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IRVINE

Toxoplasma gondii modulation of immune receptor-mediated immunity

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Pedro Morgado Flores

Dissertation Committee:
Assistant Professor Melissa Lodoen, Chair
Professor David Fruman
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2014

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Table of Contents

LIST OF FIGURES III

LIST OF TABLES IV

ACKNOWLEDGEMENTS V

CURRICULUM VITAE IX

ABSTRACT OF THE DISSERTATION XIII

CHAPTER ONE AN INTRODUCTION TO *TOXOPLASMA GONDII* IMMUNITY 1

- TOXOPLASMA GONDII 2
- IMMUNITY TO TOXOPLASMA 4
- TOXOPLASMA SECRETED PROTEINS 5
- MACROPHAGES IN IMMUNITY TO TOXOPLASMA INFECTION 7
- TOXOPLASMA AND MACROPHAGE IMMUNE RECEPTORS 11

CHAPTER TWO *TOXOPLASMA GONDII* INDUCES B7-2 EXPRESSION THROUGH ACTIVATION OF JNK SIGNAL TRANSDUCTION 13

- INTRODUCTION 14
- RESULTS 16
- DISCUSSION 34
- ACKNOWLEDGEMENTS 40

CHAPTER THREE STRAIN-SPECIFIC INDUCTION OF CD40 DURING *TOXOPLASMA GONDII* INFECTION AMPLIFIES INFLAMMATORY CYTOKINE RESPONSES 41

- INTRODUCTION 42
- RESULTS 44
- DISCUSSION 56
- ACKNOWLEDGMENTS 62

CHAPTER FOUR DEFINING THE CELL SURFACE PROTEOME OF *TOXOPLASMA GONDII*-INFECTED MACROPHAGES 63

- INTRODUCTION 64
- DISCUSSION 79

CHAPTER FIVE CONCLUDING REMARKS 82

CHAPTER SIX MATERIALS AND METHODS 90

REFERENCES 103

List of Figures

Figure 2.1. Expression of costimulatory molecules during *T. gondii* infection *in vitro* and *in vivo* 17
Figure 2.2. Characterization of B7-2 induction in *T. gondii*-infected macrophages 19
Figure 2.3. Examination of MyD88 and TRIF in *T. gondii*-induced B7-2 expression 24
Figure 2.4. Genome-wide transcriptional profiling of *T. gondii*-infected macrophages 26
Figure 2.5. B7-2 expression after MAPK inhibitor treatment and *T. gondii* infection 29
Figure 2.6. Examination of B7-2 expression on *T. gondii*-infected human monocytes 32
Figure 2.7. Effect of blocking *T. gondii*-induced B7-2 expression on T cell proliferation 33
Figure 3.1. Expression of CD40 during *T. gondii* infection 46
Figure 3.2. QTL mapping of the CD40 induction phenotype 49
Figure 3.3. Role of GRA15 in CD40 induction during *T. gondii* infection 51
Figure 3.4. Characterizing $GRA15_{II}$-expressing THP-1 cells 52
Figure 3.5. CD40 stimulation of *T. gondii*-infected macrophages 55
Figure 4.1. Surface protein isolation schematic 66
Figure 4.2. Validation of cell surface protein isolation protocol and visualization of cell surface proteins 67
Figure 4.3. Quantification of proteins unique to one sample or shared between both samples 72
Figure 4.4. FcγR expression profile of type I *T. gondii*-infected bone marrow-derived macrophages 74
Figure 4.5. Strain-specific cell surface expression of FcγR 77
Figure 4.6. Evaluating signal transduction pathways downstream of FcγR engagement 78

List of Tables

Table 3.1. Recombinant type II x III progeny induction of CD40 48

Table 4.1. List of protein hits unique to uninfected BMdM 69

Table 4.2. List of protein hits unique to infected BMdM 70

Table 4.3. List of protein in identified by mass spectrometry shared between infected and uninfected BMdM 71

Acknowledgements

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I would like to start by acknowledging my advisor and mentor, Dr. Melissa Lodoen. As some of you may know, selecting a new faculty, (especially one with no previous graduate students), as your thesis advisor is a risky proposition. You just don’t know what you will get. Yet, as I have told countless people, joining Melissa’s lab was probably the best decision I made in graduate school. I found that Melissa’s ability to somehow always find the silver lining in any situation, such as when experiments didn’t work, really eliminated the stigma associated with being the bearer of bad news. This helped establish a congenial lab environment that not only fostered open communication but also motivated all of us in the lab to learn from our mistakes and strive to grow as scientists. In addition to being an outstanding thesis advisor, Melissa is committed to providing outstanding guidance that extended beyond the lab. The sincerity and enthusiasm with which Melissa mentors everyone in the lab only made

working with her that much more rewarding. I have learned much from Melissa and I will always be thankful to her.

I would also like to acknowledge Dr. Marlene de la Cruz and Dr. Luis Mota-Bravo from the Minority Science Programs at UC Irvine for their tremendous support. As administrators, they tirelessly work to establish resources on campus to help underrepresented minority students, like myself, succeed in graduate school. I, like many before me, have directly benefited from their efforts. On top of this, their doors were always open and they were always up for an impromptu visit whenever I needed advice. For that reason they have been so much more to me and I am thankful for the mentoring relationship we have developed.

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Thank you to my friends, both old and new. Sheefteh Khalili, how was I to know that your serendipitous entrance into my life back in 2006 would be one of the most fortunate experiences of my life thus far? Your strong sense of commitment to your family, academic endeavors and work is inspiring. Being so busy, I am grateful that you had time to be a great friend. Your support, in every sense of the word, through the toughest and the brightest times these last few years has left me indebted to you. I am looking forward to being able to be there for you to celebrate the many accomplishments awaiting you. Thomas Lechuga, you were in our lab for only a quarter but that was long enough for us to build a friendship I am thankful for. To the gals that were there from the beginning, Heidi Contreras, Roxanna Ochoa, and Barbara Alcaraz, you all have provided much needed support in and out of lab as well as the occasional distraction. I would also like to thank the guys Matt Pearce, Thomas Elliott, Justin O’Neill, and Matt Rafalow, who helped me appreciate and celebrate both the small and large milestones in life. I look up to each one of you. Matt P., you are without a doubt one of the most caring and thoughtful people I know. Thomas, your passion for academia is inspiring, and I look forward to calling your Professor Elliott in the very near future. Justin, your love of the arts is contagious and one day I’ll be as cultured as you are. Matt R., what can I say, I admire your confidence; combined with your academic prowess you’ll go far.

Finally, I would like to thank my family for their unconditional love and support throughout it all. Jordan, I am thankful for the opportunity to have you be such an integral part of my life. I have learned so much about you and have enjoyed getting the opportunity to see you grow these last few years. You continuously amaze me and I am looking forward to seeing you thrive as the young adult you have become. As much as I give you slack, I'm not that bitter that you are much taller than me. My sisters, Claudia, Elidet, and Veronica, you guys are everything a brother could ask for. I know that well always be there for each other. Lastly, but not least, I want to thank my parents for raising the five of us with unconditional love and support. I would not be the person I am today without my parents. !Gracias y los quiero mucho!

Curriculum Vitae

EDUCATION

2009 – 2014 **Doctor of Philosophy**: Biological Sciences
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Ph.D. Advisor – Dr. Melissa B. Lodoen

2006-2008 **Master of Science**: Conservation Biology, Restoration and Land Management
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RESEARCH EXPERIENCE

2009 – Present Doctoral Research: Department of Molecular Biology and Biochemistry,
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Benoit ME, Clarke EV, **Morgado P**, Fraser DA, Tenner AJ. (2012) Complement protein C1q directs macrophage polarization and limits inflammasome activity during the uptake of apoptotic cells. J. Immunol. 188:5682–5693.

Morgado P, Ong Y-C, Boothroyd JC, Lodoen MB. (2011) *Toxoplasma gondii* induces B7-2 expression through activation of JNK signal transduction. Infect. Immun. 79:4401–4412.

TEACHING EXPERIENCE

Instructor

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Abstract of the Dissertation

Toxoplasma gondii modulation of immune receptor-mediated immunity

by

Pedro Morgado

Doctor of Philosophy in Biological Sciences

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Assistant Professor Melissa Lodoen, Chair

Protective immunity to *Toxoplasma gondii* depends on innate immune activation and the establishment of a robust Th1 response. Notably, however, *T. gondii* can subvert host immunity by altering the transcriptional program and the proteome of infected cells. The mechanisms by which the parasite alters the expression and function of cell surface receptors, which play a key role in immune activation, are not well understood. As critical regulators of T cell activation, the expression of the costimulatory receptors B7-1 and B7-2 are tightly controlled. We examined the molecular mechanisms governing their induction in macrophages. We found that all three strains of *T. gondii* (types I, II, and III) up-regulated the expression of B7-2. Transcriptional profiling by microarray analysis revealed nodes of regulation in infected cells, in particular the induction of MAPK signaling. Using specific inhibitors against MAPKs, we determined that parasite-induced B7-2 is dependent on JNK. We demonstrate that B7-2 induced by *T. gondii* is capable of activating T cells and promoting their proliferation.

We also examined the regulation of the CD40 receptor on the surface of *T. gondii*-infected macrophages. CD40 expression was dramatically increased only in type

II *T. gondii*-infected cells. Forward genetic analysis suggested that GRA15 from type II strains ($GRA15_{II}$) was the parasite factor responsible for the CD40-inducing phenotype. Using type I parasites and THP-1 cells stably expressing $GRA15_{II}$, we confirmed that $GRA15_{II}$ was sufficient to induce CD40. We also found that engagement of CD40 in type II-infected cells amplified the IL-12 response. These data indicate that $GRA15_{II}$ may promote parasite immunity through CD40-mediated production of IL-12.

Using a proteomic approach, we sought to determine how *T. gondii* infection globally alters the expression of cell surface proteins. Using this approach we found that the expression of FcγR is altered on infected cells, which may represent a previously undescribed immune evasion strategy by *T. gondii*. The dataset generated using this protocol may lead to the identification of novel surface markers specific to infected cells that can serve as therapeutic targets.

Collectively, our data suggest that changes in the expression of surface proteins on infected cells may profoundly shape *T. gondii* immunity.

Chapter One

An Introduction to *Toxoplasma gondii* Immunity

Toxoplasma gondii

Toxoplasma gondii is a globally distributed obligate intracellular protozoan parasite capable of infecting and establishing a lifelong chronic infection in humans. It is estimated that *T. gondii* infects ~ 30% of the human population worldwide (1). In healthy individuals, infection with *T. gondii* is typically mild or asymptomatic, due to a robust, protective immune response that effectively controls the acute infection. However, the parasite is able to evade immune detection and establish a chronic infection. Moreover, primary infection or reactivation of a latent infection in immunocompromised individuals, including transplant recipients, AIDS patients, and the developing fetus, can result in life-threatening disease (2-4).

T. gondii is a member of the phylum Apicomplexa, which includes other important human and livestock pathogens such as: *Plasmodium, Eimeria, Neospora, Babesia, Theileria* and *Cryptosporidium.* Members of this phylum are characterized by the presence of the following: (i) an apical complex important for organizing the microtubular cytoskeleton of the parasite; (ii) an inner membrane complex located below the plasma membrane; (iii) a non-photosynthetic plastid used for neutral lipid and isoprenoid synthesis; and (iv) secretory organelles named micronemes, rhoptries, and dense granules (5).

T. gondii is remarkable in that it can infect almost any nucleated cell within warm-blooded animals. Despite its ability to infect a wide range of hosts, its definitive host is restricted to felids. It is in the intestine of the cat that *T. gondii* undergoes sexual reproduction (5). Reproduction in felids results in the production and shedding of highly infectious oocysts, which contain sporozoites. In intermediate hosts, *T. gondii* exists as

tachyzoites and bradyzoites. Tachyzoites are the proliferative form of the parasite that is capable of infecting virtually any cell within the host. Tachyzoites replicate asexually by a process known as endodyogeny (6). This form is also associated with the acute stage of infection characterized by rapid proliferation and infection of host cells in a variety of organs. A parasite developmental switch that occurs with the onset of host immunity results in the conversion of *T. gondii* from the tachyzoite form to the cyst-forming bradyzoite form. It is this form that establishes tissue cysts within intermediate hosts that contributes to the transmission between other intermediate host or felids when infected hosts are consumed (7-9)**.** While humans are readily infected through the consumption of infected intermediate hosts containing tissue cysts, they generally do not contribute to *T. gondii*'s dissemination except via vertical transmission in pregnant women (10).

There are three predominant strains of *T. gondii* (referred to as type I, II, and III) in North America and Europe, which differ in their prevalence and virulence in mice (11-13)**.** The tachyzoite form has a haploid genome consisting of 14 chromosomes that encode for approximately 8,000 genes. While genetically similar, single nucleotide polymorphisms (SNPs) contribute to differences in virulence among the three strains. Type I parasites are hyper-virulent in mice, with an LD_{100} of one parasite (14). By contrast, type II and type III parasites exhibit lower virulence in mice, yet are more highly associated with human infections in the United States (15). Studies on recombinant progeny generated by crossing these three strains has shed light on the genetic loci that contribute to differences in virulence among these strains (16-21).

Immunity to Toxoplasma

Initial infection with *T. gondii* results in the rapid production of IL-12 and IFN-γ by cells of the innate immune system (22). The coordinated effort between these two cytokines helps to promote a robust innate immune response characterized by macrophage and NK cell effector functions, and the development of Th1 cell-mediated immunity (23). IL-12 is produced by neutrophils (24), dendritic cells (DC) (25), and macrophages (26) and leads to NK cell and T cell IFN-γ production, which is critical to control *T. gondii* infection (27, 28). In addition to its role in promoting a Th1 response, IFN-γ activates macrophages and enhances their antimicrobial effector functions through the production of reactive oxygen species (ROS), nitric oxide (NO), autophagy, and nutrient deprivation mechanisms (29-31). IL-12 production early during infection is also critical for controlling *T. gondii* infection (26, 32) and for coordinating adaptive immunity (24). Impaired IL-12 production by depletion of IL-12-producing cells, such as neutrophils, impairs IFN-γ and Th1 immunity, increasing the susceptibility to *T. gondii* infection (24).

Protective immunity to *T. gondii* depends on the establishment of a robust Th1 response (33). Initially, CD8+ T cells were thought to control *T. gondii* and $CD4^+$ T cells were though to play a more synergistic role (34). However, both CD4+ and CD8+ T cells play an important role in mediating *T. gondii* immunity; $CD4^+$ T cell-produced IFN-γ is critical for maintaining Th1 response and promoting $CD8^+$ T cell immunity (35). Furthermore, chronically infected mice succumb to *T. gondii* infection only after antibody dependent depletion of both $CD4^+$ and $CD8^+$ T cells (36). The importance of T cells in mediating *T. gondii* immunity in humans is evident from the increased susceptibility observed in *T. gondii*-infected immune compromised individuals that have impaired T

cell mediated immunity due to infection with HIV before treatment with antiviral therapies (4).

As such, protective immunity to *T. gondii* is dependent on the coordinated establishment of an innate and adaptive immune response. The expression of IL-12 and IFN-γ is tightly regulated, and their dysregulation results in impaired *T. gondii* control or exacerbated immunopathology (28, 30, 32, 34). The ability of *T. gondii* to modulate the expression of these cytokines in a parasite strain-specific manner has been associated with observed differences in immunity to the parasite (26), and the mechanism by which this occurs is the focus of many research groups within the field.

Toxoplasma secreted proteins

The ability of *T. gondii* to modulate host cellular functions has been largely attributed to the release and secretion of parasite-derived proteins into the host cell during and shortly after an invasion event (20, 37, 38). Secreted proteins originate from one of three secretory organelles: micronemes, rhoptries, and dense granules. Each secretory organelle has a non-redundant role during invasion, which is attributed to the distinct composition and characteristics of the proteins they contain.

T. gondii micronemes contain at least 15 characterized proteins, most of which are continuously secreted and are involved with the gliding motility observed in extracellular parasites (39) and in the initial attachment to the host cell surface (40). Microneme proteins and various rhoptry proteins, particularly those from the rhoptry neck (RON proteins) (41-44), are necessary for invasion and the formation of the parasitophorous vacuole (PV), an intracellular compartment in which the parasite resides and replicates.

In addition to their role in invasion, rhoptry proteins (ROP) are comprised of numerous well-characterized kinases and pseudokinases (20, 45), which play an important role in virulence (19, 20, 46, 47). They have been shown to directly interfere with host immune recognition strategies (47-49) and alter signaling and gene transcription (47, 50, 51). Additionally, ROP16 and ROP18 are known to directly activate STAT signaling cascades through their kinase activity in a strain specific manner (52-55). The proteins encoded by the type I and type III alleles of *ROP16* were found to directly phosphorylate STAT3 and STAT6, while that encoded by the type II allele did not.

The dense granule proteins are thought to be secreted last, shortly after invasion. They have been implicated in organizing and maintaining the integrity, structure and function of the PV (56, 57). However, recent analysis of the dense granule protein (GRA) 15 (GRA15) revealed that it is capable of inducing the activation of NF-κB signaling in a parasite strain-specific manner (58). It was demonstrated that the type II allele of *GRA15* resulted in sustained nuclear translocation of NF-κB. More recently, two additional newly described GRA were shown to interfere with host-cell processes. GRA16 was shown to localize to the host-cell nucleus and alter gene expression, whereas GRA24 initiates and sustains p38 mitogen-activated protein kinase (MAPK) (59, 60). Consequently, the secretion of these parasite factors during invasion help to establish a niche for the parasite within the cell as well as to alter host processes that may facilitate its survival and growth.

Macrophages in immunity to Toxoplasma infection

Macrophages are found in nearly all tissues. They are thought to be derived from a common myeloid progenitor cell in the bone marrow that gives rise to monocytes. Monocytes exit the bone marrow and enter the circulation in the bloodstream. Monocytes migrate from the bloodstream into tissues during steady state conditions or in response to inflammation (61). In tissues, monocytes differentiate into macrophages and dendritic cell populations (62).

Macrophages are a critical component of innate immunity. Their wide distribution within tissues and their differentiation from monocytes that are recruited from the bloodstream poises them to be amongst the first cells at the site of an infection (63). As phagocytic cells, they are essential for maintaining homeostasis through their efficient uptake and clearance of cellular debris and apoptotic cells (62). Not surprisingly, following an infection, macrophages efficiently neutralize pathogens through phagocytosis. The degradation of pathogens within the cell is facilitated by cell intrinsic microbicidal mechanisms characterized by the production of ROS and NO (64). Macrophages are a heterogeneous cell population whose activation and effector function is determined by stimuli from their microenvironment. The clearance of pathogens is initiated by the recognition of pathogen-associated molecular patterns (PAMPs) present on pathogens by Toll-like receptors (TLRs) and the intracellular pattern recognition receptors, such as NOD-like receptor (NLR)-1 and NLR2 (65, 66).

Currently, only a few known *T. gondii* ligands for TLRs that have been identified. *T. gondii* heat shock protein 70 (67) and glycosylphosphatidylinositol-anchored proteins (GPIs) (68) have been found to bind to TLR2 and TLR4. In mice, *T. gondii* profilin, which is involved in parasite motility and host-cell infection through actin binding (69), is

recognized by TLR11 (70) and a newly characterized TLR12 (71). Human peripheral blood mononuclear cells (PBMC), which lack TLR11 and TLR12, do not respond to profiling, although they are still able to respond to *T. gondii* nucleic acid through TLR8 and TLR9 (72). The importance of TLR-mediated immunity to *T. gondii* is best illustrated in mice deficient in MyD88, which is involved in all TLR signaling except TLR3. When *MyD88*$^{-/-}$ mice are infected with *T. gondii,* they fail to control the acute phase of infection as determined by the high parasite load and increased mortality rates compared to wild-type mice (73).

Engagement of TLR leads to signaling through MAPK and NF-kB pathways and the production of inflammatory cytokines, most notably IL-12 and TNF-α, in a MyD88- or TRIF-dependent manner (74). TLR engagement leads to TAK1 activation that then results in the activation of MAPKs, including extracellular signal-related kinases (ERK1/2), p38 MAPK and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (75). IL-12 production by macrophages during *T. gondii* infection is induced through p38 MAPK activation by upstream signaling molecules TRAF6 or TAB1 (76). In addition to MAPK signaling, TLR-mediated activation of TAK1 can result in the activation of NF-kB signaling and IL-12 production (77). Furthermore, the expression of TLRs varies among cell types, which may help to explain the diverse responses of different types of cells to TLR ligands.

Macrophage activation is complex and multi-factorial, depending on several factors. These include the local cytokine milieu, as well as the presence of host or microbial ligands for engagement of pattern recognition receptors (PRR). The extent to which macrophages are then activated helps to shape subsequent immune responses.

For example, classically activated macrophages have an enhanced antimicrobial capacity and elevated production of pro-inflammatory cytokines, including IL-1β, IL-6, IL-12 and IL-23 (78). These cytokines can promote the differentiation of distinct T cell subsets. Most notably, IL-12 drives Th1 cell skewing which promotes IFN-γ production. While classically activated macrophages produce IL-12 to promote naïve Th1 cells, depending on the stimuli, they can also produce IL-27, which inhibits Th1 and Th2 cells (79). These cytokines are important components of host defense but can also cause pathology. For this reason, macrophage activation and cytokine production are context dependent and thought to be tightly regulated.

Moreover, macrophages are responsive to cytokines produced by other innate or adaptive cells, most notably IFN-γ. Selective impairment of signaling through the IFN-γ receptor in macrophages during *T. gondii* infection reduces macrophage activation and *T. gondii* clearance *in vivo*, increases mortality despite the presence of IFN-γ (80). It is appreciated that IFN-γ establishes various antimicrobial programs necessary for controlling intracellular parasite infection (81-83). For example, many intracellular pathogens, including *T. gondii,* are sensitive to tryptophan deprivation. It was demonstrated that IFN-γ stimulation of macrophages results in tryptophan degradation and consequently enhanced parasite control (84, 85). Additionally, immunity-related p47 GTPases (IRGs), which are induced by IFN-γ, along with a closely related family of p67 GTPases, known as guanylate-binding proteins (GBPs), contribute to promoting macrophage control of pathogens (48, 86, 87). The exact mechanism by which IRG and GBP contribute to enhanced parasite clearance is not known. It has been demonstrated

that recruitment of IRGs to the PV enhances lysosome degradation of *T. gondii* and was preceded by indentation and breaking down of the PV membrane (88).

The induction of cell-intrinsic antimicrobial responses in macrophages can be regulated independently of IFN-γ through the engagement of cell surface receptors. The interaction between CD40 on antigen-presenting cells (APC) and CD154 (CD40L) on activated $CD4^{+}$T cells results in the establishment of antimicrobial programs independent of other established IFN-γ- and p47 GTPases-dependent antimicrobial effector functions (89, 90). One such mechanism involves autophagy, which is a cellular process that allows cells to maintain homeostasis through the degradation and recycling of damaged organelles and misfolded proteins (91). CD40 engagement on macrophages results in the production of ROS (92) as well as autophagy (93), both of which limit the survival of intracellular pathogens (89, 90, 93). Following macrophage activation, it is likely that parasite control is mediated through the concerted induction of not one antimicrobial response, but through the establishment of several programs.

The establishment of an appropriate innate immune response depends on the coordinated communication between APC and T cells (23). In addition to producing cytokines that skew T cell responses, macrophages also function as a professional APC. As APC, macrophages up-regulate major histocompatibility complex (MHC) class I and II and costimulatory molecules (including B7-1 and B7-2) in response to infection. Although macrophages are incapable of trafficking to draining lymph nodes, they are fully capable of presenting antigen and providing costimulatory signal in tissues to help sustain T cell responses. The expression of the costimulatory molecules, B7-1 and B7-

2, is tightly regulated, and the mechanisms that regulation their expression during *T. gondii* infection are not fully understood.

Toxoplasma and macrophage immune receptors

Controlling *T. gondii* infection is dependent on both innate and adaptive immunity. Cells of the innate immune system are critical for initiating the appropriate immune response to parasite infection (94)*. T. gondii*, however, has evolved numerous mechanisms for modulating host immunity, which likely contribute to its ability to establish a chronic, persistent infection in tissues of the infected host. Primary infection or the reactivation of a latent infection in immune compromised individuals can be deadly. The disease outcomes associated with *T. gondii* infection in immune-deficient hosts underscore the complex relationship between the parasite and the host immune response.

