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Impact of IL-27 on regulatory T cell responses

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Abstract
Interleukin (IL)–27 is a heterodimeric cytokine with potent inhibitory properties. Thus, mice that lack IL–27–mediated signaling develop exaggerated inflammatory responses during toxoplasmosis as well as other infections or autoimmune processes. While regulatory T (Treg) cells are critical to limit inflammation, their role during toxoplasmosis is controversial because this infection results in a dramatic decrease in the total numbers of these cells associated with reduced levels of IL–2. Because IL–27 suppresses IL–2, we initially hypothesized that it was responsible for the Treg cell “crash”. Thus, we examined the role of IL–27 and IL–2 and their effects on Treg cells during toxoplasmosis. We observed that although IL–2 production is enhanced in the absence of IL–27, this was not sufficient to rescue Treg cell frequencies during infection. Rather, our data indicated that IL–27 promoted an immunosuppressive Treg cell population that displayed a T helper 1 (TH1) phenotype, characterized by the expression of T–bet, CXCR3, IL–10 and interferon (IFN)–γ. Although IFN–γ and IL–27 can give rise to a population of T–bet+ Treg cells in vitro, there were distinct differences in the signaling and transcriptional responses of Treg cells to these cytokines. Analysis of the genes induced by IL–27 revealed that it promoted Treg cell expression of a number of inhibitory receptors such as LAG–3 and PD–1 which function to limit the magnitude of T cell responses. Taken together, these studies highlight the role of IL–27 as a central coordinator of Treg cell effector functions during inflammation.

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IMPACT OF IL-27 ON REGULATORY T CELL RESPONSES

Aisling O'Hara

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IMPACT OF IL-27 ON REGULATORY T CELL RESPONSES

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DEDICATION

For my son.

To my mother, Mary, who gave me the freedom to develop a passion for discovery.

To my father, Henry, who listened to his fourth grade daughter when she told him that her Fisher Price microscope was “crap”, and bought her the real thing.

To my husband Turney, who supports me, and believes in my abilities.
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PUBLICATIONS


ATTRIBUTIONS

This thesis contains work from publications #2 and #3, listed above.

Dr. Daniel Beiting, while in the laboratory of Dr. David Roos, assisted with the microarray experiments described in Chapters 3 and 4.

Dr. Beena John, while in the Hunter Laboratory, assisted with the experiments with *Salmonella* described in Chapter 3.

Dr. Claudia Lombana, while in the laboratory of Dr. Philip Scott, assisted with the *Leishmania major* experiments described in Chapter 3.

Dr. Guillaume Oldenhove, while in the laboratory of Dr. Yasmine Belkaid, performed the DEREG experiments described in Chapter 3.

Dr. Cristina Tato, and Barbara Joyce-Shaikh performed the IL-27 minicircle experiments while in the laboratory of Dr. Dan Cua as described in Chapters 3 and 4.

Arielle Glatman Zaretzky assisted with the IL-27 pegylated experiments in Chapter 4.

Gretchen Harms Pritchard assisted with the in vitro Treg cell experiments in Chapter 3, and the experiments in Chapter 4 with *T. gondii* infection.

Deb Lefferts assisted in generating Figures 1.3 and 5.1.
ABSTRACT

IMPACT OF IL-27 ON REGULATORY T CELL RESPONSES

Aisling O’Hara
Christopher A. Hunter

Interleukin (IL)-27 is a heterodimeric cytokine with potent inhibitory properties. Thus, mice that lack IL-27-mediated signaling develop exaggerated inflammatory responses during toxoplasmosis as well as other infections or autoimmune processes. While regulatory T (Treg) cells are critical to limit inflammation, their role during toxoplasmosis is controversial because this infection results in a dramatic decrease in the total numbers of these cells associated with reduced levels of IL-2. Because IL-27 suppresses IL-2, we initially hypothesized that it was responsible for the Treg cell “crash”. Thus, we examined the role of IL-27 and IL-2 and their effects on Treg cells during toxoplasmosis. We observed that although IL-2 production is enhanced in the absence of IL-27, this was not sufficient to rescue Treg cell frequencies during infection. Rather, our data indicated that IL-27 promoted an immunosuppressive Treg cell population that displayed a T helper 1 (TH1) phenotype, characterized by the expression of T-bet, CXCR3, IL-10 and interferon (IFN)-γ. Although IFN-γ and IL-27 can give rise to a population of T-bet+ Treg cells in vitro, there were distinct differences in the signaling and transcriptional responses of Treg cells to these cytokines. Analysis of the genes induced by IL-27 revealed that it promoted Treg cell expression of a number of inhibitory receptors such as LAG-3 and PD-1 which function to limit the magnitude of T
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CHAPTER 1: Introduction

1.1 The immunobiology of IL-27

Like many cytokines, IL-27 has pleiotropic properties that can limit or enhance ongoing immune responses depending on context. Thus, under certain circumstances, IL-27 can promote TH1 differentiation and has been linked to the activation of CD8+ T cells and enhanced humoral responses. However, IL-27 also has potent inhibitory properties and mice that lack IL-27-mediated signaling develop exaggerated inflammatory responses in the context of infection or autoimmunity. This section will review in depth the biology of IL-27, including the initial discovery, characterization, and signaling mediated by IL-27 as well as more recent insights into the molecular and cellular basis for its pleiotropic effects. Many of these advances are relevant to human diseases and highlight the potential of therapies that harness the regulatory properties of IL-27.

IL-27: subunits, receptors, sources and signaling

IL-27 is a heterodimeric cytokine composed of 2 subunits, Epstein-Barr virus-induced gene 3 (EBI3) and IL-27p28, which engages a receptor composed of gp130 and the IL-27Rα that activates Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and mitogen activated protein kinase (MAPK) signaling. There are a number of distinct structural motifs that characterize the receptor and cytokine subunits of IL-27 that highlight its evolutionary relationship to other factors (IL-6, IL-12 and IL-23) that are central regulators of cell mediated and humoral responses. Indeed,
early studies suggested that IL-27 was pro-inflammatory, but it is now recognized that this factor can limit the intensity and duration of a wide array of adaptive responses (Table 1.1). The sections below describe the parallel studies that led to the definition of the IL-27 signaling cassette and our current understanding of how it functions in the immune system. The progress in these areas has now started to translate into the clinical situation and IL-27 has been implicated in the pathogenesis of a number of inflammatory conditions and its potential role as a therapeutic target will be discussed.

**Discovery and characterization of IL-27 subunits and receptors**

A comprehensive understanding of how the various components of the IL-27 system are integrated was a consequence of multiple discovery-based approaches that identified individual receptor or cytokine subunits without an appreciation of how these distinct components were related. The section below attempts to place these events in a wider context and highlights the reports that were critical to our current understanding of how this system functions as well as newer questions about the combinatorial biology that appears inherent to this system.

**EBI3 and p28 subunits of IL-27**

EBI3 was first identified in 1996 from a subtractive hybridization screen of genes expressed in Epstein-Barr Virus (EBV) transformed B cell lines (Devergne et al., 1996). These initial studies revealed that EBI3 shares structural homology with other known members of the class I cytokine receptor family, namely IL-12p40, and the ciliary
neurotrophic factor receptor (CNTFR) (Figure 1.1). Like these proteins, EBI3 contains no membrane anchoring motifs, suggesting that it is secreted, and two cytokine binding domains containing WSXWS motifs characteristic of other cytokines in the hematopoietin receptor family (Devergne et al., 1996). Furthermore, when EBI3 was ectopically expressed in COS7 cells it accumulated in the endoplasmic reticulum and was not secreted as a monomer or homodimer (Devergne et al., 1997). This finding implied that EBI3 would pair with another subunit for secretion, and since EBI3 is related to IL-12p40, the p35 subunit of IL-12 represented a likely candidate. Indeed, when coexpressed with p35, EBI3 could form a novel secreted IL12p35/EBI3 heterodimer, (now named IL-35) (Collison et al., 2007, Devergne et al., 1997). The finding that the deletion of EBI3 was associated with altered susceptibility to oxazolone-induced colitis suggested a role in immunity, but its function as a subunit of IL-27 was not apparent for several more years.

The eventual recognition that IL-27p28 is a partner for EBI3 was a consequence of a computational approach, using the genomic databases that emerged in the late 1990s, to identify novel α-helical cytokines of the IL-6 family (Pflanz et al., 2002). One of the central structural features of cytokines such as IL-6 and IL-12p35, that bind to type I cytokine receptors, is that they have long-chain four-helix bundle motifs and this feature formed the basis to identify IL-27p28 (Bazan., 1990a). Since IL-27p28 on its own was poorly secreted and had no obvious biological properties, these findings indicated that, like IL-12p35, it may need to partner with a soluble receptor-like molecule in order to be secreted (Pflanz et al., 2002). A number of candidate molecules (soluble IL-11 receptor, CLF-1, and IL-12p40) were tested for their ability to facilitate the secretion of p28, but
only EBI3 permitted efficient secretion (Pflanz et al., 2002). This heterodimeric protein was designated IL-27 and fusion proteins linking p28 to EBI3 allowed Kastelein and colleagues to define its biological properties and showed that IL-27 could synergize with IL-12 to promote proliferation of naïve CD4+ T cells and the production of IFN-γ from natural killer (NK) cells and CD4+ T cells (Figure 1.2) (Pflanz et al., 2002). In the same study, these authors demonstrated that the orphan cytokine receptor WSX1 could bind to IL-27 and was required for the effect of IL-27 and their subsequent work revealed that gp130 constituted the second subunit of the IL-27 receptor complex (Pflanz et al., 2004).

**Regulating the expression of IL-27**

With the description of the pairing of EBI3 and IL-27p28, interest centered on what cell types were likely sources of this heterodimer. Initial studies that identified EBI3 as being induced in response to EBV was an indication that microbial stimuli were likely to be involved. Early surveys of expression patterns indicated that it was restricted to myeloid cells and expressed highly in LPS activated dendritic cells (DCs), monocytes and macrophages (Hashimoto et al., 2000, Liu et al., 2007, Smits et al., 2004, Veckman et al., 2004, Wirtz et al., 2005). The observation that other cell types including plasma cells, endothelial cells, microglia, placental trophoblasts and uterine NK cells can express IL-27p28 and EBI3 (Devergne et al., 1996, Larousserie et al., 2004, Sonobe et al., 2005, Zhang et al., 2003) implies that IL-27 may have a broad role in immune regulation in specialized environments such as the brain and uterus.

Consistent with the idea that microbial stimuli can induce IL-27, a number of different toll-like receptor (TLR) agonists (LPS, Poly (I:C), CPG, Gram-negative, and
Gram-positive bacteria) are able to induce EBI3 and IL-27p28 mRNA expression in human and mouse antigen presenting cells (Hashimoto et al., 2000, Liu et al., 2007, Smits et al., 2004, Veckman et al., 2004, Wirtz et al., 2005). Although the role of Gram-positive bacteria in promoting EBI3 expression is debated (Schuetze et al., 2005), it is clear that LPS is a potent inducer of EBI3 and IL-27p28 and its effects are further enhanced by the presence of IFN-γ (Sonobe et al., 2005). The pathways triggered by IFN-γ and LPS are distinct but facilitate the sustained expression of IL-27, which is greatly abrogated in MyD88 and NF-κB p50 deficient mice (Liu et al., 2007, Wirtz et al., 2005). In response to TLR4 signals, NF-κB binds to the IL-27p28 promoter, and although c-Rel (an NF-κB family member) was shown to associate with this NF-κB binding site, c-Rel is only partially required for the LPS induced effects (Liu et al., 2007). Following NF-κB activation, the synergistic effects of IFN-γ in this system are attributed to interferon response fragment-1 (IRF-1) and IRF-8 (also known as interferon consensus sequence-binding protein) binding IRF response elements within the IL-27p28 promoter (Zhang et al., 2010). These studies have led to a model in which early NF-κB signals initiate the transcription of IL-27p28, but sustained production requires other transcription factors such as IRF-1 and IRF-8. It should be noted that stimuli that utilize the adapter molecule MyD88 could also promote IL-27p28 expression through the activation of the mitogen activated protein kinase (MAPK) induced transcription factor AP-1. This is illustrated by reports in which incubation of human and mouse macrophages with Mycobacterium tuberculosis resulted in MyD88 dependent recruitment of AP-1, which trans-activated the IL-27p28 promoter (Zhang et al., 2011).
MyD88-independent pathways can also trigger the production of type I interferons which promote IL-27 expression. Thus, in response to TLR4 and TLR3, signals through the adaptor Toll/IL-1R-related domain-containing adaptor-inducing IFN (TRIF) (Molle et al., 2007), activates IRF3 and IRF7 (Fitzgerald et al., 2003) and the expression of IL-27p28 and EBI3 is increased. Cells that lack TRIF cannot produce robust amounts of either IL-27p28 or EBI3 mRNA in response to TLR4 ligands (Molle et al., 2007). While IRF3 is recruited to the promoter region and is required for expression of IL-27p28, it is not required for the expression of EBI3 (Molle et al., 2007, Molle et al., 2010). The promoter region of EBI3 does contain a consensus sequence for IRF7 binding (Wirtz et al., 2005), but its role in promoting EBI3 expression is unknown. Similar to the synergistic effects of IFN-γ on MyD88 dependent signals, IFN-α/β can amplify IL-27p28 expression by activating IRF1 and further enhance IL-27p28 expression through the activation of the STAT1/STAT2/IRF9 (ISGF3) complex (Molle et al., 2010, Pirhonen et al., 2007). It has been known for some time that IFN-β can promote the expression of IL-27 (Remoli et al., 2007, van Seventer et al., 2002), and that while IFN-β can promote IL-27p28 and EBI3, it can also block the expression of IL-12p40 and IL-23p19 from DCs, depending on the context of the IFN-β signals (Nagai et al., 2007). This observation is of clinical interest as type I interferon therapy has been used to ameliorate the inflammatory response during experimental autoimmune encephalitis (EAE) and multiple sclerosis (MS) (Brod et al., 1995, Knobler et al., 1984). While one mechanism by which this is thought to occur is due to suppression of IL-12, another potential result of such treatment could be increased IL-27 which could contribute to the anti-inflammatory effects of this treatment regimen (Guo et al., 2008, Karp, 2000, Sweeney et al., 2011).
Despite advances in our understanding of the types of microbial and host signals that control the production of IL-27, there is still a limited appreciation of the cell biology that allows the p28 and EBI3 subunits to dimerize and be secreted. One of the difficulties in studying this aspect of the biology of IL-27 stems from the paucity of reagents to detect the heterodimer, and new approaches are needed to define the relative contribution of cell specific expression of IL-27 to inflammatory responses and immune homeostasis. This would be facilitated by the development of more sensitive antibody reagents, cytokine reporters and/or floxed alleles of EBI3 and p28 to allow lineage specific deletion of these factors.

The IL-27 receptor

WSX-1 is a transmembrane protein that represents the alpha chain of the IL-27R (IL-27Rα), and was first identified from a human infant brain cDNA library in 1998 using screening approaches to identify receptors with homology to glycoprotein 130 (gp130) (Sprecher et al., 1998). Not only does WSX-1 have significant amino acid similarity with gp130, but it also contains the WSXWS sequence motif characteristic of class I cytokine receptors. Subsequent work that focused on cytokine receptors on immune cells cloned this receptor (termed T-cell cytokine receptor, TCCR) and noted the homology to gp130 and IL-12Rβ2 (Chen et al., 2000). These class I cytokine receptors are characterized by an extracellular cytokine binding domain of approximately 200 amino acids that consists of four N-terminal cysteines, three fibronectin type III domains and the highly conserved WSXWS motif near the C-terminus (Bazan., 1990b). In contrast to other related cytokine receptor α chains such as IL-6Rα or IL-11Rα, which
lack intrinsic tyrosine kinase activity and the ability to transduce signals but confer receptor specificity (Yamasaki et al., 1988), the cytoplasmic domain of IL-27Rα has a Box 1 motif that associates with JAK1 and JAK2, and is important for signal transduction (Sprecher et al., 1998).

Signaling by type I cytokines normally involves a heterodimeric receptor and with the identification of IL-27 and IL-27Rα as its partner it was quickly recognized that gp130 was the additional receptor component (Pflanz et al., 2004). Gp130 is a signal-transducing receptor that is utilized by at least eight different cytokines, including IL-6, IL-11, Leukemia Inhibitory Factor (LIF), Oncostatin-M (OSM), Ciliary Neurotrophic Factor (CNTF), Cardiotrophin-1 (CT-1), and Cardiotrophin-like-cytokine (CLC) (Figure 1.1) (Lupardus et al., 2011). Gp130 is present on hematopoietic and non-hematopoietic cells and its expression can vary depending on the cell’s activation status (Andersson et al., 1978, Betz and Muller., 1998, Saito et al., 1992, Taga and Kishimoto., 1997, Wang et al., 1998).

The initial studies that identified IL-27Rα reported that its mRNA was highly expressed in lymphoid tissues such as the spleen, lymph node and thymus, indicating a broad role for this receptor in immune function (Chen et al., 2000, Sprecher et al., 1998). Indeed, the use of IL-27Rα specific antibodies demonstrated that this receptor is expressed by T and B cells as well as NK cells (Villarino et al., 2005). In murine systems, the IL-27Rα is not readily apparent on accessory cells, but there are data that support the notion that macrophages or DCs are responsive to IL-27 and this is readily detected in human macrophages (Figure 1.2C) (Chen et al., 2000, Holscher et al., 2005, Kalliolias et al., 2010a, Yoshida et al., 2001). Human B cell subsets have also been analyzed for
their expression of gp130 and IL-27Rα protein and naive (IgD+CD38−) and memory (IgD·CD38+) B cells express the highest levels of the IL-27 receptor (Boumendjel et al., 2006, Larousserie et al., 2006). Germinal center (IgD·CD38+) B cells also express low levels of these receptor subunits, and there is evidence that plasma cells express IL-27Rα (Cocco et al., 2011, Larousserie et al., 2006). Consistent with these reports, stimulation of B cells through the B cell receptor or through CD40 ligation can up-regulate gp130 and IL-27Rα, suggesting that IL-27 can affect a variety of B cell subsets (Larousserie et al., 2006).

IL-27Rα is also expressed on a variety of human and mouse tumor cell lines such as HeLa cells, B16 melanoma, and Lewis Lung Carcinoma and is up-regulated on cancer cells from patients with acute myeloid leukemia (Dibra et al., 2009, Pradhan et al., 2007). However, the role of IL-27Rα expression in cancer biology is complex, with reports that attribute pro and anti-tumor activity to IL-27 (Table 1.2) (Dibra et al., 2009, Dibra et al., 2011, Pradhan et al., 2007, Yoshimoto et al., 2008). For example, the effects of IL-27Rα expression on tumors has been associated with expression of MHC class I-related chain A (MICA) which is a ligand for NKG2D, an activating receptor expressed on NK cells that which promotes cytotoxicity (Dibra et al., 2009). In contrast, work from the same group has also shown that IL-27Rα expression on different tumor cell lines inhibited effector responses and promoted tumor growth (Dibra et al., 2011). One possible explanation for this observation is that IL-27Rα may act as a homodimeric receptor and, via its Box 1 motif, can activate the JAK/STAT pathway leading to the transformation of myeloid cells (Pradhan et al., 2007). However, studies with B16 melanoma demonstrated that tumor expression of IL-27Rα mediated inhibition of tumor
growth, and these effects were IL-27 dependent (Yoshimoto et al., 2008). The studies described above appear contradictory, with IL-27 promoting growth or killing of tumors, but it seems likely that these effects are context specific, and could be shaped by the individual tumor microenvironment and how the cancer cells have evolved to evade the immune system.

Like gp130, the levels of the IL-27Rα chain are altered in response to activation (Betz and Muller., 1998). Thus, while naïve CD4+ T cells are responsive to IL-27 they express basal levels of the IL-27Rα receptor but following TCR engagement these levels are markedly increased. In contrast, resting NK cells express high levels of the IL-27Rα but activation leads to downregulation of this receptor (Villarino et al., 2005). As for many cytokines, a change in the expression of the receptor subunits is one way to mediate responsiveness, and to influence the amplitude and duration of the signals. Although it is clear that there is a dynamic regulation of IL-27Rα, our understanding of how this impacts on the biological properties of IL-27 remains poor. Nonetheless, the controlled expression of cytokine-specific alpha chains (such as IL-6Rα etc.) is one mechanism to regulate responses to gp130-family cytokines. Moreover, it is notable that the pattern of IL-27Rα expression correlates with the biological effects of IL-27 on T cells, where IL-27 is required to negatively regulate responses of activated CD4+ T cells in multiple settings (discussed below). Thus, the regulated expression of IL-27Rα is a critical variable in the role of this cytokine during inflammation and additional studies examining the regulation of receptor expression should provide insight into the biological activities of IL-27 in vivo.
**IL-27 signaling and transcriptional mediators**

Upon ligation of its receptor, IL-27 induces a signal transduction cascade that engages the JAK/STAT pathway as well as MAPK signaling. The latter events have not been extensively studied but are characterized by the activation of p38 MAPK and ERK1/2 (Owaki et al., 2006). In human naïve CD4\(^+\) T cells, the activation of MAPK induces c-Myc and the expression of cyclins D2, D3, cyclin A and the cyclin dependent kinase (CDK) 4, a process that was shown to be regulated by the IL-27 induction of Pim-1 (Charlot-Rabiega et al., 2011). These events have been most closely associated with the ability of IL-27 to promote T cell proliferation and the transition from the G0/G1 to S phase. The capacity of IL-27 to activate the JAK/STAT pathway in lymphocytes has been extensively studied and the pathways induced by IL-27 resemble those propagated by IL-6. So, although IL-27 shares properties with IL-6 such as shared receptor subunits and the ability to signal through STAT1 and STAT3, there are also distinct effects of these two cytokines. These may be explained by differences in signaling or by how cells integrate these signals depending on activation status or through the regulation of receptor expression. As mentioned earlier, the alpha chain of the IL-6 receptor does not have the capacity to signal, but gp130 and IL-27R\(\alpha\) contain multiple motifs that are important for binding to JAKs (Sprecher et al., 1998). Early studies demonstrated that IL-27R\(\alpha\) was associated with JAK1 (Takeda et al., 2003), whereas gp130 is associated with JAK1, JAK2 and tyrosine kinase 2 (TYK2) (Luticken et al., 1994, Narazaki et al., 1994, Stahl et al., 1994). Indeed, stimulation of naïve CD4\(^+\) T cells with IL-27 induces the activation of JAK1, JAK2 and TYK2 and the marked phosphorylation of STAT 1, 3 and 5 (Kamiya et al., 2004, Lucas et al., 2003, Villarino et al., 2003). Subsequent studies on T helper cell differentiation revealed that the ability of IL-27 to activate STAT1 promoted T
cell expression of the transcription factor T-bet which is associated with the development of a TH1 response (Hibbert et al., 2003, Kamiya et al., 2004, Owaki et al., 2005). Additional insights into the signaling pathways utilized by IL-27 include the ability to activate STAT3, which is required for the expression of c-Maf, a transcription factor associated with the ability of IL-27 to promote IL-10 production (Pot et al., 2009, Xu et al., 2009).

While the main events downstream of IL-27R signaling are described above, there is less known about the mechanisms that temper IL-27 signaling. SOCS proteins have a prominent role in providing negative feedback to many type I cytokines and represent obvious candidates to be involved in this process. For gp130, the activation of the STAT pathways leads to increased expression of SOCS3 that can then bind to SH2 domains in gp130 and so prevent further association with JAKs (Nicholson et al., 2000). This mechanism also appears relevant to IL-27, as IL-27 can induce SOCS proteins (Villarino et al., 2007). Furthermore, T cells from mice in which gp130 lacks the ability to interact with SOCS3 have a sustained pattern of STAT activation when stimulated with IL-27 (Silver., 2011). Nevertheless, even in this setting, IL-27 signaling is eventually downregulated but the additional mechanisms that underlie these events have not been explored.

Complexity and combinatorial biology of the IL-27 system

The sections above summarize our current understanding of the composition of the IL-27 signaling cassette, but even the initial studies provided hints that there are additional levels of complexity in this system. The observation that the p28 and EBI3
subunits of IL-27 can be secreted independently from one another suggested that they had properties distinct from their functions as IL-27 subunits (Batten and Ghilardi., 2007, Devergne et al., 1996, Pflanz et al., 2002, Stumhofer et al., 2010). Thus, the early report that EBI3 could partner with p35 (Devergne et al., 1997) has now been linked to the cytokine IL-35, and Treg cell biology (Collison et al., 2007, Collison et al., 2009, Niedbala et al., 2007). However, there are conflicting reports as to whether human Treg cells express IL-35 (Bardel et al., 2008, Collison et al., 2010), and the signals downstream of IL-35, including the constituents of the IL-35 receptor are at this point poorly understood. Other heterodimers consisting of EBI3 or p28 have also been shown to form in vitro; IL-23p19 can bind to EBI3 (Kastelein et al., 2007), and IL-27p28 has been reported to associate with cytokine-like factor (CLF), and signal through a tripartite receptor composed of gp130, IL-27Rα and IL-6Rα. Although, the in vivo significance of these novel complexes is unclear, they present potential complications for the interpretation of studies with IL-27 and IL-27Rα knockout animals.

As mentioned earlier, the pairing of the soluble IL-6Rα with IL-6 structurally resembles the heterodimeric cytokines IL-12, IL-23 and IL-27 and it has been proposed that these heterodimeric cytokines co-evolved from an ancestral IL-6 like cytokine (Trinchieri., 2003). Consequently, the well-characterized interactions of IL-6 with its receptors can be used to infer other areas that might be relevant to the biology of IL-27. For example, while the IL-6Rα chain is expressed on the surface of many cell types, the ability to generate a soluble version through alternative splicing or enzymatic cleavage of the membrane bound form allows it to pair with free IL-6 and this heterodimer can bind to gp130 and directly activate the downstream signaling cascades (Lust et al., 1992,
Novick et al., 1989). This process is referred to as trans-signaling and even in the steady state, there are basal levels of circulating soluble IL-6 receptor in the serum and synovial fluid, which are elevated during inflammation (Desgeorges et al., 1997). Relevant to this thesis, there are reports of an alternatively spliced form of IL-27Rα that gives rise to a soluble version of this alpha chain of the IL-27R (Hashimoto et al., 2009). While this has been linked to forming a complex with gp130 and CNTFR, whether this is involved in trans-signaling has not been addressed. Similarly, many cytokine systems include natural receptor antagonists, and there is a soluble form of gp130 that antagonizes the process of IL-6-mediated trans-signaling, but does not inhibit conventional IL-6 or IL-27 signaling (Scheller et al., 2005). Work from our group has revealed an immunoregulatory role for IL-27p28 independently of EBI3, whereby it can bind with low affinity to the Ig-like domain of gp130 and antagonize the ability of IL-6, IL-11 and IL-27 to signal (Stumhofer et al., 2010). Thus, analogous to the IL-1Rα and IL-4Rα the IL-27 p28 subunit may also have a role as a low affinity receptor antagonist and its possible role in human disease is discussed below. Taken together, an understanding of the unique biological properties of IL-27 and its components is emerging, as well as an appreciation of the combinatorial biology that is apparent in this family that indicate its potential to impact many aspects of the immune system.

**Pro-inflammatory properties of IL-27**

*IL-27 can promote TH1 responses*

Before it was recognized as the receptor for IL-27, two separate groups generated mice that lacked the IL-27Rα (WSX-1, TCCR) and, based on its similarity to other
immune receptors, screened these mice for altered immune phenotypes (Chen et al., 2000, Yoshida et al., 2001). Although no obvious immune defects were noticed in mice that lacked IL-27Rα, when they were challenged with intracellular pathogens they were reported to be more susceptible to *Listeria monocytogenes* and *Leishmania major* as a consequence of defects in TH1 immunity (Table 1.1). The discovery that IL-27Rα was the receptor for IL-27, and that IL-27 can enhance the proliferation of naïve CD4+ T cells, and the production of IFN-γ (Pflanz et al., 2002), supported the idea that signaling through the IL-27Rα was pro-inflammatory. A molecular basis for these events was provided by studies in which IL-27 was found to promote STAT1 activation, and the transcription factor T-bet, which induces the expression of the IL-12Rβ2 thereby sensitizing the T cell to be responsive to the signals that facilitate TH1 development (Hibbert et al., 2003, Lucas et al., 2003, Takeda et al., 2003). Similarly, IL-27 also mediates the expression of the adhesion molecules ICAM-1/LFA-1, downstream of STAT1, which also aids in the differentiation of TH1 cells (Morishima et al., 2010, Owaki et al., 2006).

