

# CCR2 Receptor Is Essential to Activate Microbicidal Mechanisms to Control *Toxoplasma gondii* Infection in the Central Nervous System

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**Chemokines comprise a structurally related family of cytokines that regulate leukocyte trafficking. Because infection with *Toxoplasma gondii* can induce an important inflammatory reaction that, if left uncontrolled, can lead to death, we investigated the role of the chemokine receptor CCR2 in *T. gondii* infection. We orally infected CCR2<sup>-/-</sup> mice with five ME-49 *T. gondii* cysts and monitored morbidity, survival, and immune response thereafter. The CCR2<sup>-/-</sup> mice displayed higher susceptibility to infection as all mice died on day 28 after infection. Despite similar Th1 responses, a more evident anti-inflammatory response was induced in the peripheral organs of CCR2<sup>-/-</sup> mice compared with wild-type C57BL/6 mice. Additionally, CCR2<sup>-/-</sup> mice presented greater parasitism and a milder inflammatory reaction in their peripheral organs with lesser CD4<sup>+</sup> and MAC-1<sup>+</sup> and greater CD8<sup>+</sup> cell migration. The parasite load decreased in these organs in CCR2<sup>-/-</sup> mice but remained uncontrolled in the central nervous system. Additionally, we observed down-regulated inducible nitric oxide synthase expression in peripheral organs from CCR2<sup>-/-</sup> mice that was associated with a small nitric oxide production by spleen macrophages. In conclusion, in the absence of CCR2, another mechanism is activated to control tissue parasitism in peripheral organs. Nevertheless, CCR2 is essential for the activation of microbicidal mediators that control *T. gondii* replication in the central nervous system. (Am J Pathol 2008, 173:741–751; DOI: 10.2353/ajpath.2008.080129)**

Chemokines play an important role in the physiopathology of toxoplasmosis in murine and *in vitro* models.<sup>1–5</sup> In the oral *Toxoplasma gondii* infection, enterocytes can initiate innate immunological events that lead to a robust inflammatory process in the gut.<sup>3</sup> Tissue samples isolated from the small intestine of *T. gondii* infected mice display a significant increase in chemokine secretion including monocyte chemoattractant protein 1 (CCL2) and interferon (IFN)- $\gamma$  inducible protein (CXCL10), and to a lesser extent, both macrophage inflammatory proteins 1 $\alpha$  and  $\beta$  (CCL3 and CCL4), as well as CXCL2 regulated on activation and normally T-cell-expressed (CCL5).<sup>6</sup> The CXCL2 secreted by enterocytes is involved in the trafficking of neutrophils and clearance of the invading parasite, since CXCL2-deficient mice fail to recruit neutrophils to the site of infection and exhibit higher parasite burden following *T. gondii* inoculation.<sup>7</sup> The expression of several chemokine receptors such as CCR1, CCR2, CCR5, and CXCR3 was observed in the ileum of *T. gondii*-infected mice, being that CCR5 expression is markedly increased in antigen-primed CD8<sup>+</sup> intraepithelial lymphocytes.<sup>8</sup> In addition to promoting cell migration by inducing a different set of chemokines and chemokine receptors, *T. gondii* produces specific chemokine-like factors, in particular cyclophilin-18 (C-18), that stimulates production of interleukin (IL)-12 through the CCR5 chemokine receptor.<sup>9</sup>

CCL2 is a chemokine that can be produced by many cells, including macrophages, dendritic cells, endothelial cells, and fibroblasts, and expression is promoted after exposure to inflammatory stimuli such as IL-1, tumor necrosis factor (TNF)- $\alpha$ , or IL-4.<sup>10</sup> CCR2 is the only known functional receptor for CCL2 expressed in monocytes and on activated and memory T cells,

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including both Th1 and Th2 cells,<sup>11,12</sup> and also has a vital role in host defense to a number of pathogens.<sup>2,13–17</sup> Likewise, the lack of CCR2 impairs the control of infectious diseases such as from *Mycobacterium tuberculosis*<sup>16</sup> and infection of *Leishmania major*.<sup>13,17</sup> Recent studies have shown the requirement of CCR2 and a substantial role for CCL2 in the recruitment of Gr-1<sup>+</sup> inflammatory monocytes in mice inoculated by intraperitoneal route with tachyzoites to the local control of *T. gondii* replication.<sup>2</sup>

Infection with *T. gondii* is naturally acquired through the oral route by ingestion of undercooked or raw meat containing cysts of the parasite or through ingestion of water or food contaminated with cysts or oocysts.<sup>18</sup> Although in immunocompetent individuals the infection with the parasite causes little or no overt signs of disease, in patients with immunodeficiency, or during congenital infection, *T. gondii* may emerge as a serious infection, which, if not treated, can lead to host death.<sup>19,20</sup> IL-12, TNF- $\alpha$ , and IFN- $\gamma$ , and the generation of reactive nitrogen intermediates show an important role as mediators of host resistance to early *T. gondii* infection in mice.<sup>20</sup> Resistance in both acute and chronic infection with *T. gondii* in the murine model is highly dependent on endogenous IFN- $\gamma$ .<sup>21</sup> Depending on genetic susceptibility in mice, the peroral infection with *T. gondii* can lead to CD4<sup>+</sup> T cell-dependent, interferon- $\gamma$ -mediated necrosis of the small intestine.<sup>22</sup> The oral route of *T. gondii* infection is thought to reflect the typical infection that occurs in naturally acquired toxoplasmosis in humans. To understand the role of CCR2 in acute and chronic *T. gondii* infection we inoculated CCR2-deficient mice with five cysts of ME-49 strain by oral route. This experimental procedure allowed us to analyze the effect of the absence of CCR2 in the morbidity, mortality, parasite multiplication, inflammatory lesions, and phenotype cell migration in peripheral organs and in the central nervous system (CNS) of *T. gondii*-infected mice. In the present investigation, we observed that the absence of CCR2 resulted in a higher susceptibility in the knockout mice with a down-regulation of microbicidal mechanisms and uncontrolled parasite burden in the CNS.

## Materials and Methods

### Animals

Female C57BL/6 (wild type; WT) and CCR2-deficient mice (CCR2<sup>-/-</sup>), 8 to 10 weeks old, were bred and maintained under standard conditions in the animal facility of the Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo-USP (Ribeirão Preto, Brazil). CCR2<sup>-/-</sup> mice were generated by W.A. Kuziel (Proc. Natl. Acad. Sci. USA 1997, Oct 94: 12053–12058 and were kindly supplied). CCR2<sup>-/-</sup> mice were backcrossed with C57BL/6 mice for over 20 generations. All procedures were in accordance with international guidelines for the use of animals and received prior approval by the animal ethics committee of the University of São Paulo.

### Parasites and Challenge Infection

The low-virulent ME-49 strain of *T. gondii* was used to infect animals. Cysts were harvested from the brains of C57BL/6 that had been inoculated 1 month beforehand with approximately 20 cysts by the intraperitoneal route. For the experimental infection CCR2<sup>-/-</sup> and C57BL/6 mice were orally infected with five cysts in a volume of 0.2 ml and mortality monitored daily for 2 months.

