The role of Toxoplasma gondii dense granule protein GRA24 in signaling and immunity during infection

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The role of *Toxoplasma gondii* dense granule protein GRA24 in signaling and immunity during infection

BY

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B.S., Biology, New Mexico State University, 2013
M.S., Biology, University of New Mexico, 2019

DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Biology

The University of New Mexico
Albuquerque, New Mexico

July 2021
DEDICATION

This work is dedicated to my nieces and nephews, may you reach every goal you set forth.
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The role of *Toxoplasma gondii* dense granule protein GRA24 in signaling and immunity during infection

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ABSTRACT

*Toxoplasma gondii* is a pathogen whose control partly depends upon Toll-like receptors (TLR) 11 and 12, that signal through MyD88. Yet, human TLR11 and TLR12 are nonfunctional, motivating us to investigate MyD88-independent immune pathways. Parasite dense granule protein GRA24 activates p38 MAPK independently of MyD88 in macrophages. Using wild-type and GRA24-deleted *T. gondii* strains, I demonstrate GRA24 activates p38 MAPK, resulting in IL-12 production, and protective immunity. Furthermore, GRA24 triggers p38 MAPK-dependent CX3CL1 production. GRA24 also induces CCL17 and Ppbp independently of p38 MAPK. Additionally, I demonstrate GRA24 downregulates CCL12. Furthermore, IL-15 is negatively regulated by GRA24 through activation of p38 MAPK activation. Moreover, I show GRA24 may play a role in CD4^+^ T cell recruitment during *T. gondii* infection. Together, this research is a prime example
of the fine balance of inflammatory events triggered by *T. gondii* to ensure persistence of both host and parasite.
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Chapter 1

Introduction: *Toxoplasma gondii* life cycle, biology, manipulation of host cell and immune recognition

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Toxoplasma gondii Life Cycle and Biology

Toxoplasma gondii is an intracellular protozoan parasite that is a global health problem as it infects 25-30% of the world population [1, 2]. Furthermore, T. gondii is considered one of the neglected parasitic infections of the United States with a rate of infection over 40 million [3]. Toxoplasma was first discovered in 1908 by Charles Nicolle and Louis Manceaux in tissues of the hamster-like rodent Ctenodactylus gundii. In 1939, T. gondii was first identified in tissues of a congenitally infected infant [4]. Healthy humans infected with T. gondii are asymptomatic. However, within infected immunocompromised hosts, the parasite undergoes unchecked replication, which can be fatal if left untreated [5].

In Europe and North America T. gondii is comprised of 3 widespread clonal lineages that differ in allelic variation and virulence. The Type I strain is the most virulent, Type II strain being less virulent and Type III strain being the least virulent. The majority of cases of human toxoplasmosis arises from infection of the Type II strain [6]. T. gondii is in the phylum Apicomplexa which share the presence of an apical complex consisting of a conoid and apical secretory organelles that are crucial for cell invasion [7]. There are a number of apicomplexans that cause detrimental diseases in the human population including malaria, caused by Plasmodium species, cryptosporidiosis, caused by Cryptosporidium species and toxoplasmosis, caused by T. gondii. It is important to elucidate the infection strategy and immune response elicited by T. gondii to understand how to fight these apicomplexan pathogens that cause detrimental effects on the human
population. *T. gondii* is a model organism for studying apicomplexans due to the availability of excellent animal models such as *Mus musculus* and ease of passaging *T. gondii* in the lab.

*T. gondii* is an obligate intracellular protozoan parasite that invades and replicates within nucleated cells [8]. *Toxoplasma* has a high transmission rate partly because of its complex life cycle. *T. gondii* infects warm-blooded animals and birds [9]. The most common route of infection is by ingestion of oocysts in contaminated food or water [1]. Within its life cycle, *T. gondii* alternates between sexual replication within the felid species (definitive host) and asexual replication within mammals and birds (intermediate host) [10]. Felids are most commonly infected by the ingestion of tissue cysts found in natural intermediate hosts such as small rodents. Infected felids shed oocysts in their feces into the environment, which are ingested by domestic animals as well as humans. Another route of infection is by the consumption of tissue cysts found in infected raw meat [11] (Figure 1.1). Once infection of the intermediate host is achieved *T. gondii* differentiates into the tachyzoite life stage, which actively invade cells of the immune system, in particular dendritic cells and monocytes [12].

The invasion of cells by tachyzoites is an active process involving the formation of a moving junction and the expulsion of secretory organelles including micronemes, rhoptries and dense granule proteins [13] (Figure 1.2). Micronemes are long thin secretory organelles located at the apical tip of the parasite. Early during
tachyzoite attachment and invasion of the host cell, micronemes (MIC) expel their contents, which interact with receptors on the host cell membrane aiding in invasion as well as virulence [14, 15]. Specifically microneme protein AMA-1 is present on the apical surface of the parasite and plays an important role in mediating intimate association of apical attachment to the host cell [16]. Following microneme secretion, rhoptry neck proteins (RONs) are secreted from the neck region of the parasite, inserting into the host cell membrane to form an anchor for the moving junction [13]. Rhoptry proteins (ROP) are secreted following RON secretion. Rhoptries discharge their contents into the nascent parasitophorous which associate with the parasitophorous vacuole membrane [17]. After RON and ROP secretion, dense granule proteins are discharged at the end of the invasion process and are involved in remodeling of the parasitophorous vacuole (GRA3, GRA5) and formation of the intravacuolar membranous network within the vacuole (GRA2, GRA4, GRA6) [18, 19]. Furthermore, some dense granule proteins (GRA15, GRA24) cross the parasite vacuolar membrane (PVM) into the host cell cytoplasm. Within the parasitophorous vacuole the tachyzoites proliferate until active egress from the host cell [20]. The newly egressed parasites enter the circulatory system disseminating throughout the body to other tissue. Once the environment within the host is unfavorable for parasite expansion, the tachyzoites differentiate into bradyzoites and undergo latent infection by encysting in the brain and muscle tissues, replicating slowly and persisting in the chronic stage of infection [21, 22]. The formation of tissue cysts ensures the parasite can be easily spread to other hosts by ingestion of infected meat. Once the environment within
the host is favorable for parasite proliferation the parasite differentiates back into the infective form, reactivating active infection [23].
Figure 1.1 *Toxoplasma gondii* Life Cycle (1) Members of the Felidae family become infected with *T. gondii* and shed oocysts into the environment where they sporulate into the infective stage. Each sporulated oocyst contains 2 sporoblasts each consisting of 4 sporozoites which are ingested by the natural intermediate hosts such as small rodents. (2) Within the natural intermediate host, the cysts differentiate into tachyzoites which give rise to bradyzoites found in long-lived tissue cysts. Re-infection of cats can occur by the ingestion of sporulated oocyst found in the environment or by the consumption of infected rodents. (3) Infection of accidental intermediate hosts such as domestic animals and humans occurs by the consumption of contaminated food and water by sporulated oocysts shed into the environment. (4) Infection of humans can also occur by ingesting meat that is infected with tissue cysts. (5) Once infection occurs in the human host, the parasite converts into the tachyzoite life stage, causing detrimental effects such as congenital diseases, ocular disease and complications particularly when the host is immunosuppressed.
Figure 1.2 Schematic of the anatomy of a tachyzoite Tachyzoites contain many organelles that play a role in the invasion process which include micronemes (MIC), rhoptries which house rhoptry neck proteins (RON) and rhoptry proteins (ROP) and dense granule proteins (GRA)
Clinical and Epidemiological Aspects of *T. gondii*

Toxoplasmosis is the disease caused by reactivation of chronic *T. gondii* infection in individuals with a weakened immune system. This opportunistic parasite has had a worldwide impact on immunocompromised individuals. *Toxoplasma* is the cause of intracerebral focal lesions resulting in toxoplasmic encephalitis. Toxoplasmic encephalitis can be fatal and represents an important concern in the AIDS community [24]. If left untreated in immunocompromised hosts, the parasite will undergo unchecked replication leading to the death of the host. Congenital infection occurs via trans-placental transmission of tachyzoites after primary maternal infection during pregnancy. The severity of the congenital defects relates to gestational age at the time of maternal infection. More severe manifestations develop when infection occurs within the first trimester. Congenital defects include hydrocephaly, microcephaly, hepatosplenomegaly, chorioretinitis, intracranial calcification and/or death of the fetus [25]. In some cases, *T. gondii* infection can cause serious disease in immunocompetent hosts. However, in most cases *T. gondii* infection in immunocompetent hosts is largely asymptomatic and resolves itself with minimal pathology but a persistent latent infection endures throughout the lifetime of the host. This leaves the patient at risk of reactivation if there is ever a deficiency in the immune system. Furthermore, it is not well understood how the human immune system is controlling the parasite during chronic infection in immunocompetent hosts.
The seroprevalence of *T. gondii* differs depending upon the geographic area. In the United States it is estimated that 8-22% of the population is infected with *T. gondii*. In Central America, South America and Europe it is estimated that infection ranges from 30-90% [26, 27]. A few states within the USA (Massachusetts, New Hampshire and Rhode Island) along with many European countries have implemented aggressive screening programs to mitigate the high rates of seroprevalence of *T. gondii* [28]. In 1972, laws in France to diagnose and treat *T. gondii* infection *in utero* were implemented. Treatment with pyrimethamine and sulphadiazine with leucovorin has almost completely eradicated severe symptomatic congenital toxoplasmosis in France.

The most effective method for the prevention of toxoplasmosis is by cooking meat to 160°F or 71°C before consuming. If meat is going to be served rare, meat should be kept at subzero for several days; cutting boards and cooking utensils that have made contact with raw meat should be sterilized. Other important measures should be taken such as washing fruits and vegetables before ingesting, avoiding raw mussels and avoiding contact with materials that could have come into contact with cat excrement. Furthermore, prophylactic treatment of seropositive patients can prevent reactivation of the latent infection. Screening of pregnant women to determine if they are seropositive or negative for *T. gondii* during gestation can be a tool to determine risk of infection and the potential outcome for congenital transmission.
**Roll of Toll-like receptors in immune recognition of Toxoplasma**

In the early 1990’s, Janeway theorized that the innate immune system uses pattern recognition receptors (PRRs) for the response to pathogen-associated molecular patterns (PAMPs) [29]. Lemaitre *et al.* 1996 described the Toll receptor as a PRR against fungi in *Drosophila melanogaster*. A few years later Janeway and Medzhitov discovered a major pattern recognition receptor in the innate immune system, Toll-like receptors (TLR) named after the Toll receptor described in *Drosophila*. Toll-like receptors detect an array of microbial molecules including RNA, DNA, proteins and lipids [30]. The TLR are proteins made up of a leucine-rich repeat (LRR) extracellular domain that mediates the recognition of a PAMP, a short transmembrane (TM) domain and an intracellular Toll/interleukin-1 receptor (TIR) signaling domain, which is required for the transmission of signal to downstream pathway components [31]. TLR function as homodimers or heterodimers. *T. gondii* parasite protein profilin is detected by TLR 11 and TLR 12, inducing the recruitment of MyD88 to the TIR domain of the receptor [32]. (Figure 1.3) IRAK1 and 4 are then recruited to the MyD88 adaptor protein. The IRAK molecules form a molecular complex with TNF receptor-associated factor (TRAF) 6, inducing the recruitment of Ubc13 and Uval. This molecular complex is an E3 ubiquitin ligase leading to the polyubiquitination of TRAF6, inducing activation of transforming growth factor-b-activated kinase-1 (TAK1) and TAK1-binding protein (TAB)1 and 2. Furthermore, TRAF6 couples the IKKγ/NEMO, IKKa and IKKβ complex which is responsible for the phosphorylation of the Ikβ inhibitor molecule causing polyubiquitination, destroying the proteasome [33]. This frees NF-κB
which translocate into the nucleus leading to the transcription of a variety of genes that are involved in immune and inflammatory response [34]. Additionally, mitogen activated protein kinases (MAPK) are activated by TAK1 activating p38 as well as JNK for the triggering of activating protein-1 AP1 composed of c-fos and JUN. AP1 translocate to the nucleus and gene transcription of proinflammatory cytokines occurs [35] (Figure 1.3).
Figure 1.3 Signaling Cascade of TLR/MyD88 induction during *T. gondii* infection. (1) *T. gondii* protein profilin is detected by TLR11 and TLR12 within the endosome. (2) The MyD88 adaptor protein is recruited to the IRF domain of TLR11 and 12. (3) This triggers IRAK1 and 4 recruitment to MyD88. (4) The IRAK molecules form a molecular complex with TRAF6, inducing the recruitment of Ubc13 and Uval. TRAF6 induces activation of transforming growth TAK1, TAB 1 and 2. (5) MAKK are activated by TAK1. (5a) MAKK activates p38 as well as JNK for the triggering of AP1 composed of c-fos and JUN. (5b) AP1 translocate to the nucleus. (5c) AP1 induces gene transcription of proinflammatory cytokines. (6) TRAF6 couples the IKKy/NEMO, IKKα and IKKβ complex. (6a) Phosphorylation of the IκB inhibitor molecule causing polyubiquitination frees NFκB. (6b) NFκB translocate into the nucleus. (6c) NFκB induces the transcription of genes that are involved in immune and inflammatory response.
Immune Response to *T. gondii*

*T. gondii* most often establishes infection through oral ingestion of tissue cysts or oocysts shed by felids. Upon release of sporozoites or bradyzoites from either oocysts or tissue cysts, parasites invade the enterocytes of the small intestine where proliferation occurs. The parasites then penetrate through the epithelial barrier into the lamina propria. Once in the lamina propria *T. gondii* infects macrophages, dendritic cells and neutrophils allowing for systemic dissemination to organs such as the liver, spleen and brain [36]. Dissemination also occurs systemically through the persistence of free parasites in the lymphatic and circulatory system. Phagocytosis of tachyzoites by macrophages and dendritic cells leads to detection of the parasite by the immune system.

The host immune response to *T. gondii* in mice is activated through endosomal TLR. Within the TLR family, TLR11/12 are the two main receptors involved in recognition of actin-binding protein profilin, a parasite protein that is necessary for host cell invasion and also serves as a PAMP activating the innate immune system. As discussed above, upon recognition of profilin by TLR11, TLR12 forms a heterodimer with TLR11 leading to the initiation of the MyD88 immune signaling cascade, inducting the downstream production of IL-12 [32]. The downstream effects of the TLR/MyD88 axis are important in the production of IL-12. If IL-12 is blocked in mice, this results in acute susceptibility of *T. gondii* with a similar effect in MyD88-deficient animals [37-39]. The main source of MyD88-dependent IL-12 production occurs in CD8α⁺ DC in the spleen as well as CD103⁺ CD11b⁻ and...
CD103+ CD11b- DC in the intestine [40]. Other sources of IL-12 are derived from macrophages, inflammatory monocytes, plasmacytoid DCs and neutrophils [40]. The production of IL-12 activates natural killer (NK) cells, CD4+ and CD8+ T cells to generate IFN-γ, which in turn activates primed monocytes to destroy the parasite (Figure 1.4). Furthermore, the induction of IL-12 triggers IFN-γ production in NK cells triggering interferon regulatory factor 8 (IRF8) signaling pathway in macrophages and CD8α+ DC [32]. TLR12 colocalizes Unc-93 homolog B1 (UNC93B1), a chaperone protein involved in the trafficking of endosomal TLRs, inducing IRF8-dependent dendritic cell IL-12 production rather than NF-κB signaling cascade. This cascade induces a more potent IL-12 response within these cell populations.

Parasite glycosylphosphatidylinositol-anchored proteins (GPI) are found on the surface of T. gondii tachyzoites. Cell surface TLR2 and TLR4 detect tachyzoite GPI activating NF-kB inducing the synthesis of tumor necrosis factor (TNF)α and CC-chemokine ligand 2 (CCL2) production by macrophages and neutrophils [41, 42]. Furthermore, endosomal TLR7 and TLR9 have also been linked in playing an important role in the innate sensing of T. gondii DNA and RNA [43]. However, the roles of TLRs 2, 4, 7, and 9 in the murine host are less important than TLR11/12. Since humans do not express the profilin-recognizing TLR, it is possible that TLR2, 4, 7 and 9 play a more prominent role in the human host.
Upon active invasion of host cell dendritic cells by *T. gondii*, the parasite resides in a parasitophorous vacuole within the cytoplasm of the cell. It is hypothesized that priming of CD8$^+$ T cells is achieved by cross-presentation of *T. gondii* derived antigen [44, 45]. This process requires the fusion of the host endoplasmic reticulum (ER) to the PVM enabling parasite antigen to be exported into the cytosol for proteasomal degradation, transport into the endoplasmic reticulum and MHC class 1 loading for cell surface antigen presentation. Priming of CD8$^+$ T cells could be a mechanism used by the parasite to promote pathogen latency. Additionally, fusion of the host ER could provide an avenue for parasite nutrient acquisition from the host [44, 45].
Figure 1.4 TLR11/12 IL-12 induction during T. gondii infection (1) Tachyzoites are phagocytosed by mononuclear phagocytes or dendritic cells. (2) A phagosome is formed around the engulfed tachyzoite which fuses with a lysosome forming a phagolysosome. (3) TLR11 and TLR12 are expressed in the membrane of the phagolysosome and detect parasite profilin. (4) The MyD88 adaptor protein is recruited to the cytoplasmic domain of the TLR heterodimer, inducing the IL-12 immune signaling cascade. (5) Secretion of IL-12 activates NK cells inducing the production and secretion of IFN-γ. Along with NK cells, CD8 and CD4 T cells as well as neutrophils are other sources of IFN-γ. (6) Activation of macrophages is triggered by the secreted IFN-γ leading to the (7) destruction of the parasite.
Mechanisms of *T. gondii* killing

Of relevance, IFN-γ has the ability to suppress *T. gondii* proliferation in infected cells by the induction of tryptophan degradation. This induces antiproliferative effects on *T. gondii* mediated by indoleamine 2,3-dioxygenase (IDO) which converts tryptophan to N-formylkynurenine, resulting in starvation of the cell due to the deficiency of tryptophan [46]. Furthermore, IFN-γ mediates parasite killing by production of nitric oxide (NO) which is dependent upon L-arginine [47]. NO has potent microbicidal effects on many intracellular pathogens including *T. gondii*. Nitric oxide intermediates are nonpolar uncharged molecules that can cross the PVM and directly interact with the parasites. NO then modifies proteins inhibiting parasite proliferation [48].

