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Interferon β selectively Inhibits Interleukin-2 (IL-2) production through cAMP responsive element modulator (CREM)-mediated chromatin remodeling

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Abstract

Interferon beta (IFNβ) 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. Here we demonstrate that in both mouse and human T cells, IFNβ specifically inhibits the production of IL-2 upon T cell receptor (TCR) engagement without affecting other cytokines or activation markers. Rather than disrupting TCR signaling, IFNβ 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 IFNβ and consequent recruitment of histone deacetylases (HDACs) to the IL-2 promoter. In accordance, ablation of CREM expression or inhibition of HDAC activity eliminates the suppressive effects of IFNβ on IL-2 production. Collectively, these findings provide a molecular basis by which IFNβ limits T cell responses.

INTRODUCTION

Type I interferons (IFNα/β) have been approved worldwide for the treatment of multiple sclerosis, yet the mechanism behinds its effectiveness has remained elusive. Treatment with IFNα/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 IFNβ is vital to improve treatment strategies. While type I interferons have been extensively studied in the context of viral or bacterial infection as part of the innate immune response, it is only recently that the importance of these cytokines in the adaptive immune response is being more appreciated (2-5). Type I interferons exert strong anti-proliferative effects on lymphocytes and thus limit immune responses by controlling the number of responding cells, but also attenuate the activity of individual T cells (6, 7). T helper (Th) lymphocytes, which play a key role in the

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development of multiple sclerosis, proliferate in response to antigen by producing IL-2 that subsequently acts in an autocrine positive feedback loop. Surprisingly, the effects of type I interferons on IL-2 production by activated T cells has hitherto not been evaluated.

In this study we investigate IL-2 production from T cells that have been exposed to type I interferons in vitro as well as in vivo. Our data reveal a novel pathway by which IFNα/β inhibit gene expression at the epigenetic level and implicate the involvement of CREM in this process. As such we provide a possible mechanism by which IFNβ functions to control MS as well as 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 Jackson Laboratory (Bar Harbor, ME). Animals were between 6 and 12 weeks 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. 6-10 week old mice were infected intravenously with 2×10^6 pfu/mouse WT LCMV Cl13. All viruses were grown, identified, and quantified as previously described (12).

Flow-cytometric analysis

For immunostaining, single cell suspensions were prepared from mouse spleen with approximately 1×10^6 cells suspended in FACS buffer (PBS pH 7.4, 1% FCS, 0.02% NaN_3) and stained for 20 min in the dark on ice. Mouse antibodies, FITC-anti-CD4 (GK1.5), PE/Cy7-anti-CD8 (53.6.7), and PE-anti-IL2 (JE56-5H4), Biotin-anti-CD44 (PGp-1), PE anti-CD25 (PC61.5) were obtained from eBioscience (San Diego, CA), as well as PE anti-human IL-2 (MQ1-l7Hl2), 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 Flow Jo (Ashland, OR). Intracellular staining was carried out 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 16 h with IFNβ (Biogen Idec, Cambridge, MA) prior to dye loading, washed, and then a baseline reading was taken for 30 seconds. Hamster anti-CD3 (eBioscience) was added at 10 μg/mL and data was collected for another min prior to the addition of 25 μg/mL donkey anti-hamster IgG (eBioscience) and 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 IFNβ (Biogen Idec) for 16 h or as indicated prior to stimulation with either 10 μg/mL anti-CD3

and 2 μg/mL anti-CD28 (eBioscience) or 5 ng/mL phorbol myristate acetate (PMA) and 500 ng/mL Ionomycin (Sigma) for 3 or 6 h and then cells were analyzed for IL-2 protein by intracellular stain or RNA by reverse transcription and quantitative PCR. Mouse IL-2 ready set go kit (eBioscience) was used to measure IL-2 released into the conditioned T cell culture medium. Human PBL's were stimulated with Dynabeads Human T cell activator CD3/CD28 (Invitrogen). Trichostatin A (Sigma) was added to cultures 1 h prior to stimulation with anti-CD3/CD28. For in vivo stimulations OTII TCR transgenic mice were intravenously injected with 100ug $Ova_{323-336}$ peptide (Anaspec) 24 hours following injection with 10000 U IFNβ (Biogen Idec). Splenic T cells were removed 4 hours following peptide injection and subjected to intracellular staining for IL-2 and analyzed by flow cytometry.

Quantitative PCR

DNase-treated RNA was isolated from activated T cells by the RNeasy method (Qiagen). cDNA was prepared with the high capacity cDNA reverse transcription kit (Applied Biosystems) and QT-PCR was performed using Taqman primers for mouse and human IL-2, mouse IL-4, and mouse IFN-γ (Applied Biosystems). Analysis was carried out on 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 μg/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).

CHART assay

Chart assay was performed as described (13). Briefly, 1×10^7 cells per sample were stimulated for 2 h with 10 μg/mL anti-CD3 and 2 μg/mL anti-CD28 following 16 h pretreatment with 1000 U/mL IFNβ (Biogen Idec). Nuclei were isolated and subsequently digested with micrococcal nuclease (Mnase), then, following 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: CACAGGTAGACTCTTTGAAAATATGTGTAA, reverse: CATGGGAGGCAATTTATACTGTTAATG, Set C forward: CCTAAATCCATTCAGTCAGTGTATGG, reverse: TGTGTGGCAGAAAGCATTACCT, Set D forward: CTTTTGTGTCTCCACCCCAAA, reverse: CACACTTAGGTGGCAGTTTTAATTCAT, Set F forward: CATGCAGAGTTTTTTGTTGTTTTCTAG, reverse: GCCTAAAGTCTCTCACAAAGAACAGA. Change in accessibility for individual primer sets was calculated as 1-[(stimulated)/(unstimulated)].

