Deficiency of Type I IFN Receptor in Lupus-Prone New Zealand Mixed 2328 Mice Decreases Dendritic Cell Numbers and Activation and Protects from Disease

Hemant Agrawal, Noam Jacob, Esther Carreras, Sandra Bajana, Chaim Putterman, Sean Turner, Barbara Neas, Alexis Mathian, Michael N. Koss, William Stohl, Susan Kovats and Chaim O. Jacob

*J Immunol* published online 7 October 2009
http://www.jimmunol.org/content/early/2009/10/07/jimmunol.0803872
Deficiency of Type I IFN Receptor in Lupus-Prone New Zealand Mixed 2328 Mice Decreases Dendritic Cell Numbers and Activation and Protects from Disease

Hemant Agrawal, Noam Jacob, Esther Carreras, Sandra Bajana, Chaim Puttermann, Sean Turner, Barbara Neas, Alexis Mathian, Michael N. Koss, William Stohl, Susan Kovats, Chaim O. Jacob

Type I IFNs are potent regulators of innate and adaptive immunity and are implicated in the pathogenesis of systemic lupus erythematosus. Here we report that clinical and pathological lupus nephritis and serum anti-nuclear Ab levels are greatly attenuated in New Zealand Mixed (NZM) 2328 mice deficient in type I IFN receptors (IFNAR). To determine whether the inflammatory environment in NZM 2328 mice leads to IFNAR-regulated changes in dendritic cells (DC), the number, activation, and function of DC subsets were compared in 2- and 5-mo-old (clinically healthy) female NZM and NZM-IFNAR−/− mice. Numbers of activated CD40high plasmacytoid DC (pDC) were significantly increased in renal lymph nodes of 2-mo-old NZM but not NZM-IFNAR−/− mice, suggesting an early IFNAR-dependent expansion and activation of pDC at disease sites. Relative to NZM spleens, NZM-IFNAR−/− spleens in 5-mo-old mice were significantly decreased in size and contained reduced numbers of conventional DC subsets, but not pDC. Splenic and renal lymph node NZM-IFNAR−/− DC analyzed directly ex vivo expressed significantly less CD40, CD86, and PD-L1 than did NZM DC. Upon activation with synthetic TLR9 ligands in vitro, splenic NZM-IFNAR−/− DC produced less IL-12p40/70 and TNF-α than did NZM DC. The limited IFNAR−/− DC response to endogenous activating stimuli correlated with reduced numbers of splenic activated memory CD4+ T cells and CD19+ B cells in older mice. Thus, IFNAR signaling significantly increases DC numbers, acquisition of Ag presentation competence, and proinflammatory function before onset of clinically apparent lupus disease. The Journal of Immunology, 2009, 183: 0000 – 0000.

Type I IFNs, including multiple IFN-α species, IFN-β, and certain other IFNs, are potent regulators of both innate and adaptive immunity (1). Activating via the two-chain IFN-α/β receptor (IFNAR), type I IFNs modulate the differentiation, proliferation, and survival of B cells, T cells, NK cells, macrophages, and dendritic cells (DC), as well as their respective cytokine production and signaling responses (1).

DC are critical for both innate and adaptive immunity, and they can be divided into plasmacytoid DC (pDC) and conventional DC (cDC) populations. Following initial activation of pDC or cDC through either TLR-dependent or TLR-independent pathways, the IFN produced can act in an autocrine-paracrine manner via IFNAR to amplify the ongoing proinflammatory response (2, 3). Thus, IFNAR expression in both pDC and cDC is likely essential for the strength and duration of inflammatory responses under both physiologic and pathophysiologic conditions. Indeed, type I IFNs and IFNAR increase the functional competence of DC, thus promoting adaptive T cell and B cell immunity (4–9).

It is well established that DC production of type I IFNs and responses to type I IFNs are central to effective antiviral immune responses (10). However, dysregulation of type I IFN-mediated pathways involving DC can also contribute to the development of systemic autoimmunity, including systemic lupus erythematosus (SLE), in susceptible hosts (10). SLE patients harbor elevated serum levels of IFN-α, and their blood leukocytes show increased expression of type I IFN-regulated genes (11–14).

Consistent with the role of type I IFN in DC survival and activation, abnormalities in DC numbers, phenotype, and function have been identified in human SLE (15–19). SLE patients harbor activated myeloid DC and monocytes in peripheral blood, likely due to abundant IFN produced by pDC consequent to chronic TLR signaling triggered by endogenous TLR7 and/or TLR9 ligands (20–22). Increased DC activation and expression of type I IFN-regulated molecules likely contribute to the breach of lymphocyte self tolerance (13, 14, 21, 23). Moreover, elevated splenic and bone marrow (BM) DC numbers and activation state are features
of SLE-prone mice, including the (New Zealand Black (NZB) × New Zealand White NZW)F₁ and New Zealand Mixed (NZM) 2328 strains, suggesting a dysregulation of the type I IFN pathways in DC in these mice (24–31).

Prior studies of IFNAR deficiency in strains of mice with incomplete SLE phenotypes (B6.Na2b, B6.BnA2 × NZW)F₁, 129 × C57Bl/6lpr, or NZB), or in chemically induced SLE in mice bearing a 129Sv background, pointed to a contribution for IFNAR signaling in autoantibody production and development of renal disease (32–35). In stark contrast, it is the absence of IFNAR signaling in MRL/lpr mice that heightens autoantibody production and end-organ disease (36). These conflicting results highlight the need to more fully assess the role for IFNAR in development and progression of SLE and to determine how IFNAR deficiency alters numbers or function of specific immune cells during SLE.