MyD88 Independent Mechanisms of Immune Recognition

Although, it is well known that immune response to *T. gondii* is dependent upon the TLR/MyD88 signaling cascade, there have been reports of MyD88-independent pathways of immunity during *Toxoplasma* infection [49]. Interestingly, humans that lack IRAK-4 which is recruited by MyD88 associated with TLR and IL-1R, do not have increased susceptibility to other microbes including *T. gondii* [50, 51]. Furthermore, mice deficient in the MyD88 gene established protective immunity to intraperitoneal and oral challenge following vaccination with *cps1-1*, an attenuated uracil auxotrophic strain of *T. gondii* [52]. Additionally, vaccination with *cps1-1* elicits anti-tumor immunity in MyD88 knockout mice [53]. It has also
been previously shown that bone marrow derived macrophages (BMDM) that were activated with IFN-γ, were able to control *T. gondii* infection in the absence of MyD88 [49]. Furthermore, MyD88 KO BMDM were able to produce IL-12 when infected with RH, a virulent strain of *T. gondii* [54].

Another relevant MyD88-independent immune response used by *T. gondii* is through inflammasome activation. Inflammasomes are intracellular sensor systems that interact with nod like receptor (NLR) in the host cell. Specifically, NLRP1 (NOD, LRR and pyrin domain containing 1) has been shown to be an inflammasome sensor for *T. gondii* in the murine host. The inflammasome induces cell death and IL-1β production during infection [55].

**T. gondii Manipulation of Host cell**

GRA6, GRA16, GRA15, GRA24, ROP16 and TgWIP

*T. gondii* has the incredible ability to invade any nucleated cell in part due to the sequential secretion of effectors from specialized secretory organelles called micronemes, rhoptries and dense granules [56]. Most dense granule proteins localize to the PVM contributing to the maturation of the PV and nutrient acquisition [57]. Some GRA proteins traffic to the host cytoplasm or nucleus interfering with host signaling pathways. GRA6 plays a central role in the formation of the nanotubule network within the PV [58]. Furthermore, GRA6 induces the transcription of nuclear factor of activated T cells 4 (NFAT4) via binding to calcium
calcineurin activator calcium modulating ligand (CAMLG) [59]. The activation of NFAT4 induces the production of chemokines CCL2 and CXCL2, which play a pivotal role in the recruitment of inflammatory monocyte and neutrophils [60]. GRA16 has been shown to impact p53 in the host cell, which is important to host cell survival during infection. GRA16 accomplishes this by increasing nuclear localization of phosphatase, tensin homolog (PTEN) and p53 dependent apoptosis by binding to herpes virus associated ubiquitin specific protease (HAUSP) in hepatocellular carcinoma cells (HCC) [61]. GRA15 expressed by Type II *T. gondii* strains has been shown to traffic to the PVM activating NF-κB, inducing production of Th1 type immune responses promoting the induction of proinflammatory cytokines [62]. Furthermore, GRA15 activates NF-κB independently of MyD88 but dependent upon the TRAF6 and the IKK complex [62].

The rhoptry protein (ROP16) is secretory parasite kinase that is secreted into the host cell cytoplasm during infection. ROP16 shortcuts the JAK/STAT signaling pathway and directly phosphorylates STAT3 and STAT6 proteins resulting in decreased IL-12p40 production in the host cell [63, 64]. Furthermore, ROP16 induces arginase-1 which under starving conditions of L-arginine *in vitro* decreased the ability for the parasite to replicate. Additionally, nitric oxide synthase utilizes L-arginine for nitric oxide mediated toxicity. Therefore, ROP16 is using this pathway to evade the immune system by inducing the downregulation of arginine production [63].
Additionally, *T. gondii* rhoptry protein TgWIP is secreted into the host cytoplasm from the PV and interacts with the WASP family verprolin homologous protein (WAVE) regulatory complex and SHP2 phosphatase. This leads to alterations in the actin dynamics within infected dendritic cells inducing host cell motility and further promotion of parasite dissemination [65]. Furthermore, immunity-related GTPases (IRG) are mediated through IFN-γ inducible proteins that are essential for host resistance against *T. gondii* [66]. Upon infection, highly coordinated loading of IRGs onto the parasitophorous vacuole membrane occurs. Initiation of the recruitment of IRGs is initiated by IRGB6 and IRGB10 which is followed by recruitment of IRGA6, IRGD and IRGM2 [67]. This leads to the disruption of the vesicle and elimination of the parasite by lysosome mediated degradation [54, 68]. Interestingly, Type I *T. gondii* strains can subvert the IRG defense mechanism by secreting ROP5, ROP17 and ROP18 which forms a complex and prevents the loading of IRGs to the parasite vacuole membrane [69, 70].

Finally, parasite GRA24 is exported from the PV into the host cytoplasm. GRA24 then binds to p38α MAP kinase triggering autophosphorylation of p38 MAPK. The GRA24/p38MAPK complex then translocate into the host nucleus. This results in the upregulation of transcription factors EGR-1 and c-FOS, inducing secretion of proinflammatory cytokines MCP-1 and IL-12 (Figure 1.5) [2]. We have recently shown the induction of IL-12 by GRA24 is independent of MyD88. Furthermore, GRA24 induces protective immunity in an MyD88-independent manner (Chapter 2) [71].
Figure 1.5 IL-12 induction by GRA24 by activation of p38 MAPK

1. *T. gondii* tachyzoites infect macrophages or dendritic cells and form a parasitophorous vacuole (PV) within the cytoplasm of the cell.
2. *T. gondii* secretes GRA24 into the host cytoplasm.
3. GRA24 binds host p38α MAPK.
4. p38α MAPK autophosphorylation.
5. The GRA24/p38α MAPK complex translocates into the nucleus.
6. Activation of EGR-1 and c-FOS by phosphorylated GRA24/p38α MAPK complex.
7. Induction of IL-12, MCP-1, triggering Th1 immune response.
The organization of this dissertation is presented in five chapters. Chapter one is an introduction to the life cycle, biology, manipulation of host cell and immune recognition of *Toxoplasma gondii* in the murine host.

Chapter two, published in "*PloS Pathogens*", focuses on the role of parasite dense granule protein GRA24 in the immune response during *T. gondii* infection. Using a genetically engineered uracil auxotroph parent strain (*cps1-1*) and a GRA24 KO strain (*cps1-1:ΔGRA24*) we discovered GRA24 modulates chemokines and cytokines during *T. gondii* infection. Furthermore, we showed GRA24 induces p38 MAPK IL-12 production as well as protective immunity independently of the MyD88/TLR cascade.

Chapter three focuses on the specific cytokines and chemokines regulated by GRA24 in C57BL/6 BMDM. We evaluated the transcription of 84 cytokine and chemokine genes by qPCR in BMDM infected with GRA24 sufficient or GRA24 deficient parasites. We found a specific subset of cytokine/chemokine genes are regulated by GRA24. We described evidence that some of these are regulated independently of p38 MAPK. Overall, this chapter recognizes GRA24 as an important parasite protein that has a role in regulating the expression of several other immune mediators in addition to IL-12.

Chapter four concentrates on the regulation of myeloid and lymphocyte cell population infiltration into the peritoneal cavity during infection by GRA24. We used
flow cytometry to look at the specific cell populations in the peritoneal cavity by intraperitoneal infection with GRA24 positive and GRA24 negative parasites. Our results showed GRA24 may have a role in regulating CD4 T cell infiltration although this effect is minor. Additionally, there was no change in the myeloid cell population when comparing WT mice infected with GRA24 KO or GRA24 positive parasites. This study potentially identifies a novel role for GRA24 in the regulation of CD4 T cells during *T. gondii* infection.

Chapter five summarizes the overall significance of these findings and discusses future directions for the continuation of this research.
References


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Chapter 2

*Toxoplasma gondii* dense granule protein GRA24 drives MyD88-independent p38 MAPK activation, IL-12 production and induction of protective immunity

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Abstract

The apicomplexan *Toxoplasma gondii* induces strong protective immunity dependent upon recognition by Toll-like receptors (TLR)11 and 12 operating in conjunction with MyD88 in the murine host. However, TLR11 and 12 proteins are not present in humans, inspiring us to investigate MyD88-independent pathways of resistance. Using bicistronic IL-12-YFP reporter mice on *MyD88*+/+ and *MyD88*−/− genetic backgrounds, we show that CD11c+MHCII+F4/80− dendritic cells, F4/80+ macrophages, and Ly6G+ neutrophils were the dominant cellular sources of IL-12 in both wild type and MyD88 deficient mice after parasite challenge. Parasite dense granule protein GRA24 induces p38 MAPK activation and subsequent IL-12 production in host macrophages. We show that *Toxoplasma* triggers an early and late p38 MAPK phosphorylation response in *MyD88*+/+ and *MyD88*−/− bone marrow-derived macrophages. Using the uracil auxotrophic Type I *T. gondii* strain *cps1-1*, we demonstrate that the late response does not require active parasite proliferation, but strictly depends upon GRA24. By i. p. inoculation with *cps1-1* and *cps1-1:Δgra24*, we identified unique subsets of chemokines and cytokines that were up and downregulated by GRA24. Finally, we demonstrate that *cps1-1* triggers a strong host-protective GRA24-dependent Th1 response in the absence of MyD88. Our data identify GRA24 as a major mediator of p38 MAPK activation, IL-12 induction and protective immunity that operates independently of the TLR/MyD88 cascade.
Author Summary

*Toxoplasma gondii* is a protozoan parasite that infects over 1 billion people worldwide. Infection with the parasite is normally asymptomatic and *Toxoplasma* co-exists with its host in the form of latent cysts in brain and muscle tissue. The balance between immune recognition and immune evasion is likely a key factor in the outcome of this host-parasite interaction. It is therefore important to understand how *Toxoplasma* triggers immunity, and in particular how the protective cytokine IL-12 is induced during infection. While Toll-like receptor (TLR)/MyD88 signaling is important in mouse resistance to *Toxoplasma*, this pathway is likely less important in human infection. Here, we report that the parasite dense granule protein GRA24 triggers p38 MAPK activation and IL-12 production independently of TLR/MyD88 signaling. We identify additional cytokines and chemokines that are regulated by GRA24 during *in vivo* infection. Our data demonstrate that GRA24 initiates a protective MyD88-independent immune response during *in vivo* infection. The GRA24 molecule provides an example of a parasite molecule whose function is induction of a host protective immune response. From the standpoint of *Toxoplasma*, this likely reflects an evolutionary adaptation to ensure host survival and simultaneously enable latency to maximize the chance of transmission.
Introduction

The intracellular protozoan *Toxoplasma gondii* is a globally distributed parasite that infects humans, companion animals, livestock and wildlife. The parasite is estimated to infect 25-30% of the human population worldwide [1]. The course of infection is characterized by two phases. In the acute phase initiated in the intestine after oral infection, the parasites disseminate widely through tissues as rapidly dividing and highly invasive tachyzoites. This is followed by a chronic, or latent, phase associated with differentiation to slowly dividing bradyzoites that form long-lived cysts in tissues of the skeletal muscle and central nervous system [2]. Infection at this stage is usually asymptomatic. Nevertheless, in immunodeficient populations cysts may reactivate resulting in uncontrolled parasite replication that can rapidly culminate in death [3]. *Toxoplasma* may also cross the placenta during pregnancy causing life-threatening disease both before and after birth [4].

*Toxoplasma* is highly effective at stimulating a protective immune response, an outcome that accounts for the normally asymptomatic nature of infection [5-7]. While clearly aiding the host, the parasite also benefits from this protective response since host survival enables establishment of latent infection. The immune response to *T. gondii* revolves around early production of IL-12 by cells such as CD8 α+ dendritic cells (DC) in the spleen as well as CD103+CD11b− and CD103−CD11b− DC in the intestinal mucosa [8-10]. The central role played by IL-12 in resistance is dramatically highlighted by the extreme susceptibility of IL-12 knockout (KO) mice to *T. gondii* infection [11]. Production of IL-12 drives early NK
cell production of IFN-γ and generation of IFN-γ-producing Th1 cells. IFN-γ ultimately controls the parasite through its ability to induce anti-Toxoplasma effector molecules such as the immunity-related GTPase (IRG) family and guanylate-binding proteins (GBP) that destroy the parasitophorous vacuole harboring intracellular tachyzoites [12-16].

The molecular and cellular basis for recognition and subsequent IL-12 production in response to *T. gondii* and other microbial pathogens has been extensively studied in mouse models. Early on, it was understood that the Toll-like receptor (TLR) adaptor molecule MyD88 is important in triggering IL-12 and promoting host resistance to *Toxoplasma* [17, 18]. Cell-specific knockout studies revealed CD11c+ DC as the major source of MyD88-dependent resistance rather than macrophages or neutrophils that can also contribute to protective IL-12 [19-21]. Amongst TLR, both TLR11 and TLR12 are major receptors involved in recognition and resistance. Together, these receptors are activated by profilin, a parasite protein required for host cell invasion that also serves as a classical pathogen-associated molecular pattern igniting innate immunity [22-27]. Other TLR, for example cell surface TLR2 and 4 and the endosomal nucleic acid receptors TLR7 and 9, have been suggested to play secondary roles in resistance to *Toxoplasma* [24, 28, 29].

While a great deal is known about initiation of immunity to *T. gondii* in mouse models, the major innate immune receptors and signaling molecules involved in human resistance to this intracellular protozoan remains an open question. This is
because in humans TLR11 is present only as a pseudogene and the TLR12 gene is entirely absent [30]. Moreover, MyD88-deficient individuals remain resistant to all but a select few pyogenic bacterial infections [31, 32]. Thus, while TLR/MyD88 signaling plays a major role in rodent resistance to *Toxoplasma* and other infections, this signaling axis appears less crucial for defense in humans.

Previously, we reported the presence of MyD88-independent pathways of immunity during *Toxoplasma* infection [33]. While MyD88 KO mice ultimately do not survive infection, the days immediately preceding death are characterized by emergence of IFN-γ -producing Th1 effectors. More strikingly, vaccination of *MyD88*−/− mice with *cps1-1*, an avirulent *Toxoplasma* uracil auxotroph, results in protective immunity to lethal challenge [33]. In parallel, we discovered that the *Toxoplasma* Type I strain RH triggers an unusual autophosphorylation pathway of p38 mitogen-activated protein kinase (MAPK) activation, and that this response drives IL-12 production in mouse bone marrow-derived macrophages [34]. This finding was later extended by others who identified the parasite dense granule secretory protein GRA24 as the activator of p38 MAPK [35, 36]. Thus, GRA24 is targeted by the parasite into the host cell where it binds and triggers allosteric autoactivation of p38 MAPK. This results in nuclear translocation and changes in host gene transcription, including upregulation of genes controlling IL-12 transcription.
In the present study, we investigate the role of GRA24 in MyD88-independent triggering of immunity both *in vitro* and *in vivo*. Using genetically engineered *cps1*-1 GRA24 KO parasites, we show that this molecule drives p38 phosphorylation and IL-12 production independently of MyD88 signaling. In addition, we identify a novel subset of cytokines and chemokines that are controlled by GRA24 during *in vivo* infection. Furthermore, we show that GRA24 triggers a strong host defense response during induction of immunity by *cps1*-1. Our results provide a striking molecular example illustrating the evolutionary adaptation of *Toxoplasma* to actively trigger inflammatory cytokine production to promote host survival, parasite latency, and transmission.
Results

Toxoplasma triggers MyD88-independent IL-12 production during in vivo and in vitro infection

To understand the MyD88-independent IL-12 response during T. gondii infection, we generated bone marrow-derived macrophages (BMDM) from MyD88+/+ and MyD88−/− mice then stimulated the cells with Type I RH tachyzoites and lipopolysaccharide (LPS). We found increasing amounts of IL-12p40 production as infection progressed in MyD88+/+ (Fig. 2.1A) and MyD88−/− (Fig. 2.1B) BMDM infected with RH compared to media control. Nevertheless, we note that the cytokine was not produced at detectable levels until 24-36 hr after infection. In contrast, the response to LPS, which signals through TLR4 and MyD88, was largely abrogated in MyD88 KO BMDM, and in WT cells the maximum response was achieved within 6 hr of stimulation (Fig. 2.1A and B). The parasites initiate egress at approximately 48 hr post-infection, likely accounting for lack of IL-12 increase between 48 and 72 hr. Although IL-12p40 levels were somewhat higher in MyD88−/− BMDM compared to MyD88+/+ BMDM infected with RH in this experiment, over biological replicates (n=3) this result was not consistent. We examined BMDM IL-12p40 production following infection with Type II strain tachyzoites that have been reported to induce a more robust IL-12 response compared to Type I strains [37]. While there were some minor differences, we found overall that IL-12p40 production was very similar following infection with RH and Type II PTG in both MyD88+/+ (Fig. 2.2A) and MyD88−/− (Fig. 2.2B) BMDM. Caspase-8 has recently been shown to contribute to IL-12 production and
antimicrobial defense to *Toxoplasma* independent of RIPK3 [38]. Therefore, we wanted to determine if RIPK3-independent caspase-8 signaling contributed to parasite-induced IL-12 in BMDM. We infected *RIPK3*<sup>−/−</sup> and *RIPK3*<sup>−/−</sup>*Caspase8*<sup>−/−</sup> BMDM with either RH or PTG tachyzoites and measured IL-12p40 in the supernatants. The results showed *RIPK3*<sup>−/−</sup> and *RIPK3*<sup>−/−</sup>*Caspase8*<sup>−/−</sup> BMDM infected with either RH or PTG produced similar amounts of IL-12p40 production compared to WT BMDM (Fig. 2.3A and 2.3B). We conclude that caspase-8 is not involved in the response to *T. gondii* triggered in BMDM.