Chromatin IP

Chromatin immunoprecipitations were performed using the Epitect Chip One Day kit (Qiagen) as described using anti-Histone H3 (acetyl K14) and anti-Histone H4 (acetyl K8) (Abcam). IP's were analyzed by quantitative PCR using primer B as in the CHART assay.

siRNA knockdown

Purified $CD4^+$ T cells were transfected by electroporation with either 25 nM CREM siRNA or control siRNA (Thermo Scientific Darmacon) and rested for 4 h prior to treatment with IFNβ. After 16 h, cells were stimulated with anti-CD3/CD28 for 3 h and RNA was isolated and quantitative PCR was used to determine IL-2 message levels. Percent inhibition was calculated as [(stimulated – inhibited)/stimulated)] ×100. Student's T test was used to determine significance.

RESULTS

IFNβ inhibits activation-induced IL-2 production in T cells

Signaling through the TCR in conjunction with co-stimulation 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 antigen specific cells (14). As IFNβ is a strong inhibitor of T cell proliferation, we chose to investigate the effects of IFNβ on IL-2 production in T cells. Upon stimulation with agonistic anti-CD3 and anti-CD28 antibodies, splenic T cells of WT 129SveV mice produce IL-2 within 5 h as detected by intracellular flow cytometry staining (Figure 1A, second panel). However, upon pretreatment with IFNβ the number of IL-2 producing cells is significantly reduced (Figure 1A, third panel), an effect specific to IFNα/β, as the type II interferon, IFNγ, had no effect on IL-2 production (Figure 1A, right panel). In addition, IL-2 production was inhibited in both $CD4^+$ (Figure 1A, upper row) as well as $CD8^+$ (Figure 1A, lower 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 IFNβ also inhibited the production of IL-2 when T cells where stimulated with the mitogens PMA and Ionomycin (Figure 1B) or Concanavalin 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 ConA was further corroborated by measuring the IL-2 concentration by Enzyme Linked Immunosorbant Assay (ELISA) analysis of the conditioned medium (Figure 1C and data not shown). Further experiments revealed that IFNβ-mediated inhibition of IL-2 production occurred on the transcriptional level, as IL-2 mRNA was also reduced as a consequence of IFNβ exposure prior to stimulation (Figure 1D). Strikingly, the inhibitory effect of IFNβ was restricted to IL-2, as neither IL-4 (Figure 1D) nor IFNγ (not shown) mRNA, nor expression of the cell surface activation markers CD25 and CD44 (Figure 1E) were subdued by IFNβ pretreatment.

Activated T cells often exhibit significant differences in their response to type I interferons as compared to naïve T cells (15). We therefore investigated next whether IFNβ could inhibit IL-2 production in already activated T cells. $CD4^+$ T cells were stimulated for 3 days

with anti-CD3/CD28 (Figure 2A, right panels), and then incubated overnight with (Figure 2A, bottom) or without (Figure 2A, top) 1000U/mL IFNβ.

Subsequently, cells were stimulated with PMA/Iono for 5 h. IFNβ significantly reduced the number of IL-2 producing cells that were naive at the time of stimulation (Figure 2A, middle plots), but failed to inhibit IL-2 production if the cells were preactivated for 3 days with anti-CD3/CD28 (Figure 2A, right panels). This finding indicates that once IL-2 transcription is activated it can no longer be suppressed by IFNβ.

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

IFNβ 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 (Treg) cells were required in order for IFNβ to inhibit IL-2 production. Indeed, it was shown recently that interferon is required for maintaining Foxp3 expression in Treg cells during infection (16). Therefore, we decided to eliminate Treg cells from the $CD4^+$ T cell cultures prior to IFNβ exposure. Although Treg cells alone had, as expected, an intrinsic inhibitory effect on IL-2 production by $CD4^+$ cells (Figure 3A, compare black bars), IFNβ was nevertheless still capable of significantly inhibiting IL-2 production even in the absence of Treg cells (Figure 3A, compare gray bars). We therefore concluded that IFNβ acts on the responding effector T cells directly to inhibit IL-2 production independent of Treg cells.

To address whether the inhibitory effect of IFNβ on IL-2 production could also be seen in human cells, we utilized human PBMCs to analyze both IL-2 production by flow cytometry, as well as IL-2 mRNA expression in response to anti-CD3/CD28 in the absence or presence of human IFNβ. Similar to their murine counterparts, human T cells, too, were inhibited in their ability to synthesize IL-2 when previously exposed to IFNβ (Figure 3B and C). Thus, IFNβ acts directly on human and murine T cells to inhibit activation-induced IL-2 production.

IFNβ−mediated inhibition of IL-2 expression requires signaling through the type I interferon receptor (IFNAR) but does not alter TCR signaling

To determine if the inhibitory effect of IFNβ on IL-2 production required known components of the canonical type I interferon signaling pathway, we employed STAT1-deficient mice which display severely impaired interferon 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 interferon (18). This was corroborated in Figure 4A wherein IFNβ inhibited IL-2 production in WT T cells (Figure 4A, left panels), but strikingly caused a significant increase in IL-2 producing cells (Figure 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 IFNβ 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 IFNβ pretreatment (Figure 4A, right panels).

IL-2 induction requires engagement of the TCR along with stimulation through the co-receptor CD28 which together activate multiple downstream signaling pathways including the MAP kinases, NFκB, PLCγ, and increases in cytoplasmic free calcium (19, 20). As IFNβ 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 IFNβ. Indeed, phosphorylation of p42/44 MAPKs, and the SAPKs p38 and JNK are not affected by IFNβ pretreatment (Figure 4B), nor is there a reduction in cFos levels (Figure 4B) or an impairment of NFκB signaling (data no shown) following IFNβ treatment. In concurrence, there were also no differences in the increase of cytoplasmic free calcium after TCR stimulation in the presence of IFNβ (Figure 4C). Furthermore, the use of various luciferase reporter constructs representing the major enhancer elements within the IL-2 promoter (e.g. NFκB, NFAT, AP-1, etc.) (21) did not indicate any interference of IFNβ on this level (data not shown). Thus, IFNβ 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 IFNβ selectively targets IL-2 without affecting the induction of other cytokines or activation markers.

