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The impact of the host CCR5, TLR2 and TLR11 on production of nitric oxide, IL-6, IL-12 and growth of *Toxoplasma gondii*

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ABSTRACT

The current study investigated the impact of the CCR5, TLR2 and TLR11 on production of nitric oxide (NO), IL-6 and IL-12 and growth of *T. gondii*, high virulent RH strain and avirulent PLK strain. All examined knockout macrophages infected with PLK strain produced significant lower levels of IL-12. On the other hand, $TLR2^{-/-}$ and $TLR11^{-/-}$ macrophages infected with RH strain produced significantly reduced levels of IL-12 as compared to wild-type macrophages. $TLR2^{-/-}$ and $TLR11^{-/-}$ macrophages infected with PLK strain significantly inhibited the production of IL-6 when compared to wild-type macrophages. Interestingly, significant reduction in the IL-6 and NO production was observed in $TLR2^{-/-}$ macrophages infected with PLK strain as compared to wild-type macrophages or to the other examined knockout macrophages. On contrary, significant increase in the NO levels was demonstrated in $TLR11^{-/-}$ macrophages infected with the RH strain. The growth of RH strain was significantly enhanced in $CCR5^{-/-}$, $TLR2^{-/-}$ and $TLR11^{-/-}$ macrophages as compared to wild-type macrophages. The highest parasite growth of both RH and PLK strains was achieved in $TLR2^{-/-}$ macrophages. In conclusion, the current study suggests that TLR2 is the most critical receptor in the host defense against *T. gondii* infection.

Keywords: *Toxoplasma gondii*, CCR5, TLR2, TLR11

INTRODUCTION

Mammalian cysteine–cysteine chemokine receptors (CCRs) and toll-like receptors (TLRs) are families of host defense receptors that are differentially expressed on many immune cells. CCRs have important roles in the positioning and the recruitment of immune cells within different host tissues (Martin-Blondel *et al*., 2016). TLRs have been identified in mice and humans as the first sensors for the recognition of its related ligands that known as pathogen associated molecular patterns (PAMPs) (O'Neill and Greene, 1998; Poltorak *et al*., 1998; Means *et al*., 2000; Takeda and Akira, 2003; Takeda *et al*., 2003). PAMP-TLR interactions activate unparalleled, but interfering Th1-Th2 chemokine-cytokine profiles revealing of a complex interaction of stimulatory signals (Agrawal *et al*., 2003; Qi *et al*., 2003). The resultant stimuli are necessary for the functional activation and maturation of tissue macrophages and dendritic cells (DCs), generation of local inflammation and sequent polarization of B and T lymphocyte responses (Wagner, 2002; Bourke *et al*., 2003; Månsson *et al*., 2006).

Toxoplasma gondii, that belonging to phylum apicomplexa, is an intracellular

protozoan parasite and can infect a broad range of warm blooded vertebrates, including humans (Luft and Remington, 1992). Previous reports demonstrated the interfering role of *T. gondii* with the host intracellular signaling leading to pro-inflammatory mediators, including interleukin-6 (IL-6), IL-12, tumor necrosis factor (TNF) and NO (Butcher *et al.,* 2001; Denkers *et al.,* 2003; Luder *et al.,* 2003; Lee *et al.,* 2006; Lang *et al.,* 2007; Whitmarsh *et al.,* 2011). *T. gondii* could establish a stable host-parasite interaction through its specific molecules that could manipulate the immune response not only to complete its life cycle, but also to protect its host. *Toxoplasma* possesses unparalleled mechanisms for initiating immune responses and cell migration in its host. An immunomodulatory effect of *T. gondii* cyclophilin (TgCyp18) was reported through triggering CCR5 in macrophages and DCs (Aliberti *et al.,* 2003; Ibrahim *et al.,* 2009; 2010; 2014). Moreover, previous study reported that *T. gondii* heat shock protein 70-induced NO release was dependent on the IL-1 receptor-associated kinase 4, myeloid differentiation protein-88 (MyD88) and TLR2 (Mun *et al.,* 2005). Purified *Toxoplasma* glycosylphosphatidylinositol (GPI) triggers TLR4 pathways (Debierre-Grockiego *et al.,* 2007). Co-operation between TLR2 and TLR4 was reported during *T. gondii* infection as both TLR2 and TLR4 deficient mice completely abolished the TNF production in response to the parasite GPI (Debierre-Grockiego *et al.,* 2007). Additionally, *T. gondii* actin binding protein, profilin, enhances the production of IL-12 via MyD88 and TLR11 (Yarovinsky *et al.,* 2005).

