Regulation of T cell responses during chronic neuroinflammation: from regulatory T cells to ICOS

Carleigh Anne O'Brien
Brownsburg, IN

B.A. (Double Major: Biology and French), DePauw University, 2013

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Department of Microbiology, Immunology, and Cancer Biology

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Abstract

Control of chronic central nervous system (CNS) infection with the parasite *Toxoplasma gondii* requires ongoing inflammatory T cell responses in the brain. With this long-lived inflammatory response required for control of the parasite, understanding how this response occurs without causing debilitating immunopathology, particularly in a largely non-regenerative tissue, is of tremendous interest. Similar to other infection models, regulatory or immunosuppressive mechanisms are in play during chronic *T. gondii* infection that limit immune-mediated pathology. To explore some of these regulatory mechanisms, we began by focusing on the canonical immunosuppressive cell type, regulatory T cells (T_{regs}). T_{regs} have been shown to play an integral role in balancing inflammatory immune responses both in CNS autoimmune disorders as well as CNS infections. The precise mechanism by which the T_{reg} population in the CNS carries out this regulation *in vivo*, however, remains unclear. We began by generally characterizing the phenotype of T_{regs} recruited to the CNS during chronic infection. We found that the T_{regs} recruited to the inflamed brain were largely T_h1-polarized, expressing Tbet, CXCR3, and IFNγ, as well as IL-10. We also interrogated the TCR clonality of the effector CD4+ T cells and T_{regs} in the CNS during chronic infection. Interestingly, while we found CD4+ effector T cells with TCR specificity for a *T. gondii*-specific MHCII tetramer, we found no T_{regs} in the CNS specific for this same reagent. Upon further characterization of the TCR clonality of these populations using TCR sequencing, we observed minimal overlap between the TCR sequences of effector CD4+ T cells and T_{regs} in the inflamed CNS, suggesting that these two populations have distinct lineages and may be recognizing largely separate antigen pools during chronic CNS infection.

We also observed differential localization of the effector CD4+ T cells and T_{regs} within the CNS. While CD4+ effector T cells were abundantly found in the brain parenchyma, T_{regs} were mainly relegated to the meninges and perivascular spaces. The meninges and perivascular spaces during chronic infection were also enriched for $MHCII^{hi}CD11c^{+}$ APCs. We hypothesized that T_{reg} interaction with these APCs was important for their local regulation of infiltrating T cell responses, perhaps serving as a "gatekeeper" to the CNS. To begin to understand what is involved in the interaction between these two cell types that might support T_{reg} suppression in the inflamed CNS, we blocked either the adhesion molecule LFA-1 or MHCII during chronic infection. Both blockade of LFA-1 and MHCII led to increased T_{reg} velocity and less extensive contact time with APCs in the CNS, suggesting that both are important for maintenance of T_{reg}:APC contact and, perhaps, continued T_{reg} suppression. Interestingly, we found that LFA-1 blockade also rapidly led to a significant depletion of APCs from the CNS, suggesting that LFA-1:ICAM interactions play a role in recruitment or maintenance of APC populations in the inflamed CNS in addition to maintaining T_{reg}:APC contact. MHCII blockade on the other hand, did not lead to loss of APCs from the CNS, but only affected T_{reg}:APC contact. These results suggest that, upon recruitment to the inflamed CNS, T_{regs} are anatomically restricted, and their interaction with APCs, through both TCR and adhesion molecule interactions, regulates their local behavior, and perhaps their suppressive capacity, during chronic CNS infection.

In addition to T_{reg}-mediated suppression of the ongoing immune response to *T. gondii* in the CNS, we also sought to understand other signals involved in regulation of chronic T cell responses in the inflamed brain. To explore the loss of suppressive

cytokine exclusively during the chronic phase of infection, we blocked IL-10 receptor (IL-10R) in chronically infected mice. Consistent with previous reports, IL-10R blockade led to severe, fatal pathology associated with widespread changes in the inflammatory response, including increased antigen presenting cell (APC) activation, expansion of CD4+ T cells, and neutrophil recruitment to the brain. We then sought to identify regulatory mechanisms contributing to IL-10 production, focusing on ICOS (inducible T cell costimulator), a molecule implicated in IL-10 production in other models. Unexpectedly, ICOS-ligand (ICOSL) blockade during chronic infection led to a local expansion of effector T cells in the brain without affecting IL-10 production or APC activation. Instead, we found that ICOSL blockade led to changes in T cells associated with their proliferation and survival. In particular, we observed increased expression of IL-2 associated signaling molecules CD25, phosphorylated STAT5, Ki67, and Bcl-2 in effector T cells in the brain, along with decreased apoptosis. Interestingly, increases in CD25 and Bcl-2 were not observed in effector T cell populations following IL-10R blockade. Also unlike IL-10R blockade, ICOSL blockade led to an expansion of both CD8+ and CD4+ effector T cells in the brain, with no expansion of peripheral T cells or neutrophil recruitment to the CNS. Overall, these data suggest that IL-10 and ICOS differentially regulate T cell responses in the brain during chronic *T. gondii* infection. Taken together, the above results suggest multiple levels of regulation are necessary to maintain a balanced immune response during chronic infection, and each of these regulatory signals must be constantly integrated to limit immune-mediated pathology.

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List of Abbreviations

AHR	Airway hypersensitivity reaction
AIDS	Acquired immunodeficiency syndrome
Aire	Autoimmune regulator
Akt	Protein kinase B
AP-1	Activator protein 1
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing a CARD
ATAC-Seq	Assay for transposase-accessible chromatin using sequencing
Atg	Autophagy related gene
Batf3	Basic leucine zipper transcriptional factor ATF-like 3
Bcl	B cell lymphoma
BMNC	Brain mononuclear cells
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CDR3	Complementarity-determining region 3
CFP	Cyan fluorescent protein
ChIP-Seq	Chromatin immunoprecipitation-sequencing
cLN	Cervical lymph nodes
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocyte

CTLA-4	Cytotoxic T lymphocyte associated protein 4
CVID	Common variable immunodeficiency
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X-C motif chemokine receptor
CX_3CR_1	CX3C chemokine receptor 1
DC	Dendritic cell
dcLN	Deep cervical lymph nodes
DEREG	Depletion of regulatory T cells
EAE	Experimental autoimmune encephalomyelitis
Ebi3	Epstein Barr virus induced 3
ELISA	Enzyme-linked immunosorbent assay
Foxp3	Forkhead box P3/scurfin
GATA3	GATA binding protein 3
GFP	Green fluorescent protein
GPI	Glycophoshatidylinositol
HA	Influenza hemagglutinin
HBV	Hepatitis B virus
HCV	Hepatitis C virus
H&E	Hematoxylin and eosin staining
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
ICAM	Intercellular adhesion molecule
ICOS	Inducible T cell costimulator

ICOSL	Inducible T cell costimulator ligand
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IRF3	Interferon regulatory factor 3
IRG	Immune-related GTPase
Jak	Janus kinase
LAG-3	Lymphocyte-activation gene 3
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph nodes
LTα	Lymphotoxin α
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MHV	Mouse hepatitis virus
MOG	Myelin oligodendrocyte protein

mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
Mtb	*Mycobacterium tuberculosis*
mTEC	Medullary thymic epithelial cell
MyD88	Myeloid differentiation primary response 88
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NLRP/NALP	NOD-like receptor
NO	Nitric oxide
NOD	Non-obese diabetic
OVA	Ovalbumin
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cell
PI3K	Phosphoinositide 3-kinase
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PRR	Pattern recognition receptor
Pru	Prugniaud
pSTAT	Phosphorylated-signal transducer and activator of transcription
pT_{reg}	Peripherally-derived regulatory T cell
RA	Retinoic acid
RAG	Recombination-activating gene
RORγt	Retinoic-acid-receptor-related orphan nuclear receptor gamma

RSV	Respiratory syncytial virus
S6K	S6 kinase
SCID	Severe combined immunodeficiency
scLN	Superficial cervical lymph nodes
STAT	Signal transducer and activator of transcription
TANK	TRAF family member associated NF-κB activator
Tbet	T-box transcription factor TBX21
TBK1	TANK-binding kinase 1
T_{conv}	Conventional T cell
TCR	T cell receptor
TE	Toxoplasmic encephalitis
T_{eff}	Effector T cell
TGF	Transforming growth factor
T_h	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TPSLM	Two-photon scanning laser microscopy
TRAF	TNF receptor associated factor
T_{reg}	Regulatory T cell
tT_{reg}	Thymus-derived regulatory T cell
YFP	Yellow fluorescent protein

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CHAPTER 1

INTRODUCTION

1.1 The Fight Against Invaders: Evolution of the Immune System

Immune responses have intricately evolved to protect hosts from a wide variety of potentially harmful pathogens[1,2]. The first line of cellular defense involves the innate immune system, largely consisting of short-lived phagocytes that can quickly differentiate and upregulate effector mechanisms in response to a large array of infectious pathogens[3]. These cells, including monocytes and macrophages, neutrophils, basophils, and eosinophils, express a wide array of receptors that recognize general molecular patterns derived from invading pathogens[3-5]. These "pattern recognition receptors" (PRRs) allow for one of the most essential aspects of the innate immune response: polyspecificity. Having a cellular repertoire able to recognize similar, invariable components of "non-self" would have allowed primitive organisms to fight a large variety of infectious pathogens, giving them the best chance at survival.

Another essential component of the innate immune response is its ability to rapidly respond to infection. While adaptive responses require clonal expansion and differentiation in mounting an inflammatory response after pathogen recognition, innate immune cells are able to quickly differentiate and produce a variety of antimicrobial molecules to begin the fight against the invading pathogen[1,3,5]. These molecules, such as reactive oxygen and nitrogen species, a host of antimicrobial enzymes, and inflammatory cytokines like IL-1, IL-6, and TNF-α, can contribute to clearance of the pathogen. These innate mechanisms, though essential, are often not enough in fighting off invading pathogens. This can easily be seen in human patients with severe combined immunodeficiency (SCID), a disorder encompassing several different mutations, all of which result in severely dysfunctional or non-existent adaptive immune responses[6].

These patients, though they have adequate innate immune responses, are ultimately unable to control otherwise mild infections, and require bone marrow transplantation, gene therapy, or other extensive medical treatments to survive[6-9]. These patients, and the subsequent discovery and characterization of the *scid* mutation in mice[10], have greatly emphasized the requirement for a functional adaptive immune response in combatting a constantly evolving array of pathogens.

1.2 The Adaptive Immune Response

Besides polyspecificity and a rapid effector response to infections, the innate immune system plays another crucial role in clearing organisms of dangerous "non-self": activation of adaptive immune responses. The adaptive immune system consists of B and T lymphocytes, so named according to the site where their progenitors were initially discovered: B cells in the Bursa of Fabricius in the chicken and T cells in the thymus of the mouse[11]. B cells and the development of antibody responses are termed the "humoral" arm of adaptive immunity, while T cells and their effector mechanisms are the "cellular" arm of adaptive immunity. Both arms have commonalities that differentiate them from the innate immune response, namely, V(D)J recombination of antigen receptors and the establishment of immune memory. Rather than expressing a variety of germline-encoded receptors that recognize common microbial molecular patterns, both B and T cells express recombined, highly specific receptors that recognize precise antigenic components of pathogens[11-14]. This recombination at the level of the genome allows for an extremely diverse receptor repertoire capable of recognizing a much more diverse array of antigens than the more slowly evolving innate immune system[1,5]. This stochastic recombination, however, comes at a price. While B and T cells are able to recognize a

more diverse array of "non-self" molecules, the frequency of antigen-specific cells expressing a certain receptor is extremely low, making a specific antigen-adaptive immune cell interaction a somewhat rare event[15-17]. This means that, when such an interaction does occur under the right inflammatory conditions, the adaptive immune cell must undergo clonal expansion and differentiation in order to produce a sufficiently large pool of effector adaptive immune cells that can then contribute to clearance of the infection[1,18].

In addition to providing a more varied repertoire of "adapted" immune cells, B and T cells are also capable of establishing memory of immune responses to pathogens. Following clonal expansion in response to an antigen, some B and T cells differentiate into long-lived cells that are able to rapidly induce a secondary response to the same antigen should the organism be re-infected[11,19-21]. These two tenets of adaptive immunity have subsequently been extensively studied in the context of many different types of inflammation, including infections, autoimmunity, and cancer. Studies into the molecular signaling events, extracellular cues, and tissue specific signals that shape the adaptive immune response have been at the forefront of the immunology field for the past sixty years, and remain a primary focus in understanding immunity as a whole. To fully understand an immune response, however, both innate and adaptive immune mechanisms must be understood, as in most cases, one cannot fully function without the other.

1.2.1 The Adaptive Immune Response: T Cell Activation

Besides recognizing the presence of an infectious and dangerous pathogen, one of the crucial roles of the innate immune system is to activate and shape the adaptive immune response. In addition to inducing expression of antimicrobial and antiviral

compounds and inflammatory cytokines, recognition of pathogens by PRRs also leads to upregulation of both MHC and costimulatory molecules on antigen presenting cells (APCs)[18,22]. T cell activation begins with recognition of its cognate antigen and MHC presented on the surface of APCs, along with a secondary costimulatory signal provided by the APC[23,24]. This "two step" T cell activation leads to the proliferation and clonal expansion of antigen specific T cells that can then carry out effector functions and lead to the elimination of invading pathogens (Figure 1.1).

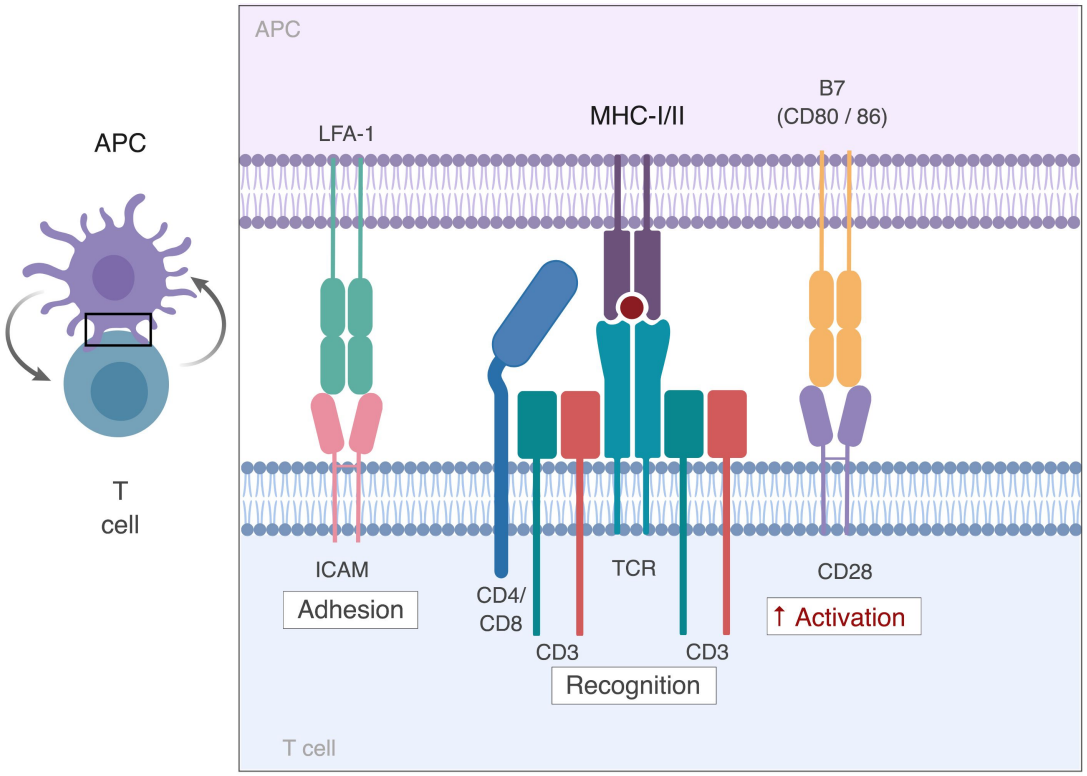


Figure 1.1 APC:T cell interactions. MHC-expressing APCs present peptide antigens to T cells. Upon antigen recognition, the CD4 or CD8 co-receptor binds to the MHC molecule to stabilize the TCR:MHC interaction. Stabilization of this APC:T cell interaction is then amplified by other molecules that promote adhesion (LFA-1:ICAM) and increased T cell activation (CD80/86:CD28). The formation of this "immunological synapse" then induces downstream signaling that leads to full activation of the T cell.

After binding of the T cell receptor (TCR) to the MHC:peptide complex, the CD4 or CD8 coreceptor binds to the MHCII or MHCI molecule, respectively. This interaction, further stabilized by the binding of adhesion molecules between the T cell and the APC, then leads to the formation of the immunological synapse, inducing complex downstream signaling, ultimately leading to the nuclear translocation of multiple transcription factors, including NF-κB, NFAT, and AP-1 that leads to the activation of the T cell[25] (Figure 1.2). These transcription factors bind to the IL-2 promoter, inducing IL-2 production that aids in T cell proliferation and expansion[25]. The activating effects of TCR stimulation are further enhanced by the necessary "second signal" of CD28 costimulation, though other costimulatory molecules have since been identified that can also provide this secondary signal, as discussed in subsequent sections[24].

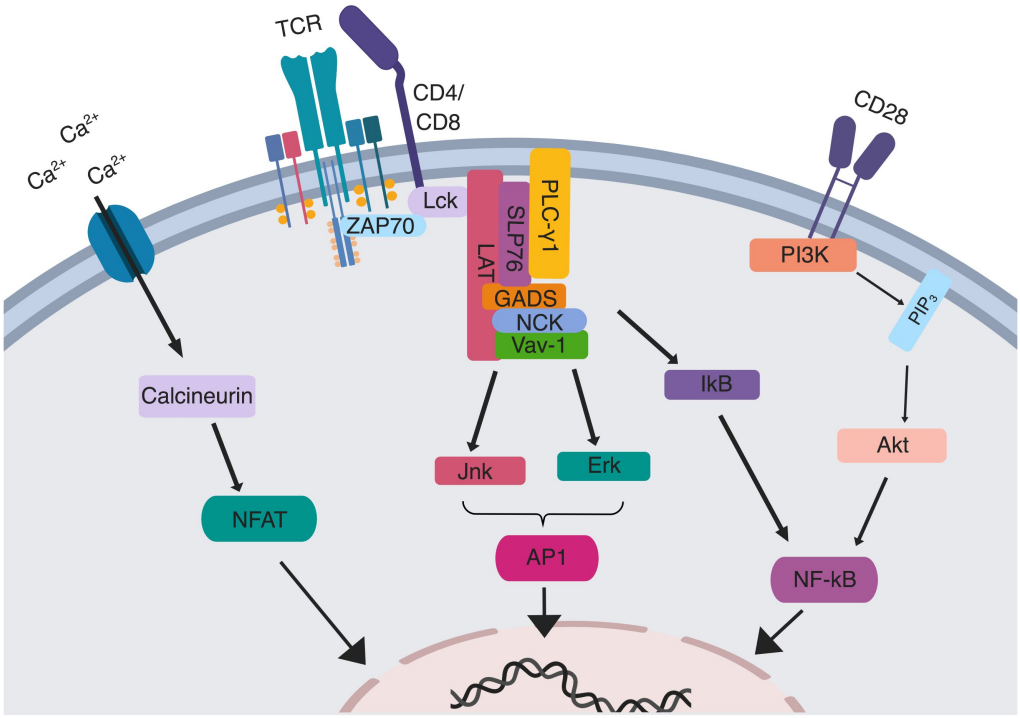


Figure 1.2. Integration of TCR, CD28, and calcium signaling leading to T cell activation. Upon TCR engagement with MHC and cognate antigen, ITAMs in the cytoplasmic tail of CD3 are phosphorylated by Lck and Zap-70. Adapter molecules LAT and SLP-76 then begin the formation of a molecular complex that leads to the activation of Jnk and Erk, which then activates AP-1. Signaling downstream of CD28 ligation includes the activation of PI3K and Akt which, along with signaling from the TCR complex activates NF-κB. Additionally, calcium influx activates calcineurin, which dephosphorylates NFAT, allowing translocation to the nucleus. The activation of NF-κB, AP-1, and NFAT and subsequent translocation to the nucleus leads to the transcription of downstream target genes (such as IL-2) that are required for full activation of the T cell and T cell expansion.

Upon binding of CD28 to CD80 or CD86 on APCs, tyrosine residues in the cytoplasmic tail of CD28 become phosphorylated and lead to the recruitment of class IA phosphoinositide 3-kinase (PI3K)[24]. PI3K recruitment and activation then results in the production of phosphatidylinositol 3,4,5-triphosphate (PIP_3), culminating in Akt activation that is required for cellular proliferation, changes in cellular metabolism, and survival[24] (Figure 1.2). Together, the addition of a secondary costimulatory signal in addition to TCR stimulation further lowers the threshold for T cell activation and prevents T cell anergy to allow for a fully functional effector T cell response.

1.2.2 The Adaptive Immune Response: T Cell Differentiation

Though two signals are sufficient to induce T cell expansion in response to antigen, it is now understood that an important "third signal" is also involved in shaping the type of effector T cell response to pathogen that occurs. Cytokines produced by innate immune cells early in infection are recognized in conjunction with signals from the TCR and costimulatory molecules, thereby both activating and "instructing" newly activated T cells as to the nature of inflammation required to eliminate the invading pathogen[26-28]. In particular, naïve CD4+ T cells activated in the context of different cytokines produced by innate or adaptive immune cells can differentiate into any number of helper cell lineages, including T_h1, T_h2, T_h17, and T_{fh}, as well as regulatory populations[29] (Figure 1.3). Multiple seminal studies have since identified the primary transcription factors involved in maintaining different T helper differentiation and lineage, as well as epigenetic changes occurring during this differentiation[29].

Each T_h lineage is generally defined by a "master" transcription factor, a set of specific cytokines produced, and the inflammatory context in which the T_h cell is playing

a role (Figure 1.3). The first T_h subsets to be identified, T_h1 and T_h2, generally represent the immune response to intracellular bacteria and viruses, and extracellular bacteria and helminths, respectively. T_h1 cells develop in the context of IL-12 and IFNγ production, which leads to the expression of the master transcription factor Tbet[29,30]. Tbet expression then serves to amplify IFNγ production from T cells, further strengthening the T_h1 response by then activating APCs and leading to the degradation of intracellular pathogens (further discussed in Sections 1.9.2 and 1.9.3). Recognition of extracellular bacterial or helminth infection and subsequent IL-4 production differentiates naïve CD4+ T cells into a T_h2 lineage by upregulation of master transcription factor GATA3[29,30]. These T_h2 cells then produce cytokines IL-4, IL-5, and IL-13, and can recruit and activate other T_h2-associated cells such as eosinophils, basophils, and mast cells[29,30]. The initial characterization of these two subsets of CD4+ helper T cells represented the beginnings of understanding how the immune system is able to fine-tune inflammatory responses to best respond to any number of invading pathogens.

Since the identification and characterization of T_h1 and T_h2 lineages, many other T_h lineages have been discovered and are still being characterized today. T_h17 cells, induced by TGF-β, IL-6, IL-21, and IL-23 and defined by expression of the master transcription factor RORγt and the cytokine IL-17, are important for responses to fungi and extracellular bacteria[29,30]. T_{fh} cells are a specialized subset essential for the maturation of B cell responses and antibody class switching. These cells develop in response to IL-6 and IL-21, and are thought to rely on the master transcription factor Bcl-6[29]. Bcl-6 upregulates expression of CXCR5 and ICOS, which allows T_{fh} cells to migrate to B cell follicles and induce antibody class switching[29,31]. Lastly, the differentiation of

peripherally derived T_{regs} (pT_{regs}) can occur in the presence of cytokines TGF-β and IL-2, which promote the expression of master transcription factor Foxp3 and expression of the high affinity IL-2Rα (CD25), respectively (further discussed in Section 1.3.2)[29,30].

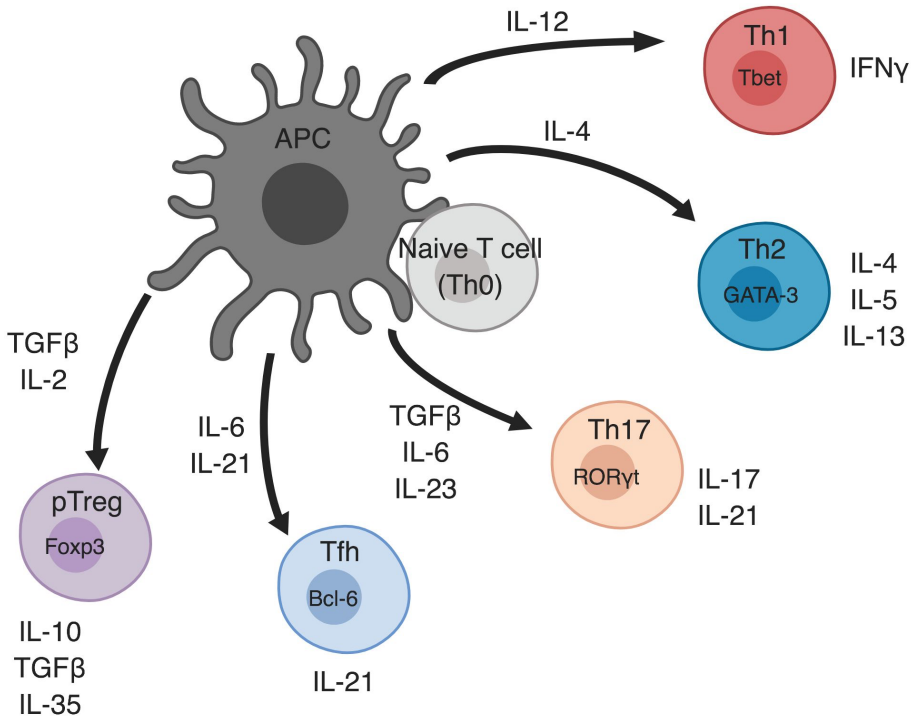


Figure 1.3. T helper cell differentiation. Upon recognition of cognate antigen and subsequent activation, naïve CD4+ (T_h0) cells respond to the cytokine milieu in the environment and differentiate into various lineages. Different cytokines that have been shown to induce differentiation into specific lineages, along with the "master" transcription factor for each lineage, are indicated. Each specific lineage T_h cell, once differentiated, then further contributes to the inflammatory environment by producing canonical T_h cytokines, which are indicated beside each T_h lineage cell.

These differing T cell lineages have been exquisitely adapted to deal with any number of potential pathogens, even remaining somewhat plastic, with the ability to produce multiple T_h subset-associated cytokines, allowing for highly specific and fine-tuned T cell responses during ongoing inflammation[29]. Continued study into both innate and adaptive immune responses to any number of pathogens further highlights the extraordinary ability of our immune system to quickly recognize, assess the danger of, and respond to a vast variety of encountered pathogens. On the other hand, dysregulation of any of these immune responses can result in immunopathology or autoimmune disease, highlighting the importance of a balanced immune response (discussed in subsequent sections).

1.3 Regulatory T Cell Development

The concept of the lack of immunological response to self, later termed immune tolerance, has been a major point of immunological study since Sir Macfarlane Burnet's discussion of "The Facts of Immunity" in 1959[11]. As the quantitative and qualitative aspects of immune recognition of non-self began to become clearer in the mid-20th century, the question of how our immune system "decides" what to respond to with regards to self versus non-self was becoming increasingly of interest. Burnet went on to postulate that instruction of lymphoid cells in the thymus is essential for this self versus non-self recognition[32]. In the coming years, clonal deletion of self-recognizing T cells in the thymus would come to be known as the major contributor to the concept of "central tolerance", namely that potentially autoreactive T cells would be deleted from the T cell repertoire in the thymus, thereby never encountering peripheral self-antigen and preventing autoimmune disease[33]. Another major mechanism of tolerance, later identified

as an immunosuppressive population of T cells, was first proposed in 1971 following the observation that the presence of thymocytes could inhibit immune responses to injected antigen[34]. Based on these (and other similar) findings, the authors proposed the existence of a thymus-derived mechanism to "shut-off" immunological responses independently of the clonal deletion mechanism of tolerance[34]. In the coming years, this "shut-off" mechanism would be identified as a population of "suppressor T cells", later called regulatory T cells (T_{regs}) that play a major role in controlling immune responses.

A major step forward in the characterization of this regulatory T cell population came with the finding that these cells were a relatively stable lineage that constitutively expressed the high affinity IL-2Rα subunit (CD25)[35] and were under the control of the master transcriptional regulator Foxp3[36,37]. Perhaps the most convincing data supporting the idea that this regulatory T cell population played an essential role in immune homeostasis came from studies in mice deficient in Foxp3 (*scurfy* mice). These Foxp3 deficient mice present with lymphoproliferative disease and multi-organ autoimmunity that is fatal in the first month of life[38,39]. The subsequent identification of the same mutation causing human immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX) syndrome[40,41] gave major momentum to the study of T_{regs} that continues to this day.

1.3.1 T_{reg} Development in the Thymus

Though clonal deletion of potentially autoreactive T cells in the thymus plays an important role in preventing T cell response to self and resulting autoimmunity, it has been shown to be an imperfect system. Self-reactive T cell clones can escape the thymus and, when present in the periphery, can be activated and result in disease[42]. Therefore,

other mechanisms of checking the immune system must be present to decrease the potential for autoimmunity. One such mechanism is the development of thymic-derived T_{regs} (tT_{regs}). tT_{regs} are a distinct CD4+ T cell population that arises in the thymus, and constitutively expresses both the master transcription factor Foxp3 and the high affinity IL-2Rα subunit CD25[35,36,43]. In the traditional model of clonal selection in the thymus, CD4 and CD8 double positive thymocytes undergo TCR gene rearrangement, resulting in an extremely broad repertoire of TCR specificities[33]. From this point in T cell development, the affinity of each TCR for MHC:peptide plays a major role in determining the fate of the thymocyte. Thymocytes with a TCR that does not bind to the MHC:peptide complex will not receive survival signals, and will quickly die from neglect, while thymocytes expressing TCRs with weak affinity for MHC:peptide will be positively selected[33].

Thymocytes with a TCR that displays strong reactivity to the MHC:peptide complex can have multiple fates. These cells represent a potentially self-reactive population that could go on to cause autoimmunity should they reach the periphery. In the commonly held view, to ensure that self-reactive T cell clones reaching the periphery are limited, thymocytes with the strongest TCR affinities either receive signals leading to apoptosis (clonal deletion) or anergy[44], or they differentiate into tT_{regs}[43,45-47]. This "TCR affinity hypothesis" was first suggested based on the observation that mice engineered to express high levels of antigen (e.g. influenza hemagglutinin (HA) or ovalbumin (OVA)) along with a TCR specific for each antigen, respectively, develop high numbers of T_{regs}, suggesting that high antigen load and strong TCR reactivity can favor tT_{reg} development over clonal deletion or anergy[47,48]. This hypothesis is also supported by a more recent

study using *Nur77-GFP* reporter mice, in which the level of GFP expression correlates to the strength of TCR binding. In this study, tT_{regs} were consistently found to express higher levels of GFP than conventional T cells (T_{conv}), suggesting increased TCR signal in tT_{regs}[49]. However, the development of tT_{regs} is likely to be more complex than a simple TCR affinity or avidity selection, as another study showed that a low affinity agonist self-peptide was sufficient to induce clonal deletion but was not sufficient to induce tT_{reg} differentiation, suggesting that a highly specific balance of TCR signal intensity, along with additional costimulatory signals like CD28 and IL-2, are required for tT_{reg} development[50,51].

Additionally, the antigen landscape in the thymus appears to play an important role in determining clonal deletion or anergy (sometimes termed recessive central tolerance) versus tT_{reg} development (sometimes termed dominant central tolerance). Using MHCII tetramers, two recent reports traced CD4+ T cell populations in cases where their cognate antigen was expressed under different promoters to achieve either a ubiquitous or tissue-restricted antigen expression pattern in medullary thymic epithelial cells (mTECs). In the case of ubiquitous antigen expression in the thymus, large decreases in CD4+ T cells were observed, demonstrating efficient clonal deletion, while when the antigen was expressed in a tissue-restricted fashion, no effect was seen on the numbers of CD4+ T cells in the thymus, yet a large proportion of these CD4+ T cells were Foxp3+, indicating differentiation into tT_{regs}[52,53]. Furthermore, *Aire* deficient mice, whose expression of tissue-restricted antigen in mTECs is severely limited, show decreased TCR diversity in T_{regs} as well as diminished tT_{reg} differentiation[54]. Overall, the intrinsic and extrinsic factors determining recessive and dominant central tolerance represent a sensitive balance

of signals including TCR affinity and avidity, the antigen landscape, and secondary signals such as costimulation and cytokines. Though much about the mechanisms of central tolerance have been described, the exact interplay of each of these signals in the context of tT_{reg} development remain to be elucidated.

1.3.2 T_{reg} Development in the Periphery

As mentioned in Section 1.2.3, T_{regs} capable of suppressing immune responses can also differentiate from naïve CD4+ T cells in the periphery. Contrary to tT_{reg} development, the conditions required for induction of pT_{regs} include suboptimal activation signals from APCs, the administration of sub-immunogenic doses of antigen, and the cytokines TGF-β and IL-2[30]. High concentrations of TGF-β, coupled with sub-optimal TCR activation and costimulation in the periphery, in part favor pT_{reg} differentiation by inducing Foxp3 expression and downregulating receptors to inflammatory cytokines like IL-6, while continued IL-2 signaling induces STAT5 activation that prevents differentiation into alternate lineages[30]. High levels of retinoic acid (RA) can also potentiate pT_{reg} induction and, through synergy with TGF-β, can even promote pT_{reg} differentiation in the context of high costimulation from APCs[30].

Overall, despite differential induction, tT_{regs} and pT_{regs} have largely been shown to have equivalent suppressive function. Indeed, tT_{regs} and pT_{regs} are equally efficient in limiting both CD4+ and CD8+ T cell proliferation *in vitro*, as well as limiting the antigen-presenting capacity of APCs[30,35,55]. The known mechanisms of T_{reg} suppression of immune responses and maintenance of tolerance are further discussed in Section 1.4.

1.4 Mechanisms of Regulatory T Cell Suppression for Maintenance of Immune Tolerance

Conventional Foxp3+ T_{regs} remain the prominent immune cell population tasked with maintaining immune tolerance and suppressing a vast variety of inflammatory responses. In order to carry out this role, T_{regs} must exert control on a wide variety of immune cell types in many different tissue environments. Since their discovery, several different mechanisms of suppression have been ascribed to this population. One of the first mechanisms of suppression attributed to T_{regs} was the ability to produce immunosuppressive cytokines like IL-10 and TGF-β[56]. In many systems, though not all, T_{regs} are the main producers of IL-10, which is a cytokine that can inhibit many aspects of an inflammatory immune response. Deletion of IL-10 exclusively in T_{regs}, though not resulting in systemic autoimmunity as seen in *scurfy* mice that lack T_{regs} altogether, leads to the development autoimmunity at environmental surfaces such as the colon and lungs[57]. These results suggest that 1) T_{reg} production of IL-10 is essential for preventing autoimmunity at mucosal surfaces but is dispensable for systemic control of inflammation and 2) other mechanisms of immune suppression must be utilized by the T_{reg} population in other tissues to maintain control of autoimmune responses. The role of IL-10 in suppressing immune responses during both homeostasis and inflammation is further discussed in Section 1.7. TGF-β is another cytokine produced by T_{regs} that can limit inflammation by skewing naïve CD4+ T cells in the periphery to become pT_{regs}, further amplifying the immunosuppressive effects of the presence of T_{regs}[58]. Lastly, the relatively newly discovered cytokine IL-35, a heterodimer consisting of the IL-12α subunit of IL-12 and the Ebi3 subunit of IL-27, can be produced by T_{regs} to both induce

more IL-35-producing pT_{regs} and confer immunosuppression by limiting effector T cell proliferation and inflammatory cytokine production[59,60].

With the initial characterization of T_{regs}' constitutive expression of the high affinity IL-2Rα subunit CD25, one of the earliest hypotheses regarding a mechanism for T_{reg} suppression of immune responses was the idea of an "IL-2 sink". This idea was mostly a result of *in vitro* observations that CD4+CD25+ T_{regs} are potent inhibitors of effector cell proliferation[61]. Knowing that T_{conv} transiently rely on IL-2 signals to proliferate, the simple explanation for these *in vitro* observations was that, as T_{conv} become activated and begin to produce IL-2, the CD25+ T_{regs} serve as a "sink" and outcompete T_{conv} for IL-2, thereby limiting their proliferative capacity. Subsequent observations (since questioned[62]) that T_{regs} *in vitro* actually limit IL-2 production from T_{conv} in a contact-dependent manner seemed to put the brakes on the "IL-2 sink" hypothesis, with the conclusion that if T_{regs} were limiting IL-2 production itself, there would be no IL-2 in the environment for which to outcompete T_{conv}[61,63]. Interestingly, nearly two decades later, recent elegant *in vivo* studies looking for a functional role for IL-2 signaling in T_{regs} brought this hypothesis somewhat back to the light. Using a mouse model to delete IL-2R exclusively from Foxp3+ T_{regs}, the study found that T_{reg} depletion of IL-2 via IL-2R was important for suppressing CD8+ T cell responses, while CD4+ T cell responses were suppressed via other mechanisms downstream of IL-2 signaling[64].

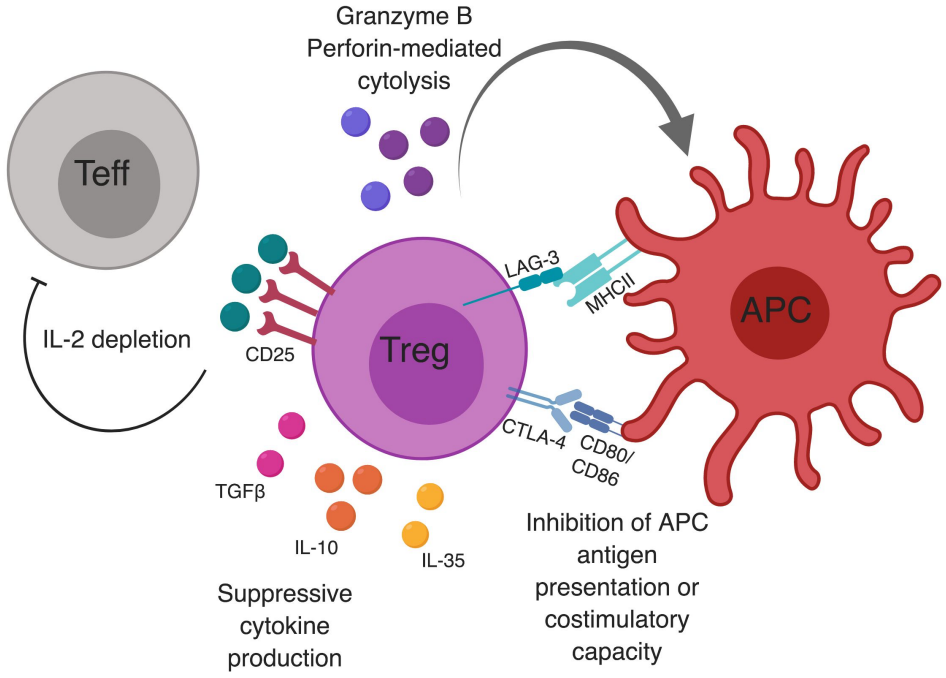


Figure 1.4. Major mechanisms of T_{reg}-mediated suppression. T_{regs} utilize many methods to suppress inflammatory immune responses. One major mechanism involves modulation of APC phenotype by CTLA-4 and LAG-3-mediated suppression of costimulatory capacity by interfering with CD80/CD86 expression or antigen presentation, respectively. T_{regs} have also been shown in some cases to induce cytolysis of target APCs by production of granzyme B and perforin. T_{regs} can also directly limit effector T cell expansion through high expression of CD25, thereby allowing them to deprive effector T cells of IL-2. Finally, production of various immunosuppressive cytokines such as IL-10, IL-35, and TGFβ, can inhibit the inflammatory capacity of many cell types.

Other molecules expressed on the surface of T_{regs} have also been shown to be an important aspect of T_{reg} immunosuppression. One of the most well-characterized of these is CTLA-4[56]. Similar to CD25, CTLA-4 is upregulated on activated effector T cells, but is constitutively expressed on T_{regs}[65]. It is essential for T_{reg} maintenance of self-tolerance, as specific deletion of CTLA-4 in Foxp3+ T_{regs} results in systemic lymphoproliferation, immunopathology in multiple tissues, and early mortality without affecting tT_{reg} development in the thymus[66]. On effector T cells, CTLA-4 is known to bind to costimulatory molecules CD80 and CD86 expressed on APCs with higher affinity than CD28[24,67,68]. Its upregulation after initial T cell activation thus prevents continued CD28 costimulation and serves as a negative regulator of T cell activation[24,67,68]. The observation that T_{regs} form long-lasting and stable contacts with DCs both *in vitro* and *in vivo*[69,70] then suggested that CTLA-4 expression on T_{regs} could be an important mechanism of suppression by inhibiting effector T cell access to CD80 and CD86 costimulation. Remarkably, CTLA-4 on T_{regs} is not only blocking effector T cell access to costimulatory molecules expressed on APCs, but subsequently was shown to be able to trans-endocytose CD80 and CD86 after CTLA-4 binding, physically forcing the downregulation of these molecules on the surface of DCs[71] (Figure 1.4). Moreover, CTLA-4 ligation of CD80 and CD86 can induce expression of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO), inducing tryptophan catabolism and increasing the threshold for T cell activation[72,73].

LAG-3 is another molecule expressed on the surface of T_{regs} after their activation. Similar to CTLA-4 binding to CD80 and CD86, LAG-3 binds to MHCII with a higher affinity than CD4[74]. Though the exact mechanism of action has yet to be elucidated,

LAG-3 deficient T_{regs} show reduced ability to limit autoimmune T cell responses, and ectopic expression of LAG-3 on CD4+ T cells confers suppressor capacity, suggesting that LAG-3 plays a role in T_{reg} suppressive function[75,76]. Additionally, recent work has shown that, similar to the CTLA-4-mediated physical uptake of CD80 and CD86 by T_{regs}, T_{regs} are also able to selectively suppress antigen specific responses by depleting MHCII:peptide complexes from the surface of DCs, decreasing their antigen presentation capacity[77]. This process was shown to be CTLA-4-independent[77], but whether this depletion of MHCII:peptide complexes is dependent on LAG-3 is still unknown.

Lastly, in the context of tumor-draining lymph nodes, T_{regs} have even been shown to induce perforin-dependent cytolysis of DCs as mechanism of immunosuppression[78], though other inflammatory contexts in which this mechanism might play an important role remains to be seen. Despite extensive studies into elucidating mechanisms of T_{reg} function, the primary mechanisms they use to suppress immune responses *in vivo* remains poorly understood. It is likely that T_{regs} employ multiple methods for immunosuppression depending on the inflammatory or tissue environment. Indeed, there is evidence that both the activation status of a T_{reg} population and the tissue in which it is located (lymphoid versus peripheral tissue) could have important implications for they types of regulatory mechanisms used[56]. Overall, since their initial discovery, the suppressive mechanisms utilized by T_{regs} to inhibit immune responses and maintain tolerance have been shown to be as varied as the inflammatory response itself. Mechanisms of regulation, both T_{reg}-dependent and T_{reg}-independent, that are at play during necessary inflammation, such as in response to an invading pathogen, remain an intriguing and open question in the

immunology field. The interplay between inflammation and regulation is further discussed in subsequent sections.

1.5 Regulatory T Cells During Infection: Friend or Foe?

As previously discussed, inflammatory immune responses to invading pathogens are absolutely essential for pathogen clearance and host survival[79]. On the other hand, this same inflammatory environment can cause damage to healthy host tissue, potentially causing severe morbidity or even mortality despite efficient clearance of the pathogen[79]. Mechanisms of immune regulation and suppression are obviously important, particularly during homeostasis, but what role do these mechanisms play during a necessary, active inflammatory response? As mentioned in Sections 1.3 and 1.4, one of the most well characterized players in immunosuppression is the T_{reg} population. This population is essential for preventing the rise of inflammatory autoimmune responses, but how exactly do they fit in during necessary immune responses to pathogens? Presumably, inflammatory responses must be allowed to occur in order to clear pathogens, yet some aspect of regulation must also be required to prevent immunopathology associated with this inflammation.

Since their discovery, T_{regs} have been of great interest in many models of ongoing inflammation. The role of T_{regs} in preventing autoimmunity cemented their initial characterization as a necessary, beneficial cell type[45,80]. However, subsequent studies in the context of infection showed a much more complex picture of T_{regs}. During infection, T_{regs} can be found in secondary lymphoid organs, but are also recruited to sites of tissue inflammation where they can carry out suppressive function[79,81]. In one study of ocular infection with herpes simplex virus (HSV), depletion of T_{regs} resulted in increased

severity of inflammatory lesion size and increased incidence of pathogenic CD4+ T cell responses[82], implicating T_{regs} in protecting the host from CD4+ T cell mediated immunopathology. Similarly, depletion of T_{regs} during CNS infection with mouse hepatitis virus (MHV), though not affecting virus-specific T cell responses or viral clearance, led to increased proliferation, inflammatory cytokine production, and infiltration into the brain of effector T cells specific for myelin oligodendrocyte protein (MOG), a CNS self-antigen[83]. On the other hand, following mucosal infection with HSV, depletion of T_{regs} led to uncontrolled viral burden, decreased IFNγ production at the site of infection, and increased mortality, suggesting that T_{regs} can also play a role in coordinating early protective anti-viral responses[84]. T_{reg} depletion prior to acute respiratory syncytial virus (RSV) infection enhanced protective immune responses and viral clearance, but the mice had increased weight loss, inflammatory cytokine production and T cell activation, as well as increased cellular infiltration into the lung[85], demonstrating that T_{regs} are a major player in controlling the delicate balance between pathogen clearance and immunopathology.

The presence of T_{regs} at a site of infection is not only associated with suppression of inflammatory responses and decreased immunopathology. In several models, T_{regs} have also been linked to the persistence of pathogen and the promotion of a chronic infection. During skin infection with *Leishmania major*, T_{regs} were linked to suppressed anti-parasitic T cell responses and inability to clear the parasite infection, leading to the establishment of a chronic infection[86]. Similarly, human patients with chronic hepatitis B (HBV) viral infection had increased frequencies of circulating T_{regs} than either healthy controls or patients with resolved HBV infection, and depletion of CD25+ T cells in these

patients resulted in increased T cell proliferation and IFNγ production after *ex vivo* antigen stimulation[87]. In patients with chronic hepatitis C (HCV) viral infection, increased infiltration of T_{regs} into the liver was correlated with decreased fibrosis, once again highlighting the potential for T_{regs} to protect from immunopathogenic responses[88]. These studies, along with many others, have squarely placed T_{regs} at the crux of inflammatory responses to pathogens, yet much still remains to be elucidated as to their tissue-dependent functions, mobilization and activation cues, as well as how different pathogens might manipulate regulatory responses to promote their own survival.

HSV (ocular)	*Protection from immunopathology	+	(82)
HSV (mucosal)	*Amplified early anti-viral T cell responses	+	(83)
MHV	*Protection from autoreactive T cell responses	+	(84)
RSV	*Decreased viral clearance *Protection from immunopathology	-/+	(85)
L. major	*Decreased parasite clearance and enhanced chronic infection	-	(86)
HBV (human)	*Suppression of T cell proliferation and cytokine production	-	(87)
HCV (human)	*Protection from fibrosis	+	(88)

Table 1.1. Summary of the role of T_{regs} in various infection models.

