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Pleiotropic IFN-Dependent and -Independent Effects of IRF5 on the Pathogenesis of Experimental Lupus

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Genetic polymorphisms of IFN regulatory factor 5 (IRF5) are associated with an increased risk of lupus in humans. In this study, we examined the role of IRF5 in the pathogenesis of pristane-induced lupus in mice. The pathological response to pristane in IRF5−/− mice shared many features with type I IFN receptor (IFNAR)−/− and TLR7−/− mice: production of anti-Sm/RNP autoantibodies, glomerulonephritis, generation of Ly6C+ monocytes, and IFN-I production all were greatly attenuated. Lymphocyte activation following pristane injection was greatly diminished in IRF5−/− mice, and Th cell differentiation was deviated from Th1 in wild-type mice toward Th2 in IRF5−/− mice. Th cell development was skewed similarly in TLR7−/− or IFNAR−/− mice, suggesting that IRF5 alters T cell activation and differentiation by affecting cytokine production. Indeed, production of IFN-I, IL-12, and IL-23 in IRF5−/− mice prevented Th cell development from occurring, whereas IL-4 increased. Unexpectedly, plasmacytoid dendritic cells (pDC) were not recruited to the site of inflammation in IRF5−/− mice. Th cell development was skewed similarly in TLR7−/− or IFNAR−/− mice, suggesting that IRF5 regulates chemokines involved in the homing of pDCs and certain lymphocyte subsets.


The transcription factor IFN regulatory factor 5 (IRF5) is a member of the IRF family with a key role in TLR-stimulated production of proinflammatory cytokines such as IL-12, IL-23, IL-6, and TNF-α (1), activation of type I IFN genes (2, 3), regulation of apoptosis (4), and development of B cells (5, 6). In humans, there are multiple IRF5 isoforms resulting from alternative splicing of the IRF5 gene (7–10). In contrast, murine IRF5 is expressed as a single transcript (11). Certain genetic polymorphisms of IRF5 are strongly associated with an increased risk of developing systemic lupus erythematosus (SLE) in humans, and the IRF5 haplotype helps to define the risk for SLE (7, 10, 12–15). IRF5 also contributes to the pathogenesis of lupus in mouse models. In the FCyRIIB−/− Yaa and FCyRIIB−/− lupus models, IRF5 is required for autoantibody production and renal disease (16). The mechanism appears to be partly independent of IFN-I production, but additional mechanisms have not been defined. IRF5 deficiency also abolishes anti-Sm/RNP Abs and reduces anti-dsDNA autoantibodies and inflammatory cytokine production while decreasing renal disease and improving survival in MRL/lpr mice (17). Although IFN-I ameliorates lupus in the MRL/lpr model (18), lupus induced by pristane is mediated by signaling through the type I IFN receptor (IFNAR) and TLR7 (19). In a recent study, autoantibody production and renal disease were abolished in pristane-treated IRF5−/− mice, an effect ascribed to a B cell-intrinsic IRF5 requirement for class switching to IgG2a, the predominant autoantibody isotype in the pristane lupus model (6). The present study was carried out to further define the mechanisms by which IRF5 influences the development of autoimmune disease in mice. We present evidence that the effects of IRF5 on the induction of autoimmune disease in pristane-treated mice is more complex than previously believed, with IFN-dependent or -independent effects on multiple cell lineages including B and T lymphocytes, monocytes/macrophages, and plasmacytoid dendritic cells (pDC).

Materials and Methods
Mice and pristane treatment
Mice were bred and maintained under specific pathogen-free conditions at the University of Florida Animal Facility. IRF5−/− mice on a C57BL/6 (B6) background were provided by Dr. Katherine Fitzgerald (University of Massachusetts, Worcester, MA) with permission from Dr. Tak Mak (University of Toronto, Toronto, ON, Canada) and were backcrossed to B6 for at least 10 generations. B6 MyD88−/− mice were provided by Dr. Lyle
Moldawer (Department of Surgery, University of Florida). TLR7−/− mice on a BALB/c background were acquired from Oriental Bioservices (Kyoto, Japan), and IFNAR−/− mice backcrossed nine generations onto a BALB/c background were provided by Dr. Joan Durbin ( Nationwide Children’s Hospital, Ohio State University, Columbus, OH), respectively. Wild-type BALB/c, B6, and BALB/C X B6 F1 CB6F1/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice received a single i.p. injection of 0.5 ml pristane (2,6,10,14-tetramethylpentadecane [TMPD]; Sigma-Aldrich, St. Louis, MO) filtered through a 0.25-μm filter or left untreated as controls. These studies were approved by the Institutional Animal Care and Use Committee.

