Studies on the Behavior of *Toxoplasma gondii* In the Intermediate Host

（トキソプラズマ原虫の中間宿主体内における挙動に関する研究）

2013

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CONTENTS

ABBREVIATIONS

AIDS: Acquired immunodeficiency syndrome

ANOVA: Analysis of variance

APC: Allophycocyanin

BAG1: Bradyzoite–specific antigen 1

CDA1: Cell division autoantigen–1

ECM: Extracellular matrix

FCS: Fetal calf serum

GFP: Green fluorescent protein

HA: Hyaluronan

ICAM–1: Intercellular adhesion molecule –1

IL–1ß: Interleukin–1ß

KOP: Mitogen activated protein kinase p38α deficient mouse embryonic fibroblast

LPS: Lipopolysaccharide

MAPK: Mitogen activated protein kinase

MFI: Mean fluorescence intensity

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate–buffered saline

PE: Phycoerythrin

PI3K: Phosphatidylinositol 3–kinase

PKBα: Protein Kinase Bα

PLK/DUAL: Dual fluorescent protein–expressing stable transgenic *Toxoplasma gondii*

PLK/GFP: GFP–expressing stable transgenic *Toxoplasma gondii*

PLK/RED: DsRed Express–expressing stable transgenic *Toxoplasma gondii*

RKOP: Revertant mutants of mitogen activated protein kinase p38α deficient mouse embryonic fibroblast

qRT–PCR: quantitative real–time PCR

T. gondii: *Toxoplasma gondii*

TNF–α: Tumor Necrosis Factor–α

GENERAL INTRODUCTION

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite that infects a broad range of intermediate hosts with the cat as the definitive host. *T. gondii* infection is established by oral ingestion of a cyst formed in the intermediate host or of an oocyst formed in the cat. In the body of an intermediate host, tachyzoite, a rapidly–growing form of the parasite released from cysts or oocysts, penetrates the intestine and establishes the acute stage of infection, characterized by rapid proliferation (76). Tachyzoites proliferate asexually and disseminate to peripheral organs where they convert to slowly growing bradyzoites. The bradyzoites are contained within a thin but rigid cyst wall that is retained within a viable host cell, which helps further shield the parasite from immune responses as well as from current drug treatments (76). Although cysts appear capable of forming in virtually any cell type *in vitro*, in infected individuals they are most prevalent in the neural and muscular tissues including the central nervous system, eyes, and skeletal and cardiac muscles (69).

In contrast, in the body of the definitive host, tachyzoites in the intestine

convert to an asexual form of the parasite that is structurally distinct from the tachyzoite and asexually proliferates. After asexual development, the sexual cycle begins and an oocyst wall is formed around the parasite after fertilization. Oocysts are then released in cat feces (27). The acute stage of *T. gondii* infection in intermediate hosts is initiated by latent cyst or oocyst infection. Latent cyst that is formed in skeletal muscle, contaminates food, particularly meat, while oocysts contaminate water and soil (39, 44). Although it is not possible to distinguish infection from latent cysts in meat versus that caused by oocysts, Sullivan and Jeffersit reported that the surge of infection in teenagers and the low prevalence in younger children indicates that ingestion of cysts in undercooked meat is an important source of transmission (77). During the acute stage, tachyzoites are disseminated to peripheral organs such as the brain which is normally protected by the blood–placenta and blood–brain barrier. Infection during pregnancy leads to vertical transmission of *T. gondii* to the neonate in both humans and domestic livestock (25, 38). Vertical transmission of *T. gondii* can cause fetal abortion and neonatal neurological diseases including ocular disease and hydrocephalus (87). In addition, parasites inside leukocytes, particularly the

CD11b– and CD11c–expressing population, have been suggested to migrate through the blood–brain barrier (19, 46, 81).

Tachyzoites reaching the brain convert to bradyzoites, a slow–growing form that develop into latent tissue cysts at 7–10 days after infection (2), and can persist throughout the life of the host. Differentiation of *T. gondii* from the tachyzoite into bradyzoite is known to predominantly occur in the brain, suggesting that the brain environment can induce bradyzoite formation. Some extracellular conditions *in vitro* can also induce a high frequency of bradyzoite conversion compared with normal culture conditions (3, 4, 74, 86). However, these conditions are unlikely to reflect the trigger of natural conversion of the parasite in the brain because conducive conditions such as high or low pH and heat shock rarely occur *in vivo*. Moreover, the trigger for natural stage–conversion remains unknown. Bradyzoites within latent cysts can reconvert into active tachyzoites and cause encephalitis in immunocompromised patients such as those with acquired immunodeficiency syndrome (AIDS). In an immunocompetent person, most infections are asymptomatic; however, some individuals do develop chorioretinitis, lymphadenitis, myocarditis or polymyositis (87).