With a growing appreciation of the broad immunoregulatory effects of IL-27 the interpretation of some of the earlier in vivo studies has been revisited. Thus, while initial reports suggested that the TCCR deficient mice were susceptible to *L. monocytogenes*, there is now a consensus that they are, in fact, better able to control this pathogen (Batten et al., 2008, Yang et al., 2008a). Furthermore, with an appreciation that IL-27 inhibits TH2 responses, the reduced TH1 response observed during *L. major* infection appears to be a secondary consequence of enhanced TH2 responses observed in the absence of IL-27 (Artis et al., 2004a). This is discussed in more detail below, but the
complex cross regulation of TH1 and TH2 responses can make it difficult to equate altered IFN-γ responses observed in the IL-27Rα deficient mice with the ability of IL-27 to promote CD4+ TH1 responses. However, one example is provided by a model of proteoglycan (PG)-induced arthritis where IL-27 is associated with the development of a pathological TH1 response (Cao et al., 2008). In this experimental system, T cell production of IFN-γ is required for disease progression and loss of the IL-27Rα leads to decreased IFN-γ and reduced disease. This is also a system in which anti-PG Abs are important for the development of arthritis and the IL-27Rα−/− mice also have a reduction in PG-specific IgG2a. Indeed, IL-27 could promote IgG2a indirectly through the production of IFN-γ (Monteyne et al., 1993), and directly through the activation of STAT1 and T-bet (Yoshimoto et al., 2004). Nevertheless, the sections below will highlight the studies in which the effects of IL-27 have been most concretely linked to promoting cell mediated and humoral responses.

Reports that IL-27 limits Foxp3+ regulatory T cell populations

It has been proposed that one pro-inflammatory property of IL-27 is its ability to antagonize the differentiation of Treg cell populations. Multiple groups have reported that when T cells were differentiated under inducible Treg cell differentiation conditions (TGF-β, IL-2) in the presence of IL-27, there was a marked decrease in the frequency of Treg cells that were generated (Huber et al., 2008, Neufert et al., 2007, Stumhofer et al., 2006). Furthermore, in the CD45RBhi transfer model of colitis, IL-27Rα−/− T cells more readily acquired Foxp3 and had less severe disease compared to wildtype (WT) CD45RBhi recipients (Cox et al., 2011). Studies by Cox and Ghilardi also demonstrated
that in an oral tolerance model where mice are fed OVA, transferred OVA specific CD4\(^+\) T cells from IL-27R\(\alpha\)^/- mice had an increase in their ability to express Foxp3, suggesting that in these experimental settings IL-27 could limit Treg cell populations in vivo. Interestingly, mice which have been engineered to over-express IL-27p28 and EBI3 have a profound defect in the peripheral homeostasis of Treg cell and succumb to a scurfy-like immuno-proliferative disease. However, this appears to be an indirect effect of the ability of IL-27 to limit production of IL-2 (Tait Wojno et al., 2011). The interpretation of this body of work is complicated by the limited amount of in vivo evidence that this occurs during normal inflammatory processes. For instance, at steady state, mice deficient in IL-27 or the IL-27R\(\alpha\) do not have increased numbers or frequency of Treg cells, and it is unclear at this point if IL-27R\(\alpha\) knockout mice have altered Treg cell homeostasis. However, given the broad anti-inflammatory properties of IL-27 and its ability to drive the expression of IL-10, discussed below, the capacity of IL-27 to directly limit Treg activities appears incongruous, and suggests additional studies are required to understand where this property of IL-27 is most biologically relevant.

**IL-27 enhances CD8\(^+\) T cell responses**

Similar to the effect on CD4\(^+\) T cells in vitro, in mouse and human CD8\(^+\) T cells IL-27 induces the activation of pSTATs 1-5, increases proliferation, T-bet expression, IFN-\(\gamma\) production and IL-12R\(\beta\)2 expression (Figure 1.2A) (Morishima et al., 2005, Morishima et al., 2010, Schneider et al., 2011). Additionally, IL-27 promotes cytotoxic T lymphocyte (CTL) responses by up-regulating the expression of perforin, granzyme and the specific lysis of target cells (Morishima et al., 2005, Schneider et al., 2011). The
efficacy of IL-27 in promoting CD8+ T cell effector function has been best illustrated by a number of in vivo studies where cancer cell lines engineered to express IL-27 promote tumor specific CTL responses, tumor regression, and, in some cases, complete remission with memory responses to subsequent challenge (Table 1.2) (Chiyo et al., 2004, Chiyo et al., 2005, Hisada et al., 2004, Salcedo et al., 2004, Shinozaki et al., 2009). In models of colon carcinoma and neuroblastoma, the ability of IL-27 to promote tumor regression was found to be dependent on CD8+ T cells, but not NK or CD4+ T cells (Hisada et al., 2004, Salcedo et al., 2004). The efficacy of IL-27 in the regression of colon carcinoma was also shown to be IFN-γ and T-bet dependent but surprisingly independent of STAT4 signals, indicating a potential role for IL-27 that is distinct from that of IL-12 (Hisada et al., 2004).

The role of IL-27 in driving CD8+ T cell responses in autoimmune and infectious disease settings is less well characterized. Initial studies showed that in the absence of IL-27Rα, CD8+ T cell responses are not defective during acute toxoplasmosis, or infection with Trypanosoma cruzi, influenza or malaria (Findlay et al., 2010, Hamano et al., 2003, Sun et al., 2011, Villarino et al., 2003). However, a subsequent report, using a mixed chimeric approach to study the CD8+ T cell response concluded that in mice infected with T. gondii or influenza, the expression of the IL-27Rα was critical for the induction of T-bet and IFN-γ (Mayer et al., 2008, Sun et al., 2011). This apparent discrepancy may be due to indirect effects of the global IL-27Rα deficiency versus those that are revealed in a competitive setting that is engineered using mixed chimeras. There is also a role for IL-27 in driving effector CD8+ T cell responses during influenza, where IL-27 receptor but not IFN-γ receptor expression is required for optimal effector
function, T-bet expression, and presence of IFN-γ+ CD8+ T cells in the lungs of infected animals (Mayer et al., 2008). These studies suggest that IL-27 does play a part in shaping the CD8+ T cell response, however it has not been extensively studied in other well characterized models of CTL responses such as LCMV and Listeria monocytogenes and it remains to be determined if it is critical in the acquisition of effector functions in these settings. In addition, the impact of reports that link IL-27 to the production of IL-10 and the related cytokine IL-21, both of which are CD8+ T cell growth factors, have not been explored in the context of CTL responses during infection or cancer.

**IL-27 and humoral responses**

Although B cells were first identified as a source of EBI3 these cells are also responsive to IL-27, and B cell subsets have differential expression of IL-27Rα and gp130 (Boumendjel et al., 2006, Yoshimoto et al., 2004). The differentiation of naïve IgM+ IgD+ B cells into class switched antibody-secreting cells following B cell receptor stimulation and CD40 ligation is a process that is shaped by factors such as IL-4, IL-6, IL-21 IFN-γ and lymphotoxin-α. Indeed, in vitro, IL-27 has been reported to promote IgG2a secretion by mouse B cells (Yoshimoto et al., 2004), but IgG1 production by human B cells (Boumendjel et al., 2006). Regardless, it should be noted that the effects of IL-27 on class switching are modest when compared to the effects of other cytokines such as IFN-γ and IL-4. Nevertheless, some of the direct effects of IL-27 on B cells have been addressed by studies in which polyclonal stimulation of naïve and GC B cells in the presence of IL-27 increase their proliferation, but IL-27 does not seem to promote formation of memory B cells (Boumendjel et al., 2006, Charlot-Rabiega et al., 2011,
IL-27 can also up-regulate B cell expression of co-stimulatory molecules such as ICAM-1, and CD86 in addition to increasing Fas/CD95 expression and modulating chemokine receptor expression (Cocco et al., 2011, Larousserie et al., 2006). Consistent with these reports, the lack of IL-27Rα has been associated with altered B cell responses in a number of experimental settings. For instance, following infection with L. monocytogenes, IL-27Rα−/− mice have diminished IgG2a responses compared to wildtype mice (Chen et al., 2000). Similarly, in the PG-induced model of arthritis, IL-27Rα−/− mice have diminished PG-specific IgG2a, as discussed previously (Cao et al., 2008). Furthermore, following airway OVA challenge, IL-27Rα−/− mice have exacerbated IgE production (Miyazaki et al., 2005). These studies suggest a potential role for IL-27 in the regulation of class switched antibody responses.

It is not clear from the studies described above whether the altered humoral responses observed in the absence of IL-27 are a consequence of direct or indirect effects of IL-27 on B cells. Indeed, IL-27 can induce CD4+ T cells to produce IL-21, which promotes B cell expression of Blimp-1 and Bcl-6 and is critical for plasma cell differentiation and B cell function (Ozaki et al., 2002, Ozaki et al., 2004, Pot et al., 2009). Ghilardi and colleagues investigated the role of IL-27 in the development of T follicular helper (TFH) cells and TFH cell dependent B cell responses in vitro and also in vivo in response to multiple immunizations with OVA conjugated to the hapten TNP. They reported that while the differentiation of TFH did not require IL-27, this cytokine did stimulate their production of IL-21 and TFH survival was increased. The in vivo production of IL-21 by CD4+ T cells appeared to be dependent on IL-27, and in IL-27Rα−/− mice this IL-21 expression was compromised in an OVA immunization model (Batten
et al., 2010). Following immunization, these mice also displayed diminished GC (B220⁺ GL-7⁺ Fas⁺) B cell responses, decreased class switched antibodies and lower levels of hapten-specific antibody production (Batten et al., 2010). Furthermore, in a pristane-induced lupus model, characterized by the development of anti-dsDNA autoantibodies, the absence of IL-27R expression resulted in less severe pathology (Batten et al., 2010). It should be noted that in other models using IL-27Rα−/− mice, such as in the studies using the OVA airway inflammation model (Miyazaki et al., 2005), there is no defect in class switched antibody production, suggesting that IL-27 is not critical for the GC reaction. Moreover, there are studies in which the over-expression of IL-27Rα in the MRL/lpr mouse model of lupus ameliorated autoantibody responses (Sugiyama et al., 2008), suggesting that IL-27Rα expression can antagonize antibody production. Taken together, these studies demonstrate that IL-27 is a key regulator of B cell responses through its ability to act directly on multiple B cell subsets as well as its effects on CD4⁺ T cells. Given the ability of IL-27 to induce class-switching and TFH development, this cytokine may be useful as an adjuvant of vaccine-induced humoral immunity, although this hypothesis has yet to be directly tested.

**Anti-inflammatory properties of IL-27**

While early reports focused on the ability of IL-27 to promote TH1 immunity, subsequent studies, primarily using parasitic systems, revealed the immunosuppressive effects of IL-27. This section will summarize that early work and how these studies led to our current understanding of the mechanisms utilized by IL-27 to limit an array of inflammatory responses.
**IL-27 and type I responses**

The sections above highlight the pro-inflammatory properties of IL-27 in promoting TH1 activity, CTL responses, inhibiting Treg cells, and promoting humoral immunity. Based on these data, one would predict that the absence of IL-27 signaling would lead to a failure to generate type I responses and humoral immunity and have enhanced Treg cell responses, and that this would be associated with a decreased ability to control pathogens. However, there is now a preponderance of in vivo evidence that this is not the case (Table 1.1). Studies with an array of parasitic (*T. gondii, L. donovani, T. cruzi, Plasmodium bergheri*) (Artis et al., 2004a, Findlay et al., 2010, Rosas et al., 2006, Villarino et al., 2003), bacterial (*M. tuberculosis*) (Holscher et al., 2005, Robinson and Nau., 2008), viral (influenza) (Sun et al., 2011), and autoimmune models of inflammation (Table 1.2) lupus (Igawa et al., 2009, Shimizu et al., 2005, Sugiyama et al., 2008), colitis (Kim et al., 2013, Sasaoka et al., 2011, Troy et al., 2009), asthma (Dokmeci et al., 2011, Miyazaki et al., 2005, Shimanoe et al., 2009, Yoshimoto et al., 2007), MS (Batten et al., 2006, Diveu et al., 2009, Fitzgerald et al., 2007) and hepatitis (Frank et al., 2010) show that IL-27 is a critical negative regulator of the pathology associated with these models.

The ability of IL-27 to limit TH1 responses is highlighted by studies which showed that IL-27Rα−/− mice infected with the protozoan parasite *T. gondii* developed a lethal CD4+ T cell mediated immune-pathology (Villarino et al., 2003). Although IL-27Rα−/− mice infected with *T. gondii* succumbed within two weeks of challenge, they efficiently controlled parasite replication, but actually had enhanced CD8+ and CD4+ T
cell responses that were associated with increased production of IFN-γ. Exacerbated TH1 responses and disease pathology are characteristic of other studies with the IL-27Rα−/− mice such as those following infection with *T. cruzi, P. bergheri* and *L. donovani* (Findlay et al., 2010, Hamano et al., 2003, Rosas et al., 2006). Initial evidence of the ability of IL-27 to limit multiple classes of T helper cells came from studies with *T. cruzi*. In this model, IL-27Rα−/− mice infected with *T. cruzi* developed enhanced TH1 and TH2 activities, and the elevated IL-4 contributes to the higher parasite burden in these mice. In contrast, the heightened TH1 activity in these mice resulted in severe immune pathology, and exacerbated IFN-γ production contributes to liver injury and lethality (Hamano et al., 2003). Recent studies looking at the role of IL-27 during infection with *P. bergheri* reveal that IL-27 can prevent CD4+ T cell-mediated pathology, which is characterized by elevated IFN-γ, IL-17, and TNF-α (Findlay et al., 2010). Similar findings have also been observed following infection of IL-27Rα−/− animals with *M. tuberculosis, L. major*, and *L. donovani* (Anderson et al., 2009, Holscher et al., 2005, Rosas et al., 2006).

The mechanism by which IL-27 limits TH1 responses to intracellular pathogens is not completely understood, especially since early studies indicated a role for promoting TH1 immunity. However, in the studies with *T. gondii*, it was clear that depletion of CD4+ T cells could rescue acute lethality in these mice, suggesting that IL-27 directly limits TH1 cells (Villarino et al., 2003). Consequently, the suppressive role of IL-27 in vivo has been attributed to some of the pleiotropic anti-inflammatory properties of IL-27, such as its ability to limit IL-2 or IFN-γ production by CD4+ T cells and promote T cell expression of the potent anti-inflammatory cytokine IL-10 (Anderson et al., 2009,
Batten et al., 2006, Diveu et al., 2009, El-behi et al., 2009, Murugaiyan et al., 2009, Stumhofer et al., 2006, Villarino et al., 2006, Villarino et al., 2010, Yoshimura et al., 2006). It is also worth noting that the inhibitory effects of IL-27 on T cells could be indirect. Studies with \textit{M. tuberculosis} suggest that IL-27Rα deficient macrophages in this model produce more IL-12p40, IL-6 and TNF-α which could manifest as exacerbated TH1 responses (Holscher et al., 2001, Holscher et al., 2005, Robinson and Nau., 2008).

Another example of the anti-inflammatory properties of IL-27 is illustrated by studies in the MRL/lpr mouse model of systemic lupus erythematosus (SLE). These mice, which have defective apoptosis (Watanabe-Fukunaga et al., 1992), develop disease characterized by anti-DNA antibodies and mixed T helper response including TH1, TH2 and TH17 cells, reviewed in (Fairhurst et al., 2006, Shin et al., 2011). MRL/lpr mice transgenic for the IL-27Rα have less severe disease characterized by reduced glomerulonephritis, decreased production of IFN-γ and IL-4, lower levels of anti-double stranded DNA antibodies, and less severe skin inflammation, associated with increased survival compared to non-transgenic animals (Sugiyama et al., 2008). Furthermore, the lack of IL-27 signals in MRL/lpr mice results in exacerbated disease characterized by more severe skin lesions resembling human SLE disease (Kido et al., 2011). These examples illustrate the broad and sometimes synergistic anti-inflammatory properties of IL-27 during TH1 responses but much of the mechanism by which this occurs is still unknown and may differ depending on the inflammatory context. Nonetheless, in other models such as TH2 and TH17 immunity it is apparent that IL-27 can exert its effects in a more direct fashion, described below.
**IL-27 and type 2 responses**

One of the earliest studies with the IL-27Rα deficient mice challenged them with *L. major*, a parasite that requires type I immunity for protection. The course of disease in these mice was characterized by a marked increase in the TH2 response, reduced production of IFN-γ, and an early but transient inability to control this infection (Yoshida et al., 2001, Zahn et al., 2005). One interpretation of these findings was that IL-27 was important for the development of TH1 immunity to this parasite. An important characteristic of this model system is that while IFN-γ is required to limit this infection, TH2 cells dominate during the early phase of this infection. As noted earlier, the complex relationship between TH1 and TH2 cells can make it difficult to interpret these early studies and additional studies suggested that that the initial failure of these mice to control *L. major* was not because IL-27 was required for the genesis of TH1 cells, but rather, that the IL-27Rα/− mice were unable to down-regulate the early TH2 response (Anderson et al., 2009, Artis et al., 2004a). Thus, when IL-27Rα/− mice were treated with α-IL-4 antibodies prior to infection with *L. major*, they developed normal parasite specific TH1 cells and control of this infection. Thus, these studies demonstrate that the early susceptibility in these mice was not due to a defect in TH1 immunity, but rather a consequence of unrestrained TH2 responses.

In the setting of other TH2-centered experimental systems, IL-27 has also emerged as a critical negative regulator and IL-27-deficient mice have enhanced protective immunity to helminth infection (Artis et al., 2004b) and increased IL-4 production in response to *T. cruzi* (Hamano et al., 2003). During the induction of
experimental asthma, IL-27 can also play a protective role by limiting TH2 responses (Dokmeci et al., 2011, Fujita et al., 2009, Miyazaki et al., 2005, Yoshimoto et al., 2007). This effect has been linked to the ability of invariant NKT cells to produce IL-27 to negatively regulate TH2 responses (Fujita et al., 2009). Finally, administration of IL-27 during experimental asthma ameliorated disease, suggesting a novel therapeutic approach for the treatment of T cell-mediated autoimmunity (Miyazaki et al., 2005, Yoshimoto et al., 2007). The molecular basis of the effect of IL-27 on developing TH2 responses are revealed by studies which showed that IL-27 antagonized expression of the master regulator of TH2 responses, GATA3, and so provided a mechanistic insight into these effects (Lucas et al., 2003).

**IL-27 inhibits TH17 responses**

The ability of IL-27 to attenuate T cell differentiation is not limited to TH1 or TH2 responses, as multiple reports have shown that IL-27 also modulates TH17 activities (Anderson et al., 2009, Diveu et al., 2009, El-behi et al., 2009, Fitzgerald et al., 2007, Liu and Rohowsky-Kochan., 2011, Murugaiyan et al., 2009, Sasaoka et al., 2011, Stumhofer et al., 2006, Troy et al., 2009, Villarino et al., 2010, Yang et al., 2008a, Yoshimura et al., 2006). TH17 cells, characterized by their production of IL-17 and IL-22, are associated with protective immunity to a number of extracellular pathogens such as *Candida albicans*. Most notably however, TH17 cells are causally associated with in a number of autoimmune disease states such as rheumatoid arthritis (characterized by pro-inflammatory auto-reactive TH17 cells (Nakae et al., 2003)), inflammatory bowel disease (TH17 cells can induce colitis (Elson et al., 2007)) and MS (TH17 cells are
associated with increased EAE scores (Komiyama et al., 2006)), also reviewed in (Korn et al., 2009).

Following infection with L. major, L. monocytogenes, or during chronic toxoplasmosis in IL-27Rα−/− or EBI3−/− mice, exacerbated disease has been associated with the increased presence of TH17 cells (Anderson et al., 2009, Stumhofer et al., 2006, Yang et al., 2008a). Of particular relevance, these studies have identified a role for IL-27 in limiting the expansion of TH17 cells during chronic inflammation, and highlight the opposing roles of IL-6 and IL-27 on the generation of TH17 cells: IL-6 promotes TH17 development whereas IL-27 inhibits IL-17 production (Batten et al., 2006, Fitzgerald et al., 2007, Stumhofer et al., 2006). Notably, IL-27p28− or WSX1-deficient mice develop more severe EAE, associated with enhanced TH17 responses, demonstrating the significance of this pathway in disease development (Batten et al., 2006, Diveu et al., 2009, Fitzgerald et al., 2007).

The mechanistic basis underlying the inhibitory effects of IL-27 during TH17 responses are not entirely clear, but several studies have provided some insights into these events. Thus, during in vitro TH17 cultures (α-CD3, α-CD28, IL-6 and TGF-β), IL-27 can block the production of IL-17 from CD4+ T cells, in a STAT1 and partially STAT3 dependent manner (Stumhofer et al., 2006). This is independent of the ability of IL-27 to up-regulate T-bet, IL-10 and Socs3, which are facets of the anti-inflammatory properties of IL-27 in other systems (Liu and Rohowsky-Kochan., 2011, Stumhofer et al., 2006, Yoshimura et al., 2006). However, in the absence of STAT1 signals there may be a role for T-bet in directly inhibiting TH17 responses, as evidenced by recent studies by Villarino et al. (Villarino et al., 2010). Furthermore, during in vitro polarization of TH17
cells, IL-27 can directly inhibit the expression of RORα and RORγt, two transcription factors associated with TH17 development and inhibit production of IL-22, a cytokine that is important for TH17 effector function (Diveu et al., 2009, El-behi et al., 2009, Yang et al., 2008a).

IL-27 can inhibit the de novo differentiation of TH17 cells, although there is some debate as to whether IL-27 can also limit fully differentiated TH17 cells. Some of the disparities in these studies may be due to the differences between mouse and human TH17 cells or the sources of the memory cell populations (El-behi et al., 2009, Liu and Rohowsky-Kochan., 2011). For instance, in the human studies, IL-27 was capable of inhibiting production of IL-17 by polyclonal CD45RO+ memory cells and when it was present during the induction phase of TH17 polarizing cultures (Liu and Rohowsky-Kochan., 2011). Similarly, during toxoplasmic encephalitis, IL-27 can block IL-17 production by effector CD4+ and CD8+ T cells isolated from the CNS of infected mice (Stumhofer et al., 2006). However, in an assay in which memory cells were isolated from mice with EAE, IL-27 could not inhibit IL-17 production (El-behi et al., 2009). Thus, while IL-27 may be a promising therapeutic target for the treatment of pathological TH17 effectors, fully understanding the effects of IL-27 on developing and existing pathogenic TH17 responses will be critical in determining how to tailor its use therapeutically.

*IL-27 promotes production of IL-10*

In addition to the broad anti-inflammatory effects of IL-27 in directly limiting TH2 and TH17 effector cells, it also supports the in vitro and in vivo generation of CD4+
and CD8+ T cells and NK cells that make IL-10 (Awasthi et al., 2007, Fitzgerald et al., 2007, Laroni et al., 2011, Stumhofer et al., 2007b). IL-10 is a potent anti-inflammatory cytokine, with a critical role in limiting immune pathology, reviewed in (Ouyang et al., 2011). Studies with protozoan parasite models such as Plasmodium spp., T. cruzi and T. gondii exemplify the requirement for IL-10 production during infection (Findlay et al., 2010, Gazzinelli et al., 1996, Hunter et al., 1997, Jankovic et al., 2007, Neyer et al., 1997, Wilson et al., 2005), and reviewed in (Couper et al., 2008). While there are many sources of IL-10, CD4+ T cells are a key factor in this process and studies from Alan Sher’s group highlighted the critical role for T cell derived IL-10 in limiting infection-induced pathology caused by T. gondii, but the signals that promote their production of IL-10 were unclear. The first indication that IL-27 was involved in the regulation of IL-10 was provided by studies in IL-27Rα−/− mice with toxoplasmic encephalitis or EAE. In these models, the enhanced pathology observed in the absence of IL-27, also correlated with reduced levels of IL-10 (Fitzgerald et al., 2007, Stumhofer et al., 2007b). These findings suggest a role for IL-27 in this process and in vitro studies established that IL-27 was able to directly promote T cell (TH1, TH2, TH17, Treg) production of IL-10 (Stumhofer et al., 2007b). These observations were part of a series of studies that solidified a role for IL-27 in this process using different experimental approaches (Awasthi et al., 2007, Fitzgerald et al., 2007, Stumhofer et al., 2007b). In the EAE studies, Fitzgerald, Rostami and colleagues used an adoptive transfer model to show that normally encephalitogenic T cells failed to induce disease when treated with IL-27 (Fitzgerald et al., 2007). This was found to be IL-10 dependent, as IL-10 knockout T cells treated with IL-27 induced disease (Fitzgerald et al., 2007). Studies from Awasthi, Kuchroo and Weiner demonstrated that DCs exposed to inducible Treg cell became
tolerogenic, and were a potent source of IL-27 that promoted the expression of IL-10 by CD4+ T cells (Awasthi et al., 2007). It should be noted that IL-6 in combination with TGF-β can also promote the production of IL-10 from CD4+ T cells (McGeachy et al., 2007, Stumhofer et al., 2007b) and that TGF-β in addition to IL-27 can augment IL-10 production by CD4+ T cells (Stumhofer et al., 2007b). These IL-27-induced regulatory cells ("TR1" cells) express IL-10 and are distinct from Treg cells in that they do not express the transcription factor Foxp3, reviewed in (Pot et al., 2010). Whether these cells represent a distinct lineage of T helper cells or are a population that transiently expresses IL-10, similar to effector cells, remains to be determined. Nonetheless, one mechanism reported to promote the development of TR1 cells has been shown to be indirect, through Foxp3+ Treg cells, and their ability to induce a regulatory subset of DCs that make IL-27, that in turn can promote TR1 cells (Awasthi et al., 2007).

In order to understand the mechanism by which IL-27 induced IL-10 in CD4+ T cells, initial studies examined the role of STAT1 and STAT3 in this process and suggested that both transcription factors were involved in the ability of IL-27 to induce IL-10 in CD4+ T cells (Stumhofer et al., 2007b). However, the role for these signaling molecules when TGF-β was present was not examined and recent studies illustrate that there are TGF-β dependent and independent pathways that can promote the development of TR1 cells. Thus when TGF-β is absent, the differentiation of TR1 cells in the presence of IL-27 is STAT1 dependent (Xu et al., 2009). In contrast, when TGF-β is present, STAT1 is not required for the IL-27 mediated induction of IL-10 (Stumhofer et al., 2007b). Understanding these events is further complicated by reports that IL-27 induces expression of the ligand-activated transcription factor aryl hydrocarbon receptor (Ahr)
and the transcription factor c-Maf, both of which trans-activate the IL-10 and IL-21 promoters (Apetoh et al., 2010). Interestingly, IL-21 has emerged as an important co-factor for IL-10. Both IL-6 and IL-27 can induce the production of IL-21 by CD4⁺ T cells, which acts as an autocrine growth that is necessary for the expansion of TR1 cells (Pot et al., 2009).

One area that these studies highlight is the relationship between IL-6 and IL-27 in promoting T cell production of IL-10. Although IL-6 may give rise to a population of cells that resemble IL-27-induced TR1 cells, whether they have redundant roles in vivo remains to be determined. Nonetheless, IL-27 contributes to the development of IL-10 producing CD4⁺ T cell populations during EAE (Fitzgerald et al., 2007), or following infection with *L. major* (Anderson et al., 2009), *T. gondii* (Stumhofer et al., 2007b), or *L. monocytogenes* (Batten et al., 2008). As the studies using IL-27Rα⁻/⁻ mice suggest non-redundant roles for IL-27 exist, it remains to be determined if there is an in vivo role for IL-6 in this process independently of IL-27.

**The role of IL-27 in other cell types**

*IL-27 and innate immunity*

While the majority of studies on IL-27 have focused on its role in the regulation of adaptive immunity, this cytokine is part of an evolutionary conserved pathway that has a prominent role in innate immunity. For example, Drosophila and *Caenorhabditis elegans* have gp130 orthologues and downstream JAK/STAT signaling associated with resistance to infection, although no orthologues of the IL-27 or IL-27R subunits have yet
been described in non-vertebrates (Huising., 2006). Nonetheless, innate cells are the major sources of IL-27 (described earlier) and multiple innate populations express gp130 and IL-27Rα. This includes NK and mast cells as well as eosinophils and macrophages (Artis et al., 2004b, Pflanz et al., 2004, Villarino et al., 2005) and these cell types show a range of biological responses to IL-27. For example, IL-27 has been linked to the ability of human eosinophils to produce cytokines and chemokines (Hu et al., 2010). However, the role of IL-27 in mast cell biology is not clear. Pflanz and colleagues demonstrated that human primary mast cells respond to IL-27, initiate STAT3 activation, and turn on the transcription of pro-inflammatory cytokines such as IL-1α, IL-1β and TNF-α, but did not enhance Fc receptor-mediated de-granulation (Pflanz et al., 2004). Furthermore, in a model of passive cutaneous anaphylaxis, IL-27Rα−/− mice have elevated mast cell protease activity (Artis et al., 2004b), suggesting that IL-27 may limit mast cell-mediated inflammation. In contrast, recent studies have highlighted that although mouse mast cells express IL-27Rα, they are largely unresponsive to this cytokine, a finding that was attributed to incomplete glycosylation of gp130 and retention within the cytoplasm (Traum et al., 2011).

