IRF4 Deficiency Abrogates Lupus Nephritis Despite Enhancing Systemic Cytokine Production

Maciej Lech,* Marc Weidenbusch,* Onkar P. Kulkarni,* Mi Ryu,* Murthy Narayana Darisipudi,* Heni Eka Susanti,* Hans-Willi Mittruecker,† Tak W. Mak,‡ and Hans-Joachim Anders*

*Department of Nephrology, Medizinische Poliklinik, University of Munich, Munich, Germany; †Institute for Immunology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; and ‡The Campbell Family Institute for Breast Cancer Research, Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, Canada

ABSTRACT
The IFN-regulatory factors IRF1, IRF3, IRF5, and IRF7 modulate processes involved in the pathogenesis of systemic lupus and lupus nephritis, but the contribution of IRF4, which has multiple roles in innate and adaptive immunity, is unknown. To determine a putative pathogenic role of IRF4 in lupus, we crossed Irf4−/− mice with autoimmune C57BL/6-(Fas)lpr mice. IRF4 deficiency associated with increased activation of antigen-presenting cells in C57BL/6-(Fas)lpr mice, resulting in a massive increase in plasma levels of TNF and IL-12p40, suggesting that IRF4 suppresses cytokine release in these mice. Nevertheless, IRF4 deficiency completely protected these mice from glomerulonephritis and lung disease. The mice were hypogammaglobulinemic and lacked antinuclear and anti-dsDNA autoantibodies, revealing the requirement of IRF4 for the maturation of plasma cells. As a consequence, Irf4−/− C57BL/6-(Fas)lpr mice neither developed immune complex disease nor glomerular activation of complement. In addition, lack of IRF4 impaired the maturation of Th17 effector T cells and reduced plasma levels of IL-17 and IL-21, which are cytokines known to contribute to autoimmune tissue injury. In summary, IRF4 deficiency enhances systemic inflammation and the activation of antigen-presenting cells but also prevents the maturation of plasma cells and effector T cells. Because these adaptive immune effectors are essential for the evolution of lupus nephritis, we conclude that IRF4 promotes the development of lupus nephritis despite suppressing antigen-presenting cells.


Systemic autoimmunity in systemic lupus erythematosus (SLE) involves a polyclonal expansion of lymphocytes that are autoreactive to multiple nuclear autoantigens. This process can cause a broad spectrum of clinical manifestations ranging from mild fever, skin rashes, and arthralgia to severe inflammation of kidney, lungs, or brain.1 The pathogenesis of SLE is based on variable combinations of genetic polymorphisms that promote loss of tolerance or tissue inflammation.2,3 For example, certain genes impair lymphocyte apoptosis and the clearance of dying cells via opsonization, phagocytosis, and digestion of self-DNA, which all increase the release of nuclear particles from secondary necrotic lymphocytes and expose them to the immune system.4 Another group of susceptibility genes enhances the immune recognition of self nucleic acids by Toll-like receptors (TLRs) in
dendritic cells (DCs), which increases the production of type I IFN\(^5,6\) and eventually the expansion of autoreactive lymphocytes.\(^7\) A third class of genetic lupus risk factors affects tissue inflammation.\(^4\)