Understanding the mechanism of latent infection of *T. gondii* is an important current issue to prevent maternal infection and subsequent vertical transmission, as well as reactivation in immunocompromised patients.

In this study, the expression of adhesion molecules on the surface of leukocytes was analyzed to gain an understanding of the mechanism by which the parasite disseminates to the brain. The trigger in the brain environment which induces bradyzoite differentiation of the parasite after reaching the brain was then investigated.

CHAPTER 1)

CD44 mediated hyaluronan adhesion of *T. gondii*–infected leukocytes

INTRODUCTION

T. gondii is an obligate intracellular apicomplexan parasite causing congenital infection and abortion, and opportunistic diseases in immunodeficient individuals. Following oral ingestion of *T. gondi* cysts or oocysts, the parasites cross the intestinal epithelium, invade the general circulation and are then transported to the peripheral organs (19, 56, 81). Notably CD11b positive and CD11c positive mouse leukocytes transport *T. gondii* tachyzoite from the intestine to the periphery including the brain (19). It is recently reported that *T. gondii*–infected peripheral mononuclear cells (PBMC) from the circulation sequester in several organs in greater numbers than non–infected PBMCs (83). It is also reported that *T. gondii* infection of monocytic cells alters the expression of certain adhesion molecules, including CD44, on their surface (84). It is possible that *T. gondii* invasion into the

circulating leukocytes promotes host cell adhesion and/or extravasation.

CD44 is a widely expressed cell adhesion molecule for which a major ligand is hyaluronan (HA), a glycosaminoglycan component of the extracellular matrix (ECM) (60, 79). HA is also produced on the surface of human and mouse vascular endothelial cells and inflammatory cytokines: both tumor necrosis factor–α (TNF–α) and interleukin–1ß (IL–1ß) upregulate HA production (43, 54, 84). It has previously been reported that leukocytes extravasate from the blood into the surrounding tissue through complementary ligand interactions between leukocytes and endothelial cells. Activation of T cells increases their binding to HA and enables CD44–mediated adhesion in both mouse and human endothelial cells (5, 10, 21, 22, 30, 67). HA is also located on the ECM and the HA–CD44 interaction is important for mesenchymal cell and leukocyte adhesion. Human PBMCs adhere to HA on the mucosal smooth muscle cell of the intestine *via* CD44 on the surface of human PBMCs (23, 24). This indicates the importance of the HA–CD44 interaction for leukocyte infiltration into peripheral tissue. Previously, it was reported that *T. gondii*–infected monocytic cells expressed significantly higher levels of CD44 than non–infected ones (82). However, it

was still unknown whether such alterations of CD44 expression by *T. gondii* invasion facilitated adhesion of *T. gondii*–infected leukocytes to HA. In this study, I investigated the effect of the alteration of CD44 expression levels of mouse and human PBMCs by *T. gondii* infection and revealed that the difference in CD44 expression level between infected and non–infected PBMCs results in selective adhesion of infected PBMCs on immobilized HA.

MATERIALS AND METHODS

Parasites.

Tachyzoites of green fluorescent protein (GFP)–expressing transgenic *T. gondii* derived from the PLK strain, PLK/GFP, (55) were passaged in Vero cells. Vero cells and tachyzoites were maintained in RPMI 1640 medium supplemented with 7.5% fetal calf serum (FCS) and 20 μg/ml gentamicin, and incubated at 37 °C in a 5% CO_2 incubator.

PBMCs, monocytes and splenocytes.

Human and mouse PBMCs were obtained from a healthy human volunteer and male C57BL/6J wild type mice using Lympholyte®–Mammal (Cedarlane, Burlington, Canada) according to the manufacturer's protocol. Human monocytes in the PBMCs were isolated by negative immunoselection using a monocyte isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. PBMCs and human monocytes were cultured in RPMI 1640 medium supplemented with 10% FCS. Splenocytes from C57BL/6 wild type mice or CD44 knockout mice with

C57BL/6 genetic background (61) were prepared by removal of the whole spleen following ether anesthesia, exsanguination and cervical dislocation in mice. Spleens were minced and pressed through a nylon mesh with a pore size of 77 µm. The filtrated cells were suspended in 9 ml of hemolytic buffer (0.155 M NH_4Cl in dH_2O and 1 ml Tris–HCl pH7.65), left for 15 min at room temperature and washed with phosphate–buffered saline (PBS) to remove erythrocytes. Mouse splenocytes were cultured in RPMI 1640 medium supplemented with 10% FCS and 20 µg/ml gentamicin. The experimental protocols using human samples and animals were reviewed and approved by the local ethics committee of Gifu University.

Tachyzoite infection.