For other granulocytes, elevated neutrophil activity has been noted in the absence of IL-27. Following cecal ligation and puncture, IL-27−/− mice are more resistant to sepsis caused by the leakage of bacteria into the peritoneal cavity and this phenotype is associated with accelerated neutrophil recruitment and reduced bacterial loads (Wirtz et al., 2006). These data sets are consonant with the idea that the absence of IL-27 allows neutrophils to reach the critical concentration that is essential for the control of bacterial growth (Li et al., 2004). Regardless, it has been difficult to distinguish whether these
enhanced granulocyte responses observed in different models were simply a secondary consequence of altered inflammatory responses or directly limited by IL-27. For example, in the setting of innate immunity, IL-27 is an antagonist of NK cell production of IL-17 (Passos et al., 2010), a cytokine that promotes neutrophil mobilization and could contribute to the increased neutrophil response observed in IL-27Rα−/− animals.

In terms of macrophages and DCs, there is experimental evidence for the ability of IL-27 to inhibit murine DCs during leishmaniasis (Wang et al., 2007) and studies with human DCs describe the ability of IL-27 to promote DC expression of B7-H1 (PD-L1), a molecule that provides suppressive signals to T cells (Karakhanova et al., 2011). While there are reports that resting mouse macrophages are not responsive to IL-27 (Kalliolias and Ivashkiv., 2008), others have found that IL-27 inhibits the ability of murine macrophages to produce IL-12 and TNF-α (Holscher et al., 2005), and can promote macrophage production of IL-10 (Iyer et al., 2010). Whether the ability of IL-10 to antagonize IL-12 production is part of an autocrine loop that allows IL-27 to directly regulate accessory cell function has not been addressed.

In human macrophages, IL-27 can inhibit their responsiveness to pro-inflammatory cytokines such as IL-1 and TNF-α by down-regulating receptor expression of their cognate receptors (Kalliolias et al., 2010a). In human monocytes, a different paradigm is emerging and Ivashkiv and colleagues have found that in these cells, IL-27 signaling is associated with a pro-inflammatory signature (Kalliolias and Ivashkiv., 2008). Consistent with this activity, IL-27 has been linked to the induction of type I IFNs that can inhibit the replication of HIV in macrophages. IL-27 can also inhibit the ability of CD14+ monocyte precursors to generate osteoclasts (Kalliolias et al., 2010b) an activity
that may be dependent on the production of type I IFNs (Greenwell-Wild et al., 2009, Imamichi et al., 2008, Kurihara and Roodman., 1990). Clearly, the studies described in this section highlight the impact of IL-27 on innate populations but dissecting cell intrinsic effects can be difficult. The ability to use lineage specific deletions of the IL-27R in different innate populations will facilitate a better dissection of the effects of IL-27 on these innate cells in vivo.

**IL-27 and non-hematopoietic cells**

Although most of the studies examining the functions of IL-27 have been centered on hematopoietic cells, there is also evidence that IL-27 can have effects on non-hematopoietic populations such as epithelial cells, fibroblasts and keratinocytes. In the cancer literature, there are studies showing that IL-27Rα is expressed on epithelial tumors derived from the colon, breast and melanocytes, and that in certain settings, IL-27 can have direct effects on these cells (Dibra et al., 2009, Dibra et al., 2011, Yoshimoto et al., 2008). In the setting of systemic sclerosis (SSc), IL-27 is hypothesized to play a role in disease development because SSc patients express elevated levels of IL-27Rα in fibroblasts and IL-27 promotes fibroblast proliferation and the production of collagen (Yoshizaki et al., 2011). Another study reported that during chronic eczema, human keratinocytes express IL-27 and respond to IL-27 which triggers increased MHC Class I expression and the production of the chemokine CXCL10 (Wittmann et al., 2009). IL-27 can also play a beneficial role in maintaining barrier function as well as stimulating intestinal epithelial cells to express the scavenger receptor DMBT1 that can bind to a variety of Gram-positive and Gram-negative bacteria and act as an antimicrobial peptide.
Taken together, the studies described in this section illustrate some of the effects of IL-27 - potentially beneficial or detrimental to the host - on non-hematopoietic cells and thus need to be taken into consideration when examining the role of IL-27 in different settings.

**Translational implications of IL-27**

*Targeting IL-27 pathways for therapies*

With the initial studies linking IL-27 to the development of TH1 responses, it made sense that neutralizing IL-27 might be a useful strategy to limit inflammatory conditions associated with increased production of IFN-γ. Alternatively, the properties of IL-27 that promote TH1 responses might be useful as part of an adjuvant to promote cell mediated immunity and, as discussed earlier, there is now a literature that supports its use in cancer vaccines. Nonetheless, despite a better understanding of the role of endogenous IL-27 as an inhibitor of inflammation in many disease settings, questions remain about which therapeutic approaches for IL-27 are most likely to be successful. For example, IL-12 and IL-27 have proven to be effective in similar mouse models of cancer, but in humans, IL-12 has been associated with severe toxicity leading to a halt in clinical trials of IL-12 (Cohen., 1995, Leonard et al., 1997, Marshall., 1995). To date there are no reports of toxicity caused by treatment of mice with IL-27 and so IL-27 may represent an attractive alternative to IL-12 as a therapeutic in the setting of cancer. Indeed, studies in which DNA minicircles that express IL-27 in combination with IL-2 were given to mice bearing neuroblastoma resulted in tumor regression, suggesting that IL-27 could be utilized in the clinical setting (Salcedo et al., 2009). Furthermore,
treatment with IL-27 can also attenuate collagen-induced arthritis (CIA) (Niedbala et al., 2008), and local delivery of an adenovirus expressing IL-27 into the ankles with CIA also resulted in improved disease scores (Pickens et al., 2011). There are also reports that use of IL-27 blocking reagents to modify disease processes, such as the use of the IL-27R-Fc fusion protein to block peritoneal sepsis (Wirtz et al., 2006). While the potential application of these findings to the clinical setting is promising, it is imperative to consider the possible negative side-effects of altering levels of bioactive IL-27. For instance, long-term ablation of IL-27 signals may also yield unwanted effects such as autoimmunity. Alternatively, administration of IL-27, while beneficial in limiting pathology, in other contexts could limit protective immune responses.

In the context of murine models of EAE, several reports have identified a role for IL-27 in limiting disease (Fitzgerald et al., 2007, Guo et al., 2008, Wang et al., 2008). In these experimental systems, IL-27 has been shown to contribute to the efficacy of type I IFN therapy, a treatment that is used in human patients to treat MS, but which is associated with significant side effects (Biggioggero et al., 2010, Sweeney et al., 2011). Although type I interferons can promote the production of IL-27, it was somewhat surprising that the therapeutic effects of type I interferon treatment were dependent on the induction of IL-27p28 (Guo et al., 2008, Shinohara et al., 2008). Thus, these findings suggest that the clinical efficacy of IFN-β in patients with MS may be attributed to its ability to induce IL-27 (or the IL-27p28 monomer). Therefore, the use of IL-27 may represent an alternative strategy to manage this condition, without the side effects associated with the type I IFNs. Indeed, when others have utilized a format that allows sustained delivery of IL-27, such as through osmotic pumps, delayed the onset of EAE,
and amelioration of established EAE was observed (El-behi et al., 2009, Fitzgerald et al., 2007). Similarly, the use of DNA minicircles to force transient high levels of IL-27 completely blocked the development of EAE (Stumhofer et al., 2010).

While IL-27 has not been used in any human therapies to date, the studies above illustrate the potential impact of IL-27 in clinical settings. One consideration for the use of any cytokine treatment is the relatively short half-lives of these proteins. In our experience treatment of naïve mice with IL-27 has had no adverse effects on the immune system (Tait Wojno et al., 2011). While the strategies described above, using transient or local expression of IL-27 have shown the remarkable therapeutic potential, in contrast, transgenic over-expression of IL-27 results in severe disease associated with defects in myelopoiesis and/or the loss of Treg cell populations (Seita et al., 2008, Wojno et al., 2011). While these latter models may not relate directly to the clinical use of IL-27, they do illustrate the complex biology of IL-27 and, as for many cytokines, the challenge is to find optimal ways to deliver IL-27 to the appropriate cellular or tissue targets. For instance, the ability of IL-27 to inhibit inflammatory pathways associated with the development of inflammatory bowel disease (IBD) and Crohn’s disease (CD) means that strategies to deliver IL-27 in the gut might be a viable therapy. Indeed, Durum and colleagues have used the Gram-positive bacteria *Lactococcus lactis* to transiently express IL-27 in the gut in a model of colitis and found remarkable protective effects (Hanson et al., 2013).

The use of nanoparticles to deliver IL-21 to T cells has been highlighted recently (Stephan et al., 2010), and development of similar approaches with IL-27 may provide platforms that allow very specific cell populations to be targeted. Cytokine
“engineering”, involving modifying the structure or route of delivery, represents another potential approach to use IL-27 therapeutically and there is precedent for this strategy with IL-6. The fusion of IL-6 with its soluble receptor results in a “hyperkine” which is capable of binding to gp130 and propagating signals that are a log fold more potent than IL-6 (Rakemann., 1999). Thus, one potential option is to engineer novel forms of IL-27 that can preferentially interact with different elements of the IL-27R, but whether a similar strategy to that used with IL-6 would be applicable to IL-27 is not known. One study utilizing a mutant form of IL-27, in which the p28 subunit could not interact with gp130, revealed that this mutant protein acted as a receptor antagonist and limited TH1-mediated liver damage (Rousseau et al., 2010). A parallel study demonstrated that IL-27p28 could act as a receptor antagonist of gp130 and transgenic expression of this subunit limited humoral responses (Stumhofer et al., 2010). Regardless, while the crystal structures of the interactions of IL-6 with gp130 and the IL-6Rα provide a template for the likely interactions of IL-27 with its receptor subunits, there is still a need for additional structure-function studies that would inform the development of altered versions of IL-27 that could act as receptor agonists or antagonists.

The role of IL-27 in human disease

Given the broad roles that cytokines play in shaping all aspects of innate and adaptive immunity, there has been a concerted effort towards identifying cytokine and cytokine receptor single nucleotide polymorphisms (SNP) associated with human disease. For instance, polymorphisms in IL-10 have been strongly linked to susceptibility to lupus, asthma and arthritis reviewed in (Hollegaard and Bidwell.,
Until recently there was a paucity of information about whether IL-27 or the IL-27R were associated with human disease, but several studies have highlighted polymorphisms in IL-27p28 that are associated with autoimmunity. One of the first SNPs identified in IL-27p28 linked to disease (g.-964A > G), was associated with susceptibility to asthma and increased IgE and eosinophilia (Chae et al., 2007). This same polymorphism in IL-27 has also been associated with susceptibility to chronic obstructive pulmonary disease (COPD) and IBD (Huang et al., 2008, Li et al., 2009). Another study utilizing genome-wide association studies and high-density SNP analysis was able associate two loci near the IL-27p28 gene, that were in linkage disequilibrium (rs8049439 and rs1968752), with the onset of CD and ulcerative colitis (UC). These studies showed that the A allele of rs1968752 was statistically associated with decreased mRNA expression of IL-27 in lymphoblastic cell lines and colonic epithelium taken from patients with early-onset CD and UC, thus providing evidence that IL-27 may play a protective role in preventing these autoimmune diseases (Imielinski et al., 2009).

However, the recent findings, which illustrate that IL-27p28 can act as a receptor antagonist, may complicate understanding how polymorphisms in this gene impact human disease. While the mechanisms by which these disease states are affected by polymorphisms in IL-27p28 have yet to be determined, studies looking at the role of STATs in human disease may be informative. For instance, a gain of function mutation in STAT1 enhanced the ability of IL-27 to suppress TH17 cells, which in turn was associated with increased susceptibility to fungal infections (Liu et al., 2011, van de Veerdonk et al., 2011). Furthermore, polymorphisms in STAT3 are associated with CD, and studies involving these patients may provide insights into the role of IL-27 in this disease (Franke et al., 2008).
1.2 The immune response to *Toxoplasma gondii*

The protozoan parasite *T. gondii* infects all warm-blooded animals and elicits a robust TH1 response for which innate sensing mechanisms and adaptive immunity are critical in mounting a protective immune response. Equally important for resistance to this parasite is the ability to limit immune pathology. This section will discuss briefly the global impact of this parasite and then the critical players in the immune response to *T. gondii*. This response is characterized by the production of the pro-inflammatory cytokines IL-12 and IFN-γ, which are absolutely critical to control parasite replication, and the anti-inflammatory cytokines IL-10 and IL-27, which are required to limit immune pathology (Figure 1.3). A perspective on the role of Treg cells during infection will also be presented.

**Global impact of infection with *T. gondii***

*T. gondii* is an obligate intracellular protozoan parasite that can infect virtually any nucleated cell and has important impacts on public health and on livestock populations (Dubey., 2008). Felines are the primary host of *T. gondii* and the only organisms where sexual reproduction of this parasite takes place, although asexual replication occurs in intermediate hosts (most mammals), and both the primary and intermediate hosts contribute to the spread of infection. Cats shed environmentally resistant oocysts in their feces that contribute to the transmission of this parasite to humans and livestock through the ingestion of contaminated water or food (Dubey., 2008, Frenkel et al., 1970). The consumption of tissue cysts in the form of raw or
undercooked meat from animals infected with *T. gondii* and congenital transmission are other ways humans are infected with the parasite (Kean et al., 1969, Wolf et al., 1939). Oral infection can only occur with the bradyzoite and oocyst forms of *T. gondii* because these are stages resistant to the harsh environment of the gut, whereas the highly replicative tachyzoite stage of the parasite does not survive this environment, and can only be used to infect animals when the oral route is bypassed (Jacobs et al., 1960). The global seroprevalence of *T. gondii* ranges from 10-20% in North America, to 40-60% in Western Europe, to rates higher than 60% in regions of South America (Pappas et al., 2009). While most immunocompetent individuals are asymptomatic (Weiss and Dubey., 2009), severe complications can arise during congenital infections and in immunocompromised individuals such as those with AIDS, or those undergoing transplants (Havelaar et al., 2007, Luft et al., 1984, Montoya et al., 2001). Cases of congenital transmission are most commonly the result of new infections acquired during pregnancy and are associated with spontaneous abortion (Havelaar et al., 2007). If the fetus does survive, a number of clinical manifestations are noted in the first year of life including chorioretinitis, intracranial calcifications, hydrocephalus, mental retardation, and even death can occur (Havelaar et al., 2007). In patients with defects in T cell-mediated immunity, reactivation of latent infection is the common cause of toxoplasmic encephalitis and disseminated disease (Luft and Remington., 1992, Weiss and Dubey., 2009). Although there is an effective commercially available vaccine for use in livestock, no such treatment for humans exists (Dubey., 2008), therefore studying the effects of this parasite on the host immune system is of medical relevance. Given that mice represent a natural intermediate host for this infection, and a number of experimental
tools are available to study both the parasite and immune response to \textit{T. gondii}, they are an ideal model to examine host-pathogen immune interactions (Dupont et al., 2012).

\textbf{Initiation of the immune response to \textit{T. gondii}}

 Upon ingestion of tissue cysts, \textit{T. gondii} converts to the rapidly replicating tachyzoite stage and disseminates throughout the host within 24 hours (Dubey., 1998). Within this period, innate accessory cells such as DCs, inflammatory monocytes and neutrophils are recruited to the site of infection, where these cells in addition to tissue resident macrophages and DCs sense the parasite and produce IL-12 (Figure 1.3) (Bliss et al., 2000, Dunay et al., 2008, Gazzinelli et al., 1994, Mashayekhi et al., 2011, Mordue and Sibley., 2003, Yarovinsky et al., 2005). Innate sensing mechanisms are required for the production of IL-12. This is most readily apparent from studies looking at mice that lack the TLR adapter protein MyD88 or endoplasmic reticulum membrane protein UNC93B, which coordinate signals for multiple TLRs (Andrade et al., 2013, Melo et al., 2010, Scanga et al., 2002). While no specific single TLR deficiency is associated with a complete defect in IL-12 production, TLRs 2, 4, 9, 11 and 12 are important in this process (Andrade et al., 2013, Benson et al., 2009, Debierre-Grockiego et al., 2007, Koblansky et al., 2013). Although the single critical cellular source of IL-12 is debated, reviewed in (Dupont et al., 2012), it is clear that IL-12 is absolutely necessary as evidenced by mouse studies in which mice that lack IL-12p40 or IL-12p35 during the acute or chronic stages succumb to infection (Gazzinelli et al., 1994, Lieberman et al., 2004, Yap et al., 2000). IL-12 is responsible for the innate production of IFN-\(\gamma\) by NK cells early in infection (Gazzinelli et al., 1993b, Gazzinelli et al., 1994, Hunter et al., 1994) and also the IFN-\(\gamma\)
made by T cells. IFN-γ is an essential mediator of anti-parasitic mechanisms and mice lacking this cytokine succumb to infection within 10 days due to an inability to control parasite burdens (Suzuki et al., 1988). IFN-γ limits parasite replication by inducing effector mechanisms in macrophages such as the production of reactive nitrogen and oxygen species and promotes the direct killing of *T. gondii* mediated by p47 GTPases and GBPs that help to disrupt the parasitophorous vacuole (Adams et al., 1990, Howard et al., 2011, Murray et al., 1985, Selleck et al., 2013, Yamamoto et al., 2012). IFN-γ also induces the production of indolamine 2,3-dioxygenase in a variety of cell types which catabolizes tryptophan, an essential nutrient for parasitic growth, thus limiting the replication of *T. gondii* (Murray et al., 1989, Pfefferkorn., 1984).

**Adaptive immunity to *T. gondii***

Mice deficient in T and B cells succumb to experimental toxoplasmosis within 2 to 3 weeks (Gazzinelli et al., 1994, Jankovic et al., 2007), underlying the importance of adaptive immunity for resistance to *T. gondii*. Generally speaking, the T cell response is initiated by antigen presenting cells, which can process and present antigens to CD4⁺ and CD8⁺ T cells in the context of MHC class II and I respectively. During *T. gondii* infection, the production of IL-12, results in the development of a TH1 response characterized by the differentiation of CD4⁺ and CD8⁺ T cells that produce IFN-γ. T cell production of IL-2 (predominately by CD4⁺ T cells) drives the clonal expansion of antigen specific T cells. In terms of survival, the relative contribution of CD4⁺ and CD8⁺ T cells to the acute phase of resistance is somewhat redundant, as both populations serve as a source of IFN-γ, mentioned in the previous section. Antibody depletion and T cell
deficient knockout models have demonstrated that mice lacking either CD4+ or CD8+ T cells survive acute infection, but develop increased susceptibility to the chronic phase, where tissue cysts are found predominately in the brain (Denkers and Gazzinelli, 1998, Schaeffer et al., 2009, Vollmer et al., 1987). CD4+ T cells provide an important means of T cell help during the priming of the CD8+ T cell response and development of antibody responses. One means whereby CD4+ T cells provide such help is through their expression of CD40L which can help to license DCs to present antigen to CD8+ T cells (Schoenberger et al., 1998) and specialized populations of TFH cells are critical in driving the germinal center B cell response and the production of antibodies. Indeed, when CD4+ T cells are depleted during the acute phase of infection, mice exhibit defective IFN-γ production by antigen specific CD8+ T cells in the CNS, lower levels of toxoplasma specific antibodies, and ultimately die at approximately 50 days post infection (Denkers and Gazzinelli, 1998, Johnson and Sayles, 2002, Lutjen et al., 2006, Vollmer et al., 1987). The role of CD4+ T cells during chronic infection is perhaps best exemplified by the clinical data from AIDS patients (Luft and Remington, 1992) and studies in mice where CD4+ T cell depletion during the chronic phase of infection resembled human disease with reactivation of parasite replication and increased areas of necrosis (Gazzinelli et al., 1992b, Vollmer et al., 1987).

While CD4+ T cells are implicated in the maintenance of CD8+ T cells and promoting antibody responses, CD8+ T cells are also critical during the chronic phase of infection. Depletion of these cells acutely appears to have limited impact, but depletion of CD8+ T cells during the chronic stage of infection does cause mortality (Gazzinelli et al., 1992b). While CD8+ T cell production of IFN-γ is important in this process,
cytotoxicity also has a role in the control of *T. gondii*. Indeed, perforin knockout mice succumb to chronic infection although their production of IFN-γ is normal (Denkers et al., 1997). Taken together, these studies demonstrate that the active immune pressure of CD4⁺ and CD8⁺ T cells are critical during the chronic phase and are important in maintaining the asymptomatic status of the host.

**Cytokine-mediated immunoregulation during toxoplasmosis**

The previous section highlighted how early studies focused on what immune networks controlled the replication of *T. gondii*, and based on this work it was clear that T cells were critical for the survival of the host in the chronic stage. Paradoxically, there was another body of work demonstrating that depletion of CD4⁺ T cells during the chronic phase ameliorated immune pathology (Israelski et al., 1989). These data were early evidence that T cells could also play pathological roles during this infection. This section will focus on the roles of two cytokines: IL-10 and IL-27 and their critical requirement in limiting T cell-mediated immune pathology during this infection.

IL-10 is a cytokine made by a variety of cell types including T cells, B cells, antigen presenting cells, and NK cells (Moore et al., 2001). Although increased IL-10 production was noted following infection with *T. gondii* (Gazzinelli et al., 1992a, Gazzinelli et al., 1993a, Khan et al., 1995), it was proposed that the parasite induced IL-10 would suppress host-killing mechanisms and promote the ability to establish chronic infection (Sher et al., 1992). However, the essential role of IL-10 in limiting T cell-mediated immune pathology was soon appreciated with studies in the IL-10 knockout mice. Thus, IL-10 knockout mice challenged with *T. gondii* efficiently control parasites
but are unable to limit the magnitude of the pro-inflammatory T cell response and succumb within 2 weeks of a CD4+ T cell-mediated response associated with increased levels of IFN-γ, IL-12 and TNF-α (Gazzinelli et al., 1996). The role of IL-10 in limiting acute T cell responses is similar to later studies looking at the role of IL-27 (discussed extensively in the first part of this chapter). IL-27 was initially characterized as a pro-inflammatory cytokine because it could promote T cell expression of the transcription T-bet and induce T cell proliferation (Pflanz et al., 2002, Takeda et al., 2003). However when IL-27R deficient animals were challenged with T. gondii, and exhibited a phenotype similar to that of the IL-10 knockout mice (Villarino et al., 2003), and it became apparent that this cytokine was a key negative regulator of T cell responses during this infection.

**The role of regulatory T cells during T. gondii infection**

Studies with mice lacking IL-10 or the IL-27R established the critical requirement of these two cytokines in limiting T cell-mediated immune pathology during toxoplasmosis. However, the role of Treg cells during this infection was unclear. Like IL-10 and IL-27, Treg cells can have important roles in dampening T cell responses, but how these were integrated was not completely understood or appreciated for this infection. Work from Sher and colleagues identified a predominant population of CD4+ IL-10+ T-bet+ T cells as the critical source of IL-10 during acute infection (Jankovic et al., 2007), and ruled out a role for Treg cells in this process by using CD25 depleting antibodies (a standard strategy at that time to target these cells). Subsequent data from the Belkaid lab and others, revealed that the frequency of Treg cells is dramatically
reduced following *T. gondii* infection (Benson et al., 2012, Oldenhove et al., 2009). These studies are part of the reason why a role for Treg cells during this infection was not appreciated. Moreover, IL-27 was thought to negatively regulate Treg cell responses, as discussed earlier. This collectively led to the idea that Treg cell functions were limited during *T. gondii* infection. The remainder of this thesis will examine this point in detail. In Chapter 2 studies are presented that will examine the loss of Treg cells during toxoplasmosis and demonstrate that although IL-27 does limit T cell expression of IL-2, this is not responsible for this phenomenon during the acute phase of this infection. In Chapter 3, the role of IL-27 in driving Treg cell heterogeneity and survival is assessed and this section demonstrates that IL-27 acts on Treg cells to promote their function during infection. Moreover, these data highlight that Treg cell are operational during toxoplasmosis. In this section, we also compare the effects the pro-inflammatory cytokine IFN-γ on Treg cells with IL-27 using transcriptional analysis and phenotypic analysis. These data demonstrate that IL-27 plays a non-redundant role in driving Treg cell function at the sites of inflammation. These studies provided the impetus to examine the role of IL-27 on Treg cell expression of a number of inhibitory receptors such as LAG-3, PD-1 and PD-L1 and will be discussed in chapter 4.
## 1.3 Tables

### Table 1.1: The role of IL-27 during infection.

<table>
<thead>
<tr>
<th>Model</th>
<th>Mouse</th>
<th>Phenotype</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td><strong>Influenza</strong></td>
<td>Mixed bone marrow chimeras with IL-27Rα+mice, EBI3^+</td>
<td>IL-27 is required for optimal CD8+ T cell effector response.</td>
<td>(Mayer et al., 2008; Sun et al., 2011)</td>
</tr>
<tr>
<td><strong>Leishmania donovani</strong></td>
<td>IL-27Rα^+</td>
<td>IL-27Rα^+ have enhanced control of the parasite but increased liver pathology due to increased TNF-α and IFN-γ production.</td>
<td>(Rosas et al., 2006)</td>
</tr>
<tr>
<td><strong>Leishmania major</strong></td>
<td>IL-27Rα^−, EBI3^−</td>
<td>Knockouts have initial TH1 defect and increased parasite burden, but no TH1 defect or defect in parasite control at later time-points.</td>
<td>(Yoshida et al., 2001; Zahn et al., 2005)</td>
</tr>
<tr>
<td><strong>L. major</strong></td>
<td>IL-27Rα^−, EBI3^−</td>
<td>Knockouts have increased TH2 response responsible for early susceptibility. Increased lesion size is associated with increased TH17 responses. Normal TH1 and pathogen clearance at later time-points.</td>
<td>(Anderson et al., 2009; Artis et al., 2004a; Zahn et al., 2005)</td>
</tr>
<tr>
<td><strong>L. major</strong></td>
<td>IL-27Rα^−</td>
<td>Enhanced antigen presentation and TH1 differentiation by IL-27Rα^- DCs.</td>
<td>(Wang et al., 2007)</td>
</tr>
<tr>
<td><strong>Listeria monocytogenes</strong></td>
<td>IL-27Rα^−</td>
<td>Defective TH1 and bacterial clearance, lower IgG2a response.</td>
<td>(Chen et al., 2000)</td>
</tr>
<tr>
<td><strong>L. monocytogenes</strong></td>
<td>IL-27Rα^−</td>
<td>IL-27Rα^− TH1 response is normal, but decreased IL-10 production by CD4^+ T cells.</td>
<td>(Batten et al., 2008)</td>
</tr>
<tr>
<td><strong>L. monocytogenes</strong></td>
<td>EBI3^−</td>
<td>TH1 response not impaired, with increased bacterial clearance, and increased TH17 response.</td>
<td>(Yang et al., 2008b)</td>
</tr>
<tr>
<td><strong>Mycobacterium bovis BCG</strong></td>
<td>IL-27Rα^−</td>
<td>No defect in IFN-γ or bacterial clearance, defective granuloma formation in IL-27Rα^−.</td>
<td>(Yoshida et al., 2001)</td>
</tr>
<tr>
<td><strong>Plasmodium berghei</strong></td>
<td>IL-27Rα^−</td>
<td>CD4^+ T cell-mediated liver pathology, enhanced TH1 response, enhanced parasite clearance in the absence of IL-27 signals.</td>
<td>(Findlay et al., 2010)</td>
</tr>
<tr>
<td><strong>Toxoplasma gondii</strong></td>
<td>Mixed bone marrow chimeras with IL-27Rα^+ mice</td>
<td>IL-27 is required for optimal CD8+ T cell effector response.</td>
<td>(Mayer et al., 2008)</td>
</tr>
<tr>
<td><strong>T. gondii</strong></td>
<td>IL-27Rα^−</td>
<td>Acute lethal and pathological TH1 response in the absence of IL-27Rα with enhanced IFN-γ, and parasite control. Increased TH17 responses during chronic toxoplasmic encephalitis.</td>
<td>(Stumhofer et al., 2006; Villarino et al., 2003)</td>
</tr>
<tr>
<td><strong>Trichuris muris</strong></td>
<td>IL-27Rα^−</td>
<td>Enhanced expulsion of parasites, increased production of TH2 cytokines, and increased mast cell activity in the absence of IL-27Rα.</td>
<td>(Artis et al., 2004b)</td>
</tr>
<tr>
<td><strong>Trypanosoma cruzi</strong></td>
<td>IL-27Rα^−</td>
<td>Increased liver pathology and production of the pro-inflammatory cytokines IFN-γ, TNF-α and IL-4.</td>
<td>(Hamano et al., 2003)</td>
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Table 1.2: The role of IL-27 in mouse models of cancer and autoimmunity.

