

B cell–derived IL-6 initiates spontaneous germinal center formation during systemic autoimmunity

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Recent studies have identified critical roles for B cells in triggering autoimmune germinal centers (GCs) in systemic lupus erythematosus (SLE) and other disorders. The mechanisms whereby B cells facilitate loss of T cell tolerance, however, remain incompletely defined. Activated B cells produce interleukin 6 (IL-6), a proinflammatory cytokine that promotes T follicular helper (T_{FH}) cell differentiation. Although B cell IL-6 production correlates with disease severity in humoral autoimmunity, whether B cell–derived IL-6 is required to trigger autoimmune GCs has not, to our knowledge, been addressed. Here, we report the unexpected finding that a lack of B cell–derived IL-6 abrogates spontaneous GC formation in mouse SLE, resulting in loss of class-switched autoantibodies and protection from systemic autoimmunity. Mechanistically, B cell IL-6 production was enhanced by IFN- γ , consistent with the critical roles for B cell–intrinsic IFN- γ receptor signals in driving autoimmune GC formation. Together, these findings identify a key mechanism whereby B cells drive autoimmunity via local IL-6 production required for T_{FH} differentiation and autoimmune GC formation.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic inflammatory disease characterized by the development of class-switched antinuclear antibodies. Multiple lines of evidence link germinal centers (GCs) with the genesis of autoantibody (autoAb)–producing plasma cells in SLE, including extensive somatic hypermutation in autoreactive B cell clones and the development of spontaneous GCs in both mouse lupus models and in human patients with lupus (Wellmann et al., 2005; Alois and Pujol-Borrell, 2006; Vinuesa et al., 2009). Importantly, rather than being downstream targets of T cell activation signals, autoreactive B cells can directly initiate breaks in T cell tolerance and spontaneous GC formation in SLE, via antigen presentation to CD4 $^{+}$ T cells in the context of MHCII (Giles et al., 2015; Jackson et al., 2016).

In addition to cognate interactions between B cells and T follicular helper (T_{FH}) cells, cytokine signals profoundly influence GC biology in autoimmunity. Although type 1 IFN signals are strongly associated with lupus disease activity, recent work has shown that dysregulated type 2 IFN (IFN- γ) signals function early in disease to promote autoimmune GC for-

mation. In independent lupus models, B and T cell–intrinsic IFN- γ receptor (IFN- γ R) activation promotes the generation of GC B cells and T_{FH} cells, respectively; suggesting that IFN- γ is critical for the initiation of spontaneous, autoimmune GCs (Lee et al., 2012; Domeier et al., 2016; Jackson et al., 2016). Importantly, these observations model longitudinal studies in human SLE showing that increased serum IFN- γ correlates with development of lupus-specific autoAb years before disease diagnosis or the development of a type 1 IFN signature. Notably, elevated serum IL-6 is also observed concurrently or before first positive autoAb in preclinical SLE, suggesting a key role for IL-6 signals in initiating breaks in B and/or T cell tolerance (Lu et al., 2016; Munroe et al., 2016).

IL-6 facilitates early T_{FH} differentiation by transiently inducing expression of the T_{FH} master transcription factor BCL-6 (Nurieva et al., 2009). Whether IL-6 is required for GC formation, however, remains controversial. For example, although early studies reported reduced GCs in IL-6–deficient mice after T–dependent antigen immunization (Kopf et al., 1998; Nurieva et al., 2008; Wu et al., 2009), antiviral GC responses were not affected by IL-6 deletion (Poholek et al., 2010; Eto et al., 2011; Karnowski et al., 2012). Rather, deletion of both IL-6 and IL-21 blocked the antiviral GC response, whereas GCs were preserved after deletion of either cytokine alone, suggesting redundant roles in T_{FH}

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Abbreviations used: autoAb, autoantibody; BCR, B cell receptor; dsDNA, double-stranded DNA; EAE, experimental autoimmune encephalomyelitis; EM, effector/memory; GC, germinal center; IC, immune complex; IFN- γ R, IFN- γ receptor; MOG, myelin oligodendrocyte glycoprotein; PNA, peanut agglutinin; Q β -VLP, Q β virus-like particle; SLE, systemic lupus erythematosus; Sm/RNP, Smith/ribonucleoprotein; T_{FH} , T follicular helper; WAS, Wiskott–Aldrich syndrome; μ MT, B cell deficient.

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differentiation (Karnowski et al., 2012). In contrast, in the BXSB.Yaa mouse lupus model, IL-6 deletion prevented T_{FH} and GC B cell expansion, resulting in loss of class-switched autoAb (Jain et al., 2016). Thus, IL-6 signals affect GC biology, but the context of antigen engagement likely influences the absolute requirement for IL-6 in promoting T_{FH} differentiation, GC development, and autoimmune pathogenesis.

Importantly, the cellular source for IL-6 responsible for systemic autoimmunity and spontaneous GCs has not been identified. In the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis, loss of B cell–derived IL-6 attenuates disease severity via reduced T_{H17} differentiation (Barr et al., 2012). However, myelin oligodendrocyte glycoprotein (MOG) antibody titers were not affected, suggesting that B cell IL-6 exerts limited effects on autoimmune GC formation. In an alternative model, B cell–intrinsic NF-κB1 deletion led to the development of autoimmune GCs that correlated with prominent B cell IL-6 production (de Valle et al., 2016). However, mixed chimera studies using that model suggested additional cell–intrinsic roles for NF-κB1 in preventing B cell–driven autoimmunity beyond IL-6 production. Thus, although B cell IL-6 production correlates with humoral autoimmunity, it remains unknown whether B cell–derived IL-6 is required for development of mouse SLE.

To dissect the B cell–intrinsic signals underlying lupus pathogenesis, we developed a chimeric model of mouse SLE in which B cells, but not other hematopoietic lineages, lack the Wiskott–Aldrich syndrome (WAS) protein (Becker-Herman et al., 2011). In this model, *Was*^{−/−} B cells initiate spontaneous humoral autoimmunity characterized by GC formation, class-switched antinuclear autoAb and immune complex (IC) glomerulonephritis, thus recapitulating several cardinal features of human SLE. An important advantage of the WAS chimera model is the ability to efficiently interrogate the B cell–intrinsic signals required for spontaneous GC formation and systemic autoimmunity. Using that strategy, we tested whether B cell–derived IL-6 is required for autoimmune GC formation. Strikingly, lack of B cell IL-6 prevented T_{FH} differentiation and the initiation of spontaneous GCs during mouse SLE, despite IL-6 production by non–B cell lineages. Further, we show that B cell IL-6 production *in vitro* is facilitated by synergistic B cell receptor (BCR), TLR7, CD40, and IFN-γ signals. Together, these findings suggest that during initial interaction between antigen-primed, autoreactive B cells and cognate CD4⁺ T cells, B cell–derived IL-6 is essential to achieve the threshold of cytokine and costimulatory signals necessary for spontaneous GC formation, leading to a break in B and T cell tolerance and systemic autoimmunity.