PBMCs, fresh isolated monocytes or splenocytes were suspended in RPMI 1640 medium supplemented with 10% FCS at a concentration of 4×10^6 cells/ml. Splenocytes were suspended in RPMI 1640 medium supplemented with 10% FCS and 20 µg/ml gentamicin at a concentration of 4×10^6 cells/ml. Intracellular tachyzoites were released from infected Vero cells by rapid extrusion through a 27–gauge needle, 3 times. Cell debris was removed by

filtration through a filter with a pore size of 5 μm (Millipore, Bedford, USA). The purified extracellular tachyzoites were suspended in RPMI 1640 medium supplemented with 10% FCS and 20 μg/ml gentamicin at a concentration of 2×10^6 tachyzoites/ml. An equal volume of the cell suspension and the extracellular tachizoite suspension were mixed and cultured at 37 °C for 24 h in humidified air with 5% CO_2. Mock–infected cells were also cultured at a concentration of 2×10^6 cells/ml without parasites for 24 h. 24 h after incubation, the cells were examined for flow cytometry or cell adhesion assays.

Flow cytometry.

T. gondii–infected and mock–infected cells were washed with PBS. The washed cells were re–suspended in PBS at a concentration of 4×10^6 cells/ml. Human IgG was added to the cell suspension at 1 μg /10^5 cells and incubated for 15 min at room temperature to prevent nonspecific attachment of antibodies to cell surface via Fc receptors. The mouse cells were stained using allophycocyanin (APC)–conjugated anti–mouse CD14 (eBioscience, San diego, USA) and phycoerythrin (PE)–conjugated rat anti–mouse CD44

antibody (BD Biosciences, Tokyo, Japan). PE–conjugated rat IgG2b, κ isotype control (BD Biosciences) was used as an isotype control for the anti–mouse CD44 antibody. The human cells were stained using APC–conjugated anti–human CD14 (BECKMAN COULTER, Brea, USA) and PE–conjugated mouse anti–human CD44 antibody. PE–conjugated mouse IgG1, κ isotype control (BD Biosciences) was used as an isotype control for the anti–human CD44 antibody. The cells were incubated for 45 minutes on ice in a dark, and washed 3 times with 4 ml PBS and re–suspended in PBS for analysis. Flow cytometry was performed on a FACSCanto II (BD Biosciences).

Cell adhesion assay.

The *T. gondii*–infected PBMCs or splenocytes were washed with PBS. The washed cells were re–suspended in RPMI 1640 medium supplemented with 10% FCS at a concentration of 1×10^6 cells/ml. Gentamicin was added into the splenocyte suspension at a concentration of 20μg/ml. For the adhesion assay, several 7–mm–diameter circles were drawn on the base of plastic petri dishes (Falcon 1007 or Falcon 351008: BD Biosciences). Then 40 μl aliquots

of 100 μg/ml hyaluronic acid sodium salt from bovine vitreous humor (Sigma, Tokyo, Japan) in PBS were adsorbed onto the surface of each circle and the petri dishes incubated for 24 hours at 4 °C. After incubation, the petri dishes were blocked using 1% bovine serum albumin in PBS for 1h at 4 °C. After blocking, the petri dishes were overlaid with the infected cell suspension and incubated at 37 °C for 90 minutes in the case of splenocytes or 120 minutes in the case of PBMCs. Unbound cells were removed by gentle washing with PBS, and the frequency of parasite–invaded cells among cells binding to the HA–coated spots was evaluated by a fluorescent microscope. For the antibody–blocking assay, infected PBMCs and monocytes were suspended in PBS at a concentration of 4×10^6 cells/ml and human IgG was added to the cell suspension at 1.0μg/10^5 cells and incubated for 15 minutes at room temperature. Then, anti–human CD44 antibody clone 5F12 (Thermo Scientific, Waltham, USA) or mouse IgG1, κ isotype control (Thermo Scientific) was added into the human derived cell suspensions. Anti–mouse CD44 antibody clone KM81 (Cedarlane) or rat IgG2a isotype control (Cedarlane) was added to the mouse derived cell suspensions. The cells were incubated for 45 minutes on ice, washed 3 times with 4 ml PBS and

suspended in RPMI 1640 medium supplemented with 10% FCS at a concentration of 1×10^6 cells/ml. The cell suspensions were used for the cell adhesion assay described above. To distinguish myeloid and non–myeloid cells, PBMC before panning and PBMCs bound to the HA–coated circle were stained using PE–conjugated rat anti–mouse CD11b antibody (BD Biosciences). The rate of tachyzoite invaded cells among the CD11b positive and negative cells were evaluated separately before and after the panning.

Statistical analysis.

Paired t–test was used for comparison between two groups. Analysis of variance (ANOVA) was used for comparisons between more than two groups. If the result of an ANOVA was significant, a post hoc, Tukey–Kramer test was performed. Statistical significance was set at $p < 0.05$.

RESULTS

CD44 expression level of mouse PBMCs and splenocytes infected with T. gondii .