Although *T. gondii* can infect almost any nucleated cell, the parasite preferentially infects myeloid cells, such as monocytes, macrophages, and dendritic cells (95). Given the important role of myeloid cells in controlling the parasite, infection of these cells has the potential to dramatically affect the immune response elicited to *T. gondii*. As noted above, macrophages are critical for controlling *T. gondii* infection through cell-intrinsic antimicrobial mechanisms that contribute to parasite clearance through the direct destruction of pathogens (26, 80). In addition, since macrophages and dendritic cells function as APC, they are instrumental in coordinating the communication with cells of the adaptive immune system and subsequently, their activation. Infection of host cells by *T. gondii* is known to globally alter the transcriptional program (96-100) and the proteome of infected cells (101, 102). Active parasite infection of APC has also been

shown to modulate their cell-surface receptor composition (96, 103, 104). Given that the induction of antimicrobial mechanisms and T cell activation are both tightly regulated, the modulation of cell-surface receptors on APC has the potential to dramatically affect the progression of the infection. The mechanisms by which *T. gondii* modulates the expression of cell-surface receptors on macrophages are not entirely understood and warrants investigation. The work presented here investigates the mechanisms involved in regulating the expression of immune receptors on the surface of *T. gondii*-infected macrophages. Collectively, this research contributes to an enhanced understanding of processes of that mediate pathogen modulation of host immunity.

Chapter Two

Toxoplasma gondii induces B7-2 expression through activation of JNK signal transduction

Introduction

A hallmark of immunity to acute infection with *T. gondii* is the production of IFN-γ and IL-12, followed by a protective T cell response that is critical for parasite control (27, 28). T cell-mediated immunity is established through the coordinated communication between APC and T cells (23). T cell activation requires two signals: 1) the interaction of the T cell receptor (TCR) with its cognate peptide:MHC complex, and 2) the engagement of costimulatory molecules. CD28 functions as the prototypical costimulatory molecule on T cells and interacts with its ligands, the B7 family members B7-1 (CD80) and B7-2 (CD86) on APCs. On naïve T cells, ligation of the TCR in the absence of costimulation results in T cell anergy (105), underscoring the importance of costimulatory engagement for initiating T cell activation. Both B7-1 and B7-2 can promote T cell proliferation and the production of IL-2 (106, 107). Once T cells become activated, their responses are attenuated by the engagement of the inhibitory receptor CTLA-4 on the T cell with B7-1 or B7-2 on the APC (108, 109). In this manner, B7-1 and B7-2 contribute to both the activation and decline of T cell responses. As critical regulators of T cell activation, the expression of B7-1 and B7-2 are tightly controlled. Since a naïve T cell may encounter self-MHC:peptide in the periphery, the additional requirement of costimulation is believed to help discriminate between self and non-self.

T. gondii is known to alter the expression profile of receptors and ligands on infected antigen presenting cells (96, 103, 104), which can impact the outcome of APC activation states and T cell-APC interactions. In the mouse, *T. gondii* infection up-regulated the expression of B7-2 but not B7-1 in macrophages (110) and dendritic cells (111). In a study comparing costimulatory ligand expression on *T. gondii*-infected

peritoneal macrophages from BALB/c and BALB/b mice, B7-2 was specifically up-regulated on BALB/c macrophages (110). As critical regulators of T cell activation, the expression of B7-1 and B7-2 are tightly controlled.

We sought to determine the signaling pathways that are induced in *T. gondii*-infected macrophages and monocytes and that specifically affect the expression of costimulatory molecules, in an effort to better understand mechanisms of parasite immune modulation and regulation of critical immune ligands.

Results

T. gondii induces B7-2 but not B7-1 on infected macrophages

Macrophages function in immunity to infection by producing cytokines and antimicrobial factors, and by serving as professional APC to T cells. Immature macrophages in tissues are highly phagocytic cells that are specialized for the uptake and processing of foreign particles. Upon exposure to PAMPs, macrophages down-regulate their phagocytic capacity and up-regulate their antigen presentation machinery. This transition is accompanied by an increased expression of MHC proteins and the costimulatory ligands B7-1 and B7-2 on the cell surface. By infecting bone marrow-derived macrophages (BMdM) with *T. gondii* and examining costimulatory ligand expression at 24 hours post-infection (hpi), we observed that B7-2, but not B7-1, was highly induced on the surface of infected cells, as compared with mock-treated cells (Fig. 2.1A). We also compared the effect of *T. gondii* on B7-1 and B7-2 expression to that of IFN-γ treatment. Interestingly, *T. gondii* infection consistently induced B7-2 to higher levels than IFN-γ treatment (Fig. 2.1A). We then investigated whether infected $CD11b^+$ (Mac-1) cells *in vivo* up-regulated B7-2 expression. Peritoneal exudate cells were harvested three days after infection of mice with type I strain parasites. By using parasites expressing GFP, we were able to examine B7-2 expression on the infected and the uninfected $CD11b^+$ cells in the population. Analysis by flow cytometry revealed the highest level of B7-2 expression in the infected, GFP^+ cell population (Fig. 2.1C). These data recapitulated the high induction of B7-2 observed specifically in infected cells *in vitro* (Fig. 2.3B, C57BL/6). We also examined *T. gondii* infection of mouse peritoneal macrophages and bone marrow-derived DC, and B7-2 was up-regulated by

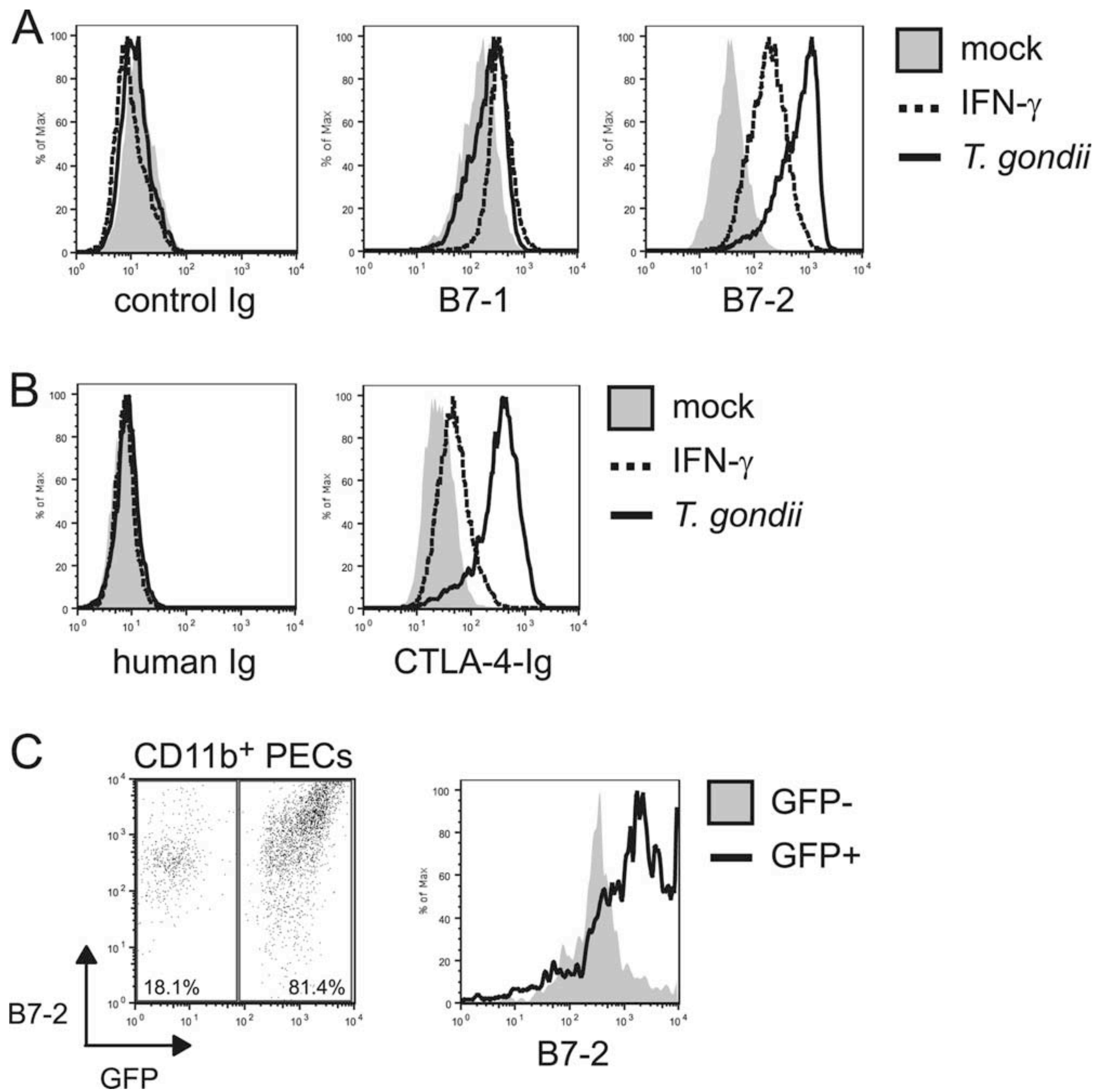


Figure 2.1. Expression of costimulatory molecules during *T. gondii* infection *in vitro* and *in vivo*

BMdM were infected with *T. gondii* (type I, RH strain) or treated with IFN-γ and stained with a control Ig, anti-B7-1, or anti-B7-2 monoclonal antibody at 24 hpi (A). (B) BMdM were infected as in panel A but were stained with a control human Ig or a CTLA-4–Ig fusion protein at 24 hpi. (C) C57BL/6 mice were infected i.p. with *T. gondii*. Peritoneal exudate cells (PECs) were harvested at 3 days postinfection, and the cells were analyzed for B7-2 expression by flow cytometry. One representative set from at least 3 independent experiments is shown for (A), (B), and (C).

T. gondii infection in both of these cell types (data not shown). These data indicate that the regulation of B7-2 expression by *T. gondii* infection occurs both *in vitro* and *in vivo*, and may be similar in different types of professional APC.

The biological function of B7-2 expression depends on its ability to successfully interact with its cognate receptors, CD28 or CTLA-4, on T cells. To evaluate whether B7-2 induced by *T. gondii* is capable of interacting with its receptor, we utilized a CTLA-4-Ig fusion protein, consisting of the extracellular domain of CTLA-4 fused to the Fc domain of human IgG1. BMdM were mock-treated, treated with IFN-γ as a positive control, or infected with type I parasites. The cells were stained with a control human IgG or the CTLA-4-Ig fusion protein and examined by flow cytometry at 24 hpi. Similar to the results observed with the anti-B7-2 monoclonal antibody, we found that CTLA-4-Ig staining of *T. gondii*-infected cells was elevated when compared to mock-treated cells or IFN-γ-treated cells (Fig. 2.1B). Parasite-induced B7-2, therefore, appears to be biologically functional, since it is capable of binding to its cognate receptor.

There are three dominant strains of *T. gondii* (type I, II, III), which differ in their global distribution, prevalence, virulence in mice and, perhaps, associated morbidity in humans (11, 20, 112). Since recent studies have demonstrated that these strains also vary dramatically in their effects on immune cell activity and function (52, 58), we sought to determine whether they differentially affected B7-2 up-regulation. BMdM were mock-infected or infected with type I, II, or III parasites, and examined by flow cytometry at 24 hpi. All three strains induced B7-2 expression (Fig. 2.2A), suggesting that the mechanism of B7-2 up-regulation during infection is likely to be conserved among these three clonal lineages of *T. gondii*.

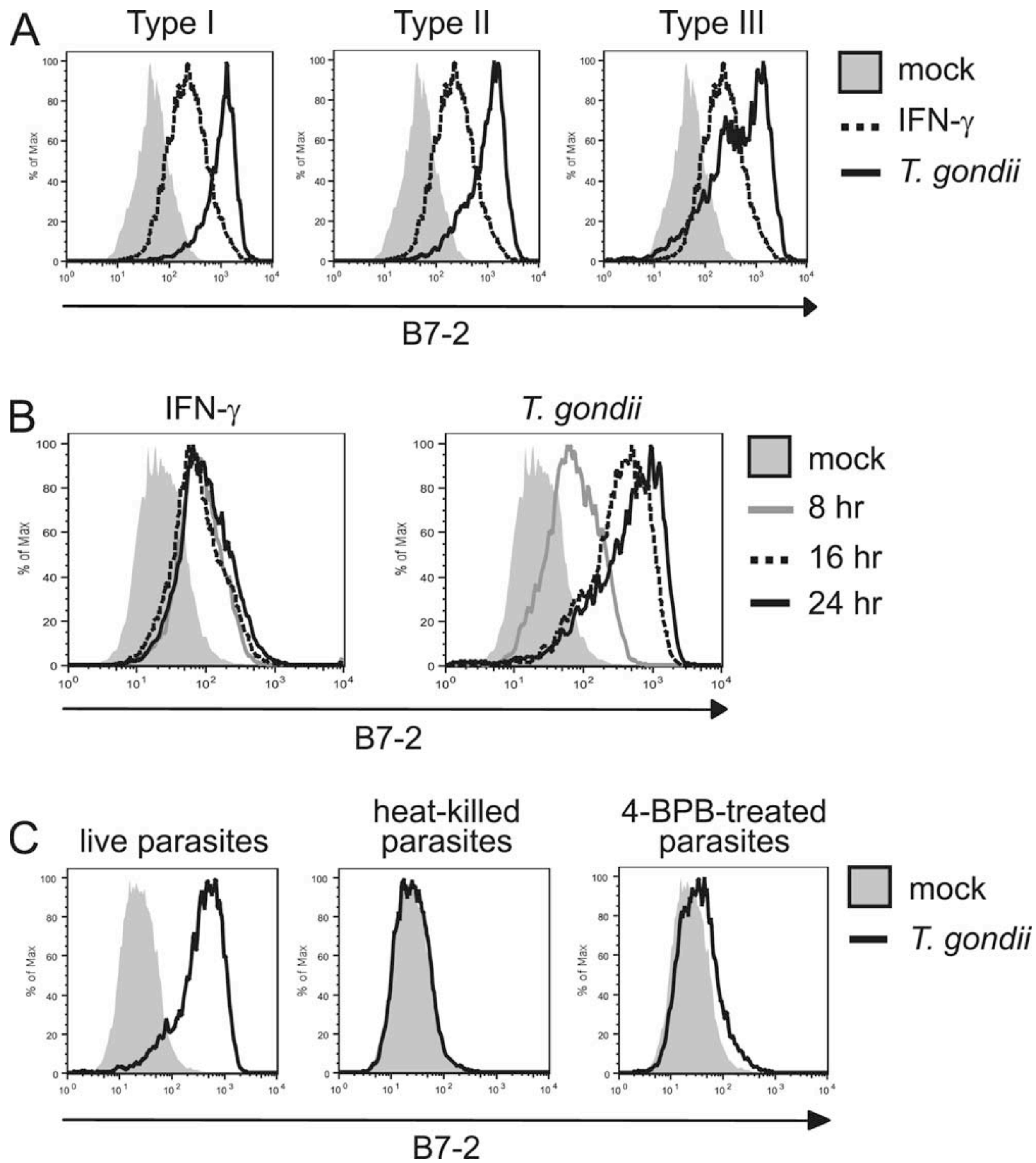


Figure 2.2. Characterization of B7-2 induction in *T. gondii*-infected macrophages

BMdM were infected with type I, II, or III parasites and examined for B7-2 expression at 24 hpi (A). (B) B7-2 expression was analyzed on *T. gondii*-infected or IFN-γ-treated BMdM at 8, 16, and 24 h post-treatment. (C) Live, heat-killed, or 4-BPB-treated type I parasites were used for infection of BMdM, and B7-2 expression was examined 24 h later. One representative set from at least 3 independent experiments is shown for (A), (B), and (C).

To determine the molecular mechanism by which the parasite induces B7-2 expression, we performed a series of experiments to understand the timing and requirements for this phenotype. A kinetic analysis would indicate whether B7-2 is induced rapidly after host cell invasion, or once the parasite has established itself in the host cell and has begun to replicate. These data would help to define the parasite factor(s) that are responsible for this effect. A time-course experiment was performed to address the kinetics of induction. BMdM were infected with type I strain parasites and harvested for B7-2 staining and flow cytometry at 8, 16, and 24 hpi. B7-2 expression began to increase as early as 8 hpi, and was almost fully induced by 16 hpi (Fig. 2.2B), suggesting that early sensing of the parasite by the macrophage, or the introduction of parasite products shortly after invasion contributed to B7-2 up-regulation. Interestingly, at 8 hours, induction of B7-2 was similar between IFN-γ treatment and *T. gondii* infection, but this represented the maximum induction for IFN-γ, whereas the infected cells showed further induction up to 24 hours. These data indicate that the pathways that are activated by IFN-γ receptor signaling and *T. gondii* infection to induce B7-2 expression are likely to differ.

To further characterize the factors required for *T. gondii*-induced B7-2 up-regulation, we investigated whether this effect required active invasion of host cell by live parasites, or if macrophage phagocytosis of parasites would also induce B7-2 expression. The results from these studies would help to distinguish whether host cell sensing of the parasite or an active parasite mechanism is responsible for this phenotype. Live or heat-inactivated *T. gondii* were added to macrophages, and the cells were examined for B7-2 expression by flow cytometry at 24 hpi. We observed that only

live parasites induced B7-2 expression on the surface of infected macrophages (Fig. 2.2C), indicating that the manner in which the parasites entered the macrophages dictated the outcome of B7-2 induction. To rule out the possibility that heat-killing the parasites impaired host cell recognition of *T. gondii*, we pre-treated the parasites with a phospholipase inhibitor called 4-bromophenacyl bromide (4-BPB). Parasites treated with this inhibitor can attach to host cells but cannot actively invade (113), so they are phagocytosed by the macrophages (data not shown). As shown in Fig. 2.2C, 4-BPB pre-treatment prevented *T. gondii* induction of B7-2. These data suggest that B7-2 up-regulation is likely due to a mechanism stimulated by actively invading parasites.

MyD88 and TRIF are not involved in T. gondii induction of B7-2 expression

A burgeoning field of research has focused on the processes whereby mammalian cells sense the presence of foreign pathogens and initiate innate immune responses. The TLRs have emerged as a large family of receptors that recognize microbial products, ranging from nucleic acids to peptidoglycan and proteins (114). TLRs associate with adaptor proteins to transduce signals into the host cell and induce inflammatory responses. MyD88 is the adaptor protein used for signaling downstream of all the TLRs, except TLR3, which uses the adaptor protein TRIF (115). Five different TLRs have been implicated in the recognition of *T. gondii*, namely TLR2, TLR4, TLR7, and TLR9 and TLR11, which is expressed in mice only (116). Given that the recognition of pathogen products by TLRs induces the expression of B7-2 in other systems (117), we tested the role of TLR signaling in *T. gondii* induction of B7-2. Macrophages deficient in the signaling adaptors MyD88 or TRIF were infected with *T. gondii* and examined for B7-2 gene expression by quantitative real-time PCR (Q-PCR), and B7-2 protein by flow

cytometry. As shown in Fig 2.3A, *T. gondii* infection of BMdM from C57BL/6 mice resulted in an increase in B7-2 transcript levels by about ten-fold compared to mock-treated cells, which is consistent with the ten-fold change in mean fluorescence intensity (MFI) of B7-2 on the surface of infected cells (Fig. 2.3B). The infection of MyD88- and TRIF-deficient macrophages with *T. gondii* similarly up-regulated B7-2, both at the transcript level (Fig. 2.3A) and on the cell surface (Fig. 2.3B). These data indicate that *T. gondii* induces B7-2 at the transcript level, and also that TLR signaling does not appear to be involved in this induction.

Microarray analysis reveals induction of genes downstream of MAPK signaling pathways

Since B7-2 expression was elevated at the level of mRNA in *T. gondii*-infected cells (Fig. 2.3A), but did not require MyD88 or TRIF, we performed genome-wide transcriptional profiling of *T. gondii*-infected macrophages, in an attempt to identify nodes of regulation of B7-2. BMdM were mock-treated or infected with the type I (RH) strain of *T. gondii*, and total RNA was harvested at 6 hpi. This time point was selected because *T. gondii* induction of B7-2 on the cell surface occurred within 8 hpi (Fig. 2.2B), and we reasoned that gene expression changes that induced this effect would be most apparent very early after infection. Furthermore, the transcriptional profiling of cells within a short time period post-infection would be more likely to reveal those transcriptional effects that occurred due to the initial infection, rather than a secondary stress response of the host cell. Labeled cDNA was hybridized to Affymetrix mouse 430 2.0 gene expression arrays to compare genes that showed increased or decreased mRNA in *T. gondii*-infected versus mock-treated cells. These arrays contain 45,101

probe set IDs corresponding to 21,635 unique known and putative genes. After standard normalization steps, a two-class unpaired t-test with Benjamini-Hochberg correction was carried out to identify differentially-expressed genes: 8127 probe sets, representing 7243 genes, were significantly different (FDR $\leq$ 1%) between mock and *T. gondii*-infected cells. A second filter for genes that changed greater than 5-fold by infection yielded 193 probe sets, representing 186 unique genes. The probe sets that met these two filtering criteria are expressed in the volcano scatter plot in red (Fig. 2.4A) and in the heat map (Fig. 2.4B). The three left columns of the heat map depict the gene expression data from mock-treated cells, and the three right columns depict that of *T. gondii*-infected cells. The filtered dataset included two probe sets for B7-2, which were induced 5.42-fold and 5.32-fold during infection (Fig. 2.4B, arrowheads).

Among the genes that met the two filtering criteria for being differentially affected by parasite infection, 91.4% (170 of 186) were up-regulated in infected cells, as compared to only 8.60% (16 of 186) that were down-regulated in infected cells (Fig. 2.4B). These data are consistent with previously published microarray experiments performed on *T. gondii*-infected human foreskin fibroblasts (HFF) and BMdM at later time points post-infection (52, 97, 98, 118), indicating that the host response to the parasite infection mostly involves up-regulation. These transcripts are likely to be induced through a combination of host-specific sensing mechanisms and active parasite-mediated effects. Indeed, there is strong evidence for the ability of *T. gondii* to alter host cell transcription (52, 97, 98, 118).

