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Murine B Cell Response to TLR7 Ligands Depends on an IFN- β Feedback Loop¹

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Abstract

Type I IFNs play an important, yet poorly characterized, role in systemic lupus erythematosus. To better understand the interplay between type I IFNs and the activation of autoreactive B cells, we evaluated the effect of type I IFN receptor (IFNAR) deficiency in murine B cell responses to common TLR ligands. In comparison to wild-type B cells, TLR7-stimulated IFNAR^{-/-} B cells proliferated significantly less well and did not up-regulate costimulatory molecules. By contrast, IFNAR1^{-/-} B cells did not produce cytokines, but did proliferate and up-regulate activation markers in response to other TLR ligands. These defects were not due to a difference in the distribution of B cell populations or a failure to produce a soluble factor other than a type I IFN. Instead, the compromised response pattern reflected the disruption of an IFN- β feedback loop and constitutively low expression of TLR7 in the IFNAR1^{-/-} B cells. These results highlight subtle differences in the IFN dependence of TLR7 responses compared with other TLR-mediated B cell responses.

The use of type I IFNs for the treatment of malignancy or viral infection can lead to lupus-like symptoms (1). Elevated serum levels of IFN- α are common in systemic lupus erythematosus (SLE)⁴ patients and associated with SLE flares (2,3). Moreover, murine models of spontaneous

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⁴Abbreviations used in this paper: SLE, systemic lupus erythematosus; IFNAR, IFN- $\alpha\beta$ receptor; WT, wild type; pDC, plasmacytoid dendritic cell; DC, dendritic cell; ODN, oligodeoxynucleotide; MZ, marginal zone; IRF, IFN regulatory factor; FO, follicular; IC, immune complex.

SLE-like disease and SLE patients develop peripheral blood gene expression profiles characterized by an “IFN- α signature” (4–6). This signature is thought to reflect high levels of IFN- α produced by plasmacytoid dendritic cells (pDC) in response to DNA- and/or RNA-associated immune complexes (7,8) through a process that depends on Fc γ R-mediated uptake and delivery of these complexes to a cytoplasmic compartment containing TLR9 and/or TLR7 (9–14). IFN- β has also been linked to autoimmune disease through its ability to raise the serum levels of the B cell survival factor BAFF (15).

All type I IFNs signal through a single receptor, a heterodimer of the IFN- $\alpha\beta$ receptor (IFNAR) 1 and IFNAR 2 subunits. To further examine the role of type I IFNs in systemic autoimmune disease, several groups of investigators have evaluated the effect of IFNAR1 deficiency on disease progression in autoimmune-prone strains of mice. Consistent with the proinflammatory properties of type I IFNs, IFNAR1 deficiency ameliorated disease manifestations in NZB mice as evidenced by less extensive hemolytic anemia and improved survival (16). These results were corroborated by studies that involved Fas-deficient 129Sv \times C57BL/6 intercrossed mice or pristane-treated 129Sv mice, where the IFNAR1-deficient mice developed lower autoantibody titers and were protected from C'-fixing immune complex deposition in the kidneys (17,18). By contrast, on an MRL/*lpr* background, IFNAR1^{-/-} mice developed higher autoantibody titers, more severe renal disease, and significantly reduced survival compared with IFNAR1^{+/+} control groups (19). These conflicting results were particularly puzzling with regard to autoantibody titers because B cells express high levels of the IFNAR1 (20) and IFN partially activates B cells, making them more sensitive to weak signals through the BCR (21).

An elevated systemic level of type I IFN, caused by a gene duplication, results in a lupus-like syndrome characterized by autoantibodies directed against RNA-associated proteins (22). The production of autoantibodies reactive to RNA-associated autoantigens in the MRL/*lpr* model has been shown to be dependent on TLR7 and, similarly, the production of anti-DNA autoantibodies has been shown to be dependent on TLR9 (23). Ligands of TLR7 and TLR9 are potent inducers of IFN- α and in vitro studies have clearly implicated TLRs in the activation of autoreactive B cells (13,24). Importantly, IFN- α has been shown to markedly enhance the in vitro proliferative response of autoreactive B cells to RNA-associated autoantigens (25) and can lower the activation threshold of autoreactive B cells to weak endogenous ligands (26). In human B cells, IFN- α produced by pDC has been shown to dramatically increase the expression levels of TLR7 and MyD88 (27). To further examine the impact of IFNAR1 deficiency on murine B cells, we compared the responses of wild-type (WT) and IFNAR1^{-/-} B cells to a panel of TLR ligands. These studies revealed an inherent and selective defect in the capacity of IFNAR1^{-/-} B cells to respond to TLR7 ligands, due to the absence of an autologous IFN- β /IFNAR feedback loop and reduced baseline expression of TLR7.

Materials and Methods

Mice

C57BL/6 (WT) mice were purchased from The Jackson Laboratory. Type I IFN receptor-deficient mice (IFNAR1^{-/-}), backcrossed more than nine generations to C57BL/6, were originally provided by Dr. J. Sprent (Garvan Institute of Medical Research, Sydney, Australia) and then intercrossed with C57BL/6 Igh^a to make the IFNAR1^{-/-} Igh^a mice. AM14 mice have been described previously (11,25,28). IFN- β ^{-/-} mice (29) were provided by Dr. E. Fish (University of Toronto, Toronto, Canada). All mice were maintained at the Boston University School of Medicine Laboratory Animal Sciences Center in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care.

B cell stimulation and analysis

B cells purified by positive magnetic bead separation with either CD45R/B220 magnetic particles (BD Biosciences) or with biotin-anti-CD23 (BD Biosciences) and anti-biotin MicroBeads or by negative magnetic bead selection using a combination of CD43 and CD11c MicroBeads (Miltenyi Biotec). Purity of B cells was validated by FACS and determined to be >98%. B cells were stimulated with the TLR ligands: 1 ng/ml Pam3Cys (EMC Microcollections), 10 μ g/ml LPS (InvivoGen), 1 μ g/ml CpG (s-oligodeoxynucleotide (ODN) 1826; Coley Pharmaceuticals), 30 ng/ml R848, or 300 ng/ml CL097 (InvivoGen). AM14 B cells, specific for IgG2a, were stimulated with the RNA-specific mAb BWR4, provided by Dr. D. Eilat (Hadassah University Hospital, Jerusalem, Israel), at a final concentration of 10 μ g/ml. The effect of type I IFN was determined by preincubating the cells with 300 U/ml IFN- α or IFN- β (PBL), unless another concentration is specifically noted, for 1 h at 37°C before adding the various ligands. B18R was obtained from eBioscience. B cell proliferation was as previously described (24). Briefly, B cells were stimulated in 96-well plates at a final concentration of 2×10^6 cells/ml for 24 h, then pulsed for 6 h with [3 H]thymidine (Amersham Biosciences). Incorporation of [3 H]thymidine was quantified via a liquid scintillation beta counter (PerkinElmer).

