Production of Inflammatory Cytokines and Nitric Oxide by Human Mast Cells Incubated with *Toxoplasma gondii* Lysate

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Abstract: The roles of mast cells in allergic diseases and helminth infections are well known. However, the roles of mast cells in *T. gondii* infection is poorly understood. This study was focused on the production of pro-inflammatory cytokines (TNF-α, IL-4), chemokines (CXCL8, MCP-1) and nitric oxide (NO) by mast cells in response to soluble lysate of *T. gondii* tachyzoites. Production of CXCL8 (IL-8), MCP-1, TNF-α and IL-4 were measured by RT-PCR and ELISA. Western blot were used for detection of CXCR-1 and CXCR2. Our results showed that *T. gondii* lysates triggered mast cells to release CXCL8, MCP-1, TNF-α, IL-4 and to produce NO. This suggests that mast cells play an important role in inflammatory responses to *T. gondii*.

Key words: *Toxoplasma gondii*, mast cell, cytokine, nitric oxide

Toxoplasma gondii is an obligate intracellular protozoan parasite with a global distribution in humans and other mammals. Although this parasite most often causes subclinical infection, primary infection during pregnancy can induce fetal damage and abortion in both humans and animals. *T. gondii* is a well-known inducer of type 1 cytokines, and while these cytokines are required to survive infection, their over-production can be harmful and even lethal [1,2].

Mast cells are abundant in the skin, mucosa of the digestive and respiratory tracts and conjunctiva. These cells provoke IgE-mediated allergic responses and are involved in the control of helminth infections of the gastrointestinal tract. They play a key role in innate and adaptive immunity [3,4] and have been shown to induce acute inflammatory reactions in mice in response to intraperitoneal injection of *T. gondii* [5]. Mast cells also protect the host when *T. gondii* is introduced by the oral route [3]. Mediators produced by activated mast cells may reduce acute inflammation and stimulate parasite clearance in *T.*

gondii infections [6]. On the other hand, *T. gondii* itself inhibits mast cell degranulation by suppressing the mobilization of intracellular Ca^{2+} by phospholipase C [7]. So, the overall effect of mast cells on *T. gondii* infections is uncertain. In addition, it is not known whether *T. gondii* lysates can induce inflammatory responses in the mast cell leukaemia cell line, HMC-1. The aim of this study was to evaluate whether *T. gondii* lysates induce the activation of HMC-1 cells to release cytokines and nitric oxide.

T. gondii (RH strain) tachyzoites were maintained by intra¬peritoneal infection of ICR mice (Osan, Korea). Tachyzoites were harvested from the mice, washed with PBS and centrifuged at low and high speeds (500 g and 3,000 g) for 5 min to remove peritoneal cells. To prepare *T. gondii* lysate, the tachyzoites were frozen and thawed 3 times and ultrasonicated at 100% amplitude, 0.75 cycles, 7-10 times, until the tachyzoites were completely disrupted. The sonicate was centrifuged at 10,000 g for 1 hr, and the supernatant was filtered through a 0.2 µm membrane (Millipore, Bedford, Massachusetts, USA). Protein concentrations were determined with the Bradford assay (Bio-Rad, Hercules, California, USA).

Human leukemic mast cells (HMC-1) were grown in IMDM supplemented with 10% FBS at 37℃ in a 5%-CO_2 incubator. To examine cytokine and iNOS mRNA expression, HMC-1

