UNIVERSITY OF CALIFORNIA, MERCED

Requirements for Immunity to Virulent Secondary Infection with the Parasite *Toxoplasma gondii*

A thesis submitted in partial satisfaction of the requirements for the degree of Master

of

Quantitative and Systems Biology

by

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ABBREVIATIONS

Cytokines
IFNγ: interferon gamma
IL-2: interleukin-2
IL-12: interleukin-12
IL-10: interleukin-10
TGFβ: transforming growth factor beta

Memory T cell effector markers
CD62L: L-selectin
KLRG1: killer cell lectin-like receptor subfamily G member 1

Immune cells
CD4: CD4 T cell
CD8: CD8 T cell
CTL: cytotoxic T lymphocyte
DC: dendritic cell
T_{CM}: central memory T cell
T_{EM}: effector memory T cell
Th1: CD4 T helpter 1 cell
Th2: CD4 T helper 2 cell
Treg: regulatory T cell

Immunoglobulins
IgA: immunoglobulin A
IgG: immunoglobulin G
IgM: immunoglobulin M

T cell exhaustion markers
CTLA-4: cytotoxic T-lymphocyte-associated protein 4
PD-1: programmed cell death protein 1
PDL-1: programmed cell death protein ligand 1
TIM-3: T cell immunoglobulin and mucin domain-containing-3

Other terms
BMDM: bone marrow derived macrophage
FACS: fluorescence-activated cell sorting
i.p.: intraperitoneal
QTL: quantitative trait loci

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Splitt, S.D. *et al*. Using CRISPR to uncover host genetic factors responsible for survival during virulent *Toxoplasma gondii* infection. Poster presented at: Quantitative and Systems Biology Annual Retreat (QSB); 2016 May 16; Merced, CA.

Splitt, S.D. and Jensen, K. Are host genetics the answer to survival against virulent *Toxoplasma gondii* strains? Poster presented at: Bay Area Microbial Pathogenesis Symposium (BAMPS); 2016 Mar 19; San Francisco, CA.

Splitt, S.D. and Risser, D.D. Sucrose Analog Sucralose is a Potent Inhibitor of Hormogonium Differentiation in *Nostoc punctiforme.* Poster presented at: Pacific Undergraduate Research and Creativity Conference (PURCC). 15th Annual PURCC Conference; 2015 Apr 25; Stockton, CA.

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ABSTRACT

Requirements for Immunity to Virulent Secondary Infection with the Parasite *Toxoplasma gondii*

By

Samantha D. Splitt

Master of Science in Quantitative and Systems Biology

University of California, Merced 2017

Professor Patricia LiWang, Chair

Parasitic infections are a global health burden. The prevention of parasitic disease has relied heavily on mass drug administration campaigns, vector control, and sanitation rather than prevention through vaccination (Mutapi et al. 2013). Among parasites that evade effective vaccine induced immunity is *Toxoplasma gondii*, a ubiquitous intracellular protozoan that infects approximately one third of the human population and nearly all warm-blooded vertebrates. The adaptive immune response is fundamental to the clearance of *T. gondii*. Previous work by our lab and others' with commonly studied *T. gondii* strains strongly implicates CD8 T cell functions as the primary immune clearance mechanism (Suzuki and Remington, 1988); however, our data also suggests important roles for CD4 T cells and B cells. Using a larger array of *T. gondii* strains sampled from around the world, we studied the immunological memory response generated in resistant and susceptible mouse genetic backgrounds to secondary infection. We found 1) three host genetic loci determine protective immunological memory responses to *T. gondii*, 2) dysfunctional marker profiles are highly expressed on both CD8 and CD4 T cells from the susceptible mouse strain, and 3) accumulation of B-1b cells in the resistant mouse strain. We present evidence that two of the genetic loci may determine the observed immune cell phenotypes. Importantly, as antibody producing cells, B cells may present an advantage in the development of effective parasite vaccines as current vaccines are conventionally used to induce antibody responses. Understanding the comprehensive immune response to *T. gondii* will provide us with both humoral and cell-mediated immune targets to prevent and treat parasitic disease.

CHAPTER 1

1. Introduction

1.1 Toxoplasmosis

Toxoplasma gondii is a ubiquitous intracellular protozoan parasite that infects nearly one third of the human population and is estimated to infect up to 30 million people in the United States (CDC, 2017). *T. gondii* infects nearly all warm-blooded vertebrates, with felines being the definitive hosts, and exhibits a great deal of genetic diversity, especially amongst South American strains (Grigg et al., 2001). *T. gondii* strains are organized according to their virulence, with type I and atypical strains being virulent, followed by type II and type III strains being relatively avirulent (Howe and Sibley, 1995; Sibley and Boothroyd, 1992; Su et al., 2003; Fux et al., 2007). It is one of five neglected parasitic infections targeted by the CDC as a priority for public health action (2017). Toxoplasmosis is most commonly transmitted through consumption of contaminated food and water. It can afflict both healthy and immunocompromised individuals and poses a notable risk to mothers who can pass the infection to their unborn babies, resulting in congenital toxoplasmosis. Although the host's immune system typically controls the parasite, rendering most infections asymptomatic, severe infection can result when the parasite evades the immune response and this can lead to miscarriage, lifelong disabilities, or death. *T. gondii* belongs to the apicomplexan phylum, including *Plasmodium spp.*, the causative agent of malaria. The immune evasion mechanisms used by human parasitic pathogens are abundant and have made these parasite infections difficult to prevent. Today we only have one parasite vaccine for malaria and its efficacy is very low (25% efficacy; RTS, 2015). Most, if not all, of our vaccines fail to elicit long-lasting effector T cell responses (Siegrist et al. 2008) which are required to kill many parasitic pathogens (Sacks, 2014; Coffman and Sher, 1992). To create better therapeutics and immunological preventions against parasitic infection we must understand both the humoral-mediated and cell-mediated immune responses elicited by these pathogens.

1.2 The importance of CD8 T cells for immunity to protozoan infections

CD8 T cells are central to protection against many protozoan pathogens of medical importance for which we have no protective vaccines. Well-known examples include protection against liver stage malaria infection (400 million infections and 500,000 deaths per year), and chronic infection with *Trypanosoma cruzi*, a kinetoplastid parasite of the heart and bowels, and the causative agent of Chagas disease (5.7 million infections and 10,000 deaths per year) (WHO, 2015). Understanding requirements for CD8 T cell-mediated immunity could have major beneficial impacts on the outcome of parasitic diseases.

Toxoplasma immunologists have long pointed to the important role of CD8 T cells during primary and secondary infection. During secondary infection, CD8 T cells confer immunity when challenged with the virulent type I RH strain (lethal dose in naïve laboratory mice = 1 tachyzoite) (Gazzinelli et al., 1991). CD8 T cells from vaccinated or immune mice can be adoptively transferred to naïve recipients and these mice are protected from challenge with RH (Gigley et al., 2009a; Suzuki and Remington, 1988). Although a variety of immune cells play a role in combating infection, the ability to clear *T. gondii* infection and to confer immunity during secondary infection is unique to CD8 T cells (Bhadra et al., 2011a). Mice vaccinated with the replication deficient CPS 1-1 strain that are depleted of CD4 T cells, but not CD8 T cells, survive challenge with the type I RH strain (Gigley et al., 2009a; Jordan et al., 2009). Even during primary infection with less virulent strains, CD8 T cells are absolutely required for host survival (Shirahata et al., 1994). CD8 T cells from primed mice adoptively transferred to naïve recipients are also protected from ileitis after oral infection (Buzoni-Gatel et al., 1997). All of these data indicate CD8 T cells are central to protection against *T. gondii* infection.

Interferon gamma (IFNγ) is a requisite for CD8 T cell defense against *T. gondii* primary infection and immunity to secondary infection (Gigley et al., 2009a). Most strain isolates of *T. gondii* can kill naïve laboratory mice because of IFNγ-resistant virulence factors (Melo et al., 2011). However, when mice are vaccinated they are protected against challenge with the virulent strain RH, suggesting that immune mice are able to overcome the IFNγ-mediated immune evasion strategies employed by *T. gondii*. While the CD8 T cell IFNγ response is necessary to clear *T. gondii* infection (Suzuki et al., 1988; Gigley et al., 2009a), it is not sufficient for all parasite strains (Jensen et al., 2015) and it is not clear how this alternative mechanism controls parasite strains that are resistant to IFNγ-mediated killing mechanisms. There are several lines of evidence that indicate an unknown alternative parasite killing mechanism other than that elicited by IFNγ, 1) *T. gondii* has many virulence factors that resist IFNγ-elicited killing mechanisms that cause lethality in naïve mice but are controlled in vaccinated mice, and 2) memory CD4 T cells make copious amounts of IFNγ during secondary infection but this cell type does not alter protection when depleted nor confer protection when transferred into a naïve recipient challenged with a virulent strain (Gigley et al., 2009a; Jordan et al., 2009; and Suzuki et al., 1989). Previous investigation of alternative pathways in the cell-mediated response excluded perforin- and FAS-FASL-mediated mechanisms as requirements for protection (Jordan et al., 2009), suggesting CD8-T-cell-triggered cytotoxicity and apoptosis of the target cell, respectively, are not required for immunity to *T. gondii*.

1.3 Other requirements for immunity to *Toxoplasma gondii*

While memory CD8 T cells are vital for secondary infection with the commonly used virulent type I RH strain, our data suggests a more expansive role for the immune response during secondary infection with diverse strain isolates. For primary infection, innate immune cells are the first line of defense against *T. gondii* infection, especially macrophages, dendritic cells, and neutrophils through toll-like receptor signaling (Plattner

et al., 2008). Upon TLR 11 signaling, dendritic cells, neutrophils, and macrophages produce the cytokine IL-12, which induces Th1 CD4 T cell IFNγ responses (Plattner et al., 2008). *T. gondii* PAMPs also trigger TLRs 2 and 4, which induce MyD88 signaling that leads to NF-κB activation and the eventual transcription of proinflammatory cytokines (Debierre-Grockiego et al., 2007). T cells are not the only IFNγ producers important for control of *T. gondii* infection—neutrophils and natural killer (NK) cells also lead to IFNγ-mediated clearing of *T. gondii* (Sturge et al., 2013). Macrophages receive cytokine signals and engage in either anti-inflammatory alternative M2 activation (which can be initiated by type I and type III strain macrophage infections *in vitro*) or pro-inflammatory M1 activation (initiated by type II strains) (Jensen et al., 2011). IFNγ is crucial for M1 activation and induces several toxoplasmicidal functions in macrophages including nitric oxide (NO)-mediated killing, inhibition of growth, and destruction of the parasitophorous vacuole through immunity-related GTPases (Yarovinsky, 2014). Like CD8 T cells, CD4 T cells are important for producing IFNγ, but they are also necessary in the promotion of B cell responses elicited by IgM, IgA, IgE, and IgG antibodies that are capable of controlling and killing *T. gondii* parasites (Kang et al., 2000; Couper et al., 2005; Dupont et al., 2012).

Several studies indicate the important role of the humoral response in immunity against *T. gondii*. In addition to increased CD8 and CD4 T cell responses following *T. gondii* infection, B cell antibody responses are routinely observed. In mice, oral infection elicits elevated levels of *T. gondii*-antigen-specific IgA, IgG, and IgM antibodies and even higher levels of antigen-specific IgG and IgM antibodies following i.p. infection (Zorgi et al., 2016). While vaccination with the type I uracil auxotroph RH strain (RH Δ*ompdc*) prolongs protection against RH challenge in B-cell-deficient Ig-μ KO mice, all mice die by day 27 following challenge (Gigley et al., 2009a). Additionally, transfer of *T. gondii*-immune serum, but not non-immune serum, into vaccinated B-cell-deficient mice significantly prolongs survival after virulent infection with *T. gondii* (Sayles et al., 2000). Furthermore, Sayles et al. show that vaccinated mice lacking Fc receptors or the fifth component of complement (C5) resist virulent infection, suggesting Fc-receptor-dependent phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), and antibody- and complement-dependent lysis of tachyzoites are not important for resistance to virulent challenge (2000). Instead, Sayles et al. suggest that B cells function protectively by producing antibodies that block infection of host cells by tachyzoites (2000). Work by the same group also showed that parasite-specific IgM, but not natural IgM, limited dissemination of tachyzoites during early acute infection by blocking host cell invasion (Couper et al., 2005). While B-1 cells are conventionally thought of as producers of natural antibody, recent evidence suggests they form plasmablasts in response to specific antigens (Covens et al., 2013). While B-cell-deficient mice do not survive virulent challenge with *T. gondii*, Chen et al. showed that B-1 cells from immunized mice rescue B-cell-deficient mice during virulent *T. gondii* infection (2003). Together, these findings suggest an important role for B cells in immunity against *T. gondii* infection.

1.4 Defining new requirements for immunity to *Toxoplasma gondii*

While there is considerable evidence that specific immune cells play an important role in combating certain strains of *T. gondii*, a complete picture of the immune requirements are not known for other more virulent strains of this parasite. By exploring the genetic diversity of both parasite strains and mice, we are situated to paint a robust picture of the requirements for immunological memory responses to this pathogen. In chapter 3, I analyze CD8 and CD4 T cell response during challenge with representative South American strains that cause lethal secondary infection. I attempt to cure disease by modulating key T cell inhibitory pathways that we found were highly expressed on these cells during virulent infection. These observations will expound upon the extent to which T cell exhaustion modulates disease outcome during a virulent *T. gondii* challenge. In particular, I present evidence that modulating these inhibitory pathways may have a limited therapeutic potential during secondary infection, and may point to other mechanisms required for clearing virulent *T. gondii* strains. In chapter 4, I explore host genetics that determine disease outcome of secondary infection with *T. gondii*. I perform a forward genetic screen using MHC congenic and recombinant inbred mouse strains. I find that MHC allele type matters for host survival to some *T. gondii* strains but not others, and that there are two genetic loci on mouse chromosomes 7 and 10 that control resistance to secondary infection. I show evidence that potentially class-switched B-1b cells are protective in resistant mice. At the end of chapter 4, I present a new model for how immunological memory might work for *T. gondii*. And finally, in chapter 5, I discuss the development, use, and limitations of a transgenic IFNγ:YFP reporter mouse that was generated to track CD8 T cell responses during *T. gondii* infection.

CHAPTER 2

2. Materials and Methods

2.1 Cells and Medium

Human foreskin fibroblasts (HFFs) monolayers were grown at 37°C, 5% CO_2 in T-25 flasks for parasite passaging in 'HFF medium' (Dulbecco's modified Eagle's medium [DMEM; Life Technologies] supplemented with 2 mM L-glutamine, 10% fetal bovine serum [FBS; Omega Scientific], 1% penicillin-streptomycin [Life Technologies], and 0.2% gentamycin [Life Technologies]. T-25 flasks were made by splitting T-175 flasks 1:50. T-175 flasks were washed with 20 mL sterile PBS and 3 mL trypsin was added. Flasks were incubated for 10 min at 37°C, 5% CO_2. Monolayers were agitated by tapping the flask and 200 mL HFF medium was added; 4 mL of cell suspension was placed in each T-25 flask. T-25 flasks were ready for passaging after 1-2 weeks.

Bone marrow-derived macrophages (BMDMs) were generated by dissecting the femurs, tibias, and fibulas from C57BL/6 or 45.1 C57BL/6 mice. The bones were placed in 10 mL of BMM (bone marrow macrophage) medium (10% FBS, 1% MEM NEAA [minimal essential medium non-essential amino acids; Life Technologies], 1% sodium pyruvate [Life Technologies], and 1% penicillin-streptomycin). Bones were crushed with mortar and pestle, rinsed with 10 mL BMM medium, crushed again, and filtered through a 70µm nylon filter. Bones were ground, rinsed, and filtered a second time. The suspension was then spun at 1500 rpm for 5 min and resuspended in 4 mL ACK red blood cell (RBC) lysis buffer (NH_4Cl at 0.15 M, $KHCO_3$ at 10 mM, EDTA at 0.1 mM) for 5 min at room temperature, quenched with 16 mL BMM medium, washed in 10 mL BMM medium and filtered through at 70µm filter. Cells were then pelleted, and resuspended BMM medium supplemented with 20% L929, plated in 8 non-tissue culture treated petri dishes (10 mL per plate), and grown to 90-100% confluency at 37°C, 5% CO_2 for 7 days. To harvest, cells were washed with sterile PBS, scraped, collected in a 50mL conical tube, and spun at 1500 rpm for 5 min. BMDMs were stored (1×10^7 cells per vial) in 10% DMSO in BMM medium at -160°C. For *in vitro* T-GREAT T cell assays and parasite assays, BMDMs were plated in 96-well plates at $1 \times 10^5 - 2 \times 10^5$ per well in BMM medium. All counting of live cells was performed by trypan blue dye exclusion.

2.2 Parasite strains

Toxoplasma gondii strains were passaged in human foreskin fibroblasts (HFFs) in 'Toxo medium' (4.5 g/L D-glucose, L-glutamine in DMEM supplemented with 1% FBS and 1% penicillin-streptomycin). The following clonal strains were used (clonal types are indicated in parentheses): RH Δ*ku80* (type I), RH1-1 (type I), GT1 (type I), Pru A7 *fLUC* Δ*hxgprt*::*HXGPRT* (5-8 B^+) (type II), Pru Δ*hxgprt* Δ*ku80* (type II), and CEP *hxgprt*- (type

III). The replication-deficient uracil auxotroph vaccine strain RH Δ*ku80* Δ*ompdc* Δ*up*::*HXGPRT* was grown in Toxo medium supplemented with 10 mM uracil. The following atypical strains were used: MAS (haplogroup HG4), GUY-MAT (HG5), FOU (HG6), GPHT (HG6), TgCATBr5 (HG7), GUY-DOS (HG10), and VAND (HG10).

2.3 Generation of GFP-expressing and tomato-expression GT1 strains

GT1 parasites were transfected with linearized plasmids for parasite expression of Td-Tomato [gifted by M. Melo, MIT] and GFP cLuciferase (Saeij et al., 2005) parasites were grown on HFF monolayers in T-25 flasks in Toxo medium for 2 weeks. Parasites were removed from the flasks by scraping; the parasites were pelleted and washed with PBS and resuspended in sterile FACS buffer (2% FBS in PBS). Fluorescent parasites where then sorted via fluorescence-activated cell sorting (FACS) into a 96-well plate. To ensure single plaque formation in at least one of the wells, the sort was titrated using the following parasite numbers: 100, 50, 25, 12, 6, 3, 2, and 1 for each well per row of 8.

2.4 Mice

Female C57BL/6J (H-2b), A/J (H-2a), C57BL/10SnJ (H-2b), B10.A-H2^{a} H2-T18^{a}/SgSnJ (H-2a), B6AF1/J F1 A/J x C57BL/6J progeny, B6.129S2-Cd8a^{tm1Mak}/J (CD8 knockout), and A/J x C57BL/6 recombinant inbred mice were purchased from Jackson Laboratories and maintained under specific pathogen free conditions. B6.SJL-*Ptprc^{a} Pepc^{b}*/BoyJ (CD45.1^{+}) mice were acquired from the Manilay laboratory (UC Merced) and were bred in-house. T-GREAT mice were obtained by crossing T57 mice (Ploegh laboratory, MIT) with GREAT mice (Reinhardt et al., 2009) [Jackson Laboratories]. T57 transnuclear mice were generated by somatic cell nuclear transfer of K^{b} TDG057$_{96–103}$ tetramer-positive CD8 T cells (C57BL/6 background), and thus 90% of peripheral CD3^{+} T cells are CD8^{+} and 80 to 90% of these have endogenously rearranged TCRα (Vα6-4) and β (Vβ13-1) loci with encoded CDR3 regions bearing specificity for K^{b}-TGD057$_{96–103}$ (Kirak et al., 2010). GREAT (interferon-gamma reporter with endogenous polyA transcript) mice have an IRES-eYFP reporter cassette inserted between the translational stop codon and 3' UTR/polyA tail of the interferon gamma gene.

2.5 Ethics Statement

Every effort was made to ensure unnecessary stress on the animals was avoided. Mouse work was performed in accordance with the National Institutes of Health *Guide to the Care and Use of Laboratory Animals*. All protocols have been reviewed and approved by UC Merced's Committee on Institutional Animal Care and Use Committee. UC Merced has an Animal Welfare Assurance filed with OLAW (#A4561-01), is registered with USDA (93-R-0518), and the UC Merced Animal Care Program is AAALAC accredited (001318).

