Rodent inflammasome activation by *Toxoplasma gondii*

by

Kimberly M. Cirelli

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Signature of Author: [Signature redacted]

Department of Microbiology
November 19, 2015

Certified by: [Signature redacted] Jeroen Saeij
Associate Professor of Biology
Thesis Supervisor

Accepted by: [Signature redacted] Kristala Jones Prather
Associate Professor of Chemical Engineering
Co-Director, Graduate Program in Microbiology
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Abstract

*Toxoplasma gondii* is an obligate intracellular pathogen capable of chronically infecting nearly all warm-blooded animals, including humans. The chronic stage is characterized by the presence of semi-dormant cysts in brain and muscle tissues. These cysts are crucial in the success of *Toxoplasma* as they are orally infectious and allow for the transmission of the parasite between hosts. As the host immune response drives cyst formation, the establishment of this chronic infection relies on the parasite’s ability to find a balance between activation of a host immune response and evasion of parasiticidal mechanisms. This balance is achieved through the modulation of host cell processes by parasite proteins secreted from specialized secretory organelles known as rhoptries and dense granules. Here, we report that *Toxoplasma* activates the inflammasomes in mice and rats. The inflammasomes are a set of cytoplasmic pattern recognition receptors (PRRs). Activation of the inflammasomes results in caspase-1 activation and the cleavage and release of the pro-inflammatory cytokines, Interleukin (IL)-1β and IL-18. IL-1β is an important mediator of local inflammation and neutrophil recruitment. IL-18 induces Interferon (IFN)-γ, which is a critical cytokine in the control of *Toxoplasma*. A form of cell death, termed pyroptosis, can accompany inflammasome activation.

The NLRP3 inflammasome is activated in mouse macrophages, leading to the secretion of IL-1β *in vitro*. The NLRP1 and NLRP3 inflammasomes play a major role in mouse survival and control of parasite replication *in vivo*. The NLRP1 inflammasome is activated in infected macrophages from rats that are able to completely clear infection. *Toxoplasma* infection leads to the secretion of active IL-1β and IL-18. Activation of the NLRP1 inflammasome leads to pyroptosis, a programmed form of cell death. Pyroptosis prevents parasite replication within the host cell and likely promotes clearance by nearby immune cells. Using a chemical mutagenesis screen, we identified three *Toxoplasma* dense granule proteins (GRAs), GRA18, GRA27 and GRA28, essential for NLRP1 inflammasome activation and pyroptosis in rat macrophages. Our work has identified *Toxoplasma gondii* as a novel activator of the rodent inflammasomes and demonstrated host cell death as a mechanism to control parasite replication. We have also identified three novel parasite proteins required for this activation, providing insight into interactions between parasite and host, which may aid in the treatment of human infection.

Thesis Advisor: Jeroen Saeij
Title: Associate Professor of Biology
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List of Abbreviations

AIM2 – Absent in Melanoma 2
ASC – Apoptosis-associated Speck-like protein containing a CARD
BBB – Blood-Brain Barrier
BMDM – Bone Marrow-Derived Macrophages
BN – Brown Norway
CARD – Caspase Activation and Recruitment Domain
Cas9 – CRISPR Associated Protein 9
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
DAMP – Damage-Associated Molecular Pattern
DC – Dendritic Cell
DS – Dextran Sulfate
GBP – Guanylate-Binding Protein
GO – Gene Ontology
GOI – Gene of Interest
GPI – Glycosylphosphatidylinositol
GRA – Dense granule protein
GSDMD – Gasdermin D
HFF – Human Foreskin Fibroblasts
HIV – Human Immunodeficiency Virus
IDO – Indoleamine 2,3-dioxygenase
IFN – Interferon
Ig – Immunoglobulin
IL – Interleukin
iNOS – Inducible Nitric Oxide Synthase
IPAF – Interleukin-1β-converting enzyme Protease-Activating Factor
IRF – Interferon Regulatory Factor
IRG – Immunity-Related Guanosine Triphosphatases
LD – Lethal Dose
LEW – Lewis
LF – Lethal Factor
LPS – Lipopolysaccharide
LRR – Leucine-Rich Repeat
LT – Lethal Toxin
MAPKK – Mitogen-Activated Protein Kinase Kinase
MHC – Major Histocompatibility Complex
MIC – Micronemal protein
NAIP – NLR family, apoptosis inhibitory protein
NFκB – Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NK – Natural Killer cell
NLR – Nod-Like Receptor
NLRC – NLR family CARD domain-containing protein
NLRP – NACHT, LRR and PYD domains-containing Protein
NO – Nitric Oxide
NOD – Nucleotide-binding Oligomerization Domain
PA – Protective Antigen
PAMP – Pathogen-Associated Molecular Pattern
PRR – Pattern Recognition Receptor
PV – Parasitophorous Vacuole
PVM – Parasitophorous Vacuole Membrane
PYD – Pyrin domain
RON – Rhopty neck protein
ROP – Rhopty protein
SD – Sprague Dawley
SNP – Single Nucleotide Polymorphism
TLR – Toll-Like Receptor
TNF – Tumor Necrosis Factor
Chapter One:
Introduction
**Toxoplasma gondii is a model intracellular pathogen**

The World Health Organization has established infectious disease as the second leading cause of death in the world, accounting for approximately 25% deaths (World Health Organization, 2004). A majority of these infectious diseases are caused by intracellular pathogens, including *Mycobacterium tuberculosis*, the causative agent of tuberculosis, Human Immunodeficiency virus (HIV), *Plasmodium*, the causative agent of malaria and *Cryptosporidium*, which causes the food-borne diarrheal disease cryptosporidiosis (McDonald et al. 2013). Studying the interaction between host and pathogen is critical in the development of more effective treatments. While bacteria and viruses have been long studied and many mechanisms through which these pathogens cause disease have been elucidated, much less is known about how eukaryotic pathogens cause disease.

*Plasmodium, Eimeria, Neospora* and *Cryptosporidium* are protozoan parasites and members of the phylum Apicomplexa. Apicomplexan parasites can infect livestock and can cause significant economical loss. *Eimeria* is a major pathogen in poultry (Chapman et al. 2013) and *Neospora* is a major cause of abortions in cattle (Mazuz et al 2014). Because of their clinical relevance, studying the host-parasite interactions of these organisms is especially important. However, these organisms are relatively difficult to study. Several species of *Plasmodium* cause human malaria, but only one has been successfully cultured in vitro. *Cryptosporidium* has only been established to grow in culture recently and is difficult to genetically engineer (Vinayak et al. 2015). *Toxoplasma gondii*, another apicomplexan parasite, serves as an excellent model for these organisms. *Toxoplasma* is a pathogen of humans and livestock, causing abortions in sheep and goats (Buxton 1990). Its genetic manipulability and tractability and ease of use in in vitro and in vivo studies make *Toxoplasma* an incredibly useful
tool in dissecting parasite biology. *Toxoplasma* has been used to identify several host and pathogen factors that determine disease outcome.

**Disease manifestation**

*Toxoplasma gondii* is an obligate intracellular parasite and a leading cause of human death due to food-borne illness in the United States (Hoffmann, Batz, and Morris 2012). *Toxoplasma* is capable of infecting all nucleated cells of all warm-blooded animals. Infection is lifelong and chronic infection is characterized by the presence of semi-dormant cysts in the muscle tissues and brain. It is estimated that one-third of the human population is chronically infected with *Toxoplasma* (Sibley and Ajioka 2008). Infection rates vary by region, with an estimated 10% of humans in the United States and 80% of those in Brazil chronically infected (Pappas et al. 2009).

Infection is usually asymptomatic in immunocompetent individuals. In immunocompromised individuals, such as HIV/AIDS patients, *Toxoplasma* is an important opportunistic pathogen and can cause brain encephalitis. During the AIDS epidemic in the 1980s, *Toxoplasma* infection of the central nervous system was diagnosed in up to 20% of patients (Velimirovic 1984). Additionally, *Toxoplasma* is a dangerous pathogen to developing fetuses in newly infected pregnant women, as the parasite is able to cross the placenta and infect the fetus. *Toxoplasma* is a leading cause of miscarriage and birth defects in humans (McLeod et al. 2012). Infection can also result in ocular disease, even in immunocompetent individuals. In Brazil, nearly 18% of examined individuals had ocular toxoplasmosis (Roberts 1999).
Toxoplasma life cycle

While the range of hosts Toxoplasma is capable of infecting is vast, the definitive host range is much narrower and limited to the members of the Felidae (feline) family. When a cat ingests an infectious form of Toxoplasma, the haploid parasite undergoes differentiation in the feline gut (Figure 1) (Sibley and Ajioka 2008). This differentiation into microgametes and macrogametes allows the mating of the two and the formation of diploid oocysts. If two distinct strains are simultaneously present, the parasites can undergo sexual recombination, resulting in numerous F1 progeny with a genome derived from both parental strains. The oocysts are shed in the feline feces into the environment, where they will undergo sporulation, forming haploid sporozoites (Dubey 2009). These oocysts are highly infectious and environmentally stable. Upon ingestion of infectious Toxoplasma (either tissue cysts or oocysts) by an intermediate host, host acids, including pepsins, digest the cyst wall, releasing the parasite. Toxoplasma sporozoites or bradyzoites released from the cysts will initially invade intestinal epithelial cells. There they will convert into tachyzoites that can cross the intestinal epithelium, in which macrophages and dendritic cells are the predominant cell type initially infected (Mordue and Sibley 2003; Suzuki et al. 2005).
The tachyzoite can infect a wealth of host cells and disseminate throughout the body of the host. Upon infection of a nucleated cell, the tachyzoite forms a non-fusogenic parasitophorous vacuole (PV), in which it can replicate. After several rounds of replication, the parasites will egress, lysing the host cell and the tachyzoites are free to invade new host cells.

The establishment of a chronic infection is key to the success of the parasite. Toxoplasma must be able to migrate from the site of infection (the gut) to distal sites, including the brain. Several models have been proposed to explain how the parasite is able to relocate and cross the Blood-Brain Barrier (BBB): (1) free tachyzoites actively cross barriers between individual host
cells (paramigration) (Dobrowolski and Sibley 1996), (2) tachyzoites may invade one part of a host cell and exit through the other side (paracellular migration) (Morisaki, Heuser, and Sibley 1995) and (3) the “Trojan horse” model, in which *Toxoplasma* exploits the trafficking ability of leukocytes (Courret et al. 2006; Lambert et al. 2006; Lambert and Barragan 2010).

When the host mounts an immune response to this acute infection, tachyzoites will convert into the slowly growing bradyzoite form. This stage of infection is characterized by the presence of the tissue cysts in nervous and muscular tissues and remains throughout the life of the host. This chronic stage of infection is critical for the success of the parasite as it is the only orally infectious stage other than the oocyst. Asexual transmission of the parasite occurs through the ingestion of infected hosts by other intermediate hosts.

**Genetic diversity**

Many different *Toxoplasma* strains have been isolated from a range of hosts throughout the world. In Europe and North America, *Toxoplasma* clonal lineages, known as types I, II and III dominate (Howe and Sibley 1995). Although these strains are genetically highly similar, they differ in specific phenotypes such as virulence in mice, growth *in vitro* and cytokine induction (Saeij, Boyle, and Boothroyd 2005). For instance, type I strains are virulent in mice (Lethal Dose (LD)\(_{100} = 1\)), while type II and type III strains are less virulent (LD\(_{50} = 10^3\) and \(10^5\), respectively) (Sibley & Boothroyd 1992). The uses of sexual crosses between strains that differ in virulence and quantitative trait locus mapping have identified specific parasite genes as modulators of host signaling and immune pathways.

South America is home to many genetically diverse *Toxoplasma* strains. Non-type I, II or III strains, termed atypical strains, can cause ocular toxoplasmosis and more severe disease than
the clonal lineages (Lehmann et al. 2006; Bossi et al. 2002). Genome-wide single nucleotide polymorphism (SNP) analysis of 26 strains, including South American strains, determined there is significant diversity between these strains (Minot et al. 2012). Another SNP analysis sorted *Toxoplasma* strains into 14 haplogroups (Su et al. 2012). The geographic regions where haplogroups are isolated are distinct. While types II and III are found predominately in Europe and Africa (Dubey et al. 2006; Al-Kappany et al. 2010; Herrmann et al. 2013), they are rarely isolated in South America (Moré et al. 2012). In North America, types II, III and 12 are most commonly found (Khan et al. 2011). Types 4, 5, 6, 8, 9 and 14 are found in South America (Su et al. 2012). This geographic diversity and differences between strains are likely due to adaptations of different strains to different hosts with distinct immune systems.

**Activation of the immune system**

The immune system is divided into two arms: innate and adaptive. The innate immune system is the first line of defense and critical in the recognition and initial response against pathogens. Adaptive immunity is responsible for immunological memory and upon re-exposure to a pathogen, leads to a faster and stronger response to eliminate the invading organism. In order for *Toxoplasma* to successfully establish a chronic infection, it must keep the host alive and therefore must be recognized by the innate immune system so a host response is mounted, but the parasite must be able to modulate this response so it is not entirely cleared by the host.

Throughout its lifetime, a host is challenged by a barrage of bacteria, viruses and parasites. In order to recognize the presence of a wide variety of these pathogens, hosts have evolved to sense conserved, often essential structures, found in many pathogens that are otherwise very different. These structures, pathogen-associated molecular patterns (PAMPs) are
sensed by pattern recognition receptors (PRRs). There are several families of PRRs that have been well characterized and studied in the context of *Toxoplasma* infection.

Toll-like receptors (TLRs) recognize a variety of ligands present on bacteria, viruses and parasites. Activation of the TLRs leads to a signal cascade that culminates in the activation of signaling pathways, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and interferon regulatory factors (IRFs). The NF-κB pathway regulates gene expression in a number of host cellular processes, including cell proliferation and the production of inflammatory cytokines, including interleukin (IL)-12 (Hayden and Ghosh 2008). IRFs are transcription factors whose target genes include type I interferons (IFNs), which are involved in an anti-viral response.

In the murine model of toxoplasmosis, TLR11 and TLR12 are primarily expressed in dendritic cells (DCs) and recognize the *Toxoplasma* essential protein, profilin (Yarovinsky et al. 2005; Koblansky et al. 2013; Raetz et al. 2013) (Figure 2). Several models have been proposed regarding the cooperation of TLR11 and TLR12 to recognize profilin in mice. It is suggested profilin recognition by TLR12 homodimers is sufficient to initiate an immune response (Koblansky et al. 2013), but an alternative model suggests a TLR11/TLR12 heterodimer is necessary for this response (Andrade et al. 2013; Raetz et al. 2013). In humans, *TLR11* is a pseudogene and *TLR12* is absent from the genome, suggesting other innate pathways are activated by *Toxoplasma* in humans.
Figure 2. Activation of the TLRs by *Toxoplasma gondii*. In macrophages and primarily dendritic cells, the *Toxoplasma* actin-binding protein, profilin, is recognized by the endosomal TLR11 and TLR12. Activation of TLR11/12 leads to the translocation of the transcription factor, IRF8, into the nucleus and the expression of several host genes, including IL-12. IL-12 stimulates T cells and NK cells to secrete IFN-γ, which acts on several cell types. IFN-γ upregulates the expression of the immune-related GTPases and the production of nitric oxide and reactive oxygen species, which aid in parasite clearance (black inhibitor). *Toxoplasma* is equipped with several parasite effectors to counter these host mechanisms (green inhibitor).

TLR2 and TLR4 sense *Toxoplasma* glycosylphosphatidylinositol (GPIs) (Debierre-Grockiego et al. 2007). TLR7 and TLR9 recognize parasite RNA and DNA respectively (Andrade et al. 2013; Melo et al. 2010). TLR3/7/8/9/11/12 reside in endosomal compartments. Localization of these TLRs to the endosomes is controlled by unc-93 homologue B1 (UNC93B1) (Kim et al. 2008; Pifer et al. 2011). Mice individually deficient in TLR3, TLR7, TLR9 or TLR11 are not more susceptible to infection than wild-type mice nor are triple TLR3/TLR7/TLR9 knockout mice. Mice deficient in UNC93B1 are hypersensitive to infection with *Toxoplasma* (Melo et al. 2010) as are TLR3/TLR7/TLR9/TLR11 quadruple knockout mice (Andrade et al. 2013).
This suggests the combined action of nucleic acid-sensing TLRs and TLR11 and TLR12 are required for full murine resistance to Toxoplasma.

Activation of the TLRs leads to the secretion of IL-12 by DCs and macrophages. IL-12 stimulates T cells and Natural Killer (NK) cells to produce Interferon (IFN)-γ. IL-1β, together with IL-12, has been found to stimulate the production of IFN-γ in NK cells (Hunter et al. 1995). IL-18 and IL-12 synergistically induce IFN-γ expression in T cells (Okamura et al. 1998). IL-1β and IL-18 are produced by myeloid cells through another set of PRRs, known as the inflammasomes.

Toxoplasma-infected TLR11-deficient mice have significantly higher frequency and total numbers of IFN-γ+ cells than wild-type mice. These cells were not T cells or NK cells and were determined to be neutrophils. Additionally, this IFN-γ production is TLR-independent as neutrophils from UNC93B1-deficient mice produce greater levels of IFN-γ than those of wild-type mice. IL-1β and Tumor Necrosis Factor (TNF), most likely from circulating monocytes and resident macrophages, regulate this IFN-γ production by neutrophils (Sturge et al. 2013). Human neutrophils produce IFN-γ (Ethuin et al. 2004). As humans do not express functional TLR11 or TLR12, neutrophil-derived IFN-γ may play a major role in human resistance to Toxoplasma. As IL-1β is activated by the inflammasomes and is a major regulator of IFN-γ production by neutrophils, it is likely that inflammasome activation by Toxoplasma plays an important role in humans.

**Interferon-gamma induced immunity to Toxoplasma gondii**

IFN-γ is a crucial cytokine in the host response to Toxoplasma (Suzuki et al. 1988). Mice deficient in IL-12 or IFN-γ succumb to acute infection by avirulent parasite strains (Gazzinelli et
IFN-γ upregulates genes encoding Major Histocompatibility Complex (MHC) molecules, allowing greater antigen presentation (Zhou 2009; Steimle et al. 1994; Yang et al. 1995). IFN-γ can also activate a wealth of cell types to mount parasiticidal effector mechanisms. IFN-γ induces the expression of the immunity-related GTPases (IRGs) and p65 guanylate binding proteins (GBPs), which play an important role in murine defense against Toxoplasma. The IRGs accumulate on the PVM and promote the destruction of the vacuole (Taylor et al. 2004). The parasite is released into the cytosol, where the parasite can be destroyed by lysosome-mediated degradation (Ling et al. 2006). Mice deficient in *Irg*1, *Irg*3 or *Igt* are acutely susceptible to parasite infection (Taylor et al. 2000; Collazo et al. 2001). GBPs are required for the recruitment of the IRGs to the PV (Yamamoto et al. 2012).

Additional host mechanisms mediated by IFN-γ include the production of nitric oxide (NO), which can inhibit parasite metabolic enzymes (Fang 2004; Adams et al. 1990). *Toxoplasma* is an arginine auxotroph and recovers the amino acid from the host cytosol. Inducible nitric oxide synthase (iNOS) utilizes L-arginine to produce NO, leading to the reduction of available arginine in the cell, which restricts parasite growth (Fox, Gigley, and Bzik 2004). In addition to these parasiticidal mechanisms, NO serves as a signal to induce bradyzoite conversion (Bohne, Heesemann, and Gross 1994). IFN-γ also induces indoleamine-2,3-dioxygenase (IDO), which converts tryptophan into N-formylkynurenine. Tryptophan auxotrophy renders *Toxoplasma* susceptible to this pathway, as a reduction in host tryptophan suppresses parasite replication (Pfefferkorn, Eckel, and Rebhun 1986). Reactive oxygen species also play a role in parasite control in mouse macrophages and human monocytes (Murray and Cohn 1979; Murray et al. 1979).
The inflammasomes

The Nucleotide-binding Oligomerization Domain (NOD)-Like Receptor (NLR) family are a set of germ line-encoded cytosolic PRRs, which contains 22 human members and 34 murine members (Proell et al. 2008; Bryant and Monie 2012). Members of the family share a leucine-rich repeat (LRR) region and a NACHT nucleotide-binding domain. NLR members can be divided according to their N-terminal region. Members of the NLRP group contain an N-terminal pyrin domain (PYD). NOD members contain caspase activation and recruitment domains (CARD). IPAF members also contain a CARD domain, but are distinct from NOD members. Proteins containing CARD are able to directly bind caspase proteins. PYD-containing proteins are unable to bind caspases directly and require a scaffold protein, apoptosis-associated speck-like protein containing a CARD (ASC or Pycard), which contains both PYD and CARD domains.

Several members of the NLR family, including NACHT, LRR and PYD domains-containing Protein (NLRP) 1, NLRP3 and NLR family CARD domain-containing protein (NLRC) 4, act as sensors that are capable of forming macromolecular complexes known as the canonical inflammasomes. Additionally, a member of the PYHIN protein family, absent in melanoma 2 (AIM2) has been identified to assemble an inflammasome. Inflammasome sensors vary in the types of ligands they recognize and their modes of activation. Despite differences in mechanisms of activation, the sensors follow the same general pathway (Figure 4). Upon recognition of a ligand, the sensors oligomerize into a large, 700kDa multimeric complex (Martinon, Burns, and Tschopp 2002). Inactive caspase-1 is recruited to this complex, in which the zymogen is cleaved. Now active caspase-1 is able to cleave two of its substrates, pro-IL-1β and pro-IL-18. In myeloid cells, pro-IL-18 is constitutively expressed (Puren, Fantuzzi, and
Pro-IL-1β expression must be induced through the activation of the transcription factor, NK-κB. A TLR agonist, typically the TLR4 agonist, lipopolysaccharide (LPS), is used to induce pro-IL-1β expression in a step known as “priming” (Guo, Callaway, and Ting 2015). Cleavage of these cytokines leads to their activation and release. IL-1β is involved in local inflammation, recruitment of neutrophils to site of infection and the induction of IFN-γ production by neutrophils and natural killer cells (Dinarello 1996; Hunter, Chizzonite, and Remington 1995). IL-18 acts on natural killer (NK) and T-cells, which release IFN-γ (Dinarello 1998).

**Figure 3. Inflammasome activation.** The inflammasomes are primarily expressed in myeloid cells. Upon recognition of an agonist, several sensors oligomerize. The multimeric complex recruits caspase-1, which is cleaved and activated. Caspase-1 cleaves pro-IL-18 and pro-IL-1β, leading to their activation and secretion.

Caspase-1 activation can also be accompanied by a programmed form of cell death, termed pyroptosis (Fink and Cookson 2005). In several gram-negative bacterial infections, a non-canonical inflammasome containing another pro-inflammatory caspase, caspase-11, plays a
major role in host resistance. Caspase-11 is activated by direct recognition of cytoplasmic LPS (Shi et al. 2014). Caspase-11 activation leads to caspase-1-dependent IL-1β release and caspase-11-dependent pyroptosis (Kayagaki et al. 2011). Very recently, gasdermin D (GSDMD) was identified as a caspase-1 and caspase-11 substrate, whose cleavage was necessary and sufficient to induce pyroptosis (Shi et al. 2015; Kayagaki et al. 2015).

Pyroptosis results in the swelling and lysis of the cell, releasing intracellular contents (Chen et al. 1996; Hersh et al. 1999; Hilbi 1998; Hilbi et al. 1997; Brennan and Cookson 2000). These contents include Damage-Associated Molecular Pattern Molecules (DAMPs), which can activate nearby cells to mount an immune response. Because of this, pyroptosis is a pro-inflammatory form of cell death (Cookson and Brennan 2001). Additionally, pyroptosis has been associated with the control of intracellular microbes. Macrophages infected with intracellular bacteria, including Salmonella, Legionella and Burkholderia, undergo pyroptosis, leading to the release of the bacterium, preventing intracellular replication and exposing the microbe to phagocytic cells, particularly neutrophils (Miao et al. 2010).

Allelic differences in human NALP1, which encodes for the NLRP1 sensor, has been linked to differences in susceptibility to congenital toxoplasmosis (Witola et al. 2011). The only identified activator of the rodent NLRP1 inflammasome is lethal toxin (LT) produced by Bacillus anthracis. Lethal toxin is composed of two proteins: lethal factor (LF) and protective antigen (PA). PA is required for entry of the toxin into the host cytosol (Milne et al. 2006), while LF is a zinc-dependent protease, which cleaves the N-terminus of mitogen-activated protein kinase kinases (MAPKKs), inhibiting their activity (Vitale et al. 1998; Pellizzari et al. 1999; Duesbery 1998). In anthrax-susceptible strains of rats, LT is also able to cleave the N-terminus of NLRP1. This cleavage is necessary and sufficient to activate the inflammasome (Levinsohn et
al. 2012). In anthrax-susceptible and resistant mice, LT cleaves NLRP1b, suggesting there are additional requirements for mouse NLRP1 inflammasome activation (Hellmich et al. 2012).