cells (1.5×10^6/well) were pretreated with IFN-γ (5 μg/ml, ProSpec, East Brunswick New Jersey, USA) for 1 hr and then stimulated with an aliquot of *T. gondii* lysate (50 μg/ml) or LPS (10 μg/ml, Sigma, St Louis, Missouri, USA). As a positive control, the HMC-1 cells were incubated with PMA (100 nm/ml) and A23187 (10 μm/ml). Total RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, California, USA) and 200 μl chloroform (Sigma Aldrich, St. Louis, Missouri, USA). cDNAs were synthesized and reverse-transcribed using RT Premix and PCR Premix (iNtRON Biotechnology, Seongnam, Korea). Primer sequences were as follows; β-actin (5′-CCA GAG CAA GAG AGG TAT CC-3′ and 5′-CTG TGG TGG TGA AGC TGT AG-3′), human IL-8 (5′-GCC AAG AGA ATA TCC GAA CT-3′ and 5′-AAA GTG CAA CCA CAT GTC CT-3′), MCP-1 (5′-TCT GTG CCT GCT GCT CAT AG-3′ and 5′-GCT GCA GAT TCT TGG GTT GT-3′), IL-4 (5′-CCC TTT CGG CAA AAT CCT CC-3′ and 5′-AGG AAT ACA GTG CAG CTT ACC A-3′), TNF-α (5′-ACT CTT CTG CCT GCT GCA CTT TGG-3′ and 5′-GTT GAC CTT TGT CTG GTA GGA GAC GG-3′), iNOS (5′-AGC ATG AGC CCC TTC ATC AAT-3′ and 5′-CTG TTT CAA CGA CCT CCG GG-3′), and GAPDH (5′-GTC AGT GGT GGA CCT GAC CT-3′ and 5′-AGG GGT CTA CAT GGC AAC TG-3′). PCR conditions were initial DNA denaturation at 94℃ for 5 min followed by 35 rounds of denaturation at 98℃ for 15 sec, and annealing for 30s at 58℃ for β-actin, GAPDH & iNOS, 56℃ for IL-8 & IL-4, 55℃ for MCP-1, and 62℃ for TNF-α, and extension at 72℃ for 35 sec.

To measure IL-8, MCP-1, TNF-α and IL-4 proteins, HMC-1 cells were pretreated with IFN-γ followed by exposure to *T. gondii* lysate or LPS for 18 hr or 24 hr. Supernatants were collected and stored at -20℃ and cytokines were measured by ELISA (BD Biosciences, San Diego, California, USA).

Western blot analysis with monoclonal antibodies was used to detect expression of IL-8 receptor (CXCR1 and CXCR2) on HMC-1 cells which were pretreated with IFN-γ for 1 hr followed by *T. gondii* lysate or LPS. After incubation, the cells were harvested and lysed in PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam, Korea). Equal amounts of protein were denatured and separated by SDS–PAGE on 10% polyacrylamide gels and transferred to Immune-Blot1 PVDF membranes (Bio-Rad, Quarry Bay, HongKong). The membranes were probed with anti-CXCR1 and anti-CXCR2 antibodies (1:1,000; Abcam, Cambridge, UK), or β-actin polyclonal antibody (1:3,000; Abcam, Cambridge, Massachusetts, USA) overnight at 4℃ followed by goat anti-rabbit IgG poly-

clonal antibody (1:10,000; ADISAB-300-J, Enzo Life Sciences, New York, USA) for 1 hr at room temperature. The blots were visualized using Chemiluminescent Sensitive Plus HRP Microwell and/or Membrane Substrate (SurModics, Minneapolis, Minnesota, USA), and signals were measured with a ChemiDoc1 (Bio-Rad).

For measuring nitric oxide, HMC-1 cells was pretreated with IFN-γ followed by *T. gondii* lysate or LPS for 3 hr, 6 hr, 18 hr or 24 hr and supernatants were collected. NO was determined as nitrite using Griess reagent [8].

Statistical analyses were performed with SPSS, version 21 (IBM, Chicago, Illinois, USA). The Mann-Whitney U test was used to compare results, and P-values < 0.05 were considered statistically significant. Data are expressed as means ± SDs of three independent experiments.

In this experiment, up-regulation of IL-8 mRNA and increased secretion of IL-8 were observed in the *T. gondii*-treated HMC-1 cells 6 hr and 18 hr post inoculation (PI), respectively. However the IL-8 levels of HMC-1 cells stimulated with LPS as positive control were significantly higher than those of the of *T. gondii*-stimulated cells. Up-regulations of MCP-1 mRNA and protein were also observed in the HMC-1 cells exposed to *T. gondii* lysate or LPS. Similarly, enhanced TNF-α mRNA expression and increased TNF-α secretion were noted in supernatants of HMC-1 cells treated with *T. gondii* lysate. IL-4 mRNA was also increased by incubation with *T. gondii* lysate for 24 hr, and IL-4 protein increased in the supernatants of treated HMC-1 cells (Fig. 1A, B). To investigate the expression of chemokine receptors for IL-8, CXCR1 and CXCR2 production was analyzed by western blotting after treatment of HMC-1 cells with *T. gondii* lysate or LPS for 1 hr. Both CXCR1 and CXCR2 increased in response to *T. gondii* lysate (Fig. 1C).