IFNβ 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 post-translational modifications of histones and/or the DNA itself primarily through acetylation and/or methylation. These epigenetic changes result in the accessibility of the gene not only to site-specific transcription factors but also to the basal transcription machinery. To determine if IFNβ pretreatment would influence the changes in chromatin structure elicited in the IL-2 locus after TCR stimulation of $CD4^+$ T cells, we employed a chromatin accessibility assay using micrococcal DNase treatment of nuclei from anti-CD3/CD28-stimulated cells with and without IFNβ 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 IFNβ. We utilized several primer sets within

the 300 bp IL-2 promoter (Figure 5A: Set B, C, and D) to determine its accessibility as well as a control primer set distant from the IL-2 locus (set F) as a control.

We observed that with IFNβ treatment alone, the promoter presented in the same closed configuration as in unstimulated cells, whereas anti-CD3/CD28 treatment lead to a substantial increase in the accessibility of the IL-2 locus. Notably, IFNβ pretreatment significantly reduced the anti-CD3/CD28 induced change in the accessibility of the IL-2 locus (Figure 5B). As 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 Figure 5C, chromatin immunoprecipitation using antibodies specific for acetylated histone 3 or acetylated histone 4 revealed a clear increase of these post-translational 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 IFNβ negated the TCR-induced histone modifications (Figure 5C). These findings suggested that interferon was either preventing acetylation of the histones within the IL-2 promoter, or was 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 interferon on IL-2 production. Indeed, we found that with increasing doses of Trichostatin A, the amount of IL-2 produced after TCR engagement increased (Figure 5D, left graph). More importantly, at higher concentrations Trichostatin A completely abrogated the inhibitory effect of IFNβ on IL-2 production (Figure 5D, right graph, average of four separate experiments). The cumulative interpretation of these studies infers that IFNβ 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 include the zinc finger transcription factors Aiolos and Ikaros, as well as Blimp and the cyclic AMP response element modulator (CREM), all of which act in a negative feedback loop to silence IL-2 expression (23-27). Our investigations did not reveal any involvement of Aiolos, Ikaros or Blimp in the inhibitory effects of IFNβ on IL-2 production (not shown). However, analysis of microarray data from anti-CD3/CD28-stimulated $CD4^+$ T cells with and without prior IFNβ exposure revealed that CREM was significantly upregulated in these T cells by IFNβ. This was confirmed by western blot analysis where there is a dramatic induction of CREM protein expression in response to 16 h IFNβ treatment (Figure 5E). Analysis by quantitative PCR (Figure 5F) revealed a STAT1 dependent induction of CREM mRNA within 5 hours, similar to that of interferon stimulated gene 15 (ISG15).

To determine whether the IFNβ-induced CREM was indeed responsible for the inhibition of IL-2 production by IFNβ, we used CREM specific siRNA to abrogate its expression in $CD4^+$ T cells that were subsequently incubated with and without IFNβ prior to stimulation with anti-CD3/CD28. As shown in Figure 5H, the control siRNA had no influence on the inhibitory effect of IFNβ on IL-2 production. In striking contrast, ablation of CREM expression (Figure 5G) completely eliminated the suppressive effects of IFNβ (Fυγνρε5H followed by intracellular stain for IL-2). Therefore, up-regulation of CREM and the

subsequent recruitment of HDACs to the IL-2 locus are responsible for inhibition of IL-2 expression in T cells by IFNβ.

Inhibition of IL-2 production in T cells from mice treated with IFNβ or infected with LCMV Clone 13

Crucially, to determine whether inhibition of IL-2 production also occurs in vivo, OTII TCR transgenic mice were injected with 10,000U IFNβ 24 hours prior to challenge with $Ova_{323-339}$ 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 hours after injection with $Ova_{323-339}$ peptide, whereas very few such IL-2 producing T cells were found in the spleens of IFNβ treated mice (Figure 6A, middle row, averages shown in figure 6B). Importantly, there was only little difference in the number of $Ova_{323-339}$ specific T cells in the spleens of interferon treated mice compared to untreated mice (Figure 6A, top row), nor was there a difference in the number of activated T cells in interferon treated versus untreated mice as determined by the upregulation of CD69, CD44 and downregulation of CD62L (Figure 6A, lower row and data not shown). These results confirm that inhibition of IL-2 production by IFNβ occurs in vivo and is thus likely a major contributing element to the efficacy of IFNβ against MS. Intriguingly, lack of IL-2 production is also observed in “exhausted” T cells derived from mice chronically infected with LCMV clone 13 (28), and it was recently shown that blocking type I interferon 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 (Figure 6C, average of multiple mice in 6D), and produced less IL-2 than T cells from uninfected mice (Figure 6E, average of multiple mice in 6F). Thus, our discovery that IFNβ 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, but also offers a molecular mechanism for the T cell exhaustion observed during chronic infections due to the substantial amounts of endogenous IFNβ.

DISCUSSION

Type I interferons have been extensively studied as part of the innate immune response, their impact on the adaptive immune system has comparatively remained rather elusive. Although it has been nearly 20 years since the first clinical trials involving interferon beta treatment for patients with multiple sclerosis, the exact mechanism by which interferon exerts its efficacy has not been resolved. Here we provide clear evidence that IFNβ is acting on T cells to specifically inhibit the production of IL-2, a cytokine vitally important for expansion of antigen specific T cells. It is consequently reasonable to conclude that inhibition of IL-2 production is one, if not the key mechanism by which IFNβ limits the number of T cells that are being activated and responding to myelin basic protein. The summary of our data unequivocally demonstrates that IFNβ 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 IFNβ. This finding could theoretically be of prognostic benefit in the treatment selection for individual MS

patients, as it is well established that not all patients respond to interferon therapy. As such, if IL-2 production by isolated T cells from a specific patient is refractory to inhibition by IFNβ 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 IFNβ in the treatment of autoimmune disorders, our findings also support the notion that IFNβ produced during infectious processes acts in a negative feedback loop that limits the expansion of the responding T cells. In support of this we observe an increase in CREM levels in T cells from mice chronically infected with LCMV Cl13 (Figure 6C), with an accompanying reduction in the IL-2 production from these animals (Figure 6E). As LCMV Cl13 induces a significant amount of interferon in the very early stages of the infection, we can extrapolate that interferon-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 impact on adaptive immune responses and could be responsible for the increased incidence of opportunistic infections following virus exposure.