The P2X7 Receptor (P2X7R) is expressed on the surface of macrophages and activated by extracellular ATP, often derived from damaged or dead cells. Macrophages infected with *Toxoplasma* are able to clear infection when activated with ATP (Lees et al. 2010). Parasite death is accompanied by host cell death. P2X7R activation leads to NLRP3 inflammasome activation through the efflux of $K^+$, although the precise mechanism of NLRP3 activation is not fully understood (Pétrilli et al. 2007).

**Rats are a better model of human toxoplasmosis**

As the host range of *Toxoplasma* is wide, there are relative differences in the susceptibility of host species to the parasite. In addition, there are differences between different strains within a species. The most commonly used host model for *Toxoplasma* is the laboratory mouse, as it is an excellent tool to study host resistance and immunology. However, the mouse may not be the most accurate model for human toxoplasmosis. Toxoplasmosis is generally asymptomatic in immunocompetent humans. In contrast, immunocompetent mice are relatively susceptible to infection. During acute infection, mice experience symptoms such as weight loss, a hunched posture and extreme lethargy. Rats are an understudied model of toxoplasmosis, but model human infection more accurately. Rats that are infected with high doses of mouse-virulent parasites not only survive infection but also do not display the common symptoms of acute infection seen in the mouse. Rats develop a chronic, asymptomatic infection.

Interestingly, a rat strain, Lewis, was identified to be completely resistant to *Toxoplasma*. Regardless of parasite strain, dosage and route of infection, Lewis rats are able to clear infection
entirely. *Toxoplasma* failed to encyst in Lewis brains and muscle tissues (Kempf et al. 1999; Sergent et al. 2005). Additionally, Lewis rats had significantly lower titers of anti-*Toxoplasma* antibodies. Neutralization of IFN-γ led to an increase in antibody titer, but failed to allow formation of tissue cysts (Sergent et al. 2005). Neutralization of IFN-γ in control rats led to an increase in *Toxoplasma*-specific antibodies and brain cysts. This suggest that IFN-γ plays a role in rat control of infection, likely through parasite replication, but Lewis rats utilize another mechanism to clear infection.

Using bone marrow chimera studies, the resistance of Lewis rats was found to be intrinsic to bone marrow-derived cells. Additionally, through the use of crosses between Lewis rats and susceptible rats, the resistance was found to be a dominant trait (Sergent et al. 2005). Utilizing F2 progeny and recombinant inbred rats, the resistance was mapped to a single 1.3cM locus on chromosome 10, termed *Toxo1* (Cavailléès et al. 2006). *Toxo1* includes approximately 250 annotated rat genes. Contained within the locus is *Nlrp1*, encoding for the NLRP1 inflammasome sensor.

**Toxoplasma effector proteins**

*Toxoplasma* is equipped with three secretory organelles that are critical in establishing an infection: the micronemes, rhoptries and dense granules. Micronemal (MICs) and rhoptry proteins (ROPs) are primarily secreted during invasion (Figure 4). Upon recognition of a host cell, MICs and rhoptry neck (RONs) proteins mediate attachment and invasion by forming a moving junction, a structure through which the parasite pulls itself (Boothroyd and Dubremetz 2008). Rhoptry bulb proteins and some dense granule (GRAs) proteins are injected into the cell upon invasion (Boothroyd and Dubremetz 2008; Rosowski et al. 2011). These proteins can
traffic to the PVM or to host cell locations, including the nucleus. The majority of GRAs are secreted from the parasite once the PV has been established and continue to be secreted during replication (Dubremetz et al. 1993).

ROPs and GRAs have been established as important parasite modulators of the host response. ROP16 from type I and III strains acts as a tyrosine kinase that directly phosphorylates and activates the host transcription factors, STAT3 and STAT6 and therefore affects host gene expression (Saeij et al. 2007; Ong, Reese, and Boothroyd 2010). Certain allelic combinations of ROP18 and ROP5, a kinase and pseudokinase respectively, counter the activity of the IRGs. GRA15 from type II parasite strains is able to activate the transcription factor, NF-κB, which leads to the expression of a wealth of host proteins, including proinflammatory cytokines (e.g. IL-12 and IL-1β) (Rosowski et al. 2011). GRA16 and GRA24 are secreted out of the PV and traffic to the host nucleus, where they change host gene expression. GRA16 binds PP2A phosphatase and the deubiquitinase HAUSP to positively regulate the tumor suppressor p53 (Bougdour et al. 2013). GRA24 interacts with p38α MAP kinase, inducing p38α’s autophosphorylation and activation. p38α activation correlates with increased expression of several transcription factors including, Egr-1 and c-Fos (Bougdour et al. 2014).
Findings presented in this thesis

In chapter II of this thesis, we explored the role the inflammasomes play in murine resistance to *Toxoplasma* infection. We determined that infected bone marrow-derived macrophages (BMDMs) prepared from mice secrete active IL-1β, but do not undergo pyroptosis. Using BMDMs from mice deficient in individual inflammasome components, NLRP3 was found to be the predominant inflammasome activated by the parasite. BMDMs deficient in NLRP1 did not show a defect in IL-1β secretion. *In vivo*, both NLRP1 and NLRP3 played a role in mouse resistance to *Toxoplasma*. Mice individually deficient in these sensors succumbed to infection earlier than their wild-type counterparts with significantly higher parasite burdens. Additionally,
while systemic IL-1β could not be detected, IL-18 was found to be a critical cytokine in the murine response to the parasite, most likely by inducing IFN-γ.

In chapter III, we noted that the Lewis rat Toxoplasma resistance locus (Toxo1) contained the Nlrp1 gene. We found that BMDMs isolated from the Toxoplasma-resistant Lewis rat, but not susceptible Sprague-Dawley (SD) rat, infected with Toxoplasma undergo pyroptosis and release active IL-1β and IL-18. Host cell death is a mechanism to prevent parasite replication, as the majority of Lewis BMDMs contain single parasites after 24 hours of infection while Toxoplasma replicates uninhibited in SD macrophages. A survey of Toxoplasma strains representing worldwide diversity found that all strains tested were able to induce pyroptosis in Lewis macrophages. Using several methods, we found Nlrp1 required for inflammasome and pyroptosis activation by Toxoplasma. Expression of Lewis Nlrp1 in rat macrophages that do not undergo pyroptosis was sufficient to sensitize the cells to infection-induced pyroptosis. Chapter II and III identify Toxoplasma as the second activator of rodent NLRP1.

In chapter IV, we designed a chemical mutagenesis screen where we isolated parasite strains unable to induce pyroptosis in Lewis rat BMDMs. Using whole genome sequencing and RNAseq, we identified the parasite genes mutated. Utilizing CRISPR/Cas9, we knocked out several candidate genes and identified three novel dense granule proteins, GRA18, GRA27 and GRA28 that are individually required for inflammasome activation. Parasites deficient in GRA18, GRA27 or GRA28 fail to induce pyroptosis in Lewis BMDMs, replicate within the Lewis macrophage and activate the Lewis inflammasomes significantly less than wild-type parasites as measured by the release of active IL-1β. The mechanism through which GRA18, GRA27 and GRA28 coordinate to activate NLRP1 is still unknown.
We have demonstrated the inflammasomes are an important innate immune pathway in the control of *Toxoplasma gondii* and identified three novel parasites genes required for NLRP1 activation. In Chapter V, we discuss how these findings have furthered our understanding of parasite-host interactions and future directions.
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Chapter Two:
Dual role for inflammasome sensors NLRP1 and NLRP3 in murine resistance to *Toxoplasma gondii*


*Authors contributed equally to the paper.

Address correspondence to Mahtab Moayeri, mmoayeri@niaid.nih.gov; Jeroen P.J. Saeij, jsaeij@ucdavis.edu; or Michael E. Grigg, griggm@niaid.nih.gov.

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Abstract

Induction of immunity that limits *Toxoplasma gondii* infection in mice is critically dependent on the activation of the innate immune response. In this study, we investigated the role of cytoplasmic nucleotide-binding domain and leucine-rich repeat containing a pyrin domain (NLRP) inflammasome sensors during acute toxoplasmosis in mice. We show that *in vitro* *Toxoplasma* infection of murine bone marrow-derived macrophages activates the NLRP3 inflammasome, resulting in the rapid production and cleavage of interleukin-1β (IL-1β), with no measurable cleavage of IL-18 and no pyroptosis. Paradoxically, *Toxoplasma*-infected mice produced large quantities of IL-18 but had no measurable IL-1β in their serum. Infection of mice deficient in NLRP3, caspase-1/11, IL-1R, or the inflammasome adaptor protein ASC led to decreased levels of circulating IL-18, increased parasite replication, and death. Interestingly, mice deficient in NLRP1 also displayed increased parasite loads and acute mortality. Using mice deficient in IL-18 and IL-18R, we show that this cytokine plays an important role in limiting parasite replication to promote murine survival. Our findings reveal *T. gondii* as a novel activator of the NLRP1 and NLRP3 inflammasomes *in vivo* and establish a role for these sensors in host resistance to toxoplasmosis.

Importance

Inflammasomes are multiprotein complexes that are a major component of the innate immune system. They contain "sensor" proteins that are responsible for detecting various microbial and environmental danger signals and function by activating caspase-1, an enzyme that mediates cleavage and release of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18. *Toxoplasma gondii* is a highly successful protozoan parasite capable of infecting a wide
range of host species that have variable levels of resistance. We report here that *T. gondii* is a novel activator of the NLRP1 and NLRP3 inflammasomes *in vivo* and establish a role for these sensors in host resistance to toxoplasmosis. Using mice deficient in IL-18 and IL-18R, we show that the IL-18 cytokine plays a pivotal role by limiting parasite replication to promote murine survival.

**Introduction**

The innate immune response plays a critical role in protecting hosts against pathogens. Activation of innate immunity occurs after pattern recognition “sensor” proteins such as the Toll-like receptors (TLRs) or nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins detect the presence of pathogens, their products, or the danger signals that they induce during active infection (Lamkanfi and Dixit 2012; Song and Lee 2012). *Toxoplasma gondii* is an intracellular protozoan parasite capable of potently activating innate immunity in the wide range of vertebrate species that it infects (Hunter and Sibley 2012; Melo, Jensen, and Saeij 2011). In mice, resistance to *T. gondii* infection is critically dependent on the TLR-associated adaptor protein MyD88, which is required for the induction of protective levels of the proinflammatory cytokines interleukin-12 (IL-12) and gamma interferon (IFN-γ) and the synthesis of nitric oxide (NO) (Khan et al. 1997; LaRosa et al. 2008; Scanga et al. 2002; Scharton-Kersten et al. 1996; Scharton-Kersten 1997; Sher et al. 2003; Suzuki et al. 1988). The activation and recruitment of inflammatory monocytes to sites of infection are protective, as infection of mice rendered deficient in Gr1⁺ inflammatory monocytes by antibody depletion results in increased susceptibility to parasite infection (Dunay et al. 2008; Robben et al. 2005). Furthermore, chemokine receptor CCR2- and MCP1 (CCL2)-knockout (KO) mice, defective in recruitment of
these cells, are also more susceptible (Dunay et al. 2008; Robben et al. 2005). Hence, induction of protective immunity against this protozoan pathogen is critically dependent on monocyte and macrophage cell activation.

Macrophages are activated when their cognate receptors detect the presence of microbial products. In the case of cytosolic NLRs, which sense the presence of microbes and/or the damage that their infection induces, activation leads to the assembly of the inflammasome, a multiprotein complex that recruits and activates caspase-1 and/or caspase-11. The murine NLRP3 inflammasome senses a wide range of bacteria, pore-forming toxins, and crystalline danger signals, including alum, amyloid clusters, cholesterol, and asbestos (Martinon, Mayor, and Tschopp 2009). In contrast, the murine NLRP1b inflammasome is more restricted; the only characterized activator is the *Bacillus anthracis* lethal toxin (LT) (Boyden and Dietrich 2006). Either multimeric complex is capable of cleaving the proform of caspase-1, which is typically associated with the rapid death of macrophages, through a process known as pyroptosis (Lamkanfi and Dixit 2012; Song and Lee 2012). Pyroptosis, unlike apoptosis, leads to lysis of the cell and release of its intracellular contents. Caspase-1 also cleaves the proinflammatory cytokines IL-1β and IL-18, allowing their secretion from cells (Lamkanfi and Dixit 2012; Song and Lee 2012). Whether the inflammasome is activated during *Toxoplasma* infection, or is capable of altering disease pathogenesis, has thus far been only inferred. An association of polymorphisms in the human Nlrp1 gene with susceptibility to congenital toxoplasmosis was recently reported (Jamieson et al. 2010; Witola et al. 2011). *T. gondii* production of cleaved IL-1β in human monocytes is dependent on both caspase-1 and the NLRP3 adaptor protein ASC (Gov et al. 2013). P2X(7) receptors, which are important in ATP-mediated activation of the NLRP3 inflammasome, have also been shown to influence parasite proliferation in human and
murine cells (Lees et al. 2010). IL-18, a key substrate of inflammasome-activated caspase-1, is known to enhance production of IFN-γ (Dinarello et al. 1998), which is a central regulator of *Toxoplasma* pathogenesis. Furthermore, *in vivo* administration of IL-1β protects mice from lethal challenge with *Toxoplasma* (Chang, Grau, and Pechère 1990) and injection of antibodies against the IL-1 receptor (IL-1R) significantly attenuates the protective effect that exogenous IL-12 confers on infected SCID mice (Hunter, Chizzonite, and Remington 1995). Thus, we hypothesized that inflammasome activation might be an important factor mediating murine host resistance to *Toxoplasma* infection.

In this study, we show that murine macrophages are not susceptible to *Toxoplasma gondii*-induced rapid pyroptosis but that NLRP3 inflammasome activation in these cells results in rapid IL-1β cleavage and release. We establish that both NLRP3 and NLRP1 are important *in vivo* regulators of parasite proliferation and that IL-18 signaling is required to mediate host resistance to acute toxoplasmosis. Our findings establish a role for two inflammasomes in the control of *Toxoplasma* infection.
Results and Discussion

*Toxoplasma* activates the inflammasome in murine macrophages without inducing cell death

Induction of protective immunity capable of controlling murine *Toxoplasma* infection is critically dependent on myeloid cell activation (Hunter and Sibley 2012). The ability of this parasite to promote caspase-1 activation and the secretion of active IL-1β has recently been established in human and rat monocytes and macrophages (Witola et al. 2011; Gov et al. 2013; Cirelli et al. 2014). To determine if *Toxoplasma* activates the inflammasome in murine macrophages, we infected unstimulated and lipopolysaccharide (LPS)-primed bone marrow-derived macrophages (BMDMs) prepared from C57BL/6J mice with type II (Pru) parasites and measured IL-1β secretion 24 hours after infection (Figure 1A). Uninfected BMDMs did not produce measurable levels of IL-1β (Figure 1A), whereas IL-1β was readily detected after infection with type II *Toxoplasma* regardless of whether the BMDMs were LPS primed or not (Figure 1A). Western blotting of infected BMDM lysates showed the presence of mature IL-1β and showed that cleavage was dependent on caspase-1/11, since infected BMDMs from caspase-1/11-deficient mice did not possess detectable levels of cleaved IL-1β (Figure 1B). The detection of mature IL-1β in the *Toxoplasma*-infected BMDMs indicated inflammasome activation, but the cells did not undergo pyroptosis over 24 hours (Figure 1C). These data support an inflammasome-mediated processing and release of mature IL-1β in the absence of pyroptosis, which have been demonstrated to occur previously (Broz et al. 2010). Interestingly, IL-18 upregulation and cleavage were not observed in *Toxoplasma*-infected BMDMs or
splenocyte lysates over a range of multiplicities of infection (MOIs) and times, and the cytokine
cell was not released from *in vitro*-infected macrophages or splenocytes (data not shown).

Because IL-1β secretion was consistently dependent on MOI (data not shown), we tested
whether parasite invasion was required for IL-1β secretion. Parasites pretreated with mycalolide 
B, an actin-depolymerizing agent that blocks invasion but allows for secretion of microneme and 
rhoptry contents, attached but induced significantly smaller amounts of IL-1β secretion 
(Figure 1D), indicating that macrophage inflammasome activation was invasion-dependent. The 
small amount of IL-1β secretion by BMDMs infected by mycalolide B-treated parasites was 
likely due to incomplete inhibition of invasion, as immunofluorescence microscopy performed 
on the same batch of treated parasites indicated that a small number had still invaded the 
BMDMs, as evidenced by their intracellular replication (data not shown).
Figure 1. *Toxoplasma* activates the inflammasome in C57BL/6 and 129S BMDCs. BMDCs were primed with 100 ng/ml LPS or left unstimulated for 2 hours and subsequently infected with type II parasites (Pru; average MOI, 1) for 24 hours. (A) Quantification of IL-1β in supernatants was performed
using ELISA. (B) IL-1β cleavage was monitored by Western blotting of cell lysates from C57BL/6NTac or caspase-1/11" BMDMs that were infected with type II parasites (Pru; MOI, 0.8) for 24 hours. The positions of both pro-IL-1β (37 kDa) and cleaved IL-1β (17 kDa) are indicated. (C) Cell viability of infected cells in panel A was determined at different time points using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Panels A and C are averages of three experiments. Error bars, + standard deviations. (D) BMDMs were primed with LPS for 2 hours and infected for 24 hours with type I parasites (RH) that were pretreated with dimethyl sulfoxide vehicle or mycalolide B (3 μM) for 20 minutes. IL-1β was measured using ELISA. Data are averages of 2 experiments. Error bars, + standard deviations. (E) C57BL/6 BMDMs were infected with the indicated strains for 24 hours. IL-1β was measured using ELISA. Data are the averages of at least 3 experiments per strain. The haplogroup to which the strain belongs is indicated above. Error bars, + standard deviations. (F) C57BL/6 BMDMs were infected for 18 hours with Pru (type II) or PruΔGRA15 (MOI, 4) for 18 hours, and microarrays were used to determine the fold change in IL-1β mRNA expression levels compared to uninfected macrophages. (G) BMDMs were infected with Pru (type II) or PruΔGRA15 for 24 hours. IL-1β was measured using ELISA. Data are representative of 3 experiments. Error bars, + standard deviations. (H) BMDMs were primed for 2 hours with LPS and then infected with indicated strains for 24 hours (MOI, 4). The haplogroup to which the strain belongs is indicated above. Data are the averages of 3 experiments. Error bars, + standard deviations. (I) IL-1β secretion from primed immortalized murine WT and caspase-1/11" macrophages, infected for 24 hours with RH. Data shown are from an experiment representative of three. Error bars, + standard deviations. (J) IL-1β secretion from BMDMs prepared from wild-type C57BL/6 mice (blue) or C57BL/6 mice lacking Nlrp1b (red), Nlrp3 (green), Nlrp1b and Nlrp3 (orange), or Asc (yellow), primed for 4 hours, and then infected with type I (RH; average MOI, 1) for 24 hours. Cytokine secretion below the detection level is indicated on the graph with arrowheads and labeled not detected (n.d.). Data are averages of four experiments. Error bars, + standard deviations. (K) BMDMs described for panel J were primed for 3 hours with LPS and then infected with type II parasites (MOI, 1.5) for 24 hours. Western blot analysis on concentrated supernatants (25-fold), probing for cleaved IL-1β (17 kDa). (L) Host cell viability in panel L was measured using the MTS assay. Error bars, + standard deviations. (M) BMDMs from C57BL/6 and 129S mice were primed with 100 ng/ml LPS for 2 hours and subsequently infected with type II parasites (Pru; average MOI, 0.7) for 24 hours. Quantification of IL-1β in supernatants was performed using ELISA. Panels L and M are the averages of 3 experiments. (N) 129S BMDMs were primed for 2 hours and infected with type II parasites (MOI, 1.6) for 24 hours. Western blot analysis on concentrated supernatants (25-fold), probing for cleaved IL-1β (17 kDa). Nig, nigericin; Tg, T. gondii.

**IL-1β secretion correlates with strain differences in NF-κB activation**

Mouse strains differ in their susceptibility to *Toxoplasma* depending on the infecting strain genotype; haplogroup 2 and 12 (HG2 and HG12) strains are relatively avirulent and readily establish chronic infections, whereas HG1 and HG4 to HG10 strains are acutely virulent. We sought to determine whether *Toxoplasma* strains differentially activate the murine macrophage inflammasome, or whether secretion of IL-1β correlated with parasite genotype
and/or pathogenesis. We infected unprimed BMDMs from C57BL/6J mice with *Toxoplasma* tachyzoites from all 12 haplogroups for 24 hours, a time point at which parasite-induced cell lysis was minimal. Cougar (HG11) and the type II strains, with the exception of DEG, induced IL-1β secretion in unstimulated BMDMs (Figure 1E). Inflammasome activation is often divided into a signal 1, which is the signal that leads to transcription of *IL-1β*, and signal 2, which is the signal that leads to the actual activation of caspase-1. Type II, but not type I or III, parasites directly activate the NF-κB transcription factor in both human and murine cells, thereby potentially providing signal 1 for the induction of *IL-1β* transcription. The secreted dense granule protein GRA15 determines this strain difference in NF-κB activation (Rosowski et al. 2011). Indeed, in murine BMDMs, type II *IL-1β* mRNA induction was partially dependent on type II GRA15 expression (Figure 1F), while IL-1β secretion of unstimulated BMDMs was completely dependent on GRA15 (Figure 1G). To determine if non-type II strains can provide signal 2, which leads to the activation of caspase-1 and subsequent cleavage and secretion of IL-1β, we prestimulated BMDMs with LPS for 2 hours and subsequently infected them with different *Toxoplasma* strains. IL-1β was now detected in the medium with no truly apparent differences between strains (Figure 1H). The IL-1β secreted into the medium also contained the cleaved active IL-1β (17 kDa) as determined by Western blot analysis (Figure S2).
Toxoplasma activation of the murine inflammasome in BMDMs is dependent on caspase-1/11 and NLRP3

To determine the components necessary for IL-1β secretion, we infected immortalized macrophages that lacked caspase-1 and -11 and showed that IL-1β secretion was completely eliminated, as expected (Figure 1I). To determine the inflammasome components necessary for IL-1β secretion, we infected BMDMs from C57BL/6 mice that lacked Nlrp1b, Nlrp3, or both Nlrp1b and Nlrp3, or the inflammasome adaptor ASC. We found IL-1β secretion by primed BMDMs upon Toxoplasma type I infection to be mostly dependent on ASC and the NLRP3 inflammasome (Figure 1J). Similar results were obtained after type II infection (Figure S3). The greatly reduced amount of cleaved active IL-1β in the supernatant of Nlrp3-deficient Toxoplasma-infected BMDMs compared to the amount present in the supernatant of wild-type (WT) or Nlrp1b-deficient infected BMDMs confirmed the importance of the NLRP3 inflammasome in Toxoplasma-mediated inflammasome activation in vitro (Figure 1K). Thus, Toxoplasma induction of IL-1β secretion by murine BMDMs is highly dependent on the NLRP3 inflammasome and requires caspase-1 activation.

Inflammasome-mediated BMDM death and cytokine processing are independent of Nlrp1a and Nlrp1b alleles

Despite the activation of caspase-1 in Toxoplasma-infected cells, we did not observe any macrophage pyroptosis. Previous reports have shown that five polymorphic Nlrp1b alleles exist among inbred mice which control sensitivity to anthrax LT-induced pyroptosis (Boyden and Dietrich 2006). To test if the >100-amino-acid (aa) differences in Nlrp1b between C57BL/6J and 129S mice were the basis for resistance to parasite-induced pyroptosis, we compared BMDMs
from the two strains. We observed no difference in cell viability between the C57BL/6J and 129S BMDMs (Figure 1L). Thus, consistent with a major role for NLRP3 inflammasome activation, BMDMs from either 129S or C57BL/6 mice produced active IL-1β (Figure 1M and N) without associated pyroptosis upon type I or II Toxoplasma strain infection. Furthermore, the fact that 129S BMDMs do not express the highly conserved NLRP1a protein (Sastalla et al. 2013) and are caspase-11 deficient (Kayagaki et al. 2011) but present a robust IL-1β response also eliminated a role for NLRP1a and caspase-11 in cytokine maturation induced by Toxoplasma.

Murine resistance to Toxoplasma infection is controlled by caspase-1/11-dependent inflammasome activation

Whether inflammasome activation is important to murine resistance to infection in vivo has not yet been established. We infected mice deleted for the caspase-1/11 genes with 10,000 type II 76K green fluorescent protein-luciferase (GFP-LUC) tachyzoites intraperitoneally (i.p.) and tested for susceptibility to acute infection by monitoring mean survival time (MST), parasite growth, dissemination, and the production of IL-1β and IL-18. In the absence of caspase-1/11 proteins, mice had a 10- to 20-fold-higher parasite load (Figure 2A and B) and were highly susceptible to acute infection (Figure 2C). In contrast, the majority of C57/BL6NTac control mice survived acute infection and established chronic infections (Figure 2C). Surprisingly, serum levels of systemic IL-1β never exceeded 10 pg/ml on day 5 (Figure 2D, graph on left) or day 9 (data not shown) for either mouse strain. IL-18 levels, however, were significantly higher following infection, ranging from 0.5 to 2.0 ng/ml in C57BL/6NTac mice on day 5 (Figure 2D),

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to strikingly high levels exceeding 10 ng/ml by day 9, compared to <200 pg/ml in caspase-1/11-deficient mice (Figure 2D) or uninfected controls (data not shown).