(+) indicates a beneficial presence of T_{regs} and (-) indicates T_{regs} play a negative role. Number in parentheses indicates the citation associated with each row.

1.5.1 Antigen Specificity of Regulatory T Cell Populations

The subject of the antigen specificity of the T_{reg} population has been a long-standing question in the immunology field, particularly in models of infection, where abundant foreign antigen is present in the environment. It was long thought that T_{regs} have a TCR specific for self-peptide. This idea was due largely to the fact that the majority of T_{regs} found in the periphery were thymically-derived tT_{regs}, and early studies using transgenic mice in which HA antigen was expressed as a representative "self-peptide" resulted in the development of a large proportion of CD25+ regulatory T cells[48]. The major caveat of these studies was that it was unclear how representative this model was for tT_{reg} development, as 1) HA is not a true self-peptide, 2) forced expression of HA in these transgenic mice was overrepresented in the thymus compared to other antigens, potentially selecting for certain T_{reg} cell clones that would not be representative in a natural setting of T_{reg} development, and 3) it was possible that the strong affinity of the HA-specific TCR for HA was able to override the lack of secondary costimulatory signals or cytokine signals normally required for tT_{reg} development. However, this hypothesis has since been supported by evidence using genetic sequencing of the TCR repertoire of T_{regs}. Using transgenic T cells expressing a fixed TCRβ chain with an endogenous, variable TCRα chain, it was found that the TCR repertoire of CD25+ T_{regs} and that of CD25- effector T cells was similarly diverse, but largely distinct from each other[89,90]. Interestingly, after retroviral expression of either CD25+ TCRα genes or CD25- TCRα genes on CD25- effector T cells and transfer of these cells into RAG-deficient animals lacking T cells or wild-type C57Bl/6 animals, effector T cells expressing CD25+ TCRα genes expanded much better than those expressing CD25-

TCRα genes, resulting in wasting disease[89,90]. These results further suggested the T_{reg} TCR repertoire could receive TCR stimulation to endogenous self-antigen in peripheral tissues and lead to the expansion of these T cells over those expressing non-T_{reg} associated TCRs.

The above studies provided some of the strongest evidence that the T_{reg} TCR repertoire responds largely to self-antigen in the periphery, but what happens during an active infection, where presumably both tT_{regs} and pT_{regs} are involved in maintaining control of an ongoing inflammatory response to foreign antigen? Despite evidence that T_{regs} express largely TCRs specific for self-antigen, several more recent studies have shown, particularly in cases of chronic infection, that a least some of the responding T_{regs} are actually specific for microbial or pathogen-derived antigen. During *Mycobacterium tuberculosis* (Mtb) infection, a small percentage of the total T_{reg} population was found to be specific for the pathogen, but even this small number was able to delay protective T cell responses early in infection, which led to early expansion of the bacterium[91]. Similarly, CNS infection with MHV revealed a small number of virus-specific T_{regs} that recognized the same viral epitopes as many effector T cells, and were able to suppress effector T cell proliferation[92]. Additionally, there is some evidence from chronic parasite infection with *Leishmania major* that shows the majority of T_{regs} at the site of infection are pathogen specific[93]. These T_{regs} proliferate in response to *L. major* antigen-loaded DCs and maintain regulatory function after *ex vivo* expansion and transfer into infected recipients[93]. Finally, in a model of influenza virus infection, MHCII-tetramer staining for a virus-specific epitope revealed a population of virus-specific T_{regs} that could suppress secondary virus-specific effector T cell responses upon reinfection[94]. Taken together,

these studies highlight the complex nature of T_{reg} responses in the face of infection. Though many of the responding T_{regs} may be activated in response to self-antigen presented after tissue damage during infection, it cannot be overlooked that pathogen-specific T_{reg} populations may arise that could be playing a role in controlling the inflammatory immune response, and it remains to be seen what implications for pathogen-specific versus self-specific T_{reg}-mediated suppression exist in these different models. The T_{reg} and effector TCR repertoire that arises in the CNS during chronic infection with the parasite *Toxoplasma gondii* is discussed further in Chapter 3.

1.5.2 Cytokine Production and T_{reg} Differentiation during Inflammation

It has now been well established that regulatory T cells play an extremely important role in suppressing inflammation during both homeostasis and infection. Under the control of master transcription factor Foxp3[36,37], they are able to carry out suppressive function in any number of inflammatory or homeostatic settings. Therefore it came as somewhat of a surprise to discover that T_{regs} can not only express the master transcription factors of other "inflammatory" T_h subsets, but in many cases rely on them for their suppressive function in a variety of immune responses. After treatment of mice with α-CD40, it was discovered that T_{regs}, while maintaining Foxp3 expression, also upregulated T_h1 transcriptional regulator Tbet and produced IFNγ[95]. These T_h1-polarized Tbet+ T_{regs} were necessary to suppress effector T cell responses to *M. tuberculosis,* and they could also rescue much of the autoimmune phenotype of *scurfy* mice, whereas Tbet-deficient T_{regs} could not[95]. It was subsequently shown that the T_h1 differentiation of T_{regs} was in response to STAT1 induction downstream IFNγ produced by effector T cells, and that

delayed induction of IL-12Rβ2 in these cells prevented full differentiation into T_h1 effector cells[96]. During acute infection with *T. gondii*, IFNγ promoted the development of T_h1-polarized T_{regs} in secondary lymphoid organs, but this T_h1 T_{reg} polarization at mucosal sites of inflammation was dependent on the cytokine IL-27[97]. In this model of infection, induction of Tbet in T_{regs} was linked to upregulation of CXCR3, a molecule thought to be important for trafficking of T_{regs} to sites of T_h1 inflammation[97].

In addition to T_h1-polarized T_{regs}, GATA-3 expressing T_{regs} have also been found in the spleen and lymph nodes of mice in steady state conditions, and addition of T_h2-associated cytokines can increase this expression without affecting Foxp3 expression[98]. Even T_h17-polarized T_{regs} have been found *in vivo*, as components of the microbiota seem to be able induce RORγt expression in colonic T_{regs} despite the paradoxical evidence that RORγt antagonizes Foxp3 in *in vitro* settings[99-101]. When T_{regs} lack RORγt expression, T_h2 responses to helminth infection are increased, while T_h2- and T_h17-mediated immunopathology is exacerbated, suggesting that T_h17-polarization of T_{regs} is important for maintaining control of immunopathologic responses at mucosal sites[99,100]. All of these studies emphasize the highly dynamic nature of the regulatory T cell population, whose phenotype remains much more plastic than originally thought while still maintaining suppressive function. Depending on a wide variety of environmental cues, from tissue residency to the cytokine milieu during inflammatory responses, specific T_{reg} subsets, with different transcriptional profiles, will arise to control very specific types of inflammation.

1.6 Tracking T cell Motility in Tissues

Widely used techniques in the immunology field, such as flow cytometry or *in vitro/ex vivo* characterization of immune cell responses, require removal of immune cells from the host environment to some degree. Though studies utilizing these techniques continue to be extremely valuable, it is important to remember that these responses are naturally occurring against the backdrop of an extremely complex and dynamic tissue environment, even in a non-inflammatory steady state. Understanding the localization, motility, and cell-cell interactions in living tissue then can provide additional insight into the complexity that is an immune response.

Being able to visualize immune responses in living tissue is a relatively recent technical advancement, but has allowed the field to confirm and better understand certain aspects of immune interactions that had previously only been hypothesized. Two of the first seminal studies using two-photon laser scanning microscopy (TPSLM) were published in 2002. In both, T cell motility was tracked in naïve, explanted lymph nodes[102,103]. It was noted that T cells in the lymph node moved relatively fast, in a "random walk" pattern, yet after antigenic challenge, antigen-specific T cells stalled and formed stationary clusters around antigen-loaded DCs[102,103]. This was the first direct *in vivo* evidence of the formation of the immunological synapse in response to antigen presentation, and provided important insight into the dynamics of early T cell activation in response to antigen[102-104]. Since this hallmark study, many groups have begun to characterize the *in vivo* dynamics of immune responses both during homeostasis and inflammation in lymphoid tissues[105-108].

Not only has TPLSM provided increased understanding of the dynamics of homeostatic T cell trafficking and initial antigen priming in lymph nodes, but *in vivo* imaging of T cell motility in inflamed tissues has also begun to increase the understanding of how different inflamed tissue environments can affect T cell response to antigen. In one study using CNS infection with the parasite *T. gondii*, it was found that CD8+ T cells recruited to the brain did not stall or noticeably interact with infected neurons or astrocytes (non-antigen-presenting cells), but significantly slowed and made transient contacts with APCs clustered around free parasite in the brain[109]. This finding provided further evidence of immunological synapse formation in an inflamed tissue in response to antigen presentation following a natural infection[109]. The behavior of CD8+ T cells in the brain during *T. gondii* infection was later mathematically modeled and described as, not random Brownian motion, but a generalized Lévy walk, allowing cytotoxic T cells to most efficiently survey the inflamed tissue to find rare target antigen[110]. In the EAE model, T cell mobility in the meninges has been studied, identifying adhesion and chemokine signals that regulate encephalitogenic T cell "crawling" along meningeal vessels preceding tissue invasion, along with observations of the same immunological synapse formation dependent on MHCII and LFA-1 in spinal cord-infiltrating encephalitogenic T cells as seen in other models[104,111,112]. Overall, these studies have allowed for the initial characterization of the complex interactions between the tissue environment and different cell types involved in regulating T cell reactivity to antigen and their resulting behavior.

1.6.1 T_{reg} Motility in Models of Inflammation

While many studies have used TPSLM to track the motility and behavior of effector T cells and different myeloid subsets[102-122], fewer studies have focused on the behavior of T_{regs} in either lymphoid or inflamed tissues. As discussed in Section 1.4 and 1.5, many suppressive mechanisms have been ascribed to T_{regs}, though it remains somewhat unclear what, if any, might be the primary ways this cell type dampens immune responses *in vivo*. The discovery that T_{regs} constitutively express high levels of immunosuppressive molecules, such as CTLA-4 and LAG-3, that can directly interact with and lead to the downregulation of costimulatory molecules on APCs[66,71,76], suggests that T_{reg}:APC interactions could play an important role in regulating effector T cell responses. Indeed, *in vitro* studies revealed that T_{regs} preferentially form stable contacts with DCs and are able to outcompete effector T cells for DC contact in this setting[123].

Evidence for this phenomenon occurring *in vivo* was provided in several models using TPSLM. In models of autoimmune diabetes using non-obese diabetic (NOD) mice, as well as experimental autoimmune encephalomyelitis (EAE), increasing numbers of antigen-specific T_{regs} in the system resulted in the formation of long-lived, stable T_{reg}:DC contacts in the draining lymph nodes, decreasing the contact time between DCs and effector T cells and leading to increased effector T cell velocity in the tissue[69,70]. These results suggested that T_{regs}, through stable contacts with DCs, could prevent potentially autoreactive T cells from accessing antigen-presenting DCs and forming the immunological synapse, thereby preventing their activation and immunopathogenic potential. Interestingly, this does not only occur in lymphoid tissues, as a subsequent study found that activated, antigen-specific T_{regs} formed transient contacts with tumor-

infiltrating DCs that correlated with DC downregulation of CD80 and increased effector CTL velocity and exhaustion[124]. Additionally, initial *in vitro* studies co-culturing T_{regs} with T_{conv} had suggested a contact-dependent form of suppression that limited the proliferation of T_{conv} independently of interactions with APCs[63,125]. Using TPSLM, this hypothesis has so far not proved to be true in *in vivo* settings, as no study has found significant direct interaction between T_{regs} and effector T cells *in vivo* during an immune response in either lymph nodes or peripheral tissues[70,124]. These studies focusing on the *in vivo* dynamics of an immune response have provided valuable insight that, when coupled with other traditional techniques, have begun to provide a clearer picture as to what occurs during both the priming and effector phases of an immune response, as well as how these responses are regulated. The *in vivo* behavior and motility of T_{regs} is discussed further in Chapter 3.

1.7 Suppression of Immune Responses through IL-10

As previously discussed, maintaining a balance between inflammation and regulation is necessary for survival. The diverse roles of the regulatory T cell population in promoting this balance was discussed in Sections 1.3-1.5, but cytokines also play a significant role in either antagonizing or supporting this equilibrium. Though several immunosuppressive cytokines have been described, IL-10 remains the most extensively studied and well-characterized immunosuppressive cytokine[126-128]. IL-10 was initially described as part of the T_h2 immune response[129], but has since been associated largely with T_{regs}, which produce large amounts of IL-10 upon stimulation[127,130,131]. Despite this initial characterization, however, it has since been reported that many cells of both the innate and adaptive immune system are capable of producing IL-10, including B cells,

NK cells, dendritic cells, monocytes and macrophages, as well as many T cell subsets[126-128]. Subsequent characterization of the IL-10:IL-10R complex led to the discovery that the IL-10R is comprised of two subunits, IL-10R1 and IL10R2[127]. IL-10R1 is expressed constitutively on most hematopoietic cells, but its expression can be induced after inflammatory activation on non-hematopoietic cells as well[127]. Expression of the IL-10R2 is constitutive on most cells and in most tissues, rendering most hematopoietic and non-hematopoietic cell types, particularly during inflammation, responsive to the suppressive effects of IL-10[127].

IL-10 signaling acts primarily through the Jak/Stat system[127]. In particular, Stat3 is recruited to the IL-10:IL-10R complex, and abolishing Stat3 in neutrophils and macrophages leads to chronic enterocolitis and prevents the ability of IL-10 to suppress macrophage proliferation[127,132]. The biological effects of IL-10 depend largely on which is the responding cell type, but IL-10 has been shown to decrease cytokine production (including downregulation of IL-1, IL-6, IL-12, IL-18, GM-CSF, TNF, and IFNγ)[127,133-137], decrease chemokine expression and leukocyte adhesion[138-140], as well as decrease costimulatory molecule expression on APCs[134,137,140-142]. Studies looking into the role of IL-10 began much like those looking into the role of T_{regs}, with the observation that mice lacking this cytokine presented with severe autoimmune reactions, as is discussed in the following section.

1.7.1 IL-10 Production and Its Role during Homeostasis

The initial roles of IL-10 were characterized in the context of immune homeostasis in the gut[143]. Disruption of gut homeostasis is thought to be the underlying cause of the symptoms seen in Crohn's disease (CD) and ulcerative colitis, yet a complete

understanding of the etiology remains unclear[143]. The IL-10 deficient mouse, developed in 1993, has since been used extensively to begin to explore the underlying pathologies of these diseases[143,144]. IL-10 deficient mice display aberrant expression of MHC on intestinal epithelia and hematopoietic cells, increased inflammation in the intestine, and severe intestinal pathology in response to endogenous microbiota-derived antigen[144-146]. Interestingly, there is evidence that lamina propria mononuclear cells from human patients with Crohn's disease or ulcerative colitis both produce less IL-10 and are less responsive to rIL-10 than either peripheral blood mononuclear cells from the same patients or lamina propria mononuclear cells from healthy controls[147]. In IL-10 deficient mice, increased intestinal pathology was first linked primarily to aberrant T_h1 responses, as a lack of IL-10 results in increased production of both IL-12 and IFNγ, with relatively normal B cell responses[148,149], and mice deficient in both IL-10 and IL-12p40 were resistant to the development of colitis[150]. This relatively simple model of colitis development in IL-10 deficient mice was soon made more complex, however, after the discovery that the IL-12p40 subunit is shared with another inflammatory cytokine, IL-23[151].

After this discovery, it was suggested that IL-23, not IL-12, was necessary and sufficient to drive colitis pathology in IL-10 deficient mice by promoting the development of CD4+ T cells that produce inflammatory cytokines IL-17 and IL-6[152]. However, several groups soon found that IL-23 could actually promote the development of both IL-17- and IFNγ-producing CD4+ T cells[153,154], and that a complex interplay between IL-23 and IL-12, and the subsequent T_h17 and T_h1 responses, was involved in colitis pathology[155]. These studies served to even further emphasize the complex cytokine

responses necessary to maintain immune homeostasis in the gut and the important and wide-ranging role of IL-10 in suppressing multiple inflammatory pathways to prevent colitis.

IL-10 production from multiple cell types in the lamina propria has been reported[143], but IL-10-producing T_{regs} are an essential source of regulation to maintain immune homeostasis in the gut. The earliest evidence for T_{reg}-derived IL-10 being the critical source came from a T-cell-transfer model of colitis, in which transfer of $CD45RB^{high}$ CD4+ T cells alone (which does not include the $CD45RB^{low}$ T_{reg} population) into immunodeficient hosts leads to intestinal immunopathology and colitis[156]. It was found that co-transfer of $CD45RB^{high}$ and $CD45RB^{low}$ CD4+ T cells prevented intestinal immunopathology (implicating the $CD45RB^{low}$ T_{reg} population as being important for prevention of colitis), while co-transfer with IL-10 deficient $CD45RB^{low}$ CD4+ T cells was not protective[157]. These findings were then supported by the development of a T_{reg}-specific IL-10 knockout mouse ($Foxp3^{Cre}/Il10^{fl/fl}$), which similarly developed immunopathology at mucosal surfaces, including the intestine, providing further evidence that T_{reg}-derived IL-10 was sufficient to protect from colitis[57]. Additionally, knockout of IL-10 from CX_3CR_1-expressing lamina propria macrophages and DCs did not result in intestinal pathology, while knockout of IL-10R in this same population resulted in the development of colitis, suggesting that production of IL-10 from lamina propria macrophages and DCs was dispensable for protection from colitis, while the ability of these cells to respond to IL-10 is required to prevent intestinal immunopathology[158]. The above studies, along with many others, have begun to piece together the mechanisms behind the development of colitis in mice, putting together a model in which the loss of

IL-10-producing T_{regs}, or the loss of myeloid cells' ability to respond to IL-10, allows for an increase in inflammatory cytokine production, including IL-6, IL-12, and IL-23, ultimately leading to aberrant T_h1 and T_h17 responses that result in pathology. Overall, these studies highlight the essential role of IL-10 in maintaining immune homeostasis in the gut, but what is the role of this immunosuppressive cytokine in the context of active infection at other tissue sites?

1.7.2 IL-10 Production and Its (Complex) Role during Infection

While IL-10 production is absolutely required to prevent the rise of pathogenic inflammatory responses in steady state conditions, an additional layer of intricacy is added when inflammatory reactions are required, as is the case in response to infection. It has become widely accepted that in cases of infection, though inflammation is necessary to control pathogen dissemination, much of the pathology observed can be attributed to the immune response itself[126]. Similar to its role in homeostasis, IL-10 production during immune responses to infection has been shown to limit T cell responses, both indirectly through modulation of APC activation or directly through limitation of T cell proliferation and cytokine production[126,127]. In infection models including *T. gondii* (discussed further in Section 1.9), *Trypanosoma cruzi, Plasmodium* spp., and *Mycobacterium* spp., depletion or deficiency of IL-10 during the course of infection results in excessive inflammation and T cell responses that lead to severe tissue damage and significant immune-mediated pathology[126]. In *T. cruzi* infection, IL-10 deficiency is correlated with increased circulating levels of TNFα, IL-12, and IFNγ as well as increased parasite clearance[159]. Despite the decrease in parasite burden, however, IL-10 deficient mice have earlier mortality, which can be reversed by administration of either

rIL-10 or a blocking IL-12 antibody, implicating the increased T_h1 inflammatory response in the absence of IL-10 in the observed fatal immunopathology[159]. Similarly, in a model of cerebral malaria infection, IL-10 has been shown to impede parasite clearance by downregulating inflammatory T_h1 responses, yet mice deficient in IL-10, though they clear parasite more efficiently, subsequently die from immune-mediated pathology[160,161]. Additionally, influenza infection requires IL-10 production from responding effector T cells in the lung that, when blocked, results in the rise of pathogenic T_h1 and T_h17 responses that cause severe injury to the lung and can be fatal[162,163].

Whereas the production of Il-10 is an obvious necessity during homeostasis and in many lethal infection models (as described above), in some cases, the production of IL-10 can so strongly inhibit anti-pathogen responses that the pathogen can escape immune control, leading to increased dissemination or chronic infections[126]. Ablation of IL-10 during infection with *L. major* results in increased IFNγ production, macrophage killing, and sterile clearance of the parasite with no reports of associated severe immunopathology[164,165]. To similar effect, infection with lymphocytic choriomeningitis virus (LCMV) Clone 13 is known to lead to a chronic infection characterized by persistent viral load and large amounts of IL-10 production[166]. Depletion of IL-10 or blockade of the IL-10R during this infection, however, leads to increased anti-viral T cell responses and clearance of the virus in the absence of immunopathology[166-168]. Taken together, the above studies accentuate the extremely complex nature of the pathogenesis of infections, where a delicate balance must be maintained in order to allow enough inflammation for pathogen control while minimizing the deleterious effects of this inflammation. Similar to the regulatory T cell population, IL-10 lies at the crux of this

balance, and its effects on pathogen control and immunopathogenic inflammatory responses are highly dependent on the context of inflammation and pathogen infection. Overall, it seems that IL-10 production during typically low- or asymptomatic infections can disrupt pathogen control and promote pathogen persistence and chronic infection, while its production during severe, highly virulent infections is necessary to prevent fatal immunopathology, regardless of its effects on pathogen control.

T. gondii	*Prevents excessive inflammation and immunopathology	+	(288)
T. cruzi	*Decreased parasite clearance *Prevents excessive inflammation and immunopathology	-/+	(159)
Plasmodium spp.	*Decreased parasite clearance *Prevents excessive inflammation and immunopathology	-/+	(160,161)
Influenza	*Prevents pathogenic T_h1 and T_h17 responses	+	(162,163)
L.major	*Prevents sterile clearance of parasite	-	(164,165)
LCMV	*Prevents viral clearance and promotes chronic infection	-	(166,167,168)

Table 1.2. Summary of the role of IL-10 in various infection models.

(+) indicates a positive role and (-) indicates a negative role for IL-10.
Numbers in parentheses indicate the citation associated with each study.

1.8 T Cell Costimulation: Inducible T Cell Costimulator

As discussed in Section 1.2, T cell activation requires not only TCR:MHC interactions, but an additional secondary costimulatory signal from APCs, for optimal activation and effector function[169,170]. This secondary signal was initially identified as an interaction between CD28 on the T cell and CD80/CD86 on the APC that is required for IL-2 production, CD25 upregulation, entry into cell cycle, as well as enhancing the expression of pro-survival Bcl family molecules[169-172]. Subsequent study has identified a host of other potent costimulatory molecules with varying effects on activation of T cells[24]. One such molecule is the inducible T cell costimulator (ICOS). ICOS is a costimulatory molecule homologous to CD28, though its expression on T cells is induced only after TCR activation, whereas CD28 is constitutively expressed on naïve T cells[173,174]. Despite the homology of these two costimulatory molecules, distinct differences in their ligand binding and downstream signaling result in nonredundant roles in modulation of T cell activation[24,175]. While CD28 binds to CD80 or CD86 on APCS, ICOS binds exclusively to ICOS-ligand (ICOSL)[176,177], which is expressed primarily on APCs but has also been shown to be upregulated on some endothelial and epithelial cell subsets during inflammation[178]. Early studies into the downstream effects induced following ICOS:ICOSL ligation reflected some of the shared homology between CD28 and ICOS, as many changes in the transcriptional profile of T cells were shared following either CD28 or ICOS ligation[179]. It soon became apparent, however, that several distinct differences between the two existed and resulted in important downstream outcomes. Though both CD28 and ICOS recruit class IA PI3K, ICOS has been shown to preferentially recruit the p50α regulatory subunit of PI3K in addition to the catalytic

p110δ subunit, leading to increased PIP_3 production at the plasma membrane and stronger Akt activation than seen following CD28 ligation[180,181] (Figure 1.5).

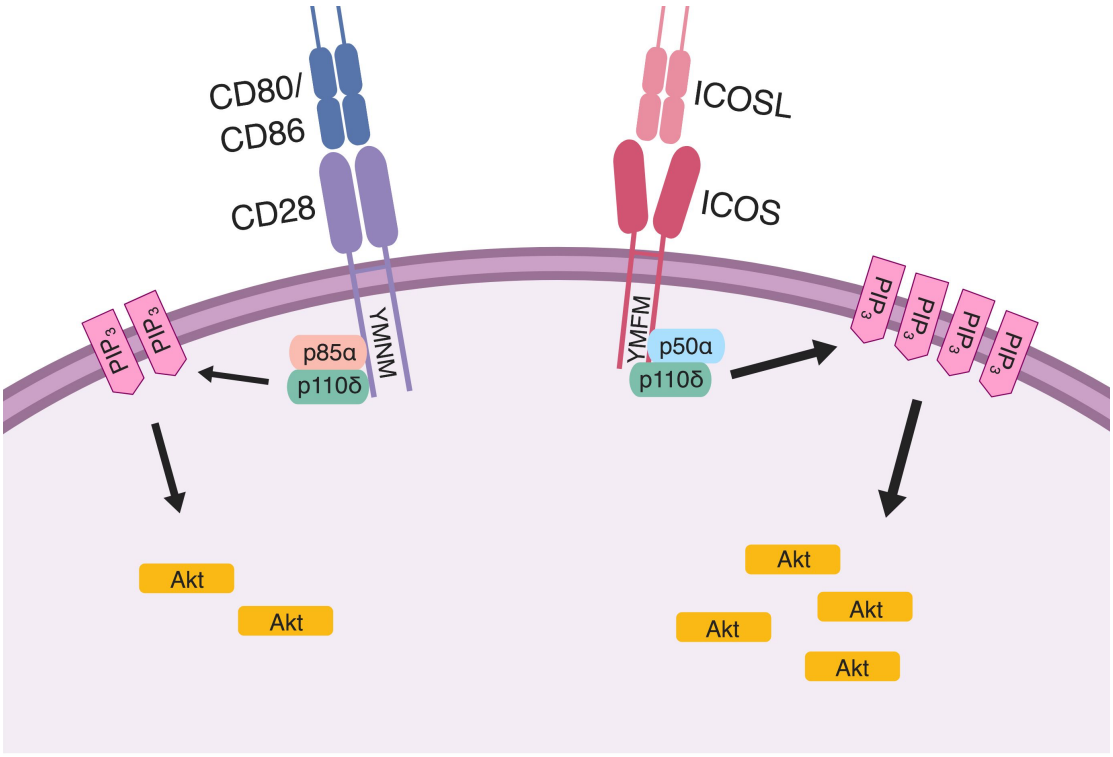


Figure 1.5. Overview of ICOS and CD28 signaling. Though both signal largely through the PI3K/Akt pathway, ICOS and CD28 signaling have distinct differences. The cytoplasmic tail of ICOS contains a YMFM motif that preferentially recruits the regulatory p50α subunit of PI3K along with the catalytic p110δ subunit. The increased recruitment of p50α leads to increased PIP_3 production at the plasma membrane, ultimately resulting in increased phosphorylation and activation of Akt (adapted from Wikenheiser and Stumhofer, 2016).

Additional differences have been noted in the downstream outcomes of ICOS ligation in comparison to CD28 ligation. While CD28 signals strongly induce IL-2 production from T cells[170], it has been discovered that ICOS lacks the signaling motif in its cytoplasmic tail required for enhanced IL-2 production, meaning ICOS stimulation is a poor inducer of IL-2 and IL-2-dependent T cell proliferation[24,173,175,177,182,183]. Conversely, ICOS is a much more potent inducer of IL-10 than CD28[24,175,182-184]. The diverse roles that ICOS plays in promoting antibody responses, T cell cytokine production, as well as regulation of immune responses is discussed in the following sections.

1.8.1 ICOS: Role in T_{fh} Differentiation and B Cell Responses

As a costimulatory molecule, much of the characterization of ICOS signaling has occurred in models of inflammation or infection requiring the activation of T cell responses. Early studies using ICOS KO or ICOSL KO mice showed an obvious defect in germinal center formation and class-switched antibody production[185,186]. It was subsequently shown that ICOS KO mice fail to generate class-switched antibody, including IgG1, IgG2A, and IgE, due to a failure of T cells to upregulate CD40L, rendering them unable to provide CD40 costimulatory "help" to B cells[187]. More recently, ICOS has been shown to promote the differentiation of follicular helper T cells (T_{fh}), which are required for the formation of functional germinal centers and selection and survival of high-affinity B cells[188-190]. During the priming of a T cell response in the secondary lymphoid organs, ICOS costimulation can induce the expression of Bcl-6, the master transcriptional regulator of T_{fh}, as well as antagonize the expression and activity of Blimp-1, preventing T cell differentiation into T_h1[191]. ICOS also promotes the

expression of c-Maf and IL-21, molecules important for supporting the differentiation and expansion of T_{fh}[192], as well as the expression of CXCR5, a chemokine necessary for trafficking of T_{fh} into the B cell follicle[189,193-195]. Overall, ICOS signaling has been shown to support the differentiation and function of T_{fh} cells, thereby serving an essential role in promoting the formation of active germinal centers and high-affinity class-switched antibody production.

1.8.2 ICOS: Role in Effector T Cell Responses

In addition to the role of ICOS in T_{fh} generation and antibody responses, early studies also noted that, at least *in vitro*, ICOS was upregulated and sustained only on T_h2 differentiated T cells, whereas T_h1 differentiated T cells only transiently upregulated ICOS, and *in vivo* vaccination models showed defects in T_h2-associated cytokine production, including IL-4, IL-5, and IL-10, while T_h1-associated cytokine production, namely IFNγ, was equivalent (or even increased) following ICOS neutralization[31,187,196]. This, along with the evidence showing the essential role of ICOS in antibody responses, led to the early hypothesis that ICOS signaling, while necessary for T_h2-mediated immunity, was dispensable for T_h1 immune responses. This idea is supported by several studies using infection models in which ICOS deficiency or neutralization resulted in enhanced T_h1 immunity. For example, ICOS signaling deficiency in mice infected with *M. tuberculosis, C. muridarum,* or *S. mansoni*, resulted in enhanced T_h1 responses, characterized by increased numbers of IFNγ+ CD4+ T cells, increased production of IL-6 and TNFα, and decreased production of regulatory cytokine IL-10[197-199]. Interestingly, these enhanced T_h1 responses correlated with better pathogen control in secondary

lymphoid organs but not in peripheral tissues, and also led to increased immunopathology[197-199].

To complicate matters, however, several studies have also reported deficient T_h1 responses in the face of ICOS signaling deficiency. In models of both *S. enterica* and *L. monocytogenes*, ICOS KO mice or ICOS-neutralized mice, respectively, displayed normal T cell activation, but impaired IFNγ production, ultimately resulting in the inability to control pathogen dissemination and persistence[200,201]. Additionally, ICOS costimulation was found to contribute to T_h1-mediated immunity during acute infection with *T. gondii*, as ICOS KO mice (on a BALB/C background) acutely infected had decreased CD4+ T cell activation, proliferation, and IFNγ production, ultimately resulting in less immunopathology during the chronic stage of infection[202]. This phenotype was only somewhat replicated, however, in ICOS KO mice on a C57Bl/6 background, which had small decreases in IFNγ production, but were only more susceptible to infection when simultaneously lacking CD28[203]. These results emphasized 1) the importance of genetic background when studying the effects of costimulatory signaling on T_h subset differentiation and effector responses, and 2) depending on the type of infection, ICOS can have both CD28-dependent and CD28-independent roles in costimulation. This latter idea has also been supported in models of nematode infection with *N. brasiliensis,* mediated by T_h2-polarized immunity and viral infection with LCMV, mediated by T_h1-polarized immunity[204]. Overall, these data present an extremely complex picture of ICOS costimulation, in which ICOS signaling can promote T_h2-mediated T cell responses, as well as either dampen or enhance T_h1-mediated T cell responses, depending on environmental context such as the type of infection or the

presence of other costimulatory signals. Its role in modulating T cell responses was even further complicated with the discovery of its importance in T_{reg} responses, as is discussed in the following section.

1.8.3 ICOS: Role in T_{reg} Survival and IL-10 Production

As mentioned in previous sections, in addition to promoting inflammatory T and B cell responses, initial characterization of ICOS included the observation that ICOS enhanced IL-10 production *in vitro*, and that ICOS+ T cells were the highest producers of IL-10 *in vivo*[175]. These first observations noting increased IL-10 production downstream of ICOS ligation provided the first glimpse into the role of ICOS in regulating immune responses as well as enhancing them. Another early hint into the regulatory capacity of ICOS was the observation that blockade of other costimulatory signals, like CD40 and CD28, though nearly completely wiping out T cell proliferation and inflammatory cytokine production, gave rise to a largely ICOS+ T cell population that produced IL-10 and had suppressive capacity when co-cultured with ICOS- T cells[205], providing some of the first evidence that ICOS can both promote IL-10 production from T cells and could be important in the differentiation or maintenance of a regulatory T cell population. It was subsequently shown that, in model of airway hypersensitivity reaction (AHR), IL-10 production from T_{regs}, as well as their overall suppressive capacity and maintenance of respiratory tolerance, was dependent on ICOS ligation[206]. Succeeding studies looking at the role of ICOS in the regulatory T cell compartment in autoimmune NOD mice noted that ICOS+ IL-10-producing T_{regs} were enriched in the pancreas, and that ICOSL antibody blockade led to a substantial loss of the T_{reg} population in the pancreas, as well as decreased IL-10 production and increased IFNγ production from the remaining T_{regs} in

the diabetic lesion, ultimately leading to the rapid onset of autoimmune diabetes[207]. Additionally, baseline differences in ICOS or ICOSL KO mice began to be reported, in which ICOS signaling deficient mice had fewer T_{regs} in secondary lymphoid organs[208], implicating ICOS signaling in not only the function of T_{regs}, but also their maintenance at steady state. Finally, ICOS was shown to be required for the maintenance of activated, $CD44^{hi}CD62L^{lo}$ effector T_{regs} by supporting their survival, as ICOSL antibody blockade in steady state led to a selective decrease in antigen-experienced $CD44^{hi}CD62L^{lo}$ T_{regs}, associated with decreased Bcl-2 expression[209].

All of the above results add an additional layer of complexity to the role of ICOS in a multitude of immune responses. This multifaceted role seems to promote immunoregulation during homeostasis and inflammation through maintenance of a functional immunosuppressive T_{reg} population as well as through production of IL-10, in addition to promoting both T_h2- and T_h1-mediated inflammatory responses, none of which is necessarily mutually exclusive. Exactly what environmental cues and molecular signals govern the outcome of ICOS signaling remains unclear. These studies highlight the need for a better understanding of these cues, and potentially how to harness an ICOS signal for therapeutic use.

1.8.4 ICOS and Common Variable Immunodeficiency

Common variable immunodeficiency (CVID) is a heterogeneous disorder in humans that results in a primary immunodeficiency disease with the hallmark symptoms of hypogammaglobulinemia and recurrent bacterial infections[210]. Though the exact cause of CVID development remains somewhat unclear, several genetic mutations have been identified that lead to the development of CVID, two of which are genetic mutations

leading to the loss of ICOS or ICOSL[211-213]. Much like ICOS KO mice, human patients with ICOS or ICOSL mutations present with extremely low class-switched serum antibody, along with decreased levels of circulating naïve B cells and nearly absent circulating memory B cells[211,212,214], however, it was also noted that CVID patients often presented with T cell abnormalities along with B cell and antibody deficiencies[215,216]. In several studies, impaired cytokine production, including IL-4, IL-5, and IL-10, from CD4+ T cells isolated from PBMCs from CVID patients was observed[215,217-219], implicating a similar defect in promotion of T cell responses in human CVID as in ICOS KO mice.

Also similar to ICOS KO mice, CVID and ICOS deficiency in humans is not at all straightforward. While all CVID patients are classified as being "immunodeficient", about 20% of them present with symptoms of autoimmunity as well[220]. As more data came to light, it was found that a subset of CVID patients do not seem to have T cell deficiencies as had been previously described, but rather showed an expansion of activated $CD44^{hi}$ T cells that produced more IFNγ than control subjects[216]. Another potentially contributing factor to the autoimmunity seen in association with CVID is a dysfunctional T_{reg} compartment. Several studies have found that in patients with CVID that also present with autoimmunity, the frequency and number of circulating T_{regs} is decreased compared to both healthy controls and CVID patients that have no obvious symptoms of autoimmunity[221-224]. These data, coupled with evidence that IL-10 production is reduced in CVID patients[217-219,221], suggests that, despite its widespread "immunodeficient" characterization in the clinic, CVID, similar to ICOS KO mice, can also present with defective regulation of inflammatory responses. Although it is unknown

if all of these CVID patients had specific mutations in ICOS, these studies emphasize the heterogeneous nature of costimulatory deficiencies, and support much of the data seen in ICOS deficient mice.

1.9 *Toxoplasma gondii*

Toxoplasma gondii is a eukaryotic parasite, first discovered in the early 1900s[225], that infects a wide range of warm-blooded hosts, including humans[226,227]. Since it's initial discovery in rodents, much work has been done to characterize the morphology, life cycle, infectivity, and host response to this parasite. To date, *T. gondii* has been isolated from domesticated sheep, pigs, cows, and goats, as well as their wild counterparts, other wild carnivores such as bears, foxes, ferrets, and raccoons, marine mammals such as sea otters, sea lions, seals, dolphins, and whales, wild ungulates, marsupials, New World monkeys, small mammals such as mice, rats, squirrels, and rabbits, and humans[226], giving it likely the widest host range of any parasite. Despite its wide range of hosts, each of the hosts listed above are considered intermediate hosts, as the parasite cannot sexually reproduce in any of them[226,227]. The only definitive host that allows for the sexual reproduction of *T. gondii* is the feline, where oocysts are found passed in the feces of cats[226].

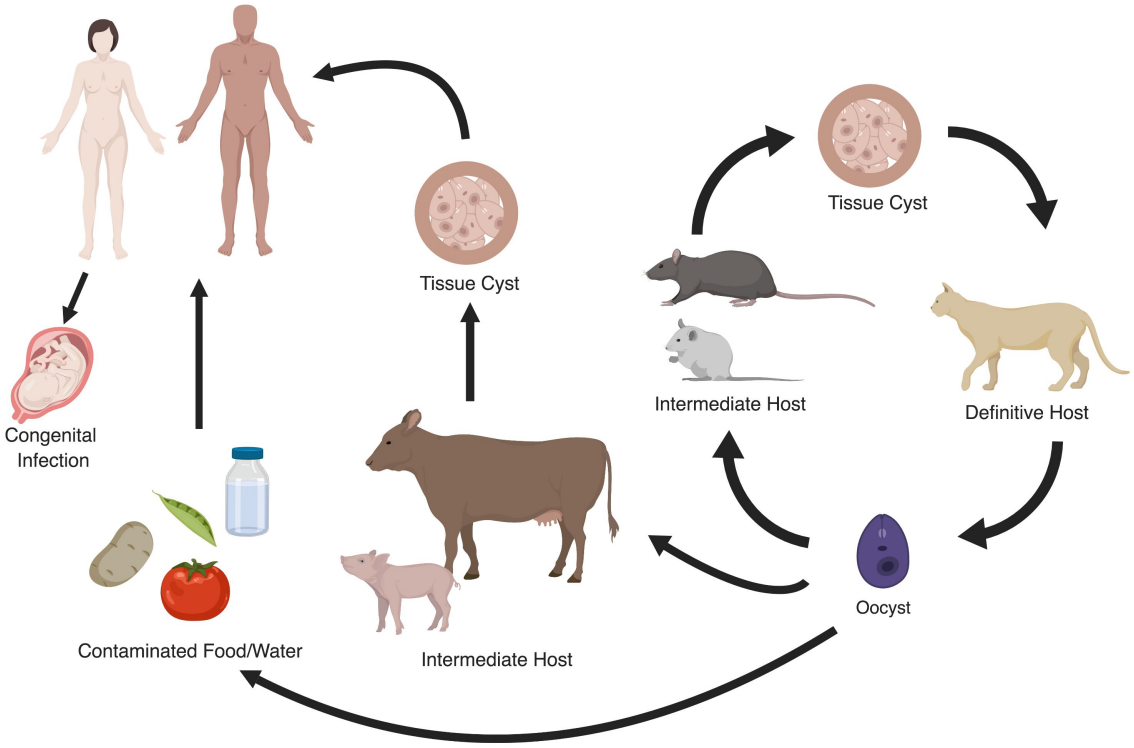


Figure 1.6. *Toxoplasma gondii* life cycle. The only discovered definitive host for the parasite is the felid family, where, upon infection, *T. gondii* can sexually reproduce in the gut. This leads to the shedding of highly infectious oocysts, which can be ingested and lead to infection in a number of intermediate hosts, such as mice, pigs, sheep, and cows. Infection in the intermediate host leads to the establishment of a chronic infection, characterized by the presence of tissue cysts in muscle and CNS tissues. Some intermediate hosts can once again be consumed by cats, where ingestion of tissue cysts will once again lead to sexual reproduction in the gut and shedding of oocysts. Humans can consume some intermediate hosts, namely domesticated animals, which also leads to a chronic infection. Though human infection is usually the result of consuming contaminated meat products, it is also possible to become infected through the consumption of food or water contaminated with infectious oocysts. Infection in humans usually remains asymptomatic in immunocompetent hosts, but primary infection during pregnancy can lead to the passing of parasite to the developing fetus, leading to potentially severe congenital infection.

The life cycle of *T. gondii* has been very well defined over the last 60 years. Three distinct infectious stages have been identified and characterized: sporozoites (in oocysts), the tachyzoites, and the bradyzoites (in tissue cysts)[226,227]. As previously mentioned, oocysts form only in the gut of felids, and are then shed in feces. Oocysts are highly resistant to environmental changes and, once shed, they become a highly infectious form of the parasite, and can be ingested and cause infection in a number of intermediate hosts[226,227] (Figure 1.6). In intermediate hosts, the parasite undergoes two stages of asexual reproduction. The tachyzoite form of the parasite is very highly replicative, and can efficiently infect any nucleated host cell by active membrane penetration[226]. After entering the host cell, the tachyzoite uses pieces of the host cell's membrane to surround itself with a parasitophorous vacuole, which helps to protect it from host defense mechansims[226]. Within the parasitophorous vacuole, the tachyzoites will continue to asexually replicate until the host cell cannot support further parasite, and lyses, releasing the tachyzoites to rapidly infect neighboring cells[226]. Somewhere along the life cycle of the parasite, the tachyzoite will convert into a much more slowly replicating form called the bradyzoite[226]. Despite extensive study into the parasite's life cycle, it is still largely unclear exactly what causes this conversion from tachyzoite to bradyzoite *in vivo*, whether intrinsic aspects of the parasite itself or whether certain tissue or environmental factors contribute, remains to be discovered. Unlike the tachyzoite, bradyzoites are housed with tissue cysts, which can vary in size from about 10 to 100 μm and house hundreds of parasites[226]. Also unlike the tachyzoites, which can be found infecting any number of host tissues, the tissue cysts that house the bradyzoites are primarily found in tissues of the central nervous system (CNS), mostly in the brain parenchyma and retinal

tissues, and, to a lesser degree, skeletal and cardiac muscle tissue[226]. Once the tissue cysts form, they can persist for the lifetime of the host, despite an extremely robust inflammatory immune response[226] (discussed in Sections 1.9.2 and 1.9.3). The tissue cysts do not remain completely dormant, however, as it has been shown that they can sporadically "reactivate", which involves the break down of the cyst wall and reconversion into the fast-replicating tachyzoite[226]. Once these reactivated parasites re-infect a neighboring cell, they can quickly convert back into a bradyzoite and form another tissue cyst if they are not cleared by the immune response[226].

1.9.1 Epidemiology and Human Infection

As mentioned in the previous section, humans represent one of the many intermediate hosts of *T. gondii*. In fact, human infection with *T. gondii* is relatively common worldwide, and it is estimated that up to a third of the world's population is seropositive[228]. This frequency of seropositivity can vary widely depending on region or country, however, ranging from 37-58% in central European countries such as Belgium, Austria, and Germany, to 51-72% in some Latin American countries such as Argentina, Brazil, and Venezuela[228]. Infection rates in the United States are on the lower end of this spectrum, averaging around 11-20%, though these rates have been declining in the last decade[229].

The acquisition of *T. gondii* infection in humans can occur through multiple routes. Though humans can acquire *T. gondii* through the fecal-oral route, typically through ingesting water contaminated with oocysts, the more common mode of transmission is consumption of contaminated meat containing tissue cysts[226,228,229] (Figure 1.6). Once ingested, the pathogenesis of infection in humans occurs much like what has been

observed in animal models (discussed in further detail in Section 1.9.2 and 1.9.3). While *T. gondii* infection in humans is very common, both the acute (tachyzoite-dominated) and chronic (bradyzoite-dominated) stages of infection typically remain asymptomatic in immunocompetent individuals[228]. Occasionally, individuals will present clinically with mild, generic symptoms, typically fever and lymphadenopathy[228]. The exception to the asymptomatic rule of acquired toxoplasmosis is ocular toxoplasmosis. Several recent studies in areas of high prevalence of *T. gondii* infection, namely Brazil and France, found several incidences of outbreaks of acute ocular toxoplasmosis in otherwise immunocompetent individuals[228]. These individuals, ranging in age from 10-57 years, presented with retinal lesions caused by uncontrolled parasite dissemination anywhere from 1 month to 3.5 years after initial infection[228]. Though the symptoms seen in these studies were severe, cases of ocular toxoplasmosis in immunocompetent individuals remain a relatively rare occurrence.

One of the most severe forms of infection with *T. gondii* occurs through congenital transmission, in which a pregnant woman becomes acutely infected with *T. gondii* sometime in her pregnancy, resulting in the parasite being transmitted to the fetus[228]. If transmission occurs early in the pregnancy, infection can result in severe symptoms such as fetal encephalomyelitis that causes spontaneous abortion or neonatal death, or fetal abnormalities including hydrocephalus, microcephaly, intracranial calcifications, or retinochoroiditis[228]. Overall, about 12-16% of early-stage congenital infections lead to death in newborns, while those that survive often present with chronic neurological deficiencies and intellectual disabilities[228]. If infection occurs later in pregnancy, however, the symptoms of congenital infection are often less severe. Infants infected in

the third trimester are often asymptomatic at birth, though they can go on to develop clinical symptoms, usually affecting the eyes (retinochoroiditis or blindness) or CNS (motor deficiencies, intellectual disability, or seizures)[228].

Though human infection typically remains asymptomatic, severe symptoms can arise if a *T. gondii*-infected individual ever becomes immunocompromised. In individuals receiving chemotherapy or radiation for cancer[230,231] or on immunosuppressive therapies following organ transplant[232-234], latent infection can become reactivated, leading to toxoplasmic encephalitis (TE) which is often fatal. The highly opportunistic nature of this pathogen, however, was highlighted during the peak of the acquired immunodeficiency syndrome (AIDS) epidemic in the 1980s-90s. Though much was still unknown about the cause of this epidemic at the time, it was observed in the clinic that many human immunodeficiency virus (HIV)-infected patients were presenting with neurological symptoms. These symptoms ranged from fever, headache, and seizures, to lethargy, dementia and coma[235-237]. In fact, it is estimated that TE led to the death of 10-30% of AIDS patients in the USA and Europe during these years[238]. On autopsy, areas of necrosis associated with free tachyzoites are noted, with very little immune reactivity, likely due to the severe immunodeficiency seen in the later stages of AIDS[238]. With the implementation of antiretroviral therapies and better screening processes for immunocompromised patients, the development of TE has, thankfully, decreased in recent years.