T. gondii can be classified into three clonal lineages such as the high virulent type I lineage, low virulent type II and the lowest virulent type III (Howe and Sibley, 1995). The acute virulence phenotype has been genetically contributed to a specific region on *T. gondii* chromosome VIII (Howe *et al.,* 1996). Despite biochemical, epidemiological, many genetic differences among *T. gondii* strains, few reports have directly focused on the extent to which these differences exert an influence on the host response during Toxoplasmosis. The aims of the current study are to investigate the initial interaction that occurs between normal (wild-type), CCR5$^{-/-}$, TLR2$^{-/-}$, TLR11$^{-/-}$ macrophages and live *T. gondii*. Here, we try to detect whether the high virulent RH strain and low virulent PLK strain stimulate different patterns of IL-6, IL-12 and NO production from these peritoneal macrophages. The current comparative study was extended to detect the impact of such NO and cytokine production on the parasite growth in our experimental model.

MATERIALS AND METHODS

Ethics statement

In this study, we strictly followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine (permission numbers 22-39, 22-40, 22-42). We used euthanize animals prior to the end of our experiments. Injection with thioglycolate medium and surgical operations were implemented under general anesthesia induced with isoflurane.

Animals

C57BL/6J female mice, six to eight weeks of age were purchased from Clea Japan (Tokyo, Japan). CCR5 knockout (CCR5$^{-/-}$) mice (B6.129P2-Ccr5^{tmlKuz}/J, Stock No. 005427) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). The TLR2$^{-/-}$ mice and TLR11$^{-/-}$ mice were the kind gift of Dr. Satoshi Uematsu and

Dr. Shizuo Akira (Osaka University). 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.

Parasites and cell cultures

T. gondii tachyzoites of RH and PLK strains were maintained on monkey kidney adherent fibroblasts (Vero cells) cultured in Eagle's minimum essential medium (EMEM, Sigma, St Louis, MO) supplemented with 8% heat-inactivated fetal bovine serum. For the purification of tachyzoites, parasites and host-cell debris were washed in cold phosphate-buffered saline (PBS), and the final pellet was resuspended in cold PBS and passed through a 27-gauge needle and a 5.0-μm-pore filter (Millipore, Bedford, MA).

Monolayer cultures of peritoneal macrophages

Mouse peritoneal macrophages were collected from wild-type and $CCR5^{-/-}$, $TLR2^{-/-}$, $TLR11^{-/-}$ mice 4 days after intraperitoneal injection of 1 ml of 4.05% brewer modified BBL™ thioglycolate medium (Becton and Dickinson, Sparks, MD), by peritoneal washing with five milliliters of cold PBS. After harvesting, the cells were centrifuged at 800 × *g* for 10 min and suspended in RPMI 1640 medium containing 10% fetal bovine serum. Then one million cells per each well of macrophage suspension were added to twenty four-well tissue culture microplates. The suspensions were kept at 37°C for 3 hrs, washed thoroughly to remove nonadherent cells, and further incubated at 37°C.

Cytokine ELISA

Macrophage culture supernatants were collected for measurement of IL-6 and IL-12 levels by ELISA (Pierce Biotechnology Inc.) according to the manufacturer's recommendations. Cytokine concentrations were calculated using standard cytokine curves run on the same plates.

Measurement of NO

Supernatant from peritoneal macrophages cultured in Dulbecco's Modified Eagle's Medium (DMEM) contain 10% FBS were collected for analysis of NO. 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. Nitrite levels were calculated with a standard absorbance curve run on the same plate.

Examination of parasite growth

The measurement of [^{3}H]-uracil uptake in the tachyzoites was done because [^{3}H]-uracil serves as a parasite-specific metabolic label for estimating viability as was previously described (Nishikawa *et al.,* 2008). In the current experiment, 1×10^6 peritoneal macrophages were infected with 1×10^6 parasites for 24 hrs. After incubation at 37 °C, [5,6-^{3}H] uracil (Moravek Biochemicals, Brea, CA) was added to the plate at 1 μCi/well, and the cell mixtures were further incubated for two hrs at 37°C. After fixation with 10% trichloroacetic acid, the cell mixtures were incubated with 0.2 N NaOH for 30 min at 37°C. The radioactivity incorporated into the parasites

was estimated using a beta counter.