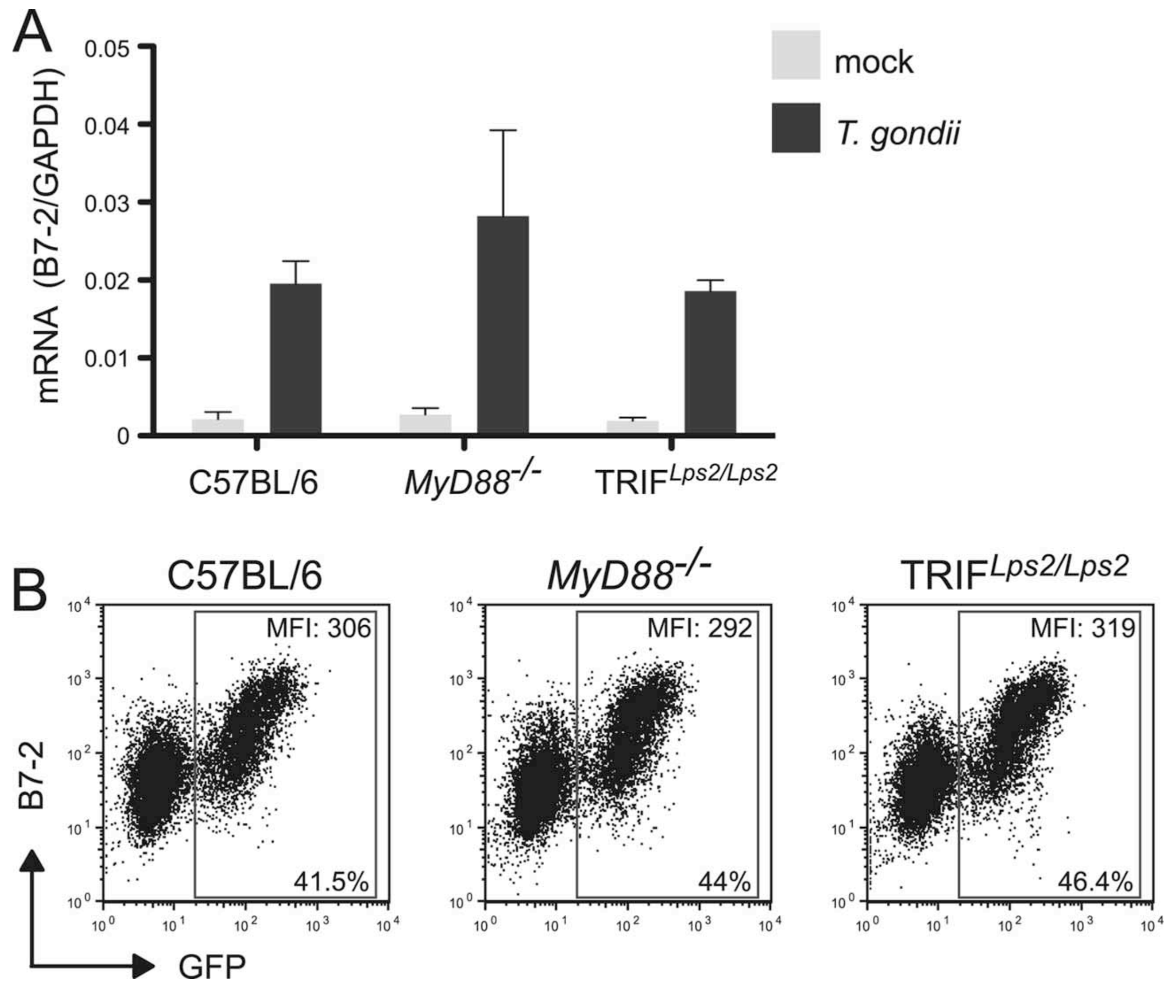
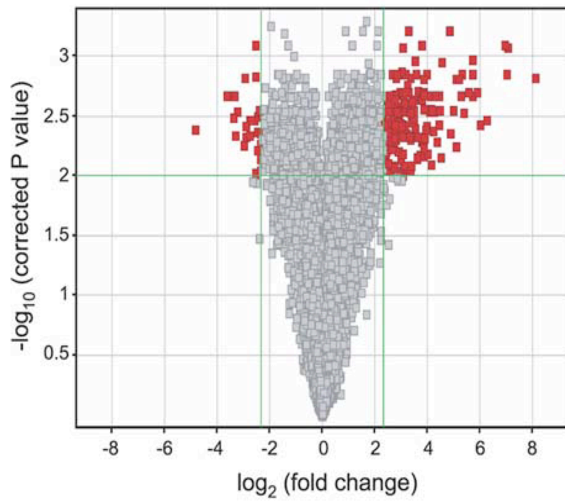


Figure 2.3. Examination of MyD88 and TRIF in *T. gondii*-induced B7-2 expression

BMdM from C57BL/6, *MyD88*$^{-/-}$, or TRIF$^{Lps2/Lps2}$ mice were infected with *T. gondii* for 24 h, and RNA was harvested for cDNA synthesis and analysis by Q-PCR for B7-2 transcript levels (A). For each condition, the expression of B7-2 relative to that of GAPDH is shown. Error bars reflect the standard deviations of triplicate samples. (B) BMdM were infected as in panel A and harvested for the analysis of B7-2 expression by flow cytometry. For each dot plot, the mean fluorescence intensity (MFI) of B7-2 expression and the percentage of cells within the gated, GFP^{+} population are shown.


A
T. gondii-infected vs. mock-treated cells
-log10 (corrected P value)
log2 (fold change)
3
2.5
2
1.5
1
0.5
-8
-6
-4
-2
0
2
4
6
8

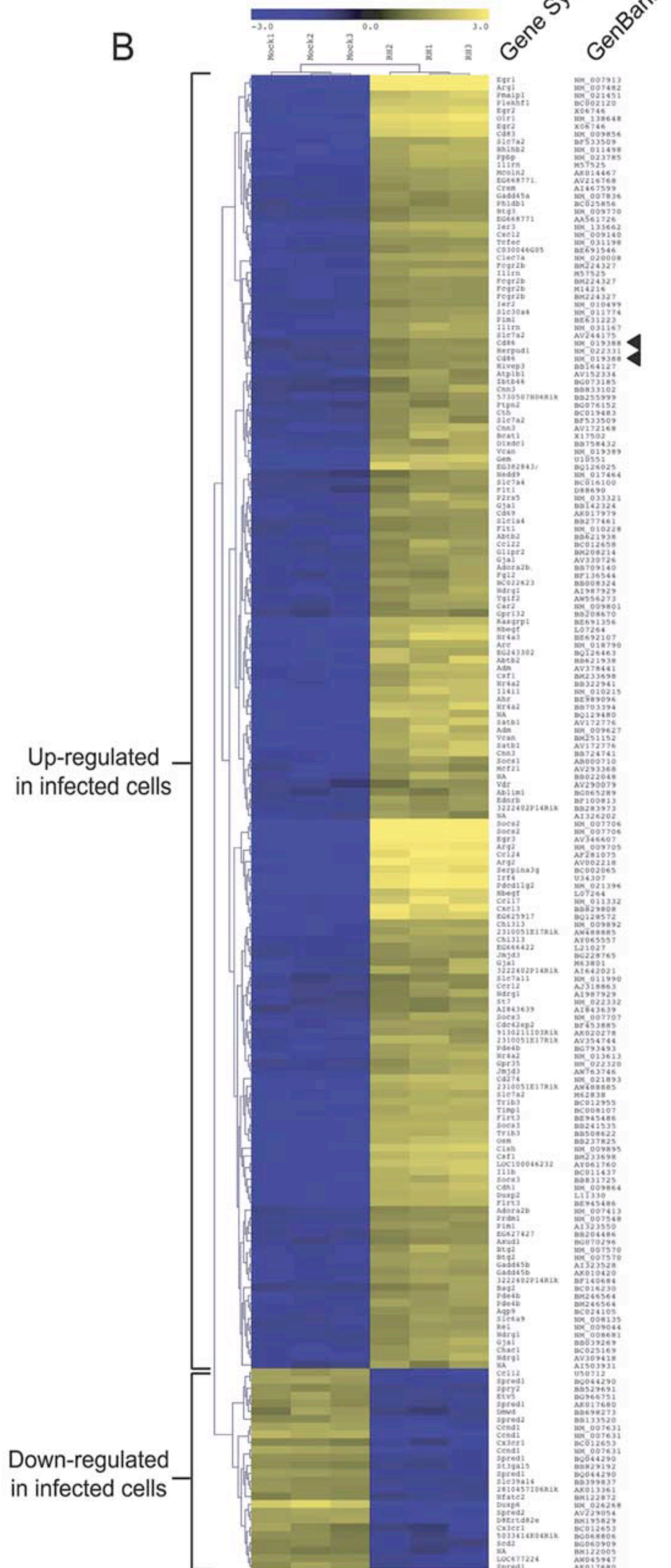

B
Gene Symbol
GenBank
-3.0
0.0
3.0
Up-regulated in infected cells
Down-regulated in infected cells

Figure 2.4. Genome-wide transcriptional profiling of *T. gondii*-infected macrophages

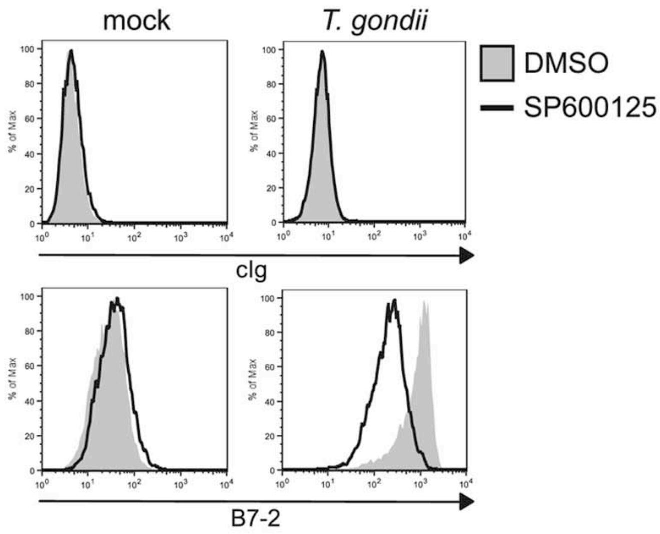
BMdM were mock treated or infected with *T. gondii* (RH strain), and RNA was harvested at 6 hpi. cDNA was synthesized and labeled for hybridization on Affymetrix mouse 430 2.0 microarrays. (A) Volcano plot of gene expression data. To identify genes significantly different in expression between mock-treated and infected cells, a two-class unpaired *t*-test with Benjamini-Hochberg FDR correction was implemented and average fold change values were calculated. Each point represents an individual probe. Green lines reflect the threshold criteria for significance (FDR, ≤1%; fold change, >5); probes meeting both these criteria are plotted in red. (B) Heat map of genes identified as significantly different between mock-treated (three left columns) and infected (three right columns) samples. The $\log_2$ gene expression values were median centered, and hierarchical clustering was performed using Euclidean distance and average linkage clustering.

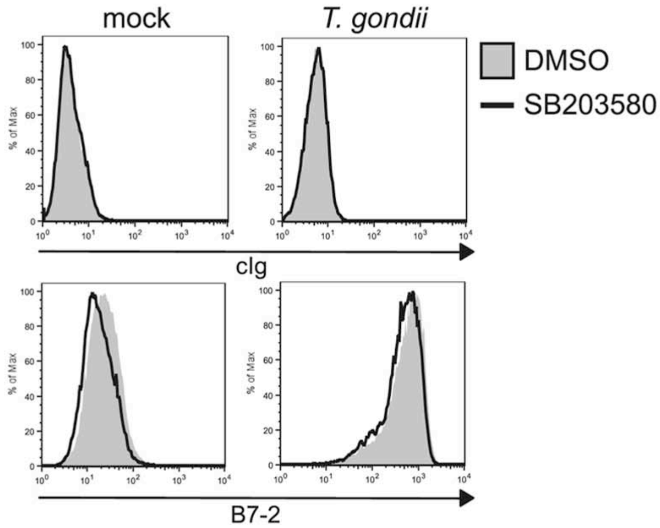
Transcriptional profiling of *T. gondii*-induced genes by microarray enabled us to evaluate nodes of gene regulation that were induced or repressed in *T. gondii*-infected cells. By using Ingenuity Pathway Analysis to reveal specific canonical pathways that were differentially regulated by infection, we observed a striking enrichment for gene sets representing signaling pathways that are involved in the maturation of APC. In particular, gene sets associated with NF-kB and MAPK signaling cascades, each of which is a major signaling nexus in immune cells, were most dramatically changed (data not shown). These data allowed us to investigate specific pathways that play a role in B7-2 induction during infection.

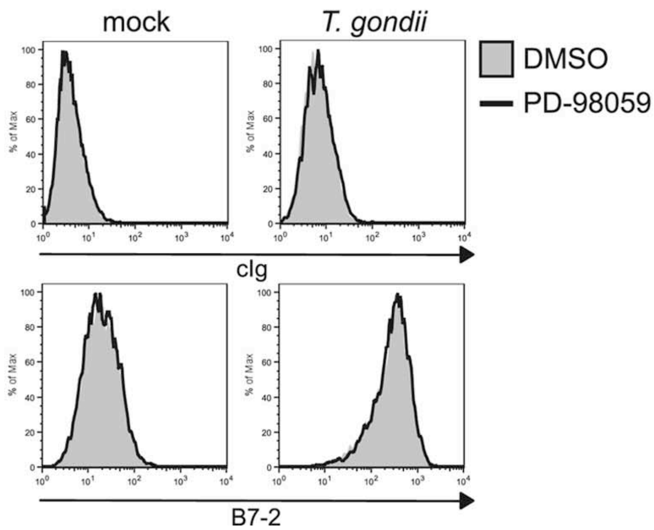
JNK is involved in T. gondii-induced B7-2 expression

Based on the analysis of the microarray data, we chose to examine the role of MAPK signaling in B7-2 induction by using specific pharmacological inhibitors. BMdM were pre-treated with inhibitors of ERK (PD-98059), JNK (SP600125), or p38 (SB203580) MAPKs and then infected with *T. gondii*. The cells were harvested and examined for B7-2 expression by flow cytometry. None of the inhibitors affected the level of B7-2 on uninfected cells, suggesting that these signaling molecules are not involved in the basal expression of B7-2 on unstimulated macrophages (Fig. 2.5A). Moreover, the treatment of BMdM with the inhibitors did not affect the ability of the parasites to infect the cells, since the percentage of infected (GFP^+) cells was unchanged in cells that were treated with the inhibitors or treated with the DMSO control (data not shown). Treatment with the ERK and p38 inhibitors had no effect on the levels of *T. gondii*-induced B7-2 on the surface of infected BMdM. In contrast, treatment of

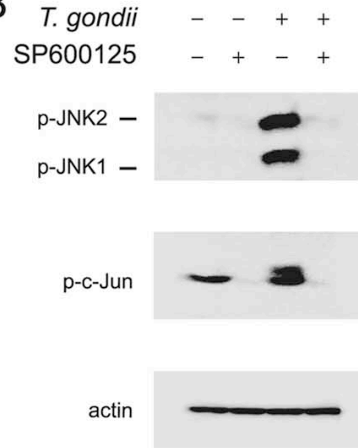
A

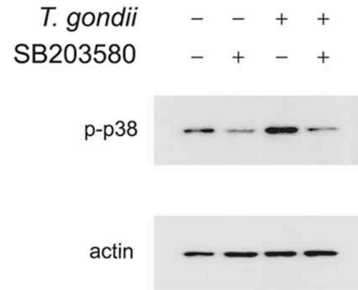






B





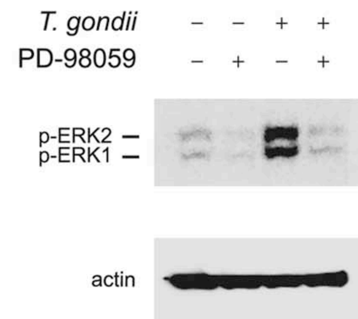


Figure 2.5. B7-2 expression after MAPK inhibitor treatment and *T. gondii* infection

BMdM were treated with DMSO or with inhibitors of JNK (SP600125), p38 (SB203580), or ERK (PD-98059) and infected with *T. gondii* (A). The cells were harvested at 24 hpi and stained with a control Ig or with anti-B7-2 monoclonal antibody. (B) BMdM were treated and infected as described above, but lysates were generated at 15 mpi and separated by SDS-PAGE for Western blotting with antibodies against p-JNK, p-c-Jun, p-ERK, p-p38, or β-actin.

BMdM with the JNK inhibitor substantially reduced *T. gondii*-induced B7-2 expression (Fig. 2.5A), in a dose-dependent fashion (data not shown).

To confirm that the MAPK inhibitors functioned effectively in our assays, we performed, in parallel, Western blotting experiments on lysates from cells that were treated with the inhibitors and infected. Infection of BMdM with *T. gondii* resulted in rapid (within 15 min) phosphorylation of ERK, p38, and JNK, as determined by immunoblotting for the phosphorylated proteins (Fig. 5B). Pretreatment with the ERK (PD-98059), JNK (SP600125), or p38 (SB203580) inhibitors, however, blocked parasite-induced phosphorylation of the respective target proteins, indicating that these reagents functioned as predicted in our assays. In addition, we examined the inhibitory effect of SP600125 on JNK phosphorylation of its downstream target, c-Jun. The phosphorylation of c-Jun was effectively inhibited by treatment with this inhibitor (Fig. 2.5B). In a time-course experiment, we found that JNK inhibition was sustained as late as 24 hpi (data not shown), the latest time point at which B7-2 levels were examined by flow cytometry in our assays. Based on these data, B7-2 up-regulation by *T. gondii* seems to be dependent on signaling through JNK.

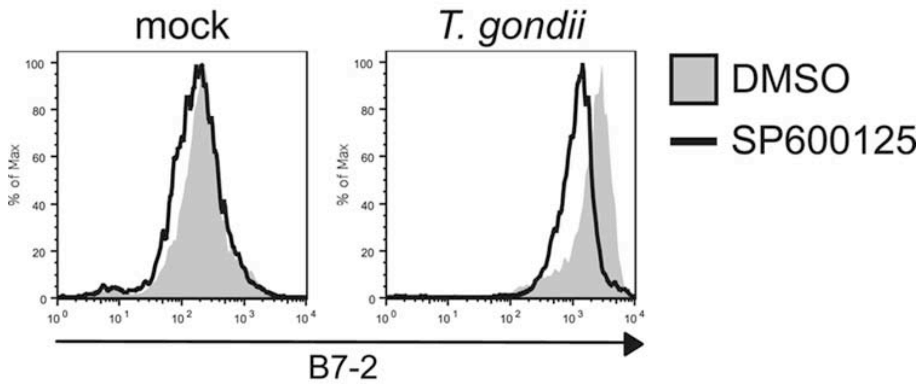
B7-2 induction in human monocytes infected with T. gondii is also dependent on JNK

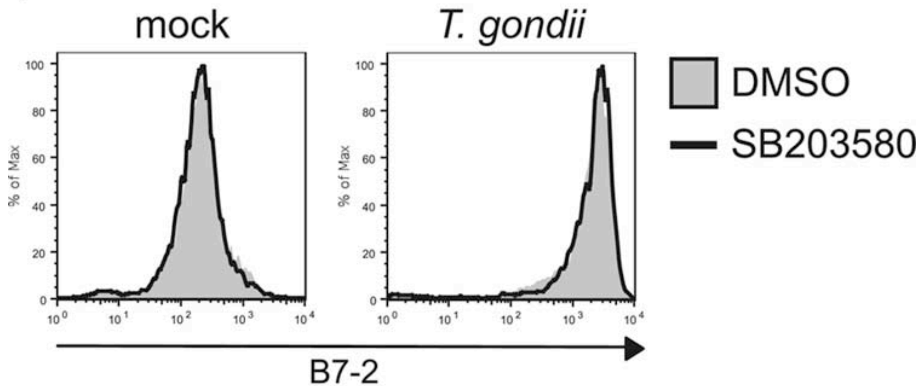
Our data have defined a role for JNK in *T. gondii*-induced B7-2 expression in mouse macrophages. To determine if a similar signaling apparatus is effective in human monocytes, we isolated monocytes from human peripheral blood mononuclear cells. Monocytes were infected with each strain (type I, II, III) of GFP-expressing parasites for 24 hr in the presence or absence of the MAPK inhibitors used above, and examined for B7-2 expression by flow cytometry. Similar to the mouse macrophages, human

monocytes up-regulated the expression of B7-2 during infection, specifically in the infected GFP$^+$ cells (Fig. 2.6). This effect was observed with all three strains of *T. gondii* (data not shown). By treating with the MAPK inhibitors prior to infection, we determined that pretreatment with SP600125 partially inhibited parasite up-regulation of B7-2 (Fig. 2.6). These data suggest that JNK also plays a role in the regulation of B7-2 expression in human monocytes during parasite infection.

Parasite-induced B7-2 costimulates naïve T cell proliferation in vitro

To assess the degree to which parasite-induced B7-2 contributes to costimulating T cell activation, we performed a proliferation assay in which infected BMdM were co-cultured with enriched splenic T cells. To focus specifically on the role of B7-2 in costimulation, T cells were cultured with infected macrophages in the presence of plate-bound anti-CD3 to stimulate the T cells through the TCR. T cells that were cultured alone did not proliferate (data not shown). While T cell proliferation was observed in the presence of uninfected macrophages, there was a dramatic increase in the level of proliferation of T cells in the presence of *T. gondii*-infected macrophages (Fig. 2.7). This increase was reflected both in the elevated percentage of T cells that had proliferated and in an increased number of cell divisions. The cell proliferation was found to be B7-2 dependent, as blockade of B7-2 using neutralizing antibodies reduced the level of T cell proliferation down to that observed during co-culture with uninfected macrophages. These data indicate that the up-regulation of B7-2 on infected macrophages can functionally costimulate T cell proliferation.





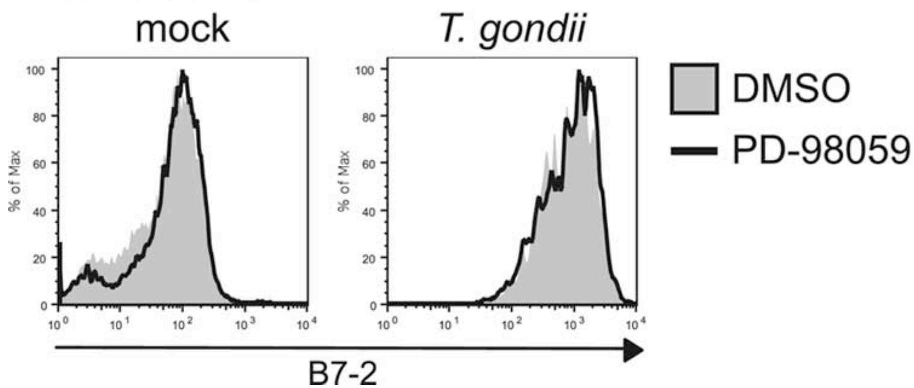


Figure 2.6. Examination of B7-2 expression on *T. gondii*-infected human monocytes

Human monocytes were harvested from PBMCs and treated with DMSO or with inhibitors of JNK (SP600125), p38 (SB203580), or ERK (PD-98059) before infection with *T. gondii*. The cells were harvested at 24 hpi and stained with a control Ig or with anti-human B7-2 monoclonal antibody.

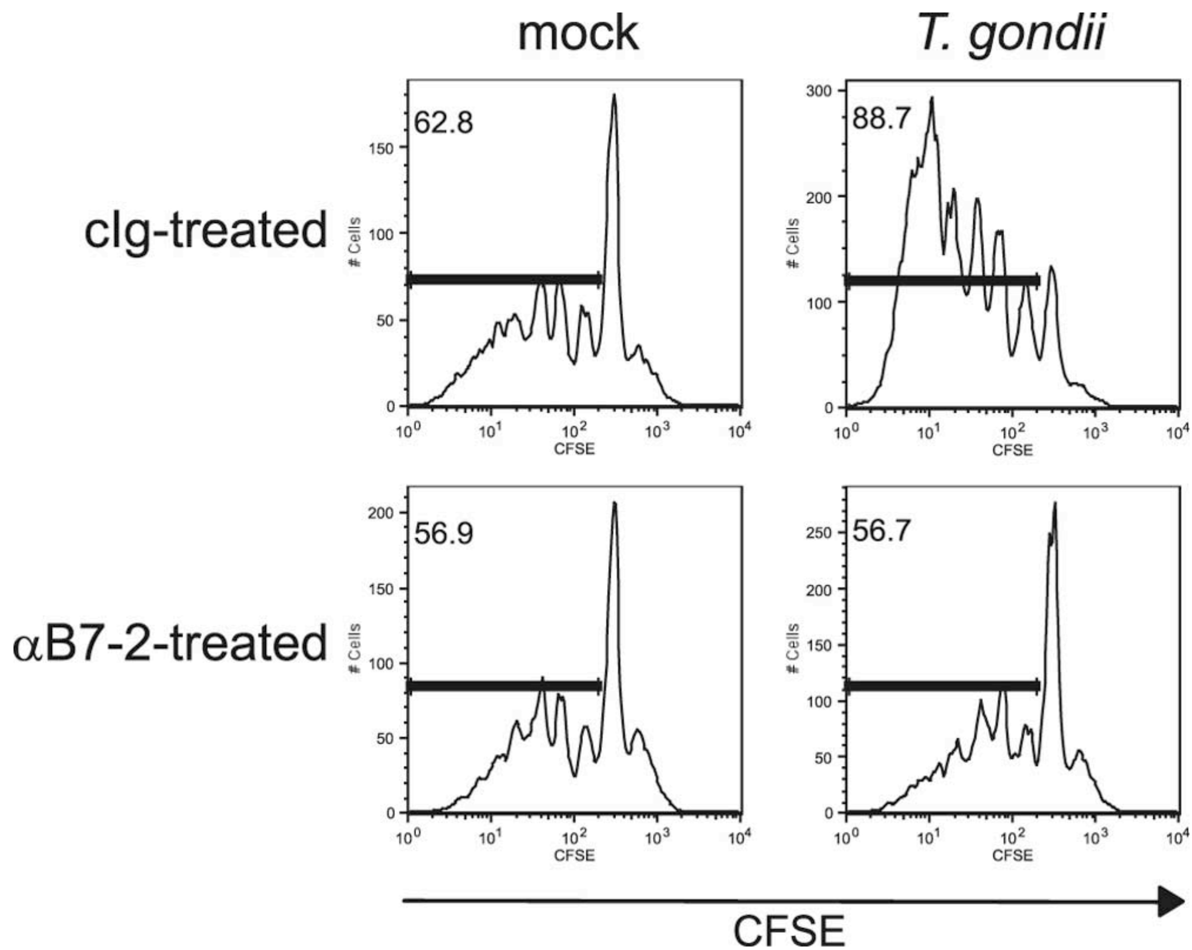


Figure 2.7. Effect of blocking *T. gondii*-induced B7-2 expression on T cell proliferation

T. gondii-infected BMdM were incubated with a control Ig or an anti-B7-2 monoclonal antibody, fixed, and then cocultured with naïve, syngeneic CFSE-labeled splenic T cells in the presence of plate-bound anti-CD3. The cells were harvested after 4 days, and proliferation was analyzed as a function of CFSE dilution by flow cytometry. For each histogram plot, the marker was set to indicate cells that had undergone division. The percentage of cells within this marker is shown in the upper left corner of the plot.