1.9.2 Mouse Model: Acute Infection and Priming of Immune Responses

In order to better understand the virulence, pathogenesis, and immune response to *T. gondii*, the mouse model is often used in the lab, as mice are a natural intermediate host for the parasite. Thanks to this model, much has been learned about the mechanisms involved in resistance to the parasite. As was alluded to in the previous sections, infection of the mouse, and other intermediate hosts, with *T. gondii* involves two distinct phases, an acute stage and a chronic stage. The acute stage is predominated by tachyzoites invading multiple peripheral tissues[239,240]. This initial infection induces a robust inflammatory response that will eventually clear the parasite from most peripheral tissues, however, the eventual conversion to bradyzoites and the formation of tissue cysts in muscle and CNS tissue establishes a chronic infection that is never completely cleared by the immune system, but requires ongoing immune surveillance and inflammation to keep the parasite in check[240].

How the parasite is initially recognized by the innate immune system has been a major area of study, though some questions still remain unanswered. Following infection with *T. gondii*, several populations of innate immune cells, including monocytes, neutrophils, and DCs, are recruited to infected tissues[241-244]. One of the most critical roles of the innate immune system in responding to *T. gondii* infection is the production of IL-12, which leads to a strongly polarized T_h1 response characterized by the production of IFNγ by NK and T cells[245-247]. The ability of these cells to sense the parasite is required for the downstream production of IL-12, along with other inflammatory cytokines that are important in shaping the early immune response to *T. gondii* infection. The early

observation that mice lacking the adaptor molecule MyD88 fail to produce IL-12 and IFNγ, cannot control parasite replication in peripheral tissues, and die early in infection[248], implicated Toll-like receptors (TLRs) in the recognition of *T. gondii* by innate immune cells. Several TLRs have been implicated in the recognition of *T. gondii* since this discovery. Glycophophatidylinositols (GPIs) derived from *T. gondii* have been shown to activate surface TLRs 2 and 4, though single or double knockouts of these receptors does not result in decreased inflammatory cytokine production or increased susceptibility to infection[249]. Additionally, TLRs 7 and 9 have been implicated in the recognition of parasite RNA and DNA, respectively, though, once again, double knockouts are not more susceptible to infection and do not recapitulate the phenotype of MyD88-deficient mice following acute infection[250]. The discovery that TLR11 is activated in response to parasite-derived profilin, and is required for IL-12 production, suggested that it may be the major innate mechanism conferring innate recognition of *T. gondii*[251]. The observation, however, that despite failing to make IL-12, the survival of TLR11-deficient mice in response to *T. gondii* infection is not impaired[251], added further confusion to the field. It was subsequently shown that TLR12 alone could also recognize parasite profilin in plasmacytoid DCs (pDCs), but that TLR11/TLR12 heterodimers were involved in other innate immune cells' recognition of profilin[252] (Figure 1.7). Interestingly, while TLR11 is not required for survival, TLR12-deficient mice acutely succumb to *T. gondii* infection, similar to MyD88-deficient mice[252]. Further study revealed that, while both TLR11 and TLR12 can bind parasite profilin, and each is required for IL-12 production, only TLR11 directly recruited MyD88 to the TLR complex[253]. Overall, there is consensus that both TLR11 and TLR12 are important for

early recognition of *T. gondii* and innate cytokine responses[248,250-253]. However, several key questions remain: 1) How does TLR12 contribute to IL-12 production and innate resistance in the absence of interaction with MyD88? 2) Why are TLR11-deficient mice not more susceptible to *T. gondii* infection despite severely decreased IL-12 production? and 3) How might the other TLRs activated in response to *T. gondii* play a role in early innate responses in conjunction with TLR11 and TLR12?

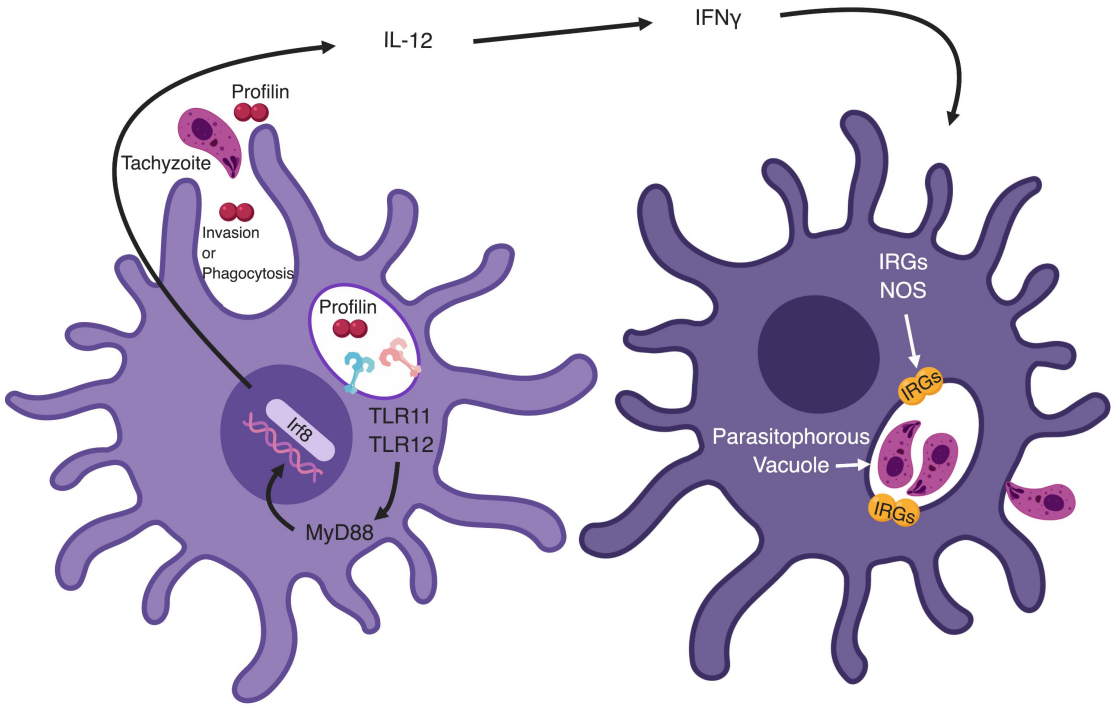


Figure 1.7. Innate recognition and response to *T. gondii* infection. Active infection or phagocytosis of the parasite (or parasite-derived products) by innate immune cells can lead to recognition through TLRs. In mice, TLR11 and TLR12 are the most well characterized members of the TLR family that are known to recognize profilin produced by the parasite, though other TLRs might also play a role in early recognition. TLR recognition leads to activation of MyD88 and Irf8, which ultimately results in IL-12 production. This IL-12 production induces potent IFNγ from NK and T cells. This IFNγ can then induce anti-parasitic mechanisms within infected cells, such as NO production and recruitment of immune-related GTPases (IRGs) to the parasitophorous vacuole, both of which can aid in destruction of the parasite.

It is also interesting to note that, while TLR11 and TLR12 play important roles in *T. gondii* recognition and early immune responses in mice, in humans, TLR11 is a pseudogene and TLR12 does not exist in the human genome[254]. For obvious reasons, studying the early responses to *T. gondii* infection in humans is difficult, but a handful of studies have provided key insight into beginning to understand human innate responses to the parasite. One study mapped "susceptibility" genes involved in the development of congenital toxoplasmosis, finding that the gene for the inflammasome component NALP1 was significantly associated with the development of congenital toxoplasmosis[255]. NALP1 knockdown in human-derived monocytes led to the acceleration of cell death and the inability of these cells to produce IL-1β, IL-18, and IL-12[255]. Additional evidence for the importance of inflammasome sensing in human infection came from another study implicating the recognition of parasite protein GRA15 in induction of IL-1β from human monocytes[256]. Knockdown of inflammasome adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) or inhibiting caspase-1 activity abrogated IL-1β production[256], further suggesting that inflammasome activation occurs in response to *T. gondii* infection and is important in the early inflammatory cytokine production in human monocytes. There is some evidence that the inflammasome is involved in innate sensing of *T. gondii* in the mouse as well. Several studies have found that inflammasomes NLRP1 and NLRP3 are activated *in vivo* in mice in response to *T. gondii* infection and are important for IL-1β and/or IL-18 production[257,258]. It was also shown that NLRP3-, ASC-, and caspase 1/11- KO mice, in addition to producing less IL-1β and IL-18, were also more susceptible to infection, as all died acutely[257]. Overall, these studies show important aspects of how the early innate

response to *T. gondii* is regulated in mice and humans, but also highlights how much is left to be uncovered in both species.

As was mentioned above, likely the most important innate-derived cytokine downstream of parasite recognition is IL-12[245-247]. Many studies have tried to determine the primary innate cell type responsible for production of IL-12, and have suggested neutrophils[259], monocytes and macrophages[244,247,260], and dendritic cells[248,251,261,262]. Of these, CD8α+ dendritic cells seem to be the most important contributor, as in studies where CD11c+ cells were depleted before infection, or studies using Batf3 knockout mice that lack CD8α+ DCs, IL-12 production was abolished and mice succumbed during the acute infection, and were rescued with transfer of WT DCs or IL-12[261,262]. Inflammatory monocytes, though not the critical source of IL-12, have still been implicated in host resistance during *T. gondii* infection, as CCR2-deficient mice have increased susceptibility after challenge[244,260,263]. It is thought that an important function of these monocytes/macrophages is destruction of the parasite through nitric-oxide (NO) production, which has been shown to inhibit parasite replication in in inflammatory monocytes *in vitro*[264-266]. Inflammatory monocytes have been shown to express inducible nitric oxide synthase (iNOS) during infection with *T. gondii*[263,265], though this cannot be their only mechanism contributing to host defense, as iNOS-deficient mice survive the acute stage of infection, while monocyte deficient mice do not[267,268]. Likely, the role of monocytes and macrophages during infection is wide-ranging, as they produce inflammatory cytokines, like IL-6, IL-1, and TNFα, that contribute to shaping early adaptive responses, as well as having roles in direct parasite killing, like production of NO[240].

Another fairly well characterized anti-parasitic mechanism utilized by macrophages is the activity of immune-related GTPases (IRGs). To date, Irgm3, Irgm1, Irgd, Irga6, and Irgb6 have all been shown to be involved in the innate host response to *T. gondii* infection[269-274]. The importance of this NO-independent mechanism of *T. gondii* resistance was emphasized in a study using mice deficient in Irgm3 who, though they had normal IL-12 and IFNγ responses, failed to control parasite and succumbed to infection acutely[273]. Though the exact mechanisms by which IRGs help in killing the parasite are still being elucidated, it appears that they can be recruited to the parasitophorous vacuole, leading to its destruction and either egress of the parasite or release of the parasite into the cytosol to be killed[275,276]. Additionally, machinery involved in autophagy has also been implicated in host resistance. Mice lacking myeloid expression of autophagy proteins Atg3, Atg5, Atg7, or Atg16L1 are all unable to control parasite replication and dissemination, succumbing early in the acute phase of infection[277-279]. Many of these Atg-deficient mice were unable to recruit IRGs to the parasitophorous vacuole in response to IFNγ, rendering them unable to control parasite replication[277,278]. All of the above studies show how important the innate immune response is in initial recognition of the parasite and parasite killing during the acute infection, but as previously discussed in Section 1.2, the other essential role of the innate response is to shape a strong adaptive immune response.

The absolute importance of the adaptive immune response to *T. gondii* has been demonstrated in a number of mouse models in which defects in B cells, or in CD4+ or CD8+ T cells exist. As mentioned above, the adaptive response to *T. gondii* is heavily T_h1-polarized, where IL-12 production early is required for IFNγ production from T

cells[247,248,262]. In the absence of IFNγ production, mice rapidly succumb to overwhelming parasite burden in peripheral tissues, dying early in the acute phase of infection[280,281]. IFNγ production has been ascribed to NK cells as well as CD4+ and CD8+ T cells during the acute infection[240,245,282,283], and is important for upregulation of many of the innate anti-parasitic mechanisms discussed above[254,264,268,269,271-276,279,284].

The priming of the T cell response to *T. gondii*, much like any other response to pathogen, involves presentation of parasite-derived antigen on MHCI or MHCII, costimulatory signals, and cytokine recognition. After initial infection with *T. gondii*, DCs expand and become activated, able to present antigen and induce proliferation of parasite-specific T cells[285-287]. This, coupled with costimulation through CD28 (or other costimulatory signals such as ICOS, as previously discussed) and IL-12 production lead to the activation, expansion, and T_h1-mediated effector functions of T cells during *T. gondii* infection. Interestingly, mice lacking members of the adaptive immune system (CD4+ and CD8+ T cells, and B cells), though they are more susceptible to *T. gondii* infection, do not succumb until the chronic phase of infection (discussed in Section 1.9.3), emphasizing the importance of early innate inflammatory responses for controlling parasite in peripheral tissues and promoting host survival acutely[240].

As was discussed in Sections 1.5 and 1.7.2, regulation of immune responses also plays an extremely important role during the course of infection, and infection with *T. gondii* is no different. Clearly T_h1-mediated inflammation is required for parasite control and host survival, but several regulatory mechanisms have also been identified in the course of *T. gondii* infection that are just as crucial for survival. As has been seen in other models of infection, IL-10 is an important mechanism of suppressing immune-mediated

pathology during acute *T. gondii* infection. IL-10-deficient mice die early in the acute infection, showing uncontrolled overproduction of IL-1β, IL-12, TNFα, and IFNγ that, despite leading to more efficient parasite clearance, resulted in CD4+ T cell-mediated lethal pathology in peripheral tissues[288]. Though IL-10 production during acute *T. gondii* infection has been attributed to both innate immune cells and adaptive immune cells, specific loss of IL-10 production in CD4+ T cells resulted in severe immunopathology and was acutely lethal, reminiscent of total IL-10 deficient mice[289] and suggestive that T cells are the critical source of IL-10. The role of IL-10 in the chronic phase of infection in the brain is further discussed in Chapter 4.

Similarly, IL-27 signaling is essential for regulation of early inflammatory responses to *T. gondii*. IL-27R deficient mice also die during acute infection from a CD4+ T cell-mediated immunopathology in peripheral tissues associated with increased IL-12 production, increased T cell activation and proliferation, and increased IFNγ production[290]. It was subsequently shown that IL-27 signaling is important for induction of IL-10 production in T cells, which limits inflammatory cytokine production[291]. Additional mechanisms of regulation have been more difficult to study during *T. gondii* infection. In trying to study the role of T_{regs}, mice acutely infected with *T. gondii* were treated with IL-2 complexes, boosting T_{reg} numbers during early infection[292]. Though the increase in T_{reg} number correlated with a rescue of lethal immunopathology in the liver and gut, increased parasite cyst burden in the brain during later stages of infection was observed[292], highlighting once again that T_{regs} seem too be involved in maintaining a balance between inflammation that controls pathogen, and immune suppression that limits immunopathology (Figure 1.8).

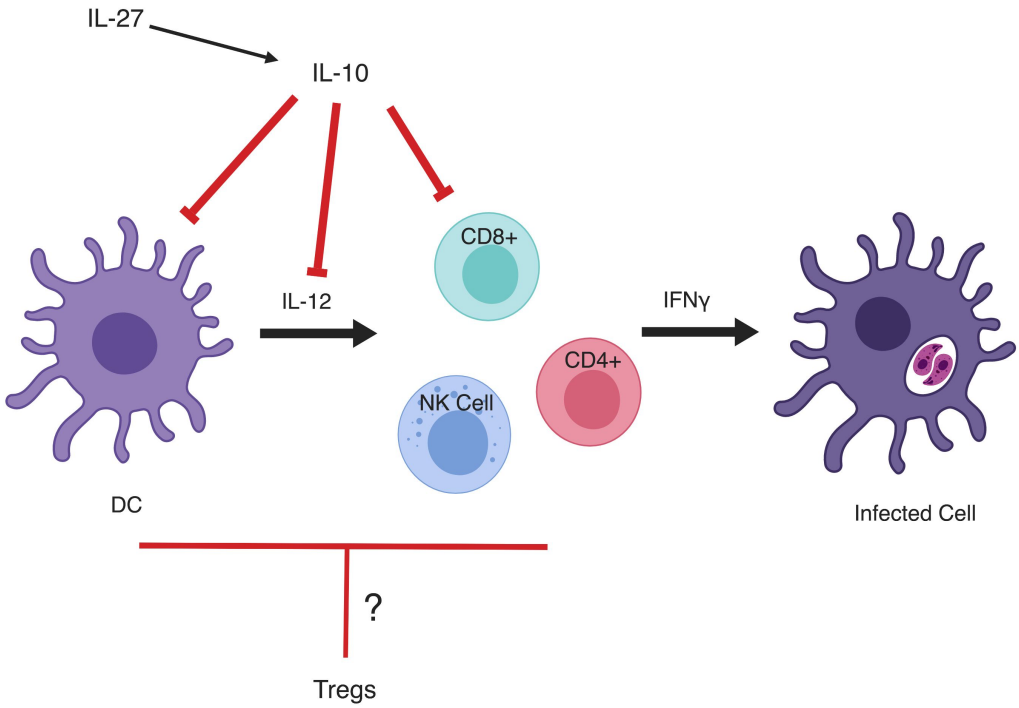


Figure 1.8. Mechanisms of regulation during infection with *T. gondii*. IL-10 is absolutely required for survival during acute stages of *T. gondii* infection, and lack of this cytokine is associated with increased production of inflammatory cytokines such as IL-12 and IFNγ, increased T cell proliferation, and exacerbated tissue pathology. Similarly, IL-27 is required for survival, and has been shown to limit effector T cell responses by inducing production of IL-10. T_{regs} likely play additional roles in regulation during *T. gondii* infection, though currently their exact contribution and the mechanisms by which they control inflammatory responses during the infection remains somewhat unclear.

Overall, the immune response to *T. gondii* infection has been fairly well characterized since its discovery in 1908. There have been many advances in our understanding of the molecular mechanisms involved in parasite recognition, innate and adaptive inflammatory responses to the parasite, and important regulatory mechanisms involved in promoting host survival during this infection. Despite these advances, however, many questions remain to answered in the field of host-pathogen interaction during *T. gondii* infection, particularly in the chronic phase of infection in the CNS, as is discussed in the following section.

1.9.3 Mouse Model: Chronic Infection and The Ongoing Immune Response

As previously discussed, the acute phase of infection with *T. gondii* induces a robust immune response that eventually clears parasite from most peripheral tissues[239]. But with the transition to the chronic phase of infection (~28 days post-infection), where parasite is largely restricted to the CNS, a continued inflammatory response, particularly supported by T cells, is required for continued pathogen control (Figure 1.9). Throughout the course of the chronic infection, peripheral immune cells (largely T cells, monocyte/macrophages, and DCs) will continuously infiltrate the normally "immune-privileged" CNS (Figure 1.9 and Figure 1.10), where they can carry out effector function to limit reactivated parasite dissemination throughout the brain[240]. The requirement for continued T cell responses in controlling chronic infection in the brain was first underscored by the resurgence of toxoplasmic encephalitis in AIDS patients in the 1980s[235,237,238]. In the late stages of the disease, when T cell numbers were severely low, many AIDS patients presented with neurological symptoms, and *T. gondii* DNA could be

detected in the cerebrospinal fluid (CSF)[293,294]. A similar phenotype is seen in the mouse model, as when chronically infected mice are depleted of both CD4+ and CD8+ T cells, they rapidly succumb to the infection, unable to control parasite reactivation and dissemination within the brain[295]. Similarly, depletion of IFNγ during the chronic phase of infection also rapidly results in death due to uncontrolled parasite dissemination in the brain[295]. These results implicated T cell production of IFNγ as crucial to maintain control of reactivated parasite in the brain and promote host survival in the chronic phase of the infection.

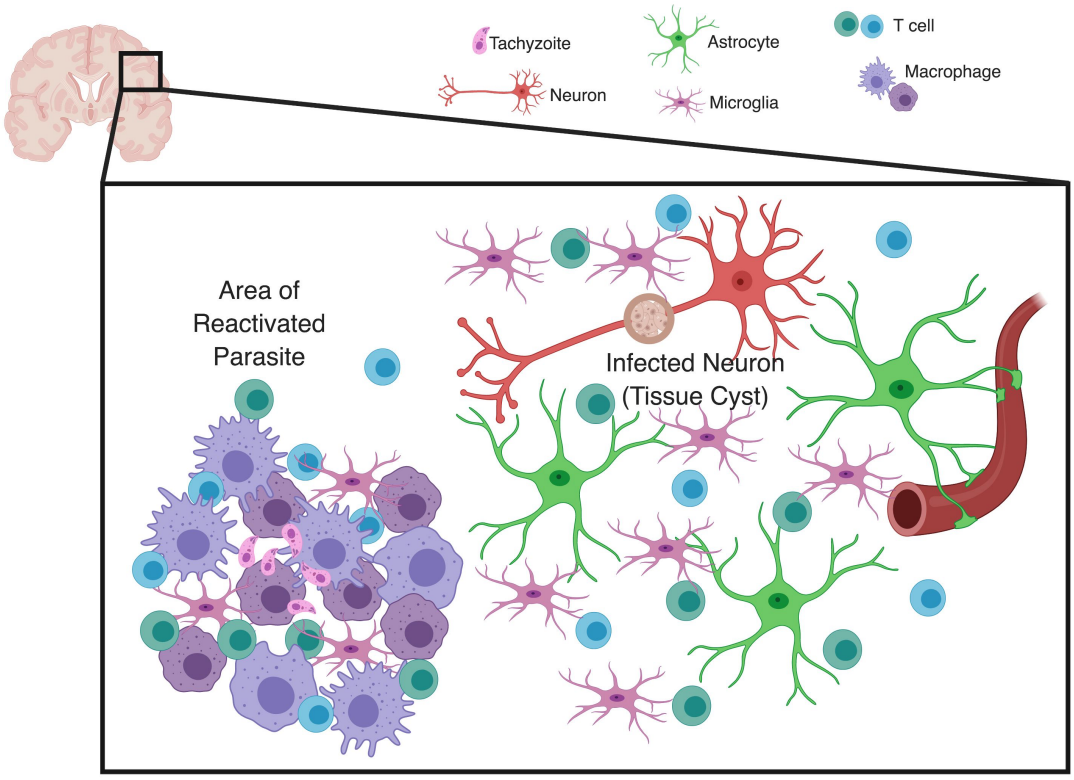


Figure 1.9. Continued immune responses in the CNS during chronic *T. gondii* infection. The chronic stage of *T. gondii* infection is characterized by the presence of latent tissue cysts, which are primarily found in neurons. It is thought that these latent tissue cysts are not actively recognized or destroyed by the surrounding immune response in the tissue; however, during the course of chronic infection, tissue cysts can stochastically "reactivate", leading to their conversion back to a fast-replicating tachyzoite. This reactivation and subsequent tissue damage is rapidly recognized by innate immune cells that can then limit further parasite dissemination within the brain.

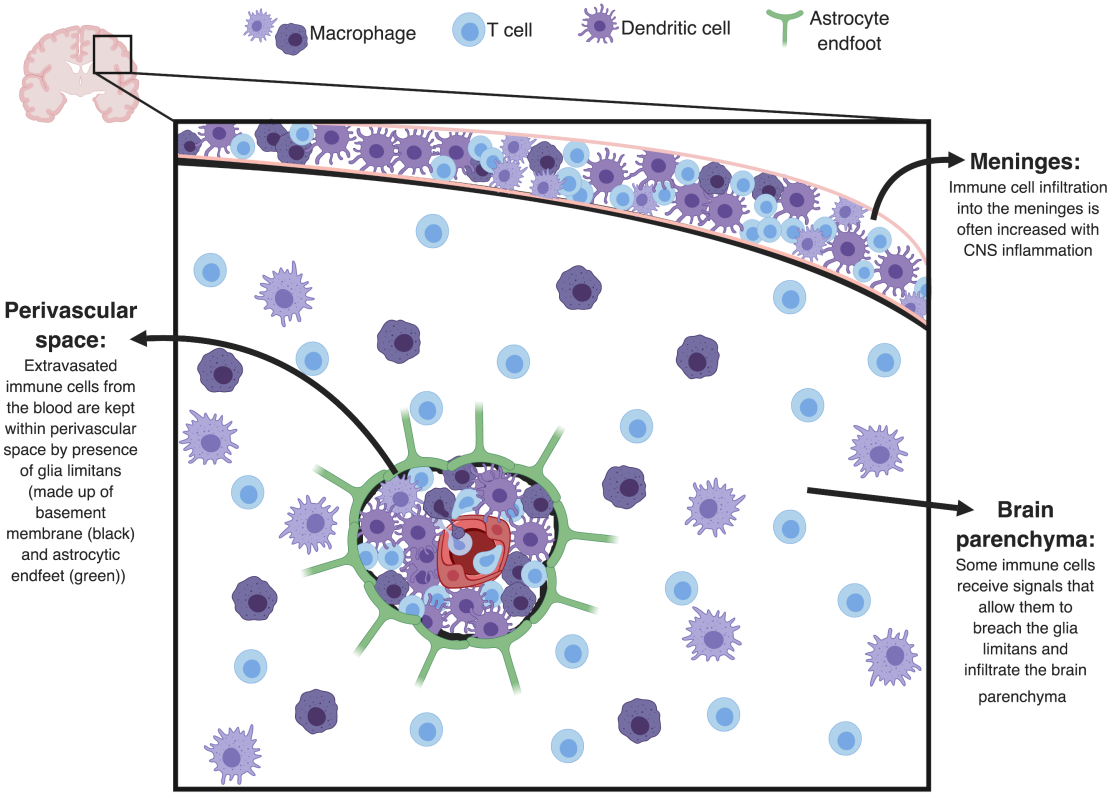


Figure 1.10. Immune cell infiltration into distinct CNS compartments during chronic infection with *T. gondii*. With parasite dissemination to the CNS during chronic infection, many different types of immune cells are recruited to the CNS tissue to maintain parasite control. In a naïve state, the meningeal compartment is the only CNS compartment that contains peripherally-derived immune cells, and recruitment to this compartment is amplified during chronic *T. gondii* infection, including increased numbers of monocyte/macrophages, dendritic cells, and T cells. During chronic infection, the *T. gondii* parasite is found largely within neurons in the brain parenchyma (see Figure 1.9). To control reactivated parasite in the brain parenchyma, peripheral immune cells are recruited to this normally "immune-privileged" site. It is thought that the vast majority of immune cells found in the brain parenchyma have infiltrated via the vasculature. Once circulating immune cells have extravagated from blood vessels, they encounter an additional barrier before infiltration in to the brain parenchyma itself. This barrier, called the glia limitans, consists of a basal lamina layer and astrocytic endfeet that "hold" these newly extravasated cells in this space. Though the exact signals required to breach this second barrier are currently unknown, many immune cells (mostly monocyte/macrophages and effector T cells) are able to fully infiltrate the brain parenchyma and contribute to parasite control. Interestingly,

unlike effector T cells and macrophages, $MHCII^{hi}CD11c^{+}$ dendritic cells are largely kept in the meningeal space and perivascular spaces (also shown in Chapter 3).

In light of these findings, it is interesting to consider that both CD4- and CD8-deficient mice survive the acute infection but succumb in the chronic infection[296,297]. This is likely due to NK cell production of IFNγ early in the infection that can compensate for the lack of T cells[245,283]. Determining whether CD4+ or CD8+ T cells are the more critical cell type in the chronic infection has been difficult. While depletion of both populations in the chronic phase is lethal, single depletion of CD8+ T cells only results in about 50% mortality, and depletion of CD4+ T cells does not increase the rate of mortality at all[295]. These results could suggest that CD8+ T cells are more important in the chronic phase, and can compensate for the loss of CD4+ T cells, but this conclusion could be clouded by the short duration of treatment or incomplete depletion when compared to the 100% lethality seen in the genetic knockouts. On the other hand, these data could also suggest that CD8+ T cells have another critical function in controlling *T. gondii* reactivation in the chronic phase, other than production of IFNγ. Perforin-mediated cytolysis of infected cells is a major mechanism of CD8+ T cell defense[298]. Interestingly, perforin-deficient mice have somewhat better survival in the chronic phase than total CD8 knockouts, about 50% mortality by day 150 post-infection in the perforin knockouts versus 100% mortality by day 50 post-infection in the CD8 knockouts, though both have significantly higher parasite burden in the brain and worse survival than WT mice[296]. These results suggest that both IFNγ production and perforin-mediated cytolysis contribute to parasite control during chronic infection.

Though CD4+ T cells clearly contribute to IFNγ production during the chronic phase[240], much of their role has been shown to be providing "help" for B cell and CD8+ T cell responses. CD4-deficient mice die around day 40 post-infection, well into the

chronic phase, with increased parasite burden in the brain but relatively normal IFNγ responses[297]. The increased parasite burden was associated with low parasite-specific IgG titers, suggesting that CD4+ T cell help is necessary for generating optimal antibody responses during *T. gondii* infection[297]. Interestingly, chronically infected CD4 knockout mice given serum from *T. gondii*-immunized mice survived longer into the chronic infection (~day 70 post-infection)[297], further supporting the idea that CD4+ T cells play an important role in mediating parasite control by promoting anti-parasite antibody responses. Additionally, the importance of humoral immune responses to *T. gondii* is highlighted in studies using μMT mice that lack B cells. Similar to CD4 knockout mice, these mice die in the chronic phase of infection with high parasite burden but otherwise normal cytokine production, and, like CD4 knockout mice, μMT mice can also be rescued with transfer of parasite-specific antibody[299]. It is important to note, however, that CD4 knockout mice given immune serum, though their survival is extended, still eventually die in the chronic phase, suggesting that there could be additional roles for the CD4+ T cell population at this stage in infection. CD4+ T cells can also provide "help" for CD8+ T cells. Interestingly, *T. gondii* infection of CD4 knockout mice induced a normal CD8+ T cell response, but these mice were not able to sustain the response into the late stages of chronic infection[300]. By 180 days post-infection, CD4 knockout animals had decreased CD8+ T cell responses, shown by cytolysis assay and IFNγ production, and transfer of CD8+ T cells from CD4 knockout mice infected for 180 days could not protect recipient mice from lethal infection, while transfer of CD8+ T cells from CD4 knockout mice infected for only 90 days could[300]. These results, coupled with the human data from AIDS patients with toxoplasmic encephalitis, begin to paint a picture of the

complex dynamics of the T cell response to chronic *T. gondii* infection. Though production of IFNγ from the CD4+ T cell population might not be absolutely required in the chronic phase, CD4+ T cells are required to maintain as strong effector CD8+ T cell response, which could explain why toxoplasmic encephalitis is only seen in late-stage AIDS patients whose CD4+ T cell numbers are so low that they likely have also begun to lose a strong CD8+ T cell response.

Innate responses must also be maintained during the course of the chronic infection. Though our understanding of the innate response during chronic *T. gondii* in the CNS is not as well understood as during the acute response, some of the same anti-parasitic mechanisms remain in use during the chronic phase of infection. IL-12, for example, continues to be made primarily from brain-infiltrating DCs during the chronic phase of infection (see Chapter 4), though there is not a consensus on whether this continued IL-12 production is necessary to maintain a strong T_h1 response in the chronic phase. Early results showed that chronically infected mice depleted of IL-12 beginning 28 days post-infection had no survival defect out to 40 days post-infection[247], suggesting that IL-12 is essential for the induction of T_h1 responses but is not required for the maintenance of this response once established. These results also could have been due to incomplete depletion of IL-12 in the chronic phase, so the same group went on to use a genetic paradigm to address this same question. In these subsequent studies, IL-12-deficient mice were given exogenous IL-12 for the first 14 days of infection to allow for the establishment of a normal T_h1 response acutely[301]. After ceasing IL-12 treatment, it was found that the short term survival of the mice was not affected, as all survived out to day 30 post-infection, suggesting that IFNγ responses can be retained for at least a short time

frame in the absence of IL-12[301]. However, monitoring survival for a longer period revealed that all the IL-12 deficient mice had died by day 60 post-infection with severely decreased IFNγ responses and increased parasite burden in the brain[301]. These results suggest that IL-12 is required for not only the establishment of IFNγ responses, but must also be maintained throughout the chronic infection to support continued effector T cell responses and IFNγ production that facilitates parasite control. This conclusion was additionally supported by the finding that resumption of IL-12 treatment to IL-12-deficient mice between days 31-42, when they still did not show obvious signs of disease, increased IFNγ production from antigen-stimulated T cells *ex vivo* and significantly decreased parasite burden in the brain[301].

Besides IL-12, CCR2-expressing monocytes themselves have been shown to be especially important in the chronic stage of infection. CCR2 knockout mice survive the acute stage of infection where they have relatively normal inflammatory responses, but die around day 28 post-infection with decreased iNOS production in the CNS and high parasite burden[263]. These results could be somewhat complicated by the fact that CCR2 knockout mice fail to release CCR2+ monocytes into the blood from the bone marrow in the first place[302,303], so exactly what role recruitment of CCR2+ monocytes plays in controlling parasite in the brain remained an open question. To better address this, another group showed that WT mice given a CCR2 blocking antibody during the chronic phase of infection had increased parasite burden in the brain and all succumbed to infection between days 40 and 50 post-infection[304]. These results strongly implicate that continued recruitment of CCR2-expressing monocytes from the blood is required to maintain control of chronic *T. gondii* infection in the CNS.

The importance of some effector mechanisms utilized by the innate immune system in the acute stage of infection, such as IRGs, have not been addressed in the chronic stage. Interestingly, however, iNOS production seems to be exclusively required in the chronic, CNS stage of the infection. As previously discussed, iNOS is induced in myeloid cells downstream of IFNγ signaling, and has been implicated in playing an important role in controlling *T. gondii* infection. Interestingly, iNOS-deficient mice survive the acute stage of infection and efficiently clear parasite from peripheral tissues, but succumb in the chronic stage with overwhelming parasite burden in the brain[268]. Similarly, chronically infected WT mice treated with an iNOS inhibitor beginning on day 35 post-infection, also succumbed due to the inability to control parasite replication and dissemination in the brain[305]. Additionally it was found that, though iNOS deficient mice had somewhat higher parasite burden in peripheral tissues during the acute stage of infection, they actually had less associated immunopathology in those tissues, suggesting that iNOS can contribute to parasite control during the acute stage, but also can contribute to immune-mediated pathology[267]. Overall, these results suggest that iNOS can aid in parasite control throughout the course of infection with *T. gondii*, but is only required for survival in the chronic, CNS stage of infection, while it actually contributes to immunopathology in peripheral tissues early in infection.

Finally, one other important anti-parasitic factor that has been identified to be crucial in the chronic stage of infection but dispensable in the acute stage is TNFR signaling. Mice lacking TNFR1/2 largely survive the acute infection but succumb 3 to 4 weeks post-infection[306,307]. These mice have largely intact IFNγ and iNOS responses, suggesting that TNF signaling in the chronic stage of infection promotes parasite control in a distinct

manner than either of these two effector mechanisms[306,307]. The anti-parasitic activity of TNF signaling was linked specifically to signaling downstream of TNFR1, while TNFR2 was dispensable for protection[306], and both TNF and another ligand of TNFR1, lymphotoxin-α (LTα), were shown to be essential for TNFR-mediated parasite control in the chronic infection[308]. Overall, the different mechanisms required to maintain control of *T. gondii* reactivation in the brain during the chronic stage of infection are only beginning to be unraveled. Additionally, though we understand several mechanisms of regulation in the acute response to *T. gondii* that are essential for protection from immunopathology, much work needs to be done to understand how the chronic inflammatory response to *T. gondii* in the brain is regulated. The topic of regulation of these chronic immune responses, and its role in preventing immunopathology in the CNS, is discussed further in Chapters 3, 4, and 6.

1.10 Project Rationale

As discussed above, our understanding of the regulatory mechanisms required to limit immunopathology during an ongoing inflammatory response are largely unknown. *Toxoplasma gondii* establishes a chronic infection in the CNS that requires ongoing peripheral myeloid and T cell infiltration into the brain to control reactivated parasite dissemination and promote host survival. Studies using genetic knockouts for immunosuppressive cytokines have made it clear that the ongoing inflammatory response, though essential for parasite control, cannot go completely unchecked, as mice lacking these cytokines rapidly succumb to CD4+ T cell-mediated immunopathology[97,291,309-311]. The overall goal of this work was to elucidate mechanisms that control the ongoing inflammatory immune response, particularly at the major site of

inflammation in the chronic stage of infection: the CNS. To address this goal, we first characterized the role of T_{regs}, known to be an essential source of immunosuppression in many models[33,55,56,312]. To this end, we began by characterizing the T_{reg} population in the brain during chronic infection, including surface marker expression, cytokine production, TCR clonality, and localization within the CNS. We hypothesized that T_{reg}-DC contact, as discussed in Section 1.4, would play an important role in maintaining the T_{reg} population in the brain, thereby allowing for this population to carry out suppressive function. Hence, we investigated the role of adhesion molecule LFA-1 and TCR:MHCII in the maintenance of T_{reg}-DC contacts in the CNS (Chapter 3). To understand what other mechanisms might be contributing to regulation of the chronic immune response during *T. gondii* infection, we also characterized the role of IL-10, a widely studied immunosuppressive cytokine[126-128], and ICOS, which has been shown to play both pro- and anti-inflammatory roles during inflammation[24,68,175]. We hypothesized that IL-10 would have systemic effects on suppression of immune responses, both at the site of inflammation in the brain and in other peripheral tissues, while ICOS, which is exclusively expressed on activated effector T cells and T_{regs}[24,68,175], could play a role in limiting immune responses solely at the major site of inflammation in the brain. On the other hand, ICOS has also been reported to have pro-inflammatory effects in some models of inflammation[24,68,175]. In these studies, we wanted to determine if ICOS costimulation supported the inflammatory T cell response in the brain, or contributed to the regulation of this response. To determine this, we used both an ICOSL blocking antibody to prevent ICOS signaling solely in the chronic phase of infection (Chapter 4), as well as mice deficient in ICOS or mice lacking ICOS-mediated PI3K signaling

(Chapter 5). Overall, the main goal of the studies presented in this work aimed to characterize three potential mechanisms of regulation of chronic immune responses in the CNS.

CHAPTER 2

MATERIALS & METHODS

2.1 Mice and Infection Model

C57BL/6, CD11c-YFP, actin-CFP, and B6.129S6-*Il10*^{tm1Flv}*/J* (*Tiger*) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Foxp3-GFP were originally obtained from Vijay Kuchroo of Harvard University and crossed to the CD11c-YFP strain. IL-10eGFP reporter (*VertX*) mice were obtained from Christopher Karp of Cincinnati Children's Hospital. ICOS KO and ICOS Y^{181}F mice were obtained from Daniel Campbell of the University of Seattle, Washington. All procedures were performed in accordance to the guidelines of the University of Pennsylvania and University of Virginia Institutional Animal Care and Use Committee. Infections used avirulent type II *Toxoplasma gondii* strains Pru or Me49. Ovalbumin-expressing Prugnauid strain parasites expressing Tomato fluorescent protein (PruOVA^{TOM}) were generated as previously described [116] and maintained by serial passage in human fibroblast (HFF) cell monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Me49 was maintained in chronically infected Swiss Webster mice (Charles River) and passaged through CBA/J mice (Jackson Laboratories) before experimental infections. Prior to infections, Pru parasites were purified from HFF culture by filtration through a 5.0μm filter (Nucleopore, Clifton, NJ), or the brains of Me49 chronically infected (4 to 8 weeks) CBA/J mice were homogenized to isolate tissue cysts. Mice were then intraperitoneally infected with either 10^3 Pru tachyzoites or 10 to 20 Me49 cysts.

2.2 Antibody Blockade Treatments

For LFA-1 blockade studies, chronically infected mice (28-35 days post-infection) were treated intraperitoneally with either 200 μg of an α-LFA-1 antibody

(BioXCell) or normal rat IgG (Sigma) four hours prior to intravital imaging or flow cytometry analysis. For MHCII and CXCR3 blockade studies, chronically infected mice were treated with 200μg of an α-MHCII or α-CXCR3 antibody (BioXCell) or control normal rat IgG (Sigma) four hours prior to intravital imaging.

For IL-10R blockade studies, chronically infected mice were treated intraperitoneally with 200 μg of a monoclonal antibody blocking the IL-10R (BioXCell) or a control rat IgG antibody (Sigma). Treatments were given every 3 days, and mice were euthanized when neurological symptoms developed between 7-10 days post-antibody treatment. For ICOSL blockade studies, chronically infected mice were treated intraperitoneally with 150 μg of an α-ICOSL (CD275) blocking antibody (BioXCell) or a control rat IgG antibody (Sigma). Antibody treatments were given every 3 days for 14 days, totaling 5 treatments.

2.3 Tissue and Blood Processing

Mice were sacrificed and perfused with 40 mL 1X PBS, and perfused brains, spleens, and lymph nodes (pooled deep and superficial cervical) were put into cold complete RPMI media (cRPMI) (10% fetal bovine serum, 1% NEAA, 1% pen/strep, 1% sodium pyruvate, 0.1% β-mercaptoethanol). Brains were then minced with a razor blade and enzymatically digested with 0.227mg/mL collagenase/dispase and 50U/mL DNase (Roche) for 1 hour at 37°C. After enzyme digestion, brain homogenate was passed through a 70μm filter (Corning). To remove myelin, filtered brain homogenate was resuspended with 20 mL 40% percoll and spun for 25 minutes at 650g. Myelin was then aspirated and the cell pellet was washed with cRPMI, then resuspended and cells were counted.

Spleens and lymph nodes were homogenized and passed through a 40μm filter (Corning) and pelleted. Lymph node cells were then resuspended in cRPMI and counted. Spleen cells were resuspended with 2 mL RBC lysis buffer (0.16 M NH_4Cl). Following RBC lysis, spleen cells were washed with media and then resuspended with cRPMI for counting.

For experiments in which peripheral blood was taken, mice were sacrificed and the right atrium was cut in preparation for perfusion. Before perfusion, 300μL blood was collected from the chest cavity. For isolation of circulating leukocytes, collected blood was put in 1mL heparin (100 USP/mL) to prevent clotting. Samples were then pelleted and resuspended in 2 mL RBC lysis buffer for 2 minutes. Samples were washed once with cRPMI and a second RBC lysis step was performed. Finally, blood cells were resuspended in cRPMI for staining and counting. For serum isolation, blood was allowed to clot overnight at 4°C. Samples were then spun at 14,000 rpm for 10 minutes to separate clotted blood from serum. After spinning, serum (supernatant) was transferred to a clean tube and stored at -80°C.

2.4 Flow Cytometry

Single cell suspensions from collected tissues were plated in a 96-well plate. Cells were initially incubated with 50μL Fc block (1μg/mL 2.4G2 Ab (BioXCell), 0.1% rat gamma globulin (Jackson Immunoresearch)) for 10 minutes at room temperature. Cells were then surface stained for CD11a (M17/4), CD3 (145-2C11), CD19 (eBio1D3), NK1.1 (PK136), ICOS (7E.17G9), ICOSL (HK5.3), MHCII (M5/114.15.2), CD25 (PC61), CD8 (S3-6.7), CD11c (N418), CD4 (GK1.5), CD80 (16-10A1), CD86 (GL1), CD45 (30-F11), CD11b (M1/70), Ly6G (1A8), and a live/dead stain for 30 minutes at

4°C. Parasite-specific cells were identified using a PE-conjugated MHCII tetramer (AVEIHRPVPGTAPPS) (National Institutes of Health Tetramer Facility). After surface staining, cells were washed with FACS buffer (1% PBS, 0.2% BSA, and 2mM EDTA) and fixed at 4°C overnight with a fixation/permeabilization kit (eBioscience) or 2% PFA. Following overnight fixation, cells were permeabilized and stained for intracellular markers Bcl-2 (3F11), Ki67 (SolA15), and Foxp3 (FJK-16S) for 30 minutes at 4°C. Cells were then washed, resuspended in FACS buffer, and run on a Gallios flow cytometer (Beckman Coulter). For Annexin V staining, surface staining of cells was performed as above, with the addition of a nuclear stain (Hoechst 33342, Life Technologies). Following surface staining, cells were washed with Annexin V binding buffer (BD Biosciences) and incubated with Annexin V-FitC (BD Biosciences) diluted in Annexin V binding buffer for 15 minutes at room temperature. Cells were then immediately run on a Gallios flow cytometer. Analysis was done using Flowjo software, v.10.

2.5 Cytokine Restimulation

Single cell suspensions were plated into a 96-well plate. For T cell cytokine restimulation, cells were incubated at 37°C with 20 ng/mL PMA and 1 µg/mL ionomycin (Sigma) in the presence of brefeldin A (Alfa Aesar). After incubation for 5 hours, cell suspensions were washed, surface stained, and fixed. Cells were then incubated with an antibody against IFNγ (XMG1.2) for 30 minutes at 4°C. To determine IL-10 production, either *VertX* or *Tiger* mice expressing eGFP under the IL-10 promoter were used. Cells from these mice were stimulated with PMA/ionomycin and stained for surface molecules as described above, then lightly fixed with 2% PFA for 30 minutes at room temperature. Cells were then permeabilized with permeabilization buffer

(eBioscience) and incubated with a biotin-conjugated α-GFP antibody (BD Biosciences) for 30 minutes at 4°C. Cells were then washed and incubated with a PE-conjugated streptavidin secondary antibody (eBioscience) for 30 minutes at room temperature. Cells were then fixed with a fixation/permeabilization kit (eBioscience) overnight at 4°C before other intracellular staining was performed. For myeloid cell cytokine restimulation, single cell suspensions were plated into a 96-well plate and incubated with brefeldin A for 5 hours at 37°C before surface and intracellular staining for IL-12 (C17.8) (eBioscience).

2.6 TCR Sequencing and Analysis

Immune cells were isolated from the meninges of *T. gondii* infected mice as previously described[313]. Cells were stained with antibodies against CD3, CD4, and Foxp3 as described above and sorted on a Becton Dickinson Infux Cell Sorter. DNA from $Foxp3^-$ and $Foxp3^+$ $CD4^+$ T cells was purified using a Qiagen DNA Micro Kit. The TCR-β CDR3 regions were sequenced with Immunoseq Assay from Adaptive Biotechnologies (Seattle, WA).

The TCR-β CDR3 sequences obtained were analyzed in the following ways: First, the presence/absence of amino acid sequences in the TCR-seq was determined, giving us a binary matrix of zeros and ones, with a one indicating that the sequence was measured at least once in that sample. The Jaccard index was used to quantify the similarity of the detected sequences in one sample versus another and was visualized with the R package *corrplot*[314]. The binary matrix was then used to create the binary heatmap showing the presence/absence of amino acid sequences. Before creating the heatmap, amino acid sequences with low counts across all samples were removed; specifically, a sequence was

removed if it did not make up 0.3% of the total measured sequences in at least one of the six samples. The heatmap plot itself was produced with the R package *pheatmap*[315]. The UpSet plot[316] used to visualize the overlap of amino acid sequences between the samples (set comparisons) was created with the R package *UpSetR*[317]. As opposed to the binary heatmap, all of the measured sequences were used to create the UpSet plot.

2.7 Immunohistochemistry

Brains from mice were embedded in OCT and flash frozen on dry ice. Samples were then stored at -20°C until cutting. For Foxp3, CD4, and laminin staining, 8μm fresh frozen sections were first lightly fixed in 25% EtOH/75% acetone solution for 15 minutes at -20°C. Sections were then blocked with a TNB buffer containing 2% normal goat serum and 0.1% triton for 1 hour at room temperature. Sections were then incubated with α-Foxp3 at 4°C overnight. A Cy3 tyramide signal amplification kit (Perkin Elmer) was then used to amplify Foxp3 staining. Following Foxp3 amplification, sections were incubated with α-laminin and α-CD4 primary antibodies at 4°C overnight. α-rabbit AF488, α-rat Cy3 and streptavidin-Cy5 were used as secondary antibodies for fluorescence staining. Finally, sections were nuclear stained with DAPI (Thermo Scientific) for 5 minutes at room temperature.

