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Regulation of Microglial Cell Phenotype and NO Production in the Mouse Brain after *Toxoplasma gondii* Infection

톡소포자충 감염 후 마우스 뇌에서 산화질소의 생성과 미세아교세포 표현형의 조절

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Regulation of Microglial Cell Phenotype and NO Production in the Mouse Brain after *Toxoplasma gondii* Infection

by

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A thesis submitted to the Department of Parasitology and Tropical Medicine in partial fulfillment of the requirement of the Degree of Master of Science at Seoul National University Graduate School, Department of Medicine (Parasitology Major)

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Approved by Thesis Committee:

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ABSTRACT

Interferon- γ (IFN-γ) polarizes microglia to M1 phenotype which is required for central nervous system (CNS) immune responses against pathogens. IFN-γ-activated M1 microglia plays a role as a defense cell in *Toxoplasma gondii* infection and simultaneously results in tissue damage related with nitric oxide (NO) during a broad range of CNS pathologies. In contrast, M2 phenotype microglia has a role in remodeling of the damaged tissues and induces Th2 type immune responses. A major goal of the present study is to understand the immunopathology in the brain attributed by T. gondii infection, the orientation of microglial activation which is induced by different *T. gondii* strains, type I (virulent; RH) and type II (avirulent; ME49), and how *T. gondii* modulates the activation of microglia to be a chronic infection without harmful events in the brain.

C57BL/6 mice were orally infected with 10 cysts of *T. gondii* type II (avirulent; ME49) strain and sacrificed at weeks 0, 1, 3, 6, 9 and 12 post-infection (PI). The brain tissues from infected mice were examined by hematoxylin and eosin stain (H-E stain) to observe histological changes in the hippocampal formation. To investigate the characteristics of immune responses in the brain, levels of proteins and mRNA were examined by cytokine arrays for IL-4, IL-10, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, tumor necrosis factor-alpha (TNF-α), IL-12p70, and by microarray analysis of mouse brain gene expression, respectively. In addition, BV2 cells (microglial cell line established from

C57BL/6 mouse brain) were cultured with *T. gondii* tachyzoites antigens (RH and ME49 strain) and confirmed the expression of cell surface markers including major histocompatibility complex II (MHC II), CD80, B7-H1, B7-DC, and CD40 using flow cytometry. Moreover, it was to determine the effects of *T. gondii* strains on relative gene expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthases (iNOS), and arginase 1 using real-time PCR.

The neuronal cell death (eosinophilic neurons) resulted in the early stage of *T. gondii* infection was observed in the hippocampal dentate gyrus region at weeks 1, 3, and 6 PI. After 6 weeks PI, the impaired region in the hippocampal dentate gyrus was gradually repaired and cells were alive. Immunohistochemistry (IHC) result stained with anti-NF-kB p65 in dentate gyrus region also showed a remarkable increase of the stained cells at 3- and 6-wks PI. In cytokine arrays, measured cytokines (IL-4, IL-10, IL-6, GM-CSF, IFN-γ, TNF-α, and IL-12p70) showed in generally increased levels with some exception of the decrease of Th2 cytokine and the increase of Th1 cytokines between 3- to 6-wks PI. In particular, inflammatory cytokines were maintained as a continued increase during the experimental period. Similarly, microarray results in the brain showed the continuous increase in proinflammatory cytokines as well as IL-10 and TGF-β gene expression during 36 wks PI. To investigate the tendency of microglial activation and NO production according to *T. gondii* strain, BV-2 cells were cultured with *T. gondii* tachyzoites antigens (RH and ME49 strain) for 20 hours. As a result, the phenotype of microglia stimulated with ME49 antigen was shifted to M1

phenotype which increased in MHC-II, CD80, CD86, B7-H1, and CD40 as shown in IFN-γ-stimulated cells, whereas the cells stimulated with RH antigen was shifted M2 phenotype which decreased in above surface markers as shown in IL-4-stimulated cells. NO production was decreased in cells cultured with RH or ME49 antigen. Moreover, a relative gene expression of iNOS and COX2 was decreased in cells cultured with *T. gondii* antigens (ME49 and RH strain) compared to control. However, arginase 1 that converts L-arginine into L-ornithine and urea was increased in cells cultured with *T. gondii* antigens.

Taken together, these findings indicate that a virulent RH strain of *T. gondii* converted BV-2 microglia to M2 phenotype, while avirulent ME49 strain to M1-like microglia in both *in vivo* and *in vitro* experiments. Accordingly, these results suggest that microglia in the brain infected with ME49 strain of *T. gondii* was activated to M1 phenotype increasing inflammatory cytokines and NF-kB expression. Although the accelerated inflammatory response resulted in neuron degeneration, IL-10 (mRNA and protein level) and TGF-b (mRNA level) increased at the same time modulated the excessive inflammatory response. In particular, the specific increase of arginase 1, and the decrease of COX2 and iNOS, decreased selectively NO production, and accordingly, it seems to be recovered from neurodegeneration of brain tissues. Taken together, neurotoxic NO production was decreased in the brain while maintaining M1-like microglial activation for continuous immune vigilance. This immunomodulation by *T. gondii* in the brain is a strategy in host-parasite interaction to protect parasite itself from detrimental NO production and

inflammation.

Keywords: ***Toxoplasma gondii*****, Microglia, Macrophage, Cytokine, Neurodegeneration, Hippocampus, Dentate gyrus**

CONTENTS

INTRODUCTION

Toxoplasma gondii, an obligate intracellular parasite of the phylum apicomplexa, infects approximately 30% of the world's population as well as a broad range of mammalian hosts (Kim and Weiss, 2004). Major strains of *T. gondii* are classified into three clonal lineages which differ in phenotypes, virulence in mice, and clinical sequelae (Boothroyd and Grigg, 2002). In most immunocompetent mice, the outcome of type I (RH) strain infections are uniformly virulent, whereas type II (ME49) and type III strains are benign (Reese et al., 2011; Saeij et al., 2005) . The chronic infection by the type II parasite is maintained by remodeling tachyzoites into bradyzoites to produce intracellular tissue cysts. The encystation of *T. gondii* starts and settles down by both intrinsic preprogramming within the parasite and a response to immune pressure (McCabe and Remington, 1988).

Life threatening toxoplasmosis in chronic infections includes encephalitis in immunocompromised individuals, such as those suffering from AIDS and congenital diseases, or being upon the chemotherapy and primary infection during pregnancy (Montoya and Remington, 2008). This continuous immune vigilance is essential to prevent reactivation of chronic diseases such as lethal toxoplasmic encephalitis. *T. gondii* is also a successful protozoan parasite due to its ability to manipulate the host immune system during chronic infection. For the successful parasitism, *T. gondii* usually induces asymptomatic chronic infection in most immunocompetent individuals (Flegr, 2007).

The central nervous system (CNS) is an immunologically privileged site.

Microglias, a type of glial cells, are macrophages that are resident in the brain and spinal cord. They are known to play key roles in infection and tissue injury as well as in normal tissue homeostasis. *T. gondii* tachyzoites migrated into the brain infects microglia, astrocytes, and neurons to escape the pressure of immune system. Then, intra-neuronal tachyzoites transform into slow replicating bradyzoites, which form cysts (Henriquez et al., 2009). The strategy of *T. gondii* for the intracellular parasitism manipulates immune signaling cascades in immune cells and changes phenotypes of macrophage to a favorable form for the parasitism. For example, *T. gondii* is generally controlled by a strong Th1 type cytokine response such as IL-12p70 (Plattner et al., 2008), TNF-α and IFN-γ (Denkers and Gazzinelli, 1998). During *T. gondii* infection, the recruited monocyte population converts to an anti-inflammatory phenotype in order to restrain excessive responses, and moreover, in the brain, pro-inflammatory myeloid cells convert in situ to regulatory populations that suppress T cell response (Murray and Wynn, 2011).

The generation of NO in brain-specific macrophages (microglia) induces an important antimicrobial mechanism, whereas it exemplifies a system that can result in collateral damage to tissue in the brain (Block et al., 2007). Type II strains of *T. gondii* induce NF-kB activation in bone marrow-derived macrophages by the secreted *T. gondii* dense granule protein, GRA15 (Robben et al., 2004; Rosowski et al., 2011). In contrast, type I strain of *T. gondii* preferentially induces macrophage activation toward STAT3 and STAT6 signaling pathway by the secreted *T. gondii* kinase, ROP16 (Saeij et

al., 2006; Saeij et al., 2007). In an experiment using human foreskin fibroblasts (HFFs), a high virulent Type I and a low virulent Type III *T. gondii* were potent activators of STAT3/STAT6, whereas a low virulent Type II strain did not sustain STAT3/STAT6 signaling (Saeij et al., 2007). The alternative activation of macrophages (M2 phenotype) is just promoted by STAT6 and PPARγ transcription factors (Odegaard et al., 2007). A key factor in M1 macrophages-mediated cytotoxicity, NO, is produced by the oxidative change of L-arginine to L-citrulline by NO synthase (NOS) (Alderton et al., 2001). The inducible form of NO synthase, iNOS, is strongly regulated by several cytokines, TNF-α and IFN-γ as inducible cytokines and TGF-β, IL-4, IL-10, and IL-13 as inhibitory cytokines (Hesse et al., 2001). Inflammatory stimuli generally induce up-regulation of iNOS in microglial cells and results in a high production of NO (Brown and Bal-Price, 2003). Brain tissues are extremely susceptible to the noxious effects of NO which is toxic substance causing a wide range of neurodegenerative and demyelinating diseases in the CNS (Smith and Lassmann, 2002). However surprisingly, neurodegeneration is not common finding during a type II *T. gondii* infection in immunocompetent Alzheimer's disease (AD) mice (Jung et al., 2012). After *T. gondii* (ME49 strain) infection, anti-inflammatory cytokines (TGF-β1 and IL-10) in Tg2576 AD mice were increased in the brain and these series of immune responses are important to regulate NO production causing tissue injury and β-amyloid plaque deposition in the cortex and hippocampus of Tg2576 mouse brains (Jung et al., 2012). However, it has not been addressed on what is the characteristic of immune mechanism regulating microglial cell

phenotype and NO production in mouse brain for a long time after *T. gondii* infection, and the immune modulation resulted in our previous study using Tg2576 mice can be reproducible in normal mice.

