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IFN Regulatory Factor 5 Is Required for Disease Development in the *FcγRIIB*$^{-/-}$*Yaa* and *FcγRIIB*$^{-/-}$ Mouse Models of Systemic Lupus Erythematosus

Christophe Richez,*[,1,2] Kei Yasuda,*[,1] Ramon G. Bonegio,* Amanda A. Watkins,* Tamar Aprahamian,* Patricia Busto,[†] Rocco J. Richards,* Chih Long Liu,[‡] Regina Cheung,[‡] Paul J. Utz,[‡] Ann Marshak-Rothstein,[†] and Ian R. Rifkin*

Polymorphisms in the transcription factor IFN regulatory factor 5 (IRF5) are strongly associated in human genetic studies with an increased risk of developing the autoimmune disease systemic lupus erythematosus. However, the biological role of IRF5 in lupus pathogenesis has not previously been tested in an animal model. In this study, we show that IRF5 is absolutely required for disease development in the *FcγRIIB*$^{-/-}$*Yaa* and *FcγRIIB*$^{-/-}$ lupus models. In contrast to IRF5-sufficient *FcγRIIB*$^{-/-}$*Yaa* mice, IRF5-deficient *FcγRIIB*$^{-/-}$*Yaa* mice do not develop lupus manifestations and have a phenotype comparable to wild-type mice. Strikingly, full expression of IRF5 is required for the development of autoimmunity, as IRF5 heterozygotes had dramatically reduced disease. One effect of IRF5 is to induce the production of the type I IFN, IFN-α, a cytokine implicated in lupus pathogenesis. To address the mechanism by which IRF5 promotes disease, we evaluated *FcγRIIB*$^{-/-}$*Yaa* mice lacking the type I IFN receptor subunit 1. Unlike the IRF5-deficient and IRF5-heterozygous *FcγRIIB*$^{-/-}$*Yaa* mice, type I IFN receptor subunit 1-deficient *FcγRIIB*$^{-/-}$*Yaa* mice maintained a substantial level of residual disease. Furthermore, in *FcγRIIB*$^{-/-}$ mice lacking *Yaa*, IRF5-deficiency also markedly reduced disease manifestations, indicating that the beneficial effects of IRF5 deficiency in *FcγRIIB*$^{-/-}$*Yaa* mice are not due only to inhibition of the enhanced TLR7 signaling associated with the *Yaa* mutation. Overall, we demonstrate that IRF5 plays an essential role in lupus pathogenesis in murine models and that this is mediated through pathways beyond that of type I IFN production. ***The Journal of Immunology*****, 2010, 184: 796–806.**

Systemic lupus erythematosus (SLE) is a systemic inflammatory autoimmune disease characterized by the production of autoantibodies and the involvement of various organ systems resulting in appreciable morbidity and mortality. The etiology of SLE is poorly understood, with disease resulting from a complex interaction between environmental and genetic factors (1–3). A large number of distinct chromosomal loci show evidence for linkage with disease or disease-related traits in human genetic studies, although it is not yet clear how each contributes to disease pathogenesis (1, 4, 5). Recently, polymorphisms in the transcription factor IFN regulatory factor 5 (IRF5) have been strongly associated in multiple studies with an increased risk of developing SLE (6–9). These polymorphisms are thought to cause the expression of novel IRF5 isoforms (6, 7) and/or an increased level of IRF5 expression by promoting the stability of the IRF5 mRNA or protein (10–12). Individuals possessing particular combinations of these polymorphisms have a greater risk of developing SLE and have higher serum IFN-α activity than individuals not possessing these combinations (11, 13).

The precise role of IRF5 in lupus pathogenesis, however, still remains incompletely defined. In addition, it is not known to what extent the level of IRF5 expression per se, as opposed to the functional effects of novel IRF5 isoforms, might contribute to disease pathogenesis. One way to address these issues is through the use of animal models where expression levels can be manipulated.

IRF5 is a member of the IRF family that collectively is involved in the regulation of innate immune responses, immune cell development, and oncogenesis (14). It is one of a number of transcription factors that participate in signaling cascades downstream of TLR3, TLR4, TLR5, TLR7, and TLR9 (15–18). Given that dysregulated TLR7 and TLR9 activation is linked to lupus pathogenesis (19), any effects of IRF5 in lupus could potentially be mediated, at least in part, through modulation of TLR-triggered events. IRF5 has also recently been linked to pathways downstream of the retinoic acid inducible gene I family, a family of proteins that recognize cytoplasmic viral RNA (20).

IRF5 is involved in the production of type I IFN (IFN-α and IFN-β) in response to TLR activation and viral infection (17, 18, 20–22). Given the potential role of type I IFN in SLE pathogenesis

*Renal Section, Department of Medicine and [†]Department of Microbiology, Boston University School of Medicine, Boston, MA 02118; and [‡]Division of Immunology and Rheumatology, Stanford University School of Medicine, Stanford, CA 94305

[1]C.R. and K.Y. contributed equally to this work.

[2]Current address: Rheumatology Department, Hôpital Pellegrin and Université de Bordeaux, Place Amélie Raba-Léon, Bordeaux, France.

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Address correspondence and reprint requests to Dr. Ian R. Rifkin, Renal Section, Department of Medicine, Boston University School of Medicine, EBRC 5th Floor, 650 Albany Street, Boston, MA 02118. E-mail address: irifkin@bu.edu

Abbreviations used in this paper: ANA, anti-nuclear autoantibody; BUN, blood urea nitrogen; IFNAR1, type I IFN receptor subunit 1; IRF5, IFN regulatory factor 5; SLE, systemic lupus erythematosus; SAM, significance analysis of microarrays; Sm/RNP, Smith/ribonucleoprotein; WT, wild-type.

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(23–25), it has been suggested that the induction of these IFNs might be the most important function of IRF5 in the context of SLE (6, 13). However, IRF5 is also involved in the production of proinflammatory cytokines such as IL-6 (15, 17, 18) that further contribute to lupus pathogenesis (26). Importantly, the extent of the IRF5 contribution to type I IFN and proinflammatory cytokine production is both cell-type and stimulus specific (15–18, 20, 22). IRF5 is also associated with apoptotic pathways in response to viral infection, DNA damage, and Fas ligand– or TRAIL-induced apoptosis and has also been shown to promote cell-cycle arrest (14, 20, 27, 28). Therefore, the effects of IRF5 on the pathogenesis of SLE could involve type I IFN induction or IFN-independent pathways (29).

To examine the role of IRF5 in the development of SLE and its potential functions beyond regulation of type I IFN expression, we have now compared the impact of deficiency of IRF5 and the type I IFN receptor subunit 1 (IFNAR1) in the C57BL/6 *Fcγ RIIB*$^{-/-}$*Yaa* and *Fcγ RIIB*$^{-/-}$ models of SLE. FcγRIIB deficiency interacts with a number of C57BL/6-specific genes to induce a spontaneous SLE-like disease, characterized by the presence of autoantibodies against chromatin and the development of lethal glomerulonephritis (30, 31). It has been proposed that this epistatic property of the *Fcγ RIIB*$^{-/-}$ B6 model mimics the multigenic nature of human SLE (31). Addition of the *Yaa* locus to the *Fcγ RIIB*$^{-/-}$ B6 model results in a marked increase in severity of the autoimmune disease (31) due to the duplication of TLR7 and other uncharacterized disease-promoting genes (32–35). Therefore, we have investigated both the *Fcγ RIIB*$^{-/-}$ and the *Fcγ RIIB*$^{-/-}$*Yaa* models.

We found that IRF5 deficiency had a much stronger influence on disease manifestations than IFNAR1 deficiency. Importantly, IRF5 heterozygotes were substantially protected from disease development, thereby demonstrating the pivotal effect of IRF5 expression levels in these lupus models.

Materials and Methods

Mice

IRF5-deficient mice backcrossed eight generations to C57BL/6 were obtained from T. Tanaguchi (University of Tokyo, Tokyo, Japan) and T. Mak (University of Toronto, Toronto, Ontario, Canada) (15). *Fcγ RIIB*$^{-/-}$*Yaa* mice on a C57BL/6 background were obtained from S. Bolland (National Institute of Allergy and Infectious Diseases, Bethesda, MD) (32). IFNAR1-deficient mice on a C57BL/6 background were obtained from J. Sprent (Garvan Institute of Medical Research, Sydney, Australia) (36). C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animal experiments were approved by the Institutional Animal Care and Use Committee at Boston University.