### Experimental Procedure and Histological Analysis

CCR2<sup>-/-</sup> and C57BL/6 mice were orally infected with five *T. gondii* cysts, and group of three mice were euthanized by cervical dislocation 8 and 23 days after infection. Blood samples were collected for serological assays, and tissue samples, such as CNS (particularly the brain), small intestine, lung, liver, and spleen were collected, fixed in 10% buffered formalin, and processed routinely for paraffin embedding and sectioning. The small intestine was cut into four pieces, and each piece was rolled on itself to make a "Swiss roll." The peripheral organs and the CNS were examined histologically. For each organ, tissue sections of 4  $\mu$ m thickness were mounted on slides and the sections stained with hematoxylin and eosin. All of the analysis were done at a magnification of 400 $\times$  in a blind manner by two investigators.

### Quantification of Tissue Parasitism by Immunohistochemistry

The tissue parasitism was quantified by immunohistochemistry as previously described.<sup>23,24</sup> Deparaffinized sections were incubated for 30 minutes at 37°C in phosphate-buffered saline (PBS) and 1% bovine serum albumin and then overnight at 4°C with polyclonal rabbit antibody against antigens of *T. gondii* ME-49 strain or nonimmune rabbit serum as control. After incubation with biotinylated sheep anti-rabbit antibodies, the sensitivity was improved with avidin-biotin complex (ABC kit, PK-4000; Vector Laboratories, Inc., Burlingame, CA). The reaction was developed with H<sub>2</sub>O<sub>2</sub> plus 3,3'-diaminobenzidine tetrahydrochloride (Zymed Laboratories Inc., CA) for 5 minutes, and the slides were examined under light microscopy. The tissue parasitism was scored by counting the number of parasitophorous vacuoles from the entire small intestine or per section in the CNS or from 40 microscopic fields in the lung, liver, and spleen (1  $\times$  400) from two histological sections of each mouse and from at least three mice per group.

### Immunohistochemical Analysis for Detection of CCL2, CCR2, CD4<sup>+</sup>, CD8<sup>+</sup>, MAC-1<sup>+</sup>, and Inducible Nitric Oxide Synthase (iNOS) Expression in Tissue Samples

To detect CCL2 and CCR2 expression by immunohistochemistry, deparaffinized sections were subjected to an

tigenic unmasking in microwave oven and then incubated for 30 minutes at 37°C in PBS/1% bovine serum albumin (Sigma Aldrich, St. Louis, MO) to reduce non-specific binding and then incubated with rat IgG anti-mouse CCL2 and goat IgG anti-CCR2 (Abcam, Inc.) at 4°C overnight. For CCL2 analysis, the slides were first incubated in PBS/saponin 0.01%. Secondary biotinylated antibodies and the streptavidin to improve the sensitivity were both from Dako Cytomation, LSAB (Dako North America). The reaction was visualized by incubating the sections with 3,3'-diaminobenzidine tetrahydrochloride (Zymed Laboratories Inc.). Control slides were incubated with nonimmune rat serum. The expression of CCL2 and CCR2 was measured by counting the positive cells in 40 microscopic fields.

To detect CD4<sup>+</sup>, CD8<sup>+</sup>, MAC-1<sup>+</sup> (monocytes/macrophages, granulocytes, and NK cells), and iNOS positive cells, tissue samples were removed, embedded in tissue-freezing medium (Tissue-Tek, Miles Laboratories), and stored in liquid N<sup>2</sup>. Five- $\mu$ m frozen tissue sections were obtained and fixed with ice-cold acetone for 10 minutes. The slides were placed in a humidified chamber, and the endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 minutes, followed by incubation with PBS plus 3% (P/V) nonfat milk (Nestle, São Paulo, Brazil). The slides were washed with PBS and incubated overnight with rat IgG anti-mouse CD4, MAC-1, and iNOS antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), rat IgG anti-mouse CD8 biotin-labeled antibodies (Caltag Laboratories), or normal rat IgG (controls) diluted 100 times in PBS. After successive rinsing with PBS, the sections were incubated for 30 minutes with biotin-labeled goat anti-rat antibody (Santa Cruz Biotechnology) and washed three times in PBS. Next, the sections were incubated with avidin-biotin-peroxidase complex (ABC kit, PK-4000; Vector Laboratories, Inc.), for 30 minutes at 37°C, the color developed with 3,3'-diaminobenzidine (Vector Laboratories, ON, Canada), and the slides counterstained with Mayer's hematoxylin. The phenotypic analysis was evaluated in 40 microscopic fields.

### *Flow Cytometry Assay (Fluorescence-Activated Cell Sorting)*

The small intestine inflammatory cell infiltrates were also evaluated by flow cytometry assay as described previously.<sup>25</sup> Viability was assessed by Trypan blue exclusion and cells were counted in a Neubauer chamber. Leukocytes obtained were incubated with the following antibodies: anti-CD8 phycoerythrin-labeled (BD Biosciences PharMingen) and anti-CD11b phycoerythrin-labeled (e-Bioscience), anti-CD4, anti-CD11c, anti-FITC-conjugated (BD Biosciences PharMingen), and anti-CD3 peridinin chlorophyll protein-conjugated (Biosciences PharMingen). Leukocytes were acquired (FACSCanto II, BD Bioscience, San Jose, CA) and analyzed by FlowJo software (TreeStar) according to size forward scatter and granularity side scatter dot plot. Single- or two-color staining was used to identify T lymphocytes (CD3<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>), phagocytes (CD11b<sup>+</sup>), and dendritic cells (CD11c<sup>+</sup>). The isotype

antibody controls used were rat IgG2a-PerCP, rat IgG2b-fluorescein isothiocyanate and rat IgG1-PE (BD Biosciences PharMingen). Results are expressed as mean  $\pm$  SEM of the percentage of each antibody specific stained subpopulation within the gated cells obtained from small intestine of five different animals per group.

### *Cytokine Measurement in the Serum Samples and Intestinal Homogenates*

The concentrations of cytokines were measured by sandwich enzyme-linked immunosorbent assay (ELISA). The small intestine tissue samples of 100 mg obtained from WT and CCR2<sup>-/-</sup> mice on day 8 of infection were homogenized with an Omni TH homogenizer in 0.5 ml of PBS containing protease inhibitors (0.1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L benzamide chloride, 10  $\mu$ g/ml aprotinin A, and 100  $\mu$ g/ml leupeptin). Each sample was then centrifuged for 10 minutes at 3000  $\times$  g, and the supernatant was used for ELISA. The IL-12p70, IFN- $\gamma$ , IL-10, and transforming growth factor (TGF)- $\beta$  (OpTEIA, BD Bioscience, San Diego, CA), TNF- $\alpha$ , IL-4, and CCL2 (Duoset R&D Systems, Minneapolis, MN), were assayed according to instructions from the manufacturer. The concentrations of cytokines in the samples were calculated from a standard curve of murine recombinant cytokine. The sensitivities of detection in the ELISAs were 31.25 pg/ml (IL-12p70), 15.63 pg/ml (IFN- $\gamma$ , IL-10, TNF- $\alpha$ , and CCL2), 62.5 pg/ml (TGF- $\beta$ ), and 3.9 pg/ml (IL-4).