![Image of Figure 2A, showing bioluminescence images of infected mice.]

![Image of Figure 2B, showing parasite load and survival.]

![Image of Figure 2C, showing percent survival over days post infection.]

![Image of Figure 2D, showing IL-18 levels in serum.]

Figure 2. Parasite load, survival, and systemic IL-18 levels in caspase-1/11-deficient mice. (A) Bioluminescence imaging (BLI) of infected caspase-1/11 knockouts and controls on days 5 to 7 following infection with 76K GFP-LUC (10,000 tachyzoites i.p.). Images shown are for 3 mice. (B) Quantifications are from 8 mice imaged/group. (C) Aggregate survival of caspase-1/11-knockout mice (n = 13/group) compared with WT C57BL/6NTac control mice (n = 10/group). (D) IL-18 measurements in serum of caspase-1/11-deficient mice on day 5 after infection were significantly different from WT (P < 0.001) when infected with 76K GFP-LUC. No detectable levels of IL-1β were detected in circulation.
ASC and NLRP3 inflammasome activation controls *Toxoplasma* proliferation and host resistance

We next investigated the role of ASC in murine susceptibility to *Toxoplasma* infection, since this adaptor protein is required to mediate the activation of multiple inflammasomes (Latz, Xiao, and Stutz 2013). If inflammasome activation controls *Toxoplasma* resistance, we hypothesized that mice rendered deficient in this protein will be more susceptible to acute infection. Asc-deficient mice consistently had ~20-fold or greater parasite loads at days 5 to 7 postinfection (Figure 3A and B) and generally succumbed to infection by days 8 to 10 (Figure 3C), in contrast to wild-type control mice, the majority of which survived acute infection at day 20 (Figure 3C). The Asc-deficient mice likewise failed to induce detectable levels of systemic IL-18 (Figure 3D) or IL-1β (data not shown) during acute infection.

To determine whether the NLRP3 inflammasome sensor is sufficient to confer this murine resistance to the *Toxoplasma* phenotype, Nlrp3<sup>−/−</sup> mice on the C57BL/6J background were infected intraperitoneally with 10,000 76K GFP-LUC tachyzoites. Nlrp3<sup>−/−</sup> mice were more susceptible than their wild-type (WT) controls and possessed 10-fold or higher parasite burdens at days 5 to 7, and the majority of mice died by day 10 postinfection (Figure 3C). These infected mice produced intermediate levels of systemic IL-18, significantly greater than those of the Asc-deficient mice but not equivalent to those of the WT (Figure 3D). They did not produce measurable levels of IL-1β (data not shown), similar to what was observed with all infections of WT mice (Figure 2D and data not shown). Infection with another type II strain (Pru GFP-LUC) produced similar results (data not shown), indicating that the phenotype was not attributable to an anomalous *Toxoplasma* clone-specific effect or the genome integration site of the GFP-LUC gene, as both type II strains activated the inflammasome *in vivo*. These results indicate that
murine resistance to acute infection with *Toxoplasma* is highly dependent on the activation of the NLRP3 inflammasome.

**Figure 3.** Parasite load, survival, and systemic IL-18 levels in ASC- and NLRP3-knockout mice following *Toxoplasma* challenge. (A) Bioluminescence imaging of 76K GFP-LUC-infected mice (various strains, 10,000 tachyzoites, i.p. route) on days 5 to 7 following infection is shown. Images shown are from two or three representative mice from 2 to 6 mice/group from one representative experiment. The experiment shown is one of 3 (WT) or 2 (ASC and NLRP3) independent experiments. (B) *P* values (*t* test) comparing luciferase activity for each knockout strain to C57BL/6J are <0.05. (C) Aggregate survival curve of ASC (*n* = 8)- and NLRP3 (*n* = 5)-knockout mice compared with WT C57BL/6J control mice (*n* = 13). (D) IL-18 measurements in serum of deficient mice on day 5 after infection. No detectable levels of IL-18 were detected in circulation.
NLRP1 inflammasome activation also controls *Toxoplasma* proliferation and host resistance

Because the infected *Nlrp3*-deficient mice still produced IL-18 at levels higher than those of *Asc*-deficient mice, we hypothesized that more than one inflammasome is activated *in vivo* to produce IL-18 during *Toxoplasma* infection. To test this prediction, we infected mice deficient at the *Nlrp1* locus encompassing *Nlrp1abc* with 10,000 76K GFP-LUC tachyzoites to determine if NLRP1 activation contributes to murine resistance during *Toxoplasma* infection. *Nlrp1abc*-deficient mice had only 3- to 5-fold-higher parasite loads than did WT mice across days 5 to 7 (*Figure 4A and 4B*). *Nlrp1abc*<sup>−/−</sup> mice also died acutely, succumbing to infection between days 16 and 22 (*Figure 4C*). The delayed MST kinetics was consistent with the decreased parasite burden in comparison to the *Nlrp3*-deficient mice. *Nlrp1abc*-deficient mice produced intermediate levels of systemic IL-18, significantly greater than those of the *Asc*-deficient mice but not equivalent to those of the WT (*Figure 4D*). No measurable levels of circulating IL-1β were found on day 5 or 9 after infection in these mice or their WT controls (data not shown).
Figure 4. Parasite load, survival, and systemic IL-18 levels in NLRP1-knockout mice following Toxoplasma challenge. (A) Bioluminescence imaging of 76K GFP-LUC-infected mice (various strains, 10,000 tachyzoites, i.p. route) on days 5 to 7 following infection is shown. Images shown are three representative mice from 4 to 6 mice/group from one representative experiment. The experiment shown is one of two WT or two NLRP1 independent experiments. (B) P values (t test) comparing luciferase activity for each knockout strain to C57BL/6NTac are <0.05. (C) Aggregate survival curve of NLRP1 (n = 10)-knockout mice compared with WT C57BL/6NTac control mice (n = 10). (D) IL-18 measurements in serum of deficient mice on day 5 after infection. No detectable levels of IL-1β were detected in circulation.
Mice deficient in signaling and secretion of IL-18 are highly susceptible to Toxoplasma infection

Although no systemic IL-1β was detectable in vivo, infection of NLRP3-deficient BMDMs showed markedly diminished levels of IL-1β (Figure 1). To test if IL-1β possesses a biologically important role locally and is capable of influencing murine resistance, we infected IL-1R−/− mice with 10,000 76K GFP-LUC tachyzoites. All IL-1R-deficient mice succumbed to infection but with a delayed kinetics compared to ASC−/−, caspase-1/11−/−, and Nlrp3-deficient mice (Figure 5C). These mice supported higher parasite loads and had intermediate levels of IL-18 in their serum compared to WT C57BL6/J mice (Figure 5A, B, and D), indicating that despite the absence of measurable IL-1β in circulation, IL-1 signaling does play a contributing role in the protection against murine toxoplasmosis.

Intriguingly, the level of IL-18 in the circulation of infected WT mice correlated with decreased parasite burden and increased survival. We hypothesized that inflammasome activation and the production of high systemic IL-18 might play an important role in the relative resistance of WT mice. To test our hypothesis, we infected IL-18−/− and IL-18R−/− mice. Both types of mice were highly susceptible to acute Toxoplasma infection and consistently had 20- to >100-fold-increased parasite loads at days 5 to 7 postinfection, greater than that found in the Asc−/− mice (Figure 5A and B). These mice typically succumbed to infection by day 8 or 9 (Figure 5C), suggesting that the presence of IL-18 is protective in C57BL/6 mice. These data indicate that the production and secretion of activated IL-18 are associated with controlling parasite proliferation and murine resistance to acute toxoplasmosis.
Figure 5. Parasite load, survival, and systemic IL-18 levels in IL-18- and IL-18R-knockout mice. (A) Bioluminescence imaging of 76K GFP-LUC-infected mice (various strains, 10,000 tachyzoites, i.p. route) on days 4 to 7 following infection is shown. Images shown are 2 to 3 representative mice from 3 to 6 mice/group. The experiment shown is one of two IL-1β, four IL-18, or four IL-18R independent experiments. (B) P values (t test) comparing luciferase activity for each knockout strain to C57BL/6J are <0.05. (C) Aggregate survival curve of IL-1R (n = 9)-, IL-18 (n = 19)-, and IL-18R (n = 13)-knockout mice compared with WT C57BL/6J control mice (n = 13). (D) IL-18 measurements in serum of deficient mice on day 5 after infection. No detectable levels of IL-1β were detected in circulation.
Discussion

Generation of a robust innate immune response is required to orchestrate murine resistance against the intracellular pathogen *Toxoplasma gondii*, as well as a wide spectrum of other pathogenic agents (Hunter and Sibley 2012). Resistance to *Toxoplasma* infection is critically dependent on the TLR-associated adaptor protein MyD88 and induction of IL-12, IFN-γ, and the synthesis of nitric oxide (NO). In this study, we show that in vivo generation of host protective immunity against *Toxoplasma* is also highly dependent on the inflammasome sensors NLRP1 and NLRP3 and the secretion of the caspase-1-dependent proinflammatory cytokine IL-18. Infection of mice deficient in NLRP3, NLRP1abc, caspase-1/11, or the inflammasome adaptor protein ASC led to decreased levels of circulating IL-18, increased parasite replication, and death. Using mice deficient in IL-18 and IL-18R, we show that this cytokine plays an important role in limiting parasite replication to promote murine survival.

IL-18, like IL-1β, has been extensively linked to both protective immune responses and disease induction. IL-18 mediates enhancement of innate resistance to acute toxoplasmosis by triggering IFN-γ induction in immune cells, especially T and NK cells, and works in synergy with IL-12. It has previously been used as a protective treatment (Cai, Kastelein, and Hunter 2000; Yap et al. 2001), and IL-18 depletion by antibodies significantly alters murine susceptibility upon infection with lethal doses of *Toxoplasma* (Mordue et al. 2001). In fact, IL-18 was at one time known as “IFN-γ-inducing factor” (Okamura et al. 1995). The inactive pro-IL-18 form is constitutively expressed in a wide range of cell types (Puren, Fantuzzi, and Dinarello 1999) but requires processing by caspase-1 to promote its secretion, as evidenced by the drastically depleted levels of IL-18 during infection of *caspase-1/11−/−* mice (Figure 2D), and promote the induction of IFN-γ production in vivo (Ghayur et al. 1997; Gu 1997).
Interestingly, Asc-deficient mice produced even less circulating IL-18, indicating that other factors such as caspase-8 (Bossaller et al. 2012) may contribute to IL-18 processing.

The role of IFN-γ in resistance to Toxoplasma is extensively documented (Hunter and Sibley 2012; Melo, Jensen, and Saeij 2011). IFN-γ activates cellular pathways that promote resistance to toxoplasmosis through multiple mechanisms, including activation of the interferon-inducible GTPases (IRG-GTPases) (Howard, Hunn, and Steinfeldt 2011; Hunn et al. 2011) and NO regulation (Scharton-Kersten 1997), processes certainly dependent on induction of IFN-γ by IL-18 produced following NLR inflammasome activation. However, IL-18 has also been linked to pathology during infection with type I strains of Toxoplasma, and IL-18 depletion resulted in enhanced survival by limiting the propathologic immune response that these virulent strains induce (Mordue et al. 2001; Gavrilescu and Denkers 2001). Hence, the balance between the protective and pathological roles of IL-18 is likely highly dependent on mouse genetics, Toxoplasma strain differences, challenge doses, routes of infection, and rates of disease progression. Previous work using a low-dose type II (PTG) infection in caspase-1-deficient mice (now known to be caspase-1/11 deficient) concluded that these mice were not altered in Toxoplasma susceptibility relative to wild-type control mice (Hitziger et al. 2005). However, this study was performed with a parasite dose that does not typically induce measurable levels of systemic IL-18 (Mordue et al. 2001), and the mice used had a mixed 129/B6 background, which itself may influence pathogenesis. Our results, using mice sufficiently backcrossed onto a C57BL/6NTac background and a parasite inoculum that induces systemic IL-18, show a role for caspase-1 and IL-18 in murine resistance. IL-18 concentrations are known to vary through the course of infection and are clearly dependent on the parasite strain and inoculum used (Mordue et al. 2001; Gavrilescu and Denkers 2001). Our results suggest that this cytokine plays a pivotal
role in mediating acute toxoplasmosis, with the cytokine playing an important early role in the control of parasite replication (Figure 5B). How exactly IL-18 mediates this protection requires further studies. Later in infection, however, high levels of IL-18 have previously been shown to cause dysregulated induction of propathologic cytokine levels that contribute to lethality in high-dose, virulent infections (Mordue et al. 2001; Gavrilescu and Denkers 2001).

The recruitment of inflammatory monocytes to sites of infection is essential to control parasite growth and dissemination in murine models of toxoplasmosis (Dunay et al. 2008; Robben et al. 2005). In rats, control of parasite proliferation and dissemination in vivo is controlled by the Toxol locus (Cavaillès et al. 2006). Our recent work showed that macrophages from Toxoplasma-resistant rat strains (e.g., LEW and SHR) undergo pyroptosis in response to inflammasome activation induced by parasite infection, and this rapid cell death is sufficient to limit parasite replication and promote sterile cure (Cirelli et al. 2014). In previous work by Miao et al., caspase-1-induced pyroptotic cell death was also identified as an innate immune mechanism to protect against intracellular pathogen infection (Miao et al. 2010). In their study, the authors used a panel of mouse strains deficient in IL-1β, IL-18, IL-1βR, or various combinations of those to show a dispensable role for IL-1β and IL-18 in the clearance of Salmonella enterica serovar Typhimurium that expresses flagellin, suggesting that innate control of bacterial infection is occurring by pyroptosis, without a requirement to induce an overt inflammatory response. In this study, we show that in vitro Toxoplasma infection of murine bone marrow-derived macrophages primarily activates the NLRP3 inflammasome, resulting in the rapid production and cleavage of IL-1β, but does not induce pyroptosis. Interestingly, although Toxoplasma-infected macrophages showed efficient caspase-1/11-dependent IL-1β cleavage and secretion, these cells did not upregulate, cleave, or secrete their preexisting pools of
IL-18. Furthermore, splenocytes also did not show any IL-18 cleavage following infection. Paradoxically, significant concentrations of IL-1β were not detected following infection in any mouse strain, whereas high levels of IL-18 were found in serum in all infection studies that we performed with different *Toxoplasma* strains. Because activation of the murine inflammasome does not affect *Toxoplasma* growth in macrophages (data not shown) and does not induce pyroptosis, our results suggest that IL-18 activation and not pyroptosis is the genetic basis for *in vivo* inflammasome-mediated control of parasite proliferation. Although the *in vivo* cellular source for this inflammasome-generated IL-18 is not known, it is likely of nonmyeloid origin, and bone marrow chimera studies should be performed to address this question. Our results also suggest that NLRP1-mediated events may be more important *in vivo* and that activation of NLRP1 may likewise occur in cells other than macrophages.

The role of inflammasome activation in the pathogenesis of *Toxoplasma* infection in human infection has recently been suggested (Jamieson et al. 2010; Witola et al. 2011). Polymorphisms in the human *NLRP1* gene are associated with susceptibility to congenital toxoplasmosis, and NLRP1 contributes to controlling parasite growth in human monocytes. A recent study in human macrophages provides compelling evidence that the inflammasome components ASC and caspase-1 regulate the release of IL-1β and that the type II allele of the parasite dense granule protein GRA15, which activates NF-κB nuclear translocation, is necessary for maximal induction of this cytokine (Gov et al. 2013). Indeed, our *in vitro* infection data show that *Toxoplasma* murine inflammasome-mediated secretion of IL-1β is strain dependent and that only parasites expressing the GRA15 type II allele, which directly activates NF-κB, were able to induce secretion of IL-1β in unprimed BMDMs (*Figure 1*). While the relative and contributing roles of IL-1β compared to IL-18 remain to be determined in the control of acute toxoplasmosis,
our preliminary studies using IL-1 receptor-knockout mice on a mixed background argue that IL-1\(\beta\) plays a less significant role in the control of parasite infection than it does in IL-18 or IL-18R knockouts (Figure 5). In vivo administration of IL-1\(\beta\) in LPS-primed caspase-1/11-deficient mice has previously been shown to increase IL-6 (Gu 1997), so it is conceivable that IL-1\(\beta\) functions locally to induce increased levels of IL-6 capable of altering inflammation-induced changes in myeloid output that impact Toxoplasma pathogenesis (Chou et al. 2012).

Of the two NLR inflammasomes activated, we found that murine resistance to acute infection was principally dependent on activation of the NLRP3 receptor both in vitro (Figure 1J) and in vivo (Figure 3). Several reports have linked P2X(7) receptor, a potent activator of the NLRP3 inflammasome, with control of acute toxoplasmosis (Jamieson et al. 2010; Lees et al. 2010; Corrêa et al. 2010; Miller et al. 2011). How and in what cell type Toxoplasma activates the murine NLRP3 inflammasome or why its activation does not lead to rapid macrophage death or IL-18 processing is enigmatic. Regulators of the NLRP3 inflammasome include ATP, the guanylate-binding protein 5 (GBP5), cellular stresses that alter calcium and potassium concentrations, redox status, and the unfolded protein response (UPR) (Wen, Miao, and Ting 2013). Importantly, Toxoplasma encodes a variety of virulence effector proteins that specifically inactivate the host endoplasmic reticulum (ER)-bound transcription factor ATF6\(\beta\) and induction of the UPR during ER stress (Yamamoto et al. 2011), affect the recruitment of 65-kDa guanylate-binding proteins (GBPs) (Niedelman et al. 2013; Selleck et al. 2013), or alter calcium and potassium efflux to signal Toxoplasma egress (Fruth and Arrizabalaga 2007), perhaps indicating that the parasite has specifically evolved effector proteins to minimize NLR inflammasome activation to alter its pathogenesis.
Our work also identified *Toxoplasma* as the second pathogen, after *B. anthracis*, whose pathogenesis is altered by expression of the murine NLRP1 inflammasome (Boyden and Dietrich 2006). We show that the *Nlrp1* locus is capable of regulating parasite proliferation in vivo, with *Nlrp1* knockout mice possessing significantly higher parasite burdens following *Toxoplasma* infection. Although the majority of NLRP1-deficient mice died acutely (Figure 4), they were, however, less susceptible to infection than were caspase-1/11, *Asc, Nlrp3, IL-18-,* or *IL-18R*-deficient mice in the same genetic background and possessed only 5- to 10-fold-higher parasite loads than in WT infections. How *Toxoplasma* activates the NLRP1 inflammasome is unclear. Activation of rodent NLRP1 inflammasomes by the *B. anthracis* lethal toxin (LT) occurs via proteolytic cleavage at a specific consensus sequence in the polymorphic N terminus of NLRP1 (Levinsohn et al. 2012; Newman et al. 2010). In human infection, NLRP1 polymorphism variants are likewise known to alter the susceptibility to congenital toxoplasmosis (Witola et al. 2011). One logical hypothesis is that the *Toxoplasma*-encoded effector molecule responsible for activation of NLRP1 is, like LT, a protease *Toxoplasma* secretes a wide range of proteases (Binder and Kim 2004; Choi, Nam, and Youn 1989; Dou and Carruthers 2011; Dou, Coppens, and Carruthers 2013; Kim 2004; Shea et al. 2007), and similar induction of IL-1β observed upon infection of primed BMDMs with any *Toxoplasma* strain suggests that the putative protease, or factor responsible for activation of NLRP1, is not likely to be *Toxoplasma* strain specific or is at least conserved among the majority of strains. Alternatively, polymorphisms in *Nlrp1* could affect the interaction with a different host “sensor” protein that serves as the adaptor for assembly and activation of the NLRP1 inflammasome, as has been previously described for the NLRC4/NAIP5/NAIP6 inflammasome recognition of flagellin (Kofoed and Vance 2011; Zhao et al. 2011).
In summary, we establish that both NLRP3 and NLRP1 are important \textit{in vivo} regulators of \textit{Toxoplasma} proliferation and that IL-18 signaling is required to mediate host resistance to acute toxoplasmosis. Our findings also indicate that innate resistance to acute toxoplasmosis is dependent on the activation of both TLR and NLR sensors that cooperate to detect the presence of pathogen products or the danger signals that they induce during active infection. The identification of the \textit{Toxoplasma} factor that mediates NLR inflammasome activation may contribute new insight into the development of therapeutic options to combat this important human pathogen.
Materials and Methods

Ethics statement

All animal experiments were performed in strict accordance with guidelines from the NIH and the Animal Welfare Act, under protocols approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (protocols LPD-8E and LPD-22E), and the MIT Committee on Animal Care (assurance number A-3125-01).

Material

Ultrapure LPS was purchased from Calbiochem/EMD Biosciences (San Diego, CA). Luciferin was purchased from Caliper Life Sciences (Hopkinton, MA). Nigericin was purchased from Invivogen (San Diego, CA). Mycalolide B was purchased from Wako (Richmond, VA).

Mice and NLRP1 expression status-based nomenclature

IL-18- and IL-18 receptor (IL-18R)-knockout mice on the C57BL/6J background (>10 backcrosses) and IL-1R-deficient mice on a partially backcrossed 129 × C57BL/6J background were obtained from Jackson Laboratories (Bar Harbor, ME). Caspase-1-knockout mice have been previously described (Sutterwala et al. 2006) and were backcrossed to C57BL/6NTac mice for 10 generations. These caspase-1-knockout mice are also deficient in caspase-11 (Kayagaki et al. 2011). Mice deleted for all three Nlrp1 genes in the murine Nlrp1abc locus (C57BL/NTac background), as well as those deleted only for Nlrp1b (C57BL/6J background), have been previously described (Kovarova et al. 2012; Masters et al. 2012). Mice deleted at
Nlrp3 (C57BL/6J background) (Mariathasan et al. 2006) and Asc (C57BL/6J background) (Sutterwala et al. 2006) have been previously described.

Parasites

Tachyzoites from luciferase-expressing type I (RH) and type II (76K or Prugniaud) *T. gondii* parasites were used for all studies. The following strains (haplogroup/type in parentheses) were used in a survey of effects on murine BMDMs: GT1 (I), ME49 (II), DEG (II), CEP (III), VEG (III), CASTELLS (IV), MAS (IV), GUY-KOE (V), GUY-MAT (V), RUB (V), BOF (VI), GPHT (VI), CAST (VII), TgCATBr5 (VIII), P89 (IX), GUY-DOS (X), VAND (X), Cougar (XI), B41 (XII), B73 (XII), RAY (XII), and WTD3 (XII). The generation of luciferase-expressing parasites using the plasmid pDHFR-Luc-GFP gene cassette has been described previously (Saeij et al. 2005). To construct the RH, Prugniaud, and 76K GFP-LUC strains, pDHFR-Luc-GFP was linearized with NotI, parasites were electroporated, and those with stable GFP expression were isolated by fluorescence-activated cell sorting and cloned by limiting dilution. Generation of Pru GRA15-knockout (KO) parasites has been previously described (Rosowski et al. 2011). All parasite strains were routinely passaged *in vitro* in monolayers of human foreskin fibroblasts (HFFs) at 37°C in the presence of 5% CO₂ and quantified by hemocytometer counts prior to infection studies. In some experiments, mycalolide B (3 μM, 20 min) was used to pretreat isolated parasites prior to washing in phosphate-buffered saline (PBS) (3×) before infections.
Cell culture

Bone marrow-derived macrophages (BMDMs) were cultured in complete Dulbecco’s modified Eagle’s medium (DMEM) with 20% L929 cell culture supernatant for 7 days. L929 mouse fibroblast cells were grown in DMEM supplemented with 10% fetal bovine serum, 10 mM HEPES, and 50 μg/ml gentamicin (all obtained from Invitrogen, Carlsbad, CA) at 37°C in 5% CO₂. BMDMs with or without LPS priming (0.1 μg/ml, 2 h) were infected with *Toxoplasma* at various multiplicities of infection (MOIs), and cell viability was assessed at 24 h using the CellTiter 96 AQueous One Solution cell proliferation assay (Promega, Madison, WI). Culture supernatants were removed for cytokine measurements by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) or Western blotting, following concentration using Amicon filters (3,000-molecular-weight cutoff) (Millipore, Billerica, MA) or Spin-X UF 500 concentrators (5,000-molecular-weight cutoff) (Corning, United Kingdom). For Western blots, anti-mouse IL-1β (Abcam, Cambridge, MA) or anti-caspase-1 antibody (Abcam, Cambridge, MA) was used as the primary antibody. Secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Immun-Star Western C substrate (Bio-Rad, Hercules, CA) and a charge-coupled device camera (Chemidoc XRS; Bio-Rad) were used for visualization. All immortalized macrophage cell lines (WT and caspase-1/11⁻/⁻) were grown in complete DMEM with 10% L929-conditioned medium.

