**Toxoplasma gondii** Cyclophilin 18 Regulates the Proliferation and Migration of Murine Macrophages and Spleen Cells

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**Toxoplasma gondii** is an intracellular parasite that shows a unique capacity to infect a variety of cell types in warm-blooded animals. It can invade and survive well inside immune cells, such as macrophages, that disseminate the parasite around the body because of their migratory properties. The aim of the present study was to evaluate the role of *T. gondii* cyclophilin 18 (TgCyp18) in the proliferation and migration of macrophages and spleen cells (mainly T lymphocytes) in order to understand the effects of TgCyp18 on the dynamics of the infection. A high dose of TgCyp18 enhanced the proliferation of macrophages and spleen cells in a cysteine-cysteine chemokine receptor 5 (CCR5)-independent way. In contrast, TgCyp18 controlled the migration of macrophages and spleen cells in dose- and CCR5-dependent manners. Our data suggest that TgCyp18 recruits cells and enhances the growth of host cells at the site of infection for maintenance of the interaction between the parasite and host.

*Toxoplasma gondii* is an intracellular parasite that shows a unique capacity to infect a variety of cell types in warm-blooded animals (20). It can invade and survive well inside immune cells, such as macrophages and dendritic cells (DCs), which disseminate the parasite around the body through their migratory properties (9, 12, 27). In the host, macrophages and lymphocytes play essential roles against pathogens. Macrophages are crucial in the initiation and regulation of T-lymphocyte responses during *Toxoplasma* infection (9, 18, 30). *Toxoplasma*-infected macrophages, DCs, and neutrophils are important sources of interleukin-12 (IL-12) early in infection (4, 7, 8, 23, 34). IL-12 acts on both natural killer (NK) cells and T cells to promote the production of interferon gamma (4, 7, 8, 23, 34). This cytokine stimulates NK cells and T cells to promote the production of interferon gamma (IFN-γ) and create a positive feedback loop whereby IFN-γ promotes classical activation of macrophages (30). Moreover, CD4+ and CD8+ T cells have been demonstrated to be important in controlling *T. gondii* infection (1, 16, 17).

Interestingly, *T. gondii* stimulates IL-12 production through a unique pathway that involves the triggering of cysteine-cysteine chemokine receptor 5 (CCR5) in DCs and macrophages by the secreted *T. gondii* cyclophilin 18 (TgCyp18) (3). TgCyp18 recruits immature mouse DCs in vitro (3). Moreover, it appears to act as a structural mimic of CCR5-binding ligands, albeit one with no sequence similarity to the known host ligands, macrophage inflammatory protein 1α (MIP-1α)/chemokine (C-C motif) ligand 3 (CCL3), MIP-1β/CCL4, regulated on activation normal T-cell expressed and secreted (RANTES)/CCL5, or monocyte chemotactic protein 2 (MCP-2)/CCL8, for this receptor (3, 6, 42). High et al. in 1994 isolated genes encoding two *Toxoplasma gondii* cyclophilins, TgCyp18 and TgCyp20, as cyclosporine-binding proteins on affinity columns (24). Both were highly similar to human cyclophilin (hCyp18) in the central core region, but TgCyp20 differed in a 7-amino-acid insertion in the same region as in the *Plasmodium falciparum* cyclophilins (24).

TgCyp18, but neither hCyp18 nor PfCyp19A, appears to induce IL-12 production by interacting directly with CCR5, an effect that is blocked by the addition of cyclosporine A (CsA) (3, 6, 42). These observations implied that structural determinants of TgCyp18, related to CsA binding, are responsible for the induction of IL-12 synthesis (6, 42). This idea was confirmed by modeling the TgCyp18 structure on that of *P. falciparum* Cyp19A (PfCyp19A) and site-directed mutagenesis of putative surface-exposed residues that were absent in PfCyp19A (42). Two of the TgCyp18 mutants, namely, 17GEH19 to 17AAA19 and 149RP150 to 149YV150, located in the N and C termini of the protein, respectively, had reduced interactions with CCR5 and reduced IL-12 induction (42). Moreover, TgCyp18 peptidyl-prolyl cis-trans isomerase (PPIase) activity was not required for its interaction with CCR5, but IL-12 induction by TgCyp18 required both CCR5 binding and PPIase enzymatic activities (42). There is also evidence that the closely related protozoan *Neospora* cyclophilin plays a role in stimulating IFN-γ production by bovine peripheral blood mononuclear cells and *Neospora caninum*-specific CD4+ T cells (40). This effect is also blocked by CsA (40). IFN-γ production induced by *N. caninum* tachyzoites is thought to be critical in controlling the acute phase of neosporosis (40).