### *Spleen Macrophage Cultures*

To isolate spleen macrophages, the WT and CCR2<sup>-/-</sup> mice were euthanized and their spleens were removed. The suspensions of spleen cells were washed in RPMI 1640 medium and treated for 4 minutes with lysing buffer (9 volumes of 0.16 mol/L NH<sub>4</sub>Cl and 1 volume of 0.17 mol/L Tris-HCl, pH 7.5). The erythrocyte-free cells were then washed three times and adjusted to 1  $\times$  10<sup>6</sup> cells/ml in RPMI supplemented with 5% fetal bovine serum. The cell suspension was distributed in triplicate in 96-well culture plate and incubated for 3 hours at 37°C in a humidified 5% CO<sub>2</sub> incubator. The nonadherent cells were removed by exhaustive washing with Hanks' solution, and the adherent cells were stimulated with IFN- $\gamma$  (1, 5, or 25 U/ml) and MCP-1 (2, 10, or 50 ng/ml) and the culture supernatants were collected after 48 hours for determination of NO production.

### *Nitrite Assay*

NO production by spleen macrophage was measured by accumulation of nitrite, nitric oxide's metabolite, in supernatants collected after 48 hours of culture, as described previously.<sup>26</sup> Briefly, 0.05 ml of Griess reagent (0.1% naphthyl ethylenediamine and 1% sulfanilamide in 2.5% phosphoric acid, prepared with reagents from Sigma) was added to 0.05 ml of supernatant, and absorbance



was read at 540 nm using an automated plate reader. Nitrite concentration was calculated from a  $\text{NaNO}_2$  standard curve.

### Statistical Analysis

The Kaplan-Meier method was used to compare the survival rates of the studied groups, and the survival curves were compared using log-rank and  $\chi^2$  tests that generate a two-tail *P* value to test the null hypothesis that they were identical in the overall groups of animals. Histological analysis, tissue parasitism, and cytokine levels produced by groups of animals were compared by using Mann-Whitney *U* test. Statistical analysis and graphs were performed using GraphPad prism version 4.0 (GraphPad Software, San Diego, CA). Values of *P* < 0.05 were considered statistically significant.

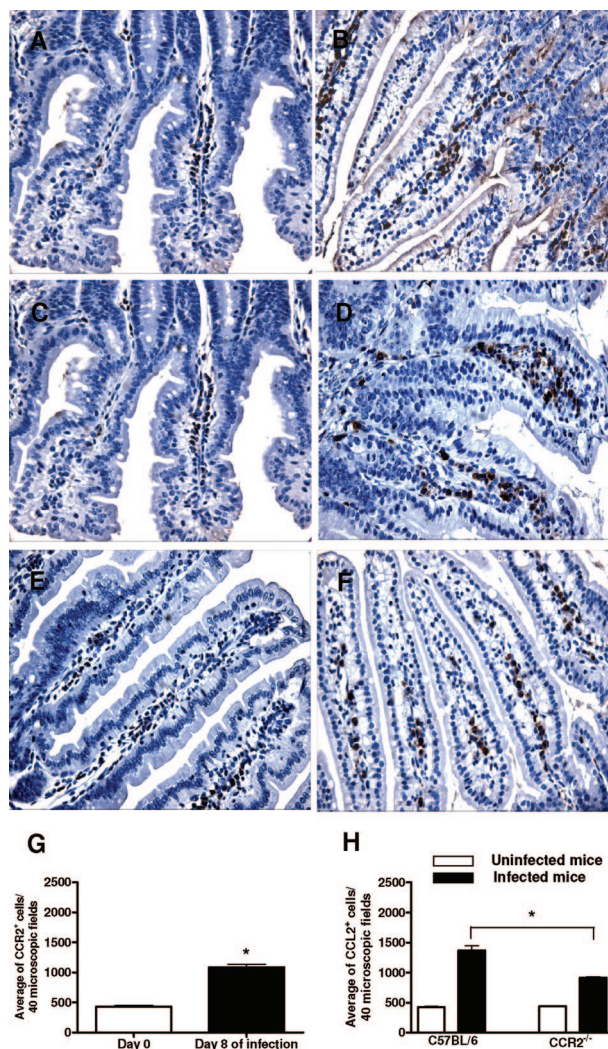
## Results

### The Expression of CCR2 and CCL2 Are Increased in Small Intestine after Oral *T. gondii* Infection

In our experiments, we evaluated the role of CCR2 in the resistance to the infection with five *T. gondii* cysts in the predominant stage of parasite transmission by the natural (oral) route of infection. Firstly, we evaluated the CCR2 and CCL2 expression in the intestinal tissue samples from WT mice uninfected or infected with *T. gondii*. We found that the number of CCR2<sup>+</sup> cells in WT mice was significantly increased on day 8 after inoculation of the parasite (Figure 1, B and G) compared with uninfected mice, which presented only a small number of CCR2<sup>+</sup> cells in the lamina propria (LP) (Figure 1A). In relation to the expression of CCL2, WT (Figure 1C) and CCR2<sup>-/-</sup> (Figure 1E) mice presented a small amount of CCL2<sup>+</sup> cells in the LP of the small intestine. However, an increase of these cells was observed on day 8 after *T. gondii* infection (Figure 1, D and F). In addition, infected WT mice presented a higher number of CCL2<sup>+</sup> cells (Figure 1D) compared with CCR2<sup>-/-</sup>-infected mice (Figure 1F), as clearly shown after quantification of these cells (Figure 1H). Next, we assayed the CCL2 production in the small intestine and in serum samples from WT and CCR2<sup>-/-</sup>-infected mice by ELISA. In accordance with previous results, the infection with *T. gondii* led to increased levels of CCL2 in the serum samples and in the small intestine at day 8 after infection (data not shown). Interestingly, systemic CCL2 production was increased in CCR2<sup>-/-</sup> compared with WT mice. Therefore, we suggest that after *T. gondii* infection the increase of CCL2 and CCR2 expression could be involved in the migration of inflammatory cells in the site of infection.