Discussion

We found that all three strains of *T. gondii* (types I, II, and III) up-regulated the expression of B7-2, but not B7-1, on the surface of mouse BMdM. Additionally, intraperitoneal injection of mice resulted in enhanced B7-2 levels on infected $CD11b^+$ cells. B7-2 induction occurred at the transcript level, required active parasite invasion, and was not dependent on MyD88 or TRIF. Functional assays demonstrated that *T. gondii*-infected macrophages stimulated naïve T cell proliferation in a B7-2-dependent manner. Genome-wide transcriptional analysis comparing infected and uninfected macrophages revealed the activation of MAPK signaling in infected cells. Using specific inhibitors against MAPKs, we determined that parasite-induced B7-2 is dependent on JNK, but not ERK or p38 signaling. We also observed that *T. gondii*-induced B7-2 expression on human peripheral blood monocytes is dependent on JNK signaling, indicating that a common mechanism of B7-2 regulation by *T. gondii* may exist in both humans and mice.

The costimulatory ligands B7-1 and B7-2 provide a critical function in the activation and attenuation of T cell-mediated immunity, through their engagement with CD28 and CTLA-4. As a result, their expression is thought to be tightly regulated. The signaling pathways that are involved in infection-induced B7-2 expression, however, are not well known. This study demonstrates the involvement of MAPK signaling in B7-2 up-regulation by *T. gondii* infection. Given the important role of costimulatory signals in initiating immune responses, these data contribute to our understanding of the molecular mechanisms that underpin host immunity to infection.

The differential effect of parasite infection on B7-1 and B7-2 in human and mouse APCs is intriguing. Although both accessory molecules serve as natural ligands for CD28 and CTLA-4, they have limited homology (26% sequence identity) (119). Functional studies suggest that they serve both overlapping and distinct functions, which contribute to the inherent complexity in this receptor-ligand system. B7-2 is expressed at higher levels on APCs, and its induction by external stimuli occurs more rapidly (120). B7-2 expression in *T. gondii*-infected cells increased at the transcript level within 6 hr of infection. These data suggest that B7-1 and B7-2 are induced by different signaling mechanisms and may explain the different effects of the parasite on the expression of these proteins. Although B7-1 is typically up-regulated with slower kinetics, its affinity for both CD28 and CTLA-4 is substantially higher than that of B7-2 for these receptors (121). Moreover, the affinities of both B7-1 and B7-2 for CTLA-4 are higher than for CD28 (121). The rapid kinetics of up-regulation and broad expression of B7-2 on APCs has led to the idea that it may be important for the initiation of immune responses. In contrast, B7-1 may play a more critical role in amplifying and sustaining T cell activation. Indeed, B7-1 has been shown to be a more potent stimulator of T cell activation (122). The differential induction of B7-1 and B7-2 in *T. gondii*-infected human and mouse cells may, therefore, have profound implications for shaping the T cell response to infection.

The *in vivo* consequences of B7-2 up-regulation by *T. gondii* remains unclear. Since CTLA-4-Ig fusion protein binding to macrophages increased after infection, parasite-induced B7-2 appears to be capable of interacting with its cognate receptor. Moreover, B7-2 up-regulation on infected cells costimulated naïve T cell activation *in*

vitro, indicating a potential biological function and role in T cell priming *in vivo*. To formally examine this possibility, however, will likely require the identification of the parasite factors responsible for B7-2 induction during infection. This would enable the generation of parasites deficient in this ability and a comparison with wild-type parasites in T cell priming *in vivo*.

Although B7-2 was rapidly up-regulated in infected cells, both as mRNA and as protein expression on the cell surface, this induction was independent of MyD88 or TRIF signaling. This suggests B7-2 induction by *T. gondii* is independent of TLR signaling. Infection of MyD88 and TRIF double knock-out macrophages would definitively demonstrate a TLR-independent mechanism of induction for B7-2 during infection, since there remains a possibility that one adaptor may compensate for the loss of the other. MyD88 has an important role in host defense against *T. gondii* infection, as MyD88-deficient mice are acutely susceptible to infection, as compared with C57BL/6 controls. TLR11 was found to be responsible for the specific recognition of a *T. gondii* protein, called profilin, and induces DC production of IL-12 during *in vivo* infection (70). Unlike *MyD88*$^{-/-}$ mice, however, *TLR11*$^{-/-}$ mice remain resistant to *T. gondii* infection (70), indicating that other defense mechanisms that utilize MyD88 are involved in controlling the parasite. Although it is perhaps surprising that MyD88 signaling is not required for B7-2 up-regulation by *T. gondii*, LPS-induced DC maturation was also found to be independent of MyD88 (123). MyD88 is also a signaling adaptor downstream of the IL-1 receptor (124). *T. gondii* induces high levels of IL-1 during infection (Hunter *et al.*, 1993, Pelloux *et al.*, 1994); however, our experiments using

MyD88$^{-/-}$ macrophages infected with *T. gondii* suggest that IL-1 receptor signaling may not contribute to B7-2 expression.

Given that B7-2 induction by *T. gondii* occurred predominantly in infected cells and required active invasion by live parasites, we reasoned that B7-2 may be up-regulated through a mechanism involving the introduction of a secreted parasite protein during invasion. *T. gondii* uses its actin-myosin cytoskeletal machinery to actively invade host cells (125). A key step in parasite invasion is the secretion of proteins from the specialized secretory organelles of the parasite, namely the rhoptries, micronemes, and dense granules. While some of these secreted proteins play a role in host cell attachment and invasion, others are translocated into the parasitophorous vacuole or the host cell cytosol (126). There is now clear evidence that secreted proteins interact with host proteins and modulate their activity (53, 55). Whether a secreted protein is involved in the induction of B7-2 transcript during infection remains to be determined, although there is certainly precedent for a rhoptry protein (ROP16) trafficking to the nucleus and altering host cell gene transcription (52). We do not believe that ROP16 is responsible for the effects observed in these studies, however, since its activity is strain-specific, and the induction of B7-2 occurs during infection with all three parasite strains. In addition, B7-2 transcript levels did not differ in macrophages that were infected with the parental type I (RH) strain or Δ*rop16* parasites (A. Shastri and J. Boothroyd, personal communication). Another possibility is that a secreted protein is sensed by the host cell through a mechanism independent of MyD88 and TRIF, leading to the up-regulation of B7-2 mRNA. This possibility, which has interesting implications for parasite modulation of APC function, awaits further study.

Transcriptional profiling by microarray analysis revealed nodes of regulation in infected cells, in particular the induction of MAPK signaling and the up-regulation of APC maturation markers. Given the early time point used in our experiments (6 hpi), it is clear that the activation of a large number of genes by these pathways occurs within a very short time-frame post-infection, and that host cells can mount a swift response to the parasite. Indeed, the detection of phosphorylated forms of JNK, ERK, and p38 MAPKs within 15 mpi indicates a rapid alteration in host processes by the parasite. These data are consistent with previous reports on MAPK activation following infection (21, 127, 128). Among the down-regulated transcripts in our analysis, many of the genes are negative regulators of MAPK signaling, which may be actively repressed by infection. Indeed, integrative genomic approaches have revealed the importance of secreted parasite kinases that can down-regulate host genes associated with MAPK signaling (21). In this way, *T. gondii* may have a global effect on MAPK signaling cascades in infected cells, by inducing positive regulators and repressing negative regulators.

The role of MAPK signaling in immunity to *T. gondii* has largely been examined in the context of macrophage IL-12 production. In macrophages, autophosphorylation of p38 is the dominant player in activating IL-12 secretion (129). In neutrophils, IL-12 and CCL2/MCP-1 production require JNK2, as PMN from *JNK2*$^{-/-}$ mice were totally deficient in the production of these cytokines (130). Neutrophil chemotaxis is also partially dependent on JNK2 (130). Using pharmacological inhibitors, we found that B7-2 up-regulation in *T. gondii*-infected cells is dependent on JNK signaling. *JNK2*$^{-/-}$ mice were recently infected with *T. gondii*, and examined for their disease resistance and

pathology. After oral infection, mice lacking JNK2 had a lower parasite burden and reduced pathology, as compared with wild-type C57BL/6 controls (131). Decreased neutrophil recruitment to the intestinal mucosa and reduced inflammation at this site accounted for the reduced pathology in the knock-out mice. Although these reports primarily focused on nutrophils, they indicate an important role for JNK2 in parasite-induced immune pathology.

To what degree is B7-2 induction during infection beneficial for parasite dissemination or for the host immune response? Costimulation is clearly important for the activation of naïve T cells, but effector T cells are less reliant on this engagement. Because B7-2 can bind to both an activating (CD28) and an inhibitory (CTLA-4) receptor, the functional consequences of B7-2 expression may vary dramatically, depending on the stage of infection in which the APC and T cell encounter one another. As we have shown, B7-2 induced by *T. gondii* is capable of activating T cells and promoting their proliferation. However, the importance of *T. gondii*-induced B7-2 *in vivo* may depend on the particular type of T cell that an infected macrophage encounters. The possibility of interaction between infected APCs and regulatory T cells (Tregs) also has interesting implications for B7-2 function. Tregs express high levels of CTLA-4 (132), and parasite induction of B7-2 may contribute to modulating the Treg population in infected animals. As we continue to develop our understanding of the intricate relationship between the parasite and the host, it will be of great value to consider the manner in which *T. gondii* alters or manipulates host responses to ensure its viability and propagation.

Acknowledgements

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Chapter Three

Strain-specific induction of CD40 during *Toxoplasma gondii* infection amplifies inflammatory cytokine responses

Introduction

A robust innate immune response is initiated rapidly following *T. gondii* infection and is responsible for establishing a first line of defense and for slowing parasite growth and dissemination. Myeloid cells such as monocytes, DC, and macrophages are among the first immune cells to migrate to the site of infection and subsequently become activated (63). CD40 is a receptor on macrophages that plays a critical role in macrophage activation and controlling *T. gondii* infection. The engagement between CD40 and CD154 (CD40L), expressed by APC and activated $CD4^{+}$T cells, respectively, results in the establishment of antimicrobial programs. One such antimicrobial program is mediated through autophagy, which is a cellular process that allows cells to maintain homeostasis through the degradation and recycling of damaged organelles and misfolded proteins (133). More recently, the induction of autophagy upon the engagement of CD40 has been shown to contribute to the clearance of intracellular pathogens such as *Leishmania major* (134, 135), and *T. gondii* (89, 136). Autophagy induced in *T. gondii*-infected cells following CD40 engagement lead to increased lysosome fusion with the parasitophorous vacuole (PV), resulting in enhanced parasite clearance (89, 90, 93). This robust innate immune response is initiated rapidly following *T. gondii* infection and contributes to the establishment of a first line of defense to slow parasite growth and dissemination.

Proper T cell priming and activation is also crucial for controlling *T. gondii* infection (137). T cell-deficient hosts succumb rapidly to infection with *T. gondii* (36). CD40 engagement has long been known to contribute to IL-12 production (138-140) and consequently plays a role in priming T cell responses. Moreover, APC production of

IL-12 shortly after *T. gondii* infection (141, 142) is necessary for priming adaptive immunity by stimulating IFN-γ production (143), which is necessary to establish a Th1 response (144). Not surprisingly, disruption of sustained IL-12 production diminishes Th1 immune responses and allows for uncontrolled parasite growth (143).

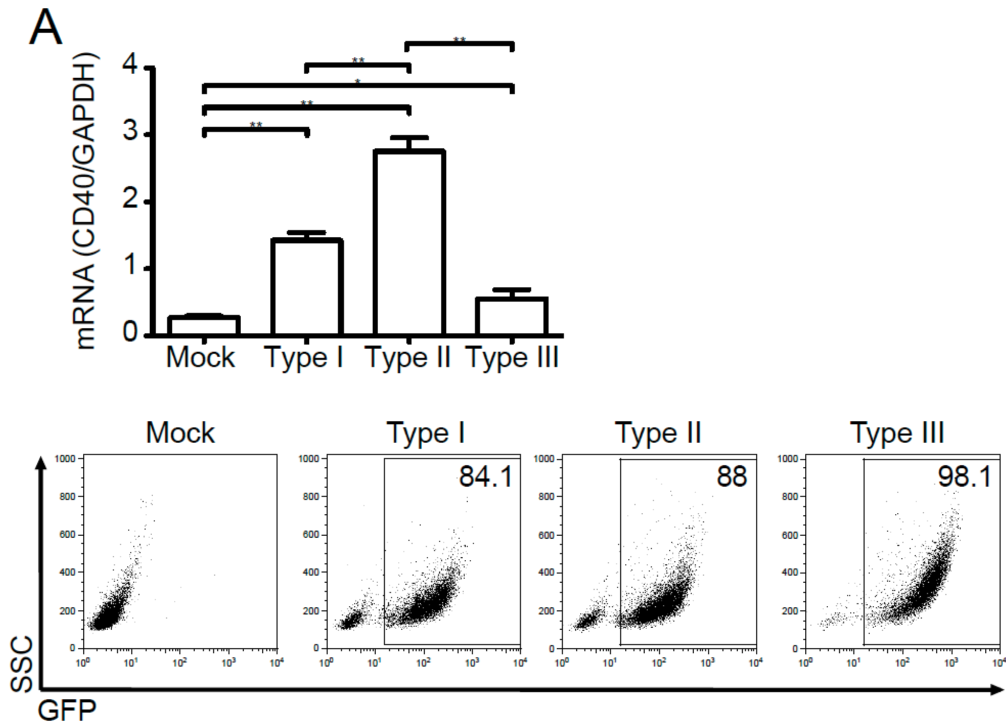
Despite its central role in mediating macrophage activation, the regulation of CD40 during *T. gondii* infection is not well understood. CD40 is expressed by macrophages as well as other APC. LPS stimulation of macrophages and microglia has been shown to induce CD40 transcription via STAT-1 and NF-κB p65 and p50 nuclear translocation and binding to the CD40 promoter (77). In addition to STAT-1 and NF-κB-mediated CD40 transcriptional activation, binding of transcription factor specificity protein 1 (Sp1) to the CD40 promoter is necessary for optimal CD40 induction in response to LPS (145). STAT-1 signaling was found to be critical for CD40 expression following IFN-γ stimulation (146), whereas IL-4 and STAT-6 inhibit CD40 expression (147). The processes that mediate the regulation of CD40 expression during infection are unknown. Here, we examined the mechanisms that regulate the expression of CD40 on the surface of *T. gondii*-infected BMdM.

Results

Type II T. gondii induces high expression of CD40 on infected macrophages

We evaluated the expression of the costimulatory molecule CD40 following *T. gondii* infection. After infecting primary mouse BMdM with type I, II or III parasites, we observed that CD40 transcript levels were increased in all infected samples at 6 hpi. However, the increase in CD40 transcript levels were significantly higher in BMdM infected with type II parasites compared to mock-infected cells (10-fold change, $P<0.05$) and to cells infected with either type I or III parasites (Fig. 3.1A). By using parasites expressing GFP, we were able to quantify the percent of infected, GFP^+ cells in each sample. At 18 hpi, the percent of cells that were infected with each of the strains was comparable (>84% of the population) (Fig. 3.1A). We then investigated whether there was a strain-specific induction of CD40 on the surface of infected cells. Flow cytometry analysis of infected BMdM at 18 hpi revealed that the highest induction of CD40 was observed in cells infected with the type II strain (Fig. 3.1B). Moreover, the expression of CD40 was specifically up-regulated in the infected, GFP^+ population. We also investigated the kinetics of CD40 induction following infection with the type II strain of *T. gondii*. CD40 cell surface protein levels were increased by 12 hpi and peaked at 18 hpi in *T. gondii*-infected BMdM compared to mock-infected cells (Fig. 3.1C). These data indicate that *T. gondii* induced CD40 expression on infected macrophages and that high CD40 induction occurred in a strain-specific manner.

The observation that high CD40 induction was strain-specific implicated a genetic basis for the phenotype. In this case, the phenotype should segregate among


A
mRNA (CD40/GAPDH)
4
3
2
1
0
**
**
*
**
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Type I
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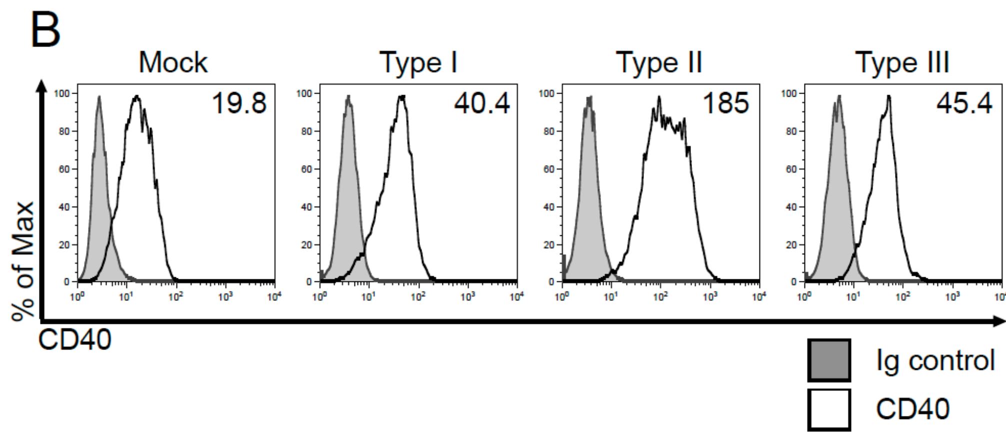
84.1
88
98.1
SSC
GFP


B
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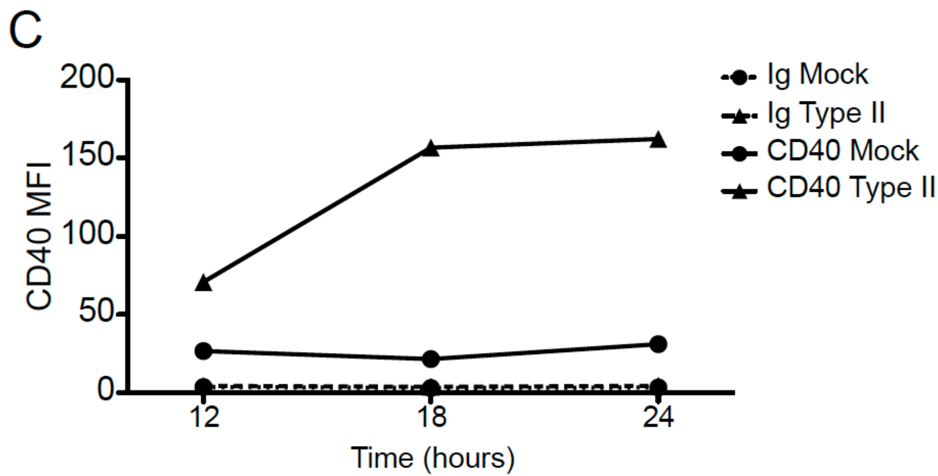

C
CD40 MFI
200
150
100
50
0
12
18
24
Time (hours)
Ig Mock
Ig Type II
CD40 Mock
CD40 Type II

Figure 3.1. Expression of CD40 during *T. gondii* infection

BMdM were mock-infected or infected with type I, type II, or type III *T. gondii* tachyzoites (A). RNA was harvested at 6 hpi for cDNA synthesis and analysis by Q-PCR for CD40 transcript levels. The expression of CD40 relative to GAPDH is shown for each condition. Error bars represent the standard deviation of technical triplicates. For each dot plot, the values within the gate indicate the percentage of GFP^+, infected cells. (B) BMdM were mock-infected or infected as in (A), harvested at 18 hpi and stained with a control-Ig (gray), or monoclonal antibodies (mAb) against CD40 (white). In each histogram plot, the numbers in the upper right corner represent the MFI of CD40 expression. (C) BMdM were mock-infected or infected with type II *T. gondii*, harvested at 12, 18 or 24 hpi, stained with control-Ig or anti-CD40 mAbs, and the mean fluorescence intensity (MFI) values for each sample were plotted. *, $P< 0.05$: **, $P< 0.01$ (Student's *t*-test). For (A), (B), and (C), one representative set from at least three independent experiments is shown.

recombinant progeny generated by a cross between the type II and III parental strains. Indeed, by comparing CD40 levels on macrophages infected with type II x III recombinant progeny lines, we found that some strains induced high CD40 levels, whereas other strains induced CD40 at a low level (Table 3.1). We analyzed CD40 levels in BMdM infected with 19 F1 recombinant progeny and used quantitative trait locus (QTL) mapping to identify the genetic locus responsible for this effect. QTL mapping identified a peak on the right arm of chromosome X (logarithm of odds score=2.87; P=0.145; Fig. 3.2A). Although the peak was not statistically significant, we chose to investigate this region of the *T. gondii* genome further, since it showed the strongest association with the high CD40 phenotype. To do this, we chose two strains from the original 19 F1 recombinant strains, as well as two additional strains (S26 and S28, (16)) that had recombinations in the right arm of chromosome X for more detailed analysis. Using the annotated genotype maps for these four strains (16), we found that strains with the type II genotype between marker AK157 and the end of chromosome X induced high CD40 expression, whereas those with the type III genotype had low CD40 induction (Fig. 3.2B). Specifically, CL13 and S30 did not induce high levels of CD40 (Fig. 3.2B) and strain S28 also only weakly up-regulated CD40 expression; however, strain S26 induced the high CD40 phenotype (Fig. 3.2B). The responsible 0.46 megabase region of chromosome X contains 91 genes, 31 of which encode putative secretory proteins, including GRA15 (58).

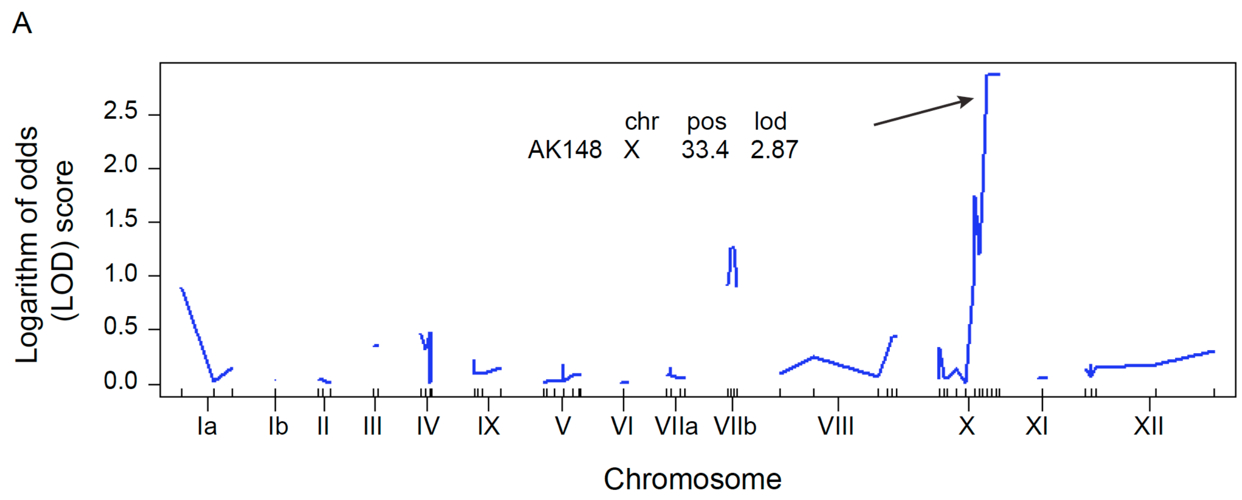
$GRA15_{II}$ induces CD40 on infected macrophages

Recent work demonstrated that the protein encoded by the type II allele of *GRA15* ($GRA15_{II}$) mediates sustained NF-κB nuclear translocation in *T. gondii*-infected

Strain	CD40 MFI	Level of induction
Mock-infected	15.3	n/a
S23	27.3	low
CL12	28.8	low
E7	33.8	low
STC7	34.2	low
S30	35.7	low
STH1	35.8	low
STH10	36.5	low
CL16	36.5	low
CL13	37.8	low
CL18	38.8	low
C96AS	41.8	low
S2T	41.9	low
S22+LC37	53.7	medium
C96C12	57.9	medium
STF3	58.5	medium
B4	78	medium
CL29	103	high
STE10	108	high
STG4	192	high

Table 3.1. Recombinant type II x III progeny induction of CD40

BMdM were mock-infected or infected with each of the progeny strains listed, stained with anti-CD40 mAbs, and examined by flow cytometry. The mean fluorescence intensity (MFI) values for CD40 on mock and infected samples are listed and were used to identify strains that highly induced CD40 expression on the cell surface.