RESULTS AND DISCUSSION

IFN-γ synergizes with BCR, TLR, and CD40 signals to promote B cell IL-6 production

Recent studies have demonstrated that IFN-γ promotes spontaneous GC formation in mouse lupus, in a manner dependent on B cell–intrinsic IFN-γR and STAT1 expression but

independent of T-box transcription factor TBX₂₁ (encoded by *Tbx21*; Domeier et al., 2016; Jackson et al., 2016). Mechanistically, IFN-γR signals synergize with BCR-, TLR7-, and CD40-dependent signals to enhance expression of the GC master regulator transcription factor BCL-6 (Jackson et al., 2016); findings that suggest IFN-γ facilitates autoimmune GC formation by initiating a GC transcriptional program. Based on those observations, we predicted that *Ifngr*^{−/−} B cells would exhibit a selective disadvantage for entry into autoimmune GCs in a competitive setting. To test that idea, we adoptively transferred congenically marked CD45.1 *Was*^{−/−} (15%) and CD45.2 *Was*^{−/−}*Ifngr*^{−/−} (15%) BM, together with B cell–deficient (μMT) BM (70%) into lethally irradiated μMT recipients. Surprisingly, we observed no competitive advantage for *Was*^{−/−} versus *Was*^{−/−}*Ifngr*^{−/−} B cells for entry into the GC compartment (Fig. 1, A and B). These findings suggested that although B cell IFN-γR signals are critical for spontaneous GC formation, *Ifngr*^{−/−} B cells are able to adopt a GC B cell phenotype and enter established autoimmune GCs. More important, these data suggest that additional events, beyond increased B cell BCL-6 expression, explain the lack of GCs after B cell–intrinsic IFN-γR deletion.

In an effort to identify an additional key signal, we examined the effect of IFN-γ on mouse B cell activation *in vitro*, focusing on the potential role for B cell–derived cytokines. Among candidate cytokines, IFN-γ synergized with BCR, TLR7, and CD40 signals to markedly increase B cell IL-6 expression in an IFN-γR-dependent, T-box transcription factor TBX₂₁–independent manner (Fig. 1 C). After IFN-γ binding, IFN-γR subunits IFNLR1 and IFNLR2 oligomerize and activate Janus family kinases JAK1 and JAK2, resulting in STAT1 phosphorylation and translocation of STAT1 dimers to the nucleus (Ramana et al., 2002). Consistent with these data, the IFN-γ–mediated increase in IL-6 production was blocked by treatment of stimulated B cells with ruxolitinib, a JAK1/JAK2 inhibitor (Verstovsek et al., 2012) and tofacitinib, a JAK1/JAK3 inhibitor (Lee et al., 2014; Fig. 1 D). In addition, *Stat1*^{−/−} mouse B cells exhibited no increase in IL-6 production in response to IFN-γ; findings that paralleled absent IFN-γ–driven peanut agglutinin (PNA) binding in stimulated *Stat1*^{−/−} B cells *in vitro* and loss of spontaneous GCs *in vivo* after B cell–intrinsic STAT1 deletion (Fig. 1, E and F; Domeier et al., 2016).

IFN-γ also markedly increased IL-6 production by primary human B cells stimulated *in vitro* with anti-IgM, R848, and CD40L. We previously demonstrated that IFN-γ increased the proportion of stimulated human B cells, exhibiting a CD38⁺CD27[−] early GC phenotype (Jung et al., 2000; Jackson et al., 2016). Notably, in human B cell cultures, this CD38⁺CD27[−] subset exhibited increased intracellular IL-6 staining compared with CD38[−]CD27[−] naive B cells (Fig. 1 G), implicating human GC B cells as a source for IL-6. In addition to increasing the proportion of CD38⁺CD27[−] B cells, IFN-γ treatment increased intracellular IL-6 mean fluorescence intensity among CD38⁺CD27[−]–gated human B cells (Fig. 1 H).

