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IFN-α/β Receptor Signaling Promotes Regulatory T Cell Development and Function under Stress Conditions

Amina Metidji,*† Sadiye Amcaoglu Rieder,* Deborah Dacek Glass,* Isabelle Cremer,‡,§ George A. Punktosdy,*1,2 and Ethan M. Shevach*1

Type I IFNs are a family of cytokines with antiviral and immunomodulatory properties. Although the antiviral effects of IFNs are well characterized, their immunomodulatory properties are less clear. To specifically address the effects of type I IFNs on T regulatory cells (Tregs), we studied mixed bone marrow chimeras between wild-type and IFN-α/β receptor (IFNAR) knockout (KO) mice, and heterozygous female mice expressing a Treg-specific deletion of the IFNAR. In these two models, IFNAR signaling promotes the development of the Treg lineage in the thymus and their survival in the periphery. IFNAR KO Tregs had a higher expression of the proapoptotic gene Bim and higher frequency of active caspase-positive cells. IFNAR KO Tregs from chimeric mice displayed a more naive phenotype, accompanied by lower levels of CD25 and phosphorylated STAT5. Therefore, in Tregs, IFNAR signaling may directly or indirectly affect phosphorylation of STAT5. In mixed chimeras with Scurfy fetal liver, Tregs derived from IFNAR KO bone marrow were unable to control T effector cell activation and tissue inflammation. Under stress conditions or in a competitive environment, IFNAR signaling may be required to maintain Treg homeostasis and function. The Journal of Immunology, 2015, 194: 4265–4276.

Type I IFNs are a family of cytokines that possess diverse properties. Type I IFNs, consisting of multiple IFN-α subtypes and a single IFN-β subtype, are derived from a single ancestral gene and are structurally related (1). They bind to a common receptor, IFN-α/β receptor (IFNAR), which is expressed on most cell types (2). IFNAR consists of two subunits, IFNAR 1 and IFNAR 2, and is associated with Janus protein tyrosine kinases (Tyk2 for IFNAR 1 and Jak1 for IFNAR2) (3, 4). Signaling through IFNAR induces a cascade of protein phosphorylation (STAT1 and STAT2) that recruits the IRF-9 to form the heterotrimeric complex, IFN-stimulated gene factor 3 translocates to the nucleus and binds to IFN-stimulated response elements to initiate the transcription of IFN gene (6, 7).

Type I IFNs were initially defined by their antiviral properties but are also potent immunomodulators that can act directly on components of the innate and adaptive immune system. Type I IFN, acting directly on T cells, can modulate their activation and/or survival (8, 9). It was reported that treatment with IFN-α/β in vitro prolonged the survival of activated T cells and increased clonal expansion and effector differentiation of CD8+ T cells (10–13). Similarly, Type I IFNs were required in vivo for clonal expansion of Ag-specific CD4+ and CD8+ T cells during T cell priming (14, 15). Given the diverse effects of IFN-α/β in the innate and adaptive immune system, it is not surprising that these cytokines play a role in several autoimmune diseases. Psoriasis and systemic lupus erythematosus are improved by the inhibition of type I IFNs (16, 17), whereas arthritis and multiple sclerosis benefit from the administration of type I IFNs (18). Although the associations between type I IFN and these diseases are established, the mechanisms responsible for the differential effects of IFN have not yet been elucidated.

A number of recent studies have examined the role of type I IFNs on Foxp3+ T regulatory cells (Tregs) in different experimental models of autoimmunity and inflammation, and reached conflicting conclusions. In the classic adoptive transfer model of inflammatory bowel disease (IBD), one study (19) demonstrated that signaling via the IFNAR was essential for maintenance of Foxp3 expression and Treg suppressor function, whereas a second study (20) demonstrated that IFNAR knockout (KO) Tregs were fully competent suppressor cells. Similarly, the transfer of the combination of wild-type (WT) CD45RBhi and WT Tregs, but not IFNAR KO CD45RBhi and IFNAR KO Tregs, induced colitis in RAG KO Trex 1 KO mice that express high levels of endogenous cytoplasmic DNA that can trigger type I IFN production. Development of disease depended on expression of the IFNAR on the WT effector cells, and not on the Tregs, as IFNAR KO T effector cells (Teffs) did not cause disease (21). In contrast, in the tumor microenvironment, signaling via the IFNAR was required for the activation of tumor-infiltrating Tregs to produce IL-10, resulting in suppression of angiogenesis and lymphoangiogenesis induced by tumor-infiltrating Th17 cells (22). Lastly, a recent study (23) demonstrated that type I IFNs directly inhibit Treg activation, proliferation, and function in vivo during acute infection with lymphocytic choriomeningitis virus (LCMV), and that a failure of

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Abbreviations used in this article: BM, bone marrow; IBD, inflammatory bowel disease; IFN, IFN-α/β receptor; KO, knockout; LCMV, lymphocytic choriomeningitis virus; MFI, mean fluorescence intensity; poly (I:C), polyinosinic-polycytidylic acid; Teff, T effector cell; Treg, T regulatory cell; WT, wild-type.

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this inhibitory effect results in impaired function of virus-specific CD4+ and CD8+ T cells and defective viral clearance.

Taken together, these studies suggest that the effects of type I IFNs on Tregs are complex and likely context dependent. In this study, we have examined the effects of type I IFNs on Treg homeostasis and suppressor function. We demonstrate that type I IFNs are essential for the development, survival, and function of Tregs in a competitive or stressed environment.