Real-time quantitative PCR
Quantitative PCR (Q-PCR) was performed as previously described (20, 21). In brief, total RNA was extracted from 10⁶ peritoneal cells using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized using the Superscript II First-Strand Synthesis kit (Invitrogen) according to the manufacturer’s protocol. SYBR Green Q-PCR analysis was performed using an Opticon II thermocycler (Bio-Rad, Hercules, CA). Amplification conditions were as follows: 95°C for 10 min, followed by 45 cycles of 94°C for 15 s, 60°C for 25 s, and 72°C for 25 s. After the final extension (72°C for 10 min), a melting-curve analysis was performed to ensure specificity of the products. Primer sequences are listed as follows: IFN-stimulated gene (ISG)-15 forward, 5′-TAAAGACCTTGCTGAGACCA-3′; IRF7 forward, 5′-ACAGAAGGGGCTTTTATC-3′; and reverse, 5′-GAGCCGCAGCATTTTCTCTTG-3′; MX-1 forward, 5′-GATCCGACTTCCCTAGATGG-3′; and reverse, 5′-CTCATCGGTGATGCAAAAACC-3′; CXCL5 forward, 5′-CCCCCTTCCTCAGCTAAGCC-3′; and reverse, 5′-TGGATCCCTGATGTC-3′; YM-2 forward 5′-CTGGTGGATAGGGTTGGGTTG-3′; and reverse, 5′-CTGGTGACGTGTCGCTT-3′; FNDC5 forward, 5′-TGCCGGCGAGCAACT-3′; and reverse, 5′-CCCATGACCTGCTTTATC-3′; Fizz1 forward, 5′-TGGTGGGAGTCTGCTCACGT-3′; and reverse, 5′-AGCTGGGTCTTCCACCTTT-3′; and IL-23 P19 forward, 5′-CATGGGGGCATTCAGGAGGTTA-3′; and reverse, 5′-GACCCACAAGGACTCAAGGA-3′.

Flow cytometry
Flow cytometry was performed as described previously (20). Before surface staining, peritoneal or peripheral blood cells were incubated with anti mouse CD16/32 (Fc Block; BD Biosciences, San Jose, CA) for 10 min. Cells were then stained with an optimized amount of primary Ab or the appropriate isotype control for 10 min at room temperature before washing and resuspending in PBS supplemented with 0.1% BSA. Ten thousand to 50,000 events per sample were acquired using a CYAN ADP flow cytometer (Beckman Coulter, Hialeah FL), analyzed with FACS Express 3 software (De Novo Software). The following conjugated Abs were used: anti-CD8-allophycocyanin, anti-ly–6G-PE, anti-B220-allophycocyanin–Cy7, anti–CD11c-allophycocyanin, anti–B220–FITC, anti–Ly–6C–FITC, CD69–FITC, CD69–PE–Cy7, CD25–PE (BD Biosciences), anti-H2–Kβ–allophycocyanin, anti–H2–Dβ1–biotin, avidin–PE–Cy7, anti–CD11b–Brilliant Violet 590, anti–pDC Ag-1 (PDCA-1)–biotin (BioLegend, San Diego, CA), anti–CD11c–allophycocyanin, anti–B220–FITC, anti–Ly–6C–FITC, CD69–PE–Cy7, and CD25–PE (BD Biosciences). In intracellular analysis, 10⁶ peritoneal cells were cytospun onto glass slides and stained using the Hema3 kit (modified Wright stain; Fisher Scientific, Pittsburgh, PA).

Intracellular cytokine staining
Intracellular IFN-γ, IL-4, and IL-17 were detected after culturing peritoneal cells or spleenocytes at a concentration of 2.5 × 10⁶ cells/ml in RPMI 1640 medium with 10% FBS, PMA (50 ng/ml), ionycin (1 μg/ml), and GolgiStop (BD Biosciences; 5 μg/ml) for 6 h at 37°C in a 5% CO2 incubator. After stimulation, cells were surface stained, fixed in 4% Formalin/Permeabilization buffer (eBioscience), washed in Perm/Wash buffer (eBioscience), and then stained with PE-labeled anti–IFN-γ, allophycocyanin-labeled anti–IL-4 (BD Biosciences), allophycocyanin-labeled anti–IL-17 (eBioscience), or isotype controls, according to the manufacturer’s protocol. Cells were washed twice in Perm/Wash buffer (eBioscience), resuspended in PBS, and analyzed by flow cytometry. Data were analyzed with FACS Express 3 software (De Novo Software).

Ig and autoantibody ELISAs
Total Ig levels were measured as described (20). Briefly, microtiter plates (Immunoblot Amino; Nunc) were coated with goat anti-mouse L-chain (κ- and λ-specific) Abs (Southern Biotechnology Associates, Birmingham, AL). Sera were diluted 1:200,000 in 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.5% BSA, 0.3% Nonidet P-40, and 0.05% NaN3. Alkaline phosphatase-conjugated goat anti-mouse polyclonal Abs against μ, γ1, γ2c, γ2b, or κ H-chain (1:1000 dilutions; Southern Biotechnology Associates) were used as secondary Abs. Ag-capture ELISAs for anti-nuclear RNP/Sm Abs were performed as described (20), using mouse sera at a dilution of 1:400 and goat anti-mouse IgG second Abs (Southern Biotechnology Associates). Levels of anti-dsDNA Abs were tested (1:400 serum dilution) by ELISA using S1 nuclelease-treated calf thymus DNA as Ag as described previously (22). Anti-U1A (subset of anti-RNP) Abs were tested by ELISA as described (23). Briefly, recombinant U1A Ag-coated wells were incubated with mouse sera (1:4000 dilution) followed by alkaline phosphatase-conjugated goat anti-mouse μ or γ1, γ2c, γ2b, or κ H-chain-specific Abs. ELISAs were developed with p-nitrophenyl phosphate substrate (Sigma-Aldrich), and OD at 405 nm (ODabs) was read using a VersaMax microplate reader ( Molecular Devices, Sunnyvale, CA).

