Z-DNA Binding Protein Mediates Host Control of *Toxoplasma gondii* Infection

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Intrinsic to *Toxoplasma gondii* infection is the parasite-induced modulation of the host immune response, which ensures establishment of a chronic lifelong infection. This manipulation of the host immune response allows *T. gondii* to not only dampen the ability of the host to eliminate the parasite but also trigger parasite differentiation to the slow-growing, encysted bradyzoite form. We previously used RNA sequencing (RNA-seq) to profile the transcriptomes of mice and *T. gondii* during acute and chronic stages of infection. One of the most abundant host transcripts during acute and chronic infection was Z-DNA binding protein 1 (ZBP1). In this study, we determined that ZBP1 functions to control *T. gondii* growth. In activated macrophages isolated from ZBP1 deletion (ZBP1^{-/-}) mice, *T. gondii* has an increased rate of replication and a decreased rate of degradation. We also identified a novel function for ZBP1 as a regulator of nitric oxide (NO) production in activated macrophages, even in the absence of *T. gondii* infection. Upon stimulation, *T. gondii*-infected ZBP1^{-/-} macrophages display increased proinflammatory cytokines compared to wild-type macrophages under the same conditions. These *in vitro* phenotypes were recapitulated *in vivo*, with ZBP1^{-/-} mice having increased susceptibility to oral challenge, higher cyst burdens during chronic infection, and elevated inflammatory cytokine responses. Taken together, these results highlight a role for ZBP1 in assisting host control of *T. gondii* infection.

Toxoplasma gondii is an obligate intracellular parasite capable of infecting any nucleated cell in warm-blooded animals. With such a large host range, *T. gondii* has become one of the most prevalent eukaryotic parasites in the world, with approximately 30% of the human population infected (1). *T. gondii* has both a sexual and an asexual cycle, with the sexual cycle occurring only in the feline intestine and the asexual cycle existing in all warm-blooded hosts. Two asexual forms of the parasite exist in infected animals, the rapidly replicating tachyzoite and the slower-growing encysted bradyzoite. During acute infection, the tachyzoite disseminates throughout the host until pressure from the immune system triggers differentiation to the slower-growing encysted bradyzoite, which signifies the establishment of a chronic lifelong infection. Cysts containing bradyzoites occur only in cells of the central nervous system and striated muscle (2). Cysts persist for the lifetime of the host and remain infectious if contaminated tissue is consumed. The most common routes of exposure to *T. gondii* are ingestion of undercooked meat containing bradyzoite cysts or consumption of unwashed food contaminated with environmentally stable oocysts (3).

Infection with *T. gondii* is typically asymptomatic but does present issues in the immunocompromised and in unborn fetuses when acquired congenitally. In immunocompetent hosts, a multitude of defenses to combat *T. gondii* infection are present, with the majority involved in production of interferon gamma (IFN- γ). The significance of IFN- γ during infection is attributed to its ability to stimulate hundreds of genes (4). These genes initiate an array of responses necessary for control of parasite growth and dissemination, including host immune cell proliferation, differentiation, and destruction of infected cells. *T. gondii* has developed strategies to evade these host immune responses. An example of *T. gondii* modulation of host cell responses is its ability to block degradation in activated macrophages (5, 6). At least part of this block is due to the ability of *T. gondii* to suppress nitric oxide

(NO) production by limiting the availability of intracellular arginine (5, 7, 8). Type I strain parasites initiate arginine starvation by secreting ROP16, a kinase that activates STAT6, resulting in expression of host arginase-1 (5). Arginase-1 degrades available host cell arginine, thus limiting the availability for NO (9). A decrease in NO synthesis would appear to be beneficial to the parasite, but *T. gondii* is an arginine auxotroph and exhibits decreased growth in media lacking arginine (5). Another example of the *T. gondii*-triggered host response is the MyD88-dependent production of interleukin 12 (IL-12) and subsequent expression of IFN- γ . The downstream effector of the MyD88 pathway that triggers IL-12 transcription is NF- κ B. Type II strains of *T. gondii* promote expression and translocation of NF- κ B to the nucleus via secretion of GRA15 (10). The promotion of this proinflammatory signal would seem detrimental to the parasite, but stimulation of this mechanism may be an adaptation of the parasite to ensure survival of the host, establishment of a stable chronic infection, and subsequent transmission of the parasite to the next host.

