Beyond Innate Immunity: Defining a Role for Interferon Regulatory Factor 3 in CD4⁺ T Cell Responses

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by

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The Dissertation of Nancy Josephine Fares-Frederickson is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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LIST OF ABBREVIATIONS

Antigen-presenting cell (APC)
cAMP responsive element modulator (CREM)
Dendritic cell (DC)
Forkhead box p3 (Foxp3)
GATA binding protein 3 (GATA-3)
Histone deacetylase (HDAC)
Interferon (IFN)
Interferon alpha/beta receptor (IFNAR)
Interferon regulatory factor (IRF)
Interferon stimulated genes (ISGs)
Interleukin (IL)
Lipopolysaccharide (LPS)
Lymphocytic choriomeningitis virus (LCMV)
MAP kinase (MAPK)
Multiple Sclerosis (MS)
Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)
Pathogen associated molecular patterns (PAMPs)
Pattern recognition receptor (PRR)
Retinoid-related orphan receptor gamma (RORγt)
Regulatory T cell (Treg)
Systemic lupus erythematosus (SLE)
T-box expressed in T cells (T-bet)
T cell receptor (TCR)
T helper cell (Th)
Toll-like receptor (TLR)
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ABSTRACT OF THE DISSERTATION

Beyond Innate Immunity: Defining a Role for Interferon Regulatory Factor 3 in CD4⁺ T Cell Responses

by

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Interferon regulatory factor 3 (IRF3) is a transcription factor found in the cytoplasm of most nucleated cells. Upon pathogen detection by pattern recognition receptors, IRF3 becomes activated and translocates to the nucleus where it mediates the production of type I interferon (IFN) and IFN-stimulated genes. The collective actions of these genes induce an antimicrobial state within
infected and neighboring cells, allowing for pathogen clearance. Whereas the type I IFN system has been studied extensively in the context of pathogen responses, it has more recently become appreciated for its role in immune cell modulation. In this report we discuss novel findings highlighting the involvement of the type I IFN system—with a focus on IRF3—in T cell development, activation, differentiation, and cytokine production.

Using a combination of in vitro assays and in vivo disease models, we demonstrate that IRF3 is a positive regulator of IL-17 and IFN-γ in CD4⁺ T cells. We show that IRF3-deficient CD4⁺ T cells have impaired production of the pro-inflammatory cytokines IL-17 and IFN-γ, and are unable to induce disease in the T cell transfer model of colitis. Unexpectedly, we observed IRF3 nuclear translocation in activated T cells. To our knowledge, this is the first description of IRF3 activation following T cell receptor stimulation. These results, which are the focus of chapter 1, reveal a previously unappreciated role for IRF3 in CD4⁺ T cell responses and extend its role beyond the innate immune response to pathogens.

Additionally, we show that type I IFN limits T cell production of IL-2—a cytokine required for T cell activation and expansion—by indirectly altering histone modifications in the IL-2 promoter to retain the locus in an inaccessible configuration. These results, which are the focus of chapter 2, help explain why type I IFN has been an effective treatment for multiple sclerosis—a T cell-mediated autoimmune condition.
INTRODUCTION

The concept of viral interference—the inhibitory effect that one viral infection exerts on the infectivity of a different virus—was first described in the early 1800s by Edward Jenner, who reported that herpes infections could prevent the development of vaccinia lesions during inoculation against smallpox [1]. In 1957, Isaacs and Lindenmann identified the factor responsible for this phenomenon. They demonstrated that cells incubated with heat-inactivated influenza virus released a soluble factor that could inhibit the growth of live virus. Due to its ability to interfere with viral replication, they named the factor interferon (IFN) [2, 3]. Since then, substantial progress has been made in understanding the biology of IFNs, including the processes that lead to IFN production, and the transcriptional activation in response to IFN signaling.

IFNs are a family of cytokines that are best known for their antiviral properties. This family can be classified into three main cytokine groups—type I, type II, and type III IFNs. The type I IFN family is the largest, and includes 12 IFN-α subtypes, IFN-β, IFN-ε, IFN-κ, and IFN-ω. The type II IFN family is composed of just one cytokine: IFN-γ. The type III IFN family includes three members: IFN-λ.1 (IL-29), IFN-λ.2 (IL-28A), and IFN-λ.3 (IL-28B). Type III IFNs are structurally distinct from type I and type II IFNs, and more closely resemble members of the IL-10 family [4]. Despite their structural differences, all IFNs exhibit antiviral properties. This dissertation focuses on type I IFNs, which can be produced by nearly every cell type.
Induction of type I IFNs is mediated through the detection of pathogen-associated molecular patterns (PAMPs) by various pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are the predominant sensors of microbial infections in mammals. TLRs recognize a broad range of PAMPs, including microbial nucleic acids and structural proteins [5]. Type I IFN production is not entirely dependent on TLR engagement, as it was demonstrated that TLR-deficient animals could still produce type I IFN in response to RNA and DNA ligands [6, 7]. These RNA and DNA ligands are detected by the cytoplasmic sensors retinoic-acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), stimulator of IFN genes (STING), and DNA-dependent activator of IRFs (DAI) [8-11].

Engagement of the abovementioned PRRs culminates in the activation of the transcription factors interferon regulatory factor 3 (IRF3) and IRF7. Serine/threonine phosphorylation by TANK-binding kinase (TBK1) and IκB kinase ε (IKKe) induces conformational changes in IRF3 and IRF7, enabling their homo- and heterodimerization and nuclear translocation [12, 13]. Once in the nucleus, IRF3 and IRF7 promote the transcription of type I IFN and IFN-stimulated genes (ISGs) by binding to interferon-stimulated response elements (ISREs) within their promoters.

Type I IFNs signal through a common receptor, known as the IFN-α/β receptor (IFNAR), which is expressed on nearly every cell type. This heterodimeric receptor consists of two subunits—IFNAR1 and IFNAR2—that are constitutively associated with Janus kinase 1 (JAK1) and tyrosine kinase 2
(TYK2) [14]. Binding of type I IFNs to IFNAR activates JAK1 and TYK2, resulting in the phosphorylation and activation of several signal transducer and activator of transcription (STAT) family members. Activated STAT1 and STAT2 associate with IRF9, forming the interferon-stimulated gene factor 3 (ISGF3) complex. ISGF3 undergoes nuclear translocation and mediates the production of ISGs by binding to ISREs within their promoters [15-17]. The collective actions of these ISGs induce an antimicrobial state in infected and neighboring cells, which enables pathogen clearance.

Whereas the type I IFN system is best known for modulating host immune responses against pathogens, type I IFNs have more recently become appreciated for their immunomodulatory functions [18]. Our group has published several reports demonstrating the involvement of the type I IFN system in T cell development and function. We initially observed that STAT1-deficiency results in dramatically heightened susceptibility to autoimmune disease [19]. Subsequent investigations revealed that mice lacking components of the type I IFN signaling system exhibit defects in T cell selection [20], and the generation and function of both natural and induced regulatory T cells. We further noted impaired development of autoantigen-presenting medullary thymic epithelial cells (mTECs) in IRF7−/−, IFNAR1−/− and STAT1−/− mice, resulting in a disrupted thymic architecture and failure in adequate negative selection [21]. Most recently, our group demonstrated that type I IFN inhibits T cell production of IL-2 through indirect chromatin remodeling via CREM up-regulation [22]. This latest publication is presented in chapter 2.
Although IRF3 and IRF7 have been studied extensively in the context of innate immune responses, their role in adaptive immunity has remained largely unexplored. In particular, the contributions of IRF3 and IRF7 to the lymphoid compartment have barely been investigated. This may be at least in part due to the fact that comparison of individual T cell subsets between wild type, IRF3<sup>-/-</sup>, IRF7<sup>-/-</sup>, IFNAR1<sup>-/-</sup> and STAT1<sup>-/-</sup> mice provided no evidence for significant differences between these strains [19, 23, 24]. Our interest into the contributions of IRF3 and IRF7 to the lymphoid compartment stems from the above-mentioned observations that mice lacking components of the type I IFN system display defects in T cell development and function that render them susceptible to autoimmune disorders [19-21]. In chapter 1 of this dissertation, we examine the role of IRF3 and IRF7—the IRFs governing type I IFN production—in T cell responses. As the majority of our findings focus on IRF3, the remainder of this introduction will highlight our current knowledge of IRF3 function within innate and adaptive immune responses.