Cytokine ELISAs
CCL2 (MCP-1) levels in peritoneal lavage fluid were measured using the Mouse MCP-1 OptEIA Set (BD Biosciences) following the manufacturer’s instructions. IL-12 p40/p70 in peritoneal lavage fluid was measured as described previously (23) using rat mAb pairs for IL-12 (BD Biosciences). After incubation with biotinylated IL-12–specific Abs, streptavidin-conjugated alkaline phosphatase (1:1000 dilution; Southern Biotechnol

Assessment of glomerulonephritis
Glomerularcellularitywascountedbythenumberofnucleiperglomerularcross-sectionafterstainingwithH&Easdescribed(24).Forassessingrenalimmunecomplexdeposition,4-μmfrozensectionswere stained with FITC rat anti-mouse complement component C3 mAb (Cedarlane Laboratories) and examined by fluorescence microscopy (24).

Bone marrow chimeras
Bone marrow chimeras were generated as described (25). Briefly, lethally irradiated (1000 rad) BALB/c × B6 F1 (CB6F1/J) mice were reconstituted with bone marrow from wild-type BALB/c (H-2Kβ and H-2Dβ1-positive) mice mixed 1:1 with bone marrow from either wild-type BALB/c or BALB/c IFNAR−/− mice (H-2Dβ1 single positive). After 6 wk, the reconstituted mice were treated with 0.5 ml pristane i.p., and 3 wk later, the presence of activated (CD4+CD69+) T cells was determined in the peritoneum and spleen.

Statistical analysis
Statistical analyses were performed using Prism 4.0 software (GraphPad, La Jolla, CA). Differences between groups were analyzed by the unpaired Student t test or the Mann–Whitney U test. The data are shown as mean ± SD for normally distributed data sets and as median and interquartile range for nonnormally distributed data sets. Student t test was used for normally distributed data and the Mann–Whitney U test for nonnormally distributed data. Normality was determined by D’agostino and Person omnibus normality test using Prism 4.0 (GraphPad). All tests were two-sided, and p < 0.05 was considered significant.

Results
Intraperitoneal pristane injection induces autoantibodies in two phases: an early phase, peaking at ∼2 wk, of IgM anti-ssDNA and chromatin autoantibodies and a later phase (3–6 mo) in which autoantibodies characteristic of SLE are produced, including IgG anti-Sm/RNP and anti-dsDNA (19). Most of these autoantibodies are IgG2a in BALB/c background mice (IgG2c in B6 background mice) or IgG2b. As pristane-induced lupus (autoantibodies and renal disease) is dependent on TLR7-stimulated IFN-1 production (20), we examined the role of IRF5, a transcription factor down-

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stream of TLR7 and MyD88, in pristane-induced IFN-I production.

IRF5 is critical for the pathogenesis of pristane-lupus

Pristane-treated B6 IRF5−/− mice (n = 14) did not produce anti-Sm/RNP autoantibodies detectable by ELISA, and only one mouse was weakly positive by immunoprecipitation (Fig. 1A, 1B). In contrast, two out of seven wild-type B6 mice treated with pristane produced high levels of anti-Sm/RNP, and another four displayed low levels of anti-Sm/RNP detectable by ELISA but not immunoprecipitation (Fig. 1A, 1B). The frequency of anti-Sm/RNP in wild-type mice detectable by immunoprecipitation was consistent with our previous studies in which these autoantibodies were found in 24% pristane-treated female B6 mice (26). IgG autoantibodies specific for the U1A protein, a subset of anti-RNP, were found in 24% pristane-treated female B6 mice (26). IgG1, IgG2b, and IgG3 anti-U1A Abs were not induced in IRF5−/− mice as reported previously (6). Nevertheless, IgG1, IgG2b, and IgG3 anti-U1A autoantibodies were not induced in IRF5−/− mice (Fig. 1C and data not shown). The low level of IgG2c (IFN-γ dependent) and high level of IgG1 (IL-4 dependent) correlated well with changes in Th cell subsets (see below).