Our laboratory has sought to determine other mechanisms of host and parasite interactions through dual-transcriptome analysis of mice infected with *T. gondii* (11). From this data set, host Z-DNA binding protein 1 (ZBP1) was shown to be highly abun-

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dant at acute and chronic time points compared to values in uninfected samples. ZBP1 also had a fold change difference of approximately 240 in a similar study in which *T. gondii*-infected mice were compared at 30 days postinfection to uninfected mice (12). Since its initial identification, ZBP1 has been implicated in the cytosolic sensing of foreign bacterial and viral DNA and subsequent activation of type I interferon pathways (13–15). Known binding partners are RIPK3 and RIPK1 through the RHIM binding domain of ZBP1 and can activate NF- κ B through IRF3 and TBK1 recruitment (13–16). In more recent years, ZBP1 has been studied in the context of virus-induced necroptosis through RIPK3, in the absence of RIPK1 (17). It has been demonstrated that ZBP1 and RIPK3 form a complex that triggers necroptosis of infected cells, with viruses lacking the M45 gene responsible for blocking the interaction of RIPK3 and ZBP1 with the RHIM domain (17). ZBP1 also initiates type I interferon production in response to viral infection, which is a critical response for clearance. Although it has been implicated in multiple host defense pathways, ZBP1 was deemed dispensable for the innate and adaptive immune response to B-DNA and DNA vaccine (18), possibly due to redundancy in DNA sensing proteins. The role of ZBP1 during parasitic infection has yet to be elucidated. In this paper, we address the significance of ZBP1 expression during *T. gondii* infection.

MATERIALS AND METHODS

qPCR. Using an Invitrogen Superscript III reverse transcriptase cDNA synthesis kit, cDNA was generated from the same RNA samples as used for previous RNA sequencing (RNA-seq) analysis (11). Quantitative PCR (qPCR) primers were IDT PrimeTime primers designed to target ZBP1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a house-keeping control. Fold changes were determined using the threshold cycle ($\Delta\Delta C_T$) method, normalized to GAPDH, and then compared to uninfected samples. For qPCR measurements in macrophages, wild-type (WT) and ZBP1^{-/-} macrophages were plated and infected at a multiplicity of infection (MOI) of 5. Two hours postinfection, macrophages were stimulated with 5 ng/ml of lipopolysaccharide (LPS) and 25 U/ml of IFN- γ , and 48 h postinfection, RNA was extracted using TRIzol. cDNA was synthesized using an Invitrogen Superscript III reverse transcriptase cDNA synthesis kit. PrimeTime qPCR primers from IDT targeting GAPDH (control), inducible nitric oxide synthase (iNOS), ZBP1, and ARG1 were used in these experiments. ZBP1, iNOS, and ARG1 transcript levels were normalized to GAPDH levels and then compared to those of uninfected wild-type and ZBP1^{-/-} naive macrophages using the $\Delta\Delta C_T$ method.

Determination of parasites per vacuole and the percent degraded parasites. Bone marrow-derived macrophages were isolated and grown in RPMI medium containing 20% L929 cell-conditioned medium as previously described (19). A total of 1×10^5 wild-type or ZBP1^{-/-} macrophages were plated on glass coverslips in RPMI medium. Cells were infected with 5×10^5 parasites per well. Two hours postinfection, the medium was changed and RPMI medium containing 5 ng/ml of LPS and 25 U/ml of IFN- γ or fresh untreated RPMI was added to the cells. For the replication assay, cells were fixed at 24 h postinfection, and for the degradation assay, cells were fixed at 48 h postinfection. Cells were incubated with chronic-infection serum, followed by Alexa Fluor 488-conjugated anti-mouse secondary antibody. Parasites were visualized using a Zeiss inverted Axiovert 200 motorized microscope with a 100 $\times$ objective (PlanApo 1.4-numerical-aperture oil PH3 objective). Slides were blinded prior to the counting. For the replication assay, a total of 6 slides for wild-type cells and 6 slides for ZBP1^{-/-} cells were quantified, with 150 vacuoles counted on each slide. The percentages of vacuoles containing 1, 2, 4, and 8 parasites were calculated based on the number of total vacuoles

counted on each slide. For the degradation assay, a total of 4 slides for wild-type cells and 4 slides for ZBP1^{-/-} cells were quantified, with 100 vacuoles counted on each slide. The percentage of vacuoles containing degraded parasites was calculated based on number of total vacuoles counted on each slide as described in reference 20.

