Type I Interferons Negatively Regulate Foxp3^{+} Regulatory T Cells during Acute Viral Infection and Autoimmunity

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Abstract

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CD4$^+$ regulatory T (Treg) cells expressing the transcription factor Foxp3 are potent anti-inflammatory cells capable of restraining immune responses to both self- and foreign-antigens. In addition to preventing autoimmunity and immunopathology, Treg cells can also inhibit immune responses during viral, bacterial, and parasitic infections. Paradoxically, signals known to drive Treg proliferation, such as IL-2 and activated dendritic cells, are also abundant during infection when Treg activity may need to be curbed. How Treg activity is restrained during infection to allow for the generation of effective immune responses remains largely unclear. Type I interferons (IFNs) are a family of cytokines that coordinately regulate many cell types during viral infection, but their effects on Treg cells remain largely unknown. Here, we demonstrate that type I IFNs directly inhibit co-stimulation-dependent Treg proliferation and activation both *in vitro* and *in vivo* during acute infection with lymphocytic choriomeningitis virus (LCMV). This inhibition is cell-intrinsic and preferentially targets CD62L^{lo}CD44^{hi} effector/memory Treg cells. Moreover, loss of the type I IFN receptor specifically in Treg cells results in functional impairment of virus-specific CD8$^+$ and CD4$^+$ T cells and inefficient viral clearance. Together, these data indicate that inhibition of Tregs by IFNs during acute viral infection is necessary for the development of robust anti-viral T cell responses.

Because overexpression of type I interferons is associated with the development of many autoimmune disorders, we also asked how chronic IFN overexpression regulates Treg cell activity and how this contributes to immune dysfunction during IFN-associated autoimmune

diseases. We show that Treg cell function is impaired in mice that chronically overproduce type I IFNs due to loss of the DNA exonuclease Trex1. However, although IFNs directly inhibited Treg cell proliferation and activation, this direct inhibition was neither required nor sufficient for development of inflammatory disease. Rather, chronic IFN expression directly promoted the expansion of conventional T cells, and inflammatory disease was completely dependent on IFNαR signaling in conventional T cells. Thus, chronic IFN expression renders effector T cells resistant to Treg-mediated suppression, thereby resulting in immune dysregulation and development of inflammatory disease.

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To my Baba and Dadi,

who taught me the value of love, laughter, and hard work.

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Chapter 1:

Introduction

The Immune System: Establishing Self-Tolerance

The vertebrate immune system has evolved to patrol the interaction of host organisms with the myriad of microbes in the environment, including viruses, bacteria, and parasites. While many of these microbes are harmless and actually beneficial to the host, others – termed "pathogens" – are capable of causing extensive damage to the host and must be kept in check. A robust immune system must be able to identify these potentially dangerous targets, selectively eliminate them through a variety of effector mechanisms, and remember the interaction to allow quick and efficient clearance upon re-exposure to the offending pathogen. The specificity of these processes is incredibly important: misidentification of self-tissue as non-self can result in collateral damage to the host and autoimmunity, while misguided attack of harmless or even beneficial foreign matter, like food or commensal bacteria, can result in allergy. Thus, one of the fundamental challenges faced by the immune system is how to distinguish self from non-self, benign from dangerous.

Self-tolerance is accomplished in part through a combination of central and peripheral mechanisms. Central tolerance is established during T cell development in the thymus, where self-reactive T cells expressing T cell receptors (TCRs) with too high affinity for self-antigen/MHC complexes are negatively selected and deleted before entering the periphery (*1*). This process is imperfect, however, in part because not all peripheral antigens are expressed in the thymus. Self-reactive T cells that escape central tolerance in the thymus, thus, must be inactivated by additional tolerance mechanisms in the periphery to prevent damage to the host. These can include programmed cell death (deletion) and induction of a functionally unresponsive state (anergy) upon encounter of self-antigen/MHC complexes in the absence of the appropriate co-stimulatory signals (*2*).

More recently, it was discovered that activation of self-reactive T cells can also be prevented by the activity of $CD4^+$ regulatory T (Treg) cells expressing the transcription factor Foxp3. These immunosuppressive cells dampen immune responses to both self- and foreign-antigens through their actions on a number of immune cells, including effector T cells, B cells, dendritic cells (DCs), natural killer cells, and macrophages (*3*). While their actions are critical for establishing self-tolerance and preventing autoimmunity, their activity can interfere with protective immune responses to pathogens. Understanding the mechanisms by which Treg cell activity is regulated to balance self-tolerance with pathogen clearance is critical to our understanding of the mechanisms that drive autoimmunity and chronic infection.

Regulatory T Cells

Similar to conventional $CD4^+$ T cells, most natural $CD4^+Foxp3^+$ regulatory T cells are produced in the thymus, although some Treg cells also arise in the periphery through the induction of Foxp3 expression in naïve conventional T cells under certain conditions, and these are referred to as “induced” Treg cells. Treg cells have been shown to play a dominant role in maintaining tolerance and immune homeostasis, as evidenced by the widespread autoimmunity that develops in Foxp3-deficient IPEX patients and *scurfy* mice, and decreased Treg cell activity has also been implicated in the development of a number of more common autoimmune and inflammatory diseases, including type-1 diabetes, rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus.

Treg cells display a TCR repertoire distinct from conventional T cells that is skewed for the high-affinity recognition of self-peptides, and this is thought to underlie their ability to regulate auto-reactive T cells in an antigen-specific manner (*4*). For example, Treg cells can compete with self-reactive T cells for binding to self-peptide/MHC complexes and can even inactivate self-antigen presenting cells (APCs) by trans-endocytosing the co-stimulatory molecules CD80 and CD86, making APCs less immunogenic and unable to efficiently activate

self-reactive T cells (*5*). Treg cells can also suppress immune responses in an antigen-independent manner, a process termed "bystander suppression." This can occur via expression of anti-inflammatory cytokines like IL-10 and TGFβ, cytolysis via granzyme B and perforin expression, and metabolic disruption via IL-2 consumption (*6*, *7*). These mechanisms allow Treg cells to regulate immune responses to both self- and foreign-antigen regardless of their own antigen-specificity. Interestingly, recent studies have demonstrated that pathogen-specific Treg cells also exist among the pool of thymically-derived Treg cells, and these cells are potent suppressors of effector responses that can delay or prevent pathogen clearance (*8*, *9*).

Immune Modulation by Treg Cells: Infection

In addition to preventing autoimmunity and maintaining self-tolerance, Treg cells can also inhibit immune responses during viral, bacterial, and parasitic infections (*10*). A major challenge Treg cells face during infection is how to maintain self-tolerance while allowing protective pathogen-specific immune responses to occur. Whereas excessive Treg cell activity can lead to chronic infection and cancer, too little Treg cell activity during infection can result in collateral autoimmunity and immunopathology and can even impair the quality of pathogen-specific response.

Some inhibition of Treg activity early during infection is critical for the development of effective pathogen-specific T cell responses. Considering the abundance of Treg cell-activating factors present during infection, like IL-2 and activated DCs, the host must employ counter-regulatory mechanisms to circumvent Treg cell activity and ensure pathogen control. Indeed, a number of acute infections induce a transient decline in Treg cell numbers that correlates with the expansion of effector T cells (*11*). This inhibition of Treg cell activity can be accomplished indirectly in some instances: for example, activation of APCs by Toll-like receptor (TLR) or CD40 stimulation protects them from Treg cell-mediated suppression (*12-14*), and pro-inflammatory cytokines such as IL-1β and IL-6 can render effector T cells resistant to Treg cell-

mediated inhibition (*15*-*17*). Alternatively, pro-inflammatory cytokines like TNFα can directly inhibit Treg cell activity (*18*).

Failure to limit Treg activity by such mechanisms can impair effector T cell responses, promoting pathogen persistence and chronic infection. Elevated Treg cell numbers, for example, are associated with higher viral burden and exaggerated liver pathology following infection with hepatitis C virus (*19*, *20*), and with lung pathology and active disease following *Mycobacterium tuberculosis* (*Mtb*) infection (*21*). In fact, recent studies have demonstrated that thymically-derived Treg cells specific for *Mtb* antigen expand early during *Mtb* infection, delay the priming of $CD4^+$ and $CD8^+$ T cells in draining lymph nodes, and block their migration to infected lungs, resulting in higher bacterial burden (*8*, *22*). In such infectious settings where excessive Treg cell activity is detrimental to the host, Treg cell depletion can restore protective immune responses. For example, Treg cell depletion enhances bacterial clearance following *Mtb* infection (*23*) and protects mice infected with *Plasmodium yoelii* from death by restoring anti-parasite effector responses (*24*).

Although Treg cells are known for their ability to interfere with effector T cell responses, several recent studies highlight an underappreciated role for Treg cells in shaping the quality of pathogen-specific responses. In a ground-breaking study, Lund et al. demonstrated that Treg cells are actually essential for the development of appropriate anti-viral T cell responses during murine infection with herpes simplex virus-2 (HSV-2) (*25*). In this study, depletion of Treg cells following intravaginal infection with HSV-2 accentuated T cell priming and proliferation in the draining lymph node, but uncontrolled T cell activation prevented effector T cell mobilization from the lymph node to the vaginal epithelium, resulting in uncontrolled viral replication and death. Interestingly, Treg cell depletion can also impair the avidity of $CD8^+$ T cell responses during *Listeria monocytogenes* infection (LM) (*26*). In this model, Treg cells promote high-avidity $CD8^+$ T cell responses to LM infection by destabilizing low-affinity T cell-DC interactions. Treg cells can also indirectly promote memory responses: following infection with the intracellular

parasite *Leishmania major*, Treg cells block the sterile eradication of the pathogen, providing a source of persistent antigen that is essential for the maintenance of protective memory responses (*27*). In other instances, the ability of Treg cells to limit effector T cell activity can be beneficial to the host by limiting immunopathology in the presence of persistent antigen. Patients with chronic HCV infection have higher numbers of Treg cells in blood and liver biopsies than uninfected patients, with Treg number inversely correlated with histological severity (*20*, *28*). However, although depletion of Treg cells enhances HCV-specific $CD8^+$ T cell function *in vitro*, sustained $CD8^+$ T cell responses correlate strongly with immunopathology and liver damage (*29*). Likewise, Treg depletion increases damage to the liver in mice chronically infected with *Schistosoma mansoni*, and Treg cells protect against inflammatory lesions in a mouse model of HSV infection (*30*, *31*). Treg cells, thus, can play a protective role during infection by regulating both the quality and quantity of effector responses to pathogens.

Taken together, these studies underscore the need for Treg cells to integrate cues from the immune environment in order to appropriately modulate their activity depending on the immune context. Too much Treg cell activity can result in immunosuppression and impaired pathogen clearance, whereas too little Treg cell activity can impair effector T cell mobilization and avidity during infection and unleash potentially fatal inflammatory and autoimmune diseases. Identifying the cellular and molecular signals that control Treg cell homeostasis and function is essential for understanding how Treg cells influence the outcome of normal and pathological immune responses.

Immune Modulation by Treg Cells: Autoimmunity

Though Treg cell suppressive activity is clearly impaired in autoimmune disorders, what factors lead to this dysfunction are still being elucidated. Treg and effector T cell-intrinsic defects may contribute to loss of tolerance, while overexpression of inflammatory mediators can activate APCs and effector T cells, making both cell types refractory to Treg cell-mediated suppression.

Interestingly, circulating numbers of Treg cells appear to be mostly normal in patients with autoimmunity. Although the data from these studies is complicated by the lack of precise markers used to identify Treg cells in humans, most studies have demonstrated no difference in the numbers of $CD4^+Foxp3^+$ Treg cells in the peripheral blood of patients with type I diabetes (T1D) and multiple sclerosis (MS) compared with healthy controls (*32*, *33*). On the other hand most studies of patients with systemic lupus erythematosus (SLE) detected a decrease in circulating numbers of Treg cells that correlated with disease activity (*34*, *35*). Of course, these findings are limited only to peripheral blood of patients and do not necessarily reflect changes in Treg abundance or activity at sites of on-going inflammation. In studies where sites of inflammation were also analyzed, Treg numbers were actually increased in the synovial fluid of patients with rheumatoid arthritis (RA) and in the inflamed lamina propria of patients with inflammatory bowel disease (IBD) (*36*, *37*). These increases in Treg abundance are likely secondary to Treg cell-activating signals abundant in inflamed tissues. However, in many cases Treg cells from autoimmune patients show functional defects in *in vitro* suppression assays. In some cases, dysfunction appears to be due to Treg cell-intrinsic defects. For example, Treg cells isolated from patients with RA were unable to suppress the activation of autologous effector T cells from healthy controls as well as control Treg cells (*38*, *39*). However, Treg cells from IBD or T1D patients were able to suppress healthy control effector T cells to similar levels as control Treg cells (*40*, *41*). In these cases, other factors including local inflammatory cytokine production, APC activation, and effector T cell resistance to suppression may indirectly subvert Treg function *in vivo*.

The resistance of effector T cells to Treg-mediated suppression has been described both in patients and in mouse models of T1D, MS, and SLE. Effector T cells isolated from T1D patients, for example, could not be suppressed by either T1D or healthy control Treg cells (*40*, *42*). Likewise, Treg cells are able to migrate to the central nervous system (CNS) during experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, but are unable to

suppress effector T cells during active disease due to the production of IL-6 and TNFα, both of which have been implicated in driving effector T cell activation and resistance to suppression (*43*). Other studies indicate that changes in APC activity and cytokine production can also subvert Treg suppressive function. Treg cells from SLE patients showed defective suppression in the presence of APCs from SLE patients, and this was linked to their production of IFNα (*44*). Similarly, DCs and monocytes from the synovial fluid of RA patients have a more activated phenotype and can induce Th17 cell polarization better than those from peripheral blood (*37*), while DCs in the CNS promote Th17 maintenance via secretion of IL-23 during EAE (*43*). Thus, despite the presence of normal numbers of functional Treg cells, a number of factors in the inflammatory milieu may still circumvent Treg-mediated suppression and drive autoimmunity.

This indirect subversion of Treg cell function may make putative therapies targeted at Treg expansion difficult to implement. Adoptive Treg cell therapy trials, involving the *ex vivo* expansion of Treg cells and reinfusion into patients, are currently underway and will help determine whether transferring greater numbers of Treg cells is sufficient to overcome Treg cell dysfunction in patients with autoimmunity, or whether extrinsic factors in the inflammatory environment will still render these expanded Treg cells ineffective.

Control of Treg Cell Homeostasis

Because of their potent immunomodulatory function, Treg cell abundance and activity must be tightly controlled in order to prevent autoimmunity whilst allowing immune responses to harmful pathogens and toxins to occur. Indeed, work over the last decade has identified several factors that control Treg cell homeostasis and function *in vivo*. Among these, the role of the cytokine IL-2 is best understood. IL-2 is produced by activated T cells and signals through a receptor complex containing the common gamma (γc) chain, the IL2/15Rβ (also known as CD122) and the IL2Rα (CD25). Treg cells were initially identified based on their constitutive expression of CD25, and the importance of IL-2 in controlling Treg cell function in the periphery

is evidenced by the lymphoproliferative disease and colitis that develop in either IL-2- or CD25-deficient mice (*45*, *46*). IL-2 is thought to drive the homeostatic proliferation and survival of peripheral Treg cells through activation of the transcription factor STAT5 and regulation of genes involved in cell proliferation, metabolism, and apoptosis (*47*), and several studies have demonstrated that Treg cells occupy a distinct homeostatic "niche" that is limited by the amount of available IL-2 (*48*). Consistent with this notion, administration of super-agonistic IL-2/anti-IL-2 immune complexes causes a dramatic expansion in the Treg cell population (*49*). IL-2 is produced by $CD4^+CD44^{hi}Foxp3^-$ effector/memory T cells, and therefore is thought to act in a paracrine fashion to promote the proliferation and survival of peripheral Treg cells (*50*). This links the size of the Treg cell compartment to the number of these effector/memory cells, thereby ensuring that autoimmunity and inflammatory diseases do not develop as a result of uncontrolled effector/memory T cell activation.

During infection, IL-2 is classically produced by pathogen-specific effector T cells upon activation in the periphery, and Treg cells are among the first cells to respond to IL-2 upon antigenic challenge *in vivo* (*51*, *52*). That Treg cells are stimulated by IL-2 early in an immune response when their activity may need to be curbed is puzzling. One possibility is that parallel activation of Treg cells with effector T cells may prevent the development of collateral autoimmunity. However, IL-2 expression varies widely in different infectious models, and reduced IL-2 production by effector T cells is thought to underlie a transient decrease in Treg cell number during infection with *Toxoplasma gondii*, *Listeria monocytogenes*, and vaccinia virus (*11*, *53*). IL-2 may also drive the proliferation of Treg cells late in infection, after the clearance of pathogen, in order to turn off the effector T cell response and limit immunopathology. For instance, in work described later in this dissertation, we have shown that Treg cells peak in proliferation and number two weeks post-infection with lymphocytic choriomeningitis virus (LCMV), correlating with the contraction of $CD8^+$ and $CD4^+$ effector T cell

responses. IL-2 expression in the spleen and STAT5 phosphorylation in Treg cells also peak at this time point, suggesting a role for IL-2 in driving Treg cell expansion late in infection.

Along with IL-2, antigen recognition also helps control Treg cell homeostasis in the periphery. Treg cells are thought to be largely self-reactive (*54*), and consistent with this they display numerous phenotypic characteristics of recently activated conventional $CD4^+$ T cells, including their constitutive expression of CD25, increased expression of activation markers such as CD44 and CD69, and high-rate of homeostatic proliferation. Interaction with self-antigen helps shape the TCR repertoire of peripheral Treg cells (*55*), and accordingly Treg cells from male mice prevent autoimmune prostatitis far more effectively than those from female animals (*56*). The link between self-antigen recognition and Treg cell homeostasis is further supported by the recent finding that changes in the frequency or activity of dendritic cells can alter Treg cell proliferation and abundance (*57*), and by the fact that mutations in molecules important for T cell activation via the TCR such as Zap-70 or CD28 result in impaired Treg cell homeostasis and function (*58*, *59*).

The importance of CD28 signaling in Treg cell development and homeostasis was first appreciated nearly a decade ago by the seemingly paradoxical finding that rather than being protected, the development of diabetes in NOD mice deficient for either CD28 or its ligands was actually accelerated, and this could be prevented by the transfer wild-type Treg cells (*60*). Indeed, like most T cells, Treg cells constitutively express CD28, and removal of CD28 co-stimulation results in decreased numbers of thymic and peripheral Treg cells (*58*). Although initial reports were hindered by potential *trans* effects of loss of CD28-mediated co-stimulation on IL-2 production by effector T cells, a recent study generated CD28-conditional knockout mice and reported the cell-intrinsic requirement for CD28 in Treg cell survival and function, as these mice developed severe autoimmunity with increased numbers of activated effector cells (*61*). Consistent with their potent antigen-presenting and costimulatory function, Bar-On *et al.* used

mixed chimeras to demonstrate that DCs were the primary cell type responsible for providing CD80/86 co-stimulation to Treg cells (*62*).

Although both IL-2- and TCR-dependent signals clearly help control Treg cell homeostasis and function in baseline conditions, the generation of strong $CD4^+$ and $CD8^+$ T cell responses to invading pathogens may require that Treg cell abundance be 'uncoupled' from these inputs. For instance, recognition of pathogen-associated molecular patterns by cytosolic and surface receptors induces dendritic cell activation, which increases their expression of MHC II and co-stimulatory ligands, and augments their antigen-presentation function. Although this is essential for the priming of pathogen-specific T cells, it may also lead to enhanced Treg cell activation, which could dampen the protective T cell response. Similarly, IL-2 produced by activated pathogen-specific $CD4^+$ T cells may increase the size of the Treg cell niche (*63*). During infection, numerous pro-inflammatory cytokines are produced, including IL-6, IFN-γ, IL-12 and type I interferons (IFNs), and these may alter Treg cell homeostasis by impinging on IL-2- and TCR-mediated signals. However, the ways in which these and other cytokines impact Treg cell abundance and activity during specific types of normal and pathogenic immune responses are still not well understood.

Type I Interferons

The type I IFNs are a group of more than a dozen closely related cytokines that are highly up-regulated during viral infection, all of which signal through an identical heterodimeric receptor (*64*). In humans and mice, the type I interferons consist of 14 IFNα subtypes and IFNω, IFNε, IFNτ, IFNκ and IFNβ. Nearly all nucleated cells can express type I IFNs upon activation of cell-intrinsic cytoplasmic sensors of DNA/RNA, like RIG-I and MDA5, which activate one or more members of the interferon regulatory factor (IRF) family of transcription factors (*65*, *66*). In addition, plasmacytoid dendritic cells secrete large amounts of type I IFNs following ligation of the nucleic acid binding toll-like receptors, TLR7 and TLR9.

All type I interferons signal through the common type I IFN receptor (IFNαR), which comprises a heterodimer of high-affinity (IFNαR2) and low-affinity (IFNαR1) chains. IFN initially binds to the high-affinity IFNαR2, with the low-affinity IFNαR1 chain subsequently recruited to initiate signaling (*67*). Assembly of the IFNαR2-IFN- IFNαR1 complex leads to phosphorylation and activation of STAT1 and STAT2, resulting in induction or repression of hundreds of IFN-stimulated genes (ISGs). These changes in gene expression act to limit viral replication in cells already infected and to render healthy cells resistant to infection (*68*). Among its many effects, type I IFNs are well known as potent inhibitors of cellular proliferation. This can help limit viral spread and has made type I IFNs clinically useful in the treatment of certain types of leukemia. The anti-proliferative effects of type I IFN are at least in part due to its ability to up-regulate both the expression and activity of the tumor suppressor p53 (*69*, *70*).

In addition to directly limiting viral replication and infection, type I IFNs also indirectly affect viral spread through their impact on the anti-viral immune response. Because nearly all nucleated cells express the type I IFN receptor, IFNs can have a wide range of effects on many different cell types. For instance, type I IFNs can potently activate dendritic cells, enhancing their antigen-presentation and production of pro-inflammatory cytokines (*71*, *72*). Additionally, type I IFNs influence B cell activation and the anti-viral antibody response (*73*), and help direct the functional differentiation of virus-specific $CD4^+$ T cells (*74*). As with most other cell types, type I IFNs have potent anti-proliferative effects on both $CD4^+$ and $CD8^+$ T cells *in vitro* (*75*). However, during murine infection with lymphocytic choriomeningitis virus (LCMV), the clonal expansion of virus-specific $CD4^+$ and $CD8^+$ T cells critically depends on their ability to respond to type I IFN (*76*, *77*). Thus, type I IFN signaling can have a dramatic and complex impact on both T and B cells that qualitatively and quantitatively tunes the adaptive immune response during viral infection.

Due in part to their anti-proliferative and pro-inflammatory effects, type I IFNs have been used clinically to treat a variety of immunological diseases. Pegylated IFNα therapy, in

combination with radiation and chemotherapy, is a common treatment for many types of cancer. This therapy has proved most effective for treating hematological malignancies, like chronic myeloid leukemia and T cell lymphomas, although some success has also been observed in the treatment of melanoma. IFNα is also used to treat chronic hepatitis B and hepatitis C infections, typically in combination with antiviral drugs. The current standard of care can treat hepatitis C infection with a 60-80% success rate, resulting in a sustained anti-viral response that can clear the virus. Unlike IFNα, IFNβ is commonly used to treat multiple sclerosis and has proved effective in reducing attacks and slowing disease progression in patients with relapsing-remitting multiple sclerosis. These anti-inflammatory effects are in stark contrast to the pro-inflammatory effects of IFNα in the treatment of cancer and chronic infection.

