INNATE IMMUNITY OF THE INTESTINAL EPITHELIUM DURING *TOXOPLASMA GONDII* INFECTION

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

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January 2011

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Cornell University 2011

The single layer of epithelial cells that line the intestinal tract provides both a physiologic and immunologic barrier for microbes and foreign antigens. Intestinal epithelial cells are differentiated into different functional subtypes and have regulatory roles in maintaining the homeostasis of the gut as well as initiating inflammatory responses during pathogenic infections. *Toxoplasma gondii* is a widespread zoonotic protozoan parasite that infects warm-blooded animals. Infection usually occurs through ingestion of *T. gondii* contaminated food or water. Therefore, natural route of infection has revealed the importance of epithelial cell response in influencing the outcome of the local and systemic immune response.

In this thesis, I examined the innate immune response of intestinal epithelial cell during *T. gondii* infection. The immediate response upon intestinal epithelial and parasite contact revealed that *T. gondii* infected epithelial cells elicited MAPK phosphorylation, NF-κB activation, and secretion of IL-8 as well as several other inflammatory cytokines and chemokines in epithelial cells. I found that activation of MAPK and production of IL-8 was dependent on the MyD88 signaling pathway, and TLR2 was sufficient for parasite induced signaling in HEK 293 transfected cells. In addition, TLR2 signaling plays an important role in modulating of the local intestinal environment by inducing migration of dendritic cells into the follicle-associated

epithelium of Peyer's patches. Using the susceptible C57BL/6 mice as a model, morphological changes of different intestinal epithelial cell subtypes in response to peroral *T. gondii* infection were further characterized. Oral infection with *T. gondii* ME49 cysts induced intestinal pathology and morphological changes of different epithelial cell subtypes. Mice deficient in TLR2 were more resistant to intestinal pathology and associated morphological changes. Parasite–derived TLR2 stimulatory molecules were found to be present in the supernatant collected from *T. gondii* infected cells. Therefore, potential biological significance of the activity in supernatants from *T. gondii* infected cells was also investigated. I found that the supernatants from *T. gondii* infected cells enhanced luminal antigen uptake and induced TLR2-dependent migration of dendritic cells to the follicle associated epithelium of Peyer's patches. Furthermore, analysis of the supernatant revealed several *T. gondii* proteins, and the GPI-anchored surface antigen-1 contributes to TLR2 stimulatory activity.

In summary, this dissertation explores the innate immune response of the intestinal epithelium during *T. gondii* infection. The signaling pathways of intestinal epithelial cells elicited by the parasite as well as the modulation of the local intestinal environment can help us better understand the immunopathogenesis of *T. gondii* infection.

BIOGRAPHICAL SKETCH

Chia-Hsin Ju was born in on the 8th of July 1978 in Kaohsiung City, Taiwan. She grew up in Taipei and attended Da-Tung Senior High School where she became interested in biology. She was admitted into Fu Jen University in Taipei and received her bachelor's degree of Food Science and Nutrition in 2001. During her college years, she enjoyed spending time in the research laboratory and developed interest in food microbiology. She was encouraged by Dr. Chih-Wei Perry Chiu to apply for graduate school abroad. In 2002, she got admission to the department of Food Science and Technology at Cornell University where she studied bacterial food borne pathogens. She got her master's degree in 2004 and then continued on to a PhD program in the same department. During this period, she took a immunology course and realized that the interaction between pathogens and the host immune system is a fascinating topic. Her interest in host immune regulation and infectious disease led her to transfer to the department of Microbiology and Immunology to continue her PhD studies, where she has spent 5 happy years in the laboratory of Dr. Leifer, further training her as an immunologist.

謹此獻給

阿嬤，謝謝您對我小時候的照顧

親愛的爸媽，謝謝您對我的疼愛與鼓勵

家葳，謝謝你這些日子以來的關懷與陪伴

ACKNOWLEDGMENTS

Foremost of all, I sincerely thank my advisor Dr. Cynthia Leifer. This thesis will not be possible without her guidance and generous support. She has been an excellent mentor in many aspects. Academically, she is a great advisor who is always available for scientific and intellectual discussions with students. Her effort in creating a fun and inspiring environment in the lab has not only helped me learn lots of new techniques, but also importantly, help me develop critical scientific thinking that facilitates my research move forward. Her encouragement and critique on experiments, writing, and presentation skills through these years has also made me become an independent scientist with confidence. Besides a supportive advisor, her enthusiasm in science, optimistic philosophy, and hard working qualities has set up a great role model for me to follow in the future.

I would also like to express my gratitude to my thesis committee members Dr. Eric Denkers, Dr. Hélène Marquis, and Dr. Theodore Clark for asking me sharp questions to make me think and giving me invaluable suggestions on my research work in each meetings. I also thank them for generously providing me several research materials to complete this thesis.

I appreciate my lab members that contribute to a dynamic atmosphere and a great working environment. I would like to acknowledge our postdocs Annapoorani Chocklingam, William Rose, and Maroof Hassan for the collaborative work and helpful comments on my research. I thank my colleague James Brooks for friendship mental support, and our technician Jody Lopez for her assistance and generosity. You all have made my life memorable during these years in Cornell.

I would like to thank many friends for their long lasting friendship. I thank Chih-Wei Tung for the enlightening scientific discussions and being there during my most difficult times. To all my best friends in Cornell and in Taiwan: Chen-Hua Chuang, Lu Huang, Chi-Ting Tsai, Chang-Shiou Wu, Chia-Pei Chen, Han-Yi Hsu, and Ya-Ying Wang. I appreciate your patience, encouragement, and keeping me happy all these years. Thank you for traveling a great distance from Taiwan to Ithaca to visit me and give me warm support.

Finally, I would like to thank my mother who always gave me faith and letting me fly high, my father who always believed in me and gave me the strongest support. Thank you for the unconditional love since I was born, and being proud of me.

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CHAPTER I

INTRODUCTION

1. Life cycle of *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular protozoan parasite that can infect warm-blooded animals, and is arguably one of the most successful parasitic organisms worldwide. It was originally discovered in 1908 by Nicolle and Manceaux, who found the parasite in tissues of the rodent *Ctenodactylus gundii*. They named this new organism (*toxon* meaning arc or bow in Greek, and *plasma* meaning life) based on the crescent-shaped morphology, and the host species in which it was originally discovered (1, 2). *Toxoplasma* belongs to the Phylum Apicomplexa, Class Sporozoasida, Subclass Coccidiasina, Order Eimeriorina, and Family Toxoplasmatidae. There is only one species of *Toxoplasma gondii* (3).

There are three different infectious stages for *T. gondii*: sporozoite-containing oocysts, tachyzoites, and bradyzoite-containing tissue cysts. Sporozoites are disseminated in the environment within oocysts, tachyzoites are rapidly replicating forms that facilitate expansion during acute infection, and bradyzoites are in a quiescent form that is maintained during chronic infection. Tachyzoites and bradyzoites are associated with the intermediate host, whereas sporozoites are only associated with its definitive host. All three stages are haploid; tachyzoites and bradyzoites divide asexually, and sporozoites are derived from meiosis (4, 5).

T. gondii has a complex life cycle consisting of a sexual cycle in felidae, the definitive hosts, and an asexual cycle in its intermediate hosts. The life cycle of *T. gondii* begins with cats that have ingested the parasite at any of the 3 infectious stages. After ingestion of tissue cysts or oocysts by cats, the cyst wall is digested by proteolytic enzymes in the small intestine. When the parasites are released they penetrate into epithelial cells and multiply as tachyzoites in the small intestine. Five

morphologically distinct asexual types are formed in the intestinal epithelial cells and eventually lead to a sexual cycle resulting in the production of oocysts (6). The prepatent period is from 3 -10 days following cyst ingestion, approximately 18 days after ingesting oocysts, and approximately 13 days after ingesting tachyzoites (6). Nearly all cats shed oocysts after ingesting tissue cysts, but only a few shed oocysts after ingesting tachyzoites or oocysts. Unsporulated oocysts are non-infectious, but they can sporulate quickly within 5 days and become infectious (7-9). Intermediate hosts and humans are infected with *T. gondii* by ingesting animal tissue cysts or food and water contaminated with oocysts. After ingestion, brazyzoites or sporozoites transform into rapidly replicating tachyzoites and disseminate out of the intestine and spread throughout the body. Vertical transmission can also occur through transplacental infection of tachyzoites from mother to fetus (Fig 1.1).

2. Clinical Aspects of *T. gondii*

Epidemiological studies have suggested as much as 1/3 of the human population may harbor *T. gondii* infection; however the seroprevalence for *T. gondii* varies from population group and geographic region (10). Most infections in immunocompetent individuals are asymptomatic, yet the individual remains infected throughout their lifetime. Toxoplasmic encephalitis is a very serious disease following reactivation of the parasite due to various mechanisms of immunosuppression. Encephalitic disease is the most important manifestation of toxoplasmosis in immunocompromised individuals such as HIV-infected patients, and organ/bone marrow transplant patients treated with immunosuppressive drugs (11). Only a minority of healthy individuals infected with *T. gondii* develop symptoms, such as fever, malaise, and swollen lymph nodes (10). In congenital transmission, the fetus becomes infected if the mother acquires the parasite for the first time during pregnancy. *T. gondii* tachyzoites can

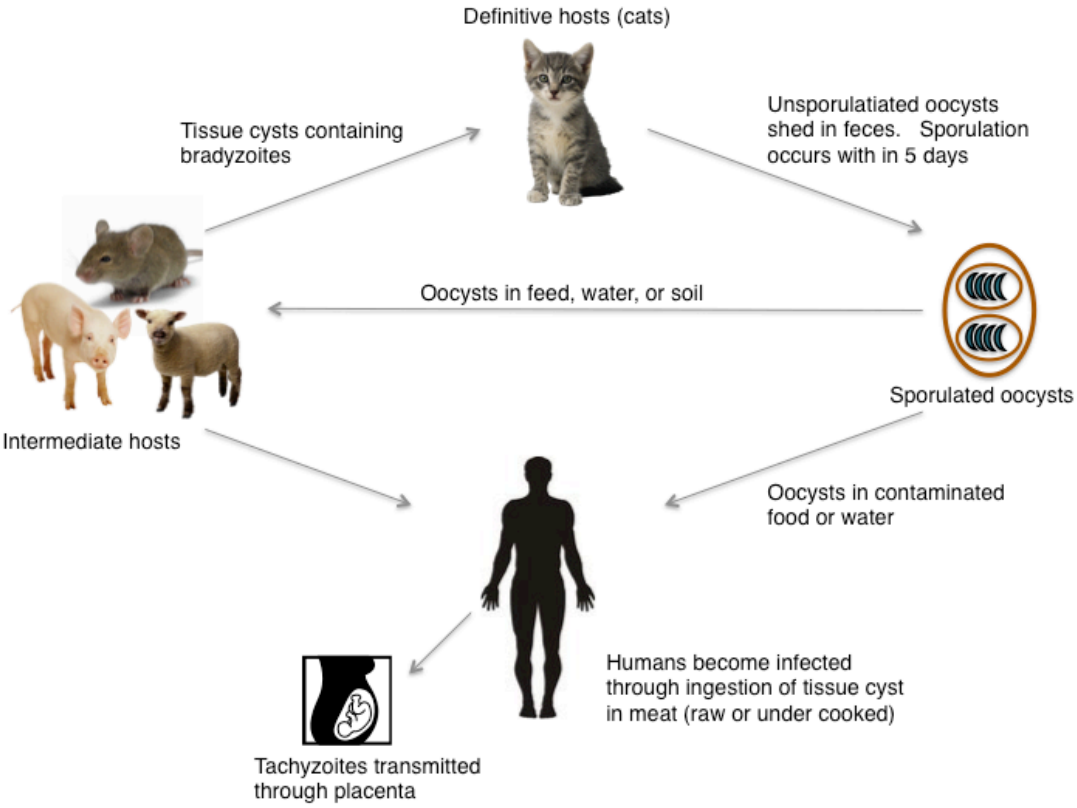

Figure 1.1. Life cycle of *Toxoplasma gondii*. *T. gondii* oocysts are only produced in the definitive host (cats). Intermediate hosts become infected after ingesting oocyst contaminated feed, soil, or water. Oocysts sporulate to an infective form rapidly in the environment. Sporulated oocysts transform into tachyzoites after ingestion and spread systemically to the brain and muscle tissue to become tissue cyst containing bradyzoites. Cats can be infected by ingesting oocysts or from intermediate hosts harboring tissue cysts. Humans can also be infected through consuming food or water contaminated with oocysts, or eating undercooked meat from infected animals. The fetus can be infected through transplacental infection.

enter the fetus through placental infection, and lead to a variety of manifestations in the fetus depending on the stage of the gestation such as damage to the central nervous cystem, and chorioretinitis. The risk of congenital infection is 10–15% when maternal infection is in the first trimester, and up to 60–90% during the third trimester (12-15). While clinical toxoplasmosis is not a major problem in livestock, *T. gondii* can cause severe disease in ovine. Abortion and neonatal mortality in dairy goats and sheep worldwide is a source of significant economic loss (16-18).

The standard drug combinations for treating toxoplasmosis are pyrimethamine, sulfadiazine, and folinic acid treatment for 4-6 weeks (10). For maternal infection, spiramycin is used during the first and second trimester, pyrimethamine, sulfadiazine, and spiramycin combinations are used for the late second and third trimester when fetus infection is confirmed with PCR analysis of the amniotic fluid. In cases of suspected infection but test negative for PCR analysis, pregnant women are treated with spiramycin prophylaxis until the 17^{th} week of pregnancy (10).

Diagnosis of *T. gondii* infection can be achieved indirectly with serological methods and directly by PCR. Indirect serological methods by detection IgG antibodies to *T. gondii* are widely used in immunocompetent individuals and pregnant women. IgG antibodies to *T. gondii* can be detected after 2 weeks of infection and persist in the host throughout their lifetime. Common methods for detecting serum IgG antibodies include: the Sabin-Feldman dye test, immunofluorescent antibody test, ELISA based assays, IgG avidity test, and agglutination and differential agglutination test (19-23). For direct detection, the *T. gondii* repetitive gene B1 is often used at targets for PCR amplification of body fluid and tissue samples (24-26).

Currently there is no vaccine to prevent *T. gondii* infection in animals and humans. The commercial vaccine "Toxovax®" is used for limiting abortion in sheep, but protection is of short duration and does not completely eradicate the parasite (27, 28). Studies on human vaccine development have focussed on antigens that stimulate Th1 immune response. Considering that *T. gondii* is a pathogen that infects the host through the oral route, it is important to develop a vaccine that stimulates mucosal immunity effectively. Recent studies on designing *T. gondii* vaccines have used defined subcellular components of the parasite, such as native antigens obtained from tachyzoites (surface antigens, rhoptry antigens, dense granule antigens, and microneme antigens) (29, 30), recombinant antigens (31), and the construction of auxotrophic *T. gondii* mutants (32). Interestingly, GPIs from *T. gondii* stimulate inflammatory responses and are potential targets for vaccine development (33).

3. Cellular biology and virulence of *T. gondii*

T. gondii is an intracellular parasite that can actively infect any nucleated cell. The three infectious stages of *T. gondii* are similar in morphology, but differ in some organelles and inclusion bodies. Both oocysts and tissue cysts differentiate into tachyzoites once they invade the host cell after ingestion by the host. Tachyzoites are the most well characterized stage, and can be clonally propagated both in vitro and in vivo. They are crescent shaped (2 × 6 μm) with a pointed anterior end and a rounded posterior end. The structure of *T. gondii* is shown in Fig 1.2. Entry of the tachyzoites into the host is an active process that involves gliding motility to approach a host cell, tight binding of host receptors during attachment, and active penetration into the host cell (34, 35). During invasion, specialized secretory organelles such as micronemes, rhoptries and dense granules are discharged from the apical end and assist in invasion and remodeling of the host cell. Micronemes secrete MIC proteins that are adhesion

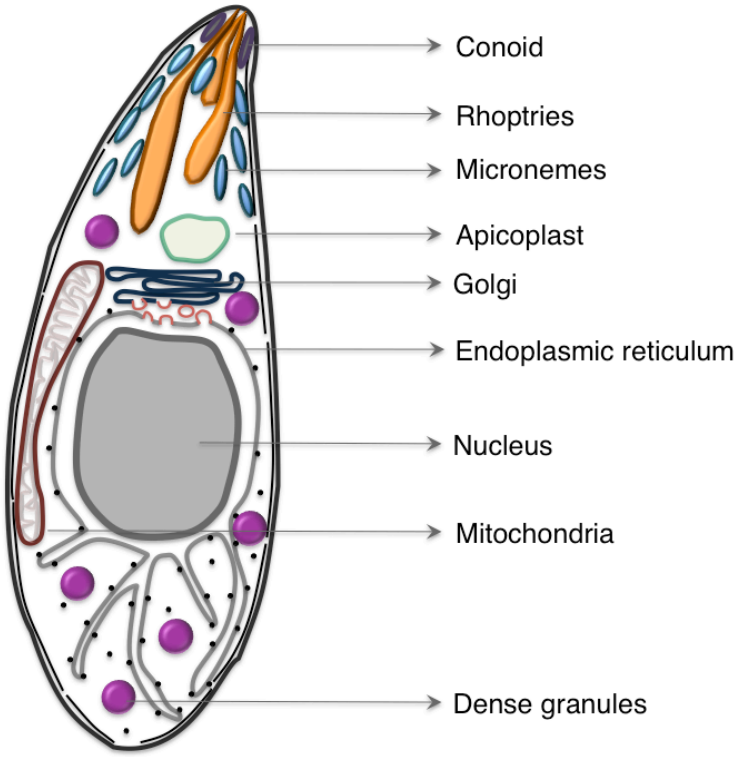

Figure 1.2. The structure of *T. gondii*. *T. gondii* has the basic features of an eukaryotic cell including a nucleus, endoplasmic reticulum, and Golgi. The conoid located at the apical end is composed of microtubule elements. The three secretory organelles, micronemes, rhoptries, and dense granules secrete various MIC, ROP, and GRA proteins, respectively, which play an important role during the invasion process. The mitochondria and apicoplast contains extra-chromosomal genomes in addition to the nucleus.

proteins important for host cell entry (36-39). Rhoptries produce ROP and RON proteins that form the moving junctions during invasion and contribute to the formation of the parasitophorous vacuole once inside the cell (40-45). Dense granules secrete GRA proteins that facilitate the formation of specialized tubules for nutrient acquisition by the parasite (46-49).

T. gondii isolates from humans and animals in the Europe and North America region belong to three clonal lineages (50, 51). In mice, the type I strains are highly virulent, whereas type II and type III are considered avirulent (52). In Europe and North America, type II strains are most commonly associated with human toxoplasmosis (53, 54), and type II (pigs, sheep) or type III (chickens) strains are mostly isolated from livestock. The three clonal lineages differ in growth rate, migration and virulence, which lead to strain-specific differences and interaction with the host. In vitro infections with different clonal lineages results in different host cell gene regulation and activation of signaling pathways (52, 55-58). Modulation signaling pathways of the host cells alters the proinflammatory cytokine production and also results in a difference of the disease outcome in mice (59, 60).

4. Immunology of *T. gondii* infection

Several studies have shown that *T. gondii* infection is predominantly controlled by T-cell-mediated immunity (61). Nude mice that lack a thymus, and therefore do not produce functional T cells, are extremely susceptible to *T. gondii* infection (62, 63). Furthermore, depletion of CD4+ and CD8+ T cells in chronically infected mice results in toxoplasmic encephalitis (64). Adoptive transfer of *T. gondii*-specific T cells from chronically infected mice to naïve mice provides protection against *T. gondii* challenge demonstrating that specific T cells alone are protective (65, 66). Infections

with *T. gondii* also activate humoral immunity. In B cell-deficient mice, although normal levels of IFN-γ, TNF-α, and inducible nitric oxide synthase (iNOS) were produced during the acute phase of infection, mice succumbed during chronic infection due to uncontrolled parasite replication as well as brain and lung pathology (67). Oral infection with *T. gondii* also induces specific secretory immunoglobulin A (IgA) in the gut, sera, and milk (68).

Primary infection with *T. gondii* elicits a strong Th1-polarized response characterized by the production of high amounts of IFN-γ, IL-12, and other proinflammatory cytokines such as TNF–α. IFN-γ is essential for controlling the acute and chronic stage of infection since depletion of IFN-γ by monoclonal antibodies or gene knockout studies increased susceptibility to infection (69, 70). During the acute stage of infection, T cells and natural killer (NK) cells are the major source for IFN-γ, and during the chronic stage production is mostly from αβ T cells (64, 71-73). Both CD4+ and CD8+ T cells are required for preventing the reactivation of parasite (64). However, while CD4+ T helper cells are important for driving CD8+ T cell differentiation, they may also play an immunopathogenic role that causes tissue damage (74).

Innate immune cells such as macrophages, dendritic cells, and neutrophils are the main producers of IL-12, which is necessary for the induction of IFN-γ to control *T. gondii* infection (Fig 1.3) (72, 75-80). Mice deficient in IL-12 are extremely susceptible to acute infection due to impaired production of IFN-g (81). In addition to IL-12 production, both macrophages and dendritic cells can present antigens to T cells. Macrophages activated by IFN-γ become effector cells with increased

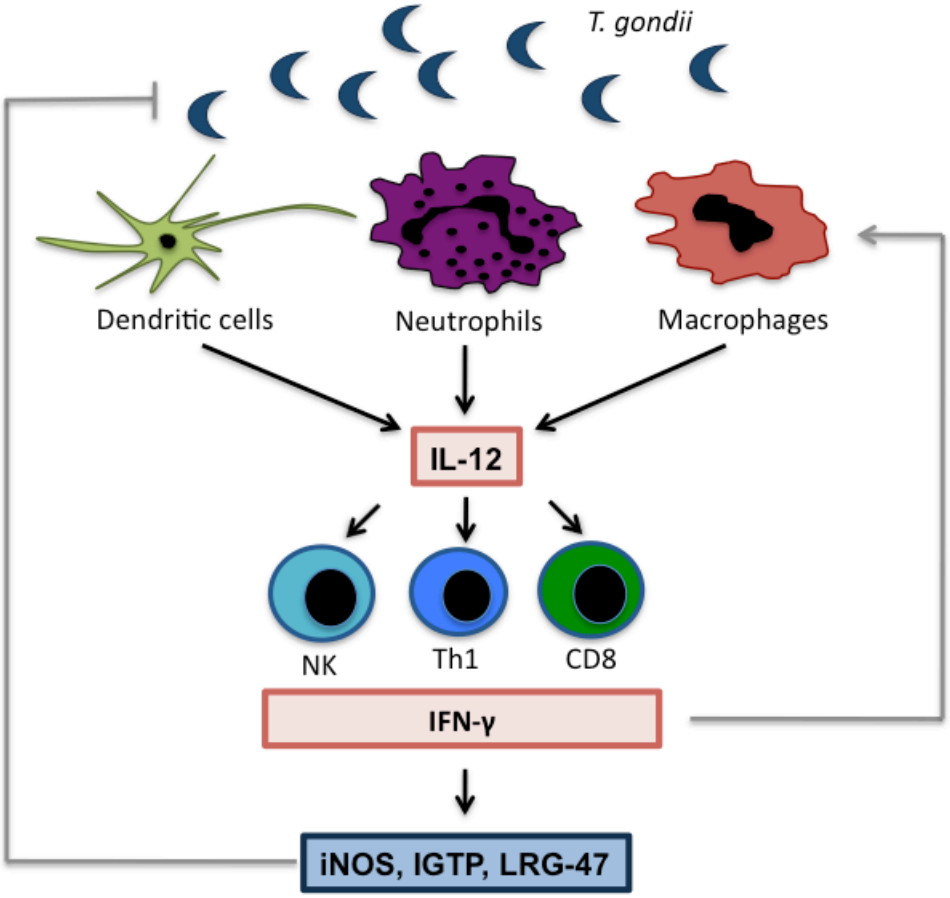

Figure 1.3. Immune response during *T. gondii* infection. Dendritic cells, neutrophils, and macrophages produce IL-12 during *T. gondii* infection. IL-12 is critical for activating natural killer (NK) cells, CD4+ Th1 cells, and CD8+ T cells to produce IFN- γ. IFN- γ is can promote the production of nitric oxide, IFN- γ -inducible guanosine triphosphate binding proteins (IGTPs), and LRG-47 that contain microbicidal activity. Macrophages primed with IFN- γ can also become effector cells against *T. gondii.*

antimicrobial activity, increased phagocytic activity, and induced production of nitric oxide (82). IFN- γ priming of dendritic cells can also limit intracellular growth of *T. gondii* (83). Neutrophils are also early-response cells and produce IL-12 and TNF-α during *T. gondii* infection (77, 79). They respond to chemoattractants such as IL-8 (MIP-2 and KC in mice), migrate rapidly to the site of infection, phagocytose parasites, and release microbicidal molecules such as reactive oxygen intermediates (ROI) and degradative enzymes (77, 84, 85). Studies have shown the essential role for neutrophils during the acute phase of *T. gondii* infection. Mice depleted of neutrophils are highly susceptible to *T. gondii* infection due to high parasite burden, a phenotype similar to IFN- γ, IL-12 and MyD88 deficient mice (84, 86, 87).

In order to prevent excessive immune response in the host, the innate immune cells can also produce anti-inflammatory cytokines such as IL-10, IL-4, and TGF-β. Mice deficient in IL-10 have increased immunopathology following infection with *T. gondii*, which correlates with increased IL-12, IFN-γ and TNF-α, despite a similar parasite burden (88). IL-10 inhibits cell-mediated immunity by downregulating the expression of costimulatory molecules required for T-cell activation. In addition to IL-10, both IL-4 and TGB-β regulate inflammation and limit cell based immunity. TGF- β synergizes with IL-10 to inhibit macrophage effector function, and IL-12-induced production of IFN-γ by NK cells (89-91).

The mucosa is a major entry site for most pathogens. Intestinal epithelial cells are the first cell type that encounters infectious agents including *T. gondii*. Therefore the intestinal epithelium is a critical site for initiation of host immunity, as well as for orchestrating the response of immune cells in the lamina propria. In this thesis, I will focus on the mucosal epithelial immune response during *T. gondii* infection.

5. Mucosal epithelial cells and immune regulation in the gut

The gut consists of the epithelial layer, the gut-associated lymphoid tissues (GALT), and the commensal flora. The single layer of epithelial cells that line the intestinal lumen is important for digestion, nutrient absorption, as well as formation of a physical barrier. Four differentiated epithelial cell types reside within the small intestinal epithelium: enterocytes, enteroendocrine cells, goblet cells, and Paneth cells. Enterocytes are absorptive cells that cover the intestinal villi. Enteroendocrine cells and goblet cells are scattered among enterocytes and function as hormone and mucus secreting cells, respectively. Paneth cells migrate to the bottom of crypts and secrete bactericidal defensins and lysozymes (Fig 1.4). The colon has a similar structure as the small intestine, but the surface epithelium contains crypts without villi, and Paneth cells are not present (92-97).

