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Absence of mitogen-activated protein kinase family member c-Jun N-terminal kinase-2 enhances resistance to *Toxoplasma gondii*

Woraporn Sukhumavasi^a, Amy L. Warren^b, Laura Del Rio Alonso^{c,†}, and Eric Y. Denkers^{c,*}

^aParasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand ^bDepartment of Veterinary Clinical and Diagnostic Sciences, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1 ^cDepartment of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA 14853-6401

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.

Keywords

Infection – protozoan; signal transduction; cytokine

Introduction

The intracellular protozoan parasite *Toxoplasma gondii* infects humans and other warm-blooded animals (Dubey, 2007). Within the host, the parasite is capable of parasitizing a wide range of hematopoietic and nonhematopoietic cells. Infection is normally asymptomatic, resulting in lifelong 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

*Correspondence: E. Denkers, Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853-6401. eyd1@cornell.edu.; Tel: 607-253-4022; Fax: 607-253-3384.

†Current address: Department of Animal Health, School of Veterinary Medicine, University of Murcia 30071, Murcia, Spain

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the fetus including birth defects and abortion, as well as major sequelae of infection later in life (Kravetz and Federman, 2005, Luft and Remington, 1992).

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 (Denkers and Gazzinelli, 1998). The importance of IFN- γ and IL-12 for disease resistance has been most clearly demonstrated using IFN- γ and IL-12 knockout (KO) mice, as well as in Stat1 and Stat4 KO animals, all of which uniformly succumb within 7-10 days of infection due to overwhelming parasite replication and dissemination (Cai, et al., 2000, Gavrilescu, et al., 2004, Lieberman, et al., 2004, Scharton-Kersten, et al., 1996). However, unregulated production of these same cytokines can be detrimental to the host, a situation leading to immunopathology and sometimes death of the host (Gazzinelli, et al., 1996). 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 (Liesenfeld, et al., 1996).

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 (Dong, et al., 2002). 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 (Weston and Davis, 2007). 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 (Ia Sala, et al., 2005, Ma, et al., 2004, Tao, et al., 2007, Utsugi, et al., 2003, Yang, et al., 1998).

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 (Kim, et al., 2005, Sukhumavasi, et al., 2007). 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 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 following protocols available from The Jackson Laboratory. 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.

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 (Sukhumavasi, et al., 2007). 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 μ m 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 μ g/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 (Bliss, et al., 1999), was added for restimulation. The cells were incubated at 37°C in a humidified 5% CO₂ incubator for 48 and 72 hr, then supernatants were recovered and stored at -20°C for ELISA.

ELISA

Production of IL-12p40 and IFN- γ was measured by ELISA as previously described (Bliss, et al., 1999).

Splenic dendritic cell enrichment

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 by a board certified pathologist (ALW) and graded based on severity of inflammation (minimal, mild, moderate, severe), number of inflammatory foci, type of inflammatory reaction, severity of necrosis (minimal, mild, moderate, severe) and intensity of *T. gondii* infection (0, no organisms; 1, 1-5 organisms; 2, 6-10 organisms; 3, 11-15 organisms; 4, >16 organisms). Ten randomly selected 40 $\times$ fields were examined using 2-4 mice per section.

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* (Sukhumavasi, et al., 2007). Nevertheless, we also found that production of IL-12p40 by bone marrow-derived macrophages did not require JNK2 (Kim, et al., 2005). 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* (Denkers and Gazzinelli, 1998), 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. 1A). We inoculated mice by oral gavage to determine if route of infection influenced the pattern of susceptibility in the absence of JNK2. Here again, wild-type (WT) animals displayed an accelerated rate of death compared to JNK2 KO mice (Fig. 1B). 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. 1C; $p = 0.039$). To determine if 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 (data 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* (Denkers and Gazzinelli, 1998). 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 at both time points during low and high dose infection (Fig. 2A and B). Along similar lines, serum IFN- γ levels were comparable between the two mouse strains (Fig. 2C 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. 3A and B). There was also a trend in increased IFN- γ production by KO cells, although differences between WT and KO were minor (Fig. 3C 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).

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 (Yarovinsky, et al., 2005). 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. 3E, and 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* (Liesenfeld, et al., 1996). Accordingly, we assessed the role of JNK2 in parasite-induced intestinal damage to the gut. WT mice (Day 10 post-infection) 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. 4A). 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. 4B). The intestinal architecture was largely intact compared to the WT counterparts. The histopathology analysis is summarized in Table 1.