IRF3 was discovered nearly 20 years ago as a regulatory component of virus infected cells [25]. This transcription factor is constitutively expressed in nearly all cell types, mainly residing in the cytoplasm under steady state conditions [26]. IRF3 possesses an amino (N)-terminal DNA-binding domain (DBD), an IRF-association domain, and a carboxy (C)-terminal auto-inhibitory (regulatory) domain. Upon pathogen detection, phosphorylation of serine residues located in the regulatory domain of IRF3 allows its homo- and heterodimerization (with IRF7), nuclear translocation, and association with
chromatin modifiers such as CREB-binding protein (CBP) and p300 [27]. Once in the nucleus, the DBD of IRF3 binds to ISRE sites (GAAANNGAAANN) within target genes to promote their transcription.

Although IRF3 was first identified in the context of viral infections, the discovery of TLRs extended the role of IRF3 to host immune responses against bacterial infections. IRF3 was found to be a major component of the signaling pathway triggered downstream of TLR4 in response to lipopolysaccharide (LPS), a component of Gram-negative bacteria [28, 29]. More recently, IRF3 has been shown to alter T helper (T\textsubscript{H}) cell differentiation by modulating the function of antigen-presenting cells (APCs). In LPS-stimulated macrophages and dendritic cells (DCs), several cytokines involved in T\textsubscript{H}1 and T\textsubscript{H}17 differentiation were found to be direct or indirect targets of IRF3 [30]. Hence, through modulation of APC function, IRF3 limits T\textsubscript{H}1 and T\textsubscript{H}17 differentiation. As T\textsubscript{H}1 and T\textsubscript{H}17 cells are drivers of T cell-mediated inflammatory conditions, these findings have implications for disease outcomes. In fact, animals lacking IRF3 expression have been shown to be more susceptible to the development of certain T cell-driven autoimmune and inflammatory conditions [30].

In this dissertation, we discuss the complex role of IRF3 in shaping T\textsubscript{H} cell cytokine responses. We show that IRF3 has distinct and opposing roles in APCs and T cells in shaping the outcome of T\textsubscript{H} cell differentiation, and we discuss the implication of these findings in immune-mediated disorders. Our discovery of a direct involvement of IRF3 in T\textsubscript{H} cell responses is novel and original, and identifies IRF3 as a key player in adaptive immune responses.
CHAPTER 1: INTERFERON REGULATORY FACTOR 3 (IRF3) IN T CELL 
ACTIVATION, DIFFERENTIATION, AND CYTOKINE PRODUCTION

Abstract

Interferon regulatory factor 3 (IRF3) plays an essential role in innate immune responses against pathogens. However, its role in adaptive immunity has remained largely unexplored. In this report we reveal a novel function of IRF3 in the control of CD4+ T cell responses. We demonstrate that whereas T cell development remains intact in IRF3-/- mice, IRF3-/- CD4+ T cells show impaired production of the pro-inflammatory cytokines IL-17 and IFN-γ. As such, IRF3-/- CD4+ T cells fail to induce disease in the T cell transfer model of colitis. The decrease in IL-17 and IFN-γ production is not due to a defect in Th17 or Th1 lineage commitment, as IRF3-/- CD4+ T cells cultured under Th17- and Th1-polarizing conditions express similar levels of RORγt and T-bet as their wild type counterparts. Instead, IRF3 appears to promote the production of these Th17- and Th1-associated cytokines on a transcriptional level. Unexpectedly, we observe IRF3 nuclear translocation in activated T cells cultured under neutral, Th17-, and Th1-polarizing conditions. As we do not detect nuclear IRF3 expression in resting T cells, these results indicate that IRF3 is activated downstream of TCR engagement. To our knowledge, this is the first description of IRF3 activation following TCR stimulation. Our findings reveal a previously unappreciated role of IRF3 in CD4+ T cell responses and extend its function beyond the innate immune response to pathogens.
**Introduction**

Interferon regulatory factors (IRFs) were first identified as transcriptional mediators of type I interferon (IFN) production and signaling. To date, 9 mammalian family members have been characterized (IRF1-9). Though IRFs were initially identified and studied in the context of type I IFN signaling and innate immune responses, it is now appreciated that they play a much broader role in host defense, including CD4\(^+\) T cell differentiation [31, 32].

After TCR activation, naïve CD4\(^+\) T cells have the ability to differentiate into one of several T helper (T\(_H\)) cell subsets based on the surrounding microenvironment. These T helper cells perform distinct biological functions and aid in the clearance of both intracellular and extracellular pathogens. CD4\(^+\) T cells were initially characterized into two subsets—T\(_{H1}\) and T\(_{H2}\) cells [33]. T\(_{H1}\) cells are defined by their production of IFN-\(\gamma\) and are important in the clearance of intracellular pathogens. T\(_{H2}\) cells are defined by their production of IL-4 and support humoral immunity against extracellular infections. More recently, a new subset of T helper cell has been identified, called T\(_{H17}\) cells [34, 35]. These cells—defined by their production of IL-17A, IL-17F, and IL-22—are now thought to be major players in the development of T cell-driven inflammatory diseases.

Several IRFs are expressed in T cells and have been shown to intrinsically modulate T helper cell differentiation. For example, IFN-\(\gamma\)-induced IRF1 activates the IL12rb1 promoter in CD4\(^+\) T cells, resulting in up-regulation of IL-12R\(\beta1\) [36]. IRF1 was additionally shown to repress IL-4 gene expression in CD4\(^+\) T cells in response to IFN-\(\gamma\), therefore skewing helper T cell differentiation toward the T\(_{H1}\)
type [37]. IRF4 promotes the expression of GATA-3 [38] and RORγt/RORα [39, 40], thereby facilitating T\textsubscript{H}2 and T\textsubscript{H}17 development, respectively. IRF8, which is expressed in activated T cells, interacts with RORγt and represses IL-17 transcription, limiting T\textsubscript{H}17 responses [41].

IRF3 was first identified nearly 20 years ago [25]. This transcription factor is constitutively expressed as an inactive monomer in the cytoplasm of most nucleated cells. Upon pathogen recognition, IRF3 becomes phosphorylated by TANK-binding kinase 1 (TBK1) or IκB kinase ε (IKKe). The resulting conformational change in IRF3 enables its dimerization and nuclear translocation, where it promotes the transcription of type I IFN and IFN-stimulated genes (ISGs).

Whereas the role of IRF3 in innate immune responses to pathogens has been well established, its role in adaptive immune responses remains less understood. Previous studies have revealed a role for IRF3 in limiting T\textsubscript{H}1 and T\textsubscript{H}17 polarization by modulating the function of antigen-presenting cells (APCs) [42, 43]. More recently, a report demonstrated that IRF3 directly interacts with RORγt in the cytoplasm of CD8\textsuperscript{+} T cells and limits its ability to bind to the IL-17 promoter, thus negatively regulating T\textsubscript{C}17 cell polarization [44].

In this study, we reveal a novel function of IRF3 in CD4\textsuperscript{+} T cell responses. We first observed an increase in IRF3 expression following CD4\textsuperscript{+} T cell activation, suggesting that IRF3 is involved in T cell responses. Using a combination of in vitro T\textsubscript{H} cell polarization assays and an in vivo model of colitis, we demonstrate that IRF3 is a positive regulator of IL-17 and IFN-γ production in
CD4+ T cells. We show that IRF3-deficient CD4+ T cells have impaired IL-17 and IFN-γ production, and are unable to induce disease in the T cell transfer model of colitis. IRF3 appears to promote IL-17 (and possibly IFN-γ) production transcriptionally, as suggested by decreased transcript levels in polarized TH cells lacking IRF3 expression. These results reveal a novel, T cell-specific function of IRF3 in pro-inflammatory immune responses.

Materials and Methods

Mice. IRF3−/− and IRF7−/− mice have been described previously [45] [46]. Wild type C57BL/6, RAG1−/−, and ROSA<sup>mT/mG</sup> mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice used in these experiments were housed in a pathogen-free environment and were bred and cared for in accordance with UCSD Animal Care Facility regulations. All studies involving animals have been approved by the UCSD Animal Subject Committee (Protocol S02194).