The (NZB × NZW)F₁-derived recombinant inbred NZM 2328 line is a spontaneous SLE model resembling the human disease more closely than other models (37). In the present work, we have generated IFNAR-deficient NZM 2328 mice to assess the role for the IFNAR in SLE disease manifestations and immune dysfunction. We herein report that development of SLE nephritis and pathogenic autoantibodies are greatly attenuated in female NZM 2328 mice lacking IFNAR. Concurrently, DC numbers and their Ag-presentation competence and proinflammatory function are considerably reduced before the onset of clinical disease, implicating IFNAR engagement on DC as a vital process in the development of SLE.

Materials and Methods

Mice

Mice were maintained at the University of Southern California (Los Angeles, CA), and the experiments were approved by the Institutional Animal Care and Use Committee. Mice lacking the α-chain of the IFN-α/β receptor (IFNAR1), encoded by the Ifnar1 gene located on the distal segment of chromosome 16) on the 129/SvEv background were provided by Dr. D. Moskophidis from the Medical College of Georgia (38). These IFN-α/β receptor (IFNAR)-deficient mice were backcrossed onto the SLE-prone NZM 2328 mice using a marker-assisted selection protocol as described (39). The studies presented herein used homozygous IFNAR knockout (KO) mice of at least the N6 generation of backcross, at which time the mice showed the NZM genotype at all markers tested.

Genomic DNA extracted from tail clippings was PCR-amplified with the primers 5′-AAGATGTTGCTGTCCCTTCTCTGTCGTCA-3′ and 5′-ATTATTAAAGAAAAGACGCAGGCGAAGTGG-3′ for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The band size for the intact IFNAR gene fragment is 150 bp, whereas the band size for the disrupted IFNAR gene is 1.3 kb.

IFN-α treatment

Aliquots of recombinant adenovirus containing the IFN-α subtype 5 under the control of the CMV promoter/enhancer was used in vivo as described (40) with minor modifications. Female mice 8–9 wk of age were injected once in the tail vein with 1–2 × 10⁸ IFN-α adenovirus particles. Negative control mice received AdCMVpLpA vector lacking a cDNA insert at 10⁴ virus particles per i.v. injection.

Serum autoantibody concentrations

Sera were assayed for levels of IgG anti-chromatin, anti-histone, and anti-dsDNA autoantibodies by ELISA as described with minor changes (41). Positive control sera from five female, 36- to 38-wk-old, (NZB × NZW)F₁ mice were combined together and assayed at 1/200 dilution on each plate, and the average OD of these sera for each autoantigen was arbitrarily set at a dilution value of 100 U. Values for the test sera were calculated as (ODserum/ODcontrol) × 100. In contrast to IgG autoantibodies, IgM autoantibody levels do not correlate with lupus development in this model (39, 41).

Assessment of nephritis

The development of proteinuria was assessed twice weekly using Albustix assay strips (Bayer) using a scale of 0–4. Severe proteinuria was defined as >300 mg/dl (3+ or more) on two consecutive examinations. For the assessment of renal histology, paraffin sections (3 μm) were stained with H&E and periodic acid-Schiff and were scored in a blinded fashion. Lupus nephritis was classified histopathologically according to the scheme developed by the World Health Organization and widely used in human SLE.

Analyses of cells by flow cytometry

For analyses of DC, spleens and lymph nodes were digested to a single-cell suspension with collagenase type D (Roche) (1 mg/ml) and DNase I (Roche) (0.1 mg/ml) in HBSS at 37°C for 30 min, after which red cells (in spleen) were lysed. BM cells were released from the spine and red cells lysed. After washing, cells were immediately processed for flow cytometry as described (42). To detect DC subsets and activation status, cells directly isolated from the spleen, lymph nodes, or BM were preincubated with anti-CD16/32 and labeled with optimally titrated mAbs in FACS buffer (PBS, 5% newborn calf serum, 0.1% sodium azide). Fluorochrome- or biotin-labeled mAbs specific for CD11c, CD11b, B220, Ly6C, CD8a, F4/80, CD19, CD115, and activated caspase-3 (rabbit IgG) were obtained from BD Biosciences. PE-labeled mAbs specific for CD40, CD86, PD-1, MHC-II (M5/114.15.2), and PDCA-1 were obtained from ebioscience or Miltenyi Biotec. PE- or FITC-conjugated streptavidin was used to detect the biotinylated mAbs. Samples were run on a FACScalibur or LSRII instrument and data were analyzed with FlowJo software.

For lymphocyte subset analyses, mouse spleen mononuclear cells, obtained after mechanical disruption and red cell lysis, were stained with combinations of fluorochrome-conjugated mAb specific for murine CD3, CD4, CD5, CD8, CD11b, CD19, CD21, CD23, CD44, CD62L, CD69, NK1.1, IgM, or IgD (BD Pharmingen and ebioscience) and analyzed by flow cytometry.

Analyses of DC production of cytokines after TLR9 stimulation

To detect production of IL-12p40/p70 in DC subsets, splenocytes or DC from BM cultures were activated for 16–18 h directly ex vivo in the presence of brefeldin A (for the final 10–12 h) with 5 μg/ml CpG-B oligodeoxynucleotides (ODN) (Integrated DNA Technologies) before testing the production of cytokines from surface marker and intracellular cytokine staining with anti-IL-12p40/p70 mAb (C15.6) (BD Biosciences). To detect production of TNF-α, splenocytes were activated for 6 h in the presence of brefeldin A before intracellular staining with anti-TNF-α mAb (MIP6-XT22) (BD Biosciences).