For Ki67 staining, fresh frozen sections were lightly fixed in 25% EtOH/75% acetone solution for 15 minutes at -20°C. Sections were then blocked in 1% PBS containing 2% goat serum (Jackson Immunoresearch), 0.1% triton, and 0.05% Tween 20 for 1 hour at room temperature, then incubated with primary antibodies against Ki67 at 4°C overnight. After primary staining, sections were washed with 1% PBS and incubated

with secondary antibodies for 30 minutes at room temperature. Finally, DAPI was used to visualize nuclei.

For pSTAT5 immunofluorescence staining, fresh frozen sections were fixed in 3.2% PFA for 20 minutes at room temperature and then permeabilized with ice cold 90% methanol for 10 minutes at -20°C[209]. Sections were then incubated with blocking buffer and an α-pSTAT5 (D47E7) (Cell Signaling Technologies) primary antibody as described above. After primary antibody staining, pSTAT5 signal was amplified using an α-rabbit biotinylated antibody (Jackson Immunoresearch) before using a streptavidin secondary antibody. All images were captured using either a Nikon Eclipse E600 microscope (Melville, NY) equipped with a Photometrics Cool Snap EZ CCD camera (Tucson, AZ), an inverted Leica DMI4000 B microscope equipped with a Hammamatsu camera (Leica), or a DMI6000 B widefield microscope with a Hamamatsu C10600 Orca R^2 digital camera (Leica), and visualized using either Metamorph or Nikon NIS Elements software. Images were then analyzed using Imaris (Bitplane) or Image J software.

For quantification of the number of Ki67- or pSTAT5-expressing cells, 12-15 equal-sized pictures were taken (40x) within each brain slice, and the numbers of Ki67+ or pSTAT5+ CD4+ or CD8+ cells were counted in each image. Numbers from each picture were then averaged and reported as the average number of Ki67+ or pSTAT5+ per 100μm^2 or 500μm^2 per mouse, respectively.

2.8 Effector T Cell Transfers

$CD4^+$ $CD25^-$ cells from mice expressing cyan fluorescent protein (CFP) under the actin promoter were isolated using enrichment columns (Miltenyi, San Diego, CA). The cells were activated with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) and

cultured with anti-IL-4 (1 μg/ml) and 20 U/ml recombinant human IL-2 (proleukin) for 4 days. One million activated CFP^+ cells were transferred to mice chronically infected with *T. gondii* and imaged seven days later.

2.9 Multiphoton Microscopy

For *ex vivo* multiphoton microscopy, mice were sacrificed by CO_2 asphyxiation and the brains were removed immediately, with minimal mechanical disruption and placed in heated chamber where specimens were constantly perfused with warmed (37°C), oxygenated media (phenol-red free RPMI 1640 supplemented with 10% FBS, Gibco). The temperature in the imaging chamber was maintained at 37°C using heating elements and monitored using a temperature control probe. Intravital imaging experiments were performed using the thinned skull technique, as previously described[318]. Briefly, mice were anesthetized and a region of skull bone of 0.5-1 mm in diameter was thinned with a dental drill and surgical blade until approximately 30μm of skull remained.

All imaging was performed using a Leica SP5 2-photon microscope system (Leica Microsystems, Mannheim, Germany) equipped with a picosecond laser (Coherent Inc., Santa Clara, CA) and external non-descan detectors that allow simultaneous detection of emissions of different wavelengths and second harmonic signals (SHG, ~460nm). Enhanced GFP, YFP, CFP, and QDots were excited using laser light of 880 or 920 nm. Images were obtained using a 20X water-dipping lens. Four-dimensional imaging data was collected by obtaining images from the *x*-, *y*-, and *z*-planes, with a *z*-thickness of 68 μm and step size of 4 μm to allow for the capture of a complete z-series every 20 seconds for period of 15 minutes. The resulting images were analyzed with

Volocity (PerkinElmer, Waltham, MA) or Imaris (Bitplane) software. Movies of T cell migration, mean migratory velocity, and cell contact duration were calculated using Imaris software.

2.10 *Toxoplasma gondii* Cyst Counts

After mincing with a razor blade, brain tissue was passed through an 18- and 22-gauge needle. 30μL of brain homogenate was then placed onto a microscope slide (VWR) and cysts were counted manually on a brightfield DM 2000 LED microscope (Leica).

2.11 ELISA

ELISAs for parasite-specific IgG were performed as previously described[319]. Briefly, Immunolon 4HBX ELISA plates (Thermofisher) were coated with 5μg soluble *Toxoplasma* antigen (STAg) diluted in 1X PBS overnight at 4°C. After antigen coating, plates were washed in 1x PBS with 0.1% Triton and 0.05% Tween, then blocked with 10% FBS for 2 hours at room temperature. After washing, serial dilutions of collected serum were added to plate wells overnight at 4°C. After incubation with serum samples, plates were washed and wells were incubated with goat α-mouse IgG, human ads HRP (Southern Biotechnology) for 1 hour at room temperature. ABTS peroxidase substrate solution (KBL) was then added to wells and immediately after a color change plates were read on an Epoch BioTek plate reader using Gen5 2.00 software.

2.12 qRT-PCR

Approximately 100mg brain tissue was put into 1mL Trizol (Ambion) in bead beating tubes (Sarstedt) containing 1mm zirconia/silica beads (BioSpec). Using a Mini-bead beater (Biospec), tissue was homogenized for 30 seconds and RNA extraction was

completed using the manufacturer's instructions (Ambion). For cDNA synthesis, a High Capacity Reverse Transcription Kit (Applied Biosystems) was used. qRT-PCR was set up using a 2X Taq-based mastermix (Bioline) and Taqman gene expression assays (Applied Biosystems) and reactions were run on a CFX384 Real-Time System (Bio-Rad). HPRT was used as a housekeeping gene for all reactions and relative expression compared to control treated animals was calculated as $2^{(-\Delta\Delta CT)}$.

2.13 Statistics

Statistical analysis comparing two different groups at a single time point was performed in Prism software (v. 7.0a) using a Student's t test. In some cases, multiple experiments from different infection dates were combined to show natural biological variation between infections. When data from multiple experiments were combined, a randomized block ANOVA was used in R v.3.4.4 statistical software. This statistical test accounted for natural variability between experimental dates by modeling the treatment (IgG vs. α-IL-10R or α-ICOSL) as a fixed effect and the experimental date as a random effect. The particular test used for each individual panel shown is specified in the figure legend. P values are displayed as follows: ns= not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. All data were graphed using Prism software. All data is presented as mean ± standard deviation unless otherwise noted in the figure legend.

CHAPTER 3

CD11c-expressing cells affect T_{reg} behavior in the meninges during CNS infection

This chapter contains unpublished data, as well as data from the following published manuscript: O'Brien CA, Overall C, Konradt C, O'Hara Hall AC, Hayes NW, Wagage S, John B, Christian DA, Hunter CA, Harris TH. CD11c-expressing cells affect T_{reg} behavior in the meninges during CNS infection. *Journal of Immunology*. 1 May 2017. Vol. 198, Issue 9. DOI 10.404

3.1 INTRODUCTION

Regulatory T cells (T_{reg} cells) have potent suppressive capacity capable of limiting effector T cell responses and immune-mediated pathology in the context of immune homeostasis as well as in infectious and non-infectious inflammatory processes. While multiple suppressive mechanisms have been attributed to T_{reg} cells[320,321], only a limited number of reports have examined T_{reg} cell behavior *in vivo,* where T_{regs} have been imaged in the bone marrow, spleen, lymph nodes in diabetes and graft-versus-host models, and in tumors[70,124,322-324]. In many CNS inflammatory conditions T_{reg} cells are recruited to the brain, where it has been proposed that their presence represents one mechanism to limit the catastrophic consequences of inflammation in this site[325,326]. For example, in mice infected intracranially with murine hepatitis virus, the depletion of T_{reg} cells leads to an increase in self-reactive T cell responses and more severe pathology in the brain[83]. While the importance of T_{reg} cells in many experimental models that involve the CNS has been demonstrated[83,327-331], the behavior of these cells within the brain remains unexplored.

Toxoplasma gondii is a protozoan parasite that establishes a chronic infection within the CNS. In mice, cytotoxic T cells and T cell-production of IFNγ are required to control parasite replication within the brain[123,247,295]. Several studies have established that T_{reg} cells contribute to the regulation of effector T cells during acute toxoplasmosis[97,160,292] and that during many intracellular infections there is the emergence of a population of T_h1-like T_{reg} cells that express Tbet, IFNγ, IL-10, and CXCR3[96,97,292], but there are open questions about the specificity of these populations[81,332]. During acute toxoplasmosis, expansion of the T_{reg} cell population is associated with an increase in

parasite burden within the brain[292,333]. These latter observations suggest that T_{reg} cells can suppress the protective T cell response required to control *T. gondii* but it is unclear if this is a general regulatory effect or mediated locally within the brain. The studies presented here reveal that, unlike parasite-specific effector T cells, during TE, T_{reg} cells were localized predominantly to the meninges and perivascular cuffs where they maintained interactions with CD11c-expressing cells, which influence the migratory behavior of T_{reg} cells. These observations suggest that T_{reg}-dendritic cell interactions are an important component of T_{reg} cell function during toxoplasmic encephalitis (TE) and this may be broadly relevant to T_{reg} cell functions in other inflammatory settings that affect the CNS.

3.2 RESULTS

Phenotype and localization of T_{reg} cells during Toxoplasmic encephalitis

To characterize T_{reg} cell responses during chronic toxoplasmosis, mononuclear cell preparations were isolated from the spleen, cervical lymph nodes (CLN), and brains of mice infected with *T. gondii.* The numbers and phenotype of these populations were assessed using flow cytometry for intracellular Foxp3 (Figure 3.1a). Based on the use of MHC class I and II tetramers that contain defined parasite epitopes, activated antigen-specific $CD4^+$ and $CD8^+$ effector cells were readily detected in the spleen and cervical lymph nodes and the T_{reg} cells represented 12-18% of the total $CD4^+$ population (data not shown). In the brains of uninfected mice, T cells are confined to the meningeal spaces [313,334] and BMNC preparations contained very few conventional effector/memory T cells, T_{reg} cells, or DC (Figure 3.1a and data not shown). During early stages of infection, a sizable population of effector $CD4^+$ T cells was recruited to the brain by day 14 (Figure 3.1a). T_{regs} comprised approximately 1.5% of the $CD4^+$ T cell compartment at day 14 and 21 post-infection. During chronic infection (day 28 post-infection), T_{reg} cells comprise approximately 8-10% of the total $CD4^+$ T cell population and these cells express high levels of CD25 (Figure 3.1b).

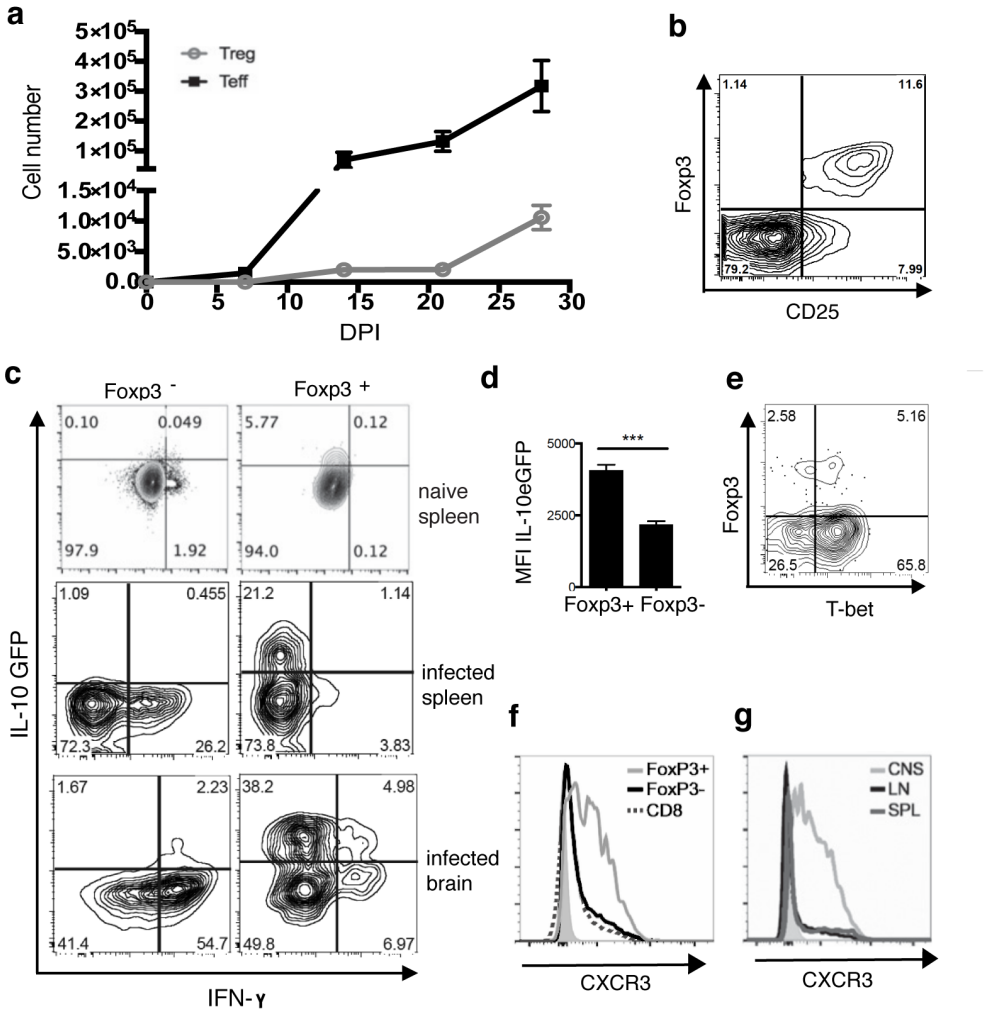


Figure 3.1. T_{reg} cells are present in the CNS during chronic *T. gondii* infection and produce cytokines. Mononuclear cells were isolated from the brains of C57BL/6 mice infected with *T. gondii* for 28 days. Numbers of Foxp3+ and Foxp3- CD4 T cells infiltrating the brain were measured on days 0, 7, 21, and 28 post-infection (a). Foxp3 and CD25 expression was measured on $CD4^+$ cells isolated from the CNS by flow cytometry on day 28 post-infection (b). IL-10 expression was measured by flow cytometry in IL-10-eGFP reporter mice on day 28 post-infection. The co-expression of IL-10 and IFNγ in $CD4^+$ T cells was measured in the naïve and infected spleen and the infected brain (c). The mean florescence intensity (MFI) of IL-10-eGFP expression in $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells isolated from the brain (d). Expression of Tbet by $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells (e). CXCR3 expression was measured on $CD4^+Foxp3^-$ ($Foxp3^-$), $CD4^+Foxp3^+$ ($Foxp3^+$), and $CD8^+$ T cells (f) and T_{reg} CXCR3 expression was compared between the CNS, spleen, and cervical lymph nodes (g). The presented data is representative of

five independent experiments with 4 animals per group (b) or two independent experiments with 3 mice per group (a, c-g). *** denotes $p<0.001$ as determined by the students t-test.

To further characterize this population, IL-10-eGFP reporter mice (*VertX* or *Tiger* mice) were infected and IL-10 and IFNγ production was assessed and compared to basal IL-10 production in the naïve spleen. We observed negligible IL-10 production from conventional $CD4^+$ T cells in the spleens of naïve and infected mice. On the other hand, T_{regs} in the spleen did produce some IL-10 at baseline (approximately 5%) that increased to nearly 20% during infection (Figure 3.1c). In the infected CNS, approximately 4% of $Foxp3^-$ $CD4^+$ cells were $eGFP^+$, whereas 45% of T_{regs} in the CNS expressed IL-10-eGFP (Figure 3.1c). While the number of $Foxp3^+$ and $Foxp3^-$ IL-10-producing cells are similar, the MFI of IL-10-eGFP was higher in the T_{reg} cells isolated from the infected brain (Figure 3.1d). Analysis of IFNγ production revealed that while 57% of effector $CD4^+$ T cells in the infected brain produced IFNγ, only 12% of the T_{regs} produced IFNγ at this site (Figure 3.1c). This analysis also highlighted the presence of a small sub-population of T_{reg} cells that produce both cytokines in the brain, which was not observed in peripheral tissues at this time point (Figure 3.1c). Consistent with the highly polarized T_h1 response that is generated against *T. gondii*, the T_{reg} cells in the brain during TE express the canonical T_h1 transcription factor T-bet (Figure 3.1e) and the Tbet-dependent chemokine receptor CXCR3 (Figure 3.1f-g). Moreover, T_{regs} isolated from the infected CNS express higher levels of CXCR3 in comparison to effector $CD4^+$ and $CD8^+$ T cells (Figure 3.1f) and the CXCR3 expression on CNS T_{regs} was higher in the brain in comparison to T_{regs} isolated from the spleen and cervical lymph nodes of infected mice (Figure 3.1g). Together these data demonstrate that a T_h1 polarized T_{reg} is recruited to the CNS during chronic *T. gondii* infection.

Specificity and sequencing of T_{reg} cells isolated from the meninges during TE

During infection with *T. gondii,* parasite-specific $CD4^+$ and $CD8^+$ T cells, as well as T_{regs} accumulate in the CNS, but little is known about the specificity of the local T_{reg} populations. To address whether the T_{regs} may be specific for parasite antigen, immune cells isolated from the CNS were stained with an MHC Class II tetramer reagent specific for the AVEIHRPVPGTAPPS peptide. Parasite-specific $CD4^+$ effector cells were detected but very few $CD4^+$ T cells were tetramer-positive and $Foxp3^+$ (0.018±0.0059%) (Figure 3.2a). Although this analysis was performed using a single tetramer, these results suggest that T_{reg} cells in the CNS may not recognize the same antigens as the *Toxoplasma*-specific effector CD4+ T cells.

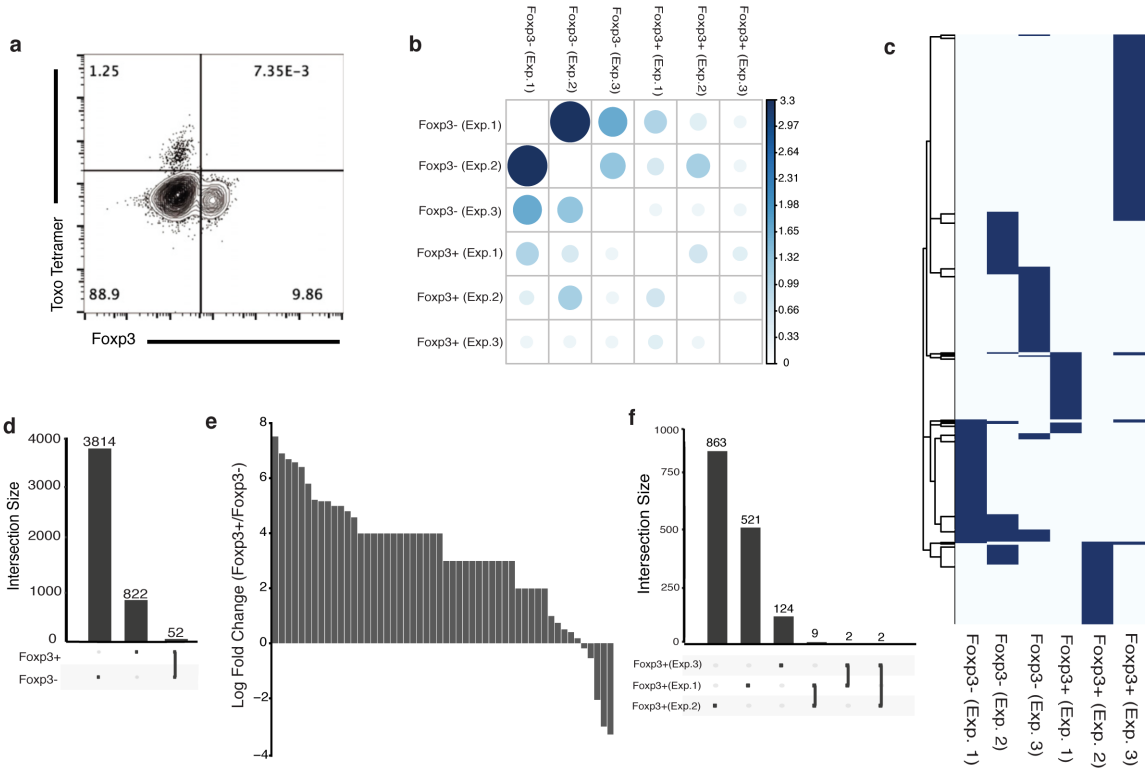


Figure 3.2. Regulatory T cells that accumulate in the CNS during *T. gondii* infection have little TCR sequence similarity with effector T cells and vary greatly between experiments. Brains from chronically infected C57Bl/6 mice were harvested and processed for flow cytometry 28 days post infection. Cells were gated on $CD3^{+}CD4^{+}$ live lymphocytes and *T. gondii* tetramer-specific effector ($CD4^{+}Foxp3^{-}$) and regulatory ($CD4^{+}Foxp3^{+}$) T cells are shown (a). T cells isolated from the meninges were FACS sorted to isolate effector ($CD4^{+}Foxp3^{-}$) and T_{reg} ($CD4^{+}Foxp3^{+}$) populations. CDR3 regions of the TCRβ chains were sequenced from each population. The resulting sequences were compared between populations and experiments to identify the degree of overlap by Jaccard index (b). Sequences that represented at least 0.3% of the population were displayed by binary heatmap (blue=present and white=absent), indicating overlap between samples (c). The amount of overlap between effector T cells ($Foxp3^{-}$) and T_{regs} ($Foxp3^{+}$) from a single experiment is shown by upset plot, where joining lines indicate sequences detected both samples, with the number of sequences indicated above the bar (d). Within the overlapping samples the ratio (fold change) of reads between $Foxp3^{+}$ and $Foxp3^{-}$ samples is depicted (e). The overlap among T_{regs} between experiments is shown by upset plot (f).

To further explore the clonality and potential specificity of the T_{regs} in the CNS, effector and regulatory $CD4^+$ T cells were sorted from meninges of infected mice and the CDR3 regions of the TCRβ gene were sequenced. The TCR sequences of effector T cells and T_{regs} from three individual experiments were compared at the amino acid level. First, the similarity of the TCR sequences was compared between samples, where the presence of a sequence was considered, but not the frequency of the sequence within the population. While many unique sequences were detected in each population, the most similarity was found in the effector $CD4^+$ T cell populations (Figure 3.2b-c and Supplementary Table 3.1). When comparing TCR sequences from effector T cells and T_{regs}, we found very little (approximately 1%) between the populations (Figure 3.2d), which is in agreement with results from MHC II tetramer staining. Moreover, if a sequence was detected in both effector and T_{reg} populations, the number of reads for that sequence was overrepresented in the T_{reg} population (Figure 3.2e). These results suggest that T_{reg} cells found in the CNS during chronic infection are rarely from the same clonal lineage as the effector population and some T_{regs} may lose Foxp3 expression and resemble effector T cells. Unexpectedly, we found that the CDR3 regions of T_{reg} TCRs had very little sequence overlap (0.2-0.65%) between three separate *T. gondii* infections (Figure 3.2b-c and 3.2f), suggesting that the T_{reg} repertoire recruited to the CNS during infection varies greatly between experimental infections. While the TCR sequences do not identify the antigen specificity of the T_{reg} cells, these experiments reveal that during each individual infection in C57BL/6 mice the presence of a unique repertoire of T_{reg} cells that is largely distinct from the effector cells arises in the CNS.

T_{reg} localization in the CNS during chronic T. gondii infection

In order to understand the spatial organization of the T_{reg} cells during TE, IHC was performed for CD4, parasite antigen, Foxp3, and laminin to identify basement membranes and thus demarcate the meninges and blood vessels and identify CD4+ T cell location within the brain. Staining for CD4 revealed the presence of T cells in the meninges and perivascular cuffs, but the largest numbers of effector T cells were present in the parenchyma (around 75.7%), where parasite cysts and replicating tachyzoites are found (Figure 3.3a). In contrast, $Foxp3^+$ cells were largely (89%) localized to the inflamed meninges and perivascular cuffs, and thus were more rarely detected in the brain parenchyma in comparison to effector $CD4^+$ T cells (Figure 3.3b-e). Together with the data presented in Figure 3.1, these data indicate that the T_{reg} cells present in the CNS during TE are T_h1 polarized like the effector population, but their distribution within the brain is distinct from effector $CD4^+$ T_h1 cells.

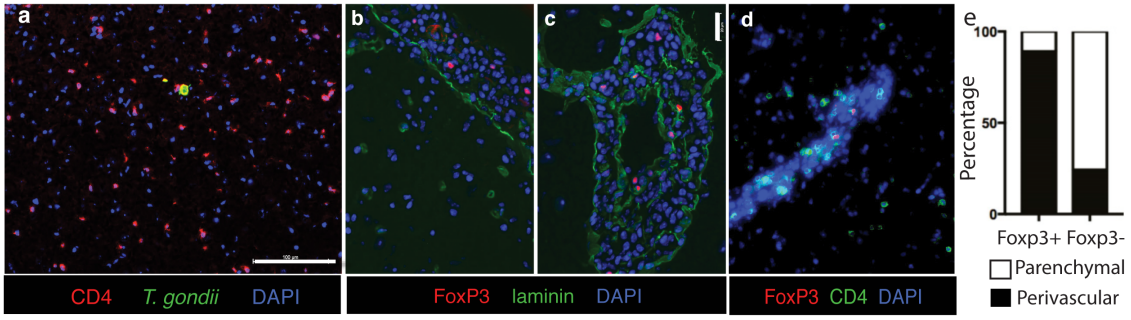


Figure 3.3. T_{regs} are absent from the brain parenchyma. Sections of brain from *T. gondii*-infected C57BL/6 mice were stained with antibodies against CD4 (red) and *T. gondii* (green) (a), Foxp3 (red) and laminin (green) to detect basement membranes (b-c), or (red) and CD4 (green) (d). DAPI was used to detect nuclei (blue). Representative quantification of parenchymal and perivascular Foxp3+ (n=163) and Foxp3- (n=189) $CD4^+$ cells is shown in (e).

Imaging T_{reg} cell populations during toxoplasmic encephalitis

Because dendritic cell (DC) populations are critical regulators of T_{reg} homeostasis and are known targets of T_{reg} suppression[75,124,321], Foxp3-GFP mice were crossed with CD11c-YFP reporter mice and mice expressing both reporters were used for intravital imaging studies (Figure 3.4a). Firstly, these studies showed that there was close association of $Foxp3^+$ cells with blood vessels in the CNS of mice with TE and that these cells were frequently co-localized with $CD11c^+$ cells (Figure 3.4a and Supplementary Video 3.1), confirming results obtained with IHC (data not shown). Next, imaging of $Foxp3^+$ cells in the CLN of infected mice revealed that they were highly motile and had many short lived interactions with $CD11c^+$ cells (Figure 3.4b-c and Supplementary Video 3.2), similar to the behavior of T_{reg} cells in the lymph nodes of uninfected mice[70]. However, the T_{reg} cells localized within the meninges of infected mice had a slower velocity and formed long-lived associations with $CD11c^+$ populations in this microenvironment in comparison to the spleen (Figure 3.4d and Supplemental Video 3.3). In contrast to naïve T cell interactions with dendritic cells during priming[107], $Foxp3^+$ cells were not stationary and did not round up while interacting with $CD11c^+$ cells but were frequently observed to move from one $CD11c^+$ cell to the next, while maintaining contact with these cells (Supplemental Video 3.3). It is relevant to note that when $CD4^+$ T cells from CFP-expressing mice were activated and expanded *in vitro* and transferred to infected Foxp3-GFP reporter mice, this $CD4^+CD44^{hi}CD62L^{lo}Foxp3^-$ population was present in the same area as T_{reg} cells but remained highly migratory (Figure 3.4e and Supplemental Video 3.4) and did not form sustained interactions with the CD11c-YFP cells (data not shown). These data highlight that the T_{reg} cells in the CNS during TE

display a pattern of behavior that is distinct from T_{reg} cell populations in the CLN or effector $CD4^+$ T cells present in the meninges (Figure 3.4f).

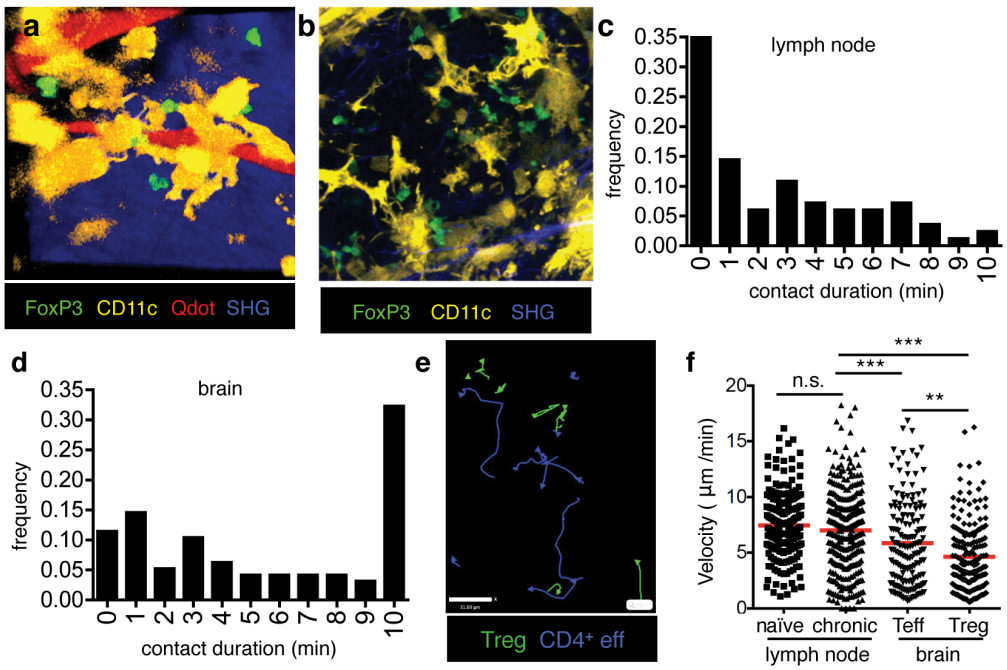


Figure 3.4. T_{regs} form long-lived contacts with $CD11c^+$ cells in the brain. Foxp3-GFP CD11c-YFP mice were infected with *T. gondii* for 28 days. Intravital imaging was performed through thinned skull (blue) to detect Foxp3-GFP (green), CD11c-YFP (yellow), and the vasculature highlighted by a fluorescent vascular tracer (red) (a). On day 28 post-infection, explant lymph nodes from CD11c-YFP x Foxp3-GFP mice were imaged by MP microscopy, with secondary harmonic generation (SHG, blue) is shown in blue (b). The duration of contact between Foxp3-GFP-expressing cells (green) and YFP-expressing cells (yellow) was measured using Volocity software (c-d). $CD4^+CD25^-$ T cells from a CFP-expressing mouse were activated *in vitro* and transferred to a chronically infected Foxp3-GFP mouse. Explant brains were imaged using multiphoton microscopy and individual cell paths of T_{regs} (green) and effector cells (blue) were tracked (e-f). The lymph nodes from naïve and chronically infected Foxp3-GFP mice were also imaged using MP microscopy. More than 100 GFP-expressing cells in each tissue from 4 or more movies and 4 independent experiments were tracked. The track velocity of T_{regs} in the lymph node and CNS and effector cells in the CNS was calculated (f). Results are denoted as non-significant (n.s.), ** for $p<0.01$, and *** for $p<0.001$ as measured by one way ANOVA with a Tukey's multiple comparison post-test.

Anti-LFA-1 treatment disrupts T_{reg} interactions with CD11c-expressing cells in the meninges

The reduced migratory phenotype of T_{reg} cells observed in the CNS during TE may be explained by prolonged interactions with $CD11c^+$ cells. Previous studies have demonstrated that treatment with α-LFA-1 antibodies leads to a loss of dendritic cells in the CNS[335]. Indeed, treatment with α-LFA-1 antibodies for four hours leads to a significant decrease in DCs ($CD3^-CD19^-NK1.1^-CD11c^+MHCII^{hi}$) in the meninges (Figure 3.5a). To determine if T_{reg} cell interactions with DCs *in vivo* affects their migratory behavior, mice were treated with LFA-1 blocking antibodies four hours prior to imaging (Supplementary Movie 3.5). This loss of dendritic cells resulted in a reduced duration of contacts between T_{reg} cells and the remaining $CD11c^+$ cells, with fewer T_{regs} maintaining contact for the duration of the imaging period and more cells making contacts of short duration (Figure 3.5b). The average contact time was significantly reduced from 11.5 to 8.5 minutes. Moreover, antibody blockade resulted in a significant increase in the speed of $Foxp3^+$ cell migration from to 2.2 to 3.3 μm/min (Figure 3.5c). Together these results suggest that interactions between T_{reg} cells and CD11c+ cells limit the migratory speed of T_{reg} cells in the CNS.

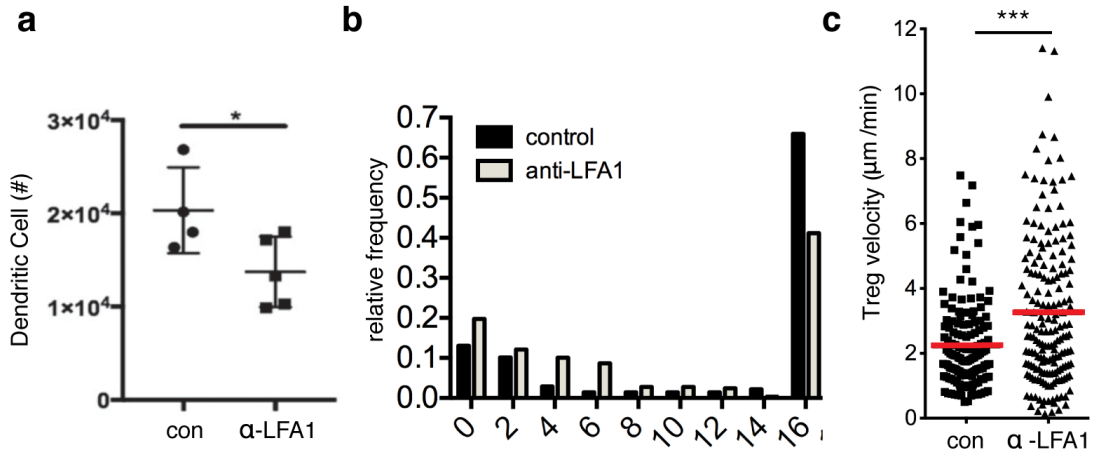


Figure 3.5. Dendritic cells shape the migratory behavior of Foxp3-GFP cells in the meninges. Foxp3-GFPxCD11c-YFP mice were chronically infected with *T. gondii*. On day 28 post-infection, mice received 200 μg of control or anti-LFA-1 blocking antibodies by intraperitoneal injection. The number of dendritic cells (live $CD3^{-}NK1.1^{-}CD19^{-}$ $CD45^{hi}$ $CD11c^{hi}MHC\ class\ II^{hi}$) remaining after four hours of antibody treatment was measured by flow cytometry (a). Explant brains were imaged 4 hours post-antibody injection. The contact duration between cell types in each condition was measured (b). The track velocity of Foxp3-GFP cells was tracked in each condition (c). 148 cells in control-treated mice and 181 cells in anti-LFA-1 treated mice were tracked from two independent experiments. * denotes $p<0.05$ and *** $p<0.001$ by student's t-test.

Blockade of MHCII increases T_{reg} velocity in the inflamed meninges.

In addition to examining the role played by adhesion molecule LFA-1 in T_{reg} motility in the inflamed CNS, we also sought to identify other factors regulating T_{reg} motility, and potentially suppressive capacity, in the CNS during chronic infection. As such, we first wanted to assess how TCR:MHCII interactions might affect T_{reg} motility. TCR engagement has been shown to be important for maintenance of T_{reg} function during homeostasis[336], but its role in T_{reg} motility in the inflamed CNS has not been addressed. To determine if TCR:MHCII interactions affected T_{reg} motility in this setting, we treated chronically infected mice with either an α-MHCII blocking antibody or control IgG antibody. After four hours, T_{regs} in these mice were imaged. Similar to the results seen following LFA-1 blockade, blockade of MHCII also resulted in a significant increase in T_{reg} velocity in the meninges, from about 6.3 μm/min to about 8.7 μm/min (Figure 3.6a). This result suggests that TCR:MHCII interactions can influence T_{reg} migratory behavior, perhaps by preventing stable contacts with dendritic cells in the meninges. In the case of LFA-1 blockade, both the role of LFA-1 in retention or recruitment of DCs to the inflamed meninges, and the role of LFA-1 in T_{reg}:DC interactions themselves could be contributing the increased velocity of T_{regs} seen in that setting. Interestingly, following α-MHCII blockade for four hours, no decreases were seen in the number of infiltrating dendritic cells in the meninges (Figure 3.6b). Likewise, we also did not observe a decrease in T_{reg} cell number in the meninges following MHCII blockade (Figure 3.6c). This suggests that, unlike LFA-1:ICAM interactions, TCR:MHCII interactions are not important for DC recruitment or retention in the inflamed tissue in this time frame, and are limiting T_{reg} motility by interfering with meningeal T_{reg}:DC contacts themselves.

These data taken together show that both LFA-1:ICAM and TCR:MHCII interactions contribute to limiting T_{reg} motility in the inflamed CNS, though LFA-1:ICAM interactions play additional roles in retention and/or recruitment of DCs to the CNS as well.

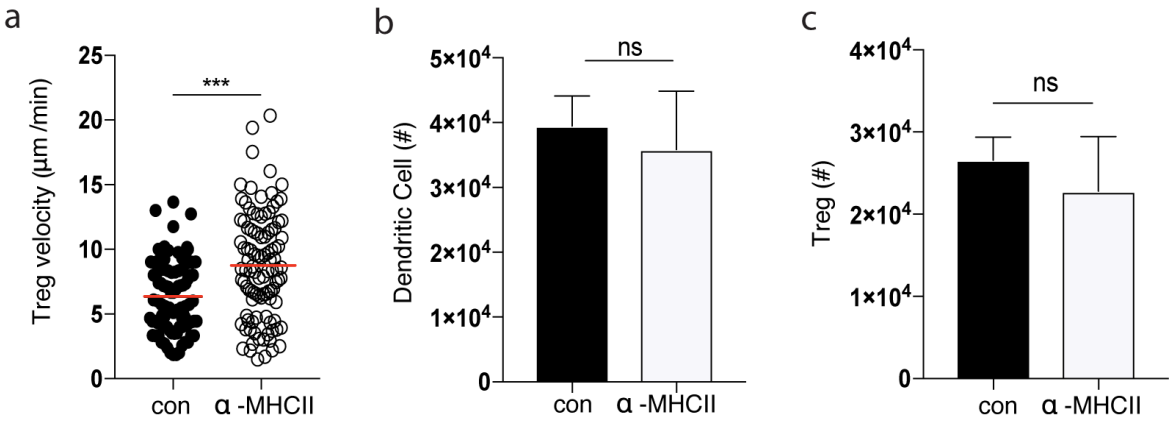


Figure 3.6. TCR:MHCII interactions are involved in limiting T_{reg} motility in the inflamed meninges. Foxp3-GFP mice were chronically infected with *T. gondii*. On day 28 post-infection, mice received 200 μg of control or anti-MHCII blocking antibodies by intraperitoneal injection. Four hours post-treatment, mice were anesthetized and their skulls were thinned for multiphoton imaging. After imaging analysis and tracking of GFP+ T_{regs}, T_{reg} velocities were calculated using Imaris software (a). 95 cells and 105 cells were counted for the control and α-MHCII-treated mice, respectively. Graph is representative of two independent experiments with three mice in each group. (b-c) Chronically infected C57Bl/6 mice were treated as above. Four hours post-treatment, BMNCs were isolated and stained for flow cytometry. The total number of CD11c+MHCIIhi dendritic cells (b) and Foxp3+ T_{regs} (c) are shown. Graphs are representative of two independent experiments with three mice in each group. *** $p<0.001$ by student's t-test, ns = not significant.

3.3 DISCUSSION

Multiple studies have associated the presence of T_{reg} cells in the CNS with the ability to limit inflammation in the context of infection (West Nile Virus, Murine Hepatitis Virus, and Coronaviruses) and autoimmunity (EAE and MS)[327-330,337-339], but little is known about the localization and behavior of T_{reg} cells in these different disease settings. In addition, in many cases it has been difficult to discern local effects within the CNS versus a role for this regulatory population in peripheral events[340]. The studies presented here reveal that during TE, unlike the effector $CD4^+$ T cells, $Foxp3^+$ T cells were restricted to the perivascular spaces and the meninges. Precedent exists for this observation, as Campbell and colleagues reported a similar localization of T_{reg} cells in the CNS in a model of EAE[341], and suggested that signaling through CXCR3 is critical to prevent T_{reg} cell entry into the inflamed brain parenchyma. Consistent with this idea, T_{reg} cells present in the CNS during TE express high levels of CXCR3 and we have observed that in CXCR3 knockout mice infected with *T. gondii*, the localization of T_{reg} cells is altered and that these populations are now present in the brain parenchyma (Harris unpublished observation). Thus, while recent studies have proposed that CXCR3 expression on T_{reg} cells during T_h1 inflammation allows these populations to access sites of T_h1 inflammation[342], these observations suggest that CXCR3 is not only involved in entry to the tissue, but can also regulate T_{reg} cell location within the CNS.

Whether the exclusion of T_{reg} cells from the brain parenchyma is biologically relevant remains unclear but this may be one mechanism that allows effector T cells present in the brain parenchyma to operate independently of the suppressive effects of T_{reg} cells and therefore better control parasite replication. In previous reports, the use of

IL-2 complexes to expand T_{reg} cell populations in acutely infected mice led to an increased parasite burden in the CNS[292,333], further suggesting an important role for T_{reg} cells in controlling effector T cell responses and resulting parasite burden. There are several possible ways that T_{reg} cells in perivascular sites might influence parasite specific effector responses in the parenchyma: In models of EAE and viral encephalitis the ability of effector T cells to interact with antigen presenting cells within these perivascular compartments allows effector T cells to proliferate and be retained in the CNS[343,344]. The ability of T_{reg} cells to limit DC activity at this site of T cell entry to the parenchyma of the brain, perhaps through the production of IL-10, may allow T_{reg} cells to serve as "gatekeepers" to the CNS.

Recent studies have shown that TCR signaling is required for T_{reg} cell suppressor capacity *in vivo*[336], but it remains unclear whether T_{reg} cell populations in the brain during TE are specific for parasite-derived antigens or whether these are self-reactive populations. While reagents to detect parasite-specific $CD4^+$ T cells are currently limited to a single tetramer reagent, our results do not indicate that T_{regs} are specific for this parasite antigen. Moreover, the results from TCR sequencing performed in this study suggest that if T_{regs} are indeed parasite-specific, they rarely share a clonal lineage with effector T cells. We also found that when a TCR sequence is shared between effector T cells and T_{regs}, more reads were typically detected in the T_{reg} population, which may suggest the loss of Foxp3 expression and acquisition of an effector T cell phenotype. In addition, unique T_{reg} clones were identified in the CNS in each independent experimental infection, suggesting that variable(s) other than infection shape the repertoire of T_{regs} in the CNS. Our results are in agreement with several studies that also did not detect

overlap in TCR sequences between regulatory and effector $CD4^+$ T cell populations in diverse settings of tissue inflammation[345-348]. Though it remains unclear what, if any, roles continued TCR:MHCII interactions play in the T_{reg} population during chronic inflammation in the CNS, our results suggest that MHCII blockade may disrupt T_{reg}:DC interactions, even on a short time scale, hinting that this interaction could be crucial in either T_{reg} survival or T_{reg} effector function in inflammatory states.

Several observations suggest that the unique behavior of T_{reg} cells in the CNS (compared with activated effector $CD4^+$ T cells in the same areas or T_{reg} cells in secondary lymphoid organs) may be a function of the sustained interactions with $CD11c^+$ populations. Indeed, several *in vitro* studies have also examined the influence of T_{reg} cells on DCs and found that T_{reg} cells decrease levels of MHC class II and expression of co-stimulatory molecules[66,75,123]. This observation is consistent with early studies that demonstrated that the ability of T_{reg} cells to interact with $CD11c^+$ cells is central to T_{reg} cell suppression of effector T cell responses[70,124,349]. In our studies, the loss of dendritic cells from the meninges led to increased T_{reg} cell velocity, suggesting that interactions between these two cell types influence T_{reg} behavior at this site. The T_{reg} interaction with DCs in this space may be important for suppressing DC function by down-regulating the co-stimulatory capacity of these antigen presenting cells or their production of cytokine, thereby regulating local effector T cell responses. Alternatively, recent studies have also demonstrated that DCs in peripheral tissues provide survival signals for T_{reg} cells that differ from those in the lymph nodes and that these cells may promote the survival of T_{reg} cells in the brain[209]. If these interactions are disrupted long-term, it is possible T_{reg} survival could be affected. Regardless, the ability to visualize how T_{reg} cells behave in

inflamed tissues and determine the cell types they interact with provides insight into how these cells operate to limit inflammatory processes and a better understanding of how local effector responses are regulated at sites of inflammation.

CHAPTER 4

IL-10 and ICOS differentially regulate T cell responses in the brain during chronic *Toxoplasma gondii* infection

This chapter contains data from the following manuscript: O'Brien CA, Batista SJ, Still KM, and Harris TH. IL-10 and ICOS differentially regulate T cell responses in the brain during chronic *Toxoplasma gondii* infection. *Journal of Immunology*. 4 February 2019. DOI: 10.4049

4.1 Introduction

Immune responses have intricately evolved to protect hosts from a wide range of potentially harmful pathogens[2,350], yet these same inflammatory responses can often cause host damage themselves. The importance of a balanced immune response is apparent in models of infection, where inflammation is required for pathogen control, yet amplified immune responses observed after depletion of regulatory T cells or immunosuppressive cytokines often lead to exacerbated tissue pathology and increased mortality[86,160,163,288,290,351]. One such immunosuppressive cytokine, IL-10, has been broadly studied in the context of both tissue homeostasis and during infection, and has been shown to play a key role in suppressing many aspects of an immune response. Production of IL-10 during immune responses to infection has been attributed to a wide variety of cell types, including T cells, dendritic cells, macrophages, NK cells, and B cells[126]. IL-10 also acts on a wide range of cell types, with one of its main roles being the downregulation of MHC and costimulatory molecules in antigen presenting cells (APCs), thereby preventing their full activation capacity and limiting T cell responses[134,141,352]. IL-10 also has direct effects on T cells, limiting IFNγ and IL-2 production, as well as T cell proliferation *in vitro*[353,354].

Infection with the eukaryotic parasite *Toxoplasma gondii* leads to widespread activation of the immune system and systemic inflammation that is required for host survival [240]. The generation of a parasite-specific adaptive immune response clears the parasite from most peripheral tissues; however, the parasite is able to encyst in the central nervous system and establish a chronic infection[227,239]. This chronic infection requires ongoing activation and infiltration of highly polarized T_h1 cells into the brain in order to

prevent extensive parasite replication and fatal disease[301,355]. However, like in other models of infection, regulation of this immune response is also required to promote host survival. In particular, IL-10 production is required for host survival during acute infection. IL-10 knockout mice succumb to CD4+ T cell-mediated immunopathology and excessive inflammatory cytokine production in the periphery early in the course of infection[288,356]. Similarly, continued IL-10 production in the chronic phase of infection is also necessary for host survival. IL-10 knockout mice given the antibiotic sulfadiazine early in the infection to limit parasite replication survive the acute phase of infection, but later present with similar CD4+ T cell-mediated fatal immunopathology in the brain[309]. Despite demonstrating the requirement for IL-10 signaling over the course of *T. gondii* infection, previous studies have not addressed what additional signals promote immune regulation in the context of chronic neuroinflammation.