IFN-regulatory factors (IRFs) form a group of transcription factors that have the potential to contribute to all of the aforementioned pathomechanisms of SLE. IRF-1 is a proinflammatory transcription factor that triggers expression of proinflammatory cytokines in tubular epithelial cells and immune cells in the postischemic kidney\(^8\) as well as in mesangial cells during lupus nephritis of MRL-(Fas)\(^{1pr}\) mice.\(^9\) IRF3 and IRF7 mediate type I IFN production upon immune recognition of viral and endogenous nucleic acids in DCs,\(^10\) including TLR7 signaling, which was shown to be an essential pathway in SLE.\(^7,11–13\) IRF5 is required for immune cell maturation and for TLR signaling, two mechanisms that contribute to SLE and lupus nephritis of Fc\(^\gamma\)RIIB\(^+\) or Fc\(^\gamma\)RIIB\(^−/−\) mice\(^14\) and in lupus secondary to pristane injection.\(^15\) Unlike other IRFs, IRF4 is not regulated by IFNs and its expression is restricted to immune cells.\(^16\) IRF4 has multiple regulatory functions in adaptive immunity.\(^16,17\) For example, IRF4 is required for the maturation of B and T cells,\(^18\) plasma cell maturation and Ig isotype switching,\(^19\) the ability of regulatory T cells to suppress Th2 responses,\(^20\) and the induction of Th17 T cells.\(^21,22\) IRF4 also regulates innate immunity because it is required for M2 macrophage polarization.\(^23\) IRF4 also serves as an inhibitor of TLR signaling via binding to MyD88, which impairs its interaction with IRF5 and other downstream signaling elements.\(^24\) For example, Irf4-deficient mice develop an exaggerated postischemic inflammatory response aggravating ischemic acute renal failure, which depends on the oxidative stress-driven induction of IRF4 in intrarenal DCs.\(^25\) As another example, bacterial products specifically induce IRF4 in DCs of the intestinal wall, a mechanism that protects mice from experimental colitis.\(^26\) Hence, IRF4 suppresses innate immunity but fosters adaptive immunity. These ambivalent immunoregulatory roles are again unique among the members of the IRF family.

Given the clear roles of IRF1, IRF3, IRF5, and IRF7 in the pathogenesis of SLE and lupus nephritis and the multiple roles of IRF4 in innate and adaptive immunity, we hypothesized a functional contribution of IRF4 to SLE and lupus nephritis. To test this concept, we generated Irf4-deficient C57BL/6\(^{lpr/lpr}\) (B6\(^{lpr}\)) mice and compared the phenotype with that of wild-type B6\(^{lpr}\) mice, an autoimmune mouse strain that develops lupus autoantibodies and SLE manifestations in kidneys and lungs.\(^27\)

Figure 1. Lack of IRF4 increases the activation of antigen-presenting cells. (A) Serum cytokine levels were determined by ELISA in B6\(^{lpr}\) mice (black bars) and B6\(^{lpr/Irf4−/−}\) mice (white bars) at 6 months of age. (B) Spleen monocytes were stimulated with 1 \(\mu\)g/ml LPS and the cytokine levels were determined by ELISA. (C-E) Spleen cells were quantified by flow cytometry using surface activation markers as indicated. Data represent mean ± SEM from 12 to 14 mice in each group. *P < 0.05, **P < 0.01 versus B6\(^{lpr}\) mice.
RESULTS

IRF4 Deficiency Increases Serum Cytokine Levels by Activating DCs and Macrophages in B6\textsuperscript{lpr} Mice

We generated Irf4\textsuperscript{-deficient} B6\textsuperscript{lpr} mice by crossing Irf4\textsuperscript{-deficient} with Fas-deficient (\textit{lpr}) C57BL/6 mice. Litters of B6\textsuperscript{lpr/irf4\textsuperscript{-/-}} mice were bred along Mendelian ratios and revealed no differences in body weight gain as compared with B6\textsuperscript{lpr} (Irf4 wild-type) mice (not shown). In phenotyping both strains at 6 months of age, we first determined serum levels of various proinflammatory cytokines by ELISA. B6\textsuperscript{lpr/irf4\textsuperscript{-/-}} mice revealed significantly higher serum levels of TNF and IL-12\textsubscript{p40} than their age-matched B6\textsuperscript{lpr} counterparts (Figure 1A), suggesting that IRF4 suppresses systemic cytokine release in B6\textsuperscript{lpr} mice. Because IRF4 has been described to suppress the activation of antigen-presenting cells, we isolated splenocytes from B6\textsuperscript{lpr} and B6\textsuperscript{lpr/irf4\textsuperscript{-/-}} mice and assessed cytokine production 24 hours after LPS stimulation \textit{in vitro}. In fact, splenocytes from B6\textsuperscript{lpr/irf4\textsuperscript{-/-}} mice produced much higher levels of IFN\gamma, IL-12p40, TNF, and MCP-1/CCL2 as compared with cells from B6\textsuperscript{lpr} mice (Figure 1B).