Cytokine-driven cultures models of DC differentiation

BM cells were cultured in RPMI 1640 with 10% FCS (Omega Scientific), 2 mM glutamine, 100 U penicillin/ml, 10 mM HEPES, 5% newborn calf serum, 0.1% sodium pyruvate. In the GM-CSF-driven model of DC differentiation, cultures were set up using total BM cells (5 × 10⁶/ml) in including red cells, as described (42). Cells were harvested on day 7 and analyzed by flow cytometry for DC surface markers. GM-CSF-induced DC were activated for 16 h from days 6–7 with 5 μg/ml CpG-B ODN. In the Flt3 Ligand (FL)-driven culture model, DC were generated from total BM cells (after red cell lysis) at 1–3 × 10⁶/ml in cultures supplemented with FL-IgGFc (400 ng/ml) as described (43). Cells were harvested on days 7–9, counted, and analyzed by flow cytometry. FL-induced DC were activated by incubation for 16 h with 10 μg/ml CpG-A ODN (Coley Pharmaceutical Group) (44).

Statistical analyses

Analyses were performed using SigmaStat software (SPSS) or SAS (version 9.1; SAS Institute). When necessary (Fig. 6, A–F and supplemental Fig. S2, C and D), raw results were log-transformed to achieve normality and/or to satisfy the equal variance test. Mean fluorescence intensity (MFI) value by the wild-type (WT, control) mean of a specific experiment. Relative MFI was a normalizing method producing an MFI rate. Data were summarized using means and SEs and displayed through box plots and bar charts. Parametric testing between two groups was performed by the t test, and parametric testing among three groups was performed by one-way ANOVA. When combining experiments for analysis, a two-way ANOVA was performed. These data were summarized using least-square means due to the unbalanced data. Survival data were analyzed by the log rank test with multiple comparisons by the Holm-Sidak method. Results were considered statistically significant when p < 0.05.

3 The online version of this article contains supplemental material.
Virus lacking the IFN-γ WT female mice (adenovirus as described in Materials and Methods). A third group of WT female mice (n = 7) labeled “control” were injected with an adenovirus lacking the IFN-α construct. Data are presented as the percentage cumulative prevalence of severe (+3, ≥300 mg/dl) proteinuria and as percentage cumulative mortality (B) at the ages shown.

**Results**

Generation of NZM 2328 mice unresponsive to IFN-α

We have transferred the IFNAR1-null mutation into the lupus-susceptible (NZB × NZW)F1-derived recombinant inbred line NZM 2328. To demonstrate that IFNAR−/− NZM 2328 mice are functionally incapable of responding to IFN-α in vivo, a replication-deficient mouse rIFN-α adenovirus was administered to WT or IFNAR−/− (IFNAR-KO) female mice at 8–9 wk of age, before any autoimmune phenotype was evident. As negative controls, any autoimmune phenotype was evident. As negative controls, mice were injected with PBS or with the adenovirus vector lacking IFN-α cDNA. As shown previously in (NZB × NZW)F1 mice (40), WT mice developed severe proteinuria within 3–6 wk after injection of the IFN-expressing adenovirus and died within 6–10 wk (Fig. 1). In contrast, IFNAR−/− mice remained completely healthy during a follow-up period of 12 wk after injection. These results conclusively confirm the inability of these IFNAR−/− NZM 2328 mice to respond to IFN-α in vivo.

**IFNAR deficiency attenuates disease development in NZM 2328 mice**

NZM 2328 females began developing severe proteinuria at 6 mo of age and began dying 1–2 mo later, with ~95% mortality by 10–11 mo (p < 0.001, Fig. 2, A and B). In contrast, >90% of IFNAR−/− females were alive at 12 mo of age, and >80% were disease-free at 15 mo of age. Histological examination of kidney sections showed milder glomerulonephritis in IFNAR−/− females than in age-matched WT female mice (Fig. 2, C and D).

By 7–9 mo of age, IFNAR−/− mice showed significant reductions in circulating levels of IgG anti-dsDNA, anti-histone, and anti-chromatin autoantibodies in comparison to corresponding levels in WT mice (p < 0.001 for each comparison, Fig. 3). Indeed, even at 10–13 mo of age, circulating autoantibody levels were much lower in IFNAR−/− mice than in WT mice (p < 0.001 for each comparison).

**Young NZM 2328 mice show an IFNAR-dependent increase in activated pDC only in renal lymph nodes (rLN)**

Altered numbers of pDC and cDC have been found in blood and target organs of SLE patients (15–19, 22, 45). Additionally, most lines of lupus-prone mice harbor elevated numbers of activated DC as disease progresses with age, although this has not been linked to IFNAR function (24–31). One important question is whether DC in lupus-prone mice are activated in young mice before disease onset, which would suggest that intrinsic defects in DC contribute to lupus susceptibility. Indeed, in previous studies of 2-mo-old lupus-prone B6.Slc3 or NZM 2410 mice, splenic DC displayed elevated levels of costimulatory molecules, although their numbers were not increased (24, 25).

To determine whether DC were activated or increased in number in young healthy NZM 2328 mice in an IFNAR-dependent manner, we used flow cytometry to analyze myeloid cells in the spleen, rLN, and BM of 2-mo-old WT NZM and NZM-IFNAR−/− mice. In these mice, numbers of total cells, cDC, pDC, monocytes, and macrophages in the spleen and BM were not different, and DC and macrophage populations in the spleen did not show evidence of activation, as assessed by costimulatory molecule expression (H. Agrawal, E. Carreras, and S. Kovats, unpublished data).

