

IFN- β Selectively Inhibits IL-2 Production through CREM-Mediated Chromatin Remodeling

Dennis C. Otero,* Nancy J. Fares-Frederickson,* Menghong Xiao,* Darren P. Baker,[†] and Michael David*[‡]

IFN- β is widely used in the treatment of multiple sclerosis, yet the mechanism facilitating its efficacy remains unclear. IL-2 production by activated T cells, including those mediating autoimmunity, and subsequent autocrine stimulation is vital for T cell expansion and function. In this study, we demonstrate that in mouse and human T cells, IFN- β specifically inhibits the production of IL-2 upon TCR engagement without affecting other cytokines or activation markers. Rather than disrupting TCR signaling, IFN- β alters histone modifications in the IL-2 promoter to retain the locus in an inaccessible configuration. This in turn is mediated through the upregulation of the transcriptional suppressor CREM by IFN- β and consequent recruitment of histone deacetylases to the IL-2 promoter. In accordance, ablation of CREM expression or inhibition of histone deacetylases activity eliminates the suppressive effects of IFN- β on IL-2 production. Collectively, these findings provide a molecular basis by which IFN- β limits T cell responses. *The Journal of Immunology*, 2015, 194: 000–000.

Type I IFNs (i.e., IFN- α/β) have been approved worldwide for the treatment of multiple sclerosis (MS), yet the mechanism(s) behind their effectiveness has remained elusive. Treatment with IFN- α/β reduces the frequency of relapses and slows the progress of disability associated with the disease; nevertheless, some patients fail to respond (1). Thus, a better understanding of the mechanism behind the efficacy of IFN- β is vital to improve treatment strategies. Type I IFNs have been studied extensively in the context of viral or bacterial infection as part of the innate immune response, but it is only recently that the importance of these cytokines in the adaptive immune response has been more fully appreciated (2–5). Type I IFNs exert strong antiproliferative effects on lymphocytes, thus limiting immune responses by controlling the number of responding cells and attenuating the activity of individual T cells (6, 7). T helper lymphocytes, which play a key role in the development of MS, proliferate in response to Ag by producing IL-2 that subsequently acts in an autocrine positive feedback loop. Surprisingly, the effects of type I IFNs on IL-2 production by activated T cells has not been evaluated previously.

In this study, we investigate IL-2 production from T cells that have been exposed to type I IFNs in vitro and in vivo. Our data reveal a novel pathway by which IFN- α/β inhibit gene expression at the epigenetic level and implicate the involvement of CREM in this process. We provide a possible mechanism by which IFN- β

functions to control MS and a possible reason for the occurrence of T cell exhaustion following virus infection.

Materials and Methods

Animals

STAT1^{-/-} (8), Tyk2^{-/-} (9), STAT5^{-/-} (10), and STAT3^{fl/fl} (11) mice have been described previously. Wild type 129SvEv, C57BL/6, and BalbC mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were between 6 and 12 wk of age at the time of the experiments. All mice used in these experiments were housed in a pathogen-free environment and were bred and cared for in accordance with University of California, San Diego Animal Care Facility regulations. Six- to ten-week-old mice were infected intravenously with 2×10^6 PFU/mouse wild type (WT) lymphocytic choriomeningitis virus (LCMV) clone 13 (Cl13). All viruses were grown, identified, and quantified as described previously (12).

Flow cytometric analysis

For immunostaining, single-cell suspensions were prepared from mouse spleen with $\sim 1 \times 10^6$ cells suspended in FACS buffer (PBS pH 7.4, 1% FCS, 0.02% NaN₃) and stained for 20 min in the dark on ice. Mouse Abs, FITC-anti-CD4 (GK1.5), PE/Cy7-anti-CD8 (53.6.7), and PE-anti-IL2 (JE56-5H4), Biotin-anti-CD44 (PGp-1), and PE-anti-CD25 (PC61.5) were obtained from eBioscience (San Diego, CA), as well as PE-anti-human IL-2 (MQ1-17H12), and FITC-anti-human CD3 (OKT3). APC-streptavidin was used as a secondary reagent to detect biotin-labeled mAbs. All samples were analyzed on a FACSCalibur (BD Biosciences) and processed using FlowJo software (Ashland, OR). Intracellular staining was completed using the Intracellular Fixation and Permeabilization Buffer with Brefeldin A (eBioscience) according to the manufacturer's directions. Intracellular calcium levels were monitored by flow cytometry after loading cells with Fluo-4 AM and Fura Red (Invitrogen), and data represent the ratio of the signal for each. CD4⁺ T cells were treated for 16 h with IFN- β (Biogen Idec, Cambridge, MA) prior to dye loading and then washed, and a baseline reading was taken for 30 s. Hamster anti-CD3 (eBioscience) was added at 10 μ g/ml, and data were collected for 1 min prior to the addition of 25 μ g/ml donkey anti-hamster IgG (eBioscience). Readings were continued for a total of 5 min.

T cell stimulation

Splenic T cells or purified CD4⁺ T cells (*Pan* T cell isolation kit, CD4⁺ CD25⁺ Regulatory T Cell Isolation Kit; Miltenyi Biotec) were treated with the indicated concentrations of IFN- β (Biogen Idec) for 16 h or as indicated prior to stimulation with either 10 μ g/ml anti-CD3 and 2 μ g/ml anti-CD28 (eBioscience) or 5 ng/ml PMA and 500 ng/ml Ionomycin (Sigma-Aldrich) for 3 or 6 h. Cells were analyzed for IL-2 protein by intracellular stain or RNA by reverse transcription and quantitative PCR.

*Division of Biological Sciences, University of California San Diego, La Jolla, CA 92093; [†]Biogen Idec, Cambridge, MA 02142; and [‡]UC San Diego Moores Cancer Center, University of California San Diego, La Jolla, CA 92093

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Address correspondence and reprint requests to Dr. Michael David, Division of Biological Sciences, University of California San Diego, Bonner Hall 3138, 9500 Gilman Drive, La Jolla, CA 92093-0322. E-mail address: midavid@ucsd.edu

Abbreviations used in this article: Cl13, clone 13; ISG15, IFN-stimulated gene 15; LCMV, lymphocytic choriomeningitis virus; MS, multiple sclerosis; siRNA, small interfering RNA; Treg, regulatory T cell; WT, wild type.

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Mouse IL-2 Ready-Set-Go Kit (eBioscience) was used to measure IL-2 released into the conditioned T cell culture medium. Human PBLs were stimulated with Dynabeads Human T cell activator CD3/CD28 (Invitrogen). Trichostatin A (Sigma-Aldrich) was added to cultures 1 h prior to stimulation with anti-CD3/CD28. For *in vivo* stimulations, OTH TCR transgenic mice were injected *i.v.* with 100 μ g Ova₃₂₃₋₃₃₆ peptide (Anaspec) 24 h after injection with 10,000 U IFN- β (Biogen Idec). Splenic T cells were removed 4 h after peptide injection and were subjected to intracellular staining for IL-2 and analyzed by flow cytometry.