In addition to its role as a physical boundary, intestinal epithelial cells are important for immune modulation in the gut (98, 99). The intestinal environment contains up to 10^{14} commensal bacteria from an estimated 500–1000 different species (100). Therefore, in order to maintain a symbiotic relationship, the intestinal environment has evolved to become tolerized to the heavy bacterial load by signaling the immune cells (stromal cells, B cells, T cells, macrophages, and dendritic cells) underlying the epithelium (101-103). Intestinal dendritic cells (DC) in the lamina propria are antigen-presenting cells that are non-inflammatory against commensal bacteria and food antigens, but are immunogenic to pathogen infections (104, 105). Intestinal epithelial cells regulate the function of mucosal DCs, and the epithelial cells are in turn controlled, at least in part, by commensal bacteria (106-109). Under normal (steady-state) conditions, intestinal epithelial cells and commensal bacteria contribute

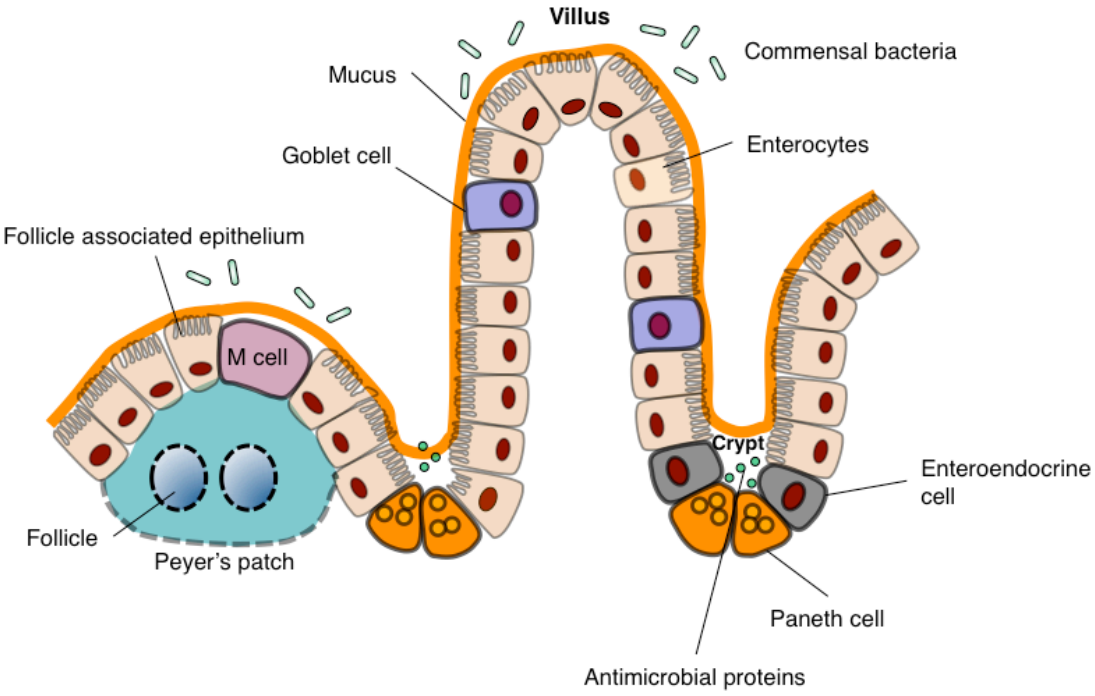

Figure 1.4. Illustration of the intestinal epithelial barrier. The small intestine is covered with a single layer of epithelial cells organized into villi and crypts. The epithelial cells are differentiated into 4 functional subtypes: absorptive encerocytes, hormone secreting enteroendocrine cells, mucus secreting goblet cells, and Paneth cells that produce antimicrobial peptides. The follicle associated epithelium overlies the Peyer's patch and lymphoid follicles, and within the follicle associated epithelium are M cells, which specialize in that uptake of luminal antigens.

to a Th2-polarizing response, as well as inducing regulatory T cells (Tregs) and IgA-producing plasma cells.During infection, the intestinal DCs are involved in the induction of effector lymphocytes such as Th1 and Th17 cells (104, 110, 111). Previous studies have proposed that these different responses are driven by different DC subsets in the mucosa. The non-inflammatory resident DCs express CD11c+CX3CR1+, and the inflammatory DCs located in the Peyer's patch express CD11c+CCR6+ (110-112). The mechanism of how intestinal epithelial cells distinguish non-pathogenic or pathogenic microbes and release appropriate cytokines is unknown; however, it is clear that intestinal epithelial cells play a key role in regulating mucosal immune response. Intestinal epithelial cells sample luminal antigens in several ways. The follicle-associated epithelium, which covers the Peyer's patches and lymphoid follicles, contains microfold (M) cells which transcytose luminal antigens to DCs in the subepithelial dome (113, 114). DCs can also directly sample antigens and microbes through dendritic processes extended between epithelial tight junctions, a process dependent on DC expression of CX3CR1 (115).

Intestinal epithelial cells also serve as sensors for microbial infection. Pathogenic microbes and their products activate signaling pathways in intestinal epithelial cells and induce the secretion of chemokines and cytokines for the attraction and activation of DCs, macrophages, neutrophils, lymphocytes, mast cells, and eosinophils (116). In the case of *T. gondii* infection, intestinal epithelial cells initiate a series of innate immunologic events that lead to a robust inflammatory process in the gut. Tissue samples from the small intestine of *T. gondii* infected mice have increased chemokines including monocyte chemotactic protein 1 (MCP-1/CCL2)), macrophage inflammatory protein 1α and β (MIP-1α/CCL3, and MIP1-β/CCL4), and MIP-2/CXCL2, chemoattractants for DCs, macrophages, neutrophils, and T cells,

respectively (117). However, *T. gondii* infection in certain strains of inbred mice leads to uncontrolled immunopathology during acute infection, which will be discussed in detail in the next few sections.

6. Toll-like receptor signaling in the intestinal epithelium

Toll-like receptors (TLR) are pattern recognition receptors that detect microbes through specific recognition of microbial structures called pathogen-associated molecular patterns (PAMPs) (118) from bacteria, fungi, viruses, and protozoa. TLRs are structurally similar with an ectodomain containing leucine-rich repeats (LRR) for ligand binding, a transmembrane domain, and an intracellular domain containing the Toll/IL-1 receptor/resistance (TIR) domain for signaling. TLRs are expressed at the cell membrane (TLR-1, -2, -4, -5, and -6) or localized in endosomes (TLR-3, -7, -8, 9). To date, 10 TLRs in humans and 13 TLRs in mice have been identified. TLRs 1 to 9 are conserved among humans and mice, but TLR10 is functional only in humans, and TLR11 is functional only in mice (119-121). TLR2 forms heterodimers with TLR1 and TLR6, and recognizes a wide spectrum of microbial components, including lipoproteins, lipoteichoic acid, lipoarabinomannan, GPI anchors, phenol-soluble modulin, zymosan, glycolipids, and peptidoglycan from various pathogens (122-125). TLR4 recognizes bacterial LPS (126), and TLR5 recognizes bacterial flagellin (127). TLR3 and TLR7-9 are intracellular receptors that recognize double-stranded RNA, single-stranded RNA, and bacterial CpG DNA respectively (120, 128-132). More recently, mouse TLR11 is identified to recognize *T. gondii* profilin (133, 134).

Dimerization of TLRs activates signaling pathways through the cytoplasmic TIR domain. MyD88 is an adapter protein that associates with the TIR domain and initiates signaling cascades after ligand binding to all TLRs except TLR3. TLR3

instead uses the adapter protein TRIF. TLRs initiate a signaling cascade resulting in activation of NF-κB, which induces inflammatory cytokines. However, activation of each individual TLR can result in difference of gene expression profile activation (119-121, 135).

Primary intestinal epithelial cells of normal mucosa express TLRs 1-9 (136-138). Although TLRs are generally expressed at low levels, they are upregulated during intestinal inflammation (136, 139, 140). Specifically within the intestine, TLR2, TLR4 and TLR5 are expressed by the follicle-associated epithelium as well as the villi and crypts of the small intestine (141). However, the expression of TLR2 and TLR4 are generally low in the intestinal epithelial cells (136). Further, TLR localization is restricted within intestinal epithelial cells. For example, human intestinal epithelial cells express TLR5 at the basolateral surface (142), whereas mouse cells express functional TLR5 at the apical surface (143). Lee and colleagues showed that, in polarized intestinal epithelial cells, TLR9 is expressed at both the apical and basolateral surface (144). This is in contrast to the endosomal localization described for TLR9 in immune cells. Interestingly, the localization is important for cellular response to CpG in epithelial cells since apical stimulation inhibited NF-κB signaling, whereas basolateral stimulation activated NF-κB signaling (144).

TLRs expressed in epithelial cells were initially thought to be hyporesponsive to their ligands (137). However, several studies have now demonstrated that epithelial cells require TLR signaling to maintain a healthy intestinal environment (102, 145, 146). Rakoff-Nahoum and colleagues have shown that TLR/MyD88 signaling in intestinal epithelial cells is crucial for gut homeostasis and protection against experimental induced epithelial injury and associated mortality (145). In the Peyer's patch, TLR2,

TLR4, and TLR5 stimulation in the follicle-associated epithelium can increase uptake of luminal antigens and promote antigen capture by subepithelial DCs (141). TLR2 stimulation in polarized intestinal epithelial cells triggers redistribution of the tight junction protein ZO-1, and phosphorylation of protein kinase C (PKC) for the biogenesis of tight junctions (147, 148). Paneth cells also respond directly to bacterial PAMPs and increase the expression of antimicrobial peptides (149). Furthermore, TLR9 stimulation induces Paneth cells degranulation (150). Bacterial stimulation through TLRs in epithelial cells induces the secretion of thymic stromal lymphopoietin (TSLP), TGF-β, and IL-25, which can decrease TLR responsiveness in dendritic cells, and limit development of Th1 and Th17 responses (151-153). TLR stimulation of epithelial cells also induces the cytokine APRIL (a proliferation of inducing ligands), which promotes plasma cell survival and class switching from IgA1 to IgA2 (154). Goblet cells secrete trefoil factor 3 (TFF3), a peptide that is important in wound healing of the intestinal epithelium, when stimulated through TLR2 (155).

7. The role of TLRs and mucosal defense against *T. gondii* infection

The importance of TLRs in host defense against *T. gondii* infection was first demonstrated in MyD88 deficient mice. Mice that lack MyD88 did not survive intraperitoneal (i.p.) infection due to uncontrolled parasite replication, and insufficient production of IL-12 and IFN-γ (156). During oral infection, MyD88 deficient mice also display defects in neutrophil recruitment, T cell IFN-γ secretion, and p47 GTPase IGTP (Irgm3) induction (157). MyD88 is a shared adapter protein for TLR and IL-1 and IL-18 receptors and phenotypes observed in MyD88 deficient mice could be due to either failure in TLR signaling, or in either IL-1 or IL-18 receptor signaling (158). However, mice deficient in the interleukin-1b converting enzyme, which lack functional IL-1 and IL-18, survived infection (159). Therefore, the phenotype

observed in the MyD88 deficient mice is most likely due to lack of TLR signaling (159).

Infection of individual TLR deficient mice revealed that multiple TLRs are important for host defense against *T. gondii* infection. In the case of TLR4, two groups have reported contradicting results. In one study, oral infection of C3H/HeJ mice, which encode a missense mutation in the TLR4 gene that destroys signaling, were more susceptible to oral infection. These mice displayed increased cyst numbers, reduced cytokine production, and enhanced pathology (160). In contrast, another group showed that TLR4-/- mice displayed reduced mortality (161). TLR2×4-/- and TLR11-/-mice survived oral infection, but showed higher cyst numbers in the brain (162). In addition to TLR2 and TLR4, TLR11 plays an important role in IL-12 production by DC in response to the *T. gondii* protein profilin (163). Surprisingly, TLR11 is dispensable for IL-12 production during oral infection (162). Lastly, *T. gondii* infection in TLR9-/- mice resulted in higher parasite burden and reduced IFN-γ production, suggesting that TLR9 is also required for development of effective T cell responses (164).

Oral infection with *T. gondii* induces severe intestinal inflammation and early mortality in C57BL/6 mice but not in BALB/c mice. The difference in disease outcome is reportedly associated with the H-2 complex. Mice with the H-2a haplotype are resistant, whereas mice with the H-2b haplotype are susceptible to oral infection (165). In the case of C57BL/6 mice, at 7 days post infection there is massive necrosis of the villi, as well as infiltration of DCs, macrophages, neutrophils, and lymphocytes (74). Excessive Th1 responses in susceptible C57BL/6 mice contribute to immunopathology mediated by CD4+ T cells (74). Recent studies have proposed that

the commensal flora contribute to Th1 cytokine production because less pathology was observed in mice lacking bacterial sensing TLRs (TLR2, TLR4, and TLR9) (162, 164). More recent studies have shown that *T. gondii* oral infection causes CCR2-dependent expansion in CD103+ intestinal epithelial lymphocytes (IEL), which possess pathologic activity during infection (166).

The mucosal epithelium plays an important role in initiating protective response during *T. gondii* infection. Following *T. gondii* oral infection, the parasite can be found in the small intestinal epithelium, lamina propria, and Peyer's Patches within 1 hour (167). This suggests that the parasite may infect the mucosal surface by direct invasion of epithelial cells. Yet, while studies have shown that high virulent *T. gondii* can utilize the host intercellular adhesion molecule (ICAM-1) for transmigration across the cellular barrier and dissemination (168), several lines of evidence suggests that intestinal epithelial cells can respond to *T. gondii* infection. In vivo studies have shown that *T. gondii* infection can induce innate immune responses such as production of antimicrobial peptides in Paneth cells (169). In vitro studies by Mennechet and colleagues have also demonstrated that the small intestinal epithelial cell line (mIC_{c12}, H2-b haplotype) synergizes with lamina propria CD4+ T cells to enhance proinflammatory responses to *T. gondii* (117).

8. Brief outline of dissertation research

The immune response elicited by infection with *T. gondii* involves both humoral and cell based immunity. While systemic responses have been extensively investigated, the knowledge regarding the local immune responses in the gut during the acute phase of infection is limited. **Chapter 2** reports the finding of early response of human intestinal epithelial response during *T. gondii* infection. In this chapter, I used an in vitro model to demonstrate that *T. gondii* infection in epithelial cells elicits different

inflammatory responses and pathways and identified TLR2 as a receptor involved in recognition of *T. gondii.* In **Chapter 3**, I examined how oral infection with *T. gondii* modulates the intestinal environment. I found that *T. gondii* oral infection in wild type and TLR2 deficient mice result in differences in goblet cell and Paneth cell numbers, as well as different pathology. Furthermore, *T. gondii* induced the movement of DCs to the follicle-associated epithelium in a TLR2 dependent fashion. This finding has led me to identify TLR2 dependent molecules derived from *T. gondii*. In **Chapter 4,** I found that supernatants from *T. gondiii* infected cells contain parasite-derived factors that trigger epithelial transport of luminal antigens and a TLR2-dependent movement of DC to the follicle-associated epithelium. Analysis of the supernatant revealed several *T. gondii* proteins, including 2 GPI-anchored proteins (SAG-1 and subtilisin) that are candidate TLR2 ligands. I found that supernatants from SAG1-deficient parasites had reduced TLR2 stimulatory activity. Finally, **Chapter 5** summarizes my results, discusses the significance of my findings, and suggests future directions for this research area.

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CHAPTER II

EARLY RESPONSE OF MUCOSAL EPITHELIAL CELLS DURING *TOXOPLASMA GONDII* INFECTION *

* Reprinted from Chia-Hsin Ju, Annapoorani Chockalingam and Cynthia A. Leifer. Early response of mucosal epithelial cells during *Toxoplasma gondii* infection. *J. Immunol.* 183;7420-7427.

Abstract

The innate immune response of mucosal epithelial cells during pathogen invasion plays a central role in immune regulation in the gut. *Toxoplasma gondii* (*T. gondii*) is a protozoan intracellular parasite that is usually transmitted through oral infection. Although much of the information on immunity to *T. gondii* has come from intra-peritoneal infection models, more recent studies have revealed the importance of studying immunity following infection through the natural per-oral route. Oral infection studies have identified many of the key players in the intestinal response; however, they have relied on responses detected days to weeks following infection. Much less is known about how the gut epithelial layer senses and reacts during initial contact with the pathogen. Given the importance of epithelial cells during pathogen invasion, this study uses an *in vitro* approach to isolate the key players and examine the early response of intestinal epithelial cells during infection by *T. gondii*. We show that human intestinal epithelial cells infected with *T. gondii* elicit rapid MAPK phosphorylation, NF-κB nuclear translocation, and secretion of interleukin (IL)-8. Both ERK1/2 activation and IL-8 secretion responses were shown to be MyD88 dependent and TLR2 was identified to be involved in the recognition of the parasite regardless of the parasite genotype. Furthermore, we were able to identify additional *T. gondii*-regulated genes in the infected cells using a pathway-focused array. Together, our findings suggest that intestinal epithelial cells were able to recognize *T. gondii* during infection, and the outcome is important for modulating intestinal immune responses.

Introduction

A single layer of intestinal epithelial cells that line the mucosal surface must prevent the entry of exogenous antigens, allow absorption of essential nutrients and yet initiate effective and appropriate immune responses when pathogens are present (1). Host defenses at mucosal surfaces include the secretion of IgA, defensins, and cytokines and chemokines. IgA and defensins prevent bacterial adherence and contribute to pathogen elimination, while cytokines and chemokines participate in gut homeostasis as well as recruitment of immune cells during infection. Epithelial cells express several innate immune receptors including nucleotide oligomerization domain (NODs) proteins and Toll-like receptors (TLRs) that participate in initiating the immune response (2). However, activation is tightly controlled to prevent pathology due to mucosal inflammation (2-6). Upon encounter with pathogenic bacteria, epithelial cells elicit a potent response that shapes the ensuing immune response (7, 8)

T. gondii is an orally acquired apicomplexan protozoan parasite (9). Human infections are usually asymptomatic, but reactivation of chronic infection in immunosuppressed individuals results in toxoplasmic encephalitis (10, 11). Serological surveys have estimated that one third of the world's population has been exposed to this parasite (12). However, there is no vaccine and therapeutic treatment regimens have significant side effects. *T. gondii* infections are controlled primarily by T lymphocytes. IL-12, and TNF-α, are critical cytokines for stimulating Th1 CD4+ T cell induced protection (13, 14), while interferon gamma (IFN-γ) plays a major role in protection through CD8+ T cells (14, 15).

TLRs are innate immune receptors that directly recognize microbial structures and initiate an inflammatory response. All TLRs, except TLR3, use the adapter molecule MyD88 to initiate the signaling cascade. MyD88 deficient mice are highly susceptible

to *T. gondii* infection due to a failure to produce IL-12 (16). Multiple TLRs have been linked to protective immunity against *T. gondii* infection. In mice, TLR11 expressed by DCs is required for secretion of IL-12 in response to stimulation with the *T. gondii* protein profilin (17, 18). TLR2 deficient mice show increased susceptibility with high dose intraperitoneal infection (19). Oral infection of mice results in intestinal inflammation, ileitis, in wild type but not mice deficient in TLR9 or TLR4 (20, 21). This suggests that TLR9 and TLR4 may play a much more important role in initiating immunity to *T. gondii* at the mucosal surface. The role of TLRs in human cell recognition of *T. gondii* infection is much less well studied. Human TLR2 can respond to glycosylphosphatidylinositols (GPIs) from *T. gondii*, but the role of TLRs during live infection of human cells, especially in the gut has not been studied.

T. gondii can infect the gut mucosa by direct invasion of epithelial cells in the small intestine (22). Therefore, epithelial cells may respond directly to *T. gondii* infection and initiate early local mucosal immune responses. This is supported by *in vitro* RNase protection studies using an immortalized mouse small intestinal enterocyte cell line, which demonstrated that the chemokines MCP-1, MIP-1 and eotaxin were induced upon infection (23). In the present study, we have investigated whether human intestinal epithelial cells respond directly to infection with *T. gondii* and by what mechanism this recognition occurs.

Materials and Methods

Cell culture and parasites

Henle 407 (human embryonic intestinal epithelial cells), HEK293 (human embryonic kidney cells), and HS27 (human foreskin fibroblast) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with containing 2 mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and 10% low endotoxin fetal bovine serum. *T. gondii* tachyzoites strain were maintained by serial 2 days passages on HS27 monolayers in DMEM. All cell cultures and parasites were routinely checked for mycoplasma by a high sensitivity PCR-ELISA based Mycoplasma detection kit (Roche, Indianapolis IN).

In vitro infections

T. gondii tachyzoites were added to Henle 407 cells and were briefly centrifuged (400 × g, 1 min) to initiate parasite and cell contact. At time points indicated in each figure legend, supernatants or cell lysates were collected for further analyses. In some experiments, 50 ng/mL wortmannin (WM) was added 2 hours prior infection.

In vivo passage of T. gondii tachyzoites

To pass the parasite through mice, C57BL/6 mice were infected with $5x10^4$ RH tachyzoites by intraperitoneal (i.p.) infection. After 3 days of infection, the parasites were collected from the peritoneal fluid and washed once with HBSS. The parasites were then expanded by one additional passage in HS27 fibroblast prior stimulation.

Antibodies

Abs specific to total and phosphorylated forms of ERK1/2, p38, and PKB (Akt) were from Cell Signaling Technology (Danvers, MA). Anti-MyD88 polyclonal Ab was

from Alexis Biotechnology (San Diego, CA). NF-κB p65 antibody was from Santa Cruz Biotechnology, Inc (Santa Cruz, CA), and AlexaFluor 594 was from Molecular Probes (Invitrogen, Carlsbad, CA). Anti-IL-18 antibodies were purchased from Abcam (Cambridge, MA)

NF-κB translocation assay

For immunofluorescence analyses, Henle 407 cells were plated at a number of 2-3 × 10^5 cells per well on sterile coverslips placed wells of a 24-well plate. Cell monolayers were infected with *T. gondii* RH-YFP tachyzoites and were then fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 in TBS (TBS-TX) for 15 min and blocked in 1% BSA in TBS-TX for 20 min. The cells were then stained with primary rabbit anti-NF-κB p65 followed by secondary antibody goat anti–rabbit IgG conjugated to AlexaFluor 594. Nuclei were stained using DAPI. Confocal images were taken with a Leica SP5 laser scanning confocal microscope using a 63×lens. Contrast and brightness of individual channels were adjusted linearly in Photoshop (Adobe). For immunoblot analyses, Henle 407 cells were plated at a number of 1.5 × 10^6 cells per well in 6-well plate and infected with *T. gondii* RH for the indicated times. Cytoplasmic and nuclear proteins were isolated according to previous protocols (24) and blotted for p65 (1:500).

RNA interference

SureSilencing human MyD88, TLR2, and TLR9 shRNA and control plasmids were purchased from SuperArray Bioscience Corp (Frederick, MD). Henle 407 cells were transfected using TransIT Transfection Reagent (Mirus Bio, Madison, WI) according to manufacturer's protocols. Transfected cells were selected with neomycin (1.0

mg/mL) for 14 days, and antibiotic-resistant individual colonies were isolated for further analysis and maintained in the presence of neomycin. For transient transfections, Henle 407 cells were transfected with shRNA plasmids by electroporation. Cells were used at 48 to 96 hr post transfection.

RNA extraction and PCR analysis

Total RNA of Henle 407 cells infected with *T. gondii* was extracted using the RNeasy mini kit (QIAGEN, Valencia, CA). Reverse transcription of the RNA (1 μg) was performed using ImProm-II™ Reverse Transcription System (Promega, Madison, WI). PCR was performed in 25 μL of a reaction mixture containing 1 μL of the reverse-transcribed RNA. The final PCR products were electrophoresed on 2% agarose gels and visualized using UV light illumination after ethidium bromide staining. Real-time PCR was performed in the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems Inc., Foster City, CA) according to the manufacture instructions. The reaction was performed using the Power SYBR Green PCR Master Mix. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal controls for each sample. The primers sequences are listed in Table 2.1. TLR and GAPDH primers are used as previously described (25, 26).

Luciferase reporter assays

HEK293 cells were plated at 1 x 10^4 cells/well in 96-well plates. Cells were transfected using TransIT Transfection Reagent (Mirus) and a total of 200 ng DNA/well consisting of human TLR2 plasmids, and NF-κB or IL-8 luciferase reporters. Cells were stimulated with TLR2 ligand, or infected with *T. gondii* tachyzoites, lysed in reporter lysis buffer (Promega), and assayed for luciferase (Promega) activity. NF-κB activity was calculated and processed by Microsoft Excel.

Gene array analysis

The commercial pathway-focused oligonucleotide microarray (OHS-011, Human Inflammatory Cytokines & Receptors Microarray) was purchased from SuperArray Bioscience Corp. The array analyses were performed using a chemiluminescence-based detection system according to the manufacturer's instructions. Images of the array were developed on X-ray films. Image data sets were scanned and analyzed using ScanAlyze (Eisen Lab), and Microsoft Excel software. Background adjustment was performed by subtracting the lowest measured value on the membrane from the values of all genes. The signals from the expression of each gene on the array were normalized against the signal from the internal housekeeping gene GAPDH to obtain the processed data sets. Fold changes were calculated as the normalized ratio of average experimental processed data sets divided by the average medium control processed data sets. Thresholds were set to select for genes up-regulated two fold or more. The original array data were deposited in National Center for Biotechnology Information GEO database under accession number GSE18085.

Cytokine ELISA

Production of IL-8 was measured with the human CXCL8/IL-8 DuoSet ELISA Development Kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical Analysis

Minitab® 15 Statistical Software (Minitab, State College, PA) was used for Student's t tests. A p value of < 0.05 was considered significant.

Table 2.1. PCR primers used in this study.

Gene	Forward	Reverse
IL-8	AGC CTT CCT GAT TTC TGC AGC TCT	AAT TTC TGT GTT GGC GCA GTG TGG
CCL20	AGT TTG CTC CTG GCT GCT TTG ATG	CTG CCG TGT GAA GCC CAC AAT AAA
CCL15	TTG GAT CCC AGG CCC AGT TCA TAA	AGC AGT CAG CAG CAA AGT GAA AGC
CCL24	ATG CCT CAA GGC AGG AGT GAT CTT	TCT TCA TGT ACC TCT GGA CCC ACT
MyD88	AGA TGA TCC GGC AAC TGG AAC AGA	AGT CAC ATT CCT TGC TCT GCA GGT
TLR1	GGC TGG CCT GAT TCT TAT AA	CTC TAG GTT TGG CAA TAA TTC A
TLR2	TCC AGC ACA CGA ATA CAC AGT	ATT GCC ACC AGC TTC CAA AG
TLR3	TGC AGC TGA CTA GGA ACT CCT T	TGC TGC AAA TCG AGA ATT TC
TLR4	TAA TAT TGA CAG GAA ACC CC	TTC ATA GGG TTC AGG GAC AG
TLR5	CCT TTT ATC GTT TCT GCA AC	GCA TCT GGA TGC AAG AAG TA
TLR6	GCT CTT TTG GGC TAA CAT TA	AGA CTG GGC TGT CTC TAA CT
TLR7	AGA TAA CAA TGT CAC AGC CG	AGT TTT GGG ACA GAT CCA GT
TLR8	AAA CAA ACT ACT CTT TTT AAC TG	AAT TTT GAC ATT CAG ATG TT
TLR9	GGA GGC TGG ATG TCA GCT GCA A	CAG GCG CAG TGC AGA GGG TT
TLR10	TCT TTA GTA AGT TGC TTT GC	CAG GGA GAT CAG TTA GAA AA
GAPDH	GAA GGT GAA GGT CGG AGT C	GAA GAT GGT GAT GGG ATT TC

Results

Intestinal epithelial cells respond to *T. gondii* infection *in vitro*

Oral infection with *T. gondii* results in ileitis in C57BL/6 mice mediated by a robust Th1 type of response (23). CD4+ T cells synergize with intestinal epithelial cells to drive the secretion of Th1 type cytokines and various chemokines. The response of intestinal epithelial cells directly to the parasite during the early stage of infection in mouse, and especially in human, remains unclear. To determine if human intestinal epithelial cells respond directly to *T. gondii*, we incubated *T. gondii* tachyzoites (RH strain) with human intestinal epithelial cell lines. Consistent with other studies on large intestine, colon cell lines did not activate mitogen-activated protein kinases (MAPKs) in response to *T. gondii* infection even though they were permissive for infection. *T. gondii* were also able to infect the human small intestinal epithelial cell line Henle 407 (Fig. 2.1). The kinetics of infected Henle 407 cells showed increased parasite invasion during the first few hours, followed by parasite replication after 8 hours as indicated by the appearance of rosettes (Fig. 2.1 arrows). Unlike colon cell lines, which have high levels of basal MAPK phosphorylation, *T. gondii* infection of Henle 407 cells induced phosphorylation of both ERK1/2 and p38 MAPKs by 15 minutes (Fig. 2.2A). ERK1/2 and p38 phosphorylation decreased at 30 minutes and was reduced nearly to baseline by 45 minutes.

NF-κB regulates proinflammatory and anti-apoptotic genes in response to pathogens. To determine whether *T. gondii* infection of Henle 407 cells results in activation of NF-κB, we infected the cells for various times with RH tachyzoites and immunoblotted cytosolic and nuclear extracts for p65 NF-κB. Translocation of NF-κB to the nucleus was observed as early as 15 minutes, peaked at 60 minutes, and was still detected two hours post infection (Fig. 2.2 C).