Serological assays

IgG isotypes and anti-Smith/ribonucleoprotein (Sm/RNP) autoantibodies were measured by ELISA established using commercially available reagents. Anti-nuclear autoantibody (ANA) titer was measured by immunofluorescence using HEp-2-coated-slides (Antibodies Incorporated, Davis, CA). Anti-dsDNA autoantibodies were measured by immunofluorescence analysis of *Crithidia lucillae* kinetoplast staining (The Binding Site, San Diego, CA). Serum cytokine levels other than IFN-α were measured by Luminex multiplex cytokine analysis at the Baylor Institute for Immunology Research Luminex Core Facility (Dallas, TX). Serum IFN-α was measured by ELISA (PBL). This ELISA has a sensitivity of 12.5 pg/ml, and samples were tested at a 1:4 dilution. Blood urea nitrogen (BUN) levels were measured using a QuantiChrom Urea Assay kit (BioAssay Systems, Hayward, CA).

Autoantigen arrays

Autoantigen arrays were performed and analyzed as described previously, using a panel of recombinant or native proteins (37). Arrays were probed with sera and bound Abs revealed using IgG/IgM-specific secondary Abs conjugated to fluorophores. The signal intensities obtained were hierarchically clustered by sample based on Pearson correlation with average linkage (38). Significance analysis of microarrays (SAM) was performed to identify statistically significant differences between autoantigen reactivities in the experimental groups (39). q Values <0.05 were considered significant. Antigens were ordered by the SAM observed score in descending order. The microarray data has been deposited in the GEO database, accession number GSE17926 (www.ncbi.nlm.nih.gov/geo/).

Histology

H&E-stained kidney sections were evaluated in a blinded manner. Randomly selected areas of cortex were digitally photographed using an RT color spot camera (Diagnostic Instruments, Sterling Heights, MI), and the images were recorded using Spot Advanced software version 4.0.9 (Diagnostic Instruments). Crescents were identified by their characteristic appearance, and 100 glomeruli from each animal were examined to determine the percentage of glomeruli with crescents. Interstitial disease was semiquantitatively scored on a scale of 0 to 3 (40). Mean glomerular cell count was determined by computer-assisted image analysis (Adobe Photoshop CS3, Adobe, San Jose, CA) of at least 25 equatorially sectioned glomeruli from each mouse.

Immunohistochemistry

Kidneys were snap-frozen in OCT (Tissue-Tek, Sakura Finetek, Torrance, CA) and stored at −80°C. Eight micrometer cryosections were cut and blocked with 1% donkey serum and then stained with Cy3-conjugated donkey anti-mouse IgG (The Jackson Laboratory) followed by FITC-conjugated goat anti-mouse C3 (Cappel Laboratories, Cochranville, PA). Stained sections were coded and then digitally photographed and analyzed in a blinded manner using a fluorescent stereomicroscope (Nikon, Melville, NY) fitted with an RT color spot camera (Diagnostic Instruments). Fluorescence intensity, representing IgG and C3 deposition, was measured as the mean luminosity in 7–10 glomeruli per mouse (Adobe Photoshop CS3, Adobe). To obtain the representative glomerular images shown in Fig. 5*E*, stained sections were digitally photographed using the Nikon TE-2000 inverted epifluorescence microscope fitted with a CoolSnap HQ camera (Photometrics, Tucson, AZ). Z-stack images were deconvolved using NIS Elements (Nikon) with Media Cybernetics deconvolution plugin.

Flow cytometry

Splenocytes were labeled with mAbs (BD Biosciences, San Jose, CA) specific for CD4, CD8, and pan-Vβ to identify T cells, CD19 and B220 to identify B cells, and CD69 and CD44 to identify activation markers. Immunofluorescence was measured with a FACScan flow cytometer (BD Biosciences) and the data analyzed with FlowJo software (Tree Star, Ashland, OR). For T cell activation marker expression, where two distinct cell populations were observed, the data are expressed as percent of cells positive, with the threshold for positivity set at the trough between the two separate populations. For B cell activation marker expression where only a single population was observed, the data are expressed as mean fluorescence intensity.

Western blot analysis

B220^{+} cells were purified from the spleens of 19-wk-old *Irf5*$^{+/+}$, *Irf5*$^{+/-}$, and *Irf5*$^{-/-}$ *Fcγ RIIB*$^{-/-}$ female mice and 13-wk-old C57BL/6 wild-type (WT) female mice using anti-mouse CD45R/B220 magnetic particles (BD Biosciences). Cells were lysed in RIPA buffer (Boston BioProducts) containing protease inhibitors (Calbiochem, EMD Chemicals, Gibbstown, NJ). Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). A 4× sample buffer (Boston BioProducts, Ashland, MA) was added to the samples, which were then denatured at 95°C for 5 min. Samples were separated by 10% SDS-PAGE, electroblotted onto a PVDF membrane (Millipore, Bedford, MA), and IRF5 and β-actin detected using rabbit anti-mouse IRF5 and rabbit anti-β-actin Abs (both from Cell Signaling Technology, Danvers, MA). IRF5 and β actin levels were quantitated using Image J (National Institutes of Health, Bethesda, MD).

Quantification of IRF5, IFIT1, and MX2 gene expression

For IRF5 gene expression, B220^{+} cells were purified from the spleens of 19-wk-old *Irf5*$^{+/+}$, *Irf5*$^{+/-}$, and *Irf5*$^{-/-}$ *Fcγ RIIB*$^{-/-}$ female mice and 13-wk-old C57BL/6 WT female mice using anti-mouse CD45R/B220 magnetic particles (BD Biosciences) and total RNA obtained using an RNeasy Micro kit (Qiagen, Valencia, CA). For IFIT1 and MX2 gene expression, kidneys were isolated from 4- to 5-mo-old *Irf5*$^{+/+}$ and *Irf5*$^{-/-}$ *Fcγ RIIB*$^{-/-}$ *Yaa* mice and 4-mo-old C57BL/6 WT mice, homogenized using a Brinkmann Polytron Homogenizer (Brinkmann Instruments, Riverview, FL), and total RNA obtained using an RNeasy Micro kit (Qiagen). A total of 150 ng of RNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and quantitative real-time PCR (Applied Biosystems StepOnePlus Instrument and software,

Applied Biosystems, Foster City, CA) using TaqMan probes and primers (Applied Biosystems) was performed to determine the expression levels of IRF5, IFIT1, and MX2 target genes. The Δ-Δ Ct threshold cycle method was used for analysis. All genes of interest were normalized against the housekeeping gene GAPDH, and changes were expressed as fold change relative to the C57BL/6 WT samples (C57BL/6 B220$^+$ splenocytes for IRF5 and C57BL/6 kidney for IFIT1 and MX2).

Statistical analysis

The Kaplan-Meier method was used to analyze the survival studies, and the log-rank test was used for statistical analysis. Two-tailed Mann-Whitney *U* tests were used for all other analyses. Bonferroni correction for multiple comparisons was performed. p values <0.05 were considered significant.

Results

IRF5 deficiency abrogates disease in the FcγRIIB$^{-/-}$ Yaa lupus model

To test the role of IRF5 in the pathogenesis of SLE, we intercrossed IRF5-deficient mice with *FcγRIIB$^{-/-}$ Yaa* mice to generate the following experimental groups: *FcγRIIB$^{-/-}$ Yaa* IRF5-sufficient male mice (*Irf5$^{+/+}$* RII.*Yaa* mice); *FcγRIIB$^{-/-}$ Yaa* IRF5-heterozygous male mice (*Irf5$^{+/-}$* RII.*Yaa* mice); and *FcγRIIB$^{-/-}$ Yaa* IRF5-deficient male mice (*Irf5$^{-/-}$* RII.*Yaa* mice). At 5 mo of age, we compared disease manifestations in these cohorts, using age- and sex-matched C57BL/6 WT mice as controls. Consistent with the previously observed phenotype of *FcγRIIB$^{-/-}$ Yaa* B6 mice (31), the *Irf5$^{+/+}$* RII.*Yaa* mice developed massive lymphadenopathy and splenomegaly. This was not observed in the *Irf5$^{-/-}$* RII.*Yaa* mice, which had lymph node and spleen weights similar to those of WT mice (Fig. 1*A*). Expression of the activation markers CD69 and CD44 on splenic T cells was markedly reduced in *Irf5$^{-/-}$* RII.*Yaa* mice compared with *Irf5$^{+/+}$* RII.*Yaa* mice, due predominantly to a decreased percentage of T cells expressing these activation markers (Fig. 1*B*). B cell expression of CD69 was also reduced in *Irf5$^{-/-}$* RII.*Yaa* mice, due predominantly to a overall reduction of expression in the total B cell population (Fig. 1*C*). All four IgG isotypes were elevated in *Irf5$^{+/+}$* RII.*Yaa* mice compared with WT mice, consistent with global B cell activation (Fig. 2*A*). However, Ab titers were much lower in the *Irf5$^{-/-}$* cohort compared with *Irf5$^{+/+}$* RII.*Yaa* mice. The reduction was particularly striking for IgG2b and IgG2c, where serum concentrations in the *Irf5$^{-/-}$* mice were similar to those found in WT C57BL/6 mice. Thus, IRF5 expression has the most dramatic effect on those isotypes associated with pathogenic autoantibodies (41).

