



IRF9 and STAT1 are required for IgG autoantibody production and B cell expression of TLR7 in mice

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A hallmark of SLE is the production of high-titer, high-affinity, isotype-switched IgG autoantibodies directed against nucleic acid-associated antigens. Several studies have established a role for both type I IFN (IFN-I) and the activation of TLRs by nucleic acid-associated autoantigens in the pathogenesis of this disease. Here, we demonstrate that 2 IFN-I signaling molecules, IFN regulatory factor 9 (IRF9) and STAT1, were required for the production of IgG autoantibodies in the pristane-induced mouse model of SLE. In addition, levels of IgM autoantibodies were increased in pristane-treated *Irf9*^{-/-} mice, suggesting that IRF9 plays a role in isotype switching in response to self antigens. Upregulation of TLR7 by IFN- α was greatly reduced in *Irf9*^{-/-} and *Stat1*^{-/-} B cells. *Irf9*^{-/-} B cells were incapable of being activated through TLR7, and *Stat1*^{-/-} B cells were impaired in activation through both TLR7 and TLR9. These data may reveal a novel role for IFN-I signaling molecules in both TLR-specific B cell responses and production of IgG autoantibodies directed against nucleic acid-associated autoantigens. Our results suggest that IFN-I is upstream of TLR signaling in the activation of autoreactive B cells in SLE.

Introduction

SLE is a chronic autoimmune disease characterized by the inflammatory destruction of many organ systems including skin, blood elements, joints, kidneys, and other tissues. A hallmark of lupus is the production of high-titer IgG autoantibodies that recognize primarily nuclear autoantigens, many of which contain nucleic acids. Several studies have indicated a pathogenic role for the type I IFN (IFN-I) system in human SLE patients. An increase in serum IFN- α in SLE patients was first noted in 1979 (1). Expression profiling of SLE patient blood reveals an IFN-I-inducible gene expression signature in peripheral blood cells (2–4) as well as increased serum levels of IFN-inducible cytokines and chemokines (5). A causative role for IFN-I in the pathogenesis of SLE is demonstrated by the observation that a subset of patients treated with recombinant IFN- α for nonautoimmune disorders develop a lupus-like syndrome that resolves when IFN- α therapy is discontinued (6).

All members of the IFN-I family, including 13 subtypes of IFN- α and IFN- β , bind to the same receptor, IFN (α and β) receptor (IFNAR). Both IFN-I and IFN- γ signal through STAT1, and *Stat1*^{-/-} mice fail to upregulate IFN-inducible genes in response to IFN-I or IFN- γ (7). IFN-I signal transduction results in activation of the IFN-stimulated gene factor 3 (ISGF3) heterotrimeric complex, which is composed of STAT1, STAT2, and IFN regulatory factor 9 (IRF9).

Nonstandard abbreviations used: FDR, false discovery rate; IFA, incomplete Freund adjuvant; IFNAR, IFN (α and β) receptor; IFN-I, type I IFN; IRF, IFN regulatory factor; ISGF, IFN-stimulated gene factor; MCTD, mixed connective tissue disease; ODN, oligodeoxynucleotide; pDC, plasmacytoid dendritic cell; RiboP, ribosomal phosphoprotein P0; RNP, ribonucleoprotein; SAM, significance analysis of microarrays; Sm, Smith antigen; ssDNA, single-stranded DNA; U1snRNP, U1 small nuclear RNP.

Conflict of interest: P.J. Utz has served as a consultant to Centocor, Biogen/Idec, Avanir Inc., and Genentech Inc.; is a member of the Scientific Advisory Board of Monogram Biosciences, XDX Inc., and Argos Therapeutics; and is a cofounder and consultant at Bayhill Therapeutics. The remaining authors have declared that no conflict of interest exists.

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Upon engagement of IFNAR by IFN-I, ISGF3 translocates to the nucleus and induces transcription of IFN-I-regulated genes. *Irf9*^{-/-} mice fail to upregulate IFN-inducible genes in response to IFN-I (8). The induction of IFN- α and IFN- β is severely impaired in these mice (9). Evidence exists that IRF9 can bind to STAT1 homodimers in response to IFN- γ stimulation (10) and that the ISGF3 complex can form in response to IFN- γ (11). The induction of guanylate binding protein was impaired in response to IFN- α , but not IFN- γ , in *Irf9*^{-/-} embryonic fibroblasts (8), suggesting that IRF9 is required for IFN-I, but not IFN- γ , signaling in vivo. Crosstalk between these 2 pathways has been well established, and both pathways play an important role in the development of autoimmunity.

The production of IgG autoantibodies directed against ribonucleoproteins (RNPs), including components of the U1 small nuclear RNP/Smith antigen (U1snRNP/Sm) complex, the Ro/La complex, and ribosomal phosphoprotein P0 (RiboP), is a hallmark of SLE and mixed connective tissue disease (MCTD). The overexpression of IFN-I-inducible genes correlates with the production of anti-RNP autoantibodies in SLE patients (4, 12, 13). In the pristane model of SLE, BALB/c mice given a single i.p. injection of the mineral oil pristane develop a lupus-like disease characterized by the production of anti-U1snRNP/Sm, anti-RiboP, and anti-DNA autoantibodies and the development of inflammatory kidney disease (14–16). Pristane treatment induces apoptosis (17), formation of peritoneal lipogranulomas that express high levels of IFN-I-inducible genes (18), and hypergammaglobulinemia characterized by high levels of the pathogenic isotype IgG2a (19). This model therefore recapitulates key features of SLE pathogenesis in humans including hypergammaglobulinemia, anti-RNP autoantibody production, kidney disease, defects in clearance of apoptotic debris, and upregulation of IFN-I-inducible genes.

Although aberrations in the IFN-I system have been associated with SLE since as early as 1979 (1), the mechanisms by which this pathway becomes activated have remained elusive until the recent

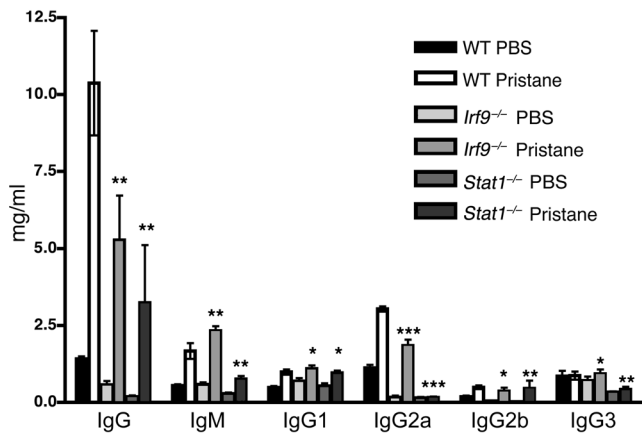


Figure 1
Serum Ig isotype levels in pristane-treated mice. Total Ig levels of individual isotypes were measured by ELISA in serum obtained 6 months after treatment with PBS or pristane. Values are mean ± SEM for each group of mice. **P* > 0.05 (not significant) versus pristane-treated WT. ***P* < 0.05, ****P* < 0.001 versus pristane-treated WT, Mann-Whitney test.

characterization of the nucleic acid-sensing TLRs: TLR7, which recognizes ssRNA; and TLR9, which recognizes dsDNA. A serum factor in SLE patients capable of inducing IFN-I production in normal blood leukocytes was first noted in 1999 (20) and was subsequently identified as circulating immune complexes containing nucleic acid-associated autoantigens (21, 22). It was later shown that DNA-containing immune complexes could induce IFN-I production in plasmacytoid dendritic cells (pDCs) through the cooperation of FcγRIIa and TLR9 (23). DNA-containing immune complexes can activate autoreactive B cells by dual engagement of the B cell receptor and TLR9 (24). Likewise, the RNA components of autoantigens, such as the U1-RNA and the Y-RNAs associated with the Ro/La complex, activate B cells and pDCs through the engagement of TLR7 (25–31). In murine SLE models, TLR9 is necessary for anti-dsDNA autoantibody production and antibody class switching to the pathogenic IgG2a and IgG2b isotypes (32, 33). TLR7 is both necessary (33) and sufficient (34, 35) for the induction of autoantibodies that recognize RNPs in lupus mouse models. It is hypothesized that ligation of TLRs by the nucleic acid components of SLE-associated autoantigens and the subsequent induction of IFN-I may drive the immune response against nucleic acid-containing autoantigens in SLE.