The results in Fig. 2A show that iNOS mRNA increased in HMC-1 cells treated with *T. gondii* lysate for 3 hr, and then gradually disappeared. In LPS-treated cells, iNOS mRNA continued to increase between 3 hr and 24 hr of incubation although at a decreasing rate. Maximal NO production in HMC-1 cells treated with *T. gondii* lysate or LPS was observed after 3 hr of stimulation (Fig. 2B).

In *T. gondii* infections, immune cells including neutrophils, monocytes and macrophages are activated, and also these cells are targets of *T. gondii* [9,10]. *T. gondii* down-regulates type I immune responses, blocking production of pro-inflammatory cytokine including IFN-γ in macrophages and dendritic cells [11]. Mast cells are abundant in the intestinal mucosa and play
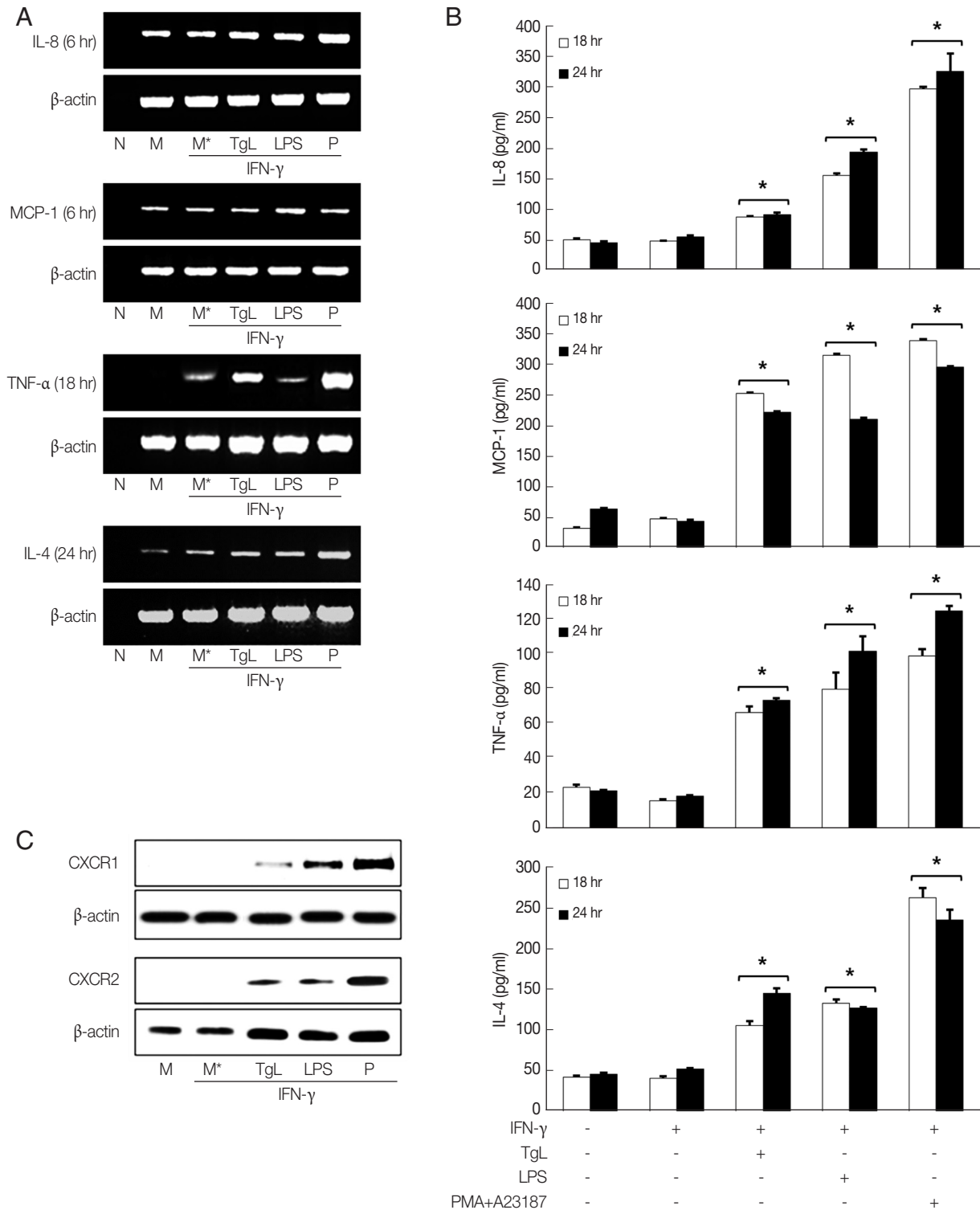
Fig. 1. Increased production of IL-8, MCP-1, TNF-α and IL-4 mRNAs and proteins by HMC-1 cells incubated with *T. gondii* lysate. HMC-1 cells were pretreated with IFN-γ for 1 hr and then stimulated with *T. gondii* lysate or LPS. mRNA expression of each cytokine (A) and cytokine level (B) were increased. CXCR1 and CXCR2 was measured by western blotting with monoclonal antibodies to each