As an intracellular pathogen, *T. gondii* has mechanisms to interfere with host signaling pathways to subvert innate immunity (18). *T. gondii* profilin induces IL-12 via toll-like receptor 11 (TLR11)/myeloid differentiation factor 88 (MyD88), promotes parasite actin assembly, and contributes to gliding motility and invasion and egress (21, 32). Glycosylphosphatidylinositol-anchored proteins on the surface of *T. gondii* trigger tumor necrosis factor alpha (TNF-α) production from macrophages in a TLR2/TLR4/MyD88-dependent manner (14). *T. gondii* has three genotypic lineages, types I, II, and III, which...
differ widely in terms of virulence and effects on host cell signaling. Recent data indicate that these differences are controlled by specific T. gondii molecules (35). Types I and III, but not type II, isoforms of the rhoptry protein 16 (ROP16) kinase induce activation of signal transducer and activator of transcription 3/6 (STAT3/6) and suppress IL-12 production from macrophages (35). Moreover, it was reported that T. gondii heat shock protein 70 (HSP70)-induced nitric oxide (NO) release is dependent on TLR2, MyD88, and interleukin-1 receptor-associated kinase 4 (IRA4K) (31), which means that T. gondii has several proteins that work through different pathways to interfere with the host immune response. The identification of such molecules and determination of how they impact host cell behavior are very important when attempting to control the parasite. Because of the strong cooperation between macrophages and spleen cells (mainly lymphocytes) during infection, we focused our study on the role of TgCyp18 on the proliferation and migration of these cells in order to determine the effect of TgCyp18 on the parasite-host interaction.

MATERIALS AND METHODS

Animals. C57BL/6J female mice (B6 mice), 6 to 8 weeks of age, were obtained from CLEA Japan (Tokyo, Japan). CCR5 knockout mice (CCR5<sup>−/−</sup>; B6.129P2-Ccr5<sup>+</sup>/Scid; stock no. 005427) were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed under specific-pathogen-free conditions in the animal facility of the National Research Center for Protozoan Diseases at the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. Animals used in this study were treated and used according to the Guiding Principles for the Care and Use of Research Animals published by the Obihiro University of Agriculture and Veterinary Medicine.

Reagents. Lipopolysaccharide (LPS) from Escherichia coli (10 μg/ml stock solution in RPMI 1640 medium), concanavalin A (ConA) from Canavalia ensiformis (5 mg/ml stock solution in RPMI 1640 medium) were obtained from Sigma (St. Louis, MO). Recombinant mouse MIP-3α (25 μg/ml stock solution in RPMI 1640 medium) was obtained from R&D Systems, Inc. (Minneapolis, MN).

Construction and expression of recombinant TgCyp18 and MTgCyp18. Construction and expression of recombinant TgCyp18 and recombinant mutant TgCyp18 (MTgCyp18), with changed amino acids of 17GEH19 to 17AAA19 and 40DGDF42 to 40YAY42, were performed as described previously (25). Wild-type and mutant TgCyp18s were expressed as glutathione S-transferase (GST) fusion proteins in the E. coli DH5α strain (Takara, Bio Inc., Japan). The GST tags of the recombinant proteins were removed with thrombin protease (GE Health care, Buckinghamshire, England) according to the manufacturer’s instructions. Recombinant proteins were dialyzed in phosphate-buffered saline (PBS) and purified with Detoxi-Gel endotoxin-removing gel (Pierce Biotechnology Inc., Rockford, IL).

For cell culture use, the proteins were filtered using a 0.45-μm low-protein-binding Supor membrane (Pall Life Sciences, Ann Arbor, MI). The purity and quantity of the proteins were detected as a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue R250 (CBBR; MP Biomedicals Inc., France) staining. The concentration was measured using a bichinchonic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL).