The present study aimed to examine the immunopathology attributed by *T. gondii* infection in normal C57BL/6 mouse brain, the orientation of microglial activation which is induced by different *T. gondii* strains, type I (virulent; RH) and type II (avirulent; ME49), and how *T. gondii* modulates the activation of microglia to be a chronic infection without harmful events in the brain. Ultimately, we should determine why neurodegeneration is not common finding during a type II *T. gondii* infection.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the Guidelines for Animal Experiments issued by the Ethics Committee of Seoul National University. The study protocol was approved by the Ethics Committee on Animal Experiments, Seoul National University (Permit Number: SNU-110315-5). All surgeries were performed under anesthesia, and all efforts were made to minimize animal suffering.

Toxoplasma gondii infection

T. gondii, ME49 strain, was maintained by injecting intraperitoneally cysts isolated from the brains of infected C57BL/6 mice (Orient Bio Animal Center, Seongnam, Gyeonggi-do, Korea). To infect C57BL/6 mice, the brains of mice infected with ME49 strain were harvested at 3 months post-infection and minced to isolate cysts. Cysts were then isolated under a microscope and 10 cysts were inoculated orally into each experimental mouse. Infected mice were raised under SPF conditions at Seoul National University College of Medicine.

In vitro Isolation of *T. gondii* tachyzoites in Vero cell culture

T. gondii RH tachyzoites were recovered from BALB/c mice infected intraperitoneally with *T. gondii* 4 days earlier with PBS (pH 7.2). To obtain

ME-49 tachyzoites, ME-49 cysts were intraperitoneally inoculated in BALB/c mice injected with 4 mg methylprednisolone acetate (Shinpoong Co., Seoul, korea). Six to eight days later PI, tachyzoites were harvested by washing the peritoneal cavity with PBS (pH 7.2). Both *T. gondii* tachyzoites were washed with PBS and cultured in Vero cells grown in complete RPMI 1640 (Welgene, Korea) media supplemented with 100 ug/ml of penicillin (Gibco), 100 ug/ml of streptomycin (Gibco), and 5% new calf serum (Lonza), The culture flasks were cultured at 37°C in 5% CO_2 incubator.

Hematoxylin and eosin staining

Brain tissues were embedded in paraffin and coronally sectioned at 10 μm through the hippocampus, mounted, and stained with hematoxylin and eosin. They were then dehydrated using a graded alcohol series, cleared in xylene, and coverslipped in Canadian balsam (Caedax, Merck). Neuronal degeneration in hippocampus was determined by detecting eosinophilic neurons under a light microscope (Olympus PM-20; Olympus, Tokyo, Japan). To count degenerative cells in the hippocampal dentate gyrus, photomicrographs were acquired with a digital camera (Leica DFC 280, Leica Microsystems, Wetzlar, Germany) attached to a microscope (BX-51; Olympus, Japan). Degenerative cells were counted using Image J software (version 1.46, National Institutes of Health, Bethesda, MD)

Immunohistochemistry

T. gondii-infected mouse brains were formalin fixed and paraffin-embedded.

Sections (4 μm: Probe-On-Plus Slides; Fisher Scientific) were de-paraffinized and rehydrated in xylene, 100%, 95%, 80%, and 70% alcohol, and DW washing. Sections were incubated overnight at 4˚C with either Iba1 (Wako Chemicals USA, Inc., Richmond, VA), p-NF-kB p65 (Santa Cruz Biotechnology, Santa Cruz, CA), or Iba1 antibody (1:1000; Wako Chemicals) for 60 min, then incubated with HRP-conjugated anti-Rabbit IgG secondary antibody (K4003, DAKO) for 30 min and treated with a diaminobenzidine-chromogen substrate solution (K3468, DAKO) substrate for staining. Slides were counterstained with hematoxylin ((Sigma-Aldrich MHS-16) and observed using light microscopy.

***In vitro* activation of BV-2 cells using recombinant cytokines**

A murine microglial cell line, BV-2, was cultured in Dulbecco's modified essential medium (DMEM; Applied Scientific, San Francisco, CA) containing 5% heat-inactivated fetal calf serum (Hyclone, Ogden, UT, USA), 4 mM L-glutamine, 0.2 mM penicillin, 0.05 mM streptomycin, and 20 mM HEPES (Sigma, St. Louis, MO, USA) at 37 °C in a CO_2 incubator. Cells were then washed twice with serum-free DMEM, and incubated for 24 or 30 h in 6 well culture plates (SPL Lifescience, Pocheon-si, Gyeonggi-do, Korea) with either 50 ng/ml lipopolysaccharide (LPS; Sigma), 20 ng/ml IL-4 (Prospec-Tany Technogene LTD, Rehovot, Israel), 40 ng/ml IL-10 (PeproTech, Rocky Hill, NJ, USA), 30 ng/ml interferon-gamma (PeproTech), or *T. gondii* (RH and ME49) tachyzoites (1:5, ET ratio). The cultured cells were then harvested for real-time PCR and flow cytometry analysis, and culture supernatants were

harvested for the determination of nitrite content.

RT-PCR

Total RNA of *T. gondii*-infected mouse brain was isolated using the RNeasy Mini kit (QIAGEN, Hilden, Germany), and then PCR was performed using MG Taq DNA polymerase (Macrogen, Seoul, South Korea). Primer sequence is as follows. GAPDH (Primer Bank ID 6679937a1) forward, 5′-AGG TCG GTG TGA ACG GAT TTG-3′ and reverse, 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′ (123 bps). *T. gondii* 18S rRNA primer was designed by NCBI Primer-blast; *T. gondii* 18S rRNA forward, 5'- CGG GTA ADG GGG AAT TAG GG-3' and reverse, 5'-AGG TGC AGG AGA AGT CAA GC-3' (722 bps). RT-PCR was performed for 35 cycles with an annealing temperature of 55°C, and the products were analyzed by 1% agarose gel electrophoresis.

Real-time PCR

Total RNAs from brain tissue samples and BV2 cells were isolated using RNeasy kit (QIAGEN). All samples were reverse transcribed using RT premix (Elpisbiotech, Daejeon, Korea). Real-time PCR was performed using the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA) and SYBR green (Enzynomics, Cheongju, Korea) was used to detect amplification products. The reaction condition is as follows; Initial denaturation at 50°C for 5 min and 95°C for 10 min, 40 amplification cycles [denaturation at 95°C for 10 s and annealing at 60°C for 30 s], followed by one cycle at 72°C for 5 min. Specific

amplification was verified by melting curve analysis and also by fractionation of PCR products on 3% agarose gel to identify fragment size (data not shown). Fold levels represent the mean (± SEM) of triplicate reactions. Data analysis was performed using iQ^{TM}5 optical system software (Bio-Rad). Primer sequences and amplicon sizes are shown in PrimerBank (http://pga.mgh.harvard.edu-/primerbank/); GAPDH (PrimerBank ID 6679937a1) forward, 5′-AGG TCG GTG TGA ACG GAT TTG-3′ and reverse, 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′ (123 bp); Arg1 (PrimerBank ID7106255a1) forward, 5′- CTC CAA GCC AAA GTC CTT AGA G -3′ and reverse, 5′- AGG AGC TGT CAT TAG GGA CAT C -3′ (185bp); Nos2 forward, 5-GTT CTC AGC CCA ACA ATA CAA GA-3’ and reverse 5’-GTG GAC GGG TCG ATG TCA C -3’ (127bp); Ptgs2 forward, 5’-TGT GAC TGT ACC CGG ACT GG-3’ and reverse 5’-TGC ACA TTG TAA GTA GGT GGA C-3’ (233bp).

Flow cytometry analysis

The following antibodies were used for flow cytometry: CD80-FITC (BD Pharmingen), CD86-PE (BD Pharmingen), PD-L1-FITC (BD Pharmingen), PD-L2-PE (BD Pharmingen) and MHC II-APC (BD Pharmingen). All staining process was carried out according to the supplier's protocol (BD Pharmingen) in PBS containing 1% BSA and 0.1% Sodium Azide, and with each antibody. Samples were analyzed by a FACS Caliber flow cytometer (BD Immunocytometry Systems) with forward/side scatter gates set to exclude nonviable cells, and the data were analyzed using the FlowJo

software (Tree Star, Ashland, OR).

Nitric oxide production in primary cultured microglia and BV-2 cells

Culture supernatants of primary cultured or BV-2 microglial cells were assayed for nitrite content, which reflects NO production, using Griess reagent (0.1% naphthylethylene diamine dihydrochloride and 1% sulfanilamide containing 2.5% phosphoric acid in equal volumes), as described previously (Jung et al., 2012).

Multiplex analyses of cytokines with the Bio-Plex system

Cytokine Profile Analysis was measured using a commercial kit, BIO-Plex cytokine assay (BIO-Plex Mouse 3-Plex Assay, Bio-Rad Laboratories, CA, USA), from the brain sample (soluble fraction of homogenate) of *T. gondii*-infected C57BL/6 mice at 0 (PBS-injected control group), 1, 3, 6, 9 and 12 weeks PI. Each set of beads was combined with a monoclonal antibody raised against GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, and TNF-α. The brain samples were analyzed by using a Bio-Plex cytokine reagent kit with Bio-Plex mouse cytokine 20-Plex Panel in the Bio-Plex 200 system (Bio-Rad Laboratories) in triplicate as directed by the manufacturer. Raw data (mean fluorescent intensities) were analyzed by Bio-Plex Manager Software (Bio-Rad Laboratories) to obtain concentration values. The *t*-test function in Bio-Plex Data Pro™ software was used to perform statistical analysis on sample data.

