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EXTENDED REPORT

NLRP3 and ASC suppress lupus-like autoimmunity by driving the immunosuppressive effects of TGF-β receptor signalling

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ABSTRACT

Objectives The NLRP3/ASC inflammasome drives host defence and autoinflammatory disorders by activating caspase-1 to trigger the secretion of mature interleukin (IL)-1β/IL-18, but its potential role in autoimmunity is speculative.

Methods We generated and phenotyped *Nlrp3*-deficient, *Asc*-deficient, *Il-1r*-deficient and *Il-18*-deficient C57BL/6-lpr/lpr mice, the latter being a mild model of spontaneous lupus-like autoimmunity.

Results While lack of IL-1R or IL-18 did not affect the C57BL/6-lpr/lpr phenotype, lack of NLRP3 or ASC triggered massive lymphoproliferation, lung T cell infiltrates and severe proliferative lupus nephritis within 6 months, which were all absent in age-matched C57BL/6-lpr/lpr controls. Lack of NLRP3 or ASC increased dendritic cell and macrophage activation, the expression of numerous proinflammatory mediators, lymphocyte necrosis and the expansion of most T cell and B cell subsets. In contrast, plasma cells and autoantibody production were hardly affected. This unexpected immunosuppressive effect of NLRP3 and ASC may relate to their known role in SMAD2/3 phosphorylation during tumour growth factor (TGF)-β receptor signalling, for example, *Nlrp3*-deficiency and *Asc*-deficiency significantly suppressed the expression of numerous TGF-β target genes in C57BL/6-lpr/lpr mice and partially recapitulated the known autoimmune phenotype of *Tgf-β1*-deficient mice.

Conclusions These data identify a novel non-canonical immunoregulatory function of NLRP3 and ASC in autoimmunity.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a paradigmatical form of systemic autoimmunity.[1] SLE is based on genetic variants that compromise lymphocyte death and their silent clearance.[2] In SLE, aberrant lymphocyte death triggers sterile inflammation, costimulation and, subsequently, autoimmunisation against nuclear autoantigens.[3] This process is driven by the adjuvant effect of endogenous nucleic acids at Toll-like receptor (TLR)-7 and TLR-9 that trigger interferon-α-dependent immunity.[4 5] Lupus nephritis results from immune complex disease and is associated with progressive infiltrates of autoreactive T cells, often forming tertiary lymphoid tissue organs like in other solid organs.[1 6 7]

Inflammasomes are danger signalling platforms within the cytosol of myeloid antigen-presenting cells that integrate various pathogen-associated or damage-associated signals into the activation of caspase-1.[8] The NACHT, LRR and PYD domains-containing protein (NLRP)-3 inflammasome is comprised of NLRP3 and of apoptosis-associated speck-like protein containing a CARD (ASC), which first interact at their caspase recruitment domain (CARD) followed by the assembly of several such heterodimers to a wheel-like structure that can activate caspase-1.[8] Caspase-1 activation has two major biological effects that relate to its enzymatic activity: First, it cleaves pro-interleukin (IL)-1β and pro-IL-18 into its mature forms. The pyretic and proinflammatory effects of IL-1R and IL-18R signalling imply an important role for caspase-1 activation in host defence as well as in autoinflammation disorders.[8–11] In addition, crystals or crystalline proteins specifically activate the NLRP3 inflammasome, which unravelled the central role of caspase-1-mediated inflammation in crystal-related disorders.[12–16] The second biological effect of caspase-1 is DNA fragmentation and programmed cell death, referred to as pyroptosis.[17 18]

While the role of the NLRP3 inflammasome in host defence and autoinflammation is obvious, its role in autoimmunity is less clear, mostly because IL-1β and IL-18 have diverse effects on the adaptive immune system.[19] Some studies support a similar proinflammatory effect on adaptive immunity, as IL-1β and IL-18 promote the proliferation and survival of Th1 and Th17 T cells and B cells,[19 20] and *Nlrp3*-deficient and *Asc*-deficient mice are resistant to experimental autoimmune encephalitis.[21 22] In contrast, NLRP3 overexpression does not induce autoimmunity,[23 24] and therapeutic IL-1 blockade does not consistently improve experimental and human systemic autoimmunity in a clinically meaningful manner, for example, in systemic lupus.[25–28]

Given its dominant proinflammatory role in innate immunity one would predict that the NLRP3 inflammasome will drive antigen-presentation, T cell priming, lymphocyte proliferation and finally, autoimmune tissue inflammation.[29 30] Furthermore, nuclear lupus autoantigens and neutrophil extracellular traps potentially contribute to lupus activity by their NLRP3 agonistic effect.[31–33] To test this concept, we generated

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Nlrp3-deficient, *Asc*-deficient, *Il-1r*-deficient and *Il18*-deficient C57BL/6-lpr/lpr (B6^{lpr}) mice. Female B6^{lpr} mice represent a mild model of spontaneous lupus-like autoimmunity or autoimmune lymphoproliferative syndrome[34] that has proven suitable to identify functional roles of immunoregulatory elements in systemic autoimmunity.[35–39] We hypothesised that based on the concept of canonical NLRP3 inflammasome signalling *Nlrp3*-deficient, *Asc*-deficient, *Il-1r*-deficient and *Il18*-deficient B6^{lpr} mice would display attenuated SLE, a hypothesis disproved by our data.

MATERIALS AND METHODS

Animal studies

Nlrp3-deficient and *Asc*-deficient mice were generated and backcrossed (F10) to the C57BL/6 strain as described.[14 40] *Il-1r*-deficient and *Il-18*-deficient mice in the same genetic background were obtained from Jackson Laboratories (Bar Harbour Maine). *Nlrp3*-deficient and *Asc*-deficient mice as well as B6$^{lpr/lpr}$ mice (B6^{lpr}) were obtained from Jackson Laboratories (Bar Harbour, Maine). Each strain was mated with B6^{lpr} mice to generate double heterozygote mice, which were then mated among each other to generate double homozygous knockout and B6^{lpr} control mice. In each individual mouse, the genotype was assured by PCR. Mice were housed in groups of five in sterile filter top cages with a 12 h dark/light cycle and unlimited access to autoclaved food and water. All mice were sacrificed by cervical dislocation at 24 weeks of age. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities.

Evaluation of autoimmune tissue injury

Spleens, lymph nodes, lungs and kidneys from all mice were fixed in 10% buffered formalin, processed and embedded in paraffin. The severity of the renal lesions was graded on periodic acid-Schiff stained sections using the indices for activity and chronicity as described.[41] Lung and kidney fibroses were assessed by Masson–Trichrom staining. Immunostaining was performed as described[42] using the following primary antibodies: antimouse IgG and antimouse IgM (1:100, M32015, Caltag Laboratories, Burlingame, California, USA), antimouse C3c (complement, GAM/C3c/FITC, Nordic Immunological Laboratories, Tilburg, Netherlands, 1:200), antimouse CD11c (BD), antimouse CD19 (antibody-online.de), antimouse CD45 (BD), antimouse MAC2 (Cedarlane, Eching, Germany), antimouse Ki67 (Dako Cytomatic, Hamburg, Germany) and antimouse neutrophil (AbD SeroTec). Negative controls included incubation with a respective isotype antibody. For quantitative analysis, glomerular cells were counted in 10 cortical glomeruli per section. Immunostainings of lungs and spleens were analysed using Adobe Photoshop CS4 extended for area calculation. Serum creatine levels were determined using Jaffe method (DiaSys Diagnostic Systems). Albuminuria was determined via mouse Albumin Quantification Set (Bethyl Laboratories, Montgomery, Texas, USA). Cleaved caspase-3 staining was done on paraffin-embedded sections using cleaved caspase-3 (Asp175) antibody (#9661, Cell signalling) in combination with antirabbit biotin and Strept-Alexa-Fluor (Vector Laboratories Burlingame, California, USA).