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<th>Model</th>
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<td>Colitis (T cell transfer model)</td>
<td>IL-27Rx⁻/⁻</td>
<td>IL-27 limits Treg populations, IL-27Rx⁻/⁻ transferred T cells induce less severe disease.</td>
<td>(Cox et al., 2011)</td>
</tr>
<tr>
<td>Colitis (T cell transfer model)</td>
<td>IL-27Rx⁻/⁻</td>
<td>IL-27 promotes Treg populations, Treg cells from IL-27Rx⁻/⁻ not protective.</td>
<td>(Kim et al., 2013)</td>
</tr>
<tr>
<td>Proteoglycan-induced arthritis</td>
<td>IL-27Rx⁻/⁻</td>
<td>IL-27 promotes TH1-mediated pathology. Reduced IFN-γ and anti-PG specific antibody in the absence of IL-27Rx.</td>
<td>(Cao et al., 2008)</td>
</tr>
<tr>
<td>Cancer</td>
<td>Tumor over-expresses IL-27</td>
<td>IL-27 promotes NK cell and CTL responses, and tumor regression.</td>
<td>(Chiy et al., 2004; Chiy et al., 2005; Hisada et al., 2004; Salcedo et al., 2004; Shinozaki et al., 2009)</td>
</tr>
<tr>
<td>Cecal ligation and puncture model of sepsis</td>
<td>EBI3⁺⁺/⁻</td>
<td>IL-27 limits neutrophil recruitment and bacterial clearance.</td>
<td>(Laroni et al., 2011; Wirtz et al., 2006)</td>
</tr>
<tr>
<td>Experimental autoimmune encephalitis (EAE)</td>
<td>IL-27Rx⁻/⁻, p28⁻/⁻</td>
<td>IL-27R/IL-27 knockouts have more severe EAE, increased TH17 responses, and decreased IL-10 production.</td>
<td>(Batten et al., 2006; Diveu et al., 2009)</td>
</tr>
<tr>
<td>EAE</td>
<td>IL-27Rx⁻/⁻</td>
<td>IL-27Rx⁻/⁻ TH1 response normal, and decreased IL-10 production by CD4⁺ T cells.</td>
<td>(Batten et al., 2008)</td>
</tr>
<tr>
<td>MRL/pr lupus</td>
<td>IL-27Rx transgenic</td>
<td>Over-expression of IL-27Rα ameliorated disease and limited autoantibody production.</td>
<td>(Sugiyama et al., 2008)</td>
</tr>
<tr>
<td>MRL/pr lupus</td>
<td>IL-27Rx⁻/⁻</td>
<td>Increased TH2-type skin inflammation.</td>
<td>(Kido et al., 2011; 2011)</td>
</tr>
<tr>
<td>OVA-airway inflammation</td>
<td>IL-27Rx⁻/⁻</td>
<td>IL-27Rx⁻/⁻ have increased airway responsiveness, and goblet cell hyperplasia, exacerbated TH1 and TH2 responses and increased serum IgE levels. NKT cell-derived IL-27 limits TH2 responses.</td>
<td>(Fujita et al., 2009; Miyazaki et al., 2005)</td>
</tr>
<tr>
<td>OVA-hapten immunization</td>
<td>IL-27Rx⁻/⁻</td>
<td>IL-27Rx⁻/⁻ have decreased IL-21 production by CD4⁺ T cells, less germinal center B cells, and less hapten-specific antibody production.</td>
<td>(Batten et al., 2010)</td>
</tr>
<tr>
<td>Pristane-induced lupus</td>
<td>IL-27Rx⁻/⁻</td>
<td>IL-27Rx⁻/⁻ have less severe disease, diminished B cell responses, and less auto-antibodies.</td>
<td>(Batten et al., 2010)</td>
</tr>
<tr>
<td>Scurfy-like disease</td>
<td>IL-27p28 and EBI3 double transgenic</td>
<td>Transgenic mice lack peripheral Treg due to suppression of IL-2 production by over-expression of IL-27.</td>
<td>(Tait Wojno et al., 2011)</td>
</tr>
<tr>
<td>Streptozotocin-induced type 2 diabetes</td>
<td>IL-27Rx⁻/⁻, EBI3⁺⁺</td>
<td>Hyperglycemia and pancreatic islet inflammation are increased in the absence of IL-2/IL-27 signals.</td>
<td>(Fujimoto et al., 2011)</td>
</tr>
</tbody>
</table>
1.4 Figures

Figure 1.1

IL-12 family

IL-23
IL-23R

IL-12R

IL-12

IL-27

IL-27R

IL-6

gp130 family

gp130

CLC
CLF

LIF-R

CNTF-R

IL-6R

EBI3

Humanin

Four-helix bundle

Cytokine receptor homology domain

Immunoglobulin domain

Fibronectin-like domain

Humanin peptide

p19

p40

p35
Figure 1.1: Structural relationship of IL-27/IL-27R to the different gp130/IL-12 family of cytokines.

The schematic cytokine and receptor complexes for IL-27, IL-6, Humanin, ciliary neurotrophic factor (CNTF), cardiotrophin-like cytokine (CLC), CLC/CNTFR, cytokine-like factor (CLF)/p28, IL-12, IL-23 and IL-35 are shown. IL-27 is a heterodimer consisting of Epstein-Barr virus-induced gene 3 (EBI3) and IL-27p28. IL-27p28 is a four-helix bundle cytokine (yellow barrels) and resembles other helical cytokines such as IL-6, CLC, IL-12p35 and IL-23p19. EBI3 has an immunoglobulin domain (diamonds) and cytokine receptor homology domain (see legend), and WSXWS motifs (yellow lines) like IL-12/IL-23p40 and has homology to the soluble receptors IL-6Rα, CNTFR and CLF. The heterodimeric IL-27 receptor consists of IL-27Rα (WSX-1, TCCR) and glycoprotein 130 (gp130), both of which are involved in signal transduction; this is in contrast to the IL-6Rα, which lacks intrinsic kinase activity. IL-27Rα has structural homology to gp130 and IL-12Rβ2. Each of the related heterodimeric cytokine receptors consist of one or more subunits with an extracellular cytokine binding domain with WSXWS motifs and fibronectin-like domains (see legend). IL-27Rα is a shared receptor sub-unit of the Humanin and CLF/p28 receptors. EBI3 and IL-12p35 form IL-35.
Figure 1.2

A. The effects of IL-27 on lymphocytes:

- **CD4+ T cell**
  - IL-27 activates T-bet, GATA3, and RORγt, promoting proliferation and Ahr.
  - IL-12p40 and IL-21 upregulated, IL-10 downregulated.

- **CD8+ T cell**
  - IL-27 activates T-bet, promoting proliferation and cytotoxicity.
  - IL-10 and IFN-γ induced.

- **NK cell**
  - IL-17 expression upregulated.
  - IL-10 downregulated.

- **B cell**
  - Antibody secretion enhanced.
  - Proliferation and co-stimulation activated.
  - Isotype switching induced.

B. The effects of IL-27 on granulocytes:

- **Mast Cell**
  - IL-17 upregulated, IL-4 downregulated.
  - TNF-α activates.

- **Eosinophil**
  - IL-17 upregulated.
  - IL-1 upregulated, IL-6 downregulated.

- **Neutrophil**
  - IL-6 downregulated.
  - IL-12p40 upregulated.

C. The effects of IL-27 on antigen presenting cells:

- **Macrophage**
  - IL-10 upregulated, IL-12p40 downregulated.
  - TNF-α upregulated, IL-6 downregulated.

- **Monocyte**
  - TNF-α, IL-10, IL-6 downregulated.

- **Dendritic cell**
  - CD80, CD86 downregulated, PD-L1 upregulated.
**Figure 1.2:** The IL-27 receptor is expressed by a variety of different cell types and can exert pro- and anti-inflammatory effects.

(A) In CD4$^+$ and CD8$^+$ T cells, as well as in natural killer (NK) cells, IL-27 promotes the expression of the transcription factor T-bet and the cytokines IL-10 and IFN-γ. IL-27 also limits production of IL-17 by CD4$^+$ T cells and NK cells. In response to IL-27, B cell subsets increase their proliferation and antibody production.

(B) In mast cells and eosinophils, IL-27 can promote pro-inflammatory responses by increasing expression of IL-1, TNF-α and IL-6, however in neutrophils IL-27 limits cytokine secretion of IL-6 and IL-12p40.

(C) The effects of IL-27 on antigen presenting cells is less well characterized however IL-27 can exert anti-inflammatory effects on macrophage and dendritic cells by decreasing their production of TNF-α and IL-12p40 while promoting the production of IL-10. However, in monocytes IL-27 negatively regulates the expression of IL-10 in response to inflammatory stimuli and following LPS stimulation, IL-27 can promote increased expression of IL-6 and TNF-α.
**Figure 1.3:** The immune response to *Toxoplasma gondii* involves resistance and regulatory mechanisms.

Innate cells such as DCs and macrophages (MΦ) produce IL-12 in response to sensing the parasite. IL-12 is required for T cells to produce pro-inflammatory cytokines such as IFN-γ, which are necessary to induce effector mechanisms that kill *T. gondii*. Immune regulation in the form of cytokines such as IL-10, IL-27, PD-1, and Treg cells are critical to limit T cell-mediated immune pathology.
Chapter 2: IL-27-mediated suppression of IL-2 does not fully account for the loss of Treg cells during infection with *T. gondii*

Abstract

Infection with *T. gondii* leads to changes in Treg cell populations that are characterized by a profound crash in cell numbers associated with infection-induced suppression of IL-2. Since IL-27 is a dominant factor in limiting IL-2 production by T cells in this infection, studies were performed to determine if the loss of Treg cells was a secondary consequence of this IL-27 activity. These studies revealed that although in the absence of IL-27, IL-2 levels were increased in mice infected with *T. gondii*, these mice still exhibited a reduction in Treg cell frequencies similar to WT mice. Additionally these studies revealed that the Treg cells that remained in WT mice expressed low levels of the IL-2Rα (CD25\textsuperscript{LO}) and that this specialized population of Treg cells continued to proliferate, whereas the Treg cells from IL-27 knockout mice had increased expression of IL-2Rα and KLRG1. While treatment with IL-2 complexes led to marked increases in Treg cell numbers this did not alter the overall kinetics of the Treg cell crash. Together, these studies indicate that during acute toxoplasmosis, the reduced production of IL-2 is not sufficient to explain the collapse of Treg cell populations.
Introduction

The cytokine IL-2 is a critical factor for the maintenance of Treg cell populations at steady state and during inflammatory responses (Almeida et al., 2002, de la Rosa et al., 2004, Fontenot et al., 2005, Malek et al., 2002, Setoguchi et al., 2005). In mice where IL-2 signaling is ablated, although Treg cells develop in the thymus, as these animals age, their Treg cell populations are dramatically decreased and these mice succumb to a lethal autoimmune disease characterized by the inflammation of multiple organs, lymphadenopathy, hemolytic anemia and autoantibody production (Sadlack et al., 1995, Suzuki et al., 1995, Willerford et al., 1995). While IL-2 has an important role in the maintenance of Treg cells at steady state, it is also implicated in Treg cell homeostasis during inflammatory responses. Recent studies have shown that during acute infection with several intracellular pathogens where T cell production of IL-2 was suppressed, this suppression was associated with a “crash” in Treg cell populations (Benson et al., 2012, Oldenhove et al., 2009). It has been proposed that this reduction in Treg cell frequencies allows for the emergence of potent effector T cell responses required for the rapid control of pathogens (Belkaid and Tarbell., 2009). At the time these studies were initiated, the cytokine IL-27 had been implicated as a negative regulator of Treg cell activities. Although mice deficient in IL-27Rα have no overt changes in their Treg cell population at steady state, IL-27 transgenic mice (through the ability of IL-27 to suppress T cell production of IL-2) have a profound reduction in Treg cell number (Tait Wojno et al., 2011). Also, as noted in the introduction, other studies suggested that IL-27 blocked Treg cell differentiation (Batten et al., 2008, Cox et al., 2011, Huber et al., 2008, Neufert et al., 2007) although those reports seemed at odds with the suppressive effects of IL-27. During inflammation many questions remain about
the impact of IL-27 on Treg cell phenotype and, given its effects on IL-2 production, we hypothesized that the ability of IL-27 to limit IL-2 underpinned the Treg cell crash.

Based on this hypothesis, a series of studies were performed to understand the effects of IL-27 on infection-induced alterations in Treg cell homeostasis. These data revealed that consistent with previous findings, infection with *T. gondii* led to a loss in Treg cell populations. In the absence of IL-27 there was increased IL-2 but despite this, there was still a crash in Treg cells. Mice that lacked IL-27p28, IL-27 EBI3, IL-27Rα or WT mice treated with IL-27R blocking antibodies exhibited similar decreases in Treg cell frequency as WT controls. Further analysis revealed that a proliferating population of CD25LO (IL-2RαLO) Treg cells was present in infected WT animals. Moreover when naïve mice and mice infected with *T. gondii* were given IL-2 complexes to expand Treg cells, the loss of Treg cell populations was comparable to control mice in sites of inflammation. Together these studies indicate that in this setting, IL-27-mediated suppression of IL-2 is not sufficient to explain the infection-induced changes in Treg cell homeostasis.

**Results**

*IL-2 production by effector CD4+ T cells is suppressed by IL-27p28 and IL-27 Ebi3 during infection with T. gondii.*

Studies in *Il27ra−/−* mice demonstrated that signals through this receptor are required to limit effector cell production of IL-2 during infection with *T. gondii* (Villarino et al., 2006); however, the role of the individual IL-27 subunits p28 and EBI3
in limiting IL-2 had not been examined. To characterize the production of IL-2 following infection with *T. gondii*, WT, *Il27*−/− and *Ebi3*−/− mice were infected with Me49 tissue cysts by the intraperitoneal (i.p.) or oral route. Consistent with previous reports (Chan et al., 1986, Oldenhove et al., 2009), following infection with *T. gondii*, WT CD4+ T cells produced 2-fold less IL-2 than naïve CD4+ T cells upon restimulation with PMA and ionomycin (Figure 2.1a and data not shown). Furthermore, CD4+ T cells from *Il27*−/− and *Ebi3*−/− animals did not show a significant decrease in IL-2 production by CD4+ T cells following infection in the same experiment (Figure 2.1a, b). To further assess T cell IL-2 production from animals that lack IL-27, cells from infected WT or knockout mice were restimulated with αCD3 or soluble *Toxoplasma* antigen (STAg) and IL-2 production was measured in culture supernatants by ELISA. Whereas cells from infected WT mice did not produce detectable levels of IL-2, robust IL-2 production by T cells from infected IL-27 knockout mice was detected in these cultures (Figure 2.1C).

*The increased IL-2 production in IL-27 knockout mice does not prevent changes in Treg cell frequency following infection with T. gondii.*

Since the suppression of IL-2 observed during acute toxoplasmosis has been proposed to be responsible for the collapse of Treg cell populations, and IL-2 production is increased in IL-27 knockout mice (Figure 2.1), it was hypothesized that the loss of Treg cells during infection would be reversed in the absence of IL-27. To test this, WT and *Il27*−/− mice were infected orally or i.p. with Me49 cysts. Following oral infection, compared to naïve mice at day 5 postinfection, there was no decrease in Treg cell frequency or numbers (Figure 2.2A,B), but by day 8, there was a reduced frequency of
Treg cells in the tissues examined (Figure 2.2A). This change also resulted in a loss of Treg cell numbers in most organs analyzed except in the lamina propria lymphocytes (LPL) (Figure 2.2B), and there were no obvious differences between WT and IL-27 knockout mice. Of note, although the number of Treg cells did appear to increase in the LP in this particular experiment, this result was variable. The loss of Treg cells was also similar between WT and IL-27 knockout animals following i.p. infection (Figure 2.2C) demonstrating that this process is not dependent on the route of infection. Moreover, the inability of the increased IL-2 present in the knockout mice to restore Treg cell frequencies is not a consequence of aberrant Treg cell development in the absence of IL-27 because similar results were observed in infected WT mice which received IL-27Rα blocking antibodies (Figure 2.2D).

**CD25LO Treg cells are present at steady state and continue to proliferate during infection with T. gondii.**

Since the infection-induced reduction of Treg cells occurred in the IL-27 knockout mice despite the presence of increased IL-2 it was possible that changes in the ability of these cells to respond to IL-2 could explain this paradox. Therefore, to determine if Treg cell expression of the IL-2Rα changes following infection, Treg cells from naïve mice and from mice infected with *T. gondii* were examined. Analysis of CD25 expression revealed that at steady state, 20-40% of Treg cells in the spleen, lymph node (LN), and peritoneum expressed low levels of the IL-2Rα (CD25LO) and this did not significantly change following infection (Figure 2.3 A, B). Analysis of the proliferation of these Treg cells by their expression of Ki67 revealed that naïve CD25LO Treg cells had
equivalent Ki67 expression as CD25^{hi} cells with approximately 15-25% of Treg cells in the spleen and the lamina propria expressing this protein (Figure 2.3C). Following infection, both CD25^{hi} and CD25^{lo} populations increased their expression of Ki67 to a similar degree, such that 30-40% of Treg cells expressed Ki67 (Figure 2.3C). These data suggest that although IL-2 expression is suppressed following infection with T. gondii, the Treg cells in these mice continue to proliferate and this does not appear to correlate with alterations in CD25 expression.

_Treg cells from infected IL-27 deficient mice exhibit a phenotype characteristic of exposure to IL-2._

In this study, the assays used to evaluate T cell production of IL-2, such as restimulation of T cells ex vivo, likely overestimated the levels of bio-available IL-2 in vivo (Finkelman et al., 2003). Rudensky and colleagues have reported that upon exposure to IL-2, Treg cells increase their expression of CD25 and the Killer cell Like Receptor Group 1 (KLRG1) (Fontenot et al 2005). In order to assess if the elevated IL-2 in the IL-27 knockout mice impacted Treg cell phenotype, Treg cells were analyzed following infection, from WT and IL-27 knockout mice. Whereas in WT mice, Treg cells did not significantly modify their expression of the IL-2Rα following infection (p > 0.05) (Figure 2.3A), Treg cells from mice which lacked IL-27 or IL-27 signals had a marked upregulation of their expression of CD25 and KLRG1 (Figure 2.4A). Furthermore, the Treg cells from IL-27 knockout mice expressed significantly higher frequencies of CD25^{+} and KLRG1^{+} cells, which is characteristic of exposure to high levels of IL-2 (Figure 2.4B). This is consistent with the observed increase in IL-2 in these infected animals and
suggests that Treg cells in these mice received IL-2 signals. Of note, IL-27Rα−/− Treg cells did not have significant increases in their CD25+ KLRG1+ populations, and this may point to a role for alternative cytokines that use this receptor subunit (Figure 1.1). Although IL-12 can promote CD8+ T cells to express KLRG1 (Yap et al., 2000) and IL-12 is elevated in the IL-27 knockout mice, it is not clear if this also contributes to this phenotype in observed in IL-27 knockout Treg cells. Given that the Treg cells from IL-27 knockout mice are lost to a similar degree as Treg cells from WT mice following infection, these data indicate that despite these IL-2 signals, these populations are still subject to decreased survival and maintenance following infection with *T. gondii.*

*Treatment with IL-2/anti-IL-2 complexes does not reverse the Treg cell crash during infection with *T. gondii.**

Previous studies have demonstrated that IL-2/anti-IL-2 complexes (IL-2C) that specifically expand Treg cells (Boyma et al., 2006) may reverse the infection-induced loss of Treg cells in mice challenged with *T. gondii* (Benson et al., 2012, Oldenhove et al., 2009). In order to determine if following oral infection IL-2C had a similar effect on Treg cell populations in this study, naïve mice and mice infected with *T. gondii* were treated with IL-2C and their Treg cell population frequencies were examined. Consistent with previous studies, treatment of naïve mice with IL-2C resulted in a two to three-fold increase in Treg cell frequency (Figure 2.5A). Surprisingly, although IL-2 did expand Treg cell frequencies in naïve and infected mice, the overall loss of Treg cells was similar in treated and untreated infected mice with an approximate four-fold decrease in Treg cells in the LP and two to three-fold decrease in the spleen regardless of IL-2C treatment.
(Figure 2.5B), which is consistent with the Treg cell crash observed in the IL-27 knockout mice.

**Discussion**

The phenomenon of the Treg cell crash following *T. gondii* infection was described in a number of studies and has been attributed to reduced availability of IL-2. However, the work presented here demonstrates that suppression of IL-2 does not fully explain this process, as IL-2C treatment did not appear to fully restore the loss of Treg cells. While we cannot fully explain the disparate results between this study and previous findings from other groups (Benson et al., 2012, Oldenhove et al., 2009) we interpret the IL-2c experiments cautiously. These experiments have only been repeated twice and further investigation into the kinetics of the loss of Treg cells following IL-2c treatment will help to clarify any inconsistencies between these data and other reports. Nonetheless, in our other experimental systems where the infection-induced loss of IL-2 was not apparent, such as infection in *Il27r−/−, Ebi3−/−, Il27ra−/−* mice or mice treated with IL-27R blocking antibodies, there was a profound loss of Treg cells similar to WT or control treated infected mice. Increased apoptosis was associated with the Treg cell crash in previous studies (Oldenhove et al., 2009) and this is consistent with our preliminary experiments demonstrating that activated caspase 3 expression increased in Treg cells following infection in WT and IL-27 knockout mice (AOH, data not shown). IL-2 was not sufficient to rescue Treg cell populations in these mice, suggesting that other mechanisms such as sustained TCR stimulus or other pro-inflammatory signals mediate the loss of the vast majority of Treg cells during *T. gondii* infection.
Treg cells at steady state exhibit heterogeneity in their expression of CD25, and there have been reports of CD25- Foxp3+ cells (Fantini et al., 2004, Nishioka et al., 2006), but it is not clear from these studies whether this indicates that different subsets of Treg cells differ in their signaling capacity in response to IL-2. Future experiments on sorted Treg cell populations could address whether CD25LO cells are refractory to IL-2 stimulation, or can become CD25HI Treg cells. To determine if enhanced IL-2 signaling was occurring in mice in which IL-2 was not suppressed during infection, we attempted to isolate Treg cells from WT and IL-27 knockout infected mice to look at basal pSTAT5 levels in these different IL-2 environments. These preliminary experiments were technically challenging but suggested that basal pSTAT5 was increased in the IL-27 knockout mice following infection (AOH, data not shown). Nonetheless, the high levels of KLRG1 and CD25 on Treg cells isolated from IL-27 knockout mice suggest that these cells received IL-2 signals in vivo, but that this was not sufficient to prevent the Treg cell crash.

The observation that a population of Treg cells that has low levels of the IL-2Rα continues to proliferate following infection suggests that factors other than IL-2 can contribute to homeostasis of these cells during infection. At steady state the IL-2Rα chain is required for Treg cell development and homeostasis (Yu et al., 2009a) but the requirement of cytokines other than IL-2 has not been completely examined during inflammatory responses. However, these data suggest that other factors drive these Treg cells to proliferate independently of IL-2. Candidate cytokines related to IL-2 include IL-7 and IL-15, which could be responsible for the continued proliferation of these cells following infection, and the mean fluorescence intensity (MFI) of IL-2Rβ, IL-2Rγ and
IL-7R all increased slightly in Treg cells in *T. gondii* infected mice (AOH, data not shown). Additionally, the antigen specificity of proliferating Treg cells from infected mice is not known but Class II tetramers could be used to evaluate whether or not TCR stimulus could also be important in the continued proliferation of these cells. Preliminary experiments using oral infection with OVA expressing parasites and mice that had received adoptively transferred OT-II cells demonstrated that these Treg cells expanded following infection (AOH, data not shown). Additionally, mice do recover their Treg cell populations after the resolution of the acute phase of infection (AOH, data not shown), and whether or not these cells arise from the population of continuously proliferating Treg cells or new thymic output is not known. However one report suggests that thymic Treg cells are resistant to the Treg cell “crash” during *T. gondii* infection (Benson et al., 2012).

Although the initial hypothesis leading up into this study - that IL-27-mediated suppression of IL-2 was the cause of the Treg cell crash during toxoplasmosis - was not validated by these experiments, these data did reveal that the remaining Treg cells in these infected mice were enriched for TH1 transcription factors, and led to new questions as to the function of the remaining Treg cells and whether they were influenced by the TH1-associated cytokines during infection. These factors will be addressed in the subsequent chapters of this thesis.
**Figure 2.1 IL-27 suppresses IL-2 production during T. gondii infection.**

(A and B) CD4+ T cells (gated on live/dead-, CD8a-, CD4+, Foxp3-) from the spleens of naïve or i.p. T. gondii infected WT or IL-27 knockout mice were analyzed for their expression of IL-2 following re-stimulation with PMA/ionomycin in the presence of Golgi inhibitors. (B) The mean percentage ± SEM of IL-2+ CD4+ T cells. The experiment has four mice per group,* p < 0.05. Data representative of > 3 independent experiments.

(C) IL-2 was measured by ELISA in the supernatants of splenocyte cultures taken from d10 T. gondii infected mice following 72 hours of restimulation in the presence of α-CD3 or soluble *Toxoplasma* antigen (STAg).
**Figure 2.2** Treg cells crash in the absence of IL-27 during acute *T. gondii* infection.

(A and B) Cells were isolated from the spleen, mLN, Peyer’s patches, and LP of naïve and *T. gondii* orally infected WT and *Il27*−/− mice. The mean ± SEM frequency (A) and number (B) of Treg cells (gated on live/dead−, TCR−β+, CD4+, Foxp3+ cells). 3 mice per group, data is representative of > 3 independent experiments.

(C) Cells were isolated from the peritoneum (PECs) of naïve or i.p. *T. gondii* infected WT or knockout mice as indicated and analyzed for their frequency of Treg cells 2 weeks postinfection. 4 mice per group, data is representative of > 3 independent experiments.

(D) Mice were treated i.p. with 200µg/mouse of anti-WSX-1 blocking antibodies to IL-27R every other day starting on day 0 until sacrifice following oral infection with *T. gondii*. 3 mice per group, spleen shown. Data is from one experiment.
Figure 2.3 CD25LO Treg cells are present at steady state and continue to proliferate during infection with T. gondii.

(A) Cells were isolated from the spleen, LN, and PECs of naïve and i.p. T. gondii infected WT mice. The frequency of Treg cells that are CD25HI or CD25LO (gated on live/dead-, CD8α-, CD4+, Foxp3+ cells). Data is representative of > 3 independent experiments.

(B) Frequency of CD25LO Treg cells in individual mice. Naïve n=3, infected n=10.

(C and D) Cells were isolated from the spleen or LP of naïve or orally T. gondii infected WT mice and analyzed for their mean ± SEM frequency of Ki67+ CD25HI Treg or Ki67+ CD25LO cells day 9 postinfection. Naïve n=1, infected n= 4 mice per group, data is representative of > 2 independent experiments.
Figure 2.4 Treg cells from infected IL-27 deficient mice exhibit a phenotype characteristic of exposure to IL-2.
Cells were isolated from the PECs (A) and spleen (B) of naïve and i.p. T. gondii infected WT mice or knockout mice as indicated. Data is representative of at least 2 independent experiments. WT, Ebi3−/− n=4; Il27ra−/−, Il27−/− n=3.

(A) The mean ± SEM mean fluorescence intensity (MFI) of CD25 (left panel) or KLRG1 (right panel) in Treg cells (gated on live/dead−, CD8α−, CD4+, Foxp3+ cells).
(B) The mean ± SEM frequency of CD25+ KLRG1+ of Treg cells in infected mice (left panel), representative FACs plots (right panel). **p < 0.01, ***p < 0.001.
Figure 2.5 IL-2c treatment does not reverse the Treg crash during *T. gondii* infection. (A and B) Cells were isolated from the LP and spleen of naïve and orally *T. gondii* infected mice; ratIgG or IL-2C treatment was given every 3 days starting at day 0. Representative of 2 experiments, n=3. (A) FACs plots of Treg cells from naïve and infected LPL (left panel) and spleen (right panel), gated on CD4+ TCR-β+ CD8- cells. (B) The mean ± SEM frequency of Treg cells in naïve and infected mice treated with ratIgG or IL-2C.
Chapter 3: The cytokines Interleukin 27 and Interferon-γ promote distinct Treg cell populations required to limit infection-induced pathology during TH1 responses

Abstract

IFN-γ promotes a population of T-bet+ CXCR3+ Treg cells that limit TH1 cell-mediated pathology. Our studies demonstrate that IL-27 also promoted expression of T-bet and CXCR3 in Treg cells. During infection with Toxoplasma gondii a similar population emerged which limited T cell responses and were dependent on IFN-γ in the periphery but IL-27 at mucosal sites. Transfer of Treg cells ameliorated the infection-induced pathology observed in Il27−/− mice and this was dependent on their ability to produce IL-10. Microarray analysis revealed that Treg cells exposed to either IFN-γ or IL-27 have distinct transcriptional profiles. Thus, IFN-γ and IL-27 have different roles in Treg cell biology and IL-27 is a key cytokine that promotes the development of Treg cells specialized to control TH1 cell-mediated immunity at local sites of inflammation.