ICOS (inducible T cell costimulator) is a costimulatory molecule expressed on activated T cells[173,357]. ICOS signaling is important in a wide variety of immune responses, including the development of T_{fh} cells and formation of germinal centers, as well as effector T cell inflammatory cytokine production[31,195,357,358]. The primary function attributed to ICOS is the amplification of effector T cell responses by serving as a costimulatory molecule similar to its family member CD28[24]. More recently, ICOS has been shown to also promote immune regulation through potent induction of IL-10 both *in vitro* and in mouse models of acute inflammation[183,184,359]. In addition to promoting production of IL-10, ICOS is also important for maintaining effector regulatory T cell populations. During homeostasis, blockade of ICOS signaling results in a loss of $CD44^{hi}CD62L^{lo}$ effector T_{regs} in the spleen[209,360]. In mouse models of diabetes and

helminth infection, a similar loss of T_{regs} is seen with a lack of ICOS signaling, in addition to decreased IL-10 production[207,361]. During acute *T. gondii* infection, ICOS signaling has been reported to amplify T cell inflammatory responses by promoting increased IFNγ production early in the infection[202,203]. ICOS appears to play a redundant role with CD28 in this setting, as mice lacking ICOS are only more susceptible to infection on a $CD28^{-/-}$ background[203]. Together, these reports highlight the context-dependent role of ICOS signaling, contingent on variables ranging from the type of inflammatory environment to stage of infection[24,31,173,183,184,195,202,203,207,209,357-361].

The role of ICOS, and its relationship to IL-10–mediated regulation of immune responses, in the context of a chronic neuroinflammatory response to a pathogen is not well understood. In this study, we first characterized what role IL-10 plays in promoting regulation of immune responses in the chronic stage of infection by blocking signaling through the IL-10 receptor. This IL-10R blockade during chronic infection led to an expansion of CD4+ effector T cells correlating with increased expression of CD80 on APCs, along with widespread immunopathology. Based on previous reports implicating a role for ICOS in stimulating IL-10 production, we then addressed the question of whether ICOS signaling can promote suppression of chronic T cell responses in the central nervous system through induction of IL-10. Surprisingly, we find that blockade of ICOS signaling during chronic infection with *T. gondii* does not lead to decreased IL-10 production from either regulatory or effector T cells, nor does it lead to impaired inflammatory cytokine production. In fact, despite maintaining IL-10 levels in the brain, blockade of ICOSL still results in a loss of T cell regulation, with two to three-fold more effector T cells found in the inflamed brain. Interestingly, this increase in effector T cells

occurred without a loss of T_{regs} in the brain and did not affect parasite burdens. We found this increase in T cell number in the brain to correlate with increased levels of CD25 and pSTAT5 expression in effector T cells following ICOSL blockade, suggesting increased responses to IL-2. Along these lines, ICOSL blockade increased T cell proliferation and expression of the survival factor Bcl-2 among the effector CD4+ and CD8+ T cell populations in the brain, and we also observed decreased Annexin V-positive T cells in the brain following α-ICOSL treatment. Interestingly, IL-10R blockade did not result in the same increases in IL-2-associated signaling molecules CD25 and Bcl-2; rather, IL-10R blockade increased the activation state of APCs. Taken together, our results suggest that ICOS signaling on T cells can suppress STAT5-induced survival signals, providing a mechanism of local suppression in the context of chronic inflammation in the brain that is distinct from IL-10-mediated regulation.

4.2 Results

Blockade of IL-10R during chronic T. gondii *infection leads to broad changes in the immune response and results in fatal immunopathology in the brain.*

During chronic infection with *T. gondii*, both effector T cells and T_{regs} recruited to the brain are capable of producing IL-10[362]. Previously published results have shown a requirement for IL-10 signaling to limit fatal immunopathology in both the acute and chronic stages of infection[288,289,309,363]. Despite the necessity for IL-10 over the course of infection with *T. gondii*, previous studies addressing the role of IL-10 in the chronic phase of infection relied on total IL-10 knockout mice, which succumb to fatal immunopathology during the first two weeks of infection[364]. In order for these mice to survive to the chronic stage of infection, the anti-parasitic drug sulfadiazine must be administered for the first two weeks of infection in order to limit parasite replication and dissemination. These previously published studies reported that, after sulfadiazine treatment, IL-10 knockout mice subsequently presented with CD4+ T cell dependent lethal immunopathology and died late in the chronic stage of disease[309]. It is still unknown, however, how a loss of IL-10 only in the chronic phase of infection influences immune responses. Thus, we treated mice with an α-IL-10R blocking antibody beginning four weeks post infection. Mice treated with an α-IL-10R blocking antibody during the chronic stage of infection presented with overt disease and became moribund between 7 to 10 days post-treatment. H&E staining of tissue sections from brains of α-IL-10R-treated mice showed increased leukocyte infiltration and associated areas of necrosis not seen in control treated animals (Figure 4.1A-B). Similar to previously published results

using IL-10 knockout mice[309], the increased numbers of immune cells in the brains of α-IL-10R-treated mice included increases in both CD4+Foxp3- T cells and infiltrating macrophages (Figure 4.1C and Supplementary Figure 4.1A). Though the increase in T cell number in the brains of α-IL-10R-treated mice predominantly came from an increase in the CD4+Foxp3- T cell compartment and not the CD8+ T cell compartment, an increased frequency of both Ki67+ CD4+ and CD8+ effector T cells was observed (Figure 4.1D). Interestingly, using an MHCII tetramer reagent to measure CD4+ T cells specific for the parasite, we observed no significant increase in the number of tetramer-positive CD4+Foxp3- T cells in the brain (Figure 4.1E). Though this analysis of antigen specificity was done using only a single parasite peptide, this result suggests that IL-10R blockade in the chronic phase of infection leads to the expansion of CD4+ effector T cells with potentially different antigen specificities, possibly through the expansion of other parasite-specific T cell clones or self-antigen-specific T cell clones.

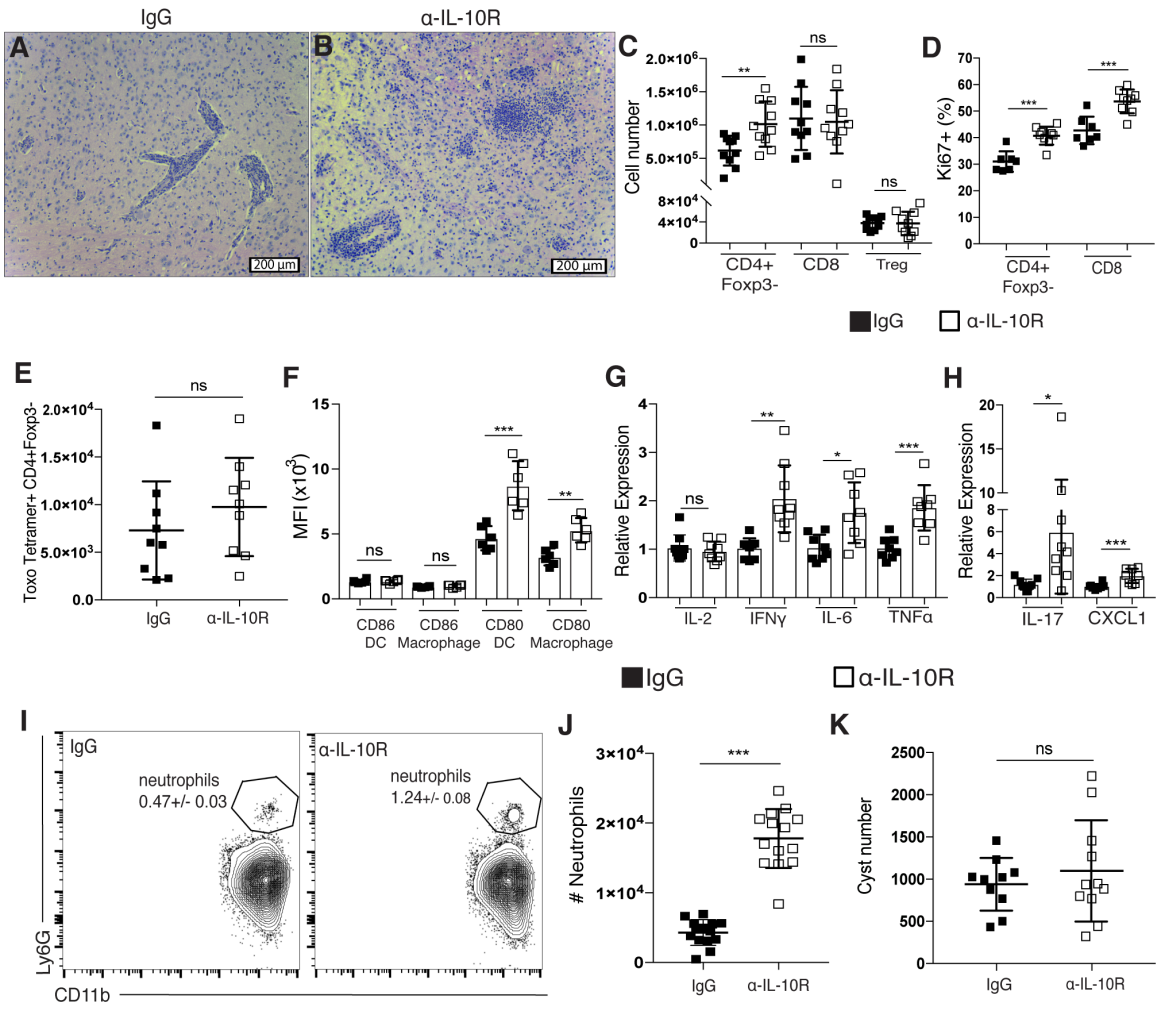


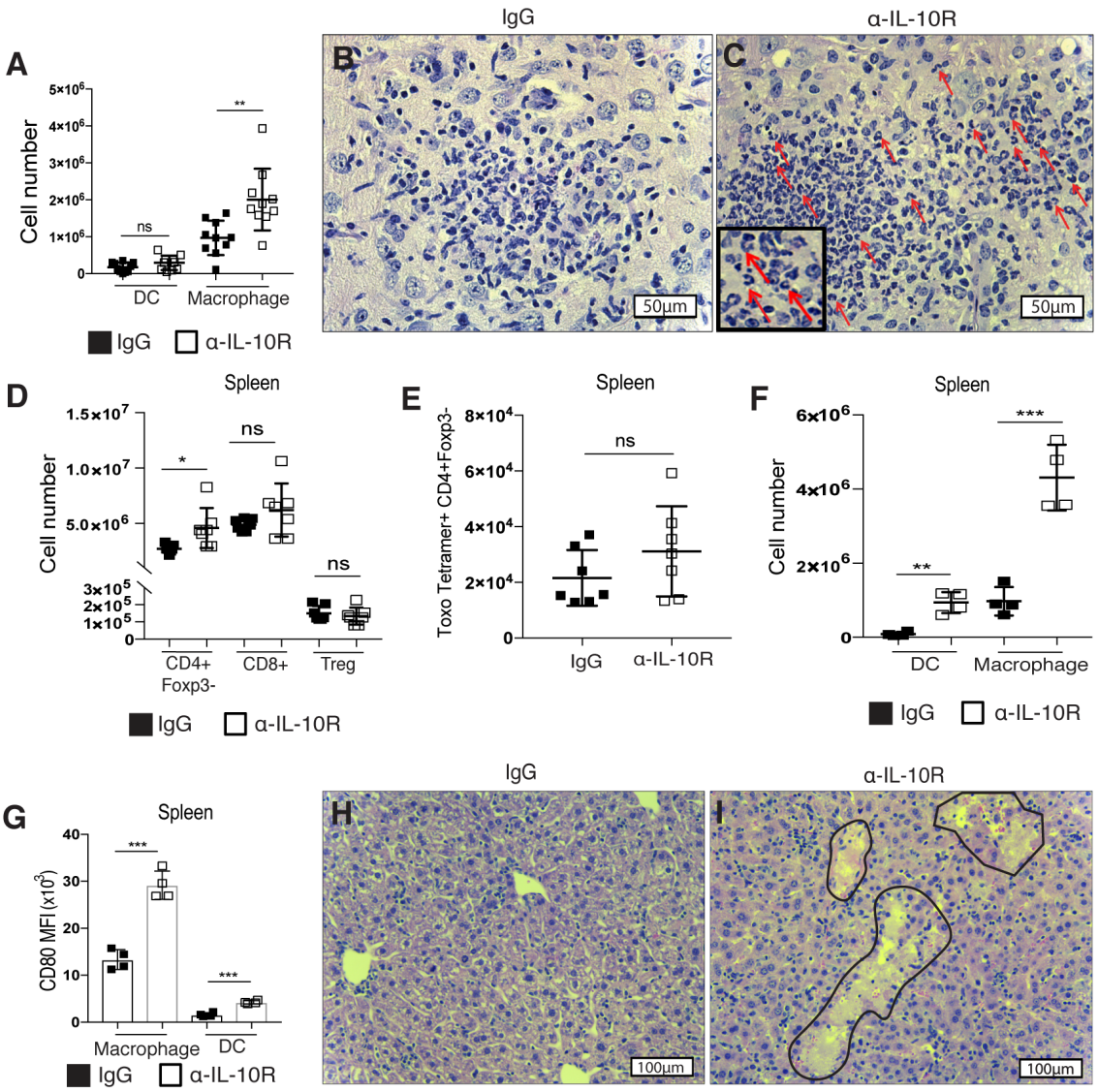
Figure 4.1. **Blockade of IL-10R during chronic *T. gondii* infection leads to broad changes in the immune response and fatal immunopathology in the brain.** (**A-K**) Rat IgG or an α-IL-10R blocking antibody was administered to chronically infected mice beginning at day 28 post-infection. (**A-B**) Representative H&E stained brain sections from a chronically infected control-treated mouse (**A**) and an α-IL-10R-treated mouse (**B**). (**C**) T cells isolated from the brain were analyzed by flow cytometry. Effector CD4+ T cells (CD4+Foxp3-), CD8 T cells, and T_{regs} (CD4+Foxp3+) were enumerated (n=5 per group, data is pooled from two independent experiments and analyzed using a randomized block ANOVA). (**D**) The frequency of Ki67+ effector T cells was measured by flow cytometry from mononuclear cells isolated from the brains of control and α-IL-10R-treated mice (n=3-5 per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA). (**E**) Parasite-specific CD4+ effector T cells were assessed by flow cytometry using an MHCII-peptide tetramer. (**F**) The mean fluorescence intensity (MFI) of CD80 and CD86 on brain-infiltrating APCs. DCs were gated on

$CD45^{hi}CD3^{-}NK1.1^{-}CD19^{-}CD11c^{+}MHCII^{hi}$ live singlet cells and macrophages were gated on $CD45^{hi}CD3^{-}NK1.1^{-}CD19^{-}CD11c^{-}CD11b+$ live singlet cells (n=6 per group, data is representative of 3 independent experiments and analyzed using Student's t test). (**G-H**) qRT-PCR was done using mRNA isolated from whole brains of chronically infected control or α-IL-10R-treated mice. Relative expression was normalized to the control (IgG-treated) group (n=4-5 per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA). (**I**) Representative flow plots showing the neutrophil population isolated from the brains of chronically infected mice. Neutrophils were gated on $CD45^{hi}CD3^{-}CD19^{-}NK1.1^{-}CD11b^{+}Ly6G^{+}$ live singlet cells. Number in plot indicates the mean frequency ± standard error. (**J**) Neutrophils were identified by flow cytometry from cells isolated from the brains of chronically infected control or α-IL-10R-treated mice (n=4-5 per group, data is pooled from three independent experiments and analyzed by randomized block ANOVA). (**K**) Total cyst numbers from the brains of chronically infected control and α-IL-10R-treated mice were counted using light microscopy (n=3-4 per group, data is pooled from three independent experiments and analyzed by randomized block ANOVA). * denotes $p<0.05$, ** denotes $p<0.01$, and *** denotes $p<0.001$ for all panels.

The increased proliferation of effector T cells in the brain correlated with an increase in the expression of costimulatory molecule CD80 on infiltrating dendritic cells and macrophages in α-IL-10R-treated mice (Figure 4.1F). The increased numbers of immune cells infiltrating the brain was associated with increased mRNA levels of many pro-inflammatory cytokines and chemokines, including IFNγ, IL-6, TNFα, IL-17, and CXCL1, demonstrating a widespread increase in inflammation in the brain in the absence of IL-10 signaling (Figure 4.1G-H). An increase in IL-17 production is notable in this model, as infection of wild-type mice with *T. gondii* typically leads to a robust T_h1-polarized immune response, characterized by IL-12 and IFNγ production that persists throughout the chronic stage, with very little production of T_h17-associated cytokines[365,366]. Indeed, production of IL-17 has been linked to a loss of IL-27-mediated regulation of immune responses during infection with *T. gondii*[291,366], suggesting a pathogenic role for IL-17 in this context.

Production of IL-17 and CXCL1 has previously been shown to enhance recruitment of neutrophils to inflamed tissues and enhance their activity in certain disease contexts[367-372]. Neutrophil recruitment to the central nervous system however, has been shown to be detrimental in many cases[119,372,373]. With the increased mRNA levels of both IL-17 and CXCL1 seen in the brain after IL-10R blockade, we wanted to assess whether more neutrophils were recruited to the inflamed brain in this context. Indeed, α-IL-10R-treated mice had a nearly three-fold increase in neutrophil numbers in the brain in comparison to control-treated mice (Figure 4.1I-J and Supplementary Figure 4.1B-C). Despite the increased T cell response in the brain with IL-10R blockade, no change in the number of parasite cysts was observed (Figure 4.1K).

The effects of IL-10R blockade during the chronic phase of infection were not limited to the inflamed brain, as increased CD4+Foxp3- effector T cells were found in the spleens of α-IL-10R-treated mice (Supplementary Figure 4.1D). Similar to what was observed in the brain, though total CD4+ effector T cells were increased in the spleen after IL-10R blockade, there was no increase in parasite tetramer-specific CD4 T cells in this tissue (Supplementary Figure 4.1E). We also observed an increase in myeloid cells in the spleen following IL-10R blockade (Supplementary Figure 4.1F). These myeloid cells were also highly activated, with higher levels of CD80 expression on APCs in the spleens of α-IL-10R-treated mice as opposed to controls (Supplementary Figure 4.1G). Large areas of necrosis were also observed in the livers of α-IL-10R-treated mice (Supplementary Figure 4.1H-I), suggesting that the increased inflammatory response seen following IL-10R blockade can contribute to extensive immunopathology in not only the brain but peripheral tissues as well. Overall, a loss of IL-10 signaling specifically during the chronic infection led to widespread immune cell activation that rapidly resulted in fatal immunopathology.



Supplementary Figure 4.1. IL-10R blockade results in increased APC activation and immunopathology in the periphery along with increased numbers of infiltrating macrophages in the brain. (**A**) Total number of infiltrating DCs and macrophages in the brains of chronically infected control and α-IL-10R-treated mice analyzed by flow cytometry (n=5 per group, data is pooled from two independent experiments and analyzed by randomized block ANOVA). (**B-C**) Representative H&E stained brain sections from chronically infected control (**B**) and α-IL-10R-treated (**C**) mice. Red arrows indicate the presence of neutrophils in the brain parenchyma. (**D**) Total T cell numbers in the spleens (n=3-4 per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA) and (**E**) parasite tetramer+ CD4 T cells in the spleens of control and α-IL-10R-treated mice (n=3-5 per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA). (**F**)

Total myeloid cell numbers in the spleens analyzed by flow cytometry (n=4 per group, data is representative of three independent experiments and analyzed using Student's t test) of control and α-IL-10R-treated mice. (**G**) The MFI of CD80 on macrophages and DCs in the spleen (n=4 per group, data is representative of two independent experiments and analyzed using Student's t test). (**H-I**) Representative H&E stained sections of the livers of chronically infected control and α-IL-10R-treated mice. α-IL-10R-treated mice show areas of necrosis (outlined in black) not seen in controls. * denotes $p<0.05$, ** denotes $p<0.01$, and *** denotes $p<0.001$ for all panels.

Blockade of ICOSL does not decrease IL-10 production during chronic infection, but leads to expanded T cell populations in the brain.

Despite clear evidence that IL-10-mediated regulation of immune responses during *T. gondii* infection is required for host survival, little is known about what signals can induce IL-10 production in activated T cells during the chronic inflammation in the brain associated with the later stages of infection. Several studies have implicated a role for ICOS in inducing IL-10 production in cases of acute inflammation[207,359]. We found ICOS-expressing T cells in the brain during chronic *T. gondii* infection, as well as infiltrating APCs expressing the ICOS-ligand (ICOSL) (Figure 4.2A-B). Additionally, ICOS was most highly expressed on the activated T cells in the brain compared to secondary lymphoid organs (Supplementary Figure 4.2A). To address whether ICOS signaling is important for T cell production of IL-10 in the brain during chronic infection, we treated chronically infected IL-10-eGFP reporter (*Tiger*) mice with the ICOSL blocking antibody. Surprisingly, following ICOSL blockade in the chronic stage of infection, we did not observe decreases in IL-10 production from either CD4+Foxp3- effector T cells or CD4+Foxp3+ regulatory T cells in the brain (Figure 4.2C), and IL-10 mRNA expression from whole brain homogenate was also not decreased (Figure 4.2D). Although IL-10 levels were not decreased after α-ICOSL treatment, we unexpectedly observed a two- to three-fold increase of both CD4+Foxp3- and CD8+ effector T cells, respectively (Figure 4.2E). This increase in effector T cell number was not due to a loss of the local regulatory T cell population, as their numbers in the brain were also increased, though to a lesser degree than the effector T cell populations (Figure 4.2E). The increased numbers of T cells in the brains α-ICOSL-treated animals could be seen

dispersed throughout the brain parenchyma (Figure 4.2F-G), though unlike α-IL-10R treated animals, ICOSL blockade was not lethal in the observed timeframe. Furthermore, we observed an increase in the number of tetramer-positive CD4+ effector T cells in the brain (Figure 4.2H), suggesting that ICOSL blockade can lead to an expansion of parasite-specific CD4+ effector T cells. We also found an increase in the number of IFNγ-producing effector T cells in the brain (Figure 4.2I), while mRNA levels of other pro-inflammatory cytokines and chemokines were not increased (Figure 4.2J). Infiltrating myeloid cell numbers were also assessed, revealing almost a two-fold increase in dendritic cells in the brain compared to control treated animals (Figure 4.2K). Though increased numbers of dendritic cells were found in the brain following α-ICOSL treatment, there was no effect on IL-12 production from either the dendritic cells or macrophages isolated from the brain (Supplementary Figure 4.2B), suggesting that continued production of IL-12 is not reliant on ICOS-ICOSL interactions. An important distinction between IL-10R blockade and ICOSL blockade in the chronic phase of infection was a difference in neutrophil recruitment. Whereas an increase in neutrophil numbers in the brain was seen in α-IL-10R-treated mice (Figure 4.1I-J), no significant increase was seen in α-ICOSL-treated mice (Figure 4.2L-M).

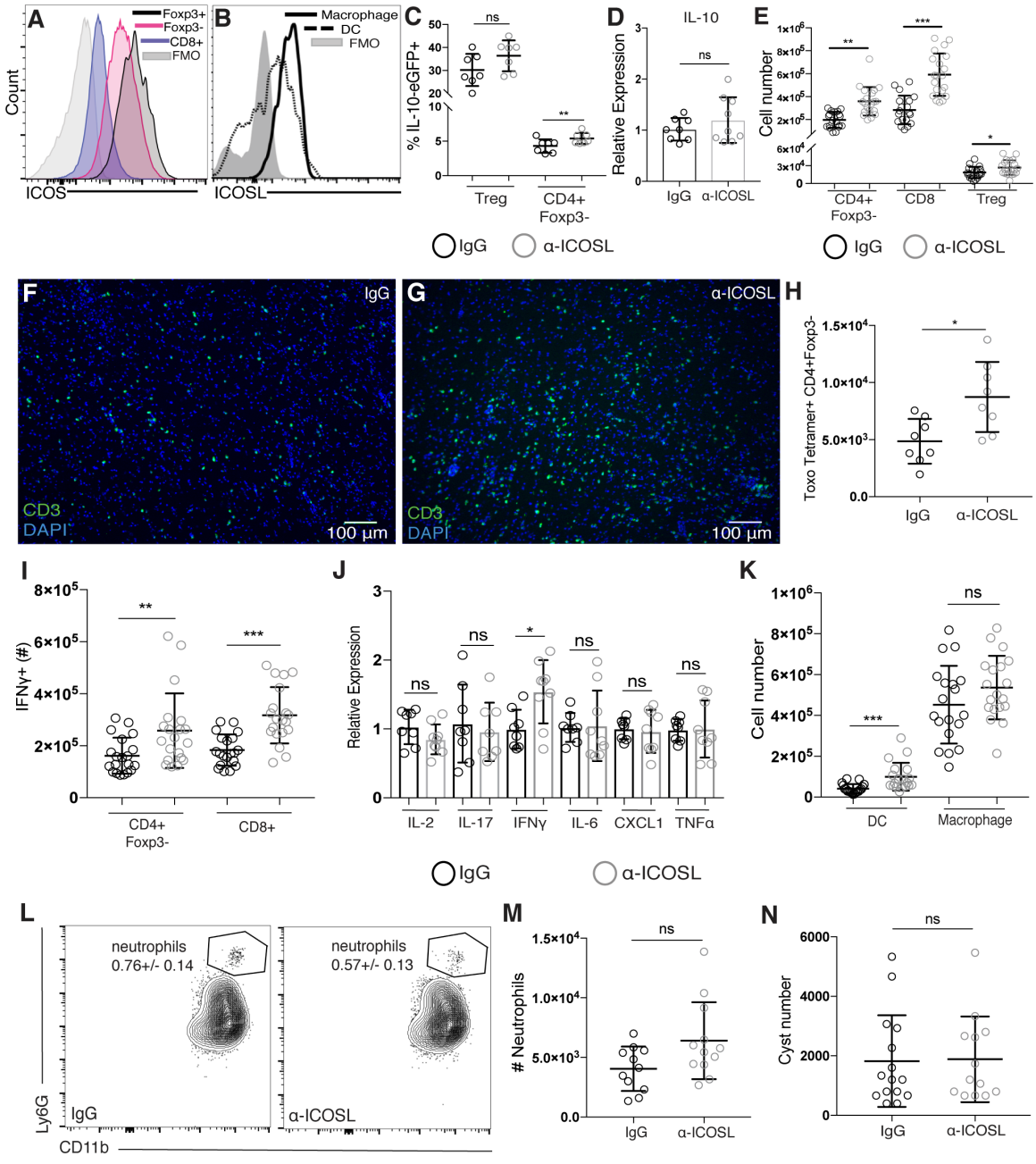
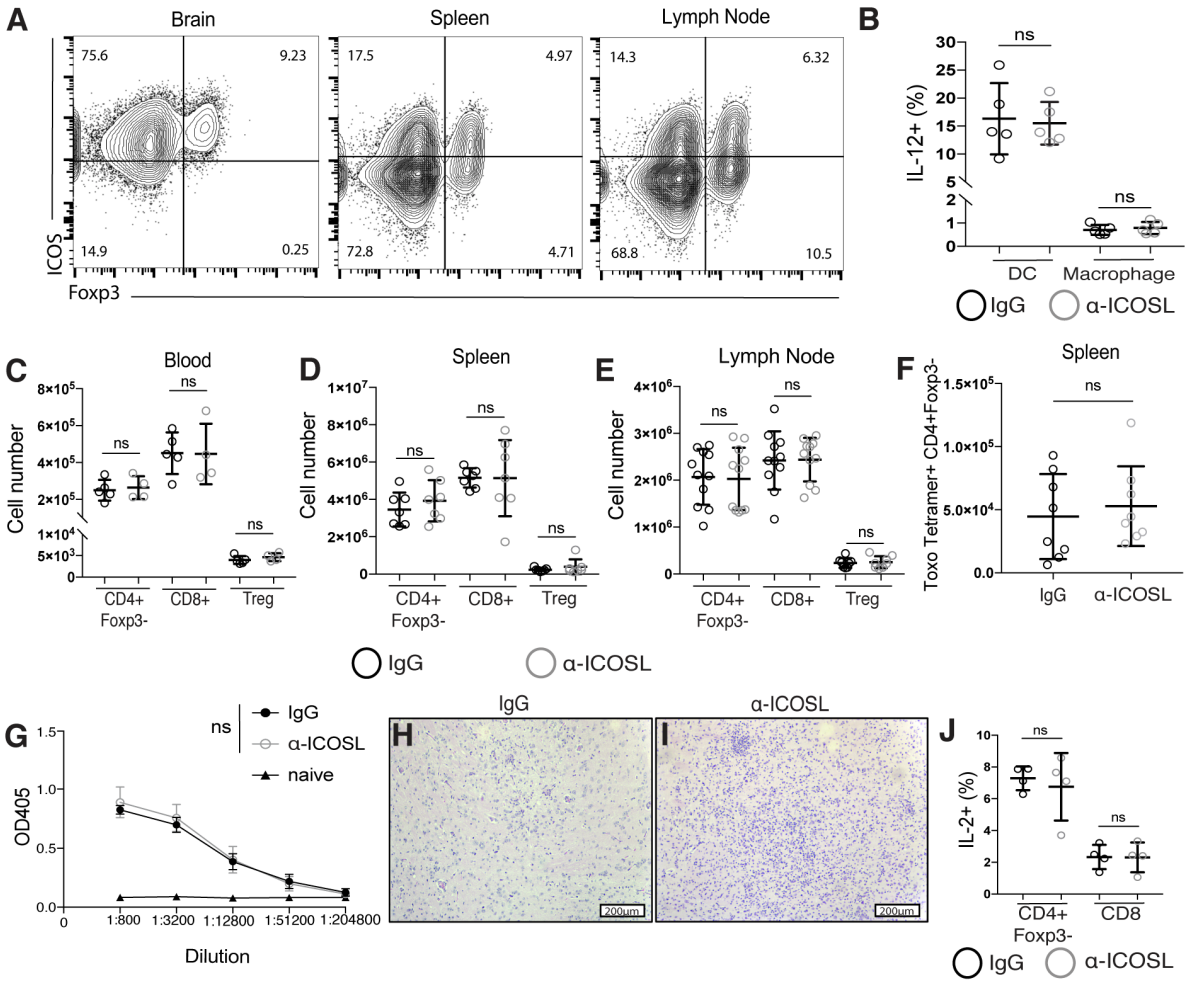


Figure 4.2. Blockade of ICOSL does not decrease IL-10 production during chronic *T. gondii* infection, but leads to expanded T cell populations in the brain. (**A**) ICOS expression on T cells and (**B**) ICOSL expression on infiltrating myeloid cells isolated from the brains of chronically infected C57BL/6 mice using flow cytometry. Immune cell populations were gated as described in Figure 4.1C. (**C**) IL-10 production was assessed using chronically infected control or α-ICOSL-treated IL-10-eGFP reporter (*Tiger*) mice. The frequency of IL-10-GFP+ CD4 effector and T_{reg} cells isolated from the brain is shown (n=3-4 per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA). (**D**) The relative

expression of IL-10 mRNA in chronically infected whole brains from C57BL/6 mice treated with control or α-ICOSL blocking antibody. Relative expression was normalized to the control (IgG-treated) group (n=4 per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA). (**E-N**) Chronically infected C57BL/6 mice were treated with an α-ICOSL blocking antibody or control rat IgG. (**E**) Total T cell numbers isolated from the brain were analyzed by flow cytometry (n=3-4 per group, representative data is pooled from 5 independent experiments and analyzed by randomized block ANOVA). (**F-G**) Representative brain sections stained for CD3 (green) from control (**F**) or α-ICOSL-treated (**G**) mice. (**H**) Parasite-specific CD4+ effector T cells were identified by flow cytometry using an MHCII-peptide tetramer. (**I**) IFNγ production from T cells isolated from the brains of control and α-ICOSL-treated mice was measured following *ex vivo* restimulation (n=3-5 per group, data is pooled from five independent experiments and analyzed using randomized block ANOVA). (**J**) qRT-PCR was done using mRNA isolated from whole brains of chronically infected mice after α-ICOSL blockade. Relative expression was normalized to the control (IgG-treated) group (n=3-5 per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA). (**K**) DC and infiltrating macrophage numbers isolated from the brains of chronically infected control or α-ICOSL-treated mice was determined by flow cytometry (n=3-5 per group, data is pooled from five independent experiments and analyzed using randomized block ANOVA). (**L**) Representative flow plots of the neutrophil population and (**M**) total numbers of neutrophils isolated from the brain. Number in plots indicates the mean frequency ± standard error (n=3-4 per group, data is pooled from three independent experiments and analyzed using randomized block ANOVA). (**N**) Total cyst numbers from the brains of chronically infected control and α-ICOSL-treated mice enumerated by light microscopy (n=4-5 per group, data is pooled from three independent experiments and analyzed using randomized block ANOVA). * denotes $p<0.05$, ** denotes $p<0.01$, and *** denotes $p<0.001$ for all panels.

Blockade of ICOSL primarily affected the T cell populations in the CNS and did not affect T cell numbers in the spleen, draining LNs, or blood of α-ICOSL-treated mice (Supplementary Figure 4.2C-E). Interestingly, though we observed a significant increase in the number of tetramer-positive CD4 effector T cells in the brain following ICOSL blockade, we did not see an increase in tetramer-positive CD4 effector T cells in the spleen at the same time point (Supplementary Figure 4.2F). ICOS signaling has also been shown to be crucial for primary antibody responses to infection[187,194,195]. In order to assess whether the increased T cell numbers found in the brains of α-ICOSL treated animals were merely a result of a change in parasite burden due to decreased antibody production, both parasite-specific serum IgG titers and brain cyst burden was measured. No change was seen in either circulating parasite-specific IgG (Supplementary Figure 4.2G) or parasite burden in the brain (Figure 4.2N). Representative H&E stained brain sections from control or α-ICOSL-treated mice reflected the increased density of infiltrating immune cells seen by flow cytometry and immunohistochemistry, but lacked severe pathology in comparison to those treated with α-IL-10R blocking antibody (Supplementary Figure 4.2H-I). Compared to α-IL-10R treated mice, fewer areas of necrosis and tissue destruction were evident following α-ICOSL treatment (Supplementary Figure 4.2H-I). Together, these results suggest that ICOS signaling limits excessive T cell responses in the brain during chronic neuroinflammation independent of changes in either IL-10 production or parasite burden.



Supplementary Figure 4.2. ICOSL blockade leads to increased numbers of DCs and IFNγ-producing CD4+ and CD8+ T cells in the brain, with no changes in peripheral T cell responses. (**A**) CD4 T cells were isolated from the brain, spleen, and cervical lymph nodes of chronically infected mice. Representative ICOS expression is shown on effector CD4 and regulatory T cells. Cells are pre-gated on $CD3^{+}CD4^{+}$ live singlets. Plots are representative of three independent experiments of 2-4 mice. (**B**) The frequency of IL-12+ DCs and infiltrating macrophages isolated from the brain and measured by flow cytometry following *ex vivo* incubation with BFA (n=5 per group, data is representative of 3 independent experiments and analyzed using Student's t test). (**C-E**) T cell numbers analyzed by flow cytometry after isolation from the blood (**C**) (n=4-5 per group, data is representative of two independent experiments and analyzed using Student's t test), spleen (**D**) (n=3-4 per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA), and cervical lymph nodes (**E**) (n=3-4 per group, data is pooled from 3 independent experiments and analyzed using randomized block ANOVA) after α-ICOSL blockade. (**F**) Parasite-specific CD4 effector T cells

were stained for flow cytometry using an MHCII-peptide tetramer (n=4 per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA). (**G**) Parasite-specific total IgG was measured by ELISA in the serum of chronically infected mice after α-ICOSL blockade (n=4-5 mice per group, data is representative of two independent experiments and analyzed using two-way ANOVA). (**H-I**) Representative H&E stained brain sections from chronically infected mice following control or α-ICOSL blockade. (**J**) IL-2 production was assessed following *ex vivo* restimulation of T cells isolated from the brains of control or α-ICOSL treated mice.* denotes $p<0.05$, ** denotes $p<0.01$, and *** denotes $p<0.001$ for all panels.

ICOSL blockade during chronic infection is associated with increases in Ki67$^+$ T cells and decreases in Annexin V$^+$ T cells in the brain.

We next wanted to determine how lack of ICOS-ICOSL interaction during chronic inflammation leads to increased numbers of T cells in the CNS. Using immunofluorescence staining for Ki67, increased numbers of proliferating CD4+ and CD8+ effector T cells were found throughout the brain after ICOSL blockade during chronic infection (Figure 4.3A-B and Figure 4.3D-E). Using flow cytometry, we confirmed this increase in the number of proliferating effector T cells in the brain (Figure 4.3C and Figure 4.3F). Distinct from IL-10R blockade, the increase in Ki67+ effector T cells in the brain following ICOSL blockade was not correlated with an increase in CD80 or CD86 expression on infiltrating APCs (Figure 4.3G). Rather, ICOSL blockade led to the upregulation of the pro-survival factor Bcl-2 in both CD4+Foxp3- and CD8+ effector T cells isolated from the brain (Figure 4.3H-I). Bcl-2 has been shown to increase T cell survival by preventing cytochrome c release from mitochondria that would normally lead to apoptosis[374,375]. The upregulation of Bcl-2 following ICOSL blockade suggested that effector T cells could have increased survival in the brain following α-ICOSL treatment. To assess this, we isolated T cells from the brain and stained them with Annexin V as a marker of apoptosis. Interestingly, we observed a significant decrease in the frequency of Annexin V-positive CD4 and CD8 T cells in the brain after α-ICOSL treatment, suggesting that fewer T cells in the brain are undergoing early apoptosis following ICOSL blockade (Figure 4.3J-L). Together, these results suggest that ICOS limits effector T cell proliferation and survival during chronic neuroinflammation.

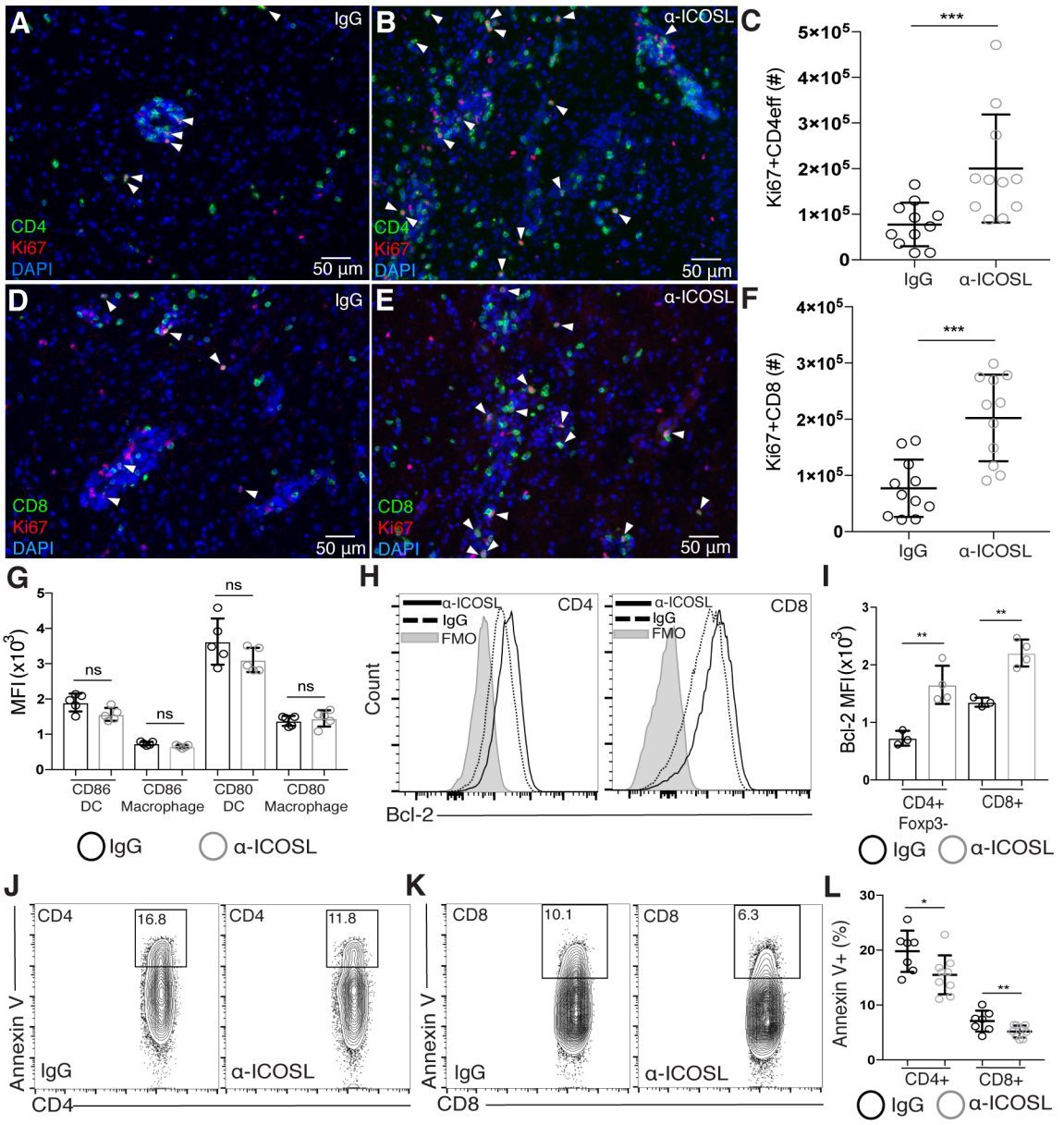


Figure 4.3. ICOSL blockade during chronic infection is associated with increases in Ki67$^+$ T cells and decreases in Annexin V$^+$ T cells in the brain. Brain sections from chronically infected control (**A, D**) and α-ICOSL-treated (**B, E**) mice were stained for CD4 or CD8 (green), Ki67 (red), and DAPI (blue). White arrowheads indicate Ki67+ CD4 or CD8 T cells. (**C, F**) The number of Ki67+ CD4 effector T cells (**C**) and CD8 T cells (**F**) in the brains of control or α-ICOSL-treated mice was analyzed by flow cytometry (n=3-4 mice per group, data is pooled from 3 independent experiments and analyzed by randomized block ANOVA). (**G**) The MFI of CD80 and CD86 on infiltrating APCs isolated from the brain after α-ICOSL or control treatment (n=5 mice per group, data is representative of 3 independent experiments and analyzed using Student's

t-test). (**H**) Representative histograms of Bcl-2 expression measured by flow cytometry on effector CD4+ and CD8+ T cells isolated from the brain. (**I**) The MFI of Bcl-2 on effector T cell populations isolated from the brains of chronically infected control or α-ICOSL-treated mice (n=3-4 per group, data is representative of 4 independent experiments and analyzed using Student's t-test). (**J-L**) CD4 and CD8 T cells were isolated from the brains of control and α-ICOSL-treated mice and stained with Annexin V for analysis by flow cytometry. Representative flow plots showing Annexin V staining on CD4 (**J**) and CD8 (**K**) T cells are shown. CD4 and CD8 T cells were pre-gated on $CD3^+$ nucleated ($Hoechst^+$), live, singlet cells. (**L**) Frequency of Annexin V^+ CD4 and CD8 T cells in the brains of control and α-ICOSL-treated mice. (n=3-5 mice per group, data is pooled from 2 independent experiments and analyzed by randomized block ANOVA). * denotes $p<0.05$, ** denotes $p<0.01$, and *** denotes $p<0.001$ for all panels.

Blockade of ICOSL increases CD25 expression and STAT5 phosphorylation in effector T cells in the brain during chronic infection.

IL-2 signaling in T cells has been shown to support both their proliferation and survival[376,377]. Thus, we wanted to determine if the increased effector T cell proliferation and survival factor Bcl-2 expression could be a result of increased response to IL-2 following ICOSL blockade. After ICOSL blockade, both CD4+Foxp3- and CD8+ effector T cells isolated from the brain expressed higher levels of CD25, and there was a two- to three-fold increase in the total number of CD25+ effector T cells in the brain compared to control treated animals (Figure 4.4A-D). In order to assess whether more of the infiltrating T cells in the brain may be responding to IL-2, we used immunofluorescence staining for phosphorylated STAT5 (pSTAT5), the main signaling molecule downstream of the IL-2R. Correlated with the increased number of CD25+ effector T cells found in the brain with ICOSL blockade, we observed increased numbers of pSTAT5-positive CD4+ and CD8+ T cells in the brains of α-ICOSL-treated animals (Figure 4.4E-J).

