FUNCTION OF JNK2 AND MYD88
DURING *TOXOPLASMA GONDII* INFECTION

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The intracellular protozoan parasite *Toxoplasma gondii* infects humans and other warm-blooded animals. Understanding the pathogenesis of infection and immunity to the parasite is important since this pathogen is capable of causing fatal disease in immunocompromised hosts. *T. gondii* infection elicits IL-12 release from innate immune cells, resulting in high level IFN-γ production. This confers resistance to infection through T cell dependent Type I immunity. Signal transduction pathways involved in IL-12 production, a little understood area, are the major focus of this dissertation.

Neutrophils are innate immune cells capable of IL-12 production during *T. gondii* infection. I found that JNK2 MAPK is the major isoform expressed in mouse neutrophils. My results show that JNK2 plays a major role in PMN IL-12 and CCL/MCP-1 production during in vitro parasite stimulation. Neutrophil chemotaxis was also partially dependent upon JNK2, whereas oxidative burst and phagocytic activities were not affected by absence of this MAPK protein.

I examined the role of JNK2 in response to *T. gondii* infection in vivo. I found that JNK2 plays a role in *T. gondii*-induced immunopathology since absence of JNK2 contributed to enhanced disease resistance associated with alleviated pathology in the small intestine and decreased neutrophil recruitment at this site. Surprisingly, and in

contrast to my in vitro data, JNK2 negatively regulated IL-12 production during in vivo infection.

Toll-like receptor adaptor protein MyD88 is important for IL-12 induction during *Toxoplasma* infection. During oral *T. gondii* infection, I found that MyD88 was essential for controlling parasite burden leading to disease resistance. In addition, I found that neutrophil recruitment and expression of IGTP, a molecule required for control of infection, was also dependent upon MyD88 signaling. Interestingly, while IL-12 was greatly diminished in the absence of this molecule, T cell dependent IFN-γ responses were nevertheless generated. Importantly, I found that MyD88 was not required for development of protective immunity to *T. gondii*. Thus, while MyD88 signaling was required for microbicidal control of infection, it was not necessary for initiation of adaptive immunity to this parasite.

In summary, this dissertation elucidates the complex roles of JNK2 and MyD88 signaling pathways in IL-12 production and resistance to *Toxoplasma* infection. Investigating the functions of these molecules in response to *T. gondii* contributes to a clearer understanding of the immunopathogenesis of infection with *Toxoplasma* and other microbial pathogens.

BIOGRAPHICAL SKETCH

Woraporn Sukhumavasi was born in Bangkok, Thailand on October 31, 1977. Her mother was influential in sparking her interested in biomedical sciences by exposing her to the laboratory environment during her childhood. With her affection for animals, she obtained Doctor of Veterinary Medicine with Honors from Chulalongkorn University in 2000. As a veterinary student, she found parasites fascinating from the field trip experiences and collection of worms from abattoirs. With strong interests in parasitology as well as teaching, she was subsequently recruited to Faculty of Veterinary Sciences, Chulalongkorn University, where she graduated, as an instructor of veterinary parasitology. After working as an instructor and a practioner for three years, her interests extended to interactions between hosts and their parasites. She received a prestigious scholarship in 2002 to pursue her Ph.D. from the Anandamahidol foundation, under The Royal Patronage of His Majesty King Bhumibol Adulyadej of Thailand and chaired by Her Royal Highness Princess Sirindhorn. Because of its distinction in immunology and infectious diseases, she then joined the Ph.D. program in field of comparative biomedical sciences at Cornell University in 2003 and worked in Dr. Eric Y. Denkers's laboratory. She was awarded the Milton L. Shifman Endowed Scholarship and with support from Dr. Eric Y. Denkers she attended an intensive summer course training in Biology of Parasitism at Woods Hole, MA, for 2 months following her Ph.D. defense. She intends to go back to serve her home country by continuing to work in veterinary parasitology. In additon to her interest in academia, she is an avid tabletennis player, a scuba diver taught by her brother and a Thai classical dancer.

I dedicate this work to:

Beloved His Majesty King Bhumibol Adulyadej, the King of Thailand, who devoted himself to bettering the lives of Thai people.

My parents, brother, relatives, friends and Apibunyopas family who always encourage and provide moral support to me unconditionally.

My teachers, instructors and professors who provide me invaluable education.

The animals that sacrificed their lifes for a better life for people and animals.

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Last but not least, I would like to express my utmost gratitude to His Majesty King Bhumibol Adulyadej and Her Royal Highness Princess Sirindhorn for their vision in recognizing the importance of education in Thailand and establishing the Anandamahidol Foundation, which provided me the scholarship to pursue this Ph.D. study.

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LIST OF ABBREVIATIONS

Ab, antibody

AP-1, activator protein 1

CCL, CC chemokine ligand

CCR, CC chemokine receptor

CpG, cytosine guanine dinucleotide

DC, dendritic cells

EGFP, enhanced green fluorescent protein

ELISA, enzyme-linked immunosorbent assay

FACS, fluorescence-activated cell sorter

FcR, Fc receptors

fMLP, formyl-methionyl-leucyl-phenylalanine

G-CSF, granulocyte colony-stimulating factor

ICAM, intercellular adhesion molecule

IEL, intraepithelial lymphocyte

IFN-γ, interferon-γ

Ig, immunoglobulin

IGTP, IFN-γ- inducible GTPase

IL, interleukin

iNOS, inducible nitric oxide synthase

IP-10, IFN-γ-inducible protein-10

JNK, c-Jun N-terminal kinase

KO, knockout

LPS, lipopolysaccharide

MAPK, mitogen-activated protein kinase

MCP, monocyte chemoattractant protein

MHC, major histocompatibility complex

MIP, macrophage-inflammatory protein

MyD88, myeloid differentiating factor 88

NADPH, nicotinamide adenine dinucleotide phosphate (reduced)

NBT, nitroblue tetrazolium

NETs, neutrophil extracellular traps

NFκB, nuclear factor κB

NK cell, natural killer cell

NO, nitric oxide

PI3K, phosphatidylinositol 3-kinase

PMA, phorbol myristate acetate

PMN, polymorphonuclear leukocyte

SCID, severe combined immunodeficiency

STAT, signal transducer and activator of transcription

TGF, transforming growth factor

Th cell, T helper cell

TLR, Toll-like receptor

TNF, tumor necrosis factor

YFP, yellow fluorescent protein

CHAPTER 1

INTRODUCTION

1. Life cycle of *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular protozoan parasite. It was initially found in tissues of a hamster-like african rodent, the gundi (*Ctenodactylus gundi*). Nicolle and Manceaux named this pathogen as a new organism in 1909 based on the morphology (*toxo* = arc or bow, *plasma* = life) and the host species in which the parasite was originally discovered (1). *T. gondii* is a coccidian belonging to phylum Apicomplexa that also includes the medically important *Plasmodium spp.* and *Cryptosporidium parvum*. *T. gondii* is the only species in genus *Toxoplasma*.

The complete life cycle of *T. gondii* was elucidated in 1970 when members of the family Felidae, including the domestic cat, were identified as definitive hosts of the parasite (2) (Fig.1.1). Most warm-blooded animals, including humans, serve as intermediate hosts. Three different infective forms of *Toxoplasma* play roles in disease transmission: sporozoite-containing oocysts, tachyzoites and bradyzoite-containing cysts. Oocysts, 10 x 12 µm, are generated by gametogenesis inside the enterocytes of the felid. In this host, there are five stages of asexual development (type A-E) of *T. gondii* within intestinal mucosa that occur before entering sexual stages to produce oocysts (3, 4). During acute infection, cats can shed millions of oocysts over a period of 7-21 days (5). They are shed in the feces into the environment and become sporulated as sporozoite-containing oocysts. Each oocyst contains 2 sporocysts with 4 sporozoites per sporocyst. Sporulation occurs outside the host within 1-21 days depending upon environmental conditions. *T. gondii* oocysts are environmentally resistant. They can survive in seawater, soil, detergent, disinfectant, refrigeration and warm water. Studies have demonstrated that oocysts remain infective (tested by bioassay in mice) when the oocysts are stored at room temperature for up to 200 days (6-9).

Toxoplasma can be transmitted to intermediate hosts through fecal-oral contamination, animal-to-animal carnivorism, consumption of infected undercooked meat and transplacental transmission. Pigs and small ruminants commonly harbor the tissue cysts, whereas the parasite is present within cattle at very low prevalence (10). Because intermediate hosts can ingest oocyst-contaminated food or water inadvertently, this route of transmission contributes to widespread infection in herbivore animals and vegetarians, and this impacts public health concerns regarding foodborne and waterborne zoonosis (1, 8, 11-13).

Upon ingestion of oocysts, sporozoites are liberated by enzymatic digestion inside the gastrointestinal tract giving rise to the haploid tachyzoite stage. The crescent-shaped tachyzoite, 2-4 µm wide and 4-8 µm long, is a rapidly dividing form of *T. gondii* associated with the acute infection. It invades virtually all nucleated cells and replicates inside a parasitophorous vacuole formed during invasion of host cells (14-16). After tachyzoite egress, contiguous cells are infected and the parasite disseminates throughout the body. Lysis of host cell leads to tissue inflammation and necrosis. In response to immune pressure and intrinsic programming within the parasite, tachyzoites transform into latent bradyzoites and become encysted in the brain, heart, and muscle. In this manner, the parasite establishes life-long infection in its host (17, 18). Cysts usually cause no immune reaction and remain in the tissues until they are consumed by the feline or human.

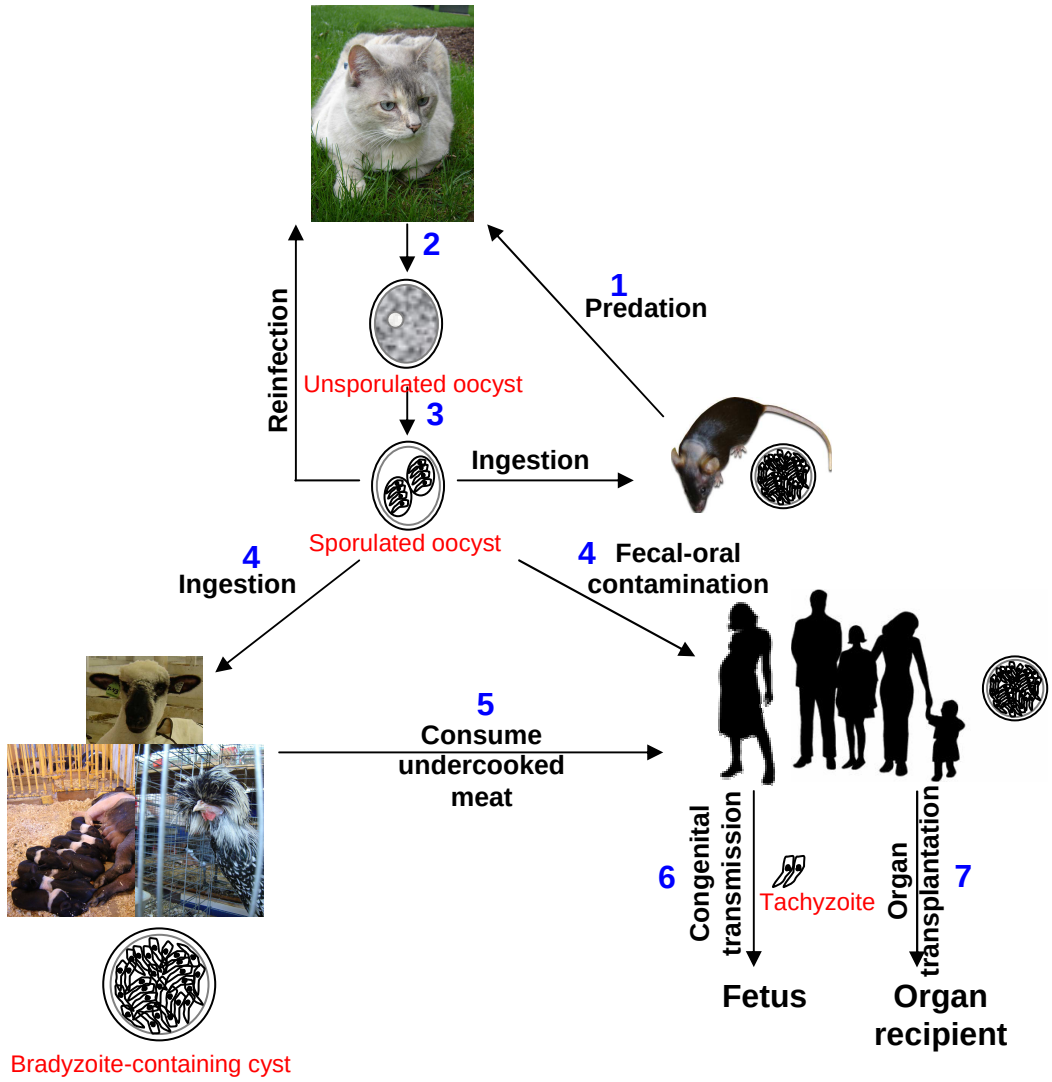

Figure 1.1. Life cycle of *Toxoplasma gondii*. Cat and feline family members are definitive hosts. They become infected by predation and carnivorism of infected rodents harboring dormant bradyzoite-containing cyst in their brains and muscles (1). Gametogenesis occurs in the feline intestinal mucosa forming unsporulated oocysts which later are shed in feces (2). The oocysts become sporulated and infective in the environment (3). *Toxoplasma* can be transmitted to intermediate hosts by ingestion of sporulated oocysts within fecally contaminated food (4), by ingestion of cyst-containing undercooked meat (5), by transplacental infection (6) or by organ transplantation from infected individuals (7).

2. Clinical and veterinary importance of *T. gondii*

The disease caused by *Toxoplasma* infection is referred to as toxoplasmosis. The disease impact raises serious concerns in both medical and veterinary health as a major zoonotic infection. Toxoplasmosis is estimated to be the third leading cause of food-related deaths in the USA following salmonellosis and listeriosis (19). The seroprevalence of *T. gondii* varies highly in human and animals throughout the world. Several factors could contribute to variable infection rates, including meat consumption behaviour, food production practices, and cat feces-related hygiene (20). In humans, the incidence of the infection is as high as 75% in El Salvador and nearly one quarter of adults and adolescents (22.5%) in USA are estimated to be infected (21). In the latter group, persons infected with *T. gondii* were more likely to be infected with *Toxocara* (22). Cases of congenital toxoplasmosis are estimated to be 400–4000 per year in the United States (23). The incidence of *Toxoplasma*-related ocular disease is estimated to be up to 1.26 million cases per year (24). However, the seroprevalence in several countries has been reduced in the past decade, including the USA, 9% seroprevalence was found between 1999-2004 compared to 1988-1994 (22.5%) (25).

In animals, *T. gondii* can cause disease in domestic livestock, companion animals, and wildlife including marine mammals. Toxoplasmosis is one of the major causes of abortion in sheep and goats in many countries, leading to economic and agricultural losses (26, 27). While *Toxoplasma* can cause epidemics of ovine abortion, clinical toxoplasmosis in cattles and horses has not yet been reported (1). Chronic toxoplasmosis is reported to alter behaviour in mice and rats by converting their aversion to feline odors into attraction, hence increasing the susceptibility of infected rodents to predation by cats. The loss of fear appears to be specific since other behaviours are not affected (28, 29). In dogs, clinical toxoplasmosis is reported to be

associated with canine distemper virus (CDV) infection probably due to the massive immunosuppression associated with CDV. However, the incidence of *T. gondii* seems to have decreased because of routine canine distemper vaccination (30). Recently, an increased incidence of toxoplasmosis in a variety of marine mammals such as sea otters, seals, and Atlantic bottlenose dolphins has been reported (31-33). Also, *T. gondii* oocysts were found entrapped within Eastern oysters and were found to be the cause of infection in otters (34). This evidence suggests that *T. gondii* oocysts pollute the marine ecosystem through contaminated freshwater runoff, as the seroprevalence in otters living in areas of maximal freshwater runoff are higher compared to the ones living in the areas of low flow (33).

In humans, acute infection in immunocompetent individuals is usually asymptomatic, although lymphadenopathy can be observed in some cases (35). In major contrast, if *Toxoplasma* infection occurs during gestation or in immunocompromised individuals such as HIV-infected patients or organ/bone marrow transplant patients undergoing immunosuppressive drug treatment, the consequences of infection is life-threatening. In congenital transmission, fetuses become parasite-positive as a result of maternal acquisition of *T. gondii* for the first time during pregnancy. Tachyzoites enter the fetal circulation through placental infection, which can have lethal consequences depending upon the stage of gestation during which infection is acquired. Early maternal infection in the first and second trimester may result in death of the fetus in utero and spontaneous abortion, whereas infection in the last trimester normally results in subclinical disease in the newborn but later development of adverse sequelae such as retinochoroiditis, blindness, hydrocephalus and mental retardation (36-38). However, when infection occurs in seropositive women during pregnancy, the risk to the fetus is minimal (39, 40). In immunocompromised individuals, reactivation of cysts during

chronic infection causes severe toxoplasmic encephalitis which is the most common manifestation in AIDS patients (41). In addition, seronegative patients may acquire *Toxoplasma* through transplanted organs of seropositive donors (42)

T. gondii infection can be diagnosed indirectly by serological methods or directly by detection of the parasite. After the first two weeks of infection and throughout the lifetime of the infected host, immunoglobulin G (IgG) can be detected by several techniques including the Sabin-Feldman dye test, IgG avidity test and agglutination, immunofluorescent antibody test, and ELISA (43-48). For IgM detection, the IgM immunosorbent agglutination assay (ISAGA) is useful for diagnosis of congenital toxoplasmosis in the cord blood or infant serum, since the maternal IgM molecule is too large to cross the blood-placenta barrier to the fetus (49, 50). For direct detection of the parasite, PCR of the highly repetitive B1 *Toxoplasma* gene has been developed to detect prenatal toxoplasmosis in amniotic fluids (51).

Standard therapy for toxoplasmosis is the combination of pyrimethamine, sulfadiazine and folinic acid. Spiramycin is recommended for use during maternal infection especially during the first and second trimester, or in combination with pyrimethamine/sulfadiazine for treatment during the third semester (18, 52). However, development of new and better anti-*Toxoplasma* drugs is required to overcome drug hypersensitivity reactions that occur in some patients (53).

The development of effective anti-*Toxoplasma* vaccines is an area of ongoing interest. Experimental vaccines have been developed using the parasite protein SAG-1 (p30) as well as the secreted microneme protein MIC3 (54-56). The trend in vaccine development for this parasite is centered on enhancing T helper type 1 (Th1) responses

by producing DNA vaccines or by genetic manipulation of parasite genes to generate attenuated strains that induce life-long immunity similar to that induced during the natural infection (57, 58). However, no commercially available vaccine has been succesfully approved for use in humans yet. The only vaccine licensed is attenuated live tachyzoite S48 strain for use in sheep to reduce the rate of abortion (59).

3. Immunology of *T. gondii* infection

Toxoplasma is well known for its ability to elicit robust protective Th1 cell-mediated immune responses characterized by high amounts of interferon-γ (IFN-γ). Loss of the IFN-γ protective response causes reactivation of cysts in chronically infected immunocompromised hosts during both human infection and in experimental animal models (60, 61). The development of toxoplasmic encephalitis during chronic infection was demonstrated by simultaneous depletion of CD4 and CD8 T cells, implicating a requirement for both cell types to prevent reactivation of chronic infection (62).

Although cell-mediated immunity is essential in disease resistance to *T. gondii* infection, humoral immunity also plays a role. In the absence of circulating antibody (Ab), μMT (B-cell deficient) mice survive acute infection but succumb later with high parasite burden in the brain and lung. Administration of anti-*T. gondii* IgG prolongs their survival suggesting that protective antibodies are important in increased survival (63). Support for this view comes from another study revealing a role for IgM in limiting parasite dissemination during acute *T. gondii* infection by utilizing IgM knockout mice (64).

Th1 response initiation has been much studied in the context of *Toxoplasma* infection. Early induction of interleukin-12 (IL-12) is an important trigger for IFN-γ-dependent control of parasite replication and is required to promote disease resistance (Fig. 1.2). The importance of IFN-γ, released from $CD4^+$ and $CD8^+$ T lymphocytes, in mediating resistance to *T. gondii* infection has been established by both antibody-mediated depletion and adoptive transfer studies (62, 65-68). In the absence of endogenous IFN-γ, the IL-12 response is not impaired, but the animals rapidly succumb to the infection with uncontrolled parasite replication (69). On the other hand, in IL-12 knockout mice, IFN-γ production is impaired and the animals rapidly succumb to infection. These results provide evidence that IL-12 is necessary in vivo to drive production of IFN-γ, which in turn allows animals to control the parasite and survive infection (70, 71). Direct proof that these cytokines mediate protection come from studies showing that administration of recombinant IL-12 and IFN-γ prolongs survival of infected mice (72, 73). In addition to its role during initiation of immunity, IL-12 is required for long-term maintenance of IFN-γ-dependent resistance against *T. gondii* infection (60).

Early studies employing *severe combined immunodeficiency* (SCID) mice that lack T and B cells led to the discovery that natural killer (NK) cells were also a source of IL-12-dependent IFN-γ during infection with *Toxoplasma* and other microbial pathogens such as *Listeria monocytogenes* and *Leishmania major*. For *T. gondii*, while NK cells can provide limited early protection in the absence of T lymphocytes, mice ultimately succumb to chronic infection (66, 74, 75).

Dendritic cells (DCs), macrophages and neutrophils are innate immune cells responsible for IL-12 production in response to *T. gondii* infection (76). Murine DCs

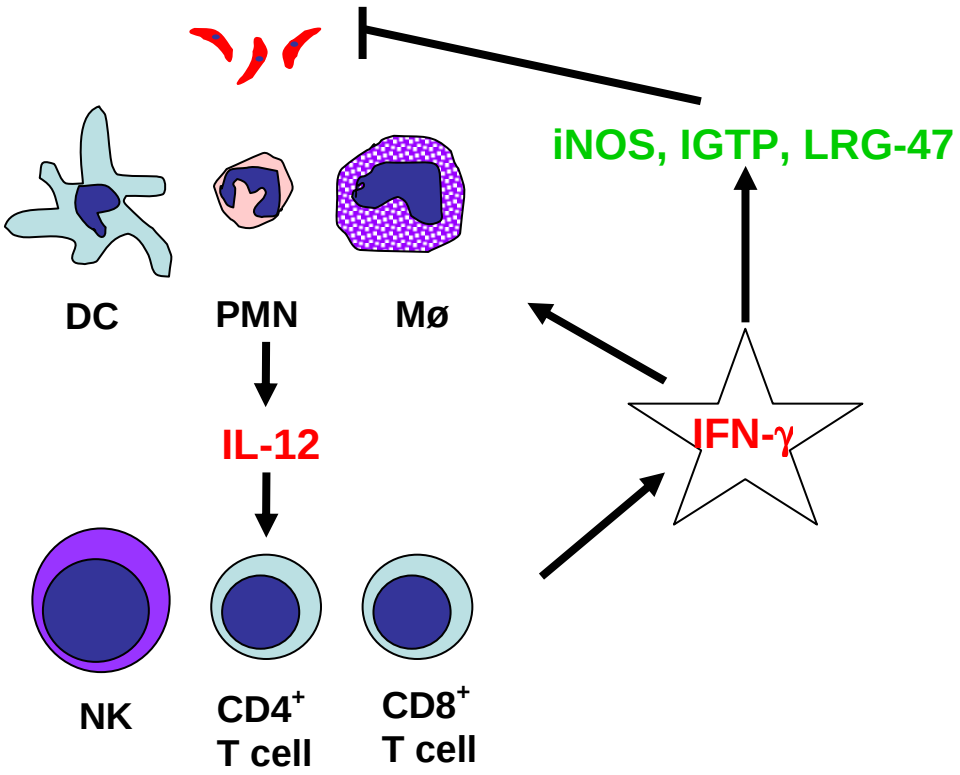

Figure 1.2. Immune response during *T. gondii* infection. Upon recognition of parasites, innate immune cells including dendritic cells (DC), neutrophils (PMN), and macrophages (Mø) release interleukin-12 (IL-12). This cytokine activates natural killer cells (NK), $CD4^+$ and $CD8^+$ T lymphocytes to produce interferon-gamma (IFN-γ) which, in turn, promote anti-toxoplasma effects through generation of nitric oxide, and induction of p47 GTPases including IGTP and LRG-47.

are believed to be the major source of *T. gondii*-induced IL-12 production in vivo without the need of IFN-γ priming or costimulation (77, 78). Studies with *Toxoplasma*-infected IL-12-enhanced green fluorescent protein (EGFP) reporter mice support this model (Egan and Denkers, unpublished). However, CD40/CD40L interaction is required for *T. gondii*-mediated IL-12 production in human DCs (79). Upon parasite recognition, DCs have been implicated in both initiating the IL-12-mediated IFN-γ response and presentation of parasite-derived antigen to MHC-restricted T cells. Infection with *T. gondii* resulted in DC activation based on high expression of activation-associated molecules including MHC class II, CD80, CD86, and CD40 on both $CD8\alpha^-$ and $CD8\alpha^+$ splenic DC and IL-12 production (80). There are several subsets of DCs identified. Recent evidence suggests that plasmacytoid DCs (usually associated with virus-induced production of Type 1 IFN) also produce high level of IL-12 dependent on Toll-like receptor (TLR) 11 (81). Also, IFN-γ-activated dendritic cells have been shown to serve as effector cells by inhibiting replication of *Toxoplasma* (82). Thus, in response to *Toxoplasma*, DC can produce IL-12, present antigen to T cells, and may be able to directly kill the parasite.

Macrophages are also an important source of IL-12 production upon parasite recognition hence bridging the innate and adaptive immunity (72, 83). IFN-γ strongly activates macrophages to become potent effector cells with high level antimicrobicidal activity. Activated macrophages are capable of engulfing pathogens and rapidly degradating them in phagolysosomes. Nitric oxide (NO) production is induced in activated macrophage against *Toxoplasma* in vitro whereas inducible nitric oxide synthase (iNOS)-deficient macrophages displayed defective microbicidal activity. However, in vivo, iNOS-deficient mice survive acute infection and control parasite growth at the site of inoculation suggesting NO-independent mechanisms operating

against the parasite during early infection (71). While this result clearly indicates that NO is not required for control of acute infection, the same study showed that iNOS knockout mice were susceptible to chronic infection. Subsequently, members of the IFN-γ-induced family of p47 GTPases have been implicated in resistance to intracellular parasite infection. The p47 GTPases IGTP and LRG-47 are required for resistance to acute *T. gondii* infection in vivo since IGTP- or LRG-47-deficient mice succumbed with kinetics similar to those of IFN-γ knockout mice (84, 85). IGTP- or LRG-47-deficient macrophages exhibited attenuated IFN-γ-induced inhibition of *T. gondii* growth indicating a role for these two molecules in control of parasite growth in macrophages (86). While these molecules are clearly important in controlling parasite survival in vitro and in vivo, the mechanisms underlying these effects are unknown.