B

Genetic Marker/Genotype

F1 Clone	AK34	AK153	AK32	AK132	L366	MIC2AP	AK154	AK157	AK148	GRA6	RC4	CD40 phenotype*
CL13	II	II	II	II	II	III	III	III	III	III	III	0.9
S26	III	III	III	III	III	III	III	III	II	II	II	3.9
S28	II	II	II	II	II	II	II	III	III	III	III	1.7
S30	II	II	II	II	II	II	III	III	III	III	III	1.9

*Fold versus mock - infected

Figure 3.2. QTL mapping of the CD40 induction phenotype

QTL scan using raw data from BMdM infected with 19 F1 progeny from a II x III cross **(**A). A non-significant logarithm of odds (LOD) peak was identified near marker AK148 (*P*=0.145). The LOD score cutoff for genome-wide significance (*P*≤0.05) was 4.50. (B) Genotypes and phenotypes of four F1 progeny used to further map the locus responsible for the CD40 induction phenotype.

cells (58) and induces the production of IL-12 in mouse macrophages (58) and IL-1β in human monocytes (148). NF-κB signaling has been found to play a role in LPS-induced CD40 expression in macrophages. To test if GRA15 mediates CD40 induction in macrophages infected with the type II strain, we infected BMdM with type I, type II, or type I parasites expressing the type II allele of *GRA15* (called type I $GRA15_{II}$). As expected, type I parasites did not induce robust CD40 cell-surface expression on infected BMdM. Expression of CD40 was increased on cells infected with type I $GRA15_{II}$ and type II parasites (Fig. 3.3A). To evaluate if human macrophage-like cells up-regulate CD40 following infection with type I, type II or type I $GRA15_{II}$ parasites, THP-1 cells differentiated with phorbol myristate acetate (PMA) were infected with each of these three parasite strains. Similar to murine macrophages, human THP-1-differentiated macrophages were found to increase CD40 expression after infection with either type II or type I $GRA15_{II}$ parasites but not with type I parasites (Fig. 3.3B). These data indicate that $GRA15_{II}$ mediates the induction of CD40 in both human THP-1 cells and mouse-infected macrophages.

$GRA15_{II}$ is sufficient for T. gondii-induced CD40 expression

To evaluate if $GRA15_{II}$ alone is sufficient to induce CD40 expression in the absence of parasite infection, we generated a retroviral vector containing the cDNA of the *$GRA15_{II}$* allele fused to the sequence for the HA epitope tag. THP-1 cells were stably transduced with this vector ($GRA15_{II}$-HA-pMX-puro) or the parental pMX-puro vector (pMX-puro) as a control. We confirmed the expression of the HA-tagged $GRA15_{II}$ by Western blot (Fig. 3.4A). Expression of $GRA15_{II}$-HA in THP-1 cells was sufficient to induce the expression of CD40, even in the absence of parasite infection (Fig. 3.4B).

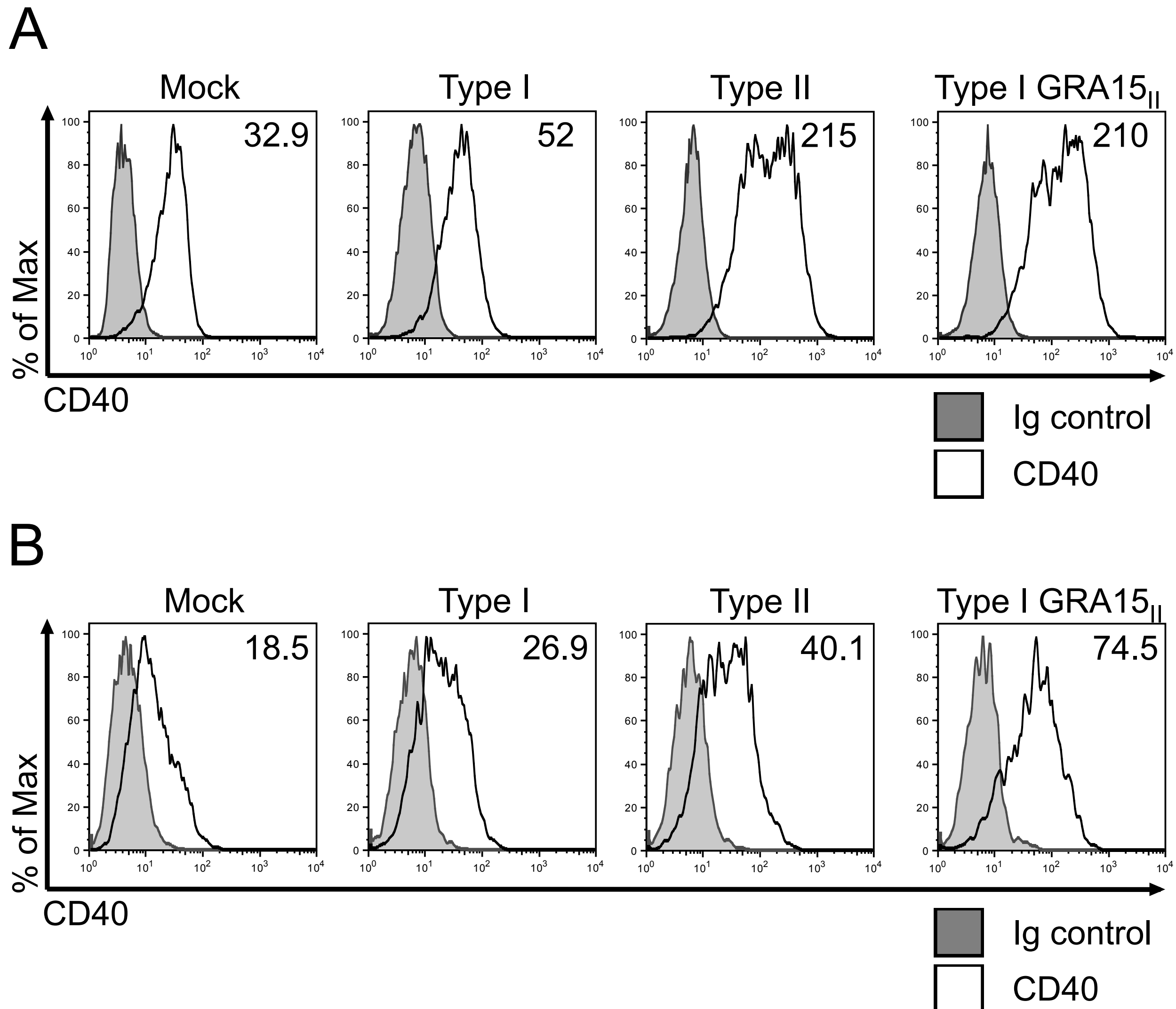


Figure 3.3. Role of GRA15 in CD40 induction during *T. gondii* infection

(A) BMdM or (B) THP-1 cells differentiated with PMA were mock-infected or infected with type I, type II, or type I parasites expressing type II GRA15 (type I $GRA15_{II}$). Cells were harvested at 18 hpi and stained with control-Ig (gray), or anti-CD40 (white) mAbs. In each histogram plot, the numbers in the upper right corner represent the MFI of CD40 expression. One representative set from at least 3 independent experiments is shown for (A) and (B).

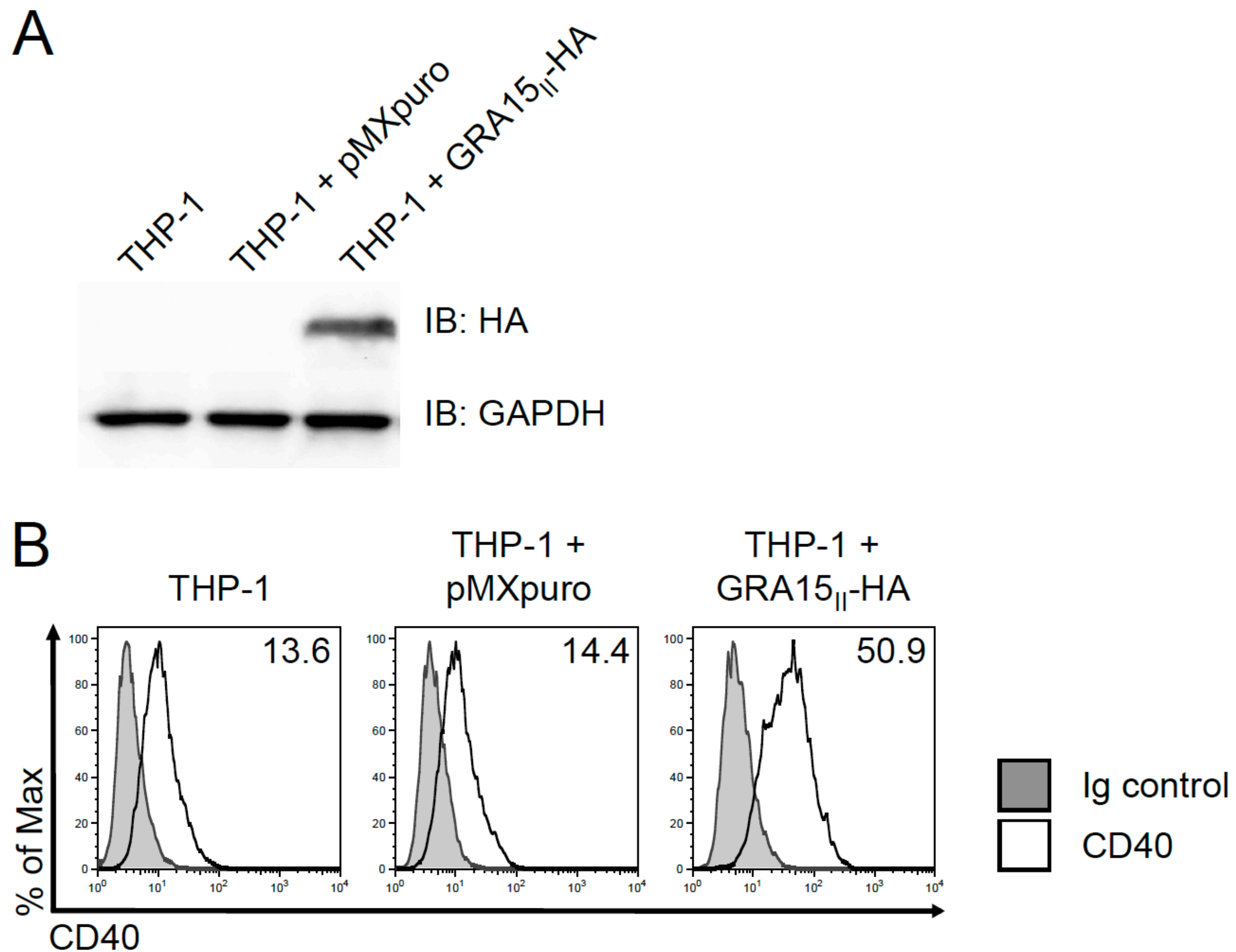


Figure 3.4. Characterizing $GRA15_{II}$-expressing THP-1 cells

A retroviral vector containing the cDNA of the type II GRA15 ($GRA15_{II}$) allele fused to the sequence for the HA epitope tag was generated ($GRA15_{II}$-HA). THP-1 cells were stably transduced with this vector ($GRA15_{II}$-HA) or the parental pMX-puro vector (pMXpuro) as a control. (A) Lysates were generated from stably transduced cells and the expression of the $GRA15_{II}$-HA was examined by immunoblotting with an anti-HA mAb or anti-GAPDH as a loading control. (B) Stably transduced cells were collected and stained with control-Ig (gray) or anti-CD40 (white) mAbs. In each histogram plot, the numbers in the upper right corner represent the MFI of CD40 expression. One representative set from four independent experiments is shown from two independent transductions for (A) and (B).

CD40 expression on infected macrophages increases IL-12 production following CD40L stimulation

In addition to their role as professional APC, macrophages are capable of eliciting cell-intrinsic effector functions through CD40 signaling. For example, CD40 and CD40L engagement has been shown to induce the transcription and secretion of IL-12p40 (149). Since CD40 levels are up-regulated on macrophages infected with type II parasites, we hypothesized that type II-infected cells may have enhanced CD40-mediated effector functions. To investigate this possibility, we examined the production of IL-12 by *T. gondii*-infected cells. We measured IL-12p40 levels in BMdM that were infected with the type I, type II, or type I $GRA15_{II}$ parasites and subsequently cultured in the presence or absence of soluble CD40L. The uninfected (GFP^-) cell population produced very little IL-12p40 when stimulated with CD40L to engage the CD40 receptor (Fig. 3.5A). As expected based on previous reports looking at IL-12 production (58), expression of the $GRA15_{II}$ allele was sufficient to induce the production of IL-12p40 in the GFP^+ cell population infected with either the type II or type I $GRA15_{II}$ parasites (Fig. 3.5A). We then evaluated the ability of infected BMdM to produce following CD40/CD40L engagement. The percent of cells producing IL-12 increased after CD40 ligation in BMdM infected with either the type II or type I $GRA15_{II}$ parasites, compared to macrophages that were not treated with soluble CD40L (Fig. 3.5A). Notably, IL-12 production following CD40L stimulation was primarily observed in the infected, GFP+ cell population. We then measured the production of the biologically active IL-12p70 heterodimer by BMdM that were mock-infected or infected with the type I, type II or, type I $GRA15_{II}$ parasites and cultured either with 293T cells or with 293T cells

expressing murine CD40L. The production of IL-12p70 by mock-infected and type I-infected BMdM was below the level of detection, regardless of whether the cells were cultured with CD40L-expressing 293T cells (Fig. 3.5B). Consistent with the IL-12p40 results, infection of BMdM with the type II or type I $GRA15_{II}$ parasites resulted in the production and secretion of IL-12p70 when cultured alone or with 293T cells. However, culturing BMdM infected with the type II or type I $GRA15_{II}$ parasites with CD40L-expressing 293T cells resulted in a significant increase in the production of IL-12p70. These data suggest that CD40 expression on macrophages infected with type II *T. gondii* contributed to an elevated production of IL-12 in response to CD40 and CD40L engagement.

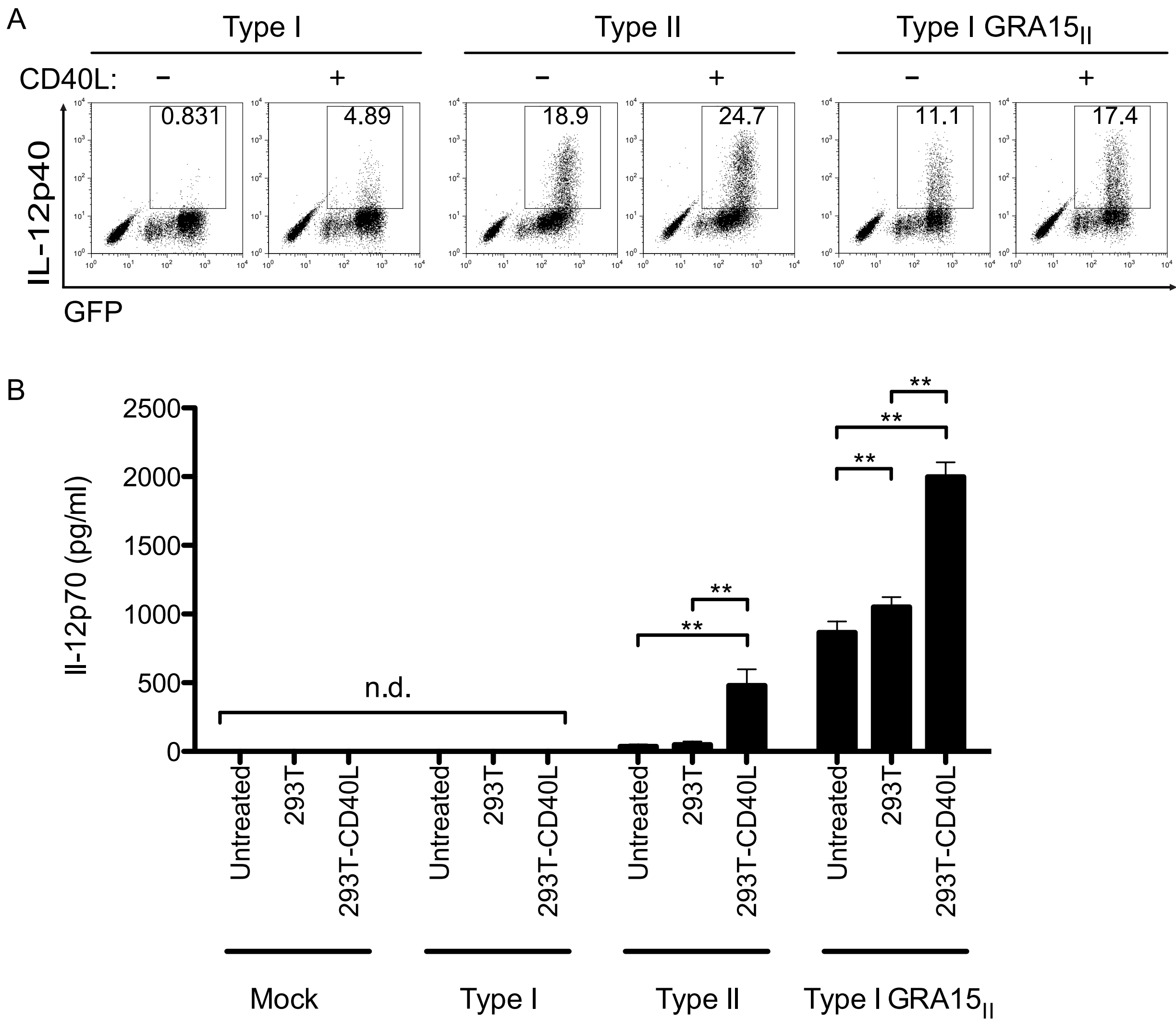


Figure 3.5. CD40 stimulation of *T. gondii*-infected macrophages

BMdM were mock-infected or infected with type I, II, or type I parasites expressing type II GRA15 (type I $GRA15_{II}$). (A) At 18 hpi, the cells were stimulated with media containing 10 µl/ml of CD40L. At 4 hrs post-CD40L stimulation, the cells were treated with Brefeldin A and then incubated for an additional 4 hrs. At 8 hrs post-CD40L stimulation, the cells were collected, permeabilized, and stained with mAb against intracellular IL-12p40. IL-12p40 levels in the uninfected (GFP^-) and infected (GFP^+) populations were analyzed by flow cytometry. The values in the gates represent the percent of IL-12-expressing GFP^+ cells in the population. One representative set of samples from at least three independent experiments is shown. (B) At 18 hpi, 293T or CD40L-expressing 293T cells were added to BMdM at a ratio of 2:1 (BMdM:293T). At 26 hpi, IL-12p70 released into the culture media was analyzed by ELISA. The error bars represent the standard deviation of biological triplicates. n.d., not detected. *, $P < 0.05$ (Student’s *t*-test).

Discussion

The establishment of host defense against infection with *T. gondii* is initiated by cells of the innate immune response, which help to shape the course of the infection. Effective parasite control requires a robust immune response without the induction of immunopathology. This is achieved through coordinated communication between innate immune cells and the regulation of signaling cascades triggered by cell surface immune receptors.

The modulation of immune receptors on macrophages during the acute stage of infection can have profound implications for the development of the ensuing immune response. This led us to investigate the mechanisms behind the observed induction of CD40 on infected macrophages following infection with *T. gondii*. It has previously been shown that CD40 is up-regulated on mouse macrophages (136) and human monocytes (150) after *T. gondii* infection. We found that there is a parasite strain specificity to this induction – the type II strain of *T. gondii* induced high expression of CD40, whereas the types I and III strains induced only low levels of CD40. The protein encoded by the *$GRA15_{II}$* allele drove CD40 expression. Furthermore, we showed that the induction of CD40 in cells infected with parasites expressing $GRA15_{II}$ contributed to the establishment of a Th1-conducive microenvironment through the production of IL-12p40 as well as IL-12p70 after stimulation with CD40L. By investigating the mechanism of CD40 induction by the type II strain of *T. gondii*, we have identified the parasite factor responsible for inducing CD40 and established a role for infected cells in the establishment of protective immunity during parasite infection.

CD40 and CD40L engagement contribute to enhanced parasite clearance through the activation of macrophages and other APC (151). CD40/CD40L interaction leads to the induction of an antimicrobial program in macrophages (93, 152) and is also critical for controlling *T. gondii* infection in mice (136). This results in a reduction in *T. gondii* burden in infected cells (90, 153). Furthermore, CD40 engagement leads to the production of IL-12, TNF-α, and NO which have been shown to play a role in host defense (139, 140, 154, 155). These cytokines and effectors produced in response to CD40 engagement are important for establishing an immune response capable of controlling intracellular pathogens through Th1- mediated immunity (134, 135).

Macrophages infected with type II or type I $GRA15_{II}$ parasites produced higher levels of IL-12 when stimulated by CD40L than unstimulated macrophages. Notably, the increase in IL-12p70 production from macrophages stimulated with CD40L (12.8- and 2.3-fold increase in type II- and type I $GRA15_{II}$-infected BMdM, respectively) was significantly greater than the observed increase in intracellular IL-12p40 signal (1.3- and 1.3-fold increase in type II- and type I $GRA15_{II}$-infected BMdM, respectively). One explanation for the difference in the magnitude of these responses is the different techniques used to evaluate IL-12 production. Whereas the intracellular cytokine assay detects IL-12p40 produced during a 4 hr period of Brefeldin A treatment, the ELISA detects accumulated IL-12p70 secreted into the supernatant over the entire 26 hr culture period. Despite these differences, the trend in IL-12 production was similar using these two assays; in that CD40L stimulation of macrophages infected with type II or type I $GRA15_{II}$ parasites induced elevated levels of IL-12.

CD40 signal strength can have an important effect on p38 and ERK MAPK signaling and IL-12 production (140, 156, 157), as well as T cell skewing through reciprocal regulation of IL-12 and IL-4 (144, 158). We demonstrate that macrophages infected with CD40-inducing *T. gondii* parasite strains, dramatically increase IL-12 production following CD40-CD40L stimulation: type II-infected macrophages cultured with CD40L-expressing cells exhibited an over 12-fold increase in IL-12p70 production compared to type II-infected cells that were cultured with the control cells. The enhanced CD40 expression may amplify the strength of the signal mediated by CD40L stimulation. Consistent with our findings, it is known that strong CD40 engagement, achieved by increasing the concentration of recombinant soluble CD40L resulted in robust Th1 skewing conditions due to preferential p38 signaling and subsequently more IL-12p40 and p70 production. In contrast, weak CD40 engagement of T cells was found to enhance Th2 immunity due to elevated ERK activation and subsequently enhanced IL-10 expression (157). Our observation that type II *T. gondii*-infected macrophages produce higher levels of IL-12p40 following CD40 engagement suggests that these infected macrophages contribute to the promotion of Th1 responses. *T. gondii* proteins secreted into infected host cells have been shown to alter host cell signaling and gene transcription (47, 50, 51) as well as antimicrobial processes (48, 49, 54). Interestingly, however, there are parasite strain-specific effects on the modulation of host-cell signaling pathways, including JAK/STAT, NF-κB, and MAPK pathways. A growing body of work has demonstrated that differences in the modulation of these host-cell signaling pathways is a result of strain-specific polymorphisms in the secreted factors released into host cells during infection (21, 45, 47, 52). Consequently, polymorphisms in

secreted parasite proteins can differentially influence macrophage polarization and effector functions: type I parasites induce an alternative activation state, whereas type II strains induce a classical activation state (159). Type I *T. gondii* actively dampen the responsiveness of infected host cells by directly activating STAT3 signaling, rendering cells refractory to IFN-γ stimulation (54). Lang *et al*. demonstrated that the suppressed responsiveness to IFN-γ is the result of chromatin remodeling following infection with the type I RH strain of *T. gondii* (51). Our data showing CD40 up-regulation by the type II strain of *T. gondii* is consistent with this paradigm and primes macrophages for a pro-inflammatory program following receptor engagement (158, 159).