NO assay. Wild-type and ZBP1^{-/-} macrophages were seeded at 1×10^4 cells per well in a 96-well plate. Three hours after plating, macrophages were infected with *T. gondii* at an MOI of 0, 2, 5, or 20. Two hours postinfection, the medium was replaced with RPMI medium containing 5 ng/ml of LPS and 25 U/ml of IFN- γ or fresh untreated RPMI. Supernatants from the cells were removed 48 and 72 h postinfection and transferred to a new 96-well plate. NO levels were determined using the Promega Griess reagent system according to the manufacturer's protocol. Absorbance was measured at 530 nm using a Synergy HT plate reader. Concentrations were determined using a standard curve generated for each reaction. Experiments were conducted with three technical replicates and three biological replicates. Statistical significance was determined using GraphPad Prism's two-way analysis of variance (ANOVA).

Mouse experiments. All animal use was approved by and in accordance with the policies of the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison. ZBP1^{-/-} mice were backcrossed 6 times to C57BL/6 mice; therefore, to generate ZBP1^{+/+} and ZBP1^{-/-} mice, we established heterozygous ZBP1^{+/-} breeding colonies. ZBP1^{+/-} mice were bred to produce wild-type, heterozygous, and knockout mice. Primers used for genotyping were WT forward (GCTCTGGGAATGACGACAGC), knockout forward (CTAAGCGCATGCTCCAGACTG), and reverse (CACTTCGTCTGCCCTCAATTAGA).

Six- to 8-week-old ZBP1^{+/+} and ZBP1^{-/-} mice were used for all studies. For acute infection, mice were infected with 5×10^3 tachyzoites by intraperitoneal (i.p.) injection. Animals were monitored daily for clinical signs of disease (ruffled fur, hunched posture, paralysis, etc.) and were euthanized if moribund. For quantification of the cyst burden, mice were infected with 1×10^3 tachyzoites by i.p. injection. Mice were sacrificed at 24 days postinfection, and their brains were removed and ground with a mortar and pestle in 1.3 ml of phosphate-buffered saline (PBS). A total of 250 μ l of the homogenized brain was spun at $3,000 \times g$ for 5 min, and then the supernatant was removed for cytokine analysis and the pellet fixed with 3.0% formaldehyde. Tissue cysts were stained with fluorescein-labeled *Dolichos biflorus* agglutinin (Vector Laboratories, Burlingame, CA), 3- to 5- μ l samples were mounted, and cyst numbers were determined by fluorescence microscopy. Samples were blinded to ensure no counting bias.

For oral-infection studies, 8-week-old CBA/J mice from Jackson Laboratory were infected with 2×10^4 tachyzoites i.p. Cyst burden was quantified as described above for 2 to 4 mice at 27 days postinfection. Then at 28 days postinfection, the brains were removed from the rest of the mice, pooled, ground with a mortar and pestle, and fed to ZBP1^{+/+}, ZBP1^{+/-}, and ZBP1^{-/-} mice at approximately 4,000 cysts/mouse. Mice were monitored daily for clinical signs of disease (ruffled fur, hunched posture, paralysis, etc.) and were euthanized if moribund.

Cytokine quantification. Wild-type and ZBP1^{-/-} bone marrow-derived macrophages were infected with *T. gondii* at an MOI of 5 and then stimulated with LPS and IFN- γ after 2 h. At 48 h postinfection, medium was removed and the cytokines were quantified using a mouse inflammation cytokine bead array (CBA; BD Biosciences), which measures IL-12p70, IFN- γ , tumor necrosis factor alpha (TNF- α), monocyte chemoattractant protein 1 (MCP-1), IL-6, and IL-10 in the same sample. Alternatively, cytokines were measured in the supernatants of the 24-day-postinfection brain preparations described above. Events were collected and gated using the BD LSR II flow cytometer and FACSDIVA software (BD Biosciences).