Introduction

IL-27 is a member of the IL-6 and IL-12 family of cytokines. Early studies described it as an inducer of the TH1 cell associated transcription factor T-bet, which enhances TH1 differentiation (reviewed in Chapter 1). However, IL-27 is also an antagonist of inflammation associated with TH1, TH2 and TH17 cell responses in
multiple settings (Stumhofer and Hunter., 2008) and the regulatory properties of IL-27 can be explained in part by its ability to limit IL-2 production, antagonize TH2 and TH17 cell responses and promote T cell production of IL-10. However, questions remain about the mechanisms used by IL-27 to limit immune pathology associated with TH1 responses, reviewed in (Stumhofer and Hunter., 2008, Yoshida and Miyazaki., 2008).

CD4+ T cells that express the transcription factor Foxp3, are an important means of immune suppression. Recent studies have demonstrated that during inflammation, specialized populations of Treg cells emerge that express transcriptional profiles similar to their effector counterparts (Esposito et al., 2010, Fujimoto et al., 2010, Koch et al., 2009). It has been suggested that this heterogeneity allows for regulation of specific types of immunity. For example, Treg cell expression of STAT3 is critical for limiting TH17 cell responses (Chaudhry et al., 2009), while expression of IRF4 allows control of TH2 cells (Zheng et al., 2009). During infections dominated by TH1 cells, Treg cells express Tbx21 and Cxcr3, genes associated with the presence of IFN-γ and expression of T-bet is required for their survival and proliferation (Koch et al., 2009). Whereas IFN-γ has been implicated in the development of this specialized Treg cell population, whether other environmental cues influence this program are unclear.

A subset of natural Treg cells (nTreg) expresses high levels of the IL-27Rα (Villarino et al., 2005). Paradoxically, there are reports that IL-27 is a direct antagonist of Treg cell conversion (Cox et al., 2011, Huber et al., 2008, Neufert et al., 2007, Stumhofer et al., 2007a). The data presented here reveal that IL-27 directly promotes Treg cell expression of T-bet and CXCR3. In mice challenged with T. gondii, or other intracellular pathogens, a population of Treg cells emerged that express T-bet, CXCR3
and IL-10, and limited T effector responses. In mice that lack IL-27, this population is reduced at primary sites of infection but not at peripheral sites, such as in the spleen, where IFN-γ had a more prominent role. However, transcriptional profiling highlighted that IL-27 appeared to have a more dominant impact than IFN-γ on Treg expression of immunosuppressive genes such as Il10. Together, these studies identify distinct roles for IL-27 and IFN-γ in driving the T-bet+ subset of Treg cells, which are specialized to control regional pathology during TH1 cell responses.

**Results**

*IL-27 promotes the expression of T-bet and CXCR3 in inducible Treg cells and natural Treg cells.*

Although previous studies have demonstrated that 30-40% of nTreg cells express the IL-27Rα, it was unclear whether this receptor was functional in Foxp3+ CD4+ T cells. In this study, we define nTreg cells as Foxp3+ CD4+ T cells isolated from mice, and “inducible” Treg cells as Foxp3+ CD4+ Treg cells generated from Foxp3+ CD25 precursors in vitro (iTreg). To address whether nTreg cells respond to IL-27, naïve CD25+ T cells or Foxp3GFP+ cells were incubated with IL-27. Whereas unstimulated cells had negligible amounts of pSTAT1 or pSTAT3, IL-27 induced pSTAT1 and pSTAT3 in 30-40% of nTreg cells, (Figure 3.1A). Similarly, IL-27 induced pSTAT1 and pSTAT3 in 50-70% of iTreg cells (Figure 3.1B). It is notable that in Treg cells, IFN-γ and IL-10 also activate STAT1 and STAT3 respectively, but this was less than with IL-27 (Figure 3.S1A). It is also relevant to note that previous reports have suggested that IL-27 antagonizes iTreg cell development (Cox et al., 2011, Huber et al., 2008, Neufert et al., 2007, Stumhofer et al., 2007a) and in our experiments the frequency of Treg cells were initially reduced in the
presence of IL-27, but Treg cells were generated and their numbers increased over time (Figure 3.S1B, C). Together, these data suggest that existing and emerging Treg cell responses can be influenced by IL-27 and that IL-27 can actually promote Treg cell expansion.

Because IL-27 induces the expression of T-bet in effector CD4+ T cells, studies were performed to determine if IL-27 had a similar effect on Treg cells. When naïve Foxp3- CD4+ T cells were used to generate iTreg cells, those cultured in the presence of IL-27 expressed elevated levels of T-bet (Figure 3.S1D). When Treg cells were differentiated in the presence of IL-27 plus α-IL-4 and α-IFN-γ (neutral conditions), it still promoted Treg cell expression of T-bet (Figure 3.1C). Thus, independent of its ability to promote IFN-γ, IL-27 promotes Treg cell expression of T-bet. It is notable that long-term TCR signaling was associated with the eventual up-regulation of T-bet in cultures of Treg cells, although the highest amounts of T-bet were always observed in the presence of IL-27 (Figure 3.S1E).

Previous studies established that activation of T-bet in Treg cells promotes CXCR3 expression (Koch et al., 2009), a chemokine receptor involved in lymphocyte migration during TH1 responses (Lord et al., 2005). When iTreg cells were generated with IL-27, there was a consistent 4-5-fold increase in CXCR3 levels and nTreg cells incubated with IL-27 for 24 to 48hr expressed high levels of T-bet and CXCR3 (Figure 3.1C). Similarly, while iTreg cells stimulated with PMA/ionomycin did not produce IL-10 or IFN-γ those generated in the presence of IL-27 expressed IFN-γ and IL-10 (Figure 3.1D). Together, these data demonstrate that IL-27 promotes cytokine production by
Treg cells and influences their proliferation, survival, and chemokine receptor expression.

IL-27-driven expansion of CXCR3⁺ Treg cells requires STAT1 and T-bet.

Because IL-27 activates STAT1 and STAT3, experiments were performed to determine which pathway contributed to the up-regulation of T-bet and CXCR3 in iTreg cells. As noted earlier, addition of IL-27 to the cultures led to the induction of T-bet in 15-30% of the iTreg cells, and a modest but consistent increase in T-bet was observed in the Stat3⁻/⁻ CD4⁺ T cells (Figure 3.S1F). However, in the Stat1⁻/⁻ Treg cell cultures, IL-27 did not induce T-bet (Figure 3.1E). In addition to T-bet, Eomesodermin (Eomes) has been shown to promote CXCR3 expression (Intlekofer et al., 2008) and polyclonal expansion of naïve cells CD4⁺ T cells in the presence of IL-27 led to increased levels of Eomes and T-bet, but in iTreg cells IL-27 did not promote Eomes (Figure 3.S1G). This finding is consistent with the idea that TGF-β1 (required for the iTreg cell cultures) suppresses Eomes but not T-bet (Narayanan et al., 2010). Moreover, Treg cells generated with IL-27 from Eomes⁻/⁻ T cells showed up-regulation of CXCR3, similar to WT Treg cells (Figure 3.1F), whereas Tbx21⁻/⁻ iTreg cells had impaired up-regulation of CXCR3 (Figure 3.1F). These data suggest a model in which the ability of IL-27 to activate STAT1 drives T-bet and CXCR3 expression in a subset of iTreg cells.
**IL-27 treatment increases Treg cell expression of T-bet and CXCR3 in vivo.**

To assess the effects of IL-27 on the phenotype of Treg cells in vivo, hydrodynamic gene delivery was used to administer DNA plasmid “minicircles” that express IL-27 (IL-27MC) (Chen et al., 2005). Mice that received IL-27MC or GFP vector-only controls were sacrificed after 4 weeks for analysis of their T cell populations. No pathology was noted in either experimental group (data not shown) and while there was increased cellularity associated with IL-27MC (Figure 3.S2A), there was no decrease in Treg cell frequency (Figure 3.S2B). While total splenic CD4^+ T cells from mice given IL-27MC had higher amounts of T-bet (Figure 3.S2C), further analysis revealed that, consistent with the ability of IL-27 to promote a population of T-bet^+ CXCR3^+ Treg cells in vitro, only the Foxp3^+ Treg cell population had higher T-bet and CXCR3 expression (Figure 3.2A-D).

**Infection with T. gondii induces an immunosuppressive T-bet^+ CXCR3^+ Treg cell population.**

Challenge with *T. gondii* results in the development of CD4^+ effector T cells that produce IFN-γ and IL-10 (Jankovic et al., 2007, Roers et al., 2004). Analysis of Treg cells from infected mice following re-stimulation with PMA/ionomycin, and/or directly ex vivo from IL-10 Vert-X reporter mice revealed that these cells were also a source of IFN-γ and IL-10 (Figure 3A, B). Following re-stimulation, a portion of these *T. gondii*-induced IL-10^+ Treg cells coexpressed IFN-γ as well as T-bet and CXCR3 (Figure 3.3C-E). Further analysis of BrdU incorporation and Ki67 expression revealed that infection promoted proliferation of these cells (Figure 3.S3A, B). The appearance of CXCR3^+ T-bet^+ Treg cells
coincided with the emergence of a population of cells that produced IL-27p28. While conventional DCs (CD11c\textsuperscript{hi} Class-II\textsuperscript{+}) produced IL-12p40 in the spleen and the LPL population, IL-27p28 production was the highest in the LPL and was made by monocytes and macrophages (CD11b\textsuperscript{+} CD11c\textsuperscript{lo}) (Figure 3.3F).

To determine if Treg cells contributed to the control of the TH1 cell response during toxoplasmosis, DEREG mice were utilized to deplete Treg cells, and analysis of effector cell proliferation and cytokine production was performed. This treatment resulted in approximately 70% loss of Treg cells (Figure 3.S3C) and an increased frequency of Ki67\textsuperscript{+} effector CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells (Figure 3.S3D). This effect was accompanied by increased T-bet and IFN-γ expression in CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in response to STAg (Figure 3.3G, Figure 3.S3E). Thus, the Treg cells present in infected mice limited the TH1 effector cell response to \textit{T. gondii}.

\textit{The role of IL-27 in regulating Treg cell populations during multiple TH1 infections.}

To determine if IL-27 was involved in generating Treg cell heterogeneity during TH1 responses, \textit{Il27}\textsuperscript{−/−} mice were challenged orally with \textit{T. gondii} and their Treg cell populations were analyzed. In these experiments the Foxp3\textsuperscript{+} CD4\textsuperscript{+} effector (CD44\textsuperscript{hi}) T cells from wildtype or \textit{Il27}\textsuperscript{−/−} mice expressed equivalent levels of T-bet or CXCR3 (Figure 3.4A) but several Treg cell populations from the \textit{Il27}\textsuperscript{−/−} mouse had reduced expression of T-bet, CXCR3 and IL-10 (Figure 3.4B-D, Figure 3.S3F). Despite the systemic levels of IFN-γ and IL-12 (Figure 3.4E), a striking deficiency in T-bet\textsuperscript{+} CXCR3\textsuperscript{+} Treg cells was observed in the gut associated lymphoid tissue (GALT). In contrast, at peripheral sites like the spleen, these populations were not affected, consistent with the increased expression of
IL-27 observed in the gut versus the spleen (Figure 3.3F). To determine whether IL-27 had a similar role in other models, WT or Ebi3^-/- mice were infected with Leishmania major or Salmonella typhimurium. These infections also induced T-bet^+ CXCR3^+ Treg cells and in the absence of IL-27 there was a defect in Treg cell expression of T-bet and CXCR3 (Figure 3.S3G, H). These results indicate a dominant role for IL-27 in Treg cell polarization at sites of ongoing TH1 cells responses.

Acute lethality in T. gondii infected Il27^-/- mice is rescued by transfer of Treg cells.

To test whether the infection-induced CD4^+ T cell-mediated pathology in Il27^-/- mice could be ameliorated by Treg cells, WT and Il27^-/- mice were infected with T. gondii, and at days 4, 7 and 10 postinfection, mice were provided with iTreg cells. This regimen was chosen because Treg cell homeostasis is altered during T. gondii infection due to increased Treg cell death (Oldenhove et al., 2009), and repeated transfer of Treg cells alleviates inflammation in several models (Darrasse-Jeze et al., 2009, Grainger et al., 2010, Mor et al., 2007, Zheng et al., 2006). While infected Il27^-/- mice developed immune pathology and succumbed to acute infection, those that received IL-27-conditioned Treg cells, or Treg cells generated under neutral conditions, were rescued (Figure 3.4F). This effect was associated with reduced numbers of effector T cells and decreased serum alanine aminotransferase (ALT) (Figure 3.S4A-C), suggesting that the transferred Treg cells limit effector cell expansion and pathology. Compared with the endogenous Treg cells, the Treg cells transferred into infected Il27^-/- mice expressed higher levels of IL-10, IFN-γ, T-bet, CXCR3, and CTLA-4 and had increased proliferation as measured by Ki67 (Figure 3.S4D-G). When Treg cells were transferred into naïve
mice, Foxp3 expression was not retained whereas in infected mice transferred cells sustained Foxp3 expression at sites of inflammation (Figure 3.S4G), suggesting that environmental cues maintain these cells. Moreover, when adoptive transfer experiments were conducted with Treg cells generated from Il10−/− mice, recipient animals succumbed rapidly to infection, indicating that IL-10 is required for Treg cell-mediated rescue of Il27−/− mice (Figure 3.4F). These data establish that Treg cells can ameliorate the pathology observed in the Il27−/− mice and are consistent with a model in which Il27−/− mice infected with T. gondii have an underlying Treg cell defect, which contributes to the development of immune pathology. Nevertheless, these data have to be interpreted cautiously. The ability of neutral Treg cells to rescue these mice may be attributed to their eventual acquisition of T-bet and CXCR3 noted following TCR stimulation (Figure 3.S1E). However, because neutral Treg cells could promote the survival of Il27−/− mice, Il27ra−/− and Ifngr1−/− iTreg cells were generated and transferred into infected Il27−/− mice to determine if expression of these receptors was required for protection (Figure 3.S5A). These studies revealed that both cytokine receptors were required to rescue Il27−/− mice and suggest that although IL-27 is required for Treg cell expression of T-bet and CXCR3 at the sites of inflammation, signals through IFN-γR1 also contribute to the rescue of these mice.

**Differential effects of IFN-γ and IL-27 on Treg cells.**

Although our studies have focused on the effects of IL-27 on Treg cells, our own data (Figure 3.S5A) indicate that IFN-γ also has a role in these events and Treg cells from the spleens (but not the gut) of infected mice have high expression of IFN-γR1 and IFN-
γR2 (Figure 3.S5B). To directly address the role of IFN-γ in generating Treg cell diversity during toxoplasmosis, infected mice were treated with α-IFN-γ starting 3 days postinfection. Neutralization of IFN-γ did not alter basal expression of T-bet or CXCR3 in Treg cells from naïve mice but in infected mice there was a significant decrease in levels of T-bet and CXCR3 in the spleen (Figure 3.5A, B). In contrast, there was no effect of IFN-γ depletion on Treg cell expression of T-bet and CXCR3 in the GALT of infected mice (Figure 3.5A, B). Furthermore, following re-stimulation, Treg cell production of IL-10 and IFN-γ was not lower in the spleen or the LPL in the absence of IFN-γ suggesting that IFN-γ is not critical for Treg cells production of these cytokines (Figure 3.5C, D).

These results suggest that the effects of IFN-γ are prominent at peripheral sites whereas the effects of IL-27 are most apparent at the local sites of inflammation.

To better understand the differential effects of IFN-γ and IL-27 on Treg cells, a series of studies were performed to directly compare their signaling and their effects on Treg cell differentiation and transcriptional responses. When Treg cells were stimulated with IFN-γ or IL-27, both cytokines induced pSTAT1 but only IL-27 activated STAT3 and STAT5 (Figure 3.6A, B). However, although the kinetics of pSTAT1 signaling was similar, IL-27 induced markedly elevated amounts of pSTAT1 (Figure 3.6A, B). Next, the effects of IFN-γ and IL-27 on Treg cells were compared in vitro. By day 5 of iTreg cell culture, IFN-γ and IL-27 treatment resulted in increased T-bet and CXCR3 expression but IL-27 had a more profound effect (Figure 3.6C-E). In nTreg cell cultures, treatment with IL-27 resulted in higher T-bet expression compared to IFN-γ treatment although the cytokine-induced expression of CXCR3 was similar (Figure 3.6G). Perhaps the most notable
difference was that while both IL-27 and IFN-γ-conditioned Treg cells produced IFN-γ, only the IL-27-conditioned Treg cells made IL-10 (Figure 3.S6A).

Finally, expression profiling of Treg cells generated during IL-27 and IFN-γ treatment was performed. To obtain sufficient cells for analysis and ensure a uniform starting population, iTreg cells were generated under neutral conditions and then cultured in neutral conditions, or with IFN-γ or IL-27 for 10 and 48 hours. Microarray analysis identified 185 genes that were differentially expressed compared to neutral controls, either early (10hr) or late (48hr) following treatment (p value ≤ 0.05). Hierarchical clustering identified groups of genes that showed similar expression patterns across the three treatments and two time-points, and revealed three distinct clusters that are regulated in different ways by IL-27 compared to IFN-γ (Figure 3.7, Figure 3.S6B). Cluster 1, (23 genes) was enriched for metabolic functional categories, and is suppressed by IL-27 while remaining relatively unchanged in the presence of IFN-γ (Figure 3.7A, B). In contrast, Cluster 2 (17 genes) is induced by IL-27, but not IFN-γ (Figure 3.7C, D). This cluster contains two subgroups: the first includes 10 genes induced early by IL-27 but were either not induced by IFN-γ, or weakly induced at 10 hours and were not sustained to 48 hours (Figure 3.7D). This subgroup includes Tbx21, Il10, Lag3, Ccr5, Il12rb1 and Il12rb2 highlighting a group of genes involved in T cell activation that are co-coordinately regulated by IL-27. The second subgroup includes 7 genes that are not affected early by either cytokine but are induced at 48hr by IL-27 (Figure 3.7D).

Finally, a third cluster of 45 genes was identified that is enhanced by IL-27 and IFN-γ, but for which IL-27 was a more potent inducer (Figure 3.7E, F). This cluster includes many canonical STAT1 target genes, further demonstrating the ability of IL-27 to act as
an activator of STAT1-dependent gene transcription in Treg cells. Thus, while IFN-γ and IL-27 can give rise to a phenotypically similar population of Treg cells, these two cytokines have distinct transcriptional effects on Treg cells that are indicative of a more complex biology and may reflect the regional effects observed in vivo.

Discussion

In the last decade, there has been a growing appreciation of the inhibitory properties of IL-27 in the setting of autoimmunity and inflammation (Stumhofer and Hunter., 2008, Yoshida and Miyazaki., 2008). Indeed, studies with Il27ra−/− mice infected with T. gondii have identified a role for IL-27 in limiting the production of IL-2, antagonizing TH17 cells and promoting effector cell production of IL-10 (reviewed in Chapter 1). In early studies it did not appear that the enhanced inflammation observed in these mice was a consequence of a defective Treg cell response, as IL-27 did not alter Treg cell activity in suppressor assays, nor are there any overt differences in the frequency of Treg cell populations in Il27ra−/− mice (Villarino et al., 2003; and this study). The data presented here highlight that the ability of IL-27 to promote a specialized population of Treg cells contributes to its suppressive activities in multiple experimental models and indicate that this is one of the many pathways that limit overt T cell-mediated inflammation in this model (Aliberti et al., 2002, Bhadra et al., 2011, Buzoni-Gatel et al., 2001, Gazzinelli et al., 1996).

It has been suggested that Treg cells have a limited role during toxoplasmosis (Jankovic et al., 2007) but the Treg cell depletion studies presented here indicate that these cells are relevant. The finding that Treg cell transfers can reverse the infection-
induced pathology observed in Il27/- mice provides additional support for the idea that Treg cells are operational during toxoplasmosis. However, these transfer experiments have to be interpreted carefully, and illustrate how understanding the mechanism by which adoptively transferred Treg cells suppress in vivo remain elusive. Although our system clearly shows that Treg cells need to express Il27ra, Ifngr1 and Il10 to rescue Il27/- mice, it is unclear how these signals are integrated. Since the transfer of activated Treg cells can rescue the Il27/- mice, it implied that signals other than IL-27 (such as IFN-γ or TCR stimulus) could be important. However, given these data, if IFN-γ or TCR were sufficient to contribute to Treg cell function in vivo we would expect that it could contribute to protection upon adoptive transfer of Il27ra/- Treg cells, which is not the case. We cannot rule out a role for IL-27Rα independently of IL-27 and/or gp130 as the IL-27Rα has the capacity to signal via JAK1-STAT1 independently of gp130 (Pradhan et al., 2007, Takeda et al., 2003). Moreover, the IL-27Rα is also a component of the receptors for cytokine-like factor-1 (CLF-1) and humanin, and there is an alternatively spliced form of IL-27Rα, which may be involved in trans-signaling (Figure 1.1). Thus, it remains to be determined if the IL-27-independent properties of IL-27Rα play a role in Treg cell function during infection. Alternatively, our mRNA expression data (Figure 3.S6C) suggest the possibility that Treg cells may also produce IL-27 implying that autocrine signaling through IL-27Rα may be important for Treg cells function.

Nevertheless, these observations raise fundamental questions about whether this TH1-like Treg cell population is derived from nTreg cells or iTreg cells, whether these cells are specific for Toxoplasma and how IFN-γR1, IL-27Rα and IL-10 contribute to their function in vivo.
Although the emphasis of these studies has been on the role of IL-27 during infection, our findings imply that IL-27 and IFN-γ act in distinct sites during infection, perhaps a consequence of local differences in the cytokine environment. However, despite the systemic elevation of IFN-γ present in infected Il27−/− mice, there is still a defect in the generation of T-bet+ Treg cells at sites of inflammation, indicating a critical role for IL-27 in mediating Treg cell expression of T-bet and CXCR3. Additionally, the ability of IL-27, but not IFN-γ, to promote the expression of IL-10 in Treg cells, highlight distinct functions of these cytokines.

The observation that in naïve mice only a subset of Treg cells expresses the IL-27 and IFN-γ receptors raises the question of whether these Treg cells subsets are “hard-wired” to deal with specific types of inflammation. Indeed, little is known about the heterogeneity in Treg cell expression of cytokine receptors, and whether this predicts their capacity to influence distinct types of inflammation. In the context of trying to understand Treg cell heterogeneity, microarray analysis of Treg cells isolated from different anatomical sites revealed that distinct subsets of Treg cells exist with non-overlapping transcriptional profiles (Feuerer et al., 2009a, Feuerer et al., 2010). Similarly, when we compared the transcriptional profiles of Treg cells treated with IFN-γ and IL-27, these data revealed at least three clusters of genes that are differentially regulated. The largest cluster consists of many known STAT1 target genes, reinforcing the notion that IL-27 is a potent inducer of STAT1-mediated transcription. It is unclear how the specific STAT1 genes that we have identified as IL-27 targets might contribute to Treg cell activity but these data will aid in the selection of candidates for future studies of Treg cell function. In addition, we have identified a subset of genes that are specifically
induced by IL-27 but not IFN-γ, providing candidate mediators for IL-27-specific Treg cell function. For instance, we found that Ly6c1 is strongly induced by IL-27. Although its role in Treg cell biology has not been addressed, it has been implicated in the function and homing of effector T cells (Jaakkola et al., 2003, Marshall et al., 2011). These data also highlight that examining a select few phenotypic markers (such as T-bet and CXCR3), may over-simplify the complex heterogeneity that exists in Treg cells during infection.

Many studies have defined how factors such as IL-2, TGF-β and Foxp3 have a prominent role in the homeostasis and function of Treg cells (Apostolou et al., 2008, Bayer et al., 2007, Fontenot et al., 2005). While there is good evidence that an ongoing immune response can limit Treg cell function and differentiation (Caretto et al., 2010, Mantel et al., 2007, Pasare and Medzhitov., 2003, Wei et al., 2007), recent reports indicate that by utilizing the same transcription factors as their effector counterparts, Treg cells may become specialized to operate in distinct inflammatory environments (Chaudhry et al., 2009, Koch et al., 2009, Lu et al., 2010, Zheng et al., 2009). These findings have led to models in which environmental cues promote the development of specialized Treg cell subsets. The finding that IL-27 promotes a specialized subset of Treg cells delineates a unique pathway by which they are influenced by the inflammatory environment. These findings may be directly relevant to human disease and it is notable that a loss of Treg cells has been observed in human patients with inflammatory bowel disease (IBD) (Eastaff-Leung et al., 2010) and a recent report linked a polymorphism in the IL-27p28 loci, associated with reduced production of IL27 transcripts, to increased susceptibility to IBD (Imielinski et al., 2009). The finding that IL-27 can profoundly
influence Treg cell populations in the gut may offer a partial explanation for the susceptibility of these particular patients to IBD.
Figure 3.1 IL-27 treatment of Treg cells induces STAT1 and STAT3 phosphorylation and the expansion of a STAT1-dependent T-bet\(^+\) CXCR3\(^+\) population.

(A and B) Natural Treg (nTreg) cells harvested from naïve mice and (B) inducible Treg (iTreg) cells generated in vitro (as described in the Experimental Procedures) were stimulated with IL-27 or media alone and phosphorylated STAT1 and STAT3 were measured by flow cytometry. Plots depict the mean percentage ± standard error of the mean (SEM) of Treg cells (gated on live/dead\(^-\), CD4\(^+\), Foxp3\(^+\) cells) expressing pSTAT1 or pSTAT3.

(C) nTreg cells or iTreg cells were cultured in the presence of neutralizing antibodies to IFN-\(\gamma\) and IL-4 (neutral conditions) and in the presence or absence of IL-27. Following 48 hours of culture, the expression of T-bet and CXCR3 was measured by flow cytometry. Plots depict the percentage of positive Treg cells (number inside gate) and the geometric mean channel fluorescence (MFI) to the left of the gate.

(D) iTreg cells were cultured in the presence or absence of IL-27 for 72 hours and subsequently re-stimulated with PMA/ionomycin in the presence of BFA and monensin Golgi inhibitors for 5 hours. The production of IFN-\(\gamma\) and IL-10 by iTreg cells was measured by flow cytometry.

(E) iTreg cells were generated from wildtype (WT) and Stat1\(^-/-\) deficient T cells in the presence or absence of IL-27 for 72 hours. Plots depict the mean percentage ± SEM of Treg cells that expressed T-bet or CXCR3.

(F) iTreg cells were generated from WT, Tbx21\(^-/-\), and CD4-Cre x Eomes\(^C^d\) mice in the presence or absence of IL-27 for 48 hours. The percentage (inside plot) and MFI (outside plot) of Treg cells expressing CXCR3 was measured by flow cytometry. All plots are representative of three independent experiments with 3 replicates per group. See also Figure 3.S1.
Figure 3.2 Ectopic expression of IL-27 increases Treg cell expression of T-bet and CXCR3. 

(A-D) WT mice were injected by hydrodynamic tail vein delivery control (eGFP) or IL-27 minicircles (IL-27MC) and examined 4 weeks after treatment. 

(A and B) T-bet (A) and CXCR3 (B) expression by Treg cells (gated on live/dead-, CD4+, Foxp3+ cells) and non-Treg T cells (gated on live/dead-, CD4+, Foxp3- cells) isolated from the spleen were measured by flow cytometry. 

(C and D) The percentage of Treg (C) and non-Treg (D) cell expression of T-bet and CXCR3 from individual mice. Data are representative of two independent experiments with five mice per group, *p < 0.05, **p < 0.01. See also Figure 3.S2.
Figure 3.3 Treg cells have a TH1 phenotype and suppress effector T cell IFN-γ-production during toxoplasmosis.

(A and B) Treg cells (gated on live/dead-, TCR-β⁺, CD4⁺, Foxp3⁺) were isolated from the lamina propria (LPL) of naïve or T. gondii infected WT mice and analyzed for their expression of IFN-γ following re-stimulation with PMA/ionomycin in the presence of Golgi inhibitors (A) or directly ex vivo without re-stimulation from Vert-X IL-10-eGFP reporter mice (B).

(C) LPL Treg cells from WT T. gondii infected mice co-express IFN-γ and IL-10 following re-stimulation with PMA/ionomycin in the presence of Golgi inhibitors.