### Absence of CCR2 Leads to an Increased Susceptibility to *T. gondii* Infection

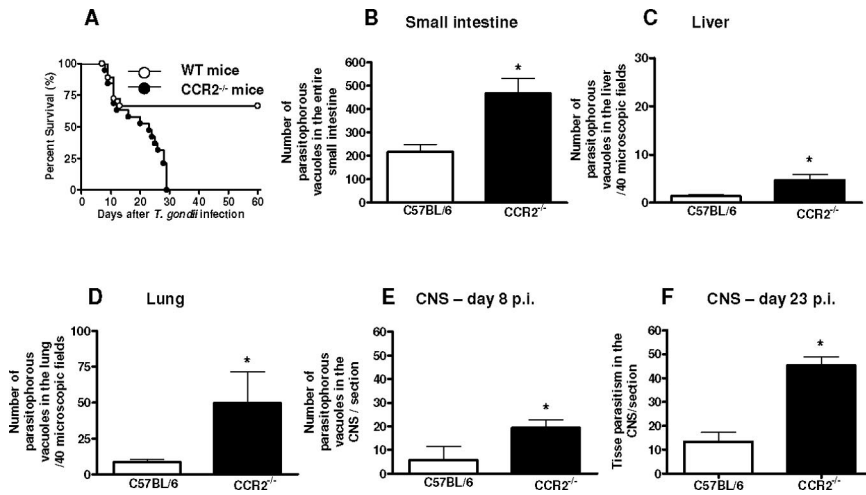
To analyze the role of CCR2 in the mortality of mice in oral *T. gondii* infection, the animals were inoculated with five *T.*



**Figure 1.** Immunohistochemical staining for CCR2 and CCL2 and quantification of positive cells in the small intestine of CCR2<sup>-/-</sup> and WT mice infected with five ME-49 *T. gondii* cysts by oral route. The CCR2 positive cells were found in the small intestine from WT noninfected mice (A), and the expression was higher on day 8 after infection (B). WT (C) and CCR2<sup>-/-</sup> (E) uninfected mice presented immunohistochemistry staining for CCL2 in the small intestine, and the expression was higher in the organ in WT (D) and CCR2<sup>-/-</sup> (F) on day 8 after infection. Number of infiltrating CCR2<sup>+</sup> cells (G) in the small intestine from WT mice and CCL2<sup>+</sup> cells (H) in the small intestine from WT and CCR2<sup>-/-</sup> mice. The positive cells were counted by immunohistochemistry in 40 microscopic fields. Data are representative of at least two independent experiments with three mice per group and two noncontiguous sections from each mouse. Original magnification,  $\times 40$ . \*, significantly different (*P* < 0.05).

*gondii* cysts by gavage. For these experiments, eight animals from each group were used, and the mortality was monitored daily until 60 days after infection. We found that 70% of WT mice survived until 60 days after infection. In contrast, the CCR2<sup>-/-</sup> mice presented profound susceptibility to the infection ( $\chi^2 = 12.47$ ; *P* = 0.0004; *df* = 1), presenting 100% mortality 28 days after *T. gondii* inoculation (Figure 2A). These findings indicate that CCR2<sup>-/-</sup> mice are highly susceptible to oral infection with *T. gondii*.

It is well known that oral infection with the ME-49 strain of *T. gondii* causes a high proliferation of parasites in the small intestine that disseminate through the blood ves-



**Figure 2.** Mortality rates and tissue parasitism of CCR2<sup>-/-</sup> and WT mice orally infected with five ME-49 *T. gondii* cysts. The mortality rate for eight mice from each group was determined (A). CCR2<sup>-/-</sup> mice were significantly more susceptible to toxoplasmosis than were WT mice ( $\chi^2 = 12.47$ ;  $P = 0.0004$ ;  $df = 1$ ). The tissue parasitism in the small intestine (B), liver (C), lung (D), and CNS (E, F) were detected by immunohistochemistry staining and scored by counting the number of parasitophorous vacuoles per 40 microscopic fields in the peripheral organs and number of parasitophorous vacuoles and cyst-like structures per section in the CNS. Data are representative of at least two independent experiments of three mice per group that provided similar results (\* $P < 0.05$ ).

sels to other organs.<sup>18</sup> To determine whether the decreased resistance of CCR2<sup>-/-</sup> mice to *T. gondii* infection was due to increased parasite replication, the small intestine, liver, lung and CNS of WT and CCR2<sup>-/-</sup> mice were analyzed for tissue parasitism. We found that CCR2<sup>-/-</sup> mice exhibited higher parasite load in the small intestine (Figure 2B), liver (Figure 2C), lung (Figure 2D), and CNS (Figure 2E) compared with WT mice at day 8 after infection. As the majority of CCR2<sup>-/-</sup> mice succumbed to *T. gondii* infection around 23 days after parasite inoculation, we evaluated the tissue parasitism in the organs of CCR2<sup>-/-</sup> and WT mice at this time of infection. We found that the tissue parasitism decreased in the peripheral organs in both lineages of mice. However, the CCR2<sup>-/-</sup> mice presented a remarkably increase in the parasite load in the CNS, showing four times more parasites compared to WT mice (Figure 2F). Therefore, CCR2<sup>-/-</sup> mice present impaired ability to control parasite replication in the CNS at the initial chronic phase of infection. Similar data were observed when we measured the amount of parasites using real-time polymerase chain reaction.

### The Absence of CCR2 Attenuates Lesions in the Peripheral Organs but Not in the CNS of *T. gondii*-Infected Mice

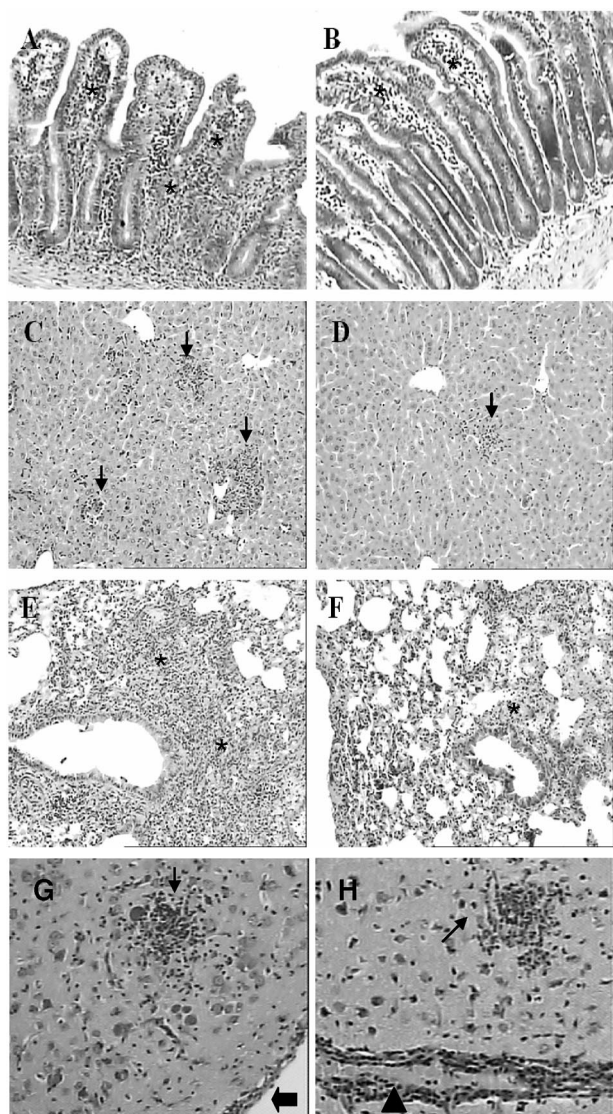
Since CCR2<sup>-/-</sup> mice are more susceptible to oral infection with five *T. gondii* cysts, we investigated whether the susceptibility was associated with histopathological lesions. On day 8 after infection the small intestine, liver, and lungs were the most inflamed organs in both CCR2<sup>-/-</sup> and WT mice. The small intestine presented a moderate inflammatory infiltration in the LP, epithelium, and submucosa (Figure 3, A and B). In some areas, a reduced length and increased thickness of the villi was also observed. The liver presented lesions that were characterized mainly by mononuclear inflammatory foci scattered by parenchyma and portal areas (Figure 3, C and D). The lungs of *T. gondii*-infected mice presented an interstitial pneumonia with mononuclear inflammatory infiltration in the alveolar septa (Figure 3, E and F). In all of

