Gain-of-function polymorphisms in the transcription factor IRF5 are associated with an increased risk of developing systemic lupus erythematosus. However, the IRF5-expressing cell type(s) responsible for lupus pathogenesis in vivo is not known. We now show that monoallelic IRF5 deficiency in B cells markedly reduces disease in a murine lupus model. In contrast, similar reduction of IRF5 expression in macrophages, monocytes, and neutrophils does not reduce disease severity. B cell receptor and TLR7 signaling synergize to promote IRF5 phosphorylation and increase IRF5 protein expression, with these processes being independently regulated. This synergy increases B cell-intrinsic IL-6 and TNF-α production, both key requirements for germinal center responses, with IL-6 and TNF-α production in vitro and in vivo being substantially lower with loss of one allele of IRF5. Mechanistically, TLR7-dependent IRF5 nuclear translocation is reduced in B cells from IRF5-heterozygous mice. In addition, we show in multiple lupus models that IRF5 expression is dynamically regulated in vivo with increased expression in germinal center B cells compared to non-germinal center B cells and with further sequential increases during progression to plasmablasts and long-lived plasma cells. Overall, a critical threshold level of IRF5 in B cells is required to promote disease in murine lupus.
Monoallelic IRF5 deficiency in B cells prevents murine lupus

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Abstract

Gain-of-function polymorphisms in the transcription factor IRF5 are associated with an increased risk of developing systemic lupus erythematosus. However, the IRF5-expressing cell type(s) responsible for lupus pathogenesis in vivo is not known. We now show that monoallelic IRF5 deficiency in B cells markedly reduces disease in a murine lupus model. In contrast, similar reduction of IRF5 expression in macrophages, monocytes, and neutrophils does not reduce disease severity. B cell receptor and TLR7 signaling synergize to promote IRF5 phosphorylation and increase IRF5 protein expression, with these processes being independently regulated. This synergy increases B cell-intrinsic IL-6 and TNF-α production, both key requirements for germinal center responses, with IL-6 and TNF-α production in vitro and in vivo being substantially lower with loss of one allele of IRF5. Mechanistically, TLR7-dependent IRF5 nuclear translocation is reduced in B cells from IRF5-heterozygous mice. In addition, we show in multiple lupus models that IRF5 expression is dynamically regulated in vivo with increased expression in germinal center B cells compared to non-germinal center B cells and with further sequential increases during progression to plasmablasts and long-lived plasma cells. Overall, a critical threshold level of IRF5 in B cells is required to promote disease in murine lupus.
Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by autoantibody production, inflammation, and tissue damage in multiple organs resulting from an overactivation of the immune system through various mechanisms (1, 2). Disease development is due to a combination of genetic and environmental factors, amongst which polymorphisms in interferon regulatory factor 5 (IRF5) have been strongly associated with an increased risk of developing SLE (3, 4). While the exact mechanism of how these polymorphisms in IRF5 lead to an increased risk of developing SLE is incompletely understood, it is thought that they lead to increased levels of IRF5 protein expression and/or functional change (3, 4). Notably, global homozygous or heterozygous deficiency of IRF5 has conferred protection in many mouse models of lupus (5-10). Thus, there is both human and mouse genetic evidence that suggests that IRF5 expression levels contribute to disease pathogenesis. However, the previous studies of global heterozygous and homozygous gene deficiencies do not address how cell type specific expression of IRF5 impacts disease and how IRF5 expression and activation is modulated in the pathogenic cell type(s) during disease development.

IRF5 is a transcription factor that plays an important role in regulating immune responses downstream of Toll like receptors (TLRs), specifically, TLR-3, 4, 7, and 9, and other innate immune receptors including nucleotide-binding oligomerization domain 2 (NOD2), retinoic acid inducible gene I (RIG-I), and dectin-1 (11-15). Of these, TLR7 has been most closely associated with disease pathogenesis in mouse lupus models (16). Similar to other IRF family members, IRF5 resides predominantly in the cytosol in latent form as a monomer in unstimulated cells. Upon activation, specific serine residues in the C-terminal region are phosphorylated, which
causes IRF5 to homodimerize or heterodimerize and undergo nuclear translocation. The IRF5 dimer then assembles with transcriptional coactivators such as CREB binding protein or p300 in the nucleus which enables promoter binding and the induction of gene transcription (17, 18).

While IRF5 is also expressed outside of the immune system, it is constitutively expressed in B cells, myeloid dendritic cells (mDCs), plasmacytoid dendritic cells (pDCs), and monocytes/macrophages (19, 20), and plays a key role in the induction of a number of pro-inflammatory cytokines and chemokines (11, 21). The extent of involvement of IRF5 in cytokine production is both cell-type and stimulus dependent (3, 20). Beyond cytokine production, IRF5 has also been linked to promoting M1 macrophage polarization (22), IgG2a/c class switching (6), and upregulation of co-stimulatory molecules and Blimp-1 in B cells (23, 24). As B cells, mDCs, pDCs, and monocytes/macrophages have all been implicated in lupus pathogenesis, it is plausible that IRF5 in any one or more of these cell types may be required for disease pathogenesis.

B lineage cells are thought to be central to the pathogenesis of lupus, although the optimal therapeutic intervention targeting this lineage is not yet established (25). Clinical trials of B cell depletion in lupus have shown variable efficacy leading to a shift in focus to developing more effective B cell-depleting strategies and also targeting long-lived plasma cell populations (26). In mouse models of lupus, B cell deficiency is protective (16). A role for B cell-intrinsic TLR signaling was demonstrated in bone marrow chimera studies showing that B cell-specific expression of the TLR adaptor protein MyD88 was required for disease development (27). Given that B cells and their expression of key molecules in the TLR signaling pathways are
important in disease development, we hypothesized that the function of IRF5 in B cells might be a central component of pathogenesis. Furthermore, the fact that heterozygous global deletion of IRF5 markedly reduces disease in lupus models suggested that loss of one allele of IRF5 in B cells might confer protection (5, 10, 28, 29).

To examine the role for B cell specific IRF5 we conditionally deleted one allele of IRF5 in the FcγRIIB−/− Yaa mouse lupus model, the model we used previously to demonstrate the effect of global homozygous and heterozygous IRF5 deletion (5). We found that heterozygous deletion of IRF5 in B cells leads to a marked reduction in disease severity. In contrast, a similar reduction of IRF5 expression in macrophages, monocytes, and neutrophils does not impact disease. Thus there is a critical threshold level of IRF5 expression in B cells that is necessary to induce disease and this suggests that future therapeutic approaches targeting IRF5 in SLE will need to effectively reduce IRF5 expression or activity in B cells.
Results

IRF5 expression in bone marrow-derived cells is required for the development of autoimmune disease.

IRF5 is expressed in both immune cells and also non-bone marrow-derived cells (19) and previous reports have suggested a role for IRF5 in non-bone marrow-derived cells in lupus pathogenesis (30). To evaluate the relative contribution of IRF5 expression in bone marrow-derived cells to disease pathogenesis in the FcγRIIB−/−Yaa model, we performed bone marrow chimera studies. Bone marrow from FcγRIIB−/−Yaa IRF5+/+ or FcγRIIB−/−Yaa IRF5 -/- mice was transferred into irradiated C57BL/6 (B6) recipients. Disease parameters were evaluated 28 weeks after transfer. Mice that received the FcγRIIB+/−Yaa IRF5+/+ bone marrow developed splenomegaly, anti-nuclear autoantibodies (ANA) and kidney disease whereas mice that received the FcγRIIB−/−Yaa IRF5−/- bone marrow did not. These studies demonstrated that IRF5 expression in bone marrow-derived cells is sufficient to drive the lupus like disease observed in FcγRIIB+/−Yaa mice (Supplemental Figure 1).

Generation of FcγRIIB−/−Yaa mice with a B cell specific deletion of IRF5.