Interestingly, several studies suggest that IFNβ may be differentially regulated and functionally distinct from IFNα. IFNβ shares as little as 30% amino acid sequence identity with IFNα and can be produced in different contexts compared to those that result in IFNα production. For example, IFNβ is the only type I IFN induced by lipopolysaccharide (LPS) and is selectively produced in response to macrophage colony-stimulating factor and RANKL (*78*, *79*). This differential regulation may be explained in part by the fact that the *Ifnb* promoter has other elements in addition to IRF binding sites, including sites for NF-κB and AP-1. In addition, IFNβ and IFNα exhibit many functional differences. IFNβ is more potent than IFNα in inducing the apoptotic and anti-proliferative pathways required for control of tumor cell growth (*80*), and IFNβ alleviates the exacerbations of multiple sclerosis, whereas IFNα does not. Interestingly, a recent study demonstrated that IFNβ can specifically bind to IFNαR1 independently of IFNαR2 and transduce signals independently of Jak-STAT pathways, perhaps explaining the molecular basis underlying these functional differences (*81*).

Although essential for proper anti-viral immunity, excessive type I IFN production has been associated with a variety of organ-specific and systemic autoimmune disorders[3]. This is best demonstrated in SLE, in which patients accumulate autoantibodies against DNA, RNA and

other nuclear and nucleolar antigens. Indeed, SLE is associated with a type I IFN gene expression "signature" indicated by over-expression of numerous ISGs in peripheral blood leukocytes of SLE patients (*82*, *83*). Current models of SLE development posit that defects in the handling and degradation of cellular nucleic acids result in the activation of TLR7, TLR9 and/or the cytoplasmic nucleic acid sensors, leading to excessive type I IFN production and loss of tolerance to nuclear antigens. Consistent with this model, mutations in proteins responsible for the clearance and degradation of apoptotic cells, or the processing of cytoplasmic DNA lead to development of autoimmunity in various mouse models and have been associated with development of SLE in humans (*84*). Moreover, development of SLE, colitis and type-1 diabetes have all been reported in individuals receiving type I IFN therapeutically, supporting a direct role for type I IFNs in autoimmune pathogenesis (*85*, *86*).

Regulation of Treg Cells by Type I IFNs

Despite the dramatic effects that type I IFNs have on conventional $CD4^+$ and $CD8^+$ effector T cells, and the clear association between over-production of type I IFNs and development of autoimmunity, the ways in which type I IFNs impact the development, homeostasis and function of $Foxp3^+$ Treg cells are largely unexplored.

Clinical studies and results from mouse models have provided conflicting data on IFN's role in regulating Treg cells. On one hand, IFNα therapy was shown to decrease Treg cell frequency in patients with melanoma and renal cell carcinoma, and this correlated with improved tumor rejection (*87*, *88*). Likewise, IFNα was shown to improve viral clearance in patients with chronic hepatitis C virus, though conflicting reports have revealed both increases and decreases in Treg cell frequency following therapy (*89-91*). Treg cells were also found to be significantly decreased in patients with SLE (*92*), and patients receiving IFN therapeutically for HCV and MS have occasionally been reported to develop autoimmune side effects like ulcerative colitis and type I diabetes (*85*, *86*), supporting a causative role for IFN in autoimmune

pathogenesis. Consistent with this, plasma from SLE patients was shown to inhibit human Treg cell suppressive function *in vitro* in an IFN-dependent manner, although this may be secondary to IFN's ability to inhibit IL-2 production by effector T cells (*93*).

On the other hand, type I IFNs have been reported to have anti-inflammatory and pro-proliferative effects on Treg cells in other contexts. For example, IFNβ treatment markedly improved the frequency of Treg cells in patients with relapsing–remitting multiple sclerosis and mice with experimental autoimmune encephalitis, resulting in lower relapse rates and slower disease progression (*94*-*97*). Likewise, IFNs were also implicated in promoting IL-10 production by tumor-infiltrating Treg cells, as *Ifnar1*$^{-/-}$ mice exhibited significantly reduced accumulation of IL-10^{+} Treg cells and heightened Th17 responses in solid tumors (*98*). More recently, two studies demonstrated that chronic, but not acute, infection with LCMV drives heightened and prolonged expression of type I IFNs that is associated with immune dysfunction and Treg cell expansion (*99*, *100*). However, neither of these studies demonstrated that Treg cell expansion during LCMV infection is IFN-dependent, and in fact work by Pundosky et al. revealed that expansion of Treg cells during chronic LCMV infection actually depends on expression of endogenous retroviral superantigens that are induced upon infection (*101*). Altogether, these studies fail to distinguish how IFNs *directly* regulate Treg cell activity and whether this direct regulation varies in different inflammatory contexts.

Questions to Address

The effects of type I IFNs on Treg cells, thus, are incredibly complex. IFNs have been shown to exert both pro- and anti-proliferative effects that may vary based on contextual factors, such as the timing or duration of signaling, the tissue being acted on, or indirect effects on other cell types. For example, IFNβ and IFNα are known to differ in their induction and signaling pathways, and it is possible that these differences may underlie the distinct pro- and anti-inflammatory properties of these subtypes in clinical settings. Alternatively, the duration of IFN

signaling may also alter its functional properties, as acute IFN expression promotes anti-viral T cell responses and viral clearance during acute LCMV infection, but prolonged IFN expression drives immunosuppression during chronic LCMV infection. Moreover, because IFNs can have varying effects on a wide range of cell types, it is unclear from existing studies how IFNs *directly* regulate Treg cell activity. In fact, several studies have now demonstrated that the protective effect of IFNs during experimental autoimmune encephalomyelitis depend on IFNαR expression on DCs but not on T cells, and that the enhanced proliferation of Treg cells may be secondary to activation of co-stimulatory molecules like GITRL on DCs (*102-104*).

It is important, therefore, to understand exactly how IFNs regulate Treg cell activity, both directly and indirectly, in a more controlled setting. In Chapter 3, we investigate how type I IFNs directly impact Treg cell activity during acute viral infection. Using LCMV as an infectious model system, we show that type I IFNs directly inhibit Treg cell proliferation during acute viral infection and that this transient inhibition of Treg cells is necessary for the generation of optimal antiviral T cell responses. In Chapter 4, we investigate how dysregulated IFN over-expression regulates Treg cell activity, both directly and indirectly, and how this in turn contributes to immune dysfunction during type I IFN-associated autoimmunity. Using a well-established *in vivo* assay of Treg cell function, we show that Treg cell function is impaired in mice that chronically overproduce type I IFNs due to loss of the DNA exonuclease Trex1, and that chronic IFN expression drives inflammatory disease by impairing Treg cell function both directly and indirectly through its effects on conventional T cells.

Chapter 2:

Materials and Methods

Mice

C57BL/6J (B6), CD45.1^{+} B6 congenic, B6.129P2-*Tcrb*^{tm1Mom} *Tcrd*^{tm1Mom}/J (TCRβδ$^{-/-}$), and B6.129S2-*Cd28*^{tm1Mak}/J (*Cd28*$^{-/-}$) mice were purchased from The Jackson Laboratory. C57BL/6NTac-*IL15*^{tm1Imx} N5 (*Il15*$^{-/-}$) mice were purchased from Taconic Farms. Foxp3^{GFP} and Foxp3^{DTR} mice were provided by A. Rudensky (Memorial Sloan-Kettering Cancer Center). *Ifnar1*$^{-/-}$ mice were provided by K. Murali-Krishna (Emory University) and crossed to *Cd28*$^{-/-}$ and Foxp3^{GFP} mice. *Rag2*$^{+/-}$*Trex1*$^{+/-}$ mice were provided by Daniel Stetson (University of Washington) and bred to generate *Rag2*$^{-/-}$*Trex1*$^{+/-}$ and *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice. All mice were housed and bred at the Benaroya Research Institute (Seattle, WA), and all experiments were performed in accordance within the guidelines of the Benaroya Research Institute Animal Care and Use Committee.

Virus/infections

LCMV Armstrong 53b was grown in baby hamster kidney (BHK) cells and titered on Vero cells as described (Ahmed et al., 1984). Mice were infected intra-peritoneally with 2x10^{5} plaque-forming units (PFU).

Mixed bone marrow chimeras

Bone marrow cells were depleted of CD4^{+} and CD8^{+} cells using anti-CD4 and anti-CD8 microbeads (Miltenyi Biotech) and injected intravenously into lethally irradiated (1000 Rad) TCRβδ$^{-/-}$ mice. Chimeras received 4-8×10^{6} cells of a 1:1 mixture of WT (CD45.1^{+}) and *Ifnar1*$^{-/-}$ (CD45.2^{+}) bone marrow.

Cell isolation

Cell suspensions were prepared from spleen and peripheral lymph nodes by tissue disruption with glass slides and filtered thru a 40-μM filter. After dissection and removal of Peyer's patches, intestinal intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were isolated as follows. The intestinal epithelium was stripped, as previously described (*105*, *106*), and the remaining intestinal pieces were washed three times in CMF solution (HBSS, Ca^{2+} and Mg^{2+} free, 100 mM HEPES (Sigma-Aldrich), 250 mM sodium bicarbonate (Fisher Scientific, Pittsburgh, PA), and 2% FBS (HyClone, Logan, UT)). The tissue was then placed into a flask containing CMF with 1 mM DTT and shaken for 20 min at 37°C. The IEL containing supernatant was removed and transferred into 50-ml centrifuge tubes and pelleted by centrifugation. The cells were resuspended in 44% Percoll (Sigma-Aldrich) and layered onto a 67% Percoll cushion in a 15-ml polycarbonate centrifuge tube. The tubes were centrifuged (2800 rpm) for 20 min at room temperature. The IEL were removed from the 44/67% Percoll interface and washed with RPMI 1640. For LPL isolation, immediately following incubation with DTT, HBSS with 0.5M EDTA was added to the intestinal tissue pieces and was incubated an additional 30 min on ice. Intestinal pieces were washed with RPMI and added to 50 ml of RPMI plus 100 μl 0.5 M $MgCl_2$, 100 μl 0.5 M $CaCl_2$, and 150 U/ml collagenase (Roche). Samples were stirred at 37°C for 1 h, and the released cells were then filtered through nitex. Cells isolated from the lamina propria were pelleted, resuspended in 44% Percoll (GE Healthcare) in RPMI, layered over 67% Percoll, and spun at 2,800 rpm for 20 min. Lymphocytes were isolated from the interface and used for subsequent flow cytometry analyses.

Flow cytometry and cell sorting

For surface staining, cells were incubated at 4°C for 30 minutes in staining buffer (HBSS, 2% FBS) with the following directly conjugated antibodies for murine proteins (from Biolegend unless otherwise specified): anti-CD4 (RM4-5), -CD44 (IM7), -CD25 (PC61.5), -CD45.1 (A20), -

CD45.2 (104), -CXCR3 (CXCR3-173), -CD62L (MEL-14), -PD-1 (29F.1A12), -ICOS (15F9), -GITR (YGITR-765), -IFNAR1 (MAR1-5A3), -CTLA-4 (UC10-4B9, eBioscience), -CD8 (53-6.7, eBioscience), -IFNAR2 (237526; R&D Systems), -CD45RB (C363.16A, eBioscience), -CD69 (H1.2F3, BD), IgG1 κ isotype (MOPC-21), and IgG2a isotype (20102; R&D Systems). For intracellular staining, cells were surface stained as described, washed and permeabilized for 20 minutes with eBioscience Fix/Perm buffer at 4°C. Cells were stained for 30 minutes at 4°C with the following antibodies: anti-IFN-γ (XMG1; eBioscience), -Foxp3 (FKJ-16s; eBioscience), -Ki-67 (B56; BD Biosciences), and -STAT1 N-terminus (1/Stat1; BD Biosciences) in PermWash staining medium (eBioscience). For intracellular cytokine staining following restimulation, cells were stimulated with LCMV peptides in 96-well U-bottomed plates (Costar, Cambridge, MA) with 10μg/mL monensin in 0.2ml of complete RPMI (RPMI plus 2.05mM L-glutamine, 10% (vol/vol) fetal calf serum, 50units/l of penicillin, 50mg/mL of streptomycin, 50mg/mL gentamycin, 1mM sodium pyruvate, 1mM HEPES, 50mM b-mercaptoethanol) for 5 hours at 37°C, 5%CO_2 prior to staining. GP_{33-41} (KAVYNFATC) and GP_{61-80} (GLKGPDIYKGVYQFKSVEFD) peptides were used at 0.1ug/ml and 10ug/ml, respectively. For tetramer staining, MHC class I tetramers of H-2D^b complexed with LCMV GP_{33-41} (Fred Hutchinson Cancer Research Center Immune Monitoring Lab) were produced as previously described (Murali-Krishna et al., 1998). Biotinylated complexes were tetramerized using PE-conjugated streptavidin (Molecular Probes). Splenocytes were surface stained as described, washed, and stained for 30 minutes at 37°C with tetramer in staining buffer. PE-conjugated I-A^b/GP_{66-77} MHC class II tetramers were a generous gift from Marion Pepper (Univ. of Washington). Cells were stained with class II tetramer for 1 hr at RT, with addition of surface stain markers in the last 20 minutes, and washed. Data were acquired on LSRII flow cytometers (BD Biosciences) and analyzed using FlowJo software (Treestar). For cell sorting experiments, $CD4^+$ cells were enriched using CD4

Dynabeads (Invitrogen), stained for desired cell surface markers, and isolated on a FACS Aria (BD Biosciences).

Phospho-STAT staining

Cells were stimulated for 30 minutes at 37°C, 5%CO_2 in complete RPMI with recombinant murine IFNβ (PBL Biomedical Laboratories) for pSTAT1 staining or recombinant murine IL-2 (Peprotech) for pSTAT5 staining. Cells were harvested and fixed for 20 minutes in BD Fix/Perm buffer at RT (BD Biosciences), washed with BD Perm Wash buffer, and fixed in 90% ice cold methanol for 30 minutes. Cells were washed with BD Perm/Wash and stained with antibodies against cell surface and intracellular markers, including pSTAT5 (Y694; BD Biosciences) and pSTAT1 (Y701; BD Biosciences) in BD Perm Wash for 45 minutes at RT. For direct *ex vivo* pSTAT staining, ~1/5 of each spleen was ground between glass slides in BD Fix/Perm buffer, left for 20 minutes at RT, washed, fixed in 90% methanol and stained as described.

In vitro *suppression and Treg cell proliferation assays.*

For Treg cell proliferation assays, $CD4^+CD25^+$ Treg cells (>90% pure) were isolated from spleens and LNs of WT or *Ifnar1*$^{-/-}$ mice by magnetic separation using CD4 Dynabeads (Invitrogen) and CD25 microbeads (Mitenyi Biotech). Treg cells were incubated for 9 minutes at 37°C in 5 μM Cell Proliferation Dye (CPDye) eFluor 670 (eBioscience) in PBS and washed with 100% FBS. Antigen-presenting cells (APCs) were isolated from spleens of WT or *Ifnar1*$^{-/-}$ mice by depleting splenocytes of T cells using anti-CD4 and anti-CD8 microbeads (Miltenyi Biotech). In each culture well, CPDye-labeled $CD4^+CD25^+$ Treg cells were incubated with irradiated (2,500 Rad) APCs at a 1:1 ratio and stimulated with 0.15 μg/ml soluble anti-CD3 (2C11) and 500U/ml recombinant IL-2 (Peprotech) in the presence or absence of recombinant IFNβ (PBL Biomedical Laboratories) for 72 hours at 37°C, 5%CO_2. For suppression assays, $CD4^+Foxp3^{GFP+}$ Treg cells were sorted (>95% pure) from spleens and LNs of WT and *Ifnar1*$^{-/-}$

mice on a FACS Aria (BD Biosciences). $CD4^+CD25^-$ T_{conv} (>95% pure) were isolated from spleens and LNs of *Ifnar1*$^{-/-}$ mice by magnetic separation using CD4 Dynabeads (Invitrogen) and CD25 microbeads (Miltenyi Biotech). $CD4^+CD25^-$ cells were incubated for 9 minutes at 37°C in 5 μM Cell Proliferation Dye (CPDye) eFluor 670 (eBioscience) in PBS, and washed with 100% FBS. In each culture well, CPDye-labeled $CD4^+CD25^-$ T_{conv} were incubated with equal numbers of irradiated (2,500 Rad) *Ifnar1*$^{-/-}$ APCs with or without addition of Treg cells at the indicated ratios, and stimulated with 0.15 μg/ml soluble anti-CD3 (2C11) in the presence or absence of 50U/ml recombinant IFNβ (PBL Biomedical Laboratories) for 72 hours at 37°C, 5%CO_2. Data were acquired on LSRII flow cytometers (BD Biosciences). Division index was calculated from CPDye dilution profiles using FlowJo software (Treestar). Percent suppression was calculated as: [(T_{conv} division index without Treg cells)-(T_{conv} division index with Treg cells)]/(T_{conv} division index without Treg cells).

Treg cell adoptive transfers and antibody treatments

$CD4^+CD25^+$ Treg cells (>90% pure) were isolated from spleens and LNs of $CD45.1^+$ WT, $CD45.2^+$ *Ifnar1*$^{-/-}$, or *Ifnar1*$^{-/-}$*Cd28*$^{-/-}$ mice by magnetic separation using CD4 Dynabeads (Invitrogen) and CD25 microbeads (Miltenyi Biotech). 5-10x10^5 $CD4^+CD25^+$ Treg cells were injected intravenously into each recipient $CD45.1^+CD45.2^+$ mouse. For transfer of $CD44^{hi}$ Treg cells, Foxp3^{GFP} mice were pre-treated with IL-2 complex prior to Treg cell isolation: 50ug anti-IL-2 (JES6, BioXCell) was incubated with 1.5ug recombinant murine IL-2 (carrier free, eBioscience) in PBS overnight at 4°C and injected into donor mice intra-peritoneally on days 0, 2, and 4. Mice were sacrificed on day 6 for sorting of $CD4^+$Foxp3$^{GFP}CD44^{hi}CD62L^{lo}$ Treg cells. 5x10^5 sorted Treg cells were transferred per mouse. Mice were infected one day post-transfer. For IL-2, ICOSL, and B7-1/B7-2 blockade, recipient mice were injected intra-peritoneally with a mixture of 100ug anti-IL-2 (JES6, BioXCell) and 100ug anti-IL-2 (S4B61, BioXCell), with 100ug anti-ICOSL (HK5.3, BioXCell), with a mixture of 100ug anti-B7-1 (16-10A1, BioXCell) and 100ug

anti-B7-1 (GL-1, BioXCell), or with 100ug rat IgG (Sigma) prior to Treg cells transfer (day -1) and every two days thereafter.

Quantitative PCR.

Fractions of spleens (<5mg) and sorted cells were stabilized in RNALater. RNA extraction was performed using RNeasy columns (Qiagen) and cDNA was generated using Omniscript RT Kit (Qiagen) according to the manufacturer's instructions. Expression of *Ifnb* and *Socs1* were assessed with Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas) and normalized to expression of *Gapdh* using the following primers: *Ifnb*, 5'-CTCCACCACAGCCCTCTC-3' and 5'-CATCTTCTCCGTCATCTCCATAG-3'; *Socs1*, 5'-CTGCGGCTTCTATTGGGG-3' and 5'-AAAAGGCAGTCGAAGGTC-3'; and *Gapdh*, 5'-CCAGTATGACTCCACTCACG-3' and 5'-GACTCCACGACATACTCAGC-3'. Determination of viral load by qPCR was performed as has been previously described (McCausland and Crotty, 2008). Briefly, 1ug of RNA was used in a 20ul cDNA reaction with SuperScript III Reverse Transcriptase (SSIII, Invitrogen), dNTPs and RT buffer from the Omniscript RT Kit (Qiagen), and reverse GP primer at 55°C for 1 hour. 5ul of cDNA was used as template for a 25ul qPCR reaction using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) and GP primers: 5'-CATTCACCTGGACTTTGTCAGACTC-3' and 5'-GCAACTGCTGTGTTCCCGAAAC-3'. Amplification was done for 40 cycles, with each cycle consisting of two steps: 95 °C, 15 sec; 60 °C, 30 sec. Standard curves were generated using linearized pSG5-GP plasmid. For quantitation of miR-155 levels, total RNA was isolated from sorted cells using Trizol (Invitrogen). 1ug RNA was used for cDNA synthesis using SSIII reverse transcriptase kit (Invitrogen) followed by real-time PCR using miR-155-specific TaqMan miRNA Assay (Applied Biosystems). Expression was normalized to U6 snRNA (Applied Biosystems). All qPCR analysis was performed in an Applied Biosystems 7900HT Real Time PCR System.

Treg cell replacement in Foxp3^{DTR} mice

Donor $CD4^+CD25^+$ Treg cells (>90% $Foxp3^+$) were isolated from spleens and LNs of WT or *Ifnar1*$^{-/-}$ mice as described above. 2-3x10^6 donor Treg cells were transferred intravenously into each $Foxp3^{DTR}$ mouse. $Foxp3^{DTR}$ recipients were treated daily with 5ug/kg diphtheria toxin (Calbiochem) beginning at the time of transfer. Mice were infected with LCMV 7-8 days post-transfer.

In vivo killing assay

Splenocytes from congenically marked ($CD45.1^+$) mice were incubated for 9 minutes at 37°C with either 5 μM ("$CPDye^{hi}$") or 1uM ("$CPDye^{lo}$") Cell Proliferation Dye eFluor 670 (eBioscience) in PBS, and washed with 100% FBS. $CPDye^{hi}$-labeled splenocytes were pulsed for 1 hour at 37°C, 5% CO_2 with 1uM GP_{33-41} peptide in PBS; $CPDye^{lo}$-labeled splenocytes were unpulsed. Pulsed and unpulsed splenocytes were washed and mixed at a 1:1 ratio, and 10^7 cells were injected intravenously into each mouse. Mice were sacrificed 1 hour post-transfer. Percent killing was calculated as: 100 – ([%peptide-pulsed splenocytes in infected mice / %unpulsed splenocytes in infected mice) / (%peptide-pulsed in uninfected mice / %unpulsed in uninfected mice)] X 100).

Colitis induction

$CD4^+CD25^{hi}$ Treg cells were FACS sorted from spleens and peripheral lymph nodes of B6 or *Ifnar1*$^{-/-}$ mice. $CD4^+Foxp3^{GFP-}CD25^-CD45RB^{hi}$ Tconv cells were FACS sorted from spleens and peripheral lymph nodes of $Foxp3^{GFP}$ or $Foxp3^{GFP}$*Ifnar1*$^{-/-}$ mice. *Rag2*$^{-/-}$*Trex1*$^{+/-}$ or *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice (8-12 weeks old) were then injected intravenously with 1x10^5 Tconv and 2x10^5 Treg cells of the indicated genotype. Mice were weighed just prior to T cell transfer (time 0) and 1-2 times per week thereafter. Percent weight change was calculated as: (weight at time X – weight at time 0) / (weight at time 0). All mice in the same experimental group were sacrificed when any individual mice showed clinical signs of severe disease or 20 percent weight loss.

Histology and colitis scoring

Colon sections were immersion fixed in 10% neutral buffered formalin, paraffin embedded, cut into 5 µm sections, and stained with hematoxylin and eosin by the Benaroya Research Institute Histology Core. Sections were scored semiquantitatively from 0 to 4 for colitis severity in a blinded fashion (*107*). A grade of 0 was given when there were no changes observed. Changes typically associated with other grades are as follows: grade 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; grade 3, mild to moderate inflammatory cell infiltrates that were sometimes transmural, often associated with ulceration, with moderate epithelial hyperplasia and mucin depletion; grade 4, marked inflammatory cell infiltrates that were often transmural and associated with ulceration, with marked epithelial hyperplasia and mucin depletion; and grade 5, marked transmural inflammation with severe ulceration and loss of intestinal glands.