Flow cytometry

Antimouse CD3, CD4, CD8, CD25 (BD Pharmingen, Heidelberg, Germany), foxP3 (BioLegend, San Diego, USA), CD11c (BD), F4/80 (AbDSeroTec, Düsseldorf, Germany), CD19 (AbD), B220, CD21, CD23, IgD and IgM, antimouse κ light chain and CD138 (BD) have been stained to differentiate T cell and B cell subsets as well as dendritic cells and macrophages. Major histocompatibility complex II (MHCII), (eBioscience, San Diego, USA), CD44, CD86 (BD) and CD69 (Caltag Laboratories, Buckingham, UK) were used as activation markers. Intracellular labelling (foxP3 and κ light chain) was done using Cytofix/Cytoperm kit

(BD), following the manufacturer's instructions. Each immune cell subset was defined at least by two different markers. Annexin/ propidium iodide fluorescence-activated cell sorting (PI FACS) was performed with annexinV-FITC-apoptosis-detection-kit (BD-Bioscience) following the manufacturer's instructions. Respective isotype antibodies were used to demonstrate specific staining of cell subpopulations. Cell counting beads (Invitrogen) were used for determining cell numbers by FACS.

Real-time quantitative PCR

Real-time RT-PCR was performed on total spleen mRNA as previously described.[43] SYBR Green Dye detection system was used for quantitative RT-PCR on Light Cycler 480 (Roche, Mannheim, Germany). Gene-specific primers (300 nM, Metabion, Martinsried, Germany) were used as listed in online supplementary table S1. Controls consisting of ddH2O were negative for target and housekeeper genes. 18S rRNA was used as a housekeeper. The PCR Arrays were performed in both 96-well plates according to the manufacturer's protocol (Quiagen, SABiosciences, Germany). Five housekeeping genes including actin β, β-2 microglobulin, glyceraldehyde-3-phosphate dehydrogenase, β-glucuronidase and heat shock protein 90 α (cytosolic), class B member 1 were used. Standard controls on each array for genomic DNA contamination, RNA quality and general PCR performance were included.

Autoantibody and plasma cytokine analysis

Serum (auto-) antibody levels were determined by ELISA as described.[37 44] C57BL/6 10 week mouse serum was used as negative control. The horseradish peroxidase (HRP) conjugated antimouse IgG was used as secondary antibody. Serum cytokine levels and cell culture supernatants were determined using the commercial ELISA kits.

In vitro experiments

Bone marrow cells from wild type and knockout mice were cultured with 20 ng/mL mouse recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF)/M-CSF (R&D Systems, Wiesbaden, Germany) in Roswell Park Memorial Institute (RPMI) medium for 7 days to generate >90% CD11c dendritic cell subsets (DCs). On day 7, cells were harvested, suspended in fresh medium and seeded at 1×10^6 cells/well; 1500 µL/well in 12-well plates. Stimuli were ultrapure lipopolysaccharide (LPS) 1 µg/mL (Invivogen, San Diego, California, USA), CpG-DNA 1668 10 µg/mL (Invivogen, San Diego, California, USA), imiquimod 1 µg/mL (Sequoia Research Products Ltd, Oxford, UK) and CpG (ODN 1668; Invivogen, San Diego, USA) for 24 h. TGF-β (1 ng/mL R&D Systems, Minneapolis, Minnesota, USA) stimulation of bone marrow-derived dendritic cells (BMDCs) was done for 30–45 min before they were harvested in radioimmunoprecipitation assay (RIPA) buffer, supplemented with phosphatase inhibitor cocktail (Sigma Aldrich, St Louis, USA) and complete protease inhibitor cocktail (Roche, Germany). CD19 B cells were isolated from spleens of female B6^{lpr} mice using B Cell Isolation Kit, followed

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by depletion of dead cells via the dead cell removal kit (Miltenyi, Bergisch Gladbach, Germany). Purity as determined by FACS analysis revealed over 80% purity for each isolate. Total thymocytes were isolated according to standard protocols from control and *Nlrp3*-deficient or *Asc*-deficient B6^{lpr} mice. For the differentiation of FoxP3+ regulatory T cells, CD4CD25^{negative} cells were purified from the spleen by two-step magnetic cell sorting (MiltenyiBiotec). Cells were cultured in complete RPMI medium with 0.0001% of 2-mercaptoethanol in flat bottom 24-well plates (1×10^6 cells in 1 mL) and activated with anti-CD3-CD28 beads (Invitrogen, Darmstadt, Germany) at a bead-to-cell ratio of 1:5 in the presence of TGF-β (5 ng/mL, R&D Systems, Minneapolis, Minnesota, USA) and IL-2 (10 ng/mL, Peprotech Hamburg, Germany). After 72 h, the cells were stained with antimouse CD4-PE (BD) and antimouse FoxP3-Pacific Blue (eBioscience) antibodies for flow cytometry analysis. FoxP3 was detected using the eBioscience Treg cell staining kit. Analysis was performed using an FACS Canto II flow cytometer (BD) and FlowJo software (TreeStar, Ashland, Oregon, USA). For analysis of regulatory T cell function 5×10^4 CD4CD25^{negative} T effector cells were co-incubated with either 5×10^3 or 5×10^4 CD4CD25 regulatory T cells in the presence of 5×10^4 CD4-depleted splenocytes for 3 days. Expression of CD69 on T cells and secretion of IL-2 was determined by flow cytometry and ELISA, respectively. SMAD2/3 translocation in DCs was assessed after the staining procedure with anti-SMAD2/3 (Cell signalling, Boston, Massachusetts, USA) following 45 min of stimulation with TGF-β and compared using Adobe Photoshop CS4 Extended.

Western blotting

Proteins were extracted from whole spleen lysates or cultured BMDCs homogenised in RIPA buffer (Sigma Aldrich) containing phosphatase and proteinase inhibitor. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting were performed following standard procedures, using rabbit antiphosphoSMAD2 (Ser465/467), anticleaved-caspase-3 (Cell Signalling, Danvers, USA) or rabbit anticaspase-1 (Santa Cruz, Heidelberg, Germany) as primary antibodies. The signal of rabbit anti-β-actin monoclonal antibody (Cell Signalling) served as a loading control. Secondary antibody incubation was done with HRP conjugated antirabbit IgG (Cell Signalling) prior to detection with enhanced chemiluminescence plus chemiluminescent kit (GE Healthcare Life Sciences, Freiburg, Germany).

Statistical analysis

One-way analysis of variance followed by post hoc Bonferroni's test was used for multiple comparisons using GraphPad Prism V.4.03. Single groups were compared by unpaired two-tailed Student t test. Data were expressed as mean±SEM. Statistical significance was set at a p value of <0.05 (*); p value of <0.01 (**); p value of <0.001 (***) in comparison with control animals.

RESULTS

NLRP3 and ASC, but not IL-1R and IL-18, protect B6^{lpr} mice from kidney and lung injury

Lupus nephritis is the most common organ manifestation of SLE[1] and B6^{lpr} mice develop mild glomerulonephritis.[34] Lack of NLRP3, ASC, IL-1R or IL-18 alone did not cause any kidney phenotype up to 12 months of age. We generated *Nlrp3*-deficient, *Asc*-deficient, *Il-1*-deficient *r* and *Il-18*-deficient B6^{lpr} mice that bred at Mendelian ratios and common litter sizes. At 6 months of age *Nlrp3*-deficient and *Asc*-deficient B6^{lpr} mice revealed diffuse mesangioproliferative glomerulonephritis and periglomerular inflammation in kidneys of B6^{lpr} mice, while lack of IL-1R or IL-18 had no effect on kidney disease (figure 1). The composite activity score for lupus nephritis was significantly increased only in B6^{lpr} *Asc*−/− and B6^{lpr} *Nlrp3*−/− mice (figure 1B) and there was a non-significant trend towards more albuminuria (see online supplementary figure S1A). CD45 cell and Mac2 macrophage numbers inside the glomerular tufts were significantly increased in these mutant strains only (figure 1A, C, D). Only B6^{lpr} *Nlrp3*−/− and B6^{lpr} *Asc*−/− mice showed increased glomerular deposits of complement factor C3c and intrarenal mRNA expression of tumour necrosis factor (TNF)-α (not shown, figure 1E), although glomerular IgG deposits remained unaffected (figure 1F). Serum creatine levels were increased only in B6^{lpr} *Asc*−/− and B6^{lpr} *Nlrp3*−/− mice, although this reached statistical significance only for *Nlrp3*-deficiency (figure 1G). Lung disease is another organ manifestation of SLE in humans[1] but usually does not affect B6^{lpr} mice.[34] Nevertheless, *Nlrp3*-deficient as well as *Asc*-deficient B6^{lpr} mice developed significant peribronchial mononuclear CD3 T cell infiltrates, while these were absent in wild type B6^{lpr} mice and *Il-1r*-deficient and *Il18*-deficient B6^{lpr} mice (see online supplementary figure S2A, B). Neither kidneys nor lungs developed significant interstitial fibrosis in any of the mouse strains (see online supplementary figure S1B) and also the low neutrophil numbers as well as IL-17 gene mRNA expression levels were genotype-independent (see online supplementary figure S3A–D). Together, lack of NLRP3 or ASC aggravates autoimmune nephritis and lung disease in B6^{lpr} mice, while *Il-1r-deficient* and *Il-18*-deficient B6^{lpr} mice displayed no such phenotype. These findings imply an immunoregulatory role of ASC and NLRP3 in systemic autoimmunity that is independent of IL-1 and IL-18.