2.6 Primary infection and serotyping

Parasite injections were prepared by scraping T-25 flasks containing vacuolated HFFs and sequential syringe lysis first through a 25G needed followed by a 27G needle. The parasites were spun at 400 rpm for 5 min and the supernatant was transferred, followed by a spin at 1700 rpm for 7 min. The parasites were washed with 10 mL phosphate-buffered saline (PBS), spun at 1700 rpm for 7 min, and resuspended in PBS. For chronic infections or adoptive transfer experiments, mice were infected intraperitoneally (i.p.) with 10^4 tachyzoites in 200 µL PBS with CEP *hxgprt-*; for direct infection of T-GREATs or for adoptive transfer experiments, Pru Δ*hxgprt* Δ*ku80* or RH Δ*ku80* Δ*ompdc* were used at the same dose. Parasite viability of the inoculum was determined by plaque assay following i.p. infections. 100 or 300 tachyzoites were plated in HFF monolayers grown in a 24-well plate and 4-6 days later were counted by microscopy (4x objective).

Between 30-35 days following chronic infection, 50 uL of blood was harvested from mice from the tail vein, collected in tubes containing 5 uL 0.5M EDTA, and placed on ice. The blood was pelleted at 10,000 rpm for 5 min; blood plasma was collected from the supernatant and stored at -80°C. To evaluate seropositivity of the mice, HFFs were grown on coverslips and infected with GFP-expressing Pru A7 *fLUC* Δ*hxgprt*::*HXGPRT* (5-8 B^+) or RH1-1 overnight, fixed 18 hours later with 3% formaldehyde in PBS, permeabilized with 3% bovine serum albumin, 0.2M Triton X-100-0.01% sodium azide, incubated with a 1:100 dilution of collected blood plasma for 2 hours at room temperature, washed with PBS, and detected with Alexa Fluor 594-labeled secondary antibodies specific for mouse IgG [Life Technologies]. Seropositive parasites were observed by immunofluorescence microscopy.

2.7 Secondary infections and assessment of parasite viability

Seropositive mice were challenged with 5 x 10^4 syringe-lysed parasites and weighed every 3-4 days for survival experiments or euthanized on D5 for FACS experiments. Parasite viability for each strain was determined by plaque assay following completion of injections.

2.8 Genetic linkage analysis

Quantitative trait loci (QTL) analysis was performed with the program R/QTL [Bowman]. LOD scores for each marker were calculated. For all LOD scores >2, 1,000 permutations were performed to obtain the LOD threshold at a P value of ≤0.05, which was considered statistically significant.

2.9 Brain superinfection assays

Brains from chronically infected mice (CEP *hxgprt*-) that survived secondary challenge were dissected, rinsed in PBS, placed in 5 mL PBS in a 10mL syringe, and passed through a 21G needle several times. The suspension was spun at 1700 rpm for 7 min and resuspended in 1 mL of PBS. For rederivation, 200 uL of the brain homogenate was used to inoculate T-25 flasks containing HFF monolayers in Toxo medium. Two weeks later, infected HFFs were syringe-lysed and plated on new HFF monolayers to encourage parasite growth. Once HFFs were fully vacuolated, parasites were passaged onto new HFF monolayers in mycophenolic acid – xanthine (MPA xanthine) medium which selects for parasites encoding a functional *HXGPRT* (i.e. the challenging strains).

2.10 Cell isolation, in *vitro* recall infections, and FACS analysis

To isolate peritoneal exudate cells (PECs) by peritoneal lavage, 4mL of PBS and 3mL of air were injected into the peritoneal cavity with a 27G needle. After shaking, the puncture was expanded with scissors and the PEC wash was poured into a conical tube. PEC washes were filtered through a 70µm cell strainer, pelleted, and washed with FACS buffer (PBS with 1% FBS) before staining. Peripheral lymph nodes (PLNs; axillary, brachial, and inguinal) were dissected and crushed through 70µm cell strainers, pelleted, and washed in FACS buffer. Spleens and mesenteric lymph nodes (MLNs) were dissected and crushed through 70µm cell strainers, pelleted, resuspended in ACK RBC lysis buffer, quenched with 'T cell medium' (10% FBS in RPMI 1640 with GlutaMAX, antibiotics, 10 mM HEPES, 1 mM sodium pyruvate [Life Technologies], and 1.75 µl β-mercaptoethanol per 500 ml [MP Biomedicals]).

For FACS analysis, all preparations were done on ice, and cells were blocked in FACS buffer containing Fc Block anti-CD16/32 [2.4G2; BD Biosciences], 5% normal hamster serum, and 5% normal rat serum [Jackson ImmunoResearch] prior to staining with fluorophore-conjugated monoclonal antibodies (mAbs). The following mAbs (1:100 staining dilutions) were purchased from eBioscience: anti-CD11c-eFlour 450 (N418), anti-CD45.2-eFlour 450 (104), anti-CD4-eFlour 450 (GK1.5), anti-CD62L-eFlour 450 (MEL-14), anti-CD11b-FITC (M1/70), anti-KLRG1-FITC (2F1), anti-CD8α-FITC (53-6.7), anti-PD-1-PE (J43), anti-IFNγ-PE (XMG1.2), anti-TIM-3-PE (RMT3-23), anti-CD8α-PE (53-6.7), anti-CD137/41bb-PE (17B5), anti-PD-1-APC (J43), anti-Ly6G-APC (1A8-Ly6g), anti-CD8α-APC (53-6.7), anti-CD19-PerCP/Cy5.5 (ebio1D3), anti-CD3-eFlour 710 (17A2), anti-CD3-eFlour 780 (17A2), anti-CD4-PECy7 (GK1.5), and anti-Ly6C-PECy7 (HK1.4). The following mAbs were purchased from BioLegend: anti-CD8α-BV510 (53-6.7), anti-41bbL-PE (TKS-1), anti-CD45.1-PECy7 (A20), anti-CD23-Pacific Blue (B3B4), anti-CD21/CD35-FITC (7E9), anti-CD19-PE (6D5), anti-CD5-APC (53-7.3), anti-CD45R/B220-APC/Cy7 (RA3-6B2), and anti-IgM-PECy7 (RMM-1). The following antibodies were purchased from BD Biosciences: anti-CD152/CTLA-4-PE (UC10-4F10-11), and anti-CD11b-BUV395 (M1/70). Other FACS reagents included the viability dyes

propidium iodide [Sigma] at a final concentration of 1 ug/mL and fixability dye 506 [eBioscience] at 1:100, the stimulatory antibodies anti-CD28 [37.51; BD Biosciences] and anti-CD3ε in NA/LE buffer (no azide, low endotoxin buffer) [145-2C11, BD Biosciences], and Calbrite™ APC beads [BD Biosciences].

For *in vitro* recall, peritoneal lavage was performed on chronic and challenged (day 5 following secondary infection) A/J and C57BL/6 mice. PECs were filtered through a 70µm cell strainer, washed in FACS buffer, and 6 x 10^5 PECs or splenocytes per well (96-well plate) were plated in T cell medium. Cells were infected with the type I RH strain at an MOI (multiplicity of infection) of 0.2 for 18 hr; 3µg/mL brefeldin A, 1x [eBioscience] was added for the last 5 hr of infection. 96-well plates were placed on ice, cells were harvested by pipetting and washed with FACS buffer, blocked, and stained for surface markers (described above). Next, cells were fixed with BD Cytofix/Cytoperm and permeabilized with BD Perm/Wash solution [BD Pharmingen]. Cells were then stained with anti-IFNγ-PE (XMG1.2) on ice for 1 hr. Cells were then washed once with BD Perm/Wash solution, once in FACS buffer, and analyzed by FACS.

For *in vitro* stimulation of T-GREAT T cells, 2 x 10^5 BM macrophages were plated in a 96-well plate and 1ug/mL soluble anti-CD3ε antibodies were added for 1 hr prior to adding 5 x 10^5 purified T-GREAT splenic- and PLN-derived T cells to the culture. Cells were harvested 24 hr later and analyzed by FACS.

2.11 Adoptive transfers

CD8 T-GREAT cells were isolated by negative selection using an EasySep™ Mouse $CD8^+$ T Cell Isolation Kit, according to the manufacturer's protocol. Cells were then washed with PBS, resupended in PBS, and kept on ice for injection. Adoptive transfer was performed by injecting 1 x 10^6 – 5 x 10^6 45.2^+ T-GREAT CD8 T cells intravenously through the tail vein (200 uL) or intraorbitally (100 uL) into $45.1^+/45.2^+$, 45.1^+, or CD8 knockout mice. For intraorbital injections, mice were placed under anesthesia using isofluorane. Recipient mice were then injected 2-12 hours later with 1 x 10^4 CEP *hxgprt*- type III or ME49 type II parasites. On days 8, 9, or 35 PECs and spleens from recipient mice were evaluated via FACS analysis for CD8 T-GREAT expansion.

2.12 Neutralization

C57BL/6 mice were chronically infected with 10^4 CEP *hxgprt*- type III parasites followed by challenge 35 days later with 5 x 10^4 virulent GT1 type I parasites. Mice were injected with 200µg of blocking antibodies [anti-TIM-3 (RMT3-23), anti-PDL-1 (B7-H1), and rat IgG2b isotype control, all purchased from BioXCell] at days 1, 3, 5, 7, 10, and 13 post challenge. Antibody injections were prepared in 200µL of sterile PBS. Mouse survival and weight was monitored for 27 days.

CHAPTER 3

3.1 Introduction

3.1.1 CD4 and CD8 T cell exhaustion

T cell exhaustion is a state of T cell dysfunction characterized by hyporesponsiveness, wherein T cells produce fewer cytokines and cytotoxic molecules, lower expression of activating receptors, and increase expression of inhibitory receptors (Wherry, 2011). T cell exhaustion was first characterized in chronic viral infection models (Zajac et al., 1998), but is now widely studied in cancer, bacterial infection, and parasitic infection models (Bhadra et al., 2011a; Stager, et al., 2009; Butler et al., 2012; Virgin et al., 2009). The molecular signature of CD4 and CD8 exhaustion has been thoroughly defined in recent years and several markers have been identified to distinguish these dysfunctional T cell states (Wherry et al., 2007; Crawford et al., 2014). Several key markers have been identified that label CD8 or CD4 T cells as exhausted: 1) high expression of the inhibitory receptors PD-1, LAG-3, BTLA, 2B4, CTLA-4, TIM-3, and others, 2) lowered expression of the costimulatory receptors 4-1bb, ICOS, and OX40, and 3) differential expression of the transcription factors Tbet, Eomes, BLIMP-1, and others (Wherry and Kurachi, 2015; Wherry et al., 2007; Crawford et al., 2014; Sakuishi et al., 2010). Exhausted T cells may carry most, or a portion of, these markers; marker expression varies with disease model, progression of disease state, and cell type.

3.1.2 T cell exhaustion during *Toxoplasma gondii* infection

CD4 and CD8 T cells both play important roles in protection during *T. gondii* infection; however, the requirement for CD8 T cells and their IFNγ response, above all other cell types, has been shown to be necessary through depletion and adoptive transfer studies in C57BL/6 mice using the highly passaged type I lab strain RH (Gigley et al., 2009a; Suzuki et al., 1988). CD8 T cell exhaustion during chronic *T. gondii* infection is defined by loss of polyfunctionality: loss of cytotoxicity (measured by granzyme B production, GzmB) and loss of production of the pro-inflammatory cytokines IFNγ and TNFα; exhausted CD8 T cells exist on a spectrum in which they are able to produce two, one, or none of these functional indicators (Bhadra et al., 2011a; Długołńska and Grzybowski, 2013; Hwang, S. et al., 2016). CD8 T cell exhaustion during *T. gondii* infection has been predominantly defined by elevated expression of the inhibitory receptor PD-1 along with decreased effector functions like IFNγ, TNFα, and GzmB production (Bhadra et al., 2011a; Długołńska and Grzybowski, 2013). Bhadra et al. were able to rescue exhausted CD8 T cells and parasite recrudescence following blockade of the PD-1 ligand (2011a). Later

Exhaustion Marker	Immunological Function
PD-1 (CD279) Programmed cell death protein 1	Inhibitory (Ishida, Y. et al., 1992; Freeman, G.J. et al., 2000). Promotes apoptosis of antigen-specific T cells and reduces apoptosis of Tregs. Downregulates inflammatory cytokine production and induces suppressive cytokines (i.e. IL-10).
TIM-3 (HAVCR2) T-cell immunoglobulin and mucin-domain containing-3	Inhibitory (Monney et al., 2002; Zhu et al., 2005). Found on IFNγ-producing CD8 CTLs and CD4 Th1 cells. Suppresses TCR activation (Tomkowicz et al., 2015). Induces alternative activation (suppressive) and represses classical activation (inflammatory) of macrophages (Zhang et al., 2017). Also found on Tregs in cancer models, where its presence induces suppression (Anderson, 2014).
CTLA-4 (CD152) Cytotoxic T-lymphocyte-associated protein 4	Inhibititory (Brunner et al., 1999). Upregulates on activated T cells and constitutively expressed on Tregs. Binds CD80/86 on APCs with greater affinity than its costimulatory counterpart CD28 (Dyck and Mills, 2017).
2B4 (CD244)	Inhibitory/Stimulatory. Mostly found on memory CD8 T cells and NK cells and its ligand, CD48, is expressed on hematopoietic cells. Decreases survival of memory T cells during secondary LCMV infection (West et al., 2011). Activates and represses lymphocytes depending domain binding (McNerney et al., 2004).
41bb (CD137)	Stimulatory. Mostly expressed on activated T cells, moreso on CD8 T cells than CD4 T cells. Enhances T cell proliferation and survival, cytolytic activity, and IL-2 secretion (Wang et al., 2009; Long et al., 2015).

Table 1. Description of common markers associated with T cell exhaustion and some of their functions.

work by the same group showed CD4 T cell exhaustion is more pronounced—greater expression of inhibitory receptors, and decreased expression of costimulatory receptors and the cytokines IFNγ and TNFα—than CD8 T cell exhaustion during chronic *T. gondii* infection, and that this is mediated by the transcription factor BLIMP-1 (Hwang et al., 2016). Hwang et al. showed that deletion of BLIMP-1 from CD4 T cells not only restores CD4 T cell function, but also CD8 T cell function (2016).

While primary infection with avirulent *T. gondii* strains can induce protective memory responses to virulent strains upon secondary infection, this is not true for all strains—namely, the atypical strains (Jensen et al., 2015). We hypothesized that susceptibility to secondary infection may be due to dysfunctional T cell responses caused by more virulent atypical *T. gondii* strains. Moreover, we also predicted that by correcting the T cell dysfunction, mouse survival to lethal secondary infection may be enhanced. We tested these hypotheses with the following experiments.

3.2 Results

3.2.1 During secondary infection, virulent *Toxoplasma gondii* strains elicit an enhanced CD4 and CD8 T cell exhaustion marker profile

We first evaluated the exhaustion profiles of CD8 T cells using several conventional exhaustion markers (Table 1) following secondary infection with virulent atypical *T. gondii* strains because these cells play a dominant role in controlling *T. gondii* infection. Our model is to first prime C57BL/6 mice intraperitoneally (i.p.) with the type III avirulent *T. gondii* strain CEP *hxgprt*- and then, 35 days later, mice are reinfected with the atypical strains, MAS and GUY-DOS, or the highly passaged type I lab strain RH. In naïve mice, it is known that these strains cause a lethal primary i.p. infection (Su et al., 2003; Fux et al., 2007; Jensen et al., 2015); however, during secondary infection MAS and GUY-DOS, but not RH, produce a lethal outcome in C57BL/6 mice (Jensen et al., 2015).

The T cell populations we analyzed were designated by CD62L (L-selectin) and KLRG1 (killer cell lectin-like receptor G1). CD62L is a cell adhesion molecule found on lymphocytes and is important for homing lymphocytes into the T cell zone of secondary lymphoid tissues; CD62L is found on naïve T cells and central memory T cells (T_{CM}) (Masopust and Schenkel, 2013). Memory T cells, or antigen-experienced T cells, are important for mounting a swift and robust response to secondary encounters with an antigen. T_{CM} cells, like naïve T cells, express CCR7, a chemokine receptor that engages with the ligands CCL19/21 in the peripheral lymphoid organs, thereby homing these cells to these tissues (Ohl et al., 2003). Additionally, T_{CM} cells produce a very minimal repertoire of cytokines, namely IL-2, but following differentiation into effector T cells in response to TCR triggering by antigen, these cells also produce large amounts of IL-4 and IFNγ (Sallusto et al., 2003). Effector memory (T_{EM}) cells are active, cytokine-producing cells that, like effector T cells, do not express CD62L or CCR7; instead, they express inflammatory chemokine receptors that home these cells to the site of infection. KLRG1 is a transmembrane protein found on lymphocytes that marks highly activated, terminally differentiated effector T cells and T_{EM} cells (Robbins et al., 2003; Wilson et al., 2010). T_{EM} and T_{CM}, designated using CD62L and KLRG1, have been noted to have different requirments for IL-12 during differentiation and the ability to make IFNγ during *T. gondii* infection (Wilson et al., 2008; Wilson et al., 2010). It should be noted that these studies often use tetramers to define their memory populations, which are then correlated to surrogate markers—CD62L and KLRG1 in this case. However, we have not used tetramer staining so the population that we loosely define as T_{CM}-like and T_{EM}-like also includes other cells, namely naïve T cells in the $CD62L^+$ population and effector T cells in the $CD62L^-$ population. Using this staining approach, we noted that the frequencies of $CD62L^-$ T cells isolated from the peritoneal cavity increase following chronic infection. However, on day 5 of secondary infection the relative frequencies of each population remained constant (Fig. 1, A and B).

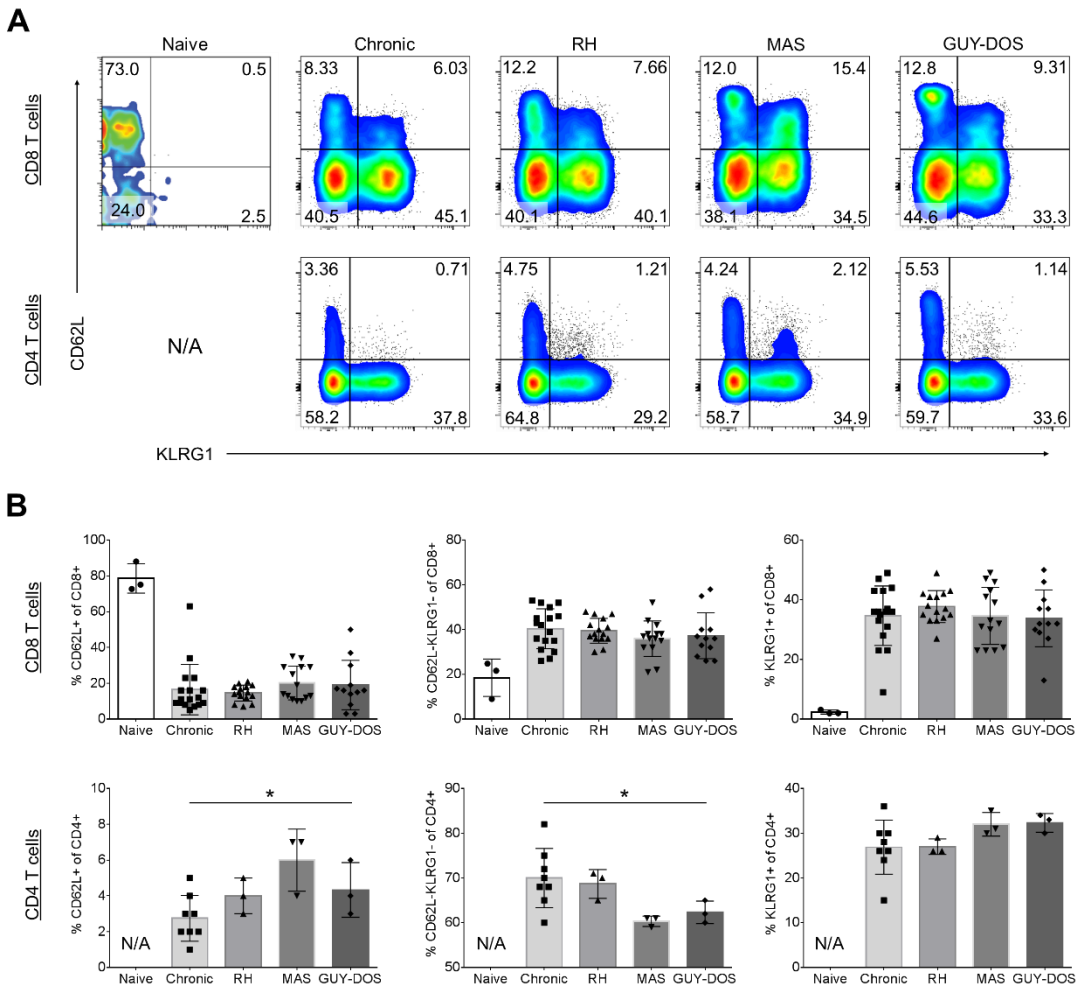


Figure 1. Frequencies of effector populations following chronic and secondary infections with *Toxoplasma gondii*. A) Representative FACS plots of PEC CD4 and CD8 T cells and their expression of CD62L and KLRG1 from naïve C57BL/6 mice, from mice that were infected with the the type III CEP *hxgprt*- strain and allowed to progress to chronic infection for 35-45 days, or from chronically infected mice that were challenged with the indicated *T. gondii* strains on day 5. B) Quantificiation of the relative frequencies of the indicated populations; each dot represents the data from one mouse. P values were calculated using ANOVA testing; *P < 0.05 was considered significant.