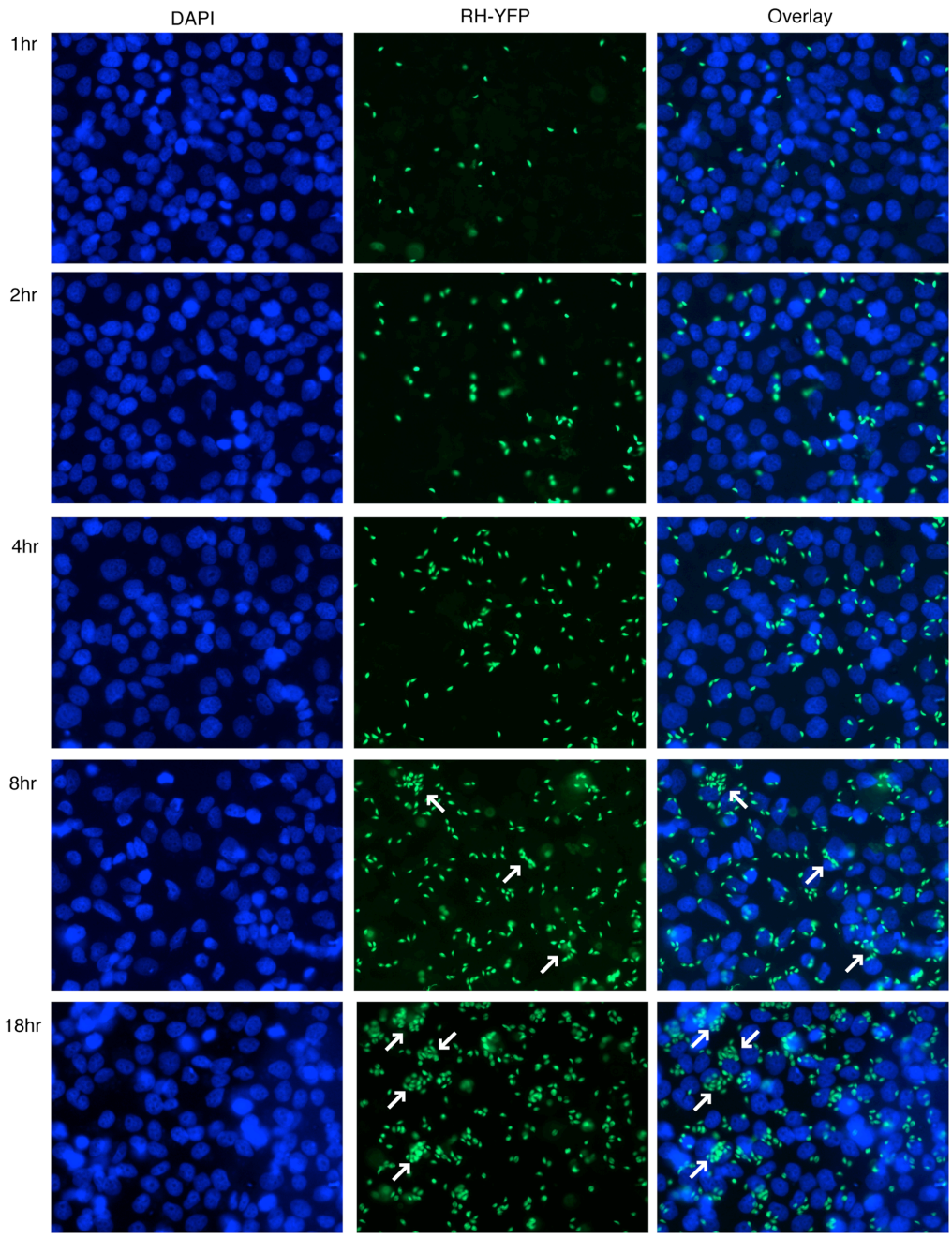


Figure 2.1. *In vitro* infection of Henle 407 cells. Henle 407 cells were plated on cover slips and infected with RH-YFP *T. gondii* tachyzoites. At each indicated time, cells were fixed with 3% paraformaldehyde and observed under 40X objective. Arrows indicate parasite replicating rosettes forming as early as 8 hr post infection.

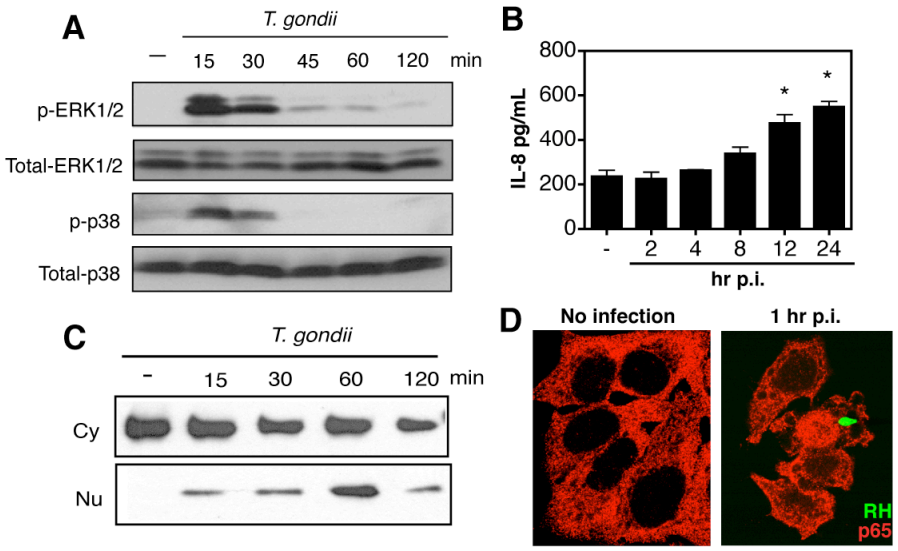


Figure 2.2. Intestinal epithelial cells respond to *T. gondii* infection. (A) Activation of MAPK in *T. gondii* infected Henle 407 cells. Henle 407 cells were incubated with RH tachyzoites (parasite to cell ratio 6:1) for the indicated times. Whole cell lysates were collected for Western blot analysis by immunoblotting with antibodies against total and phosphorylated forms of ERK/1/2 and p38 MAPK. (B) IL-8 secretion in *T. gondii* infected Henle 407 cells. IL-8 levels in infected culture supernatants were collected at indicated time points and assayed by ELISA (triplicate assays, error bar = SD). Experiments were performed 3 times with similar results. *, $p < 0.05$ compared with IL-8 levels of uninfected cells. C and D, translocation of NF-κB to the nucleus. Henle 407 cells were infected with RH or RH-YFP parasites for the indicated times. NF-κB p65 localization was visualized by Western blot (C) or immunofluorescence staining (D) after probing with anti–NF-κB p65 (red). In uninfected cells, p65 was present in the cytoplasm, and infection with *T. gondii* resulted in nuclear localization of p65.

Infection with transgenic parasites expressing YFP (yellow fluorescent protein) allowed the visualization of infected cells. Staining for p65 NF-κB demonstrated that infected, but not uninfected, cells translocated NF-κB to the nucleus (Fig. 2.2 D). Early immune defense against *T. gondii* involves recruitment of several innate immune cell populations including neutrophils, macrophages, dendritic cells and eosinophils (27-30). Neutrophils, recruited by IL-8 (CXCL1/KC, CXCL2/MIP-2, and CXCL5-6/LIX homologues in mice), are often the first cell type recruited to the area of infection and provide the initial source of IL-12 that triggers Th1 T cell mediated immunity (27, 31). Therefore, we next tested if epithelial cells could participate in the recruitment of neutrophils by secreting IL-8 in response to *T. gondii* infection. Henle 407 cells produced significant levels of IL-8 at 12 hours after infection with *T. gondii* (Fig. 2.2 B). IL-8 levels continued to rise up to 24 hours post infection. Taken together these data demonstrate that human small intestinal epithelial cells directly respond to infection with *T. gondii* tachyzoites by activating MAPK and NF-κB signaling cascades as well as producing chemokines that actively participate in the innate immune response to *T. gondii*.

***T. gondii* infected intestinal epithelial cells express several inflammatory genes**

Oral infection studies have demonstrated an increase in several cytokines and chemokines in response to *T.gondii* infection; however, the mixed population of the intestinal mucosa did not allow for the determination of the relative role that specific cell populations play in the production of these mediators. To address the role of intestinal epithelial cells to modulate the cytokine environment early following infection, we used specific pathway arrays to identify cytokines and chemokines induced 4 hours after exposure to *T. gondii*. At this time point most cells have become infected, but the parasites have not replicated. Consistent with previous studies on late

responses, infected epithelial cells expressed higher levels of proinflammatory chemokines including macrophage inflammatory protein-1a (MIP-1α/CCL3) and -2 (MIP-2/CXCL2), RANTES/CCL5, monocyte chemotactic protein-2 (MCP-2/CCL8), -3 (MCP-3/CCL7), and INF-γ-inducible protein-10 (IP-10) (Table 2.2). The induced cytokines and chemokines included ones that induce neutrophil chemotaxis (IL-8, MIP-2/CXCL2), homing of mucosal DCs (CCL20), and migration of DCs to sites of infection (MIP-1α/CCL3 and RANTES/CCL5) (27) (32). Of note, IL-18, which enhances IL-12 mediated immune responses to *T. gondii*, was highly induced in infected epithelial cells (33-35). Real-time PCR analysis confirmed the upregulation of several neutrophil and monocyte chemoattractants including IL-8, CCL15, CCL20, and CCL24 upon *T. gondii* infection (Fig. 2.3). Together, these data demonstrate that human intestinal epithelial cells induce chemotactic and inflammatory mediators capable of modulating the local immune response early (minutes to hours) after infection.

Epithelial cell response to *T. gondii* is PI-3 kinase independent

T. gondii infection of mouse macrophages induces MAPK and protein kinase B (PKB, also known as Akt) activation through a Gi-dependent PI-3 kinase signaling pathway (36). Similar to macrophages, phosphorylation of Akt occurred in Henle 407 cells 60 minutes after *T. gondii* infection (Fig. 2.4 A). The PI-3 kinase inhibitor wortmannin completely blocked *T. gondii* induced phosphorylation of Akt, but only slightly reduced the phosphorylation of ERK1/2 and p38 at 15 minutes and had no detectable effect on ERK1/2 at 60 minutes (Fig. 2.4 A). However, *T. gondii* infection of wortmannin treated cells still resulted in an induction of IL-8 secretion with similar fold induction as untreated cells (Fig. 2.4 B) Real-time PCR analysis demonstrate

Table 2.2. Pathway specific microarrays revealed upregulation of several *T. gondii* induced inflammatory cytokines, chemokines, and receptors. Henle 407 cells were infected with RH parasites and total RNA were collected at 4 hr post infection. RNA from infected and uninfected cells was compared using a pathway-focused oligonucleotide microarrays containing 113 genes involved in the inflammatory response. Genes that were upregulated 2 fold or more after infection are shown.

Cytokines and cytokine receptors			**Chemokines and chemokine receptors**		
Gene	***Genbank no.***	***Fold change***	***Gene***	***Genbank no.***	***Fold change***
Il3	NM_000588	10.5	*Ccl3*	NM_002983	3.9
Il8	NM_000584	2.3	*Ccl5*	NM_002985	4.7
Il9	NM_000590	2.5	*Ccl7*	NM_006273	2.7
Il11	NM_000641	2.2	*Ccl8*	NM_005623	3.2
Il12a	NM_000882	2.3	*Ccl11*	NM_002986	5.9
Il18	NM_001562	8.5	*Ccl13*	NM_005408	3.8
Il1rn	NM_000577	2.9	*Ccl15*	NM_032965	6.2
Il1r2	NM_004633	2.1	*Ccl20*	NM_004591	5.8
Il8rb	NM_001557	2.9	*Ccl23*	NM_005064	4.5
Il13ra2	NM_000640	2.1	*Ccl24*	NM_002991	11.1
Il15ra	NM_002189	3.9	*Cxcl2*	NM_002089	2.1
Il18r1	NM_003855	7.9	*Cxcl3*	NM_002090	3.6
Mif	NM_002415	3.6	*Cxcl10*	NM_001565	5.6
Tnf	NM_000594	2.9	*Cxcl13*	NM_006419	2.4
Others			*Ccr2*	NM_000648	2.5
Gene	***Genbank no.***	***Fold change***	*Ccr3*	NM_001837	2.6
Cd40lg	NM_000074	2.9	*Ccr6*	NM_004367	2.4
Cebpb	NM_005194	3.4	*Ccr7*	NM_001838	3.1
Crp	NM_000567	2.4	*Ccr9*	NM_006641	6.3
Edg3	NM_005226	6.7	*Blr1*	NM_001716	2.8
Rps27a	NM_002954	2.8	*Pf4*	NM_002619	2.5
Spp1	NM_000582	4.4			

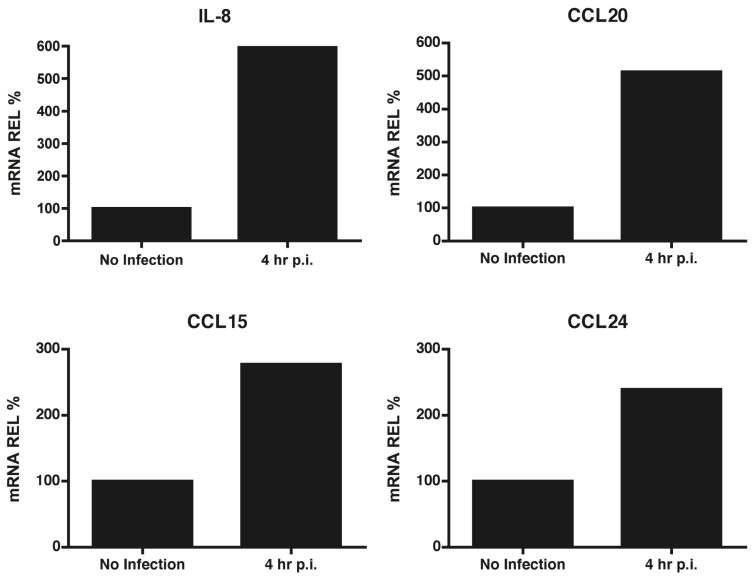


Figure 2.3. Cytokine and chemokine gene induction by *T. gondii* infection. Human IL-8, CCL15, CCL20, and CCL24 gene transcripts levels measured by Real-Time PCR analysis of RNA collected 4 hours post *T. gondii* infection. The data are normalized to GAPDH and compared against uninfected expression levels (REL, relative expression levels).

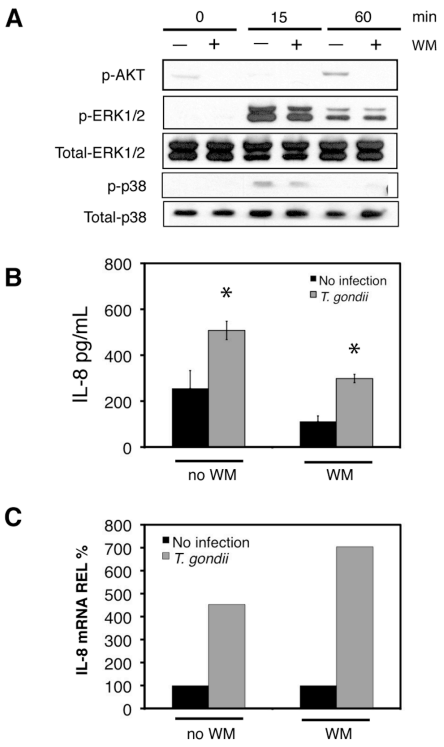


Figure 2.4. *T. gondii* induced MAPK activation and IL-8 secretion in intestinal epithelial cells is PI 3-kinase signaling independent. (A) Henle 407 cells were preincubated 2 hr with or without PI3-kinase inhibitor wortmannin (WM, 50 ng/mL) followed by infection with *T. gondii* (parasite to cell ratio 6:1). At the indicated time points, cell lysates were collected for Western blot analysis. Antibodies against phosphorylated forms of AKT and ERK1/2 were blotted simultaneously, and the same blot was srtipped and reprobed with antibodies against total ERK1/2. (B) Henle 407 cells were pretreated with or without wortmannin and infected with *T. gondii* as described above. IL-8 levels in infected culture supernatants were collected at 24 hours post infection and assayed by ELISA (triplicate assays, error bar = SD). *, $p < 0.05$ compared with IL-8 levels of uninfected cells. (C) Henle 407 cells were pretreated with or without wortmannin and infected with *T. gondii* for 4 hours. Analysis of IL-8 and GAPDH transcripts was performed by Real-Time PCR.

that IL-8 mRNA upregulation was not affected by wortmannin in infected or uninfected cells (Fig. 2.4 C). This suggests that while the steady state production of IL-8, but not mRNA production is dependent on PI-3 kinase, *T. gondii* induced upregulation is independent of PI-3 kinase.

Epithelial cell response to *T. gondii* infection is MyD88 dependent

TLR9 has been implicated in the host response to oral infection with *T. gondii* since TLR9 deficient mice fail to develop ileitis that is observed in wild type mice (21). Both hematopoietic and non-hematopoietic cells express TLR9, and experiments using bone marrow chimeras suggest that both compartments are critical for the host response. However, it is unclear if non-hematopoietic cells, such as epithelial cells, respond directly to *T. gondii* through TLR9, or if the response is secondary to commensal bacterial leak into the lamina propria following damage to the epithelium (37-39). To determine if TLR9, or other TLRs, play a role in early response of epithelial cells to *T. gondii*, we depleted MyD88 from Henle 407 cells by stable expression of a short hairpin RNA (shRNA) against MyD88. All TLRs, except TLR3, are dependent on MyD88 for signal transduction. Control shRNA transfected Henle 407 cells expressed similar levels of MyD88 protein as non-transfected cells. However, cells transfected with the MyD88 shRNA expressed significantly less MyD88 protein, confirming the effect of the RNAi mediated knockdown (Fig. 2.5 A). Upon *T. gondii* infection, cells deficient in MyD88 had a significantly reduced level of ERK1/2 phosphorylation compared to control cells (Fig. 2.5 B). Furthermore, MyD88 deficient cells failed to induce IL-8 upon exposure to *T. gondii* (Fig 2.5 C). MyD88 is also an adapter protein for IL-1 and IL-18. Since IL-18 is induced upon *T. gondii* infection, and contributes to small intestinal pathology in C57BL/6 mice (33, 34), it remains possible that the MyD88 dependence is via IL-18 signaling. However, in

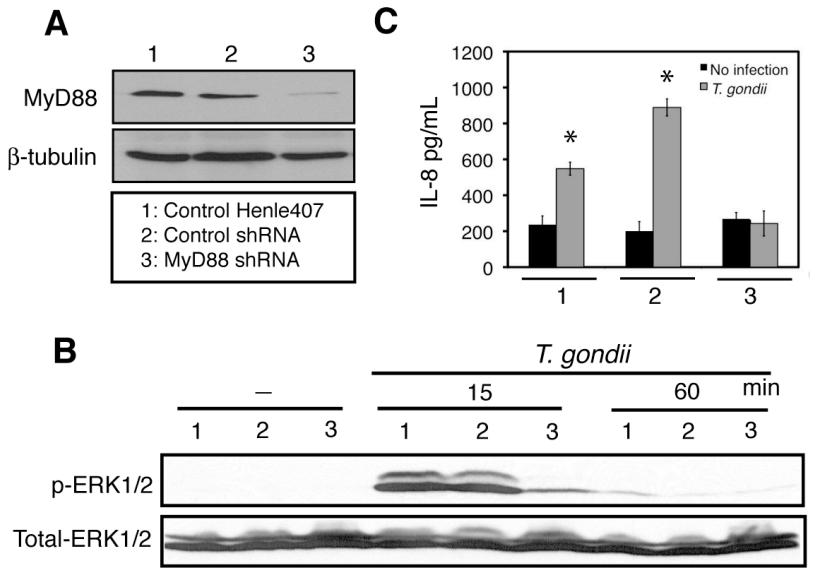


Figure 2.5. ***T. gondii*** **induced MAPK activation and IL-8 secretion is MyD88 dependent.** (A) Henle 407 cells were either untransfected (1), transfected with control shRNA plasmids (2), or MyD88 shRNA plasmids carrying the MyD88 RNA interference sequence (3). Cell lysates from the stably transfected cell lines were collected for immunoblotting with antibodies against MyD88 or β-tubulin. (B) Activation of ERK1/2 in *T. gondii* infected MyD88 knockdown Henle 407 cells. Control Henle 407 cells (1), control shRNA cells (2), and MyD88 shRNA cells (3) were infected with RH tachyzoites (parasite to cell ratio 6:1) at the indicated time points. Cell lysates were collected for immunoblotting with antibodies against total and phosphorylated forms of ERK1/2. (C) Control Henle 407 (1), control shRNA (2), and MyD88 shRNA cells (3) were infected with *T. gondii* (parasite to cell 6:1). Supernatants from each sample were collected for IL-8 ELISA analysis. (Triplicate assays, error bar = SD).

Henle 407 cells, the bioactive form of IL-18 was not detected until 6 hours post *T. gondii* infection (Fig 2.6). This suggested that while IL-18 mRNA level is regulated after infection, the post-translational cleavage to generate the bioactive form of IL-18 did not occur until later. Therefore, MyD88 plays a critical role in the response of Henle 407 small intestine epithelial cells to *T. gondii* infection most likely through a TLR.

T. gondii activates TLR2 on intestinal epithelial cells

Similar to other studies on primary human small intestine cells, Henle 407 cells expressed most of the TLRs except TLR8 (40) (Fig 2.7 A). Stimulation with TLR ligands, or phorbol-12-myristate-13-acetate (PMA) as a control, induced phosphorylation of ERK1/2 and p38 within 15 minutes (TLR1/2: Pam3Cys; TLR2/6: Malp-2; TLR3: polyI:C; TLR4: LPS; TLR5: flagellin; TLR7: loxoribine; TLR9: CpG DNA) (Fig. 2.7 B). To specifically identify which human TLR was involved in *T. gondii* recognition, we reconstituted HEK293 cells with each human TLR independently and measured activation of NF-κB following stimulation with positive control ligands, soluble *Toxoplasma* antigen (STAg) or live *T.gondii* infection using a luciferase reporter. While each positive control ligand stimulated NF-κB activation in the respective TLR expressing cells, only TLR2 expression was permissive for NF-κB response to live *T.gondii* infection (Fig. 2.8 A). STAg failed to activate NF-κB, suggesting that live infection was required. To confirm the role of TLR2 in Henle 407 cell response to *T. gondii* infection, we knocked down TLR2 expression using shRNA. Transient transfection with TLR2 shRNA depleted the mRNA relative expression levels to 6% of wild type (Fig. 2.8 B).

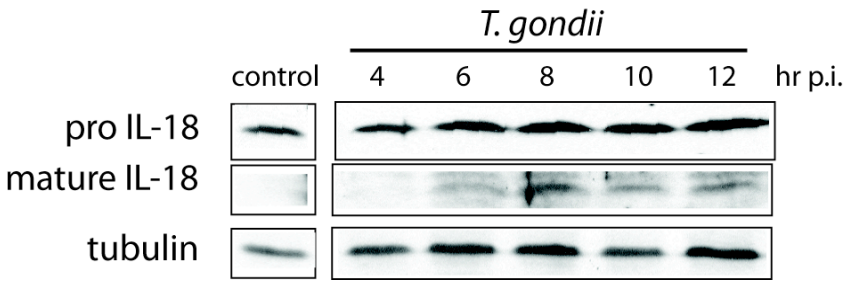


Figure 2.6. The mature form of interleukin (IL)-18 is induced after 6 hours post infection. Henle 407 cells were uninfected (control) or infected with RH tachyzoites (parasite to cell ratio 6:1) and cell lysates were collected at 4, 6, 8, 10, and 12 hours post infection. Immunoblot analysis was performed by immunoblotting with antibodies against IL-18 or β-tubulin. The mature form of IL-18 is detected as early as 6 hours post infection.

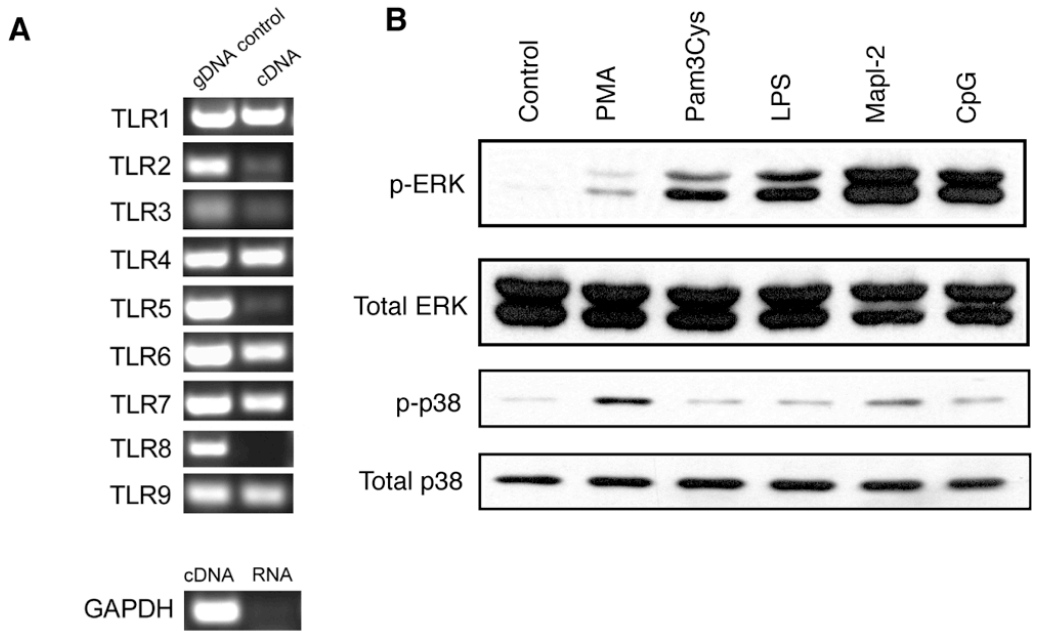
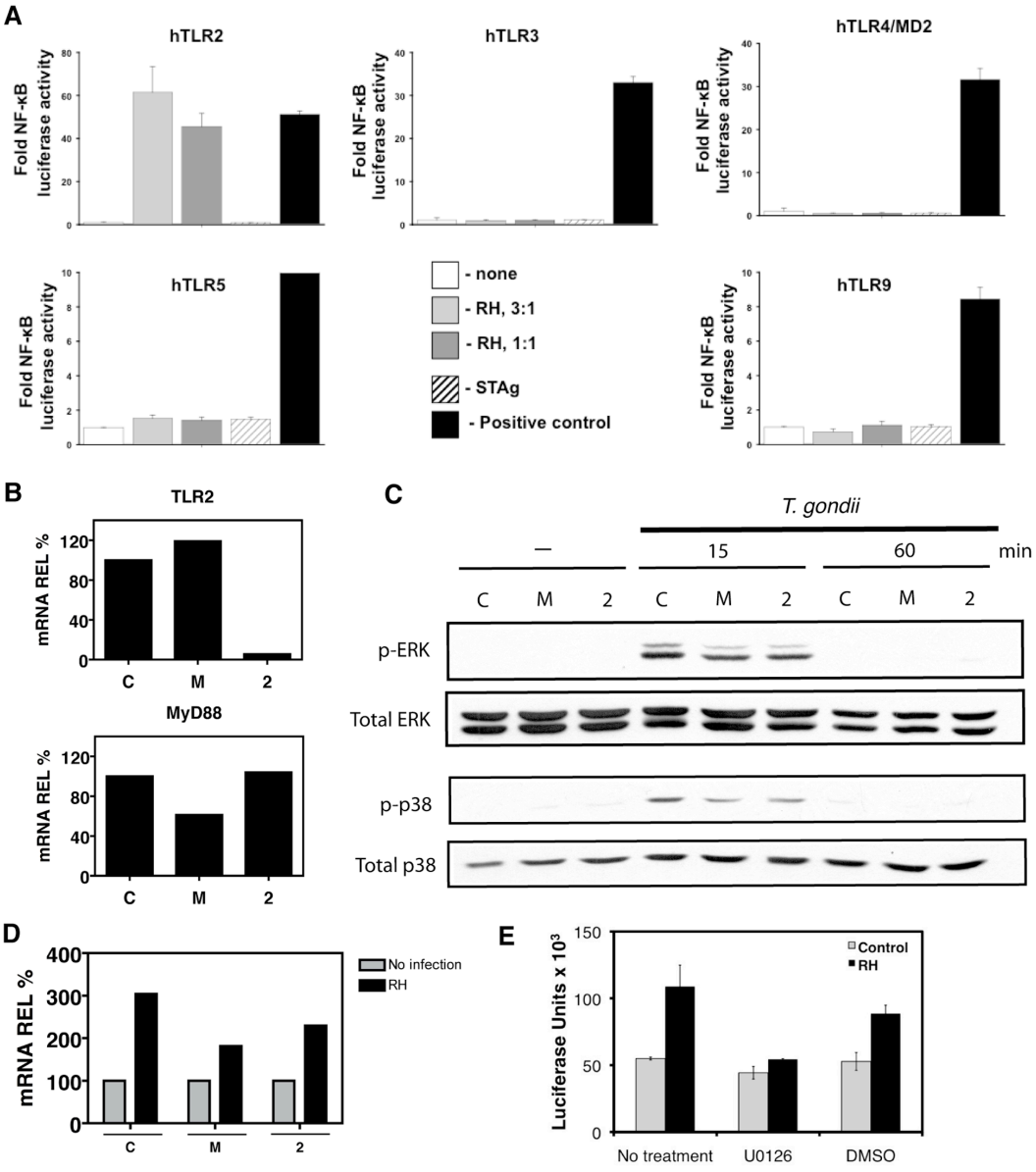


Figure 2.7. TLR genes are expressed in Henle 407 cells. (A) Total RNA was collected from Henle 407 cells, reverse transcribed to cDNA and then amplified for human TLR or GAPDH by PCR (cDNA). Genomic DNA was used as a positive control (gDNA control). (B) Henle 407 cells were treated with 10 ng/mL PMA as positive control or TLR1/2, TLR4, TLR2/6 and TLR9 ligands (1 μg/mL Pam3Cys, 100 ng/mL LPS, 1 μg/mL Malp-2, and 5 μg/mL CpG respectively). Total cell lysate were collected and immunoblotted for total and phosphorylated forms of ERK1/2 and p38.