NLRP3 and ASC, but not IL-1R and IL-18, suppress lymphoproliferation in B6^{lpr} mice

To unravel this unexpected finding, we first evaluated *Nlrp3*-deficient, *Asc*-deficient, *Il-1r*-deficient and *Il-18*-deficient mice for spontaneous autoimmunity, but up to 6 months of age antinuclear antibodies, anti-dsDNA IgG or rheumatoid factor could not be detected (not shown). Flow cytometry profiling of spleen monocyte and lymphocyte populations did not reveal any differences between the genotypes at 6 weeks and 24 weeks of age (not shown). Next we carefully assessed the *Nlrp3*-deficient, *Asc*-deficient, *Il-1r*-deficient and *Il-18*-deficient B6^{lpr} mice. Consistent with a dysfunctional inflammasome, *Nlrp3*-deficient or *Asc*-deficient dendritic cells or macrophages of B6lpr mice secreted reduced amounts of IL-1β in vitro (see online supplementary figure S3). In addition, activated caspase-1 mRNA and protein levels were strongly reduced in spleens of *Nlrp3*-deficient and *Asc*-deficient B6^{lpr} mice as compared with those of B6^{lpr} control mice (figure 2A, D). This was remarkable as other cell death-related mRNAs were increased in these two mouse strains (figure 2A). Caspase-3 mRNA showed the strongest induction but caspase-3 protein cleavage was not increased in western blots of spleen isolates (figure 2D). Similarly, immunostaining or cleaved caspase-3 positive splenocytes did not reveal any differences among the genotypes (see online supplementary figure S5A), arguing against enhanced splenocyte apoptosis in *Nlrp3*-deficient or *Asc*-deficient B6^{lpr} mice. In fact, annexin IV flow cytometry revealed that splenocyte apoptosis remained unaffected by the genotype (see online supplementary figure S5B). However, lack of NLRP3 and ASC caused massive splenomegaly and lymphadenopathy at 6 months of age, while

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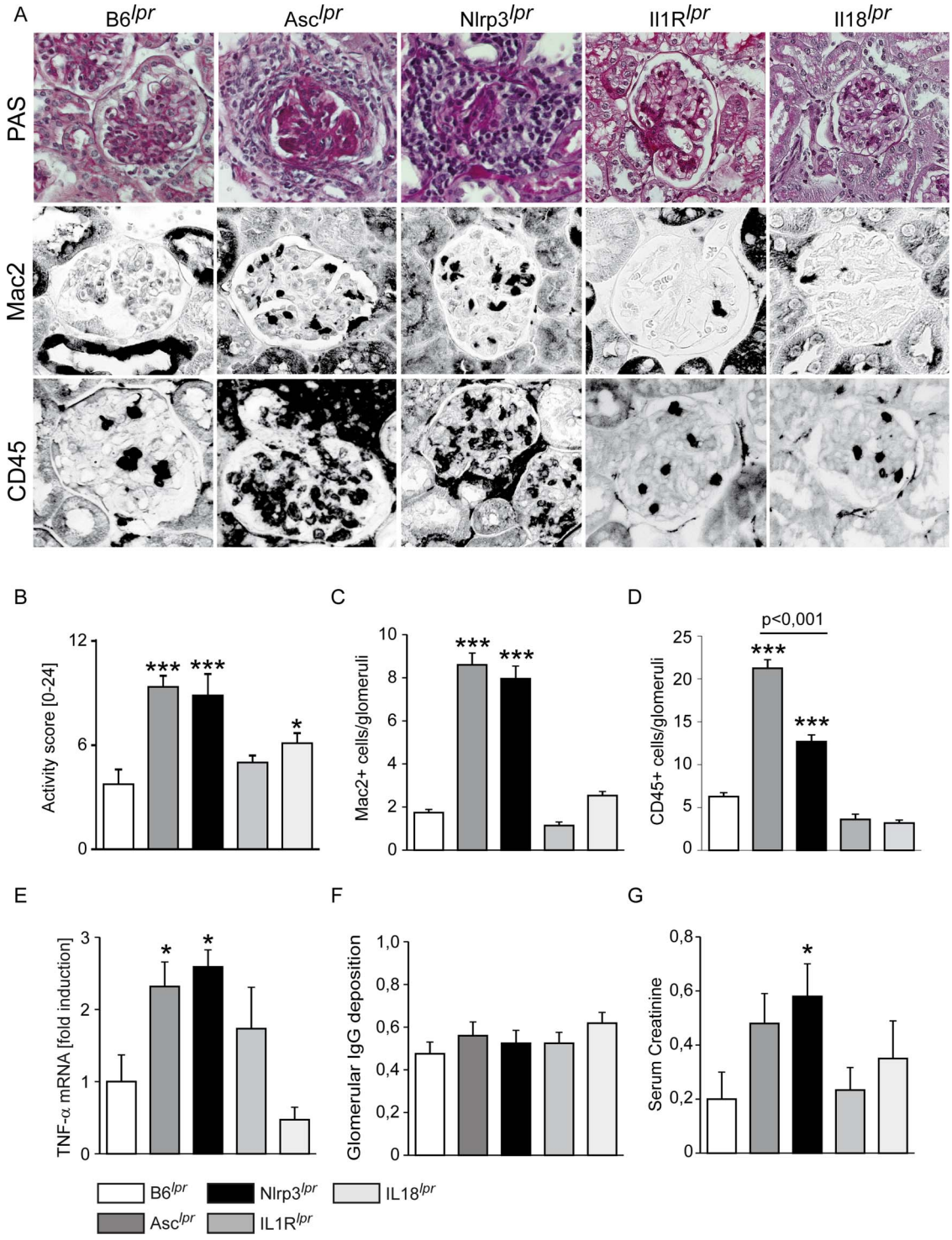

Figure 1 Immune complex nephritis in B6^{lpr} mice. (A and B) Renal sections were stained with periodic acid-Schiff (PAS) or Mac2 antibodies and signal intensity was scored applying a semiquantitativem score or quantified in glomeruli, respectively. Data are shown as means of at least 40 scored hpf from at least eight mice in each group; *p<0.05, ***p<0.001. (C) Kidney mRNA was isolated from B6^{lpr}/Asc−/−, B6^{lpr}/Nlrp3−/−, B6^{lpr}/Il-18−/−, B6^{lpr}/Il-1r−/− and B6^{lpr} mice. mRNA levels in kidney were analysed for tumour necrosis factor (TNF)-α. Data are shown as means of the ratio of the specific mRNA versus that of 18S rRNA, *p<0.05. (D) Renal sections immunostaining-score was also performed for immunoglobin G (IgG). Data are shown as means of quantification of at least 40 glomeruli from at least eight mice in each group; *p<0.05. (E) Serum creatine was measured in all three groups of mice. The histogram presents means±SEM of at least 10 mice in each group, *p<0.05 versus B6^{lpr} mice.

total body weight was phenotype-independent (figure 2B, C). Spleen Ki-67 staining displayed a massive increase in spleen cell proliferation in *Nlrp3*-deficient and *Asc*-deficient B6^{lpr} mice compared with B6^{lpr} controls (see online supplementary figure S5C). This was consistent with increased total numbers of spleen and lymph node cells quantified by flow cytometry, while leucocyte subset percentages remained constant (data not shown). Thus, *Nlrp3*-deficiency, *Asc*-deficiency, *Il-1r*-deficiency and *Il-18*-deficiency do not cause autoimmunity by itself, but aggravated nephritis in B6^{lpr} mice lacking only NLRP3 or ASC