Monolayer cultures of peritoneal macrophages. Mouse peritoneal macrophages were collected from B6 and CCR5<sup>−/−</sup> mice 4 days after intraperitoneal injection of 1 ml of 4.05% brewer modified BBL thioglycolate medium (Becton Dickinson, Sparks, MD) by peritoneal washing with 5 ml of cold PBS. After harvesting, the cells were centrifuged at 800 g for 10 min and suspended in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum (FBS). The macrophage suspension was then added to 24-well tissue culture microplates at a density of 10<sup>6</sup> cells/well. The suspensions were incubated at 37°C for 3 h, washed thoroughly to remove nonadherent cells, and further incubated at 37°C. The purity of the peritoneal macrophage cultures was quantified by flow cytometry using anti-CD11b and anti-CD3 monoclonal antibodies (MAb). The percentage of CD11b<sup>+</sup> cells was 99.4 ± 0.17% at 0 h and 98.6 ± 1.01% at 24 h (n = 3). Moreover, the percentage of CD3<sup>+</sup> cells was 0.02% ± 0% at 0 h and 0.04% ± 0.06% at 24 h. These results indicate the high purity of the peritoneal macrophages used in this study.

Spleen cell isolation. B6 and CCR5<sup>−/−</sup> mice were sacrificed and their spleens were aseptically removed. The splenocytes were suspended in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. The mononuclear cells (the majority of which are lymphocytes) were isolated by using Histopaque 1077 (Sigma) according to the manufacturer’s instructions.

II. ELISA

Measurement of nitric oxide. Supernatants from peritoneal macrophages cultured in Dulbecco’s modified Eagle’s medium (DMEM) contain 10% FBS were collected for analysis of NO. Nitrate and nitrite production in the culture medium was measured using a nitrite/nitrate assay kit (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer’s recommendations.

Sandwich ELISA for detection of secreted TgCyp18. TgCyp18 secretion from extracellular parasites was determined as described previously (11). Purified T. gondii tachyzoites (3 × 10<sup>7</sup>) were incubated in 1.5 ml of GIT medium (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) at 37°C. Before transfer of the parasite suspensions from ice to 37°C for the secretion assay, 250 μl was removed and processed as the time zero reading. The rest of the parasite suspension was incubated at 37°C in a water bath. After 15, 30, 60, and 120 min of incubation, 250-μl aliquots of the parasite suspensions were removed. The culture supernatants were centrifuged sequentially (760 × g for 10 min at 4°C and then 7,000 × g for 10 min at 4°C), together with the ascitic fluid from the in vivo experiment, and subjected to a sandwich ELISA.

Rabbit anti-TgCyp18 polyclonal immunoglobulin G (IgG) was purified using protein A chromatography columns (Bio-Rad Laboratories). Protein concentrations of the purified protein were measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). One microgram of the IgG diluted in 0.05 M carbonate buffer (pH 9.6) was used as the capture antibody to coat microtiter plates at 4°C overnight. Blocking was performed with a blocking solution (3% PBS-SM [PBS containing 3% skim milk], pH 7.2) at 37°C for 2 h. The plates were incubated at 37°C for 30 min with each supernatant in triplicate. After washing six times with a washing solution (0.05% Tween 20 in PBS), anti-TgCyp18 mouse serum diluted 1:100 in blocking solution was added to each well as a detection antibody. After further washing, the plates were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Amersham Pharmacia Biotech) diluted 1:2,500 in blocking solution. Binding was visualized with substrate solution [0.3 mg/ml 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid), 0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H<sub>2</sub>O<sub>2</sub>]. The absorbance at 415 nm was measured as a unit with a MTP-500 microplate reader (Corona Electric, Tokyo, Japan). The TgCyp18 concentration of each sample was calculated by standardization with recombinant TgCyp18.

Parasite infection of mice. Parasites purified from an in vivo culture were washed in sterile PBS, and 10<sup>7</sup> tachyzoites were inoculated intraperitoneally into B6 mice. After the indicated time periods, peritoneal cells were collected from the peritoneal cavities of naive or parasite-infected mice by peritoneal washing with 5 ml of cold PBS. After washing, the cells were centrifuged at 800 × g for 10 min and suspended in cold PBS. The supernatants were used to measure the TgCyp18 secretion.

Macrophage or spleen cell proliferation assay. Macrophages (5 × 10<sup>6</sup>) from B6 and CCR5<sup>−/−</sup> mice were cultured for 24 h with TgCyp18, MTgCyp18, GST, or LPS. Spleen cells (5 × 10<sup>6</sup>) from B6 and CCR5<sup>−/−</sup> mice were cultured for 48 h with TgCyp18, MTgCyp18, GST, or ConA. Then, 10 μl of cell counting kit 8 reagent (Dojindo Laboratories, Japan) was added. After 3 h of incubation at 37°C in 5% CO<sub>2</sub>, the optical density at 450 nm was determined (Corona Electric, Tokyo, Japan).