We have previously used autoantigen microarrays to profile the autoantibody response in human and murine autoimmune diseases (16, 36–41). In the current study, we used this technology to profile the autoantibody response in pristane-treated mice deficient for the IFN-I signaling molecules IRF9 and STAT1. Our results demonstrated that the development of IgG autoantibodies directed against prominent, clinically assayed SLE and MCTD autoantigens was dependent on IRF9 and STAT1. Pristane-treated *Irf9*^{-/-} mice developed significantly higher titers of IgM autoantibodies, indicating a potential role for this molecule in isotype switching. B cells from *Irf9*^{-/-} and *Stat1*^{-/-} mice exhibited specific defects in expression of TLR7 and response to TLR7 ligands. We demonstrated what we believe to be a novel role for IFN-I signaling molecules in TLR-specific B cell responses and the development of autoantibodies in vivo.

Results

Hypergammaglobulinemia. To examine the role of signaling molecules downstream of the IFNAR in development of SLE, we treated WT, *Irf9*^{-/-}, and *Stat1*^{-/-} mice, all on the BALB/c background, with pristane or PBS as a negative control. IFN-I stimulates isotype switching to all subclasses of IgG (42) and, in combination with IL-6, induces B cell maturation into plasma cells that secrete high levels of IgG (43). In addition, IFN-γ, which also signals through STAT1, is critical for isotype switching to the pathogenic IgG2a isotype (44). Pristane treatment induced hypergammaglobulinemia in WT mice; however, levels of total serum IgG as well as IgG2a were significantly decreased in pristane-treated *Irf9*^{-/-} and *Stat1*^{-/-} mice (Figure 1). Interestingly, pristane-treated *Irf9*^{-/-} mice developed significantly higher levels of total serum IgM, consistent with a role for IFN-I signaling in isotype switching. In contrast, levels of serum IgM in pristane-treated *Stat1*^{-/-} mice were significantly decreased compared with those in pristane-treated WT mice. There were no differences in the serum levels of IgG1, IgG2b, or IgG3 between pristane-treated WT and *Irf9*^{-/-} mice; however, *Stat1*^{-/-} mice had significantly decreased levels of IgG2b and IgG3.

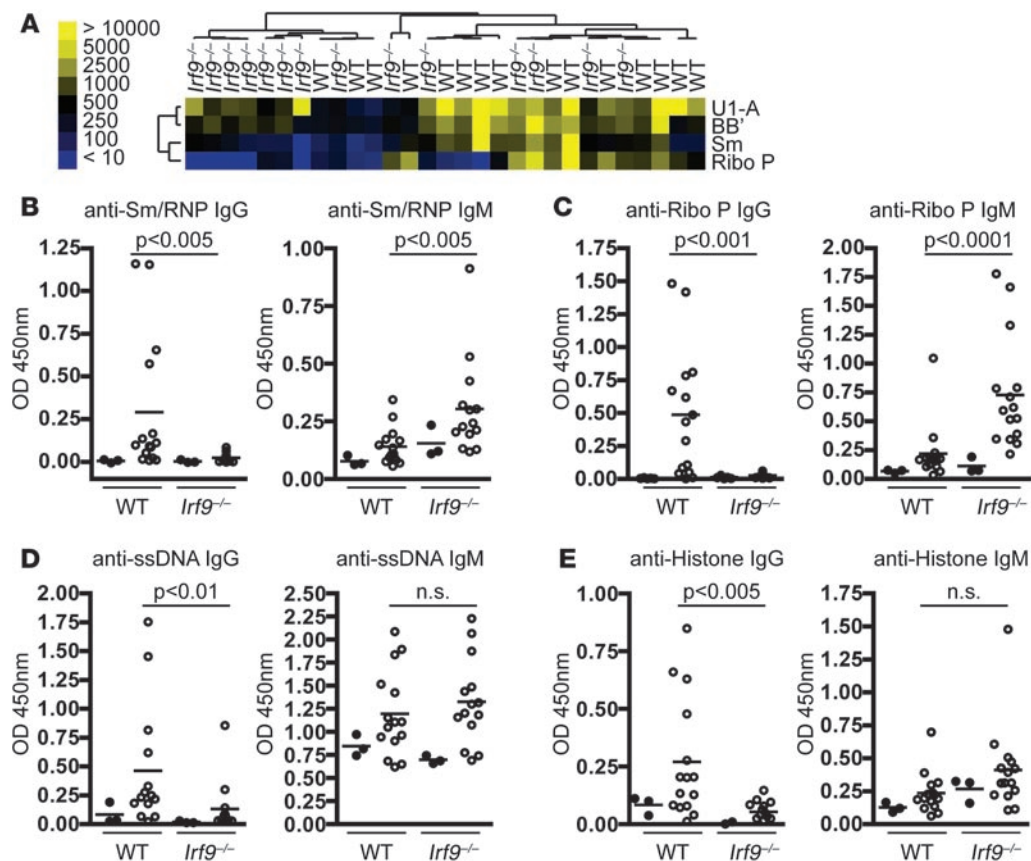
Autoantibody production. We used autoantigen microarrays to profile the autoantibody response in WT, *Irf9*^{-/-}, and *Stat1*^{-/-} mice treated with pristane or PBS. The lupus arrays contained 616 features consisting of more than 50 proteins, peptides, and nucleic acids that are candidate autoantigens in human and murine SLE. We used a hierarchical clustering program (45) to order individual mice on the basis of similarity of autoantibody profiles. Cluster results were displayed as a heatmap (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI30065DS1) using Java Treeview software (46). We used the significance analysis of microarrays (SAM) algorithm (47) to determine statistically significant differences in array reactivity between groups of animals. Pairwise SAM comparison of PBS-treated mice with pristane-treated WT mice revealed significantly increased reactivity to 15 antigens in pristane-treated WT mice (Supplemental Figure 2). SAM identified many autoantigens known to be targeted in this model, including single-stranded DNA (ssDNA), histones, components of the Sm/RNP complex (BB', Sm/RNP, U1A, U1C, and U1-70kD), and RiboP. Next, we compared the autoantibody profiles of pristane-treated WT mice with those of pristane-treated *Irf9*^{-/-} mice. SAM identified reactivity to 4 antigens as significantly increased in serum from pristane-treated WT mice compared with pristane-treated *Irf9*^{-/-} mice (false discovery rate [FDR], <0.05). These antigens included 3 components of the Sm/RNP complex (U1A, BB', and Sm) and RiboP. Interestingly, all 4 polypeptides are components of RNA-associated autoantigen complexes.