Despite the fact that macrophages possess strong antimicrobial activity, these innate immune cells serve as a perfect niche for intracellular *Toxoplasma* replication. There are several parasite-mediated subversion mechanisms that are likely to contribute to enhanced survival of *Toxoplasma* inside infected non-activated macrophages (87), and there is evidence that the parasite hijacks these cells for use as “Trojan horses” to establish infection in the host (88). Macrophage proinflammatory cytokine production is actively suppressed by infection with *Toxoplasma*, and this may allow time for the parasite to replicate and disseminate to other cells. When macrophages are infected with *T. gondii*, production of IL-12 and tumor necrosis factor (TNF)-α are suppressed within 24 hr in part through the blockade of nuclear factor κB (NFκB) activity (89). *T. gondii* also down-regulates major histocompatibility complex (MHC) II gene expression and antigen presentation in bone-marrow derived macrophages by interfering with IFN-γ-induced signal transducer and activator of transcription (STAT)

1 nuclear translocation (90, 91). Furthermore, *T. gondii* interferes with pathways leading to apoptosis (92). For example, the parasite exploits host G_i-protein-dependent phosphatidylinositol (PI) 3-kinase signaling to prevent induction of apoptosis in infected macrophages (93). The ability to trigger strong innate and adaptive immue responses, yet at the same time suppress certain immune functions in infected cells is likely to be key to success of *Toxoplasma* as a parasitic microorganism.

4. Immunopathology during *T. gondii* infection: Induction and prevention

Toxoplasma induces strong Th1 cytokine responses resulting in parasite killing, and long-term immunity to reinfection. However, in certain cases uncontrolled production of proinflammatory cytokines triggered by infection can cause severe immune-mediated pathology in addition to tissue damage associated with parasite replication and lytic egress (94).

Several studies demonstrate that $CD4^+$ T cells are a major contributor to immunopathology during *Toxoplasma* infection. Early studies showed ameliorated pathologic lesions in the brain following antibody-depletion of $CD4^+$ T lymphocytes during chronic toxoplasmosis (95). Further support for a role for $CD4^+$ T cells comes from an ocular toxoplasmosis model indicating mild inflammatory responses with low parasite burden in the eyes of CD4-deficient compared to WT mice after intraocular *T. gondii* infection (96). The immunopathogenic role of $CD4^+$ T cells was further demonstrated by oral infection of C57BL/6 mice with *T. gondii*. In this case, mice develop fatal ileal necrosis dependent upon $CD4^+$ T cell IFN-γ production (97). In addition to IFN-γ, TNF-α and nitric oxide were found to contribute to gut pathology based on elevated mRNA levels of TNF and iNOS in $CD4^+$ lamina propria mononuclear cells of infected mice as well as studies in iNOS and TNF Receptor

knockout mice (98). Furthermore, lamina propria $CD4^+$ T cells synergize with intestinal epithelial cells to induce inflammatory chemokines including macrophage-inflammatory protein (MIP)-2, monocyte chemoattractant protein (MCP)-1, MCP-3, MIP-1α, MIP-1β and IFN-γ inducible protein (IP)-10 suggesting that $CD4^+$ T cells and enterocytes interact to promote inflammation in the intestinal mucosa (99). Together, these findings emphasize the adverse effect of proinflammatory cytokines and chemokines, in the pathogenesis of Th1 immunopathology during *Toxoplasma* infection.

As an early IFN-γ producer during acute *T. gondii* infection, NK cells may also play a role in immunopathology during *Toxoplasma* infection. A recent study revealed the importance of chemokine receptor CCR5 for immune-mediated pathology and this was associated with NK cell trafficking during *Toxoplasma* infection. In the absence of CCR5, hepatic and ileal tissue architecture from infected mice was less affected relative to WT mice, and this was associated with a defective NK cell influx into infected tissue. Adoptive transfer of NK cells from WT into naive $CCR5^{-/-}$ mice orally challenged with *T. gondii* results in extensive intestinal necrosis, strongly arguing for an immunopathogenic role of NK cells via CCR5-mediated recruitment (100). Moreover, there is evidence that NKT cells participate in pathological inflammatory responses during *T. gondii* infection in the intestine because NKT cell-deficient mice display resistance in lethal ileitis development (101). Thus, in addition to $CD4^+$ T cells, NK and NKT cells are also implicated in *T. gondii*-mediated immunopathology.

In addition to IFN-γ, TNF-α, NO and IFN-γ-induced chemokines, IL-12 and IL-18 are thought to contribute to immune-mediated gut pathology. Thus, treatment of mice with anti-IL-12- or IL-18 antibody following *T. gondii* oral infection prevents ileal necrosis.

However, IL-12, but not IL-18, seems to play the dominant role in controlling parasite replication as, higher parasite burdens and early death are observed in IL-12p35/p40, but not IL-18 knockout mice (102).

In several key aspects, *T. gondii*-induced immunopathology resembles human inflammatory bowel diseases in which proinflammatory cytokines triggered by intestinal flora are thought to play an important role (103). Recent data reveal the contribution of commensal intestinal microflora to ileal inflammation during *Toxoplasma* oral infection. Gnotobiotic mice, in which cultivable gut bacteria were eliminated, do not develop ileitis following *T. gondii* infection. This suggests that endogenous bacteria exacerbate development of gut immunopathology triggered by *T. gondii* (104). Subsequently, TLR4 signaling was identified as a key event in *E. coli* recognition following *T. gondii* infection based on improved gut pathology in $TLR4^{-/-}$ mice as well as polymyxin B treatment to eliminate bacterial lipopolysaccharide in WT mice (105). Thus, the bacteria-host interaction appears to be an important player in cytokine-dependent immunopathology triggered by *Toxoplasma*.

While the above studies clearly show that infection can result in overproduction of proinflammatory cytokines resulting in immunopathology, it is also clear that the host possesses immunoregulatory mechanisms to prevent immune-mediated pathology during *T. gondii* infection. The major anti-inflammatory mediators include the cytokines IL-10, IL-27, TGF-β, and the lipid mediator lipoxin. IL-10 is produced by macrophages, DC, B cells, Th2 cells and regulatory T cells (106). The significance of IL-10 as an immunomodulatory cytokine during *T. gondii* infection was discovered by infecting $IL\text{-}10^{-/-}$ mice intraperitoneally with *Toxoplasma*. $IL\text{-}10^{-/-}$ mice succumb within 2 weeks and death is associated with extensive liver necrosis as well as highly

elevated serum IL-12, IFN-γ and TNF-α. Depletion of $CD4^+$ T cells rescues the knockout mice suggesting that IL-10 is dependent upon this cell type (107). There is also evidence that IL-10 is involved in protection against gut pathology in *Toxoplasma* resistant mice such as the BALB/c strain, because BALB/c background IL-10 knockout mice develop intestinal pathology that is similar to the C57BL/6 strain (108). The protective role of IL-10 was also demonstrated in an ocular toxoplasmosis model. In common with findings in the gut of $IL\text{-}10^{-/-}$ mice, intracameral injection with *Toxoplasma* induces cellular infiltration and necrosis in the eye tissues of both mouse strains that is increased in severity in the absence of IL-10 (109). Collectively, these studies have led to the view that IL-10 is the major anti-inflammatory cytokine that protects against immunopathology during *Toxoplasma* infection.

Nevertheless, other cytokines play a similar role. IL-27 has recently been identified as an important regulator of inflammatory cytokines during infection with *T. gondii* and other microbial pathogens. Although this molecule can play a role in inducing Th1 responses in certain situations, for *Toxoplasma* infection, IL-27 is required to prevent toxoplasmic encephalitis. Thus, in IL-27 Receptor knockout mice, the recently identified Th17 T cell subset emerges as a mediator of IL-17-dependent pathology in the central nervous system (110).

In addition to IL-10 and IL-27, TGF-β plays role in preventing immunopathology during oral *Toxoplasma* infection. Intraepithelial lymphocytes (IELs) serve as a major source of this cytokine. Adoptive transfer of primed IELs prevents the mice from severe hemorrhagic enteritis post challenge with *Toxoplasma*. In addition, depletion of TGF-β with antibody results in ileitis in normally resistant mouse strains. Also, co-culture of antigen-primed IELs with enterocytes in the presence of neutralizing TGF-β

antibody results in increased enterocyte inflammatory chemokine production supporting a role of TGF-β in downregulating intestinal pathology (111).

Lipoxin A_4 (LXA_4), an eicosanoid, is produced during acute *T. gondii* acute infection. This molecule inhibits parasite-induced IL-12 production by inducing a refractory state in DCs so that they are unable to generate IL-12. Infection of mice deficient in 5-lipoxygenase, an enzyme required for LXA_4 biosynthesis, results in extensive immunopathology indicating an anti-inflammatory role of lipoxin (112, 113). Thus, while proinflammatory cytokines are needed to control *T. gondii* and survive infection, the host has multiple mechanisms to regulate production of these potent mediators that can themselves be lethal when overproduced.

5. Neutrophil biology and role during *Toxoplasma* infection

Neutrophils are major antimicrobial effectors in the innate immune system. Paul Ehrlich first demonstrated this cell type based on staining of multilobular nuclei and cytoplasmic granules in 1905. Given the name by neutral cytoplasmic staining with basic hematoxylin and acid eosin dyes, neutrophils can be distinguished from other granulocytes in which eosinophil and basophil granules are stained in red and blue, respectively (114). Neutrophils are the most abundant leukocytes in the blood. Although they are terminally differentiated and possess a relatively short lifespan in the circulation, vast numbers of polymorphonuclear leukocytes (PMNs) exit the bone marrow to enter the circulation, followed by apoptotic death 8-20 hr later. Nevertheless, several inflammatory cytokines such as TNF-α, IFN-γ, IL-6, G-CSF prolong their lifespan in vitro, suggesting that these cells may be more long-lived during infection and inflammation in vivo (115).

PMNs serve as the first line of defense by rapidly migrating from the blood circulation to the site of infection where they adhere to local capillaries. To transmigrate across the endothelial barrier into the tissues requires that vascular endothelium upregulates expression of E-selectin in response to inflammatory signals. This provides interaction with L-selectin on the neutrophil surface promoting rolling of PMN along the endothelium. The β_2-integrin CD11b/CD18 on neutrophils further engages endothelial adhesion molecules such as ICAM-1 rendering firm adhesion and initiating transmigration (116, 117).

Neutrophil development is tightly regulated. Differentiation and maturation of PMNs takes place in bone marrow originating from pluripotent hematopoietic stem cells. These differentiate into myeloid progenitor cells that are committed to the neutrophilic lineage. The different maturation stages leading to mature neutrophils are, in progressive order of differentiation: myeloblast, promyelocyte, myelocyte, metamyelocyte and band neutrophil which then give rise to mature neutrophil. Production of PMNs is stimulated by granulocyte colony-stimulating factor, G-CSF (118). During myeloid cell differentiation, neutrophil granules are formed sequentially, a process initiated during the early promyelocyte stage and continuing throughout the various stages of neutrophil development.

Neutrophils contain three types of granules termed azurophil (primary), specific (secondary) and gelatinase (tertiary). The granules are classified according to their protein content and differential ability to be exocytosed after neutrophil activation. Gelatinase granules are more readily exocytosed than specific granules. Azurophil granules undergo limited exocytosis in response to stimulation, instead contributing primarily to intracellular degradation of pathogens in the phagolysosome. Azurophil

granules are also referred to as peroxidase-positive granules because they are characterized by a high content of myeloperoxidase. In addition, azurophil granules contain defensins, bactericidal permeability-increasing protein, serine proteases such as neutrophil elastase and cathepsin G. Specific granules and gelatinase granules are peroxidase-negative. The specific granules are composed of several potent antimicrobial substances including lactoferrin, hCAP-18 (a cathelicidin family member), and collagenase (matrix metalloprotease-9). Gelatinase granules are important primarily as a reservoir of matrix degrading enzymes and membrane receptors required during PMN extravasation. As implied by the name, this type of granule predominantly contains gelatinase. In addition to these granules, mature neutrophils contain secretory vesicles, constituting a reservoir of membrane-associated receptors important for the early phase of the neutrophil-mediated inflammatory response. Examples include the β_2-integrin CD11b/CD18, the formyl-methionyl-leucyl-phenylalanine (fMLP) receptor and the lipopolysaccharide (LPS)/lipoteichoic acid co-receptor CD14 (119, 120). Together, the contents of these cytoplasmic granules are important in the antimicrobial arsenal enabling neutrophils to fight microbial infection.

The phagocytic function of neutrophils was discovered by Elie Metchnikoff in 1883. Neutrophils are extremely efficient at engulfing pathogens into phagosomes which fuse with intracellular granules to form a phagolysosome. In the phagolysosomes, microorganisms are killed by a combination of oxygen-independent and oxygen-dependent mechanisms (121). The oxygen-dependent mechanisms involves generation of reactive oxygen species, also known as the respiratory burst. Upon PMN activation, assembly of transmembrane and cytosolic subunits of the NADPH oxidase complex is formed at the phagosomal membrane leading to electron transfer to molecular oxygen.

This process produces superoxide which forms hydrogen peroxide and serves as a substrate for myeloperoxidase to generate hypochlorous acid (122). The latter molecule is thought to be the most bactericidal oxidant produced by neutrophils (123). In humans, the importance of NADPH oxidase has been demonstrated in chronic granulomatous disease patients, because inactive NADPH oxidase renders patients extremely susceptible to bacterial infections.

In addition to degranulation and NADPH oxidase-mediated intracellular killing, it was recently discovered that neutrophil extracellular traps (NETs) contribute to killing of extracellular bacteria and pathogenic yeast (124, 125). NETs, released by neutrophils, contain granule proteins, DNA and associated histones. In sum, phagocytosis, exocytosis of granules or secretory vesicles, production of reactive oxygen species, and NETs are important mechanisms for PMN function in killing microbes.

PMNs are also capable of producing several cytokines and chemokines involved in recruitment and activation of immune effector cells. A number of studies have demonstrated the presence of preformed cytokines and chemokines in PMN, including IL-12 and MIP-2 (126, 127). Stimuli such as LPS, fMLP, bacteria and virus infection stimulate the release of cytokines and chemokines from neutrophils (128). *T. gondii* also triggers cytokine and chemokine production from PMNs (115). For example, after exposure to *Toxoplasma* soluble lysate antigen, PMNs upregulate expression of TNF-α and IL-12 as well as the chemokines MIP-1α and MIP-1β (129, 130). This suggests that PMN may be an important source of inflammatory mediators during early *Toxoplasma* infection.

Many studies have suggested an essential role for neutrophils in the context of bacterial and fungal infection. This is based upon in vivo depletion experiments using an antibody against the Gr-1 molecule that is expressed at high levels on PMNs. During acute *T. gondii* infection, Gr-1-depleted mice succumb to infection within two weeks, and this is accompanied by failure to induce a protective Th1 response (131, 132). The latter observation suggests that neutrophils may play a role in immune response induction, in addition to being directly microbicidal. However, caution is warranted with this interpretation because recent findings reveal that other cell types, including DC and monocyte subpopulations, also express Gr-1 (133).

6. Role of mitogen-activated protein kinase pathways in the immune response

Part of this thesis examines the role of mitogen-activated protein kinases (MAPK) in the immune response to *T. gondii*. In response to a variety of extracellular stimuli, these evolutionarily conserved enzymes relay, amplify, and integrate signals from cell surface receptors leading to an intracellular cascade resulting in transcriptional or post-transcriptional regulation of specific target genes. MAPKs are activated by phosphorylation, whereas MAPK phosphatases reverse phosphorylation and return the MAPK to an inactive state.

The MAPK themselves are the terminal members of a three-component linear kinase cascade (Fig. 1.3). The activation loop of MAPK is activated by dual phosphorylation on a conserved tripeptide Thr-Xxx-Tyr motif by an upstream MAPK kinase (MKK or MAP2K). The MAP2K is activated by dual phosphorylation of an upstream kinase termed MKKK or MAP3K. Different upstream signals can lead to activation of MAP3K (134, 135).

Three major families of MAP kinases have been identified. These are the p38 MAP kinases (p38α, p38β, p38γ, p38δ), the extracellular signal regulated protein kinases (ERK1, ERK2), and the c-Jun N-terminal kinases (JNK1, JNK2, JNK3). A fourth MAPK, ERK5, was later identified (136). These MAPKs are activated by specific MAP2K: MEK1/2 for ERK1/2, MKK3/6 for p38, MKK4/7 for JNKs. In turn, these MAP2K can be activated by a wide variety of upstream MAP3K, hence creating the diversity of MAPK signaling. In addition to this prototypic cascade of MAPK activation, p38α MAPK can be autophosphorylated in dependence upon interaction with TAB1 (137).

Despite their diverse range of activating stimuli, MAPK signaling cascades confer remarkable response specificity. Several mechanisms have been implicated in this unique property (135). First, scaffold proteins have been identified for each MAPK family (138). These proteins sequester kinases and insulate them from inappropriate activation, hence enhancing signaling fidelity. Also, stimulus-selective phosphorylation of scaffold proteins determines the components that can bind. For example, JIP-1, a scaffold protein for the JNK pathway, ties together MLK1 (a MAP3K), MKK7 (a MAP2K), and the MAPK JNK into a specific signaling module (139). Second, MAPK have specificity-determining motifs, in addition to the active site, to bind with other proteins through highly specific docking sites composed of a docking groove, an acidic common docking (CD) and a Glu-Asp (ED) pocket. These allow the docking domain (D) of MAPK-interacting proteins to interact with the MAPK more specifically. Such proteins include scaffold proteins, substrates, MAP2Ks and phosphatases. The crystal structure of p38 MAPK bound to docking domains of a p38 substrate and a p38-activating enzyme clearly demonstrate these

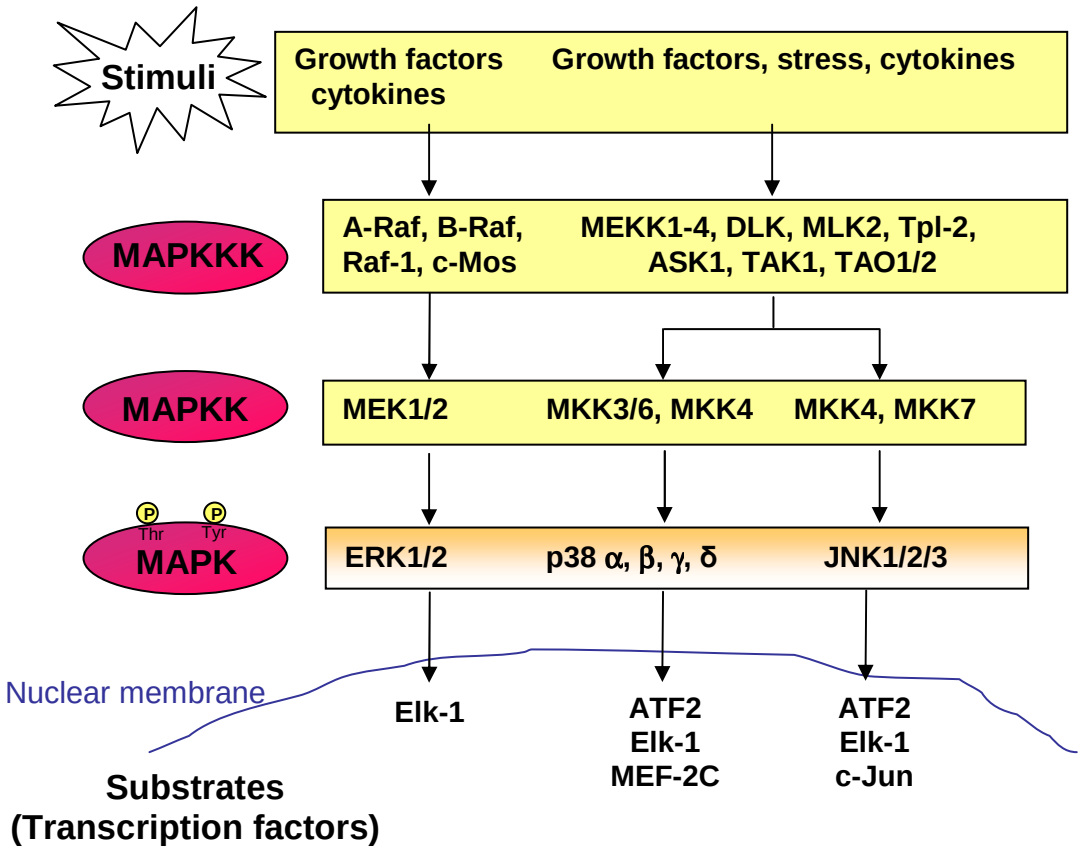

Figure 1.3. Components of mitogen-activated protein kinase (MAPK) signaling pathways. Three families of MAPK include ERK1/2, p38α/β/γ/δ, and JNK1/2/3. A fourth MAPK, ERK5 is not shown. Each MAPK family is composed of three modules activated in series: MAPKKK, MAPKK, MAPK. The activation of MAPK cascades is initiated by diverse stimuli. MAPK is activated by dual phosphorylation on a conserved tripeptide motif Thr-Xxx-Tyr by upstream MAPK, which itself is phosphorylated by a diverse group of MAPKKK. Upon MAPK activation, it is translocated into the nucleus to phosphorylate its downstream substrate.

important sites (140). Another mechanism that provides exclusive MAP kinase activation is sequential physical interaction between members of a given cascade. For example, JNK1/2 is bound by the N-terminal extension of MKK4 which also interacts with the catalytic domain of MEKK1. Each interaction is disrupted on activation of the downstream kinase (141).

MAPKs play diverse roles in a wide variety of cell functions including cell proliferation, cytokine production, cell migration, cell survival and apoptosis. In general, it is believed that ERK activation is mainly involved in cell survival and proliferation, contributing to neoplasia if the activation is dysregulated. In contrast, p38 and JNK MAPK are linked to induction of apoptosis and inflammatory responses, implicating them in immune regulation (142). However, the actual roles of each MAPK cascade are complex being highly cell type- and context of stimuli-dependent. Targeted disruption of MAPK modules through genetic manipulation and use of inhibitors provides specific insights into MAP kinase functions (135).

The role of MAPK is a growing area of interest in both innate and adaptive immunity (142). In the innate immune response, for example, MKK3 and p38 mediate IL-12 production in LPS-activated macrophages and CD40L-activated DCs (143). In the adaptive immune response, ERK, p38, and JNK have been shown to play several roles including early thymocyte development, $CD4^+$ and $CD8^+$ T cell activation and differentiation (144). Also, the p38 signaling cascade has been implicated in Th1 differentiation and cytokine production (145).

JNK is activated primarily by cytokines and exposure to environmental stress such as UV irradiation, chemicals, heat or osmotic shock. Activated JNK molecules

phosphorylate substrates including transcription factors c-Jun and AP-1. Other substrates of JNK includes JunB, JunD, ATF2 and antiapoptotic bcl-2 family members (146, 147). In common with p38, JNK is implicated in T-cell activation and IL-2 expression, as demonstrated in Jurkat T cells (148). Furthermore, $CD4^+$ T cells from JNK2 knockout mice fail to differentiate into Th1 cells because of a defect in IFN-γ production during early stages of differentiation, suggesting a role of JNK2 in Th1 development (149).

7. Toll-like receptors and their role during *T. gondii* infection

The hallmark of host immunity is the ability to distinguish self from non-self to combat invading pathogens. To initiate host defense, innate immune cells utilize pattern recognition receptors (PRRs) that detect conserved structures among microorganisms. These molecular signatures are named pathogen-associated molecular patterns (PAMPs). The molecules that possess PAMPs usually have an essential role for microbial survival. In mammalian cells, three major PRRs have been identified. These are the Toll-like receptors (TLRs), NLR family (NOD and NALPs) (150) and RNA helicase retinoic acid inducible gene-I (RIG-I) (151). The latter two are intracellular recognition receptors detecting microbial components in the cytoplasm such as peptidoglycan or double-stranded RNA, respectively.

TLRs are mammalian homologs of the Toll protein that was originally discovered as a determinant of dorsal-ventral polarity during embryogenesis and later as a molecule involved in anti-fungal defense in *Drosophila* (152). To date, 11 human and 13 mouse TLRs have been identified. Each TLR recognizes distinct PAMPs derived from various microbes, including bacteria, viruses, protozoa and fungi (153-155). TLRs

function as a dimer in PAMP recognition. TLR1 and TLR2 form a heterodimer to sense bacterial triacylated lipopeptides but TLR2 can also heterodimerize with TLR6 to recognize bacterial diacylated lipopeptides. Homodimerization occurs with TLR4, TLR9 and presumably TLR3 and TLR5 to detect LPS, unmethylated CpG motifs, viral dsRNA and bacterial flagellin, respectively (156).

TLRs contain a leucine rich repeat domain and a key cytoplasmic signaling region, termed the Toll/IL-1 receptor (TIR) domain (Fig. 1.4). Upon ligand recognition by TLR, downstream signaling molecules including the adaptor proteins MyD88, TIRAP/Mal, TRIF/TICAM1, and TRAM/TICAM2 mediate the coupling to several protein kinases (157). This leads to activation of transcription factors in the NFκB and MAPK pathways responsible for proinflammatory cytokine and chemokine production as well as co-stimulatory molecule expression on antigen-presenting cells.