Flow cytometry. Phycoerythrin (PE)-labeled anti-mouse CD11b MAb, CCR5, CD3e (CD3e chain; 145-2C11), and Fc block were purchased from BD Biosciences (San Jose, CA).

After washing with cold PBS, 1 × 10<sup>6</sup> cells (macrophages or spleen cells) were suspended in cold PBS containing 0.5% bovine serum albumin (BSA) and treated with Fc block to avoid non-specific adherence of MAb to Fc receptors. Cells were subsequently incubated with PE-labeled anti-mouse MAb for 30 min at 4°C followed by a final washing step with cold PBS. Labeled macrophages (5 × 10<sup>4</sup>) or spleen cells (1 × 10<sup>5</sup>) were examined using an EPICS XL flow cytometer (Beckman Coulter, Hialeah, FL).

Migration assays. Cell migration was assessed using 24-well chemotaxis chambers with 8-μm inserts (Nunc) and polycarbonate filters (Falcon, Beckton Dickinson, Franklin Lakes, NJ). Macrophages or spleen cells (1 × 10<sup>5</sup>) from B6 and CCR5<sup>−/−</sup> mice suspended in 0.4 ml of RPMI 1640 supplemented with 10% FBS were applied to the upper compartment of the chamber. In the lower chamber, TgCyp18, MTgCyp18, or MIP-3α (R&D Systems) in 1.2 ml RPMI 1640 supple-
mented with 10% FBS was added as a chemoattractant. Chambers were incubated at 37°C for 24 h. The cells that migrated into the lower compartment were counted by microscopy. Each treatment was performed in triplicate. Results are expressed as the percentage of macrophages or spleen cells in the lower compartment in relation to the number of total macrophages or spleen cells used in the experiment as follows: percentage of migrating cells = [(number of cells in the lower chambers)/(total cell number)] × 100.

Statistical analysis. Data are expressed as the means ± standard deviations. Various assay conditions were evaluated by using the independent Student t test and/or an analysis of variance (ANOVA) test followed by a post hoc analysis of group differences that was performed with the least significant differences (LSD) test. P values of <0.05 were considered statistically significant, and P values of <0.01 were considered highly statistically significant.

RESULTS

IL-12 p40 and NO production in response to TgCyp18 and physiologically relevant doses of TgCyp18. TgCyp18 has been shown to stimulate macrophages to produce IL-12 and NO in a CCR5-dependent manner (25). However, the dose-dependent effect of TgCyp18 has not yet been elucidated. We investigated the production of IL-12 p40 and NO by peritoneal macrophages treated with different concentration of TgCyp18. As shown in Fig. 1A and B, the higher dose (50 μg/ml) of TgCyp18 showed potent effects on the production of IL-12 p40 and NO. Next, we examined the effects of physiologically relevant doses of TgCyp18 using in vitro and in vivo models (Fig. 1C and D). TgCyp18 was secreted from extracellular tachyzoites in a time-dependent manner (Fig. 1C), with detectable endogenous levels of 216.3 ± 17.5 pg/ml from 5 × 10^6 parasites after 120 min. Moreover, higher levels of secreted endogenous TgCyp18 (24.57 ± 7.1 and 37.74 ± 2.1 ng/ml) were detected in the ascitic fluid from mice infected with T. gondii tachyzoites (1,000 tachyzoites) at 5 and 8 days postinfection, respectively. These results suggested that
huge numbers of tachyzoites were required to secrete a threshold concentration of TgCyp18 capable of IL-12 and NO production. Thus, TgCyp18 might have paracrine effects (locally or systemically) at the site of infection.