Quantitative PCR

DNase-treated RNA was isolated from activated T cells with the RNeasy method (Qiagen). cDNA was prepared with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and quantitative PCR was performed using Taqman primers for mouse and human IL-2, mouse IL-4, and mouse IFN- γ (Applied Biosystems). Analysis was performed with a Step One Plus real time PCR system (Applied Biosystems). mRNA abundance was determined by relative quantification and normalized to GAPDH.

Western blot

Splenic T cells were treated as above but then stimulated with 10 μ g/ml anti-CD3 for 2, 5, 20, and 60 min. Cells were lysed and subjected to SDS-PAGE and Western blot. Blots were probed for phospho-p44/42, Phospho-cJun, Phospho-p38, total p38, and total cFos (Cell Signaling).

Chromatin accessibility by real-time PCR assay

Chromatin accessibility by real-time PCR assay was performed as described (13). Briefly, 1×10^7 cells per sample were stimulated for 2 h with 10 μ g/ml anti-CD3 and 2 μ g/ml anti-CD28 following 16 h pretreatment with 1000 U/ml IFN- β (Biogen Idec). Nuclei were isolated and subsequently digested with micrococcal nuclease and then, after DNA purification, real-time PCR was used to determine relative amounts of specific sequences within the IL-2 promoter. Primer sequences were used as published: set B forward: 5'-CACAGGTAGACTCTTTGAAAATATGTGTAA-3'; reverse: 5'-CATGGGAGGCAATTTACTGTTAATG-3'; set C forward: 5'-CCTAAATCCATTCAGTCAGTGTATGG-3'; reverse: 5'-TGTGTGGCAGAAAGCATTACCT-3'; set D forward: 5'-CTTTTGTGTCTCCACCCAAA-3'; reverse: 5'-CACACTTAGGTGGCAGTTTAATTCAT-3'; set F forward: 5'-CATGCAGAGTTTTTGTGTTTCTAG-3'; reverse: 5'-GCCTAAAGTCTCTCACAAAGAACAGA-3'. Change in accessibility for individual primer sets was calculated as $1 - (\text{Stimulated}/\text{Unstimulated})$.

Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed using the EpiTect ChIP OneDay Kit (Qiagen) as described using anti-histone H3 (acetyl K14) and anti-Histone H4 (acetyl K8; Abcam). Immunoprecipitations were analyzed with quantitative PCR using primer B as in the Chromatin accessibility by real-time PCR assay.

Small interfering RNA knockdown

Purified CD4⁺ T cells were transfected by electroporation with either 25 nM CREM small interfering RNA (siRNA) or control siRNA (Thermo Scientific Dharmacon) and rested for 4 h prior to treatment with IFN- β . After 16 h, cells were stimulated with anti-CD3/CD28 for 3 h, RNA was isolated, and quantitative PCR was used to determine IL-2 message levels. Percent inhibition was calculated as $[(\text{Stimulated} - \text{Inhibited})/\text{Stimulated}] \times 100$. Student *t* test was used to determine significance.

Results

IFN- β inhibits activation-induced IL-2 production in T cells

Signaling through the TCR in conjunction with costimulation through CD28 results in the activation of T cells. One of the earliest highly induced genes following T cell activation is IL-2, which acts in an autocrine fashion to promote the proliferative expansion of Ag-specific cells (14). As IFN- β is a strong inhibitor of T cell proliferation, we chose to investigate the effects of IFN- β on IL-2 production in T cells. Upon stimulation with agonistic anti-CD3 and anti-CD28 Abs, splenic T cells of WT 129SvEv mice produce IL-2 within 5 h, as detected by intracellular flow cytometry staining (Fig. 1A, *second panel*). However, upon pretreatment with IFN- β , the number of IL-2-producing cells is significantly

reduced (Fig. 1A, *third panel*), an effect specific to IFN- α/β , as the type II IFN, IFN- γ , had no effect on IL-2 production (Fig. 1A, *right panel*). In addition, IL-2 production was inhibited in CD4⁺ (Fig. 1A, *top row*) and CD8⁺ (Fig. 1A, *bottom row*) T cells, although the latter do not produce significant amounts of IL-2 upon stimulation. The effect was not unique to anti-CD3/CD28 stimulation, as IFN- β also inhibited the production of IL-2 when T cells were stimulated with the mitogens PMA and ionomycin (Fig. 1B) or Con A (not shown). Inhibition of IL-2 release by IFN in a dose-dependent manner into the culture media of splenic T cells stimulated with anti-CD3/CD28, PMA/ionomycin, or Con A was further corroborated by measuring the IL-2 concentration with ELISA analysis of the conditioned medium (Fig. 1C and data not shown). Additional experiments revealed that IFN- β -mediated inhibition of IL-2 production occurred on the transcriptional level, as IL-2 mRNA was also reduced because of IFN- β exposure prior to stimulation (Fig. 1D). Strikingly, the inhibitory effect of IFN- β was restricted to IL-2, as neither IL-4 (Fig. 1D) nor IFN- γ (not shown) mRNA, nor expression of the cell surface activation markers CD25 and CD44 (Fig. 1E), were subdued by IFN- β pretreatment.

Activated T cells often exhibit significant differences in their response to type I IFNs as compared with naive T cells (15). We therefore investigated next whether IFN- β could inhibit IL-2 production in already activated T cells. CD4⁺ T cells were stimulated for 3 d with anti-CD3/CD28 (Fig. 2A, *right panels*) and then incubated overnight with (Fig. 2A, *bottom panels*) or without (Fig. 2A, *top panels*) 1000 U/ml IFN- β . Subsequently, cells were stimulated with PMA/ionomycin for 5 h. IFN- β significantly reduced the number of IL-2-producing cells that were naive at the time of stimulation (Fig. 2A, *middle plots*), but failed to inhibit IL-2 production if the cells were preactivated for 3 d with anti-CD3/CD28 (Fig. 2A, *right panels*). This finding indicates that once IL-2 transcription is activated, it can no longer be suppressed by IFN- β .

As ongoing IL-2 production in already activated T cells was resistant to suppression by IFN- β , we wanted to determine the window of opportunity for IFN- β to inhibit IL-2 production through pretreatment of T cells with IFN- β prior to stimulation. To this end, splenic T cells were stimulated with anti-CD3/CD28 after 12, 6, or 3 h of IFN- β exposure, respectively. In addition, IFN- β was added at the time of stimulation (0 h), or 3, 6, and 12 h after TCR engagement. Cultures were maintained for an additional 36 h, and IL-2 was measured in the conditioned medium by ELISA. As shown in Fig. 2B, the addition of IFN- β simultaneously or after TCR stimulation had little effect on IL-2 production; however, when T cells were pretreated with IFN- β for as little as 3 h prior to stimulation, a dramatic decrease in the amount of IL-2 was registered. This result strongly suggested that new gene expression and protein synthesis were required for inhibition of IL-2 production by IFN- β . Unfortunately, experiments using cycloheximide were inconclusive because of the sensitivity of primary T cells to the agent (data not shown).