CD44 expression in freshly isolated leukocytes is unknown. Therefore, CD44 expression of *T. gondii* infected PBMCs and splenocytes was examined. To distinguish parasite–invaded and non–invaded cells in infected cell cultures using flow cytometry, I used a GFP–expressing transgenic *T. gondii*, PLK/GFP (Fig. 1–1 A). In addition, CD14 positive and negative cells were distinguished and examined (Fig. 1–1 B–E). As shown in Fig. 1–1 B and C, parasite–invaded PBMC showed a significantly higher level of CD44 expression than non–invaded PBMCs, regardless of whether they were initially CD14 positive or negative. Parasite–invaded cells in the splenocyte culture also showed a significantly higher CD44 expression level compared to non–invaded cell within the same culture, regardless of whether they were initially CD14 positive or negative (Fig. 1–1 D and E).

Contribution of CD44 to adhesion of T. gondii–infected mouse leukocytes to HA.

To investigate whether the higher expression level of CD44 in parasite–invaded cells facilitated the adhesion of the cells to immobilized HA, a mixture of parasite–invaded and non–invaded mouse PBMCs were added to plastic plates coated with HA, and the ratio of parasite–invaded cells among HA–adhering cells was determined. The infection rate of HA–adhering cells was higher than that of the population as a whole, indicating that parasite–invaded PBMCs were enriched on the immobilized HA (Fig. 1–2 A). The enrichment of parasite–invaded PBMCs was reduced by the addition of anti–CD44 antibody (Fig. 1–2 B). I also showed that anti–CD44 antibody blocked enrichment of parasite–infected cells on both CD11b positive and negative PBMCs (Fig. 1–2 C and D). Whereas parasite–invaded splenocytes from wild type mice were enriched on immobilized HA (2.36 ± 0.75 times, $p<0.05$), parasite–invaded splenocytes from the CD44 knock out mice were not enriched on the immobilized HA at all (Fig. 1–2 E).

Contribution of CD44 to adhesion of T. gondii–infected human leukocytes to HA.

When CD44 expression of parasite–invaded and non–invaded human PBMCs in the same culture was checked separately, in common with mouse PBMCs, parasite–invaded cells showed significantly higher levels of CD44 expression than non–invaded cells, regardless of whether they were CD14 positive or negative (Fig. 1–3 A and B). Therefore, to investigate whether CD44 also contributes to adhesion of parasite–invaded human PBMCs on immobilized HA, the adhesion assay using freshly isolated human PBMCs was examined. As shown in Fig. 1–4 A, the infection rate of HA–adhering PBMCs was higher than that of the population as a whole. It also revealed that parasite–invaded CD11b negative PBMCs were enriched on immobilized HA (Fig. 1–4 D, 1.61 ± 0.26 times, $p<0.05$). Although CD11b positive population is a minor component in total PBMCs, the majority of HA–adhering parasite–invaded cells were CD11b (Fig. 1–4 B). The adherence of parasite–invaded human PBMCs was significantly blocked by anti–CD44 antibody (Fig. 1–4 C). It was confirmed that the non–specific mechanical effects of binding of the antibody on the surface of infected cells

to interfere its adherence to HA by using anti–CD45 antibody (data not shown). As a result, anti–CD45 antibody did not show inhibition of infected cell adhesion, indicating that the observed effect of anti–CD44 antibody is not due to mechanical effect of attachment to cell surface but CD44 antigen specific manner. To examine the effect of anti–CD44 antibody on CD11b positive and negative PBMCs separately, the adhered PBMC and the PBMC stock before adhesion were stained using PE–conjugated rat anti–mouse CD11b antibody and the rates of tachyzoite infection of CD11b positive and negative cells were determined. As shown in Fig. 1–4 D, the anti–CD44 antibody blocked the adherence of parasite–invaded CD11b negative cells. However, it could not be evaluated that the effect on the adherence of parasite–invaded CD11b positive cells because only a few CD11b positive cells adhered on immobilized HA in the presence of anti–CD44 or isotype control antibody. To overcome this technical problem, monocytes which are considered to be major CD11b positive population in human PBMC (34, 53, 64) were once purified from freshly isolated human PBMCs and then performed the adhesion assay using enough number of monocytes in the presence of the anti–CD44 antibody. Although treatment with isotype control

antibody diminished the enrichment of parasite–invaded human monocytes, the ratio of infected cells among HA–adhering monocytes was significantly lower in the presence of anti–CD44 antibody when compared with those in the isotype control antibody (Fig. 1–4 E). This result indicates that anti–CD44 antibody specifically inhibits the adhesion of parasite–infected human monocytes to immobilized HA.