Materials and Methods

Mice

WT C57BL/6, Mx1-Cre, Rosa/eYFP, Foxp3-YFP-Cre, and Scurfy mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Congenic C57BL/6 (CD45.1), IFNAR KO, and RAG1 KO mice were obtained from Taconic Farms (Germantown, NY). IFNAR KO mice were kindly provided by Ulrich Kalinke (Paul-Ehrlich Institut, Langen, Germany) and crossed to Foxp3-YFP-Cre. All other mice were bred in-house. The Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases approved all experiments.

Bone marrow chimeras

Recipient mice were lethally irradiated with two doses of 550 rad of total body irradiation. Recipients were injected i.v. on the same day with bone marrow (BM) cells (1 × 10^6) or fetal liver cells (2 × 10^5) from donor mice and allowed to reconstitute for 6–8 wk. The fetal liver cells were obtained to detect active pan-caspase was purchased from eBioscience. A total of 1 × 10^6 cells were stimulated for 4 h with cell stimulation mixture plus IFN-γ (XMG1.2) staining, cells were stimulated for 4 h with cell stimulation mixture plus pro-TNF protein transport inhibitor (eBioscience) and stained intracellularly. A kit to detect active pan-caspase was purchased from eBioscience. A total of 1 × 10^6 spleenocytes were stained with FITC-Z-VAD-FMK or allophycocyanin-Z-VAD-FMK for 45 min at a 37°C incubator with 5% CO₂. The data were acquired on an LSRII instrument (BD Biosciences) and analyzed using FlowJo software (TreeStar). For cell sorting experiments, cells were labeled with anti-CD4 (L3T4) beads and purified on the AutoMACS Cell Separator (Miltenyi Biotec), stained for desired cell-surface markers, and sorted using FACSAria flow cytometers (BD Biosciences).

Flow cytometry and cell sorting

A single-cell suspension was generated from spleens or thymus. Cell-surface staining was performed with the following directly conjugated anti-murine Abs (from BD Biosciences or eBioscience unless otherwise specified): anti-CD4 (RM4-5), -CD8 (53-6.7), -CD19 (1D3), -CD25 (7D4), -CD44 (IM7), -CD45.1 (A20), -CD45.2 (104), -CD62L (MEL-14), -CD69 (H1.2F3), -CD101 (IGSF2), -CD103 (2E7), -ICOS (7E.17G9). For intracellular staining, cells were surface stained and then permeabilized with FixPerm buffer (eBioscience). Cells were then washed and stained with Abs against Foxp3 (FJK-16s), -GFP (Life Technology). For IFN-γ (XMG1.2) staining, cells were stimulated for 4 h with cell stimulation mixture plus pro-caspase transport inhibitor (eBioscience) and stained intracellularly. A kit to detect active pan-caspase was purchased from eBioscience. A total of 1 × 10^6 spleenocytes were stained with FITC-Z-VAD-FMK or allophycocyanin-Z-VAD-FMK for 45 min at a 37°C incubator with 5% CO₂. The data were acquired on an LSRII instrument (BD Biosciences) and analyzed using FlowJo software (TreeStar). For cell sorting experiments, cells were labeled with anti-CD4 (L3T4) beads and purified on the AutoMACS Cell Separator (Miltenyi Biotec), stained for desired cell-surface markers, and sorted using FACSAria flow cytometers (BD Biosciences).

p-STAT staining

To assess p-STATS levels directly ex vivo, we immediately disrupted spleens and fixed them with Cytofix/Cytoperm buffer (BD). After incubation for 12 min in a 37°C incubator with 5% CO₂, the cells were washed, resuspended in 1 ml Perm Buffer II (BD), and incubated on ice for 30 min. After an additional wash, cells were stained for surface and intracellular Ags, including p-STAT1 (pY701; Cell Signaling Technology) or p-STAT5 (pY694; Cell Signaling Technology), for 45 min on ice. In some experiments, cells were stimulated with different concentrations of mouse IFN-β (PBL IFN Source) and mouse IFN-a2 (eBioscience) for 15 min.

BrdU staining

Mice were injected with 1 mg BrdU i.p. and sacrificed 24 h later. BrdU incorporation was detected in spleenocytes using BrdU Flow kit (BD Biosciences), and Ki-67 staining was performed with Flow kit (BD Biosciences).

ELISA

Mice were injected i.p. with 100 μg of the synthetic dsRNA homolog polyinosinic-polycytidylic acid [poly (I: C)] (Invivogen) and sacrificed 24 h later. The IFN-α level was measured in serum using a mouse IFN-α ELISA kit (PBL IFN Source).

mRNA isolation, cDNA production, and real-time PCR

RNA extraction was performed using RNeasy columns (Qiagen), and cDNA was generated using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions. Presynthesized TaqMan Gene Expression Assays (Applied Biosystems) were used to amplify Bim (Mm00437796_m1), Bcl-2 (Mm00477631_m1), PUMA (Mm00519268_m1), Bax (Mm00432051_m1), Mcl-1 (Mm00725832_s1), Bcl-xL (Mm00437783_m1), Bad (Mm00432042_m1), and Bak-1 (Mm00432045_m1). Actb was used as an internal control, and target gene values are expressed relative to Actb. Real-time PCR was conducted with the ABI Prism 7900HT, using TaqMan Universal PCR Master Mix (Applied Biosystems).