Figure 2.8. Human TLR2 is important for recognition of *T. gondii*. (A) HEK293 cells were transfected with different human TLRs and an NF-κB luciferase reporter plasmid. The transfected HEK293 cells were stimulated with TLR ligands, STAg (soluble *Toxoplasma* antigen), or infected with live RH tachyzoites. Cell lysates were collected and assayed for luciferase activity (Triplicate assays, error bar = SD). (B-D) Henle 407 cells were transiently transfected with shRNA plasmids carrying TLR2 (2), MyD88 (M) RNA interfering sequences, or no DNA control (C). (B) TLR2 and MyD88 mRNA levels as measured by Real-time PCR. (C) Total cell lysates from cells infected with RH tachyzoites (parasite to cell ratio 6:1) for the indicated time points were immunoblotted with antibodies against total and phosphorylated forms of ERK1/2 and p38. (D) IL-8 levels from cells in B as measured by Real-time PCR following 4 hours infection with *T. gondii* RH tachyzoites. (E) HEK293 cells were transfected with TLR2 and an IL-8 promoter-luciferase reporter construct. Luciferase activity from cells left in media alone (control), or infected with tachyzoites (RH strain). Cells were pretreated with ERK1/2 inhibitor U0126, or DMSO as a solvent control, prior to infection. (Triplicate assays, error bar = SD)


A
hTLR2
hTLR3
hTLR4/MD2
hTLR5
hTLR9
Fold NF-κB luciferase activity
- none
- RH, 3:1
- RH, 1:1
- STAg
- Positive control
B
TLR2
MyD88
mRNA REL %
C
M
2
C
T. gondii
15
60
min
p-ERK
Total ERK
p-p38
Total p38
D
No infection
RH
mRNA REL %
E
Luciferase Units x 10³
Control
RH
No treatment
U0126
DMSO

Knockdown of TLR2 reduced the activation of ERK1/2, p38, and IL-8 induction in response to *T. gondii* infection (Fig. 2.8 C and D). Transient transfection of MyD88 shRNA gave similar results compared to stably transfected cells (Fig. 2.8 C and D compared to Fig. 2.5). TLR2 knockdown in Henle 407 cells does not completely block the response of MAPK activation and IL-8 induction. Therefore, the response is almost entirely MyD88 dependent, but only partially dependent on TLR2. Attempts to combine TLR2 with other TLRs, including TLR4 and TLR9, in our HEK293 based stimulation assay did not result in further increases in NF-κB activation. TLR2 was sufficient for IL-8 production since its expression in HEK293 cells permitted induction of an IL-8 regulated luciferase reporter (Fig. 2.8 E). Pretreatment with ERK1/2 inhibitor U0126 inhibited IL-8 luciferase. These data demonstrate that TLR2 induced IL-8 production was through the ERK1/2 pathway (Fig. 2.8 E). In our system, TLR9 was neither necessary nor sufficient for epithelial cell response to *T. gondii* infection (Fig. 2.9 and Fig. 2.8 A). Taken together, *T. gondii* induced IL-8 is dependent on ERK1/2, and MyD88, and that TLR2 is both necessary and sufficient for their response.

Genotype of *T. gondii* does not influence epithelial cell response

T.gondii strains have been classified into 3 clonal lineages that differ in their pathogenicity in mouse models (41). Several strains of each clonal lineage were tested for their ability to induce epithelial cell response and activate TLR2. Henle 407 cells phosphorylated ERK1/2 in response to all strains from each of the three lineages (Fig. 2.10 A; Type 1: GT; Type 2: PT-G, CC, DEG; and Type 3: VEG). The various strains differed dramatically in their capacity to induce phosphorylation of p38, but there was no correlation with genotype (Fig. 2.10. A). All strains induced NF-κB activation through TLR2 (Fig. 2.10. B). The type I RH strain had a lower capacity to induce NF-

κB when compared to another Type I strain, GT-1. RH tachyzoites grown in cell cultures have reduced virulence when compared to those passaged in mice (42). Similarly, we observed that RH tachyzoites passaged in mice induced significantly more TLR2-dependent NF-κB activation than those passaged through fibroblasts *in vitro*. The ability of live *T. gondii* to activate cellular responses through TLR2 was not unique to human cells, since cells transfected with mouse TLR2 responded similarly (Fig 2.11). Together, we conclude that TLR2 dependent activation of signaling cascades by *T. gondii* is not genotype dependent.

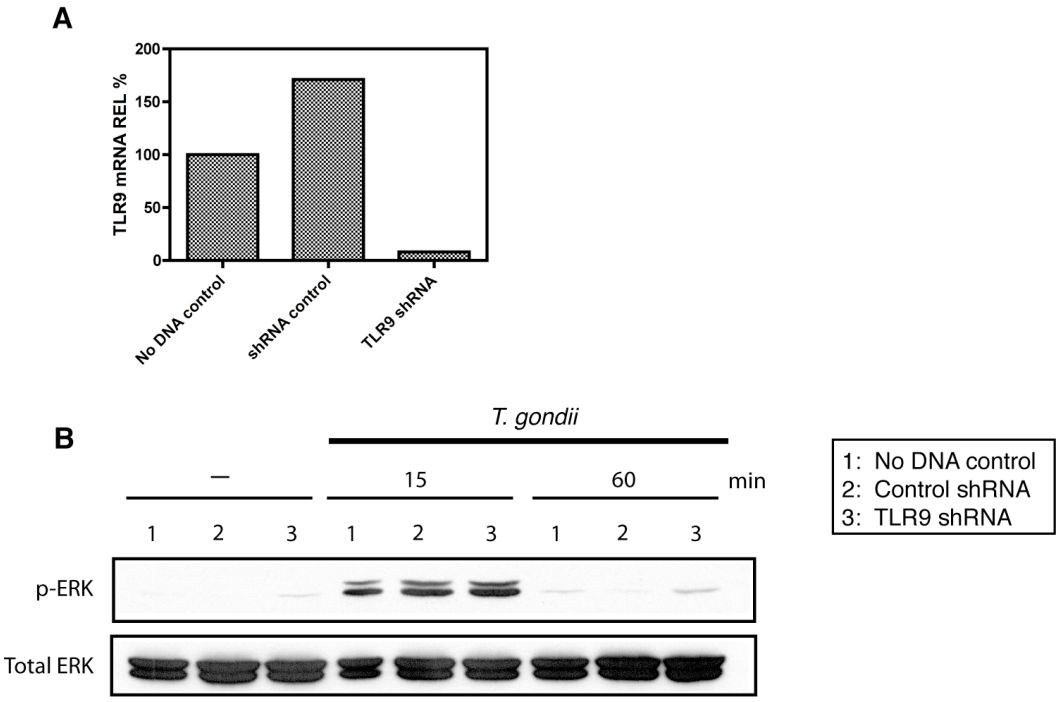


Figure 2.9. TLR9 was despensible for epithelial cell response to *T. gondii*. (A) Henle 407 cells were either untransfected (no DNA control), transiently transfected with control shRNA plasmids (shRNa control), or TLR9 shRNA plasmids carrying the TLR9 RNA interference sequence (TLR9 shRNA). TLR9 mRNA levels were measured by Real-time PCR. (B) Total cell lysates from cells infected with RH tachyzoites (parasite to cell ratio 6:1) for the indicated time points were immunoblotted with antibodies against total and phosphorylated forms of ERK1/2.

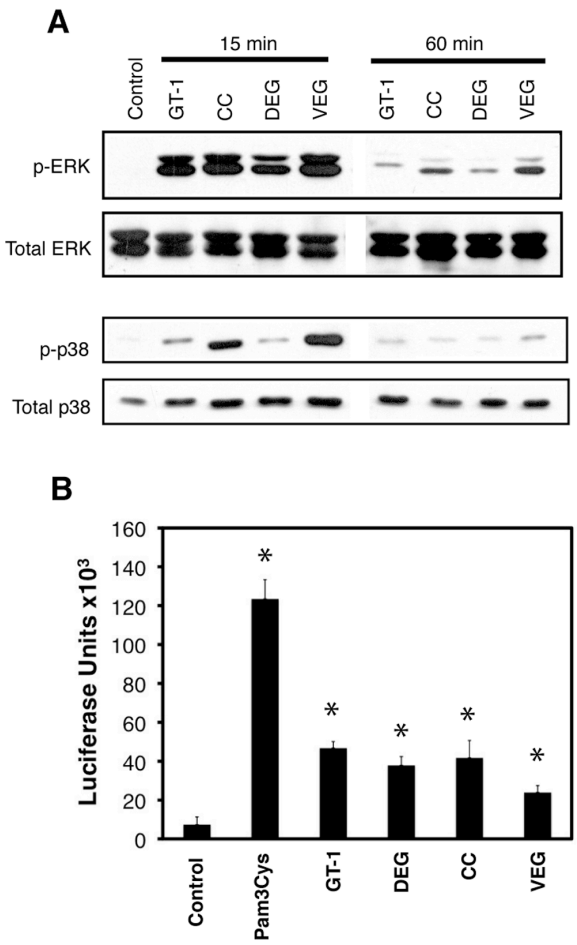


Figure 2.10. MAPK activation and TLR2 dependent response to *T. gondii* is not strain type specific. (A) Henle 407 cells were infected with, GT-1 (Type I), CC, DEG (Type II), and VEG (Type III) *T. gondii* strains for the indicated time points. Total cell lysates were collected for immunoblotting with antibodies against total and phosphorylated forms of ERK1/2 and p38. (B) HEK293 cells were transfected with human TLR2 and an NF-κB luciferase reporter plasmid. The transfected HEK293 cells were stimulated with TLR2 ligand Pam3Cys or infected with different *T. gondii* tachyzoite strains. Cell lysates were collected at 18 hr and assayed for luciferase activity. (Triplicate assays, error bar = SD).

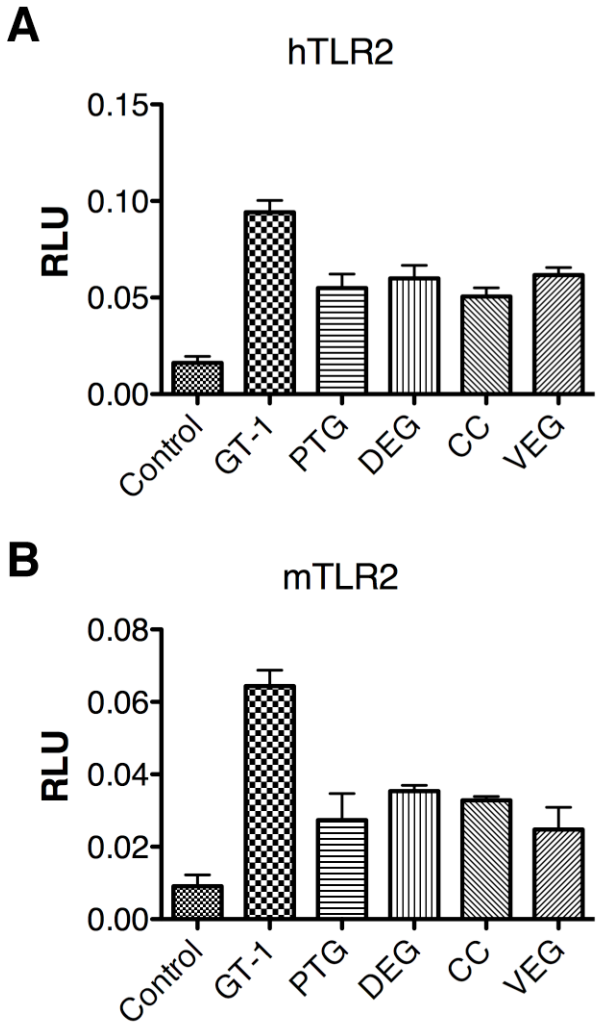


Figure 2.11. ***T. gondii*** **activates both human and mouse TLR2.** HEK293 cells were transfected with an NF-κB luciferase reporter plasmid, pSV-β-galactosidase reporter plasmid, and human or mouse TLR2 plasmids. The transfected HEK293 cells were infected with different *T. gondii* tachyzoite strains. Cell lysates were collected at 8 hr and assayed for luciferase and β-galactosidase activity. NF-κB activity (relative light units, RLU) was calculated by dividing the luciferase counts by the control β-galactosidase. (Triplicate assays, error bar = SD).

Discussion

In the present study, we evaluated the initial cellular responses of human intestinal epithelial cells to *T. gondii* infection. This type of study is critical for identifying the very early innate immune responses to parasitic infection of the intestinal mucosa. Using an *in vitro* model, where an isolated cell type is directly exposed to the infectious agent offers the advantage over mixed cell populations in identifying the response of a specific cell type. Most studies on the immune response to *T. gondii* infection have used a peritoneal challenge model for this orally acquired pathogen. More recently, studying the natural route of infection has revealed the importance of epithelial cell response in influencing the outcome of the local and systemic immune response (43, 44). Therefore, it is highly relevant to study the response of these cells during *T. gondii* infection, which occurs both locally in the intestine and systemically.

Very little is known about the human intestinal response to *T. gondii* infection; therefore, a major finding of this study is that human small intestinal epithelial cells directly respond to *T. gondii* within minutes to activate signaling cascades. By examining the response of the cells most likely to first encounter the pathogen, we can begin to uncover the early responses that may limit, or induce, the spread of *T. gondii* to other tissues such as muscle and brain, where a persistent infection results.

The neutrophil chemoattractant IL-8 is upregulated both at the protein and mRNA level within hours. Several additional cytokines and chemokines are also upregulated at the mRNA level within 4 hours. During mouse infections with *T. gondii*, neutrophils are critical for host defense and are one of the first cells recruited to the site of infection. They play a key role in the recruitment and activation of macrophages and DCs (27, 31, 45). Therefore, our findings that the human small

intestine epithelial cell line, Henle 407, directly responds to *T. gondii* infection suggests that *in vivo* epithelial response would modulate the local inflammatory environment to initiate host defense against infection.

This series of studies also elucidates the molecular mechanism for epithelial response to *T. gondii* infection. We show that, similar to macrophages and DCs, epithelial cells activate the MAPK pathway. The inability of MyD88 deficient epithelial cells to secrete IL-8 and activate ERK1/2 during infection suggests that TLRs play a critical role in the initiation of mucosal inflammatory process. Human TLR2 responds to live *T. gondii* infection in our heterologous reconstitution assay. However, knocking down TLR2 with short hairpin RNAs in intestinal epithelial cells only partially reduced the activation of ERK1/2. So while TLR2 contributes to epithelial response to *T. gondii* infection, there is likely an interaction with additional TLRs or other receptors that we could not detect in our assay. In fact, preliminary examination of the dependence of several cytokines and chemokines on MyD88 and TLR2 using shRNA knockdown revealed a complex pattern. While several genes were TLR2 dependent (IL-8, CCL10, CCL15), a few were TLR2 independent, MyD88 dependent (CCL5 (RANTES) and CCL11). IL-18 and CCL20 did not depend on either MyD88 or TLR2. TLR4 and TLR9 are candidates to work in concert with TLR2 for the production of cytokines and chemokines since mice deficient in these TLRs have reduced intestinal pathology during oral *T. gondii* infection. Furthermore, TLR9 in both hematopoietic and nonhematopoietic compartments is important for efficient T cell responses to oral infection (21). However, in our *in vitro* system, knocking down TLR9 in Henle 407 cells or reconstituting TLR9 in HEK293 cells does not affect MAPK or elicit NF-κB response to *T. gondii*. Commensal bacteria are present in the intestine and are capable of activating TLR9 (46-48). Therefore, it is likely that the TLR9 dependent pathology

induced during oral infection with *T. gondii* is secondary to epithelial damage and recognition by TLR9 of bacteria that penetrate the epithelial barrier.

Ligands for TLR2 include lipopeptides, lipoproteins and GPIs. GPI anchored proteins are abundant on the surface of *T. gondii* tachyzoites, and GPIs from *Trypanosoma cruzi, Plasmodium falciparum* activate TLR2 (49, 50). *T. gondii* GPIs also stimulate cytokine production in macrophages through TLR2 and TLR4 (51, 52). Direct studies on the role of TLR2 in human intestinal epithelial cells during *T. gondii* infection are lacking. Our data suggest that activation of TLR2 requires live parasites, or at least components not present in STAg, or damaged or lost during the preparation of STAg. The molecular component of *T. gondii* that intestinal epithelial cells recognize via TLR2 remains unknown.

The molecular mechanisms by which *T. gondii* activates epithelial cells and macrophages are different. In macrophages, *T. gondii* exploits Gi-protein mediated signaling to activate PI-3 kinase that leads to Akt and ERK1/2 activation, a process that is independent of MyD88 signaling (36). We show that inhibition of PI-3 kinase had no effect on the ability of epithelial cells to respond to infection. *T. gondii* infection of macrophages fails to induce NF-κB nuclear translocation and in fact inhibits activation in response to TLR ligands such as LPS (53, 54). In epithelial cells, NF-κB translocation to the nucleus was not impaired. Similar results in infected murine embryonic fibroblasts (MEFs) have been reported (55, 56). Both ERK1/2 and p38 kinases were activated by *T. gondii* infection of epithelial cells; however, activation was almost entirely dependent on MyD88 but only partially dependent on TLR2. Additional innate immune receptors or other recognition mechanisms present in the intestinal epithelial cells may cooperate to regulate the immune response of *T.*

gondii infection. These other receptors are unlikely to be TLRs since attempts to co-express other TLRs with TLR2 did not enhance the response in our HEK293 based stimulation assay.

There are three clonal lineages of *T. gondii* that differ in their ability to induce cytokines and in virulence. Low virulence type II parasites show higher induction level of IL-12p40, IL-10, IL-1b, and IL-6, where as high virulence type I parasites attract more neutrophils during infection (57, 58). We predicted that ability to activate TLR2 might correlate with lower virulence due to an increased activation of the immune response especially since NF-κB activation and cytokine secretion in immune cells correlates with genotype (59). However, all types elicited ERK1/2 and p38 MAPK activation through TLR2. Although the strains varied in the level of activation, there was no correlation with genotype. Virulence and cytokine induction differences among strains are not due to ability to activate epithelial cells via TLR2.

In summary, this study demonstrates that human intestinal epithelial cells directly respond to *T. gondii* infection via MyD88 and TLR2 driven ERK1/2 kinase and NF-κB signaling pathways. An interesting question for future studies is how epithelial cells cross talk and influence immune cells during infection. Our preliminary data point to a complex pattern of cytokine regulation, in which TLR2 is important but not the whole story. Understanding the local immune response against pathogens in the intestine will provide insight into the development of intestinal disorders, mechanisms for enhancing immune response to infection, or targets for vaccine development.

Acknowledgements

We thank Jitender P Dubey and Eric Denkers for the providing the parasites. We gratefully thank Hélène Marquis and Eric Denkers for providing intellectual discussions on this manuscript.

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CHAPTER III

TOXOPLASMA GONDII MODULATES THE LOCAL INTESTINAL ENVIRONMENT THROUGH TLR2

Abstract

Toxoplasma gondii is an intracellular pathogen that infects the host through the oral route. The intestinal epithelial layer not only functions as a physical barrier between the lamina propria and commensal microbes, but is also important for host defense during pathogen invasion. The intestinal epithelium is composed of various cell types such as enterocytes, goblet cells, and Paneth cells. We have previously shown a role for TLR2 in intestinal epithelial cells in initiating immune responses during *T. gondii* infection using an in vitro model. This study describes the kinetics of *T. gondii* infection-induced modulation of the local intestinal epithelium. Our study shows that oral infection with *T. gondii* causes TLR2 dependent pathology, as manifested by decreased numbers of goblet cells and Paneth cells, as well as secretion of Paneth cell granules to the crypt lumen. RNA transcript analysis of the infected tissues demonstrates that in addition to the cytokines that were previously characterized to cause pathology in the gut, several chemokines involved in immune cell trafficking were also highly upregulated after infection. By using a surgical ligated ileal loop model, subepithelial dendritic cells located in the Peyer's patch were shown to migrate to the follicle-associated epithelium within 90 minutes after live parasite infection. These responses were not observed in TLR2 deficient mice. In addition to DC migration, our in vitro studies using murine epithelial cell lines showed upregulation of the tight junction protein ZO-1 and defensin mRNA transcripts within 6 hours after *T. gondii* infection. Taken together, our findings suggest that *T. gondii* infection triggers epithelial cell responses at different stages of infection. These data support a model where TLR2 is important in modulating immune cell trafficking as well as inducing pathology.

Introduction

The intestinal epithelium is important for maintaining gut homeostasis and initiating appropriate immune responses when encountering pathogens (1). To perform their various functions, the epithelial cells consist of different subtypes. Enterocytes are absorptive cells that are the most abundant cell type (2). Goblet cells and enteroendocrine cells are found scattered throughout the epithelium and secrete mucins and hormones respectively (2). Paneth cells are located at the base of the crypt and contain lysozymes and defensins packed in eosinophilic granules (2). M (membranous or microfold) cells sample luminal antigens and are found near lymphoid structures including Peyer's patches (2-10).

Toll-like receptors (TLRs) are expressed by many cells types in the intestine and act as sensors for detecting conserved molecular PAMPs from both commensal and pathogenic microbes (11-15). In healthy hosts, TLR signaling is essential and activated at basal levels to maintain epithelial integrity, cell proliferation, tolerance and regulation of mucosal immune functions (16-18). The Peyer's patch plays a central role in immune surveillance of the small intestine and is an important inductive site for mucosal immunity (19-21). The Peyer's patch is covered with specialized follicle associated epithelium (FAE) containing M cells and enterocytes. The FAE secretes various cytokines that attracts dendritic cells to the subepithelial dome (SED) (22-25). In vivo studies have shown that stimulation with TLR agonists enhance M cell uptake and induce the migration of DC to the FAE (26-28).

Toxoplasma gondii is an intracellular protozoan parasite that infects humans and animals worldwide (29, 30). Transmission of this parasite is mostly through ingestion of contaminated soil or meat (31, 32). Oral infection with *T. gondii* in susceptible

C57BL/6 mice results in severe inflammation in the small intestine. This immunopathology is mainly caused by Th1 type CD4+ T cells, which mediate the increase in proinflammatory cytokines such as TNF-α, IFN-γ and nitric oxide (33, 34). The characteristics of *T. gondii* induced ileal inflammation in these mice also resemble the acute phase of inflammatory bowel disease in humans (33, 35-37). The mechanism of *T. gondii* induced Th1 pathology is thought to initiate through epithelial damage that leads to translocation of luminal bacteria (38-40). In combination with DCs that have acquired *T. gondii* antigens, the bacterial triggered DCs produce IL-12 and activate Th1 lymphocytes (39, 41). Several mouse strains including TLR9 deficient and TLR2×4 double deficient mice were shown to have less pathology but higher parasite burden (39, 42). In TLR9 deficient mice, oral infection with *T. gondii* also resulted in decreased IFN-γ production and release of a-defensins (cryptins) into the crypt lumen (43). However, it is not known whether other TLRs participate in the recognition of *T. gondii* during infection. Whether *T. gondii* infection directly regulates epithelial cell barrier integrity, defensin secretion or modulates the FAE function is also not known.

In our previous study, we demonstrated that epithelial cells are involved in the recognition of *T. gondii* through TLR2. Here, we ask how oral infection with of *T. gondii* elicits a response of the intestinal microenvironment at different stages of infection. The result of infection by *T. gondii* may be caused by combination of direct or indirect consequences. In this study, we focus on the direct response of the intestinal epithelial cells triggered by the parasite. The role of TLR2 in initiating DC migration in the Peyer's patch and subsequent parasite-induced pathology was characterized using TLR2 deficient mice.

Materials and Methods

Experimental Animals

MyD88$^{+/+}$ and MyD88$^{-/-}$ mice originally generated by S. Akira (Osaka University, Osaka, Japan) and provided by Dr. Eric Denkers (Cornell University, Ithaca, NY) were generated by heterozygous breeding. TLR2 $^{-/-}$ (*Tlr2*^{tm1Kir}) on a C57BL/6 background and wild type (C57BL/6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice of both sexes were used at 8-12 weeks of age. The mice were bred and housed under specific pathogen-free conditions at the Transgenic Mouse Core Facility, Cornell University College of Veterinary Medicine, overseen by the Institutional Animal Care and Use Committee.

Parasite, cyst isolation, and oral infection

Type II low-virulence *T. gondii* ME49 cysts were obtained from chronically infected Swiss Webster mice. To obtain the brain cysts, animals were sacrificed and the brains were homogenized in sterile PBS. The brain homogenate was passaged through an 18G needle. For oral infection studies, mice were gavaged with 20-30 cysts in a volume of 200 µl in PBS. The weight of the mice was monitored daily and the percentage of weight loss was compared to the initial weight on day 0.

Histochemical stains and microscopy

Animals were sacrificed at 7 or 11 days post-infection. Small intestine tissues were collected, preserved in 10% neutral-buffered formalin, and submitted to the Histology Unit, College of Veterinary Medicine (Cornell University, Ithaca, NY) to be processed into paraffin-embedded blocks. The tissue sections were cut with a microtome (5µm), and then stained with hematoxylin and eosin (H&E), periodic-acid Schiff (PAS) and Lendrum's phloxine tartrazine (Lendrum's) for identification of crypt-villus units,

goblet cells and Paneth cells respectively. An AxioImager M1 microscope (Carl Zeiss MicroImaging, Inc.) was used to examine the sections and images were captured at 20× or 63× objective (200× or 630× magnification) using an Axiocam HRc (Carl Zeiss MicroImaging, Inc.). Villus length was quantified by measuring from the crypt-villus junction to the apical cell surface at the tip of intact crypt-villus units using the length measurement function in the AxioVision Rel. 4.8 software (Carl Zeiss MicroImaging, Inc.). Morphological differences were achieved by quantifying goblet cells per villus, Paneth cells per crypt, and granules per Paneth cell. Measurements for each quantitative outcome were collected from 48 intact crypt-villus units analyzed from the distal to proximal ileum.