Colitis induction. Spleens and/or lymph nodes from 8-12 week old donors were disrupted and pooled. CD4+ T cells were purified by magnetic separation (STEMCELL Technologies, Vancouver, BC) and CD4+CD45RB<sup>hi</sup> cells were further sorted using a FACSARia (BD Biosciences, San Jose, CA). 4x10<sup>5</sup> T cells were transferred (i.p.) into RAG1−/− recipients. Mice were weighed twice weekly and monitored for signs of disease.

Histology. Colon tissues were fixed for 24 hours in 10% buffered formalin and transferred to 70% ethanol. Tissues were paraffin embedded and stained with hematoxylin and eosin (H&E) at the UCSD Cancer Center Histology Core.
Blinded histopathological analysis was performed using the following scoring criteria (adapted from [47]): 1) degree of inflammation in lamina propria (score 0–3); 2) goblet cell loss (score 0–2); 3) abnormal crypts (score 0–3); 4) presence of crypt abscesses (score 0–1); 5) mucosal erosion and ulceration (score 0–1); 6) submucosal spread to transmural involvement (score 0–3). Total histopathological score was calculated by combining the scores for each of the parameters for a maximum score of 13.

**Image acquisition.** H&E images were acquired on a Nikon Eclipse E800 (10x objective lens, NA 0.25) equipped with a CRI Nuance FX multispectral imaging system (2.10.0). Scale bars were created with ImageJ.

**Isolation of colon lamina propria (LP) T cells.** Colons were washed in RPMI 1640 and cut into 1 cm pieces. Tissues were incubated (37°C, 20 minutes, 200 rpm) in 10 mL RPMI containing penicillin/streptomycin (P/S), 5% FBS, 5 mM EDTA, 20 mM HEPES, and 1M DTT. Tissues were then washed 3x in RMPI containing P/S, 2 mM EDTA, 20 mM HEPES. Tissues were further cut into smaller pieces and incubated (37°C, 30 minutes, 200 rpm) in 10 mL RPMI containing P/S, 20 mM HEPES, 0.1 mg/mL Liberase TL (Roche, Risch-Rotkreuz, Switzerland), and 0.05% DNase1. Enzymes were inactivated by the addition of 10 mL cold RPMI complete (P/S, 10% FBS, 20 mM HEPES, β-ME, non-essential amino acids). Supernatants were poured through a 70 μm filter and the tissue was agitated with a syringe plunger to extract the remaining cells. Cells were resuspended in 47% percoll (1.124) and spun at 1500 rpm at 4°C for 10 minutes. LP T cells were collected from the cell pellet.
**T<sub>reg</sub> suppression assays.** Conventional (CD4<sup>+</sup>, CD25<sup>-</sup>) and regulatory (CD4<sup>+</sup>CD25<sup>+</sup>) T cells were isolated by magnetic separation (Miltenyi, Bergisch Gladbach, Germany). T<sub>conv</sub> cells were labeled with CFSE and activated with 0.5 μg/mL α-CD3 (eBioscience, San Diego, CA) and 1 μg/mL α-CD28 (eBioscience) soluble antibodies in the presence or absence of T<sub>reg</sub> cells. T<sub>conv</sub> proliferation was assessed by CFSE dilution at the indicated time points.

**T cell polarization.** Naïve CD4<sup>+</sup> T cells were enriched by magnetic separation (STEMCELL Technologies). Cells were stimulated with 5 μg/mL plate-bound α-CD3 (eBioscience, San Diego, CA) and 1 μg/mL soluble α-CD28 (eBioscience) antibodies in RPMI complete (RPMI 1640, 10% FBS, non-essential amino acids, sodium pyruvate, β-ME, penicillin/streptomycin/glutamate) for 96 hours. Th1/Th2 control: IL2 (50 U/mL), α-IL12 (10 μg/mL), α-IL4 (10 μg/mL), α-IFNγ (10 μg/mL). Th1: IL2 (50 U/mL), IL12 (100 U/mL), α-IL4 (10 μg/mL). Th2: IL2 (50 U/mL), IL4 (20 ng/mL), α-IL12 (10 ng/mL), α-IFNγ (10 μg/mL). Th17/iTreg control: IL2 (50 U/mL). Th17: TGFβ (2 ng/mL), IL6 (10 ng/mL). iTreg: TGFβ (5 ng/mL), IL2 (50 U/mL). Cells were re-stimulated with 5 ng/mL phorbol myristate acetate (PMA) and 500 ng/mL ionomycin (Sigma-Aldrich, St. Louis, MO) for 4-5 hours before flow cytometric analysis. All cytokines used in these experiments were purchased from PeproTech (Rocky Hill, NJ). Blocking antibodies were obtained from Bio X Cell (West Lebanon, NH).

**Flow cytometric analysis.** Single cell suspensions were prepared in FACS buffer (PBS pH 7.4, 1% FBS, 0.02% NaN<sub>3</sub>) and stained for 15 minutes in
the dark on ice. Intracellular staining was performed using the Intracellular Fixation and Permeabilization Buffer Set (with Brefeldin A) and the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer’s instructions. CD3ε (145-2C11), CD4 (GK1.5), CD45RB (C363.16A), IFN-γ (XMG1.2), T-bet (eBio4B10), IL-4 (BVD6-24G2) Gata-3 (TWAJ), IL-17A (eBio17B7), Rorgt (B2D), Foxp3 (FJK-16s), α4β7 (DATK-32) (eBioscience). All samples were analyzed on a FACSCalibur (BD Biosciences) and processed using Flow Jo (Tree Star, Ashland, OR).

**Quantitative PCR.** RNA was isolated using an RNeasy kit (QIAGEN, Hilden, Germany) and cDNA was synthesized using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA). Quantitative PCR analysis was performed using iTaq Universal Probes Supermix (Bio-Rad, Hercules, CA) and TaqMan probes (Thermo Fisher Scientific, Waltham, MA): Rn18s (Mm03928990_g1), Irf3 (Mm00516784_m1), Irf7 (Mm00516793_g1), Ifng (Mm01168134_m1), T-bet (Mm00450960_m1), Rorc (Mm01261022_m1), Il17a (Mm00439618_m1), Il17f (Mm00521423_m1).

**Calcium mobilization.** T cells were loaded with Fluo-4 AM and Fura Red dyes according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). A baseline reading was taken for 30 seconds. 10 μg/mL of hamster α-CD3 antibody (eBioscience) was added and data was collected for 1 minute prior to the addition of 25 μg/mL α-hamster IgG antibody (eBioscience). Readings were continued for a total of 5 min. Data represent the ratio of Fluo-4 AM/Fura Red.
**TCR (MAPK and NF-κB) signaling.** T cells were purified by magnetic separation (STEMCELL Technologies) and serum-starved overnight in RPMI 1640 + 0.2% FBS. Cells were incubated for 30 minutes on ice with 5 μg/mL α-CD3 (eBioscience) and 1 μg/mL α-CD28 (eBioscience) antibodies followed by 15 minutes on ice with 10 μg/mL of IgG crosslinking antibody (eBioscience). Samples were transferred to a 37°C water bath and incubated for the indicated times. Signaling was terminated by the addition of cold media.

**Preparation of cytoplasmic and nuclear extracts.** T cells were washed 1x with PBS, resuspended in cytoplasmic lysis buffer* (20 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl2), and incubated for 20 minutes on ice. Samples were treated with NP-40 (0.5% final concentration), vortexed briefly, and centrifuged at 12,000 x g for 30 seconds at 4°C. Cytoplasmic extracts were cleared and stored at -80°C. Pellets containing the nuclei were washed 3x in cytoplasmic lysis buffer (12,000 x g, 30 seconds, 4°C), resuspended in nuclear extraction buffer* (20 mM Hepes, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl2, 25% glycerol), and incubated on ice for 30 minutes with periodic vortexing. Samples were centrifuged at full speed for 5 minutes at 4°C and nuclear lysates were stored at -80°C. *DTT (1 mM final), protease inhibitors, and phosphatase inhibitors were added to buffers before use.

**Western blotting.** T cells were lysed as described above and subjected to SDS-PAGE and Western blot. Blots were probed for IRF3 (D83B9, #4302), Histone H3 (D2B12, #4620), p44/42 MAPK (137F5, #4695), phospho-p44/42 MAPK (Thr202/Tyr204, #9101), p38 MAPK (D13E1, #8690), phospho-p38 MAPK
Thr180/Tyr182, #9211, IκB-α (#9242)β-actin (13E5, #4970), and GAPDH (14C10, #2118) (Cell Signaling Technology, Danvers, MA).