IL-2 immune complex treatment

WT + IFNAR KO chimera and IFNAR KO × Foxp3-YFP-Cre/YFP-Cre mice were treated with IL-2 immune complexes, as previously described (24). In brief, IL-2/anti–IL-2 mAb complexes were prepared by mixing recombinant murine IL-2 (1 μg; Peprotech) with JES6-1 (5 μg) at the optimal 1:2 molar ratio and incubated for 10 min at room temperature. Immune complexes were then diluted in PBS and injected i.p. for 5 d consecutively. Expansion of T cells was assessed 2 h after the last injection in the spleen and the thymus.

H&E staining

Organs were fixed in 10% neutral-buffered formalin (Sigma-Aldrich) for 24 h and then transferred to a solution of 70% ethanol. Fixed tissues were embedded in paraffin and stained with H&E by American Histolabs (Gaithersburg, MD).

Statistical analysis

Data were statistically analyzed, as indicated in the figure legends, using GraphPad Prism. Values were considered statistically significant when p < 0.05. All data are presented as the mean ± SD.

Results

Tregs respond to type I IFN in vitro and in vivo

The ability of both CD4+ and CD8+ Teffs to respond to type I IFN is well established (25, 26), but the role of type I IFN in Treg function remains poorly understood. Although all cells express the IFNAR and can potentially respond to type I IFNs, we initially assessed the ability of Tregs to directly respond to type I IFN ex vivo by measuring the phosphorylation of STAT1 after a 15-min stimulation with rIFN-α2 or rIFN-β. The response of Tregs was similar to CD4+ and CD8+ Teffs over a range of cytokine concentrations (data not shown).

We then addressed a potential role for type I IFN signaling in Treg function by assessing the relative ability of different T cell subpopulations to respond to type I IFN in vivo. Injection of mice with the synthetic dsRNA homolog poly(I:C) has been shown to induce large amounts of type I IFN in a TLR-3–dependent manner (27). Previous studies have shown that stimulation of lymphocytes with type I IFN rapidly induces the expression of CD69 (28, 29). We initially measured CD69 expression on various T lymphocyte subpopulations 24 h after poly(I:C) injection as a readout for the response to type I IFN. CD4+, CD8+, and CD4+ Foxp3+ T cells uniformly expressed high levels of CD69 (Fig. 1A) after treatment of the mice with poly(I:C). As a negative control, CD69 upregulation was not observed in any cell population after treatment of IFNAR KO mice with poly(I:C) (data not shown). IFN-α was detected in the serum at 24 h after poly(I:C) injection, but not 72 h after injection, indicating that the secretion of IFN-α was transient with this dose of poly(I:C) (Fig. 1B).

Finally, we generated mice in which cells that had responded to type I IFN could be identified by detection of a fluorescent marker. We crossed mice expressing the Cre-recombinase under the control of the Mx-1 gene promoter to mice expressing a LoxP-Stop-LoxP-eYFP cassette inserted into the ROSA26 gene locus (termed Mx-1/Cre × ROSA/eYFP). Because Mx-1 is a type I IFN inducible gene (30), cells that have responded to type I IFN will excise the stop codon and be permanently marked by eYFP expression. Injection of poly(I:C) into Mx-1/Cre × ROSA/eYFP mice resulted...
in eYFP expression in all T cell subpopulations (Fig. 1C). In this assay, the highest level of induction of YFP expression was seen in CD8+ (57%) T cells, but Foxp3+ (38%) responded to a greater extent than CD4+Foxp3+ (25%) T cells, suggesting that Tregs may be more sensitive to type I IFN in this model. The average MFI of eYFP expression on T cells. Data are representative of one experiment from three independent experiments with three to five mice per group. Error bars in all panels represent the mean ± SD. (B) Data were analyzed by one-way ANOVA with p = 0.0002. (D) Data were analyzed by two-way ANOVA. ***p ≥ 0.001, ****p ≥ 0.0001, **p < 0.0001.

**FIGURE 1.** WT Tregs respond to type I IFN in vivo. WT C57BL/6 and Mx-1/Cre × ROSA/eYFP mice were injected with poly(I:C). (A) WT mice were sacrificed 24 h later, and spleen cells from noninjected (shaded histogram) and injected (black line) mice were analyzed by flow cytometry (n = 5) for expression of CD69 on CD4+Foxp3−, Foxp3+, and CD8−. (B) The concentration of IFN-α was measured by ELISA in the serum of mice treated or not with poly(I:C) at 24 and 72 h (each symbol represents data from one animal). (C) Mx-1/Cre × ROSA/eYFP mice were injected with poly(I:C) every other day for 5 d and sacrificed 2 d later. Spleen cells from noninjected (shaded histogram) and injected (black line) mice were analyzed by flow cytometry (n = 5) for expression of eYFP gated on CD4+ Foxp3−, Foxp3+, and CD8−. (D) The average MFI of eYFP expression on T cells. Data are representative of one experiment from three independent experiments with three to five mice per group. Error bars in all panels represent the mean ± SD. (B) Data were analyzed by one-way ANOVA with p = 0.0002. (D) Data were analyzed by two-way ANOVA. ***p ≥ 0.001, ****p ≥ 0.0001, **p < 0.0001.

