EFFECTS OF TOXOPLASMA GONDII ON HOST CELL SIGNALING: FOCUS ON CHROMATIN REMODELING

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Jin Leng

August 2009

EFFECTS OF TOXOPLASMA GONDII ON HOST CELL SIGNALING: FOCUS ON CHROMATIN REMODELING

Jin Leng, Ph. D.

Cornell University 2009

Toxoplasma gondii is an important opportunistic protozoan parasite. Macrophages infected with *Toxoplasma gondii* cannot upregulate many pro-inflammatory cytokines, including tumor necrosis factor (TNF-α), upon stimulation with LPS and other Toll-like receptor (TLR) ligands. However, LPS induction of IL-10 is not affected by the parasite. Here, we employed chromatin immunoprecipitation (ChIP) to examine TNF-α promoter activity of infected macrophages. During LPS stimulation, *Toxoplasma* blocked recruitment of RNA polymerase II to the TNF-α promoter. In addition, binding of transcription factors NFκB, CREB and c-Jun was inhibited by infection. Analysis of chromatin structure surrounding the TNF promoter showed that LPS-induced Ser^{10} phosphorylation and $Lys^{9/14}$ acetylation of histone H3 (modifications associated with increased transcriptional activity) were inhibited by *Toxoplasma*. Immunofluorescence assays revealed that global Ser^{10} histone H3 phosphorylation levels were also blocked by *T. gondii.* Low level IL-10 gene induction by LPS that was not inhibited by the parasite did not require chromatin remodeling. However, high level IL-10 production induced by the combination of LPS and immune-complex (IC) stimulation was associated with histone H3 covalent modification. Remodeling of chromatin at the IL-10 promoter induced by LPS plus IC was also inhibited by *Toxoplasma*. Our results argue that *Toxoplasma* targets chromatin remodeling to inhibit cytokine gene transcription, and as such the data reveal a new mechanism by

which an intracellular pathogen incapacitates cytokine production during infection. We also performed further studies and obtained evidence that both MyD88 dependent and MyD88 independent TLR signaling were inhibited by the parasite. Strain comparison studies also showed that Type I parasite strains have the most potent inhibitory effects in interfering with host signaling.

BIOGRAPHICAL SKETCH

Jin Leng was born in a small town of Hubei province, China in 1979. He became interested in biomedical sciences, especially immunology when he saw people dying from severe infectious diseases like rabies. He then chose biochemistry as his undergraduate major when he was admitted into Nankai University, China in 1997. He obtained his bachelor's degree of Biochemistry in 2001. After that, he was granted a scholarship for studying for his master's degree of Biochemistry at the Institute for Molecular Biology of Nankai University until 2004. During this period, he took part in the preliminary development of a protein vaccine against Epstein Barr virus.

After spent seven years at Naikai, Jin decided to pursue his PhD degree in the United States. In 2004 fall, he was admitted to the department of microbiology and immunology at Cornell University. In the summer of 2005, he joined Dr. Eric Y Denkers' lab. His research was initially related with the influence of *Toxoplasma gondii* infection on different Toll like receptor signaling pathways. After passing the exam for admission to candidacy for PhD, he focused his research on the mechanism of TNF inhibition by *Toxoplasma* and stepped into the study of parasite interference on host cell epigenetics.

Jin was awarded a Genomics Scholarship from Cornell University Center for Vertebrate Genomics in 2007 for his ground breaking work in how pathogens can target the host cell's ability to remodel chromatin during gene induction.

Jin married with Tianyi Wang in the summer of 2005 and they had a baby girl named Jasmine born in Ithaca in June 2007.

ACKNOWLEDGMENTS

I sincerely thank my thesis advisor: Dr. Eric Y Denkers, who is the best advisor I could ever have. During the four years in his laboratory, he has always provided me with generous support and enlightening suggestions in every aspect of my research. I benefited a lot from his scientific insights, his creative ideas of experimental design, his academic writing and presentations. Even during the time when I was frustrated by disappointing results, he still encouraged me and helped me in trouble shooting. This thesis would not be possible without his advisory.

I appreciate all past and current members in Denkers' lab. Dr. Barbara A Butcher taught me various experiment techniques from the very first day of my joining the lab. She always provided valuable suggestions during my lab meeting presentations. She also contributed to my first paper. Dr. Leesun Kim also taught me some experiment skills and was always ready to offer help when I got questions during my research. Dr. Charlotte E Egan was a great person to work with and contributed to my paper. I also want to thank my fellow graduate students: Alison Bierly, Woraporn Sukhumavasi, Anne Gordon and Delbert Abdallah for their help and suggestions for the past four years. I enjoyed all those discussions upon our research. I also appreciate all the technical support provided by Mozammal Hossain.

I thank Dr. Theodore G Clark and Dr. Cynthia A Leifer as my special committee members. In the past four years, they had always provided me with valuable suggestions about my research and my future career plans.

I want to specially thank Dr. David M Mosser, who kindly agreed to become my

external examiner for my thesis defense and came all the way from Maryland to Ithaca. I also got help from the experts in his lab about the study of immune complex.

Finally, I would like to thank my wife, Tianyi Wang, whose love and support accompanied me and gave me faith through the hard times; my parents in law, Mr. Yanquan Wang and Mrs. Qingmei Sun, who always treated me like their own son; my parents for continuous love and encouragement, and last but not the least my daughter Jasmine as a gift of life.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

Introduction

1. Life cycle of *Toxoplasma gondii*

Toxoplasma gondii is an intracellular apicomplexan protozoan parasite. It belongs to the phylum Apicomplexa and is the only species in genus *Toxoplasma*. It was originally identified in the small North African rodent *Ctenodactylus gundi*, hence its name (1). *T. gondii* is one of the most successful parasites with an approximate infection rate of 20-50% in the human population around the world. High infection has been related to a preference in consuming raw or undercooked meat and a climate favoring the survival of oocysts in certain countries (2).

Members of the cat family (Felidae) are the only known definitive hosts for the sexual stages of *T. gondii*. Cats become infected with *T. gondii* by ingesting tissue cysts or oocysts from eating rats or mice. The parasites are then released from the cysts and invade epithelial cells of the small intestine where they undergo an asexual followed by a sexual cycle and form oocysts. Oocysts are excreted with cat feces. The unsporulated oocysts take 1 to 5 days after excretion to sporulate and become infective. Oocysts are resistant to disinfectants, freezing temperatures and drying conditions but are killed at $70^{\circ C}$ for 10 minutes (3).

Human and animal infections are naturally acquired by ingestion of undercooked meat containing *Toxoplasma* cysts or ingestion of the oocysts from fecally contaminated food (Figure 1.1). Organ transplantation or blood transfusion in humans also transmits the infection in certain conditions. Women who become infected during pregnancy can also transmit the parasite to the fetus. Within a normal immunocompetent host, replication of the parasites is suppressed and the parasites form tissue cysts, most commonly in skeletal muscle, myocardium, and central nerve system and normally remain quiescent throughout the life of the host (2).

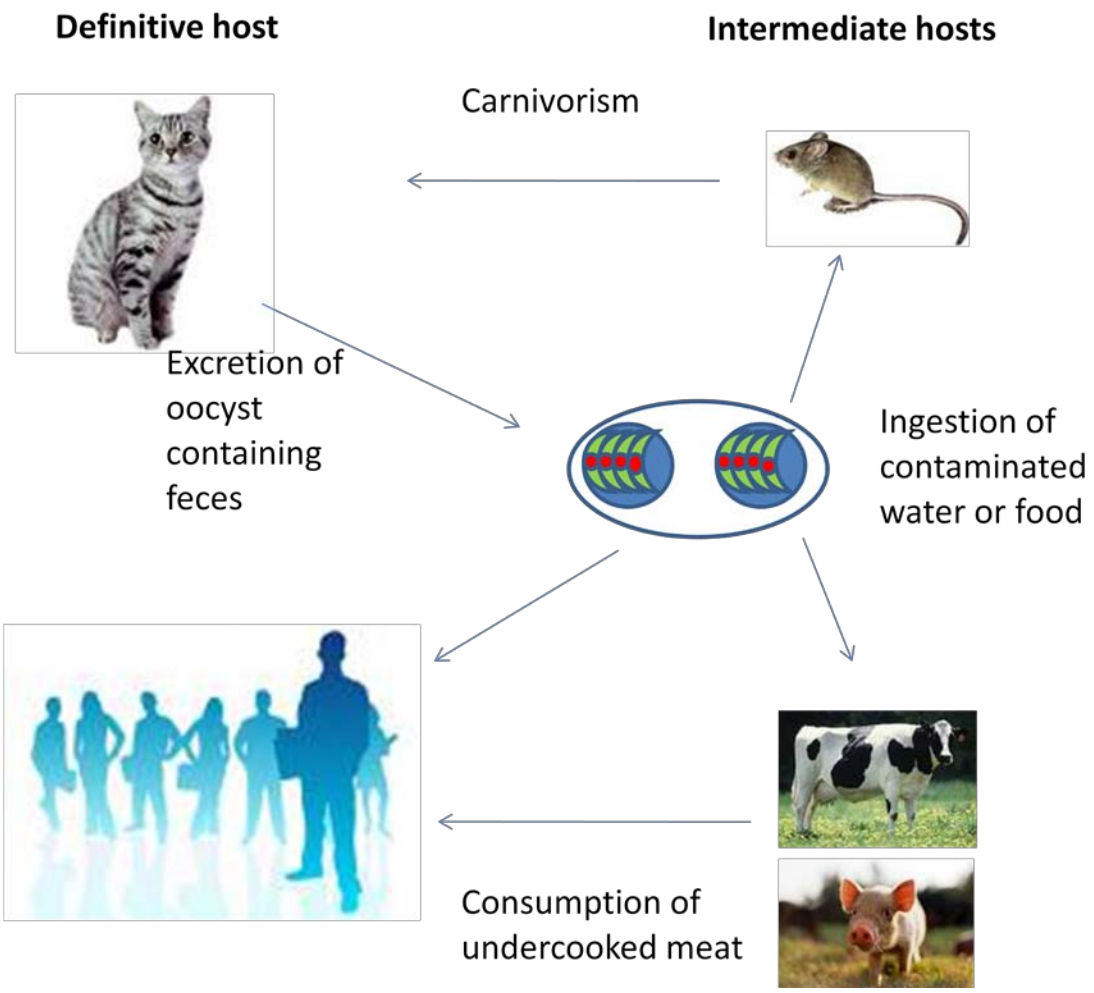


Figure 1.1 Natural ways of Toxoplasma gondii infection. Cats are the only definitive host for the sexual stage of T. gondii. Wild rodents, including mice, as a major reservoir of infection, transmit the infection to cats during carnivorism. Oocysts are shed in the cat's feces, allowing chances for infection of other species including human beings through accidental ingestion of oocyst contaminated water or food. Humans can also become infected by consuming undercooked meat containing parasite cysts.

2. Public health and *T. gondii* infection

Infection by *Toxoplasma* is comprised of acute stages and chronic stages. The acute infection often incurs flu-like symptoms which fade within a few days to months, leading to the chronic stage when the replication of parasites is suppressed by the host immune system. During latent infection, the parasite forms tissue cysts found in skeletal muscle and central nervous system and thus remains quiescent (2).

However, within immunosuppressed hosts, such as AIDS patients and organ transplant patients on immunosuppressive therapy, *Toxoplasma* is a dangerous opportunistic pathogen (4, 5). Reactivation of a persisting infection can result in life-threatening toxoplasmosis with encephalitis, necrotic lesions within the central nervous system (CNS) or retinochorioiditis. In the United States alone, between 10% and 40% of HIV-infected patients are estimated to test positive for antibodies against *T. gondii* and some studies suggested that 24-47% of *T gondii*-seropositive AIDS patients eventually developed toxoplasmic encephalitis (6, 7).

When a woman with no previous exposure to *T. gondii* becomes infected during pregnancy, the parasite can cross the placenta and infect the fetus. The premature immune system of the fetus cannot restrict the replication of the parasite within different fetal tissues, thus leading to hydrocephalus or microcephaly, intracranial calcification and chorioretinitis, with the possibility of spontaneous abortion or intrauterine death (8, 9).

During the acute stage of infection, medications include antibiotic treatment with pyrimethamine, sulfadiazine, clindamycin and spiramycin. Pyrimethamine and sulfadiazine are used in combination to interfere with folic acid synthesis needed for

DNA and RNA synthesis in many species, including protozoan parasites. Clindamycin and spiramycin are used to interfere with protein synthesis of the parasite. In people with latent *toxoplasmosis*, the cysts become immune to these treatments. Medications that are prescribed include atovaquone and clindamycin for killing the cysts in AIDS patients. However, clearance of latent infection is very difficult and not guaranteed, as the antibiotics are hard to reach the bradyzoites in sufficient concentration (10).

3. Biology of *T. gondii*

T. gondii is a unicellular parasite that infects virtually any nucleated cell (11). The parasite structure is shown in Figure 1.2. During the cell invasion, the parasite first uses microneme proteins (MICs) to attach to the host through the binding of specific receptors (12, 13). Rhoptries, which are composed of a rhoptry neck and a rhoptry bulb, are then discharged. Rhoptries contain numerous enzymes that are released during the invasion process. A moving junction (MJ) is then assembled by the rhoptry neck proteins and microneme proteins to facilitate the movement of parasite, which helps the formation of the parasitophorous vacuole (PV) (14, 15). PV formation includes glycosylphosphatidylinositol (GPI)-anchored proteins, while excluding other host membrane proteins (14). Therefore, the PV is resistant to acidification and is non-fusigenic with host lysosome (16, 17). Dense granule proteins are discharged after PV formation and form the intravacuolar network (IVN) of tubular membranes inside the PV (18, 19). Within the protective environment of PV, the parasite obtains nutrients by forming pores in the PVM that permit diffusion of small molecules (20). The parasite multiplies within the PV asexually by a specialized form of binary fission called endodyogeny and eventually lyses the host cell after 3 to 5 replication cycles of 6 to 8 hours each (21, 22). The escape from the parasitophorous vacuole and host cell membrane requires both calcium and protease activity (23-26). More recently, egress

has been found to involve host cell calpain proteases where disruption of calpain activity by siRNA or genetic deletion prevented the egress of *T. gondii* from mammalian fibroblasts and genetic complementation restored the parasite egress (27).

The natural populations of *Toxoplasma* in Europe and North America have been divided into three types by restriction fragment length polymorphism (RFLP) (28). They are found to have approximately 99% genetic identity. Only two separate alleles exist at any given locus of the three strains. This suggests that they are progenitors of a single cross between two parental strains (29). All three types are found in both humans and animals and they differ in virulence. Type I strains are the most virulent with a LD100 of a single parasite in the mouse model, while Type II and Type III strains are less virulent with LD100 of more than 1000 parasites. Therefore, only Type II and Type III strains establish stable chronic infection in mouse models and Type II strains are found to be the most common in human toxoplasmosis and chronically infected animals (30). Studies investigating virulence and pathogenicity showed that infection by Type I strains in mice results in more rapid dissemination of parasites throughout the body and higher tissue burden compared with other strains (31). The higher mobility and ability to penetrate across the intestinal epithelium of Type I strains may contribute to their high virulence (32).

During infection, rhoptry proteins have been found to be important parasite effectors for manipulating host signaling pathways. ROP18 contains a kinase domain and displays a divergent expression profile between parasite strains (33). ROP18 lacks expression in Type III strains probably because an extra 2.1kb sequence exists near the start codon in Type III strains (34). Studies found that when ROP18 from a Type I strain is expressed in a Type III strain, the parasite gains a much higher intracellular

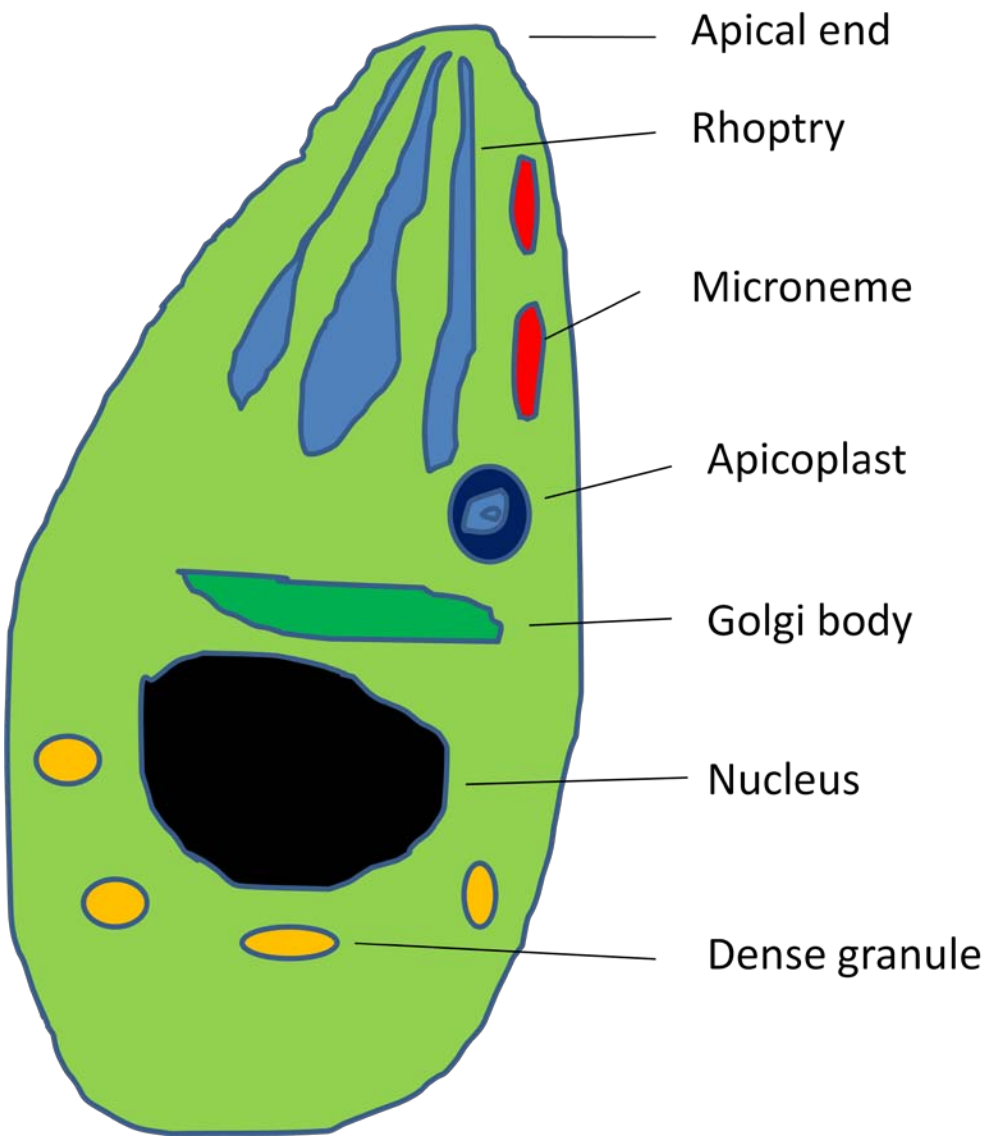


Figure 1.2 Structure of Toxoplasma gondii. Toxoplasma contains three secretory organelles: micronemes, rhoptries and dense granules. During cell attachment and invasion, micronemes are first discharged followed by rhoptries. Dense granules are released after parasite invades the cell.

replication rate and a higher virulence causing rapid death of infected mice (35). In natural isolates, ROP18 expression level directly correlates with virulence and intracelluar growth rate. However, a host protein substrate has not yet been identified while a 70-kDa protein from a tachyzoite lysate is found to be phosphorylated by ROP18 (33). This suggests that ROP18 acts on the parasite itself.

Another rhoptry protein, ROP16, also has a kinase domain but is found to be more related with modulation of host signaling. The rapid and sustained STAT3 tyrosine phosphorylation triggered by *T. gondii* infection requires ROP16 even though ROP16 seems to act indirectly because it is a putative serine/threonine kinase (36). ROP16 contains a nuclear localization signal and accumulates within the host nucleus soon after infection, suggesting that it is acting on some yet to be indentified host molecule. Type II parasites lack ROP16 and this likely contributes to low virulence of Type II since STAT3 phosphorylation is not sustained upon infection, and a high level of IL-12 production is induced, which helps maintain the survival of both host and Type II parasite (34, 37).

4. *Toxoplasma* and host immunity

The major cell type studied in my thesis are macrophages because they are readily infected by *Toxoplasma* and used by the parasite as reservoirs of infection and early dissemination. Macrophages are an important component of the innate immune system and play a key role in defense against infection. These cells are poised to respond to microbial antigens and possibly host 'danger' signals that sense presence of infection. Macrophages are located throughout tissues and their precursors circulate in the blood as undifferentiated monocytes. They probably participate in first encounter with invading microbial pathogens, yet they also serve as targets of infection for many

intracellular microorganisms (38, 39). Therefore, complex biological relationships probably occur between macrophages and intracellular pathogens that reside within these cells.

Macrophages are armed with multiple effector functions. They are potent phagocytes that may rapidly degrade the microorganisms. Nitric oxide production is another macrophage antimicrobial defence mechanism. Cells can upregulate the enzyme inducible nitric oxide synthase (iNOS) leading to high level production of nitric oxide. This short-lived, but highly reactive, molecule interferes with multiple metabolic pathways required for microbial survival. Similar to nitric oxide production, macrophages can undergo an oxygen-dependent respiratory burst during infection, which kills microorganisms through generation of toxic intermediates such as superoxide and hypochlorate. Microbial products, especially bacterial endotoxin such as LPS, can activate macrophages and elicit high level antimicrobial activity and cytokine production (40, 41).

Macrophages are also important in bridging innate and acquired immunity. They are capable of producing high amounts of IL-12 and presenting antigenic peptides with MHC and co-stimulatory molecules such as CD80/CD86. The polarizing signal (IL-12), antigen triggering, and co-stimulation comprise three signals necessary to drive Th1-type acquired immunity (42). Thus, macrophages may also be important in triggering the Th1 response that is crucial to control microbial infection.

Upon infection, *T. gondii* elicits an early IL-12 response from innate immune cells such as DC, macrophages and neutrophils. This IL-12 response is critical in the control of infection in that it promotes activation of Th1 T cells, NK cells and CD8 T

cells which produce IFNγ. IFNγ is the major effector molecule in limiting infection during both acute and chronic stages. It mediates its protective function through STAT1, which generates nitric oxide, and induces IGTP and LRG-47 to kill the intracellular parasite (Figure 1.3) (43, 44). It has been shown that absence of IL-12, IFNγ or T cells leads to rapid death during *Toxoplasma* infection in mouse models (45-47). Mice deficient in the iNOS gene are more susceptible to *T. gondii* infection (47). The IFNγ responsive genes, such as IGTP and LRG-47 are also essential in surviving acute infection as both IGTP and LRG-47 knockout (KO) mice succumb with similar kinetics as IFNγ and IL-12 deficient mice (48, 49).

In order to establish long term infection, *Toxoplasma* needs to avoid elimination from the host while eliciting an immune response to keep the host alive. Also, an immune response too vigorous can lead to lethal immunopathology. Overproduction of cytokines such as IL-12, TNFα and IFNγ leads to the death in IL-10 KO mice during non-lethal infection (50-52). C57BL/6 mice are susceptible to oral infection largely due to severe gut pathology mediated by CD4+ T cells and IFNγ (53). In this regard, in vitro studies showed that *Toxoplasma* actively suppresses proinflammatory responses during intracellular infection, a response that might enable self survival and replication and avoid immunopathologic damage to the host. Infected macrophages do not produce TNFα, and the IL-12 response is only induced after approximately 24 hrs. The cells also lose the ability to respond to LPS stimulation. Inhibition is specific to infected cells and requires active invasion by the parasite (54, 55).

Interference with mitogen-activated protein kinase (MAPK) activation, NFκB nuclear translocation, and activation of STAT3 have been implicated in the inhibition by the parasite. Specifically, *Toxoplasma* infected cells display defects in LPS-induced

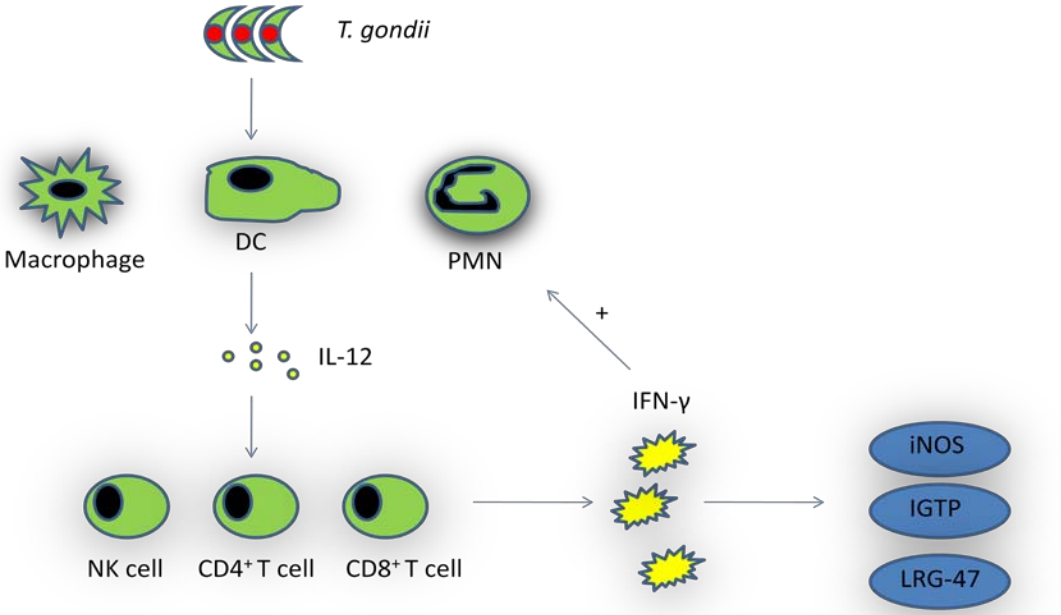


Figure 1.3 T. gondii and host immunity. Upon infection, the innate immune system first reacts to the parasite. Macrophages, DCs and PMNs produce IL-12, which activates NK cells, CD4+ T cells and CD8+ cells. These cells produce IFN-γ. IFN-γ mediates anti-Toxoplasma effects by inducing iNOS, IGTP and LRG-47. It also potentiates the innate immune system by activating the killing mechanisms of macrophages.

MAPK phosphorylation and NFκB nuclear translocation (56). Cell invasion by *Toxoplasma* also induces rapid and sustained activation of host STAT3. This mediates inhibition of IL-12 and TNFα as *Toxoplasma* is not able to suppress the cytokine production in the absence of STAT3 (57).

Studies comparing *T. gondii* Type I and Type II strains showed that there are strain differences in parasite elicited MAPK activation and IL-12 production. The Type I RH strain triggers MAPK activation and IL-12 production in a MyD88-dependent pathway, while induction of IL-12 by the Type II ME49 strain involves both MyD88-dependent and MyD88-independent pathways (58). IL-12 production by parasite infected macrophages is generally higher when the cells are infected with Type II strains (37).

During chronic infection, *Toxoplasma* preferentially forms cysts in the brain. A balance of host immune responses and the parasite's evasion of such responses must be established to maintain this chronic stage of infection. IFN-γ has been found to be essential to control the infection in the brain. A variety of cytokines are induced by infection from microglia, astrocytes, and neurons, which either promote or suppress inflammatory responses (59-61). A delicate interplay between the host and the parasite maintains the chronic infection. The parasite strain, host genotype and route of infection are all involved in this interplay.

Most of our studies examined the effects of parasite infection on macrophage signaling after overnight infection followed by LPS stimulation. This is biologically relevant because natural infection leads to damage of small intestine and leakage of bacterial products such as LPS. Macrophages that are recruited by the infection are

then encountering stimulants coming from bacteria. Therefore, our studies mimic the natural in vivo situation upon infection.