IFN- β inhibits IL-2 production independent of regulatory T cells and occurs in human PBLs

As all previous experiments were conducted with either total splenic T cell populations or the purified CD4⁺ subset, the possibility existed that regulatory T cells (Tregs) were required for IFN- β to inhibit IL-2 production. Indeed, it was shown recently that IFN is required for maintaining Foxp3 expression in Tregs during infection (16). Therefore, we decided to eliminate Tregs from the CD4⁺ T cell cultures prior to IFN- β exposure. Although Tregs alone had, as expected, an intrinsic inhibitory effect on IL-2 production by CD4⁺ cells (Fig. 3A, compare black bars), IFN- β

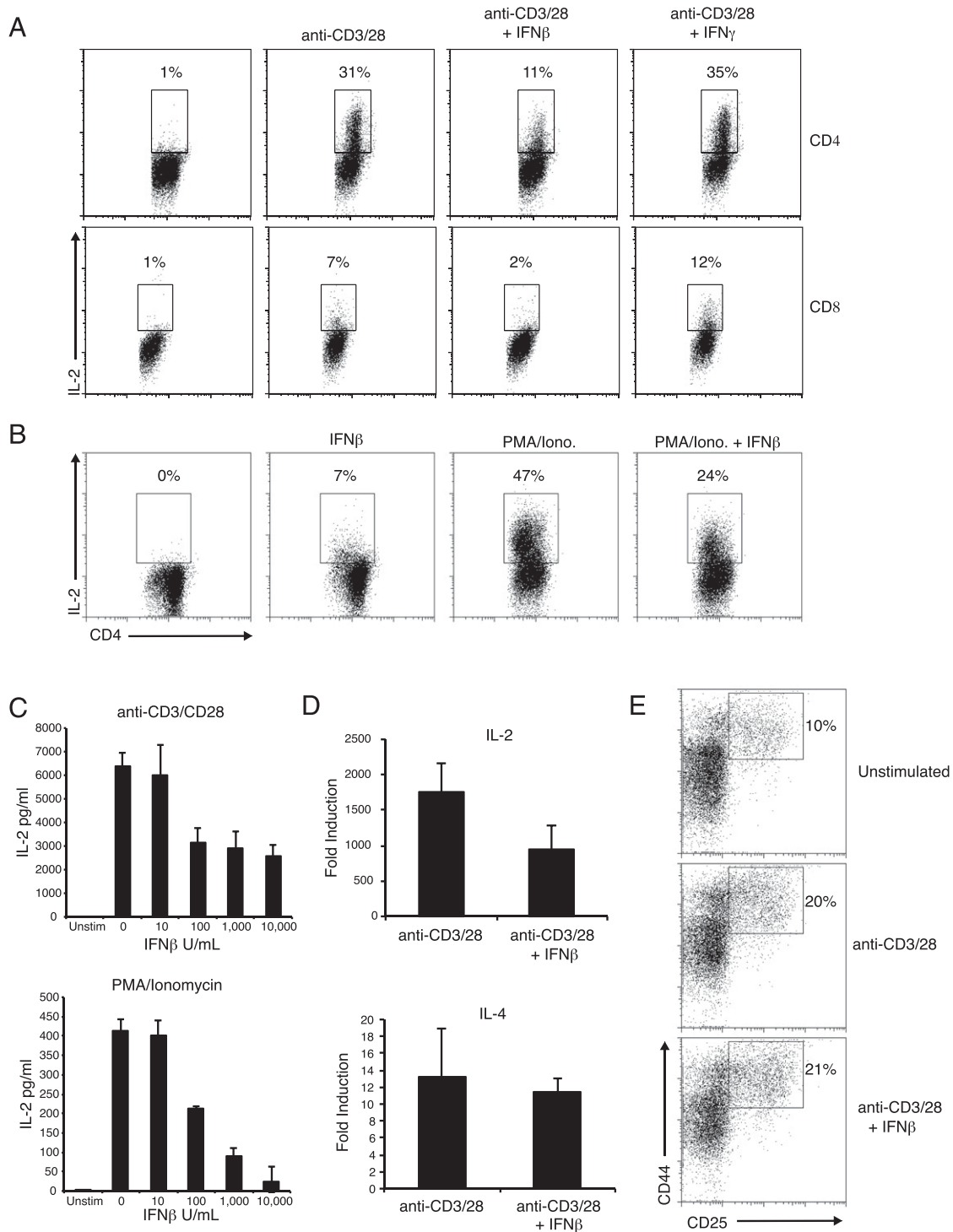


FIGURE 1. Specific inhibition of IL-2 expression by IFN- β . **(A)** Splenocytes from WT 129/SvEv mice were treated with or without IFN- β or IFN- γ for 16 h and then stimulated with 10 μ g/ml immobilized anti-CD3 and 2 μ g/ml anti-CD28 for 5 h followed by intracellular staining for IL-2. Upper plots were gated on live CD4⁺ cells, and lower plots were gated on live CD8⁺ cells; they are representative of at least five experiments. **(B)** Splenocytes from WT 129/SvEv mice were treated for 16 h with IFN- β and stimulated with PMA/ionomycin for 5 h followed by intracellular stain for IL-2. Plots are gated on live CD4⁺ cells. **(C)** Splenocytes were stimulated with the indicated mitogens and cultured for 48 h in the presence of increasing doses of IFN- β . IL-2 in the conditioned medium was quantified by ELISA ($n = 3$). **(D)** CD4⁺ splenocytes were treated for 16 h with IFN- β prior to stimulation with anti-CD3/anti-CD28. Six hours later, RNA was purified and quantitative PCR was used to measure IL-2 and IL-4 mRNA levels ($n = 3$). **(E)** CD4⁺ splenocytes were treated as in (C), stained for CD25 and CD44 expression and analyzed by flow cytometry.

was nevertheless still capable of significantly inhibiting IL-2 production, even in the absence of Tregs (Fig. 3A, compare gray bars). We therefore concluded that IFN- β acts on the responding effector T cells directly to inhibit IL-2 production independent of Tregs.