Studying the role of TLRs in the context of infectious diseases can be achieved by targeted deletion of single or multiple TLRs as well as by using MyD88$^{-/-}$ mice. The latter mouse strain has been used extensively to investigate the role of TLRs in response to infection since most of TLRs (except TLR3) utilize this adaptor protein. However, to interpret results with MyD88 knockout mice, caution should be exercised since MyD88 is also a component of the IL-1R family signaling pathway, rendering these mice impaired in IL-1- and IL-18-mediated responses (158). Nevertheless, the role of TLR signaling has been implicated in several models of infectious disease. For example, TLR2, TLR4 and TLR9 are involved in recognition of *Mycobacterium tuberculosis*, *Candida albicans*, and *Trypanosoma cruzi* through recognition of pathogen lipid associated molecules (159). For the malaria parasite *Plasmodium falciparum*, recognition appears to occur through TLR9 interaction with hemozoin-

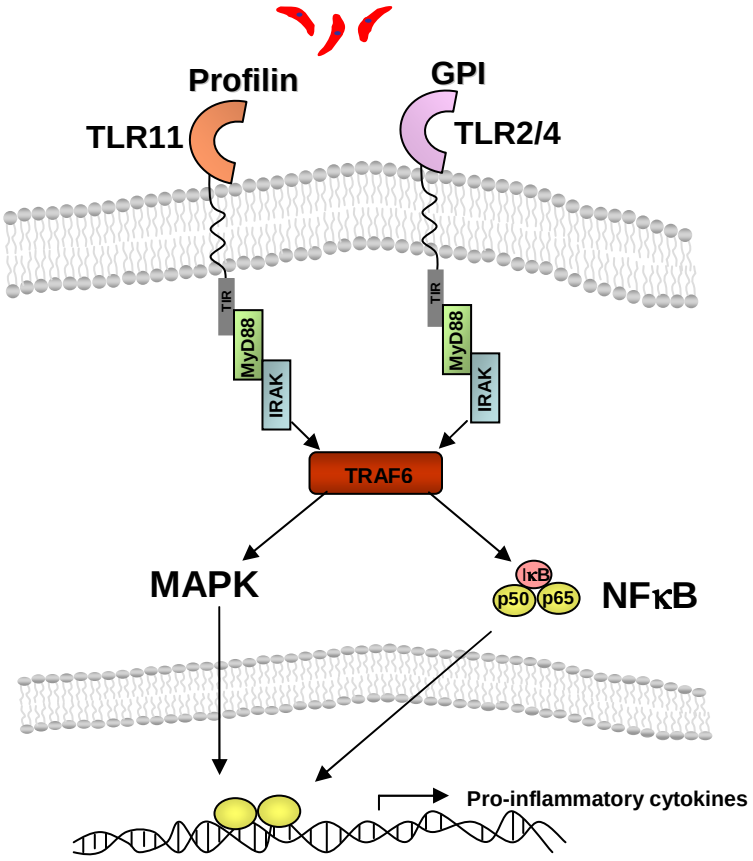

Figure 1.4. Toll-like receptor (TLR) recognition of *T. gondii*. TLR11 and TLR2/4 on the surface of innate immune cells specifically recognize *T. gondii* pathogen associated molecular pattern (PAMPs): profilin and glycosylphosphatidylinositol (GPI) protein anchors, respectively. These TLRs function as a dimer in PAMP recognition. The ligands are detected by interacting with the N-terminal leucine-rich repeats of TLRs. TLR-ligand interaction triggers the C-terminal Toll-interleukin-1 receptor (TIR) domain-dependent association with MyD88 and other adapter proteins (not shown). This association recruits members of the IL-1 receptor-associated kinase (IRAK) 1 and 4 which are sequentially phosphorylated, causing them to dissociate from the receptor complex, then associate with TRAF6. This activates NFκB and mitogen-activated protein kinase (MAPK). TLR activation results in expression of pro-inflammatory cytokine genes such as IL-12, TNF-α, and IL-6.

bound malaria DNA (154, 160).

The role of TLRs in the response to *T. gondii* has also been studied. In the absence of MyD88, intraperitoneally-infected mice succumb to infection associated with high parasite load in the peritoneal cavity and defective IL-12 and IFN-γ responses (161). Furthermore, interleukin-1β converting enzyme $(ICE)^{-/-}$ mice, lacking functional IL-1 and IL-18, are able to control *T. gondii* replication and survive i.p. infection indicating that signal transduction through MyD88 is mediated by TLRs, but not IL-1R family members (162). Also, during high parasite dose i.p. infection, $TLR2^{-/-}$ mice succumb to *Toxoplasma*, suggesting a role for TLR2 (163). Subsequently, parasite surface glycosylphosphatidylinositols (GPIs) were identified as *T. gondii* ligands for TLR2 and TLR4 (164). Furthermore, TLR9 knockout were found to be resistant to Toxoplasma-induced pathology during oral infection, although whether this was due to altered responses to endogenous gut flora or the parasite itself was not determined (165).

In addition to the involvement of TLR2, 4, and 9 in the response to *Toxoplasma*, recent studies reveal that a parasite profilin-like protein (TgPRF) serves as a ligand for TLR11 (166). This TLR, which is not expressed in humans, was previously demonstrated to recognize uropathogenic bacteria (167). In the absence of TLR11, mice display increased susceptibility and impaired IL-12 production. In addition, TgPRF was further demonstrated to be a TLR11-dependent IL-12 inducer by using a genetically engineered *Toxoplasma* conditional knockout of TgPRF (168). Collectively these studies show that multiple TLRs play a role in immunity and disease resistance during *Toxoplasma* infection.

8. Brief outline of dissertation research

Toxoplasma infection induces strong Th1 immune responses, a response that is largely believed to be dependent upon induction of IL-12. During acute *Toxoplasma* infection, DCs, macrophages and PMNs are major sources of IL-12. Although TLR adaptor protein MyD88 is required for PMN IL-12 production during *T. gondii* infection, the downstream MAPK signaling molecules involved have not been investigated.

JNKs have been implicated in both positive and negative regulation of IL-12 production. **Chapter 2** reports the finding that JNK2 is the major JNK/SAPK isoform expressed in mouse neutrophils. In addition, this chapter demonstrates that PMN JNK2 is required for IL-12 and MCP-1 production in response to in vitro *T. gondii* stimulation. These findings prompted me to investigate the role of JNK2 in vivo during *Toxoplasma* infection, as described in **Chapter 3**. To my surprise, I found that JNK2 plays role in *T. gondii*-mediated immunopathology, and in negative regulation of IL-12 in vivo. Another signaling molecule that is important in innate immunity, and in particular early IL-12 production, is TLR adaptor protein MyD88. **Chapter 4** describes immune responses occurring during oral infection in the absence of MyD88. We found that MyD88 signaling is important in PMN recruitment and p47 GTPase Irgm3/IGTP expression in intestinal mucosa. Interestingly, while in vivo IL-12 production was heavily dependent upon MyD88, IFN-γ production reached normal levels and mice developed strong protective immunity. Finally, **Chapter 5** summarizes the results and discusses the relevance of the above findings.

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CHAPTER 2

MOUSE NEUTROPHILS REQUIRE JNK2 MAPK FOR *TOXOPLASMA GONDII*-INDUCED IL-12p40 AND CCL2/MCP-1 RELEASE*

* Reprinted from Woraporn Sukhumavasi, Charlotte E. Egan and Eric Y. Denkers. Mouse neutrophils require JNK2 MAPK for *Toxoplasma gondii*-induced IL-12p40 and CCL2/MCP-1 release. *J. Immunol*. 179:3570-3577.

Abstract

The MAPK family member c-Jun N-terminal kinase (JNK)/stress-activated MAPK (SAPK) is involved in extracellular stress and proinflammatory cytokine responses, including production of cytokines such as IL-12. The JNK1 and 2 isoforms are widely expressed, but JNK3 is largely restricted to tissues of the brain, testis and heart. In this study, we focus on mouse neutrophils, a cell type in which JNK/SAPK expression and activity has been given little study. We used Western blot analysis to examine expression patterns of JNK/SAPK in wild-type and *Jnk2*$^{-/-}$ polymorphonuclear leukocytes (PMN). Surprisingly, neutrophils displayed a major deficiency in JNK1 expression, in contrast to macrophages that expressed high levels of both JNK1 and JNK2 MAPK. JNK1 expression was steadily reduced during the neutrophil maturation in bone marrow. We used PMN infection with the protozoan parasite *Toxoplasma gondii* to determine whether neutrophil JNK2 was functional. The parasite induced rapid JNK2 phosphorylation and intracellular FACS staining demonstrated preferential activation in infected neutrophils. Use of *Jnk2*$^{-/-}$ neutrophils revealed that this MAPK family member was required for PMN IL-12p40 and CCL2/MCP-1 production. The chemotactic response displayed a minor JNK2 dependence but phagocytosis and oxidative burst activity did not require this MAPK. These findings are important because they demonstrate 1) a previously unrecognized unusual JNK expression pattern in mouse neutrophils, 2) JNK2 in PMN is activated by *Toxoplasma* invasion and 3) a requirement for JNK2 in PMN IL-12p40 and CCL2/MCP-1 production in response to a microbial pathogen.

Introduction

Neutrophils are well-known as acute inflammatory cells that rapidly accumulate in high number at sites of infection, largely in response to the chemokine IL-8/CXCL8 in

humans and IL-8/CXCL8-like chemokines in mice (1-3). In the classical view of polymorphonuclear leukocyte (PMN) function, these cells exert anti-microbial activity through phagocytosis, granule exocytosis and production of reactive oxygen intermediates (4). This is followed by neutrophil apoptotic death and phagocytic removal by macrophages. More recently, neutrophil extracellular traps (NETs) composed of chromatin and granule proteins have been reported to contribute to killing of extracellular bacteria (5, 6).

Although PMN possess an abbreviated lifespan under steady-state conditions, their half-life is extended by cytokines including TNF-α, suggesting that the cells can function for extended periods in a proinflammatory milieu (7, 8). Neutrophils themselves also produce proinflammatory cytokines and chemokines that can be released from preformed stores or produced de novo (9-13). The ability of neutrophils to produce immunoregulatory cytokines such as IL-12 and TNF-α suggests that the cells play a role in shaping development of the acquired immune response to infection. Recent studies have found Ag-bearing PMN in secondary lymphoid organs that have the potential to influence generation of Th1 lymphocytes through IL-12 release (14). Increasingly, there is evidence for crosstalk between PMN and dendritic cells (15). Neutrophils infected with the protozoan parasite *Toxoplasma gondii* release TNF-α which, in turn, activates dendritic cells (9, 16). Similar neutrophil-DC interactions occur in humans, and this cross-talk is mediated by direct cell contact and glycosylation-dependent interaction between neutrophil MAC-1 and DC-SIGN expressed by dendritic cells (17). In addition, DC can acquire antigen from neutrophils through direct physical contact for presentation to T lymphocytes (18). These combined observations strongly argue for an immunoregulatory function for neutrophils, in addition to their well-known microbicidal activity (19).

Neutrophils are a source of IL-12 during infection, but little is known regarding intracellular signaling molecules involved in PMN production of this cytokine. During *Toxoplasma* infection, the TLR adaptor molecule MyD88 is required for parasite-induced IL-12 in macrophages, DC and PMN (20). Neutrophils also produce the monocyte chemoattractant CCL2/MCP-1, a response dependent upon MyD88 and TLR2 (21). Members of the MAPK family influence IL-12 production in cells such as macrophages and dendritic cells. In particular, p38 and JNK/stress activated protein kinase (SAPK) promote IL-12 production, although in some cases JNK is reported to negatively regulate IL-12 (22-25). ERKs 1 and 2 have been implicated in negative regulation of IL-12 synthesis in macrophages (22, 23, 26, 27). However, little is known regarding MAPK in mouse neutrophil function.

In this study, we investigated JNK/SAPK function in murine PMN. Of the three JNK isoforms, JNK1 and JNK2 are widely expressed but JNK3 is limited to tissues of the brain, testis and heart (22). Unexpectedly, we found that neutrophil JNK expression is largely restricted to JNK2, unlike macrophages which express both JNK1 and JNK2. Using PMN from *Jnk2*$^{-/-}$ mice, we show a major requirement for JNK2 in neutrophil IL-12 and CCL2/MCP-1 production. Chemotaxis was partially dependent upon JNK2. In contrast, no JNK2 requirement for PMN respiratory burst and phagocytic activity was observed. These results reveal novel aspects of MAPK expression in mouse neutrophils, and they show a critical contribution of JNK2 in PMN production of certain chemokines and cytokines during parasite infection.

Materials and Methods

Mice

Female C57BL/6 mice 6-8 weeks old were purchased from Charles River Breeding Laboratories or Taconic Farms. Homozygous JNK2 knockout mouse (*Mapk9*^{tm1Flv}) breeding pairs were obtained from The Jackson Laboratory. They were maintained in the transgenic mouse core facility at the College of Veterinary Medicine, Cornell University (Ithaca, NY), an institution accredited by the American Association for Accreditation of Laboratory Animal Care.

Parasites

T. gondii tachyzoites of the RH strain and transgenic Yellow Fluorescent Protein (YFP)-expressing RH tachyzoites (kindly provided by D. Roos, University of Pennsylvania) were passaged in human foreskin fibroblasts twice a week in medium consisting of DMEM (Mediatech) supplemented with 1% bovine growth serum (Hyclone), 100 U/ml Penicillin and 100 µg/ml Streptomycin (both from Invitrogen Life Technologies). Tachyzoites freshly egressed from fibroblasts were washed with PBS and resuspended in complete DMEM (cDMEM), consisting of 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 30 mM HEPES (all purchased from Invitrogen Life Technologies), 10% bovine growth serum and 0.05 mM 2-ME in DMEM.

Mouse neutrophil isolation

To obtain neutrophils, mice were intraperitoneally injected with 10% thioglycolate (BD Microbiology Systems), then peritoneal exudate cells (PEC; consisting of 60-70% PMN) were collected 18 h later. Cells were washed and resuspended in PBS. Neutrophils were purified from PEC by continuous Percoll gradient centrifugation.

One hundred percent Percoll (Amersham Biosciences) was adjusted to pH 7.4 with 10 M HCl. The PEC preparation was mixed with the pH-adjusted Percoll at a ratio 1:9, then the mixture was transferred to a 10 ml polycarbonate tube (Beckman Coulter). Ultracentrifugation was performed at 60,650 x *g* for 65 min at 4°C with a 50 Ti rotor (Beckman Coulter). The neutrophil-enriched layer was harvested using a gel loading pipette tip. Cells were washed with PBS and resuspended in cDMEM. More than 90% PMN purity was routinely obtained, as determined by morphology after differential staining (Fisher Scientific). PMN viability, monitored by trypan blue exclusion was >98%. Cells were counted at least three times to ensure accurate quantitation.

Magnetic labeling of bone marrow cells and cell sorting

Mouse bone marrow single-cell suspension was prepared followed by magnetic labelling with CD11b MicroBeads (Miltenyi Biotec). Positive selection for $CD11b^{+}$ cells was performed twice by autoMACS Separator according to the manufacturer's instruction (Miltenyi Biotec). Enriched-$CD11b^{+}$ bone marrow cells were then stained with PerCP-Cy5.5-conjugated anti-Gr-1 mAb and PE-conjugated anti-CD11b mAb (BD Pharmingen). A FACSAria fluorescence-activated cell sorter (BD Biosciences) was used to isolate the bone marrow $CD11b^{+}$ cells into 3 different populations based on the surface expression level of CD11b and Gr-1: $CD11b^{int}$ $Gr\text{-}1^{int}$; $CD11b^{low}$ $Gr\text{-}1^{high}$; and $CD11b^{high}$ $Gr1^{high}$.

Mouse bone marrow-derived macrophage preparation

Macrophages were generated from mouse bone marrow in L929 supernatant containing M-CSF as described previously (28).

Flow cytometry

For intracellular staining of phosphorylated JNK, YFP-expressing tachyzoites were incubated with PEC (15:1 ratio of parasites to cells). At various times, cells were transferred to a 96-well plate and washed with ice-cold PBS containing 1% bovine growth serum and 0.01% sodium azide (FACS buffer). Samples were fixed with 3% paraformaldehyde for 15 min followed by incubation with anti-CD16/32 (2.4G2) to block nonspecific antibody binding to FcγRIII/II. Surface staining for Gr-1 was performed for 15 min with PerCP-Cy5.5-conjugated anti-Gr-1 mAb (BD Pharmingen). For phenotypic analysis of purified neutrophils, cells were co-stained with the following PE-conjugated Ab: anti-F4/80 (Invitrogen Life Technologies), anti-CD11c (BD Biosciences), anti-CD11b (BD Biosciences), anti-MHC class II (eBioscience), anti-CD62L (eBioscience), anti-CD80 (eBioscience), and anti-CD86 (BD Biosciences). For intracellular staining of phospho-JNK, cells were permeabilized with 0.075% saponin in PBS for 30 min followed by blocking with FACS buffer for 10 min. Intracellular staining was accomplished with Alexa Fluor 647-conjugated phospho-SAPK/JNK (T183/Y185) mouse mAb (Cell Signaling Technology) for 30 min at room temperature. Samples were acquired on a FACSCalibur (BD Biosciences) cytometer collecting 10,000 Gr-1^+ events. Data were analyzed using FlowJo software (Tree Star).

Immunoblot Analysis

PMN and tachyzoites were added to a 24-well plate and infection was synchronized by brief centrifugation (550 x *g*, 3 min, 4ºC). After incubation for various times (37ºC, 5% CO_2), supernatants were removed and cell lysates were prepared for immunoblot analysis. Bone marrow-derived macrophages were used as a control for JNK

activation by incubation for 20-30 min with 100 ng/ml LPS (*Escherichia coli* strain 055:B5; Sigma-Aldrich).

For immunoblot analysis, samples were lysed with SDS-PAGE reducing sample buffer and passed through a 27-guage needle three times to reduce sample viscosity. Lysates were boiled for 5 min, run on a 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane. Membranes were blocked with 3% nonfat dry milk (Nestle) in PBS for 20 min. They were probed with a rabbit anti-total JNK polyclonal antibody (Upstate Cell Signaling Solutions) or anti-phospho-JNK according to the manufacturer's recommendation (Cell Signaling Technology). HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology) was used as a secondary Ab. Bands were visualized by developing with luminol chemiluminescent substrate (Cell Signaling Technology) and exposure to autoradiographic film.

Cytokine/chemokine ELISA

For cytokine protein measurement, neutrophils from WT and *Jnk2*$^{-/-}$ mice were resuspended in cDMEM and plated in a 96-well plate. Tachyzoites were added and infection was synchronized by brief centrifugation. The cells were incubated at 37ºC in humidified 5% CO_2 for varying times, then supernatants were recovered for ELISA. Production of IL-12p40 and CCL2/MCP-1 was measured by ELISA as previously described (21).

Multiplex bead immunoassay

Neutrophil supernatants from infected cultures were subjected to Multiplex bead immunoassay according to the manufacturer's instruction (BioSource International). The following 19 mouse cytokines and chemokines were measured using a Luminex

100 instrument (Qiagen): IL-12p40/p70, CCL2/MCP-1, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN-γ-inducible protein 10, KC, MIG, MIP-1α, TNF-α and vascular endothelial growth factor (VEGF).

Oxidative burst assay

PMN were stimulated with PMA (200 ng/ml) and fMLP (10^{-6} M) (EMD Biosciences) in the presence of 0.2% NBT (Sigma-Aldrich). After 30 min (37ºC, 5% CO_2), cells were solubilized with 10% SDS and 0.1 N HCl, then sample OD (570 nm) were measured on a spectrophotometer.

Phagocytosis assay

PMN were incubated (1 h, 37ºC, 5% CO_2) with 1 µm fluorescent yellow-green latex beads (Sigma-Aldrich) that had been pre-opsonized with normal mouse serum for 1 h. Internalization of beads was determined by flow cytometry, using a FACSCalibur (BD Biosciences) flow cytometer and data were analyzed with FlowJo software (Tree Star).

Chemotaxis assay

Assays for chemotaxis used a 96-well chemotaxis chamber (3-µm pore size; Neuroprobe). The assay was set up as described previously (16), using 45,000 neutrophils, the chemoattractants fMLP (10^{-6} M) and KC (100 ng/ml; AbD Serotec) were used in the study. The assay was terminated after 45 min at 37ºC, then the number of migrated cells was enumerated by microscopy.

Statistics

The difference of means was compared using Student's *t* test with $p < 0.05$ considered statistically significant. All experiments were repeated at least 2 times.

Results

Isolation of mouse peritoneal neutrophils

To obtain purified neutrophils for in vitro studies, we elicited peritoneal cell recruitment by i.p. injection of 10% thioglycolate and collected exudate cells 18 h later. PEC were routinely composed of 60-70% neutrophils and 20-25% macrophages, with small numbers of eosinophils, mast cells and lymphocytes (Fig. 2.1*A*). To purify neutrophils, PEC were subjected to ultracentrifugation over a Percoll continuous density gradient. This procedure resulted in cell preparations composed of >90% neutrophils (Fig. 2.1*B*). We performed flow cytometry to further assess the phenotype of the Gr-1$^+$ neutrophil population. As shown in Fig. 2.1*C*, the cells expressed high levels of CD11b and CD62L, but lacked expression of CD11c and MHC class II. The neutrophil population was also negative for F4/80 (data not shown). Interestingly, the cells expressed intermediate levels of costimulatory molecule CD86, and low levels of CD80. Although we are uncertain as to the functional significance of this result, expression of costimulatory molecules by neutrophils isolated from humans has also been reported (29, 30).

Diminished expression of JNK1 in mouse neutrophils

The MAPK family members are implicated in proinflammatory cytokine responses, and we previously reported a requirement for JNK in macrophage IL-12 production (31). Because neutrophils produce IL-12 during microbial stimulation, we assessed the status of JNK1/2 in these cells. Fig. 2.2 shows levels of total JNK in macrophages compared to purified neutrophils. As expected, WT macrophages expressed high levels of 46- and 54-kDa protein corresponding to JNK1 and JNK2. In marked contrast, neutrophils were almost completely devoid of JNK1. JNK3 migrated with a mass of 54-kDa, and therefore comigrated with JNK2. However, using neutrophils

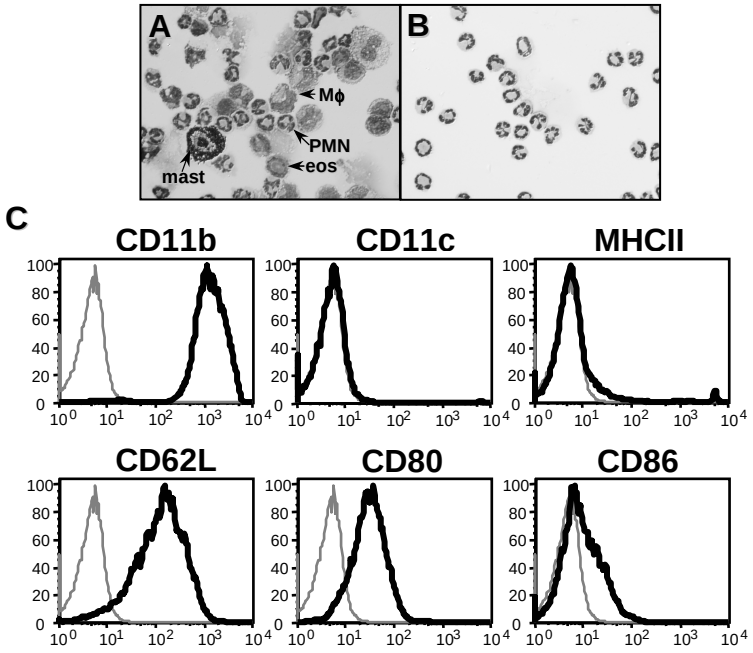

Figure 2.1. Mouse neutrophils from thioglycolate-elicited peritoneal cells before and after purification. *A*, PEC were obtained by peritoneal lavage with ice-cold PBS 18 h after i.p. thioglycolate injection. The cells were comprised of 60-70% PMN, 20-25% macrophages (Mø), low numbers of eosinophils (eos), mast cells (mast) and lymphocytes (data not shown). *B*, PEC were subjected to PMN purification over a continuous Percoll gradient immediately following harvest. More than 90% neutrophil purity was routinely obtained. *C*, Purified neutrophils were stained with anti-Gr-1 Ab and a panel of cell surface markers. Expression of CD11b, CD11c, CD62L, CD80, CD86 and MHC class II (thick black line in each histogram) is shown on the gated Gr-1^+ population relative to staining with isotype controls (thin gray lines in each histogram).

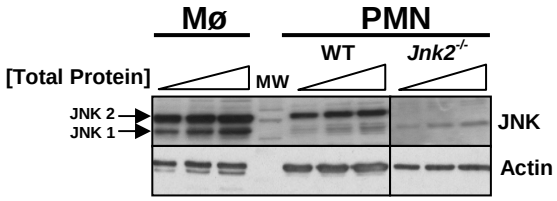

Figure 2.2. JNK2 is the major JNK/SAPK isoform expressed in mouse neutrophils. Total cell lysates of bone marrow-derived macrophage (Mø) and highly purified PMN from WT or *Jnk2*$^{-/-}$ mice were subjected to immunoblot analysis using a JNK-specific polyclonal antibody, and an actin-specific antibody as a protein loading control. The amount of macrophage and PMN protein loaded was 5, 10, 15 µg/lane. Positions of JNK1 and 2 isoforms are marked by arrows. This experiment was repeated twice with similar result. MW, Molecular weight marker.

from *Jnk2*$^{-/-}$ mice, we established that the 54-kDa band corresponded to JNK2 protein (Fig. 2.2).

Down-regulation of JNK1 during neutrophil maturation

We next sought to determine where JNK1 expression was down-modulated during neutrophil differentiation in the bone marrow. PMN and their precursors in bone marrow can be distinguished by morphology and expression of surface markers Gr-1 and CD11b (32). Accordingly, we enriched for CD11b^{+} bone marrow cells using immunomagnetic beads, then sorted cells into 3 different populations consisting of CD11b^{int} Gr-1^{int}, CD11b^{low} Gr-1^{high}, and CD11b^{high} Gr1^{high} populations (Fig. 2.3*A*, *a-c*, respectively). Each population was subjected to Diff-Quick staining (Fig. 2.3*B*). In agreement with previous results (32), the morphology of CD11b^{int} Gr-1^{int} cells was consistent with promyelocyte/myelocyte-stage cells (Fig. 2.3*B*, a) whereas the CD11b^{low} Gr-1^{high} and CD11b^{high} Gr1^{high} displayed morphology consistent with immature PMN and multilobular nucleated mature PMN (Fig. 2.3*B*, b and c, respectively). To examine the expression levels of JNK1 in each population, Western blot analysis was performed on protein lysates from the sorted bone marrow cell populations, as well as on purified peritoneal neutrophils from WT and *Jnk2*$^{-/-}$ mice and bone marrow-derived macrophages for comparison. Fig. 2.3*C* shows a steady decline in JNK1 and JNK2 expression associated with neutrophil maturation. However, based on densitometric analysis, the decrease in JNK1 was more drastic. Thus, the JNK2:JNK1 ratio in promyelocyte/myelocytes, immature PMN, and mature PMN was 1.9, 3.7 and 7.3, respectively. In elicited neutrophils obtained from the peritoneal cavity, the JNK2:JNK1 ratio was 15.8. In contrast, JNK1 was expressed at a higher level in macrophages (JNK2:JNK1 = 1.6) and CD11b^{-} bone marrow cells (JNK2:JNK1 = 1.7) (Fig. 2.3*C*).