5. Toll-like receptors and *Toxoplasma*

Toll-like receptors (TLR) play an essential role in sensing and responding to pathogens by the innate immune system (62, 63). Signalling through TLR leads to induction of proinflammatory cytokines and chemokines, as well as anti-microbial molecules such as nitric oxide generated by inducible nitric oxide synthase. To date, 11–13 TLR molecules have been identified in mammals. Most are expressed on the cell surface, but some are expressed in intracellular compartments (TLR3, 7, 8 and 9) (64). One of the best characterized is TLR4, which, along with MD2, serves as a receptor for Gram-negative bacterial LPS. With the exception of TLR3, activation through these receptors requires recruitment of adaptor molecule MyD88, in turn resulting in recruitment of IL-1 receptor-associated kinases 1 and 4. These molecules form a complex with TNF receptor-associated factor (TRAF) 6, resulting in interaction with Uva1 and Ubc13, leading in turn to ubiquitination of TRAF6. Then, ubiquitinated TRAF6 activates transforming growth factor-β-activated kinase (TAK) 1. Interacting with TAK1-binding proteins 1 and 2, TAK1 serves as a mitogen-activated kinase kinase kinase, triggering the MAPK cascade. The TAK1 molecule also activates the IκB kinase complex. This results in phosphorylation-dependent ubiquitination and degradation of IκBα, enabling nuclear translocation of NFκB (63).

Studies showed that both NFκB and MAPK pathways downstream of TLR signaling are inhibited by *T. gondii* infection even though the parasite increases TLR4 expression. During early infection of macrophages, the parasite is found to block nuclear translocation of NFκB in response to LPS despite the fact that IκBα undergoes

normal degradation (54, 56, 65). When the cells are stimulated with LPS 6 hr or more after infection, nuclear translocation of NFκB is restored, indicating that the inhibitory effect on NFκB is transient (56). Infected cells also display impaired MAPK activation triggered by LPS, particularly p38 MAPK, which is required for LPS-triggered TNFα and IL-12 production. This is not due to defective upstream signaling as the kinase for p38, MAPK kinase 3/6, is activated and remains so upon infection (56). Nonresponsiveness of infected macrophages to LPS restimulation resembles LPS tolerance in some aspects. However, a study comparing the two processes found them to be distinct. Parasite infection induces sustained activation of the MAPK kinase MKK3/6. In contrast, during LPS stimulation of LPS-tolerized cells, phosphorylation of MKK3/6 is defective. Futhermore, IκBα is resistant to TLR4-induced degradation in LPS-tolerized macrophages, whereas in *T. gondii* infected cells this molecule undergoes normal degradation following LPS exposure (66). It is currently not clear whether *Toxoplasma* blocks activation of MAPK through the activity of host or parasite phosphatases, or by other phosphatase-independent mechanisms.

There is also evidence that type II *T. gondii* strains themselves induce NFκB activation, and as mentioned previously, IL-12 production induced by the type II ME49 strain is partially dependent upon MyD88 (37, 67). *Toxoplasma* itself also mediates a rapid but transient activation of MAPK pathways including SAPK/JNK, p38 and ERK1/2 during macrophage infection and p38 and SAPK/JNK MAPK activation contributes to parasite induced IL-12 production (68, 69). However, as mentioned above, subsequent stimulation with LPS fails to result in robust activation normally associated with TLR stimulation.

Toxoplasma tachyzoite glycosylphosphatidylinositol lipid anchors activate TLR2 and TLR4. Also, *T. gondii* profilin induces high level TLR11-dependent IL-12 production in mouse DCs, although this TLR molecule is not expressed in humans (70).

Studies performed on mice deficient in TLR signaling pathways revealed their importance in survival of *T. gondii* infection. MyD88$^{-/-}$ mice succumb to infection while mice defective in production of IL-1 and IL-18 resist infection normally (71, 72). This indicates the unique importance of TLR signaling since IL-1 and IL-18 signaling also involves MyD88. TLR2$^{-/-}$ mice displayed increased susceptibility to high dose *Toxoplasma* infection (73). However, upon low-dose infection, knock out of either TLR-2 or TLR-4 had no effect in survival. Genetic deletion of both TLR2 and TLR4 did lead to an increase in cyst burden (74). This suggests that there is some functional redundancy between TLR2 and TLR4. Mice deficient in TLR11 had increased brain cyst numbers but still survived (75).

6. Histone modification and chromatin remodeling

A large part of my thesis focuses on histone modification in *Toxoplasma* infected cells. Inducible gene expression is now understood to involve two types of regulatory cascades. One type of transduction cascade leads to transcription factor activation, most often involving kinase signalling, to enable binding to target DNA sites on gene promoters. The particular pattern of transcription factors activated plays a role in determining the specificity of genes induced. At the same time, it is now appreciated that chromatin structure itself is subject to regulation, inasmuch as signalling leading to covalent modification of histones plays a role in determining the activity of transcription factors (Figure 1.4) (76, 77).

Histones undergo posttranslational modifications which alter their interaction with DNA and nuclear proteins. The H3 and H4 histones have long N-terminal tails protruding from the nucleosome which can be covalently modified at several places. Modifications of the tail include methylation, acetylation, phosphorylation, ubiquitination, sumoylation, citrullination, and ADP ribosylation. The core of the histones (H2A and H3) can also be modified. Combinations of modifications are thought to constitute a code, the so-called "histone code". Histone modifications act in diverse biological processes such as gene regulation, DNA repair and chromosome condensation (mitosis) (78).

DNA methylation and histone post-translational modifications lead to the recruitment of protein complexes that both positively and negatively regulate transcription. All histone post transcriptional modifications are reversible. Histone deacetylases (HDACs) remove acetyl groups and Ser/Thr phosphatases remove phosphate groups. Ubiquitin proteases remove mono-ubiquitin from H2B. Arginine methylation is altered by deiminases, which convert the side chain to citrulline . The functional consequences of histone post transcriptional modifications can be direct, causing structural changes to chromatin, or indirect, acting through the recruitment of effector proteins (79).

Acetylation of the lysine residues at the N terminus of histone proteins removes positive charges, thereby reducing the affinity between histones and DNA. This makes it easier for RNA polymerase and transcription factors to access the promoter region. Therefore, in most cases, histone acetylation enhances transcription while histone deacetylation represses transcription. Histone acetylation is catalyzed by histone acetyltransferases (HATs) and histone deacetylation is catalyzed by histone

deacetylases (denoted by HDs or HDACs). Several different forms of HATs and HDs have been identified. Among them, CBP/p300 is probably the most important, since it can interact with numerous transcription regulators (80, 81).

Phosphorylation of serine 10 in histone H3 has been shown to correlate with gene activation in mammalian cells and with the induction of transcription during heat-shock response in Drosophila. Quiescent fibroblasts treated with epidermal growth factor undergo rapid serine 10 phosphorylation, coincident with the induction of early response genes such as c-fos (82). This phosphorylation is catalyzed by MSK-1 and MSK-2, two kinases downstream of p38 and ERK MAPKs (83).

In mammalian cells, H3 phosphorylation is mediated via the ERK or p38 MAP kinase pathways depending on the stimulus used, but not the JNK/SAPK pathway (84, 85). It is known that inflammatory signals induce p38 mitogen-activated protein kinase−dependent phosphorylation and phosphoacetylation of histone H3, which selectively occurred on the promoters of a subset of stimulus-induced cytokine and chemokine genes. p38 activity was shown to be required to enhance the accessibility of the cryptic NFκB binding sites within H3 phosphorylated promoters (86-89). This indicates that p38-dependent H3 phosphorylation may mark promoters for increased NFκB recruitment. The regulation of histone H3 phosphorylation at Ser10 is therefore of special interest. Two kinases downstream of p38 and ERK MAPK, MSK-1 and MSK-2 have been suggested to directly phosphorylate histone H3 when the cell is treated with a mitogen signal or exposed to stresses (83, 90). More recently, histone H3 has been found to be a novel substrate for MAP Kinase Phosphatase-1 (MKP-1) which normally dephosphorylates MAP kinases (91, 92).

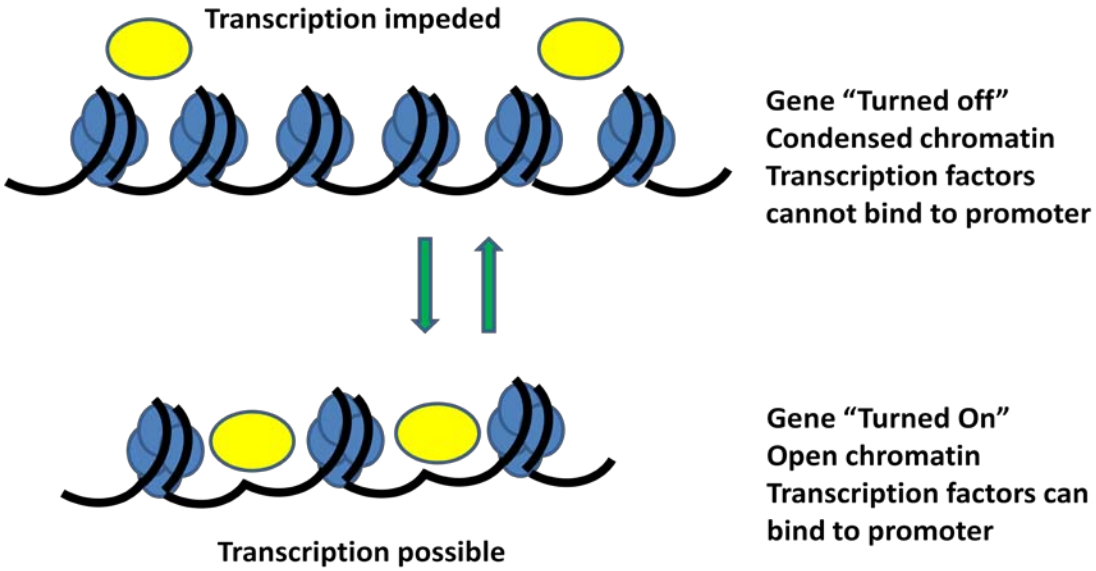


Figure 1.4 Chromatin structure and gene transcription. Under silent condition, the gene promoter region remains condensed, DNA remains methylated, and histone tails remain suppressively modified. Transcription factors can not bind to the promoter region and transcription is not initiated in this case. Upon activation signal, histone tails undergo modifications that opens up the chromatin structure. DNA becomes unmethylated. Transcriptional machinery binds to the promoter and starts gene transcription.

7. Brief outline of dissertation research

Toxoplasma infection inhibits LPS-triggered IL-12 and TNFα production in macrophages. After about 12 hr post infection, the block in IL-12 production is lifted and the parasite itself induces IL-12. However, TNFα production remains potently suppressed. The molecular mechanism of TNFα inhibition in macrophages is not yet understood. **Chapter 2** reports that *Toxoplasma* prevents the binding of transcription factors such as NFκB, c-Jun and CREB to the TNFα promoter region by interfering with histone modifications on both distal and proximal regions. Specifically, the parasite blocks the phosphorylation of histone H3 at Ser 10 and acetylation of histone H3 at Lys9/Lys14 triggered by LPS. These findings led to the study in **Chapter 3** where I found that *Toxoplasma* inhibits production of high level IL-10 induced by immune complex in the presence of LPS in macrophages through a similar mechansim where the histone modifications required for transcription initiation is blocked by the parasite. **Chapter 4** describes experiments showing that *Toxoplasma* is able to block different TLR ligand induced IL-12 responses in macrophages. Moreover, the parasite also inhibits type I interferon production triggered by MyD88-independent TLR-3 signaling. Parasite strain comparison studies show that only Type I RH strain inhibits TNFα in dendritic cells and RH strain most potently blocks LPS-induced phosphorylation of histone H3 at Serine 10 compared with Type II and Type III strains. Finally, **Chapter 5** summarizes the results in this thesis and discusses the significance of the above findings.

REFERENCES

1. Dubey, J. P. 2007. The history and life-cycle of *Toxoplasma gondii*. In *Toxoplasma gondii. The model apicomplexan: Perspective and methods*. L. M. Weiss, and K. Kim, eds. Academic Press, San Diego. 1-17.
2. Montoya, J. G., and O. Liesenfeld. 2004. Toxoplasmosis. *Lancet* 363:1965-1976.
3. Dubey, J. P. 1998. Advances in the life cycle of *Toxoplasma gondii*. *Int. J. Parasitol.* 28:1019-1024.
4. Liesenfeld, O., S. Y. Wong, and J. S. Remington. 1999. Toxoplasmosis in the setting of AIDS. In *Textbook of AIDS medicine*. J. G. Bartlett, T. C. Merigan, and D. Bolognesi, eds. Williams and Wilkins, Baltimore. 225-259.
5. Slavin, M. A., J. D. Meyers, J. S. Remington, and R. C. Hackman. 1994. *Toxoplasma gondii* infection in marrow transplant recipients: a 20 year experience. *Bone Marrow Transplant.* 13:549-557.
6. Luft, B. J., and J. S. Remington. 1992. Toxoplasmic encephalitis in AIDS. *Clin. Infect. Dis.* 15:211-222.
7. Grant, I. H., J. W. Gold, M. Rosenblum, D. Niedzwiecki, and D. Armstrong. 1990. Toxoplasma gondii serology in HIV-infected patients: the development of central nervous system toxoplasmosis in AIDS. *AIDS* 4:519-521.
8. Hohlfeld, P., F. Forestier, S. Marion, P. Thulliez, P. Marcon, and F. Daffos. 1990. *Toxoplasma gondii* infection during pregnancy: T lymphocyte subpopulations in mothers and fetuses. *Pediatr. Infect. Dis. J.* 9:878-881.
9. Kravetz, J. D., and D. G. Federman. 2005. Toxoplasmosis in pregnancy. *Am J Med* 118:212-216.
10. Joynson, D. H., and T. J. Wreghitt. 2001. *Toxoplasmosis: a comprehensive clinical guide*. Cambridge University Press, Cambridge, UK.

11. Kim, K., and L. M. Weiss. 2004. Toxoplasma gondii: the model apicomplexan. *Int J Parasitol* 34:423-432.

12. Carruthers, V. B. 1999. Armed and dangerous: Toxoplasma gondii uses an arsenal of secretory proteins to infect host cells. *Parasitol Int* 48:1-10.

13. Carruthers, V. B., S. Hakansson, O. K. Giddings, and L. D. Sibley. 2000. *Toxoplasma gondii* uses sulfated proteoglycans for substrate and host cell attachment. *Infect. Immun.* 68:4005-4011.

14. Mordue, D. G., N. Dessai, M. Dustin, and L. D. Sibley. 1999. Invasion by *Toxoplasma gondii* establishes a moving junction that selectively excludes host cell plasma membrane proteins on the basis of their membrane anchoring. *J. Exp. Med.* 190:1783-1792.

15. Carruthers, V., and J. C. Boothroyd. 2006. Pulling together: an integrated model of Toxoplasma cell invasion. *Curr Opin Microbiol*.

16. Sibley, L. D., R. Lawson, and J. L. Krahenbuhl. 1985. Phagosome acidification blocked by intracellular *Toxoplasma gondii*. *Nature* 315:416-419.

17. Joiner, K. A., S. A. Fuhrman, H. M. Miettinen, L. H. Kasper, and I. Mellman. 1990. *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science* 249:641-646.

18. Walker, M. E., E. E. Hjort, S. S. Smith, A. Tripathi, J. E. Hornick, E. H. Hinchcliffe, W. Archer, and K. M. Hager. 2008. Toxoplasma gondii actively remodels the microtubule network in host cells. *Microbes Infect* 10:1440-1449.

19. Magno, R. C., L. Lemgruber, R. C. Vommaro, W. De Souza, and M. Attias. 2005. Intravacuolar network may act as a mechanical support for Toxoplasma gondii inside the parasitophorous vacuole. *Microsc Res Tech* 67:45-52.

20. Schwab, J. C., C. J. M. Beckers, and K. A. Joiner. 1994. The parasitophorous vacuole membrane surrounding intracellular *Toxoplasma gondii* functions as a molecular sieve. *Proc. Natl. Acad. Sci. USA* 91:509-513.

21. Black, M. W., and J. C. Boothroyd. 2000. Lytic cycle of Toxoplasma gondii. *Microbiol Mol Biol Rev* 64:607-623.

22. Hu, K., T. Mann, B. Striepen, C. J. Beckers, D. S. Roos, and J. M. Murray. 2002. Daughter cell assembly in the protozoan parasite Toxoplasma gondii. *Mol Biol Cell* 13:593-606.

23. Pomel, S., F. C. Luk, and C. J. Beckers. 2008. Host cell egress and invasion induce marked relocations of glycolytic enzymes in Toxoplasma gondii tachyzoites. *PLoS Pathog* 4:e1000188.

24. Nagamune, K., L. M. Hicks, B. Fux, F. Brossier, E. N. Chini, and L. D. Sibley. 2008. Abscisic acid controls calcium-dependent egress and development in Toxoplasma gondii. *Nature* 451:207-210.

25. Fruth, I. A., and G. Arrizabalaga. 2007. Toxoplasma gondii: induction of egress by the potassium ionophore nigericin. *Int J Parasitol* 37:1559-1567.

26. Caldas, L. A., W. de Souza, and M. Attias. 2007. Calcium ionophore-induced egress of Toxoplasma gondii shortly after host cell invasion. *Vet Parasitol* 147:210-220.

27. Chandramohanadas, R., P. H. Davis, D. P. Beiting, M. B. Harbut, C. Darling, G. Velmourougane, M. Y. Lee, P. A. Greer, D. S. Roos, and D. C. Greenbaum. 2009. Apicomplexan parasites co-opt host calpains to facilitate their escape from infected cells. *Science* 324:794-797.

28. Howe, D. K., and L. D. Sibley. 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human diseases. *J. Infect. Dis.* 172:1561-1566.

29. Sibley, L. D., and J. C. Boothroyd. 1992. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature* 359:82-85.

30. Sibley, L. D., D. G. Mordue, C. Su, P. M. Robben, and D. K. Howe. 2002. Genetic approaches to studying virulence and pathogenesis in *Toxoplasma gondii*. *Phil. Trans. R. Soc. Lond.* 357:81-88.

31. Mordue, D. G., F. Monroy, M. La Regina, C. A. Dinarello, and L. D. Sibley. 2001. Acute toxoplasmosis leads to lethal overproduction of Th1 cytokines. *J Immunol* 167:4574-4584.

32. Barragan, A., and L. D. Sibley. 2002. Transepithelial migration of *Toxoplasma gondii* is linked to parasite migration and virulence. *J. Exp. Med.* 195:1625-1633.

33. El Hajj, H., M. Lebrun, S. T. Arold, H. Vial, G. Labesse, and J. F. Dubremetz. 2007. ROP18 Is a Rhoptry Kinase Controlling the Intracellular Proliferation of Toxoplasma gondii. *PLoS Pathog* 3:e14.

34. Saeij, J. P., J. P. Boyle, S. Coller, S. Taylor, L. D. Sibley, E. T. Brooke-Powell, J. W. Ajioka, and J. C. Boothroyd. 2006. Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science* 314:1780-1783.

35. Taylor, S., A. Barragan, C. Su, B. Fux, S. J. Fentress, K. Tang, W. L. Beatty, H. E. Hajj, M. Jerome, M. S. Behnke, M. White, J. C. Wootton, and L. D. Sibley. 2006. A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen Toxoplasma gondii. *Science* 314:1776-1780.

36. Saeij, J. P., S. Coller, J. P. Boyle, M. E. Jerome, M. W. White, and J. C. Boothroyd. 2007. Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature* 445:324-327.

37. Robben, P. M., D. G. Mordue, S. M. Truscott, K. Takeda, S. Akira, and L. D. Sibley. 2004. Production of IL-12 by macrophages infected with Toxoplasma gondii depends on the parasite genotype. *J. Immunol.* 172:3686-3694.

38. Silverman, M. S. 1970. The macrophage in cellular and humoral immunity. *J Reticuloendothel Soc* 8:105-123.

39. Allison, A. C. 1978. Macrophage activation and nonspecific immunity. *Int Rev Exp Pathol* 18:303-346.

40. Lohmann-Matthes, M. L. 1981. The macrophage as a cytotoxic effector cell. *Haematol Blood Transfus* 27:49-57.

41. Nathan, C. F., H. W. Murray, and Z. A. Cohn. 1980. The macrophage as an effector cell. *N Engl J Med* 303:622-626.

42. O'Garra, A., N. Hosken, S. Macatonia, C. A. Wenner, and K. Murphy. 1995. The role of macrophage- and dendritic cell-derived IL12 in Th1 phenotype development. *Res Immunol* 146:466-472.

43. Alexander, J., T. M. Scharton-Kersten, G. Yap, C. W. Roberts, F. Y. Liew, and A. Sher. 1997. Mechanisms of innate resistance to Toxoplasma gondii infection. *Philos Trans R Soc Lond B Biol Sci* 352:1355-1359.

44. Sher, A., E. Y. Denkers, and R. T. Gazzinelli. 1995. Induction and regulation of host cell-mediated immunity by Toxoplasma gondii. *Ciba Found Symp* 195:95-104; discussion 104-109.

45. Scharton-Kersten, T. M., T. A. Wynn, E. Y. Denkers, S. Bala, E. Grunvald, S. Hieny, R. T. Gazzinelli, and A. Sher. 1996. In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to Toxoplasma gondii while failing to control acute infection. *J Immunol* 157:4045-4054.

46. Scharton-Kersten, T., C. Contursi, A. Masumi, A. Sher, and K. Ozato. 1997. Interferon consensus sequence binding protein-deficient mice display impaired

resistance to intracellular infection due to a primary defect in interleukin 12 p40 induction. *J Exp Med* 186:1523-1534.

47. Scharton-Kersten, T. M., G. Yap, J. Magram, and A. Sher. 1997. Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen Toxoplasma gondii. *J Exp Med* 185:1261-1273.

48. Collazo, C. M., G. S. Yap, S. Hieny, P. Caspar, C. G. Feng, G. A. Taylor, and A. Sher. 2002. The function of gamma interferon-inducible GTP-binding protein IGTP in host resistance to *Toxoplasma gondii* is Stat1 dependent and requires expression in both hematopoietic and non-hematopoietic cellular compartments. *Infect. Immun.* 70:6933-6939.

49. Collazo, C. M., G. S. Yap, G. D. Sempowski, K. C. Lusby, L. Tessarollo, G. F. Vande Woude, A. Sher, and G. A. Taylor. 2001. Inactivation of LRG-47 and IRG-47 reveals a family of interferon-g-inducible genes with essential, pathogen-specific roles in resistance to infection. *J. Exp. Med.* 194:181-187.

50. Gazzinelli, R. T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent upon $CD4^+$ T cells and accompanied by overproduction of IL-12, IFN-g, and TNF-a. *J. Immunol.* 157:798-805.

51. Wilson, E. H., U. Wille-Reece, F. Dzierszinski, and C. A. Hunter. 2005. A critical role for IL-10 in limiting inflammation during toxoplasmic encephalitis. *J Neuroimmunol* 165:63-74.

52. Neyer, L. E., G. Grunig, M. Fort, J. S. Remington, D. Rennick, and C. A. Hunter. 1997. Role of interleukin-10 in regulation of T-cell-dependent and T-cell-independent mechanisms of resistance to *Toxoplasma gondii*. *Infect. Immun.* 65:1675-1682.

53. Liesenfeld, O., J. Kosek, J. S. Remington, and Y. Suzuki. 1996. Association of CD4+ T cell-dependent, interferon-gamma-mediated necrosis of the small intestine with genetic susceptibility of mice to peroral infection with Toxoplasma gondii. *J Exp Med* 184:597-607.

54. Butcher, B. A., L. Kim, P. F. Johnson, and E. Y. Denkers. 2001. *Toxoplasma gondii* tachyzoites inhibit proinflammatory cytokine induction in infected macrophages by preventing nuclear translocation of the transcription factor NFkB. *J. Immunol.* 167:2193-2201.

55. Butcher, B. A., and E. Y. Denkers. 2002. Mechanism of entry determines ability of *Toxoplasma gondii* to inhibit macrophage proinflammatory cytokine production. *Infect. Immun.* 70:5216-5224.

56. Kim, L., B. A. Butcher, and E. Y. Denkers. 2004. *Toxoplasma gondii* interferes with lipopolysaccharide-induced mitogen-activated protein kinase activation by mechanisms distinct from endotoxin tolerance. *J. Immunol.* 172:3003-3010.

57. Butcher, B. A., L. Kim, A. D. Panopoulos, S. S. Watowich, P. J. Murray, and E. Y. Denkers. 2005. IL-10-independent STAT3 activation by Toxoplasma gondii mediates suppression of IL-12 and TNF-alpha in host macrophages. *J Immunol* 174:3148-3152.

58. Kim, L., B. A. Butcher, C. W. Lee, S. Uematsu, S. Akira, and E. Y. Denkers. 2006. *Toxoplasma gondii* genotype determines MyD88-dependent signaling in infected macrophages. *J. Immunol.* 177:2584-2591.

59. Kang, H., and Y. Suzuki. 2001. Requirement of non-T cells that produce gamma interferon for prevention of reactivation of *Toxoplasma gondii* in the brain. *Inect. Immun.* 69:2920-2927.

60. Schluter, D., N. Kaefer, H. Hof, O. D. Wiestler, and M. Deckert-Schluter. 1997. Expression pattern and cellular origin of cytokines in the normal and *Toxoplasma gondii*-infected murine brain. *Am. J. Pathol.* 150:1021-1035.

61. Halonen, S. K., G. A. Taylor, and L. M. Weiss. 2001. Gamma interferon-induced inhibition of Toxoplasma gondii in astrocytes is mediated by IGTP. *Infect Immun* 69:5573-5576.

62. Akira, S., and T. Takeda. 2004. Toll-like receptor signaling. *Nat. Rev. Immunol.* 4:499-511.

63. Kawai, T., and S. Akira. 2007. TLR signaling. *Semin Immunol* 19:24-32.

64. Uematsu, S., and S. Akira. 2008. Toll-Like receptors (TLRs) and their ligands. *Handb Exp Pharmacol*:1-20.

65. Shapira, S. S., K. Speirs, A. Gerstein, J. Caamano, and C. A. Hunter. 2002. Suppression of NF-kB activation by infection with *Toxoplasma gondii*. *J. Infect. Dis.* 185:S66-72.

66. Kim, L., B. A. Butcher, and E. Y. Denkers. 2004. Toxoplasma gondii interferes with lipopolysaccharide-induced mitogen-activated protein kinase activation by mechanisms distinct from endotoxin tolerance. *J Immunol* 172:3003-3010.

67. Kim, L., B. A. Butcher, C. W. Lee, S. Uematsu, S. Akira, and E. Y. Denkers. 2006. Toxoplasma gondii genotype determines MyD88-dependent signaling in infected macrophages. *J Immunol* 177:2584-2591.

68. Valere, A., R. Garnotel, I. Villena, M. Guenounou, J. M. Pinon, and D. Aubert. 2003. Activation of the cellular mitogen-activated protein kinase pathways ERK. p38 and JNK during *Toxoplasma gondii* invasion. *Parasite* 10:59-64.

69. Kim, L., L. Del Rio, B. A. Butcher, T. H. Mogensen, S. R. Paludan, R. A. Flavell, and E. Y. Denkers. 2005. p38 MAPK autophosphorylation drives

macrophage IL-12 production during intracellular infection. *J Immunol* 174:4178-4184.

70. Yarovinsky, F., and A. Sher. 2006. Toll-like receptor recognition of Toxoplasma gondii. *Int J Parasitol* 36:255-259.