Decreased inflammatory cytokine production in IRF5−/− mice

Pristane-treated lupus is abolished in the absence of signaling through the IFNAR (24). Consistent with the importance of IFN-I in the pathogenesis of this disease, peritoneal exudate cells from

![FIGURE 1. Absence of autoantibodies in IRF5−/− mice. IRF5−/− and control B6 mice (B6 mice, n = 7; IRF5−/− mice, n = 14) were treated with pristane, and 6 mo later, serum autoantibody and Ig levels were evaluated. Sera collected from mice before pristane (TMPD) injection served as a control (Ctr). (A) Anti-Sm/RNP autoantibody levels (ELISA) in IRF5−/− and B6 control mice. (B) Immunoprecipitation of 35S-radiolabeled protein components A–G of U1 small RNP’s by sera from six B6 and six IRF5−/− sera. Positions of molecular mass markers (kDa) are indicated on the right. (C) Anti-U1A IgG1, IgG2b, and IgG2c autoantibodies levels (ELISA) in IRF5−/− and control mice (B6 mice, n = 7; IRF5−/− mice, n = 14). (D) IgG anti-dsDNA autoantibody levels (ELISA) in IRF5−/− and control mice (B6 mice, n = 6; IRF5−/− mice, n = 8). (E) Serum levels of total IgM, IgG1, IgG2c, IgG2b, and IgG3 in pristane-treated IRF5−/− and B6 mice (B6 mice, n = 7; IRF5−/− mice, n = 14). *p < 0.05, **p < 0.01, ***p < 0.001 by Mann–Whitney U test. ND, Not detected.

![FIGURE 2. Attenuated renal disease in pristane-treated IRF5−/− mice. (A) Top panel, Kidneys from IRF5−/− mice and wild-type B6 controls 6 mo after pristane treatment were sectioned and stained with H&E. Bottom panel, Renal tissue was embedded in OCT, and 4-μm sections were stained with FITC-conjugated anti-mouse complement component C3 mAb. Original magnification ×200. (B) Glomerular cellularity was evaluated by counting the number of nuclei per glomerular cross-section (n = 5 mice/group, eight glomeruli per mouse). ***p < 0.001, Student t test.](http://www.jimmunol.org/DownloadedFrom)
IRF5<sup>−/−</sup> mice expressed markedly lower levels of the ISGs IRF7, ISG15, and Mx1 (Fig. 3A). Similarly, protein levels of the IFN-I-inducible chemokine MCP-1 (CCL2) in peritoneal lavage fluid were significantly lower in IRF5<sup>−/−</sup> mice (Fig. 3B). The levels of other proinflammatory cytokines (IL-12 p40/p70 and IL-6) involved in the pathogenesis of pristane-lupus also were decreased in peritoneal lavage fluid (Fig. 3C) and expression of IL-23 p19 mRNA was decreased in pristane-treated IRF5<sup>−/−</sup> mice versus controls (Fig. 3D). Taken together, these data suggest that deficiency of IRF5 attenuates lupus in pristane-treated mice by affecting the production of IFN-I and other key cytokines (IL-12 and IL-23) that link innate immunity to the activation of Th1 and Th17 cells, respectively (27). Production of IL-4 (Fig. 4) and the chemokine CXCL5, which recruits neutrophils to peritoneal cavity cells, respectively (27), was similar in IRF5<sup>−/−</sup> mice and controls, suggesting that the effects of IRF5 deficiency on cytokine production were selective for Th1 cells and not due to an overall attenuation of the inflammatory response (Fig. 3D).

**IRF5 deficiency alters the chronic inflammatory response to pristane**

We have shown previously that a subset of inflammatory monocytes expressing CCL2 and high levels of the surface marker Ly6C<sup>+</sup> is recruited to the peritoneum in response to MCP-1 and is a major source of IFN-I in pristane-treated mice (29). The total number of peritoneal cells in pristane-treated IRF5<sup>−/−</sup> mice was slightly lower than in wild-type mice but much higher than in untreated mice, indicating that pristane could induce an inflammatory response in IRF5<sup>−/−</sup> mice (Fig. 5A). The effect of IRF5 deficiency on the recruitment of Ly6C<sup>hi</sup> monocytes (CD11b<sup>+</sup>Ly6C<sup>hi</sup>Ly6G<sup>−</sup>) also was examined (Fig. 5B–E). As shown previously in TLR7<sup>−/−</sup> and IFNAR<sup>−/−</sup> mice, peritoneal exudates from pristane-treated IRF5<sup>−/−</sup> mice contained few Ly6C<sup>hi</sup> monocytes in comparison with peritoneal exudates of control-treated B6 controls (Fig. 5B, R1, 5C, 5D). In contrast, the peritoneal exudates from IRF5<sup>−/−</sup> mice contained many Ly6C<sup>lo</sup> monocytes (Fig. 5B, R3, 5C, 5D). Peritoneal neutrophils were largely unaffected (Fig. 5B, R2, 5D). Gating on all monocytes (CD11b<sup>+</sup>, Ly6G<sup>−</sup>) and analyzing fluorescence intensity of Ly6C<sup>hi</sup> staining underscored the dramatic difference in the profile of peritoneal monocyte populations in IRF5<sup>−/−</sup> mice versus controls (Fig. 5C). In addition, monocytes in the peritoneal exudates had different morphological features: those in B6 mice had the appearance of immature monocytes, whereas those in IRF5<sup>−/−</sup> mice were larger and more granular, consistent with a more mature phenotype (Fig. 5E). Because Ly6C<sup>hi</sup> monocytes are a major source of IFN-I production in the inflamed peritoneum of pristane-treated mice (29), these results are consistent with the low IFN-I expression following pristane treatment (Supplemental Fig. 1A).