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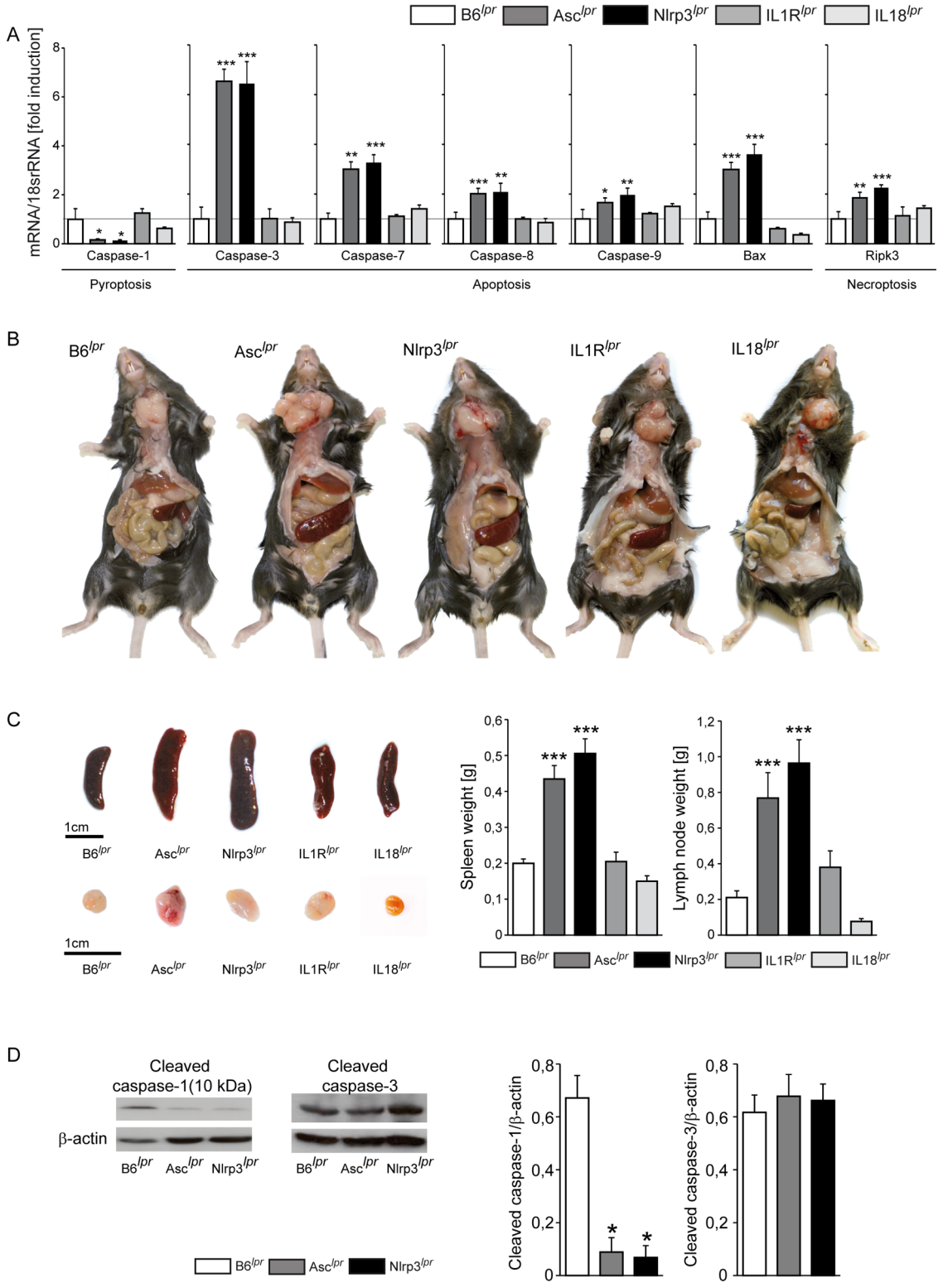

Figure 2 Lack of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) or NACHT, LRR and PYD domains-containing protein (NLRP3) and lymphoproliferation in B6^{lpr} mice. (A) RNA was isolated from B6^{lpr}/Asc−/−, B6^{lpr}/Nlrp3−/−, B6^{lpr}/Il-18−/−, B6^{lpr}/Il-1r−/− and B6^{lpr} mice for real-time PCR analysis. Data are expressed as means of the ratio of the specific mRNA versus that of 18S rRNA ±SEM and presented as fold induction to B6^{lpr} gene expression level; *p<0.05; **p<0.01; ***p<0.001 versus B6^{lpr} mice. (B and C) At 6 months of age *Asc*-deficient and *Nlrp3*-deficient B6^{lpr} but not *Il-18*-deficient and *Il-1R*-deficient B6^{lpr} mice revealed massive hyperplasia of cervical, axillar and mesenteric lymph nodes as well as splenomegaly. Data are means±SEM from at least 15 mice in each group, *p<0.05; ***p<0.001. Images of B and C are representative for at least 15 mice in each group. (D) Protein samples were prepared from spleens of 6 month-old B6^{lpr}/Asc−/−, B6^{lpr}/Nlrp3−/− and B6^{lpr} mice. Cleaved caspase-1 and cleaved caspase-3 western blots indicate the quantitative (50 μg protein load per lane) protein expression. Histograms represent the ratio of cleaved caspase-1 and cleaved caspase-3 to the respective β-actin expression. Data are means±SEM from at least three independent experiments, *p<0.05 versus B6^{lpr} mice.

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was associated with hyperproliferation of lymphocytes despite lack of caspase-1 activation and was independent of IL-1R and IL-18. Impaired lymphocyte apoptosis could be excluded as a causative mechanism for this phenotype (see online supplementary figure S5B).

NLRP3 and ASC suppress the activation of antigen-presenting cells in B6^{lpr} mice

Given the proinflammatory effects of the NLRP3 inflammasome in innate immunity,[8 45] we expected less activation of dendritic cells and macrophages in mice. However, spleen cell flow cytometry rather revealed an expansion of activated (MHC II+) CD11c cells and F4/80+ cells in *Nlrp3*-deficient or *Asc*-deficient B6^{lpr} mice (figure 3A and see online supplementary figure S6A). This was associated with increased mRNA expression levels of numerous NF-κB-dependent proinflammatory mediators (figure 3B). TLR signalling per se was not genotype-dependent. TNF-α induction in spleen dendritic cells upon stimulation with agonists to TLR4, TLR7 and TLR9 for 24 h was identical for all genotypes (figure 3C). Also the uptake rate of dextran particles by spleen mononuclear cells was genotype-independent (see online supplementary figure S6B). Together, although we expected *Nlrp3*-deficiency or *Asc*-deficiency to reduce caspase-1/IL-1β-dependent inflammation in B6^{lpr} mice, lack of NLRP3 and