Because IRF5 has been linked to proinflammatory cytokine and type I IFN production (15, 21), serum cytokine levels were measured. This analysis revealed a decrease in serum levels of IL-6 and IL-10 from *Irf5$^{-/-}$* RII.*Yaa* mice compared with *Irf5$^{+/+}$* RII.*Yaa* mice, whereas there were no differences in serum levels of IL-12 p70 and IFN-γ (Fig. 2*B*). IFN-γ concentrations remained elevated in all *FcγRIIB$^{-/-}$ Yaa* groups compared with controls. Hence, despite a marked overall reduction in immune cell activation, IRF5 deficiency does not abrogate all components of the autoimmune phenotype. The reduction in IL-6 and IL-10 may be relevant for lupus pathogenesis, as both cytokines contribute to B cell activation and autoantibody production and correlate with disease activity in human studies (26). IFN-α was not detected in sera using an ELISA with a level of sensitivity of 50 pg/ml (data not shown).

Measurement of serum IFN-α by ELISA is, however, not sufficiently quantitative, and there is often evidence of induction of type I IFN-induced genes in PBMCs of lupus patients in situations where no serum type I IFN is detected by ELISA (42). We therefore measured mRNA expression of the type I IFN-regulated genes IFIT1 and MX2 (42, 43) in B220$^+$ splenocytes and kidney from additional cohorts of *Irf5$^{+/+}$* RII.*Yaa*, *Irf5$^{-/-}$* RII.*Yaa*, and C57BL/6 WT mice. No increase in IFIT1 or MX2 expression was seen in B220$^+$ splenocytes from *Irf5$^{+/+}$* or *Irf5$^{-/-}$* RII.*Yaa* mice compared with WT mice (data not shown). However, an approximate 3-fold induction of both IFIT1 and MX2 was seen in kidneys from *Irf5$^{+/+}$* RII.*Yaa* mice compared with WT mice, whereas no induction was seen in kidneys from *Irf5$^{-/-}$* RII.*Yaa* mice (Fig. 2*C*). Thus, there is evidence for IRF5-dependent type I IFN expression in *FcγRIIB$^{-/-}$ Yaa* mice, albeit at low levels and only at a site of severe inflammation.

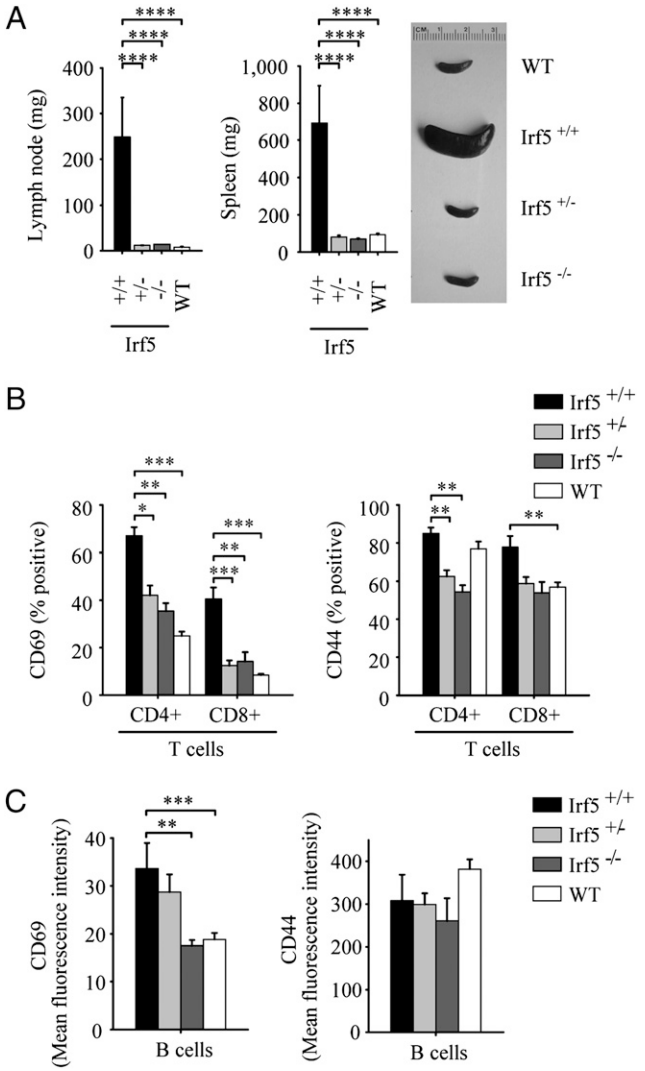

FIGURE 1. Lymphadenopathy, splenomegaly, and lymphocyte activation is reduced in IRF5-deficient RII.*Yaa* mice. *A*, Lymph node and spleen weights from *Irf5$^{+/+}$* (n = 12), *Irf5$^{+/-}$* (n = 12), and *Irf5$^{-/-}$* (n = 14) RII.*Yaa* mice and WT mice (n = 12) were measured at 5 mo of age. Representative spleens are shown in *right panel*. *B* and *C*, CD69 and CD44 expression on splenic T cells (*B*) and B cells (*C*) from 5-mo-old *Irf5$^{+/+}$* (n = 6), *Irf5$^{+/-}$* (n = 10), and *Irf5$^{-/-}$* (n = 9) RII.*Yaa* mice and WT mice (n = 11). Data are presented as mean ± SEM. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$ by Mann-Whitney *U* test.

Autoantibodies directed against nuclear components, in particular DNA/protein or RNA/protein macromolecular complexes, are a diagnostic feature of SLE and contribute to disease pathogenesis (3). As expected, *Irf5$^{+/+}$* RII.*Yaa* mice produced high titers of ANAs as measured by immunofluorescence on HEp2 cells (Fig. 3*A*). However, ANAs were almost totally absent from the sera of *Irf5$^{-/-}$* RII.*Yaa* mice (Fig. 3*A*) as were Abs to ribonucleoprotein (Sm/RNP) (Fig. 3*B*) and dsDNA (Fig. 3*C*).

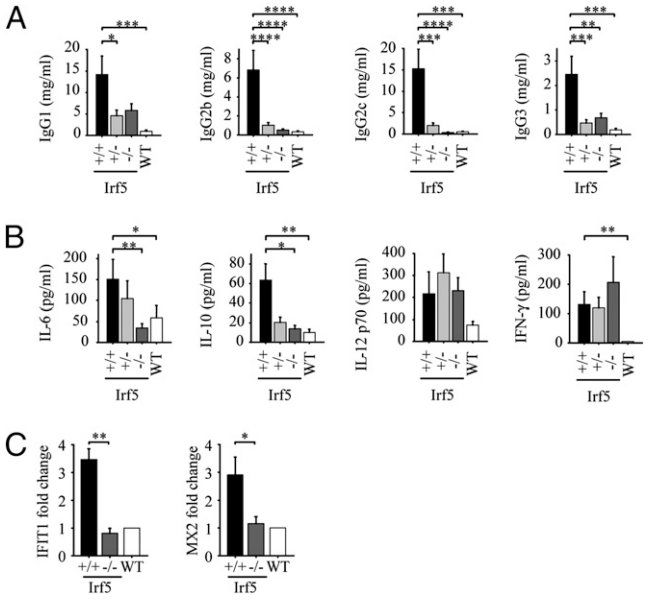

FIGURE 2. Decreased serum IgG and cytokine levels in IRF5-deficient RII.*Yaa* mice. *A* and *B*, $Irf5^{+/+}$ ($n = 12$), $Irf5^{+/-}$ ($n = 12$), and $Irf5^{-/-}$ ($n = 14$) RII.*Yaa* mice and WT mice ($n = 12$) were analyzed at 5 mo of age. *A*, Serum IgG isotype concentrations. *B*, Serum cytokine levels. *C*, IFIT1 and MX2 mRNA expression in kidneys of 4- to 5-mo-old $Irf5^{+/+}$ ($n = 5$) and $Irf5^{-/-}$ ($n = 5$) RII.*Yaa* mice shown as fold change relative to expression in kidneys of 4-mo-old C57BL/6 WT mice. Data are presented as mean ± SEM. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$ by Mann-Whitney *U* test.