Cell cultures and parasites

The mIC_{cl2} cells are mouse intestinal epithelial cells derived from the small intestine of a transgenic C57BL/6 mouse described previously (44). Cells were grown in tissue culture flasks in a modified defined medium (DMEM/HAM's F12 (1/1, v/v) supplemented with 2% fetal bovine serum, 5 μg/ml insulin, 10 ng/ml epidermal growth factor, 5 μg/ml human transferrin, 50 nM dexamethasone, 30 nM sodium selenate, 1 nM triiodothyronine, 100 IU/ml penicillin-streptomycin, 1% fungizone, and 20 mM HEPES buffer at 37°C in 5.2% CO_2/air atmosphere. Human foreskin fibroblasts (HS27) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with containing 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and 10% low endotoxin fetal bovine serum. *T. gondii* tachyzoites strain were maintained by serial 2 days passages on HS27 monolayers in DMEM. All cell cultures and parasites were testd negative for mycoplasma by a high sensitivity PCR-ELISA based Mycoplasma detection kit (Roche, Indianapolis IN).

Surgical ligated intestinal loops

Mice were anesthetized by intraperitoneal (i.p.) injection of avertin (tribromoethanol in *t*-amyl alcohol; 200 mg/kg animal weight), and a 3- to 5-cm segment of ileum containing a Peyer's patch was ligated and injected as protocols described previously (45). Loops were injected with approximately 200 µl of complete DMEM or live parasites resuspended in complete DMEM. The animals were sacrificed after 90 minutes, and Peyer's patches were excised, washed in cold PBS, immediately embedded in Tissue-Tek OCT-embedding medium (Sakura, Torrance, CA), frozen in 2-methylbutane (Sigma-Aldrich, St. Louis, MO) cooled with liquid nitrogen, and stored at –80°C until sectioned.

Immunohistochemistry of frozen tissue sections

Immunohistochemistry was performed on fresh frozen Peyer's patch tissue sections. Cryosections (3-5 µm) of frozen Peyer's patches were cut on a cryostat model Microm HM 525, mounted on Superfrost Plus microscope slides, air-dried overnight, and fixed with cold acetone for 2 minutes. Cryosections were blocked using PBS containing 2% goat serum. The following primary antibodies were used: biotin-conjugated rat anti-mouse CD11c (BD Pharmingen), and rabbit anti-mouse laminin (MP Biomedicals, Solon, OH). As secondary reagents, the following were used: streptavidin-Alexa-488 or -647 for CD11c detection, and goat anti-rabbit-Alexa-488 or 647 (Invitrogen, Carlsbad, CA) for laminin detection. All stained tissues were mounted with Prolong Gold antifade reagent containing DAPI (Invitrogen, Carlsbad, CA).

RNA extraction and Real Time PCR

Total RNA were extracted using the RNeasy mini kit (QIAGEN, Valencia, CA) and DNase I treated. The cDNA was synthesized from 1 μg of RNA using SuperScript III Reverse Transcriptase Kit with the supplied Olige-dT primers (Invitrogen, Carlsbad, CA). Real-time PCR was performed on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with the SYBR green detection reagent (40 cycles at 95°C for 10 s and at 60°C for 1 min). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for each sample. Primers used in this study are designed as previous studies and listed in Table 3.1 (46-48). Percentage of relative expression level (REL%) was calculated using the $2^{-\Delta\Delta Ct}$ method. For infected tissue Real-time mRNA analysis, PCR arrays (PAMM-073, Th17 for Autoimmunity & Inflammation PCR Array) were purchased from SABioscience Corp. The signals from the expression of each gene on the array were normalized against the signal from the internal housekeeping gene. Fold regulation for each gene was calculated as the processed data set from infected sample divided by processed data set from uninfected sample. Thresholds were set to select for genes up-regulated 3 fold or more.

Immunofluorscent staining of mIC_{c12} cells and concofal microscopy

Murine small intestinal mIC_{c12} cells were plated at 2-3 × 10^5 cells per well on sterile coverslips placed in 24-well plates. Cell monolayers were polarized for 6 days and infected with *T. gondii* RH-YFP tachyzoites apically. The cells were fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 in TBS (TBS-TX) for 15 min and blocked in 1% BSA in TBS-TX for 20 min. Tight junctions were stained

Table 3.1. Real time PCR primers for mouse alpha and beta defensins.

Gene	Forward	Reverse
mAD-1	GTC GCT GAA CAT GGA GAC CA	GAA GTG GTC ATC AGG CAC CA
mAD-2	CTG CTC ACC AAT CCT CCC A	GCC TGG ACC TGA AAG GAC AGT A
mAD-3	GAG CAG CCA GGG GAA GAC	ACC AGA TCT CTC AAC GAT TCC TCT
mAD-4	TTC ATG AAA AAT CTT TGA GAG GTT TGT	GCA GTA CAA AAA TCG TAT TCC ACA AGT
mAD-5	GGA CCT GCA GAA ATC TTT TTT TAA CTT	CTC AGA GCC GAT GGT TGT CA
mBD-1	GAA CAC GGT ACA CAG GCT TCC	CCT GAA TCA CAG ATG TCC AAG
mBD-2	CTC TCT GGA GTC TGA GTG CCC	AGG ACG CCT GGC AGA AGG AGG
mBD-3	GAT TGG CAG TTG TGG AGT TCC	GTC CAT CTT CAT GGA GGA GCA
mBD-4	ACA TGC ATG ACC AAT GGA GCC	CAT CTT GCT GGT TCT TCA TCT
mBD-5	GCT GCT GTG TCC ACT TGC AA	TGC CTC CAA TCA TAC AGC AAC T
GAPDH	ACT CCA CTC ACG GCA AAT TCA ACG G	AGG GGC GGA GAT GAT GAC CC

with primary rabbit anti-ZO-1 (Invitrogen) followed by secondary antibody goat anti–rabbit IgG conjugated to AlexaFluor 594 (Invitrogen). Samples were mounted with Prolong Gold antifade reagent containing DAPI. Confocal images were taken with a Leica laser scanning confocal microscope using a 63×lens. Contrast and brightness of individual channels were adjusted linearly in Adobe Photoshop CS5.

Statistical Analysis

Measurement of 48 crypt-villus units for quantitative morphological analyses provides a power of 0.8 to detect a difference of 0.414 standard deviations at a significance level of 0.05. Statistical analyses were performed using GraphPad Prism Software (La Jolla, CA). Differences between groups were analyzed by unpaired *t*-test. Values for $*p < 0.05$ were considered significant.

Results

T. gondii oral infection in mice induces an inflammatory response and morphologic changes in the small intestine

Oral infection with *T. gondii* results in Th-1 type immunopathology in the small intestine of inbred C57BL/6 mice (33, 49). Intestinal epithelial cells are important for responding to *T. gondii* oral infection and these responses involve TLRs (43, 50). Yet, little is known about how the intestinal environment changes during the early contact with the parasite and about early progression of the infection. The intestinal epithelium integrity is maintained by the stem cells in the crypt which differentiate into different subtypes: the absorptive enterocytes, mucus producing goblet cells, hormone producing enteroendocrine cells, and the antimicrobial peptide producing Paneth cells (2). To characterize the effect of *T. gondii* infection and the morphologic changes in the intestinal epithelium, wild type C57BL/6 mice were orally infected with type II low virulence ME49 cysts. The mice were monitored for weight changes over time (Fig 3.1 A). At day 7, the mice were sacrificed and the small intestine was collected for histological analysis. In agreement with previous studies, wild type mice continuously lose weight beginning at day 3 post infection (Fig 3.1 A). Histological analyses of the terminal ileum showed severe ileitis in the infected wild type mice characterized by villus blunting, crypt abscesses and inflammatory infiltrate in the mucosa (Fig 3.1 B, C). Additionally, quantification of ileal pathology showed a significant reduction in villus length, and in the number of both goblet cells and Paneth cells when compared to uninfected mice (Fig 3.1 D, E, and F). Together, these results suggest that *T. gondii* infection changes the intestinal villus architecture as well as goblet cells and Paneth cell numbers.

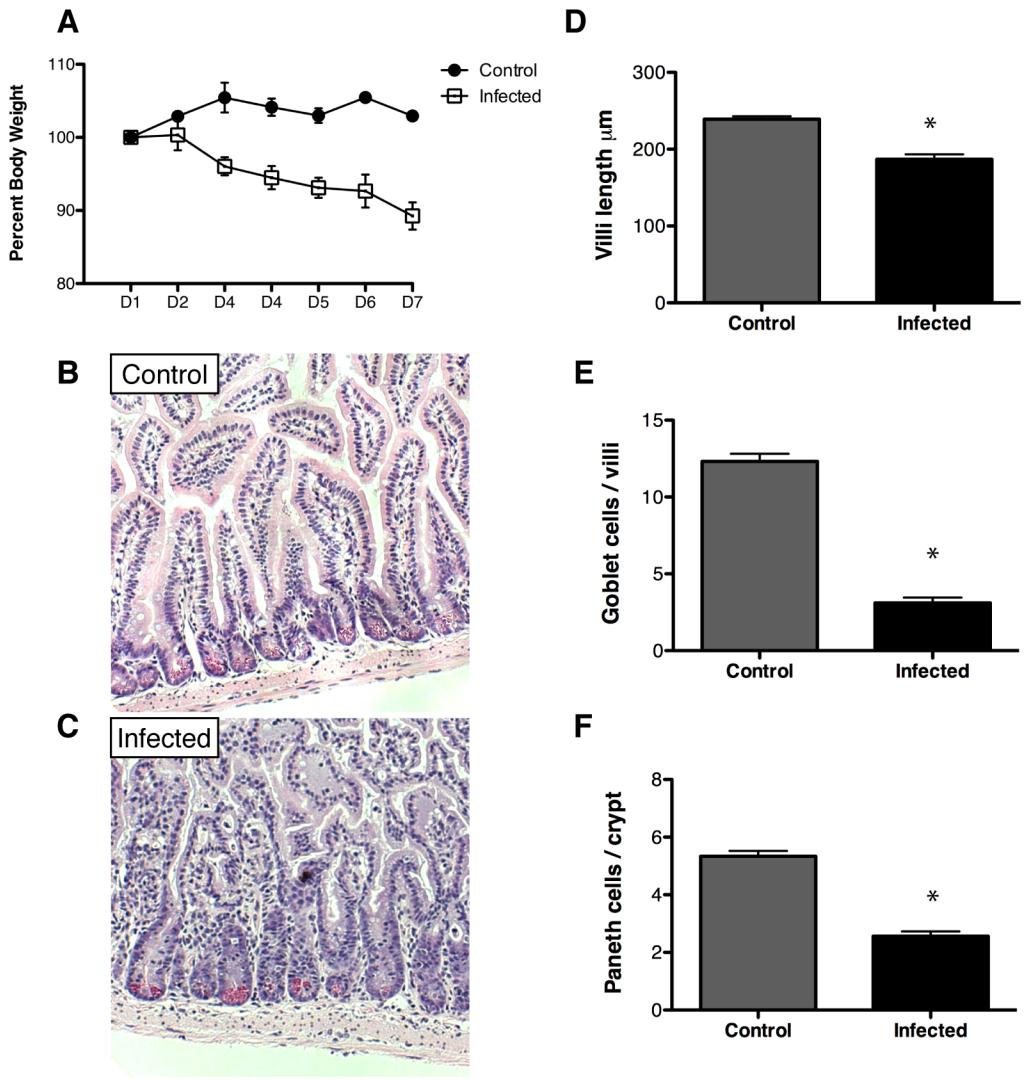

Figure 3.1. Oral infection with *T. gondii* alters numbers of goblet cells, Paneth cells, and villi length in the small intestine. WT (C57BL/6) mice were orally infected with *T. gondii* ME49 cysts. After 7 days, small intestines were collected and subjected to histologic analyses with H&E, PAS, and Lendrum's staining. Infected mice showed histopathological changes in the infected mice (C) when compared to the PBS control (B). Staining and quantification showed shortened villi (D), decreased goblet cells (D) and Paneth cells (F) in *T. gondii* infected mice.

Oral infection with *T. gondii* induces chemoattractant cytokines

The development of acute ileitis during *T. gondii* infection is characterized by over production of IFN-γ, TNF-α, and IL-12 and extensive immune cell infiltration to the gastric mucosa (33, 49). Analysis of mRNA expression in the infected intestine tissue at day 7 confirmed that IFN-γ and TNF-α were highly expressed following infection (Table 3.1, IFN- γ, 34.1 fold; TNF- α, 5.5 fold). It is notable that TLR4 and MyD88 transcripts were also upregulated after infection, since these innate immune effects have been found highly expressed at the terminal ileum of ulcerative colitis and Crohn's disease patients due to the accumulation of gram-negative bacteria (51-54). In addition to the Th-1 type proinflammatory genes, several chemokines such as CCL2, CCL7, and CXCL5, important for monocyte, T cell, and dendritic cell trafficking, were also highly upregulated during infection. Therefore, these results suggest that the small intestine responds to *T. gondii* infection by upregulating chemoattractants for innate immune cell recruitment to the site of infection.

***T. gondii* induces migration of dendritic cells to the FAE in a MyD88 dependent manner**

CD4+ and CD8+ T cell immune responses are required for the host to survive *T. gondii* infection, and activation of these cells depends on the encounter between antigen presenting cells and the parasite (34). The Peyer's patches are secondary lymphoid tissues in the small intestine and are the major inductive site for mucosal adaptive immunity.

Table 3.1. A list of genes determined by Real-Time PCR induced by *T. gondii* in the small intestine at day 7 after oral infection. Fold regulation greater than 3 are shown.

Gene	Genbank No.	Fold Regulation
Ccl2	NM_011333	111.0
Ccl7	NM_013654	85.9
Ifng	NM_008337	34.1
Cxcl5	NM_009141	13.6
Tlr4	NM_021297	10.9
Icam1	NM_010493	9.2
Mmp3	NM_010809	7.9
Cd34	NM_133654	7.3
Il27	NM_145636	7.2
Il10	NM_010548	6.7
Tnf	NM_013693	5.5
Traf6	NM_009424	5.4
Cd40lg	NM_011616	5.3
Cd4	NM_013488	5.3
Cd8a	NM_001081110	5.1
Myd88	NM_010851	4.9
Cxcl1	NM_008176	4.7
Il12rb2	NM_008354	4.7
Socs1	NM_009896	4.5
Mmp13	NM_008607	3.7
Il1b	NM_008361	3.6
Cd28	NM_007642	3.4
Il21	NM_021782	3.1
Il7r	NM_008372	3.0

Dendritic cells (DCs) located in the subepithelial dome region of the Peyer's patch are critical for initiating the mucosal T cell responses. Previous studies have shown that TLRs regulate the follicle associated epithelium (FAE) to promote antigen capture by DCs (26). TLR/MyD88 signaling is also critical for initiating defense against *T. gondii* infection (55-58). To determine whether *T. gondii* have a TLR dependent effect on FAE function and DC migration, a surgical ligated intestinal loop containing a Peyer's patch was injected with *T. gondii* tachyzoites in MyD88$^{-/-}$ or control wild type littermates. Peyer's patch tissue samples were collected after 90 minutes and analyzed for CD11c+ DCs in the FAE. Results showed that during live *T. gondii* infection, the parasites induced migration of DCs into the FAE in wild type mice, but this was not observed in the MyD88-deficient mice (Fig 3.2 A and B). These results suggest that *T. gondii* directly regulates FAE function and induces DC migration dependent on TLR signaling.

Intestinal epithelial cells upregulate tight junction proteins and defensins in response to *T. gondii* infection.

The intestinal epithelium is an important physical barrier for separating the commensal bacteria from the lamina propria, as well as for secreting antimicrobial peptides to maintain the homeostasis of the gut. Intestinal epithelial cells express several TLRs including TLR2, TLR4, and TLR9 that are linked to initiating immune response during *T. gondii* oral infection (42, 59-61). Stimulation of TLR2 in colonic epithelial cells are important for the biogenesis of tight junction proteins (17, 62). Secretion of α- defensin during *T. gondii* infection is dependent on TLR9 (43). Here, we use the mouse intestinal epithelial cell line mIC$_{c12}$ to study the tight junction response and induction of defensins during *T. gondii* infection. The mIC$_{c12}$ cells are a crypt-like murine small intestinal cell line that polarized within 6 days (44). The epithelial

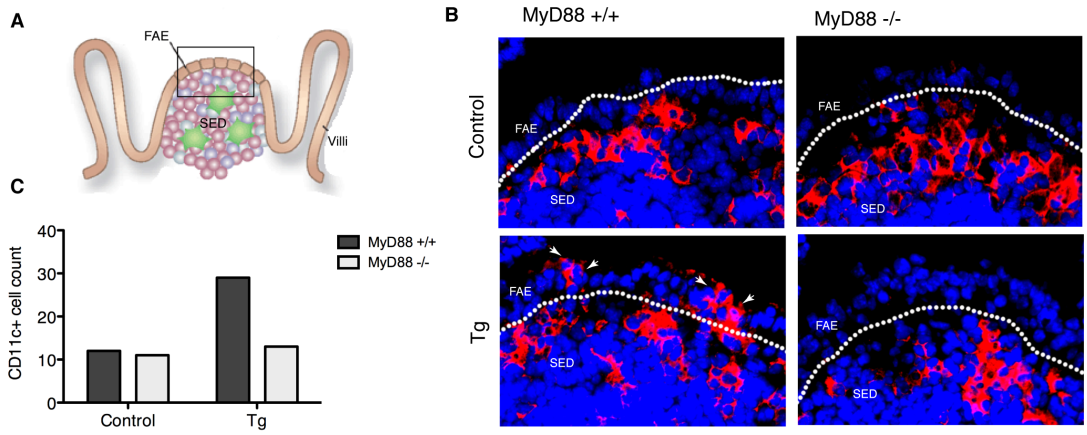

Figure 3.2. ***T. gondii*** **induce the migration of dendritic cells (DCs) into the follicle-associated epithelium (FAE) in a MyD88-dependent manner.** (A) Model of Peyer's patch structure (63). (B) Frozen tissue sections stained with anti- CD11c+ (red) and DAPI (blue) showed increased DCs in the FAE after exposure to *T. gondii* tachyzoites. Images shown are region corresponding to the illustration in (A). Dotted line defines the basal limit of the FAE as determined by staining for laminin. Representative images show that the migration of CD11c+ DCs (arrows) was induced upon exposure to *T. gondii* in MyD88 +/+ mice, but not in MyD88 deficient mice. (C) Quantitative analysis of DCs that have migrated to the FAE in control or *T. gondii* infected Peyer's patch. Data are from one experiment with one mouse for each treatment.

monolayer was infected with RH-YFP tachyzoites for 6 hours, which allows the parasites to invade into the host cells but not to complete a replication cycle, and then stained for the tight junction marker ZO-1. Confocal microscopy images showed higher intensity in ZO-1 staining when cells were infected with *T. gondii* parasites (Fig 3.3 A). This was also observed in polarized mIC_{c12} monolayers treated with flagellin, but not with Pam3Cys or CpG treatment (Fig 3.3 A). The epithelial monolayer eventually looses the tight junction barrier after 24 hours of infection (Fig 3.3 B); however, this might be due to multiple replication cycles of the parasites that causes the lost of barrier integrity.

The crypt epithelial cells of the villus are know to secrete several antimicrobial peptides as a defense mechanism to pathogens (64). To test what types of defensins were induced following *T. gondii* infection, total RNA from mIC_{c12} cells was collected at 6 hours post infection and the expression level of alpha and beta defensins was analyzed by real-time PCR. Results show that infection with *T. gondii* tachyzoites induced mouse α-defensin-3 (4.5 fold), as well as low levels of β-defensin-5 (1.9 fold) (Fig 3.4 A, B). Taken together, these results suggest that *T. gondii* infection causes epithelial cells to enhance tight junctions and upregulate defensins within few hours of infection.

***T. gondii* induced migration of dendritic cells to the FAE is dependent on TLR2**

We previously showed that intestinal epithelial cells respond to *T. gondii* directly via TLR2. Therefore, we next asked whether DC migration in the Peyer's patch depends on TLR2. Surgical ligated loops in TLR2 deficient and control wild-type mice were either injected with control media or RH tachyzoites. After 90 minutes Peyer's patches were collected, sectioned, and immunofluorescently stained for laminin and the DC

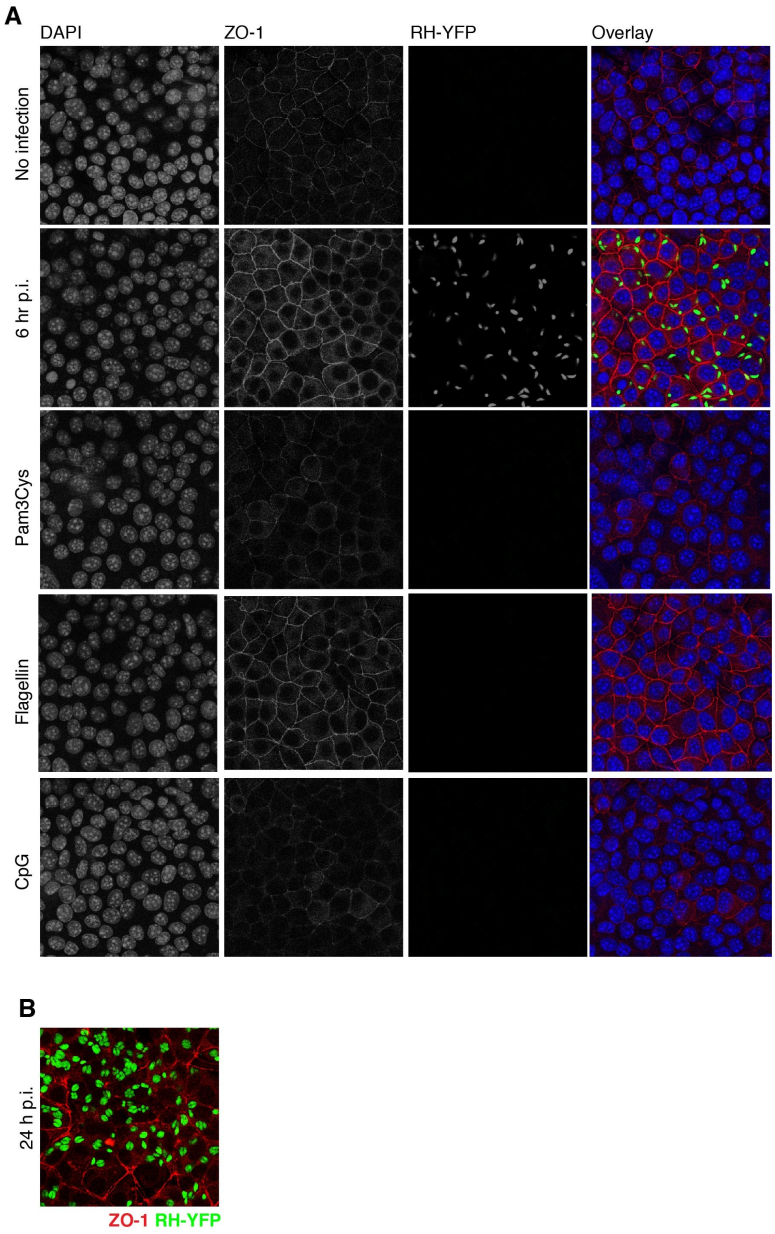

Figure 3.3. *T. gondii* infection of mouse small intestinal epithelial cells regulates tight junctions. (A) Immunofluorscent staining of tight junction marker ZO-1 in mIC_{c12} cell after infection with RH-YFP tachyzoites infection. The intensity of ZO-1 staining was increased 6 hours after parasite infection when compared to uninfected controls. (B) mIC_{c12} cell ZO-1 is disrupted after 24 hours post infection.

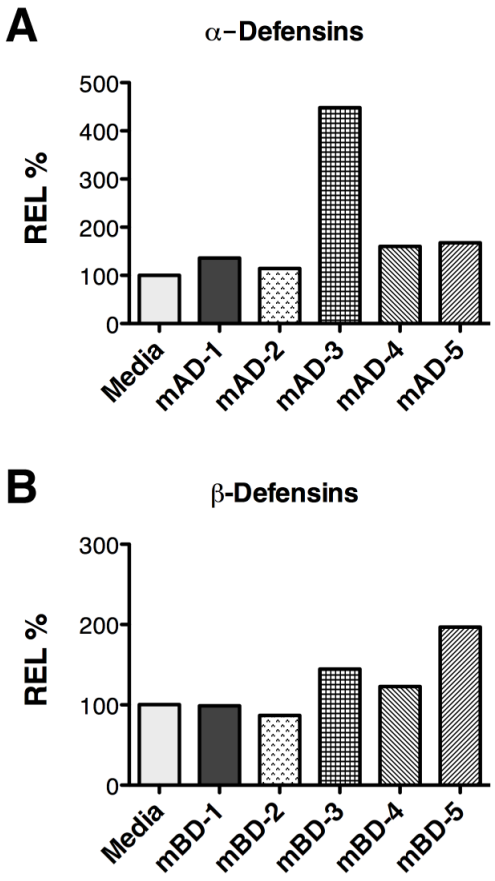

Figure 3.4. *T. gondii* infection of small intestinal epithelial cells induce mouse a-defensins-3 and b-defensin-5 upregulation of mRNA transcripts. Mouse alpha (A) and beta (B) defensin gene transcript levels was measured by Real-Time PCR analysis 6 hours post *T. gondii* infection. The data are normalized to GAPDH and compared against uninfected expression levels (REL, relative expression levels).

marker CD11c. Although live *T. gondii* induce migration of DCs to the FAE in wild type mice, DC migration was not seen in the TLR2 deficient mice (Fig 3.5 A, B). Taken together, these results suggest that the migration of DCs to the FAE induced by *T. gondii* in the small intestinal Peyer's patch is dependent on TLR2.

Oral infections with *T. gondii* in TLR2 deficient mice is characterized by less gut pathology, and maintenance of Paneth cell numbers and granules

Our study showed that DC migration is dependent on TLR2. We next examined whether TLR2 influenced the outcome of parasite-induced pathology. To further investigate the role of TLR2 during infection, we orally infected wild type and TLR2 deficient mice with ME49 cysts to and monitored weight loss over time. TLR2 deficient mice were resistant to *T. gondii* induced weight loss when compared to wild type infected mice (Fig 3.6 A). Histological analyses of the terminal ileum showed reduced length of villi, reduced Goblet cell numbers and a dramatic reduction in Paneth cell nembers in infected wild type mice. However, in TLR2 deficient mice there was no reduction in villus length, and a much less dramatic reduction in Goblet and Paneth cell numbers (Fig 3.6 D, E, F). Closer examination of the ileal crypts in the uninfected TLR2 knockout mice demonstrated that, in the absence of infection, they have fewer Paneth cell numbers when compared to wild type mice (Fig 3.6 F). (Fig 3.5 D, E, and F). Taken together, our results demonstrate that TLR2 plays a role in developing *T. gondii*-induced intestinal pathology by modulating effector cell numbers and function.

