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NF- κ B1 contributes to T cell-mediated control of *Toxoplasma gondii* in the CNS

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Abstract

In this study, the role of NF- κ B1 was examined during toxoplasmosis. While wildtype BALB/c mice generated protective responses, NF- κ B1^{-/-} mice developed Toxoplasmic encephalitis, characterized by increased parasite burden and necrosis in the brain. Susceptibility was primarily associated with a local decrease in the number of CD8⁺ T cells and IFN- γ production, while accessory cell function appeared intact in NF- κ B1^{-/-} mice. Consistent with these findings, T cell transfer studies revealed that NF- κ B1^{-/-} T cells provided SCID mice less protection than wildtype T cells. These results demonstrate an intrinsic role for NF- κ B1 in T cell-mediated immunity to *T. gondii*.

Keywords

Toxoplasma gondii; NF- κ B; CD8⁺ T cell

Introduction

Many aspects of the immune response are dependent on the NF- κ B family of transcription factors: RelA, RelB, c-Rel, NF- κ B1, and NF- κ B2. NF- κ B1 (p50) is processed from the p105 protein and regulated by inhibitor of NF- κ B (I κ B) proteins. Stimuli, including TLR ligands, TNF- α , or T cell receptor engagement lead to the nuclear localization of NF- κ B1 hetero- or homodimers which regulate gene expression (Beinke and Ley, 2004; Mason et al., 2004a; Zhang and Ghosh, 2001). The importance of NF- κ B1 in the generation of protective immunity and autoimmunity has been demonstrated using mice deficient in this subunit. While NF- κ B1^{-/-} mice develop normally, they have several immune defects, including decreased T and B cell proliferation, antibody production, and macrophage expression of IL-10 (Artis et al., 2003; Beinke and Ley, 2004; Cao et al., 2006; Corn et al., 2003; Mason et al., 2004a; Sha et al., 1995; Snapper et al., 1996; Sriskantharajah et al., 2009). Initial studies demonstrated that NF- κ B1^{-/-} mice are more susceptible to the bacterial pathogens *Listeria monocytogenes* and *Streptococcus pneumoniae*, but were more resistant to

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encephalomyocarditis virus, due to increased apoptosis of infected cells (Schwarz et al., 1998; Sha et al., 1995). Later, cell-specific roles for NF- κ B1 in the generation of protective immune responses to parasites were identified. The failure of NF- κ B1^{-/-} mice to control *Leishmania major* was attributed to defects in CD4⁺ T cell proliferation, which was associated with a reduced protective Th1 cell response (Artis et al., 2003). On the other hand, NF- κ B1 was shown to be essential for dendritic cell (DC)-mediated induction of Th2 responses (Artis et al., 2005). Additionally, NF- κ B1^{-/-} mice develop severe colitis in response to *Trichuris muris* infection, characterized by increased IFN- γ production (Artis et al., 2002). Consistent with the identified defects in the generation of CD4⁺ T cells responses, NF- κ B1^{-/-} mice fail to generate autoreactive T cells and do not develop experimental autoimmune encephalomyelitis (Hilliard et al., 1999). Taken together, these studies demonstrate that the absence of NF- κ B1 differentially impacts the generation of a variety of immune responses.

T. gondii is a protozoan parasite that infects approximately one-quarter of the U.S. population and roughly one-third of the world population. Infection can be acquired by ingestion of oocysts, consumption of undercooked meat, and congenitally. Despite the development of protective immunity, parasites persist in a latent cyst form in multiple tissues, most prominently the brain. In immuno-competent individuals, the chronic phase is asymptomatic, but persons with acquired immune-deficiencies in T cell function develop Toxoplasmic encephalitis when parasites reactivate within the brain (Hill et al., 2005; Hunter and Remington, 1994; Luft and Remington, 1992).

Consistent with the role of NF- κ B in the recognition and control of infections, several pathogens interfere with this signaling pathway in order to subvert the host immune response (Tato and Hunter, 2002). Relevant to these studies, multiple groups have described the ability of *T. gondii* to disrupt NF- κ B activation, by preventing the phosphorylation and nuclear translocation of RelA to the nucleus of infected macrophages (Butcher et al., 2001; Shapira et al., 2005; Shapira et al., 2002). Overall, the inhibition of NF- κ B signaling by *T. gondii* leads to reduced capacity of infected cells to produce proinflammatory cytokines required for resistance to infection with this organism (Butcher et al., 2001).

Although this parasite interferes with immune responses generated through NF- κ B, several studies from this laboratory have identified essential roles of NF- κ B subunits in the immune response to *T. gondii*. Resistance to *T. gondii* is generated by innate recognition of the parasite leading to production of IL-12 which stimulates NK and T cells to secrete IFN- γ (Gazzinelli et al., 1994; Liu et al., 2006; Scanga et al., 2002; Scharton-Kersten et al., 1995). IFN- γ -mediated effector responses are required for clearance of the parasite during the acute phase of infection (Lieberman et al., 2004; Scharton-Kersten et al., 1996; Taylor et al., 2004). Once infection progresses to the chronic phase, current models suggest that IFN- γ and CD4⁺ and CD8⁺ T cells are required to limit parasite replication in the brain (Gazzinelli et al., 1992; Suzuki et al., 1989). The NF- κ B family has been implicated in all of these processes and, not surprisingly, this infection leads to global activation of NF- κ B (Shapira et al., 2002). Previous work from this laboratory identified roles for NF- κ B2, c-Rel, and RelB as well as a T cell-intrinsic requirement for NF- κ B in resistance *T. gondii* (Caamano et al., 1999; Caamano et al., 2000; Mason et al., 2002; Mason et al., 2004b; Tato et al., 2003). Moreover, NF- κ B1 has been implicated in the regulation of NK cell proliferation and IFN- γ production during Toxoplasmosis (Tato et al., 2006). The studies presented here demonstrate that while NF- κ B1-deficient mice are able to control the early phase of infection, a T cell-intrinsic role for NF- κ B1 is essential for long-term resistance to the parasite.