Enumeration of lymphocytes

Absolute numbers of lymphocytes in various tissues was determined using Polybead polystyrene nonfluorescent microspheres (15 um, Polysciences, Inc.). Briefly, 100ul of the cell suspension to be counted was mixed with 100ul of a fixed concentration (C_B) of Polybeads (one drop of Polybeads per ml of PBS) in a FACS tube. Without washing, the samples were acquired on a FACS Calibur (BD) and were quantified using the appropriate gates. Beads and lymphocytes were identified by their distinct forward- and side-scatter characteristics. The ratio of lymphocyte gate events (n_L) to bead gate events (n_B) was determined and used to calculate the concentration (C) of the original cell suspension as follows: $C = (n_L / n_B) * C_B$.

Statistics

All data are presented as the mean values ± SEM. Statistical significance was determined by one-way ANOVA with Tukey post-test, two-tailed unpaired t-test, or two-tailed unpaired t-test as indicated in figure legends. Statistical significance was established at the levels of *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$.

Chapter 3:

Type I IFNs Directly Inhibit Treg Cells During Acute LCMV Infection to Allow Optimal Antiviral Responses

Introduction

Excessive Treg cell activity interferes with efficient pathogen clearance and control in a variety of infectious diseases (*108*). Paradoxically, many of the signals known to drive Treg cell proliferation, like IL-2 and recognition of self-antigen, are also abundant during infection when Treg cell activity may need to be curbed. For example, IL-2 is produced by activated pathogen-specific $CD4^+$ T cells (*63*), and recognition of pathogen-associated molecular patterns drives dendritic cell activation, resulting in increased antigen presentation and expression of MHC class II and co-stimulatory ligands. While this is essential for the priming of pathogen-specific T cells, it could also lead to enhanced Treg cell activation, which could dampen protective T cell responses.

As a result, counter-regulatory mechanisms may be necessary to limit the activity of Treg cells during infection in order to allow for the generation of protective immune responses. This can be accomplished indirectly in some instances: for example, activation of APCs by TLR or CD40 stimulation protects them from Treg cell-mediated suppression (*109*), and pro-inflammatory cytokines such as IL-1β and IL-6 can render effector T cells resistant to Treg cell-mediated suppression (*110*, *111*). Additionally, effector T cells can limit Treg cell activity via IL-2 deprivation (*11*), and pro-inflammatory cytokines like TNFα can directly inhibit Treg cells (*18*).

Type I IFNs are known to regulate a number of cell types to coordinate effective antiviral immune responses, but their overexpression is associated with the development of autoimmunity. Previous studies have provided conflicting results regarding the impact of type I IFNs on Treg cells (*93*, *112-115*), and have generally not used experimental systems to examine the direct effects of IFNs on Treg cell homeostasis and function. Thus, the influence of

type I IFN signaling on Treg cell function, and the importance of this for the generation of effective antiviral immune responses, remains poorly understood.

Here, we demonstrate that type I IFNs directly inhibit co-stimulation-dependent Treg cell proliferation and activation both *in vitro*, and *in vivo* during acute infection with LCMV. This inhibition is cell-intrinsic and preferentially targets $CD62L^{lo}CD44^{hi}$ effector/memory Treg cells that potently inhibit effector T cell responses. Selective loss of IFNαR expression on Treg cells during LCMV infection results in functional impairment of virus-specific $CD8^+$ and $CD4^+$ T cells and inefficient viral clearance. Together, these data indicate that transient inhibition of Treg cells by IFNs during acute viral infection is necessary for the development of optimal antiviral T cell responses.

Results

Treg cells are dynamically regulated during acute viral infection

In C57BL/6 mice, infection with the Armstrong strain of LCMV causes acute viremia and induces potent $CD4^+$ and $CD8^+$ T cell responses that result in viral clearance by ~8 days post-infection (dpi). To determine how Treg cell activity is modulated during viral infection, we first monitored Treg cell proliferation and abundance over the course of LCMV-Armstrong infection. Whereas at 2 dpi there was little change in Treg cell abundance and phenotype, Treg cell numbers dropped progressively in the spleens of infected mice by 4 and 7 dpi (Fig 3.1a). This correlated with a significant decline in Treg cell proliferation at these time points, as measured by the percentage of cells expressing the cell cycle-associated nuclear antigen Ki-67, a common marker of cell proliferation that is expressed by cells in the G1, S, G2 and M phases of the cell cycle, but not by quiescent cells in G0 (Fig 3.1a).

This reduction in Treg cell abundance correlated inversely with $CD4^+Foxp3^-$ and $CD8^+$ T effector cell responses, which peaked in proliferation and number at 7 dpi, consistent with

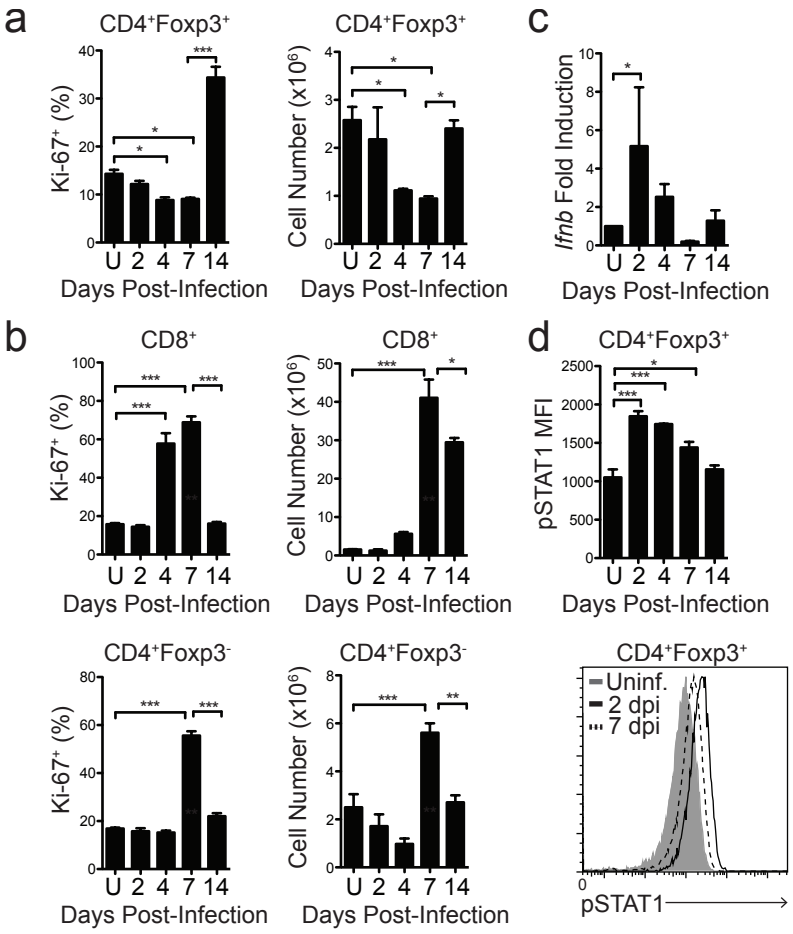

Figure 3.1. Treg cells are dynamically regulated during LCMV infection. a) Proportion of Ki-67$^+$ cells among CD4$^+$Foxp3$^+$ Treg cells (left) and absolute number of CD4$^+$Foxp3$^+$ Treg cells (right) in spleens of mice left uninfected (U) or at various days post-infection (dpi) with LCMV-Armstrong. b) Proportion of Ki-67$^+$ cells (left) and absolute number (right) of CD4$^+$Foxp3$^-$ T cells (bottom) and CD8$^+$ T cells (top) in spleens of mice infected with LCMV-Armstrong. c) Fold induction of *Ifnb* mRNA from whole spleens of mice infected with LCMV-Armstrong relative to uninfected controls. *Ifnb* mRNA expression was normalized to *Gapdh* expression. d) Median fluorescence intensity (MFI) (top) and representative staining (bottom) of phospho-STAT1 in gated CD4$^+$Foxp3$^+$ Treg cells directly *ex vivo* from spleens of mice infected with LCMV-Armstrong. For all panels, n=3-4 mice per group. Statistical significance was determined using one-way ANOVA with Tukey post-test (a-d). *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$. Data are representative of two independent experiments with 3-4 mice per time point.

published findings (Fig 3.1b) (*116*, *117*). After viral eradication, Treg cells displayed a proliferative burst at 14 dpi, which correlated with a significant recovery in Treg cell numbers and a reduction in the proliferation and number of $CD4^+Foxp3^-$ and $CD8^+$ T cells (Fig 3.1a, b).

LCMV provokes a robust type I IFN response that peaks at ~2 dpi and is required for antigen-specific T cell expansion and for viral clearance (Fig 3.1c) (*118*). Treg cells analyzed directly *ex vivo* from infected spleens showed increased phosphorylation of STAT1 that peaked by 2 dpi and gradually declined in intensity, closely mimicking the kinetics of IFNβ induction in infected spleens (Fig 3.1d). Interestingly, STAT1 phosphorylation in Treg cells preceded the decline in Treg cell number and proliferation, suggesting that type I IFN signaling may play a role in inhibiting Treg cells during infection.

IFNβ directly inhibits Treg cell proliferation in vitro

To determine whether type I IFNs could be responsible for the attrition of Treg cells we observed during acute LCMV infection, we tested the effect of IFNβ on Treg cell proliferation *in vitro*. For this, we isolated $CD4^+CD25^+$ Treg cells from WT or *Ifnar1*$^{-/-}$ mice that lack functional IFNαR expression, labeled them with CFSE, and cultured them with *Ifnar1*$^{-/-}$ antigen-presenting cells (APCs), soluble anti-CD3, and IL-2 in the presence or absence of recombinant IFNβ. After 72h of culture, both WT and *Ifnar1*$^{-/-}$ Treg cells proliferated robustly in the absence of IFNβ. However, increasing concentrations of IFNβ substantially reduced the proliferation of WT but not *Ifnar1*$^{-/-}$ Treg cells (Fig 3.2a, data not shown). Under physiological conditions, however, both APCs and Treg cells will be exposed to IFNs during infection, and the sum of IFNs' effects on both cell populations may affect Treg proliferation differently. Thus, to determine how IFNs affect Treg proliferation in this setting, we cultured WT or *Ifnar1*$^{-/-}$ Treg cells with WT APCs in the presence or absence of IFNβ for 72 hours. Interestingly, IFNβ inhibited the proliferation of WT Treg cells but not *Ifnar1*$^{-/-}$ Treg cells to the same extent whether in the presence of WT or

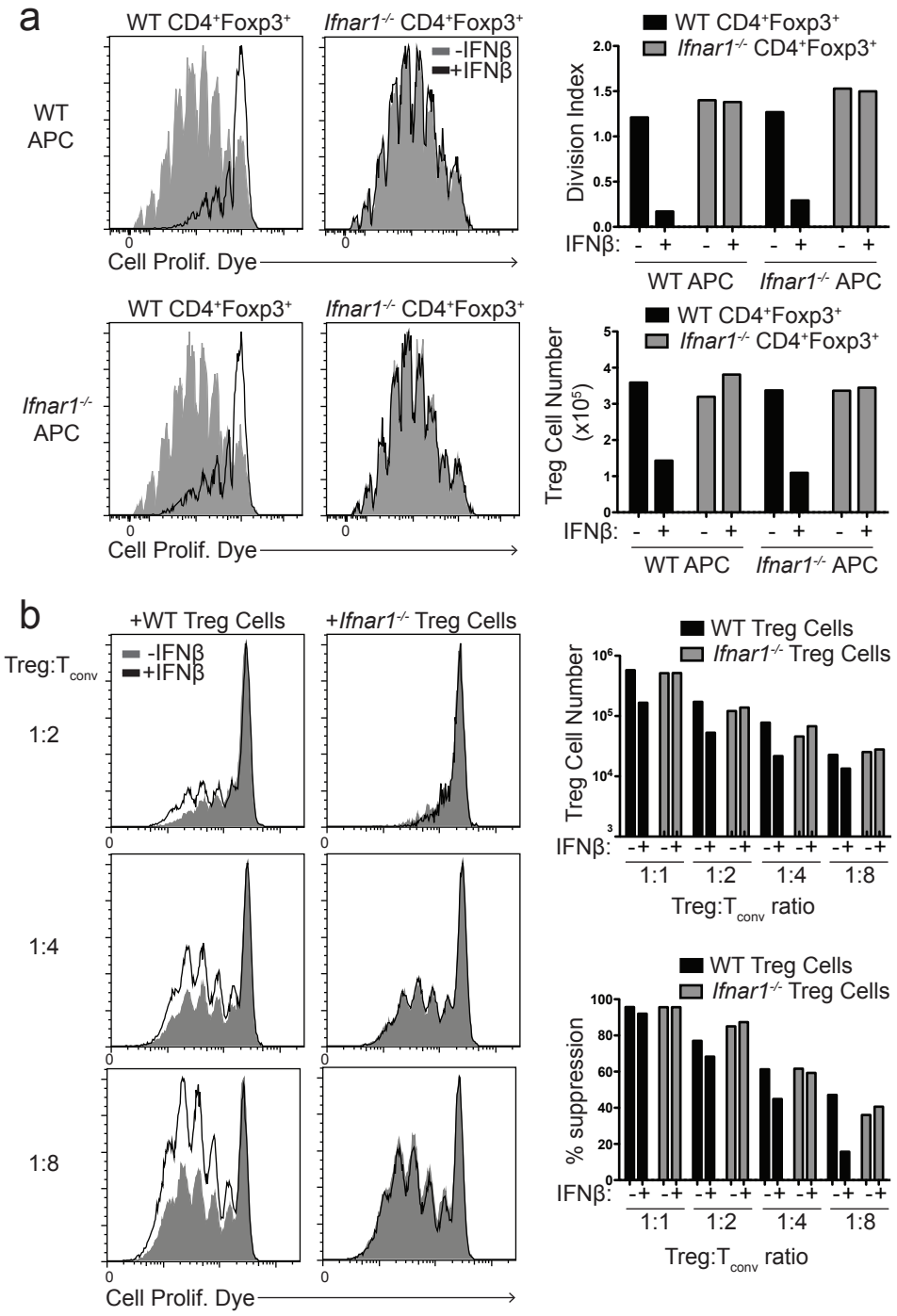

Figure 3.2. Type I IFNs directly inhibit Treg cell proliferation and activity *in vitro*. a) Left: Treg cell proliferation assay showing cell proliferation dye dilution of gated WT (left) or *Ifnar1*$^{-/-}$ (right) CD4^{+}Foxp3^{+} Treg cells cultured with WT (top) or *Ifnar1*$^{-/-}$ APCs (bottom) in the absence (gray) or presence (black) of 50 units (U)/ml IFNβ after 72h of culture with soluble CD3 and IL-2. Right: division index (top) and absolute number (bottom) of WT (black) or *Ifnar1*$^{-/-}$ (gray) Treg cells after 72h of culture in the indicated conditions. b) Left: *in vitro* suppression assay showing cell proliferation dye dilution of *Ifnar1*$^{-/-}$ CD4^{+}Foxp3^{-} conventional T cells (Tconv) in the presence of WT or *Ifnar1*$^{-/-}$ Treg cells at the indicated Treg:Tconv ratios in the absence (gray) or presence (black) of 50U/ml IFNβ after 72h of culture with soluble CD3 and irradiated *Ifnar1*$^{-/-}$ APCs. Right: absolute number of and percent suppression by WT (black) or *Ifnar1*$^{-/-}$ (gray) Treg cells at decreasing Treg:Tconv ratios after 72h culture in the absence ("-") or presence ("+") of 50U/ml IFNβ. Data are representative of three independent experiments.

Ifnar1$^{-/-}$ APCs, suggesting that inhibition was due to a direct effect of IFNβ on Treg cells and not due to an indirect effect on APCs (Fig 3.2a).

Next, we asked if addition of IFNβ could impair Treg cell function in a standard *in vitro* suppression assay. To ensure that the effects of IFNβ were restricted to Treg cells, we isolated CD4^{+}CD25^{-} conventional T cells (T_{conv}) and APCs from *Ifnar1*$^{-/-}$ mice. In the absence of added IFNβ, T_{conv} proliferation was suppressed equivalently by the addition of sorted WT or *Ifnar1*$^{-/-}$ CD4^{+}Foxp3^{gfp+} Treg cells. However, addition of IFNβ substantially inhibited suppression by WT Treg cells but not by *Ifnar1*$^{-/-}$ Treg cells (Fig 3.2b). This inhibitory effect was magnified at decreasing Treg:T_{conv} ratios, suggesting that the effect of IFNs is most apparent when Treg cell activity is already limited, and may be due to the impaired proliferation of Treg cells in these cultures. In fact, IFNβ-treated WT Treg cells showed a substantial reduction in cell number at the end of the culture period, whereas *Ifnar1*$^{-/-}$ Treg numbers were unaffected by the addition of IFNβ, suggesting that decreased Treg cell numbers may underlie the decline in suppression of T_{conv} proliferation (Fig 3.2b). Consistent with this, culture conditions with similar numbers of WT Treg cells (e.g. 1:4 with IFN compared to 1:8 without IFN) demonstrated similar suppression of T_{conv} proliferation (~40%), regardless of the presence of IFNβ (Fig 3.2b). This suggests that reduced suppression was due to a decline in Treg cell number, rather than an inhibition in Treg cell activity or function on a per cell basis.

Type I IFNs directly inhibit Treg cell proliferation and activation during LCMV infection

To determine if type I IFNs act directly on Treg cells to inhibit their activation during viral infection, we set up mixed bone marrow chimeras by transferring a 1:1 mixture of bone marrow from CD45.2^{+} *Ifnar1*$^{-/-}$ and CD45.1^{+} WT donor mice into irradiated T cell-deficient TCRβδ$^{-/-}$ recipients. After full hematopoietic reconstitution, WT and *Ifnar1*$^{-/-}$ Treg cells were present at equal numbers and were phenotypically similar, with ~15-20% of each population Ki-67^{+}, indicating that *Ifnar1*$^{-/-}$ Treg cells were not intrinsically different from their WT counterparts (Fig

3.3a, b). However, at both 4 and 7 dpi, there was a dramatic decline in the number of WT Treg cells but not *Ifnar1*$^{-/-}$ Treg cells (Fig 3.3a; data not shown), consistent with our *in vitro* results (Fig 3.2a). Additionally, at both 4 and 7 dpi there was a dramatic increase in Ki-67 expression among *Ifnar1*$^{-/-}$ Treg cells compared to WT Treg cells in the same animals (Fig 3.3b; data not shown). This pattern was unique to Treg cells, as *Ifnar1*$^{-/-}$ effector CD8^{+} and CD4^{+}Foxp3^{-} T cells examined in the same mixed bone marrow chimeric mice showed impaired proliferation compared to their WT counterparts, consistent with previous studies demonstrating type I IFNs are required for the expansion of virus-specific T cells (Fig 3.3c) (*119*, *120*). Moreover, *Ifnar1*$^{-/-}$ Treg cells displayed a more activated phenotype, with a greater proportion expressing the chemokine receptor CXCR3, and elevated surface expression of the activation markers CD44 and ICOS (Fig 3.4a). Expression of inhibitory receptors important for Treg cell suppressive function, such as CTLA-4, PD-1, and GITR, was also increased on *Ifnar1*$^{-/-}$ Treg cells (Fig 3.4a), altogether indicating that type I IFNs directly inhibit Treg cell proliferation, activation and function during LCMV infection.

The decline in the number of WT Treg cells in the spleens of infected mice could reflect a selective redistribution of Treg cells to non-lymphoid tissues. Specifically, recent studies have posited a role for IFNs in the distribution of Treg cells to the mucosa during inflammatory colitis (*121*). However, at 7 dpi we did not detect any change in the number or proliferation of WT Treg cells in the intestinal lamina propria (LP) (Fig 3.4b, c), suggesting that WT Treg cells from the spleen are not redistributing to the gut. Rather, there was a massive and significant increase in the proliferation and number of *Ifnar1*$^{-/-}$ Treg cells in the LP, and the ratio of *Ifnar1*$^{-/-}$:WT Treg cells approached 6:1 in the LP, compared with 3:1 in the spleens of the same animals (Fig 3.3a, 3.4b, c). Thus, these data suggest that IFNs do not simply induce Treg cells to re-distribute to non-lymphoid/mucosal tissues, but that they directly inhibit Treg cell proliferation and accumulation in both lymphoid and non-lymphoid tissues during LCMV infection.

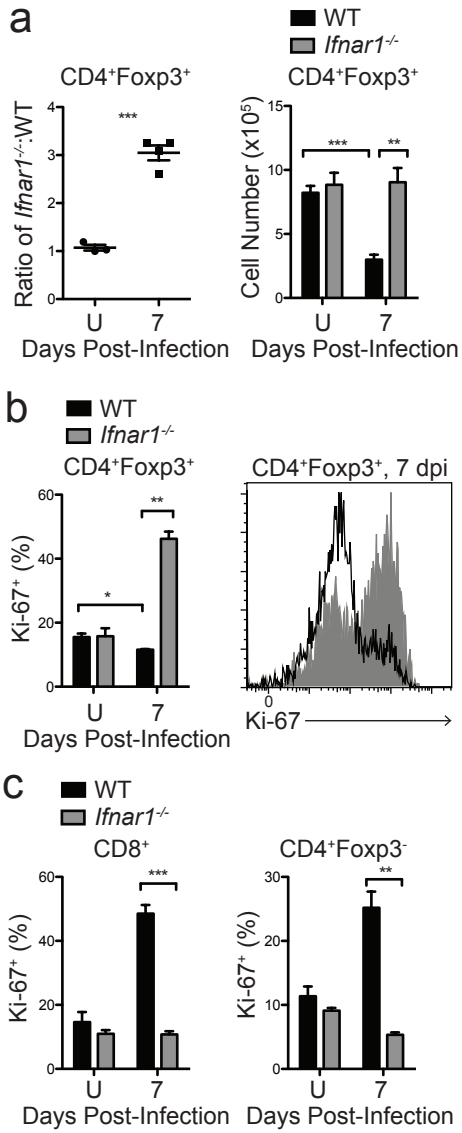

Figure 3.3. Type I IFNs directly inhibit Treg cell proliferation during LCMV infection. a) Ratio of *Ifnar1*$^{-/-}$:WT CD4^{+}Foxp3^{+} Treg cells (left) and absolute number of WT (black) and *Ifnar1*$^{-/-}$ CD4^{+}Foxp3^{+} Treg cells (right) in spleens of mixed bone marrow chimeric (BMC) mice left uninfected or 7 dpi with LCMV Armstrong. b) Summary (left) and representative flow cytometry analysis (right) of Ki-67 expression by WT (black) and *Ifnar1*$^{-/-}$ (gray) CD4^{+}Foxp3^{+} Treg cells from spleens of mixed BMC mice left uninfected or 7 dpi. c) Summary of Ki-67 expression by WT (black) and *Ifnar1*$^{-/-}$ (gray) CD8^{+} and CD4^{+}Foxp3^{-} T cells from spleens of mixed BMC mice left uninfected or 7 dpi. Statistical significance was determined using two-tailed paired t-test when comparing WT and *Ifnar1*$^{-/-}$ cells within the same BMC mouse. Two-tailed unpaired t-test was used when comparing cells from different mice. N=4 per group. Data are representative of 6 independent experiments with 3-4 mice per group. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$.