(D) T-bet and CXCR3 expression by Treg cells isolated from the mesenteric lymph nodes (mLN) of naïve (shaded histogram) or T. gondii infected mice (solid line).

(E) The mean percentage ± SEM of T-bet and CXCR3 expressing Treg cells in the mLN of naïve and infected mice are depicted. Data are representative of > 5 experiments, n=5, * p < 0.05.
**Figure 3.3** Treg cells have a TH1 phenotype and suppress effector T cell IFN-γ-production during toxoplasmosis (continued).

(F) Cells were isolated from the spleen and LPL of naïve and infected mice and incubated with Golgi inhibitors for 6-8 hours. IL-12p40 and IL-27p28 expression was measured in live/dead-, CD19-, B220-, NK1.1-, CD3- cells that are CD11b<sup>+</sup>CD11c<sup>int</sup> (top panel) or CD11c<sup>hi</sup> MHC Class II<sup>hi</sup> (bottom panel).

(G) WT and DEREG mice were infected with *T. gondii* and treated with diphtheria toxin (DT) on days 2-9 of infection. On day 9 postinfection, T cells were isolated from the lamina propria and spleen by CD90.2 expression and cultured with media alone, dendritic cells, or dendritic cells pulsed with STAg. Treg cells (gated on live/dead-, TCR-β<sup>+</sup>, CD4<sup>+</sup>, Foxp3<sup>+</sup> cells) were stained for T-bet and IFN-γ expression. These data are representative of 3 experiments, n ≥ 3 per experiment. See also Figure 3.S3.
Figure 3.4 TH1 Treg cell development during T. gondii infection is IL-27-dependent and iTreg cells can rescue acute pathology in Il27−/− mice.

(A-D) Cells were isolated from the spleen, mLN, Peyer’s patches, and LPL of naïve and T. gondii infected WT and Il27−/− mice. T-bet and CXCR3 expression were measured on non-Treg CD4+ T cells (gated on live/dead−, TCR-β+, CD4+, Foxp3− cells) (A) and Treg cells (gated on live/dead−, TCR-β+, CD4+, Foxp3+ cells) (B), Peyer’s patches shown. The MFI of Treg cell T-bet (C) and CXCR3 (D) expression from individual mice are shown for all tissues. Data are representative of > 5 experiments, * p < 0.05, ** p < 0.01, ***p < 0.001.
**Figure 3.4** TH1 Treg cell development during *T. gondii* infection is IL-27-dependent and iTreg cells can rescue acute pathology in Il27−/− mice (continued).

(E) Levels of IL-27p28, IL-12p40, and IFN-γ were measured in the serum of WT and Il27−/− mice on day 9 postinfection by ELISA.

(F) WT and Il27−/− mice were infected i.p. with *T. gondii* and received PBS or 2-4 × 10^6* WT Treg cells cultured in neutral conditions (2 experiments, n=5), WT Treg cells cultured with IL-27 (TH1 Treg cells) (4 experiments, n=10), or Il10−/− Treg cells cultured with IL-27 (2 experiments, n=8). Mice received Tregs on day 4, 7, and 10 postinfection. See also Figure 3.S4.
Figure 3.5 IFN-γ promotes the TH1 Treg cell phenotype in the periphery, but not sites of inflammation.

(A-D) Mice were infected with T. gondii and treated with anti-IFN-γ or control antibodies (rat IgG) on day 3, 5, 7, and 9 postinfection.

(A) On day 10, cells were isolated from the spleen and LPL of naïve and infected mice and T-bet and CXCR3 expression by Treg cells were measured by flow cytometry (gated on live/dead-, TCR-β+, CD4+, Foxp3+ cells).

(B) Plots depict the MFI of T-bet and CXCR3 expression by Treg cells (presented as mean ± SEM), * p < 0.05, ** p < 0.01.

(C) Spleen and LPL cells were re-stimulated with PMA/ionomycin in the presence of Golgi inhibitors and analyzed for IL-10 and IFN-γ expression within Treg cells.

(D) Plots depict the frequency of Treg cells expressing IL-10 and IFN-γ following re-stimulation. Plots are representative of 2 experiments, n=5. See also Figure 3.S5.
Figure 3.6 IFN-γ and IL-27 have distinct effects on Treg cells.
(A) Naïve Treg cells were isolated from naïve mice and treated with media alone, IFN-γ, or IL-27. Levels of pSTAT1, 3, and pSTAT5 were measured over time by flow cytometry. Results were normalized to media control. Plots are representative of 2 experiments, n=2.
(B) Treg cells were generated under neutral conditions for seven days and stimulated as in (A).
(C-E) Treg cells were generated under neutral conditions or with IL-27 or IFN-γ (without blocking antibodies to IFN-γ and IL-4) and T-bet and CXCR3 expression was measured on day 5 (C) in replicate cultures (D) and over time (E). Plots are representative of three experiments, **p < 0.01, ***p < 0.001.
(F and G) Treg cells were isolated and cultured under neutral conditions or treated with IFN-γ or IL-27 and monitored for T-bet and CXCR3 expression on day 5 of culture (F) and over time (G). Plots are representative of 3 independent experiments. See also Figure 3.S6.
**Figure 3.7** IL-27 induces a gene expression profile in Treg cells that is distinct from the profile induced by IFN-γ.

(A-F) iTreg cells were cultured under neutral conditions for seven days, harvested, and then exposed to neutral, IL-27 or IFN-γ Treg culture conditions for 10 or 48 hours. mRNA was isolated and microarray analysis was performed as described in the experimental procedures. Hierarchical clustering analysis was performed on genes that were differentially regulated 1.5 fold or greater, compared to neutral conditions, by either IL27 or IFN-γ after 10hr or 48hr culture, p < 0.05. Three clusters of genes are shown as heatmaps. (A) Genes down-regulated most strongly by IL-27; (C) genes up-regulated by IL-27 but not IFN-γ; and (E) genes induced by both cytokines, but to a greater degree by IL-27. Heatmap color indicates log2 expression value. Clusters are also represented by line graphs showing the fold changes in gene expression (B, D, F). Each line shows gene expression changes for a single gene. Several genes had more dramatic changes in expression at either the 10 hr or 48 hr timepoints and thus were grouped as sub-group 1 “early” and sub-group 2 “late”, respectively. See also Figure 3.36.
Supplemental Figures

A. Media, IFN-γ, IL-27, IL-10, IFN-γ + IL-27

B. IL-2, IL-27

C. Foxp3+ CD4+ Cells (%)

D. Foxp3+ CD4+ Cells (#)

E. Day 6 culture: Neutral, IFN-γ, IL-2, IL-27

F. Wildtype, Stat1−/−, CD4-Cre x Stat3fl/fl, Wildtype, Stat1−/−, CD4-Cre x Stat3fl/fl

G. 24 Hours neutral, neutral + IL-27

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**Figure 3.S1 IL-27 promotes the generation and expansion of TH1 Treg cells in vitro.**

(A) Natural Treg (nTreg) cells (gated on live/dead−, CD4+, Foxp3+ T cells) were rested and stimulated with indicated cytokines at 50ng/mL for 15-20 minutes at 37°C, followed by immediate fixation and analysis for pSTAT1 and pSTAT3 expression.

(B) CD25− CD4+ T cells were isolated from naïve WT mice and cultured under inducible Treg (iTreg) cell culture conditions (TGF-β, IL-2, αCD3, and αCD28) with or without the indicated cytokines or blocking antibodies to IFN-γ and IL-4 (neutral). The frequency of iTreg cells was measured at 48 hours (left panel) and over time (right panel) (gated on live, CD4+, Foxp3+, CD25+ T cells), shown as percentage of total CD4+ T cells.

(C) Graphs depict the total number (left) and fold expansion (right) of iTreg cells in each culture condition in the presence or absence of IL-27 at day 5.

(D) Graph depicts the number of Tbet+ iTreg cells (gated on live, CD4+, Foxp3+, T-bet+ T cells) at day 5 in various iTreg culture conditions (TGF-β, αCD3, αCD28, with or without the indicated cytokines and blocking antibodies).

(E) Representative FACs plots of T-bet and CXCR3 expression by iTreg cells on day 6 are shown for neutral and IL-27 iTreg cell cultures compared to Treg cells isolated from naive mice.

(F) CD25- CD4+ T cells were isolated from naïve WT, Stat1−/− and CD4-Cre x Stat3fl/fl mice and cultured under iTreg cell differentiation conditions for 72 hours. The frequency of iTreg cells (left panel, gated on live, CD4+ T cells) and iTreg cells expression of T-bet (right panel, gated on live, CD4+, CD25+, Foxp3+ T cells) was measured by flow cytometry.

(G) CD4+ T cells were stimulated with αCD3, αCD28, IL-2, αIFN-γ and αIL-4 with or without IL-27 (left panel), and with TGF-β (right panel), and expression of Eomes was measured by flow cytometry on day 5. All data are representative of >3 independent experiments.
Figure 3.S2 IL-27 minicircle treatment.

(A) Cellularity in the liver and spleen was enumerated following IL-27MC or control MC (eGFP) treatment at 4 weeks.

(B and C) The frequency of Foxp3+ CD4+ T cells (B) and total CD4+ T cell expression of T-bet was measured by flow cytometry at 4 weeks post MC treatment. Data are representative of 2 independent experiments, (*, p < 0.05; **, p < 0.01).
**Figure 3.S3** Characterization of Treg cells during multiple TH1 infections.

(A) BrdU incorporation by T-bet+ Treg cells was measured following *T. gondii* infection by flow cytometry, LPL shown from naïve and day 9 infected mice, (gated on Foxp3+, CD4+, live/dead- cells).

(B) Treg cell expression of Ki67 following oral *T. gondii* infection (gated on Foxp3+, CD4+, live cells), day 9 shown.

(C-F) WT or DEREG mice were infected orally with 10 cysts of ME49. Two days postinfection, DT was administrated daily until day 9 to deplete CD4+Foxp3+ Treg cells.

(C) Treg cell frequencies in spleen, MLN and LP compartments at day 6 postinfection (day 4 of DT treatment).

(D) Cells were stained for TCR-β, CD4, CD8, Foxp3, and Ki67 to determine effector T cell proliferation after Treg cell depletion.
Figure 3.S3 Characterization of Treg cells during multiple TH1 infections (continued).

(E) Purified CD90.2\(^+\) T cells were re-stimulated overnight with dendritic cells and STAg. Golgi transport inhibitor was added for the last 6 hours. Cells were stained for TCR-\(\beta\), CD4, Foxp3, T-bet and IFN-\(\gamma\). Dot plots illustrate T-bet and IFN-\(\gamma\) intracellular staining of CD8\(^+\) T cells from DEREG mice in spleen and LP compartments. Error bars represent the SDs of the means of 3 individual mice. Experiment shown was performed 3 times, (\(^*\), \(p < 0.05\); \(\ast\), \(p < 0.01\); \(\ast\ast\), \(p < 0.001\)).

(F) Treg cell production of IL-10 from WT and Il27\(^{-/-}\) mice acutely infected i.p. with T. gondii was measured following re-stimulation with PMA/ionomycin in the presence of Golgi inhibitors for 5 hours, spleen shown. Data are representative of 3 experiments.

(G) WT or Ebi3\(^{-/-}\) mice were inoculated intradermally into the ear with 10\(\mu\)L of PBS containing 1x10\(^6\) L. major promastigote metacyclics and monitored weekly for lesion size. Mice were sacrificed 6 weeks postinfection. Ear Treg cell expression of T-bet and CXCR3 was examined by flow cytometry. N=5, (\(^*\), \(p < 0.05\); \(\ast\), \(p < 0.01\)).

(H) WT and Ebi3\(^{-/-}\) mice were infected with 1X10\(^8\) Salmonella typhimurium bacteria by oral gavage. Treg cell expression of T-bet and CXCR3 in gut tissues were analyzed by flow cytometry 12 days postinfection, Peyer’s patches shown. N=4, (\(^*\), \(p < 0.05\)).
Figure S4 Adoptively transferred iTreg limit effector cell expansion.

(A-C) II27-/- mice were infected i.p. with 20 cysts ME49 and given i.v. PBS or TH1 iTreg as described in the Experimental Procedures and analyzed day 14 postinfection, n=5 per group.

(A) The effector cell phenotype and cytokine production of CD4+ Foxp3- T cells, Foxp3+ CD4+ Treg cells, and CD8+ T cells in T. gondii infected II27-/- mice given PBS or TH1 Treg cells was determined by flow cytometry directly ex vivo (CD62L, CD44, T-bet) or following re-stimulation with PMA/ionomycin in the presence of Golgi inhibitors for 5 hours (IFN-γ), spleen shown.

(B) CD4+ Foxp3- T cell and CD8+ T cell counts were enumerated from the pooled lymph nodes (LNs) and spleens of recipient animals.

(C) Serum alanine aminotransferase levels were measured from the serum of recipient animals on day 0, day 5, and 10-postinfection (n=5).
Figure 3.54 Adoptively transferred iTreg limit effector cell expansion (continued).

(D-G) Fate of transferred TH1 Treg cells. 4x10⁶ (CD45.1⁺) TH1 Treg cells were generated as described in the Experimental Procedures and transferred into naïve Il27-/- mice or Il27-/- mice that had been infected 4 days previously with 20 cysts ME49 T. gondii i.p., n=5 per group.

(D) Phenotype of TH1 Treg cells was determined prior to adoptive transfer by flow cytometry, (gated on live, CD4⁺ T cells).

(E) IL-10 and IFN-γ expression by transferred (CD45.1⁺) and endogenous (CD45.2⁺) Treg cells was determined following re-stimulation with PMA/ionomycin for 5 hours in the presence of Golgi inhibitors.

(F) Phenotype of transferred (open histogram) versus endogenous (shaded histogram) Treg cells, (gated on CD25⁺ Foxp3⁺ CD4⁺ live cells) spleen shown.

(G) Expression of Foxp3 in the transferred cells was determined by flow cytometry, (gated on CTLA4⁺ Foxp3⁺ CD4⁺ live cells) spleen and mLN shown.
Figure 3.S5 IFN-γR1 and IL-27Rα expression by adoptively transferred iTreg cells is required to rescue *T. gondii* infected *Il27*−/− mice.

(A) *Il27*−/− mice were infected with *T. gondii* and received PBS or 2-4 x 10^6 WT Treg cells cultured in conditions (4 experiments, n=15), *Il27ra*−/− Treg cells cultured with IL-27 (2 experiments, n=13), or *Ifngra*−/− Treg cells cultured with IL-27 (2 experiments, n=19). Mice received Tregs on day 4, 7, and 10 postinfection, **p < 0.01, ***p < 0.001.

(B) Spleen and mLN Treg cell expression of IFN-γR1 and IFN-γR2 was determined by flow cytometry on cells isolated from naive and *T. gondii* infected WT and *Il27*−/− mice, day 9 postinfection.
Figure 3.S6 Comparison of IL-27 and IFN-γ-conditioned iTreg cells.
(A) Expression of IL-10 and IFN-γ by iTreg cells in each culture condition were measured by flow cytometry on day 7 of following re-stimulation with PMA/ionomycin in the presence of Golgi inhibitors for 5 hours (gated on live, CD4+, Foxp3+ cells). Data are representative of 2 independent experiments. (B) Gene ontology (GO) enrichment analysis from microarray-based expression profiling of IL-27 or IFN-γ-conditioned Treg cells. Fold enrichment is shown for each GO term. The number at the end of each bar indicates number of genes associated with each term. For description of gene clusters, see hierarchical clustering analysis in Figure 7. (C) Log2 expression values of Ebi3 and Il27 were determined by microarray analysis.
Chapter 4: IL-27 promotes Treg cell expression of inhibitory receptors

Abstract

Multiple inhibitory receptors that regulate T cell activation have emerged as critical modulators in infectious, cancer and autoimmune settings, but little is known about the factors that regulate their expression. IL-27 has emerged as an activator of Treg cells and transcriptional analysis of Treg cells revealed that IL-27 selectively induced a unique signature of inhibitory molecules (Ly6c, TIM-3, CD200R, LAG-3, Protein S, PD-1 and PD-L1). In vitro and in vivo studies confirmed that IL-27 treatment promoted Treg cell expression of these molecules, while studies using mice infected with Toxoplasma gondii showed that endogenous IL-27 was also important in this process. These findings identify a key role for IL-27 in coordinating Treg cell expression of receptors involved in limiting inflammatory processes.

Introduction

Numerous studies have demonstrated the importance of diverse inhibitory receptors, such as CTLA-4, PD-1, PD-L1, TIGIT, LAG-3, CD200R, TIM-3, Ly6c and PROS1, as a means of limiting T cell activation in the context of chronic infection, anti-tumor immunity, and autoimmune responses (Carrera Silva et al., 2013, Joller et al., 2012, Keir et al., 2008, Odorizzi and Wherry., 2012, Peggs et al., 2009). Effector T cell expression of some of these receptors (PD-1, LAG-3, TIM-3, CTLA-4) is linked to the
process of “exhaustion”, and their blockade can lead to augmented effector responses (Butler et al., 2012, Curran et al., 2010, Fourcade et al., 2010, Peggs et al., 2009, Woo et al., 2012). This information now forms the basis for therapies that target inhibitory receptors in order to augment the immune response and thereby promote anti-viral and anti-tumor responses (Bettini et al., 2011, Fourcade et al., 2010, Golden-Mason et al., 2009, Jones et al., 2008, Keir et al., 2008, Monney et al., 2002, Odorizzi and Wherry, 2012, Okazaki et al., 2011, Sanchez-Fueyo et al., 2003, Woo et al., 2012). Consistent with the concept of immune exhaustion, repeated antigenic stimulation is one mechanism that leads to increased expression of inhibitory receptors such as PD-1, TIM-3 and LAG-3 by lymphocytes (Agata et al., 1996, Fourcade et al., 2010, Golden-Mason et al., 2009, Huang et al., 2004, Petrovas et al., 2007). Treg cell populations also have a prominent role in limiting many types of immune response, and their ability to express LAG-3, TIM-3, PD-1 and PD-L1 been linked to the inhibition of T cell effectors (DiPaolo et al., 2007, Francisco et al., 2009, Gupta et al., 2012, Huang et al., 2004). However, little is known about whether Treg cells coordinate the expression of multiple inhibitory receptors or the factors that influence inhibitory receptor expression.

Several reports have highlighted the idea that environmental cues, such as IFN-γ, can lead to the specialization of Treg cells to limit different classes of inflammation (Campbell and Koch., 2011, Chaudhry and Rudensky., 2013). For example, IL-27 is a major stimulus for T cell production of IL-10 (Awasthi et al., 2007, Batten et al., 2008, Fitzgerald et al., 2007, Stumhofer et al., 2007b) and directly inhibits TH2 and TH17 cells (Kastelein et al., 2007), but IL-27 also promotes the development of a T-bet+ CXCR3+ Treg cell population during multiple infections that limits immunopathology (Chapter
Recent work has also demonstrated that IL-27 promotes effector CD4$^+$ and CD8$^+$ T cells to express PD-L1 (Hirahara et al., 2012). The studies presented here reveal that IL-27 stimulates Treg cells to express a unique signature of genes encoding inhibitory receptors including Protein S, TIM-3, LAG-3, CD200R, Ly6c, PD-1, and PD-L1 and that IL-27 treatment, in vitro and in vivo promoted the expression of these immunosuppressive proteins, in addition to the molecule TIGIT. Moreover, endogenous IL-27 was required for optimal Treg cell expression of multiple inhibitory receptors during infection with the parasite *T. gondii*. Together, these data identify IL-27 as a central regulator of multiple inhibitory mechanisms utilized by Treg cells to limit inflammation.

**Results**

*IL-27 induces the transcription of genes encoding inhibitory receptors in Treg cells.*

To investigate the mechanisms by which IL-27 could promote Treg cell activity, microarray data generated previously by this laboratory (GSE38686) was analyzed to determine the relative expression of inhibitory receptors on Treg cells under neutral conditions versus those that had been exposed to IL-27 or IFN-γ (Chapter 3). These data revealed that genes encoding the inhibitory molecules Ly6c, LAG-3, PD-1, PD-L1, TIM-3, CD200R, and Protein S were all induced by IL-27 and, to a lesser extent, by IFN-γ (Figure 4.1A, B). In contrast, other genes involved in Treg cell function such as *Foxp3* were relatively unchanged by IL-27 treatment (data not shown). *Lag3, Pdcd1*, and *Cd274* were selectively induced early by IL-27, at 10 hours post stimulation (Figure 4.1A and data not shown), but not by Treg cells generated under neutral conditions or treated
with IFN-γ. Whereas for genes encoding Ly6c, TIM-3, CD200R and Protein S, their highest expression was observed preferentially in the IL-27 treated Treg cells at 48 hours (Figure 4.1B, and data not shown). To test whether IL-27 treated Treg cells resemble exhausted T cells, gene set enrichment analysis (GSEA) was used to compare our arrays with Immunological Signatures Collection of the Molecular Signatures Database (MSigDB) (Subramanian et al., 2005). This collection includes microarray datasets of many immunological signatures, including those of T cells with gene signatures of exhaustion such as occurs during chronic LCMV infection (West et al., 2011, Wherry et al., 2007). This analysis revealed that 46 signatures were enriched two-fold or greater (P < 0.05) in IL-27 treated Treg cells, and were related to T cell activation and STAT1 signaling, but not to exhausted effector T cells (data not shown). These data suggest that IL-27 Treg cells are a novel population of Treg cells distinct from exhausted T cells

In vitro treatment of Treg cells with IL-27 leads to their expression of inhibitory molecules.

To investigate whether the ability of IL-27 to promote gene expression of inhibitory receptors was also observed at the protein level, various Treg cell populations were treated with different cytokines and the expression of these molecules was assessed by flow cytometry. In vitro differentiated iTreg cells were generated from naïve CD4+ T cells using IL-2 plus TGF-β in the presence of IL-27 or IFN-γ, with or without the presence of cytokine neutralizing antibodies. IL-27 treatment resulted in Treg cells that expressed an increased frequency and MFI of PD-1, PD-L1, Ly6c, LAG-3, TIGIT, and CD200R (Figure 4.2A). For most of the markers tested, IFN-γ could also induce the
expression of these molecules, however IL-27 consistently induced a higher frequency and MFI of these receptors than IFN-γ treatment with the exception of PD-L1, where it was equivalent. Moreover, although IL-27 can promote IFN-γ (Pflanz et al., 2002), the neutralization of IFN-γ did not abrogate the effects of IL-27 on inhibitory receptor expression (data not shown).

It is notable that although the microarray data indicated that IL-27 promoted TIM-3 (Figure 4.1A) this was not observed in the experiments described in Figure 4.2A. However, similar to the microarray conditions, when a homogeneous starting population of iTreg cells were restimulated under neutral, IFN-γ or IL-27 conditions, and then cells were restimulated in the presence of anti-CD3 and IL-27 or IFN-γ, TIM-3 was more robustly upregulated in the presence IL-27 (Figure 4.2B). Furthermore, FACS sorted Foxp3GFP+ or column sorted CD25+ Treg cells isolated directly ex vivo from the lymph nodes of naïve mice also demonstrated a similar induction of the inhibitory molecules PD-1, PD-L1, Ly6c, LAG-3, TIGIT, and CD200R following stimulation in the presence of IL-27 after 48 hours (data not shown). One of the novel targets of IL-27 in the microarray was the surface receptor Protein S that has recently been described as a potent inhibitor of effector T cell responses (Carrera Silva et al., 2013). The detection of Protein S is technically challenging and requires acetone fixation, which precluded co-analysis of multiple inhibitory receptors at the same time. Nonetheless, analysis of protein S expression by Treg cells revealed that relative to neutral treated Treg cells, IL-27 promoted Protein S expression on approximately 20% of Treg cells, whereas IFN-γ only had a modest effect (Figure 4.2C).
Given that IL-6, IL-10, IFN-α and IL-12 have also been reported to influence Treg cell populations (Bettelli et al., 2006, Chaudhry et al., 2011, Koch et al., 2012, Lee et al., 2012, Oldenhove et al., 2009) experiments were performed to determine if these cytokines could also influence Treg cell expression of inhibitory receptors. These data revealed that while these cytokines had no effect on the expression of Protein S, TIM-3, PD-1, PD-L1, TIGIT, or CD200R. However, IL-12 and IFN-α could promote a modest increase in Ly6c and LAG-3 but not to the extent of IL-27 alone, and there was no synergistic increase when either IL-12 or IFN-α were added to Treg cells in the presence of IL-27 (data not shown).

While IL-27 was able to induce the expression of multiple inhibitory receptors on Treg cells it was unclear which ones were coexpressed by subsets of Treg cells. To do this, Simplified Presentation of Incredibly Complex Evaluations (SPICE) analysis of iTreg cells generated in the presence of IL-27 was used. This analysis revealed that IL-27 promoted more heterogeneity and coexpression of the inhibitory receptors PD-1, PD-L1, LAG-3 and Ly6c than iTreg cells generated in the presence of IFN-γ (Figure 4.2D). This IL-27-induced heterogeneity was not lost when IFN-γ was neutralized, and there was not an additive effect having IFN-γ present in IL-27 cultures (data not shown). SPICE analysis also revealed that IL-27 produced a LAG-3+ PD-1+ PD-L1+ Ly6c+ population of Treg cells that was largely absent in the other cultures (Figure 4.2D).
In vivo expression of IL-27 promotes Treg cell expression of multiple inhibitory proteins.

At steady state Treg cells express high levels of the IL-27 receptor alpha and appear poised to respond to IL-27 (Villarino et al., 2005). Therefore, to assess the effects of IL-27 on existing Treg cells, mice were either treated with IL-27MC or pegylated IL-27 (IL-27pg) to determine the effects of IL-27 treatment in vivo on Treg cell expression of inhibitory receptors. Administration of IL-27pg resulted in increased Treg cell expression of PD-1, PD-L1, LAG-3, TIGIT and Protein S by approximately two-fold (Figure 4.3 A, B). When IL-27MC were used, a similar increase in Treg cell expression of inhibitory receptors was observed with Treg cell expression of LAG-3, Ly6c, PD-1 and TIM-3 increasing two to three-fold relative to control MC treatment (Figure 4.3C), (of note, at the time of this thesis dissertation, these experiments are still ongoing and not all markers (CD200R, Protein S, TIGIT) were included in both the IL-27pg and IL-27MC experiments). SPICE analysis of Treg cells from IL-27pg treated mice revealed that Treg cells coexpressed more inhibitory receptors than PBS treated controls (Figure 4.3D). Furthermore, LAG-3 was coexpressed with PD-1, PD-L1 and TIGIT exclusively in the IL-27pg treated mice indicating that IL-27 can coordinate expression of multiple inhibitory receptors on Treg cells at steady state.

Infection with Toxoplasma gondii results in IL-27-dependent Treg cell expression of inhibitory molecules.

Lastly, to determine if endogenous IL-27 contributes to Treg cell expression of inhibitory receptors an in vivo model of toxoplasmosis was utilized in which IL-27, Treg
cells and the PD-1 pathways are all known to be operational (Bhadra et al., 2011, Hall et al., 2012, Villarino et al., 2003). Following i.p. challenge with *T. gondii*, inflammatory monocytes produce IL-27p28 predominantly in the peritoneum while this was less apparent in the LN or spleen (Figure 4.4A, and data not shown). Treg cells isolated from this site demonstrated a two to three-fold increase expression of the molecules LAG-3, Ly6c, PD-1 and PD-L1 (Figure 4.4B). Analysis of individual inhibitory receptor expression on Treg cells isolated from i.p. infected WT or *Il27rα−/−* mice revealed that in the PECs, Treg cells expressed less Ly6c, PD-1, PD-L1 and TIM-3 in the absence of IL-27Rα, however not all trends were significant; no decrease in CD200R or TIGIT was observed in this organ (Figure 4.4C). In the spleens of these animals Treg cells from IL-27Rα−/− mice expressed less PD-1, PD-L1 and CD200R, whereas there was no change in LAG-3, Ly6c, TIGIT or TIM-3 in these organs (Figure 4.4C). In non Treg CD4⁺ T cells in these mice, a different pattern of inhibitory receptor expression was observed with non Treg cells expressing increased levels of PD-1, LAG-3, CD200R and TIGIT (data not shown). This is consistent with previous reports of a role for WSX-1 in limiting LAG-3 and TIM-3 expression in effector cells during malaria, and may be a consequence of enhanced IL-12 signaling in the absence of IL-27 as shown in this report (Villegas-Mendez et al., 2013). To further investigate the role of IL-27 in Protein S expression during infection, WT and *Il27−/−* mice were infected i.p. and their Treg cell populations were analyzed. Relative to Treg cells isolated from naïve mice, there was an infection induced increase in Protein S expression in the peritoneum but not the spleen (data not shown). This infection-induced increase in Protein S was found to be dependent on IL-27 because Protein S expression was significantly decreased in the Treg cells isolated from the peritoneum of *Il27−/−* mice (Figure 4.4D). To assess the coexpression of multiple
inhibitory receptors on Treg cells, SPICE analysis was performed for the markers PD-1, LAG-3, Ly6c, TIGIT, and TIM-3. These data revealed that Treg cells in the peritoneum had increased heterogeneity and marker coexpression relative to the spleen (Figure 4.5A), consistent with the high levels of IL-27 observed in the peritoneum (Figure 4.5A). In the WT peritoneum >25% of Treg cells coexpressed PD-1, LAG-3, TIGIT, and TIM-3 whereas this population was largely absent in the PECs of Il27ra−/− mice. Together, these studies identify a key role for endogenous IL-27 as a factor that promotes Treg cell expression of inhibitory receptors at local sites of inflammation.