DISCUSSION

Here, it was revealed that CD44 dependent selective adhesion of *T. gondii*–infected mouse and human leukocytes to immobilized HA. It has been reported that leukocytes infected with viruses gain a higher migratory ability than non–infected leukocytes (12, 40, 42, 45, 65, 66, 72, 73). Although the mechanism of higher migratory ability is not yet well–known, it has been shown that measles virus–infected monocytes show higher ability to adhere to endothelial cells via up–regulation of leukocyte function–associated antigen–1 expression on the surface of the infected monocyte (40). In this study, it was showed that CD44 expression level between parasite–invaded and non–invaded cells controls adhesion of an obligate intracellular parasite, *T. gondii*. Although the CD44 expression level of total cells in the infected PBMC culture was higher than that of mock infected culture, similar increasing of CD44 expression was also induced by Vero cell debris, a host cell for parasite maintenance culture (data not shown). However, overall, parasite–invaded cells expressed significantly higher levels of CD44 than non–invaded cells (Fig. 1–1 B–E, Fig. 1–3 A and B). It is possible that the

parasite preferentially invades cells with relatively higher CD44 expression level while there still remains possibility that CD44 expression is up–regulated by parasite invasion.

CD44 represents a heterogeneous group of proteins generated by alternate splicing of a single gene. Alternative splicing of 10 variant exons of a single CD44 gene produces numerous isotypes and each of the variant isotypes has different affinity to HA (70, 71). However, *T. gondii* did not change the splicing pattern of CD44 mRNA of human myeloid leukemia cells, THP–1 (data not shown). Considering that the parasite–invaded THP–1 cells also selectively adhere to HA (82), splicing alteration is not absolutely necessary for host leukocyte control by *T. gondii*. Compared with non–infected THP–1 cells, the parasite–invaded THP–1 cells produced more mRNA coding for the standard form of CD44 (CD44s, not include variant exon) (data not shown). This standard form of CD44 does not contain any variant exons. The amount of CD44 on the surface of parasite–invaded cells might, therefore, be the main reason for their selective adherence to HA.

In this study, I used CD14 or CD11b as markers to distinguish myeloid cells from non–myeloid cells. Although a very small population of B cells and

basophils are CD14 positive, a major population of CD14 positive cells in human PBMC is monocyte/macrophage (1, 47). It is also known that the majority population of CD14 negative PBMCs and splenocytes is lymphocyte. Therefore, CD14 positive and negative populations found in this study basically represent monocyte/macrophages and lymphocytes. It was reported previously that the parasite–invaded myeloid leukemia cells express a higher level of CD44 than non–invaded cells (82). However, the effect of *T. gondii* infection on non–myeloid cells had not been determined. This is the first report that showed similar phenomenon on non–myeloid cells by *T. gondii* invasion. CD11b positive cells in human PBMCs include monocyte/macrophages, granulocytes, natural killer (NK) cells, and minor subsets of CD5+ B cells and CD8+ T cells (34, 53, 64). Considering that granulocytes were removed during PBMC preparation, the CD11b positive population in human PBMCs shown in this study basically represents monocyte/macrophages and NK cells. Mouse CD11b is also known to be a myeloid marker and 40%–60% of CD11b positive cell in the mouse PBMCs are monocytes (48). In this study, it was revealed that the majority of HA–adhered parasite–invaded human PBMCs were CD11b positive,

although the CD11b positive population in whole human PBMCs is minor (Fig. 1–4 B). It has been reported that CD11b positive myeloid cells are a major transporter of *T. gondii* to the brain and anti–CD11b antibody inhibits transport of parasite into the brain, suggesting that such leukocytes migrate out of the circulation (19). The extremely effective adhesion ability of CD11b positive parasite–invaded cells might be a reason why CD11b positive cells, a minor population in general circulating leukocytes, can be a major transporter of *T. gondii* to the brain.

It is known that TNF–α, a major cytokine secreted during *T. gondii* infection (13, 63), upregulates CD44 expression of leukocytes (7, 36, 51). There is a possibility that cytokines secreted by parasite–invaded cells up–regulate CD44 expression level of infected cells and neighboring cells by autocrine or paracrine action. However, parasite–invaded cells showed higher expression of CD44 than non–invaded cells in the same culture. Although parasite might preferentially invade into cells with relatively higher CD44 expression level, there is also a possibility that parasite invasion and/or autocrine action is necessary for effective up–regulation of CD44 expression.

Using a mouse model, it was reported recently that parasite–invaded leukocytes remain in solid organs more effectively than non–invaded ones (83). The difference in CD44 expression level between parasite–invaded cell and non–invaded neighboring cells might facilitate effective delivery of parasites from the general circulation into solid organs. It is important to study adhesion ability of parasite–invaded leukocyte to HA–producing endothelial cells and/or HA rich ECM to understand the intracellular pathogen delivery system.