Microarray analysis

Microarray analyses were performed as previously described (Jensen et al. 2011).
Mouse infections

Mice (male and female, 8 to 12 weeks old) were infected intraperitoneally (i.p.) with either 10,000 (76K) or 1,200 (Pru) type II tachyzoites diluted in 400 µl of phosphate-buffered saline. Mice were imaged on successive days (typically days 4 to 9) postinfection, and parasite burden was quantified by firefly luciferase activity using an IVIS BLI system from Caliper Life Sciences. Mice were injected i.p. with 3 mg of D-luciferin substrate (prepared in 200 µl of PBS) and imaged for 5 min to detect photons emitted, as previously described (Saeij et al. 2005). Mice were bled by tail vein at day 5 and/or day 9 after infection. Blood collection was performed in either serum collector or Microtainer EDTA tubes (Sarstedt, Newton, NC). IL-1β and IL-18 were measured by ELISA (R&D Systems, Minneapolis, MN).
Supplementary Figure S1. Type II parasites activate the inflammasome without inducing cell death. BMDMs were primed with 100 ng/ml LPS or left unstimulated for 2 hours and subsequently infected with type II parasites (Pru; MOI, 0.4) for 24 h. (A) Quantification of IL-1β in supernatants was performed using ELISA. (B) Cell viability of infected cells in panel A was determined at different time points using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay.
Supplementary Figure S2. Type I and type IV parasites induce cleavage of pro-IL-1β. C57BL/6 BMDCs were primed with 100 ng/ml LPS for 2 hours and infected with type I (RH) or type IV (MAS) parasites (MOI, 5) for 24 hours. Western blot analysis was performed on concentrated supernatant (25-fold), probing for pro-IL-1β (37 kDa) and active IL-1β (17 kDa).
Supplementary Figure S3. Type II parasites activate the Nlrp3 inflammasome. IL-1β secretion from BMDMs prepared from wild-type C57BL/6 mice (blue) or C57BL/6 mice lacking Nlrp1b (red), Nlrp3 (green), or Nlrp1b and Nlrp3 (orange) primed for 4 h and then infected with type II parasites (Pru; MOI, 0.8) for 24 hours. The figure represents one experiment. Error bars, ± standard deviations.
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Chapter Three:
Inflammasome sensor NLRP1 controls rat macrophage susceptibility to Toxoplasma gondii

Kimberly M. Cirelli, Gezahegn Gorfu, Musa A. Hassan, Morton Printz, Devorah Crown, Stephen H. Leplla, Michael E. Grigg, Jeroen P.J. Saeij, Mahtab Moayeri

Address correspondence to Michael E. Grigg, griggm@niaid.nih.gov; Jeroen P.J. Saeij, jsaeij@ucdavis.edu; or Mahtab Moayeri, mmoayeri@niaid.nih.gov.

Kimberly M. Cirelli contributed to experiments in Figure 1B and D, 2, 3, 4A, C-G, 5B, E, F, 6E-G, S1, S2, S3, S4, S5 and S6. Gezahegn Gorfu contributed to experiments in Figure 4B. Musa A. Hassan contributed to experiments in Figure 2, S3 and S4. Mahtab Moayeri contributed to experiments in Figure 1B-E, 2, 5A-D, F, 6A-D, H and I, S7 and S8.


Supplementary Data is available online at http://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1003927#s5
Abstract

Toxoplasma gondii is an intracellular parasite that infects a wide range of warm-blooded species. Rats vary in their susceptibility to this parasite. The Toxo1 locus conferring Toxoplasma resistance in rats was previously mapped to a region of chromosome 10 containing Nlrp1. This gene encodes an inflammasome sensor controlling macrophage sensitivity to anthrax lethal toxin (LT) induced rapid cell death (pyroptosis). We show here that rat strain differences in Toxoplasma-infected macrophage sensitivity to pyroptosis, IL-1β/IL-18 processing, and inhibition of parasite proliferation are perfectly correlated with NLRP1 sequence, while inversely correlated with sensitivity to anthrax LT-induced cell death. Using recombinant inbred rats, SNP analyses and whole transcriptome gene expression studies, we narrowed the candidate genes for control of Toxoplasma-mediated rat macrophage pyroptosis to four genes, one of which was Nlrp1. Knockdown of Nlrp1 in pyroptosis-sensitive macrophages resulted in higher parasite replication and protection from cell death. Reciprocally, overexpression of the NLRP1 variant from Toxoplasma-sensitive macrophages in pyroptosis-resistant cells led to sensitization of these resistant macrophages. Our findings reveal Toxoplasma as a novel activator of the NLRP1 inflammasome in rat macrophages.
Inflammasomes are multiprotein complexes that are a major component of the innate immune system. They contain “sensor” proteins that are responsible for detecting various microbial and environmental danger signals and function by activating caspase-1, an enzyme that mediates cleavage and release of the pro-inflammatory cytokines, IL-1β and IL-18. *Toxoplasma gondii* is a highly successful protozoan parasite capable of infecting a wide range of host species that have variable levels of resistance. Rat strains have been previously shown to vary in their susceptibility to this parasite. We report here that rat macrophages from different inbred strains also vary in sensitivity to *Toxoplasma*-induced lysis. We find that NLRP1, an inflammasome sensor, whose only known agonist is anthrax LT, is also activated by *Toxoplasma* infection. In rats there is a perfect correlation between NLRP1 sequence and macrophage sensitivity to *Toxoplasma*-induced rapid cell death, inhibition of parasite proliferation, and IL-1β/IL-18 processing. *Nlrp1* genes from sensitive rat macrophages can confer sensitivity to this rapid cell death when expressed in *Toxoplasma* resistant rat macrophages. Our findings suggest *Toxoplasma* is a new activator of the NLRP1 inflammasome.
Introduction

*Toxoplasma gondii* is an obligate intracellular parasite, for which different host species or strains within a species display variable susceptibilities. Different *Toxoplasma* strains also differ in virulence within the same host, suggesting variation in effectors among parasite strains and/or their impact in various hosts. Host innate immunity is known to play a critical role in susceptibility to infection. In mice, for example, resistance to *Toxoplasma* infection is critically dependent on the induction of IL-12, which subsequently induces IFN-γ, the main mediator of toxoplasmicidal activities (Melo, Jensen, and Saeij 2011).

Rats, like humans, are quite resistant to *Toxoplasma* infection when compared to mice. However varying levels of resistance also exist among rat strains. The resistance of the Lewis (LEW) strain is characterized by total clearance of the parasite, failure to develop cysts and the absence of a strong antibody response. Fischer (CDF) and Brown Norway (BN) rats, however, are susceptible to chronic infection and develop transmissible cysts in their brain and muscle tissue (Cavaillès et al. 2006; Sergent et al. 2005). Resistance in rats is a dominant trait and is linked to myeloid cell control of parasite proliferation (Cavaillès et al. 2006; Sergent et al. 2005).

Linkage analyses of LEWxBN F2 progeny was previously used to map *Toxoplasma* resistance in rats to a single genetic locus, termed *Toxo1*, within a 1.7-cM region of chromosome 10 (Cavaillès et al. 2006). We noted that this locus overlaps with the locus that controls rat and macrophage sensitivity to the anthrax lethal toxin (LT) protease. Inbred rat strains and their macrophages exhibit a perfectly dichotomous phenotype in response to LT: animals either die rapidly (<1 hour) or exhibit complete resistance to the toxin (Newman et al. 2010). Only macrophages from LT-sensitive rat strains undergo rapid caspase-1 dependent death (pyroptosis). The HXB/BXH recombinant inbred (RI) rat collection, developed from the
SHR/Ola and BN-Lx congenic parental strains (Pravenec et al. 1996; Pravenec et al. 1989; Printz et al. 2003), with opposing LT sensitivities, was used to map anthrax toxin susceptibility to a single locus at 55.8–58.1 Mb of rat chromosome 10. SNP analyses and sequence correlation to phenotype implicated the inflammasome sensor *Nlrp1* (nucleotide-binding oligomerization domain, leucine-rich repeat protein 1) as the likely susceptibility locus. NLRP1 is a member of the NLR cytosolic family of pathogen-associated molecular pattern molecule (PAMP) sensors, the activation of which leads to recruitment and autoproteolytic activation of caspase-1, followed by cleavage and release of the proinflammatory cytokines IL-1β and IL-18. NLR-mediated activation of caspase-1 is typically accompanied by rapid death of macrophages through a process known as pyroptosis (Lamkanfi and Dixit 2012; Song and Lee 2012). NLRP1 sequences from 12 inbred rat strains show a perfect correlation between sensitivity and the presence of an N-terminal eight amino acid (aa) LT cleavage site (Newman et al. 2010; Levinsohn et al. 2012). Proteolytic cleavage by LT activates the NLRP1 inflammasome in rat macrophages leading to rapid caspase-1 dependent cell death (pyroptosis) and cytokine processing (Levinsohn et al. 2012).

We hypothesized that the *Toxo1* locus could be *Nlrp1* as the macrophage is an important carrier of the parasite (Mordue and Sibley 2003; Suzuki et al. 2005) and inflammasome-mediated pyroptosis of this cell could impact in vivo parasite dissemination. The recent association of polymorphisms in the human *NLRP1* gene with susceptibility to congenital toxoplasmosis, evidence that P2X(7) receptors influence parasite proliferation in mouse cells, and the finding that IL-1β responses in *Toxoplasma* infected human monocytes are dependent on caspase-1 and the inflammasome adaptor protein ASC all suggest that the inflammasome plays a role in
determining the outcome of *Toxoplasma* infection in humans and mice (Lees et al. 2010; Witola et al. 2011; Gov et al. 2013).

Our results indicate that rat strain macrophages exhibit dichotomous susceptibilities to *Toxoplasma*-induced rapid lysis and associated cytokine processing in a manner correlated with NLRP1 sequence. We go on to show that *Nlrp1* knockdown in *Toxoplasma*-sensitive macrophages protects against this cell death while overexpression of certain variants of the gene in resistant macrophages can sensitize these cells to the parasite-induced pyroptosis. Our findings establish *Toxoplasma* as the second known activator of the inflammasome sensor NLRP1 and suggest a mechanism of host resistance involving activation of this sensor.
Results

NLRP1 sequence in inbred rats correlates with macrophage cell death, parasite proliferation and IL-1β/IL-18 release

The Toxo1 locus on chromosome 10, which controls rat resistance to toxoplasmosis, maps within a region containing the inflammasome sensor Nlrp1 gene. NLRP1 was previously shown to control rat macrophage sensitivity to pyroptosis by the anthrax protease LT. Sequencing of twelve inbred rat strains revealed five highly homologous variants, two encoding NLRP1 protein sensitive to LT-mediated cleavage activation (NLRP1variant1,2), and three which encode LT-resistant proteins (NLRP1variant3,4,5) (Figure 1A). We noted that rat strains encoding NLRP1variant1,2 historically support parasite proliferation in myeloid cells while rat strains encoding NLRP1variant5 do not (Cavaillès et al. 2006). Therefore we investigated whether macrophages from rats expressing different NLRP1 variants also differed in inflammasome activation and pyroptosis upon parasite infection. Inflammasome activation was assessed by monitoring cell death and cleavage of pro-IL-1β (37 kD) with subsequent secretion of mature active IL-1β (17 kD). We infected BMDMs from LT-sensitive CDF, BN or SD (NLRP1variant1,2) rat strains and LT-resistant LEW and SHR (NLRP1variant5) rat strains with luciferase-expressing Type I (RH) and Type II (76K, or PRU) Toxoplasma strains at various MOIs. BMDM viability measurements showed that NLRP1variant5-expressing macrophages underwent a rapid cell death after Toxoplasma infection starting at 3 hours and completed by 24 hours whereas the majority of the NLRP1variant1,2-expressing macrophages remained viable and supported Toxoplasma growth even 24 hours after infection (Figure 1B–D). The parasite itself did not contribute significantly to MTT or LDH signals (Figure S1, panels A, B) and DAMPs
from lysed host cells also did not induce cell death (Figure S1 panels C, D). Results were unaltered when cells were pre-treated with LPS (100 ng/ml) prior and throughout infection (Figure S1 panels C, D). Fischer F344/NTac (NLRP1variant2) macrophages also showed resistance similar to that of Fischer CDF macrophages (data not shown). Both NLRP1variant1,2 and NLRP1variant5-expressing macrophages were fully responsive to nigericin-induced NLRP3 activation (Figure S2 and (Newman et al. 2010)), indicating fully functional inflammasome assembly and caspase-1 function in these rat strains.
Figure 1. NLRP1 sequence in inbred and RI rats correlates with rapid macrophage death.

(A) Sequence map of rat NLRP1 variants. This diagram was modified from (Newman et al. 2010). Vertical black lines indicate amino acid polymorphisms relative to the protein encoded by allele 1. Approximate NACHT, LRR and CARD domain locations relative to polymorphisms are shown. Macrophage sensitivity to LT-induced pyroptosis for the listed rat strains is from (Newman et al. 2010) and Toxoplasma sensitivities are from this work. (B–E) Viability measurements for rat BMDMs from LEW, SHR (expressing NLRP1variant5); CDF, BN or SD (expressing NLRP1variant1,2); or RI rat strains following infection with Toxoplasma Type I (RH) or Type II (76K or PRU) (MOI, 3) by MTT measurements. Data shown are average from three independent experiments with SD (triplicate wells/experiment/condition), except RI strains, which are averages from two experiments (triplicate wells/experiment/condition). Viability values were calculated relative to MTT measurements for uninfected control cells at each time point, which were set at 100%. P-values comparing all NLRP1variant1,2-expressing strains to NLRP1variant5-expressing strains are <0.001.
We next tested macrophages from three rat strains (HXB1, HXB15 and HXB29) from the HXB/BXH recombinant inbred (RI) rat collection previously used to map LT sensitivity (Newman et al. 2010). These strains have chromosome 10 crossover points closely flanking the Nlrp1 locus, as indicated by SNP analyses. We found that macrophages from the RI strain HXB1, an LT-resistant strain, were sensitive to Toxoplasma Type I (RH) and Type II (76K) infection-induced lysis while the macrophages from the other two strains, which are LT-sensitive, were resistant to parasite induced rapid death (Figure 1E). These rats allowed us to reduce the Toxol locus from the previous 54.2 Mbp–61.8 Mbp region to 54.2 Mbp–59.2 Mbp (Figure S3). We performed SNP and haplotype analyses for the CDF (F344/Crl), F344/NTac, BN (all strains with macrophages resistant to Toxoplasma-induced lysis) and the SHR strain (a strain with macrophages sensitive to Toxoplasma-induced lysis) and further narrowed the region determining resistance to 55.3–59.2 Mbp (between SNPs rs63997836 and rs106638778) (Figure S3). This region contained 133 genes of which 21 contained non-synonymous SNPs that were present in F334 and/or SHR rats, where genotype correlated with Toxoplasma resistance phenotype. To further narrow down the list of possible candidate genes, we performed whole transcriptome sequencing on BMDM from the LEW (pyroptosis-sensitive macrophages), BN (pyroptosis-resistant macrophages) and SD (pyroptosis-resistant macrophages) strains. We determined which genes were expressed in unstimulated and LPS-stimulated LEW BMDM (which are sensitive to parasite induced pyroptosis under both conditions), and contain SNPs that correlate with the resistance phenotype. Sixty-five of the 133 genes in the fine-mapped region were expressed (fragments per kilobase of transcript per million mapped reads >2) but only five of these contained non-synonymous SNPs that distinguished LEW from SD/BN (Dataset S1 and Figure S4). Although there were also differences in gene expression levels between
LEW and SD/BN macrophages, none of the genes were expressed higher (1.5 fold) in both the non-stimulated and LPS stimulated LEW macrophages compared to the SD/BN macrophages (Dataset S1). By combining all analyses, we were able to narrow down the possible candidate genes to *Aurkb* (Aurora kinase B1, 55.7 Mbp, 1 SNP), *Neurl4* (neutralized homolog 4, 56.7 Mbp, 1 SNP), *Cxcl16* (chemokine C-X-C ligand 16, 57.3 Mbp, 1 SNP) and *Nlrp1* (6 SNPs). Figure 2 summarizes the above described mapping steps. Of these four genes, *Nlrp1* was the most likely candidate to be *Toxo1*; it contained the highest number of non-synonymous SNPs and is a known activator of the inflammasome. Our fine-mapping analyses combined with the established perfect correlation between sensitivity to *Toxoplasma* induced macrophage cell death and the NLRP1 N-terminal sequence in inbred and RI rats (Newman et al. 2010), which was in turn inversely correlated to rat resistance to chronic, transmissible *Toxoplasma* infection suggested that the *Toxo1* locus could be the *Nlrp1* gene.
Figure 2. Summary flow diagram for mapping of rat macrophage sensitivity to four candidate genes. Methods for reducing the number of candidates at each stage are listed to the right and explained in detail in the Results section. Detailed SNPs and gene lists for each stage can be found in Supporting Figures S3, S4 and Dataset S1.

A survey of Toxoplasma strains that are genetically distinct from the archetypal I, II and III strains (Minot et al. 2012; Su et al. 2012) showed that they all induced NLRP1 variant-dependent rapid cell death (Figure 3).
Figure 3. NLRP1 variant-dependent rapid cell death is induced by many different parasite strains. Viability as measured by LDH release for BMDMs from SD (NLRP1\textsuperscript{1,2} variant) or LEW (NLRP1\textsuperscript{3} variant) infected with strains representing global diversity for 24 hours (MOI, 0.5–1 depending on strain, n = 4 wells/strain). P-values comparing LEW and SD <0.05 for all strains except MAS, CAST, GPHT and GUY-MAT.

Because cell death was consistently dependent on MOI, we tested whether parasite invasion was required for cell death, as *Toxoplasma* can secrete effectors from its rhoptry organelles directly into the host cytoplasm. Parasites treated with Mycalolide B, a drug that blocks invasion but allows for secretion of microneme and rhoptry contents, attached but were unable to kill BMDMs, indicating that macrophage sensitivity to cell death was invasion-dependent (Figure 4A). Mycalolide B did not affect the viability of parasites or their ability to secrete rhoptry contents as verified by the observation that every cell with an attached mycalolide-B-treated parasite also had protein kinase ROP16 activation of STAT6 (Figure S5). Because *Toxoplasma* needs host cells for replication and the parasite replicates equally well in fibroblasts from different rat strains (Cavaillès et al. 2006), we hypothesized that rapid macrophage cell death prevents *Toxoplasma* replication. We therefore investigated parasite
proliferation in BMDMs from the different rat strains. *Toxoplasma* burden, as measured by bioluminescence, was significantly higher in infected NLRP\(^{\text{variant1,2}}\)-expressing BMDMs than NLRP\(^{\text{variant5}}\)-expressing cells (Figure 4B, 4C). This difference was independent of *Toxoplasma* strain but perfectly correlated with NLRP1 sequence and continued to increase over time only in the cell death-resistant BMDMs from *Toxoplasma* susceptible rat strains (Figure 4C). Similarly, GFP signal indicative of parasite load was higher in resistant cells from these rat strains (data not shown). Parasite proliferation was independent of LPS-priming (data not shown) and more parasites/vacuole were detected in NLRP\(^{\text{variant1,2}}\)-expressing macrophages compared to Nlrp\(^{\text{variant5}}\)-expressing cells (Figure 4D). Although only ~10% of sensitive LEW (NLRP\(^{\text{variant5}}\)) BMDMs were intact after 24 hours of infection (Figure 4E left panels), 90% of these surviving cells contained single parasites (Figure 4E right panels). Nearly 100% of resistant SD, BN or CDF (NLRP\(^{\text{variant1,2}}\)) BMDMs were intact after 24 hours, and >60% of those infected contained multiple parasites per vacuole (Figure 4D, 4E). To determine if parasites released from lysed cells were viable, we measured the parasite's ability to reinvade macrophages by adding an antibody specific for the *Toxoplasma* surface protein, SAG1, to the medium of pre-infected BMDMs. We found that ~35% of intracellular parasites in the sensitive LEW BMDMs were coated with the SAG1 antibody while only 5% were coated in resistant cells, demonstrating that some fraction of parasites released from rat BMDMs that rapidly lyse remain viable and capable of re-invasion (Figure S6). We verified that SAG1 was not shed upon invasion by immunofluorescence, where 100% of parasites were stained for SAG1 when infected SD BMDMs were fixed and permeabilized at 18 hours post-infection (Figure S6). Supernatants from lysed *Toxoplasma*-sensitive BMDMs also did not contribute to the rapid pyroptosis of resistant macrophages (Figure 4F) or alter parasite proliferation within these cells (Figure 4G).
Figure 4. NLRP1-variant dependent macrophage death depends on parasite invasion and controls parasite proliferation. (A) Viability of LEW BMDMs infected with Mycalolide-treated (3 μM, 15 minutes) RH tachyzoites (MOI 1) after 24 hours as measured by MTS assay (P-value comparing Mycalolide group to untreated = 0.0002). (B, C) Radiance emission analyses of metabolically active, viable Type II Toxoplasma 76K parasites (B, graph MOI 3, 6 hours; inset shows representative plate from one experiment) or Type I RH parasites (C, MOI 1 over 48 hours) in BMDMs from different rat strains. P-value comparing NLRP1variant1,2-expressing strains to NLRP1variant5-expressing strains are <0.01 in I by t-test and <0.0001 in J by two-way ANOVA. (D) Number of parasites/vacuole in infected BMDMs (24 hours, MOI 3) as assessed by microscopy is shown. CDF, BN infections were with 76K, and SD, LEW infections were with RH. Between 50–100 vacuoles counted per experiment. Average values from 3 experiments are shown for all strains, except SD (n = 2). P-values are <0.01 (two-way ANOVA) when comparing NLRP1variant1,2-expressing strains to NLRP1variant5-expressing strains. (E) Left panels show light microscopy images of CDF and LEW monolayers infected with 76K (MOI 6, 6 hours). Right panels show fluorescence microscopy image of single SD and LEW BMDMs infected with RH (MOI 1, 2 hours). Blue is Hoechst stained nucleus, green are GFP-expressing parasites. Dividing parasites in SD cells (upper right) or a single parasite in LEW cells (lower right) are shown. (F) LEW BMDMs were infected with PRU (MOI 3) and at 5 hours post infection culture supernatants from dying cells was spun, filtered and transferred to similarly infected (PRU, MOI 3) CDF BMDMs. Viability of CDF BMDMs was assessed at 10 hours post-infection by MTT staining. All values were calculated relative to uninfected control BMDMs (G) SD BMDMs were infected with RH parasites (2 hours, MOI 1), washed with PBS and medium replaced with fresh media, media from RH-infected (24 hours, MOI 1) or uninfected LEW BMDMs. Parasites/vacuole counted at 24 hours. P-values >0.1 (ns) for comparison of any of three groups for 1, 2, 4 and 8 parasites/vacuole counts (by two-way ANOVA).
To investigate whether *Toxoplasma* infection induced maturation and secretion of IL-1β and IL-18 in an NLRP1 sequence-dependent manner, we measured secreted levels of these cytokines in the different rat strains. In the absence of LPS priming, Type II strain-infected BMDMs did not produce IL-1β (data not shown), but low levels of IL-18 were measurable by 6 hours (PRU) and 24 hours (76K) of infection in an NLRP1 variant-dependent manner. Thus in the unprimed situation, both 76K and PRU produced a much higher response in the LEW macrophages (expressing NLRP1<sup>variant5</sup>) when compared to infection of CDF macrophages (expressing NLRP1<sup>variant2</sup>) with the same Type II strain (Figure 5A). After LPS-priming, high levels of IL-1β and IL-18 secretion also correlated with NLRP1 sequence and macrophage sensitivity to rapid lysis (Figure 5B, 5C). Furthermore, the HXB1 (NLRP1<sup>variant5</sup>), HXB15 and HXB29 (NLRP1<sup>variant1</sup>) RI strains also produced IL-1β after infection in a manner correlated with NLRP1 sequence and macrophage sensitivity to *Toxoplasma* (Figure 5D). No IL-1β or IL-18 release was measurable from uninfected controls at any time point for any of the experiments shown in Figures 5A–D (data not shown). If parasites were treated with Mycalolide B, there was a significant reduction in cytokine production (Figure 5E) indicating that parasite invasion was necessary for inflammasome activation. Finally, cleavage of IL-1β and IL-18 was detected in cell lysates from LPS-primed, 76K or PRU-infected LEW, but not infected CDF and SD BMDMs, and cleavage correlated with cytokine secretion (Figure 5F). Nigericin activation of the NLRP3 inflammasome in both *Toxoplasma*-sensitive (LEW, NLRP1<sup>variant5</sup>-expressing) and CDF or SD (NLRP1<sup>variant1</sup>-expressing) BMDMs confirmed previous findings that no general defect in the caspase-1 pathway was present in rats (Figure S1, 5F) and (Newman et al. 2010)). Together these findings indicate a perfect correlation between sensitivity to *Toxoplasma*-induced macrophage cell death, decreased parasite proliferation, IL-1/IL-18 processing, rat resistance
to *Toxoplasma* infection and NLRP1 sequence (Newman et al. 2010), suggesting that the Toxo1 locus could be assigned to the Nlrp1 gene.