receptor after 1 hr of treatment with *T. gondii* lysate. PMA +A23187 was used as positive control. M, culture supernatant of HMC-1 alone; M*, culture supernatant of HMC-1 treated with IFN-γ; TgL, *T. gondii* lysate. *$P < 0.05$ vs HMC-1 treated with IFN-γ.
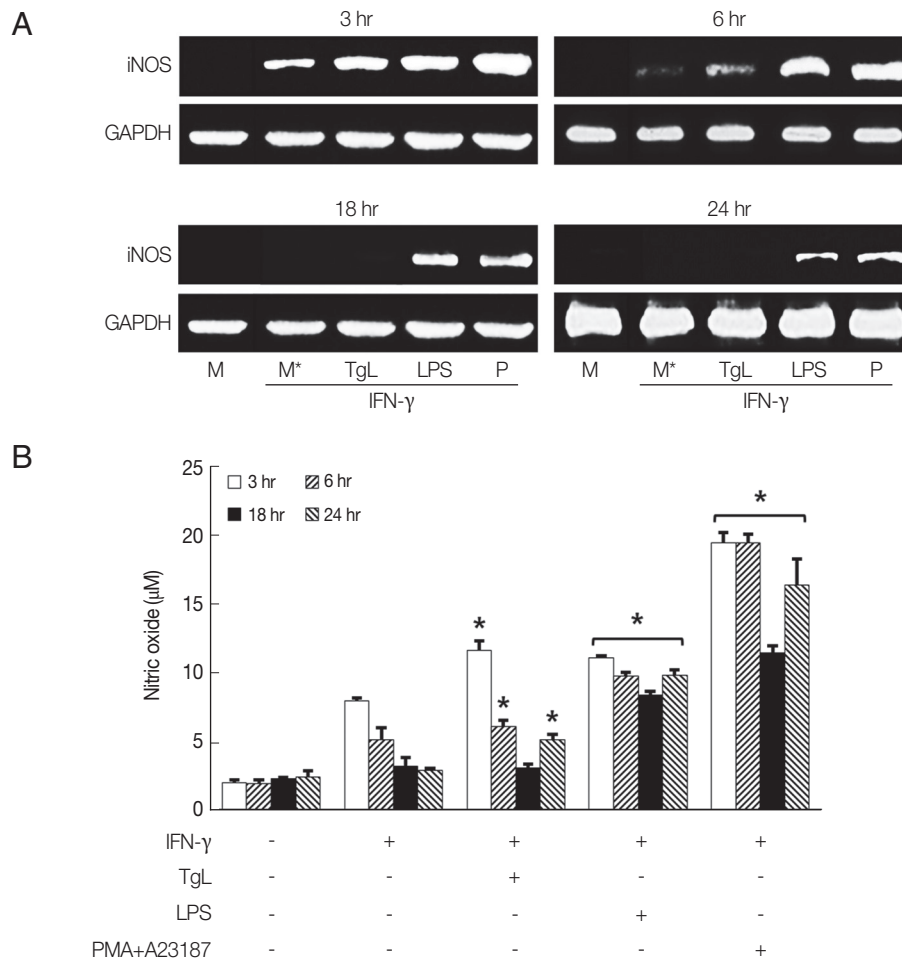
Fig. 2. iNOS mRNA and nitric oxide (NO) productions were up-regulated by HMC-1 cells stimulated with *T. gondii* lysate. HMC-1 cells were pretreated with IFN-γ for 1 hr and then stimulated with *T. gondii* lysate or LPS. iNOS mRNA (A) and NO production (B) were determined after 3 hr, 6 hr, 18 hr, and 24 hr of incubation. NO production was measured with Griess reagent. PMA +A23187 was used as positive control. M, culture supernatant of HMC-1 cells alone; M*, culture supernatant of HMC-1 cells treated with IFN-γ; TgL, *T. gondii* lysate. *$P < 0.05$ vs HMC-1 cells treated with IFN-γ.

an important role in protective immune responses to oral infection by *T. gondii* [3]. Exposure to *T. gondii* causes mast cell to activate, degranulate and release mediators, proteases and cytokines [12]. Increases in mast cells numbers may be involved in the balance between inflammation and wound healing [13].