71. Scanga, C. A., J. Aliberti, D. Jankovic, F. Tilloy, S. Bennouna, E. Y. Denkers, R. Medzhitov, and A. Sher. 2002. Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells. *J Immunol* 168:5997-6001.

72. Hitziger, N., I. Dellacasa, B. Albiger, and A. Barragan. 2005. Dissemination of *Toxoplasma gondii* to immunoprivileged organs and role of Toll/interleukin-1 receptor signalling for host resistance assessed by in vivo bioluminsecence imaging. *Cell. Micro.* 6:837-848.

73. Mun, H.-S., F. Aosai, K. Norose, M. Chen, L.-X. Piao, O. Takeuchi, S. Akira, H. Ishikura, and A. Yano. 2003. TLR2 as an essential molecule for protective immunity against *Toxoplasma gondii* infection. *Internat. Parasitol.* 15:1081-1087.

74. Debierre-Grockiego, F., M. A. Campos, N. Azzouz, J. Schmidt, U. Bieker, M. G. Resende, D. S. Mansur, R. Weingart, R. R. Schmidt, D. T. Golenbock, R. T. Gazzinelli, and R. T. Schwarz. 2007. Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from Toxoplasma gondii. *J Immunol* 179:1129-1137.

75. Yarovinsky, F., S. Hieny, and A. Sher. 2008. Recognition of Toxoplasma gondii by TLR11 prevents parasite-induced immunopathology. *J Immunol* 181:8478-8484.

76. Margueron, R., P. Trojer, and D. Reinberg. 2005. The key to development: interpreting the histone code? *Curr Opin Genet Dev* 15:163-176.

77. Berger, S. L. 2007. The complex language of chromatin regulation during transcription. *Nature* 447:407-412.

78. Wang, Y., W. Fischle, W. Cheung, S. Jacobs, S. Khorasanizadeh, and C. D. Allis. 2004. Beyond the double helix: writing and reading the histone code. *Novartis Found Symp* 259:3-17; discussion 17-21, 163-169.

79. Shindo, N., and T. Hirota. 2009. Histone modification: A new era of targeting epigenetics. *Rinsho Ketsueki* 50:282-288.

80. Yasui, K., T. Matsuyama, and T. Ito. 2005. [Transcriptional regulation by post-translational modification of histone]. *Seikagaku* 77:498-504.

81. Fuchs, J., D. Demidov, A. Houben, and I. Schubert. 2006. Chromosomal histone modification patterns--from conservation to diversity. *Trends Plant Sci* 11:199-208.

82. Cheung, P., K. G. Tanner, W. L. Cheung, P. Sassone-Corsi, J. M. Denu, and C. D. Allis. 2000. Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. *Mol Cell* 5:905-915.

83. Davie, J. R. 2003. MSK1 and MSK2 mediate mitogen- and stress-induced phosphorylation of histone H3: a controversy resolved. *Sci STKE* 2003:PE33.

84. Zhong, S. P., W. Y. Ma, and Z. Dong. 2000. ERKs and p38 kinases mediate ultraviolet B-induced phosphorylation of histone H3 at serine 10. *J Biol Chem* 275:20980-20984.

85. Dunn, K. L., and J. R. Davie. 2005. Stimulation of the Ras-MAPK pathway leads to independent phosphorylation of histone H3 on serine 10 and 28. *Oncogene* 24:3492-3502.

86. Clayton, A. L., S. Rose, M. J. Barratt, and L. C. Mahadevan. 2000. Phosphoacetylation of histone H3 on c-fos- and c-jun-associated nucleosomes upon gene activation. *EMBO J* 19:3714-3726.

87. Lee, Y. J., and S. D. Shukla. 2007. Histone H3 phosphorylation at serine 10 and serine 28 is mediated by p38 MAPK in rat hepatocytes exposed to ethanol and acetaldehyde. *Eur J Pharmacol* 573:29-38.

88. Saccani, S., S. Pantano, and G. Natoli. 2002. p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. *Nat Immunol* 3:69-75.

89. Clayton, A. L., and L. C. Mahadevan. 2003. MAP kinase-mediated phosphoacetylation of histone H3 and inducible gene regulation. *FEBS Lett* 546:51-58.

90. Soloaga, A., S. Thomson, G. R. Wiggin, N. Rampersaud, M. H. Dyson, C. A. Hazzalin, L. C. Mahadevan, and J. S. Arthur. 2003. MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. *EMBO J* 22:2788-2797.

91. Pages, G. 2009. MAP kinase phosphatase-1: a link between cell signaling and histone phosphorylation. Focus on "Histone H3 as a novel substrate for MAP kinase phosphatase-1". *Am J Physiol Cell Physiol* 296:C233-234.

92. Kinney, C. M., U. M. Chandrasekharan, L. Yang, J. Shen, M. Kinter, M. S. McDermott, and P. E. Dicorleto. 2009. Histone H3 as a novel substrate for MAP kinase phosphatase-1. *Am J Physiol Cell Physiol* 296:C242-249.

CHAPTER TWO

Toxoplasma gondii Prevents Chromatin Remodeling Initiated by Toll-Like Receptor-Triggered Macrophage Activation*

*This Chapter is reprinted from "*Toxoplasma gondii* prevents chromatin remodeling initiated by TLR-triggered macrophage activation" by J. Leng, B. A. Butcher, C. E. Egan, D. S. Abdallah, E. Y. Denkers. *The Journal of Immunology*, 2009 Jan 1;182(1):489-97.

ABSTRACT

Macrophages infected with the opportunistic protozoan *Toxoplasma gondii* are unable to upregulate many pro-inflammatory cytokine genes, including tumor necrosis factor (TNF-α), upon stimulation with LPS and other Toll-like receptor (TLR) ligands. Here, we examined the influence of *T. gondii* on transcription factors associated with TNF-α transcription, as well as phosphorylation and acetylation of histone H3 at distal and proximal regions of the TNF-α promoter. During LPS stimulation, we found that *Toxoplasma* blocks nuclear accumulation of transcription factor c-Jun, but not that of CRE binding protein (CREB) or NFκB. However, chromatin immunoprecipitation studies revealed that binding of all these transcription factors to the TNF promoter was decreased by *T. gondii* infection. Furthermore, the parasite blocked LPS-induced Ser^{10} phosphorylation and Lys^{9}/Lys^{14} acetylation of histone H3 molecules associated with distal and proximal regions of the TNF-α promoter. Our results show that *Toxoplasma* inhibits TNF-α transcription by interfering with chromatin remodeling events required for transcriptional activation at the TNF promoter, revealing a new mechanism by which a eukaryotic pathogen incapacitates proinflammatory cytokine production during infection.

INTRODUCTION

The opportunistic intracellular protozoan *Toxoplasma gondii* is a potent trigger of Th1 cytokines, a response that enables host survival and long-term parasite persistence (1, 2). Proinflammatory cytokine induction must be tightly regulated, because when overproduced these mediators cause immunopathology and host death. For *T. gondii*, this is exemplified during infection of IL-10 knockout mice that succumb from an inability to down-regulate parasite-induced proinflammatory cytokine production (3). From the perspective of the parasite, preventing overinduction of cytokines such as TNF-α, IL-12 and IFN-γ has the dual benefit of keeping the host alive to allow persistence while avoiding immune-mediated elimination.

In recent years, it has become clear that *Toxoplasma* takes an active role in interfering with intracellular signaling leading to proinflammatory mediators including IL-12, TNF-α, and nitric oxide (4-8). The exact molecular mechanisms by which this occurs remain largely unknown, although interference with mitogen-activated protein kinase (MAPK) activation, NFκB translocation as well as activation signal transducer and activator of transcription (STAT)-3 has been implicated (4, 9-13). In our studies, we have focused on *T. gondii*-infected macrophages, where a large panel of LPS-responsive genes, including IL-12p40 and TNF-α, is suppressed (5). For the case of IL-12, inhibition is relieved after approximately 12 hr, but TNF-α remains potently suppressed throughout the time course of infection (14). Because of the dramatic effect of *Toxoplasma* on macrophage TNF-α production, we chose to focus in detail on induction of this mediator to gain insight into how the parasite interferes with host cell signaling.

Regulation of TNF-α production is complex, and is controlled in a tissue-specific and

stimulus-specific manner (15-17). The primary control step of TNF-α gene expression resides in transcription initiation (18, 19). Studies have established that NFAT, ATF-2, Jun, Ets/Elk, and Sp-1 transcription factors and CBP/p300 coactivator proteins are involved in regulation of TNF-α transcription (16, 19). NFκB binding to distal κB sites is also important for maximal induction of TNF-α (20). Downstream of these events, production of TNF-α protein is also dependent upon regulation of mRNA splicing, regulation of mRNA half-life, and regulation of mRNA translation (21, 22).

Transcriptional initiation of many genes, including TNF-α, requires changes in higher order chromatin structure surrounding the promoter (23-25). In general, the promoter region undergoes stimulus-induced chromatin remodeling to allow access of transcription factors and RNA polymerase II machinery. Chromatin remodeling usually includes histone phosphorylation, acetylation and methylation, accompanied by nucleosome disassembly (26). The particular pattern of modification, also known as the histone code, determines whether a gene is in an active or inactive conformation (27). Among the histone modifications, phosphorylation of histone H3 at serine 10, acetylation of histone H3 at lysines 9 and 14, and methylation of histone H3 at lysine 4 are associated with gene activation.

Here, we show that macrophage infection by *T. gondii* inhibits recruitment of RNA polymerase II to the TNF-α promoter during LPS stimulation. Focusing on TNF-associated transcription factors, we found that nuclear accumulation of c-Jun, but not NFκB p65 or cAMP-responsive element-binding protein (CREB), was blocked by the parasite. Despite LPS induction of nuclear NFκB and CREB in infected cells, the parasite prevented recruitment of each to their respective sites on the TNF promoter. Furthermore, *Toxoplasma* interfered with LPS-induced histone H3 phosphorylation

and acetylation surrounding the TNF-α promoter, providing an explanation for the inability to recruit transcription factors and RNA polymerase II. These results show that *T. gondii* targets the histone modification machinery to prevent TNF-α transcription, and they provide a likely explanation for the widespread suppressive effects of the parasite on proinflammatory genes induced by LPS and possibly other stimuli.

MATERIALS AND METHODS

Mice and Parasites

C57BL/6 female mice (6-8 wk of age) were purchased from The Jackson Laboratory. The mice were kept under specific pathogen-free conditions at the Transgenic Mouse Facility, Cornell University College of Veterinary Medicine. The facility is overseen by an Institutional Animal Care and Use committee. *T. gondii* parasite strains RH, CC, ENT and DEG were maintained by biweekly passage on human foreskin fibroblast monolayers in DMEM supplemented with 1% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. In some experiments, we employed transgenic RH strain tachyzoites expressing tandem copies of the gene encoding yellow fluorescent protein (kindly provided by D. Roos and B. Striepen). Parasite cultures were tested for *Mycoplasma* every 6-8 wk using a highly sensitive PCR-based ELISA (Roche Diagnostics).

Cell Culture

Bone marrow cells were flushed from femur and tibia and cultured in cDMEM consisting of DMEM supplemented with 10% FCS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20% supernatant from L929 cells, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The cells were supplemented with fresh macrophage medium on Day 3. After 5 days of culture, nonadherent cells were removed, adherent monolayers were washed in ice-cold PBS, and cells were harvested by gentle pipetting in DMEM supplemented with 1% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Infection of macrophages was accomplished by adding tachyzoites to cell cultures followed by brief centrifugation (200 x g for 3 min) to synchronize contact between cells and parasites. In most cases, LPS (100 ng/ml) was added 12 hr after infection. Cells were recovered at varying times as indicated, depending upon the

assay performed.

Semi-Quantitative Real-time PCR

Real-time PCR were performed with a Power SYBR green kit according to the manufacturer's instructions (Cat. 4367659, Applied Biosystems). The primers employed were as follows. TNF RNA pol II site 1 forward: GAAAAGCAAGCAGCCAACCA; TNF RNA pol II site 1 reverse: CGGATCATGCTTTCTGTGCTC; TNF RNA pol II site 2 forward: ACAGAAAGCATGATCCGCGA; TNF RNA pol II site 2 reverse: GCCACAAGCAGGAATGAGAAGA; TNF forward: CCTTGTTGCCTCCTCTTTTGC; TNF reverse: TCAGTGATGTAGCGACAGCCTG; IL-10 forward: CCTGGCTCAGCACTGCTAT; IL-10 reverse: GCTCTTATTTTCACAGGGGAGAA; TNF promoter proximal forward: CCCCAACTTTCCAAACCCTCT; TNF promoter proximal reverse: CCCTCGGAAAACTTCCTTGGT; TNF promoter distal forward: GGCTTGTGAGGTCCGTGAATT; TNF promoter distal reverse: CCCTCGGAAAACTTCCTTGGT; IL-10 promoter forward: GCAGAAGTTCATTCCGACCA; IL-10 promoter reverse: GGCTCCTCCTCC CTCTTCTA; GAPDH forward: CCTGAACAGAACAGCAATGGCT; GAPDH reverse: GCTTGACGGTGTCTTTTGCCT.

Cytokine ELISA

IL-10 and TNF-α in cell cultures were measured using commercial kits according to the manufacturer's recommendations (R and D Systems).

Immunoblotting

The following antibodies were employed in immunoblotting studies: anti-phospho-c-Jun (cat. # 2361, Cell Signaling), anti-total-c-Jun (cat. # 9165, Cell Signaling), anti-phospho-CREB (cat. # 9191, Cell Signaling), anti-total CREB (cat. # 9197, Cell Signaling), anti-PARP (cat. # 9542, Cell Signaling), anti-NFκB p65 (cat. # SC-109, Santa Cruz Biotechnology), anti-phospho-histone H3 (Ser^{10}) (cat. # 9701, Cell Signaling), anti-acetyl-histone H3 ($Lys^{9/14}$) (cat. # 9677, Cell Signaling), anti-total H3 (cat. # 9715, Cell Signaling) and anti-phospho-MSK1 (cat. # 9591, Cell Signaling). Cells (2 x 10^6/sample) were lysed in reducing SDS sample buffer, and DNA was sheared by forcing samples 5 times through a 27-gauge needle. In some experiments, nuclear and cytoplasmic portions of cell lysates were separated by using a nuclear extract kit (Active Motif). After 5 min at 100ºC, samples were separated by 10% SDS-PAGE, and proteins were subsequently electrotransferred onto nitrocellulose membranes. Membranes were then blocked in 5% nonfat dry milk containing 0.1% Tween 20 in Tris-buffered saline, pH 7.6 (TBST), for 1 hr at room temperature, followed by incubation with antibody reconstituted in 5% BSA in TBST overnight at 4°C. After washing blots in TBST, primary antibodies were detected with a horseradish peroxidase-conjugated secondary antibody in TBST containing 5% nonfat dry milk for 1 h at room temperature. After washing in TBST, protein bands were visualized using a chemiluminescence based detection system (Cell Signaling).

Chromatin Immunoprecipitation (ChIP)

The following ChIP grade antibodies were employed for immunoprecipitation: anti-RNA pol II (cat. # 39097, Active Motif), anti-NFκB p65 (cat. # SC-109, Santa Cruz Biotechnology), anti-phospho-CREB (cat. # 9197, Cell Signaling), anti-phospho-c-Jun (cat. # 2361, Cell Signaling), anti-phospho-histone H3 (Ser10) (cat. # 9701, Cell

Signaling), anti-acetyl-histone H3 (Lys9/Lys14) (cat. # 9677, Cell Signaling). Assays were performed using the ChIP-IT enzymatic express kit (Active Motif) according to the manufacturer's instructions. Briefly, cells (1.5 x 10^7/sample) were fixed using 1% formaldehyde at room temperature for 10 min. Fixation was stopped by adding glycine to the mixture. The cells were then collected by scraping in buffer containing PMSF (100 mM). After brief centrifugation, the cells were resuspended in lysis buffer (Active Motif) with a protease inhibitor cocktail (Active Motif) and incubated for 30 min on ice. The cells were then resuspended in cell digestion buffer (Active Motif) and subjected to enzymatic digestion for 10 min at 37ºC. The reaction was stopped with addition of 0.5 M EDTA. Antibodies were added into the sheared chromatin preparations and the mixture was incubated with Protein G Magnetic Beads (Active Motif) overnight at 4ºC. The precipitated DNA-protein-antibody complexes were then washed and the crosslinking was reversed by incubation at 65ºC for 4 hr. Proteinase K was added to digest protein and DNA subsequently was purified using ethanol extraction, air dried and redissolved in 100 µl H_2O. The retrieved DNA was then subjected to real-time RT-PCR amplification using promoter-specific primers.

Transcription Factor DNA-binding ELISA

Presence of NFκB p65 in nuclear extracts was determined by binding to plate-bound target oligonucleotides exactly according to the manufacturer's instructions (Active Motif).

Immunofluorescence Microscopy

Macrophages were plated onto coverslips and infected with RH strain tachyzoites at a parasite to cell ratio of 4: 1. After overnight incubation, cells were treated with LPS (100 ng/ml) for 30-60 min. Coverslips bearing macrophages were fixed with 3% PFA

(20 min, room temperature) then permeabilized with methanol for 10 min at -20ºC. Coverslips were blocked with 5% normal goat serum in PBST for 60 min, then rabbit anti-phospho-H3 (Ser 10) antibody (cat. # 9701, Cell Signaling) or rabbit anti-acetyl-histone H3 ($Lys^{9/14}$) (cat. # 9677, Cell Signaling) was added in PBST to the coverslips, and cells were incubated overnight at 4ºC. Coverslips were washed with PBS then goat anti-rabbit antibody conjugated to Alexa Fluor 594 and anti-*Toxoplasma* p30 conjugated to FITC was added and cell were incubated 2 hr at 4ºC. After washing in PBS, coverslips were mounted with ProLong Antifade containing DAPI (Molecular Probes). Images were collected with a BX51 fluorescence microscope (Olympus) equipped with a DP 70 camera using DP controller software (Ver.1.1.1.65, Olympus) and DP manager software (Ver.1.1.1.71, Olympus).

Flow Cytometry

Peritoneal cells from infected mice were washed in cDMEM, resuspended at 1 x 10^7/ml and plated in a 24-well plate. Brefeldin A (Golgi plug, BD Biosciences) was added into the culture to block the protein transport. The cells were then stimulated with LPS. After 6 hours of incubation, the cells were washed in FACS wash buffer (1% BSA in PBS) and plated 2 x 10^6 per well in 96-well plate. F4/80 APC antibody (cat. # MF48005, Caltag Laboratories) was added and incubated for 20 min at 4ºC. The cells were then washed twice with FACS wash buffer and incubated with 200 µl Fix/Permeabilization buffer (BD Biosciences) for 15 min. The cells were washed again and resuspended in antibody cocktail containing anti-TNF PE (cat. # 554419, BD Biosciences) and anti-p30 FITC (cat. # 12-132, Argene). After 1 hr of incubation, cells were washed and resuspended in FACS wash buffer. Samples were analyzed on a FACScalibur flow cytometer (BD Biosciences) collecting at least 50,000 events. The data were subsequently analyzed using FlowJo software (Tree Star).

Statistics

The statistical significance of the data was determined by unpaired Student's *t* test. Values of $p < 0.05$ were considered significant. All experiments were performed at least three times.

RESULTS

T. gondii potently and specifically inhibits TLR-induced TNF-α production in macrophages

Previously we established that *T. gondii* infection inhibits LPS induced production of TNF and other proinflammatory mediators in mouse macrophages (5, 14). In order to confirm and extend these results, we employed the type I *T. gondii* RH strain to infect bone marrow-derived macrophages, cells that are readily invaded by the parasite (Fig. 2.1A and B). When infected cells were subjected to LPS/TLR4 triggering, TNF-α cytokine release was potently suppressed (Fig. 2.1C). Parasite-induced suppression of TNF-α was maintained for up to 36 hr post-LPS stimulation (data not shown). This was not a nonspecific cytotoxic effect, because LPS-induced IL-10 production was unaffected by parasite infection (Fig. 2.1D). We also examined cytokine mRNA levels during LPS stimulation of infected cells. In accord with the cytokine protein data, LPS-induced TNF mRNA induction was inhibited by pre-infection with *T. gondii* (Fig. 2.1E). In contrast, there was no consistent effect of the parasite on IL-10 mRNA during LPS stimulation (Fig. 2.1F).

T. gondii inhibits TNF-α induction by multiple TLR ligands and multiple parasite strains interfere with TNF-α induction

To examine whether *T. gondii* blocks TNF release triggered through other TLR pathways in addition to that initiated by LPS, we used Pam3Cys (TLR2 ligand) and CpG oligodinucleotides (TLR9 ligand) to stimulate infected macrophages. Figure 2.2A shows that both TLR2 and TLR9-induced TNF responses were inhibited by RH strain infection. We also examined the activity of agonists directed at TLR3, 5, 7, and 8, and while none of these induced significant amounts of TNF-α, IL-12 production was suppressed during triggering through these TLR (data not shown).

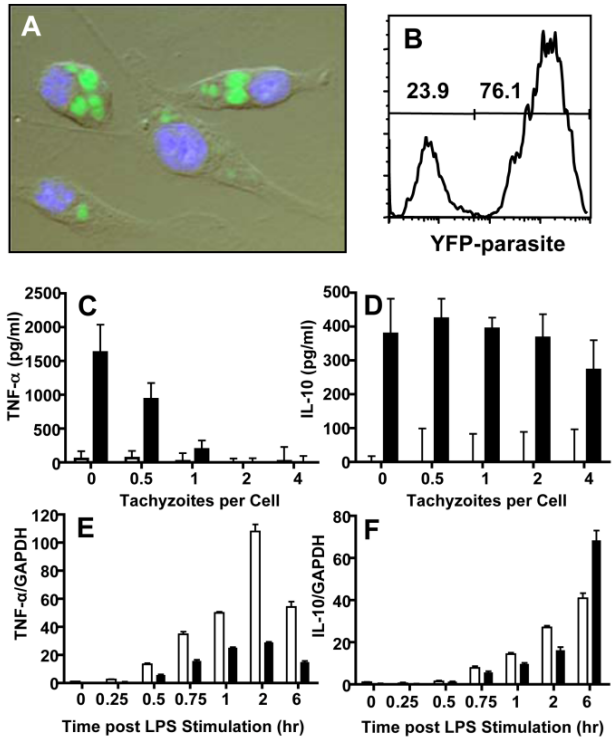


Figure 2.1 *T. gondii* inhibits LPS-induced TNF-α, but not IL-10 production. (A and B) Bone marrow-derived macrophages were infected (4: 1 ratio of tachyzoites to cells) with a *T. gondii* RH strain genetically engineered to express tandem copies of yellow fluorescence protein (YFP). After 12 hr, cells were prepared for fluorescence microscopy (A) and flow cytometry (B). (C and D) 12 hr-infected macrophages were stimulated with LPS (closed bars) or cultured in medium alone (open bars), and then supernatants were collected 6 hr later for TNF-α (C) and IL-10 (D) ELISA. (E and F) Macrophages were infected (4: 1 ratio of parasites to cells) then 12 hr later subjected to LPS stimulation. At the indicated time-points, RNA was extracted, reverse transcribed into cDNA and real time PCR was performed for TNF-α (E) and IL-10 (F) transcripts. The data are normalized to GAPDH expression levels. In E and F, closed and open bars indicate LPS-induced responses of infected and noninfected cells, respectively.

Next, we compared highly virulent Type I *Toxoplasma* strains with low virulence Type II strains to ask whether inhibition was specific for RH or whether other strains could suppress TNF-α production. As shown in Figure 2.2B, although RH was the most potent suppressor of TNF-α, another Type I strain (ENT) also blocked TNF production. In addition, the Type II strains CC and DEG also possessed suppressive activity.

T. gondii blocks RNA polymerase II recruitment to the TNF gene

Regulation of TNF-α is complex, with control being exerted at transcriptional and post-transcriptional levels. In order to determine if *Toxoplasma* acts at the transcriptional level to interfere with TNF-α production, we examined recruitment of RNA polymerase II to the TNF-α gene start codon using ChIP analysis with 3 independent sets of primers. As shown in Fig. 2.3A, LPS stimulated RNA polymerase II recruitment to the TNF-α promoter. In contrast, parasite infection alone failed to trigger recruitment. Importantly, when cells were pre-infected with *T. gondii*, LPS stimulation failed to induce upregulation of RNA polymerase II activity at the TNF-α promoter. In order to substantiate this finding, we switched to a real time PCR-based approach to examine ChIP products using two additional primer sets for amplification. In both cases, while LPS induced increased RNA polymerase II recruitment to the promoter, parasite infection blocked this response (Fig. 2.3B and C). We conclude that *T. gondii* prevents TNF-α protein release, at least in part, through interference with recruitment of RNA polymerase II to the TNF gene transcription start site.

T. gondii blocks LPS-induced nuclear accumulation of c-Jun, but not CREB or NFκB p65

In order to elucidate the molecular basis by which *T. gondii* inhibits TNF-α gene induction, we first focused on transcription factors that target the TNF promoter (Fig.

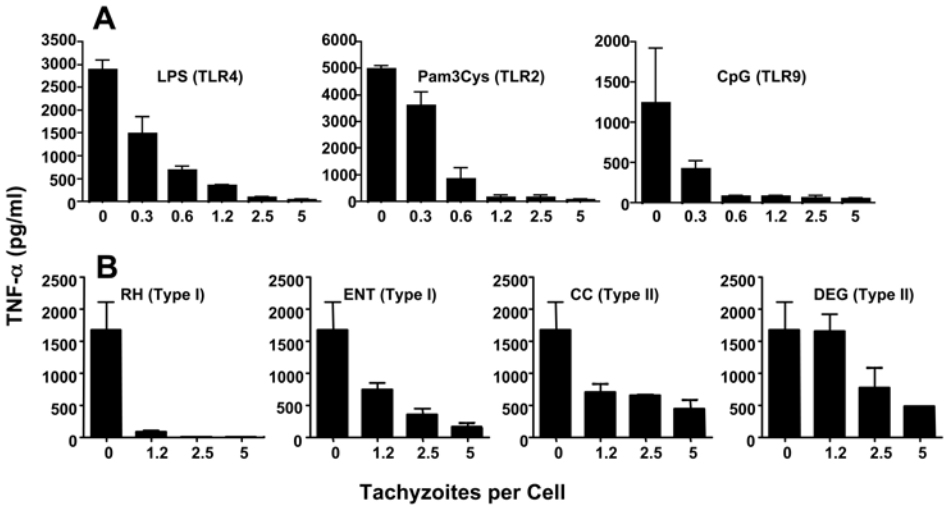


Figure 2.2 *T. gondii* inhibits TNF-α induction by multiple Toll-like receptor ligands and multiple parasite strains inhibit TNF-α induction. (A) Bone marrow-derived macrophages were infected with RH strain tachyzoites for 12 hr, followed by 6 hr stimulation with LPS, Pam3Cys or CpG. Supernatants were collected for subsequent TNF ELISA. (B) Macrophages were infected with RH, CC, ENT or DEG strain tachyzoites for 12 hr, followed by 6 hr stimulation with LPS. Supernatants were collected for subsequent TNF ELISA.