Materials and Methods

Mice and infections

NF- κ B1^{-/-} mice were originally provided by Rodrigo Bravo at Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ) and bred in University Laboratory Animal Resources facilities at the University of Pennsylvania. Mice were crossed onto the BALB/c background for greater than nine generations, bred as heterozygotes, and genotyped by PCR. Age and sex-matched knockout mice were used in experiments with wildtype littermates serving as controls. BALB/c SCID and CBA mice were purchased from Taconic Farms, Inc. (Germantown, NY). All procedures were performed in accordance to the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee. For infections, the Me49 strain *T. gondii* parasites were isolated from chronically infected CBA mice. Brains were removed and homogenized by serial needle passage in PBS. Parasite cysts were enumerated by light microscopy. Mice were infected with 20 cysts i.p.

Real-time PCR

To measure the amount of parasite DNA in the brain and liver, real-time PCR was utilized as previously described (Wilson et al., 2005). Briefly, approximately 50 mg of tissue was digested and DNA was purified using the High Pure PCR template preparation kit (Roche, Mannheim, Germany). Real-time PCR specific for the *Toxoplasma* B1 repeat region was used to quantify the amount of parasite DNA from 500 ng of DNA purified from tissue (Wilson et al., 2005). For the analysis of gene expression, brain tissue was placed in Trizol (Invitrogen, Carlsbad, CA) and mRNA was extracted as instructed by the manufacturer. Purified RNA was treated with DNase I to eliminate any contamination with genomic DNA (Promega, Madison, WI). cDNA was generated using Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed using primers for *iNOS* and normalized to HPRT (Qiagen, Germantown, MD). Samples, standard amounts of purified *T. gondii* DNA, or cDNA were amplified using Power SYBR[®] Green PCR Master Mix and a 7500 Fast Real-Time PCR System. Analysis was performed with system software v1.3.1 (Applied Biosystems, Warrington, UK).

Histology and Immunohistochemistry

For histological examination of brain tissue, the organ was excised and placed in 4% buffered formalin. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Sections were examined by light microscopy on a Nikon Eclipse E600 scope and images captured using a Nikon DS-Fi1 camera run by NIS Elements software (Nikon, Melville, NY). For immunohistochemistry, organs were embedded in OCT and flash frozen. Six μ m sections were cut using a Microm cryostat. Sections were fixed with a solution of 75% acetone and 25% ethanol. Anti-CD4 (5 μ g/ml), anti-CD8 (5 μ g/ml) (eBioscience, San Diego, CA), anti-I/A I/E (BD Pharmingen), anti-iNOS (10 μ g/ml), anti-Ki67 (3.25 μ g/ml) (Abcam, Cambridge, MA), anti-Me49 (1:4000), and anti-GFAP (10 μ g/ml) (Invitrogen) antibodies were used. Anti-rabbit Alexa 488 (Invitrogen) and anti-rat Cy3 (Jackson ImmunoResearch, West Grove, PA) were used as secondary antibodies for fluorescence staining. DAPI (Invitrogen) was used to visualize nuclei. To detect the expression of iNOS, a biotinylated goat anti-rabbit antibody was used, followed by Vectastain ABC reagent (Vector Laboratories, Burlingame, CA). DAB was used as an HRP substrate that created a brown precipitate and slides were counterstained with hematoxylin. Images were captured using light microscopy or standard fluorescence microscopy using a Nikon Eclipse E600 microscope (Melville, NY) equipped with a Photometrics Cool Snap EZ CCD camera (Tucson, AZ). Nikon NIS Elements software was used to capture and overlay images. The percentage of Ki67⁺ cells was determined by scoring at least 150 T cells from images from two separate experiments.

Cell purification, flow cytometry, and intracellular cytokine staining

Spleens were dissected, dissociated, and subjected to hypotonic red blood cell lysis to generate a cell suspension. The remaining cells were enumerated and used in subsequent assays. Brain mononuclear cells (BMNCs) were isolated as previously described (Wilson et al., 2005). Briefly, perfused brains were cut into small pieces, passed through an 18 gauge needle, and digested with collagenase/dispase and DNase (Roche) for 90 minutes. Following the digestion, the cells were washed and strained through a 70 μ m filter. Subsequently, cells were resuspended in 60% percoll, overlaid with 30% percoll, and centrifuged at room temperature for 25 minutes at 2000 rpm. BMNCs were collected from the interface, washed, and enumerated. For T cell recall responses, splenocytes were plated at 500,000 cells/well and BMNCs at 200,000 cells/well. Cells were stimulated with medium alone, 25 μ g/ml soluble *Toxoplasma* antigen (STAg), or 1 μ g/ml soluble anti-CD3 antibody (BD Pharmingen) for 48 hours at 37° C with 5% CO₂. IFN- γ and IL-12p40 were measured by ELISA from the culture supernatant or serum, as previously described (Caamano et al., 1999; Sander et al., 1989). For flow cytometry, cells were washed with FACS buffer (1X PBS, 0.2% BSA, and 2mM EDTA) and incubated in F_c block (0.1 μ g/ml 24G2 antibody) for 15 minutes prior to surface staining with CD4-FITC, CD8-PerCp Cy5.5, CD44-APC, and CD62L-APC Alexa 750 (eBioscience). Dendritic cells were identified as CD3⁺, NK1.1⁺, CD19⁺, CD45⁺, and CD11c⁺ (all antibodies from eBioscience) and stained with anti-K^d-PE or anti-I/A-PE (BD) to assess MHC class I and II expression, respectively. For intracellular cytokine staining, 5 $\times$ 10⁵ splenocytes and 2 $\times$ 10⁵ BMNCs were plated in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum, 1% sodium pyruvate, 1% non-essential amino acids, 0.1% β -mercaptoethanol, 100 U of penicillin/ml and 100 μ g/ml of streptomycin (Gibco, Invitrogen Incorporation, Grand Island, NY). Cells were stimulated with PMA (50 ng/ml) and ionomycin (500ng/ml) in the presence of brefeldin A (10 μ g/ml) for four hours at 37° C with 5% CO₂. Cells were washed, incubated with F_c block, and surface stained with anti-CD4-FITC and anti-CD8-PerCp Cy5.5 (eBioscience). The cells were fixed with 2% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) for 10 minutes. For intracellular staining, the cells were permeabilized with 0.1% saponin (Sigma, St. Louis, MO) in FACS buffer and stained with anti-IFN- γ -PE (eBioscience). All flow cytometry was performed on a FACSCanto using FACSDIVA 6.0 software (BD Biosciences, San Jose, CA). Analysis was performed using FloJo software (Treestar Inc., Ashland, OR).