Statistical analysis

The GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA) was used. Data are presented as means ± standard deviation. Statistical analyses were performed with two-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for group comparisons. A P value < 0.05 was considered statistically significant.

RESULTS

Alteration in IL-6 and IL-12 levels in response to *T. gondii* infection in macrophages

In the current study, we investigated the production of IL-6 and IL-12 by peritoneal macrophages infected with high virulent RH strain and avirulent PLK strain to demonstrate the role of CCR5, TLR2 and TLR11 (Fig. 1). Interestingly, significant reduction in the IL-6 production was observed in TLR2$^{-/-}$ macrophages infected with both RH and PLK strains and in TLR11$^{-/-}$ macrophages infected with PLK strain when compared to wild-type B6 macrophages. Moreover, CCR5$^{-/-}$, TLR2$^{-/-}$ and TLR11$^{-/-}$ macrophages infected with PLK strain produced significant lower levels of IL-12 as compared to wild-type macrophages infected with PLK strain. TLR2$^{-/-}$ and TLR11$^{-/-}$ macrophages infected with RH strain significantly reduced levels of IL-12 as compared to wild-type macrophages infected with RH strain. Interestingly, TLR2$^{-/-}$ macrophages infected with RH or PLK strain produced significant lower levels of IL-6 and IL-12 as compared to other infected groups. Altogether, TLR2 seems to be the main controller of both IL-6 and IL-12 among the examined receptors during *T. gondii* infection.

Alteration in the production of NO in response to *T. gondii* infection in macrophages

Because of the main role of NO for the anti-*Toxoplasma* effect in activated macrophages, we examined the NO production in wild-type, CCR5$^{-/-}$, TLR2$^{-/-}$, TLR11$^{-/-}$ macrophages (Fig. 1). TLR11$^{-/-}$ macrophages infected with RH strain significantly augmented the production of NO as compared to wild-type macrophages infected with RH strain while no significant differences were detected in the production of NO in TLR11$^{-/-}$ macrophages infected with PLK strain compared to wild-type macrophages infected with PLK strain. Moreover, no significant differences were detected in the production of NO in CCR5$^{-/-}$ macrophages infected with RH or PLK strain as compared to the infected wild-type macrophages. TLR2$^{-/-}$ macrophages infected with PLK strain, but not RH strain, significantly reduced the NO production as compared to the infected wild-type macrophages. Altogether, TLR2 seems to play critical role in the NO production during *T. gondii* infection, especially PLK strain.

Parasite growth in wild-type, CCR5$^{-/-}$, TLR2$^{-/-}$ and TLR11$^{-/-}$ macrophages

In order to study the role of CCR5, TLR2, TLR11 on *T. gondii* growth, macrophages from wild-type and different knockout mice were collected and infected with high virulent RH strain or avirulent PLK strain (Fig. 2). The growth of RH strain was significantly enhanced in all kinds of knockout macrophages as compared to wild-type macrophages. Avirulent strain, PLK, showed higher parasite growth in TLR2$^{-/-}$ macrophages than wild-type macrophages. These results suggested that TLR2

seems to have the priority in controlling the parasite growth among the examined receptors.