In order to study the role of IRF5 expression specifically in B cells we first generated an IRF5 floxed allele on the C57BL/6 genetic background by introducing loxP sites flanking exons 3-6 including a neo cassette flanked by Frt sites to the 5’ end of exon 3 (Supplemental Figure 2). Mice were then bred to the Flpe deleter mice to remove the neo cassette. The resulting IRF5F/F mice were intercrossed with Mb1cre/wt and FcγRIIB−/−Yaa mice, generating FcγRIIB−/−Yaa mice with a heterozygous deletion of IRF5 in B cells (Mb1cre/wt x IRF5+/+ mice; termed IRF5ΔB) and littermate control FcγRIIB−/−Yaa mice with no deletion of IRF5 in B cells (Mb1wt/wt x IRF5+/+...
mice; termed $IRF5^{F/+}$). We chose to focus on the heterozygous deletion of $IRF5$ in B cells given our previous finding that global heterozygous $IRF5$ deletion protected mice from disease to a similar extent as homozygous deletion, a finding that has been confirmed in other mouse lupus models (5, 10, 28, 29).

In Mb1cre-expressing animals, the cre transgene is driven by the CD79$\alpha$ promoter and results in deletion of the floxed allele in 95% of B cells (31). In some cases, the Mb1cre transgene has been reported to induce ectopic, germline deletion of floxed genes (32). Therefore, it was important to ensure that the deletion mediated by Mb1cre in our system was restricted to the B cell lineage.

To evaluate $IRF5$ deletion, we first used flow cytometry to sort mature B cells (B220+, CD93-) and myeloid cells (B220-, CD3-, CD11b+, Ly6G-) from $IRF5^{F/+}$ mice expressing or not expressing Mb1cre and performed western blot on the B cell and myeloid cell populations. Mb1cre mediated deletion of $IRF5^{F/+}$ mice resulted in an approximate 50% reduction in IRF5 expression in B cells (Figure 1, A and B). This is consistent with the reduction we observed in the B cells of $IRF5$ germline heterozygous mice (Supplemental Figure 3, A and B). Importantly there was no difference in IRF5 protein expression in myeloid cells from the $IRF5^{bB}$ and $IRF5^{F/+}$ mice. To corroborate the western blot studies and extend the analysis to specific IRF5-expressing immune cell types not captured in the myeloid gating strategy, we measured IRF5 expression by intracellular flow cytometry (Figure 1, C-E). This confirmed that IRF5 reduction in $IRF5^{F/+} Fc\gamma RIIB^{-Yaa}$ mice expressing Mb1cre was restricted to B cells as the mean fluorescence intensity (MFI) of IRF5 was reduced by approximately 50% in B cells but was not
reduced in monocytes, neutrophils or plasmacytoid dendritic cells (pDCs) (Figure 1, C-E). The gating strategy used to define each immune cell population is shown in Supplemental Figure 3C. Figure 1D represents MFI values from two separate mice of each genotype from one experiment. Figure 1E is a compilation of MFI values from six $IRF5^{B}$ mice normalized to the $IRF5^{F/+}$ littermate controls from the same experiment. The intracellular staining for IRF5 was specific as cells from $IRF5$ germline knockout mice stained with the anti-IRF5 antibody had a similar staining profile as the isotype control antibody (Supplemental Figure 3D). Taken together, these data indicate that we successfully generated $FcγRIIB^{−/−}$ $Yaa$ mice with B cell specific deletion of one allele of $IRF5$.

Monoallelic deletion of IRF5 in B cells reduces splenomegaly and T cell activation in $FcγRIIB^{−/−}$ $Yaa$ mice.

Splenomegaly is seen in 10-45% of SLE patients and in multiple mouse models of lupus like disease (33). Therefore, we evaluated the effect of heterozygous deletion of $IRF5$ in B cells on splenomegaly and found that it was markedly reduced and to the same extent as that seen in global $IRF5$ heterozygous $FcγRIIB^{−/−}$ $Yaa$ mice (Figure 2A). No reduction in splenomegaly was seen in $FcγRIIB^{−/−}$ $Yaa$ mice expressing Mb1cre alone or the $IRF5$ floxed allele alone. The reduction in spleen weight also correlated with a reduction in overall spleen cell number (Figure 2B). There was no significant difference in the number of CD3+ T cells and CD19+ B cells between the genotypes, indicating that the splenomegaly in this model is due to the expansion of a non-B, non-T cell population (Supplemental Figure 4).
We next evaluated the effect of heterozygous deletion of *IRF5* in B cells on T cell activation using flow cytometry to assess T cell activation based on CD44 and CD62L expression. In both the CD4 (Figure 2, C-E) and CD8 (Figure 2, H-J) T cell populations, we found a dramatic reduction in the percent and number of effector/memory (CD62L⁻CD44⁺) T cells in the *IRF5ΔB* mice as compared to the *FcγRIIB⁺/⁺*Yaa mice and the littermate *FcγRIIB⁺/⁻*Yaa mice expressing Mb1cre alone or the *IRF5* floxed allele alone. Notably, as with splenomegaly, no impact on T cell activation was seen in *FcγRIIB⁺/⁻*Yaa mice expressing Mb1cre alone or the *IRF5* floxed allele alone, as compared to the *FcγRIIB⁺/⁺*Yaa mice. Conversely, the percent and number of naive (CD62L⁺CD44⁻) T cells in the *IRF5ΔB* mice was greatly increased as compared with the other experimental groups for both the CD4⁺ (Figure 2, F and G) and CD8⁺ (Figure 2, K and L) populations. These data indicate that heterozygous deletion of *IRF5* in B cells markedly reduces splenomegaly and prevents T cell activation in *FcγRIIB⁺/⁻*Yaa mice and that the *Mb1cre* allele or the *IRF5* floxed allele alone have no impact on these measurements.

*Monoallelic deletion of IRF5 in B cells reduces serum autoantibodies, splenic plasmablasts and bone marrow plasma cells.*

Anti-nuclear autoantibodies are a hallmark of SLE and are thought to contribute to disease pathogenesis (34). We first evaluated the effect of B cell specific heterozygous deletion of *IRF5* on serum IgG isotype levels and found that this significantly reduced the levels of all four IgG isotypes, IgG1, IgG2b, IgG2c and IgG3 (Figure 3A). We next measured serum ANA and found that, as expected, *IRF5F/+* mice had a high ANA titer as measured by immunofluorescence on HEp2 cells (Figure 3B). However, there was a marked reduction of ANA titer in the *IRF5ΔB* mice (Figure 3B). This reduction was seen both for autoantibodies directed against DNA-
containing autoantigens (anti-nucleosome) (Figure 3C) and for autoantibodies directed against RNA-containing autoantigens (anti-Sm/RNP) (Figure 3D).

To determine how B cell specific heterozygous deletion of IRF5 might cause the observed reduction in serum IgG and autoantibody levels we evaluated plasmablast numbers in the spleen and plasma cell percentages in the bone marrow. It has been recently reported that B cell intrinsic IRF5 expression is necessary for human B cell differentiation into plasmablasts in vitro (35) and IRF5-deficient mice have fewer plasma cells (23). We found that there was an appreciable population of plasmablasts in the spleen of IRF5F/+ mice at 5 months of age and that this plasmablast population was greatly reduced in the IRF5ΔB mice (Figure 3E). Similarly, there was a large reduction in plasma cells in the bone marrow compartment of the IRF5ΔB mice as compared with the IRF5F/+ mice at 5 months of age (Figure 3F). These data explain the reduction of IgG and autoantibodies observed with the heterozygous deletion of IRF5 in B cells in the FcγRIIB−/−Yaa mice and demonstrate for the first time that B cell intrinsic IRF5 is necessary for plasmablast and plasma cell development in a mouse model of lupus.

Monoallelic deletion of IRF5 in B cells reduces disease manifestations in the kidney.