Effects of TgCyp18 on macrophage and spleen cell proliferation. The mitogenic effects of TgCyp18 and MTgCyp18 were evaluated in peritoneal macrophages and spleen cells. Since serial doses of TgCyp18 (0.01, 0.1, 1, and 10 μg/ml) did not have significant effects on the proliferation of these cells (data not shown), we examined the effects of higher concentration of TgCyp18 (25 and 50 μg/ml) (Fig. 2). Significant proliferative responses were observed after stimulation with 25 μg/ml of TgCyp18 in wild-type macrophages (P < 0.05) compared to CCR5−/− macrophages (Fig. 2A). The proliferative responses were not significant after stimulation with 50 μg/ml of TgCyp18 or with 25 and 50 μg/ml of MTgCyp18 in wild-type macrophages compared to CCR5−/− macrophages. Although the result from the 25 μg/ml TgCyp18 stimulation in wild-type macrophages may give some evidence about the dependence on CCR5, the results of the 50 μg/ml of TgCyp18 and both doses of MTgCyp18 give clear-cut evidence that the responses are independent of CCR5. The MTgCyp18 results also give evidence about the role of other receptors and TgCyp18. This observation is important toward determining the active binding site on TgCyp18 that is responsible for macrophage proliferation. It may also suggest that cytokine induction (e.g., IL-12), as shown by Yarovinsky et al. 2004 (42), is dependent on this site (19GEH19 and 19RP150) but not the macrophage proliferation site. A previous report demonstrated that treating RAW 264.7 macrophages with LPS inhibited their proliferation (10). Our results demonstrated that no significant differences were detected upon treatment of B6 or CCR5−/− macrophages with LPS compared to controls (complete medium or GST). The difference between our results and the previously mentioned report may be related to the use different types of cells and different methods of detection. On the level of macrophages, although significant proliferative responses were observed after stimulation with both TgCyp18 and MTgCyp18 in wild-type and CCR5−/− macrophages compared to controls (complete medium or GST), these effects were marginal, suggesting that the effects of TgCyp18 on macrophage growth might be questionable under physiological conditions.

On the level of spleen cells, significant proliferative responses were elicited after incubation of wild-type or CCR5−/− spleen cells with both doses (25 and 50 μg/ml) of TgCyp18 or MTgCyp18 (P < 0.01) compared to controls (complete medium or GST) (Fig. 2B). Moreover, high proliferative responses were observed with a known inducer of proliferation, ConA. Furthermore, a significant proliferative response was observed after stimulation with 25 or 50 μg/ml of TgCyp18 in CCR5−/− spleen cells (P < 0.05) compared to stimulation with the same doses in wild-type spleen cells. On the other hand, no significant proliferative response was demonstrated after stimulation with MTgCyp18 in CCR5−/− spleen cells. Taken together, treatment of macrophages or spleen cells with TgCyp18 resulted in the enhancement of macrophage and spleen cell proliferation in a CCR5-independent manner.

Effects of TgCyp18 on CCR5 expression by macrophages and spleen cells. To determine the quality of the recombiant proteins in altering CCR5 expression on macrophages and spleen cells, the expression of CCR5 on macrophages and spleen cells after contact with TgCyp18 and MTgCyp18 was examined by flow cytometry (Fig. 3). After 24 h of incubation with TgCyp18, expression of CCR5 on macrophages and spleen cells was enhanced in comparison to the control groups (complete medium or GST). Although incubation of macrophages with MTgCyp18 showed a slight enhancement in the expression of CCR5 compared to the control groups, treatment of spleen cells with MTgCyp18 failed to enhance the expression of CCR5 compared to the control groups. Interestingly, clear enhancements of the expression of CCR5 were observed in LPS-treated macrophages and ConA-treated spleen cells. A recent report suggested that CCR5-mediated neuron-glia signaling functions to protect neurons by suppressing microglia toxicity through downregulation of the expres-