T cell transfer

T cells were purified from the spleens and lymph nodes of wildtype BALB/c mice and NF- κ B1^{-/-} mice using a T cell enrichment column (R&D Systems, Minneapolis, MN). The cells were determined to be greater than 90% pure by flow cytometric analysis (data not shown). 2 $\times$ 10⁶ T cells in PBS were transferred i.p. to BALB/c-SCID mice.

Statistical analysis

Significance was determined using student's t-test. $p \leq 0.05$ was considered significant. Analysis was performed using Prism software (GraphPad Software, Inc.).

Results

NF- κ B1 deficient mice succumb to Toxoplasmic encephalitis

To determine the role of the NF- κ B subunit, NF- κ B1, in the immune response to *T. gondii*, wildtype BALB/c and NF- κ B1-deficient mice were injected i.p. with 20 cysts of the Me49 strain. Whereas the wildtype BALB/c were resistant to infection, the knockout mice succumbed approximately 3–4 weeks post-infection (Figure 1A). Quantification of parasite

DNA at day 22 post-infection revealed a two-log increase in parasite burden in the brain and liver of the NF- κ B1^{-/-} mice (Figure 1B). Histological analysis indicated that the most prominent pathology was evident in the brain, with only limited indicators of pathology in the liver. In NF- κ B1^{-/-} mice, extensive regions of parasite replication, necrosis, and some inflammatory infiltrate were present in the brain (Figure 1C). While a significant difference in parasite burden was observed during the chronic phase, clearance of parasites from the peritoneal cavity on day 10 post-infection was evident in wildtype and knockout mice (data not shown). These results indicate that NF- κ B1-deficient mice survive the acute phase of infection, but succumb as a consequence of the presence of large numbers of parasites in the brain.

NF- κ B1 is not required for accessory cell responses to *T. gondii*

Multiple studies have implicated astrocytes in the local control of *T. gondii* in the CNS (Wilson and Hunter, 2004). Therefore, the function of astrocytes in the absence of NF- κ B1 was addressed. As an indicator of astrocyte activation *in vivo*, GFAP expression was assessed by immunohistochemistry. Widespread astrocyte activation was observed in NF- κ B1^{-/-} and wildtype mice (Figure 2A). In addition, when primary astrocyte and macrophage cultures were treated with IFN- γ and TNF- α and infected with *T. gondii*, NF- κ B1^{-/-} cells were able to control the parasites as well as wildtype cells (Supplemental Figure 1). These results suggest that NF- κ B1 deficiency does not appreciably affect the ability of astrocytes or macrophages to respond to infection *in vivo* or control parasite replication *in vitro*.

Several NF- κ B-regulated molecules are induced in the brain during chronic *T. gondii* infection, including inducible nitric oxide synthase (iNOS), MHC class I, and MHC class II, which are all required for resistance to *T. gondii* (Gazzinelli et al., 1993; Scharton-Kersten et al., 1997; Schluter et al., 1999). Levels of these molecules were measured in the brains of wildtype and NF- κ B1^{-/-} mice on day 22 post-infection. Immunohistochemical staining of brain sections identified numerous regions of iNOS production associated with parasite replication (Figure 2B). The marked increase in parasite burden in the NF- κ B1^{-/-} mice correlated with increased iNOS staining in comparison to wildtype mice. The relative amount of *iNOS* transcript was also elevated in the brains of NF- κ B1^{-/-} mice in comparison to wildtype mice (Figure 2C). Following infection, the expression levels of MHC class I and II molecules increase on antigen presenting cells (APCs). Using flow cytometry, MHC class I and II expression was measured on microglia, macrophages, and dendritic cells from the spleen and CNS to assess the activation status of APCs. While MHC class I and II levels were decreased on dendritic cells (DCs) in the brain (Figure 2D), expression was equivalent on macrophages and microglia from the CNS and splenic DCs and macrophages (data not shown). Immunohistochemical staining demonstrated the association of MHC class II positive cells near regions of parasite replication in wildtype and knockout mice (Figure 2E). While MHC class I and II expression were largely intact and higher levels of iNOS were present, NF- κ B1^{-/-} mice fail to limit parasite replication. Expression of IFN- γ -induced GTPases required for parasite control were also assessed by real-time PCR. During chronic infection, higher levels of *ifi47* and *igtp* were measured in the knockout mice versus wildtype mice (Supplemental Figure 2). Thus, it is likely that additional NF- κ B1-dependent immune responses are altered in the knockout mice, which ultimately leads to increased susceptibility to *T. gondii*.

Reduced numbers of CD8⁺ T cells are present in the brains of NF- κ B1^{-/-} mice

The control of *T. gondii* in the CNS is dependent on local T cell production of IFN- γ , leading to the activation of anti-parasitic effector mechanisms (Gazzinelli et al., 1992; Suzuki et al., 1988). Given a role for NF- κ B1 in the regulation of T cell responses (Beinke and Ley, 2004), studies were performed to assess if the absence of this transcription factor

affects the local T cell response. Therefore, T cells in the CNS were examined on day 22 post-infection by immunohistochemistry. These studies revealed that, as in wildtype mice, CD4⁺ and CD8⁺ T cells from NF- κ B1^{-/-} mice were observed in close proximity to parasites (Figure 3A and 3B). Since NF- κ B1 affects T cell proliferation, brain sections from wildtype and knockout mice were stained with antibodies to Ki67, a marker used to detect cells in active cell cycle (Barber et al., 2006; Scholzen and Gerdes, 2000). Approximately 30% of T cells in the brains of wildtype mice were Ki-67⁺. The majority of CD4⁺ (56%) and CD8⁺ (62%) T cells in the brains of NF- κ B1^{-/-} mice were Ki-67⁺ (Figure 3C), although the overall number of T cells was decreased (Figure 4A). Thus, despite having a higher percentage of T cells in the cell cycle, NF- κ B1^{-/-} mice appeared to have fewer T cells in the brain. Based on histology, T cell recruitment to the CNS and activation of cell cycle are not defective in NF- κ B1^{-/-} mice, but survival or proliferation deficits may lead to reduced T cell populations during chronic infection.