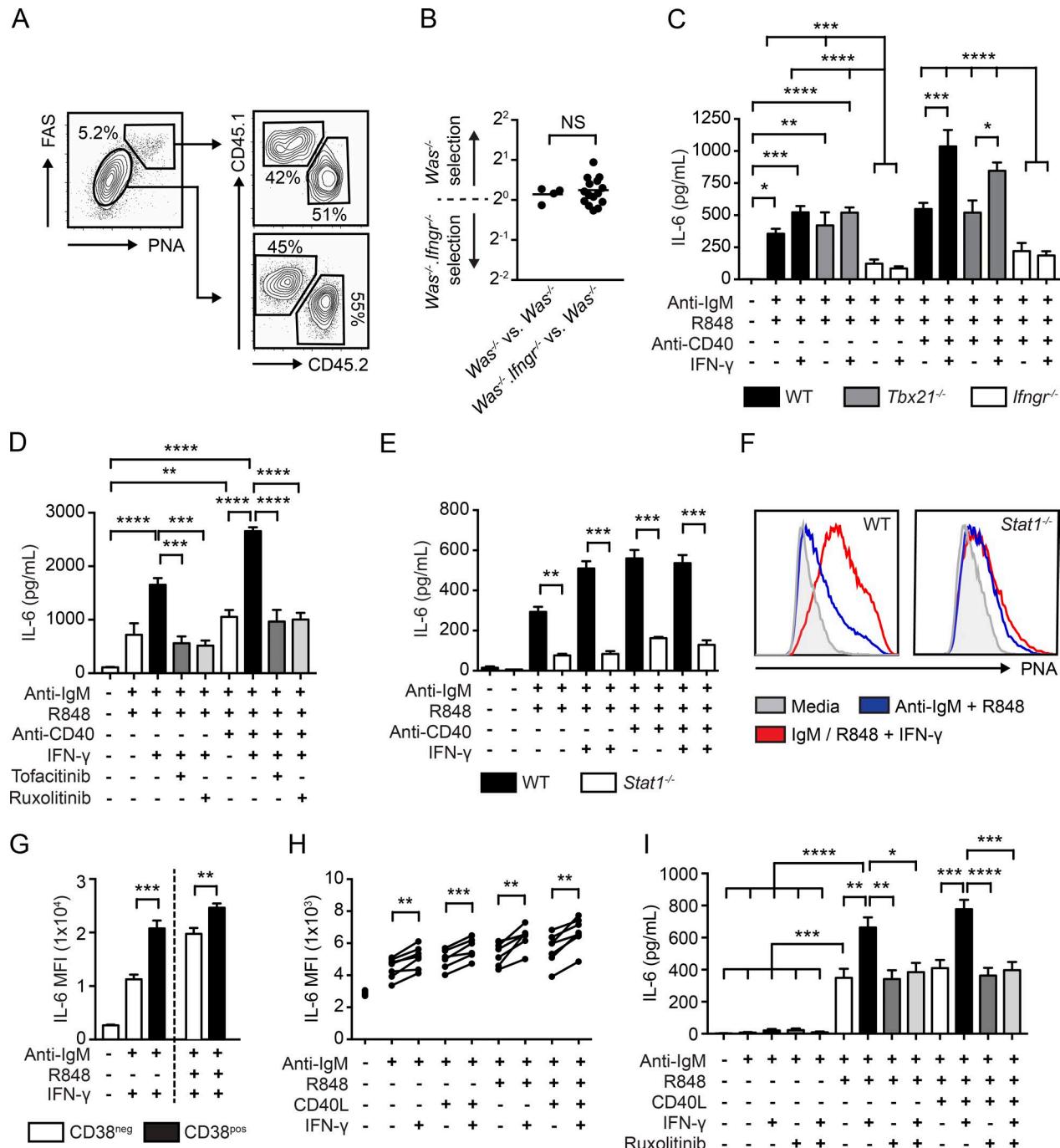


Figure 1. IFN-γ synergizes with B cell activation signals to promote IL-6 production by mouse and human B cells. (A) Representative FACS plots showing CD45.1⁺ Was^{-/-} versus CD45.2⁺ Was^{-/-}/Ifngr^{-/-} B cells in GC and non-GC B cell compartments. (A, left) Gated on CD19⁺ B cells. (A, right) Gated on PNA⁻FAS⁺ GC B cells (top) and PNA⁻FAS⁻ non-GC B cells (bottom). Number equals percentage within gate. (B) Selection of CD45.1⁺ Was^{-/-} versus CD45.2⁺ Was^{-/-}/Ifngr^{-/-} B cells into the GC compartment. NS, not significant. (C) IL-6 from splenic WT (black), *Tbx21*^{-/-} (gray), and *Ifngr*^{-/-} (white) B cells cultured for 48 h with anti-IgM, R848, anti-CD40 and/or IFN-γ. (D) IL-6 production by stimulated mouse B cells with or without ruxolitinib or tofacitinib (500 nM). (E) IL-6 in WT (black) and *Stat1*^{-/-} (white) B cells stimulated as indicated. (F) Surface PNA binding in WT (left) and *Stat1*^{-/-} (right) splenic B cells stimulated as indicated. (G) IL-6 intracellular staining at 72 h in cultured human B cells, gated as CD38⁻CD27⁻ "naïve" (white) or CD38⁺CD27⁺ "GC" (black) B cells. (H) IL-6 mean fluorescence intensity by intracellular staining in human CD38⁺CD27⁻ B cells stimulated for 24 h. (I) IL-6 from human B cells stimulated for 72 h with or without ruxolitinib (500 nM). (B-E and G-I) Error bars indicate means \pm SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001, by two-tailed Student's t test (B); by one-way ANOVA and Tukey's multiple comparison test (C-E and I); or by paired two-tailed Student's t test (G and H). (H) Data shown as paired analysis of different stimulation conditions from individual human donors. (A and B) Data representative of three independent Was^{-/-} versus Was^{-/-}/Ifngr^{-/-} competitive chimeras. (C-H) Data representative of at least two independent experiments.

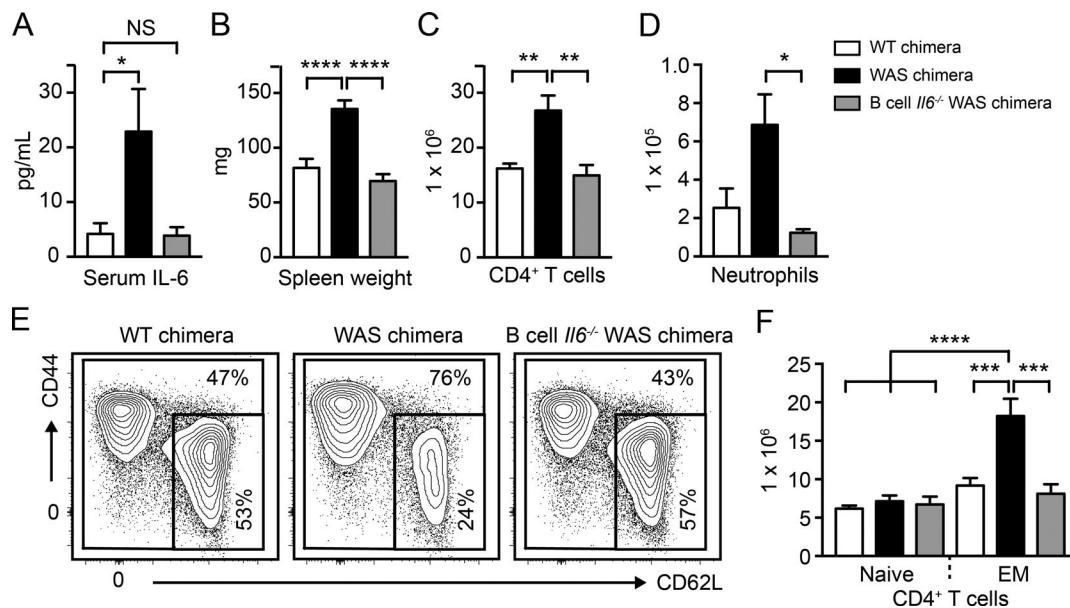


Figure 2. B cell-derived IL-6 promotes systemic inflammation in SLE. (A) Serum IL-6 levels in WT, $Was^{-/-}$, and B cell-intrinsic $Was^{-/-}Il6^{-/-}$ chimeras. (B–D) Spleen weight (B), number of splenic CD4⁺ T cells (C), and CD11b⁺GR1⁺ neutrophils (D) in indicated chimeras. (E) Representative FACS plots (gated on CD4⁺ T cells) showing naive (CD44^{lo}CD62L^{hi}) and effector/memory (CD44^{hi}CD62L^{lo/hi}) CD4⁺ T cells. The percentages are those within the gate. (F) Number of naive and effector/memory CD4⁺ T cells. (A–F) Error bars indicate means \pm SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001, by one-way ANOVA and Tukey's multiple comparison test. Data are representative of four WT (white, n = 11), four $Was^{-/-}$ (black, n = 13), and two B cell-intrinsic $Was^{-/-}Il6^{-/-}$ (gray, n = 6) independent chimeras.