The renal disease in lupus patients and in many mouse lupus models is characterized by immune complex deposition, complement activation, and glomerular inflammation with resultant glomerular crescent formation and cellular injury, often accompanied by tubulointerstitial disease (36). To assess whether deletion of one allele of IRF5 in B cells might impact renal disease, we measured glomerular inflammation and injury as well as interstitial disease in kidneys from 4-5 month-old IRF5F/+ and IRF5ΔB mice. The IRF5F/+ mice all had glomerular
crescents and/or necrosis while these features were essentially absent in the \( IRF5^{\Delta B} \) mice (Figure 4, A and B). The more severe renal disease in the \( IRF5^F/+ \) mice was also evident as assessed by the overall glomerular injury score and interstitial disease (Figure 4, C and D), and by the extent of immune complex and complement deposition (Figure 4, E and F).

**Deletion of IRF5 in macrophages, monocytes and neutrophils does not reduce disease severity in \( Fc\gamma RIIB^{-/-} \) Yaa mice.**

Although our data demonstrated that IRF5 expression in B cells was required for disease pathogenesis, this did not exclude the possibility that IRF5 expression in other bone marrow-derived cells might also be required. To address this possibility, we deleted \( IRF5 \) in macrophages, monocytes and neutrophils in the \( Fc\gamma RIIB^{-/-} \) Yaa model using the LysMcre system (37, 38). We found substantial reductions in IRF5 expression in peritoneal macrophages and splenic monocytes and neutrophils in the \( IRF5^F/F \ Fc\gamma RIIB^{-/-} \) Yaa mice expressing LysMcre (termed \( IRF5^{\Delta M} \)) as compared with littermates not expressing LysMcre (termed \( IRF5^F/F \)), but no difference in IRF5 expression in B cells (Figure 5A). However, we found no difference in spleen size, spleen cell number, autoantibody levels, or renal disease between the \( IRF5^F/F \) mice and their \( IRF5^{\Delta M} \) littermates at 5 months of age (Figure 5, B-D).

**Critical threshold level of IRF5 in B cells is necessary for the formation of spontaneous germinal center B cells, T follicular helper cells and age associated B cells in \( Fc\gamma RIIB^{-/-} \) Yaa mice early in disease pathogenesis.**

Our data show that the \( IRF5^{\Delta B} \) mice are protected from the development of lupus disease manifestations. To better understand mechanistically how IRF5 expression in B cells was
contributing to this phenotype we evaluated mice at 8-10 weeks of age prior to the appearance of overt signs of disease.

Spontaneous formation of autoimmune germinal center (GC) B cells and the presence of T\textsubscript{FH} have been shown in several mouse lupus models. These same populations are also present in human SLE (39) and it is thought that the interplay between GC B cells and T\textsubscript{FH} is important for the subsequent generation of pathogenic autoantibodies (40). We found that in mice as young as 8 weeks of age there was an appreciable population of CD19\textsuperscript{+}, CD95\textsuperscript{+}, CD38\textsuperscript{-} cells (GC B cells) in the spleen of \textit{FcyRIIB\textsuperscript{−/−}Yaa} and \textit{IRF5\textsuperscript{F/+}} mice (Figure 6, A and B). Monoallelic deletion of \textit{IRF5} in B cells dramatically reduced the number of GC B cells, comparable to the reduction observed in mice that have global homozygous and heterozygous \textit{IRF5} deficiency (Figure 6, A and B). Concordantly, we observed a similar marked reduction in splenic T\textsubscript{FH} cells (CD3\textsuperscript{+}, CD4\textsuperscript{+}, CXCR5\textsuperscript{+}, PD-1\textsuperscript{+}) in the \textit{IRF5\textsuperscript{Δ}B} mice as compared to the \textit{FcyRIIB\textsuperscript{−/−}Yaa} mice and the littermate \textit{FcyRIIB\textsuperscript{−/−}Yaa} mice with the \textit{IRF5} floxed allele alone (Figure 6, C and D).

Age-associated B cells (ABC) are a population of germinal center-derived memory B cells that are CD11c\textsuperscript{+}Tbet\textsuperscript{+} and are thought to be pathogenic in autoimmune disease (41). It was recently shown that IRF5 expression in B cells was required for the in vitro differentiation of naïve B cells to ABC, and that global heterozygous \textit{IRF5} deficiency resulted in a reduction in ABC in the SWEF-deficient mouse model in vivo (29). We identified ABC as CD19\textsuperscript{+}, B220\textsuperscript{+}, CD93\textsuperscript{-}, CD43\textsuperscript{-} CD21\textsuperscript{-}, CD23\textsuperscript{-}, CD11c\textsuperscript{+}, Tbet\textsuperscript{+} (Figure 6E) (41, 42), and found a large reduction in the number of splenic ABC in the \textit{IRF5\textsuperscript{Δ}B} mice (Figure 6F). The reduction in the number of ABC in the \textit{IRF5\textsuperscript{Δ}B} mice was similar to the reduction observed in mice with one or two copies of \textit{IRF5}
globally deleted (Figure 6F). Taken together these data indicate that there is a critical threshold level of IRF5, specifically in B cells, that is necessary for the formation of spontaneous germinal center B cells, T<sub>FH</sub> and ABC in the FcγRIIB<sup>−/−</sup>Yaa lupus model early in disease pathogenesis.

**Monoallelic deletion of IRF5 reduces IL-6 and TNF-α production from B cells and reduces serum IL-6 and TNF-α levels in vivo.**

We next wanted to investigate how IRF5 expression in B cells drives the development of spontaneous GC B cells. B cell derived IL-6 has been shown to be necessary for the development of spontaneous GCs in a mouse model of lupus and has also been suggested to be important in the development of experimental autoimmune encephalomyelitis (43, 44). B cell-derived soluble TNF is required for the development of germinal centers in response to T-dependent antigens and for an efficient humoral immune response (45, 46). B cell-derived soluble TNF has also been shown to mediate disease severity in a mouse model of collagen induced arthritis via control of pathogenic autoantibody production (47). To evaluate how IRF5 monoallelic deficiency might impact B cell intrinsic IL-6 and TNF-α production induced by the different pathways critical for GC B cell formation, we isolated B cells from 8-week-old FcγRIIB<sup>−/−</sup>Yaa, FcγRIIB<sup>−/−</sup>Yaa IRF5<sup>+</sup>− or FcγRIIB<sup>−/−</sup>Yaa IRF5<sup>−/−</sup> mice and stimulated the B cells with various combinations of anti-IgM antibody to model B cell receptor (BCR) activation, anti-CD40 antibody to model T cell co-stimulation, and the TLR7 ligand R848 or TLR9 ligand CpG-B. We found that a modest degree of IL-6 and TNF-α was induced by R848 or CpG-B alone. A substantial increase in IL-6 and TNF-α production was observed with R848 or CpG-B in combination with anti-IgM (Figure 7A). There was a gene dosage dependent impact of IRF5 on IL-6 and TNF-α in that heterozygous deficiency of IRF5 reduced IL-6 and TNF-α production by 40-60% in all
conditions tested and homozygous deletion reduced IL-6 and TNF-α by 70-80% (Figure 7, B and C). Notably, B cells from mice with B cell-specific heterozygous deletion of IRF5 by Mb1cre showed a similar reduction in IL-6 and TNF-α as observed in B cells from mice with germline heterozygous deficiency of IRF5 (Figure 7, D and E). IL-12p40 production was also reduced in IRF5 heterozygous B cells in response to most stimuli combinations, but effects on RANTES, MIP-1α, and MIP-1β were more variable (Supplemental Figure 5).

We next evaluated if B cell specific deletion of IRF5 impacted the level of serum IL-6 and/or TNF-α in the FcγRIIB−/−Yaa model. B cell-specific deletion of IL-6 has been shown to reduce serum IL-6 in another lupus model (43). We found that serum levels of both IL-6 and TNF-α were indeed decreased in 5-month-old IRF5ΔB mice compared to the FcγRIIB−/−Yaa controls (Figure 7F). These data suggest that there is a synergistic effect of both TLR7 and TLR9 signaling with BCR signaling on the production of IL-6 and TNF-α and that B cell intrinsic IRF5 plays a critical role in mediating the induction of IL-6 and TNF-α in all combinations of stimuli.