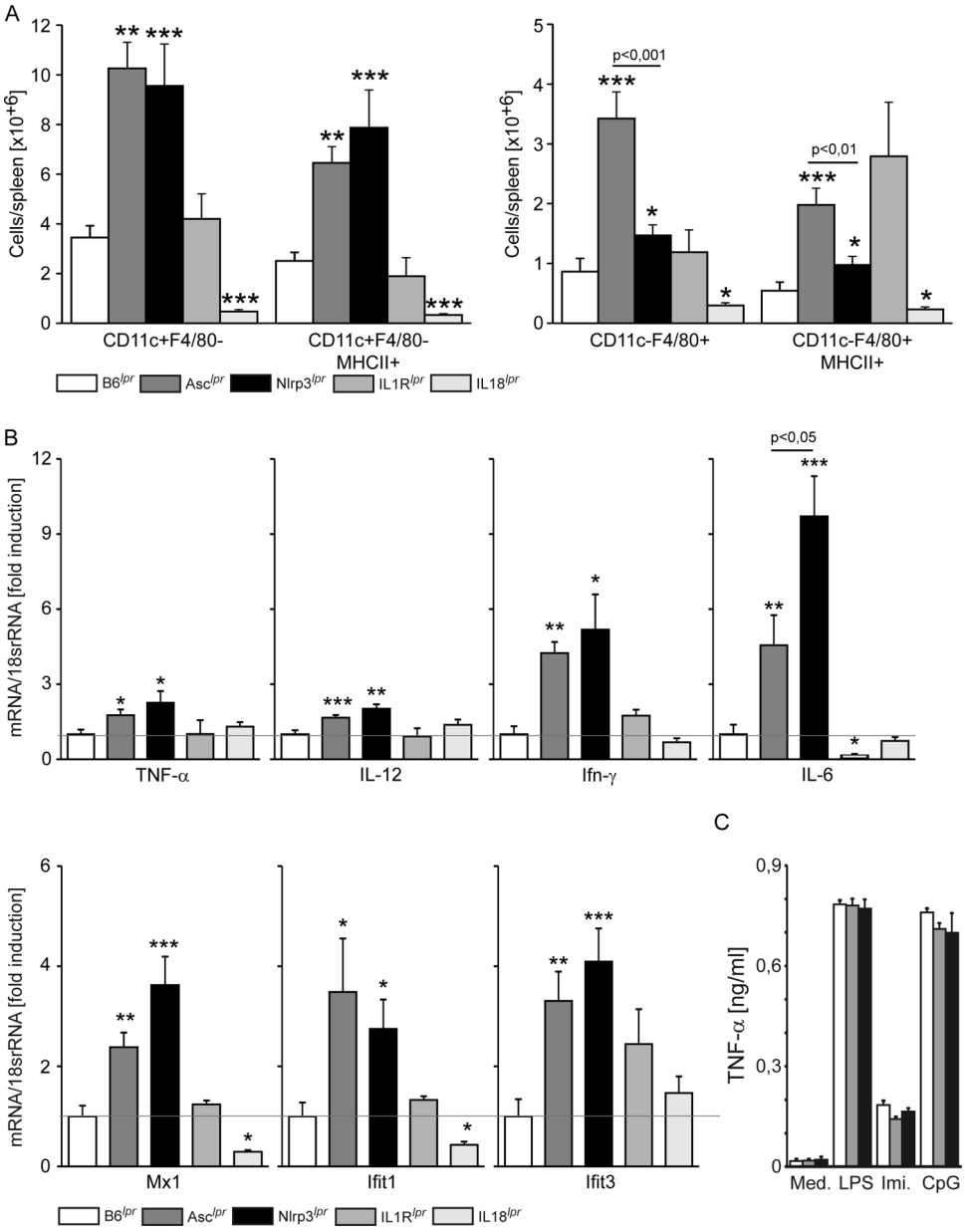

Figure 3 Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), NACHT, LRR and PYD domains-containing protein (NLRP3) and myeloid cell activation. (A) The total number of F4/80−/CD11c dendritic cells and CD11c-/F4/80 macrophages and their activation in spleens was quantified by flow cytometry in all investigated genotypes of B6^{lpr} mice as described in methods. Data represent means ±SEM from at least 10 mice in each group. *p<0.05; **p<0.01; ***p<0.001 versus B6^{lpr} mice. (B) RNA was isolated from spleens and mRNA levels in spleen were analysed for NF-κB genes expression, proinflammatory genes and type I interferon dependent genes. Expression levels were quantified by real-time PCR and presented as fold induction to controls. Data are shown as means of the ratio of the specific mRNA versus that of 18S rRNA, *p<0.05; **p<0.01; ***p<0.001. (C) Spleen-derived dendritic cells were stimulated with lipopolysaccharide (LPS), imiquimod (Imi) and CpG as described in methods. Levels of tumour necrosis factor (TNF)-α were determined in supernatants. Data represent means±SEM.

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ASC (but not of IL-1R or IL-18) was rather associated with increased antigen-presenting cell activation and expression of proinflammatory mediators.

NLRP3 and ASC suppress the expansion of lymphocytes in B6^{lpr} mice

How does the activation of antigen-presenting cells in *Nlrp3*-deficient and *Asc*-deficient B6^{lpr} mice affect lymphocyte subsets? CD4 and CD8 T cells as well as CD3CD4/CD8 double negative T cells, a cell population abnormally expanded in SLE and thought to contribute SLE activity in B6^{lpr} mice,[46 47] were all increased and more activated in *Nlrp3*-deficient and *Asc*-deficient B6^{lpr} mice, as indicated by the activation markers CD69 and CD44 (figure 4A,B,D). CD4/CD25/FoxP3+ 'regulatory' T cells were significantly increased only in B6^{lpr} *Asc*−/− mice (figure 4C). However, Nlrp3-deficiency and Asc-deficiency did not at all affect the capacity of Treg cells to suppress T cell proliferation; further the TGF-β-induced differentiation of Tregs from CD4 T cells was not impaired (figure 4E,F). *Nlrp3*-deficiency and *Asc*-deficiency also increased the numbers of naïve B cells, marginal zone B cells, follicular B cells, B1 cells with increased surface expression of the activation marker MHC-II and CD19CD69 B cells (figure 5A,B). This was associated with increased mRNA expression of Bcl6, while other factors that drive the proliferation and survival of B cells and plasma cells were not much affected (figure 5B). Also in vitro activation of TLR4, TLR7 and TLR9 in CD19 B cells was genotype-independent (not shown). In fact, CD138 plasma cells were not significantly expanded in both mutant mouse strains (figure 5C). In contrast, lack of IL-1R or IL-18 did not significantly increase these lymphocyte counts. *Il-18*-deficiency rather reduced them. When analysing lymphocyte data in percentage of all splenocytes no genotype-related differences were seen, which implies unspecific lymphoproliferation. Finally, we phenotyped lymphocyte precursors in bone marrow and thymus of *Nlrp3*-deficient and *Asc*-deficient B6^{lpr} mice and B6^{lpr} controls. There was no consistent alteration of lymphocyte maturation that could explain the observed phenotype in adult mice (data not shown). Together, *Nlrp3*-deficiency or *Asc*-deficiency (but not IL-1R or IL-18) deficiency is associated with abnormal expansion of T cells (including CD4/CD8 double positive T cells) and on several B cell subsets (but not plasma cells) in B6^{lpr} mice. Also the numbers of bone marrow plasma cells were not affected by the genotypes (see online supplementary figure S6C). This implies that NLRP3 and ASC have an immunosuppressive effect on T cells and B cells in B6^{pr} mice that is independent from their capacity to trigger IL-1 and IL-18 secretion.

Lack of NLRP3 or ASC has little effects on systemic inflammation and autoantibody production in B6^{lpr} mice

As *Nlrp3*-deficiency and *Asc*-deficiency had not significantly affected plasma cell counts in B6^{lpr} mice, we questioned whether enhanced lymphoproliferation and T cell and B cell activation at all translated into more IgG and autoantibody production or systemic inflammation. Plasma levels of multiple proinflammatory and immunoregulatory cytokines were largely genotype-independent except for a significant increase of IL-12p40 and IL-23 in *Nlrp3*-deficient B6^{lpr} mice (figure 5E). Interferon-α/β, IL-1β and IL-10 plasma levels were undetectable in all genotypes. Also plasma IgG levels were comparable in all strains (figure 5D). Among the lupus autoantibodies only rheumatoid factor and anti-dsDNA IgG were significantly increased in B6^{lpr} *Nlpr3*−/− mice (figure 5D), the latter confirmed by *Crithidia luciliae* assay (not shown). Despite some trends of higher antibody levels in individual mice of the mutant strains there was no statistically significant difference among the genotypes for the other lupus autoantibodies tested (figure 5D). Thus, the suppressive effect of NLRP3 and ASC on T cells and B cells in B6^{lpr} mice does not much affect plasma cytokine and antibody levels.