**Tregs from WT, IFNAR KO, and IFNARYFP−/Foxp3−/Cre/eYFP−/Cre mice are similar in terms of phenotype and number**

We next compared the numbers and phenotype of Tregs in WT, IFNAR KO, and IFNARYFP−/Foxp3−/Cre/eYFP−/Cre mice. All strains had equal percentages of splenic CD4+ T cells that expressed the Treg markers CD25 and Foxp3 (Fig. 2A), and the absolute numbers of Treg were also similar (Fig. 2B). The absolute numbers of Tregs were also similar in the thymus of these mice (Fig. 2C). Furthermore, Tregs from WT and IFNARYFP−/Foxp3−/Cre/eYFP−/Cre mice were similar in terms of the levels of expression of various T cell markers (namely, CD44, CD46, CD62L, CD69, CD101, CD103, ICOS, CD25, and p-STAT5; Fig. 2D). Because type I IFNs may regulate the survival of CD4+ and CD8+ T cells, we also compared the expression of several proapoptotic and antiapoptotic genes (Bim, Puma, Bax, Bad, Bak-1, Bcl-2, Bcl-xL, and Mcl-1) in WT Tregs and Tregs with a selective deletion of the IFNAR. The only significant difference observed was that IFNAR KO Tregs had elevated levels of Bim expression (Fig. 2E), raising the possibility that the increased levels of Bim may increase the susceptibility of these cells to apoptosis.

**IFNAR KO Tregs are at a competitive disadvantage in a mixed BM chimera**

We set up a model to test the fitness of IFNAR KO Tregs in a competitive environment using mixed BM chimeras. We reconstituted lethally irradiated RAG KO mice with an equal mixture of IFNAR KO and WT BM cells that could be differentiated by a congenic marker. Control mice were reconstituted with two congenically marked WT BM populations. When we examined the thymus of chimeric mice 8 wk after reconstitution, we noted a decrease in the ratio (<1) of IFNAR KO T cells to WT T cells in all T cell compartments (double-positive, CD4+Foxp3−, CD4+ Foxp3− single-positive, and CD8+ single-positive thymocytes), indicating that the reduction in IFNAR KO T cells was not Treg specific (Fig. 3A). In the spleen of the WT (CD45.2) + WT (CD45.1) chimeric mice, equal levels of reconstitution were seen in all lymphocyte compartments (Fig. 3B). In the IFNAR KO (CD45.2) + WT (CD45.1) reconstituted mice, all cell populations were significantly skewed toward WT (indicated by a ratio <1). The average level of skewing for total splenocytes, B cells, CD4+, and CD8+ was ~2-fold. However, IFNAR KO Tregs were underrepresented by ~8-fold compared with WT Tregs in these same mice. Taken together, these results suggest that type I IFNs play a role in the development of all T cell subpopulations in the thymus and also play a major role in the survival of Tregs in the periphery in the competitive environment of the mixed chimera.

To further analyze the mechanisms responsible for the decreased survival of the IFNAR KO Tregs, we measured the level of apoptosis directly ex vivo in the various T cell compartments of the chimeric mice. We measured active pan-caspase activity in cells by staining with a FITC-labeled general caspase inhibitor (Z-VAD-FMK). IFNAR KO Tregs had a significantly higher percentage of active pan-caspase–positive cells than WT Tregs in the same chimeric mouse (Fig. 3C). Importantly, the percentages of active pan-caspase–positive cells WT CD4+ and CD8+ T cells and IFNAR KO CD4+ and CD8+ T cells in these same mice were similar, suggesting that IFNAR KO Tregs were unique in their requirement for type I IFN signaling in the periphery. As it has recently been shown that Tregs require the antiapoptotic factor Mcl-1 in a Treg repopulation model after diphtheria toxin–induced depletion (32), we also tested the expression of a panel of proapoptotic and antiapoptotic molecules. The only difference observed between the WT and IFNAR KO Tregs isolated from the chimeric mice was that IFNAR KO Tregs expressed higher levels of the proapoptotic gene, Bim. This result is similar to that seen in Tregs derived from the parental IFNAR KO mice (Fig. 3D).

Because major prosurvival effects of type I IFN signaling on Teffs are mediated during the process of T cell activation (13) and Tregs display an activated phenotype in naive mice based on CD44 expression, we evaluated the possibility that the pronounced underrepresentation of IFNAR KO Tregs in the chimeric environment was secondary to preferential death of activated Tregs. IFNAR KO Tregs displayed a more naive phenotype with lower levels CD44 and higher levels CD62L expression than WT Tregs. Furthermore, the percentages of IFNAR KO Tregs expressing the activation markers CD69, CD101, CD103, and ICOS were also lower than WT Tregs. Most importantly, IFNAR KO Tregs expressed lower levels of CD25 and displayed markedly lower levels of p-STAT5 (Fig. 3E). The average MFI was also signifi-
sitionally different for the markers of activation, demonstrating a more naive phenotype of IFNAR KO cells in the chimeras (Fig. 3F). The decreased survival of the IFNAR KO Tregs was not compensated by an increase in homeostatic proliferation, because the levels of Ki-67 staining and BrdU incorporation were identical in WT and IFNAR KO Tregs derived from the chimeric mice (Fig. 3G). Collectively, these studies are consistent with a require-ment for a type I IFN–mediated signal, which is required for the survival of activated Tregs in the competitive environment, and it also raises the possibility that IFNAR KO Tregs that develop in a chimeric environment may be functionally defective.