Next we determined the activation state of various DC subsets in spleens of 6-month-old mice by flow cytometry. IRF4 deficiency increased the total numbers of CD11c/CD4-positive DCs and CD4/CD8double-negative CD11c DCs (Figure 1C). IRF4 was required to suppress DC activation because B6\textsuperscript{lpr/irf4\textsuperscript{-/-}} mice displayed higher numbers of CD40-positive CD11c DCs and MHCII-positive F4/80/CD11c DCs (Figure 1D). In addition, lack of IRF4 increased the numbers of CD206-positive macrophages in spleens; most of them were MHCII positive, indicating a classically activated (M1) macrophage phenotype (Figure 1E). Alternatively activated CD206-positive macrophages remained in a minor splenocyte population in 6-month-old B6\textsuperscript{lpr/irf4\textsuperscript{-/-}} mice (Figure 1E). Taken together, lack of IRF4 increases systemic cytokine production and the activation of antigen-presenting cells in B6\textsuperscript{lpr} mice, indicating an immunosuppressive role for IRF4 in these aspects of innate immunity.

Lack of IRF4 Prevents Lupus Nephritis in B6\textsuperscript{lpr} Mice

Next we questioned whether the aggravated systemic inflammation in B6\textsuperscript{lpr/irf4\textsuperscript{-/-}} mice is associated with an aggravation of lupus nephritis. At 6 months of age, B6\textsuperscript{lpr} mice developed diffuse proliferative glomerulonephritis that was associated with diffuse mesangial matrix expansion, mesangial cell proliferation, and occasional tuft adhesions (Figure 2). Tuft necrosis and crescent formation were absent. On immunostaining, diffuse proliferative glomerulonephritis was associated with extensive glomerular IgM and IgG deposits presenting in mesangial (IgG) and capillary (IgM/IgG) staining patterns (Figure 2).
Staining for complement factor 9 showed mainly mesangial positivity (Figure 2), altogether indicating diffuse proliferative lupus-like immune complex glomerulonephritis as the renal manifestation of spontaneous autoimmunity in B6\textsuperscript{pr} mice. Lack of IRF4 completely abrogated this phenotype because age-matched B6\textsuperscript{lpr/Irf4\textsuperscript{-/-}} mice developed hardly any glomerular Ig and complement deposits or glomerular abnormalities as shown by light microscopy (Figure 2). This was also evident by morphometrical assessment of the activity and chronicity index for lupus nephritis (Figure 3). Some renal sections showed a slight increase in activated mesangial cells in B6\textsuperscript{lpr/Irf4\textsuperscript{-/-}} mice, but this was not a consistent finding. Abnormalities of the vascular or tubulointerstitial compartment were absent in both mouse strains. Because of the moderate renal lesions, plasma creatinine levels were only mildly elevated in B6\textsuperscript{pr} mice and there was a nonsignificant trend toward lower levels in B6\textsuperscript{lpr/Irf4\textsuperscript{-/-}} mice (Figure 3). Albuminuria was absent in both strains (not shown).

The abrogated lupus nephritis phenotype in B6\textsuperscript{lpr/Irf4\textsuperscript{-/-}} mice was associated with lower mRNA expression levels of the proinflammatory chemokines CCL2, CCL5, CXCL2, and CXCL10 (Figure 4A), which correlated with a significant reduction of Mac2-positive glomerular macrophages (Figure 4B). Together, lack of IRF4 protects B6\textsuperscript{pr} mice from lupus nephritis.

**Lack of IRF4 Prevents Autoimmune Lung Disease in B6\textsuperscript{pr} Mice**

Autoimmune lung disease is another manifestation of SLE. At 6 months of age, B6\textsuperscript{pr} mice displayed focal areas of peribronchial and perivascular lymphocyte infiltrates (Figure 5). Such infiltrates were not detected in age-matched B6\textsuperscript{lpr/Irf4\textsuperscript{-/-}} mice, indicating that lack of IRF4 protects B6\textsuperscript{pr} mice not only from lupus nephritis but also from autoimmune lung disease.