We also assessed IL-12p40 production in peritoneal exudate cells (PEC) and splenocytes (SPL) in *MyD88*<sup>+/+</sup> and *MyD88*<sup>−/−</sup> mice that were infected by intraperitoneal (i. p.) inoculation with either Type I RH or Type II PTG tachyzoites. There was a strong IL-12p40 response in the *MyD88*<sup>+/+</sup> PEC and SPL infected with both RH and PTG parasite strains (Fig. 2.1C). The IL-12 response was less robust in PEC and SPL from infected MyD88 KO mice. Nevertheless, there was clear IL-12 production elicited by both parasite strains independent of MyD88 (Fig. 2.1C). Together, our results identify an alternative MyD88-independent pathway of IL-12p40 production triggered by Type I and Type II strains of *Toxoplasma* during both *in vitro* and *in vivo* infection.
Figure 2.1 *Toxoplasma gondii* triggers production of MyD88-independent IL-12 during *in vivo* and *in vitro* infection. Bone marrow-derived macrophages (BMDM) from wild-type (A, *MyD88*+/+) and MyD88 knockout (B, *MyD88*−/−) mice were cultured in medium (C), infected with RH strain tachyzoites (1:1 ratio of parasite to cells) or stimulated with LPS (100 ng/ml). At the indicated time points, supernatants were collected for cytokine ELISA. (C) *MyD88*+/+ and *MyD88*−/− mice (n = 2 per group) were infected by intraperitoneal inoculation with 10^3 RH or PTG strain tachyzoites. Four days later, peritoneal exudate cells (PEC) and splenocytes (SPL) were isolated and cultured with no further stimulation. Supernatants were collected for cytokine ELISA at the indicated time points (hr). Data shown are the means ± SD of cells cultured in triplicate. Statistical significance was assessed using Student’s t test (* p < 0.05, *** p < 0.001). These experiments were performed three times with the similar result.
Figure 2.2 IL-12 responses to *Toxoplasma* strains PTG and RH in WT and MyD88 KO BMDM. BMDM were generated from C57BL/6 (A) and *MyD88*−/− (B) mice and infected with either Type I RH or Type II PTG tachyzoites at a 1:1 ratio of parasites to cells. Supernatants were collected for cytokine ELISA at the indicated time points. NI, non-infected BMDM, supernatants were collected at 24 hr. Data shown are the means ± SD of cells cultured in triplicate. Statistical significance was analyzed using two way ANOVA with Tukey's multiple comparisons test (*** p > 0.001). This experiment was repeated two times and yielded similar results.
Figure 2.3 Caspase 8 is not involved in Toxoplasma-induced IL-12 production in BMDM. BMDM were generated from C57BL/6 (WT), RIPK3−/− and RIPK3−/−caspase-8−/− mice and infected with either Type I RH (A) or Type II PTG (B) tachyzoites at a 1:1 ratio of parasites to cells. Supernatants were collected for cytokine ELISA at the indicated time points. NI, non-infected BMDM, supernatants were collected at 24 hr. Data shown are the means ± SD of cells cultured in triplicate, n = 2 mice per group. Statistical significance was assessed using two way ANOVA with Tukey’s multiple comparisons test (** p > 0.01).
Dendritic cells, neutrophils and macrophages are sources of IL-12 production in *T. gondii* infected MyD88 deficient mice

To understand the cells involved in IL-12 production in MyD88 deficient mice during *T. gondii* infection, we utilized IL-12eYFP reporter mice, a bicistronic reporter strain in which cells expressing IL-12 also express eYFP [39]. We crossed *MyD88^{+/+}IL-12p40^{eYFP/eYFP}* with *MyD88^{-/-}* animals to generate strains expressing IL-12eYFP on *MyD88^{+/+}* and *MyD88^{-/-}* backgrounds. The reporter mouse strains were infected with RH tachyzoites by i. p. inoculation and we then used flow cytometry to measure percentages and total cell numbers in the peritoneal cavity five days post infection. Our data show similar numbers of cells in the peritoneal cavity of *MyD88^{+/+}IL-12p40^{eYFP/eYFP}* and *MyD88^{-/-}IL-12p40^{eYFP/eYFP}* infected mice at this time point (Fig. 2.4A). There were also similar numbers of DC in both mouse strains, but there was a 3-5 fold decrease in neutrophils (PMN) and macrophages (MØ) in the absence of MyD88 (Fig. 2.4A). In line with the data shown in Fig. 2.1C, IL-12-positive cells in *MyD88^{-/-}* mice were present at approximately 30% of the levels in the IL-12 reporter *MyD88^{+/+}* animals (Fig. 2.4B and C). In Fig. 2.4D we examined the relative contributions of DC, PMN and MØ to the IL-12-positive populations in *MyD88^{+/+}* and *MyD88^{-/-}* mice. While each cell type contributed to the IL-12-positive population, DC contributed the largest portion in the WT strain. In contrast, the proportions were more evenly divided in the KO mice (Fig. 2.4D). By staining for intracellular tachyzoites we determined the percent infection in each of the IL-12-positive populations (Fig. 2.4E). In the *MyD88^{+/+}* reporter strain, most of the IL-12-positive DC, PMN and MØ were uninfected. Interestingly, the majority of IL-12-
positive cells were infected on the MyD88−/− background, regardless of identity (Fig. 2.4E). Collectively, these data show that the cell types producing IL-12p40 in both MyD88 sufficient and MyD88 deficient mice are dendritic cells, neutrophils and macrophages. In addition, the MyD88-independent IL-12 that is produced derives predominantly from infected cells.
Figure 2.4 Multiple cell types produce MyD88-independent IL-12 during *in vivo* infection. IL-12 reporter mice on a wild type (MyD88<sup>+/+</sup>/IL-12<sup>p40<sup>eYFP/eYFP</sup>) and MyD88 knockout (MyD88<sup>−/−</sup>/IL-12<sup>p40<sup>eYFP/eYFP</sup>) background were infected by intraperitoneal injection with 10<sup>3</sup> RH strain tachyzoites. On day 5 post-inoculation, peritoneal exudate cells were collected for *ex vivo* analysis. (A) Number of PEC, dendritic cells (DC), neutrophils (PMN) and macrophages (MØ) present in the peritoneal cavity. (B) Percentage of IL-12<sup>eYFP</sup> positive cells in the peritoneal cavity of one representative infected MyD88<sup>+/+</sup>/IL-12<sup>p40<sup>eYFP/eYFP</sup>, one representative infected MyD88<sup>−/−</sup>/IL-12<sup>p40<sup>eYFP/eYFP</sup>, and one representative noninfected MyD88<sup>+/+</sup>/IL-12<sup>p40<sup>eYFP/eYFP</sup> mouse. (C) Percent IL-12 positive cells in the peritoneal cavities of WT and MyD88 KO IL-12 reporter mice. (D) Number of IL-12 positive cells amongst DC, PMN and MØ isolated from MyD88<sup>+/+</sup> and MyD88<sup>−/−</sup> reporter mice. (E) Percent of infected cells that are positive for IL-12. In these experiments, DC were defined as MHCII<sup>+</sup>, CD11c<sup>+</sup>, F4/80<sup>−</sup> cells; PMN were defined as Ly6G<sup>+</sup> cells; MØ were defined as F4/80<sup>+</sup> cells. Values are the means ± SEM of 2 pooled independent experiments, n=6 per group. Each symbol represents an individual mouse. Statistical significance was assessed using Student's t test (* p < 0.05, ** p < 0.01, *** p < 0.001) (A, C and D). Mann-Whitney test was used to determine statistical significance of IL-12-positive MyD88 WT vs. KO DC in E. These experiments were performed three times with similar results.
Parasite GRA24 plays a key role in MyD88-independent p38 MAPK-dependent IL-12 production

*T. gondii* dense granule protein GRA24 has been shown to trigger p38 MAPK-dependent IL-12 [35]. To examine the contribution of GRA24 to MyD88-independent IL-12, we assessed IL-12 production by the uracil auxotroph strain *ΔompdcΔup* (designated as *cps1-1*) and the corresponding GRA24 knockout strain *ΔompdcupΔgra24* (designated as *cps1-1:Δgra24*) [40, 41]. Inclusion or exclusion of exogenous uracil enabled us to determine the influence of tachyzoite proliferation on IL-12 production and MAPK activation. Fig. 2.5 shows images of *cps1-1* (panel A) and *cps1-1:Δgra24* (panel C) on fibroblasts in the presence of uracil. At this time point (48 hr post-infection), cells were heavily infected and parasite egress was readily observed with uracil supplementation. In parallel cultures without uracil (Fig.2.5B and D), growth of both strains was restricted to ≤2 parasite divisions. Percent infection and average parasites per vacuole were measured in the *cps1-1* and *cps1-1:Δgra24* infected fibroblasts in the presence and absence of exogenous uracil (Fig. 2.5E). We found that presence or absence of GRA24 had no significant effect on infection or replication rate whether or not exogenous uracil was provided.
Figure 2.5 Both \textit{cps1-1} and \textit{cps1-1:Δgra24} tachyzoites require supplemental uracil for sustained replication. Tachyzoites of strains \textit{cps1-1} or \textit{cps1-1:Δgra24} were inoculated onto human foreskin fibroblast monolayers in the presence (A and C) or absence (B and D) of exogenous uracil. Two days after infection, monolayers were fixed and stained with FITC conjugated anti-\textit{Toxoplasma} antibody (green), phalloidin-Texas red to stain host cell actin, and DAPI (blue) to stain nuclei. Images were collected under 40x magnification. (E) Percent infection and the average parasites per vacuole were quantified in \textit{cps1-1} and \textit{cps1-1:Δgra24} infected fibroblasts in the presence and absence of exogenous uracil. This experiment was repeated twice with the same result. Data shown are the mean counts of three independent experiments. Statistical significance was assessed using Mann-Whitney test comparing \textit{cps1-1} (+/−) exogenous uracil and \textit{cps1-1:Δgra24} (+/−) exogenous uracil (E) ( *** p < 0.001).
MyD88+/+ and MyD88−/− BMDM were inoculated with either cps1-1 and cps1-1:Δgra24 tachyzoites and supernatants were collected 48 hr later for cytokine assay (Fig. 2.6A). In the presence of exogenous uracil, proliferating cps1-1 parasites triggered IL-12p40 in both WT and KO BMDM (Fig. 2.6A, left panels). Strikingly, these responses failed to occur using GRA24-negative parasites. Because parasite-induced IL-12 was not produced until 24-36 hr post-infection, it was possible the response was tied to tachyzoite proliferation. Accordingly, the experiment was conducted in the absence of exogenous uracil. Even when proliferation was restricted, cps1-1 tachyzoites maintained the ability to induce IL-12 in dependence upon GRA24 (Fig. 2.6A, right panels). We also generated two independent GRA24 complementation mutants, designated cps1-1:gra24C1 and cps1-1:gra24C2, and measured IL-12p40 production in BMDM. As expected, restoration of GRA24 expression in cps1-1:Δgra24 parasites also reestablished production of IL-12 (Fig. 2.6B).

We next examined the influence of GRA24 on p38 MAPK activation in the context of cps1-1 infection of BMDM. Interestingly, infection triggered an early wave of p38 phosphorylation (≤ 15 min post infection) followed by dephosphorylation and a later wave that occurred 36 hr after infection (Fig. 2.6C). Both responses occurred independently of MyD88, but the second wave was strictly dependent upon GRA24 (Fig. 2.6C). Type I Toxoplasma is known to activate both STAT3 and ERK1/2 [42-44]. Accordingly, we examined the influence of MyD88 and GRA24 on these responses. We found neither host MyD88 nor parasite GRA24 influenced ERK1/2
phosphorylation (Fig. 2.7A) or STAT3 phosphorylation (Fig. 2.7B). The experiment in Fig. 2.6C was carried out in the presence of uracil, and we therefore wondered if the second wave of p38 activation was driven by parasite proliferation and possibly host cell lysis and reinfection of new cells. Accordingly, we examined cps1-1-driven p38 MAPK activation under proliferation permissive (+ uracil) and proliferation non-permissive (- uracil) conditions (Fig. 2.6D). Even when parasite replication was prevented by exclusion of uracil, we saw the same two-wave pattern of p38 MAPK phosphorylation.

Production of GRA24-dependent IL-12 correlated most closely with the late wave of p38 phosphorylation, which was also dependent upon GRA24. To formally demonstrate that late p38 phosphorylation was the key event in cps1-1-induced IL-12 production, we employed timed addition of the small molecule p38 MAPK inhibitor SB202190 [45]. Addition of the inhibitor 1 hr prior to infection (“Early add”) completely prevented IL-12 production during infection (Fig. 2.6E), as measured in 48 hr supernatants. Addition of the inhibitor 10 hr after infection (“Late add”) also almost completely blocked the IL-12 response. Thus, the second wave of p38 activation stimulated by GRA24 underlies delayed IL-12 production in Toxoplasma infected BMDM.
Figure 2.6 GRA24 controls MyD88-independent p38 MAPK-dependent IL-12 production. (A) MyD88<sup>+/+</sup> and MyD88<sup>-/-</sup> BMDM were infected with cps1-1 or cps1-1:Δgra24 tachyzoites at the indicated parasite to cell ratios. Cultures were initiated with (+) and without (-) exogenous uracil and supernatants were collected 48 hr after infection. C, control non-infected cells. (B) MyD88<sup>-/-</sup> BMDM were inoculated with cps1-1, cps1-1:Δgra24, cps1-1:gra24C1 or cps1-1:gra24C2 tachyzoites at a 2:1 parasite to cell ratio. Supernatants were collected 48 hr post infection for IL-12 measurement. Production of IL-12 was significantly reduced in cells infected with cps1-1:Δgra24 in pairwise comparisons with cps1-1 and the two complementation mutants. (C) cps1-1 or cps1-1:Δgra24 tachyzoites were inoculated onto MyD88<sup>+/+</sup> and MyD88<sup>-/-</sup> BMDM at a ratio of 2:1. Cultures were briefly centrifuged to initiate parasite and cell contact, then lysates were prepared for Western blot assay at the indicated time points after infection (hr). (D) WT BMDM were infected with cps1-1 parasites in the presence and absence of uracil, then lysates were prepared for Western blot analysis as in (C). (E) WT BMDM were infected with cps1-1 tachyzoites in the presence and absence of p38 MAPK inhibitor SB202190 (10 µM). Supernatants were collected 36 hr after infection for cytokine ELISA. The inhibitor was added either 1 hr prior to infection (“Early add”) or 10 hr after infection (“Late add”). Data shown are the means ± SD of cells cultured in triplicate. Statistical significance was evaluated using Student’s t test (* p < 0.05, *** p < 0.001). These collective experiments were repeated with the similar result two times.
Figure 2.7 GRA24 does not control phosphorylation of ERK1/2 or phosphorylation of STAT3 triggered by *Toxoplasma*. BMDM from *MyD88*+/− and *MyD88*−/− mice were infected with *cps1-1* or *cps1-1:Δgra24* tachyzoites at a 1:1 ratio of parasites to cells. Cell lysates were prepared for Western blot analysis at the indicated time points.
GRA24 regulates IL-12p40 and other cytokines and chemokines during *in vivo* infection

To determine if GRA24 controls production of IL-12p40 during *in vivo* infection, *MyD88*<sup>+/−</sup> and *MyD88*<sup>−/−</sup> mice were infected with either *cps1-1* or *cps1-1:Δgra24* by i. p. injection. Four days post infection, PEC were harvested and cultured without further stimulation. IL-12p40 was measured in the supernatants at 24 hr, 48 hr and 72 hr. In line with the BMDM responses, *cps1-1* infected *MyD88*<sup>+/−</sup> PEC produced approximately twice as much IL-12p40 compared to the *cps1-1:Δgra24* infected mice (Fig. 2.8A). Furthermore, there was a near complete ablation of IL-12 production in MyD88 KO mice infected with the GRA24 deletion mutant (Fig. 2.8A). To determine if GRA24 controls expression of other chemokines and cytokines, we used a proteomic array to screen a total of 111 cytokines, chemokines and related immune mediators released by PEC isolated from WT mice infected with *cps1-1* and *cps1-1:Δgra24*. Figure 2.8B shows that overall, a large collection of cytokines and chemokines were up-regulated in PEC isolated from mice infected with each parasite strain. A schematic showing the coordinates of each cytokine and chemokine on the proteomic array is shown in Fig. 2.9. A collection of immune-related factors were up-regulated similarly during infection with the two parasite strains. Figure 2.8C shows factors up-regulated 20-fold or greater above background by both parasite strains. A subset of cytokines and chemokines were clearly up-regulated in dependence upon GRA24, including IL-12p40 and CCL17 (Fig. 2.8D). Interestingly, we also identified a subset of immune-related mediators whose expression was negatively regulated by GRA24.
including CXCL1 and CXCL2 (Fig. 2.8E). As independent confirmation of these results, we used ELISA to measure CCL17. The result shows CCL17 production is clearly dependent upon GRA24 (Fig. 2.8F). Together, these results demonstrate that GRA24 positively and negatively regulates distinct sets of cytokines and chemokines during \textit{in vivo} \textit{T. gondii} infection.
**Figure 2.8** GRA24 controls the up-regulation of IL-12p40 and other cytokines and chemokines during *T. gondii* infection. (A) *MyD88*+/+ and *MyD88*−/− mice (n = 3 per group) were infected by intraperitoneal injection of 10^6* cps1-1 or cps1-1:Δgra24* tachyzoites. Four days later, cells were harvested from the peritoneal cavity and cultured without further stimulation for the indicated times. (B) *MyD88*+/+ mice (n = 2 per group) were infected by i. p. inoculation with 10^6* cps1-1 or cps1-1:Δgra24* tachyzoites. Four days later, peritoneal exudate cells were harvested and cultured for 72 hr. The supernatants were harvested and prepared for cytokine proteome array. (C) Cytokine mean pixel intensity of the proteomic cytokine array in (B) that were equivalent between *MyD88*+/+ mice infected with *cps1-1* and *cps1-1:Δgra24*, and that were greater than 20 fold up-regulated above background. (D) Cytokines up-regulated in *MyD88*+/+ mice infected with *cps1-1* relative to *cps1-1:Δgra24* in the cytokine proteome array. (E) Cytokines in the proteomic array that were up-regulated in *cps1-1:Δgra24* relative to *cps1-1* in infected *MyD88*+/+ mice. (F) *MyD88*+/+ mice (n = 2 per group) were infected, cells were collected and cultured as in (B). The supernatants were harvested and CCL17 was measured by ELISA. Data shown are the means ± SD of cells cultured in triplicate. Statistical significance was assessed using Student’s t test (** p < 0.001, *** p < 0.001). These collective experiments were repeated 2-3 times.
Figure 2.9. **Cytokine Proteome Array Coordinates.** Diagram indicating the coordinates of each cytokine and chemokine included on the cytokine proteome array.
GRA24 plays a role in protective immunity independent of the MyD88 adaptor protein

Vaccination with \( \text{cps1-1} \) induces a strong Th1 response and protective immunity in both \( \text{MyD88}^{+/+} \) and \( \text{MyD88}^{-/-} \) mice [33]. We wanted to determine the role of GRA24 in the response. Accordingly, we employed a vaccination protocol involving two sequential inoculations with \( \text{cps1-1} \) and \( \text{cps1-1:Δgra24} \) parasite strains. Two weeks after the final inoculation, a splenocyte cytokine recall assay was performed using soluble tachyzoite antigen (STAg). The results (Fig. 2.10A) revealed that vaccination of WT mice with either \( \text{cps1-1} \) or \( \text{cps1-1:Δgra24} \) tachyzoites induced a strong IFN-\( \gamma \) response. However, the splenic recall response in \( \text{cps1-1:Δgra24} \) vaccinated MyD88 KO mice was significantly weaker than the corresponding \( \text{cps1-1} \) response (Fig. 2.10A). Production of IFN-\( \gamma \) during \( T. \ gondii \) infection is strongly dependent upon IL-12 in wild type mice [46]. To determine if the IFN-\( \gamma \) recall response in \( \text{cps1-1} \) vaccinated \( \text{MyD88}^{-/-} \) mice was dependent upon IL-12, we treated \( \text{MyD88}^{-/-} \) mice with a depleting anti-IL-12p40 mAb or rat immunoglobulin isotype control over the course of an i. p. infection. One week following infection, splenocytes were subjected to an \textit{in vitro} recall assay with STAg. The results (Fig. 2.10B) show that \textit{in vivo} depletion of IL-12 substantially reduces the IFN-\( \gamma \) response relative to the Ab control-treated mice. We also examined the IFN-\( \gamma \) response in mesenteric lymph nodes in the vaccinated mice. Interestingly, production of IFN-\( \gamma \) was completely dependent upon MyD88 at this location (Fig. 2.10C).
To determine if GRA24 was responsible for protective immunity elicited by \textit{cps1-1}, we determined how \textit{cps1-1} vaccination with or without GRA24 impacted systemic parasite burden in MyD88 deficient mice following challenge infection with virulent RH strain tachyzoites. Nine days after challenge, spleen, liver, lungs and MLN were collected and parasite burden was measured by quantitative PCR measurement of the \textit{Toxoplasma} B1 gene relative to the host argininosuccinate lyase gene. As shown in Fig. 2.10D, there was a dramatic increase in parasite burden in all four organs in the \textit{cps1-1:Δgra24} vaccinated mice compared to the \textit{cps1-1} vaccinated mice.