IFNAR<sup>fl/fl</sup>× Foxp3<sup>YFP-Cre/YFP-Cre</sup> Tregs display the same development and survival defect as IFNAR KO Tregs from the chimeric mice

One problem with interpretation of the earlier studies is that they were performed in irradiated chimeras. To further examine the phenotype of the IFNAR KO Tregs under more physiologic conditions, we crossed female IFNAR<sup>fl/fl</sup>× Foxp3<sup>YFP-Cre/YFP-Cre</sup> mice to make IFNAR<sup>fl/fl</sup>× Foxp3<sup>WT/WT</sup> mice to produce heterozygous IFNAR<sup>fl/fl</sup>× Foxp3<sup>YFP-Cre/YFP-Cre</sup> female mice. Because genes on the X chromosome are subject to random inactivation of one allele, female mice that are IFNAR<sup>WT/WT</sup> and heterozygous for YFP-Cre...
FIGURE 3. IFNAR KO Tregs are at a competitive disadvantage in a mixed BM chimera. RAG KO mice were lethally irradiated and reconstituted for 8 wk with a 1:1 mixture of WT and IFNAR KO congenically marked BM. (A) Thymic cells from CD45.1 WT+CD45.2 WT (black dots) and CD45.1 WT+CD45.2 IFNAR KO (white dots) chimera were stained for CD4+CD8−, CD4+Foxp3−, CD4+Foxp3+, and CD4+CD8+. Graph represents the ratio of CD45.2 WT or IFNAR KO to CD45.1 WT (each dot represents one mouse). (B) Spleen cells were also stained for B cells (CD19+) and CD4+Foxp3−, CD4+Foxp3+, and CD8+ T cells, and the ratio of CD45.2 WT or IFNAR to KO CD45.1 was determined. (C) Active pan-caspase activity was measured by staining with a FITC-labeled general caspase inhibitor (Z-VAD-FMK) on CD4+Foxp3−, CD4+Foxp3+, and CD8+ cells. Black dots represent WT cells and white dots IFNAR KO cells (each dot represents a single chimeric mouse). (D) Relative expression of apoptotic genes in sorted CD4+CD25+ Tregs from WT and IFNAR KO mixed chimeras (data are representative of three biological replicates with 10 mice each time). (E) Expression of activation markers CD44, CD62L, CD69, CD103, CD101, ICOS, CD25, and p-STAT5 on WT (shaded histogram) and IFNAR KO (black line) gated splenic CD4+Foxp3+ T cells. Dashed line is isotype control for p-STAT5 staining. (F) Average MFI expression of different markers of activation on CD4+Foxp3+ cells from WT+IFNAR KO chimera. (G) Chimeric mice were injected with 1 mg BrdU 24 h before sacrifice. Levels of BrdU incorporation and Ki-67 positivity were determined in CD4+Foxp3+ CD45.1 WT and CD45.2 IFNAR KO in the spleen. Data are representative of one experiment from at least three independent experiments with five to eight mice per group. Error bars in all panels represent the mean ± SD. Statistical significance was determined using two-way ANOVA. *p < 0.01, **p ≤ 0.001, ***p ≤ 0.0001, ****p < 0.0001.
have 50% of Tregs that use the WT allele and 50% of Tregs that use the Foxp3YFP-cre allele, and thus are marked by YFP in both the thymus and peripheral lymphoid tissues (Fig. 4A and 4B). A marked defect in the development of IFNAR KO (YFP) Tregs in the thymus of IFNARf/f × Foxp3YFP-Cre/WT mice was observed (Fig. 4A). Similarly, in secondary lymphoid tissues, IFNAR KO (YFP) Tregs represented only ~20% of total Tregs as early as 2 mo of age (Fig. 4B). Thus, in this competitive, but physiologically normal, environment, a marked defect in Treg development and/or survival was seen in the absence of type I IFN signaling. Survival of IFNAR KO Tregs in the periphery was also impaired as IFNAR KO (YFP) Tregs had a significantly higher percentage of active pan-caspase-positive cells than WT (YFP) Tregs in the same mouse, and the increase in cell death was also associated with greater level of Bim expression (Fig. 4C). Similar to the results seen in the mixed chimera studies, IFNAR KO Tregs in the heterogeneous female mice displayed a more naive phenotype with lower levels of CD44 and higher levels of CD62L expression and lower levels of CD101 and ICOS expression. Lower levels of CD25 and markedly lower levels of p-STAT5 were also seen in the IFNAR KO Tregs (Fig. 4D). The average MFI expression was significantly different for CD44, CD62L, and p-STAT5, demonstrating a more naive phenotype of IFNAR KO cells in the heterogeneous females (Fig. 4E).

**IFNAR KO Tregs fail to control T cell activation and tissue inflammation in a Scurfy disease model**

We assessed the function of IFNAR KO Tregs in an autoimmune disease model by testing their capacity to rescue the Scurfy disease phenotype. Scurfy mice lack Tregs because of a mutation in foxp3 and are known to develop a fatal lymphoproliferative syndrome with multiorgan inflammation (33, 34). The Scurfy disease phenotype can be induced in adult mice by transfer of fetal liver cells from a day 15 Scurfy fetus into lethally irradiated RAG KO mice. Cotransfer of a source of functional Tregs (e.g., WT BM) controls disease development in this model. We therefore compared the capacity of CD45.1 WT and CD45.1 IFNAR KO BM to prevent disease when cotransferred with CD45.2 scurfy fetal liver cells. After 4 wk of reconstitution, splenocytes were stained for CD44 to measure the overall level of T cell activation. The levels of CD44 expression were high on CD4+ and CD8+ T cells in the group that received Scurfy fetal liver cells alone (Fig. 5A). Reconstitution with WT BM prevented this level of Teff activation, but reconstitution with IFNAR KO BM failed to control the Teff activation. Surprisingly, the absolute numbers of Tregs were similar in the groups reconstituted with WT or IFNAR KO BM (Fig. 5B). Using congenic markers, we were able to distinguish between the effectors derived from the Scurfy and WT or IFNAR KO donors. IFNAR KO Tregs were much less efficient at controlling the expansion of Scurfy cells (Fig. 5C). Mice reconstituted with IFNAR KO BM also exhibited greater levels of inflammation in liver and lung (Fig. 5D). Although this result is consistent with a functional defect of the IFNAR in Tregs, interpretation of the experiment is confounded because the Teffs also lack expression of the IFNAR. To demonstrate a specific defect of the IFNAR in Tregs, we cotransferred Scurfy fetal liver with BM from IFNARf/f × Foxp3YFP-Cre/YFP-cre mice (Fig. 5E). Tregs with a conditional deletion of the IFNAR were also much less efficient in controlling Teff activation in the chimera as measured by CD44 expression, by expansion of CD4+ or CD8+ Teffs, or by suppression of IFN-γ production (Fig. 5F and 5G) even though the absolute number of Foxp3+ T cells was the same in mice reconstituted with WT and IFNAR KO Tregs (Fig. 5H).