We next evaluated the cell surface expression of several inhibitory markers and one activation marker associated with T cell exhaustion in the LCMV model (Wherry et al., 2007; Crawford et al., 2014)—PD-1, TIM-3, CTLA-4, 2B4, and 4-1bb—on T cells isolated from the peritoneal cavity (Table 1 and Fig. 1, left), as this site exhibits a robust T cell response to i.p. infection with *T. gondii* (Wilson et al., 2010). PECs (peritoneal exudate cells) from mice challenged with atypical strains exhibited increased expression of PD-1, TIM-3, and 4-1bb on CD8 T cells—most strikingly in the $CD62L^{-}KLRG1^{+/-}$ (T_{EM}-like) populations—compared to chronic and RH-challenged mice (Fig. 1, left).

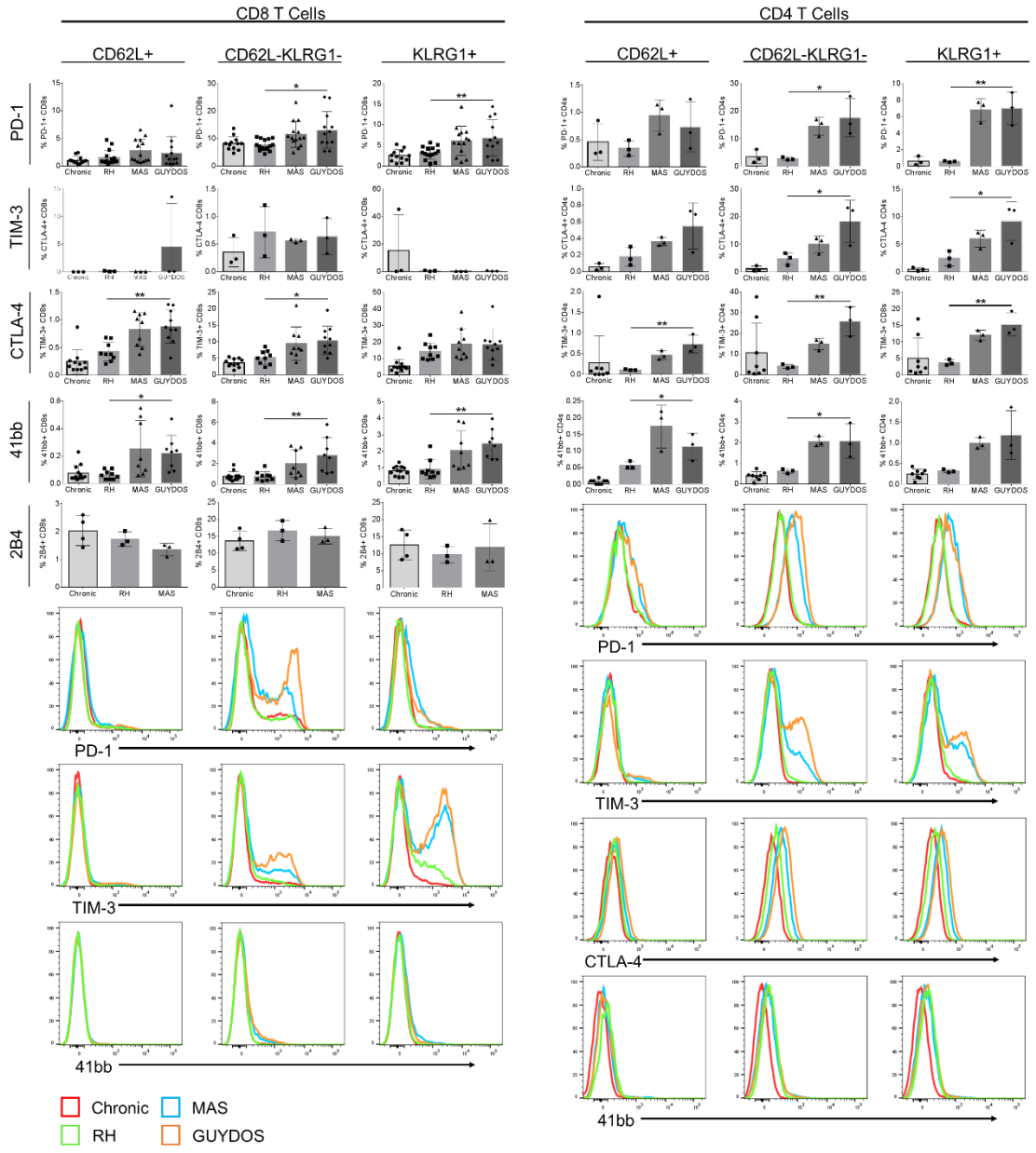


Figure 2. Expression of exhaustion markers on CD4 and CD8 T cells is enhanced following challenge with virulent strains of *Toxoplasma gondii*. PECs were harvested from C57BL/6 mice that were chronically infected with *T. gondii* (type III CEP *hxgprt*- strain), or on D5 post secondary infection (i.p.) with indicated *T. gondii* strains. The frequency of $CD62L^{+}KLRG1^{-}$ ($CD62L^{+}$), $CD62L^{-}KLRG1^{-}$, or $CD62L^{-}KLRG1^{+}$ ($KLRG1^{+}$) that express the indicated marker among total $CD8^{+}CD3^{+}$ PECs (left, top) or total $CD4^{+}CD3^{+}$ PECs (right, top) are shown; each dot is representative of an individual mouse. P values were obtained by ANOVA testing of the challenged mice; *P values < 0.5 and **P < 0.01 are considered significant. Representative histogram plots of $CD62L^{+}$, $CD62L^{-}KLRG1^{-}$, or $KLRG1^{+}$ on $CD8^{+}CD3^{+}$ $CD19^{-}$ PECs (left, bottom) or $CD4^{+}CD3^{+}$ $CD19^{-}$ PECs (right, bottom) that express the indicated exhaustion marker are shown. Chronic/no challenge (red), RH (green), MAS (blue), and GUY-DOS (orange). Cummulative data from four separate experiments for CD8 T cell data (left, top and bottom); cumulative data from two separate experiments for CD4 T cell data, (right, top and bottom).

We similarly evaluated the same surface marker profile on CD4 T cells in the PECs (Fig. 1, right). PEC CD4 T cells showed the same general trend as the CD8 T cells in increasing exhaustion marker expression in response to challenge with atypical strains, although there were two notable differences. First, CD4 T cell expression differences following secondary infection with atypical strains compared to cells from chronic and RH-challenged mice were more apparent. Second, CTLA-4, an inhibitory receptor more highly expressed on CD4 T cells during exhaustion in the LCMV model (Crawford et al., 2014) and constitutively expressed on regulatory T cells (Egen et al., 2002) was elevated in the CD4 CD62L- (T_{EM}-like) population in response to atypical strain challenge (Fig. 1, right). We did not look at 2B4 expression on CD4 T cells.

Additionally, we evaluated coexpression of exhaustion markers during chronic and secondary infection with *T. gondii*. We observed that PD-1 expression correlated strongly with several other exhaustion markers—CTLA-4, TIM-3, and 4-1bb—on both CD4 and CD8 T cells (Fig. 3). Again, we found this trend to be more apparent in CD4 T cells than in CD8 T cells.

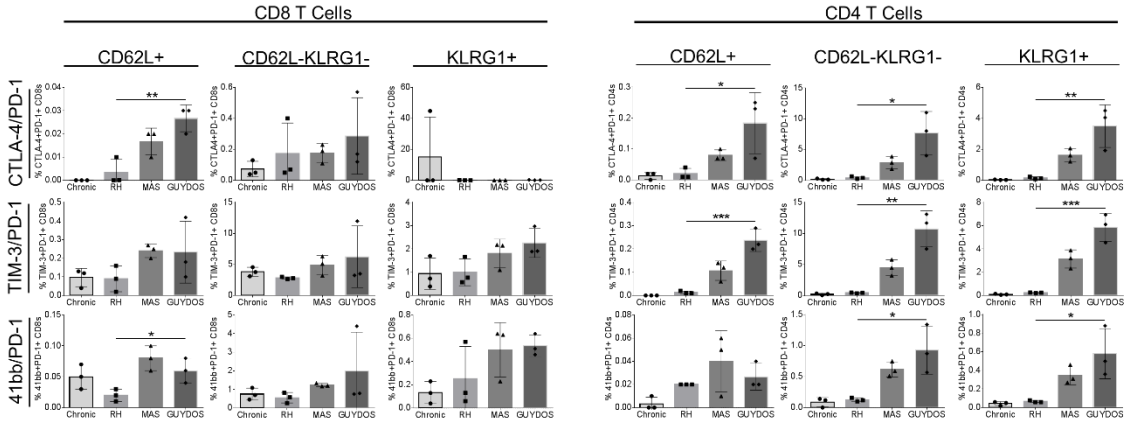


Figure 3. Co-expression of exhaustion markers on CD4 and CD8 T cells is enhanced following challenge with virulent strains of *Toxoplasma gondii*. PECs were harvested from C57BL/6 mice that were chronically infected with *T. gondii* (type III CEP *hxgprt*- strain), or on D5 post secondary infection (i.p.) with indicated *T. gondii* strains. The frequency of $CD62L^{+}KLRG1^{-}$ ($CD62L^{+}$), $CD62L^{-}KLRG1^{-}$, or $CD62L^{-}KLRG1^{+}$ ($KLRG1^{+}$) that express the indicated marker among total $CD8^{+}CD3^{+}$ PECs (left) or total $CD4^{+}CD3^{+}$ PECs (right) are shown; each dot is representative of an individual mouse. P values were obtained by ANOVA testing of the challenged mice; *P < 0.5, **P < 0.01, and ***P < 0.001 are considered significant.. Cummulative data from four separate experiments for CD8 T cell data (left); cumulative data from two separate experiments for CD4 T cell data (right).

3.2.2 Despite exhaustion profiles in both CD4 and CD8 T cells, only CD8 T cells show impaired IFNγ production

To assess whether CD4 and CD8 T cells made IFNγ, the critical effector cytokine required for survival to *T. gondii* infection (Yarovinsky, 2014), its expression was measured by *in*

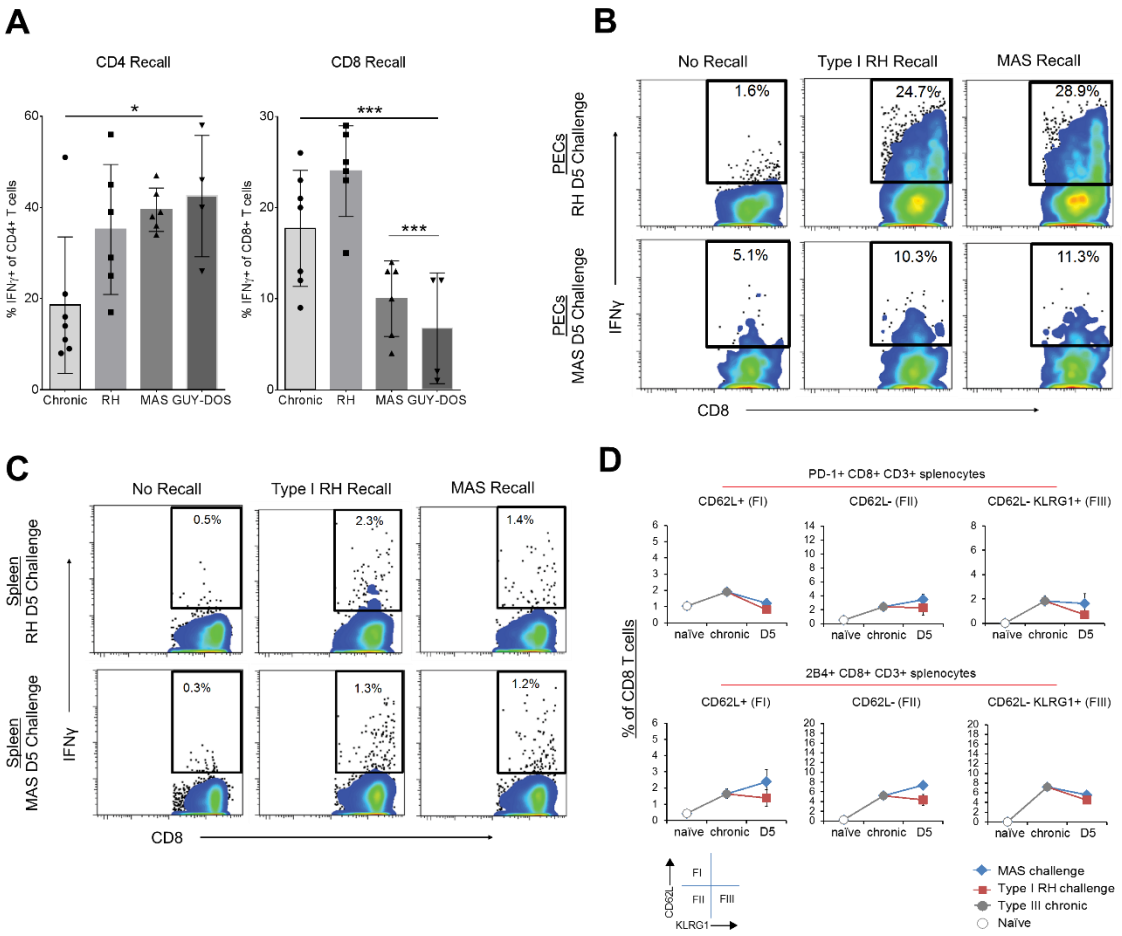


Figure 4. CD8 T cells are less capable of producing IFNγ following following challenge with virulent atypical *Toxoplasma gondii* strains, while CD4 T cells are not impaired. *In vitro* recall was performed on PECs harvested from C57BL/6 mice challenged with the indicated *T. gondii* strain and were infected (recalled) with one of the following *T. gondii* strains, type I RH or MAS; 24 hours later, intracellular staining for IFNγ and FACS analysis was performed. A) Quantification of IFNγ expression in CD4 (left) or CD8 (right) T cells following no challenge, challenge with type I RH, or challenge with the atypical strains MAS and GUYDOS. P values were calculated by ANOVA testing; *P < 0.05, **P < 0.01, and ***P < 0.001 were considered significant. B) Representative FACS plots showing intracellular IFNγ expression on CD8 T cells following RH or MAS challenge, during no recall, RH recall, or MAS recall. The IFNγ response was independent or strain type used in the *in vitro* recall assay. Data is representative of 2-3 experiments; n = 3-8 mice per experiment. C) CD8 T cell splenocytes were analyzed for IFNγ as in panel B. D) Average frequency of the indicated populations among total CD8 T cell splenocytes are shown; +/- SEM, results are compiled from 1-2 experiments.

vitro recall and intracellular FACS analysis. PECs were harvested from C57BL/6 mice following secondary challenge with the atypical strains MAS or GUY-DOS, the highly passaged lab strain RH, or no secondary challenge. PECs were then infected with *T. gondii* and evaluated 16 hours later for IFNγ expression. CD8 T cells challenged with virulent atypical, but not the RH, strains displayed a diminished capacity to produce IFNγ (Fig. 4A, right and Fig. 4B). In contrast, despite CD4 T cells exhibiting an exhausted surface marker profile, their IFNγ expression was not impaired, but rather, increased following challenge (Fig. 4A, left).

Additionally, we evaluated the spleen following virulent challenge and observed very little IFNγ response or exhaustion marker profile differences between RH and MAS secondary challenges (Fig. 4, C and D). It should also be noted that low splenic responses as compared to the PECs are also observed following vaccination with a replication-deficient type I RH strain (Gigley et al., 2009b) and this may represent a general feature of i.p. injections with less virulent *T. gondii* strains (Jensen et al., 2015).

3.2.3 Neutralization of PDL-1 and TIM-3 fails to rescue exhausted CD8 T cells following virulent challenge

PD-1—PDL-1 neutralization proved effective in rescuing CD8 T cell function during chronic infection with type II ME49 *T. gondii* in a previous study (Bhadra et al., 2011a),

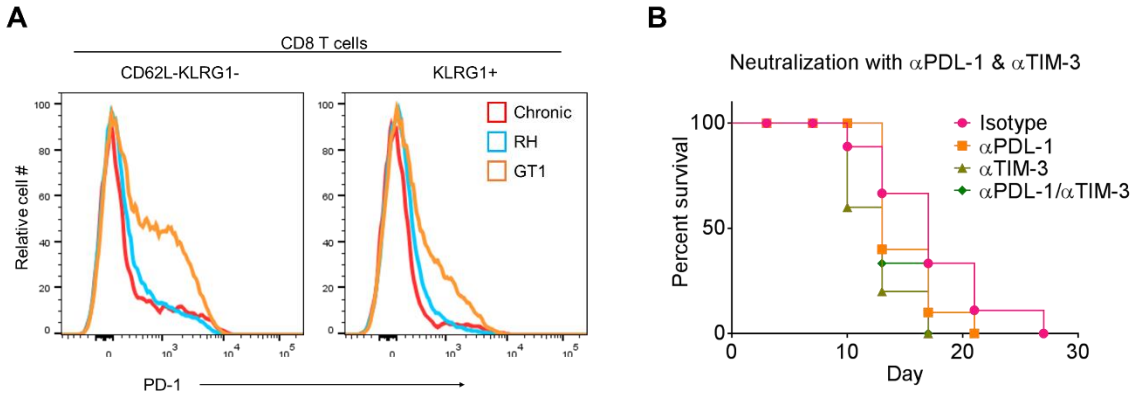


Figure 5. Neutralization with αTIM-3 and αPDL-1 blocking antibodies fails to rescue C57BL/6 mice following virulent secondary challenge. C57BL/6 mice were primed with 1 x 10^4 type III avirulent *T. gondii* CEP *hxgprt-* parasites; then, 35 days later, mice were challenged with 5 x 10^4 type I virulent GT1 *T. gondii* parasites. A) Representative FACS histograms showing PD-1 expression in T_{EM}-like $CD3^+CD8^+$ $CD19^-$ $CD62L^-KLRG1^{+/-}$ populations during chronic infection, RH challenge, and GT1 challenge. B) Following GT1 challenge, mice were injected (i.p.) with 200 µg of the following monoclonal antibodies: rat IgG2b isotype (n = 9), αPDL-1 (n = 10), αTIM-3 (n = 5), or αPDL-1/αTIM-3 (n = 9) on days 1, 3, 5, 7, 10, and 13 after challenge. Survival was monitored daily. Cummulative data from two separate experiments are plotted, with the exception of αTIM-3 which is the result of one experiment. For each therapeutic cohort, P values were calculated by Log-rank (Mantel-Cox) testing against the isotype control; no P values were significant. $P < 0.05$ was considered significant.

but has not been tested during secondary challenge with virulent *T. gondii* strains. To test whether we could reverse T cell exhaustion following virulent challenge and promote host survival, we administered a neutralizing antibody regimen by blocking the pathways of the inhibitory receptors most highly expressed on C57BL/6 mice in our experiments (Fig. 2), TIM-3 and PDL-1. Although we did not measure PDL-1 expression, treatment with blocking antibodies against the PD-1 ligand is the convention (Keir et al., 2008). In this experiment, the GT1 type I strain was used as it induces a lethal secondary infection in C57BL/6 mice (Jensen et al., 2015), and induces expression of PD-1 on CD62L^{-} T_{EM}-like CD8 T cells following challenge (Fig. 5A). Blocking these pathways failed to rescue mice (Fig. 5B). Whether the mice died from parasite burden or cytotoxicity is not known.

3.3 Discussion

3.3.1 CD4 and CD8 T cell function are intertwined during secondary infection with virulent *Toxoplasma gondii*

Our data suggest that both CD4 and CD8 T cells express markers associated with T cell exhaustion following challenge with virulent *T. gondii* strains. However, the individual impact of each of these cell types in this context is not yet resolved. CD4 T cell help to CD8 T cells conventionally occurs by activation of antigen-presenting cells (APCs) through CD40-CD40L interactions. However, it has also been shown that CD40 expression on CD8 T cells facilitates direct CD4 T cell help and that this is necessary for CD8 T cell memory (Bourgeois et al., 2002). Bhadra et al. suggests the CD40-CD40L pathway plays an important role in the rescue of exhausted CD8 T cells during chronic *T. gondii* infection (2011b). Although inducing CD40-CD40L interactions had minimal effects in reinvigorating exhausted CD8 T cells during chronic *T. gondii* infection, cotreatment of both αCD40L and αPDL-1 rescued CD8 T cells more effectively than αPDL-1 treatment alone (Bhadra et al., 2011b). Our data might suggest an important role for CD4 T cell help, that fails to be propagated while the cells are exhausted. Further studies should be conducted to assess the function of the CD40-CD40L pathway during virulent secondary challenge with *T. gondii*.