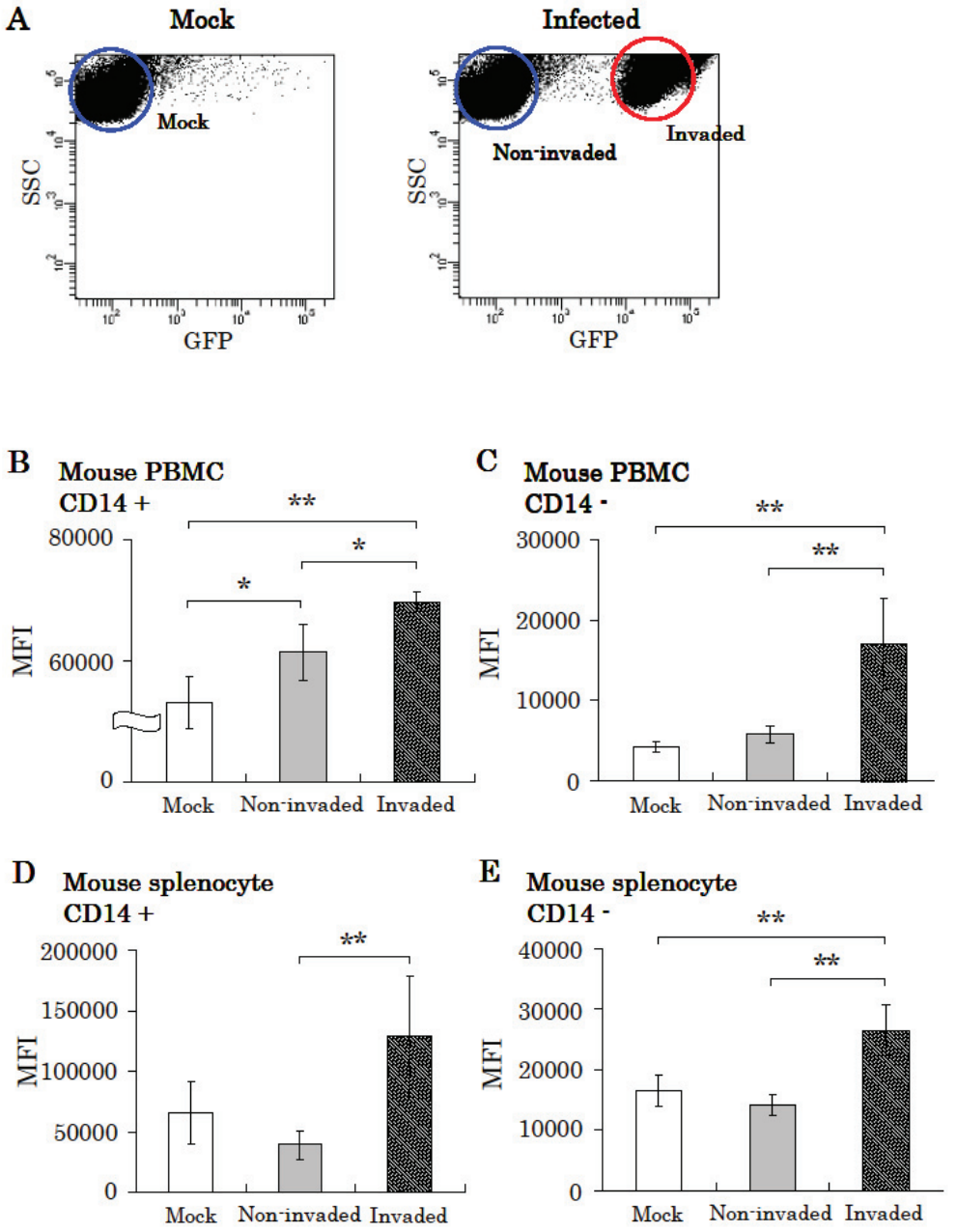


Fig.1–1 CD44 expression of *T. gondii*–infected mouse leukocytes.

(A) Distinction between parasite–invaded cells and non–invaded cells. Green fluorescence of cells in mock–infected and PLK/GFP tachyzoite–infected cultures detected by flow cytometry (left and right panel,

respectively). (B, C) CD44 expression level of CD14 positive and negative PBMCs (B and C, respectively). (D, E) CD44 expression level on CD14 positive and negative splenocytes (D and E, respectively). Results are presented as the mean ± S.D. of mean fluorescence intensity (MFI). ANOVA was used for comparisons between more than two groups. If the result of an ANOVA was significant, a post hoc, Tukey–Kramer test was performed. The data represent the average and S.D. of 4 independent experiments (*$p < 0.05$, **$p < 0.01$).