**IRF5 deficiency alters the Th cell response to pristane**

IRF5 has a critical role in the development of Th1 responses to *Leishmania donovani* infection (30). Therefore, we examined the Th cell response to pristane. There were significant differences in the Th subsets present in the peritoneum 2 wk after pristane injection. Intracellular cytokine staining revealed that IRF5<sup>−/−</sup> mice had fewer total peritoneal exudate cells producing IFN-γ, whereas IL-4—and, to a lesser degree, IL-17—producing cells were increased (Fig. 4A, 4B). Although the IL-4—producing cells in IRF5<sup>−/−</sup> mice were primarily T lymphocytes, the cells producing IL-17 were CD4<sup>+</sup>CD8<sup>−</sup> (data not shown). To clarify whether the
apparent Th cell skewing is due to the lack of IRF5, IFN-I, or other inflammatory cytokines such as IFN-γ and IL-12, we investigated Th cell subsets in IFNAR2−/− and TLR7−/− mice and found that both IFN-γ- and IL-4-producing (but not IL-17) cells were decreased in these strains (Supplemental Fig. 1B). As in IRF5−/− mice, the ratio of Th1 to Th2 cells (defined as the ratio of IFN-γ+ to IL4+ T cells) was lower in IFNAR2−/− and TLR7−/− versus their wild-type controls (Fig. 4D). Nevertheless, the cytokine pattern exhibited the same trend in both strains, and the cytokine pattern was quite different (Fig. 4D). Nevertheless, the cytokine pattern exhibited the same trend in both strains, and the cytokine pattern was quite different (Fig. 4D). The recent observation that IRF5 promotes M1 macrophage polarization (32), along with the greatly reduced IL-12 (a major product of M1 macrophages) and IL-23 levels in peritoneal washings from IRF5−/− mice (Fig. 3) led us to hypothesize that deficiency of IRF5 may polarize monocyte/macrophage differentiation toward the M2 pathway in pristane-treated mice. We therefore examined the expression of Ym1, Mzm2, and Fizz1, genes characteristically expressed by alternatively activated (M2) macrophages (Supplemental Fig. 1D). We did not find a significant difference in the expression of these genes in peritoneal exudate cells from pristane-treated IRF5−/− mice versus controls. Taken together, these data suggest the altered Th cell ratio seen in IRF5−/− mice may reflect decreased IFN-1 production and altered inflammatory cytokine production.

**Decreased lymphocyte activation in IRF5−/− mice**

The percentages and total cell counts of B220+, CD4+, and CD8+ lymphocytes in peritoneal exudates of pristane-treated mice were similar in IRF5−/− and wild-type mice (not shown). However, the percentage of CD4+, CD8+, and B220+ peritoneal exudate cells expressing the activation marker CD69 was decreased in IRF5−/− mice versus controls (Fig. 6A). Interestingly, the percentage of activated peritoneal CD69+CD4+ cells was highly correlated with the percentage of Ly6C+ monocytes (Fig. 6B), suggesting that IFN-I production, which also correlates strongly with numbers of Ly6C+ monocytes (29), might promote lymphocyte activation in pristane-treated mice. Although CD69 is an ISG (33), the presence of two distinct populations of CD4+ T cells, CD69+ and CD69− in IFN-I−/− mice may reflect decreased surface expression of CD69 (Fig. 6A). Gated on the CD4+CD69+ population, ~30% of B6 T cells produced IFN-γ (Th1) and ~8% produced IL-4 (Th2) versus ~20% cells producing IFN-γ and IL-4, respectively, in IRF5−/− mice (Fig. 6C).

To further verify the role of IRF5-stimulated IFN-I in CD4+ T cell activation, we examined bone marrow chimeras. Bone marrow cells from wild-type BALB/c × B6 F1 (CB6F1/J) mice (bearing both H2-Kb and H2-Dd) were mixed 1:1 with bone marrow cells from wild-type BALB/c or BALB/c IFNAR2−/− mice (H2-Dd single positive) and injected into lethally irradiated CB6F1/J recipients. Six weeks after reconstitution, pristane was administered to the reconstituted mice, and 3 wk later, peritoneal lymphocyte activation was examined (Fig. 6D). T cells derived from the CB6F1/J donor are H2-Kb, whereas T cells derived from BALB/c or IFNAR2−/− donors are H2-Kd. In the control group, there is a mixture of CB6F1/J and BALB/c donor cells, and T cells from both donors became activated (CD69+) after pristane injection. In mice reconstituted with CB6F1/J and IFNAR2−/− cells, only the CB6F1/J-derived T cells were activated, whereas IFNAR2−/−-derived T cells remained CD69− (Fig. 6D, left panel). In contrast to the peritoneum, CD69...
expression on CD4+ peripheral blood and splenic T cells was comparable in B6 and IRF5−/− mice (Supplemental Fig. 1E). Together, these results suggest that: 1) CD4+ T activation is both IFN-I and IRF5 dependent; 2) the T cells must express IFNAR endogenously; and 3) the exposure of T cells to IFN-I is local IFN-I and IRF5 dependent; 2) the T cells must express IFNAR and IL-4 are indicated (*p < 0.05, **p < 0.01, ***p < 0.001 by Mann–Whitney U test). PEC, Peritoneal exudate cells.