Although TIM-3 is highly expressed on CD4 and CD8 T cells following virulent challenge, previous studies in viral models show that the state of progression of T cell exhaustion matters during rescue, and perhaps, T cells, following virulent secondary *T. gondii* infection, are *too* exhausted to be rescued. Exhausted T cells that are T-bet^{hi} and PD-1^{mid} are more inclined to be rescued by inhibitory receptor blockade, while exhausted T cells that are EOMES^{hi} and PD-1^{hi} are more prone to die following blockade (Wherry and Kurachi, 2015). Additional blocking antibodies against other inhibitory receptors—for example, CTLA-4, should be tested following virulent secondary challenge with *T. gondii*. Anti-CTLA-4 treatment has proven effective in restoring exhausted CD4 T cell function in several cancer studies (Dempke et al., 2017). Costimulatory receptor agonists to enhance CD40 and 4-1bb, for example, might also be explored (Long et al., 2015).

3.3.2 T cell function may be insufficient for protection against secondary infection with virulent *Toxoplasma gondii*

While CD8 T cells play an absolutely necessary role in survival against challenge with some *T. gondii* strains, like RH, there are likely additional requirements for immunity to more virulent *T. gondii* strains. If restorative treatments for T cell exhaustion fail, then this suggests susceptibility to virulent secondary challenge may not be controlled *only* by exhaustion. Although the exhausted T cell phenotype correlates with increasing parasite virulence, it may not be the driving factor for host susceptibility. To uncover other potential requirements for immunity to virulent secondary challenge, we turned to mouse genetics.

CHAPTER 4

4.1 Introduction

4.1.1 *Toxoplasma gondii* seeks a balanced infection in its many hosts

Toxoplasma gondii infects a wide breadth of hosts ranging from humans to birds to sea-dwelling animals. Because of this host diversity, *T. gondii* has garnered a highly adaptable profile suited to living in diverse niches. Globally, *T. gondii* exhibits great genetic diversity that substantially impacts strain pathogenicity. Strains can be relatively benign and easily cleared, disease-causing and requiring treatment, or even lethal. For *T. gondii* to complete its lifecycle, however, it is important that during acute infection it is virulent enough to remain in the host but not too virulent to kill the host. The balanced infection that *T. gondii* seeks is one that generates a stable chronic infection. At this stage, the parasite developmentally switches to a slow dividing form and produces a tissue cyst structure around itself. The tissue cyst is orally infectious and resistant to immune attack and antibiotics. Completion of the lifecycle occurs when *T. gondii* tissue cysts are consumed by felines, often through a rodent meal, where conditions are suitable for sexual reproduction of the parasite. Oocysts, infectious to intermediate hosts, are then shed by felines through the feces, thereby continuing the lifecycle. Therefore, the balance between the parasite and the host is maintained by a constant battle between parasite virulence factors and the host immune system. Furthermore, a balanced infection in one host may be different for another host. This is evidence by the observation that *T. gondii* strains differ dramatically in virulence in laboratory mice (Howe and Sibley, 1995) and correlate with the severity of human toxoplasmosis (Khan et al., 2006; McLeod et al., 2012; Grigg et al., 2001; Gilbert et al., 2008). This led to the hypothesis that different parasite strains have adapted to certain intermediate host niches (Boothroyd, 2009), defined by host genetics (Lilue et al., 2013). This hypothesis has been expanded to postulate that in endemic regions, such as South America, *T. gondii* has adapted to immune hosts (Jensen et al., 2015), and as such, certain strains of *T. gondii* may have virulence factors tailored to modulate adaptive immunity to establish a secondary chronic infection. However, this hypothesis was derived from observations made in C57BL/6 mice, which were highly susceptible to secondary infection with most virulent parasite strains tested (Jensen et al., 2015).

Here, we hypothesize that mouse genetics will influence the ability of mice to withstand virulent *T. gondii* secondary infection, and control the ability of *T. gondii* to form tissue cysts in challenged survivors. Furthermore, we hypothesize that mouse genetics will drive qualitatively different immunological memory responses and this will determine disease outcome. By studying these differences, and determining the genes and loci that promote mouse survival to secondary infection, we aim to uncover new requirements for immunity to *T. gondii* and define the adaptive niche that may have selected for virulent *T. gondii*

strains. In this chapter, a forward genetic screen and FACS analysis was used to understand how genetics impact immunity to *T. gondii*. The results herein, suggest a role for B-1 cell responses that are controlled genetically and their response correlates with host resistance to *T. gondii*.

4.2 Results

4.2.1 Mouse genetics influence the immunological memory response to virulent challenge with atypical *Toxoplasma gondii* strains

We utilized C57BL/6 mice and A/J mice to assess whether their genetic differences correlated with host resistance to secondary infection with *T. gondii*. *T. gondii* infection has been extensively studied in the C57BL/6 background. Previous data from Jensen et al. show that when C57BL/6 mice are immunized with the avirulent type III CEP *hxgprt*- strain, mice survive secondary challenge with type I (RH), type II, and type III infection, but are very susceptible to "atypical" South American strains (2015). In contrast, we found that A/J mice were resistant to challenge with virulent atypical *T. gondii* strains (Fig. 7A). These results suggest that at least one or more genetic loci control resistance to secondary infection with *T. gondii* strains.

4.2.2 The MHC locus offers protection against some strains of *Toxoplasma gondii*, but not all

Disease outcome of toxoplasmosis in human populations is associated with host genotypes, including the MHC locus (McLeod et al., 1989). Polymorphisms in the MHC locus also confer susceptibility to chronic infection in mice (McLeod et al., 1989). To evaluate the impact of the MHC locus on the differences in mouse susceptibility to secondary infection, we performed a similar experiment with C57BL/10 (B10) and C57BL/10.A (B10.A) mice, both of which are of the C57BL/6 background (Fig. 7A). B10 mice are closely related to C57BL/6 mice and differ by 310 non-synonymous SNPs and 159 exonic structural variants (Doran et al., 2016). The MHC congenic B10.A strain differs from B10 mice only at the MHC locus. B10.A mice encode the A/J MHC locus (H-2a) in place of the C57BL/6 MHC locus (H-2b). The H-2a locus encodes the MHC class I L^d which promotes resistance to cyst formation in type II chronically infected mice (Brown et al., 1995), likely due to the presentation of the polymorphic and protective type II GRA6 epitope (Blanchard et al., 2008). Although the type III strain used to induce chronic infection in our model does not encode the GRA6 polymorphism required for epitope presentation by L^d (Lopez et al., 2015), mice with the H-2a haplotype on average had less cysts and weighed more during type III chronic infection compared to mice with the H-2b locus ($p < 0.04$; Fig. 6).

As predicted, B10 mice challenged with atypical *T. gondii* strains succumbed to infection. However, B10.A mice were highly susceptible (> 70% mortality) to challenge with certain atypical strains (VAND, GUY-DOS, GPHT, FOU) but displayed varying degrees of resistance to challenge with other atypical strains (TgCATBr5, MAS, GUY-MAT), indicating that the MHC locus confers resistance to certain *T. gondii* strains but not all in this system. Importantly, these data also indicate that the A/J genetic background encodes non-MHC-linked genes that control resistance to secondary infection with atypical *T. gondii* strains (Fig. 7A).

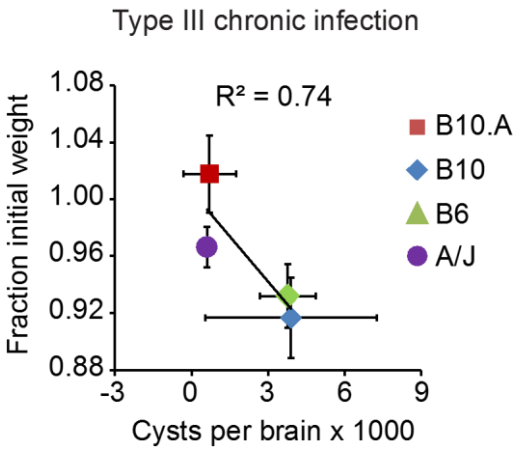


Figure 6. Brain cyst burden and weight during chronic infection in A/J, C57BL/6, C57BL/10, and C57BL/10.A mice. A/J, C57BL/6 (B6), C57BL/10 (B10), and C57BL/10.A (B10.A) mice were injected with the type III strain *CEP hxgprt*- and allowed to progress to chronic infection. Plotted (+/- SEM) is the average cyst number (x 1000) in the brain vs. the average fraction of initial weight, where 1 is the normalized weight on the day of type III injection; the regression value (R^2) is indicated. Results are from 3 to 5 mice for cyst numbers (day 42 of chronic infection), and 5-12 mice for weight measurements (day 30 of chronic infection) per mouse strain.

4.2.3 Genetic mapping reveals two potential loci that determine host resistance to type I GT1 secondary infection

To identify non-MHC loci that promote resistance to secondary infection, we chose to analyze the type I GT1 *T. gondii* strain, as this strain generates a lethal secondary infection in B10 and B10.A, but not in A/J mice or first filial generation A/J x C57BL/6 (F1) mice (Fig. 7B). Furthermore, unlike the highly passaged lab type I RH strain, GT1 elicits a lethal infection in C57BL/6 mice vaccinated with a uracil auxotroph vaccine strain (Jensen et al., 2015). Our results testing F1 mice with secondary GT1 challenge indicate that the genetic loci protecting the resistant A/J strain are possibly haplo-sufficient, and likely, dominantly inherited. Interestingly, while both the A/J and F1 mice are resistant to challenge, the A/J mice exhibited far less superinfection in the brains of survivors (Fig. 7C and Table 2). Our data implies that virulence may be required to superinfect genetically resistant hosts like A/J mice, and can be entirely *balanced* in slightly less resistant hosts like F1 (A/J x C57BL/6) mice, in which no other symptoms—weight loss, dehydration, or lethargy (not

shown)—were noticed while nearly 90% superinfection rates were observed (Fig. 7C and Table 2). Superinfection may be a significant mechanism impacting the parasite population structure of *T. gondii*, because this presents two distinct *T. gondii* strains in a single meal consumed by the definitive host (Jensen et al., 2015). Following consumption, sexual recombination in the feline intestinal tract can lead to the production of genetically distinct parasite progeny. Thus, in the process of identifying A/J loci that determine immunity to secondary infection, the adaptive niche for the selection of virulent *T. gondii* strains may further be defined.

Using the GT1 strain, a similar experiment was performed on 26 recombinant inbred (RI AxB, BxA) A/J x C57BL/6 mice strains, and survival to primary and secondary infection was assessed (Table 3). The RI mice contain an assortment of homozygous A/J and C57BL/6 alleles, which allows for the genetic mapping of loci that contribute to various phenotypes. Genetic mapping of secondary infection mortality revealed two distinct Quantitative Trait Loci (QTL) peaks with logarithm of the odds (LOD) scores greater than 3 on chromosomes 7 (LOD = 3.57, imputed marker between rs8261820 and rs8261944) and 10 (LOD = 3.29, imputed marker between rs13480776 and rs13480777) (Fig. 8, A and B). Permutation testing (n = 1000) revealed neither of these two genetic loci reached significance ($p < 0.05$, LOD = 5.5). Reanalysis of McLeod et al.'s data derived from the same set of RI mice, which identified the MHC H-2a locus on chromosome 17 as a host resistance factor against tissue cyst generation during chronic infection, also produced a non-significant QTL peak (rs13482999, LOD = 2.65; n = 1000 permutations, n.s.; not shown). Nonetheless, non-significant QTL peaks controlling complex traits can still lead to the identification of causal variants within a QTL region, as occurred for the identification of the H-2L^d gene as the resistance factor against type II tissue cyst generation (Brown et al., 1996). We also assessed superinfection in RI mice, although we did not genetically map this data; 52% of survivors were superinfected (Table 4).

Given the major effects were dominated by two genetic loci, candidate genes within the location of the QTL peak were explored. A highly polymorphic gene candidate within the QTL region on chromosome 7 and closest to the genetic marker with the highest LOD score is *nfkbid*, NF-κB inhibitor delta (also known as IκBNS) (Table 5). The NFκB signaling pathway functions in almost all cell types, and regulates genes important for both the innate and adaptive immune responses (Hayden et al., 2006). NFκB has many roles—it functions to induce the expression of proinflammatory molecules and functions in the activation and development of B and T lymphocytes (Livolsi et al., 2001; Gerondakis and Siebenlist, 2010). IκBNS is a member of the atypical NF-κB inhibitors, called nuclear IκBs, reflecting their restricted cellular localization. Unlike classical inhibitors of NF-κB, atypical NF-κB inhibitors can modulate NF-κB to induce or repress transcription (Schuster et al., 2013). Previous work has shown that mice deficient in IκBNS signaling are deficient in B-1 B cell development and lack the ability to respond to T-independent type II antigens, antigens that engage with the B cell receptor (BCR) directly and induce B cell responses in the absence of CD4 T cell help (Touma et al., 2011; Arnold et al., 2012; Pedersen et al., 2014). In general, NF-κB null mice have low levels of circulating IgM antibodies, an immunoglobulin important in the primary immune response to a variety of infections

(Obukhanych and Nussenzweig, 2006; Notley et al., 2010). Pedersen et al. showed that mutations in IκBNS reduce levels of circulating IgM, B-1 cells, and marginal zone (MZ) B cells, (2016). IκBNS is also important for activation-associated cytokines in T cells including IL-2 and IFNγ (Touma et al., 2007). Interestingly, in RNA expression data from whole spleens C57BL/6 mice express greater *nfkbid* than A/J mice (Immgen.org; Heng et al., 2008) (Fig. 8). Whether these expression differences are observed during *T. gondii* challenge is unknown and warrants further investigation.

Mouse Strain	Chronic Infection	Secondary Infection	Heterologous Brain Infection?
C57BL/10	CEP *hxgprt*-	GUYMAT	1/1
Total C57BL/10			1/1 = 100%
C57BL/10.A	CEP *hxgprt*-	TgCATBr5	2/2
C57BL/10.A	CEP *hxgprt*-	GUYMAT	2/2
C57BL/10.A	CEP *hxgprt*-	MAS	1/2
C57BL/10.A	CEP *hxgprt*-	FOU	0/1
Total C57BL/10.A			5/7 = 71%
A/J	CEP *hxgprt*-	GUYMAT	0/4
A/J	CEP *hxgprt*-	TgCATBr5	0/5
A/J	CEP *hxgprt*-	GUYDOS	4/5
A/J	CEP *hxgprt*-	GT1	3/12
A/J	CEP *hxgprt*-	VAND	4/6
A/J	CEP *hxgprt*-	FOU	1/5
A/J	CEP *hxgprt*-	GPHT	0/5
A/J	CEP *hxgprt*-	MAS	0/5
Total A/J			11/40 = 27%
F1	CEP *hxgprt*-	GT1	8/9
Total F1			8/9 = 88%

Table 2. Superinfection of chronically infected mice challenged with virulent *Toxoplasma gondii* strains. Chronically infected C57BL/10, C57BL/10.A, A/J, F1 (A/J x C57BL6) mice were challenged with the indicated *T. gondii* strain; then, 35-45 days after secondary challenge, brains from surviving mice were homogenized in PBS and used to inoculate HFF monolayers. The resultant parasite cultures were then tested for the presence of the challenging strain (numerator = # of mice positive for challenge strain in brain, denominator = n mice tested that exhibited parasite growth in the HFF monolayer).
[1]Parasite growth was observed in mycophenolic acid / xanthine medium, which selects for parasites encoding a functional *HXGPRT* (i.e. the challenging strains).

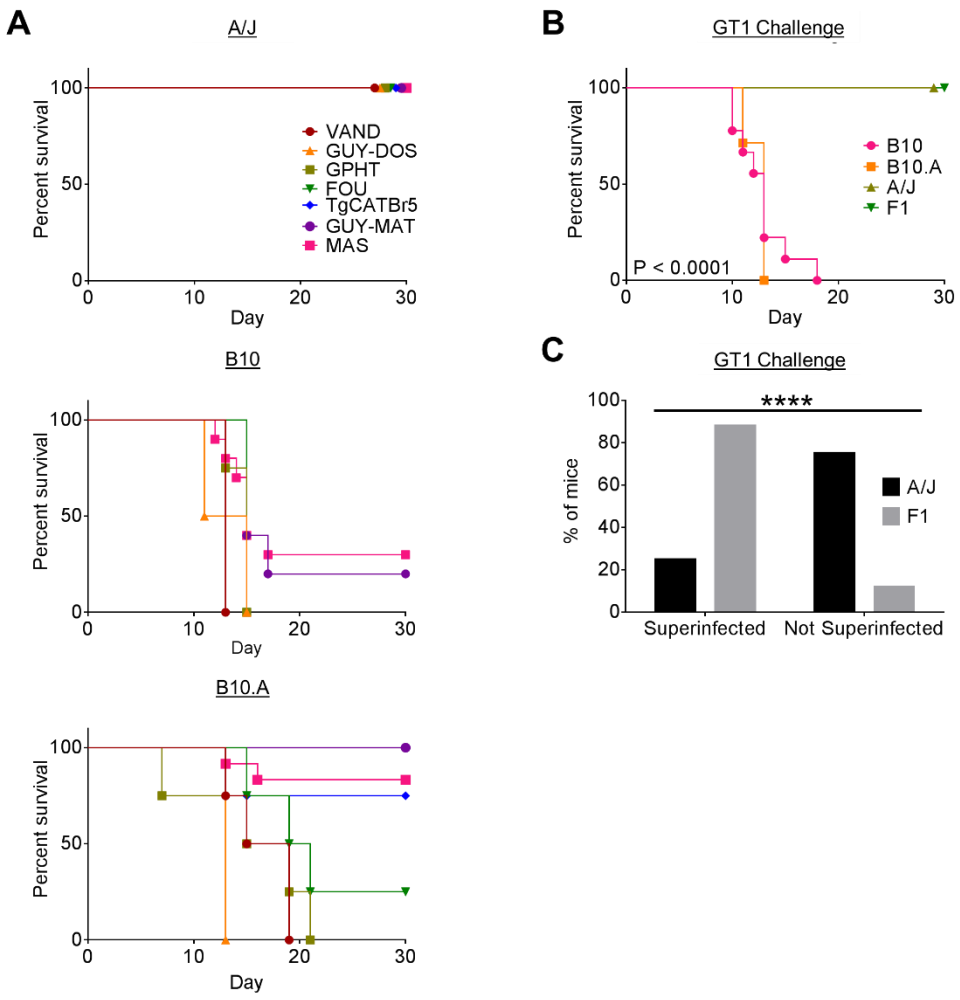


Figure 7. **MHC is a protective factor against some *Toxoplasma gondii* strains, but not all.** All mice were primed with 1 x 10^4 type III avirulent *T. gondii* CEP *hxgprt-* parasites; then, 35 days later, mice were challenged with 5 x 10^4 of the indicated strain of *T. gondii* parasites. A) Survival of A/J [VAND (n = 7), GUYDOS (n = 5), GPHT (n = 5), FOU (n = 5), TgCATBr5 (n = 5), GUYMAT (n = 4), and MAS (n = 7)], B10 [VAND (n = 4), GUYDOS (n = 4), GPHT (n = 4), FOU (n = 4), TgCATBr5 (n = 4), GUYMAT (n = 4), and MAS (n = 12)], and B10.A [VAND (n = 4), GUYDOS (n = 4), GPHT (n = 4), FOU (n = 4), TgCATBr5 (n = 4), GUYMAT (n = 5), and MAS (n = 10)] mice following secondary infection with virulent atypical *T. gondii* strains. Results are from 1-2 experiments. Log-rank (Mantel-Cox) testing was performed to calculate P values. For B10 mice, P values were calculated against the most lethal strain, VAND; P = n.s. (GUY-DOS), 0.0404 (GPHT), 0.0082 (FOU), n.s. (TgCATBr5), 0.0047 (GUY-MAT), and 0.0229 (MAS). For B10.A mice, P values were calculated against the most lethal strain, GUY-DOS; P = 0.0404 (VAND), n.s. (GPHT), 0.0082 (FOU, TgCATBr5, GUY-MAT), and 0.0009 (MAS). B) Survival of B10 (n = 5), B10.A (n = 5), A/J (n = 12), and F1 (A/J x C57BL/6, n = 9) mice following secondary infection with the virulent *T. gondii* strain GT1 (top) and percent of superinfection in A/J (n = 12), F1 (A/J x C57BL/6, n = 9), with the virulent *T. gondii* type I strain, GT1. C) To evaluate superinfection, brains of mice were taken from secondary challenge survivors, emulsified, and grown in MPA-xanthine selection medium, which selects against the *hxgprt-* type III CEP strain used for chronic infection. The percent of mice for which the presence of the secondary parasite strain detected is plotted. P values were calculated by Fisher's exact test; ****P < 0.0001.