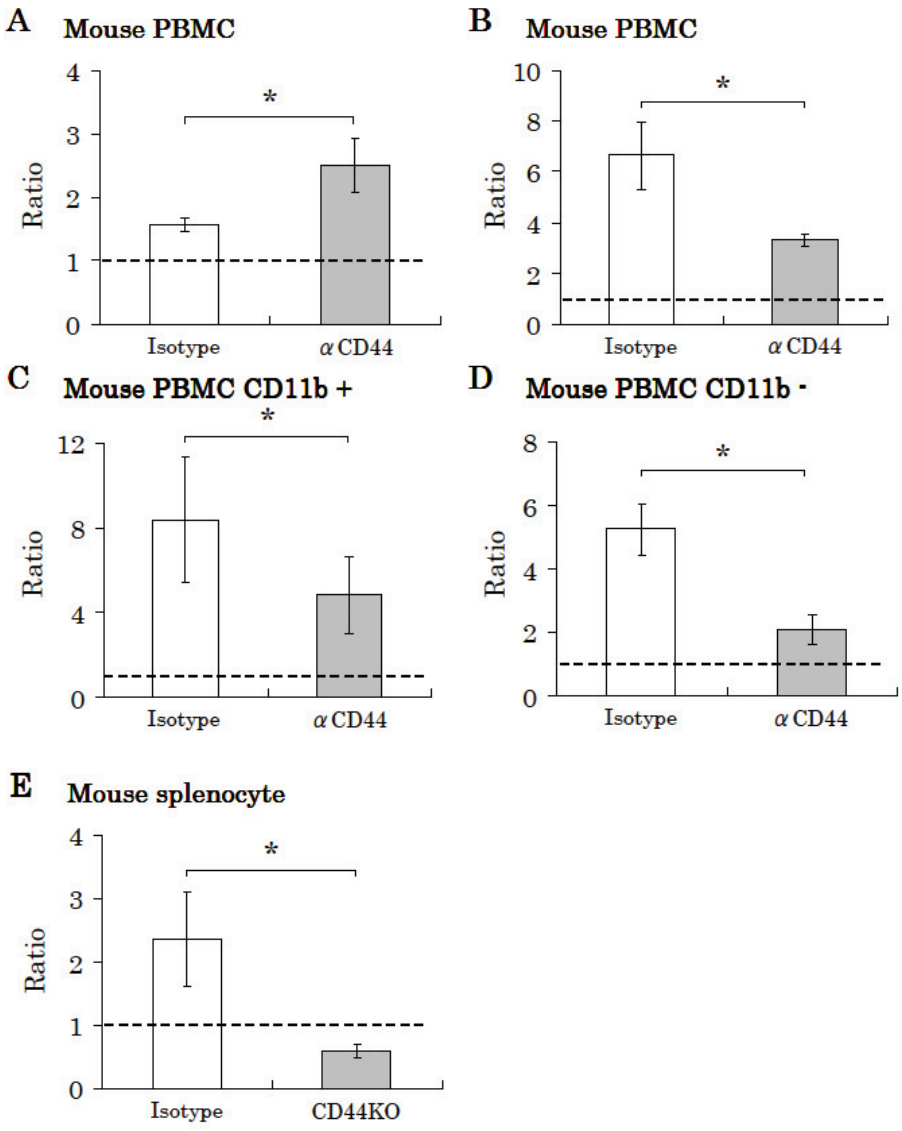


Fig.1–2 Contribution of CD44 of *T. gondii*–infected mouse leukocyte adhesion to HA.

Infected mouse PBMCs or splenocytes were added on immobilized hyaluronan and infection rate of adhering cells was determined. Results are presented as fold change compared with infection rate of population as a

whole. (A) Infection rate of HA–coated and non–coated plate tested with mouse PBMCs. (B) Infection rate of HA–coated plate tested with mouse PBMCs in the presence of anti–CD44 antibody or isotype control. (C, D) Infection rate of HA–coated plate tested with mouse CD11b positive and negative PBMCs in the presence of anti–CD44 antibody or isotype control (C and D, respectively). (E) Infection rate of HA–coated plate tested with splenocytes of wild type and CD44 knock out mouse. Dashed lines indicate ratio = 1.0. Paired t–test was used for comparisons between two groups. The data represent the average and S.D. of 3 or 4 independent experiments (A–D and E, respectively, $*p < 0.05$, $**p < 0.01$).

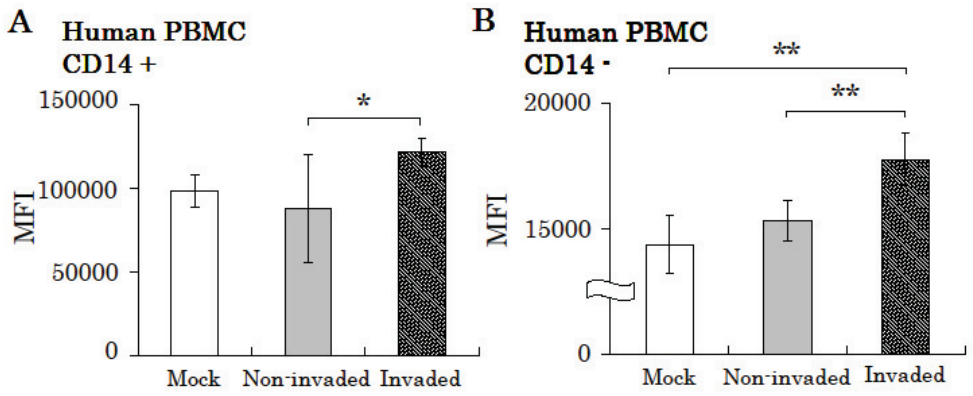


Fig.1–3 CD44 expression of *T. gondii*–infected human PBMCs.