For the cell mixture experiments, cells were cultured in 48-well plates at a final concentration of 1.5×10^6 cells/ml for 24 h. For some of the cultures, the allotype-disparate cells were mixed at a 1:1 ratio before stimulation; in other wells, the cells were combined at 1:1 volume ratio after stimulation but before flow cytometric analysis. IgM allotype was determined with mouse anti-IgM^a-FITC and mouse anti-IgM^b-PE (BD Biosciences). Analysis was conducted with FlowJo software (Tree Star). The following Abs were used to characterize splenic B cell populations: B220-Pacific Blue, CD21-FITC, CD23-PE-Cy7, IgM-PE, and AA4.1-allophycocyanin (eBioscience).

Cytokine assays

Cytokine secretion by in vitro-stimulated B cells and dendritic cells (DC) was measured at 24 h. The IFN- α ELISA has been previously described (9). For the IL-6 and IL-10 ELISAs, plates were coated with anti-murine IL-6 or anti-murine IL-10 (BD Biosciences) at 1 μ g/ml for 16 h. Samples were added for 4 h and detected with biotin-anti-IL-6 or biotin-anti-IL-10 (BD Biosciences) at 0.5 μ g/ml. The plate was developed with streptavidin-HRP (BD Biosciences) and tetramethylbenzidine liquid substrate system (Sigma-Aldrich). Standard curve was generated using recombinant mouse IL-6 (BD Biosciences) or recombinant mouse IL-10 (BD Biosciences).

Type I IFN production was determined using a bioassay as previously described (30). Briefly, L929 cells were pretreated with increasing concentrations of mouse IFN- β (PBL) (to generate a standard curve) or supernatants from mock-treated or TLR ligand-treated B cells for 3 h. Then the cells were challenged with vesicular stomatitis virus at a multiplicity of infection of 0.1. Cells were fixed and stained with crystal violet at 30 h after infection. The extent of protection was determined by reading at 595 nm.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from WT and IFNAR1^{-/-} B cells, reverse transcribed, and analyzed by qPCR. Data are the means \pm SEM of three (WT) and five (IFNAR1^{-/-}) independent experiments done in triplicate. For IFN- β , IFN- α 2, IFN- α 4, and GAPDH, standard commercial TaqMan probes were used (Applied Biosystems). For TLR9 and TLR7, the following custom primers were used: TLR9f, 5'-ACTTCGTCCACCTGTCCAAC-3' and TLR9r, 5'-TCATGTGGCAAGAGAAGTGC-3' and TLR7f, 5'-GGAGCTCTGTCCTGAGTGG-3' and TLR7r, 5'-GTCTGTGGTCAGGGCATT-3'. Samples were normalized to GAPDH and

represented as fold change over medium control for IFN genes or fold change over WT for TLRs using the $\Delta\Delta\text{CT}$ method previously described (31).

Immunoprecipitation of TLR7 and UNC93B

Immunoprecipitation of TLR7 and UNC93B was conducted as previously described (32). Naive B cells were purified from spleens of WT and IFNAR1^{-/-} mice using CD43 beads (Miltenyi Biotec) after removal of RBC via hypotonic lysis. Both CD43-negative (naive B cells) and CD43-positive cells were metabolically labeled with [³⁵S]methionine/cysteine for 4 h in the presence or absence of IFN- α (2000 U/ml; PBL) and lysed in a buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and protease inhibitors. TLR7 and UNC93B were immunoprecipitated using anti-TLR7 (Imgenex) and anti-UNC93B Abs (32), respectively. Immunoprecipitates were subjected to 10% SDS-PAGE and proteins were visualized by fluorography.

Results

Among their many regulatory functions, type I IFNs play a critical role in the amplification of TLR signaling cascades. To determine whether type I IFNs played a significant role in the regulation of TLR-mediated B cell responses, we stimulated IFNAR1^{-/-} B cells and WT controls with a panel of TLR ligands. IFNAR1 deficiency had no major effect on B cell proliferation in response to TLR2 (Pam3Cys), TLR4 (LPS), or TLR9 (CpG s-ODN1826 (CpG)) ligands. However, the response of the IFNAR1^{-/-} B cells to the TLR7 ligand R848 was significantly reduced relative to WT B cells (Fig. 1A).

DC populations isolated from IFNAR1^{-/-} mice exhibit dramatically reduced levels of inflammatory cytokine production in response to both TLR7 and TLR9 ligands (9). These data point to a critical IFN-dependent positive feedback loop in the regulation of TLR-dependent DC responses. B cells also respond to TLR ligands by producing cytokines (33). To determine how IFNAR1 deficiency affects cytokine production, WT and IFNAR1^{-/-} B cells were stimulated for 24 h with the same panel of TLR ligands and then the culture supernatants were assayed for IL-6 and IL-10. In addition to reduced proliferation, the R848-stimulated IFNAR1^{-/-} cells made only low levels of cytokines. Unexpectedly, even though the IFNAR1^{-/-} B cells proliferated normally in response to LPS and CpG, these cells made markedly reduced levels of cytokine (Fig. 1B). Therefore, IFNAR1 deficiency compromises the ability of B cells to fully respond to TLR engagement.