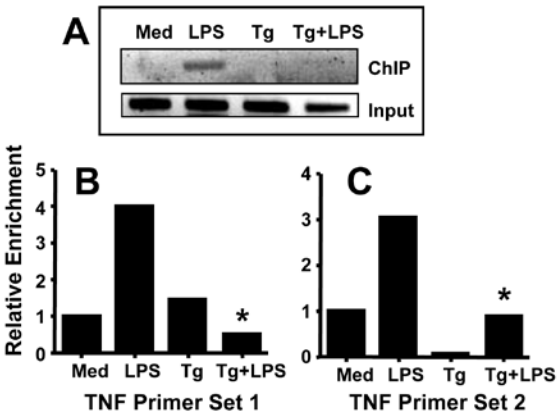


Figure 2.3 *T. gondii* blocks RNA polymerase II recruitment to the TNF gene. Macrophages were infected with RH strain tachyzoites for 12 hr, followed by 30 min stimulation with LPS. Nuclear extracts were prepared and subjected to chromatin immunoprecipitation (ChIP) assay using antibody against RNA polymerase II. (A) ChIP DNA was amplified by standard PCR methodology using primers spanning the 5' region of the TNF gene, and input DNA was amplified as a control. (B and C) ChIP DNA was amplified by real-time PCR using two different primer sets spanning the 5' end of the TNF gene. The data was normalized to input DNA and amplification was expressed as relative enrichment compared to cells in medium (defined as 1). Med, cells incubated in medium alone; Tg, cells infected with *Toxoplasma*. *, $p < 0.01$ comparing LPS and Tg+LPS.

2.4A). Previous studies have established that both proximal and distal regions of the promoter are important for maximal TNF induction (16, 19, 20). The distal region (-500 to -900) contains four NFκB binding sites, and of these there is evidence that the κB2 and κB3 sites are the most active (20). The proximal region (0 to -200) contains binding sites for multiple transcription factors including CREB, ATF-2, c-Jun, Ets/Elk, Egr-1 and Sp1 (Fig. 4A). CREB, ATF-2 and c-Jun each recognize a conserved cAMP-responsive element (CRE) palindrome. CBP/p300 serves as a platform to bring all the transcription factors together to form an enhanceosome and also functions as a histone acetylase (28, 29).

We chose to examine the activation and binding properties of CREB, c-Jun and NFκB p65 upon TLR4 triggering of infected and noninfected cells. In Fig. 2.4B, we examined total and phosphorylated levels of c-Jun and CREB in macrophage nuclear extracts from noninfected and infected cells stimulated with LPS. Stimulation through TLR4 led to strong phosphorylation of c-Jun that was apparent at 30 min and sustained for at least 2 hr. Interestingly, in cells pre-infected with *Toxoplasma*, levels of nuclear phospho-c-Jun activation were lower. Most strikingly, we found a major decrease in total nuclear c-Jun following LPS stimulation of infected macrophages (Fig. 2.4B). LPS stimulation also resulted in modestly increased amounts of phospho-CREB in the nuclei of non-infected macrophages, but, with the possible exception of the 120 min time point, *T. gondii* pre-infection failed to have a major effect on phosphorylated or total nuclear CREB levels (Fig. 2.4B).

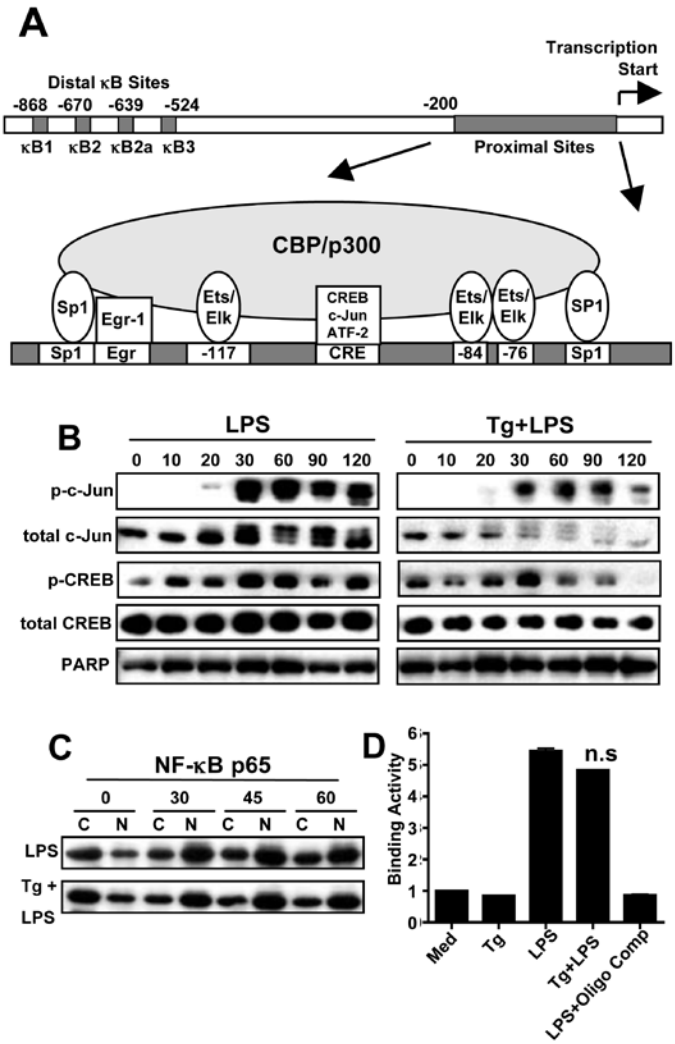
Next, we examined the influence of *T. gondii* on LPS-induced NFκB nuclear translocation. We and others previously reported that the parasite interferes with nuclear accumulation of this transcription factor, but this block was not sustained

beyond 6 hr of infection (9). In Fig. 2.4C, we show that LPS induces nuclear accumulation of NFκB p65 within 30 min of stimulation that was maintained for at least 60 min. In accord with previous data, 12-hr pre-infected cells responded with similar rapid NFκB p65 translocation in response to TLR4 triggering (Fig. 2.4C). We then assessed the binding activity of nuclear NFκB p65 after LPS stimulation in the presence and absence of infection. As shown in Fig. 2.4D, LPS induced a 5-fold increase in NFκB p65 binding activity, as measured in a binding assay using plate-bound κB consensus target oligonucleotides. As expected, parasite infection alone failed to increase nuclear NFκB binding activity, and presence of tachyzoites in macrophages did not significantly diminish the LPS-induced increase in nuclear p65 binding (Fig. 2.4D). We attempted a similar assay to assess CREB binding activity, but were unable to obtain consistent results (data not shown).

T. gondii blocks binding of NFκB p65 and phosphorylated c-Jun to the TNF promoter

We next determined the influence of *Toxoplasma* on recruitment of NFκB p65, phospho-CREB, and phospho-c-Jun to the TNF-α promoter region in live cells using ChIP assays. As shown in Fig. 2.5A, LPS stimulated strong binding of p65 to the TNF distal promoter region. In sharp contrast, *T. gondii* infection potently blocked recruitment of p65 to κB sites on the TNF-α promoter. Thus, even though NFκB translocated to the nucleus normally in infected cells (Fig. 2.4C), and even though this transcription factor was functional based upon in vitro binding assays (Fig. 2.4D), it was prevented from binding to its target promoter sequence (Fig. 2.5A). We observed a similar pattern when we examined the effect of *Toxoplasma* on phospho-CREB recruitment to the TNF promoter, although in this case parasite-mediated inhibition did not reach statistical significance. When we examined phospho-c-Jun, we found that LPS triggered strong binding to the TNF promoter and that this response was

Figure 2.4 *T. gondii* blocks LPS-induced nuclear translocation of c-Jun, but not that of CREB or NFκB p65. (A) Schematic map of the mouse TNF promoter showing distal and proximal sites involved in transcriptional initiation. (B) Macrophages were either infected or left uninfected for 12 hr and then subjected to LPS stimulation for the indicated time periods (min). Western blotting for total and activated forms of c-Jun and CREB was performed on nuclear lysates. Blotting for the nuclear enzyme PARP was performed to confirm equal protein loading. (C) Cytoplasmic and nuclear extracts were prepared from infected and noninfected macrophages stimulated with LPS for the indicated time periods (min). The extracts were subsequently probed with antibody to NFκB p65. In (D) the in vivo binding capability of nuclear NFκB p65 was determined using an ELISA-based method to measure transcription factor binding to solid-phase target oligonucleotides. In this panel, addition of soluble target oligonucleotide (Oligo Comp) blocked p65 binding, confirming the specificity of the assay. Med, cells cultured in medium alone; Tg, *T. gondii* infected cells. n.s., not significant comparing LPS and Tg+LPS.


A
Transcription Start
Distal κB Sites
-868 -670 -639 -524
-200
κB1 κB2 κB2a κB3
Proximal Sites
CBP/p300
Sp1
Egr-1
Ets/ Elk
CREB c-Jun ATF-2
Ets/ Elk
Ets/ Elk
SP1
Sp1
Egr
-117
CRE
-84
-76
Sp1
B
LPS
Tg+LPS
0 10 20 30 60 90 120
p-c-Jun
total c-Jun
p-CREB
total CREB
PARP
C
NF-κB p65
0 30 45 60
C N C N C N C N
LPS
Tg + LPS
D
Binding Activity
n.s
Med
Tg
LPS
Tg+LPS
LPS+Oligo Comp

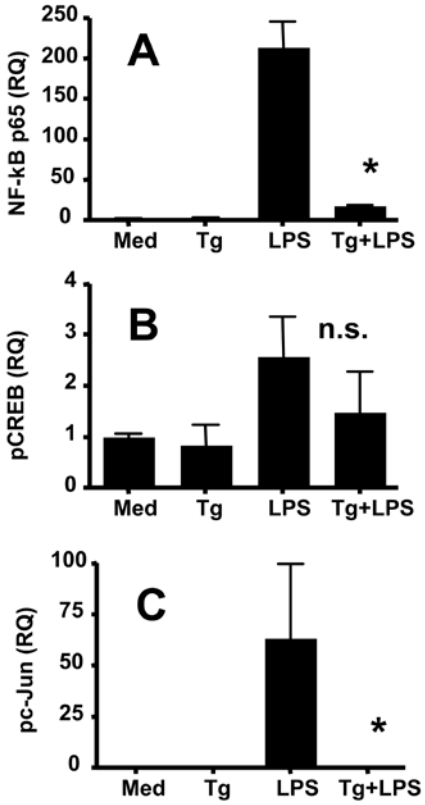


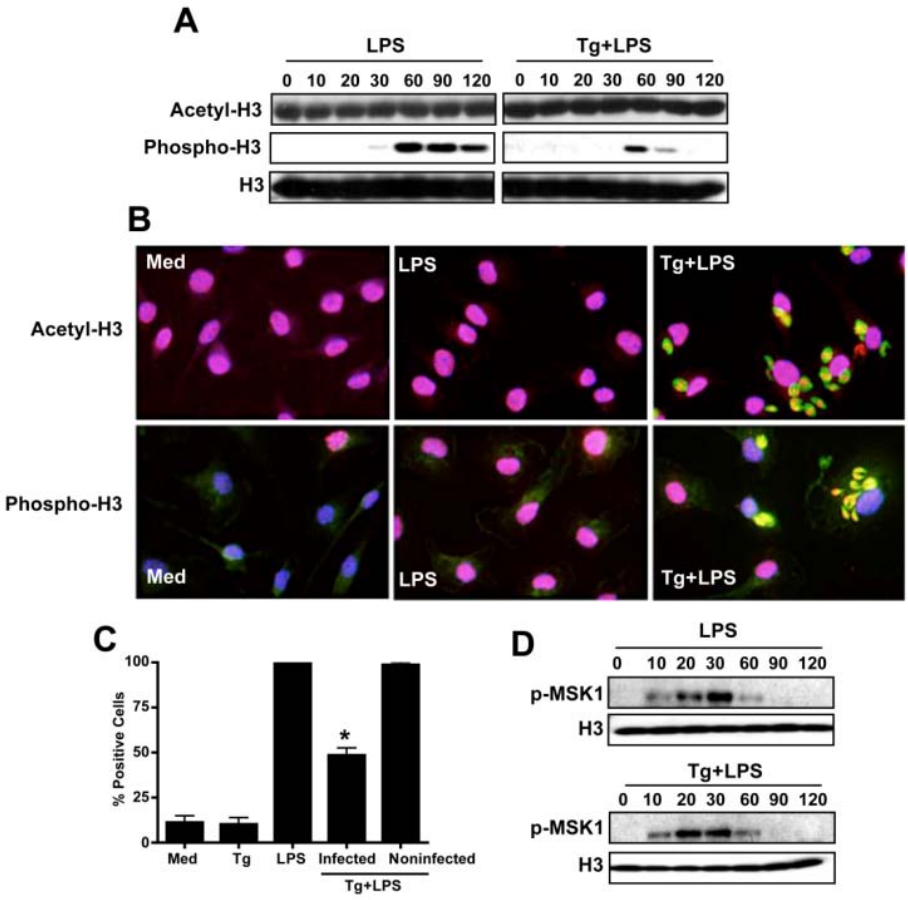
Figure 2.5 *T. gondii* blocks LPS-induced binding of phosphorylated c-Jun and NFκB p65 to the TNF promoter. Bone marrow-derived macrophages were infected with RH strain tachyzoites then 12 hr later subjected to 30 min LPS stimulation. ChIP assays were performed using antibodies specific for NFκB p65 (A), phospho-CREB (B) and phospho-c-Jun (C). The immunoprecipitated DNA was amplified by real-time PCR using primers spanning either proximal (for c-Jun and CREB) or distal (for NFκB p65) sites of the TNF promoter. The results were normalized to the input DNA and expressed as Relative Quantitation (RQ) where medium is defined as 1. Med, medium; Tg, cells infected with *T. gondii*. n.s., not significant; *, $p < 0.01$ comparing LPS and Tg+LPS.

strongly down-modulated by prior *T. gondii* infection (Fig. 2.5C). While the latter result was not entirely unexpected based upon decreased levels of total c-Jun in the nucleus during infection, it is notable that substantial amounts of phosphorylated nuclear c-Jun remained in infected cells (Fig. 2.4B).

Toxoplasma interferes with histone H3 modification associated with gene transcriptional activation

The finding that *T. gondii* potently blocked recruitment of transcription factors to the TNF-α promoter without interfering with activation or nuclear translocation suggested that the parasite might target chromatin structure rather than transcription factors per se. Therefore, we sought to determine the influence of *Toxoplasma* on $Lys^{9/14}$ acetylation and Ser^{10} phosphorylation of histone H3, modifications that are associated with chromatin decondensation and gene activation (30). We found that even nonstimulated macrophages expressed high amounts of acetylated histone H3, and levels were not changed by LPS stimulation in the presence or absence of infection (Fig. 2.6A). These results were confirmed by immunofluorescence microscopy (Fig. 2.6B). However, we found that LPS induced phosphorylation of histone H3 within 60 min of stimulation, and this response was strongly inhibited in parasite-infected macrophages (Fig. 2.6A). Likewise, immunofluorescence microscopy confirmed inhibition of histone H3 phosphorylation in TLR4-triggered infected cells, in contrast to noninfected macrophages that were stimulated with LPS (Fig. 2.6B and 2.6C). The kinase MSK1 elicits histone H3 phosphorylation on the Ser^{10} residue (31, 32). Therefore, we asked whether LPS triggered activation of MSK1, and, in turn, whether *T. gondii* blocked the response. While LPS stimulated rapid activation of MSK1, the response was unaffected by prior infection with *Toxoplasma* (Fig. 2.6D). We also examined histone H3 Thr^{3} and Ser^{28} modification, but found little or no effect of

Figure 2.6 *T. gondii* globally blocks LPS-induced phosphorylation of histone H3 at Ser 10 but has no effect on Lys 9/14 histone H3 acetylation. (A) Macrophages were infected then 12 hr later subjected to LPS stimulation for indicated time periods (min). Total cell lysates were extracted and subjected to Western blotting using antibodies specific to phospho-Ser10 histone H3, acetyl-Lys9/14 and total H3. In (B), fluorescence microscopy was employed to examine $Lys^{9/14}$ acetylation (top panels) and Ser^{10} phosphorylation (bottom panels) of histone H3 in infected and noninfected cells stimulated for 30 min with LPS. Macrophages were stained with antibody specific for acetylated or phosphorylated histone H3 as indicated (Red) and *Toxoplasma* p30 (Green). Nuclei are stained with DAPI (Blue). In (C), cells that were positive for phospho-Ser10 at histone H3 staining were counted in each group of cells (approximately 200 cells per condition). *, $p < 0.01$ comparing infected and non-infected cells. (D) 12-hr-infected and control macrophages were stimulated with LPS (100 ng/ml) and cell lysates were prepared at the indicated time points (min). Immunoblotting for phospho-MSK1 was subsequently performed. As a control for protein loading, levels of histone H3 were assessed in the same samples. Med, medium; Tg, *T. gondii*.


A
LPS
Tg+LPS
0 10 20 30 60 90 120
0 10 20 30 60 90 120
Acetyl-H3
Phospho-H3
H3
B
Med
LPS
Tg+LPS
Acetyl-H3
Phospho-H3
Med
LPS
Tg+LPS
C
% Positive Cells
100
75
50
25
0
*
Med
Tg
LPS
Infected
Noninfected
Tg+LPS
D
LPS
0 10 20 30 60 90 120
p-MSK1
H3
Tg+LPS
0 10 20 30 60 90 120
p-MSK1
H3

Toxoplasma on the phosphorylation status of these residues (data not shown).

We next wanted to specifically examine the status of histone H3 modification surrounding distal and proximal regions of the TNF-α promoter during macrophage activation in the presence and absence of infection, since global levels of H3 phosphorylation and acetylation might not be reflective of histone modification at the TNF promoter. Therefore, we examined histone modifications at this site by ChIP assay. We found that LPS induced elevated levels of phosphorylation of histone H3 at serine 10 at both distal and proximal TNF promoter regions (Figure 2.7A and 2.7C). Notably, this response was blocked by *Toxoplasma* infection. Furthermore, we also examined acetylation of histone H3 at lysine 9 and lysine 14 at proximal and distal locations of the TNF promoter (Figure 2.7B and 2.D). While LPS induced H3 acetylation at both sites, this response was blocked in infected macrophages. Finally, we examined the status of histone H3 surrounding the IL-10 promoter, an LPS-induced gene that is not affected by *Toxoplasma* (Fig. 2.1). In this case, there was no evidence for histone H3 modification following TLR4 stimulation as measured by phosphorylation (Fig. 2.7E) or acetylation (Fig. 2.7F), and infection with *T. gondii* had no influence in the presence or absence of LPS. Therefore, while global levels of acetylated histone H3 are not affected by *Toxoplasma*, the parasite down-regulates acetylated H3 at the TNF promoter. In contrast, the parasite blocks LPS-induced Ser^{10} H3 phosphorylation at the global level and this extends to the activity at the TNF promoter.

Toxoplasma suppresses TNF-α production during in vivo infection

We wanted to determine if parasite suppression of bone marrow-derived macrophage TNF-α production extended to in vivo infection. Therefore, we assessed production of

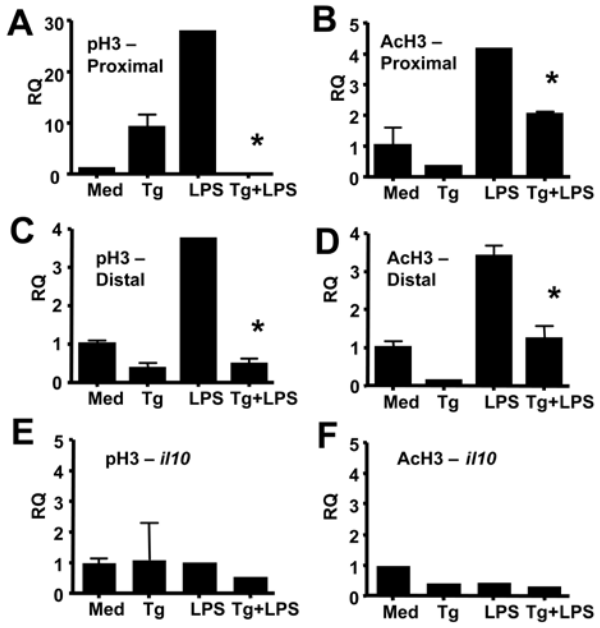


Figure 2.7 *T. gondii* blocks LPS-induced histone H3 modification at the TNF promoter. Macrophages were infected with *T. gondii* then 12 hr later subjected to LPS stimulation for 30 min. The cells were fixed with formaldehyde and ChIP assay performed using antibodies specific for Ser^{10} phosphorylated (A and C) or $Lys^{9/14}$ acetylated (B and D) histone H3. The resulting DNA was amplified by real time PCR using primers spanning either proximal (A and B) or distal (C and D) sites of the TNF promoter region. As a control, the same ChIP DNA was amplified using primers spanning IL10 promoter region (E and F). The results were normalized to the input DNA and expressed as Relative Quantitation (RQ), with medium defined as 1. Med, cells incubated in medium alone; Tg, cells infected with *Toxoplasma*. *, $p < 0.05$ comparing LPS and Tg+LPS.

this cytokine by inflammatory macrophages recruited to the peritoneal cavity during infection. Mice were inoculated by i.p. injection with *Toxoplasma*, and peritoneal wash-out cells were collected 12 hrs later. The cells were cultured in vitro in medium or LPS, and TNF-α production was assessed in infected and noninfected populations of F4/80-positive macrophages (Fig. 2.8A). As shown in Fig. 2.8B, in cells cultured without further stimulation, 13% of noninfected macrophages produced TNF-α. In contrast, only 3% of infected macrophages produced this cytokine in the same overall population. When F4/80-positive macrophages were stimulated ex vivo with LPS (Fig. 2.8C), 84% of noninfected macrophages produced TNF-α. Strikingly, in the infected cell population, only 25% stained positive for TNF-α in response to ex vivo TLR4 triggering. We note that in the F4/80-positive population, a distinct subset of cells harbors low parasite levels and can respond to LPS (Fig. 2.8C), a distribution pattern that we do not see in vitro (Fig. 2.1B). We do not know the origin of these macrophages at present. One possibility is that these are cells that were newly infected during the in vitro response to LPS. Another possibility is that this population represents a macrophage subset that is less permissive to *T. gondii* replication and is also less sensitive to parasite-induced inhibition of TNF-α. Regardless, our data for the first time show that *Toxoplasma* shuts down proinflammatory signaling in an in vivo situation.

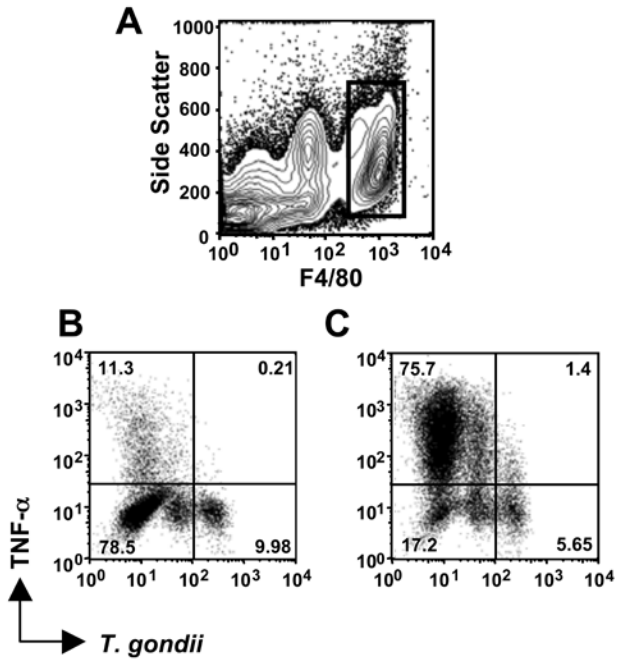


Figure 2.8 *T. gondii* inhibits TNF-α production during in vivo infection of F4/80-positive macrophages. Mice were infected by intraperitoneal injection of 10^6 RH strain tachyzoites. After 12 hr, cells in the peritoneal cavity were collected and cultured for 6 hr in either medium alone or LPS in the presence of Brefeldin A. Cells were then subjected to surface staining for F4/80 and intracellular staining for TNF-α and *Toxoplasma* p30 (SAG-1) to detect tachyzoites. A, F4/80-positive cells in the peritoneal cavity. The rectangle delineates cells selected for analysis. B, TNF-α and infection levels in F4/80-positive cells cultured in medium. C, TNF-α and infection levels in F4/80-positive cells stimulated in vitro with LPS.

DISCUSSION

Microbial pathogens such as *Toxoplasma* must evade the potent antimicrobial effects of proinflammatory mediators produced by the innate immune system if they are to establish persistent infection in the host. For intracellular pathogens, direct interference with gene transcription is a highly effective strategy to achieve this end (33, 34). Inflammatory gene expression is controlled by multiple regulatory signal transduction cascades (35). First, gene-specific transcription factors must be activated and translocated to the nucleus. Second, it is becoming increasingly realized that in many cases chromatin structure surrounding target genes must be modified to allow access of transcription factors (36-38). There are many examples of pathogens that target pathways leading to transcription factor activation. A more limited number of recent studies have found that some bacterial pathogens epigenetically affect gene function (39-42). This study is the first example of a eukaryotic pathogen that targets regulatory cascades controlling chromatin structure to subvert host cell function.

The four core histones (H2A, H2B, H3 and H4) that make up the nucleosome are arranged as an octamer around which DNA is wound. The N termini of the histones are subject to covalent modification that can be associated with repression or induction of genes (36-38). Transcriptional activation is strongly associated with histone H3 modification. The pattern of chemical changes is complex but among the modifications, phosphorylation of Ser^{10} and acetylation of Lys^{9} and Lys^{14} are linked to changes in chromatin structure that permit access of transcription factors (30).

Our results for the first time show that *T. gondii* prevents chromatin remodeling surrounding the TNF-α promoter by interfering with phosphorylation and acetylation of histone H3 that would otherwise be induced by TLR signaling. We found that

Toxoplasma blocks Ser^{10} phosphorylation of total histone H3 in the nucleus, as well as at distal and proximal TNF-α promoter regions. Our studies in addition show that *T. gondii* interferes with LPS-induced acetylation of histone H3 at Lys^{9} and Lys^{14} surrounding the TNF promoter. Interestingly, this was a gene-specific effect because global levels of acetylated H3 were constitutively high in macrophages, and neither LPS nor the combination of *Toxoplasma* and LPS altered these levels. Ser^{10} phosphorylation of histone H3 can recruit several histone acetyl transferases to catalyze acetylation of Lys^{9} and Lys^{14} (43, 44). Therefore, it is possible that the effects of *Toxoplasma* on histone H3 acetylation at the TNF promoter are indirectly mediated by inhibition of H3 Ser^{10} phosphorylation. Insofar as chromatin modification is a prerequisite to allow transcription factor access at many proinflammatory genes, our results may provide a unifying model for the parasite's ability to simultaneously down-regulate production of several proinflammatory mediators in addition to TNF-α (5, 14).

Although *Toxoplasma* down-modulates many TLR4-inducible genes, some escape inhibition. Most prominently, we found that LPS-triggered IL-10 is not blocked by the parasite. We also found no evidence that LPS altered chromatin modification surrounding the IL-10 promoter. Thus, the relatively low amount of LPS-triggered IL-10 compared to TNF-α appears to be produced independently of histone H3 Ser^{10} phosphorylation or $Lys^{9/14}$ acetylation, and therefore escapes inhibition by the parasite. In contrast, there is evidence that high level IL-10 transcription induced by other stimuli such as combined treatment with LPS and immune complexes involves chromatin remodeling at the IL-10 promoter (45). We are currently examining whether these responses are likewise affected by *Toxoplasma.*

While interference with chromatin remodeling is likely to be a potent parasite mechanism to manipulate the host cell transcriptome, we also found evidence for interference with c-Jun activity. Thus, total levels of nuclear c-Jun rapidly decreased following LPS stimulation of infected macrophages. One interpretation of this result is that TLR4 triggering causes phosphorylation of nuclear c-Jun, and *Toxoplasma* increases nuclear export of the activated form of this transcription factor. Unlike the results of the 12 hr infections reported here, the parasite also prevents nuclear accumulation of NFκB during short-term infection. Whether this reflects decreased nuclear import or increased export is unclear (4, 10, 46).