To extend our analysis to a more comprehensive panel of autoantigens, we analyzed sera from all cohorts with multiplexed autoantigen microarrays composed of a broad panel of autoantigens found in various autoimmune conditions (37). This demonstrated highly significant differences between sera from the $Irf5^{+/+}$ and $Irf5^{-/-}$ RII.*Yaa* mice (Fig. 3*D*). The analysis confirmed the marked reduction in anti-Sm/RNP and anti-dsDNA autoantibodies in the $Irf5^{-/-}$ RII.*Yaa* mice shown by ELISA and Crithidia immunofluorescence respectively (Fig. 3*B*, 3*C*). The analysis further demonstrated a reduction in autoantibodies directed against a variety of other autoantigen targets such as centromere proteins A and B, histones, liver cytosol type 1 Ag, collagen, thyroperoxidase, β-2 glycoprotein I, Mi-2 Ag, and Pm/Scl 100 (Fig. 3*D*). Despite the previous association of TLR9 and TLR7 with the generation of autoantibodies reactive with DNA- and RNA-associated autoantigens, respectively (44), many of these autoantibodies identified by microarray do not bind DNA or RNA macromolecules and are not known to be regulated by TLR7 or TLR9. This indicates either that TLR7 and TLR9 control the production of a greater range of autoantibodies than is currently appreciated or that the IRF5 regulation of autoantibody production is not simply through its involvement in TLR7 and TLR9 signaling pathways.

Renal disease in human lupus as well as in animal models of the disease is characterized by immune complex deposition and complement activation, with a proliferative glomerulonephritis leading to an increase in glomerular cell number (45). Glomerular crescents and interstitial disease are indicators of more severe renal injury (45). All these features were strongly evident in the $Irf5^{+/+}$ RII.*Yaa* mice as expected (31, 34). In contrast, $Irf5^{-/-}$ RII.*Yaa* mice exhibited a renal phenotype indistinguishable from that of WT mice, apart from small amounts of glomerular IgG and complement C3 deposition (Fig. 4*A*–*C*). To evaluate whether these abnormalities in renal pathology were sufficiently severe to cause renal failure, we measured serum levels of BUN. Normal serum BUN in C57BL/6 mice is <30 mg/dl, with elevated levels indicating a decrease in renal function (46, 47). $Irf5^{+/+}$ RII.*Yaa* mice had high BUN levels (Fig. 4*D*), similar to those seen in another severe mouse model of lupus, MRL-*lpr* (48). In contrast, $Irf5^{-/-}$ RII.*Yaa* mice had BUN levels similar to WT C57BL/6 mice (Fig. 4*D*).

To determine whether the decrease in disease severity would translate into differences in survival, we bred new cohorts of $Irf5^{+/+}$ and $Irf5^{-/-}$ RII.*Yaa* mice and monitored them until the time of death or until they met predetermined criteria for euthanasia. $Irf5^{+/+}$ RII.*Yaa* mice had a median survival of 27 wk, consistent with previous reports (31) (Fig. 4*E*). In contrast, >90% of mice in the $Irf5^{-/-}$ cohort were alive at the conclusion of the experiment at 40 wk of age.

IRF5 heterozygote FcγRIIB$^{-/-}$ Yaa mice also develop minimal disease manifestations

Human IRF5 polymorphisms are predicted to modulate expression levels, and therefore $Irf5^{+/-}$ mice were included in our study to evaluate the effect of gene dosage. Remarkably, the $Irf5^{+/-}$ RII.*Yaa* mice exhibited only minimal evidence of disease as documented by the absence of splenomegaly, lymphadenopathy, and lymphocyte activation (Fig. 1*A*, 1*B*), IgG titers comparable to $Irf5^{-/-}$ RII.*Yaa* mice (Fig. 2*A*), and greatly reduced autoantibody production (Fig. 3*A*–*D*). The $Irf5^{+/-}$ RII.*Yaa* mice developed limited renal disease as detected by increased glomerular cell number, but there was no detectable increase in glomerular crescents, or interstitial disease (Fig. 4*A*, 4*B*). Moreover, the extent of complement deposition in the $Irf5^{+/-}$ RII.*Yaa* mice was not significantly greater than that observed in the $Irf5^{-/-}$ mice (Fig. 4*C*), and $Irf5^{+/-}$ RII.*Yaa* mice had normal serum BUN levels (Fig. 4*D*). Notably, the survival rate of the $Irf5^{+/-}$ mice was comparable to that of the $Irf5^{-/-}$ mice at 40 wk (Fig. 4*E*). Thus, IRF5 heterozygosity was sufficient to prevent the development of any major clinical phenotype.

IRF5 deficiency also abrogates disease in FcγRIIB$^{-/-}$ mice lacking Yaa

Mice bearing the *Yaa* mutation have duplication of ~17 X-chromosome–specific genes, a number of which may contribute to autoimmunity on the appropriate genetic background (32–35). As IRF5 is known to be involved in signaling cascades downstream of at least one of these genes, TLR7 (16, 17), it was important to determine whether the observed beneficial effects of IRF5 deficiency were mediated predominantly through downregulation of the enhanced function of genes associated with the *Yaa* mutation. Therefore, we evaluated the effect of IRF5 deficiency in female $Fc\gamma RIIB^{-/-}$ (RII) mice that lack *Yaa* but nevertheless develop severe autoimmune disease, albeit at an older age than $Fc\gamma RIIB^{-/-}$ *Yaa* mice (30).

At 8 mo of age, $Irf5^{+/+}$ RII mice exhibited lymphadenopathy and splenomegaly (Fig. 5*A*), whereas $Irf5^{+/-}$ and $Irf5^{-/-}$ RII mice had lymph node and spleen weights (Fig. 5*A*) comparable to those of B6 WT mice (Fig. 1*A*). Effects on IgG isotype were similar to those observed in the $Fc\gamma RIIB^{-/-}$ *Yaa* (RII.*Yaa*) model (Fig. 2*A*), with $Irf5^{+/-}$ and $Irf5^{-/-}$ RII mice having marked reductions in serum levels of IgG2b, IgG2c, and IgG3 as compared with $Irf5^{+/+}$ RII mice (Fig. 5*B*). Serum IgG1 levels were only modestly reduced in $Irf5^{-/-}$ RII mice, and no difference in IgG1 levels was seen between the $Irf5^{+/-}$ and $Irf5^{+/+}$ RII mice, indicating that the effects of IRF5 on IgG production are not due simply to a global inhibition of B cell activation. Strikingly, ANA production was almost completely abolished in $Irf5^{-/-}$ RII mice and markedly reduced or absent in the $Irf5^{+/-}$ RII mice (Fig. 5*C*). The >100-fold reduction in ANA titer (Fig. 5*C*) as compared with the 2–7-fold