**IRF4 Is Required for the Production of Lupus Autoantibodies in B6\textsuperscript{pr} Mice**

Lupus nephritis is a manifestation of immune complex disease in systemic autoimmunity. Glomerular immune complex deposits can develop in situ when circulating autoantibodies bind to nuclear particles that have deposited along the glomerular capillaries. Alternatively, circulating immune complexes get deposited along the glomerular filtration barrier. In any case, lupus autoantibodies represent an essential element in the pathogenesis of lupus nephritis; therefore, we next examined the effect of the Irf4 genotype on lupus autoantibody formation. At the age of 6 months, B6\textsuperscript{pr} mice displayed significant hypergammaglobulinemia and antinuclear antibodies (ANAs) as well as autoantibodies specifically directed against double-stranded DNA (dsDNA) or the Smith antigen (Figure 6). By contrast, IRF4-deficient B6\textsuperscript{pr} mice had low serum IgG levels, and ANAs, dsDNA, and Smith autoantibodies were absent (Figure 6). Thus, IRF4 is required for the production of lupus autoantibodies in B6\textsuperscript{pr} mice.

**Lack of IRF4 Reduces Plasma Cells in B6\textsuperscript{pr} Mice**

Serum IgG and pathogenic autoantibodies are derived from Ig-producing plasma cells. Given the low levels of circulating IgG and the absence of lupus autoantibodies in B6\textsuperscript{lpr/Irf4\textsuperscript{-/-}} mice, we performed spleen cell flow cytometry for CD138 and...
light chains to quantify plasma cells in 6-month-old B6
\(lpr\) and B6\(lpr/Irf4\) mice. Lack of IRF4 was associated with a drastic
reduction of the absolute numbers of spleen plasma cells on
flow cytometry (Figure 7A). By contrast, the total numbers of
mature B cells were not affected by the Irf4 genotype, although
a shift from follicular- to marginal-zone B cells was observed in
B6\(lpr/Irf4\) versus B6\(lpr\) mice (Figure 7A). The comparable
numbers of mature B cells on flow cytometry were consistent
with identical staining patterns for IgM-positive cells in mice
of both genotypes (Figure 7B). We therefore conclude that lack of IRF4 abrogates
lupus nephritis because B6\(lpr/Irf4\) mice are plasma cell deficient and no longer pro-
duce those autoantibodies that cause immune complex glomerulonephritis.

Lack of IRF4 Reduces Th1 and Th17
Effector T Cells in B6\(lpr\) Mice
The various T cell subsets are essential reg-
ulators of autoimmune disease. Regulatory
T cells suppress the expansion of autoreac-
tive T cells and autoantigen-specific Th1 or
Th17 effector T cells that promote inflam-
matory tissue injury.\(^{29,30}\) We therefore
quantified T cell subsets in the spleens of
6-month-old B6\(lpr\) and B6\(lpr/Irf4\) mice by
flow cytometry. The total number of CD3-
positive T cells was not affected by the
Irf4 genotype, but CD8 T cells were slightly re-
duced and CD4/CD25/Foxp3-positive “reg-
ulatory” T cells were increased in B6\(lpr/Irf4\)
versus B6\(lpr\) mice (Figure 8A). We identified
Th1 and Th17 CD4 T cells by intracellular
staining for IFN\(\gamma\) or IL-17, respectively,
and found that lack of IRF4 substantially
reduced both of these subsets in B6\(lpr\) mice
(Figure 8B). By contrast, IL-17 positivity in
CD4/CD8 double-negative T cells was in-
dependent of the Irf4 genotype (not
shown). Consistent with the latter finding,
IL-17 and IL-21 were substantially reduced
in the serum of 6-month-old B6\(lpr/Irf4\) mice
(Figure 8C). Together, IRF4 defi-
ciency impairs the maturation of Th1 and
Th17 T cells as well as that of autoantibody-
producing plasma cells, which is associated
with an abrogation of lupus nephritis in
B6\(lpr\) mice.