In parallel, IL-6 concentrations in culture supernatants were markedly increased by IFN- γ treatment. Finally, that IFN- γ -dependent increase in B cell IL-6 production was inhibited by ruxolitinib and tofacitinib treatment (Fig. 1 I and not depicted). Together, these data demonstrate that IFN- γ synergizes with BCR-, TLR-, and CD40-dependent signals to directly enhance IL-6 production by the mouse and human B cells.

B cell-derived IL-6 promotes systemic inflammation in mouse lupus

The prominent IL-6 production by activated B cells in response to signals known to promote spontaneous GC formation suggested a role for this proinflammatory cytokine in lupus pathogenesis. Notably, serum IL-6 levels were markedly increased in autoimmune WAS chimeras (Fig. 2 A), in keeping with human studies showing elevated serum IL-6 levels in patients with SLE (Linker-Israeli et al., 1991; Gröndal et al., 2000; Lu et al., 2016). To test whether B cell-derived IL-6 is required for SLE, we generated WAS B cell chimeras, in which IL-6 deletion was limited to the B cell compartment (by reconstituting lethally irradiated μ MT recipients with 20% WT, $Was^{-/-}$ or $Was^{-/-}Il6^{-/-}$ BM, together with 80% μ MT BM). After reconstitution, all B cells were donor derived (either WT, $Was^{-/-}$, or $Was^{-/-}Il6^{-/-}$), with minimal WAS chimerism in the myeloid and T cell lineages (unpublished data; Becker-Herman et al., 2011).

Notably, B cell-intrinsic IL-6 deletion eliminated the immune activation characteristic of the WAS chimera model (Becker-Herman et al., 2011; Jackson et al., 2014, 2016).

Although WAS chimeras develop prominent splenomegaly, spleen size in B cell-intrinsic $Il6^{-/-}$ WAS chimeras was equivalent to WT controls, an observation explained by the lack of myeloid and T cell expansion in the absence of B cell-derived IL-6 (Fig. 2, B–D). In keeping with proinflammatory roles for IL-6, the proportion of CD4⁺ T cells exhibiting an effector/memory phenotype was markedly increased in WAS chimeras, whereas that T cell activation was abrogated by B cell IL-6 deletion (Fig. 2, E and F). Strikingly, despite intact IL-6 production by non-B cell lineages in this model, serum IL-6 levels were markedly reduced in B cell IL-6-deficient WAS chimeras (Fig. 2 A). These combined findings indicate that B cells comprise the dominant source for IL-6 during humoral autoimmunity and/or that B cell IL-6 initiates an inflammatory cascade that promotes IL-6 secretion by activated, non-B cell lineages.

B cell-derived IL-6 initiates spontaneous autoimmune GCs

Disease development in the WAS chimera model depends on the formation of spontaneous autoimmune GCs responsible for generation of pathogenic, class-switched, antinuclear autoAb. As previously shown, autoreactive B cells activated by dual BCR and TLR ligands initiate GC formation by presenting self-antigens to cognate CD4⁺ T cells (Jackson et al., 2014, 2016). Based on those observations, we hypothesized that B cell-derived IL-6 might be critical for GC formation by facilitating BCL-6 expression and T_{FH} differentiation during initial interactions with cognate CD4⁺ T cells.

In keeping with that hypothesis, lack of B cell–derived IL-6 abrogated the expansion of CXCR5⁺PD-1⁺ T_{FH} cells (Fig. 3, A and B). In parallel with the loss of T_{FH} cells, the proportion of PNA⁺FAS⁺ GC B cells was markedly reduced compared with autoimmune WAS chimeras and was equivalent to WT chimera controls (Fig. 3, C and D). We confirmed those findings by immunofluorescence staining demonstrating prominent PNA⁺ GCs in splenic sections from diseased WAS chimeras that were markedly reduced in the absence of B cell–derived IL-6 (Fig. 3 E). We next assessed the effect of the lack of spontaneous GCs on serum autoAb titers. Notably, although anti–double-stranded DNA (dsDNA) and Sm/RNP IgM titers were modestly affected by B cell IL-6 deletion, lack of B cell–derived IL-6 abrogated class-switched IgG autoAb, including autoAb of the pathogenic IgG2c subclass (Fig. 3, F and G).

Because these observations contrasted with redundant roles for IL-6 in GC formation in nonautoimmune settings (Poholek et al., 2010; Eto et al., 2011; Karnowski et al., 2012), we examined whether *Was*^{−/−}*Il6*^{−/−} B cells were able to form GCs in response to immunization with Q β virus-like particles (Q β -VLPs) containing ssRNA, a model of RNA virus infection (Hou et al., 2011). Using fluorescently labeled Q β -VLPs, we first confirmed that WT Q β -specific B cells are recruited into GCs after Q β -VLP immunization, in a manner dependent on CD40:CD40 ligand interactions with CD4⁺ T cells (Fig. S1). We next immunized WAS and B cell-intrinsic *Il6*^{−/−} WAS chimera cohorts with Q β -VLPs at 6–8 wk posttransplant, a time point after B cell reconstitution, but before the development of humoral autoimmunity (Jackson et al., 2016). Strikingly, an equivalent proportion of Q β -specific B cells adopted a PNA⁺FAS⁺ GC phenotype in the absence of B cell–derived IL-6 (Fig. 3, H and I). Thus, absent autoimmune GCs after B cell-intrinsic IL-6 deletion in the WAS model cannot be explained by defective activation responses of *Was*^{−/−}*Il6*^{−/−} B cells. Rather, our findings uncover a critical, context-specific role for B cell–derived IL-6 in facilitating the formation of spontaneous, autoimmune GCs necessary for the generation of class-switched autoAbs.