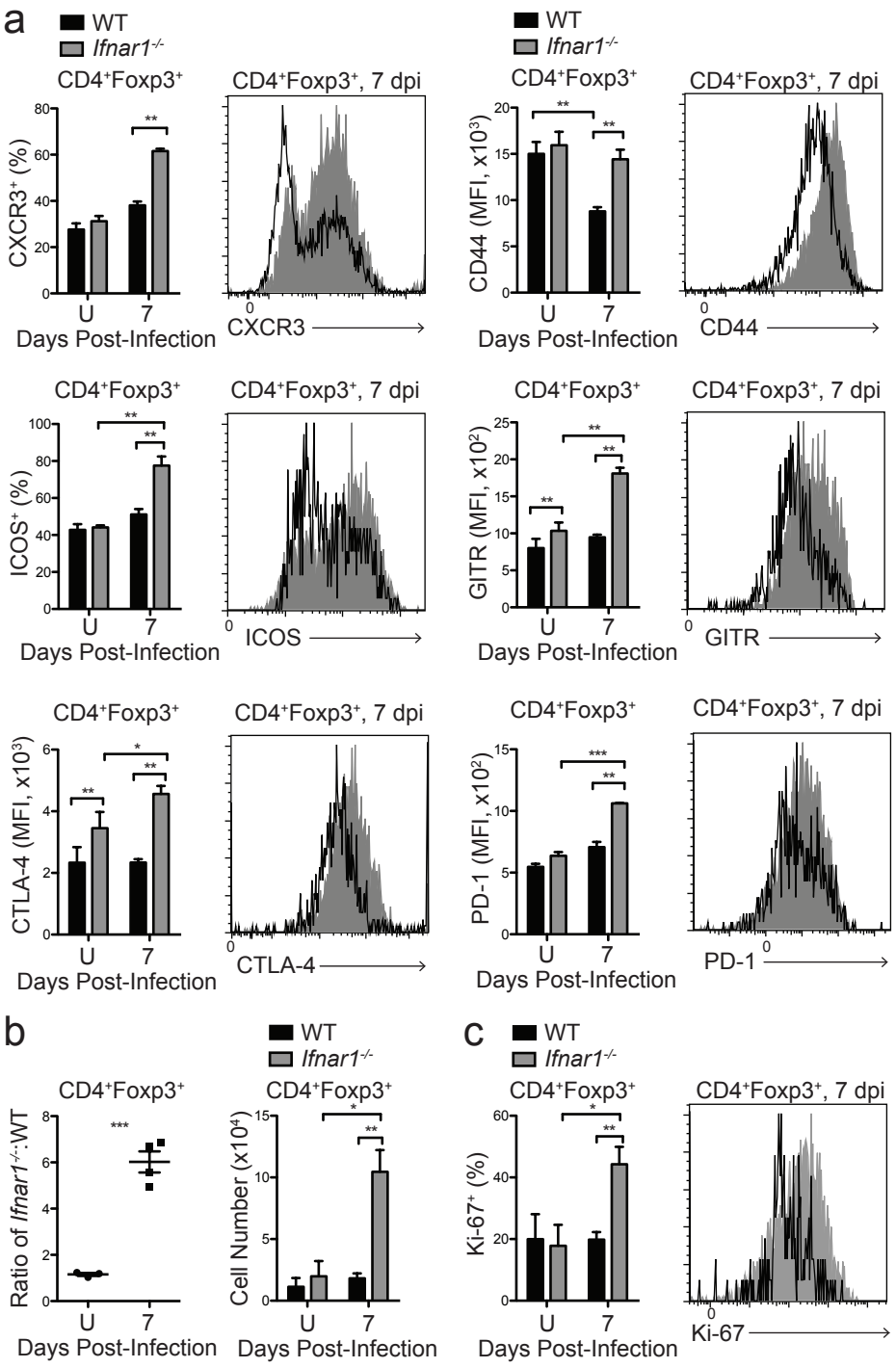

Figure 3.4. Type I IFNs directly inhibit Treg cell activity and accumulation in non-lymphoid tissues during LCMV infection. a) Representative flow cytometry analysis and summaries of the indicated markers expressed by WT (black) and *Ifnar1*$^{-/-}$ (gray) CD4^{+}Foxp3^{+} Treg cells in spleens of mixed bone marrow chimeric (BMC) mice left uninfected or 7 dpi with LCMV-Armstrong. b) Ratio of *Ifnar1*$^{-/-}$:WT CD4^{+}Foxp3^{+} Treg cells (left) and absolute number of WT (black) and *Ifnar1*$^{-/-}$ CD4^{+}Foxp3^{+} Treg cells (right) in small intestinal lamina propria (SI-LP) of mixed BMC mice left uninfected or 7 dpi with LCMV Armstrong. c) Summary (left) and representative flow cytometric analysis (right) of Ki-67 expression by WT (black) and *Ifnar1*$^{-/-}$ (gray) CD4^{+}Foxp3^{+} Treg cells from SI-LP of mixed BMC mice left uninfected or 7 dpi. Statistical significance was determined using two-tailed paired t-test when comparing WT and *Ifnar1*$^{-/-}$ cells within the same BMC mouse. Two-tailed unpaired t-test was used when comparing cells from different mice. N=4 per group. Data are representative of 6 (a) or 2 (b, c) independent experiments with 3-4 mice per group. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$.

Recent studies have demonstrated that pathogen-specific natural Treg cells are potent suppressors of effector responses and can delay or prevent pathogen clearance (*8*, *9*). To determine if loss of type I IFN responsiveness allowed for the expansion of LCMV-specific Treg cells, we used tetramer staining to track cells specific for the immunodominant I-A^b/GP_{66-77} epitope in our mixed bone marrow chimeric mice. LCMV infection provoked a robust response to the GP_{66-77} peptide only among WT-derived $CD4^+Foxp3^-$ effector T cells (Fig 3.5). As expected, GP_{66-77}-specific cells were largely absent from the population of *Ifnar1*$^{-/-}$ $CD4^+Foxp3^-$ T cells, consistent with the requirement for IFN in the expansion of antigen-specific effector $CD4^+$ T cells during LCMV infection (*119*). However, only ~1-2% of either WT or *Ifnar1*$^{-/-}$ Treg cells were I-A^b/GP_{66-77}-specific, indicating that the majority of Treg cells during LCMV infection are not specific for the immunodominant I-A^b/GP_{66-77} epitope. Moreover, although *Ifnar1*$^{-/-}$ Treg cells are hyperproliferative, activated, and accumulate during LCMV infection, this was not due to an expansion of Treg cells specific for the I-A^b/GP_{66-77} epitope.

Type I IFNs preferentially inhibit $CD62L^{lo}CD44^{hi}$ effector Treg cells

Like effector T cells, Treg cells can be divided into distinct subsets based on their expression of CD44 and CD62L (Fig 3.6a) (*122*, *123*). $CD62L^{hi}CD44^{lo}$ ("$CD44^{lo}$") Treg cells have a quiescent phenotype, undergo minimal homeostatic proliferation and recirculate through secondary lymphoid tissues. By contrast, $CD62L^{lo}CD44^{hi}$ ("$CD44^{hi}$") effector Treg cells are highly proliferative and express high levels of functional immunosuppressive molecules such as IL-10, GITR and CTLA-4 (*124*). Interestingly, the reduction in Treg cell number we observed during LCMV infection was restricted primarily to the $CD44^{hi}$ Treg cell subset, whereas the number of $CD44^{lo}$ Treg cells did not significantly change (Fig 3.6a). The selective decline in $CD44^{hi}$ Treg cells was IFN-dependent, as the number of $CD44^{hi}$ Treg cells was reduced among WT but not *Ifnar1*$^{-/-}$ Treg cells in mixed bone marrow chimeric mice 7 dpi, whereas the number of $CD44^{lo}$ Treg cells remained unchanged among both WT and *Ifnar1*$^{-/-}$ Treg cells (Fig 3.6b),

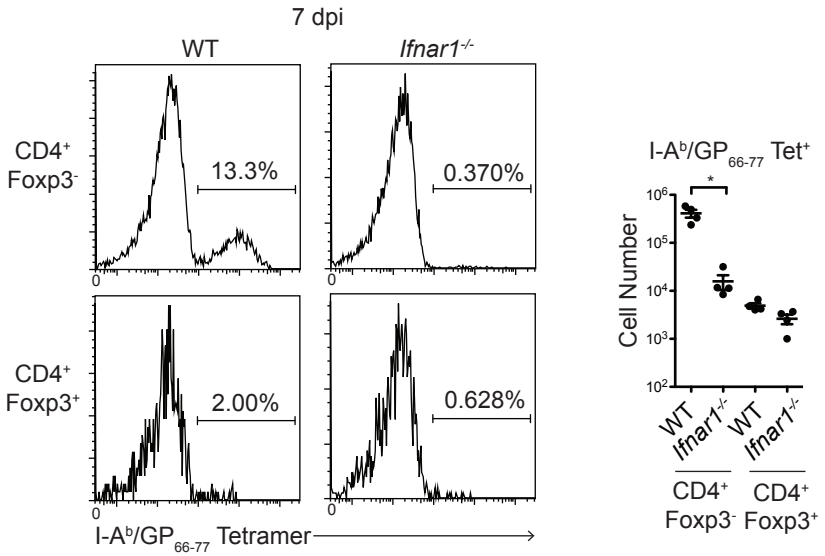

Figure 3.5. Neither WT nor *Ifnar1*$^{-/-}$ Treg cells are GP_{66-77} specific during LCMV infection. Left: Representative histograms showing I-A^b/GP_{66-77} tetramer staining among gated WT (left) or *Ifnar1*$^{-/-}$ (right) $CD4^+Foxp3^-$ T cells (top) and $CD4^+Foxp3^+$ T cells (bottom) from spleens of the same mixed bone marrow chimeric mouse 7 dpi with LCMV-Armstrong. Numbers represent frequency of tetramer-positive cells among the indicated populations. Right: Absolute number of the indicated tetramer-positive populations in spleens of chimeric mice 7 dpi with LCMV-Armstrong. Statistical significance was determined using two-tailed paired t-test. N=4 per group. Data are representative of 2 independent experiments with 3-4 mice per group. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$.

suggesting that type I IFNs preferentially act on the effector Treg cell population. Consistent with this hypothesis, $CD44^{hi}$ Treg cells showed a significantly greater degree of STAT1 phosphorylation than $CD44^{lo}$ Treg cells *in vivo* during LCMV infection and *in vitro* following stimulation with IFNβ (Fig 3.6c, 3.7a). By contrast, STAT1 phosphorylation was reduced in $CD44^{hi}$ effector $CD8^{+}$ and $CD4^{+}Foxp3^{-}$ T cells (Fig 3.7a). Although both $CD44^{lo}$ and $CD44^{hi}$ Treg cells showed comparable expression of IFNαR1, $CD44^{hi}$ Treg cells expressed consistently higher amounts of IFNαR2 and total STAT1 protein (Fig 3.7b). Additionally, $CD44^{hi}$ Treg cells expressed lower amounts of *Socs1* mRNA, a negative regulator of type I IFN signaling, as well as higher amounts of *miR-155*, a negative regulator of Socs1 (Fig 3.7c). Taken together, these changes in the IFNαR signaling machinery likely account for the higher responsiveness of $CD44^{hi}$ Treg cells to type I IFNs.

On most cells, type I IFNs are pro-apoptotic and anti-proliferative, and this is thought to underlie much of their antiviral effects. To determine if IFNαR signaling results in attrition of $CD44^{hi}$ Treg cells during infection, we asked how LCMV infection impacted the recovery of sorted $CD44^{hi}$ WT and *Ifnar1*$^{-/-}$ Treg cells following adoptive transfer into congenically-marked recipients. Indeed, we recovered significantly fewer of the transferred $CD44^{hi}$ WT Treg cells from infected mice, whereas the number of *Ifnar1*$^{-/-}$ $CD44^{hi}$ Treg cells did not change following infection (data not shown). These results were consistent with the IFNαR-dependent loss of $CD44^{hi}$ Treg cells we observed in our mixed bone marrow chimeric mice upon LCMV infection (Fig 3.6b) and indicate that $CD44^{hi}$ Treg cells undergo IFN-dependent attrition during LCMV infection. Moreover, the proliferation of WT $CD44^{hi}$ Treg cells was substantially lower than that of *Ifnar1*$^{-/-}$ $CD44^{hi}$ Treg cells in LCMV-infected mixed bone marrow chimeras (Fig 3.7d), indicating that IFNs also have direct anti-proliferative effects on these cells. Thus, a combination of IFN's anti-proliferative and pro-apoptotic effects on $CD44^{hi}$ effector Treg cells likely accounts for the loss of Treg cells observed during LCMV infection.

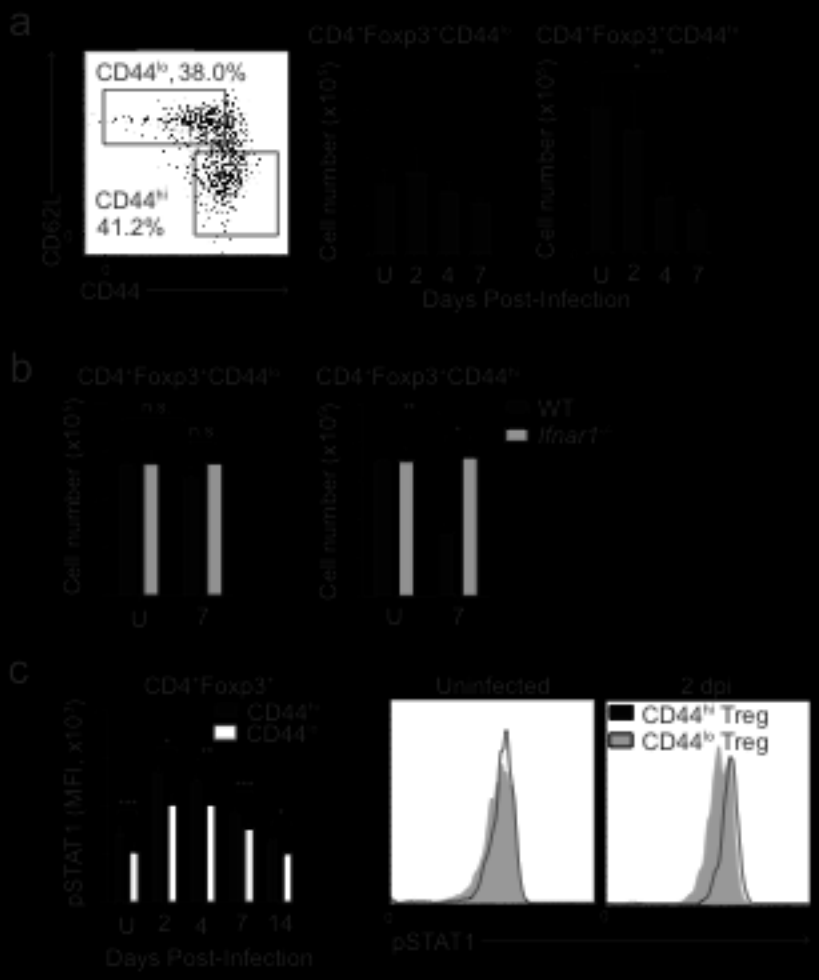

Figure 3.6. Type I IFNs preferentially inhibit CD62L^{lo}CD44^{hi} effector/memory Treg cells. a) Left: representative gating of CD44^{lo} and CD44^{hi} Treg cells. Right: absolute number of CD44^{lo} and CD44^{hi} CD4$^+$Foxp3$^+$ Treg cells in spleens of mice infected with LCMV-Armstrong. Data are representative of two independent experiments. N=4 mice per group. Statistical significance was determined using one-way ANOVA with Tukey post-test. b) Absolute number of CD44^{lo} (left) and CD44^{hi} (right) CD4$^+$Foxp3$^+$ Treg cells in mixed bone marrow chimeric mice left uninfected or 7 dpi. Data are representative of 6 independent experiments with 3-4 mice per group. Statistical significance was determined by two-tailed paired t-test. Two-tailed unpaired t-test was used when comparing cells from different mice. c) Representative histograms (right) and MFI summary (left) of phospho-STAT1 in CD44^{lo} (white) and CD44^{hi} (black) CD4$^+$Foxp3$^+$ Treg cells in spleens of mice infected with LCMV-Armstrong. Data are representative of two independent experiments. N=4 mice per group. Statistical significance was determined using two-tailed paired t-test. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$.

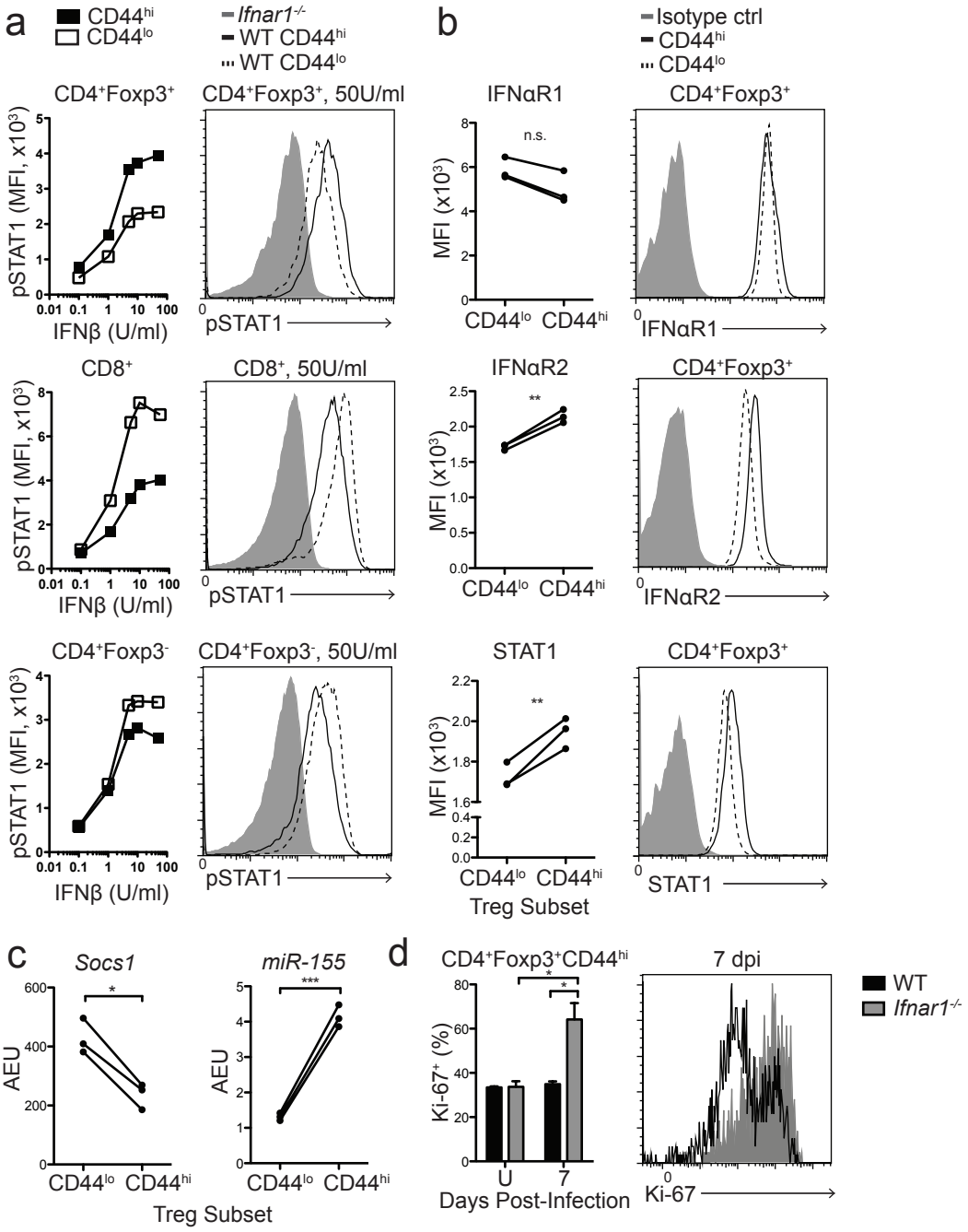

Figure 3.7. CD62L^{lo}CD44^{hi} effector/memory Treg cells are more responsive to type I IFNs *in vitro*. a) Representative histograms (right) and MFI summary (left) of phospho-STAT1 in *Ifnar1*$^{-/-}$, WT CD44^{lo} and WT CD44^{hi} Treg cells, CD8^{+}, and CD4^{+}Foxp3^{-} T cells stimulated for 30 minutes with IFNβ. Data are representative of 3 independent experiments. b) Representative histograms (right) and MFI summary (left) of IFNαR1, IFNαR2, and STAT1 in CD44^{lo} and CD44^{hi} CD4^{+}Foxp3^{+} Treg cells determined by flow cytometry. N=3 mice per group. Data are representative of three independent experiments. Statistical significance was determined using two-tailed paired t-test. c) *miR-155* microRNA and *Socs1* mRNA expression in sorted CD44^{lo} and CD44^{hi} CD4^{+}Foxp3^{+} Treg cells, expressed in arbitrary expression units (AEU) normalized to *U6* and *Gapdh* expression, respectively. Data are representative of two independent experiments with three mice each. Statistical significance was determined using two-tailed paired t-test. d) Summary (left) and representative histogram (right) of Ki67 expression by WT (black) and *Ifnar1*$^{-/-}$(gray) CD44^{hi} Treg cells from mixed bone marrow chimeric mice left uninfected (black) or infected with LCMV for 7 days (gray). N=4 per group. Data are representative of 6 independent experiments. Statistical significance was determined by two-tailed paired t-test. Two-tailed unpaired t-test was used when comparing cells from different mice. *, p<0.05; **, p<0.005; ***, p<0.0001.

Enhanced proliferation of Ifnar1$^{-/-}$ Treg cells during LCMV infection is ICOSL- and CD28-dependent

To better define the mechanism of IFN's anti-proliferative effects on Treg cells, we determined whether IFNs blocked Treg cell proliferation in response to different activating signals. IL-2 and the closely related cytokine IL-15 can drive Treg cell proliferation both *in vitro* and *in vivo* (*49*, *125*, *126*), and both are expressed abundantly during LCMV infection by effector T cells and as an interferon-stimulated gene, respectively (*127*, *128*). However, pre-treatment of Treg cells for 30 minutes with IFNβ did not affect IL-2-induced phosphorylation of STAT5 (data not shown). Additionally, using blocking antibodies and genetically deficient mice, we determined that neither IL-2 nor IL-15 was required for the enhanced proliferation of *Ifnar1*$^{-/-}$ Treg cells during LCMV infection (Fig 3.8a, b).