To address whether the inhibitory effect of IFN- β on IL-2 production could also be seen in human cells, we used human PBMCs to analyze IL-2 production by flow cytometry and IL-2 mRNA expression in response to anti-CD3/CD28 in the absence or presence of human IFN- β . Similar to their murine counterparts,

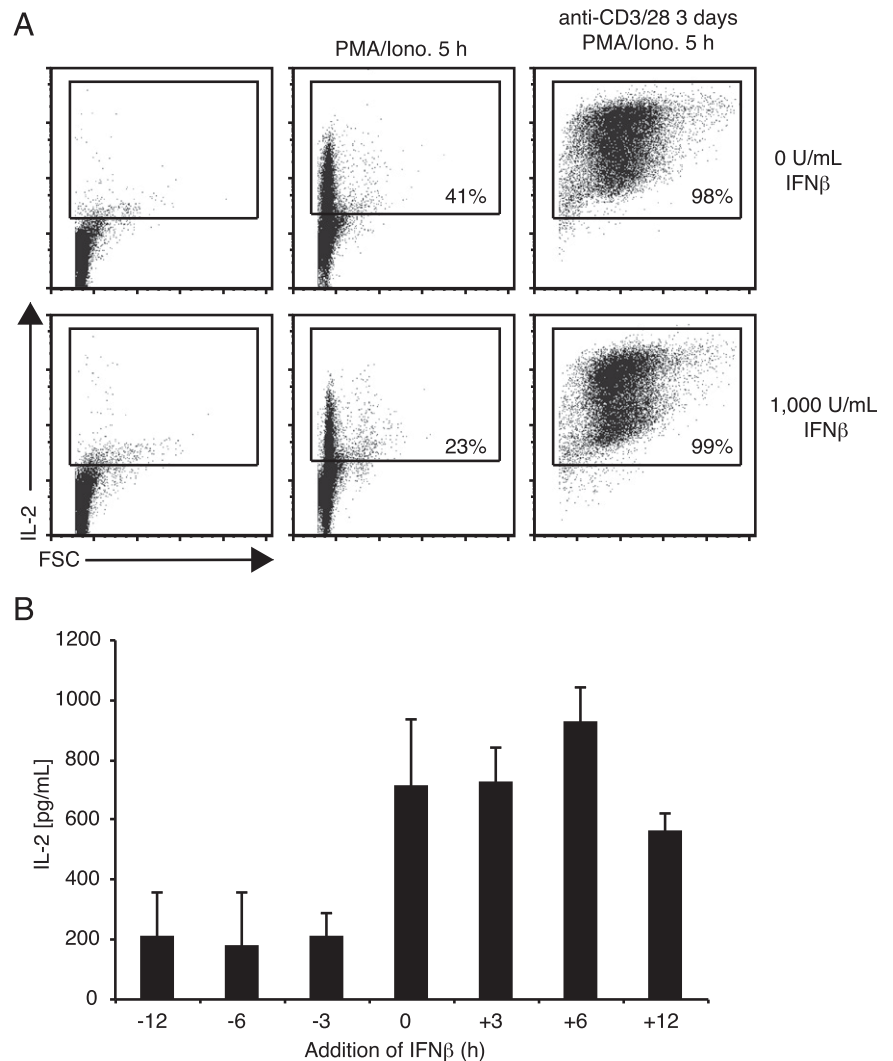


FIGURE 2. Inhibition of IL-2 expression requires pretreatment with IFN- β . **(A)** Purified CD4⁺ splenic T cells were treated with IFN- β for 16 h (*lower plots*) before (*middle plots*) or after (*right plots*) 3 d in culture with anti-CD3 and anti-CD28 and then restimulated with PMA/ionomycin for 5 h followed by intracellular stain for IL-2. **(B)** Splenocytes were cultured for a total of 4 d following stimulation with anti-CD3 and anti-CD28. IFN- β was added up to 12 h before or 12 h after stimulation. The amount of IL-2 in the conditioned medium was measured with ELISA ($n = 3$).

human T cells were inhibited in their ability to synthesize IL-2 when previously exposed to IFN- β (Fig. 3B, 3C). Thus, IFN- β acts directly on human and murine T cells to inhibit activation-induced IL-2 production.

IFN- β -mediated inhibition of IL-2 expression requires signaling through the type I IFNR but does not alter TCR signaling

To determine whether the inhibitory effect of IFN- β on IL-2 production required known components of the canonical type I IFN signaling pathway, we used STAT1-deficient mice that display severely impaired IFN responses. The role of STAT1 in murine T cells is well documented (2, 3, 8, 17), and we previously reported that the absence of STAT1 in T cells results in an unexpected mitogenic response to IFN (18). This report is corroborated in Fig. 4A, (left panels), wherein IFN- β inhibited IL-2 production in WT T cells, but strikingly caused a significant increase in IL-2-producing cells (Fig. 4A, *middle panels*) and the total amount of IL-2 produced in the absence of STAT1 (data not shown). Furthermore, IL-2 production was unaffected by IFN- β pretreatment of Tyk2^{-/-} T cells, whereas IL-2 release from T cells isolated from Cre^{Lck}/STAT3^{loxP} and STAT5-deficient mice was still inhibited by IFN- β pretreatment (Fig. 4A, *right panels*).

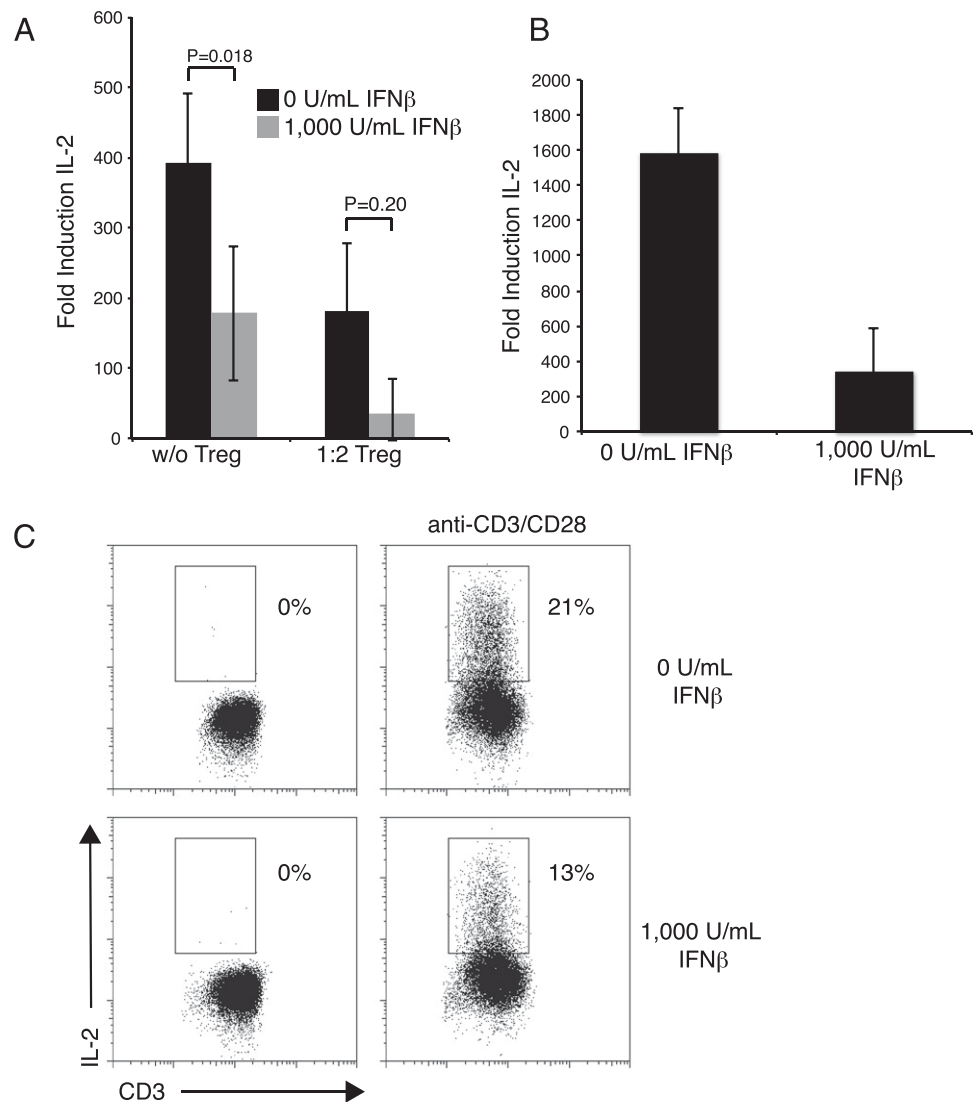
IL-2 induction requires engagement of the TCR along with stimulation through the coreceptor CD28, which together activate multiple downstream signaling pathways including the MAPKs,