**TLR7 and BCR stimulation synergize in the induction of IRF5 phosphorylation.**

Phosphorylation of IRF5 is necessary for IRF5 dimerization and nuclear translocation (48). To determine whether the increased IL-6 and TNF-α production observed with TLR7 and BCR co-stimulation might be mirrored by changes in IRF5 phosphorylation, we stimulated isolated B cells from FcγRIIB−/−Yaa mice with the different combinations of anti-IgM, R848, and anti-CD40. We chose to use R848 as TLR7 gene dosage is essential for the accelerated disease in the FcγRIIB−/−Yaa mice and TLR7 is important for pathogenesis in other mouse models of lupus (16, 49). We did not detect any phosphorylation of IRF5 after stimulation with anti-IgM alone or anti-
CD40 alone, consistent with the lack of IL-6 and TNF-α production observed in Figure 7A. However, we could readily detect phosphorylation after stimulation with R848 alone for two hours (Figure 8A). The ratio of phosphorylated to unphosphorylated IRF5 significantly increased three-fold with the addition of anti-IgM to R848 (Figure 8B). Interestingly, the addition of anti-CD40 stimulation did not increase IRF5 phosphorylation in any of the stimulus combinations. Moreover, we demonstrated that B cells from C57BL/6 mice harboring the kinase dead mutation of interleukin-1 receptor-associated kinase 4 (IRAK4) completely lack IRF5 phosphorylation in all stimulation conditions (Supplemental Figure 6A). These data indicate that TLR7 stimulation alone is sufficient to induce IRF5 phosphorylation in B cells and that the BCR and TLR7 synergize to increase IRF5 phosphorylation.

**TLR7-induced IRF5 phosphorylation and nuclear translocation is reduced in B cells with monoallelic deletion of IRF5.**

As TLR7-induced IL-6 and TNF-α production is reduced in B cells with monoallelic deletion of IRF5 (Figure 7, A, B and D), we hypothesized that this would be associated with a reduction in IRF5 nuclear translocation. We first examined the extent of IRF5 phosphorylation in splenic B cells from FcγRIIB<sup>-/-</sup>Yaa WT and IRF5<sup>+/−</sup> mice 2 hours after stimulation with anti-IgM, anti-CD40, and R848 alone or in combination. We found that the general pattern of IRF5 phosphorylation, as assessed by phospho-Tag gel, and the ratio of phosphorylated to unphosphorylated IRF5 did not differ between the groups (Supplemental Figure 6, B and C). However, as the total amount of IRF5 in B cells from the FcγRIIB<sup>-/-</sup>Yaa IRF5<sup>+/−</sup> mice is about half of that in the WT, we infer that the total amount of phosphorylated IRF5 is less in the IRF5<sup>+/−</sup> mice under these stimulation conditions. More importantly, we directly compared the ratio of
nuclear IRF5 expression in B cells from the WT and \( IRF5^{+/-} \) mice following R848 stimulation and found that nuclear IRF5 expression was reduced by approximately half in the \( IRF5^{+/-} \) mice (Figure 8, C and D).

IRF5 expression is increased in activated B cells in vitro and in germinal center B cells, splenic plasmablasts and bone marrow plasma cells in vivo.

Our data in Figures 3 and 6 suggested that there is a critical threshold level of IRF5 required for the generation of autoimmune GC B cells and plasma cells. We therefore hypothesized that IRF5 expression levels might be dynamically regulated after B cell activation. To determine if the expression level of IRF5 changes after B cell activation, we stimulated naïve B cells isolated from \( FcγRIIB^{-/-}Yaa \) mice using various combinations of anti-IgM, anti-CD40 and R848 and measured IRF5 expression levels by intracellular flow cytometry after 24 hours. In contrast to their lack of effect on IRF5 phosphorylation, anti-IgM and anti-CD40 alone increased IRF5 expression about two-fold, similar to the increase induced by R848 alone (Figure 9, A-C). The various stimulus combinations induced a greater increase in IRF5 expression than the individual stimuli alone. To determine whether these findings were specific to B cells from \( FcγRIIB^{-/-}Yaa \) mice, we repeated these studies using naïve B cells isolated from C57BL/6 mice and found a similar increase in IRF5 expression in C57BL/6 B cells (Figure 9, D and E). This indicates that the increase in IRF5 expression observed in B cells under these stimulation conditions is not a unique feature of \( FcγRIIB^{-/-}Yaa \) B cells only.

The increase in IRF5 expression after activation with anti-IgM, anti-CD40 and R848 in vitro suggested the possibility that IRF5 might be increased in GC B cells in \( FcγRIIB^{-/-}Yaa \) mice in
vivo as TLR7 activation is thought to be important for spontaneous germinal center formation in other lupus models (50). To test this hypothesis, we evaluated IRF5 expression in splenic germinal centers of 8-10 week old FcγRIIB−/−Yaa mice. We observed an approximately 1.5-fold increase in the MFI of IRF5 in GC B cells as compared to non-GC B cells (Figure 10, A and B). Next, we evaluated the levels of IRF5 in plasmablasts as these cells are thought to either be derived from the germinal center or from an extrafollicular response (1) and it was recently reported that human plasmablasts express higher levels of IRF5 as compared to naïve B cells (35). We observed a roughly 2.5-fold increase in the MFI of IRF5 in plasmablasts as compared to non-plasmablast B cells (CD19+) (Figure 10, A and C). Furthermore, we did a similar analysis comparing the splenic CD19+ B cells with plasma cells in the bone marrow and observed an increase in IRF5 MFI in plasma cells at least as large as that observed in the splenic plasmablasts (Figure 10, A and D).

To determine whether our findings were specific to the FcγRIIB−/−Yaa model or were more generally relevant, we measured changes in IRF5 expression in GC B cells, plasmablasts, and plasma cells in two additional mouse lupus models, (NZBxNZW)F1 and MRL-lpr. We found that IRF5 expression was increased in these models at least as much as had been observed in the FcγRIIB−/−Yaa model (Figure 10, E-G). In addition, to determine whether our findings were restricted to lupus models or whether they might reflect germinal center responses more broadly, we measured changes in IRF5 expression in GC B cells and plasmablasts in C57BL/6 mice 14 days after immunization with the hapten NP coupled to chicken gamma globulin. We found an increase in IRF5 expression in these cell types similar to that observed in the three lupus models (Figure 10, E and F).
Taken together, these data suggest that IRF5 expression levels are dynamically regulated in the germinal center compartment as well as during plasmablast differentiation and that this increased IRF5 expression is maintained in long-lived plasma cells.
Discussion

Global homozygous or heterozygous deficiency of IRF5 markedly reduces disease severity in mouse lupus models (5-9). However, it is not known which IRF5-expressing cell type(s) is involved in lupus pathogenesis. In this report we demonstrate that heterozygous deficiency of IRF5 in B cells results in a marked reduction in lupus disease manifestations. In contrast, similar reduction of IRF5 expression in macrophages, monocytes, and neutrophils does not reduce disease severity. While these findings do not exclude a role for IRF5 in other cell types in disease pathogenesis, they do demonstrate that there is a critical threshold level of IRF5 expression in B cells that is absolutely required.