In addition to cytokine-driven proliferation/survival, recognition of self-antigen via stimulation of the TCR and co-receptors like CD28 and ICOS is also known to positively regulate Treg cell homeostasis (*57*, *58*). Therefore, we hypothesized that during infection increased antigen-presentation and expression of co-stimulatory ligands by pathogen-activated DCs drives the activation and proliferation of *Ifnar1*$^{-/-}$ Treg cells. Indeed, the number and proliferation of *Ifnar1*$^{-/-}$ Treg cells was significantly reduced 7 dpi after B7-1/B7-2 blockade compared with IgG-treated controls (Fig 3.8c). This inhibition was not secondary to impaired IL-2 signaling caused by reduced activation of effector T cells, as STAT5 phosphorylation in Treg cells was not decreased following B7-1/B7-2 blockade (data not shown). To confirm that enhanced proliferation of *Ifnar1*$^{-/-}$ Treg cells required Treg cell-intrinsic B7/CD28 co-stimulation, we compared the proliferation of *Ifnar1*$^{-/-}$ and *Ifnar1*$^{-/-}$*Cd28*$^{-/-}$ Treg cells transferred into WT recipients 4 dpi. Similar to our results with B7 blockade, loss of CD28 expression completely blocked the enhanced proliferation and accumulation of *Ifnar1*$^{-/-}$ Treg cells during infection (Fig 3.8d), indicating that the hyper-proliferation of *Ifnar1*$^{-/-}$ Treg cells during LCMV infection is dependent on CD28-costimulation. However, *Ifnar1*$^{-/-}$ Treg cells still proliferated roughly 2-fold

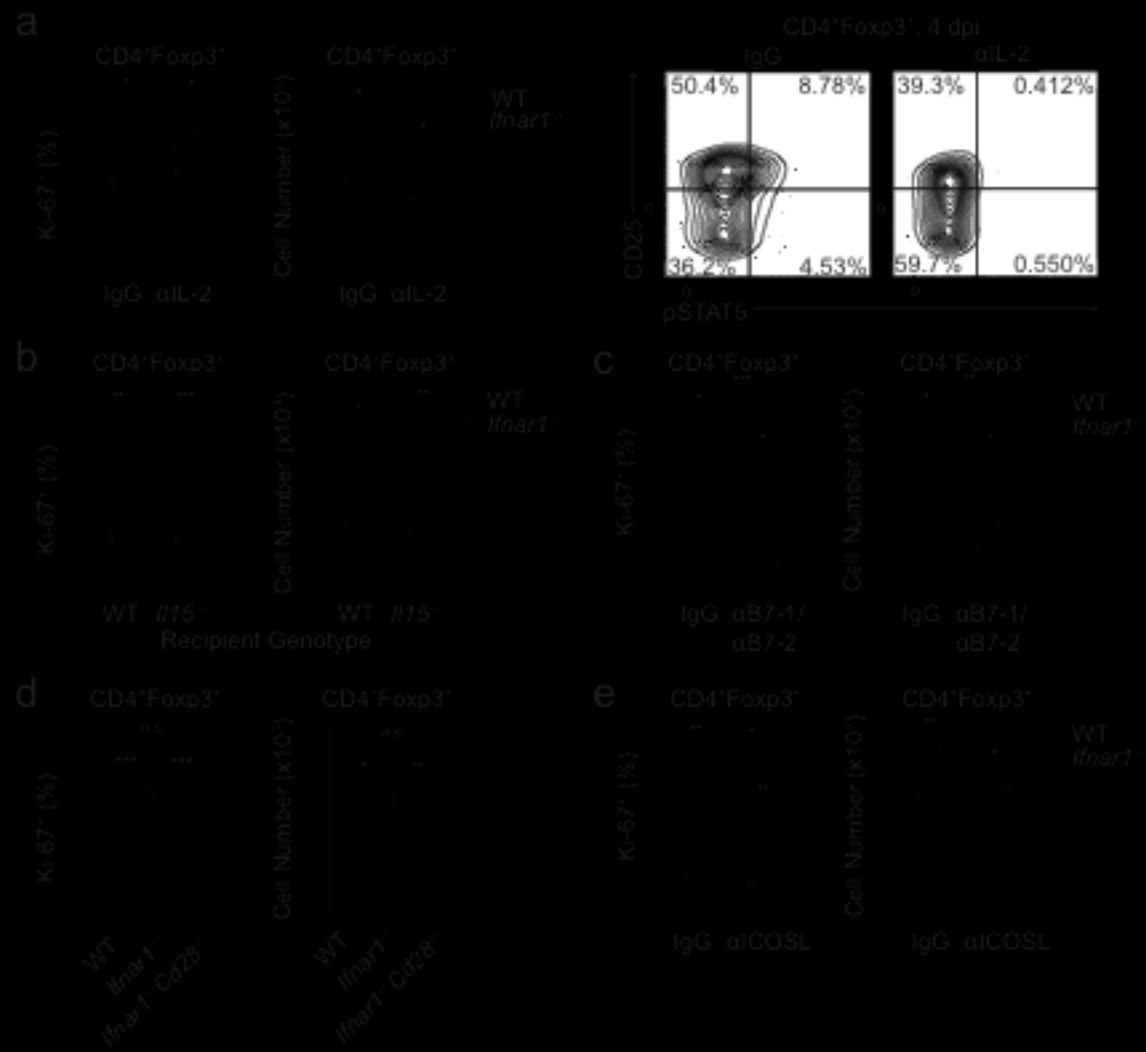

Figure 3.8. Enhanced proliferation of *Ifnar1*$^{-/-}$ Treg cells is ICOSL- and CD28-dependent. a) Left: summary of Ki-67 expression and absolute number of transferred WT (black) and *Ifnar1*$^{-/-}$ (open squares) CD4$^+$Foxp3$^+$ Treg cells in WT mice 4 dpi with LCMV-Armstrong and treated with IgG or blocking anti-IL-2 antibody (αIL-2). Right: representative flow cytometric analysis showing pSTAT5 and CD25 expression by total CD4$^+$Foxp3$^+$ Treg cells in spleens of mice 4 dpi treated with IgG or αIL-2. Numbers represent frequency of the indicated quadrant among total CD4$^+$Foxp3$^+$ Treg cells. b) Summary of Ki-67 expression and absolute number of transferred WT (black) and *Ifnar1*$^{-/-}$ (open squares) CD4$^+$Foxp3$^+$ Treg cells in WT or *Il15*$^{-/-}$ recipient mice 4 dpi with LCMV-Armstrong. c) summary of Ki-67 expression and absolute number of transferred WT (black) and *Ifnar1*$^{-/-}$ (open squares) CD4$^+$Foxp3$^+$ Treg cells in WT mice 4 dpi with LCMV-Armstrong and treated with IgG or blocking anti-B7-1/B7-2 antibodies (αB7-1/B7-2). d) summary of Ki-67 expression and absolute number of transferred WT, *Ifnar1*$^{-/-}$, or *Ifnar1*$^{-/-}$ *Cd28*$^{-/-}$ Treg cells in spleens of WT mice 4 dpi with LCMV-Armstrong. e) Summary of Ki-67 expression and absolute number of transferred WT (black) and *Ifnar1*$^{-/-}$ (open squares) CD4$^+$Foxp3$^+$ Treg cells in WT mice 4 dpi treated with IgG or blocking anti-ICOSL antibody (αICOSL). a-c, e: statistical significance was determined using paired two-tailed t-test when comparing WT and *Ifnar1*$^{-/-}$ Treg cells in the same recipient mice or unpaired two-tailed t-test when comparing Treg cells in different mice. d: statistical significance was determined using one-way ANOVA with Tukey post-test. For all panels, n=3 per group. Results are representative of 2 independent experiments. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$.

more and were present at higher number than WT Treg cells in both control and B7-1/B7-2-blocked mice, indicating that the ability of type I IFNs to inhibit Treg cell proliferation is independent of any direct effects on CD28 signaling. Consistent with this, IFNβ had no effect on CD3/28-dependent phosphorylation of the downstream PI3K/Akt substrate S6 in Treg cells (data not shown), indicating that type I IFNs do not inhibit Treg cells by directly modifying this important co-stimulatory signaling pathway.

Stimulation through the TCR and CD28 leads to up-regulation of the inducible co-stimulatory molecule ICOS, which can assume many of the functions of CD28 in promoting T cell expansion/survival (*129*). As ICOS expression was significantly up-regulated on *Ifnar1*$^{-/-}$ Treg cells during infection (Fig 3.4a), we also asked if enhanced ICOS-ICOSL interactions contributed to the hyper-proliferation of *Ifnar1*$^{-/-}$ Treg cells by transferring WT and *Ifnar1*$^{-/-}$ Treg cells into mice, treating them the an α-ICOSL blocking antibody HK5.3 (*130*, *131*), and infecting them with LCMV. Blockade of ICOS signaling partially inhibited the enhanced proliferation of *Ifnar1*$^{-/-}$ Treg cells, indicating that up-regulation of ICOS contributes to CD28-dependent activation of *Ifnar1*$^{-/-}$ Treg cells during infection (Fig 3.8e). Together, these data demonstrate that although LCMV infection results in a co-stimulatory environment highly favorable for Treg cell expansion, this is prevented by the direct action of type I IFNs on Treg cells.

IFN-mediated inhibition of Treg cells is necessary for optimal antiviral T cell responses

Finally, to determine what effect IFN-mediated inhibition of Treg cells has on the generation of antiviral T cell responses, we modified a previously described Treg cell "replacement" protocol in which Foxp3^{DTR} mice were treated daily with diphtheria toxin (DT) to deplete mice of endogenous Treg cells and reconstituted with Treg cells from either WT or *Ifnar1*$^{-/-}$ donors (*132*). By 7 days post-transfer, both WT and *Ifnar1*$^{-/-}$ Treg cells were present at similar frequencies in spleen and peripheral lymph nodes that were comparable to those in found in unmanipulated mice (Fig 3.9a; not shown). Moreover, mice reconstituted with *Ifnar1*$^{-/-}$

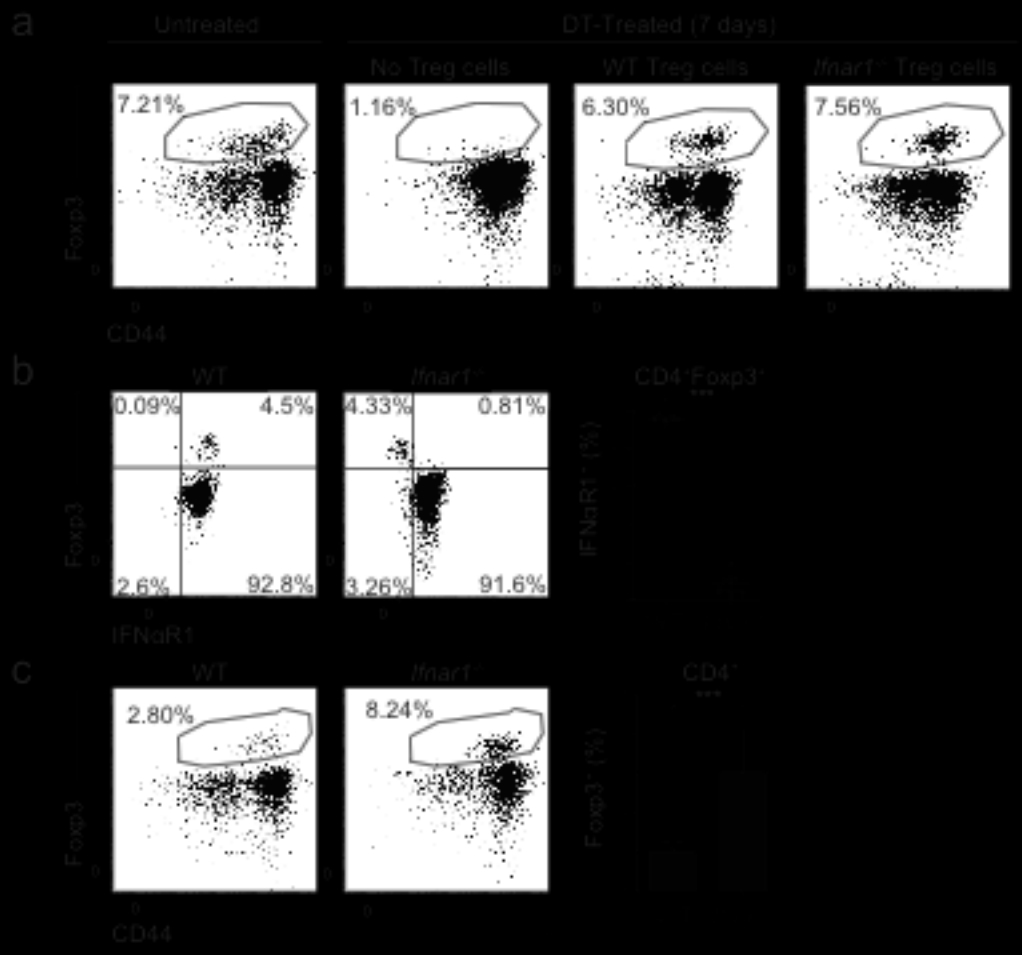

Figure 3.9. Treg "replacement" of Foxp3^{DTR} mice selectively eliminates IFNαR1 expression on Treg cells. a) Representative flow plots showing gated CD4$^+$ T cells from spleens of Foxp3^{DTR} mice left untreated (left) or treated daily for 7 days with 5μg/kg diphtheria toxin (DT). DT-treated mice were reconstituted with either no Treg cells (middle left), WT Treg cells (middle right), or *Ifnar1*$^{-/-}$ Treg cells (right) at the start of DT treatment. Numbers represent frequency of Foxp3$^+$ Treg cells among gated CD4$^+$ T cells. b) Representative flow cytometry analysis showing IFNαR1 expression on gated CD4$^+$ T cells (left) and summary of IFNαR1 expression on CD4$^+$Foxp3$^+$ Treg cells (right) from peripheral blood of Foxp3^{DTR} mice replaced with WT or *Ifnar1*$^{-/-}$ Treg cells one day prior to infection with LCMV. Numbers represent frequency of the indicated quadrants among total CD4$^+$ T cells. N=5 per group. Data are representative of three independent experiments. c) Representative flow plots (left) and summary (right) showing frequency of Foxp3$^+$ Treg cells among gated CD4$^+$ T cells in WT- and *Ifnar1*$^{-/-}$-replaced Foxp3^{DTR} mice 7 dpi with LCMV-Armstrong. Numbers represent frequency of CD4$^+$Foxp3$^+$ Treg cells among total CD4$^+$ T cells. N=10 per group; data are summarized from 3 independent experiments. For all panels, statistical significance was determined using a two-tailed unpaired t-test. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$.

Treg cells showed a selective absence of IFNαR1 expression on Treg cells (Fig 3.9b). However, by 7 dpi, Treg cells in the *Ifnar1*$^{-/-}$-replaced mice were present at significantly higher frequency than those in WT-replaced mice (Fig 3.9c). To understand how these changes in Treg cell abundance may impact the antiviral effector T cell response, we examined the abundance and function of LCMV-specific effector T cells in these mice. The absolute number of CD8^{+} T cells specific for the immunodominant D^b/GP_{33-41} epitope as assessed by tetramer staining was not significantly different between WT- and *Ifnar1*$^{-/-}$-replaced mice (Fig 3.10b). However, the proportion and number of CD8^{+} T cells producing IFN-γ upon restimulation with GP_{33-41} peptide was significantly reduced in mice reconstituted with *Ifnar1*$^{-/-}$ Treg cells, and similar results were observed in CD4^{+}Foxp3^{-} T cells stimulated with the GP_{61-80} peptide (Fig 3.10a). Additionally, mice reconstituted with *Ifnar1*$^{-/-}$ Treg cells showed a reduced ability to kill GP_{33-41} peptide-pulsed splenocytes in an *in vivo* cytotoxicity assay (Fig 3.10c, d), and had significantly higher levels of viral RNA at 7 dpi (Fig 3.10e). Thus, type I IFN-dependent inhibition of Treg cells is essential for the generation of optimal antiviral T cell responses and normal viral clearance.

Discussion

A major challenge Treg cells face during infection is how to maintain self-tolerance while allowing pathogen-specific immune responses to occur. Although excessive Treg cell activity is associated with chronic infection and failure to clear pathogens, too little Treg cell activity during infection can result in autoimmunity and widespread effector T cell activation that impairs pathogen-specific responses (*26*, *133*, *134*).

Interestingly, type I IFNs did not globally inhibit Treg cells during LCMV infection, but instead induced a selective decrease in CD44^{hi} Treg cells. Unlike their quiescent CD44^{lo} counterparts, which reside primarily in secondary lymphoid tissues and effectively inhibit T cell priming, CD44^{hi} Treg cells proliferate robustly, abundantly express suppressive effector molecules such as CTLA4, IL-10, and ICOS, and are able to migrate to sites of infection and

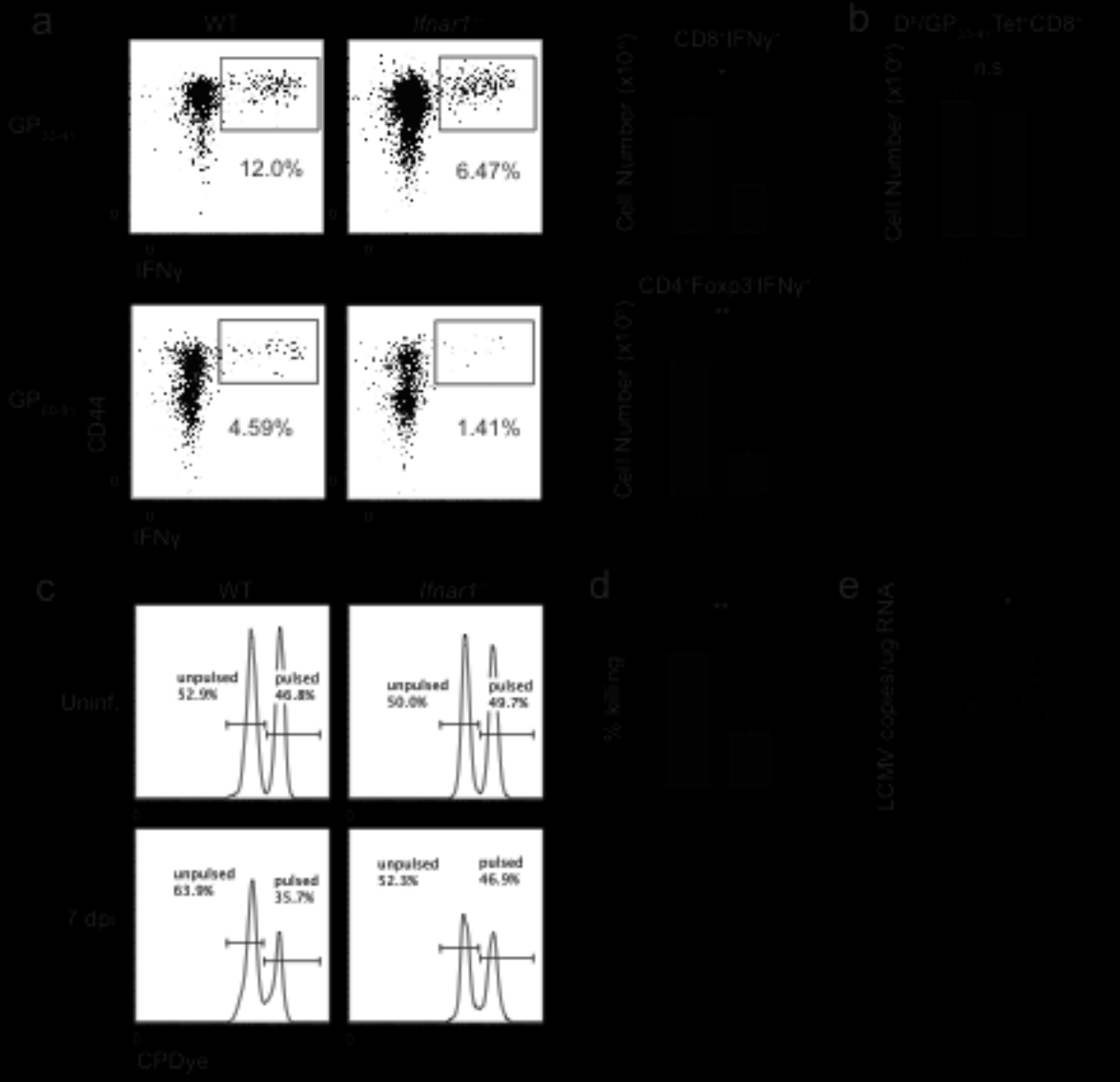

Figure 3.10. Inhibition of Treg cells by type I IFNs is necessary for optimal antiviral immune responses. a) Left: Representative flow cytometric analysis of IFN-γ production by gated $CD8^+$ T cells (top) and gated $CD4^+Foxp3^-$ T cells (bottom) by intracellular cytokine staining 7 dpi in WT- and *Ifnar1*$^{-/-}$-replaced $Foxp3^{DTR}$ mice following 5h stimulation of whole splenocytes with GP_{33-41} (top) or $GP_{276-286}$ (bottom) peptide. Numbers represent frequency of IFN-γ$^+$ cells among $CD8^+$ (top) and $CD4^+Foxp3^-$ (bottom) T cells. Right: absolute number of LCMV peptide-specific IFN-γ$^+CD8^+$ (top) and IFN-γ$^+CD4^+Foxp3^-$ T cells (bottom). b) Absolute number of $CD8^+$ T cells staining positively for D^b/GP_{33-41} tetramer as assessed by flow cytometry. c) Representative flow cytometric histograms from *in vivo* cytotoxicity assay showing frequency of CPDye^{low}-labeled GP_{33-41} peptide-"pulsed" splenocytes relative to CPDye^{high}-labeled "unpulsed" splenocytes in WT- and *Ifnar1*$^{-/-}$-replaced $Foxp3^{DTR}$ mice left uninfected or 7 dpi, 1hr after transfer of splenocytes. d) Summary of percent killing of peptide-pulsed splenocytes in WT- and *Ifnar1*$^{-/-}$-replaced $Foxp3^{DTR}$ mice 7 dpi. e) LCMV *GP* RNA expression measured by qPCR in infected spleens and normalized to a standard curve generated using an LCMV-GP plasmid. N=10 per group; data are summarized from 3 independent experiments. For all panels, statistical significance was determined using a two-tailed unpaired t-test. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$.

inflammation via expression of a broad array of tissue-homing receptors (*124*, *135*). Thus, they are poised to inhibit the activity of primed effector T cells. Consistent with this, when Treg cells were made resistant to IFN-mediated inhibition, there was no change in the absolute number of virus-specific $CD8^+$ T cells, suggesting there was no difference in T cell priming or clonal expansion. Rather, LCMV-specific $CD8^+$ T cells were selectively impaired in their effector functions, failing to produce IFN-γ and kill viral peptide-pulsed APCs as efficiently as in mice with WT Treg cells. Several studies have demonstrated that Treg cells can regulate $CD8^+$ and $CD4^+$ T cell effector function independent of their ability to inhibit T cell proliferation via anti-inflammatory cytokines such as TGFβ and IL-10 (*136*-*139*). Interestingly, IL-10 and TGFβ production are significantly enhanced in $CD44^{hi}$ Treg cells (*124*, *140*), and thus our data are consistent with a model in which specific inhibition of $CD44^{hi}$ effector Treg cells allows pathogen-specific effector T cells to carry out their protective functions, whereas the priming of self-reactive T cells in lymphoid tissues remains blocked by $CD44^{lo}$ Treg cells, thereby helping avoid the development of collateral autoimmunity.

That Treg cells and effector T cells both rely on similar signals, like IL-2 and TCR/co-receptor signaling, for their activation and proliferation has made it difficult to understand how these functionally opposed cell types are differentially regulated. However, during LCMV infection, the ability of type I IFNs to enhance the clonal expansion of antigen-specific effector T cells while inhibiting the activation and proliferation of Treg cells is one way in which the activities of effector and Treg cells can be separated. Although LCMV infection results in a co-stimulatory environment that is favorable for both effector T cell and Treg cell proliferation, the parallel expansion of Treg cells is prevented by the direct action of type I IFNs on Treg cells. The precise molecular mechanisms by which IFNs inhibit Treg cell proliferation are not well defined, but our results suggest that IFNs exert direct pro-apoptotic and anti-proliferative effects on Treg cells. There is some evidence for cross-talk between IFNαR and TCR/co-receptor pathways, as several signaling components such as Zap70, CD45 and Lck have been shown to

interact with IFNαR, and this interaction was required for the anti-proliferative effects of IFNα in Jurkat T cells (*141*). Although IFNβ had no effect on CD3/28-dependent phosphorylation of the downstream PI3K/Akt substrate S6 in Treg cells (data not shown), it is possible that TCR/co-receptor signaling may modulate the IFNαR signaling pathway in Treg cells. The anti-proliferative effects of type I IFNs in conventional T cells appear to be STAT1-dependent (*142*, *143*). However, STAT1 expression is reduced in virus-specific $CD8^+$ T cells, and this changes the ratio of different STAT proteins activated by IFNαR signaling, turning this from an anti-proliferative into a pro-proliferative signal required for the optimal expansion of virus-specific cells (*144*, *145*). An inverse mechanism may be at play in Treg cells, where TCR signaling modulates components of the IFNαR signaling pathway to sensitize activated Treg cells to the anti-proliferative effects of type I IFNs. Indeed, we show that $CD44^{hi}$ Treg cells expressed higher levels of IFNαR2, STAT1, and *mir-155* and lower levels of *Socs1*, than $CD44^{lo}$ Treg cells, consistent with their higher responsiveness to IFNs *in vitro* and *in vivo* during LCMV infection.