Toxoplasma is sequestered within a parasitophorous vacuole, but recent findings show that the parasite delivers molecules to the host cell to modify signal transduction (47). During host cell invasion, apical organelles called rhoptries discharge and at least some rhoptry proteins enter the host cell. The ROP16 molecule, a rhoptry protein with predicted kinase activity, is released during invasion and targets STAT3/6 for activation (12). STAT3 activation by *T. gondii* has previously been implicated in suppression of IL-12 by the parasite (11). In addition a protein phosphatase 2C released by the parasite is targeted to the host cell nucleus, although in this case host target molecules have not yet been identified (48). Nevertheless, the emerging opinion is that *T. gondii* takes an active part in manipulation of the host cell (13, 47, 49), and the results of the present study provide strong mechanistic evidence for this view.

Toxoplasma inhibits signaling through TLR, yet the parasite itself expresses its own TLR ligands. Tachyzoite profilin activates TLR11 and glycosylphosphoinositol (GPI) anchors on the parasite surface are capable of triggering TLR2 and TLR4 (50, 51). The parasite cannot survive without profilin, which is used in invasion (52). Likewise, the parasite is not viable without the ability to synthesize GPI-anchored proteins (53).

Possibly for these reasons *T. gondii* has adopted potent strategies to down-modulate proinflammatory signaling that would otherwise result from recognition of its own essential molecules by host pattern recognition receptors.

Several bacterial pathogens target proinflammatory signaling pathways in macrophages. The *Yersinia* virulence proteins YopP/J block phosphorylation of IκB and MAPK kinase, effectively terminating both NFκB and MAPK signal transduction (54-57). Along similar lines *Bacillis anthracis* lethal factor (LF) enzymatically cleaves MAPK kinases (58). Some bacterial pathogens can also affect chromatin structure. *Mycobacerium tuberculosis* downregulates histone acetylation around the *MHC2TA* locus, and *Listeria monocytogenes* dephosphorylates histone H3 and deacetylates histone H4 during early infection (39-41). It has recently been reported that *Shigella flexneri* escapes inflammatory chemokine induction through effects of the OspF protein on histone H3 phosphorylation (42). Intracellular protozoans are now also emerging as master evaders of immunity (34). *Toxoplasma* has negative effects on MAPK, NFκB as well as STAT1 signaling pathways, although the mechanisms involved are less well defined (9, 59, 60). Our present studies reveal gene-specific targeting of chromatin structure as a new and profound strategy used by this intracellular protozoan to manipulate host cell responses.

REFERENCES

1. Alexander, J., and C. A. Hunter. 1998. Immunoregulation during toxoplasmosis. *Chem. Immunol.* 70:81-102.
2. Denkers, E. Y., and R. T. Gazzinelli. 1998. Regulation and function of T cell-mediated immunity during *Toxoplasma gondii* infection. *Clin. Microbiol. Rev.* 11:569-588.
3. Gazzinelli, R. T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent upon $CD4^+$ T cells and accompanied by overproduction of IL-12, IFN-g, and TNF-a. *J. Immunol.* 157:798-805.
4. Butcher, B. A., L. Kim, P. F. Johnson, and E. Y. Denkers. 2001. *Toxoplasma gondii* tachyzoites inhibit proinflammatory cytokine induction in infected macrophages by preventing nuclear translocation of the transcription factor NFkB. *J. Immunol.* 167:2193-2201.
5. Lee, C. W., S. Bennouna, and E. Y. Denkers. 2006. Screening for *Toxoplasma gondii* regulated transcriptional responses in LPS-activated macrophages. *Infect. Immun.* 74:1916-1923.
6. Luder, C. G. K., M. Algner, C. Lang, N. Bleicher, and U. Gross. 2003. Reduced expression of the inducible nitric oxide synthase after infection with *Toxoplasma gondii* facilitates parasite replication in activated murine macrophages. *Internat. J. Parasitol.* 33:833-844.
7. Lang, C., U. Gross, and C. G. Luder. 2006. Subversion of innate and adaptive immune responses by Toxoplasma Gondii. *Parasitol Res.*
8. Denkers, E. Y., L. Kim, and B. A. Butcher. 2003. In the belly of the beast: subversion of macrophage proinflammatory signaling cascades during

Toxoplasma gondii infection. *Cell. Micro.* 5:75-83.

9. Kim, L., B. A. Butcher, and E. Y. Denkers. 2004. *Toxoplasma gondii* interferes with lipopolysaccharide-induced mitogen-activated protein kinase activation by mechanisms distinct from endotoxin tolerance. *J. Immunol.* 172:3003-3010.
10. Shapira, S., O. Harb, J. Margarit, M. Matrajt, J. Han, A. Hoffmann, B. Freedman, M. J. May, D. S. Roos, and C. A. Hunter. 2005. Initiation and termination of NFkB signaling by the intracellular protozoan parasite *Toxoplasma gondii*. *J. Cell Sci.* 118:3501-3508.
11. Butcher, B. A., L. Kim, A. Panopoulos, S. S. Watowich, P. J. Murray, and E. Y. Denkers. 2005. Cutting Edge: IL-10-independent STAT3 activation by *Toxoplasma gondii* mediates suppression of IL-12 and TNF-a in host macrophages. *J. Immunol.* 174:3148-3152.
12. Saeij, J. P., S. Coller, J. P. Boyle, M. E. Jerome, M. W. White, and J. C. Boothroyd. 2007. Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature* 445:324-327.
13. Denkers, E. Y., B. A. Butcher, L. Del Rio, and L. Kim. 2004. Manipulation of mitogen-activated protein kinase/nuclear factor-kB-signaling cascades during intracellular *Toxoplasma gondii* infection. *Immunol. Rev.* 201:191-205.
14. Butcher, B. A., and E. Y. Denkers. 2002. Mechanism of entry determines ability of *Toxoplasma gondii* to inhibit macrophage proinflammatory cytokine production. *Infect. Immun.* 70:5216-5224.
15. Falvo, J. V., B. M. Brinkman, A. V. Tsytsykova, E. Y. Tsai, T. P. Yao, A. L. Kung, and A. E. Goldfeld. 2000. A stimulus-specific role for CREB-binding protein (CBP) in T cell receptor-activated tumor necrosis factor alpha gene expression. *Proc Natl Acad Sci U S A* 97:3925-3929.
16. Falvo, J. V., A. M. Uglialoro, B. M. Brinkman, M. Merika, B. S. Parekh, E. Y.

Tsai, H. C. King, A. D. Morielli, E. G. Peralta, T. Maniatis, D. Thanos, and A. E. Goldfeld. 2000. Stimulus-specific assembly of enhancer complexes on the tumor necrosis factor alpha gene promoter. *Mol Cell Biol* 20:2239-2247.

17. Tsai, E. Y., J. V. Falvo, A. V. Tsytsykova, A. K. Barczak, A. M. Reimold, L. H. Glimcher, M. J. Fenton, D. C. Gordon, I. F. Dunn, and A. E. Goldfeld. 2000. A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter in vivo. *Mol Cell Biol* 20:6084-6094.
18. Goldfeld, A. E., and T. Maniatis. 1989. Coordinate viral induction of tumor necrosis factor alpha and interferon beta in human B cells and monocytes. *Proc Natl Acad Sci U S A* 86:1490-1494.
19. Tsytsykova, A. V., and A. E. Goldfeld. 2002. Inducer-specific enhanceosome formation controls tumor necrosis factor alpha gene expression in T lymphocytes. *Mol Cell Biol* 22:2620-2631.
20. Kuprash, D. V., I. A. Udalova, R. L. Turetskaya, D. Kwiatkowski, N. R. Rice, and S. A. Nedospasov. 1999. Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. *J. Immunol.* 162:4045-4052.
21. Osman, F., N. Jarrous, Y. Ben-Asouli, and R. Kaempfer. 1999. A cis-acting element in the 3'-untranslated region of human TNF-alpha mRNA renders splicing dependent on the activation of protein kinase PKR. *Genes Dev* 13:3280-3293.
22. Dumitru, C. D., J. D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J. H. Lin, C. Patriotis, N. A. Jenkins, N. G. Copeland, G. Kollias, and P. N. Tsichlis. 2000. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* 103:1071-1083.

23. Lee, J. Y., N. A. Kim, A. Sanford, and K. E. Sullivan. 2003. Histone acetylation and chromatin conformation are regulated separately at the TNF-alpha promoter in monocytes and macrophages. *J Leukoc Biol* 73:862-871.

24. Garrett, S., K. Dietzmann-Maurer, L. Song, and K. E. Sullivan. 2008. Polarization of primary human monocytes by IFN-gamma induces chromatin changes and recruits RNA Pol II to the TNF-alpha promoter. *J Immunol* 180:5257-5266.

25. Sullivan, K. E., A. B. Reddy, K. Dietzmann, A. R. Suriano, V. P. Kocieda, M. Stewart, and M. Bhatia. 2007. Epigenetic regulation of tumor necrosis factor alpha. *Mol Cell Biol* 27:5147-5160.

26. Shivaswamy, S., A. Bhinge, Y. Zhao, S. Jones, M. Hirst, and V. R. Iyer. 2008. Dynamic remodeling of individual nucleosomes across a eukaryotic genome in response to transcriptional perturbation. *PLoS Biol* 6:e65.

27. Berger, S. L. 2007. The complex language of chromatin regulation during transcription. *Nature* 447:407-412.

28. Ait-Si-Ali, S., S. Ramirez, F. X. Barre, F. Dkhissi, L. Magnaghi-Jaulin, J. A. Girault, P. Robin, M. Knibiehler, L. L. Pritchard, B. Ducommun, D. Trouche, and A. Harel-Bellan. 1998. Histone acetyltransferase activity of CBP is controlled by cycle-dependent kinases and oncoprotein E1A. *Nature* 396:184-186.

29. Martinez-Balbas, M. A., A. J. Bannister, K. Martin, P. Haus-Seuffert, M. Meisterernst, and T. Kouzarides. 1998. The acetyltransferase activity of CBP stimulates transcription. *EMBO J* 17:2886-2893.

30. Li, B., M. Carey, and J. L. Workman. 2007. The role of chromatin during transcription. *Cell* 128:707-719.

31. Dyson, M. H., S. Thomson, M. Inagaki, H. Goto, S. J. Arthur, K. Nightingale,

F. J. Iborra, and L. C. Mahadevan. 2005. MAP kinase-mediated phosphorylation of distinct pools of histone H3 at S10 or S28 via mitogen- and stress-activated kinase 1/2. *J Cell Sci* 118:2247-2259.

32. Soloaga, A., S. Thomson, G. R. Wiggin, N. Rampersaud, M. H. Dyson, C. A. Hazzalin, L. C. Mahadevan, and J. S. Arthur. 2003. MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. *Embo J* 22:2788-2797.

33. Rosenberger, C. M., and B. B. Finlay. 2003. Phagocyte sabotage: disruption of macrophage signalling by bacterial pathogens. *Nat. Rev. Mol. Cell Biol.* 4: 385-396.

34. Sacks, D., and A. Sher. 2002. Evasion of innate immunity by parasitic protozoa. *Nature Immunol.* 3:1041-1047.

35. Muegge, K. 2002. Preparing the target for the bullet. *Nat Immunol* 3:16-17.

36. Cheung, P., C. D. Allis, and P. Sassone-Corsi. 2000. Signaling to chromatin through histone modifications. *Cell* 103:263-271.

37. Jenuwein, T., and C. D. Allis. 2001. Translating the histone code. *Science* 293:1074-1080.

38. Saccani, S., S. Pantano, and G. Natoli. 2002. p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. *Nat Immunol* 3:69-75.

39. Hamon, M. A., E. Batsche, B. Regnault, T. N. Tham, S. Seveau, C. Muchardt, and P. Cossart. 2007. Histone modifications induced by a family of bacterial toxins. *Proc Natl Acad Sci U S A* 104:13467-13472.

40. Wang, Y., H. M. Curry, B. S. Zwilling, and W. P. Lafuse. 2005. Mycobacteria inhibition of IFN-gamma induced HLA-DR gene expression by up-regulating histone deacetylation at the promoter region in human THP-1 monocytic cells.

J Immunol 174:5687-5694.

41. Pennini, M. E., R. K. Pai, D. C. Schultz, W. H. Boom, and C. V. Harding. 2006. Mycobacterium tuberculosis 19-kDa lipoprotein inhibits IFN-gamma-induced chromatin remodeling of MHC2TA by TLR2 and MAPK signaling. *J Immunol* 176:4323-4330.

42. Arbibe, L., D. W. Kim, E. Batsche, T. Pedron, B. Mateescu, C. Muchardt, C. Parsot, and P. J. Sansonetti. 2007. An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses. *Nat Immunol* 8:47-56.

43. Cheung, P., K. G. Tanner, W. L. Cheung, P. Sassone-Corsi, J. M. Denu, and C. D. Allis. 2000. Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. *Mol Cell* 5:905-915.

44. Lo, W. S., R. C. Trievel, J. R. Rojas, L. Duggan, J. Y. Hsu, C. D. Allis, R. Marmorstein, and S. L. Berger. 2000. Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14. *Mol Cell* 5:917-926.

45. Zhang, X., J. P. Edwards, and D. M. Mosser. 2006. Dynamic and transient remodeling of the macrophage IL-10 promoter during transcription. *J Immunol* 177:1282-1288.

46. Shapira, S. S., K. Speirs, A. Gerstein, J. Caamano, and C. A. Hunter. 2002. Suppression of NF-kB activation by infection with *Toxoplasma gondii*. *J. Infect. Dis.* 185:S66-72.

47. Ravindran, S., and J. C. Boothroyd. 2008. Secretion of proteins into host cells by Apicomplexan parasites. *Traffic* 9:647-656.

48. Gilbert, L. A., S. Ravindran, J. M. Turetzky, J. C. Boothroyd, and P. J. Bradley.

2007. Toxoplasma gondii targets a protein phosphatase 2C to the nuclei of infected host cells. *Eukaryot Cell* 6:73-83.

49. Laliberte, J., and V. B. Carruthers. 2008. Host cell manipulation by the human pathogen Toxoplasma gondii. *Cell Mol Life Sci* 65:1900-1915.

50. Debierre-Grockiego, F., M. A. Campos, N. Azzouz, J. Schmidt, U. Bieker, M. G. Resende, D. S. Mansur, R. Weingart, R. R. Schmidt, D. T. Golenbock, R. T. Gazzinelli, and R. T. Schwarz. 2007. Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from Toxoplasma gondii. *J Immunol* 179:1129-1137.

51. Yarovinsky, F., D. Zhang, J. F. Anderson, G. L. Bannenberg, C. N. Serhan, M. S. Hayden, S. Hieny, F. S. Sutterwala, R. A. Flavell, S. Ghosh, and A. Sher. 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science* 308:1626-1629.

52. Plattner, F., F. Yarovinsky, S. Romero, D. Didry, M. F. Carlier, A. Sher, and D. Soldati-Favre. 2008. Toxoplasma profilin is essential for host cell invasion and TLR dependent induction of interleukin-12. *Cell Host and Microbe* 14:77-87.

53. Wichroski, M. J., and G. E. Ward. 2003. Biosynthesis of glycosylphosphatidylinositol is essential to the survival of the protozoan parasite Toxoplasma gondii. *Eukaryot Cell* 2:1132-1136.

54. Mukherjee, S., G. Keitany, Y. Li, Y. Wang, H. L. Ball, E. J. Goldsmith, and K. Orth. 2006. Yersinia YopJ acetylates and inhibits kinase activation by blocking phosphorylation. *Science* 312:1211-1214.

55. Ruckdeschel, K., J. Machold, A. R. Roggenkamp, S. Schubert, J. Pierre, R. Zumbihl, J.-P. Liautard, J. Heesemann, and B. Rouot. 1997. *Yersinia enterocolitica* promotes deactivation of macrophage mitogen-activated protein kinases extracellular signal-regulated kinase-1/2, p38, and c-Jun NH_2-terminal

kinase. *J. Biol. Chem.* 272:15920-15927.

56. Ruckdeschel, K., O. Mannel, K. Richter, C. A. Jacobi, K. Trulzsch, B. Rouot, and J. Heesemann. 2001. *Yersinia* outer protein P of *Yersinia enterocolitica* simutaneously blocks the nuclear factor-kB pathway and exploits lipopolysaccharide signaling to trigger apoptosis. *J. Immunol.* 166:1823-1831.

57. Viboud, G. I., and J. B. Bliska. 2005. Yersinia outer proteins: role in modulation of host cell signaling responses and pathogenesis. *Annu Rev Microbiol* 59:69-89.

58. Park, J. M., F. R. Greten, Z.-W. Li, and M. Karin. 2002. Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. *Science* 297:2048-2051.

59. Kim, S. K., A. E. Fouts, and J. C. Boothroyd. 2007. Toxoplasma gondii dysregulates IFN-gamma-inducible gene expression in human fibroblasts: insights from a genome-wide transcriptional profiling. *J Immunol* 178:5154-5165.

60. Luder, C. G. K., W. Walter, B. Beuerle, M. J. Maeurer, and U. Gross. 2001. *Toxoplasma gondii* down-regulates MHC class II gene expression and antigen presentation by murine macrophages via interference with nuclear translocation of STAT1a. *Eur. J. Immunol.* 31:1475-1484.

CHAPTER THREE

Toxoplasma gondii Inhibits Chromatin Remodeling at the IL-10 Promoter Triggered by Immune Complex plus LPS

ABSTRACT

IL-10 is an important immune regulatory cytokine. Macrophages stimulated with LPS produce low amounts of IL-10. Previously, we found that the opportunistic protozoan *Toxoplasma gondii* does not influence IL-10 production in macrophages upon LPS stimulation, the latter of which does not incur chromatin remodeling. However, we find here that infection does block the high level of IL-10 production in macrophages stimulated with LPS plus immune complex. In this study, we also found that LPS plus immune complex stimulation triggers chromatin remodeling at the IL-10 promoter and that infection blocks chromatin remodeling. Specifically, the parasite inhibits the Ser^{10} phosphorylation and Lys^{9}/Lys^{14} acetylation on the IL-10 promoter. Our results show that *Toxoplasma* inhibits IL-10 transcription by interfering with chromatin remodeling events required for transcriptional activation on the IL-10 promoter.

INTRODUCTION

Macrophages play a key role in innate immunity. When activated by bacterial products such as LPS, they produce a variety of inflammatory cytokines including TNF, IL-1, IL-6 and IL-12. This inflammatory response is critical in clearing the infection. To avoid immunopathological damage to the host, inflammation needs to be tightly controlled by the immune system. Anti-inflammatory regulatory cytokines such as IL-10 and TGF-β can prevent the overproduction of inflammatory cytokines such as TNF and IL-12 (1, 2).

Monocytes, including macrophages, are the main producers of IL-10. This cytokine has been shown to have pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th1 cytokines, MHC class II antigens, and costimulatory molecules on macrophages (3, 4). IL-10 also displays a potent ability to suppress the antigen presentation capacity of antigen presenting cells (5). On the other hand, IL-10 also enhances B cell survival, proliferation, and antibody production (6). This cytokine can block NF-κB activity, and is involved in the regulation of the JAK-STAT signaling pathway (7). Knockout studies in mice suggested the function of this cytokine as an essential immunoregulator during *Toxoplasma* infection, as the knock out mice succumb to immunopathology due to uncontrolled inflammatory responses (8-10).

Activated macrophages produce inflammatory cytokines such as TNFα and the production of IL-10 normally follows the induction of inflammatory cytokines to prevent their overproduction. It has been shown the LPS induced synthesis of IL-10 is very low (11). However, in the presence of immune complex, activated macrophages produce large amounts of IL-10 (12). The mechanism of this superinduction of IL-10

has caused much interest. Recently, it has been shown that while LPS alone does not induce chromatin structural changes at the IL-10 locus, immune complex does induce a transient chromatin remodeling at the IL-10 promoter with an increase in phosphorylation of histone H3 at Ser^{10} and acetylation of histone H3 at Lys^{14} (13, 14). It was therefore proposed that LPS activates MAPK pathways and transcription factors such as NFκB, while immune complex induces remodeling of chromatin structure on the IL-10 locus. The combination of these two factors superinduces IL-10 in the macrophages.

The opportunistic intracellular protozoan *Toxoplasma gondii* actively inhibits a large panel of immune cytokine genes upon LPS stimulation including IL-12p40 and TNFα (15, 16). It has been shown that *T. gondii* inhibits the TNFα response by blocking histone modifications required for TNFα gene transcription (11). We also found that *T. gondii* infection does not influence the low level of IL-10 induction by LPS. It was therefore interesting to investigate whether the parasite is able to block the superinduction of IL-10 by LPS plus immune complex, especially since this superinduction requires chromatin remodeling on the IL-10 locus.

In this study, we show that *T. gondii* inhibits the IL-10 response superinduced by LPS and immune complex, but not the low level of IL-10 induced by LPS alone. Interestingly, by examining the histone modifications occuring at the IL-10 locus, we found that the parasite is able to block the phosphorylation of histone H3 at Ser^{10} and acetylation of histone H3 at Lys^{9} and Lys^{14} induced by immune complex. These results provide further support for the idea that *T. gondii* targets chromatin structure to suppress the cytokine gene transcription. This is possibly a unifying strategy for the parasite's suppressive effect.

MATERIAL AND METHODS

Mice and parasite

C57BL/6 female mice (6–8 wk of age) were purchased from The Jackson Laboratory. The mice were kept under specific pathogen-free conditions at the Transgenic Mouse Facility, Cornell University College of Veterinary Medicine. The facility is overseen by an Institutional Animal Care and Use Committee. *T. gondii* parasite RH strain was maintained by biweekly passage on human foreskin fibroblast monolayers in DMEM supplemented with 1% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Parasite cultures were tested for *Mycoplasma* every 6–8 wk using a highly sensitive PCR-based ELISA (Roche Diagnostics).

Cell culture

Bone marrow cells were flushed from femur and tibia and cultured in complete DMEM consisting of DMEM supplemented with 10% FCS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20% supernatant from L929 cells, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cells were supplemented with fresh macrophage medium on day 3. After 5 days of culture, nonadherent cells were removed, adherent monolayers were washed in ice-cold PBS, and cells were harvested by gentle pipetting in DMEM supplemented with 1% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Infection of macrophages was accomplished by adding tachyzoites to cell cultures followed by brief centrifugation (200 x *g* for 3 min) to synchronize contact between cells and parasites. In most cases, immune complex and LPS were added 12 h after infection. Cells were recovered at varying times as indicated, depending upon the assay performed.

Immune Complex

IgG-opsonized erythrocytes (E-IgG) were generated by incubating sheep red blood cells (SRBC, Lampire Biological Laboratories) with anti-SRBC IgG (MP Biomedicals) at nonagglutinating titers for 30 min at room temperature while rotating. Nonagglutinating titers were determined by titrating IgG concentrations. Opsonized cells were washed once in HBSS (Invitrogen Life Technologies) and resuspended in complete medium. E-IgG were added to macrophages at a ratio of 10:1 E-IgG to macrophages.

Semiquantitative real-time PCR

Real-time PCR was performed with a Power SYBR green kit according to the manufacturer's instructions (catalog no. 4367659; Applied Biosystems). The primers used were as follows: IL-10 forward CCTGGCTCAGCACTGCTAT; IL-10 reverse: GCTCTTATTTTCACAGGGGAGAA; GAPDH forward: CCTGAACAGAACAGCAATGGCT; GAPDH reverse: GCTTGACGGTGTCTTTTGCCT; IL-10 nucleosome 2 forward: GCAGAAGTTCATTCCGACCA; IL-10 nucleosome 2 reverse: GGCTCCTCCTCCCTCTTCTA.

Cytokine ELISA

IL-10 in cell cultures was measured using commercial kits according to the manufacturer's recommendations (R&D Systems).

Immunoblotting

The following Abs were used in immunoblotting studies: anti-phospho-histone H3 (Ser^{10}; catalog no. 9701; Cell Signaling). Cells (2×10^6/sample) were lysed in reducing

SDS sample buffer, and DNA was sheared by forcing samples five times through a 27-gauge needle. In some experiments, nuclear and cytoplasmic portions of cell lysates were separated by using a nuclear extract kit (Active Motif). After 5 min at 100°C, samples were separated by 10% SDS-PAGE and proteins were subsequently electrotransferred onto nitrocellulose membranes. Membranes were then blocked in 5% nonfat dry milk containing 0.1% Tween 20 in TBS, pH 7.6 (TBST), for 1 hr at room temperature, followed by incubation with Ab reconstituted in 5% BSA in TBST overnight at 4°C. After washing blots in TBST, primary Abs were detected with a HRP-conjugated secondary Ab in TBST containing 5% nonfat dry milk for 1 hr at room temperature. After washing in TBST, protein bands were visualized using a chemiluminescence-based detection system (Cell Signaling).

Chromatin immunoprecipitation (ChIP)

The following ChIP grade Abs were used for immunoprecipitation: anti-phospho-histone H3 (Ser^{10}; catalog no. 9701; Cell Signaling), and anti-acetyl histone H3 (Lys^{9}/Lys^{14}; catalog no. 9677; Cell Signaling). Assays were performed using the ChIP-IT enzymatic express kit (Active Motif) according to the manufacturer's instructions. Briefly, cells (1.5×10^{7}/sample) were fixed using 1% formaldehyde at room temperature for 10 min. Fixation was stopped by adding glycine to the mixture. The cells were then collected by scraping in buffer containing PMSF (100 mM). After brief centrifugation, the cells were resuspended in lysis buffer (Active Motif) with a protease inhibitor mixture (Active Motif) and incubated for 30 min on ice. The cells were then resuspended in cell digestion buffer (Active Motif) and subjected to enzymatic digestion for 10 min at 37°C. The reaction was stopped with addition of 0.5 M EDTA. Abs were added into the sheared chromatin preparations and the mixture was incubated with Protein G Magnetic Beads (Active Motif) overnight at 4°C. The

precipitated DNA-protein-Ab complexes were then washed and the cross-linking was reversed by incubation at 65°C for 4 h. Proteinase K was added to digest protein and DNA subsequently was purified using ethanol extraction, air dried, and redissolved in 100 µl of H_2O. The retrieved DNA was then subjected to real-time RT-PCR amplification using promoter-specific primers.

RESULTS

T. gondii inhibits high level IL-10 production induced by LPS plus immune complex, but does not influence the low level of IL-10 induced by LPS alone.

Previously, we showed that *T. gondii* infection inhibits LPS induced TNFα production in macrophage while it has no effect on the IL-10 response induced by LPS (Chapter 2). To confirm this result, we used the type I strain *T. gondii* RH strain to infect the macrophages. When these macrophages were stimulated with LPS, we found that the cells produced ~1000 pg/ml IL-10 and this response was not influenced by *T. gondii* infection (Figure 3.1). When we used LPS to stimulate the macrophages in the presence of immune complex, we found that the cells produced three-fold more IL-10. However, when macrophages were infected with *Toxoplasma*, this super-induction response was blocked. In accord with the cytokine protein data, we found that LPS plus immune complex induced IL-10 mRNA production was also blocked by the parasite (Figure 3.2). In contrast, we found that LPS induced IL-10 mRNA was left inact in the presence of infection (data not shown).