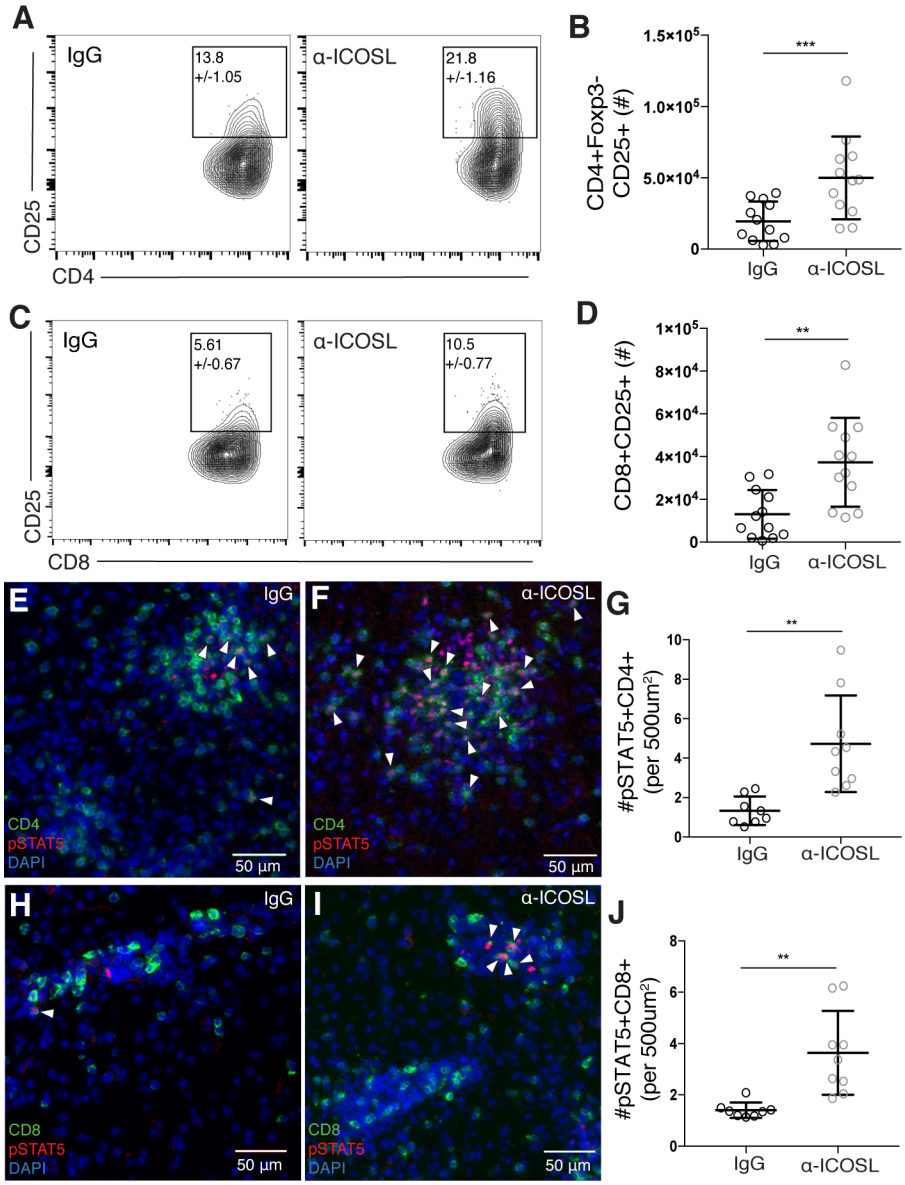
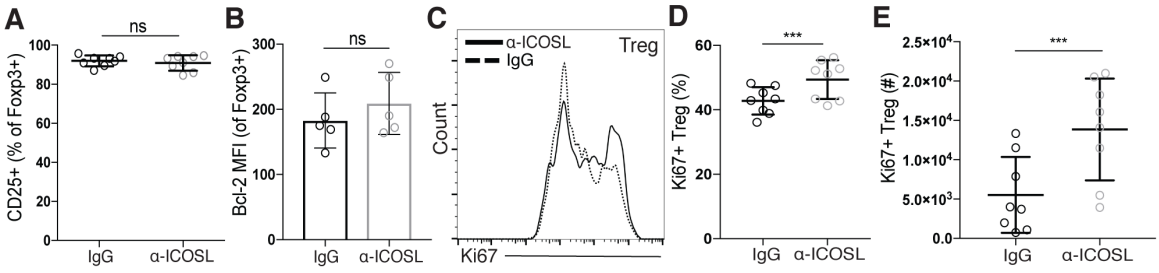


Figure 4.4. ICOSL blockade increases CD25 expression and STAT5 phosphorylation in effector T cells in the brain during chronic infection. (**A-D**) T cells were isolated from the brains of chronically infected control or α-ICOSL-treated mice. Representative flow plots of CD25+CD4+ effector T cells (**A**) and CD25+CD8+ effector T cells (**C**) are shown. Number in gate indicates the mean frequency of CD25+ cells ± standard error. (**B**) Total number of CD25+ CD4+ effector T cells and (**D**) total number of CD25+ CD8+ T cells isolated from the brain (n=4 per group, data is pooled from 3 independent experiments and analyzed by randomized block ANOVA). (**E-F and H-I**) Brain sections from chronically infected control (**E, H**) and α-ICOSL-treated (**F, I**) mice were stained for CD4 or CD8 (green), pSTAT5 (red) and DAPI (blue). White arrowheads indicate pSTAT5+ CD4 or CD8 T cells. (**G**) The number of pSTAT5+CD4+ and (**J**)

number of pSTAT5+CD8+ T cells were quantified per 500 μm^2 (n=4-5 mice per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA). * denotes $p<0.05$, ** denotes $p<0.01$, and *** denotes $p<0.001$ for all panels.

The increases in IL-2-associated signaling molecules occurred independently of increased IL-2 production from effector T cells (Supplementary Figure 4.2J), suggesting that similar amounts of IL-2 are available in the brain environment after α-ICOSL or control treatment. Interestingly, T_{regs} were increased in number in the brain following ICOSL blockade (Figure 4.1E), but unlike effector T cells, T_{reg} expression of CD25 and Bcl-2 was unaffected after α-ICOSL treatment (Supplementary Figure 4.3A-B). On the other hand, we did observe increases in Ki67+ T_{regs} in the brain after α-ICOSL treatment (Supplementary Figure 4.3C-E). These results indicate that ICOSL blockade may differentially affect regulatory and effector T cells populations in the inflamed brain. Taken together, these data suggest that after ICOSL blockade, effector T cells may maintain higher levels of CD25 and pSTAT5, which in turn could support increased proliferation, Bcl-2 expression, and decreased apoptosis of effector T cells during chronic inflammation in the brain.



Supplementary Figure 4.3. ICOSL blockade leads to more Ki67+ T_{regs} in the brain independent of changes in T_{reg} expression of CD25 or Bcl-2. (**A-E**) T cells were isolated from the brains of chronically infected C57BL/6 mice following control or α-ICOSL treatment and stained for flow cytometry. (**A**) Frequency of CD25+ T_{regs} (n=4 per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA) and (**B**) MFI of Bcl-2 in T_{regs} from control and α-ICOSL treated mice (n=5 per group, data is representative of five independent experiments and analyzed using Student's t-test). (**C**) Representative histogram showing Ki67 expression in T_{regs} and (**D**) Frequency of Ki67+ T_{regs} isolated from the brains of control and α-ICOSL treated mice (n=3-5 per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA). (**E**) Number of Ki67+ T_{regs} in the brains of control and α-ICOSL treated mice (n=3-5 per group, data is pooled from two independent experiments and analyzed using randomized block ANOVA). * denotes $p<0.05$, ** denotes $p<0.01$, and *** denotes $p<0.001$ for all panels.

IL-10R blockade does not affect Bcl-2 or CD25 expression on T cells in the brain during chronic infection.

While increased expression of both Bcl-2 and CD25 was seen on effector T cells in the brain following ICOSL blockade, no change in Bcl-2 expression was seen in effector T cell populations isolated from the brains of α-IL-10R-treated mice (Figure 4.5A-B). Additionally, increased levels of CD25 were not observed to the same degree with α-IL-10R treatment, as there were no changes in the levels of CD25 expression on CD4+Foxp3- T cells, and only a slight increase in CD25 expression on the CD8+ T cell population (Figure 4.5C).

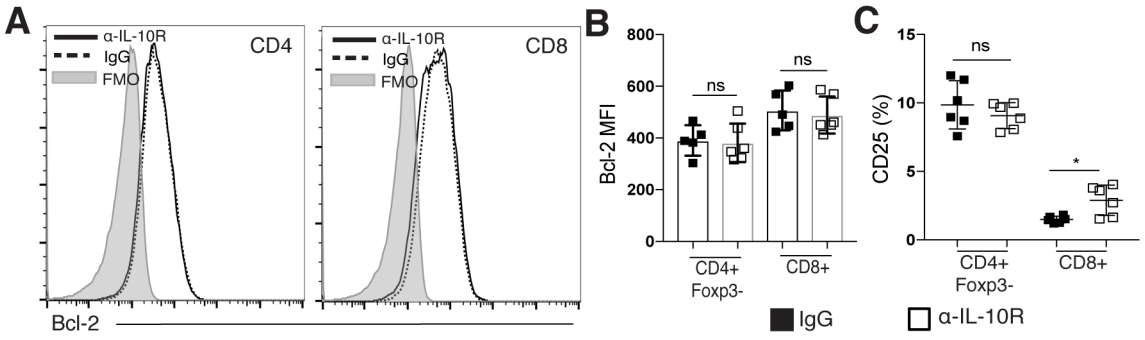


Figure 4.5. **IL-10R blockade does not affect Bcl-2 or CD25 expression on T cells in the brain during chronic infection.** (**A-C**) Effector T cell populations isolated from the brain were analyzed by flow cytometry following α-IL-10R blockade. (**A**) Representative histograms showing Bcl-2 expression on effector CD4+ and CD8+ T cells isolated from the brains of chronically infected mice treated with control or α-IL-10R blocking antibody. (**B**) The MFI of Bcl-2 on effector T cells isolated from the brains of control or α-IL-10R-treated mice (n=5-6 per group, data is representative of three independent experiments and analyzed using Student's t-test). (**C**) The frequency of CD25+ effector T cells isolated from the brains of chronically infected mice after control or α-IL-10R treatment (n= 6 per group, data is representative of two independent experiments and analyzed using Student's t-test). * denotes $p<0.05$, ** denotes $p<0.01$, and *** denotes $p<0.001$ for all panels.

Overall, though both IL-10R blockade and ICOSL blockade resulted in increased numbers of effector T cells in the brain, IL-10R blockade correlated with increased APC activation and inflammatory cytokine mRNA expression, while ICOSL blockade correlated with upregulation of IL-2-associated signaling molecules and decreased apoptosis in effector T cells. These data further support that ICOS and IL-10 signaling pathways can differentially promote regulation of effector T cell responses in the brain during chronic infection.

4.3 Discussion

Inflammation is required to promote clearance of pathogens, but preventing excessive inflammation during immune responses to infection is necessary to avoid immune-mediated tissue damage[240,288,310]. In cases of chronic infection, this balance between inflammation and regulation must be maintained over long periods of time[309,366], but many of the signals required to maintain control of ongoing immune responses are not well understood. Our results identify one such signal, ICOS, whose expression on activated T cells in the chronically infected brain provides a regulatory signal preventing overabundant T cell accumulation in the inflamed CNS. We show that ICOSL blockade is associated with increased expression of IL-2-associated signaling molecules CD25, pSTAT5, and Bcl-2, as well as effector T cell accumulation in the brain.

ICOS, similar to its family member CD28, was initially characterized for its ability to amplify both B cell antibody production and T cell inflammatory cytokine production in cases of acute inflammation[31,187,358]. The pro-inflammatory role for ICOS signaling was subsequently supported by human data, as humans carrying mutations in the ICOS gene are included in the class of mutations known as common variable immunodeficiency (CVID)[211,212,214]. Patients with CVID have increased susceptibility to bacterial infections, and are diagnosed based on severely decreased class-switched antibody production, further implicating ICOS as important for T cell-dependent B cell responses[214]. Interestingly, further data from patients with CVID began to emerge highlighting widespread immune system abnormalities outside of the B cell compartment, particularly splenomegaly, a loss of naïve CD4+ T cells and expansion of activated CD4+ T cells, and increased T cell inflammatory cytokine production[210,212,378].

Additionally, despite being defined as an immunodeficiency disease, about 20% of CVID patients also present with autoimmune complications, though the pathogenesis of this autoimmunity remains unclear[220].

Studies regarding the role of ICOS in promoting regulation of immune responses largely come from mouse models, where ICOS has been shown to both support T_{reg} populations and promote IL-10 production[207,209,359,361]. Our observation of increased effector T cell numbers in the brain following ICOSL blockade suggests a loss of regulation that could occur if the T_{reg} population is decreased or defective after α-ICOSL treatment. Surprisingly, we found no evidence of a local T_{reg} defect following ICOSL blockade, suggesting that T_{regs} in the brain during chronic infection rely on signals other than ICOS to support their survival, proliferation, and accumulation in the tissue, as well as their production of IL-10. Many activated effector T cells in the brain continue to produce IL-2 in the chronic phase of infection with *T. gondii*, so it is possible that the largely CD25+ T_{regs} in the CNS rely mainly on IL-2 signals for their maintenance in an inflamed tissue, whereas other signals are required during homeostasis when lower levels of IL-2 would be present in the absence of an ongoing effector T cell response. We also observed an increase in DC numbers in the brain after α-ICOSL treatment that could be potentiating the increased T cell response. Though this could be a contributing factor, we do not anticipate that the increased DC number in the brain is the primary driver of the T cell expansion we observe with ICOSL blockade because the activation status of the DCs, including MHC and costimulatory molecule expression is not increased.

One of the main differences observed between IL-10R blockade and ICOSL blockade in the chronic phase of infection was the disparity in the magnitude of the

immune response. IL-10R is expressed more broadly than ICOS, which may explain the widespread inflammation and lethality of IL-10R versus ICOSL blockade. IL-10R is expressed by most hematopoietic cells, but can also be induced on non-hematopoietic cells such as fibroblasts and endothelial cells, rendering them also able to respond to IL-10 in inflammatory settings[127,139,379]. In the context of chronic *T. gondii* infection, this could explain why changes were seen in myeloid and T cell subsets in both the brain and peripheral tissues following α-IL-10R blockade. On the other hand, ICOS is only expressed on activated T cells during chronic *T. gondii* infection, and its highest levels of expression were found in the inflamed brain. This more limited expression pattern of ICOS compared to IL-10R could potentially explain the more local and specific response to ICOSL blockade.

Another interesting aspect of the immunological phenotype seen with IL-10R blockade during chronic infection was the differential effects on the accumulation of CD8+ and CD4+ effector T cell populations in the brain. The accumulation of CD4+ effector T cells, but not CD8+ T cells, is in agreement with a previously published study examining T cell responses in IL-10 knockout mice[309]. In the current study, we also examined the phenotype of APCs and the proliferative capacity of T cells in the brain. Following IL-10R blockade, local APCs in the brain upregulate CD80, so it is possible that the CD4+ effector T cells infiltrating the brain are interacting with the highly activated local MHCII+ APCs more so than the CD8+ effector T cells, leading to their increased accumulation. It is interesting to note that, of the brain-infiltrating APCs isolated during chronic *T. gondii* infection, only a small fraction of them are classic CD8α+ cross-presenting DCs that could interact in a TCR-dependent fashion with

infiltrating CD8+ T cells[117]. This could suggest that the activated CD8+ effector T cells in the brain rely less on local restimulation through TCR-MHC and costimulatory interactions, and are therefore less affected by extrinsic mechanisms of suppression through APCs in this context. Overall, it is largely unknown what kinds of secondary signals, such as TCR-MHC or costimulatory interaction, are required for activated T cells to carry out effector function and survive at a distal site of inflammation after initial priming in secondary lymphoid organs, though it is likely these requirements differ in some way for CD8+ and CD4+ T cells. Though the overall number of CD8+ effector T cells in the brain is not increased, we still observed an increased frequency of Ki67+ CD8+ T cells in the brain after IL-10R blockade, suggesting that this population is still responsive to IL-10-mediated suppression; perhaps through an intrinsic response to IL-10 that limits their proliferative capacity. IL-10 has been shown to be able to directly inhibit proliferation of CD8+ T cells *in vitro*[354], as well as control the threshold of their response to antigen upon initial activation[380]. Much is still unknown about the direct effects of IL-10 on activated CD8+ T cells that could contribute to their regulation, though these data further suggest that CD8+ effector T cells may be differentially regulated from the CD4+ effector T cell compartment in the brain.

Two well-characterized inhibitory co-receptors are CTLA-4 and PD-1, both of which have been shown to carry out their inhibitory effect at least partially through inhibition of the PI3K/Akt signaling pathway[67,381]. In this light, it is interesting to consider ICOS, which is a potent activator of the PI3K/Akt pathway[175,181], as also providing an inhibitory signal to T cells during chronic inflammation. Initial Akt activation induced during priming has been associated with increased T cell responses,

both through promotion of T cell proliferation and survival[172,382,383]. However, more recent reports have shown that constitutive Akt activation in CD8+ T cells is associated with decreased expression of CD122 and Bcl-2, and can promote the development of short-lived effector T cells over the development of memory T cells, while constitutive STAT5 signaling can maintain Bcl-2 expression and favor the development of memory precursor cells[384,385]. These results emphasize that the fate of T cells is extremely sensitive to both the level and duration of signaling cascades like PI3K/Akt. During the chronic neuroinflammation seen with *T. gondii* infection, continued activation of Akt downstream of ICOS in activated T cells in the brain could potentially serve as an intrinsic mechanism of controlling T cell responses by downregulating IL-2-associated signaling molecules and driving effector T cells in the brain to be short-lived effectors rather than memory precursors. Indeed, our data support the hypothesis that increased IL-2 signaling in activated effector T cells in the inflamed brain provides survival signals, like upregulation of Bcl-2, which limits T cell apoptosis and allows for T cell accumulation in the tissue. Continued signaling through ICOS, then, might normally promote short-lived effector T cells by providing a strong PI3K/Akt signal that inhibits expression of IL-2-associated signaling molecules, leading to apoptosis.

Overall, we provide evidence that ICOS costimulation provides an inhibitory signal to antigen-experienced effector T cells in the chronically inflamed brain. These data postulate a regulatory role for ICOS in T cells during chronic neuroinflammation that is distinct and more specific than the suppressive role of IL-10. Altogether, while we show that IL-10 is absolutely required for preventing exaggerated immune responses and immunopathology, other regulatory signals like ICOS are also likely at play during

chronic inflammation that provide more fine-tuned suppression of ongoing immune responses without affecting IL-10-mediated suppression.

CHAPTER 5

Genetic Deficiencies in ICOS: Additional functions during homeostasis and infection with *Toxoplasma gondii*

This chapter contains preliminary, unpublished data generated by Carleigh O'Brien.

5.1 Rationale

Based on the early findings described in Chapter 4 regarding loss of ICOS signaling, we sought a genetic model of ICOS deficiency for additional support of the results found following ICOSL antibody blockade during chronic infection with *T. gondii*. To do this, we analyzed the phenotype of both constitutive ICOS KO and ICOS $Y^{181}F$ mice (hereby referred to as ICOS YF) during chronic infection with *Toxoplasma gondii*. ICOS YF mice express normal levels of ICOS on activated T cells, but contain a Y181F mutation in the cytoplasmic tail, preventing recruitment of and signaling through $PI3K^{386}$. For reasons discussed below, we ultimately chose to use the ICOSL antibody blockade for the majority of our studies, as it was found to be superior to the genetic models of ICOS deficiency discussed in this chapter. Despite the caveats of the following genetic models discussed below, we found that constitutive deficiency in ICOS or ICOS-mediated PI3K signaling resulted in widespread changes in the immune system during both homeostasis and *Toxoplasma gondii* infection.

As discussed in Section 1.8, the role of ICOS on T cells is extensive and highly context-dependent. ICOS signaling has been shown to be important in mediating germinal center formation and antibody class switching in B cells, promoting both T_{reg} survival and cytokine production, as well as effector T cell function [24,31,68,173,183,184,195,202,203,207,357-361]. Based on this published literature, we hypothesized that genetic deficiency in ICOS or inability to signal through PI3K downstream of ICOS ligation from birth would affect immune responses both at baseline and during *T. gondii* infection. Thus, in the following studies, we directly compared and characterized the

basic phenotype of ICOS KO and ICOS YF mice during chronic *T. gondii* infection, as well as during homeostasis in the absence of active inflammation.

5.2 Results and Discussion

ICOS KO and ICOS YF mice chronically infected with T. gondii *have increased numbers of T cells in the brain and increased expression of CD25.*

Given the results discussed in Chapter 4 with ICOSL blockade during chronic *T. gondii* infection, we hypothesized that both ICOS KO and ICOS YF mice would have increased effector T cell responses in the brain, correlated with increased IL-2-associated signaling molecules CD25 and Bcl-2. Similar to WT mice, ICOS KO and ICOS YF mice survived the acute phase of infection with no overt signs of disease, but between 30 and 35 days post-infection, both ICOS KO and ICOS YF mice appeared sicker than their WT counterparts (lack of grooming, some neurological symptoms) (unpublished observation). Both ICOS KO and ICOS YF mice had increased numbers of effector CD4+ and CD8+ T cells in the brain (ICOS KO mice had about 3x as many effector T cells, while ICOS YF mice had about 2x as many effector T cells) during chronic infection (Figure 5.1A). Interestingly, while ICOS KO mice also had increased numbers of T_{regs} in the brain compared to WT mice, ICOS YF mice did not (Figure 5.1A). Similar to what was seen following ICOSL blockade during the chronic phase of infection, both CD4+ and CD8+ effector T cells had increased expression of CD25 on their surface (Figure 5.1B), suggesting that these cells had increased capacity to respond to IL-2 in the brain environment. Also similar to the phenotype following ICOSL blockade, increased Bcl-2 expression in CD4+ effector T cells was correlated with the increased CD25 expression in the brain (Figure 5.1C), suggesting that CD4+ effector T cells that lack ICOS signaling could instead maintain IL-2 signaling that could support their survival. However, unlike ICOSL blockade, no change in Bcl-2 in the CD8+ effector T cell population was

observed in the brain (Figure 5.1C). These results suggest that complete genetic deficiency in ICOS or ICOS-mediated PI3K signaling can support CD4+ T cell expansion in the inflamed brain, perhaps through increased response to IL-2, while CD8+ T cells lacking ICOS or ICOS-mediated PI3K signaling, though they maintain somewhat higher levels of CD25, do not have the associated upregulation of Bcl-2 as seen following short-term ICOSL blockade. Despite no observed increases in Bcl-2 expression in the ICOS KO or ICOS YF CD8+ T cells in the inflamed brain, this population was still expanded, suggesting ICOS-mediated effects independent of increased CD8+ T cell survival that led to their accumulation in the brain.

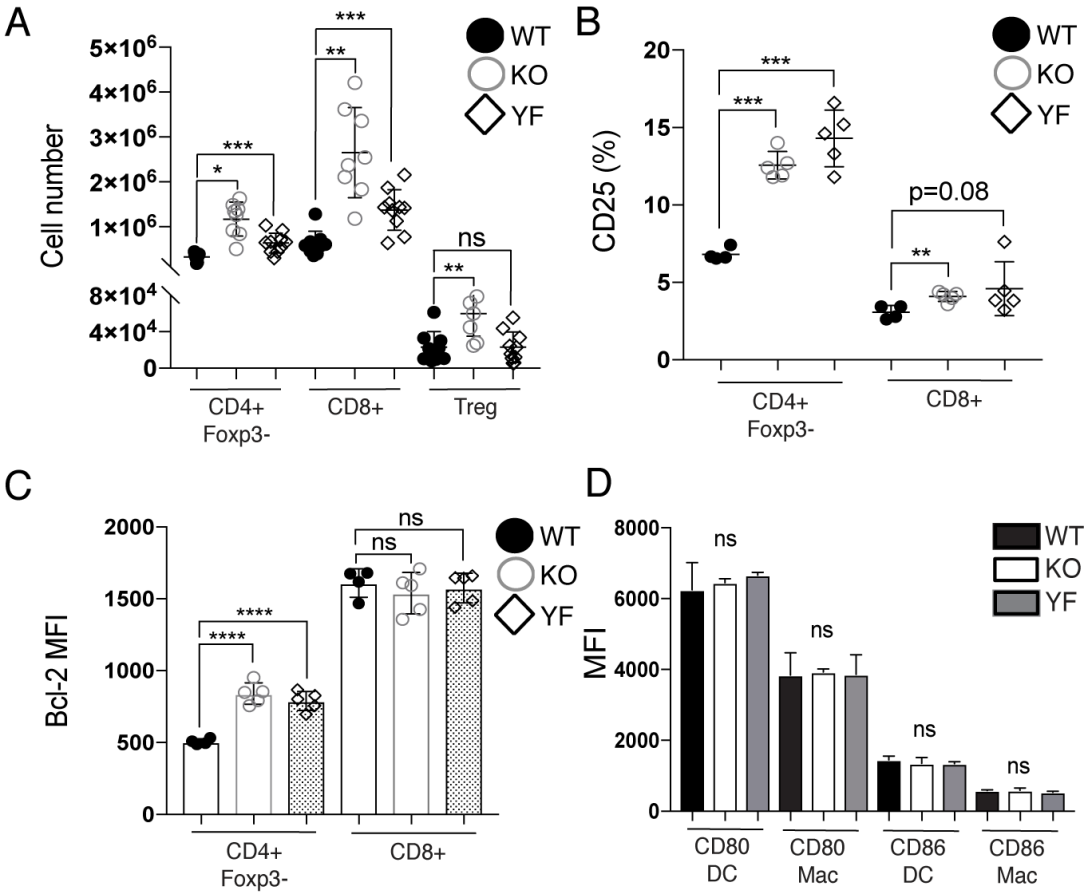


Figure 5.1. ICOS KO and ICOS YF mice chronically infected with *T. gondii* have increased numbers of T cells in the brain and increased expression of CD25. WT, ICOS KO, and ICOS YF mice were infected with *T. gondii*. 28 days post-infection, BMNC were isolated and analyzed by flow cytometry. **(A)** Total numbers of effector CD4+ T cells (CD4+Foxp3-), effector CD8+ T cells, and T_{regs} (CD4+Foxp3+) isolated from the brain (n=3-5 per group, data is pooled from two independent experiments and analyzed using a randomized block ANOVA). **(B)** Frequency of CD25+ effector CD4+ T cells and CD8+ T cells in the brain. (n=4-5 per group, data is representative of two independent experiments and analyzed using one-way ANOVA). **(C)** MFI of Bcl-2 on effector CD4+ and CD8+ T cells in the brain (n=4-5 per group, data is representative of a single experiment and analyzed using one-way ANOVA). **(D)** MFI of CD80 and CD86 on macrophages and DCs isolated from the brains of chronically infected mice. Populations pre-gated as previously described (n=4-5 per group, data is representative of a single experiment and analyzed using one-way ANOVA). * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$, and **** denotes $p<0.0001$ for all panels.

This differential effect on Bcl-2 expression in CD4+ and CD8+ effector T cells in ICOS KO versus ICOS YF mice could be due to differences in the response of these populations to IL-2. Though CD4+ and CD8+ effector T cells have been shown to activate STAT5 to equivalent levels downstream of IL-2R signaling, the downstream targets of pSTAT5 in these two populations could be highly dependent on the environmental context, particularly in an inflammatory state as seen during chronic *T. gondii*. This could lead to differential transcriptional outcomes that could reflect the difference in Bcl-2 expression seen in each population. IL-2R signaling has also been shown to have differential effects in other signaling pathways in CD4+ and CD8+ effector T cells. For example, S6K has been shown to become more activated in CD8+ T cells downstream of IL-2R signaling compared to CD4+ T cells[387,388]. As pS6K can promote cell growth, this could explain the differential sensitivity to IL-2 seen in CD8+ and CD4+ T cells[387,388]. Therefore, with a lack of ICOS or ICOS-mediated PI3K signaling, it is possible that increased responsiveness to IL-2 in the CD4+ effector T cell population in the brain results in increased Bcl-2 and survival, while increased responsiveness to IL-2 in the CD8+ T cell population in the brain could lead to expansion through increased proliferation instead. Indeed, preliminary results have shown an increase in the frequency of proliferating CD8+ effector T cells in the brain in both ICOS KO and ICOS YF mice during chronic infection.

It is also interesting to consider the differential effects on Bcl-2 expression in the CD8+ T cell population in mice lacking ICOS or ICOS-mediated PI3K signaling compared to the short-term ICOSL blockade described in Chapter 4. Whereas short-term blockade of ICOSL during chronic infection led to increased Bcl-2 expression in both

CD4+ and CD8+ effector T cell populations in the brain (Figure 4.3), this effect was not seen in the CD8+ effector T cell population in the brain of ICOS KO and ICOS YF mice. This difference could be a result of lacking ICOS signaling completely during the entirety of the T cell activation process. It is currently unknown how initial ICOS-mediated costimulation affects T cells immediately upon TCR activation versus how this costimulation might affect an already activated T cell at a site of inflammation. The outcome of ICOS signaling has been shown to be highly dependent on the environmental and cell-intrinsic context[24,68,175]. It is likely that the stimulatory signals (and inhibitory signals) a naïve T cell receives immediately upon activation in a secondary lymphoid tissue would be very different from the signals they would encounter after activation and trafficking to a site of inflammation. As such, it would not be surprising that differential signaling inputs from the TCR, other costimulatory or co-inhibitory molecules, or even cytokine signals, might affect the downstream outcome of ICOS costimulation early versus late in a T cell's life. From the results described above, it seems that particularly in the CD8+ T cell population, lacking ICOS signaling from initial activation (as would be the case in ICOS KO and ICOS YF mice), though it still leads to an expansion of this population, could result in a somewhat different phenotype to that seen after short-term ICOSL blockade, which could be affecting highly activated effector T cells that did receive ICOS costimulation early in their life. Similar to the short-term ICOSL blockade, the expansion of effector T cells occurred independently of increased CD80 or CD86 expression on brain-infiltrating APCs (Figure 5.1D). Together, these data suggest that the effect of a loss of ICOS or ICOS-mediated PI3K signaling results in T cell expansion in

the inflamed brain, without additional costimulatory signals from APCs, during chronic *T. gondii* infection.

Both ICOS KO and ICOS YF mice have a T_{reg} defect in the brain and spleen during chronic T. gondii *infection.*

The expansion of effector T cells seen in the brains of ICOS KO and ICOS YF mice during chronic *T. gondii* infection could be due to an intrinsic effect of the loss of ICOS signaling, but could also be explained by an extrinsic lack of suppression in the inflamed CNS. ICOS has been shown to be important for T_{reg} survival and maintenance of an effector T_{reg} population both during homeostasis and in models of inflammation[206-208,360,361]. Therefore, we assessed the basic phenotype of the T_{reg} population in the CNS during chronic *T. gondii* infection in ICOS KO and ICOS YF mice. We found a decreased frequency of T_{regs} in the brain in both ICOS KO and ICOS YF mice, as both had about half the WT frequency of T_{regs} (Figure 5.2A-D). Interestingly, however, of the T_{regs} that were in the brain during chronic *T. gondii* infection, WT and ICOS KO T_{regs} maintained the same level of Foxp3 expression by MFI, but ICOS YF T_{regs} in the brain had significantly decreased Foxp3 expression (Figure 5.2E). Similarly, while both WT and ICOS KO T_{regs} were capable of maintaining CD25 expression in the inflamed brain environment, significantly fewer ICOS YF T_{regs} expressed CD25 (Figure 5.2F).

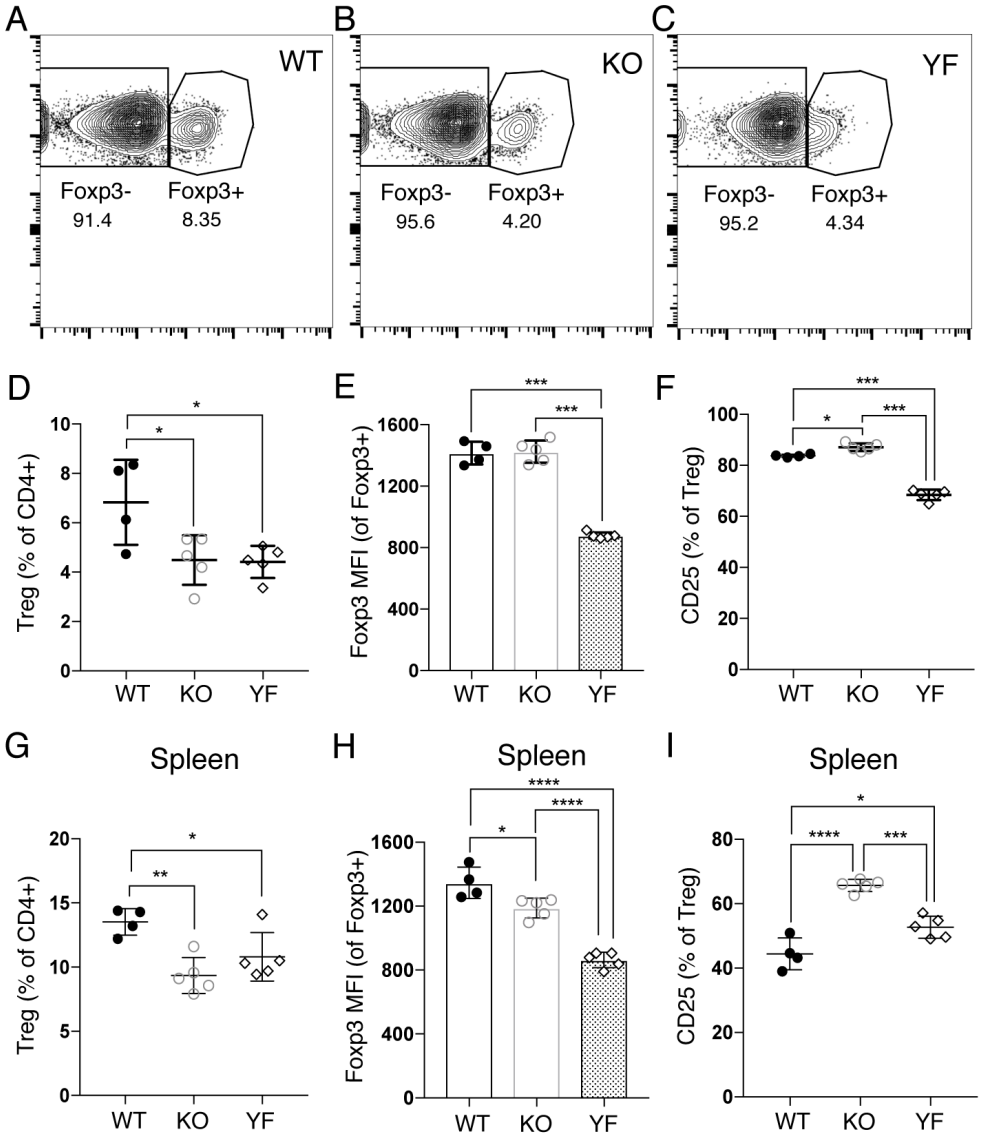


Figure 5.2. Both ICOS KO and ICOS YF mice have a T_{reg} defect in the brain and spleen during chronic *T. gondii* infection. (A-F) BMNCs were isolated from chronically infected WT, ICOS KO, and ICOS YF mice and analyzed by flow cytometry. **(A-C)** Representative flow cytometry plots are shown indicating the decrease in T_{reg} frequency observed in ICOS KO and ICOS YF mice. **(D)** Total T_{reg} frequency in the brain (n=4-5 mice, data is representative of two independent experiments and analyzed using one-way ANOVA). **(E)** MFI of Foxp3 in the Treg population isolated from the brain (n=4-5 mice, data is representative of two independent experiments and analyzed using one-way ANOVA). **(F)** Frequency of CD25+ Tregs in the brain (n=4-5 mice, data is representative of two independent experiments and analyzed using one-way ANOVA). **(G-I)** T cells from the spleens of chronically infected WT, ICOS KO, and ICOS YF mice were isolated and analyzed using flow cytometry. **(G)** T_{reg} frequency in the spleen (n=4-5

mice, data is representative of two independent experiments and analyzed using one-way ANOVA). **(H)** Foxp3 MFI on T_{regs} in the spleen (n=4-5 mice, data is representative of two independent experiments and analyzed using one-way ANOVA). **(I)** Frequency of CD25+ T_{regs} in the spleen (n=4-5 mice, data is representative of two independent experiments and analyzed using one-way ANOVA). * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$, and **** denotes $p<0.0001$ for all panels.

The difference in maintenance of Foxp3 and CD25 expression in T_{regs} completely lacking ICOS and those lacking ICOS-mediated PI3K signaling is surprising, as PI3K is largely considered the main downstream signaling molecule activated with ICOS ligation[24,68,169,175,177,180,183,386]. The observation that ICOS KO T_{regs} and ICOS YF T_{regs}, though both have population defects as a whole in the inflamed brain (Figure 5.2D), have differential capacity to maintain Foxp3 and CD25 expression among the surviving T_{regs} suggests that different intracellular signaling events are occurring when there is a total lack of ICOS compared to a lack of only ICOS-mediated PI3K signaling. In early studies into the differences in CD28 and ICOS signaling, besides the increased PI3K-Akt activation following ICOS ligation compared to CD28 ligation, it was also noted that mitogen-activated protein kinase (MAPK) family members were differentially activated, resulting in decreased p46 c-Jun activation downstream of ICOS ligation versus CD28 ligation[182]. This is supported by a study showing a difference in the ability of ICOS KO and ICOS YF effector T cells to confer graft-versus-host (GVH) disease. In this study, ICOS YF effector T cells were able to potentiate TCR-mediated calcium flux, while total ICOS KO T cells could not[389]. This potentiated calcium flux was associated with increased inflammatory cytokine production and increased pathology and mortality in mice receiving ICOS YF T cells compared to those receiving ICOS KO T cells[389]. These data further suggest that PI3K-independent signaling pathways downstream of ICOS could play important roles T cell responses.

More recently, a unique motif called IProx was identified in the cytoplasmic tail of ICOS[390]. This motif has homology to TNFR-associated factors TRAF2 and TRAF3, and was shown to recruit and activate the serine/threonine-protein kinase TBK1[390]. TBK1

activation downstream of ICOS ligation was shown to be dispensable for early polarization of T_{fh} cells, but the full differentiation and maturation into germinal center T_{fh} was inhibited after deletion of the IProx motif or depletion of TBK1 in CD4+ T cells[390]. These studies suggest that PI3K-independent signaling pathways can be activated following ICOS ligation, and these pathways may explain some of the differences between the phenotypes of ICOS KO and ICOS YF mice. TBK1 is a member of the inhibitor of NF-κB kinase (IKK) family, and its role has largely been studied in innate immune cell response to infection, where it has been shown to activate interferon-regulatory factor 3 (IRF3) and promote Type I interferon production[391-393]. Additionally, one study investigating the role of TBK1 in T cells found that mice lacking TBK1 specifically in T cells had increased numbers of $CD44^{hi}CD62L^{lo}$ activated T cells producing IFNγ in the spleen during steady state[394]. It was subsequently shown that TBK1 induced the ubiquitination-dependent degradation of Akt, hence why T cells lacking TBK1 showed an accumulation of Akt and increased activation[394]. These results implicate TBK1 as an important negative regulator of the Akt-mTOR pathway in T cells. Interestingly, Akt inhibition has been shown to preferentially inhibit T_{reg} maintenance in a cancer model[395], suggesting that T_{regs} could rely heavily on Akt activation to maintain their suppressive phenotype, and perhaps preferential TBK1 activation downstream of ICOS in ICOS YF mice could be forcing the degradation of Akt and inhibiting T_{reg} maintenance during chronic inflammation with *T. gondii*. However, the role of either TBK1-mediated IRF3 activation or TBK1-mediated inactivation of Akt is unstudied in T_{regs}, but both could potentially contribute to the differential phenotypes seen in the T_{reg} populations of ICOS KO and ICOS YF mice during chronic infection in the CNS.

To determine whether the T_{reg} defect was only present in the active site of inflammation in the brain, we also analyzed the phenotype of T_{regs} in the spleen during chronic infection. Similar to what was observed in the brain, there was a decreased frequency of T_{regs} among the CD4+ T cell population in the spleen (Figure 5.2G). Interestingly, however, we observed that both the ICOS KO and ICOS YF T_{reg} populations in the spleen did not maintain WT levels of Foxp3, though the decrease in Foxp3 MFI was more significant in the ICOS YF T_{reg} population than in the ICOS KO T_{reg} population (Figure 5.2H). Somewhat surprisingly, both the ICOS KO and the ICOS YF mice had more CD25+ T_{regs} in the spleen (Figure 5.2I). This is in contrast to the brain at the same time point, where ICOS KO mice had slightly more CD25+ T_{regs} in the brain than WT mice, but ICOS YF mice had significantly fewer CD25+ T_{regs} in the brain. These data suggest that, while ICOS KO T_{regs} in the spleen express less Foxp3 than WT T_{regs}, they can overcome this deficit in Foxp3 expression at the site of inflammation in the brain, while maintaining higher levels of CD25. In contrast, ICOS YF T_{regs} express less Foxp3 than both WT and ICOS KO T_{regs} in the spleen, but unlike ICOS KO T_{regs} are unable to overcome this deficit in the inflamed brain. ICOS YF T_{regs} also lose the ability to maintain CD25 expression in the inflamed brain, though they express more than WT mice in the spleen. Overall, these results suggest that ICOS expression and ICOS-mediated PI3K signaling are important for maintaining Foxp3 expression, though not CD25 expression, in secondary lymphoid organs during chronic inflammation, but that specific loss of ICOS-mediated PI3K signaling is associated with the inability to maintain both Foxp3 and CD25 at the distal site of inflammation in the brain. On the other hand, T_{regs} completely lacking ICOS signaling can overcome a deficit in Foxp3 at the site of

inflammation and maintain high levels of CD25. These data further suggest that PI3K-independent signaling molecules may be at play in T_{regs} from ICOS YF mice that somehow inhibit the ability to maintain Foxp3 and CD25. Furthermore, these data suggest that the overall decrease in the frequency of T_{regs} in the inflamed brain observed in ICOS KO and ICOS YF mice, could be a result of slightly different intracellular mechanisms resulting from the complete lack of ICOS signaling or only the loss of PI3K signaling downstream of ICOS.

ICOS KO and ICOS YF mice are severely impaired in parasite-specific IgG production and have increased cyst burden in the brain compared to WT mice.

As discussed in Section 1.8.1, ICOS not only plays a role in T_{regs} and effector T cells, but also plays an essential role in the formation of germinal centers and the production of class-switched antibody[31,185,193-196,214,396]. Besides defects in the T_{reg} population and the potential for loss of T_{reg}-mediated suppression in ICOS KO and ICOS YF mice, increased parasite burden as a result of inefficient parasite-specific antibody formation could also contribute to the increased effector T cell responses seen in these mice. To address this question, we first looked for parasite-specific total IgG levels in the serum of chronically infected WT, ICOS KO, and ICOS YF mice.

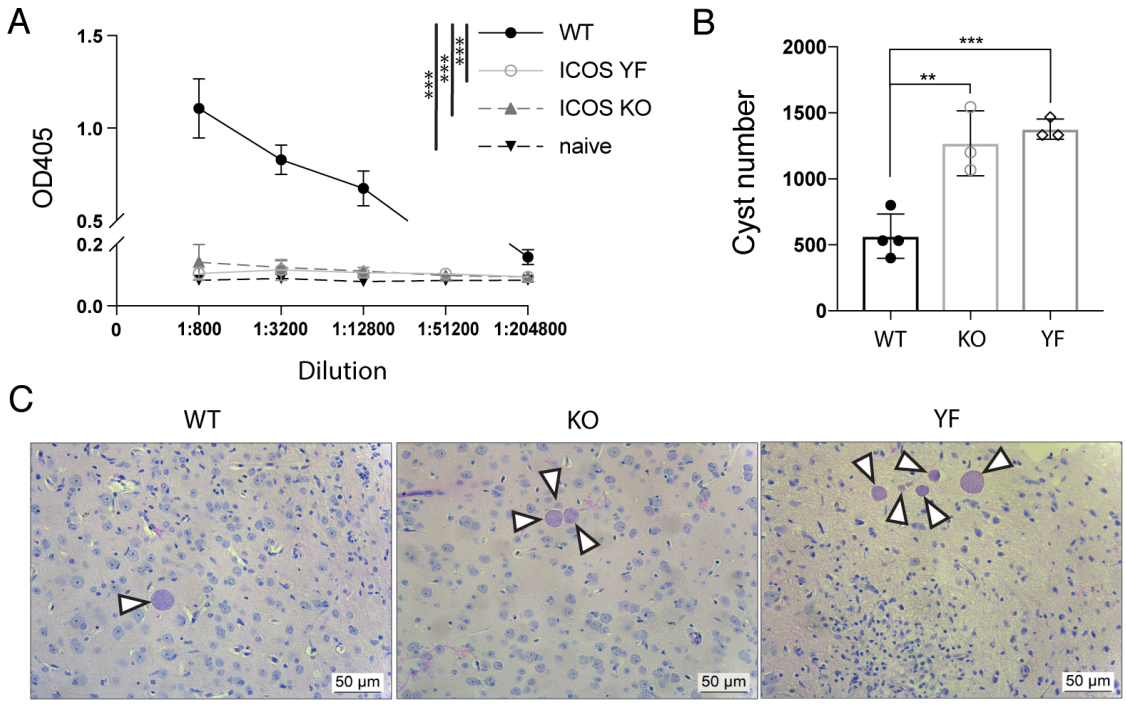


Figure 5.3. ICOS KO and ICOS YF mice have severely impaired parasite-specific IgG production and have increased cyst burden in the brain compared to WT infected mice. (A) Parasite-specific total IgG was measured by ELISA in the serum of chronically infected WT, ICOS KO, and ICOS YF mice (n=3-4 mice per group, data is representative of a single experiment and analyzed using two-way ANOVA). **(B)** Total cyst numbers from the brains of chronically infected WT, ICOS KO, and ICOS YF mice were enumerated by light microscopy (n=3-4 mice per group, data is representative of a single experiment and analyzed using one-way ANOVA). **(C)** Representative H&E stained brain sections from chronically infected WT, ICOS KO, and ICOS YF mice. White arrows indicate individual parasite cysts ranging in size. Scale bar is 50µm. * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$, and **** denotes $p<0.0001$ for all panels.

Similar to what has been previously been published regarding antibody responses in ICOS KO and ICOS YF mice, both showed extremely diminished levels of parasite specific IgG in the serum compared to WT mice during chronic *T. gondii* infection (Figure 5.3A). μMT mice that lack B cells succumb to *T. gondii* infection in the chronic phase with high parasite levels found in the brain, suggesting that antibody responses are crucial in controlling parasite burden in the later stages of infection in the CNS, or that early control of parasite dissemination by antibody responses prevents excessive dissemination to the brain in the chronic phase[299]. We hypothesized that ICOS KO and ICOS YF mice, with their inability to produce parasite-specific IgG, would also have increased parasite burden in the brain. Indeed, both ICOS KO and ICOS YF mice had significantly increased cyst burden in the brain (Figure 5.3B). This increase was reflected in H&E stained histology sections from the brains of chronically infected WT, ICOS KO, and ICOS YF mice. Interestingly, while chronically infected WT mice typically have single cysts in isolation from others, ICOS KO and ICOS YF mice both had increased incidence of cyst "clusters", where multiple cysts were found in close proximity to each other in the brain (Figure 5.3C). These results suggest the inability to control reactivated parasite in the brain, which can then quickly re-encyst in closely neighboring cells. Taken together, these data suggest that ICOS KO and ICOS YF mice cannot produce parasite-specific class switched antibody, likely through inability to differentiate T_{fh} cells and form germinal centers, which can contribute to increased parasite burden in the brain during chronic infection.

ICOS KO and ICOS YF mice have baseline defects in Foxp3 expression and T_{reg} frequency and expanded effector T cell populations in the spleen.

Based on the above results, genetic deficiency in ICOS or ICOS-mediated PI3K signaling has wide-ranging effects on multiple compartments of the immune system. It was possible that some of the T cell abnormalities, including a decreased T_{reg} population and expanded effector T cell populations might be detected at baseline before any active inflammation. To address this, we analyzed the T cell populations in secondary lymphoid organs during homeostasis in adult WT, ICOS KO, and ICOS YF mice. Importantly, the baseline studies using ICOS KO and ICOS YF mice were not done side-by-side, so direct comparisons between ICOS KO and ICOS YF mice should not be made in this case. During homeostasis, ICOS KO mice and ICOS YF mice have a decrease in T_{reg} frequency in the spleen compared to age-matched WT controls (Figure 5.4A and Figure 5.4C). Interestingly, the remaining T_{regs} present in the spleen expressed less Foxp3 by MFI than WT mice (Figure 5.4B and Figure 5.4D), suggesting that the defect in Foxp3 expression in ICOS KO and ICOS YF T_{regs} in the spleen during chronic *T. gondii* infection could stem from a baseline defect present before any active inflammation. Additionally, both CD4+ and CD8+ effector T cell populations were significantly expanded in ICOS KO and ICOS YF mice compared to controls (Figure 5.4E-F). Overall, the decrease in T_{reg} frequency and expanded effector CD4+ T cell population resulted in a skewing of the CD4+ effector:T_{reg} ratio in the spleen in both ICOS KO and ICOS YF mice (Figure 5.4G-H). Overall, these data indicate that mice lacking ICOS or ICOS-mediated PI3K signaling have baseline defects in T_{reg} maintenance, which could allow for expansion of effector CD4+ and CD8+ T cells before the onset of inflammation. It

also suggests that the inflammation seen with chronic *T. gondii* infection could simply be a result of amplification of some of the defects seen in the T_{reg} population at baseline, specifically the decreased Foxp3 expression and decreased frequency in the spleen.