Lack of B cell IL-6 production protects against IC glomerulonephritis

Lupus nephritis is characterized by glomerular IC deposition, resulting in complement activation and the recruitment of inflammatory cells into inflamed glomeruli (Lech and Anders, 2013). To assess whether the lack of class-switched autoAbs in B cell-intrinsic *Was*^{−/−}*Il6*^{−/−} chimeras correlated with protection from renal disease, we first quantified glomerular IC deposition by immunofluorescence staining. Notably, whereas glomerular IgM deposition did not differ between genotypes, lack of B cell–derived IL-6 abrogated class-switched IgG and IgG2c antibody deposits (Fig. 4, A–C). In keeping with critical roles for IgG2c subclass antibodies in promoting complement activation (Nimmerjahn and Ravetch, 2005), C3 complement deposition was prevented by B cell-intrinsic IL-6 de-

letion (Fig. 4 D). Finally, although WAS chimeras developed prominent glomerulonephritis characterized by glomerular basement-membrane thickening, mesangial expansion, and glomerular hypercellularity, inflammatory changes were absent in B cell IL-6-deficient chimeras (Fig. 4 E). Together, these findings demonstrate that loss of B cell–derived IL-6 is sufficient to prevent IC glomerulonephritis in this mouse model of lupus nephritis.

In summary, our study provides novel insight into the mechanisms whereby B cells promote humoral autoimmunity by demonstrating that B cell–derived IL-6 is critical for spontaneous GC formation. Based on these data, we propose a model in which naïve, autoreactive B cells are first activated by dual BCR and TLR signals after recognition of nucleic acid-containing self-ligands. Subsequently, BCR/TLR-primed B cells migrate to the T–B border to present antigens to cognate CD4⁺ T cells. During these initial B:T interactions, we propose that B cell–derived IL-6 facilitates transient expression of the T_{FH} master regulator BCL-6 in cognate CD4⁺ T cells. Importantly, B cell IL-6 production is markedly enhanced by IFN- γ , likely derived locally from either activated T_H1 cells or from T_H1-biased CXCR3⁺ T_{FH} cells (Morita et al., 2011; Bentebibel et al., 2013). During autoimmunity, we predict that B cell–derived IL-6 facilitates CD4⁺ T cell activation and BCL-6 expression above a threshold required for T_{FH} differentiation and spontaneous GC formation. In this context, decreased local IL-6 levels likely contribute to absent GCs after B cell-intrinsic IFN- γ R deletion (Domeier et al., 2016; Jackson et al., 2016), although whether *Ifngr*^{−/−} B cells produce less IL-6 in vivo during autoimmunity has not yet been formally tested.

Importantly, although B cell–derived IL-6 is critical during this initial GC-dependent priming phase, autoreactive T cells likely sustain and propagate autoimmunity, via the production of IL-21 by cognate T_{FH} cells and by the activation of additional naïve, autoreactive B cell clones, events which may occur independently of B cell TLR and, possibly, B cell–derived cytokine signals (Giles et al., 2017). In addition, although direct interactions between autoreactive B cells and cognate CD4⁺ T cells are likely critical in this setting, B cell–derived IL-6 might also indirectly affect autoimmune GC formation, by, e.g., promoting the activation of DCs that facilitate T_{FH} differentiation.

An important question is how to reconcile these findings with preserved GC development in IL-6-null mice after infectious antigen challenge. In particular, IL-6 is redundant for antiviral GCs (Karnowski et al., 2012), despite the predicted similar requirement for B cell-intrinsic TLR7 signals in initiating GCs in SLE and after influenza infection (Hou et al., 2011; Jackson et al., 2014). Using the identical chimeric mouse model, we highlight the distinct requirements for B cell–derived IL-6 in facilitating the formation of GCs in autoimmunity versus responses to RNA-containing VLP immunization. We anticipate that, although multivalent viral surface epitopes and viral nucleic acid can promote robust BCR