**Results**

**Activated CD4⁺ T cells up-regulate IRF3 expression.** Our group has previously shown that IFNAR⁻/⁻ and STAT1⁻/⁻ mice exhibit disturbances in T cell development and function that render them susceptible to autoimmunity [19, 20]. To investigate whether the IRFs controlling type I IFN production also play a role in T cell responses, we first assessed the expression of IRF3 and IRF7 in CD4⁺ T cells. We observed an increase in IRF3 message (Figure 1-1A) and protein levels (Figure 1-1B) following CD4⁺ T cell activation. This increase was not observed in the closely related IRF7 (Figure 1-1A). Of note, we did not observe IRF3 nuclear translocation during the time points examined (data not shown). Since these results suggested that IRF3 might play a role in CD4⁺ T cell responses, we next addressed the functional consequences of IRF3 expression in T cell development and function.

![Figure 1-1. IRF3 up-regulation following T cell activation.](image)

Naive CD4⁺ T cells were activated with plate-bound α-CD3 (5 μg/mL) and soluble α-CD28 (1 μg/mL) antibodies for the indicated times. A) IRF3 transcript levels (mean ± SEM) were assessed by quantitative PCR. Results were pooled from 5 independent experiments. B) IRF3 protein levels were detected by western blot (representative of 3 independent experiments). Statistical significance was determined by Student’s t-test (**P≤.01, ***P≤.001).
**T cell development remains intact in IRF3-deficient mice.** To examine the role of IRF3 in T cell development, we assessed T cell populations in IRF3−/− mice. We observed that CD4+ and CD8+ T cell proportions in the lymphoid organs of IRF3−/− mice were roughly similar to that of wild type mice (Figure 1-2A-C, left panels). However, IRF3−/− mice harbored significantly increased T cell numbers in the thymus (Figure 1-2A, right panel). Interestingly, this increase in T cell number was not as apparent in the spleen (Figure 1-2B, right panel) and was not observed in the lymph nodes (Figure 1-2C, right panel). In further assessing T cell populations, we observed that resting T cells from IRF3−/− mice showed similar expression of cell surface activation markers as their wild type counterparts (Figure 1-2D). Combined, these results indicate that T cell development remains intact in mice lacking IRF3 expression, however peripheral T cell homeostasis/persistence may be altered.

Signaling through Toll-like receptor 3 and 4 (TLR3 and TLR4) has been shown to enhance CD4+ T cell survival, though the mechanisms governing this survival remain unclear [48, 49]. Given that both TLR3 and TLR4 signal through IRF3, one explanation for the discrepancy between T cell numbers in the thymus and the periphery could be that tonic signaling through TLR3 and TLR4 enhances T cell homeostasis in the periphery, where T cells are more likely to encounter TLR3 and TLR4 ligands. However, we did not pursue this issue further during the course of this study.
Figure 1-2. T cell development remains intact in IRF3−/− mice. A-D) Tissues from age- and sex-matched mice were processed into single-cell suspensions and stained with the indicated antibodies (n=7-8 mice/group). D) Representative histograms are shown (gated on CD4+ T cells). Statistical significance was determined by Student’s t-test (*P≤.05, **P≤.01).
IRF3$^{-/-}$ T cells exhibit enhanced proliferation. We next addressed the functional consequences of IRF3 expression in CD4$^+$ T cell responses using in vitro assays for T cell proliferation and T$_{reg}$ cell-mediated suppression. We observed that IRF3$^{-/-}$ T cells are more proliferative than their wild type counterparts as determined by CFSE dilution profiles (Figure 1-3A). Interestingly, we also observed that IRF3$^{-/-}$ conventional T cells appear to be resistant to T$_{reg}$ cell-mediated suppression (Figure 1-3B, lower panels). In contrast, IRF3$^{-/-}$ T$_{reg}$ cells exhibited normal behavior, as indicated by their ability to inhibit the proliferation of wild type conventional T cells (Figure 1-3B, top right panel).

The reason for the observed hyperproliferation of IRF3$^{-/-}$ T cells remains elusive. Engagement of the TCR, along with stimulation through the CD28 co-receptor, activates multiple downstream signaling pathways including the MAP kinases (MAPK), NF-κB, and increases in cytoplasmic free calcium [50, 51]. We detected normal rates of apoptosis and MAPK signaling in IRF3$^{-/-}$ T cells following TCR stimulation (Figure 1-4A,B). However, we did observe slightly enhanced calcium mobilization and NF-κB signaling (as determined by IκB$\alpha$ degradation) following TCR engagement (Figure 1-4C,D). These results could explain the increased proliferation observed in IRF3$^{-/-}$ T cells, though further investigation is needed to fully elucidate the mechanism behind these findings.
Figure 1-3. IRF3−/− T cells show increased proliferation and decreased susceptibility to T_reg cell-mediated suppression compared to wild type T cells. Naïve CD4+ conventional T cells (T_conv) were CFSE-labeled and activated with soluble α-CD3 (0.5 μg/mL) and α-CD28 (2.5 μg/mL) antibodies in the presence (B) or absence (A) of regulatory T cells (T_reg). Proliferation was assessed after 72 hours. Results are representative of 3 individual experiments.
IRF3-/- T cells show impaired IFN-γ and IL-17 production. Since several IRFs have been shown to intrinsically modulate T<sub>H</sub> cell differentiation [31, 32], we next assessed whether IRF3 is also involved in the process of T<sub>H</sub> cell differentiation. We co-cultured wild type (mT/mG) and IRF3-/- naïve CD4<sup>+</sup> T cells under T<sub>H</sub>1-, T<sub>H</sub>2-, T<sub>H</sub>17-, and T<sub>reg</sub>-polarizing conditions. As we wanted to exclude the involvement of IRF3 within APCs during T<sub>H</sub> cell differentiation, we activated T
cells using agonistic \( \alpha \)-CD3/CD28 antibodies in lieu of APCs. We observed a decrease in the percentage of IRF3\(^{-/-}\) T cells producing IFN-\( \gamma \) and IL-17 as compared to wild type T cells, which was more profound for the latter mentioned cytokine (Figure 1-5A,C). IL4 production and Foxp3 up-regulation appeared normal (Figure 1-5B,D), indicating that the decrease in IFN-\( \gamma \) and IL-17 production was not due to a complete disturbance in \( T_H \) cell differentiation, nor the result of pan-cytokine suppression. As this manuscript was in preparation, another group reported that IRF3 expression was essential in both APCs and T cells for optimal IFN-\( \gamma \) and IL-17 production [52]. These findings support our conclusion that IRF3 intrinsically modulates IFN-\( \gamma \) and IL-17 production in T cells.

To determine whether the decrease in IFN-\( \gamma \) and IL-17 production was due to impaired \( T_H1 \) and \( T_H17 \) lineage commitment, we looked at T-bet and \( ROR_\gamma t \) levels in wild type and IRF3\(^{-/-}\) T cells polarized under \( T_H1 \)- and \( T_H17 \)-conditions, respectively. The percentage of wild type and IRF3\(^{-/-}\) T cells expressing T-bet and \( ROR_\gamma t \) was comparable between wild type and IRF3\(^{-/-}\) T cells, indicating that IRF3\(^{-/-}\) T cells are capable of responding to polarizing stimuli (Figure 1-5E,F). However, the MFI of \( ROR_\gamma t \) in \( ROR_\gamma t^+ \) IRF3\(^{-/-}\) T cells appears to be slightly lower than that of wild type T cells (Figure 1-5F). This slight decrease in \( ROR_\gamma t \) expression may partly account for the reduced IL-17 production seen in IRF3\(^{-/-}\) T cells. Overall, these results indicate that the decrease in IFN-\( \gamma \) and IL-17 production does not appear to be a defect in lineage commitment, but rather a defect in the production of \( T_H1 \)- and \( T_H17 \)-associated cytokines.
Figure 1-5. IRF3−/− T cells display normal lineage commitment but impaired cytokine production under Th1- and Th17-polarizing conditions. Naive CD4+ T cells from wild type (mT/mG) and IRF3−/− mice were co-cultured under Th1-, Th2-, Th17-, and Treg-polarizing conditions. Cytokine production and transcription factor expression were assessed after 96 hours. Results were pooled from 6 (A,C), 4 (B,D) or 2 (E,F) independent experiments. Numbers in flow plots represent the percentage of positive cells within each population. Bar graphs represent mean ± SEM. Statistical significance was determined by Student’s t-test (*P≤.05, **P≤.01).