NLRP3 and ASC regulate TGF-β signalling in B6^{lpr} mice

What could be the reason for this unexpected aggravation of nephritis-related autoimmunity of the *Nlrp3*-deficient and *Asc*-deficient B6^{lpr} mice? Among the known IL-1-independent and IL-18-independent effects of NLRP3 and ASC,[45 48–50] only their role in TGF-βR signalling (until now documented for TGF-β-induced epithelial–mesenchymal transition in kidney epithelial cells[50]) has the potential to explain NLRP3-related and ASC-related immunosuppression. In fact, of *Nlrp3*-deficient or *Asc*-deficient B6^{lpr} mice revealed less Smad-2 phosphorylation on western blots of spleen isolates (figure 6A), which was associated with a reduction in TGF-β1-induced nuclear translocation of Smad 2/3 in *Asc*-deficient or *Nlrp3*-deficient bone marrow dendritic cells as compared with wild type dendritic cells (figure 6B). In addition, ASC and NLRP3 were needed in dendritic cells for full TGF-β1-mediated suppression of IL-6 production upon LPS stimulation (figure 6C). As a further proof of the concept that ASC and NLRP3 are needed for TGF-R signalling, spleen mRNA profiling by PCR array revealed that lack of ASC or NLRP3 suppressed the expression of most TGF-β target genes in B6^{lpr} mice (figure 6D). We selected the most regulated genes and validated their lower spleen mRNA expression levels in comparison with B6^{lpr} mice by RT-PCR (figure 6D). Spleen TGF-R chain mRNA expression levels and serum TGF-β levels excluded an upstream defect of TGF-R signalling, in fact, TGFβR1 and TGFβR2 chains were rather induced than suppressed in *Nlrp3*-deficient or *Asc*-deficient B6^{lpr} mice (figure 6E, F). We conclude that ASC and NLRP3 are needed for TGF-β1-mediated Smad-2/3 phosphorylation in dendritic cells and subsequent TGF-β target gene expression in spleens of B6^{lpr} mice. Hence, lack of ASC or NLRP3 (but not of IL-1R or IL-18) impairs the immunoregulatory effect of TGF-β1 signalling in B6^{lpr} mice.

DISCUSSION

We had hypothesised that lack of NLRP3 or ASC would attenuate systemic autoimmunity and lupus-like nephritis in B6^{lpr} mice, mainly because canonical NLRP3 inflammasome activation via caspase-1, IL-1β and IL-18 should elicit proinflammatory effects.[8 11 19] However, our data disprove this concept and rather document that NLRP3 and ASC limit the expansion and activation of antigen-presenting cells and of T cells and B cells and, subsequently, autoimmune tissue inflammation in B6^{lpr} mice. Interestingly, lupus autoantibody production and immune complex disease were hardly affected by lack of NLRP3 or ASC, implying an immunoregulatory role of NLRP3 and ASC mainly on cellular immunity. Obviously, the NLRP3 inflammasome has previously unknown immunoregulatory effects in the context of experimental SLE. Until now, aggravation of disease in *Nlrp3*-deficient mice has rarely been reported. For example, NLRP3 suppresses antitumor immunity and age-related macula degeneration.[51 52]

The NLRP3 inflammasome contributes to host defence and to autoinflammatory disorders consistently via the proinflammatory effects of caspase-1 activation in mononuclear phagocytes. The proinflammatory effects of caspase-1 are threefold: via pyroptosis, a programmed form of inflammatory cell death[11] and via secretion

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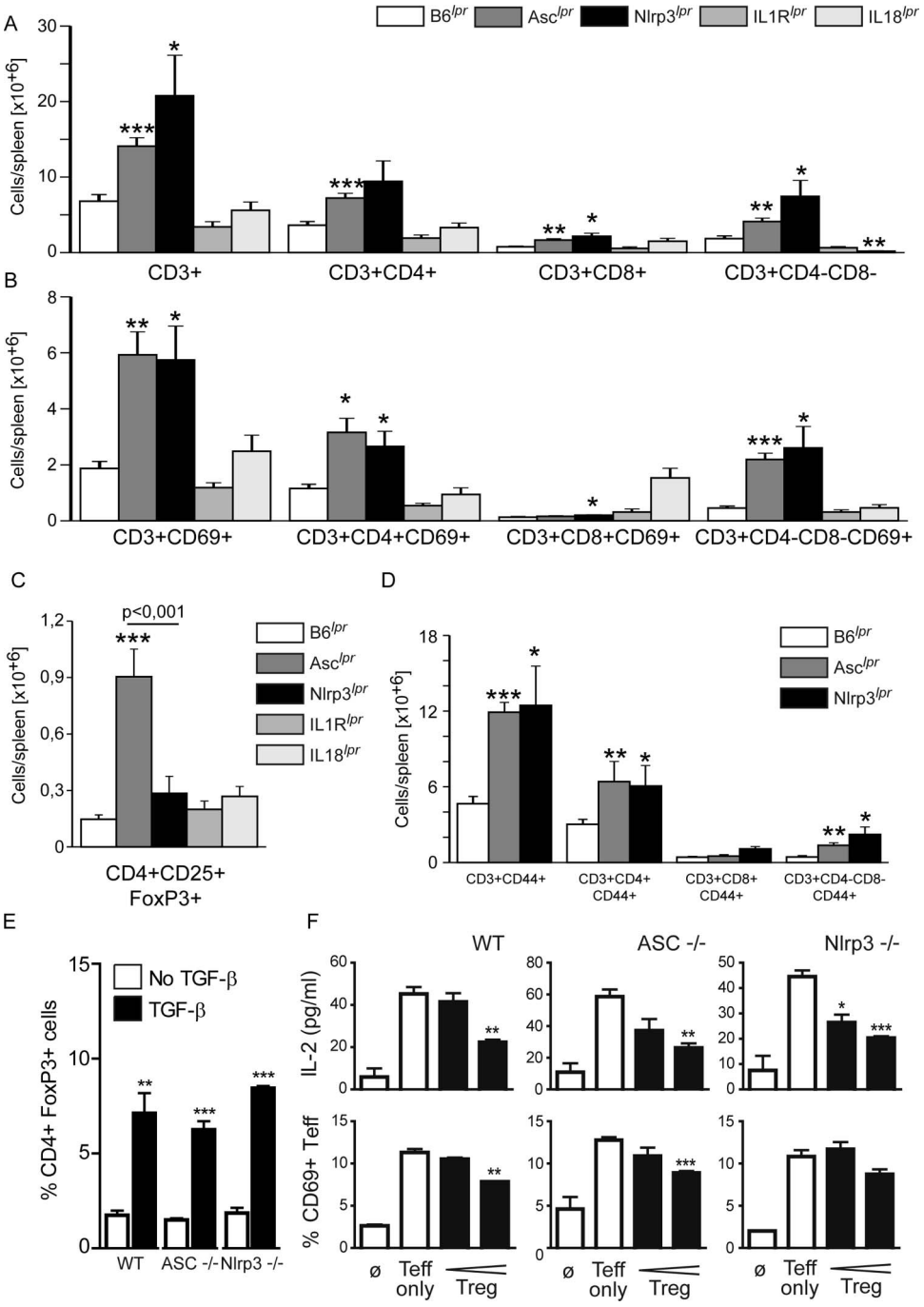

Figure 4 Characterisation of T cell subsets and regulatory T cell function. (A–D) Flow cytometry was used to determine the total number of distinct T cell subsets in spleens of 6 month-old B6^{lpr}/Asc−/−, B6^{lpr}/Nlrp3−/−, B6^{lpr}/Il-18−/−, B6^{lpr}/Il-1r−/− and B6^{lpr} mice. The histogram presents means±SEM of at least 15 mice in each group. *p<0.05, **p<0.01, ***p<0.001 versus B6^{lpr} mice. (E) CD4CD25^{negative} cells (1×10^6) that were isolated from the spleen of the indicated mice were stimulated with anti-CD3-CD28 beads and tumour growth factor (TGF)-β in the presence of interleukin (IL)-2. The percentage of CD4FoxP3 cells in the culture was determined on day 3 by flow cytometry. Conditions without TGF-β were treated with beads and IL-2 only. Error bars indicate SEM of n=3, significance was calculated in comparison with cultures with beads alone: **p<0.01; ***p<0.001. (F) CD4CD25 and CD4CD25^{negative} cells were isolated from the spleen of the indicated mice. For analysis of regulatory T cell function, CD4CD25^{negative} were stimulated with anti-CD3-CD28 beads in the presence of increasing numbers of CD4CD25 cells. After 3 days, IL-2 secretion and expression of CD69 and CD44 by the T cells were determined. Ø, T effector cells without stimulation, Teff only, conditions without CD4CD25 cells, significance was calculated in comparison with cultures with Teff only. Error bars indicate SEM of n=3, significance was calculated in comparison with cultures with beads alone: *p<0.05; **p<0.01; ***p<0.001.