Different B cell subsets are known to produce distinct sets of cytokines. For example, marginal zone (MZ) B cells were found to secrete most of the IL-10 elicited by CpG activation (34). To rule out the possibility that the defect of cytokine production observed in IFNAR1^{-/-} B cells was due to a skewed ratio of splenic B cell subsets, we analyzed the distribution of the different B cell compartments in the spleen by flow cytometry. The ratio of immature (B220⁺AA4.1⁺) and mature (B220⁺AA4.1⁻) B cells was the same in WT and IFNAR1^{-/-} spleens (Fig. 2A). Immature B cells can be further divided into three transitional B cell stages (termed T1–T3) based on CD23 and IgM expression (35). The immature B cell compartment in IFNAR1^{-/-} spleens did not differ from that of WT (Fig. 2B). The mature B cell compartment in the spleen consists of two subsets, follicular (FO) and MZ B cells. These populations are distinguished by the expression of CD21 and CD23. FO B cells are CD21^{low}CD23^{high}, whereas MZ B cells are CD21^{high}CD23^{low}. Again, no difference in B cell compartments was evident comparing WT and IFNAR1^{-/-} spleens (Fig. 2C). Therefore, the reduced level of cytokine production by IFNAR1^{-/-} B cells was not due to the absence of a particular B cell subset.

Although IL-6 is considered a proproliferative cytokine, the inability to make IL-6 is unlikely to account for the weak proliferative response of the R848-stimulated B cells since the

proliferative response of the CpG- and LPS-stimulated IFNAR1^{-/-} cells was normal. We therefore reasoned that one potential difference between WT and IFNAR1^{-/-} B cells could be the ability of WT B cells to produce a proproliferative cytokine, other than IL-6, in response to the TLR7 ligand. To test this possibility, we used a coculture system in which WT Igh^b B cells were combined with IFNAR1^{-/-} Igh^a B cells at a 1:1 ratio and then stimulated for 24 h with the panel of TLR ligands. As a control, the parental strains were stimulated independently at the same final concentration and then combined at a 1:1 volume basis after the 24-h stimulation period, immediately before flow cytometric analysis. Activated cells were identified on the basis of forward vs side scatter and the percentage of Igh^a and Igh^b cells was determined with the appropriate IgM allotype-specific reagents. As expected, the WT and IFNAR1^{-/-} B cells responded comparably to Pam3Cys, LPS, and CpG as indicated by an Igh^a to Igh^b ratio close to 1 (Fig. 3). The same results were obtained when the WT Igh^b B cells and IFNAR1^{-/-} Igh^a B cells were combined before stimulation; again both populations contributed comparably to the TLR2, TLR4, and TLR9 responses. However, the majority of the activated cells recovered from the R848-stimulated cultures were Igh^b. These data clearly demonstrate that IFNAR1^{-/-} B cells have a significantly decreased proliferative response to R848, which cannot be rescued by coculture with WT cells.

The reduced response of the IFNAR1^{-/-} B cells to R848 pointed to the absence of an IFN-mediated positive feedback loop in which R848 initially stimulated low level secretion of type I IFN by either the B cells or small contaminating population of DC. However, we were unable to detect IFN- α in culture fluids obtained from either WT or IFNAR1^{-/-} R848-stimulated B cells using an ELISA that could detect as little as 80 pg/ml IFN- α and readily detected IFN- α secreted by pDC obtained from the same mice (data not shown). Although it was reported that culturing B cells for 3 days with 5% pDC can enhance the B cell TLR7 responses (27), our cultures have <0.5% CD11c-positive cells; therefore, we have ruled out pDC as having a significant effect in our cultures. Furthermore, B cells rigorously purified by positive selection with either anti-B220-coated magnetic beads, anti-CD23-coated magnetic beads, or by double-negative selection, with anti-CD43 beads, followed by anti-CD11c beads, all responded comparably to the TLR7 ligand CL097, an R848 derivative (data not shown).

To determine whether or not the B cell cultures were producing a small amount of type I IFN, below the limit of detection of our ELISAs, we used two more sensitive techniques. First, we measured activation of type I IFN genes by qRT-PCR. A robust up-regulation of IFN- β , IFN- α 2, and IFN- α 4 was seen, primarily in response to the TLR7 ligand CL097 (Fig. 4A). Second, we used a bioassay to test for the presence of bioactive type I IFN. The bio-assay showed that both TLR7 and TLR9 ligands stimulated B cells to produce similar, albeit small, amounts of type I IFN (Fig. 4B). Combined, these assays show that B cells produce type I IFN in response to TLR7 or TLR9 stimulation.

To assess the importance of the type I IFN produced by B cells stimulated with a TLR7 ligand, we decided to test the effect of a potent IFNAR antagonist, B18R, on the CL097 response of WT B cells. B18R is a protein originally derived from the Western Reserve vaccinia virus strain that has neutralizing activity for human, mouse, pig, rat, and cow type I IFNs (36) and thereby blocks effective engagement of the IFNAR heterodimer. Consistent with the data obtained with the IFNAR1^{-/-} B cells, B18R did not lower the response of WT B cells to LPS or CpG. However, unexpectedly, B18R also failed to inhibit the CL097 proliferative response unless added to the cultures at microgram concentrations (Fig. 5A). To further investigate the inhibitory activity of B18R, we determined the minimal concentration of B18R that could block the proliferation of IgG2a-reactive AM14 B cells, preincubated with either IFN- α or IFN- β , to RNA-associated immune complexes (IC). This response had been previously shown to be highly type I IFN dependent (25).

Both IFN- α and IFN- β bind to the IFN receptor and we found that on a per unit basis, both forms of IFN promoted the response of AM14 B cells to RNA-associated ICs to a comparable extent (Fig. 5B). However, despite previous studies demonstrating that B18R could bind both IFN- α and IFN- β with comparable affinity (36), B18R blocked the IFN- α enhanced RNA IC response much more effectively than the IFN- β -enhanced RNA IC response; IFN- β inhibition required a 100-fold higher concentration of B18R than that required for IFN- α inhibition (Fig. 5C).

