Interferon-α Treatment of Female (NZW × BXSB)F₁ Mice Mimics Some but Not All Features Associated With the Yaa Mutation

Meera Ramanujam,¹ Philip Kahn,¹ Weiqing Huang,¹ Haiou Tao,¹ Michael P. Madaio,² Stephen M. Factor,³ and Anne Davidson¹

Objective. Male (NZW × BXSB)F₁ mice develop antiphospholipid syndrome (APS) and proliferative glomerulonephritis that is markedly accelerated by the Yaa locus encoding an extra copy of Tlr7. Female (NZW × BXSB)F₁ mice with only 1 active copy of Tlr7 develop late-onset glomerulonephritis but not APS. Because a major function of Toll-like receptor 7 is to induce type I interferons (IFNs), our goal was to determine whether IFNα can induce or accelerate the manifestations of systemic lupus erythematosus (SLE) in female (NZW × BXSB)F₁ mice.

Methods. Eight-week-old female (NZW × BXSB)F₁ mice were injected with a single dose of adenovirus expressing IFNα. Mice were monitored for the development of thrombocytopenia and proteinuria. Sera were tested for anticardiolipin and anti-Sm/RNP antibodies. Mice were killed at 17 or 22 weeks of age, and their kidneys and hearts were examined histologically and by immunohistochemistry. Spleen cells were phenotyped, and enzyme-linked immunospot assays for autoantibody-producing B cells were performed.

Results. IFNα markedly accelerated nephritis and death in female (NZW × BXSB)F₁ mice. A significant increase in spleen cell numbers associated with a striking increase in the number of activated B and T cells was observed. Marginal-zone B cells were retained. IFNα-induced increased titers of autoantibodies were observed, but thrombocytopenia was not observed. Cardiac damage was milder than that in male mice.

Conclusion. IFNα accelerates the development of renal inflammatory disease in female (NZW × BXSB)F₁ mice but induces only mild APS and does not induce thrombocytopenia. The effect of IFNα on SLE disease manifestations is strain dependent. These findings are relevant to our understanding of the physiologic significance of the IFN signature.

The pathogenic role of interferon-α (IFNα) in systemic lupus erythematosus (SLE) has been inferred from findings that IFNα can induce lupus-like symptoms and from the discovery that peripheral blood mononuclear cells from patients with active lupus show dysregulated expression of a group of IFNα-induced genes (1). In NZB/NZW mice, administration of exogenous IFNα accelerates SLE-like disease that has characteristics similar to those observed in the model of spontaneous SLE (2).

IFNα is induced by the ligation of Toll-like receptors (TLRs) that are expressed on B cells and plasmacytoid dendritic cells and are specific for nucleic acid antigens (3). TLR-7 overexpression induces SLE in mice, and its depletion modulates some of the manifestations of SLE (4). In human SLE, the IFN signature has been associated with active and severe disease (5) and with antibodies to RNA-associated antigens (6).

Male (NZW × BXSB)F₁ mice carry 2 active copies of Tlr7 (7) and have an accelerated form of lupus nephritis. In addition, they are the only SLE-prone mice
in which the antiphospholipid syndrome (APS) develops spontaneously (8,9). In female mice with a single active copy of Tlr7, nephritis develops late in life, but APS does not develop (8). To determine whether exogenous IFNα is sufficient to confer the disease phenotype associated with Tlr7 reduplication, we administered an adenovirus expressing IFNα to female (NZW × BXSB)F1 mice. Although accelerated nephritis developed in the mice, IFNα was not sufficient to induce full-blown APS.

**MATERIALS AND METHODS**

**IFNα adenovirus treatment.** (NZW × BXSB)F1 mice (The Jackson Laboratory, Bar Harbor, ME) were bred in our facility. Thirty-five female F1 mice were treated at 8 weeks of age with a single intravenous injection of 10⁹ particles of AdIFNα (Qbiogene, Irvine, CA). Thirty control subjects received the same dose of ß-galactosidase–expressing adenovirus (AdLacZ) or no treatment. Fifteen mice received the TLR-7 agonist imiquimod at a dosage of 25 µg intraperitoneally 3 times weekly for 6 weeks. Mice were tested for proteinuria every 2 weeks (Multistick; Fisher Scientific, Pittsburgh, PA) and bled periodically for serologic analysis. Platelets were counted at 8, 17, and 22 weeks of age, using a Coulter counter (Beckman Coulter, Fullerton CA). Groups of 6–8 mice were killed at 17 weeks or 22 weeks of age, and the remaining mice were observed for proteinuria onset and survival. These experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committees of Columbia University and the Feinstein Institute.