In contrast, the total number of rLN cells was significantly reduced in 2-mo-old IFNAR−/− mice relative to WT mice (Fig. 4A). While total numbers of cDC (CD11c⁺B220⁻) and pDC (CD11c⁺B220⁺PDCA-1⁻) in rLN were not different (Fig. 4, B and C, and supplemental Fig. S1), rLN in IFNAR−/− mice did harbor a reduced percentage and number of activated CD40high...
pDC (Fig. 4, D and E). These data show that before activation of DC in other lymphoid organs, pDC in rLN are preferentially activated via an IFNAR-dependent pathway. This local pDC activation, as well as the general increase in rLN cell numbers, likely reflects early IFNAR-dependent renal specific disease activity that occurs before onset of more systemic disease in NZM mice.

By 5 mo of age, numbers of total cells, cDC, and pDC in rLN were significantly increased, relative to numbers at 2 mo of age, in WT but not IFNAR−/− mice (Fig. 4, A–C). An increase in numbers of activated CD40high pDC was observed in rLN of both WT and IFNAR−/− mice, although the numbers continued to be significantly higher in WT mice (Fig. 4, D and F). A similar expansion of activated pDC was not found in cutaneous lymph nodes (S. Bajana and S. Kovats, unpublished data), suggesting that pDC preferentially accumulate proximal to active disease sites.

**Numbers of splenic cDC, but not pDC, are reduced in 5-mo-old IFNAR-deficient NZM 2328 mice**

We also quantified splenic DC subsets in ~5-mo-old mice, before their development of severe proteinuria or elevated serum autoantibodies. Relative to WT NZM 2328 spleens, which are notably larger than in age-matched healthy C57BL/6 spleens (H. Agrawal and S. Kovats, unpublished data), NZM-IFNAR−/− spleens contained significantly fewer cells (p < 0.0001, Fig. 5A). These data suggest that 5-mo-old NZM 2328 IFNAR−/− mice do not have the splenomegaly often observed in lupus-prone mice.

Conventional CD11cint/B220−MHC-II+ DC (cDC) were subdivided into CD8α− lymphoid (LDC) or CD8α+ myeloid (MDC), while pDC were identified as CD11cint/B220+PDCA-1+ (supplemental Fig. S1). Relative to WT spleens from 5-mo-old mice, IFNAR−/− spleens contained reduced numbers of cDC (MDC and LDC) (Fig. 5B), although the frequency of cDC was identical in WT and IFNAR−/− mice, being 1.3% vs 1.2% of total live splenocytes in this example (supplemental Fig. S1). The ratio of MDC to LDC was not altered. The ~40% reduction in cDC numbers was comparable to the reduction in total splenocyte numbers. In contrast, the frequency of pDC was elevated in IFNAR−/− mice (in this example, 0.84% vs 0.4% of total live splenocytes), with the result that total pDC numbers did not differ significantly in the two lines of mice (Fig. 5B and supplemental Fig. S1). Numbers of splenic CD11b+F4/80−CD11clow macrophages tended to decrease in the IFNAR−/− mice, although this difference did not quite reach significance (p = 0.0556) (Fig. 5C). These data show that IFNAR-mediated events lead to increased numbers of splenic cDC, but not pDC, before onset of overt clinical disease or accumulation of...
autoantibodies. This contrasts with previous observations in mice bearing a nonautoimmune 129Sv background, in which no differences in organ size or the frequency of DC in various organs in NZM-IFNAR−/− mice. Spleens were digested with collagenase, red cells were lysed, and total viable cell numbers were determined by hemocytometer counts.

Reduction of numbers of total splenocytes, splenic macrophages, and splenic cDC, but not pDC, in 5-mo-old IFNAR−/− mice. A, IFNAR deficiency reduces the splenomegaly characteristic of NZM 2328 mice. Splenocytes from IFNAR-KO mice were cultured in vitro for 18 h with CpG-B ODN (5 μg/ml), with brefeldin A added for the last 10 h, before detection of IL-12 p70 production (black line) as compared with unstimulated cells (shaded histogram), and the means ± SEM values for WT and IFNAR-KO mice. B, Splenic DC from IFNAR-KO mice were less activated in vivo. Splenic DC subsets, as gated in supplemental Fig. S1, were analyzed directly ex vivo for surface expression of (A) CD40, (B) CD86, and (C) PD-L1. D–F, Splenic DC from IFNAR-KO mice showed less up-regulation of costimulatory molecules after in vitro activation by TLR9 ligands. Splenic DC were stimulated for 16 h in vitro with CpG-B ODN (5 μg/ml) and DC subsets analyzed for expression of (D) CD40, (E) CD86, and (F) PD-L1. For A–F, shown are means ± SEM of relative MFI values of each costimulatory molecule on the indicated splenic DC subsets in WT (open bars) or IFNAR-KO (filled bars) mice. The data were compiled from three to four independent experiments, each with groups of four to five mice (20 wk of age), using a statistical normalization method. Raw data were log-transformed to achieve normality and/or to satisfy the equal variance test. *, p < 0.05; **, p < 0.01; $$$, p < 0.001; nd, not determined. G, IFNAR-KO LDC produced less IL-12 p40/70 than did WT LDC. Splenocytes were activated in vitro for 18 h with CpG-B ODN (5 μg/ml), with brefeldin A added for the last 10 h, before detection of IL-12 p70 production by surface and intracellular staining. Shown are representative histograms of IL-12 p70 expression (black line) as compared with unstimulated cells (shaded histogram), as well as the means ± SEM values for WT and IFNAR-KO DC, n = 4–5; $$$, p < 0.0001. Data are representative of three independent experiments. H, IFNAR-KO LDC produced less TNF-α than do WT LDC. Splenocytes were activated in vitro for 6 h with CpG-B ODN (5 μg/ml), in the presence of brefeldin A, before detection of TNF-α. Shown are representative histograms of TNF-α expression (black line) as compared with unstimulated cells (shaded histogram), and the means ± SEM values for WT and IFNAR-KO DC, n = 4; *, p = 0.039. Data are representative of two independent experiments.