RESULTS

ZBP1 is highly abundant during acute and chronic *T. gondii* infection. Our laboratory previously generated an *in vivo* RNA-

TABLE 1 Comparison of ZBP1 transcripts as determined by RNA-seq and qPCR

Method	Fold change of transcripts between forebrains of ^a :	
	Acutely infected vs uninfected mice	Chronically infected vs uninfected mice
RNA-seq	300	1,000
qPCR	480	1,500

^a Fold change of ZBP1 transcript levels between acutely infected versus uninfected and chronically infected versus uninfected mouse forebrains was analyzed through RNA-seq analysis, as described previously (11). To verify the dramatic increase in ZBP1 transcripts between each sample, qPCR using the same cDNA samples was performed. Primers targeting ZBP1 and GAPDH were used. qPCR fold change was determined by normalization to GAPDH and then comparison to the values for uninfected samples using the $\Delta\Delta C_T$ method.

seq time course of the forebrains of mice during acute and chronic *T. gondii* infection (11). Differential expression analysis was conducted and comparisons were made between the brains of uninfected mice and the brains of mice 10 and 28 days postinfection. One of the highest differentially expressed transcripts between uninfected and infected time points was ZBP1. The average values for fold change between acute versus uninfected and chronic versus uninfected time points were 300 and 1,000, respectively (Table 1). qPCR was conducted on these samples to verify the increase in abundance of ZBP1 transcripts between each experimental time point. Values for fold change of ZBP1 transcript levels were similar between qPCR and RNA-seq analysis (Table 1).

ZBP1 expression is altered by *T. gondii* in activated macrophages. ZBP1 was originally described as a product highly upregulated in response to LPS and IFN- γ stimulation in macrophages (21). Because upregulation of ZBP1 was highest in macrophages stimulated with both LPS and IFN- γ (21), we characterized the expression of ZBP1 by qPCR in LPS- and IFN- γ -stimulated macrophages with and without *T. gondii* infection. In wild-type uninfected macrophages stimulated with LPS and IFN- γ , an 11.5-fold increase in ZBP1 expression was detected compared to the value for uninfected naive macrophages (Fig. 1A). In wild-type macrophages infected with *T. gondii* and stimulated, only a 5-fold increase in expression of ZBP1 was observed. As expected, there was no expression of ZBP1 in macrophages from ZBP1^{-/-} mice. These results confirm that ZBP1 is upregulated by IFN- γ in macrophages and that macrophages do not initiate expression of ZBP1 in response to *T. gondii* infection alone. The decrease in ZBP1 transcript levels between infected and uninfected macrophages was significant, suggesting that while IFN- γ induces expression of ZBP1, *T. gondii* infection can counteract that induction.

Absence of ZBP1 leads to increased parasite replication and decreased parasite degradation in activated macrophages. To determine if ZBP1 contributes to *T. gondii* growth in macrophages, a replication assay was conducted. Bone marrow-derived macrophages were plated on glass coverslips, infected with *T. gondii* at an MOI of 5, and stimulated with LPS and IFN- γ 2 h postinfection. After 24 h, cells were fixed and an immunofluorescence assay (IFA) was used to determine the number of parasites per vacuole. The number of total parasites within the 150 random vacuoles was quantified; ZBP1^{-/-} stimulated macrophages contained significantly more parasites than wild-type stimulated

macrophages (Fig. 1B). In wild-type macrophages, there was a statistically higher percentage of single parasite vacuoles: 44%, compared to 28% ZBP1^{-/-} cells. Only 13% of wild-type cells contained vacuoles with 4 parasites, whereas 24% in ZBP1^{-/-} cells contained vacuoles with 4 parasites. To analyze whether the difference in parasite numbers was intrinsic to stimulation, we compared the numbers of parasites per vacuole in naive wild-type and ZBP1^{-/-} macrophages. There was no statistical difference in the numbers of parasites per vacuole between wild-type and ZBP1^{-/-} naive macrophages (data not shown). These data suggest that ZBP1 has an influence on parasite growth and/or stability in activated, but not naive, macrophages.