IRF3 transcriptionally regulates IL-17 production. Given the role of IRF3 as a transcription factor, we next assessed whether IRF3 promotes IFN-γ and IL-17 transcription. We observed that IRF3−/− Th1-polarized cells showed only slightly decreased Ifng transcript levels compared to wild type T cells (Figure 1-6A). In contrast, Il17a and Il17f transcript levels were noticeably decreased in
IRF3−/− T\textsubscript{H}17-polarized cells (Figure 1-6B), suggesting that IRF3 directly or indirectly controls IL-17 production transcriptionally. This conclusion is supported by our observation that IRF3 undergoes nuclear translocation in T\textsubscript{H}17-polarized cells (Figure 1-6C). Interestingly, we also detect nuclear IRF3 expression under neutral and T\textsubscript{H}1-polarizing conditions (Figure 1-6C), suggesting that events downstream of TCR stimulation may be activating IRF3. To our knowledge, this is the first description of IRF3 activation following TCR engagement.

Figure 1-6. IRF3 transcriptionally regulates IL-17 production. Naive CD4\textsuperscript{+} T cells from wild type and IRF3−/− mice were cultured under T\textsubscript{H}0-, T\textsubscript{H}1- and T\textsubscript{H}17-polarizing conditions for 96 hours. A-B) T\textsubscript{H}1- and T\textsubscript{H}17-associated transcripts were assessed by quantitative PCR. Results were pooled from 2 independent experiments. C) Cytoplasmic and nuclear fractions were analyzed by Western blot. Results are representative of 3 individual experiments. Bar graphs represent mean ± SEM. Statistical significance was determined by Student’s t-test (*P≤.05).
**IRF3⁻/⁻ T cells do not induce colitis due to impaired IL-17 and IFN-γ production.** Since a conditional IRF3⁻/⁻ mouse model is unavailable, we employed the T cell transfer model of colitis to assess the role of IRF3 specifically within CD4⁺ T cells in vivo. In this model, RAG⁻/⁻ mice receive a single injection of naïve CD4⁺ T cells which mediate the development of colitis—characterized by progressive weight loss and colonic tissue destruction [53]. Interestingly, we observed that mice receiving IRF3⁻/⁻ CD4⁺ T cells did not exhibit signs of colitis, such as weight loss (Figure 1-7A). Upon histological examination, the colons from these mice displayed limited signs of inflammation as compared to mice that received wild type CD4⁺ T cells (Figure 1-7B,C). In contrast, CD4⁺ T cells deficient in the closely related IRF7 were fully capable of mediating disease (Figure 1-7D). These results suggest a T cell-intrinsic function for IRF3 in T cell-driven inflammatory disease, likely due to its role in IFN-γ and IL-17 production.

Interestingly, whereas IRF3 and IRF7 are thought to play functionally redundant roles in innate immune responses, mainly offset temporally, we observe that IRF3 and IRF7 have distinct functions within adaptive immunity. We have previously shown that type I IFN production driven by IRF7, but not IRF3, is essential for the development of medullary thymic epithelial cells—the cell type responsible for mediating negative selection of auto-reactive T cells [21]. In contrast, this study shows that CD4⁺ T cells up-regulate IRF3, but not IRF7, following TCR engagement, and that CD4⁺ T cells lacking IRF3, but not IRF7, fail to induce disease in the T cell transfer model of colitis.
Figure 1-7. IRF3−/− T cells do not induce colitis. A-C) 4x10⁵ naive CD4⁺ T cells (CD45RB⁺CD25⁻) from wild type or IRF3−/− mice were injected (i.p.) into RAG−/− hosts. Mice were monitored for weight loss (mean ± SEM) and colon histology was assessed by H&E staining at the indicated time (n=4-5 mice/group, representative of 4 independent experiments). D) 4x10⁵ naive CD4⁺ T cells from wild type or IRF7−/− mice were injected (i.p.) into RAG−/− hosts. Colon histology was assessed by H&E staining 5 weeks post T cell transfer (n=4 mice/group). Statistical significance was determined by Student’s t-test (*P≤.05). Scale bars = 0.1 mm.
To validate our in vitro findings showing impaired IFN-γ and IL-17 production in IRF3−/− T cells, we co-transferred naïve CD4+ T cells from wild type (mT/mG) and IRF3−/− mice into RAG−/− hosts (Figure 1-8A). Mice receiving this mixed population of CD4+ T cells showed signs of colitis, indicating that IRF3−/− T cells are intrinsically unable to mediate disease rather than actively inhibiting the development of colitis (Figure 1-8B). To rule out a migration defect, we assessed T cell expression of α4β7 integrin—the integrin required for T cell migration into the colon—in the mesenteric lymph nodes and looked at T cell populations in the colon. IRF3−/− T cells were able to up-regulate α4β7 expression and migrate to the colon similarly to wild type T cells (Figure 1-8C,D), excluding the possibility of a migration defect.

We next assessed cytokine production by T cells from the gut-draining lymph nodes. IRF3−/− CD4+ T cells isolated from the mesenteric lymph nodes of colitic RAG−/− hosts showed impaired production of IL-17 and, to a lesser extent, IFN-γ compared to their co-transferred wild type counterparts (Figure 1-8E). As such, we conclude that IRF3−/− CD4+ T cells could not induce colitis due to an inability to produce the proper inflammatory cytokines.

Of note, we observed a decreased proportion of IRF3−/− CD4+ T cells in the mesenteric lymph nodes as compared to wild type T cells, even though equal numbers of cells were initially injected (Figure 1-8F). As mentioned earlier, signaling through TLR3 and TLR4 has been shown to enhance CD4+ T cell survival, though the mechanisms governing this survival remain unclear [48, 49]. Since both of these TLRs signal through IRF3, it is possible that TLR activation
and subsequent signaling through IRF3 may be required for adequate T cell persistence in this disease model. In addition to impaired IL-17 and IFN-\(\gamma\) production, this potential decrease in CD4\(^+\) T cell survival/persistence could partly account for the reduced symptoms of colitis seen in mice receiving IRF3\(^{-/-}\) CD4\(^+\) T cells.
Figure 1-8. Impaired in vivo IFN-γ and IL-17 production in IRF3−/− T cells. 4x10^5 naive CD4+ T cells (CD45RBhiCD25−) from wild type (mT/mG) and IRF3−/− mice were co-injected (i.p.) into RAG−/− hosts. A) Experimental design. B) H&E staining of colon sections. C) α4β7 integrin staining in the mesenteric lymph nodes. D) T cell populations in the colon. E,F) Cytokine production and distribution of CD4+ T cells isolated from the mesenteric lymph nodes. Results were pooled from 2 independent experiments for a total of 8 mice. Bar graphs represent mean ± SEM. Statistical significance was determined by Student’s t-test (**P≤.001). Scale bars = 0.1 mm.
**Discussion**

Previous studies investigating the role of IRF3 in the context of autoimmunity have produced opposing results. Mice deficient in IRF3 have been shown to be more susceptible to both DSS-induced colitis [54] and EAE [55]. However, unlike the current study, these studies were performed in mice lacking IRF3 expression in all cells. In alignment with our current results, a recent study using a T cell transfer model of EAE showed that mice receiving pre-activated, myelin-specific IRF3−/− T cells did not develop EAE due to decreased IFN-γ and IL-17 production [56]. However, the T cells used for these experiments were obtained from total IRF3−/− mice immunized with MOG33-35 peptide in complete Freund’s adjuvant (CFA), obscuring any interpretation of IRF3 function specifically within the T cell compartment.