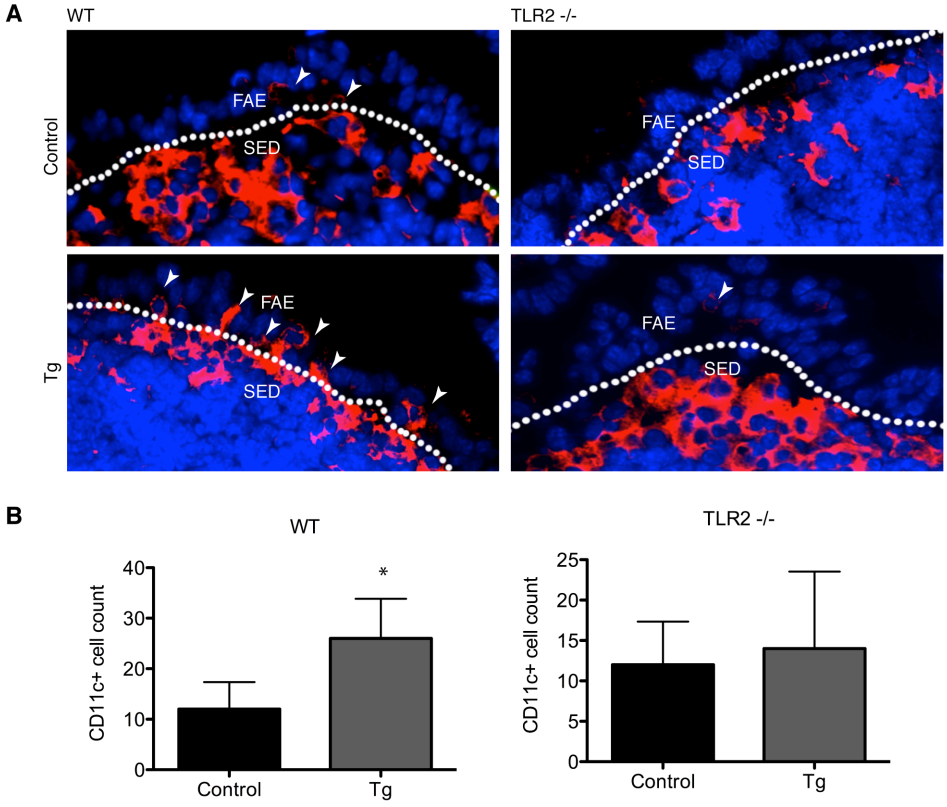

Figure 3.5. *T. gondii* induced migration of dendritic cells (DCs) into the follicle-associated epithelium (FAE) is dependent on TLR2. (A) Peyer's patch from wild type or TLR2 deficient mice were collected after 90 minutes of intraluminel injection of control (media) or live *T. gondii* parasites. Frozen tissue sections were stained with anti- CD11c+ (red), DAPI (blue). The dotted line defines the basal limit of the FAE as determined by laminin staining. Representative images show that the migration of CD11c+ DCs (arrows) was induced upon exposure to *T. gondii* in wild type mice, but not in TLR2 deficient mice. (B) Quantitative analysis of DC that has migrated to the FAE in control or *T. gondii* infected Peyer's patch.

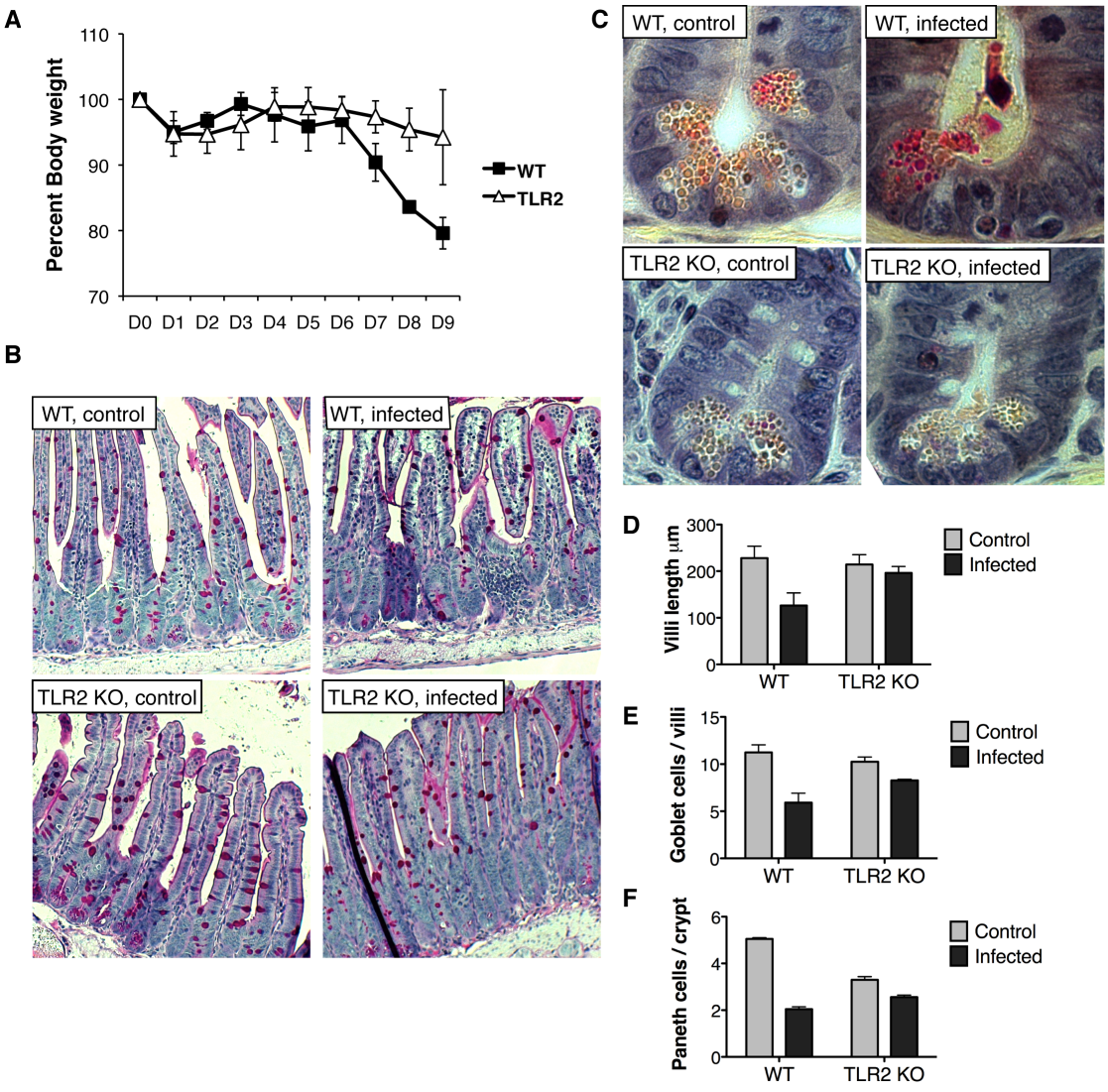

Figure 3.6. TLR2 deficient mice are more resistant to *T. gondii* induced pathology. Wild type (C57BL/6) and TLR2 deficient mice were orally infected with 20 ME49 cysts. (A) Following oral infection, TLR2 deficient mice showed less weight loss compared with wild type controls. (B, C) Small intestines were isolated at day 9 and subjected to PAS for goblet cell identification and Lendrum's stain for and Paneth cells. TLR2 deficient mice showed less histopathological changes, as well as less decrease in goblet cells and Paneth cells. Representative images of the crypt Paneth cells were shown. (D, E, and F) Quantification of villi length, goblet cell, and Paneth cell numbers.

Discussion

The intestinal epithelium is a strategic place to respond to pathogenic invasion as well as orchestrate signaling cascades for host protection and control inflammation. In our previous study we demonstrated that TLR2 involved in activating inflammatory responses during *T. gondii* infection. Here, we demonstrate that TLR2 modulates the intestinal epithelial function during *T. gondii* infection, which leads to changes in the intestinal microenvironment.

The present work demonstrates that oral infection of susceptible mice C57BL/6 mice with low virulence ME49 cysts results in intestinal inflammation, reduced goblet cells, Paneth cells, and discharge of granules into the crypt lumen. The *T. gondii*-induced pathology in theses mice is similar to inflammatory bowel disease (IBD) in human (33, 65). Crohn's disease and ulcerative colitis are the two main groups of IBD. In the healthy intestine, secretion of protective factors such as mucins and antibacterial peptides in the epithelium can effectively control the commensal load in the intestine to avoid inflammation (66, 67). Human IBD is often characterized by heavy loads of bacteria in the mucus that adheres or invades the intestinal epithelial cells, which leads to breakdown of the epithelial barrier function (68). The reduction of goblet cells in mice infected with *T. gondii* observed in this study is similar to what is observed in ulcerative colitis patients (69). The depletion of goblet cells results in a thinner mucus layer can increase the epithelial cell-bacteria contact and therefore exacerbate the inflammatory response in the small intestine. Paneth cells contain granules that secrete antimicrobial peptides to maintain homeostasis of the intestine. We show that oral infection results in decrease of Paneth cell numbers after *T. gondii* infection. In previous studies, *T. gondii* infection induces microflora changes and accumulation of gram-negative bacteria at the inflamed sites of the intestine (37, 70). It is possible that

the reduction of Paneth cell numbers during *T. gondii* infection results in decreased defensin production and alters the luminal bacterial composition.

Acute ileitis induced by *T. gondii* may involve multiple TLR and multiple epithelial cell types. In the case of TLR2 deficient mice, they are more resistant to *T. gondii*-indued pathology as demonstrated by a milder reduction in goblet cell, Paneth cell, and less discharge of granules into the lumen of the small intestine crypt. This result is similar to other oral infection studies done in transgenic mice lacking bacterial sensing TLRs (TLR2×4 and TLR9) or chemokine receptors (CCR2), where less intestinal inflammation and IL-12 production was observed (39, 40). The emerging proposed model is that immunopathology induced by *T. gondii* infection is caused by immune reaction against the luminal bacteria rather than the parasite itself. Interestingly, we observed a lower number of Paneth cells, as well as fewer granules discharge into the crypt lumen in TLR2 deficient mice. Similar reduction of Paneth cells is also observed in MyD88 deficient mice (Rose and Leifer, unpublished observations). It is not clear whether reduced Paneth cell numbers result in differences of gut microbiota content in the TLR2 or MyD88 deficient mice. However, it is possible that proper TLR signaling through the commensal bacteria in the small intestine is required for the differentiation and function of Paneth cells.

TLR2 may play multiple and opposite roles in regulating the intestine at different times following infection. The TLR2 agonist synthetic Pam_3Cys-SK4 (PCSK) enhances ZO-1 junctions in human colonic intestinal cell lines and ameliorate damage to the epithelial barrier in DSS colitis models (17, 62, 71). In our study using the murine small intestinal cell line mIC_{c12}, direct stimulation with bacterial flagellin or live tachyzoites for 6 hours also enhanced tight junction in the epithelial cells. The effect of *T. gondii* infection was more dramatic than that observed with TLR2 or

TLR9 ligands. Since live infection was more dramatic than direct TLR2 stimulation, it is likely that invasion of parasites into the cells activates other intracellular pathways that upregulates ZO-1 independent of TLRs. However, ZO-1 tight junctions are eventually disrupted after several replication cycles in the intestinal epithelial cells, suggesting that higher permeability through the epithelial barrier may facilitate bacterial leakage into the lamina propria.

Defensins are cationic anti-microbial peptides that are important effectors in intestinal innate immunity. Foureau and colleagues have demonstrated that *T. gondii* induces α-defensin production and release by Paneth cells via a TLR9-dependent induction of type I IFNs (43). In our study, we show that early and direct contact of *T. gondii* with crypt–like small intestinal epithelial cells induced rapid induction of a-defensin-3 and β-defensin-5. Defensins are able to kill other protozoan parasites such as *Giardia lamblia* (72), but whether they play a direct role in eliminating *T. gondii* is not known. α-defensins have biological effects in epithelial cells that enhance mucosal protection in addition their antimicrobial activities. α-defensin-3 forms pores in the apical membrane of intestinal epithelial cells and lead to chloride secretory response. Such response would lead to salt and water secretion in epithelial cells, and flush the bacteria off the epithelial surface (73). Another study also reported that α -defensin-3 induces secretion of the neutrophil chemoattractant IL-8 through Ca^{2+}-dependent activation of the p38 and NF-κB signaling (74).

The Peyer's patch is the primary site for induction of mucosal immune responses. Dendritic cells in the Peyer's patch are known to have the ability to prime T cells and help IgA B cell differentiation, as well as uptake antigens transcytosed from the FAE and activate T and B cell in the interfollicular regions (75, 76). Movement of DCs

beneath the FAE into the intraepithelial layer promotes rapid capture of antigens and microorganisms. In the current study, we showed that *T. gondii* tachyzoites promote the migration of DCs to the FAE within 90 minutes in a TLR2 dependent manner. *T. gondii* possesses TLR2 (GPIs) and TLR11 (profilin) ligands (60, 77). However, more recent studies have shown that TLR11 is expressed intracellularly (observations by F. Yarovinsky). Therefore, it is possible that DC migration during *T. gondii* infection depends on TLR2 expressed on the surface of FAE, but the production of IL-12 from DCs is dependent on TLR11 or bacteria sensing TLRs (TLR2, TLR4, and TLR9) in order to initiate Th1-polarizing response.

Several studies have reported the cross talk of epithelial cells and dendritic cells in the intestine. In the lamina propria, villous epithelial cell TLR signaling upon exposure to microbial stimuli induces DC sampling of the lumen (78-80). In the Peyer's patch, a subset of DCs also responds to FAE secreted cytokines and sample antigens transported by M cells (81). However, there are several subsets of Peyer's patch DCs, which are distinguished based on their surface markers and functional activity. $CD11b^{+}$ $CD8a^{-}$ $CCR6^{+}$ DC primarily mount a Th2 responses, whereas $CD11b^{-}$ $CD8a^{+}$ $CCR6^{-}$ and $CD11b^{-}$ $CD8a^{-}$ $CCR6^{+}$ DC activate Th1 responses (82). In this study, it is not clear whether the FAE upregulates chemokine mRNA and protein synthesis, or secretes other chemotactic molecules, to induce DC migration within 90 minutes. An alternative possibility is that *T. gondii* antigens cross the FAE and directly induce the DC movement within the Peyer's patch. Regardless of the exact mechanism, our data demonstrate that the innate response to *T. gondii* enhances DC migration in the Peyer's patch in a TLR2 dependent manner and that this response may promote adaptive immune responses in the intestinal mucosa. Further studies are required to identify the subset of DCs in the Peyer's patch that are recruited to the FAE, whether

they initiate a Th1 response during *T. gondii* infection, and whether the effect on these cells is direct, or via *T. gondii* activation of the FAE.

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CHAPTER IV

TLR2 STIMULATORY MOLECULES FROM *TOXOPLASMA GONDII* INFECTED SUPERNATANTS INDUCE DENDRITIC CELL MIGRATION INTO THE FOLLICLE-ASSOCIATED EPITHELIUM OF PEYER'S PATCHES

Abstract

Toxoplasma gondii is an obligate intracellular parasite that has multiple surface antigens as well as cytoplasmic proteins that can initiate host inflammatory responses. In this study, we show that supernatants collected from *T. gondii* infected cells were capable of activating immune responses in intestinal epithelial cells. Similar to live parasite infections, the supernatant from infected cells contains parasite-derived mediators that trigger NF-κB activity through TLR2. Glycosylphosphatidylinositols (GPIs) isolated from *T. gondii* were previously demonstrated to activate TLR2 and TLR4. Therefore, we used nano-LC-MS/MS to identify *T. gondii* GPI anchored proteins in supernatants, and found two candidate proteins: surface antigen 1 (SAG1) and subtilisin-like protein (TgSUB1). The TLR2 stimulatory activity was reduced in supernatants from SAG1 deficient parasites, but not the TgSUB1 deficient parasites. *T. gondii* SAG1 were previously shown to be involved in inducing ileitis in susceptible C57BL/6 mice. Therefore we tested whether the supernatants from *T. gondii* infected cells, which contains SAG1, promote capture of incoming antigens by dendritic cells in the Peyer's patch. Using a surgical ligated loop model, we show that the supernatant from *T. gondii* infected cells enhances the transepithelial transport of fluorescent microbeads and induces dendritic cell migration to the follicle-associated epithelium. Furthermore, dendritic cell migration induced by supernatant from infected cells was dependent on TLR2.

Introduction

The obligate intracellular parasite *Toxoplasma gondii* is a widespread protozoan parasite that belongs to the phylum Apicomplexa that infects a variety of nucleated mammalian and avian cell types (1). Individuals are infected with the parasite by ingesting contaminated food or water from oocysts or tissue cysts containing bradyzoites. Once in the small intestine, the bradyzoites convert to rapidly multiplying tachyzoites and invade target cells in the gut (2). In most cases, with the exception of a certain inbred strain of mice, *T. gondii* does not cause a CD4+ T cell mediated immunopathology during the acute phase of infection (3, 4).

In order to induce cell-mediated protective immunity against *T. gondii* in intermediate hosts, numerous studies on *T. gondii* vaccine designs were based on attenuated parasite or DNA vaccines encoding proteins from parasite secretory organelles such as dense granule proteins GRA-1, -2, -4, -6, -7, rhoptry proteins ROP-1, -2, and microneme proteins MIC-1, -2, -3, -4 (5-12). Other *T. gondii* derived antigen molecules including heat shock protein-70, -30 (13-15), M2AP (MIC2-associated protein), AMA1 (apical membrane antigen-1) and SAG-1, -2 (surface antigen-1, -2) are also strong inducers of immunity (10, 16). However, protective immune responses triggered by these antigens varies, and little is known about whether these antigens play a role in initiating response at the mucosal surface during infection.

During the acute phase of *T. gondii* infection, intestinal epithelial cells can initiate series of immunologic events that leads to a robust inflammatory response in the gut. Studies have shown that these responses involve Toll-like receptors (TLRs). In MyD88 deficient mice, oral infection with *T. gondii* demonstrates a defect in neutrophil recruitment to the site of infection, early IFN-γ production by T cells, and

induction of p47 GTPase IGTP (Irgm3). (17). This suggests that early TLR signaling at the mucosal surface during oral infection is important for triggering protective responses. *T. gondii* possesses several TLR ligands including profilin and cell surface GPI anchors that activate TLR11, and TLR2 and TLR4 respectively (18, 19). One of the *T. gondii*-derived antigens that induces strong immunopathology at the ileum is the GPI-anchored protein SAG1 (20). SAG1 is exclusively expressed on the surface of tachyzoites and can induce dominant antibody responses, and high IFN-γ production by T lymphocytes (21, 22). Our previous study demonstrated that intestinal epithelial cell response to *T. gondii* infection relies on TLR2 signaling, and oral infection of *T. gondii* in TLR2 deficient mice develop less pathology (Chapter 3). TLR2 is expressed in the follicle-associated epithelium covering lymphoid follicles of the mucosa-associated lymphoid tissues (MALT) of Peyer's patches in mice (23). The function of the follicle associated epithelium is distinct from other types of mucosal epithelial cells because the follicle associated epithelium contains M cells that transport and deliver luminal antigens into MALT, where adaptive mucosal immune responses are initiated. In our study, we show that *T. gondii*–derived factors in the supernatants elicit inflammatory response similar to live parasite infection. This response is dependent on the parasite GPI-anchored protein SAG1. Similar to live parasite infection, supernatant from *T. gondii* infected cells enhance the transport of luminal antigens, and induce a TLR2 dependent dendritic cell migration to the follicle associated epithelium.

Materials and Methods

Experimental Animals

MyD88$^{+/+}$ and MyD88$^{-/-}$ mice originally generated by S. Akira (Osaka University, Osaka, Japan) and provided by Dr. Eric Denkers (Cornell University, Ithaca, NY) were generated by crosses of heterozygous mice. TLR2 $^{-/-}$ (*Tlr2*^{tm1Kir}) with a C57BL/6 background and wild type (C57BL/6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR11$^{-/-}$ and TLR2×11$^{-/-}$ mice were provided by Dr. Felix Yarovinsky (University of Texas Southwestern Medical Center, Dallas, TX). All mice were used at 8-12 weeks of age. The mice were bred and housed under specific pathogen-free conditions at the Transgenic Mouse Facility, Cornell University College of Veterinary Medicine, overseen by the Institutional Animal Care and Use Committee.

Cell culture, parasite, and supernatant preparation

Henle 407 (human embryonic intestinal epithelial cells), HEK293 (human embryonic kidney cells), and HS27 (human foreskin fibroblast) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and 10% low endotoxin fetal bovine serum. *T. gondii* tachyzoites strain were maintained by serial 2 day passage on HS27 monolayers in DMEM. Wild-type RHΔ (24), Δsag1 (*sag1* deficient parasite), and the sag1-complemented M34 tachyzoites were provided by Dr. Michael Grigg (NIH, MD). Tachyzoites lacking subtilisin (DSUB1) that are complemented with control vector or the full-length TgSUB1 were provided by Dr. Kami Kim (25) (Albert Einstein College of Medicine, NY). All cell cultures and parasites were tested negative for mycoplasma by a high sensitivity PCR-ELISA based Mycoplasma detection kit (Roche, Indianapolis IN). Supernatants from

T. gondii infected cells were prepared by in vitro infections of *T. gondii* tachyzoites to HS27 fibroblasts or Henle 407 cells (MOI 30:1) for 20-24 h, supernatants were collected and passed through a 0.22 μm pore size filter. Control supernatants were collected from non-infected cells. Supernatants from *Listeria monocytogenes* and mock infection control supernatant were prepared by Dr. Hélène Marquis (Cornell University, Ithaca, NY).

NF-κB translocation assay

For immunofluorescence analyses, Henle 407 cells were plated at 2-3 × 10^5 cells per well on sterile coverslips placed in wells of a 24-well plate. Cell monolayers were infected with *T. gondii* RH-YFP tachyzoites and were then fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 in TBS (TBS-TX) and blocked in 1% BSA in TBS-TX. The cells were then stained with primary rabbit anti-NF-κB p65 followed by secondary antibody goat anti–rabbit IgG conjugated to AlexaFluor 594. Nuclei were stained using DAPI. Confocal images were taken with a Leica SP5 laser scanning confocal microscope. For immunoblot analyses, Henle 407 cells were plated at a number of 1.5 × 10^6 cells per well in 6-well plate and stimulated with *T. gondii* infected supernatants for the indicated times. Cytoplasmic and nuclear proteins were isolated according to previous protocols (26) and blotted for NF-κB p65.

RNA extraction and PCR analysis

Total RNA was extracted using the RNeasy mini kit (QIAGEN, Valencia, CA). Reverse transcription of the RNA (1 μg) was performed using SuperScript III Reverse Transcriptase Kit with the supplied Olige-dT primers (Invitrogen, Carlsbad, CA). Real-time PCR was performed on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with the SYBR green detection reagent

(40 cycles at 95°C for 10 s and at 60°C for 1 min). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for each sample. Percentage of relative expression level (REL%) was calculated using the $2^{-\Delta\Delta Ct}$ method. Primers for IL-8 and GAPDH were used as described in chapter 2.

In vitro infections

T. gondii tachyzoites or supernatants from infected cells were added to Henle 407 cells and were briefly centrifuged (400 × g, 1 min) to initiate parasite and cell contact. At time points indicated in each figure legends, cell lysates were collected and immunoblotted for total and phosphorylated forms of ERK1/2, and p38, (Cell Signaling Technology, Danvers, MA).

Luciferase reporter assays

HEK293 cells were plated at 1 x 10^4 cells/well in 96-well plates. Cells were transfected using TransIT Transfection Reagent (Mirus) and a total of 200 ng DNA/well consisting of different TLR plasmids, and a NF-κB luciferase reporter. Cells were stimulated with TLR ligands, or infected with *T. gondii* tachyzoites, lysed in reporter lysis buffer (Promega), and assayed for luciferase (Promega) activity. NF-κB activity was calculated and processed by Graphpad Prism Software.

Surgical ligated intestinal loops

Mice were anesthetized by intraperitoneal (i.p.) injection of avertin (tribromoethanol in *t*-amyl alcohol; 150 mg/kg animal weight), and a 3- to 5-cm segment of ileum containing a PP was ligated and injected as protocols described previously (27). Loops were injected with approximately 200 ml of sample supernatants. For measuring follicle associated epithelium transport of microparticles, sample

supernatants were injected in combination with fluoresbrite plain yellow-green 0.2-μm microbeads (Polyscience, Werrington, PA). After 60 (for microbeads) or 90 (for DC migration) minutes, animals were sacrificed, and PP were excised, washed in cold PBS, immediately embedded in Tissue-Tek OCT-embedding medium (Sakura, Torrance, CA), frozen in 2-methylbutane (Sigma-Aldrich, St. Louis, MO) cooled with liquid nitrogen, and stored at –80°C until sectioned.

Immunohistochemistry

Immunohistochemistry was performed on fresh frozen Peyer's patch (PP) tissue sections. Cryosections (3-5 μm) of frozen PP were cut on a cryostat model MICROM HM 525, mounted on Superfrost Plus microscope slides, air-dried overnight, and fixed with cold acetone for 2 minutes. Cryosections were blocked using PBS containing 2% goat serum. The following primary antibodies were used: biotin-conjugated rat anti-mouse CD11c (BD Pharmingen, San Diego, CA), and rabbit anti-mouse laminin (MP Biomedicals, Solon, OH). As secondary reagents, the following were used: streptavidin-Alexa-488 or -647 (Molecular Probes) for CD11c detection, and goat anti-rabbit-Alexa-488 or 647 (Molecular Probes, Carlsbad, CA) for laminin detection. All stained tissues were mounted with Prolong Gold antifade reagent containing DAPI (Invitrogen, Carlsbad, CA).

Microscopy and image analysis

Fluorescence images were collected on an AxioImager M1 microscope (Carl Zeiss MicroImaging, Inc.) using a 20× objective (200× magnification). AxioVision Rel. 4.8 software was used for image capture and processing. Brightness and contrast was adjusted using Adobe Photoshop CS5 software, and changes were applied equally across the entire images.

Analysis of DC migration and statistics

The numbers of intraepithelial CD11c+ DCs within the follicle associated epithelium were quantified by the following method: Peyer's patches from each mouse were sectioned, and four non-contiguous sections from the central region of each Peyer's patch that included the apex of one or more follicle domes were selected. The laminin staining was used as reference for drawing dotted lines to represent the basal limit of the epithelium. All CD11c+ cells located within the follicle associated epithelium as well as cells that associated with the basal limit of the epithelium were counted. Data were expressed as mean DCs per follicle associated epithelium sample. Thus, data from each treatment group represented the average of at least 4 separated segments of follicle associated epithelium. Statistical analyses were performed using GraphPad Prism Software (La Jolla, CA). The difference between control and *T. gondii*-infected supernatant were analyzed using an unpaired *t* test. Differences with $^{*}p < 0.05$, were considered statistically significant.

Protein identification by nano LC-MS/MS

Supernatants from infected cells were collected as described above and concentrated by a 30, 000 Da MWCO centricon. The supernatant proteins were separated by 10% SDS-PAGE and visualized by Coomassie Blue stain. The protein analysis of the 1D gel sample was performed at The Proteomics and Mass Spectrometry Core Facility, Institute for Biotechnology and Life Sciences (Cornell University, Ithaca, NY). The MS/MS data generated were submitted to Mascot 2.2 for database searching using Mascot local server and the search was performed to query to NCBInr (Taxonomy: Eucaryota) database (downloaded on July 28th, 2008 with 2,073,350 entries after taxonomy filter) with one missed cleavage site by trypsin allowed. The peptide tolerance was set to 1.5 Da and MS/MS tolerance was set to 0.6 Da.

Carbobamidomethyl modification of cysteine and a methionine oxidation were set as variable modifications. Only significant scores for the peptides defined by Mascot probability analysis greater than "identity" with 95% confidence interval were considered for the protein identifications.

Results

T. gondii induced nuclear translocation of NF-κB is not restricted to infected cells

In our previous study, we showed that *T. gondii* induced NF-κB nuclear translocation in epithelial cells infected with tachyzoites (28). However, this is not restricted to infected cells, but also in adjacent non-infected cells (Fig 4.1 A). Soluble factors in the supernatant may be capable of inducing NF-κB translocation to the nucleus. To test this, we collected supernatants from cells that were infected with live tachyzoites. The supernatant was filtered to exclude live parasites (Fig 4.1 B) and then applied to Henle 407 cells, a human small intestinal epithelial cell line. At the indicated time points, cytoplasmic and nuclear proteins were collected and blotted for p65 NF-κB. Results show that without live infection, the supernatants collected from *T. gondii* infected cells are capable of activating NF-κB nuclear translocation (Fig 4.1 C).

Supernatants from *T. gondii* infected cells induce inflammatory responses similar to live infection

To test whether supernatants collected from *T. gondii* infected cells elicited inflammatory responses similar to live infection in epithelial cells, we examined MAPK activation and IL-8 mRNA induction by Henle 407 cells stimulated with supernatants from infected cells. Similar to live parasite infection, intestinal epithelial cells responded to supernatants collected from *T. gondii* infected cells by phosphorylating ERK and p38 MAPKs as early as 15 minutes (Fig 4.2 A) and

upregulated IL-8 mRNA transcripts at 4 h post infection (Fig 4.2 B). Taken together, these data demonstrate that supernatants collected from *T. gondii* infected cells elicited inflammatory response with kinetics similar to live parasite infection.