**FIGURE 6.** IFNAR−/− DC are less activated in vivo and show a reduced capacity for activation upon TLR9 stimulation in vitro. A–C, Splenic DC from IFNAR-KO mice were less activated in vivo. Splenic DC subsets, as gated in supplemental Fig. S1, were analyzed directly ex vivo for surface expression of (A) CD40, (B) CD86, and (C) PD-L1. D–F, Splenic DC from IFNAR-KO mice showed less up-regulation of costimulatory molecules after in vitro activation by TLR9 ligands. Splenic DC were stimulated for 16 h in vitro with CpG-B ODN (5 μg/ml) and DC subsets analyzed for expression of (D) CD40, (E) CD86, and (F) PD-L1. For A–F, shown are means ± SEM of relative MFI values of each costimulatory molecule on the indicated splenic DC subsets in WT (open bars) or IFNAR-KO (filled bars) mice. The data were compiled from three to four independent experiments, each with groups of four to five mice (20 wk of age), using a statistical normalization method. Raw data were log-transformed to achieve normality and/or to satisfy the equal variance test. *, p < 0.05; **, p < 0.01; $$$, p < 0.001; nd, not determined. G, IFNAR-KO LDC produced less IL-12p40/70 than did WT LDC. Splenocytes were activated in vitro for 18 h with CpG-B ODN (5 μg/ml), with brefeldin A added for the last 10 h, before detection of IL-12p70 production by surface and intracellular staining. Shown are representative histograms of IL-12p70 expression (black line) as compared with unstimulated cells (shaded histogram), as well as the means ± SEM values for WT and IFNAR-KO DC, n = 4–5; $$$, p < 0.0001. Data are representative of three independent experiments. H, IFNAR-KO LDC produced less TNF-α than do WT LDC. Splenocytes were activated in vitro for 6 h with CpG-B ODN (5 μg/ml), in the presence of brefeldin A, before detection of TNF-α. Shown are representative histograms of TNF-α expression (black line) as compared with unstimulated cells (shaded histogram), and the means ± SEM values for WT and IFNAR-KO DC, n = 4; *, p = 0.039. Data are representative of two independent experiments.

**FIGURE 5.** Reduction of numbers of total splenocytes, splenic macrophages, and splenic cDC, but not pDC, in 5-mo-old IFNAR−/− mice. A, IFNAR deficiency reduces the splenomegaly characteristic of NZM 2328 mice. Splenocytes were digested with collagenase, red cells were lysed, and total viable cell numbers were determined by hemocytometer counts. Shown are the values for individual 5-mo-old mice with the mean value indicated by the bar. The mean ± SEM for WT spleens was 2.36 ± 0.104 × 10^8 (n = 15) and for IFNAR-KO spleens was 1.43 ± 0.089 × 10^8 (n = 17); $$$, p < 0.0001. B, Numbers of splenic cDC but not pDC were reduced in IFNAR-KO mice. Splenic DC subsets were identified using multiparameter flow cytometry according to the gating scheme shown in supplemental Fig. S1. Shown are the means ± SEM numbers of each DC subset, n = 15–17; $$$, p < 0.0001; ns, not significant. C, Numbers of splenic macrophages tend to be reduced in IFNAR-KO mice. Shown are the means ± SEM, n = 5; p = 0.0556. D, IFNAR deficiency alters the percentages of myeloid cells that express activated caspase-3. Shown are the means ± SEM of each cell subset, n = 5; *, p < 0.05 and **, p < 0.01.

DC in SLE patients and murine models of lupus often show elevated costimulatory molecule expression (e.g., CD86, CD40), reflecting their increased activation state and hyperstimulatory capacity (17, 24, 25, 28, 31, 45). To determine the effects of IFNAR deficiency on the activation status of splenic DC in 5-mo-old mice, the DC subsets identified in Fig. 5 were assessed directly ex vivo (without culture or additional stimulation) for surface expression of the costimulatory molecules CD40, CD86, and PD-L1 (Fig. 6, A–C). Relative to WT DC, IFNAR−/− cDC subsets, especially LDC, showed decreased CD40 expression (∼30–40% reduction in MFI), whereas pDC expression of CD40 was variable and not significantly different in the two lines of mice (Fig. 6A). IFNAR−/− cDC subsets (especially LDC) and pDC also showed decreased expression of CD86 and PD-L1 (∼20–40% reduction in MFI, Fig. 6, B and C). Surface MHC class II levels on splenic DC and CD86 levels on splenic macrophages were not different in the two lines of mice (H. Agrawal and S. Kovats, unpublished data). These data
show that in 5-mo-old NZM 2328 mice, IFNAR promotes an activated splenic DC phenotype before development of overt clinical lupus. In contrast, pDC and cDC in 129Sv and 129Sv-IFNAR−/− mice did not differ in their low expression of CD40 or CD86 costimulatory molecules when analyzed directly ex vivo (46). Thus, the difference in costimulatory molecule expression between NZM and NZM-IFNAR−/− DC is due to IFNAR-regulated activation of DC in vivo by endogenous stimulators that arise even before clinical lupus develops in NZM mice.