To quantify the number and activation status of T cells following infection, T cells were isolated and analyzed by flow cytometry during the acute (day 10 post-infection) and chronic (day 22 post-infection) stage of disease. Early during infection there were no differences found in CD4⁺ T cell number or activation status (CD44^{hi} and CD62L^{lo}). In addition, there was a trend toward fewer total and activated CD8⁺ T cells isolated from the spleens of NF- κ B1^{-/-} mice, but no significant differences were detected (results not shown). Later, during chronic infection, more dramatic defects in the T cell compartment were revealed. While the total number of CD4⁺ T cells in the spleen and brain were not dramatically altered at this timepoint, there was a significant reduction in the number of CD8⁺ T cells in the spleen (Figure 4A, p=0.0475) and brain (Figure 4B, p=0.0108). Additionally, there were significant decreases in activated CD4⁺ and CD8⁺ T cells in the spleen by percentage (Figure 4C) and number (Figure 4D). The most striking difference in activated T cell numbers in the CNS was in the CD8⁺ T cell compartment (Figure 4E, p=0.01). These results confirmed previous observations that NF- κ B1^{-/-} mice have a defective T cell response in the brain, predominantly in the CD8⁺ compartment and also revealed a CD4⁺ T cell defect in the spleen.

T cell-intrinsic role for NF- κ B1 in resistance to *T. gondii*

Control of *T. gondii* during the acute and chronic stages of infection relies on a strong Th1 response. Thus, the ability of the NF- κ B1 knockout mice to mount a Th1 response during the acute (day 10) and chronic (day 22) stage was examined. Serum levels of IL-12p40 and IFN- γ were measured by ELISA on day 10 post-infection. NF- κ B1^{-/-} mice displayed increased levels of IL-12 p40 and decreased levels of IFN- γ (Figure 5A and 5B). These results suggest that the defect in IFN- γ production during the acute phase was not caused by a reduction in IL-12 production by innate immune cells.

As an additional readout of T cell function, splenic T cell recall responses to soluble *Toxoplasma* antigen (STAg) and anti-CD3 were measured on day 10 and 22 post-infection. Following a 48-hour incubation with antigen, cells from the knockout mice produced less IFN- γ in response to STAg than wildtype mice (Figure 5C and 5D). IFN- γ production was also assessed by intracellular cytokine staining. Cells from the spleen and brain isolated on day 22 post-infection were stimulated with PMA and ionomycin for 4 hours *ex vivo* in the presence of brefeldin A. Flow cytometric analysis revealed that T cells from the knockout mice were capable of making IFN- γ ; in fact, a higher percentage of T cells were producing cytokine in comparison to wildtype mice (Figure 6A). Despite this, the numbers of cytokine-producing CD8⁺ T cells were diminished in spleens and significantly reduced in the brains (p=0.0076) of NF- κ B1^{-/-} mice, while the number of CD4⁺ T cells producing IFN- γ was comparable to wildtype mice (Figure 6B and 6C). These data suggest that T cells from NF- κ B1^{-/-} mice are capable of producing IFN- γ , but the severe Toxoplasmic encephalitis in

NF- κ B1^{-/-} mice is associated with a marked defect in the number of CD8⁺ T cells producing this cytokine in the brain.

The studies described above identify a major role for NF- κ B1 in the regulation of T cell-mediated responses to *T. gondii*, but it was unclear whether this was a secondary consequence of reduced APC function or was T cell intrinsic. To directly distinguish this, a transfer model was utilized. Cells isolated from the spleen and lymph nodes of uninfected wildtype and NF- κ B1^{-/-} mice were enriched for T cells and 2×10^6 T cells were transferred i.p. to SCID mice one day prior to infection with *T. gondii*. SCID mice that were not given T cells succumbed to infection prior to day 20, while SCID mice that received T cells from WT mice survived for approximately 40 days. Mice that received NF- κ B1 deficient T cells demonstrated an intermediate phenotype, where the majority of the mice survived 25 days post-infection (Figure 6D). On day 10 post-transfer, IFN- γ levels were measured in the serum of mice receiving T cell transfer. Recipients of NF- κ B1^{-/-} T cells had less systemic IFN- γ in comparison to mice receiving wildtype T cells (Figure 6E). This defect in cytokine production may underlie the increased susceptibility of SCID mice receiving NF- κ B1 deficient T cells. Together these data identify a T cell-intrinsic role for NF- κ B1.

Discussion

The results presented here establish a role for NF- κ B1 in T cell-mediated control of *T. gondii* in the CNS. Given the ubiquitous role of NF- κ B transcription factors in the immune response, it was surprising that NF- κ B1 deficient mice survived acute infection. Several immune parameters remained intact or were enhanced in NF- κ B1^{-/-} mice, and thus may contribute to the early control of the parasite. For example, it has been previously reported that NK cells from NF- κ B1^{-/-} mice infected with *T. gondii* are hyper-proliferative and produce more IFN- γ than their wildtype counterparts (Tato et al., 2006). In addition, the data presented here indicate increased levels of IL-12 are found in the serum of NF- κ B1^{-/-} mice. Previous reports have demonstrated that NF- κ B1 homodimers and NF- κ B1 dimers with RelA and c-Rel are capable of binding the *iL-12p40* promoter (Sanjabi et al., 2000). Given the role of NF- κ B1 homodimers as transcriptional repressors (May and Ghosh, 1997), the results presented here are consistent with a negative regulatory role of NF- κ B1 on IL-12p40 production. Nevertheless, despite enhanced innate responses in the absence of NF- κ B1, the knockout mice ultimately fail to control parasite replication in the brain. Due to the role of NF- κ B in autoimmune disease and cancer, NF- κ B inhibitors have been developed and are currently in clinical trials (Karin et al., 2004). Based on the findings of this study, reactivation of latent *T. gondii* in the CNS may be a negative side effect of such therapies.