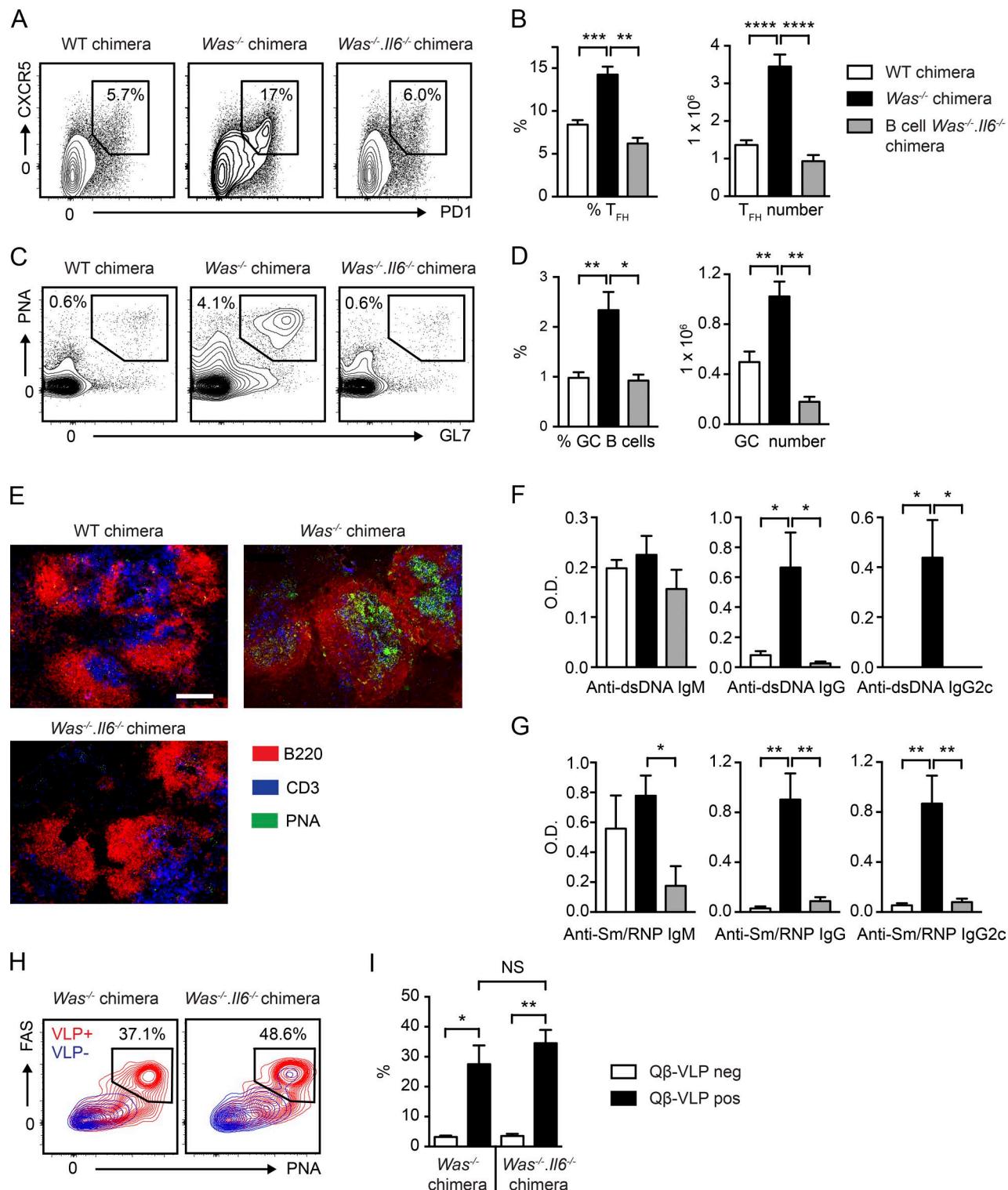


Figure 3. B cell IL-6 production initiates spontaneous, autoimmune GCs. (A and B) Representative FACS plots (gated on splenic CD4⁺ T cells) showing PD1⁺CXCR5⁺ T_{FH} cell percentage (left) and total number (right) of splenic T_{FH} cells in indicated chimeras. (C) Representative FACS plots (gated on splenic CD19⁺ B cells) showing loss of PNA⁺GL7⁺ GC B cells after B cell-intrinsic *Il6*^{-/-} deletion. (D) Percentage (left) and total number (right) of splenic GC B cells in indicated chimeras. (E) Representative splenic sections stained with B220 (red), PNA (green), and CD3 (blue). Bar, 150 μ m. (F and G) Anti-dsDNA (F) and anti-Sm/RNP (G) IgM, IgG, and IgG2c autoAb at 12 (F) and 24 (G) wk after transplant. (H) Representative overlaid FACS plots showing PNA⁺FAS⁺ GC phenotype in Q β -VLP⁺ (red) versus Q β -VLP⁻ (blue) B cells in *Was*^{-/-} (left) and B cell-intrinsic *Was*^{-/-}/*Il6*^{-/-} (right) chimeras, 12 d after Q β -VLP immunization. Percentages

and TLR signals in virus-specific B cells, disease development in autoimmunity is first initiated by low-affinity, autoreactive B cells that have escaped developmental B cell tolerance. Consistent with that idea, humoral autoimmunity in the WAS chimera model is a stochastic process driven by modestly increased BCR and TLR signaling in *Was*^{-/-} B cells (Becker-Herman et al., 2011). In this manner, we propose that B cell-derived IL-6 is critical for the initiation of spontaneous GCs but is redundant in the setting of antiviral GC responses.

Notably, significant complexity exists regarding the contributions of B cell-derived IL-6 to the pathogenesis of systemic autoimmunity. For example, although B cell IL-6 potentiated EAE severity, lack of B cell IL-6 production exerted no effect on anti-MOG antibody titers induced by immunization with MOG peptide emulsified in complete Freund's adjuvant and pertussis toxin (Barr et al., 2012). These data suggest that, although robust adjuvants can overcome the requirement for B cell IL-6 in autoantibody production, B cell-derived IL-6 remains critical for pathogenic T cell activation and T_H17 differentiation in EAE. Thus, specific roles for B cell IL-6 production in the pathogenesis of systemic autoimmunity are context dependent and likely differ among autoimmune models.

Despite these caveats, our data mirror recent findings in human SLE, wherein the initial development of class-switched autoAbs correlates with increased serum IFN- γ and IL-6 levels. These events occur years before the onset of clinical SLE and also predate increases in the lupus-associated type-1 IFN and B cell activating factor of the TNF family (Lu et al., 2016; Munroe et al., 2016). Thus, the immune mechanisms underlying initial breaks in B and T cell tolerance and progression of systemic autoimmunity are likely distinct, with B cells fulfilling critical roles in orchestrating initial breaks in tolerance. In addition, although most frequently studied in the context of SLE, this paradigm likely also has relevance to other human autoimmune diseases, including type 1 diabetes and rheumatoid arthritis, diseases characterized by the development of spontaneous GCs within inflamed tissues and by the serial accumulation of disease-specific autoAbs years before the onset of clinical disease (Pihoker et al., 2005; Aloisi and Pujol-Borrell, 2006; Vinuesa et al., 2009; Deane et al., 2010).

MATERIALS AND METHODS

Mice

CD45.1 and CD45.2 C57BL/6, μ MT, *Was*, *Il6*^{-/-}, *Ifngr*^{-/-}, *Tbx21*^{-/-}, and *Stat1*^{-/-} mice and the relevant mouse crosses were bred and maintained in the specific pathogen-free an-

imal facility of Seattle Children's Research Institute (Seattle, WA). All animal studies were conducted in accordance with Seattle Children's Research Institute institutional animal care and use committee-approved protocols.

BM transplantation

BM was harvested from the femora and tibiae of C57BL/6 ("WT"), *Was*^{-/-}, *Was*^{-/-}*Ifngr*^{-/-}, *Was*^{-/-}*Il6*^{-/-}, and μ MT mice. Single cell suspensions were depleted for CD138⁺ cells (130-098-257; Miltenyi Biotec). CD138-depleted WT, *Was*^{-/-}, *Was*^{-/-}*Ifngr*^{-/-} or *Was*^{-/-}*Il6*^{-/-} donor BM was mixed with μ MT BM (15:15:70 ratio for competitive chimeras; 20:80 ratio for WAS chimeras), and 6×10^6 total BM was injected retro-orbitally into lethally irradiated (450 centigray 2 times) μ MT recipients. Resulting BM chimeras were bled every 8–12 wk from posttransplant date by retro-orbital puncture and sacrificed at 24 wk after transplant. Data are representative of at least two independent experimental cohorts for each chimera.