Because we had previously determined that decreased parasite growth in activated macrophages could be directly related to parasite degradation (20), we assessed the ability of stimulated wild-type and ZBP1^{-/-} macrophages to degrade intracellular *T. gondii*. Using IFA and differential interference contrast (DIC) to visualize *T. gondii* within stimulated macrophages, parasites were verified as being intracellular and classified as degraded based on the lack of consistent staining around the membrane of the parasites (20). A total of 100 vacuoles were counted, and the percentage of degradation was quantified. Over 60% of parasites within wild-type activated macrophages appeared degraded, whereas only 30% of parasites were degraded in activated ZBP1^{-/-} macrophages (Fig. 1C). These data suggest that ZBP1 has a key role in the pathway that leads to *T. gondii* degradation in activated macrophages.

NO production is decreased in ZBP1^{-/-} activated macrophages. Generation of reactive nitrogen species, such as nitric oxide (NO), by macrophages is critical for control of *T. gondii* infection (22, 23). Macrophage production of NO induces degradation of intracellular *T. gondii*, with detectable levels of NO at 48 h poststimulation (24). Because of the enhanced degradation of *T. gondii* in stimulated wild-type macrophages, compared to ZBP1^{-/-} macrophages, we assessed NO production in *T. gondii*-infected cells. Wild-type and ZBP1^{-/-} bone marrow-derived macrophages were infected with *T. gondii* at an MOI of 0, 2, or 5. Two hours postinfection, macrophages were primed and stimulated with LPS and IFN- γ , and 48 h postinfection, a Griess reaction was performed to quantify production of NO. Wild-type cells produced significantly higher levels of NO in response to stimulation than did ZBP1^{-/-} cells (Fig. 2A). There was a significant decrease in NO production ZBP1^{-/-} macrophages, even in the absence of *T. gondii* infection (MOI of 0), indicating the role of ZBP1 in the NO production pathways.

To evaluate whether the decrease in NO production in ZBP1^{-/-} macrophages is due to a difference in transcriptional expression of inducible nitric oxide synthase (iNOS), the enzyme that catalyzes the conversion of L-arginine into NO, qPCR was performed. Uninfected and infected wild-type and ZBP1^{-/-} macrophages, with and without stimulation, were grown for 48 h prior to RNA extraction. No difference in levels of iNOS transcript expression was observed between wild-type and ZBP1^{-/-} infected or uninfected stimulated macrophages (Fig. 2B). A decrease in iNOS transcript levels was seen between infected and uninfected wild-type and ZBP1^{-/-} macrophages, indicating that *T. gondii* infection can inhibit transcription of iNOS independently of ZBP1.

A decrease in NO production can also be attributed to reduction in the availability of the substrate of iNOS, L-arginine. *T. gondii* upregulates expression of arginase-1 (ARG1), an enzyme

FIG 1 ZBP1 is upregulated and influences parasite replication in activated macrophages. (A) Wild-type (WT) and ZBP1^{-/-} macrophages were infected with *T. gondii* at an MOI of 5 (infected) or left uninfected. Two hours later, the medium was changed with no additives (naive) or with 5 ng/ml of LPS and 25 U/ml of IFN- γ (stimulated). At 48 h postinfection, RNA was extracted, cDNA was synthesized, and qPCR was performed using primers targeting ZBP1 and the GAPDH housekeeping gene. Using the $\Delta\Delta C_T$ method, ZBP1 transcript levels were normalized to GAPDH transcripts and then normalized to uninfected naive cells and the fold change displayed on the y axis. Thus, uninfected naive cells have a fold change of 1. qPCR was performed in duplicate, and RNA was collected from 3 independent experiments. *, $P = 0.0123$ as determined by 2-way ANOVA of stimulated versus naive samples. (B) *T. gondii* was cultured in wild-type and ZBP1^{-/-} macrophages and stimulated with LPS and IFN- γ .

that competes with iNOS for L-arginine in the cell, in order to decrease the ability of cells to produce NO (5). qPCR was also performed to determine if the decrease in NO production in ZBP1^{-/-} macrophages was due to an increase of ARG1 expression (Fig. 2C). Infected ZBP1^{-/-} macrophages increased ARG1 expression at levels similar to those of wild-type macrophages, indicating the reduced NO levels in ZBP1^{-/-} macrophages is not due to ZBP1 influence on ARG1 expression.