**IFNAR KO Tregs fail to phosphorylate STAT5 after exposure to poly(I:C)**

One of the most prominent abnormalities of the IFNAR KO Tregs derived from either mixed BM chimeras or from heterozygous female mice was a decrease in the basal level of p-STAT5 and CD25. It was also of interest to determine whether a similar abnormality existed in the Scurfy treatment model in which the absolute numbers of Tregs were similar in mice reconstituted with WT or IFNAR KO BM or BM from IFNARf/f × Foxp3YFP-Cre/YFP-cre donors both displayed lower levels of p-STAT5 and CD25 when compared with Tregs from mice reconstituted with WT BM (Fig. 6A and 6B).

STAT1 and STAT2 are the most important mediators of the response to type I IFNs. However, type I IFNs can also activate both STAT3 and STAT5 (35–37). We initially assessed the ability of Tregs to directly phosphorylate STAT5 ex vivo after a 15-min stimulation with different concentrations of rIFN-β. We were not able to detect any difference in the levels of p-STAT5 on WT or IFNAR KO Tregs (data not shown). We then directly tested whether type I IFNs might mediate STAT5 phosphorylation in vivo by injecting both WT and IFNAR KO mice with poly(I:C) and measured the levels of p-STAT5 on Tregs 6 h later. Although the basal level of STAT5 phosphorylation was identical in Tregs derived from WT or IFNAR KO mice when injected with PBS (Fig. 7A), a marked increase in p-STAT5 was seen in WT Tregs, but not in IFNAR KO Tregs after injection with poly(I:C) (Fig. 7B). WT, but not IFNAR KO Tregs, were able to phosphorylate STAT1 after poly(I:C) injection. Taken together, these results suggest that under certain conditions in vivo, type I IFNs can augment the ability of Tregs to respond to IL-2, and positively modulate the survival and function of Tregs.

**IFNAR KO Tregs expand after IL-2 immune complex treatment in the thymus, but not in the spleen**

Because the defective homeostasis of IFNAR KO Tregs appeared to be secondary to a decrease in the activation of STAT5, we next evaluated whether treatment with IL-2 immune complexes in vivo could rescue the defective survival of IFNAR KO T cells in WT + IFNAR KO mixed BM chimeras. Mice were treated with IL-2 immune complexes for 5 d (24, 38) and the ratio of WT and IFNAR KO T cells was measured in the thymus and the spleen of the treated mice. Surprisingly, IL-2 complex treatment completely restored the defect in thymic development of not only Tregs, but also resulted in reconstitution of all the other T lymphocyte compartments (Fig. 8A). Although short-term IL-2 immune complex treatment also normalized the percentages of IFNAR KO CD4+Foxp3− and CD8+ T cells in the periphery, it failed to modulate the numbers of IFNAR KO Tregs in the spleen (Fig. 8B). IL-2 immune complex treatment also restored Treg development in the thymus of heterozygous female IFNARf/f × Foxp3YFP-Cre/WT mice (Fig. 8C), but had no effect on IFNAR KO Tregs in the spleen (Fig. 8D). These data demonstrate that treatment with IL-2 complexes can completely restore the developmental defect of IFNAR KO T cells in the thymus but failed to enhance Treg survival in the periphery.

**Discussion**

Previous studies have suggested that the effects of type I IFNs on both conventional T cells and Tregs are complex and potentially context dependent. Although we and others (19) have shown the phenotype and in vitro suppressor function (data not shown) of
Tregs from mice with a global deletion of the IFNAR is normal, it is likely that any defect in Treg function would be offset by the defect in type I IFN signaling in Teffs in IFNAR KO mice. To specifically address the effects of type I IFNs on Tregs, we studied mixed BM chimeras between WT and IFNAR KO mice and heterozygous female mice expressing a Treg-specific deletion of the IFNAR. Taken together, studies in all of these models suggest that under conditions of stress and competition (irradiated chimeras) or competition alone (heterozygous females), IFNAR signaling is required for development of the Treg lineage in the thymus and potentially for survival of Tregs in the periphery.