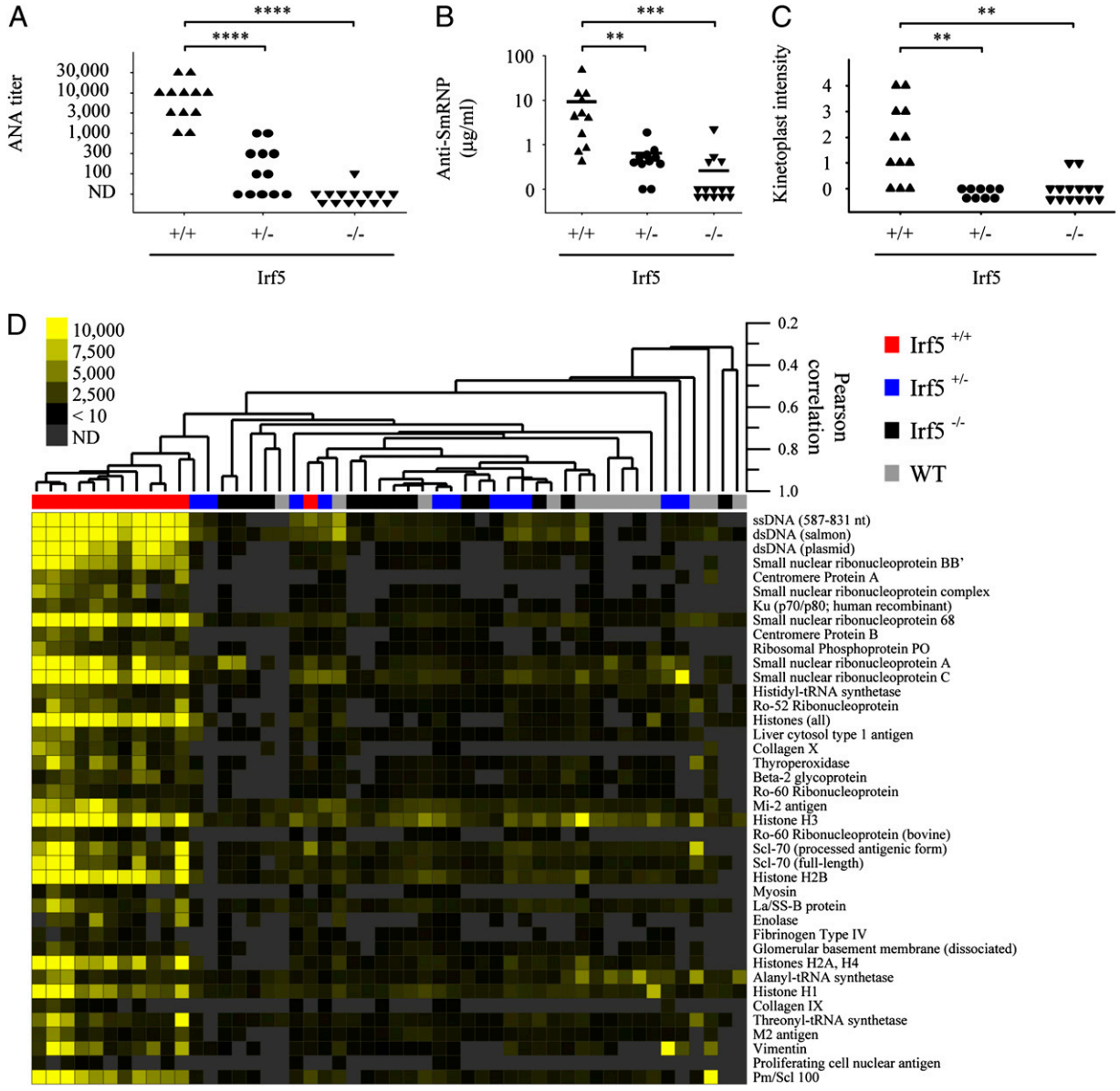

FIGURE 3. Decreased autoantibody production in IRF5-deficient RII.*Yaa* mice. Sera from $Irf5^{+/+}$ (n = 11–12), $Irf5^{+/-}$ (n = 9–12), and $Irf5^{-/-}$ (n = 14) RII.*Yaa* mice were analyzed at 5 mo of age. *A*, ANA titers. ND, not detected. *B*, Antiribonucleoprotein (Sm/RNP) autoantibody levels. *C*, Anti-dsDNA autoantibodies determined by kinetoplast staining intensity. Bars represent median values (*B*). *D*, Autoantigen array analysis was performed on sera from 5-mo-old $Irf5^{+/+}$, $Irf5^{+/-}$, and $Irf5^{-/-}$ RII.*Yaa* mice and from WT mice. Samples are arranged by hierarchical clustering and displayed as a heat map. SAM identified significant differences between $Irf5^{+/+}$ lupus mice and the other experimental groups ($q < 0.0001$; false discovery rate = 0 for all 40 Ags shown). Ags are ordered by the SAM observed score in descending order. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ by Mann-Whitney U test.

reduction in IgG titer (Fig. 5*B*) in $Irf5^{-/-}$ RII mice suggests that the effect on autoantibody production is at least partly specific and is not purely due to effects on IgG levels. Development of renal disease was also substantially IRF5-dependent, with marked reductions in glomerular hypercellularity, crescent formation, interstitial disease, and glomerular IgG and complement deposition observed in the $Irf5^{+/-}$ and $Irf5^{-/-}$ RII mice, although the extent of reduction in renal disease was less complete in the IRF5 heterozygotes (Fig. 5*D–F*). Overall, these results demonstrate that IRF5 deficiency markedly abrogates disease in $Fc\gamma RIIB^{-/-}$ mice lacking *Yaa*. This indicates that the beneficial effects of IRF5 deficiency in the $Fc\gamma RIIB^{-/-}$ *Yaa* model are not mediated solely through effects on the enhanced TLR7 signaling resulting from the *Yaa* mutation.

Given the surprising finding that the IRF5 heterozygous RII and RII.*Yaa* mice were largely protected from disease development, it was important to measure IRF5 expression levels. We measured IRF5 mRNA and protein in $B220^+$ splenocytes from $Irf5^{+/+}$, $Irf5^{+/-}$ and $Irf5^{-/-}$ RII mice and WT C57BL/6 mice. This demonstrated that IRF5 expression in $Irf5^{+/-}$ RII mice is ~40% of that in $Irf5^{+/+}$ RII mice, with no expression being seen in $Irf5^{-/-}$ RII mice (Fig. 5*G*). IRF5 protein expression in $Irf5^{+/+}$ RII mice is similar to that in WT C57BL/6 mice. Thus, normal levels of IRF5 are sufficient to promote disease in RII mice, whereas a 60% reduction in IRF5 expression is protective.

IFNAR1 deficiency does not affect autoantibody levels but partially reduces end-organ disease in the $Fc\gamma RIIB^{-/-}$ Yaa lupus model

All type I IFNs act through a single cell surface type I IFN receptor (49–51). To determine the extent to which the protective effect of IRF5 deficiency was linked to its effects on type I IFN expression, we examined the disease phenotype of $Fc\gamma RIIB^{-/-}$ *Yaa* mice that