Recombinant Inbred Strain	Primary Infection Mortality	Secondary Infection Mortality
AXB1	0	100
AXB2	0	0
AXB4	100	N/A
AXB5	50	0
AXB6	100	N/A
AXB8	0	50
AXB10	100	N/A
AXB12	0	0
AXB13	100	N/A
AXB15	0	0
AXB19	0	100
AXB23	0	100
AXB24	0	100
BXA1	50	100
BXA2	0	100
BXA4	0	0
BXA7	0	100
BXA8	0	0
BXA11	0	100
BXA12	50	0
BXA13	0	0
BXA14	0	100
BXA16	0	100
BXA24	0	50
BXA25	0	0
BXA26	0	0

Table 3. Primary and secondary infection mortality of recombinant inbred (A/J x C57BL/6) lines following secondary challenge with GT1. 26 strains (n = 2) of RI mice were primed with 1 x 10^4 type III avirulent CEP *hxgprt- T. gondii* parasites; then, 35 days later, mice were challenged with 5 x 10^4 virulent type I GT1 *T. gondii* parasites. Primary infection mortality indicates death following CEP *hxgprt-* infection and secondary infection mortality indicates death following GT1 infection.

Mouse Strain	Chronic Infection	Secondary Infection	Heterologous Brain Infection?
AXB2	CEP *hxgprt-*	GT1	0/2
AXB5	CEP *hxgprt-*	GT1	1/1
AXB8	CEP *hxgprt-*	GT1	1/1
AXB12	CEP *hxgprt-*	GT1	2/2
AXB15	CEP *hxgprt-*	GT1	1/2
BXA4	CEP *hxgprt-*	GT1	0/1
BXA8	CEP *hxgprt-*	GT1	2/2
BXA12	CEP *hxgprt-*	GT1	1/1
BXA13	CEP *hxgprt-*	GT1	0/1
BXA25	CEP *hxgprt-*	GT1	1/2
BXA26	CEP *hxgprt-*	GT1	0/2
RI lines			9/17 = 52%

Table 4. Superinfection of chronically infected recombinant inbred (A/J x C57BL/6) mice challenged with virulent *Toxoplasma gondii* strains. 35-45 days after secondary challenge, brains from the indicated RI mice were homogenized in PBS and used to inoculate HFF monolayers. The resultant parasite cultures were then tested for the presence of the challenging strain (numerator = # of mice positive for challenge strain in brain, denominator = n mice tested that exhibited parasite growth in the HFF monolayer).

[1]Parasite growth was observed in mycophenolic acid / xanthine medium, which selects for parasites encoding a functional *HXGPRT* (i.e. the challenging strains).

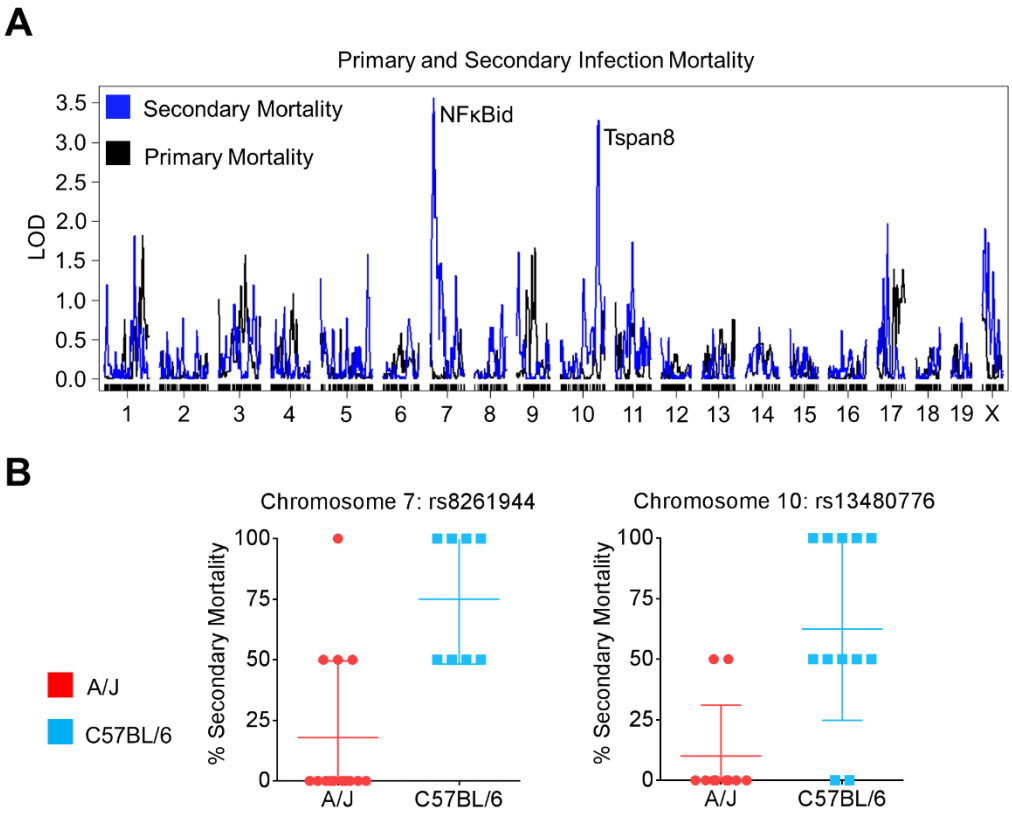


Figure 8. Two genetic loci on chromosomes 7 and 10 confer host resistance to virulent secondary *Toxoplasma gondii* infection. 26 recombinant inbred (A/J x C57BL/6) strains (n = 2) mice were primed with 1 x 10^4 type III avirulent *T. gondii* CEP *hxgprt*- parasites; then, 35 days later, mice were challenged with 5 x 10^4 virulent type I GT1 *T. gondii* parasites. A) LOD scores for each marker were calculated and the running LOD scores of primary and secondary infection mortality is plotted. Quantitative trait loci (QTL) analysis was performed with the program R/QTL (Bowman). For all LOD scores > 2, 1,000 permutations were performed to obtain the LOD threshold at a P value of < 0.05, which was considered statistically significant. Primary infection (black) indicates chronic infection with CEP *hxgprt*- and secondary infection (blue) indicates secondary infection with GT1. B) Effect plots of the genetic markers closest to the maximal LOD scores for chromosomes 7 and 10 is plotted. A/J (red) and C57BL/6 (blue); each point indicates mortality of one RI strain (n = 2) mice.

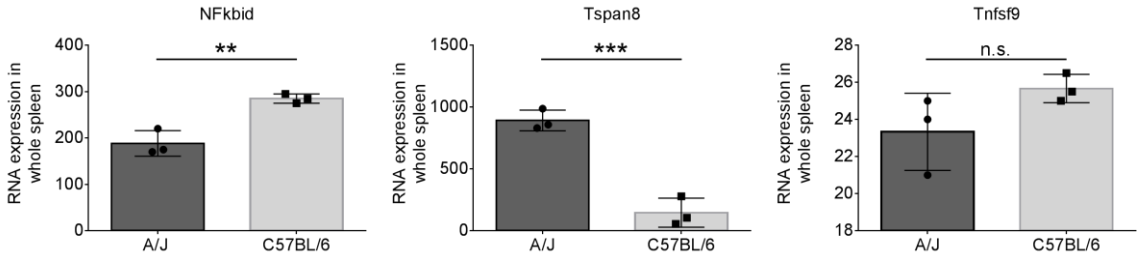


Figure 9. Genetically mapped candidate genes exhibit expression differences between A/J and C57BL/6 mice. RNA was isolated from whole spleen in C57BL/6 mice and A/J mice and the normalized microarray chip intensities for the indicated genes are plotted (adapted from Immgen.org; Heng et al., 2008). Gene expression data was averaged from 3 separate experimental data sets for each gene.

The second gene candidate, *Tspan8* (tetraspanin 8), was found on chromosome 10; it is highly polymorphic between A/J and C57BL/6 mice, and is 6-fold more highly expressed in A/J mice than C57BL/6 mice in whole spleens (Hemler, 2005; Fig. 8 and Fig. 9). Tetraspanins are a group of cell surface proteins that function in motility, proliferation, activation, and growth. Tetraspanins, namely *Tspan8*, have been shown to be important mediators of cancer metastasis; melanoma cancers show higher expression of *Tspan8* (Berthier-Vergnes et al., 2011). Tetraspanin 8 interacts with integrins, which are known to be highly expressed on immune cells, and plays a role in lymphocyte function (Hemler, 2005; Immgen.org; Heng et al., 2008). Perhaps tetraspanin 8 functions to assist immune cell migration during *T. gondii* infection. Further investigation should be done to determine *Tspan8* expression in the context of *T. gondii* infection in A/J and C57BL/6 mice. Another potential, highly polymorphic, gene candidate near the peak on chromosome 10 is NeST, (*Tmevpg1*) a long non-coding RNA that controls microbial susceptibility and epigenetic activation of the IFNγ locus (Gomez et al., 2013). However, this gene is not directly within the confidence interval of the QTL.

Of note, we found a small QTL peak (LOD = 1.97, rs3709414) on chromosome 17. Originally, we thought that an anti-sense transcript (*Gm11110*) to 4-1bbL (*Tnfsf9*) might be a likely candidate controlling expression of this costimulatory ligand found on antigen presenting cells that is polymorphic between A/J and C57BL/6 mice. 4-1bbL interacts with 4-1bb, a costimulatory receptor located predominantly on CD8 T cells. 4-1bbL is a member of the tumor necrosis factor family and associates with TIRAP (Toll-interleukin 1 receptor adaptor protein) and signals through TRAF (TNF receptor associated factor) to activate NF-κB, a transcription factor important for microbial killing (Wang et al., 2009). However, we found 4-1bbL and 4-1bb expression to be very low or not expressed, without any obvious differences, on all immune cells that we looked at in both A/J and C57BL/6 mice (data not shown). It is unclear whether 4-1bb signaling differences exist between A/J and C57BL/6 mice.

Chromosome 7	Start	End	MGI ID	Feature Type	Symbol	Name	NS-SNP	UTR-SNP	Syn-SNP	Intron SNP	Region SNP	Non-coding Transcript SNP	Sum Mutation
LOD ≤ 2.5	30291728	30308367	MGI:1923936	protein coding gene	Clip3	CAP-GLY domain containing linker protein 3	0	1	2	23	3	0	29
	30308717	30314304	MGI:2142037	protein coding gene	Alkbh6	alkB homolog 6	0	1	0	3	4	1	9
	30314807	30319046	MGI:2141950	protein coding gene	Syne4	spectrin repeat containing, nuclear envelope family member 4	0	0	0	1	2	0	3
	30321406	30323895	MGI:1915582	protein coding gene	Sdhaf1	succinate dehydrogenase complex assembly factor 1	0	2	0	0	13	0	15
	30321740	30323472	MGI:3767226	protein coding gene	E130208F15Rik	RIKEN cDNA E130208F15 gene	0	0	0	0	6	0	6
	30355489	30362772	MGI:2442512	protein coding gene	Lrfn3	leucine rich repeat and fibronectin type III domain containing 3	0	0	2	6	0	0	8
	30410916	30413705	MGI:5591188	lncRNA gene	Gm32029	predicted gene, 32029	0	0	0	6	5	0	11
	30413760	30417585	MGI:1277211	protein coding gene	Tyrobp	TYRO protein tyrosine kinase binding protein	0	0	0	10	6	0	16
	30417712	30419854	MGI:1344360	protein coding gene	Hcst	hematopoietic cell signal transducer	0	0	1	8	7	0	16
LOD ≥ 3.0	30421417	30422808	MGI:5683874	CpG island	Cpgi18799	CpG island 18799	0	0	0	0	7	0	7
	30421732	30428746	MGI:3041243	protein coding gene	Nfkbid	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, delta	2	13	2	39	23	0	79
	30423094	30423532	MGI:5683875	CpG island	Cpgi18800	CpG island 18800	0	0	0	0	2	0	2
	30434982	30445535	MGI:88046	protein coding gene	Aplp1	amyloid beta (A4) precursor-like protein 1	0	0	3	35	2	0	40
LOD ≤ 2.5	30447534	30457690	MGI:2442334	protein coding gene	Kirrel2	kin of IRRE like 2 (Drosophila)	1	1	0	7	7	0	16
	30456855	30457472	MGI:5683879	CpG island	Cpgi18804	CpG island 18804	0	0	0	0	1	0	1
	30458315	30487223	MGI:1859637	protein coding gene	Nphs1	nephrosis 1, nephrin	0	0	3	48	0	0	51
	30462164	30465591	MGI:2675468	antisense lncRNA gene	Nphs1os	nephrosis 1 homolog, nephrin, opposite strand	0	5	0	6	8	5	24
	30465354	30466232	MGI:5683880	CpG island	Cpgi18805	CpG island 18805	0	0	0	0	3	0	3

Table 5. List of polymorphic genes within the confidence interval of mouse chromosome 7. All genes on mouse chromosome 7 that have at least one SNP between A/J and C57BL/6 and are between genetic markers with calculated LOD scores ≥ 2.5. The gene boundaries (in base pairs), MGI ID, symbol, gene feature type, and name for each gene is given. Additionally, the type of SNP is indicated as well as the total number of SNPs associated with each gene. All data is derived from MGI (Mouse Genome Informatics). SNP, single nucleotide polymorphism; NS, non-synonymous; UTR, untranslated region; Syn, synonymous.

Chromosome 10	Start	End	MGI ID	Feature Type	Symbol	Name	NS-SNP	UTR-SNP	Syn-SNP	Intron SNP	Region SNP	Non-coding Transcript SNP	Sum Mutation
LOD 1.7 ≤ 2.5	115802415	115869521	MGI:1921893	unclassified gene	4930422I22Rik	RIKEN cDNA 4930422I22 gene	0	0	0	0	63	0	63
	115817284	115849893	MGI:2384918	protein coding gene	Tspan8	tetraspanin 8	0	2	3	55	4	0	64
LOD = 3.28	116018213	116274932	MGI:109559	protein coding gene	Ptprr	protein tyrosine phosphatase, receptor type, R	0	0	0	2	0	0	2
LOD 1.7 ≤ 2.5	116485165	116581511	MGI:1919318	protein coding gene	Cnot2	CCR4-NOT transcription complex, subunit 2	0	0	0	1	0	0	1
	117070129	117148474	MGI:1100860	protein coding gene	Frs2	fibroblast growth factor receptor substrate 2	0	0	0	2	0	0	2
	117344673	117380015	MGI:1913948	protein coding gene	Cpsf6	cleavage and polyadenylation specific factor 6	0	3	0	19	2	0	24
	117377336	117378526	MGI:5621351	lncRNA gene	Gm38466	predicted gene, 38466	0	0	0	0	3	1	3

Table 6. List of polymorphic genes within the confidence interval of mouse chromosome 10. All genes on mouse chromosome 10 that have at least one SNP between A/J and C57BL/6 and are between genetic markers with calculated LOD scores ≥ 1.7. The gene boundaries (in base pairs), MGI ID, symbol, gene feature type, and name for each gene is given. Additionally, the type of SNP is indicated as well as the total number of SNPs associated with each gene. All data is derived from MGI (Mouse Genome Informatics). SNP, single nucleotide polymorphism; NS, non-synonymous; UTR, untranslated region; Syn, synonymous.

4.2.4 Evaluating immunological differences between resistant A/J mice and susceptible C57BL/6 mice

FACS analysis on isolated PECs was performed to assess a comprehensive view of the immune response differences between A/J and C57BL/6 mice in response to secondary infection with the virulent strain GT1. We investigated neutrophils, macrophages, dendritic cell subsets, CD4 and CD8 T cell effector populations and exhaustion marker expression, as well as conventional B-2 and B-1 B cells and their IgM expression. We found high numbers and frequencies of most of these immune cell populations—neutrophils, macrophages, dendritic cells, and CD4s—in C57BL/6 mice (Fig. 10); however, we found A/J mice had more than two times the number of CD8 T cells (Fig. 11A) and showed greater numbers of B-2 cells and B-1b cells (Fig. 17).

4.2.5 C57BL/6 mice have reduced numbers of CD8 T cells and display a more exhausted T cell profile than A/J mice, but their T cells are functionally similar

In the PECs, A/J mice have greater numbers (Fig. 11, A and C) and frequencies (Fig. 12, left) of CD8 T cells than C57BL/6 mice by a factor of more than 2, while C57BL/6 mice have greater numbers (Fig. 11, B and D) and frequencies of CD4 T cells (Fig. 12, right); this trend also applies to each memory and effector memory population of CD4 and CD8 T cells (Fig. 11, C and D). Generally, A/J mice display greater proportions of $CD62L^{-}$ T_{EM}-like and/or effector CD8 T cells while C57BL/6 mice display greater proportions of T_{CM}-like and/or naïve $CD62L^{+}$ CD8 T cells following secondary infection (Fig. 11, E). From our previous data (chapter 3), we knew that CD4 and CD8 T cells in C57BL/6 mice express exhaustion markers during secondary infection with virulent *T. gondii* strains. To evaluate

exhaustion profile differences in A/J and C57BL/6 mice, we evaluated PD-1, CTLA-4, TIM-3, and 4-1bb expression via FACS analysis (Fig. 13). While some exhaustion markers were more highly expressed in C57BL/6 CD8 T cells, namely TIM-3, CD4 T cells exhibited a more striking inhibitory receptor expression profile (Fig. 13). This is interesting considering the greater numbers of CD4 T cells present in C57BL/6 mice. Increases in exhaustion markers also tended to be higher in T_{EM}-like than in T_{CM}-like populations.

We then tested C57BL/6 and A/J CD4 and CD8 T cells functionally by performing *in vitro* recall experiments to look at IFNγ production. *Ex vivo* PECs were infected with the type I RH *T. gondii* strain (recalled) followed by intracellular staining and FACS analysis. Despite exhaustion marker differences, there was no striking difference in IFNγ expression between A/J and C57BL/6 mice (Fig. 14). IFNγ is not the only readout for T cell dysfunction—cytotoxic molecules and other proinflammatory cytokines are often used to assess function—so other functional readouts should be performed. While these results did not exclude the possibility that T cell exhaustion plays an important role in secondary infection susceptibility, they potentially suggested that another immune mechanism was at play in host resistance.

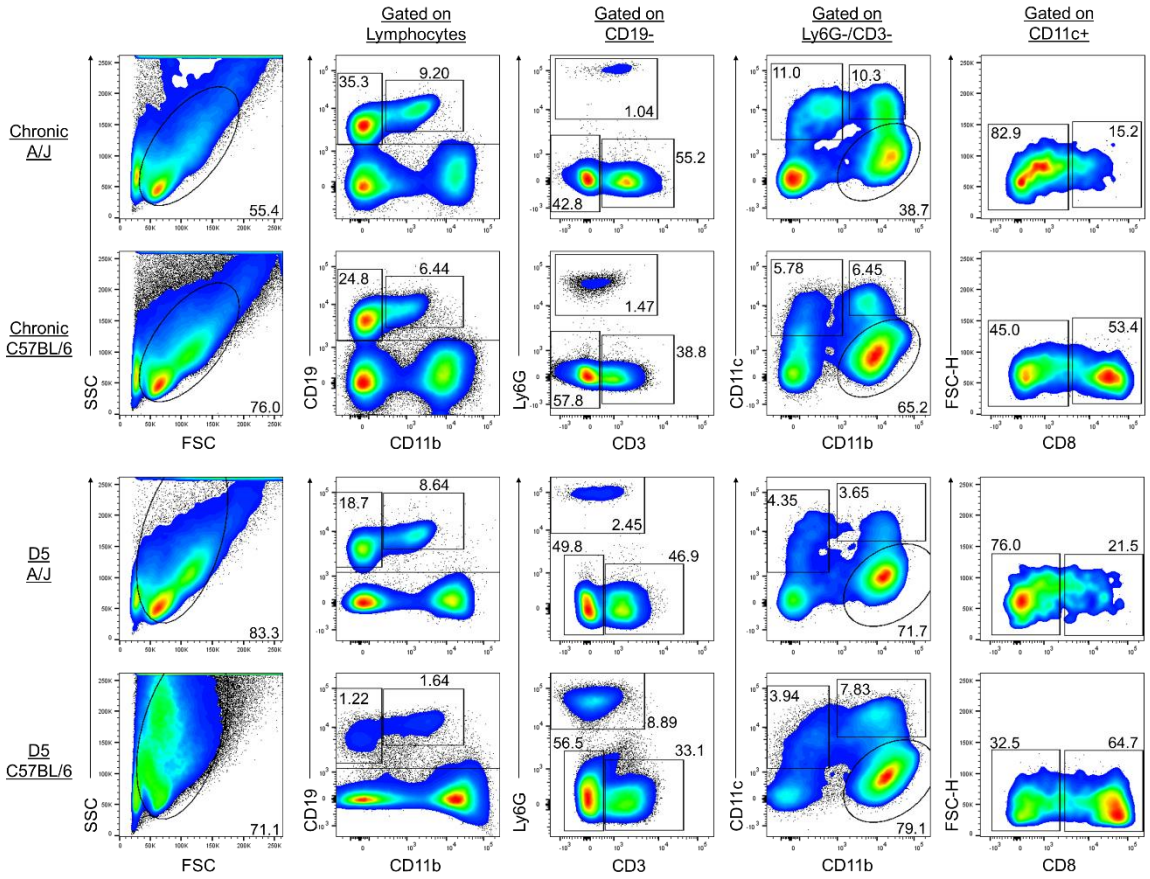


Figure 10. Following challenge, A/J mice have greater frequencies of lymphocytes, while C57BL/6 mice have greater frequencies of innate immune cells and antigen-presenting cells. Representative FACS plots from chronic and day 5 post challenge A/J and C57BL/6 mice**.** A/J (n = 10) and C57BL/6 (n = 10) mice were primed with 1 x 10^4 type III avirulent *T. gondii* CEP *hxgprt*- parasites; then, 35 days later, A/J (n = 5) and C57BL/6 (n = 5) mice were challenged with 5 x 10^4 virulent type I GT1 *T. gondii* parasites. On day 5 (D5) post challenge, PECs and spleen (data not shown) were harvested from chronic and D5 mice, washed with FACS buffer, stained on ice, and analyzed via FACS. Moving from left to right, gating strategy shown does not include exclusion of doublets.