the peripheral organs analyzed on day 8 after infection, the lesions were more severe in WT (Figure 3, A, C, and E) compared to CCR2<sup>-/-</sup> mice (Figure 3, B, D, and F). The CNS was also analyzed for histopathological changes on day 8 after *T. gondii* inoculation, but no significant inflammatory lesions were observed in this period of infection. On day 23 after *T. gondii* infection, the inflammatory lesions were partially controlled in the peripheral organs in both lineages of mice. However, in this phase of infection the CNS presented important inflammatory alterations, which were characterized by diffuse infiltrates of mononuclear cells, glial nodules, vascular cuffing by lymphocytes, and focal infiltrates in the meninges. The lesions were similar in the CNS in WT (Figure 3G) and CCR2<sup>-/-</sup> mice (Figure 3H).

### CCR2 Modulates CD4<sup>+</sup> and MAC-1<sup>+</sup> Cell Migration in Response to *T. gondii* Infection

To determine the subsets of infiltrating cells in the small intestine on day 8 and in the CNS on day 23 after *T. gondii* inoculation, we counted the number of CD4<sup>+</sup>, CD8<sup>+</sup>, and MAC-1<sup>+</sup> cells in WT and CCR2<sup>-/-</sup>-infected mice by immunohistochemistry (Figure 4). In accordance with histological analysis, there was a reduction in MAC-1<sup>+</sup> (Figure 4, A and B) and CD4<sup>+</sup> (Figure 4, A and C) cell migration in the small intestine of CCR2<sup>-/-</sup> compared to WT mice. In contrast, CCR2<sup>-/-</sup> showed an increased number of CD8<sup>+</sup> cells in this organ when compared to WT mice (Figure 4, A and D). The presence of a leukocyte subpopulation within the small intestine was also demonstrated by fluorescence-activated cell sorting analysis. Confirming our immunohistochemical data, CD3<sup>+</sup>CD4<sup>+</sup>T and CD11b<sup>+</sup>CD11c<sup>-</sup> cells were higher in the small intestine in WT compared to CCR2<sup>-/-</sup> mice. In contrast, CD3<sup>+</sup>CD8<sup>+</sup> T cells were higher in the intraepithelial cell subpopulation in the organs from CCR2<sup>-/-</sup> compared to WT mice (Figure 4E). In the CNS, the same pattern of cell migration was observed on day 23 after infection (Figure 4, F and G). These results indicate that CCR2 is involved in CD4<sup>+</sup> and MAC-1<sup>+</sup> but not CD8<sup>+</sup> cell migrations in the sites of *T. gondii* replication.





**Figure 3.** Histological changes in the small intestine, liver, lung, and CNS of  $CCR2^{-/-}$  and WT mice infected perorally with *T. gondii*. WT (A, C, E) and  $CCR2^{-/-}$  (B, D, F) mice were inoculated with *T. gondii* and the peripheral organs were collected on day 8 after infection. Observed were inflammatory infiltrates in the lamina propria, epithelium, and submucosa (asterisks) in the small intestine (A, B); inflammatory foci (arrows) in the parenchyma of the liver (C, D); and inflammatory cell infiltration within the alveolar walls (asterisks) in the lung (E, F). The inflammatory reaction was milder in the peripheral organs of  $CCR2^{-/-}$  (B, D, F) compared to WT mice (A, C, E). The CNS from WT (G) and  $CCR2^{-/-}$  mice (H) was analyzed on day 23 after infection. The lesions characterized by glial nodules (small arrows), vascular cuffing (arrowheads) and inflammatory cells in the meninges (large arrows) were similar in both lineages of mice in this period of infection. Slides were stained with hematoxylin and eosin. Original magnification,  $\times 10$ .

### *T. gondii* Infection Induces an Enhanced and Concomitant Proinflammatory and Anti-Inflammatory Immune Response in $CCR2^{-/-}$ Mice

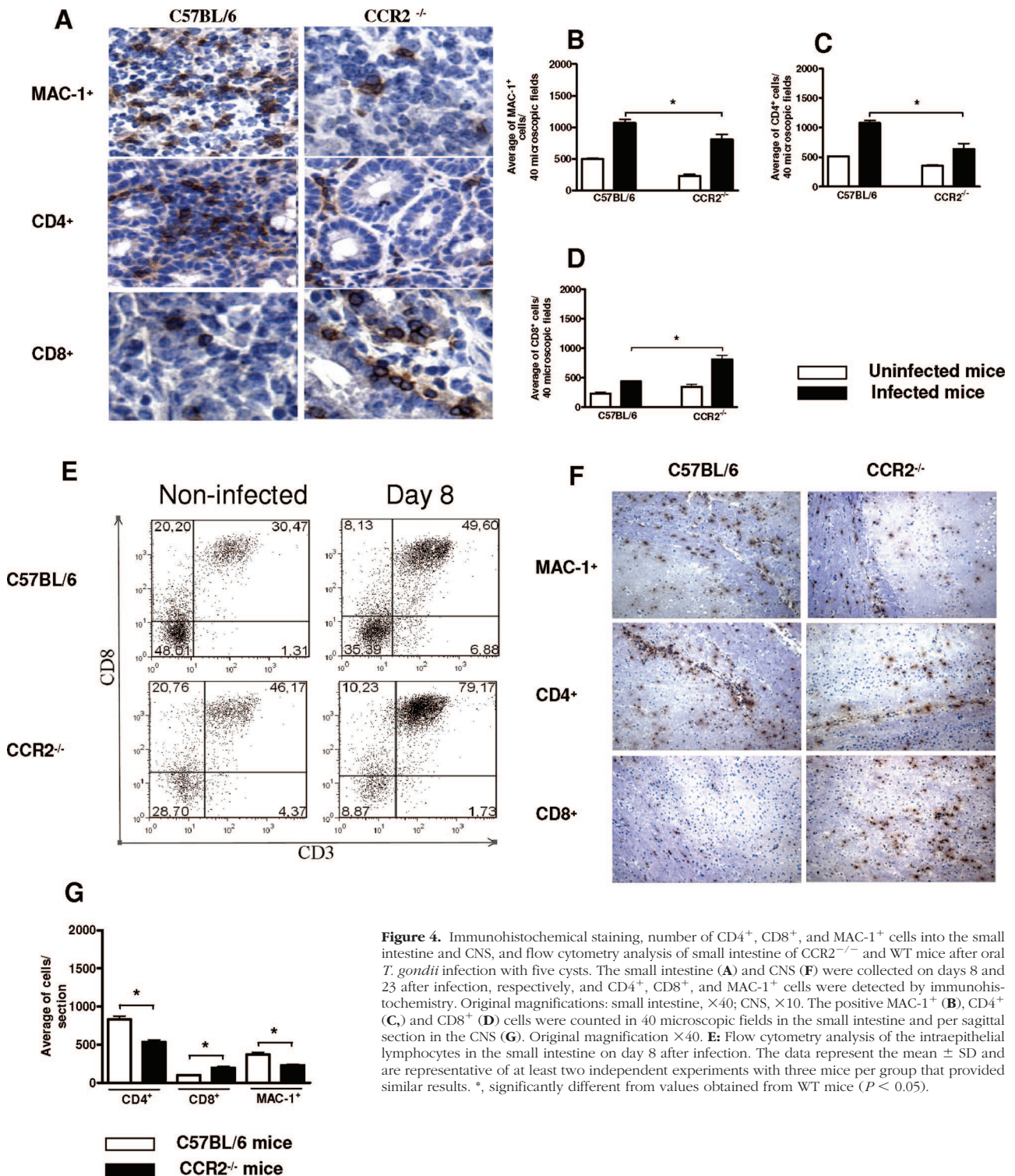
Since  $CCR2^{-/-}$  mice proved to be more susceptible to *T. gondii* infection, we wanted to know whether systematic and local cytokine production could be involved in the susceptibility. The proinflammatory IL-12, IFN- $\gamma$ , and TNF- $\alpha$  and anti-inflammatory IL-4, IL-10, and TGF- $\beta$  were

