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Role Of Il-27 And Tcr Stimulation In Inhibitory Receptor Expression

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Abstract

The adaptive immune response is necessary for control of pathogen burden in a wide range of infections. However, in the absence of active regulatory mechanisms, this protective response can lead to immune pathology. The cytokine interleukin (IL)-27 is required for control of exaggerated immune responses during toxoplasmosis and other infections and autoimmune settings. Multiple regulatory pathways have been found to be controlled by IL-27. However, there are gaps in our knowledge of the mechanisms by which IL-27 limits T cell responses. The present work focuses on the ability of IL-27 to promote expression of inhibitory receptors on T cells. The studies presented here establish that TCR and cytokines have distinct and complementary roles in promoting inhibitory receptor expression. In vitro, IL-27, type I IFN, and IFN-g induced expression of PD-L1 and Sca-1 on naïve murine CD4+ and CD8+ T cells in the absence of TCR stimulation. TCR stimulation induced expression of multiple inhibitory receptors and IL- 27 combined synergistically with TCR stimulation to further upregulate Ly6C, LAG-3, CTLA-4, TIGIT and TIM-3. This IL-27-mediated inhibitory expression was STAT1- dependent. The response to TCR stimulation was graded and thus a stronger TCR stimulus resulted in greater inhibitory receptor expression. In vivo, during infection with Toxoplasma gondii, IL-27 was required for full expression of Ly6C, PD-L1, LAG-3, CTLA-4, and TIGIT by parasite-specific T cells in the lung, a local site of infection, but not in the spleen. STAT1 was also required for full expression of LAG-3, CTLA-4, and TIGIT at local sites of infection. Taken together, these studies suggest a model in which inhibitory receptor expression on T cells is a graded regulatory pathway that is upregulated by exposure to increasing levels of TCR stimulation and cytokines present at sites of inflammation.

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ROLE OF IL-27 AND TCR STIMULATION IN INHIBITORY RECEPTOR EXPRESSION

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ROLE OF IL-27 AND TCR STIMULATION IN INHIBITORY RECEPTOR

EXPRESSION

Dedication

To my mother, who believes in me unconditionally.

To my father, who taught me to do things that scare me.

To my sister, my friend through life's challenges.

To Naomi, my love, my inspiration.

Acknowledgments

ABSTRACT

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EXPRESSION

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Chapter 1: Introduction

Abstract

The adaptive immune response is necessary for control of pathogen burden in a wide range of infections. However, in the absence of active regulatory mechanisms, this protective response can cause immune pathology. The parasite *Toxoplasma gondii* provides a well-characterized model to study immune regulation because it is a natural mouse pathogen, and infection with this parasite induces a protective type I response that becomes lethal in the absence of appropriate immune regulation. Therefore, toxoplasmosis has been utilized to understand immunoregulatory mechanisms that are broadly applicable to inflammatory processes. Indeed, studies from this laboratory have highlighted that the cytokine IL-27 is required for control of CD4-mediated immunopathology during toxoplasmosis. There are gaps in our knowledge about the mechanisms by which IL-27 limits T cell responses, and the present work focuses on the ability of IL-27 to promote T cell expression of inhibitory receptors to limit the immune response. The sections below will review 3 main topics: the protective and pathological response to *T. gondii*, the biology of IL-27, and what was known of the biology of inhibitory receptors at the time these studies were initiated.

1.1   Immune response to toxoplasmosis

Pathogenesis of Toxoplasma gondii and impact on human health

Toxoplasma gondii is an intracellular protozoan parasite capable of infecting any warm-blooded vertebrate. In the chronic phase of infection, the parasite forms cysts in

muscle and brain tissue that are controlled but not cleared by the host immune system. Consumption of these tissue cysts allows transmission of infection from a chronically infected animal to a new host (Kean et al., 1969). The parasite also undergoes sexual reproduction in infected feline hosts and is shed as highly infectious oocysts in cat feces (Frenkel et al., 1970). After infection, tissue cysts and oocysts convert into a quickly replicating tachyzoite form of the parasite, which can infect any nucleated cell. Following a systemic phase of the infection, immune pressure forces the parasites to convert to the slowly-replicating bradyzoite stage of their life cycle that once again form tissue cysts (Hunter and Sibley, 2012).

Approximately 60 million people in the USA are infected with *T. gondii*, which is a Class B Biodefense pathogen and an NIAID Emerging/Re-emerging Pathogen that is a major cause of food-borne mortality (Mead et al., 1999). Toxoplasmosis is relevant to human health due to congenital transmission and its adverse impact on fetal development (Ajzenberg, 2012; Torgerson and Mastroiacovo, 2013), as well as ocular disease in immunocompetent individuals (Carme et al., 2002; Desguerre et al., 1993; Glasner et al., 1992; McLeod et al., 2006; Montoya and Liesenfeld, 2004; Pappas et al., 2009). Additionally, patients with acquired defects in T cell function have increased susceptibility to reactivation of this chronic infection (Gaines et al., 1973; Israelski et al., 1993; Israelski and Remington, 1993; Slavin et al., 1994; Young and McGwire, 2005). Therefore, there is a longstanding interest in understanding the role of inhibitory receptors in limiting the T cell response to this infection.

The mouse is a natural host for *T. gondii* that has provided many clinically relevant insights into the pathogenesis of infection (Beaman et al., 1994; Hunter and

Remington, 1994; Khan et al., 1999; Leiva et al., 1998; Luft and Remington, 1988; Reichmann et al., 2000; Suzuki et al., 1996; Wilson and Hunter, 2003; Wilson et al., 2010). For example, the increased susceptibility of T cell-deficient patients to toxoplasmosis (Pappas et al., 2009) tells us that T cells are important for resistance to infection, and studies in mice demonstrate that T cells are required for survival during toxoplasmosis primarily due to their role in production of IFN-γ. In addition, CD4^{+} T cells promote Ig antibody class switching, and CD8^{+} T cells are responsible for perforin-mediated killing of infected cells (Bhadra et al., 2011; Denkers et al., 1997; Hunter and Sibley, 2012; Johnson, 2002). However, as discussed below, active mechanisms of immune regulation are required to prevent the dominant Th1-type response from becoming pathological (Aliberti, 2005; Gazzinelli et al., 1996; Villarino et al., 2003).

Early immune response to T. gondii

Infection of mice with *T. gondii* leads to production of IL-12 by a range of myeloid cells (Dupont et al., 2012), but BATF3^{+} DC1 cells are a particularly important source during toxoplasmosis (Mashayekhi et al., 2011). The mechanisms by which the immune system initially detects *T. gondii* are not fully defined, but the adapter protein MyD88 downstream of multiple TLRs is required for production of IL-12 and control of parasite burden (Scanga et al., 2002). Other studies have shown that TLR11 recognizes a profilin-like protein that *T. gondii* produces during infection and promotes IL-12 production (Yarovinsky et al., 2005). This IL-12 induces NK cells and T cells to produce IFN-γ, which is critical for resistance to *T. gondii*. Thus, when mice deficient in IFN-γ or IL-12 are infected with *T. gondii*, they rapidly succumb to this challenge, dying 7-10 days

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after infection due to overwhelming parasite burden (Gazzinelli et al., 1994; Suzuki et al., 1988). These types of studies were important in helping to understand the role of IL-12 and IFN-γ in resistance to many intracellular pathogens.

Balance between resistance and tolerance during toxoplasmosis

While a strong Th1 immune response is required for resistance to many intracellular infections and control of parasite replication, multiple active regulatory pathways are necessary to prevent this immune response from becoming pathologic. Indeed, infection of WT mice with *T. gondii* leads to systemic inflammation and weight loss, and in some cases this can lead to the development of severe CD4$^+$ T cell dependent immune pathology (Liesenfeld, 1996; Liesenfeld et al., 1997). Our group and others have identified multiple regulatory mechanisms (discussed below) involving regulatory T cells, IL-10, IL-27, and PD-1 that promote tissue tolerance during toxoplasmosis. In doing so, these mechanisms allow for persistence of the parasite but limit immunopathology, which promotes host survival. This balance allows a detente between a largely asymptomatic infection and immune hyperactivity (Figure 1.1). The broader relevance of the mechanisms that regulate immunopathology during toxoplasmosis is illustrated by the role of IL-10 and IL-27 in limiting the immune response in diverse settings such as the MRL/*lpr* model of lupus, respiratory syncytial virus, collagen-induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE), and colitis (Batten et al., 2006; Bettelli et al., 1998; Fitzgerald et al., 2007; Muallem et al., 2017; Persson et al., 1996; Sugiyama et al., 2008; Yin et al., 2002; Zhu et al., 2018; Zhu et al., 2016).

Regulatory T cells in toxoplasmosis

During acute infection with *T. gondii*, the numbers of CD4^{+} FoxP3^{+} regulatory T cells (Tregs) collapse, due partially to a decrease in IL-2 production (Oldenhove et al., 2009). This likely contributes to the development of immunopathology in these mice, but infection of DEREG mice, which lack Treg cells entirely, reveal that the remaining Tregs contribute to immune suppression in this system (Hall et al., 2012a). Tregs suppress the immune response by a range of mechanisms that include production of IL-10, sequestration of IL-2 through expression of the high-affinity IL-2 receptor, and the expression of inhibitory receptors that suppress the immune response by multiple mechanisms (Josefowicz et al., 2012). Recent studies have found that Treg cells can be subsetted into populations that roughly mirror the T helper cell subsets. Treg counterparts have been described for Th1 (Hall et al., 2012a; Koch et al., 2009), Th2 (Zheng et al., 2009), Th17 (Chaudhry et al., 2009), and Tfh (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011) cells, and Tregs are specialized to suppress the T helper cells they most closely resemble. For example, a CXCR3^{+} T-bet[+] Treg population is found to limit the development of Th1-mediated inflammatory disease in mice (Koch et al., 2009). IL-27 promotes the generation of this CXCR3^{+} T-bet[+] Treg population during toxoplasmosis (Hall et al., 2012a), suggesting that IL-27 can limit immunopathology through differentiation of Tregs optimized to limit the Th1 response. IL-27 also causes Tregs to produce IL-10 (Stumhofer et al., 2007), a cytokine necessary for limiting immunopathology during toxoplasmosis

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Cytokines that limit immunopathology during toxoplasmosis

IL-10 is central to limit immunopathology in multiple infectious systems. During toxoplasmosis, T cells are an essential source of IL-10, but IL-10 is also produced by NK cells, monocytes, and macrophages (Hedrich and Bream, 2010; Perona-Wright et al., 2009; Roers et al., 2004; Wagage et al., 2014). IL-10 limits myeloid cell production of IL-1, IL-12, and TNF-α, which all contribute to production of IFN-γ and control of toxoplasmosis. Therefore, IL-10 was initially expected to be a factor that would allow parasite persistence and it was initially a surprise to find that during acute toxoplasmosis, IL-10^{-/-} mice develop lethal CD4^{+} T cell-mediated immunopathology characterized by high expression of IFN-γ, IL-12, and TNF-α (Gazzinelli et al., 1996; Neyer et al., 1997). Blockade of CD28 and CD40 rescues IL-10^{-/-} mice during toxoplasmosis, demonstrating that costimulation is required for this immunopathology (Villegas et al., 2000). It is notable that IL-10 can limit productive immune responses, as loss of IL-10 leads to improved parasite control in SCID mice, and during chronic infection neutralization of IL-10 enhances control of parasite burden in the CNS (Deckert-Schluter et al., 1997; Neyer et al., 1997). However, IL-10^{-/-} mice that are treated with sulfadiazine to allow them to progress to chronic infection develop CD4^{+} T cell-mediated immunopathology in the CNS during chronic toxoplasmosis (Wilson et al., 2005). These studies demonstrate that while IL-10 can promote parasite replication and persistence in some circumstances, it is essential to prevent the development of immunopathology.

TGF-β has long been regarded as immunoregulatory, and TGF-β and IL-2 promote Treg responses. In addition, TGF-β limits production of IFN-γ by NK cells during toxoplasmosis and inhibits control of parasite burden in SCID mice, in which NK

cells are required for IFN-γ production (Hunter et al., 1995). It also limits production of

TNF-α by macrophages and prevents their effective control of the parasite (Langermans

et al., 2001). Treatment with exogenous TGF-β decreases IFN-γ levels in the cerebral

spinal fluid and limits T cell recruitment to the brain, which is accompanied by outgrowth

of cysts in the brain and increased mortality (Schluter et al., 1998). However, the

suppressive effects of TGF-β produced by intraepithelial lymphocytes were found to limit

the development of ileitis during oral toxoplasmosis (Buzoni-Gatel et al., 2001). Thus,

like IL-10, production of TGF-β can promote parasite persistence but can also have a host

protective activity.

1.2   The Immunobiology of Interleukin-27

IL-27 is an IL-6/IL-12 family cytokine that acts on lymphocytes to modify the

immune response in pleiotropic ways. It can promote T cell survival and proliferation,

but also upregulates production of IL-10 and differentiation of Th1-like Tregs. It has been

found to be essential in limiting immunopathology in diverse inflammatory contexts. This

section will describe the components involved in IL-27 signaling and its role in

modulating the immune response during toxoplasmosis and other infections.

Structure, sources, receptor, signaling

IL-27 is a heterodimeric cytokine composed of Epstein Barr Virus-Induced gene 3

(EBI-3) and p28. The EBI-3 subunit was first described as a secreted protein that was

predicted to have a role in immune signaling due to its homology with IL-12p40

(Devergne et al., 1996). The p28 subunit was identified by a computational sequence

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alignment algorithm in an attempt to identify novel IL-6-related cytokines (Pflanz et al., 2002). IL-27 was identified by a computational approach that predicted the interaction of EBI-3 with p28 (Pflanz et al., 2002). Monocytes, macrophages, and dendritic cells are considered to be the primary source of IL-27 (Hall et al., 2012a; Pflanz et al., 2002). Expression of IL-27 in these myeloid populations is induced by a range of microbial stimuli: LPS, poly(I:C), CpG, and gram-negative bacteria (Hashimoto et al., 2000; Liu et al., 2007; Smits et al., 2004; Wirtz et al., 2005). Type I IFN and IFN-γ also promote expression of IL-27 (Pirhonen et al., 2007; Remoli et al., 2007; Sonobe et al., 2005; van Seventer et al., 2002). This upregulation of IL-27 by inflammatory stimuli allows it to be upregulated and limit immunopathology at sites of inflammation.

IL-27 signals through a heterodimeric cytokine receptor composed of gp130 and IL-27Rα. gp130 was first identified in a screen of glycoproteins upregulated on activated T cells (Andersson et al., 1978), but it is expressed by diverse hematopoietic and non-hematopoietic populations (Taga and Kishimoto, 1997). The gp130 chain serves as a common receptor chain for other cytokines, such as IL-6, oncostatin M (OSM), IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and cardiotrophin (CT)-1 (Heinrich et al., 1998). The IL-27 receptor α-chain (IL-27Rα) was identified in a search for class I cytokine receptors with DNA sequence similarity to that of gp130 (Sprecher et al., 1998). IL-27Rα is also known as TCCR (T cell cytokine receptor) and WSX-1, in reference the C-terminus WSXWS sequence motif it contains, which is conserved among class I cytokine receptors. IL-27Rα is expressed primarily by T cells, B cells and NK cells (Pflanz et al., 2004). Its expression is upregulated on T cells when they are activated but is high on NK cells and is down regulated when they are activated

(Villarino et al., 2005). Signaling through the IL-27 receptor results in phosphorylation of STAT1, STAT3, p38 MAPK and ERK1/2 (Owaki et al., 2006; Pflanz et al., 2004).

While the focus of this thesis is on IL-27, the EBI-3 and p28 proteins have also been described to have functions distinct from their role as subunits of IL-27. EBI-3 has been reported to bind IL-12p35 and form the cytokine IL-35 (Collison et al., 2007), while the p28 molecule functions in the absence of EBI-3 as a receptor antagonist of gp130 that blocks signaling by IL-27 and IL-6. Thus, IL-27p28 has been shown to limit the development of germinal center B cells and the production of class-switched antibody after immunization (Stumhofer et al., 2010), limit Th1 and Th17 responses during experimental autoimmune uveitis (EAU) and experimental autoimmune encephalomyelitis (EAE) (Chong et al., 2014), and prolong survival of cell grafts into muscle (Shimozato et al., 2009). While several studies highlight the role of p28 as a receptor antagonist for gp130, there are reports that p28 alone is able to transduce a signal through this receptor (Airoldi et al., 2016; Petes et al., 2018).

Additional functions have also been described for the IL-27Rα subunit of the IL-27 receptor. For example, there are reports that IL-27Rα can complex with gp130 and CNTFRa to form a receptor for Humanin that limits neuronal cell death associated with Alzheimer's disease (Hashimoto et al., 2009). IL-27Rα also complexes with gp130 and IL-6Rα to from a receptor for a p28-CLF heterodimer that modulates the function of NK cells and T cells (Crabe et al., 2009). Additionally, IL-27Rα can be shed by T and B cells into the blood and functions as an endogenous antagonist to IL-27 by binding and preventing it from signaling (Dietrich et al., 2014). However, the phenotypes seen in *Il27ra*^{-/-} mice have largely been found to phenocopy those seen in *Il27p28*^{-/-} and *Ebi3*^{-/-}

mice (Hall et al., 2012a; Liu et al., 2017; Muallem et al., 2017; Villarino et al., 2003), suggesting that the primary role of these molecules in these systems is as subunits of IL-27 or the IL-27 receptor.

Roles of IL-27 in the immune response

When IL-27 was first described, it was found to promote NK and T cell proliferation and production of IFN-γ in vitro (Pflanz et al., 2002). Subsequent studies found IL-27 capable of promoting expression of CXCR3, T-bet, and the IL-12Rα in T cells, which increases IFN-γ production when cells are cultured with IL-12 (Lucas et al., 2003; Takeda et al., 2003; Yoshida et al., 2001). In addition to the homology of IL-27 to IL-6 and IL-12, and its ability to phosphorylate STAT1 and STAT3, these findings led to the conclusion that the primary role of IL-27 was to promote Th1 responses (Murphy and Reiner, 2002).

IL-27 and toxoplasmosis

During toxoplasmosis, IL-27 is produced primarily by monocytes, peaking at day 4-8 of infection (Kugler et al., 2013; Stumhofer et al., 2010). A study published in 2003 established a role for IL-27 in limiting the development of immunopathology during toxoplasmosis (Villarino et al., 2003). IL-27Rα-deficient mice were found to succumb to toxoplasmosis after only 2 weeks of infection. Parasite burden in *Il27ra*^{-/-} mice was low, serum levels of IFN-γ were high, and depletion of CD4^{+} T cells rescued the mice, leading to the conclusion that the increased susceptibility of *Il27ra*^{-/-} mice was due to CD4^{+} T cell-mediated immunopathology. This system has been used to identify multiple

mechanisms by which IL-27 limits the immune response: it suppresses production of IFN-γ (Villarino et al., 2003) and IL-2 (Villarino et al., 2006), limits the differentiation of Th17 cells (Stumhofer et al., 2006), promotes IL-10 production by Tregs and Tr1 cells (Awasthi et al., 2007; Fitzgerald et al., 2007; Stumhofer et al., 2007), and promotes the generation of T-bet[+] CXCR3^{+} Tregs that are specialized for suppressing Th1 responses (Hall et al., 2012a; Koch et al., 2009) (Figure 1.2). While these studies are informative, there are still gaps in our understanding of the mechanisms controlling these regulatory pathways and as discussed below, inhibitory receptors might provide a link between the roles of IL-27, IL-10 and IL-17 during toxoplasmosis.

IL-27 limits immunopathology in other infections

IL-27 has been found to limit immunopathology in several other infection systems. It limits IFN-γ production during infection with *Plasmodium berghei*, *Leishmania donovani*, and *Mycobacterium tuberculosis* (Findlay et al., 2010; Holscher et al., 2005; Rosas et al., 2006), limits IL-4 during *Trichuris muris* and *L. donovani* infection, and limits both IFN-γ and IL-4 during infection with *Trypanosoma cruzi* (Artis et al., 2004a; Artis et al., 2004b; Hamano et al., 2003). CD4^{+} T cell depletion reduces the immunopathology in *Il27ra*^{-/-} mice during infection with *P. berghei* and *L. donovani*, similar to what is seen during toxoplasmosis. In the context of Sendai virus infection, IL-27 deficiency leads to immunopathology in the lungs, associated with influx of eosinophils and alternatively activated macrophages (Muallem et al., 2017). This is accompanied by a decrease in CD4^{+} T cells that coproduce IFN-γ and IL-10 and an increase in CD4^{+} T cells that coproduce IFN-γ and IL-13 or IL-17. IL-27 also limits

immunopathology in the lung during respiratory syncytial virus infection (Pyle et al., 2017). Depletion of IL-27 is associated with increased numbers of virus-specific CD4$^+$ and CD8$^+$ T cells, increased production of IFN-γ, and decreased production of IL-10. Deficiency of the EBI-3 subunit of IL-27 results in T cell and macrophage infiltration into the CNS during infection with coronavirus (Tirotta et al., 2013), which is associated with increased IFN-γ production and lethal encephalomyelitis. While these studies demonstrate a broad role for IL-27 in limiting immunopathology, its common role in these diverse infections is to prevent the activation and cytokine production of CD4$^+$ and CD8$^+$ T cells from becoming dysregulated.

1.3   Inhibitory receptors and infection

A robust immune response is required to limit the survival and spread of infectious organisms, and primary or acquired immunodeficiencies are generally associated with increased susceptibility to infection. However, active suppressive mechanisms are required to ensure that this protective response does not cause collateral damage. In the absence of appropriate regulation, immune responses can become pathologic during acute infection, during chronic infection - such as in leprosy and Chagas disease - and even after resolution of infection, as in Guillain-Barre syndrome. As discussed above, some mechanisms include IL-27, IL-10, and regulatory T cells, and in recent years there has been an increased appreciation of the role of a wide variety of cell surface molecules expressed by immune cells in limiting the immune response. The term "inhibitory receptor" has been used to describe these molecules functionally, not by structural or sequence homology with each other, and they utilize a broad range of

mechanisms to modulate the immune system (Chen and Flies, 2013). Inhibitory receptors (IR) can function cell intrinsically, inhibiting the cell on which they are expressed, or they can inhibit target cells in a cell extrinsic fashion as they often function to inhibit signaling by antigen receptors or costimulatory molecules. In current models, TCR stimulation transiently upregulates the expression of multiple inhibitory receptors by T cells, while repeated TCR stimulation upregulates their expression during chronic infection or cancer and gives rise to an "exhausted" phenotype. A broad overview of IR function in diverse inflammatory environments has been reviewed elsewhere (Chen and Flies, 2013); this section will focus on those receptors that have well-defined roles in limiting the immune response during infection.

Role of inhibitory receptors during infection

During chronic inflammation in response to tumors or infection, T cells are found to develop an exhausted phenotype. This has been most thoroughly described on CD8^{+} T cells during infection with the clone 13 strain of LCMV. This chronic infection causes virus-specific CD8^{+} T cells to upregulate PD-1 and other inhibitory receptors, lose their ability to proliferate, and decrease production of IFN-γ and TNF-α (Wherry et al., 2007). The significance of these pathways is illustrated by the finding that PD-L1-deficient mice control infection with the clone 13 strain of LCMV but succumb to infection after 6-12 days due to immunopathology (Barber et al., 2006; Mueller et al., 2010). Antibody blockade of PD-L1 restores the ability of CD8^{+} T cells to produce cytokines and control viral titers without resulting in overt immunopathology (Barber et al., 2006). Exhausted CD8^{+} T cells also upregulate LAG-3 and TIM-3 during LCMV and coblockade of PD-L1

and LAG-3 or PD-1 and TIM-3 reverses exhaustion more effectively than blockade of any of these inhibitory receptors alone, leading to increased degranulation, IFN-γ production, and control of virus burden (Blackburn et al., 2009; Jin et al., 2010). These principles are applicable to a range of murine and clinical settings, including human immunodeficiency virus (HIV) (Day et al., 2006; Jones et al., 2008; Kaufmann et al., 2007), hepatitis C virus (HCV) (Urbani et al., 2006) and hepatitis B virus (HBV) (Boni et al., 2007).