In addition to analyzing the data using hierarchical clustering and a rigorous statistical algorithm, we validated all array findings using

Table 1
Immunoprecipitation of the Sm/RNP complex

Genotype	Treatment	<i>n</i>	Sm/RNP (no.)
WT	PBS	4	0 (0%)
WT	Pristane	15	8 (53%)
<i>Irf9</i> ^{-/-}	PBS	5	0 (0%)
<i>Irf9</i> ^{-/-}	Pristane	15	0 (0%) ^A
<i>Stat1</i> ^{-/-}	PBS	6	0 (0%)
<i>Stat1</i> ^{-/-}	Pristane	11	0 (0%) ^A

^A*P* < 0.01 versus pristane-treated WT, Fisher's exact test.

**Figure 2**

Pristane-treated *Irf9*^{-/-} mice lack isotype-switched IgG autoantibodies specific for nucleic acid-associated autoantigens but produce high titers of IgM anti-RNP autoantibodies. (A) Individual arrays were incubated with 1:300 dilutions of serum obtained from WT and *Irf9*^{-/-} mice 6 months after treatment with pristane. Pairwise SAM was used to determine antigen features with statistically significant differences in array reactivity between sera derived from pristane-treated WT and *Irf9*^{-/-} mice (FDR, <0.05; fold change, >3). Hierarchical clustering of samples based on reactivity to antigens with statistically significant differences is displayed as a heatmap and dendrogram. (B–E) Sera obtained from WT and *Irf9*^{-/-} mice 6 months after treatment with PBS (filled circles) or pristane (open circles) were analyzed for levels of IgG and IgM anti-Sm/RNP (B), anti-RiboP (C), anti-ssDNA (D), and anti-histone (E) autoantibodies by ELISA. Data represent absorbance values for individual mice minus background. Horizontal bars represent mean OD values for each group. *P* values were determined by Mann-Whitney test.

conventional techniques including immunoprecipitation and ELISA (see Supplemental Figure 3 for correlation plots of ELISA versus array values). WT mice treated with pristane develop autoantibodies capable of immunoprecipitating components of the Sm/RNP complex (15). Because SAM identified reactivity to 3 components of this complex as significantly increased in pristane-treated WT versus pristane-treated *Irf9*^{-/-} mice, we examined the ability of serum autoantibodies to immunoprecipitate the Sm/RNP complex. Sera from 8 of 15 (53%) pristane-treated WT mice immunoprecipitated this complex (Table 1); in striking contrast, all serum samples from *Irf9*^{-/-} ($n = 15$) and *Stat1*^{-/-} ($n = 11$) mice treated with pristane failed to immunoprecipitate the Sm/RNP complex.

We also used conventional ELISA to determine the titers of autoantibodies directed against the 2 RNA-associated complexes identified as significant on the arrays. IRF9 was required for the development of IgG autoantibodies directed against Sm/RNP and RiboP (Figure 2, B and C). Interestingly, in contrast to the phenotype for IgG, the titer of IgM autoantibodies directed against these 2 complexes was significantly increased in pristane-treated *Irf9*^{-/-} mice (Figure 2, B and C). The secondary reagent used for the array stud-

ies recognizes both IgG and IgM antibodies. Therefore, the high titers of IgM autoantibodies in pristane-treated *Irf9*^{-/-} mice may explain the weak reactivity to the 4 SAM-identified autoantigens in this group of mice (Figure 2A). These findings identify an IRF9-dependent defect in isotype switching from IgM to pathogenic IgG autoantibodies directed against RNA-associated autoantigens.

Pristane-treated WT mice also develop high titers of IgG anti-ssDNA and anti-histone autoantibodies (48). We examined the production of autoantibodies directed against these DNA-associated autoantigens using conventional ELISA. IgG anti-ssDNA and anti-histone autoantibody levels were significantly decreased in pristane-treated *Irf9*^{-/-} mice compared with pristane-treated WT mice (Figure 2, D and E). Although the titer of IgM autoantibodies directed against DNA-associated autoantigens was increased in pristane-treated compared with PBS-treated *Irf9*^{-/-} mice, there was no significant increase in IgM titers of anti-ssDNA or anti-histone autoantibodies between pristane-treated WT and *Irf9*^{-/-} mice (Figure 2, D and E). We also tested for the presence of anti-dsDNA autoantibodies in the serum of pristane-treated WT and *Irf9*^{-/-} mice using *Crithidia luciliae* immunofluorescence assays. At 9 months following pristane