Interestingly, by simply blocking interferon signaling in LCMV Cl13 infected mice one can prevent or revert T cell exhaustion and induce clearance of the virus (29, 30). We propose this occurs by ‘restoring’ IL-2 production in antigen specific T cells which would otherwise be inhibited by interferon produced during the innate response to the virus. This model also emphasizes a possible link between chronic viral infection and human autoimmune diseases, as reduced T cell responses, increased systemic interferon levels and elevated CREM expression have all been noted in patients suffering from systemic lupus erythematosus (SLE) (31-33), although no direct connection between high interferon levels and CREM expression in lupus patients has been suggested until now. Thus, even though type I interferon is used to treat one form of autoimmune disease (MS) and is also involved in the pathogenesis of another (SLE), both may involve the same mechanism, namely CREM mediated inhibition of T cell responses as a consequence of type I interferon exposure.

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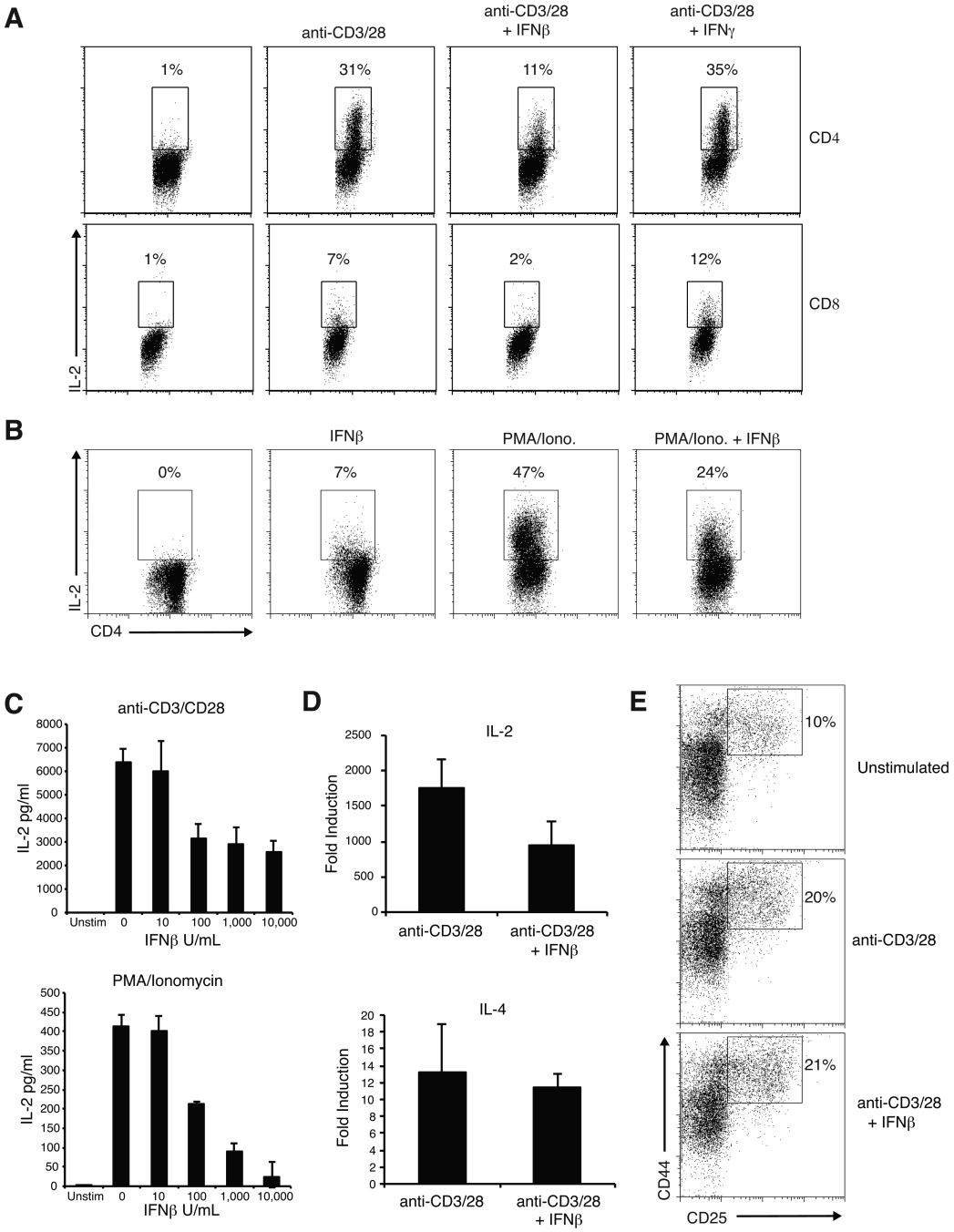

Figure 1. Specific inhibition of IL-2 expression by IFNβ

(**A**) Splenocytes from wild type 129/SvEv mice were treated with or without IFNβ or IFNγ for 16 h and then stimulated with 10 μg/mL immobilized anti-CD3 and 2 μg/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 and are representative of at least five experiments. (**B**) Splenocytes from wild type 129/SvEv mice were treated for 16 h with IFNβ 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 IFNβ. IL-2 in the conditioned medium was quantified by ELISA (n=3). (**D**) $CD4^+$ splenocytes were treated for 16 h with IFNβ prior to stimulation with anti-CD3/anti-CD28. 6 h 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.