NF- κ B, PLC γ , and increases in cytoplasmic free calcium (19, 20). Because IFN- β inhibited IL-2 production even in response to stimulation with PMA and ionomycin, we concluded that TCR proximal signaling events are likely not compromised by IFN- β . Indeed, phosphorylation of p42/44 MAPKs, the stress-activated protein kinase p38, and JNK is not affected by IFN- β pretreatment (Fig. 4B), nor is there a reduction in cFos levels (Fig. 4B) or an impairment of NF- κ B signaling (data not shown) after IFN- β treatment. In concurrence, there were also no differences in the increase of cytoplasmic free calcium after TCR stimulation in the presence of IFN- β (Fig. 4C). Furthermore, the use of various luciferase reporter constructs representing the major enhancer elements within the IL-2 promoter (e.g., NF- κ B, NFAT, AP-1) (21) did not indicate any interference of IFN- β on this level (data not shown). Thus, IFN- β does not attenuate IL-2 production through interference with TCR signaling or activation of the major transcription factors that control IL-2 transcription. These facts are also in line with the observation that IFN- β selectively targets IL-2 without affecting the induction of other cytokines or activation markers.

IFN- β induces changes in chromatin remodeling of the IL-2 locus through induction of Crem

Gene expression depends on changes in the chromatin structure at the specific gene locus which, in turn, is regulated by posttranslational modifications of histones or the DNA itself, or both,

FIGURE 3. IFN- β inhibition of IL-2 expression is independent of Tregs and is reproduced in human peripheral blood leukocytes. **(A)** CD4⁺ T cells were depleted of CD25⁺ Tregs and then treated for 16 h with IFN- β , followed by stimulation with anti-CD3/anti-CD28 for 4 h in the presence or absence of CD25⁺ Tregs. IL-2 mRNA was measured with quantitative PCR ($n = 3$). **(B)** Human PBLs were stimulated with anti-CD3/anti-CD28 Dynabeads with and without IFN- β pretreatment, and RNA was collected after 3 h stimulation and analyzed for IL-2 message by quantitative PCR ($n = 3$). **(C)** Human PBLs were stimulated as in (B), except that cells were stained for intracellular IL-2 after 5 h (representative of five experiments).



primarily through acetylation, methylation, or both. These epigenetic changes result in the accessibility of the gene only to site-specific transcription factors and the basal transcription machinery. To determine whether IFN- β pretreatment would influence the changes in chromatin structure elicited in the IL-2 locus after TCR stimulation of CD4⁺ T cells, we used a chromatin accessibility assay using micrococcal DNase treatment of nuclei from anti-CD3/CD28-stimulated cells with and without IFN- β pretreatment. Changes in the amount of recovered DNA reflect whether a particular locus is in a closed formation or is open to DNase digestion (22). Purified CD4⁺ T cells were stimulated with anti-CD3/CD28 for 4 h with or without prior exposure to IFN- β . We used several primer sets within the 300 bp IL-2 promoter (Fig. 5A, sets B, C, and D) to determine its accessibility and a control primer set distant from the IL-2 locus (Fig. 5A, set F) as a control.

We observed that with IFN- β treatment alone, the promoter presented in the same closed configuration as in unstimulated cells, whereas anti-CD3/CD28 treatment led to a substantial increase in the accessibility of the IL-2 locus. Notably, IFN- β pretreatment significantly reduced the anti-CD3/CD28 induced change in the accessibility of the IL-2 locus (Fig. 5B). Because changes in chromatin structure are regulated by histone modifications, we next determined the acetylation status of histones within the IL-2 promoter. As shown in Fig. 5C, chromatin immunoprecipitation using Abs specific for acetylated histone 3 or

acetylated histone 4 revealed a clear increase of these posttranslational modifications within the IL-2 promoter after TCR engagement or PMA/ionomycin stimulation. In accordance with the chromatin accessibility studies, pretreatment of the cells with IFN- β negated the TCR-induced histone modifications (Fig. 5C). These findings suggested that IFN was either preventing acetylation of the histones within the IL-2 promoter or promoting their deacetylation via recruitment of histone deacetylases. To distinguish these possibilities, we chose to use the histone deacetylase inhibitor trichostatin A to test its influence on the inhibitory effect of IFN on IL-2 production. Indeed, we found that with increasing doses of trichostatin A, the amount of IL-2 produced after TCR engagement increased (Fig. 5D, left panel). More importantly, at higher concentrations trichostatin A completely abrogated the inhibitory effect of IFN- β on IL-2 production (Fig. 5D, right panel; average of four separate experiments). The cumulative interpretation of these studies infers that IFN- β is selectively recruiting histone deacetylases to the IL-2 locus to maintain it in a closed, transcriptionally inactive configuration.

Histone acetylation is an important regulator of gene expression following TCR stimulation, and several negative regulatory factors have been shown to recruit deacetylases to the IL-2 locus. These factors include the zinc finger transcription factors Aiolos and Ikaros, as well as Blimp and CREM, all of which act in a negative feedback loop to silence IL-2 expression (23–27). Our inves-