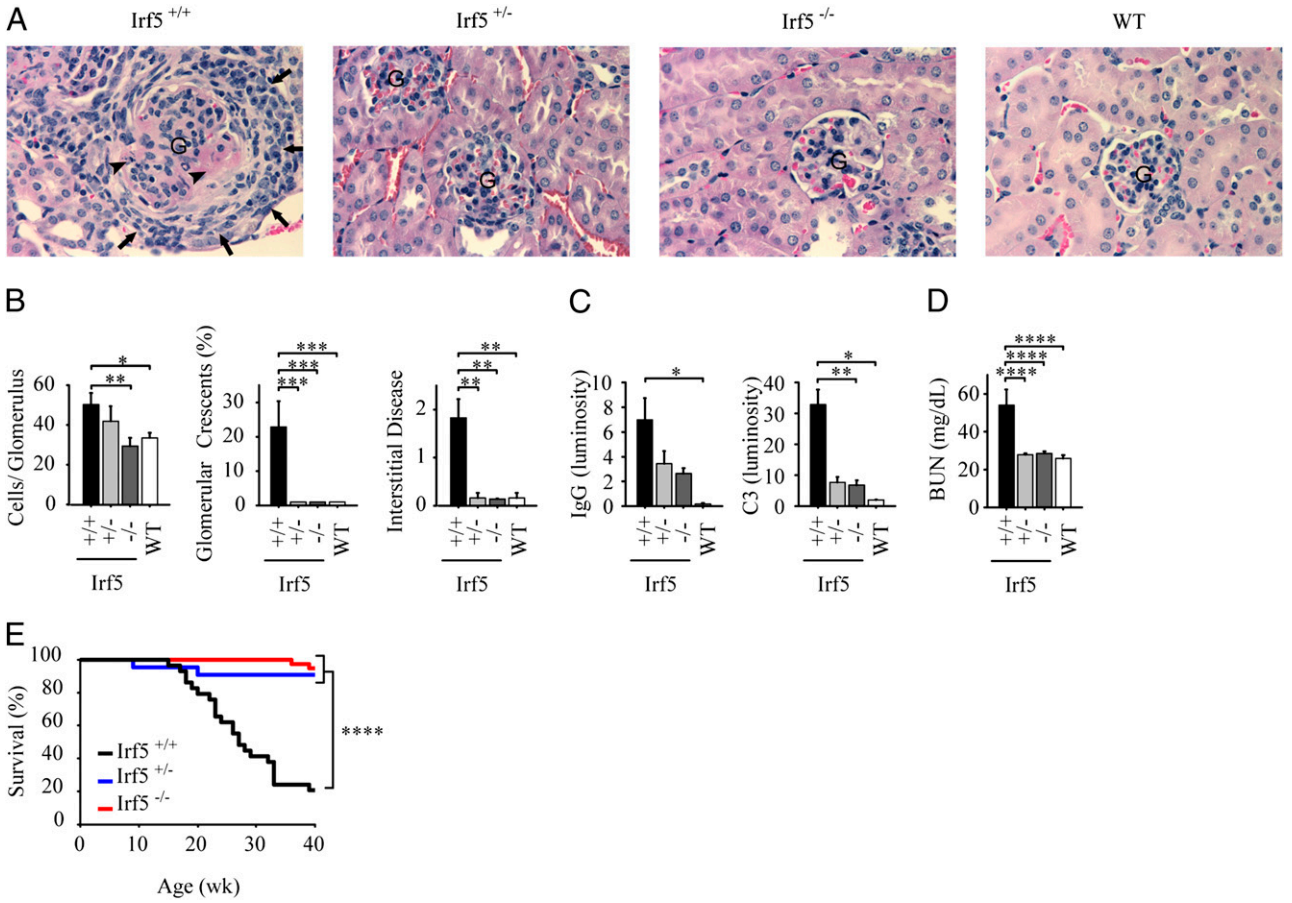

FIGURE 4. Decreased renal disease and enhanced survival in IRF5-deficient RII.*Yaa* mice. *A* and *B*, $Irf5^{+/+}$ ($n = 12$), $Irf5^{+/-}$ ($n = 12$), and $Irf5^{-/-}$ ($n = 14$) RII.*Yaa* mice and WT mice ($n = 12$) were analyzed at 5 mo of age. *A*, Representative renal histology. G indicates glomerulus. Arrows indicate cellular crescent. Arrowheads indicate necrotic areas within glomerulus (H&E, original magnification ×200). *B*, Quantitation of renal disease as shown by cell number per glomerulus, percentage of glomeruli with crescents, and interstitial disease score. *C*, Glomerular IgG and complement C3 deposition measured by fluorescence intensity (luminosity) in $Irf5^{+/+}$ ($n = 6$), $Irf5^{+/-}$ ($n = 3$), and $Irf5^{-/-}$ ($n = 6$) RII.*Yaa* mice and WT mice ($n = 4$). *D*, Serum BUN levels in $Irf5^{+/+}$ ($n = 10$), $Irf5^{+/-}$ ($n = 11$), and $Irf5^{-/-}$ ($n = 14$) RII.*Yaa* mice and WT mice ($n = 10$). Data are presented as mean ± SEM. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$ by Mann-Whitney *U* test. *E*, $Irf5^{+/+}$ (black line, $n = 29$), $Irf5^{+/-}$ (blue line, $n = 22$), and $Irf5^{-/-}$ (red line, $n = 38$) RII.*Yaa* mice were observed until the time of death. $****p < 0.0001$, log-rank test.

lacked the IFNAR1 chain of the IFNAR and were therefore unable to respond to type I IFN (36, 52). A similar approach to assessing the role of type I IFN in lupus pathogenesis has been used by other investigators in a number of different mouse lupus models, with variable effects on disease outcome (25).

In contrast to the $Irf5^{-/-}$ mice, there were no significant differences in serum levels of IgG isotypes between $Ifnar1^{+/+}$ and $Ifnar1^{-/-}$ RII.*Yaa* mice (Fig. 6*A*). There were also no significant differences in serum ANA titer, anti-Sm/RNP Ab levels, or autoantigen microarray profiles (Fig. 6*B–D*). Nevertheless, both lymph node and spleen sizes were smaller in the $Ifnar1^{-/-}$ RII.*Yaa* mice relative to the $Ifnar1^{+/+}$ RII.*Yaa* mice (Fig. 6*E*), although spleen size in the $Ifnar1^{-/-}$ RII.*Yaa* mice (426 ± 50 mg) was larger than in the $Irf5^{-/-}$ RII.*Yaa* mice (70 ± 3 mg; Fig. 1*A*; $p < 0.0001$ for comparison of $Ifnar1^{-/-}$ and $Irf5^{-/-}$). Renal disease was also less severe in the $Ifnar1^{-/-}$ than in the $Ifnar1^{+/+}$ RII.*Yaa* mice as shown by a reduction in glomerular crescent formation ($p = 0.04$) and a trend toward a reduction in cell number per glomerulus ($p = 0.10$) and interstitial disease ($p = 0.07$) (Fig. 6*F*). Nevertheless, substantial residual renal disease remained in the $Ifnar1^{-/-}$ RII.*Yaa* mice, with an increase in all these measures of renal injury compared with $Irf5^{-/-}$ RII.*Yaa* or WT C57BL/6 mice (Fig. 4*B*). Furthermore, the amount of glomerular IgG and complement deposition and the degree of serum BUN elevation was similar in $Ifnar1^{-/-}$ and $Ifnar1^{+/+}$ RII.*Yaa* mice (Fig. 6*G*, 6*H*).

The effects of IFNAR1 deficiency on survival were also determined. $Ifnar1^{-/-}$ RII.*Yaa* mice did survive longer than $Ifnar1^{+/+}$ RII.*Yaa* mice (Fig. 6*I*), confirming that the $Fc\gamma RIIB^{-/-}$ *Yaa* model is at least in part type I IFN-dependent. However, $Ifnar1^{-/-}$ RII.*Yaa* mice (Fig. 6*I*) did not survive as long as either the $Irf5^{-/-}$ or $Irf5^{+/-}$ RII.*Yaa* mice (Fig. 4*E*) ($p < 0.0001$ and $p = 0.00014$, respectively). Thus, overall, in contrast to IRF5 deficiency or heterozygosity, IFNAR1 deficiency did not affect autoantibody production and only partially ameliorated end-organ disease.

Discussion

A large number of genes have been associated with SLE in human genetic studies (1, 4, 5); however, their biological roles in disease pathogenesis are incompletely understood. In this report, we demonstrate that deficiency of a single gene, IRF5, robustly associated with an increased risk of developing human lupus, abrogates disease in the $Fc\gamma RIIB^{-/-}$*Yaa* and $Fc\gamma RIIB^{-/-}$ mouse models of SLE.

The initial reports of the strong association of IRF5 polymorphisms with SLE (6, 7) have now been confirmed in multiple studies in different population groups (9, 11, 12, 53–55). It is not yet clear exactly how these polymorphisms affect IRF5 protein production and function, although the polymorphisms are predicted to result in an increased level of IRF5 expression or activity