measured in serum samples and intestinal homogenates on day 8 after infection. The levels of IFN- $\gamma$  were significantly higher in infected WT and  $CCR2^{-/-}$  mice compared to noninfected control mice (Figure 5A). Elevated levels of IFN- $\gamma$  were observed in serum samples from  $CCR2^{-/-}$  and WT mice, and it was found to be higher in  $CCR2^{-/-}$  compared to WT mice (Figure 5A). In contrast, the levels of TNF- $\alpha$  were higher in WT compared to  $CCR2^{-/-}$  mice (Figure 5D), and infected WT mice showed higher levels of the cytokine compared to noninfected mice. Regarding anti-inflammatory cytokines in serum samples, the infection with the parasite induced an increased IL-10 production in both lineages of mice, and the infection did not systemically alter TGF- $\beta$  (Figure 5, B and C).

*T. gondii* infection induced increased IL-12, IFN- $\gamma$ , and TNF- $\alpha$  production in small intestine homogenates in both  $CCR2^{-/-}$  and WT mice (Figure 5, E–G). Interestingly, the production of IL-12 and TNF- $\alpha$  locally was higher in  $CCR2^{-/-}$  compared to WT mice (Figure 5, E and G). IL-4 and IL-10 levels were also increased in the small intestine when animals were infected with *T. gondii*, and  $CCR2^{-/-}$  showed more elevated levels of IL-4 than those observed in WT mice (Figure 5H). In accordance with the smaller inflammatory cell infiltrates in the small intestine, the production of TGF- $\beta$ , a cytokine that is known to control the exacerbated inflammatory immune response in the gut,<sup>27</sup> was lower in WT compared to  $CCR2^{-/-}$  mice (Figure 5J), and infected knockout mice showed higher levels of the cytokine compared to noninfected animals. These data suggest that despite the higher systemic production of proinflammatory cytokines (IFN- $\gamma$ ) in the small intestine (IL-12 and TNF- $\alpha$ ) of  $CCR2^{-/-}$  mice, the higher production of anti-inflammatory cytokines locally could counteract the detrimental effect of an exacerbated proinflammatory immune response at this site of infection.

### *CCR2* Modulates iNOS Expression and NO Production in *T. gondii* Infection

Reactive nitrogen intermediates, including nitric oxide (NO), have been identified in the control of protozoan replication.<sup>28,29</sup> Thus, we investigated the expression of iNOS, the enzyme that induces NO production, by immunohistochemistry in the small intestine on day 8 and in the CNS on day 23 after *T. gondii* infection. A high number of cells from WT mice expressing iNOS in the small intestine on day 8 (Figure 6A) and in the CNS on day 23 after infection (Figure 6C) probably are macrophages and microglial cells, respectively, in accordance with previous data that showed that these cells express iNOS in *T. gondii* infection.<sup>30</sup> Surprisingly, in  $CCR2^{-/-}$  mice, cells expressing iNOS was seldom found in the organs from this lineage of mice (Figure 6, B and D), indicating that this pathway of parasite control is not functional in this lineage. In accordance with the iNOS tissue expression, the spleen macrophages



**Figure 4.** Immunohistochemical staining, number of CD4<sup>+</sup>, CD8<sup>+</sup>, and MAC-1<sup>+</sup> cells into the small intestine and CNS, and flow cytometry analysis of small intestine of CCR2<sup>-/-</sup> and WT mice after oral *T. gondii* infection with five cysts. The small intestine (**A**) and CNS (**F**) were collected on days 8 and 23 after infection, respectively, and CD4<sup>+</sup>, CD8<sup>+</sup>, and MAC-1<sup>+</sup> cells were detected by immunohistochemistry. Original magnifications: small intestine, ×40; CNS, ×10. The positive MAC-1<sup>+</sup> (**B**), CD4<sup>+</sup> (**C**), and CD8<sup>+</sup> (**D**) cells were counted in 40 microscopic fields in the small intestine and per sagittal section in the CNS (**G**). Original magnification ×40. **E**: Flow cytometry analysis of the intraepithelial lymphocytes in the small intestine on day 8 after infection. The data represent the mean ± SD and are representative of at least two independent experiments with three mice per group that provided similar results. \*, significantly different from values obtained from WT mice ( $P < 0.05$ ).