In the current study we found that IL-8 and MCP-1 secretion was up-regulated after 18 hr exposure to *T. gondii* lysate. This result is in agreement with our previous report that infection with *T. gondii* tachyzoites leads to increased secretion of IL-8 and MCP-1 by HeLa cells and fibroblasts [14]. Up-regulation of TNF-α and IL-4 mRNAs was observed in HMC-1 cells treated with *T. gondii* lysate for 18 hr and 24 hr, respectively. Pro-

duction of both cytokines increased in *T. gondii* lysate- or LPS-treated HMC-1 cells indicating that *T. gondii* lysate induces inflammatory effect in the cells. *T. gondii* lysate alone did not increase cytokine production in HMC-1 cells. However, pretreatment of IFN-γ resulted in increased cytokine production in the cells. Therefore, IFN-γ was thought to be acted as a trigger for HMC-1 cells in cytokine production by *T. gondii* lysate.

The functions of chemokines induced by intracellular pathogens include leukocyte recruitment, cell mediated immunity and anti-protozoal activity. IL-8 induces a rapid and transient increase of Ca^{2+} in mast cells. HMC-1 cells express the CXCL8 chemokine receptors, CXCR1 and CXCR2 [15-18]. Increased levels of these receptors were observed on HMC-1

cells stimulated with *T. gondii* lysate for 1 hr (Fig. 1C).

The release of mediators by mast cell activation plays an important role in modulating inflammation during *T. gondii* infection [6,16]. In protozoal infections these mast cells are characterized by degranulation and the production of pro-inflammatory mediators, such as TNF-α [13]. The mast cell activator, compound 48/80, up-regulated Th1 cytokine mRNA (IFN-γ, IL-12p40, TNF-α), and the mast cell stabilizer, disodium cromoglycate, increased Th2 cytokine mRNA (IL-4, IL-10) [12].

Mast cells are effector cells involved in clearance of parasites including *Trichomonas vaginalis* (*T. vaginalis*) [6]. We observed that rat peritoneal mast cells (RPMCs) were involved in the inflammatory response to *T. vaginalis* infection. Increased release of histamine and TNF-α were observed in RPMC-stimulated *T. vaginalis* ESP or live trichomonads [19]. Activation of mast cells has been reported to trigger production of cytokines and inflammatory mediators by protozoan parasites such as *Plasmodium* sp., *Leishmania* sp., and *T. gondii* [12].

Several protozoan parasites have evolved strategies to evade NO-mediated anti-microbicidal activity. Inducible nitric oxide synthase (iNOS)-derived NO can inhibit leucocyte adhesion in the micro-vasculature during ongoing inflammation. *T. gondii* infection partially inhibits NO production by murine macrophages and thus promotes parasite survival in phagocytic cells [12,20,21]. *Leishimania* sp. and *Trypanosoma cruzi* are known to inhibit iNOS expression [22,23]. We investigated HMC-1 cells were treated with *T. gondii* lysate to determine whether *T. gondii* infection affects NO production. Increased iNOS mRNA and NO production were observed at 3 hr PI and it then declined by 6 hr.

Mast cells induce recruitment of other immune cells to initiate inflammation. In the present study, we observed that *T. gondii* lysate stimulated the production of chemokines (CXCL8, MCP-1), cytokines (TNF-α, IL-4) and NO production in HMC-1 cells. Therefore, activated mast cells by *T. gondii* lysate may be involved in innate and adaptive immune responses against *T. gondii* infection.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this study.

REFERENCES

1. Henderson WR Jr, Chi EY. The importance of leukotrienes in mast cells-mediated *Toxoplasma gondii* cytotoxicity. J Infect Dis 1998; 177: 1437-1443.

2. Długońska H. *Toxoplsma gondii* and mast cells. Ann Parasitol 2014; 60: 235-238.

3. Cruz A, Mendes ÉA, de Andrade MV, do Nascimento VC, Cartelle CT, Arantes RM, Melo JR, Gazzinelli RT, Ropert C. Mast cells are crucial in the resistance against *Toxoplasma gondii* oral infection. Eur J Immunol 2014; 44: 2949-2954.