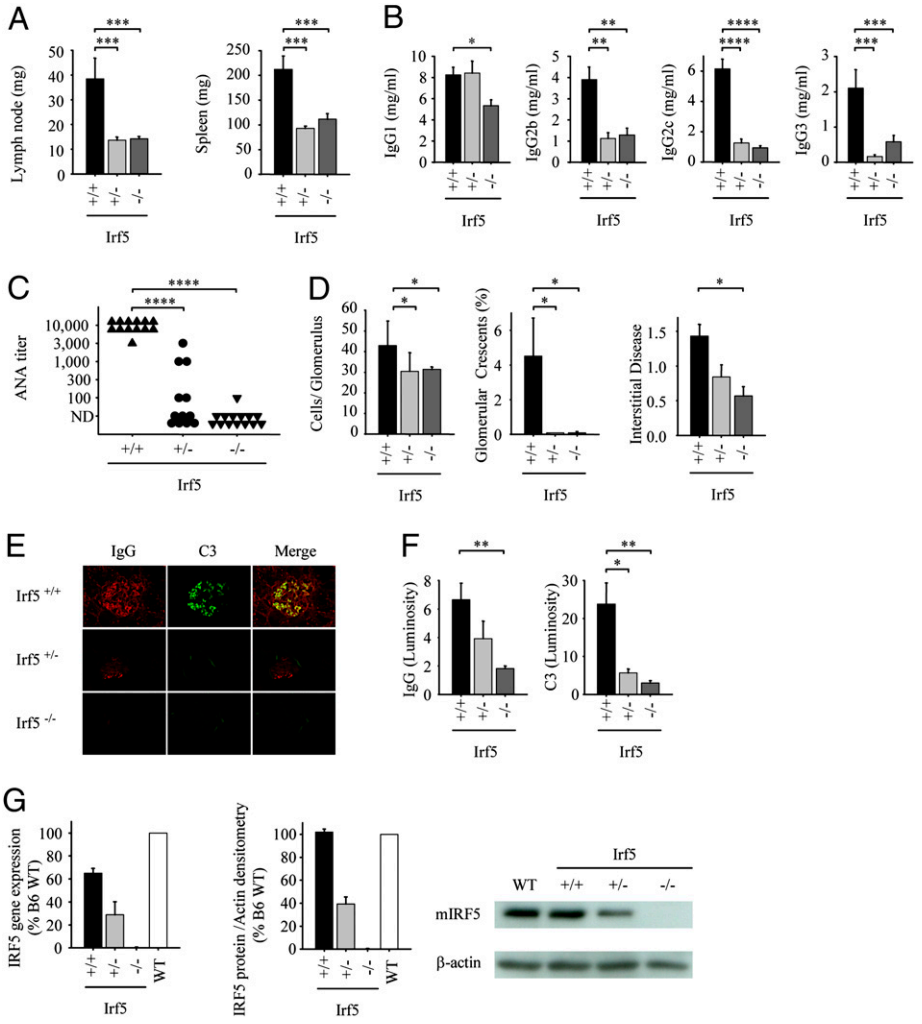

FIGURE 5. IRF5 deficiency reduces disease manifestations in RII mice lacking *Yaa*. *A–F*, All analyses were performed at 8 mo of age. *A*, Lymph node and spleen weights from $Irf5^{+/+}$ (n = 16), $Irf5^{+/-}$ (n = 16), and $Irf5^{-/-}$ (n = 23) RII female mice. Serum IgG isotype concentrations (*B*) and serum ANA titers (*C*) from $Irf5^{+/+}$ (n = 13), $Irf5^{+/-}$ (n = 12), and $Irf5^{-/-}$ (n = 14) RII female mice. *D*, Quantitation of renal disease in $Irf5^{+/+}$ (n = 14), $Irf5^{+/-}$ (n = 19), and $Irf5^{-/-}$ (n = 21) RII female mice as shown by cell number per glomerulus, percentage of glomeruli with crescents, and interstitial disease score. Representative examples (*E*) and quantitation (*F*) of glomerular IgG and complement C3 deposition measured by fluorescence intensity (luminosity) in $Irf5^{+/+}$ (n = 6), $Irf5^{+/-}$ (n = 6), and $Irf5^{-/-}$ (n = 6) RII female mice. *G*, IRF5 mRNA (*left panel*, RT-PCR) and protein (*middle panel*, Western blot) expression in $B220^{+}$ splenocytes from 19-wk-old $Irf5^{+/+}$ (n = 3), $Irf5^{+/-}$ (n = 3), and $Irf5^{-/-}$ (n = 3) RII female mice and 13-wk-old C57BL/6 WT mice (n = 3). A representative Western blot is shown in the *right panel*. Data are presented as mean ± SEM. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$ by Mann-Whitney U test.

(1, 29). In addition to the polymorphisms that confer risk, there appear to be IRF5 variants that confer protection (11, 12). Human IRF5, unlike mouse IRF5, is expressed in multiple spliced variants, and some of these are transcriptionally inactive and may function as dominant negative mutants (18, 56). A genetic model has been proposed of an SLE risk haplotype carrying multiple mutations of IRF5 (1, 11).

We found an unexpectedly strong requirement for IRF5 gene dose in disease pathogenesis. In animal models of SLE, it is common for gene-targeted heterozygotes to express a phenotype similar to the WT controls or to express an intermediate phenotype. For example, $Tlr9^{+/-}$ MRL/*lpr* mice have a survival rate similar to $Tlr9^{+/+}$ mice and do not resemble $Tlr9^{-/-}$ mice (44). Similarly, $MyD88^{+/-}$ mice on both the MRL-*lpr* and $56R^{+}FcγRIIB^{-/-}$ backgrounds have phenotypes comparable to $MyD88^{+/+}$ mice and quite different from their $MyD88^{-/-}$ counterparts (41, 57). In contrast, $Irf5^{+/-}$ mice on both the $FcγRIIB^{-/-}$ *Yaa* and $FcγRIIB^{-/-}$ backgrounds display a phenotype comparable to $Irf5^{-/-}$ mice and develop only very limited disease manifestations. This demonstrates that IRF5 expression levels are important in regulating disease activity and may help explain how IRF5 polymorphisms that modulate expression levels could increase the risk of developing SLE.

Our findings are consistent with IRF5 contributing to lupus pathogenesis at least in part through its role in TLR signaling. Autoantibodies in SLE are thought to be pathogenic with the predominant autoantigenic targets being protein–nucleic acid complexes, either chromatin or small nuclear ribonucleoproteins (3). In animal models, TLR9 contributes to the development of antichromatin autoantibodies and TLR7 to the development of antiribonucleoprotein autoantibodies, although they have opposing effects on disease severity, with TLR9 deficiency unexpectedly aggravating disease in most models and TLR7 deficiency partially ameliorating disease (41, 44, 58–62). The ultimate effects of TLR7 and TLR9 engagement are mediated through the downstream activation of a number of transcription factors including IRF5, IRF7, NF-κB, and AP-1 (63).

In our study, IRF5-deficient mice did not develop autoantibodies against either chromatin or ribonucleoprotein. The absolute requirement for IRF5 in autoantibody production and overall disease development was unexpected and suggests that IRF5 plays a critical and nonredundant role in TLR7 and TLR9 signaling in SLE. Alternatively, it may be that IRF5 contributes to autoantibody production and disease development through TLR7- and TLR9-independent pathways. This latter possibility would be consistent with our microarray data showing that IRF5 deficiency greatly reduces the production of a wide range of autoantibodies in addition to those directed against RNA- or DNA-associated autoantigens. It will be necessary to explore these alternatives in future studies by, for example, comparing the phenotype of TLR7/9-deficient lupus models with the phenotype of lupus models deficient both in TLR7/9 and IRF5.

IRF5 plays an important role in proinflammatory cytokine production following TLR activation and viral infection (15–18, 20–22). In our study, serum levels of IL-6 and IL-10 were greatly