IL-10 promoter chromatin remodeling induced by immune complex plus LPS is blocked by T. gondii

It has been shown that immune complex induces chromatin remodeling at the IL-10 locus. Stimulation induces a transient increase in the phosphorylation of histone H3 at Ser^{10} followed by an increase in the acetylation of histone H3 at Lys^{14}. This process enables remodeling of chromatin structure at the IL-10 locus and allows the binding of activated transcription factors at the IL-10 promoter. We therefore sought to investigate the influence of *T. gondii* infection on the histone modifications at the IL-10 locus induced by immune complex. We used the type I strain *T. gondii* RH strain to infect macrophages. The cells were then stimulated with LPS alone or a combination

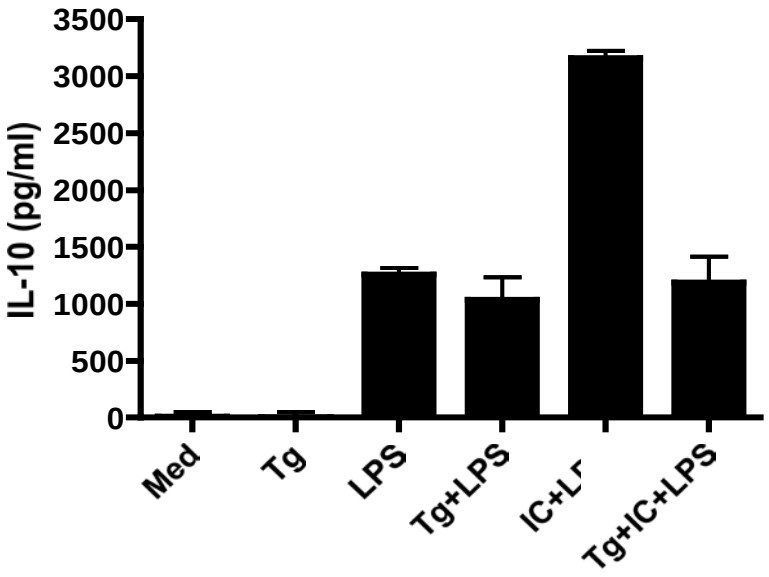


Figure 3.1 *Toxoplasma* inhibits IL-10 protein superinduced by LPS and immune complex. Macrophages were infected with *T. gondii* RH strain tachyzoites at a parasite to cell ratio of 2:1. After 18 hr, cells were treated with either LPS (10 ng/ml) or immune complex plus LPS. The supernatants were collected 6 hr later and cytokine ELISA was performed to measure IL-10 concentrations. Med, medium; Tg, *T. gondii*; IC, immune complex.

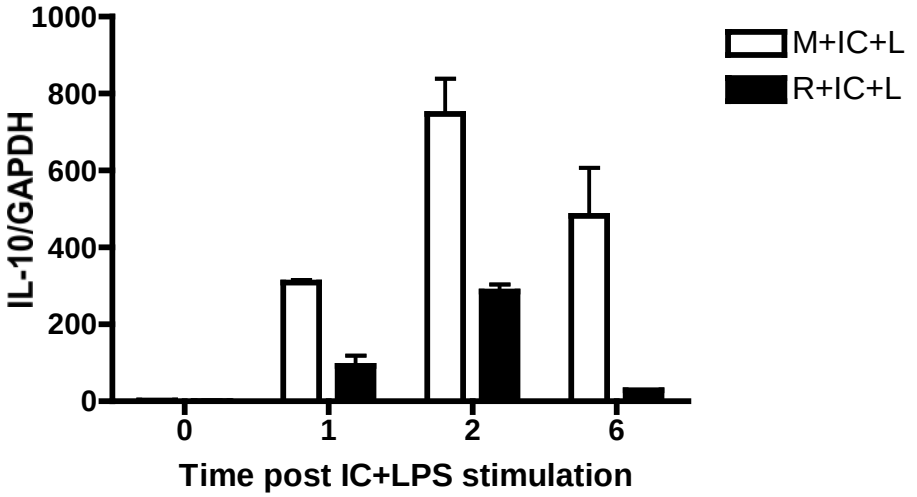


Figure 3.2 *Toxoplasma* inhibits IL-10 mRNA superinduced by LPS and immune complex. Macrophages were infected with *T. gondii* RH strain tachyzoites at a parasite to cell ratio of 2:1 for 18 hr. The cells were then subjected to immune complex plus LPS (10ng/ml) stimulation. RNA was then extracted at indicated time points (hr) and reversed transcribed into cDNA. Real time PCR was performed to measure the IL-10 mRNA. GAPDH was used as an internal control. M, medium; IC, immune complex; R, RH strain; L, LPS.

of LPS and immune complex. We chose to examine the phosphorylation of histone H3 at Ser^{10} at 30 minutes post stimulation and the acetylation of histone H3 at Lys^{9}/Lys^{14} at 60 minutes post stimulation as these two time points have been shown to be the peak time for these two histone modifications, respectively (13). Using a chromatin immunoprecipitation (ChIP) assay, we found that while LPS alone does not induce any chromatin changes on the IL-10 locus, LPS combined with immune complex induces an increase of both modifications at 30 and 60 minutes post stimulation, respectively. However, infection with *T. gondii* inhibits both histone modifications induced by LPS and immune complex, while having no effects on LPS stimulated cells (Figure 3.3 and 3.4).

T. gondii inhibits global phosphorylation of histone H3 at Ser^{10} induced by LPS or Immune Complex

Previously in Chapter 2 we showed that *T. gondii* infection blocks LPS induced global phosphorylation of histone H3 at Ser^{10} in macrophages. We were then interested in examining whether immune complex in the presence of LPS induced global phosphorylation of histone H3 at Ser^{10} and whether this response is influenced by the parasite. When macrophages were stimulated with LPS at 10 ng/ml, we detected an increased global level of phosphorylation of histone H3 at Ser^{10} in cell lysates, which starts 30 min post stimulation and lasts up to 120 min post stimulation. Upon infection with RH, phosphorylation of histone H3 at Ser^{10} in cell lysates is greatly decreased. With a combination of immune complex and LPS, we are able to induce a similar response in macrophages, and as we expected, immune complex and LPS induced phosphorylation of histone H3 at Ser^{10} was also blocked by *T. gondii* infection (Figure 3.5).

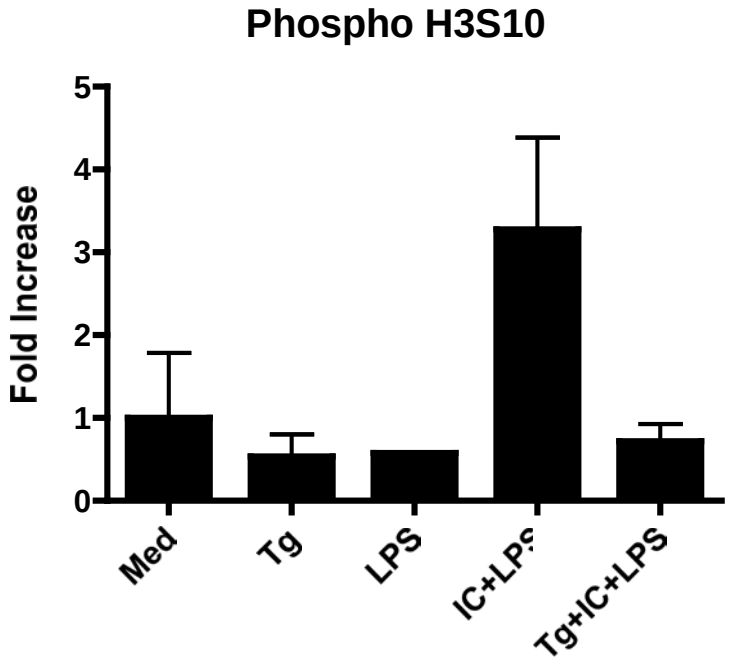


Figure 3.3 *Toxoplasma* infection blocks phosphorylation of histone H3 at Ser 10 at the IL-10 promoter induced by LPS and immune complex. Macrophages were infected with *T. gondii* RH strain tachyzoites at a parasite to cell ratio of 2:1. After overnight infection, the cells were then subjected to either LPS (100 ng/ml) or immune complex plus LPS stimulation. The nuclei from each sample was extracted at 30 mins post stimulation and then subjected to ChIP assay using antibody against phosphorylated H3 at Ser^{10} and primers specific for the IL-10 promoter. The results were normalized to corresponding input controls. Med, medium; Tg, *T. gondii*; IC, immune complex.

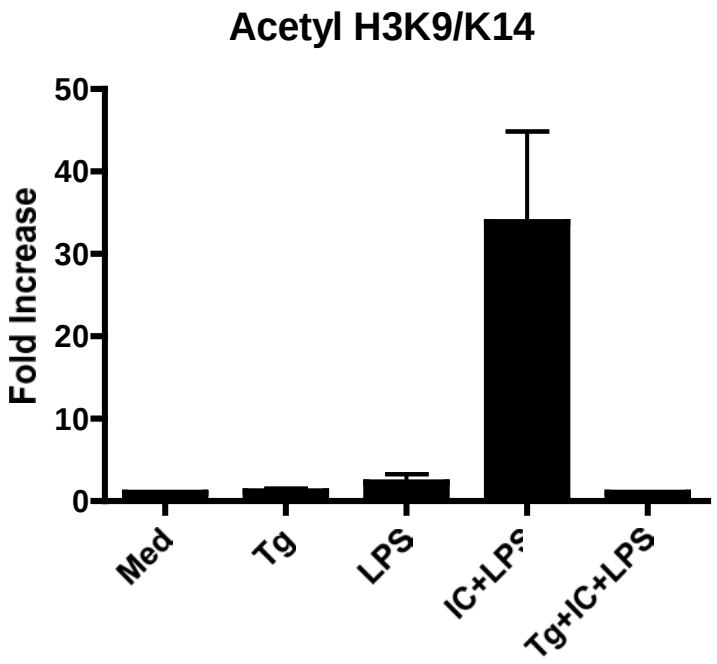


Figure 3.4 *Toxoplasma* infection blocks acetylation of histone H3 at Lys9/Lys14 at the IL-10 promoter induced by LPS and immune complex. Macrophages were infected with *T. gondii* RH strain tachyzoites at a parasite to cell ratio of 2:1. After overnight infection, the cells were then subjected to either LPS (100ng/ml) or immune complex plus LPS stimulation. The nuclei from each sample was extracted at 60 mins post stimulation and then subjected to ChIP assay using antibody against acetylated H3 at Lys^{9}/Lys^{14} and primers specific for the IL-10 promoter. The results were normalized to corresponding input controls. Med, medium; Tg, *T. gondii*; IC, immune complex.

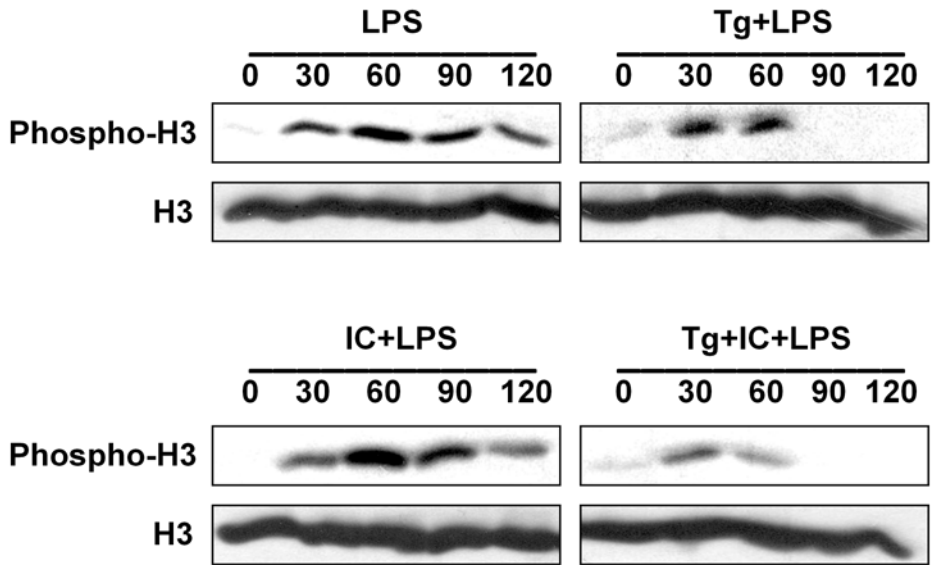


Figure 3.5 *Toxoplasma* blocks immune complex and LPS induced global phosphorylation of H3 at Ser^{10}. Macrophages were infected with *T. gondii* RH strain tachyzoites at a parasite to cell ratio of 2:1. 18 hr later, the cells were subjected to either LPS (10 ng/ml) or immune complex plus LPS stimulation. The cells were lysed at indicated time points (mins) and cell lysates were subjected to Western Blotting assay using antibody against phosphorylated H3 at Ser^{10}. Total H3 was used to control the protein loading amounts. Tg, *T. gondii*; IC, immune complex.

Only Type I Toxoplasma RH strain inhibits IL-10 induced by Immune Complex plus LPS

Up to now we have confirmed that *Toxoplasma* Type I RH strain inhibits IL-10 super-induction by immune complex in the presence of LPS. We were then interested to know whether other *T. gondii* strains, especially Type II and Type III strains, have a similar effect on the immune complex induced IL-10 response. In these experiments, we used Type II PTG and Type III VEG tachyzoites to infect macrophages and then subjected the infected cells to immune complex stimulation. To our surprise, the tested Type II and Type III strains did not have an obvious inhibitory effect on immune complex induced IL-10 compared to Type I RH strain (Figure 3.6). Instead, we observed a synergistic effect of PTG on IL-10 production at low MOI no matter whether the cells were treated with LPS or immune complex plus LPS. Although we have not yet tested additional strains, our results suggest that only the Type I strain inhibits IL-10 super-induction by immune complex and LPS. We need to validate these preliminary results by performing more repeats.

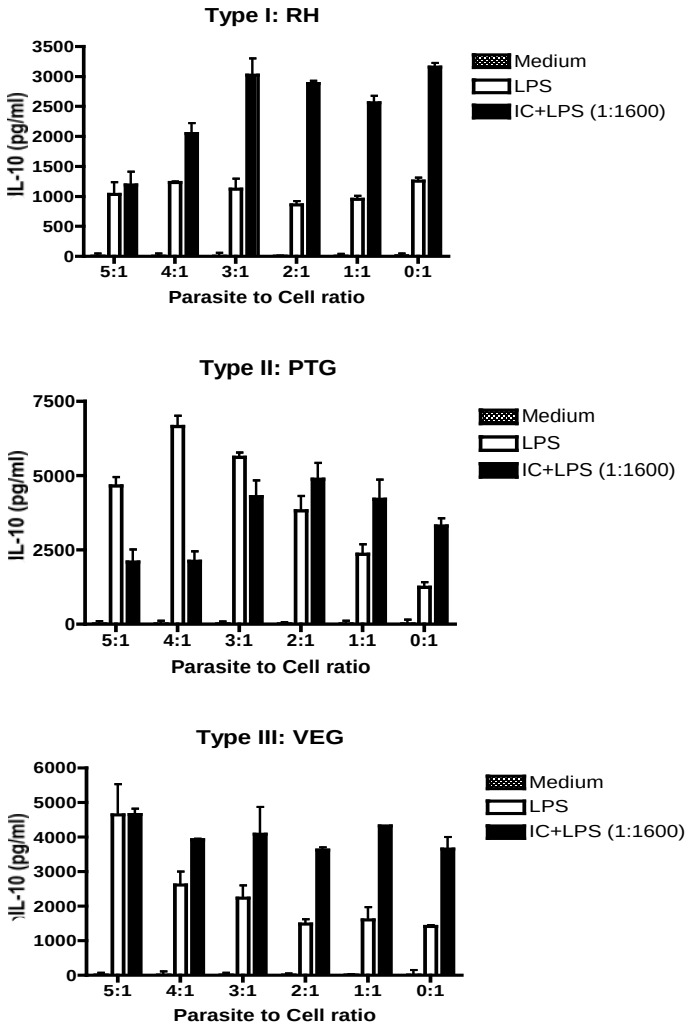


Figure 3.6 *Toxoplasma* Type II and Type III strains do not inhibit IL-10 super-induction by immune complex and LPS. Macrophages were infected with *T. gondii* RH, PTG or VEG strain tachyzoites at indicated parasite to cell ratios. The cells were then subjected to either LPS (100 ng/ml) or immune complex plus LPS stimulation. Supernatants were collected 6h later and subjected to cytokine ELISA to measure the IL-10 concentrations. IC, immune complex.

DISCUSSION

The opportunistic intracellular parasite *T. gondii* actively suppresses the proinflammatory responses in macrophages. In Chapter 2, we showed that the parasite inhibits the LPS-induced TNFα response in macrophages by interfering with chromatin remodeling at the TNF promoter region required for transcriptional initiation. In this way, the parasite prevents the binding of activated transcription factors to the promoter region.

In the previous study, we also found that LPS induced a low amount of IL-10 production in macrophages, and *T. gondii* did not block this response. In terms of chromatin remodeling, we found that LPS did not induce any chromatin structural changes at IL-10 locus and the parasite had no influence on the IL-10 locus (11). Since macrophage activation in the presence of immune complex induces a high level of IL-10 and this response requires chromatin remodeling on the IL-10 locus, we were intrigued to investigate whether *T. gondii* inhibits IL-10 superinduced by LPS and immune complex. Furthermore, it was of interest to know if the parasite blocks histone modifications on the IL-10 locus upon superinduction with LPS and immune complex.

In this study, we found that *T. gondii* inhibits high level IL-10 production induced by LPS plus immune complex, but does not influence the low level of IL-10 induced by LPS alone. Moreover, immune complex/LPS induced IL-10 promoter chromatin remodeling was also blocked by the parasite. In some ways, these results are within our expectations as we previously found that the parasite uses a similar strategy to inhibit the TNFα response induced by LPS.

The immune complex induced IL-10 response is found to involve FcγR ligation followed by ERK and p38 activation (14). ERK activation leads to phosphorylation of histone H3 Ser^{10}. However, when p38 or ERK activity is inhibited, the IL-10 response is blocked indicating that both MAPKs are required for super-induction of IL-10. Our study did not examine the effect of *T. gondii* on activation of p38 or ERK by immune complex and LPS, but it remains possible that the parasite also interferes with these two MAPKs.

Even though the *Toxoplasma* parasitophorous vacuole evades fusion with the host lysosome, the parasite still manages to deliver some molecules to the host cell nucleus although the exact functions of these molecules are still under investigation. For example, PP2C-hn, a parasite phosphatase, is injected into the host nuclei after invasion. Although disruption of PP2C-hn had no obvious effects on parasite growth, it is still possible that some host elements are modulated by this phosphatase (17). Another parasite rhoptry protein, ROP16, is found to accumulate inside host nuclei shortly after invasion. ROP16 contains a kinase domain and is indirectly related with the rapid and sustained tyrosine phosphorylation of host STAT3 after infection (18, 19), because ROP16 is a putative serine/threonine kinase.

Combining the findings in this Chapter and Chapter 2, it is reasonable to speculate that some parasite effector proteins target the host nucleus, especially host chromatin structure to fulfill their functions such as shutting down some sets of genes while activating others to benefit the parasite's intracellular survival. Another possibility is that the parasite can act in the cytoplasm by interfering with activation and/or nuclear translocation of host effectors.

We also observed in this study that *Toxoplasma* is able to block the global phosphorylation of histone H3 at Ser 10 induced by immune complex and LPS. This is within our expectations since we obtained similar data with LPS stimulation. The parasite's ability to shut down the global phosphorylation of histone H3 at Ser^{10} is of great significance as the epigenetic signal of phosphorylation of histone H3 at Ser^{10} is shown to be important for transcription of many genes, including those controlled by NFκB. Studies showed that phosphorylation of histone H3 at Ser^{10} allows binding access to NFκB on some cytokine and chemokine gene promoters, such as IL-8 (20, 21). Therefore, *Toxoplasma*'s ability to inhibit phosphorylation of histone H3 at Ser^{10} might be involved in its ability to shut down a large panel of inflammatory genes. Future studies will be required to further evaluate this hypothesis.

By comparing all three types of *Toxoplasma*, we found that only Type I RH strain clearly inhibits IL-10 superinduction by immune complex and LPS. However, we are not yet able to conclude the effects of Type II PTG and Type III VEG strains on IL-10 superinduction. This is interesting because Type I strains are the most virulent during in vivo infection and Type II and Type III are more moderate.

With the future identification of parasite molecules that are delivered into host cell, we can expect to see more functions to be discovered. In particular, we can expect to determine molecules involved in the modulation of host signaling and possibly in the interference of host chromatin remodeling.

REFERENCES

1. Fiorentino, D. F., A. Zlotnick, T. Mosmann, M. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* 147:3815-3822.
2. Oswald, I. P., R. T. Gazzinelli, A. Sher, and S. L. James. 1992. IL-10 synergizes with IL-4 and transforming growth factor-b to inhibit macrophage cytotoxic activity. *J. Immunol.* 148:3578-3582.
3. Bai, X. F., J. Zhu, G. X. Zhang, G. Kaponides, B. Hojeberg, P. H. van der Meide, and H. Link. 1997. IL-10 suppresses experimental autoimmune neuritis and down-regulates TH1-type immune responses. *Clin Immunol Immunopathol* 83:117-126.
4. Mosmann, T. R., and K. W. Moore. 1991. The role of IL-10 in crossregulation of TH1 and TH2 responses. *Immunol Today* 12:A49-53.
5. Fiorentino, D. F., A. Zlotnik, P. Vieira, T. R. Mosmann, M. Howard, K. W. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 146:3444-3451.
6. Itoh, K., and S. Hirohata. 1995. The role of IL-10 in human B cell activation, proliferation, and differentiation. *J Immunol* 154:4341-4350.
7. Wehinger, J., F. Gouilleux, B. Groner, J. Finke, R. Mertelsmann, and R. M. Weber-Nordt. 1996. IL-10 induces DNA binding activity of three STAT proteins (Stat1, Stat3, and Stat5) and their distinct combinatorial assembly in the promoters of selected genes. *FEBS Lett* 394:365-370.
8. Suzuki, Y., A. Sher, G. Yap, D. Park, L. Ellis Neyer, O. Liesenfeld, M. Fort, H. Kang, and E. Gufwoli. 2000. IL-10 is required for prevention of necrosis in the small intestine and mortality in both genetically resistant BALB/c and

susceptible C57BL/6 mice following peroral infection with *Toxoplasma gondii*. *J. Immunol.* 164:5375-5382.

9. Gazzinelli, R. T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent upon $CD4^+$ T cells and accompanied by overproduction of IL-12, IFN-g, and TNF-a. *J. Immunol.* 157:798-805.
10. Wilson, E. H., U. Wille-Reece, F. Dzierszinski, and C. A. Hunter. 2005. A critical role for IL-10 in limiting inflammation during toxoplasmic encephalitis. *J Neuroimmunol* 165:63-74.
11. Leng, J., B. A. Butcher, C. E. Egan, D. S. Abdallah, and E. Y. Denkers. 2009. Toxoplasma gondii prevents chromatin remodeling initiated by TLR-triggered macrophage activation. *J Immunol* 182:489-497.
12. Anderson, C. F., J. S. Gerber, and D. M. Mosser. 2002. Modulating macrophage function with IgG immune complexes. *J. Endotoxin Res.* 8:477-481.
13. Zhang, X., J. P. Edwards, and D. M. Mosser. 2006. Dynamic and transient remodeling of the macrophage IL-10 promoter during transcription. *J Immunol* 177:1282-1288.
14. Lucas, M., X. Zhang, V. Prasanna, and D. M. Mosser. 2005. ERK activation following macrophage FcgammaR ligation leads to chromatin modifications at the IL-10 locus. *J Immunol* 175:469-477.
15. Butcher, B. A., and E. Y. Denkers. 2002. Mechanism of entry determines ability of *Toxoplasma gondii* to inhibit macrophage proinflammatory cytokine production. *Infect. Immun.* 70:5216-5224.

16. Lee, C. W., S. Bennouna, and E. Y. Denkers. 2006. Screening for Toxoplasma gondii-regulated transcriptional responses in lipopolysaccharide-activated macrophages. *Infect Immun* 74:1916-1923.

17. Gilbert, L. A., S. Ravindran, J. M. Turetzky, J. C. Boothroyd, and P. J. Bradley. 2007. Toxoplasma gondii targets a protein phosphatase 2C to the nuclei of infected host cells. *Eukaryot Cell* 6:73-83.

18. Saeij, J. P., J. P. Boyle, S. Coller, S. Taylor, L. D. Sibley, E. T. Brooke-Powell, J. W. Ajioka, and J. C. Boothroyd. 2006. Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science* 314:1780-1783.

19. Saeij, J. P., S. Coller, J. P. Boyle, M. E. Jerome, M. W. White, and J. C. Boothroyd. 2007. Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature* 445:324-327.

20. Arbibe, L., D. W. Kim, E. Batsche, T. Pedron, B. Mateescu, C. Muchardt, C. Parsot, and P. J. Sansonetti. 2007. An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses. *Nat Immunol* 8:47-56.

21. Cerutti, H., and J. A. Casas-Mollano. 2009. Histone H3 phosphorylation: universal code or lineage specific dialects? *Epigenetics* 4:71-75.

CHAPTER FOUR

Molecular Characterization of Immunosuppression by *Toxoplasma gondii*: Further Studies

ABSTRACT

In this study, we further examined the characteristics of suppression mediated by *Toxoplasma gondii*. We examined the influence of *T. gondii* infection on both MyD88 dependent and independent TLR signaling pathways in macrophages. During stimulation with various ligands of different TLR, we found that *T. gondii* blocks the IL-12 response induced by these ligands. In addition, we examined the MyD88 independent TLR signaling pathways triggered by TLR3 and TLR4 that lead to Type I Interferon production. These responses were also inhibited by the parasite. We also compared the effects of different parasite strains on TNF production in dendritic cells. We found that only Type I strain parasites inhibited TNF production in dendritic cells. We then examined the effects of different parasite strains on the global level of phosphorylation of histone H3 at Ser^{10} in macrophages and found that the Type I RH strain has the most potent inhibitory effect compared to Type II and Type III strains.

INTRODUCTION

Innate immunity is critical in the immune system for sensing and reacting to microbial infection. Toll like receptors (TLRs) play an essential role in recognizing the pathogen-associated molecular patterns (PAMPs) (1). TLRs have evolved into more than thirteen individual receptors to recognize a variety of pathogen molecules. As of today, a total of eleven TLRs have been identified within human beings and thirteen in mice, recognizing bacterial products like LPS, virus components such as ssRNA, DNA, and proteins such as bacterial flagellin (2).

Most TLRs depend on an important adapter protein, MyD88, for their signal transduction. However, TLR3 is an exception in that its signaling transduction does not involve MyD88. TLR4 has also been shown to trigger both MyD88 dependent and independent pathways upon ligand binding. Specifically, the MyD88 dependent TLR4 pathway leads to the activation of mitogen-activated protein kinase (MAPK) and NFκB pathways, initiating inflammatory cytokine production. MyD88 independent TLR4 and TLR3 pathways signal through TIR-domain-containing adapter-inducing interferon-β (TRIF), which leads to activation of the transcription factor IRF3 and induces IFN-inducible genes and IFN-β (3).

Previously, our laboratory has shown that *T. gondii* actively interferes with macrophage function during intracellular infection. Specifically, the parasite is able to inhibit LPS-triggered TLR4 signaling leading to IL-12 and TNFα production. The infected cells are not responsive to LPS stimulation and TLR4 signal transduction is impaired (4, 5). We were therefore interested in investigating whether *T. gondii* infection has any effects on signaling pathways triggered by other TLR ligands. We

were especially interested in understanding the influence of parasite infection on TLR signaling that involves MyD88 independent pathways.

There is evidence that *T. gondii* preferentially uses cells of innate immunity, especially macrophages and DCs, as reservoirs of infection and early dissemination (6-9). Therefore we were interested in expanding our studies to examine the effects of parasite infection on dendritic cell (DC) signaling. Furthermore, previous studies indicated strain specific differences in parasite induced IL-12 responses (10). Therefore, we also wanted to compare parasite strain types I, II and III in terms of interference in DC signaling.