DISCUSSION
IRFs contribute to SLE and lupus nephritis
in various ways. IRF1 acts as a nonredun-
dant transcription factor that promotes the
expression of many proinflammatory genes in immune and
nonimmune cells.\(^{8,9}\) IRF3 and IRF7 mediate the expression of
type I IFNs upon activation of innate viral nucleic acid sensors
that, in lupus, can also be activated by endogenous nucleic
acids and lupus autoantigens.\(^{7,12}\) IRF5 is required for immune
cell maturation and for TLR signaling, two mechanisms that
contribute to human SLE and lupus nephritis of Fc\(\gamma RII\) mice.\(^{14}\) Here we show that IRF4 contributes to
SLE and lupus nephritis in a different manner. On one side,

\[\text{Figure 6. IRF4 deficient B6}^{lpr}\text{ mice lack hypergammaglobulinemia and autoantibody production. (A) B6}^{lpr/Irf4}\text{ and B6}^{lpr}\text{ mice were bled at the end of the study to determine serum levels of }\text{IgG, IgM, anti-Smith, and anti-dsDNA autoantibodies by ELISA. (B) ANAs were detected by staining of Hep2 cells using plasma dilutions of 1:40 as described in Concise Methods. Note the homogenous nuclear staining pattern using plasma from B6}^{lpr}\text{ that was not detectable with plasma from B6}^{lpr/Irf4}\text{ mice.}\]
IRF4 suppresses innate immune recognition and therefore IRF4 deficiency enhances the activation of antigen-presenting cells including the production of NFκB-dependent proinflammatory cytokines. Although this process should enhance autoimmunity and autoimmune tissue inflammation, IRF4-deficient B6^{lpr} mice still remain protected from glomerulonephritis because IRF4 has a nonredundant role in the maturation of plasma cells and Th1 and Th17 effector T cells, which protects B6^{lpr} mice from the evolution of autoimmunity and immune complex disease.

We have recently shown that IRF4 potently suppresses the activation of DCs in postischemic kidneys, a process that limits local cytokine production and thereby prevents excessive renal pathology and acute renal failure. This immunosuppressive effect of IRF4 is related to its inducible expression in DCs, which blocks the interaction of IRF5 with the TLR adaptor MyD88 and thereby suppresses the local expression of NFκB-dependent proinflammatory cytokines during bacterial or sterile forms of inflammation. Consistent with this concept, spleen DCs and macrophages in Irf4-deficient B6^{lpr} mice were more activated and produced more proinflammatory cytokines as compared with their wild-type B6^{lpr} counterparts. IRF4-deficient splenocytes also showed increased IFN-γ production by natural killer (NK)1.1 cells, whereas IRF4 deficiency did not affect IFN-γ producing T cells. This process should be sufficient to enhance autoimmunity and autoimmune tissue injury because enhanced antigen-presentation and co-stimulation is usually sufficient to increase the expansion of autoreactive B and T cells in lupus. For example, lack of the TLR signaling inhibitor single immunoglobulin IL-1R-related molecule was similarly associated with activated DCs and increased production of lupus autoantibodies and subsequent immune complex glomerulonephritis in B6^{lpr} mice. In addition, we recently observed the same for IL-1 receptor-associated kinase M, a factor that suppresses TLR signaling in DCs at the kinase level (unpublished observation). However, increased activation of antigen-presenting cells and massively increased serum cytokine levels were not associated with more autoimmunity or more severe lupus nephritis in B6^{lpr/Irf4/-} mice, most likely because of the lack of autoimmunity and immune complex disease.