The immunomodulatory effects of type I IFNs are incredibly complex, and several studies have suggested that IFNs may actually promote Treg activity in other contexts, particularly during multiple sclerosis and inflammatory colitis. IFNβ is a commonly prescribed treatment for relapsing-remitting multiple sclerosis, and IFNβ treatment markedly enhanced the function of Treg cells in these patients (*113*, *146*). However, these studies fail to distinguish the direct effect of type I IFNs on Treg cells from their indirect effects on other cell types. In fact, several studies have now demonstrated that the protective functions of IFNs during experimental autoimmune encephalomyelitis depend on IFNαR expression on DCs but not on T cells, and that the enhanced proliferation of Treg cells may be secondary to activation of co-stimulatory molecules like GITRL on DCs (*102-104*). However, contrary to our results, a recent study demonstrated a direct role for IFN in the maintenance of Treg cells in the mucosa during inflammatory colitis (*121*). This discrepancy may be due to differences in the timing and extent of type I IFN expression in the different models used, as IFNs can exert opposing effects when

expressed acutely or chronically. For instance, during LCMV infection, transient IFN production helps promote viral clearance, whereas heightened and prolonged IFN expression promotes viral persistence and immunosuppression (*99*, *100*). Indeed, in the colitis studies mice were given pegylated IFNα for several weeks, whereas IFNs are only acutely induced for 2-4 days during acute LCMV infection. As excessive type I IFN production is associated with a number of organ-specific and systemic autoimmune disorders, such as systemic lupus erythematosus, psoriasis and Sjögren's syndrome (*147*), it will be essential to determine how IFNs directly and indirectly modulate Treg cell activity in a variety of normal and pathological contexts.

Our data elucidate a novel antiviral mechanism of type I IFNs during acute LCMV infection and further highlight the degree to which Treg cell activity is sensitive to external cues in the immune environment. Further studies will be required to determine whether prolonged IFN production impacts Treg cell function, and how this may in turn contribute to immune dysfunction in chronic infection and type I IFN-associated autoimmune diseases.

Chapter 4:

Chronic IFN Expression Impairs Treg Suppressive Function Indirectly through Its Effects on Conventional T Cells

Introduction

Although Treg suppressive activity is clearly impaired in autoimmune disorders, what factors lead to this dysfunction are still being elucidated. Treg and effector T cell-intrinsic defects may contribute to loss of tolerance; additionally, overexpression of inflammatory mediators can directly inhibit Treg cell activity and can activate antigen-presenting cells and effector T cells, making both cell types refractory to Treg cell-mediated suppression. Several pro-inflammatory cytokines, including IL-6, IL-1β, and TNFα, have been shown to subvert Treg cell activity, both directly and indirectly (*18*, *110*, *111*, *148-150*). While these cytokines may play a protective role during acute infection by circumventing Treg cell activity, their overexpression is largely associated with Treg cell dysfunction and autoimmunity.

Although essential for proper anti-viral immunity, excessive type I IFN production has been associated with a variety of organ-specific and systemic autoimmune disorders (*147*). In the previous chapter, we demonstrated that type I IFNs inhibit Treg cell activation and proliferation both *in vitro* and *in vivo* during acute LCMV infection, and that this transient inhibition is necessary for the generation of optimal antiviral T cell responses. However, the immunomodulatory effects of type I IFNs are incredibly complex, and recent studies have suggested that type I IFNs may exert opposing effects based on contextual factors, such as the timing and extent of their expression (*99*, *151*). Thus, how dysregulated IFN production regulates Treg cells in the context of autoimmunity is less clear.

Here, we asked how chronic IFN expression regulates Treg cell activity, both directly and indirectly, and how this in turn contributes to immune dysfunction during type I IFN-associated autoimmune diseases. Using a well-established *in vivo* assay of Treg cell function, we show that Treg cell function is impaired in mice that chronically overproduce type I IFNs due

to loss of the DNA exonuclease Trex1. Treg cell dysfunction in these mice completely depended on type I IFN signaling in T cells. Although IFN overexpression directly inhibited Treg cell proliferation and activation, this inhibition was not required for the onset of inflammatory disease. Rather, chronic IFN expression directly promoted the expansion of conventional T cells, and inflammatory disease was completely dependent on IFNαR signaling in conventional T cells. Thus, chronic IFN expression drives inflammatory disease by impairing Treg cell function *in vivo* both directly and indirectly through its effects on conventional T cells.

Results

Treg cell function is impaired in Trex1$^{-/-}$ mice due to IFNαR signaling in T cells

To assess how dysregulated overproduction of type I IFNs impacts Treg cell activity, we have used mice lacking Trex1, a 3'→5' cytoplasmic DNA exonuclease that is ubiquitously expressed and functions to degrade endogenous retroelements and other cytoplasmic DNA (*152*, *153*). Trex1 is a critical negative regulator of the IFN-stimulatory DNA (ISD) response, and mutations in Trex1 cause an accumulation of endogenous cytoplasmic DNA that triggers type I IFN production, inflammation and autoimmunity in mice and humans (*152-155*). Importantly, autoimmunity in *Trex1$^{-/-}$* mice is entirely dependent on lymphocyte function and type I IFN expression, as *Trex1$^{-/-}$Rag2$^{-/-}$* and *Trex1$^{-/-}$Ifnar1$^{-/-}$* double-deficient animals are completely protected from disease development (*153*, *156*). Importantly, although *Rag2$^{-/-}$Trex1$^{-/-}$* mice remain healthy, they display chronically elevated type I IFN production as early as 8 days after birth (*156*).

We used a well-described T cell transfer model of colitis as a sensitive assay of Treg cell function to assess how overproduction of type I IFNs influences Treg cell function in *Trex1$^{-/-}$* mice. Colitis caused by adoptive transfer of CD4^{+}Foxp3^{-}CD45RB^{hi} conventional T cells (Tconv) into *Rag2$^{-/-}$* hosts can be prevented by co-transfer of purified CD4^{+}CD25^{+} Treg cells (*107*, *157*, *158*). Whereas co-transfer of WT Tconv cells and WT Treg cells prevented colitis in all *Rag2$^{-/-}$*

Trex1$^{+/-}$ animals as expected, colitis still occurred in the *Rag2*$^{-/-}$*Trex1*$^{-/-}$ recipients. *Rag2*$^{-/-}$*Trex1*$^{-/-}$ recipients exhibited clinical signs of colitis, including hunching, diarrhea, and anal inflammation, and gained significantly less weight than *Rag2*$^{-/-}$*Trex1*$^{+/-}$ recipients as early as 4 weeks post-transfer (Fig 4.1a; not shown). Moreover, *Rag2*$^{-/-}$*Trex1*$^{-/-}$ recipients developed significant colon inflammation, with higher numbers of intraepithelial (IEL) and lamina propria lymphocytes (LPL) in the colon compared to *Rag2*$^{-/-}$*Trex1*$^{+/-}$ recipients (Fig 4.1a). Consistent with this, histological analysis of colons showed leukocytic infiltrate in the LP, depletion of goblet cells, and moderate epithelial cell hyperplasia, as well as disrupted colonic architecture in *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice compared to *Rag2*$^{-/-}$*Trex1*$^{+/-}$ mice (Fig 4.1b). Altogether, these data clearly demonstrate that loss of Trex1 significantly impairs the ability of Treg cells to suppress inflammatory colitis *in vivo*.

Trex1-deficiency results in the dysregulation of many cellular processes, such as the clearance of extranuclear DNA, the activation of the ISD response, and the production of type I IFNs. To identify whether overproduction of IFNs, specifically, was responsible for the impairment of Treg cell function in Trex1-deficient mice, we sorted Tconv and Treg cells from *Ifnar1*$^{-/-}$ mice that lack the ability to signal through the type I IFN receptor. We then co-transferred *Ifnar1*$^{-/-}$ Tconv and *Ifnar1*$^{-/-}$ Treg cells into *Rag2*$^{-/-}$*Trex1*$^{+/-}$ or *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice. Interestingly, neither *Rag2*$^{-/-}$*Trex1*$^{+/-}$ nor *Rag2*$^{-/-}$*Trex1*$^{-/-}$ recipients developed colitis, showing comparable weight gain, similar IEL and LPL numbers, and normal colon crypt architecture (Fig 4.1c, d). Thus, although IFNs are capable of acting on many different cell types, their direct effects on the transferred T cells specifically were required for the impairment of Treg cell function in *Trex1*$^{-/-}$ mice.

IFN signaling in Tconv, but not Treg, cells is required for inflammatory disease in Trex1$^{-/-}$ *mice*

Type I IFNs could be subverting Treg cell function in Trex1-deficient mice through their effects on Treg cells, Tconv cells, or both. Both Tconv and Treg cells express the type I IFN receptor, and IFNs can have both pro- and anti-proliferative effects on both T cell populations

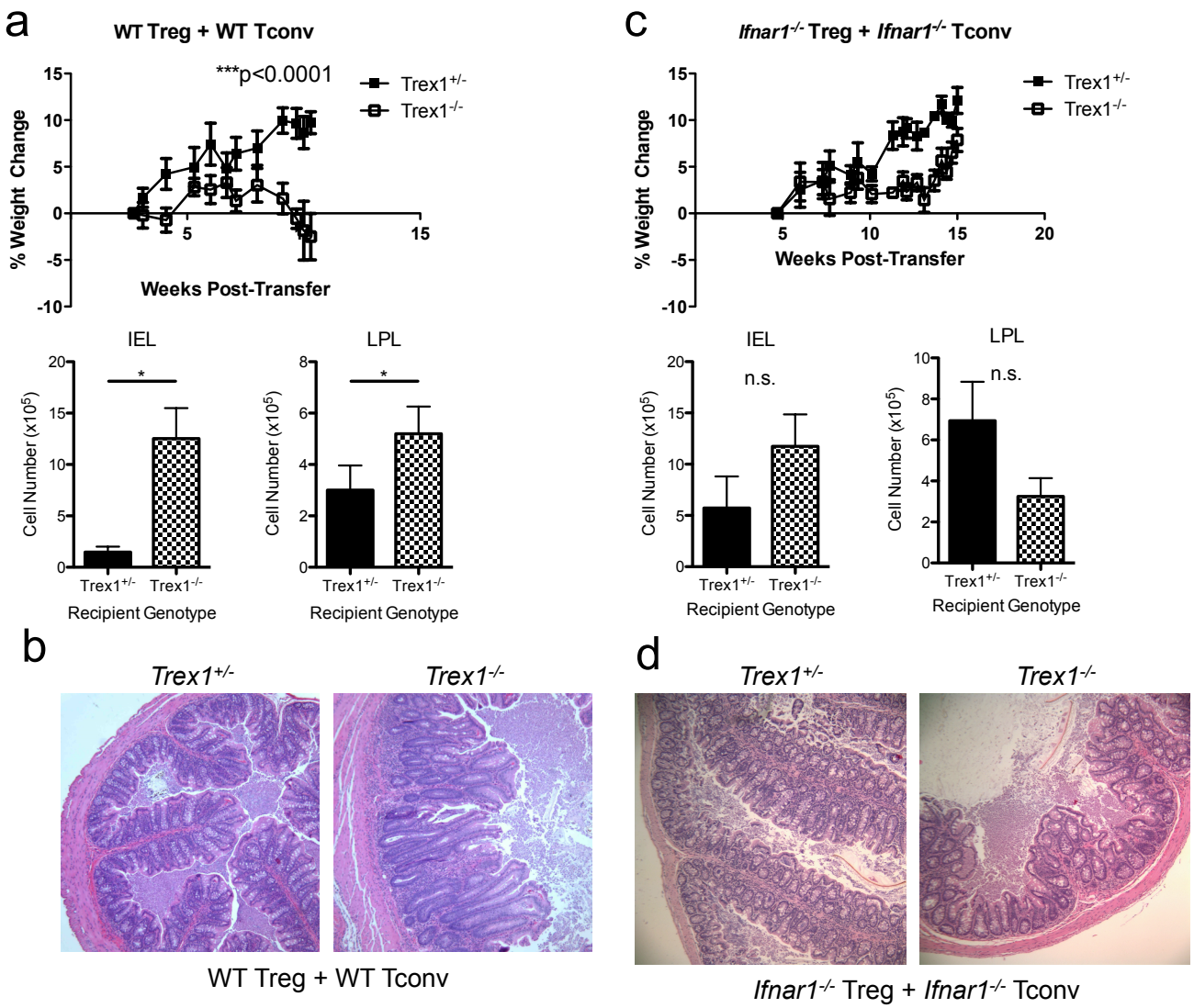

Figure 4.1. Treg suppressive function is impaired in *Trex1*$^{-/-}$ mice due to IFNαR signaling in T cells. a) Top: percent weight change in *Rag2*$^{-/-}$*Trex1*$^{+/-}$("Trex1$^{+/-}$", black squares) and *Rag2*$^{-/-}$*Trex1*$^{-/-}$("Trex1$^{-/-}$", open squares) mice at various time points after co-transfer of WT CD4^{+}CD25^{+} Treg and WT CD4^{+}Foxp3^{gfp-}CD45RB^{hi} Tconv cells. Bottom: absolute number of intraepithelial (IEL) and lamina propria lymphocytes (LPL) in the colons of *Rag2*$^{-/-}$*Trex1*$^{+/-}$ (black) and *Rag2*$^{-/-}$*Trex1*$^{-/-}$(checkered) recipient mice at time of sacrifice. b) Representative H&E staining of cross-sections of intermediate to distal colon from *Rag2*$^{-/-}$*Trex1*$^{+/-}$ and *Rag2*$^{-/-}$*Trex1*$^{-/-}$ recipients of WT Treg and WT Tconv cells at time of sacrifice. c) Top: percent weight change in *Rag2*$^{-/-}$*Trex1*$^{+/-}$ ("Trex1$^{+/-}$", black squares) and *Rag2*$^{-/-}$*Trex1*$^{-/-}$("Trex1$^{-/-}$", open squares) mice at various time points after co-transfer of *Ifnar1*$^{-/-}$ CD4^{+}CD25^{+} Treg and *Ifnar1*$^{-/-}$ CD4^{+}Foxp3^{gfp-}CD45RB^{hi} Tconv cells. Bottom: absolute number of intraepithelial (IEL) and lamina propria lymphocytes (LPL) in the colons of *Rag2*$^{-/-}$*Trex1*$^{+/-}$ (black) and *Rag2*$^{-/-}$*Trex1*$^{-/-}$(checkered) recipient mice at time of sacrifice. d) Representative H&E staining of cross-sections of intermediate to distal colon from *Rag2*$^{-/-}$*Trex1*$^{+/-}$ and *Rag2*$^{-/-}$*Trex1*$^{-/-}$ recipients of *Ifnar1*$^{-/-}$ Treg and *Ifnar1*$^{-/-}$ Tconv cells at time of sacrifice. Statistical significance was determined using unpaired two-tailed Student's t-test. Data are representative of two independent experiments with 3-4 mice per group. *, p<0.05; **, p<0.005; ***, p<0.0001.

that likely vary based on contextual factors, such as antigen specificity, activation status, and Foxp3 expression.

To determine whether IFNs' effects on Tconv and/or Treg cells was responsible for the loss of Treg cell suppression in Trex1-deficient mice, we co-transferred different combinations of WT and *Ifnar1*$^{-/-}$ Treg and Tconv cells into *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice and monitored colitis development. Consistent with our previous findings, *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice receiving WT Tconv and WT Treg cells developed rapid weight loss as early as 3 weeks post-transfer and suffered the most severe weight loss of all experimental groups by the time of sacrifice (Fig 4.2a, d). Moreover, they exhibited the highest colitis scores of any group, characterized by severe goblet cell depletion, leukocytic infiltrate, and disrupted crypt architecture in the colon (Fig 4.3a, b). By contrast, and consistent with our previous findings, *Rag2*$^{-/-}$*Trex1*$^{-/-}$ receiving *Ifnar1*$^{-/-}$ Tconv and *Ifnar1*$^{-/-}$ Treg cells were completely protected from weight loss and showed significantly fewer lymphocytic infiltrates in the epithelia and LP of the colon compared to recipients of WT Tconv and WT Treg cells (Fig 4.2a). Transfer of *Ifnar1*$^{-/-}$ Tconv and *Ifnar1*$^{-/-}$ Treg cells also significantly protected *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice from colon inflammation, as these mice showed the lowest colitis scores of any group, with fewer leukocytic infiltrates, reappearance of goblet cells, and restored crypt architecture (Fig 4.3a, b).

We next asked whether IFNαR signaling in Treg cells, specifically, was responsible for the loss of Treg suppressive function in Trex1-deficient mice. Surprisingly, all *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice developed colitis regardless of whether they received WT or *Ifnar1*$^{-/-}$ Treg cells in combination with WT Tconv (Fig 4.2b), indicating that loss of Treg function in Trex1-deficient mice does not depend on IFNs' direct effects on Treg cells. Transfer of WT Tconv cells with either WT or *Ifnar1*$^{-/-}$ Treg cells induced weight loss with similar kinetics and magnitude (Fig 4.2b, d) and similarly severe colitis, with no difference in the numbers of IEL or LPL in the colon (Fig 4.2b, 4.3a, b). Thus, WT and *Ifnar1*$^{-/-}$ Treg cells do not differ significantly in their ability to suppress colon inflammation in Trex1-deficient mice.

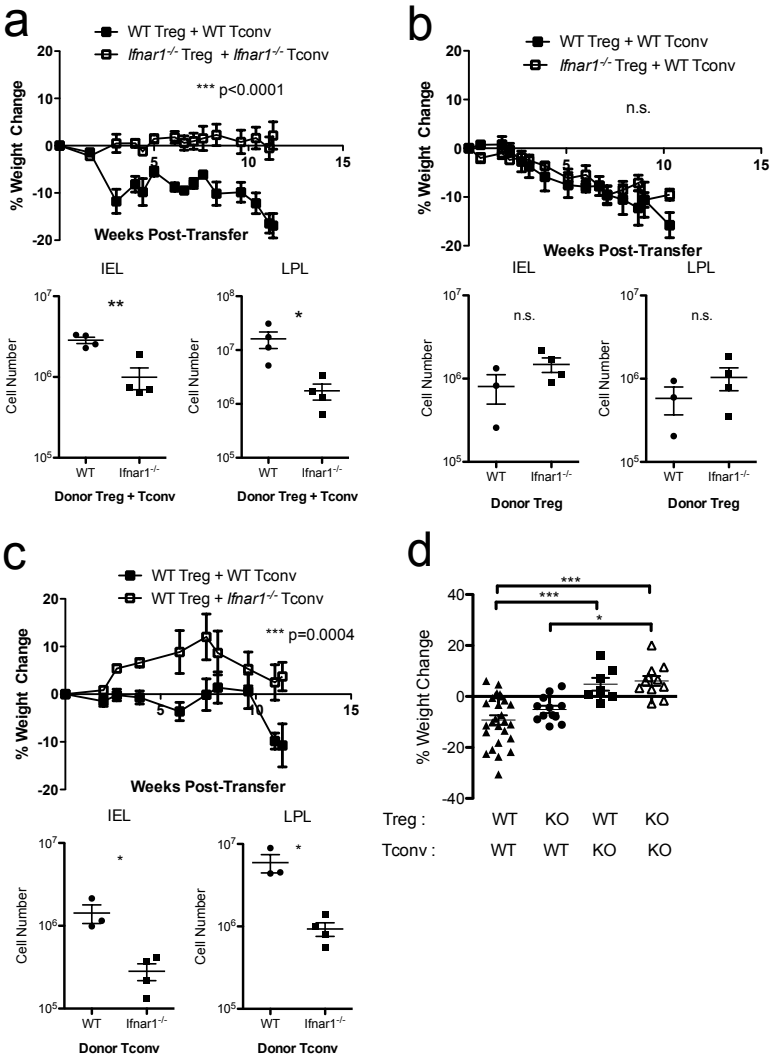

Figure 4.2. Treg dysfunction in *Trex1*$^{-/-}$ mice depends on IFNαR signaling in Tconv, but not Treg, cells. a-c) Top: percent weight change in *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice at various time points after co-transfer of WT Treg + WT Tconv cells (black squares) or co-transfer of: (open squares): (a) *Ifnar1*$^{-/-}$ Treg + *Ifnar1*$^{-/-}$ Tconv cells; (b) *Ifnar1*$^{-/-}$ Treg + WT Tconv cells; or (c) WT Treg + *Ifnar1*$^{-/-}$ Tconv cells. Bottom: absolute number of intraepithelial (IEL) and lamina propria lymphocytes (LPL) in the colons of the indicated *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice. Data are representative of 2-3 independent experiments with 3-4 mice per group. d) Summary of percent weight change at time of sacrifice in *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice receiving the indicated WT or *Ifnar1*$^{-/-}$ ("KO") Treg and Tconv cells. Statistical significance was determined using unpaired two-tailed Student's t-test (a-c) or one-way ANOVA with Tukey post-test (d). *, p<0.05; **, p<0.005; ***, p<0.0001.

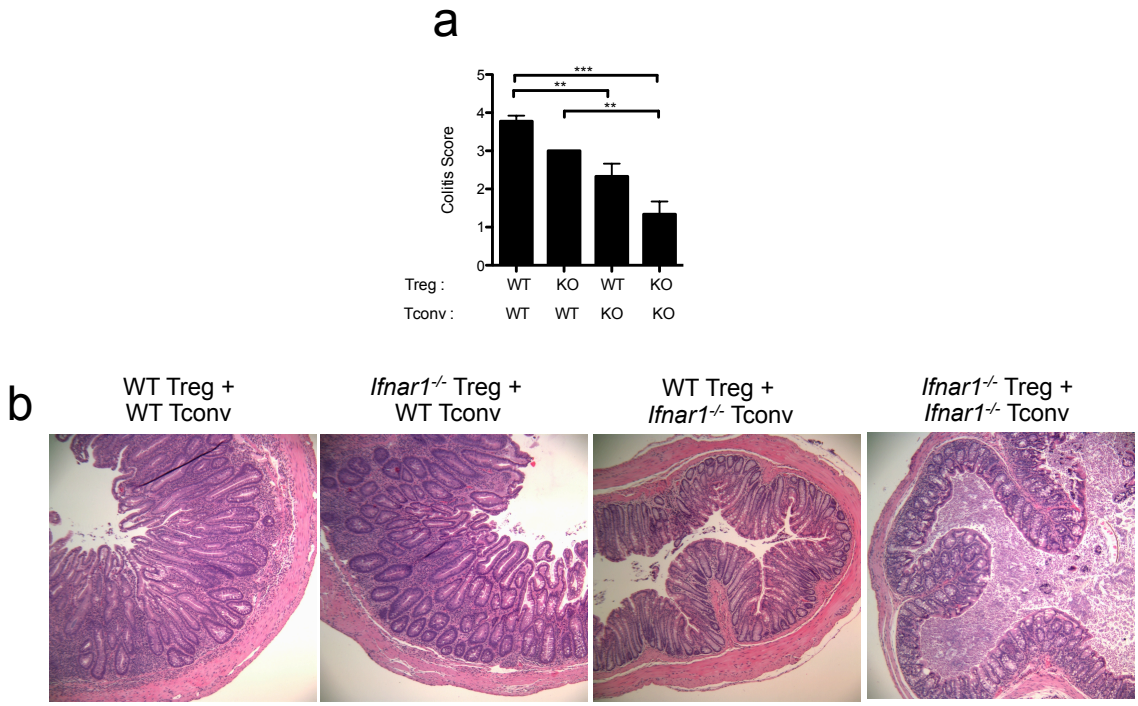

Figure 4.3. Treg dysfunction in *Trex1*$^{-/-}$ mice depends on IFNαR signaling in Tconv, but not Treg, cells. a) Summary of colitis scores based on histological analysis of colon cross-sections from *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice receiving the indicated WT or *Ifnar1*$^{-/-}$ ("KO") Treg and Tconv cells. Data are summarized from 7 independent experiments with 3-4 mice per group. b) Representative H&E staining of cross-sections of intermediate to distal colon from *Rag2*$^{-/-}$*Trex1*$^{-/-}$ recipients of the indicated Treg and Tconv cells. Statistical significance was determined using one-way ANOVA with Tukey post-test. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$.