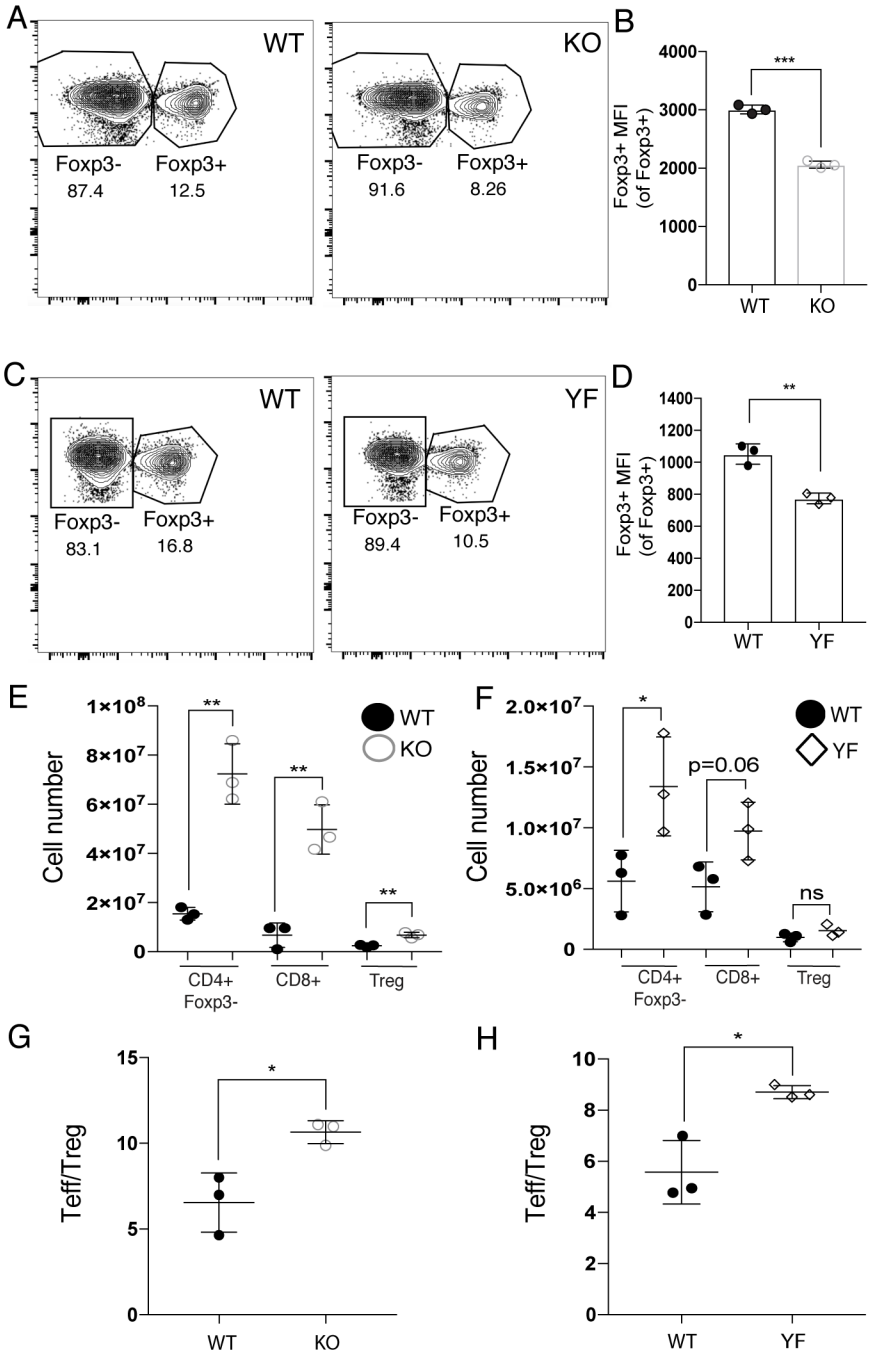


Figure 5.4. ICOS KO and ICOS YF mice have baseline defects in Foxp3 expression and T_{reg} frequency, as well as expanded effector T cell populations in the spleen. (A) Representative flow plots showing the decrease in T_{reg} frequency in the spleens of ICOS KO mice compared to WT controls. **(B)** MFI of Foxp3 in T_{regs} in the spleen of uninfected WT and ICOS KO mice (n=3 per group, data is representative of a single experiment and analyzed using Student's t test). **(C)** Representative flow plots showing the decrease in T_{reg} frequency in the spleens of ICOS YF mice

compared to WT controls. **(D)** MFI of Foxp3 in T_{regs} in the spleen of uninfected WT and ICOS YF mice (n=3 per group, data is representative of a single experiment and analyzed using Student's t test). Effector CD4+ T cells (CD4+Foxp3-), CD8+ T cells, and T_{regs} (CD4+Foxp3+) were enumerated in the spleens of uninfected ICOS KO **(E)** and ICOS YF **(F)** mice. The T_{eff}/T_{reg} ratio in the spleens of uninfected ICOS KO **(G)** mice and ICOS YF **(H)** mice was calculated.

Overall, these results point to ICOS expression and ICOS-mediated PI3K signaling being an important part of the immune response to *T. gondii*, as well as contributing to T cell homeostasis in the absence of ongoing inflammation. The T_{reg} defects seen at baseline were likely amplified after infection with *T. gondii*. It has been reported that during the acute stage of infection with *T. gondii*, the T_{reg} population "crashes" and allows for the rapid expansion of effector T cell populations that contribute to the inflammation required to control parasite replication and dissemination in peripheral tissues[292,397]. This "T_{reg} crash" is reflected in the timing of T_{reg} recruitment to the brain during the chronic stage of infection, as T_{reg} numbers remain low in the early chronic phase until rescuing their numbers and remaining at about 5-8% of the CD4+ T cell population in the brain for the remainder of the infection[362]. It is likely then, in the case of chronic infection of ICOS KO and ICOS YF mice, that this crash cannot be overcome, as the baseline defects in T_{reg} frequency (seen in both ICOS KO and ICOS YF mice) and the inability to maintain Foxp3 and CD25 expression (seen in ICOS YF mice) prevents the T_{reg} population from ever being fully rescued. This skewed T_{eff}:T_{reg} ratio, that begins during homeostasis in the spleen, could then only be amplified with inflammation and a decrease in T_{reg}-mediated suppression. Additionally, the inability to produce sufficient antibody responses and control parasite burden in the brain could also result in amplified effector T cell responses in an attempt to control the increased parasite burden.

These effects on antibody production, parasite burden, and the T_{reg} population don't discount the intrinsic effects of a loss of ICOS or ICOS-mediated PI3K signaling in effector T cells themselves. As seen in Chapter 4, short-term blockade of ICOS signaling

using an ICOSL blocking antibody exclusively in the chronic phase of *T. gondii* infection, results in seemingly intrinsic effects on the IL-2 signaling pathway in effector T cells independent of changes in the T_{reg} compartment or parasite burden. Similar to what was seen during the short-term ICOSL antibody blockade, ICOS KO and ICOS YF effector T cells in the brain during chronic infection had upregulated CD25, supporting the idea of ICOS-mediated effects in effector T cells themselves. Unfortunately, using mice lacking ICOS or ICOS-mediated PI3K signaling from birth, it is difficult separate out the effects of the loss of these signaling pathways in multiple immune compartments in the context of chronic inflammation. For this reason, we decided that blocking ICOS signaling during only the chronic infection, after primary antibody responses, the bulk of initial T cell activation, parasite clearance in peripheral tissues, and the "T_{reg} crash", was the best strategy to identify the short-term effects of a loss of ICOS signaling on an ongoing T cell response.

CHAPTER 6

Conclusions, Future Directions, and Final Remarks

6.1 Summary

In this work, we sought to better understand the regulatory mechanisms at play during chronic neuroinflammation. During any infection, inflammatory innate and adaptive immune responses must occur in order to control the invading pathogen, yet this same inflammatory response can cause "bystander" pathology and damage that can be as severe (or worse) than the infectious pathogen itself. Therefore, it is likely that multiple regulatory mechanisms are involved during the course of inflammation to help limit immune-mediated pathology that may occur. Particularly in the case of ongoing inflammation, as occurs in cases of chronic infection, a delicate balance must be maintained for long periods of time between sustaining enough of an inflammatory effector response to control the pathogen, and at the same time limit as much as possible the negative effects of this inflammation on host tissues.

In order to study some of the regulatory mechanisms involved during chronic inflammation in the CNS, we used infection with the parasite *Toxoplasma gondii*, a parasite that naturally disseminates to the CNS and establishes a chronic infection. This chronic infection requires ongoing T cell recruitment and infiltration into the brain in order to control the parasite and limit spread of the parasite throughout the brain parenchyma. Inflammation in the CNS can very rapidly have devastating consequences, as has been observed in cases of CNS infection, stroke, MS, and other models of neurodegeneration[398,399]. As such, it is crucial to better understand how this inflammation can be regulated at multiple levels for potential therapeutic development.

We initially sought to characterize the T_{reg} population that arises during chronic infection with *T. gondii*. We found that the T_{regs} recruited to the CNS during the chronic

stage of infection were T_h1 polarized, expressing IL-10 as well as canonical markers of T_h1 inflammation IFNγ, Tbet, and CXCR3. Additionally, we found the TCR repertoire that arises in the brain at this stage of infection was remarkably distinct from that of the effector CD4+ T cell population. We found only about 1% overlap between the TCR sequences in the T_{reg} and effector T cell populations arising in the brain during a single infection, suggesting that the T_{reg} population may not be parasite-specific. It has been shown that tT_{reg} populations are largely specific for self-antigen[50,52,54,83,400,401], though there is also evidence of pathogen-specific T_{regs} arising during infection[93,94,402]. In addition to data showing very little overlap in TCR sequence between T_{regs} and effector T cells in the same infection, we also observed extremely low overlap between T_{reg} TCR sequences arising during different infections. Over the course of three separate *T. gondii* infections, while we found up to 8% overlap in the TCR sequences among the effector T cell population arising in the brain (with multiple sequences arising in all three infections), the largest TCR sequence overlap between T_{reg} populations arising in the CNS was only ~0.4%, and no sequences appeared in all three infections assessed. This great variability between infections further supports the idea that the T_{reg} TCR specificity could arise from variables independent of the infection (possibly self-antigen).

TCR signaling has been shown to be important for T_{reg} suppressive function *in vivo*[336], as have interactions with DCs[69,71,76,124], but the role of these interactions in the inflamed CNS is still currently unclear. To begin to assess what contributes to maintaining T_{reg}:DC contact in the CNS during chronic *T. gondii* infection, we blocked either TCR:MHCII interactions or LFA-1:ICAM interactions during the chronic stage of infection. Following either blockade, we found that interactions between T_{regs} and DCs in

the brain were shortened, and T_{reg} velocity was increased, suggesting that both TCR:MHCII and LFA-1:ICAM interactions are involved in maintaining contact between T_{regs} and DCs in the CNS. Overall, these results suggest that T_{reg}:DC interaction is mediated by both TCR:MHCII interactions and LFA-1:ICAM. Taken together, these studies identified and characterized important factors in T_{reg}:DC interactions that could allow them to carry out their suppressive function in the inflamed brain (Figure 6.1).

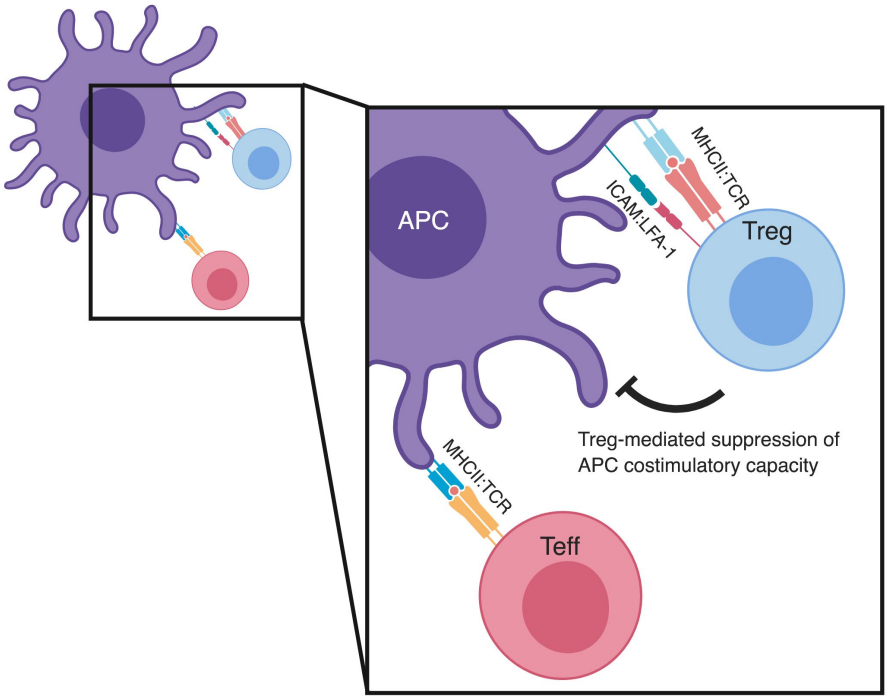


Figure 6.1. Model of T_{reg}:APC interactions that could contribute to T_{reg}-mediated suppression of inflammatory T cell responses. Observations that T_{regs} make long-lived, stable contacts with APCs in the brain led us to hypothesize that this interaction is important for local T_{reg}-mediated suppression during chronic *T. gondii* infection. We found that inhibition of LFA-1:ICAM interactions and TCR:MHCII interactions led to increased T_{reg} velocity and decreased contact time with APCs in the CNS, implicating these two molecules in as important for maintaining this contact. Though whether interfering with this T_{reg}:APC contact in this context inhibits T_{reg}-mediated suppression remains unknown, further study could provide insight into the role these interactions play during chronic inflammatory T cell responses in the CNS.

During the course of a chronic inflammatory response, it is likely that multiple mechanisms of regulation are in place. To better understand other regulatory mechanisms involved during chronic *T. gondii* infection, we also characterized the role of the canonical anti-inflammatory cytokine IL-10. In support of previously published literature[309], we found that IL-10 was essential to the survival of chronically infected mice, and suppressed many aspects of both systemic and local inflammation during the chronic phase of infection. In the brain, IL-10R blockade resulted in increased costimulatory molecule expression on APCs, increased proliferation and accumulation of CD4+ effector T cells, and increased inflammatory cytokine production (including IFNγ, TNF, and IL-6). Interestingly, we also observed an increase in neutrophil chemoattractants IL-17 and CXCL1 in the brain after IL-10R blockade, along with significantly increased neutrophil infiltration into the brain parenchyma. Increased APC activation and T cell accumulation was also seen in secondary lymphoid organs following IL-10R blockade. Overall, the increased inflammatory environment resulting from a loss of IL-10-mediated suppression led to immunopathology and necrosis in both the brain and the liver, which likely contributed to the rapid mortality of chronically infected mice treated with the α-IL-10R blocking antibody. These studies emphasized the necessary role for continued IL-10 signaling during the chronic phase of infection in limiting many aspects of the ongoing inflammatory response (Figure 6.2).

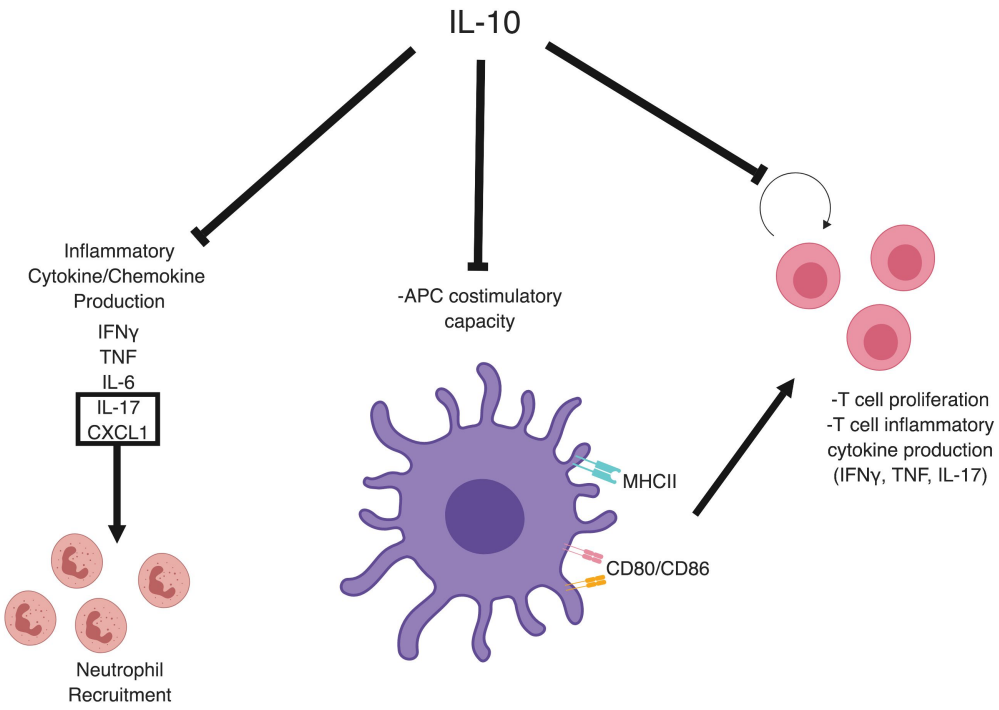


Figure 6.2. The many immunosuppressive effects of IL-10 during chronic *T. gondii* infection. IL-10 is expressed by both T cells and myeloid cells during chronic *T. gondii* infection, and its effects are extensive. We found that without IL-10 signaling, expression of costimulatory molecules on the surface of APCs was increased, potentially allowing them to amplify inflammatory effector T cell responses. Accordingly, we observed increased T cell proliferation in the brain and secondary lymphoid organs after IL-10R blockade, as well as increased inflammatory cytokine production. Interestingly, we found that lack of IL-10 signaling led to increases in IL-17 and CXCL1 expression, both of which have been shown to mediate neutrophil recruitment. Indeed, we observed significant increases in neutrophil numbers in the brain following IL-10R blockade, which likely contributed to the severe immunopathology seen in mice after IL-10R blockade.

IL-10 suppresses immune responses on a large scale during chronic infection, but it is still unknown what signals are required to maintain IL-10 production from effector or regulatory T cells during ongoing inflammatory responses. ICOS signaling has been shown to be a potent inducer of IL-10 in activated T cells[24,68,174,175,182,184,207,219] (discussed in Section 1.8). Because we found that the majority of both effector and regulatory T cells in the brain during chronic *T. gondii* infection express ICOS, we initially hypothesized that ICOS could be required for continued IL-10 production from T cells in the inflamed brain, thereby promoting local regulation at the primary site of inflammation. To address this question, we treated chronically infected mice with an α-ICOSL blocking antibody to prevent ICOS:ICOSL ligation. Surprisingly, we found no defect in IL-10 production from either the T_{reg} or effector T cell populations following ICOSL blockade, yet we still observed an expansion of effector T cell populations in the inflamed brain, suggesting that ICOS:ICOSL ligation normally acts to limit effector T cell expansion independently of promoting IL-10 production. To further distinguish IL-10R blockade from ICOSL blockade, we found that ICOSL blockade, though it resulted in increased numbers of IFNγ-producing T cells in the brain, did not lead to widespread changes in inflammatory cytokine or chemokine production, and we did not observe increased neutrophil recruitment to the inflamed brain. Additionally, while IL-10R blockade increased APC activation and T cell inflammatory responses in both secondary lymphoid organs and the inflamed brain, ICOSL blockade only resulted in increased T cell responses at the tissue site of inflammation, with no changes observed in APC activation in secondary lymphoid organs or the inflamed brain, or T cell responses in secondary lymphoid organs. These results suggested that IL-10 acts on a systemic level to

suppress the inflammatory responses of multiple cell types, while ICOS:ICOSL suppresses T cell responses exclusively at the local site of inflammation.

To further understand what was leading to expanded effector T cell populations in the brain following ICOSL blockade, we assessed both proliferation and survival markers. After ICOSL blockade in chronically infected mice, we observed both increased numbers of proliferating T cells and upregulation of IL-2-associated signaling molecules CD25, pSTAT5, and Bcl-2, suggesting that ICOS signaling normally suppresses IL-2-mediated survival and proliferation (Figure 6.3). Indeed, we also observed decreased Annexin-V staining on T cells in the brain following ICOSL blockade, suggesting that a lack of ICOS signaling can decrease rates of early T cell apoptosis in an inflamed tissue. Interestingly, though ICOS signaling has been implicated in supporting effector T_{reg} populations[206-208,361], we observed no changes in CD25 or Bcl-2 expression among the activated T_{reg} population in the inflamed brain and, in fact, this population was also expanded, though to a lesser degree than the effector T cell populations. Taken together, these results suggest that, during chronic neuroinflammation, ICOS normally inhibits intrinsic IL-2-associated signaling pathways and limits the survival and expansion of effector T cells. In this way, we have identified a novel role for ICOS signaling in regulating long-term inflammatory T cell responses, specifically at the local site of inflammation, independently of supporting the local T_{reg} population or IL-10 production (Figure 6.3).

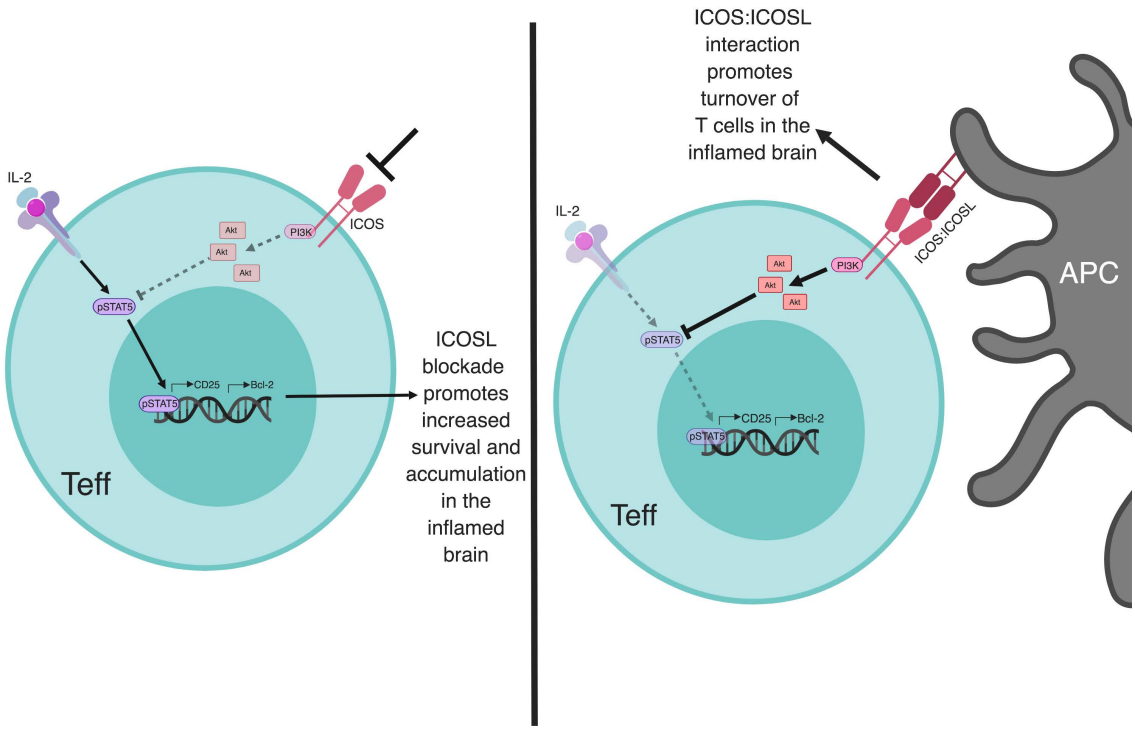


Figure 6.3. Model of ICOS-mediated regulation of effector T cell responses in the CNS during chronic *T. gondii* infection. ICOSL blockade during chronic infection likely decreases the amount of activated Akt within effector T cells infiltrating the inflamed brain. This loss of Akt is correlated with increased response to IL-2 and subsequent increased survival and accumulation of effector T cells in the CNS (left panel). Our observations following ICOSL blockade during chronic infection suggest that ICOS signaling on activated effector T cells in the brain inhibits T cell responses to IL-2, limiting pSTAT5, CD25 and Bcl-2 expression. In this way, it is possible that additional PI3K/ Akt activation downstream of ICOS on activated effector T cells further "pushes" them towards a short-lived effector T cell phenotype, thereby promoting a homeostatic rate of turnover of effector T cells at the site of inflammation during chronic infection (left panel). Indeed, we found decreased Annexin V staining following ICOSL blockade, suggesting that ICOS signaling can promote effector T cell apoptosis.

Finally, we also wanted to determine the effects of genetic deficiency in ICOS or ICOS-mediated PI3K signaling, both during homeostasis and during chronic infection with *T. gondii*. Similar to what has been reported in human patients with CVID (including those with known mutations in ICOS or ICOSL)[211-214,217,221-224], we found that chronically infected mice lacking ICOS (ICOS KO) or ICOS-mediated PI3K signaling (ICOS YF) had multiple abnormalities in the immune response to *T. gondii*. In our preliminary studies, we found increased numbers of effector T cells in the brain, as well as increased CD25 expression among both CD4+ and CD8+ effector T cell populations. Unlike in our ICOSL blockade studies, however, we found significant T_{reg} defects in secondary lymphoid organs and in the inflamed brain. Both ICOS KO and ICOS YF mice had a decreased frequency of T_{regs} in the spleen and brain during the chronic stage of infection. Interestingly, however, the T_{regs} that were present in the brain of ICOS KO mice maintained WT levels of Foxp3 and CD25, while the T_{regs} present in the brain of ICOS YF mice failed to maintain WT levels of Foxp3 and CD25. These results suggest that ICOS and ICOS-mediated PI3K signaling is important for maintaining normal T_{reg} numbers during chronic infection, but hints at potentially interesting differences in how this maintenance is sustained depending on what signaling pathways downstream of ICOS are available to the T_{reg} population (Figure 6.4). Additionally, we found that both ICOS KO and ICOS YF mice failed to generate parasite-specific IgG responses, likely contributing to the increased parasite cyst burden that was observed in the brain during the chronic stage of infection. Overall, these results suggest that multiple immune defects, including decreased antibody responses, increased parasite burden in the brain,

and a failure to maintain an adequate T_{reg} population, could be contributing factors in the increased effector T cell responses seen in both ICOS KO and ICOS YF mice.

We also wanted to determine whether the observed changes in the immune response were a result of infection, or whether there were immune abnormalities already present in homeostatic conditions. When we assessed the baseline phenotype of ICOS KO and ICOS YF mice, we found already largely expanded effector T cell populations in the spleen, correlating with a skewing of the T_{eff}:T_{reg} ratio, suggesting that ICOS and ICOS-mediated PI3K signaling is important for maintaining a T_{reg} population even in the absence of active inflammation. Interestingly, we found that, of the T_{regs} present in the spleen at baseline, both ICOS KO T_{regs} and ICOS YF T_{regs} did not maintain WT levels of Foxp3. These results suggest that, in the absence of inflammation, ICOS and ICOS-mediated PI3K signals are required to maintain normal levels of Foxp3 expression in T_{regs}, but during an ongoing inflammatory response, total ICOS KO T_{regs} can rescue Foxp3 levels (though not completely rescue T_{reg} frequency), while ICOS YF T_{regs} cannot. These results suggest that alternative signaling pathways activated downstream of ICOS besides PI3K can differentially regulate the core "T_{reg} identity" genes, while complete lack of ICOS signaling may not, though both are important for maintaining overall T_{reg} numbers (Figure 6.4).

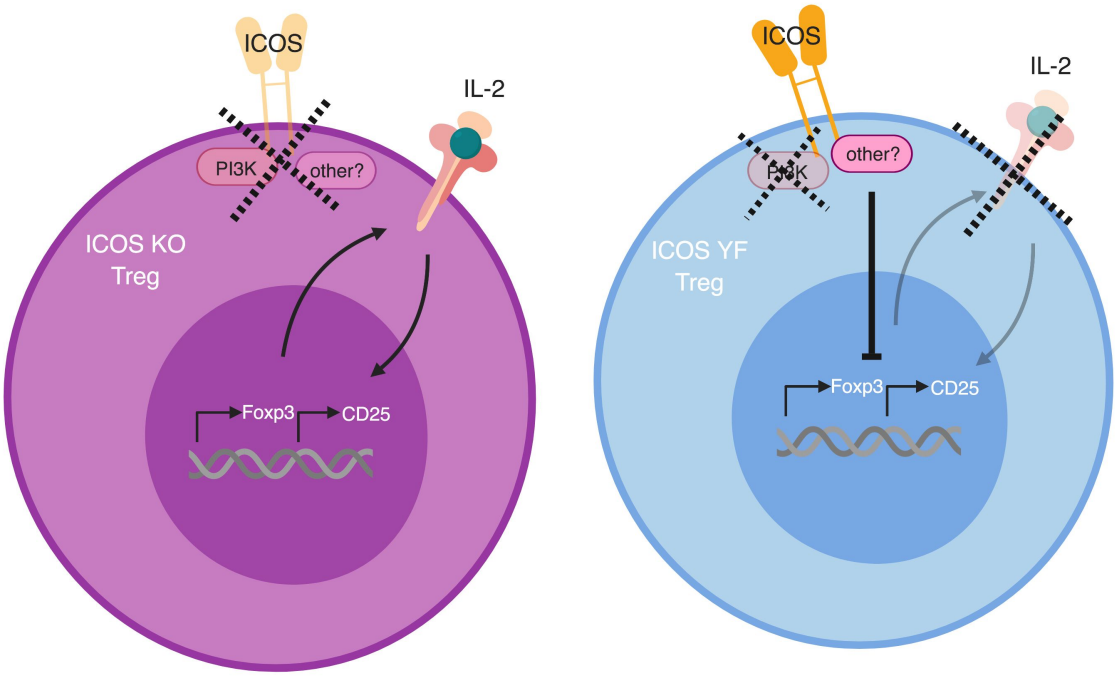


Figure 6.4. Basic phenotype of ICOS KO T_{regs} and ICOS YF T_{regs} in the CNS during chronic infection. While both ICOS KO and ICOS YF T_{regs} are decreased in number during chronic *T. gondii* infection in the CNS, of the remaining T_{regs} that are recruited to the inflamed tissue, ICOS KO T_{regs} are able to overcome baseline deficiency in Foxp3 expression and maintain WT levels of both Foxp3 and CD25 during chronic infection, while ICOS YF T_{regs} cannot. These data suggest that other signaling pathways downstream of ICOS are in play that could impact the ability of T_{regs} to maintain a fully functional "T_{reg} identity".

By studying a chronic CNS infection, we were able to better characterize several distinct regulatory mechanisms in use during a chronic T cell response in the CNS. Importantly, these findings provide insight into 1) how T_{reg} populations are maintained in the inflamed CNS environment, 2) how effector T cells contribute to regulating themselves, and 3) potential signaling pathways important for maintaining both T_{reg} numbers and T_{reg} identity during both homeostasis and active, chronic inflammation. These findings emphasize that multiple regulatory mechanisms are in play during chronic T cell responses that can potentially be used in therapeutic treatment for many inflammatory CNS diseases. Overall, it is essential that we better understand the complicated dynamics of chronic inflammatory responses and all the different levels of regulation that are present in order to prevent, as much as possible, immune-mediated pathology. Better understanding of these multiple levels of both cellular and molecular regulation will provide clues as to how immune responses can be manipulated to either increase or decrease certain inflammatory components of the response, providing fine-tuned control of inflammation.

6.2 Herding Cats: How to Get T_{regs} to Stay and Play

Since the discovery of T_{regs} being an essential means of regulating immune responses, understanding the many ways in which they carry out regulation has been of utmost interest, particularly for the development of therapeutics involving manipulation of the T_{reg} population. As discussed in Sections 1.4 and 1.5, T_{regs} have a multitude of ways to suppress immune responses, yet which of these mechanisms is the most important in different *in vivo* settings still remains unclear. It is likely that the mechanisms T_{regs} utilize to suppress immune responses are highly context-dependent,

varying based on such factors as the tissue in which they reside, the level and type of inflammation present in the local environment, and what other cell types are present in the tissue with which the T_{reg} can interact. Direct interactions with other cell types, particularly APCs, as a means of suppressing immune responses has been of interest in the field since the observation that T_{regs} both express high levels of co-inhibitory receptors like CTLA-4 and LAG-3[65,74,76], and that T_{regs} form long-lasting, stable contacts with APCs both *in vitro*[123] and *in vivo*[69-71,362,403] was discovered. These stable interactions between T_{regs} and APCs have been shown to be important for mediating T_{reg} suppression through allowing for downregulation of costimulatory molecules on the surface of APCs, or through preventing effector T cell access to APCs[66,71,123,124]. Interestingly, though CTLA-4 has been shown to be required for costimulatory molecule downregulation in these T_{reg}:APC aggregates, it is dispensable for the formation of the aggregates themselves. What then initiates and stabilizes these contacts *in vivo*? And how does the tissue environment affect these contacts?

The role of T_{reg}:APC interactions in the context of neuroinflammation is potentially an important mechanism of suppression, as inflammatory immune responses infiltrating into the CNS must be tightly regulated in order to prevent excessive damage to this delicate (and largely non-regenerative) tissue. We showed that the adhesion molecule LFA-1 was involved in the maintenance of T_{reg}:APC interactions during the chronic neuroinflammation seen during *T. gondii* infection (Chapter 3), which supports a previous observation that LFA-1 knockout mice have worse EAE disease associated with fewer T_{regs} in the brain and expansion of inflammatory effector T cell responses[404]. These results together suggest T_{reg} recruitment and/or longevity in the CNS is dependent on

LFA-1. We additionally observed in our LFA-1 blockade study that LFA-1 is required to maintain APC populations in the inflamed CNS as well, and as APC numbers were diminished in the hours following blockade, T_{reg} numbers followed suit. The timing of the loss of APCs versus T_{regs} (with loss of APCs preceding loss of T_{regs} in the CNS) is interesting, as it suggests that T_{regs} could rely on contact with APCs in the CNS for their survival and/or recruitment and subsequent maintenance in the tissue. It is difficult, however, to delineate specifically in which tissue LFA-1 interactions between these two cell types is most important. In the previously mentioned study of EAE, total LFA-1 knockout mice were used, and it was observed that T_{reg} frequencies in the spleen were also decreased[404], implying that LFA-1 is required for maintaining T_{reg} populations in peripheral tissues potentially in addition to the inflamed CNS. With our LFA-1 blockade studies, we found that short-term blockade (4 hours) led to slight decreases in T_{reg} frequency (~8% to ~5% of total $CD4^+$) in the spleen before any defect was observed in the CNS. These results suggest that LFA-1 could be involved in maintenance of T_{reg} populations in the spleen during chronic *T. gondii* infection as well. Defects in the T_{reg} population in peripheral tissues could subsequently lead to defects in CNS-infiltrating T_{regs}, as continued recruitment from secondary lymphoid organs is necessary to maintain immune cell populations in the CNS during chronic infection[117]. It remains unclear however, whether 4 hours is long enough to see the effects of recruitment of T_{regs} to the CNS from secondary lymphoid organs, or whether the dynamics of depletion of APCs and T_{regs} from the CNS truly reflects the necessity of a CNS-specific LFA-1-dependency for T_{reg} maintenance in in the context of neuroinflammation. Likely, LFA-1 contacts between T_{regs} and APCs play a role in T_{reg}-mediated suppression in multiple tissues,

including secondary lymphoid organs and the inflamed tissue itself. These contacts with APCs are also likely multifaceted, potentially both promoting the survival of T_{regs} themselves and allowing for T_{reg} suppression of APC costimulatory capacity (Figure 6.5).

In addition to adhesion molecules, our results also implicate TCR:MHCII interactions as being important in maintaining contact between T_{regs} and APCs in the inflamed CNS. TCR engagement has been shown to be required for maintenance of T_{reg} suppressive capacity, as inducible ablation of the TCR in Foxp3+ T_{regs} prevents their activation into effector T_{regs} and expression of genes associated with actively suppressive T_{regs}, such as Irf4, LAG-3, CTLA-4, and IL-10[336]. Though TCR signals are clearly important for initial activation of T_{reg} cells and their subsequent control of effector T cell responses, the requirement for "secondary" TCR signals after initial activation remains unclear. It is thought that, once T cells are activated through the TCR in secondary lymphoid organs and then traffic to a distal site of tissue inflammation, they must once again "see" their cognate antigen in order to be retained in the tissue and carry out effector function. Indeed, effector CD8+ T cells have been shown to require continued parasite-derived antigen recognition for their maintenance in the brain during chronic *T. gondii* infection, as non-antigen-specific CD8+ T cells, though they can briefly infiltrate the inflamed tissue, are not retained[109].

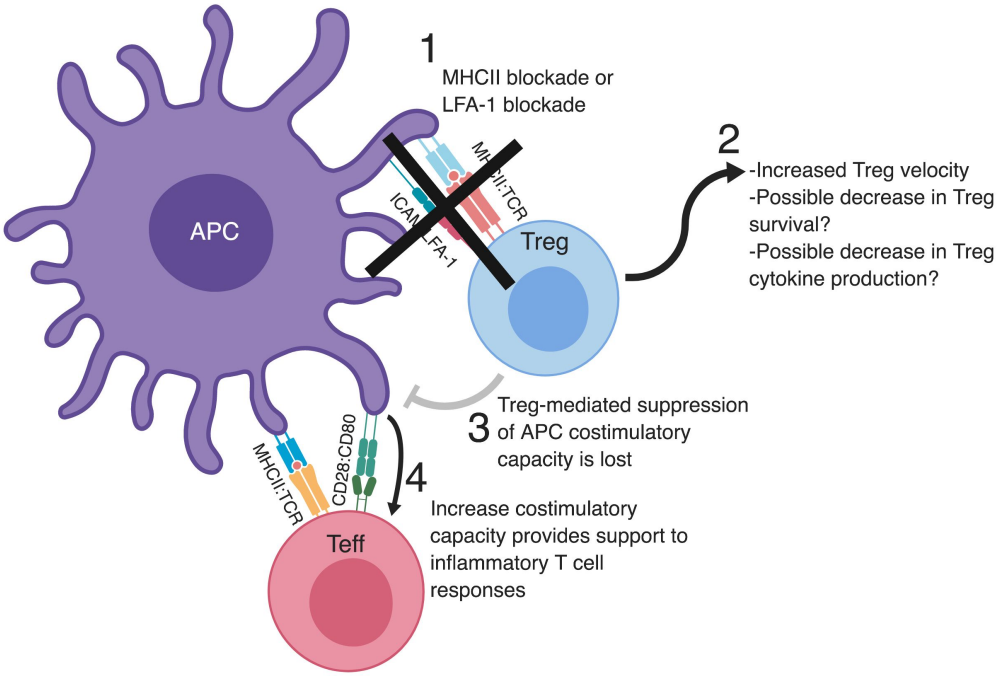


Figure 6.5. Model of the potential effects of MHCII or LFA-1 blockade on the T_{reg}-mediated suppression of immune responses in the inflamed CNS. Following either blockade of LFA-1 or MHCII, T_{reg} velocities are increased and contact time with APCs in the CNS are decreased. We hypothesize that when these T_{reg}:APC interactions are disrupted, T_{reg}-mediated suppression of APC costimulatory capacity is lost, which can allow for increased costimulation of CNS-infiltrating effector T cells. It is also possible that disruption of this interaction leads to decreased T_{reg} survival or immunosuppressive cytokine production, as APCs could be providing essential survival or costimulatory signals to the activated CNS T_{reg} population.

It is currently unknown whether this requirement for TCR:MHCII-mediated interaction applies similarly to the T_{reg} population in the brain during chronic infection, though we hypothesize that it does. The results from the α-MHCII blockade studies during chronic *T. gondii* infection (Chapter 3) suggest that T_{reg}:APC contacts are mediated, at least partially, by TCR:MHCII contact, as T_{regs} under the condition of MHCII blockade had significantly increased velocities, suggesting less stable interactions with CNS APCs. Interestingly, short-term blockade (4 hours) of MHCII did not lead to depletion of either APCs or T_{regs} from the CNS, suggesting that, unlike adhesion molecule LFA-1, TCR:MHCII contact is not necessarily required for short-term maintenance of APC and T_{reg} populations in the inflamed tissue. We would hypothesize that this disruption of TCR:MHCII-mediated contact between activated T_{regs} and APCs would inhibit the suppressive function of T_{regs} in the CNS in some capacity (Figure 6.5). As previously discussed, interactions with APCs and downregulation of costimulatory molecules is an important mechanism of suppression utilized by activated T_{regs}[66,71,124], as is IL-10 production, which has also been shown to be dependent on TCR activation[336]. It is still unclear, however, if continued TCR:MHCII ligation is necessary for either of these suppressive mechanisms to be carried out at sites of inflammation, or whether T_{regs} that have previously been activated in secondary lymph nodes can continue to modulate APC activation and produce IL-10 at distal sites of inflammation independent of further TCR ligation. Blockade studies in this model of chronic *T. gondii* infection do carry the caveat of being systemic, so MHCII blockade will affect T_{reg}:APC interactions in both secondary lymphoid organs and the CNS. They also are not specific to T_{regs}, as effector CD4+ T cells will also be affected by MHCII blockade. These caveats make longer

blockade studies difficult, as interpretations can be clouded by the fact that effector CD4+ T cell activation through the TCR will also be affected, although CD8+ T cell responses should remain intact.

Subsequent studies could further characterize the interaction time between T_{regs} and CNS APCs following short-term MHCII blockade, as well as further characterize the activation status of CNS APCs (particularly the expression of costimulatory molecules) and the production of IL-10 on the part of the T_{regs} following MHCII blockade. If continued use of these suppressive mechanisms requires additional TCR ligation in the inflamed CNS after initial activation in secondary lymphoid organs, it is possible that we could observe decreased CD80/CD86 expression on APCs or decreased IL-10 production from T_{regs} in the CNS. CD44 expression could be used as a marker to track what percentage of the T_{reg} population in the CNS has been "activated", as we typically see nearly 100% $CD44^{hi}$ T cells in the CNS during chronic infection. If MHCII blockade begins to inhibit T_{reg} activation in secondary lymphoid organs, we would expect to see either fewer $CD44^{hi}$ T_{regs} in the CNS or fewer T_{regs} in the CNS altogether. This could give insight into what regulatory mechanisms mediated by activated effector T_{regs} are dependent on continued TCR ligation in the inflamed CNS. On the other hand, working in such a short time frame of blockade might not be enough time to see these effects even with the immediate disruption of T_{reg}:APC contacts, meaning that it could not be ruled out that T_{regs} utilize contact with APCs in the CNS to regulate chronic inflammatory responses. Overall, the importance of local T_{reg}:APC contacts in the inflamed CNS remains an unanswered, but important, question. Understanding the mechanisms of T_{reg} suppression at local sites of inflammation could provide clues as to how to modulate T_{reg}

activity at specific sites of inflammation while retaining T_{reg}-mediated regulation systemically, which could limit immunopathology associated with total T_{reg} depletion or systemic lack of T_{reg} function.

Another interesting question arising from the studies into the importance of TCR:MHCII interactions occurring between T_{regs} and APCs during chronic infection with *T. gondii* deals with the antigen specificity of the T_{reg} population recruited to the CNS. As previously discussed in Section 1.5.1, the antigen specificity of T_{regs} in the context of pathogen infection can largely depend on the infection model. Although pathogen-specific T_{regs} have been found in models of Mtb, MHV, influenza, and *L. major* infection[91-94], we found essentially no T_{regs} present in the CNS with specificity for the available *T. gondii* tetramer (Chapter 3). Additionally, there was very little overlap between the TCR repertoires of the effector CD4+ T cell population and T_{reg} population recruited to the brain during the chronic stage of infection (Chapter 3). These results suggest that the T_{reg} population as a whole has TCR specificity for an entirely different antigen pool than the effector CD4+ T cell population. It is thought that most, if not all, tT_{regs} are specific for self-antigen and are responsible for preventing autoimmune responses that might arise during homeostatic states[30,50,52,54,55]. Interestingly, in cases of autoimmune reactions in the CNS, such as EAE, the T_{reg} repertoire has been shown to also be largely distinct from that of the still-autoreactive effector T cell repertoire[346,347], suggesting distinct developmental lineages for these two cell types. It is even more interesting, therefore, that in the face of chronic parasite-derived antigen presentation, only very few (to no) T_{regs} in the CNS could be specific for the parasite during chronic *T. gondii* infection. Although the chronic stage of infection with *T. gondii* is characterized

by the presence of "latent" parasite cysts, stochastic reactivation throughout the life of the host causes local tissue damage, so it is likely presentation of self-antigen also occurs alongside parasite-derived antigen. Perhaps then, this presentation of self-antigen could support the recruitment and maintenance of the T_{reg} population in the CNS. If this is the case, this presentation of self-derived antigen could also represent added danger to the host, if it also could support the recruitment and maintenance of self-specific effector T cells in addition to the parasite-specific effector T cells. In this model, the self-specific T_{reg} population might play the essential role of limiting autoreactive T cell responses that might arise during this chronic stage of neuroinflammation as a result of continued damage to self tissue, perhaps by out-competing autoreactive effector T cells for "space" on the APC or by modulating the costimulatory capacity of self-presenting APCs in the CNS. This has been shown to be the case in a model of viral encephalomyelitis, as depletion of T_{regs} in this model did not affect viral-specific T cell responses or viral clearance, but instead led to increased CNS tissue damage associated with the recruitment of autoreactive (MOG)-specific effector T cells[83]. Although the antigen-specificity of the T_{regs} in this case was not assessed, these results suggest that T_{regs} recruited to the CNS during infection could play a specific role in limiting autoreactive T cell responses rather than pathogen-specific T cell responses. How this exclusive regulation of self-specific effector T cells is carried out while preserving the protective anti-pathogen T cell responses remains an open and intriguing question. A more recent report has shown that T_{regs} are able to limit antigen-specific effector T cell responses by actively depleting peptide:MHCII molecules from the surface of APCs through trans-endocytosis[77]. This idea suggests a model in which self-specific T_{regs} recruited to the CNS during chronic

infection may be able to interact with self-antigen-presenting APCs and actively deplete these self-peptide:MHCII complexes, while leaving intact parasite-derived peptide:MHCII expression, thereby preventing the infiltration, maintenance, or further activation of any self-specific effector CD4+ T cells. Though complete depletion of T_{regs} is difficult during the chronic stage of *T. gondii* infection (we have found a maximum depletion of about 80-85% for a period of about 36 hours), it would be interesting to know if any autoreactive T cell responses could arise and be recruited to the inflamed CNS during the chronic stage of disease, and whether these responses are specifically regulated by the T_{reg} population.

Overall, these studies bring up interesting questions, and emphasize how much more there is to be learned when it comes to T_{reg}-mediated suppression of immune responses. It is now well established that the main role of T_{regs} is to suppress all different kinds of inflammatory immune responses, and we understand quite a lot about different mechanism T_{regs} can use to carry out this essential role (both *in vivo* and *in vitro*). However, much is still unknown about the dynamics of this regulation during active and ongoing immune responses. If T_{regs} or T_{reg} functions are to be harnessed for therapeutics, it is necessary to understand how T_{regs} act and carry out suppression in a multitude of contexts. What is the importance of T_{reg}:APC interactions versus anti-inflammatory cytokine production? What is the balance between what might be more specific suppression (such as suppression of APCs presenting a certain antigen pool) and non-specific suppression (such as production of anti-inflammatory cytokine that can be recognized by many cell types regardless of specificity)? What happens when one is inhibited versus the other? Can we separate the importance of the T_{reg}:APC interaction in

modulating the costimulatory phenotype of the APC versus the effects of this interaction on supporting the survival and maintenance of the T_{reg} population itself? These questions are interesting because it is important to consider that the T_{reg}:APC interaction is likely playing roles on both sides of the coin. Several studies have shown this interaction is important for downregulation of costimulatory capacity on the side of the APC[66,71,76,124], but there is also significant evidence that this interaction supports the maintenance of the T_{reg} population as well[206-208,336,361,404], and it is probable that both "sides" are important in regulating an ongoing immune response. Additional intricacies must also be considered when the inflammatory environment comes into play. Does the tissue in which the T_{reg} is located play a role in the mechanisms of suppression? How do different inflammatory (or anti-inflammatory) cues integrate to influence the T_{reg} population at a particular site, and can this environment "instruct" the T_{reg} population as to which suppressive mechanisms should be in use? Though these are difficult questions to address, understanding how each variable occurring during an inflammatory immune response impacts the potential for T_{reg}-mediated suppression can add to our overall understanding of the multi-level regulation occurring during any immune response.