Both IRF4 and IRF8 have been shown to intrinsically modulate IL-17 production in CD4+ T cells. IRF4−/− CD4+ T cells display decreased RORγt and increased Foxp3 levels under Th17-polarizing conditions, resulting in decreased IL-17 production [39, 40]. In contrast, IRF8 was shown to silence IL-17 transcription in part through its interaction with RORγt [41]. IRF3 and IRF8 have been shown to interact in human blood monocytes [57]. It is plausible that IRF3 and IRF8, both of which are induced in activated T cells, interact during Th17 differentiation. This interaction may prevent IRF8 from associating with RORγt and inhibiting IL-17 transcription. Additionally, IRF3 was shown to bind to RORγt within CD8+ T cells [44]. It is also plausible that IRF3 may interact with RORγt in
CD4+ T cells and limit IRF8 binding. A third possibility is that IRF3 may directly bind to and activate the IL-17 promoter. This possibility is supported by our observation that IRF3 can be detected in the nuclei of T\textsubscript{h}17-polarized cells. Further studies will address the molecular pathways associated with IRF3 regulation of IL-17 production.

While the type I IFN system has been studied overwhelmingly in the context of pathogen response, it is now appreciated that this system plays an important role in immune modulation [18]. In this study, we focus on the role of IRF3 and IRF7 (the IRFs governing type I IFN production) in T cell responses. We show that CD4+ T cells lacking IRF3, but not IRF7, fail to induce colitis due to disturbances in IL-17 and IFN-\textgamma production.

It was previously reported that IRF3\textsuperscript{−/−} mice are more susceptible to the development of certain autoimmune disorders, likely due to increased T\textsubscript{h}1 and T\textsubscript{h}17 differentiation as a result of altered cytokine production within IRF3\textsuperscript{−/−} APCs [42, 43]. In our studies we limited IRF3-deficiency to the T cell compartment by employing a disease model in which only T cells were transferred into RAG\textsuperscript{−/−} recipients, and used in vitro differentiation assays in which naïve CD4+ T cells were activated with agonistic \textgammaline-CD3/CD28 antibodies instead of APCs. Using these approaches we discovered that IRF3 was a positive regulator of IL-17 and IFN-\textgamma production within CD4+ T cells.

Since we limited IRF3-deficiency to the T cell compartment and used co-transferred and co-cultured wild type and IRF3\textsuperscript{−/−} CD4+ T cells in our experiments, we can rule out that a defect in type I IFN production was responsible for the
decreased IL-17 and IFN-γ production observed in IRF3−/− T cells. In fact, type I IFN has been shown to limit IL-17 production within CD4+ T cells [35], further eliminating this possibility.

Collectively, our results show that IRF3 positively regulates IL-17 and IFN-γ production in CD4+ T cells, independent of its role in type I IFN production. These findings reveal a novel function of IRF3 in CD4+ T cells and suggest that IRF3 could be a potential therapeutic target for T cell-mediated inflammatory diseases.

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CHAPTER 2: TYPE I INTERFERON IN T CELL RESPONSES

Abstract

Interferon beta (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. Here we demonstrate that in both mouse and human T cells, IFN-β 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, 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 up-regulation of the transcriptional suppressor CREM by IFN-β 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 IFN-β on IL-2 production. Collectively, these findings provide a molecular basis by which IFN-β limits T cell responses.

Introduction

Type I interferons (IFN-α/β) have been approved worldwide for the treatment of multiple sclerosis, yet the mechanism 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 [58]. Thus, a better understanding of the mechanism
behind the efficacy of IFN-β 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 [18, 20, 21, 59]. 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 [60, 61]. T helper (Th) lymphocytes, which play a key role in the 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 IFN-α/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 IFN-β 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+− [24], Tyk2+− [62], STAT5+− [63], and STAT3fl/fl [64] 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. Six- to ten-week-old mice were infected intravenously with 2 x 10^6 PFU/mouse wild type (WT) lymphocytic choriomeningitis virus (LCMV) clone 13 (Cl13). All viruses were grown, identified, and quantified as described previously [65].

**Flow-cytometric analysis.** For immunostaining, single-cell suspensions were prepared from mouse spleens with ~ 1 x 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 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 IFN-β (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 IFN-β (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 lonomycin (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 100 μg Ova323-336 peptide (Anaspec) 24 hours following injection with 10000 U IFN-β (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 [66]. 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 IFN-β (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,
Set B reverse: CATGGGAGGCAATTATACTGTTAATG,
Set C forward: CCTAAATCCATTCAGTCAGTGTATGG,
Set C reverse: TGTGTGGCAGAAAGCATTACCT,
Set D forward: CTTTTGTGTCTCCACCCCAAA,
Set D reverse: CACACTTAGGTGGCAGTTTTAATTCAT,
Set F forward: CATGCAGAGTTTTTTGTTGTTTTCTAG,
Set F 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 IFN-β. 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 \(\frac{\text{stimulated} - \text{inhibited}}{\text{stimulated}}\) ×100. Student's 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 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 [67]. 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 antibodies, splenic T cells of WT 129SveV mice produce IL-2 within 5 h as detected by intracellular flow cytometry staining (Figure 2-1A, second panel). However, upon pretreatment with IFN-β the number of IL-2 producing cells is significantly reduced (Figure 2-1A, third panel), an effect specific to IFN-α/β, as the type II
interferon, IFN-\(\gamma\), had no effect on IL-2 production (Figure 2-1A, right panel). In addition, IL-2 production was inhibited in both CD4\(^+\) (Figure 2-1A, upper row) as well as CD8\(^+\) (Figure 2-1A, 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 IFN-\(\beta\) also inhibited the production of IL-2 when T cells where stimulated with the mitogens PMA and Ionomycin (Figure 2-1B) or Concanavalin A (not shown). Inhibition of IL-2 release by IFN-\(\beta\) 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 2-1C and data not shown). Further experiments revealed that IFN-\(\beta\)-mediated inhibition of IL-2 production occurred on the transcriptional level, as IL-2 mRNA was also reduced as a consequence of IFN-\(\beta\) exposure prior to stimulation (Figure 2-1D). Strikingly, the inhibitory effect of IFN-\(\beta\) was restricted to IL-2, as neither IL-4 (Figure 2-1D) nor IFN-\(\gamma\) (not shown) mRNA, nor expression of the cell surface activation markers CD25 and CD44 (Figure 2-1E) were subdued by IFN-\(\beta\) pretreatment.
Figure 2-1. Specific inhibition of IL-2 expression by IFN-β. (A) Splenocytes from wild type 129/SvEv mice were treated with or without IFN-β or IFN-γ 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 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. 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.
Activated T cells often exhibit significant differences in their response to type I interferons as compared to naïve T cells [68]. We therefore investigated next whether IFN-β could inhibit IL-2 production in already activated T cells. CD4+ T cells were stimulated for 3 days with anti-CD3/CD28 (Figure 2-2A, right panels), and then incubated overnight with (Figure 2-2A, bottom) or without (Figure 2-2A, top) 1000 U/mL IFN-β. Subsequently, cells were stimulated with PMA/Iono for 5 h. IFN-β significantly reduced the number of IL-2 producing cells that were naive at the time of stimulation (Figure 2-2A, middle plots), but failed to inhibit IL-2 production if the cells were preactivated for 3 days with anti-CD3/CD28 (Figure 2-2A, 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 Figure 2-2B, the addition of IFN-β simultaneously or after TCR stimulation had little effect on IL-2 production, yet, 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 strongly suggested that new
gene expression and protein synthesis were required for inhibition of IL-2 production by IFN-β. Unfortunately, experiments utilizing cycloheximide were inconclusive due to the sensitivity of primary T cells to the agent (data not shown).

**Figure 2-2. Inhibition of IL-2 expression requires pre-treatment 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 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. IFN-β 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).
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 (T_{reg}) cells were required in order for IFN-β to inhibit IL-2 production. Indeed, it was shown recently that interferon is required for maintaining Foxp3 expression in T_{reg} cells during infection [69]. Therefore, we decided to eliminate T_{reg} cells from the CD4⁺ T cell cultures prior to IFN-β exposure. Although T_{reg} cells alone had, as expected, an intrinsic inhibitory effect on IL-2 production by CD4⁺ cells (Figure 2-3A, compare black bars), IFN-β was nevertheless still capable of significantly inhibiting IL-2 production even in the absence of T_{reg} cells (Figure 2-3A, compare gray bars). We therefore concluded that IFN-β acts on the responding effector T cells directly to inhibit IL-2 production independent of T_{reg} cells.