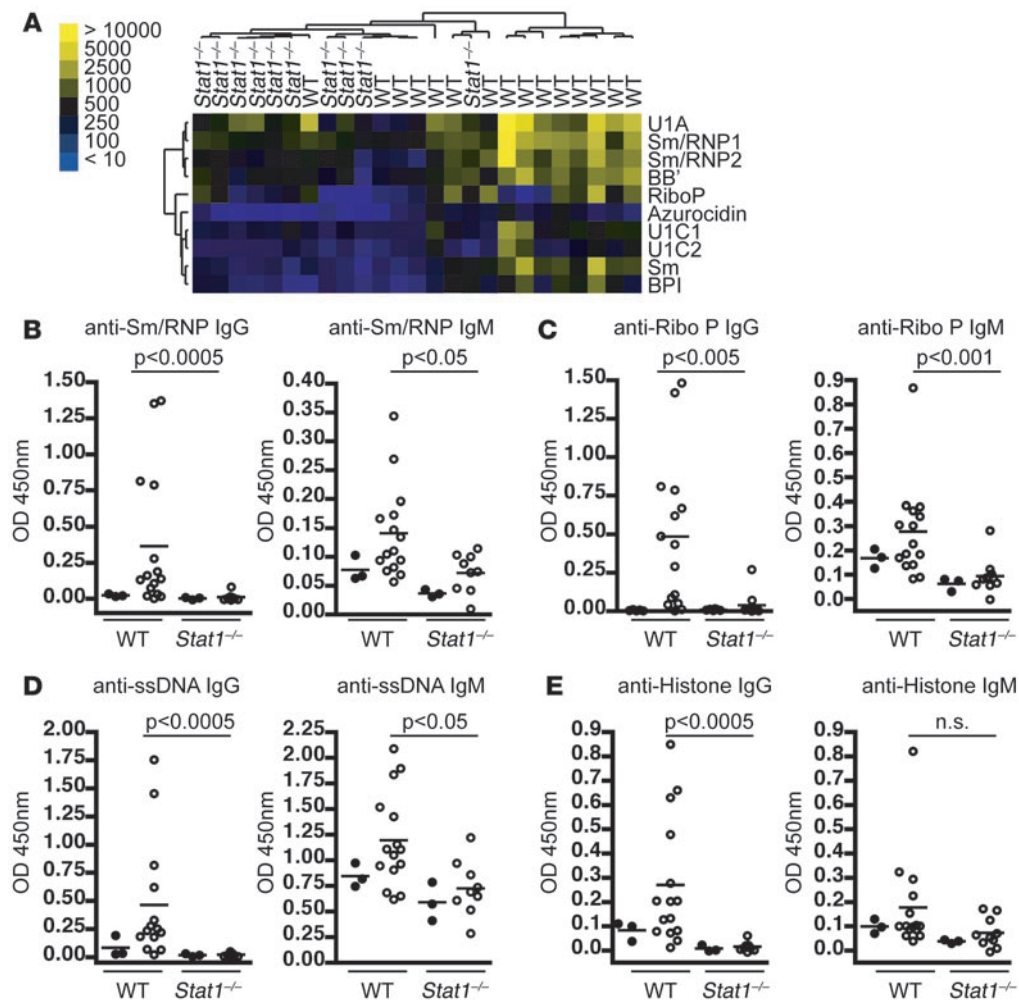


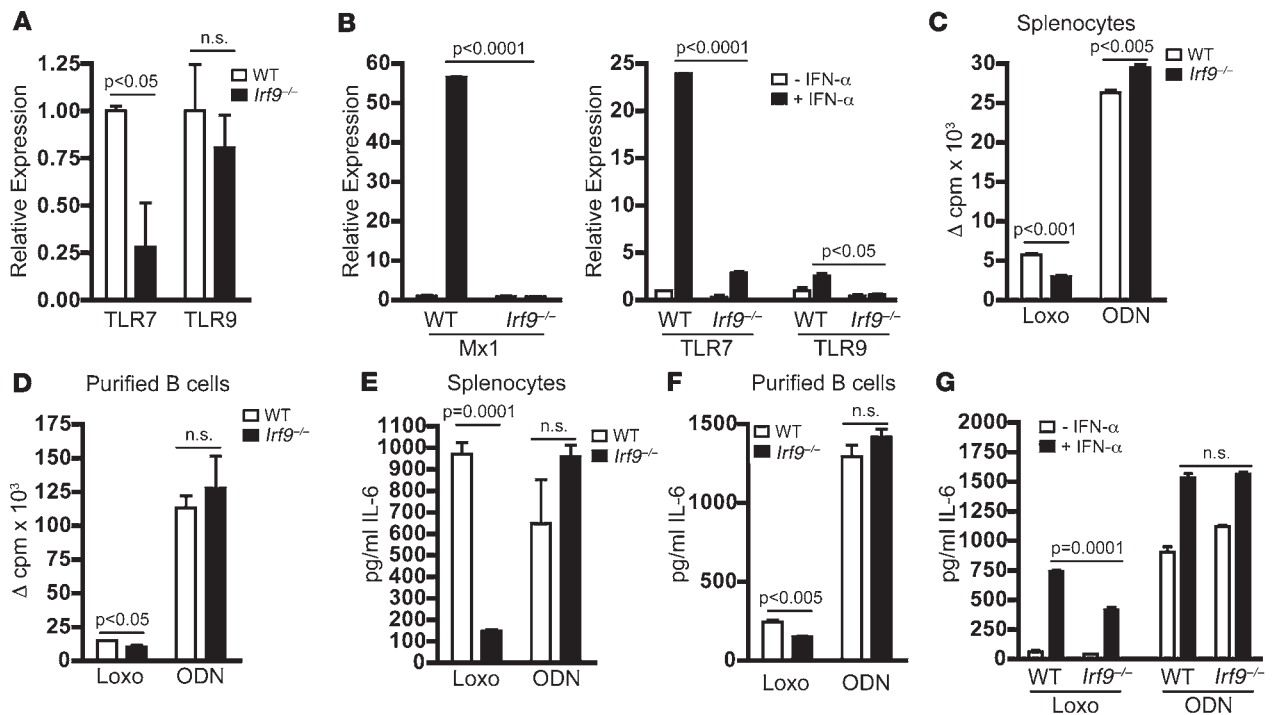
Figure 3
 Pristine-treated *Stat1*^{-/-} mice lack IgG and IgM autoantibodies specific for nucleic acid-associated autoantigens. (A) Individual arrays were incubated with 1:300 dilutions of serum obtained from WT and *Stat1*^{-/-} mice 6 months after treatment with pristane. Pairwise SAM was used to determine antigen features with statistically significant differences in array reactivity between sera derived from pristane-treated WT and *Irf9*^{-/-} mice (FDR, <0.05; fold change, >3). Hierarchical clustering of samples based on reactivity to antigens with statistically significant differences is displayed as a heatmap and dendrogram. (B–E) Sera obtained from WT and *Stat1*^{-/-} mice 6 months after treatment with PBS (filled circles) or pristane (open circles) were analyzed for levels of IgG and IgM anti-Sm/RNP (B), anti-RiboP (C), anti-ssDNA (D), and anti-histone (E) autoantibodies by ELISA. Data represent absorbance values for individual mice minus background. Horizontal bars represent mean OD values for each group. P values were determined by Mann-Whitney test.

treatment, serum from 2 of 15 (13%) WT mice demonstrated positive staining of the *C. luciliae* kinetoplast, whereas no serum samples from *Irf9*^{-/-} mice were positive (D.L. Thibault and P.J. Utz, unpublished observations). Therefore, we conclude that IRF9 is required for the production of isotype-switched IgG autoantibodies against both RNA- and DNA-associated targets. Titers of IgG isotypes directed against Sm/RNP and ssDNA are shown in Supplemental Figure 4.

We next examined the production of autoantibodies in pristane-treated *Stat1*^{-/-} mice. The SAM algorithm revealed reactivity to several components of the Sm/RNP complex (U1A, Sm/RNP, BB', U1C, and Sm) as well as RiboP, azurocidin, and BPI as significantly increased in pristane-treated WT mice versus pristane-treated *Stat1*^{-/-} mice (FDR, <0.05; Figure 3A). Conventional ELISA studies revealed that, unlike the phenotype for pristane-treated *Irf9*^{-/-} mice, pristane-treated *Stat1*^{-/-} mice had significantly lower levels of both IgG and IgM autoantibodies against Sm/RNP, RiboP, and ssDNA versus pristane-treated WT mice (Figure 3, B–D). Levels of IgG autoantibodies against total histone proteins were also significantly decreased in pristane-treated *Stat1*^{-/-} mice; however, there was no difference in the levels of IgM anti-histone autoantibodies (Figure 3E). Taken together, these data demonstrate a critical role for IFN-I signaling molecules in the development of high-titer, isotype-switched, IgG autoantibodies directed against prominent nucleic acid-containing SLE autoantigens. They further reveal an

important role for IRF9, but not STAT1, in isotype switching to IgG in response to RNA-associated autoantigens.

TLR7 and TLR9 expression. The nucleic acid components of SLE autoantigens have previously been shown to activate autoreactive B cells through the nucleic acid-sensing TLR7, which recognizes RNA, and TLR9, which recognizes DNA. One study demonstrated that IFN-I secreted by pDCs induces the expression of TLR7 but not TLR9 in human naive B cells (49). The proliferation of autoreactive B cells in response to RNA-associated immune complexes is enhanced by IFN-I, presumably due to its ability to enhance TLR7 expression (29). We hypothesized that there may be defects in the expression of TLR7 and TLR9 in B cells from *Irf9*^{-/-} mice. Splenic B cells were purified from WT and *Irf9*^{-/-} mice, RNA was extracted, and the relative expression of TLR7 and TLR9 normalized to GAPDH was determined by real-time quantitative PCR. B cells from *Irf9*^{-/-} mice expressed significantly lower levels of TLR7 mRNA than did WT B cells; however, there was no significant difference in the expression of TLR9 (Figure 4A). We next examined the ability of IFN-I to enhance the expression of TLRs in B cells. Purified B cells from WT or *Irf9*^{-/-} mice were cultured in the presence or absence of recombinant IFN- α . As expected, the induction of expression of a known IFN-I-inducible gene, *Mx1*, was completely abolished in B cells from *Irf9*^{-/-} mice following IFN- α treatment (Figure 4B). The expression of TLR7 was upregulated more than 20-fold in IFN- α -treated B cells from WT mice, but