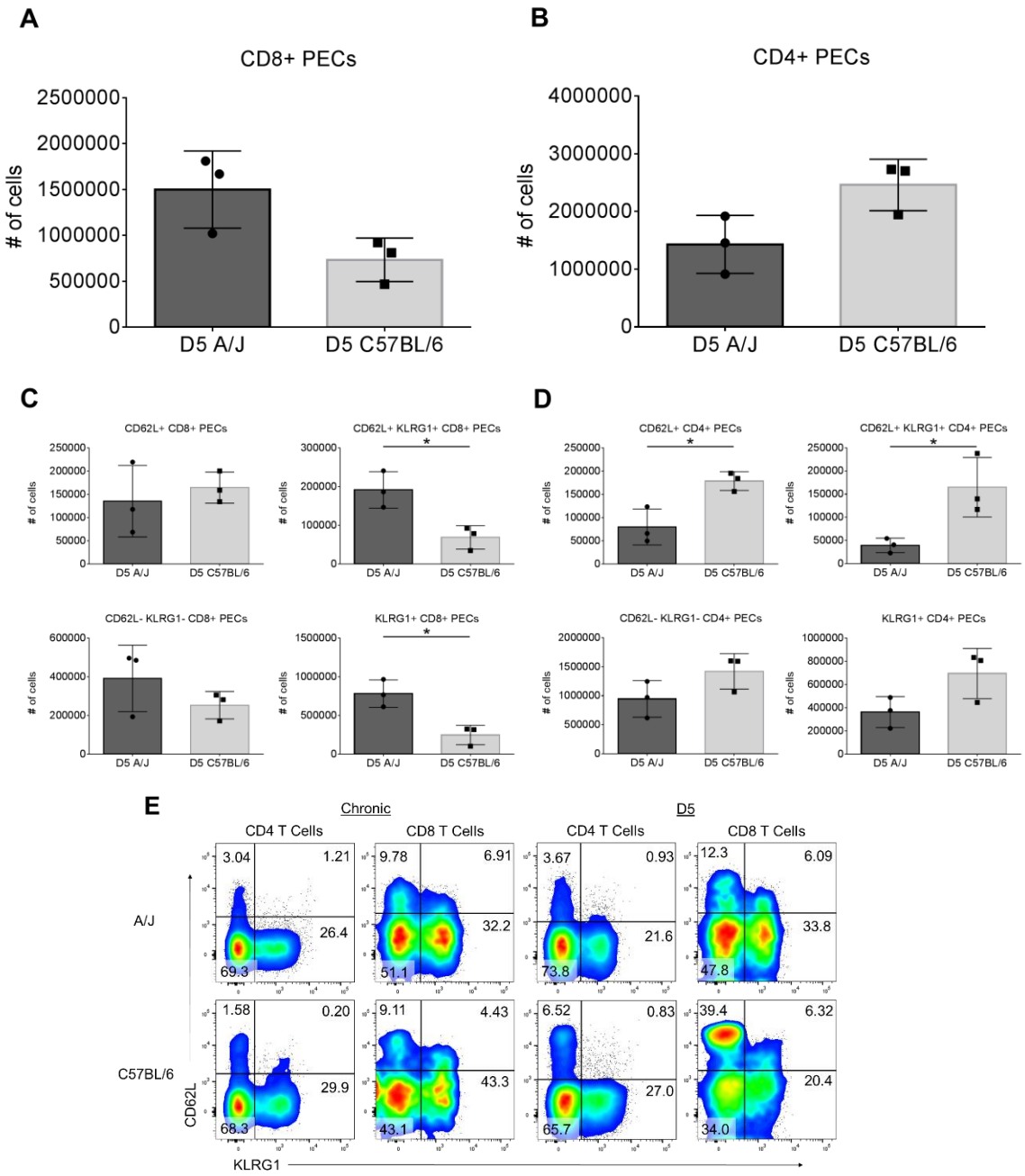


Figure 11. Following challenge, A/J mice have greater numbers of CD8 T cells and display greater frequencies of CD8 T cell effectors following virulent challenge. *Figure legend continued on next page.*

Figure 11 (continued). A-E) A/J and C57BL/6 mice were primed with 1 x 10^4 type III avirulent *T. gondii* CEP *hxgprt*- parasites (chronic); then, 35 days later, A/J and C57BL/6 mice were challenged with 5 x 10^4 virulent type I GT1 *T. gondii* parasites (D5). A-D) Graphs display data from one experiment showing quantification of CD8 and CD4 T cell effector populations from D5 A/J (n = 3) and D5 C57BL/6 (n = 3) PECs. 5mL of PBS containing 5 x 10^4 APC-conjugated CalBrite beads (BD Biosciences) was injected into peritoneal cavities of mice. PEC cells were isolated, washed in FACS buffer, stained, and analyzed by FACS. T_{CM}-like ($CD62L^+$), T_{EM}-like ($CD62L^-KLRG1^-$), and activated T_{EM}-like ($KLRG1^+$). A) Total numbers of $CD3^+CD8^+$ PECs, B) total numbers of $CD3^+CD4^+$ PECs, C) total numbers of indicated CD8 and D) CD4 T cell populations. P values were calculated by student's t-test, unpaired; *P < 0.05. E) Representative FACS plots from chronic and D5 A/J and C57BL/6 mice. Pre-gating was performed to include lymphocytes and to exclude doublets and $CD19^+$ cells.

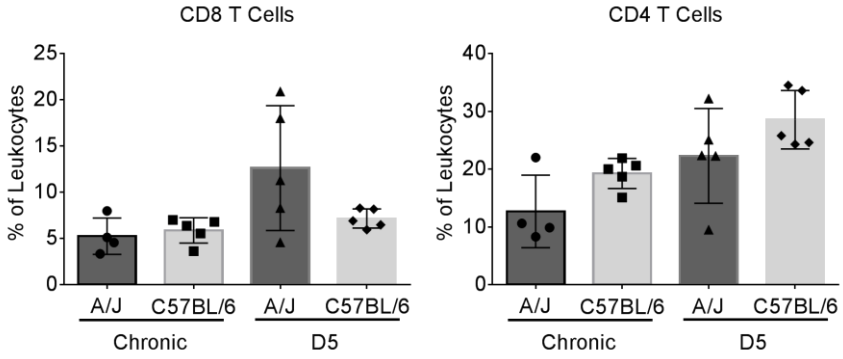
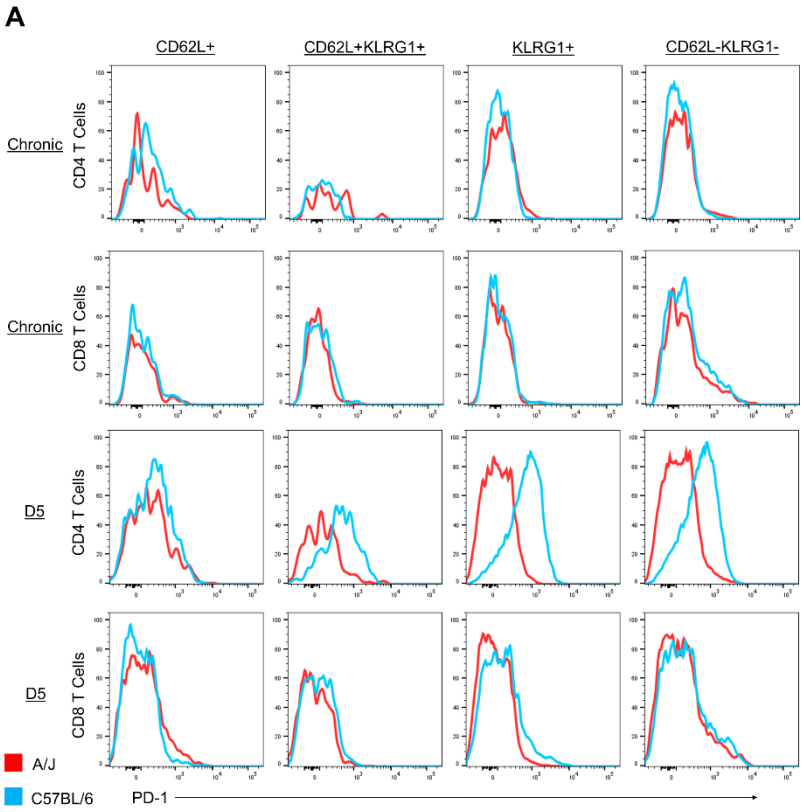


Figure 12. Following challenge, A/J mice have higher frequencies of CD8 T cells while C57BL/6 mice have higher frequencies of CD4 T cells following virulent challenge. A/J and C57BL/6 mice were primed with 1 x 10^4 type III avirulent *T. gondii* CEP *hxgprt*- parasites (chronic); then, 35 days later, A/J and C57BL/6 mice were challenged with 5 x 10^4 virulent type I GT1 *T. gondii* parasites (D5). Graphs display data from one experiment showing percent of total leukocytes that are CD8 T cells (left) and CD4 T cells (right) in A/J (n = 3 per group) and C57BL/6 (n = 3 per group) PECs during chronic and secondary infection with *T. gondii.* Pre-gating was performed to include $CD3^+$ lymphocytes and to exclude doublets and $CD19^+$ cells. P values were calculated by student's t-test, unpaired, comparing chronic A/J to chronic C57BL/6 and D5 A/J to D5 C57BL/6; P values were not significant; P < 0.05 was considered significant.


A
CD62L+
CD62L+KLRG1+
KLRG1+
CD62L-KLRG1-
Chronic
CD4 T Cells
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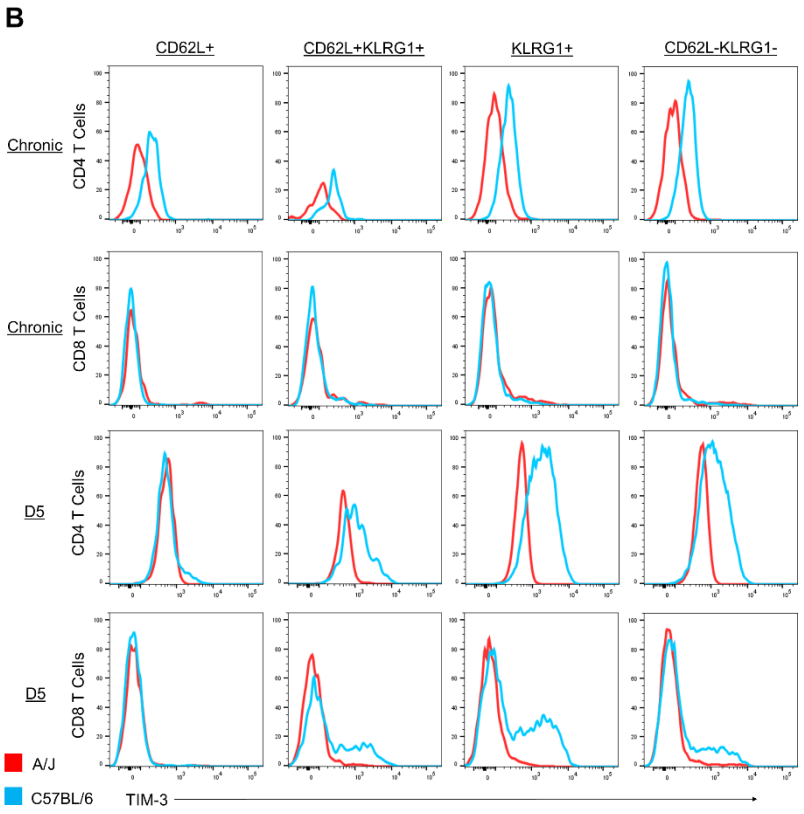
C57BL/6
PD-1


B
CD62L+
CD62L+KLRG1+
KLRG1+
CD62L-KLRG1-
Chronic
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C57BL/6
TIM-3

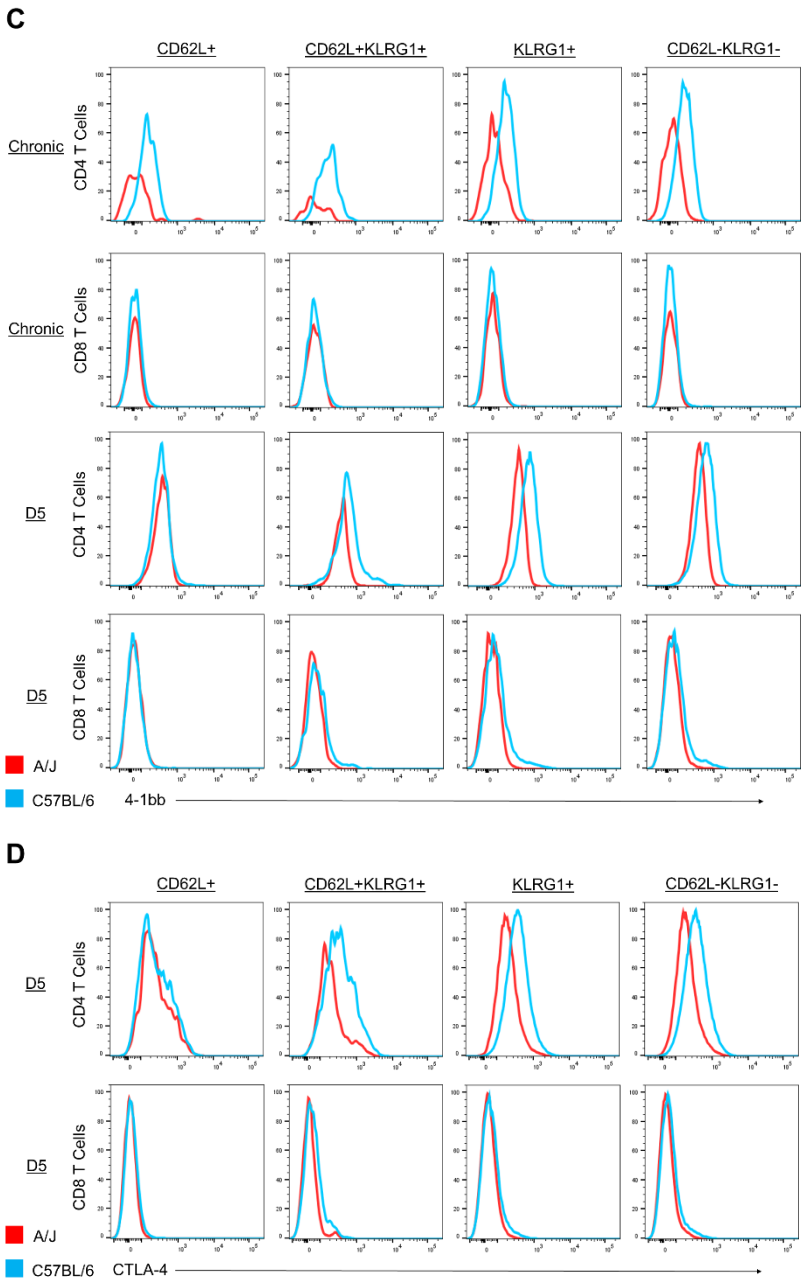


Figure 13. C57BL/6 mice display exhausted marker profiles on CD4 and CD8 T cells following virulent challenge with *Toxoplasma gondii.* Exhaustion marker expression on CD4 and CD8 T cells from A/J and C57BL/6 PECs during chronic and day 5 following secondary infection with type I *T. gondii* GT1 parasites. Representative histogram plots are depicted from one experiment comparing A/J (n = 3 mice per group) and C57BL/6 (n = 3 mice per group), A) PD-1 expression, B) TIM-3 expression, C) 4-1bb expression, and D) CTLA-4 expression. Pre-gating was performed to include $CD3^+$ lymphocytes and to exclude doublets and $CD19^+$ cells.

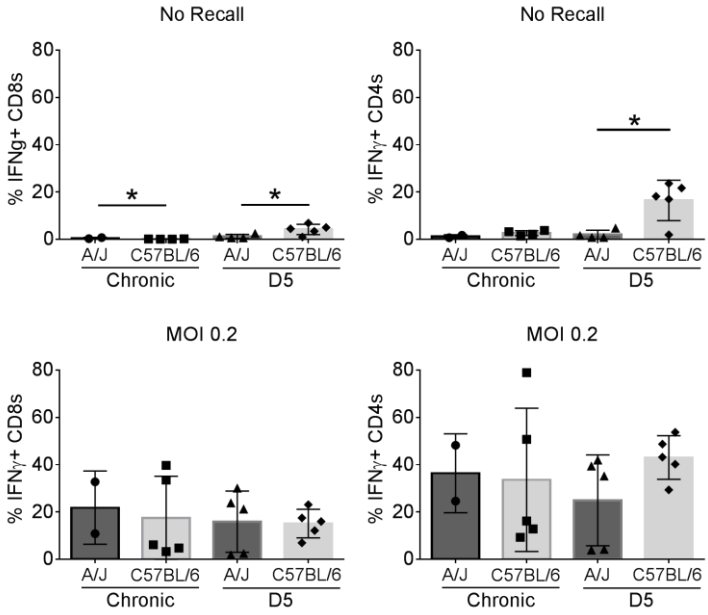


Figure 14. Despite exhaustion profile differences, T cells in A/J and C57BL/6 mice generate similar IFNγ responses. *In vitro* recall of IFNγ in CD4 and CD8 T cells from A/J and C57BL/6 mice during chronic and secondary infection with *T. gondii.* A/J and C57BL/6 mice were primed with 1 x 10^4 type III avirulent *T. gondii* CEP *hxgprt*- parasites (chronic); then, 35 days later, A/J and C57BL/6 mice were challenged with 5 x 10^4 virulent type I GT1 *T. gondii* parasites (D5). PECs were harvested from chronic and D5 mice, plated, and infected or "recalled" with type I RH *T. gondii* parasites at an MOI (multiplicity of infection) of 0.2. FACS analysis was then performed on surface stained and intracellularly stained (IFNγ-PE) recall PECs and spleens (data not shown). A) Percent of unstimulated IFNγ^+ CD8$^+$ PECs (left) and percent of IFNγ^+ CD4$^+$ PECs (right) in chronic and D5 A/J and C57BL/6 mice. B) Percent of recalled IFNγ^+ CD8$^+$ PECs (left) and percent of IFNγ^+ CD4$^+$ PECs (right) in chronic and D5 A/J and C57BL/6 mice. P values were calculated by student's t-test, unpaired; *P < 0.05.