The lack of lupus-like autoimmunity as indicated by the absence of ANAs and other lupus autoantibodies was the most prominent phenotype of B6^{lpr/Irf4/-} mice. Our analysis identified an almost complete absence of plasma cells, which was also illustrated by hypogammaglobulinemia. IRF4 has a nonredundant role in plasma cell maturation and Ig class switch recombination while germinal center B cell formation remains intact. As such, Irf4-deficient mice are generally unable to induce humoral immune responses to antigen exposure, which obviously includes the production of ANAs in experimental lupus. These data document that innate immune activation is necessary but not sufficient to cause lupus nephritis because autoantibody-mediated immune complex disease is an essential com-

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**Figure 7.** IRF4 is required for plasma cells' maturation. Flow cytometry was used to determine the total number of distinct B and plasma cell subsets (A) in spleens of 6-month-old B6^{lpr} mice (black bars) and B6^{lpr/Irf4/-} mice (white bars). The histogram presents mean ± SEM of 12 to 14 mice in each group. *P < 0.05, **P < 0.001 versus B6^{lpr} mice. (B) IgM immunostaining of spleens identifies mature B cell distribution in mice of both genotypes. Original magnification, ×100.
ponent for the development of lupus nephritis. B6<sup>lpr/Irf4</sup>−/− mice also lacked IFNγ-producing Th1 T cells and IL-17-producing Th17 T cells, both of which have been shown to contribute to glomerulonephritis and lupus nephritis in <i>lpr</i> mutant lupus mice. IRF4 is required for the maturation of T cells, especially for T cell priming toward Th17 cells, because IRF4 phosphorylation by ROCK2 is required for the synthesis of IL-17 and of IL-21. As such, B6<sup>lpr/Irf4</sup>−/− mice lack the major effector T cell populations that are involved in autoimmune tissue injuries including lupus nephritis beyond the production of lupus autoantibodies by plasma cells.

In summary, IRF4 has nonredundant biologic effects for evolution of immune complex glomerulonephritis like the other members of the IRF family, namely IRF1, IRF3, IRF5, and IRF7. However, the mechanisms by which IRF4 contributes to autoimmune tissue injury differ from those of the other IRF family members. Although IRF4 deficiency activates antigen-presenting cells and induces systemic inflammation, lack of IRF4 also severely impairs plasma cell maturation and subsequent autoantibody production as well as the maturation of Th1 and Th17 effector T cells. As a consequence, IRF4 deficiency protects from the evolution of autoimmunity and lupus-like immune complex glomerulonephritis. It is therefore very likely that these immunoregulatory roles of IRF4 contribute to other lupus disease models in a similar manner. Together, we conclude that IRF4 is essential for the development of lupus-like autoimmunity and immune complex glomerulonephritis despite its suppressive effect on innate immunity.

**CONCISE METHODS**

**Animal Studies**

<i>Irf4</i>-deficient mice were generated, genotyped, and backcrossed to the C57BL/6J strain for ten generations as described previously. B6<sup>lpr/Irf4</sup>−/− and B6<sup>lpr</sup> mice (Charles River) were mated to generate B6<sup>lpr/Irf4</sup>−/+ mice, which were then mated among each other to generate B6<sup>lpr/Irf4</sup>−/+ and B6<sup>lpr/Irf4</sup>−/− mice as described. Littermate females were used for all experimental procedures. In each individual mouse, the genotype was assured by PCR. Mice were housed in groups of five mice in sterile filter-top cages with a 12-hour dark/light cycle and unlimited access to autoclaved food and water. One cohort of mice was sacrificed at 24 weeks of age and one cohort was followed until 52 weeks of age. All experimental procedures were performed according to the German animal care and ethics legislation and were approved by the local governmental authorities.