Figure 5. NLRP1-variant dependent cytokine cleavage and secretion. IL-18 (A, C) and IL-1β (B, D) from LPS-primed (0.1 μg/ml, 1 hour) (B, C, D) or unprimed (A) rat BMDMs following *Toxoplasma* infection (MOI 3 for 76K and 3 and 5 for PRU). All infections are with strain 76K unless otherwise indicated with the additional exception that SD BMDMs in panel B were infected with PRU. Results shown are averages from three experiments with SD shown, except measurements for PRU infections in panel A which are the averages of four experiments, two with MOI 3 and two with MOI 5 and those for the RI rats, which are from two independent experiments (triplicate wells/experiment/time point). No IL-1 of IL-18 release was measurable from uninfected controls at any time point for any of the experiments in A–D. P-values in (A) comparing CDF and LEW groups in (A) and (C) are <0.001 by two-way ANOVA. In (B) and (D), all P-values comparing NLRP1 variant3 expressing strains to the NLRP1 variant2-expressing strains are <0.001 in all comparison combinations, by two-way ANOVA (E) IL-1β measurements from LPS-primed LEW BMDMs infected with Mycalolide-treated (3 μM, 15 minutes) RH tachyzoites (MOI 1) after 24 hours; P-value comparing Mycalolide group to untreated is
Western blot analyses for IL-18 and IL-1β in cell lysates and culture supernatants (indicated by “S”) of 76K-infected CDF and LEW BMDMs (MOI 3, 4 hours) (left panels) or PRU infected LEW and SD BMDM cell lysates (MOI 3, 24 hours) (right panels). NLRP3 agonist nigericin (40 μM, 4 hours) was used as a positive control for inflammasome activation in the gel shown on the right. In the left pair of gels, supernatants (no concentration, mixed 1:1 with SDS loading buffer) were loaded and Westerns were visualized using IR-dye conjugated secondary antibodies and the LiCOR Odyssey. Cell lysates were also run, with processed IL-1β and IL-18 shown with arrowheads in these gels, and pro-forms shown by red arrow. In the right gel, cell lysates are shown in Westerns visualized by chemiluminescence using a charge-coupled device camera. The unprocessed form of IL-1β is shown as the 37-kD band, and the mature form is labeled 17 kD.

*Nlrp1* knockdown provides protection against *Toxoplasma*-induced pyroptosis

We utilized two methods to knock down expression of rat *Nlrp1* (designated as *Nlrpla* in the rat genome) to determine if NLRP1 mediates *Toxoplasma*-induced rat macrophage pyroptosis. First, an siRNA nucleofection approach was utilized. Only 20–35% of rat BMDMs can be transfected with this method, as assessed by control nucleofections with GFP expression vector and confirmed in parallel nucleofections in our current studies (data not shown). We found that there was a significant protection against LEW macrophage death in cells transfected with *Nlrp1* siRNA, compared to control siRNA, under conditions where 100% of BMDMs succumbed (*Figure 6A and 6B*). The 20–30% difference in viability was correlated with the number of successfully transfected cells, as reflected by the all-or-none nature of the protection in individual cells assessed by microscopy (*Figure 6A, inset*). Surviving LEW BMDMs remaining attached after longer periods of infection were verified to contain dividing GFP-expressing *Toxoplasma gondii* by fluorescence microscopy (*Figure 6C, D*), and viability was verified by MTT-staining (*Figure 6D, left panel*). Nonsurviving cells were completely detached from monolayers. A second method of knockdown by lentiviral delivery of a homologous mouse *Nlrplb* shRNA was used to achieve a 2.2-fold reduction in *Nlrp1* expression compared to controls infected with a scrambled shRNA. Expression of *Nlrp1* was assessed by qPCR and standardized against actin levels (*Figure 6E*). Knockdown correlated with increased parasite...
proliferation and a higher number of vacuoles with more than one parasite (~60%), compared to the macrophages treated with a scrambled control (35%) (Figure 6F). Host cell viability was also increased by 30% in the shRNA knockdown condition (Figure 6G).
Figure 6. *Nlrp1* knockdown provides protection against *Toxoplasma*-induced pyroptosis and overexpression of NLRP1variant sensitizes resistant macrophages. (A) Viability of LEW BMDMs nucleofected with Nlrp1 siRNA pool or control siRNA (CR) 24 hours or 48 hours prior to infection with PRU (MOI 3) as measured by MTT assay at 5 hours post infection. Average from 6 separate nucleofection experiments (24 hours, n = 3, 48 hours, n = 3) are shown (triplicate wells/condition/experiment). P-values comparing Nlrp1 siRNA to controls is <0.001. Microscopy images of MTT stained nucleofected cells from representative 24 hours and 48 hours knockdown experiments are also shown. (B) Viability of LEW BMDMs nucleofected with Nlrp1 siRNA pool or control siRNA (CR) 36 hours prior to infection with PRU (MOI 1) as measured by MTT signal at 24 hours post-infection. Average of 4 separate nucleofections are shown (triplicate wells/condition/nucleofection experiment) (C,
D) *Toxoplasma* division in individually surviving nucleofected LEW BMDMs from (B) at 24 hours post-infection. In C cells were fixed prior to microscopy, while in D cells were MTT-stained and fluorescence microscopy performed with no fixing. Note that all non-transfected or control siRNA transfected LEW macrophages which have succumbed are not present in these fields (detached by 24 hours), while the MTT-negative ghosts and organelles of these lysed cells can be seen in parallel experiments at the earlier 5–6 hour time points, as shown in panel A. (E–G) Knockdown by the alternative lentiviral shRNA method was confirmed in LEW BMDMs by qPCR (E) and parasites per vacuole counts (F) and viability by MTS assay (G) were assessed in Nlpr1-knockdown LEW BMDMs after RH strain infection (MOI 0.5). P-values by t-test comparing knockdown to controls is 0.03 for C and 0.01 for D. (H) Viability of LEW and CDF BMDMs nucleofected with full length HA-tagged NLRP1 constructs at 24 hours prior to infection with PRU (MOI 5) was measured by MTT assay at 5 hours post-infection. Cell lysates from nucleofected cells were made at 32 hours post-transfection and analyzed by Western using anti-HA antibody. Superscripts indicate the NLRP1 construct or vector that was transfected into the cell. Graph shows average from two nucleofection studies, with duplicate wells/condition/experiment. Lysates are from one of these nucleofections. There is no significant difference between any of the nucleofected LEW cells. The P-value comparing the CDF cells (expressing NLRP1 variant2) transfected with LEW (NLRP1 variant1) to CDF cells nucleofected with vector or CDF (NLRP1 variant2) is <0.0005. Presence of MTT-negative cells was also verified by microscopy for each well. Similar data is also shown in Figure S8, with anthrax LT control treatments. (I) Representative microscopy images of MTT viability staining for LEW and CDF BMDMs nucleofected with full length HA-tagged NLRP1 constructs 36 hours prior to infection with PRU (MOI 3) or treatment with LT (PA + LF, each at 1 μg/ml). MTT staining was performed on *Toxoplasma*-infected cells at 8 hours post-infection and on LT-treated cells at 5 hours post-infection. Superscripts indicate the NLRP1 construct or vector that was transfected into the cell.

**Overexpression of NLRP1 variant 5 sensitizes CDF BMDMs, but not fibroblasts and mouse macrophages, to *Toxoplasma*-induced pyroptosis**

We next overexpressed HA-tagged NRLP1 variant2 and NLRP1 variant 5 constructs (Levinsohn et al. 2012) in rat BMDMs by nucleofection to test if this alters susceptibility to parasite-induced pyroptosis. The efficiency of transfection ranged from 25–40% in BMDMs in individual nucleofections (as assessed by monitoring of a co-transfected GFP construct in control cells). The LEW BMDMs did not gain resistance when transfected with the resistant CDF NLRP1 variant2, but were sensitized to treatment with anthrax LT, confirming expression of the CDF NLRP1 variant2 in a subpopulation of nucleofected cells (Figure S7). There was a significant sensitization to parasite-induced pyroptosis in CDF cells transfected with the LEW NLRP1 variant5 (Figure 6H, Figure S7), while these cells remained almost 100% susceptible to
rapid lysis by LT (Figure S8). Microscopy confirmed cell death for both *Toxoplasma*-infected CDF cells expressing LEW NLRP1\textsuperscript{variant5} and LT-treated LEW cells expressing the CDF NLRP1\textsuperscript{variant2} (Figure 61). These results confirm that the LEW NLRP1\textsuperscript{variant2} -mediated sensitivity to *Toxoplasma* is dominant, much in the manner the resistance of LEW rats to the parasite was previously shown to be a dominant trait (Cavailles et al. 2006). They also re-confirm that the sensitivity to anthrax LT, mediated by the CDF NLRP1\textsuperscript{variant2} is a dominant trait. Interestingly, fibroblast HT1080 lines expressing these rat NLRP1 constructs (Levinsohn et al. 2012) were not sensitized to *Toxoplasma*-induced pyroptosis even when transiently transfected and confirmed to express caspase-1 along with NLRP1 (Figure S8, panel A). These results confirmed that a macrophage cofactor or the macrophage cellular environment is required for parasite-induced pyroptosis. Furthermore, infection of mouse macrophage cell lines stably expressing rat NLRP1 constructs also did not result in sensitization to *Toxoplasma* (Figure S8, panel B), suggesting the presence of other factors in murine macrophages, or the BMAJ macrophage cell line, that result in a dominant resistance to pyroptosis or the absence of a factor needed for interaction with rat NLRP1 and subsequent pyroptosis. All tested mouse macrophages from any inbred strain, to date, have been resistant to *Toxoplasma*-induced pyroptosis (data not shown and Figure S8, panel C). The competition of endogenous murine NLRP1a and NLRP1b proteins for co-factors required for pyroptosis in the mouse macrophage may explain this resistance.

Together, the results presented in this work indicate that *Nlrp1* expression contributes to the ability of BMDMs from rats resistant to *Toxoplasma* infection to control parasite replication, most likely because of its role in mediating *Toxoplasma*-induced macrophage pyroptosis.
Discussion

The Toxol locus that controls rat susceptibility to toxoplasmosis (Cavaillès et al. 2006) was previously mapped to a region of rat chromosome 10 containing the inflammasome sensor Nlrp1. In this work we identify Toxoplasma as a novel pathogen activator of the NLRP1 inflammasome. Until this work, anthrax LT was the only known activator of this inflammasome sensor (Newman et al. 2010; Levinsohn et al. 2012; Hellmich et al. 2012). We now demonstrate that like LT, rapid Toxoplasma-induced rat macrophage cell death is a pyroptotic event for which sensitivity correlates to NLRP1 sequence. Type I, Type II and a variety of genetically diverse T. gondii strains induce rapid pyroptosis in macrophages derived from inbred rats expressing NLRP1\textsuperscript{variant5}, while macrophages from BMDMs expressing NLRP1\textsuperscript{variant1,2} are resistant to the parasite. This is the inverse of what is known for LT, where NLRP1\textsuperscript{variant1,2} confers sensitivity (Newman et al. 2010). In rats, macrophage sensitivity to Toxoplasma-induced cell death inversely correlates with whole animal resistance to infection. Rat strains historically susceptible to chronic Toxoplasma infection (e.g., CDF, BN, SD; NLRP1\textsuperscript{variant1,2}) have pyroptosis-resistant macrophages whereas resistant rats that cure infection (e.g., LEW, SHR; NLRP1\textsuperscript{variant5}) harbor macrophages that undergo parasite-induced pyroptosis. This suggests that the ability of the macrophage to allow parasite proliferation and possibly dissemination is linked to resistance to parasite-induced macrophage pyroptosis. Similar findings were previously described for mouse Nlrp1b-mediated control of anthrax infection. Mice resistant to Bacillus anthracis have macrophages expressing Nlrp1b variants which confer macrophage sensitivity to anthrax LT, and resistance is linked to the IL-1β response induced by toxin (Moayeri et al. 2010; Terra et al. 2011). The idea of control of parasite proliferation at the macrophage level is supported by findings that macrophages are among the first cell types to be infected when an
animal ingests *Toxoplasma* cysts or oocysts (Mordue and Sibley 2003; Suzuki et al. 2005) and innate immune cells are used to traffic from the site of infection to distant sites such as the brain (Lambert and Barragan 2010).

In parallel to the consequences for parasite proliferation after NLRP1 activation, the pro-inflammatory cytokines, IL-1β and IL-18, which are substrates of caspase-1, are cleaved and released following inflammasome activation. We demonstrate that these events only take place after infection of pyroptosis-sensitive macrophages in a manner correlating with NLRP1 sequence. It is possible that the release of these cytokines of the innate immune system could also play a role in controlling toxoplasmosis. IL-18 was at one time known as “IFN-inducing factor” and the role of IFN-γ in resistance to *Toxoplasma* is extensively documented (Melo, Jensen, and Saeij 2011; Hunter and Sibley 2012). Treatment of resistant LEW rats with anti-IFN-γ antibodies does not reverse resistance but results in a much stronger antibody response, while anti-IFN-γ antibody treatment in susceptible rats causes an increase in parasite burden (Sergent et al. 2005). Altogether these findings suggest that IL-18, (through actions by IFN-γ) could be important for inhibition of *Toxoplasma* replication in rats, but that the cytokine's actions do not necessarily prevent parasite dissemination. On the other hand, it is important to note that as *Toxoplasma* can replicate and form cysts in many cell types that do not undergo pyroptosis, macrophage death may play a role strictly in dissemination. Thus, we suggest the combined consequences of inflammasome activation, macrophage cell death and IL-1/IL-18 secretion, on both dissemination and parasite proliferation, may ultimately result in resistance to *Toxoplasma*.

The only difference between the NLRP1 proteins from *Toxoplasma*-resistant and *Toxoplasma*-sensitive inbred strains is an 8 aa polymorphic region in the N-terminus of the protein, in a region of unknown function (Newman et al. 2010). LT cleaves
NLRP\textsuperscript{variant1,2} proteins to activate this sensor and induce pyroptosis, while NLRP\textsuperscript{variant5} is resistant to cleavage (Levinsohn et al. 2012). How \textit{Toxoplasma} activation of NLRP1 varies between rat strains based on an 8 aa sequence difference is unclear. The similar induction of pyroptosis we observed with numerous \textit{Toxoplasma} strains suggests that the factor activating NLRP1 is unlikely to be parasite strain specific, or at least is conserved among multiple strains.

One logical hypothesis is that the parasite-encoded effector molecule responsible for activation of NLRP1 is, like LT, a protease, but one which targets the LT-cleavage resistant sequence found in NLRP\textsuperscript{variant5}. \textit{Toxoplasma} secretes a large number of proteases (Choi, Nam, and Youn 1989; Dou and Carruthers 2011; Dou, Coppens, and Carruthers 2013; Kim 2004; Shea et al. 2007). It is unlikely that such a secreted protease could be derived from the rhoptries, because rhoptry secretion into the host cell was not sufficient to induce cell death. To date, we have been unable to observe any cleavage of NLRP1 in \textit{Toxoplasma} infected fibroblasts, which overexpress an HA-tagged variant of the protein (data not shown). It has also been recently shown that \textit{Toxoplasma} can secrete effectors post invasion beyond the parasitophorous vacuole membrane (Bougdour et al. 2013) and these could be candidate effectors for NLRP1 activation.

An alternative hypothesis to the parasite causing direct cleavage of NLRP1 is that the N-terminal polymorphic region of rat NLRP1 affects this protein's interaction with a different host 'sensor' acting as adaptor for the inflammasome, much in the manner described for the NLRC4/NAIP5/NAIP6 inflammasome recognition of flagellin (Kofoed and Vance 2011; Zhao et al. 2011). This unknown adaptor would interact with \textit{Toxoplasma} or its effectors in all macrophages but may be limited by its ability to interact with the N-terminus of NLRP\textsuperscript{variant1,2} in rat BMDMs, or alternatively it could act as a direct inhibitor with specificity for these variants. The likelihood of a proteolytic activation of NLRP1 is also reduced when
considering the finding that mouse ortholog NLRP1b proteins harbor an LT-cleavage site similar to rat proteins (Hellmich et al. 2012) but are highly resistant to *Toxoplasma*-induced pyroptosis in a manner independent of NLRP1b sequence or LT sensitivity (Figure S8). Furthermore, mouse macrophages could not be sensitized by rat NLRP1 overexpression. This finding was in contrast to the sensitization of the same cells to LT-mediated cell death (Levinsohn et al. 2012), suggesting resistance of mouse macrophages to *Toxoplasma*-induced pyroptosis was dominant to any NLRP1-mediated effect, or (less likely) that co-factors required for parasite-mediated activation were only present in rat cells. Alternatively, the endogenous *Toxoplasma* non-responsive NLRP1a and NLRP1b proteins in mouse macrophages could compete in a dominant manner with expressed rat NLRP1 for co-factors required for pyroptosis. Interestingly, human NLRP1 does not contain an LT cleavage site in its N-terminus (Moayeri, Sastalla, and Leplla 2012). Instead human NLRP1 contains a pyrin domain required for association with the adaptor protein ASC (Faustin et al. 2007), which does not appear to play a role in NLRP1-mediated rodent cell death (Nour et al. 2009; Broz et al. 2010). SNPs prevalent in this N-terminal region of human NLRP1 have been correlated with the severity of human congenital toxoplasmosis (Witola et al. 2011). In those studies, knockdown of *NLRP1* in human monocytic lines led to reduced cell viability after *Toxoplasma* infection, perhaps by allowing uncontrolled division of the parasite. Unlike our findings in rat cells, a protective role for human NLRP1 against macrophage death was suggested. It seems likely that the cell death observed in these human cell studies, which occurred over a period of days, differs from NLRP1-mediated rapid pyroptosis of rat cells, which occurs over a period of hours. Future studies are required to determine the mechanism of NLRP1 action in human cells.
In summary, we have established that *Toxoplasma gondii* is a new activator for the NLRP1 inflammasome. The identification of *T. gondii* as the second pathogen to activate the NLRP1 inflammasome raises the question whether this parasite activates the sensor via a novel mechanism, or whether proteolytic cleavage is required, in a manner similar to anthrax LT.
Materials and Methods

Ethics statement

All animal experiments were performed in strict accordance with guidelines from the NIH and the Animal Welfare Act, approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (approved protocols LPD-8E and LPD-22E) and the MIT Committee on Animal Care (assurance number A-3125-01).

Materials

Ultra-pure lipopolysaccharide (LPS), nigericin (Calbiochem/EMD Biosciences, San Diego, CA and Invivogen, San Diego, CA), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St Louis, MO), Mycalolide B (Wako USA, Richmond, VA) were purchased. LT consists of two polypeptides, protective antigen (PA) and lethal factor (LF). Endotoxin-free LF and PA were purified from B. anthracis as previously described (Park and Leppla 2000). Concentrations of LT refer to equal concentrations of PA+ LF (ie, LT 1 μg/ml is LF+PA, each at 1 μg/ml).

Rats

Brown Norway (BN/Crl; BN), Fischer CDF (F344/DuCrl; CDF), Lewis (LEW/Crl; LEW), Spontaneously Hypertensive Rat (SHR/NCrl; SHR) and Sprague Dawley (SD) rats (8–12 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and used as
source of bone marrow. Certain experiments utilized F344/NTac rats from Taconic Farms (Germantown, NY). The recombinant inbred (RI) rat strains HXB1, HXB15 and HXB29 are derived from the progenitor strains BN-Lx and SHR/Ola (Pravenec et al. 1996; Pravenec et al. 1989; Printz et al. 2003). The microsatellite marker genotypes and linkage maps used in mapping LT sensitivity using the HXB/BXH RI collection have been described (Newman, Printz, et al. 2010b).

Parasites

Tachyzoites from Type I (RH) and Type II (76K or Prugniaud [PRU]) strains expressing luciferase and GFP from the plasmid pDHFR-Luc-GFP gene cassette (Saeij et al. 2005) were used for most experiments. The following strains (haplogroup/type in parentheses) were used in a survey of effects on rat macrophages: GT1 (I), ME49 (II), DEG (II), CEP (III), VEG (III), CASTELLS (IV), MAS (IV), GUY-KOE (V), GUY-MAT (V), RUB (V), BOF (VI), GPHT(VI), CAST (VII), P89 (IX), GUY-DOS (X), VAND (X), Cougar (XI), RAY (XII), WTD3 (XII). All parasite strains were routinely passaged in vitro in monolayers of human foreskin fibroblasts (HFFs) at 37°C in the presence of 5% CO₂, spun and washed prior to quantification by hemocytometer counts. In some experiments, Mycalolide B (3 μM, 15 minutes) or DMSO was used to pretreat isolated parasites prior to washing in PBS (3×) before infections. The viability of these Mycalolide B- or DMSO-treated parasites was assessed in each experiment by adding them to a monolayer of HFFs and staining for STAT6 activation induced by the parasite secreted rhoptry kinase ROP16. Mycalolide B-treated parasites were able to secrete ROP16 but could no longer invade. In other experiments parasites were lysed using cell lysis solution (Abcam,
Cambridge, MA) to assess LDH activity. Parasite viability and health differed from experiment to experiment, accounting for variations in experimental results that are reflected in standard deviations for pooled studies.

Cell culture, nucleofection, toxicity, cytokine measurement, Western and microscopy studies

BMDMs were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 30–33% L929 cell supernatants as previously described (Newman et al. 2010; Wickliffe, Leplla, and Moayeri 2008), or with minor modification (20% fetal bovine serum, 50 µg/ml penicillin and 50 µg/ml streptomycin). NLRP1-expressing HT1080 or macrophage BMAJ lines and their growth conditions have been previously described (Levinsohn et al. 2012). The c-myc tagged rat caspase-1 gene was synthesized by GeneArt (Regensburg, Germany) and cloned into pcDNA(3.1)+ vector for expression in HT1080 cells by transfection with TurboFect (Fermentas, Glen Burnie, MD) using manufacturer's protocols. HA-tagged LEW and CDF NLRP1 expressing constructs used in BMDM nucleofection experiments have been described (Levinsohn et al. 2012c). Endotoxin-free control vector or various NLRP1 expressing constructs were purified (Endofree kit, Qiagen, Germantown, MD) and nucleofected (1.2–3.0 µg/1×10⁶ cells/nucleofection) into rat BMDMs using the Amaxa Nucleofector (Lonza, Walkersville, MD) (kit VPA-1009, program Y-001). Nucleofections were performed at −24, −36, −48, and −72 hours prior to infections with parasite. Toxicity and viability assays were modified from previously described methods (Newman et al. 2010; Wickliffe, Leplla, and Moayeri 2008). Briefly, animal-derived BMDMs with or without LPS priming 0.1 µg/ml, 1 h)
were infected with *Toxoplasma* at various multiplicities of infection (MOIs) or treated with anthrax LT (1 μg/ml) and cell viability was assessed at different time points by one of three methods. 1) MTT staining (0.5 mg/ml) was performed as previously described (Newman et al. 2010; Wickliffe, Leppla, and Moayeri 2008); 2) MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]) was used to measure viability with the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to manufacturer protocol; 3) Lactate dehydrogenase (LDH) release assays were performed in select experiments according to manufacturer protocol (Roche Diagnostics, Mannheim, Germany). For luciferase assays, cells were lysed in 1× Lysis Reagent (Promega) and luciferin (Caliper Life Sciences, Hopkinton, MA) added prior to luciferase activity readings. In all experiments culture supernatants were removed for cytokine measurements by ELISA (R&D Systems, Minneapolis, MN and Abnova Corporation, Walnut, CA) or Western blotting, with or without concentration using Amicon filters (3000 Molecular weight cutoff) (Millipore, Billerica, MA). Cell lysates were made from infected cells as previously described (Newman et al. 2010; Wickliffe, Leppla, and Moayeri 2008). Anti-rat IL-1β (Abcam or Santa Cruz BT, Santa Cruz, CA), anti-rat IL-18 (Santa Cruz BT) or anti-HA antibody (Roche Diagnostics) were used as primary antibodies. Secondary IR-dye conjugated or HRP-conjugated antibodies were from Rockland (Gilbertsville, PA), Licor Biosciences (Lincoln, NE) or Jackson Immunoresearch (West Grove, PA). Immun-Star Western C substrate (BioRad, Hercules, CA) and a charge-coupled device camera (Chemidoc XRS, BioRad) or the Odyssey Infrared Imaging System (Licor Biosciences) was used for Western visualization depending on the secondary antibody used for detection. For select microscopy studies phase contrast images of MTT-stained cells were acquired on a Nikon Eclipse TE2000-U microscope without cell fixation followed by
fluorescence image collection for the same field. For other fluorescence microscopy studies
nucleofected cells were plated on poly-lysine (Sigma, St. Louis, MO) treated coverslips prior to
infection and fixed (4% paraformaldehyde, Electron Microscopy Sciences, Hatfield, PA), with or
without permeabilization (0.1% TritonX-100). Immunostaining was with anti-HA antibody
(Roche Diagnostics) and Alexa Fluor 594 secondary antibody (Invitrogen). For
immunofluorescence staining of surface antigen (SAG)-1 or assessment of STAT6
phosphorylation, cells were fixed (3% formaldehyde) and permeabilized (0.2% TritonX-100 or
100% ethanol) followed by staining with a rabbit polyclonal antibody against human pSTAT6
(Santa Cruz BT, Santa Cruz, CA) or rabbit polyclonal antibody against Toxoplasma surface
antigen (SAG)-1. Alexa Fluor 594 secondary antibodies were used for detection as has been
described (Rosowski and Saeij 2012).