Increased levels of proinflammatory cytokines in ZBP1^{-/-} macrophages. To assess whether a lack of ZBP1 leads to defects in cytokine production after stimulation with LPS and IFN- γ , the mouse inflammation CBA was used to determine levels of IL-12, TNF- α , IL-10, MCP-1, and IL-6. Wild-type and ZBP1^{-/-} bone marrow-derived macrophages were infected with *T. gondii* at an MOI of 5 and then stimulated with LPS and IFN- γ 2 h postinfection. At 48 h postinfection, ZBP1^{-/-} macrophages had increased levels of TNF- α , MCP-1, and IL-6 compared to those of wild-type cells (Fig. 3). There was no difference in IL-12 or IL-10 levels between stimulated wild-type and ZBP1^{-/-} macrophages. While IFN- γ is part of the mouse inflammation array, levels were saturated due to the activation of the macrophages with IFN- γ . These results indicate that the levels of the proinflammatory molecules TNF- α , MCP-1, and IL-6 are higher in ZBP1^{-/-} macrophages.

ZBP1^{-/-} mice have increased inflammatory cytokines and cyst counts during chronic infection. Because ZBP1 was highly abundant in mouse brains, compared to uninfected controls (Table 1), we determined whether ZBP1 influences outcome of *T. gondii* acute and chronic infections. To examine acute infection, wild-type and ZBP1^{-/-} mice were infected with 5×10^3 parasites by i.p. inoculation. No significant differences were seen in the susceptibilities of wild-type and ZBP1^{-/-} mice to this high dose of parasites (data not shown).

We then compared the cyst burdens during chronic infection of wild-type and ZBP1^{-/-} mice infected with a lower dose (1×10^3). ZBP1^{-/-} mice had a statistically higher cyst burden at 28 days postinfection than wild-type mice (Fig. 4A). We measured the cytokine levels in these brains and made observations similar to those for the macrophages, where ZBP1^{-/-} mice had increased levels of TNF- α , MCP-1, and IL-6 compared to those of wild-type mice (Fig. 5). We also saw higher levels of IFN- γ in the ZBP1^{-/-} mice, which we were not able to measure in our stimulated macrophages. There was no difference in IL-12 or IL-10 levels between infected wild-type and ZBP1^{-/-} mouse brains. These *in vivo* results were similar to the tissue culture results of increased parasitemia and inflammatory cytokines.

ZBP1^{-/-} mice have decreased resistance to *T. gondii* after oral challenge. ZBP1 was highly abundant in the small intestine of mice (21, 25), possibly to assist in the control of oral pathogens. To

At 24 h postinfection, the number of total parasites was determined by adding together the numbers of vacuoles containing 1, 2, and 4 parasites per vacuole from a total of 150 vacuoles. Three technical replicates were collected from two independent experiments. ***, $P = 0.0005$ as determined by paired *t* test. (C) Degradation of *T. gondii* is decreased in stimulated ZBP1^{-/-} macrophages. WT and ZBP1^{-/-} macrophages were infected with *T. gondii* (MOI of 5), stimulated with LPS and IFN- γ 2 h later, and fixed 48 h postinfection. Degradation was determined based on presence of intact vacuole staining by IFA as described in reference 20. One hundred vacuoles were counted, and the percentage of intact versus degraded parasites was counted in duplicate in three independent experiments. ***, $P = 0.0006$ as determined by paired *t* test.

FIG 2 NO production is decreased in stimulated ZBP1^{-/-} macrophages. (A) Wild-type and ZBP1^{-/-} macrophages were infected with *T. gondii* at an MOI of 0, 2, 5, or 20. At 2 h postinfection, cells were stimulated, and at 48 h postinfection, NO levels in cell supernatants were determined by Griess reaction. Three technical replicates were collected from three independent experiments. ***, $P = 0.0001$ as determined by 2-way ANOVA of all data. (B and C) To determine the mechanism of NO reduction in ZBP1^{-/-} cells, qPCR was performed targeting iNOS (B) and ARG1 (C) transcripts. Levels of iNOS and ARG1 transcripts were normalized to GAPDH and then to naive conditions for wild-type (black bars) and ZBP1^{-/-} (gray bars) macrophages using the $\Delta\Delta C_T$ method. The fold change for stimulated versus naive cells is displayed on the y axis. qPCR was performed in duplicate in two independent experiments.

test this hypothesis, we fed wild-type and ZBP1^{-/-} mice approximately 4,000 *T. gondii* cysts each and monitored their health. ZBP1^{-/-} mice showed an increase in susceptibility to lethal oral doses of *T. gondii* compared to that of wild-type mice (Fig. 4B)