FIG. 2. TgCyp18-induced macrophage and spleen cell proliferation. (A) Macrophages (5 × 10⁵) from B6 and CCR5−/− mice were cultured for 24 h with 25 or 50 μg/ml of TgCyp18, MTgCyp18, or GST or 10 ng/ml LPS. (B) Spleen cells (5 × 10⁵) from B6 and CCR5−/− mice were cultured for 48 h with 25 or 50 μg/ml of TgCyp18, MTgCyp18, or GST or 5 μg/ml ConA. Then, 10 μl of Cell Counting kit 8 reagent was added. After 3 h of incubation at 37°C, the optical density was determined at 450 nm. The percentage of growth was calculated by dividing each value (control or tested) by the average mean of the control samples (nontreated cells, complete medium) multiplied by 100. Each value represents the mean ± the standard deviation of triplicate samples. The results are representitive of three repeated experiments with similar results. Statistical significance was calculated compared to the control groups (complete medium or GST) with an ANOVA and follow-up test (LSD). **, high significance (P < 0.01). In addition, the statistical difference between the wild-type B6 and CCR5−/− cells upon stimulation with the same dose of TgCyp18 was calculated using an independent Student t test. *, significance at P < 0.05.
cellular immunity is an important immune defense mechanism against intracellular pathogens. During *T. gondii* infection, production of high levels of IL-12 and IFN-γ by the innate immune system elicits a very strong adaptive Th1-biased CD4+ and CD8+ T-cell-mediated immune response (30). Secreted products from *T. gondii* can extensively modify its host cell environment (33). *Toxoplasma* possesses two mechanisms to trigger IL-12 production from DCs (3, 18, 37). One is the interaction of *T. gondii* profilin with TLR11, triggering signaling pathways via the MyD88 pathway (18, 37, 43). The other is through an 18-kDa cyclophilin, TgCyp18, which is released by extracellular tachyzoites, triggering IL-12 production through binding to CCR5, a transmembrane receptor expressed by multiple cell types that transmit signals through a Gi-like protein (3, 4, 18, 33). Both CCR5−/− and MyD88−/− mice display defects in IL-12 production after *T. gondii* infection (2, 28, 37). The present study aimed to clarify the immunomodulatory role of TgCyp18 in relation to CCR5 on the proliferation and migration of macrophages and spleen cells (mainly lymphocytes) in order to understand the immunobiology of the immune response toward *T. gondii* as one step on the way to controlling the parasite.

The results of the present study demonstrated that treatment with TgCyp18 enhanced expression of CCR5 on mac-

**FIG. 3.** CCR5 expression on macrophages and spleen cells after incubation with TgCyp18. (A) Peritoneal macrophages (1 × 10^6) from B6 mice were incubated with 50 μg/ml TgCyp18, MTgCyp18, or GST or 10 ng/ml LPS for 24 h. (B) Spleen cells (1 × 10^6) from B6 mice incubated with 50 μg/ml TgCyp18, MTgCyp18, or GST or 5 μg/ml ConA for 24 h; the cells were subjected to flow cytometry. Shaded profiles correspond to cells labeled with isotype controls. Numbers correspond to the percentage of labeled cells. Data are representative of two separate experiments with similar results.
In the lower chamber, 0.25 g/ml of TgCyp18 was added as chemoattractant. Chambers were incubated at 37°C for 24 h. The cells that migrated into the lower compartment were counted by microscopy. The percentage of migrating cells shown represents the mean ± the standard deviation of triplicate samples. Statistical significance was calculated compared to the complete medium control group using an ANOVA and an LSD follow-up test. * P < 0.05; ** P < 0.01. (C and D) TgCyp18-induced cell migration in a CCR5-independent manner. Macrophages (C) or spleen cells (D) (1 × 10^6) from B6 and CCR5^−/− mice were applied to the upper compartment of the chamber. In the lower chamber, 0.25 μg/ml MIP-3α, 50 μg/ml MTgCyp18, or 50 μg/ml MTgCyp18 was added as chemoattractant. Chambers were incubated at 37°C for 24 h. The cells that migrated into the lower compartment were counted by microscopy. The percentage of migrating cells represents the mean ± the standard deviation of triplicate samples. The results are representative of two repeated experiments with similar results. Statistical significance was calculated compared to the complete medium control group using an ANOVA and LSD follow-up test. * P < 0.05; ** P < 0.01. The statistical difference between the wild-type B6 and CCR5^−/− cells upon stimulation with the same dose of TgCyp18 was calculated using an independent Student t test (* P < 0.05). In addition, the difference between TgCyp18 and MTgCyp18 wild-type B6-stimulated cells was significant (* P < 0.05).

![Figure 4](http://cvi.asm.org/)