We previously reported that heterozygous deficiency of IRF5 reduces disease in the FcγRIIB−/−Yaa mouse lupus model (5). Subsequently it was demonstrated that global heterozygous deficiency of IRF5 also reduced disease in the Lyn-deficient mouse lupus model, the gld.apoE-deficient mouse lupus model, and in the Swap70-deficient Def6-deficient mouse lupus model (10, 28, 29). In the Swap70-deficient Def6-deficient model, deletion of the second IRF5 allele specifically in CD21-expressing cells or in CD11c-expressing cells did not lead to further reductions in disease manifestations. In this report, we identified B cells as a critical cell type in which heterozygous deficiency of IRF5 is protective and thus sought to uncover possible mechanisms that might explain the profound protective effect of a 50 percent reduction in IRF5 expression in B cells. We found that IL-6 and TNF-α production by B cells from IRF5 heterozygous mice was reduced by about half in response to combinations of stimuli that are thought to be involved in B cell activation in the germinal center in lupus (51). In contrast, the expression of co-stimulatory molecules involved in B cell-T cell interactions and germinal center
B cell activation such as CD80, CD86, MHC class II, and ICOS-ligand did not differ between B cells from IRF5 wildtype and IRF5 heterozygous mice (Supplemental Figure 7). B cell intrinsic IL-6 and TNF-α production have been shown to be important in germinal center formation in mice immunized with T-dependent antigens, and in germinal center formation and disease pathogenesis in lupus and other autoimmune models (43-47). It is thus plausible that the reduction in IL-6 and TNF-α production observed in our studies may explain, at least in part, the reduction in disease manifestations seen in the B cell-specific IRF5 heterozygous mice.

In the inactivated state, IRF5 resides in the cytoplasm as a monomer. IRF5 activation requires phosphorylation of the monomer leading either to IRF5 homodimerization, or to heterodimerization of IRF5 with other IRF or NF-κB family members, followed by nuclear translocation and the induction of target gene transcription (17). We found that the amount of IRF5 in the nucleus after TLR7 activation is approximately 50% lower in B cells from IRF5 heterozygous FcγRIIB−/− Yaa mice than in B cells from IRF5–sufficient FcγRIIB−/− Yaa mice. Mechanistically, this may explain the reduced production of IL-6 and TNF-α by the IRF5 heterozygous B cells.

The IRF5 polymorphisms associated with an increased risk of developing SLE and other autoimmune diseases are in the non-coding region of the IRF5 gene and show some association with increased IRF5 expression and/or functional change (3, 20). We found that BCR activation alone or CD40 activation alone increased IRF5 protein about two-fold but did not induce IRF5 phosphorylation. Thus, increased IRF5 expression, per se, does not result in IRF5 activation. In contrast, TLR7 activation alone induces both increased IRF5 expression and IRF5
phosphorylation, and BCR activation, while not able to induce IRF5 phosphorylation independently, synergizes with TLR7 activation to further increase both IRF5 expression and phosphorylation. These findings are not just restricted to B cells from an autoimmune prone background but are also observed in B cells from C57BL/6 mice. Intriguingly, we also observed that IRF5 phosphorylation in all stimulation conditions is IRAK4 dependent. Consistent with these in vitro findings, we observed an increase in IRF5 expression in GC B cells in vivo early in disease development, prior to the appearance of overt signs of autoimmunity, with even larger increases evident in plasmablasts and bone-marrow plasma cells. We corroborated these findings in three different lupus mouse models, and also in C57BL/6 mice immunized with a T-dependent antigen, suggesting that the increase in IRF5 in these compartments during B cell activation could be a general feature in B cell biology.

IRF5 exerts a number of effects in B cells including class-switching to IgG2a/IgG2c by binding to the IgG2a promoter, regulation of the transcription factor Blimp1, and regulation of T-bet-expressing age-associated B cells (6, 23, 29). While these B cell-intrinsic effects likely make significant contributions to disease pathogenesis our data suggest that there are also critical roles for B cell-intrinsic IRF5 more proximally in the autoimmune response. The generation of high-affinity autoantibodies and long-lived plasma cells in lupus is dependent on the development of a robust germinal center response with TLR7 and TLR9 signaling in B cells likely being required for this response (51). We found a dramatic reduction in the number of GC B cells prior to the onset of overt autoimmune disease in the mice with heterozygous deficiency of IRF5 in B cells. This was accompanied by an equally marked reduction in the number of T follicular helper (T_{FHV}) cells, a cell type that provides the signals to B cells that are required for germinal center
formation, affinity maturation, and the development of most high-affinity autoantibodies and memory B cells (52, 53). This suggests that B cell-intrinsic IRF5 may be required for the generation of T<sub>FH</sub> cells in our lupus model. Early T<sub>FH</sub> cell differentiation is regulated by IL-6, inducible costimulator (ICOS), IL-2 and T cell receptor (TCR) signal strength in mouse models (52). This early T<sub>FH</sub> cell differentiation is generally thought to be the result of naïve T cell interactions with dendritic cells. However, B cells can be the dominant antigen-presenting cells that activate naïve CD4+ T cells in certain circumstances and B cells activated by TLR9 and TLR7 ligands in the context of viral infection are sufficient to induce T<sub>FH</sub> development in the absence of dendritic cells (54).

In summary, we have demonstrated that there is a critical threshold level of IRF5 in B cells that is required for disease development in the FcγRIIB<sup>−/−</sup>Yaa mouse lupus model and that IRF5 nuclear translocation is substantially reduced in B cells from IRF5 heterozygous mice following TLR7 activation. We also show in multiple lupus models and in immunization studies, that the level of IRF5 expression is dynamically regulated throughout the B cell activation process, increasingly progressively from germinal center B cells to mature plasma cells. Furthermore, IRF5 phosphorylation is increased in a synergistic manner by activation signals believed to be important in the generation of autoreactive germinal center B cells. The fact that a 50% reduction in B cell IRF5 expression is sufficient to largely abrogate disease development, suggests that targeting IRF5 in B cells may be an effective therapeutic approach in lupus.
Methods

**Generation of IRF5 floxed mice.**

The vector to target IRF5 was a pEZ-Frt-LoxP-DT (courtesy of Klaus Rajewsky). Three DNA fragments of the C57BL/6 mice IRF5 locus were inserted into the cloning site of the vector pEZ-Frt-LoxP-DT; 1.6 kb of the region upstream of the IRF5 exon 3, 2 kb of the region between exon 3 to exon 6, and 2.8 kb of the region downstream of exon 6 (Supplemental Figure 2). The linearized vector was electroporated into embryonic stem cells derived from C57BL/6 mice in the Transgenic Mouse Core at Brigham and Women’s Hospital, Boston, and correctly targeted ES cell colonies were identified by PCR. Selected ES cells were injected into blastocysts. Chimeras were bred with C57BL/6 mice to obtain mice with the conditional IRF5 allele in the germ-line as determined by PCRs. The mice were further crossed with ROSA26:FLPe knock in mice (Jackson Cat#9086) to delete the neomycin resistant gene between frt sites. IRF5 LoxP positioning was confirmed by PCR and southern blotting analysis.

**Animals.**

WT (IRF5+/+) FcγRIIB−/− Yaa, IRF5−/- FcγRIIB−/− Yaa, and IRF5+/− FcγRIIB−/− Yaa mice were all generated as previously described (5). FcγRIIB−/− Yaa Mb1cre/+ IRF5flox/+ (IRF5ΔB) were generated by crossing IRF5flox/flox mice on the FcγRIIB−/− Yaa background. Mice that expressed cre under the direction of the Ig Alpha promoter (mb1-cre mice) were kindly provided by Michael Reth (University of Freiburg, Freiburg, Germany) (32) and were also crossed on to the FcγRIIB−/− Yaa background. Mb1cre/+ FcγRIIB+/− Yaa males were then crossed to FcγRIIB−/− Yaa IRF5flox/flox females. The final experimental and littermate control mice were generated by breeding Mb1cre/+ FcγRIIB−/− Yaa males with FcγRIIB−/− Yaa IRF5flox/+ females. B6.SJL-Ptprcα
Pepc<sup>b</sup>/BoyJ (“CD45.1”) mice were purchased from the Jackson Laboratory. Mice that expressed cre under the direction of the LysM promoter (LysMcre mice) were purchased from the Jackson Laboratory (B6.129P2-Lyz<sup>2</sup>m1(cre)If/J; strain number 004781) (37). The final experimental and littermate control mice were generated in a similar manner to the Mb1cre study detailed above, except that the LysMcre/+ <i>FcyRIIB<sup>−/−</sup>Yaa</i> males were bred with <i>FcyRIIB<sup>−/−</sup>Yaa IRF5flox/flox</i> females, not <i>FcyRIIB<sup>−/−</sup>Yaa IRF5flox/+</i>. Mice deficient in interleukin-1 receptor-associated kinase 4 activity (<i>IRAK-4<sup>KN/KN</sup></i>) were kindly provided by Shizuo Akira (Osaka University, Osaka, Japan) (55). (NZBxNZW)F1 and MRL-<i>lpr</i> mice were purchased from the Jackson Laboratory (stock numbers 100008 and 000485 respectively). All mice used in this study, apart from (NZBxNZW)F1 and MRL-<i>lpr</i>, were backcrossed more than 9 generations onto the C57BL/6 background.