IFNAR deficiency alters DC apoptosis

The increase in splenic cDC numbers in 5-mo-old WT mice could be due to increased survival mediated by IFNAR signaling. Indeed, DC in B6.Sle3 mice were less apoptotic than in WT B6 DC after a period of in vitro culture (25). Apoptosis is characterized by the accumulation of the active form of caspase-3 (48). Additionally, activated caspase-3 levels decrease after DC stimulation by TLR ligands, thus facilitating endosomal trafficking that leads to new peptide-MHC-II complex formation (49). Therefore, we hypothesized that DC in WT NZM mice might contain less activated caspase-3 if they are chronically activated and longer lived than DC in IFNAR−/− mice. We used intracellular flow cytometry directly ex vivo to measure the percentage of DC and macrophages bearing activated caspase-3 (48). Relative to WT cells, a greater percentage (~2-fold) of IFNAR−/− LDC and macrophages contained active caspase-3 (Fig. 5D). These data are consistent with the reduced numbers of activated cDC in IFNAR−/− mice. In contrast, a greater percentage of WT pDC contained active caspase-3 (Fig. 5D). This may account for the absence of elevated pDC numbers in WT spleens, despite the increase in spleen size.

NZM-IFNAR−/− DC show a reduced capacity for activation upon TLR9 stimulation in vitro

Signaling through TLR7 and TLR9 by engagement of endogenous nucleic acid ligands may contribute importantly to the inflammatory state in SLE (21). Indeed, SLE patients have elevated serum levels of the proinflammatory cytokine IL-12 (50). Thus, we determined whether IFNAR expression in NZM DC influences TLR9-induced signaling pathways that lead to increased cell surface costimulatory molecules and production of inflammatory cytokines.

Splenocytes were incubated 16 h in culture with the TLR9 ligand CpG-B ODN. Relative to WT DC, IFNAR−/− MDC, LDC, and pDC showed less increase in surface CD40, CD86, and PDL1 expression after activation with TLR9 ligands, with the most marked difference noted in LDC (Fig. 6, D–F). Intracellular cytokine staining showed that a reduced percentage of IFNAR−/− LDC were capable of producing IL-12p40/p70 and TNF-α (Fig. 6, G and H); similar results were obtained with MDC (H. Agrawal and S. Kovats, unpublished data). IL-6 production by WT and IFNAR−/− cDC did not differ (H. Agrawal and S. Kovats, unpublished data). These data are consistent with earlier reports using 129Sv mice that IFNAR signaling promotes cDC and pDC activation in response to synthetic TLR9 ligands in vitro or in vivo (46, 47). Thus, in NZM 2328 mice, IFNAR affects the magnitude of DC activation and proinflammatory cytokine production in response to stimulation via TLR9, possibly contributing to the ability of DC to promote breaches in T cell tolerance.

NZM WT bone marrow cells have an IFNAR-dependent reduction in DC precursors responsive to FL and GM-CSF in vitro

Recent reports showed that IFN-α acting via IFNAR stimulates the proliferation of normally quiescent BM hematopoietic stem cells in vivo (51, 52). Chronic IFN-α stimulation of hematopoietic stem cells led to their depletion and compromised their function, including the ability to repopulate the BM of irradiated mice. Therefore, we hypothesized that elevated type I IFN and IFNAR signaling in NZM WT mice would lead to decreased numbers of functional hematopoietic stem cells, which might reduce the frequency of DC precursors in BM.

We used two cytokine-driven (GM-CSF and FL) DC development models to study the differentiation potential of BM DC precursors in WT and IFNAR−/− mice. Since GM-CSF and FL regulate DC differentiation in vivo, these cytokine-driven culture models have typically been used to determine factors that modulate DC differentiation (53). Indeed, published reports assessing DC differentiation in these culture models showed that IFN-α or IFNAR inhibited MDC differentiation in noninflammatory conditions (4, 32, 55).

The FL-driven model yields pDC (CD11c+CD220PDCA-1+) and cDC (CD11c+CD220CD11b+ MDC and CD11c+CD220CD11blow− LDC) (43). After differentiation of WT or IFNAR−/− BM cells for 8 days in FL-containing medium, numbers of total live cells in IFNAR−/− cultures were increased relative to WT cultures (supplemental Fig. S2A). The numbers of each DC subset also were significantly increased in IFNAR−/− cultures. DC in these cultures were not activated, as determined by their low level of CD40 and CD86 expression (supplemental Fig. S2, C and D, and data not shown). These data suggest that NZM WT mice have an IFNAR-dependent impairment in DC precursor activity, perhaps caused by elevated amounts of IFN-α already present in 5-mo-old mice.

In contrast to effects on differentiation, IFN-α can promote the survival of developed pDC in culture (4). Upon activation of DC in FL-driven cultures with the TLR9 ligand CpG-A ODN, the total numbers of live cells in IFNAR−/− BM cultures were reduced relative to WT cultures (supplemental Fig. S2B). Numbers of IFNAR−/− pDC and LDC were significantly reduced, while numbers of MDC were not significantly different (supplemental Fig. S2B). These data show that in the presence of activating stimuli, IFNAR signaling promotes increased numbers of DC, perhaps due to enhancement of cell survival.