**Total IgG levels and antibodies to cardiolipin and Sm/RNP.** Enzyme-linked immunosorbent assay (ELISA) plates (Falcon Labware, Lincoln Park, NJ) were coated with unlabeled goat anti-mouse IgM, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology, Birmingham, AL) overnight at 4°C. After blocking, the plates were incubated with dilutions of serum for 1 hour at 37°C, followed by horseradish peroxidase–conjugated goat anti-mouse isotype-specific antibodies and
substrate solution (KPL, Gaithersburg, MD). Standard curves were established using serial dilutions of purified antibody of the appropriate isotype (Sigma-Aldrich, St. Louis, MO).

Sm/RNP (Arotec Diagnostics Ltd., Wellington, New Zealand) was coated onto Falcon plates at 1 μg/ml in phosphate buffered saline (PBS). An ELISA was performed according to the manufacturer’s instructions. Anticardiolipin antibody titers were measured as previously described (9). A high-titer serum sample was run in serial dilution on each plate as a quantitation control.

Enzyme-linked immunospot (ELISpot) assay. ELISpot assays for total immunoglobulin-secreting cells and for anticardiolipin antibody–secreting B cells were performed on isolated spleen and bone marrow cells, as previously described (9). A high-titer serum sample was run in serial dilution on each plate as a quantitation control.

Flow cytometry analysis. Spleens were analyzed for B cell and T cell markers as previously described (10), using antibodies to CD4, CD8 (Caltag, Burlington, CA), and CD19. Splenic dendritic cells were identified using phycoerythrin (PE)–conjugated anti-CD11b and fluorescein isothiocyanate (FITC)–conjugated anti-CD11c. B cell subsets were identified as follows: follicular (CD19+IgM+IgD+), marginal-zone (CD19+CD21highCD23low), class-switched (CD19+IgMlow IgDlow), and immature (CD19+IgMhighIgDlow). CD4+ T cells were classified as naive (CD62LhighCD44low) or memory (CD62LlowCD44high). Activated T cells and B cells were defined as CD69high. Unless otherwise stated, all antibodies were obtained from BD PharMingen (San Diego, CA).

Histologic analysis of kidneys and hearts. Scoring of hematoxylin and eosin–stained sections of the kidneys and hearts was performed using a 1–4-point scale for glomerular damage and interstitial inflammation, as previously described (9). Histologic analyses were performed by observers who were blinded to the treatment group of the mice.

Cryosections of kidney were stained with FITC–conjugated anti-mouse IgG2a, PE-conjugated anti-mouse IgD (Southern Biotechnology), F4/80 (Invitrogen, Carlsbad, CA), CD4, CD19, or CD11c (BD PharMingen) in 2% bovine serum albumin/PBS containing 0.5% anti-mouse CD16/CD32 (BD PharMingen) for 1 hour at room temperature. Slides were counterstained with 4',6-diamidino-2-phenylindole (Invitrogen), and images were captured using a digital CCD camera system connected to a Zeiss microscope (Zeiss, Thornwood, NY).

Statistical analysis. Proteinuria and survival data for the entire group are shown in Figure 1 and were analyzed using Kaplan-Meier curves and log rank tests. Data in the bar graphs are shown as the mean +1 SD. Comparisons in Figure 2 and
Table 1 were performed using the Mann-Whitney test. *P* values less than or equal to 0.05 were considered significant.

**RESULTS**

Administration of a small dose of AdIFNα markedly accelerated disease onset in female (NZW × BXSB)F1 mice; proteinuria occurred 4–12 weeks after the virus injection, followed rapidly by death (for both proteinuria and death, *P* < 0.0001, IFNα versus controls) (Figure 1A). Serum IFNα levels were below the threshold of detectability in treated mice. However, 2 weeks after virus injection, there was a significant increase in the expression of several IFN-inducible genes in the spleens of treated mice, to levels comparable with those of proteinuric male mice (data not shown). Imiquimod had no effect on autoantibody production, proteinuria onset, or mortality, and the imiquimod-treated mice were not studied further.