The pathways that mediate CD40 induction after *T. gondii* infection were unknown. The fact that CD40 was induced in a strain-specific manner enabled us to use QTL to identify a genomic region that associates with CD40 induction. QTL analysis indicated that the genomic locus responsible for CD40 induction contained the recently described gene, *GRA15* (TGME49_275470) that encodes the dense granule protein GRA15. GRA15 was the first *T. gondii* dense granule protein shown to modulate host cell NF-κB signaling due to sustained nuclear translocation of p65 (58). The $GRA15_{II}$ allele encodes a protein consisting of 635 amino acids, while the type I allele of *GRA15* contains a frame-shift mutation that leads to a premature stop codon and a final protein product of 312 amino acids. Additionally, the type I and type III alleles of *GRA15* contain an insertion/deletion mutation near the C-terminus of the protein, leading to an early termination signal, along with 5 other amino acids that are polymorphic (58). We demonstrate that the $GRA15_{II}$ is sufficient to induce CD40 expression even in the absence of parasite infection.

Surprisingly, we observed that not all type II strains induce CD40 expression. Whereas infections with independently obtained ME49 strains highly up-regulated CD40, the *Prugniaud* strain (160) did not. It has been previously reported that there are phenotypic differences between different type I lines (161) and between different type II lines (162). Yang *et al.* demonstrated that phenotypic differences in NF-κB activation and IL-12 production between type I parasites was partially explained by polymorphisms in GRA15 (161). However, GRA15 from the *Prugniaud* strain can induce other NF-κB-dependent pathways, including the production of IL-12 (58) and IL-1β (148). One possible explanation for the observed differences in CD40 induction between type II parasite isolates is that CD40 expression is regulated by additional parasite factors that differ in function or expression level between the type II strains we have evaluated. CD40 expression induced by the various recombinant progeny was not strictly binary, suggesting that while CD40 induction is primarily dependent on GRA15, it may be influenced by additional parasite factors. Indeed, QTL analysis revealed the presence of a second peak on chromosome VIIb (Fig. 3.2A). There is precedent for dual regulation of host pathways among secreted proteins, such as ROP5 and ROP18. Strain differences in the parasites' susceptibility to killing by IFN-γ-stimulated mouse embryonic fibroblasts was dependent on the expression of both virulent polymorphic genes encoding ROP5 (type I and III) and ROP18 (type I and II) (47). In addition, the protein encoded by the virulent allele of *ROP16* in type I parasites has been shown to antagonize the activity of GRA15 (58). Further work characterizing additional parasite factors that enhance or inhibit GRA15 activity may help to explain the differences in CD40 induction observed in distinct type II isolates.

Here we report a novel function for GRA15 in CD40 induction and demonstrate that CD40 signaling amplifies the IL-12 response in type II-infected macrophages and may contribute to the Th1 environment established during infection with type II strains of *T. gondii.*

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Chapter Four

Defining the cell surface proteome of *Toxoplasma gondii*-infected macrophages

Introduction

Infection of host cells by *T. gondii* is known to alter the transcriptional program (96-100) and the proteome of infected cells (101, 102). In Chapter 2 we demonstrated that the up-regulated B7-2 on infected APC is functionally active and co-stimulates T cell proliferation. Additionally, in Chapter 3 we showed that the expression of the co-stimulatory molecule CD40, induced by *T. gondii* in a strain-specific manner, contributes to IL-12 production by infected cells following engagement with its ligand, CD40L. These data suggest that changes in the expression of proteins on the surface of infected cells can have a significant impact on intercellular communication and play a role in mediating *T. gondii* immunity. To date, it has not been reported how the parasite globally alters the expression of cell surface proteins with roles in eliciting and regulating immune responses. We hypothesize that the cell surface protein profile will dramatically differ between infected and uninfected cells. Identifying proteins with altered expression during infection is a first step towards investigating the mechanisms by which proteins of immunological interest are regulated during *T. gondii* infection. These findings would contribute to a better understanding of the interactions between *T. gondii* and its host.

In addition, this approach also has the potential to help us identify novel surface markers specific to infected cells that can serve as targets for antibody-dependent therapies, similar to strategies currently being used to target and eliminate various cancers. To more comprehensively determine how *T. gondii* infection alters the surface expression of receptors and ligands on macrophages and to identify potentially novel markers of infected cells, we performed a proteomic analysis of the surface of infected and uninfected cells.

Results

To investigate how *T. gondii* infection alters the macrophage surface proteome, we began by isolating the surface proteins from uninfected and type I *T. gondii*-infected BMdM using a protocol adapted from de Miguel *et al.* 2010. The adapted protocol is depicted in Fig. 4.1. BMdM were infected with the type I *T. gondii* for 18 hrs and uninfected BMdM were cultured in parallel to generate a control sample. To isolate cell surface proteins on infected and uninfected BMdM we labeled extracellular, membrane bound proteins by incubating the cells with the labeling reagent sulfo-NHS-SS-biotin. Two useful features of sulfo-NHS-SS-biotin are that it is cell impermeable, minimizing the labeling of intracellular proteins, and that the disulfide bridge allows for the cleavage and elution of biotin-labeled proteins from a streptavidin resin with the use of a reducing agent. After incubation of the cells with the labeling reagent, the cells were lysed, and the cell lysates were incubated with streptavidin-coated resin to pull down biotin-labeled surface proteins.

To confirm that protein labeling protocol enriched for surface proteins, we performed an immunoblot for an intracellular protein, ERK. As expected, ERK was present in the total cell lysate and was diminished in the fraction enriched for the surface proteins (Fig. 4.2A). Additionally, we observed enrichment of a cell surface protein, B7-2, in the surface fraction samples (Fig. 4.2B), further confirming that our surface biotinylation protocol resulted in the selective enrichment of cell surface proteins. The isolated surface proteins were eluted from the biotin resin by adding β-mercaptoethanol, a reducing agent that cleaves the disulfide bridge. Following elution, the samples were separated by SDS-PAGE and visualized by silver stain (Fig. 4.2C).

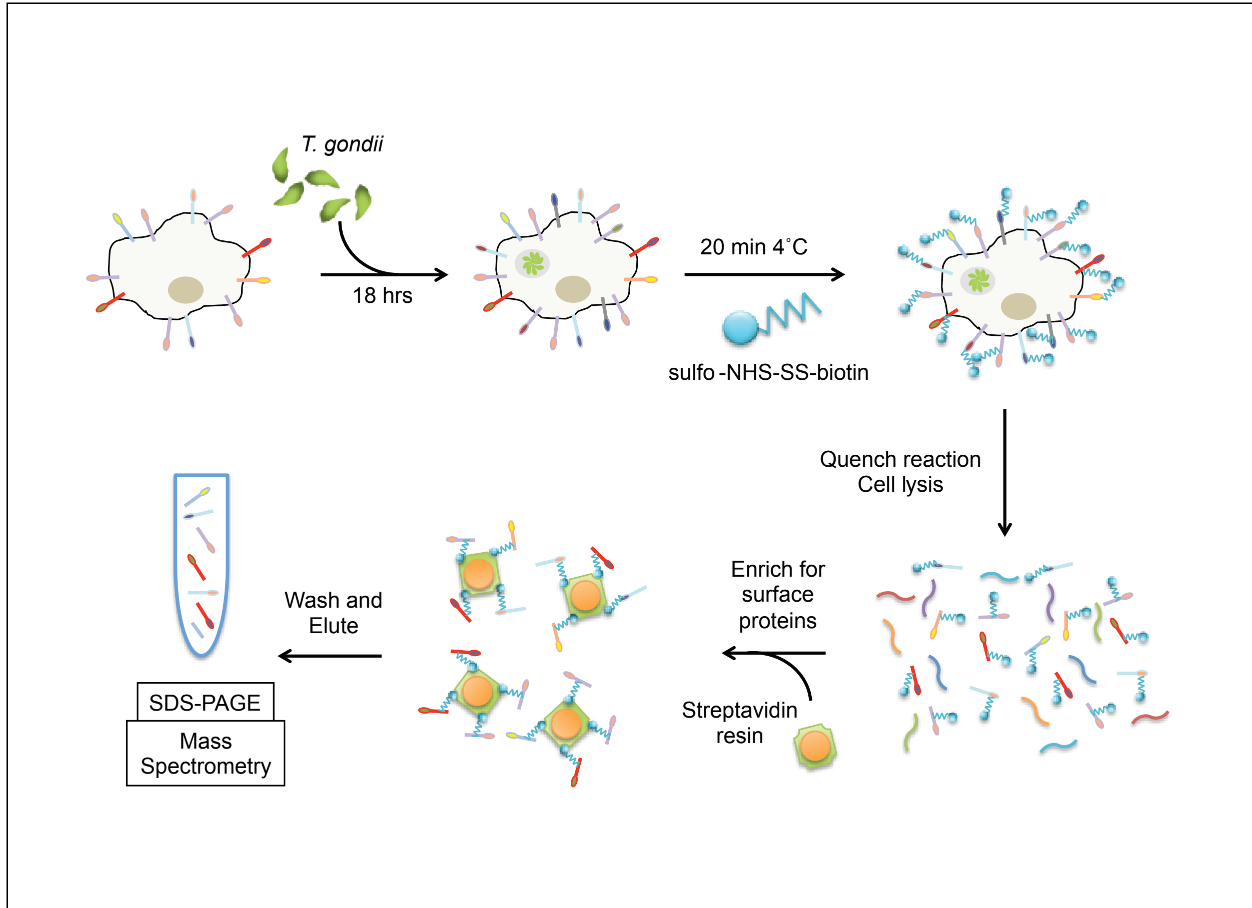

Figure 4.1. Surface protein isolation schematic

BMdM were incubated with sulfo-NHS-SS-biotin to allow the reactive NHS group to covalently link with primary amines on proteins. The reaction was quenched by adding 100 molar excess of tris-HCl and several washes with PBS were done to remove excess biotin reagent. The cells were lysed in a lysis buffer containing a cocktail of protease inhibitors to maintain protein integrity. The total cell lysate was incubated with streptavidin-coated resin. Following incubation, the resin, which contains bound surface proteins, was washed to remove un-specifically bound proteins. SDS loading buffer containing dithiothreitol (DTT) was then used to elute proteins bound to the resin. Eluted proteins were then run on polyacrylamide gels, and visualized by coomassie blue staining before being sent to the UCI Mass Spectrometry facility on campus for analysis.

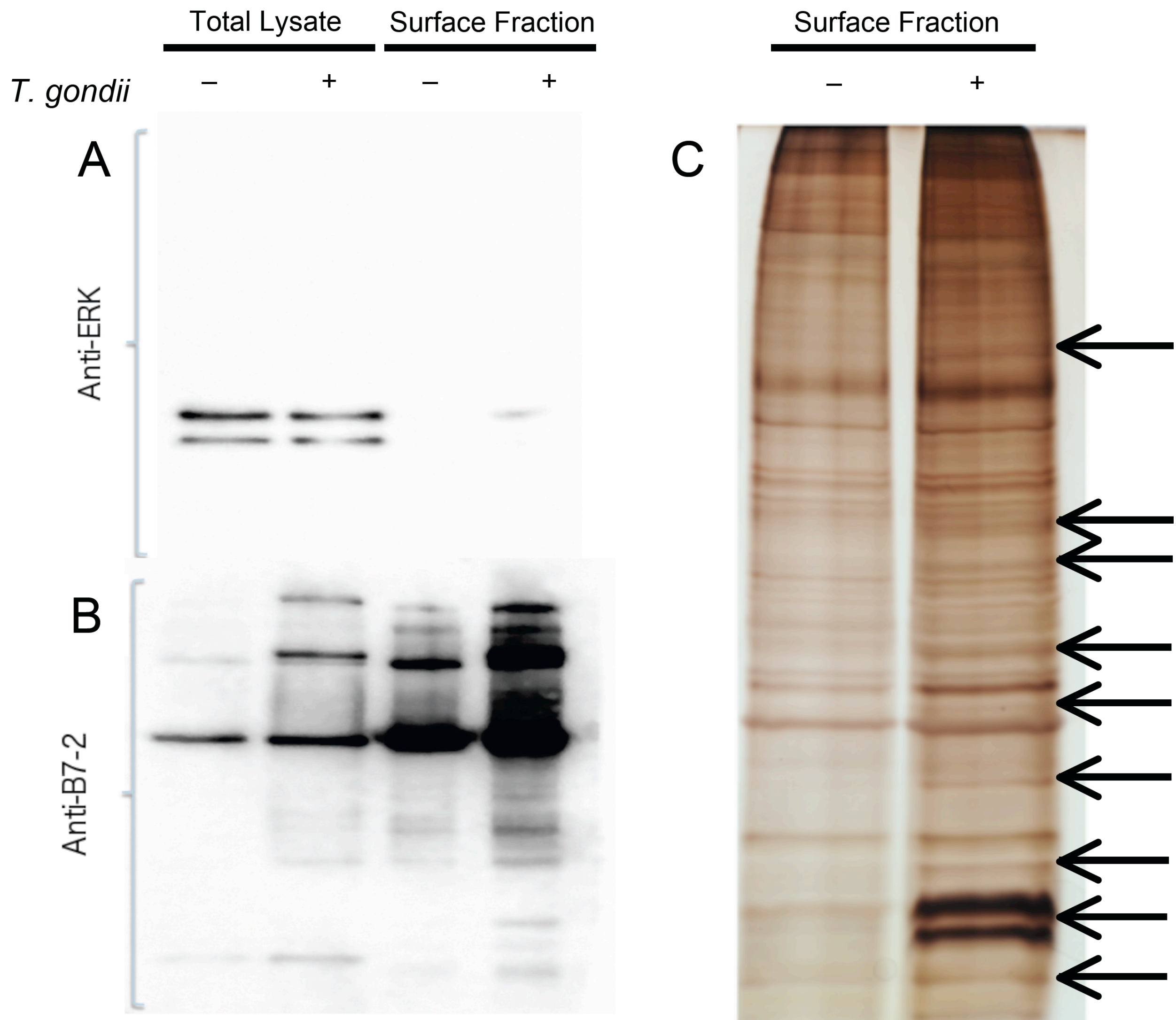

Figure 4.2. Validation of cell surface protein isolation protocol and visualization of cell surface proteins

To evaluate the level of intracellular labeling, equal cell equivalent aliquots of total lysate and the surface protein fraction were separated by SDS-PAGE and immunoblotted for ERK (A). Additionally, samples were immunoblotted for B7-2 to assess the enrichment of surface proteins (B). Surface fraction from both infected and uninfected cells was separated by SDS-PAGE and visualized by silver stain (C). Of particular interest are proteins bands that are unique to only one sample (←).

The stained gels were sent to the UCI Mass Spectrometry Facility for processing, and following an in-gel trypsin digest, the digested proteins were eluted and lyophilized. The proteins were then reconstituted in acetonitrile water and analyzed by LC-MS/MS using a Quadropole Waters Quattro Premier XE UPLC triple quadropole instrument.

The mass spectrometry hits were analyzed using the Universal Protein Resource database, which contains protein sequences and functional information for known proteins. Among the mouse proteins we identified, 35 out of 55 were known or predicted cell surface proteins, indicating that the surface biotinylation approach utilized allowed for successful enrichment and identification of cell surface proteins. The identified proteins were grouped based on whether they were found exclusively in the uninfected or the infected cell samples (Table 4.1 and 4.2, respectively), or were shared between both samples (Table 4.3, Fig. 4.3). The surface proteins identified by LC-MS/MS and found to be unique to one sample were prioritized for subsequent analysis. Although this initial dataset is not comprehensive (and not quantitative), the characterization of the surface of infected versus uninfected macrophages has never been reported.
Of particular interest were those proteins that were unique to either the infected or uninfected sample. An analysis of the proteins identified by mass spectrometry indicated that the expression of Fc-gamma receptors (FcγR) was differentially affected by type I *T. gondii* infection. FcγRI (CD64), a high affinity FcγR, was identified in only the uninfected sample, whereas FcγRII (CD32) and FcγRIII (CD16), both low affinity FcγR, were detected only in the infected sample. When we cross-referenced the proteomics dataset with our microarray dataset generated from type I *T. gondii*-infected BMdM, we found that FcγRI was significantly reduced at the RNA level in infected cells,

Table 4.1. List of protein hits unique to uninfected BMdM

Accession	Description	Peptides	Theoretical Peptides	Coverage (%)
AHSA1_MOUSE	Activator of 90 kDa heat shock protein ATPase homolog 1	3	29	11.2426
ATPB_MOUSE	ATP synthase subunit beta mitochondrial	10	37	30.8129
CD166_MOUSE	CD166 antigen	10	44	18.5249
EF2_MOUSE	Elongation factor 2	15	69	19.2308
HA12_MOUSE	H 2 class I histocompatibility antigen D D alpha chain	4	30	19.4521
FCGR1_MOUSE	High affinity immunoglobulin gamma Fc receptor I	10	26	30.4455
IMB1_MOUSE	Importin subunit beta 1	12	62	14.6119
ITA6_MOUSE	Integrin alpha 6	10	89	14.4821
CD14_MOUSE	Monocyte differentiation antigen CD14	8	21	34.4262
CD14_MOUSE	Monocyte differentiation antigen CD14	3	21	11.4754
KPYM_MOUSE	Pyruvate kinase isozymes M1 M2	15	45	34.275
IQGA1_MOUSE	Ras GTPase activating like protein IQGAP1	13	118	11.7079
RTN4_MOUSE	Reticulon 4	8	89	17.1256
SFXN3_MOUSE	Sideroflexin 3	6	24	21.4953
SLAF5_MOUSE	SLAM family member 5	8	31	17.6292
SQRD_MOUSE	Sulfide quinone oxidoreductase mitochondria	3	45	11.5556
SDCB1_MOUSE	Syntenin 1	2	20	26.7559
LY9_MOUSE	T lymphocyte surface antigen Ly 9	7	52	13.9144
ECHA_MOUSE	Trifunctional enzyme subunit alpha mitochondrial	12	50	23.329

Table 4.2. List of protein hits unique to infected BMdM

Accession	Description	Peptides	Theoretical Peptides	Coverage (%)
THIKA_MOUSE	3 ketoacyl CoA thiolase A peroxisomal	5	25	20.7547
CH60_MOUSE	60 kDa heat shock protein mitochondrial	9	53	26.8761
CAP1_MOUSE	Adenylyl cyclase associated protein 1	4	41	13.7131
ANXA1_MOUSE	Annexin A1	11	31	34.6821
ARI3B_MOUSE	AT rich interactive domain containing protein 3B	11	48	15.8451
CMC2_MOUSE	Calcium binding mitochondrial carrier protein Aralar2	7	51	12.8698
RPN2_MOUSE	Dolichyl diphosphooligosaccharide protein glycosyltransferase	12	38	36.6086
ESYT1_MOUSE	Extended synaptotagmin 1	14	85	15.1099
LEG3_MOUSE	Galectin 3	4	12	19.3182
HA1B_MOUSE	H 2 class I histocompatibility antigen K B alpha chain	7	25	33.3333
FCGR2_MOUSE	Low affinity immunoglobulin gamma Fc region receptor II	5	22	16.9697
FCGR3_MOUSE	Low affinity immunoglobulin gamma Fc region receptor III	2	18	11.8774
CD68_MOUSE	Macrosialin	3	16	14.1104
PCCB_MOUSE	Propionyl CoA carboxylase beta chain mitochondrial	7	42	22.1811
PDIA3_MOUSE	Protein disulfide isomerase A3	10	55	20.198
AT1B3_MOUSE	Sodium potassium transporting ATPase subunit beta 3	9	20	44.2446
VIME_MOUSE	Vimentin	5	47	12.8755

Table 4.3. List of protein in identified by mass spectrometry shared between infected and uninfected BMdM

Accession	Description
4F2_MOUSE	4F2 cell surface antigen heavy chain
AMPN_MOUSE	Aminopeptidase N
ATPA_MOUSE	ATP synthase subunit alpha mitochondrial
CMC1_MOUSE	Calcium binding mitochondrial carrier protein Aralar1
CD180_MOUSE	CD180 antigen
CD44_MOUSE	CD44 antigen
HA11_MOUSE	H 2 class I histocompatibility antigen D B alpha chain
HA1L_MOUSE	H 2 class I histocompatibility antigen L D alpha chain
ITA5_MOUSE	Integrin alpha 5
ITAM_MOUSE	Integrin alpha M
ITB1_MOUSE	Integrin beta 1
ITB2_MOUSE	Integrin beta 2
CD47_MOUSE	Leukocyte surface antigen CD47
ADCL1_MOUSE	Neutral cholesterol ester hydrolase 1
PLSL_MOUSE	Plastin 2
CD36_MOUSE	Platelet glycoprotein 4
PTPRC_MOUSE	Receptor type tyrosine protein phosphatase C
SN_MOUSE	Sialoadhesin
AT1A1_MOUSE	Sodium potassium transporting ATPase subunit alpha 1
SHPS1_MOUSE	Tyrosine protein phosphatase non receptor type substrate 1

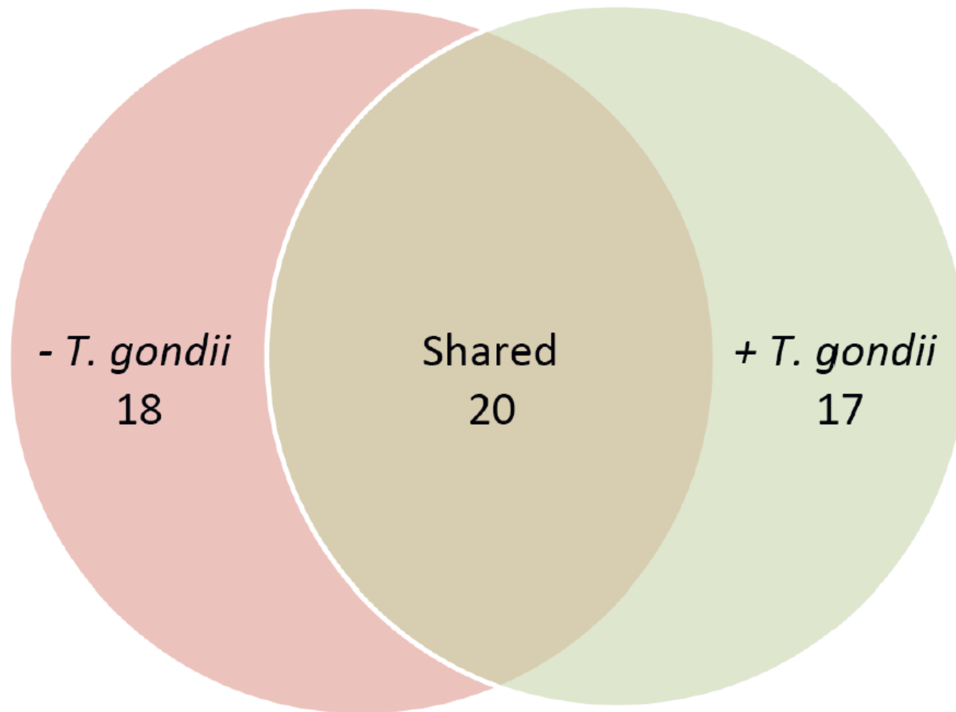

Figure 4.3. Quantification of proteins unique to one sample or shared between both samples

Of the 55 protein hits obtained, 18 were found exclusively in the uninfected sample. The infected sample contained 17 unique proteins. Additionally, 20 of the proteins identified were shared.

whereas FcγRII was among the most highly up-regulated genes in infected macrophages (96) .