of IL-1β and IL-18, which trigger inflammation via IL-1R and IL-18R signalling systemically and in many tissues.[9 10] As expected, *Nlrp3*-deficiency and *Asc*-deficiency (but not *Il-1r*-deficiency or *Il-18*-deficiency) reduced caspase-1 activation in B6^{lpr} mice, hence, the associated activation of myeloid antigen-presenting cells and the expression of numerous proinflammatory mediators are inconsistent with what has been demonstrated in experimental infection or sterile inflammation.[11 19–23 31–33] So by which mechanisms could *Nlrp3*-deficiency and *Asc*-deficiency drive lymphoproliferation, immune cell activation and lupus

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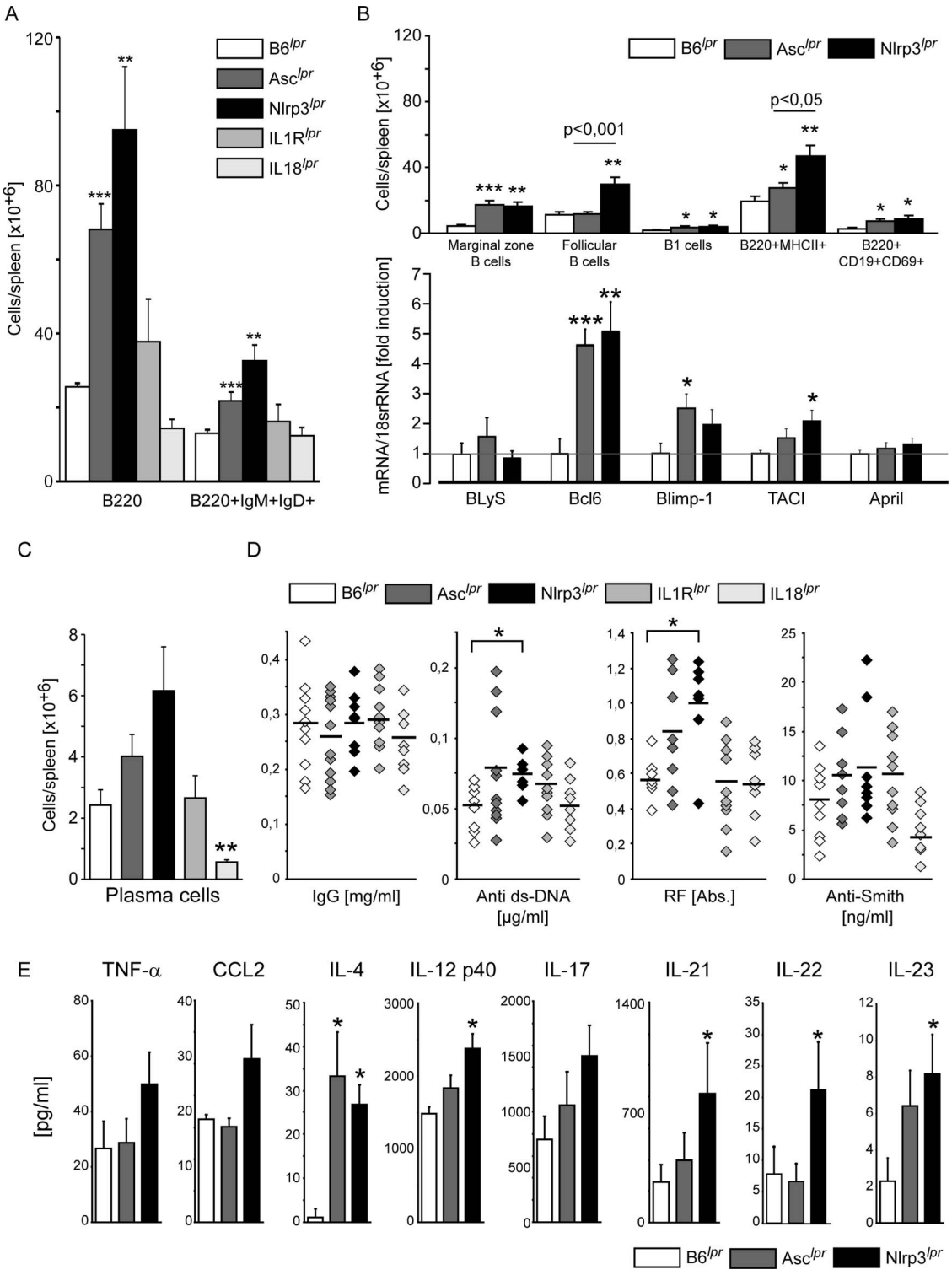

Figure 5 B cell subsets and antibody production in B6^{lpr} mice. (A and C) The total number of spleen B220 cells, B220/IgM/IgD mature B cells and κ-light chain/CD138 plasma cells were quantified in 6 month-old B6^{lpr}, B6^{lpr}Asc−/−, B6^{lpr}Nlrp3−/−, B6^{lpr}/Il-18−/− and B6^{lpr}/Il-1r−/− mice) by flow cytometry. The histogram presents means±SEM of at least 15 mice in each group; *p<0.05, **p<0.01, ***p<0.001 versus B6^{lpr} mice. (B) The total number of spleen B220+ cells and B cell subsets were quantified in 6 month-old B6^{lpr}, B6^{lpr}Asc−/− and B6^{lpr}Nlrp3−/− mice (B220/IgM/IgD mature B cells; B220 CD21high/CD23low marginal zone B cells; B220 CD21low/CD23high follicular B cells; B1-cells and B220/CD19/CD69 B cells) by flow cytometry. The histogram presents means±SEM of at least 15 mice in each group; *p<0.05, **p<0.01, ***p<0.001 versus B6^{lpr} mice. (B) mRNA levels in spleen were analysed for B cell survival and stimulatory factors. Expression levels were quantified by real-time PCR and presented as fold induction to controls. Data are shown as means of the ratio of the specific mRNA versus that of 18S rRNA, *p<0.05; **p<0.01; ***p<0.001 versus B6^{lpr} mice. (D) Mice from all groups were bled at month 6 to determine serum levels of immunoglobin G (IgG) and ds-DNA autoantibody, autoantibodies against Smith antigen or rheumatoid factor (RF) by ELISA. Data show individual mice from each group. The mean is indicated by a bar; *p<0.05 versus B6^{lpr} mice. (E) B6lpr, B6lprAsc−/− and B6lprNlrp3−/− mice were bled at month 6 to determine serum levels of interleukin (IL)-4, IL-12, IL-17, IL-21, IL-22, IL-23, monocyte chemoattractant protein-1 and tumour necrosis factor (TNF)-α. Same grey scale for groups was used as for 6D. Data represent means±SEM from at least eight mice in each group. *p<0.05.