T cell exhaustion is also characteristic of chronic infection with intracellular parasites. At the chronic stage of toxoplasmosis, CD8$^+$ T cells upregulate PD-1 and begin to lose the ability to produce IFN-γ and granzyme B and to kill infected cells (Bhadra et al., 2012; Bhadra et al., 2011; Wilson et al., 2009). Antibody blockade of PD-L1, a ligand for PD-1, reinvigorates the function of parasite-specific polyfunctional CD8$^+$ T cells, prevents recrudescence of the parasite, and leads to improved survival. During acute infection with *Leishmania donovani*, T cell responses are amplified with blockade of CTLA-4 and during chronic *L. donovani* infection, exhausted T cells are reinvigorated with PD-L1 blockade (Joshi et al., 2009; Murphy et al., 1998). During blood-stage *Plasmodium falciparum* infection, CD4$^+$ T cells express LAG-3 and PD-L1 and have limited ability to produce IFN-γ, TNF-α, and IL-2. Co-blockade of LAG-3 and PD-L1 restores CD4$^+$ T cell cytokine production and decreases parasitemia (Butler et al., 2011).

In other infectious systems, blockade of IR function results in immunopathology. Infection of mice with *Plasmodium berghei* ANKA results in cerebral malaria associated with T cell expression of CTLA-4 and PD-1. Blockade of CTLA-4 and PD-L1 increases migration of CD8$^+$ T cells to the brain and results in increased incidence of cerebral

malaria and decreased survival (Hafalla et al., 2012). Blockade of the inhibitory receptor CD200R during infection with influenza virus results in increased IFN-γ production by T cells and immunopathology in the lung, which delays resolution of infection and worsens outcome (Snelgrove et al., 2008). A complementary study examined the role of CD200R by treatment with CD200-Fc, which binds CD200R agonistically. During herpes simplex virus infection, which typically results in immunopathology in the cornea, CD200-Fc treatment limits production of IL-12 and IFN-γ, and limits infiltration of inflammatory cells into the cornea, ameliorating disease. (Sarangi et al., 2009). These studies demonstrate that inhibitory receptors can limit both protective and pathological immune responses in diverse infectious settings.

Regulation of inhibitory receptor expression

Given the ability of IRs to limit immunopathology, limit productive immune responses, and promote T cell exhaustion, it is important to understand the signals that regulate their expression. At the time that the studies described here were initiated, the induction of IRs was thought to be primarily controlled by TCR stimulation. Indeed, as shown in Chapter 2 and 3, TCR stimulation induces expression of diverse IRs on T cells. During chronic inflammation, repeated TCR stimulation results in further upregulation of inhibitory receptors in the phenomenon of immune exhaustion that is seen during chronic infection and cancer (Wherry and Kurachi, 2015). Other studies have found that expression of inhibitory receptors by T cells is not only a result of TCR stimulation, but depends on the cytokine milieu present when the T cell is activated. Studies largely performed *in vitro* have found a role for Type I IFN, IL-2, IL-7, IL-15, IL-21, and IL-27

in driving the expression of PD-1, PD-L1, LAG-3, TIM-3, and TIGIT by a variety of T cell populations (Boivin et al., 2015; Bruniquel et al., 1998; Chihara et al., 2018; Kinter et al., 2008). Fewer studies have been performed during infection, but IL-2 was found to promote PD-1 expression by CD4$^+$ and CD8$^+$ T cells in HIV patients (Kinter et al., 2008).

Inhibitory receptors are also expressed by diverse cell lineages beyond T cells and multiple signals have been found that upregulate their expression by myeloid cells. Stimulation of human monocytes with LPS or the TLR7/8 agonist R848 early in differentiation upregulates PD-L1 and PD-L2 and results in tolerogenic immature DCs that produce IL-6 and IL-10 (Wolfle et al., 2011). Stimulation with poly(I:C) or LPS upregulates PD-L1 on peripheral blood DCs as well (Karakhanova et al., 2010). Studies performed in non-infectious systems have found that that IFN-β, IL-2, IL-7, IL-15, IL-21, and IL-27 have roles in promoting PD-1 or PD-L1 in myeloid populations (Karakhanova et al., 2011; Kinter et al., 2008; Schreiner et al., 2004). While less thoroughly studied, non-hematopoietic cell inhibitory receptor expression has also been identified that regulates the immune response. IFN-γ drives expression of PD-L1 on endothelial cells, tumor cell lines, and retinal pigment epithelium, limiting NK cell killing and T cell infiltration into sites of inflammation (Bellucci et al., 2015; Eppihimer et al., 2002; Ke et al., 2010).

1.4   Summary

The immune system utilizes multiple active regulatory mechanisms to limit the collateral damage that can accompany a strong immune response. Infection of mice with *T. gondii* provides a sensitive system to examine these pathways, as several of them (IL-

10, IL-27, TGF-β) have been found to be required to avoid lethal immunopathology during toxoplasmosis. Therefore, this system has been useful to identify mechanisms by which IL-27 limits the immune response. The following chapters describe the impact of IL-27 on T cell expression of multiple IR, a class of molecules that could link multiple immunoregulatory pathways during toxoplasmosis.

Figures
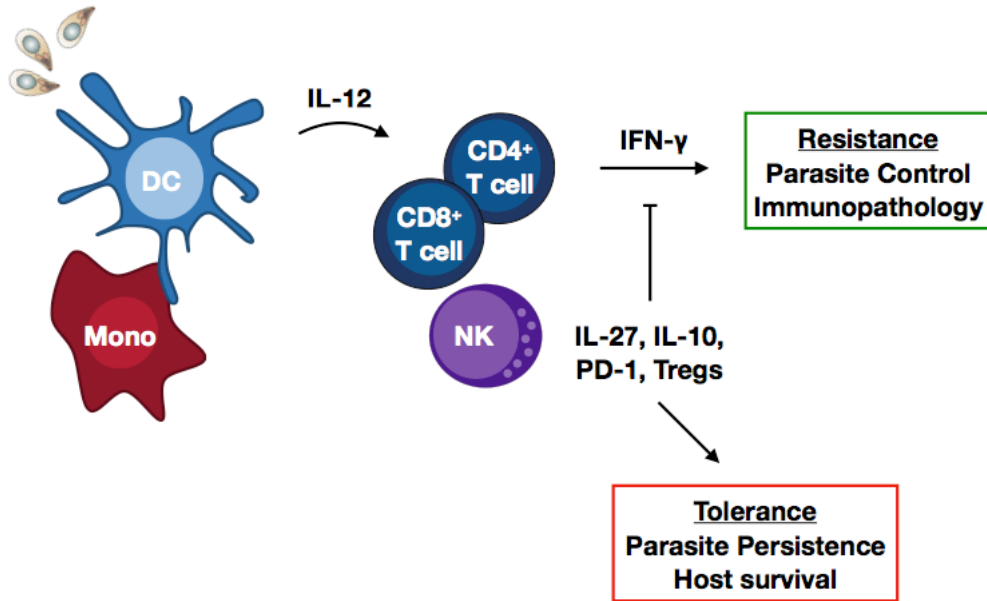
Figure 1.1 Balance of parasite resistance and tissue tolerance during toxoplasmosis.
In response to infection with T. gondii, myeloid cells produce IL-12, which induces
production of IFN-γ by NK cells and T cells as part of a Th1 immune response required
to control parasite replication. However, several active regulatory mechanisms, namely
IL-27, IL-10, PD-1 and regulatory T cells, help limit this immune response and prevent it
from becoming pathological.
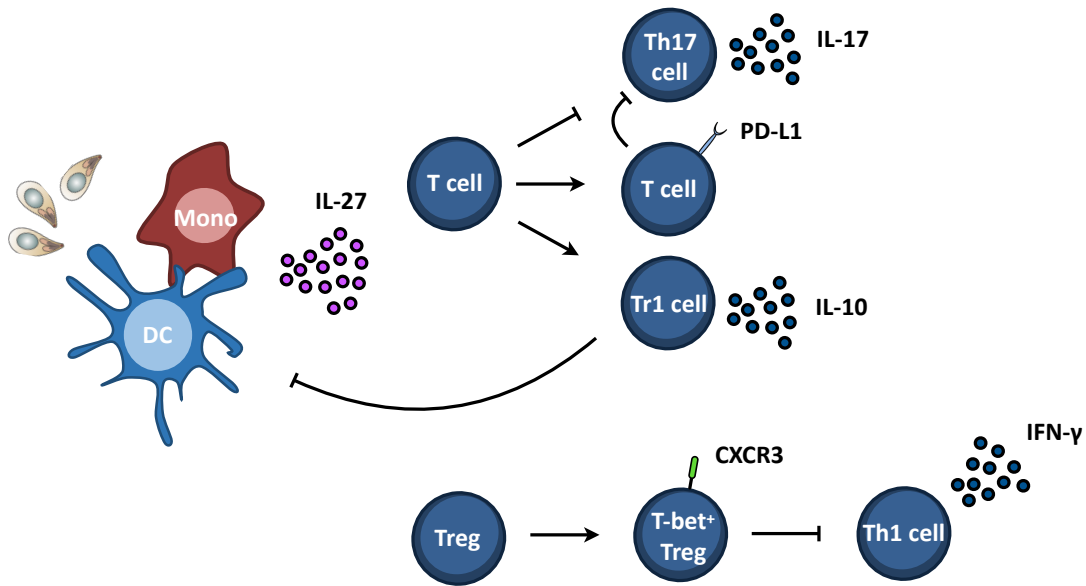
Figure 1.2 Role of IL-27 during toxoplasmosis. During toxoplasmosis, IL-27 is produced by inflammatory monocytes and dendritic cells. It promotes differentiation of T-bet+CXCR3+ Tregs that are specialized for controlling Th1 inflammation. It also promotes upregulation of PD-L1 on T cells, which limits Th17 differentiation. IL-10 also promotes differentiation of Tr1 cells, which produce IL-10. IL-10 limits activation of myeloid cells and is necessary for limiting immunopathology during toxoplasmosis.

Chapter 2: Cytokine- and TCR-mediated regulation of T cell expression of Ly6C and Sca-1

Abstract

Ly6C and Sca-1 (Ly6A/E) are Ly6 family GPI-anchored surface molecules that are differentially expressed by multiple immune populations. Ly6C expression has been used to distinguish short-lived effector CD4$^+$ T cells from memory precursor effector cells, whereas Sca-1 has been used in the identification of stem-like memory T (Tscm) cells. The present study examines the expression patterns of these molecules and establishes that, *in vitro*, IL-27, type I IFN and IFN-γ are potent inducers of Ly6C and Sca-1 in naïve murine CD4$^+$ and CD8$^+$ T cells, while TGF-β limits their expression. The induction of Ly6C and Sca-1 by IL-27 and IFN-γ is dependent on STAT1, but not STAT3 or T-bet. *In vivo*, at homeostasis, Ly6C and Sca-1 expression was not restricted to effector cells, but was also found at various levels on naïve and memory populations. However, in response to infection with *Toxoplasma gondii*, pathogen-specific T cells expressed high levels of these molecules and in this context, endogenous IL-27 and IFN-γ were required for the expression of Ly6C but not Sca-1. Together, these findings highlight the TCR-dependent and cytokine-mediated signals that modulate T cell expression of Ly6C and Sca-1 *in vitro* and *in vivo* during infection.

Introduction

The T cell response to infection or immunization involves the generation of minimally differentiated memory cells as well as highly differentiated effector cells

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(Joshi and Kaech, 2008). Effector T cells produce the cytokines, granzymes, and other molecules necessary for immediate pathogen control, while central memory T cells are long-lived and can differentiate into effector cells upon rechallenge (Joshi and Kaech, 2008; Kaech and Cui, 2012; Murali-Krishna and Ahmed, 2000). The identification of surface molecules that distinguish effector and memory T cell populations has allowed researchers to track the expansion, evolution and contraction of the T cell response during infection and has provided insights into how these cells operate. For example, central memory cells upregulate CD44 and express CD62L, which keeps them localized to lymphoid organs. Effector cells are also CD44^{hi} but lose expression of CD62L, allowing them to home to sites of inflammation. While these definitions have proven useful to define naïve, effector and memory T cells, additional markers, including KLRG1, CXCR3 and Ly6C have been used to further sub-divide these populations (Chu et al., 2016; Gerlach et al., 2016; Hand et al., 2007; Marshall et al., 2011; Peters et al., 2014). In current models, a subset of highly-differentiated, short-lived effector cells (SLECs), which for CD8$^+$ T cells are often identified by expression of KLRG1 (Hand et al., 2007), are specialized to control acute infection. These cells produce high levels of cytokines and granzymes and survive poorly upon adoptive transfer to naïve hosts. Less-differentiated CD8$^+$ T cells, identified during acute toxoplasmosis as CXCR3$^+$KLRG1$^-$, exhibit the longevity, proliferative capacity and differentiation potential typical of memory cells (Chu et al., 2016).

Ly6C and Sca-1 (Ly6A/E) are members of a family of 21 Ly6-like proteins in mice, with 20 Ly6 family homologues in humans (Kong and Park, 2012). Expression of Ly6C has been used to identify highly differentiated effector CD4$^+$ T cells (Hu et al.,

2015; Marshall et al., 2011; Walunas et al., 1995). Among virus-specific effector CD4$^+$ T cells, Ly6C$^+$ cells produced more cytokines and effector molecules than Ly6C$^-$ cells (Hu et al., 2015; Marshall et al., 2011). Conversely, Sca-1, in combination with the IL-2R beta chain (CD122) and Bcl-2, has been used to identify mature CD8$^+$ T cells with stem-like properties, termed stem cell memory (Tscm) cells (Gattinoni et al., 2011; Gattinoni et al., 2009; Zhang et al., 2005).

The finding that Ly6C is preferentially expressed by CD4$^+$ SLECs during acute infection suggests that identification of the factors that modulate Ly6C expression could yield insights into the signals that control the development of memory and effector populations. For example, sustained TCR signals have been found to skew T cells toward terminal differentiation and away from memory development (Joshi and Kaech, 2008; Lanzavecchia and Sallusto, 2002), while multiple cytokines influence the development of effector and memory populations (Obar et al., 2011). Relevant to this report, type I interferons, IFN-γ, and IL-27 have previously been implicated in promoting Ly6C$^+$ and Sca-1$^+$ populations in CD4$^+$, CD8$^+$ and regulatory T cells (Dumont et al., 1986; Hall et al., 2012a; Liu et al., 2013; Schlueter et al., 2001; Zhu et al., 2016). However, previous studies have not been able to distinguish whether these stimuli induce Ly6C and Sca-1 expression or simply promote the outgrowth of Ly6C$^+$ or Sca-1$^+$ populations. Furthermore, extant reports have not examined how these cytokines intersect with TCR signaling to impact Ly6C and Sca-1 expression and what roles these signals play in modulating the expression of these molecules *in vivo*. In utilizing sorted Ly6C$^-$Sca-1$^-$ populations, the present study was able to show that TCR stimulation alone induces Sca-1

on CD4$^+$ and CD8$^+$ T cells but is not sufficient to induce Ly6C on CD4$^+$ T cells. The cytokines IL-27, IFN-γ and type I IFN were found to broadly promote the expression of Ly6C and Sca-1, while TGF-β inhibited this expression. This cytokine-mediated induction of Ly6C and Sca-1 was largely STAT1-dependent and is not dependent on STAT3 or T-bet. A survey of naïve and antigen-experienced T cells in a range of differentiation states found that Ly6C expression is not restricted to effector cells and that Sca-1 expression is not limited to naïve/memory-like cells. Furthermore, *in vivo* studies during infection with *Toxoplasma gondii* identified a role for endogenous IL-27 and IFN-γ in promoting effector cell expression of Ly6C. Together, these studies provide new insights into the signals that modulate the development of Ly6C$^+$ and Sca-1$^+$ T cell populations but indicate caution in the use of these molecules as markers of highly-differentiated effector cells (Ly6C) and minimally-differentiated stem cell memory cells (Sca-1).

Results

IL-27 promotes expression of Ly6C and Sca-1 on CD4$^+$ and CD8$^+$ T cells

Given previous studies that implicated IL-27 in the regulation of Ly6C on Treg cells (Hall et al., 2012a) and Sca-1 on CD8$^+$ T cells *in vitro* and CD4$^+$ T cells *in vivo* (Liu et al., 2013; Zhu et al., 2016), initial experiments were performed to determine the relationship between IL-27 signaling and TCR stimulation in modulating expression of these molecules. Multiple experiments using bulk CD4$^+$ or CD8$^+$ splenocyte cultures showed that IL-27 in combination with TCR potently promotes T cell expression of

Ly6C and Sca-1 (Fig 2.S1). Because subpopulations of splenic CD4$^+$ and CD8$^+$ T cells express Ly6C and/or Sca-1, these experiments were repeated with sort-purified Ly6C$^-$ Sca-1$^-$ naïve (CD44^{lo}CD62L$^+$) T cells (>90% purity). Cells were then labeled with CFSE and cultured in the presence or absence of plate-bound anti-CD3 and soluble anti-CD28 stimulation (henceforth referred to as TCR stimulation) with IL-2, IL-27, and neutralizing anti-IFN-γ and anti-IL-4 antibodies (Fig 2.1A). After three days of culture in the absence of TCR stimulation or IL-27, the cells did not express Ly6C. Culture of CD4$^+$ or CD8$^+$ T cells with IL-27 alone induced modest Ly6C expression. TCR stimulation on its own induced robust proliferation (as seen by CFSE dilution) but did not induce expression of Ly6C on naïve CD4$^+$ T cells, and induced Ly6C on a small percentage of CD8$^+$ T cells. However, when naïve CD4$^+$ and CD8$^+$ T cells were provided TCR stimulation combined with IL-27, there was a synergistic effect on Ly6C expression, which was apparent even during early divisions. It is notable that among CD4$^+$ T cells, TCR stimulation alone did not induce Ly6C expression, even in those cells that had proliferated. These results demonstrate that Ly6C is not a general activation marker on CD4$^+$ T cells, but in these experiments requires TCR activation in the presence of IL-27.

When the role of IL-27 and TCR stimulation in the regulation of Sca-1 was examined, each stimulus alone was sufficient to promote high expression of Sca-1 by CD4$^+$ and CD8$^+$ T cells (Fig 2.1B). However, TCR stimulation in the presence of IL-27 resulted in further induction of Sca-1 expression, which was upregulated in early divisions and maintained as cells divided. We previously reported that IL-2 downregulates the IL-27 receptor (Villarino et al., 2005), raising the possibility that the

exogenous IL-2 used in these cultures might limit the effect of IL-27. However,

exogenous IL-2 did not limit expression of Ly6C and Sca-1 by CD4$^+$ T cells and

enhanced their expression by CD8$^+$ T cells (Fig 2.S2). These results demonstrate that IL-

27 has a major impact on Sca-1 and Ly6C expression, and that Sca-1 is more readily

induced by these stimuli than Ly6C.

Expression of Ly6C and Sca-1 is modulated by multiple cytokines

Given the impact of IL-27 on Ly6C and Sca-1, studies were performed to assess

whether other cytokines (IFN-γ, type I IFN, IL-6, IL-12, IL-4, IL-10, IL-7, IL-15, and

TNF-α) modulate expression of these molecules. Ly6C$^-$Sca-1$^-$ naïve (CD62L$^+$CD44^{lo}) T

cells were sort purified as described above and were given TCR stimulation in the

presence of the different cytokines for 72 hours before being assayed for expression of

Ly6C or Sca-1. Across multiple experiments, IFN-γ, type I IFN, and IL-12 induced

expression of Ly6C on naïve Ly6C$^-$ CD4$^+$ T cells, though not as robustly as IL-27 (Fig

2.2A). For naïve CD8$^+$ T cells, both IFN-γ and type I IFN induced expression of Ly6C

but type I IFN was consistently the strongest inducer of Ly6C in these experiments (Fig

2.2B). Stimulation of naïve CD4$^+$ or CD8$^+$ T cells with TCR alone induced high

expression of Sca-1, but this was not further upregulated by cytokines (data not shown).

IL-4, IL-10, IL-7, IL-15 and TNF-α did not modulate expression of Sca-1 or Ly6C on

CD4$^+$ or CD8$^+$ T cells (Fig 2.2, bar charts). Although there is variation in the impact of

individual cytokines on different T cell populations, when taken together, these data

identify two groups of related cytokines (the interferons and the IL-6 family members) that utilize similar signaling pathways that promote T cell expression of Ly6C and Sca-1.

Requirements for IL-27-mediated upregulation of Ly6C and Sca-1

There are several common elements to the signaling pathways used by the cytokines that most robustly induced expression of Ly6C and Sca-1, specifically the ability to activate STAT1 and to upregulate expression of T-bet (Hall et al., 2012b; Lazarevic et al., 2013; Platanias, 2005). We previously reported that Ly6C expression is T-bet-dependent *in vivo* during toxoplasmosis (Harms Pritchard et al., 2015), but culture of T-bet$^{-/-}$ T cells demonstrated that T-bet is not required for IL-27-mediated induction of Ly6C and Sca-1 *in vitro* (Fig 2.S3A, B). These findings agree with an earlier study that found that stimulation with anti-CD3/28 antibodies overcomes the need for T-bet in the induction of Ly6C (Sullivan et al., 2003). A single experiment using T cells from STAT3$^{fl/fl}$ x CD4-Cre mice suggested that STAT3 is not required for IL-27-mediated expression of Ly6C or Sca-1 on CD4$^+$ or CD8$^+$ T cells (Fig 2.S3C, D). In contrast, when naïve CD8$^+$ Ly6C$^-$Sca-1$^-$ T cells from STAT1$^{-/-}$ mice were given TCR stimulation in the presence of IL-27 or IFN-γ, the induction of Ly6C was found to be almost entirely STAT1-dependent (Fig 2.3A), as was the induction of Sca-1 (Fig 2.3B). However, the type I IFN-mediated induction of Ly6C and Sca-1 was not STAT1-dependent in this system (Fig 2.3A, B). Similar results were seen for CD4$^+$ T cells (Fig 2.3C, D). These results demonstrate a key role for STAT1 in IL-27 and IFN-γ-mediated induction of Ly6C and Sca-1.

TGF-β antagonizes Ly6C and Sca-1 expression

While multiple cytokines promote expression of Ly6C and Sca-1, this screening process revealed that TGF-β was a potent inhibitor of the expression of these molecules. The addition of TGF-β reduced IL-27-mediated expression of Ly6C on CD4$^+$ T cells (Fig 2.4A, C) and CD8$^+$ T cells (Fig 2.4B, C), which is similar to results from a previous study that used P14 cells in the context of LCMV infection (Casey et al., 2012). Addition of TGF-β also limited proliferation of CD4$^+$ and CD8$^+$ T cells, as illustrated by the reduced dilution of CFSE (Fig 2.4A, B, D, E). This was true in the presence and absence of IL-27 and is consistent with the ability of TGF-β to limit the proliferation and differentiation of naïve T cells into terminally-differentiated effector cells (Gorelik and Flavell, 2002). Addition of TGF-β also reduced TCR-mediated induction of Sca-1 on CD4$^+$ T cells (Fig 2.4D, F) and on CD8$^+$ T cells (Fig 2.4E, F), but did not affect the expression of Sca-1 in the presence of IL-27. The relatively modest effect that TGF-β has on IL-27-mediated expression of Sca-1 is consistent with the data in earlier figures that Sca-1 is more robustly expressed than Ly6C, and also indicates that the inhibitory effects of TGF-β is most closely associated with reduced TCR signaling.

Analysis of Ly6C and Sca-1 expression on diverse T cell populations

Ly6C has been used to identify terminally-differentiated effector CD4$^+$ T cells during infection (Hu et al., 2015; Marshall et al., 2011; Walunas et al., 1995), but its expression during homeostasis is not well described. To determine which T cell

populations express Ly6C under homeostatic conditions, a survey of uninfected SPF mice was conducted. The expression of high levels of CD44 was used to identify antigen-experienced cells (Budd et al., 1987) and CD62L was used to identify cells that home to lymph nodes, which are primarily naïve and central memory cells (Lefrancois, 2006). In this setting, 20% of naïve (CD62L$^+$CD44^{lo}) and 10% of memory (CD62L$^+$CD44^{hi}) CD4$^+$ T cells expressed Ly6C and only 10% of CD4$^+$ T cells with an effector phenotype (CD62L$^-$CD44^{hi}) were Ly6C$^+$ (Fig 2.5A). For CD8$^+$ T cells, 20% of naïve (CD44^{lo}CD62L$^+$) CD8$^+$ T cells expressed Ly6C, while approximately 30% of CD44^{hi}CD62L$^-$ CD8s and 90% of CD44^{hi}CD62L$^+$ cells expressed Ly6C. Thus, Ly6C expression was concentrated on the antigen-experienced cells, but further subsetting based on T cell expression of CD25, KLRG1, CD127, CD69 or CD49d indicated that Ly6C expression is not restricted to a particular effector/memory T cell population (data not shown). However, it is notable that in this survey, the majority of CD122$^+$ CD8$^+$ T cells expressed Ly6C, regardless of their expression of CD62L and CD44 (Fig 2.S4). CD122 is the beta subunit of the IL-2 and IL-15 receptors and along with CD44 and Ly6C, is upregulated on T cells undergoing homeostatic proliferation (Cho et al., 2000; Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000).