Antibodies

Anti-mouse antibodies used in this study included: B220 (RA3-6B2), CD19 (1D3), CD4 (RM4-5), CXCR5 (2G8), and Fas (Jo2) from BD; CD62L (MEL-14), CD11c (N418), Gr-1 (RB6-8C5), Ly5.1 (A20), Ly5.2 (104), CD11b (M1/70), GL7 (GL-7), and PD-1 (J43), from eBioscience; goat anti-mouse IgM⁻, IgG⁻, IgG2c⁻ horseradish peroxidase-conjugated, unlabeled, or isotype from SouthernBiotech; CD19 (6D5), CD24 (M1/69), CD23 (B3B4), CD21 (7E9), CD44 (IM7), CD4 (RM4-4), Ly5.2 (104) from BioLegend; PNA (Fl-1071) from Vector Laboratories. Anti-human antibodies used in this study include CD38 (HIT2) and CD27 (L128) from BD; CD19 (HIB19) and IgM (MHM-88) from BioLegend; and IL-6 (MQ2-12A5) from eBioscience.

Flow cytometry

For mouse studies, single-cell splenocyte suspensions were obtained, as previously described (Becker-Herman et al., 2011) and incubated with fluorescence-labeled antibodies for 20 min at 4°C. Data were collected on a LSR II (BD) and analyzed using FlowJo software (Tree Star). For human studies, cells were stained with a fixable viability dye for 10 min at room temperature, followed by incubation with human TruStain FcX (BioLegend) for 10 min at 4°C. Surface antibodies were stained for 20 min at 4°C followed by incubation with transcription factor fixation/permeabilization solution (BD) for 40 min at 4°C. Intracellular antibodies were then stained for 40 min at 4°C in permeabilization

are Q β -VLP⁺ B cells within the PNA⁺FAS⁺ gate. (I) Percentage of PNA⁺FAS⁺ B cells in Q β -VLP⁺ (black) versus Q β -VLP⁻ (white) B cells in indicated chimeras. (B, D, F, G, and I) Error bars indicate means \pm SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; NS, not significant, by one-way ANOVA and Tukey's multiple comparison test. (A–G) Data representative of four WT (white, n \geq 10), four *Was*^{-/-} (black, n \geq 12), and two B cell-intrinsic *Was*^{-/-}*Il6*^{-/-} (gray, n = 6) independent chimeras. (H and I) Representative FACS plots (H) and combined data (I) from two independent, B cell-intrinsic, chimera experiments (*Was*^{-/-} [n = 4] and *Was*^{-/-}*Il6*^{-/-} [n = 5]).

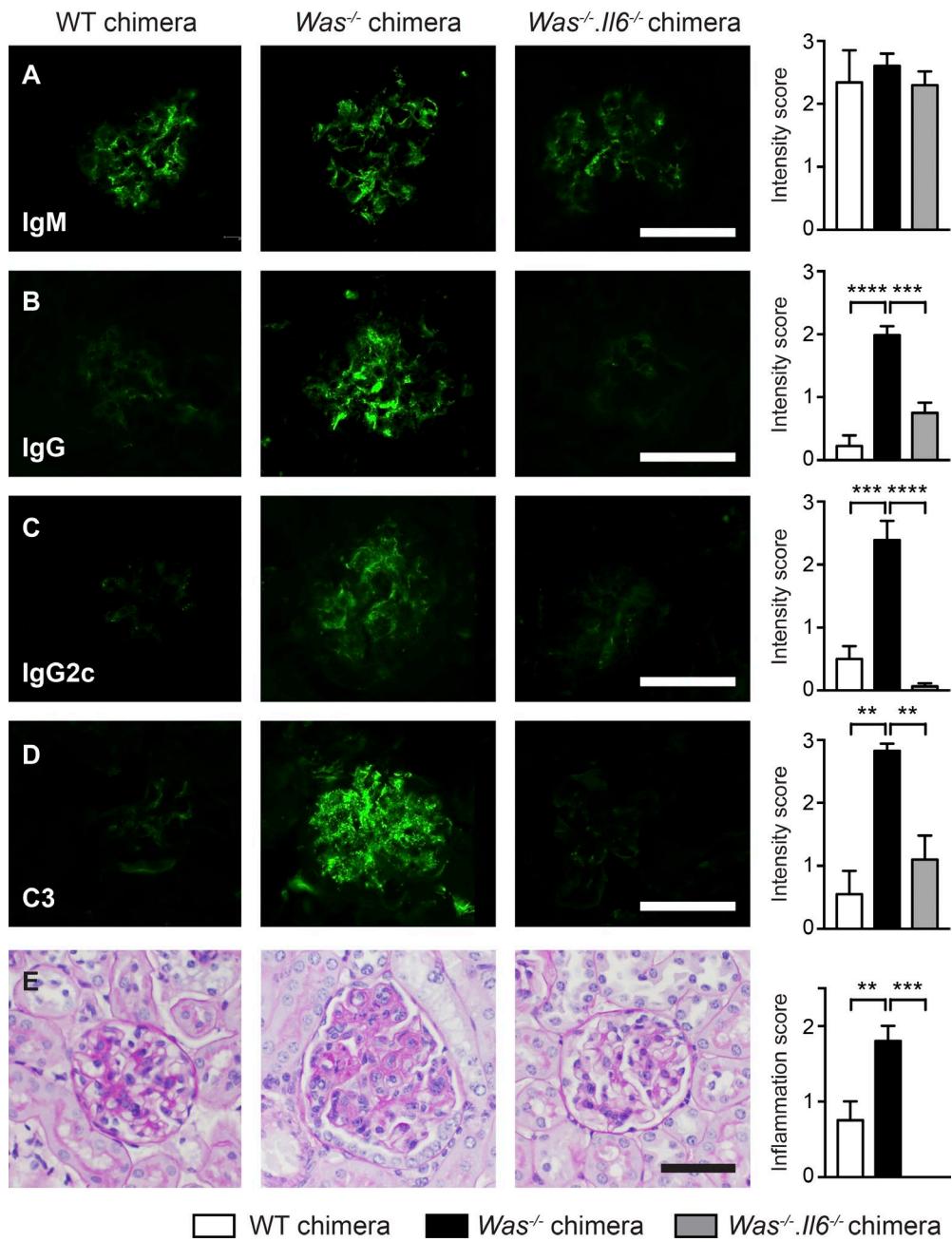


Figure 4. Development of IC glomerulonephritis requires B cell-derived IL-6. (A–D) Immunofluorescence staining for glomerular (A) IgM; (B) IgG; (C) IgG2c; (D) C3. (Left) Representative images. (Right) Intensity of glomerular immunofluorescence staining. (E, left) Representative kidney sections stained with periodic acid–Schiff. (E, right) Glomerular inflammation score. (A–E) Data representative of ≥ 2 independent chimeras, including WT (white, $n = 3$ [A–D], $n = 4$ [E]), *Was*^{-/-} (black, $n = 5$ [A–E]), and B cell–intrinsic *Was*^{-/-}*Il6*^{-/-} (gray, $n = 5$ [A–D], $n = 4$ [E]) chimeras. Scoring by observers blinded to genotype. Error bars indicate means \pm SEM. Bars, 50 μ m. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by one-way ANOVA and Tukey's multiple comparison test.

buffer (BD). Data collected with human samples were run on a FACSCanto II (BD).