**Figure 4**

IRF9 is required for expression of, and activation through, TLR7 in B cells. (A) B cells were purified from WT or *Irf9*^{-/-} mice using magnetic beads. RNA was extracted, and the relative expression of TLR7 and TLR9 normalized to GAPDH was measured by real-time quantitative PCR using the 2^{-ΔΔCt} method. (B) Purified B cells were cultured in the presence or absence of 1,000 IU/ml IFN- α for 4 hours. RNA was extracted, and the relative expression of Mx1, TLR7, and TLR9 normalized to GAPDH was measured as in A. (C and D) Whole splenocytes (C) or splenic B cells purified using magnetic beads (D) were cultured with 1 mM loxoribine (Loxo) or 1 μ M ODN1826 (ODN). Cells were pulsed with [³H]TdR at 16 hours and harvested at 24 hours, after which incorporated radioactivity was measured. Data represent the difference in mean cpm (Δ cpm) between stimulated and unstimulated triplicate wells \pm SEM. (E and F) Whole splenocytes (E) or purified B cells (F) were cultured as in C and D. Cells were harvested at 24 hours following stimulation, and the concentration of IL-6 in the supernatant was measured by ELISA. (G) Purified B cells were pretreated with 1,000 IU/ml IFN- α for 24 hours before treatment with loxoribine or ODN1826. The concentration of IL-6 in the supernatant was measured by ELISA after 24 hours in culture with TLR ligands. *P* values were determined by Student's *t* test.

this upregulation was almost completely inhibited in B cells from *Irf9*^{-/-} mice (Figure 4B). In contrast to results previously obtained in human B cells (49), IFN- α enhanced the expression of TLR9 in WT B cells approximately 3-fold. This upregulation was abolished in *Irf9*^{-/-} B cells. Therefore, IRF9 mediates the IFN-I-inducible expression of TLR7 and TLR9 in murine B cells. We also examined the expression of TLR7 and TLR9 in B cells from *Stat1*^{-/-} mice. Similar to the phenotype for *Irf9*^{-/-} B cells, *Stat1*^{-/-} B cells failed to upregulate the expression of TLR7 and TLR9 following IFN- α stimulation (Supplemental Figure 5). We conclude that IRF9 and STAT1 are critical for the expression of nucleic acid-sensing TLRs in B cells and are required for the induction of TLR expression by IFN-I.

TLR7 and TLR9 activation. To determine whether differences in TLR7 and TLR9 expression had functional consequences on *Irf9*^{-/-} B cell activation, we examined the ability of *Irf9*^{-/-} B cells to respond to TLR7 and TLR9 agonists. We stimulated whole splenocytes and purified B cells from WT and *Irf9*^{-/-} mice with a TLR7-activating ssRNA analog, loxoribine, or a TLR9-activating CpG-containing oligodeoxynucleotide (ODN), ODN1826. Both proliferation (Figure 4, C and D) and IL-6 secretion (Figure 4, E and F) were significantly decreased in *Irf9*^{-/-} splenocytes and purified B cells in response to the TLR7 agonist. Whole splenocytes from *Irf9*^{-/-} mice proliferated slightly more in response to the TLR9 ligand (Figure 4C), whereas

no difference in B cell proliferation (Figure 4D) or secretion of IL-6 by whole splenocytes (Figure 4E) or purified B cells (Figure 4F) was observed in response to this ligand. These data indicate functional defects in TLR7-specific B cell responses in *Irf9*^{-/-} mice. Because IFN- α induced very high levels of TLR7 expression in B cells from WT mice but not *Irf9*^{-/-} mice, we examined the secretion of IL-6 in response to TLR ligands following treatment with IFN- α . Although pretreatment with IFN- α increased the level of IL-6 secretion by purified B cells in response to loxoribine in both WT and *Irf9*^{-/-} mice, these levels were significantly lower in B cells from *Irf9*^{-/-} mice than in those from WT mice (Figure 4G). IFN- α pretreatment enhanced B cell secretion of IL-6 in response to ODN1826; however, there was no difference between WT and *Irf9*^{-/-} mice.

Stat1^{-/-} B cells also displayed defects in responses to TLR7 agonists (Supplemental Figure 5). In particular, *Stat1*^{-/-} splenocytes and purified B cells proliferated less and secreted less IL-6 upon stimulation with loxoribine compared with cells from WT mice. Unlike *Irf9*^{-/-} mice, splenocytes and purified B cells from *Stat1*^{-/-} mice proliferated significantly less and secreted less IL-6 in response to a CpG-containing TLR9 agonist (Supplemental Figure 5, C-F). *Stat1*^{-/-} B cells pretreated with IFN- α failed to upregulate secretion of IL-6 upon stimulation with both TLR7 and TLR9 agonists (Supplemental Figure 5G). We have therefore identified

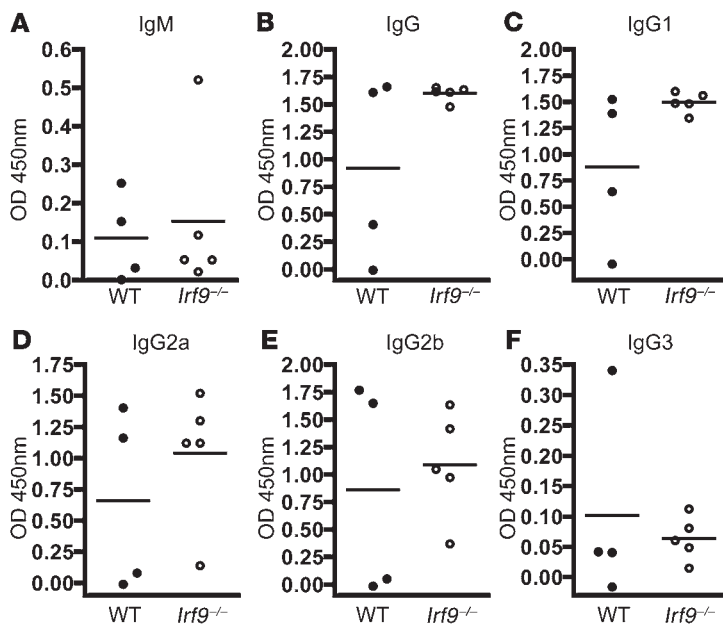


Figure 5
Irf9^{-/-} mice mount an effective IgG antibody response when immunized with OVA in CFA. WT and *Irf9*^{-/-} mice were immunized with OVA in CFA on day 0 and boosted with OVA in IFA on day 14. Sera were obtained on day 28 and analyzed for levels of anti-OVA IgM (A), IgG (B), IgG1 (C), IgG2a (D), IgG2b (E), and IgG3 (F) antibodies by ELISA using isotype-specific secondary antibodies. Data represent absorbance values for individual mice. Horizontal bars represent mean OD values for each group.

deficiencies in TLR expression and in TLR7-specific responses in B cells that lack critical IFN-I signaling molecules. Our data suggest that IRF9 and STAT1 are central mediators of TLR7- and TLR9-specific B cell responses and that activation of these pathways may lead to antigen-specific IgG autoantibody responses in vivo.

Adjuvant-enhanced antibody responses. Recently it has been suggested that adjuvant-enhanced antibody responses do not require TLR signaling in vivo (50). Because *Irf9*^{-/-} mice exhibited specific defects in isotype switching to IgG in the pristane model, we asked whether there were global defects in isotype switching or whether this defect in IgG antibody response was limited to nucleic acid-containing self antigens with the capacity to ligate TLR7 and/or TLR9. We immunized WT and *Irf9*^{-/-} mice with OVA in CFA on day 0, boosted with OVA in incomplete Freund adjuvant (IFA) on day 14, and measured anti-OVA antibody titers on day 28. There were no significant differences in levels of IgM, IgG, IgG1, IgG2a, IgG2b, or IgG3 anti-OVA antibodies between WT and *Irf9*^{-/-} mice (Figure 5). We conclude that unlike the phenotype for TLR-dependent autoantigens, B cells from *Irf9*^{-/-} mice are capable of isotype switching to all subtypes of IgG in response to a foreign antigen together with a strong stimulus such as CFA. These data also demonstrate that there are no global defects in B cell activation, B cell signaling, B cell maturation, affinity maturation, or isotype switching in *Irf9*^{-/-} mice in vivo.