Although IFNs do not appear to directly inhibit Treg suppressive function *in vivo*, they may indirectly subvert Treg function through their effects on Tconv cells. To test this, we transferred either WT or *Ifnar1*$^{-/-}$ Tconv cells together with WT Treg cells into *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice and monitored disease. Whereas recipients of WT Tconv cells developed colitis as expected, recipients of *Ifnar1*$^{-/-}$ Tconv were significantly protected from wasting disease, showing no significant weight loss (Fig 4.2c, d). Additionally, recipients of *Ifnar1*$^{-/-}$ Tconv were partially protected from colon inflammation, with lower numbers of IEL and LPL in the colon and significantly lower colitis scores (Fig 4.3a, b). Histological analysis revealed lower numbers of leukocytic infiltrates in the LP and less goblet cell depletion in the colons of *Rag2*$^{-/-}$*Trex1*$^{-/-}$ that received *Ifnar1*$^{-/-}$ Tconv, although there was still evidence of moderate epithelial cell hyperplasia (Fig 4.3b).

Altogether, these results indicate that *Rag2*$^{-/-}$*Trex1*$^{-/-}$ recipients of *Ifnar1*$^{-/-}$ Tconv were protected to the greatest degree from weight loss and colitis, regardless of the presence of WT or *Ifnar1*$^{-/-}$ Treg cells (Fig 4.2d, 4.3a). By contrast, recipients of WT Tconv developed similarly severe weight loss and colitis, again regardless of the presence of WT or *Ifnar1*$^{-/-}$ Treg cells (Fig 4.2d, 4.3a). Thus, IFNs appear to indirectly subvert Treg suppressive function in Trex1-deficient mice by directly acting on Tconv cells, as inflammatory disease was completely dependent on IFNαR signaling in Tconv cells but not in Treg cells.

IFNs directly inhibit Treg cell proliferation and activation

Although IFNs' direct effects on Treg cells did not appear to contribute to Treg cell dysfunction and colitis development in *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice, we wondered whether IFNs had any effect on the cellular phenotype of Treg cells in these mice. IFNs are classically known for their anti-proliferative properties, and in the previous chapter we showed that IFNs have direct anti-proliferative effects on Treg cells during acute infection. Interestingly, *Ifnar1*$^{-/-}$ Treg cells transferred into *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice consistently proliferated at higher levels in the IEL and LPL

of the colon compared to transferred WT Treg cells, as measured by the percentage of cells expressing the cell cycle-associated nuclear antigen Ki-67, a common marker of cell proliferation (Fig 4.4a). This was true in the presence of either WT or *Ifnar1*$^{-/-}$ Tconv, indicating that IFNs directly inhibit Treg cell proliferation in the mucosa. Moreover, *Ifnar1*$^{-/-}$ Treg cells also displayed a more activated phenotype than WT Treg cells in the IEL and LPL, with a greater proportion expressing the chemokine receptor CXCR3 and elevated expression of the activation marker CD44 (Fig 4.4b, c). By contrast, *Ifnar1*$^{-/-}$ Treg cells expressed lower levels of CD69 than WT Treg cells in the gut, consistent with the ability of type I IFNs to induce CD69 expression in T cells (*159*, *160*). Thus, IFNs appear to directly inhibit Treg cell proliferation and activation, although this inhibition is not required for the IFN-dependent loss of Treg function in Trex1-deficient mice.

IFN signaling in Tconv directly promotes Tconv accumulation and reduces Treg frequency in the colon

Because IFNs appeared to directly inhibit Treg cell proliferation and activation, we next asked how IFNs affected the numbers of Treg and Tconv cells in the colons of *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice. Interestingly, the ratio of Treg to Tconv cells in the epithelia and LP of the colon correlated closely with the degree of weight loss and colitis severity (Fig 4.2d, 4.3a; 4.5a). The presence of WT or *Ifnar1*$^{-/-}$ Treg cells did not appear to significantly influence the final ratio of Treg to Tconv cells in the gut, reflecting the similar degree of colitis that developed in these mice. Consistent with this, recipients of WT or *Ifnar1*$^{-/-}$ Treg cells showed similar numbers of Treg and Tconv cells among the IEL and LPL of the colon (Fig 4.5b, c). By contrast higher ratios of Treg to Tconv cells were associated with greater protection from inflammatory disease, with the highest ratios observed in recipients of *Ifnar1*$^{-/-}$ Tconv cells, regardless of the presence of WT or *Ifnar1*$^{-/-}$ Treg cells. Higher Treg to Tconv cell ratios in recipients of *Ifnar1*$^{-/-}$ Tconv cells were due to a significant decline in the numbers of Tconv in the IEL and LPL, with little to no change in the

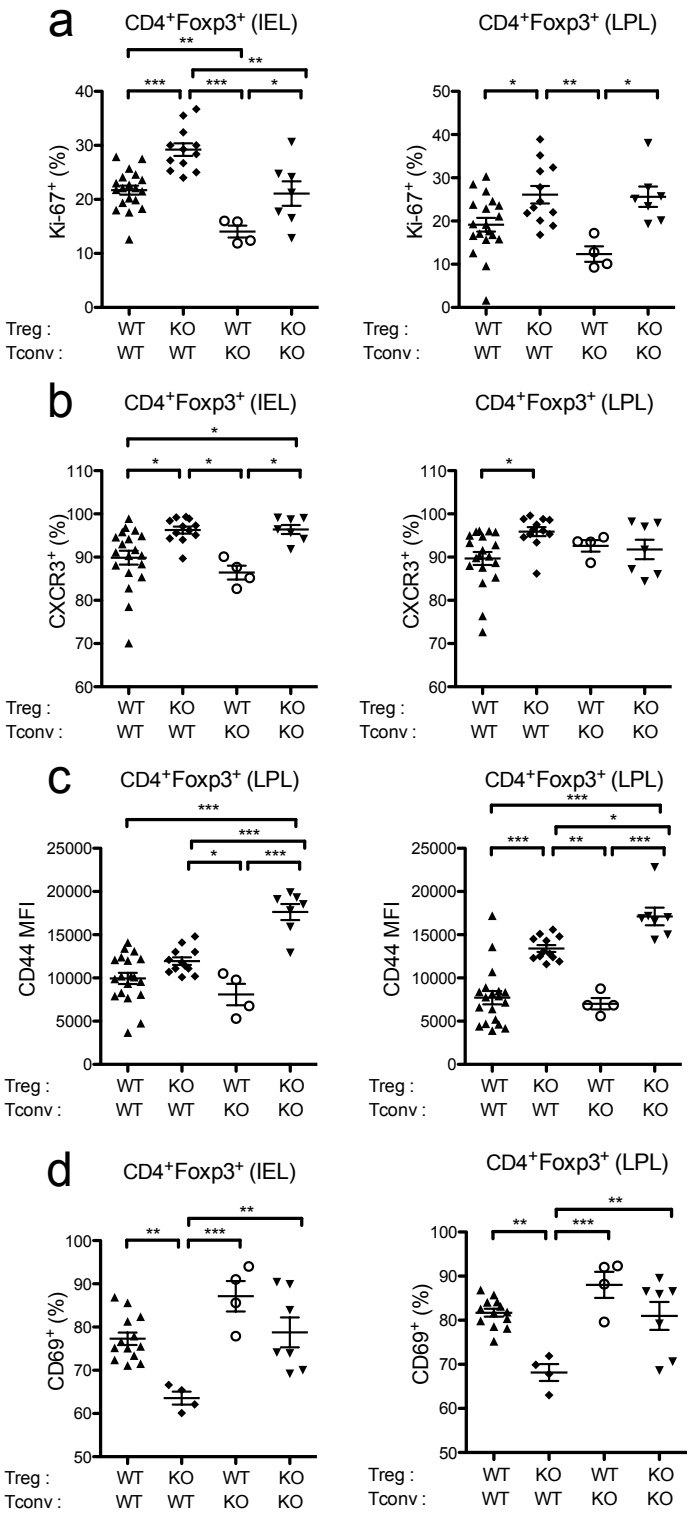

Figure 4.4. IFNs directly inhibit Treg cell proliferation and activation in *Trex1*$^{-/-}$ mice. Summary of Ki-67 (a), CXCR3 (b), CD44 (c), and CD69 (d) expression by CD4$^+$Foxp3$^+$ Treg cells in the IEL (left) and LPL (right) in the colons of *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice receiving the indicated combinations of WT or *Ifnar1*$^{-/-}$ (knockout, "KO") Treg and Tconv cells. Data are summarized from 7 independent experiments 3-4 mice per group. Statistical significance was determined using one-way ANOVA with Tukey post-test. *, p<0.05; **, p<0.005; ***, p<0.0001.

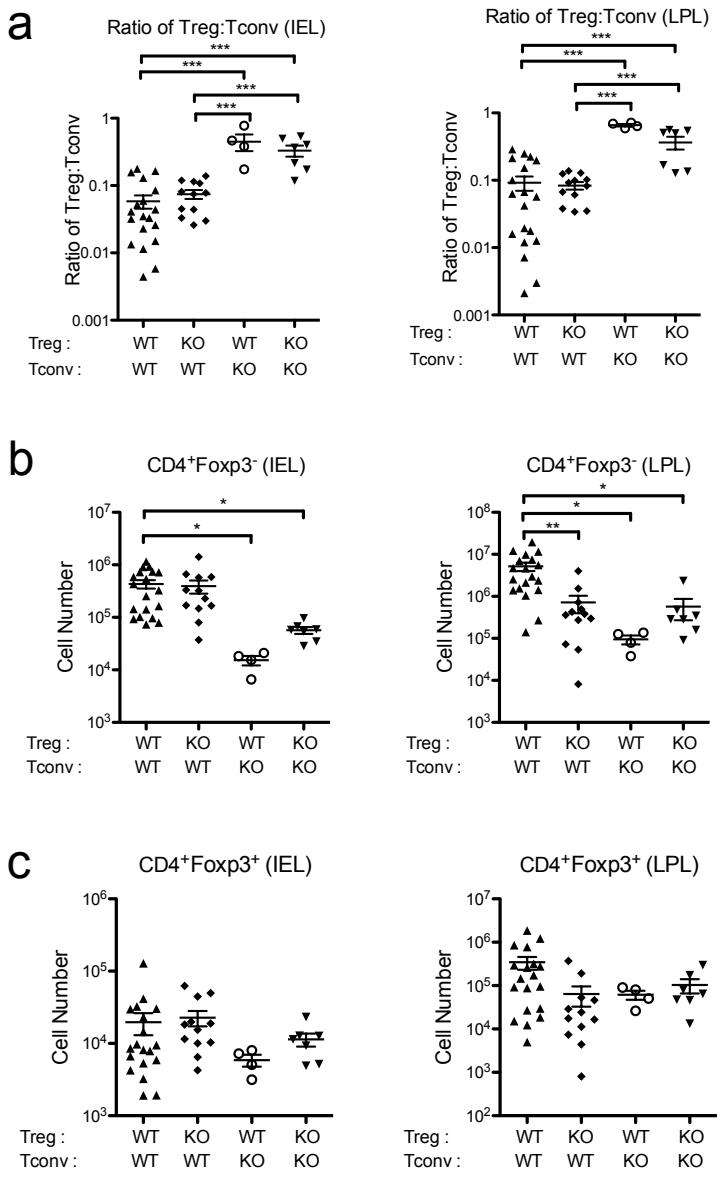

Figure 4.5. IFNs directly promote Tconv accumulation in the colons of *Trex1*$^{-/-}$ mice. a) Summary of the ratio of Treg to Tconv cells in the IEL (left) and LPL (right) in the colons of *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice receiving the indicated combinations of WT or *Ifnar1*$^{-/-}$ (knockout, "KO") Treg and Tconv cells. b, c) Summary of the absolute number of CD4^{+}Foxp3^{-} Tconv cells (b) and CD4^{+}Foxp3^{+} Treg cells (c) in the IEL (left) and LPL (right) in the colons of *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice receiving the indicated combinations of WT or *Ifnar1*$^{-/-}$ (knockout, "KO") Treg and Tconv cells. Data are summarized from 7 independent experiments 3-4 mice per group. Statistical significance was determined using one-way ANOVA with Tukey post-test. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$.

numbers of Treg cells (Fig 4.5b, c). Altogether, these results suggest that Tconv accumulation in the colon depends on IFNs' direct effects on Tconv but not on its effects on Treg cells.

Discussion

The effects of type I IFNs are incredibly complex and vary based on a number of contextual factors. For example, while they exert STAT1-dependent anti-proliferative effects on $CD4^+$ and $CD8^+$ T cells, they can have pro-proliferative effects on antigen-activated T cells that down-regulate STAT1 expression and are required for the expansion of virus-specific T cells during acute LCMV infection (*119*, *120*, *145*, *161*). Additionally, two recent studies demonstrated that IFNs can have opposing pro- and anti-inflammatory effects based on the extent and duration of their expression during acute and chronic infection, respectively (*99*, *151*). Whereas IFNs were pro-inflammatory and promoted viral clearance when expressed acutely, they were immunosuppressive and delayed viral clearance when their expression was prolonged during chronic infection. Thus, although we demonstrated that IFNs transiently inhibit Treg cell proliferation during acute LCMV infection to allow optimal viral clearance, it has remained unclear what effect prolonged IFN expression has on Treg cell function *in vivo*.

Here, we demonstrate that chronic overexpression of IFNs due to loss of Trex1 also inhibits Treg cell function, both directly and indirectly, and drives the development of inflammatory disease *in vivo*. As seen during acute LCMV infection, chronic IFN expression directly inhibited Treg cell proliferation in the gut, as *Ifnar1*$^{-/-}$ Treg cells consistently proliferated more and exhibited a more activated phenotype than WT Treg cells in Trex1-deficient mice. However, unlike during acute LCMV infection, this direct inhibition of Treg cell proliferation did not affect Treg cell numbers in the gut and was not required for the overall loss of Treg cell suppressive function observed in Trex1-deficient mice. Whereas during acute LCMV infection *Ifnar1*$^{-/-}$ Treg cells were able to suppress antiviral T cell responses better than WT Treg cells, in Trex1-deficient mice *Ifnar1*$^{-/-}$ Treg cells were no better at suppressing Tconv cell expansion or

inflammatory colitis than WT Treg cells. This difference may be due to differences in the way IFNs inhibit Treg cells directly in these two models: while IFNs inhibited both proliferation and accumulation of Treg cells during LCMV infection, they only inhibited Treg cell proliferation without affecting their accumulation in the guts of Trex1-deficient mice, suggesting differences in the survival of Treg cells in these two models. IL-2, which is a critical factor for Treg cell survival that inhibits apoptosis via promotion of Bcl2 and Mcl1 activity (*162*, *163*), is increased in the guts of *Trex1*$^{-/-}$ mice and thus may promote Treg survival despite the anti-proliferative effects of IFNs (data not shown). Consistent with this, as IL-2 signaling is known to promote expression of the high-affinity IL-2 receptor chain CD25, Treg cells transferred into *Rag2*$^{-/-}$*Trex1*$^{-/-}$ mice had elevated levels of surface CD25 expression compared to those transferred into *Rag2*$^{-/-}$*Trex1*$^{+/-}$ mice, suggesting they received more IL-2 signaling in Trex1-deficient mice (data not shown). Finally, although the direct inhibition of Treg cells by IFNs was not required, it is possible that this inhibition may still contribute to the loss of Treg cell function in Trex1-deficient mice. Although Trex1-deficient mice receiving *Ifnar1*$^{-/-}$ Tconv cells with WT Treg cells were protected significantly from colitis, there was still evidence of epithelial cell hyperplasia in their colons that was not present in the colons of mice receiving *Ifnar1*$^{-/-}$ Tconv cells with *Ifnar1*$^{-/-}$ Treg cells, suggesting that *Ifnar1*$^{-/-}$ Treg cells may protect from some aspects of inflammatory colitis better than WT Treg cells.

Interestingly, the loss of Treg cell suppressive function in Trex1-deficient mice was primarily due to the indirect effects of IFNs on Tconv cells. *Ifnar1*$^{-/-}$ Tconv cells were significantly impaired in their ability to accumulate in the guts of Trex1-deficient mice. The resistance of effector T cells to Treg-mediated suppression has been described both in patients and in mouse models of many autoimmune disorders, including type I diabetes, multiple sclerosis, and SLE. This often occurs as a result of pro-inflammatory cytokine signaling in Tconv cells. For example, Treg cells are able to migrate to the central nervous system during experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, but are unable to suppress effector T cells

during active disease due to the production of IL-6 and TNFα, both of which have been implicated in driving effector T cell activation and resistance to suppression (*164*). Other studies indicate that Treg cells from healthy controls show defective suppression in the presence of APCs from SLE patients, and this was linked to their production of IFNα (*165*). Thus, despite the presence of normal numbers of Treg cells, a number of factors in the inflammatory milieu may still circumvent Treg cell-mediated suppression and drive autoimmunity. Activation of Tconv cells by IFNs in Trex1-deficient mice, therefore, may render them resistant to suppression by Treg cells. The greater extent of IFN signaling in Trex1-deficient mice compared to during LCMV infection may also make Tconv cells more resistant to suppression, even by *Ifnar1*$^{-/-}$ Treg cells.

Chapter 5:

Concluding Remarks

Here, we have used a number of experimental approaches and genetic tools to precisely determine how type I IFNs directly impact the homeostasis and function of Treg cells in two disparate inflammatory contexts. Despite the conflicting roles for IFN in regulating Treg cell activity in the literature, we demonstrate that IFNs exert a similar inhibitory, anti-proliferative effect on Treg cells, both in the context of acute viral infection and during the development of type I IFN-dependent autoimmunity. This inhibition by IFNs is protective during acute infection: although LCMV infection produces a co-stimulatory environment that favors the proliferation of Treg cells, IFNs act directly on Treg cells to prevent this expansion, thereby allowing $CD8^+$ and $CD4^+$ effector T cells to function optimally and clear the virus. However, when IFN expression is dysregulated and overexpressed, it chronically impairs Treg cell function *in vivo* and can drive the development of inflammatory disease.

As dysregulated Treg activity has been implicated in the progression of cancer, autoimmunity, and infectious disease, understanding how Treg cell homeostasis is regulated in these immune contexts has the potential to provide us with many opportunities for therapeutic intervention. Indeed, Treg cell depletion has proved beneficial for the treatment of cancer in some cases (*166*), and Treg cell-based adoptive therapy is currently under development for the treatment of autoimmunity. Considering that administration of type I IFN is a commonly prescribed treatment for chronic infection, cancer, and even multiple sclerosis (*167*), it is crucial to understand how type I IFNs regulate Treg cell activity, both directly and indirectly, in these different contexts in order to tailor the effectiveness of this treatment option. For example, our results suggest that IFNβ therapy for patients with multiple sclerosis may actually directly inhibit Treg cell activity and that, in light of the results of Prinz et al., targeting IFNβ administration to

$CD11c^+$ DCs may enhance the protective effects of IFNβ while limiting its potentially harmful, pro-inflammatory effects on Treg cells (*168*).

Although important for immunity to viral infections, type I IFNs are strongly linked to the development of certain autoimmune diseases, such as psoriasis and SLE. In addition, there have been several reports of autoimmune and inflammatory diseases developing in patients receiving type I IFN therapeutically. Thus, our work demonstrating that type I IFNs inhibit Treg cell function both when expressed acutely and chronically has important implications for understanding the development of SLE and other type I IFN-associated autoimmune diseases. Further work will be required to determine the mechanisms by which IFNs specifically inhibit Treg cells and whether this varies between different inflammatory contexts.

References

1. K. A. Hogquist, T. A. Baldwin, S. C. Jameson, Central tolerance: learning self-control in the thymus, *Nat.Rev.Immunol.* **5**, 772–782 (2005).

2. A. Schietinger, P. D. Greenberg, Tolerance and exhaustion: defining mechanisms of T cell dysfunction, *Trends in Immunology* **35**, 51–60 (2014).

3. A. Schmidt, N. Oberle, P. H. Krammer, Molecular mechanisms of treg-mediated T cell suppression, *Front Immunol* **3**, 51 (2012).

4. Becoming self-aware: the thymic education of regulatory T cells,, – (2010).

5. O. S. Qureshi *et al.*, Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4, *Science* **332**, 600–603 (2011).

6. How regulatory T cells work, **8**, 523–532 (2008).

7. Mechanisms of suppression by suppressor T cells, **6**, 338–344 (2005).

8. S. Shafiani *et al.*, Pathogen-specific Treg cells expand early during mycobacterium tuberculosis infection but are later eliminated in response to Interleukin-12, *Immunity* **38**, 1261–1270 (2013).

9. J. Zhao *et al.*, IFN-γ- and IL-10-expressing virus epitope-specific Foxp3(+) T reg cells in the central nervous system during encephalomyelitis, *Journal of Experimental Medicine* **208**, 1571–1577 (2011).

10. Y. Belkaid, K. Tarbell, Regulatory T cells in the control of host-microorganism interactions (*), *Annu. Rev. Immunol.* **27**, 551–589 (2009).

11. A. Benson *et al.*, Microbial infection-induced expansion of effector T cells overcomes the suppressive effects of regulatory T cells via an IL-2 deprivation mechanism, *The Journal of Immunology* **188**, 800–810 (2012).

12. A. Ballesteros-Tato, B. Leon, F. E. Lund, T. D. Randall, CD4+ T helper cells use CD154-CD40 interactions to counteract T reg cell-mediated suppression of CD8+ T cell responses to influenza, *The Journal of experimental medicine* **210**, 1591–1601 (2013).

13. Suppression of mature dendritic cell function by regulatory T cells in vivo is abrogated by CD40 licensing, **180**, 1405–1413 (2008).

14. CD40 ligation releases immature dendritic cells from the control of regulatory CD4+CD25+ T cells, **19**, 877–889 (2003).

15. IL-1 beta breaks tolerance through expansion of CD25+ effector T cells, **176**, 7278–7287 (2006).

16. C. Pasare, R. Medzhitov, Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells, *Science* **299**, 1033–1036 (2003).

17. Signaling through the Adaptor Molecule MyD88 in CD4(+) T Cells Is Required to Overcome

Suppression by Regulatory T Cells, **40**, 78–90 (2014).

18. H. Nie *et al.*, Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-α in rheumatoid arthritis, *Nature medicine* **19**, 322–328 (2013).

19. F. Bolacchi *et al.*, Increased hepatitis C virus (HCV)-specific CD4+CD25+ regulatory T lymphocytes and reduced HCV-specific CD4+ T cell response in HCV-infected patients with normal versus abnormal alanine aminotransferase levels, *Clinical and experimental immunology* **144**, 188–196 (2006).

20. R. Cabrera *et al.*, An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection, *Hepatology* **40**, 1062–1071 (2004).

21. Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis, **173**, 803–810 (2006).

22. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis, **207**, 1409–1420 (2010).

23. Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis, **204**, 2159–2169 (2007).

24. H. Hisaeda *et al.*, Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells, *Nature medicine* **10**, 29–30 (2004).

25. J. M. Lund, L. Hsing, T. T. Pham, A. Y. Rudensky, Coordination of early protective immunity to viral infection by regulatory T cells, *Science* **320**, 1220–1224 (2008).

26. L. Pace *et al.*, Regulatory T cells increase the avidity of primary CD8+ T cell responses and promote memory, *Science* **338**, 532–536 (2012).

27. Y. Belkaid, C. A. Piccirillo, S. Mendez, E. M. Shevach, D. L. Sacks, CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity, *Nature* **420**, 502–507 (2002).

28. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection, **38**, 1437–1448 (2003).

29. D. G. Bowen, C. M. Walker, Adaptive immune responses in acute and chronic hepatitis C virus infection, *Nature* **436**, 946–952 (2005).