Microarray and analysis

Total RNA of *T. gondii*-infected mouse brain was isolated using the RNeasy Mini, RNA isolation kit (Qiagen) according to the manufacturer's protocol. The samples were pooled (n=3). Microarray analysis was performed by Macrogen Inc. (Seoul, Korea) using an Illumina MouseRef-8 v2 Expression BeadChip (Illumina, San Diego, CA, USA) following manufacturer's recommendations. Briefly, the total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, TX, USA) to yield biotinylated cRNA. Biotinylated cRNA were prepared from 0.55 ug total RNA using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin,TX). Following fragmentation, 0.75 ug of cRNA were hybridized to the Illumina HumanHT-12 Expression Beadchip according to the protocols provided by the manufacturer. Arrays were scanned using the Illumina Bead Array Reader Confocal Scanner Array data export processing, and analysis was performed using Illumina GenomeStudio v2011.1 (Gene Expression Module v1.9.0). The data were analyzed by R statistical language v. 2.15.1. Hierarchical cluster analysis was performed using Permut Matrix EN software.

Statistical analysis

All statistical analyses were carried out using the Microsoft office Excel (Version 2010). The Student's unpaired two-tailed *t*-test was used for this experiment. Differences between groups were considered significant when $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, $****p < 0.001$, $*****p < 0.0005$. Data are presented as means ± SD.

RESULTS

Degeneration of neuronal cells in mice brain infected with ME49 *T. gondii* strain

The body weight of C57BL/6J male mice (n=6, 7-wk-old) infected with 10 cysts of *T. gondii* (ME49) was measured every week, and mice were sacrificed at weeks 0, 1, 3, 6, 9, and 12 PI. To determine the neuronal cell damage caused by *T. gondii* infection, histopathologic changes in the hippocampal region were examined by hematoxylin and eosin (H-E) staining. As shown in Figure 1 A, no cell death was observed in the dentate gyrus of uninfected mice. However, *T. gondii* infection resulted in neuronal cell death (eosinophilic neurons) in the dentate gyrus at weeks 1 (74%), 3 (83%), 6 (80%), and 9 (50%) PI suggesting the decrease in the neuronal degeneration of brain cells after 9-wk PI (Fig. 1A). Neuronal degeneration was remarkably reduced at 12 weeks PI (28%), (Fig. 1C).

The infiltration of microglial cells was increased from 3 weeks PI (Fig. 1B). Iba-1-positive cells (shown in brown color) were increased from 3 weeks after infection in hippocampal formation of *T. gondii*- infected mouse brain. Iba-1-positive cells (shown in brown) became thicker and larger at 3 weeks PI compared to cells at weeks 0 and 1 PI (Fig. 1B).

Both groups showed a gradual increase in body weight during the experimental period. However, mice with type II parasites (ME49) showed a less body weight gain compared to the control mice. During the first three weeks, infected mice did not show any change in body weight compared to

control mice. A significant transient body weight reduction was observed in the infected mice at 3 weeks PI (Fig. 1D). Except the fluctuation of body weight in the early stage of infection, it was no longer fluctuated with continuing increase in both groups.

This study shows that the infected mice with developing brain cysts in an acute stage of infection (at weeks 1, 2, and 3 PI) were characterized by weight loss and neuronal cell death (Fig. 1C-D). However, a chronic phase (at weeks 3, 6, 9, and 12 PI) of toxoplasmosis was a period of recovery from both the neuronal cell death and body weight loss.

Pro-inflammatory cytokines were increased in the brain with a chronic toxoplasmosis

To investigate the kinetics of immune response in type II (ME49) *T. gondii*-infected mouse brain, cytokines and mRNA levels in the brain cortex were analyzed using Bio-Plex cytokine array kit and Microarray at weeks 0, 1, 3, 6, 9, and 12 PI. The value of each cytokine and gene expression at each experimental point was compared to that of PBS-injected control mice (Fig. 1A). The result of microarray was processed by a cluster analysis to identify gene expression at each time point studied. Considering the 58 cytokines-related genes, it was observed that many genes related with inflammation were increased (Figure 2A).

Among the cytokines, TNF-α, IL-12p70, IFN-γ, IL-4, GM-CSF, IL-6, and IL-10 showed a similar kinetic pattern during experimental period suggesting a rapid increase during weeks 0 to 3 (or 6) PI and a decrease during weeks 3

(or 6) to 12 PI (Fig. 2B & C). In this result, IL-4 production is remarkably increased at week 3 PI with statistically significance and after then slowly decreased (Fig. 2B).

In this result, all cytokines studied by cytokine array were increased at the early stage of *T. gondii* infection regardless of Th1 or Th2 cytokines (Fig. 2B & C). However, pro-inflammatory cytokines, GM-CSF, IL-6, IFN-r, IL-12, and TNF-α**,** were the highest at 6 weeks PI during experimental period. After then, the concentration of pro-inflammatory cytokines was slightly decreased and maintained higher than cytokine levels in the early stage of infection at weeks 0 and 1 PI.

NF-kB activation is increased when *Toxoplasma* was detected in the brain.

To investigate the increase of NF-kB in *T. gondii*-infected mouse brain, the dentate gyrus region of the hippocampus was examined by immunohistochemistry using a phosphorlyated-NF-kB p65 antibody (Fig. 3). When *T. gondii* cysts were orally infected, the migration of *Toxoplasma* into the brain was appeared at 3 week PI (Fig. 3B). The time period for NF-kB activation in the brain was corresponding when *T. gondii* appeared in the brain (Fig. 3A).

Toxoplasma* mediates the change of microglial cell phenotype in *in vivo* and *in vitro

Surface markers destined phenotype of microglial cells were determined in ME49-infected mouse brain (Fig. 4A). The infected mouse brain was

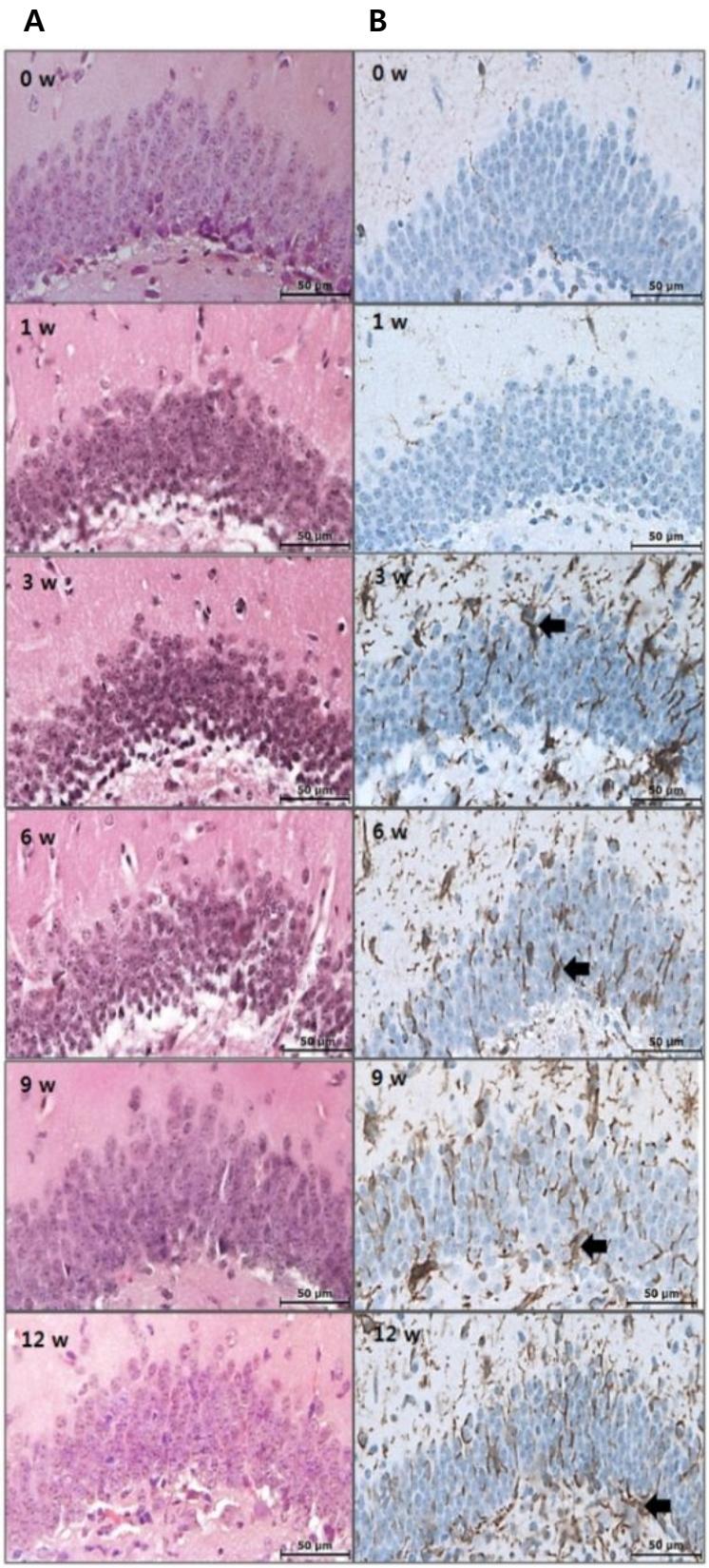
harvested at weeks 1, 3, 6, 9, 12, 24, and 36 PI, and then performed by microarray analysis (Fig. 4A). The result shows that MHC class II antigens (H2-Eb1, H2-Aa, and H2-Ab1), CD80, CD86, B7-H1, and CD40 were increased in the *T. gondii*-infected mouse brain (Fig. 4A). Moreover, to determine the change of microglial cell phenotype according to *Toxoplasma* strains (a virulent RH or an avirulent ME49 strain), BV2 cells were cultured with antigen of RH or ME49 *T. gondii* tachyzoites. The phenotype was determined by data in microarray and flow cytometry analysis (FACS) (Fig. 4). Markers determining the phenotype of microglia cells are as follows: MHC Class II, CD80, CD86, B7-H1, B7-DC, and CD40 (Fig. 4B). To determine the change of above surface markers, BV2 cells were cultured for 24 hours with either culture condition of untreated (control), or treated with recombinant IL-4, recombinant IFN-γ, RH tachyzoites (1:5 ET ratio), and ME49 tachyzoites (1:5 ET ratio) (Fig. 4B). The treatment of IFN- γ induced the expression of surface marker toward classically activated macrophages, whereas the treatment of IL-4 resulted in alternative macrophage activation. BV2 cells treated with *T. gondii* RH tachyzoites also showed a similar pattern when treated with recombinant IL-4 (Fig. 4B). In MHC II, an classically activated microglial cell marker, expression, BV2 cells cultured with type I (ME49) tachyzoite antigen or IFN-γ induced up-regulation of MHC II compared with treatment of type I (RH) tachyzoite antigen or recombinant IL-4 (Fig. 4B). Besides, the treatment of ME49 tachyzoite antigen or recombinant IFN-γ increased both CD80 and CD86 (Fig. 4B). Furthermore, CD40 were also up-regulated on BV2 cells treated with ME49 tachyzoite

antigen or recombinant IFN-γ, whereas RH- or IL-4-treated BV2 cells was down-regulated in CD40 expression (Fig. 4B). PD-L1 (B7-H1), a marker which is expressed on classically activated microglia, was remarkably increased on BV2 cells stimulated with IFN-γ and ME49 antigen. On the contrary, PD-L2 (B7-DC), an alternatively activated microglia marker, was increased on BV2 cells treated with IL-4 and RH antigen (Fig. 4B).