To further elucidate the impact of GRA24 during vaccination in induction of protective immunity, we monitored survival in lethally challenged animals. Non-vaccinated \textit{MyD88}^{+/+} and \textit{MyD88}^{-/-} mice died by day 10 after challenge (Fig. 2.10E and F). In contrast, \textit{MyD88}^{+/+} mice vaccinated with \textit{cps1-1} and \textit{cps1-1:Δgra24} were resistant to challenge (Fig. 2.10E). While WT mice vaccinated with \textit{cps1-1:Δgra24} appeared slightly less resistant, this result was not statistically significant. \textit{MyD88}^{-/-} mice vaccinated with \textit{cps1-1} were resistant to RH inoculation, with animals beginning to succumb at approximately 3 weeks post-challenge. In striking contrast MyD88 KO animals vaccinated with the GRA24 deletion mutant were significantly more susceptible to challenge (Fig. 2.10F). We also note that this group of mice appeared more resistant than non-vaccinated counterparts, indicating the presence of protective mechanisms that involve neither GRA24 nor MyD88. Regardless, these collective results demonstrate that GRA24 elicits
MyD88 independent immunity, thereby limiting systemic dissemination and enhancing host survival.
A  

IFN-γ (pg/mL)  

B  

MyD88−/−  

C  

IFN-γ (pg/mL)  

D  

Parasite genomes/Host genomes  

E  

MyD88+/+  

F  

MyD88−/−
Figure 2.10 GRA24 induces protective immunity in the absence of MyD88. (A) MyD88<sup>+/+</sup> and MyD88<sup>−/−</sup> animals (n=5-6 mice per group) were i. p. inoculated at Day 0 and Day 14 with 10<sup>6</sup> cps1-1 or cps1-1:Δgra24 tachyzoites, then at day 28 splenocytes were isolated and subjected to *in vitro* stimulation with soluble tachyzoite antigen (STAg; 50 µg/ml). Supernatants were collected at 72 hr for cytokine ELISA. Each point represents a single mouse. (B) MyD88<sup>−/−</sup> mice (n=3 mice per group) were administered IL-12 neutralizing antibody or isotype control antibody during the course of infection initiated by inoculation with 5 x 10<sup>6</sup> cps1-1 tachyzoites. At Day 10 post-infection, splenocytes were isolated and stimulated with STAg at the indicated concentrations (µg/ml), then 72 hr later supernatants were collected for IFN-γ assay. NI, splenocytes from noninfected mice. M1, M2, M3 are individual MyD88<sup>−/−</sup> mice treated with the isotype control and M4, M5, M6 are individual MyD88<sup>−/−</sup> mice treated with the IL-12 neutralizing antibody. (C) Mesenteric lymph node cells were pooled from vaccinated mice, and subjected to STAg re-stimulation and cytokine assay as in (A). C, control cells cultured in medium alone. (D) MyD88<sup>−/−</sup>/IL-12p40<sub>eYFP/eYFP</sub> mice (n=7 per group) were vaccinated by i. p. inoculation with cps1-1 or cps1-1:Δgra24 tachyzoites. Two weeks after the final inoculation, mice were challenged with 10<sup>3</sup> RH tachyzoites. Nine days post challenge tissues were harvested from each individual mouse and processed independently for parasite quantitation by qPCR. In E and F, MyD88<sup>+/+</sup> and MyD88<sup>−/−</sup> animals were vaccinated by i. p. inoculation with cps1-1 or cps1-1:Δgra24 tachyzoites as in (D). Two weeks after the final inoculation, mice were challenged by subcutaneous injection with 10<sup>3</sup> RH strain tachyzoites, and survival was monitored. Data shown are the means ± SD of cells cultured in triplicate (A, B and C). Statistical significance was evaluated using Student’s t test (A, C, D). A Mann-Whitney test was used to measure statistical significance between isotype controls and the corresponding anti-IL-12 concentrations (B). Log rank Mantel-Cox test was used to measure the statistical significance between the survival curves. (E and F). The asterisks in F indicate MyD88<sup>−/−</sup> mice vaccinated with the cps1-1:Δgra24 strain were significantly more susceptible than cps1-1 vaccinated mice, and that animals vaccinated with the cps1-1:Δgra24 strain were significantly more resistant than the nonvaccinated group. For the results shown, * p < 0.05, ** p < 0.01, and *** p < 0.001. These collective experiments were repeated with the same result two to three times.
Discussion

The TLR/MyD88 signaling module in mice underlies resistance to a large number of pathogens, including *T. gondii*. However, the emerging realization that this axis of immunity is likely less critical for anti-microbial defense in humans has prompted significant interest in identifying MyD88-independent pathways of resistance. Here, we identify an MyD88-independent pathway of immunity to *T. gondii* that relies upon host p38 MAPK phosphorylation initiated by parasite dense granule protein GRA24. Activation of p38 MAPK by GRA24 triggers production of IL-12 during *in vitro* and *in vivo* models of infection. We also identified a novel subset of cytokines and chemokines whose expression during *in vivo* infection was upregulated by GRA24, and a unique subset that was down-regulated by this dense granule protein. In a vaccination model employing the uracil auxotrophic *Toxoplasma* strain *cps1-1*, we found that GRA24 drives an MyD88-independent Type I cytokine response associated with production of IFN-γ and protection from lethal challenge infection.

Evidence for an unusual pathway of *T. gondii*-driven p38 MAPK autophosphorylation during *in vitro* infection of mouse macrophages was first uncovered several years ago [34]. GRA24 was subsequently identified as the critical parasite effector protein using in silico methods to predict proteins capable of both entering the parasite secretory pathway and targeting the host cell nucleus [35, 47, 48]. GRA24 is unusual in that it has no homology to genes in other Apicomplexa. Interestingly, the closely related apicomplexan *Neospora caninum*
uses a distinct pathway to phosphorylate host p38 MAPK dependent upon G-protein-coupled receptor signaling [49]. In contrast to our results, p38 MAPK activation by *N. caninum* is associated with IL-12 down-regulation and evasion of host immunity.

Like other dense granule proteins, GRA24 is composed of intrinsically disordered regions that are predicted to favor its activity as a host-directed effector molecule [50]. The protein is exported across the parasitophorous vacuole membrane using a translocation pathway involving parasite proteins MYR1 and ASP5 [51-53]. GRA24 subsequently binds to p38α MAPK, triggering allosteric autoactivation, nuclear translocation and upregulation of transcription factors such as Egr-1 and c-fos [35, 54]. Despite a relatively clear picture of GRA24 function inside the host cell, our understanding of the importance of this parasite molecule in MyD88-independent pathways of Th1-induction and protective immunity has been relatively limited.

Our *in vitro* infection experiments revealed a bi-phasic pattern of p38 MAPK activation in which the kinase was activated in a GRA24-independent manner within minutes of infection, followed by a second GRA24-dependent activation occurring 24-36 hr later. The second wave occurred even when replication and egress was prevented by excluding exogenous uracil, a result that argues against cell lysis and secondary infection as the stimulus for GRA24-dependent p38 MAPK phosphorylation. While we do not understand the underlying basis for this pattern,
it may be noteworthy that GRA24 appears in the host cell nucleus only at later points in infection [35]. Regardless, it appears that the delayed wave of GRA24-dependent p38 MAPK phosphorylation is the critical driver of IL-12. First, IL-12 is only produced at later time points (36-48 hr) of BMDM infection, in contrast to LPS that triggers a response within the first 6 hr of stimulation. Second, addition of a pharmacological p38 MAPK inhibitor during the eclipse phase of parasite-induced p38 phosphorylation (10 hr post-infection) potently blocked subsequent IL-12 secretion.

The cause and significance of the early MyD88-independent p38 MAPK activation event is unclear at present. However, it is interesting to note that *Toxoplasma*-triggered STAT3 and STAT6 activation follows a similar pattern, with an early ROP16-independent phase followed by a later phase dependent upon this parasite kinase [42]. In this case, early STAT3 activation is dependent upon a FAK-Src-STAT3 signaling pathway associated with invasion [55]. We are currently examining whether this pathway also triggers early p38 MAPK activation.

We were able to induce an MyD88-independent protective immune response that depended upon GRA24 activity in a vaccine strain of the parasite. Yet, we also observed that even in the absence of this dense granule protein there remained a significant MyD88-independent protective response. The latter result provides strong evidence for the existence of other pathways of protection that rely on neither host TLR/MyD88 nor parasite GRA24. A recent study employing human
cells revealed that peripheral blood monocytes and dendritic cells produce IL-12 only after phagocytosis of live tachyzoites, a process not generally associated with involvement of TLR or MyD88 [56]. It has also been shown that TLR11-independent inflammasome activation triggers Th1 immunity during Toxoplasma infection and the neutrophils can provide an early source on IFN-γ independently of TLR signaling [57, 58]. It is possible that these phenomena contribute to MyD88-independent activation of immunity in the mouse model employed here. It has also been reported that the T. gondii dense granule protein GRA15 possesses proinflammatory cytokine-inducing activity. GRA15 is trafficked to the parasitophorous vacuole membrane where it activates NFκB signaling independently of MyD88. This in turn leads to induction of IL-12 [59]. However, GRA15 from Type I parasite strains (including the cps1-1 strain used here) does not display this activity and is therefore unlikely to account for cps1-1-induced, GRA24-independent immunity in MyD88−/− mice.

Dense granule proteins GRA24 and GRA15 are members of a growing family of Toxoplasma host-directed effectors that target signaling to remodel cell function [60]. Parasite GRA6 activates host transcription factor NFAT4 through binding to calcineurin activator calcium-modulating ligand (CAMLG) [61]. GRA6-dependent NFAT4 activation results in production of the chemokines CCL2 and CXCL2 which together promote recruitment of inflammatory monocytes and neutrophils. Because these cells are targets of infection, they may play a role in parasite dissemination. By interacting with herpesvirus-associated ubiquitin-specific
protease (HAUSP) and the phosphate PP2A-B55, GRA16 alters steady-state levels of host p53, which may be important in host cell survival under conditions of stress associated with intracellular infection [47]. Through secretion of dense granule protein TgIST, *Toxoplasma* potently suppresses STAT1-induced gene expression. In the infected cell, this results in generalized nonresponsiveness to the anti-microbial effects of IFN-γ [62, 63].

Rhoptries contain host-directed effector molecules that are released early during invasion. Rhoptry protein ROP16 is a host-directed kinase that is injected into the host cytoplasm during invasion where it activates both STAT3 and STAT6 [42, 64]. In macrophages, this results in deviation to an M2 phenotype and down-regulation of IL-12 [65]. The *Toxoplasma* rhoptry protein TgWIP is secreted into the host cell where it interacts with the WAVE regulatory complex and SHP2 phosphatase. This impacts actin dynamics in dendritic cells, enhancing host cell motility that in turn promotes parasite dissemination [66]. Together, these studies form the basis for our expanding understanding of *T. gondii* as a microbial parasite equipped with a sophisticated toolbox used during infection to deal with the host immune system.

GRA24 is an effector molecule that the parasite deploys to activate signaling leading to IL-12. In one sense this appears counter-intuitive given that this cytokine is critical in activation of immunity leading to parasite elimination [5, 46, 67]. However, mice lacking IL-12 succumb extremely rapidly to infection before parasite latency is established [11]. Our findings support the model that GRA24
induces MyD88-independent IL-12 production to promote survival of the host in acute infection and parasite survival and transmission by promoting latency. The ability to establish a balance between excessive immunity and inadequate immunity is likely a major factor accounting for the evolutionary success of *Toxoplasma.*
**Materials and Methods**

**Ethics Statement**

All experiments performed in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Protocols were approved by the Institutional Animal Care and Use Committee at the University of New Mexico (Animal Welfare Assurance Number A4023-01). All efforts were made to reduce animal suffering during this study.

**Mice**

Female and male mice (6-12 weeks of age) were used in these studies. Female C57BL/6 mice (5 weeks of age) were purchased from Taconic Biosciences Inc. (Rensselaer, NY). Female and male (5 weeks of age) B6.1292(SPJ)-MyD88<sup>tm1.1Defr/J</sup> (MyD88<sup>-/-</sup>) and B6.129-IL12b<sup>tm1.1Kly/J</sup> (IL-12p40<sup>eYFP/eYFP</sup>) mouse strains were purchased from The Jackson Laboratory (Bar Harbor, ME). The MyD88<sup>-/-</sup> and IL-12p40<sup>eYFP/eYFP</sup> mouse strains were crossed in house to generate a MyD88<sup>-/-</sup>/IL-12p40<sup>eYFP</sup> strain. The MyD88<sup>-/-</sup>, IL-12p40<sup>eYFP</sup> and MyD88<sup>-/-</sup>/IL-12p40<sup>eYFP</sup> strains were bred in-house in the University of New Mexico Department of Biology Animal Research Facility. Mouse genotyping was performed by Transnetyx, Inc. (Cordova, TN).

**Parasites**

Confluent human foreskin fibroblast monolayers (HFF; ATCC, Manassas, VA) were infected with *T. gondii* Type I (RH) or Type II (PTG) strains and passaged...
weekly into confluent HFF monolayers. HFF were grown to confluence in Dulbecco’s Modified Eagle’s Medium (DMEM) (VWR, Randor, PA) supplemented with 1 mM glutamine and 10% bovine growth serum (FBS; HyClone, Logan, UT), 100 U/ml penicillin (ThermoFisher Scientific, Waltham, MA) and 0.1 mg/ml streptomycin (ThermoFisher Scientific). Construction of the uracil auxotroph strains \( \Delta ompdc \Delta up \) (designated as \( cps1\text{-}1 \)) and \( \Delta ompdc \Delta up \Delta gra24 \) (designated as \( cps1\text{-}1: \Delta gra24 \)) has previously been described [40, 41].

The \( cps1\text{-}1: \Delta gra24 \) strain was complemented with a C-terminal HA-tagged copy of GRA24 to independently isolate complemented strains \( cps1\text{-}1:gra24C1 \) and \( cps1\text{-}1:gra24C2 \). The gra24C targeting plasmid was constructed by recombining 5 PCR fragments using forward (F) and reverse (R) primers to synthesize the 5’ target region (F = TTGGGTAACGCGAGGTTTTCCCCAGTCACGACGGTTTAAACCTAGGACAGATGTCCTCATCTCAGCGTCC; R = GGTGCAGACGTTCGCTGCTG), the N-terminal region (F = GGTTGATGAGAGCGGTCCAGC; R = GGGATTATTGTGCCGGGTTGGC), the middle region (F = GGTTCGGACGACGCTCTGCTGTATGTC), the C-terminal region (F = TCGGTGTCTCGCTGACATACAGAG; R = GACTTTGTCTCGCTCCTTGTAGTCCGATTACCCTTAGGTTGGGTTTTAAGCAGT), and the 3’ target region (F = CTGGAGTCTTGTAGATCCCTTACCTTAGGTTGGGTTTTAAGCGATG), and the 3’ target region (F = CTGGAGTCTTGTAGATCCCTTACCTTAGGTTGGGTTTTAAGCGATG).

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Complemented strains *cps1-1:gra24C1* and *cps1-1:gra24C2* were isolated after selection in 6-thioxanthine and uracil as previously described [40], and insertion of HA-tagged GRA24 at the GRA24 locus was confirmed using primer pairs (F = CAGCCAACAACGACACTCAGCG; R = GTTGGCCTACCATGTGCTGAACC), and (F = ACCCATACGATGTCCAGATTACGC; R = CAGGCAACGCGGTGA TCCAC).

The mutant parasites were maintained in DMEM (VWR) supplemented with 300 µM uracil, 1% FBS (ThermoFisher Scientific), 100 U/ml penicillin (ThermoFisher Scientific) and 0.1 mg/ml streptomycin (ThermoFisher Scientific). Parasite propagation was maintained by passaging weekly onto a confluent HFF monolayer. To verify that all uracil auxotroph *T. gondii* strains maintained dependence upon exogenous uracil, the parasites were passaged into growth medium without supplemental uracil once a month. Parasites were tested for *Mycoplasma* contamination every 3 months employing the MycoProbe detection assay (R & D Systems, Minneapolis, MN).

**Infections and Vaccination**

*Toxoplasma* was administered to mice by intraperitoneal injection of tachyzoites into mice 6-12 weeks of age. Animals were vaccinated by two intraperitoneal injections (10⁶ tachyzoites) of *cps1-1* or *cps1-1:Δgra24* administered two weeks apart. Two weeks after the final vaccination mice were challenged by subcutaneous injection of 10³ RH strain tachyzoites.
Cell Culture

Bone marrow was isolated from the tibias of donor mouse strains C57BL/6, *MyD88*<sup>−/−</sup>, *RIPK3*<sup>−/−</sup> and *RIPK3*<sup>−/−</sup>*Caspase8*<sup>−/−</sup>. A 10 ml syringe loaded with a 27 Ga needle was used to flush the bone marrow out of the tibias into a 50 ml conical tube. The media containing the bone marrow was subsequently passed through an 18 Ga needle. The bone marrow suspension was plated into five petri dishes, and cells were allowed to differentiate into bone marrow derived macrophages (BMDM) in DMEM (VWR) supplemented with 10% BGS (HyClone), nonessential amino acids (ThermoFisher Scientific), 100 U/mL penicillin (ThermoFisher Scientific), 0.1 mg/mL streptomycin (ThermoFisher Scientific) and L929 culture supernatant as previously described [42]. BMDM were harvested after five days of differentiation for use in experiments.