**FIGURE 6.** Role of IRF5 in lymphocyte activation following pristane treatment. Two weeks after pristane injection, peritoneal lymphocytes were analyzed by flow cytometry. (A) Left panel, flow cytometry of CD4+ and CD69 (gated on CD11b+ cells). Right panel, Percentage of CD4+, CD8+, and CD220+ cells that were positive for CD69 was determined. (B) Correlation of CD69+ CD4+ T cells with the percentage of Ly6C+ monocytes. (C) Intracellular staining of IFN-γ and IL-4 in B6 and IRF5−/− mice. Peritoneal lymphocytes were gated on the CD4+ population and then gated on the CD69+ and CD69− populations, respectively. Percentages of cells expressing IFN-γ and IL-4 are indicated (n = 4/group). (D) Left panel. Peritoneal cells from bone marrow chimeric mice were gated on the CD11b+CD4+ population, and CD69 expression was analyzed by flow cytometry in cells that were H2-Kb+ (CB6F1/J-derived) or H2-Kb− (BALB/c or BALB/c IFNAR−/−-derived). Right panel. Quantification of CD69+CD4+ T cells as a percentage of strain-specific total CD4+ T cells (n = 4 to 5/group). Data are representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 by Mann–Whitney U test.

**FIGURE 7.** Decreased pDCs in the peritoneum of IRF5−/−, but not TLR7−/− or IFNAR−/−, mice. Two weeks after pristane injection, peritoneal exudate and bone marrow pDCs were quantified by flow cytometry. (A) pDC staining. CD11b+CD11c−B220−PDCA-1+ pDCs in the peritoneal cavity of IRF5−/− mice was strikingly reduced in comparison with wild-type B6 mice (Fig. 7A). A similar reduction of pDCs in peritoneal exudates was seen in MyD88−/− mice, but not in IFNAR−/− or TLR7−/− mice (Fig. 7B). This discrepancy appeared to be limited to the pDC population, as myeloid dendritic cells (CD11b+CD11c−CD8α−) were found at comparable levels in the peritoneal exudates of IRF5−/− and wild-type mice (Fig. 7C). In contrast to the peritoneal exudates, the percentage of pDCs in the bone marrow was similar in IRF5−/− and wild-type controls, both before pristane treatment and 2 wk after treatment (Fig. 7D). CCR7 is expressed on T cells and dendritic cells, and its ligands, CCL19 and CCL21, are essential for the homing of pDCs to lymph nodes (34). Therefore, we examined the expression of CCL19/CCL21 and found that both were decreased considerably in peritoneal exudate cells from IRF5−/− versus control mice (Fig. 7E). Thus, defective pDC migration in IRF5−/− mice is associated with decreased production of chemokines that mediate pDC homing. In view of the MyD88 dependence of pDC migration (Fig. 7B) and the ability of TLR ligands to enhance CCR7 expression (34), we examined CCR7 expression levels on bone marrow and peritoneal pDCs from IRF5−/− mice 2 wk after pristane injection. The fluorescence intensity of B220+CD11b−CD11c−B220−PDCA-1+ pDCs expressing CCR7 was similar in bone marrow from B6 and IRF5−/− mice (Fig. 7F). Unexpectedly, the fluorescence intensity of CCR7 was lower in peritoneal pDCs from B6 mice than in B6 bone marrow pDCs, whereas IRF5−/− peritoneal pDCs (although few in number) exhibited similar fluorescence intensity to that in IRF5−/− bone marrow pDCs. Although the explanation is unclear, this could be due to CCL19/CCL21 blocking an epitope of the CCR7 receptor recognized by the mAb in the B6 controls. These data suggest that MyD88 expression enhances pDC migration to the peritoneum by increasing IRF5-regulated CCL19/CCL21 and not by increasing CCR7 expression.

**Discussion**

Genetic polymorphisms of IRF5 are strongly linked to susceptibility to SLE in humans (9, 10). Recent studies also link IRF5 to pleiotropic effects of IRF5 on lupus pathogenesis.
the pathogenesis of autoantibodies in both human SLE (35) and pristane-induced lupus (6) and in MRL mice (17). Although it has been suggested that the primary effect of IRF5 in pristane-lupus may involve isotype switching of pathogenic autoantibodies (6), our data strongly suggest that the influence of IRF5 on the development of autoimmune disease in pristane-lupus is multifactorial. In the present studies, IRF5 not only affected autoantibody production, isotype switching, and the development of renal disease, but also modulated the polarization and activation of T cells as well as the differentiation and migration of inflammatory monocytes/macrophages and pDCs.