6.3 Hold Your Horses: With Chronic Inflammation Comes Chronic Regulation

6.3.1 Part 1: The Major Players

As mentioned in the previous section, regulation is a multi-tiered process during both the initiation of an immune response and during ongoing inflammation. Many different cell types and signaling pathways are involved in this multi-tiered regulatory response, and each plays an important role in maintaining the crucial balance between

inflammation and suppression. Some players in this multi-level model of regulation are what one might term "major" players. These players include those factors that are absolutely essential for survival; lack of these players leads to complete loss of control over inflammation, and often rapidly results in fatal immunopathology. T_{regs} fall into this category, as exemplified by the phenotype resulting from the loss of T_{regs} during homeostasis (which is fatal)[38-40,80] or the loss of T_{regs} during infection (which at the very least often leads to severe immunopathology)[82,83,85,292,405,406]. The immunosuppressive cytokine IL-10 is another of these major players. Similar to a loss of the T_{reg} population (though not as extreme), loss of IL-10 during homeostasis also leads to severe immunopathology (specifically at mucosal sites)[143,144,149,158], and its loss during infection can cause immunopathology and tissue damage that, in some cases, is more severe than the pathogen itself[159-161,288,309].

In the context of chronic *T. gondii* infection, IL-10 production is absolutely required during the induction of immune responses to the parasite in order to control inflammation on a large scale to prevent immune-mediated tissue damage[288]. Similarly, we (and others[309]) have now shown that this IL-10 production must be continued throughout the chronic stage of infection (Chapter 4). From our studies into the regulatory role of IL-10 during chronic *T. gondii* infection, it is clear that IL-10 has wide-ranging effects on the inflammatory response. We observed expansion of macrophages and CD4+ T cells in both secondary lymphoid organs and in the inflamed brain, as well as an overall change in the cytokine milieu in the brain (Figure 6.2). As discussed in Section 1.9.2, the immune response to *T. gondii* is heavily T_h1-polarized, with very little to no involvement of T_h2- or T_h17-associated cytokines[240]. It is interesting, therefore, that

a lack of IL-10 signaling leads not only to increases in IFNγ, but also increased IL-17 production in the brain, suggesting that IL-10 normally limits T_h17 responses during *T. gondii* infection. However, the precise mechanism of this suppression of IL-17 production from effector T cells during the course of *T. gondii* infection is still unknown. It been reported that both IL-10 and its major downstream signaling molecule STAT3 are required in T_{regs} to suppress T_h17 responses in the gut, as without expression of IL-10R1, IL-10R2, or STAT3, T_{regs} are unable to limit Th17 responses and immune-mediated colitis[407-409]. Once again, however, it is still somewhat unclear what exactly leads to this effect. In some studies, IL-10R2-deficient T_{regs} are unable to maintain Foxp3 expression, leading to the development of "exT_{regs}" that then contribute to intestinal pathology[409]. On the other hand, studies using mice with IL-10R1-deficient T_{regs} show equivalent (or even more) Foxp3 expression, along with increased expression of markers of activated effector T_{regs}, such as CD25, GITR, ICOS, and CTLA-4, though these T_{regs} are still incapable of suppressing pathogenic T_h17 responses in the gut[408]. This discrepancy could be due the differing components of the IL-10R (the ligand-binding component versus the signaling component) being targeted. Though both are clearly required to prevent the rise of T_h17 inflammatory responses, whether instability in the T_{reg} population when lacking IL-10-mediated signaling plays a significant role in T_{reg}-mediated suppression remains unclear. It has also been shown that IL-10R signaling is important to maintain IL-10 production from T_{regs}[408], which (once again) puts T_{regs} squarely on the balance of inflammation and regulation by serving as an "amplifier" of IL-10 mediated suppression of pathogenic T_h17 responses. Our results showing an increase in IL-17 mRNA in the inflamed brain following IL-10R blockade support the idea that IL-10 signaling can limit T_h17

responses, though, because of the systemic effect of our IL-10R blockade, it is difficult to narrow down the main players in IL-10-mediated suppression during chronic infection. Interestingly, though we did observe a decrease in the frequency of T_{regs} in the brain, we did not observe defects in total T_{reg} numbers or Foxp3 expression among the remaining T_{reg} population following IL-10R blockade, suggesting that a loss of Foxp3 expression in T_{regs} is not correlated with the loss of regulation in this setting. It is still unknown, however, exactly what role T_{regs} might play in supporting or amplifying IL-10-mediated suppression in the brain during chronic *T. gondii* infection. STAT3-dependent genes have been implicated in maintaining T_{reg}-mediated suppressive capacity independent of affecting total T_{reg} number or Foxp3 expression[407], further implicating IL-10 in supporting T_{reg}-mediated suppression in addition to promoting T_{reg}-independent suppression of immune responses. Perhaps the loss of IL-10 signaling in T_{regs}, though it does not seem to affect Foxp3 expression or overall number in our model of chronic infection, could be important in supporting the expression of certain T_{reg} genes that are necessary for suppressing both T_h17 and T_h1 inflammatory responses.

Subsequent studies could further characterize the gene expression of the T_{reg} population in the brain following IL-10R blockade to determine what role, if any, continued IL-10 signaling plays in maintaining T_{reg}-mediated suppressive capacity in the inflamed brain. Ideally, using inducible Cre-mediated recombination to delete expression of IL-10R only in T_{regs} (or alternatively only in subsets of myeloid cells) and only in the chronic stage of infection would begin to provide answers to exactly what role continued IL-10 signaling plays in supporting a suppressive T_{reg} population during chronic infection, and how big of a role T_{regs} play in IL-10-mediated suppression. Unfortunately,

based on previous data obtained by our lab, the available Foxp3 inducible Cre mouse likely has infection-induced excision of target genes, meaning that we could not control the timing of IL-10R excision during the course of infection.

Due to the limitations of our model system, teasing apart what contribution T_{regs} have in IL-10-mediated suppression during chronic infection in the CNS will remain difficult to assess. It is, however, unlikely that the loss of IL-10 signaling in T_{regs} alone accounts for the severe and fatal phenotype seen in mice treated with IL-10R blockade. Indeed, nearly all hematopoietic cells express the IL-10R[127], and in cases of inflammation, non-hematopoietic cells can also upregulate the IL-10R[139,379]. Based on the effects of IL-10R blockade on multiple cell types and in multiple tissues, this immunosuppressive cytokine is likely acting on many levels to regulate the chronic inflammatory response to *T. gondii* infection. The upregulation of costimulatory molecules on APCs in the absence of IL-10 signaling that we observed in the spleen and brain has been reported in other models of IL-10 deficiency[134,137,140,158], and, at baseline, IL-10R signaling is also necessary in macrophages and neutrophils to prevent colitis[158]. These data together suggest that IL-10 signaling in both myeloid and T_{reg} cells is necessary at baseline to prevent colitis, so in the context of the chronic inflammation we observe during *T. gondii* infection, it is likely that IL-10 is involved in influencing the inflammatory capacity of multiple cell types. A bone marrow chimera experiment using a head cover could provide insight into whether IL-10R expression is important on hematopoietic cells or brain-resident cells as a whole.

Additionally, IL-10 has been shown to have direct effects on T cell proliferation, cytokine production, and sensitivity to antigen[354,356,380,410], further emphasizing the

widespread effects of IL-10 on regulating inflammatory responses. Interestingly, we saw somewhat different effects on the accumulation of CD4+ and CD8+ effector T cells in the brain following IL-10R blockade. Though both populations had increased frequencies of proliferating cells, only CD4+ effector T cells increased in number in the brain (Chapter 4). This differential accumulation could be due to several reasons. Despite increased proliferation with loss of IL-10 signaling, CD8+ T cells might rely more on environmental survival factors, such as IL-2, than CD4+ T cells for survival. Several studies have suggested that, though both CD4+ and CD8+ T cells will respond to IL-2, CD8+ T cells are especially sensitive to environmental IL-2[64,411]. If the ability to scavenge IL-2 is a limiting factor in CD8+ T cell survival, they might have a faster turnover rate, preventing their accumulation in the tissue. Though there was a small increase in the frequency of CD25+ CD8+ T cells following IL-10R blockade (Chapter 4), the overall number of CD25+ T cells was not increased. This suggests that, despite increased proliferation, CD8+ T cells might be turning over faster than the CD4+ effector T cell population, resulting in no overall increase in the number of CD8+ T cells in the brain.

Additionally, it is possible that CD4+ effector T cells could rely more on interaction with MHCII-expressing APCs for costimulatory or survival signals in the inflamed brain. Because IL-10R blockade affected CD80 expression on APCs in the brain, it is possible that, if CD4+ effector T cells rely on this signal for survival in the tissue, this population is then better able to accumulate in the inflamed brain after IL-10R blockade. Very few of the myeloid cells infiltrating the brain during chronic infection are classical cross-presenting DCs that might highly interact with CD8+ T cells[117],

potentially suggesting that CD8+ T cells could be less affected by changes in the myeloid population in this context. As a consequence, rather than suppressing CD8+ T cells through suppression of APC costimulatory capacity, IL-10 could normally be suppressing CD8+ T cells through an intrinsic mechanism. This suggests a potential model in which IL-10 affects the proliferative capacity of both effector T cell populations, but the added effect of increased costimulatory capacity of APCs could be providing increased proliferative and survival signals to CD4+ effector T cells, leading to overall increases in CD4+ effector T cell number but not CD8+ T cell number in the CNS. Finally, though we did not observe a defect in overall T_{reg} numbers in the brain, the frequency of T_{regs} among the total CD4+ T cell population was significantly decreased in the CNS following IL-10R blockade, resulting in a significant "skewing" of the CD4+ T_{eff}:T_{reg} ratio. Though it likely doesn't account entirely for the accumulation of CD4+ effector T cells, it is possible that some T_{regs} in the brain lose Foxp3 expression entirely and convert to a Foxp3-CD4+ effector T cell (an "exT_{reg}"), which could also contribute to the increased CD4+ effector T cell number over the CD8+ effector T cell number. Indeed, our TCR sequencing data suggests that some conversion from a Foxp3+ T_{reg} to a Foxp3- effector CD4+ T cell does occur in the CNS during chronic infection. Though we found very little overlap in TCR clones between the effector CD4+ T cell population and the T_{reg} population in the CNS, of the overlapping clones that did exist, the TCR sequences were heavily overrepresented in the T_{reg} population (Chapter 3). These data could suggest that certain TCR sequences arise in the T_{reg} population and are recruited to the CNS during chronic infection, where a small proportion of them then lose Foxp3 expression entirely and become effector CD4+ T cells, which would explain the reason that

particular TCR clone is overrepresented in the T_{reg} population. It is possible, with the increased inflammatory environment in the CNS following IL-10R blockade, that this rate of conversion is increased, which could even further increase the effector CD4+ T cell number. An interesting subsequent study to determine the rate of conversion of T_{regs} to effector T cells following IL-10R blockade would be to congenically mark the T_{reg} population with fluorescence before infection and then determine how many Foxp3- CD4+ T cells retain the fluorescent signal in the CNS after IL-10R blockade. Overall, it is possible that any one of the above mechanisms could explain the exclusive increase in the CD4+ effector T cell population, but certainly the differential effects on the CD4+ and CD8+ effector T cell populations highlights that significantly different effects in different T cell populations is a potential outcome when manipulating major players in regulation. The extrinsic and intrinsic signaling pathways involved in these differential responses remain a major question and hold significant implications for potential therapeutics involving regulatory or suppressive players.

6.3.2 Part 2: The Minor (But Still Important) Players

While both T_{regs} and IL-10 represent major players in the regulation of inflammatory immune responses to infection, depletion or inhibition of either in human disease could have serious deleterious effects. IL-10 is likely too broad a regulatory mechanism to ever be targeted for depletion or inhibition on a systemic level, as serious autoimmunity associated with increased inflammation would be expected when IL-10 signaling is lacking for any extended period of time. Depletion or inhibition of T_{regs} is a more promising strategy for immunotherapy, particularly in models of cancer, where T_{reg} depletion has shown some mild anti-tumor effects, and can potentially serve to amplify

anti-tumor responses when given in conjunction with other immunotherapies such as immune checkpoint blockade[412,413]. However, T_{reg} depletion as an immunotherapeutic also carries with it the possibility of autoimmunity, particularly in cases where long term depletion or inhibition would be required[412,413]. Regardless, a better understanding of the *in vivo* intricacies, including kinetics and tissue-specific effects, of both IL-10- and T_{reg}-mediated suppression are needed in order to more precisely manipulate their suppressive action to limit associated immunopathology.

At the same time, understanding other mechanisms of immune regulation, those involved on a somewhat smaller scale, are also important in providing potential for immunotherapies that allow more specific manipulation of inflammatory responses while limiting associated immunopathology. One of the most interesting (and somewhat still unanswered) questions in the field of immunology is how exactly does the activity of activated effector T cells differ in the secondary lymphoid organs versus the actual inflamed tissue? It has been established that much, if not all, of the initial activation of a classical T cell response occurs in secondary (or tertiary in some cases) lymphoid organs. And once that activation occurs, the activated T cells proliferate and then traffic to the site of inflammation and carry out effector function. Presumably, the inflamed tissue provides very distinct signals to these incoming effector T cells than they received in the secondary lymphoid organ in which they were activated. These environmental cues might differ greatly depending on the type of tissue that is inflamed or infected, the type of infection itself, the presence of PAMPs or DAMPs, the type of inflammatory cytokine production occurring, and the types of immune cells already present in the tissue. Upon T cell entry into this extremely variable battleground, how are all of the signals the T cell

encounters going to be integrated and affect their response? This is a loaded question; yet an essential one to answer if we hope to provide "tailored" manipulation of immune responses that might provide the ideal balance of inflammation and suppression when responding to a wide variety of insults.

We focused on a model of chronic infection of the CNS with *T. gondii*, a model in which long-term inflammatory responses in a delicate tissue are required. It has now been well established that the major players of IL-10 and T_{regs} are important over the course of *T. gondii* infection to prevent fatal immunopathology[288,292,309, (Chapter 4)], but what other regulatory mechanisms might be involved, particularly local mechanisms used in the inflamed brain in the chronic stage of infection? We have identified one such "minor" immunoregulatory player: ICOS. As discussed in Section 1.8, ICOS signaling is involved in a whole host of immune responses, including promoting inflammatory responses through amplifying effector T cell inflammatory cytokine production and B cell antibody production, as well as being involved in immunoregulation through supporting the survival of T_{reg} populations and promoting IL-10 production[24,175]. We have identified an additional role for ICOS specifically in activated effector T cells during chronic *T. gondii* infection in the CNS, that is, inhibiting IL-2-associated signaling in effector T cells and promoting apoptosis in the inflamed tissue (Chapter 4). Our data suggest that, by inhibiting IL-2-associated signaling, ICOS signaling promotes increased turnover of both CD4+ and CD8+ effector T cells in the inflamed brain, thereby preventing their over-accumulation in the tissue.

While IL-10 and ICOS both promote regulation, there are some notable differences in the magnitude and downstream effects of each during chronic *T. gondii*

infection. As discussed above, our data show that lack of IL-10 signaling leads to increased immunopathology not only in the inflamed brain, but also in the liver, which suggests that IL-10 is required for systemic regulation of inflammation during the chronic stage of infection. This increased systemic immunopathology is associated with increased APC activation, effector T cell proliferation, and inflammatory cytokine production both in secondary lymphoid organs and in the inflamed brain, further highlighting the systemic regulatory effects of IL-10. On the other hand, a lack of ICOS signaling appears to only affect effector T cell responses in the inflamed brain itself, without showing the same systemic effects as IL-10. We observed no differences in T cell responses in secondary lymphoid organs, as well as no increase in APC activation in either the brain or secondary lymphoid organs (Chapter 4). These data suggest that ICOS signaling specifically carries out regulation of effector T cell responses at the local site of inflammation, rather than affecting their initial activation in secondary lymphoid tissues. This differential effect on the magnitude of inflammation seen with lack of IL-10 versus lack of ICOS signaling could be due to expression patterns of each of these molecules. As discussed in the previous section, the IL-10R is very widely expressed on both hematopoietic and non-hematopoietic cells during inflammation, so it is not surprising that IL-10 serves to regulate inflammation on a systemic level. ICOS, on the other hand, is only upregulated after T cell activation, meaning that the bulk of ICOS signaling might occur at the site of active inflammation, in the case of chronic *T. gondii* infection: the brain. Indeed, we observed the highest levels of ICOS expression in the brain during chronic infection, which could explain the more local and specific effect of ICOSL blockade versus IL-10R blockade. Additionally, following ICOSL blockade, we did not

observe the same changes in inflammatory cytokine production or APC activation as was seen after IL-10R blockade, further emphasizing the more specific effects of ICOS-mediated regulation.

Finally, despite the significantly increased numbers of effector T cells in the inflamed brain following ICOSL blockade, this treatment was not fatal in the observed timeframe. This is likely because ICOSL blockade, unlike IL-10R blockade, did not lead to systemic increases in inflammation or immunopathology, nor did it result in changes in the cytokine milieu or increased neutrophil infiltration into the inflamed brain. These results suggest that ICOS signaling, unlike IL-10 signaling, has the ability to "fine-tune" local effector T cell responses in an inflamed tissue independently of affecting other aspects of inflammation. This is a significant result when considering therapeutics, as it is often the case when targeting the major players in immunosuppression that the associated drastic increase in inflammation could have serious autoimmune effects. Therefore, being able to more precisely fine-tune specific aspects of a given immune response holds promise for limiting autoimmunity associated with increasing inflammation. In this case, blockade of ICOS signaling during chronic inflammation could allow for increased effector T cell survival and effector function at a local site of inflammation without associated autoimmunity in other tissues that might be seen when manipulating the major players in immunosuppression. Of course, because ICOS signaling plays a role in other aspects of an immune response, particularly in the formation of T_{fh} cells, germinal centers, and promotion of antibody class switching, this must be taken into account when considering therapeutics specifically targeting ICOS or ICOSL. In the case of *T. gondii* infection, we did not observe differences in circulating parasite-specific IgG following

ICOSL blockade, likely because this treatment was started in the chronic phase of infection, after the initiation and amplification of B cell responses has already occurred. In acute infection settings, blockade of ICOS or ICOSL could potentially disrupt T cell-dependent B cell responses, so blockade in these settings might not be a good strategy. Likewise, T_{reg} dependency or IL-10 production dependency on ICOS signaling would have to be monitored in cases of ICOS or ICOSL blockade, as both T_{reg} survival and IL-10 production have been linked to ICOS ligation[184,206-209,361]. We did not observe changes in the T_{reg} population or IL-10 production in the inflamed brain following ICOSL blockade (Chapter 4), suggesting that perhaps in cases of chronic inflammation, the T_{reg} population depends on other signals for survival and IL-10 production. Overall, however, these results stress the importance of building a better understanding of the minor players in immunoregulation: those that play a role in fine-tuning immune responses on a single or more localized level, as they, along with the major players, contribute to the overall picture of an immune response.

Our studies into the role of ICOS in T cell responses during chronic *T. gondii* infection in the inflamed brain suggest that ICOS signaling serves as a co-inhibitory signal rather than a costimulatory signal in this context. Yet one major unanswered question stemming from these studies deals with the signaling pathways involved in ICOS-mediated regulation of effector T cell responses. As discussed in previous chapters, it is thought that ICOS signals primarily through the PI3K-Akt pathway after ligation, in fact inducing more potent activation of Akt than its family member CD28[175,180,389]. Other known co-inhibitory receptors CTLA-4 and PD-1 have been shown to mediate their immunosuppressive activity on T cells at least partially through inhibition of PI3K

signaling, thereby limiting the effects of other activating signals[67,381]. In this context, it was initially surprising to note that our results suggest that potentially potent PI3K-Akt activation downstream of ICOS during chronic neuroinflammation could lead to a similar outcome as inhibiting PI3K-Akt signaling downstream of CTLA-4 or PD-1. Although it has been well documented that initial PI3K-Akt activation downstream of CD28 costimulation is essential for early priming and expansion of effector T cells[172,382,383], more recent studies have shown that constitutive Akt activation in CD8+ T cells correlates with downregulation of IL-2R component CD122 and survival factor Bcl-2, thereby promoting the differentiation of CD8+ T cells towards a short-lived effector phenotype rather than a longer-lived memory phenotype[385]. Conversely, constitutive STAT5 expression in CD8+ T cells was associated with maintenance of Bcl-2 expression and promoted the formation of CD8+ memory precursors[385]. Taken together, these results suggest that T cells are extremely sensitive to both the magnitude and duration of signaling cascades like PI3K-Akt. In this light, continued activation of PI3K-Akt downstream of ICOS signaling could serve as an intrinsic negative feedback mechanism by interfering with continued IL-2 signaling once an effector T cell reaches the inflamed brain, thereby providing the final push towards a short-lived effector T cell and preventing the over-accumulation of longer-lived inflammatory memory-like T cells (Figure 6.6). Indeed, our results suggest that sustained IL-2 signaling in activated effector T cells in the inflamed brain following ICOSL blockade can maintain expression of survival signals (like Bcl-2), and limit apoptosis, allowing for accumulation of effector T cells in the tissue (Chapter 4). Overall, these data then suggest a model in which effector T cells become activated in secondary lymphoid tissues, upregulate ICOS expression on

their surface, and traffic to the inflamed brain. Upon interaction with ICOSL-expressing APCs in perivascular spaces or the brain parenchyma, additional PI3K-Akt activation downstream of ICOS ligation in the effector T cell inhibits continued IL-2-associated signaling, driving the cell towards a short-lived effector T cell. By driving incoming effector T cells towards this short-lived effector T cell fate, ICOS signaling then serves as a regulatory mechanism by promoting a constant rate of apoptosis and turnover of the effector T cell population (Figure 6.6).

Another mechanism through which ICOS-mediated PI3K-Akt signaling could enhance the full differentiation into short-lived effector T cells is through maintaining glycolytic metabolism in the T cell. It has been shown that, as CD8+ T cells transition to a memory phenotype, they switch from glycolytic metabolism to fatty acid oxidation, which is mediated by the down regulation of mTORC1[414,415]. As mTOR is activated by Akt[416], it is possible that additional Akt activation induced upon ICOS ligation supports mTORC1 activation and prevents the effector T cell from transitioning to a more memory-like phenotype in the inflamed brain (Figure 6.6).

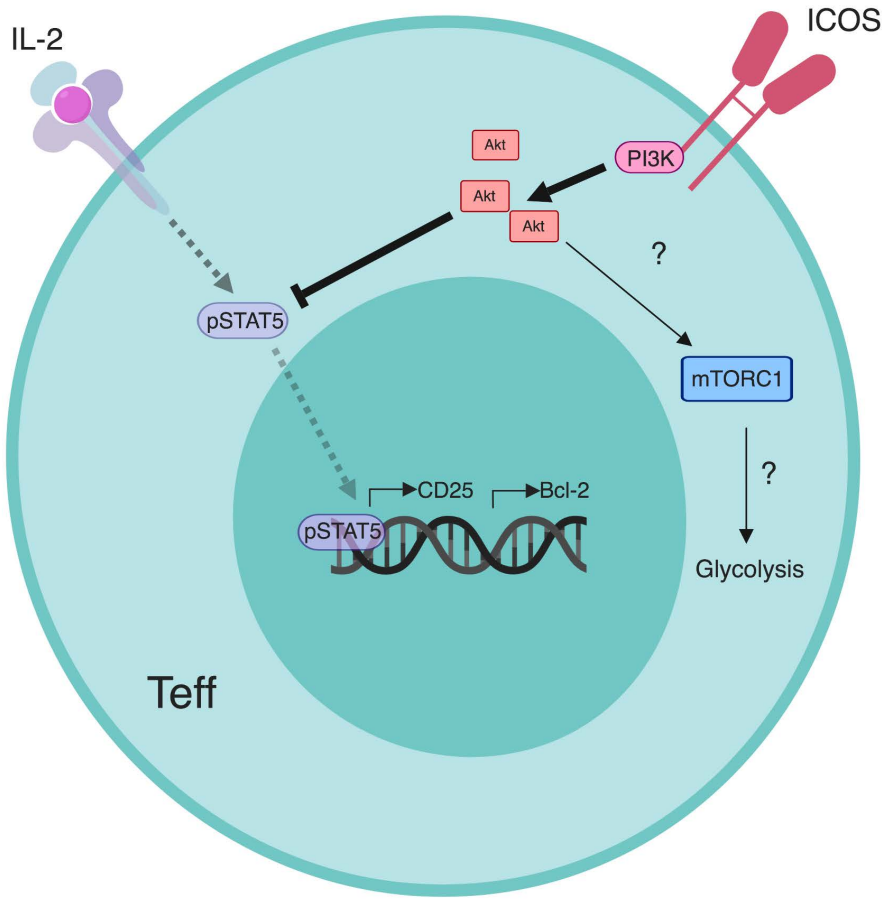


Figure 6.6. Model of the integration of IL-2 and ICOS signaling in activated effector T cells in the inflamed brain. Our data suggests that continued ICOS signaling during chronic infection in the brain interferes with IL-2 responses. We hypothesize that maintenance of strong PI3K/Akt activation in effector T cells inhibits signaling through pSTAT5, thereby leading to the downregulation of CD25 and Bcl-2. This interference with IL-2 signaling then promotes the full differentiation into a short-lived effector T cell, leading to apoptosis and rapid turnover of inflammatory effector T cells in the inflamed brain. Additionally, continued Akt activation could support continued glycolytic activity, further supporting a short-lived effector T cell rather than a longer-lived inflammatory memory-like cell, though this has yet to be confirmed in our model.

The above results suggest that effector T cell responses rely on precisely balanced PI3K-Akt and STAT5 signaling cascades during ongoing inflammation. But how exactly might continued PI3K-Akt signaling downstream of ICOS interfere with IL-2-associated signaling molecules to promote a short-lived effector phenotype? This question remains unanswered, but further elucidation using ChIP-Seq to interrogate pSTAT5-dependent gene expression, and how this gene expression might change with increased PI3K-Akt activation, could begin to tease apart how these two prominent signaling cascades are connected and how perturbing the balance between them changes effector T cell gene expression. It is not unlikely that increased PI3K-Akt signaling in an effector T cell could greatly affect the activation of other transcription factors that might interfere with pSTAT5 signaling, or other regulatory mechanisms, like microRNAs, that could modulate pSTAT5-dependent gene expression. Additionally, RNA-Seq or ATAC-Seq could be utilized to obtain a better understanding of the overall gene expression changes or changes in the available chromatin in effector T cells following ICOSL blockade compared to control-treated effector T cells. These kinds of data could provide more insight into how changes in the availability of ICOS signaling globally affect STAT5-dependent genes in activated effector T cells, and how the transcriptional landscape is changed in effector T cells lacking ICOS costimulation.

Finally, the role of ICOS signaling in T_{regs} also remains of interest. Following short-term ICOSL blockade during the chronic phase of *T. gondii* infection, we did not observe obvious defects in the T_{reg} population (Chapter 4), despite previously published evidence that ICOS signaling is important for supporting an effector T_{reg} population[206-209,361]. However, in mice lacking ICOS (ICOS KO) or ICOS-mediated PI3K signaling

(ICOS YF) from birth, defects in T_{reg} responses both during chronic *T. gondii* infection and at baseline were observed. This suggests that ICOS signaling during homeostasis is important for maintenance of a T_{reg} population, and this baseline defect is unable to be overcome during chronic *T. gondii* infection. In the context of our short-term ICOSL blockade studies, the activated T_{reg} population in the inflamed brain was not affected, suggesting that in the absence of a baseline defect in T_{reg} frequencies, short-term blockade of ICOS signaling has no effect on the T_{reg} population, perhaps because this population relies on other signals, like IL-2, for their maintenance in the inflamed brain.

Perhaps the most intriguing finding from our studies using ICOS KO and ICOS YF mice was the difference in phenotype seen in the T_{reg} population in each of the two mouse strains. While both showed about half the WT frequency of T_{regs} in the brain during chronic *T. gondii* infection, we found that, of the remaining T_{regs} in the tissue, ICOS KO T_{regs} expressed WT levels of Foxp3 and increased levels of CD25, while ICOS YF T_{regs} had significantly decreased expression of Foxp3 and CD25 in the brain (Chapter 5). Though these results are preliminary, they suggest that signaling downstream of ICOS, other than the well-described PI3K-Akt signaling pathway, could drastically impact the ability of the T_{reg} population to maintain T_{reg}-associated markers like Foxp3 and CD25. Though this differential phenotype has yet to be explored, a recent study has described a novel "IProx" motif in the cytoplasmic tail of ICOS that activates the serine/threonine kinase TBK1[390]. ICOS KO mice lack both the downstream PI3K-Akt- and TBK1-mediated signaling, while ICOS YF mice only lack downstream PI3K-Akt signaling, and could potentially preferentially activate TBK1 when the PI3K binding site is unavailable (Figure 6.7). It is entirely unknown what role TBK1 has in T_{regs}, and only

very little is known about its role in T cells in general. TBK1 has been most thoroughly characterized in innate immune cells, where it has been shown to be important in the activation of Irf3 and subsequent Type I interferon responses to viral infection[391-393]. In T cells, one study found that mice lacking TBK1 specifically in T cells accumulated $CD44^{hi}CD62L^{lo}$ activated, cytokine producing T cells in the spleen during steady state[394]. This group went on to show that TBK1 could induce the ubiquitination-dependent degradation of Akt in T cells, explaining why T cells lacking TBK1 accumulated Akt and displayed an increased activation phenotype[394]. These results implicate TBK1 as a potentially important, and largely understudied, negative regulator of the Akt-mTOR pathway in effector T cells. Interestingly, T_{regs} also seem reliant on a delicate balance of Akt activation. On one hand, Foxp3 expression has been shown to rely on initial TCR-induced activation of Akt, yet prolonged Akt activation inhibits Foxp3 expression and T_{reg} differentiation[417,418]. Conversely, another study has shown that Akt inhibition can preferentially inhibit T_{reg} maintenance in a cancer model[395], suggesting that T_{regs} could rely on at least some level of Akt activation to maintain their suppressive phenotype. Putting these data together, it is possible that preferential TBK1 activation downstream of ICOS in ICOS YF mice could be leading to increased degradation of Akt, thereby inhibiting T_{reg} maintenance during chronic inflammation with *T. gondii* (Figure 6.7). This is highly speculative, however, as the role of either TBK1-mediated IRF3 activation or TBK1-mediated inactivation of Akt is unstudied in T_{regs}, but either or both of these pathways could be contributing to the differential phenotypes seen in the T_{reg} populations of ICOS KO and ICOS YF mice during chronic infection in the CNS. Subsequent studies could begin to describe the transcriptional landscape of ICOS KO and ICOS YF T_{regs}, in

addition to interrogating phenotypic aspects such as demethylation at the Foxp3 locus (important for maintenance of Foxp3 expression), as well as better understanding of the suppressive capacity of both ICOS KO and ICOS YF T_{regs} during chronic inflammation.

Using western blotting, levels of activated TBK1 could be assessed in ICOS YF and WT T_{regs}, as well as levels of activated Akt. Based on previously published data suggesting TBK1 is a negative regulator of Akt, we would hypothesize that if increased TBK1 levels are found in ICOS YF T_{regs} compared to WT T_{regs}, levels of activated Akt would be decreased. If this is the case, subsequently knocking down TBK1 in ICOS YF T_{regs} could potentially rescue both Akt levels and expression of T_{reg} identity genes like Foxp3 and CD25. The general role of TBK1 could also be assessed, ideally using Foxp3-Cre^{ERT2}/$TBK1^{fl/fl}$ mice to knockout TBK1 exclusively in T_{regs} while not altering its expression in effector T cells. Further characterization of both ICOS YF and TBK1 KO T_{regs} could provide insight into the delicate balance of the Akt signaling pathway required to maintain a strong T_{reg} identity. Finally, assessing how TBK1 KO Tr_{egs} respond in an inflammatory setting (i.e. *T. gondii* infection) would provide insight into their suppressive capacity in an *in vivo* setting. For a summary of future directions regarding ICOS YF T_{regs} and the role of TBK1 in T_{regs}, see Figure 6.8.

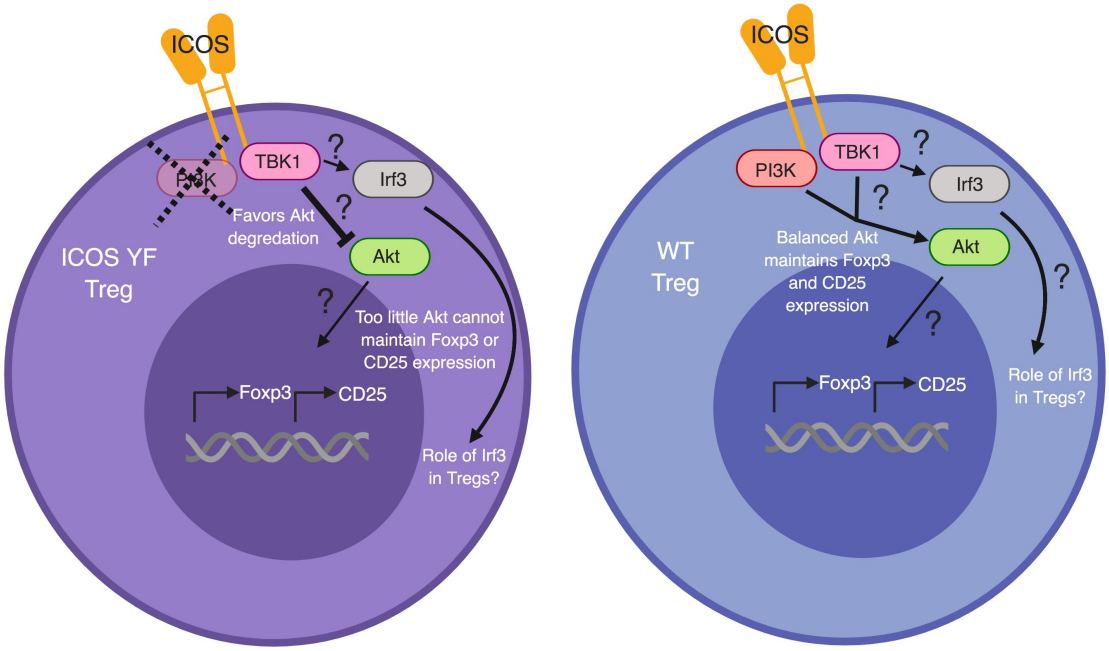
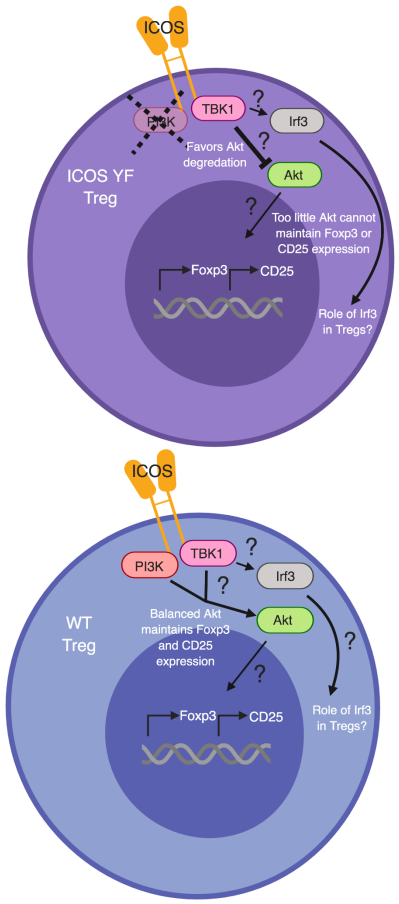


Figure 6.7. Speculative model of the role of ICOS signaling in ICOS YF and WT T_{regs}. ICOS YF T_{regs}, which lack PI3K-mediated signaling, fail to maintain WT levels of "T_{reg} identity" genes, particularly Foxp3 and CD25. Though ICOS signaling has been characterized as acting largely through PI3K, an additional motif (IProx) has recently been identified that binds and activates TBK1. When lacking PI3K binding sites, ICOS YF T_{regs} may preferentially bind TBK1. Though the role of TBK1 is unknown in T_{regs}, it has been shown both to induce the ubiquitination of Akt and activate Irf3 in T cells and myeloid cells, respectively. Possible roles of Irf3 in T_{regs} are also unknown, but it is possible that TBK1-induced degradation of Akt disrupts a delicate balance of PI3K/Akt signaling that is required for T_{reg} identity (left). This could suggest a model in which, in WT T_{regs}, both PI3K and TBK1 bind and are activated downstream of ICOS signaling. Activation of both could help maintain the "right" balance of Akt activation that allows a T_{reg} to maintain Foxp3 and CD25 expression. Because constitutive Akt activation has been shown to decrease Foxp3 expression, TBK1 recruitment and activation may be an intrinsic negative regulatory mechanism in T_{regs} to maintain this delicate balance.



1. Is TBK1 activated after ICOS ligation in WT and YF Tregs?
   a. Determine p-TBK1 levels after ICOS stimulation in WT and YF Tregs
   b. Determine PI3K:TBK1 ratio in WT and YF Tregs after ICOS stimulation

2. What is the role of TBK1 in Tregs?
   a. Characterize TBK1 KO Tregs (Foxp3-CreERT2/TBK1 fl) during homeostasis and during chronic *T. gondii* infection

3. What are the effects on Akt levels in YF Tregs?
   a. If increased TBK1 is associated with decreased Akt in YF Tregs, can this be rescued with constitutive Akt transduction?
   b. Determine effects of Akt levels on "Treg identity" genes. Is this mediated by TBK1 in YF Tregs (using TBK1 knockdown)?

4. Is Irf3 activated dowstream of ICOS ligation in YF Tregs?

5. What is the role of Irf3 in Tregs?
   a. Characterize Irf3 KO Tregs during homeostasis and chronic *T. gondii* infection

Figure 6.8. Future directions to characterize ICOS YF T_{regs} and their inability to maintain a strong "T_{regs} identity". It is possible that ICOS YF T_{regs} preferentially recruit and activate TBK1 downstream of ICOS ligation in the absence of recruitment of PI3K. To determine this, levels of p-TBK1 could be determined in both ICOS YF and ICOS WT T_{regs}. Based on previously published studies, we would hypothesize that ICOS YF T_{regs} would preferentially activate TBK1, leading to decreased activated Akt levels. Activated Akt levels could be determined in ICOS YF T_{regs} compared to WT T_{regs} using western blotting. If it is true that ICOS YF T_{regs} activate TBK1 and show decreased Akt levels, we could then try to rescue Akt levels and T_{regs} identity gene expression (Foxp3 and CD25) using transduction of active Akt, knockdown of TBK1, or transduction of WT ICOS. To determine if TBK1 plays a role in T_{regs} in general, TBK1 KO T_{regs} could be studied both during homeostasis and during chronic *T. gondii* infection in the brain. Ideally, using a Foxp3-Cre^{ERT2}/$TBK1^{fl/fl}$ mouse would allow us to study the loss of TBK1 exclusively in T_{regs} without altering effector CD4+ or CD8+ T cells. Because ICOS YF T_{regs} display an inability to maintain a strong "T_{regs} identity", TBK1 KO T_{regs} would be assessed for their expression of T_{regs} identity genes such as Foxp3 and CD25, though RNA-sequencing could be used to observe global changes in the T_{regs} transcriptome in ICOS YF and TBK1 KO T_{regs}

compared to WT. If TBK1 is a negative regulator of Akt, in TBK1 KO T_{regs} we might expect increased levels of activated Akt, which could either have no effect on T_{regs} identity genes, or could increase Akt levels to an extent that T_{regs} identity is lost (as has been previously reported using strong Akt activation) similar to what is seen in ICOS YF T_{regs}. These studies using ICOS YF and TBK1 KO T_{regs} could provide insight into the delicate balance of Akt activation that is required for maintenance of T_{regs} identity. Finally, TBK1 has also been shown to activate Irf3, whose role in T cells is currently undescribed. First, we would determine whether Irf3 is activated downstream of ICOS ligation in ICOS YF T_{regs}. The role of Irf3 in T_{regs} during both homeostasis and during chronic *T. gondii* could be determined using Irf3 KO T_{regs}.

6.4 Final Remarks

The immunology field is constantly advancing in its understanding of the molecular and cellular basis of immune responses in a wide variety of inflammatory contexts. The two "competing" sides of these immune responses, namely inflammation and immune suppression, have long been of interest. On one hand, inflammatory responses are required to control infections and cancer, but on the other hand, this inflammation is often associated with immune-mediated damage of self-tissues or even the rise of auto-reactive immune responses. Particularly in cases of infection, where some level of inflammation is required to control invading pathogens, studying how the immune response maintains balance between inflammation and limiting immunopathology can help us better understand this delicate equilibrium and potentially lead to better, more precise therapeutics. Much work has been done to identify and characterize the effects of major players, like IL-10 and regulatory T cells, that contribute to this balance. These players provide large-scale regulation and suppression of immune responses by acting in a systemic way, carrying out their suppressive mechanisms on multiple cell types and in multiple tissue sites. These regulatory factors are clearly important for prevention of severe immunopathology both during homeostasis and during ongoing inflammatory responses.

Besides these major players, though, how are immune responses regulated on a more refined scale? Regulation of inflammatory immune responses is likely occurring on multiple levels, and no single factor is responsible for the entirety of a regulatory response during inflammation (Figure 6.8). In this work, I aimed to identify and characterize two major players in immune regulation (IL-10 and T_{regs}) and better

understand signals that contributed to the "fine-tuning" of a chronic T cell response (ICOS). This is just the beginning, however, in trying to illuminate the complex molecular underpinnings that result in a given T cell response. During any immune response, immune cells are constantly bombarded by different signals, both pro-inflammatory and anti-inflammatory. Regulatory or anti-inflammatory factors, whose expression or production are likely impacted by a vast amount of variables themselves, from timing to tissue specificity in the immune response, must then constantly be integrated by an immune cell to the finally result in some phenotypic response. This integration of signals is also going to vary widely based on the type of immune cell that is responding, what type of tissue the immune cell is in, and what other cell types are in the vicinity. Understanding how this signal integration occurs in different cell types can help us further explain the heterogeneity of different immune responses both on a single cell level as well as a population level.

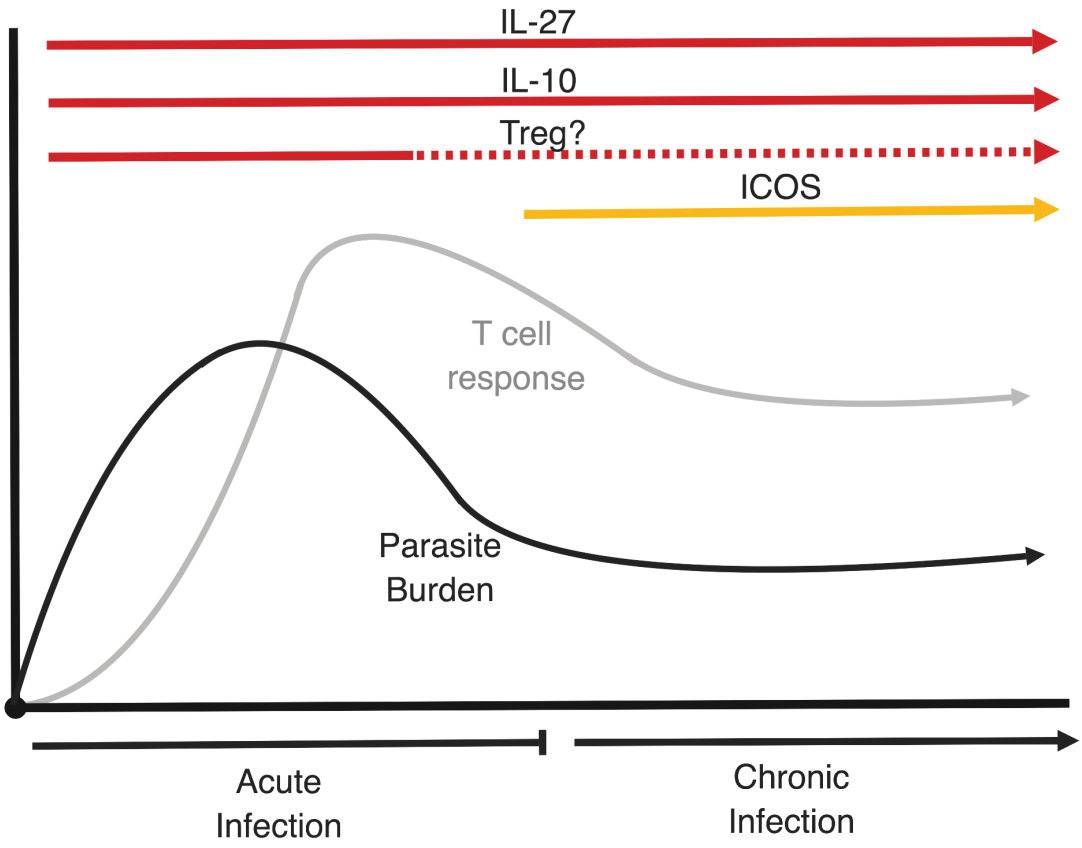


Figure 6.8. Multiple levels of regulation involved throughout the course of *T. gondii* infection. In the context of chronic infection with *T. gondii*, it is likely that multiple regulatory mechanisms are in play to maintain a "balanced" immune response that allows for long-term parasite control while also limiting, as much as possible, immune-mediated tissue damage. IL-27 and IL-10 (solid red line) are required for prevention of fatal immunopathology during both the acute and chronic stages of infection. These two immunosuppressive cytokines act to prevent excessive inflammation on a systemic scale. Though the exact role of T_{regs} (dotted red line) is yet somewhat unknown during *T. gondii* infection, it is likely that this immunosuppressive T cell population is also important throughout the course of infection to limit inflammatory responses in both secondary lymphoid tissues and in the brain. Besides these major players, minor players are also contributing to the long-term regulation of chronic immune responses. ICOS signaling (yellow line) is important for limiting effector T cell responses to IL-2, thereby promoting their apoptosis and rapid turnover in the tissue. Unlike IL-10 and IL-27, ICOS promotes regulation on a smaller scale, serving to suppress effector T cell responses locally in the inflamed brain. Additional minor players are likely to be involved in chronic inflammatory responses, and

understanding all of these regulatory players, and how their signals might interact, is important for building a lager picture of what is involved in complexities of chronic inflammation.

In understanding how immune responses are regulated on multiple levels, from the major players to the minor players, we can begin to identify immune regulatory targets that will allow us to more precisely and specifically manipulate immune responses while limiting immune-mediated pathology. Further work using the newest technologies, such as bulk RNA-Seq and single-cell RNA-Seq, will help in elucidating the molecular pathways involved in large-scale and small-scale regulation of T cell responses, how different environmental signals are integrated to maintain a balanced immune response, and what environmental and molecular signals lead to dysregulated immune responses, which can provide essential information that could lead to better immunotherapeutics for a variety of diseases.

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