Discussion

While the expression of inhibitory receptors such as PD-1, PD-L1 and LAG-3 have been most prominently described in the context of CD8+ T cell exhaustion during chronic infection (Wherry., 2011) the role of these molecules in Treg cell function is not as well characterized. Understanding what the inhibitory receptors do to Treg cells is still not understood, but in light of our GSEA analysis comparing IL-27-induced Treg cells to the transcriptional profiles of exhausted effector cells, these populations appear distinct from exhausted T cells. Rather, it seems likely that these receptors on Treg cells are markers of Treg functionality. Also, Treg cells have been proposed to suppress by many mechanisms (Vignali et al., 2008), but the coordinated regulation of Treg suppressive effector molecules is poorly understood. While IL-27 has been linked in early reports to the inhibition of Treg populations (Huber et al., 2008, Neufert et al., 2007), reports from this laboratory and others demonstrate that IL-27 also promotes Treg survival (Kim et al., 2013) as well as expression of IL-10, T-bet, and CXCR3 (Hall et al., 2012). Thus,
taken together with the data presented here it appears that IL-27 is a central coordinator of multiple pathways that Treg cells use to modulate the immune response.

While there have been reports about the function of Lag-3 in Treg cell-mediated suppression (Huang et al., 2004, Liang et al., 2008), for many of the receptors examined here, relatively little is known about their function in Treg cells. One of the genes most profoundly regulated by IL-27 is the glycoprotein Ly6c. This molecule is expressed by many cell types, and early work provided evidence that engagement of Ly6c expression was associated with reduced T cell production of IL-2 (Yamanouchi et al., 1998). Indeed, dysregulation of T cell expression of Ly6c is associated with the development of diabetes and lupus like disease in NOD and NZB mice respectively (Herold et al., 1990, Philbrick et al., 1990). While these early studies point to a potential regulatory role for Ly6c in T cells, there have been a paucity of studies addressing this further, although a population of IL-10$^+$ Treg cells that express high levels of Ly6c has been identified in the abdominal fat of lean mice (Feuerer et al., 2009b). More recent studies identify a role for TIM-3 on Treg cells that is associated with enhanced Treg cell function such as IL-10 production and an enhanced Treg cell suppressor phenotype in cancer and transplant tolerance (Gupta et al., 2012, Sakuishi et al., 2013). While CD200$^{-/-}$ mice succumb to a lethal T cell-mediated pathology when infected with influenza, the mechanism of suppression was attributed to CD200R on macrophages (Snelgrove et al., 2008). Although T cells express CD200R (Rijkers et al., 2008, Wright et al., 2003), and increased CD200R expression is associated with dysfunctional T cell phenotypes during chronic antigen stimulus (Caserta et al., 2012), it is unclear how this molecule functions as an inhibitory receptor for Treg cells.
One theme common to the some of the inhibitory receptors examined here is that they are able to dampen T cell responses indirectly by targeting DC functions. For example, while PD-1 can limit T cell receptor signals, it can also act in an extrinsic manner to limit DC activation and cytokine production (Kuipers et al., 2006, Okazaki et al., 2001, Sheppard et al., 2004). In addition, T cell expression of PD-L1 can bind to CD80 to further inhibit T cell activation (Butte et al., 2007). Similarly, TIGIT expressed on T cells binds to the poliovirus receptor on DCs and promotes their production of IL-10, which in turn limits T production of IFN-γ (Yu et al., 2009b). LAG-3 (a CD4 homologue) also plays important roles in the ability of DCs to provide costimulation and present antigen (Bayry et al., 2007, Huang et al., 2004, Liang et al., 2008) while more recent work has shown that Protein S also limits DC activation (Carrera Silva et al., 2013). Thus, several studies have highlighted the key role for Treg cells in the control of DC function and these accessory cells represent likely targets for Treg cells. Consistent with this idea, DCs isolated from infected mice lacking IL-27 have increased expression of activation markers such as CD80, and CD86 as well as increased Class II expression and IL-12 production (Figure 4.5B). Our data derived from mice infected with *T. gondii* suggests a model in which Treg cells are acting at the local site of inflammation to limit the pro-inflammatory activities of DCs but we can’t also rule out a role for their ability to limit T cell priming in the LN.

The concept of Treg cell plasticity is relatively new, but it is clear that Treg cells are specialized to operate in varied inflammatory environments. What is not clear is whether the Treg cell populations examined in vivo that express multiple inhibitory receptors, are antigen specific for toxoplasma, maintain this plasticity, and where they
function. In the broader context of human disease, polymorphisms to the IL27 gene have been associated with susceptibility to lupus, arthritis, cancer, IBD, MS, COPD and asthma (Chae et al., 2007, Huang et al., 2008, Imielinski et al., 2009, Li et al., 2009, Sweeney et al., 2011). Based on data from mouse models, targeting IL-27 could promote positive outcomes in these settings. Thus, IL-27 is an ideal candidate for targeted therapies aimed at modulating Treg cell activities in human disease.
**Figures**

![Heatmap of inhibitory receptors](image)

**Figure 4.1** IL-27 induces the transcription of genes encoding inhibitory receptors in Treg cells. 
(A-B) iTreg cells were cultured under neutral conditions for seven days, harvested, and then exposed to neutral, IL-27 or IFN-γ Treg culture conditions for 10 or 48 hours. mRNA was isolated and microarray analysis was performed as described in the experimental procedures. Heatmap of a priori analysis of inhibitory receptors generated using GenePattern.
Figure 4.2 In vitro treatment of Treg cells with IL-27 leads to their expression of inhibitory molecules. (A-C) iTreg cells were cultured under iTreg conditions with indicated cytokines for 72 hours and analyzed for their expression of inhibitory receptors, or (B) were cultured under neutral conditions for one week and restimulated in either neutral or IL-27 conditions for 72 hours. (D) SPICE analysis from 3 different FACS panels for inhibitory receptor expression on Treg cells from iTreg cultures at 72 hours treated as indicated in figure legends. Pie slice color corresponds to the number of coexpressed inhibitory receptors. Pie arcs denote individual marker expression. Data is representative of >2 experiments.
Figure 4.3 In vivo expression of IL-27 promotes Treg cell expression of multiple inhibitory proteins.

(A & B) Mice were treated with PBS or pegylated IL-27 (IL-27pg) for 12 days and analyzed for their expression of inhibitory receptors. Treg cells were isolated from the spleen and bone marrow of treated mice. Bone marrow shown. Gated on Foxp3<sup>+</sup> CD4<sup>+</sup> CD8a<sup>-</sup> Live cells. Experiment was performed once, N=3 IL-27pg, N=1 PBS.

(C) Mice were treated with IL-27MC for 14 days as in the experimental procedures. Spleen and bone marrow Treg were isolated and analyzed for their expression of inhibitory receptors. Spleen shown. Data is representative of 2 independent experiments, N=5/ group. ** p < 0.01, ***p < 0.001. (D) SPICE analysis of Treg cells in (A) from 2 different FACS inhibitory receptor panels as noted in the figure legends.
Figure 5.4

WT Il27ra-/- PECs

Spleen

Ly6c

0 50 100 150 200 250 300 350

***

PECs

PD-1

0 250 500 750 1000 1250

PECs

PD-L1

0 1000 2000 3000 4000 5000

***

PECs Spleen

TIM3

0 50 100 150 200 250 260

***

PECs Spleen

CD200R

0 100 200 300 400

**

PECs Spleen

TIGIT

0 1000 2000 3000 4000

PECs Spleen

Sample:

PECs Il27ra-/-

PECs WT

PECs no primary control

Protein S

% of max

6500 5500 4500 3500 2500 1500 500

***

WT Il27ra-/-
Figure 4.4 Endogenous IL-27 expression promotes Treg cell inhibitory receptor expression during T. gondii infection.

(A-D) Mice were infected i.p. with T. gondii and analyzed at day 14 post infection. **p < 0.01, ***p < 0.001. Treg cells gated on live, CD4+, CD8-, Foxp3+ cells.


(B) Infection induced Treg cell expression of various inhibitory receptors. Data is representative of 2 independent experiments.

(C) Inhibitory receptor expression in infected WT and Il27ra−/− mice. WT, N=5; Il27ra−/−, N=4. Data is representative of 2 independent experiments.

(D) WT or Il27−/− mice were analyzed for their expression of Protein S, PECs shown N=5. Data is representative of 2 independent experiments.
Figure 4.5 Endogenous IL-27 expression promotes coexpression of multiple inhibitory receptors on Treg cells and limits DC activation during T. gondii infection.

(A) WT and Il27ra/- mice were infected i.p. with T. gondii and analyzed at day 14 post infection. Analysis of the markers PD-1, LAG-3, TIM-3, TIGIT and Ly6c were performed on Treg cells (gated on live, CD4+ Foxp3+ CD8a- T cells). Data is representative of 2 experiments. PECs shown.

(B) Dendritic cells produce more IL-12p40 and express higher levels of MHC class II in the absence of IL-27. LP cells from WT or Il27/- mice were incubated in the presence of Golgi inhibitors for 6-8 hours and stained for their expression of IL-12p40 (left panel), or directly ex vivo for DC markers. Gated on live, CD3-, CD19-, NK1.1-, CD11cINT-HI, Ly6G-, cells. Representative of > 2 independent experiments, N=4, ** p < 0.01.
Chapter 5: Discussion and future directions

Abstract

This section will discuss the main findings of this thesis in the context of recent advances in the field of Treg cell and cytokine biology and our understanding of how inflammatory environments influence Treg cell function. The findings that Treg cells are operational during toxoplasmosis, and that IL-27 promotes Treg cell proliferation, function, and inhibitory receptor expression have contributed the field of Treg cell biology. However, they have led to many questions about the basic mechanisms whereby IL-27 shapes Treg cells, and whether the effects of IL-27 on Treg cell function are unique to TH1 inflammation or are relevant to other types of T helper cell responses. It is still not known whether different subsets of Treg cells are hardwired to respond to cytokines upon exit from the thymus, or expression of cytokine receptors occurs later in the periphery. There are also basic questions about the role of antigen specificity in Treg cells that operate during infection, and how Treg cells use inhibitory receptors to suppress T cell responses. Thus, this section is organized to address three main themes that have emerged from this thesis work: the role of Treg cells during T. gondii infection, the effects of IL-27 on Treg cells, and unresolved questions about how heterogeneous Treg cell populations arise.
5.1 The role of Treg cells during infection with *T. gondii*

*Treg cells are operational during infection with *T. gondii.*

In 2009, there was a good rationale for the conclusion that Treg cells played a minor role in limiting immune pathology during toxoplasmosis: Treg cells are dramatically decreased during this infection (Oldenhove et al., 2009) and (Silver and Hunter unpublished observations) and CD25 antibody-mediated depletion approaches suggested Treg cells were not critical immune regulators in this setting (Jankovic et al., 2007). Moreover, at the time, the cytokines IL-10 and IL-27 were considered to be two of the main players in limiting immune pathology, because they can directly (IL-27) and indirectly (IL-10) dampen T cell responses, potentially obviating the need for Treg cell-mediated suppression (Gazzinelli et al., 1996, Villarino et al., 2003). Additionally, IL-27 was thought to limit Treg cell populations (discussed in subsequent sections of this chapter). Given the global cellular destruction mediated by the rapid replication and dissemination of this parasite, it seemed logical to limit Treg cells in order to allow for the emergence of a strong IFN-γ and IL-12 driven TH1 response to protect the host.

In retrospect, there were limitations to the approaches used in these early studies to deplete Treg cells during infection, and none of these prior studies directly addressed whether Treg cells were operating in this infection. Nonetheless, this work (in particular anti-CD25 depletion) led to the conclusion that Treg cells were not a relevant source of IL-10 during *T. gondii* infection (Jankovic et al., 2007). Subsequent studies revealed that treatment with anti-CD25 depleting antibodies during toxoplasmosis was only effective at targeting \( \sim 40\% \) of Treg cells, and effector TH1 and effector CD8\(^+\) T cells which express CD25 during acute activation, were also depleted (Couper et al., 2009).
Although Treg cell frequencies decreased by approximately 90% during infection with *T. gondii* (Chapter 2), the remaining Treg cells appeared to share characteristics with TH1 cells (Oldenhove et al., 2009). Whether these cells were functional and represented a specialized population of TH1-adapted Treg cells was not clear. Although the studies in Chapter 2 demonstrated that Treg cell homeostasis is altered during infection, the finding that the reduced production of IL-2 could not completely account for this loss, and the fact that Treg cells still proliferated suggested that this population may have unappreciated suppressive capacities.

To determine if Treg cells were functional during toxoplasmosis, the studies in Chapter 3 tested whether Treg cells could dampen T cell responses in vivo. In a specific Treg cell depletion approach to determine if Treg cells limited T cell responses, *T. gondii* infected DEREG mice were depleted of their Treg cells and their effector cell responses were analyzed (experiments were performed in collaboration with Yasmine Belkaid’s lab). These data showed that these Treg cells were one factor responsible for limiting CD4$^+$ and CD8$^+$ T cell proliferation and their production of IFN-$\gamma$ during infection (Chapter 3). Moreover, in another assay of Treg cell function, the adoptive transfer of Treg cells could ameliorate the infection-induced CD4$^+$ T cell-mediated pathology in the IL-27p28 knockout mice (Chapter 3). The recipient mice still developed a robust TH1 response, but the magnitude of their response was greatly attenuated in comparison to untreated IL-27p28 knockout mice (Chapter 3). Since in this model mice can also be rescued by depleting their CD4$^+$ T cells (Villarino et al., 2003), these data support a model where during normal infection in WT mouse, Treg cells limit pathological CD4$^+$ T cell responses. However, it should be noted that these cells do not act in isolation, and
are simply one of several inhibitory mechanisms important in limiting pathology during this infection. For example, these include NK cell production of IL-10, and T cell production of TGF-β, as well as the suppressive mechanisms mediated by the lipoxin A4 pathway (Aliberti et al., 2002, Hunter et al., 1995, Neyer et al., 1997).

*Does altered Treg cell phenotype during infection correlate with enhanced function?*

While these studies indicate that Treg cells are operational during toxoplasmosis, how these Treg cells respond to infection and mediate their suppressive effects is still a matter of conjecture. Recent reviews suggest that Treg cells can suppress using over 30 different mechanisms (Vignali et al., 2008) and the studies in Chapter 3 demonstrated that they needed to express multiple cytokine receptors (IFN-γR1, IL-27Rα) and produce IL-10 to rescue these mice. However, throughout the course of this thesis work, we observed a number of infection-induced changes in Treg cells and these data suggest that it’s not about the number of the Treg cells but rather their phenotype, that is important for their suppressive activities. As mentioned previously, despite the decreased number of Treg cells, the remaining Treg cell populations undergo proliferation, as measured by their expression of Ki67 and incorporation of BrdU (Chapters 2, 3), implying that infection-induced factors such as IL-27 may promote a subset of specialized Treg cells to persist in this inflammatory environment. While IL-2Rα expression is not altered during this response, Treg cells increased their expression of IL-2Rβ, IL-2Rγ and IL-7R following infection (AOH, data not shown) suggesting that other cytokines such as IL-7 or IL-15 may also be involved in the survival of a subset of Treg cells. In agreement with studies describing phenotypic characteristics of Treg cells operating during other TH1
responses (Koch et al., 2009), we found that following infection with *T. gondii*, Treg cells exhibit many of these same TH1 characteristics (T-bet⁺, CXCR3⁺) and were likely to represent specialized TH1-adapted Treg cells. CXCR3 is a target of the transcription factor T-bet and is a chemokine receptor involved in lymphocyte homing to sites of inflammation, which is one example of how IL-27 may influence Treg cell behavior. Additionally, these Treg cells also produce IL-10 and IFN-γ (Chapter 3) and showed an infection-induced increase in multiple inhibitory receptors (Chapter 4). However the function of these molecules in Treg cells and IL-27’s role in this process have yet to be determined. For instance, although Treg cells isolated from infected mice express T-bet and CXCR3 it is not clear these factors are required for their function during *T gondii* infection. Indeed one recent study from this laboratory highlights that CXCR3 is only partially required for the recruitment of CD8⁺ T cells to sites of inflammation during toxoplasmosis, but was critical for the behavior of these cells in seeking their targets (Harris et al., 2012). Therefore, utilizing specific deletion approaches within Treg cells such as with Foxp3-cre mice will be critical to dissect whether infection-induced Treg cell-associated transcription factors, cytokines, chemokine receptors and inhibitory receptors are important for their immunosuppressive function during toxoplasmosis. Also, technologies that answer questions regarding Treg cell antigen specificity, such as the use of MHC Class II tetramers and well as parasites that express model antigens such as OVA will be important tools. Moreover, the use of two-photon imaging to monitor Treg cell interactions during infection provide an opportunity to assess how these pathways affect Treg localization and behavior during this infection.
5.2 The role of IL-27 in Treg cell biology

*IL-27 does not limit Treg cell responses.*

At the onset of my thesis studies (2009), a paradigm existed in Treg cell biology that environmental cues such as those from cytokines were important in disarming Treg cell populations. This could be beneficial if Treg cells interfered with the immune system’s ability to clear pathogens or fight off tumors, but also detrimental in the case of progressive autoimmunity where Treg cells are critical to limit pathological responses. Perhaps the best example of this phenomenon was from the pivotal studies that identified the pro-inflammatory cytokine IL-6 as a factor that could interfere with the ability of Treg cells to suppress DC activation of T cells (Pasare and Medzhitov., 2003). This initial finding was supported by subsequent studies from Chen Dong and colleagues demonstrating that IL-6 could downregulate Foxp3 in existing Treg cells (Yang et al., 2008b), and work from Bluestone and colleagues, that showed in a fate mapping Foxp3 reporter system, that Treg cells can lose their expression of Foxp3 and become pathogenic participants in the setting of diabetes (Zhou et al., 2009).

As noted elsewhere, there were a number of reasons to suspect that IL-27 negatively regulated Treg cell responses: IL-27 signals in a manner similar to IL-6 (through pSTAT1 and pSTAT3), IL-27 limits T cell production of IL-2, a critical factor for Treg cell homeostasis, and additionally, a number of studies looking at Treg cell differentiation in vitro suggested that Treg cell frequencies were limited in the presence of IL-27 (Awasthi et al., 2007, Stumhofer et al., 2007b). Nonetheless, when experiments were performed to test the role of IL-27 in Treg homeostasis during *T. gondii* infection (Chapter 2), they demonstrated that IL-27-mediated suppression of IL-2 was not the
underlying cause of the Treg cell “crash”. This provided the first clue that IL-27 may be an environmental cue for Treg function during infection and raised questions about the role of IL-2 in Treg homeostasis during inflammatory responses.

While our initial studies on Treg cell homeostasis during infection were underway, a model was emerging largely based on the work of Rudensky and Campbell, about how inflammation impacts Treg cells. Using a Foxp3-Cre-Lox system to specifically delete transcription factors in Treg cells, mice that lacked Treg cell expression of the transcription factors STAT3 or IRF4 developed spontaneous TH17 or TH2-mediated disease, respectively (Chaudhry et al., 2009, Zheng et al., 2009). Moreover, work from Dan Campbell’s group demonstrated in a number of TH1-mediated disease settings that IFN-γ promoted Treg cells expression of T-bet which was required to limit pathology (Koch et al., 2009). These studies showed that Treg cells needed to integrate environmental cues to function in specific environments. However, at this point, it was not clear if IL-27 fit into this facet of Treg cell biology and whether or not this cytokine was beneficial to Treg cell function.

Integrating the results in Chapter 2 and the model that environmental signals such as cytokines are responsible for Treg specialization, a new hypothesis was developed: that IL-27 deficient mice succumbed to a lethal T cell pathology during toxoplasmosis in part because they lacked the specific TH1-associated Treg cell population required to limit the magnitude of their CD4+ T cell response. The studies in Chapter 3 demonstrated that IL-27 signaled in Treg cells through STAT1, 3, and 5 and promoted a population of T-bet+ CXCR3+ IL-10+ Treg cells in vitro. While STAT1 is required for T-bet and CXCR3 expression in response to IL-27, the finding that this
cytokine also promoted STAT3 and STAT5, two transcription factors associated with cell survival and proliferation (O'Shea et al., 2002), are consistent with the notion that IL-27 can promote Treg cell survival in environments where IL-2 is suppressed. While these studies did not address the relative contribution of IL-27 to the survival of Treg cells during infection, they do point to a role for driving the appropriate phenotype of Treg cells required to function in this inflammatory environment. Together, these data suggested that IL-27 might promote TH1 Treg cells in vivo. To test this, WT and IL-27 deficient mice were infected with T. gondii, and mice lacking IL-27 had a defect in TH1 Treg in the GALT. Additionally, adoptively transferred Treg cells were able to limit the magnitude of the CD4+ and CD8+ T cell responses in T. gondii infected Il27−/− mice and prolong their survival (discussed above) consistent with the idea that a Treg cell defect contributes to the lethal pathology in these mice. Collectively, these studies placed IL-27 as a central regulator of Treg cell activities and firmly established that IL-27 did not limit Treg cell responses in this model.

*IFN-γ and IL-27: distinct and overlapping roles in Treg cell biology.*

The findings that IL-27 promoted a population of Treg cells that expressed T-bet and CXCR3 was reminiscent of the data from Campbell’s group, in which IFN-γ and STAT1 were implicated as the primary factors responsible for this population in several models. In contrast, in our studies, IL-27 was found to be important at sites of inflammation where monocyte production of IL-27p28 production was most pronounced. Because mice lacking IL-27 had a defect in TH1 Treg cells in these sites, despite the presence of elevated levels of IFN-γ, one trivial explanation could be that Treg
cells differ in their expression of the IFN-γR at different anatomical sites. Indeed, a population of Treg cells isolated from the spleen of infected animals expressed both IFN-γR1 and IFN-γR2, whereas this population was not present in the GALT Treg cells (Chapter 3). The studies in Chapter 3 compared directly the effects of IFN-γ to IL-27 and revealed that while IL-27 and IFN-γ can both drive T-bet and CXCR3 in vitro and in vivo, IL-27 had distinct effects, presumably as a consequence of its ability to also engage STAT3 and STAT5. Furthermore, IL-27’s effects were most prominent at the sites of inflammation, whereas the influence of IFN-γ appeared most prominent in sites such as the spleen.

Our adoptive transfer studies revealed that Treg cells needed to express the IFN-γR1 to rescue the acute pathology in the IL-27 knockout mice, and this points to a non-redundant role for IFN-γ in Treg cell function during TH1 responses. While IL-27 can promote IL-10, in Treg cells, IFN-γ does not have this capacity and it is not clear what are the distinct functions of Treg cells that get IFN-γ signals versus IL-27 signals. In other words, why have compartmentalized Treg cell responses? Transcriptional analysis of our microarray data set for genes that are differentially regulated by IFN-γ and IL-27 (Chapter 3) reveal that the genes in cluster 1 are suppressed by IL-27 but sustained in IFN-γ treated Treg cells. These genes are associated with metabolic function, whereas the clusters 2 and 3 which are enriched with IL-27 treatment are associated with a more differentiated, TH1 like Treg cell that also expresses multiple inhibitory receptors. However, little is known about the relationship between these IFN-γ-induced and IL-27-induced Treg cells. One notion is that IFN-γ signals could maintain Treg cells in a less differentiated, transiently activated state such that these cells survive the resolution of
acute inflammation, whereas IL-27 could promote a highly specialized, and perhaps short-lived Treg effector cell population that is important in rapidly dampening immune pathology. None of these ideas have been formally tested, and the longevity of specialized Treg cell populations is not known. However, a paradigm similar to that for T cells, where short-lived effector cell populations are critical for immediate responses, whereas long lived memory populations are important for recall responses to subsequent immune challenge, could also exist for Treg cells.

**IL-27 promotes Treg cell expression of inhibitory receptors.**

The transcriptional analysis of Treg cells exposed to IL-27 or IFN-γ revealed that Treg cells exposed to either cytokine had distinct transcriptional profiles. While some molecules with regulatory functions such as LAG-3, Ly6c and IL-10 were enriched by IL-27 to the extent that their expression exceeded our arbitrary 1.5 fold cutoff, other genes such as CXCR3 fell just below this cutoff, yet were still obviously influenced by IL-27 as evidenced by our numerous in vitro and in vivo experiments. With this in mind, in the studies described in Chapter 4, we took an a priori approach to our analysis and selected a set of genes of known inhibitory receptors to interrogate our array data in an attempt to understand how IL-27 could be influencing Treg cell function during infection. This analysis revealed that the expression of genes for the inhibitory receptors LAG-3, PD-1, PD-L1, TIM-3, CD200R, and Protein S were increased by IL-27 relative to IFN-γ and neutral treatments. Our validation studies looking at protein expression in vitro and in vivo revealed that IL-27 could drive their expression on Treg cells. However, how these multiple inhibitory receptors function in Treg cells is still not understood. Although this
constellation of receptors have described functions in CD8+ T cell exhaustion, their T cell intrinsic mechanisms of inducing decreased functionality and cell death in CD8+ T cells are not consistent with a beneficial role for these receptors in Treg cell biology.

Additionally, we compared our Treg cells to the MSigDb immunological signatures collection (Bild and Febbo., 2005, Subramanian et al., 2005). This comparison revealed that IL-27 treated Treg cells do not share the same transcriptional profiles as exhausted cells, suggesting cell-type specific differences in how these molecules could function intrinsically in Treg cells. For instance while TIM-3 can induce cell death in CD8+ T cells (Zhu et al., 2005), in Treg cells it limits immune responses (Gupta et al., 2012, Sakuishi et al., 2013). As discussed in Chapter 4, like IL-10, the inhibitory receptors LAG-3, TIGIT, Protein S, PD-1 and PD-L1 all have reported roles in limiting DC activation, pro-inflammatory cytokine production or antigen presentation. This coupled with the finding that IL-27 exposure can promote their expression has important implications for our understanding of how Treg cells operate in vivo and how IL-27 coordinates these activities. A current view of how they may be functioning is illustrated in (Figure 5.1).

Future studies are warranted that test directly the cell intrinsic and cell extrinsic functions of these inhibitory receptors on Treg cells. For most of these molecules (LAG-3, TIM-3, TIGIT, PD-1, PD-L1, Ly6c and CD200R) blocking antibodies are available that could be used in in vitro assays testing Treg cell function on DCs. These reagents will also be useful for in vivo assessment of Treg cell behavior using imaging approaches during infection and dissecting how each of these receptors function. Moreover, the increasing availability of conditional knockout mice for each of these receptors will allow for more definitive experiments. While the sections above highlight the impact of inhibitory receptors on DCs, studies in CD8+ T cells have highlighted the cell intrinsic properties of
these receptors, and much is still unknown about these effects in Treg cells. For this assessment, known ligands could be used to measure the functional differences of Treg cells following inhibitory receptor engagement like the studies using Gal-9 to assess the function of TIM-3 in Treg cells (Gupta et al., 2012).

**IL-27 modulates Treg cell activities, implications in mice and men.**

Since the initial reports that Treg cells express high levels of the IL-27 receptor (Villarino et al., 2005) there have been a limited number of studies that address its role in promoting Treg cell function. With an appreciation for how IL-27 plays broadly anti-inflammatory roles in disease settings (Table 1.1), these data have implications in our understanding of how exacerbated immune responses manifest in the absence of IL-27. We suggest that each of these models in which IL-27 is critical to limit immune pathology be reexamined in the context of the effects of IL-27 on Treg cell function. For instance, there are multiple parasitic infections in which IL-27 is associated with limiting pathology (*T. cruzi, P. berghei, L. donovani*) (Findlay et al., 2010, Hamano et al., 2003, Rosas et al., 2006). It is tempting to speculate that in the absence of IL-27 signals, defective Treg cell activities during these infections may also contribute to immune pathology.