T. gondii antigens decreased iNOS gene expression as well as nitric oxide (NO) production in BV2 microglial cells

The defense mechanism of macrophages against pathogen is usually depended on metabolites secreted from classically activated macrophages. To investigate NO production regulated by treatment of inflammatory cytokine and *T. gondii* antigen, BV2 microglia cells were cultured with recombinant IL-4, recombinant IFN-γ, type I (RH) *T. gondii* antigen, or type II (ME49) tachyzoite antigen. As expected, BV2 cells stimulated with 30 ng/ml IFN-γ increased nitrite production, whereas, the addition of IL-4 or *T. gondii* antigen (RH or ME49) decreased nitrite production despite the addition of IFN-γ (Fig. 5A). In other words, cells incubated with ME49 *T. gondii* antigen for 6 hours before it was treated with IFN-γ resulted in significantly a less nitrite production compared to the incubation with RH antigen (Fig. 5A). To determine the mechanism which NO production from microglial cells was decreased by the addition of ME49 *T. gondii* antigen, the level of Arg1 mRNA expression was examined by real-time PCR (Fig. 5B). Arg1 was more increased by the addition of not RH but ME49 antigen. At this time, cox-2

(cyclooxygenase-2) and iNOS mRNA levels were decreased by ME49 antigen as well as RH antigen (Fig. 5B). These results suggest that *Toxoplasma* could use the arginase metabolic pathway with a priority to promote its own growth within cells. Above results were also confirmed by the increased mRNA level of Arg1 in the brain tissue infected with ME49 *T. gondii* (Fig. 5C).


A
B
0 w
1 w
3 w
6 w
9 w
12 w
50 μm

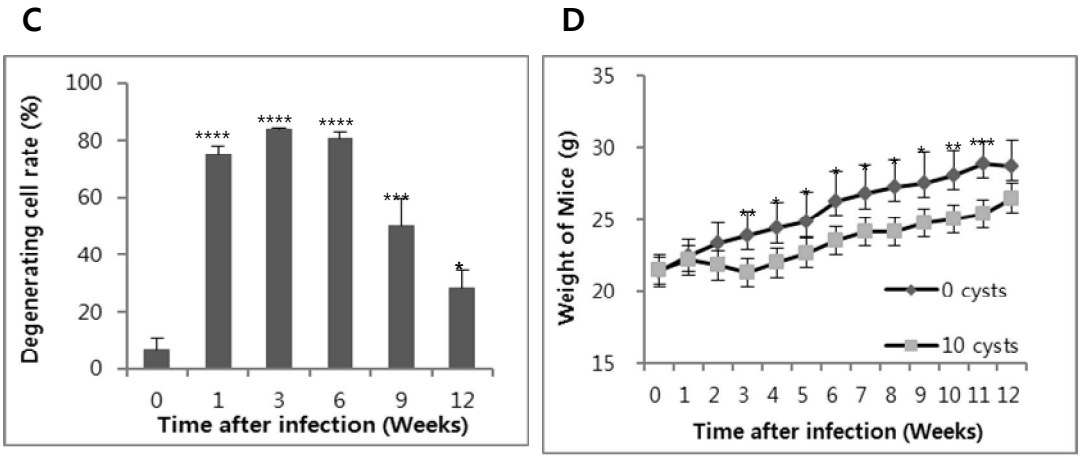
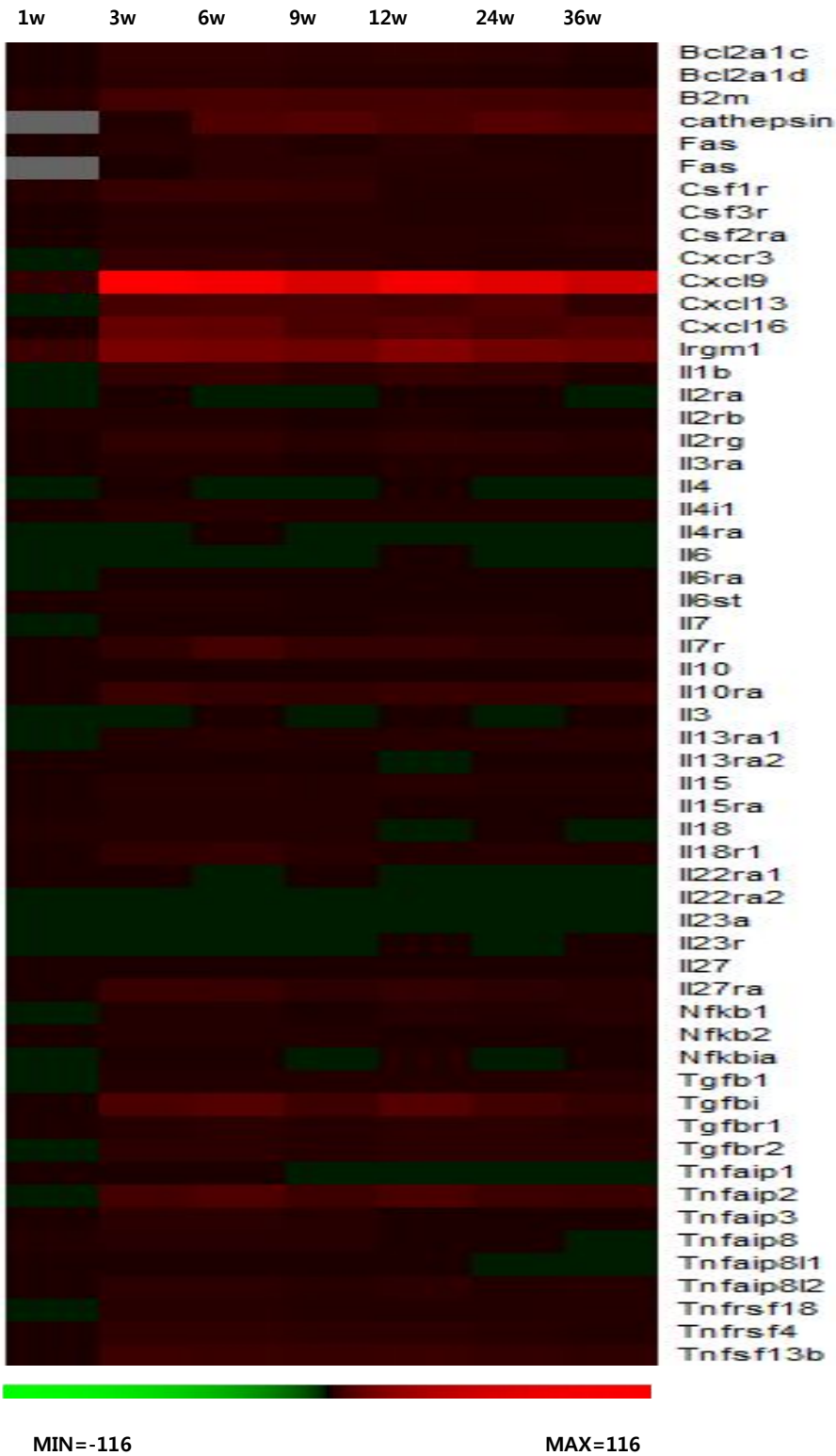


Figure 1. *T. gondii* infection resulted in a spontaneous neuronal degeneration and microglia activation in the brain, and the reduction of body weight in C57BL/6 mice.