**Bone marrow chimera generation.**

Bone marrow cells were isolated from <i>FcyRIIB<sup>−/−</sup>Yaa IRF5<sup>+/+</sup></i> and <i>FcyRIIB<sup>−/−</sup>Yaa IRF5<sup>−/−</sup></i> donors and washed once in PBS followed by RBC lysis using RBC lysing buffer (Sigma R7757). Donor marrow cells (10<sup>6</sup> cells per mouse) were injected into the tail-veins of B6.SJL-Ptpr<sup>a</sup> Pepc<sup>b</sup>/BoyJ (“CD45.1”) recipient mice following cesium irradiation of recipient mice with two doses of 500 Rad separated by 3 to 4 hours. Recipient mice were subsequently placed in irradiated cages and water was supplemented with Sulfamethoxazole/Trimethoprim at 2/0.4 mg/ml. Immune cell engraftment was subsequently confirmed using flow cytometry of PBMC and antibodies specific for CD45.1 and CD45.2 (Biolegend).

**Immunization**
8-10 week old C57BL/6 mice were immunized intraperitoneally with 50 μg 4-hydroxy-3-nitrophenylacetyl coupled to chicken gamma globulin at a substitution rate of 16 (NP-CGG, Biosearch Technologies) in alum (56). Spleens were harvested at day 14.

**B cell isolation and stimulation.**

Resting B cells were purified from the spleens of WT FcγRIIB−/− Yaa, IRF5−/− FcγRIIB−/− Yaa, IRF5+/− FcγRIIB−/− Yaa, and IRF5ΔB mice by negative selection using MACS Cell Separation with anti-CD43 beads (Miltenyi Biotec, Bergisch Glasbach, Germany). Cells were plated at 1-2x10^5 per well in duplicate or triplicate in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and stimulated with 0.05μM R848 (InvivoGen, San Diego, CA), 10 μg/mL anti-IgM (F(ab’)2 fragment; μ chain specific), (Jackson Immunoresearch, West Grove, PA), 2 μg/mL anti-CD40 antibody (HM40-3, BD Biosciences) alone or in combination. Supernatants were collected 24 hours later and cytokines evaluated by ELISA (R&D Systems, Minneapolis, MN; BioLegend, San Diego, CA) and Procartaplex multiplex (Invitrogen, Waltham, MA). Cells were evaluated by flow cytometry for activation markers and intracellular levels of IRF5.

**Western blot and phospho-tag immunoblot analysis.**

Splenic B cells were lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO) that included phosphatase and protease inhibitors (Sigma-Aldrich). For standard western blot analysis, samples were separated on a 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA) using MOPS buffer (Invitrogen). For phospho-tag immunoblot analysis, SDS-PAGE was performed using SuperSep Phos-tag 7.5% precast gel, (Fujifilm Wako Chemicals USA Corporation). Samples were
transferred to nitrocellulose membranes using the iBlot (Invitrogen) and probed with anti-IRF5 antibody (ab181553, Abcam, Cambridge, United Kingdom) and anti-β actin antibody (8H10D10, Cell Signaling Technology, Danvers, MA). Samples were imaged using the Odyssey CLx (LICOR, Lincoln, NE) and quantified using ImageStudio.

Preparation of cytosolic and nuclear protein fractions.

Cytosolic and nuclear protein fractions were prepared using a slight modification of the protocol described by Liou et al (57). Splenic B cells were washed once with cold PBS and resuspended in buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, and 0.5 mM DTT) with 0.1% Triton X-100, phosphatase inhibitors (Sigma) and protease inhibitors (Calbiochem). Cells were lysed on ice for 10 minutes. After centrifugation, the supernatant was saved as the cytosolic fraction. The nuclear pellet was washed twice with buffer A to remove any residual cytosolic fraction proteins and was then resuspended in buffer C (20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, and 0.5 mM DTT) with protease inhibitors on ice. The nuclear pellet was sonicated briefly before incubation on ice for 20 minutes. The insoluble debris were removed by centrifugation and the supernatant was saved as the nuclear fraction. The protein concentration was determined with Pierce BCA Protein Assay Kit (Life Technologies).

Flow cytometry.

Splenocytes and bone marrow cells were treated with RBC lysing buffer. Splenocytes were labeled with antibodies against CD4 (GK1.5), CD8 (53-6.7) and CD3 (17A2) to identify T cell populations, against CD19 (ID3) and B220 (RA3-6B2) to identify B cells, and against CD44 (IM7), CD62L (MEL-14) to identify naive T cells, activated T cells and memory T cells. Biotin
labeled anti-CXCR5 (2G8) was used in combination with streptavidin-BV711, CD3, PD-1 (J43) and CD4 to identify T_{FH}. ABC B cells were identified by first gating on CD19 and B220, then gating out CD43 (S7) and CD93 (AA4.1) positive cells, followed by gating on CD21 (7G6) and CD23 (B3B4) negative cells. Finally, CD11c (HL3) and Tbet (eBio4B10) delineated Tbet+ ABC. The FoxP3 fix/perm from eBioscience was used for the intracellular staining of Tbet. For staining of spleen plasmablasts, antibodies against CD3, CD19, B220, CD44 and CD138 were used. For staining of bone marrow plasma cells, antibodies against CD4, CD8, Gr-1 and F4/80 were used to gate out non-B cells, and plasma cells (CD138 (281-2)+B220−) were identified using antibodies against B220 and CD138. Bone marrow plasma cells were also identified using same stain as used for spleen. Antibodies to CD19, CD38 (90), CD95 (Jo1) and B220 were used to define germinal center B cells. Antibodies to CD80 (16-10A1), CD86 (GL-1), CD275 (HK5.3) and MHCII (M5/114.15.2) were used to evaluate activation. B cells were first gated on CD19 and live cells (live/dead, 65-0866-14, Ebioscience). Antibodies were from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA) or BioLegend (San Diego, CA). Immunofluorescence was measured with a LSRII (BD Bioscience). Sorting experiments were done on BD FACs Aria III (BD Bioscience). The data was analyzed using FlowJo software (Tree Star, Ashland, OR).

*Intracellular flow cytometry.*

B cells or splenocytes were first stained with extracellular antigens. Cells were then fixed for 15 min at room temperature with BioLegend fixative. Cells were then washed with BioLegend permeabilize buffer and then cells were incubated with anti-IRF5 (W16007B, BioLegend) or isotype control (R&D systems, IC006P) diluted in 1X perm buffer. To identify neutrophils,
monocytes and plasmacytoid dendritic cells, splenocytes were stained with antibodies against CD11b (M1/70), Ly6c (HK1.4), Ly6G (1A8), PDCA1 (eBio927), CD19 and CD3.

**Histology.**

At the end of the experiment at 4-5 months of age, kidneys were harvested from *FcγRIIB*−/− *Yaa IRF5F/+ control and *FcγRIIB*−/− *Yaa IRF5A/B* mice and fixed in formalin or snap frozen in OCT compound. Formalin fixed and paraffin embedded kidneys were sectioned at 8 μm then stained with H&E. Kidney disease was assessed by a blinded investigator (RGB). To this end randomly selected areas of cortex were examined and at least fifty glomeruli from each animal were scored. Glomerular and interstitial disease was evaluated as previously described (5, 58, 59).