The enhanced parasite replication and associated areas of necrosis in the brains of NF- κ B1^{-/-} mice are reminiscent of the phenotypes observed in mice that lack iNOS or TNF- α signaling (Deckert-Schluter et al., 1998; Gazzinelli et al., 1993; Scharton-Kersten et al., 1997; Yap et al., 1998). Since TNF- α signaling is required for the optimal induction of IFN- γ -mediated control of intracellular *T. gondii* (Sibley et al., 1991), cell-intrinsic NF- κ B1 may be required in for parasite control in infected macrophages and astrocytes. However, the use of *in vitro* studies, where exogenous TNF- α and IFN- γ was provided to cells infected with *T. gondii*, did not reveal an inability of NF- κ B1 deficient cells to control parasite replication. Moreover, astrocyte activation in infected knockout mice, assessed by expression of GFAP, appeared to be equivalent to wildtype mice. Consistent with these results, more iNOS was detected in the brains of NF- κ B1^{-/-} mice in comparison to wildtype mice. In addition, MHC class I and II expression was not decreased on NF- κ B1^{-/-} macrophages isolated from the brain. Taken together, these results suggested that the increased susceptibility of NF- κ B1^{-/-} mice to TE was not due to an intrinsic defect in the effector functions of two commonly infected cell types, macrophages and astrocytes.

The ability of T cells, particularly CD8⁺ T cells, to produce IFN- γ is an essential element of the immune response necessary for the local control of *T. gondii* in the CNS (Gazzinelli et al., 1992; Suzuki et al., 1989; Suzuki et al., 1988). Characterization of the T cell response in chronically infected NF- κ B1^{-/-} mice revealed a marked decrease in the number of activated and cytokine-producing CD8⁺ T cells in the spleen and brain. When assessing T cells in the brain by immunohistochemistry, it was apparent that NF- κ B1^{-/-} T cells were able to traffic to the CNS to regions of parasite replication and expressed Ki67, a marker of active cell cycle. Consistent with the idea that accessory cell functions are intact in NF- κ B1^{-/-} mice, T cells were activated during acute infection and there was no significant decrease in CD8⁺ T cells in the knockout mice at this stage of disease. However, T cell transfer to SCID mice confirmed that NF- κ B1^{-/-} T cells were not able to provide as much protection as wildtype T cells. Together, these results support the idea that the increased susceptibility of NF- κ B1^{-/-} mice to TE is associated with an intrinsic role for NF- κ B1 in the regulation of T cell functions.

An important question these studies raised is how NF- κ B1 influences the CD8⁺ T cell response. Given the prominent role of NF- κ B in T cell proliferation and survival, one possible explanation is that the absence of NF- κ B1 these processes are affected, leading to the loss of parasite-specific T cells (Beinke and Ley, 2004; Li and Verma, 2002; Mason et al., 2004a). Based on the histological analysis of proliferation using the marker Ki67, more cells in the brains of NF- κ B1^{-/-} mice were in active cell cycle in comparison to T cells in brains of wildtype mice. These results suggest that NF- κ B1 may impact cell survival. Alternatively, the role of NF- κ B1 may not be intrinsic to the CD8⁺ T cells, as altered CD4⁺ T cell responses could impact CD8⁺ T cells. This piece of data is analogous to the situation in AIDS patients that develop TE, where a loss of CD4⁺ T cells is associated with a decrease in CD8⁺ T cell numbers and function (Hunter and Remington, 1994; Luft and Remington, 1988; Luft and Remington, 1992). In support of this hypothesis, previous studies addressing the role of NF- κ B1 during parasitic infections have described defects in CD4⁺ T cells. For example, NF- κ B1^{-/-} CD4⁺ T cells were shown to have proliferative defects in response to *L. major* infection and polyclonal stimulation *in vitro* (Artis et al., 2003). In addition, mice lacking the ability to process the NF- κ B1 precursor protein, p105, have T cell-intrinsic defects in CD4⁺ T cell proliferation following stimulation with anti-CD3 and anti-CD28, which was not attributed to apoptosis but rather lower IL-2 production and a failure to enter S-phase (Sriskantharajah et al., 2009). In support of this idea, it appears that the CD4⁺ cell responses induced early during infection in NF- κ B1^{-/-} mice were intact, but there was a significant decrease in activated CD4⁺ T cells in the spleen but not the brain during TE. However, these studies do not dissect the impact of NF- κ B1 on the individual role of CD4⁺ and CD8⁺ T cells. Additional experiments will be required to gain an understanding of whether NF- κ B1 has a role in promoting CD4⁺ T cell functions that are required to either generate or maintain parasite-specific CD8⁺ T cells.

Previous work from this laboratory has addressed the individual roles of NF- κ B2, c-Rel, RelB, and I κ B- α degradation during *T. gondii* infection (Caamano et al., 1999; Caamano et al., 2000; Hilliard et al., 2002; Mason et al., 2004b; Tato et al., 2003). These studies have shown that the absence of a single family member or the inability to degrade I κ B- α causes unique immune defects and that the remaining family members fail to completely compensate for the deficiency. For example, RelB^{-/-} mice do not survive the acute phase of infection, while NF- κ B2^{-/-} and c-Rel^{-/-} mice succumbed to TE. In each of these knockout strains aberrant T cell function and reduced production of IFN- γ was associated with increased susceptibility, similar to what was observed with NF- κ B1^{-/-} mice. While many previous studies have identified a critical role for NF- κ B1 in the control of CD4⁺ T cell mediated immunity to a variety of parasitic infections, this study provides the first indication that NF- κ B1 impacts the CD8⁺ T cell population. One difference between the current study

and those previously published on the role of NF- κ B in resistance to infection is the genetic background of the mice. Previous studies have utilized mice on the C57BL/6 background, while the mice in this study are on the BALB/c background. Since resistance to *T. gondii* in BALB/c mice is critically dependent on CD8⁺ T cell responses generated by antigens presented by L^d, it is perhaps not that surprising that defects in the CD8⁺ T cell population were identified in NF- κ B1^{-/-} mice on this genetic background (Blanchard et al., 2008). Nevertheless, the results presented in this study identify a previously unrecognized T cell-intrinsic role for NF- κ B1 in the control of *T. gondii* in the CNS. These studies not only identify a role for this transcription factor in the generation of CD8⁺ T cell population but also complete the assessment of the role of individual NF- κ B family members in the immune response to *T. gondii*.