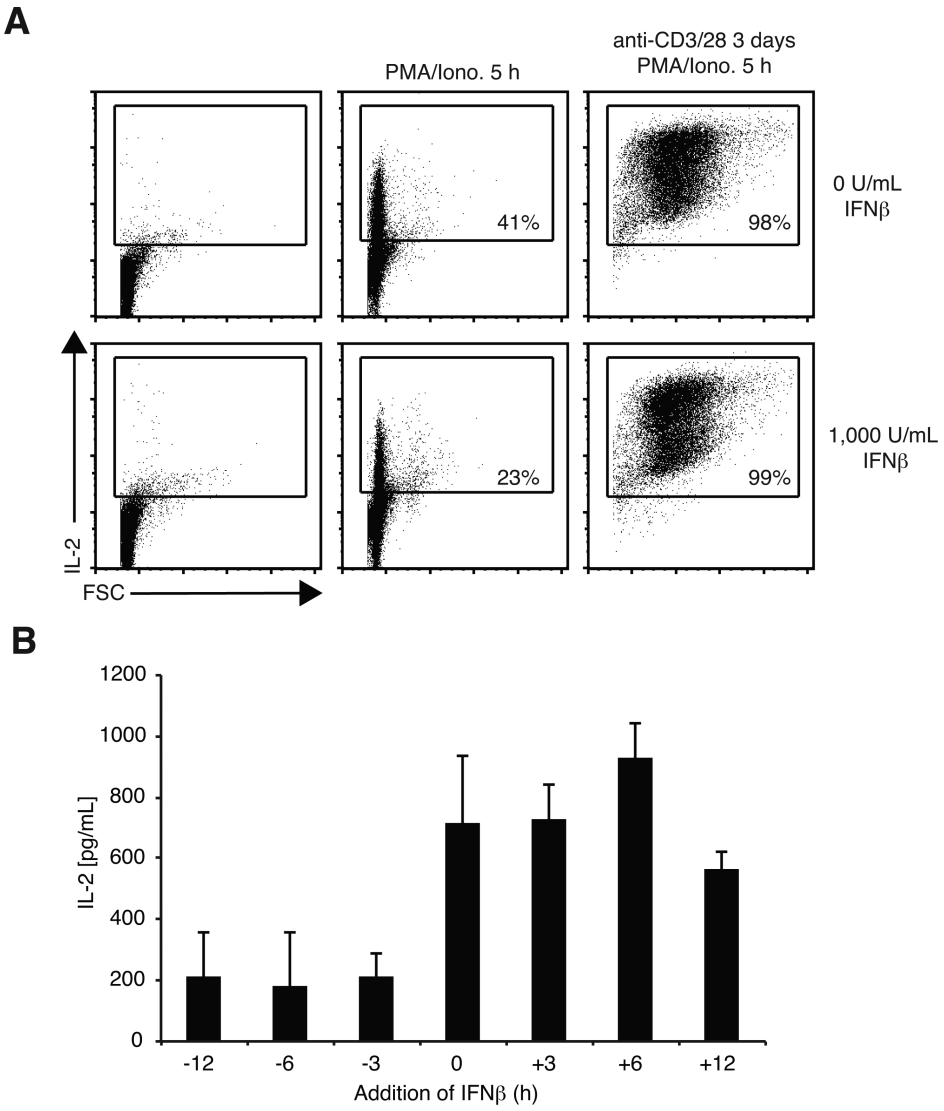

Figure 2. Inhibition of IL-2 expression requires pre-treatment with IFNβ

(A) Purified $CD4^+$ splenic T cells were treated with IFNβ for 16 h (lower plots) before (middle plots) or after (right plots) 3 days in culture with anti-CD3 and anti-CD28 and then re-stimulated with PMA/Ionomycin for 5 h followed by intracellular stain for IL-2. (**B**) Splenocytes were cultured for a total of 4 days following stimulation with anti-CD3 and anti-CD28. IFNβ was added up to 12 h prior to or up to 12 h post stimulation. The amount of IL-2 in the conditioned medium was measured by ELISA (n=3).