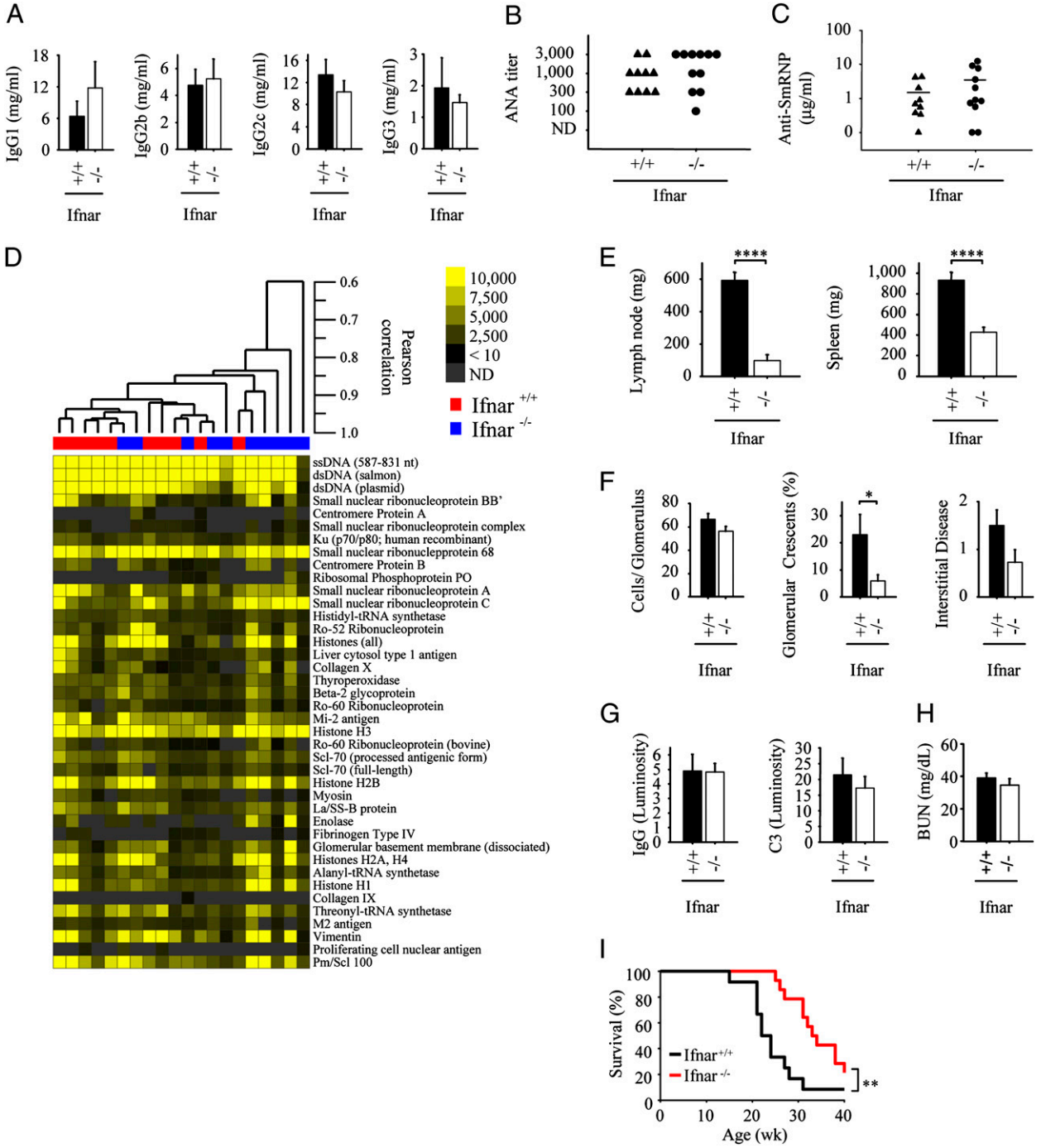

FIGURE 6. Autommune disease parameters in IFNAR1-deficient RII.*Yaa* mice. *Ifnar1*$^{+/+}$ ($n = 10$) and *Ifnar1*$^{-/-}$ ($n = 11$) RII.*Yaa* mice were analyzed at 5 mo of age. *A*, Serum IgG isotype concentrations. *B*, ANA titers in serum. ND, not detected. *C*, Antiribonucleoprotein (Sm/RNP) autoantibody levels in serum. *D*, Autoantigen array analysis was performed on sera. Samples are arranged with hierarchical clustering and displayed as a heat map. Ags are ordered using the order defined by the SAM observed score in Fig. 3*D*. SAM identified no significant differences between the *Ifnar1*$^{+/+}$ and *Ifnar1*$^{-/-}$ groups. *E*, Lymph node and spleen weights. *F*, Quantitation of renal disease as shown by cell number per glomerulus, percentage of glomeruli with crescents, and interstitial disease score. *G*, Glomerular IgG and complement C3 deposition in *Ifnar1*$^{+/+}$ ($n = 6$) and *Ifnar1*$^{-/-}$ ($n = 6$) RII.*Yaa* mice. *H*, Serum BUN levels in *Ifnar*$^{+/+}$ ($n = 8$) and *Ifnar*$^{-/-}$ ($n = 10$) RII.*Yaa* mice. Bars represent median values (*C*). Data are presented as mean ± SEM. $*p < 0.05$; $**p < 0.01$; $****p < 0.0001$ by Mann-Whitney *U* test. *I*, *Ifnar1*$^{+/+}$ (black line, $n = 12$) and *Ifnar1*$^{-/-}$ (red line, $n = 14$) RII.*Yaa* mice were observed until the time of death. $**p = 0.0043$, log-rank test.

reduced in the IRF5-deficient *Fc*γ*RIIB*$^{-/-}$ *Yaa* mice. IL-6 has been linked to lupus pathogenesis in both animal models and in human disease (64). Serum levels of IL-10 correlate with disease activity in human lupus (65, 66) and may contribute to pathogenesis through enhancement of B cell autoantibody production (67). In a preliminary study, treatment of lupus patients with an anti–IL-10 mAb reduced disease activity (68). Thus, IRF5 could contribute to lupus pathogenesis in part through promoting the production of both IL-6 and IL-10.

Type I IFN is thought to play an important role in SLE pathogenesis with the major source of type I IFN derived from plasmacytoid dendritic cells activated through TLR9 and TLR7 by DNA- or

RNA-containing immune complexes, respectively (23, 25, 69–71). IRF5 was originally identified as a regulator of type I IFN expression in human cell lines (21, 72), a finding confirmed in subsequent human cell line studies (16). IRF5 has also been shown to participate in type I IFN production in murine experimental systems both in vitro and in vivo (17, 18, 20, 73, 74). In addition, high serum IFN-α is a heritable risk factor for human lupus, and the IRF5 lupus risk haplotype is associated with higher serum IFN-α activity in lupus patients (13, 75). A central issue regarding the mechanism of IRF5 action in SLE is the extent to which induction of type I IFN by IRF5 is responsible for disease pathogenesis.

In our lupus model, we observed a modest protective effect of IFNAR1 deficiency on survival as has been seen in certain other (76–78), but not all (79), lupus mouse models. IRF5 played a role in mediating the effects of type I IFN as the low level expression of the type I IFN-induced genes IFIT1 and MX2 seen in the kidneys of IRF5-sufficient *Fcγ RIIB*$^{-/-}$ *Yaa* mice was not evident in the IRF5-deficient *Fcγ RIIB*$^{-/-}$ *Yaa* mice. However, we observed a far more profound effect of IRF5 deficiency on disease manifestations compared with IFNAR1 deficiency. This does not exclude an important role for IRF5 in the induction of type I IFN in SLE, but it clearly demonstrates the involvement of IRF5 in additional pathogenic signaling cascades independent of type I IFN production, at least in these models. The extent to which these IRF5-mediated type I IFN-independent pathogenic pathways are involved in human lupus remains to be determined, and it is certainly possible that the relative contribution of the type I IFN-dependent pathway may be greater in human lupus than in certain mouse models. Determining the relative contributions of these IRF5-mediated pathways in human lupus will be important, not only in terms of understanding disease pathogenesis but also because it relates directly to therapeutic approaches to treat the disease. If the major role of IRF5 in SLE pathogenesis is through type I IFN production, then inhibition of IRF5 as a therapy would likely not be more efficacious than type I IFN inhibition. However, if the IRF5-mediated type I IFN-independent pathway(s) does play a substantial role, then targeting IRF5 may confer additional therapeutic benefit. Another unresolved issue relating to heritable risk factors for lupus, such as high serum IFN-α levels or enhanced IRF5 function, is whether they are involved primarily in disease initiation or whether they also play a role in ongoing disease activity (25). Clinical trials of type I IFN inhibition in SLE are currently in progress and may help to resolve this question as it relates to IFN-α. Our current study examined the role of IRF5 in disease initiation and development, but the role of IRF5 in ongoing disease activity could be addressed in future studies in *Fcγ RIIB*$^{-/-}$ *Yaa* mice by inhibiting IRF5 expression after disease onset.

All type I IFNs act through a single cell surface type I IFN receptor (49–51). The IFNAR is comprised of two chains designated IFNAR1 and IFNAR2 (51, 80). Ligand-induced cross-linking of IFNAR1 and IFNAR2 induces a pleiotropic cellular response (81). IFNAR1 is necessary for signaling and also participates in ligand binding (52, 80, 81). Studies using IFNAR1-deficient mice have demonstrated that IFNAR1 is essential for responses to multiple IFN-α subtypes as well as IFN-β (36, 52). We used IFNAR1-deficient mice to evaluate the contribution of type I IFN to disease development in our model. Although it is difficult to definitively exclude the possibility of residual type I IFN signaling in IFNAR1-deficient mice, there is no evidence at present in the literature as far as we are aware that such residual signaling, if present, has a biologically important effect in vivo.

In addition to its role in cytokine production, IRF5 is also associated with apoptosis. IRF5 expression can be induced by the tumor suppressor p53, suggesting a connection between IRF5 and p53-induced proapoptotic pathways (82). Like p53, IRF5 stimulates the cyclin-dependent kinase inhibitor p21 while repressing cyclin B1 and stimulates the expression of the proapoptotic genes Bak 1, Bax, caspase 8, and DAP kinase 2 (72, 83). IRF5 promotes cell cycle arrest and apoptosis independently of p53 (84). IRF5 is required for Fas-induced apoptosis in hepatocytes and dendritic cells but not in thymocytes (27) and is required for DNA damage-induced apoptosis in embryonic fibroblasts (20). Given the strong association between dysregulated apoptosis and apoptotic material clearance and SLE (85–87), it is certainly conceivable that IRF5 regulation of apoptotic pathways could contribute to disease pathogenesis in SLE.

In summary, we have shown that IRF5 plays an essential role in disease pathogenesis in the *Fcγ RIIB*$^{-/-}$ *Yaa* and *Fcγ RIIB*$^{-/-}$ mouse lupus models. Although IRF5 contributes to type I IFN production in *Fcγ RIIB*$^{-/-}$ *Yaa* mice, it is likely that in this model, the major effects of IRF5 are mediated through type I IFN-independent pathways, possibly through inhibition of the production of IL-6 and IL-10. In addition, even IRF5 heterozygous mice are substantially protected from disease development, indicating that a certain threshold level of IRF5 is required for disease development. It will be important to evaluate whether IRF5 deficiency has similar effects in other mouse models of SLE. It will also be particularly important to determine in future studies whether IRF5 is involved in disease onset and/or disease progression and whether manipulation of IRF5 levels can reverse established disease. If IRF5 is involved in disease progression in multiple models and if inhibiting IRF5 can reverse established disease, this would suggest that IRF5 might be a key therapeutic target in lupus, particularly because a partial reduction in the level of IRF5 could have a meaningful effect on disease severity.

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Disclosures

The authors have no financial conflicts of interest.

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