When a similar survey was performed to assess the expression of Sca-1 on naïve, effector, and memory T cells in SPF mice, 20% of naïve (CD44^{lo}CD62L$^+$) CD4$^+$ T cells expressed Sca-1, while 80% of CD44^{hi}CD62L$^-$ effector CD4$^+$ T cells were Sca-1$^+$ (Fig 2.5B). Similarly, minimal Sca-1 expression was seen in CD44^{lo}CD62L$^+$ CD8$^+$ T cells, while the highest Sca-1 expression by CD8$^+$ T cells (15%) was seen in the CD44^{hi}CD62L$^-$ population (Fig 2.5B). These findings indicate that Sca-1 was enriched

on CD44^{hi} CD4$^+$ and CD8$^+$ T cells, but in this survey was not exclusively expressed by any particular effector/memory population examined.

Ly6C and Sca-1 are upregulated on pathogen-specific T cells during toxoplasmosis

The above survey utilized established surface markers to distinguish antigen-experienced and naïve cells, but in this setting, it is difficult to determine how expression of Ly6C or Sca-1 correlates with previous antigen exposure. To examine an effector population with a well-defined history, mice were infected with *T. gondii* and the expression of Ly6C and Sca-1 on parasite-specific T cells during acute toxoplasmosis was examined. Mice were infected intraperitoneally with *T. gondii* and spleens and peritoneal exudate cells (PECs) were harvested 10 days post-infection. Toxoplasma-specific T cells were identified by staining with parasite-specific MHCI or MHCII tetramers in combination with high expression of LFA-1 (Dupont et al., 2014). At day 10 post infection, the majority of parasite-specific CD4$^+$ and CD8$^+$ T cells in the peritoneum expressed Ly6C, demonstrating that toxoplasmosis promotes Ly6C expression by parasite-specific T cells (Fig 2.6A).

A recent study (Chu et al., 2016) proposed that during toxoplasmosis, minimally-differentiated memory CD8$^+$ T cells that are CXCR3$^+$KLRG1$^-$ give rise to an intermediate CXCR3$^+$KLRG1$^+$ population that in turn downregulates CXCR3 when it differentiates into terminally-differentiated effector cells. Consistent with this previous report, at day 10 of infection, 20% of splenic parasite-specific CD8$^+$ T cells were CXCR3$^+$KLRG1$^-$, 60% were CXCR3$^+$KLRG1$^+$ and less than 10% were CXCR3$^-$KLRG1$^+$. This analysis was extended to CD4$^+$ T cells, in which 20% were

CXCR3$^+$KLRG1$^-$, 30% were CXCR3$^+$KLRG1$^+$ and 30% were CXCR3$^-$KLRG1$^+$ (Fig 2.6B). 70% of parasite-specific CD4$^+$ T cells expressed Ly6C (Fig 6A). When the cells were subsetted by expression of CXCR3 and KLRG1, Ly6C was expressed by 60% of CXCR3$^+$KLRG1$^-$, 75% of CXCR3$^+$KLRG1$^+$, and 70% of CXCR3$^+$KLRG1$^-$ parasite-specific CD4$^+$ T cells. Similar results were seen for CD8$^+$ T cells, as Ly6C was expressed by 90% of CXCR3+KLRG1$^-$, 95% of CXCR3+KLRG1$^+$, and 85% of CXCR3$^-$KLRG1$^+$ parasite-specific CD8$^+$ T cells. Therefore, Ly6C expression on these individual subsets was not exclusive to the KLRG1$^+$CXCR3$^-$ population for CD4$^+$ or CD8$^+$ T cells, demonstrating that Ly6C and KLRG1 are not interchangeable markers of differentiation (Fig 2.6C).

When a similar analysis was performed for Sca-1, 85% of parasite-specific CD4$^+$ and CD8$^+$ T cells expressed Sca-1 (Fig 2.6D). When these cells were subsetted by their expression of KLRG1 and CXCR3, approximately 90% of the CXCR3$^+$KLRG1$^-$ and CXCR3$^+$KLRG1$^+$ populations expressed Sca-1 in both CD4$^+$ and CD8$^+$ T cells. 70% of CXCR3$^-$KLRG1$^+$ CD4$^+$ T cells and 60% of CXCR3$^-$KLRG1$^+$ CD8$^+$ T cells expressed Ly6C, indicating that Sca-1 is present at a lower frequency on more highly differentiated cells. Nonetheless, Sca-1 is more widely expressed during toxoplasmosis than KLRG1 or CXCR3, and does not enable the ready differentiation of distinct antigen-experienced T cell populations in this experimental system.

IL-27 and IFN-γ promote T cell expression of Ly6C during toxoplasmosis

IL-27 and IFN-γ are key cytokines during toxoplasmosis (Suzuki et al., 1988; Villarino et al., 2003) and are two of the strongest inducers of Ly6C and Sca-1 *in vitro*. To determine the contribution of IL-27 and IFN-γ signaling to the expression of Ly6C and Sca-1 during infection, WT and IL-27 receptor (*Il27ra*)-deficient mice were infected with *T. gondii*. Mice were also treated with an isotype antibody or a neutralizing anti-IFN-γ antibody on day 3 and 6 of infection. When peritoneal tetramer-positive T cells were examined at day 9 of infection, the percentage of parasite-specific CD4$^+$ T cells expressing Ly6C was substantially lower in *Il27ra*-deficient mice than in WT mice. Ly6C levels were significantly reduced in CD8$^+$ T cells as well, providing evidence that IL-27 promotes the Ly6C$^+$ population in this system (Fig 2.7A, B). Additionally, neutralizing IFN-γ resulted in less Ly6C expression by CD4$^+$ T cells but in *Il27ra*-deficient mice did not result in a complete ablation of Ly6C expression. In contrast, the absence of the IL-27 receptor did not limit the expression of Sca-1 by parasite-specific T cells. Surprisingly, the blockade of IFN-γ increased Sca-1 expression in both WT and *Il27ra$^{-/-}$* mice, possibly because the absence of IFN-γ leads to a marked increase in parasite replication and antigen load that might lead to increased T cell activation. Together, these studies establish that IL-27 and IFN-γ are involved in the regulation of Ly6C expression during toxoplasmosis, but in this setting they were not required for maximal Sca-1 expression.

Discussion

Studies to understand the functions of Ly6 molecules have been performed since the 1970s, but questions still remain about their functions and the factors that influence their expression (Shevach and Korty, 1989). The association of Ly6C expression with short-lived effector T cells has been reported in multiple experimental systems (Hu et al., 2015; Marshall et al., 2011) while the combination of Sca-1, CD122 and Bcl-2 can be used to identify CD8$^+$ stem cell memory (Tscm) cells (Gattinoni et al., 2011; Gattinoni et al., 2009; Zhang et al., 2005). A previous study showed that treatment of mice with IL-27 promotes the development of a memory precursor population of tumor antigen-specific CD8$^+$ T cells, characterized by high expression of Bcl-6, SOCS3, and Sca-1 (Liu et al., 2013). However, the expression patterns of Ly6C and Sca-1 have not been compared and there is a limited appreciation of how cytokine and TCR-mediated signals are integrated to promote their expression. The *in vitro* experiments performed here utilized a defined population of naïve Ly6C$^-$ CD4$^+$ and CD8$^+$ T cells to address any concerns that the stimuli used here might preferentially expand a Ly6C$^+$ population. This approach showed quite modest effects of IL-27, the interferons, or TCR alone on Ly6C expression, but the combination of cytokine plus TCR synergistically promoted the expression of Ly6C by a subset of activated CD4$^+$ and CD8$^+$ T cells. That this activity was STAT1-dependent correlated well with the range of cytokines that could modulate Ly6C, while cytokines that predominantly utilize STAT3, STAT4 or STAT6 had minimal effects. TGF-β was the only signal identified that suppressed the induction of Ly6C and Sca-1, which

correlates with its ability to suppress T cell activation and proliferation (Gorelik and Flavell, 2000; Kehrl et al., 1986).

Since Ly6C and Sca-1 have been used as markers to identify T cells at different stages of differentiation, it was notable that the expression of these two molecules was upregulated by the same cytokine signals, in the same cells. Sca-1 was potently induced by either TCR stimulation or cytokine signaling and consequently appeared to be more widely expressed than Ly6C. Indeed, a survey to determine if either of these molecules could be associated with different effector or memory populations found that it was difficult to link them to memory-like or terminally-differentiated effector cells based on differential expression of KLRG1 and CXCR3. The ability of these cytokines to induce Ly6C and most notably Sca-1 in the absence of TCR stimulation suggest the need for caution in using these molecules alone to identify Ag-experienced populations.

Infection with *T. gondii* is dominated by the generation of parasite-specific CD4$^+$ and CD8$^+$ T cells that produce IFN-γ, but this is also a system in which endogenous IL-27 is required to limit the inflammatory response (Villarino et al., 2003). While the loss of either cytokine signal during infection reduces Ly6C expression, it was relevant to note that in the setting of IL-27R-deficiency, there are markedly elevated levels of IFN-γ (Villarino et al., 2003), but IFN-γ blockade did not result in a further reduction in the numbers of parasite-specific effectors that expressed Ly6C. The observation that IL-27 was more important in driving Ly6C in CD4$^+$ T cells than in CD8$^+$ T cells during toxoplasmosis is consistent with the larger effect of interferons in promoting Ly6C in

CD8$^+$ T cells seen *in vitro*. Nevertheless, the *in vivo* studies presented here indicate that

IL-27 and IFN-γ are not redundant in promoting Ly6C expression during toxoplasmosis.

 While the section above focuses on the regulation of Ly6C, the *in vitro* and *in vivo* studies identified common pathways that influence expression of Ly6C and Sca-1, but also highlighted some notable differences. Again, the ability to utilize a defined, naïve, Sca-1$^-$ starting population helped establish the profound impact of TCR stimulation alone or in combination with cytokines on Sca-1 expression. As seen for Ly6C, not every cytokine was a potent inducer of Sca-1 and those that activated STAT1 seemed dominant, although the use of STAT1-deficient cells does indicate the presence of additional pathways that are involved in this process. Potential STAT1-independent pathways relevant to the ability of IL-27 and type I IFNs include p38 MAPK and ERK1/2 (Hall et al., 2012b; Platanias, 2005). However, unlike Ly6C, Sca-1 expression was not attenuated by the loss of IL-27 or IFN-γ in *in vivo* studies. This contrasts with an earlier report using a model of colitis, in which *in vivo* administration of an AAV vector encoding IL-27 was associated with reduced inflammation and increased expression of Sca-1 by CD4$^+$ T cells (Zhu et al., 2016). This contradiction likely reflects the complexity in trying to distinguish a role for endogenous IL-27 or IFN-γ in a systemic infection, in which many signals including TCR and other cytokines readily promote Sca-1 expression, versus a dominant signal provided by overexpression of IL-27.

 There have been few studies that have directly addressed the function of Ly6C and Sca-1 in the immune response. Ly6C has been proposed to be involved in T cell homing to secondary lymphoid organs, possibly through an association with LFA-1

(Hanninen et al., 1997; Hanninen et al., 2011; Jaakkola et al., 2003), and ImageStream analysis of *T. gondii*-specific effectors shows co-localization of Ly6C and CD11a (a subunit of LFA-1) on the surface of these cells (Harms Pritchard et al., 2015). IL-27 has been proposed to modulate T cell homing through upregulation of ICAM-1 and selectin ligands on conventional CD4^{+} T cells (Ebel et al., 2015; Hall et al., 2012a; Owaki et al., 2005), as well as CXCR3 on Tregs (Hall et al., 2012a). Thus, upregulation of Ly6C may be an additional mechanism by which IL-27 and/or interferons modulate T cell trafficking. There is also a literature that suggests a regulatory role for Ly6C and Sca-1 in limiting the T cell response. A mutation in the promoter of Ly6C reduces its expression in NOD, NZB/W and ST mice, which are strains that spontaneously develop autoimmune diseases (Philbrick et al., 1990). Moreover, in the context of TCR stimulation, antibodies that crosslink Ly6C or Sca-1 on the surface of T cells limit their ability to produce IL-2 and proliferate (Codias et al., 1992; Fleming and Malek, 1994; Yamanouchi et al., 1998). Furthermore, in mice genetically engineered to lack Sca-1 expression, T cells exhibit enhanced proliferation in response to TCR stimulation (Stanford et al., 1997). Additional evidence for a regulatory function of Sca-1 is the finding that transgenic overexpression of Sca-1 limits T cell proliferation (Codias et al., 1992; Fleming and Malek, 1994; Henderson et al., 2002) and suppresses lymphoproliferation and autoimmunity in *lpr/lpr* mice (Henderson et al., 2002). Together, these findings suggest that Ly6C and/or Sca-1 may have a role in limiting T cell responses. A suppressive function for Ly6C and Sca-1 would complement reports that IL-27 promotes inhibitory pathways including IL-10 and LAG-3 (Awasthi et al., 2007; Do et al., 2015; Hirahara et al., 2012; Karakhanova et al., 2011; Mascanfroni et al., 2013; Moon et al., 2013; Stumhofer et al., 2007), and that IL-27

and IFN-γ promote expression of PD-L1 (Bellucci et al., 2015; Hirahara et al., 2012; Ke et al., 2010). Additional studies are needed to determine whether Ly6C and Sca-1 primarily function to promote T cell activation and migration, or if any of the shared immune-regulatory effects of IL-27 and the IFNs are mediated through the induction of Ly6C and/or Sca-1.

Figures
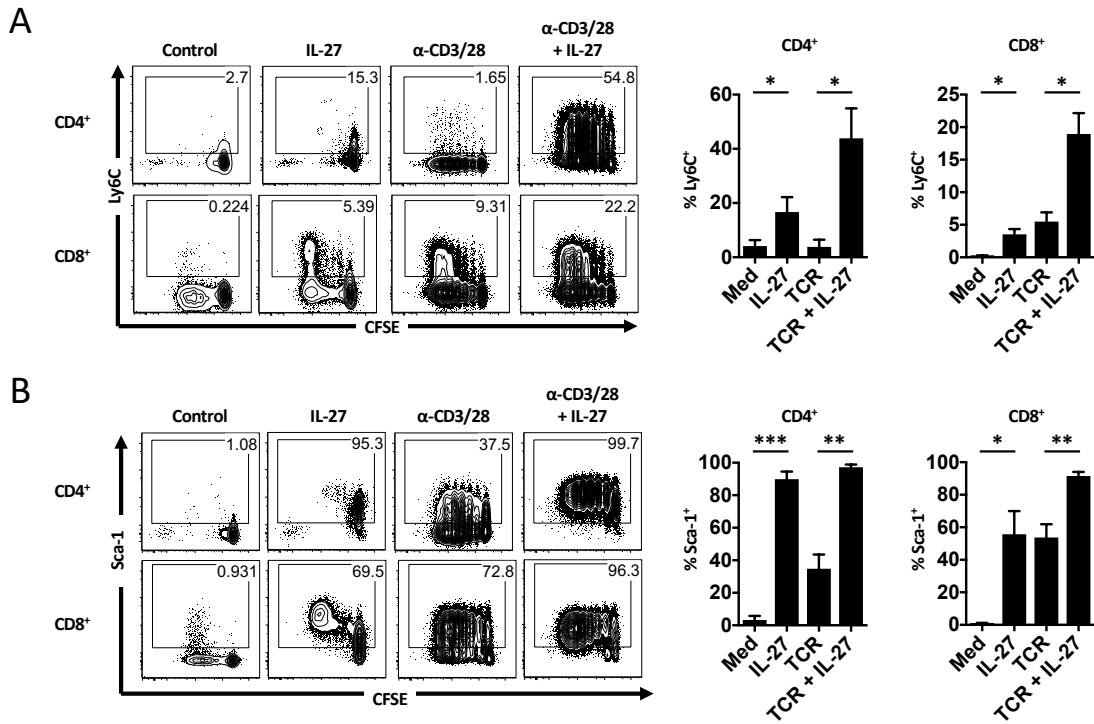
Figure 2.1 IL-27 induces Ly6C and Sca-1 on CD4$^+$ and CD8$^+$ T cells. Naive Ly6C$^-$ Sca-1$^-$ CD4$^+$ or CD8$^+$ T cells were sorted and stained with CFSE before a 3 day culture in the presence or absence of TCR stimulation and/or IL-27. A-B. Induction of Ly6C (A) or Sca-1 (B) on naive CD4$^+$ or CD8$^+$ T cells. *Left*, representative flow cytometry plots. *Right*, bar charts summarize the results of four experiments. Error bars indicate SEM. * p < 0.05, ** p < 0.01, *** p < 0.001
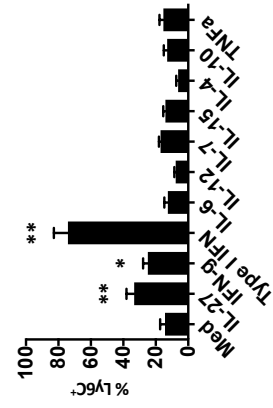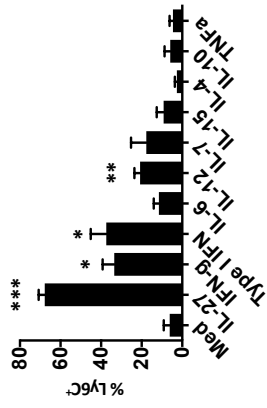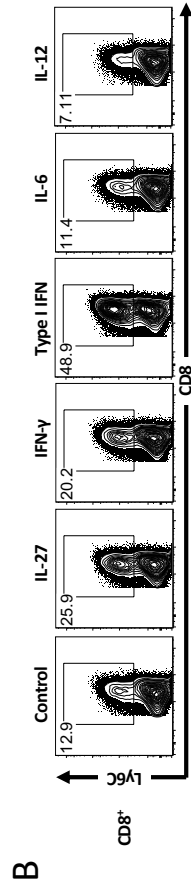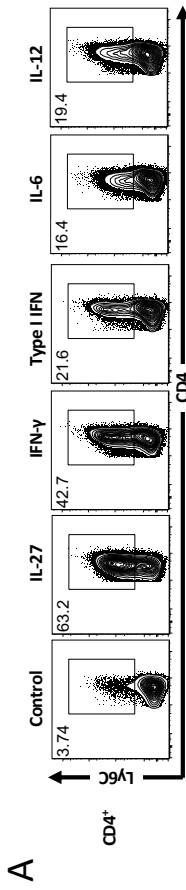
Figure 2.2 Ly6C expression is modulated by multiple cytokines *in vitro*. Naive Ly6C$^-$

Sca-1$^-$ CD4$^+$ or CD8$^+$ T cells were sorted and stained with CFSE before a 3 day culture in

the presence of TCR stimulation and Type I IFN, IL-6, IL-12, IL-4, IL-10, IL-7, or IL-15.

A-B. Expression of Ly6C by CD4$^+$ (A) and CD8$^+$ (B) T cells. **C**. Expression of Sca-1 by

CD4$^+$ T cells by a panel of cytokines. *Left*, representative plots for select cytokines.

Right, bar charts summarize the results of four experiments. Error bars indicate SEM. * p

< 0.05, ** $p < 0.01$, *** $p < 0.001$
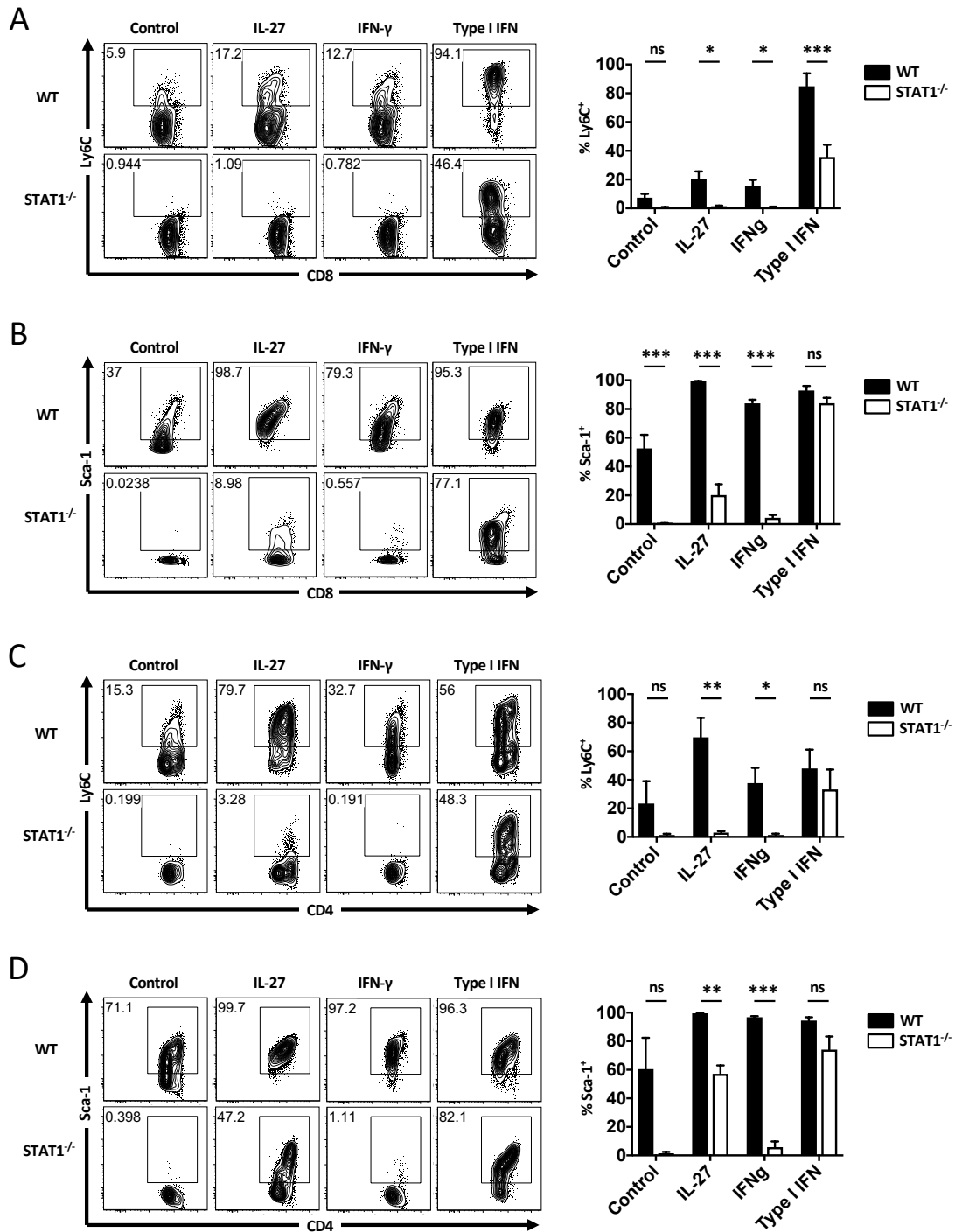
Figure 2.3 Signaling pathways involved in cytokine-mediated Ly6C and Sca-1 expression *in vitro*. WT or STAT1$^{-/-}$ splenocytes were enriched for T cells by negative-selection bead enrichment before a 3 day culture in the presence or absence of TCR stimulation and/or IL-27, IFN-γ or Type I IFN. **A, B.** Expression of Ly6C (A) or Sca-1 (B) by WT and STAT1$^{-/-}$ CD8$^+$ T cells. **C, D.** Expression of Ly6 (C) or Sca-1 (D) by WT and STAT1$^{-/-}$ CD4$^+$ T cells. *Left*, representative plots. *Right,* bar charts summarize the results of three experiments. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