**Effect of IRF5 deficiency on autoantibody production and renal disease**

IRF5 promotes B cell maturation in part by modulating the expression of Blimp-1, a master regulator of plasma cell maturation (5). As they age, IRF5−/− mice have more CD19−B220− plasmablasts and a decreased number of CD138+ B220+ plasma cells (5). The effects on serum Ig levels are complex: serum levels of IgG subclasses in young naive IRF5−/− mice are similar to wild-type, whereas in older mice, IgG1, IgG2a/IgG2c, and IgG2b are decreased. Following immunization with T cell-dependent or -independent Ags, IRF5−/− mice have a reduced Ag-specific IgG1 response, although IgG2a and IgG2b were not examined (5). In a previous study on pristane-induced lupus, the production of IgG2a/IgG2c and IgG2b anti-Sm and anti-dsDNA Abs was abolished in IRF5−/− mice (6). Our data are generally consistent with these observations, although we have not been able to detect anti-dsDNA Abs in pristane-treated wild-type B6 mice (Fig. 1) (19). Strikingly, despite increased levels of total IgG1 in IRF5−/− mice, IgG1 anti-Sm/RNP autoantibodies were not produced, suggesting that IRF5 may affect the regulation of tolerance to the Sm/RNP autoantigens.

Although wild-type B6 mice are relatively resistant to the development of glomerulonephritis following pristane treatment (19), we confirmed that glomerular cellularity and renal immune complex deposits were decreased in IRF5−/− mice versus wild-type controls.

**IRF5 is critical for inflammatory cytokine production and recruitment of Ly6C<sup>hi</sup> monocytes**

Intrapерitoneal injection of pristane induces the production of inflammatory cytokines, influx of immune cells, and a chronic inflammatory response, culminating in the development of a lupus-like disease (20). IRF5 deficiency had critical effects on the i.p. production of multiple cytokines implicated in the pathogenesis of pristane-lupus, including IL-12, IL-23, and IL-6 (Fig. 3). Although this could be related to overall differences in cytokine production, it should be pointed out that the gene expression data reflect the presence of several cell types in the peritoneal exudate, so that differences in cytokine production also could be related to altered composition of the peritoneal exudate cells, as exemplified by the predominance of Ly6C<sup>hi</sup> monocytes in controls versus Ly6C<sup>hi</sup> monocytes in IRF5−/− mice (Fig. 5A). IL-17 production by non-T cells also was affected by the absence of IRF5, but the present studies did not identify the peritoneal cell subset(s) producing IL-17 following pristane treatment. This will require further investigation.

Although the role of IRF5 in the induction of IFN-I is tissue specific and apparently of minor importance in some cases (1, 36), IRF5 appears to be essential for the expression of IFN-I-regulated genes in the pristane model (Fig. 3A). IRF5−/− mice also failed to produce the IFN-I-inducible chemokine CCL2 (MCP-1, Fig. 3B), which mediates the recruitment of Ly6C<sup>hi</sup> monocytes through CCR2 (21). Similar to the findings in IFNAR<sup>−/−</sup> mice, the small number of monocytes migrating to the peritoneal cavity of pristane-treated IRF5−/− mice rapidly acquired a Ly6C<sup>lo</sup> phenotype (Fig. 5) indicative of a more mature phenotype and enhanced phagocytic capacity. Taken together with our previous findings (21), IRF5 functions downstream of TLR7, MyD88, IL-1R–associated kinase (IRAK)-4, IRAK-1, and IRAK-2 to promote the production of IFN-I, MCP-I, and other proinflammatory chemokines and cytokines (21). In contrast, granulocyte migration was unaffected by the absence of IRF5 (Fig. 5D), suggesting that IRF5 does not participate in the IL-1α–dependent pathway of granulocyte recruitment in this model (28).

**IRF5 deficiency alters T cell activation**

TLR7-mediated IRF5 activation is necessary for protective Th1 responses to *L. donovani* (30). Our data illustrate that TLR7 and IRF5 also contribute to the activation and polarization of Th1 cells in pristane-treated mice (Figs. 4, 6). Peritoneal exudates from wild-type mice contained two distinct subsets of CD4<sup>+</sup> T cells: one CD69<sup>+</sup> and the other CD69<sup>+</sup> (Fig. 4C). The CD69<sup>+</sup> subset was largely absent in IRF5−/− mice. The effect of IRF5 on T cell activation could be either T cell intrinsic or mediated via its effects on IFN-I production. The IFNAR dependence in bone marrow chimera experiments (Fig. 6D) and the correlation between the number of CD69-expressing T cells and type I IFN-producing Ly6C<sup>hi</sup> monocytes (Fig. 6B) provide evidence that the effect of IRF5 on T cell activation by pristane is mediated, in part, by IFNAR signaling. Although CD69 is an IFN-I–responsive gene (33), the absence of CD69<sup>+</sup>CD4<sup>+</sup> T cells in pristane-treated IRF5−/− mice may reflect more than just the low levels of IFN-I. In pristane-treated B6 mice, one population of CD4<sup>+</sup> T cells expressed CD69, but another subset had no detectable CD69 staining, suggesting that CD69 expression may have significance beyond being a marker of IFN-I exposure (Fig. 6A). The fact that intracellular IFN-γ staining was preferentially seen in the CD69<sup>+</sup> CD4<sup>+</sup> subset provides strong evidence that the CD69<sup>+</sup> T cells were not only exposed to IFN-I but also activated (Fig. 6C). Thus, our data suggest that IRF5-induced IFN-I promotes T cell activation, consistent with previous observations that IFN-I also promotes the survival of activated T cells (37).