RNA knockdown studies

NLRP1 knockdown was achieved by two methods. First, siGENOME SMARTpool
siRNA set of four, targeting rat Nlrp1a (D-983968-17, D-983968-04, D-983968-03, D-983968-
02;

| target sequences of GGUCUGAACAUAAGCGA, CCACGGUGUUCCAGACAAA, GCAUUACGUUCUCU
CAUGU, GCAGUACGCAGUCUCUGUA) and siGENOME non-targeting siRNA pool (D-
001206-14-05,

| target sequences of UAAGGCUAUGAAGAGAUAC, AUGUAUUGGCCUGUAUUAG, AUGAACGUGAAUU
GCUCAA, UGGUUUACAUGUCGACUAAA) were obtained from Thermo Sciences-Dharmacon
(Pittsburgh PA). siRNA pools were nucleofected (200 nM) into rat BMDMs (day 5 or 6 of
differentiation) using the Amaxa Nucleofector (Lonza, Walkersville, MD) (kit VPA-1009, program Y-001) at -24, -36, -48, and -72 hours prior to infection. Alternatively, on day 2 of differentiation BMDMs were infected with high-titer lentivirus (Broad Institute RNAi consortium) encoding shRNA against target sequence TGATCTACTATCGAGTCAATCdesigned against murine Nlrp1b with high homology (18 out of 21 nucleotides, perfect seed sequence identity) to rat Nlrp1a or the control shRNA with sequence (GCTTATGTCGAATGATAGCAA or GTCGGCTTACGGCGGTGATTT). Puromycin selection (6 μg/ml) of lentivirus infected cells, followed by qPCR analysis (Nlrp1a primers were 5’CATGTGATTTGGACCTGACG’3, 5’TCTTTTGCTGCAAGTTTCCT’3, actin primers were 5’GTCGTACCACTGGCATTGTG’3, 5’CTCTCAGCTGTGGTGAA’3) verified knockdown. Expression of Nlrp1a was normalized against actin expression levels.

Whole transcriptome sequencing and SNP analyses

SNP and haplotype analyses for the HXB, SHR, F334 and LEW rats were performed based on data and genome analysis tools at the Rat Genome Database (RGD), Rat Genome Database Web Site, Medical College of Wisconsin, Milwaukee, Wisconsin (http://rgd.mcw.edu/). Any gene within the region fine mapped using the above haplotype analysis that contained at least one non-synonymous SNP was identified using Ensembl’s Biomart engine and the rat short variation (SNPs and indels) (Rnor_5.0) dataset. We then used the variant distribution tool on the RGD website to identify which SHR strain genes contained at least one SNP difference from F344 and BN strains. Nucleotide positions correspond to the RGSC3.4 assembly. Further fine
mapping analyses were performed by whole transcriptome sequencing and novel SNP identification. RNA (Qiagen RNeasy Plus kit) was isolated from unprimed and LPS-primed (100 ng/ml) LEW and SD BMDMs or LPS-primed BN BMDMs. mRNA purified by polyA-tail enrichment (Dynabeads mRNA Purification Kit, Invitrogen) was fragmented into 200–400 bp, and reverse transcribed into cDNA before Illumina sequencing adapters (Illumina, San Diego, CA) were added to each end. Libraries were barcoded, multiplexed into 5 samples per sequencing lane in the Illumina HiSeq 2000, and sequenced from both ends (60 bp reads after discarding the barcodes). Sequences were mapped to the Rat genome (rn4) using Bowtie (2.0.2) (Langmead et al. 2009) and Tophat (v2.0.4) (Trapnell et al. 2012). To identify SNPs from the RNAseq data in the interval fine mapped above, Bam files were processed with samtools (0.1.16, r963:234) mpileup function, with m4 as reference sequence. Read pileups were processed across all five samples using VarScan.v2.2.11 and the mpileup2snp function (parameters: -min-coverage 2 –min-reads2 1 –min-var-freq 0.01 –p-value 0.05 –variants). Resulting variant positions were annotated using UCSC Genome Browser’s “Variant Annotation Integrator”. SNPs identified between 5 samples (2 SD, 2 LEW, 1 BN) were filtered for concordance and homozygosity between the two independent LEW samples and BN having the same nucleotide as the reference genome (which is from BN), and subsequently filtered for non-synonymous SNPs where LEW differed from BN and SD. It should be noted that not all known LEW SNPs in Nlrp1 are discovered using this procedure as the N-terminal NLRP1 region contains a stretch of eight amino acids that differ between LEW and BN and our procedure for mapping reads to the genome does not allow for that many mismatches. Similar problems lead to underreported Nlrp1 SNPs in the RGD website.
Supplementary Data

Supplementary Figure S1. Parasite-derived MTT signal and LDH levels. (A) CDF BMDMs were infected PRU (MOI 1 or 3) and MTT assessed at 6 hours post-infection relative to uninfected controls (B) RH parasites at shown MOI were lysed in the absence of cells using the same volume to lyse uninfected BMDM monolayer used in typical experiments and LDH levels measured (C, D) Primed or unprimed (LPS 100 ng/ml, 2 hours) LEW BMDMs were infected with RH (MOI 0.5 or 1.0, as indicated) or treated with LEW macrophages or HFFs that had been syringe-lysed and prepared in parallel to parasites. The volume of cell lysates added to LEW BMDMs is equivalent to the volume of parasites added at the MOI indicated in parentheses. Viability and IL-1β release were then assessed 24 hours post infection.
**Supplementary Figure S2. Activation of the NLRP3 inflammasome by nigericin in CDF and LEW rats.** CDF or LEW BMDMs were pre-treated with LPS (1 μg/ml, 2 hours) followed by either LT (1 μg/ml LF+1 μg/ml PA, 90 minutes) or nigericin (10 μM, 1 hour). In a separate experiment, SD BMDMs were LPS treated (100 ng/ml, 2 hours) and either infected with RH strain (MOI 0.5, 6 or 8 hours), or treated with nigericin (40 μM, 4 hours). Supernatants were Amicon-concentrated prior to Western blotting. The unprocessed form of IL-1β is 37 kD. The mature cleaved form is 17 kD.
Supplementary Figure S3. Fine-mapping of the Toxol region using whole transcriptome sequencing, SNP and haplotype analyses. Table was generated using SNPlotyper tool at RGD. Alternative SNP annotations can be found at that site. Shaded area indicates the new boundaries for Toxol locus based on comparison of the inbred and RI rat strain SNP genotypes for the 7 rat strains BN, F344 (CDF), LEW, SHR, HXB1, HXB15 and HXB59.
Supplementary Figure S4. Whole transcriptome analyses of LEW, SD and BN rats. Summary of genes expressed in both LPS primed and unprimed conditions are shown for which non-synonymous SNPs (NS) existed. For each SNP, comparison of Toxoplasma-resistant and Toxoplasma-sensitive rat genotype correlation to phenotype was then used to narrow Toxo1 to four candidates, in red.

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The table lists the genes, their start and end positions, expression levels (LEWIS-NS, LEWIS-LPS, SD-NS, SD-LPS, BN-LPS), non-synonymous SNP (NS SNP) information, and the differentials between SHR vs F334/BN and SD/BN vs LEWIS.
Supplementary Figure S5. Parasites treated with Mycalolide B are able to secrete ROP16 and induce activation of pSTAT6. HFFs were infected with GFP-expressing type I parasites that were pretreated with 3 μM Mycalolide B or vehicle control for 15 minutes. Cells were infected for four hours and then fixed with 3% formaldehyde, permeabilized with 100% ethanol and blocked. A rabbit antibody against human pSTAT6 was used as the primary antibody, followed by a goat-anti-rabbit antibody conjugated to Alexa Fluor 594. Green = Parasite, Blue/Pink = Hoechst, Red = p-STAT6.
Supplementary Figure S6. Parasites released from lysed macrophages can reinvade other cells. A) SD or LEW BMDMs were infected with GFP-expressing RH (2 hours), washed three times with PBS and the media was replaced with fresh media containing rabbit anti-SAG1 antibody. After 24 hours, cells were fixed, permeabilized and stained with Alexa Fluor 594 goat anti-rabbit antibody. Parasites are green, while SAG1 is red. The quantification of SAG1-antibody coated parasites was performed with a minimum of 50 vacuole counts per condition from 3 experiments. (B) Parasites do not shed SAG1 upon invasion of SD BMDMs. Cells were infected with GFP-expressing RH for 18 hours, cells were fixed, permeabilized and stained with a rabbit anti-SAG primary antibody followed by Alexa Fluor 594 goat anti-rabbit antibody. SAG1 was detected on 100% of parasites in any infected cells. Green = parasite, Red = SAG1, Blue = Hoechst.
Supplementary Figure S7. Overexpression of Nlrp1 variants confers sensitivity to Toxoplasma and LT. Viability of LEW and CDF BMDMs nucleofected with full length HA-tagged NLRP1 constructs at 36 hours prior to infection with PRU (MOI 1) was measured by MTT assay at 8 hours post-infection. Viability of similarly nucleofected cells was measured 5 hours after treatment with anthrax LT (PA + LF, each at 1 μg/ml). Superscripts indicate the NLRP1 construct or vector that was transfected into the cell. Graph shows average from three independent nucleofections per condition.
Supplementary Figure S8. Viability of different cell lines and BMDMs overexpressing rat NLRP1 following infection with Toxoplasma. (A) HT1080 fibroblast cells or (B) BMAJ mouse macrophage cell lines expressing full length HA-tagged NLRP1\textsuperscript{variant2} (CDF sequence) or NLRP1\textsuperscript{variant5} (LEW sequence) were tested for viability following Toxoplasma infection. Infections were with Type I (RH and Type II (76K) strains (MOI 5) were performed and viability was assessed 24 hours post-infection. Details on constructions of these lines can be found in (Levinsohn et al. 2012). In select experiments myc-tagged caspase-1 was also transfected 24 hours prior to infection. Values graphed are mean ± SD, n = 3 wells/treatment. (C) Various mouse macrophage cell lines and BMDMs from mouse strains were tested for susceptibility to infection as described above. RAW264.7 cells were not tested with the RH strain. There is no statistical difference between any of the groups or treatments in these studies.
Acknowledgments

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References


Chapter Three:
Addendum
Results and Discussion

*Neospora caninum* activates the inflammasome in Lewis macrophages

As every strain of *Toxoplasma* was able to induce pyroptosis in Lewis macrophages (Chapter Four, Figure 3), we were interesting in determining if related parasite species were able to activate the rat inflammasome. *Neospora caninum* is another member of the phylum Apicomplexa and a close relative of *Toxoplasma* (Dubey et al. 1988). *Neospora* and *Toxoplasma* share many biological features, including the ability to reproduce both sexually and asexually and move between definitive and intermediate host. *Neospora* has a more limited host range than *Toxoplasma* and cannot infect humans (McCann et al. 2008). Despite these differences in host range, *Toxoplasma* and *Neospora* have similar genomes with conserved gene content (Reid et al. 2012).

To determine if *Neospora* was capable of activating the rat inflammasome, we infected Lewis and Sprague-Dawley (SD) BMDMs. *Neospora* was able to induce pyroptosis in both primed and unprimed Lewis BMDMs to the same level as *Toxoplasma* (Figure 1A). Primed SD macrophages retained almost 100% viability when infected with *Toxoplasma*. Interestingly, we observed a 20% decrease in cell viability in both unprimed and primed SD BMDMs infected with *Neospora*. 
Figure 1. Neospora caninum is able to activate the inflammasomes in Lewis BMDMs. (A and B) Lewis and Sprague-Dawley (SD) BMDMs were primed with LPS (100ng/ml) for 2-4 hours or left untreated and then infected with the indicted parasite species (Toxoplasma RH or Neospora NC-I, MOI 1, 18 hours). Lewis data is average of 2 experiments. SD data is 1 experiment. (A) Cell viability was measured via MTS assay. Error bars, SD. (B) IL-1β release measured by ELISA. Error bars, SD.

As expected, host cell death was accompanied by the release IL-1β from primed Lewis BMDMs infected Neospora and Toxoplasma at comparable levels (Figure 1B). Neospora-infected primed Lewis macrophages released more IL-1β than primed SD macrophages. However, Neospora infection induced more IL-1β secretion than Toxoplasma infection. This suggests that Neospora may be able to activate the inflammasome and induce host cell death in macrophages from both strains of rats to varying degrees.
Knockdown of caspase-11 provides protection against *Toxoplasma*-mediated pyroptosis

Pyroptosis has been linked to two inflammatory caspases, caspase-1 and caspase-11. Caspase-11 is a member of the noncanonical inflammasome that is activated by Gram-negative bacteria that reach the host cytosol. Caspase-11 directly senses cytosolic lipopolysaccharide (LPS) (Hagar et al. 2013; Kayagaki et al. 2013). Activation of caspase-11 leads to the cleavage of gasdermin D (GSDMD), a cytoplasmic protein with no known physiological role (Kayagaki et al. 2015; Shi et al. 2015). Cleavage is sufficient to initiate pyroptosis. Caspase-11 is unable to cleave pro-IL-1β (Wang et al. 1996). In some bacterial infections, such as *E.coli* and *Vibrio cholera*, caspase-11 is involved in the activation of caspase-1 through the NLRP3 inflammasome (Kayagaki et al. 2011). The exact mechanism through which caspase-11 leads to caspase-1 activation in this model has yet to be elucidated.

We were unable to knockdown *Casp1* in Lewis macrophages using lentiviral shRNAs and attempts to block caspase-1 activity using chemical inhibitors did not inhibit *Toxoplasma*-induced pyroptosis (Figure 2A). Interestingly, caspase-1 inhibition resulted in a significant reduction in IL-1β released from infected macrophages compared to untreated cells (Figure 2B), suggesting *Toxoplasma*-mediated host cell death is caspase-1-independent, while IL-1β secretion is caspase-1-dependent.
Figure 3. Chemical inhibition of caspase-1 does not prevent *Toxoplasma*-induced pyroptosis. (A) Lewis BMDMs were treated with caspase-1-specific inhibitors Z-WEHD-FMK (WEHD, 50µM) or Z-YVAD-FMK (YVAD, 50µM) for two hours or left untreated and then infected with *Toxoplasma* (RH, MOI 1, 18 hours). Cell viability measured via MTS assay. Error bars, ±SD. Data using WEHD is average of five experiments, YVAD is average of two experiments. (B) IL-1β release measured by ELISA. Lewis BMDMs were primed with LPS (100ng/ml, 2-4 hours) prior to treatment with inhibitors (50µM, 2 hours) and infection (MOI 1, 18 hours). Error bars, ± SD. Data using WEHD is average of five experiments, YVAD is one experiment. ***p<0.005, paired t-tests.

We were next interested to determine the role, if any, caspase-11 plays in *Toxoplasma*-induced host cell death. *Casp11* gene expression in Lewis BMDMs was knocked down by at least 60% using three individual shRNAs (Figure 3A). Knockdown was accompanied by an increase in host cell viability. At least 75% of macrophages from each *Casp11*-specific shRNA condition survived parasite infection, compared to 45% of cells treated with control shRNA (Figure 3B). This suggests caspase-11 plays a role in parasite-induced pyroptosis in Lewis macrophages. We previously demonstrated this death was due to the NLRP1 inflammasome (Chapter 3). Although preliminary, this may implicate the canonical NLRP1 inflammasome in a caspase-11-dependent form of pyroptosis. Thus far, only NLRP3 has been found to be downstream of caspase-11 activation.
Figure 3. Knockdown of Caspase-11 protects Lewis BMDMs from Toxoplasma-mediated host cell death. (A) Knockdown by lentiviral shRNA was confirmed by qPCR. (B) Lewis BMDMs treated with indicated shRNA were infected with Toxoplasma (RH, MOI 0.5, 18 hours) and viability was measured via MTS assay. Data is 1 experiment. Error bars, SD.
Materials and Methods

Animals

Lewis (LEW/Crl; LEW) and Sprague Dawley (SD) rats (6-8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and used as source of bone marrow.

Parasites and cells

Human foreskin fibroblasts (HFFs) were grown in DMEM, supplemented with 1% heat inactivated FBS and 50μg/ml each of penicillin and streptomycin and 20μg/ml gentamycin. Parasites were maintained in vitro by serial passage in HFF monolayers and grown in DMEM, supplemented with 1% heat inactivated FBS and 50μg/ml each of penicillin and streptomycin.

Toxoplasma gondii tachyzoites from Type I (RH) and Neospora caninum NC-I were used in experiments. Lewis BMDMs were prepared as previously described (Cirelli et al. 2014). Cell Viability experiments were performed as previously described (Cirelli et al. 2014).

Reagents

DMEM was obtained from Invitrogen. Antibiotics were purchased from Life Technologies Corporation. FBS was purchased from PAA. Lipopolysaccharide (LPS) was purchased from Calbiochem/EMD Biosciences. CellTiter 96 AQueous One Solution Cell Proliferation Assay was obtained from Promega. Rat IL-1β DuoSet ELISA, Z-YVAD-FMK and Z-WEHD-FMK were purchased from R&D Systems.
Knockdown experiments

High-titer lentivirus (Broad Institute RNAi consortium) encoding shRNA against murine *Casp4* (also known as *Casp11*) was used to infect Lewis BMDMs on day two of differentiation. The target sequences of the shRNAs are: #1 – GCTCTTGTTCATCTTTGATA (20/21 bases match to rat *Casp4*), #2 – CCGTACACGAAAGGCTTAT (20/21 match), #3 – AGCAACTGAATCTCATTTCTT (19/21 match). Cells were selected with puromycin (6μg/ml). Knockdown was confirmed by qPCR. Actin primers: 5’ GTCGTACCAGCCATTGTG ‘3 and 5’ CTCTCAGCTGTGGTGGTGA ‘3. *Casp4/11* primers: 5’ TGGACTCAGGCAGCCAC ‘3 and 5’ CTAATGTCAATGTAGCC ‘3. *Casp4/11* gene expression was normalized against actin expression levels.
References


Chapter Four:
Three novel *Toxoplasma gondii* dense granule proteins are required for Lewis rat NLRP1 activation

Kimberly M. Cirelli, Vincent Butty, Musa A. Hassan, Jeroen P.J. Saeij

Kimberly M. Cirelli contributed to Figures 1, 2, 3, S1, S2, S3. Vincent Butty and Musa Hassan contributed to Figures 2A.
Abstract

*Toxoplasma gondii* is an intracellular parasite that can form a lifelong chronic infection in hosts, characterized by cysts in muscle tissues and the brain. The Lewis rat is unique in its ability to completely clear *Toxoplasma* infection and prevent the establishment of a chronic infection. Previous findings established that Lewis macrophages undergo rapid cell death, known as pyroptosis, when infected with *Toxoplasma*. Pyroptosis was controlled by the NLRP1 inflammasome. We have used a chemical mutagenesis screen to identify *Toxoplasma* genes involved in NLRP1 inflammasome activation. We isolated several parasite mutants that induce significantly less pyroptosis in Lewis macrophages and reduced IL-1β secretion. Utilizing whole genome sequencing of our mutants, we identified single nucleotide polymorphisms and deletions. We then used CRISPR/Cas9 to knockout individual candidate genes. We have identified novel *Toxoplasma* dense granule proteins, GRA18, GRA27 and GRA28, which are individually required for inflammasome activation *in vitro*. Strains deficient in GRA18, GRA27 or GRA28 display the same phenotype as the mutants isolated from the screen. Complementation of mutants with wild-type allele of *Gra18, Gra27* or *Gra28* is sufficient to restore ability to induce pyroptosis in Lewis macrophages.
Introduction

*Toxoplasma gondii* is an obligate intracellular parasite that infects all warm-blooded animals (Hill and Dubey 2002). Among its different hosts, there are natural differences in susceptibility to the parasite. Rats and humans are relatively resistant to *Toxoplasma*. Most members of both species are asymptomatic upon infection, but the parasite establishes a chronic lifelong infection by developing into cysts in brain and muscle tissues. However, the Lewis rat strain, can clear the parasite and fails to develop this chronic infection (Sergent et al. 2005). This resistance was mapped to a single locus, *Toxo1* (Cavaillès et al. 2006) and correlated with induction of pyroptosis by *Toxoplasma in vitro*. *Toxoplasma*-induced cell death in Lewis macrophages was determined to be controlled by *Nlrp1*, which encodes for the NLRP1 inflammasome sensor (Cirelli et al. 2014; Gorfu et al. 2014).

The inflammasomes are a family of cytosolic pattern recognition receptors (PRRs). Activation of the sensor, leads to the formation of a multimeric complex and the recruitment and proteolytic activation of pro-caspase-1. Caspase-1 cleaves pro-IL-1β and pro-IL-18, resulting in their release from the cells. Caspase-1 activation can be accompanied by host cell death, termed pyroptosis (Lamkanfi and Dixit 2012; Kayagaki et al. 2015; Shi et al. 2015). Pyroptosis has been established as a host mechanism to clear intracellular pathogens, particularly *Salmonella typhimurium* and *Legionella pneumophila* (Miao et al. 2010; Miao et al. 2011). NLRP1 activation in Lewis bone marrow-derived macrophages (BMDMs) results in the release of IL-18 and rapid death of the host cell, releasing *Toxoplasma* into the extracellular space before parasite replication can occur. In conditions where pro-IL-1β expression is induced in macrophages (e.g. LPS-primed), infected cells also release bioactive IL-1β (Cirelli et al. 2014). As macrophages are among the predominant cell type infected upon an oral infection, it’s likely that macrophage
pyroptosis is a host mechanism to prevent parasite proliferation and dissemination (Mordue and Sibley 2003; Lambert and Barragan 2010).

The specific stimuli that can activate the inflammasomes and their mechanism of activation vary. NLR family CARD domain-containing protein 4 (NLRC4) recognizes NLR family, apoptosis inhibitory protein (NAIP) proteins bound to bacterial components, namely flagellin and type III secretory system proteins (Kofoed and Vance 2011; Zhao et al. 2011). Anthrax Lethal Toxin (LT) is a protease and a direct activator of rat NLRP1 (Newman et al. 2010). LT cleaves the N-terminus of NLRP1 in LT-susceptible rat macrophages. This cleavage is sufficient to activate the inflammasome and induce pyroptosis (Levinsohn et al. 2012). Because the types of ligands and modes of activation of the inflammasomes are varied, we chose to take an unbiased approach to identify the Toxoplasma gene product(s) required for Lewis NLRP1 inflammasome activation. Using a mutagenesis screen followed by whole genome sequencing we identified three novel Toxoplasma dense granule proteins (GRAs) required for NLRP1 inflammasome activation in Lewis rat macrophages. Parasite strains deficient in individual proteins induce significantly less pyroptosis and IL-1β processing in vitro.
Results

A mutagenesis screen isolates parasites that do not activate the NLRP1 inflammasome

We previously found that upon infection of Lewis bone marrow-derived macrophages (BMDMs) by Toxoplasma the NLRP1 inflammasome is activated leading to rapid host cell death and the control of Toxoplasma replication (Cirelli et al. 2014). To identify Toxoplasma gene(s) required for NLRP1 inflammasome activation, we designed a chemical mutagenesis screen to enrich for parasites that fail to induce pyroptosis in Lewis BMDMs (Figure 1A). Five independent populations of chemically mutagenized type I (RH) parasites were used to infect unprimed Lewis BMDMs for two hours. Extracellular parasites were washed from cells and media was replaced with fresh media that contained the glycosaminoglycan, dextran sulfate. Dextran sulfate acts as a glycan competitor and prevents host cell invasion by extracellular parasites (Carruthers et al. 2000). Parasites that retain the ability to activate the NLRP1 inflammasome are released from the lysed cell into the supernatant, where the parasite is coated with dextran sulfate, blocking invasion into a new host cell. Mutated parasites unable to induce pyroptosis are able to replicate within the surviving macrophage. After 24 hours of infection, surviving cells were washed, thereby removing the extracellular parasites capable of inducing pyroptosis from the population. The parasites within the macrophages were then allowed to continue replication until their natural egress from the macrophages.