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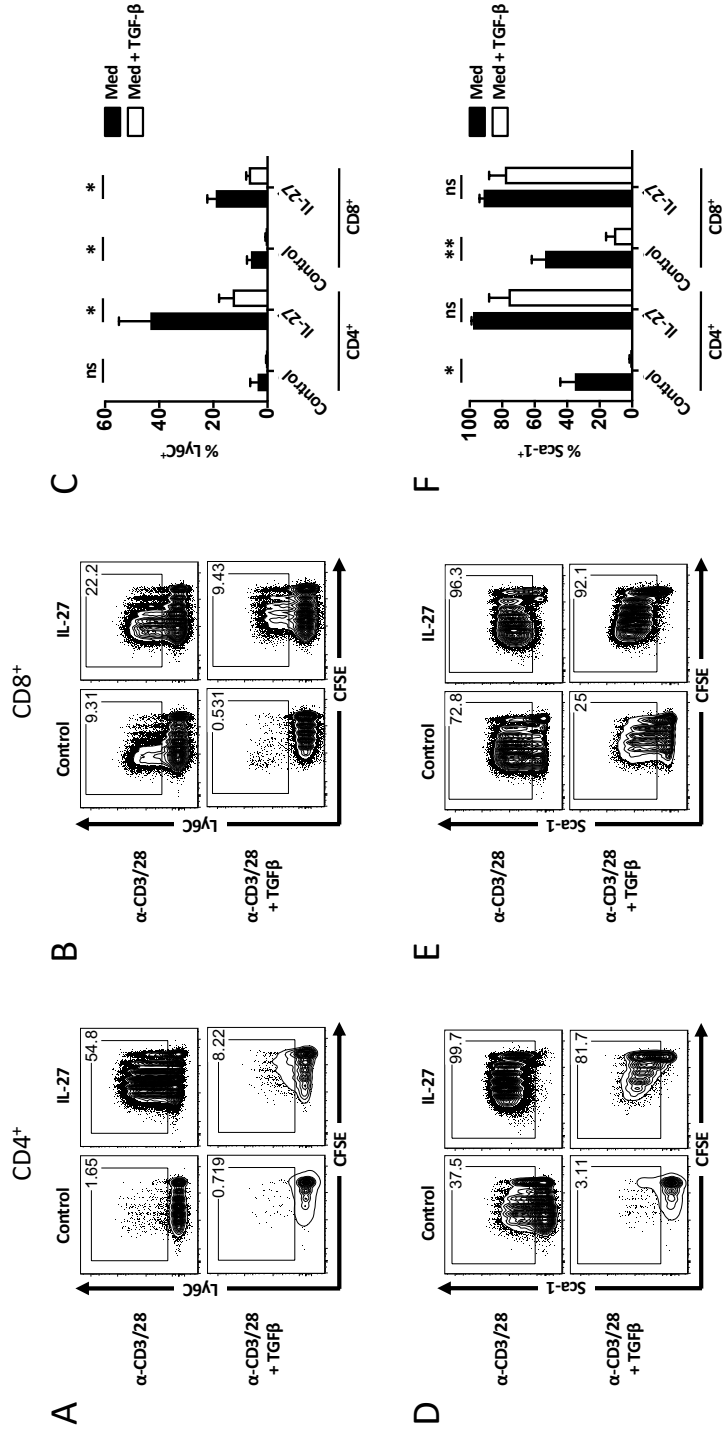
Figure 2.4 TGFβ inhibits induction of Ly6C and Sca-1. Naive Ly6C⁻Sca-1⁻ CD4⁺ or CD8⁺ T cells were sorted and stained with CFSE before a 3 day culture with TCR stimulation in the presence or absence of IL-27 and/or TGFβ. **A, B.** Expression of Ly6C by CD4⁺ (A) and CD8⁺ (B) T cells after culture. **C, D**. Expression of Sca-1 by CD4⁺ (C) and CD8⁺ (D) T cells after culture. *Left*, representative plots. *Right,* bar charts summarize the results of four experiments. Error bars indicate SEM. $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$
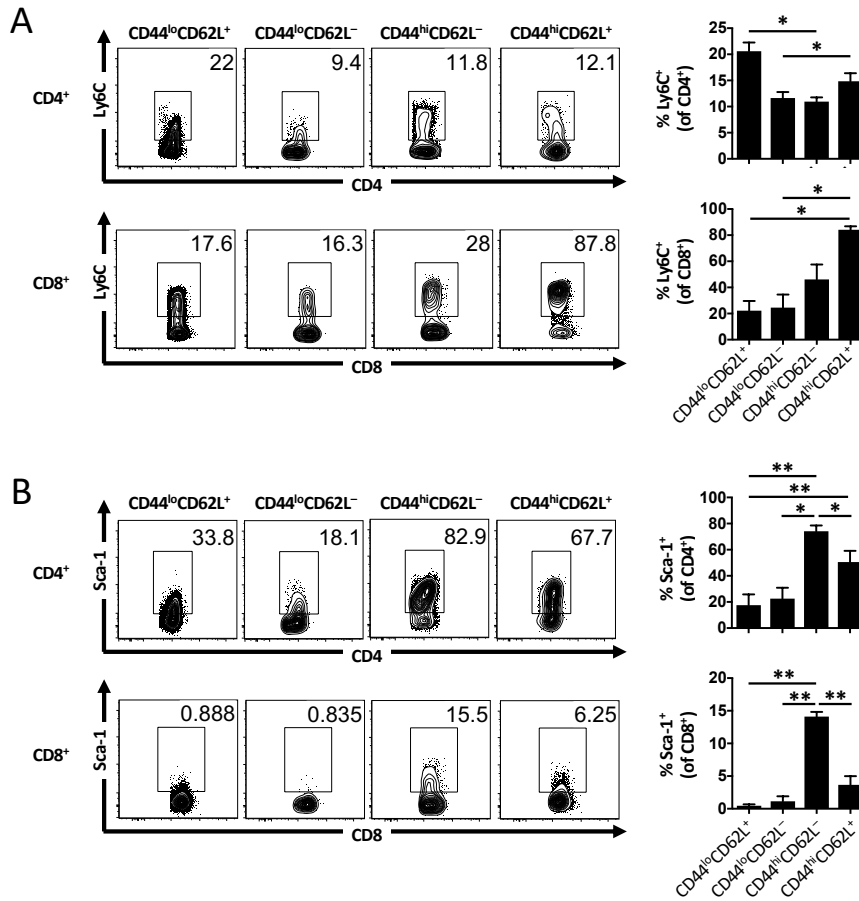
Figure 2.5 Expression of Ly6C and Sca-1 by naïve, effector and memory populations. Splenocytes from WT C57BL/6 mice were harvested and analyzed for expression of Ly6C and Sca-1. **A.** Expression of Ly6C by CD4$^+$ (top) and CD8$^+$ (bottom) T cell populations. **B.** Expression of Sca-1 by CD4$^+$ (top) and CD8$^+$ (bottom) T cell populations. *Left*, representative plots. *Right,* bar charts summarize the results of three experiments. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$

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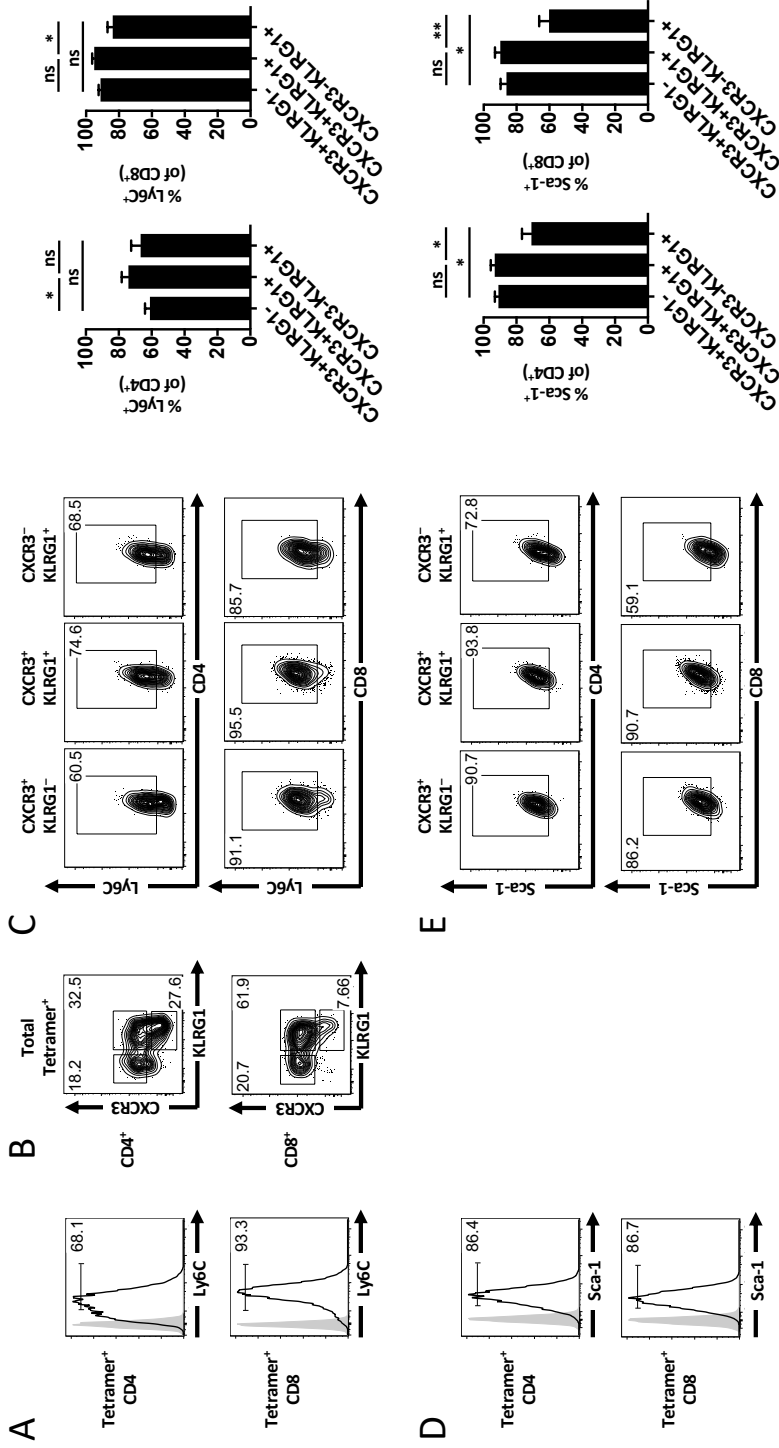
Figure 2.6 Toxoplasmosis upregulates Ly6C and Sca-1 in T cells. WT C57BL/6 mice were infected with 20 *T. gondii* cysts intraperitoneally. Splenocytes were harvested and analyzed at day 9 of infection. *T. gondii*-specific T cells were identified by MHCI and MHCII tetramers. **A, D.** Expression of Ly6C (A) or Sca-1 (D) by CD4$^+$ and CD8$^+$ T cells. **B**. Expression of CXCR3 and KLRG1 by *T. gondii*-specific CD4$^+$ and CD8$^+$ T cells. **C, E.** Expression of Ly6C (C) or Sca-1 (E) by different populations of T cells, distinguished by expression of CXCR3 and KLRG1. *Left*, representative plots. *Right,* bar charts summarize results from one of two experiments, *n* = 3-4 mice per experiment. Error bars indicate SEM. * p < 0.05, ** p < 0.01
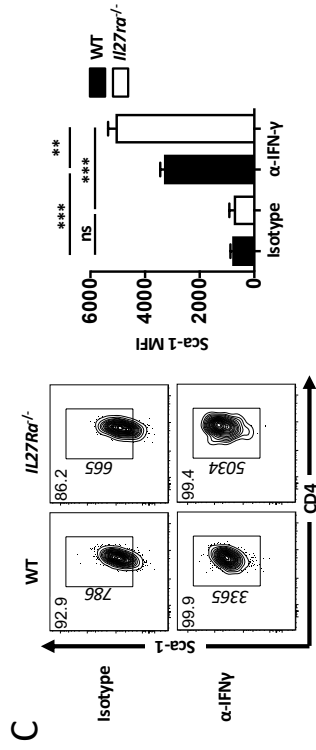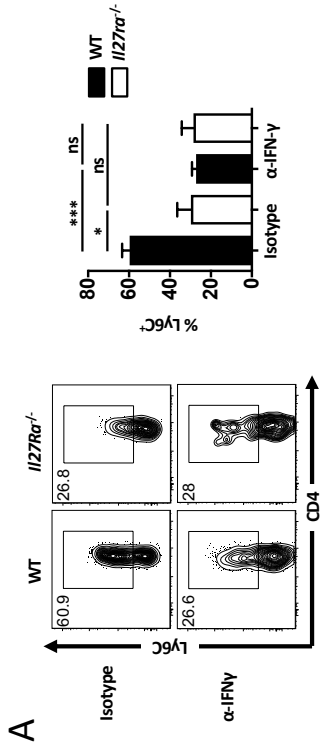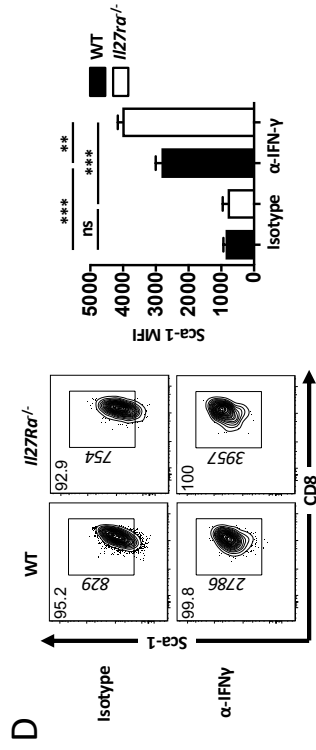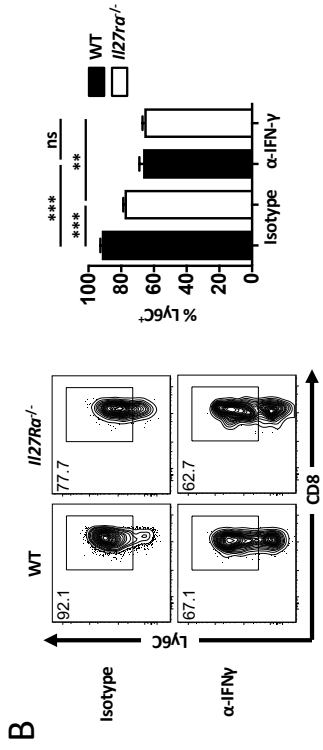
Figure 2.7 IL-27 and IFN-γ promote expression of Ly6C *in vivo* during

toxoplasmosis. WT or *Il27ra*$^{-/-}$ mice were infected with 20 *T. gondii* cysts

intraperitoneally and a subset of mice were treated with anti-IFN-γ antibody on day 3 and

6 of infection. Peritoneal cells were harvested 9 days after infection and *T. gondii*-

specific cells were identified using MHCI and MHCII tetramers. Ly6C expression by

tetramer$^+$ CD4$^+$ (**A**) or CD8$^+$ (**B**) T cells was examined. Sca-1 expression by tetramer$^+$

CD4$^+$ (**C**) or CD8$^+$ (**D**) T cells was also examined. *Left*, representative plots with MFI

indicated by vertical italic numbers. *Right,* bar charts show representative results from

one of two experiments, *n* = 2-4 mice per group, per experiment. Error bars indicate

SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Supplementary Figures
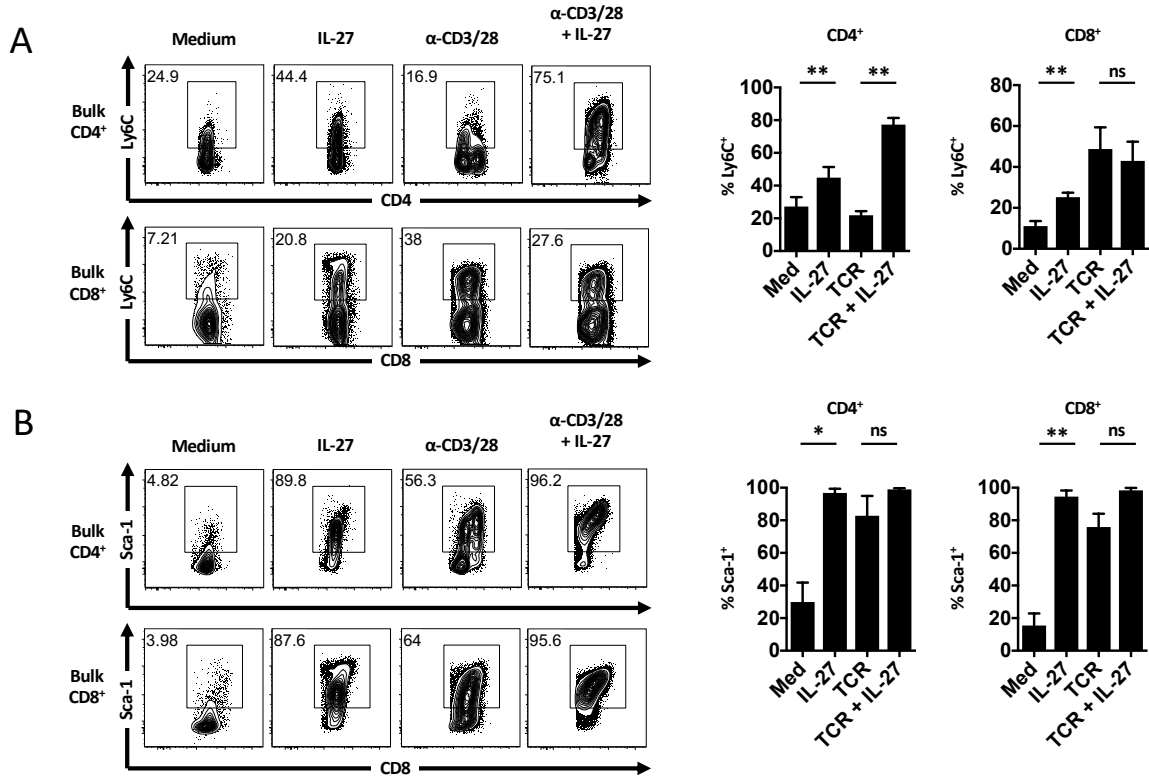
Figure 2.S1 IL-27 and TCR stimulation promote Ly6C+ and Sca-1+ populations in bulk T cell cultures. Spleens and lymph nodes (axillary, brachial, and inguinal) were harvested and enriched for T cells using an R&D T cell column and then separated into CD4+ and CD4− (primarily CD8+) T cells using the Miltenyi CD4+ T cell enrichment kit. The cells were then cultured for 3 days in the presence or absence of anti-CD3/28 stimulation and/or IL-27. **A-B.** Expression of Ly6C (**A**) and Sca-1 (**B**) on CD4+ and CD8+ T cells after culture. *Left*, representative flow cytometry plots. *Right*, bar graphs summarize the results of three experiments. Error bars indicate SEM. * p < 0.05, ** p < 0.01
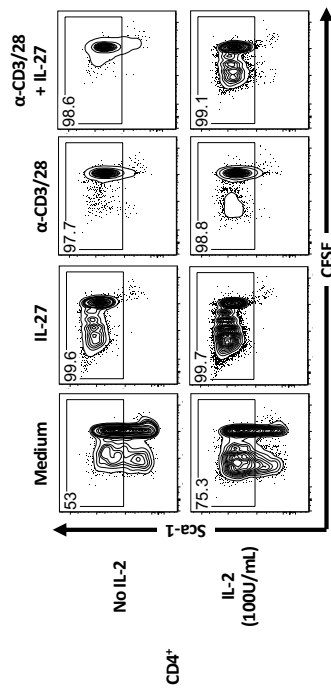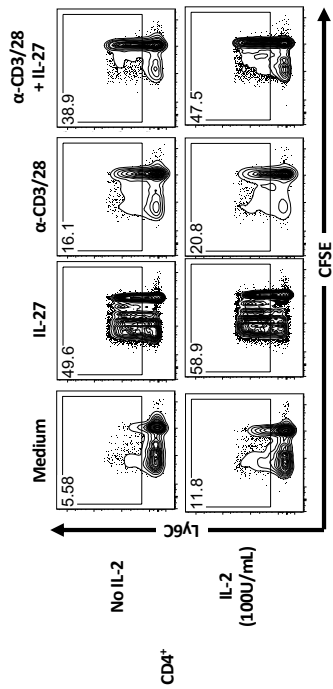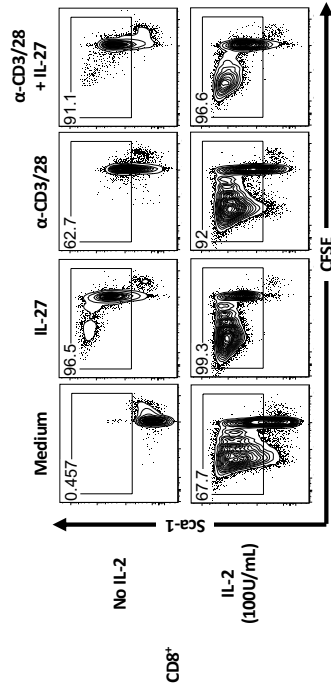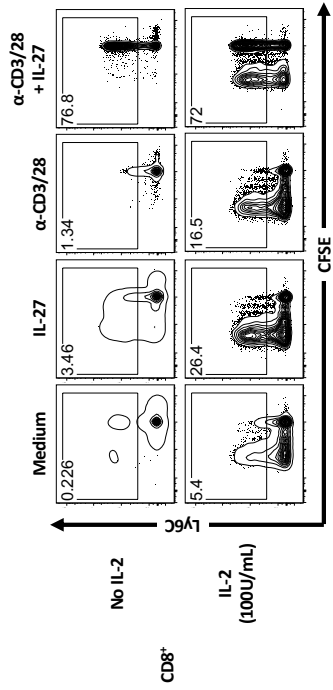
Fig 2.S2 IL-2 does not limit Ly6C and Sca-1 induction on T cells. Naive Ly6C⁻Sca-1⁻ CD4$^+$ and CD8$^+$ T cells were sorted and stained with CFSE before a 3 day culture in the presence or absence of anti-CD3/28 stimulation and/or IL-27 and/or IL-2. **A-B.** Expression of Ly6C (**A**) and Sca-1 (**B**) on CD4$^+$ T cells after culture.
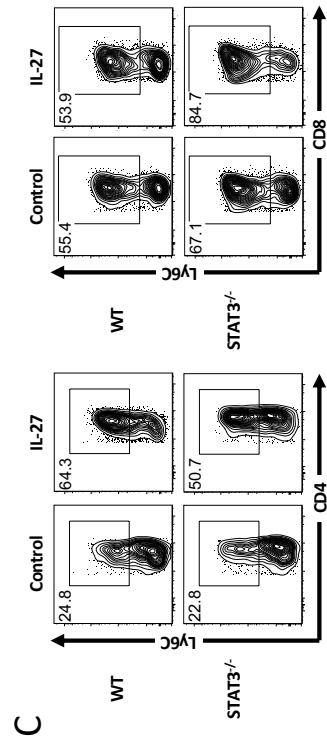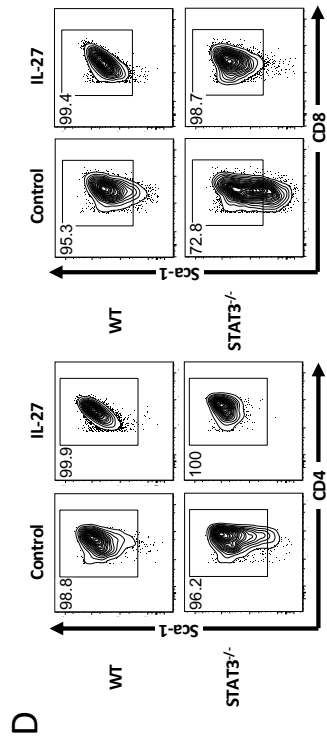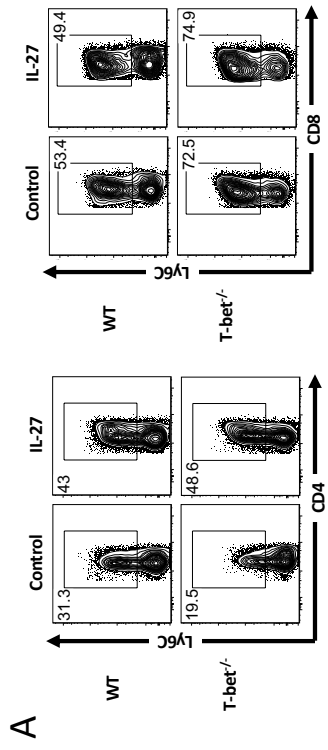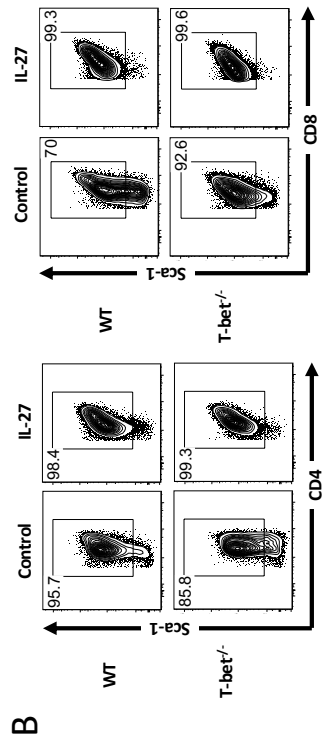
Fig 2.S3 Requirement for T-bet and STAT3 in Ly6C and Sca-1 expression *in vitro*.