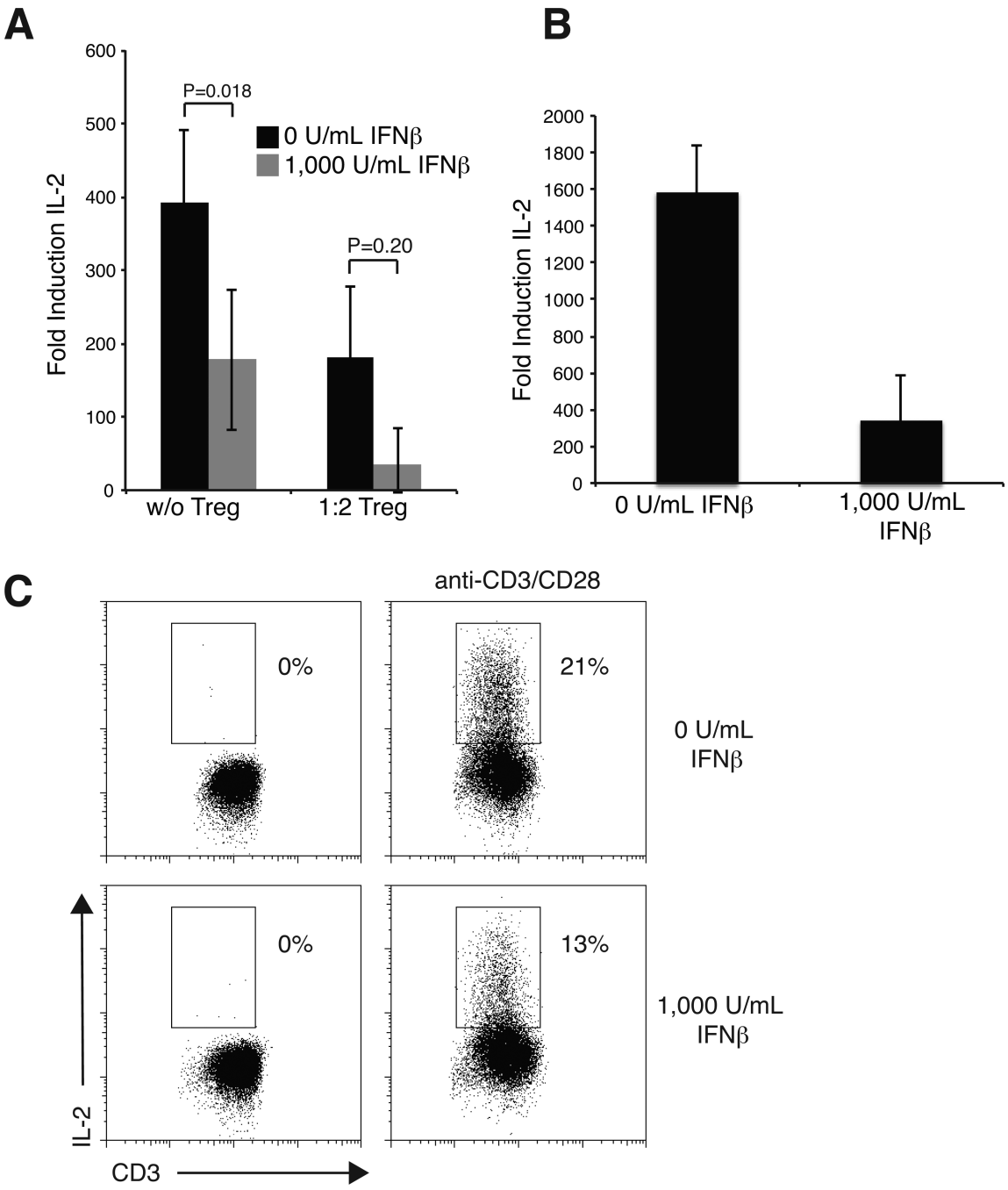

Figure 3. IFNβ inhibition of IL-2 expression is independent of Treg cells and is reproduced in human peripheral blood leukocytes

(**A**) $CD4^+$ T cells were depleted of $CD25^+$ T_{reg} cells and then treated for 16 h with IFNβ followed by stimulation with anti-CD3/anti-CD28 for 4 h in the presence or absence of $CD25^+$ Treg cells. IL-2 mRNA was measured by quantitative PCR. (n=3) (**B**) Human PBLs were stimulated with anti-CD3/anti-CD28 dynabeads with and without IFNβ 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).