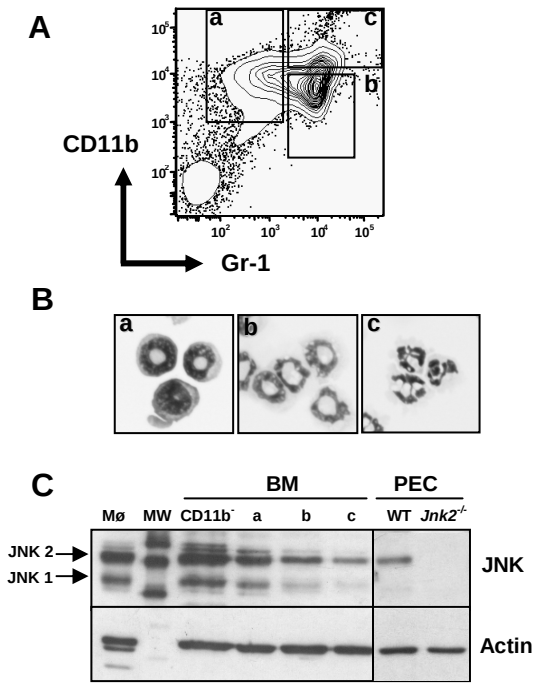

Figure 2.3. JNK expression in wild-type mice during neutrophil maturation. *A*, $CD11b^+$ bone marrow cells were enriched by magnetic sorting then further stained and FACS-sorted based on the expression level of CD11b and Gr-1. The gated populations define: *a*, promyelocytes/myelocytes; *b*, immature neutrophils; and *c*, mature neutrophils. *B*, Sorted populations *a-c* were subjected to cytospin and Diff-Quick staining to determine the cell morphology. *C*, Bone marrow (BM) $CD11b^-$ cells as well as populations *a-c* were subjected to Western Blot analysis probing with JNK polyclonal Ab. These were compared with lysates of bone marrow-derived macrophages (Mø) and WT and *Jnk2*$^{-/-}$ neutrophils isolated from the peritoneal cavity after thioglycolate elicitation. The same membrane was later stripped and reprobed with actin Ab. Data shown are representative of two independent experiments.

Neutrophil JNK2 is activated during microbial infection

We used *T. gondii*, an intracellular pathogen that activates JNK during macrophage infection (31, 33), to examine whether neutrophil JNK2 undergoes similar activation. Purified PMN were incubated with tachyzoites, and intracellular tachyzoites (an average of 1 per cell) were subsequently observed within 20 min of infection (Fig. 2.4*A*). At 18 h post-infection, intact intracellular tachyzoites were apparent, although rarely more than four per cell (Fig. 2.4*B)*. Absence of any overt signs of parasite death or degradation suggests that live tachyzoites actively invade PMN rather than being internalized by neutrophil phagocytosis. During infection of PMN, the parasite triggered JNK2 phosphorylation and the response was maximal between 20 and 30 min (Fig. 2.4*C*). This result correlates with the kinetics of *T. gondii*-mediated JNK activation in infected bone marrow-derived macrophages (28).

Rapid JNK activation in *T. gondii*-infected, but not bystander PMN

Phosphorylation of neutrophil JNK2 could be induced by secreted products of extracellular tachyzoites, by products released during or shortly after invasion, or the combination of the two. To address this issue, we used YFP-expressing *T. gondii* to infect PEC that were subsequently stained with anti-Gr-1 Ab. This enabled us to distinguish infected and noninfected Gr-1^+ populations (Fig. 2.5, *A* and *B*). To examine the JNK2 phosphorylation in each population, intracellular phospho-specific JNK staining was also performed. The intensity of phosphorylation between infected and noninfected Gr-1^+ PMN (thick lined-boxes in Fig. 2.5*B*) was compared with Gr-1^+ neutrophils unexposed to parasites (thin-lined box in Fig. 2.5*A*). Interestingly, JNK phosphorylation occurred predominantly in infected neutrophils, with little or no activation in the bystander noninfected population (Fig. 2.5*C*). In this situation,

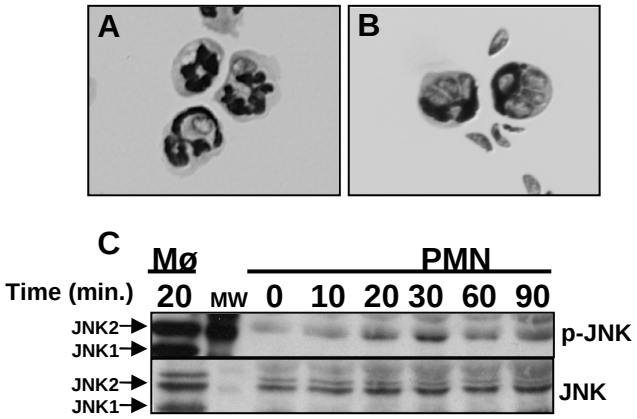

Figure 2.4. *T. gondii* infection and activation of JNK in mouse neutrophils. Neutrophils were infected with tachyzoites, and cells were examined 20 min (*A*) and 18 h post-infection (*B*). *C*, PMN were stimulated with tachyzoites for the period of time indicated. Cell lysates were subjected to immunoblot analysis probing with polyclonal antibody specific for phospho-JNK (p-JNK). The membrane was then stripped and reprobed with total JNK Ab. LPS-stimulated bone marrow-derived macrophages were used as a positive control for JNK activation. Data shown are representative of three independent experiments.

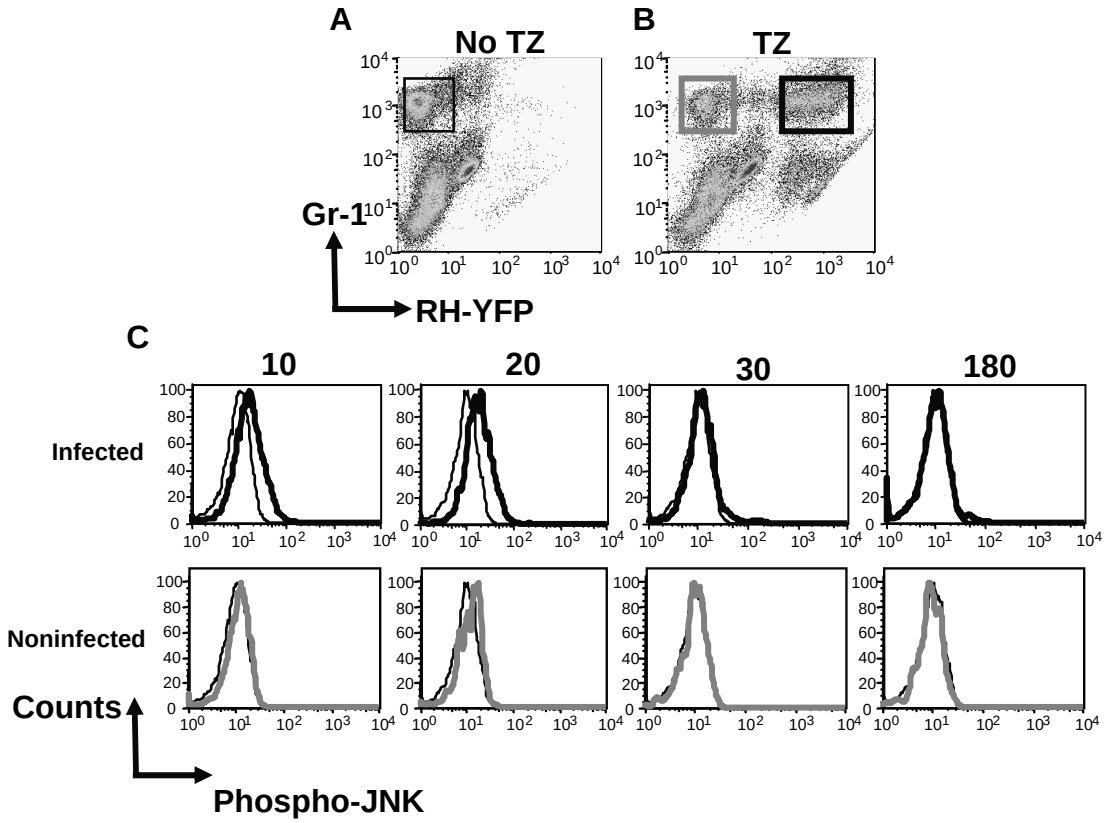

Figure 2.5. JNK activation occurs predominantly in *T. gondii* infected-Gr-1$^+$ PMN. *A* and *B*, Use of YFP-expressing tachyzoites allows identification of infected and noninfected PMN. Thioglycolate elicited PEC were stained with anti-Gr-1 Ab in the absence of tachyzoite (TZ) infection (*A*). Alternatively, cells were infected, and flow cytometric analysis performed 20 min post-infection (*B*). *C*, infected (rectangle in *B*) and noninfected (square in *B*) neutrophils were subjected to phospho-specific JNK staining and analyzed by flow cytometry at the indicated time points (min) after in vitro infection. The thick lines show phospho-JNK staining in infected and noninfected populations compared with phospho-JNK staining in PMN not exposed to parasites (thin line in *C*, and area defined in *A*). This experiment was repeated three times with equivalent results.

activation occurred with rapid kinetics, reaching maximal levels at 20 min then shifting back to basal level by 30 min postinfection.

T. gondii-induced IL-12p40 and CCL2/MCP-1 production in mouse PMN requires JNK2

Previously, we found that PMN produce both IL-12p40 and CCL2/MCP-1 in response to *Toxoplasma* (21). Therefore, we examined the role of JNK2 in production of these mediators of inflammation. Neutrophils from WT and *Jnk2*$^{-/-}$ mice were incubated with *T. gondii* tachyzoites at a multiplicity of infection 0.5:1 and supernatants were subsequently collected for ELISA. In WT PMN, IL-12p40 production was detectable at 4 hours, then peaked and maintained at ~1.6 ng/ml for up to 21 hours (Fig. 2.6*A*). The kinetics of CCL2 production differed, in that this chemokine was not detected until 8 hours post-infection, and its production increased steadily throughout the time course (Fig. 2.6*B*). These results are consistent with our previous data suggesting that IL-12p40 and CCL2/MCP-1 production during *T. gondii* infection are independently regulated (21). In sharp contrast to the behavior of WT neutrophils, *Jnk2*$^{-/-}$ PMN failed to produce detectable levels of either IL-12p40 or CCL2/MCP-1 (Fig. 2.6). We extended these studies by using a Multiplex Bead Immunoassay to assay neutrophil supernatants for 19 cytokines and chemokines (listed in *Material and Methods*). In this assay, we confirmed that *Toxoplasma* stimulates JNK2-dependent IL-12 and CCL2/MCP-1. In addition, we found that the neutrophils release MIP-1α and VEGF in a manner largely independent of infection and , more importantly, without a requirement for JNK2 (Fig. 2.6*C*).

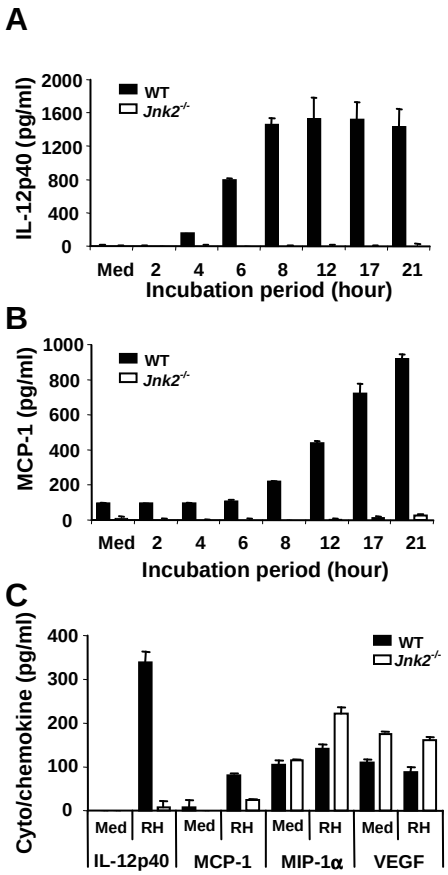

Figure 2.6. JNK2 is required for *T. gondii* mediated IL-12p40 and CCL2/MCP-1 production. Thioglycolate-elicited, Percoll gradient-purified neutrophils from WT and *Jnk2*$^{-/-}$ mice were incubated with *T. gondii* at a multiplicity of infection of 0.5:1. At the indicated time points, supernatants were collected for measurement of IL-12p40 (*A*) and CCL2/MCP-1 (*B*) production by ELISA. *C*, Out of 20 cyto/chemokines measured from the supernatants by Multiplex Bead Immunoassay, IL-12p40, CCL2/MCP-1, MIP-1α, and VEGF were detected. Med, levels of cytokine/chemokine in neutrophil supernatants after culture for 21 h in medium alone. Data shown are representative of three independent experiments.

Neutrophil respiratory burst, phagocytosis, and chemotaxis in the absence of JNK2

In addition to the role of JNK2 in IL-12p40 and CCL2/MCP-1 production, we asked whether this MAPK was involved in other neutrophil functions. To address this question, we performed three assays of PMN function, namely, oxidative burst activity, phagocytosis, and chemotaxis. As shown in Fig. 2.7*A*, both WT and *Jnk2*$^{-/-}$ neutrophils produced an equivalent oxidative burst in response to PMA and fMLP. Second, we determined the ability of WT and *Jnk2*$^{-/-}$ PMN to phagocytose opsonized 1-µm fluorescent beads. As shown in Fig. 2.7*B*, WT and *Jnk2*$^{-/-}$ neutrophils were able to phagocytose the beads to the same extent (WT neutrophils, 78% bead-positive; *Jnk2*$^{-/-}$ neutrophils, 82% bead-positive). In these experiments, we confirmed by microscopy that cell-associated fluorescence reflected internalization of microbeads rather than simple adsorption to the neutrophil surface (Fig. 2.7*B*, *inset*). Third, we used fMLP and the IL-8-related chemokine KC to induce PMN chemotaxis across a transwell membrane. As shown in Fig. 2.7*C*, there was a minor decrease in fMLP-stimulated transmigration in the absence of JNK2 that was not statistically significant ($p = 0.07$). However, although KC induced weaker chemotaxis, the response was reduced by ~50% in the absence of JNK2 ($p < 0.01$). In sum, we could not detect a requirement for JNK2 in neutrophil phagocytosis or oxidative burst, but there was a partial requirement for this MAPK molecule in neutrophil chemotaxis.

Discussion

The results of this study demonstrate that expression pattern of JNK molecules in mouse PMN is almost completely restricted to JNK2. This contrasts with macrophages, which express both JNK1 and JNK2. We were able to trace down-regulation of JNK1 protein during development of neutrophils through promyelocyte/myelocyte stages to

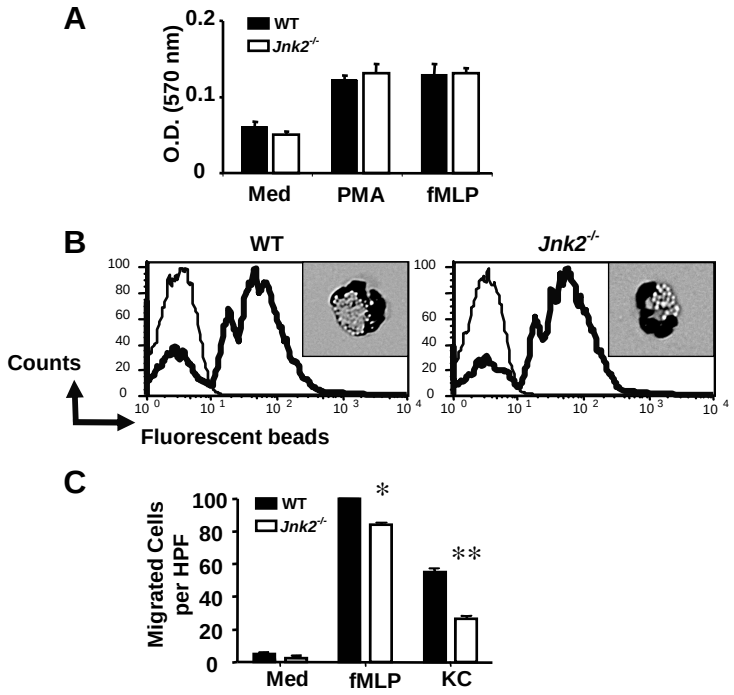

Figure 2.7. Neutrophil oxidative burst, phagocytosis and chemotaxis proceed normally in the absence of JNK2. *A*, Oxidative burst. PMN were stimulated with PMA (0.2µg/ml) and fMLP (10^{-6} M) along with NBT solution for 20 min at 37ºC. Cells were solubilized in 10%SDS 0.1N HCl. OD was measured at 570 nm. *B*, Phagocytosis. PMN were incubated 1 h with 1 µm green fluorescent beads precoated with normal mouse serum. FACS analysis was used to examine phagocytosis of beads by PMN. *Inset*, bead-phagocytosed PMN. *C*, Chemotaxis. PMN were cultured in a 3-µm pore transwell membrane in contact with fMLP (10^{-6} M) and KC (100 ng/ml) in the lower chamber. Number of migrated cells was determined after 40 min by counting under the microscope at least 3 different high power fields (HPF). Each of these experiments was performed twice with the same result. *, $p = 0.07$ and **, $p < 0.01$ (WT vs JNK2 knockout). Each of these experiments was performed twice with the same result.

immature neutrophils to mature neutrophils in the bone marrow. Infection of PMN with *T. gondii* resulted in JNK2 phosphorylation in a manner kinetically similar to that occurring in macrophages. Using PMN isolated from *Jnk2*$^{-/-}$ mice, which are devoid of all JNK isoforms, we demonstrated a requirement for JNK2 in infection-induced IL-12p40 and CCL2/MCP-1 production. Nevertheless, production of MIP-1α and VEGF was normal in the absence of JNK2. We detected a partial JNK2 requirement in the neutrophil chemotactic response and indeed others have also found involvement of JNK in neutrophil chemotaxis (34, 35)

We previously reported that *Toxoplasma* induces both IL-12p40 and CCL2 in mouse neutrophils (21, 36). The importance of IL-12 during infection with this opportunistic pathogen has long been recognized (37, 38). Absence of IL-12 results in uncontrolled parasite proliferation and death of the host. More recent data suggests that this is the result of Th1 cell secretion of IL-10, rather than complete failure to generate IFN-γ-positive Th1 T lymphocytes as originally proposed (39). The role of CCL2 during *T. gondii* infection is currently under investigation. However, the chemokine appears to be involved in recruiting a novel population of Gr-1^{+} monocytes capable of microbicidal activity against *Toxoplasma* and other intracellular pathogens (40, 41). The importance of these cells is suggested by the finding that mice lacking CCR2, the receptor for CCL2, are susceptible to *T. gondii* (40).

In the absence of an Ab capable of specifically depleting neutrophils in vivo, or neutrophil-specific cytokine/chemokine knockout mice, it is difficult to directly address the role of neutrophil-derived cytokines and chemokines such as IL-12 and CCL2 in the innate response to infection. Nevertheless, PMN accumulate at high numbers at sites of infection and in secondary lymphoid organs during the early

response to *Toxoplasma* (42). Given the fact that neutrophils produce a large battery of cytokines and chemokines during in vitro stimulation with *Toxoplasma* and other stimuli (11, 13), it seems likely that production of these mediators plays a role in the outcome of infection in vivo, either through direct effects on T lymphocytes or by influencing other immune effectors such as dendritic cells (16-18).

This study is the first to demonstrate a requirement for JNK in mouse neutrophil IL-12p40 and CCL2 production. Previously, we reported that neutrophil production of both mediators, like production of IL-12 by dendritic cells and macrophages, is dependent upon TLR adaptor molecule MyD88 (20, 21). We found that TLR2 was required for CCL2 induction, and indeed others have reported that absence of this TLR increases susceptibility to *T. gondii* (21, 43). Interestingly, neutrophil IL-12 did not require TLR2. It is possible that TLR11, recently identified as a mouse receptor for parasite profilin involved in DC IL-12 production (44), also drives neutrophil IL-12 release. Further evidence that regulation of IL-12 and CCL2 differs in neutrophils comes from the combined findings that IL-10 down-regulates IL-12 but not CCL2 and an IFN-γ-STAT1 pathway induces CCL2 but not IL-12p40 secretion (21). In the present study, circumstantial evidence for independent regulation comes from the disparate kinetics of IL-12 and CCL2 release during neutrophil infection (Fig. 2.6).

Although we did not examine responses of human neutrophils, other studies suggest that JNK/SAPK is regulated differently than in mouse PMN. Several reports indicate presence of JNK1 in human neutrophils, although it has also been reported that JNK2 is the dominant isoform in these cells (45-49). More interestingly, it has been reported that inflammatory cytokine production in human PMN is independent of JNK and the downstream transcription factor complex AP-1 (50). Although this particular study did

not specifically examine either IL-12p40 or CCL2, a variety of PMN chemical activation stimuli failed to induce nuclear translocation of JNK or activation of downstream Jun/Fos proteins. Nevertheless, we note that another group reported a JNK requirement for CCL2 production by human PMN (46), a result in line with our findings here.

In several infection models there is evidence that JNK1 and JNK2 fulfill distinct functions in the immune response. For example, JNK1 is involved in activated T cell survival during infection with lymphocytic choriomeningitis virus, whereas JNK2 controls proliferation of virus-specific $CD8^+$ T lymphocytes (51, 52). The concept that there are noncompensatory functions for JNK1 and JNK2 is also suggested by reports that Th1 effector cell generation is defective in the absence of JNK2, and that lack of JNK1 confers susceptibility to *Leishmania major* (53, 54). Interestingly, a recent report implicated JNK2 in the response to *Plasmodium falciparum* glycosylphosphatidylinositol and pathogenesis of cerebral malaria (55). There is also evidence for complex interplay between JNK1 and JNK2 insofar as JNK2 has been reported to bind to c-Jun inducing its degradation, whereas JNK1 functions as the major c-Jun kinase following cell stimulation (56). Collectively, these studies indicate nonredundant functional roles for JNK molecules in the immune system.

In the absence of an ability to artificially enforce JNK1 expression in neutrophils, it is difficult to determine why this MAPK family member is down-regulated during the neutrophil differentiation program. Neutrophils are well known to be preprogrammed for accelerated apoptotic cell death, a response largely unique to this cell type. Although some studies indicate a role for JNK in promoting programmed cell death (57, 58), others indicate that JNK signaling suppresses apoptosis (59-61). Therefore, it

is possible that down-modulation of JNK1 could be involved in promoting an apoptotic death program in neutrophils.

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CHAPTER 3

ABSENCE OF MITOGEN-ACTIVATED PROTEIN KINASE FAMILY MEMBER c-JUN N-TERMINAL KINASE-2 ENHANCES RESISTANCE TO *TOXOPLASMA GONDII**

* Reprinted from Woraporn Sukhumavasi, Amy L. Warren, Laura del Rio Alonso and Eric Y. Denkers. Submitted for publication.

Abstract

The function of mitogen-activated protein kinase (MAPK) family member c-Jun N-terminal kinase (JNK)-2 in resistance and pathology during infection has not been greatly studied. Here, we employed *Jnk2*$^{-/-}$ mice to investigate the role of JNK2 in resistance and immunity during oral infection with the protozoan pathogen *Toxoplasma gondii*. We found increased host resistance in the absence of JNK2 as determined by lower parasite burden and increased host survival. Lack of JNK2 also correlated with decreased neutrophil recruitment to the intestinal mucosa and less pathology in the small intestine. In the absence of JNK2, IL-12 production was slightly but significantly increased in restimulated splenocyte populations as well as in purified splenic dendritic cell cultures. These results provide evidence that expression of JNK2 plays a role in *T. gondii*-induced immunopathology, at the same time in promoting susceptibility to this parasitic pathogen.

Introduction

The intracellular protozoan parasite *Toxoplasma gondii* infects humans and other warm-blooded animals (1, 2). Within the host, the parasite is capable of infecting a wide range of hematopoeitic and nonhematopoietic cells. Infection is normally asymptomatic, resulting in life-long infection associated with quiescent cysts in the brain and skeletal muscle tissue. However, toxoplasmic encephalitis may emerge as a life-threatening condition in immunocompromised populations, and congenital infection can have severe consequences to the fetus including birth defects and abortion, as well as major sequelae of infection later in life (3, 4).

Under normal circumstances, *Toxoplasma* elicits robust protective immunity that is likely to underlie the normally asymptomatic nature of infection. Induction of host

resistance to *T. gondii* requires early stimulation of IL-12 production from innate immune cells leading to IFN-γ-dependent control of parasite replication (5, 6). The importance of IFN-γ and IL-12 for disease resistance has been most clearly demonstrated using IFN-γ and IL-12 knockout mice, as well as in Stat1 and Stat6 knockout animals, all of which uniformly succumb within 7-10 days of infection due to overwhelming parasite replication and dissemination (7-10). However, unregulated production of these same cytokines can be detrimental to the host, a situation leading to immunopathology and sometimes death of the host (11, 12). A striking example of this phenomenon occurs during oral infection of the C57BL/6 mouse strain, which develop severe ileal necrosis mediated by $CD4^+$ T cell IFN-γ overproduction (13-15).

The role of host cell signal transduction in immunity to infection is an area of intense interest. In particular, the mitogen-activated protein kinase (MAPK) family has been demonstrated to control gene expression and immune function, and can play roles in positive and negative regulation of proinflammatory cytokine production (16). There are three major groups of MAPK in mammalian cells: p38, extracellular signal-regulated protein kinases (ERK), and c-Jun N-terminal kinases (JNK), also known as stress-activated protein kinases (SAPK). The JNK signaling pathway is activated primarily by cytokines and exposure to environmental stress, and is believed to play important roles in both innate and adaptive immune responses (17-19). Three JNK-encoding genes have been identified in mammals, namely, JNK1, 2 and 3. JNK1 and JNK2 are ubiquitously expressed whereas JNK3 is selectively expressed in neuronal tissues. The JNK2 molecule has been implicated in positive and negative regulation of IL-12 synthesis, depending on the study, as well as promotion of Th1 and $CD8^+$ T lymphocyte responses (20-24).

To determine the function of JNK2 in response to *Toxoplasma*, we recently employed *Jnk2*$^{-/-}$ mice as a source of cells during in vitro infection. We found that while JNK2 was required for neutrophil production of IL-12p40 and CCL2/MCP-1, macrophage IL-12 release did not require this MAPK signaling molecule (25, 26). Here, we evaluated the function of JNK2 in immunity and resistance during in vivo infection with *Toxoplasma*. Although we found no major differences in production of IFN-γ in the absence of JNK2, dendritic cell IL-12 production was slightly, but significantly, elevated in *Jnk2*$^{-/-}$ animals. Host resistance was increased in the absence of JNK2, and this correlated with lower parasite burden in the small intestine and decreased neutrophil recruitment into the gut mucosa. In line with these observations, gut pathology was alleviated in the absence of JNK2. Thus, while *Jnk2*$^{-/-}$ mice displayed only minor changes in proinflammatory cytokine responses during *T. gondii* infection, deletion of the JNK2 molecule nevertheless promotes resistance to this parasitic pathogen.