(**A, B**) Spleens and lymph nodes (axillary, brachial, and inguinal) from WT or T-bet$^{-/-}$ mice were harvested, enriched for T cells using an R&D T cell column and then separated into CD4$^+$ (**A**) and CD4$^-$ (primarily CD8$^+$) (**B**) T cells using the Miltenyi CD4$^+$ T cell enrichment kit. The cells were then cultured for 3 days in the presence or absence of anti-CD3/28 stimulation and/or IL-27. (**C, D**) Spleens from STAT3$^{fl/fl}$ x CD4-Cre mice or littermate controls were harvested, enriched for T cells using an R&D T cell column and then separated into CD4$^+$ (**C**) and CD4$^-$ (primarily CD8$^+$) (**D**) T cells using the Miltenyi CD4$^+$ T cell enrichment kit. The cells were then cultured for 3 days in the presence or absence of anti-CD3/28 stimulation and/or IL-27.
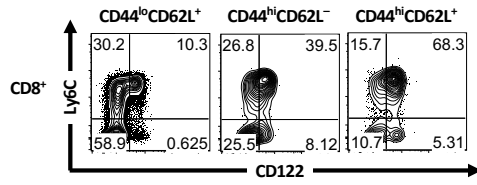
Fig 2.S4. Expression of Ly6C and Sca-1 by naïve, effector and memory populations.

Splenocytes from WT C57BL/6 mice were harvested and analyzed for expression of

Ly6C and Sca-1. CD122+ CD8+ T cells express high levels of Ly6C.

Chapter 3: IL-27 and TCR stimulation promote T cell expression of multiple inhibitory receptors

Abstract

Inhibitory receptors (IR) are a diverse group of cell surface molecules that modulate T cell function in response to inflammatory stimuli, but there are gaps in our knowledge of the cell extrinsic factors that regulate their expression during infection. The present study found that *in vitro*, IL-27 alone induced expression of PD-L1 but synergized with TCR stimulation to enhance LAG-3, CTLA-4, and TIGIT and this activity was STAT1-dependent. Other cytokines, most notably IL-12 and type I IFN alone could also promote PD-L1, but their impact on TCR-mediated effects was less apparent. *In vivo*, infection with *Toxoplasma gondii* resulted in parasite-specific effector T cells present in the spleen and at sites of infection that expressed high levels of IR. The absence of IL-27 did not affect IR expression by parasite-specific CD4$^+$ and CD8$^+$ effector T cells in the spleen, but at local sites of infection was required for maximal PD-L1, LAG-3, CTLA-4, and TIGIT expression. These findings indicate a role for IL-27 in bystander and pathogen-specific T cell expression of multiple IR at local sites of inflammation.

Introduction

Inhibitory receptors (IR) are a diverse group of cell surface molecules expressed by T cells and other immune populations that are defined functionally, by their ability to limit the immune response (Chen and Flies, 2013). These diverse receptors are spread

across the immunoglobulin, TNF and Ly6 families of proteins and limit immune function by a variety of mechanisms. Numerous studies have demonstrated the importance of inhibitory receptors such as PD-L1, PD-1, LAG-3, and CTLA-4 to limit T cell function in the context of chronic infection, anti-tumor immunity, and other inflammatory responses (Blackburn et al., 2009; Odorizzi and Wherry, 2012; Pardoll, 2012). In the setting of parasitic infection, blockade of these molecules has been used to augment CD8^{+} and CD4^{+} T cell responses during leishmaniasis, toxoplasmosis, and malaria, leading to improved cytokine production, cytotoxicity, and control of parasite burden (Bhadra et al., 2011; Butler et al., 2011; Murphy et al., 1998). This strategy has also been used to reinvigorate the CD8^{+} T cell response and promote anti-tumor immunity, leading to durable reductions in tumor burden in model systems and clinical disease (Pardoll, 2012).

While there are many examples that illustrate the impact of inhibitory receptors on effector T cell responses, there remains a knowledge gap about the factors that influence T cell expression of these molecules during infection. Studies of T cell exhaustion have demonstrated that repeated stimulation through the TCR is one mechanism that induces expression of these IR by T cells (Agata et al., 1996, Fourcade et al., 2010, Golden-Mason et al., 2009, Huang et al., 2004, Petrovas et al., 2007). *In vitro* studies have found that during T cell activation, IFN-β promotes TIM-3 expression by murine CD4^{+} T cells (Boivin et al., 2015), and that IL-2, IL-7, IL-15, and IL-21 promote PD-1 and PD-L1 on human CD4^{+} and CD8^{+} T cells (Kinter et al., 2008). There remain significant gaps in our understanding of which cytokines affect the ability of different T cell subsets to express different combinations of IR and the significance of these pathways *in vivo,* during infection.

IL-27 is an IL-12-family cytokine that signals through STAT1 and STAT3 and can limit the intensity and duration of the T cell response in a variety of infectious and inflammatory conditions (Yoshida and Hunter, 2015). For example, in parasitic systems, IL-27 limits Th1, Th2, and Th17 responses by promoting production of IL-10 and differentiation of Th1-like Tregs (Anderson et al., 2009; Artis et al., 2004b; Bancroft et al., 2004; Hall et al., 2012a; Hamano et al., 2003; Stumhofer et al., 2006; Stumhofer et al., 2007; Villarino et al., 2003). Consistent with its ability to promote Treg activity, IL-27 upregulates LAG-3 on Tregs *in vitro* and has been implicated in the regulation of LAG-3 in a murine model of colitis (Do et al., 2015). For conventional CD4$^+$ and CD8$^+$ T cells, IL-27 has been found to promote expression of PD-L1, LAG-3, TIM-3, Ly6C, and Sca-1 *in vitro* (DeLong et al., 2018; Hall et al., 2012a; Hirahara et al., 2012; Liu et al., 2013; Ma et al., 2017; Zhu et al., 2015; Zhu et al., 2016). It remains unclear if IL-27 or other cytokines have a broad ability to promote effector T cell expression of multiple IR such as LAG-3, PD-1, TIGIT, and CTLA-4, and how TCR and cytokine signals are integrated to modulate IR expression during infection.

Previous studies from this laboratory examining the transcriptome of Treg cells stimulated in the presence of IL-27 highlighted its ability to enhance expression of several IR (Hall et al., 2012a). To distinguish whether this activity was broadly relevant to different T cell subsets, *in vitro,* studies using Treg cells and naive CD4$^+$ and CD8$^+$ T cells confirmed that IL-27 alone could induce PD-L1 expression but its effects on LAG-3, CTLA-4, and TIGIT were TCR-dependent and these effects were STAT1-dependent. Other cytokines, notably IFN-γ, type I IFN and IL-12 also upregulated expression of some inhibitory receptors but were not as potent as IL-27. To assess the impact of

endogenous IL-27 on these pathways, IR expression was examined in IL-27p28-deficient mice during acute toxoplasmosis. This infection resulted in the generation of effector T cells that expressed multiple inhibitory receptors, but neither IL-27 nor STAT1 was required for their expression in the spleen. However, IL-27 and STAT1 were required for optimal IR expression by parasite-specific T cells at sites of parasite replication and inflammation. Together, these findings highlight the ability of IL-27 to upregulate multiple IR during T cell priming and establish a role for endogenous IL-27 in promoting T cell expression of multiple IR in peripheral sites of infection during toxoplasmosis.

Results

IL-27 promotes expression of inhibitory receptors by T cells in vitro

Previous studies from this laboratory using transcriptional profiling of Treg cells stimulated with IL-27 highlighted its ability to enhance transcripts of a variety of IR (Hall et al., 2012a). To distinguish whether this activity was broadly relevant to different T cell subsets, naïve (CD44^{lo}CD62L^{+}Ly6C^{-}Sca1^{-}) CD4^{+} or CD8^{+} T cells were sort-purified, stained with CFSE to assess proliferation, and cultured with or without anti-CD3 and anti-CD28 antibody stimulation in the presence or absence of IL-27. IL-27 alone upregulated PD-L1 on CD4^{+} T cells while IL-27 combined with TCR stimulation synergized to upregulate LAG-3, TIGIT, and TIM-3 (Fig 3.1). IL-27 did not increase activation-induced expression of CTLA-4 or PD-1 (Fig 3.1 and data not shown). Similar results were seen with FoxP3^{+} regulatory T cells (Fig 3.S1). Similarly, for naive CD8^{+} T cells, IL-27 induced expression of PD-L1 in the absence of anti-CD3/28 simulation and further enhanced anti-CD3/28-induced expression of LAG-3, CTLA-4, TIGIT, and TIM-

3 but not PD-1 (Fig 3.2 and data not shown). The most prominent and robust effects of IL-27 in this assay were on conventional CD4$^+$ and CD8$^+$ T cell and their expression of PD-L1, LAG-3, and CTLA-4, while induction of TIGIT and TIM-3 was modest and there was no impact on PD-1. In the absence of TCR stimulation, STAT1 was required for IL-27-mediated expression of PD-L1 in CD8$^+$ T cells (Fig 3.S3). In the presence of TCR stimulation, STAT1 was found to contribute to the expression of LAG-3, CTLA-4, TIGIT, and TIM-3. These data suggest a key role for STAT1 in promoting IL-27-mediated inhibitory receptor expression.

Because of the observed synergy between anti-CD3/28 and IL-27 stimulation, studies were performed to assess whether varying the strength of the TCR signal impacted IR expression. To examine this, naïve CD8$^+$ T cells were cultured in the presence of soluble anti-CD28 with increasing concentrations of plate-bound anti-CD3. Cells were then assessed for expression of PD-L1, LAG-3, and CTLA-4. Stimulation with 0.1 g/mL anti-CD3 was sub-optimal and did not result in T cell proliferation or inhibitory receptor expression (Fig 3.3A-C). 1 g/mL anti-CD3 upregulated expression of each inhibitory receptor, as shown in Fig 1. Increasing anti-CD3 to 10 g/mL did not further increase expression of PD-L1 (Fig 3.3A, open circles), but there was enhanced expression of LAG-3 and CTLA-4 (Fig 3.3B-C), indicating that the expression of these IR correlates with the strength of TCR signal. In the presence of IL-27, three different patterns of expression were observed. As expected, IL-27 induced PD-L1 in T cells that had not received anti-CD3 stimulation, but it is noteworthy that in the context of TCR stimulation, PD-L1 expression is lowest in cells that had most diluted CFSE (Fig 3.3A). In contrast, IL-27 upregulated expression of LAG-3 at both 1 g/mL and 10 g/mL of

anti-CD3 stimulation. A third pattern was seen for CTLA-4: IL-27 upregulated CTLA-4 at 1 g/mL anti-CD3, but at 10 g/mL anti-CD3, IL-27 was not required for maximal CTLA-4 expression. Therefore, the ability of IL-27 to promote IR expression varies depending on the level of TCR stimulation and the inhibitory receptor in question.

Coexpression of inhibitory receptors in vitro

The finding that IL-27 upregulates expression of inhibitory receptors on some but not all cells *in vitro* led us to examine whether inhibitory receptor expression is stochastically upregulated across these cells, or if a subset of cells co-expresses a disproportionately high number of inhibitory receptors. To examine this, we first used the expression of each inhibitory receptor to calculate the amount of coexpression that would be predicted if the molecules were independently expressed (Fig 3.S2A). For example, if 60% of the cells in a given sample express Protein A and 20% express Protein B, 12% of the cells (60% x 20%) will express both Protein A and Protein B if these proteins are independently regulated. We then used boolean analysis in Flowjo to determine the actual coexpression of these molecules in our samples. Cells cultured without IL-27 or α-CD3/28 stimulation expressed few inhibitory receptors and had correspondingly low predicted inhibitory receptor coexpression (Fig 3.S2B, gray bars). Stimulation with α-CD3/28 upregulates individual IR and IL-27 further augments this upregulation, leading to an increase in the predicted coexpression of these molecules (Fig 3.S2B, gray bars). However, we found that the actual coexpression of these molecules is much higher than would be predicted if these molecules were independently expressed (Fig 3.S2B, black bars). For example, in cells stimulated with anti-CD3/28 + IL-27, stochastic distribution

of inhibitory receptors would result in 6% of cells expressing all 6 inhibitory receptors, while in these cultures, 29% of cells were expressing all 6 inhibitory receptors. This analysis demonstrates that in this system, IR expression is not distributed stochastically among cells, but stimulation with TCR and IL-27 results in a disproportionate concentration of IR on a subset of cells and suggests that this process is coordinated.

Multiple cytokines modulate inhibitory receptor expression in vitro

Given the impact of IL-27 on IR expression, a survey was performed to examine the ability of the related type I cytokines IFN-γ, type I IFN, IL-6 and IL-12 to modulate IR expression by naive CD8$^+$ T cells. In the absence of anti-CD3/28 stimulation, IFN-γ, type I IFN, and IL-12 (but not the closely-related IL-6) upregulated PD-L1 expression similar to IL-27 in CD8$^+$ T cells (Fig 3.4A). However, in the presence of anti-CD3/28 stimulation, only IL-12 upregulated expression of LAG-3 in CD8$^+$ T cells and none of the other cytokines had any discernible effect on expression of CTLA-4, TIGIT, TIM-3 or PD-1 (Fig 3.4B and data not shown). This screening process also revealed that in cells stimulated with anti-CD3/28, TGF-β limited expression of PD-L1 (Fig 3.4C). Similarly, in cells stimulated with anti-CD3/28 in the presence of IL-27, TGF-β limited expression of LAG-3, CTLA-4, TIGIT, and TIM-3, but not PD-L1. As PD-L1 is the only inhibitory receptor in this panel induced by IL-27 in the absence of anti-CD3/28 stimulation, these findings are consistent with reports that suggest that TGF-β has a role in limiting TCR-mediated signaling (Chen et al., 2003; Robinson and Gorham, 2007).

IL-27 promotes expression of PD-L1, LAG-3, CTLA-4 and TIGIT during toxoplasmosis

In the absence of IL-27, infection with *T. gondii* results in increased levels of T cell activation and high circulating levels of IFN-γ (Villarino et al., 2003; Villarino et al., 2006). It was uncertain whether these aberrant T cell responses would be associated with increased expression of IR as overall indicators of T cell activation or if IL-27 would be required for IR expression. Therefore, to examine an effector population with a well-defined history, mice were infected with *T. gondii* and inhibitory receptor expression was examined on parasite-specific T cells from the spleens and lungs 10-12 days post infection. *T. gondii*-specific T cells were identified by staining with MHCI or MHCII tetramers in combination with high expression of LFA-1 (Dupont et al., 2014). In uninfected mice (thin lines), splenic Tregs (Fig 3.5A), CD4$^+$ T cells (Fig 3.5B) and CD8$^+$ T cells (Fig 3.5C) expressed low levels of each of the inhibitory receptors examined with the exception of CTLA-4, which was basally expressed by Tregs in uninfected mice. At day 10 post infection, expression of PD-L1, LAG-3, CTLA-4, and TIGIT were upregulated by each of these T cell populations (Fig 3.5A-C, thick lines).

A recent study (Chu et al., 2016) proposed that during toxoplasmosis, minimally-differentiated memory CD8$^+$ T cells that are CXCR3$^+$KLRG1$^-$ give rise to an intermediate CXCR3$^+$KLRG1$^+$ population that in turn downregulates CXCR3 when it differentiates into terminally-differentiated effector cells. At day 10 of infection, splenic CD8$^+$ T cells were analyzed by their expression of KLRG1 and CXCR3 (Fig 3.5D). Inhibitory receptor expression was examined on these different populations and the highest inhibitory receptor expression was seen on the less-differentiated KLRG1$^-$CXCR3$^+$ and the intermediate KLRG1$^+$CXCR3$^+$ cells. The highly-differentiated KLRG1$^+$CXCR3$^-$ cells were consistently the population with the lowest expression of

inhibitory receptors (Fig 3.5E, dashed line). This finding indicates that during

toxoplasmosis, inhibitory receptors are upregulated early after T cell priming, but that as

cells differentiate into terminally-differentiated effector cells, they downregulate these

molecules.

To determine if endogenous IL-27 contributes to T cell expression of inhibitory

receptors, WT and $Il27p28^{-/-}$ mice were infected with *T. gondii* as above and inhibitory

receptor expression was assessed on T cells from the spleen and lung at day 11-12 of

infection. In the spleen, IR expression between WT and $Il27p28^{-/-}$ mice was largely

comparable, although decreased expression of CTLA-4 was detected in $Il27p28^{-/-}$ CD4$^+$ T

cells (Fig 3.S5). In the lung, expression of PD-1 and TIM-3 were also comparable in WT

and $Il27p28^{-/-}$ mice (data not shown). However, in the lung, expression of PD-L1, CTLA-

4 and TIGIT was decreased on tetramer$^+$ $Il27p28^{-/-}$ CD4$^+$ T cells compared to WT cells

(Fig 3.6A). Expression of LAG-3 was not significantly lower on $Il27p28^{-/-}$ CD4$^+$ T cells.

Among parasite-specific CD8$^+$ T cells in the lung, expression of PD-L1, LAG-3, and

TIGIT was lower on $Il27p28^{-/-}$ cells than on WT cells. Expression of CTLA-4 was not

significantly lower on $Il27p28^{-/-}$ CD8$^+$ T cells. These results suggest that IL-27

contributes to expression of PD-L1, LAG-3, CTLA-4 and TIGIT *in vivo*, during

toxoplasmosis.

To examine the role of STAT1 in inhibitory receptor expression *in vivo*, during

toxoplasmosis, STAT1$^{fl/fl}$ x CD4-Cre mice were utilized in order to eliminate STAT1

signaling from T cells. These mice were infected with *T. gondii* as above and peritoneal

exudate cells, spleens and lungs were examined for inhibitory receptor expression at day

7 of infection. LFA-1^{hi} CD4$^+$ T cells were examined due to low numbers of tetramer$^+$

CD4$^+$ T cells. As seen in *Il27p28$^{-/-}$* mice, inhibitory receptor expression was intact on parasite-specific T cells in the spleen. However, on LFA-1^{hi} CD4$^+$ T cells from the peritoneum, expression of LAG-3, CTLA-4, and TIGIT was decreased in the STAT1-deficient population (Fig 3.S4A). Similarly, on peritoneal tetramer$^+$ CD8$^+$ T cells, expression of LAG-3 and TIGIT was lower in STAT1-deficient cells (Fig 3.S4B). In the lung, STAT1-deficient parasite-specific CD4$^+$ and CD8$^+$ T cells were sometimes found to express decreased levels of LAG-3, CTLA-4 and TIGIT, but this trend was not consistent across experiments (data not shown). These findings demonstrate a role for one or more STAT1-signaling cytokines in upregulating inhibitory receptor expression during acute toxoplasmosis.

Discussion

The present study found that, *in vitro*, IL-27 promoted T cell expression of PD-L1, similar to previous reports (Hirahara et al., 2012), but synergized with TCR signals to promote LAG-3, CTLA-4, TIGIT and TIM-3 on T cells. This is consistent with previous work that found that IL-27 caused CD4$^+$ and CD8$^+$ T cells to upregulate Ly6C and Sca-1, molecules ascribed IR functions (DeLong et al., 2018; Liu et al., 2013; Zhu et al., 2016). The present study found that PD-L1, LAG-3, CTLA-4, and TIGIT were upregulated during acute toxoplasmosis and that IL-27 was required for maximal IR expression in the lung, a peripheral site of infection, but not in the spleen. Therefore, while our *in vitro* results demonstrated that IL-27 is able to upregulate IR expression in naive cells during priming, the *in vivo* results suggest an additional role for IL-27 and STAT1 in modulating IR expression in effector cells in the periphery. These effects of IL-27 on IR expression

during infection can also be seen in other experimental systems. IL-27-Fc administration promoted Treg expression of PD-1 and CTLA-4 *in vivo* during collagen-induced arthritis (Moon et al., 2013). IL-27 was also found to be required for maximal expression of PD-1, LAG-3, TIGIT, and TIM-3 on CD4$^+$ and CD8$^+$ T cells in the context of B16F10 melanoma (Chihara et al., 2018). In agreement with the present study, these authors found that IL-27 did not promote PD-1 expression *in vitro*. Induction of inhibitory receptors by IL-27 could help explain previous studies reporting better tumor control in IL-27-deficient mice (Sauer et al., 2008; Zhu et al., 2015).

Heterogeneity was noted in these *in vitro* cultures and the examination of inhibitory receptor coexpression gave insights into possible causes for this. Inhibitory receptor expression was found to not be upregulated stochastically, but was concentrated on a subset of cells. It is possible that heterogeneity in the initial cell populations results in some cells being more responsive to stimulation with α-CD3/28 and IL-27. It is also possible that signaling molecules, such as STAT1, bind regulatory elements for multiple inhibitory receptors so signals that drive one inhibitory receptor will also promote upregulation of other inhibitory receptors. This could also give insight into the high coexpression of inhibitory receptors seen in other systems (Blackburn et al., 2009). Post-hoc analysis of ChIP-Seq data from a study examining the effect of IL-27 on anti-CD3/28-stimulated T cells (Hirahara et al., 2015) indicated that STAT1 binds to the gene region or directly upstream of the genes for PD-L1, LAG-3, CTLA-4, TIGIT, TIM-3, Ly6C and Sca-1 (data not shown). Culture with IL-27 appeared to increase STAT1 binding to each of these genes except for PD-L1. It is therefore possible that STAT1

binding to these gene regions is responsible for the high degree of inhibitory receptor coexpression we see in cells stimulated through the TCR in the presence of IL-27.

The finding that inhibitory receptor expression is further upregulated with increasing TCR stimulation demonstrates that induction of this regulatory pathway is graded. This is supported by the finding that cytokines present at sites of inflammation further upregulate inhibitory receptor expression, suggesting that inhibitory receptor expression is finely tuned to prevent highly activated, pathogen-specific cells at sites of infection from causing pathologic collateral damage. This suggests a model in which inhibitory receptors are a mechanism of negative feedback, expressed on the most highly activated T cells to prevent them from becoming pathologic.

The expression of IR has been most prominently described in the context of CD8$^+$ T cell exhaustion during chronic infection (Blackburn et al., 2009; Wherry et al., 2007) and their expression patterns during acute infection are less well characterized. In the present study, *Il27p28$^{-/-}$* and CD4-Cre x STAT1$^{fl/fl}$ mice displayed deficient IR expression in peripheral sites of infection, where we know IL-27 is produced, and not in the spleen, suggesting that cytokines control inhibitory receptor expression primarily at peripheral sites during toxoplasmosis. Future studies will examine whether the upregulation of these inhibitory receptors is a pathway by which IL-27 limits immunopathology.

Inhibitory receptor blockade has been found to increase T cell cytokine production, cytotoxicity, and control of parasite burden during toxoplasmosis, leishmaniasis, and malaria (Bhadra et al., 2011; Butler et al., 2011; Joshi et al., 2009), infections in which IL-27 plays a key role in limiting immunopathology (Findlay et al., 2010; Hafalla et al., 2012). Therefore, it is possible that IL-27 limits immunopathology in

these systems through induction of inhibitory receptors. Recombinant, Fc-tagged inhibitory receptors are a powerful tool for elucidating the roles of IR *in vivo*. It is notable that in IL-27Rα-deficient mice, CTLA-4-Ig limits *T. gondii*-induced immunopathology (Stumhofer et al., 2006). These results must be interpreted with care, but they support a model in which IL-27 limits immunopathology through upregulation of CTLA-4. Similar experiments utilizing LAG-3-Fc, PD-L1-Fc and TIGIT-Fc could examine the ability of these IR to reverse immunopathology in IL-27-deficient mice.