Kidney disease and plasmacytoma development. The development of proteinuria, a measure of kidney disease, was assessed in WT, *Irf9*^{-/-}, and *Stat1*^{-/-} mice treated with PBS and pristane (Table 2). As expected, about half of WT mice primed with pristane developed proteinuria over the course of 12 months. None of the pristane-treated *Irf9*^{-/-} mice developed proteinuria, suggesting that IRF9 may

play a role in the development of nephritis. One *Stat1*^{-/-} mouse treated with pristane developed proteinuria, and this mouse also developed very severe ascites as the result of plasmacytoma induction. Following the completion of the study at 12 months, kidneys were harvested from the remaining mice, sectioned, and stained with hematoxylin and eosin, after which the extent of kidney damage was scored according to standard NIH activity and chronicity indices (51) in a blinded manner by a single investigator (see Supplemental Table 1 for detailed description of extent and type of kidney pathology). The overall extent of kidney damage was not severe in the pristane-treated WT group, which had an average activity score of 0.23 out of a possible 4 (Supplemental Table 1). None of 6 pristane-treated *Irf9*^{-/-} or 5 pristane-treated *Stat1*^{-/-} mice examined developed any changes in activity index. Taken together, these data suggest a role for IFN-I signaling molecules in the end-organ pathogenesis of lupus nephritis.

One major caveat of these findings was that a high percentage of pristane-treated *Irf9*^{-/-} mice developed fatal ascites prior to the conclusion of the study as a result of plasmacytoma induction. Approximately 20% of BALB/c mice primed with a single injection of pristane develop plasmacytomas driven by chronic inflammation in the peritoneal cavity (52). The development of ascites in pristane-treated WT, *Irf9*^{-/-}, and *Stat1*^{-/-} mice was plotted as a Kaplan-Meier curve (Supplemental Figure 6). Induction of plasmacytoma was confirmed by cytological analysis of ascites fluid (D.L. Thibault and P.J. Utz, unpublished observations). The median survival time of pristane-treated *Irf9*^{-/-} mice was 11 months, and 67% of the mice developed fatal ascites before

the conclusion of the study. *Stat1*^{-/-} mice displayed a greatly accelerated course of ascites development (median survival time, 8 months), and 65% of mice developed ascites over the course of 12 months. The anti-neoplastic properties of IFNs have been well documented, and STAT1 mediates the antiproliferative and proapoptotic effects of both IFN-Is and IFN- γ (53, 54). Recombinant IFN- α has been used successfully to treat a variety of malignancies (55). The higher rate of plasmacytoma induction in both *Irf9*^{-/-} and *Stat1*^{-/-} mice is therefore not surprising given their susceptible BALB/c genetic background, although the mechanism of this induction remains to be elucidated. It is possible that some *Irf9*^{-/-} mice may have gone on to develop proteinuria or kidney damage had they survived. WT mice primed with pristane, however, did not develop the extensive kidney pathology seen in other spontaneous models of SLE such as MRL/lpr and (NZB \times NZW)F1. We are in the process of backcrossing *Irf9*^{-/-} and *Stat1*^{-/-} mice to these models to more carefully assess the contribution of these molecule to end-organ kidney damage.

Discussion

Since the original clinical and serological description of MCTD by Sharp and colleagues in this journal (56), many hypotheses have been put forward regarding the mechanisms by which high-titer IgG autoantibodies against the U1snRNP and other nucleic acid-containing autoantigens become immunogenic. Cross-reactivity with viral antigens such as U1-70K and the retroviral protein p30gag (57), coassociation of RNPs with viral antigens such as EBER1 and EBER2 (58), and apoptosis-specific modifications of many components of the U1snRNP (59) have all been suggested to play important roles in the immunogenicity of the U1snRNP. Our present results do not



Table 2
Development of proteinuria

Genotype	Treatment	n	Proteinuria (no.) ^A
WT	PBS	4	0 (0%)
WT	Pristane	15	7 (47%)
<i>Irf9</i> ^{-/-}	PBS	5	0 (0%)
<i>Irf9</i> ^{-/-}	Pristane	15	0 (0%) ^B
<i>Stat1</i> ^{-/-}	PBS	6	0 (0%)
<i>Stat1</i> ^{-/-}	Pristane	11	1 (9%) ^C

^ADefined as 2 consecutive readings of 300 mg/dl or higher. ^B*P* < 0.01 versus pristane-treated WT, Fisher's exact test. ^CNS versus pristane-treated WT.

refute these models, but identify a central, *in vivo* role for 2 molecules downstream of IFNAR in the production of IgG autoantibodies capable of immunoprecipitating RNP complexes targeted in MCTD and SLE, including the U1snRNP and RiboP.

In the current study, we describe the autoantibody response in mice deficient for IRF9 and STAT1, critical downstream mediators of IFN-I signaling. Our initial screen using autoantigen microarrays followed by rigorous statistical analysis using SAM revealed 4 significantly different antigens between pristane-treated WT and *Irf9*^{-/-} mice, all of which are associated with RNPs. Further validation using conventional techniques demonstrated that IRF9 and STAT1 were required for the development of IgG autoantibodies to several different nucleic acid-associated complexes targeted in this model. In contrast, *Irf9*^{-/-} mice mounted an effective IgG antibody response against a foreign antigen when immunized with the strong adjuvant CFA. *Irf9*^{-/-} and *Stat1*^{-/-} B cells had specific defects in expression of, and activation through, the nucleic acid-sensing TLRs, suggesting that defects in TLR7- and TLR9-dependent B cell responses may account for the lack of isotype-switched IgG autoantibodies.

The ISGF3 complex, composed of STAT1, STAT2, and IRF9, has also been shown to form upon activation by IFN- γ (10, 11). ISGF3 formation in response to IFN- γ treatment occurred at higher levels following pretreatment with IFN- α , which upregulates the expression of STAT1 and IRF9, or in the presence of highly overexpressed STAT1 and IRF9 (10). The *in vivo* relevance of this pathway has not yet been established, and it is possible that IRF9 mediates signals through both the IFNAR and the IFN- γ receptor. To more directly address the role of the IFN-I pathway in the development of autoimmunity, we have initiated similar studies in pristane-treated mice deficient for the IFNAR2 chain of the IFNAR. Preliminary data suggest that pristane-treated *Ifnar2*^{-/-} mice have a phenotype similar to that seen in pristane-treated *Irf9*^{-/-} mice (D.L. Thibault and P.J. Utz, unpublished observations). Specifically, the production of IgG autoantibodies, and the expression of nucleic acid-sensing TLRs in B cells, was completely dependent on IFNAR2. Levels of total serum IgM were significantly increased in pristane-treated *Ifnar2*^{-/-} mice, and these mice developed high titers of IgM autoantibodies, suggesting a defect in isotype switching similar to that seen in *Irf9*^{-/-} mice. In addition, pristane-treated *Ifnar1*^{-/-} mice failed to develop anti-RNP autoantibodies; however, the expression of TLRs and the isotypes of the autoantibodies were not assessed in this study (60). This differs from the autoantibody phenotype seen in pristane-treated IFN- γ -deficient mice, in which IFN- γ was not absolutely required for the development of high titers of IgG autoantibodies capable of precipitating the Sm/RNP complex (ref. 61 and D.L. Thibault, K.L.