**FIG. 4.** The effects of TgCyp18 on macrophage and spleen cell migration. (A and B) Macrophages (A) or spleen cells (B) (1 × 10^6) from wild-type mice were applied to the upper compartment of the chamber. In the lower chamber, serial concentration of TgCyp18 (0.1, 1, 10, and 50 μg/ml) were added as chemoattractant. Chambers were incubated at 37°C for 24 h. The cells that migrated into the lower compartment were counted by microscopy. The percentage of migrating cells shown represents the mean ± the standard deviation of triplicate samples. Statistical significance was calculated compared to the complete medium control (0 μg/ml) based on an ANOVA and an LSD follow-up test. * P < 0.05; ** P < 0.01. (C and D) TgCyp18-induced cell migration in a CCR5-independent manner. Macrophages (C) or spleen cells (D) (1 × 10^6) from B6 and CCR5^−/− mice were applied to the upper compartment of the chamber. In the lower chamber, 0.25 μg/ml MIP-3α, 50 μg/ml MTgCyp18, or 50 μg/ml MTgCyp18 was added as chemoattractant. Chambers were incubated at 37°C for 24 h. The cells that migrated into the lower compartment were counted by microscopy. The percentage of migrating cells represents the mean ± the standard deviation of triplicate samples. The results are representative of two repeated experiments with similar results. Statistical significance was calculated compared to the complete medium control group using an ANOVA and LSD follow-up test. * P < 0.05; ** P < 0.01. The statistical difference between the wild-type B6 and CCR5^−/− cells upon stimulation with the same dose of TgCyp18 was calculated using an independent Student t test (* P < 0.05). In addition, the difference between TgCyp18 and MTgCyp18 wild-type B6-stimulated cells was significant (* P < 0.05).

In the present study, the purities of the spleen cell cultures were quantified by flow cytometry using anti-CD3 and anti-CD11b monoclonal antibodies. The percentage of CD3^+ cells was 90.6 ± 0.85% (n = 3). Moreover, the percentage of CD11b^+ cells was 0.17 ± 0.05%. These results indicate the high purity of the lymphocytes (the majority of cells are T lymphocytes) used in this study. * T. gondii* possesses superantigen-like properties that contribute to T-cell proliferation and activation (15, 16). Incubation of *ex vivo* spleen cells cultured with tachyzoite lysates or irradiated parasites resulted in vigorous T-cell proliferation (15). Furthermore, previous studies on human peripheral blood T lymphocytes from unexposed donors also suggested that *T. gondii* acts as a strong T-lymphocyte mitogen, inducing high proliferative responses (39). TgCyp18 and MTgCyp18 stimulate the proliferation of spleen cells in wild-type and CCR5^−/− spleen cells (Fig. 2B). Our data demonstrated that treatment of spleen cells with TgCyp18 enhanced their proliferation in a CCR5-independent manner. It has been reported that TgHSP70 induces splenic B-cell proliferation in a TLR4-dependent manner (5), indicating that *T. gondii* has several proteins that act as mitogens to induce lymphocyte proliferation. Furthermore, a significant proliferative response was observed after stimulation with 25 or 50 μg/ml of TgCyp18 in CCR5^−/− spleen cells compared to stimulation with the same doses in wild-type spleen cells (P < 0.05). In contrast, no significant proliferative response was demonstrated after stimulation with 25 or 50 μg/ml of MTgCyp18 in CCR5^−/− spleen cells compared to wild-type spleen cells. Interestingly, this effect was supported by the result with MTgCyp18, where the induced mutations (17GEH19 to 17AAA19 and 149RP150 to 149V150) located in the N and C termini of TgCyp18, respectively, reduced interactions with CCR5 (42). Altogether, TgCyp18 could control CCR5 expression on macrophages and spleen cells.

Moreover, TgCyp18 enhanced the proliferation of macrophages and spleen cells in a CCR5-independent way. Treatment with TgCyp18 and MTgCyp18 enhanced the proliferation of wild-type and CCR5^−/− macrophages (Fig. 2A). A previous report revealed that cyclophilin A (CypA), which shows functional similarity to TgCyp18, contributes to macrophage proliferation through activation of the Raf-1/MEK/extracellular signal-regulated kinase pathway and regulation of cyclin-dependent kinase activities, which are required for cell cycle progression (36). Cytokines and/or soluble factors might contribute to the cell proliferation via a CCR5-independent manner, but determination of the potential roles of the factors is needed.