**Immunohistochemistry**

Mouse kidneys were snap-frozen in OCT (Tissue-Tek, Sakura Finetek, Torrance, CA) and stored at -80°C. Seven micrometer cryosections were cut and fixed with methanol and acetone (1:1), blocked with 1% BSA, and then stained with Alexa Fluor 594 conjugated donkey anti-mouse IgG (Invitrogen) and FITC conjugated goat anti-mouse C3 (Cappel Laboratories, Cochranville, PA) at 4°C overnight. After washing with Tris-buffered saline, the stained sections were scanned with a Nikon Deconvolution Wide-Field Epifluorescence System. Fluorescence intensity, representing IgG and C3 deposition, was measured using ImageJ and analyzed with GraphPad Prism (San Diego, CA). Representative images were acquired using a Zeiss LSM 710 confocal microscope.

**Serological Assays.**
IgG isotypes and IgM were measured by ELISA established using antibodies from BD Biosciences (San Jose, CA) and Southern Biotech (Birmingham, AL). Anti-nuclear autoantibody (ANA) titer was measured by immunofluorescence using HEp-2-coated slides (INOVA Diagnostics, Inc. San Diego, California) as described (5, 59). The anti-nucleosome antibody ELISA was developed using nucleosome antigen ATN02-05 (2µg/ml, Arotec Diagnostics Limited) and HRP-conjugated anti-mouse IgG antibody (1:3000, Sigma-Aldrich). Anti-nucleosome antibody (PL2-3) was used as the positive control for quantification. The anti-Sm/RNP ELISA was developed using Sm/RNP antigen ATR01 (2µg/ml, Arotec Diagnostics Limited) and HRP-conjugated anti-mouse IgG antibody (1:3000, Sigma-Aldrich). Anti-Sm/RNP antibody (Y2) was used as the positive control for quantification.

Statistics.

P values were calculated using a two-tailed, unpaired, Welch’s t-test or a one-way ANOVA with Tukey post hoc test. Details about the use of these tests for particular experiments are provided in each individual figure legend. P values < 0.05 were considered significant. Statistics were calculated using GraphPad Prism (San Diego, CA).

Study Approval.

All animal studies were performed under protocols that were approved by the Institutional Animal Care and Use Committees at Boston University and the University of Massachusetts Medical School.
**Author contributions**