Pristane-induced lupus is augmented by a Th1 response, as both autoantibody production and renal disease are attenuated in pristane-treated IFN-γ-deficient mice, whereas disease is exacerbated in IL-4−/− mice (38). The current study shows that in wild-type mice, the predominance of a Th1 response occurs as early as 2 wk after pristane injection (Fig. 4). This profile was limited to the inflamed peritoneal cavity, a site of IFN-I production, and it was not apparent in the spleen (Supplemental Fig. 1). Interestingly, the Th1/Th2 ratio was strikingly altered in IRF5−/− mice, mainly due to increased numbers of IL-4–producing cells (Fig. 4). Similar findings were evident in IFNAR−/− and TLR7−/− mice (Fig. 6, Supplemental Fig. 1), suggesting that the shift toward a Th2 response in IRF5−/− is due to impairment of IFN-I production rather than an intrinsic effect of IRF5 expression in T cells.

**IRF5 deficiency has an IFN-I–independent effect on pDCs**

An unexpected finding of this study was the near absence of pDCs in the inflamed peritoneal cavity of IRF5−/− or MyD88−/− mice 2 wk after TMPD injection (Fig. 7). In striking contrast, a normal number of pDCs were present in the peritoneum of pristane-treated TLR7<sup>−/−</sup> or IFNAR<sup>−/−</sup> mice, indicating that the lack of peritoneal pDCs was not a consequence of low IFN-I levels. The numbers of pDCs in the bone marrow of IRF5−/− mice were...
similar to controls, indicating that IRF5 deficiency does not cause decreased production of pDCs but instead results in defective recruitment of these cells to the inflamed peritoneum. The observation that CCL19 and CCL21 expression was abolished in the peritoneal cavity of IRF5−/− mice following pristane injection (Fig. 7E) strongly suggests that IRF5 regulates the expression of these chemokines. In contrast, the expression of CCR7 was similar or possibly increased in IRF5−/− mice (Fig. 7F), suggesting that IRF5 affects pDC migration mainly through regulation of CCL19 and CCL21 and not via the CCR7 receptor. The mechanism for chemokine modulation by IRF5 is unclear. This could be explained by either direct transcriptional regulation at the promoter region of CCL19/CCL21 or by indirect activation downstream of inflammatory mediators (other than IFN-I) regulated by IRF5 and MyD88. As CCL19 and CCL21 also play a role in the recruitment of naive and central memory T cells, future studies to further characterize the T cell populations found in the peritoneal cavity of IRF5−/− mice (Fig. 7F) might provide insights into new insights from fine mapping and genome-wide association studies. Nat. Rev. Genet. 10: 285–357.


Seth, S., L. Oberdorfer, R. Hyde, K. Hoff, V. Thiers, T. Werbs, S. Schmitz, and R. Forster. 2011. CCR7 essentially contributes to the homing of plasmacytoid
Figure S1

A, Two weeks after pristane injection, myeloid subsets (monocytes and neutrophils) in PBMC were analyzed by flow cytometry. Black bars, B6 controls; gray bars, IRF5−/− mice (N=4 mice per group). The dashed line indicates levels in untreated B6 controls (N=3). Left: Percentages of CD11b+Ly6C+ monocytes, CD11b+Ly6Cint neutrophils, and CD11b+Ly6C− “mature” monocytes. Not significant by Mann-Whitney test.

B, Two weeks after pristane injection, BALB/c IFNAR−/−, BALB/c TLR7−/−, or wild type BALB/c peritoneal cells (PEC) were stimulated for 6 hrs with PMA + ionomycin and stained intracellularly for IFNγ, IL-4, or IL-17 (N = 4-6 mice per group). Percentage of total PEC expressing cytokines is shown. * P < 0.05; ** P <0.01; *** P <0.001 by Mann-Whitney test.

C, Two weeks after pristane injection, spleen cells were stimulated for 6 hrs with PMA + ionomycin and stained intracellularly for IFNγ, IL-4, or IL-17 (N = 3-4 mice per group). There was no significant difference in staining between IRF5−/− mice and controls. Not significant by Mann-Whitney test.

D, Expression of Fizz 1, Yml, and Ym2 (markers of M2 macrophage polarization) was measured in peritoneal exudate cells of B6 and IRF5−/− mice by Q-PCR (N=6 mice per group). NS, not significant by Mann-Whitney test.

E, CD69+CD4+ peripheral blood T cells (percentage of total CD4+ T cells) and CD11b+CD11c+B220PDCA-1+ pDCs (percentage of PBMC) and CD69+CD4+ T cells (percentage of CD4+ splenocytes) were quantified by flow cytometry 2 weeks after pristane treatment. (N = 3-4 mice per group). * P < 0.05; ** P <0.01; *** P <0.001 by Mann-Whitney test; ns, not significant.