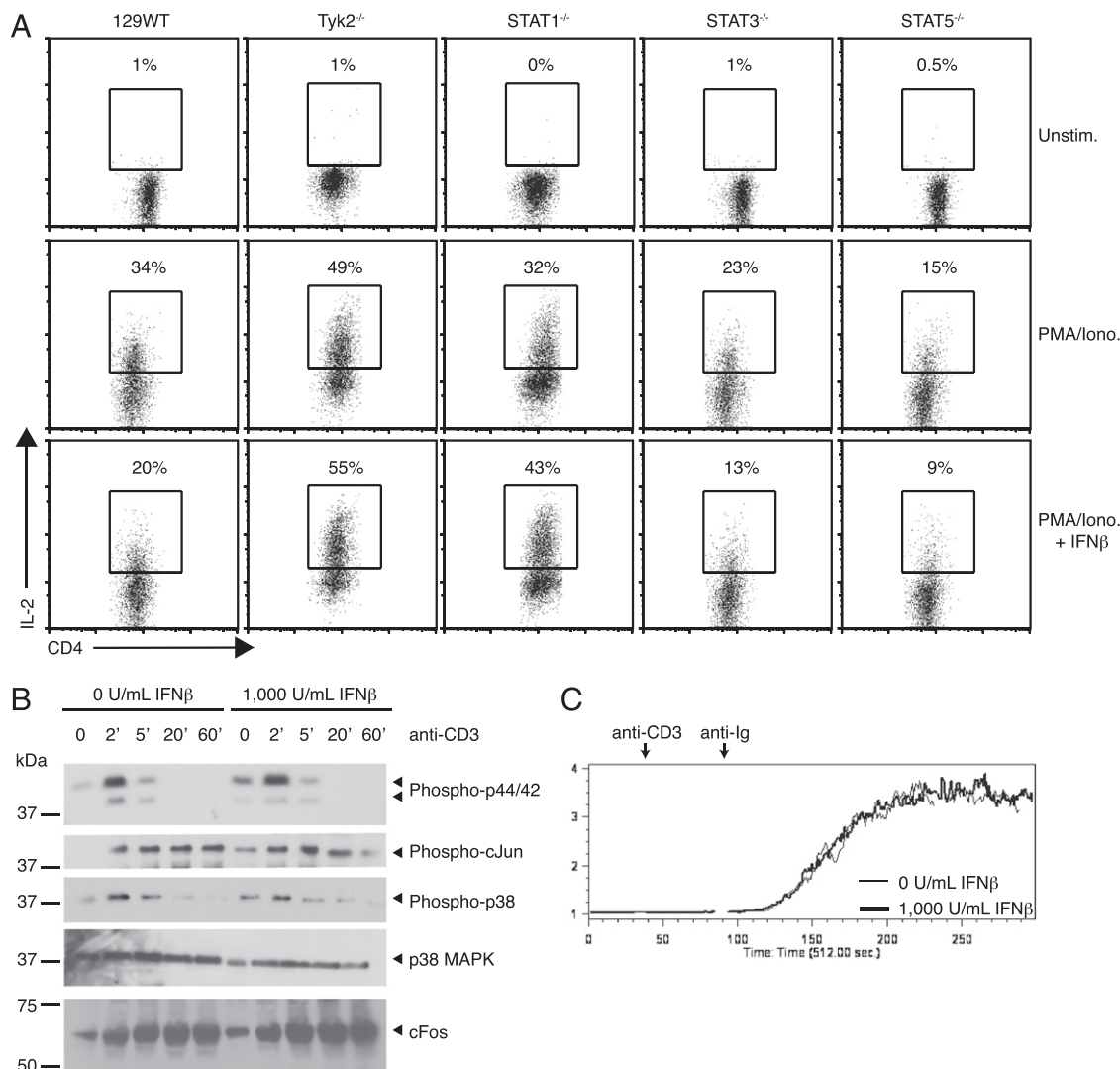


FIGURE 4. Inhibition of IL-2 by IFN- β in naive T cells is dependent on STAT1, but does not affect proximal signaling through the TCR. **(A)** Splenocytes from WT and STAT1^{-/-}, Tyk2^{-/-}, STAT3^{-/-}, and STAT5^{-/-} mice were treated with IFN- β for 16 h and then stimulated with PMA/ionomycin for 5 h followed by intracellular IL-2 stain. Plots are gated on CD4⁺ cells. Representative of three to five mice each. **(B)** Splenic T cells were treated for 16 h with IFN- β and then stimulated with anti-CD3/CD28 for the indicated time. Western blots of whole cell lysates were performed and probed for the indicated phosphorylated proteins. **(C)** Splenic T cells, treated for 16 h with IFN- β and then loaded with Fluo-4 and Fura Red, were run on flow cytometer for 30 s to establish baseline, and then stimulated with anti-CD3. After 1 min, a crosslinking Ab was added to cells to induce calcium flux (representative of at least five experiments).

tigations did not reveal any involvement of Aiolos, Ikaros, or Blimp1 in the inhibitory effects of IFN- β on IL-2 production (data not shown). However, analysis of microarray data from anti-CD3/CD28-stimulated CD4⁺ T cells with and without prior IFN- β exposure revealed that CREM was significantly upregulated in these T cells by IFN- β . This finding was confirmed by Western blot analysis, where there was a dramatic induction of CREM protein expression in response to 16 h IFN- β treatment (Fig. 5E). Analysis with quantitative PCR (Fig. 5F) revealed a STAT1-dependent induction of CREM mRNA within 5 h, similar to that of IFN-stimulated gene 15 (ISG15).

To determine whether the IFN- β -induced CREM was indeed responsible for the inhibition of IL-2 production by IFN- β , we used CREM-specific siRNA to abrogate its expression in CD4⁺ T cells that were subsequently incubated with and without IFN- β prior to stimulation with anti-CD3/CD28. As shown in Fig. 5H, the control siRNA had no influence on the inhibitory effect of IFN- β on IL-2 production. In striking contrast, ablation of CREM expression (Fig. 5G) completely eliminated the suppressive effects

of IFN- β (Fig. 5H) as determined by intracellular staining for IL-2. Therefore, upregulation of CREM and the subsequent recruitment of histone deacetylases to the IL-2 locus are responsible for inhibition of IL-2 expression in T cells by IFN- β .

Inhibition of IL-2 production in T cells from mice treated with IFN- β or infected with LCMV Cl13

To determine whether inhibition of IL-2 production also occurs in vivo, OTII TCR transgenic mice were injected with 10,000 U IFN- β 24 h prior to challenge with Ova₃₂₃₋₃₃₉ peptide to trigger T cell activation. A significant number of IL-2-producing T cells could be found in the spleens of OTII mice 4 h after injection with Ova₃₂₃₋₃₃₉ peptide, whereas few such IL-2-producing T cells were found in the spleens of IFN- β -treated mice (Fig. 6A, middle row; averages shown in Fig. 6B). Importantly, there was little difference in the number of Ova₃₂₃₋₃₃₉-specific T cells in the spleens of IFN-treated mice compared with untreated mice (Fig. 6A, top row), nor was there a difference in the number of activated T cells in IFN-treated versus untreated mice, as deter-