The kidneys of IFNα-treated mice contained intense IgG deposits. Infiltrating F4/80-positive macrophages were located in the interstitium and around glomeruli, whereas CD11c-positive dendritic cells were located within the glomerular tuft (Figures 1B and D). This pattern is similar to that observed in male (NZW × BXSB)F1 mice (11). In contrast, few glomerular or interstitial infiltrates were present in 22-week-old control mice (Figures 1C and E). Renal damage in 17–22-week-old IFNα-treated mice was significant compared with that in untreated controls (for the glomerular score, *P* < 0.001; for the interstitial score, *P* < 0.01) (Figure 1F). Mild cardiac damage was also observed in the IFNα-treated mice but not in the untreated controls (Figure 1G). In contrast, thrombocytopenia was not observed (Figure 1H), despite the presence of high titers of antiphospholipid autoantibodies in the serum.

Serum IgG2a levels increased significantly in the IFNα-treated mice compared with controls (mean ± SD 1,036.2 ± 452.9 versus 433.4 ± 258.9 μg/ml; *P* < 0.0072 at 12 weeks). The difference was no longer significant at 17 weeks. In contrast, serum IgG1 levels were significantly lower in the IFNα group than in the control group at both 12 weeks and 17 weeks (59.8 ± 31.5 versus 142.3 ± 71.5 μg/ml [*P* < 0.001] and 116.1 ± 91.0 versus 209.6 ± 95.5 μg/ml [*P* < 0.02], respectively). Autoantibodies to both cardiolipin (*P* < 0.004 at 14 weeks and *P* < 0.03 at 18–20 weeks) and Sm/RNP (*P* < 0.02 at 12 weeks and *P* < 0.04 at 16 weeks) arose earlier in the IFNα-treated mice than in untreated age-matched controls. The maximal autoantibody titers reached were comparable with those in male mice (Figures 2A and B). Low-titer anti-double-stranded DNA (anti-dsDNA) antibodies arose late in both treated and untreated mice (results not shown).

In accordance with these data, ELISpot analysis of spleens revealed an increase in both the frequency (*P* < 0.04 at 17 weeks and *P* < 0.0001 at 22 weeks) and the total number of IgG-secreting cells (*P* < 0.003 at 17 weeks and *P* < 0.0001 at 22 weeks) and anticardiolipin.
antibody–secreting B cells ($P < 0.0001$ at 22 weeks) in IFNα-treated mice compared with controls (Figures 2C and D). The number of anticardiolipin-producing B cells also increased in the bone marrow (mean ± SD 3.93 ± 0.99 per $10^5$ cells in IFNα-treated mice versus 1.37 ± 0.87 per $10^5$ cells in controls at 17 weeks [$P < 0.02$]; 9.93 ± 0.48 in IFNα-treated mice versus 3.52 ± 2.92 in controls at 22 weeks [$P < 0.03$]).

Phenotypic analysis of spleen cells revealed a marked increase in spleen cell number in the treated mice compared with controls, with a significant increase in the number of CD11b-positive cells and activated CD4 T cells and B cells (Table 1). The number of circulating monocytes in the blood was increased 2-fold (data not shown). All B cell subsets were expanded in the treated mice. This is in contrast to male (NZW × BXSB)F$_1$ mice, in which the overexpression of TLR-7 results in loss of the marginal-zone subset and a marked increase in the follicular-to–marginal zone B cell ratio (Kahn P and Davidson A: unpublished observations). There was also a significant increase in the number of class-switched B cells and plasma cells in the spleens of IFNα-treated mice (Table 1). Germinal centers appeared earlier in the spleens of IFNα-treated mice compared with untreated controls, but the spleens became disorganized, with loss of follicular architecture and germinal centers as the mice aged, similar to what was observed in male mice (Figures 2E–J).

**DISCUSSION**

The association of the IFN signature with SLE in humans and the ability to induce accelerated SLE with IFNα in some murine models of SLE have led to the hypothesis that IFNα is an important pathogenic cytokine in SLE and a target for therapy (3). IFNα accelerates SLE in female NZB/NZW mice, in which it induces early development of anti-dsDNA antibodies and nephritis (2). We have shown in NZB/NZW mice that IFNα rapidly induces germinal centers that generate short-lived plasma cells producing pathogenic IgG2a autoantibodies in a T cell–independent manner, whereas pathogenic IgG3 autoantibodies are generated in a T cell–dependent manner (Davidson A and Liu S: unpublished observations). In other mouse models, however, type I IFNs have protective effects with respect to SLE initiation, indicating strain heterogeneity (12).