Peritoneal exudate cells (PEC) were collected by filling the peritoneal cavity with sterile PBS then using a 21 Ga needle to collect the PBS containing PEC. The cells were re-suspended in cDMEM consisting of DMEM (VWR) supplemented with 10% bovine growth serum (HyClone), 1% sodium pyruvate (ThermoFisher Scientific), 1% Non-Essential Amino Acids (ThermoFisher Scientific), 3% HEPES (ThermoFisher Scientific), 0.0001% 2-mercaptoethanol (Sigma, St. Louis, MO), 100 U/ml penicillin (ThermoFisher Scientific) and 0.1 mg/ml streptomycin (ThermoFisher Scientific). Splenic single cell suspensions were prepared by homogenizing spleens, then lysing red blood cells with RBC ACK lysis buffer (ThermoFisher Scientific) as previously described [33]. Removal of cell debris was
accomplished by filtration through a 40 μm cell strainer. Cells were washed in PBS and re-suspended in cDMEM. Mesenteric lymph node (MLN) single cell suspensions were prepared by crushing the tissue through a 40 μm cell strainer. The cells were re-suspended in cDMEM. Cells were stimulated with soluble tachyzoite lysate antigen (STAg) and supernatants were collected for cytokine ELISA 72 hr later. STAg was prepared from RH strain tachyzoites by sonication of parasites at 0°C in the presence of protease inhibitors, followed by centrifugation at 10,000 x g. Supernatant was collected and dialyzed into PBS and stored in aliquots at -80°C.

In Vivo IL-12 Depletion
Mice were i. p. injected with 0.5 mg anti-mouse IL-12p40 mAb (BioXCell, New Hampshire, Catalog #BE0051) or normal rat gamma globulin (Jackson ImmunoResearch, West Grove PA, Catalog #012000002) at Day 0. On Day 1, mice were i. p. inoculated with 5 x 10^6 cps1-1 or cps1-1:Δgra24 tachyzoites. On Days 3 and 6, mice received further injections with anti-IL-12 mAb or control Ab (0.5 mg per mouse). On Day 11 splenocytes were isolated for in vitro cytokine assay.

Flow Cytometry
Single cell suspensions of peritoneal exudate cells were stained with primary antibodies including anti-CD11c eFluor610 (Catalog # 61011482, eBioscience, San Diego, CA), anti-MHCII AlexaFluor 647 (Catalog # 107618, BioLegend, San Diego, CA), anti-F4/80 Brilliant Violet 711 (Catalog # 123147, BioLegend), and anti-Ly-6G PE/Cy7 (Catalog #127618, BioLegend) for 20 min at 4°C in FACS buffer
Cells were then fixed in 3.7% formaldehyde (MilliporeSigma, Burlington, MA) for 15 min and then permeabilized in permeabilization buffer (0.1% saponin in PBS). Intracellular \textit{T. gondii} tachyzoites were detected by staining with monoclonal anti-\textit{T. gondii} (TP3) AlexaFluor 700 (Catalog # NB1102570, Novus Biologicals, Centennial, CO) for 45 min at 4°C in permeabilization buffer. All samples were run on a four laser (violet, blue, yellow, red) Attune NxT flow cytometer (ThermoFisher Scientific) and the data were analyzed using FlowJo v.10 software (FlowJo, Ashland, OR).

**Cytokine ELISA**

The production of IL-12p40 and IFN-\(\gamma\) were measured using a murine IL-12/IL-23p40 ELISA Kit (Invitrogen, Carlsbad, CA) and a IFN-\(\gamma\) ELISA Kit (Invitrogen) according to the manufacturer’s instruction. Briefly, 96 well ELISA plates (Corning Costar, Corning, NY) were coated with anti-cytokine capture antibody in coating buffer (Invitrogen) and incubated overnight at 4°C. The plates were washed 3 times in PBS containing 0.05% Tween-20 (PBST) then blocked with diluent (Invitrogen) for 1 hr at room temperature. After washing the plates once in PBST, sample supernatants as well as recombinant standard was added and incubated overnight at 4°C. The plates were washed 5 times in PBST then anti-cytokine biotin detection antibody was added and plates were incubated at room temperature for 1 hr. The plates were washed 5 times in PBST, avidin-horseradish peroxidase was added and plates were incubated for 30 min at room temperature. The plates were washed 5 times in PBST and 3,3’,5,5’-Tetramethylbenzidine (TMB) was added and
plates incubated for 20 min. The reaction was quenched with 2 M H$_2$SO$_4$ and plates were read at 450 nm on an iMark ELISA reader (Bio-Rad, Hercules, CA).

**Western Blot Analysis**

BMDM were plated overnight in a 24-well tissue culture plate, then parasites were added at a 3:1 ratio of tachyzoites to cells. The plates were centrifuged at 63 x g for 3 min to initiate contact between parasites and cells. Lysates were collected using 200 µl SDS lysis buffer and passaged 3 times through a 27 Ga needle to shear DNA. The samples were boiled for 5 min and subjected to Western blot analysis using primary anti-phospho-p38 antibody (Catalog #4511, Cell Signaling, Danvers, Massachusetts), anti-phosphoERK1/2 (Catalog #4370, Cell Signaling), and anti-phosphoSTAT3 (Catalog #4074, Cell Signaling). Samples were also probed for total p38 (Catalog #9218, Cell Signaling), total ERK1/2 (Catalog #4695, Cell Signaling) and total STAT3 (Catalog #4904, Cell Signaling). Lysates were loaded into a 10% acrylamide protein gel (Bio-Rad). Proteins were transferred onto a nitrocellulose membrane then blocked with Tris-buffered PBS containing 5% nonfat dry milk and 0.05% Tween 20 for 2 hr. The nitrocellulose membranes were washed 3 times before the primary antibody was added in Tris-buffered PBS containing 5% BSA and 0.05% Tween 20 for overnight incubation on a rocking platform at 4°C. The nitrocellulose membranes were washed 6 times, then horseradish peroxidase-linked anti-rabbit antibody was added and membranes were incubated for 2 hr at room temperature on a rocking platform. A
chemiluminescent substrate system (ThermoFisher Scientific) was used to detect the bound antibodies.

**Cytokine Proteome Array**

Cytokine and chemokine expression was measured using the Murine Proteome Profiler Mouse XL Cytokine Array (R&D Systems, Minneapolis, MN). To obtain samples, C57BL/6 mice were i. p. inoculated with either $cps1-1$ or $cps1-1:\Delta gra24$ ($10^6$ tachyzoites per mouse). Four days post infection the peritoneal exudate cells were collected by filling the peritoneal cavity with sterile PBS then using a 21 Ga needle to collect the cell suspension. The PECs were plated in cDMEM at $2 \times 10^6$ cells per well and were incubated for 72 hr. Following incubation, the supernatants were collected and the Murine Proteome Profiler Mouse XL Cytokine Array was used according to the manufacturer’s directions. In brief, nitrocellulose membranes were blocked for 1 hr then sample supernatants were added to the membranes. After overnight incubation, membranes were washed and a detection antibody cocktail (R&D Systems) was added. The membranes were incubated with the detection antibody for 1 hr, washed, and streptavidin-horseradish peroxidase was added. After 30 min incubation, membranes were washed three times (10 min per wash) and spots visualized by addition of enhanced chemiluminescence reagent. The membranes were imaged on a Chemi Touch Imaging System (Bio-Rad), and semi-quantitative analysis was accomplished using ImageJ software.
qPCR for Parasite Load

DNA was extracted from tissues using the DNeasy Blood and Tissue Kit (Qiagen Inc. Valencia, CA) following the manufacturer’s instruction. Quantitative PCR was performed on the indicated tissue targeting the conserved *Toxoplasma* B1 gene and the murine argininosuccinate lyase (ASL) gene [21]. The extracted DNA from each tissue was normalized to 125 ng of DNA for each 20 μl reaction. A 5 μM primer working solution containing the forward and reverse primers for B1 (forward 5’-GGA-GGA-CTG-GCA-ACC-TGG-TGT-CG-3’, reverse 5’-TTG-TTT-CAC-CCG-GAC-CGT-TTA-GCA-3’) and a 5 μM working solution containing the forward and reverse primers for ASL (forward 5’-TCT-TCG-TTA-GCT-GGC-AAC-TCA-CCT-3’, reverse 5’-ATG-ACC-CAG-CAG-CTA-AGC-AGA-TCA-3’) was made. 2.5 μl of primer solution of B1 or ASL was added to 10 μl SYBR green (Bio-Rad), 2.5 μl molecular biology grade water and 4 μl of template for a total volume of 20 μl. The reactions were carried out using a Bio-Rad CFX96 Real Time System C1000 Touch thermal cycler with the following thermal cycling conditions: 98°C for 5 min, 95°C for 5 sec, 60°C for 30 sec followed by 40 cycles of 95°C at 5 sec and 60°C for 30 sec. A melting curve analysis was performed to ensure specificity of amplification. Quantification of the number of B1 and ASL gene copy numbers was accomplished by using a quantitative standard curve. Bio-Rad CFX manager version 3 software was used to quantify B1 and ASL gene copy number. Non-infected tissues were used as negative controls and molecular grade water was used as a negative template control.
Immunofluorescence

HFF were grown to confluency on glass coverslips for five days. The confluent HFFs were infected with tachyzoites in 1% HFM in the presence or absence of uracil (0.3 mM). After 48 hrs, coverslips were washed in PBS, then fixed with 3.7% PFA for 20 min. The cells were washed in permeabilization buffer (PB; 0.1% saponin in PBS) then blocked in PB containing 5% normal mouse serum (Invitrogen) for 1 hour. After blocking, the cells were washed with PBS and stained by the addition of goat anti-Toxoplasma FITC-conjugated antibody (Catalog #PA17253, Invitrogen) and Texas Red-X phalloidin (Catalog #T7471, Invitrogen) diluted in PB containing 5% bovine serum albumin (VWR) for 1 hr. The cells were washed 3 times in PB and 4 times in PBS. Finally, the coverslips were dried then mounted onto slides using DAPI containing mounting media (Catalog #P36962, Invitrogen). Imaging was accomplished with a BX53 fluorescence microscope (Olympus America, Inc, Waltham, MA) and DP manager software (Olympus).

Quantification of Immunofluorescence staining

For the quantification of percent infection, 100-150 cells were counted per field of view (n=10 fields per treatment). Infected cells were counted and percent infection was calculated. To quantify average number of tachyzoites per vacuole, number of parasites in each parasitophorous vacuole (20-30 per field of view) was counted and average number of tachyzoites per vacuole was calculated.
**Statistical Analyses**

Student t-test was used to assess the significant difference between groups. A p value <0.05 was considered significant. A two way ANOVA with a Tukey’s multiple comparisons test was used to compare three groups across multiple time points. A Mann-Whitney test was used to assess statistical significance in the *in vivo* IL-12 depletion experiment. Survival after challenge with a virulent RH strain was assessed using a Kaplan-Meier curve and the statistical significant difference between groups was calculated using the Log-Rank test with the use of GraphPad Prism Software. All experiments were repeated 2-3 times.

**Acknowledgements**

We thank Dr. I. Brodsky (University of Pennsylvania) for generously providing tibias from *RIPK3*−/− and *RIPK3*−/−*Caspase8*−/− mice. We also thank Dr. E. Casadei for expert discussion and advice, and we gratefully acknowledge the services of the staff of the Animal Research Facility in the UNM Department of Biology.
References


Baba M, Batanova T, Kitoh K, Takashima Y. Adhesion of Toxoplasma gondii tachyzoite-infected vehicle leukocytes to capillary endothelial cells triggers


Chapter 3

p38 MAPK activation by *Toxoplasma gondii* secretory effector protein GRA24: Role in cytokine regulation
Abstract

The apicomplexan *Toxoplasma gondii* induces strong protective immunity in part dependent upon recognition by Toll-like receptors (TLR) 11 and 12 operating in conjunction with MyD88 in the murine host. However, these TLR are not present in humans, prompting us to investigate MyD88-independent signaling pathways of resistance. We have previously shown that parasite dense granule protein GRA24 induces p38 MAPK activation and subsequent IL-12 production in bone marrow derived mouse macrophages (BMDM). By i. p. inoculation with *cps1-1* and *cps1-1:Δgra24*, we identified unique subsets of chemokines and cytokines that were up and down regulated by GRA24. To gain deeper insight into genes regulated by GRA24, we employed qPCR to evaluate expression of transcripts for 84 cytokine and chemokine genes in BMDM infected with GRA24-positive compared to GRA24-negative parasites. Our results identify a specific signature of genes regulated by this dense granule protein. Furthermore, we employed qPCR to analyze the role of GRA24 role in the regulation of a select set of lncRNAs recently found in our lab to be upregulated by *Toxoplasma*. Taken together, our data identify GRA24 as a parasite protein that regulates expression of several other immune mediators in addition to IL-12.
Introduction

The protozoan parasite *Toxoplasma gondii* is an apicomplexan that infects most species of warm-blooded animals and birds [1]. It is estimated that *T. gondii* has infected 25-30% of the human population worldwide [2]. Infection mainly occurs through eating undercooked contaminated meat, accidental ingestion of oocysts from infected cat feces or through congenital transmission during pregnancy [2]. Upon infection, *T. gondii* has two phases, an acute phase and a latent/chronic phase. The acute phase is initiated in the intestine after oral infection which leads to the dissemination of invasive tachyzoites to the host tissues [3]. The latent stage follows the acute stage. Within the chronic phase, the tachyzoites differentiate into bradyzoites, the slowly dividing life stage. Bradyzoites are contained within a cyst structure and reside in the skeletal muscle and central nervous system tissue of the host [4]. Immunocompetent hosts infected with *T. gondii* are asymptomatic [5]. However, *T. gondii* is especially problematic in immunocompromised populations such as AIDS patients, where the parasites may reactivate from cysts resulting in unchecked parasite replication which can lead to toxoplasmic encephalitis and death [6].

*T. gondii* elicits a strong innate immune response that has been extensively studied in mice. Specifically, parasite profilin is detected by Toll like receptors (TLR) 11 and 12, initiating Interleukin 12 (IL-12) dependent immune responses [7]. The IL-12 cascade is induced via the MyD88- dependent signaling pathway [8]. IL-12 is essential in the induction of NK and CD4+ T cell interferon gamma (IFN-γ)
production which activates macrophages and leads to the destruction of the parasite [9, 10]. Splenic CD8α+ dendritic cells (DC) as well as intestinal CD103+ CD11b− and CD103− CD11b− DC are the main sources of MyD88-dependent IL-12 [11]. IFN-γ is important in activating macrophages for the destruction of the parasite. Furthermore, IFN-γ induces anti-Toxoplasma gondii effector molecules such as immunity-related GTPase (IRG) family and guanylate binding proteins (GBP) that disassemble the parasitophorous vacuole containing tachyzoites within the cytoplasm of infected cells [12-14].

We previously described the presence of an MyD88-independent pathway of immunity during T. gondii infection [15]. Although, MyD88 knockout mice (KO) infected with T. gondii succumb to death approximately 10 days after infection, we found that there is elicitation of an IFN-γ Th1 immune response in the days preceding death. Indeed, vaccination of MyD88 KO mice with the avirulent uracil auxotroph cps1-1 T. gondii strain induces protective immunity to lethal challenge with the virulent RH T. gondii strain [16]. Furthermore, RH has been shown to trigger IL-12 production by phosphorylation of p38 mitogen activated protein kinase (MAPK). Shortly after, dense granule protein GRA24 was discovered to be responsible for binding and triggering allosteric autoactivation of host p38 MAPK, inducing translocation into the nucleus and causing changes in host gene transcription [17]. This leads to the upregulation of genes including IL-12. Interestingly, we have shown that parasite GRA24 induces IL-12 production by p38 MAPK activation in MyD88 KO BMDM [18]. Additionally, we have shown that
GRA24 induces protective immunity independent of the MyD88 adaptor protein [18].

Long non-coding (lnc) RNAs are a group of non-translated non-ribosomal RNA that are defined as non-protein-coding transcripts that are greater than 200 nucleotides long. Such RNAs account for up to 85% of the transcriptome [19, 20]. LncRNAs are involved in gene regulation at the transcriptional and post transcriptional level and are known to be transcriptional coactivators, recruit chromatin modifiers, function as miRNA sponges, regulate splicing and stabilize mRNA [21-24]. Very little is understood about the role lncRNAs play in the host immune response to pathogens. Recently, it was shown lncRNA designated NONSHAT022487 was upregulated in macrophages infected with *T. gondii*. A correlation analysis was performed and NONSHAT022487 was shown to play a role in the suppression of UNC93B1 which is involved in TLR11 and TLR12 recognition of *T. gondii*. Moreover, *T. gondii* induces the expression of lncRNAs in regulating anti-inflammatory and pro-inflammatory responses [25]. Furthermore, studies in human retinal Müller glial cell infected with *T. gondii*, showed MIR17HG-lnc was significantly changed when comparing to infections with Type I and Type II *T. gondii* strains [26]. MIR17HG is involved in the regulation of Th17 immune responses [26, 27]. The production of IL-17 and IL23 have been implicated in the pathogenesis of ocular toxoplasmosis [26, 28]. Additionally, Menard, *et al.* revealed 282 host lncRNAs were regulated by both the virulent RH *T. gondii* strain as well as the less virulent PTG strain [25].
During invasion, tachyzoites secrete rhoptry protein ROP16 into the host cell cytosol. ROP16 localizes to the host cell nucleus and modulates the expression of genes. Expression of Csf1-lnc and Socs2-lnc were shown to be dependent upon ROP16. Csf-1-lnc is associated with the protein coding gene Csf1 which triggers differentiation of hematopoietic stem differentiation into macrophages [29]. Moreover, Socs-2 lnc which overlaps with Socs-2 gene has been implicated in anti-inflammatory responses to *T. gondii* [30]. Since these specific IncRNA were dependent upon ROP16 we wanted to elucidate if GRA24 is playing a role in modulating the expression of a subset of host lncRNA during infection. Additionally, GRA24 contains a nuclear localization sequence which indicates a possible role for host nuclear translocation and the potential for modulating lncRNA [17]. Due to GRA24 contains polymorphisms between the allele shared by types II and allele characterizing type I lineages, we hypothesize GRA24 is playing a role in the modulation of host lncRNA and virulence during *T. gondii* infection [17].