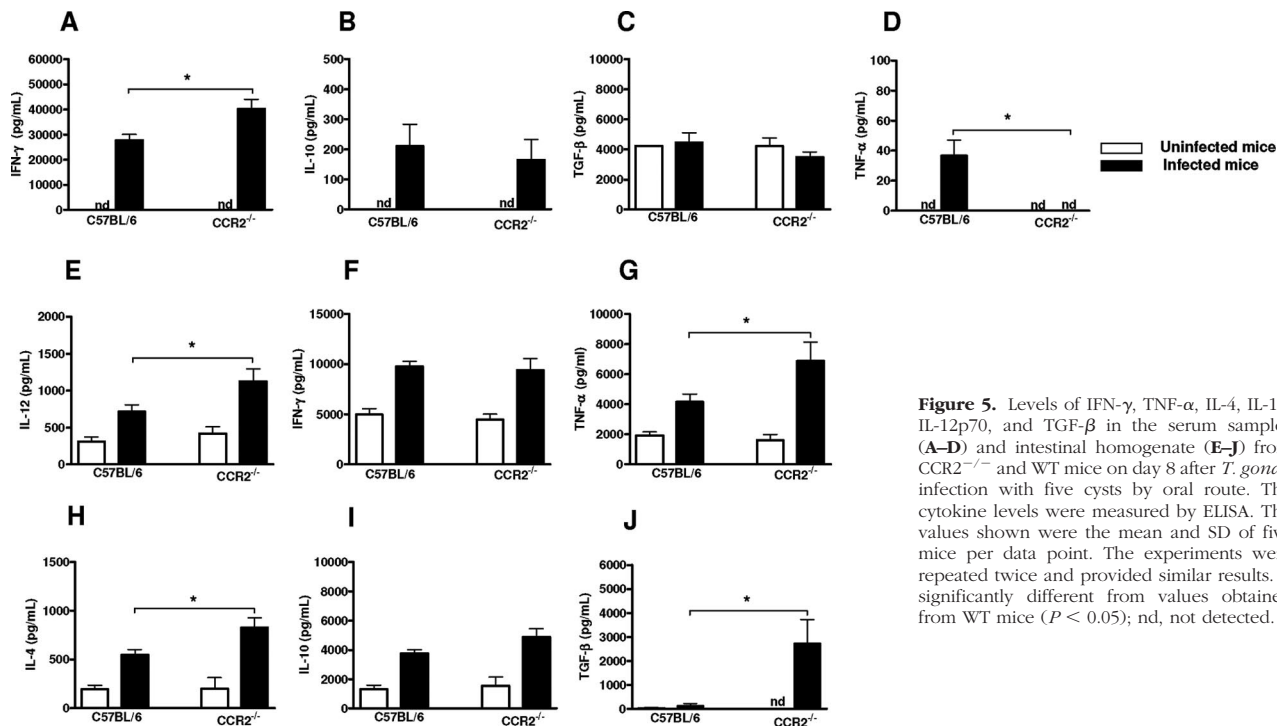
from CCR2<sup>-/-</sup> mice showed a very low NO production when stimulated with IFN-γ or CCL2 (data not shown).

### Discussion

The dominant immune response during toxoplasmosis is a Th1 response that results in a prominent leukocyte

migration to the site of infection, but if uncontrolled, the response may lead to immunopathology. In the present investigation, we wanted to determine the role of CCR2 in cell migration and control of the parasite in peripheral organs and CNS of *T. gondii*-infected mice. For this purpose, CCR2<sup>-/-</sup> mice were orally infected with five cysts of the parasite and the morbidity, survival, and immune





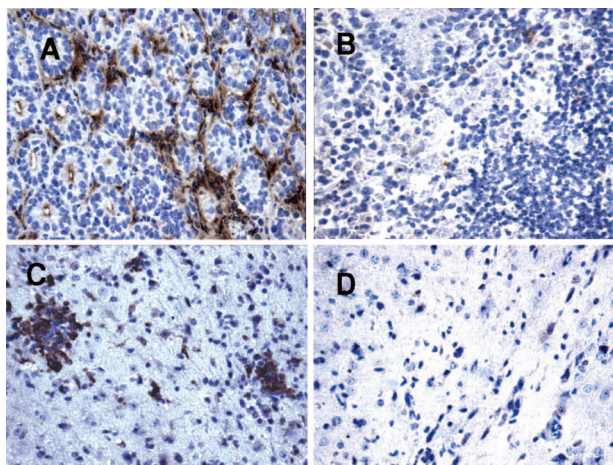
**Figure 5.** Levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-4, IL-10, IL-12p70, and TGF- $\beta$  in the serum samples (A–D) and intestinal homogenate (E–J) from CCR2<sup>-/-</sup> and WT mice on day 8 after *T. gondii* infection with five cysts by oral route. The cytokine levels were measured by ELISA. The values shown were the mean and SD of five mice per data point. The experiments were repeated twice and provided similar results. \*, significantly different from values obtained from WT mice ( $P < 0.05$ ); nd, not detected.

response were evaluated. In accordance with previous studies, we observed a high inflammatory cell infiltration expressing CCR2<sup>B</sup> and CCL2 in the ilea of *T. gondii*-infected C57BL/6 mice in the acute phase, indicating that this chemokine and chemokine receptor are involved in inflammatory cell migration to the small intestine. In parallel, the CCL2 concentration was elevated in the serum samples and in the small intestine homogenate in the same period of infection. The levels of the chemokine in the sera were higher in CCR2<sup>-/-</sup> compared to WT mice, which is consistent with the fact that CCR2 is the primary receptor for CCL2 in the mouse<sup>31</sup> and that in the absence

of the receptor, the concentration of the unbinding chemokine in the serum samples increases.

CCR2<sup>-/-</sup> mice presented accelerated death associated with high parasite rates in the peripheral organs and CNS in acute phase and also in the CNS at the initial phase of chronic infection. As we have previously shown, there is parasite dissemination in the peripheral organs of the C57BL/6, TNFp55 receptor<sup>-/-</sup>, and iNOS<sup>-/-</sup> mice infected with *T. gondii*; the decreased parasite load in the majority of these sites is at the beginning of the chronic phase of infection but not in the CNS of knockout mice.<sup>32</sup> Similarly, in our present work, it was also observed that the tissue parasitism was decreased in the peripheral organs from WT and CCR2<sup>-/-</sup> mice but increased with the progression of infection in the CNS of CCR2<sup>-/-</sup> mice. Thus, in the peripheral organs, microbicidal mechanisms that are independent of CCR2 are important to control the parasite, but in the CNS, the chemokine receptor seems to be essential to control *T. gondii* replication.

It is well known that mice lacking the CCR2 chemokine receptor present defects in macrophage recruitment.<sup>15</sup> Oral infection with *T. gondii* induces an important inflammatory reaction in the small intestine, liver, lung, and CNS in C57BL/6 mice that is controlled in peripheral organs from day 14 of infection and onward.<sup>33</sup> In our experiments, it was verified that on day 8 after *T. gondii* infection the inflammatory reaction in peripheral organs was milder in the knockout compared to WT mice, indicating that the CCR2 is important to promote inflammatory cell migration to these sites. Interestingly, in the small intestine from CCR2<sup>-/-</sup> mice the CCL2<sup>+</sup> cells were lower compared to WT mice, reflecting a smaller cell migration in this lineage of mice and the dependence of the receptor on recruitment of the inflammatory cells. In this organ on day 8 after



**Figure 6.** Immunohistochemical staining for iNOS in the small intestine and in the CNS of WT (A, C) and CCR2<sup>-/-</sup> (B, D) mice infected with five ME-49 *T. gondii* cysts by oral route. The animals were infected and the small intestine were collected on day 8 (A, B) and the CNS on day 23 (C, D) after infection for iNOS immunostaining. Original magnification,  $\times 40$ .