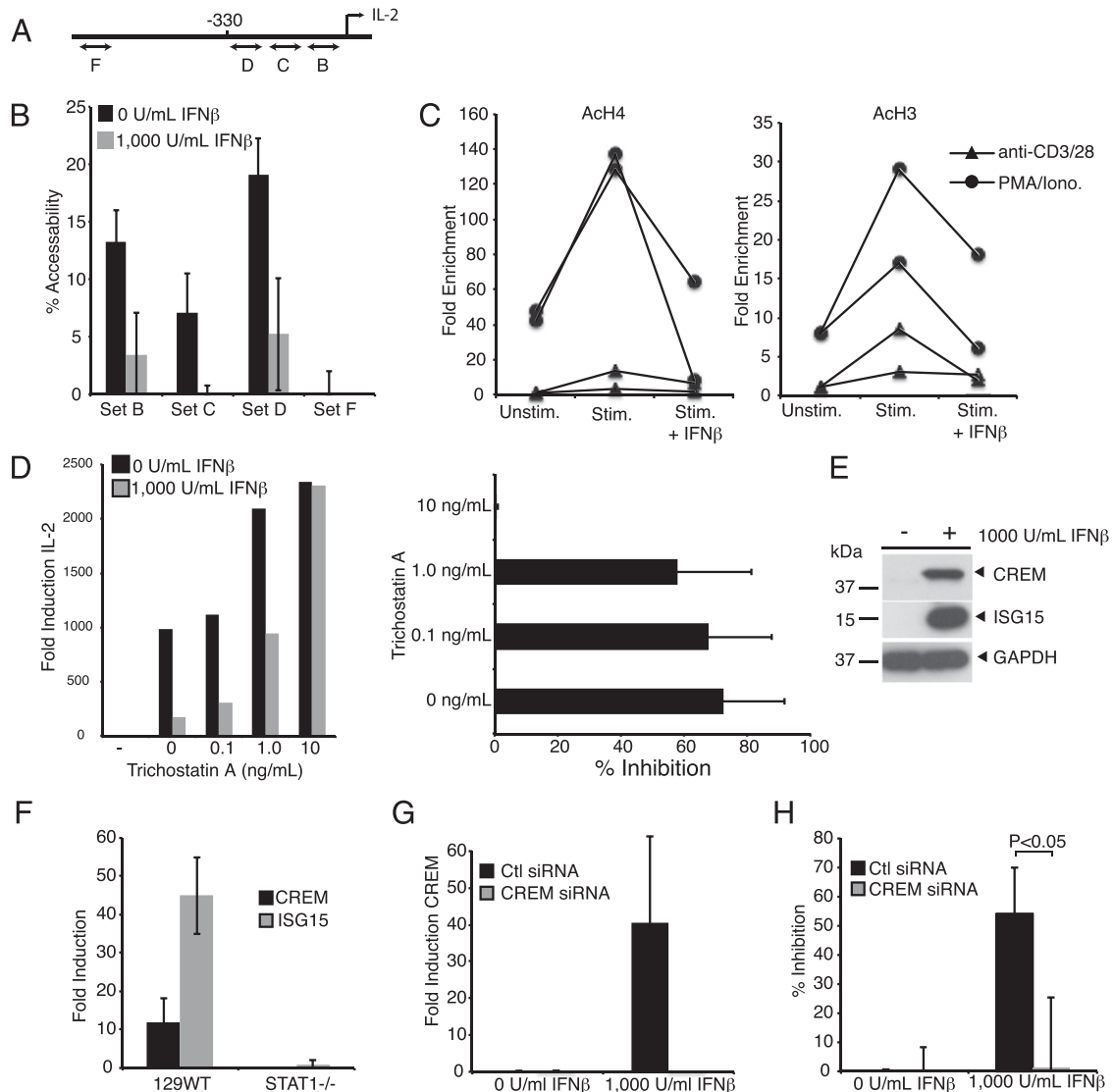


FIGURE 5. IFN- β affects chromatin remodeling of the IL-2 promoter through histone deacetylase activity and CREM. **(A)** Diagram showing location of primers in the IL-2 promoter used in the subsequent figures. **(B)** Chromatin accessibility of the IL-2 promoter–enhancer. Purified CD4⁺ T cells were stimulated with anti-CD3/anti-CD28 for 4 h with and without IFN- β pretreatment. Chromatin accessibility was determined by quantitation of the quantitative PCR products obtained with the indicated primer sets ($n = 4$). **(C)** Chromatin immunoprecipitations using antiacetylated H3 and antiacetylated H4 Abs and primers within the IL-2 promoter–enhancer following stimulation of CD4⁺ T cells with either anti-CD3/CD28 or PMA/ionomycin. **(D)** IL-2 mRNA was quantitated from CD4⁺ T cells following 3 h anti-CD3/CD28 stimulation with or without IFN- β pretreatment. Increasing doses of trichostatin A were added to cells 1 h prior to stimulation. The *left graph* is a representative of three independent experiments, the average percent inhibition of which is shown on the *right graph*. **(E)** CD4⁺ T cells were treated with IFN- β for 16 h, and Western blots of cell lysates were probed for CREM, ISG15, and GAPDH as a loading control. **(F)** mRNAs for CREM and ISG15 were measured in CD4⁺ T cells from 129 WT or STAT1^{-/-} mice stimulated with IFN- β for 5 h as measured with quantitative PCR. **(G and H)** CD4⁺ T cells were transfected with control or CREM-specific siRNA prior to treatment with IFN- β for 16 h and subsequent stimulation with anti-CD3/CD28 for 3 h. **(G)** CREM mRNA and **(H)** IL-2 mRNA were measured with quantitative PCR. **(H)** The average percent inhibition of IL-2 production was determined from four independent experiments.

mined by the upregulation of CD69, CD44 and downregulation of CD62L (Fig. 6A, *bottom row*, and data not shown). These results confirm that inhibition of IL-2 production by IFN- β occurs *in vivo* and is thus likely a major contributing element to the efficacy of IFN- β against MS. Intriguingly, lack of IL-2 production is also observed in exhausted T cells derived from mice chronically infected with LCMV Cl13 (28), and it was recently shown that blocking type I IFN rescued T cell function in such chronically infected mice (29, 30). In concurrence, T cells from LCMV Cl13–infected mice (day 9) expressed substantially elevated levels of CREM, as visualized by intracellular staining and flow cytometry (Fig. 6C; average of multiple mice in Fig. 6D), and produced less IL-2 than T cells from uninfected mice did (Fig. 6E; average of

multiple mice in Fig. 6F). Thus, our discovery that IFN- β can inhibit IL-2 production in activated T cells via induction of CreM *in vitro* and *in vivo* is not only relevant for its beneficial effects in MS; it also offers a molecular mechanism for the T cell exhaustion observed during chronic infections caused by the substantial amounts of endogenous IFN- β .