We also studied how IFNAR deficiency impacted GM-CSF-mediated DC differentiation from monocytes and myeloid precursors, a pathway important for generation of tissue DC during homeostasis and inflammation in vivo (53). The GM-CSF-driven model yields myeloid (CD11c+CD11b+) DC, comprising ~50–80% of the cells in culture on day 7 (42). In the absence of activating stimuli, the total number of cells in culture on day 7 was higher in IFNAR−/− (mean ± SE, 2.6 ± 0.19 × 10⁶, n = 7) than in WT (mean ± SE, 1.7 ± 0.22 × 10⁶, n = 5) cultures (p = 0.014) (S. Bajana and S. Kovats, unpublished data). Both the frequency and absolute numbers of MDC were increased in IFNAR−/− cultures (supplemental Fig. S2E), as we observed in FL-driven cultures. These data are consistent with an IFNAR-dependent decrease in numbers of GM-CSF-responsive myeloid progenitors in NZM BM and in published reports (4, 32, 54, 55).

IFNAR−/− DC in cytokine-responsive BM cultures are less activated by TLR9 ligands

To compare the ability of DC derived from WT or IFNAR−/− BM cells in FL-driven cultures to become activated by TLR9 ligands, we assessed surface levels of CD40 and CD86 after incubation with CpG-A ODN. Relative to WT DC, IFNAR−/− MDC, LDC, and pDC showed significantly reduced levels of CD40 or CD86 after incubation with TLR9 ligands, as we observed with splenic DC (supplemental Fig. S2, C and D).

WT and IFNAR−/− DC from GM-CSF-driven cultures also were activated for 16 h with the TLR9 ligand CpG-B ODN, followed by
assessment of IL-12p40/70 production by intracellular flow cytometry. A greater percentage of WT DC produced IL-12p40/70, consistent with a role for IFNAR signaling in IL-12 production (supplemental Fig. S2F). While baseline levels of surface MHC-II, CD40, and CD86 did not significantly differ between the two DC populations, WT DC showed slightly higher expression of these molecules than did IFNAR−/− DC (S. Bajana and S. Kovats, unpublished data). These experiments with BM-derived MDC are consistent with our results with freshly isolated splenic DC in showing that IFNAR deficiency on the NZM 2328 background leads to reduced DC activation in response to synthetic TLR9 ligands, as well as the endogenous TLR9 ligands present in vivo.

**IFNAR-deficient NZM 2328 mice harbor reduced lymphocyte numbers, with a marked reduction in activated memory CD4+ T cells**

Increased numbers of activated DC promote autoreactive B and T cell responses in murine autoimmune models (25, 56). Autocrine IFN-α and IFN-β acting via IFNAR enhance DC survival and activation, thereby increasing the ability of DC to stimulate adaptive T cell immunity and enhance humoral immunity by increasing B cell proliferation and Ig class switching (4–9). IFNAR signaling also may have direct effects in lymphocytes (57). To determine whether IFNAR signaling and/or increased numbers of activated DC led to altered numbers or phenotypes of lymphocytes in NZM 2328 mice, we compared numbers of B and T cell subsets in WT and IFNAR−/− mice at 5 mo of age before development of overt clinical disease, and at 8–12 mo of age, when clinical disease is apparent in WT mice. At 5 mo of age, WT mice had greater numbers of CD19+ B cells (p = 0.026, Fig. 7A), which included an increase in CD21+CD23+ follicular B cells (supplemental Fig. S3). However, at 5 mo of age, no differences in numbers of CD4+ or CD8+ T cells were found between WT and IFNAR−/− mice (Fig. 7, B and C). By 8–9 mo, the number of CD19+ B cells was significantly increased in WT but not IFNAR−/− mice (p = 0.007, Fig. 7A), which included increased numbers of CD5+ B cells (supplemental Fig. S3). These increased B cell numbers in WT mice are consistent with the increased production of anti-nuclear autoantibodies that we also observed in WT mice at this age (Fig. 3).

Notably, 8- to 9-mo-old WT mice had a significantly expanded population of activated memory CD4+CD44highCD62Llow T cells, which was not present in the IFNAR−/− mice (Fig. 7E). Numbers of naive CD4+CD44lowCD62Lhigh T cells were not different in WT and IFNAR−/− mice at either age 5 or 8–9 mo (Fig. 7D). These data suggest that the increased numbers of activated DC observed at 5 mo of age (before onset of clinical disease) in WT mice lead to the priming and subsequent maintenance of activated memory CD4+ T cells that ultimately fuel onset and maintenance of overt clinical disease.

**Discussion**

We have shown that IFNAR deficiency on the NZM 2328 background significantly reduces the incidence of lupus nephritis, autoantibody production, and mortality. Our analyses of DC before the onset of clinical disease in 2- and 5-mo-old NZM 2328 mice show that IFNAR signaling promotes increased numbers of activated pDC in the rLN and cDC in the spleen. As disease progresses, these activated DC correlate with elevated numbers of activated effector CD4+ T cells and production of pathogenic autoantibodies. These data implicate IFNAR engagement in DC as a critical factor at early preclinical stages of lupus disease.