The relatively poor capacity of B18R to block both the CL097 response of WT B cells and the IFN- β -enhanced RNA IC response of AM14 B cells suggested that IFN- β was a critical component in the B cell response to the TLR7 ligand and perhaps also required for CpG-stimulated B cell cytokine production. To test this premise, IFN- $\beta^{-/-}$ B cells were stimulated with a panel of TLR ligands and compared to WT B cells with regard to proliferation and cytokine production. As predicted, the IFN- $\beta^{-/-}$ B cells proliferated normally in response to the TLR2, TLR4, and TLR9 ligands but the response to the TLR7 ligand was suboptimal, although not as low as the IFNAR1 $^{-/-}$ B cells (Fig. 6A, *left*). This response could be restored in the IFN- $\beta^{-/-}$ B cells by the addition of exogenous type I IFN, which had no effect on the IFNAR1 $^{-/-}$ B cell response (Fig. 6A, *right*). In addition, the CL097-stimulated IFN- $\beta^{-/-}$ B cells failed to produce IL-6, again reflecting the response of IFNAR1 $^{-/-}$ B cells. Unlike the IFNAR1 $^{-/-}$ B cells, the IFN- $\beta^{-/-}$ B cells did make IL-6 in response to CpG, but the level of cytokine production was increased by the addition of exogenously provided IFN- β (Fig. 6B) or exogenously provided IFN- α (data not shown). Activation markers on these B cells mirrored the proliferative response. IFNAR1 $^{-/-}$ B cells and IFN- $\beta^{-/-}$ B cells failed to up-regulate CD86 in response to CL097 (Fig. 6C). The ability to up-regulate activation markers and to produce cytokine in response to the TLR7 ligand could be restored by the addition of IFN- β to the IFN- $\beta^{-/-}$ B cell cultures (Fig. 6C) or by the addition of IFN- α (data not shown).

We next considered the possibility that IFNAR1 $^{-/-}$ B cells respond poorly to TLR7 ligands because they express reduced levels of TLR7. TLR7 and TLR9 expression levels were initially assessed by real-time PCR. Based on RNA, TLR9 expression levels were comparable in the WT and IFNAR1 $^{-/-}$ B cells, while the TLR7 levels in the IFNAR1 $^{-/-}$ mice were ~10–20% of the WT level (Fig. 7A). TLR7 levels were further evaluated by TLR7 immunoprecipitation from purified metabolically labeled B cells. Although barely detectable, the level of TLR7 in the IFNAR1 $^{-/-}$ B cells was significantly below that of WT mice (Fig. 7B). TLR7 levels could be dramatically up-regulated by IFN- α in the WT B cells. Notably, the level of TLR7 in the non-B cell fraction of the IFNAR1 $^{-/-}$ and WT spleens was comparable. The level of UNC93B, an endoplasmic reticulum protein required for TLR7 activity (37), was not decreased in the IFNAR1 $^{-/-}$ B cells. Overall, the data demonstrate a selective defect in TLR7 expression in IFNAR1 $^{-/-}$ B cells.

Discussion

Type I IFNs play a critical role in the antiviral responses initially triggered by pattern recognition receptors, as shown by the observations that IFNAR1 $^{-/-}$ mice are more susceptible to a range of viral infections (38,39). Abs bound to viral determinants form immune complexes that can further enhance the response of DC to viruses and even elicit an IFN response from otherwise noninterferonogenic viruses (40,41). The humoral component of these antiviral responses underscores the importance of B cells in the type I IFN-dependent immunity. In the case of vesicular stomatitis virus and presumably other RNA viruses, Ab-mediated enhancement of type I IFN production is dependent on TLR7 (42). Type I IFNs are also considered a pathogenic factor in systemic autoimmune diseases such as SLE, where immune complexes that incorporate DNA- and RNA-associated autoantigens have been implicated in pDC and B cell activation through pathways involving both TLR9 and TLR7. In the current

study, we now identify specific defects in IFNAR1^{-/-} B cells that compromise responses to both TLR7- and TLR9-dependent ligands. These observations are consistent with previous studies demonstrating that type I IFNs directly promote the activation of autoreactive B cells and thereby presumably contribute to both autoantibody production as well as other B cell effector functions (26,43). Moreover, previous studies have also shown that STAT1, a key molecule in the IFNAR signaling cascade, is required for high titer serum IgG, and the pathogenic isotype IgG2a in particular, in a pristane-induced model of SLE (44). The data further indicate that IFNAR1-associated feedback loops are important for the activation of autoreactive B cells even before Abs are produced.

Hartmann and colleagues (27) previously reported that human peripheral blood-derived B cell responses to a TLR7 ligand required an exogenous source of IFN- α , provided in their studies by cocultured pDC. Our data also demonstrate that murine B cell responses to TLR7 ligands depend on type I IFN. However, in contrast to the Hartmann studies (27), our inhibitor experiments, as well as the comparison of IFN- β ^{-/-} and WT B cells, indicate that IFN- β produced by the B cells per se is sufficient for a robust response. Even though the proliferation of IFN- β ^{-/-} B cells in response to the TLR7 ligand was not as severely decreased as the IFNAR1^{-/-} B cells, the failure to produce cytokine and up-regulate activation markers mirrored the IFNAR1^{-/-} B cells completely. The difference between the human and murine studies may reflect either a distinct capacity of murine B cells to produce IFN- β or some other distinguishing feature of the culture conditions.

IFNAR1 deficiency had the most dramatic impact on responses to TLR7 ligands such as R848 and CL097. Whereas WT B cells exhibited a robust proliferative response, produced high levels of cytokine, and up-regulated the costimulatory molecule CD86, the IFNAR1^{-/-} B cells proliferated poorly, did not produce IL-6, and failed to up-regulate CD86. One factor likely to at least partially account for the weak R848/CL097 B cell response is the reduced level of TLR7 expression that is particularly apparent in IFNAR1^{-/-} splenic B cells. In addition, type I IFN rapidly increases the expressed level of TLR7 in R848-stimulated WT cells, but not in R848-stimulated IFNAR1^{-/-} cells. The low basal level of TLR7 expression in the IFNAR1^{-/-} cells suggests that an endogenous ligand, perhaps apoptotic debris or a component of the natural microflora, provides a stimulus that induces type I IFN production that in turn engages IFNAR and thereby raises the baseline level of TLR7 expression in WT mice. The existence of such an endogenous ligand is consistent with the observation that IFNAR1^{-/-} neonatal mice are protected from the multiorgan, sterile inflammatory response that ensues in mice that fail to express the suppressor of cytokine signaling 1 (45). An IFN- α /IFNAR-dependent increase in basal TLR7 levels might also explain why IFN- β ^{-/-} B cells proliferate somewhat more strongly in response to the TLR7 ligand than IFNAR1^{-/-} B cells.