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 (Sukhumavasi, et al., 2007). 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. Peyer's patches of WT animals contained greater numbers of MPO-positive cells relative to KO counterparts (Fig. 5A and B, respectively). Similarly, we found increased numbers of MPO-positive cells in the lamina propria and submucosa of intestinal sections in WT (Fig. 5C) compared to JNK2 KO mice (Fig. 5D). Therefore, expression of JNK2 promotes susceptibility to *Toxoplasma*-induced inflammatory gut pathology. At the same time, neutrophil recruitment to the gut is at least in part dependent upon JNK2, but we do not yet know if neutrophils are a cause of pathology at this site.

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 display 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 (Denkers and Gazzinelli, 1998), 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 (Ia Sala, et al., 2005). 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 (Ma, et al., 2004, Sukhumavasi, et al., 2007). 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 (Sabapathy, et al., 1999). It was also suggested that JNK2 expression in CD4⁺ T cells was required for differentiation into Th1 effectors (Yang, et al., 1998). 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 (Arbour, et al., 2002, Conze, et al., 2002). 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 (Tao, et al., 2007). Because optimal resistance to *Toxoplasma* requires IFN- γ production by CD8⁺ T lymphocytes (Denkers and Gazzinelli, 1998), it is possible that enhanced CD8⁺ activity in the absence of JNK2 accounts for increased resistance of *Jnk2*^{-/-} mice. However, in vitro restimulation experiments suggest that parasite-induced production of IFN- γ by CD8⁺ T lymphocytes is equivalent in *Jnk2*^{+/+} and *Jnk2*^{-/-} animals (data not shown).

A striking feature of the present results is that intestinal pathology induced by oral *Toxoplasma* infection was alleviated in *Jnk2*^{-/-} mice. At the same time, parasite numbers were decreased and infiltration of neutrophils was reduced. It is possible that in this situation, neutrophils contribute to pathology rather than directly killing parasites in wild-type 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 (Liesenfeld, et al., 1996). 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 (Lu, et al., 2006). Infection with the latter induces cerebral pathology that, like oral infection with *T. gondii*, is mediated by proinflammatory cytokines (Engwerda, et al., 2005). 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 (Pedra, et al., 2008). 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 (Sukhumavasi, et al., 2007). JNK signaling is also involved in neutrophil recruitment to the lung induced by lipopolysaccharide and positive air pressure (Arndt, et al., 2005, Li, et al., 2004). 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 (Kasper, et al., 2006). 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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Fig. 1.

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).

Fig. 2.

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. These results were repeated twice with the same result.

Fig. 3.

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 medium (Med) or soluble tachyzoite antigen (ST; 3 μ g/ml). Supernatants were subsequently collected for IL-12p40 (A and B) and IFN- γ (C and D) ELISA. These results were repeated twice with the same result. E, 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 (ST; 25 μ g/ml). After 18 hr, the supernatants were collected for IL-12p40 ELISA. These results were repeated twice with the same result.

Fig. 4. *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. Ileal sections of infected WT and KO mice are shown in panel A and B, respectively. Arrows in A point to areas of villus tip damage. In B, scale bar = 200 μ m. Data shown are one representative experiment of two.

Fig. 5.

Decreased numbers of MPO-positive cells in the absence of JNK2 in intestinal tissues during 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 μ m.

Table 1

Histopathology in the gastrointestinal tract of *Jnk2^{+/+}* and *Jnk2^{-/-}* mice

Days Post Infection ^a	<i>Jnk2^{+/+}</i>			<i>Jnk2^{-/-}</i>		
	Inflammation ^b	Necrosis	Organisms	Inflammation	Necrosis	Organisms
4	17.5 ± 8.5	0 ± 0	0 ± 0	1 ± 1	0 ± 0	0 ± 0
7	276 ± 53.4	2 ± 0	2.2 ± 0.7	151 ± 51.3	0.7 ± 0.2	0 ± 0
10	648 ± 64	3 ± 0	2 ± 1	171 ± 15	2 ± 0	0 ± 0

^aMice were orally infected with 100 ME49 cysts on Day 0.

^bThe inflammatory score was obtained by multiplying the number of inflammatory foci per section multiplied by the average severity of inflammation.