In this study, we further investigated the role of GRA24 in the expression of cytokine and chemokines within the host cell as well as the role of GRA24 in the regulation of a select subset of host IncRNAs recently identified in our lab to be upregulated by *Toxoplasma*. Using genetically engineered *cps1-1* and *cps1-1:Δgra24 T. gondii* strains, we examined the role of GRA24 in expression of 84 immune related genes in a pathway-specific PCR array. Additionally, by using a small molecule p38 MAPK inhibitor we addressed the possibility that GRA24
regulates a subset of chemokines and cytokines independently of p38 MAPK. We failed to obtain evidence that GRA24 regulates expression of a subset of IncRNAs and micro-RNAs. Nevertheless, our results show that GRA24 controls expression of several cytokines and chemokines in addition to IL-12, and that a subset of these appear to be regulated in a p38 MAPK-independent manner.
Results

**GRA24 controls the upregulation of cytokines and chemokines in T. gondii infected C57BL/6 BMDM**

We have previously shown that GRA24 controls the upregulation as well as the downregulation of certain cytokines and chemokines in peritoneal exudate cells of infected mice [18]. To gain further insight into GRA24 in controlling the expression of other cytokines and chemokine, we employed a commercial PCR array to interrogate the expression status of 84 cytokines and chemokine genes. We generated bone marrow derived macrophages (BMDM) from C57BL/6 mice and infected with *cps1-1* or *cps1-1:Δgra24* tachyzoites for 48 hr. The supernatants were collected and measured for IL-12p40 as well as CCL17 by enzyme linked immunosorbent assay (ELISA) and the cells were collected for qPCR analysis. As expected, BMDM infected with *cps1-1* produced two to three times more IL-12p40 than the BMDM infected with *cps1-1:Δgra24* (Figure 3.1A). Furthermore, there was a significant decrease in the production of CCL17 in the GRA24 KO infected BMDM compared to the BMDM infected with the *cps1-1* parent strain (Figure 3.1B). Of the 84 genes screened in the cytokine/chemokine array, a subset of genes were differentially expressed in the BMDM infected with *cps1-1* vs. the GRA24 knockout strain. A table of the $2^{\Delta\Delta CT}$ values shows the differential expression of C-X3-C Motif Chemokine Ligand 1 (CX3CL1), C-C Motif Chemokine Ligand 17 (CC17) and Pro-platelet basic protein (Ppbp) (Figure 3.1C). Figure 3.1D shows a heat map of the differentially expressed genes. Our results show
CX3CL1, CCL17 and Ppbp are down regulated in BMDM infected with GRA24 KO parasites (Figure 3.1D). Interestingly, CCL12 was much more upregulated in the samples infected in the absence of GRA24 in comparison to samples infected with GRA24 sufficient parasites (Figure 3.1C and D). There was a non-significant trend for altered IL-12 expression when comparing cps1-1 infected BMDM to GRA24 KO infected BMDM. Although the IL-12 data were non-significant in the qPCR data (p = 0.062), the IL-12 ELISA data in Figure 3.1A clearly confirms a significant decrease in the supernatants of GRA24 KO infected BMDM. Together, these results indicate GRA24 regulates the expression of a subset of cytokines and chemokines in BMDM in addition to IL-12.
Figure 3.1 GRA24 controls the upregulation of cytokines and chemokines in WT BMDM. C57BL/6 BMDM were infected with $5 \times 10^5$ cps1-1 or cps1-1:Δgra24 tachyzoites for 48 hr. (A) Supernatants were measured for IL-12p40 and (B) CCL17 by ELISA. Cells were collected for RNA extraction and qPCR analysis. (C) $2^{\Delta \Delta CT}$ values of WT BMDM infected with cps1-1 or cps1-1:Δgra24. (D) Heat map showing the gene expression of cytokines and chemokines in WT BMDM infected with cps1-1 or cps1-1:Δgra24. M1, M2 and M3 indicate individual experiments. Data shown are the means ± SD of cells cultured in triplicate. Statistical significance was assessed using a Student’s t test ( ** p<0.01, *** p<0.001, Statistical significance of heat map data *p<0.05).
p38 MAPK inhibition down regulates cytokines and chemokines in the presence of GRA24

To examine whether GRA24 regulates the expression of cytokines and chemokines independently of p38 MAPK, we utilized a p38 MAPK inhibitor (SB202190) during *in vitro* *T. gondii* infection. BMDM were pre-incubated with SB202190 for one hour before inoculating the cells with *cps1-1* tachyzoites. The plates were incubated for 48 hr and supernatants were collected and measured for IL-12p40 production by ELISA to confirm the activity of the inhibitor. As shown in Figure 3.2A and as expected, there was a substantial decrease in the production of IL-12p40 in the BMDM infected with *cps1-1* in the presence of SB202190 in 4 independent experiments. We collected cells from these experiments and extracted RNA for cDNA synthesis. The profile of cytokine and chemokine gene expression was determined using the same PCR array employed in Figure 3.1. Figure 3.2B and Table 3.1 shows the downregulation of many cytokines and chemokines including C-X-C Motif Chemokine Ligand 5 (CXCL5), C-C Motif Chemokine Ligand 5 (CCL7) and interleukin 10 (IL-10). Interestingly, interleukin 15 (IL-15) was upregulated in the presence of the p38 MAPK inhibitor. These results indicate the regulation of IL-15 is negatively regulated by p38 MAPK. Alternatively, C-C Motif Chemokine ligand 12 (CCL12) is downregulated in the *cps1-1* infected mice in the presence of SB202190 indicating a dependence upon p38 MAPK signaling (Figure 3.2B). This is a seemingly paradoxical result because we also found the absence of p38 activating GRA24, CCL12 expression increases (Figure 3.1C and D)
Figure 3.2 Inhibition of p38 MAPK down regulates cytokines and chemokines in the presence of GRA24

C57BL/6 BMDM were infected with 5X10^5 cps1-1 or tachyzoites for 48 hr in the presence and absence of p38 MAPK inhibitor SB202190. (A) Supernatants were measured for IL-12p40 by ELISA. Cells were collected for RNA extraction and qPCR analysis. (B) Heat map of the differential expression of cytokines and chemokines in BMDM infected with cps1-1 in the presence and absence SB202190. M1, M2, M3 and M4 indicate individual experiments. Data shown are the means ± SD of cells cultured in triplicate. Statistical significance was assessed using a Student’s t test (** p<0.01, *** p<0.001, **** p<0.0001, Statistical significance of heat map data *p<0.05).
Table 3.1 Table showing $2^{\Delta \Delta CT}$ values of differentially expressed cytokines and chemokines genes in the presence and absence of SB202190 with a p value $\leq 0.05$ WT BMDM were infected with 5X10^5 *cps1-1* with and without SB202190 for 48 hr and qPCR was performed. CT values are normalized to the most stable house-keeping gene Glucuronidase Beta (Gusb).

<table>
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<th>Cytokine/Chemokine</th>
<th>$cps1-1$ Without Inhibitor $2^{\Delta \Delta CT}$</th>
<th>$cps1-1$ With Inhibitor $2^{\Delta \Delta CT}$</th>
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<tr>
<td></td>
<td>M1</td>
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GRA24 role in the regulation of IncRNA in WT BMDM

*T. gondii* has been shown to modulate the expression of host IncRNAs [25]. We were therefore interested in determining if GRA24 controls IncRNA expression. Of the 282 IncRNAs that were shown to be co-regulated with *T. gondii* infection, 33 of these IncRNAs were shared in both RH and PTG infected BMDM [31]. Of these IncRNAs, we chose six for these studies that were immune related non-protein coding genes which could suggest a possible regulatory role for these IncRNAs in the immune response. The list includes Socs2-Inc, CsF-1-Inc, IL1rn-Inc, Ifi44-Inc and Mir17hg. Socs2 is a suppressor of cytokine synthesis that may be involved in anti-inflammatory responses during *T. gondii* infection [30]. CsF1-Inc is involved with protein coding gene csf1, a cytokine that drives the differentiation of stem cells into the macrophage lineage [29]. IL1rn-Inc is associated with Il1rn, an antagonist cytokine that inhibits interleukin 1 (IL-1) activities and modulates IL-1 immune response [32]. Ifi44-Inc is associated with Ifi44 which is an interferon-alpha inducible protein associated with viral infection [33]. Mir17hg encodes the mir17 microRNA gene cluster implicated in the down regulation of host cell apoptosis during *T. gondii* infection.

To investigate if GRA24 modifies the expression of these select IncRNA, we infected BMDM with cps1-1 or cps1-1:Δgra24. Using qPCR, we examined the fold change of a subset of IncRNA that are both regulated in RH and PTG *T. gondii* strains. Our results show there were no differentially expressed IncRNAs in *cps1-1* and *cps1-1:Δgra24* infected BMDM including CSF-1-Inc, L1rn-Inc and Socs2-Inc.
(Figure 3.3A). Furthermore, there was no change in the gene expression of IFI44-
Inc or Mir17hg-Inc in the cps1-1 and cps1-1:Δgra24 infected BMDM (Figure 3.3B).
Additionally, a multiplicity of infection (MOI) of 8:1 yielded a 70-74% infection rate
(Figure 3.3C). These results suggest that GRA24 does not play a role in
modulating these six specific host IncRNA during *T. gondii* infection.
Figure 3.3 GRA24 does not play a role in the regulation of IncRNA in WT BMDM

C57BL/6 BMDM were infected with 8X10^6 \( cps1-1 \) or \( cps1-1:∆gra24 \) tachyzoites for 8 hr. Cells were collected for RNA extraction and qPCR analysis. (A) Fold change of qPCR analysis of CSF1-Inc, IL1m-Inc and SOCS2-Inc (B) Fold change of qPCR analysis of IFI44-Inc, Mir17hg, D43RIK. (C) Table indicating % infection of WT BMDM infected with \( cps1-1 \) and \( cps1-1:∆gra24 \) M1, M2 and M3 indicate individual experiments. Fold change is relative to NI samples. Statistical significance was assessed using a Student’s t test (NS \( p>0.05 \)).
Discussion

The p38 MAPK pathway plays an important role in response to stress stimuli including T. gondii infection [17, 34]. Here we identify GRA24 as an important activator for the production of cytokines and chemokines during T. gondii infection. Activation of p38 MAPK by GRA24 induces the production of a subset of cytokines and chemokines including CX3CL1 during in vitro infection. Furthermore, we have shown CCL17 and Ppbp are being regulated by GRA24 in a p38 MAPK independent manner. Figure 3.1D shows the downregulation of CCL17 and Ppbp in BMDM infected with GRA24 KO parasites. Additionally, CCL17 and Ppbp are upregulated in BMDM infected with GRA24 sufficient parasites. When we infected BMDM with GRA24 sufficient parasites and inhibited p38 MAPK in Figure 3.2B, the expression of CCL17 and Ppbp were not significantly altered when comparing to samples lacking the inhibitor. These results indicate CCL17 and Ppbp are regulated by GRA24 independently of p38 MAPK. Additionally, we have shown IL-15 is upregulated during infection with GRA24 sufficient parasites but only when p38 MAPK activity is blocked by chemical inhibition.

During T. gondii infection, GRA24 is secreted into the host cell and binds p38α MAPK which triggers autophosphorylation and nuclear translocation into the host nucleus. Within the nucleus, the GRA24/p38 MAPK complex upregulates transcription factors c-Fos and Egr-1 which are involved with the induction of proinflammatory cytokines including IL-12 and MCP-1 [17]. Interestingly, Neospora caninum, a closely related apicomplexan, uses G-protein-coupled receptor
signaling to phosphorylate host p38 MAPK [49]. In contrast to what we have found, p38 MAPK activation by *N. caninum* downregulates the host immune response to evade detection in BMDM [35].

Of relevance, *T. gondii* secretes other effectors such as ROP16 and GRA15 that directly induce signaling responses in macrophages and other cell types through signal transducer and activator of transcription (STAT) and NF-κB. Further ensuring host survival, GRA16 is secreted from the parasitophorous vacuole and interacts with p53 in the host cell, increasing nuclear localization of phosphatase, tensin homolog (PTEN) and p53 dependent apoptosis by binding to herpes virus associated ubiquitin specific protease (HAUSP) in hepatocellular carcinoma cells (HCC) ensuring host survival during infection [36].

We examined cytokines and chemokines that are regulated by GRA24. The cytokine and chemokine qPCR array enabled us to examine the expression of almost 100 immune-related genes and we have identified four chemokines that were differentially regulated when comparing infection with *cps1-1* and *cps1-1:Δgra24* *T. gondii* strains. These include CX3CL1 and CCL17 which we believe play an important role in the adaptive immune response to *T. gondii* due to their role in chemotaxis of T lymphocytes [37, 38]. Furthermore, our results are consistent with prior studies on *in vivo* peritoneal exudate cell responses to *T. gondii*. For example, the expression level of CCL17 was upregulated in the proteome array in mice infected with *cps1-1* compared to mice infected with *cps1-
in our previous study [18]. Together these data further indicate GRA24 plays a role in triggering adaptive immunity during *T. gondii* infection [18, 39].

Interestingly, the qPCR data in this study shows the upregulation of CCL12 in BMDM infected with the GRA24 KO *T. gondii* strain. CCL12 has been described as a chemotactic factor that attracts eosinophils, monocytes and lymphocytes [40]. When we used the small molecule p38 MAPK inhibitor, CCL12 was downregulated in BMDM infected with GRA24 sufficient tachyzoites. An explanation for this response could be that GRA24 downregulates CCL12 by a p38 MAPK independent pathway. Alternatively, p38 MAPK could be regulate an unknown pathway which would lead to the upregulation of CCL12. Furthermore, Pro-platelet basic protein (Ppbp), a chemoattractant and activator of neutrophils is significantly downregulated in BMDM infected with *cps1-1:Δgra24* expression does not change in the presence of p38 MAPK inhibitor (data not shown) [41]. This implies that GRA24 regulates Ppbp independently of p38 MAPK. Additionally, IL-15 is upregulated in BMDM infected with *cps1-1* in the presence of the p38 MAPK inhibitor, suggesting GRA24-independent negative regulation by parasite-activated p38 MAPK.

IncRNAs that are involved in the host immune response have been shown to be differentially expressed during *Toxoplasma* infection [25, 42]. It has been previously shown that parasite kinase ROP16 controls the upregulation of Csf1-Inc and Socs2-Inc [25]. To elucidate whether GRA24 could be another effector
molecule used by *T. gondii* to modulate host lncRNAs, we specifically examined Csfs1-lnc and Soc2-lnc expression patterns in BMDM infected with *cps1-1* and *cps1-1:Δgra24* *T. gondii* strains. Our results showed there was no change in IncRNA expression when comparing infection with *cps1-1* and the *cps1-1:Δgra24* *T. gondii* strains. We further tested Mir17hg-lnc and D43rik since they were both upregulated in the RH and PTG strains and are immune related protein coding genes which could suggest a possible regulatory role for these IncRNAs in the immune response during *T. gondii* infection. Again, our results showed no differential expression between *cps1-1* and GRA24 KO infected BMDM. These results suggest GRA24 is not modulating these particular lncRNAs during *T. gondii* infection.

One explanation for not seeing regulatory effects of lncRNA by GRA24 could be that there are other parasite secretory molecules such as micronemes or rhoptry neck proteins that are regulating the gene expression of host IncRNA during infection. Additionally, the secretory molecules could form complexes and induce the up or down regulation of host IncRNA. One prime example is GRA18 that is released into the host cell binds the beta catenin destruction complex driving beta catenin up-regulation and host cell gene expression [43]. Moreover, we performed a preliminary analysis and only screened six lncRNA. Use of RNAseq in infected BMDM with *cps1-1* or *cps1-1:Δgra24* will shed light on whether GRA24 is playing a role in the expression of other host IncRNA.
Overall, the studies in this chapter expand our knowledge of GRA24 in the activation of cytokines and chemokines during *T. gondii* infection. Our future work will investigate the role of GRA24 in directing the infiltration of lymphocyte and myeloid cell populations into the peritoneal cavity during *T. gondii* infection. These studies expand our understanding of *T. gondii* as an intracellular parasite that is capable of manipulating the host immune system to ensure survival.
Materials and Methods

Ethics Statement

All experiments performed in this research were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee at the University of New Mexico (Animal Welfare Assurance Number A4023-01). During this study, all efforts were made to reduce animal suffering.

Mice

Mice (6-12 weeks of age) were used in these studies. C57BL/6 female mice (5 weeks of age) were ordered from Taconic Biosciences Inc. (Rensselaer, NY). Animals were housed in the University of New Mexico Department of Biology animal facility.

Parasites

cps1-1 and cps1-1:Δgra24 tachyzoites were maintained as previously described [18]. In brief, human foreskin fibroblasts (HFF, ATCC, Manassas, VA) were grown to confluency in Dulbecco's Modified Eagle's Medium (DMEM; VWR, Randor, PA) supplemented with 1 mM glutamine and 10% bovine growth serum (FBS; HyClone, Logan, UT), 100 U/ml penicillin (ThermoFisher Scientific, Waltham, MA) and 0.1 mg/ml streptomycin (ThermoFisher Scientific). Construction of the uracil auxotroph strains ΔompdcΔup (designated as cps1-1) and ΔompdcΔupΔgra24 (designated as cps1-1:Δgra24) has previously been described [44]. Parasites were passaged
weekly in DMEM supplemented with 1 mM glutamine and 1% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 300 μM uracil. To ensure uracil auxotroph *T. gondii* parasite strains maintain dependence upon uracil, the parasites were passaged once a month in media without supplemental uracil. Parasites were tested for Mycoplasma contamination every 3 months using the MycoProbe detection assay (R & D Systems, Minneapolis, MN).

**Bone Marrow Derived Macrophage**

C57BL/6 female mouse (6-8 weeks of age, The Jackson Laboratory) tibias were extracted and used as a source of bone marrow cells. Macrophages were derived by 5-day culture in L2929-containing media as previously described [18, 45]. One day prior to infection, BMDM were harvested and plated on a 24 well tissue culture plate.

**qPCR Assay**

2E10⁶ BMDM were plated overnight in 24 well tissue culture plate. 10⁶ *cps1-1* and *cps1-1:agra24* were added to the BMDM for 48 hr in the presence of uracil containing media. Cells were collected for RNA extraction (RNeasy Plus mini kit Catalog #: 74134, Qiagen, Germantown, MD) and cDNA synthesis (Superscript VILO Catalog #: 11754-050, Invitrogen, Waltham, MA). qPCR was performed using RT² profiler PCR Array Mouse Cytokines and Chemokines (Catalog #: PAMM-150Z, Qiagen) according to manufacturer’s directions. RT² profiler PCR Array plates were loaded with cDNA and read on a Bio-Rad CFX96 PCR thermocycler. CFX Manager software v3.1 was used for data analysis. 10⁶ BMDM
were plated overnight in 24 well tissue culture plate. The following day 10μm p38 MAPK inhibitor SB202190 (Catalog #:559397, Millipore, Billerica, MA) was resuspended in uracil containing media and placed on the BMDM for 1 hr. 5E10^5 cps1-1 were added to the BMDM in the presence and absence of SB202190 for 48 hr. Supernatants were collected for measurement of IL-12p40 by ELISA. Cells were collected for RNA extraction (RNeasy Plus mini kit Catalog #: 74134, Qiagen) and cDNA synthesis (Superscript VILO Catalog #: 11754-050, Invitrogen). RNA for each sample was quantified using a NanoDrop ND-1000 (ThermoFisher Scientific). qPCR was performed using RT^2 profiler PCR Array Mouse Cytokines and Chemokines (Catalog #: PAMM-150Z, Qiagen) according to manufacturer’s directions. RT^2 profiler PCR Array plates were loaded with cDNA and read on a Bio-Rad CFX96 PCR thermocycler. CFX Manager software v3.1 was used for data analysis.

