. For detection of serum auto-antibodies against the basement membrane IF microscopy on frozen sections of normal murine skin was performed. In brief, the sections of murine skin were incubated with diluted mouse serum followed by detection of bound antibody with a secondary antibody specific for mouse IgG1 (clone A85-1; BD Pharmingen). All slides were mounted using Fluoromount imaging medium (Sigma) and analyzed using a Zeiss AxioVert system with AxioCam IcC1 camera and Axiovision 4.8 software.