Materials and Methods

Mice

Mapk9^{tm1Flv} (*Jnk2*$^{-/-}$) mice on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, ME). A breeding colony was established and genotype confirmed by PCR of tail snip DNA according to protocols available from The Jackson Laboratory (Fig. 3.1). The animals were maintained under specific-pathogen-free conditions in the transgenic mouse core facility at the College of Veterinary Medicine, Cornell University, overseen by the Institutional Animal Care and Use Committee (IACUC). Female progeny between 6-12 wk of age were age-matched with C57BL/6 mice (Taconic Farms; Hudson, NY) for experiments.

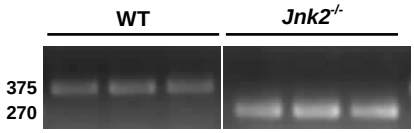

Figure 3.1. Genotypic analysis of WT and *Jnk2*$^{-/-}$ mice. Primers spanning a portion of the *Jnk2* gene were used to amplify the predicted 375 and 270 base sequence associated with the normal and deleted gene, respectively. Each lane represents an individual mouse.

Parasites and infections

Cysts of the low virulence ME49 strain were harvested from brains of chronically infected Swiss-Webster mice. Infections were initiated by oral gavage or i.p. injection in a volume of 0.2 ml in PBS. *T. gondii* tachyzoites of the RH and PTG strain (clonally derived from ME49) were maintained by passaging twice a week on human foreskin fibroblast monolayers as described previously (26). A PCR-ELISA-based kit (Roche Applied Science, Mannheim, Germany) was used to confirm absence of *Mycoplasma* contamination in the parasite cultures every two months.

Cell culture

Splenic single cell suspensions were prepared by lysing red blood cells using red blood cell lysing buffer (Sigma-Aldrich, St.Louis, MO). Debris was removed by passing the cell suspensions through a 70 µm cell strainer. The cell number and viability was determined by trypan blue exclusion. After washing the cells in PBS, they were resuspended in DMEM supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 30 mM HEPES (all from Invitrogen, Grand Island, NY), 10% bovine growth serum (Hyclone, Logan, UT) and 0.05 mM β-mercaptoethanol. The cells were plated in triplicate in 96-well plates (10^7/ml). Soluble tachyzoite antigen (STAg), prepared and described previously (27), was added for restimulation. The cells were incubated at 37ºC in a humidified 5% CO_2 incubator for 48 and 72 hr, then supernatants were recovered and stored at -20ºC for ELISA.

ELISA

Production of IL-12p40 and IFN-γ was measured by ELISA as previously described (27).

Splenic dendritic cell isolation

Dendritic cells from spleens were magnetically labeled with Pan DC MicroBeads (Miltenyi Biotec Inc., Auburn, CA) following the manufacturer's protocol. Enriched DC were isolated employing an autoMACS Separator (Miltenyi Biotec). Cells obtained in this manner were routinely 60-70% CD11c positive, as determined by flow cytometric analysis.

Histopathological examination and immunohistochemistry

Tissues were collected, preserved in 10% neutral buffered formalin and submitted to the Histology Unit at the College of Veterinary Medicine, Cornell University to be embedded in paraffin blocks for H&E staining and immunohistochemistry. Lesions were scored in blind manner and graded based on severity of inflammation (minimal, mild, moderate, severe), number of inflammatory foci, distribution of inflammation, type of inflammatory reaction, severity of necrosis and intensity of *T. gondii* infection.

Statistical analysis

Student's t-test was employed to analyze statistical differences between groups. p values < 0.05 were considered significant. Survival curves were analyzed using a logrank test.

Results

Increased resistance to *T. gondii* in the absence of JNK2

We previously found a requirement for MAPK family member JNK2 in IL-12p40 production by mouse neutrophils during in vitro stimulation with *T. gondii* (26). Nevertheless, we also found that production of IL-12p40 by bone marrow-derived macrophages did not require JNK2 (25). Because IL-12 is well known to be required

for induction of Th1 responses and because this type of immunity is required in host defense against *Toxoplasma* (5, 28), we assessed the role of JNK2 during in vivo infection with this pathogen. Surprisingly, in the absence of JNK2, mice were more resistant during i.p. infection with the low virulence ME49 *T. gondii* strain (Fig. 3.2A). We also inoculated mice by oral gavage to determine if route of infection influenced the pattern of susceptibility in the absence of JNK2. Here again, WT animals displayed an accelerated rate of death compared to *Jnk2* KO mice (Fig. 3.2B). We also terminated an i. p. infection experiment at Day 30 post-infection for brain cyst enumeration. In line with the survival data, brains of *Jnk2* KO mice contained fewer cysts than WT counterparts (Fig. 3.2C; $p = 0.039$).

To determine if the decreased parasite number associated with lack of JNK2 was also apparent in the intestine during acute infection, we isolated tissues from orally inoculated animals and performed immunohistochemical staining for *T. gondii*. Parasite numbers were elevated in the intestinal mucosa of WT relative to *Jnk2* KO mice infected with 20 cysts (Fig. 3.3A and B, respectively) as well as with 100 cysts (data not shown). Increased parasite numbers were also apparent in Peyer's patches of WT compared to KO animals (Fig. 3.3C and D, respectively) as well as in mesenteric lymph nodes (not shown).

Serum cytokine levels during *T. gondii* infection in WT and *Jnk2*$^{-/-}$ mice

We next investigated production of IL-12p40 and IFN-γ, cytokines that together play a central role in resistance to *Toxoplasma* (6, 29). Accordingly, we orally infected mice with 20 and 100 ME49 cysts and measured serum cytokine levels on Day 4 and Day 7 post-infection. Levels of IL-12p40 between WT and KO mice were indistinguishable

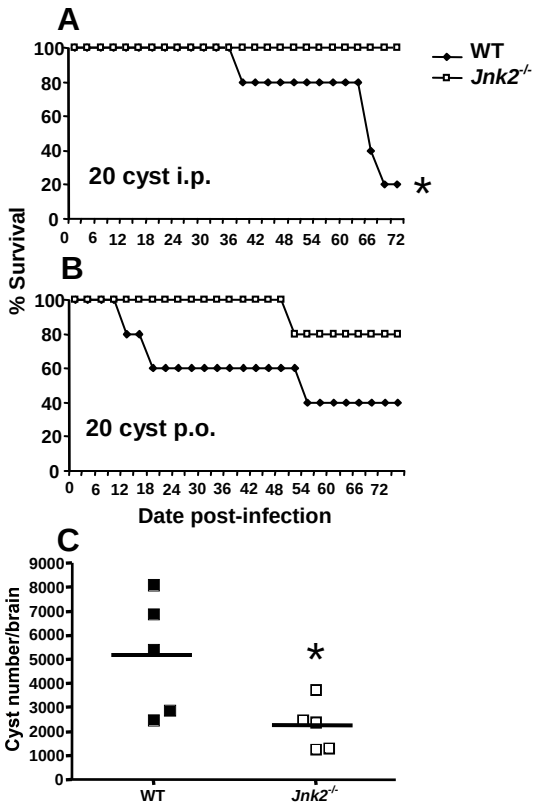

Figure 3.2. Increased resistance to *T. gondii* infection in the absence of JNK2. Wild-type and *Jnk2* knockout mice (n = 5 per group) were infected by i.p. (panel A) or oral (panel B) administration of 20 ME49 cysts. At Day 30 after i. p. infection, brains were removed from surviving mice and cyst numbers were enumerated (panel C). *, $p < 0.05$.

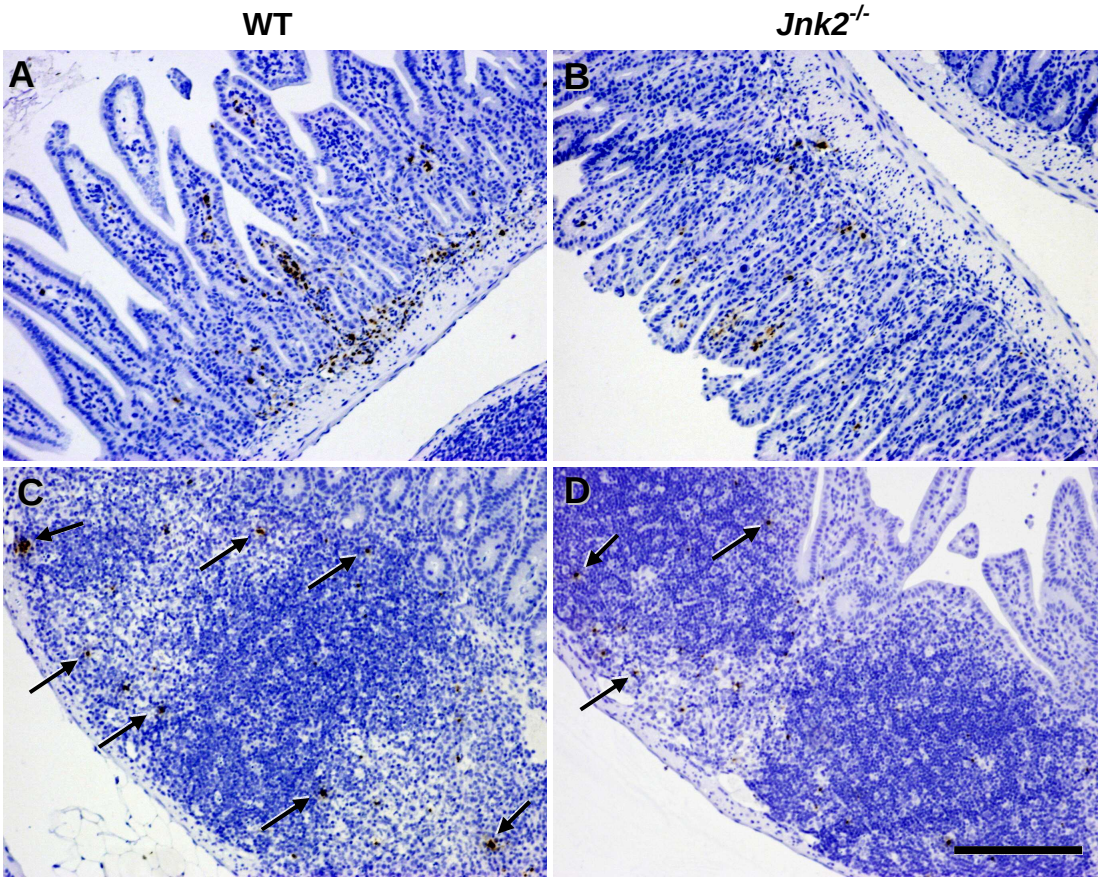

Figure 3.3. **Decreased parasite numbers in *Jnk2* KO intestinal tissues during oral infection.** WT and KO mice were infected with 20 ME49 cysts and 7 days later intestinal tissues were isolated, sectioned and subjected to immunohistochemical staining for *Toxoplasma*. Panel A and C shows the parasite staining in WT intestinal mucosa and Peyer's patches, respectively, compared to the KO in panel B and D. Scale bar = 200 µm.

at both time points during low and high dose infection (Fig. 3.4A and B). Along similar lines, serum IFN-γ levels were comparable between the two mouse strains (Fig. 3.4C and D). Nevertheless, at Day 4 post-infection with 20 cysts, *Jnk2* KO mice displayed elevated IFN-γ levels compared to WT animals, although this difference was not apparent in the high dose infection.

Splenic IL-12p40 and IFN-γ responses of WT and *Jnk2*$^{-/-}$ mice during acute infection

In addition to examining the role of JNK2 in IL-12p40 and IFN-γ production at the systemic level, we sought to determine whether this MAPK was involved in the splenic cytokine response during infection. We found a slight but significant increase in IL-12 production in cells from uninfected, as well as Day 4 and Day 7-infected KO mice relative to WT counterparts (Fig. 3.5A and B). There was also a trend in increased IFN-γ production by KO cells, although differences between WT and KO were minor (Fig. 3.5C and D). We also examined responses of mesenteric lymph node cells but could not find any consistent differences between WT and KO mice over the course of several experiments (data not shown).

Parasite-induced IL-12p40 induction in splenic CD11c^{+} cells from wild-type and *Jnk2*$^{-/-}$ mice

Splenocyte IL-12 production during *T. gondii* infection is believed to derive in large part from resident DC responding to parasite profilin, a ligand of Toll-like receptor 11 (30-32). To examine the role of JNK2 in DC responses to *Toxoplasma*, we employed immunomagnetic labeling to isolate an enriched population of CD11c^{+} cells from spleens of noninfected mice, which were then cultured with STAg, as well as live RH and PTG (clonally derived from ME49) strain tachyzoites. As shown in Fig. 3.6, and

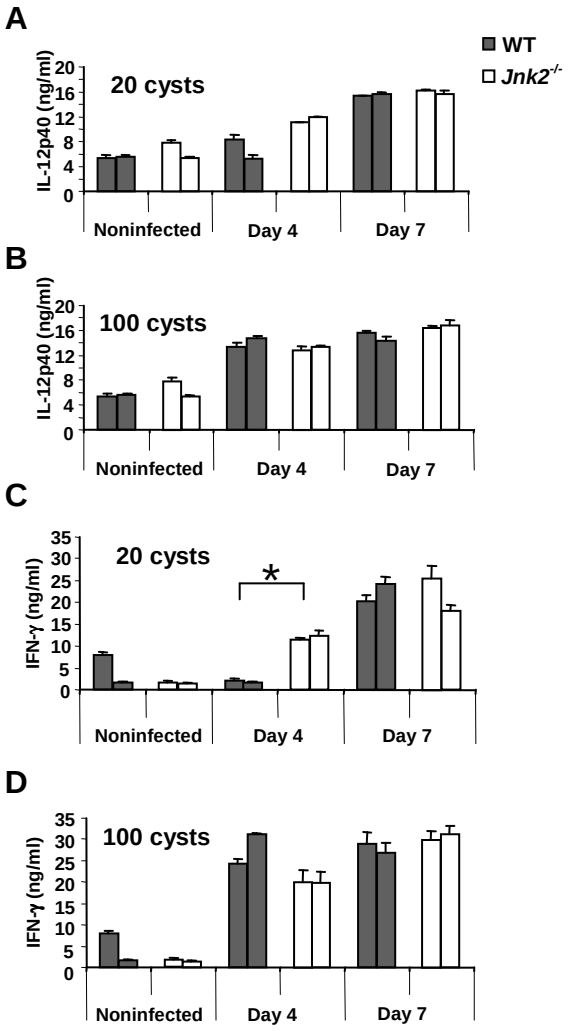

Figure 3.4. Serum cytokine response during *T. gondii* infection in wild-type and *Jnk2*$^{-/-}$ mice. Mice were orally infected with 20 (panels A and C) or 100 (panels B and D) cysts and serum was collected from individual mice for IL-12p40 (A and B) and IFN-γ (C and D) ELISA. The data shown are the mean ± SD of triplicate samples. These results were repeated twice with the same result. *, $p < 0.05$.

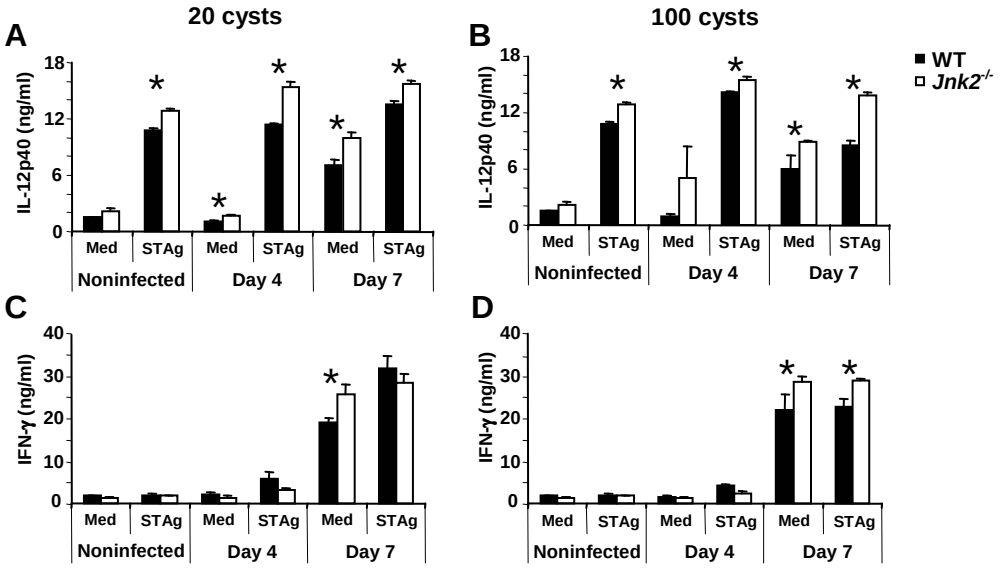

Figure 3.5. Splenic IL-12p40 and IFN-γ responses of wild-type and *Jnk2*$^{-/-}$ mice during acute infection. Mice were orally infected with 20 (A and C) or 100 (B and D) *Toxoplasma* cysts. Splenocytes were prepared at the indicated times post-infection and cultured with soluble tachyzoite antigen (3 µg/ml). Supernatants were subsequently collected for IL-12p40 (A and B) and IFN-γ (C and D) ELISA. The data shown are the mean ± SD of triplicate samples. These results were repeated twice with the same result. *, $p < 0.05$.

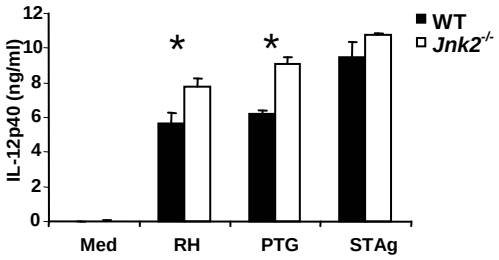

Figure 3.6. Parasite-induced IL-12p40 induction in splenic CD11c^{+} cells from wild-type and *Jnk2*$^{-/-}$ mice. Following immunomagnetic selection of CD11c^{+} DC, cells were inoculated with type I RH and type II PTG tachyzoites at a multiplicity of infection of 0.5:1, or cultured with soluble tachyzoite antigen (25 µg/ml). After 18 hr, the supernatants were collected for IL-12p40 ELISA. The data shown are the mean ± SD of triplicate samples. These results were repeated twice with the same result. *, $p < 0.05$.

consistent with the results using whole splenocyte populations, absence of JNK2 resulted in a small but significant elevation in IL-12p40 release.

Jnk2$^{-/-}$ mice are resistant to *T.gondii*-induced ileitis

Previous studies have established that C57BL/6 mice succumb to severe inflammatory pathology in the small intestine after oral infection with *T.gondii* (13, 15, 33). Accordingly, we assessed the role of JNK2 in parasite-induced intestinal damage to the gut. As shown in Fig. 3.7A and C, WT mice displayed multifocal areas of inflammation and necrosis. In these animals, 50-70% of the small intestine was affected, with inflammatory cell infiltration in lamina propria and submucosa. Loss of villi tips was also apparent in some areas (Fig. 3.7C). In contrast, lesions in *Jnk2*$^{-/-}$ were much less severe, with fewer foci of inflammation in the entire intestinal section (average of 57 foci in KO compared to 162 foci in WT) (Fig. 3.7B). The intestinal architecture was largely intact compared to the WT counterparts (Fig. 3.7D).

Jnk2$^{-/-}$ mice display decreased infiltration of myeloperoxidase-positive cells in intestinal tissues during oral infection

Our previous data demonstrated a partial requirement for JNK2 in neutrophil chemotaxis induced by the IL-8-related chemokine KC in vitro (26). To evaluate the role of JNK2 in neutrophil recruitment in vivo, we performed immunohistochemical staining for myeloperoxidase (MPO), an enzyme expressed at high level in azurophil granules of neutrophils (34). Peyer's patches of WT animals contained greater numbers of MPO-positive cells relative to KO counterparts (Fig. 3.8A and B, respectively). Similarly, we found increased numbers of MPO-positive cells in the lamina propria and submucosa of intestinal sections in WT (Fig. 3.8C) compared to *Jnk2* KO mice (Fig. 3.8D). Therefore, expression of JNK2 promotes susceptibility to

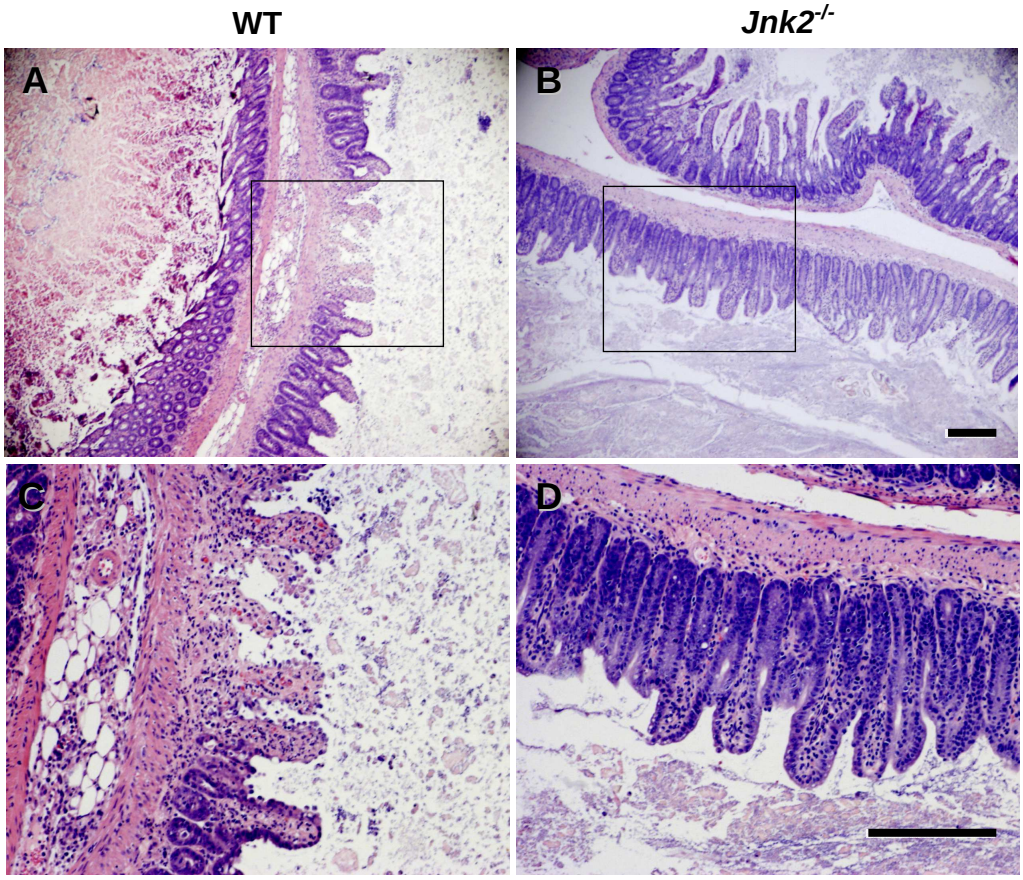

Figure 3.7. *Jnk2*$^{-/-}$ mice are resistant to *T. gondii*-induced ileitis. WT and *Jnk2*$^{-/-}$ mice (2 mice per group) were orally infected with 100 *Toxoplasma* cysts. At Day 10 postinfection, the small intestines were harvested, fixed and subjected to H&E staining. Low magnification imaging of ileal sections of infected WT mice are shown in panel A and B, respectively. The regions delineated by the rectangles were imaged at higher magnification in panels C and D. Scale bar = 200 µm. Data shown are one representative experiment of two.

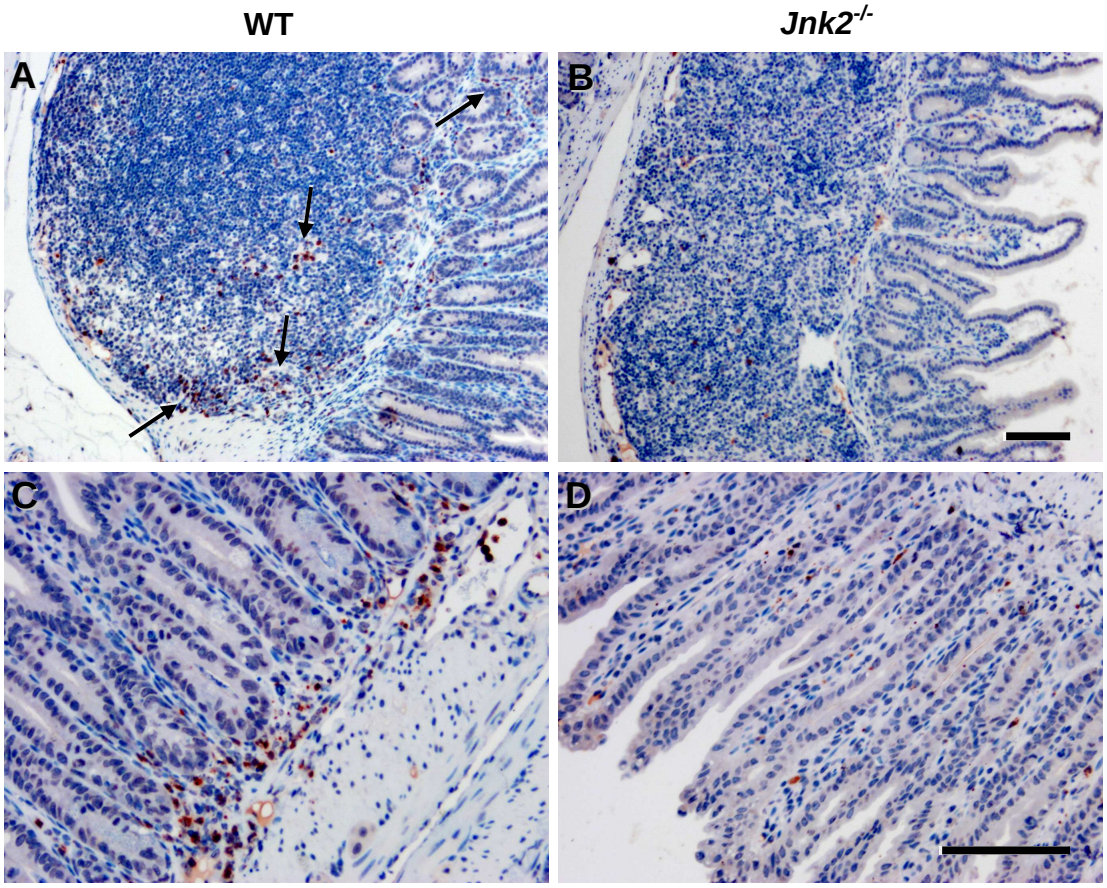

Figure 3.8. Decreased numbers of MPO-positive cells in the absence of JNK2 in intestinal tissues uring oral infection. Small intestines were collected from WT (A and C) and *Jnk2*$^{-/-}$ (B and D) mice 7 days after oral infection. Sections were then subjected to immunohistochemical staining with an anti-MPO polyclonal serum. Panels A and B show MPO staining of Peyer's patches from WT and KO, respectively. Panel C and D illustrate MPO staining of WT and KO gut mucosa. Scale bar denotes 100 µm.