4.2.6 Innate cells are more enhanced in C57BL/6 mice compared to A/J mice following infection with *Toxoplasma gondii*

To broaden our view of the immune system beyond T cells, we investigated the response of macrophages and neutrophils during chronic infection and virulent challenge. Higher frequencies of macrophages and neutrophils were observed in C57BL/6 mice than in A/J mice during both chronic and secondary infections. (Fig. 15A). Both cell types play important roles in regulating the immune response to *T. gondii*, especially during primary infection; however, macrophage and neutrophil frequencies were specifically elevated following challenge (Fig. 15A). Macrophages are the preferred immune cell type infected by *T. gondii*, and as such, play an important role in the control and clearance of the parasite (Murray, 2011). Although macrophages are important in clearing *T. gondii*, they may play

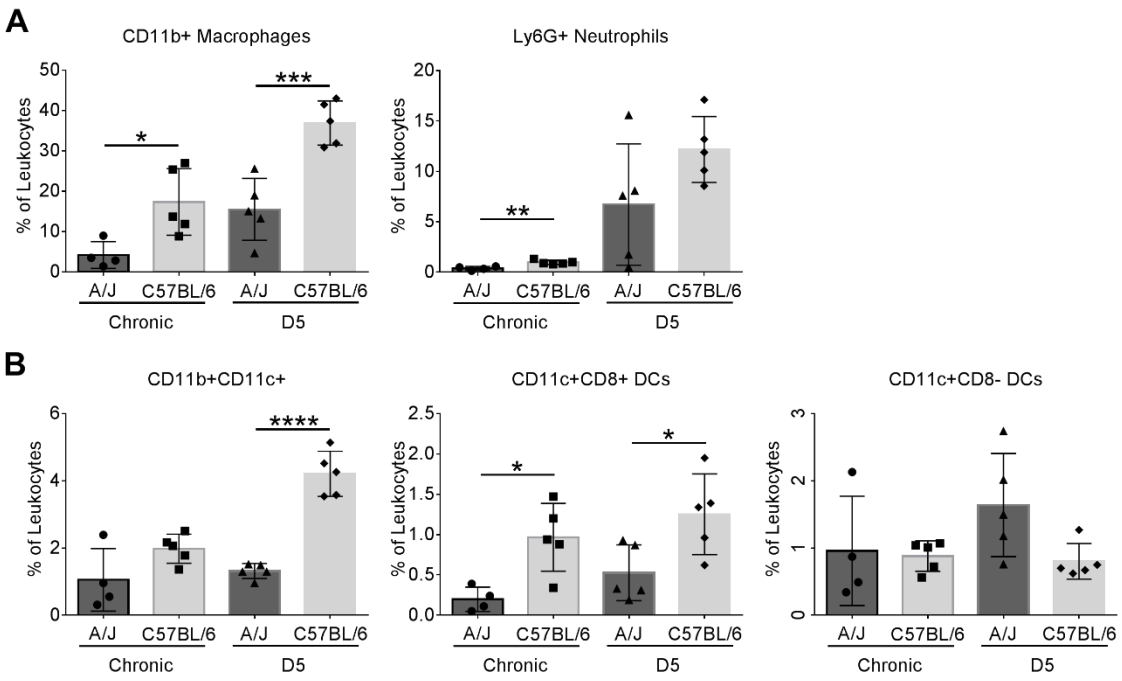


Figure 15. The innate response of C57BL/6 mice is more robust compared to A/J mice during *Toxoplasma gondii* infection. A/J and C57BL/6 mice were primed with 1 x 10^4 type III avirulent *Toxoplasma gondii* CEP *hxgprt*- parasites (chronic); then, 35 days later, A/J and C57BL/6 mice were challenged with 5 x 10^4 virulent type I GT1 *T. gondii* parasites (D5). PECs and spleen (data not shown) were stained and FACS analyzed; n = 5 for all groups, except A/J chronic, where n = 4. A) Percent of leukocytes that are $CD11b^+$ ($CD11c^-$ $CD19^-$) macrophages (left) and $Ly6G^+$ ($CD19^-$) neutrophils (right). B) Percent of three different $CD11c^+$ ($CD19^-CD3^-Ly6G^-$) $CD11b^+$ (left), $CD8^+$ (middle), or $CD8^-$ (right) dendritic cell (DC) subsets in the PECs of A/J and C57BL/6 mice during chronic and secondary infection with *T. gondii*. P values were calculated by student's t-test, unpaired; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

a counterproductive role in C57BL/6 mice, where macrophages are more largely polarized towards alternative activation (Hassan et al., 2015). While neutrophils are known to produce IFNγ in response to *T. gondii* infection (Sturge et al., 2013), they have also been shown to increase parasite spread by acting as motile vessels that cross intestinal epithelium in C57BL/6 mice, although *T. gondii* was administered orally in this case (Coombes et al., 2013). The spread of *T. gondii* throughout the host is facilitated by its exploitation of multiple different migratory immune cells (Courret et al., 2006; Lambert et al., 2006).

We also observed greater frequencies of $CD11b^+CD11c^+$ and $CD11c^+CD8^+$ dendritic cells in C57BL/6 mice during both chronic and secondary infection, and greater frequencies of $CD11c^+CD8^-$ dendritic cells in A/J mice (Fig. 15B). Like neutrophils, DCs have been shown to distribute *T. gondii* to different tissues, including immune-privileged organs like the brain, eye, and placenta (Montoya and Liesenfeld, 2004; Sanecka and Frickel, 2012). CD11c-expressing and CD11b-expressing immune cells, likely monocytes, have also been shown to transport *T. gondii* into the brain (Courret et al., 2006; Sanecka and Frickel, 2012). DCs are important in protecting against *T. gondii* through their innate functions in

recognizing TLRs and producing IL-12 and also through their adaptive functions in antigen presentation to T cells. IL-12 production drives Th1 development and induces IFNγ responses, which elicits immune cell effector functions (Melo et al., 2011). Absence of $CD8^+$ DCs, achieved with $Batf3^{-/-}$ mice, decreased production of IL-12 and IFNγ (Mashayekhi et al., 2011). In addition, $CD8^+$ DCs are important for cross presentation of antigens derived from intracellular pathogens (Theisen and Murphy, 2017).

4.2.7 B cell populations are deficient in C57BL/6 mice compared to A/J mice following virulent *Toxoplasma gondii* challenge

We also analyzed the response of the B-cell compartment to secondary infection (Fig. 10). B-2 cells, or conventional B cells, are generated in the bone marrow and comprise the humoral arm of the adaptive immune system; they make antibodies, present antigens, produce cytokines, form memory cells, and predominate in the spleen (Pieper et al., 2013). B-1 cells, or innate-like lymphocytes, self-renew and are generated early in development within the fetal liver (Baumgarth, 2011). B-1 cells produce natural antibodies (i.e. IgM), predominate in the body cavities, and have been considered to lack the ability to form memory, although this has been disputed (Yang, Y. et al., 2012). During chronic infection C57BL/6 mice and A/J mice show similar frequencies of B-2 and B-1 cells, but after challenge the frequency of both B cell lineages appears to collapse in C57BL/6 mice while A/J mice retain their B cell frequencies (Fig. 16, left).

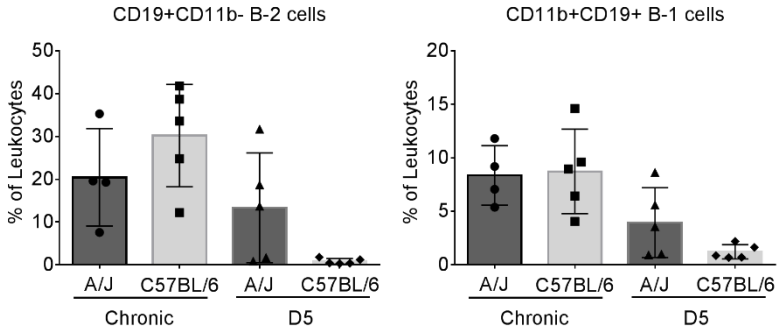


Figure 16. Frequencies of B-1 and B-2 cell populations are diminished in C57BL/6 mice following virulent challenge with *Toxplasma gondii*. Frequency of leukocytes of $CD19^+CD11b^-$ B-2 (left) and $CD19^+CD11b^+$ B-1 (right) cells in the PECs of A/J and C57BL/6 mice during chronic and secondary infection with *T. gondii*. A/J and C57BL/6 mice were primed with 1 x 10^4 type III avirulent *T. gondii* CEP *hxgprt-* parasites (chronic); then, 35 days later, A/J and C57BL/6 mice were challenged with 5 x 10^4 virulent type I GT1 *T. gondii* parasites (D5). PECs and spleen (data not shown) were stained and FACS analyzed; n = 5 for all groups, except A/J chronic, where n = 4. P values were calculated by student's t-test, unpaired; $P < 0.05$ was considered significant.

To assess both cell numbers and frequency, and to identify B cell populations more thoroughly, we performed a secondary challenge experiment with A/J and C57BL/6 mice followed by FACS analysis to evaluate B-2 cells, B-1a cells, B-1b cells, and their IgM expression (Fig. 17, A and B). B-1 cells are divided into two lineages, B-1a cells and B-1b

cells, both of which are derived from the fetal liver during early development (Hayakawa et al., 1985; Baumgarth, 2011). Both B-1a and B-1b cells reside predominantly in the pleural and peritoneal cavities, whereas B-2 cells predominantly reside in the spleen and lymph nodes. B-1a cells have polyreactive and self-reactive BCRs; they undergo positive selection on self antigens and are implicated in autoimmunity (Hayakawa et al., 1983). B-1b cells have polyreactive BCRs but their developmental pathway is unclear (Baumgarth, 2011). B-1 cells produce most of the circulating IgM, an immunoglobulin important in the activation of complement, neutralization of antigens, agglutination, precipitation, and enhancement of phagocytic clearance of dead and dying cells (Grönwall et al., 2012). They also produce natural IgA and IgG in a T-independent manner, in response to self-antigens (Berland et al., 2002). On day 5 following secondary challenge, C57BL/6 mice had similar numbers of B-1a cells in the PECs (Fig. 17B). For each B cell subset, IgM^+ cell differences were observed between A/J and C57BL/6 mice, with C57BL/6 mice expressing more IgM (Fig. 17B). However, the most striking difference between A/J and C57BL/6 mice was an increased population of B-1b IgM^{low} cells in A/J mice (Fig. 17B). Because we only performed surface staining for IgM, it is unclear whether internal IgM is present.

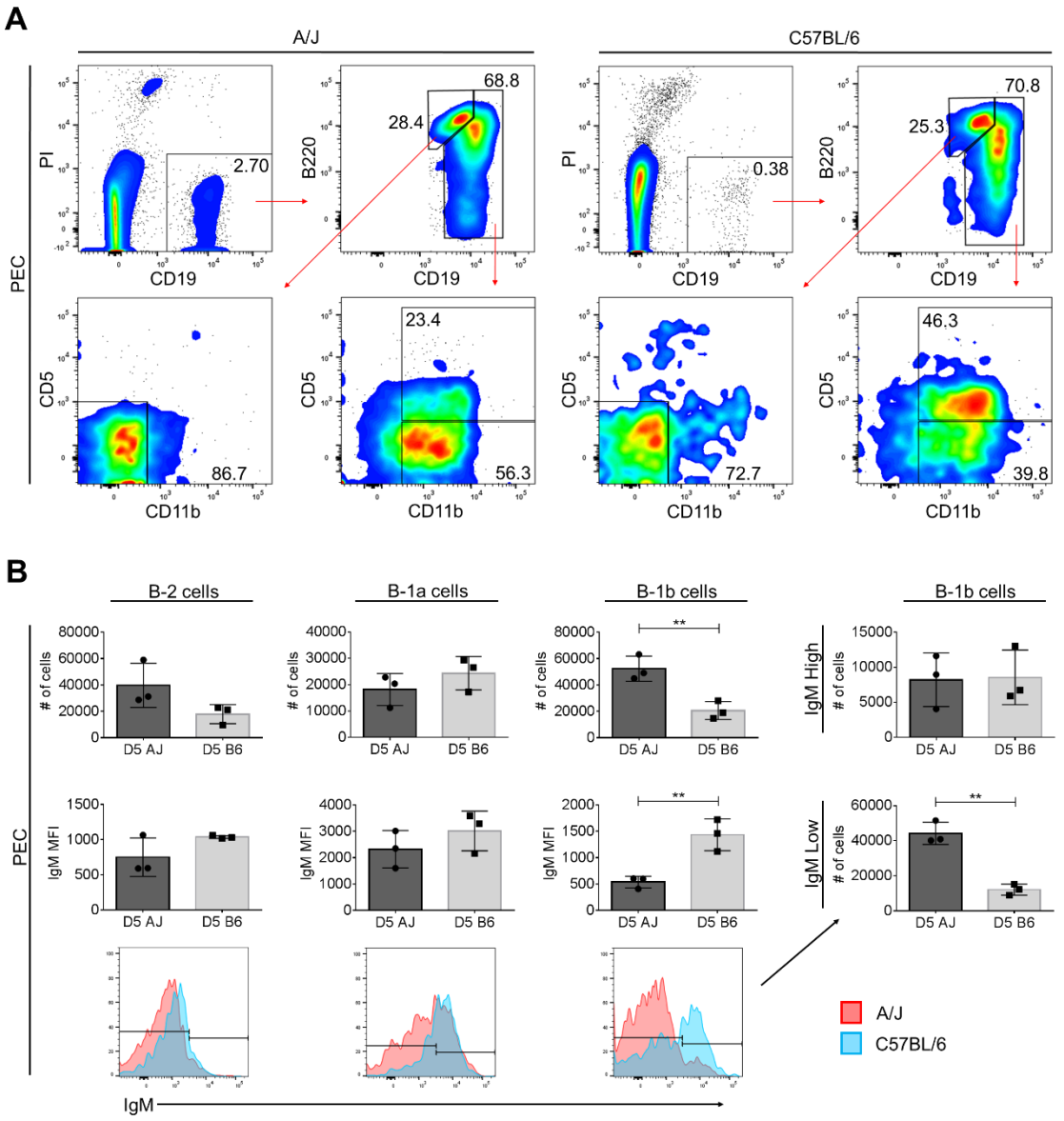


Figure 17. A/J mice have enhanced B-1b cell production following virulent secondary challenge with *Toxoplasma gondii*. A) Gating strategy and frequencies of B-2 ($B220^{hi}$ $CD19^{+}$ $CD5^{-}$ $CD11b^{-}$), B-1a ($B220^{int\text{-}hi}$, $CD19^{hi}$, $CD5^{+}$ $CD11b^{+}$), and B-1b ($B220^{int\text{-}hi}$, $CD19^{hi}$, $CD5^{-}$ $CD11b^{+}$) populations in the PECs. B) Quantitification of B-2, B-1a, and B-1b cells in the PECs and their relative IgM expression (Mean Flourescence Intensity, MFI). Histograms are from representative samples (left); A/J (red) and C57BL/6 (blue). Quantification of IgM^{high} and IgM^{low} B-1b cells in the PECs (right). For A and B, PECs of A/J and C57BL/6 mice during secondary infection with *T. gondii* (GT1) were analyzed; n = 3 A/J and n = 3 C57BL/6. Student's t-test, unpaired, was used to calculate P values. *P < 0.05 and **P < 0.01 were considered significant.

4.3 Discussion

4.3.1 Immunological niche adaptation of *Toxoplasma gondii*

As one of the most successful microorganisms on Earth, *T. gondii* infects myriad hosts, To do this, the parasite must be adaptable to changing immune environments. Some *T. gondii* strains, as shown in the secondary challenge experiments in Chapter 4, are better suited to coexist with certain hosts over others (Fig. 7). In order for *T. gondii* to promote niche adaptability it must be genetically diverse. *T. gondii* genetic divsersity can be achieved when sexual reproduction occurs in the definitive host, a feline. This can occur when the feline consumes a superinfected meal—prey infected with two or more *T. gondii* parasite strains. Interestingly, although F1 (A/J x C57BL/6) and A/J mice are both resistant to secondary infection with GT1, F1 mice are more likely (89% compared to 27%) to be superinfected. It seems that in nature, hosts that are analogous to F1 mice might be better suited to promote *T. gondii* strain diversity through superinfection, We hypothesize that strains which are better able to evade B-1b cell responses may be better suited superinfect, which in turn would promote expansion of strains with virulence factors to evade this cell type. One such candidate are the GPI-linked surface associated glycoproteins (SAG, or SRS), which represent the most polymorphic genes in *T. gondii's* genome. There are over 100 SRS genes and transgenic expression of SRS29C in the RH strain was shown to render it less virulent in naïve mice (Wasmuth et al., 2012). In light of our results, we hypothesize this surface antigen may be preferentially targeted by early B-1 cell antibody responses. Future experiments are aimed to test this hypothesis.

4.3.2 T cells and B cells are protective against virulent challenge with *Toxoplasma gondii*

While the well-established protective role of CD8 T cells is not being refuted by these A/J-C57BL/6 differences during virulent challenge, as evidenced by elevated CD8 T cell numbers in A/J mice (Fig. 10A), the requirements for immunity are certainly broader than was originally hypothesized. However, the ability of T cells to produce IFNγ does not appear to account for mouse differences in generating immunological memory responses to *T. gondii*. This was demonstrated both by the ability of CD4 and CD8 T cells to produce similar amounts of IFNγ in response to parasite stimulation (Fig. 11B). Unexpectedly, B cell populations were significantly different between A/J and C57BL/6 mice following virulent secondary challenge (Fig. 17). While B cells overall were more abundant in A/J mice, a specific population of B-1b IgM^{low} cells was increased in A/J mice by approximately 4 times that of C57BL/6 mice (Fig. 17B). We originally hypothesized that the resistant mouse would express more IgM, but similar expression, although slightly greater in C57BL/6, was observed. It may not be that the B-1b IgM^{low} cells are less functional; rather, they could be expressing a different immunoglobulin isotype such as IgG or IgA. In a study examining the roles of B-1a and B-1b cells during infection with

Streptococcus pneumoniae, Haas et al. demonstrated that B-1b cells generate induced IgM and IgG3 responses while B-1a cells produce low levels of natural IgM antibody—these two B cell subsets work together to facilitate innate and adaptive immune responses (2005). The elevated B-1b IgM^{low} population we observed could represent an induced, IgG3-producing cell, as seen by Haas et al. To test this, we are currently staining B-1b cells from infected mice for surface and intracellular IgG3 expression. We are also working on invasion assays to assess whether the addition of different immunoglobulins, like IgG3 or IgM, inhibits *T. gondii* invasion into host cells or promotes opsonization.

NF-κB is an important inducer of T and B cell development and activation. NF-κBid, or IκBNS, can induce or repress lymphocyte activation. Interestingly, Pedersen et al. showed that B-1a cells are absent and B-1b cells are greatly reduced in mice deficient in IκBNS (2014), an additional indicator that NF-κB is a likely gene candidate under the QTL peak on chromosome 7. We will be evaluating *nfkbid* expression in different A/J and C57BL/6 immune cell populations, in particular B-1 cells, where we expect expression to be higher in A/J mice.

Tspan8 is important for signal transduction, lymphocyte activation, and cell migration. This protein is found in many cell types, but is especially highly expressed on DCs (Immgen.org, Heng et al., 2008). Perhaps the expression of *Tspan8*, which is 6 times more highly expressed in A/J mice, facilitates migration of immune cells to the site of infection or allows antigen-presenting cells to migrate to and interact with lymphocytes. We will also be evaluating expression differences of this protein between A/J and C57BL/6 mice and potentially administering blocking antibodies to antagonize tetraspanin 8 signaling.

4.3.3 Resistance versus tolerance to virulent challenge with *Toxoplasma gondii*

An unusual phenomemon in A/J mice following virulent secondary challenge is the very quick initial clearance of the parasite followed by a short resurgence of the parasite (Fig. 18). From this, one could draw the conclusion that the ability of A/J mice to survive is not merely due to a resistance mechanism, but also due to a tolerance mechanism. While the spike in frequency of innate responders is indicative of failure to control parasite burden in C57BL/6 mice, these data might also suggest that macrophages, neutrophils, and some dendritic cell subsets might serve a detrimental role following virulent secondary infection with *T. gondii*. In addition to greater frequencies of innate immune cells, C57BL/6 mice also produced greater amounts of CD4 T cells. Intestinal inflammation is a well-known trait in C57BL/6 mice following *T. gondii* infection, that is driven by Th1 responses that induce lesions and allow for gram negative bacteria to enter the lamina propria (Liesenfield et al., 1996; Raetz et al., 2013; Egan et al., 2012). The IFNγ-producing Th1 cells are the likely population comprising the $CD3^{+}CD4^{+}$ cells observed in C57BL/6 mice following challenge. It is unlikely these cells are Tregs because they largely produce IFNγ and their CTLA-4 expression is not typical of that seen on a Treg ($CTLA4^{hi}$). Regardless, the

distribution of various CD4 T cell subsets should be investigated in our model. Increases in CD4 T cells in C57BL/6 mice could be the result of inflammatory Th1 expansion or enhanced Treg expansion due to increased parasite burden.

Furthermore, FAS ligand expression on B-1a cells has been shown to induce apoptosis of CD4 T cells during *Schistosoma* infection (Lundy and Boros, 2002). While CD8 T cells, B-1b cells, and B-2 cells likely play a protective role in A/J mice, C57BL/6 mice might be particularly susceptible to challenge not only because of an inadequate adaptive response, but also due to an overcompensatory, tissue-damaging innate response.