To address whether the inhibitory effect of IFN-β 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 IFN-β. Similar to their murine counterparts, human T cells, too, were inhibited in their ability to synthesize IL-2 when previously exposed to IFN-β (Figure 2-3B and C). Thus, IFN-β acts directly on human and murine T cells to inhibit activation-induced IL-2 production.
Figure 2-3. IFN-β inhibition of IL-2 expression is independent of T_{reg} 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 IFN-β followed by stimulation with anti-CD3/anti-CD28 for 4 h in the presence or absence of CD25^+ T_{reg} 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 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).
IFN-β-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 IFN-β 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 [19-21, 24], and we previously reported that the absence of STAT1 in T cells results in an unexpected mitogenic response to interferon [70]. This was corroborated in Figure 4A wherein IFN-β inhibited IL-2 production in WT T cells (Figure 2-4A, left panels), but strikingly caused a significant increase in IL-2 producing cells (Figure 2-4A, 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 (Figure 2-4A, second panels), whereas IL-2 release from T cells isolated from CreLck/STAT3loxp and STAT5-deficient mice was still inhibited by IFN-β pretreatment (Figure 2-4A, 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, NF-κB, PLC-γ, and increases in cytoplasmic free calcium [50, 51]. As 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, and the SAPKs p38 and JNK are not affected
by IFN-β pretreatment (Figure 2-4B), nor is there a reduction in cFos levels (Figure 2-4B) or an impairment of NF-κB signaling (data not shown) following 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 (Figure 2-4C). Furthermore, the use of various luciferase reporter constructs representing the major enhancer elements within the IL-2 promoter (e.g. NF-κB, NFAT, AP-1, etc.) [71] 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.
Figure 2-4. Inhibition of IL-2 by IFN-β 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 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 3-5 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 carried out 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 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).
**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 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 IFN-β 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 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 [72]. Purified CD4⁺ T cells were stimulated with anti-CD3/CD28 for 4 h with or without prior exposure to IFN-β. We utilized several primer sets within the 300 bp IL-2 promoter (Figure 2-5A: 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 IFN-β 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, IFN-β pretreatment significantly reduced the anti-CD3/CD28 induced change in the accessibility of the IL-2 locus (Figure 2-5B). 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 2-5C, 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 IFN-β negated the TCR-induced histone modifications (Figure 2-5C). 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 2-5D, left graph). More importantly, at higher concentrations Trichostatin A completely abrogated the inhibitory effect of IFN-β on IL-2 production (Figure 2-5D, right graph). 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 include the zinc finger transcription factors Aiolo 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 [73-77]. Our investigations did not reveal any involvement of Aiolos, Ikaros or Blimp in the inhibitory effects of IFN-β on IL-2 production (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 up-regulated in these T cells by IFN-β. This was confirmed by western blot analysis where there is a dramatic induction of CREM protein expression in response to 16 h IFN-β treatment (Figure 2-5E). Analysis by quantitative PCR revealed a STAT1 dependent induction of CREM mRNA within 5 hours, similar to that of interferon stimulated gene 15 (ISG15) (Figure 2-5F).

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 Figure 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 (Figure 2-5G) completely eliminated the suppressive effects of IFN-β (Figure 2-5H), followed by intracellular stain for IL-2, data not shown. 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 IFN-β.
Figure 2-5. 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 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 IFN-β 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 is shown on the right. (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 129WT or STAT1⁻/⁻ mice stimulated with IFN-β 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 IFN-β 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. The average % inhibition of IL-2 production was determined from 4 independent experiments.
Inhibition of IL-2 production in T cells from mice treated with IFN-β or infected with LCMV Clone 13. 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 hours prior to challenge with Ova\textsubscript{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\textsubscript{323-339} peptide, whereas very few IL-2 producing T cells were found in the spleens of IFN-β-treated mice (Figure 2-6A, middle row, averages shown in Figure 2-6B). Importantly, there was only little difference in the number of Ova\textsubscript{323-339} specific T cells in the spleens of interferon-treated mice compared to untreated mice (Figure 2-6A, top row), nor was there a difference in the number of activated T cells in interferon-treated versus untreated mice as determined by the up-regulation of CD69, CD44 and downregulation of CD62L (Figure 2-6A, lower 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 clone 13 [78], and it was recently shown that blocking type I interferon rescued T cell function in such chronically infected mice [79, 80]. In concurrence, T cells from LCMV CI13-infected mice (day 9) expressed substantially elevated levels of CREM as visualized by intracellular staining and flow cytometry (Figure 2-6C, average of multiple mice in Figure 2-6D), and produced less IL-2 than T cells from uninfected mice (Figure 2-6E, average of multiple mice in Figure 2-6F). 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, but also offers a molecular mechanism for the T cell exhaustion observed during chronic infections due to the substantial amounts of endogenous IFN-β.
Figure 2-6. Inhibition of IL-2 production in T cells from mice injected with IFN-β or infected with LCMV Cl13. (A) OTII TCR transgenic mice were injected with 100µg Ova\textsubscript{323-339} 24 h after i.v. injection of 10,000 U IFN-β. 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 V\textalpha\textbeta\textgamma\textdelta 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\textsuperscript{+} cells are shown. (D) Graph represents % CREM positive CD4\textsuperscript{+} T cells from 5 uninfected and 7 LCMV Cl13 infected mice stained as in (C). (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 (E).
Discussion

Type I interferons have been extensively studied as part of the innate immune response, yet 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 IFN-β 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 IFN-β limits the number of T cells that are 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. 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 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 we observe an increase in CREM levels in T cells from mice chronically infected with LCMV Cl13 (Figure 2-6C), with an accompanying reduction in the IL-2 production from these animals (Figure 2-6E). 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 [78]. 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 [79, 80]. 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) [81-83], 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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Chapter 2, in full, is a reprint of the material as it appears in *J Immunol* 2015; 194:5120-5128. IFN-β Selectively Inhibits IL-2 production through CREM-mediated chromatin remodeling. Otero, Dennis C.; Fares-Frederickson, Nancy J.; Xiao, Menghong; Baker, Darren P.; David, Michael. Nancy J. Fares-Frederickson was a co-author on this study.
CONCLUDING REMARKS AND REMAINING QUESTIONS

While the type I IFN system has been studied overwhelmingly in the context of innate immune responses against viral infections, it is now appreciated that type I IFNs play an important role in immune cell modulation in both health and disease [18]. Previous work from our group has shown that mice lacking components of the type I IFN signaling axis display defects in thymic processes that render them susceptible to the development of autoimmune conditions [19-21]. Most recently, our group published a report demonstrating the inhibitory effect of IFN-β on T cell responses [22]. These recent results are discussed in chapter 2.

The focus of this report, as discussed in chapter 1, highlights the role of IRF3 in T cell development and function. We observed that CD4+ T cells up-regulate IRF3 expression following TCR engagement (Figure 1-1), prompting us to investigate the functional consequences of IRF3 expression in T cell development and function. We discovered that T cell development remains intact in IRF3−/− mice, as indicated by normal T cell numbers and expression of cell-surface activation markers (Figure 1-2). In testing T cell responses, we observed that IRF3−/− T cells are more proliferative and less sensitive to Treg cell-mediated suppression than their wild type counterparts, though the reason behind these findings remains elusive (Figure 1-3, Figure 1-4). In further examining effector T cell responses, we discovered that T cells lacking IRF3 are compromised in their ability to produce IL-17 and IFN-γ when polarized under Th17 or Th1 conditions, respectively. This defect in cytokine production is not the result of a disturbance
in T\textsubscript{H}17 or T\textsubscript{H}1 lineage commitment, as these cells up-regulated ROR\textgamma t and T-bet to the same extent as their wild type counterparts. Additionally, this does not appear to be a pan-cytokine defect, as IL-4 production remains intact in IRF3\textsuperscript{-/-} T cells polarized under T\textsubscript{H}2 conditions (Figure 1-5).

We confirmed our in vitro cytokine production results in an in vivo setting using the T cell transfer model of colitis. We observed that IRF3\textsuperscript{-/-} T cells failed to induce inflammation and tissue destruction in this disease model (Figure 1-7). IRF3\textsuperscript{-/-} T cells recovered from the mesenteric lymph nodes of colitic mice receiving a mixed T cell population showed decreased IL-17 and IFN-\gamma production compared to wild type T cells (Figure 1-8). This defect in cytokine production is likely why IRF3\textsuperscript{-/-} T cells fail to induce disease in this model.