Supernatants from *T. gondii* infected cells activate TLR2

We have previously shown that TLR2 is involved in epithelial recognition of *T. gondii* (28). Therefore, we next ask whether supernatants from *T. gondii* infected cells contain soluble factors that activate TLRs. HEK293 cells were transfected with different TLRs and an NF-κB luciferase reporter. While each positive control ligand stimulated NF-κB activation in the respective TLR expressing cells, supernatants from *T. gondii* infected cells activated NF-κB only in TLR2 transfected cells (Fig. 4.3). To determine whether the TLR2 stimulatory activity derived from either the parasite, or secretory proteins from epithelial cells, we incubated *T. gondii* tachyzoites with Henle 407 cells or media alone for 4 hours. Supernatants were collected and tested for TLR2 activity by luciferase reporter assay. Results show TLR2 activity in supernatants from *T. gondii* with or without Henle 407 cells (Fig 4.4). Furthermore, the activity of supernatants from *T. gondii* infected cells was not genotype dependent (Fig 4.5). Importantly, supernatants from cells infected with *Listeria monocytogenes* did not activate TLR2. Taken together, these data demonstrate that *T. gondii* infected supernatants contain *T. gondii*-derived soluble mediators that activate TLR2 (Fig 4.5).

Supernatants from *T. gondii* infected cells contain several parasite and host derived molecules

We next characterized the *T. gondii* infected supernatant. To estimate the size of the molecule contained in the supernatant with stimulatory activity, the supernatants were filtered through a 30kDa molecular weight cut off (MWCO) centricon filter. The

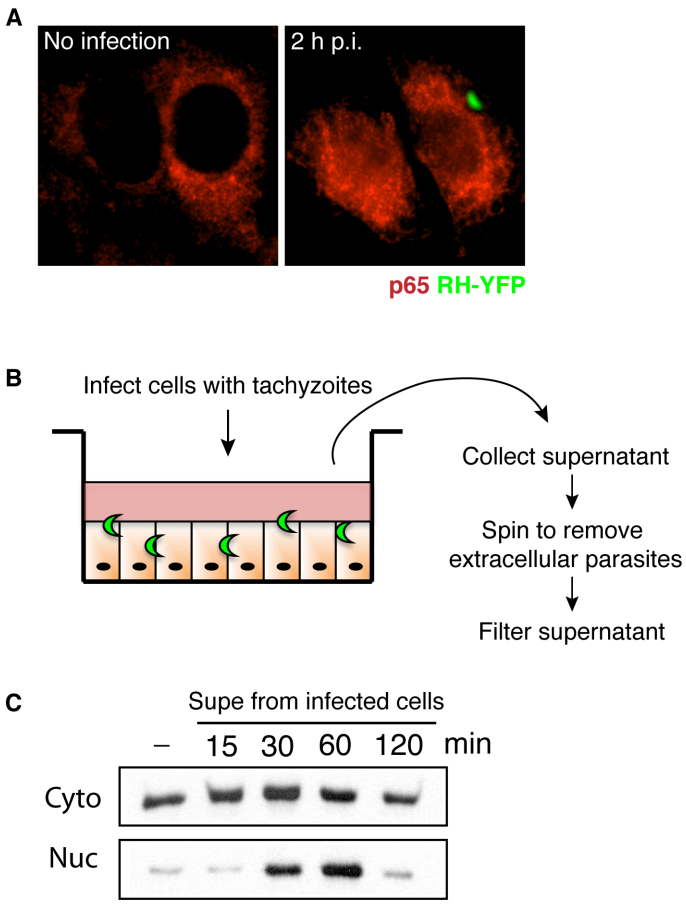


Figure 4.1. *T. gondii* induced nuclear translocation of NF-κB was not restricted to infected cells. (A) Henle 407 cells were infected with RH-YFP parasites for 2 hours. NF-κB p65 localization was visualized by immunofluorescence staining with anti–NF-κB p65 (red). In uninfected cells, p65 was present in the cytoplasm, incubation with parasites for 2 hours resulted in nuclear localization of p65 in both infected and non-infected cells. (B) Schematic procedure for collecting supernatants from *T. gondii* infected cells. (C) Supernatants from infected cells were added to Henle 407 cells for the indicated times. Cells were separated into cytoplasmic and nuclear fractions and analyzed for p65 by immunoblot.

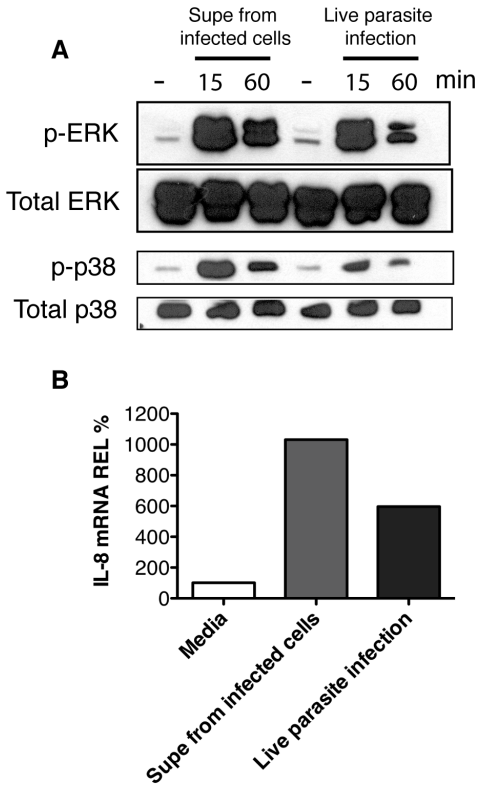


Figure 4.2. Supernatants from *T. gondii* infected cells induce an inflammatory response. (A) Activation of MAPK in intestinal epithelial cells by supernatants from infected cells or live parasites. Henle 407 cells were incubated with supernatants from RH infected cells or directly with RH tachyzoites for the indicated times. Whole cell lysates were collected for immunoblot analysis of total and phosphorylated forms of ERK/1/2 and p38 MAPK. (B) Human IL-8 gene transcript levels were measured by real-time qPCR analysis 4 hours post stimulation. The data are normalized to GAPDH and compared against uninfected expression levels (REL, relative expression levels).

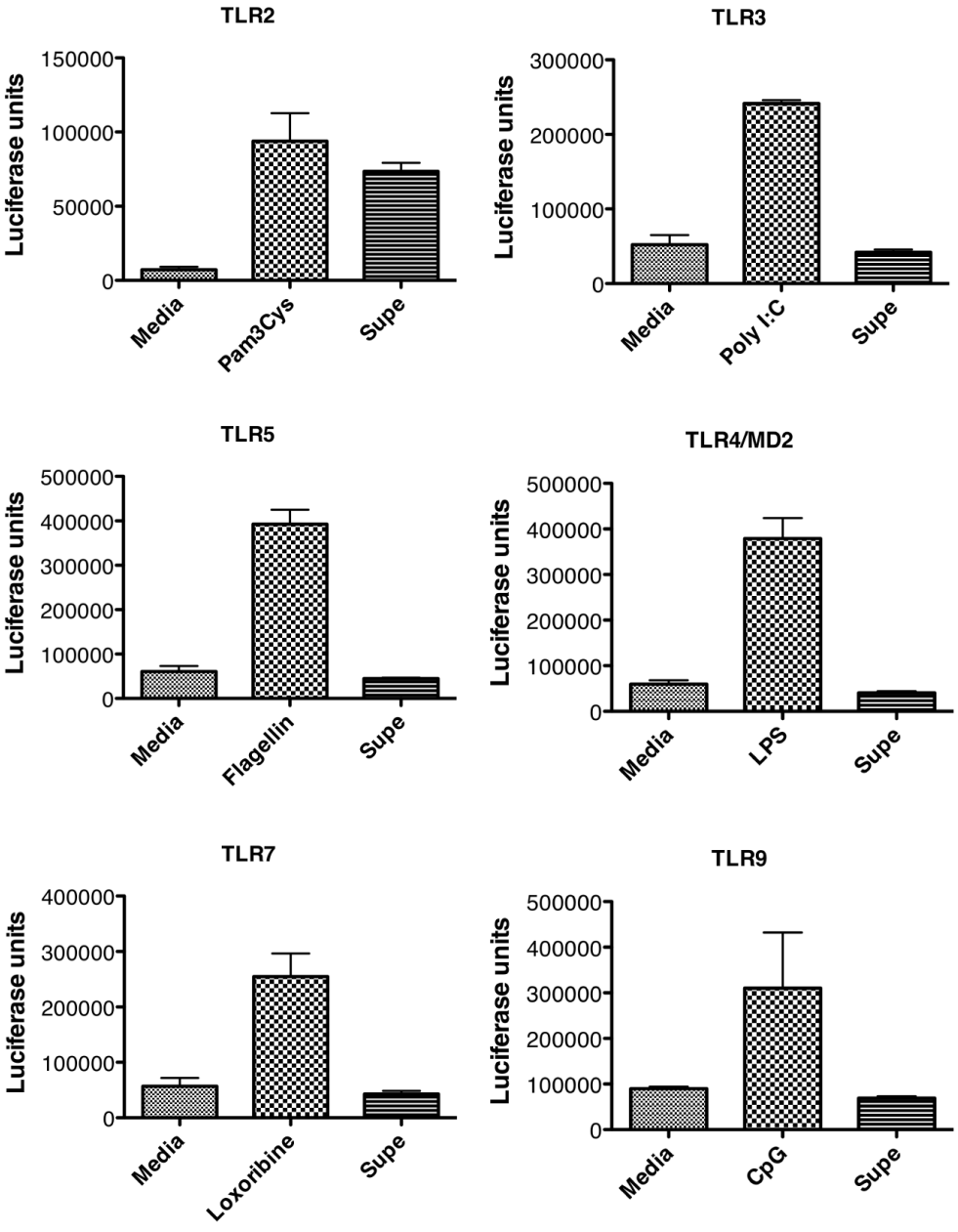


Figure 4.3. Supernatants from *T. gondii* infected cells activate TLR2. HEK293 cells were transfected with different human TLRs and an NF-κB luciferase reporter plasmid. The transfected HEK293 cells were stimulated with respective TLR ligands as positive controls or supernatants from infected cells. Cell lysates were collected and assayed for luciferase activity (Triplicate assays, error bar = SD).

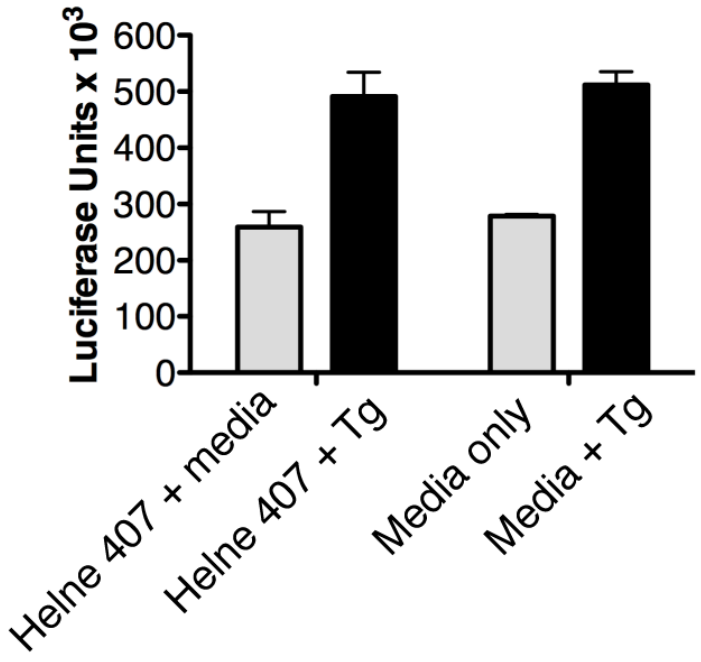


Figure 4.4. TLR2 activity in supernatants is parasite derived. *T. gondii* tachyzoites were either incubated with host cells or media alone. After 4 hours of incubation, supernatants were collected and added on to HEK 293 cells transfected with TLR2 and NF-κB luciferase reporter plasmids. HEK 293 cells lysates were assayed for luciferase activity (triplicate assays; error bar, SD).

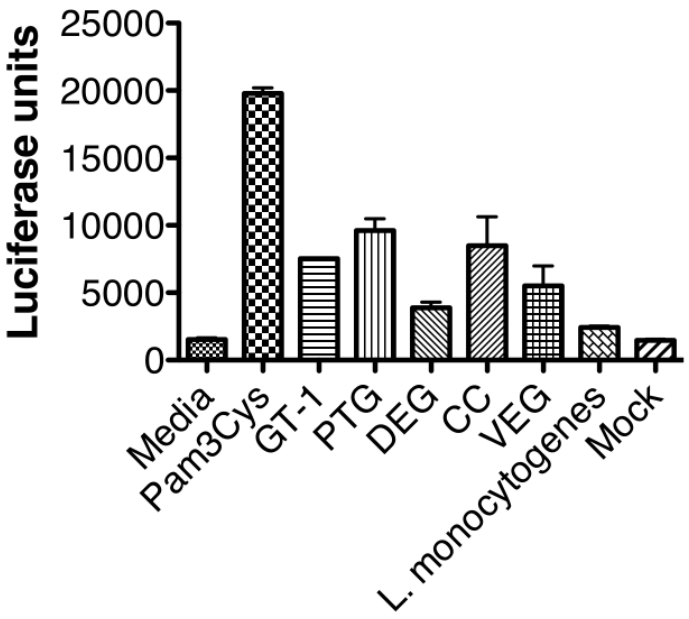


Figure 4.5. Supernatants from cells infected with different genotypes of *T. gondii*, but not *Listeria monocytogenes,* activate TLR2. HEK 293 cells transfected with TLR2 and NF-κB reporter plasmids were stimulated with supernatants collected from Henle 407 cells infected with different *T. gondii* strains. *L. monocytogenes* and mock infection control supernatants do not have TLR2 stimulating activity (triplicate assays; error bar, SD).

activity of the supernatant was 30kDa or greater (Fig 4.6 A). The supernatant (10 mg of total protein) was then separated by SDS-PAGE and visualized by Coomassie blue staining (Fig 4.6 B). Six protein sections from the 1-dimensional gel were excised and subjected to nano LC-MS/MS analysis as described in the material and methods. The proteomic analysis revealed 10 *T. gondii* proteins and 26 *Homo sapiens* proteins present in the supernatants from *T. gondii* infected cells (Table 4.1 and Table 4.2). Several identified proteins were from *T. gondii* apical secretory organelles involved in host cell invasion; for example, MIC-2 from micromemes and GRA-3, -5, -7, -8 proteins from dense granules. The surface antigen 1 (SAG1) and subtilisin-like protein (TgSUB1) are important for host cell attachment and proteolytic processing during invasion, respectively, and were also detected in the supernatant. Previous studies done by Debierre-Grocckeigo and colleagues showed that glycosylphosphatidylinositol (GPIs) purified from *T. gondii* tachyzoites are dependent on TLR2 and TLR4 for proinflammatory cytokines production in macrophages (18, 29). In this study, we have also shown that live *T. gondii* or infected supernatants trigger inflammatory responses through TLR2 in intestinal epithelial cells. Therefore, we suspected the *T. gondii* related mediator, which signals through TLR2, was a GPI-anchored protein. Of the *T. gondii* proteins identified by nano LC-MS/MS analysis, SAG1 and TgSUB1 were GPI-anchored proteins (30, 31). Since the cells used for culturing *T. gondii* were human foreskin fibroblasts, it was possible that *T. gondii* induced inflammatory cytokine secretion by these cells. Of the proteins identified from human origin, none of the proteins were known to activate TLRs; however, human TNF-α was detected. TNF-α is an inflammatory cytokine that activates the NF-κB signaling pathway through the TNF receptor. Indeed, were able to detect very slight levels of TLR2 independent activity through the NF-κB luciferase reporter assay in the supernatant collected from infected cells. Therefore, our data demonstrated that

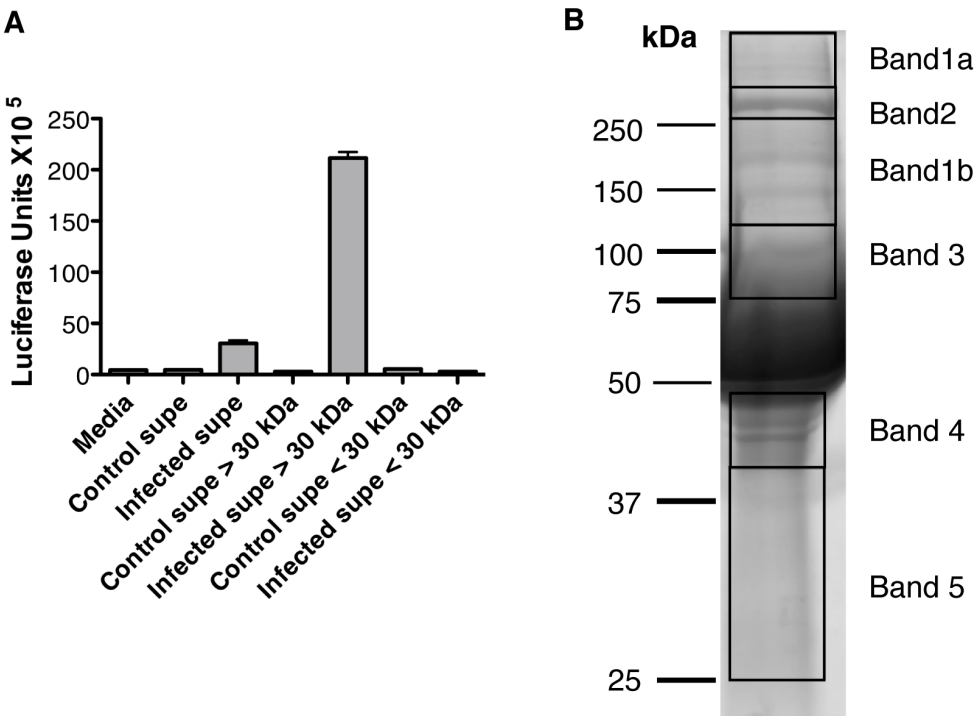


Figure 4.6. The TLR2 stimulating activity in supernatants from infected cells is greater than 30 kDa. (A) Supernatants from *T. gondii* infected or uninfected cells were filtered through molecular weight cut off centricons and tested for TLR2 dependent NF-κB activity using the luciferase reporter system. The TLR2 activity is in the concentrated fraction greater than 30 kDa. Small amounts of proteins smaller than 30 kDa may be present in the concentrated supernatant. (B) Supernatants from *T. gondii* infected cells containing 10 mg of total protein was separated by 1D SDS-PAGE. Proteins on the gel were visualized by Coomassie blue stain. Six sections were excised for nano-LC-MS/MS analysis and were referred to as band 1a, 1b, 2, 3, 4, and 5. Between band 3 and 4 was not collected due to the high amount of bovine serum at this molecular weight.

Table 4.1. A list of *T. gondii* proteins identified by nano LC-MS/MS from supernatants collected from infected cells.

Accession No.	Protein Description	Protein mass (Da)	Peptide Sequence
gil161913	28kd antigen	28,018	VAEQLFR
gil1703160	Actin	41,881	CDVDIR
gil2507039	Dense granule protein 5 precursor GRA 5	12,969	TAVGLAAAVVAVVSLLR
gil22652337	Dense granule protein GRA3	24,227	RQPFMSSVK
gil2062409	Dense granule protein GRA7	25,347	ASVESQLPR
gil5901701	Dense granule protein GRA8	28,509	IPYGGAAPPR
gil133990372	Membrane skeleton protein IMC2A	168,163	VPPPEAEGR
gil1923217	Micronemal protein MIC2	82,567	TRYNDPPPQGAGR
gil15419013	Subtilisin-like protein	85,034	TPPSAPSPSPR
gil123186979	Surface antigen 1	32,519	LTVPIEK

Table 4.2. A list of *Homo sapien* proteins identified by nano LC-MS/MS from supernatants collected from infected cells.

Accession No.	Protein Description	Protein mass (Da)	Peptide Sequence
gil4502049	aldo-keto reductase family 1, member B1	35,830	TTAQVLIR
gil4757756	annexin A2 isoform 2	38,580	TPAQYDASELK
gil230867	Chain R, Twinning In Crystals Of Human Skeletal Muscle D- Glyceraldehyde-3-Phosphate Dehydrogenase	35,853	VGVDGFGR
gil1199487	collagen binding protein 2	46,506	KPAAAAAPGTAEK
gil87196339	collagen, type VI, alpha 1 precursor	108,462	IALVITDGR
gil4503571	enolase 1	47,139	YNQLLR
gil31397	fibronectin precursor	256,529	ISCTIANR
gil53791219	filamin A	277,332	AGVAPLQVK
gil119593150	filamin A, alpha (actin binding protein 280), isoform CRA_a	248,350	VNVGAGSHPNK
gil5901956	follistatin-like 1 precursor	34,963	GAQTQTEEEMTR
gil4504165	gelsolin isoform a precursor	85,644	RTPITVVK
gil285975	human rab GDI	50,632	VPSTEAEALASSLMGLFEK
gil642534	lumican	38,375	ILGPLSYSK
gil4505763	phosphoglycerate kinase 1	44,586	YAEAVTR
gil189778	pigment epithelial-differentiating factor	46,300	ELLDTVTAPQK

Table 4.2. (Continue)

Accession No.	Protein Description	Protein mass (Da)	Peptide Sequence
gil189578	plasminogen activator inhibitor 1	45,045	TPFPDSSTHR
gil35825	pregnancy zone protein	163,733	ATVLNYLPK
gil1418930	prepro-alpha2(I) collagen	129,258	GVVGPQGAR
gil642908	procollagen C-proteinase enhancer protein	47,916	VFDLELHPACR
gil17028367	Similar to gelsolin (amyloidosis, Finnish type)	30,995	YIETDPANR
gil180665	skin collagenase precursor	54,058	ADVDHAIEK
gil40317626	thrombospondin 1 precursor	129,300	GPDPSSPAFR
gil339992	tumor necrosis factor	41,975	LTSALDELLQATR
gil30102	type I collagen	41,496	AQPENIPAK
gil28872	unnamed protein product	46,397	EFVEEFIWPAIQSSALYEDR
gil31189	unnamed protein product	23,182	HLACLPR

supernatant collected from infected cells mainly contains parasite derived TLR2 molecules as well as small amounts of TNF-α that triggers a TLR2 independent pathway.

Supernatants collected from SAG1 deficient parasite infected cells have reduced TLR2 stimulatory activity

To test whether SAG1 or TgSUB1 activates TLR2, we next tested the supernatant activity collected from SAG1 and TgSUB1 deficient parasites. SAG1 has been previously identified as the immunodominant surface antigen of *T. gondii* that is related to virulence and important for the initial stage of host invasion (32-34). The TgSUB1 is a GPI-anchored serine protease that is important for the proteolytic processing of microneme proteins (MIC) and is released from the surface of the parasite during invasion. The TgSUB1 protein can also be secreted into the media with other micronemal proteins after host cell invasion. Here, we analyzed the TLR2 stimulatory activity of infected supernatant from either SAG1-deficient parasites or TgSUB1-deficient parasites. Both knockout strains do not have defects in growth when cultured in vitro (25). Results show that supernatants from SAG1-deficient parasites have reduced TLR2 activity when compared to the parent RHD and the SAG1 complemented M34 strain (Fig 4.7 A). However, infected supernatants from TgSUB1 deficient parasites complemented with either full length or control vector do not demonstrate reduced TLR2 stimulatory activity (Fig 4.7 B). Therefore, these data suggests that the GPI-anchored protein SAG1 is important in activating TLR2.

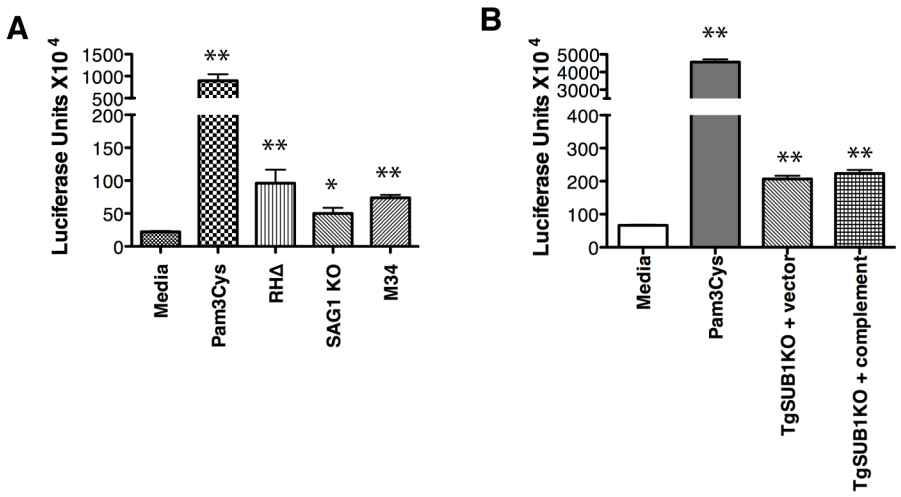


Figure 4.7. *T. gondii* tachyzoites deficient in the SAG1 gene have reduced TLR2 stimulating activity. (A) HEK 293 cells transfected with TLR2 and an NF-κB luciferase reporter plasmids were stimulated with the TLR2 ligand Pam3Cys, or supernatants from RHΔ (wild type parent strain), SAG1 KO (RHΔ lacking SAG1 gene), or M34 (SAG1 KO complemented with SAG1) infected cells. HEK 293 cells lysates were assayed for luciferase activity. (B) Similar to (A) except for supernatants from cells infected with TgSUB1 KO complemented with control vector, or TgSUB1 KO complemented with full length TgSUB1 were used to stimulate the HEK 293 transfected cells. HEK 293 cells lysates were assayed for luciferase activity (triplicate assays; error bar, SD). *P<0.05, **P<0.01, media vs. treatment.

Supernatants from *T. gondii* infected cells enhance microparticle transport and induce DC movement in the follicle- associated epithelium of Peyer's patches in vivo

Mucosal immune responses to antigens are initiated at sites containing organized lymphoid tissues such as Peyer's patches. To test whether supernatants collected from *T. gondii* infected cells enhance M cell function, ligated intestinal loops were injected with fluorescent latex microbeads mixed with infected or control supernatants. After 60 minutes, the Peyer's patch tissue samples were harvested and embedded in embedding medium. The sections from frozen tissue were stained with anti-laminin to visualize the boundary between the epithelium and the connective tissue. Results showed that more microbeads were detected in the subepithelial dome of Peyer's patch incubated with supernatants from infected cells (Fig 4.8). The follicle associated epithelium also attracts DCs to the subepithelial dome region to enhance capture of antigens. DC movement in the subepithelial dome is observed as early as 90 minutes post stimulation with TLR agonists (23). Therefore, to test whether supernatants from *T. gondii* infected cells induced the movement of DCs in the subepithelial dome, supernatant from infected and non-infected cells were injected into the lumen of the intestinal ligated loop and Peyer's patch samples were collected after 90 minutes. The frozen embedded tissue sections were stained for anti-CD11c+ and anti-laminin. There was a significant increase in the number of CD11c+ DCs in association with or migrated into the follicle associated epithelial layer when ileal loops were exposed to supernatants from infected cells compared to the non-infected cells (Fig 4.9 A, right top and right bottom panel). Together, these results demonstrate that supernatants from *T. gondii* infected cells enhance transport and induce rapid migration of DCs to the follicle associated epithelial in the Peyer's patch.

Migration of DCs to the follicle associated epithelium in response to supernatants from *T. gondii* infected cells is MyD88 dependent

We next asked whether the movement of subepithelial DCs induced by supernatants from infected cells was dependent on TLRs. Intestinal ligated loops of $MyD88^{+/+}$ or $MyD88^{-/-}$ mice were injected with supernatants collected from infected or non-infected cells, and tissues were harvested after 90 minutes. The infected supernatant induced migration of DCs to the follicle associated epithelium in $MyD88^{+/+}$ mice, but not in $MyD88^{-/-}$ mice (Fig 4.9 A). Quantitative analysis demonstrated that $MyD88^{-/-}$ had similar counts as wild type mice treated with control supernatant (Fig 4.9 B). These results suggest that *T. gondii* infected supernatants were able to induce a TLR-dependent DC migration in the Peyer's patch.