Toxoplasma-induced inflammatory gut pathology, and that this is associated with JNK2-dependent neutrophil recruitment to the site of infection.

Discussion

The function of MAPK family member JNK2 in innate and acquired immunity is unclear, and few studies have addressed the role of this molecule during the response to infection. In this study we found that mice lacking JNK2 displayed enhanced resistance to *T. gondii* infection initiated by both i. p. and oral infection. During oral infection, *Jnk2*$^{-/-}$ mice were resistant to parasite-mediated damage to the ileum, and the animals displayed decreased neutrophil recruitment to the intestinal mucosa. Insofar as IL-12 and IFN-γ play central roles in resistance to this parasitic pathogen (5), our initial hypothesis was that production of one or another of these two cytokines would be upregulated by absence of JNK2-mediated signal transduction. However, for the case of IFN-γ, levels of this Th1-associated cytokine were equivalent in WT and KO mice undergoing infection. Although IL-12 production by splenocytes as well as purified splenic DC was, indeed, higher in the absence of JNK2, the differences were marginal and it seems unlikely that this is the cause of increased resistance of *Jnk2*$^{-/-}$ mice.

There is no clear consensus on the role of JNK molecules in IL-12 synthesis. In the present study, we found evidence that JNK2 negatively regulates IL-12p40 production during infection. In line with these data, others have reported that G_i-protein mediated IL-12 downregulation is dependent upon JNK signaling in human monocytes (21). Nevertheless, our own recent studies show a requirement for JNK2 in neutrophil IL-12 production, and it has also been reported that LPS-induced IL-12 production by the human promonocytic cell line THP-1 is mediated by JNK signaling (22, 26). Taking

these results together, it seems probable that the role of JNK in IL-12 synthesis varies depending on cell type and possibly conditions of stimulation.

Initial reports employing *Jnk2*$^{-/-}$ mice indicated that JNK2 was important for T lymphocyte activation (35). It was also suggested that JNK2 expression in CD4^{+} T cells was required for differentiation into Th1 effectors (20). Other more recent studies suggest that JNK2 signaling negatively regulates expansion and effector function of CD8^{+} T cells. For example, absence of JNK2 led to increased IL-2 production and expansion of virus-specific CD8^{+} T cells during infection with both influenza virus and lymphocytic choriomeningitis virus (36, 37). In an OT-1 system using ovalbumin-specific CD8^{+} T cells on a *Jnk2*$^{-/-}$ background, it was found that CD8^{+} T cells produced increased amounts of IFN-γ and elevated levels of T-bet and eomesodermin, transcription factors that are required for effector CTL generation (23). Because optimal resistance to *Toxoplasma* requires IFN-γ production by CD8^{+} T lymphocytes (38-40), it is possible that enhanced CD8^{+} activity in the absence of JNK2 accounts for increased resistance of *Jnk2*$^{-/-}$ mice. We are currently evaluating the function of CD8^{+} T cells on a *Jnk2*$^{-/-}$ background during *T. gondii* infection.

A striking feature of the present results is that intestinal pathology induced by oral *Toxoplasma* infection was alleviated in *Jnk2*$^{-/-}$ mice. Damage to the gut during *T. gondii* infection is mediated by proinflammatory mediators that include IL-12p40, TNF-α, IFN-γ and nitric oxide (13, 15). Nevertheless, IL-12 levels were higher in the absence of JNK2, and we could find no consistent difference in TNF-α or IFN-γ levels during infection in the absence of JNK2. This was also the case for production of nitric oxide (data not shown). Regardless, it is interesting to note that *Jnk2* deletion has similarly been found to confer resistance to *Plasmodium berghei* ANKA (41).

Infection with the latter induces cerebral pathology that, like oral infection with *T. gondii*, is mediated by proinflammatory cytokines (42, 43). Along similar lines, it was recently found that JNK2 deficiency increases resistance to *Anaplasma phagocytophilum*, and this was associated with increased IFN-γ production by natural killer (NK) and NKT cells (44). Together, these data point to a role for JNK2 in down-modulation of proinflammatory responses during microbial infection.

Finally, absence of JNK2 also resulted in defective neutrophil recruitment to the small intestine during oral infection. In a previous study, we found that JNK2 signaling was required for neutrophil chemotaxis in vitro (26). JNK signaling is also involved in neutrophil recruitment to the lung induced by lipopolysaccharide and positive air pressure (45, 46). In addition to direct effects on chemotaxis, activation of JNK is necessary for neutrophil adhesion to endothelial cells that is involved in recruitment to sites of infection or inflammation (47). Whether JNK2-dependent neutrophil recruitment during oral *Toxoplasma* infection contributes to pathology in the gut is currently under investigation in our laboratory.

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CHAPTER 4

TOLL-LIKE RECEPTOR ADAPTOR MYD88 IS ESSENTIAL FOR PATHOGEN CONTROL BUT NOT ADAPTIVE IMMUNITY DURING *TOXOPLASMA GONDII* INFECTION*

*Reprinted from Woraporn Sukhumavasi, Charlotte E. Egan, Amy L. Warren, Gregory A. Taylor, Barbara A. Fox, David J. Bzik and Eric Y. Denkers. Submitted for publication.

Abstract

Toll-like receptor (TLR)/MyD88 activation is important in host resistance to *Toxoplasma gondii* during i. p. infection, but the function of this signaling pathway during oral infection, in which mucosal immunity assumes a predominant role, has not been examined. Here, we show that *MyD88*$^{-/-}$ mice fail to control the parasite and succumb within two weeks of oral infection. Early during infection, T cell IFN-γ production, recruitment of neutrophils and induction of p47 GTPase Irgm3/IGTP in the intestinal mucosa were dependent upon functional MyD88. Unexpectedly, these responses were MyD88-independent later during acute infection. In particular, CD4^{+} T cell IFN-γ reached normal levels independently of MyD88, despite continued absence of IL-12 in these animals. Intraperitoneal vaccination of *MyD88*$^{-/-}$ mice with an avirulent *T. gondii* uracil axotroph elicited robust IFN-γ responses and protective immunity to challenge with a high virulence *T. gondii* strain. Our results demonstrate that MyD88 is required to control *Toxoplasma* infection, but that the parasite can trigger adaptive immunity without the need for this TLR adaptor molecule.

Introduction

Toll-like receptors (TLR) have emerged as a major family of pattern recognition molecules involved in sensing infectious nonself molecules and possibly endogenous molecules of the host. There are 11-13 TLR in mice and humans that recognize diverse molecules including bacterial lipopolysaccharide, lipopeptides, unmethylated CpG oligodinucleotides, as well as single and double-stranded RNA (1, 2). Signaling through TLR is complex, but all TLR (with the exception of TLR3) use the adaptor molecule MyD88 to initiate intracellular signal transduction (3-5). Most prominent among these signaling pathways are NFκB and mitogen-activated protein kinase (MAPK) cascades leading to induction of proinflammatory cytokines such as IL-12

and TNF-α. MyD88 is also used to relay signals emanating from IL-1 and IL-18 receptors. As such, MyD88 represents a bottleneck through which most TLR, as well as IL-1R/IL-18R, initiated signals must pass.

The importance of MyD88 in infectious disease models has been firmly established using genetically engineered *MyD88*$^{-/-}$ mice. Animals lacking MyD88 display decreased resistance to bacteria such as *Mycobacterium tuberculosis*, *Staphyloccus aureus*, *Listeria monocytogenes*, and *Brucella abortus* (6-9). It is also becoming clear that TLR and MyD88 are important in the innate immune response to parasitic protozoans (10). Thus, MyD88 KO mice are increased in susceptibility to infections with *Toxoplasma gondii*, *Plasmodium berghei*, *Leishmania major* and *Trypanosoma cruzi* (11-15). TLR ligands have been identified for several protozoan species, including *T. gondii*, *Plasmodium*, and *T. cruzi* (16-18). Nevertheless, while knock out of MyD88 may have dramatic effects on resistance to bacterial and protozoan infection, inactivation of genes encoding individual TLR often has little or no impact. This has led to the view that the response to any given pathogen is likely to involve multiple TLR that act in concert to provide optimal resistance to infection (19-21).

There is evidence that TLR-MyD88 signaling is important, not only in innate immune responses, but also in initiation of acquired immunity (22). For example, MyD88 transduced signaling leads to upregulation of costimulatory molecules on DC that is required to activate naïve T cells, and T cell antigen-specific responses are optimized when microbial peptides are presented within the context of TLR ligands (23, 24). Nevertheless, the role of TLR-MyD88 in initiation of adaptive immunity during infection with complex pathogens is less certain. In some cases MyD88 is not necessary, such as in the generation of *Listeria monocytogenes*-specific $CD8^+$ T cell

responses and during acquired immunity to *Borrelia burgdorferi* (25-27). In other cases, MyD88 seems to play a prominent role in adaptive immunity, for example in the humoral immune response to polyoma virus infection and the antiviral $CD8^+$ response to lymphocytic choriomeningitis virus (28, 29).

In this study we focus on infection with the intracellular protozoan *T. gondii*, a parasite known for its ability to elicit strong protective Th1 responses, but that causes serious disease in immunocompromised individuals (30, 31). It has previously been shown that *MyD88*$^{-/-}$ animals rapidly succumb to *Toxoplasma* in a model involving i. p. injection of parasites (14). TLR signaling is strongly implicated in this situation because mice defective in IL-1β-converting enzyme display normal resistance to infection despite an inability to produce functional IL-1 or IL-18 (15). Tachyzoite profilin and glycosylinositolphosphatidyl lipid moieties have recently been identified as parasite ligands of TLR11 and TLR2/4, respectively (32-34). Furthermore, for the case of profilin there is evidence that signaling through TLR11 promotes an immunodominant T cell response to peptides derived from this molecule (35).

Here, we show that MyD88 is required to survive oral infection with *Toxoplasma*. Early during infection *MyD88*$^{-/-}$ animals displayed defects in neutrophil recruitment to mucosal tissues and induction of Irgm3/IGTP, a molecule required to survive acute infection. Surprisingly, T cell-derived IFN-γ production, a key event in adaptive immunity to the parasite, reached normal levels later during acute infection. To test the ability of MyD88 KO mice to generate protective immunity to *T. gondii* we employed a genetically engineered avirulent parasite strain that invades but cannot replicate in host cells. Vaccination with this mutant elicited fully functional Th1 cell differentiation and immunity to challenge infection. Our results show that during *T.*

gondii infection MyD88 signaling is essential for host microbicidal function, but that it is dispensable for the adaptive immune response to this intracellular pathogen.

Materials and Methods

Mice

Female mice (6-12 wks of age) were used throughout these studies. *IL-12p40*$^{-/-}$ mice and C57BL/6 controls were purchased from The Jackson Laboratory. *MyD88*$^{+/+}$ and *MyD88*$^{-/-}$ mice (generated by S. Akira, Osaka University, and kindly provided by Dr. E. Pearlman, Case Western Reserve University) were generated by crosses of heterozygous mice. To genotype F_1 litters, tail snips were lysed in the Lysis Reagent with Proteinase K according to the manufacturer's instructions (Viagen Biotech, Los Angeles, CA). DNA in digests were subjected to RT-PCR analysis to determine the mouse genotype using a protocol kindly supplied by Dr. E. Pearlman (Case Western Reserve University). Primers employed for sequence amplification were 5'-TGG-CAT-GCC-TCC-ATC-ATA-GTT-AAC-C-3' (MyD88F), 5'-GTC-AGA-AAC-AAC-CAC-CAC-CAT-GC-3' (MyD88R), and 5'-ATC-GCC-TTC-TAT-CGC-CTT-CTT-GAC-G-3' (MyD88Neo). WT and KO status of the animals was confirmed after the mice were euthanized in experiments. Littermate female age-matched mice between 6-12 wk of age were used for experiments. The mice were housed under specific pathogen-free conditions at the Transgenic Mouse Facility, College of Veterinary Medicine, Cornell University overseen by the Institutional Animal Care and Use Committee.

Parasites and Infections

Cysts of the low virulence ME49 strain were obtained from brains of chronically infected Swiss-Webster mice. Oral infections were carried out by intubation with a

blunt-ended needle. Except where noted, 20 cysts in a volume of 0.2 ml PBS were administered. Tachyzoites of the RH and *cps1-1* strain were maintained in vitro on human foreskin fibroblasts. For the case of *cps1-1*, 0.3 mM uracil was added to the growth medium. Vaccination with *cps1-1* was accomplished by i. p. injections of 2 x 10^4, 2 x 10^5 and 2 x 10^5 tachyzoites at biweekly intervals. Two weeks after the final injection, animals were challenged by subcutaneous injection of 2000 RH strain tachyzoites.

Histopathology

Tissues were collected and preserved in 10% neutral buffered formalin and submitted to the Histology Unit, College of Veterinary Medicine, Cornell University to be processed into paraffin embedded blocks for H & E staining. Lesions were scored in blind manner and graded based on severity of inflammation (minimal, mild, moderate, severe), number of inflammatory foci, distribution of inflammation, type of inflammatory reaction and severity of necrosis.

Immunohistochemistry

Sections of formalin-fixed paraffin-embedded tissues were stained with a rabbit anti-*Toxoplasma* antiserum by the Histology Unit, College of Veterinary Medicine, Cornell University. Other sections were stained with rabbit anti-MPO or rabbit anti-Irgm3/IGTP (59) using a standard immunoperoxidase staining protocol. Briefly, sections were de-paraffinised and rehydrated by serial immersion in xylene and graded alcohols, then finally water. Exogenous peroxidase activity was quenched by incubation in 0.5% H_20_2 followed by microwave treatment in citrate buffer (0.1 M citric acid in PBS, pH 6.0) to unmask antigens. Sections were blocked in 10% normal serum in casein blocking reagent (Vector Labs) (20 min, 20°C). Primary antibody was

diluted in PBS with casein blocking reagent, and applied to the tissue (2 hrs, 37°C). Sections were washed 4 times in PBS with 0.4% Brij® (Sigma). A biotinylated secondary antibody was diluted in PBS and incubated with the tissue (20 min, 20°C). Sections were washed 3 times (PBS with 0.4% Brij®). Biotin was detected using streptavidin-conjugated peroxidase (20 min, 20°C). Slides were subsequently washed 3 times (PBS with 0.4% Brij®) and visualized with 3-amino-9-ethylcarbazole (AEC) chromagen (Vector Labs) (10 min, 20°C). Sections were rinsed in water, counterstained in Gill's Hematoxylin (Vector Labs), mounted and examined by bright-field microscopy. The specificity of Irgm3/IGTP staining was confirmed by examination of tissues from *Irgm3/Igtp*$^{-/-}$ mice.

Flow cytometry

For phenotypic analysis of splenocyte and mesenteric lymph node cells, cell surface staining was accomplished as previously described (60) using the following fluorochrome conjugated mAb: anti-CD11c-PE, anti-Gr-1-PerCP-Cy5.5, anti-B220-PE, anti-CD8-PE-Cy7, anti-CD4-FITC, anti-CD44-APC (all from BD Pharmingen), anti-F480-APC, anti-CD3-APC (all from Invitrogen), anti-CD62L-PE, anti-CD25-PE, anti-CD69-PE (all from eBioscience). Intracellular cytokine staining was accomplished by incubating single cell suspensions in cDMEM (DMEM supplemented with 10% bovine growth serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.05 mM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, and 30 mM HEPES) with or without STAg or *cps1-1*. Golgiplug (BD Biosciences) was added for the last 4 hr of culture. Cells were washed and surface stained for CD4 and CD8 (20 min, 4°C). Cells were then fixed and permeabilized in cytofix/cytoperm solution (BD Biosciences) according to the manufacturer's instructions. Cells were washed in Perm Wash (BD Biosciences) and

resuspended in intracellular staining buffer containing 10% normal mouse serum, 10% rat IgG and optimal concentrations of fluorochrome-tagged anti-cytokine antibodies in Perm Wash (60 min, 4°C). Cells were washed twice in Perm Wash, resuspended in FACS buffer and immediately analyzed on a FACScalibur flow cytometer (BD Biosciences) collecting at least 50,000 events per sample. Data were subsequently analyzed using FlowJo software (Tree Star).

Cell culture

Single cell suspensions were prepared and red blood cells were removed by using RBC lysis buffer according to manufacturer's protocol (Sigma-Aldrich). Cell debris was removed by filtering through a 40 µm cell strainer. Cells were washed with PBS and resuspended in cDMEM. Viable cell number was determined by trypan blue exclusion. For cytokine protein measurement, cells were plated in a 96-well plate (10^7/ml). Soluble tachyzoite antigen (STAg) or *cps1-1* tachyzoites were added and cells were incubated at 37ºC in humidified 5% CO_2 for 48 and 72 hrs, then supernatants were recovered for ELISA.

ELISA

Cytokine ELISA for IFN-γ and IL-12/23p40 were carried out as previously described (61).

Statistical analyses

Student's t test was employed to analyze statistical differences between groups. P values < 0.05 were considered significant. Survival curves were analyzed using a logrank test. All experiments were repeated a minimum of 2-3 times.

Results

MyD88 is required to survive oral infection with *T. gondii*

It was previously shown that genetic inactivation of Toll/IL-1R adaptor molecule MyD88 rendered mice highly susceptible to i. p. infection with *Toxoplasma* (14, 15). We sought to determine if lack of MyD88 also resulted in susceptibility during infection initiated by the natural route of infection, namely, through oral inoculation. As shown in Fig. 4.1A, *MyD88*$^{-/-}$ mice, but not *MyD88*$^{+/+}$ littermates, uniformly succumbed to infection after oral administration of low virulence ME49 cysts. Interestingly, in parallel groups of mice i. p. infected with the same cyst number, *MyD88*$^{-/-}$ mice succumbed at a slightly, but significantly ($p = 0.0015$), accelerated rate (Fig. 4.1B). In H & E staining of gut and Peyer's patches we failed to detect major differences in pathology of WT and KO mice, but in livers we found MyD88-dependent inflammatory infiltrates (Fig. 4.2).

Death during i. p. infection of *MyD88*$^{-/-}$ animals is associated with uncontrolled tachyzoite replication and dissemination (14, 15). Likewise, we found greater numbers of parasites during low dose oral infection of *MyD88*$^{-/-}$ relative to *MyD88*$^{+/+}$ littermates. In WT mice, low numbers of tachyzoites were present in lamina propria (Fig. 4.3A) and Peyer's patches (Fig. 4.3C) of the small intestine. In contrast, high parasite numbers were present at these locations in KO animals (Fig. 4.3B and D, respectively). Regions of lymphocyte depletion were also apparent in Peyer's patches of KO mice (Fig. 4.3D) and these areas were markedly less extensive in WT animals (Fig. 4.3C). These differences were even more striking during high dose ME49 infection, where uncontrolled parasite replication was apparent in Peyer's patches of *MyD88*$^{-/-}$ but not *MyD88*$^{+/+}$ littermates (Fig. 4.4).

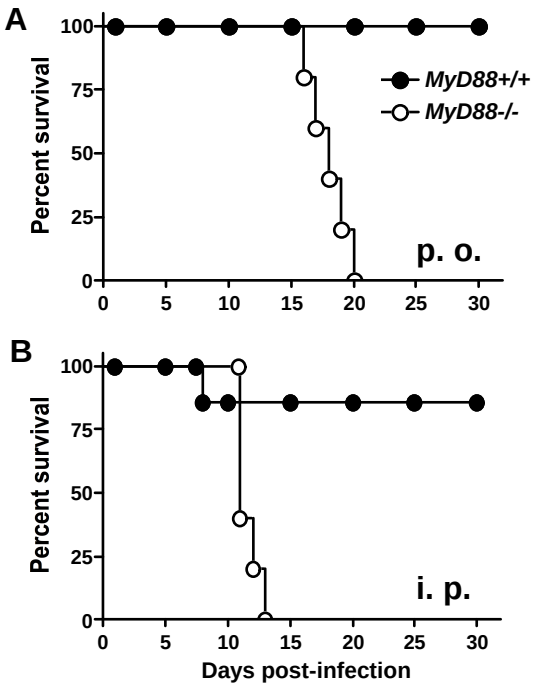

Figure 4.1. MyD88 is required to survive oral infection with *Toxoplasma gondii.* Groups of *MyD88*$^{-/-}$ and *MyD88*$^{+/+}$ littermates (5 per strain) were orally and i. p. infected at the same time with 20 cysts of the ME49 strain. Panel A, oral infection; panel B, i. p. infection. Closed symbols, *MyD88*$^{+/+}$ mice; open symbols, *MyD88*$^{-/-}$ mice.

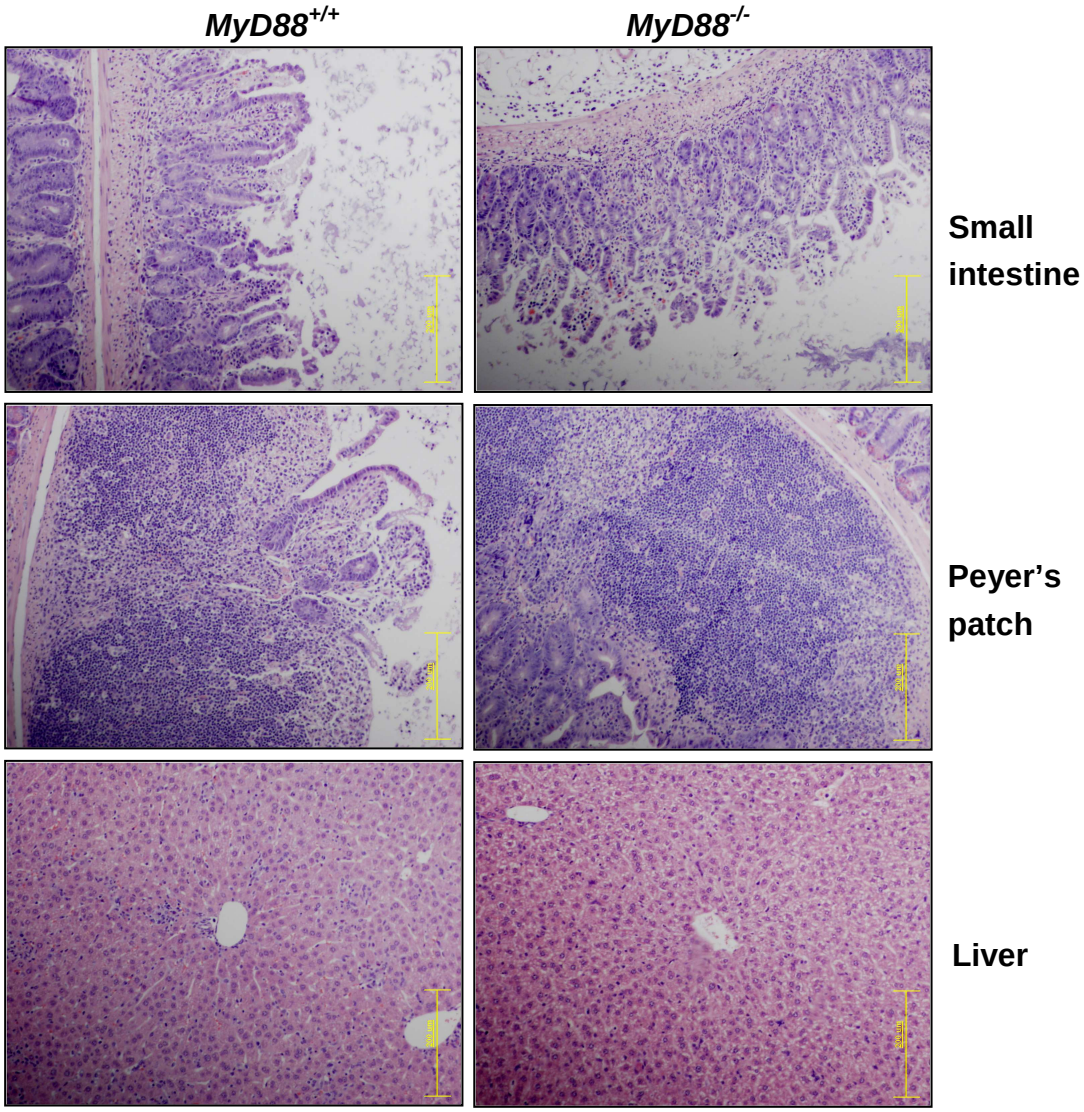

Figure 4.2. Pathology of *MyD88*$^{-/-}$ and *MyD88*$^{+/+}$ mice during oral infection. Mice (2 per group) were orally infected with 20 ME49 cysts, then at Day 7 post-inoculation the indicated tissues and organs were harvested for staining by H & E. In both strains, histopathological changes were present in gastrointestinal tracts, particularly in the Peyer's patches, and livers. The bar in D denotes 200 µm.