Increased numbers of DC bearing high levels of costimulatory molecules accumulate with age in most murine models of lupus, including the MRL/lpr, NZB, (NZB × NZW)F1, BXSB, (NZB × BXSB)F1, NZM 2328, NZM 2410, B6.Sle1, and B6.Sle3 strains (24–31). The increased DC numbers could be due to new DC differentiation or to extended DC survival mediated by endogenous TLR ligands and IFNAR signaling. New DC arise during inflammation after differentiation from inflammatory monocytes recruited to lymphoid organs and tissues (53). Additionally, TLR9 activation induces lymphoid progenitors to switch their developmental program and differentiate to myeloid cells, including DC.
and macrophages (58). Interestingly, elevated numbers of activated DC, which accumulate due to increased new development or extended survival during chronic inflammation, have been shown to promote loss of self tolerance. An extended lifespan of activated DC can induce SLE-like autoimmunity in C57BL/6 mice, as shown by experiments that inhibited DC apoptosis after stimulation by TLR ligands in vivo (56, 59). Similarly, lupus disease in mice with multiple extra copies of the TLR7 gene was associated with elevated numbers of DC, consistent with chronic TLR7 signaling inducing new DC development or extended DC survival (60). Our data show that in the context of the inflammation and chronic TLR signaling in NZM 2328 mice, IFNAR likely acts via one of these mechanisms to promote the increased numbers of activated cDC and pDC. Interestingly, the effect of IFNAR on activated DC numbers is specific to lupus-prone mice, since this effect of IFNAR deficiency was not observed in 129Sv mice (47).

In young (2-mo-old) mice, we identified an IFNAR-dependent population of CD40hi pDC that preferentially accumulated in rLN; a general increase in rLN size was also IFNAR-dependent. This was notable since we did not observe populations of activated DC or macrophages in the spleen or BM at this age. These data suggest that an early event during preclinical stages of lupus nephritis is the recruitment of pDC to the kidney or rLN, leading to the accumulation of activated pDC at these sites. We provide evidence that IFNAR has an important role in this tissue-specific accumulation, consistent with reports that IFNAR signaling increases pDC activation, survival, and migration (4, 46). In human SLE patients, elevated numbers of pDC are observed in kidney or skin but not in blood, which also suggests that pDC are preferentially recruited to active disease sites (15, 50, 61, 62).

In 5-mo-old WT NZM mice, pDC numbers in rLN increased, yet splenic pDC numbers remained comparable in NZM WT and IFNAR−/− mice. The most likely explanation is that IFNAR signaling does promote pDC survival and activation in NZM 2328 mice, but the activated pDC are preferentially recruited to target tissues such as kidney and their draining lymph nodes as we have noted. It is also possible that activated splenic pDC are converted to cDC, as occurs during viral infection or upon in vivo exposure to type I IFN (63, 64).

In NZM 2328 mice, IFNAR promotes activated cDC and pDC phenotypes in vivo before development of severe lupus symptoms. In contrast, pDC and cDC in unstimulated 129Sv and 129Sv-IFNAR−/− mice did not differ in low expression of CD40 or CD86 costimulatory molecules (46). This difference in costimulatory molecule expression between NZM and NZM-IFNAR−/− DC is likely due to activation of DC in vivo by endogenous stimulators that occur as lupus begins to develop in NZM mice. Similar comparisons of NZM2410 to BALB/c mice (24) or B6.Sle1 and B6.Sle3 to B6 mice (25, 28) showed that DC in lupus-prone mice exhibit a hyperstimulatory phenotype before disease onset.

We found that IFNAR deficiency had a profound effect on activation of pDC and CD8α+ LDC. During homeostasis, LDC and pDC help to maintain T cell self tolerance (53, 65). Upon bacterial or viral infection, LDC are critical for priming of naive T cells. Activated pDC also may have a significant role in Ag presentation to T cells at sites of inflammation and in local lymph nodes (66). Thus, the IFNAR-regulated LDC and pDC activation that occurs before overt clinical disease is likely to contribute to the loss of T cell tolerance necessary to initiate the adaptive immune phase of lupus disease. Indeed, our analyses of 8- to 9-mo-old mice showed an expansion of activated memory T cells and marginal zone B cells in WT but not in IFNAR−/− mice.

Our data with NZM 2328 mice are consistent with prior reports of the effect of IFNAR deficiency in spontaneous models with partial lupus phenomenology or in experimental lupus-like models. Deficiency of IFNAR in 129Sv mice inhibited autoantibody production, severe renal disease, and expression of type I IFN-stimulated genes in a chemically induced (2,6,10,14-tetramethylpentadecane) lupus model (35). NZB mice lacking the IFNAR α-chain had significantly reduced anti-erythrocyte autoantibodies, hemolytic anemia, and anti-DNA Abs (32). IFNAR-deficient B6.Na2b (B6.Na2 × NZWF1), and 129 × C57BL/6/lpr mice showed decreased autoantibody production and renal disease and did not develop splenomegaly or lymphadenopathy (33, 34). We now show that disease protection in these IFNAR-deficient lupus models may occur because sustained DC activation does not occur, which prevents chronic lymphocyte responses. Paradigmatically, IFNAR deficiency in MRL/lpr mice worsened lymphoproliferation, autoantibody production, and end-organ disease, suggesting that type I IFNs may suppress autoimmunity induced by Fas deficiency on the MRL genetic background (36). These distinct effects of IFNAR deficiency on lupus disease in different models could relate to the various mechanisms by which DC induce tolerance. While “immature” DC lacking costimulatory molecules or inflammatory cytokines promote tolerance by failing to activate lymphocytes, in some settings activated “mature” DC are required to induce T regulatory cells (67). Thus, it is possible that disease-suppressing T regulatory cells in MRL/lpr mice are dependent on IFNAR signaling in DC, and this mechanism is less critical in NZM 2328 mice.

NZM 2328 mice exhibit a disease course and sex bias that most closely resembles human SLE. Thus, our finding that IFNAR deficiency decreases numbers of activated DC and reduces lupus severity and mortality provides additional rationale for human SLE therapy involving IFNAR blockade or anti-IFN-α mAbs.

Disclosures

The authors have no financial conflicts of interest.

References