Discussion

Type I IFNs have been studied extensively as part of the innate immune response, but their effect on the adaptive immune system has comparatively remained rather elusive. Although it has been nearly 20 y since the first clinical trials involving IFN- β treatment for patients with MS, the exact mechanism by which IFN exerts its

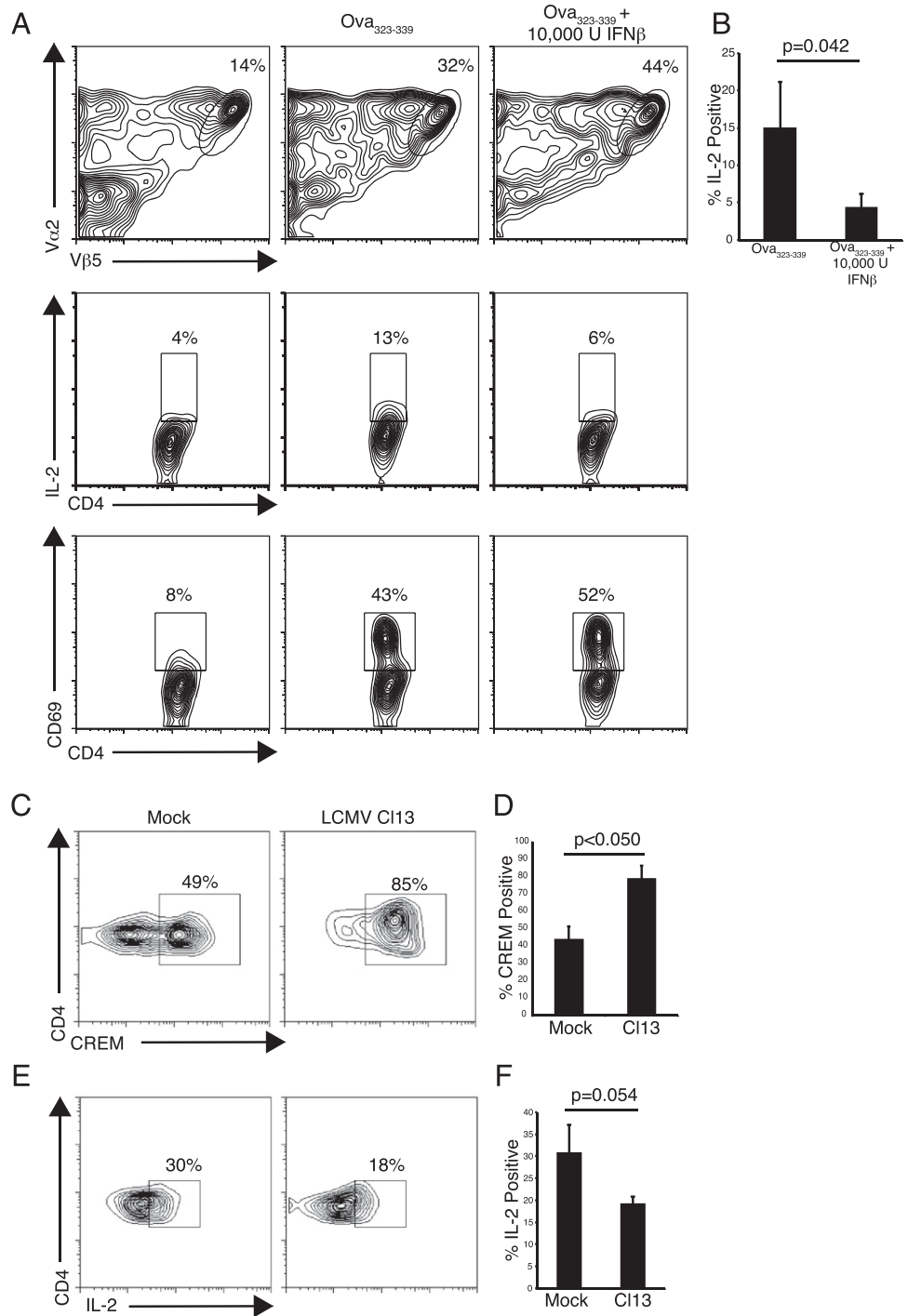


FIGURE 6. Inhibition of IL-2 production in T cells from mice injected with IFN- β or infected with LCMV CI13. **(A)** OTII TCR transgenic mice were injected with 100 μ g Ova₃₂₃₋₃₃₉ 24 h after i.v. injection of 10,000 U IFN- β . Splenic T cells were collected 4 h after peptide injection and subjected to intracellular stain for IL-2. Cells were also analyzed for surface expression of V α 2/V β 5 TCR chains and the activation marker CD69. **(B)** The average number of IL-2-positive OTII T cells from three independent experiments. **(C)** Single-cell suspensions from spleens of day 9 LCMV CI13-infected mice were subjected to intracellular stain for CREM. CD4⁺ cells are shown. **(D)** The percent CREM-positive CD4 T cells from five uninfected and seven LCMV CI13-infected mice stained as in (B). **(E)** Single-cell suspensions from spleens of day 9 LCMV CI13-infected mice were stimulated with anti-CD3/CD28 beads for 5 h and then stained intracellularly for IL-2. **(F)** The percent of IL-2-positive CD4 T cells from four mice each uninfected and LCMV CI13-infected mice stained as in (D).

efficacy has not been resolved. In this study, we provide clear evidence that IFN- β is acting on T cells specifically to inhibit the production of IL-2, a cytokine that is vitally important for expansion of Ag-specific T cells. It is reasonable to conclude that inhibition of IL-2 production is one, if not the key, mechanism by which IFN- β limits the number of T cells being activated and responding to myelin basic protein. The summary of our data unequivocally demonstrates that IFN- β induction of CREM is required for the recruitment of histone deacetylases to the IL-2 locus and the subsequent transcriptional silencing of the IL-2 gene. Highly intriguing is the selectivity of this process, as we did not observe any suppression of other cytokines or cell surface activation markers by IFN- β . Theoretically, this finding could be of prognostic benefit in the treatment selection for individual

patients with MS, as it is well established that not all patients respond to IFN therapy. As such, if IL-2 production by isolated T cells from a specific patient is refractory to inhibition by IFN- β in vitro, alternative treatments could be considered at a much earlier time point.

Beyond their contribution to a better understanding of the mechanism underlying the efficacy of IFN- β in the treatment of autoimmune disorders, our findings also support the notion that IFN- β produced during infectious processes acts in a negative feedback loop that limits the expansion of the responding T cells. In support of this conclusion, we observed an increase in CREM levels in T cells from mice chronically infected with LCMV CI13 (Fig. 6C), with an accompanying reduction in the IL-2 production from these animals (Fig. 6E). Because LCMV CI13 induces

a significant amount of IFN in the early stages of the infection, we can extrapolate that IFN-induced CREM is responsible for the reduced IL-2 production in T cells from chronically infected mice (28). This effect—often referred to as T cell exhaustion—has a profound effect on adaptive immune responses and could be responsible for the increased incidence of opportunistic infections following virus exposure.

Interestingly, by simply blocking IFN signaling in LCMV C113-infected mice, one can prevent or revert T cell exhaustion and induce clearance of the virus (29, 30). We propose that this occurs by restoring IL-2 production in Ag-specific T cells that would otherwise be inhibited by IFN produced during the innate response to the virus. This model also emphasizes a possible link between chronic viral infection and human autoimmune diseases, because reduced T cell responses, increased systemic IFN levels, and elevated CREM expression have all been noted in patients with systemic lupus erythematosus (31–33), although no direct connection between high IFN levels and CREM expression in patients with lupus has been suggested until now. Thus, although type I IFN is used to treat one form of autoimmune disease (i.e., MS) and is also involved in the pathogenesis of another (i.e., systemic lupus erythematosus), both may involve the same mechanism—namely, CREM-mediated inhibition of T cell responses as a consequence of type I IFN exposure.

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Disclosures

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