As mentioned in Chapter 1, there is a growing body of evidence in studies of human patients linking polymorphisms in or reduced production of IL-27 to human disease such as Crohn’s disease, colitis, asthma, RA, and lupus (Chae et al., 2007, Imielinski et al., 2009, Li et al., 2009, Li et al., 2010, Sweeney et al., 2011, Tanida et al., 2011). In autoimmunity, Treg cells are critical immune modulators and important in
limiting disease pathogenesis, therefore the link to IL-27 in these diseases suggests there may be a strong IL-27/Treg cell component to limiting pathology. Therapies that utilize IL-27 to target Treg cells in these settings may prove to be beneficial for the treatment of a variety of autoimmune diseases. In support of a role for IL-27 in driving Treg cell function to limit autoimmunity, Kronenberg’s group has identified that Treg cells need to express the IL-27Rα to ameliorate disease in a mouse model of colitis (Kim et al., 2013). To the best of my knowledge there are limited studies analyzing the effects of IL-27 on human Treg cells, but at least two studies provide evidence that a similar role for IL-27 in human Treg biology may exist. For example, DC production of IL-27 in response to apoptotic cells in the setting of cancer was associated with enhanced Treg cell responses in limiting tumor specific CTLs (Sekar et al., 2012). In the other study, while IL-6 blocked human Treg cell-mediated suppression in vitro in a STAT3 dependent manner, although IL-27 also signaled through STAT3, it did not limit human Treg cell activities in this assay (Goodman et al., 2011). Nevertheless, more extensive analysis of the effects of IL-27 on human Treg cells, analogous to the mouse studies presented here, is warranted.

5.3 What is Treg cell heterogeneity? Remaining questions

Treg cells express the master regulators.

The idea that lineage specifying transcription factors are expressed in a mutually exclusive fashion came from early studies that identified key lineage-specifying transcription factors such as T-bet as necessary for different T helper cell programs (Szabo et al., 2000). In more recent years, this model has shifted away from the concept of the “master regulator” as being solely associated with one type of T cell response
(Oestreich and Weinmann., 2012). This is a common theme in T helper cell biology with recent studies from Amy Weinmann illustrating the complex relationship between T-bet, Bcl-6 and Blimp-1 and the downstream expression of TH1- or TFH-associated targets (Oestreich et al., 2012). As evidence, the concept that Treg cells can express multiple transcription factors in addition to their “master regulator” Foxp3, has been central in the field of Treg biology to understanding how Treg cells function during inflammation. It is clear that Foxp3 can be coexpressed with what seem to be opposing transcription factors (T-bet, Gata3, IRF4, Bcl-6). While it is now accepted that Treg cells need to express various transcription factors to do their jobs in different types of T cell responses, it is not clear how these factors, that promote inflammatory responses in conventional CD4+ T cells, operate within the confines of a Treg cell (a transcriptional landscape dominated by Foxp3) (Chen et al., 2006).

A number of factors are likely involved in how transcription factors function in the context of Treg cells, but it is clear that Foxp3 plays a dominant role in how these paired factors function under these constraints. One dynamic that will influence how Treg cells integrate other transcription factor signals likely has to do with the different epigenetic landscape of Foxp3+ cells (either nTreg or iTreg cells) relative to conventional CD4+ T cells (Ohkura et al., 2013). Directly related to our studies, it has been shown that while STAT1 signals in conventional CD4+ T cells lead to the expression of IL-12Rβ2, the reduced ability of Treg cells to express IL-12Rβ2 is associated with repressive H3K27me3 histone modifications of this promoter (Koch et al., 2012). Another way Foxp3 can influence how transcription factors function in Treg cells is likely due to its direct interaction with other transcription factors. Indeed, studies revealed that the N-terminus
of Foxp3 can bind IRF4 or HIF1α with markedly different effects: where binding to HIF1α impedes Treg cell differentiation by promoting proteosomal degradation of Foxp3, whereas binding to IRF4 facilitates regulation of TH2 and TH17 responses by promoting IRF4 targets such as CTLA4, IL-10 and TIGIT (Darce et al., 2012). The epigenetic landscape of Foxp3+ cells, and the ability of Foxp3 to partner with various transcription factors will likely affect how IL-27 signals are integrated in Treg cells. Another layer of complexity to how IL-27 signaling affects Treg cells is that this cytokine not only induces the phosphorylation of STAT1 but also STAT3 and STAT5. Work from John O’Shea’s group has delineated that different STAT signaling downstream of cytokines and can further alter the transcriptional landscape of cells by changing the accessibility of DNA cis enhancer elements during T cell responses (Vahedi et al., 2012). Thus, there are many factors to take into account when trying to understand how IL-27 affects Treg cell function, and future studies to delineate this would benefit from a proteomic approach. For instance, beyond the transcriptional analyses used to compare the effects of IFN-γ to IL-27 used in this thesis work, it is not clear if any novel Foxp3 binding partners are present upon exposure to IL-27. To assess this, mass spectrometry-based approaches using tagged Foxp3 as bait, such as those recently published from the Rudensky lab (Rudra et al., 2012), may help to identify novel IL-27-induced Foxp3 binding partners. To further assess what genes are accessed by IL-27 signaling, ChipSeq analysis of where STAT proteins bind in Treg cells, and comparisons to IFN-γ and IL-6 STAT binding signatures will aid in the identification of novel IL-27 targets.
**Specialized Treg cell subsets during TH2 and TH17 immunity, a role for IL-27?**

While our studies focused on whether Treg cells are influenced by IL-27 during a TH1 response, the influence of IL-27 on Treg cells in other settings such as during TH2 or TH17 responses is not clear. Unlike IL-27’s role in limiting TH1 responses, where IL-27 can promote T-bet expression, IL-27 is operational during helminth infection and mouse models of EAE where it directly inhibits TH2 and TH17 responses by downregulating the transcription factors associated with their development (Artis et al., 2004b, Diveu et al., 2009, El-behi et al., 2009, Lucas et al., 2003, Yang et al., 2008a). In the case of TH2 transcription factor expression in Treg cells, GATA3 has been reported to promote Treg cell homeostasis and suppressive function (Wang et al., 2011) and suppress alternate Treg cell fates such as their expression of T-bet or RORγt in a STAT6 independent manner (Wohlfert et al., 2011). This latter report argues for a role for GATA3 in maintaining the Treg lineage, but whether or not IL-27 signaling helps to alleviate this GATA3-mediated suppression of other transcription factors such as T-bet is not clear. However, other studies support a negative role for GATA3 in Treg cell biology where strong STAT6 signals downstream of IL-4 can both induce GATA3 and limit Foxp3 expression (Hadjur et al., 2009, Mantel et al., 2007, Wei et al., 2007). IL-27 signals in this setting could help to limit the loss of Foxp3+ due to induction of GATA3. In human patients with respiratory syncytial virus, IL-4 signals are associated with increased GATA3 and decreased Treg cell function and increased susceptibility to develop allergic asthma (Krishnamoorthy et al., 2012). It is intriguing to think of IL-27 playing a potential beneficial role in limiting this pathology since polymorphisms in IL-27p28 are associated with asthma (Chae et al., 2007). What is clear is that the
transcription factor IRF4 is important for promoting Treg cell functions during TH2 responses (Darce et al., 2012, Zheng et al., 2009), but how or if IL-27 impacts on this pathway is an open question.

In the case of TH17 cells, TGF-β induced Foxp3 as well as IL-27 are known to antagonize RORγt expression (Yang et al., 2008a, Zhou et al., 2008), so a role for RORγt in Treg cell function seems somewhat contradictory. However, what seems more plausible is a role for IL-27 in Treg cell specialization during TH17 responses. Using their Foxp3-Cre-lox system to delete transcription factors specifically within Foxp3+ cells, Rudensky and colleagues demonstrated that STAT3 expression and IL-10R expression are required in Treg cells to prevent spontaneous autoimmunity mediated by TH17 cells (Chaudhry et al., 2009, Chaudhry et al., 2011). Since this thesis work has demonstrated that IL-27 can promote both STAT3 and the transcriptional upregulation of IL-10R in Treg cells, this may provide one mechanism by which IL-27 influences Treg cells to limit pathological TH17 responses.

Are Treg cells hardwired to respond to different cytokine signals?

With the early recognition by Mosmann and Coffman that T cells exist in heterogeneous subsets (Mosmann et al., 1986), came multiple models to explain how this heterogeneity can be generated, and today we are now faced with a similar question for Treg cells. At several points in this thesis we show that Treg cells are varied in their responsiveness to cytokine signals. This was shown initially in the studies in chapter 2 where a subset of Treg cells were able to increase their expression of IL-2 receptor family members following infection and also by the results in chapter 3 whereby heterogeneous
STAT activation was observed in Treg cells in response to either IL-27 or IFN-γ.

Moreover, at steady state there were differences in IFN-γR2 expression in spleen Treg cells versus LN Treg cells and this suggests that there may be an inherent difference in cytokine receptor expression in different anatomical locations. Work from Diane Mathis’s group has highlighted the unique transcriptional profiles of Treg cells in different sites such as the adipose tissue compared to the spleen and LNs, and these data are available for analysis through the Gene Expression Omnibus (Cipolletta et al., 2012, Feuerer et al., 2010). In parallel to the detection of cytokine receptors by flow cytometry, analysis of microarray data may provide an easy starting point for future signaling-based assays that would determine if Treg cells are hardwired during their development to respond differently to cytokines in different sites. This mining-based approach may help to reveal factors that are important for the ability of Treg cells to maintain immune homeostasis in different sites.

**The fate of highly specialized Treg cell populations: memory and antigen specificity.**

With the recognition that IL-27 coordinately regulates the expression of multiple inhibitory receptors in Treg cells, shown by our SPICE analysis of Treg cells (Chapter 4), this raises new questions about what this functionality implies during immune responses. In conventional CD4+ and CD8+ T cells there has been a concerted effort to understand the different stages of T cell differentiation and their function in immunological memory and the corresponding phenotypic and functional markers of these states (Kaech et al., 2002, Kaech and Cui., 2012, Mueller et al., 2013). Much of the impetus behind these studies was to understand the factors leading to good vaccination
strategies with robust secondary responses upon encounter with repeated immunological insult. Although there are some reports that self antigen specific nTreg cells can form memory-like populations (Rosenblum et al., 2011), it is not clear that Treg cells need to participate in memory responses, or whether highly specialized populations of Treg cells, such as the ones described in this thesis, have properties of long lived memory or short lived effector cells. If Treg cells are efficient to limit the magnitude of the primary response, do they also have to be present to prevent pathology later on? These questions about Treg cell memory give rise to more basic questions about the role of antigen specificity of TH1 Treg cells that emerge during inflammation or infection. Our studies do not address whether the populations generated in vivo are induced or thymically derived, specific for \textit{T. gondii}, or bystander suppressors that are influenced by the cytokine environment in specific tissues, regardless of their antigen specificity. Nonetheless, these are important questions because TCR receptor signals are likely to influence the functional characteristics and longevity of these cells. A recent report from Kevin Urdahl's group report that Treg cells specific for \textit{Mycobacterium tuberculosis} expand initially but then contract greatly (Shafiani et al., 2013). Another recent study identified long-lived antigen specific Treg cells as having tolerogenic roles during pregnancy and these Treg cells displayed memory responses in subsequent pregnancies (Rowe et al., 2012). Understanding how IL-27 influences these Treg cell responses (especially as IL-27 p28 and Ebi3 are expressed in the specialized environment of the uterus (Devergne et al., 1996, Zhang et al., 2003)) will be important when thinking about its use as a potential therapy to treat human disease.
Concluding remarks

While IL-27 was initially described as a pro-inflammatory cytokine that contributes to TH1 immunity, subsequent work from many groups firmly established its role as a potent anti-inflammatory factor in a number of settings, and data from human patients has identified that polymorphisms in IL-27 are frequently associated with human autoimmune disease. With this in mind, it was not clear at the onset of this thesis how IL-27 limited Treg cell responses and much of the early work suggesting that IL-27 blocked Treg cell function was paradoxical given the phenotypes observed in settings where Treg cells are operational. With the growing appreciation that Treg cells functionally specialize in different types of inflammatory environments, and that cytokine signals are crucial in this process, a potential role for IL-27 was identified and tested as part of this thesis. Consequently, we now recognize that IL-27 is one of the many factors promoting Treg cell functions, particularly at sites of inflammation. Although there has been a greater acceptance of IL-27 in promoting Treg cell functions, how IL-27 does this is still not known, but we have identified that it coordinately regulates a number of potential Treg cell effector molecules involved in their suppression and this has lead to new areas of study for the Hunter laboratory in identifying the cellular and molecular basis for IL-27’s effects.
Figure 5.1 Proposed model of coordinated regulation by IL-27-induced Treg cells. Treg cells respond to IL-27 and signals through pSTAT1 and pSTAT3 and this promotes Treg cell expression of T-bet, CXCR3, IL-10 and multiple inhibitory receptors (LAG-3 and PD-1 shown). The coordinated expression of these molecules by Treg cells function to dampen DC activation via multiple mechanisms: direct interaction of DCs and Treg cells through PD-1 and LAG-3, and indirectly through IL-10.
Chapter 6: Materials and methods

Mice and T. gondii infection

Il27−/− mice were generated by Lexicon Pharmaceuticals, Inc. (Hall et al., 2012). Ebi3−/− mice are described elsewhere (Yang et al., 2008a). Wildtype C57BL/6J (WT), IL-10−/−, Swiss Webster and CBA/CaJ mice were purchased from Jackson laboratory. Tbx21−/− and CD4-Cre x EomesΔfox reporter mice were provided by S. Reiner. Stat1−/− mice were purchased from Taconic labs. Il27ra−/− mice were provided by C. Saris (Amgen), Foxp3GFP reporter mice were obtained from V. Kuchroo, Stat3Δfox mice were obtained from L. Heninghausen, and Vert-X IL-10 reporter mice were provided by C. Karp, and bred in our facility. All mice were housed in a specific-pathogen free environment at the University of Pennsylvania School of Veterinary Medicine in accordance with federal and institutional guidelines. The ME49 strain of T. gondii was maintained in Swiss Webster and CBA/CaJ, and used as a source of tissue cysts for oral (100 cysts) or i.p. (20 cysts) infections. For Treg cell depletion experiments, WT or DEREG mice (Lahl et al., 2007) were infected orally with 10 cysts of ME49. Two days postinfection, mice were injected with diphtheria toxin (DT) (Calbiochem) in endotoxin-free PBS. 1 µg of toxin was injected i.p. for 7 consecutive days. For the depletion of IFN-γ, WT mice were infected orally and starting at day 3 postinfection treated with 2mg anti-mouse IFN-γ (clone XMG1.2, BioXcell), or ratIgG control (Sigma) every 2 days.
Salmonella typhimurium infection

*Salmonella typhimurium* SL3261 (aroA mutant) was grown overnight in Luria-Bertani broth, washed twice and resuspended in PBS after determining bacterial concentrations using a spectrophotometer. WT and *Ebi3*−/− mice were infected with 1X10⁸ bacteria by oral gavage, immediately following administration of 100 µl of a 3% NaHCO₃ solution. Gut tissues were analyzed 12 days postinfection.

Leishmania major infection

*L. major* (WHO /MHOM/IL/80/Friedlin wildtype *L. major*) promastigotes were grown to the stationary phase in Schneider’s Drosophila medium (GIBCO) supplemented with 20% heat-inactivated fetal bovine serum (Invitrogen), 2 mM l-glutamine, 100 U of penicillin and 100 mg of streptomycin per mL. Infective-stage promastigotes (metacyclics) were isolated from 4-5 days old stationary culture by density gradient separation by Ficoll (Sigma) (Spath and Beverley, 2001). WT and *Ebi3*−/− mice were inoculated intradermally into the ear with 10 µL of PBS containing 2x10⁶ *L. major* metacyclics. Lesion development was measured weekly by ear thickness with digital caliper (Fisher Scientific). Parasites burden in lesion tissues were detected using a limiting dilution assay. Freeze-thawed antigen (FTAg) was obtained from stationary-phase promastigotes of *L. major* subjected to several freeze thawed cycles.
Regulatory T cell differentiation

For iTreg cells, CD4+ CD25−, or Foxp3GFP− cells were separated by FACS or MACs sorting (Miltenyi). Cells were rested for 30 min in complete RPMI (cRPMI) media (1% penicillin/streptomycin, 2mM L-glutamine, 10% fetal bovine serum, 0.1% beta-mercaptoethanol, 1% non-essential amino acids, 1mM sodium pyruvate and 20mM HEPES) (GIBCO). Cells were cultured at 1x10^6 cells/mL in αCD3-coated (1µg/mL; clone 145-2C11; eBioscience) 96-well U-bottom plates (Costar) in cRPMI containing αCD28 (1µg/mL; clone 37.51; eBioscience), recombinant human (rHu) TGF-β2 (5ng/mL, eBioscience), with or without (rHu IL-2; 100U/mL; Proleukin), anti-IFNγ (10µg/mL; clone XMG1.2) and anti-IL-4 (10 µg/mL; clone 11B11) blocking antibodies, recombinant mouse (rMu) IL-27 (50ng/mL; Amgen), and with or without rMu IFN-γ (50ng/mL; R&D). Media was added every 2 days with initial cytokines and neutralizing antibodies. For nTreg cells, sorted Foxp3+ cells were cultured on plate-bound αCD3 in media containing αCD28, 100U/mL rHu IL-2, 5ng/mL rHu TGF-β2, with or without neutralizing antibodies to IFN-γ or IL-4, with rMu IL-27, or rMu IFN-γ.

Isolation and flow cytometric analysis of immune populations

Single cell suspensions from the spleens, mesenteric lymph nodes (mLN) and Payer’s patches were prepared using standard methods. For the analysis of lamina propria lymphocytes (LPL), small intestines were collected in PBS at 4°C, cut longitudinally, and fecal contents were removed in PBS. Epithelial cells were stripped
(5mM EDTA and 1mM DTT) in cRPMI, followed by digestion (0.16U/mL Liberase TL) (Roche) for 30 min at 37°C, and processed for lymphocytes.

Cells were stained in FACS buffer (0.5% BSA, 2mM EDTA in PBS) with Fc block (2.4g2, BD) containing live/dead fixable Amcyan (Invitrogen), using the following surface antibodies: CD4 Percc-Cy5.5 or eFluor650® NC (RM4-5, eBioscience), CD8a PE-Texas Red® or PE-CF594 (53-6.7, Abcam, BD), CD44 PE-Cy7 (IM7, eBioscience), CD62L APC-eFluor780® (MEL-14, eBioscience), TCR-β Alexa Fluor700® (H57597, BioLegend), CD3 eFluor450® (17A2, eBioscience), CD25 APC-eFluor780® (PC61.5, eBioscience), TIM-3 PE (8b.2C12, eBioscience), Ly6c Percc-Cy5.5 (HK1.4, eBioscience), LAG-3 eFluor450® (C9B7W, eBioscience), CD200R APC (OX110, eBioscience), TIGIT APC (GIGD7, eBioscience), KLRG PE-Cy7 (2F1, eBioscience), PD-L1 PE (M1H5, BD), PD-1 PE-Cy7 (RMP1-30, BioLegend), CD127 PE-Cy7 (A7R34, eBioscience), CD132 PE (4G3, BD), CD122 APC (TM-b1, eBioscience), CXCR3 PE and APC (220803, R&D) and PE-Cy7 (CXCR3-173, BioLegend). All intracellular staining was done using the Foxp3/transcription factor staining buffer set (eBioscience) for Foxp3 Alexa Fluor488® and eFluor450® (FJK-16s, eBioscience), T-bet eFluor660® and FITC (4B10, eBioscience and BioLegend respectively), Ki67 Alexa Fluor488® and Alexa Fluor647® (B56, BD), CTLA-4 PE (UC10-4F10-11, BD), and EOMES PE (Dan11mag, eBioscience).

To detect cytokine production, isolated cells were cultured in cRPMI in six replicates at 1x10^6 cells/mL in a 96-well U-bottom plate, with PMA and ionomycin for 5hr with Brefeldin A (Sigma) and monensin (BD) golgi inhibitors. Cells were rinsed, stained for surface markers at 4°C, and fixed with 4% PFA in PBS for 10 min at RT.
Intracellular cytokines were detected by staining in FACs buffer containing 0.5% saponin (Sigma), IL-10 APC (JES5-16E3 , eBioscience), IFNγ PE-Cy7 or Percp-Cy5.5 (XMG1.2, eBioscience), IL-2 PE (JES6-5H4, BD), and Foxp3. Intracellular IL-10 was detected ex vivo using Vert-X reporters (Madan et al., 2009) and antibodies for GFP (polyclonal rabbit anti-GFP, eBioscience and FITC-conjugated rat anti-rabbit Jackson Immunoresearch) with staining for Foxp3.

To detect surface cytokine IFN-γ receptor expression, a 3-step surface stain was used. Cells were rinsed in FACS buffer and pelleted. Cell pellets were resuspended in Fc block, live/dead fixable Amcyan, and purified IFN-γR2 antibody (MOB-47, BD), and incubated at RT for 15 minutes. Cells were rinsed with FACS buffer and incubated with biotinylated anti-armenian hamster IgG (eBioscience) at RT for 15 minutes. Cells were washed twice with FACS buffer and stained for IFN-γR1 PE (2E2, eBioscience) and streptavidin PE-Cy7 (eBioscience) on ice. Cells were fixed and intracellular stained for Foxp3, as described, using the Foxp3 staining kit (eBioscience).

**STAT phosphorylation assays**

NTreg cells were isolated ex vivo, and iTreg cells were generated in vitro. Before stimulation, cells were washed in 0.5% BSA RPMI and rested at 4°C for 20 min. WT and Il27ra−/− Treg cells were incubated with 50ng/mL of the following cytokines: rMu IL-27; rMu IL-10 (R&D); and rMu IFN-γ for 20 min or over various time points at 37°C in the presence of Amcyan fixable live/dead marker, followed by immediate fixation on ice in 4% PFA for 20 min. Following PBS rinse, cells were permeabilized in 90% methanol on
ice for 1 hour or stored at -20°C overnight. Staining was performed in Fc block with BD PhosFlow antibodies to pSTAT1 (pY701) PE, pSTAT3 (pY705) Alexa Fluor488®, and pSTAT5 (pY694) Alexa Fluor647® with antibodies to T-bet, Foxp3, TCR-β, CD4 and CD25.

*Flow cytometry for BrdU incorporation and transcription factor expression*

To detect incorporation of BrdU, WT mice were naive or infected orally with 100 cysts ME49 and were pulsed with 800µg i.p. BrdU (BD) on days 4 and 5 postinfection. Mice were sacrificed on day 8-10 postinfection and BrdU incorporation was detected using FITC BrdU Flow Kit (BD) followed by intracellular staining for Foxp3 and T-bet using the Foxp3/transcription factor kit (eBioscience).

*Production and delivery of IL-27 minicircle DNA*

The p2øC31.RSV.hAAT.bpA plasmid was provided by Dr. Zhi-Ying Chen (Stanford University, Stanford, CA) and the vector modified to include unique 5’ PmeI and 3’ PacI restriction sites flanking hAAT for directional cloning of cDNA’s. PCR amplification was used to place 5’ PmeI and 3’ PacI cloning sites on the linked mIL-27 cDNAs which were ligated with the modified minicircle plasmid. Minicircle DNA was produced as described (Chen et al., 2005) with minor modifications for overnight cultures, Terrific broth containing 100ug/ml ampicillin was inoculated and incubated for 18hr shaking at 270 rpm. Endotoxin free Qiagen megaprep kits were used for DNA purification which was resuspended endotoxin-free Tris EDTA. Minicircle DNA was
dialyzed in Midi MWCO 3.5kDa tubes overnight against Tris EDTA and minicircle DNA was verified by restriction digestion and sequencing. For a hydrodynamics-based transfection procedure (Liu et al., 1999), 20mg of minicircle DNA in 2ml of Ringer’s solution was administered via tail vein injection within 5-8 seconds.

**ELISAs and LPL dendritic cell intracellular cytokine detection**

IL-27p28 (DuoSet, R&D), IL-12p40 (C17.8 and C15.6 biotin), IL-2 (JES6-5H4 and JES6-1A12 biotin, eBioscience) and IFN-γ (AN18 and R4-6A2 biotin, eBioscience) were measured by ELISA. To detect intracellular IL-27p28 and IL-12p40 LPL dendritic cells (LpDCs) were enriched using the 1-Step 1.077/265 gradient (Accurate Chemical & Scientific Corp.) and incubated for 6-8hr at 37ºC in the presence of Brefeldin A and Monensin and then surface stained in Fc block including Amcyan live/dead dye, for MHC Class II I-A I-E Alexa Fluor 700® (M5/114.15.2, BioLegend), CD11c PE-Cy7 (N418, eBioscience), CD11b PerCP-Cy5.5 (M1/70, eBioscience), NK1.1 Pacific Blue (PK136, BioLegend), CD3 eFluor450®, and CD19 eFluor450® (1D3, eBioscience), and then fixed with 4% PFA. Cytokines were detected by staining for IL-12p40 PE (C15.6, BD) and IL-27p28 Alexa Fluor 647® (clone MM27-7B1, BioLegend) for 30 min in 0.5% Saponin.

**Adoptive transfer studies**

WT or Il27−/− animals were infected i.p. with 20 cysts ME49 and monitored for morbidity. Neutral Treg cells were generated from naïve WT CD25−CD4+ T cells or TH1 Treg cells were generated from naïve WT or Il10−/− CD4+ CD25− T cells. At day 4
postinfection, mice received either i.v. PBS or 2-4x10^6 Treg cells followed by 2 injections of Treg cells on day 7 and 10, and were monitored for survival.

*Il27/-* animals were infected i.p. with 20 cysts Me49. TH1 Treg cells were generated, as described, from naïve WT, *Il27ra/-*, or *Ifngr1/-* CD25- CD4+ T cells. At day 4 postinfection, mice received 2-4x10^6 Treg cells followed by 2 injections of Treg cells on day 7 and 11, and were monitored for survival. For phenotypic analysis of effector cells, mice that had received PBS or TH1 Treg cells were sacrificed from each group at 2 weeks postinfection. Serum was taken from naïve mice and days 5 and 10 postinfection. Serum alanine aminotransferase (ALT) was detected by the Pathobiology department at the University of Pennsylvania School of Veterinary Medicine. For fate experiments, WT C57BL/6 CD45.1 congenic TH1 iTreg cells were transferred on day 4 postinfection. Mice were sacrificed 12 days later for phenotypic analysis of transferred Treg cells using CD45.1 PerCP-Cy5.5 (A20, eBioscience), and CD45.2 eFluor® 450 (104, eBioscience) to detect endogenous Treg cells.

*Administration of IL-2 complexes*

IL-2 complexes were formed by incubating 1.5 μg carrier free recombinant mouse IL-2 (eBioscience) and 25 μg of functional grade purified anti-mouse IL-2 (Clone: JES6-1A12, eBioscience) (per mouse) for 5 min at room temperature. Naïve or *T. gondii* infected mice were given IL-2 + αIL2 complexes or 25μg ratIgG (Sigma) i.p. for 5 days.
Administration of pegylated IL-27

Mice were treated with 10ug PEGylated IL-27 (Amgen) in 0.004% Tween or with 0.004% Tween alone i.p daily for 12 days and sacrificed on day 12.

Gene expression profiling, Hierarchical clustering and Ontology Enrichment analysis

For whole genome expression microarray, iTreg cells were generated under neutral conditions for seven days to ensure a homogeneous starting population of Treg cells. Cells were harvested and then exposed to neutral, IL-27 or IFN-γ Treg culture conditions for 10 or 48 hours. RNA was isolated using RNeasy Plus (Qiagen) and quality was assessed by Bioanalyzer (Agilent). Biotin labeled complementary RNA (cRNA) was made using the Illumina TotalPrep RNA amplification kit. An Illumina MouseWG-6 version 2 expression beadchip was hybridized with cRNA from three biological replicates and scanned on a beadscan unit. Data were quantile normalized and differential expression analysis was carried out using GenomeStudio v1.8 software (Illumina). Genes were considered differentially regulated by IL-27 or IFN-γ if expression level changed ≥1.5 fold compared to neutral controls with a corresponding diffscore ≥13 or ≤-13 (equivalent to a p value of ≤ 0.05). Data was deposited on the Gene Expression Omnibus (GEO) database for public access (GSE38686). Hierarchical clustering (Eisen et al., 1998) and heat map tools available on GenePattern (Reich et al., 2006) were used to analyze and display microarray data. Gene ontology (GO) enrichment analysis was done using the Database for Visualization and Integrative Discovery (DAVID) (Dennis et al., 2003) with enrichment defined relative to entire microarray. Only GO terms shared by
three or more genes and which had an enrichment p value of < 0.05 were considered for analysis.

**Statistical analysis**

Statistical significance was determined by a two-tailed unpaired Student’s t-test. Error bars indicate standard deviation of the mean. For p values: *, p < 0.05; **, p < 0.01; ***, p < 0.001.


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