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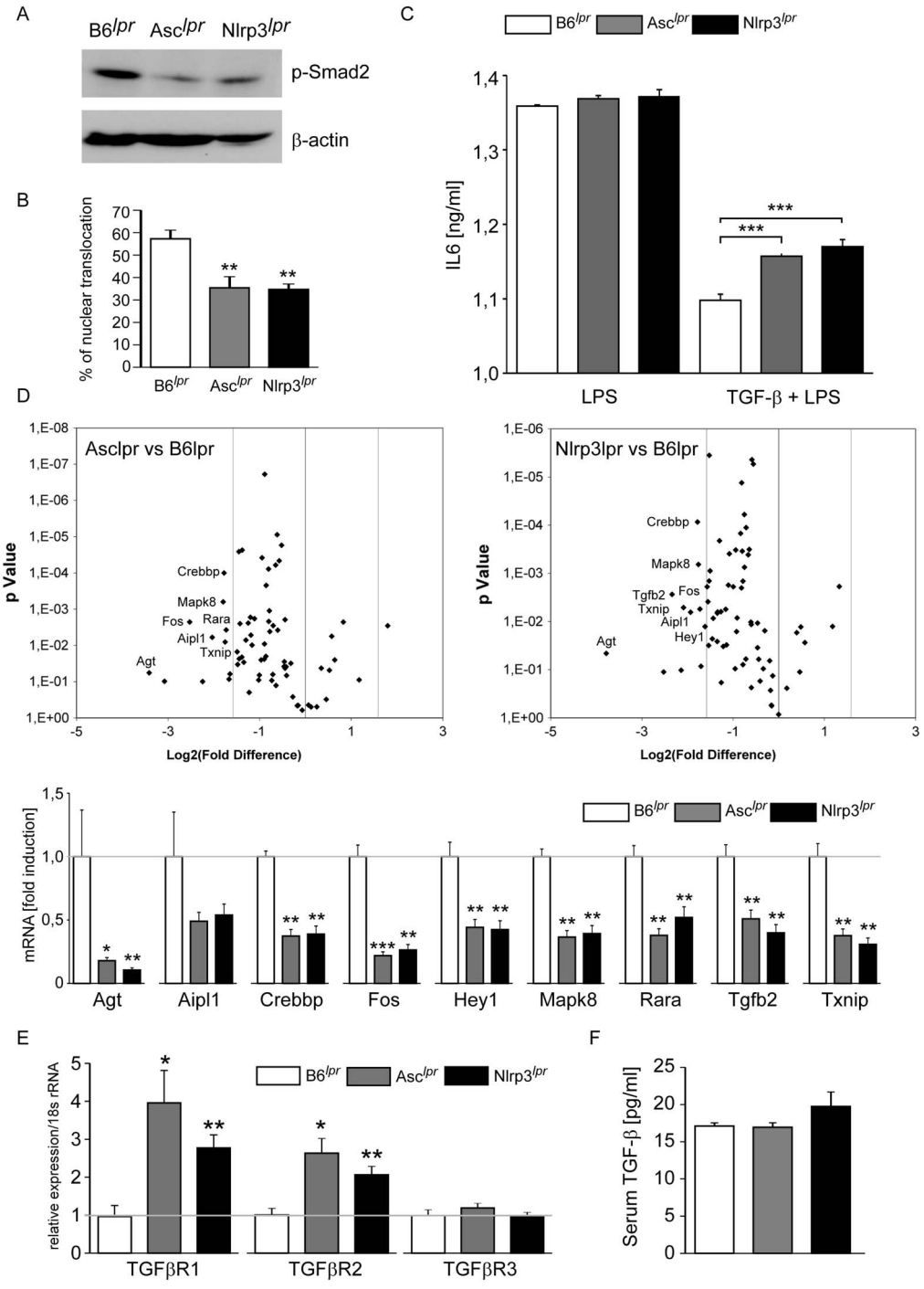

Figure 6 Lack of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and NACHT, LRR and PYD domains-containing protein (NLRP3) and tumour growth factor (TGF)-β signalling in B6^{lpr} mice. (A) Western blot analysis shows Smad2 phosphorylation and represents one of three independent experiments. (B) Smad2/3 translocation to the nucleus was quantified by signal intensity. (C) Bone marrow-derived dendritic cells were stimulated with lipopolysaccharide (LPS) alone or with LPS and TGF-β. Levels of interleukin (IL)-6 were determined in supernatants. Data represent means±SEM; ***p<0.001. (D) mRNA levels in spleen were analysed for TGF-β-dependent signalling by Qiagen RT^2 PCR profiling array. Results are presented as volcano plots. Expression levels of significantly different expressed genes were quantified by real-time PCR and presented as fold induction to controls. Data are shown as means of the ratio of the specific mRNA versus average of five different housekeeping genes as indicated in the Materials and Methods section, *p<0.05; **p<0.01; ***p<0.001. (E) RNA was isolated from B6^{lpr}/Asc−/−, B6^{lpr}/Nlrp3−/− and B6^{lpr} mice for real-time PCR analysis. Data are expressed as means of the ratio of the specific mRNA versus that of 18S rRNA±SEM and presented as fold induction to B6^{lpr} gene expression level; *p<0.05; **p<0.01; versus B6^{lpr} mice. (F) Mice from all groups were bled at month 6 to determine serum levels of TGF-β by ELISA. Data show means±SEM from at least eight mice in each group.

nephritis in SLE-like autoimmunity in an IL-1R-independent and IL-18-independent manner?

Our data point towards impaired TGF-βR signalling, a process that is known to control autoimmunity because TGF-β is an important negative regulator of the immune system.[53] TGF-β is produced by leucocytes of all lineages to control the differentiation, proliferation and activation in autocrine and paracrine manners. As such, TGF-β is a guardian factor of

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immune tolerance and T cell-mediated autoimmunity.[54 55] For example, mice deficient in either TGF-β1 or TGF-R display spontaneous lethal autoimmunity.[56–58] Our model did not display lethality, which is consistent with our in vitro data that suggest a moderate modulatory effect of *Nlrp3*-deficiency or *Asc*-deficiency rather than a complete blockade of TGF-β signalling. TGF-β1-mediated immunosuppression mostly affects dendritic cells, macrophages and T cells,[55 59] which is consistent with our own observations in *Nlrp3*-deficient and *Asc*-deficient B6^{lpr} mice. For example, autoimmunity of *Tgf-β1*-deficient mice depends on self-antigen presentation via enhanced MHC class II expression,[60 61] a phenomenon also observed by us in *Nlrp3*-deficient and *Asc*-deficient B6^{lpr} mice. In fact, dendritic cell-specific deletion of TGF-βRII is sufficient to induce spontaneous multiorgan autoimmunity,[62] which documents that TGF-βR signalling in dendritic cells is sufficient to control autoimmunity and does not necessarily require TGF-βR-mediated immunosuppression from regulatory T cells.[55 57] This is important as we could not detect any effect of *Nlrp3*-deficiency and *Asc*-deficiency on regulatory T cell control of T cell proliferation.

In contrast to the prominent effect of *Nlrp3*-deficiency and *Asc*-deficiency on cellular immunity, spleen plasma cell counts and immunoglobulin or autoantibody production were affected to the same extent inB6^{lpr} mice, except for somewhat higher dsDNA antibody and rheumatoid factor levels in *Nlrp3*-deficient mice. Therefore, immune complex disease, as determined by glomerular immune complex deposits, was genotype-independent. Immune complex-deposition is the diagnostic hallmark and a sine qua non for lupus nephritis,[39 63] but disease activity is also determined by infiltrating effector T cells and macrophages.[64 65] The same applies to autoimmune lung disease inB6^{lpr} mice, which is rather determined by de novo tertiary lymphoid tissue formation in the peribronchial compartment, than by immune complex disease.[36] Therefore, lung disease and nephritis could still be markedly aggravated in *Nlrp3*-deficient and *Asc*-deficient B6^{lpr} mice, despite negligible effects on humoral immunity.

A similar contradictory phenotype had been found for TLR9-deficient B6^{lpr} mice,[66] which was confirmed in several other lupus nephritis mouse models.[35 67 68] Before, numerous in vitro and in vivo studies had documented the proinflammatory and B cell mitogenic effect of TLR9 signalling,[69–72] especially when stimulated with self-DNA from immune complexes.[37 73–75] Later, it was discovered that this unexpected phenotype of TLR9-mutant mice related to a TLR7 inhibitory effect during the early phase of SLE,[76 77] while TLR9 blockade in the subsequent progression phase of SLE attenuated autoantibody production and inflammatory organ manifestations.[78–81] Obviously, immunostimulatory pattern recognition receptors can elicit unexpected outcomes in autoimmunity when they specifically modulate immune tolerance and adaptive immunity in lupus. There were very few phenotypical differences between *Nlrp3*-deficient and *Asc*-deficient B6^{lpr} mice that may indicate specific immunoregulatory functions of ASC and NLRP3 independent from each other.

In summary, in B6^{lpr} mice NLRP3 and ASC, the two components of the NLRP3 inflammasome, are needed to avoid excessive lymphoproliferative syndrome and autoimmune tissue injury, but this effect is independent of their canonical role in IL-1 and IL-18 secretion. This unexpected non-canonical function of NLRP3 and ASC seems to relate to their role in TGF-βR signalling, for example, in Smad2/3 phosphorylation. TGF-β target gene expression is needed to regulate the activation and expansion of myeloid antigen-presenting cells as well as most T cell and B cell subsets. Together we conclude, the NLRP3 inflammasome suppresses cellular autoimmunity in systemic lupus nephritis of B6^{lpr} mice, which defines another biological function of NLRP3 and ASC beyond pathogen recognition.[82]

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