Histological changes in the hippocampal formation of the brain were observed by H&E staining for 12 weeks after 10 cysts of *T. gondii* (ME49) were infected. (A) Neuronal degeneration in the dentate gyrus was compared between *T. gondii*-uninfected (0 week) and infected mice (at weeks 1, 3, 6, 9, and 12). (B) The distribution of microglia in the dentate gyrus was examined by immunostaining with Iba1 antibody. (C) The rate which cells were degenerated in the dentate gyrus of hippocampal formation is represented by percentages of degenerative cells among all cells. (D) Body weight variation was examined during the infection period. Body weights of postnatal 7 weeks mice (n=6) were measured every week after 10 cysts of *T. gondii* (ME49) were infected. (*$p < 0.05$, **$p < 0.01$, *** $p < 0.005$, ****$p < 0.0001$)

A


1w
3w
6w
9w
12w
24w
36w
Bcl2a1c
Bcl2a1d
B2m
cathepsin
Fas
Fas
Csf1r
Csf3r
Csf2ra
Cxcr3
Cxcl9
Cxcl13
Cxcl16
Irgm1
Il1b
Il2ra
Il2rb
Il2rg
Il3ra
Il4
Il4i1
Il4ra
Il6
Il6ra
Il6st
Il7
Il7r
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Il3
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Il13ra2
Il15
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Il22ra1
Il22ra2
Il23a
Il23r
Il27
Il27ra
Nfkb1
Nfkb2
Nfkbia
Tgfb1
Tgfbi
Tgfbr1
Tgfbr2
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Tnfaip2
Tnfaip3
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Tnfsf13b
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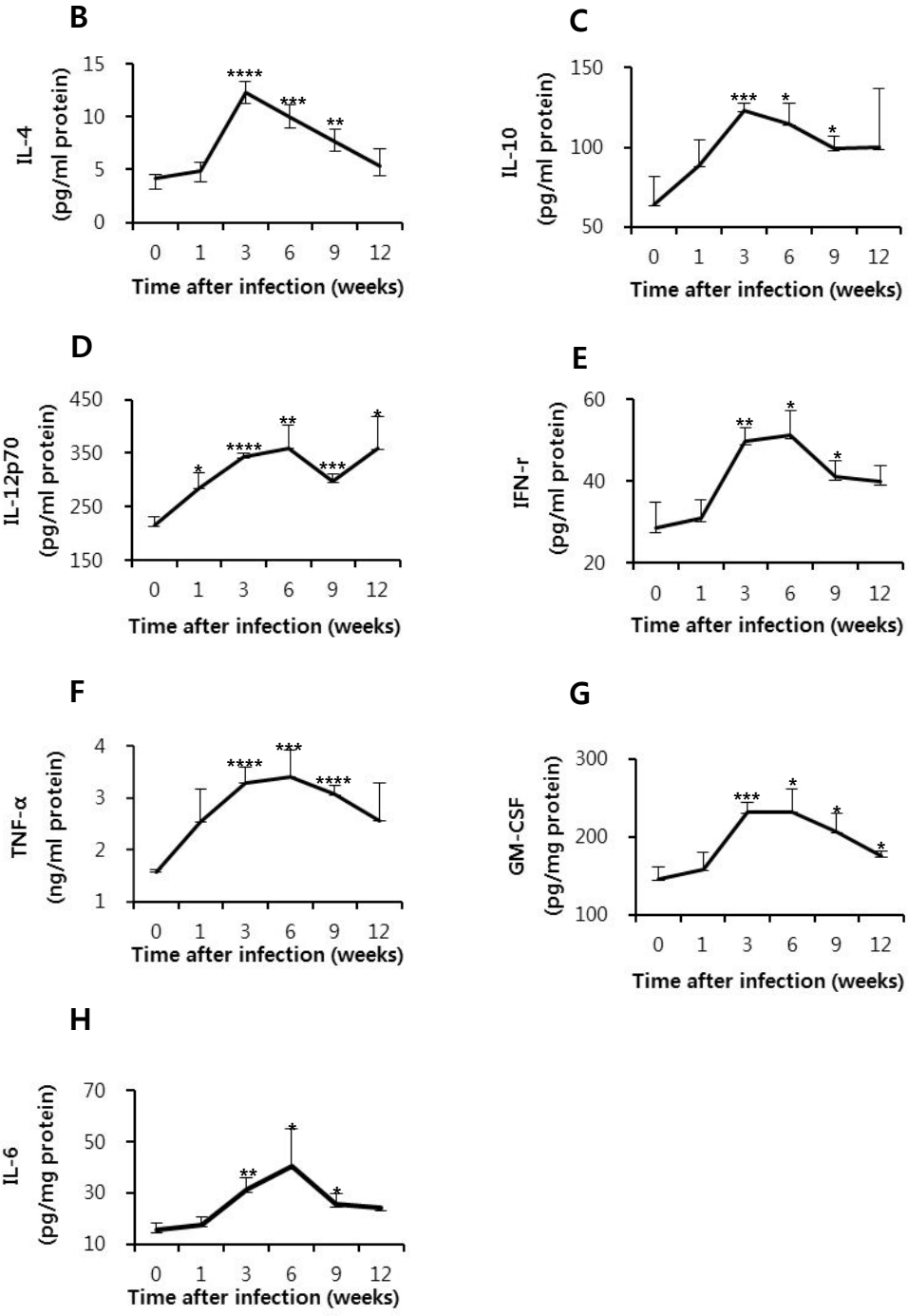
MAX=116


B
IL-4
(pg/ml protein)
15
10
5
0
****
***
**
0 1 3 6 9 12
Time after infection (weeks)
C
IL-10
(pg/ml protein)
150
100
50
***
*
*
0 1 3 6 9 12
Time after infection (weeks)
D
IL-12p70
(pg/ml protein)
450
350
250
150
*
****
**
***
*
0 1 3 6 9 12
Time after infection (weeks)
E
IFN-r
(pg/ml protein)
60
40
20
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0 1 3 6 9 12
Time after infection (weeks)
F
TNF-α
(ng/ml protein)
4
3
2
1
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0 1 3 6 9 12
Time after infection (weeks)
G
GM-CSF
(pg/mg protein)
300
200
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0 1 3 6 9 12
Time after infection (weeks)
H
IL-6
(pg/mg protein)
70
50
30
10
**
*
*
0 1 3 6 9 12
Time after infection (weeks)

Figure 2. Kinetics of immune gene expression and cytokine level in *T. gondii* (ME49)-infected C57BL/6 mouse brain. (A) The red or green colors in pooled microarray indicate up- or down-level in gene expression, respectively. (n=3) (B-H) Kinetics of cytokine levels (IL-4, IL-6, IL-10, IL-12p70, GM-CSF, IFN-γ and TNF-α) for 12 weeks PI in *T. gondii*-infected mouse brain were analyzed by a Bio-Plex cytokine array kit (n=3). The cytokine levels are represented as a mean ± SD. (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.005$, ****$p < 0.001$).

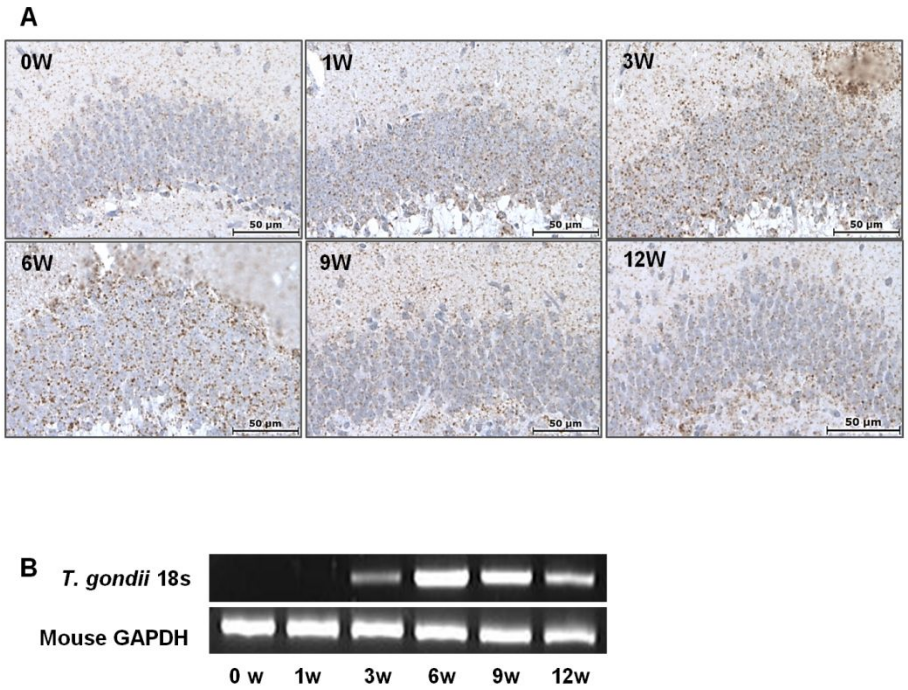

Figure 3. Immunohistochemistry (IHC) of the dentate gyrus region using a phosphorylated-NFkB p65 antibody, and RT-PCR analysis of *T. gondii* 18S rRNA gene expression in *T. gondii* (ME49)-infected mouse brain. (A) Images show a significant difference in the amount of NF-κB expression during 12 weeks PI in dentate gyrus region of the hippocampus. (B) *T. gondii* 18S rRNA gene expression was detected from 3 weeks after *T. gondii* were infected. The amplified products were visualized by EtBr staining after 1% agarose gel electrophoresis. The results of suboptimal amplification cycles (35 cycles) are shown.

A

Accession No	GeneSymbol	1/N	3/N	6/N	9/N	12/N	24/N	36/N
NM_010382.2	H2-Eb1	3.64	51.25	53.68	47.28	50.58	41.96	62.81
NM_010378.2	H2-Aa	2.35	21.50	24.60	22.14	11.83	10.45	8.97
NM_207105.2	H2-Ab1	5.50	71.40	80.51	69.77	69.77	54.67	65.02
NM_001042605.1	Cd74	7.05	89.10	93.47	86.00	78.08	67.35	86.00
NM_009853.1	Cd68	-1.027	2.9148	3.173	2.4679	3.3767	2.7615	4.5406
NM_009855.2	Cd80	-1.04	1.16	1.19	1.08	1.18	1.08	1.14
NM_009856.1	Cd83	-1.30	1.12	1.13	1.22	1.13	1.10	1.18
NM_019388.2	Cd86	1.137	6.4038	7.0529	5.038	6.1993	5.7408	3.1626
NM_021893.2	B7-H1	6.30	19.51	21.38	18.91	23.01	22.08	16.30
NM_021396.1	B7-DC	1.07	1.19	1.21	1.08	1.05	1.09	1.10
NM_170702.2	Cd40	1.11	5.73	4.40	3.60	4.16	3.66	2.04
NM_011346.1	L-selectin(CD62L)	1.14	1.98	2.25	1.55	1.89	1.92	1.59
NM_009151.2	Selplg/CD162	1.36	2.59	2.38	2.03	1.96	1.93	1.85
NM_053094.1	Cd163	1.4081	1.0452	-1.013	1.154	-1.012	-1.075	-1.075
NM_010493.2	Icam1	1.37	5.47	4.32	3.78	4.46	4.16	4.38
NM_009917.2	Ccr5	-1.46	2.60	3.27	2.26	3.65	2.75	3.04
NM_007719.2	Ccr7	1.1078	1.251	1.2126	1.0979	-1.024	1.019	1.0322
NM_011905.2	Tlr2	-1.13	7.61	6.51	5.32	6.31	5.49	4.83
NM_021297.2	Tlr4	-1.10	1.63	1.59	1.39	1.68	1.35	1.52
NM_010851.2	Myd88	-1.015	2.7038	2.6132	2.0208	2.4531	1.9865	1.693