With respect to macrophages, while the marginal effect on TgCyp18 MODULATES HOST CELLULAR IMMUNITY 1327...
production of IL-12 p40, NO, and cellular proliferation induced by high doses of TgCyp18 could be questionable, there are other possible interpretations to our results. First, we interpreted that the activity of our recombinant TgCyp18 relative to native TgCyp18 might be reduced. Therefore, higher doses of recombinant TgCyp18 expressed in E. coli would be equivalent to lower doses of native protein with high activity. Aliberti et al. in 2003 reported that low IL-12-inducing activity of recombinant TgCyp18 was observed compared to native TgCyp18, that was included in the soluble tachyzoite extract (3). Those authors explained that the reduced activity of recombinant TgCyp18 expressed in E. coli relative to native TgCyp18 resulted from a missing cofactor rather than improper expression (3). It is also possible that a threshold concentration of TgCyp18 is needed to enhance the production of NO and trigger cellular proliferation. We found low levels of secreted endogenous TgCyp18 from extracellular parasites that appeared to be time dependent (Fig. 1C). Aliberti et al. proved that TgCyp18 was actively secreted in a time- and temperature-dependent fashion (3). Moreover, we demonstrated higher levels of secreted endogenous TgCyp18 upon challenge with the tachyzoites (Fig. 1D). Therefore, these lower levels compared to the doses used in our experiments suggest that huge numbers of tachyzoites (secreted axenically through many cycles) are required to secrete a threshold dose of TgCyp18 capable of having effects on NO and IL-12 production and cell proliferation. Thus, this possibility indicates that TgCyp18 might have paracrine effects (locally or systemically) at the site of infection.

Diana et al. in 2005 demonstrated that T. gondii-excreted/secreted antigens induced the recruitment and migration of human DCs in a CCR5-dependent fashion (19). Other studies in mice have reported that T. gondii can activate DCs and trigger their migration to the spleen to activate T-cell proliferation or to potentiate parasite dissemination (27, 38). Moreover, TgCyp18, which is similar in action to MIP-1β, which binds CCR5, can attract mouse DCs in vitro (3). Furthermore, it has been reported that the secretory form of CypA possesses chemotactic activity, regulating the migration of DCs, monocytes, neutrophils, eosinophils, B lymphocytes, and T lymphocytes through its interaction with CD147, a type I transmembrane glycoprotein (13, 26, 41, 44). Our study showed that TgCyp18 elicited macrophage and spleen cell migration in a dose- and CCR5-dependent fashion. In contrast, CCR5−/− cells showed insignificant migratory responses upon incubation with the same dose of TgCyp18. Although treatment of macrophages and spleen cells with MTgCyp18 failed to attract CCR5−/− cells, the incubation of wild-type cells with MTgCyp18 showed significant migratory responses (P < 0.05). Two explanations may help to clarify the MTgCyp18 migratory response. First, the induced mutation in TgCyp18 to produce MTgCyp18 might enhance partial binding of MTgCyp18 with other receptors rather than CCR5 on macrophages and spleen cells. This partial binding might be responsible for these migratory properties related to MTgCyp18. It is also possible that the binding between the CCR5 and MTgCyp18 was not blocked completely. Yarovsky et al. in 2004 reported that two amino acid sequences (17G7E19 to 17AA19 and 149RP150 to 149YY150) detected one by one, respectively, had reduced interactions with CCR5 without complete blocking of binding (42). This minimal binding with CCR5 might be responsible for the MTgCyp18 migratory properties. A previous study demonstrated that CCR5 plays an important role in the migration of intraepithelial CD8+ T cells and the regulation of the inflammatory response following T. gondii infection (29). Altogether, TgCyp18 appears to control cellular migration in a dose- and CCR5-dependent manner.

Because the effects of TgCyp18 are very similar in macrophages and spleen cells, the question comes up whether TgCyp18 will exert the same effects in every cell type. Thus, the discussed role of TgCyp18 may not be specific for cells of the immune system but a more general cell biological effect. Further studies will be required to estimate the underlying mechanisms responsible for the induction of proliferation and migration to demonstrate this hypothesis.

In conclusion, our data suggested that T. gondii, through producing TgCyp18, has the ability to recruit macrophages to the site of infection, to invade them, and to use their migratory properties. T. gondii enhances the proliferation of macrophages to enable the egressed parasites to find more host cells. In the same way, the parasite may control the migration and proliferation of lymphocytes in order to control the parasite infection and protect the host. T lymphocytes have been demonstrated to be important in controlling T. gondii infection (1, 16, 17). The ability of T. gondii to survive within the host and establish a successful infection while under attack from potent immune responses implicates a balance between parasite immune evasion strategies and the host immune response. The data presented herein, concerning the ability of TgCyp18 to enhance the proliferation and migration of macrophages and lymphocytes, provide new evidence for the complex interaction between the parasite and the host. Further investigation is required to understand how T. gondii controls its own fate in the face of this potent immune response.

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