Previously (Chapter 2 and Chapter 3), we established that the *T. gondii* RH strain inhibits global phosphorylation of histone H3 at Ser^{10} in macrophages induced by LPS and/or immune complex and LPS. In this study, we wanted to examine whether other parasites, in particular Type II and Type III strains, have the similar effects on global phosphorylation of histone H3.

In this study, we found that *T. gondii* inhibits the IL-12 response triggered by all tested TLR ligands. Moreover, by using MyD88 knockout mice, we found that both MyD88 dependent and independent signaling in macrophages is inhibited by the parasite, as Type I interferon production triggered by either TLR3 ligand Poly IC or TLR4 ligand LPS is blocked by infection. We also found that in DCs, LPS induced TNF responses are inhibited by Type I RH strain infection, but not by Type II or Type III strains. In addition, the Type I RH strain displays the most potent inhibitory effect on LPS induced global phosphorylation of histone H3 at Ser^{10}.

MATERIAL AND METHODS

Mice and parasites

C57BL/6 female mice (6–8 wk of age) were purchased from The Jackson Laboratory. TLR2/4 knock out mice were provided by Dr. David Russell (Cornell University). The mice were kept under specific pathogen-free conditions at the Transgenic Mouse Facility, Cornell University College of Veterinary Medicine. The facility is overseen by an Institutional Animal Care and Use Committee. *T. gondii* parasite strains RH, PTG and VEG were maintained by biweekly passage on human foreskin fibroblast monolayers in DMEM supplemented with 1% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. In some experiments, we used transgenic RH strain tachyzoites expressing tandem copies of the gene encoding yellow fluorescent protein (provided by D. Roos, University of Pennsylvania, Philadelphia, PA, and B. Striepen, University of Georgia, Athens, GA). Parasite cultures were tested for *Mycoplasma* every 6–8 wk using a highly sensitive PCR-based ELISA (Roche Diagnostics).

Cell culture

Bone marrow cells were flushed from femur and tibia and cultured in complete DMEM consisting of DMEM supplemented with 10% FCS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20% supernatant from L929 cells, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cells were supplemented with fresh macrophage medium on day 3. After 5 days of culture, nonadherent cells were removed, adherent monolayers were washed in ice-cold PBS, and cells were harvested by gentle pipetting in DMEM supplemented with 1% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Infection of macrophages was accomplished by adding tachyzoites to cell cultures followed by brief centrifugation (200 x *g* for 3 min) to synchronize contact between cells and parasites. In most cases, toll like receptor

ligands were added 12 h after infection. Cells were recovered at varying times as indicated, depending upon the assay performed. Dendritic cells were derived from bone marrow by flushing from femur and tibia and cultured in complete DC low medium consisting of RPMI supplemented with 10% FCS, 0.1% of 50uM 2-βME, 20ng/ml GM-CSF and 100 U/ml penicillin, and 0.1 mg/ml streptomycin. On day 3, the cells were fed with 9ml per plate of DC low 2- βME medium containing GM-CSF. On day 6, the cells were fed with 7-8ml of DC high 2- βME medium containing GM-CSF. On day 8, GM-CSF was directly added into the medium. Dendritic cells were collected on day 9 and infected as mentioned above.

Reagents

Toll like receptors ligands were purchased from *Invitrogen* and used according to the manufacturer's instructions (Mouse TLR1-9 Agonist kit).

Cytokine ELISA

IL-10 in cell culture supernatants was measured using commercial kits according to the manufacturer's recommendations (R&D Systems). Interferon beta in cell culture supernatants was measured by an ELISA based assay developed by Dr. Melissa Lodoen from Stanford University.

RT-PCR

RNA was extracted from the cell samples by a Invitrogen RNA mineasy Kit and then reverse transcribed into cDNA as PCR templates. The PCR was performed as described in Chapter 2.

Immunoblotting

The following antibodies were employed in immunoblotting studies: anti-phospho-histone H3 (Ser^{10}) (cat. # 9701, Cell Signaling), anti-tri-methyl-histone H3 (Lys^4) (cat. # 9751, Cell Signaling), anti-mono-methyl-histone H3(Lys^4) (cat. # 9723, Cell Signaling), anti-acetyl-histone H4 (Lys^8) (cat. #2594, Cell Signaling) anti-total H3 (cat. # 9715, Cell Signaling). Cells (2×10^6/sample) were lysed in reducing SDS sample buffer, and DNA was sheared by forcing samples 5 times through a 27-gauge needle. In some experiments, nuclear and cytoplasmic portions of cell lysates were separated by using a nuclear extract kit (Active Motif). After 5 min at 100ºC, samples were separated by 10% SDS-PAGE, and proteins were subsequently electrotransferred onto nitrocellulose membranes. Membranes were then blocked in 5% nonfat dry milk containing 0.1% Tween 20 in Tris-buffered saline, pH 7.6 (TBST), for 1 hr at room temperature, followed by incubation with antibody reconstituted in 5% BSA in TBST overnight at 4°C. After washing blots in TBST, primary antibodies were detected with a horseradish peroxidase-conjugated secondary antibody in TBST containing 5% nonfat dry milk for 1 h at room temperature. After washing in TBST, protein bands were visualized using a chemiluminescence based detection system (Cell Signaling).

RESULTS

T. gondii inhibits IL-12 responses induced by a diverse array of TLR ligands.

Our previous studies show that *Toxoplasma* infection blocks LPS induced TNFα and IL-12p40 responses triggered through TLR4. We wanted to test whether the response to other TLR ligands was also suppressed. We first determined the optimal concentration for each individual TLR ligand to stimulate macrophages by serial titration (data not shown). Then we used the *Toxoplasma* Type I RH strain to infect the macrophages and then subjected these cells to stimulation with each TLR ligand. We found that TLR3 and TLR5 ligands induced moderate IL-12 production, while all other TLR ligands induced higher amounts of IL-12p40. However, infected macrophages were unable to upregulate IL-12 in response to stimulation with each of these TLR ligands (Figure 4.1). To rule out the possibility of endotoxin contamination in the preparations of TLR ligands, we used macrophages derived from TLR2/4 double knock out mice and found that the IL-12 responses we observed could not be attributed to contamination by endotoxin or bacterial lipids that engage TLR2 and TLR4 (Figure 4.2).

T. gondii inhibits MyD88 independent signaling pathways

Since TLR3 signals through an MyD88 independent pathway to induce Type I interferon, we next examined whether *T. gondii* could block this response. Poly IC was used as a TLR3 ligand to stimulate macrophages. By RT-PCR, we found that Poly IC induced IFN- β upregulation was inhibited by *Toxoplasma*. We also found that Poly IC induced IL-12 mRNA was blocked by the infection (Figure 4.3.A). We used ELISA to examine the protein level of IFN-beta and found similar results where *T. gondii* blocked the Poly IC induced IFN-beta response even at a low parasite to cell

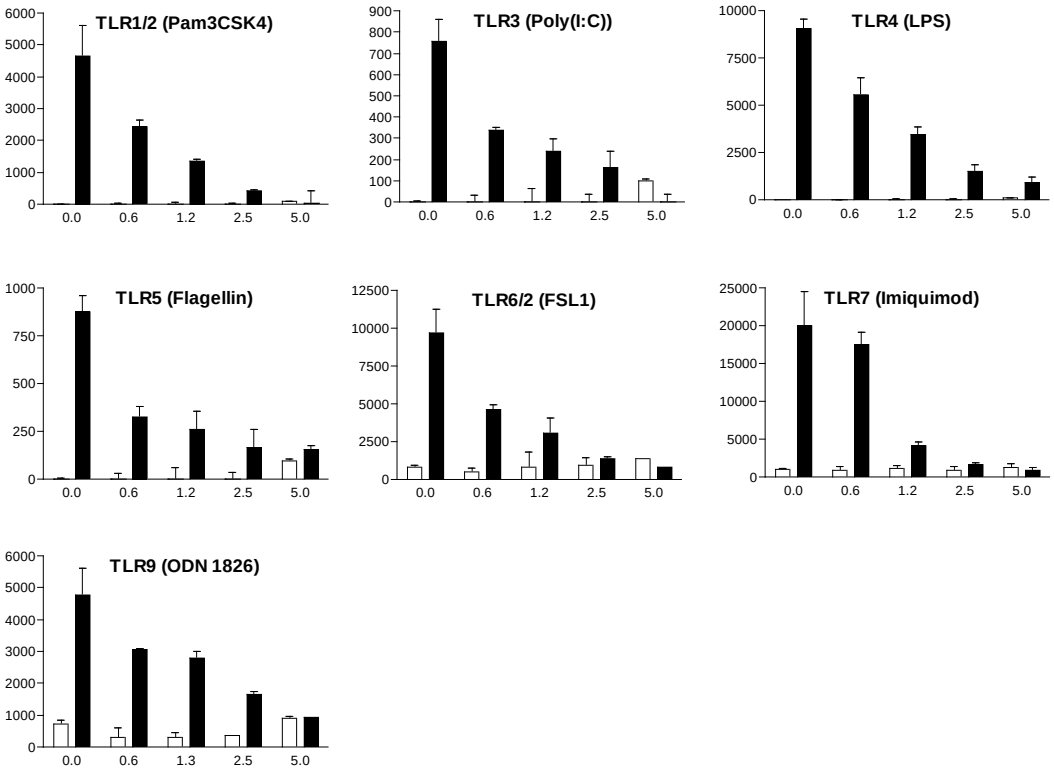


Figure 4.1 *T. gondii* inhibits IL-12 responses induced by all tested TLR ligands. Macrophages were infected with the *T. gondii* RH strain at the indicated parasite to cell ratio for 2 hr. The cells were then either treated with (black bar) or without (white bar) with the indicated TLR ligands. The optimal TLR ligand concentrations were previously determined by titration on macrophages. Supernatants were collected 6 hr later and subject to ELISA to measure the IL-12 p40 concentrations. Y, IL-12 p40 (pg/ml); X, parasite to cell ratio.

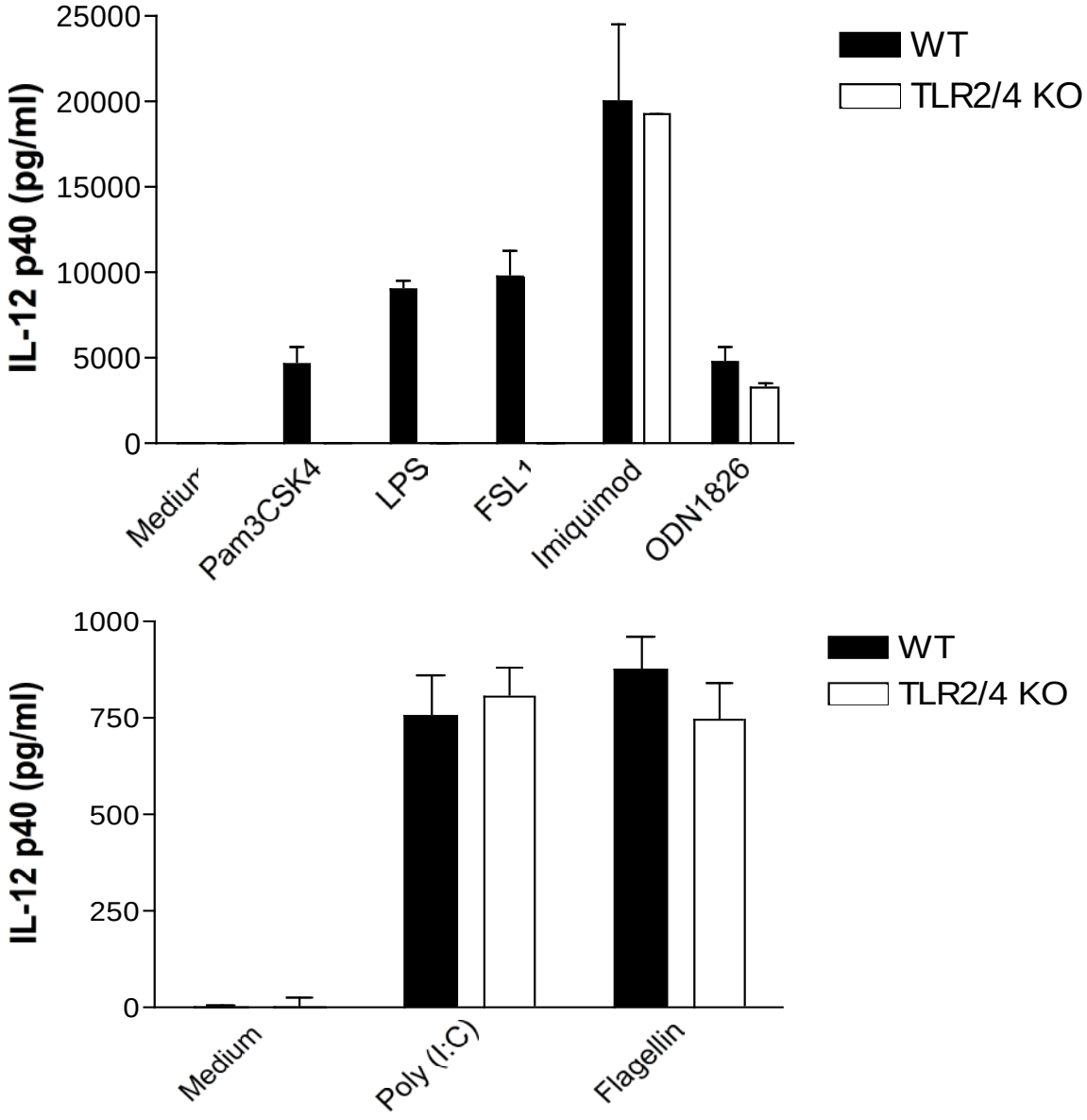


Figure 4.2 IL-12 responses induced by various TLR ligands are not due to contamination with bacterial ligands for TLR2 and TLR4. Macrophages from either WT or TLR2/4 double knock out mice were treated with indicated TLR ligands.

6 hr later, supernatants were collected and subjected to cytokine ELISA to determine the IL-12 p40 concentrations.

ratio (1.5:1). We used macrophages from MyD88$^{-/-}$ mice to confirm that the responses were MyD88 independent (Figure 4.3.B).

T. gondii Type I RH strain inhibits TNFα triggered by LPS in dendritic cells.

Previously we found that LPS induced TNFα in macrophages is blocked by *T. gondii* RH strain. In this experiment, we wanted to examine the effect of parasite infection on the LPS induced TNFα response in dendritic cells, as well as whether there were the differences between three parasite strains. Here, we used *T. gondii* Type I RH strain, Type II PTG strain and Type III VEG strain to infect bone marrow derived dendritic cells. When uninfected DCs were stimulated with LPS, these cells produced high amounts of TNFα. We observed that RH strain blocked TNF response, but PTG and VEG did not have inhibitory effects (Figure 4.4), even though all three parasite strains infected the cells at similar rates.

Toxoplasma Type I RH strain inhibits global phosphorylation of histone H3 at Ser^{10} induced by LPS more strongly than PTG and VEG strains.

Previously we established that the *Toxoplasma* RH strain blocked global phosphorylation of histone H3 at Ser^{10}. This corresponded to the parasite's ability to decrease histone modifications on TNFα and IL-10 promoters. Therefore, we were interested to know whether Type II and Type III parasite strains have similar effects on global phosphorylation of histone H3. In this experiment, we observed that Type I RH strain potently inhibited phosphorylation of histone H3 at Ser^{10} triggered by LPS. Type II PTG strain also blocked phosphorylation of histone H3 at Ser^{10}, but not as strong by the RH strain. Type III VEG strain did not seem to have any inhibitory

effect on pH3S10 (Fig 4.5). We also performed immunofluorescence staining on infected macrophages and observed similar effects (data not shown).

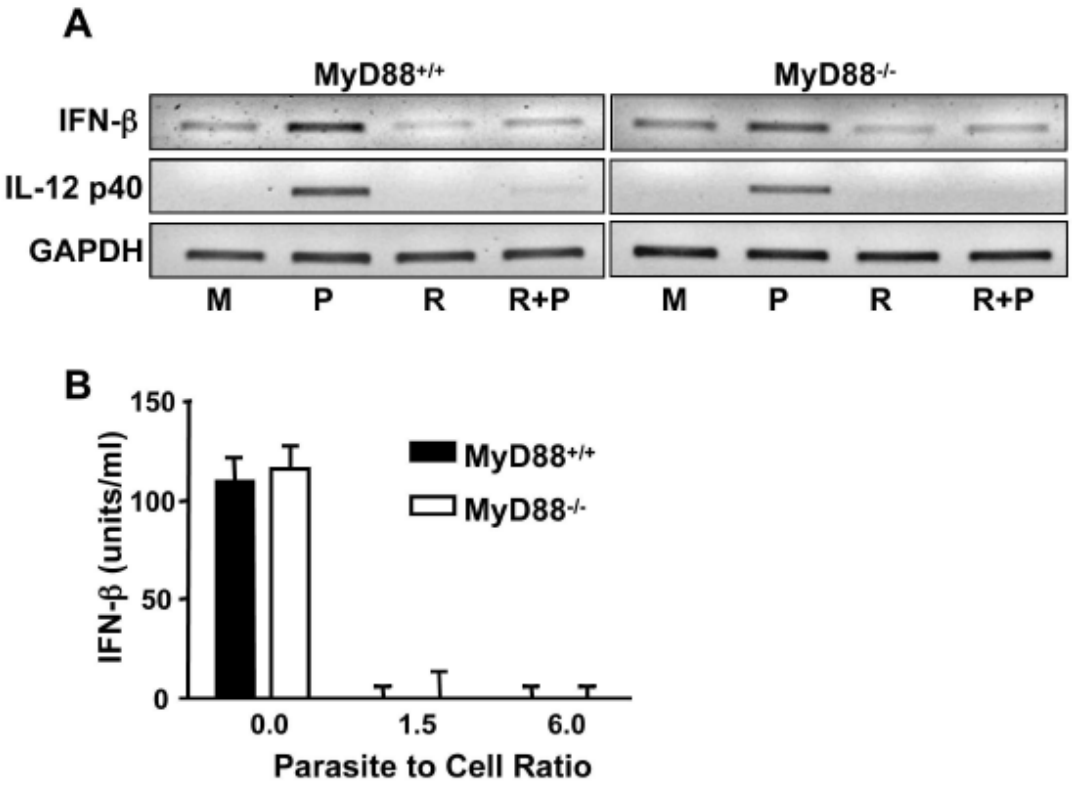


Figure 4.3 *T. gondii* inhibits MyD88 independent type I interferon response. In A, Macrophages from MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were infected with RH at a parasite to cell ratio of 3:1 for 18 hr. The cells were then subjected to LPS stimulation (100 ng/ml) for 30 mins. RNA was extracted from the cells and reversed transcribed into cDNA. PCR was performed to examine the expression of IFNβ and IL-12 p40. GAPDH was used as an internal control for PCR reactions. In B, macrophages were treated similarly and then supernatants were collected and cytokine ELISA was

performed to determine the IFNβ concentration. M, medium; P, Poly IC stimulated; R, RH infected; R+P, RH infected plus Poly IC stimulated.

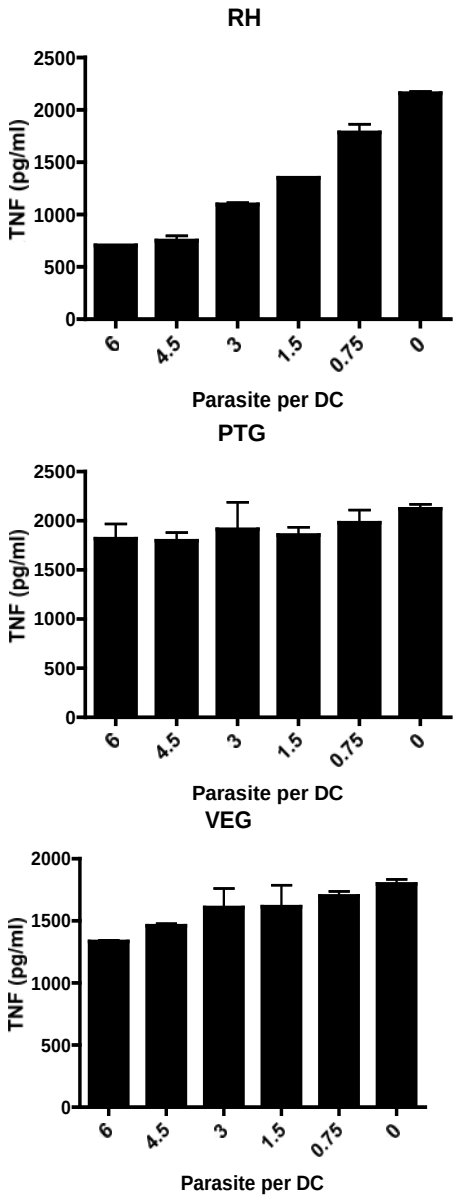


Figure 4.4 RH but not PTG or VEG inhibits TNFα response in dendritic cells. DCs were infected with RH, PTG and VEG at indicated parasite to cell ratio for 2 hr. The cells were then subjected to LPS stimulation (100 ng/ml). Supernatants were collected 6 hr later and cytokine ELISA was performed to determine the TNFα

concentration. The infected cell percentages in this experiment were calculated as: RH 45%, PTG 40%, VEG 38% (3:1).

Effect of T. gondii infection on other histone modifications.

The ability of the *T. gondii* RH strain to inhibit phosphorylation of histone H3 at Ser^{10} prompted us to examine other histone modifications. In this study, we performed Western blotting on cell lysates of bone marrow-derived macrophages using antibodies against other histone modifications including acetylated histone H4 at Lys^{8}, mono-methylated histone H3 at Lys^{4} and tri-methylated histone H3 at Lys^{4}. We consistently observed that unstimulated cells contained these modifications and we could not detect any difference between LPS treated and non-treated cells, nor did we observe any effect from parasite infection (Figure 4.6).

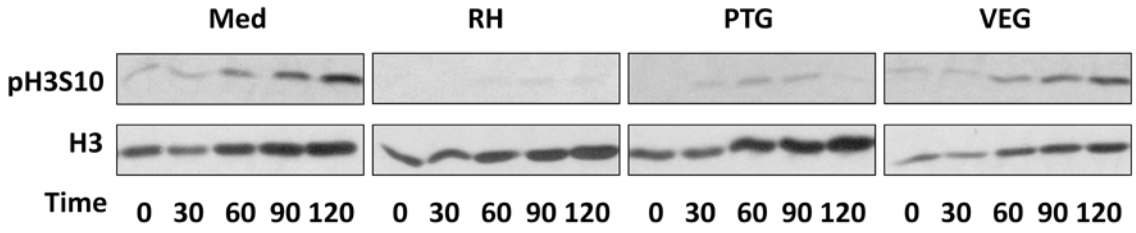


Figure 4.5 Effect of different parasite strains on global phosphorylation of histone H3 at Ser^{10} induced by LPS. Macrophages were infected with *T. gondii* RH, PTG and VEG strains at a parasite to cell ratio of 3:1 for 18 hr. The cells were then treated with LPS at 100 ng/ml for the indicated time periods (mins). The cells were lysed and Western blotting was performed to examine the level of phosphorylated H3 at Ser^{10}. Total H3 was blotted for controlling equal protein loading. In this experiment, the infected cell percentages were calculated as: RH 92%, PTG 86%, VEG 87%.

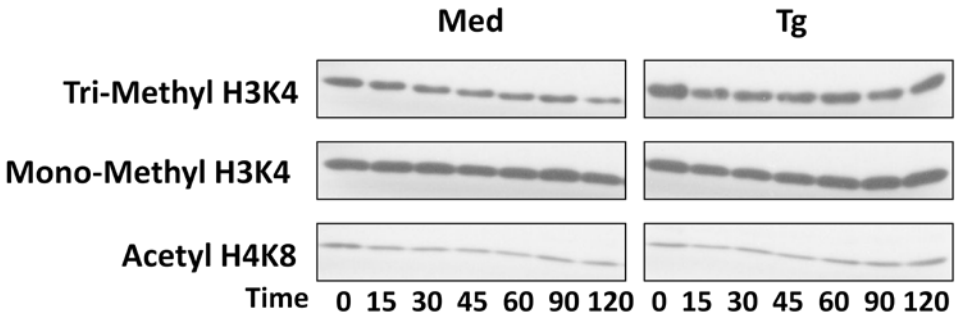


Figure 4.6 Effect of *T. gondii* infection on additional histone modifications. Macrophages were infected with the *T. gondii* RH strain at a parasite to cell ratio of 3:1 for 18 hr. The cells were then treated with LPS at 100 ng/ml for the indicated time periods (mins). Cell lysates were collected and Western blotting was performed to examine the levels of acetylated histone H4 at Lys^{8}, mono-methylated histone H3 at Lys^{4} and tri-methylated histone H3 at Lys^{4}. Med, medium; Tg, *T. gondii*.

DISCUSSION

As one essential component of innate immunity, Toll like receptors (TLRs) as well as their downstream signaling pathways are widely studied. Most TLRs use MyD88 as their adapter protein while TLR3 signaling is MyD88 independent, and requires an alternate adapter molecule called TRIF. TLR4 is a unique receptor because it uses both MyD88 dependent and independent pathways (11).

The intracellular protozoan parasite *T. gondii* has been previously found to inhibit TLR4 signaling and shut down the LPS- induced TNFα and IL-12 responses (4, 12). In this study, we found that *T. gondii* infection blocks all TLR ligand induced IL-12 responses. Moreover, the parasite inhibited Type I interferon responses stimulated by Poly IC, a TLR3 ligand. The parasite therefore seems to have a universal inhibitory effect on all TLR signaling pathways, including those that do not involve MyD88. *T. gondii* shuts down IL-12 responses possibly for its intracellular survival because IL-12 is essential in mounting an efficient immune response to clear the infection (13).

Toxoplasma itself contains pathogen-associated molecular patterns (PAMPs) that can be recognized by TLRs. The parasite glycosylphosphatidylinositol (GPI) moieties are recognized by TLR2 and TLR4 (14, 15). *T. gondii* profilin was also found to be a TLR11 ligand, even though humans do not have a functional TLR11 (16-18). The inhibition of TLR signaling can therefore be speculated to be necessary for the parasite to evade immune responses triggered by host recognition of its own molecules.

Previous gene microarray studies performed in our lab show that *T. gondii* does not inhibit every gene induced by LPS in macrophages (19). For example, the parasite does not inhibit the IL-10, CCL2 and TGFβ induced by LPS. Since we only looked at IL-12 induction in this study, it is reasonable to speculate that the infection does not shut down all genes induced by each TLR signaling. Future studies focusing on individual TLR are required to validate this speculation.

We also observed that in dendritic cells, the LPS-induced TNFα response is inhibited by *T. gondii* Type I RH strain, but not by Type II PTG or Type III VEG strains. Also, the Type I RH strain most potently inhibited LPS triggered global phosphorylation of histone H3 at ser^{10}. This is interesting because Type I strains are generally more virulent and have faster replication/dissemination rate compared to the other two strains. It is possible that the Type I strain is more capable of interfering with host signaling (20, 21).

We examined the influence of *T. gondii* infection on other histone modifications such as acetylated histone H4 at Lys^{8}, mono-methylated histone H3 at Lys^{4} and tri-methylated histone H3 at Lys$^{4.}$ Unfortunately, we were unable to draw conclusions from our data because unstimulated cells already carried those histone modifications. With the complexity of the histone code, it remains possible that *T. gondii* influences other histone modifications that we did not examine here.

We have been working on the exact molecular mechanism by which *T. gondii* blocks LPS induced global phosphorylation of histone H3 at Ser^{10}. In this study, we examined two upstream kinases that are responsible for phosphorylation of histone H3 at Ser^{10}, MSK-1 and MSK-2 (22, 23). However, we could not detect any effect by the

parasite on the activation of MSK-1(Figure 2.6, Chapter 2) and antibodies to MSK-2 proved to be ineffective (data not shown). It is possible that the parasite uses its own effector molecule, for example a parasite phosphatase, to fulfill this inhibition or possibly the parasite could utilize its own kinase to activate a host phosphatase. With the recent identification of parasite effectors being injected into the host nucleus, the kinase ROP16 and the phosphatase PP2C-hn, we hope to identify the relevant molecules in the future (24-26).