After five to nine rounds of selection, single parasites were cloned from the populations and individual clones were tested for their inability to induce pyroptosis. Eleven mutants were isolated and determined to induce significantly less pyroptosis in Lewis BMDMs. By sequence analysis, we determined several clones were identical. Overall, seven unique mutant strains were
isolated. We chose to focus on four independent clones (#1-4). At least 75% of Lewis macrophages infected with any mutant strain survived, which is in contrast to only 25% of BMDMs that survived infection with the wild-type strain (Figure 1B). As expected, survival of the host cell was linked with the ability of the parasite to replicate within the macrophage. After 24 hours of infection, 80% of the surviving macrophages infected with wild-type parasites contained only single parasites compared to those cells infected with each mutant strain, in which only 25% of infected cells contained single parasites (Figure 1C).
Figure 1. Isolation of Toxoplasma parasites that do not induce pyroptosis. (A) Schematic of mutagenesis screen. DS is Dextran Sulfate. (B) Lewis BMDMs were infected with indicated strains (MOI = 1, 24 hours). Macrophage viability was measured via 3-(4,5-dimethylthiazol-3-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay. Data shown is average of 7 experiments. Error bars, + SD. **p<0.005, ****p<0.0001, paired t-test (C) Number of parasites per vacuole in infected BMDMs (MOI = 0.5, 24 hours) as measured by microscopy. Between 50-100 vacuoles counted per experiment. Average values from 3 experiments. Error bars, +SD. P-values are <0.0001, two-way ANOVA comparing mutants to wild-type. (D) Western blot probing for IL-1β on concentrated (20X) supernatants on LPS-primed (100ng/ml, 2 hours) infected with indicated strains (MOI = 1, 24 hours). Image is representative of 2 experiments, pro-IL-1β is 37kD, active IL-1β, aspecific band is represented by asterisk and indicates similar loading of samples. Mutant 4 was not tested. (E) Lewis BMDMs were primed with LPS (100ng/ml, 2-4 hours) and infected with indicated strains (MOI =1, 24 hours). Viability measured by MTS assay. Data shown is the average of 4 experiments. Mutant 4 was not tested. Error bar, + SD. ***p<0.0005, paired t-test.
Inflammasome activation is marked by cleavage and secretion of the pro-inflammatory cytokines, IL-1β and IL-18. To induce expression of pro-IL-1β, BMDMs were primed with LPS prior to infection with *Toxoplasma*. To measure active IL-1β, we subjected the supernatants of infected BMDMs to Western blotting, probing for both the inactive (37kD) and bioactive (17kD) forms of IL-1β. We found a strong decrease in the amount of cleaved, active IL-1β secreted from macrophages infected with each of the mutant strains, compared to wild-type (*Figure 1D*). Significant amounts of the inactive form of the cytokine were also measured in the supernatants. We noted that LPS-primed macrophages infected with mutant parasites survived less than unprimed macrophages, but still significantly more so than wild-type infected BMDMs (*Figure 1E*). This increase in host cell death of primed cells most likely explains the large fraction of inactive IL-1β released from the cell.

*Toxoplasma* also activates the inflammasomes in mouse macrophages. Several reports have implicated both NLRP3 and/or NLRP1 activation in the murine response to control parasite replication. Although the role of pyroptosis, if any, in this resistance is not fully understood (Gorfu et al. 2014; Ewald, Chavarria-Smith, and Boothroyd 2014). Unprimed or LPS-primed C57Bl/6 macrophages showed no significant difference in host cell survival after infection with wild-type or any mutant strain (*Figure 2A*). Mouse macrophages infected with mutant strains secreted more IL-1β than wild-type infected cells (*Figure 2B*). These results suggest that the inability of the mutants to activate NLRP1 inflammasomes is a rat-specific phenomenon.
Figure 2. Mutants activate the inflammasome in mouse BMDMs. (A and B) C57BL/6 BMDMs were primed with LPS (100ng/ml, 3 hours) or left unprimed and infected with indicated strains (MOI 1, 24 hours). (A) Cell viability as measured via MTS assay. (B) IL-1β secretion as measured by ELISA. Error bars, + SD. Data are from 1 experiment.
Identification of mutated genes

To identify the genes mutated in each clone, we performed Illumina whole genome sequencing on each strain. Each clone had at least five non-synonymous mutations (Table 1). Two clones (mutant #3 and #4) shared one mutated gene, TgGTJ_226380 (Figure 3A). Mutations in both clones resulted in a premature stop codon. Mutants #1 and #2 did not have any genes mutated in common with any of the other isolated strains. To identify the causative mutations in mutants #1 and #2, we established a set of criteria to narrow the list of possible genes. The inflammasomes are expressed within the cytoplasm of host cells. We hypothesized that a Toxoplasma secreted protein that can interact with host cytosolic proteins might be recognized by NLRP1 or modulate the activity of the inflammasome. We therefore chose to focus on genes whose protein products contained predicted signal peptides. Additionally, we previously tested numerous strains of Toxoplasma that are genetically distinct from the clonal types I, II and III for their ability to activate the inflammasome. All strains tested were able to induce pyroptosis (Chapter 3, Figure 3). We therefore focused on genes that were expressed (FPKM>10) across all strains that we tested using a previously published RNAseq dataset (Minot al. 2012). Using these criteria, we narrowed the list of candidate genes in mutants #1 and #2 to three genes each (Figure 2A).
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Table 1. List of all identified non-synonymous mutations. "Ref" is reference nucleotide(s) in wild-type strain (GT1 v9.0). "Sub" is nucleotide variant(s). "Mut" is mutant clone number.
We individually disrupted each candidate gene in RH using CRISPR/Cas-9 and tested the resulting strains for their inability to induce pyroptosis (Sidik et al. 2014). Knockout of most genes resulted in no difference in inflammasome activation compared to wild-type (Figure 3B). Parasites that contained single disruption of three different genes, TgGT1_226380, TgGT1_237015 and TgGT1_236870, induced significantly less host cell death upon infection compared to wild-type parasites. Complementation of the knockouts with the disrupted gene restored their ability to induce host cell death (Figure 3C).

We have previously shown that multiple strains of Toxoplasma and Neospora caninum are capable of inducing pyroptosis in Lewis macrophages (Chapter 3, Figure 3 and Chapter 3 Addendum, Figure 1). We saw similar results when these genes were disrupted in type II (ME49) parasites. Lewis macrophages infected with parasites deficient in each of these genes retained 100% of cellular viability compared to 20% of wild-type infected cells. (Figure 3D).

Homologs of TgGT1_226380, TgGT1_237015 and TgGT1_236870 were identified only in two members of the Sarcocystidae family, Neospora caninum and Hammondia hammondi (Figure 4). The predicted protein products of these genes lack predicted functional domains. The resulting proteins each have two predicted transmembrane domains and several alpha helices (Figure 5).
Figure 3. Three genes are individually required to induce pyroptosis in BMDMs. (A) List of genes containing non-synonymous polymorphisms that fulfill candidate gene criteria in isolated mutants. (B) Toxoplasma parasites were individually knocked out for candidate genes using CRISPR/Cas-9, with the exception of Sub1. Lewis BMDMs were infected with indicted strains (MOI = 1, 24 hours) and cell viability was measured using MTS assay. Two clones deficient in each individual gene was tested at least twice. Graph is average of one clone per gene. Error bars, ± SD. (C and D) Cell viability as assessed by MTS assay of Lewis BMDMs infected with indicated strains (MOI = 1, 24 hours). Each strain has been tested in at least three experiments, with the exception of ME49ΔGRA28, which has been tested once. Error bars, ± SD. ***p<0.0005, ****p<0.0001, paired t-test.
Figure 4. TgGT1_226380, TgGT1_237015 and TgGT1_236870 have homologs in Neospora and Hammondia. Alignments of primary peptide sequences using Profile Alignemnt (PRALINE), scoring amino acid conservation. (A) Alignment of Toxoplasma gondii TgGT1_226380, TgME49_226380 and TgVEG_226380, Hammondia hammondi HHA_226380, Neospora caninum NCLIV_046580 and NCLIV_047520. (B) Alignment of Toxoplasma TgGT1_237015, TgME49_237015 and TgVEG_237015, Hammondia HHA_237015 and Neospora predicted protein BN1204_050915. (C) Alignment of Toxoplasma TgGT1_236870, TgME49_236870 and TgVEG_236870, Hammondia HHA_236870 and Neospora NCLIV_050780. The legend above depicts color scheme. Mutations are marked by red asterisks above mutated base.
Figure 5. *TgGTI* _226380, TgGTI_237015 and TgGTI_236870 have transmembrane domains and several alpha helices*. PSIPRED was used for secondary structure prediction (Jones 1999). Helices, red. Strands, blue. TmHMM2.0 was used for transmembrane domain (TM) prediction (Krogh et al. 2001). TM are marked with black lines above region, mutations marked with red asterisks above mutated base.
We C-terminally tagged each gene product with a hemagglutinin (HA)-tag and confirmed expression of the protein using immunofluorescent microscopy and Western blot (Figure 6A). We performed microscopy studies to determine the subcellular localization of each protein. The protein products of TgGT1_226380, TgGT1_237015 and TgGT1_236870 each localized with the dense granule protein, GRA7 and therefore were named GRA18, GRA27 and GRA28, respectively (Figure 6B).

**Figure 6. TgGT1_226380, TgGT1_237015 and TgGT1_236870 are dense granule proteins.** (A and B) Strains individually knocked out in each gene were generated using CRISPR/Cas9 and complemented with HA-tagged wild-type version of gene. (A) HFFs were infected with HA-expressing parasites for 36 hours. Extracellular parasites were removed and washed with PBS prior to lysing ("Ex"). Remaining infected cells were lysed ("In"). SAG-1 is used as parasite loading control. Predicted size: GRA18, 42.6kD. GRA27, 29.3kD. GRA28, 23.8kD. (B) HFFs were infected with strains expressing HA-tagged GRA17, GRA27 or GRA28 for 24 hours and subjected to IF with antibodies indicated. The images represent single deconvoluted focal slice. (scale bar = 10 μm).

Primed BMDMs were more sensitive to parasite-induced pyroptosis with strains deficient in GRA18, GRA27 or GRA28 compared to unprimed macrophages. We did not see this difference in primed macrophages infected with wild-type or complemented strains (Figure 7A).

To confirm the mutation of these genes were responsible for the failure to activate the inflammasome in our chemically mutagenized parasites, we expressed the wild-type allele of the
gene in each mutant. Addition of wild-type GRA18, GRA27 and GRA28 in their respective mutant was sufficient to restore pyroptosis induction (Figure 7B). Macrophages primed with LPS were more sensitive to infection with mutants (Figure 1E, 7C).

Figure 7. Addition of wild-type version of gene is sufficient to restore ability to induce pyroptosis.
(A) Lewis BMDMs were primed with LPS (100ng/ml, 2-4 hours) or left untreated and infected with indicated strains (MOI =1, 24 hours). Viability measured by MTS assay. Graph is average of 2 experiments. Error bar, + SD. (B) Lewis BMDMs infected with indicated strains (MOI =1, 24 hours). Viability measured by MTS assay. Data is average of 3 experiments. Error bar, + SD. ***p<0.0005, ****p<0.0001. (C) Lewis BMDMs were primed with LPS (100ng/ml, 2-4 hours) or left unprimed and infected with indicated strains. Data is 1 experiment. Error bar, + SD. Data is from 1 experiment.
As expected, 80% of macrophages infected with RHΔGRA18, RHΔGRA27 or RHΔGRA28 contained multiple parasites, whereas upwards of 60% of wild-type and the complemented strain-infected BMDMs contained only single parasites (Figure 8A). Similarly, only ~30% of macrophages infected with mutant strains expressing wild-type GRA18, GRA27 or GRA28 allowed replication of *Toxoplasma*, in contrast to ~80% of mutant-infected BMDMs (Figure 8B).

To determine if complementation of the mutants was sufficient to restore IL-1β cleavage and activation, we infected LPS-primed BMDMs and measured IL-1β secretion. Infection with RHΔGRA18 or RHΔGRA28 resulted in a significant decrease in secreted IL-1β compared to wild-type and their complemented counterparts (Figure 8C). Expression of wild-type GRA18, GRA27 or GRA28 in mutant strains was sufficient to induce a significant increase in IL-1β released from primed BMDMs (Figure 8D). When we probed for active IL-1β, we observed an increase in the active 17kD fragment secreted from macrophages infected with the complemented strains compared to their mutant counterparts (Figure 8E). Additionally, primed BMDMs infected with ME49ΔGRA27 and ME49ΔGRA28 secreted less active IL-1β than wild-type infected cells (Figure 8F).
Figure 8. GRA18, GRA27 and GRA28 are required for inflammasome activation. (A and B) Number of parasites per vacuole of infected Lewis BMDMs (MOI = 0.5, 24 hours) as measured by microscopy. Between 50-100 vacuoles were scored per experiment. (A) is average of 4 experiments. (B) is average of 2 experiments. Error bars, + SD. P-values are <0.01 comparing mutant strains to wild-type or complemented strains (Two-way ANOVA). (C and D) IL-1β as measured by ELISA from LPS-primed (100ng/ml, 2-4 hours) Lewis BMDMs infected with indicated strains (MOI = 1, 24 hours). Data is average of 3 experiments. Error bars, + SD. *p<0.05, **p<0.001, ***p<0.0005. (E) Western blot of IL-1β on concentrated supernatants (25X) BMDMs primed with LPS (100ng/ml, 3 hours) infected with indicated strains (MOI = 1, 24 hours). Image is representative of 2 experiments. (F) Western blot probing for IL-1β on concentrated supernatants (20X) of Lewis BMDMs that were primed (100ng/ml, 3 hours) or left unprimed and infected with indicated strains (MOI = 1, 24 hours). Image is representative of 1 experiment.
Strains deficient in GRA18 or GRA27 do not establish chronic infection in Lewis rats

In chapter three, we hypothesized that *Toxoplasma* utilizes the macrophage as a vehicle for dissemination away from the initial site of infection to distal sites, including the brain. Activation of the NLRP1 inflammasome in Lewis rats results in pyroptosis. Host cell death results in the destruction of the niche *Toxoplasma* requires for proliferation and trafficking throughout the body. In chapter four, we identified three parasite genes, GRA18, GRA27 and GRA28, required for activation of the NLRP1 inflammasome and pyroptosis in macrophages *in vitro*. We hypothesized strains deficient in these genes will fail to induce pyroptosis in macrophages *in vivo*, allowing the parasite to replicate and move to the brain. Parasites lacking GRA18, GRA27 or GRA28 would then be able to convert into the dormant bradyzoite stage and establish a lifelong chronic infection.

To test this, we infected Lewis rats and Brown Norway rats with wild-type ME49-RFP (ME49), ME49ΔGRA18 and ME49ΔGRA27, intraperitoneally. RH parasites do not readily form orally infectious cysts in mice or rats, while the ME49 strain does (data not shown). After four weeks, we harvested brains and determined the presence or absence of cysts via rederivation *in vitro*. Preliminary results suggest that individual deletion of GRA18 or GRA27 is not sufficient to establish a chronic infection in Lewis rats. Wild-type ME49 parasites were able to form cysts in the control Brown Norway rats, in which *Toxoplasma* readily establishes a chronic infection (Sergent et al. 2005) but not in Lewis brains. Neither ME49ΔGRA18 nor ME49ΔGRA27 were rederived from Lewis brains. Zero Brown Norway rats infected with ME49ΔGRA18 contained cysts within brain tissue. One out of two ME49ΔGRA27-infected Brown Norway rats contained cysts (Table 2).
Table 2. Lewis rats infected with ME49ΔGRA18 or ME49ΔGRA27 are not chronically infected. Lewis and Brown Norway rats were infected with $3 \times 10^6$, i.p. for 30 days. Brains were harvested and brain suspension was placed onto HFFs for parasite rederivation. Table is one experiment.

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<th>Strain</th>
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The failure of the ME49ΔGRA18 and ME49ΔGRA27 to form cysts as well as wild-type in the susceptible Brown Norway rats suggests that these genes are required for establishing a chronic infection. To successfully infect a host chronically, *Toxoplasma* must replicate, traffic to distal sites, sense an immune response and convert from the quickly dividing tachyzoite into the semi-dormant bradyzoite. To determine if parasites lacking GRA18, GRA27 or GRA28 are able to replicate properly, we measured plaque area formed in human foreskin fibroblasts (HFF) monolayers. Measuring plaque area accounts for the overall growth of parasites *in vitro*, including parasite replication, egress and reinfection. We found no significant differences in plaque area between HFFs infected with wild-type parasites and RHΔGRA18, RHΔGRA27 or RHΔGRA28 (Figure 9). This data, in addition to our findings that RHΔGRA18, RHΔGRA27 and RHΔGRA28 are able to replicate within the Lewis macrophage suggest that the absence of these genes do not play a role in parasite growth.
Figure 9. RHAGRA18, ΔGRA27 and ΔGRA28 do not show a growth defect in vitro. Confluent HFFs were infected with the indicated parasites for 5 days. The area of at least 30 plaques per experiment was measured. Data is average of 2 experiments. Error bars, ± SD.

GRA27 and GRA28 activate type I interferon response pathways

Toxoplasma must activate the immune system in order to convert from tachyzoite to bradyzoite. Several Toxoplasma proteins have been established as regulators of host gene expression, including immunological pathways. We were interested in determining if GRA18, GRA27 or GRA28 act as modulators of host gene expression and performed RNAseq on Brown Norway BMDMs infected with our knockout and complemented strains. 172 transcripts were differentially regulated by GRA18, GRA27 and/or GRA28 (Figure 10A, Table 3). Gene ontology (GO) analysis found immune system process (p-value = 6.31 x 10^-8) pathways as significantly enriched among these transcripts (Figure 10B).
Among the genes differentially regulated were several type I IFN-regulated genes, including *Glp2*, which encodes for a ubiquitin-like modifier protein, *Ifitm3*, encoding for an interferon-induced transmembrane protein and *Irf7*, a transcription factor typically activated by TLR3, TLR4, TLR7 and TLR8 (Schoenemeyet et al. 2005; Doyle et al. 2002). GRA27 and GRA28-complemented strains induced greater expression of *Glp2*, *Irf7* and *Ifitm3* in Brown Norway macrophages than their mutant counterparts (Figure 10C). Interestingly, macrophages infected with GRA18-deficient parasites expressed these genes to a lesser degree than the complemented strains, suggesting GRA18 may act as a negative regulator of the interferon-response.

We observed a two-fold reduction in expression of *Il1b*, which encodes for pro-IL-1β, in macrophages infected with RHAGRA28 compared to those infected with its complement (Table 3). Unlike pro-IL-18, pro-IL-1β is not constitutively expressed in myeloid cells. Inflammasome activation and release of active IL-1β requires two distinct signals: priming and recognition of an agonist by the sensor. *Toxoplasma* supplies both signals. The mechanism through which GRA28 induces pro-IL-1β expression should be explored further. Interestingly, GRA28 also leads to increased *Il1r2* expression. This gene codes for the IL-1 receptor, type II (IL-1R2), which acts as a decoy IL-1 receptor (Colotta et al. 1993). IL-1R2 binds IL-1, but does not signal. *Toxoplasma* activation of IL-1R2 may be a mechanism the parasite uses to prevent excessive IL-1β signaling, which can lead to IFN-γ production and parasite clearance.
Figure 10. GRA18, GRA27 and GRA28 are involved in IFN-regulated signaling. Brown Norway BMDMs were infected with indicated strains for 18 hours (MOI 1). Strains used were RHΔGRA18, RHΔGRA18 + GRA18, RHΔGRA27, RHΔGRA27 + GRA27, Mutant #2 and Mutant #2 + GRA27. Total RNA was extracted and subjected to RNAseq. Differentially regulated genes were identified after normalization and filtering genes that had at least a two-fold difference in expression between strains. Data is one experiment. (A) Heat map of differentially expressed rat genes. (B) Visualization of gene ontology analysis of genes in Table 3. Map created with REVIGO (Supek et al. 2011). Intensity of red indicates p-value, size of bubble indicates the frequency of GO term and line thickness between nodes indicates similarity. (C) Gene expression of representative interferon-regulated genes.
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Table 3. List of differentially expressed rat genes. Data was filtered for genes with FPKM ≥5 in at least one sample and a minimum of 2-fold difference in expression between any mutant and its complement. Log fold change using formula $\log_{2}\text{fold} = \log_{2}(\text{mutant}) - \log_{2}(\text{complement})$. Data is sorted by Log$_2$ fold change of GRA27, followed by GRA28. Data is one experiment.
Discussion

In this work, we have identified three novel dense granule proteins, GRA18, GRA27 and GRA28, which are required for *Toxoplasma*-induced pyroptosis and IL-1β activation and release in Lewis BMDMs. Previous work demonstrated this phenomenon was mediated by *Nlrp1* (Cirelli et al. 2014). Several dense granule proteins have been established as players in host modulation. Type II GRA15 is sufficient to induce host NF-κB nuclear translocation and activation (Rosowski et al. 2011). GRA16 and GRA24 are exported out of the parasitophorous vacuole into the nucleus to modulate host gene expression (Bougdour et al. 2013). GRA6 is a regulator of the host transcription factor, NFAT4 (Ma et al. 2014). The mechanism through which GRA18, GRA27 and GRA28 coordinate to activate NLRP1 remains to be determined.

GRA18, GRA27 and GRA28 do not have significant homology to known proteins from organisms outside of the family, Sarcocystidae, which includes *Neospora* and *Hammondia*. *Neospora caninum* is able to induce pyroptosis in Lewis BMDMs (Chapter 3 – Addendum, Figure 1), suggesting the function of these proteins is conserved. Genetically diverse *Toxoplasma* strains are able to induce pyroptosis in Lewis BMDMs, supporting this hypothesis. Interestingly, strains that fail to activate the NLRP1 inflammasome in Lewis macrophages do not show a deficiency in activation of the mouse inflammasomes. It is likely GRA18, GRA27 and GRA28 have other functions important to the success of the parasite and determining these functions would of interest.

Cleavage of NLRP1 is required for the activation of the inflammasome by Anthrax LT. GRA18, GRA27 and GRA28 do not have predicted protease domains. They do share predicted transmembrane domains and several helices (Figure 5). A potential model of activation is GRA18, GRA27 and GRA28 localize within the parasitophorous vacuole membrane, with C-
termini facing the host cytosol, where they mediate a host immune response. This has been demonstrated by GRA6, whose C-terminus interacts with host cytosolic protein CAML, which leads to the activation of NFAT4 (Ma et al. 2014). Our finding that individual deletion of these genes is sufficient to significantly reduce pyroptosis and IL-1β cleavage supports a model where the proteins exist within a complex. These proteins may directly interact with or modify NLRP1 or facilitate the recognition of an unknown *Toxoplasma* protein. Human fibroblasts engineered to express Lewis NLRP1 and caspase-1 or mouse macrophages that express only Lewis NLRP1 fail to undergo pyroptosis when infected with *Toxoplasma*, suggesting that additional co-factors were required for inflammasome activation (Cirelli et al. 2014). GRA18, GRA27 and GRA28 may interact and modify a rat-specific protein that is sensed by NLRP1, similar to NLRC4 recognition of a NAIP5/NAIP6/flagellin complex (Kofoed and Vance 2011; Zhao et al. 2011).

We found LPS-primed Lewis macrophages consistently underwent more pyroptosis when infected with mutant or individual knockout strains. We previously observed LPS stimulation of Lewis BMDMs induces a two-fold increase in *Nlrp1* and *Casp1* and a four-fold increase in *Casp11*. This increase in expression of key inflammasome components may lower the threshold of activation. Single knockout strains most likely have low levels of the *Toxoplasma* factor that is recognized by NLRP1. Generating double and triple knockout strains may further reduce the available activating factor and prevent pyroptosis completely in primed macrophages.

Preliminary *in vivo* experiments found individual deletion of GRA18 and GRA27 is not sufficient to form cysts in Lewis rats. Curiously, these strains show a defect in establishing a chronic infection in Brown Norway rats, in which *Toxoplasma* can readily form cysts. There are several possibilities for this reduction in virulence of these strains. Strains deficient in genes may not infect cells and replicate at rates comparable to wild-type strains. *In vitro* experiments
measuring growth in human foreskin fibroblasts showed no significant difference in parasite replication, although in future experiments parasite growth should be measured in Brown Norway macrophages to confirm this finding.