FIG 3 Expression of proinflammatory cytokines is increased in ZBP1^{-/-} macrophages. Wild-type and ZBP1^{-/-} macrophages infected with *T. gondii* at an MOI of 5, stimulated after 2 h with LPS and IFN-γ, and grown for an additional 48 h. Cytometric bead arrays were used to determine levels of IL-12, IFN-γ, TNF-α, IL-10, MCP-1, and IL-6 from supernatants. Two biological replicates were used for the analysis. TNF-α (A) and IL-6 (B) levels were significantly higher in ZBP1^{-/-} supernatants. *, $P = 0.029$ and 0.035 for panels A and B, respectively, as determined by *t* test.

($P = 0.05$). The heterozygous mice (ZBP1^{+/-}) displayed a phenotype that was in between those of the wild-type and ZBP1^{-/-} mice, suggesting a gene dosage effect of ZBP1 in the control of *T. gondii*. Overall, these animal studies mirror the results seen in tissue culture, namely, increased parasite growth and cytokine

FIG 4 ZBP1^{-/-} mice have higher cyst burdens and are more susceptible to oral *T. gondii* infection. ZBP1^{+/-} mice were bred to each other to produce the ZBP1^{+/+} (wild type), ZBP1^{+/-}, and ZBP1^{-/-} mice used in these studies. (A) Wild-type and ZBP1^{-/-} mice were infected with 1×10^5 *T. gondii* organisms and sacrificed at 28 days postinfection, and the cyst burdens were quantified in brain homogenates. Shown are the pooled results from two independent experiments with 9 wild-type and 14 ZBP1^{-/-} mice. (B) Wild-type, ZBP1^{+/-}, and ZBP1^{-/-} mice were fed brains of mice chronically infected with *T. gondii* for 28 days, receiving approximately 4,000 cysts each, and time to death was determined. Shown are the pooled results from two independent experiments with 6 wild-type, 16 ZBP1^{+/-}, and 12 ZBP1^{-/-} mice. Statistical significance was determined by log rank test, which showed a *P* value of 0.05 for WT versus ZBP1^{-/-} mice.

production in the absence of ZBP1. They suggest that ZBP1^{-/-} mice have increase in proinflammatory cytokines (Fig. 3 and 5) but are not able to control the parasite burden or that increased parasitemia leads to higher cytokine production.

DISCUSSION

We previously used RNA-seq to determine the host and parasite transcriptomes during *T. gondii* acute and chronic infection (11), with the ultimate goal of understanding the mechanisms involved in establishment and maintenance of infection. From this data set, the host-specific gene ZBP1 was highly expressed in the brains of mice during acute and chronic infection (11). The gene for ZBP1 was described over a decade ago as upregulated in macrophages in response to LPS and IFN- γ (21). Since its discovery, the function of ZBP1 in classically activated macrophages has not been elucidated. In this study, we have identified ZBP1 as another activation product that is manipulated by *T. gondii* upon infection because the LPS and IFN- γ induction of ZBP1 is reduced in *T. gondii*-infected macrophages (Fig. 1A). We determined that an absence of ZBP1 in activated macrophages leads to a defect in parasite

FIG 5 Expression of proinflammatory cytokines is increased in the brains of ZBP1^{-/-} infected mice. Wild-type and ZBP1^{-/-} mice were infected with 1×10^5 *T. gondii* organisms and sacrificed at 28 days postinfection, and the cytokine levels were measured from the supernatant of brain homogenates. Shown are the pooled results from two independent experiments with 8 wild-type and 12 ZBP1^{-/-} mice. Statistical significance was determined by a two-tailed, independent *t* test and is indicated by double or single asterisks. *P* values were as follows: for TNF- α , 0.0018; for IL-6, 0.0172; for MCP-1, 0.0461; and for IFN- γ , 0.0161.

degradation and an increase in parasite growth. We have also identified a novel function for ZBP1 as a regulator of NO production in activated macrophages, even in the absence of infection. Finally, we have demonstrated that these phenotypes in ZBP1^{-/-} tissue culture macrophages translate to increased parasite numbers and mortality in ZBP1^{-/-} mice. Overall, we have found that *T. gondii* has increased survival in ZBP1^{-/-} macrophages through lack of degradation. As macrophages are manipulated by *T. gondii* for use in dissemination throughout the host, we hypothesize that this increase in parasite survival leads to higher parasitemia in many organs, including those in immunoprivileged areas (26).