IL-27 regulates dendritic cell function by multiple pathways. It promotes T cell expression of IL-10 (Awasthi et al., 2007; Pot et al., 2009; Stumhofer et al., 2007), which in turn limits DC production of IL-12 (Couper et al., 2008). IL-27 can also directly limit DC maturation, as well as upregulate DC expression of CD39, which decreases extracellular ATP and limits T cell differentiation (Mascanfroni et al., 2013). T cells expressing LAG-3 and CTLA-4 limit the ability of DCs to upregulate CD80/86 and prime T cells. (Bayry et al., 2007; Huang et al., 2004; Liang et al., 2008; Qureshi et al., 2011; Wing et al., 2008). CTLA-4 can function through induction of IDO-expressing tolerogenic DCs or through blockade of CD28-B7 interactions (Bluestone et al., 2006; Grohmann et al., 2002). The present findings, that IL-27 promotes expression of LAG-3, and CTLA-4, highlight the ability of IL-27 to coordinate multiple inhibitory functions within a single T cell. This activity may endow T cells with multiple mechanisms that limit DC activities and help to explain the potent suppressive effects of IL-27 in multiple disease settings.

In humans, checkpoint blockade has been found to improve the anti-tumor response and blockade of PD-L1, PD-1 and CTLA-4 are now FDA approved cancer

therapies. The finding that cytokines upregulate multiple IRs suggests that modulation of cytokine signaling could be an additional approach for manipulating inhibitory receptor expression. The IL-27 receptor signals through JAK1, JAK2, STAT1 and STAT3. The finding that STAT1 contributes to expression of multiple inhibitory receptors suggests that this pathway could be targeted to modulate inhibitory receptor expression. Janus-activated kinases (JAKs) are part of the STAT signaling cascade of IL-27, IL-12, IFN-γ, and type I IFN signaling and JAK molecules are readily druggable. JAK inhibitors have shown efficacy in limiting the immune response during autoimmunity (Schwartz et al., 2017). It is possible that in another disease context, such as cancer, inhibiting JAK signaling could limit IR expression and enhance the T cell response.

Figures
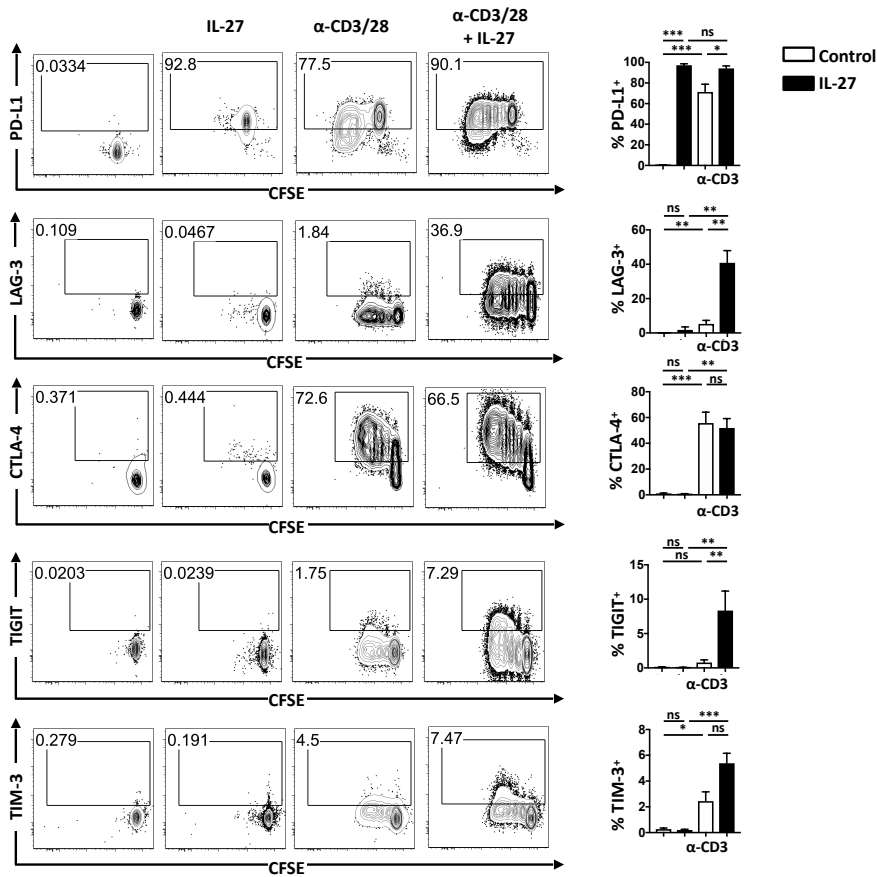
Figure 3.1 IL-27 induces expression of multiple inhibitory receptors by CD4$^+$ T cells. Naïve, Ly6C$^-$Sca-1$^-$ CD4$^+$ and T cells were sort purified and cultured in the presence or absence of α-CD3/28, in the presence or absence of IL-27. After an 85-hour culture, cells were analyzed for expression of PD-L1, PD-1, LAG-3, and CTLA-4. Representative FACS plots (left). Bar charts (right) show combined results from 4 independent experiments. Error bars indicate SEM. Statistical significance was determined by using Student's ratio t test. ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001

Figure 3.2 IL-27 induces expression of multiple inhibitory receptors by CD8$^+$ T cells. Naïve, Ly6C$^-$Sca-1$^-$ CD8$^+$ and T cells were sort purified and cultured in the presence or absence of α-CD3/28, in the presence or absence of IL-27. After an 85-hour culture, cells were analyzed for expression of PD-L1, PD-1, LAG-3, and CTLA-4. Representative FACS plots (left). Bar charts (right) show combined results from 4 independent experiments. Error bars indicate SEM. Statistical significance was determined by using Student's ratio t test. ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001
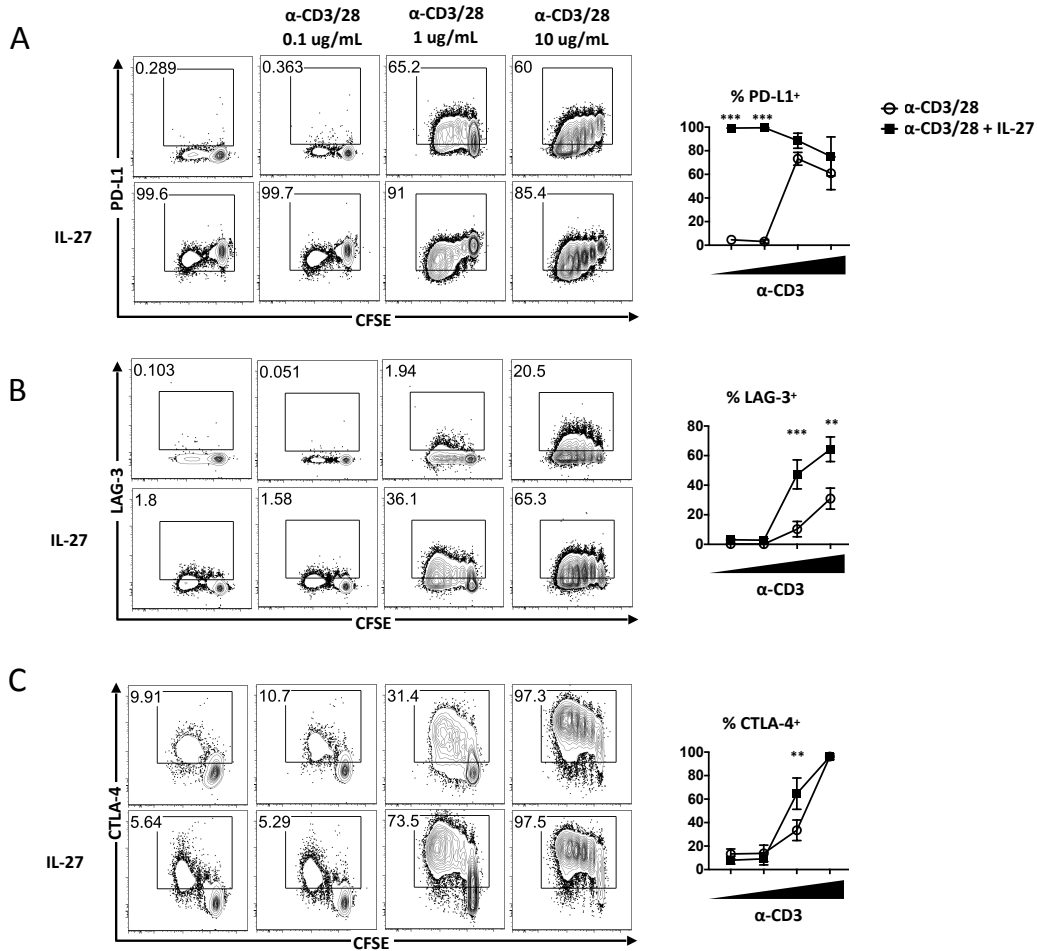
Figure 3.3 Role of IL-27 in expression of PD-L1, LAG-3 and CTLA-4 depends on strength of α-CD3/28 signaling. Naïve Ly6C− Sca-1− CD8+ T cells were sorted and cultured in the absence of α-CD3/CD28 or stimulated with 0, 0.1ug/mL, 1ug/mL or 10ug/mL plate-bound α-CD3 and 1μg/mL soluble α-CD28 for 84 hours. Cells were assessed for expression of PD-L1 (A), LAG-3 (B) and CTLA-4 (C). Representative flow plots (left). Graphs display the results of 3-5 independent experiments (right). Error bars indicate SEM. Statistical significance of differences between IL-27-treated and non-IL-27-treated cells was determined using a paired Student's t test. * p < 0.05, ** p < 0.01, *** p < 0.001
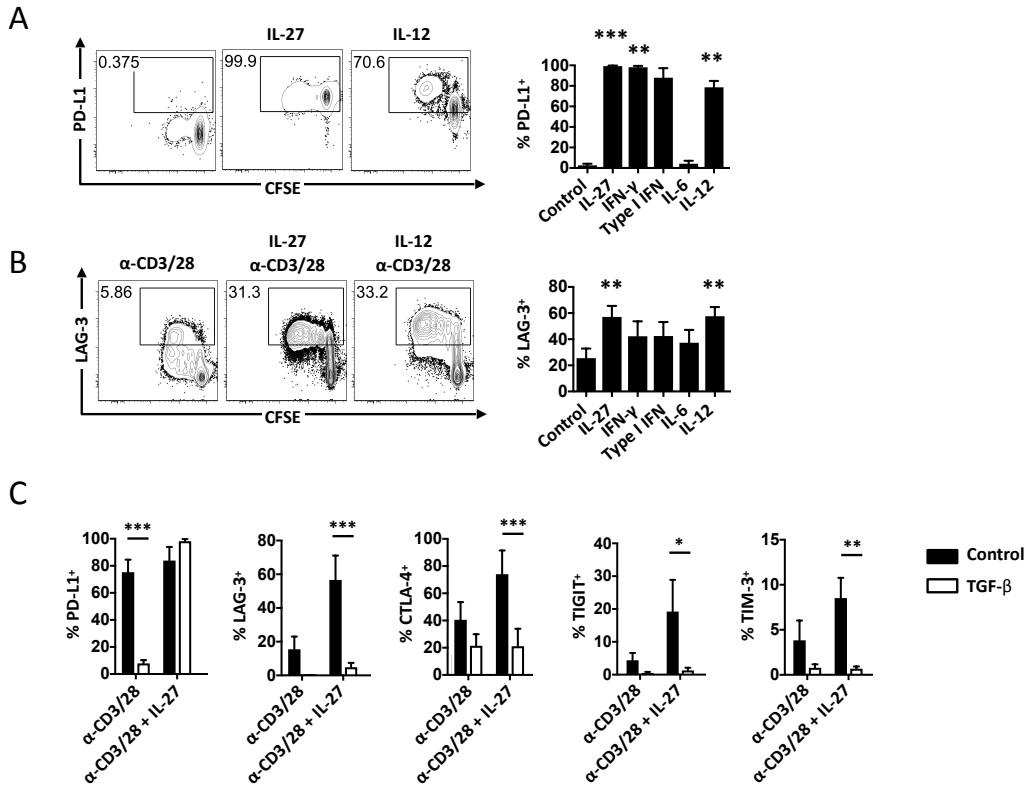
Figure 3.4 Modulation of inhibitory receptors by multiple cytokines. (A) Sorted naive CD8$^+$ T cells were cultured with the indicated cytokines and assessed for expression of PD-L1. (B) Sorted naive CD8$^+$ T cells were cultured with anti-CD3/28 stimulation and the indicated cytokines and were assessed for expression of LAG-3. Representative flow plots for control, IL-27 and IL-12 conditions (left). Summary bar charts (right). (C) TGF-β inhibits IL-27-mediated inhibitory receptor expression in naïve CD8$^+$ T cells. Bar charts summarize results of 1-4 independent experiments. Error bars indicate SEM. Statistical significance determined by paired Student's t test. ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001
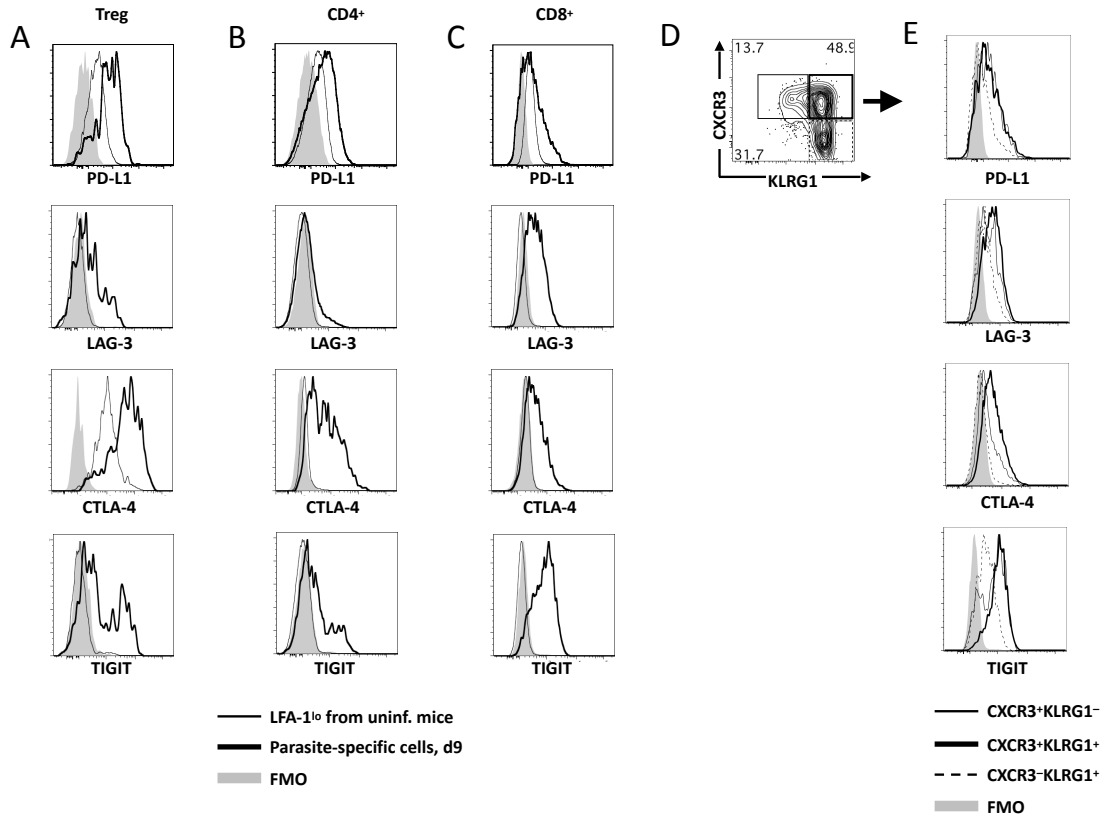
Figure 3.5 Inhibitory receptor expression is upregulated during toxoplasmosis. (A-C). Inhibitory receptor expression by splenic LFA-1^{hi} Tregs (A), tetramer$^+$ CD4$^+$ T cells (B) or tetramer$^+$ CD8$^+$ T cells (C) at day 10 of infection (thick lines) compared to corresponding LFA-1^{lo} cells from uninfected mice (thin lines). (D) Expression of CXCR3 and KLRG1 by tetramer$^+$ CD8$^+$ T cells. (E) Expression of PD-L1, PD-1, LAG-3 and CTLA-4 by WT splenic tetramer$^+$ CD8$^+$ T cells.

A

WT   *Il27p28-/-*

% PD-L1+
% LAG-3+
% CTLA-4+
% TIGIT+

** ns * **

79 / 55.8 — PD-L1 vs CD4
33 / 27.2 — LAG-3 vs CD4
53.1 / 35.3 — CTLA-4 vs CD4
55.5 / 33.5 — TIGIT vs CD4

B

WT   *Il27p28-/-*

% PD-L1+
% LAG-3+
% CTLA-4+
% TIGIT+

** *** ns **

63.4 / 36.4 — PD-L1 vs CD8
51.6 / 29.2 — LAG-3 vs CD8
30.4 / 24.2 — CTLA-4 vs CD8
73.5 / 54.3 — TIGIT vs CD8