For directed analysis of specific transcripts, C57BL/6 BMDM (10^6) were plated in a 24 well tissue culture plate overnight then infected with 8E10^6 cps1-1 and cps1-1:∆gra24 for 6 hr. Cells were collected for RNA extraction using a RNeasy Plus mini kit (Catalog #: 74134, Qiagen) and cDNA synthesis using Superscript VILO (Catalog #: 11754-050, Invitrogen). Total RNA was quantified using a NanoDrop-1000. Quantitative PCR was performed on the CSF1-mRNA, SOCS2-Inc, IFi44-Inc, Mir17hg and D43Rik. All were normalized to the expression of the ppia house keeping gene using the SYBR green method (Catalog #: 1725274, BioRad, Hercules, CA) and CFX96 PCR machine. Expression relative to noninfected
control samples were calculated using the \( \Delta \Delta \text{Ct} \) method. The primer sequences used in this study were: Ppia (F-GCA-TGT-GGT-CTT-TGG-GAA-GGTG and R-GGG-TAA-AAT-GCC-CGC-AAG-TCAA), CSF1-mRNA (F-CGG-TAG-TGA-TGG-AGT-GTG-GCTT and R-CTG-CCT-GTA-CCT-CTG-GAT-TGCT), IL1rn-mRNA (F-TGT-GTG-CCT-TAC-AGG-GTG-AACA and R-TTG-ATG-GCA-TCT-CCC-AAG-GCTT), SOCS2-lnc (F-AGA-TGG-CTC-AGT-TAC-CTT and R-ACC-TGA-CCA-ATT-CTG-CTC-AGCT), IFi44-lnc (F-GGT-GGG-CTG-TGA-AGA-TGGA and R-AAG-TTG-AAC-CGA-GAA-GCC-TGGG), Mir17hg (F-GGT-GGC-CAC-TCT-GTT-AAT-GTGC and R-TAA-CTG-CAG-CTT-CTC-CAG-ACCC), D43Rik (F-TTC-TCA-ATA-CAA-CGC-CCC-AGGT and R-AAA-AGG-GGG-AAG-GAT-TGG-GGAG).

**Cytokine ELISA**

IL-12p40 and CCL17 production was measured using a murine IL-12/IL-23p40 ELISA kit (Invitrogen) and a CCL17 ELISA kit (R & D Systems) according to the manufacturer's instructions. In brief, 96 well high binding ELISA plates (Corning Costar, Corning, NY) were coated with anti-cytokine capture antibody in coating buffer and incubated in the 4°C overnight. The next day, plates were washed 5 times in PBS containing 0.05% Tween-20 (PBST) then blocked with diluent (Invitrogen) for 1hr at room temperature. Plates were then washed 3 times in PBST, and sample supernatants as well as recombinant standard was added and incubated overnight in the 4°C. The plates were washed 5 times in PBST and avidin-horseradish peroxidase was added. Plates were incubated for 30 min at
room temperature. The plates were washed 5 times in PBST and 3,3′,5,5′-Tetramethylbenzidine (TMB) was added. The plates were incubated for 20 min. The reaction was quenched with 2 M H$_2$SO$_4$ and plates were read at 450 nm on an iMark ELISA reader (Bio-Rad, Hercules, CA).

**Measurement of percent infection**

BMDM were grown on glass coverslips for 24 hr. The BMDM containing coverslips were infected with *cps1-1* or *cps1-1:Δgr24* in the presence of uracil (0.3mM). After 6 hr coverslips were washed in PBS, then fixed in 3.7% paraformaldehyde for 20 min. The cells were washed with permeabilization buffer (PB; 0.1% saponin in PBS). Cells were then blocked for 1 hr with PB containing 5% normal mouse serum (Invitrogen). After blocking, the cells were washed with PBS and stained with goat anti-*Toxoplasma* FITC-conjugated antibody (Catalog #PA17253, Invitrogen) diluted in PB containing 5% bovine serum albumin (VWR, Randor, PA) for 1 hr. The cells were washed in PB 3 times and PBS 4 times. Finally, the coverslips were air dried and mounted onto slides using DAPI containing mounting media (Catalog #P36962, Invitrogen). Images were taken using BX53 fluorescence microscope (Olympus America, Inc, Waltham, MA) and DP manager software (Olympus). Over 300 cells and five fields of view were used to calculate percent infection.
Statistical Analyses

Student’s t-test was used to evaluate significant differences between groups. A p value <0.05 was used to indicated significant. All experiments were performed 2-3 times.
References


Chapter 4

The role of GRA24 in cell recruitment during *in vivo Toxoplasma gondii* infection
Abstract

The intracellular protozoan *Toxoplasma gondii* induces a strong Th1 immune response. In our previous studies, we showed that *T. gondii* dense granule protein GRA24 controls production of IL-12 and a select subset of chemokines during *in vitro* and *in vivo* infection. To further investigate if these responses impacted cell recruitment during early infection, we employed flow cytometry to evaluate migration of immune cell populations into the peritoneal cavity during infection with GRA24 sufficient and GRA24 deficient *T. gondii* strains. While overall cell recruitment was similar using the two *Toxoplasma* strains, we obtained evidence for a role of GRA24 in recruitment of CD4⁺ T lymphocytes.
Introduction

*T. gondii* is a food-borne pathogen found in domesticated animals within the United States [1]. Infection occurs when meat contaminated with tissue cysts are consumed [2]. Infection can also occur after ingesting oocysts from contaminated cat litter or consuming contaminated food or water [2]. Toxoplasmosis is a public health problem worldwide, with over a million people infected in the USA alone, 3,000 individuals develop symptomatic disease annually in the United States [3]. In pregnant women, infection can cause congenital problems in the fetus. Furthermore, infection in immunocompromised hosts such as AIDS patients can lead to neurological problems [4]. This is due to the reactivation of the latent form of the parasite which can cause encephalitis and death [5]. *T. gondii* infection has been associated with mental illnesses such as schizophrenia and suicidal inclinations in asymptomatic individuals [6-9]. Additionally, *T. gondii* has also been shown to be linked to causing seizures [10].

Parasite protein profilin, a key contributor to host cell invasion and virulence, activates the immune system by Toll like receptors (TLR) 11 and 12 which initiates interleukin 12 (IL-12) production [11, 12]. Nevertheless, *T. gondii* also triggers IL-12 production independently of TLR/MyD88 by phosphorylation of p38 mitogen activated protein kinase (MAPK) [13]. The dense granule protein GRA24 was shown to bind and trigger allosteric autoactivation of host p38 MAPK, inducing translocation into the nucleus and causing changes in host gene transcription [14]. The changes in host gene transcription leads to the upregulation of IL-12.
Furthermore, we have shown IL-12 upregulation by GRA24 results in strong protective immunity [15]. CD8α+ dendritic cells (DC) as well as CD103+ CD11b− and CD103− CD11b− DC are the main sources of IL-12 [16, 17]. It is unknown if GRA24 is responsible for the induction of IL-12 in these specific cell populations.

NK cells as well as CD4 and CD8 T cells are a major source of interferon gamma (IFN-γ) which activates macrophages for parasite destruction [18, 19]. IL-12 directly activates T cells driving a strong T cell mediated IFN-γ response. IFN-γ production by T cells during acute infection has been associated with protection during intraperitoneal infection with the type II strain ME49 T. gondii strain [20]. Furthermore, inoculation with the cps1-1 uracil auxotroph T. gondii strain, elicited a long-lasting CD8+ T cell response [21]. IFN-γ is indispensable for the induction of immunity-related GTPase (IRG) family and guanylate binding proteins (GBP) that are key in disassembling the parasitophorous vacuole that contain tachyzoites within infected host cells [22-24].

Myeloid cells are key players in the innate immune system during T. gondii infection. For example, neutrophils play an important role in protection. They engulf pathogens by phagocytosis, after which phagosomes containing microbes fuse with lysosomes result in pathogen destruction [25]. Additionally, neutrophils release neutrophil extracellular traps (NETs) consisting of DNA studded with histones and laced with antimicrobial peptides that kill microbial pathogens [26]. Furthermore, neutrophils have been shown to release NETs during T. gondii
infection which ensnare and kill the protozoa [27]. Macrophages phagocytose dead or opsonized parasites where degradation occurs in lysosomes [28]. During intracellular infection, macrophages that are primed by IFN-γ limits parasite replication of tachyzoites within the parasitophorous vacuole by upregulating iNOS and p47 GTPases [23, 29, 30]. Additionally, interactions of macrophages with activated T cells through CD40/CD40L ligation also contributes to parasite control [31]. Activated macrophages are also able to present parasite derived antigen to activated CD4+ and CD8+ T cells through MHC I and II [32].

In this study, we investigated the role of GRA24 in recruitment of myeloid and lymphoid cells into the peritoneal cavity during T. gondii infection. Using cps1-1 and cps1-1:Δgra24 T. gondii strains, we determined the role of GRA24 in directing infiltration of dendritic cells, neutrophils, macrophages, B cells, CD4 and CD8 T cells. We failed to see a change in the myeloid cell populations in WT mice infected with GRA24 sufficient vs. GRA24 deficient parasites. Nevertheless, we saw a small decrease in CD4+ T cell infiltration in the mice infected with cps1-1:Δgra24 infected mice. These data indicate GRA24 may play a role in directing T cell migration into the peritoneal cavity during T. gondii infection.
Results

The influence of GRA24 on recruitment of dendritic cells, neutrophils and macrophages during T. gondii infection

*T. gondii* dense granule protein GRA24 has been shown to regulate cytokine and chemokine production during *T. gondii* infection (Chapter 3). To further examine if GRA24 influences myeloid cell infiltration into the peritoneal cavity during *T. gondii* infection, C57BL/6 mice were infected by i. p. inoculation with $10^6$ *cps1-1* or *cps1-1:*Δ*gra24*. Seven days post infection (dpi), peritoneal exudate cells (PEC) were collected and stained for CD11c+, MHCII+, F4/80− dendritic cells (DC), F4/80+ macrophages (MO) and Ly6G+ neutrophils (PMN). We quantified total cell infiltration into the peritoneal cavity 7 dpi and found there was a significant increase in the total cell population in the *cps1-1* and *cps1-1:*Δ*gra24* infected mice when compared to uninfected (UI) (Figure 4.1A). To further investigate this response, we used flow cytometry to assess specific myeloid cell populations recruited into the peritoneal cavity 7 dpi. Additionally, we wanted to investigate if GRA24 influenced recruitment of specific cell populations. Figure 4.1B shows the gating strategy used for the flow cytometry analysis of CD11c+, MHCII+, F4/80− DC, Ly6G+ PMN and F4/80+ MO cell populations shown in Figures 4.1C and D. Our results show there was no significant difference in the total number if infiltrating MO, DC or PMN cell populations in the *cps1-1* and GRA24 KO infected WT mice (Figure 4.1C). However, there may be a small increase in the DC frequency in the GRA24 KO infected mice compared to the *cps1-1* infected mice (*cps1-1*, 16.3%; *cps1-1:*Δ*gra24*, 24.2%) although this result was not statistically significant over multiple
mice (Figure 4.1D). Additionally, there may be a slight decrease in the neutrophil population in the mice infected with GRA24 KO tachyzoites when compared to \textit{cps1-1} infected mice (\textit{cps1-1}, 9.0\%; \textit{cps1-1:Δgra24}, 1.9\%) although, again, this result was not statistically significant (Figure 4.1D). Interestingly, a CD11c\textsuperscript{+}MHCII\textsuperscript{-} population is present in both \textit{cps1-1} and GRA24KO infected mice. However, there is no significant difference in the CD11c\textsuperscript{+}MHCII\textsuperscript{-} population when comparing \textit{cps1-1} and \textit{cps1-1:Δgra24} infected mice (Data not shown). As reported in Patente et al. 2019, the CD11c\textsuperscript{+}MHCII\textsuperscript{-} cell population may be immature dendritic cells [33]. There was no difference in the CD11c\textsuperscript{+}MHCII\textsuperscript{-} cell population between \textit{cps1-1} and GRA24 KO infected mice which further supports our result that GRA24 is not influencing the migration of DC into the peritoneal cavity. Together, these results indicate that GRA24 does not have a major influence in the recruitment of myeloid cells into the peritoneal cavity during \textit{T. gondii} infection.

**The impact of GRA24 on the infiltration of B cell, CD4 and CD8 T cell populations during \textit{T. gondii} infection**

GRA24 upregulates CCL24, CX3CL1 and IL15 which are chemoattractants and activators of T cells (Chapter 2). Interestingly, CCL12, a chemoattractant for eosinophils, monocytes and lymphocytes, was upregulated in the BMDM infected with GRA24 KO parasites [34], To further elucidate the role of GRA24 in the recruitment of lymphocytes, C57BL/6 mice were infected with 10\textsuperscript{6} \textit{cps1-1} or \textit{cps1-1:Δgra24} tachyzoites by intraperitoneal inoculation. One week after infection, PEC were collected and stained for CD19\textsuperscript{+} B cells, CD4\textsuperscript{+} T cells and CD8\textsuperscript{+} T cell
populations. Our results show a small decrease in the infiltration of total cells of the \textit{cps1-1}:\textit{\textless}gra24 infected mice compared to the \textit{cps1-1} infected mice (Figure 4.2A). Additionally, consistent with the results in Figure 4.1A, there was an increase in cell infiltration in both of the \textit{cps1-1} and \textit{cps1-1}:\textit{\textless}gra24 infected mice when comparing to UI. Figure 4.2B shows the gating strategy for the analysis of B cell, CD4 and CD8 T cell populations in Figure 4.2C and D. We quantified total B cell, CD4 and CD8 T cell populations 7 dpi in the presence vs. absences of GRA24 and found that there was not a significant change in the B cell or CD8 T cell populations (Figure 4.2C). Interestingly, there was a significant decrease in the CD4 T cell populations in mice infected with GRA24 KO parasites when comparing to \textit{cps1-1} infected mice (Figure 4.2C). Additionally, this result was consistent with the frequencies of CD4 T cells showing a small decrease in the \textit{cps1-1}:\textit{\textless}gra24 infected mice when comparing to \textit{cps1-1} (\textit{cps1-1}, 27.3%; \textit{cps1-1}:\textit{\textless}gra24, 21.0%). Together, these studies show GRA24 does not influence the infiltration of B cell or CD8 T cells but does have a significant impact on the infiltration of CD4 T cells during \textit{T. gondii} infection.
Figure 4.1 GRA24 does not influence dendritic cell, neutrophil or macrophage infiltration into the peritoneal cavity 7 days post infection. C57L/6 mice were infected with $10^6$ *cps1-1* or *cps1-1:*Δgra24 by intraperitoneal inoculation. Seven days post infection, peritoneal exudate cells (PEC) were collected and stained for CD11c⁺, MHCII⁺, F4/80⁺ dendritic cells (DC), F4/80⁺ macrophages (MO) and Ly6G⁺ neutrophils (PMN). (A) Quantification by flow cytometry of total PEC in uninfected (UI), *cps1-1* and *cps1-1:*Δgra24 infected mice. (B) Gating strategy for the flow cytometry analysis of CD11c⁺, MHCII⁺, F4/80⁺ DC, Ly6G⁺ PMN and F4/80⁺ MO cell populations (C) Quantification of the absolute cell numbers of DC, PMN and MO populations. (D) Percentage of DC, PMN and MO cell populations isolated from the peritoneal cavity of UI, *cps1-1* and *cps1-1:*Δgra24 infected mice. Values are the means ± SEM of 1 representative experiment, n=3 per group. Each symbol represents an individual mouse. Statistical analysis was performed using a Student’s t test (NS p>0.05, ***p<0.001). These experiments were performed two times with similar results.
Figure 4.2 GRA24 does not influence B cell, CD4 or CD8 T cell infiltration into the peritoneal cavity 7 days post infection. C57L/6 mice were infected with $10^6$ *cps1-1* or *cps1-1:Δgra24* tachyzoites by intraperitoneal inoculation. 7 dpi, PEC were collected and stained for CD19+ B cells, CD4+ CD4 T cells and CD8+ CD8 T cell populations. (A) Quantification by flow cytometry of total PEC in uninfected (UI), *cps1-1* and *cps1-1:Δgra24* infected mice. (B) Gating strategy for the flow cytometry analysis of CD19+ B cells, CD4+ CD4 T cells and CD8+ CD8 T cell populations. (C) Quantification of the absolute cell numbers of B cell, CD4 and CD8 T cell populations. (D) Percentage of B cell, CD4 and CD8 T cell populations isolated from the peritoneal cavity of UI, *cps1-1* and *cps1-1:Δgra24* infected mice. Values are the means ± SEM of 1 representative experiment, n=3 per group. Each symbol represents an individual mouse. Statistical analysis was performed using a Student’s t test (*p<0.05, **p<0.001). These experiments were performed two times with similar results.
Comparison of cell recruitment 2 days and 7 days post infection

To examine whether GRA24 regulates the infiltration of cell populations during early infection, mice were i. p. infected with $cps1-1$ or $cps1-1:gra24$ tachyzoites, samples were collected 2 dpi, cells were analyzed as in Fig. 4.1 and Fig. 4.2, and the results were compared to the Day 7 data. Our results showed a proportional decrease in B cell and macrophage populations in the mice infected $cps1-1$ and $cps1-1:gra24$ at 7 days post infection when compared to 2 days post infection (Figure 4.3A). Furthermore, as expected, there was an increase in the CD4 and CD8 T cell populations in both $cps1-1$ and GRA24 KO infected mice at 7 days post infection relative to 2 days post infection. Interestingly, the neutrophil and dendritic cell populations were increased at 2 days post infection compared to 7 days post infection in both $cps1-1$ and $cps1-1:gra24$ infected mice (Figure 4.3A). Additionally, at 2 dpi there was a non-significant decrease in infiltrating macrophages in GRA24 KO infected mice compared to $cps1-1$ infected mice (Figure 4.3A). Together, these data indicate there is a difference in cell populations that infiltrate into the peritoneal cavity at 2 days post infection and 7 days post infection during *T. gondii* infection.
Figure 4.3 Infiltration of DC, PMN, MO, B cell, CD4 and CD8 T cells into the peritoneal cavity in *cps1-1* and *cps1-1:Δgra24* infected mice 2 dpi and 7 days post infection. C57BL/6 mice were infected with $10^6$ *cps1-1* or *cps1-1:Δgra24* tachyzoites by intraperitoneal inoculation. Two days and seven days post infection, PEC were collected and stained for DC, PMN, MO, B cells, CD4 T cells and CD8 T cell as described in figure 4.1 and 4.2. (A) Pie charts indicating the percentage of DC, PMN, MO, B cell, CD4 and CD8 T cell populations into the peritoneal cavity.
Discussion

The results presented in this chapter demonstrate GRA24 does not play a significant role in recruitment of myeloid cell populations during *T. gondii* infection, at least in this analysis. Additionally, GRA24 does not appear to have major regulatory effects on B cells or CD8+ T cell infiltration. Nevertheless, GRA24 may significantly impact migration of CD4+ T cells into the peritoneal cavity.