ZBP1 has been shown to initiate transcription of type I interferons; however, type I interferons are not critical for survival during *T. gondii* infection. Mice lacking the IFN- α /IFN- β receptor do not succumb to *T. gondii* infection as severely as IFN- γ ^{-/-} mice (27–29). Our data support the nonessential role of type I interferons during *T. gondii*, as no IFN- α transcripts were detected in stimulated wild-type or ZBP1^{-/-} macrophages (data not shown). These data suggest that ZBP1 does not function in the type I interferon response during *T. gondii* infection.

The reduced degradation of *T. gondii* in ZBP1^{-/-} activated macrophages lead us to investigate the role of the NO production pathways during infection. NO production during *T. gondii* infection has dichotomous roles. An absence of NO has been deemed dispensable for survival of *T. gondii* during acute infection, but tight regulation of NO production is critical, as too much can lead to substantial tissue damage (30, 31). While IL-12^{-/-} and IFN- γ ^{-/-} mice rapidly succumb to *T. gondii* during acute infection at low doses, iNOS^{-/-} mice do not begin to succumb until the early chronic stage, at 20 days postinfection (31). The role of iNOS has not been studied in the context of lethal doses of *T. gondii*, as the wild-type controls from this study did not begin to die of infection until 12 weeks postinoculation (31). These studies have also not yet addressed the potential role of NO during lethal oral challenge of *T. gondii* infection (30, 31). We conducted experiments with wild-type and ZBP1^{-/-} mice that were orally fed 4,000 cysts from the brains of mice infected with *T. gondii* for 4 weeks, simulating ingestion of a lethal challenge of *T. gondii*. Our data suggest a role for NO production in response to acute toxoplasmosis. Future experiments will elucidate the precise role of ZBP1 in the production of NO in activated macrophages as well as determine the contributions of ZBP1 during chronic *T. gondii* infection.

T. gondii blocks parasite degradation by reducing NO production in activated macrophages (5, 6). *T. gondii* accomplishes this through multiple strategies, including downregulating transcription of the enzyme iNOS, which is required for NO production (32). *T. gondii* also suppresses NO production by limiting the availability of the substrate required for NO production, L-arginine (5, 7, 8). This limitation is accomplished through *T. gondii*-mediated upregulation of ARG1, an enzyme that degrades intracellular arginine. The mechanism of action has been studied for type I strains, in which parasites initiate arginine starvation by secreting ROP16, a kinase that activates STAT6, resulting in expression of ARG1 (5). ARG1 upregulation leads to limiting the availability of L-arginine and thus NO synthesis (9). The mechanism of ARG1 upregulation and iNOS downregulation in cells infected with type II parasites is currently unknown. A decrease in NO synthesis would appear to be beneficial to the parasite, but *T. gondii* is an arginine auxotroph and exhibits decreased growth in

media lacking arginine (5). This is an example of *T. gondii* triggering a response that leads to reduction in parasite growth for the purpose of long-term survival of the parasite within the host. While NO plays a role during acute infection, it is most important during chronic infection, as iNOS knockout mice succumb to *T. gondii* in the early stages of chronic infection (20 to 25 days) (31).

Our data show that the downregulation of NO in ZBP1^{-/-} macrophages is not due to transcriptional differences in iNOS or ARG1 (Fig. 2B and C). However, iNOS expression is induced by TNF- α (33), which shows a statistically significant increase in ZBP1^{-/-} in both infected macrophages and mouse brains. We hypothesize that this increase in TNF- α seen in ZBP1^{-/-} macrophages is in response to the increased number of *T. gondii* organisms in the absence of ZBP1, perhaps due to the decrease in parasite degradation. ZBP1^{-/-} cells produce higher levels of IFN- γ , TNF- α , IL-6, and MCP-1, possibly as a means to initiate antimicrobial actions against *T. gondii* infection. The higher levels of IL-6, which can act in either proinflammatory or anti-inflammatory mechanisms, in stimulated ZBP1^{-/-} macrophages will need to be studied further to elucidate whether IL-6 acts to stimulate or dampen the cytokine response.

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