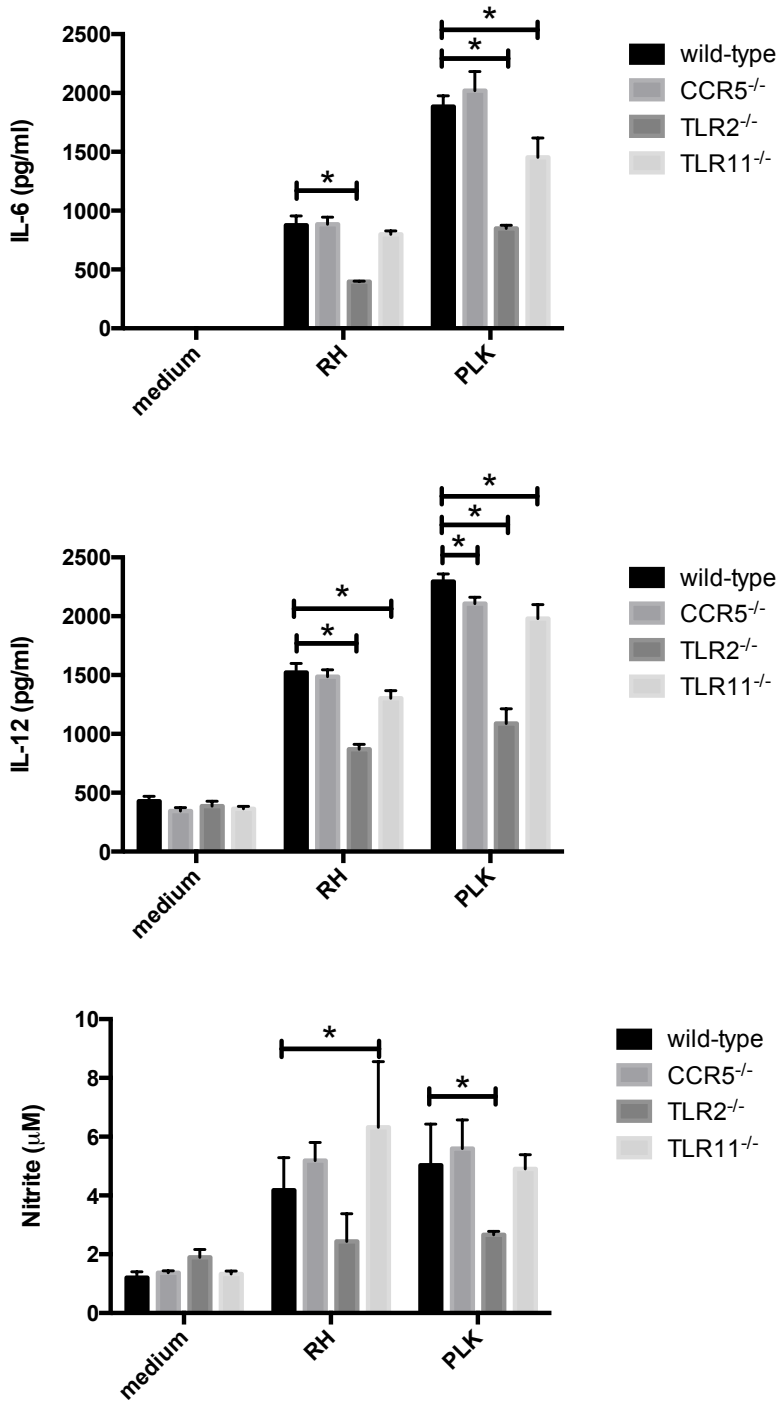


Fig. 1. Production of IL-6, IL-12 and NO (nitrite) in wild-type, CCR5$^{-/-}$, TLR2$^{-/-}$ and TLR11$^{-/-}$ macrophages infected with *T. gondii*. Peritoneal macrophages (1×10^6) from wild-type, CCR5$^{-/-}$, TLR2$^{-/-}$ and TLR11$^{-/-}$ mice were infected with 2×10^5 *T. gondii* RH strain or PLK strain for 24 hrs, and the supernatants were collected for measuring the production of IL-6, IL-12 and NO. Each value represents the mean ± the standard deviation of quadrate samples. * indicates statistically significant differences by one-way ANOVA plus Tukey–Kramer post-hoc analysis (P<0.05).