FcγR are instrumental in orchestrating cellular effector functions induced by engagement with immune complexes (IC) opsonized with IgG (reviewed in (163)). Of the four known FcγR in mice, three (FcγRI, FcγRIII, FcγRIV) are activating receptors due to immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmic tail of the signal transducing γ chain. FcγRII, which has an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail, is the only inhibitory Fcγ receptor. It is also notable that the down-regulation of the high affinity FcγRI is not a general feature of macrophage activation; additional microarray data from our lab has revealed that FcγRI is up-regulated in response to IFN-γ stimulation of macrophages.

Due to the role FcγR play in mediating APC activation and immune function, we focused on these proteins as a means to validate our MS data. We began by analyzing FcγR transcript levels in infected and uninfected BMdM by Q-PCR. Analysis of transcript levels of FcγRI, FcγRII and FcγRIII in type I-infected BMdM at 6 hpi revealed a statistically significant induction in FcγRII expression compared to uninfected samples (Fig. 4.4A). Additionally, no difference in transcript levels were observed between infected and uninfected cells for FcγRI and FcγRIII. We then measured the cell surface expression of FcγRs at 6, 12, 18, and 24 hpi. Expression of FcγRI was found to decrease as early as 12 hpi and continued to gradually decrease out to 24 hpi (Fig. 4.4B). Conversely, the cell-surface expression of FcγRII/III increased following infection, reaching a peak at 18 hpi and subsequently decreased slightly by 24 hpi.

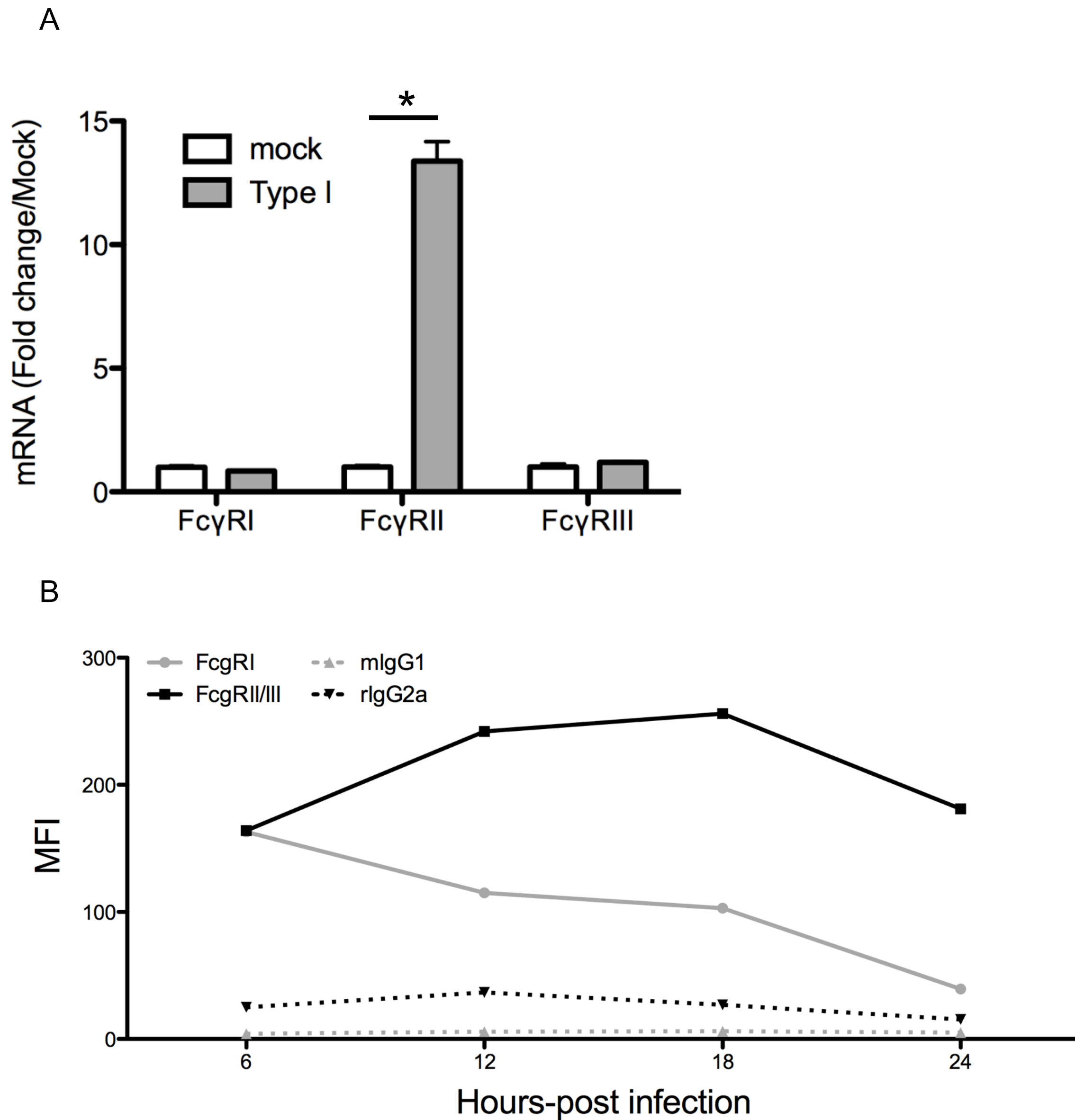

Figure 4.4. FcγR expression profile of type I *T. gondii*-infected bone marrow-derived macrophages

BMdM were mock infected or infected with type I parasites. A) At 6 hours-post infection cell lysates were generated and RNA was extracted and reverse transcribed into cDNA, which was subsequently used as the template for Q-PCR. Transcript levels of FcγRI, FcγRII and FcγRIII are shown. Values represent fold change over mock following normalization to GAPDH. Error bars represent the standard deviation of technical replicates. Data shown is representative of 2 experiments. * $p < 0.05$. B) At 6, 12, 18, and 24 hours post infection cells were analyzed for surface expression of FcγRI (isotype: mouse IgG1) and FcγRII/III (rat IgG2a) by flow cytometry. The mean fluorescence intensity (MFI) is plotted for both antibodies of interest and their respective isotype controls.

Given that *T. gondii* can alter the expression of host immune receptors in a strain-specific manner, we evaluated the induction of FcγRs on BMdM infected with type I, type II or type III parasites. We found that FcγRI is not induced by any of these three *T. gondii* strains at 18 hpi by using flow cytometric analysis of infected cells (Fig. 4.5). Interestingly, we found that similar to type I-infected cells, type III parasites dramatically increased FcγRII/III cell surface expression at 18 hpi. These data indicate that there is a parasite strain-specificity to the effects of infection on expression of the FcγR.

Having found that FcγR, which are instrumental in orchestrating cellular activation, are differentially modulated by *T. gondii* infection, we examined whether *T. gondii* may alter FcγR-mediated immune responses. FcγRI engagement has been shown to be important for establishing the activation state of APC by regulating the production of numerous cytokines, such as IL-10, IL-12, TNF-α, IL-1b, and IL-8 (164, 165) as well as MAPK signaling pathways (166-168). Given the marked decrease in the expression of the high affinity activating receptor FcγRI, and the induction of the low affinity inhibitory receptor FcγRII in infected cells, we hypothesized that, FcγR signaling may be reduced in response to FcγR engagement in type I-infected cells.

To address this possibility we examined the activation of ERK and JNK, two of the canonical MAPK pathways known to be activated downstream of FcγR engagement. To evaluate ERK and JNK activation, BMdM were infected with type I *T. gondii* and at 18 hpi stimulated with IgG2a-opsonized latex beads. At 15 minutes post-stimulation, cell lysates were generated for Western Blot analysis. Densitometry analysis of immunoblots showing phosphorylated ERK (p-ERK) revealed that mock-infected cells respond to IgG2a opsonized beads by increasing p-ERK activation (Fig.

4.6A). However, p-ERK was not detected in infected cells in response to FcγR engagement. Infected cells also displayed impaired JNK activation following stimulation with IgG2a-opsonized beads (Fig. 4.6B). These data suggest that *T. gondii* infection may reduce the FcγR-mediated activation of infected macrophages through both ERK and JNK.

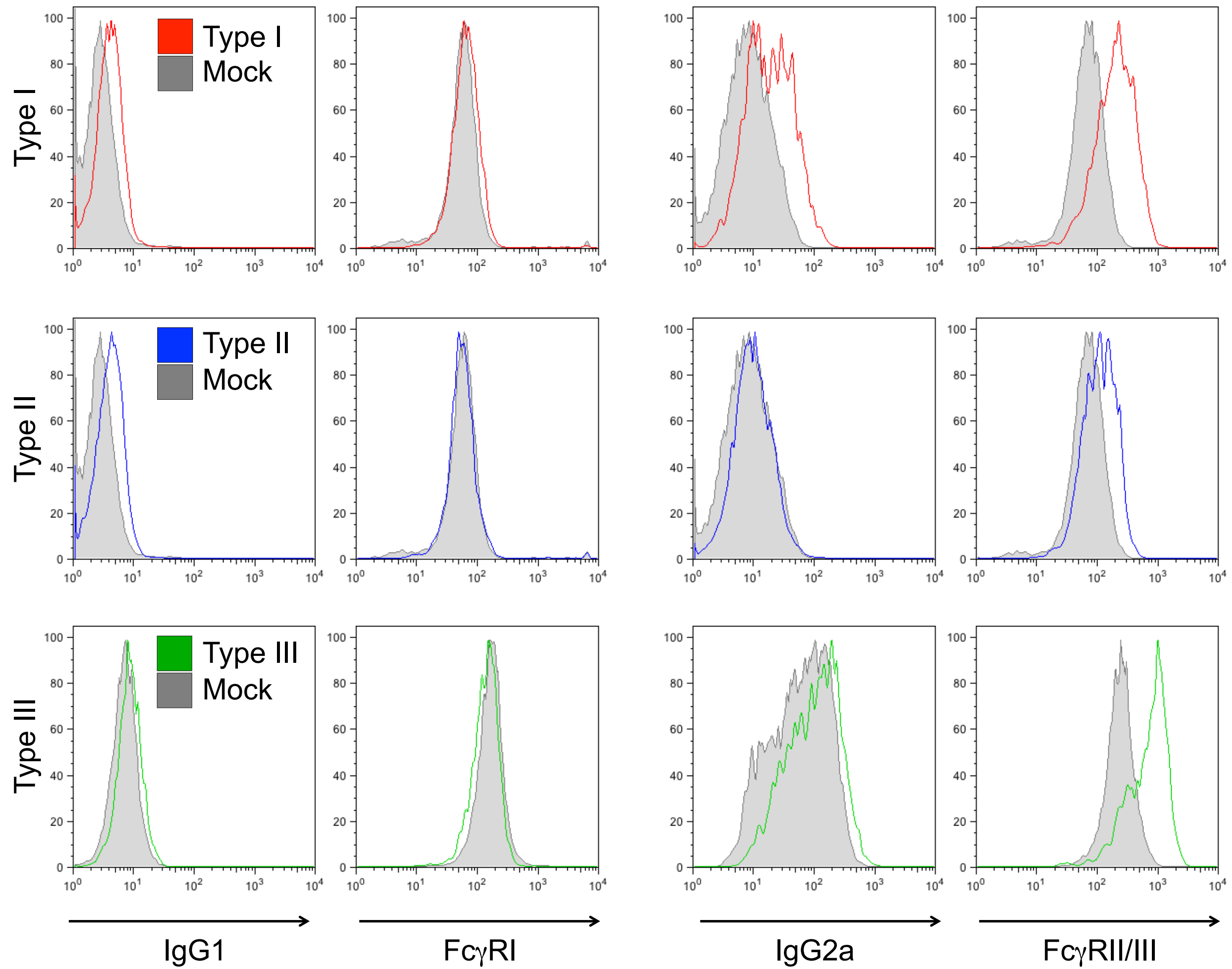

Figure 4.5. Strain-specific cell surface expression of FcγR

BMdM were mock infected (gray) or infected with type **I** (red), type **II** (blue), or type **III** (green) *T. gondii* parasites. At 18 hpi, cells were harvested and cell surface expression of FcγR were evaluated by flow cytometry using antibodies against FcγRI (isotype: mouse IgG1) and FcγRII/III (isotype: rat IgG2a). Data shown are representative of three independent experiments.

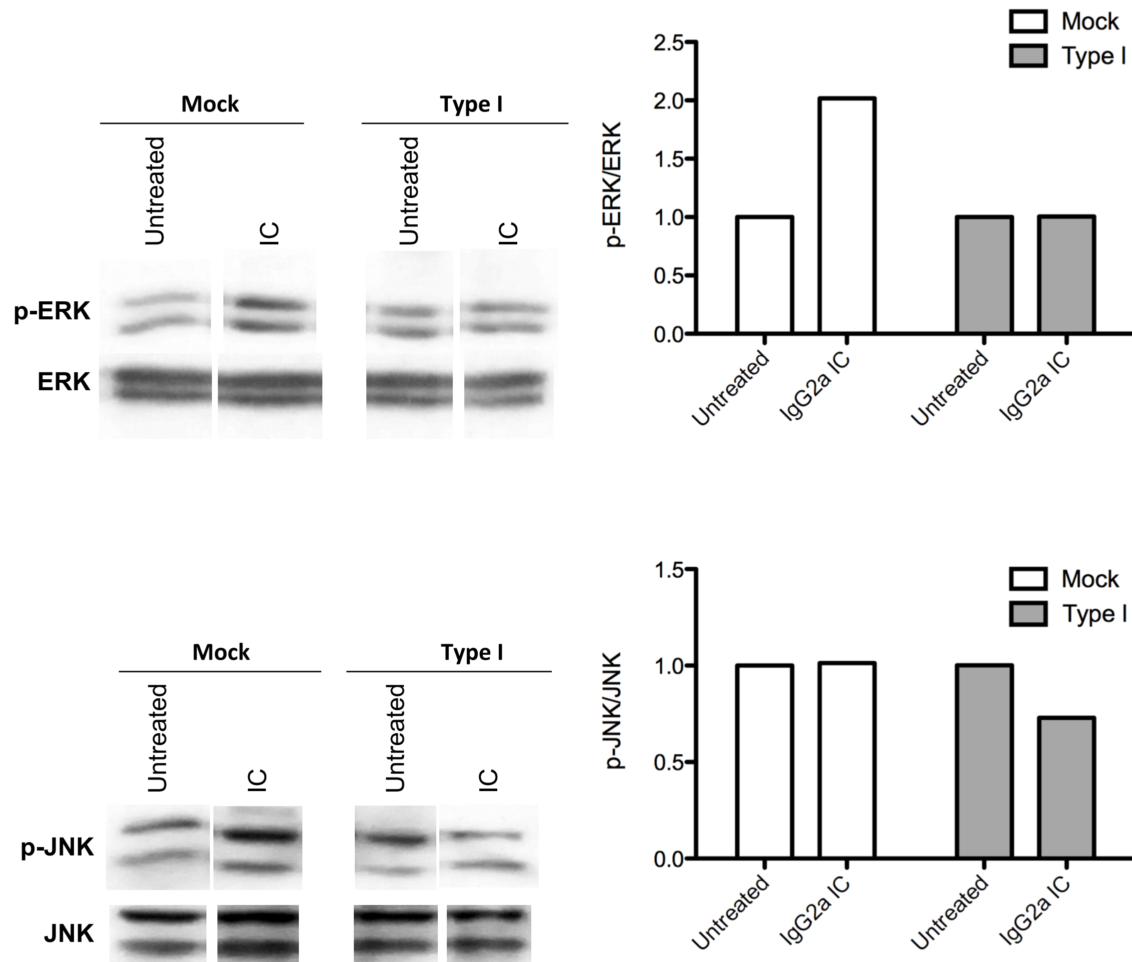

Figure 4.6. Evaluating signal transduction pathways downstream of FcγR engagement

BMdM were mock infected or infected with type I *T. gondii* parasites. At 13 hours-post infection cells were serum starved and then stimulated with immune complexes (IC) at 18 hours-post infection at a ratio of 1:50 for 15 min. IC were generated by opsonizing latex beads with mouse IgG2a. Cell lysates were harvested 15 min-post stimulation, separated by SDS-PAGE and immunoblotted for (A) phosphorylated ERK (p-ERK) and total ERK or (B) phosphorylated JNK (p-JNK) and total JNK (B). Levels of phosphorylated ERK and JNK were quantified by densitometry and normalized to total ERK or JNK, relative to mock.

Discussion

T. gondii has been shown to alter the proteome of infected cells (101, 102). Here, we describe a protocol to characterize the cell surface proteome of macrophages infected with *T. gondii*. The dataset generated using this protocol indicates that this approach can be useful for the isolation of cell surface proteins. From the list of proteins unique to infected cells we chose to validate the expression of FcγRs due to their important role in immune activation. Using a microarray dataset generated from BMdM infected with type I parasites (RH) we were able to confirm that FcγR expression is only altered in *T. gondii*-infected BMdM. More specifically, FcγRII transcripts are significantly increased in infected cells. We were able to confirm that the expression of FcγR on infected cells is also altered at the protein level, with infected cells having increased FcγRII/III expression at the cell surface. Given the role FcγR play in mediating cellular activation, we evaluated if enhanced expression of the inhibitory receptor FcγRII had an effect on the activation of signal transduction pathways following IC engagement. We demonstrate that infected macrophages have impaired MAPK activation following *T. gondii* infection. Furthermore, altered FcγR expression is strain specific, with the type I and type III parasites inducing the expression of the inhibitory FcγR. These results suggest that altered FcγR expression on infected cells may be employed as a previously undescribed immune evasion strategy by *T. gondii*. Evaluating the role FcγR play in mediating *T. gondii* immunity as well as the effect of altered FcγR expression has on modulating parasite immunity is of particular interest and warrants further investigation.

In addition to identifying the FcγR receptors in our proteomic analysis, we also identified other cell surface proteins that are differentially affected by *T. gondii* infection. Analysis of the mass spectrometry hits using the Universal Protein Resource database revealed that macrosialin and galectin-3, both of which are proteins with known immune-regulatory functions, were up-regulated by infection. Macrosialin, expressed by mice and the homologue of human CD68 is a lectin binding protein specifically expressed in monocytes and macrophages that is differentially glycosylated in response to various stimuli (169). The rapid infiltration of $CD68^+$ inflammatory monocytes and macrophage-like cells to the site of *T. gondii* infection has been reported (170, 171). The extent to which macrosialin expression and glycosylation is altered during *T. gondii* infection has yet to be described. Galectin-3 is a member of the lectin family of proteins containing a carbohydrate-binding protein domain that recognizes β-galactosides and is associated with regulating various aspects of inflammation (172). It was recently demonstrated that galectin-3 binds to *T. gondii*-derived glycosylphosphatidylinositols (GPIs) and that it might serve as a co-receptor for TLR 2 and TLR4 in addition to the canonical co-receptor CD14 (68). Furthermore, galectin-3 may have a modulatory role in inflammatory responses. Galectin-$3^{-/-}$ mice had increased IL-12 production and a more Th1-polarized response to *T. gondii* infection compared to wild-type mice (173). How galectin-3 transcriptional and protein expression are altered and the role this receptor plays in modulating macrophage activation during *T. gondii* infection has yet to be investigated.

A current interest in the lab is to improve upon the current surface proteome protocol to increase the repertoire of cell-surface proteins identified for investigation.

While the current protocol permits the identification of proteins that are present or absent on the surface of infected or uninfected macrophages, this analysis is not quantitative. We are greatly interested in quantifying protein expression in the experimental samples to be able to draw conclusions about the extent to which surface protein expression is altered between infected and uninfected cells. This can be achieved by using stable isotope labeling by amino acids in cell culture (SILAC), in which the relative abundance of proteins can be determined among 2-3 samples (174, 175). With this technique, the cells are cultured in the presence of light (N^{14} or C^{12}) or heavy isotope (N^{15} or C^{13})-labeled amino acids. The incorporation of at least one light or heavy labeled amino acids corresponds to a predictable shift in the mass spectra for each peptide. Subsequently, light and heavy isotope-labeled samples can be combined. Relative protein quantification is achieved by taking the ratio of the abundance of peptides with the same amino acid composition but with different mass (174, 175).

This work will help us move towards better understanding the intricate relationship between *T. gondii* and macrophages. Moreover, this approach may shed light on previously unappreciated strategies utilized by *T. gondii* to alter macrophage effector functions. This approach could be used to elucidate the molecular mechanisms regulating the expression of selected proteins. More importantly, the dataset generated from this work may be used as a way to screen for proteins that uniquely identify infected cells. Identification of such proteins would be ideal therapeutic targets.

Chapter Five

Concluding Remarks

Macrophages play a key role in mediating *T. gondii* immunity. In addition to cell intrinsic mechanisms that directly contribute to parasite control, macrophages play a central role in coordinating the adaptive immune response (26). Proteins expressed on the surface of macrophages enable the cells to respond adequately. As a successful pathogen, *T. gondii* has evolved immune evasion strategies that target a variety of macrophage effector functions, including altering the expression of cell surface proteins. We report here that infected macrophages display modified expression of several immune relevant cell surface proteins. The observed modulation of surface proteins has the potential to alter the ability of infected macrophages to respond to stimuli. This has significant implications for modulating cell intrinsic mechanisms of host defense as well as intercellular communication.

We found that *T. gondii*-infected macrophages have altered expression of the costimulatory protein, B7-2. The enhanced expression of B7-2 on macrophages is characteristic of macrophage activation and contributes to T cell activation. We found that B7-2 is induced in macrophages infected with type I, type II or type III parasites at both the RNA and protein level. The induction of B7-2 by the three strains we evaluated suggests that B7-2 induction in *T. gondii*-infected macrophages is a general response to active parasite infection. While B7-2 expression may be initiated by the host cell through intracellular recognition of parasite antigen, this is unlikely as heat killed parasites that are subsequently taken up by phagocytosis do not induce B7-2 expression. Rather, B7-2 expression is increased in response to active parasite infection. However, whether the enhanced B7-2 phenotype is a result of a single or multiple parasite factors that interact with host signaling pathways is not know. 4-BPB-treated parasites, which can attach but

are unable to secrete rhoptry proteins (113) and are unable to invade do not stimulate enhanced B7-2 expression. Because 4-BPB treated parasites are still able to secret micronemes into the host cytosol when they attach, our results strongly suggest that if a secreted parasite protein is necessary for B7-2 induction, then it is most likely a rhoptry protein.

We demonstrate that induction of B7-2 following *T. gondii* infection is partially dependent on JNK MAPK signaling. Because MAPK signaling is downstream of TLR engagement, we investigated the role TLRs play in regulating B7-2 expression during *T. gondii* infection. We found B7-2 expression is not altered in macrophages deficient in either of the two TLR adaptor proteins, MyD88 and TRIF. This suggests the presence of a secreted parasite factor in the host cell cytosol that may trigger JNK activation independent of MyD88 and TRIF. While TLRs function as cell surface PRR, macrophages also express intracellular PRRs. The NOD (nucleotide-binding oligomerization domain) proteins NOD1 and NOD2 are members of the nucleotide-binding oligomerization domain receptor (NLR) family of proteins and are expressed by macrophages. NOD1 and NOD2 have been shown to contribute to B7-2 and B7-1 expression in DCs (176), which differs from *T. gondii*-infected macrophages that preferentially induce B7-2 expression. While NOD1 and NOD2 are typically associated with bacterial antigen sensing, Shaw *et al.* demonstrated that *T. gondii*-infected NOD2$^{-/-}$ mice had impaired parasite clearance and lower IFN-γ production (177). This would be consistent with impaired or diminished T cell activation, which could result from reduced costimulatory molecule expression. Furthermore, NOD2 engagement on macrophages results in p38 and JNK MAPK activation (178). The possibility that *T. gondii* sensing and

B7-2 induction is mediated through intracellular PRR, such as through NOD1 or NOD2, is intriguing and warrants investigation.

Unlike B7-2-induced expression by *T. gondii*, which we believe to be a host cell sensing mechanism in response to active parasite invasion, CD40 induction appears to be an example of parasite-mediated modulation of immunity. The work presented here further contributes to our understanding of the role GRA15 plays in mediating *T. gondii* strain-specific differences. We show that the protein encoded by the type II allele of *GRA15* is sufficient to induce the expression of the activation marker CD40 on infected cells. We also demonstrate that engagement of CD40 on infected macrophages further contributes to parasite immunity through the enhanced production of IL-12. The production of IL-12 by the type II strain either through direct NF-kB activation (58) or through engagement of induced CD40 on infected cells contributes to the establishment of an immune response that ultimately helps control *T. gondii* infection.