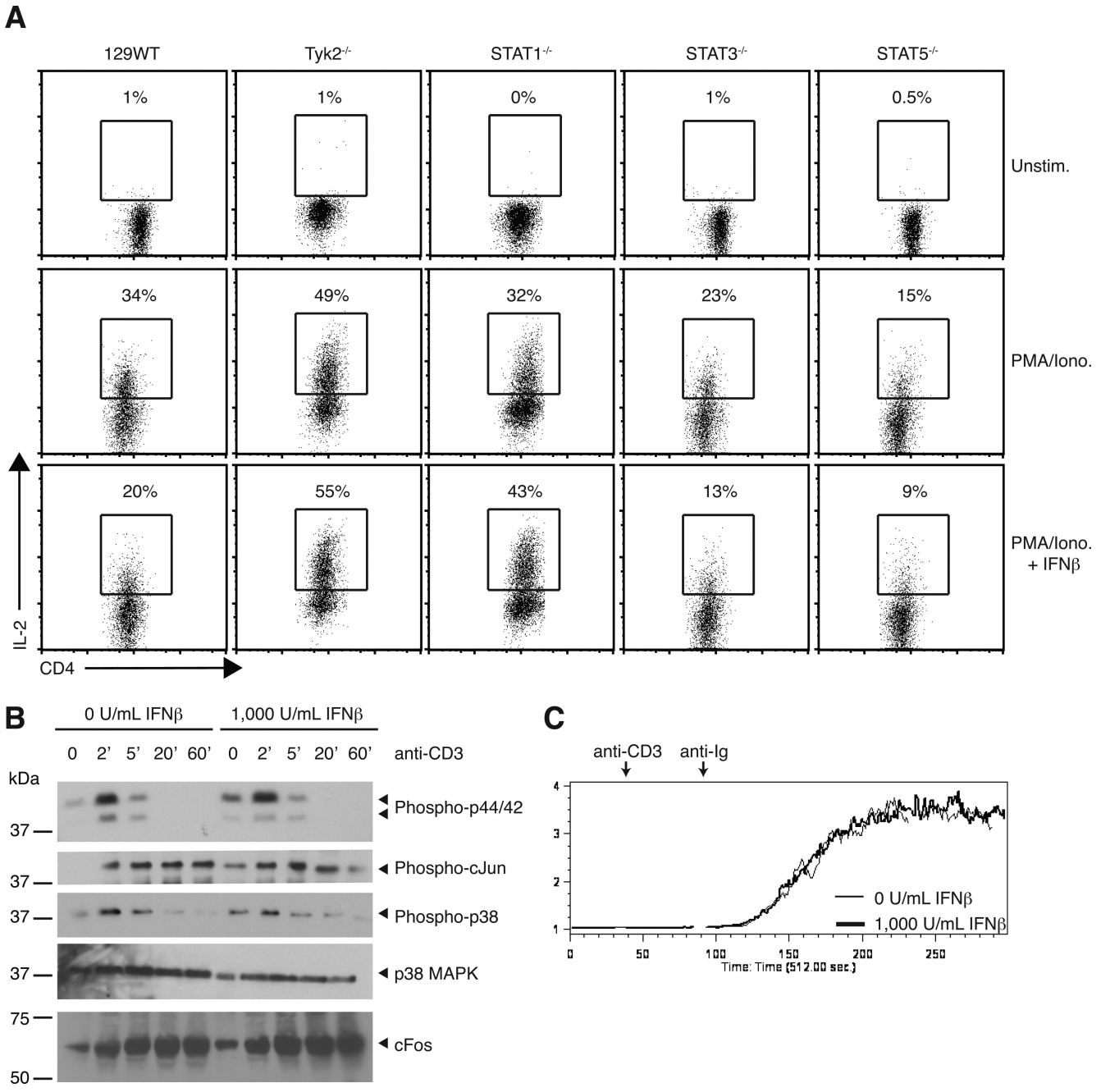

Figure 4. Inhibition of IL-2 by IFNβ in naive T cells is dependent on STAT1 but does not affect proximal signaling through the T cell receptor

(**A**) Splenocytes from wild type and STAT1$^{-/-}$, Tyk2$^{-/-}$, STAT3$^{-/-}$ and STAT5$^{-/-}$ mice were treated with IFNβ 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 3-5 mice each. (**B**) Splenic T cells were treated for 16 h with IFNβ and then stimulated with anti-CD3/CD28 for the indicated time. Western blots of whole cell lysates were carried out and probed for the indicated phosphorylated proteins. (**C**) Splenic T cells, treated for 16 h with IFNβ and then loaded with Fluo-4 and Fura Red, were run on flow cytometer for 30 sec to

establish baseline, and then stimulated with anti-CD3. After 1 min, a crosslinking antibody was added to cells to induce calcium flux (representative of at least five experiments).

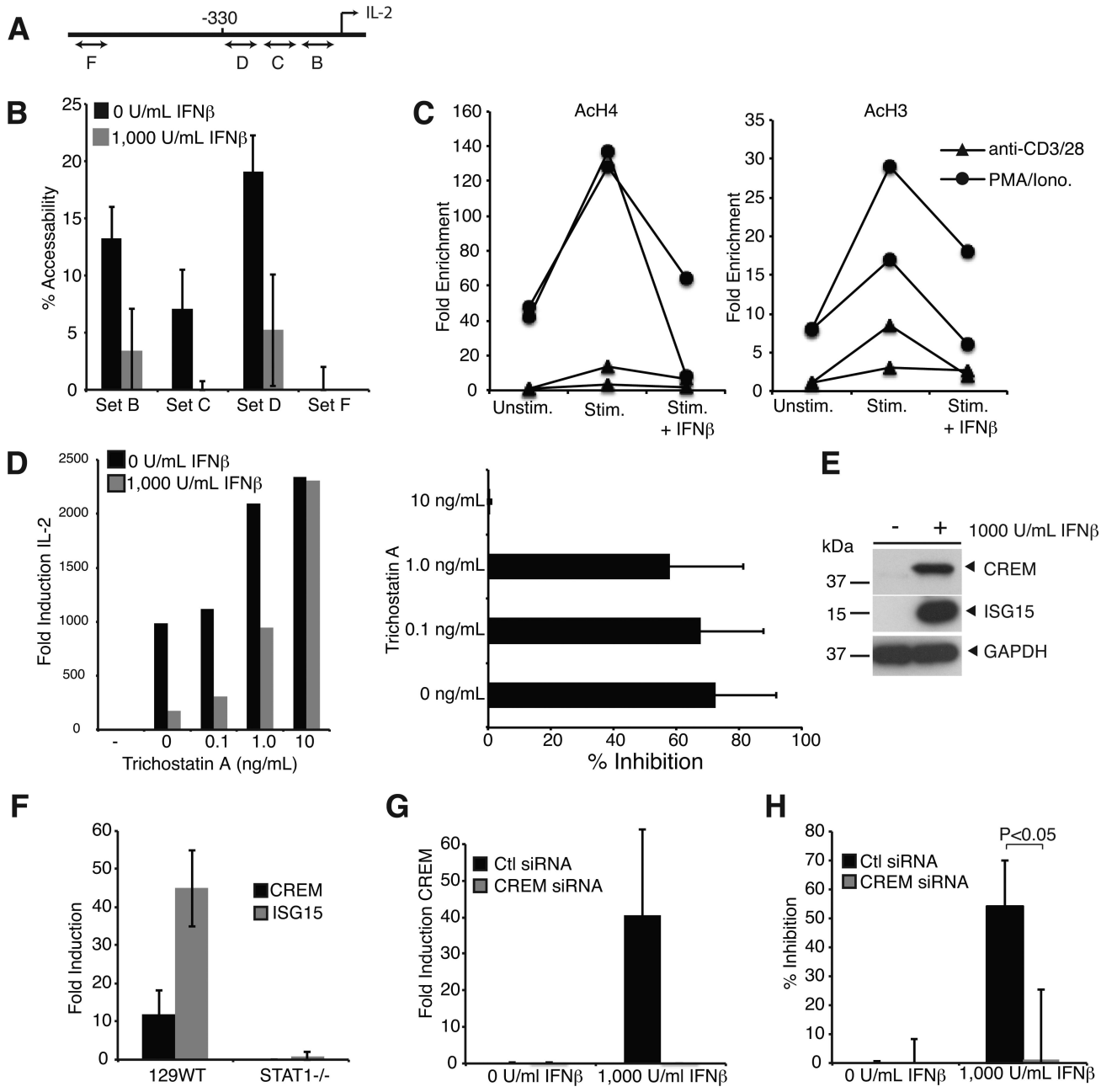

Figure 5. IFNβ affects chromatin remodeling of the IL-2 promoter through histone deacetylase activity and CREM

(**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 IFNβ pretreatment. Chromatin accessibility was determined by quantitation of the qPCR products obtained with the indicated primer sets. (n=4) (**C**) Chromatin IPs using anti-acetylated H3 and anti-acetylated H4 antibodies 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 IFNβ pre-treatment. Increasing doses of Trichostatin A were added to cells 1 h prior to stimulation. Left graph is a representative of three independent experiments the average % inhibition of which in shown on the right. (**E**) $CD4^+$ T cells were treated with IFNβ 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 129WT or $STAT1^{-/-}$ mice stimulated with IFNβ for 5 h as measured by quantitative PCR. (**G**) and (**H**) $CD4^+$ T cells were transfected with control or CREM-specific siRNA prior to treatment with IFNβ for 16 h and subsequent stimulation with anti-CD3/CD28 for 3 h. (**G**) CREM mRNA and (H) IL-2 mRNA was measured by quantitative PCR. (**H**) The average % inhibition of IL-2 production was determined from 4 independent experiments.