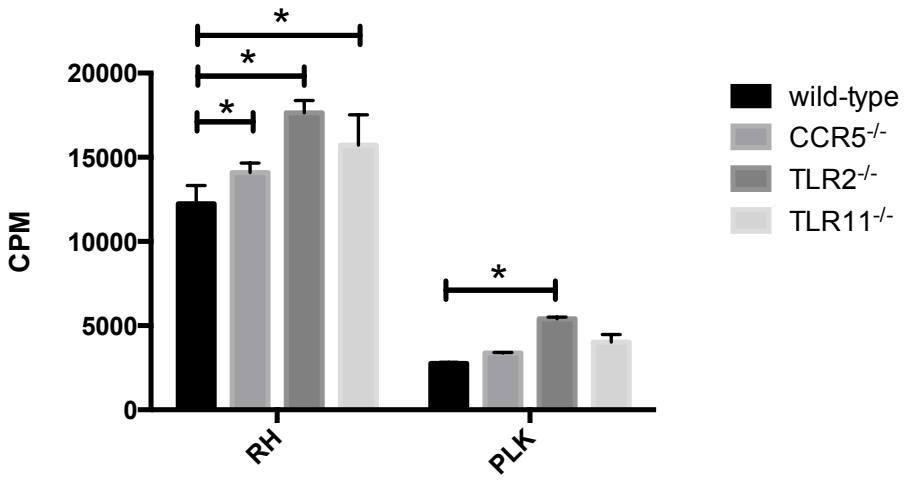


Fig. 2. The intracellular growth of *T. gondii* in macrophages obtained from wild-type, CCR5$^{-/-}$, TLR2$^{-/-}$ and TLR11$^{-/-}$ mice. Peritoneal macrophages (1×10^6) from wild-type, CCR5$^{-/-}$, TLR2$^{-/-}$ and TLR11$^{-/-}$ mice were infected with *T. gondii* RH strain or PLK strain (1×10^6) for 24 hrs, After the indicated period, [^{3}H]-uracil uptake in tachyzoites was measured. Each value represents the mean ± the standard deviation of quadrate samples. * indicates statistically significant differences by one-way ANOVA plus Tukey–Kramer post-hoc analysis (P<0.05).

DISCUSSION

Realization the basis of successful long-term interaction between *Toxoplasma* and their hosts is achieved through the clarification of the molecular mechanisms underlying the regulation of the protective immune response (Denkers, 2003). In the present study, virulent strain, RH, revealed higher parasite growth in wild-type and all examined knockout macrophages when compared to PLK strain (avirulent strain). Toxoplasmosis pathogenesis induced by different *T. gondii* strains are related to the growth levels of the parasite strains. Therefore, type I virulent strain achieves higher numbers and become widely disseminated than those of type II avirulent strain (Gavrilescu and Denkers, 2001). Moreover, in the present study, infection of wild-type, CCR5$^{-/-}$, TLR2$^{-/-}$ and TLR11$^{-/-}$ peritoneal macrophages with PLK strain resulted in higher levels of IL-6 and IL-12 production than infection with RH strain. Consistent with this observation, Robben *et al*. (2004) reported that low virulent type II strain parasites induced the production of higher levels of IL-12 and other pro-inflammatory cytokines accompanied by more effective activation of nuclear factor-kappa B than infection with type I strain parasites. It has been demonstrated that macrophages with inflammatory properties reside to *T. gondii* infected sites and play a critical role in the local control of the parasites (Mordue and Sibley, 2003). Early induction of IL-6 and IL-12 by type II PLK strain parasites may contribute to better control of the parasite replication and diminished virulence.

T. gondii has several antigens which work through different pathways to instruct and subvert host-cell responses through its interaction with host receptors. The recognition of the cytokine induced in response to the host receptors is an essential element to acquire comprehensive understanding of the host-parasite interaction. Pro-inflammatory cytokines like IL-6 and IL-12 play critical role in immunity against *T. gondii* (Scharton-Kersten *et al.,* 1995; Alexander *et al.,* 1997; Denkers and Gazzinelli, 1998; Lieberman and Hunter, 2002). In the current study, CCR5$^{-/-}$, TLR2$^{-/-}$ and TLR11$^{-/-}$ peritoneal macrophages infected with PLK produced significant lower levels of IL-12 as compared to wild-type macrophages. Moreover,

TLR2$^{-/-}$ and TLR11$^{-/-}$ peritoneal macrophages infected with RH strain produced significantly reduced levels of IL-12 as compared to wild-type macrophages. *Toxoplasma* parasites can trigger the production of IL-12 through several different pathways. One of these pathways is mainly dependent upon the secreted TgCyp18 released from the extracellular tachyzoites, which enhances IL-12 production through its binding to the host CCR5 (Aliberti *et al*., 2003; Ibrahim *et al*., 2009). Another pathway depends on *T. gondii* profilin that enhances the IL-12 production via MyD88 and TLR11 (Yarovinsky *et al*., 2005). Moreover, *Toxoplasma* GPI is recognized by TLR2 and TLR4 (Debierre-Grockiego *et al*., 2007). After five days of *T. gondii* infection, TLR2-defiecient mice showed dramatically decreased levels of IL-12 in plasma samples and from peritoneal macrophages when compared to wild-type or TLR4-defiecient mice (Mun *et al*., 2003). IL-6 has been reported to be essential for the host immunity against *T. gondii* (Suzuki *et al*., 1997). Our study also revealed that significant reduction in the production of IL-6 was recorded in TLR2$^{-/-}$ macrophages infected with either *T. gondii* strains (RH or PLK) and that reduction of IL-6 levels was also detected in TLR11$^{-/-}$ macrophages infected with PLK strain when compared to wild-type macrophages. Human TLR5 seems to be evolutionarily the oldest relatives to mouse TLR11. Recent report suggests that function and microbial ligand affinity is conserved between human TLR5 and mouse TLR11 and demonstrated that *T. gondii* profilin, which is a TLR11 ligand in mice, triggers IL-6 production from human peripheral blood monocytes in a TLR5-sensitive manner (Salazar Gonzalez *et al*., 2014). More recent *in vitro* study demonstrates that *T. gondii* oocyst lysate antigen that recognized by TLR2-transfected human embryonic kidney cells can induce the IL-6 production from splenocytes and bone marrow-derived dendritic cells (Wagner *et al*., 2016). Altogether, TLR2 and TLR11 could control IL-6 production during *T. gondii* infection.