![Figure 8](https://example.com/image.png)
Flow Cytometry
Anti-mouse CD3, CD4, CD8, and CD25 antibodies (BD Pharmingen, Heidelberg, Germany) were used to detect CD3+CD4-CD8- double-negative T cells and CD4+CD25+ regulatory T cell populations in spleens. Anti-CD11c was used to identify DCs, and the activation of CD11c-positive cells was assessed by co-staining for CD40 and MHCII (BD Pharmingen, Heidelberg, Germany). Anti-mouse B220, CD21, CD23, IgD, and IgM antibodies (BD Pharmingen) were used to detect mature B cells (B220+ IgD+ IgM-), marginal-zone B cells (B220+ CD21high CD23low), and follicular B cells (B220+ CD21low CD23high). Spleen B1 cells were identified as CD19+ IgM+B220+ CD5- CD43-. Anti-mouse CD19, CD5, and CD43 were used as the secondary antibody. Anti-nuclear antibodies were detected using anti-mouse IgG (Rockland, Gilbertsville, PA) was used for detection. Horseradish-peroxidase-conjugated goat anti-mouse IgG was used as the secondary antibody. Anti-nuclear antibodies were detected on Hep2 slides (1:50 diluted serum; BioRad Laboratories, Redmond, WA). Sections were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA).

Real-Time Quantitative (TaqMan) Reverse-Transcriptase PCR
Real-time reverse-transcriptase PCR was performed on mRNA from mouse organs as described previously. The SYBR Green Dye detection system was used for quantitative real-time PCR on a Light Cycler 480 (Roche, Mannheim, Germany). All of the technical steps were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines. Controls consisting of double-deionized water were negative for target and housekeeper genes. Gene-specific primers (300 nM, Metabion, Martinsried, Germany) were designed and further analyzed in silico to target all known possible transcripts of interest. Primers are listed in Table 1.

Statistical Analysis
One-way ANOVA followed by post hoc Bonferroni’s test was used for multiple comparisons using GraphPad Prism, version 4.03. Single groups were compared by unpaired two-tailed t test. Data were expressed as mean ± SEM. Statistical significance was assumed at P < 0.05.

Table 1. Primers used for real-time RT-PCR

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Autoantibody Analysis
Serum antibody levels were determined by ELISA as described. For anti-dsDNA antibody, NUNC maxisorp ELISA plates were coated with poly-l-lysine (Trevigen, Gaithersburg, MD) and mouse dsDNA. After incubation with mouse serum, dsDNA-specific IgG and serum IgG and serum IgM levels were detected by ELISA (Bethyl Laboratories, Montgomery, TX). For anti-Smith antibodies, NUNC maxisorp ELISA plates were coated with Smith antigen (Immunovision, Springdale, AR). A horseradish-peroxidase-conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA) was used for detection. Horseradish-peroxidase-conjugated anti-mouse IgG was used as the secondary antibody. Anti-nuclear antibodies were detected on Hep2 slides (1:50 diluted serum; BioRad Laboratories, Redmond, WA). Sections were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA).

Evaluation of Autoimmune Tissue Injury
Lungs, spleens, livers, lymph nodes, and kidneys from all mice were fixed in 10% buffered formalin, processed, and embedded in paraffin. Sections (2 μm) for periodic acid–Schiff stains were prepared following routine guidelines. The severity of the renal lesions was graded using the activity and chronicity indices for human lupus nephritis as described. Autoimmune lung injury was scored semiquantitatively (0 to 4) by assessing the extent of peribronchial, perivascular, or interstitial lymphocyte infiltrates as described. For IgM staining of the spleen sections, anti-mouse IgM-μ-chain specific antibodies (Vector, Burlingame, CA) were used.

Complement component C9 antibody (kindly provided from Mohamed R. Daha, University of Leiden, The Netherlands) was used at a 1:50 dilution, and secondary goat anti-rabbit biotinylated antibody (Vector, Burlingame, CA) was used at a 1:300 dilution. Serum cytokine levels were determined by ELISA following the manufacturer’s protocols (IL-12p40: OptEia, BD, Heidelberg, Germany; TINF: BioLegend, San Diego, CA; IL-4: OptEia, BD, Heidelberg, Germany). Plasma creatinine levels were determined using a commercial assay kit (DiaSyst).
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DISCLOSURES

None.

REFERENCES

30. Ohnmacht C, Pullner A, King SB, Drexler I, Meier S, Brocker T, Voehringer D: Constitutive ablation of dendritic cells breaks self-tolerance