Graham, and P.J. Utz, unpublished observations). These data suggest that IRF9 mediates signals downstream of the IFNAR, but not downstream of the IFN- γ receptor, and that IFN-I drives the autoimmune response to RNA-associated autoantigens *in vivo*.

Molecules involved in the negative regulation of both TLR and IFN signaling pathways have previously been identified. In particular, the TAM receptors, which include Tyro3, Axl, and Mer, are pleiotropic inhibitors of TLR- and cytokine-induced signaling, and control the induction of SOCS-1 by IFN- α (62). Both *Socs1*^{-/-} and TAM receptor triple-knockout mice develop spontaneous autoimmunity (63, 64), highlighting the critical role of negative regulation of the TLR and IFN pathways in the development of autoimmunity. The TAM receptors have previously been shown to be directly associated with IFNAR1, and *Stat1*^{-/-} mice display defects in TAM receptor function (62). TAM-mediated induction of the molecule Twist results in the suppression of TNF- α production (65), demonstrating a mechanism by which IFN-I mediates the suppression of TNF- α . The role of these molecules in the development of autoimmunity in the pristane model, as well as their function in *Irf9*^{-/-} mice, will need to be assessed in future studies.

The 9 known members of the IRF family play roles in the induction of IFN-regulated genes and of IFN-I themselves (66). Recent publications have highlighted important potential roles for members of the IRF family in the pathogenesis of SLE. A common haplotype of IRF5 is a genetic risk factor for human SLE (67–69). Both IRF7 and IRF5 are critical for DC production of IFN- α , IFN- β , and IL-6 by anti-RNP immune complexes and by conventional TLR7 and TLR9 ligands (70, 71). MRL/*lpr* mice lacking the IRF1 gene develop less severe disease compared with WT mice (72). Finally, female mice deficient for IRF4-binding protein develop a lupus-like disease characterized by hypergammaglobulinemia, autoantibody production, and immune complex-mediated glomerulonephritis (73). To our knowledge, a role for IRF9 has not previously been addressed in the contexts of SLE or TLR signaling. Our present results suggest this molecule is a critical mediator of B cell responses, including autoantibody production, isotype switching, and the expression of and activation through nucleic acid-sensing TLRs.

The pristane model offers a unique opportunity to study the role of the IFN-I pathway in the development of murine SLE. Several recent studies have examined the contributions of TLRs and IFN-I to SLE disease in the MRL/*lpr* model (33, 74, 75). DNA microarray analysis of MRL/*lpr* splenocyte subsets and kidneys clearly demonstrate an IFN- γ -regulated gene expression profile, whereas genes induced by IFN-I are not upregulated in MRL/*lpr* mice (76). This observation may explain why MRL/*lpr* mice deficient for the IFNAR1 chain of the IFNAR actually develop more severe disease: the lack of IFN-I signaling may further drive the IFN- γ response (75). In contrast to the MRL/*lpr* model, several IFN-I-inducible genes are upregulated in pristane-induced ectopic lymphoid tissue and peripheral blood mononuclear cells (18, 60), which mirrors the gene expression profile seen in human lupus patients (2–4, 12, 13). In human SLE patients, high expression of these genes correlates with the production of anti-nucleoprotein and, in particular, anti-RNP autoantibodies (12, 13). (NZB \times NZW)F1 mice do not develop autoantibodies that recognize RNA-containing autoantigens, although administration of pristane to these mice resulted in an accelerated disease course and induced the production of anti-RNP autoantibodies (77). Therefore, disease pathogenesis in the pristane model, especially in regard to dysregulation of the IFN-I pathway, more closely resembles human SLE when compared with the spontaneous models described above. To more



carefully assess the contribution of this pathway to lupus nephritis, further studies in these spontaneous models will be necessary.

Several recent studies have described the ability of nucleic acid-containing MCTD and SLE autoantigens to stimulate autoreactive B cells through nucleic acid-sensing TLRs. Here we demonstrated that IFN-I signaling components controlled the ability of B cells to upregulate the expression of TLR7 and TLR9 upon stimulation with IFN- α . The expression of TLR7 in particular was influenced by IFN-I, because its expression was enhanced more than 20-fold upon incubation with IFN- α in WT B cells. Interestingly, although B cell expression of TLR7 and secretion of IL-6 in response to a TLR7 agonist was dramatically reduced in *Irf9*^{-/-} mice following treatment with IFN- α (Figure 4, B and G), TLR7 expression and activation were slightly enhanced by IFN- α in the absence of IRF9. Residual TLR7 expression following IFN- α treatment was sufficient for the secretion of lower levels of IL-6, suggesting that expression of TLR7 is partially IRF9 independent in B cells. We propose that residual expression of TLR7 may drive the partial activation of autoreactive B cells and the subsequent production of IgM-specific autoantibodies.

IRF9 is absolutely required for isotype switching to pathogenic, high-titer, high-affinity IgG autoantibodies. This is not caused by global defects in isotype switching in *Irf9*^{-/-} B cells, because there was no defect in adjuvant-enhanced isotype switching to all subtypes of IgG against a foreign antigen (Figure 5). Unlike pristane-treated *Irf9*^{-/-} mice, pristane-treated *Stat1*^{-/-} mice did not develop high titers of total serum IgM or IgM-specific autoantibodies. Both PBS- and pristane-treated *Stat1*^{-/-} mice had very low serum titers of all antibody isotypes except for IgG1, and B cells from *Stat1*^{-/-} mice displayed a decreased ability to respond to multiple stimuli, including TLR7 and TLR9, and decreased crosslinking of the B cell receptor by anti-IgM (78). These data suggest that there may be global defects in B cell activation in *Stat1*^{-/-} mice that are not limited to TLR7-specific responses; however, further studies to characterize potential defects are necessary. B cell stimulation by CpGs through TLR9 and MyD88 promotes T cell-independent isotype switching to IgG2a through direct induction of the transcription factor T-bet (32, 79, 80). Because TLR7 also signals through MyD88, it is thought that signaling through TLR7 promotes a similar IgG2a response. In support of these studies, *Irf9*^{-/-} mice immunized with OVA using a TLR7 agonist as an adjuvant failed to produce IgG2a OVA-specific antibodies in a pilot study (D.L. Thibault and P.J. Utz, unpublished observations); however, a larger cohort of mice is necessary to confirm these findings. The lack of isotype switching to IgG in *Irf9*^{-/-} mice is not due to the inability of *Irf9*^{-/-} B cells to upregulate T-bet, as T-bet was highly induced in *Irf9*^{-/-} B cells following incubation with IFN- α together with TLR agonists (D.L. Thibault and P.J. Utz, unpublished observations). IFN-I together with IL-6 promotes B cell maturation to antibody-secreting plasma cells (43), providing further support for a role for IRF9 in B cell isotype switching. Further studies are necessary to dissect the complex role for IFN-I signaling in TLR7- and TLR9-dependent and -independent B cell responses and to determine the role of this pathway in other cell types in SLE pathogenesis.