All rats are relatively resistant to Toxoplasma. While type I parasites have an LD$_{100} = 1$ parasite in the common laboratory mouse and will succumb to infection within one week due to parasitemia, rats can be infected with over one million of the same strain and fail to show symptoms of an acute infection. This suggests rats have defense mechanisms, which are more effective at preventing the parasite expansion seen in mice. The host mounts an immune response upon recognition of Toxoplasma and uses several identified mechanisms to clear infection, particularly those who are induced by IFN-γ. Anti-IFN-γ treatment led to an increase in cyst numbers and antibody titers in Brown Norway rats (Sergent et al. 2005). Parasite ROP18 and ROP5, have been found to be critical in Toxoplasma’s ability to counter the IFN-γ-inducible IRGs (Niedelman et al. 2012; Behnke et al. 2012). GRA18, GRA27 or GRA28 may play a role in antagonizing a rat defense pathway and deletion of any of these genes renders the parasite susceptible to these mechanisms.

The “Trojan Horse” model of parasite dissemination hypothesizes Toxoplasma exploits the ability of leukocytes to traffic to help its movement throughout the body. The intracellular environment offers the parasite an advantage as it is protected from the immune system. Dendritic cells, monocytes and natural killer cells infected with Toxoplasma display a hypermotility phenotype (Harker et al. 2013; Lambert et al. 2009; Ueno et al. 2015). These cells move faster and across greater distances. Parasite-infected DCs transferred into mice resulted in faster dissemination of the parasite to distant sites (Lambert et al. 2006). These data support the “Trojan horse” hypothesis. The ability to induce hypermobility in leukocytes is conserved among
strains (Lambert et al. 2009), but the parasite genes involved are unknown. GRA18, GRA27 or GRA28 may be involved in successful parasite dissemination.

In summary, we have established GRA18, GRA27 and GRA28 are required for NLRP1 activation by *Toxoplasma gondii*. The identification of these genes raises further questions about the mechanisms through which NLRP1 is activated and the function of these proteins.
Materials and Methods

Ethics statement

All animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Animal Welfare Act, approved by the MIT Committee on Animal Care (assurance number A-3125-01).

Reagents

N-ethyl-N-nitrosourea and ethyl methanesulfonate were purchased from Sigma-Aldrich. CellTiter 96 AQueous One Solution Cell Proliferation Assay was obtained from Promega. Dextran sulfate sodium salt was obtained from Santa Cruz Biotechnology. Rabbit anti-IL-1β (ab9722; 1:1000) was purchased from Abcam. Rat anti-HA (3F10; 1:5000) antibody was obtained from Roche. Secondary HRP-conjugated antibodies were purchased from Jackson ImmunoResearch. Alexa Fluor 448 and 594 secondary antibodies were obtained from Invitrogen. Rat and mouse IL-1β DuoSet ELISA were purchased from R&D Systems. Lipopolysaccharide (LPS) was purchased from Calbiochem/EMD Biosciences.

Animals

Lewis (LEW/Crl; LEW) rats were purchased from Charles River Laboratories (Wilmington, MA) at 6-8 weeks old. C57BL/6J mice were purchased from Jackson Laboratories at 8 weeks old.
**Cell Culture**

Tachyzoites from Type I (RH) expressing luciferase and GFP from pDHFR-Luc-GFP were used for mutagenesis. RH parasites lacking GFP, luciferase and HXGPRG were used for CRISPR studies. Type II (ME49) engineered to express RFP were a gift from Dr. Michael Grigg and used in *in vivo* experiments. RHΔSub1 were a kind gift from Dr. Vern Carruthers and generated as described (Lagal et al. 2010). HFFs were maintained as previously described (Rosowski et al. 2011). Lewis BMDMs were prepared as previously described (Cirelli et al. 2014). Cell Viability experiments and parasites per vacuole counts were performed as previously described (Cirelli et al. 2014).

**Western Blot**

Western blots probing for IL-1β were performed on infected cell supernatants concentrated using Amicon filters (3kD molecular weight cutoff) (Millipore, Billerica, MA).

**Mutagenesis Screen**

Intracellular wild-type RH parasites expressing GFP and Luciferase were treated with *N*-ethyl-*N*-nitrosourea (ENU, 40uM), ethyl methanesulfonate (EMS, 100uM) or dimethyl sulfoxide (DMSO) for 4 hours. Parasites were washed three times with PBS, syringe lysed and allowed to infect fresh HFFs. For selection, Lewis BMDMs were infected with parasite populations (MOI = 0.2 – 0.3) for two hours. Non-invading parasites were removed by washing cells with PBS three times. Media was replaced with DMEM containing 30mg/ml dextran sulfate. At 24 hours post-infection, extracellular parasites were removed by washing cells with PBS five times. Cells were
scrapped into fresh DMEM and overlaid onto fresh HFFs. Populations were selected for five to nine rounds. Parasites were cloned via serial dilution.

Freshly lysed parasites were washed with 50ml PBS and filtered through 5μm syringe filter (Millipore) to remove host cells. Parasite DNA was isolated using Qiagen DNeasy Blood & Tissue Kit according to manufacturer’s protocol. Parasite RNA was isolated from HFFs infected for 48 hours using Qiagen RNeasy Mini Kit. Illumina sequencing was performed on Illumina HiSeq 2000 or MiSeq. Reads were aligned using type I GT1 (v9.0) as reference genome.

**Generation of parasite strains**

Individual knockout of candidate genes was performed using CRISPR-Cas9. Sequences targeting candidate genes were cloned into the pSS013 Cas9 vector (Sidik et al. 2014). The sequences are available in **Supplementary Table 1**. Plasmid containing gRNAs were co-transfected with XhoI (New England Biolabs) -linearized pTKOatt (Rosowski et al. 2011) into wild-type RH parasites at ratio 10:1. 24 hours post-transfection, populations were selected with mycophenolic acid (50μg/ml) and xanthine (50μg/ml) and cloned by limiting dilution. Knockout was assessed by polymerase chain reaction (**Supplementary Figure 1**).

Complemented strains were generated by cloning gene with its putative promoter (~2000 bp upstream of start codon) with C-terminal hemagglutinin (HA)-tag sequence into pENTR using TOPO cloning (Invitrogen) and then into pTKOatt using LR recombination (Invitrogen). Prior to transfection, plasmids were linearized using a restriction enzyme with a unique restriction site. Plasmid was co-transfected with plasmid containing dihydrofolate reductase (DHFR) resistance cassette at ratio of 20:1. 24 hours post-transfection, populations were selected
with pyrimethamine (1μM) and cloned by limiting dilution. Presence of tagged gene was determined by immunofluorescent assay (IFA) and Western blot.

**Immunofluorescent Microscopy**

Cells were fixed with 100% ice cold methanol for 5 minutes and permeabilized with 0.2% TritonX-100. Colocalization studies were performed with anti-GRA7 or anti-ROP1 and anti-HA antibodies. Alexa Fluor 488 and 594 secondary antibodies were used, respectively as previously described (Rosowski et al. 2011).

**Plaque Assays**

HFFs were grown to confluency in a 24 well plate. 100 parasites were added to each well and incubated for 5 days at 37°C. The number of plaques was counted using a microscope. Plaques were photographed using a digital camera (Coolsnap EZ; Roper Scientific) connected to an inverted microscope (Eclipse Ti-S; Nikon) and plaque size was measured using NIS-Elements software (Nikon).

**Rat infection and rederivation**

Tachyzoites were grown in HFFs and mechanically removed from host cells by passage through a 27-gauge needle, followed by a 30- gauge needle. Parasites were washed three times with PBS, quantified and diluted in PBS. Rats were infected using 27-gauge needle. After 30 days of infection, rats were sacrificed. Brains were harvested and homogenized in PBS by passaging though 21-gauge needle. 1/10th of brain suspension was added to confluent HFFs in T-25 flasks in DMEM, supplemented with 1% heat-inactivated FBS, penicillin and streptomycin.
and incubated at 37°C for 4 weeks. Parasite growth *in vitro* was usually observed around 2 weeks post-inoculation.

**RNAseq**

Brown Norway BMDMs were infected for 18 hours with parasites (MOI =1) in 6 well plates. Plaque assays were performed at time of infection to determine viability and actual MOI of each strain. Total RNA was isolated using Qiagen RNeasy Plus kit. RNA was prepared for Illumina sequencing according to protocols. Sequencing was performed on Illumina HiSeq 2000. Reads were mapped to the rat genome (RGSC3.4). To identify the pathways modulated by GRA18, GRA27 and/or GRA28, we focused on rat genes with FPKM ≥5 in at least one sample and at least a two-fold difference in expression between any mutant and its complemented strain. GENE-E was used to determine hierarchical clusters. PANTHER (pantherdb.org) was used to investigate enrichment. REVIGO was used to visualize gene ontology enrichment.
### Supplementary Figures

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<td>Forward primer within gene to determine KO</td>
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Supplementary Table 1. Sequences of primers used in generating knockout and complemented strains. HA-tag is bolded.
Supplementary Figure 1. PCR confirming knockout of candidate genes. Genomic DNA was isolated from clones and used as template. Knockout was determined by failure to amplify gene of interest. DNA quality was assessed by amplifying another Toxoplasma gene, either another candidate gene or the B1 gene.
Acknowledgements

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Chapter Five:

Conclusions and Future Directions
Summary

The work in this thesis demonstrated that the parasite *Toxoplasma gondii* activates the NLRP1 and NLRP3 in the murine model. Deficiency of NLRP3, but not NLRP1, in bone marrow-derived macrophages leads to a significant reduction in active IL-1β secretion *in vitro*. Inflammasome activation was not accompanied by pyroptosis. Mice deficient in NLRP1 or NLRP3 were more susceptible to infection than wild-type mice. NLRP3 deficient mice had significantly lower levels of circulating IL-18 than wild-type mice. IL-18 and IL-1R deficient mice were also highly susceptible to infection, with significantly higher parasite loads. These findings establish *Toxoplasma* as an activator of two murine inflammasomes, which play a role in mouse resistance to parasite infection.

Additionally, we presented *Toxoplasma* as the second identified activator of the rat NLRP1 inflammasome. Infection with the parasite leads to the secretion of active IL-1β and IL-18 from BMDMs from certain strains of rats. In contrast to our findings in murine macrophages, infection of rat macrophages led to pyroptosis and the control of parasite replication. This phenotype was abrogated when NLRP1 expression was reduced using RNAi.

To identify the parasite protein product(s) involved in rat NLRP1 activation, we presented a forward genetic screen used to isolate parasites that fail to induce pyroptosis in rat macrophages and are able to replicate. Infection with these parasites resulted in significantly less active IL-1β release. We identified three novel dense granule proteins, GRA18, GRA27 and GRA28 as individually required for NLRP1 inflammasome activation *in vitro*.
Discussion and Future Directions

The success of *Toxoplasma gondii* relies on its remarkable ability to reach a delicate balance between immune activation and immune evasion. It must be sensed by the immune system, where host mechanisms prevent excessive parasite replication, which could result in host death before transmissible tissue cysts are formed. The parasite actively induces a strong immune response. However, if the host mounts too strong of a response, infection will be completely cleared. To prevent this unfavorable outcome, *Toxoplasma* has also evolved to evade these host immune mechanisms. It is unlikely that *Toxoplasma* can establish an optimal balance between immune activation and immune evasion in each of the different hosts it is capable of infecting, resulting in differences in host susceptibility to toxoplasmosis. This balance is demonstrated by inflammasome activation in mice. NLRP1 and NLRP3 activation by *Toxoplasma* leads to the activation of IL-18, which can lead to the production of IFN-γ. Induction of this cytokine and its downstream mechanisms leads to the control of the parasite and its conversion into the semi-dormant bradyzoite in muscle tissues. In contrast, activation of the Lewis inflammasome by *Toxoplasma* in rat macrophages leads to host cell death, preventing replication and resulting in the complete clearance of the parasite.

*Toxoplasma* is capable of infecting all warm-blooded animals and must be equipped to modulate the immune response of each host. As the parasite evolved, host immune pathways evolved as well. Vertebrates often differ in their TLR and IRG repertoire. For example, while TLR11 and TLR12 play an important role in the sensing of *Toxoplasma* in mice, humans express neither, leading to the need for additional mechanisms for parasite control. Additionally, the IRG system utilized by many rodents to control *Toxoplasma* infection is not present in humans. Substantial differences in host defense mechanisms have also been demonstrated. IRG proteins
are highly polymorphic between strains of mice, determining their susceptibility to *Toxoplasma* (Lilue et al. 2013). Our work demonstrates the difference between host mechanisms as *Toxoplasma* infection in Lewis BMDMs leads to rapid cell death, while infection of Brown Norway, Sprague-Dawley or murine BMDMs does not.

**How are the inflammasomes activated in the murine model?**

We found a role for primarily NLRP3 and to a lesser extent, NLRP1 in the resistance of mice to *Toxoplasma*. The mechanism of activation of NLRP3 is controversial. NLRP3 activation requires a priming step, which upregulates the expression of both NLRP3 and pro-IL-1β (Bauernfeind et al. 2009). NLRP3 inflammasome activation can occur in response to both external and endogenous stimuli. Extracellular ATP activates NLRP3 through the activation of the purinergic receptor, P2X7R, leading to potassium efflux (Kahlenberg and Dubyak 2004; Surprenant et al. 1996). Interestingly, *Toxoplasma* relies on potassium and calcium alterations as a signal for parasite egress.

Regulation of NLRP3 includes the guanylate-binding protein 5 (GBP5) (Shenoy et al. 2012), which is recruited to the PVM and plays a role in parasite elimination (Winter et al. 2011). The mechanism through which GBP5 regulates NLRP3 activation is unclear although several models have been proposed. GBPs may mediate in the lysis of the PV and allow for the release of PAMPs, which are recognized by NLRP3 (Meunier et al. 2014). Another model suggests that GBP5 can directly interact with NLRP3 and directly activates the inflammasome (Shenoy et al. 2012). Recently, a third model has been proposed where PAMPs induce GBP5 oligomerization and this aids in NLRP3 oligomerization (Finethy et al. 2015).
Cellular stress, including changes in cell volume (Compan et al. 2012), an unfolded protein response (UPR) (Menu et al. 2012; Kim et al. 2014), and reactive oxygen species (ROS) (Heid et al. 2013) are able to activate NLRP3. As *Toxoplasma* extensively modifies the host cell, it is possible the parasite indirectly activates the inflammasome by inducing cellular damage. Some strains of *Toxoplasma* have been found to recruit the host mitochondria to the PV (Jones and Hirsch 1972; Pernas et al. 2014). Perhaps the process of sequestering host mitochondria damages the organelle, resulting in the release of ROS. Additionally, infection of mouse macrophages results in respiratory burst (Wilson, Tsai, and Remington 1980).

We cannot rule out the possibility that *Toxoplasma* directly activates NLRP3 or differentially activates NLRP3. Although the strains tested were able to induce active IL-1β secretion from LPS-primed mouse BMDMs with no significant differences (*Chapter 2, Figure 1H*), we did not perform a comprehensive survey of *Toxoplasma* strains. It is possible that testing several more strains may allow us to identify a strain unable to activate the NLRP3 inflammasome. By performing a sexual cross between this strain with a strain that can activate NLRP3, we can isolate a number of progeny that differ in phenotype. We can then conduct a quantitative trait locus analysis and determine the genomic regions that correlate with the phenotype.

**How do GRA18, GRA27 and GRA28 activate the rat NLRP1 inflammasome?**

Our genetic studies have demonstrated that GRA18, GRA27 and GRA28 are required for NLRP1 activation *in vitro*. It is now of interest to determine how GRA18, GRA27 and GRA28 interact with each other, whether in a complex or within a pathway, and how they are able to activate NLRP1. GRA18, GRA27 and GRA28 each have predicted transmembrane domains. We
We hypothesize that these proteins reside within the parasitophorous vacuole membrane, facing into the host cytoplasm.

We are interested in determining whether expression of one or more of these genes is sufficient to activate NLRP1. Two approaches could be used to test this. The proteins can be recombinantly expressed and purified from *E. coli* and transfected individually or in combination into Lewis BMDMs using a liposomal transfection reagent, DOTAP, which will delivery the proteins into the host cytosol. This method has successfully been used to identify flagellin as an activator of NLR4 in mouse macrophages (Franchi et al. 2006). There are several potential limitations of this method. Purified proteins may contain bacterial components that themselves can activate immune pathways, including the inflammasomes, in rat macrophages. Transfecting a purified GRA that is unrelated to GRA18, GRA27 or GRA28 can serve as a control for possible contamination. Additionally, this method may not be feasible if the proteins require post-translational modifications for NLRP1 activation. A more reasonable method is ectopically expressing the protein(s) using an inducible system. However, this approach may not work if GRA18, GRA27 or GRA28 require processing by other *Toxoplasma* proteins for proper function. Attempts at generating a Lewis macrophage cell line that behaves like primary macrophages has not been fruitful. We have created several independent immortalized macrophage cell lines using J2 virus, which uses v-raf and v-myc to induce cell proliferation (Blasi et al. 1989). Immortalized macrophages did not undergo pyroptosis when infected with *Toxoplasma* and allowed for parasite replication. Additionally, these cells do not secrete IL-1β when primed with LPS and infected or treated with the NLRP3 agonist, nigericin. We found *Nlrp1*, *Casp1* and *Casp11* expression was reduced dramatically in these cell lines compared to BMDMs as
measured by qPCR, which may explain why the inflammasomes are not properly activated in these cells.

As inflammasome sensors are expressed in the host cytoplasm, it is most likely a parasite protein that is trafficked out of the parasitophorous vacuole that is recognized by NLRP1. We did not detect export of GRA18, GRA27 or GRA28 into the host cytosol as measured by immunofluorescent microscopy. To determine the precise location of these proteins, we plan to perform cell fractionation studies of infected cells using ultracentrifugation and determine if these proteins are associated with the PVM (Neudeck et al. 2002; Sibley et al. 1995). While this method has been successful for identifying the location of proteins within the PVM, a more precise and sensitive method utilizes immunoelectron microscopy. This approach will allow us to study the subcellular localization of the proteins in situ.

As GRA18, GRA27 and GRA28 potentially interact with several other parasite proteins and host proteins, it will be interesting to determine these unknown interactors. We have performed immunoprecipitations of GRA27 and GRA28 from infected HFFs, but have not been able to IP GRA18. Although immunoprecipitations using whole infected cells allowed for the pull down of nearly all of GRA27 and GRA28, this experimental setting may yield misleading results if submitted for mass spectrometry. As dense granule proteins are continually translated during replication within the cell, a fraction of our proteins of interest (POIs) that we isolate are located within the dense granule organelle. Within this organelle are a wealth of GRAs, which do not normally interact with our POIs when secreted from the parasite. By performing immunoprecipitations of POIs remaining in the dense granule, we may detect other GRAs that are not biologically relevant. Therefore, it is worth performing a step prior to immunoprecipitation to remove intact secretory organelles. Another method involves expressing
GRA18, GRA27 or GRA28 recombinantly and immobilizing these proteins onto a matrix. We can isolate interacting proteins by allowing whole host cell or parasite lysates to incubate with POIs and identify the proteins via mass spectrometry.

As it is unlikely that GRA18, GRA27 or GRA28 are directly recognized by NLRP1, it is still of interest to identify the direct activator. Using the mutagenesis screen we described in Chapter 4, we isolated seven independent clones. We identified the causative mutations in four of these isolates. The three remaining clones have been sequenced and do not have mutations in Gra18, Gra27 or Gra28 nor do they share mutated genes with each other (Chapter 4, Table 1). By focusing on genes with predicted signal peptides, we can narrow this list to four candidate genes, TgGT1_309160 (Mutant #5), TgGT1_203230 (Mutant #6), TgGT1_408760 (Mutant #7) and TgGT1_410880 (Mutant #7). Using CRISPR/Cas9, we can knock these genes out with relative ease. One or more of these genes may not play a role in activation of the Lewis inflammasome. It is possible the causative mutations in these clones are within genes that encode for proteins that do not contain predicted signal peptides. We can then expand our candidate list to include all genes expressed by all Toxoplasma strains. We potentially can identify three more Toxoplasma genes required for NLRP1 activation. By expanding this set of proteins, we are more likely to find the activator or elucidate the pathway leading to the recognition of the activator.

Although we have focused on genetic approaches to identify the NLRP1 activator, a biochemical approach may provide a more direct method. BioID utilizes a promiscuous biotin ligase, BirA*. When fused to a POI, BirA nonspecifically biotinylates proteins within close proximity (Roux, Kim, and Burke 2013; Roux et al. 2012). Modified proteins can be isolated using streptavidin-coupled beads. A major advantage of this approach is this method will allow
for the identification of transient interactors. Additionally, BirA*-fused proteins can be transfected in a cell line, such as HEK293 cells, to generate a stable cell line. These cells will not undergo pyroptosis as human cell lines engineered to express NLRP1 and caspase-1 do not die when infected with *Toxoplasma* (Chapter 3, Figure S8). We have fused BirA* to Lewis NLRP1, but have failed to construct a stable HEK293 line expressing this protein. Once a cell line expressing the construct is cloned, cells can be infected with *Toxoplasma* and isolated modified proteins will be submitted for mass spectrometry. As we found no difference in IL-1β secretion between murine macrophages infected with wild-type and strains deficient in GRA18, GRA27 or GRA28, this method may also be used to identify parasite proteins that may interact with murine NLRP1.

**Why are GRA18, GRA27 and GRA28 conserved among strains?**

A survey of many *Toxoplasma* strains that represent worldwide diversity demonstrated that all tested strains of the parasite were able to induce pyroptosis in Lewis BMDMs (Chapter 3, Figure 3). This finding demonstrates a well-conserved phenomenon. As induction of pyroptosis leads to the control of parasite replication, it is likely the Lewis rat and other rat strains that are resistant to *Toxoplasma* evolved to recognize features of the parasite that are widely conserved. In addition, a close relative of *Toxoplasma, Neospora* is able to induce pyroptosis in the same cells, suggesting that some rats have adapted to identify conserved parasite features (Chapter 3 – Addendum, Figure 1). As GRA18, GRA27 and GRA28 share homology to proteins in *Hammondia hammondi*, it will be interesting to test the ability of this species to induce pyroptosis in rats.
New parasite strains are a result of sexual recombination of parental strains in felines. As the parasite can infect a wealth of intermediate hosts with different host mechanisms, selection of a strain is heavily determined by the host immune system. Polymorphisms in parasite effectors have been well documented to determine virulence in laboratory mice. Conservation of GRA18, GRA27 and GRA28 suggests a selective pressure on these proteins and indicate that they may play an important role in the success and transmission of the parasite. The genes are not essential in tachyzoites as we were able to generate strains deficient in each gene. A preliminary experiment in susceptible rats suggests these genes may play a role in establishment of a chronic infection, but future experiments must be conducted in both mouse and rats.

We performed preliminary RNAseq experiments on Brown Norway rat macrophages infected with strains deficient in GRA18, GRA27 or GRA28 and their complemented control strains. Expression of several type I interferon-stimulated genes, including Ifi27, Isg12(b) and Glp2 were reduced in cells infected with strains deficient in GRA27 or GRA28 compared to control strains (Chapter 4, Table 3). While type I IFN is an established inducer of these genes, only two Toxoplasma strains, BOF and COUGAR, have been identified to induce type I IFN (Melo et al. 2013). IFN-independent pathways can induce interferon-stimulated genes. (Noyce et al. 2011). Toxoplasma may activate one of these alternative pathways. In addition to the type I IFN-inducible genes, we found genes involved in leukocyte chemotaxis significantly enriched (p-value = 1.2 x 10^{-6}). These genes included Cxcl1, Cxcl2, Cxcl10 and Cxcl11. This suggests GRA27 and GRA28 play a role in activating an immune response other than the inflammasomes.
Why are rats so resistant to *Toxoplasma*?

While we have focused on the extraordinary resistance of the Lewis rat to *Toxoplasma*, we are also interested in determining why all rat strains are resistant to the parasite. Through the use of neutralizing antibodies, IFN-γ was determined to be important in the control of the parasite in susceptible rats (Sergent et al. 2005). We have performed *in vitro* experiments testing the role of IFN-γ in control of the parasite in nonimmune cells. Preliminary experiments confirmed that IFN-γ plays a role, as the parasite formed fewer and smaller plaques in rat fibroblast monolayers prestimulated with IFN-γ than those untreated.

Polymorphisms exist in IRG proteins between mice strains. The most studied laboratory mouse is relatively susceptible, with a single type I parasite able to kill a mouse within a week. This virulence is determined by the ability of parasite effectors, ROP18 and ROP5 to counter the host IRG system. The IRG system in wild mice is polymorphic and in some strains, not susceptible to the actions of ROP18 and ROP5, allowing these strains to survive infection with *Toxoplasma* (Lilue et al. 2013). Rat IRG proteins are similar to the IRGs in these resistant wild mice, suggesting that the IRGs may play a major role in rat resistance. We are generating rat fibroblasts that do not express IRGM, an important regulator of IRG oligomerization on the PVM, and ATG5, an ubiquitin ligase necessary for autophagy and important for IRG formation (Zhao et al. 2008; Ohshima et al. 2014). Using these cell lines, we plan to determine if the IRGs play a role in the control of *Toxoplasma* in rat non-immune cells.
References