The difference between the IFNAR1^{-/-} and WT B cells for other TLR responses was more subtle; both populations proliferated comparably in response to the CpG or LPS, but only the WT cells made IL-6 and IL-10. Expression of TLR9 was not affected by IFNAR deficiency and therefore could not account for the diminished CpG-induced cytokine response. Therefore, the type I IFN feedback is involved in multiple stages of the cytokine production pathway. Interestingly, the IFNAR1^{-/-} B cell response to CpG is similar to that previously reported for IRF-5^{-/-} B cells; IRF-5^{-/-} B cells also proliferate normally in response to CpG but fail to produce IL-6 (46). IFN regulatory factor (IRF) 5 is a transcription factor downstream of both TLR7 and TLR9, reported to regulate the expression of proinflammatory cytokines (47). The current data suggests that the role of IRF-5 on B cell responses to TLR7 and TLR9 may be to integrate TLR signaling with an IFNAR1-dependent feedback loop.

The specific role of IFN- β in the response to the TLR9 ligand was less clear-cut. Whereas IFNAR1^{-/-} CpG-stimulated B cells did not make IL-6, IFN- β -deficient B cells did produce

reasonably high levels of cytokine. One possible explanation for this discrepancy is that TLR9 ligands induce the production of a type I IFN other than IFN- β that promotes IL-6 production. In pDC, CpG induces the production of both IFN- α and IFN- β , but R848 only induces IFN- β ; IL-6 production in both cases is highly dependent on IFNAR expression (9). Although pDC are thought to be the primary producers of type I IFNs, CpG-stimulated B cells have been shown to produce type I IFN through a pathway dependent on IRF-3 and therefore different from the pathway used by CpG-stimulated pDC, which is IRF-3 independent (48). Although we could not detect IFN- α or IFN- β in culture supernatants by ELISA (data not shown), IFN concentrations near the limit of detection of the ELISAs, 10–30 U/ml of either, could more than triple the proliferative response to endogenous RNA immune complexes (Fig. 6B), and type I IFN may be rapidly absorbed by the cells. It is therefore likely that the small amount of type I IFN produced in response to TLR ligand B cell cultures are producing (Fig. 4) is sufficient to trigger a positive feedback loop that allows for optimal B cell activation.

We previously reported that the in vitro response of IgG2a-reactive AM14 B cells to RNA-associated immune complexes was markedly enhanced by the addition of IFN- α to the cultures (25). This enhancement most likely reflects the capacity of IFN- α to both up-regulate the level of TLR7 expression and lower the BCR signaling threshold (26). We now show that the response to RNA ICs can also be promoted by IFN- β . In this assay system, B18R, a vaccinia virus-derived inhibitor of type I IFN, could block the activity of IFN- α much more effectively than it blocked the activity of IFN- β . Even though it had been previously shown that B18R binds IFN- α and IFN- β comparably, it took an ~100-fold higher concentration of B18R to block the enhancing activity of 100 U/ml IFN- β as it did to block the same amount of IFN- α (36). The difference is most likely due to the ~100-fold higher binding affinity of IFNAR for IFN- β compared with IFN- α subclasses of type I IFN (49). B18R appears to function as a soluble IFN receptor and therefore competes for binding to the natural receptor. These data indicate that B18R may be a useful reagent for discriminating IFN- α and IFN- β responses.

The response of purified B cells to the potent synthetic TLR7 ligand R848, and its derivative CL097, required IFNAR, but, unlike stimulation by endogenous RNA ICs, did not require the addition of exogenous type I IFN. These responses were dependent on production of an endogenously produced type I IFN, IFN- β . Although the SLE IFN signature is largely attributed to pDC-produced IFN- α , the role of IFN- β to the disease process also needs to be considered. Tissue damage, either as a result of viral infection or sterile injury could be the source of apoptotic debris and thus autoantigen, which in turn induces IFN- β production. In addition to autoantibody production, both IFN- α and IFN- β are also likely to promote critical B cell effector functions such as cytokine production and Ag presentation that contribute to both the initiation and recurrence of systemic autoimmune diseases.

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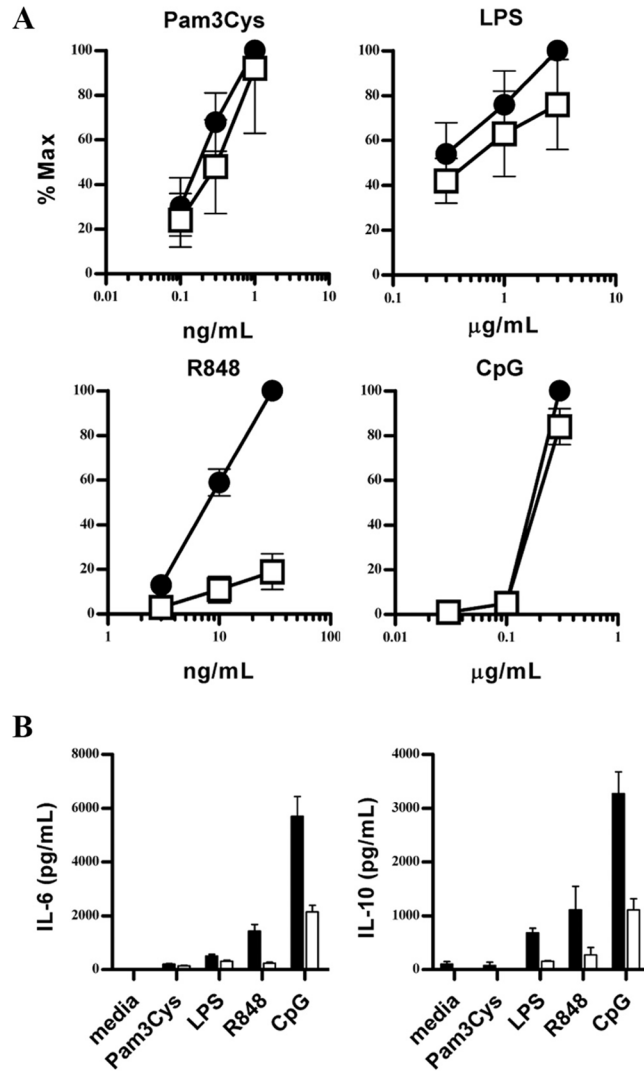


FIGURE 1. WT and IFNAR1^{-/-} B cell responses to TLR ligands. *A*, Purified splenic B cells from WT (●) and IFNAR1^{-/-} (□) mice were stimulated with Pam3Cys (TLR2), LPS (TLR4), R848 (TLR7), or CpG 1826 (TLR9) and proliferation was measured by [³H]thymidine uptake. Data are presented as the percent cpm obtained with top dilution of TLR ligand for WT control. Average ± SEM of four experiments. *B*, TLR-induced cytokine production by WT and IFNAR1^{-/-} B cells. Levels of IL-6 (*left*) and IL-10 (*right*) in the culture supernatant of TLR-stimulated WT (■) and IFNAR1^{-/-} (□) B cells were measured by ELISA. Average ± SEM of four experiments.