In summary, our data demonstrate what we believe to be a novel role for 2 downstream mediators of IFN-I signaling in the generation of IgG autoantibody responses against nucleic acid-associated autoantigens. We propose that IFN-I, which is produced by pDCs in vivo following pristane treatment, promotes the expression of TLR7 and TLR9 in B cells through the ISGF3, allowing for the acti-

vation of autoreactive B cells by MCTD- and SLE-associated autoantigens and the production of high-titer, high-affinity IgG autoantibodies. A schematic outlining this proposed model is depicted in Supplemental Figure 7. Pristane treatment has previously been shown to induce apoptosis both in vitro and in vivo, providing a potential source of lupus-associated autoantigens (17). Our data suggest that IFN-I signaling is upstream of TLR responses in the activation of autoreactive B cells. Finally, specific inhibitors of IFN-I and TLRs are in preclinical or early-stage clinical trials for the treatment of SLE in human patients. Our results suggest that patients could be selected for clinical trials and monitored for response to therapy based on autoantibody profiles and provide a clear rationale for pursuing these therapeutic approaches.

Methods

Mouse and treatment. BALB/c mice were obtained from the Department of Laboratory Animal Medicine at Stanford University School of Medicine. *Irf9*^{-/-} mice backcrossed onto the BALB/c background to the N8 generation were purchased from RIKEN BioResearch Center. *Stat1*^{-/-} mice backcrossed onto the BALB/c background to the N9 generation were a generous gift from J. Durbin (Ohio State University, Columbus, Ohio, USA). All mice used in this study were maintained under standard conditions at the Stanford University Research Animal Facility. Female mice of 8–10 weeks of age were given a single 0.5-ml i.p. injection of pristane (Sigma-Aldrich) or 0.5 ml PBS. Sera were collected before injection and at 4-week intervals throughout the duration of the experiment. Proteinuria was monitored by dipstick analysis using Albustix (Bayer) on a monthly basis. All animal experiments were approved by, and performed in compliance with the guidelines of, the Institutional Animal Care and Use Committee of Stanford University.

Lupus autoantigen microarrays. Autoantigens were diluted to 0.2 mg/ml in PBS and printed in ordered arrays on nitrocellulose-coated FAST slides (Whatman) using a VersArray ChipWriter Pro Robotic Arrayer (Bio-Rad). Individual arrays were circumscribed with a hydrophobic marker pen and blocked with PBS containing 3% fetal calf serum and 0.05% Tween 20 (Sigma-Aldrich) overnight at 4°C. Arrays were probed with 450 μ l mouse serum diluted 1:300 for 2 hours at 4°C, followed by washing and incubation with a 1:2,000 dilution of cyanine-3-conjugated goat anti-mouse IgG/IgM secondary antibody (Jackson ImmunoResearch Laboratories). Arrays were scanned using a GenePix 4000B scanner (Molecular Devices). Detailed protocols and lists of antigens have been published previously (41).

The median pixel intensities of each feature were determined using GenePix Pro version 6.0 software (Molecular Devices), and background values were subtracted. Data were expressed as normalized median net digital fluorescence units, representing the median values from 8 replicate antigen features on each array normalized to the median intensity of 8 goat anti-mouse Ig features. SAM (47) was applied to the dataset to identify antigens with statistically significant differences in array reactivity between groups of mice. A hierarchical clustering algorithm was applied (45), and data were displayed using Java Treeview software (46).

ELISAs. All serological analysis was performed on sera obtained 6 months following treatment with pristane or PBS. For anti-ssDNA ELISAs, Nunc Maxisorp plates (Nalgene) were coated with 10 μ g/ml calf thymus DNA (Sigma-Aldrich). For anti-histone, anti-Sm/RNP, anti-RiboP, and anti-OVA ELISAs, Maxisorp plates were coated with purified whole histones (ImmunoVision), Sm/RNP (Diarect), RiboP (Diarect), or OVA (Pierce) at a concentration of 1 μ g/ml overnight at 4°C. Wells were incubated with mouse sera diluted 1:250 in PBS containing 3% FBS and 0.05% Tween-20 followed by incubation with HRP-conjugated goat anti-mouse IgG, IgM, IgG1, IgG2a, IgG2b, or IgG3 secondary antibody (Jackson ImmunoResearch Laboratories) at a dilution of 1:5,000. Tetramethylbenzidine substrate (Pierce)



was added, and OD values were determined at 450 nm. Absorbances from blank wells (no serum added) were subtracted.

To determine levels of total serum Ig isotypes, plates were coated with 5 µg/ml goat anti-mouse Ig (heavy and light chain) antibody (Southern Biotechnology) overnight at 4°C. Wells were incubated with 1:5,000,000 dilution for IgG, or 1:500,000 dilution for all other isotypes, of sera in PBS containing 3% FBS and 0.05% Tween-20 followed by isotype-specific HRP-conjugated secondary goat anti-mouse antibody (Southern Biotechnology). Standard curves were constructed using mouse Ig isotype standards (Southern Biotechnology).

Real-time quantitative PCR. Splenocytes were harvested from age- and sex-matched WT, *Irf9*^{-/-}, and *Stat1*^{-/-} mice. B cells were negatively selected using magnetic beads (Miltenyi Biotech). Cells were more than 95% pure, as assessed by flow cytometry (B220⁺biotin⁻). B cells were cultured in RPMI supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-ME (5 × 10⁻⁵ M), and FBS (10%) in the presence or absence of 1,000 IU/ml recombinant IFN-α (Calbiochem) for 4 hours. RNA was extracted using RNeasy Mini kit (Qiagen). RNA (10 ng) was amplified using 1-step QuantiTect SYBR Green RT-PCR (Qiagen) and 0.5 µM forward and reverse primers using an Opticon2 continuous fluorescence detector (MJ Research). The fold change in expression of each transcript normalized to GAPDH was determined by the 2^{-ΔΔCt} method. QuantiTect Primer Assay sets for murine TLR7, TLR9, and GAPDH were purchased from Qiagen. Primer sequences for Mx1 were as follows: 5'-GATCCGACTTCACCTCCAGATGG-3' (forward) and 5'-CATCTCAGTGGTAGTCAACCC-3' (reverse).

Proliferation assay. Whole splenocytes, or B cells purified as described above, were harvested from age- and sex-matched WT, *Irf9*^{-/-}, or *Stat1*^{-/-} mice and stimulated with 1 µM ODN1826 or 1 mM loxoribine (both from Invivogen) in triplicate. Cells were pulsed with 1 µCi [³H]TdR (Amersham) 16 hours following stimulation and harvested 24 hours following TLR stimulation. Incorporated radioactivity was measured using a betaplate scintillation counter.

IL-6 production. Whole splenocytes or B cells were purified, cultured, and stimulated as described above. After 24 hours, supernatants were assayed for the production of IL-6 by sandwich ELISA using a commercially available kit (BD Biosciences – Pharmingen). For IFN-α pretreatment studies, purified B cells were incubated in the presence or absence of 1,000 IU/ml recombinant murine IFN-α (Calbiochem) for 24 hours. TLR ligands were

added as described above, and IL-6 concentration in the supernatant was determined 24 hours following stimulation.

Adjuvant enhanced antibody responses. Age- and sex-matched WT and *Irf9*^{-/-} mice were immunized i.p. with 25 µg Imject OVA (Pierce) in PBS emulsified in CFA (Sigma-Aldrich) on day 0. Mice were boosted with 25 µg Imject OVA in PBS emulsified in IFA (Sigma-Aldrich) on day 14. Serum was collected from the mice on day 28 and assayed for the production of anti-OVA antibodies by ELISA.

Statistics. Differences between and among groups were determined by Fisher's exact test, Student's *t* test, or Mann-Whitney test as indicated in the figure and table legends. A *P* value less than 0.05 was considered significant.

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