(NZW × BXSB)F$_1$ mice develop autoantibodies to cardiolipin and to RNA-associated antigens together with inflammatory glomerulonephritis, immune-mediated thrombocytopenia, and a thrombotic vasculopathy that affects the small coronary arteries, leading to myocardial infarcts, myocardial fibrosis, and a dilated cardiomyopathy (8,9,13). Male mice have markedly accelerated disease because they carry the $Yaa$ locus, a reduplication of a portion of the X chromosome that includes Tlr7 (7). Tlr7 recognizes single-stranded RNA, is required for the production of autoantibodies to RNA-associated autoantigens (14), and appears to be responsible for much of the phenotype associated with the Yaa locus (4).

Ligation of TLR-7 on plasmacytoid dendritic cells is a potent stimulus for secretion of IFNα as well as other cytokines, including tumor necrosis factor α, interleukin-12 (IL-12), and IL-6 (14). We therefore sought to determine whether the accelerating effects of TLR-7 overexpression could be mimicked by administration of IFNα. We first showed that the TLR-7 agonist imiquimod did not accelerate disease in female (NZW × BXSB)F$_1$ mice, consistent with the finding that optimal TLR-7 agonism requires the presence of IFNα (15). In contrast, IFNα accelerated the onset of anticardiolipin and anti-Sm/RNP autoantibodies and caused early mortality due to nephritis. However, the IFNα-treated female mice developed only mild cardiac disease, and they did not become thrombocytopenic. Like male (NZW × BXSB)F$_1$ mice, the IFNα-treated female mice had marked splenomegaly, an increase in the number of activated B cells and T cells, and an increase in the number of plasma cells; however, they did not lose their marginal-zone B cells.

Thus, the loss of marginal-zone B cells in male (NZW × BXSB)F$_1$ mice and in female mice overexpressing TLR-7 (4) is not mediated through IFNα and may be attributable to a TLR-7–mediated intrinsic defect in B cell selection. The differences in B cell selection between male and female mice may be one reason for the failure to develop antiplatelet antibodies and the milder APS in the treated females. Alternatively, TLR-7 ligation induces many cytokines in addition to IFNα that may cooperatively contribute to disease phenotype and severity. Finally, it is possible that other genes in the Yaa locus could contribute to a more severe disease phenotype in male mice.

The accelerating effects of IFNα are clearly different between NZB/NZW and (NZW × BXSB)F$_1$ mice. In both strains, IFNα recapitulates the autoantibody profile of the spontaneous disease (2), with early onset of B cell and T cell activation. In NZB/NZW mice, IFNα induces abundant germinal centers, large numbers of short-lived plasma cells in the spleens with lack of expansion of these cells in the bone marrow, and high
serum levels of IgG2a and IgG3 that deposit in the kidneys (Davidson A and Liu S; unpublished observations). In (NZW × BXSB)F1 mice, it accelerates the development of large germinal centers, followed by splenic disorganization, similar to what is seen in males, an increase in anticardiolipin-producing B cells in the bone marrow, and an increase in only serum levels of IgG2a.

These data show in sum that the effects of IFNα do not fully recapitulate the B cell or disease phenotype associated with TLR-7 overexpression, and that its effects on disease phenotype are strain dependent. Further characterization of the effects of excess IFNα in different lupus-prone mice models and on responses of these mice to therapy will increase our understanding of the physiologic significance of the IFN signature in humans.

**AUTHOR CONTRIBUTIONS**

Dr. Davidson had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

*Study design,* Ramanujam, Kahn, Davidson.

*Acquisition of data,* Ramanujam, Kahn, Huang, Tao, Davidson.

*Analysis and interpretation of data,* Ramanujam, Kahn, Huang, Madaio, Factor, Davidson.

*Manuscript preparation,* Ramanujam, Kahn, Davidson.

*Statistical analysis,* Ramanujam, Kahn, Davidson.

**REFERENCES**