AP, KY, UK, RGB, and IRR designed the study. AP, KY, ACB, VS, PS, KN, BKH, YX, YT, and RGB performed the experiments and analyzed the data. GAV optimized experimental protocols. AP and IRR wrote the manuscript. AP, KY, VS, PS, BKH, GAV, YX, UK, YT, RGB, and IRR read and commented on various drafts of the manuscript. For the two co-first authors, the assignment of authorship order was made after discussion with, and with the full agreement of, both co-first authors. The assignment was based on their overall contributions to the various components of the experimental work and manuscript preparation, recognizing that both co-first authors made very substantial and critical contributions to the manuscript.
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Figure 1. B cell specific reduction of IRF5 expression in IRF5 B mice. All analyses were done in 8-10-week-old FcγRIIB−/−Yaa mice. (A) Representative western blot of IRF5 protein expression in sorted splenic B cells (CD19+) and myeloid cells (CD11b+Ly6G−) from IRF5F/+ and IRF5 B mice. All lanes were run on the same gel but were noncontiguous. (B) Expression of IRF5 in B cells and myeloid cells from IRF5 B mice normalized to IRF5 F/+ (n=4). **P<0.01, NS non-significant analyzed using two-tailed, unpaired, Welch’s t-test. (C) Representative flow cytometry plots of intracellular IRF5 expression in CD19+ B cells, CD11b+ Ly6G+ monocytes, CD11b+ Ly6G+ neutrophils and CD11b− PDCA1+ Ly6G+ plasmacytoid dendritic cells (pDCs) from IRF5F/+ and IRF5 B mice (representative experiment of 3 individual experiments, n=2 for each genotype). (D) Mean fluorescence intensity (MFI) values of IRF5 in B cells, monocytes, neutrophils and pDCs from IRF5F/+ and IRF5 B mice normalized to the IRF5F/+ littermate control in each experiment (n=6). Data are means +/- SEM, analyzed using one-way ANOVA with Tukey post hoc test; ****P < 0.0001.
Figure 2: Splenomegaly and T cell activation is reduced in IRF5ΔB mice. All analyses were done in 5-month-old FcγRIIB−/−Yaa mice. (A) Spleen weights from WT (n=10), mb1cre (n=13), IRF5F/+ (n=16), IRF5ΔB (n=8) and IRF5 +/- (global heterozygous deletion, n=5) mice. (B) Splenic cell counts from WT (n=5), mb1cre (n=5), IRF5F/+ (n=14) and IRF5ΔB (n=8) mice. (C) Representative flow cytometry plots of CD4+ CD62L+ CD44+ (effector/memory) and CD62L+CD44- (naive) T cells from spleen of IRF5F/+ and IRF5ΔB mice. (D and E) Percentage and number of CD62L- CD44+ CD4+ T cells from WT (n=5), mb1cre (n=5), IRF5F/+ (n=15) and IRF5ΔB (n=8) mice. (F and G) Percentage and number of CD62L+ CD44+ CD4+ T cells. (H) Representative flow cytometry plots of CD8+ CD62L- CD44+ (effector/memory) and CD62L+CD44- (naive) T cells from spleen of IRF5F/+ and IRF5ΔB mice. (I and J) Percentage and number of CD8+ CD62L- CD44+ CD8+ T cells from WT (n=5), mb1cre (n=5), IRF5F/+ (n=15) and IRF5ΔB (n=8) mice. (K and L) Percentage and number of CD62L+ CD44+ CD8+ T cells. Data are means ± SEM, analyzed using one-way ANOVA with Tukey post hoc test; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS non-significant.
Figure 3. Serum IgG, auto-antibodies and plasma cells are reduced in IRF5ΔB mice. All analyses were done in 5-month-old FcγRIIB−/− Yaa mice. (A-D) IRF5 F/+ (n=11) and IRF5ΔB mice (n=5-7) were analyzed. (A) Serum IgG isotype concentrations. (B) Serum anti-nuclear autoantibody (ANA) titers. (C) Serum anti-nucleosome IgG concentration. (D) Serum anti-Sm/RNP IgG concentrations. (E) Representative flow cytometry plots and total numbers of splenic plasmablasts in IRF5 F/+ (n=15) and IRF5ΔB (n=8) mice. (F) Representative flow cytometry plots and percentages of bone marrow plasma cells in IRF5 F/+ (n=15) and IRF5ΔB (n=8) mice. Data are means +/- SEM, analyzed using two-tailed, unpaired, Welch's t-test; **P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 4. Decreased renal disease in IRF5ΔB mice. All analyses were done in 5-month-old FcγRIIB−/−Yaa mice. (A) Representative renal histology of IRF5F+/+ and IRF5ΔB mice. Red arrow depicts necrotic cells; black arrow depicts cellular crescent. Images were taken at 20X magnification. (B-D) Quantification of renal disease by (B) percentage of glomeruli with crescents or necrosis, (C) Glomerular injury score and (D) Interstitial disease. IRF5F+/+ (n=11) and IRF5ΔB (n=8). (E) Representative examples and (F) quantitation of glomerular IgG and complement C3 deposition measured by fluorescence intensity in 11-14 glomeruli per mouse from 5 mice per group. All scored glomeruli are shown. Images were taken at 20X magnification. Data are means +/-SEM, analyzed using two-tailed, unpaired, Welch’s t-test; ***P < 0.001, ****P < 0.0001.
Figure 5. LysMCre mediated deletion of IRF5 does not reduce disease in FcyRIIB−/−Yaa littermates. All analyses were done in FcyRIIB−/−Yaa littermates that either did not express LysMCre (IRF5 F/F) or did express LysMCre (IRF5ΔM). (A) Flow cytometry quantitation of intracellular IRF5 expression (MFI values) in neutrophils (CD11b+ Ly6G+), monocytes (CD11b+ Ly6C+) and B cells (CD19+) from spleen, and in peritoneal macrophages (F4/80+ CD11b+) in 8-10-week-old FcyRIIB−/−Yaa littermates (n=4 or 5). Data are means +/-SEM, analyzed using two-tailed, unpaired, Welch’s t-test. ***P < 0.001, ****P < 0.0001, NS non-significant. (B-D) All analyses were done in 5-month-old FcyRIIB−/−Yaa littermates. (B) Spleen weight and cell counts from IRF5 F/F (n = 10-12) and IRF5ΔM mice (n = 10-12). (C) Serum anti-nuclear autoantibody (ANA) titers, serum anti-nucleosome IgG concentration and serum anti-Sm/ RNP IgG concentration (n = 5-10). (D) Quantification of renal disease by glomerular injury score, percentage of glomeruli with crescents or necrosis, and interstitial disease (n = 10). Data are means +/- SEM, analyzed using two-tailed, unpaired, Welch’s t-test. NS non-significant.
Figure 6. Germinal center B cells, T-follicular helper cells and Tbet positive B cells are reduced in the spleen of IRF5$\Delta$B mice. Spleen cells from 8-10-week-old FoxRIIB$^+$Yaa WT (n=8), IRF5F/+ (n=10), IRF5$\Delta$B (n=8), IRF5 +/ (global heterozygous deletion, n=7) and IRF5+/- (global homozygous deletion, n=7) mice were analyzed. (A and B) Representative flow cytometry plots and total numbers of CD95+ CD38- germinal center B cells (gated on CD19+B220+ cells). (C and D) Representative flow cytometry plots and total numbers of CXCR5+ PD-1+ TFH T cells (gated on CD3+CD4+). (E) Upper panel indicates CD23-CD21- B cells (gated on B220+ CD19+ CD43+ CD93 cells); lower panel indicates Tbet+ CD11c+ ABC B cells gated on the CD23- CD21- B cells shown in the upper panel. A representative example is shown. (F) Total number of Tbet+ CD11c+ ABC. Data are means +/- SEM, analyzed using one-way ANOVA with Tukey post hoc test; *P < 0.05, **P < 0.01, ***P < 0.001, NS non-significant.
Figure 7. Reduced IRF5 expression in B cells decreases IL-6 and TNF-α production in vitro and serum IL-6 and TNF-α is reduced in IRF5Δ B mice. (A-C) B cells were isolated from the spleen of FcγRIIB−/−Yaa mice at 8-10 weeks of age and stimulated for 24 hours with anti-IgM, anti-CD40, R848, and CpG-B alone or in combination. (A) Representative experiments showing mean IL-6 and TNF-α production by B cells from WT, IRF5 Δ/Δ and IRF5 Δ/Δ mice (n=2 for each genotype). (B and C) IL-6 (n=6) and TNF-α (n=3) production after R848 stimulation (B) and CpG-B stimulation (C) by B cells from WT, IRF5 Δ/Δ and IRF5 Δ/Δ mice normalized to the WT control in each experiment. (D and E) IL-6 and TNF-α production by B cells from IRF5Δ Δ B mice normalized to the littermate IRF5F+/+ control in each experiment (n=4 for each genotype). Data are means ± SEM, analyzed using two-way ANOVA with Tukey post hoc test; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (F) Mean serum IL-6 and TNF-α levels from 5-month old IRF5 B (n=8) and littermate IRF5F+/+ (n=8) mice. Data are means ± SEM, analyzed using two-tailed, unpaired, Welch’s t-test; *P < 0.05, ***P < 0.001.
Figure 8. TLR7 signaling is required for IRF5 phosphorylation and IRF5 nuclear translocation is reduced in B cells from FoyR11B−/−Yaa IRF5+/− mice. (A and B) B cells were isolated from the spleens of FoyR11B−/−Yaa mice at 8-10 weeks of age. (A) B cells were stimulated with anti-IgM, anti-CD40, and R848 alone or in combination for 2 hours and the protein lysate analyzed using phospho-Tag gel (upper panel) or standard gel (lower panels). B cells isolated from an IRF5-deficient (IRF5−/−) mouse is shown in the first lane. p-IRF5 denotes phosphorylated IRF5. A representative example of seven individual experiments is shown. (B) Ratio of p-IRF5 to unphosphorylated IRF5 (u-IRF5). p-IRF5 intensity was normalized to the intensity of unphosphorylated IRF5 (lowest band of IRF5 on p-Tag gel as shown in A) (n=7). (C and D) B cells from FoyR11B−/−Yaa (WT) and FoyR11B−/−Yaa IRF5+/− mice were stimulated for 2 hours with R848, or not stimulated (untreated), and IRF5 was probed in the nuclear and cytoplasmic fractions. (C) A representative experiment of 4 individual experiments is shown and (D) Ratio of IRF5 expression in nucleus relative to the WT following R848 stimulation; nuclear IRF5 intensity in each sample was first normalized to its own loading control (histone) (n=5). Data are means ±/SEM, analyzed using one-way ANOVA with Tukey post hoc test; *P < 0.05, **P < 0.01, ***P < 0.001; NS non-significant.
Figure 9. IRF5 expression is increased in activated B cells in vitro. (A-E) Splenic B cells were isolated from 8-10 week old FcyRIIB−/− Yaa or C57BL/6 mice and were either not stimulated or were stimulated with anti-IgM, anti-CD40, and R848 alone or in combination for 24 hours. (A) Intracellular IRF5 levels were measured using flow cytometry. A representative experiment of 6 individual experiments using B cells from FcyRIIB−/− Yaa mice is shown. (B and D) Mean fluorescence intensity (MFI) of IRF5 with and without stimulation in B cells from FcyRIIB−/− Yaa mice (B) and C57BL/6 mice (D) (n=2 per strain) and (C and E) fold change of IRF5 expression normalized to unstimulated control in B cells from FcyRIIB−/− Yaa mice (C) and C57BL/6 mice (E) (n=6 per strain). Data are means +/-SEM, analyzed using one-way ANOVA with Tukey post hoc test; **P < 0.01, ****P < 0.0001.
Figure 10. IRF5 expression is increased in germinal center B cells, splenic plasmablasts, and bone marrow plasma cells in vivo. (A-G) Flow cytometry performed on splenocytes and bone marrow from FcyRIIB−/−Yaa mice at 8-10 weeks of age. (A) Representative examples of IRF5 expression in germinal center (GC) B cells (CD38− CD95+ CD19+) and non-GC B cells (CD38− CD95−) (upper panel); plasmablasts (PB) (CD44+ CD138+) and CD19+ CD138−B cells (non-PB) (middle panel); and bone marrow plasma cells (BM PC) (CD44+ CD138+) and non PB B cells from spleen (lower panel). (B) Fold change in IRF5 expression in GC B cells normalized to non-GC B cells (n=9). (C) Fold change in IRF5 expression in PB normalized to non-PB (n=8). (D) Fold change in IRF5 expression in PB normalized to spleenic non-PB (n=8). (E-G) IRF5 expression in NZB/W mice (n=6), MRL/fpr mice (n=6), and NP-CGG immunized C57BL/6 (n=6) mice. (E) Fold change of IRF5 expression in germinal center B cells. (F) Fold change of IRF5 expression in plasmablasts. (G) Fold change of IRF5 expression in plasma cells. Black histogram = isotype control in non-GC B cells, non-PB or BM PC; grey tinted histogram = isotype control in GC B cells, PB or BM PC; blue histogram = IRF5 expression in non-GC B cells non-PB; red histogram = IRF5 expression in GC B cells, PB or BM PC. Data are means +/- SEM, analyzed using two-tailed, unpaired, Welch’s t-test; *P<0.05, **P<0.01.