Supplementary Material

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References

- Artis D, Kane CM, Fiore J, Zaph C, Shapira S, Joyce K, Macdonald A, Hunter C, Scott P, Pearce EJ. Dendritic cell-intrinsic expression of NF-kappa B1 is required to promote optimal Th2 cell differentiation. *J Immunol* 2005;174:7154–7159. [PubMed: 15905559]
- Artis D, Shapira S, Mason N, Speirs KM, Goldschmidt M, Caamano J, Liou HC, Hunter CA, Scott P. Differential requirement for NF-kappa B family members in control of helminth infection and intestinal inflammation. *J Immunol* 2002;169:4481–4487. [PubMed: 12370384]
- Artis D, Speirs K, Joyce K, Goldschmidt M, Caamano J, Hunter CA, Scott P. NF-kappa B1 is required for optimal CD4+ Th1 cell development and resistance to *Leishmania major*. *J Immunol* 2003;170:1995–2003. [PubMed: 12574369]
- Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006;439:682–687. [PubMed: 16382236]
- Beinke S, Ley SC. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. *Biochem J* 2004;382:393–409. [PubMed: 15214841]
- Blanchard N, Gonzalez F, Schaeffer M, Joncker NT, Cheng T, Shastri AJ, Robey EA, Shastri N. Immunodominant, protective response to the parasite *Toxoplasma gondii* requires antigen processing in the endoplasmic reticulum. *Nat Immunol* 2008;9:937–944. [PubMed: 18587399]
- Butcher BA, Kim L, Johnson PF, Denkers EY. *Toxoplasma gondii* tachyzoites inhibit proinflammatory cytokine induction in infected macrophages by preventing nuclear translocation of the transcription factor NF-kappa B. *J Immunol* 2001;167:2193–2201. [PubMed: 11490005]
- Caamano J, Alexander J, Craig L, Bravo R, Hunter CA. The NF-kappa B family member RelB is required for innate and adaptive immunity to *Toxoplasma gondii*. *J Immunol* 1999;163:4453–4461. [PubMed: 10510387]
- Caamano J, Tato C, Cai G, Villegas EN, Speirs K, Craig L, Alexander J, Hunter CA. Identification of a role for NF-kappa B2 in the regulation of apoptosis and in maintenance of T cell-mediated immunity to *Toxoplasma gondii*. *J Immunol* 2000;165:5720–5728. [PubMed: 11067930]
- Cao S, Zhang X, Edwards JP, Mosser DM. NF-kappaB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. *J Biol Chem* 2006;281:26041–26050. [PubMed: 16835236]

- Corn RA, Aronica MA, Zhang F, Tong Y, Stanley SA, Kim SR, Stephenson L, Enerson B, McCarthy S, Mora A, Boothby M. T cell-intrinsic requirement for NF-kappa B induction in postdifferentiation IFN-gamma production and clonal expansion in a Th1 response. *J Immunol* 2003;171:1816–1824. [PubMed: 12902482]
- Deckert-Schluter M, Bluethmann H, Rang A, Hof H, Schluter D. Crucial role of TNF receptor type 1 (p55), but not of TNF receptor type 2 (p75), in murine toxoplasmosis. *J Immunol* 1998;160:3427–3436. [PubMed: 9531303]
- Gazzinelli R, Xu Y, Hieny S, Cheever A, Sher A. Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*. *J Immunol* 1992;149:175–180. [PubMed: 1351500]
- Gazzinelli RT, Eltoun I, Wynn TA, Sher A. Acute cerebral toxoplasmosis is induced by in vivo neutralization of TNF-alpha and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. *J Immunol* 1993;151:3672–3681. [PubMed: 7690809]
- Gazzinelli RT, Wysocka M, Hayashi S, Denkers EY, Hieny S, Caspar P, Trinchieri G, Sher A. Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with *Toxoplasma gondii*. *J Immunol* 1994;153:2533–2543. [PubMed: 7915739]
- Hill DE, Chirukandoth S, Dubey JP. Biology and epidemiology of *Toxoplasma gondii* in man and animals. *Anim Health Res Rev* 2005;6:41–61. [PubMed: 16164008]
- Hilliard B, Samoilova EB, Liu TS, Rostami A, Chen Y. Experimental autoimmune encephalomyelitis in NF-kappa B-deficient mice: roles of NF-kappa B in the activation and differentiation of autoreactive T cells. *J Immunol* 1999;163:2937–2943. [PubMed: 10453042]
- Hilliard BA, Mason N, Xu L, Sun J, Lamhamedi-Cherradi SE, Liou HC, Hunter C, Chen YH. Critical roles of c-Rel in autoimmune inflammation and helper T cell differentiation. *J Clin Invest* 2002;110:843–850. [PubMed: 12235116]
- Hunter CA, Remington JS. Immunopathogenesis of toxoplasmic encephalitis. *J Infect Dis* 1994;170:1057–1067. [PubMed: 7963693]
- Karin M, Yamamoto Y, Wang QM. The IKK NF-kappa B system: a treasure trove for drug development. *Nat Rev Drug Discov* 2004;3:17–26. [PubMed: 14708018]
- Li Q, Verma IM. NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2002;2:725–734. [PubMed: 12360211]
- Lieberman LA, Banica M, Reiner SL, Hunter CA. STAT1 plays a critical role in the regulation of antimicrobial effector mechanisms, but not in the development of Th1-type responses during toxoplasmosis. *J Immunol* 2004;172:457–463. [PubMed: 14688355]
- Liu CH, Fan YT, Dias A, Esper L, Corn RA, Bafica A, Machado FS, Aliberti J. Cutting edge: dendritic cells are essential for in vivo IL-12 production and development of resistance against *Toxoplasma gondii* infection in mice. *J Immunol* 2006;177:31–35. [PubMed: 16785494]
- Luft BJ, Remington JS. AIDS commentary. Toxoplasmic encephalitis. *J Infect Dis* 1988;157:1–6. [PubMed: 3121758]
- Luft BJ, Remington JS. Toxoplasmic encephalitis in AIDS. *Clin Infect Dis* 1992;15:211–222. [PubMed: 1520757]
- Mason N, Aliberti J, Caamano JC, Liou HC, Hunter CA. Cutting edge: identification of c-Rel-dependent and -independent pathways of IL-12 production during infectious and inflammatory stimuli. *J Immunol* 2002;168:2590–2594. [PubMed: 11884420]
- Mason NJ, Artis D, Hunter CA. New lessons from old pathogens: what parasitic infections have taught us about the role of nuclear factor-kappaB in the regulation of immunity. *Immunol Rev* 2004a; 201:48–56. [PubMed: 15361232]
- Mason NJ, Liou HC, Hunter CA. T cell-intrinsic expression of c-Rel regulates Th1 cell responses essential for resistance to *Toxoplasma gondii*. *J Immunol* 2004b;172:3704–3711. [PubMed: 15004174]
- May MJ, Ghosh S. Rel/NF-kappa B and I kappa B proteins: an overview. *Semin Cancer Biol* 1997;8:63–73. [PubMed: 9299584]