B

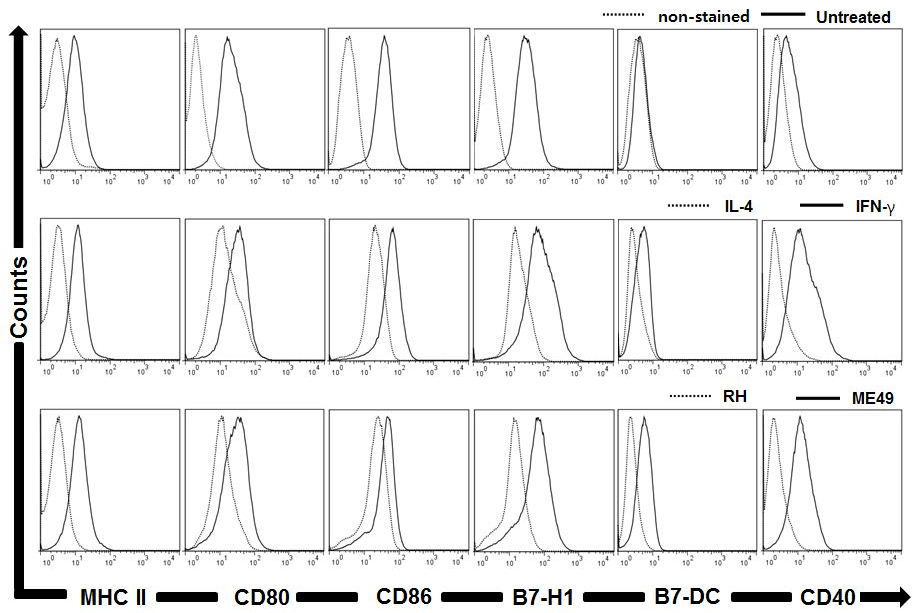

Figure 4. Microarray result in *T. gondii*-infected mouse brain and FACS analysis of BV-2 cells for the change of microglia phenotype.

(A) Microglia surface gene expression in type II (ME49) *T. gondii*-infected mouse brain was compared to it in non-infected control mice. The fold difference of pooled sample (n=3) are shown in the ratio of PBS-injected group versus *T. gondii*-infected groups (at 1, 3, 6, 9, 12, 24, and 36 weeks). Red colors indicate the increase of mRNA expression, whereas green colors indicate the decrease of gene expression. (B) BV-2 cells were cultured with IL-4 (20 ng/ml), IFN-γ (20 ng/ml), RH (1:5 in ET ratio), and ME49 tachyzoites (1:5 in ET ratio). After the culture for 24 h, cells were stained with antibodies of surface markers related with microglia activation such as MHC Class II, CD80, CD86, B7-DC, B7-H1, and CD40, and examined by flow cytometry.

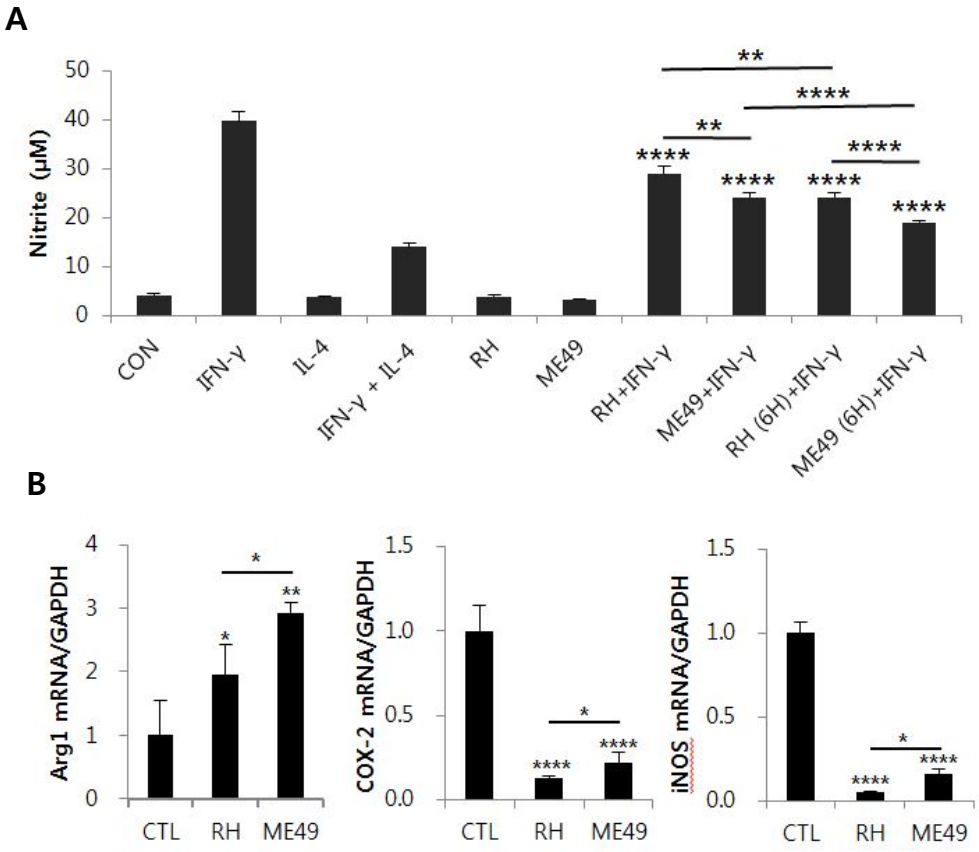

Figure 5. NO production in BV-2 cells cultured with cytokines or *T. gondii* tachyzoite (RH and ME49). In addition, gene expressions related with microglial cell activation were examined by real-time PCR and microarray analysis on the brain cortex during *T. gondii* infection. (A) BV2 cells were incubated for 24 h in the presence of IFN-γ (30 ng/ml), IL-4 (20 ng/ml), *Toxoplasma* RH (1:5, ET ratio), or ME49 (1:5, ET ratio), and then, the culture supernatants were analyzed for nitrite concentration. Data represent mean ± SD. (B) Relative abundance of COX-2, *iNOS,* and *ARG1* transcripts in Control (left), RH (center), and ME49 (right) cultured with BV2 cells for 24 hr. (C) Gene expressions related with microglia cell phenotype

was analyzed by cDNA microarray. The red characters indicate the increased gene expression; (-) indicates a decreased expression. ($p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.005$, $p^{****} < 0.001$)

C

Accession No	GeneSymbol	1W	3W	6W	9W	12W	24W	36W
NM_010927.3	Nos2	−1.02	1.14	1.10	1.13	1.09	1.03	−1.06
NM_011198.3	Ptgs2	−1.06	−1.03	−1.05	−1.10	−1.07	−1.08	−1.05
NM_007707.2	Socs3	1.01	3.24	3.47	2.87	4.04	2.71	1.44
NM_009283.3	Stat1	2.15	9.26	9.64	8.25	11.12	10.49	9.26
NM_213659.2	Stat3	1.67	3.35	3.99	2.79	2.28	1.81	1.41
NM_007719.2	Ccr7	1.11	1.25	1.21	1.10	−1.02	1.02	1.03
NM_011888.2	Ccl19	1.10	2.53	2.40	1.80	2.27	1.84	1.85
NM_011337.2	Ccl3	1.02	2.51	2.04	1.86	1.70	1.67	1.49
NM_013652.2	Ccl4	1.11	14.67	12.56	9.20	8.04	5.91	2.78
NM_013653.2	Ccl5	1.26	43.00	43.24	25.92	12.22	7.00	5.91
NM_021274.1	Cxcl10	3.25	30.33	21.92	17.81	22.35	17.16	10.76
NM_008518.1	lymphotoxin B	−1.01	2.11	2.27	1.53	1.64	1.45	1.68
NM_016850.2	Irf7	2.43	9.40	6.16	5.37	5.12	4.52	4.47
NM_009421.3	Traf1	1.06	1.96	2.18	1.65	1.58	1.40	1.34
NM_008390.1	Irf1	4.67	19.10	17.16	13.19	15.21	15.14	7.96
NM_007482.2	Arg1	1.12	2.21	3.38	1.74	1.95	1.16	1.24
NM_009896.2	Socs1	1.16	1.45	1.28	1.35	1.16	1.21	1.11
NM_009284.2	Stat6	1.03	1.38	1.47	1.41	1.20	1.33	1.20
NM_009892	Chi3l3	1.14	1.99	1.01	1.03	1.94	1.24	1.14
NM_145126.1	Chi3l4	−1.02	−1.08	−1.05	−1.13	−1.04	−1.09	−1.14
NM_010796.2	Mgl1	1.04	1.01	1.03	−1.04	1.05	1.03	1.07
NM_145137.1	Mgl2	−1.08	−1.15	−1.19	−1.11	−1.13	−1.08	−1.19
NM_019577.4	Ccl24	−1.02	1.04	1.01	1.05	1.07	−1.08	1.01
NM_008969.3	Ptgs1	1.08	1.10	1.06	1.02	1.06	1.14	−1.01
NM_013674.1	Irf4	1.16	1.64	1.79	1.79	2.01	2.02	1.96
XM_984804.1	Clec7a	−1.01	−1.11	1.03	−1.03	−1.03	1.01	1.06
NM_020509.3	Retnla (Fizz1)	−1.02	−1.09	−1.00	1.03	−1.00	−1.04	1.06
NM_011332.2	Ccl17	1.29	1.33	1.27	1.31	1.36	1.13	1.14
NM_011146.2	PPPARg	1.06	1.05	1.08	1.01	1.06	1.11	1.00