**FIGURE 4.** IFNAR^fl/fl^ × Foxp3^YFP-cre/wt^ Tregs display the same development and survival defect as IFNAR KO Tregs from the chimeric mice. (A) Thymic and (B) spleen cells were isolated from IFNAR^fl/fl^ × Foxp3^YFP-cre/wt^ and IFNAR^WT/WT^ × Foxp3^YFP-Cre/WT^ female mice, and CD4+ T cells were analyzed for YFP and Foxp3 expression by flow cytometry (left panels). Graph displays the absolute number of Foxp3+ cells (right panel) from YFP^- WT and YFP^+ IFNAR KO. (C) Active pan-caspase activity was measured (left panels) by staining with an allophycocyanin-labeled general caspase inhibitor on YFP^- WT (black dots) and YFP^+ IFNAR KO (white dots) Tregs (each dot represents a single mouse). Relative expression of apoptotic gene Bim in sorted CD4^+CD25^ Tregs from YFP^- WT and YFP^+ IFNAR KO Tregs (right panel) (data are representative of three biological replicates with five mice each time). (D) Expression of activation markers CD44, CD62L, CD69, CD103, CD101, ICOS, CD25, and p-STAT5 on YFP^- WT (shaded histogram) and YFP^+ IFNAR KO (black line) gated splenic CD4^+Foxp3^ T cells. Dashed line is isotype control for p-STAT5 staining. (E) Average MFI expression of different markers of activation on CD4^+Foxp3^ cells from IFNAR^fl/fl^ × Foxp3^YFP-cre/wt^ mice. Data are representative of one experiment from three independent experiments with five to six mice per group. Error bars in all panels represent the mean ± SD. (A–C) Data were analyzed by unpaired Student t tests. **p ≤ 0.001, ****p ≤ 0.0001. (E) Data were analyzed by two-way ANOVA. **p ≤ 0.001, ****p < 0.0001.
FIGURE 5. IFNAR KO Tregs fail to control T cell activation and tissue inflammation in a Scurfy disease model. RAG KO mice were lethally irradiated and reconstituted with Scurfy fetal liver cells alone or with a mixture of Scurfy fetal liver and WT, IFNAR KO, or IFNAR^fl/fl x Foxp3^YFP-Cre/YFP-Cre BM. (A) Spleen cells were isolated from the three chimeras (Scurfy alone, Scurfy+WT, and Scurfy+IFNAR KO), and CD44 expression was measured on CD4+ and CD8+ T cells. (B) Absolute number of CD4^+Foxp3^+ cells from Scurfy+WT and Scurfy+IFNAR KO chimeras. (C) Absolute number of CD4^+Foxp3^+ and CD8^+ Scurfy cells in the three different groups. (D) Representative H&E staining of transverse sections of liver and lung (original magnification ×20; scale bars, 50 μm). (E) Spleen cells were isolated from the three chimeras Scurfy alone, Scurfy+WT, and Scurfy+IFNAR^fl/fl x Foxp3^YFP-Cre/YFP-Cre, and CD44 expression was measured on CD4+ and CD8+ T cells. (F) Total number of CD4^+Foxp3^+ and CD8^+. (G) Spleen cells were stimulated for 4 h, and percentage of IFN-γ+ cells was measured on CD4^+Foxp3^+ and CD8^+ cells. (H) Absolute number of CD4^+Foxp3^+ cells from Scurfy+WT and Scurfy+IFNAR^fl/fl x Foxp3^YFP-Cre/YFP-Cre chimeras. Data are representative of one experiment from at least three independent experiments with five mice per group. Error bars in all panels represent the mean ± SD. Statistical significance was determined using two-way ANOVA. *p < 0.01, **p ≤ 0.001, ***p > 0.0001, ****p < 0.0001.
Although some of our data are compatible with a requirement for type I IFN signaling only for the development of Tregs in the thymus, our analysis of the phenotype of the IFNAR KO peripheral Tregs suggests an additional role for signaling via the IFNAR for survival in the periphery in a competitive environment. First, surviving IFNAR KO Tregs in both the mixed chimeras and the heterozygous females express high active pan-caspase and the proapoptotic factor, Bim, consistent with a propensity of the IFNAR KO T cells to undergo apoptotic cell death. More importantly, the surviving IFNAR KO Tregs display a less activated phenotype that is markedly different from the subsets of Tregs present in normal mice. A number of studies (39, 40) have suggested that Tregs can be divided into two phenotypically distinct subpopulations based on the differential expression of CD44 and CD62L: “naive-like” Tregs (CD44hiCD62Llo) and “effector memory” Tregs (CD44loCD62Lhi). Other groups have termed these subsets central Tregs and effector Tregs (41, 42). In addition to CD44 and CD62L, a number of other markers can be used to characterize these two populations. The naive Tregs express higher levels of CD25 and Bcl2 and higher levels of p-STAT5 (42), whereas the effector Tregs are undergoing cell cycling and express higher levels of several activation Ags, including CD69, ICOS, PD-1, and CD103. The IFNAR KO Tregs isolated from the competitive environments in many respects resemble the naive Treg subpopulation, with the notable exception being that they express lower levels of CD25 and p-STAT5. It has recently been demonstrated that TCR signals are required for the transition from the naive Treg state to the effector Treg state, and that only effector Tregs manifest suppressor function in vivo (43). Our data suggest that in the absence of type I IFN signaling, naive Tregs cannot respond to TCR signaling and transition to the fully activated suppressor-effector Treg, or die when they become activated.