74
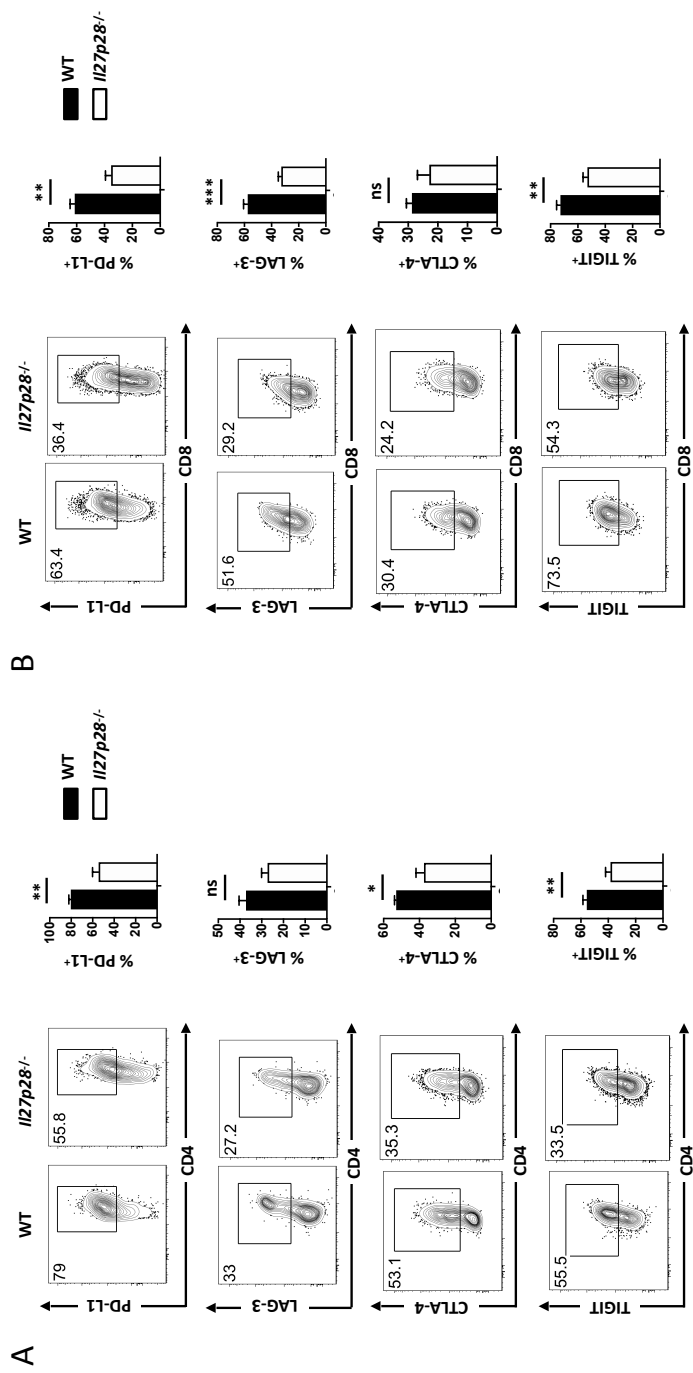
Figure 3.6 IL-27 contributes to inhibitory receptor expression by lung T cells during toxoplasmosis. A-B. WT and *Il27p28$^{-/-}$* mice were infected with 20 Me49 cysts i.p. for 11-12 days. Inhibitory receptor expression by WT and *Il27p28$^{-/-}$* lung tetramer$^+$ CD4$^+$ (A) and CD8$^+$ (B) T cells. 4-5 mice per group, data representative of four independent experiments. Error bars indicate SEM. * p < 0.05, ** p < 0.01, *** p < 0.001
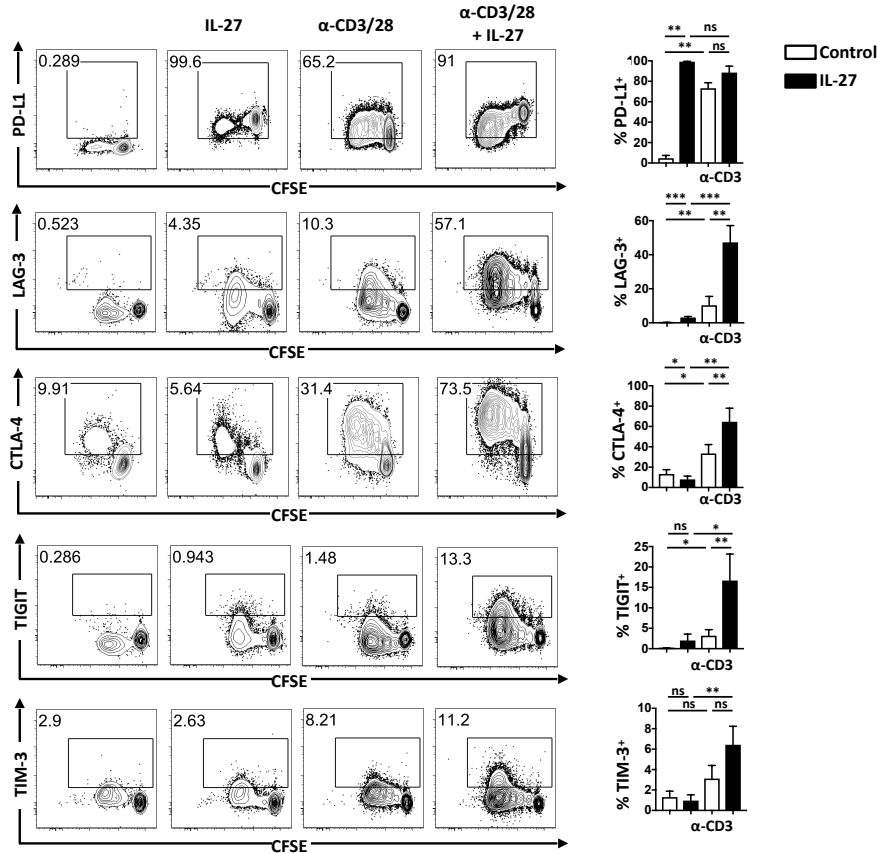
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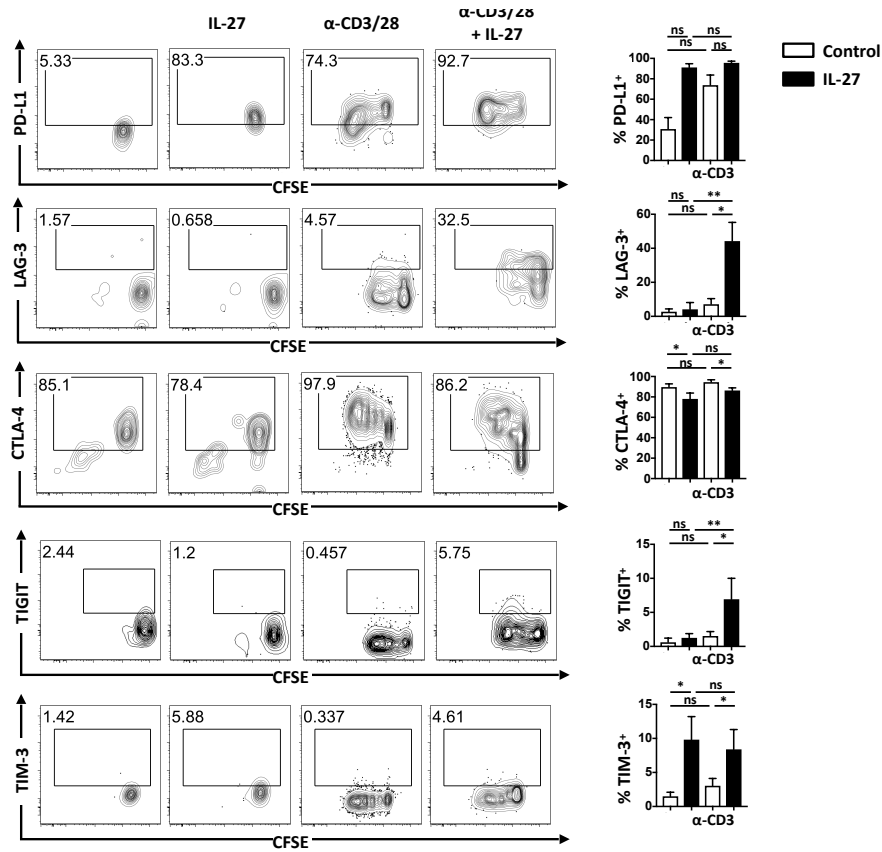
Figure 3.S1 IL-27 induces expression of multiple inhibitory receptors by regulatory T cells. Naïve, Ly6C⁻Sca-1⁻ CD4⁺ and T cells were sort purified and cultured in the presence or absence of α-CD3/28, in the presence or absence of IL-27. After an 85-hour culture, cells were analyzed for expression of PD-L1, PD-1, LAG-3, and CTLA-4. Representative FACS plots (left). Bar charts (right) show combined results from 4 independent experiments. Error bars indicate SEM. Statistical significance was determined by using Student's ratio t test. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 3.S2 TCR stimulation in the presence of IL-27 leads to high coexpression of inhibitory receptors *in vitro*. (A) Hypothetical data to explain the approach. If the expression of two molecules is entirely independent, knowledge of the fraction of cells expressing each molecule enables prediction of the fraction of cells expressing 0, 1, or both molecules. If the experimental results do not match this prediction, this is evidence that expression of these molecules is not independent. (B) Bulk CD4$^+$ splenocytes were cultured for 84 hours in the indicated conditions and the cells were assessed for expression of PD-L1, LAG-3, CTLA-4, TIGIT, PD-1, and Ly6C by flow cytometry. Predicted coexpression of the 6 inhibitory receptors in these conditions was made based on the expression of individual inhibitory receptors (gray bars). The actual coexpression of these molecules was determined using a boolean analysis in Flowjo (black bars).
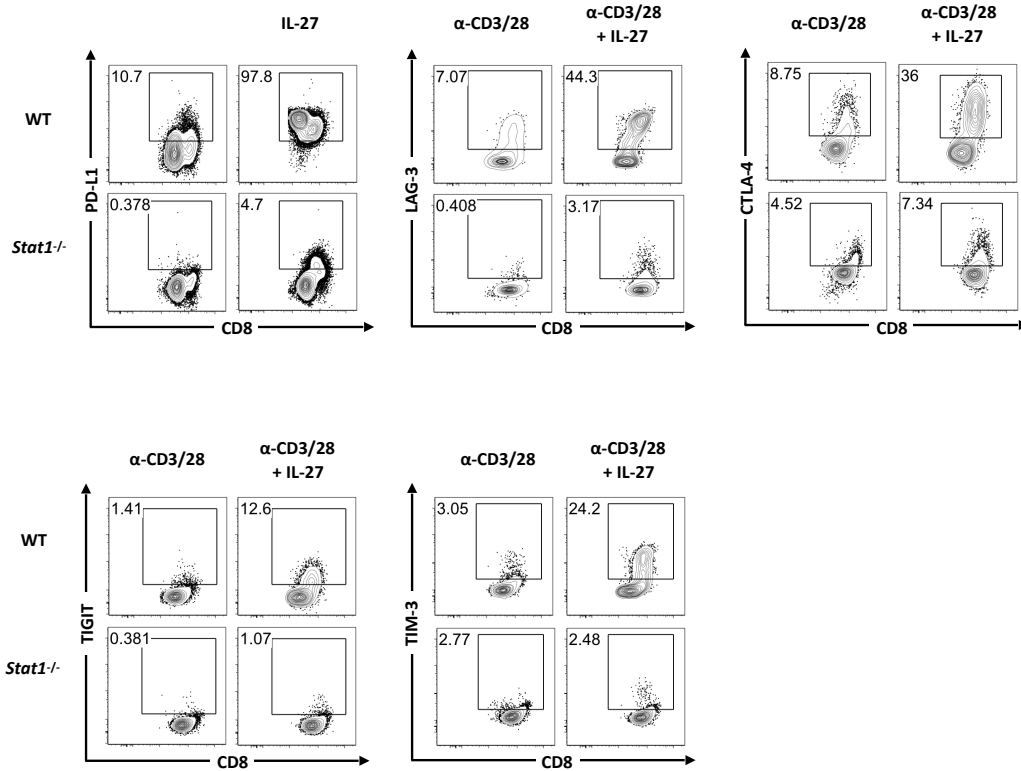
Figure 3.S3 STAT1 contributes to IL-27-mediated inhibitory receptor expression *in vitro*. Requirement for STAT1 for cytokine-mediated inhibitory receptor expression in sorted naive Ly6C⁻ Sca-1⁻ CD8⁺ T cells. Results representative of 3 independent experiments.
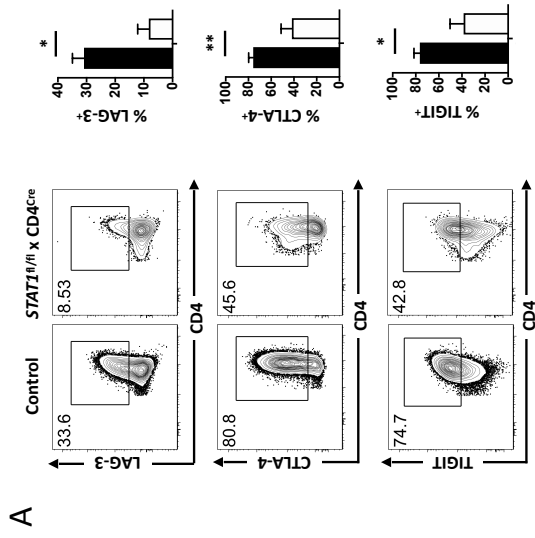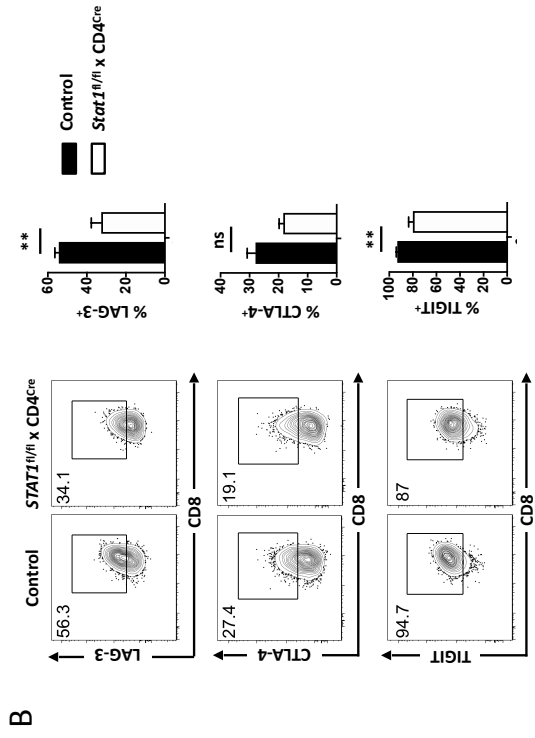
Figure 3.S4 STAT1 contributes to IL-27-mediated inhibitory receptor expression *in vivo*, during toxoplasmosis. *Stat1$^{fl/fl}$ x CD4^{Cre}* mice or Control (*Stat1$^{fl/fl}$* x *Wt, Stat1$^{wt/wt}$* x *CD4^{Cre}, or Stat1$^{fl/wt}$ x CD4^{Cre}*) mice were infected with 20 Me49 cysts i.p. for 10-11 days. Inhibitory receptor expression was examined on LFA-1^{hi} CD4$^+$ T cells (A) and tetramer$^+$ CD8$^+$ T cells (B), from the peritoneum. Bar charts display the data from one experiment with 5 mice. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$
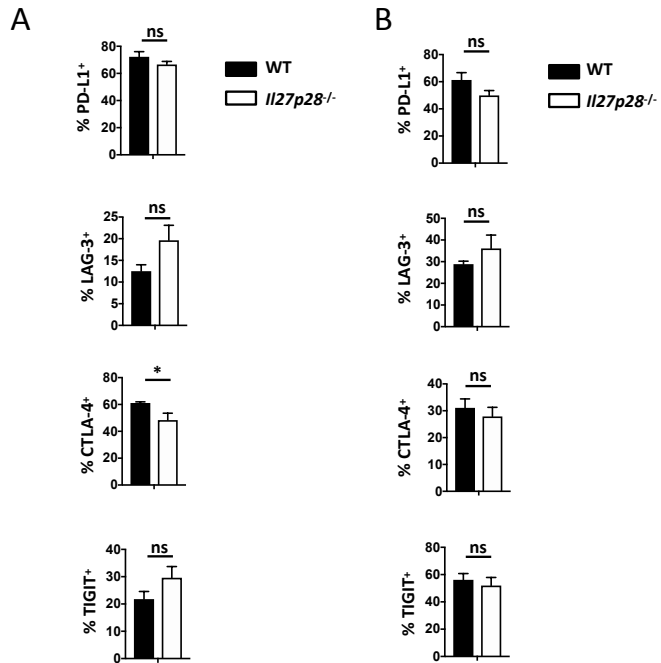
Figure 3.S5 IL-27 not required for splenic inhibitory receptor expression during toxoplasmosis. A-B. WT and *Il27p28*^{-/-} mice were infected with 20 Me49 cysts i.p. for 11-12 days. Inhibitory receptor expression by WT and *Il27p28*^{-/-} splenic tetramer⁺ CD4⁺ (A) and CD8⁺ (B) T cells. 4-5 mice per group, data representative of four independent experiments. Error bars indicate SEM.

Chapter 4: Discussion and future directions

Abstract

IL-27 modulates the immune response in diverse ways. At the time these studies were initiated, little was known about the role of IL-27 in promoting inhibitory receptor (IR) expression, although it was known that IL-27 could promote PD-L1 on bystander T cells, independently of TCR signals (Hirahara et al., 2012). The studies presented here establish that TCR stimulation and cytokine signaling have distinct and complementary roles in promoting IR expression (Fig 4.1), which aids in our understanding of the role of IL-27 and other cytokines in promoting IR expression. The role of co-stimulation in IR induction was not evaluated in these studies, but is likely to be important, as co-stimulation enhances TCR signal strength and signals through TCR-independent pathways. A better understanding of the signaling networks required for IR induction will help better understand why IL-27 appears more potent than closely-related cytokines like IL-6 and IL-12. The implications of these findings extend beyond infection and highlight that additional studies are needed to determine if Ly6 molecules function as IRs *in vivo*. The contribution of IR to IL-27-mediated suppression of pathologic or protective immune responses should be examined as well during acute infection and other inflammatory conditions. Finally, the finding that IL-27 and STAT1 contribute to IR expression *in vitro* and *in vivo* suggest that strategies that target cytokine signaling could modulate IR expression, which may be useful therapeutically.

4.1   Roles of inhibitory receptors in modulating inflammation

The role of Ly6C in the immune response

The Ly6 family of proteins is ancient, with homologous proteins found in mice and humans, and related proteins found in species as distantly related as squid (Gumley et al., 1995; Williams et al., 1988). Sca-1 is one of 23 Ly6-like molecules encoded on chromosome 15 in the mouse (Loughner et al., 2016) and at least three of these molecules, Ly6C, Ly6G and Ly6A, are highly expressed by hematopoietic cells. Ly6C is widely used to identify different populations of cells, for example, as a marker of short-lived effector cells during acute viral infection (Hu et al., 2015; Marshall et al., 2011). However, as shown in Chapter 2, Ly6C is also expressed by naïve and central memory T cells. Thus, its expression is not restricted to the short-lived effector population and the as yet unclear function of Ly6C is likely relevant for broader populations of T cells than just terminally-differentiated effector cells.

As discussed in Chapter 2, there is a literature that suggests that Ly6C functions as an IR on T cells (Codias et al., 1992; Fleming and Malek, 1994; Yamanouchi et al., 1998), but Ly6C is a GPI-anchored protein with no intracellular domain. If it does transduce a signal, it does so through association with another cell surface molecule, likely as part of a lipid raft (Jaakkola et al., 2003). I found in Chapter 3 that IL-27 and TCR stimulation, signals that upregulate Ly6C, promote expression of multiple well characterized IRs. This is consistent with a model in which Ly6C is one member of a cassette of IRs upregulated by T cells in response to these stimuli. Studies to examine the function of Ly6C have utilized antibodies to block its interaction with potential ligands or to

crosslink it and transduce a signal (Fleming and Malek, 1994; Hanninen et al., 1997; Hanninen et al., 2011; Jaakkola et al., 2003). These studies have yielded valuable information, but the field would benefit from the generation of a mouse with a targeted deletion of Ly6C.

Spontaneous mutations that limit expression of Ly6C are present in NOD, NZB/W, and ST/bJ mice, strains that spontaneously develop autoimmune diseases (Philbrick et al., 1990). It is tempting to speculate that the absence of Ly6C might promote autoinflammatory responses. However, other mutations, particularly those at MHC loci, are critical for disease development in these mice, making it difficult to isolate the contribution of Ly6C (Pearson et al., 2016; Perry et al., 2011). Two adjacent genes, Ly6c1 and Ly6c2, encode the Ly6C protein and are both transcribed (Heng et al., 2008), so both genes will need to be targeted in order to eliminate expression of the protein. One useful approach would be to delete one gene and flox the other gene to make a conditional knockout mouse. Breeding this mouse with mice that express Cre recombinase by cell populations of interest would enable investigation of the function of Ly6C on different immune populations that include inflammatory monocytes and T cells. This would reveal whether loss of Ly6C expression by a particular cell population results in the development of autoimmune disease, affects tolerance to infection, or has another immune function.

The finding in Chapter 2 that IL-27 drives expression of Ly6C initially suggested that IL-27 might support the generation of Ly6C$^+$ short-lived effector CD4$^+$ T cells, reminiscent of the cells described *in vivo* during infection with LCMV (Marshall et al., 2011). However, preliminary examination of T cells after culture with IL-27 revealed that

Ly6C$^+$ T cells did not produce more cytokines than Ly6C- T cells (data not shown). This observation indicates that Ly6C in isolation may not be an effective marker of short-lived effector cells. KLRG1 and CXCR3 have been used to distinguish specific differentiation stages of CD8$^+$ T cells during toxoplasmosis (Chu et al., 2016). The least differentiated parasite-specific cells in this model are CXCR3$^+$KLRG1$^-$. These differentiate into a CXCR3$^+$KLRG1$^+$ intermediate population that forms a proliferative pool that feeds a terminally-differentiated CXCR3$^-$KLRG1$^+$ effector population. When parasite-specific CD4$^+$ and CD8$^+$ T cells were separated by expression of KLRG1 and CXCR3, Ly6C was not enriched on any of these subpopulations (Chapter 2). The finding that there is no clear correlation between expression of KLRG1 and/or CXCR3 and Ly6C highlights the requirement for multiple markers in the identification of different stages of T cell differentiation. Nevertheless, further studies will be necessary to determine the significance of Ly6C expression in different contexts. Ly6C expression aids in identification of short-lived effector cells in two viral infections, but it is not known what populations express Ly6C in other contexts such as Th2-skewed inflammation. Our finding that Ly6C is expressed by diverse T cell populations during toxoplasmosis suggests that its utility as a marker of SLECs may be restricted to distinct inflammatory contexts.

The role of Sca-1 in the immune response

Similar to Ly6C, the role of Sca-1 (Ly6A/E) in the immune response is unclear, although antibody-mediated cross-linking of Sca-1 on T cells limits cell proliferation and production of IL-2, consistent with the idea that Sca-1 functions as an IR on T cells

(Codias et al., 1992; Fleming and Malek, 1994). Further support for this idea is provided by a report that Sca-1-deficient T cells proliferate at a higher rate when stimulated with anti-CD3 antibodies or allogeneic cells (Stanford et al., 1997). *In vivo*, Sca-1-deficient mice have dysregulated development of certain hematopoietic populations and Sca-1-deficient bone marrow is limited in its ability to serially reconstitute lethally irradiated mice (Ito et al., 2003). Despite these defects, Sca-1-deficient mice immunized with ovalbumin develop normal antibody responses (Jones et al., 2016) and the T cell response to LCMV is intact (Whitmire et al., 2009). However, there is still limited information about the role of Sca-1 in other models and it is also possible that there is redundancy in the function of Ly6 molecules that is masked in the single molecule knockout mouse.

Sca-1, in combination with the IL-2Rβ-chain (CD122) and Bcl-2, can be used to identify mature CD8$^+$ T cells with stem-like properties, termed memory stem cells (Tscm) (Gattinoni et al., 2011; Gattinoni et al., 2009; Zhang et al., 2005). These are minimally-differentiated cells with the ability to survive and differentiate upon adoptive transfer to naive recipients. They constitute a very different population of T cells than the short-lived effector cells identified by Ly6C expression. It was therefore surprising that we found that Sca-1 and Ly6C were upregulated by the same signals *in vitro*. I also showed in Chapter 2 that the vast majority of parasite-specific CD4$^+$ and CD8$^+$ T cells express Sca-1 during infection, which is inconsistent with studies that describe Sca-1 as a marker of Tscm during GVHD (Zhang et al., 2005). These differences suggest that there are signals present during infection, such as cytokines, antigen presentation and overall levels of inflammation, that differ from those present in this model of GVHD. Again, the

discrepancies between these reports highlight the necessity of using multiple markers to distinguish stages of T cell differentiation and emphasizes the need to verify these markers in different experimental systems.

The role of inhibitory receptors during infection

The findings in Chapters 2 and 3 established that IL-27 contributes to the expression of CTLA-4, PD-L1, LAG-3, TIGIT, and Ly6C during toxoplasmosis, most notably at sites of inflammation. However, the impact of IL-27-mediated IR expression on the overall immune response during infection has not been fully elucidated and it is not known if the ability of IL-27 to promote IRs contributes to its protective effects. Previous studies have shown that treatment with CTLA-4-Ig limits the T cell response directly by blocking CD28 co-stimulation, but also as an agonist that upregulates indoleamine 2, 3 dioxygenase (IDO) activity in dendritic cells (DC). This pathway depletes tryptophan from the microenvironment, which limits T cell activation (Bluestone et al., 2006; Grohmann et al., 2002). The Hunter laboratory has shown that administration of CTLA-4-Ig reduces the immunopathology that develops during toxoplasmosis in IL-27Rα-deficient mice (Stumhofer et al., 2006). While this observation must be interpreted with care, it supports the hypothesis that upregulation of CTLA-4 by IL-27 is a mechanism that limits immunopathology. IL-27Rα-deficient mice also develop immunopathology during infection with *P. berghei* (Findlay et al., 2010) and blockade of CTLA-4 and PD-L1 in WT mice increases immunopathology during *P. berghei* infection (Hafalla et al., 2012). A similar pattern is seen in *L. donovani* infection, in which IL-27 limits immunopathology and inhibitory receptor blockade results in a more effective

immune response (Joshi et al., 2009; Murphy et al., 1998). It is therefore likely that the ability of IL-27 to promote IR expression is relevant to other infection models and a survey of these other infection systems would be helpful to determine circumstances in which IL-27 or other cytokines have a similar role.

One of the major themes of studies on IL-27 is that it has diverse, pleiotropic effects on the immune response. The Hunter laboratory has reported that it promotes several inhibitory pathways during toxoplasmosis including production of IL-10, differentiation of Th1-like Tregs, and I have added the expression of multiple IRs. Each of these pathways has been found to play a role in limiting the immune response during toxoplasmosis, but it can be difficult to parse apart the effects of distinct regulatory mechanisms and determine their role within a larger related regulatory network. PD-L1, LAG-3, CTLA-4 and TIGIT were upregulated by IL-27 *in vitro* and their expression was impaired in IL-27-deficient mice during toxoplasmosis. Antibody blockade of one or more of these molecules during acute toxoplasmosis might reveal the degree to which absence of IRs is responsible for the pathological phenotype seen in IL-27-deficient mice. A complementary approach would be to attempt to ameliorate immunopathology in IL-27-deficient mice through treatment with recombinant Fc-tagged inhibitory receptors. As described above, we have found that CTLA-4-Fc limits immunopathology in this system, and commercially available LAG-3-Fc, PD-L1-Fc and TIGIT-Fc could be used to determine whether exogenous IRs would prove sufficient to prevent immune pathology. In particular, TIGIT has been found to upregulate IL-10 production by DCs (Yu et al., 2009) and it would be of interest to determine if TIGIT-Fc promotes IL-10 production

during toxoplasmosis. These types of approaches would provide further insight into the importance of IL-27-mediated expression of IRs in limiting immunopathology.

4.2   Factors controlling inhibitory receptor expression

Signaling pathways involved in inhibitory receptor expression

The studies in Chapter 3 demonstrated a role for STAT1 in cytokine-mediated inhibitory receptor expression, both *in vitro* and *in vivo* during toxoplasmosis. However, IFN-γ, type I IFN, IL-6, and IL-12 also signal through STAT1 and culture with these cytokines did not phenocopy all the effects of IL-27 in these studies. It is therefore likely that signaling pathways other than STAT1 are responsible for these differences. For example, type I IFN also signals through STAT2, IL-6 signals through STAT3, and IL-12 signals through STAT4. Multiple studies have been performed to determine the contribution of different signaling molecules to IL-27 signaling. In addition to STAT1, IL-27 signals through STAT3, ERK1/2, p38 MAPK, T-bet, c-MAF, and Blimp1 (Heinemann et al., 2014; Owaki et al., 2006; Owaki et al., 2008; Pflanz et al., 2004; Pot et al., 2009). These signaling molecules make differential contributions to the various effects of IL-27 signaling. STAT1, ERK1/2, p38 MAPK, and T-bet contribute to upregulation of IL-12Rβ and Th1 differentiation (Owaki et al., 2006). STAT1 upregulates SOCS3 and thereby limits CD28-mediated IL-2 production (Owaki et al., 2006), and STAT3 contributes to IL-27-mediated proliferation but is dispensable for IL-27-mediated Th1 differentiation (Owaki et al., 2008).

It is not clear from these studies why IL-27 has fundamentally different effects on the immune response from other cytokines, such as IL-6, that share signaling pathways

with IL-27. A recent study directly addressed this question and found that while STAT3 was required for genes upregulated by both IL-27 and IL-6, STAT1 was primarily required for genes that are uniquely upregulated by IL-27 (Hirahara et al., 2015). These results led to the conclusion that STAT1 is required for the different outcomes seen by IL-27 and IL-6 signaling. This is consistent with the finding in Chapter 3 that STAT1 is required for IL-27-mediated inhibitory receptor expression, as this is an effect of IL-27 that is not shared by IL-6. Indeed, post-hoc analysis of ChIP-Seq data from a study examining the effect of IL-27 on anti-CD3/28-stimulated T cells (Hirahara et al., 2015) indicated that STAT1 binds to the gene region or directly upstream of the genes for PD-L1, LAG-3, CTLA-4, TIGIT, TIM-3, Ly6C and Sca-1. Culture with IL-27 appeared to increase STAT1 binding to each of these genes except for PD-L1. It is therefore possible that STAT1 binding to these gene regions, alone or as part of a complex, contributes to the high degree of coordinate IR expression we see in cells stimulated through the TCR in the presence of IL-27. However, this same study found that IL-27 signaling results in STAT1 binding directly upstream of *Pdcd1* (PD-1), which is not upregulated by IL-27. This paradox demonstrates that STAT1 binding is not sufficient to result in IR upregulation in response to IL-27 and suggests that other transcription factors are required in this process. Indeed, as indicated above, IL-27 signals through Blimp1 and c-Maf, and in the context of B16F10 melanoma, Blimp1 is required for full expression of PD-1, TIM-3, and PROCR, and c-Maf is required for full expression of PD-1, TIM-3, TIGIT, and PROCR (Chihara et al., 2018). The finding in Chapter 3 that TGF-β limits inhibitory receptor expression supports a role for Blimp1 in this process, as TGF-β limits

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Blimp1 expression (Neumann et al., 2014). Blimp1 and c-Maf also contribute to IL-10

production by T cells, suggesting a central role for these transcription factors in the

coordination of multiple inhibitory pathways (Neumann et al., 2014).