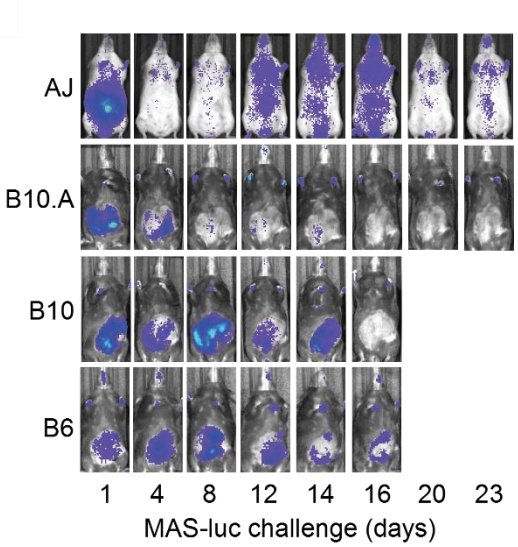


Figure 18. Resistance and tolerance might be required for survival with virulent *Toxoplasma gondii* strains. *T. gondii* parasite burden in A/J, B10.A, B10, and C57BL/6 mice following secondary infection with the virulent atypical strain MAS. Luciferase imaging shows representative mice that were primed with 1 x 10^4 type III avirulent *T. gondii* CEP *hxgprt*- parasites (chronic); then, 35 days later, A/J and C57BL/6 mice were challenged with 5 x 10^4 parasites of the virulent atypical MAS-luc (luciferase) *T. gondii* strain. B10 and C57BL/6 mice died by day 16, while A/J and B10.A survived. Images were taken under isofluorane anesthesia.

CHAPTER 5

5.1 Introduction

5.1.1 Development of T-GREAT, a transgenic IFNγ reporter mouse

We developed a mouse model to track IFNγ production by CD8 T cells during *T. gondii* infection because of its known critical role in mounting a parasite-clearing immune response. To do this, we crossed T57 mice (Kirak et al., 2010) with GREAT (interferon-gamma reporter with endogenous polyA transcript) mice (Reinhardt et al., 2009), which generated a new mouse line named T-GREAT. T57 transnuclear mice were generated by somatic cell nuclear transfer of K^b $TDG057_{96–103}$ tetramer-positive CD8 T cells on the C57BL/6 background, and thus 90% of peripheral $CD3^+$ T cells are $CD8^+$ and 80 to 90% of these have endogenously rearranged TCRα (Vα6-4) and β (Vβ13-1) loci with encoded CDR3 regions bearing specificity for the *T. gondii* antigen K^b-$TGD057_{96–103}$ (Kirak et al., 2010). GREAT mice have an IRES-eYFP reporter cassette inserted between the translational stop codon and 3' UTR/polyA tail of the interferon gamma gene. T-GREAT mice are homozygous for each of these three loci.

5.1.2 Use of TGREATs

The ability to track CD8 T cell activation and function through IFNγ responses could allow one to understand how hosts manage immune responses to different strains of *T. gondii*. Because IFNγ is a requisite for *T. gondii* clearance but is insufficient for the clearance of some *T. gondii* strains, the T-GREATs were intended for the exploration of immune responses concurrent with the IFNγ response—through transcriptional analysis of T-GREAT CD8 T cells in different infection contexts. Additionally, T-GREAT mice were intended for *in vitro* interrogation of killing of various *T. gondii* strains in the presence of T cell agonists and antagonists or following genetic modification with CRISPR-Cas9. The T-GREATs were also developed to evaluate regulators of the CD8 T cell IFNγ response during secondary infection by performing a forward genetic screen using a lenti-V2 CRISPR-Cas9 library. However, the use T-GREATs in these contexts proved to be challenging as outlined below.

5.2 Results

5.2.1 T-GREATs produce trackable CD8 T cell IFNγ responses to *Toxoplasma gondii*, but fail to persist in recipient hosts longterm

We confirmed the presence of the desired rearranged T57 TCRα and TCRβ chains, and GREAT reporter by PCR (data not shown), and performed FACS analysis to confirm the functionality of the IFNγ:YFP bicistronic reporter (Fig. 19). For naïve T-GREAT cells to flux IFNγ, an antigen-presenting cell (i.e. bone marrow derived macrophages, BMDMs) had to be present, likely for costimulation, as there was no IFNγ:YFP signal detected in response to plate bound anti-CD3ε and anti-CD28 stimulation (not shown). Although IFNγ fluxed *in vitro* in the presence of parasite-infected BMDMs (data not shown), IFNγ expression was much more substantial with CD3 stimulation and BMDMs.

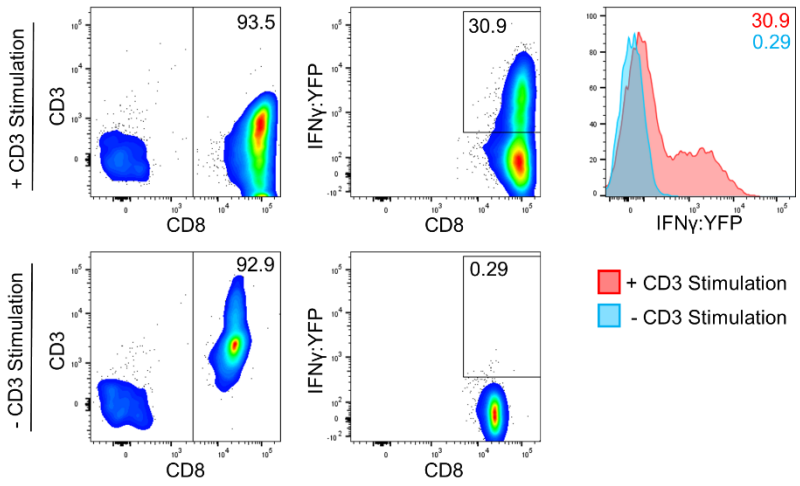


Figure 19. T-GREAT mice successfully produce IFNγ:YFP in response to stimulation. Splenocytes and lymph nodes were isolated, filtered, washed, negatively selected for CD8 T cells, and plated on bone-marrow derived macrophages. Soluble CD3 was added when the CD8 T cells were added. 24 hours later, cells were stained and analyzed by FACS. Representative FACS plots, previously gated on live (PI^-) $CD19^-$ cells, showing gating strategy of *in vitro* stimulated (top) and unstimulated (bottom) $CD8^+$ T-GREAT (T57 x GREAT IFNγ:YFP reporter**)** cells and histogram plots of IFNγ:YFP expression of CD3-stimulated (red) and –unstimulated (blue) T-GREAT cells.

To evaluate T-GREAT CD8 T cell expansion and IFNγ production *in vivo*, we performed adoptive transfer experiments by transferring (i.v.) 5 x 10^6 45.2^+ T-GREAT CD8 T cells into 45.1^+ (data not shown) or $45.1^+/45.2^+$ mice, followed by injection with type II ME49 or type III CEP *hxgprt-* (Fig. 20). Peritoneal exudate cells (PECs), spleen (SPL), peripheral lymph nodes (PLNs, axillary, brachial, and inguinal), and mesenteric lymph nodes (MLNs) were isolated on day 9 following injection (Fig. 20, A-D), and PECs and SPL were isolated on day 35 post injection (Fig. 20E). Transferred CD8 T cells expanded significantly in the

PECs (Fig. 20A), SPL (Fig. 20B), and MLNs (Fig. 20D), but expanded very little in the PLNs (Fig. 20C). Furthermore, transferred CD8 T cells expanded more substantially in the presence of a more virulent type II *T. gondii* infection (Fig. 20, right) compared to a less virulent type III *T. gondii* infection (Fig. 20, left). Additionally, production of IFNγ by transferred CD8 T cells was considerably higher during type II infection. However, by day 35, the CD8 T cell transfer population was barely detectable in recipient mice (Fig. 20E). Additionally, we were only able to evaluate the potential for day 35 expansion in recipients infected with type III *T. gondii*, as type II was lethal by day 35 in the same situation. To test whether clonal competition prevented longterm expansion, T-GREAT CD8 T cells were also transferred into CD8 knockout mice and compared to CD45.1 recipients. However, similar numbers of T-GREATs were detected in the PECs and spleens and neither recipient supported longterm expansion of T-GREATs. Furthermore, on day 9 of primary infection we had limited success sorting transfered CD8 T-GREAT cells, as the amount of cells sorted was minimal—approximately 1.35×10^5 cells for type II infection and 2.5×10^4 cells for type III per mouse (PEC)—a small fraction of the 5×10^6 CD8 T-GREAT cells we originaly injected (data not shown).

Because we were unable to get sufficient numbers of activated (IFNγ:YFP$^+$) CD8 T-GREATs, we attempted direct injection of T-GREAT mice with the replication-deficient uracil auxotroph type I strain RH *Δompdc* (vaccine strain) or the type II ME49 strain. We then sorted PECs and SPL from these mice. Although expansion was observed, cell numbers were still too low (~3×10^5 sorted cells) to be used for *in vitro* killing assays (data not shown). However, we were able to isolate RNA from IFNγ$^+$ and IFNγ$^-$ CD8 T-GREAT cells for future transcriptional evaluation.

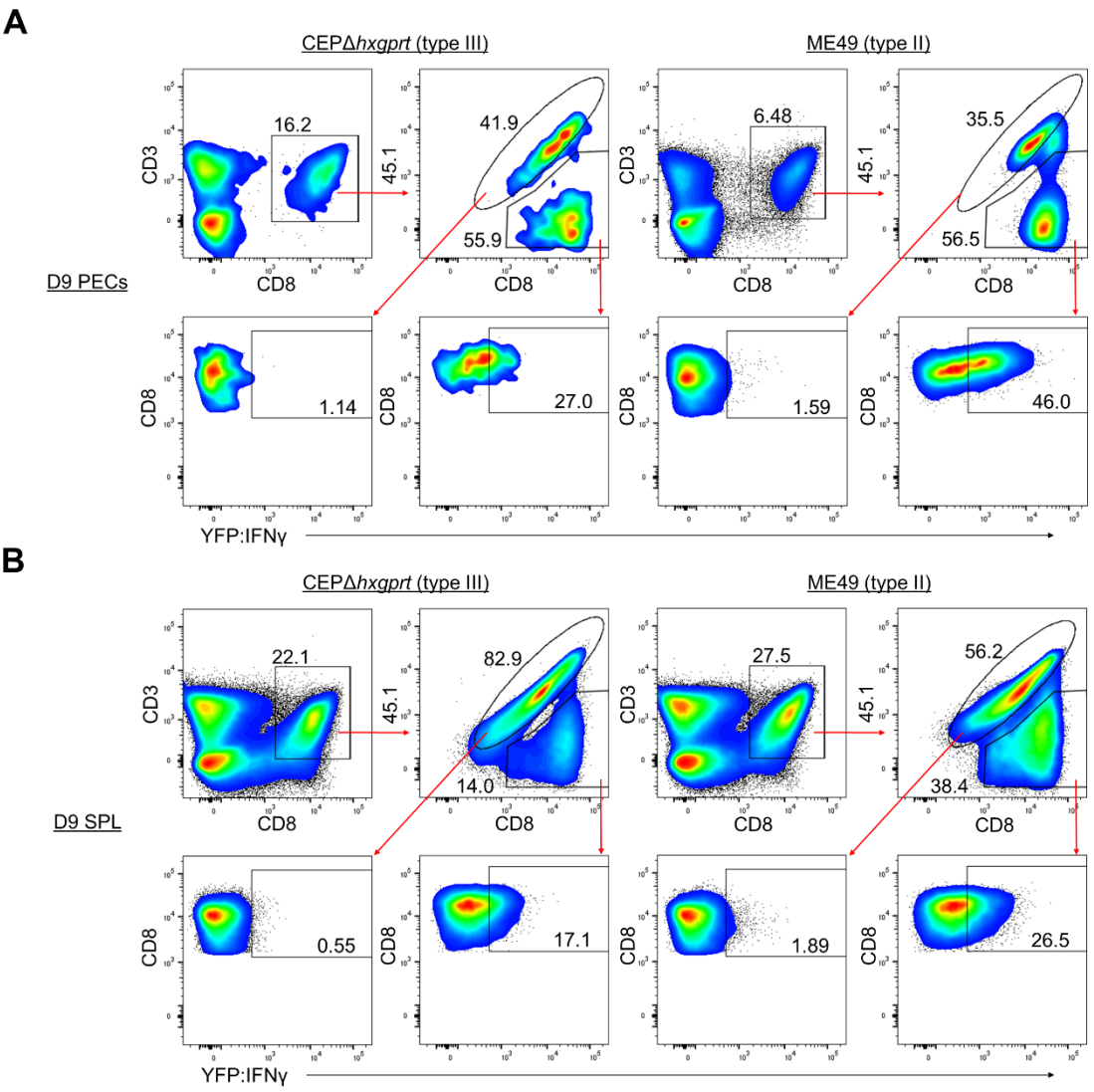
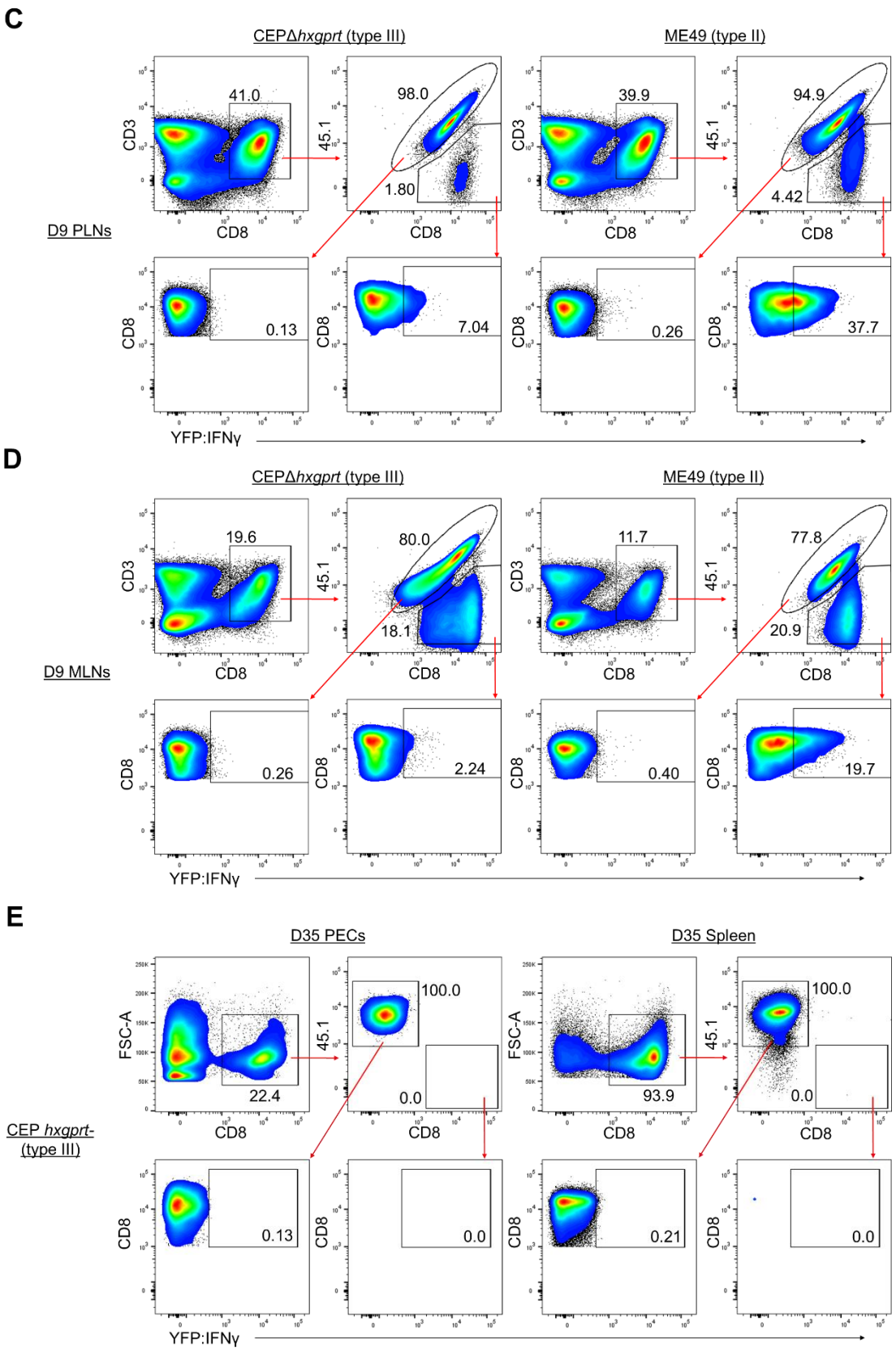


Figure 20. Adoptively transferred T-GREAT CD8 T cells successfully expand by day 9 in a recipient host following infection with type II and type III *Toxoplasma gondii* parasites, but the population collapses by day 35. Representative FACS plots showing transfer of CD8 T-GREATs in A) peritoneal exudate cells (PECs), B) spleen (SPL), C) peripheral lymphnodes (PLNs; axillary, brachial, and inguinal), and D) mesenteric lymph nodes (MLNs) of recipient mice on day 9 post infection with type II ME49 or type III CEP *hxgprt- T. gondii* strains. Spleens and lymph nodes from 6-8 week old female T-GREAT mice were isolated, filtered, washed, negatively selected for CD8 T cells, and washed in PBS. 5 x 10^6 45.2^+ T-GREAT CD8 T cells were injected intravenously into the tail vein of heterozygous $45.1^+/45.2^+$, 6-8 week old female mice. 12 hours later, 1 x 10^4 type II ME49 or type III CEP *hxgprt- T. gondii* parasites were injected. 9 days later, cells from indicated organs were isolated, stained, and analyzed by FACS on an Aria III. E) Similarly, 45.2^+ T-GREAT CD8 T cells were adoptively transferred into into 45.1 recipients, infected with the type III CEP *hxgprt-* strain and analyzed by FACS on day 35.


C
CEPΔhxgprt (type III)
ME49 (type II)
D9 PLNs
CD3
CD8
45.1
41.0
98.0
1.80
39.9
94.9
4.42
0.13
7.04
0.26
37.7
YFP:IFNγ
D
CEPΔhxgprt (type III)
ME49 (type II)
D9 MLNs
19.6
80.0
18.1
11.7
77.8
20.9
0.26
2.24
0.40
19.7
YFP:IFNγ
E
D35 PECs
D35 Spleen
CEP hxgprt-
(type III)
FSC-A
22.4
100.0
0.0
93.9
0.13
0.21
YFP:IFNγ

5.3 Discussion

5.3.1 K^b-TGD057$_{96–103}$ is a subdominant T cell receptor antigen which limits the use of T-GREATs

While T-GREATs are a useful tool for tracking IFNγ production at early timepoints during primary infection, its use for tracking IFNγ responses during secondary infection have proved difficult. The inability of CD8 T cells to expand efficiently is likely due to T cell receptor (TCR) affinity problems or inefficient antigen presentation (McMurtrey et al., 2013). Weak TCR:antigen interactions result in more rapid dissociation rates, reduced activation signals, and thus reduced clonal expansion (Stone et al., 2009). The Tgd057 antigen is subdominant; other immunodominant *T. gondii* antigens have been identified (Blanchard et al., 2008; Sanecka et al. 2016). However, these transgenic mice and epitopes are specific to the BALB/c mouse background (H-2d). To get optimal numbers of T-GREAT cells to assay killing of *T. gondii*, one would need a mouse on the susceptible C57BL/6 background with transgenic CD8 T cells specific for an immunodominant antigen, otherwise T-GREATs are of limited use for functional assays *ex vivo*. Ongoing experiments are underway to generate large numbers of activated T-GREAT cells, or "blasts," by adding peptide and allowing to expand for 7 days *in vitro*. As expected, these T-GREAT blasts flux more IFNγ:YFP in response to stimulation and such blasts are being assayed for their ability to kill *T. gondii* parasite.

Conclusion

Both the innate and adaptive immune responses are robust during *T. gondii* infection. While several immune cells play important roles in clearing this parasite, two key players emerge in fighting this infection—T cells and B cells, specifically CD8 T cells and B-1b cells. Although CD8 T cells can control many *T. gondii* strains by IFNγ-mediated responses, several virulent, IFNγ-resistant strains elude this pathway. However, some hosts, like A/J mice, are able to clear *T. gondii* presumably by mechanisms other than the IFNγ response. Recent evidence points to extracellular killing of *T. gondii* by the cytotoxic molecule granzyme B (Dotiwala et al., 2016). One potential mechanism could be the induction of egress of *T. gondii* by CD8 T cells, where *T. gondii* becomes exposed to granzyme B or is inhibited by antibodies produced by B-1b cells. Opsonization by antibodies may promote the targeted killing of *T. gondii* or may block invasion of the parasite into a new host cell. To develop effective treatment and prevention against toxoplasmosis, it is important that we evaluate both the adaptive and humoral immune response to this ubiquitous and dynamic parasite.

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