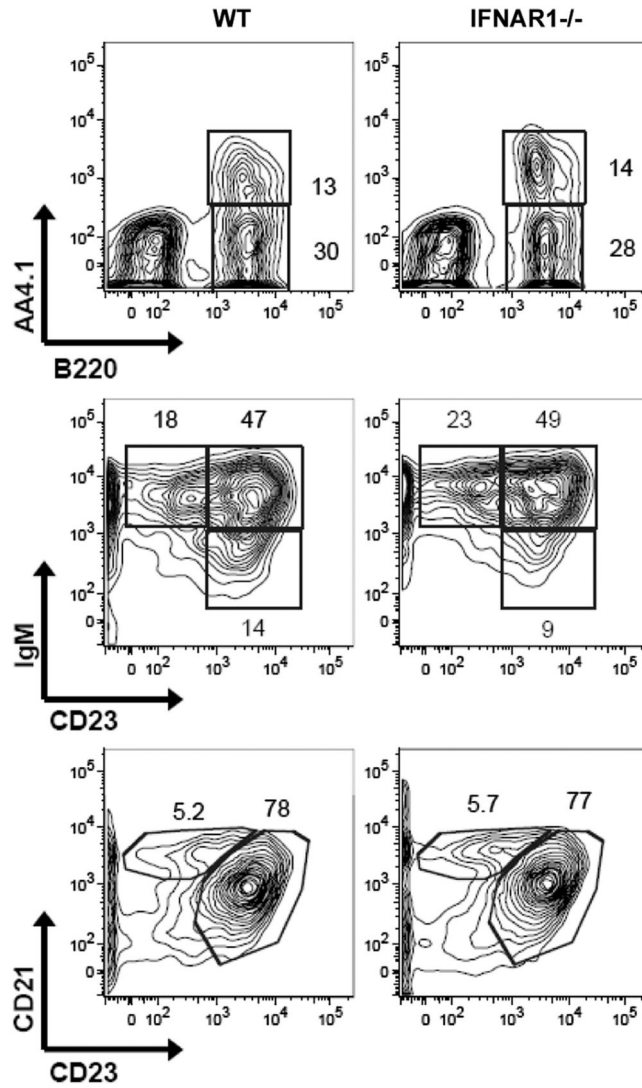


FIGURE 2.

Decreased cytokine production of $IFNAR1^{-/-}$ B cells is not due to abnormal composition of mature B cell compartments. Splenic B cells were separated into immature ($B220^{+}AA4.1^{+}$) and mature ($B220^{+}AA4.1^{-}$) B cell populations (*top*). Immature B cells were further analyzed for transitional (T) B cell stage 1 ($IgM^{high}CD23^{-}$), T2 ($IgM^{high}CD23^{+}$), and T3 ($IgM^{low}CD23^{+}$) (*middle*). The two mature B cell populations, MZ and FO B cells, were distinguished using surface markers CD21 and CD23 (*bottom*). Representative contour plots of three independent experiments are shown.