- Sander B, Cardell S, Heremans H, Andersson U, Moller G. Detection of individual interleukin 4- and gamma interferon-producing murine spleen cells after activation with T-cell mitogens. *Scand J Immunol* 1989;30:315–320. [PubMed: 2506638]
- Sanjabi S, Hoffmann A, Liou HC, Baltimore D, Smale ST. Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. *Proc Natl Acad Sci U S A* 2000;97:12705–12710. [PubMed: 11058167]
- Scanga CA, Aliberti J, Jankovic D, Tilloy F, Bennouna S, Denkers EY, Medzhitov R, Sher A. Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by dendritic cells. *J Immunol* 2002;168:5997–6001. [PubMed: 12055206]
- Scharton-Kersten T, Denkers EY, Gazzinelli R, Sher A. Role of IL12 in induction of cell-mediated immunity to *Toxoplasma gondii*. *Res Immunol* 1995;146:539–545. [PubMed: 8839159]
- Scharton-Kersten TM, Wynn TA, Denkers EY, Bala S, Grunvald E, Hieny S, Gazzinelli RT, Sher A. In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to *Toxoplasma gondii* while failing to control acute infection. *J Immunol* 1996;157:4045–4054. [PubMed: 8892638]
- Scharton-Kersten TM, Yap G, Magram J, Sher A. Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen *Toxoplasma gondii*. *J Exp Med* 1997;185:1261–1273. [PubMed: 9104813]
- Schluter D, Deckert-Schluter M, Lorenz E, Meyer T, Rollinghoff M, Bogdan C. Inhibition of inducible nitric oxide synthase exacerbates chronic cerebral toxoplasmosis in *Toxoplasma gondii*-susceptible C57BL/6 mice but does not reactivate the latent disease in *T. gondii*-resistant BALB/c mice. *J Immunol* 1999;162:3512–3518. [PubMed: 10092808]
- Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 2000;182:311–322. [PubMed: 10653597]
- Schwarz EM, Badorff C, Hiura TS, Wessely R, Badorff A, Verma IM, Knowlton KU. NF-kappaB-mediated inhibition of apoptosis is required for encephalomyocarditis virus virulence: a mechanism of resistance in p50 knockout mice. *J Virol* 1998;72:5654–5660. [PubMed: 9621024]
- Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. *Cell* 1995;80:321–330. [PubMed: 7834752]
- Shapira S, Harb OS, Margarit J, Matrajt M, Han J, Hoffmann A, Freedman B, May MJ, Roos DS, Hunter CA. Initiation and termination of NF-kappaB signaling by the intracellular protozoan parasite *Toxoplasma gondii*. *J Cell Sci* 2005;118:3501–3508. [PubMed: 16079291]
- Shapira S, Speirs K, Gerstein A, Caamano J, Hunter CA. Suppression of NF-kappaB activation by infection with *Toxoplasma gondii*. *J Infect Dis* 2002;185(Suppl 1):S66–72. [PubMed: 11865442]
- Sibley LD, Adams LB, Fukutomi Y, Krahenbuhl JL. Tumor necrosis factor-alpha triggers antitoxoplasmal activity of IFN-gamma primed macrophages. *J Immunol* 1991;147:2340–2345. [PubMed: 1918966]
- Snapper CM, Zelazowski P, Rosas FR, Kehry MR, Tian M, Baltimore D, Sha WC. B cells from p50/NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription, and Ig class switching. *J Immunol* 1996;156:183–191. [PubMed: 8598461]
- Sriskantharajah S, Belich MP, Papoutsopoulou S, Janzen J, Tybulewicz V, Seddon B, Ley SC. Proteolysis of NF-kappaB1 p105 is essential for T cell antigen receptor-induced proliferation. *Nat Immunol* 2009;10:38–47. [PubMed: 19060899]
- Suzuki Y, Conley FK, Remington JS. Importance of endogenous IFN-gamma for prevention of toxoplasmic encephalitis in mice. *J Immunol* 1989;143:2045–2050. [PubMed: 2506275]
- Suzuki Y, Orellana MA, Schreiber RD, Remington JS. Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science* 1988;240:516–518. [PubMed: 3128869]
- Tato CM, Hunter CA. Host-pathogen interactions: subversion and utilization of the NF-kappa B pathway during infection. *Infect Immun* 2002;70:3311–3317. [PubMed: 12065467]
- Tato CM, Mason N, Artis D, Shapira S, Caamano JC, Bream JH, Liou HC, Hunter CA. Opposing roles of NF-kappaB family members in the regulation of NK cell proliferation and production of IFN-gamma. *Int Immunol* 2006;18:505–513. [PubMed: 16481345]