IL-27 impacts T cells in peripheral tissues during toxoplasmosis

The *in vitro* studies presented in Chapters 2 and 3 establish that IL-27 is capable

of upregulating expression of multiple IRs on naive cells as they are primed by antigen

presenting cells. *In vivo*, T cell priming takes place in the spleen, lymph nodes, and local

secondary lymphoid structures. A report from the Kedl lab found that after immunization

with a sub-unit vaccine, splenic T cell responses in IL-27Rα-deficient mice are deficient

7 days after immunization, which is an example of a positive role for IL-27 during T cell

priming (Pennock et al., 2014). However, at day 10 of intraperitoneal infection with *T.

gondii*, IL-27 is produced at higher levels in the lung than in the spleen (data not shown)

and during oral toxoplasmosis, IL-27 is produced at higher levels in the intestinal lamina

propria than in the spleen (Hall et al., 2012a). This helps explain findings that the effect

of IL-27 deficiency on T cell phenotype during toxoplasmosis and other infections such

as Sendai virus is also more apparent in peripheral sites of infection than in the spleen

(DeLong et al., 2018; Hall et al., 2012a; Muallem et al., 2017). For example, in Chapter

2, the effect of IL-27 on Ly6C expression was most obvious in the peritoneum and in

Chapter 3, the effect of IL-27 on IR expression was most pronounced in the lungs. These

results are consistent with a model in which IL-27 is capable of upregulating IR

expression during priming, as demonstrated in the *in vitro* experiments presented here,

but activated T cells in the periphery are also influenced by local levels of IL-27 (Figure 4.2).

The finding that IL-27 contributes to IR expression more at peripheral sites of infection than in secondary lymphoid organs highlights the need for additional studies to examine the factors that control IR expression on antigen-experienced cells. The *in vitro* studies presented here demonstrate that TCR stimulation of naïve T cells is required for significant expression of PD-1, CTLA-4, LAG-3, TIGIT, and TIM-3 by naive T cells during priming, but it is possible that on antigen-experienced cells, which express increased levels of the IL-27 receptor (Villarino et al., 2005), IL-27 is sufficient for induction of inhibitory receptors. Indeed, a previous report found that although IL-2, IL-7, IL-15, and IL-21 have no effect on PD-1 expression by naive T cells, these cytokines alone are sufficient to induce PD-1 on antigen-experienced populations (Kinter et al., 2008). Elucidation of factors that control inhibitory receptor expression on antigen-experienced cells would help explain the requirement for IL-27 for full inhibitory receptor expression in peripheral tissues and would give insight into the importance of DC antigen presentation and cytokine production by in this process.

There are several approaches to distinguish the role of IL-27 during priming from its role in the periphery. For example, adoptive transfer of antigen-experienced splenic T cells from acutely-infected mice to infection-matched WT or IL-27-deficient mice would elucidate the importance of IL-27 after priming. If IR expression by transferred T cells in peripheral sites was found to be IL-27-dependent, that would suggest a role for IL-27 in the periphery, after T cell priming. Alternative approaches are available to control IL-27 signaling temporally, to remove IL-27 signaling after initial T cell priming has taken

place. This would help distinguish the effects of IL-27 during priming from those on antigen-experienced cells, especially in the periphery. Mice with a floxed IL-27Rα gene could be crossed with mice expressing a Cre recombinase-estrogen receptor fusion protein. Administration of tamoxifen would then be used to delete the IL-27Rα at day 3-4 of infection. Alternatively, IL-27-depleting antibodies could be used to neutralize IL-27 in WT mice after T cell priming has occurred. A complementary approach would be to administer IL-27 to IL-27-deficient mice at different time points during infection. These studies would need to be interpreted with care, as T cells could recirculate to secondary lymphoid organs at later time-points of infection, but these approaches would give insight into the role of IL-27 in modulating the T cell response at different stages of infection.

Upregulation of inhibitory receptor expression with increasing TCR stimulation

The finding that IR expression is responsive to increasing TCR stimulation (Chapter 3) demonstrates that induction of this regulatory pathway is graded and suggests that IR expression is concentrated on the most highly stimulated cells. Increasing TCR stimulation during T cell priming results in increased chromatin accessibility (Tu et al., 2017), which likely allows increased transcription of inhibitory receptor genes. This may be one way in which T cells can respond to low levels of stimulation with minimal inhibition, but as stimulatory signals increase, regulatory signals are coordinately elevated in order to prevent T cell hyper-activation.

The experiments to address the impact of TCR signal strength in Chapter 3 were performed using titrated amounts of anti-CD3 antibodies, which activate the TCR signaling cascade in an experimentally useful but artificial manner. It is therefore not

clear from these studies if IR expression is influenced by the affinity of individual TCR:pMHC interactions, the total avidity of the T cell - DC synapse, the duration of the T cell - DC synapse, the number of times a T cell forms a synapse with antigen-presenting cells, or a combination of these. The affinity of an individual TCR:pMHC interaction is not determined by the inflammatory environment, but the avidity and duration of the T cell - DC synapse, as well as the number of times a T cell encounters a cognate DC, depend to a great extent on the level of inflammation and antigen present during an immune response. TLR ligands and cytokines such as IFN-γ promote DC maturation, while IL-10 limits DC expression of CD80/86 and MHC molecules. Chemokines promote migration of DCs and T cells to lymph nodes, and antigen availability impacts the number of pMHC complexes available for T cells to bind. The frequency of T cell - DC interactions and the avidity of the T cell synapse therefore depend on the cytokines, chemokines and antigen load present during a given infection. As such, it is necessary for the immune system to employ regulatory mechanisms that limit T cell activation in response to diverse inflammatory signals. Control of IR expression through strength of TCR stimulation and cytokine signaling provides graded regulation proportional to exposure to these inflammatory stimuli.

The role of TCR stimulation in IR expression could be examined *in vivo* through utilization of Nur77 reporter mice (Moran et al., 2011). In T cells, Nur77 expression is transiently induced upon TCR stimulation and is not affected by cytokine signaling (Ashouri and Weiss, 2017). During toxoplasmosis, if IR expression by parasite specific cells in the periphery is concentrated on Nur77-reporting cells, that would indicate the need for recent TCR stimulation for full IR expression. If Nur77 expression does not

correlate with IR expression, that would indicate that repeated TCR stimulation is not required for persistent IR expression *in vivo.*

Role of co-stimulation in inhibitory receptor expression

The studies in Chapter 3 found that inhibitory receptor expression varied with modulation of TCR stimulation, and an important focus for future studies will be on the role of co-stimulation in this process. The best described co-stimulatory interaction is between CD28 and CD80/86. This interaction enhances TCR signal strength and also engages signaling pathways distinct from those used by the TCR (Acuto and Michel, 2003; Boomer and Green, 2010). The studies in Chapter 3 predict that signals that modulate TCR signal strength will affect inhibitory receptor expression, which means that CD28 signaling is likely important in this process. Additionally, CD28 stimulation promotes production of IL-2, and IL-2 has been found to promote inhibitory receptor expression (Fraser et al., 1991; Kinter et al., 2008). CTLA-4-Ig is a CD28 antagonist that effectively limits T cell co-stimulation *in vivo*. Treatment with CTLA-4-Ig limits the immunopathology that develops during toxoplasmosis in IL-27Rα-deficient mice (Stumhofer et al., 2006), indicating that co-stimulation is necessary for development of immunopathology in this system. Variations on this experiment could be used to elucidate the role of co-stimulation on inhibitory receptor expression during infection and other inflammatory environments.

4.3   Targets of inhibitory receptors

IL-27 acts on T cells to limit dendritic cell function

IL-27 can limit Th2 and Th17 cell function directly, through inhibition of GATA-3 and RORγt (Diveu et al., 2009; Lucas et al., 2003), but it can also have indirect effects on DCs that limit subsequent T cell activation. Multiple surface-bound and secreted products of activated T cells modulate DC phenotype. IFN-γ upregulates DC expression of IL-12, MHCII, CD80/86, and CD40, while IL-10 limits expression of these molecules and promotes tolerogenic DCs (Bhattacharyya et al., 2004; Boks et al., 2012; De Smedt et al., 1997; Sheng et al., 2013). Tregs can limit DC maturation through binding of LAG-3 and MHCII (Liang et al., 2008). T cells also promote tolerogenic DCs through expression of CTLA-4, which stimulates IDO activity (Grohmann et al., 2002; Onodera et al., 2009), and TIGIT, which promotes DC production of IL-10 (Yu et al., 2009). IL-27 modulates T cell expression of IL-10, IFN-γ and IRs and may thereby modify dendritic cell function indirectly, through T cells. Conversely, DCs can modulate T cell responses through binding cell surface molecules as well as through production of cytokines such as IL-27. An example of the complexity of this system is a study finding that Tregs induce DC production of IL-27, which in turn drives differentiation of IL-10-producing Tr1 cells, which limit DC activation (Awasthi et al., 2007). My own preliminary *in vitro* data found that culture with IL-27 improved the ability of Tregs to limit DC upregulation of CD80/86 and MHCII in response to LPS stimulation (data not shown). These preliminary studies are promising, but more work is required to understand the role IL-27 plays in T cell-mediated inhibition of DC function.

Impact of IL-27 on bystander cells

The finding that IL-27 can drive expression of Sca-1 and PD-L1 in the absence of TCR stimulation (DeLong et al., 2018; Hirahara et al., 2012) raises the question of what role IR expression plays on bystander T cells. Sca-1$^+$ T cells have not been described to inhibit target cells, but PD-L1$^+$ CD4$^+$ T cells limit Th17 differentiation of target naïve CD4$^+$ T cells (Hirahara et al., 2012). Nonetheless, relatively little is known about the role of bystander cells in modulating the immune response during infection. PD-L1 binds two ligands: PD-1, which is mainly expressed by T cells, and CD80, which is expressed by antigen presenting cells and activated T cells. Exogenous PD-L1 limits T cell proliferation and cytokine production directly, by binding CD80 on the T cell (Butte et al., 2007). However, dendritic cells also express CD80, and the finding that IL-27 induces PD-L1 expression by bystander cells suggests a mechanism by which PD-L1 expression could be induced at peripheral sites of infection early, before the full antigen-primed T cell response can form. If these PD-L1-expressing T cells bind CD80 and limit dendritic cell function at sites of inflammation, this could directly limit T cell responses in the periphery and also be a mechanism to shut down antigen presentation in secondary lymphoid organs at later time-points of infection, due to reduced activation of the dendritic cells that migrate to secondary lymphoid organs from sites of infection. This hypothesis could be examined *in vivo* during infection by treating mice with anti-PD-L1 antibodies to block interaction of PD-L1 with its ligands, which might increase DC activation. Alternatively, administration of PD-L1-Fc could be used to augment PD-L1 signaling, which might limit DC activation in secondary lymphoid organs and peripheral

sites of infection and decrease T cell activation. Similar experiments could be performed *in vitro* with isolated T cells and DCs to control for the influence of other cell populations. These studies would help link IL-27, inhibitory receptors, and IL-10 in a network of regulatory pathways that limits immune pathology.

4.4   Therapeutic applications

Therapies to modulate inhibitory receptor expression

As noted at many points in this thesis, IR blockade has been found to improve T cell cytokine production, cytotoxicity, and control of parasite burden during toxoplasmosis, leishmaniasis, and malaria, leading to improved outcomes in mice (Bhadra et al., 2011; Butler et al., 2011; Hafalla et al., 2012; Joshi et al., 2009; Murphy et al., 1998). The impact of IR blockade has been extended and has also been found to improve the anti-tumor response. This has therapeutic implications, as blockade of PD-L1, PD-1 and CTLA-4 are now FDA approved cancer therapies. The finding that cytokines play an important role in controlling IR expression suggests that modulation of cytokine signaling could be an additional approach for manipulating IR expression. Induction of IRs by IL-27 could help explain why, in some cancer models, the absence of IL-27 leads to enhanced tumor control. In the context of B16F10 melanoma, better tumor control in IL-27-deficient mice has been attributed to enhanced CD8^{+} T cell responses and reduced TIM-3 expression (Sauer et al., 2008; Zhu et al., 2015). Subsequent work found that, similar to the work presented here, IL-27 promotes expression of LAG-3, TIGIT, and TIM-3 on CD4^{+} and CD8^{+} T cells *in vitro* (Chihara et al., 2018). In agreement with the findings in Chapter 3, Chihara, et al. found that IL-27 did not promote

PD-1 expression *in vitro*. That study did find that expression of LAG-3, TIGIT and TIM-3 was deficient in Il27p28-deficient mice in the context of B16F10 melanoma (Chihara et al., 2018). It is uncertain whether this is directly relevant to humans, but in principle, blockade of IL-27 signaling in humans may provide a way to limit expression of multiple IRs simultaneously.

Administration of IL-27 has shown therapeutic potential in a variety of pre-clinical models including colitis, experimental autoimmune encephalomyelitis (EAE), and collagen-induced arthritis (CIA). Purified IL-27 has a short biological half-life, so pegylation of IL-27 protein has been used to extend its biological half-life and function. Gene therapy is another approach and administration of IL-27 expression through hydrodynamic transfection of plasmids encoding IL-27 has been shown to induce high production of IL-27. Gene therapy with IL-27 can also be administered by adeno-associated viral vector, which promotes expression of PD-L1 and limits development of colitis (Zhu et al., 2016), EAE (Zhu et al., 2018), and CIA (Pickens et al., 2011). Administration of IL-27 could therefore upregulate multiple regulatory pathways including IR expression to limit inflammation during autoimmune disease. However, in other contexts, the overexpression of IL-27 has pro-inflammatory effects and can limit growth of B16F10 melanoma directly and through NK cells (Oniki et al., 2006; Yoshimoto et al., 2008). The role of IL-27 in modulating disease is therefore context dependent, and there are potential therapeutic applications for both augmenting and limiting IL-27 signaling.

Summary

The studies presented here demonstrate distinct and complementary roles for IL-27 and TCR stimulation in the upregulation of multiple inhibitory receptors. This may be a mechanism by which precise modulation of these pathways provides levels of regulation that allow for productive immune responses while preventing them from becoming pathologic. During toxoplasmosis, IL-27 and STAT1 promote inhibitory receptor expression by T cells at peripheral sites of infection. This has important therapeutic implications, as inhibitory receptor expression is dysregulated in multiple disease contexts and drugs that are currently used to inhibit cytokine signaling could affect immune regulation through modulation of inhibitory receptor expression. These findings demonstrate a prominent role for IL-27 in a model in which inhibitory receptor expression on T cells is a graded regulatory pathway that is upregulated by exposure to increasing levels of TCR stimulation and cytokines present at sites of inflammation.

Figures
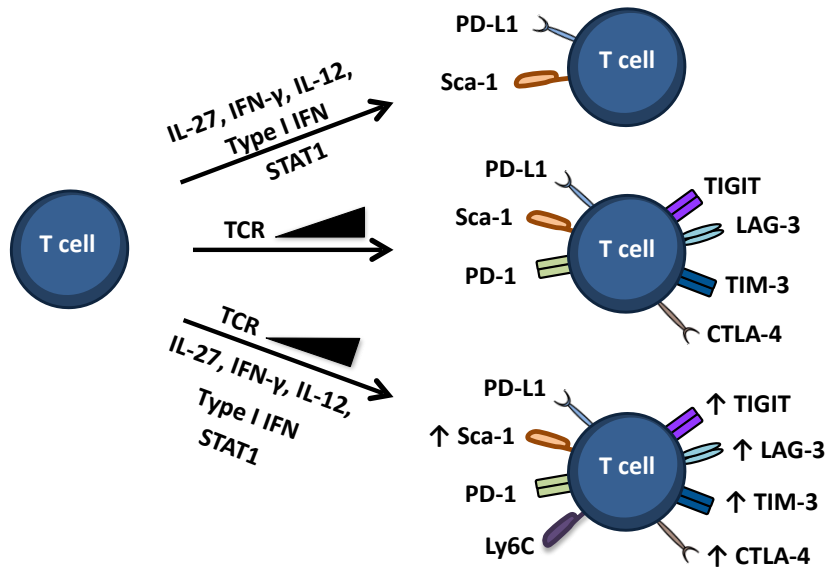
Figure 4.1 Cytokine- and TCR-mediated induction of inhibitory receptors. The studies presented here demonstrate distinct and complementary roles for cytokines and TCR signaling in modulating inhibitory receptor expression. IL-27 or other cytokines alone are able to upregulate PD-L1 and Sca-1, while TCR signaling upregulates PD-L1, Sca-1, PD-1, LAG-3, and CTLA-4. IL-27 and related cytokines augment TCR-mediated upregulation of Sca-1, LAG-3, Ly6C, and CTLA-4.
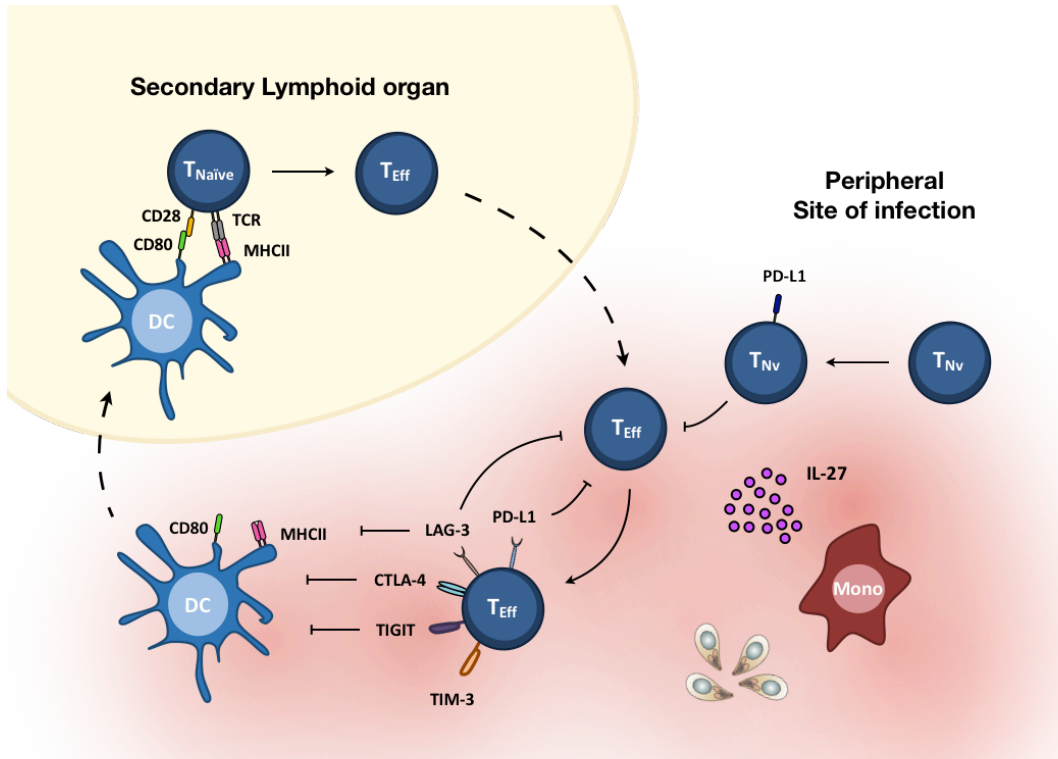
Figure 4.2 Proposed model of IL-27-mediated immune regulation during toxoplasmosis. The studies presented here demonstrate that during toxoplasmosis, IL-27 is required for full inhibitory receptor expression in the lung, a peripheral site of infection. CTLA-4 and LAG-3 inhibit dendritic cell maturation and TIGIT promotes IL-10 production in DCs, suggesting that IL-27 can act on antigen-experienced effector T cells in the periphery to reduce DC activation and antigen presentation in secondary lymphoid organs.

Chapter 5: Materials and Methods

Maintenance and care of experimental animals

Six week-old female C57BL/6 controls were purchased from Taconic. Mice deficient in Il27ra/WSX-1 (C57BL/6 background) were generated as described (Yoshida et al., 2001) and were originally provided by Amgen (Thousand Oaks, CA, USA). Mice deficient in IL-27p28 (C57BL/6 background) were originally provided by Janssen Research & Development, LLC (Spring House, PA, USA). STAT1$^{-/-}$ mice (129S6/SvEv-Stat1tm1Rds) and 129S6 control mice were purchased from Taconic. Mice were housed and bred in specific pathogen-free (SPF) facilities in the Department of Pathobiology at the University of Pennsylvania in accordance with institutional guidelines. The Me49 Strain of *T. gondii* was prepared from chronically infected CBA/ca mice and experimental animals were infected intraperitoneally with 20 cysts.

Cell sorting and in vitro cell culture

Splenocytes from C57BL/6 mice were obtained by mechanically dissociating the spleen, filtering it through a 40 m nylon strainer, and lysing red blood cells with ACK lysis buffer. T cells were enriched using a Mouse CD3$^+$ T Cell Enrichment Column (R&D Systems MTCC-25). Cells were then stained with Live/dead fixable Aqua dead cell stain (ThermoFisher L34957), anti-CD4 (GK1.5, Biolegend 100447), anti-CD8 (53-6.7, BD Biosciences 562283), anti-CD44 (IM7, eBioscience 0441-82), anti-CD62L (MEL-14, eBioscience 47-0621-82), anti-Ly6C (HK1.4, eBioscience 45-5932-82), and

anti-Sca-1 (D7, eBioscience 56-5981-82) antibodies and were sorted on a FACSAria II flow cytometer (BD Biosciences). Cells were plated in tissue culture-treated round-bottom 96-well plates, 1-2 x 10^5 per well in 200 uL RPMI supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin, 1 mM sodium pyruvate, 1x MEM non-essential amino acids (Gibco), 55 M 2-Mercaptoethanol. The tissue culture plates were precoated with 1 g/mL anti-CD3 (145-2C11, BioXCell) for 3 hours at 37 degrees and excess anti-CD3 was rinsed off with PBS. Cells were stimulated in the presence of anti-CD28 (37.N.51.1, 1 g/mL), IL-2 (Proleukin, 100 U/mL), anti-IFN-γ (XMG1.2, BioXcell, 1 g/mL) (except when exogenous IFN-γ was tested) and anti-IL-4 (11B11, BioXcell, 1 g/mL). Recombinant IL-27 (Amgen) was used at a concentration of 50 ng/mL, TGF-β (eBioscience) was used at 5 ng/mL, and Universal type I IFN (PBL Assay Science) was used at a concentration of 2000 U/mL. IFN-γ (R&D Systems), IL-6 (eBioscience), IL-12 (eBioscience), TNF-α (eBioscience), IL-10 (eBioscience), and IL-7 (Peprotech) were used at 10 ng/mL. IL-15 (Peprotech) and IL-15Ra-Fc (R&D Systems) were incubated at 37 degrees for 30 minutes at a ratio of 2:9. The resulting IL-15 complexes were used at 55 ng/mL (10 ng/mL IL-15, plus 45 ng/mL IL-15Ra).

Flow cytometric analysis

Cells were stained with the reagents used for cell sorting, described above, as well as antibodies specific for CTLA-4 (UC10-4B9, Biolegend 106306), PD-L1 (10F.9G2, Biolegend 124319 or 124314), LAG-3 (C9B7W, Biolegend 125210 or eBioscience 48-2231-82), PD-1 (29F.1A12, Biolegend 135220 or J43, eBioscience 25-9985-82), TIGIT (GIGD7, eBioscience 50-9501-82), and TIM-3 (RMT3-23, Biolegend 119704 or

199721). CTLA-4 staining was performed after fixation and permeablization of the cells. For analyses after infection, splenocytes were harvested as detailed above and peritoneal exudate cells were harvested by intraperitoneal lavage with 7 mL PBS. MHC-I monomers loaded with peptide (SVLAFRRL) from the *T. gondii* protein Tgd-057 were kindly provided by E. John Wherry (University of Pennsylvania) and tetramerized by incubation with streptavidin-conjugated PE or APC. Some experiments utilized PE- and APC-conjugated MHC-I tetramers loaded with the Tgd-057 peptide that were provided by the National Institutes of Health Tetramer Facility. PE- or APC-conjugated MHC-II tetramers loaded with the AS15 peptide AVEIHRPVPGTAPPS were also provided by the National Institutes of Health Tetramer Facility.

Cells were collected on an LSRFortessa or LSRFortessa X-20 (BD Biosciences) and analysis was performed with FlowJo (TreeStar). Cells were gated on lymphocytes (by forward scatter (FSC) and side scatter (SSC)), singlets (by FSC-W vs FSC-H and SSC-W vs SSC-H), and live cells (by exclusion of Aqua Dead Cell Stain). CD4$^+$ T cells were gated CD4$^+$CD8$^-$FoxP3$^-$ and CD8$^+$ T cells were gated CD8$^+$CD4$^-$.

Statistical Analysis

Statistical significance was determined using GraphPad Prism software, using a paired, unpaired, or ratio Student's t test, as indicated. When the ratio t test was used, 0.1 was added to zero values to make them non-zero. P values less than 0.05 were considered significant.

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