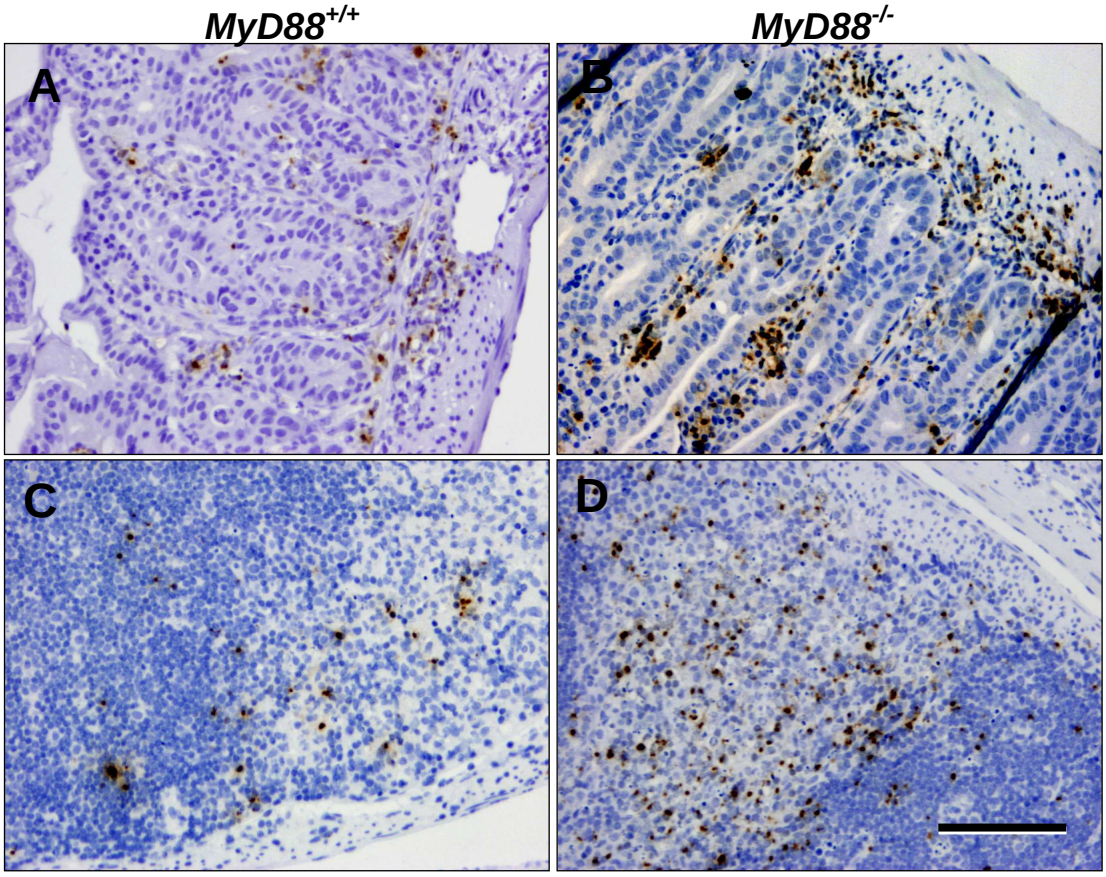

Figure 4.3. Parasite number in gut mucosal tissue during oral infection is increased in the absence of MyD88. Wild-type (A and C) and knockout (B and D) mice were infected with 20 ME49 cysts and gut tissues were collected 7 days later. Samples were subjected to immunohistochemical staining using an anti-*Toxoplasma* antiserum. A and B, villi and underlying lamina propria. B and D, Peyer's patches. The bar in D denotes 100 µm.

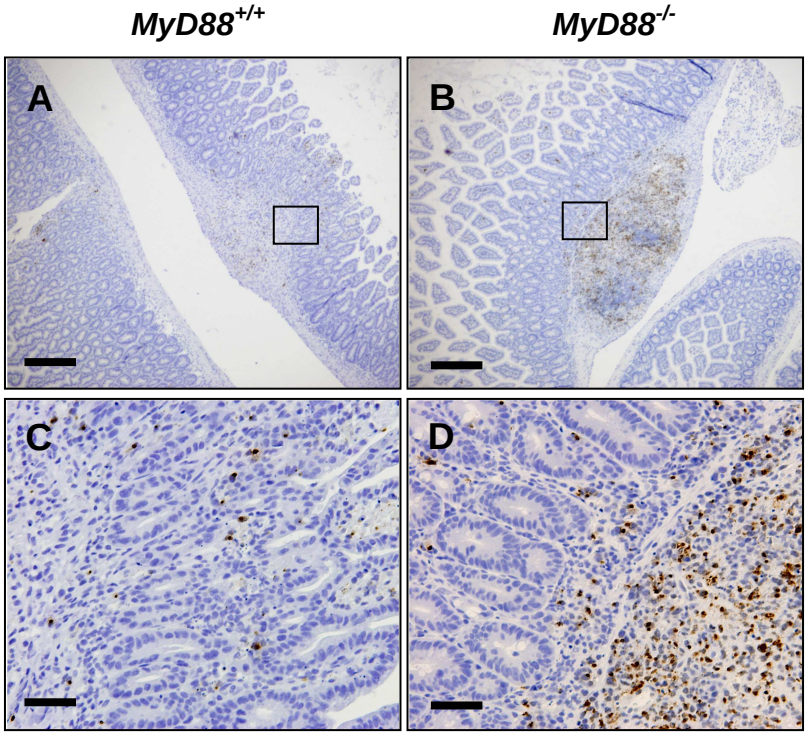

Figure 4.4. Increased parasite numbers in Peyer's patches in *MyD88*$^{-/-}$ mice during high dose oral infection. Wild-type (A and C) and MyD88 knockout (B and D) mice were orally infected with 100 ME49 cysts. Ten days later, small intestines were isolated and subjected to immunohistochemical staining with an anti-*Toxoplasma* antiserum. The regions delineated by rectangles in A and B were imaged at high magnification in C and D. In A and B, the bar denotes 250 µm; in C and D the bar denotes 50 µm.

Early but not late neutrophil recruitment is dependent upon MyD88

We next examined mesenteric lymph node and spleen cell populations of WT and KO mice undergoing oral *T. gondii* infection. In mesenteric lymph nodes of Day 4-infected animals, there was a striking decrease in Gr-1^+ neutrophil recruitment in KO relative to WT animals, but populations of macrophages (MØ), DC, as well as B and T lymphocytes were normal (Fig. 4.5A). Nevertheless, neutrophil numbers in mesenteric lymph nodes recovered in *MyD88*$^{-/-}$ mice by day 7 post-infection (Fig. 4.5B). Similarly, neutrophil numbers in spleens of Day 4-infected KO animals were less than in WT littermates (Fig. 4.5C), but this difference was minimal at day 7 post-infection (Fig. 4.5D). We next stained sections of small intestine with antibody to myeloperoxidase (MPO), an enzyme that is expressed at high level in PMN. We found an influx of MPO^+ cells into the lamina propria of WT (Fig. 4.5E) but not MyD88 KO (Fig. 4.5F) animals at day 4 post-infection. In day 7-infected mice, lamina propria neutrophils were recruited in greater number in WT mice relative to day 4 (Fig. 4.5G), and this response did not require functional MyD88 (Fig. 4.5H). We, and others, previously reported rapid recruitment of Gr-1^+ neutrophils into the peritoneal cavity early during i. p. infection with high virulence RH strain tachyzoites (36, 37). In Fig. 4.5I we show that neutrophil recruitment in this model is also highly MyD88-dependent.

IL-12/23p40 production is MyD88-dependent, whereas IFN-γ production is delayed but reaches normal levels in the absence of MyD88

During in vitro parasite antigen stimulation of splenocytes (Fig. 4.6A-C) and mesenteric lymph node cells (Fig. 4.6D-F), production of IL-12/23p40 was highly MyD88-dependent using as a cell source noninfected (A and D), Day 4 (B and E) and Day 7 (C and F) orally infected mice. Nevertheless, in splenocytes at all time points

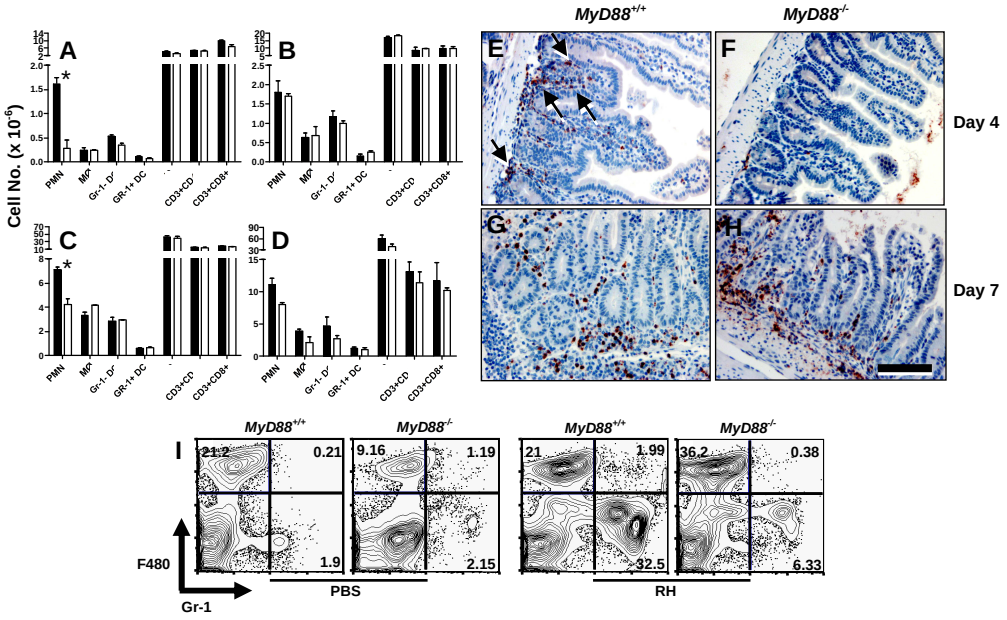

Figure 4.5. Neutrophil recruitment depends upon MyD88 during early infection. Mesenteric lymph node (A and B) and spleen (C and D) cells were prepared from *MyD88*$^{-/-}$ and *MyD88*$^{+/+}$ littermates on Day 4 (A and C) and Day 7 (B and D) post oral infection with ME49 (20 cysts). Cells were subjected to flow cytometric analysis and total cell numbers were subsequently calculated (2 mice per group). Closed bars, *MyD88*$^{+/+}$; open bars, *MyD88*$^{-/-}$. PMN, Gr-1^{+}F480^{-} cells; MØ, Gr-1^{-}F480^{+} cells; DC, CD11c^{+} cells; B, B220^{+} cells. *, p <0.05. In panels E-F, small intestines were collected from *MyD88*$^{+/+}$ (E and G) and *MyD88*$^{-/-}$ (F and H) mice at Days 4 (E and F) and 7 (G and H) after oral ME49 infection. Sections were subsequently subjected to immunohistochemical staining with an anti-myeloperoxidase (MPO) antiserum. Arrows in panel E point to areas of infiltrating MPO^{+} cells. Bar denotes 100 µm. In I, wild-type and knockout mice (n = 2 per strain) were infected by i. p. injection of 10^{6} RH strain tachyzoites. Peritoneal cells were collected 6 hr later for flow cytometric analysis.

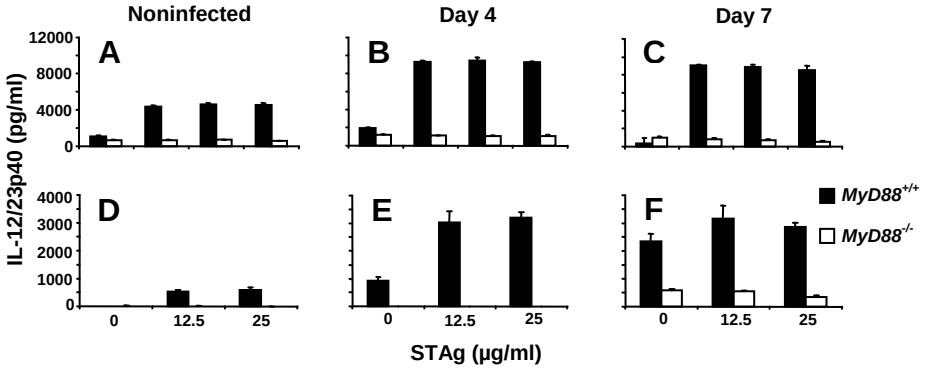

Figure 4.6. Production of IL-12/23p40 during infection is highly MyD88 dependent. Spleen (A-C) and mesenteric lymph nodes (D-F) were collected from noninfected, Day 4 and Day 7 orally infected mice (20 ME49 cysts). Single cell suspensions were prepared and cells were cultured with the indicated amounts of soluble tachyzoite lysate antigen (STAg). Closed bar, cells from wild-type mice; open bar, cells from knockout animals.

low but detectable levels of MyD88-independent p40 could be detected, and at day 7 post-infection residual IL-12 was apparent in mesenteric lymph node cultures from *MyD88*$^{-/-}$ animals.

Next, we measured production of IFN-γ, a cytokine long recognized as the major mediator of resistance to *T. gondii* (38). As shown in Fig. 4.7, antigen-induced IFN-γ release by splenocytes (A) and mesenteric lymph node cells (B) was strictly dependent upon MyD88 at Day 4 post-infection. This result was confirmed and extended by intracellular cytokine staining of soluble tachyzoite lysate (STAg)-stimulated cells (Fig. 4.7C). Thus, in the spleen (SPL), a small proportion CD4^{+} T cells produced IFN-γ in dependence upon MyD88 (0.4 vs. 0.03% for WT and KO, respectively). Interestingly, there was a strong MyD88-dependent IFN-γ response by splenic CD8^{+} T cells under the same conditions (10.0 vs. 0.57% for WT and KO, respectively). However, in mesenteric lymph node (MLN) cultures, the STAg-induced IFN-γ response was relatively minor, and most of the MyD88-dependent IFN-γ derived from CD4^{+} T lymphocytes. It is of further interest to note evidence for a default to the Th2 pathway in *MyD88*$^{-/-}$ mesenteric lymph node CD4^{+} T cells (0.09 vs. 0.56% IL-4-positive in WT and KO, respectively) that was not apparent in splenic cultures (Fig. 4.7C). Thus, as previously reported in an i. p. model of *Toxoplasma* soluble antigen injection (39), there is an increase in the Th2 response in the absence of MyD88, but here we show this response is weak and is restricted to mesenteric lymph nodes during oral infection.

When we examined IFN-γ responses at day 7 post-oral infection, strikingly different results were obtained compared to the Day 4 response. Thus, at Day 7, splenocytes (Fig. 4.8A) and mesenteric lymph node cells (Fig. 4.8B) released high levels of IFN-γ

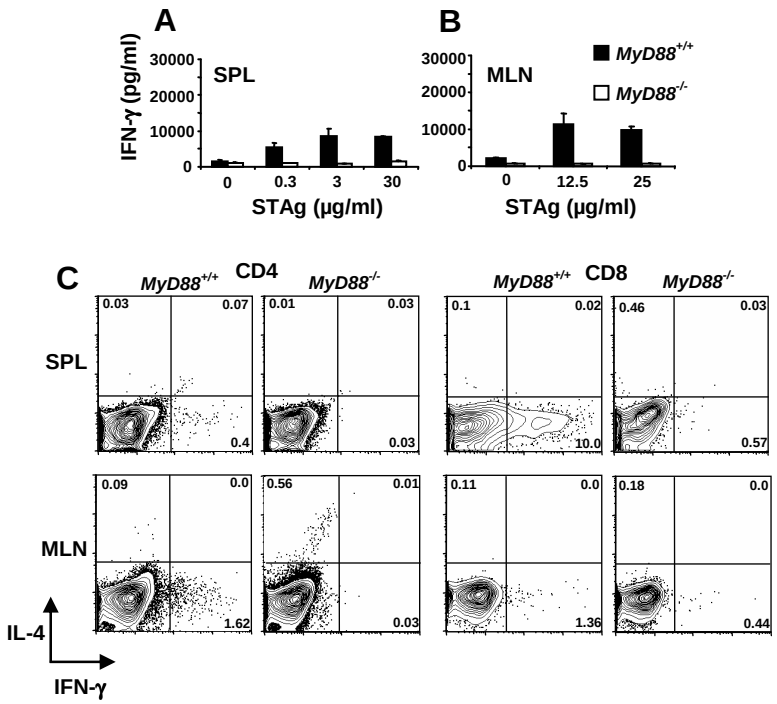

Figure 4.7. Early acute production of IFN-γ during oral *Toxoplasma* infection requires MyD88. Spleen cells (A) and mesenteric lymph node cells (B) from mice orally infected 4 days previously with 20 ME49 cysts were cultured with the indicated amounts of tachyzoite lysate antigen. Closed bar, *MyD88*$^{+/+}$ cells; open bar, *MyD88*$^{-/-}$ cells. (C) Spleen and mesenteric lymph node cells from Day 4 orally infected animals were subjected to CD4 and CD8 surface staining and intracellular IFN-γ and IL-4 staining 72 hr after STAg restimulation (25 μg/ml). Cytokine expression was evaluated after gating on CD4^{+} and CD8^{+} T cells. The results show one representative mouse out of a total of three from each strain.

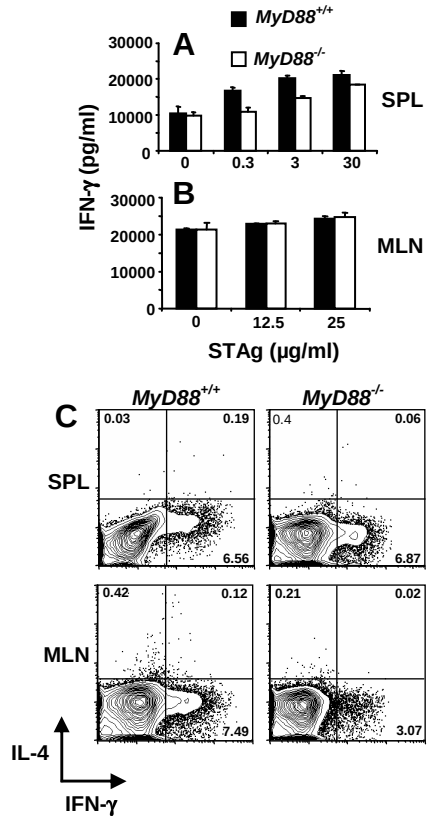

Figure 4.8. Delayed IFN-γ production and emergence of Th1 T cells does not require MyD88. Spleen (A) and mesenteric lymph node (B) cells from Day 7 orally infected wild-type (closed bars) and knockout (open bars) mice were cultured with the indicated amounts of parasite lysate antigen. (C) *MyD88*$^{+/+}$ and *MyD88*$^{-/-}$ cells from spleen and mesenteric lymph nodes of Day 7 orally infected mice were cultured for 6 hr without further antigen stimulation in the presence of Brefeldin A. The cells were subsequently stained for CD4 and intracellular IFN-γ and IL-4. The results show one representative mouse out of a total of three from each strain.

independently of MyD88 and without the need for further in vitro antigen stimulation. In the presence of parasite antigen, these responses were only modestly increased. In Fig. 4.8C, we determined the source of IFN-γ by ex vivo staining splenocytes and mesenteric lymph node cells without further antigen stimulation. In this case, there was a vigorous MyD88-independent splenic $CD4^+$ T cell response (Fig. 4.8C; 6.56 and 6.87% for WT and KO, respectively). In mesenteric lymph nodes, there was also a strong $CD4^+$ T lymphocyte IFN-γ response, although this was partially MyD88 dependent (7.49 vs. 3.07% for WT and KO, respectively). In both spleen and mesenteric lymph nodes, the IFN-γ response by $CD8^+$ T cells was extremely weak (data not shown).

MyD88$^{-/-}$ mice display a defect in Irgm3 (IGTP) expression in gut mucosa at Day 4 but not Day 7 post infection

Previously it was found that IFN-γ-dependent expression of p47 GTPase family member Irgm3/IGTP is necessary to survive *Toxoplasma* infection (40). Here, we determined the expression pattern of this molecule in small intestines of orally inoculated *MyD88*$^{-/-}$ and *MyD88*$^{+/+}$ mice. Interestingly, at day 4 post-infection we detected expression of Irgm3 in endothelial cells in basement membrane-submucosal regions of WT mice (Fig. 4.9A and C) and this response was absent in mice lacking MyD88 (Fig. 4.9B and D). However, at day 7 after infection, small intestine epithelial cells of WT mice were strongly positive for Irgm3, and this response was identical in tissues from MyD88 KO animals (Fig. 4.9). In sum, during early (day 4) oral infection, IL-12/23p40 and T cell IFN-γ expression was dependent upon MyD88. Similarly, neutrophil recruitment and Irgm3 expression, two responses previously implicated in resistance to *Toxoplasma* (40, 41), also relied on functional MyD88. Nevertheless, with the exception of IL-12/23p40 expression, each of these immune

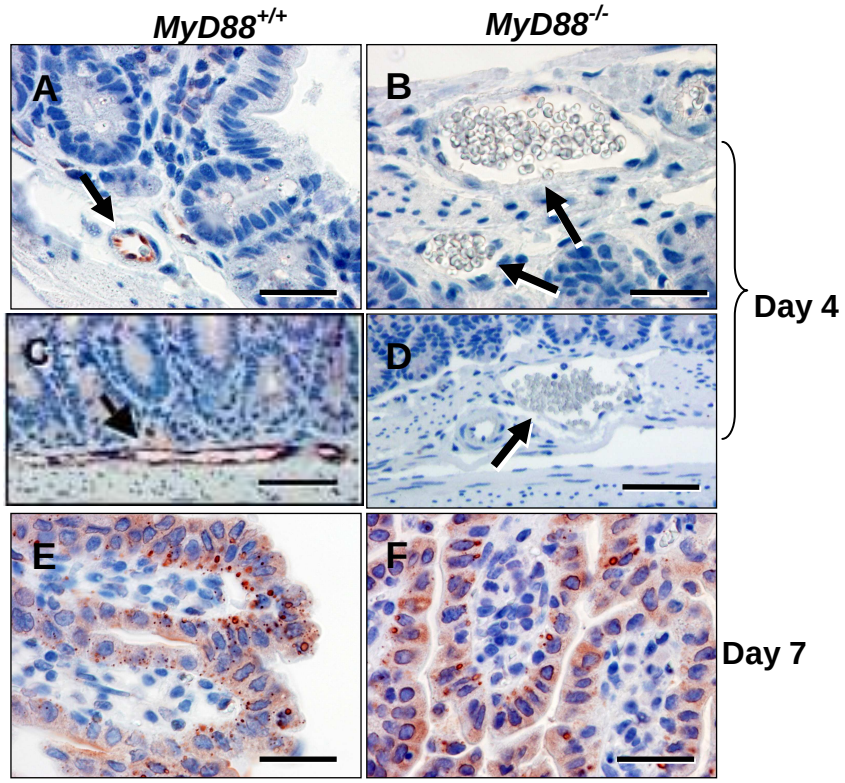

Figure 4.9. Early endothelial cell expression of Irgm3/IGTP is dependent upon MyD88. *MyD88*$^{+/+}$ (Panels A, C and E) and *MyD88*$^{-/-}$ (panels B, D and F) were orally infected and small intestine tissue was collected 4 (panels A-D) and 7 (panels E and F) days later. Endothelial cells in WT (arrows in panels A and C) but not KO (arrows, panels B and D) stained positive for Irgm3/IGTP. At day 7 post-infection, epithelial cells of both WT (panel E) and KO (panel F) were Irgm3/IGTP positive. The results show one representative mouse out of a total of three from each strain. In panels A, B, E and F the bar denotes 20 µm. In panels C and D the bar indicates 40 µm.

responses reached normal levels without the need for MyD88 by day 7 after infection.

MyD88 is not required for protective immunity to *Toxoplasma*

Although the delayed T cell IFN-γ response did not require MyD88 during oral infection (Fig. 4.8), and although $CD4^+$ and $CD8^+$ T lymphocyte expression of activation marker CD69 was identical in the presence and absence of MyD88 (Fig. 4.10), KO mice were unable to control infection and the animals succumbed to *Toxoplasma* (Fig. 4.1). This precluded us from rigorously testing whether *MyD88*$^{-/-}$ mice developed a protective immune response to challenge infection, which is known to depend upon T cell production of IFN-γ in normal mice (30). To circumvent this limitation, we employed *Toxoplasma* mutant *cps1-1*. This RH-derived parasite strain is a uracil auxotroph that invades but does not replicate in host cells. Infection of mice with *cps1-1* results in nonpersistent infection that fails to cause pathology associated with wild type *T. gondii* infection (42). Importantly, *cps1-1* vaccination has been shown to induce protective immunity to challenge infection (42, 43). As shown in Fig. 4.11A (graph a), both *MyD88*$^{+/+}$ and *MyD88*$^{-/-}$ mice vaccinated with *cps1-1* developed protective immunity to challenge with RH strain parasites. In contrast, nonvaccinated mice succumbed within two weeks of infection with this highly virulent parasite strain. We also vaccinated *IL-12/23 p40*$^{-/-}$ mice with *cps1-1* (graph b). In this case, the animals failed to develop a protective immunity to RH challenge.

We examined naïve ($CD62L^{hi}CD44^{lo}$), Effector/Effector Memory ($CD62L^{lo}CD44^{hi}$), and Central Memory ($CD62L^{hi}CD44^{hi}$) splenic T cell populations after *cps1-1* vaccination of *MyD88*$^{+/+}$ and *MyD88*$^{-/-}$ animals. As shown in Fig. 4.11B, there were no major differences between WT and KO populations amongst $CD4^+$ (a and b) or $CD8^+$ (c and d) T cell populations. To distinguish between Effector and Effector

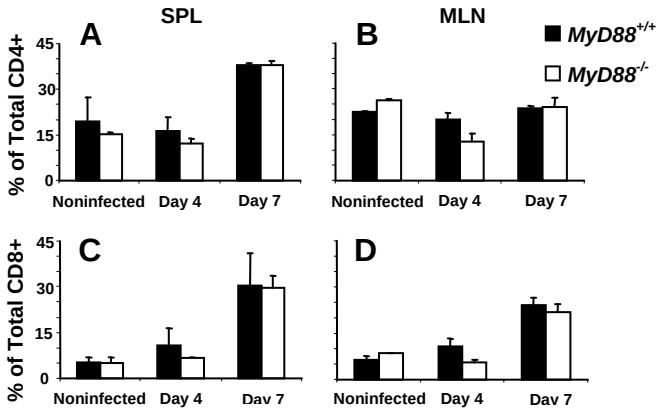

Figure 4.10. Normal expression of CD69 in the absence of MyD88 during *T. gondii* infection. A-B, expression of CD69 on $CD4^+$ T cells in the spleen and mesenteric lymph node, respectively, was examined in noninfected, Day 4, and Day 7 orally infected $MyD88^{+/+}$ (closed bars) and $MyD88^{-/-}$ (open bars) mice. C-D, expression of CD69 on $CD8^+$ T cells in the spleen and mesenteric lymph node, respectively. Two animals were used at each time point.