DISCUSSION

A chronic infection with *T. gondii*, ME49 strain, can cause severe pathology (encephalitis) in the brain of susceptible mice (Suzuki et al., 1989; Suzuki and Joh, 1994). C57BL/6 mice infected orally with ME49 strain induced necrosis caused by IFN-γ in the ilea in the early stage of infection (Liesenfeld et al., 1996). During the penetration of host tissue by *T. gondii*, the parasite is actively across biological barriers such as the intestine, the blood-brain barrier (BBB), and the placenta (Barragan and David Sibley, 2003). Those biological barriers are to regulate the permeability of fluids and thus maintain a homeostatic balance by allowing separation of apical and basal fluid compartments (Barragan and David Sibley, 2003). If the BBB is disrupted, it allows leukocytes to traverse the vessel wall, and then, inflammatory cells and *Toxoplasma* increasingly migrate into the CNS (Barragan and David Sibley, 2003). In general, the invasion of immune cells across the BBB is highly restricted and carefully regulated (Barragan and David Sibley, 2003). An invasion of activated white blood cells can create a predominantly proinflammatory local environment in the CNS, leading to immune-mediated pathology of the nervous tissue (Webb and Muir, 2008). *T. gondii* actively infects leukocytes, thus trafficking of leukocytes can contribute to dissemination of intracellular parasites by a 'Trojan horse'-type mechanism (Barragan and David Sibley, 2003). Under this circumstance, *T. gondii* migrated into the brain may give rise to the inflammatory response in CNS. However, there is little-or-no vascular response to CNS inflammation,

and therefore no recruitment of neutrophils. Only local, resident microglia show a rapid response becoming “activated”, but nonlocal microglia and other macrophages do not migrate into the inflamed area until several days after inflammation begins (Brown and Bal-Price, 2003). Before *T. gondii* (ME49) migrates into the brain, the early immune control of the host against *T. gondii* requires the production of pro-inflammatory cytokine, IL-12 which stimulates natural killer (NK) cells and the production of IFN-γ in the spleen (Hunter et al., 1994). *Toxoplasma* actively infects leukocytes, and then can be disseminated by the infected leukocytes or a direct penetration via paracellular route through BBB into the brain (Barragan and David Sibley, 2003). In the CNS, *Toxoplasma* antigens are presented by microglia and brain endothelial cells, and it follows a cascade inflammatory reaction (Webb and Muir, 2008). In this situation, brain endothelial cells and microglia produce inflammatory cytokines and nitric oxide, and more and more, resulted in BBB dysfunction and neurodegeneration by an excessive amount of NO (Guix et al., 2005; Webb and Muir, 2008). Our microarray result showed the increase of IL-12, IFN-γ, and TNF-α in the brain at the early stage of infection when the parasites migrate into the brain. It has been known that IL-12-producing cells in the brain are CD11b+ brain cells. Among them, CD11b+CD11c+-double positive cells (brain DC population) mainly produce IL-12, whereas CD11b+CD11c- cells (microglia) predominantly produced TNF-α, GM-CSF, and NO (Fischer and Reichmann, 2001). However, in our previous study, the infection of *T. gondii* ME49 strain in Alzheimer’s disease (AD) mice induced a reduction of NO due to a relative increase of anti-inflammatory cytokines,

IL-10 and TGF-β. Accordingly, we suggested the favorable effects of the immunosuppression induced by *T. gondii* infection on the pathogenesis and progression of AD in Tg2576 mice (Jung et al., 2012). However, the time point investigating immune characteristics was relatively a chronic phase of toxoplasmosis, and the observed anti-inflammatory response might be the late stage of immune responses during the chronic toxoplasmosis. Usually, because IL-10 plays a vital role in controlling the inflammatory response during acute *T. gondii* infection (Wilson et al., 2005), it needs to be understood on the kinetics of cytokine profile and neurodegeneration by inflammatory responses related with NO production in the acute phase of toxoplasmosis. Accordingly, the present study investigated on the change of microglia phenotype and NO production affecting neuronal cell degeneration during the early stage of *T. gondii* infection.

In the present study, *T. gondii* infection resulted in the increased neuronal degeneration in the hippocampal formation during experimental period up to 12 weeks PI with a tendency decreasing from 9 weeks PI. The activated microglia stained with Iba-1 antibody was increased from 3 weeks PI with appearance of neuronal degeneration. At this time, various gene levels related with immune responses were also increased in the brain, and the result in cytokine array also showed increases of IL-4, IL-10, IL-12, IFN-γ, TNF-α, GM-CSF, and IL-6 in protein levels. In the present study, mRNA level of anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 which control excessive brain inflammation (Henriquez et al., 2009; Park et al., 2005) were increased from 3 weeks PI, and continuously maintained during experimental

period of 36 weeks PI. In addition, pro-inflammatory cytokine, IL-12, was also highly increased during the whole experimental period with a slight decrease at 9 weeks PI. Levels of IFN-γ and TNF-α which play an important role in early protective immunity against *Toxoplasma* were also up-regulated during whole experimental period with slight decrease from 6 weeks PI. In this study, the early increase of IFN-γ and TNF-α may be inevitable to have anti-*Toxoplasma* activities such as microglia activation, and to trigger the transformation of tachyzoites to bradyzoite stage to avoid immune attack of the host. Our new finding is co-induction of inflammatory and anti-inflammatory cytokines in the brain tissue from the period when *Toxoplasma* migrated into the brain. Moreover, neurodegeneration in the hippocampal formation was appeared at a similar infection period and tissue regions which the activation of microglial cells was shown. Accordingly, our result strongly suggests that *Toxoplasma* infection in the brain induces simultaneously both inflammation and anti-inflammation although the brain is immune-privileged organ. However, it should be described how *Toxoplasma* induces the immune modulation which it protects both parasite itself and host brain from the inflammation. Moreover, it should be answered why neuronal cells were gradually recovered during microglial cell activation.

In this study, the infection of ME49 *T. gondii* induced the activation of NF-κB in the brain. In particular, the time when 18S rRNA of *T. gondii* was detected in the brain was consistent with the time of NF-kB activation. In other words, the oxidative/nitrosative stress increased by macrophage activation can also lead to activation of redox-sensitive transcription factors

such as nuclear factor κB (NF-κB) and activator protein 1 (AP-1), amplifying the inflammatory response and tissue injury (Laskin et al., 2011). Accordingly, our result suggests that the neuronal degeneration in the hippocampal formation was resulted in microglial cells and NF-kB activation. However, the brain tissue damage resulted from the excessive anti-*Toxoplasma* responses may be threatened both host and parasite, and this process will promote the immune modulation toward favorable to parasite survival in host-parasite relationship (Jung et al., 2012).

When exposed to inflammatory stimuli, microglial cells produce the inducible form of NO synthase (iNOS), and the NO subsequently produced is critically required to construct protective host responses against foreign pathogens (Guix et al., 2005; Suzuki, 2002). However, this immune response eventually damages host tissues, and in particular, NO is a key mediator of glia-induced neuronal cell death (Brown and Bal-Price, 2003; Guix et al., 2005). In particular, the neurotoxicity in the brain attributed by the excess production of cytotoxic factors such as NO, superoxide, and TNF-α was caused by classically activated microglia (Brown and Bal-Price, 2003).

However, this study showed the decrease of iNOS gene expression and NO production in response to *T. gondii* antigen (RH and ME49). Moreover, the neurodegeneration after infection was decreased after 6 weeks PI although it has microglial activation with the increase of Iba1-stained cells. To answer on the discrepancy, we examined the change in the phenotype of microglia after the stimulation of *T. gondii* antigens (RH or ME49) using BV2 microglial cells. The FACS result showed that although it has a few exceptions, almost

surface marker of microglia showed an alternatively activated phenotype in response to either RH antigen or recombinant IL-4, whereas the response against ME49 antigen was more closed to classical activated phenotype like it in addition of recombinant IFN-γ.

Among surface markers determining microglia phenotype, the major histocompatibility complex class II (MHC II) plays an important role in $CD4^+$ T cell activation during *T. gondii* infection (Suzuki, 2002). A commonly accepted marker profile determining M1 phenotype is CD68+ (pan macrophages)/CD80+, whereas M2 phenotype are characterized by CD68+/CD163 (Badylak et al., 2008). The CD80 (B7-1) and CD86 (B7-2) expressed on the 'professional' antigen-presenting cells (APC) in the lymphoid system are counter receptors for the T cell antigens, CD28/CTLA-4. The B7/CD28 interaction plays an important costimulatory signal in the decision between functional activation and clonal anergy of T cells, and B7-1 and B7-2 were undetectable in astrocytes under unstimulation or IFN-γ/GM-CSF-treated conditions (Satoh et al., 1995). The up-regulation of PD-L1 (B7-H1) depends on TLR4 and STAT1, whereas PD-L2 (B7-DC) expression depends on IL-4Rα and STAT6 (Loke and Allison, 2003). The inhibition of T cell proliferation by anti-B7-H1 mAb was due to enhance IFN-γ-induced NO production by macrophages, which augmented NO production by macrophages, suggesting a critical role for B7-H1 on macrophages in regulating IFN-γ production by naive $CD4^+$ T cells and, hence, NO production by macrophages (Yamazaki et al., 2005). Besides, CD40 molecule also plays an important role in promoting inflammatory responses by

macrophages/microglia, since the interaction with its cognate ligand, CD154 (CD40L), leads to secretion of cytokines such as IFN-γ and TNF-α, and neurotoxins (Tan et al., 1999). If CD40 expression has been suppressed, that may attenuate inflammation and neuronal damage within the CNS, which will ultimately be of benefit in neuroinflammatory diseases. Therefore, blocking the CD40-CD40L pathway in the CNS resulted in decreased clinical manifestations of multiple sclerosis and Alzheimer's disease in the respective rodent models of these diseases (Becher et al., 2001; Okuno et al., 2004). Accordingly, we investigated on changes of MHCII, CD80, CD86, B7H1, CD40 on microglial cells to determine the CNS response against *T. gondii* infection.