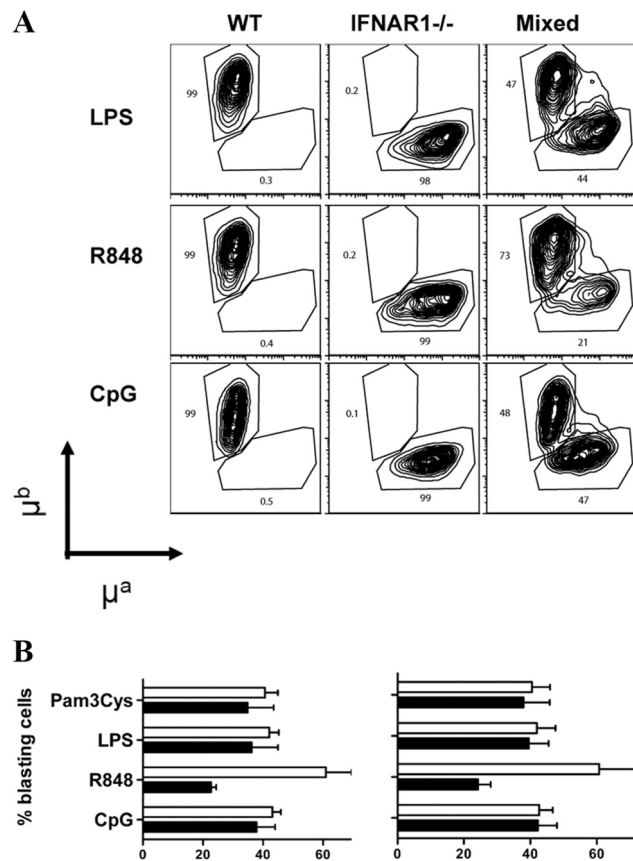
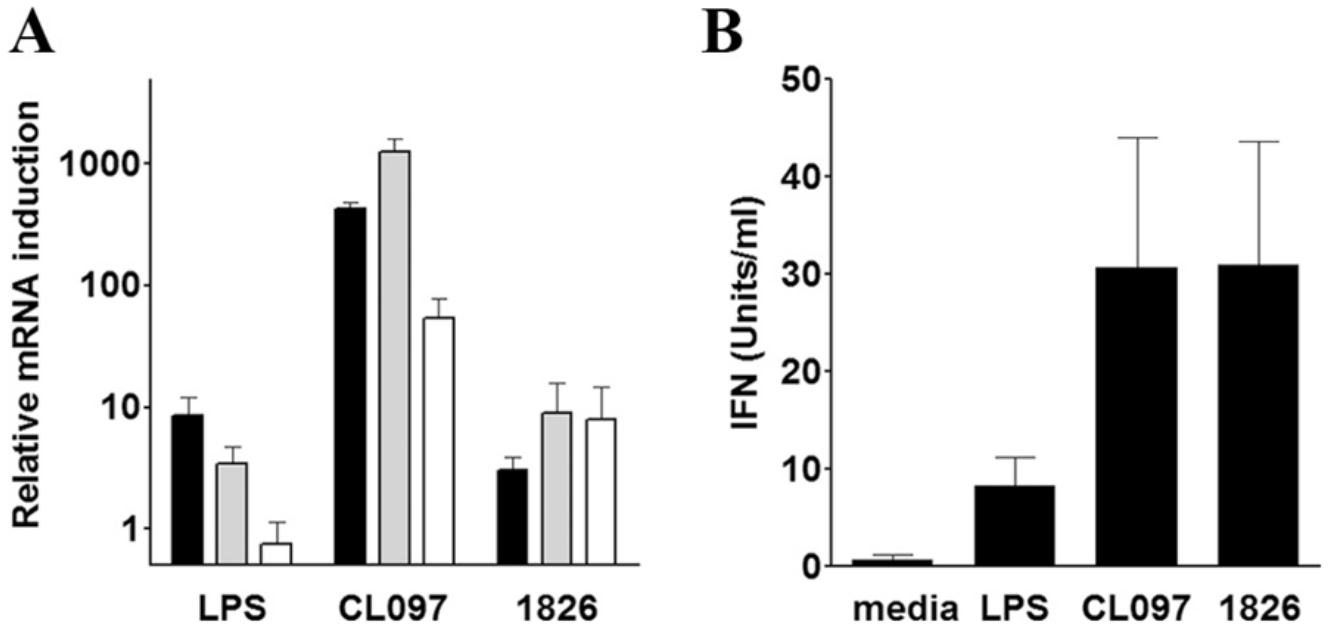
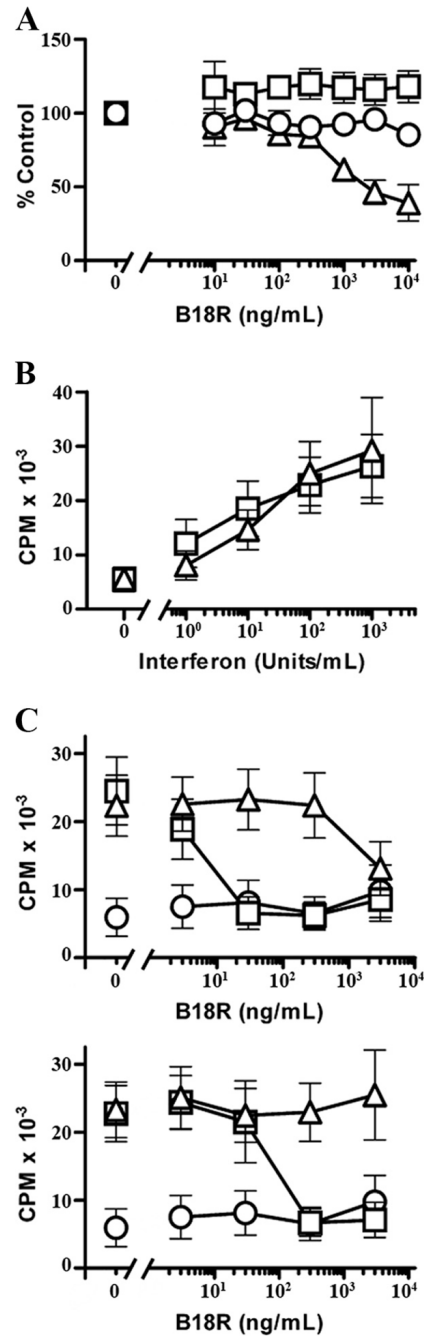


FIGURE 3. IFNAR1^{-/-} B cell proliferation cannot be rescued by soluble factors produced by WT B cells. Purified splenic B cells from IFNAR1^{-/-} Igh^a B cells, WT C57BL/6 Igh^b B cells, or a 1:1 mixture of the IFNAR1^{-/-} and WT B cells were stimulated with a panel of TLR ligands. Activated cells were identified on the basis of forward vs side scatter and parental origin of the activated cells was determined by staining with the allotype-specific reagents. *A*, Representative flow cytometric staining profile of the mixed population stimulated with LPS (*top*), R848 (*middle*), or CpG (*bottom*). *B*, The allotype distribution of cells mixed before stimulation with either Pam3Cys, LPS, CpG ODN1826, or R848 (*left*) or combined 1:1 on the basis of input number just before analysis (*right*). Average \pm SEM of four experiments.