REFERENCES

1. Krutzik, S. R., P. A. Sieling, and R. L. Modlin. 2001. The role of Toll-like receptors in host defense against microbial infection. *Curr. Opin. Immunol.* 13:104-108.
2. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21:335-376.
3. Akira, S. 2003. Toll-like receptor signaling. *J. Biol. Chem.* 278:38105-38108.
4. Butcher, B. A., and E. Y. Denkers. 2002. Mechanism of entry determines ability of *Toxoplasma gondii* to inhibit macrophage proinflammatory cytokine production. *Infect. Immun.* 70:5216-5224.
5. Kim, L., B. A. Butcher, and E. Y. Denkers. 2004. *Toxoplasma gondii* interferes with lipopolysaccharide-induced mitogen-activated protein kinase activation by mechanisms distinct from endotoxin tolerance. *J. Immunol.* 172:3003-3010.
6. Bierly, A. L., W. J. Shufesky, W. Sukhumavasi, A. E. Morelli, and E. Y. Denkers. 2008. Dendritic cells expressing plasmacytoid marker PDCA-1 are Trojan horses during Toxoplasma gondii infection. *J Immunol* 181:8485-8491.
7. Chtanova, T., M. Schaeffer, S. J. Han, G. G. van Dooren, M. Nollmann, P. Herzmark, S. W. Chan, H. Satija, K. Camfield, H. Aaron, B. Striepen, and E. A. Robey. 2008. Dynamics of neutrophil migration in lymph nodes during infection. *Immunity* 29:487-496.
8. Courret, N., S. Darche, P. Sonigo, G. Milon, D. Buzoni-Gatel, and I. Tardieux. 2006. CD11c and CD11b expressing mouse leukocytes transport single *Toxoplasma gondii* tachyzoites to the brain. *Blood* 107:309-316.

9. Lambert, H., N. Hitziger, I. Dellacasa, M. Svensson, and A. Barragan. 2006. Induction of dendritic cell migration upon Toxoplasma gondii infection potentiates parasite dissemination. *Cell Microbiol* 8:1611-1623.

10. Kim, L., B. A. Butcher, C. W. Lee, S. Uematsu, S. Akira, and E. Y. Denkers. 2006. *Toxoplasma gondii* genotype determines MyD88-dependent signaling in infected macrophages. *J. Immunol.* 177:2584-2591.

11. Fitzgerald, K. A., D. C. Rowe, and D. T. Golenbock. 2004. Endotoxin recognition and signal transduction by the TLR4/MD2-complex. *Microbes Infect.* 15:1361-1367.

12. Leng, J., B. A. Butcher, C. E. Egan, D. S. Abdallah, and E. Y. Denkers. 2009. Toxoplasma gondii prevents chromatin remodeling initiated by TLR-triggered macrophage activation. *J Immunol* 182:489-497.

13. Denkers, E. Y., and R. T. Gazzinelli. 1998. Regulation and function of T-cell-mediated immunity during Toxoplasma gondii infection. *Clin Microbiol Rev* 11:569-588.

14. Debierre-Grockiego, F., M. A. Campos, N. Azzouz, J. Schmidt, U. Bieker, M. G. Resende, D. S. Mansur, R. Weingart, R. R. Schmidt, D. T. Golenbock, R. T. Gazzinelli, and R. T. Schwarz. 2007. Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from Toxoplasma gondii. *J Immunol* 179:1129-1137.

15. Campos, M. A. S., I. C. Almeida, O. Takeuchi, S. Akira, E. P. Valente, D. O. Procópio, L. R. Travassos, J. A. Smith, D. T. Golenbock, and R. T. Gazzinelli. 2001. Activation of Toll-Like Receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. *J. Immunol.* 167:416-423.

16. Denkers, E. Y., and B. Striepen. 2008. Deploying parasite profilin on a mission of invasion and danger. *Cell Host Microbe* 3:61-63.

17. Lauw, F. N., D. R. Caffrey, and D. T. Golenbock. 2005. Of mice and man: TLR11 (finally) finds profilin. *Trends Immunol* 26:509-511.

18. Yarovinsky, F., D. Zhang, J. F. Anderson, G. L. Bannenberg, C. N. Serhan, M. S. Hayden, S. Hieny, F. S. Sutterwala, R. A. Flavell, S. Ghosh, and A. Sher. 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science* 308:1626-1629.

19. Lee, C. W., S. Bennouna, and E. Y. Denkers. 2006. Screening for Toxoplasma gondii-regulated transcriptional responses in lipopolysaccharide-activated macrophages. *Infect Immun* 74:1916-1923.

20. Ajzenberg, D., A. L. Banuls, C. Su, A. Dumetre, M. Demar, B. Carme, and M. L. Darde. 2004. Genetic diversity, clonality and sexuality in Toxoplasma gondii. *Int J Parasitol* 34:1185-1196.

21. Howe, D. K., and L. D. Sibley. 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human diseases. *J. Infect. Dis.* 172:1561-1566.

22. Davie, J. R. 2003. MSK1 and MSK2 mediate mitogen- and stress-induced phosphorylation of histone H3: a controversy resolved. *Sci STKE* 2003:PE33.

23. Soloaga, A., S. Thomson, G. R. Wiggin, N. Rampersaud, M. H. Dyson, C. A. Hazzalin, L. C. Mahadevan, and J. S. Arthur. 2003. MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. *EMBO J* 22:2788-2797.

24. Gilbert, L. A., S. Ravindran, J. M. Turetzky, J. C. Boothroyd, and P. J. Bradley. 2007. Toxoplasma gondii targets a protein phosphatase 2C to the nuclei of infected host cells. *Eukaryot Cell* 6:73-83.

25. Saeij, J. P., J. P. Boyle, S. Coller, S. Taylor, L. D. Sibley, E. T. Brooke-Powell, J. W. Ajioka, and J. C. Boothroyd. 2006. Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science* 314:1780-1783.

26. Saeij, J. P., S. Coller, J. P. Boyle, M. E. Jerome, M. W. White, and J. C. Boothroyd. 2007. Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature* 445:324-327.

CHAPTER FIVE

Discussion*

This Chapter is based in part on the manuscript “Dysregulation of macrophage signal transduction by *Toxoplasma gondii*: past progress and recent advances” by J. Leng, B. A. Butcher & E. Y. Denkers. *Parasite Immunology*, in press.

1. Summary of Findings

As one of the most successful parasites that infects around 20-50% of human population, *Toxoplasma gondii* is a master at manipulating host cell functions to enable its survival, replication and transmission. During in vivo infection, a reservoir of infection are cells of innate immunity, particularly macrophages and dendritic cells (1-5). This thesis focused on the manipulation of macrophage functions by *T. gondii*.

In Chapter 2 and 3, the role of *T. gondii* infection on host cell chromatin remodeling was investigated on both the TNFα and IL-10 promoter. I found that *T. gondii* is able to block the phosphorylation of histone H3 at Ser^{10} and acetylation of histone H3 at Lys^{9}/Lys^{14} at both proximal and distal regions of TNF promoter. Therefore, even though transcripition factors like NFκB are normally activated and translocated into the nucleus, they are not able to bind to the promoter region to start the transcription. Similar results were found on the IL-10 locus when the macrophages were activated in the presence of immune complex.

In Chapter 4, I found that *T. gondii* infection blocked IL-12 induction by virtually all tested TLR ligands. Furthermore, the MyD88 independent Type I interferon production triggered by TLR3 ligand Poly IC, and TLR4 ligand LPS, was also inhibited by the parasite. I also for the first time examined suppression in dendritic cells (DCs). I obtained evidence that Type I but not Type II and Type III parasites blocked TNFα responses induced by LPS. Strain comparison studies also showed that Type I parasites most potently inhibited LPS induced global phosphorylation of histone H3 at Ser^{10} in macrophages.

2. Inhibition of Toll-like receptor signaling by *T. gondii*

After invasion of macrophages, *Toxoplasma* actively down-regulates a large panel of pro-inflammatory cytokines and chemokines that are normally induced by TLR signaling (6, 7). We observed in particular that LPS-triggered IL-12p40 and TNFα are suppressed in infected cells. While the parasite itself eventually initiates IL-12 synthesis, TLR4-triggered production of TNFα remains potently suppressed. The ability of *T. gondii* to block LPS triggered responses requires active invasion. Thus, heat inactivated tachyzoites do not display suppressive activity, and when parasite entry is prevented by cytochalasin D blockade of actin polymerization, suppressive activity is also lost (8). The suppressive activity of *Toxoplasma* on LPS-induced TNFα requires parasite survival within the host cell. This is evidenced by the fact that drug induced tachyzoite inactivation after invasion restores the ability of cells to respond to TLR4 triggering. We also recently found that signalling through other TLRs is blocked during infection. Importantly, this includes TLR3, an intracellular receptor for double-stranded RNA that, unlike other TLR, signals in a manner independent of MyD88. Suppression of TLR signalling does not appear to be restricted to RH strain tachyzoites because other Type I, as well as Type II strain parasites also blocks LPS induction of TNFα in macrophages (9).

The block in TLR signalling has also been reported to occur in bone marrow-derived DCs (10). In this case, infected immature DCs fail to mature in response to LPS triggering, and the cells were deficient in their ability to activate T cells. During in vivo infection, we also obtained evidence that *Toxoplasma* blocks cytokine production in infected cells. Infected macrophages collected from the peritoneal cavity following i.p. parasite inoculation are suppressed in their ability to produce TNFα, and infected DCs in the spleen are defective in IL-12 production (1, 9). Thus, suppression of TLR

signalling by *T. gondii* appears to be a general phenomenon that occurs in several cell types.

Deactivation of TLR signalling by *Toxoplasma* may indicate the need for the parasite to avoid triggering these pathways by the parasite's own TLR ligands. Thus, the *Toxoplasma* profilin molecule TGPRF activates TLR11 and parasite glycosylphosphoinositol (GPI) moieties associated with tachyzoite surface proteins possess the ability to activate TLR2 and TLR4 (11, 12). As both profilin and GPI synthesis are essential for survival, the parasite may be under evolutionary pressure to block TLR signalling during intracellular infection. In this regard, the immunodominant $CD4^+$ T-cell response to TGPRF characterized by Yarovinsky et al. might result from recognition of this TLR11 ligand by noninfected antigen presenting cells. It is also possible that blocking TLR signalling is a means to prevent proinflammatory responses that would otherwise be triggered by exposure to gut flora now known to occur during oral *T. gondii* infection (13, 14). Another possibility is that inability to respond to TLR ligands reflects a general nonresponsiveness of cells to proinflammatory signals no matter what the initiating stimulus, rather than being specific for TLR pathways.

There is evidence that other protozoans target NFκB and MAPK pathways. For example, data suggest that *Leishmania* down-regulates proinflammatory signaling through induction of SHP1, a phosphatase that plays a role in deactivation key components of both Jak/Stat and TLR pathways (15, 16). Recently, it has been reported that *Leishmania* proteases cleave kinases involved in p38 MAPK and NFκB activation (17, 18). Infection with *T. cruzi* is also reported to induce macrophage TLR nonresponsiveness through induction of host cell phosphatase activity (19). Taken

together, while it is clear that infection with *Toxoplasma* and other protozoans interferes with the ability to respond to TLR ligands, there is not yet a definitive picture of how this is accomplished in any case.

3. Interference with host chromatin remodeling by *T. gondii*

Inducible gene expression is now understood to involve two types of regulatory cascades. One type of transduction cascade leads to transcription factor activation, most often involving kinase signalling, to enable binding to target DNA sites on gene promoters. The particular pattern of transcription factors activated plays a role in determining the specificity of genes induced. At the same time, it is now appreciated that chromatin structure itself is subject to regulation, inasmuch as signalling leading to covalent modification of histones plays a role in determining the activity of transcription factors (20).

Toxoplasma suppresses TNFα production in bone marrow-derived macrophages, and this is associated with decreased recruitment of RNA polymerase II to the promoter (9). This finding links suppression of TNFα release to decreased transcription rather than downstream effects such as diminished mRNA stability, altered RNA splicing or decreased translation. In a close examination of the TNF promoter during TLR4 stimulation of *T. gondii* -infected macrophages, we concluded that the parasite targets chromatin modification rather than transcription factor activation (9). In 12 hr infected macrophages, subsequent LPS-triggered NFκB nuclear translocation was unaffected by the parasite. In addition, activation and nuclear translocation of c-Jun and CREB, two other TNF-associated transcription factors, appeared largely normal in infected cells. However, NFκB as well as c-Jun and CREB were unable to bind to the TNF promoter. This is explained by the ability of the parasite to inhibit

Ser^{10} phosphorylation and Lys^{9}/Lys^{14} acetylation of histone H3 at the TNF promoter, modifications associated with increased transcriptional activity (21). Thus, evidence indicates that chromatin structure surrounding the TNF promoter remains in a closed state in infected cells, and the transcription factors therefore cannot gain access to their binding sites. In turn, RNA polymerase II is not recruited to the TNF promoter and transcription is not initiated (9). Because *Toxoplasma* does not affect low level IL-10 production triggered by TLR4, we examined histone H3 modification at the promoter for the gene encoding IL-10. In this case, LPS itself did not induce histone H3 modification, and the parasite therefore had no effect at the promoter for IL-10. Yet, when IL-10 was super-induced with the combination of immune complex and LPS (22, 23), histone H3 phosphorylation and acetylation occurred, and *Toxoplasma* simultaneously blocked chromatin modification and high level IL-10 production.

Given these results, it is tempting to speculate that targeting chromatin modification in the host cell provides an explanation for the ability of *Toxoplasma* to simultaneously down-regulate a large panel of proinflammatory genes (7). Rather than targeting specific transcription factors (which conceptually would seem to be a relatively inefficient way to shut down multiple genes), the parasite may achieve the same effect by blocking inducible modification of H3 and possibly other histone molecule. Such an inhibitory mechanism would also account for the ability of Toxoplasma to down-modulate signaling though all TLR..

How *Toxoplasma* influences host histone modification is unclear, but the host cell nucleus is emerging as a target for parasite effector proteins. Whether the putative kinase ROP16 or the phosphatase PPC2-hn, parasite proteins that traffick to the host cell nucleus (24, 25), influence histone modification either directly or indirectly

remains to be determined. It is also possible that other parasite kinases or phosphatases may be delivered into the host nucleus to actively interfere with host gene transcription by targeting chromatin remodelling.

While *Toxoplasma* is the only intracellular protozoan identified to date that is capable of interfering with host cell chromatin modification, it is possible that other parasitic protozoans possess similar properties. In this regard, a growing number of bacterial pathogens have recently been reported to interfere with host chromatin remodeling required for gene expression (26). *Shigella flexneri* injects OspF, a dually specific serine–threonine phosphatase, into epithelial cells followed by relocalization of the protein to the host nucleus (27). Here, OspF blocks histone H3 Ser^{10} phosphorylation, thereby preventing induction of a subset of NFκB-responsive genes including IL-8. The OspF molecule appears to act by dephosphorylating ERK and p38, MAPK that lie upstream of histone H3 phosphorylation. Studies with OspF null Shigella suggest that lack of this protein results in early IL-8-dependent neutrophil recruitment in vivo, resulting in attenuation of infection (27).

Listeria monocytogenes, another well-known bacterial pathogen, was also recently found to dephosphorylate histone H3 on Ser^{10} and deacetylate histone H4 during early infection (28). This relates to a decreased level of transcription of a subset of host genes, including several key immune-related genes. The *Listeria* virulence factor, listeriolysin O, was identified as the major protein required for dephosphorylation of histone H3 and deacetylation of histone H4. Interestingly, other toxins of the same family such as *Clostridium perfringens* perfringolysin and *Streptococcus pneumoniae* pneumolysin have also been found to dephosphorylate histone H3 at Ser^{10}.

Other evidence for pathogen interference with chromatin structure comes from studies on *Mycobacterium tuberculosis*. This opportunistic pathogen inhibits IFN-γ-induced MHC class II gene expression by upregulating histone deacetylation at the HLA-DR promoter region in human THP-1 monocyte/macrophage cells. The activity is mediated by increased expression of Sin3A, a corepressor required for MHC class II repression by histone deacetylase (29). A related study showed that a 19-kDa lipoprotein from mycobacteria, LpaH, inhibited acetylation of histone H3 and H4 at the CIITA promoter. LpaH also blocked recruitment of Brahma-related gene 1, a chromatin remodelling protein, to the promoter (30). The effect of LpaH is believed to be mediated through TLR2-induced p38 or ERK MAPK signalling.

More recently, a virus protein encoded by white spot syndrome virus, ICP11, has been identified as a histone-binding DNA mimic that disrupts host cell nucleosome assembly (31). ICP11 is believed to compete with host DNA for histone proteins, in particular the H2A variant H2A.x, which functions in repairing DNA double-strand breaks. Host cell chromatin is therefore vulnerable to DNA damage, leading to disruption of the nuclear transcription machinery. This may facilitate the host cell death caused by infection, promoting transmission of the virus.

Based upon these examples and our findings in *Toxoplasma*, there are strong suggestions that the host cell chromatin modification machinery represents an Achille's heel for pathogen exploitation. Interfering with inducible histone modification potentially allows the pathogen to simultaneously down-modulate a large panel of host genes using a common mechanism. We do not yet know precisely how *Toxoplasma* – and possibly other intracellular protozoans – achieve this aim. However,

studies with bacterial and viral pathogens may provide us with clues as to how *T. gondii* interferes with host chromatin modification during intracellular infection.

4. Final conclusions and future directions

The manipulation of the host cell by *T. gondii* has been widely studied and appreciated in recent years. The findings in my thesis provide more details in how *Toxoplasma* interferes with host signaling. *Toxoplasma* has been shown in these studies to target chromatin remodeling to inhibit TNFα and IL-10 transcription. It is possible that the parasite utilizes this strategy to inhibit cytokine gene transcription for its own benefits.

In this regard, we would like to propose the following mechanisms by which *T. gondii* inhibits a large panel of inflammatory cytokines: during invasion and formation of PV, the parasite injects kinases and phophatases into host nucleus and these molecules either act directly (dephophorylate histone) or indirectly (phophorylate some host phosphatase to fulfill the same functions) on the host chromatin. The chromatin structures surrounding gene promoters therefore remains in a condensed state and transcription machinery cannot obtain access to these promoters.

In the future, there are still questions to be answered. First, as mentioned above, we do not know yet how exactly the parasite manages to interfere with host chromatin remodeling. Specifically, we do not know if the parasite is producing a molecule to fulfill this function or if the parasite is hijacking a host molecule to accomplish this. With the discovery of more and more parasite molecules that are delivered into the host cell, we can expect to solve this question in the future. Second, we know that *Toxoplasma* does not inhibit every cytokine during infection, so an obvious question is that whether the interference with host chromatin remodeling is restricted to specific

gene clusters on the chromosome. It would then be interesting to study the relationship between *T. gondii* suppressed genes and *T. gondii* enhanced genes. Future studies will be required to address these important questions.

REFERENCES

1. Bierly, A. L., W. J. Shufesky, W. Sukhumavasi, A. E. Morelli, and E. Y. Denkers. 2008. Dendritic cells expressing plasmacytoid marker PDCA-1 are Trojan horses during Toxoplasma gondii infection. *J Immunol* 181:8485-8491.
2. Chtanova, T., M. Schaeffer, S. J. Han, G. G. van Dooren, M. Nollmann, P. Herzmark, S. W. Chan, H. Satija, K. Camfield, H. Aaron, B. Striepen, and E. A. Robey. 2008. Dynamics of neutrophil migration in lymph nodes during infection. *Immunity* 29:487-496.
3. Courret, N., S. Darche, P. Sonigo, G. Milon, D. Buzoni-Gatel, and I. Tardieux. 2006. CD11c- and CD11b-expressing mouse leukocytes transport single Toxoplasma gondii tachyzoites to the brain. *Blood* 107:309-316.
4. Lambert, H., N. Hitziger, I. Dellacasa, M. Svensson, and A. Barragan. 2006. Induction of dendritic cell migration upon Toxoplasma gondii infection potentiates parasite dissemination. *Cell Microbiol* 8:1611-1623.
5. Ng, L. G., A. Hsu, M. A. Mandell, B. Roediger, C. Hoeller, P. Mrass, A. Iparraguirre, L. L. Cavanagh, J. A. Triccas, S. M. Beverley, P. Scott, and W. Weninger. 2008. Migratory dermal dendritic cells act as rapid sensors of protozoan parasites. *PLoS Pathog* 4:e1000222.
6. Butcher, B. A., L. Kim, P. F. Johnson, and E. Y. Denkers. 2001. *Toxoplasma gondii* tachyzoites inhibit proinflammatory cytokine induction in infected macrophages by preventing nuclear translocation of the transcription factor NFkB. *J. Immunol.* 167:2193-2201.
7. Lee, C. W., S. Bennouna, and E. Y. Denkers. 2006. Screening for Toxoplasma gondii-regulated transcriptional responses in lipopolysaccharide-activated macrophages. *Infect Immun* 74:1916-1923.

8. Butcher, B. A., and E. Y. Denkers. 2002. Mechanism of entry determines ability of *Toxoplasma gondii* to inhibit macrophage proinflammatory cytokine production. *Infect. Immun.* 70:5216-5224.

9. Leng, J., B. A. Butcher, C. E. Egan, D. S. Abdallah, and E. Y. Denkers. 2009. Toxoplasma gondii prevents chromatin remodeling initiated by TLR-triggered macrophage activation. *J Immunol* 182:489-497.

10. McKee, A. S., F. Dzierszinski, M. Boes, D. S. Roos, and E. J. Pearce. 2004. Functional inactivation of immature dendritic cells by the intracellular parasite *Toxoplasma gondii*. *J. immunol.* 173:2632-2640.

11. Debierre-Grockiego, F., M. A. Campos, N. Azzouz, J. Schmidt, U. Bieker, M. G. Resende, D. S. Mansur, R. Weingart, R. R. Schmidt, D. T. Golenbock, R. T. Gazzinelli, and R. T. Schwarz. 2007. Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from Toxoplasma gondii. *J Immunol* 179:1129-1137.

12. Yarovinsky, F., D. Zhang, J. F. Anderson, G. L. Bannenberg, C. N. Serhan, M. S. Hayden, S. Hieny, F. S. Sutterwala, R. A. Flavell, S. Ghosh, and A. Sher. 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science* 308:1626-1629.

13. Heimesaat, M. M., S. Bereswill, A. Fischer, D. Fuchs, D. Struck, J. Niebergall, H. K. Jahn, I. R. Dunay, A. Moter, D. M. Gescher, R. R. Schumann, U. B. Gobel, and O. Liesenfeld. 2006. Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with Toxoplasma gondii. *J Immunol* 177:8785-8795.

14. Heimesaat, M. M., A. Fischer, H. K. Jahn, J. Niebergall, M. Freudenberg, M. Blaut, O. Liesenfeld, R. R. Schumann, U. B. Gobel, and S. Bereswill. 2007.

Exacerbation of murine ileitis by Toll-like receptor 4 mediated sensing of lipopolysaccharide from commensal Escherichia coli. *Gut* 56:941-948.

15. Abu-Dayyeh, I., M. T. Shio, S. Sato, S. Akira, B. Cousineau, and M. Olivier. 2008. Leishmania-Induced IRAK-1 Inactivation Is Mediated by SHP-1 Interacting with an Evolutionarily Conserved KTIM Motif. *PLoS Negl Trop Dis* 2:e305.
16. Forget, G., C. Matte, K. A. Siminovitch, S. Rivest, P. Pouliot, and M. Olivier. 2005. Regulation of the Leishmania-induced innate inflammatory response by the protein tyrosine phosphatase SHP-1. *Eur J Immunol* 35:1906-1917.
17. Cameron, P., A. McGachy, M. Anderson, A. Paul, G. H. Coombs, J. C. Mottram, J. Alexander, and R. Plevin. 2004. Inhibition of lipopolysaccharide-induced macrophage IL-12 production by Leishmania mexicana amastigotes: the role of cysteine peptidases and the NF-kappaB signaling pathway. *J Immunol* 173:3297-3304.
18. Halle, M., M. A. Gomez, M. Stuible, H. Shimizu, W. R. McMaster, M. Olivier, and M. L. Tremblay. 2009. The Leishmania surface protease GP63 cleaves multiple intracellular proteins and actively participates in p38 mitogen-activated protein kinase inactivation. *J Biol Chem* 284:6893-6908.
19. Ropert, C., M. Closel, A. C. Chaves, and R. T. Gazzinelli. 2003. Inhibition of a p38/stress-activated protein kinase-2-dependent phosphatase restores function of IL-1 receptor-associate kinase-1 and reverses Toll-like receptor 2- and 4-dependent tolerance of macrophages. *J Immunol* 171:1456-1465.
20. Muegge, K. 2002. Preparing the target for the bullet. *Nat Immunol* 3:16-17.
21. Berger, S. L. 2007. The complex language of chromatin regulation during transcription. *Nature* 447:407-412.

22. Lucas, M., X. Zhang, V. Prasanna, and D. M. Mosser. 2005. ERK activation following macrophage FcgammaR ligation leads to chromatin modifications at the IL-10 locus. *J Immunol* 175:469-477.

23. Zhang, X., J. P. Edwards, and D. M. Mosser. 2006. Dynamic and transient remodeling of the macrophage IL-10 promoter during transcription. *J Immunol* 177:1282-1288.

24. Gilbert, L. A., S. Ravindran, J. M. Turetzky, J. C. Boothroyd, and P. J. Bradley. 2007. Toxoplasma gondii targets a protein phosphatase 2C to the nuclei of infected host cells. *Eukaryot Cell* 6:73-83.

25. Saeij, J. P., S. Coller, J. P. Boyle, M. E. Jerome, M. W. White, and J. C. Boothroyd. 2007. Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature* 445:324-327.

26. Hamon, M. A., and P. Cossart. 2008. Histone modifications and chromatin remodeling during bacterial infections. *Cell Host Microbe* 4:100-109.

27. Arbibe, L., D. W. Kim, E. Batsche, T. Pedron, B. Mateescu, C. Muchardt, C. Parsot, and P. J. Sansonetti. 2007. An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses. *Nat Immunol* 8:47-56.

28. Hamon, M. A., E. Batsche, B. Regnault, T. N. Tham, S. Seveau, C. Muchardt, and P. Cossart. 2007. Histone modifications induced by a family of bacterial toxins. *Proc Natl Acad Sci U S A* 104:13467-13472.

29. Wang, Y., H. M. Curry, B. S. Zwilling, and W. P. Lafuse. 2005. Mycobacteria inhibition of IFN-gamma induced HLA-DR gene expression by up-regulating histone deacetylation at the promoter region in human THP-1 monocytic cells. *J Immunol* 174:5687-5694.

30. Diaz-Silvestre, H., P. Espinosa-Cueto, A. Sanchez-Gonzalez, M. A. Esparza-Ceron, A. L. Pereira-Suarez, G. Bernal-Fernandez, C. Espitia, and R. Mancilla. 2005. The 19-kDa antigen of Mycobacterium tuberculosis is a major adhesin that binds the mannose receptor of THP-1 monocytic cells and promotes phagocytosis of mycobacteria. *Microb Pathog* 39:97-107.

31. Wang, H. C., T. P. Ko, Y. M. Lee, J. H. Leu, C. H. Ho, W. P. Huang, C. F. Lo, and A. H. Wang. 2008. White spot syndrome virus protein ICP11: A histone-binding DNA mimic that disrupts nucleosome assembly. *Proc Natl Acad Sci U S A* 105:20758-20763.