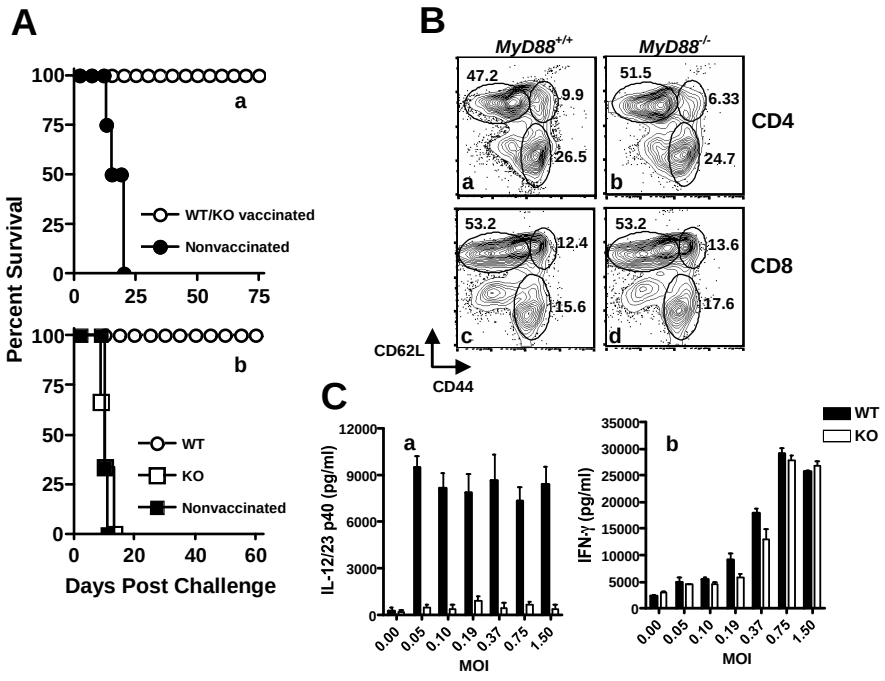

Figure 4.11. Adaptive immunity to *T. gondii* is functional in the absence of MyD88. In panel A, mice were vaccinated by i. p. injection of *Toxoplasma* uracil auxotroph *cps1-1* then challenged two weeks later by subcutaneous injection of RH strain tachyzoites. In graph a, MyD88 WT and MyD88 KO animals were used. In graph b, IL-12p40 WT and IL-12p40 KO mice were subjected to vaccination and challenge. Panel B, two weeks following *cps1-1* vaccination, splenic $CD4^+$ (a and b) and $CD8^+$ (c and d) T lymphocytes from WT (a and c) and MyD88 KO (b and d) animals were assessed for expression of CD62L and CD44 to identify populations of naïve ($CD62L^{hi}CD44^{lo}$), Effector/Effector Memory ($CD62L^{lo}CD44^{hi}$), and Central Memory ($CD62L^{hi}CD44^{hi}$) cells. In panel C, two weeks after vaccination with *cps1-1*, splenocytes *MyD88*$^{+/+}$ and *MyD88*$^{-/-}$ mice were subjected to in vitro stimulation with the indicated multiplicity of infection (MOI) of *cps1-1*. Supernatants were assessed populations that both possess a $CD62L^{lo}CD44^{hi}$ surface phenotype, we assessed CCR7 from production of IL-12/23p40 (graph a) and IFN-γ (graph b).

Memory populations that both possess a $CD62L^{lo}CD44^{hi}$ surface phenotype, we assessed CCR7 that is expressed at high level on Effector but not Effector Memory subpopulations. Here again, we found no difference on these populations amongst WT and KO T cells (Fig. 4.12).

In splenocyte cultures from *cps1-1* vaccinated mice, production of IL-12/23p40 was barely detectable in cells from MyD88 KO compared to the response of WT cells (Fig. 4.11C, graph a). In contrast, the IFN-γ recall response of *MyD88*$^{-/-}$ cells was indistinguishable from that of cells from WT littermates (Fig. 4.11C, graph b). We further probed the source of IFN-γ employing intracellular cytokine staining. Both CD4+ and CD8+ T cells from KO and WT mice contributed to IFN-γ production during in vitro recall with *cps1-1* parasites (Fig. 4.13). Thus, *cps1-1* vaccination of *MyD88*$^{-/-}$ mice results in protective adaptive immunity that, by the parameters measured, appears identical to that induced in *MyD88*$^{+/+}$ animals.

Discussion

TLR-MyD88 signaling is required for resistance to several protozoan pathogens, and parasite ligands that possess TLR activating capability are increasingly being identified (10). For the case of *Toxoplasma,* parasite profilin and tachyzoite surface GPI anchors are recognized by TLR11 and TLR2/4 respectively (18, 32, 33). In vitro studies using models of i. p. infection or i. v. tachyzoite injection have shown that MyD88 is required to control infection, and that the immune response to the parasite deviates from a Th1 to a Th2 response in the absence of this TLR adaptor (14, 39).

Here, we examined how lack of MyD88 signaling impacts host defense in animals undergoing oral infection with *T. gondii*. As during i. p. infection, orally infected

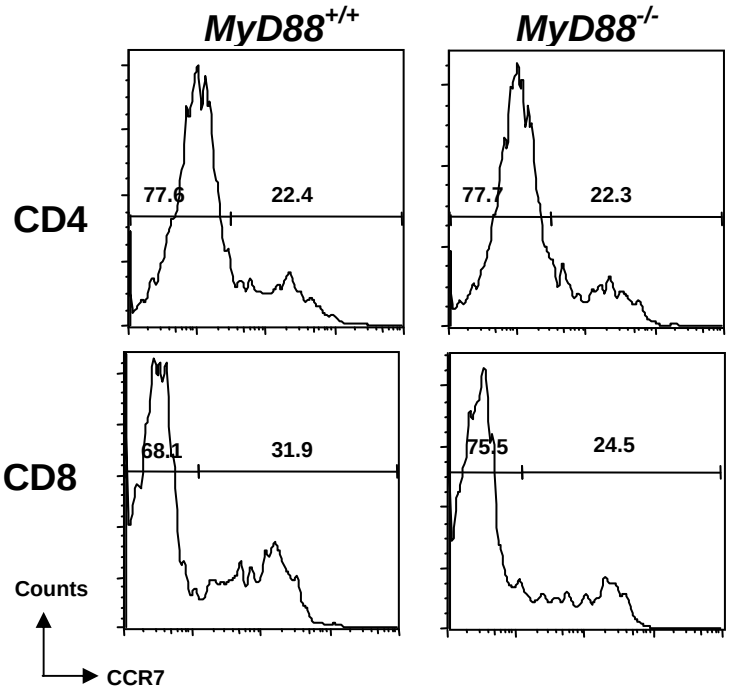

Figure 4.12. Normal distribution of CCR7 on Effector/Effector Memory T cell populations in *cps1-1* vaccinated *MyD88*$^{-/-}$ mice. Splenocyte populations from mice vaccinated with *cps1-1* were subjected to flow cytometric analysis with antibodies specific for CD4, CD8, CD44, CD62L and CCR7. $CD4^+$ and $CD8^+$ Effector/Effector Memory ($CD44^{hi}CD62L^{lo}$) were assessed for CCR7 expression to distinguish Effector ($CCR7^+$) from Effector Memory ($CCR7^-$) subpopulations.

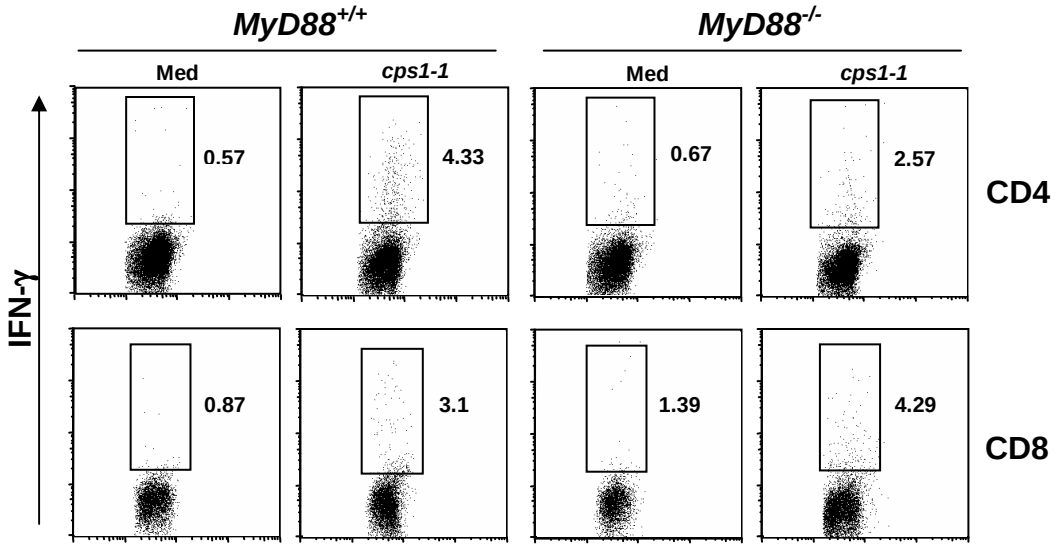

Figure 4.13. Vaccination with *cps1-1* generates splenic CD4$^+$ and CD8$^+$ T cells that contribute to IFN-γ production in *MyD88*$^{+/+}$ and *MyD88*$^{-/-}$ mice. Splenocytes from *cps1-1*-vaccinated WT and KO animals (3 per group) were cultured for 48 hr in medium alone or with *cps1-1* at a ratio of 0.5 parasites per cell. Brefeldin A was added during the last 4 hr of culture. Cells were subsequently recovered for intracellular IFN-γ staining and surface staining for CD4 and CD8.

MyD88$^{-/-}$ mice displayed early death associated with uncontrolled parasite replication. Like other studies employing the i. p. infection model, the IL-12 response was severely curtailed during oral infection of MyD88 KO mice. Early during oral infection of *MyD88*$^{-/-}$ animals, we found that neutrophil recruitment to mucosal tissues was defective, as was production of T cell IFN-γ and induction of Irgm3/IGTP, an IFN-γ-inducible p47 GTPase required to survive acute *T. gondii* infection (40). Importantly, later during acute infection MyD88-independent Th1 responses emerged. The appearence of an apparently intact Th1 response in the absence of MyD88 prompted us to examine whether we could elicit protective immunity in the absence of this signaling adaptor. Accordingly, we vaccinated mice with the avirulent *cps1-1 Toxoplasma* strain. This treatment stimulated protective immunity and strong IFN-γ production independently of MyD88. Taken together, our data suggest MyD88 signaling exerts anti-*Toxoplasma* activity at a critical early time in infection, but that it is not required to induce strong Th1-based immunity to this parasitic pathogen.

Our results were unexpected because it has been reported that MyD88 signaling plays a role in shaping adaptive immunity to *T. gondii*. For example, during i. p. *Toxoplasma* infection, defective T cell IFN-γ production was observed during acute infection (14). Following i. v. injection of soluble tachyzoite lysate antigen, the immune response in *MyD88*$^{-/-}$ animals deviates from a Th1 to Th2 cytokine response following i. v. injection of soluble tachyzoite lysate antigen (39). In addition, i. v. injection of parasite profilin has been shown to trigger a TLR11/MyD88-dependent Th1 response to the profilin molecule itself (35). Recently, it was found that MyD88 expression in T cells was required for IFN-γ production and survival during i. p. *T. gondii* infection (44). We do not completely understand why *MyD88*$^{-/-}$ animals undergoing oral *T. gondii* infection develop strong, albeit delayed, Th1 responses,

when other routes of infection and antigen delivery elicit MyD88-dependent Th1 responses. It seems possible that animals succumb with insufficient time to generate a Th1 response during i. p. infection. Another possibility is that DC populations in mucosal tissues, or possibly signals elicited by gut flora in combination with *Toxoplasma*, play a role in triggering MyD88-independent Th1 immunity during oral infection (45, 46). For the case of protective immunity induced by *cps1-1* vaccination, it is might be that multiple exposure to low doses of nonvirulent tachyzoites induces MyD88-independent adaptive immunity that is not elicited by i. p. infection with a single dose of parasites.

Our results suggest that the major role for MyD88 during oral *Toxoplasma* infection is in anti-microbial effector activity. This is in line with several other groups. For example, T cell-dependent antibody responses induced by a panel of adjuvants proceeded normally in the absence of MyD88 and TRIF signaling (47). Similarly, MyD88-deficient mice mount normal protective antibody responses and inflammatory arthritis during *Borrelia burgdorferi* infection (27). During infection with the fungal pathogen *Aspergillus fumigatus* Th1 responses in infected airways do not require MyD88, although T-bet induction was enhanced by MyD88 signaling in the draining lymph nodes (48). It was also found that *Listeria* infection induces a protective $CD8^+$ T cell response in the absence of MyD88 signaling (25, 26). During aerosol infections with *Mycobacterium tuberculosis*, there is evidence both for and against a role for MyD88 in adaptive immunity, depending on the study (9, 49-52). Regardless, in most of these cases, and as during oral *Toxoplasma* infection, while acquired immunity could proceed without MyD88, this adaptor molecule was clearly required for pathogen control during innate immunity.

At present we do not know why *MyD88*$^{-/-}$ mice are unable to control *T. gondii* infection. Recruitment of neutrophils to mucosal tissues was defective in the knockout mice during early stages of infection, and this possibly underlies the susceptibility of these animals. Others have also found that neutrophil recruitment during infection relies upon MyD88-dependent signaling (53, 54). Previously, we reported that depletion of neutrophils with an anti-Gr-1 antibody resulted in susceptibility to oral *T. gondii* infection (41). Therefore, it is possible that defective neutrophil recruitment during early infection allows increased parasite survival in the intestine, ultimately leading to death of the animals. Nevertheless, we interpret results obtained using anti-Gr-1 mAb with caution, since the Gr-1 epitope is also expressed by other types of myeloid cells (55).

Our data for the first time show tissue localization of IFN-γ-inducible Irgm3/IGTP, a molecule required to survive acute *Toxoplasma* infection (40). Interestingly, Irgm3/IGTP was expressed in endothelial cells of the intestinal submucosa in an MyD88-dependent manner during early infection. Later, this p47 GTPase was highly expressed in epithelial cells of the small intestine in both *MyD88*$^{+/+}$ and *MyD88*$^{-/-}$ mice. It is difficult to understand how early MyD88-dependent Irgm3/IGTP expression confined to endothelial cells could contribute to resistance to *T. gondii* infection, which is uniformly spread throughout mucosal tissues. Although the function of Irgm3/IGTP and other p47 GTPases is not well understood, these molecules have been implicated in autophagic destruction of the parasitophorous vacuole in tachyzoite-infected macrophages and astrocytes (56, 57). However, we have so far not detected differences in IFN-γ-mediated macrophage killing of tachyzoites in the presence and absence of MyD88 (Fig. 4.14).

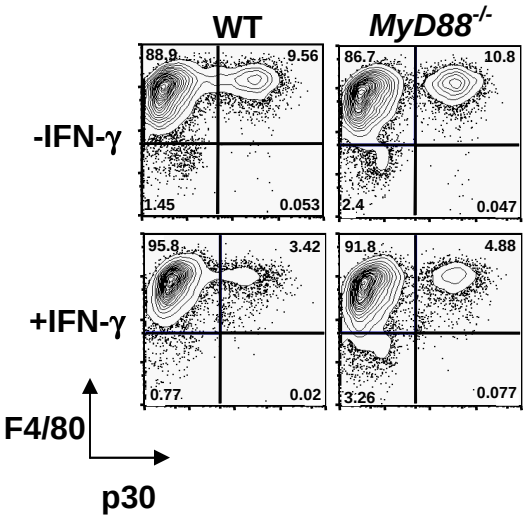

Figure 4.14. IFN-γ-activated macrophages efficiently control infection in the absence of MyD88. Bone marrow-derived macrophages were subjected to 18 hr activation with IFN-γ or incubation in medium alone. Cells were subsequently infected with PTG strain tachyzoites, and infection levels were determined 12 hr later by flow cytometry, assessing levels of the tachyzoite surface p30 antigen in F4/80-positive macrophages.

Strong MyD88-independent T cell IFN-γ responses were elicited during both oral ME49 infection and *cps1-1* vaccination. Inasmuch as production of IL-12 in both cases was minimal, this raises the question of how T cell IFN-γ responses are generated in these cases. Two related possibilities are that residual MyD88-independent IL-12 is sufficient to drive Th1 differentiation, or that more robust MyD88-independent IL-12 responses occur at sites other than the draining lymph nodes and spleen that were examined in this study. It is also possible that Th1 responses are triggered independently of IL-12 in the absence of MyD88. However, at least for the case of *cps1-1*-induced immunity, absence of IL-12/23p40 resulted in lack of a protective response, a result that argues for IL-12 as a mediator of protection in *cps1-1* vaccinated *MyD88*$^{-/-}$ animals. In this regard, we have found that bone marrow-derived macrophages infected with the RH *Toxoplasma* strain produce IL-12 independently of signaling through MyD88 (58), yet the same parasite strain clearly triggers MyD88-dependent IL-12 in splenic DC (our unpublished results and refs. 14, 18). Further work will be required to determine host mediators involved in generating MyD88-independent Th1 responses during *Toxoplasma* infection.

In sum, our results argue that MyD88-dependent signaling is crucial in anti-microbial effector function against *T. gondii*. Nevertheless, the view that this adaptor molecule plays an essential role in bridging innate and adaptive immunity to *Toxoplasma* may require revision. Other signaling molecules and recognition systems are also likely to be important determinants of adaptive immunity to this parasite. Identifying such molecules will provide important insight into immune recognition of *Toxoplasma* and other microbial pathogens.

Acknowledgements

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CHAPTER 5

DISCUSSION

1. Summary of findings

Toxoplasma gondii infection elicits strong Th1 immunity. IL-12-dependent IFN-γ production is essential for resistance to *T. gondii* infection (1-3). Increased host susceptibility to microbial infection in Gr-1^{+}-depleted mice suggests a role for this granulocyte population in providing resistance during *Toxoplasma* and other microbial infections (4-6). The neutrophil is a source of IL-12, and it may be released from pre-formed stores or synthesized de novo (7). The JNK signaling pathway has been implicated in IL-12 production from macrophages and dendritic cells (8). In Chapter 2 I found that JNK2 is the major isoform expressed in mouse neutrophils. Importantly, JNK2 plays a major role in PMN IL-12 and CCL2/MCP-1 production in response to *Toxoplasma* infection. In contrast, JNK2 is not required for oxidative burst and phagocytic activity in mouse PMN, although I found a partial role for the MAPK in chemotaxis.

In Chapter 3 I examined the in vivo response to *Toxoplasma* in the absence of JNK2. I hypothesized that JNK2-deficient mice would be more susceptible to *T. gondii* infection as a result of reduced IL-12 production by PMN. Surprisingly, JNK2 deletion confered resistance to *Toxoplasma*, and this was associated with lower parasite burden in the brain and intestinal tissues. Also, I found that immune-mediated gut pathology was alleviated in the absence of this MAPK along with decreased PMN number in intestinal tissues. In contrast to in vitro data, IL-12 production from splenocytes and splenic DCs cultured with parasite molecules was slightly higher in the absence of JNK2. IFN-γ was also found slightly increased in splenocytes cultured with parasite antigens. Thus, this evidence leads me to believe that JNK2 expression contributes to enhanced susceptibility during *Toxoplasma* infection.

Since JNK2 does not seem to be required for IL-12 induction during *T. gondii* infection in vivo, I was interested in finding other signaling molecules that were needed for IL-12 production and resistance to in vivo infection with this parasite. MyD88 is a key component of the TLR signaling pathway that is responsible for IL-12 synthesis in response to intraperitoneal *Toxoplasma* infection (9). In Chapter 4, we examined the role of this adaptor molecule in the context of oral infection, the natural mode of transmission. In common with i. p. infection (9), MyD88 was also required for resistance to *T. gondii* introduced by oral infection. Decreased PMN recruitment was observed in the absence of MyD88. While IL-12 responses were highly dependent upon this adaptor molecule, splenic $CD4^+$ and $CD8^+$ IFN-γ responses reached normal levels in the absence of MyD88. Furthermore, $MyD88^{-/-}$ mice vaccinated with an attenuated parasite strain developed protective immunity to lethal challenge infection, and this was associated with a strong Th1 response. Thus, while MyD88 is required for controlling *T. gondii* replication it is not needed for development of protective immunity against *Toxoplasma* infection.

2. Role of JNK2 in response to *T. gondii* infection in vitro and in vivo

The JNK signaling pathway has been widely implicated in diverse physiological processes including embryonic development, cell survival, apoptosis, proliferation, and inflammatory responses (10). In the immune response, there is evidence that JNK signaling is required for polarized Th1 differentiation and effector cytokine production (11). However, the role of JNK appears to be dependent on the context of cell type and biological function. As previously reported from our lab and in contrast to PMN, JNK2 is not required for *Toxoplasma*-induced IL-12 production in bone-marrow derived macrophages. However, a pharmacological JNK specific inhibitor, SP600125, inhibits parasite-triggered IL-12 release in macrophages (12). This suggests that other

JNK, most probably JNK1, plays a role in *T. gondii*-induced IL-12 induction. As shown in Chapter 2 and 3, the defect of PMN IL-12 production in the absence of JNK2 in vitro does not reflect the overall in vivo IL-12 response during infection of JNK2 KO mice. In this case, I found evidence that JNK2 negatively regulates IL-12 production during *Toxoplasma* infection, although the effects were marginal. Nevertheless, my findings are in common with others in human monocytes, in which JNK downregulates G_i-protein-mediated IL-12 production (13).

Studies in humans suggest that neutrophil JNK functions differently compared to responses in mouse neutrophils. For example, JNK in human PMN remains unphosphorylated after PMN incubation with several activation stimuli. Also, inhibition of JNK with SP600125 does not inhibit inflammatory cytokine gene expression in human PMN suggesting that this MAPK is not important for induction of inflammatory mediators (15). However, another study demonstrated that human PMNs triggered with LPS express CCL2 through JNK activation, correlating with my findings (16).

The role of JNK in adaptive immunity has been studied in several infectious disease models. During influenza and lymphocytic choriomeningitis virus infection, JNK2 negatively regulates expansion and effector function of $CD8^+$ T cells (17, 18). Since resistance to *Toxoplasma* infection requires a $CD8^+$ IFN-γ response (19, 20), this might explain why $JNK2^{-/-}$ mice are more resistant during *T. gondii* infection. In common with our result, disruption of JNK2 confers enhanced survival in response to *Plasmodium berghei* ANKA infection. However, unlike my results this was associated with lower systemic TNF-α production (21). In addition, JNK-mediated negative regulation of IFN-γ production was demonstrated during *Anaplasma phagocytophilum*

infection (22). Together, my results and those of others suggest that the predominant role of JNK2 during microbial infection in vivo is to downregulate proinflammatory responses.

In addition to a role in inflammatory responses, JNK is implicated in cell migration in innate immune cells, including neutrophils. I also found that JNK2 plays a role in PMN chemotaxis in response to the IL-8-related chemokine, KC. In line with the in vitro data, I found decreased PMN recruitment in vivo in the absence of JNK2 during early *T. gondii* infection. Studies by other groups support the role of JNK in chemotaxis and recruitment during infection and inflammation. For example, the JNK signaling pathway is required for TNF-α-mediated E-selectin expression in endothelial cells which is essential for PMN extravasation to the site of infection (23). JNK also mediates platelet factor 4-induced neutrophil adhesion to endothelial cells (24). Furthermore, inhibition of the JNK pathway disrupts PMN actin assembly after LPS stimulation in the lung, leading to reduce pulmonary neutrophil accumulation (25). JNK is also activated during CCR7 ligand-induced DC migration (26). Collectively, these studies suggest an important function for JNK in cell migration.

3. Role of MyD88 in IL-12 induction during *T. gondii* oral infection

TLR adaptor molecule MyD88 has been implicated in innate recognition and resistance to several protozoan pathogens including *Toxoplasma*, *Plasmodium berghei*, *Leishmania major* and *Trypanosoma cruzi* (27). Several protozoan TLR ligands have now been identified. During *Toxoplasma* infection, parasite profilin and glycosylphosphatidylinositol (GPI) protein anchors have emerged as ligands for TLR11 and TLR2/4, respectively (28-30).

Although MyD88 is often proposed as being required for adaptive immunity, we clearly show this is not the case for *T. gondii* infection. These findings are supported by studies with other microbial pathogens including *Aspergillus fumigatus* (31), *Borrelia burgdorferi* (32), and *Listeria monocytogenes* (33). It is possible that residual MyD88-dependent IL-12 during infection with *Toxoplasma* and other microbial pathogens is sufficient to drive the adaptive immune response.

We revealed a requirement for MyD88 signaling in PMN recruitment during early *T. gondii* infection. The defect in PMN recruitment in the absence of MyD88 is in line with other infectious disease models including *Chlamydia pneumoniae* and *Citrobacter rodentium* (36, 37). Also, the IL-1R-MyD88 signaling pathway has been shown to be involved in neutrophil recruitment in sterile inflammatory responses (38). Whether *Toxoplasma*-triggered MyD88-dependent neutrophil recruitment is a consequence of TLR or IL-1R signaling is not yet known.

4. Future directions and unanswered questions

Several questions are raised by my findings. Despite the fact that JNK2 is required for *T. gondii* induced-IL-12 production in mouse neutrophils in vitro, the studies on JNK2 function in mice have failed to demonstrate a corresponding role in the response to *Toxoplasma* infection. Instead, my data suggests that JNK2 is a negative regulator for IL-12 production in vivo, and *T. gondii*-induced intestinal pathology in $JNK2^{-/-}$ mice is alleviated. Nevertheless I did not observe a consistent JNK2 requirement for in vivo TNF-α and nitric oxide, the key players for *T. gondii*-mediated-immunopathology. Further analysis of cytokine and chemokine expressed by intraepithelial lymphocytes and cellular infiltrates isolated from infected ileum might provide explanation of why $JNK2^{-/-}$ mice are more resistant to *Toxoplasma* infection. In addition, $CD8^{+}$ T cell

activity in JNK2$^{-/-}$ mice might be enhanced and contribute to disease resistance during *T. gondii* infection, a possibility that could be addressed in future studies.

Since I found decreased PMN recruitment to the small intestines was associated with relieved pathology in the absence of JNK2, it is tempting to speculate that PMN might play a role in damage to the intestinal mucosa. Because I found *Toxoplasma* triggered JNK2-dependent CCL2/MCP-1 production in PMN in vitro, it might be possible that JNK2 might be required for CCL2 induction in vivo during *Toxoplasma* infection. This could contibute to enhanced recruitment of other cells responsible for immune-mediated gut pathology. In this regard, it is of interest that our lab has found that genetic deletion of CCR2, the receptor for CCL2, also results in resistance to *Toxoplasma*-induced ileitis during oral infection (Egan and Denkers, manuscript in preparation).

Resistance to *Toxoplasma* infection requires IFN-γ production, a response that is widely viewed as being dependent upon IL-12. Yet, despite minimal levels of IL-12, a strong protective Th1 response emerged in the absence of MyD88. It is possible that only residual amounts of IL-12 are required for Th1 induction, a possibility that could be addressed by in vivo depletion with blocking antibody. It is also possible that there are major sources of MyD88-independent IL-12 that escaped detection on our studies. For example, the activity of lamina propria DC, a major subset of professional antigen presenting cells, was not determined in these studies. Regardless, a question remaining is what drives IL-12 in the absence of MyD88. Possibly TLR3 signaling (which unlike other TLR does not involve MyD88) is required. It is also possible that other intracellular sensors of infections, such as Nod1 and Nod2, are involved. Future work will be required to address these important issues.

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