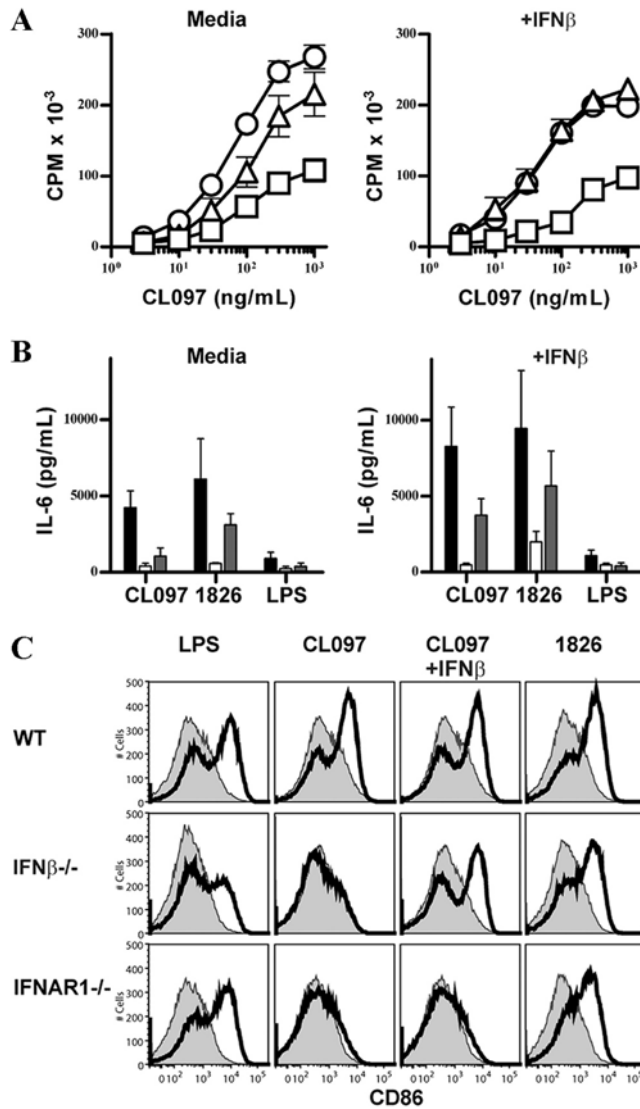
**FIGURE 4.**

Type I IFN production by B cells. *A*, Expression levels of type I IFN mRNA in B cells treated for 1 h with the indicated TLR ligands were measured by qRT-PCR. ■, IFN- β ; ▣, IFN- $\alpha 2$; and □, IFN- $\alpha 4$. Average \pm SEM of three experiments. *B*, TLR-induced production of functional type I IFN. Type I IFN in the culture supernatant of B cells, stimulated for 6 h with indicated TLR-ligands, was measured by IFN bioassay. Average \pm SEM of five experiments.

**FIGURE 5.**

Capacity of B18R to block TLR7-dependent B cell responses. *A*, WT B cells were stimulated with sODN1826 (□), LPS (○), or CL097 (△) in the presence of increasing concentrations of B18R. Data are presented as the percentage of the uninhibited proliferative response measured by uptake of [³H]thymidine. *B*, AM14 B cells were primed with either IFN- α (□) or IFN- β (△) and stimulated with 10 μ g/ml BWR4 (anti-RNA mAb) in the presence of the indicated amounts of B18R. Average \pm SEM is shown, $n = 5$. *C*, AM14 B cells were stimulated with BWR4 in the presence of medium (○) and either 100 U/ml (*top*) or 1000 U/ml (*bottom*) of IFN-

α (\square) or IFN- β (Δ) in the presence of increasing concentrations of B18R. Average \pm SEM is shown, $n = 4$. Proliferation in all experiments was measured by [^3H]thymidine uptake.

**FIGURE 6.**

Exogenously produced IFN- β enhances the B cell response to synthetic TLR7 ligands. *A*, WT (\circ), IFN- β ^{-/-} (Δ), or IFNAR1^{-/-} (\square) B cells were stimulated with increasing concentrations of CL097 in the absence (*left*) or presence of 300 U/ml IFN- β (*right*). Proliferation was measured by [³H]thymidine uptake. Data represent the average \pm SEM for four experiments. *B*, WT (\blacksquare), IFNAR1^{-/-} (\square), or IFN- β ^{-/-} (\blacksquare) B cells were stimulated with 300 ng/ml CL097 in the absence (*left*) or presence (*right*) of 300 U/ml IFN- β . IL-6 concentration in the culture supernatants at 24 h was determined by ELISA. *C*, B cells from WT (*top row*), IFN- β ^{-/-} (*middle row*), and IFNAR1^{-/-} (*bottom row*) mice were stimulated with LPS (*left*), CL097 (*middle left*), CL097 + IFN- β (*middle right*), or CpG (*right*). CD86 expression was measured by flow cytometry. Unstimulated B cells (shaded histogram) served as controls.

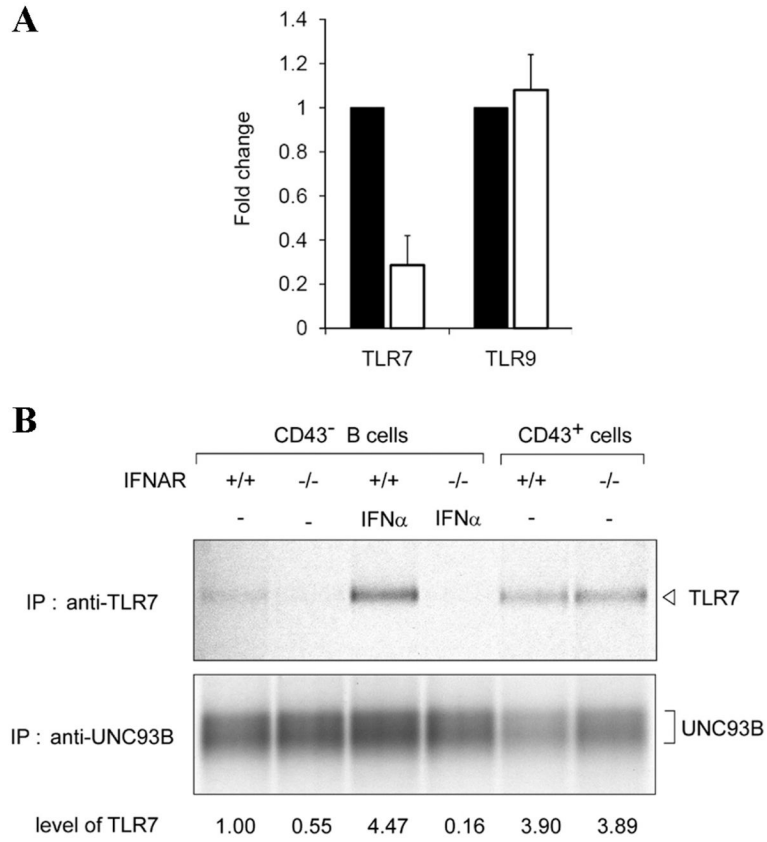


FIGURE 7. TLR7 expression in WT and IFNAR1^{-/-} cells. *A*, Levels of TLR7 and TLR9 expression in WT (■) and IFNAR1^{-/-} (□) B cells measured by qRT-PCR. *B*, Spleen cells from WT and IFNAR1^{-/-} mice were separated on the basis of CD43 expression and the B cells were metabolically labeled with [³⁵S]methionine for 4 h in the presence or absence of IFN- α . TLR7 and UNC93 were immunoprecipitated, subjected to 10% SDS PAGE, and detected by fluorography.