Strain-specific induction of the surface molecule CD40 illustrates the divergent strategies that different *T. gondii* strains may utilize to modulate host immune responses. It is becoming clear that strain-specific differences contribute dramatically to macrophage activation states and the elicited immune response to the parasite (159). For instance, type I parasites are capable of activating STAT3 and STAT6 signaling and suppressing pro-inflammatory cytokine production due to the type I allele of ROP16 (52). As a result, IFN-γ mediated antimicrobial programs are diminished in type I infected macrophages. The ability of type I parasites to actively reprogram infected cells towards an alternatively activated state contributes to that strain's ability to proliferate and disseminate effectively in infected mice.

Conversely, type II-infected macrophages are characterized as having a classically activated phenotype. Unlike type I-infected macrophages, type II-infected macrophages do not have impaired IL-12 production. As such, type II-infected mice are capable of mounting a more robust Th1 mediated response and are better able to control parasite infection. The type II allele of GRA15 has been shown to mediate IL-12 production and promote classical macrophage activation. While type I parasites are capable of suppressing host immunity, it comes at a cost, as the parasites are considerably more virulent than type II parasites. As such, infection with a single parasite is capable killing a mouse. By contrast, acute infections with type II parasites are better controlled in mice, and the parasites establish tissue cysts. This in turn may facilitate the dissemination of the type II parasite in the wild and is a possible reason for why this strain is more commonly associated with human infection (15).

While we show that $GRA15_{II}$ is sufficient to induce CD40 expression in transfected THP-1 cells, QTL analysis indicated the presence of additional loci as being associated with the high CD40 induction phenotype. It is possible that additional secreted proteins, yet to be identified, play a role in regulating CD40 induction. It is reasonable to suspect that multiple secreted proteins work together to regulate host signaling pathways, including those that lead to CD40 expression. For example, ROP18, a kinase known to lead to IRG phosphorylation (48, 179), was recently shown to negatively regulate NF-κB signaling by promoting p65 degradation (180). The protein encoded by the type I allele of *ROP18* was found to phosphorylate p65 at serine 468, leading to its degradation via the ubiquitin-dependent degradation pathway. Interestingly, unlike type III parasites, which have an insertion in the *ROP18* promoter

that inhibits its expression, type II parasites express *ROP18*. This suggest that despite the expression of full length ROP18 by both type I and type II parasites, additional differences between the two strains further regulate the ability of type II parasites to sustain NF-κB signaling. It would be interesting to see if differences at the genomic level or the expression of ROP18 account for the observed differences in the CD40 induction phenotype between different type II strains, such as the PRU and ME49 strains, as well as amongst the type II and type III recombinant strains that exhibited an intermediate or high CD40-inducing phenotype.

In addition to examining specific immune receptors on macrophages, we also developed a protocol to evaluate in an unbiased way how the cell surface protein repertoire is altered in parasite-infected macrophages. Similar to the B7-2 and CD40 work in which we evaluated protein levels at 12-24 hpi, we collected the cell surface protein fraction at 18 hpi to allow the cells sufficient time to respond to infection, thereby maximizing the differences in the cell surface protein repertoire between the infected and uninfected samples. We observed a substantial restructuring of the cell surface proteome of *T. gondii*-infected macrophages. Our protocol is amenable to identifying proteins that are unique to infected or uninfected cells. The data set generated using our protocol led us to identify FcγRs as being differentially regulated between infected and uninfected macrophages. Amongst the proteins present in only the infected cell sample were the low affinity and inhibitory FcγRs, FcγRIII and FcγRIIb, respectively. While FcγR have been implicated in contributing to *T. gondii* immunity (181), we are the first to identify and characterize the strain-specific modulation of FcγR expression on

infected cells. We found that type I parasites induced the low affinity and inhibitory FcγRs, while the type II parasites did not.

Furthermore, this led us to identify FcγR as a novel mechanism by which *T. gondii* may regulate macrophage activation states. To investigate how altered FcγR expression affects the ability of macrophages to respond to FcγR engagement we stimulated with opsonized targets. We found that infected cells had impaired MAPK signaling. Because FcγR mediated activation is dependent on the ratio of activating and inhibitory receptors (182), the enhanced expression of the low affinity and inhibitory FcγR on infected cells suggests that type I parasites actively suppress macrophage activation. These results are consistent with the ability of type I parasites to induce alternatively-activated macrophages.

This protocol, with further modifications, also has the potential to help us identify novel surface markers specific to infected cells that can serve as targets for antibody-mediated therapies. This therapeutic approach has been used to treat patients with B cell lymphomas using the anti-CD20 monoclonal antibody rituximab (183). Additionally, clinical trials to treat certain cancers expressing high levels of the surface protein CD47 with a monoclonal antibody against CD47 (184, 185) are expected to begin soon. To facilitate the identification of novel and unique cell surface markers on infected cells, we are looking to improve our protocol to incorporate measures that would allow us to more accurately quantifying the different expression levels of specific proteins between infected and infected macrophages. To this end, we are looking to incorporate stable isotope labeling by amino acids in cell culture (SILAC), which is commonly and successfully used for quantitative mass spectrometry analysis (174, 186-188).

The work presented here has elucidated novel mechanisms regulating the expression of cell surface proteins on infected macrophages. We demonstrate that the signaling molecule JNK is involved in regulating the expression of the costimulatory molecule B7-2 in *T. gondii*-infected macrophages. We also identified the parasite factor that induces CD40 expression. We further contribute to our understanding of the role GRA15 plays in mediating the ability of type II parasites to induce classically activated macrophages. Finally, we have initiated the development of a protocol to help generate a robust dataset that can be used to identify cell surface proteins that have altered expression in infected cells that may also facilitate the identification of potentially novel therapeutic targets for *T. gondii*-infected cells.

Chapter Six

Materials and Methods

Host cell culture

Human foreskin fibroblasts (HFF) were cultured in D-10% medium: Dulbecco's Modified Eagle's Medium (DMEM; Thermo Scientific, Logan, UT) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Mouse BMdM from C57BL/6 mice were generated as previously described (105) and cultured in D-10% medium supplemented with 10% M-CSF for 6-7 days before *T. gondii* infection assays. Femurs from *MyD88*$^{-/-}$ (123) and *TRIF*$^{Lps2/Lps2}$ (189) mice were kindly provided by Dr. Anthony DeFranco (UCSF). For the IFN-γ experiments, macrophages were treated with 100 U/ml of rmIFN-γ (eBioscience, San Diego, CA) for 24 hours (hrs) before harvest and staining for flow cytometry.

Human monocytes were isolated from PBMCs using counterflow elutriation, as previously described (190). The monocyte population was > 90% pure, as determined by CD11b^{+}CD14^{+}CD3^{-} staining by flow cytometry. Freshly isolated monocytes were used immediately for infection experiments.

THP-1 cells were cultured in R-10% medium: RPMI 1640 (Thermo Scientific, Logan, UT) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Where indicated, THP-1 cells were stimulated with 5 mM phorbol myristate acetate (PMA) for 24 hrs, washed, and then cultured in fresh R-10% medium for an additional 24 hrs.

All mammalian cell cultures were maintained in incubators set at 37˚C with 5% CO_2. Cell lines and parasite strains were tested monthly for *Mycoplasma* contamination and confirmed to be negative.

Parasite strains and infections

The following *T. gondii* strains were used for infections: type I strains RHΔ*hpt* and RHgfpluc (128), type II strain Prugniaud A7 (129) or ME49fLuc (generously provided by Dr. John Boothroyd), type III strain CΔLuc123 (this was the CEP strain described in (98), which was transfected with cDNAs encoding green fluorescent protein (GFP) and click beetle luciferase), transgenic type I stably expressing the type II allele of *GRA15* (type I $GRA15_{II}$) (58). All parasite strains constitutively express GFP and were maintained by serial passage in confluent HFF monolayers as previously described and grown in D-10% medium at 37˚C with 5% CO_2 (148).

BMdM were infected with syringe-lysed, washed parasites at an MOI of 2, or the medium was replaced for the mock-treated controls. At 2 hours post-infection (hpi), the cells were washed with 1X PBS and the medium was replaced with fresh D-10%. At 6, 8, 16, or 24 hpi, the cells were scraped, resuspended, and prepared for the corresponding applications detailed bellow. Human monocyte infections were performed as described above for mouse macrophages, except that the cells were not washed at 2 hpi.

In vivo infection

Female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were infected with syringe-lysed, washed RHgfpluc parasites (10^5 parasites/mouse) in sterile PBS by intraperitoneal (i.p.) injection. Mock-infected mice received an i.p. injection of PBS (200 μl/mouse). Mice were monitored daily and sacrificed at three days post-infection. Peritoneal exudate cells were collected by peritoneal lavage, and red blood cells were lysed prior to staining and analysis of cells by flow cytometry (described below). *In vivo* experiments were performed three times with similar results. A representative

experiment is shown. All research involving mice was carried out in compliance with the Institutional Animal Care and Use Committee at UCI.

Antibodies and flow cytometry

Peritoneal exudate cells, BMdM, THP-1 cells, and human monocytes that were infected with *T. gondii* or treated with IFN-γ were resuspended in FACS wash (PBS with 2% FCS) containing anti-Fc receptor antibody (for mouse: clone 2.4G2; BD Bioscience, San Jose, CA; for human: human Fc receptor binding inhibitor; eBioscience, San Diego, CA) and incubated on ice for 10 minutes (min). The cells were pelleted by centrifugation and resuspended in FACS wash containing the following biotinylated primary antibodies: control Ig, anti-B7-1 (clone 16-10A1), anti-B7-2 (mouse: clone GL1 or human: clone IT2.2, eBioscience, San Diego, CA), anti-mouse CD40 (clone 3/23; BioLegend, San Diego, CA), or with the following directly conjugated antibodies: anti-human CD40-PE (clone 5C3; BioLegend, San Diego, CA), anti-mouse-FcγRI-APC (clone X54-5/7.1; BioLegend, San Diego, CA), anti-mouse-FcγRII/III-APC (clone 93; BioLegend, San Diego, CA). The cells were stained with the primary antibodies on ice for 30 min and washed. BMdM and human monocytes were then resuspended in FACS wash containing streptavidin-PE, while peritoneal lavage cell samples were resuspended in FACS wash containing streptavidin-PE-Cy5 and anti-CD11b-PE (clone M1/70) conjugated antibodies. Samples were incubated on ice for 15 min, and washed. After the final wash, the infected cells were fixed with 4% paraformaldehyde. For intracellular cytokine staining, the cells were fixed with 2% PFA for 20 min at room temperature (RT), permeabilized with 0.5% saponin for 10 min at RT, and stained with

anti-IL-12p40-PE (clone C17.8; eBioscience, San Diego, CA) or a mouse IgG1-PE control antibody on ice for 30 min.

All flow cytometry samples were examined using a FACSCalibur cytometer with CellQuest software (BD Biosciences, San Jose, CA) for acquisition and FlowJo software (Treestar, Ashland, OR) for analysis. For the mock-treated cells, the histograms depict the total cell population. Since GFP-expressing *T. gondii* were used for the infection and flow cytometry experiments, the histograms for the infected cells depict those cells that fell within the GFP^{+} gate. All the flow cytometry experiments were performed more than three times. Representative experiments are shown.

Q-PCR

BMdM from C57BL/6 mice, $MyD88^{-/-}$, or $TRIF^{Lps2/Lps2}$ mice were infected as described above. At 6 or 24 hpi, RNA was harvested using the RNEasy kit (Qiagen, Germantown, MD) and was treated with DNase I (Invitrogen, Carlsbad, CA). cDNA was generated and used as template in quantitative real-time PCR (Q-PCR) with primers specific for mouse B7-2: (sense) GCCCATTTACAAAGGCTCAA and (anti-sense) TGTTCCTGTCAAAGCTCGTG, for mouse CD40: forward primer (5'-GCTATGGGGCTGCTTGTTGA-3') and reverse primer (5'-ATGGGTGGCATTGGGTCTTC-3'), for mouse FcγRI: forward primer (5'-GGGAAGACACCGCTACACAT-3') and reverse primer (5'-GGAGATGACACGGATGCTCT-3'), for mouse FcγRII: forward primer (5'-CTGGAAGAAGCTGCCAAAAC-3') and reverse primer (5'-CCAATGCCAAGGGAGACTAA-3'), and for mouse FcγRIII: forward primer (5'-TTGGACACCCAGATGTTTCA-3') and reverse primer (5'-

GTCTGCAAAAGCAAACAGCA-3'). Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization using the following primers: forward (5'-GCATGGCCTTCCGTGTTC-3') and reverse (5'-GATGTCATCATACTTGGCAGGTTT-3'). Q-PCR was performed in triplicate using a Bio-Rad iCycler and SYBR green detection (Bio-Rad, Hercules, CA).

The data from the Q-PCR were analyzed using the threshold cycle ($2^{-\Delta\Delta C_T}$) method (191). The values obtained for B7-2, CD40, FcγRI, FcγRII and FcγRIII expression were normalized to those of GAPDH, and the data are expressed as a ratio of mRNA levels. Error bars reflect the standard deviation from triplicate samples. In all Q-PCR assays, cDNA generated in the absence of reverse transcriptase (-RT) was used as a negative control to detect contaminating genomic DNA. No amplification was observed in the -RT samples or in samples containing water in the place of DNA template.

Microarrays and data analysis

Confluent monolayers of BMdM were mock-treated or infected with RHΔ*hpt* parasites, as described above, and total RNA was harvested at 6 hpi using TRIzol (Invitrogen, Carlsbad, CA). The experiment was performed as biological triplicates. cDNA was synthesized and labeled using the Affymetrix 3' IVT One-Cycle Target Labeling kit, according to the manufacturer's instructions. Hybridization to Affymetrix mouse 430 2.0 GeneChip oligonucleotide arrays was performed by the Stanford Protein and Nucleic Acid Facility (Stanford, CA). Background adjustment, quantile normalization, and summarization were performed using the Robust Multichip Average algorithm implemented in Agilent GeneSpring GX v11.0 (Agilent Technologies, Santa Clara, CA).

The dataset of 45,101 probe sets, representing 21,635 unique genes, was analyzed using the volcano plot analysis pipeline in GeneSpring. A two-class unpaired test with Benjamini-Hochberg multiple-testing correction was performed to determine differences in expression between mock and infected samples that were statistically significant ($\leq$1% FDR). The dataset was then further filtered to select probe sets in which there was a greater than 5-fold difference in mock versus infected cells. These probe sets are represented visually in a volcano scatter plot in red. MultiExperiment Viewer (www.tm4.org/mev) was used to generate the heat maps of the filtered dataset and to perform hierarchical clustering. Euclidean distance was used for the distance metric, with average linkage clustering. The complete microarray dataset was deposited in the Gene Expression Omnibus (GEO) database repository.

Inhibitor assays

BMdM were cultured in serum-free medium for 6 hrs prior to infection, and then treated with dimethyl sulfoxide (DMSO) as a vehicle control, or with the following MAPK inhibitors, beginning at 2 hrs prior to infection: 10 μM PD-98059 (ERK), 10 μM SP600125 (JNK), or 1 μM SB203580 (p38). These concentrations were selected based on their specificity, as published in the literature, and on dose-response experiments that we performed to select the lowest concentration that showed specific effects on the target (data not shown). The cells were harvested at 15 min post-infection (mpi) for the generation of lysates for Western blotting, or at 24 hpi for antibody staining and flow cytometry. All the inhibitors were purchased from Enzo Life Sciences (Plymouth Meeting, PA) and resuspended in DMSO.

Western blotting

BMdM were treated with MAPK inhibitors and infected, as described above. BMdM were collected at 15 mpi, or transduced THP-1 cells were washed with ice-cold 1X PBS, and cell lysates were generated by the addition of 2X Laemmli sample buffer containing 10% β-mercaptoethanol. The lysates were separated by SDS-PAGE and transferred to PVDF for immunoblotting. The membranes were blotted with antibodies against β-actin or against phosphorylated JNK, p38, ERK, or c-Jun. The membranes were blotted with a horseradish peroxidase (HRP)-conjugated antibody against the HA epitope tag (Cell Signaling, Danvers, MA). For membranes immunoblotted with unconjugated primary antibodies, HRP-conjugated secondary antibodies were used. All Western blotting antibodies were obtained from Cell Signaling (Danvers, MA). The membranes were developed with Amersham ECL Prime (GE Healthcare, Waukesha, WI) and detected using a Nikon camera as previously described (192).

T cell proliferation assay

BMdM were infected as described above. The cells were then resuspended in PBS with 2% FCS (FACS wash) containing anti-Fc receptor antibody (clone 2.4G2, BD Bioscience, San Jose, CA) and incubated on ice for 10 min. Cells were washed and resuspended in FACS wash containing control Ig or neutralizing anti-B7-2 (clone GL1) and incubated on ice for 30 min then washed. Cells were fixed in 2% paraformaldehyde then washed a total of 3 times in FACS wash to remove any residual paraformaldehyde. BMdM were then resuspended in T cell culture media (R-10% additionally supplemented with 55 μM β-mercaptoethanol, 5 mM HEPES buffer, 100 μM non-

essential amino acids, and 1 mM sodium pyruvate) before coculturing with isolated T cells.

Splenic T cells were enriched by negative selection using LS columns and biotin-conjugated antibodies against MHC class II, Ter-119, CD11b, CD11c, CD19, CD45R, CD49b, and CD105, following the manufacturers instructions (Miltenyi Biotec, Auburn, CA). Briefly, splenocytes were prepared by mechanically separating spleens through a cell strainer (40 μM) and lysing of red blood cells using ACK. Splenocytes were then incubated with the biotin-conjugated antibody cocktail followed by incubation with magnetic MicroBeads conjugated anti-biotin Ig isotype antibodies. The cell suspension was then passed through a LS column in the presence of a strong magnetic field and enriched T cells were eluted. The purity of enriched T cells was >90% as determined by flow cytometry.

T cells were labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) in PBS containing 0.05% bovine serum albumin for 15 min at 37˚C. Cells were washed twice in medium containing 10% FCS. CFSE-labeled T cells (0.5 x 10^6 cells/well) were placed in wells of a 96-well plate containing 1 μg/ml of plate-bound anti-CD3 antibody (clone 17A2, BioLegend, San Diego, CA) and in the presence of infected or uninfected BMdM that were fixed and coated with control Ig or anti-B7-2 antibodies for 4 days.

Quantitative trait locus (QTL) mapping

CD40 mean fluorescence intensity values generated from BMdM infected with 19 F1 progeny derived from crosses between a type II strain and a type III strain (14, 193) were analyzed using R/qtl (194) and the existing *T. gondii* genetic map (16). QTL peaks

were identified using the marker regression method (mr). Overall significance level was determined using 1000 permutations of the genotype data.

Retroviral transduction

The type II *T. gondii* GRA15 (GRA15$_{II}$) open reading frame (ORF) with a C-terminal hemagglutinin (HA) epitope tag was cloned into the BamHI and EcoRI sites of the pMX-puro retroviral vector to generate the vector IP9. This construct was generated by performing nested-primer PCR using the following primers: forward primer, GRA15-BamHI (5'-GCTTCA GGATCCATGATAATTCGGTGGCTTGGGTATCTTACGG-3'); reverse primer 1, GRA15-HA: (5'-TCTGGGACGTCGTATGGGTATGGAGTTACCGCTGATTGTGTGTCCC-3'); and reverse primer 2, GRA15-HA-EcoRI: (5'-GTAATGGAATTCTCAAGCGTAGTCTGGGACGTCGTATGGGTATGGAGTTA-3'). Phoenix-E packaging cells were transfected with IP9 (GRA15$_{II}$-HA-pMX-puro), the parental pMX-puro plasmid as a negative control using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Retroviral supernatants were harvested on days 2 and 3 post-transfection and centrifuged at 1200 revolutions per minute (rpm) for 10 min to clarify the supernatant and remove cell debris. THP-1 cells were infected with retrovirus by centrifugation at 2,500 rpm for 2 hrs at 25°C in the presence of 8 μg/ml hexadimethrine bromide (Sigma-Aldrich, St. Louis, MO). At 40 hrs post-infection, the transduced cells were placed in selection media containing 2 μg/ml puromycin (Thermo Fisher Scientific, Logan, UT). Western blotting

was performed after 5 days in puromycin selection to confirm expression of $GRA15_{II}$-HA.

Transient transfection

Human embryonic kidney 293T (293T) cells were used to generate CD40L-expressing transfectants (293T-CD40L). 293T cells were transfected with the CD40L-MIEG-hCD4 plasmid containing the complete coding sequence for the murine CD40L (Addgene plasmid 40355; Cambridge, MA) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. As a control, 293T cells were transfected with only Lipofectamine 2000. Transfected 293T and 293T-CD40L cells were cultured for 48 hrs at 37°C before being added to BMdM.

CD40L stimulation assays

BMdM at day 6 of culture were infected with type II, or transgenic type I $GRA15_{II}$ parasites, as described above. For cells stimulated with soluble CD40L, at 18 hpi the cells were washed with PBS and resuspended in fresh media containing 10 µl/ml of CD40L (195) with or without 10 µg/ml of anti-CD40L blocking antibody (clone MR-1; BioXCell, West Lebanon, NH). At 4 hrs after CD40L stimulation, the cells were treated with Brefeldin A (eBioscience, San Diego, CA) and cultured for an additional 4 hrs. The cells were then harvested and examined by flow cytometry for IL-12p40 using intracellular cytokine staining, as described above. Alternatively, 293T or CD40L-expressing 293T cells were added at a ratio of 1:2 (293T:BMdM) to BMdM infected with type I, II, or type I $GRA15_{II}$ parasites at 18 hpi without any media change. At 26 hpi, the cell culture supernatants were collected to measure IL-12p70 by ELISA (BioLegend, San Diego, CA).

Cell surface protein isolation and mass spectrometry

BMdM were mock infected or infected with type I (RH) parasites at an MOI of 2 for 18 hr. Cells were washed with PBS and incubated with 250 µg/ml (4.1 $x10^{-4}$ M) sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin, Thermo Scientific, Logan, UT) dissolved in PBS for 20 min at 4˚C. Cells were washed with PBS and incubated with 50 mM Tris-HCl, pH 7.4, which quench any remaining sulfo-NHS-SS-biotin. Cells were then lysed in lysis buffer (150 mM NaCl, 50 mM TEA, 1% NP-40, and 1X protease inhibitor (Roche, South San Francisco, CA), and the lysate was homogenized by passing through a syringe fitted with a 27-guage needle. Lysates were then incubated with High Capacity Streptavidin Agarose Resin (Thermo Scientific, Logan, UT) for 2 hrs at RT. The streptavidin resin was washed with PBS to remove unbound protein. Bound protein was then eluted by incubating the streptavidin resin with PBS containing 100 mM β-ME for 5 min at 95˚C. Eluted protein was concentrated using a Amicon Ultra-0.5 10K device (Millipore, Billerica, MA) following the manufacturers instructions. Lysates were separated by SDS-PAGE and immunoblotted for B7-2 or ERK as described above. The eluted and concentrated lysates were also visualized by silver stained using Silver Stain Plus Kit (Bio-Rad, Hercules, CA) following the manufacturer’s instructions. The stained gels were sent to the UCI Mass Spectrometry Facility for processing, and following an in-gel trypsin digest, the digested proteins were eluted and lyophilized. The proteins were then reconstituted in acetonitrile water and analyzed by LC-MS/MS using a Quadropole Waters Quattro Premier XE UPLC triple quadropole instrument. The mass spectrometry hits were analyzed using the Universal Protein Resource database, which contains protein sequences and functional information for known proteins from mouse and human.

Immune complex stimulation

BMdM were mock infected or infected with the type I (RH) strain at a MOI of 2. At 14 hpi, media was replaced with serum-free culture media and incubated for an additional 4 h. At 18 hpi, cells were stimulated with neutravidin beads (Invitrogen, Carlsbad, CA) that had been opsonized with 4 μg of IgG2a (clone eBM2a; eBioscience, San Diego, CA) and incubated at 37˚C for 15 min. Lysates were collected and immunoblotted for total and phosphorylated ERK and JNK as described above.

Statistical analysis

Unpaired, two-tailed Student's t-test was used to determine statistical significance. Differences were considered significant when $P<0.05$.

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