Human subjects

All procedures followed were in accordance with the ethical standards of the responsible committee on human ex-

perimentation and with the Helsinki Declaration of 1975 as revised in 2008, and were approved by Benaroya Research Institute's Institutional Review Board. PBMC were derived from subjects participating in the Benaroya Research Institute control registry. Control participants were selected based on a lack of personal or family history of autoimmune disease.

Written informed consent was obtained from all subjects before their participation.

In vitro stimulations

Mouse. Splenic B cells were purified from WT, *Ifngr*^{-/-}, *Tbx21*^{-/-}, and *Stat1*^{-/-} mice by CD43-microbead depletion (Miltenyi Biotec). Purified B cells were cultured in RPMI-1640 medium (supplemented with 10% FCS, 1% penicillin-streptomycin, sodium pyruvate, Hepes, glutaMAX, and 0.1% β -ME) at 37°C for 48 h. B cells were seeded at a density of 10⁶ cells/well in a 96-well plate with or without R848 (5 ng/ml); anti-mouse IgM F(ab')₂ fragment (1 μ g/ml, Jackson ImmunoResearch, Inc.); anti-mouse CD40 (1 μ g/ml, SouthernBiotech); recombinant mouse IFN- γ (200 U/ml, BioLegend); and ruxolitinib (500 nM) or tofacitinib (500 nM). B cell surface markers were evaluated by flow cytometry. Cell proliferation was evaluated by Cell Trace Violet (Thermo Fisher Scientific) dilution. IL-6 in the supernatants and serum was measured by mouse IL-6 ELISA (eBioscience).

Human. Human total B cells were purified with a human B cell isolation kit II (Miltenyi Biotec). Total B cells were plated at 5 \times 10⁴ in a 96-well plate for 24 or 72 h with combinations of anti-IgM (10 μ g/ml; Jackson ImmunoResearch, Inc.), CD40L (5 μ g/ml; PeproTech), R848 (3.75 μ g/ml; Thermo Fisher Scientific), IFN- γ (10 ng/ml; R&D Systems) and ruxolitinib (500 nM). Cells were analyzed at 24- and 72-h time points. Supernatant IL-6 was measured by ELISA (eBioscience).

Measurement of cytokines and autoantibodies

Serum and supernatant IL-6 levels were measured with the mouse IL-6 ELISA Ready-SET-Go! kit (88-7064-22; eBioscience). For specific autoAb ELISAs, 96 well Nunc-Immuno MaxiSorp plates (Thermo Fisher Scientific) were precoated overnight at 4°C with calf thymus dsDNA (100 μ g/ml; D3664-5X2MG; Sigma-Aldrich) or Sm/RNP (5 μ g/ml; ATR01-10; Arotec Diagnostics). Plates were blocked for 1 h with 1% BSA in PBS before addition of diluted serum for 2 h. Specific antibodies were detected using goat anti-mouse IgM-, IgG-, or IgG2c-horseradish peroxidase (1:2,000 dilution; SouthernBiotech), and peroxidase reactions were developed using OptEIA TMB substrate (BD) and stopped with sulfuric acid. Absorbance at 450 nm was read using a SpectraMax 190 microplate reader (Molecular Devices).

VLP immunization

Mice were immunized i.p. with 20 μ g Q β -VLPs, and spleens were harvested for flow analysis on d 12 after immunization. Q β -specific B cells were identified ex vivo using Alexa Fluor 647-labeled Q β -VLPs, as described (Hou et al., 2011).

Spleen and kidney immunofluorescence staining

Mouse spleens and kidneys were embedded in OCT compound and frozen over dry ice. 5- μ m sections were cut on a

cryostat, mounted on Superfrost Excell slides (Thermo Fisher Scientific), and fixed in -20°C acetone for 20 min. After rehydration in staining buffer (PBS, 1% goat serum, 1% BSA, 0.1% Tween-20), slides were stained with: B220-PE (BD), CD3-APC (Thermo Fisher Scientific), and PNA-FITC (spleen; Vector Laboratories) or IgM-FITC (Jackson ImmunoResearch), IgG-FITC (Sigma-Aldrich), IgG2c-FITC (SouthernBiotech), or C3-FITC (kidney; MP Biomedicals). Images were acquired using a Leica Biosystems DM6000B microscope, a Leica Biosystems DFL300 FX camera, and Leica Biosystems Application Suite Advanced Fluorescence software. For glomerular immune complex quantification, images were obtained using a constant exposure and scored from 0 to 3 by two independent observers blinded to genotype.

Histopathology

Kidneys were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μ m, and stained with periodic acid-Schiff, according to standard practices. Histology images were acquired using a Nikon OptiPhot-2 microscope and a Canon Eos 5D Mark II camera. Glomerular inflammation was scored as 0⁺, minimal mesangial expansion, consistent with radiation injury; 1⁺, focal glomerular changes with moderate mesangial expansion, glomerular basement membrane thickening/reduplication, and glomerular hypercellularity; or 2⁺, diffuse glomerular changes with severe mesangial expansion, glomerular basement membrane thickening/reduplication, and glomerular hypercellularity. Pathology was scored by observers blinded to genotype.

Statistical evaluation

The p-values were calculated using the two-tailed Student's *t* test and the one-way ANOVA, followed by Tukey's multiple comparison test (GraphPad Software).

Online supplemental material

Fig. S1 shows development of Q β -specific GC B cells in WT, but not *Cd40*^{-/-}, mice after Q β -VLP immunization.

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Author contributions: T. Arkatkar, S.W. Du, and H.M. Jacobs performed the animal experiments, analyzed data, and contributed to the writing of the manuscript. T. Arkatkar performed mouse *in vitro* experiments. E.M. Dam performed and J.H. Buckner directed *in vitro* studies using human B cells. B. Hou generated the single-stranded RNA-containing QB-VLPs. D.J. Rawlings and S.W. Jackson directed the project and wrote the manuscript.

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