infection, the inflammatory infiltrates were composed of CD4<sup>+</sup>, CD8<sup>+</sup>, and MAC-1<sup>+</sup> cells; the CD4<sup>+</sup> and MAC-1<sup>+</sup> cells infiltrate less and CD8<sup>+</sup> cells infiltrate more in CCR2<sup>-/-</sup> compared to WT mice. The infection of C57BL/6 mice with 100 *T. gondii* cysts by oral route induces a CD8<sup>+</sup> and TCR- $\gamma\delta$  T cell migration in the epithelia of the small intestine, whereas in the LP the majority of infiltrated cells are CD4<sup>+</sup> and TCR- $\alpha\beta$  T cells.<sup>34</sup> Additionally, it was determined that the CD4<sup>+</sup> T cell phenotype in the LP is the cell population responsible for the severe histopathological changes in the intestine, leading to tissue necrosis and subsequent mortality on day 7 of infection.<sup>22,31</sup> In other experiments, it was verified that CD8<sup>+</sup> T lymphocytes in the intestinal epithelia are able to modulate the inflammatory activity of CD4<sup>+</sup> T cells from LP.<sup>27</sup> In our studies, we observed smaller CD4<sup>+</sup> T lymphocyte and MAC-1<sup>+</sup> cell infiltrations in the small intestine of CCR2<sup>-/-</sup> mice that were associated with a smaller inflammatory reaction, in contrast to greater CD8<sup>+</sup> T lymphocyte migrations to this site. Small intestinal intraepithelial and lamina propria lymphocytes migrate to thymus-expressed chemokine (TECK/CCL25), and this attraction is mediated by CCR9, a chemoattractant receptor expressed at high levels by essentially all CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in the small intestine.<sup>35</sup> *In vivo*-primed effector CD8 $\alpha\beta$ <sup>+</sup> T cells displayed regionalized differences in their entry to the small intestine epithelium with enhanced CCR9-independent entry to the ileum.<sup>36</sup> CCR1, CCR2, CCR5, and CXCR3 are increased in *Toxoplasma*-infected ileum, and in particular, CCR5 expression was markedly increased in *Toxoplasma*-primed CD8<sup>+</sup> intraepithelial T lymphocytes.<sup>8</sup> Our results demonstrate that despite the absence of CCR2, other chemokine receptors are involved in CD8<sup>+</sup> T lymphocyte migrations to the small intestine. Thus, smaller CD4<sup>+</sup> cell migration to the LP, in addition to a greater CD8<sup>+</sup> cell migration to the epithelia, could contribute to the decrease in inflammatory lesions in the small intestine in knockout mice.

Because our experiments demonstrate the greater susceptibility of CCR2<sup>-/-</sup> mice, we hypothesized an impaired development of Th1 protective immune response in infected knockout mice. The results of the present experimental work show that the IL-12 and IFN- $\gamma$  normally developed in infected CCR2<sup>-/-</sup> mice; the IFN- $\gamma$  in serum samples and IL-12 and TNF- $\alpha$  levels in the small intestine are higher than those observed in WT mice. Previous studies have demonstrated that CCR2<sup>-/-</sup> mice have markedly reduced T cell IFN- $\gamma$  responses as well as defects in clearance of intracellular pathogens.<sup>15,31</sup> The immune response pattern of infected animals depends on the biological features of the involved infectious organisms, and in the case of *Toxoplasma*, the parasite is known to induce a strong Th1 immune response. As we demonstrated, despite the absence of CCR2, the parasite induced a Th1 type response. Th1 CD4<sup>+</sup> T cells express CCR2, CCR5, and CXCR3;<sup>37</sup> thus, our data suggest that in the absence of CCR2, other chemokine receptors or other immune mechanisms are involved in the Th1 immune response activation. We also observed that the IL-4 and TGF- $\beta$  were present in higher levels in the

small intestine of CCR2<sup>-/-</sup> compared to WT infected mice. The elevated levels of IL-4 and TGF- $\beta$  are in accordance with the smaller cell migration and inflammatory changes verified in the small intestine of the knockout mice, and TGF- $\beta$  is particularly associated with the modulation of CD4<sup>+</sup> T cell activity in the LP.<sup>27</sup> Thus, in addition to the smaller cell migration observed in the small intestine in the absence of CCR2, the secretion of IL-4 and TGF- $\beta$  might contribute to the small inflammatory changes at this site in CCR2<sup>-/-</sup> mice. Th2 CD4<sup>+</sup> T cells express CCR2, CCR3, CCR4, and CCR8,<sup>37</sup> and in the absence of CCR2 other chemokine receptors or immune mechanisms could be involved in Th2 activation. Therefore, we can conclude that both Th1 and Th2 immune mediators are activated by *T. gondii* infection in the absence of CCR2 receptors.

As the inflammatory reaction and parasite multiplication were decreased in the peripheral organs in the absence of the CCR2, we investigated the morbidity of the major important site of *T. gondii* infection in the initial chronic phase, the CNS. The inflammatory reaction in the CNS was similar in both lineages of mice on day 23 after infection, when the majority of knockout mice were dying, which suggests that other mechanisms including adhesion molecules and other chemokines and chemokine receptors in addition to CCR2-CCL2 are involved in cell migration to this organ. Accordingly, in toxoplasmic encephalitis in C57BL/6 mice, there is a significant intracerebral transcription of CXCL10, CXCL9, CCL5, CCL2, CCL3, and CCL4, which are dependent on IFN- $\gamma$ , and these levels were parallel with increasing numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as well as macrophages, granulocytes, and B cells.<sup>5</sup> Despite similar inflammatory lesions in the CNS on day 23 after infection, the CCR2<sup>-/-</sup> mice presented an uncontrolled tissue parasitism. As we observed in the small intestine, the CNS from CCR2<sup>-/-</sup> mice also presented a smaller CD4<sup>+</sup> and MAC-1<sup>+</sup> cell migration. This is in accordance with previous studies that have demonstrated the important role of these cell phenotypes to activate microbicidal mechanisms in the CNS in toxoplasmic encephalitis.<sup>38,39</sup> Since iNOS expression associated with NO production is an important microbicidal mechanism against *T. gondii* replication, we verified the iNOS expression in the CNS and the NO production by spleen macrophages. Interestingly, the iNOS expression was almost absent in CCR2<sup>-/-</sup> mice. This is in accordance with previous studies that show that dendritic cells (Tip-DC) CD11b<sup>INT</sup> CD11c<sup>+</sup> producing TNF- $\alpha$  and iNOS are absent in CCR2-deficient mice with urinary tract infections.<sup>40</sup> In addition, in our experiments the spleen macrophages from CCR2<sup>-/-</sup> mice, despite being stimulated with IFN- $\gamma$ , did not produce NO sufficiently, showing the important role of CCR2 in inducing iNOS expression and consequently the NO production. In conclusion, despite the tissue parasitism being partially controlled in the peripheral organs, the microbicidal mechanisms are not effective in the CNS of CCR2<sup>-/-</sup> mice and parasite replication remains unchecked in this site in the absence of CCR2, contributing to a greater susceptibility in the animals.

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