4. Rodríguez NE, Wilson ME. Edsinophils and mast cells in leishmaniasis. Immunol Res 2014; 59: 129-141.

5. S Ferreira GL, Mineo JR, Oliveira JG, V Ferro EA, Souza MA, D Santos AA. *Toxoplsma gondii* and mast cell interactions in vivo and in vitro: experimental infection approaches in *Calomys callosus* (Rodentia, Cricetidae). Microbes Infect 2004; 6: 172-181.

6. Huang B, Huang S, Chen Y, Zheng H, Shen J, Lun ZR, Wang Y, Kasper LH, Lu F. Mast cells modulate acute Toxoplasmosis in murine models. PLoS One 2013; 8: 10; e77327.

7. Smith NL, Abi Abdallah DS, Butcher BA, Denkers EY, Baird B, Holowka D. *Toxoplasma gondii* inhibits mast cell degranulation by suppressing phospholipase Cγ-mediated Ca²⁺ mobilization. Front Microbiol 2013; 4: 179.

8. Park GC, Ryu JS, Min DY. The role of nitric oxide as an effector of macrophage-mediated cytotoxicity against *Trichomonas vaginalis*. Korean J Parasitol 1997; 35: 189-195.

9. Bierly AL, Shufesky WJ, Sukhumavasi W, Morelli AE, Denkers EY. Dendritic cells expressing plasmacytoid marker PDCA-1 are Trojan horse during *Toxoplasma gondii* infection. J Immunol 2008; 181: 8485-8491.

10. Lambert H, Barragen A. Modelling parasite dissemination: host cell subversion and immune evasion by *Toxoplasma gondii*. Cell Microbiol 2010; 12: 292-300.

11. Leng J, Butcher BA, Denkers EY. Dysregulation of macrophage signal transduction by *Toxoplsma gondii*: past progress and recent advances. Parasite Immunol 2009; 31: 717-728.

12. Lu F, Huang S. The roles of mast cells in parasitic protozoan infections. Front Immunol 2017; 8: 363.

13. Vukman KV, Lalor R, Aldridge A, O'Neill SM. Mast cells: new therapeutic target in helminth immune modulation. Parasite Immunol 2016; 38: 45-52.

14. Lee EJ, Heo YM, Choi JH, Song HO, Ryu JS, Ahn MH. Suppressed production of pro-inflammatory cytokines by LPS-activated macrophages after treatment with *Toxoplasma gondii* lysate. Korean J Parasitol 2008; 46: 145-151.

15. Inamura H, Kurosawa M, Okano A, Kayaba H, Majima M. Expression of the interleukin-8 receptors CXCR1 and CXCR2 on cord-blood-derived cultured human mast cells. Int Arch Allergy Immunol 2002; 128: 142-150.

16. Gaddi PJ, Yap GS. Cytokine regulation of immunopathology in toxoplasmosis. Immunol Cell Biol 2007; 85: 155-159.

17. Denney CF, Eckmann L, Reed S. Chemokine secretion of human cells in response to *Toxoplasma gondii* infection. Infect Immun 1999; 67: 1547-1552.

18. Nilsson G, Mikovits JA, Metcalfe DD, Taub DD. Mast cell migratory response to interleukin-8 is mediated through interaction with chemokine receptor CXCR2/interleukin-8RB. Blood 1999; 93: 2791-2797.

19. Im SJ, Ahn MH, Han IH, Song HO, Kim YS, Kim HM, Ryu JS. Histamine and TNF-α release by rat peritoneal mast cells stimulated with *Trichomonas vaginalis*. Parasite 2011; 18: 49-55.

20. Seabra SH, de Souza W, DaMatta RA. *Toxoplasma gondii* partially inhibits nitric oxide production of activated murine macrophages. Exp Parasitol 2002; 100: 62-70.

21. Lüder CG, Algner M, Lang C, Bleicher N, Gross U. Reduced expression of the inducible nitric oxide synthase after infection with *Toxoplasma gondii* facilitates parasite replication in activated murine macrophages. Int J Parasitol 2003; 33: 833-844.

22. Pakianathan DR, Kuhn RE. *Trypanosoma cruzi* affects nitric oxide production by murine peritoneal macrophages. J Parasitol 1994; 80: 432-437.

23. Proudfoot L, Nikolaev AV, Feng GJ, Wei WQ, Ferguson MA, Brimacombe JS, Liew FY. Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of *Leishmania* lipophosphoglycan in murine macrophages. Proc Natl Acad Sci USA 1996; 93: 10984-10989.