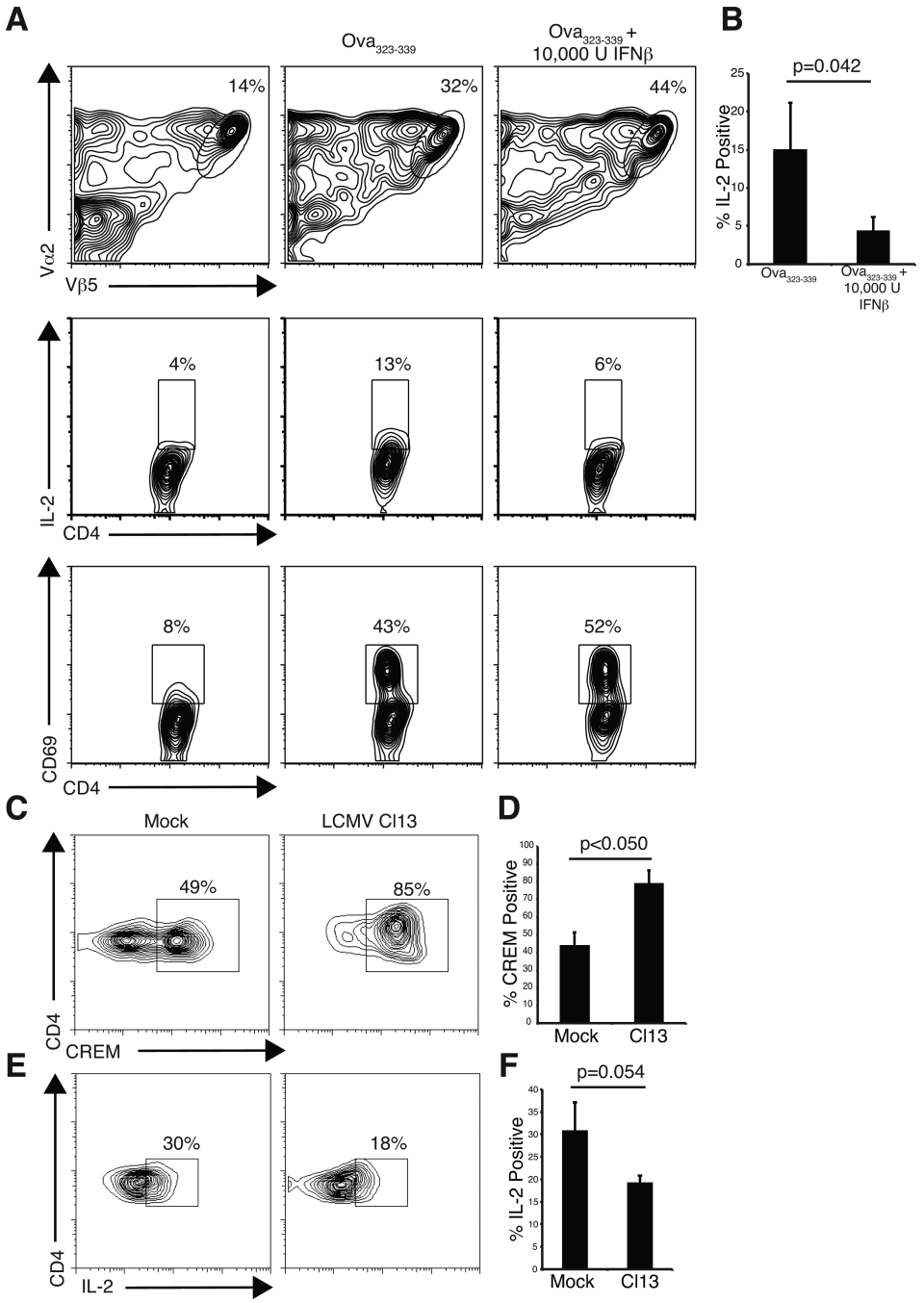

Figure 6. Inhibition of IL-2 production in T cells from mice injected with IFNβ or infected with LCMV Cl13

(**A**) OTII TCR transgenic mice were injected with 100ug $Ova_{323-339}$ 24 h after I.V. injection of 10,000 U IFNβ. Splenic T cells were collected 4 h following peptide injection and subjected to intracellular stain for IL-2. Cells were also analyzed for surface expression of Vα2/Vβ5 TCR chains as well as the activation marker CD69. (**B**) Graph represents average number of IL-2 positive OTII T cells from three independent experiments. (**C**) Single cell suspensions from spleens of Day 9 LCMV Cl13 infected mice were subjected to

intracellular stain for CREM. $CD4^+$ cells are shown. (**D**) Graph represents % CREM positive CD4 T cells from 5 uninfected and 7 LCMV Cl13 infected mice stained as in (b). (**E**) Single cell suspensions from spleens of Day 9 LCMV Cl13 infected mice were stimulated with anti-CD3/CD28 beads for 5 h and then intracellular stained for IL-2. (**F**) Graph represents % IL-2 positive CD4 T cells from 4 mice each uninfected and LCMV Cl13 infected mice stained as in (d).