DC migration in response to supernatants from *T. gondii* infected cells is absent in TLR2-deficient mice

We next examined whether supernatants from *T. gondii* infected cells induced DC migration to the follicle associated epithelium in a TLR2 dependent manner. WT and TLR2 deficient mouse surgical ligated loops were injected with supernatant from infected or non-infected cells for 90 minutes. Although supernatants from *T. gondii* infected cells induce DC migration in wild type mice, TLR2 deficient failed to shown any DC migration (Fig 4.10 A, B)). Importantly, DC migration in response to infected supernatant was not dependent on the interaction of profilin with TLR11, since DC migration was not impaired in TLR11 deficient mice and TLR2×11 double deficient mice recapitulated the phenotype observed in TLR2 deficient mice (Fig 4.10 C, D). Taken together, these results demonstrate that supernatants from *T. gondii* infected cells play a role in modulating follicle associated epithelium function and DC migration in a TLR2dependent manner.

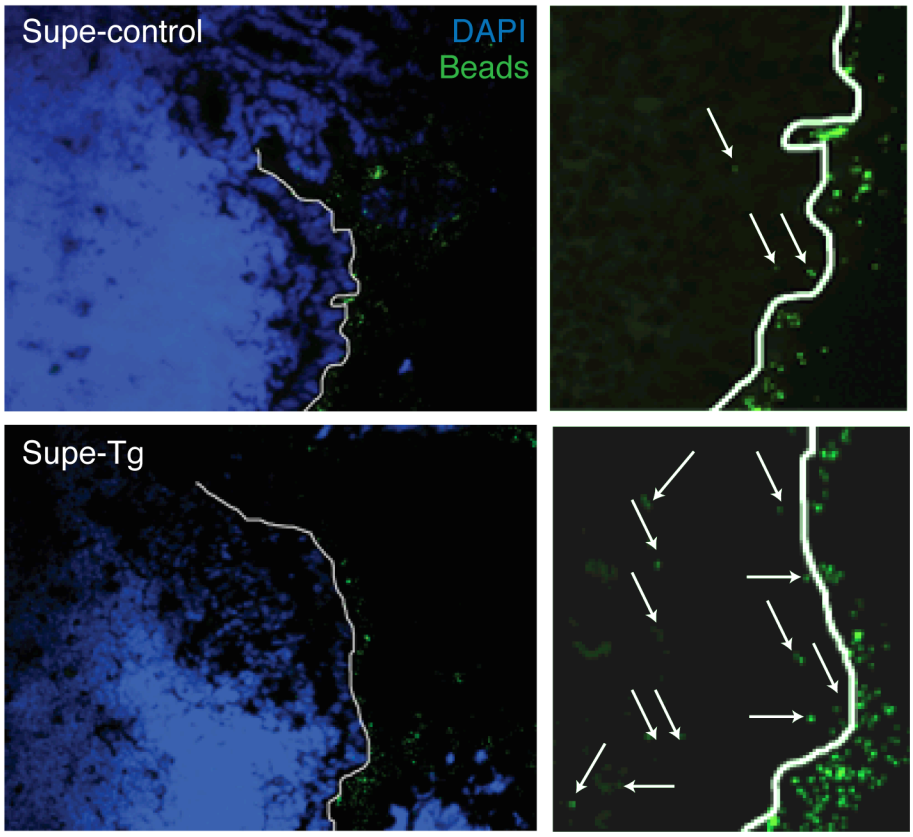
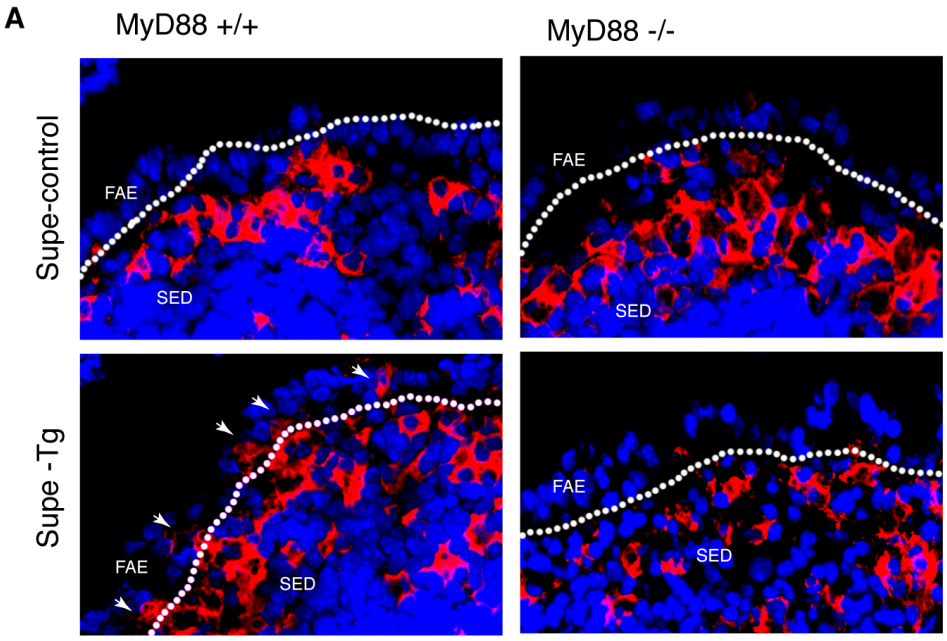


Figure 4.8. Supernatants from *T. gondii* infected cells enhance uptake of fluorescent latex beads. Ligated intestinal loops were injected with fluorescent latex beads mixed with equal volumes of supernatants collected either from infected cells (supe-Tg) or non-infected (supe-control) cells. Supernatants from *T. gondi* infected cells induce an increased uptake of fluorescent latex beads across the FAE at 60 minutes (arrows), indicative of increased M cell activity. The white line defines the basal limit of the epithelium as determined by laminin staining, and arrows indicate some of the beads on the SED side of the FAE.

Figure 4.9. Supernatants from *T. gondii* infected cells induce the migration of dendritic cells (DCs) into the FAE in a MyD88-dependent manner. (A) Peyer's patch from wild type or MyD88 deficient mice were collected after 90 minutes of intraluminel injection of control (supe-control) or supernatant from *T. gondii* infected cells (supe-Tg). Frozen tissue sections stained with anti- CD11c+ (red) and DAPI (blue) showed increased numbers of DCs in the FAE after exposure to supernatants. The dotted line defines the basal limit of the FAE as determined by laminin staining. Representative images show migrated CD11c DCs (arrows) to the FAE was induced upon exposure to supernatant from infected cells (supe-Tg) in $MyD88^{+/+}$ mice, but not in MyD88 deficient mice. (B) Quantitative analysis of DCs that have migrated to the FAE in control (supe-Tg) or supernatants from infected cells (supe-Tg). Data are from one experiment with one mouse for each treatment.


A
MyD88 +/+
MyD88 -/-
Supe-control
Supe -Tg
FAE
SED
FAE
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SED
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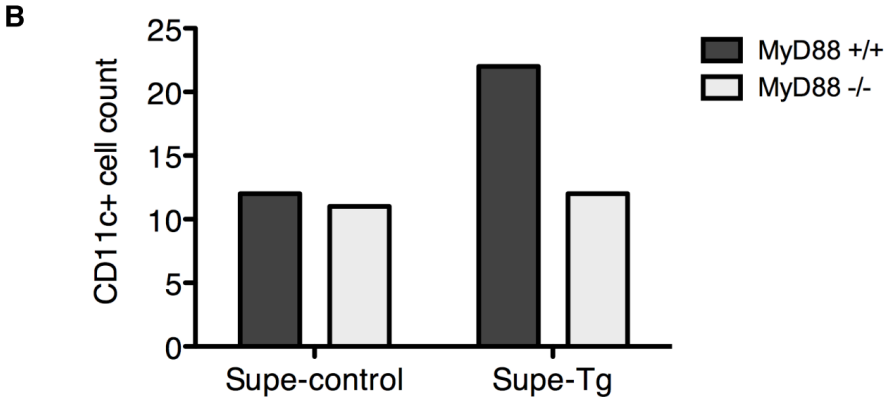
B
CD11c+ cell count
25
20
15
10
5
0
Supe-control
Supe-Tg
MyD88 +/+
MyD88 -/-

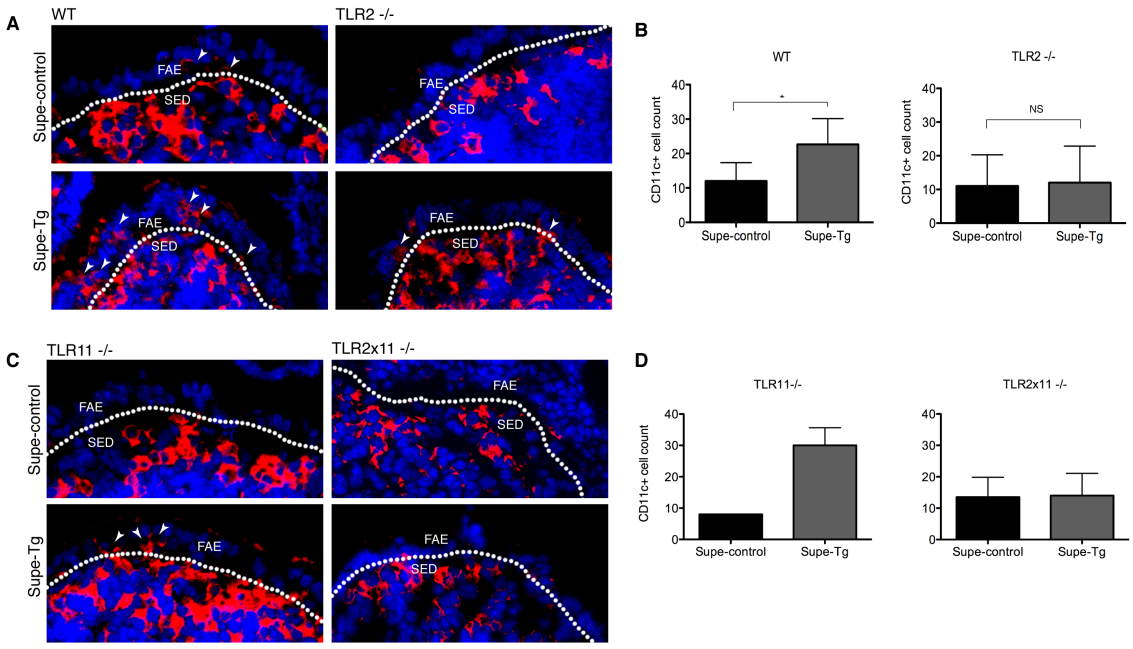

Figure 4.10. Supernatants from *T. gondii* infected cells induce migration of dendritic cells into the FAE is involves is TLR2 dependent. (A) Peyer's patch from wild type or TLR2 deficient mice were collected after 90 minutes of intraluminel injection of control (supe-control) or supernatant from *T.gondii* infected cells (supe-Tg). Frozen tissue sections stained with anti-CD11c+ (red) and DAPI (blue) showed increased DCs in the FAE after exposure to supernatants. The dotted line defines the basal limit of the FAE as determined by laminin staining. Representative images show that the migration of CD11c+ DCs (arrows) was induced upon exposure to supernatants from infected cells (supe-Tg), but not in TLR2-/- mice. (B) Quantitative analysis of DC migration into the FAE in WT or TLR2-/- mice (n=4). Significance between DC migration between WT and TLR2-/- mice with different treatments are indicated (*, $p < 0.05$). (C) Similar experiments as described in (A) were performed in TLR2×11-/- and TLR11-/- mice. (D) Quantitative analysis of DC migration to the FAE in TLR11-/- and TLR2×11-/- mice (n=2).

Discussion

TLR2 was previously shown to play a role in host protection at low dosage of *T. gondii* infection (35). In the chapter 3, we showed that oral infection with *T. gondii* in TLR2 deficient mice also resulted in less inflammation in the ileum, and less DC migration in the Peyer's patches after infection. This suggests that early signaling in the mucosa may influence the outcome of the disease. TLR2 dependent cytokine production during *T. gondii* infection also differs among cell types. For example, we previously reported that IL-8 secretion depends on TLR2 in intestinal epithelial cells (28). Studies with innate immune cells demonstrated that production of CCL2 by neutrophils and macrophages, and production of IL-12 in macrophages also depended on TLR2 (36). In this study, we further demonstrate that the GPI anchored SAG1 of *T. gondii* tachyzoites plays a role in initiating inflammatory responses in intestinal epithelial cells, and DC movement to the follicle-associated epithelium of the Peyer's patches, effects dependent on TLR2.

Interactions between epithelial cells and DCs at mucosal surfaces are thought to provide a crucial link between innate and adaptive mucosal immunity (37-39). In chapter 3, we showed that intralumenal injection with live *T. gondii* tachyzoites had a rapid, TLR2-dependent effect on the movement of DCs to the follicle associated epithelium within 90 minutes. Here, we demonstrate that the stimulatory component from *T. gondii* is secreted into the supernatant of infected cells, and these *T. gondi*-derived mediators promote antigen uptake and induce DC migration to the mucosal surface in a TLR2-dependent manner. This response is consistent with previous studies done by Chabot and colleagues using TLR2 ligands as stimulus (23). Therefore, we speculate the TLR2-dependent DC migration would result in enhanced antigen capture followed by migration to the interfollicular region (IFR). The

maturation of DCs is likely triggered by TLR2 dependent interaction with *T. gondii* derived antigens, such as SAG1, which stimulates high levels of IL-12 resulting in IFN-g secretion by T cells. In support of this hypothesis, studies by Liesenfeld and colleagues have shown that high levels of locally produced IFN-g were observed in the Peyer's patches of mice infected with *T. gondii* (40). However, in a study using bone marrow derived DCs, heat killed or soluble tachyzoite antigen induced DC maturation, but live parasite infection did not (41). Peyer's patches contain different subsets of DCs in the subepithelial dome region. $CD11b^{+}$ $CD8a^{-}$ DCs respond to CCL20 and CCL9, which are secreted by the follicle associated epithelium, while the $CD11b^{-}$ $CD8a^{-}$ DC subset expresses CX3CR1 and are in close contact with M cells. The CD11b-CD8a- DCs sample antigens by penetrating tight junctions between epithelial cells (42). Thus, it would be interesting to analyze which DC subset in the Peyer's patches is responding to *T. gondii* derived antigens and the subsequent T cell activation. In addition, while both live parasite and supernatants containing *T. gondii*-derived mediators trigger DC migration, the outcome of T cell activation could be different. *T. gondii* may subvert DCs dissemination (41, 43).

T. gondii possesses multiple molecules that activate the MyD88-signaling pathway. Therefore, immune activation during *T. gondii* infection may involve multiple TLR ligands. Two *T. gondii* molecules that activate TLRs have been identified to date. *T. gondii* GPI anchors as well as core glycans and lipid moieties trigger NF-κB activation through TLR2 and TLR4 (18, 29). Another TLR ligand derived from *T. gondii* is profilin, which induces DC production of IL-12 through TLR11 (19, 44). Although we observed reduced TLR2 activity in the SAG1-deficient parasites, DC migration may be mediated by many GPI-anchored proteins rather than just one specific antigen. Since SAG1 is very abundant on the surface of tachyzoites, depleting SAG1 in the

parasites results in reduced TLR2 stimulatory activity. In contrast, TgSUB1 is less abundant and deficient parasites did not demonstrate less stimulatory activity. Importantly, the migration of DCs in response to the *T. gondii* secreted factor is a TLR2 dependent event because we observed a defect of DC movement in TLR2×11 double deficient mice, but not in the TLR11 deficient mice. We have not analyzed the cytokine production from the Peyer's patch DCs; however, while TLR2 dependent DC migration to the follicle associated epithelium might enhance antigen uptake, it is possible that the production of IL-12 in DCs still relies on TLR11 dependent signaling.

The initial site of infection for *T. gondii* is the mucosal surface; therefore, a strong inducer that stimulates mucosal immunity is important for developing effective vaccines against toxoplasmosis. SAG1 is a strong inducer of immunity and is associated with virulence, as demonstrated by higher transcription levels in the virulent *T. gondii* strains (33). Kasper and colleagues demonstrated that SAG1 induced the expression of several chemokines that are important for inducing acute ileitis in susceptible C57BL/6 mice. This suggests that SAG1 induces strong IFN-g production (20). In vitro, SAG1, but not other *T. gondii* secretory proteins, directly induces the secretion of MCP-1/CCL2 (45). Due to these immunostimulatory features, is often a candidate for designing DNA based vaccines to prevent toxoplasmosis (22, 46-49). However, most of the immunizations in these studies are inoculated intramuscularly or intranasaly to generate CD8+ T cell response, but not through the intestinal mucosa. Further studies are needed to define the role of SAG1 in inducing effective Th1 response in the intestinal mucosa. Our data suggest that SAG1 may act very early during infection and would therefore act as both an innate immune stimulator and antigen to which adaptive immunity is directed. This information may provide insight

for generating protective response against toxoplasmosis and benefit both medical and veterinary fields.

Acknowledgements

We thank Eric Denkers for the MyD88$^{-/-}$ mice, Felix Yarovinsky for the TLR11$^{-/-}$ and TLR2×11$^{-/-}$ mice, Kami Kim for the SUB1 deficient parasites, and Michael Grigg for the SAG1 deficient parasites.

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CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

1. Summary of findings

In Chapter 2, the response of intestinal epithelial cells and signaling pathways during *T. gondii* infection was examined. By using an in vitro system, the immediate response upon intestinal epithelial and parasite contact was measured. Results from this chapter revealed that *T. gondii* infected epithelial cells elicited MAPK phosphorylation, NF-kB activation, and secretion of IL-8 as well as several other inflammatory cytokines and chemokines. Specifically, activation of MAPK and production of IL-8 was dependent on the MyD88 signaling pathway. However, while knocking down TLR2 in epithelial cells did not complete abrogate signaling, TLR2 (both mouse and human) was sufficient for parasite induced signaling in HEK 293 transfected cells. Furthermore, the epithelial cells responded to *T. gondii* regardless of their genotype and virulence.

In Chapter 3, modulation of the local intestinal environment and the morphological changes of different intestinal epithelial cell subtypes in response to peroral *T. gondii* infection were characterized. Using an intestinal ligated loop model, live *T. gondii* tachyzoites induced a TLR2-dependent migration of CD11c+ cells, which are mostly dendritic cells, into the follicle-associated epithelium. Direct infection of *T. gondii* in polarized intestinal epithelial cells enhanced barrier function and upregulated a subgroup of defensin mRNA transcripts. Oral infection of C57BL/6 mice with *T. gondii* ME49 cysts induced intestinal pathology and morphological changes such as reduced numbers of goblet cells and Paneth cells as well as secretion of Paneth cell granules to the crypt lumen. Interestingly, mice with similar genetic background but lacking TLR2 were more resistant to intestinal pathology and associated morphological changes observed following *T. gondii* infection in wild type mice.

In Chapter 4, the TLR2 stimulatory molecules derived from *T. gondii* were investigated. I found that supernatants from *T. gondii* cells activated signaling pathways in intestinal epithelial cells, a response similar to live parasite infection. Furthermore, supernatants collected from *T. gondii* infected cells activated NF-κB through TLR2. Previous studies have shown that glycosyphosphatidylinositol (GPI) anchors derived from *T. gondii* are ligands for TLR2 and TLR4 (1). The analyses of the supernatant revealed two GPI-anchored proteins from *T. gondii*: surface antigen 1 (SAG1) and subtilisin (TgSUB1). Supernatants collected from cells infected with SAG1-deficient parasites had reduced stimulatory TLR2 activity. The potential biological significance of the activity in supernatants from *T. gondii* infected cells was also addressed. Supernatants from *T. gondii* infected cells enhanced the uptake of antigens from the luminal of the small intestine and induced TLR2-dependent migration of dendritic cells to the follicle-associated epithelium of Peyer's patches.

2. The role of intestinal epithelial cells in initiating inate immunity during *T. gondii* infection

It is often questioned whether *T. gondii* infects intestinal epithelial cells or crosses epithelial barriers to gain access to deeper tissues (2, 3). To cause systemic infection, the parasite must cross the intestinal epithelium, basement membrane, and lamina propria. However, early studies on the kinetics of invasion and dissemination of *T. gondii* showed that parasites can be detected in the Peyer's patch, lamina propria, and epithelial cells of the distal ileum after 1 hour oral ingestion of *T. gondii* cysts (4). Dissemination of the parasite to the periphery, lung, and liver was observed 24-48 hours later (4). This suggests that during the first 24 hours, the intestinal epithelial cells may be a target for parasite invasion and replication. Therefore, signals initiated by intestinal epithelial cells are critical for the cross talk with the innate immune cells

underlying the lamina propria. In this thesis, I initially used an in vitro model to study the intestinal epithelial response upon *T. gondii* infection. Consistent with previous studies, I have also observed the induction of cytokines and chemokines important for neutrophil, dendritic cell, and macrophage chemotaxis.

One of my interesting findings is the importance of TLR2 signaling during *T. gondii* infection. TLR2 expressed in different epithelial cell types might play different roles during different stages of infection. While signaling of MAPK and IL-8 is only partially dependent on TLR2 in enterocytes (results from chapter 2), the gate keeping function in the FAE and DC migration is dependent on TLR2 (results from chapter 3). The migration pattern of Peyer's patch DCs is similar to previous reported using TLR ligands as stimulus (5, 6). However, the reason for parasite induced DC migration towards the mucosal surface still remains unclear. There are at least 2 possible explanations for parasite induced DC migration. First, the effect is due to a host defense mechanism. In the case of *Salmonella typhimurium*, a subset of DCs expressing CCR6+ reside in the Peyer's patch and were recruited to the subepithelial dome regions upon bacterial invasion. These cells were responsible for the rapid local activation of pathogen-specific T cells (7). Whether this is the case during *T. gondii* infection remains to be explored. Second, induction of DC migration to the mucosal surface might be induced by *T. gondii* to cause migration of leukocytes. These leukocytes would then be infected and used as Trojan horses to disseminate the organism in the host while avoiding immune attack, an evasion mechanism that has been proposed by previous studies (8, 9).

While the results in this thesis showed the importance of TLR2 in the "early" signaling of *T. gondii* at the Peyer's patch and epithelial cells, there is still a missing

link in how *T. gondii* induced TLR2-dependent early signaling events can effect the disease outcome. *T. gondii*-induced ileitis is only observed in mice on the C57BL/6 genetic background, whereas other mouse strains are resistant to this pathology. C57BL/6 mice lacking TLR2 display different levels of pathology during oral challenge with *T. gondii* when raised in different facilities (results in chapter 3 compared to results published by Yarovinsky and colleagues (10). Emerging studies have now demonstrated that bacterial recognition through TLRs can shape the intestinal function through recognition of commensals (11-16). Differences in the composition of the gut commensals can modulate the mucosal immune balance as well as the immune responses against intestinal pathogens. This is supported by the recent identification of a specific commensal member in the small intestine, a non-culturable *Clostridia*-related species named segmented filamentous bacteria (SFB). The colonization of SFB can coordinate the maturation of intestinal T cell adaptive functions in the gut mucosa (17-19). SFB is also know to adhere to the Peyer's patch in addition to the ileal mucosa and may play a role in initiation of T cell responses. It has been shown that colonizing SFB can induce IL-17, IL-10, CD40L, and IFN-γ mRNA transcripts in the Peyer's patch (19). Whether the colonizing of SFB in different TLR deficient mice results in mucosal susceptibility to pathogens is not yet know. However, it is possible that the presence of SFB, which can skew the mucosal effector T cell balance, plays a role in shaping the mucosal immune response during *T. gondii* infection. Future studies on analyzing the gut microbiome in wild type, TLR knockout mice, as well as mice raised in different environments can help us understand how commensals participate in regulating mucosal functions, which may be importance of explaining the difference in disease outcome of *T. gondii* infections.

3. *T. gondii* derived TLR ligands and their role in initiating mucosal immunity

Two TLR ligands derived from *T. gondii* have been identified to date. *T. gondii* GPI-anchored proteins activate TLR2/4, and *T. gondii* derived profilin-like protein is recognized by TLR11 (1, 20). In chapter 4, my results suggest that the dominant GPI-anchored surface protein (SAG1) of *T. gondii* was important for NF-κB activation via through TLR2. These observations raised the possibility that *T. gondii* possesses different molecules that are recognized at different stages by the host innate immune system. The activation of TLR2 in the mucosal epithelum during *T. gondii* infection may be important for mucosal immune responses, whereas *T. gondii* profilin is important for IL-12 production through TLR11 stimulation in dendritic cells (20).

The surface protein of tachyzoites SAG1 is highly immunogenic. Studies published by Kasper and colleagues showed that SAG1 knockout parasites have a reduced ability to induce ileitis following intralumenal infection, and that the infection is associated with increased parasite burden and decreased innate and adaptive immune responses. Infection with SAG1 knockout parasites also affects the capacity of intestinal epithelial cells to secrete chemokines (21). This study supports my findings on the reduced TLR2 stimulatory activity, and a defect in DC movement in the Peyer's patch in mice lacking TLR2 when treated with supernatants from SAG1-deficient parasites. This suggests that while TLR2 recognizes *T. gondii* GPI-anchored proteins, due to the dominance of SAG1 present on the surface of the parasite, *T. gondii* SAG1 could be the main factor contributing TLR2 signaling.

4. Future directions

My finding of intestinal epithelial response during *T. gondii* infection raises several interesting questions. It is not clear how TLR2-dependent migration of DCs to the

follicle associated epithelium is triggered. There are at least two possibilities that can be addressed: (1) The TLR2 expressed in the follicle associated epithelium enterocytes are activated upon encountering the parasite, which causes rapid short-term release of chemoattractants, such as CCL20, to recruit DCs in the subepithelial dome, and (2) the effect is solely dependent on the DCs with in the Peyer's patch. The first possibility requires signaling to upregulate gene transcription, followed by translation, folding, processing, secretion of chemokines, diffusion of those chemokines and activation of the CD11c+ cells, all within the 90 minutes of the experiment. While this seems unlikely, it is possible that the epithelial cells have prestored chemokines or other non-transcription dependent signaling molecules that could act as chemoattractants. Therefore, the possibility that factors secreted by *T. gondii* penetrate the epithelial junctions and act directly on the dendritic cells. It would be interesting to test the effect of DC migration using TLR2$^{flox/flox}$ Villin-cre or TLR2$^{flox/flox}$ CD11c-cre mice during *T. gondii* infection to determine whether the effect is directly on the DCs.

My results from in vitro and in vivo infection of *T. gondii* revealed the induction of several cytokine and chemokine mRNA transcripts. Defect or overexpression of certain cytokines can affect innate immune cell homing and maturation and alter the disease outcome. Most studies on *T. gondii*-induced pathology have focused on TLR deficient mice. However, the associated intestinal immunopathololology can also involve other cytokines/receptors as well. This is shown by the resistant phenotype of *T. gondii*-induced pathology in CCR2-deficient mice (22). The functional role of these inflammatory cytokines upregulated in epithelial cells during during *T. gondii* infection can be addressed in future studies. For example, the chemokine MIP3-a/CCL20 expressed at high levels in the follicle associated epithelium is the

predominant chemokine that attract and maintain the abundance of CCR6+ DCs to the subepithelial dome region of the Peyer's patch (23, 24). This gene was also upregulated during early infection of *T. gondii* in intestinal epithelial cells (results in chapter 2). Therefore, it is possible that depletion of this chemokine would result in decreased clusters of DCs in the subepithelial dome region and initiation of T cell response during infection, thus increasing susceptibility to *T. gondii* infection.

The Peyer's patch has been considered the major inductive site for IgA, and the DCs in the Peyer's patch play an important role in this production. Mice orally infected with *T. gondii* develop intestinal IgA antibodies to SAG1 (25). I have shown that both live *T. gondii* and *T. gondi*-derived molecules induce DC migration within Peyer's patches. This raises the possibility that the migrated DCs participate in the uptake of antigens. However, immunohistochemical and functional analysis of the Peyer's patch DCs has revealed three distinct populations. DCs in the subepithelial dome (SED) dominantly express CD11b, DCs in the interfollicular region (IFR) express CD8a, and a double negative DC subset are localized in subepithelial dome region, IFR and follicle associated epithelium (26-28). These three DC subsets possess distinct abilities to induce CD4+ Th1 or Th2 differentiation. Therefore, future work needs to be done on further analysis of the DCs subsets that have migrated to the follicle associated epithelium. This information will help us understand the mechanism of immune induction in the Peyer's patch during *T. gondii* infection.

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