In Chapter 3 we showed C-X3-C motif chemokine ligand 1 (CX3CL1) is highly upregulated in BMDM infected with GRA24 sufficient parasites. Due to its chemotactic effects on T cells and monocytes we hypothesize this chemokine may be responsible for the infiltration of CD4+ T cells into the peritoneal cavity during *T. gondii* infection [35, 36]. Additionally, C-C motif chemokine ligand 17 (CCL17) was highly induced in *cps1-1* infected BMDM and much less induced in GRA24 KO infected BMDM. CCL17 has been described to induce chemotaxis of T cell populations which may also be playing a role in the infiltration of CD4+ T cells during infection. Interestingly, C-C motif chemokine ligand 12 (CCL12), a chemoattractant for eosinophils, monocytes and lymphocytes, was upregulated in BMDM infected with GRA24 KO parasites (Chapter 3) indicating GRA24 downregulates this chemokine during *T. gondii* infection [34]. Additionally, T cell chemoattractants CCL7 and CCL24 are downregulated in BMDM infected with *cps1-1* in the presence of p38 MAPK inhibitor [37, 38]. Thus, GRA24 plays a role in the regulation of these chemokines in a p38 MAPK manner. In contrast, interleukin 15 (IL-15), known to regulate T cell and NK cell activation and
proliferation, was upregulated in BMDM infected with *cps1-1* in the presence of p38 MAPK inhibitor [39]. Our results indicate p38 MAPK is downregulating IL-15 during *T. gondii* infection resulting in dampening of T cell and NK cell activation and proliferation.

The limitations of this study include the scope of the cell populations we screened. We performed a basic scan of the major cell types that infiltrate the peritoneal cavity during intraperitoneal inoculation with *T. gondii*. We stained for F4/80 CD11c+, MHCII+, F4/80− dendritic cells, F4/80+ macrophages, Ly6G+ neutrophils, CD19+ B cells, CD4+ CD4 T cell and CD8+ CD8 T cell populations. We did not see a significant change in the infiltration of the myeloid population or the B cell and CD8+ T cell populations during i.p infection when we compared the *cps1-1* and GRA24 KO infected mice. However, we did see a small decrease in the CD4+ T cells in the GRA24 KO infected BMDM 7 days post infection.

To address these limitations, we will perform a more thorough analysis of the specific CD4+ T cell populations that infiltrate the peritoneal cavity. We will look at αβ and γδ T lymphocytes populations to see if GRA24 is playing a role in the infiltration of these specific cell populations. Furthermore, we will look at naïve CD4 T cell populations that exhibit high CD62L and low CD44 markers and memory CD4 T cell populations that express low CD62L and high CD44 markers.
To further analyze the specific myeloid cell populations, we will examine CD8α+ cDC as well as tissue-resident CD103+ cDC. Additionally, we will differentiate between M1 macrophages using CD80, CD86 and iNOS markers and M2 macrophages using CD163 and CD206 markers to see if GRA24 is playing a role in the polarization of macrophage populations.

Oral infection of *T. gondii* induces the recruitment of inflammatory monocytes (Gr1+ Ly6C+ Ly6G- F4/80+ CD11b+ CD11c-) into the small intestine [40]. To further investigate the myeloid cell populations during i. p. infection of *T. gondii*, and to investigate if GRA24 is playing a role in the infiltration of inflammatory monocytes found in the intestine, I can examine peritoneal exudate cells for inflammatory monocytes expressing as defined by a Ly6C+ Ly6G- F4/80+ CD11b+ CD11c- phenotype. Furthermore, I can screen for non-inflammatory CD11b+CD115+Ly6Clow monocytes. Additionally, I will investigate if GRA24 is triggering recruitment of CD8α+ DC as well as CD103+ CD11b- and CD103- CD11b- DC into the peritoneal cavity.

Overall, the studies in this chapter reveal GRA24 may play a role in the recruitment of CD4+ T cells during *T. gondii* infection. Moreover, our results showed GRA24 does not play a major role in recruitment of macrophages, dendritic cells, neutrophils, B cells and CD8+ T cells.
Materials and Methods

Ethics Statement

In these studies, experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee at the University of New Mexico Department of Biology (Animal Welfare Assurance Number A4023-01). All efforts were made to reduce the suffering of animals.

Mice

C57BL/6 female mice (6-8 weeks of age) were used in these studies and ordered from Taconic Biosciences Inc. (Rensselaer, NY). Animals were housed in the animal facility at the University of New Mexico Department of Biology.

Parasites

Parasite strains *cps1-1* and *cps1-1:Δgra24* were maintained as previously described [15]. In brief, human foreskin fibroblasts (HFF, ATCC, Manassas, VA) were grown to confluency in Dulbecco’s Modified Eagle’s Medium (DMEM; VWR, Randor, PA) that was supplemented with 10% bovine growth serum (FBS; HyClone, Logan, UT), 1 mM glutamine, 100 U/ml penicillin (ThermoFisher Scientific, Waltham, MA) and 0.1 mg/ml streptomycin (ThermoFisher Scientific). Construction of the uracil auxotroph strains Δ*ompdcΔup* (designated as *cps1-1*) and Δ*ompdcΔupΔgra24* (designated as *cps1-1:Δgra24*) has previously been described [41]. Parasites were passaged weekly in DMEM supplemented with 1
mM glutamine and 1% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 300 μM uracil. To ensure uracil auxotroph T. gondii parasite strains maintain dependence upon uracil, the parasites were passaged once a month in media in the absence of supplemental uracil. Every 3 months, parasites were tested for Mycoplasma contamination using the MycoProbe detection assay (R & D Systems, Minneapolis, MN).

**Nexcelom Cellometer**

Peritoneal exudate cell samples were mixed in Viastain Acridine Orange/Propidium Iodide (AO/PI, Catalog #: CS2-0106) reagent 1:1. 20μl of sample and AO/PI was loaded into the cell counting chambers. The counting chambers were inserted into the Nexcelom Cellometer (Lawrence, MA) for counting. Analysis was performed using the immune cells, low RBC option. Four fields of view are counted and averaged for cell number.

**Flow Cytometry**

Peritoneal exudate cells were stained with Zombie Aqua Viability kit (Catalog#: 423101, Biolegend) in PBS at room temperature for 8 min. Following viability staining, cells were stained with primary antibodies including anti-CD11c PE efluor 610 (Catalog#:61011482, Invitrogen, Waltham, MA), anti-MHCII Alexa Fluor 647 (Catalog#:107618, Biolegend, San Diego, CA), anti- Ly6G Pe Cyanine 7 (Catalog#:127618, Biolegend), anti-F4/80 Brilliant Violet 711 (Catalog#:123147, Biolegend), anti-CD4 Percp Cy5.5 (Catalog#:100434 Biolegend), anti-CD8
Brilliant Violet 711 (Catalog#:100747, Biolegend), anti-CD19 APC (Catalog#:550992, BD Pharmingen, San Diego, CA) in FACS buffer (1% BGS, 0.01% NaN₃ in PBS) for 45 min in the 4° C. Cells were then fixed in 3.7% paraformaldehyde (MilliporeSigma, Burlington, MA) for 15 min. Samples were run on an Attune NxT flow cytometer (ThermoFisher Scientific) and data was analyzed using FlowJo v.10 software (FlowJo, Ashland, OR).

**Statistical Analyses**

Significant differences were evaluated using a Student’s t-test. A p value of <0.05 indicated significant. In these studies, all experiments were performed 2-3 times.
References


Chapter 5

Discussion
Summary of finding and future directions

The MyD88/TLR signaling cascade in mice is responsible for resistance to many invading pathogens including *Toxoplasma gondii* [1]. However, the discovery that the MyD88/TLR cascade is not activated during a subset of anti-microbial defenses including *T. gondii* in humans, has been a motivating factor in identifying MyD88-independent pathways of resistance [2]. Moreover, understanding the mechanisms used by the parasite to manipulate the host immune response will bring us a step closer in understanding how to best protect ourselves against this and other pathogens. The aim of this dissertation is to understand the role of GRA24 in innate and adaptive immune responses during *T. gondii* infection.

In **Chapter 2**, I discovered GRA24 triggers the production of IL-12 by inducing early p38 MAPK activation independently of the MyD88 signaling cascade in BMDM. In the absence of GRA24 there was a significant decrease in the production of IL-12 in WT and MyD88 KO BMDM. As expected, when we blocked p38 MAPK then measured IL-12 production in the supernatants of BMDM infected with GRA24 sufficient parasites, there was a significant decrease in the IL-12 response. Additionally, *T. gondii* has been shown to induce signal transducer and activator of transcription (STAT)3 and STAT6 activation through the secretion of ROP16. Similar to the two waves of p38 MAPK activation stimulated by GRA24, STAT3/6 activation was a biphasic process. Prolonged STAT3/6 activation was mediated by ROP16 although initial STAT3 phosphorylation was not dependent upon ROP16 [3]. Early STAT3 activation was dependent upon FAK-Src-STAT3.
signaling which is associated with invasion [4]. It would be interesting to know if this pathway is involved in early p38 MAPK activation.

Additionally, we observed GRA24 induces MyD88-independent protective immunity. However, in the absence of GRA24 there remained a significant MyD88-independent protective immune response. This result highlights the existence of other pathways of resistance that are not dependent upon the host TLR/MyD88 cascade or parasite GRA24. A study on human cells showed that peripheral blood monocytes and dendritic cells produce IL-12 after phagocytosis of live tachyzoites which is a process that does not involve TLR or the MyD88 adaptor protein [5]. Furthermore, inflammasome activation that is independent of TLR11, triggers Th1 immunity during *T. gondii* infection and the neutrophils can trigger IFN-γ production independent of TLR signaling [6, 7]. There is a possibility that these phenomena contribute to MyD88-independent induction of immunity in mice. Additionally, GRA24 induces the upregulation of a subset of cytokines and chemokines during *in vivo* infection including CCL17 and CCL12. These chemokines are chemoattractants for T cells. The upregulation of CCL17 and CCL12 induced by GRA24 may play a role in T cell recruitment which could be a factor in promoting protective immunity seen in MyD88 KO mice. Thus, this study supports the model that GRA24 triggers MyD88-independent immunity to promote survival of the host to ensure parasite survival and transmission.
Future work should focus on testing to see if ROP16 is responsible for early activation of p38 MAPK activation that occurs independently of GRA24. I could employ ROP16 sufficient and ROP16 KO parasites to infect WT and MyD88 KO BMDM and measure p38 MAPK activation by western blot analysis. This study would give us insight into other pathways of resistance responsible in MyD88-independent immunity. Additionally, it would be interesting to investigate if CCL12 and CCL17 play a role in triggering protective immunity in GRA24 KO MyD88 deficient mice. This can be done by depleting CCL12 and CCL17 during a survival study and comparing *cps1-1* and GRA24 KO infected mice in WT and MyD88 KO mice. This research could illuminate other MyD88 independent pathways of resistance that could potentially be responsible for immunity in humans due to nonfunctional MyD88 adaptor molecules.

It is well known cytokines and chemokines are potent molecules that regulate inflammation and modulate cellular activities such as cell growth, survival and differentiation [8]. In **Chapter 3**, I presented data demonstrating GRA24 triggers the induction of cytokines and chemokines during *in vitro T. gondii* infection. The activation of p38 MAPK by GRA24 triggers a subset of cytokines and chemokines including CX3CL1. CX3CL1 induces chemotaxis of T lymphocytes which we speculate may play an important role in the protective immune response described in Chapter 2. Additionally, we observed CCL17, chemoattractant of T cells and Ppbp, a chemoattractant of neutrophils, were less induced in BMDM infected with GRA24 KO parasites. Additionally, these proteins are upregulated in GRA24
positive infected BMDM. However, when we infected BMDM with GRA24 sufficient parasites in the presence of p38 MAPK inhibitor the expression was not altered when comparing to samples absent of the inhibitor. This indicates GRA24 regulates CCL17 and Ppbp independently of p38 MAPK. CCL12 has been described as a chemotactic factor that attracts eosinophils, monocytes and lymphocytes [9]. We observed BMDM infected with GRA24 positive parasites in the presence of p38 MAPK inhibitor downregulated CCL12. We speculate GRA24 is downregulating CCL12 in a p38 MAPK independent manner. Alternatively, p38 MAPK could regulate an unknown pathway which would lead to the upregulation of CCL12. Additionally, we observed Ppbp was less induced in BMDM infected with GRA24 KO parasites and expression did not change in the presence of p38 MAPK inhibitor. Again, we venture GRA24 is regulating Ppbp independently of p38 MAPK. Interestingly, we detected the upregulation of IL-15 in BMDM infected with cps1-1 in the presence of the p38 MAPK inhibitor. This suggests GRA24-independent negative regulation by parasite-activated p38 MAPK.

Future investigations should include an ELISA of CCL12 in the supernatant of the BMDM infected with cps1-1, cps1-1:Δgra24 and cps1-1 +/- p38 MAPK inhibitor to elucidate if our result is consistent with our qPCR data. Additionally, by employing a proteome array of the supernatants of the BMDM infected with cps1-1 and GRA24 KO parasites in the presence and absence of p38 MAPK inhibitor could further our understanding of proteins regulated by GRA24 independently of p38 MAPK. Furthermore, it would be interesting to investigate the role of the subset of
cytokines and chemokines regulated by GRA24 in cell migration and protective immunity during *T. gondii* infection in both WT and MyD88 KO mice.

The data presented in **Chapter 4**, indicate GRA24 may influence the migration of CD4+ T cells into the peritoneal cavity during i. p. infection with *T. gondii*. We observed GRA24 does not have an effect on the migration of macrophages, dendritic cells or neutrophils. Additionally, we found that GRA24 does not trigger the infiltration of B cells or CD8+ T cells into the peritoneal cavity during i. p. infection. As observed in Chapter 3, chemokines CX3CL1 and CCL17 are highly induced by GRA24 in BMDM. We speculate due to the chemotactic activity on T cells, CX3CL1 and CCL17 may play a role in the infiltration of CD4+ T cells into the peritoneal cavity during *T. gondii* infection. Additionally, I showed in Chapter 3 that CCL12, a chemoattractant for eosinophils, monocytes and lymphocytes, was upregulated in BMDM infected with GRA24 KO parasites indicating GRA24 downregulates this chemokine during *T. gondii* infection [9]. Additionally, as observed in Chapter 3, the inhibitor SB202190 blocks production of T cell chemoattractants CCL7 and CCL24 in *cps1-1*-infected macrophages indicating GRA24 modulates these cytokines in a p38 MAPK-dependent manner. In contrast, IL-15 known to regulate T cell and NK cell activation, was triggered in BMDM infected with *cps1-1* in the presence of p38 MAPK inhibitor. We speculate p38 MAPK downregulates IL-15 in response to *T. gondii* infection to dampen T cell and NK cell activation and proliferation.
Additionally, GRA24 has been shown to increase the protein level of (C-C Motif Chemokine Ligand 2) CCL2, a chemokine that has chemotactic activity for monocytes and basophils [10, 11]. Mice lacking CCL2 or its receptor CCR2 were not able to recruit inflammatory Gr-1+ monocyte into tissues that hosted *T. gondii* [12]. Mice infected with the GRA24 KO parasite strain failed to control parasite replication in the intestine. These data show GRA24 is playing a role in resistance to acute infection partially through the regulation of CCL2. Moreover, CXCL10 secretion has been shown to be induced by GRA24 [13]. CXCL10 is required for regulating *T. gondii* tachyzoite burden. Depletion of CXCL10 inhibits T cell recruitment and effector function in mice. With these data in mind, I speculate that during peroral infection with GRA24 sufficient parasites there would be more T cell recruitment into the intestine than what I saw in the peritoneal cavity. Additionally, since peroral infection is the natural route of infection this would cause a dysbiosis in the microbiota in the intestine [14]. This in turn would trigger Th1-type immunopathology in the intestine inducing infiltration and activation of CD4+ T cell [15].

Future investigations could examine more specifically cell populations found in the peritoneal cavity during infection with GRA24 sufficient and deficient parasites at later time points. I will differentiate between naïve CD4 T cell populations of the CD62L<sup>high</sup> CD44<sup>low</sup> phenotype and memory CD4 T cell populations of the CD62L<sup>low</sup> CD44<sup>high</sup> phenotype. Additionally, it would be interesting to examine the CD8α<sup>+</sup> cDC as well as tissue-resident CD103<sup>+</sup> cDC. Furthermore, examination of M1
macrophages of the CD80+CD86+ phenotype and M2 macrophages of the CD163+CD206+ phenotype, will elucidate if GRA24 plays a role in the polarization of these cell populations. Moreover, I can examine inflammatory monocytes of the Ly6C+ Ly6G− F4/80+ CD11b+ CD11c− phenotype and non-inflammatory monocytes of the CD11b+CD115+ Ly6Clow phenotype. Additionally, it would be interesting to compare the infiltration of cells and the overall immune response in mice infected with GRA24 KO parasites on a Type I and Type II parasite background. Together, these experiments will bring us a step closer in understanding what role GRA24 plays in the migration of cells during T. gondii infection.

My research can advance interventions for T. gondii infection in humans by helping us understand what cytokines and chemokines are being regulated by GRA24 independently of MyD88. My research showed GRA24 induces CCL17 and CCL12 in an MyD88 independent manner. With these data we would use liposomes to perform targeted delivery of CCL17 and CCL12 to enhance the recruitment of T cells to the site of infection. Additionally, we would sort T cells and prime them by plating with DC infected with cps1-1 in the absence of exogenous uracil so replication does not occur within the cell. We would then activate the primed T cells by supplementation with IL-12 and IL-2 and do an adoptive transfer into AIDs patients. The primed T cells would activate B cells for the induction of an antibody response. Furthermore, I show GRA24 induces IL-12 production in the absence of MyD88. Targeted delivery of recombinant GRA24 to the site of infection in AIDs patients to elicit an IL-12 response which would activate neutrophils and natural
killer cells to trigger IFN-γ production. This would lead to the activation of macrophages leading to the destruction of the parasite. Additionally, by understanding GRA24 and its role in immunity, we can potentially target other dense granule proteins that are structurally similar that are imbedded in the tissue cyst wall to combat the latent stage of infection.

Overall, the research presented in this thesis sheds new light on the important role played by GRA24 in the early immune response to Toxoplasma. The research in this thesis gives us a better understanding of the MyD88 independent pathways of resistance that may be applied to the human immune system aiding in how to best fight the parasite. Furthermore, the data presented showcase the complex mechanism used by T. gondii to manipulate the host immune system to safeguard survival of both host and parasite.
References


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