- Tato CM, Villarino A, Caamano JH, Boothby M, Hunter CA. Inhibition of NF-kappa B activity in T and NK cells results in defective effector cell expansion and production of IFN-gamma required for resistance to *Toxoplasma gondii*. *J Immunol* 2003;170:3139–3146. [PubMed: 12626571]
- Taylor GA, Feng CG, Sher A. p47 GTPases: regulators of immunity to intracellular pathogens. *Nat Rev Immunol* 2004;4:100–109. [PubMed: 15040583]
- Wilson EH, Hunter CA. The role of astrocytes in the immunopathogenesis of toxoplasmic encephalitis. *Int J Parasitol* 2004;34:543–548. [PubMed: 15064118]
- Wilson EH, Wille-Reece U, Dzierszynski F, Hunter CA. A critical role for IL-10 in limiting inflammation during toxoplasmic encephalitis. *J Neuroimmunol* 2005;165:63–74. [PubMed: 16005735]
- Yap GS, Scharton-Kersten T, Charest H, Sher A. Decreased resistance of TNF receptor p55- and p75-deficient mice to chronic toxoplasmosis despite normal activation of inducible nitric oxide synthase in vivo. *J Immunol* 1998;160:1340–1345. [PubMed: 9570552]
- Zhang G, Ghosh S. Toll-like receptor-mediated NF-kappaB activation: a phylogenetically conserved paradigm in innate immunity. *J Clin Invest* 2001;107:13–19. [PubMed: 11134172]

Figure 1. NF-κB1^{-/-} mice succumb to Toxoplasmic encephalitis

(A) NF-κB1^{-/-} and wildtype litter mate controls were infected with 20 cysts of Me49 i.p. NF-κB1^{-/-} mice (diamonds) survived approximately 24 days in comparison to wildtype BALB/c mice (squares), which survive greater than 100 days post-infection. (B) Knockout (ko) mice had an approximately two-log increase in parasite burden in the brain and liver in comparison to wildtype mice (wt). Data is presented as the mean ± SEM, * $p \leq 0.05$. (C) Knockout mice had severe pathology in the brain 22 days post-infection, characterized by regions of inflammatory cell infiltrate and parasite replication, which were less apparent in wildtype mice.

Figure 2. GFAP, iNOS, and MHC molecules are upregulated in the CNS of NF-κB1^{-/-} mice
 (A) Immunohistochemical analysis of brain sections demonstrated widespread gliosis by GFAP staining (red) and large numbers of parasites (green) in the knockout mice. Nuclei were stained with DAPI (blue). (B) IHC analysis of iNOS expression in the brains of wildtype and knockout mice demonstrated that knockout mice (KO) have increased levels of iNOS (brown) during the chronic phase of infection. iNOS was associated with parasite replication (arrows) in wildtype and knockout mice. (C) *iNos* gene expression was also measured by real time PCR of mRNA isolated from whole brain homogenate on day 22 post-infection. Knockout mice (KO) had higher levels of *iNOS* transcript in comparison to wildtype (WT) mice. (D) Flow cytometric analysis of MHC class I and II expression on

dendritic cells (DCs) purified from the brain revealed that DCs from knockout mice (gray line) express less MHC molecules than DCs from wildtype mice (black line). Solid histograms represent negative staining controls. (E) MHC class II (red) expression was also assessed by IHC and demonstrated that MHC class II expression was associated with regions of parasite replication (green).

Figure 3. T cells infiltrate the CNS during Toxoplasmic encephalitis in NF-κB1^{-/-} mice
 (A) Immunohistochemical analysis of brain sections from wildtype BALB/c (WT) and NF-κB1^{-/-} (KO) mice detected uncontrolled parasite replication (green) in the knockout animals during chronic infection. CD4⁺ and CD8⁺ T cells (red) accumulate in regions of parasites (green) in both wildtype and knockout animals. Nuclei were stained with DAPI (blue). (B) CD8⁺ T cells were also observed in close proximity to infected cells in knockout animals, indicated by arrow. (C) T cell proliferation in the CNS was assessed by immunohistochemical staining for Ki67. CD4⁺ and CD8⁺ T cells (red) were positive for Ki67 (green) in wildtype (WT) and knockout (KO) mice, with more T cells detected in wildtype mice. Arrows indicate Ki67⁺ cells.

Figure 4. NF- κ B1^{-/-} mice have reduced numbers of activated T cells during chronic *T. gondii* infection

T cells populations were assessed by flow cytometry on day 22 post-infection with 20 cysts of Me49 strain *T. gondii*. The CD8⁺ T cell population was significantly reduced in the spleens (A) and brains (B) of NF- κ B1^{-/-} mice (KO) in comparison to wildtype BALB/c mice (WT). In addition, the percentage and number of activated (CD44^{hi} CD62L^{lo}) CD4⁺ and CD8⁺ T cells was significantly lower in the spleens of knockout mice during chronic infection (C and D). Significantly fewer activated CD8⁺ T cells were isolated were brain during chronic *T. gondii* infection (E). Data is representative of three individual experiments with five mice in each group. Data is presented as mean $\pm$ SEM. *, $p \leq 0.05$ and, **, $p \leq 0.01$.

Figure 5. NF- κ B1^{-/-} mice fail to mount a strong IFN- γ response during *T. gondii* infection
 Serum levels of IL-12p40 (A) and IFN- γ (B) were measured by ELISA on day 10 post-infection. Knockout animals have increased serum levels of IL-12p40 and reduced levels of IFN- γ . Recall responses of splenic T cells on day 10 (C) and 22 (D) post-infection demonstrated decreased IFN- γ production by cells from knockout animals (striped bars) in response to soluble *Toxoplasma* antigen (STAg) in comparison to wildtype mice (solid bars). Results are representative of three independent experiments with an n=3.

Figure 6. Fewer IFN- γ -producing T cells are generated in NF- κ B1^{-/-} mice and NF- κ B1^{-/-} T cells provide less protection to SCID mice than wildtype T cells

Intracellular cytokine staining of T cells from the brain and the spleen on day 22 post-infection demonstrated that T cells from knockout mice are capable of producing IFN- γ (A). Fewer IFN- γ producing CD8⁺ T cells were present in the spleen (B) and brain (C). Data is representative of three individual experiments with an n=5. Data is presented as mean $\pm$ SEM. **, p $\leq$ 0.01. To assess for T cell-intrinsic defects in NF- κ B1^{-/-} mice 2×10^6 purified T cells from wildtype BALB/c or NF- κ B1^{-/-} mice were transferred i.p. to BALB/c SCID mice and infected the following day with *T. gondii*. The NF- κ B1^{-/-} T cells provide a lesser degree of protection than wildtype T cells to SCID mice (D). Less IFN- γ is detected in the serum of SCID mice receiving NF- κ B1^{-/-} T cells (E).