In general, type I IFNs signal via STAT1 or STAT2; however, other studies (36, 37, 44) suggest that type I IFNs signal via STAT3 or STAT5. The decreased expression of CD25 and p-STAT5 on the surviving IFNAR KO Tregs in our chimera and heterozygous female models raised the possibility that one role of type I IFNs was to complement the prosurvival effects of IL-2 in certain situations by signaling through STAT5 directly or by increasing the ability of Tregs to respond to IL-2, resulting in enhanced activation of the STAT5 signaling pathway. Indeed, we could directly demonstrate that injection of poly(I:C) into WT but not IFNAR KO mice resulted in induction of p-STAT5. As an alternative approach, we tested whether short-term administration of IL-2 immune complexes would restore Treg homeostasis. Surprisingly, IL-2 treatment of the WT + IFNAR KO chimeras completely restored the developmental defect of all thymic lineages and restored peripheral CD4+ and CD8+ percentages to that seen in WT + WT chimeras, but had no effect on the defect in Treg percentages in the periphery. Similar results were observed in heterozygous females, although the reconstitution of Treg development in the thymus was less complete. It is unclear whether our failure to enhance Treg numbers in the periphery is secondary to the short course of therapy we used or whether type I IFNs impart a prosurvival effect in addition to that mediated by IL-2.

Absence of the IFNAR on Tregs had no effect on the in vitro suppressive function of Tregs (unpublished observations). We therefore used what we believe is a very sensitive model to assay for a defect in the suppressive function of Tregs by generating mixed chimeras between Scurfy fetal liver and WT, IFNAR KO, or Treg conditional IFNAR KO BM. IFNAR KO Tregs were much less efficient in their ability to suppress Scurfy cells than Tregs derived from WT BM, but certainly had some suppressive capacity because the chimeras did not develop as severe of a disease as that seen in Scurfy mice. The defective function of the Tregs in the Scurfy + IFNAR KO chimeras was not secondary to a developmental defect.
of the Treg lineage as the absolute numbers of Tregs were identical in Scurfy + WT and Scurfy + IFNAR KO chimeras. It is likely that the inflammatory environment in these chimeras, including the production of IL-2, compensated for some of the defects present in the development and/or survival of the IFNAR KO Tregs. These results are consistent with the observation that Tregs from IFNAR KO mice failed to protect mice from the development of colitis when cotransferred with CD45RBhi cells into RAG1 KO recipients, and that the administration of type I IFNs reduced colitis by increasing the number of Foxp3+ Tregs (19). In contrast, we (unpublished observations) and Kole et al. (20) have shown that IFNAR KO Tregs were able to protect mice from developing IBD in the same experimental model. The difference between these studies may be explained by differences in endogenous commensal bacteria and a high level of stress induced by inflammation in the studies of Lee et al. (19). Indeed, in our animal facility, which is identical to that used by Kole et al. (20), we failed to observe induction of Mx-1 gene expression after transfer of CD45RBhi cells into RAG KO recipients, suggesting that type I IFNs are not induced under our experimental conditions, and hence play no role in Treg function in IBD in our animal facility.

Collectively, our studies and the study of the effects of type I IFNs in a tumor model (22) support the concept that type I IFNs primarily promote Treg suppressor function. The one experimental model in which type I IFNs appear to abrogate Treg suppressor function is acute LCMV infection (23). In this model, a marked depletion of Tregs was observed between days 2 and 7 postinfection, and this depletion of Tregs was required for optimal CD4+ and CD8+ T responses to the virus. The addition of type I IFN also reduced the suppressor function of Tregs in the standard coculture assay, although this effect appeared to be secondary to the death of the Tregs rather than a modulatory effect on a known suppressor mechanism. The one major difference between the studies of Srivastava et al. (23) and our studies is that this group observed equal reconstitution of WT and IFNAR KO Treg populations in mixed BM chimeras. Although they used TCR-b/d KO mice as recipients of the BM, whereas we used RAG KO mice, it is unlikely that this could account for the differences between the two studies. Furthermore, our results in unmanipulated heterozygous female mice with a Treg-specific deletion of the IFNAR are identical to the results we observed in the mixed BM chimeras. More importantly, it appears that the target Treg subpopulation that was susceptible to the effects of type I IFNs was different in the two studies. As noted earlier, we believe our data indicate a requirement for type I IFN signaling in the maturation of Tregs from the naive state to the activated effector state. In contrast, in the studies of Srivastava et al. (23), the population targeted by type I IFNs was the activated, cycling CD44hi effector Treg. Thus, the effects of type I IFNs on Tregs may be Treg subset specific.

Our findings could have important therapeutic implications for a variety of diseases. IFN-β has been one of the major therapies for MS, and numerous mechanisms have been proposed for its therapeutic effects including a shift in cytokine balance from a Th1 to a Th2 response (45). However, in EAE, the animal model of MS, the major protective effects of IFNAR signaling were mediated by the IFNAR expressed on myeloid cells and not on B or
T lymphocytes (46). In preliminary studies we have observed that mice with a Treg-specific deletion of the IFNAR are highly susceptible to EAE induced by MOG35–55 and frequently succumb from disease by day 15 postinduction. Lastly, two recent studies have demonstrated that inhibition of IFNAR signaling either genetically or by Ab blockade in chronic infection with LCMV led to a substantial reduction in virus titers and enhanced antiviral CD4+ T cell responses (47, 48). Although the cellular target for the negative immunoregulatory effects of IFN in the chronic infection model were not determined, one possibility that is consistent with our results is that IFN in chronic viral infection potentiates Foxp3+ Treg function. Taken together, the role of type I IFNs in differentially regulating the function of both Teffs and Tregs is certainly context dependent, and careful studies will be needed to determine the optimum situations for the positive or negative manipulation of IFNAR signaling in T cells in different diseases.

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Disclosures

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