30. The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells, **172**, 3157–3166 (2004).

31. CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions, **172**, 4123–4132 (2004).

32. T. Brusko, M. Atkinson, Treg in type 1 diabetes, *Cell Biochem.Biophys.* **48**, 165–175 (2007).

33. Circulating CD4+CD25+ T regulatory cells are not altered in multiple sclerosis and unaffected by disease-modulating drugs, **24**, 155–161 (2004).

34. Inverse correlation between CD4+ regulatory T-cell population and autoantibody levels in

paediatric patients with systemic lupus erythematosus, **117**, 280–286 (2006).

35. Global natural regulatory T cell depletion in active systemic lupus erythematosus, **175**, 8392–8400 (2005).

36. Paradoxically increased FOXP3+ T cells in IBD do not preferentially express the isoform of FOXP3 lacking exon 2, **57**, 2846–2855 (2012).

37. Proinflammatory mediator-induced reversal of CD4+,CD25+ regulatory T cell-mediated suppression in rheumatoid arthritis, **56**, 732–742 (2007).

38. Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis, **105**, 19396–19401 (2008).

39. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy, **200**, 277–285 (2004).

40. The effector T cells of diabetic subjects are resistant to regulation via CD4+ FOXP3+ regulatory T cells, **181**, 7350–7355 (2008).

41. FoxP3(+)CD4(+)CD25(+) T cells with regulatory properties can be cultured from colonic mucosa of patients with Crohn's disease, **141**, 549–557 (2005).

42. Increased resistance to CD4+CD25hi regulatory T cell-mediated suppression in patients with type 1 diabetes, **154**, 353–359 (2008).

43. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation, **13**, 423–431 (2007).

44. Dysfunctional CD4+,CD25+ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-alpha-producing antigen-presenting cells, **58**, 801–812 (2008).

45. B. Sadlack *et al.*, Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene, *Cell* **75**, 253–261 (1993).

46. D. M. Willerford *et al.*, Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment, *Immunity* **3**, 521–530 (1995).

47. J. D. Fontenot, J. P. Rasmussen, M. A. Gavin, A. Y. Rudensky, A function for interleukin 2 in Foxp3-expressing regulatory T cells, *Nat Immunol* **6**, 1142–1151 (2005).

48. The biology of interleukin-2, **26**, 453–479 (2008).

49. O. Boyman, M. Kovar, M. P. Rubinstein, C. D. Surh, J. Sprent, Selective stimulation of T cell subsets with antibody-cytokine immune complexes, *Science* **311**, 1924–1927 (2006).

50. R. Setoguchi, S. Hori, T. Takahashi, S. Sakaguchi, Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization, *The Journal of experimental medicine* **201**, 723–735 (2005).

51. M. Long, A. J. Adler, Cutting edge: Paracrine, but not autocrine, IL-2 signaling is sustained

during early antiviral CD4 T cell response, *Journal of immunology* **177**, 4257–4261 (2006).

52. The initial phase of an immune response functions to activate regulatory T cells, **183**, 332–339 (2009).

53. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection, **31**, 772–786 (2009).

54. C.-S. Hsieh *et al.*, Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors, *Immunity* **21**, 267–277 (2004).

55. Antigen-specific peripheral shaping of the natural regulatory T cell population, **205**, 3105–3117 (2008).

56. The role of physiological self-antigen in the acquisition and maintenance of regulatory T-cell function, **212**, 170–184 (2006).

57. G. Darrasse-Jeze *et al.*, Feedback control of regulatory T cell homeostasis by dendritic cells in vivo, *Journal of Experimental Medicine* **206**, 1853–1862 (2009).

58. Q. Tang *et al.*, Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells, *Journal of immunology* **171**, 3348–3352 (2003).

59. S. Tanaka *et al.*, Graded attenuation of TCR signaling elicits distinct autoimmune diseases by altering thymic T cell selection and regulatory T cell function, *The Journal of Immunology* **185**, 2295–2305 (2010).

60. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes, **12**, 431–440 (2000).

61. An obligate cell-intrinsic function for CD28 in Tregs,, – (2013).

62. L. Bar-On, T. Birnberg, K. W. Kim, S. Jung, Dendritic cell-restricted CD80/86 deficiency results in peripheral regulatory T-cell reduction but is not associated with lymphocyte hyperactivation, *European journal of immunology* **41**, 291–298 (2011).

63. M. Long, A. J. Adler, Cutting edge: Paracrine, but not autocrine, IL-2 signaling is sustained during early antiviral CD4 T cell response, *Journal of immunology* **177**, 4257–4261 (2006).

64. G. Trinchieri, Type I interferon: friend or foe? *Journal of Experimental Medicine* **207**, 2053–2063 (2010).

65. B. Barnes, B. Lubyova, P. M. Pitha, On the role of IRF in host defense, *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* **22**, 59–71 (2002).

66. R. Medzhitov, Toll-like receptors and innate immunity, *Nat.Rev.Immunol.* **1**, 135–145 (2001).

67. P. Lamken *et al.*, Functional cartography of the ectodomain of the type I interferon receptor subunit ifnar1, *Journal of molecular biology* **350**, 476–488 (2005).

68. A. N. Theofilopoulos, R. Baccala, B. Beutler, D. H. Kono, Type I interferons (alpha/beta) in

immunity and autoimmunity, *Annu. Rev. Immunol.* **23**, 307–336 (2005).

69. Transcriptional role of p53 in interferon-mediated antiviral immunity, **205**, 1929–1938 (2008).

70. Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence, **424**, 516–523 (2003).

71. C. A. Biron, Interferons alpha and beta as immune regulators--a new look, *Immunity* **14**, 661–664 (2001).

72. T. Luft *et al.*, Type I IFNs enhance the terminal differentiation of dendritic cells, *Journal of immunology* **161**, 1947–1953 (1998).

73. Type I IFN receptor signals directly stimulate local B cells early following influenza virus infection, **176**, 4343–4351 (2006).

74. Type I interferons and T helper development, **21**, 484–489 (2000).

75. E. F. 3. Petricoin *et al.*, Antiproliferative action of interferon-alpha requires components of T-cell-receptor signalling, *Nature* **390**, 629–632 (1997).

76. G. A. Kolumam, Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection, *Journal of Experimental Medicine* **202**, 637–650 (2005).

77. C. Havenar-Daughton, G. A. Kolumam, K. Murali-Krishna, Cutting Edge: The direct action of type I IFN on CD4 T cells is critical for sustaining clonal expansion in response to a viral but not a bacterial infection, *Journal of immunology* **176**, 3315–3319 (2006).

78. S. Gessani, F. Belardelli, A. Pecorelli, P. Puddu, C. Baglioni, Bacterial lipopolysaccharide and gamma interferon induce transcription of beta interferon mRNA and interferon secretion in murine macrophages, *Journal of virology* **63**, 2785–2789 (1989).

79. H. Takayanagi *et al.*, RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-beta, *Nature* **416**, 744–749 (2002).

80. R. Deonarain *et al.*, Critical roles for IFN-beta in lymphoid development, myelopoiesis, and tumor development: links to tumor necrosis factor alpha, *Proceedings of the National Academy of Sciences of the United States of America* **100**, 13453–13458 (2003).

81. N. A. de Weerd *et al.*, Structural basis of a unique interferon-β signaling axis mediated via the receptor IFNAR1, *Nat Immunol* **14**, 901–907 (2013).

82. E. C. Baechler *et al.*, Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus, *Proceedings of the National Academy of Sciences of the United States of America* **100**, 2610–2615 (2003).

83. L. Bennett *et al.*, Interferon and granulopoiesis signatures in systemic lupus erythematosus blood, *The Journal of experimental medicine* **197**, 711–723 (2003).

84. Recent insights into the genetic basis of systemic lupus erythematosus, **10**, 373–379 (2009).

85. C. Gota, L. Calabrese, Induction of clinical autoimmune disease by therapeutic interferon-alpha, *Autoimmunity* **36**, 511–518 (2003).

86. C. Mavrogiannis *et al.*, Ulcerative colitis associated with interferon treatment for chronic hepatitis C, *Journal of hepatology* **34**, 964–965 (2001).

87. P. A. Ascierto *et al.*, Regulatory T cell frequency in patients with melanoma with different disease stage and course, and modulating effects of high-dose interferon-alpha 2b treatment, *Journal of translational medicine* **8**, 76 (2010).

88. K. Tatsugami, M. Eto, S. Naito, Influence of immunotherapy with interferon-alpha on regulatory T cells in renal cell carcinoma patients, *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* **30**, 43–48 (2010).

89. J. R. J. Burton *et al.*, Prospective analysis of effector and regulatory CD4+ T cells in chronic HCV patients undergoing combination antiviral therapy, *Journal of hepatology* **49**, 329–338 (2008).

90. B. Soldevila *et al.*, A prospective study of T- and B-lymphocyte subpopulations, CD81 expression levels on B cells and regulatory CD4(+) CD25(+) CD127(low/-) FoxP3(+) T cells in patients with chronic HCV infection during pegylated interferon-alpha2a plus ribavirin treatment, *Journal of viral hepatitis* **18**, 384–392 (2011).

91. M. Akiyama *et al.*, Relationship between regulatory T cells and the combination of pegylated interferon and ribavirin for the treatment of chronic hepatitis type C, *Intervirology* **53**, 154–160 (2010).

92. S. Barath *et al.*, The severity of systemic lupus erythematosus negatively correlates with the increasing number of CD4+CD25(high)FoxP3+ regulatory T cells during repeated plasmapheresis treatments of patients, *Autoimmunity* **40**, 521–528 (2007).

93. A. Golding, A. Rosen, M. Petri, E. Akhter, F. Andrade, Interferon-alpha regulates the dynamic balance between human activated regulatory and effector T cells: implications for antiviral and autoimmune responses, *Immunology* **131**, 107–117 (2010).

94. A. A. Vandenbark *et al.*, Interferon-beta-1a treatment increases CD56bright natural killer cells and CD4+CD25+ Foxp3 expression in subjects with multiple sclerosis, *Journal of neuroimmunology* **215**, 125–128 (2009).

95. C. de Andres *et al.*, Interferon beta-1a therapy enhances CD4+ regulatory T-cell function: an ex vivo and in vitro longitudinal study in relapsing-remitting multiple sclerosis, *Journal of neuroimmunology* **182**, 204–211 (2007).

96. A. Namdar, B. Nikbin, M. Ghabaee, A. Bayati, M. Izad, Effect of IFN-beta therapy on the frequency and function of CD4(+)CD25(+) regulatory T cells and Foxp3 gene expression in relapsing-remitting multiple sclerosis (RRMS): a preliminary study, *Journal of neuroimmunology* **218**, 120–124 (2010).

97. S. A. Brod, D. K. Burns, Suppression of relapsing experimental autoimmune encephalomyelitis in the SJL/J mouse by oral administration of type I interferons, *Neurology* **44**,

1144–1148 (1994).

98. C. A. Stewart *et al.*, Interferon-dependent IL-10 production by Tregs limits tumor Th17 inflammation, *The Journal of clinical investigation* **123**, 4859–4874 (2013).

99. E. B. Wilson *et al.*, Blockade of chronic type I interferon signaling to control persistent LCMV infection, *Science* **340**, 202–207 (2013).

100. J. R. Teijaro *et al.*, Persistent LCMV infection is controlled by blockade of type I interferon signaling, *Science* **340**, 207–211 (2013).

101. G. A. Punkosdy *et al.*, Regulatory T-cell expansion during chronic viral infection is dependent on endogenous retroviral superantigens, *Proceedings of the National Academy of Sciences of the United States of America* **108**, 3677–3682 (2011).

102. M. Prinz *et al.*, Distinct and nonredundant in vivo functions of IFNAR on myeloid cells limit autoimmunity in the central nervous system, *Immunity* **28**, 675–686 (2008).

103. A. Dann *et al.*, Cytosolic RIG-I-like helicases act as negative regulators of sterile inflammation in the CNS, *Nat. Neurosci.* **15**, 98–106 (2012).

104. M. Chen *et al.*, IFN-β induces the proliferation of CD4+CD25+Foxp3+ regulatory T cells through upregulation of GITRL on dendritic cells in the treatment of multiple sclerosis, *Journal of neuroimmunology* **242**, 39–46 (2012).

105. L. Lefrancois, N. Lycke, Isolation of mouse small intestinal intraepithelial lymphocytes, Peyer's patch, and lamina propria cells, *Curr Protoc Immunol* **Chapter 3**, Unit 3.19–3.19.16 (2001).

106. T. Goodman, L. Lefrancois, Intraepithelial lymphocytes. Anatomical site, not T cell receptor form, dictates phenotype and function, *The Journal of experimental medicine* **170**, 1569–1581 (1989).

107. C. Asseman, S. Mauze, M. W. Leach, R. L. Coffman, F. Powrie, An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation, *The Journal of experimental medicine* **190**, 995–1004 (1999).

108. Y. Belkaid, Regulatory T cells and infection: a dangerous necessity, *Nature Publishing Group* **7**, 875–888 (2007).

109. J. Hänig, M. B. Lutz, Suppression of mature dendritic cell function by regulatory T cells in vivo is abrogated by CD40 licensing, *Journal of immunology* **180**, 1405–1413 (2008).

110. B. J. O'Sullivan *et al.*, IL-1 beta breaks tolerance through expansion of CD25+ effector T cells, *Journal of immunology* **176**, 7278–7287 (2006).

111. C. Pasare, R. Medzhitov, Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells, *Science* **299**, 1033–1036 (2003).

112. N. Mozzillo, P. Ascierto, Reduction of circulating regulatory T cells by intravenous high-dose interferon alfa-2b treatment in melanoma patients, *Clin. Exp. Metastasis* **29**, 801–805

(2012).

113. A. Namdar, B. Nikbin, M. Ghabaee, A. Bayati, M. Izad, Effect of IFN-beta therapy on the frequency and function of CD4(+)CD25(+) regulatory T cells and Foxp3 gene expression in relapsing-remitting multiple sclerosis (RRMS): a preliminary study, *Journal of neuroimmunology* **218**, 120–124 (2010).

114. L. Pace *et al.*, APC activation by IFN-alpha decreases regulatory T cell and enhances Th cell functions, *The Journal of Immunology* **184**, 5969–5979 (2010).

115. C. H. Riley *et al.*, Increase in circulating $CD4^+CD25^+Foxp3^+$ T cells in patients with Philadelphia-negative chronic myeloproliferative neoplasms during treatment with IFN-α, *Blood* **118**, 2170–2173 (2011).

116. K. Murali-Krishna *et al.*, Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection, *Immunity* **8**, 177–187 (1998).

117. D. Homann, L. Teyton, M. B. Oldstone, Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory, *Nature medicine* **7**, 913–919 (2001).

118. U. Müller *et al.*, Functional role of type I and type II interferons in antiviral defense, *Science* **264**, 1918–1921 (1994).

119. C. Havenar-Daughton, G. A. Kolumam, K. Murali-Krishna, Cutting Edge: The direct action of type I IFN on CD4 T cells is critical for sustaining clonal expansion in response to a viral but not a bacterial infection, *Journal of immunology* **176**, 3315–3319 (2006).

120. G. A. Kolumam, S. Thomas, L. J. Thompson, J. Sprent, K. Murali-Krishna, Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection, *The Journal of experimental medicine* **202**, 637–650 (2005).

121. S. E. Lee *et al.*, Type I interferons maintain Foxp3 expression and T-regulatory cell functions under inflammatory conditions in mice, *Gastroenterology* **143**, 145–154 (2012).

122. J. Huehn *et al.*, Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells, *The Journal of experimental medicine* **199**, 303–313 (2004).

123. B. Min *et al.*, Gut flora antigens are not important in the maintenance of regulatory T cell heterogeneity and homeostasis, *European journal of immunology* **37**, 1916–1923 (2007).

124. E. Cretney, A. Kallies, S. L. Nutt, Differentiation and function of Foxp3(+) effector regulatory T cells, *Trends in Immunology* **34**, 74–80 (2013).

125. R. A. Clark, T. S. Kupper, IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin, *Blood* **109**, 194–202 (2007).

126. J. Xia, W. Liu, B. Hu, Z. Tian, Y. Yang, IL-15 promotes regulatory T cell function and protects against diabetes development in NK-depleted NOD mice, *Clin. Immunol.* **134**, 130–139 (2010).

127. M. A. Williams, A. J. Tyznik, M. J. Bevan, Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells, *Nature* **441**, 890–893 (2006).

128. X. Zhang, S. Sun, I. Hwang, D. F. Tough, J. Sprent, Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15, *Immunity* **8**, 591–599 (1998).

129. T. R. Simpson, S. A. Quezada, J. P. Allison, Regulation of CD4 T cell activation and effector function by inducible costimulator (ICOS), *Curr. Opin. Immunol.* **22**, 326–332 (2010).

130. Y. S. Choi *et al.*, ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6, *Immunity* **34**, 932–946 (2011).

131. H. Iwai *et al.*, Involvement of inducible costimulator-B7 homologous protein costimulatory pathway in murine lupus nephritis, *Journal of immunology* **171**, 2848–2854 (2003).

132. J. H. Rowe, J. M. Ertelt, M. N. Aguilera, M. A. Farrar, S. S. Way, Foxp3(+) regulatory T cell expansion required for sustaining pregnancy compromises host defense against prenatal bacterial pathogens, *Cell host & microbe* **10**, 54–64 (2011).

133. R. B. Fulton, D. K. Meyerholz, S. M. Varga, Foxp3+ CD4 regulatory T cells limit pulmonary immunopathology by modulating the CD8 T cell response during respiratory syncytial virus infection, *The Journal of Immunology* **185**, 2382–2392 (2010).

134. J. M. Lund, L. Hsing, T. T. Pham, A. Y. Rudensky, Coordination of early protective immunity to viral infection by regulatory T cells, *Science* **320**, 1220–1224 (2008).

135. K. Siegmund *et al.*, Migration matters: regulatory T-cell compartmentalization determines suppressive activity in vivo, *Blood* **106**, 3097–3104 (2005).

136. R. J. DiPaolo, D. D. Glass, K. E. Bijwaard, E. M. Shevach, CD4+CD25+ T cells prevent the development of organ-specific autoimmune disease by inhibiting the differentiation of autoreactive effector T cells, *Journal of immunology* **175**, 7135–7142 (2005).

137. T. R. Mempel *et al.*, Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation, *Immunity* **25**, 129–141 (2006).

138. N. Sarween *et al.*, CD4+CD25+ cells controlling a pathogenic CD4 response inhibit cytokine differentiation, CXCR-3 expression, and tissue invasion, *Journal of immunology* **173**, 2942–2951 (2004).

139. D. K. Sojka, D. J. Fowell, Regulatory T cells inhibit acute IFN-γ synthesis without blocking T-helper cell type 1 (Th1) differentiation via a compartmentalized requirement for IL-10, *Proceedings of the National Academy of Sciences of the United States of America* **108**, 18336–18341 (2011).

140. M. Firan, S. Dhillon, P. Estess, M. H. Siegelman, Suppressor activity and potency among regulatory T cells is discriminated by functionally active CD44, *Blood* **107**, 619–627 (2006).

141. E. F. 3. Petricoin *et al.*, Antiproliferative action of interferon-alpha requires components of T-cell-receptor signalling, *Nature* **390**, 629–632 (1997).

142. J. F. Bromberg, C. M. Horvath, Z. Wen, R. D. Schreiber, J. E. J. Darnell, Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma, *Proceedings of the National Academy of Sciences of the United States of America* **93**, 7673–7678 (1996).

143. Y. Tanabe *et al.*, Cutting edge: role of STAT1, STAT3, and STAT5 in IFN-alpha beta responses in T lymphocytes, *Journal of immunology* **174**, 609–613 (2005).

144. M. P. Gil, R. Salomon, J. Louten, C. A. Biron, Modulation of STAT1 protein levels: a mechanism shaping CD8 T-cell responses in vivo, *Blood* **107**, 987–993 (2006).

145. M. P. Gil *et al.*, Regulating type 1 IFN effects in CD8 T cells during viral infections: changing STAT4 and STAT1 expression for function, *Blood* **120**, 3718–3728 (2012).

146. C. de Andres *et al.*, Interferon beta-1a therapy enhances CD4+ regulatory T-cell function: an ex vivo and in vitro longitudinal study in relapsing-remitting multiple sclerosis, *Journal of neuroimmunology* **182**, 204–211 (2007).

147. R. Baccala, D. H. Kono, A. N. Theofilopoulos, Interferons as pathogenic effectors in autoimmunity, *Immunological reviews* **204**, 9–26 (2005).

148. A. Doganci *et al.*, The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo, *The Journal of clinical investigation* **115**, 313–325 (2005).

149. E. J. Wehrens *et al.*, Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells, *Blood* **118**, 3538–3548 (2011).

150. X. Valencia *et al.*, TNF downmodulates the function of human CD4+CD25hi T-regulatory cells, *Blood* **108**, 253–261 (2006).

151. Persistent LCMV infection is controlled by blockade of type I interferon signaling, **340**, 207–211 (2013).

152. Y. G. Yang, T. Lindahl, D. E. Barnes, Trex1 exonuclease degrades ssDNA to prevent chronic checkpoint activation and autoimmune disease, *Cell* **131**, 873–886 (2007).

153. D. B. Stetson, J. S. Ko, T. Heidmann, R. Medzhitov, Trex1 prevents cell-intrinsic initiation of autoimmunity, *Cell* **134**, 587–598 (2008).

154. M. Morita *et al.*, Gene-targeted mice lacking the Trex1 (DNase III) 3“-->5” DNA exonuclease develop inflammatory myocarditis, *Molecular and cellular biology* **24**, 6719–6727 (2004).

155. G. Rice *et al.*, Heterozygous mutations in TREX1 cause familial chilblain lupus and dominant Aicardi-Goutieres syndrome, *American journal of human genetics* **80**, 811–815 (2007).

156. A. Gall *et al.*, Autoimmunity initiates in nonhematopoietic cells and progresses via lymphocytes in an interferon-dependent autoimmune disease, *Immunity* **36**, 120–131 (2012).

157. S. Read *et al.*, Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo, *Journal of immunology* **177**, 4376–4383 (2006).

158. C. Mottet, H. H. Uhlig, F. Powrie, Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells, *Journal of immunology* **170**, 3939–3943 (2003).

159. S. Sun, X. Zhang, D. F. Tough, J. Sprent, Type I interferon-mediated stimulation of T cells by CpG DNA, *The Journal of experimental medicine* **188**, 2335–2342 (1998).

160. L. R. Shiow *et al.*, CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs, *Nature* **440**, 540–544 (2006).

161. M. P. Gil, R. Salomon, J. Louten, C. A. Biron, Modulation of STAT1 protein levels: a mechanism shaping CD8 T-cell responses in vivo, *Blood* **107**, 987–993 (2006).

162. W. Pierson *et al.*, Antiapoptotic Mcl-1 is critical for the survival and niche-filling capacity of Foxp3^{+} regulatory T cells, *Nat Immunol* **14**, 959–965 (2013).

163. D. Tischner *et al.*, Defective cell death signalling along the Bcl-2 regulated apoptosis pathway compromises Treg cell development and limits their functionality in mice, *J. Autoimmun.* **38**, 59–69 (2012).

164. T. Korn *et al.*, Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation, *Nature medicine* **13**, 423–431 (2007).

165. B. Yan *et al.*, Dysfunctional CD4+,CD25+ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-alpha-producing antigen-presenting cells, *Arthritis and rheumatism* **58**, 801–812 (2008).

166. J. Shimizu, S. Yamazaki, S. Sakaguchi, Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity, *Journal of immunology* **163**, 5211–5218 (1999).

167. D. Goldstein, J. Laszlo, Interferon therapy in cancer: from imaginon to interferon, *Cancer research* **46**, 4315–4329 (1986).

168. M. Prinz *et al.*, Distinct and nonredundant in vivo functions of IFNAR on myeloid cells limit autoimmunity in the central nervous system, *Immunity* **28**, 675–686 (2008).