Our result shows that BV microglia cells were already activated from the increase of MHCII, CD80, CD86, B7H1, and CD40. That is reliable that BV2 cell line is a kind of activated microglia. However, in our experiment, when IL-4 was added to the BV2 cell culture, the expression of microglia activation marker was slightly decreased compared to the un-stimulated BV2 cells. According to our expectations, IFN-γ-treated BV2 cells were changed as an activated type of microglial cells based on the surface marker. At this time, the treatment of *T. gondii* RH antigen showed a similar expression of surface markers as like IL-4 was treated, whereas the stimulation with ME49 antigen induced the increase of MHC II, CD80, B7-H1, and CD40 as similar as the treatment of IFN-γ. Accordingly, our result demonstrated that microglia cells in CNS may be differently regulated by type I or type II of *T. gondii* strains to be M2 or M1 activation, respectively. Above mentioned, our result showed an

increase of NF-kB expression in the brain cells when *T. gondii* was migrated into the brain. This result is also consistent with other findings which cells infected with type I strain of *T. gondii* slightly induces NF-κB p65 translocation into the nuclear, but the infection of type II shows much higher level in nuclear p65 compared to type I-infected cells (Rosowski et al., 2011). In particular, it has been known that a predominance of NF-κB and STAT1 activation promotes M1 polarization of macrophage, resulting in cytotoxic and inflammatory responses (Mantovani, 2006). Accordingly, our study suggests that microglia activated in the brain of ME49 *T. gondii*-infected mouse change to M1 phenotype.

NO secreted by M1 microglia is much more harmful under pathological conditions that involve the production of reactive oxygen species (ROS), such as superoxide anions, due to the formation of peroxynitrite (Guix et al., 2005). However, our result showed that massive degenerated neuronal cells appeared from 3 weeks PI was rather decreased from 9 weeks PI although it was induced by M1 phenotype microglial cells. As a reason, we found it on microarray data. Our data shows that iNOS gene was not induced, but Arginase gene was increased more and more in the brain infected with ME49 strain of *T. gondii*. Actually, NO production was decreased by the treatment of *T. gondii* antigens (RH and ME49) in vitro study. Accordingly, we convinced that the reduction of neurodegeneration may be related with the increase of arginase as well as the decrease of iNOS gene expression and NO production although the FACS result showed the activation of microglial cells to M1 phenotype.

As a factor determining macrophage phenotype, the alternative activation (M2) of macrophages is depended on *Toxoplasma*, ROP16, to activate the STAT6 pathway, while the classical activation of macrophages is due to the unique ability of its GRA15 protein to activate NF-kB pathway and elicit proinflammatory cytokines (Jensen et al., 2011; Rosowski et al., 2011). As a mechanisms, ROP16 in RH strain of *T. gondii* has an intrinsic tyrosine kinase activity and directly phosphorylates tyrosine residue for STAT6 activation (Ong et al., 2010) which followed by IL-4 mediated anti-inflammatory response, enhanced MHC class II antigen expression, and reduced proinflammatory cytokine secretion. (Ohmori and Hamilton, 1998; Stein et al., 1992). In general, numerous parasites, bacteria, and viruses are able to control to inhibit NF-κB signaling in order to prevent or delay the innate or adaptive immune response (Tato and Hunter, 2002). However, in this study, NF-kB-stained cells were increased with Iba-I-stained microglial cell in the hippocampal dentate gyrus region with neurodegeneration after *T. gondii* infection. This inflammatory response might be harmful in the host with time pass, however, neurodegeneration was rather decreased. In particular, the treatment of *T. gondii*-derived antigens selectively decreased the gene expression of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) in BV2 cell culture. At this time, mRNA level of arginase 1 which is the endogenous competitive inhibitor of iNOS, was significantly increased. In general, the gene expression of arginase 1 is selectively increased in alternatively activated macrophage. This enzyme is related to the hepatic urea cycle and usually acts for the disposal of excess nitrogen resulting from amino

acid and nucleotide metabolism (Meijer et al., 1990). It was also demonstrated that arginase I expression in macrophages is a susceptibility factor during *Toxoplasma* infection, and Arg1 -/- macrophages produce more NO in response to inflammatory stimuli (El Kasmi et al., 2008). As a neurodegenerative disease in the brain, Alzheimer's disease resulted in microglia activation by amyloid-β deposition, were increasingly by neurotoxic factors such as NO, TNF-α, and superoxide, and continually became a serious in response to neuronal damage (reactive microgliosis) which is then toxic to neighboring neurons. This perpetuating cycle of neuron death was discontinued through a decrease of NO production by *T. gondii* infection (Jung et al., 2012). Taken above result, the brain and BV-2 microglia cells stimulated with ME49 strain of *T. gondii* induced the characteristics of M1 phenotype in microglia and neurodegeneration, and simultaneously, the increase of IL-4 and IL-10 controlling inflammatory responses at the early stage of infection, the selective down-regulation of iNOS gene and NO production, and finally a selective up-regulation of arginase 1 enzyme to prevent further excessive neurodegeneration and inflammation. These results shows that although the infection of *T. gondii* in the immune privileged brain starts a protective inflammatory reaction against pathogen by endogenous microglia activation, neurodegeneration shown with the progression of inflammatory immune response are soon recovered by the decrease of cytotoxic NO production through the increase of selectively arginase 1. This immune modulation may be directed to give a favorable effect in neurodegenerative disease such Alzheimer's disease.

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국문 초록

인터페론 감마에 의하여 활성화된 뇌내 미세아교세포는 M1 표현형으로 나타난다. 미세아교세포의 M1 표현형은 톡소포자충 감염 시에도 나타나는 현상으로 산화질소를 분비하여 뇌 손상을 유발시킨다. 반면 M2 표현형은 손상된 조직을 회복하는데 중요한 역할을 한다. 톡소포자충은 감염 숙주와 독성에 따라서 1형, 2형, 3형으로 분류된다. 그 중 1형과 3형은 stat6를 활성화 하여 대식세포의 M2 표현형으로 유도한다. 반면에 2형의 톡소포자충 (ME49)은 감염 후 NF-kB를 활성화 시키며 오랜 기간 만성적으로 뇌 내에 감염되어 있다. 2형의 만성 감염 시 그 면역반응의 양상에 대해서는 아직 많은 연구가 진행 되고 있다. 이 논문의 목표는 톡소포자충의 종마다 다르게 나타나는 미세아교세포의 활성화 정도를 알아보고 어떻게 톡소포자충이 뇌 내 미세아교세포에 영향을 주는지 알아보았다.

이 연구를 위해 C57BL/6 마우스에 톡소포자충의 2형 (ME49)을 감염 시키고 0주, 1주, 3주, 6주, 9주, 12주의 일정 시간 후에 뇌에서 면역반응과 관련된 단백질과 mRNA를 사이토킨어레이(IL-4, IL-10, IL-6, GM-CSF, IFN-r, TNF-α, IL-12p70)와 마이크로어레이를 이용하여 분석하였다. 또한 BV2 미세아교 세포주를 이용하여 톡소포자충에 의해서 생성되는 산화질소의 분비양과 세포막 표면에 발현되는 MHCII, CD80, CD86 B7-H1, B7-DC, CD40의 증감을 확인하였다.

연구 결과, 감염 초기 1주부터 6주까지의 마우스 뇌 내에서 M1표현형의 미세아교세포에 의한 뇌세포의 심한 손상을 확인하였다. 3주부터 증가한 미세아교세포를 발견하였다. 그러나 감염 6주를 지나 손상된 뇌세포는 점차 회복되는 조직 염색 결과를 얻었다. mRNA를 이용한 마이크로어레이에서는 36주까지 염증성 인자들이 발현된 것을 확인 하였고, 사이토카인 어레이에서는 염증성 사이토킨의 발현양이 12주에서 조직 손상이 심한 3주와 6주보다는 줄었지만 0주보다는 증가한 상태로 유지되는 것을 확인 하였다.

BV2 미세아교 세포주를 이용한 실험에서 톡소포자충의 1형인 RH주 보다 2형인 ME49주와 함께 배양 시 미세아교세포의 표면에 M1 표현형의 인자들이 더 많이 발현 하였다. 그러나 주요 조직 손상 인자인 산화질소는 1형과 2형 모두에서 감소 되었고, 2형인 ME49에서 보다 더 많은 감소를 확인 하였다. 이는 BV2 세포주의 mRNA를 이용한 real-time PCR에서 산화질소 생산을 촉진하는 cox-2와 iNOS의 발현이 감소하였고, 산화질소의 생성을 억제하는 arg1의 발현이 증가함을 발견하였다. ME49를 감염시킨 마우스의 마이크로어레이 결과에서도 cox-2와 iNOS의 발현에는 정상 마우스와 차이를 보이지 않았으나, arg1은 3주와 6주에서 증가하였다.

따라서, 이 연구결과로부터 알 수 있듯이 마우스에 감염된 톡소포자충의 1형(RH주)은 대식세포를 M2 표현형으로 유도하는 반면, 2형(ME49주)은 미세아교세포의 표면 인자의 발현을 보면 M1 표현

형으로 유도하나, 이 때 arginase 1의 발현을 동시에 유도하여 M1형의 주요 특징인 산화질소의 분비를 줄여 M1-like 표현형으로 유도시킨다. 이는 2형 (ME49) 톡소포자충 감염에 의해 미세아교세포의 활성과 염증인자의 지속적인 발현에도 불구하고 초기 감염 후 손상된 뇌조직이 점차 회복될 수 있는 것은 산화질소의 선택적 감소 때문이다. 이러한 기생충의 숙주 면역 조절 기전은 기생충 자신의 삶의 터전인 숙주를 기생충 자신에 의해 나타나는 극심한 세포독성 면역반응으로부터 숙주를 보호하고, 기생충 자신 또한 숙주에서 지속적인 삶을 유지하기 위하여 오랜 시간 동안 발전되어 온 기생충-숙주 관련성으로 생각된다.

주요어 : 톡소포자충, 미세아교세포, 대식세포, 사이토카인, 뇌신경 손상, 산화질소 생성, 마이크로어레이 유전자 발현, 사이토킨어레이

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