Previous investigations into the role of IRF3 in inflammation and autoimmunity have produced mixed results. IRF3 has been shown to promote certain inflammatory conditions such as sepsis, meta-inflammation, and obesity-induced insulin resistance [84, 85]. In contrast, IRF3 has been shown to play a protective role against the development of certain autoimmune disorders such as EAE [55]. These opposing disease outcomes are likely due to differences in the cell populations and soluble mediators that drive inflammation in these different settings. In the case of sepsis or meta-inflammation, both of which are mediated by IRF3-driven pro-inflammatory molecules such as IL-6, IL-12, and TNF-\alpha, IRF3\textsuperscript{-/-} mice are protected from disease progression. In contrast, IRF3 was shown to limit T\textsubscript{H}1 and T\textsubscript{H}17 development by modulating cytokine production by APCs [42, 43], likely explaining why IRF3\textsuperscript{-/-} mice are more susceptible to certain
TH1- and TH17-mediated conditions such as EAE. Our findings add a layer of complexity to the role of IRF3 in T cell-driven inflammatory conditions. In contrast to previous studies, which employed global IRF3 knockout animals, our study limited IRF3-deficiency to the T cell compartment by employing a disease model in which T cells were transferred into recipient RAG−/− hosts. In this setting, both wild type and IRF3−/− naïve T cells differentiate into TH1 and TH17 cells in the presence of wild type APCs. Additionally, we used in vitro assays where T cells were activated with agonistic antibodies against CD3 and CD28 instead of with APCs. Using these approaches we discovered that IRF3 is a positive regulator of IL-17 and IFN-γ production within CD4+ T cells, and drives disease progression in TH1- and TH17-mediated inflammatory disorders.

One caveat of our study is that the T cells used in our experiments were obtained from complete IRF3 knockout mice. Since these T cells came from an IRF3-deficient environment, we cannot rule out the possibility that IRF3-mediated, T cell-extrinsic factors during development and homeostasis could later affect mature T cell responses. As there is not an IRF3 conditional knockout animal currently available, this question is best addressed by using T cells obtained from mixed bone marrow chimeras. In this setting, IRF3−/− T cells develop alongside wild type T cells in a wild type environment. If we can show that IRF3−/− T cells generated in a wild type environment are still unable to induce colitis due to cytokine production defects, we can conclude that IRF3 functions in a T cell-intrinsic manner to promote IL-17 and IFN-γ production. This matter will be the subject of future investigations.
The findings presented in this study demonstrate a novel function of IRF3 within T cell responses. While our experiments focused on the role of IRF3 in CD4^+ T cells—mainly cytokine production—our findings encourage broader investigations of IRF3 function within T cells. In Figure 1-1 we demonstrate that IRF3 expression is up-regulated following T cell activation through the TCR, and in Figure 1-6 we show that IRF3 can be detected in the nuclei of T cells polarized under T_H0, T_H1, and T_H17 conditions. Combined, these results suggest that IRF3 is mobilized following T cell activation.

Our findings raise the question as to which signals promote IRF3 nuclear translocation in CD4^+ T cell responses. IRF3 activation and nuclear localization occurs after TLR3 and TLR4 engagement during pathogen responses. CD4^+ T cells have been shown to express TLRs, including both TLR3 and TLR4 [48, 49]. One possibility is that TLR engagement on CD4^+ T cells promotes IRF3 nuclear localization during T cell responses. While this is a possibility in our colitis model, where CD4^+ T cells are likely to encounter TLR ligands in the gut and gut-draining lymph nodes, the same may not hold true in our in vitro experiments, where purified T cells are presumably cultured under sterile conditions.

Another possibility is that TCR stimulation promotes IRF3 nuclear translocation. If this were the case, IRF3 nuclear translocation would likely occur within a few hours of TCR engagement. As discussed in Figure 1-1, we did not detect nuclear IRF3 expression within 24 hours of TCR stimulation. However, we did observe nuclear IRF3 expression in T cells polarized under T_H0 conditions—activated T cells supplemented with IL-2—after 96 hours (Figure 1-6). T cells
produce IL-2 within a couple hours following TCR engagement. If IL-2 promotes IRF3 nuclear translocation, we would expect to see nuclear IRF3 expression within 24 hours of TCR engagement. Our results suggest that a different signal promotes IRF3 nuclear translocation following TCR engagement.

Recently, a body of evidence has emerged showing that endogenous danger signals—which are released by stressed and dying cells—can induce type I IFN production through IRF3 or IRF7 activation [86, 87]. These include signals such as DNA damage and reactive oxygen species, which can induce type I IFN in a cell-autonomous manner [88, 89]. We propose that the above mentioned endogenous danger signals, which accumulate during T cell responses [90-93], could be promoting IRF3 activation and nuclear translocation following T cell activation. This hypothesis could explain why we did not detect nuclear IRF3 expression until later time points following T cell stimulation.

While we observed IRF3 nuclear translocation during CD4^+ T cell responses, we did not assess IRF3 activation status in our cells. Canonically, IRF3 is activated following phosphorylation at serines 385 and 386, and the serine/threonine cluster between amino acids 396 and 405 [94, 95]. These phosphorylation events induce a conformational change in IRF3 that allow for its dimerization and nuclear translocation. One question that remains unanswered is whether IRF3 is canonically activated or whether phosphorylation occurs at different sites during T cell responses. Alternatively, it is possible that IRF3 does not need to be phosphorylated to undergo nuclear translocation during T cell responses, and that a different post-translational modification activates IRF3 in
The answers to these questions could reveal novel modes of IRF3 activation and nuclear translocation.

The studies presented in this report focus on the role of IRF3 in CD4⁺ T cells. Since T- and B-lymphocytes exhibit similar signaling events downstream of TCR and B cell receptor (BCR) engagement, it is possible that IRF3 is mobilized during CD8⁺ T cell and B cell responses as well. A recent study investigating the role of IRF3 in CD8⁺ T cells demonstrated that CD8⁺ T cell memory responses are impaired in IRF3⁻/⁻ mice infected with TMEV due to a decrease in granzyme B recall responses [96]. A second study demonstrated that IRF3 inhibits IL-17 production in CD8⁺ T cells (T_c17 cells) by interacting with RORγt and preventing it from binding to IL-17 enhancer regions [44]. These results present a striking contrast with our findings showing that IRF3 promotes IL-17 production in CD4⁺ T cells. The reason behind these opposing results remains elusive. One possibility is that IRF3 has distinct functions within CD4⁺ and CD8⁺ T cells. However, the above-mentioned study shows that IRF3 interacts with RORγt in both CD4⁺ and CD8⁺ T cells, suggesting instead that there may be qualitative or quantitative differences in the signaling pathways and transcriptional networks involved in T_H17 and T_C17 differentiation. Elucidating the role of IRF3 within different lymphocyte populations will expand our understanding of IRF3 function within adaptive immune responses.

Our results identify IRF3 as a positive regulator of IL-17 and IFN-γ production within CD4⁺ T cells and suggest that other IRF3-driven pro-inflammatory cytokines, such as IL-6 and TNF-α, could also be regulated in an
IRF3-dependent, T cell-autonomous manner. These findings present IRF3 as a potential therapeutic target for T cell-mediated inflammatory diseases. Currently available IRF3 inhibitors are primarily those of the stilbene family. Piceatannol and related stilbene derivatives have been shown to confer protection against LPS-induced septic shock due to their modulation of IRF3 activation and subsequent inhibition of IRF3-driven pro-inflammatory mediators such as IL-6, TNF-α, macrophage chemoattractant protein 1 (MCP-1) and intracellular adhesion molecule 1 (ICAM-1) [97]. However, as the mechanism involving IRF3 regulation of IL-17 and IFN-γ expression has not been fully elucidated, it is unclear whether these currently available IRF3 inhibitors would modulate IRF3 function in a manner that would inhibit IL-17 and IFN-γ production in T cells. Additionally, previous reports have suggested that deletion of IRF3 within APCs is detrimental for the outcome of T cell-mediated inflammatory diseases, as IRF3-deficiency within APCs enhances IL-17 and IFN-γ responses in T cells. Future work should focus on elucidating the cell-specific requirements of IRF3 in pro-inflammatory responses in order to design safe and effective treatments for T cell-mediated inflammatory diseases.
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