In our comparative study, TLR11$^{-/-}$ macrophages infected with *T. gondii* (RH strain) significantly increased the production of NO as compared to wild-type macrophages while no significant differences were detected in the production of NO in TLR11$^{-/-}$ macrophages infected with *T. gondii* (PLK strain). Significant reductions in the NO levels were detected in TLR2$^{-/-}$ macrophages infected with PLK strains when compared to wild-type macrophages or to the other examined knockout macrophages. Chen *et al*. (2014) suggest that TLR11 may play a biological role on the level of NO-mediated antimicrobial activities. The authors of the previous study demonstrate the ability of *T. gondii* profilin for the expression of IL-12 and IFN-γ in a time-dependent manner while it can not induce TNF-α, IL-6, and IFN-β expressions. Both TNF-α and IFN-γ are required for the induction of NO production and reactive nitrogen intermediates (James, 1995; Stephan *et al*., 1995), and NO can directly kill invading pathogens including *Toxoplasma* parasites. The ability of TLR11 to up-regulate the IFN-γ without the up-regulation of TNF-α might refer to its NO-arresting properties of TLR11. Therefore, the NO level was increased in TLR11$^{-/-}$ macrophages infected with *T. gondii* (RH strain) in the current study. Furthermore, Mun *et al*. (2005) demonstrate that *T. gondii* HSP70 has the capability to induce the release of NO through TLR2/MyD88 pathway. Altogether, among the examined receptors TLR2 is critical controller of pro-inflammatory mediators such as IL-6, IL-12 and NO as TLR2$^{-/-}$ macrophages infected with either RH or PLK strain produced significant lower levels of IL-6, IL-12 and NO.

Finally, our study showed that *T. gondii* (RH or PLK strain) growth was increased in CCR5$^{-/-}$, TLR2$^{-/-}$ and TLR11$^{-/-}$ macrophages compared to wild-type macrophages. Several previous studies come in agreement with the current data.

CCR5 control the parasite growth through its interaction with TgCyp18 (Ibrahim *et al*., 2009). The parasite load is significantly higher in TLR2$^{-/-}$ and TLR11$^{-/-}$ mice than wild-type mice, suggesting that these TLRs are necessary for resistance to murine toxoplasmosis (Mun *et al*., 2003; Yarovinsky *et al*., 2005; Debierre-Grockiego *et al*., 2007). In the current study, the highest parasite growth that recorded in TLR2$^{-/-}$ macrophages was accompanied by the lowest levels of IL-6, IL-12 and NO. The detected alteration in the pro-inflammatory mediators such as IL-12, IL-6 and NO may be the main reason for the observed changes in the parasite growth. Pro-inflammatory mediators initiate and maintaine the host protective immune response against *T. gondii* (Liew and Cox., 1991; Bohne *et al*., 1994; Khan *et al*., 1994; Suzuki *et al*., 1997; Yap *et al*., 2000). Previous reports have been proved the *in vivo* role for inducible nitric oxide synthase in toxoplasmosis pathology and parasite development (Khan *et al*., 1997; Scharton-Kersten *et al*., 1997; Schlüter *et al*., 1999). Inhibition of NO formation by the NO synthase inhibitor L-NMMA clarifies the important role of NO for the *toxoplasma*-static effects in activated macrophages (Liew and Cox., 1991**;** Sibley *et al*., 1991; Langermans *et al*., 1992). Moreover, several *in vitro* studies report that NO induces subsequent parasite stasis and conversion (Bohne *et al*., 1994; Soête and Dubremetz. 1996).

In conclusion, the current study highlights that TLR2 is the most critical receptor in the resistance against *T. gondii* infection. The immune inflammatory response could be considered as a double-edged weapon related with host-defense against various microbes and potential self-damaging. The balance between these aspects controls the physiological status of the hosts. The exact molecules that contributed to the host receptors and biological impact on the production of the pro-inflammatory mediators would be worthy of investigation in detail in the future studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

This manuscript is approved by all authors, and if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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