(A, B) CD44 expression level of CD14 positive and negative PBMCs (A and B, respectively). ANOVA was used for comparisons between more than two groups. If the result of an ANOVA was significant, a post hoc, Tukey–Kramer test was performed. The data represent the average and S.D of 5 independent experiments (*$p < 0.05$, **$p < 0.01$).

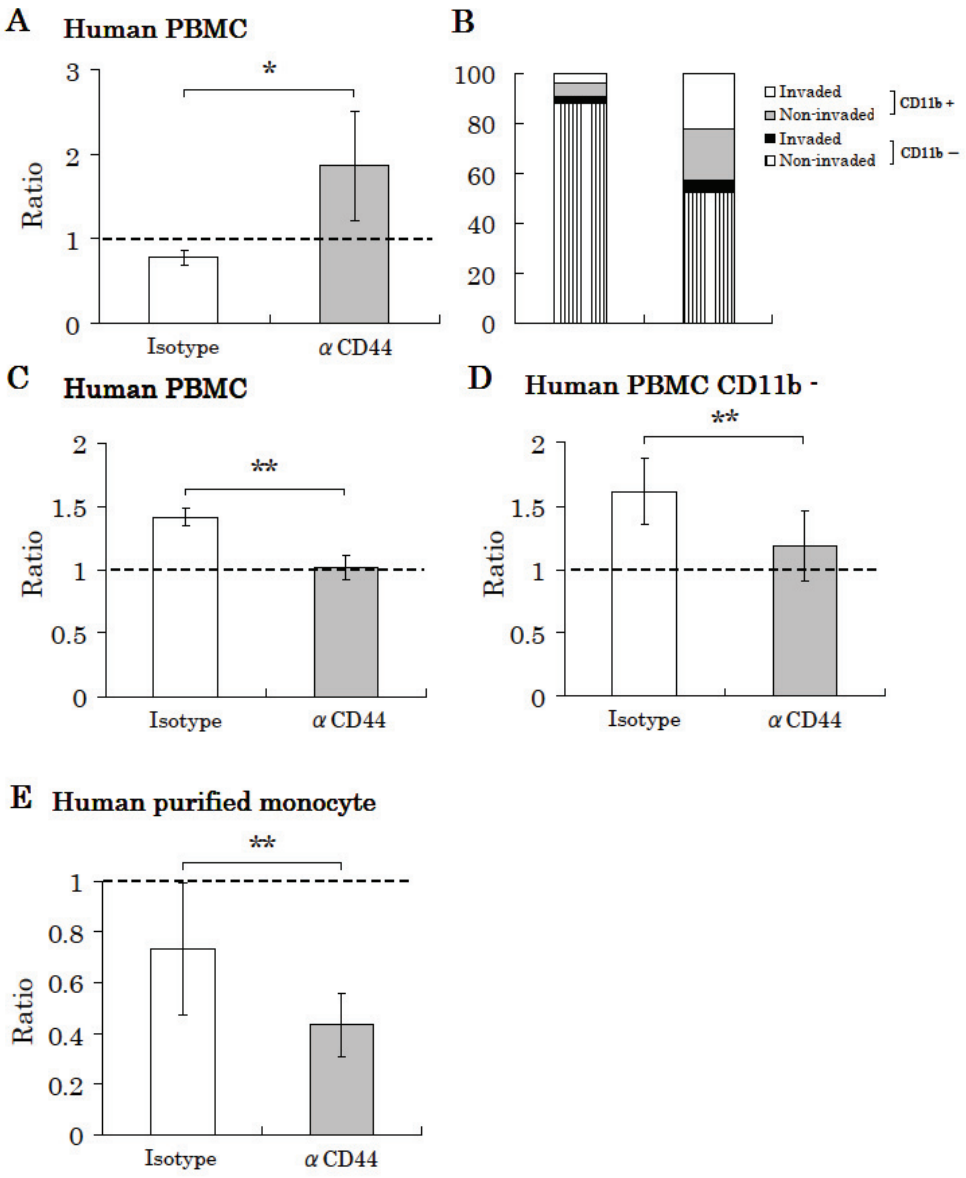


Fig.1–4 Contribution of CD44 of *T. gondii*–infected human leukocytes to adhesion to HA.

Infected human PBMCs or monocytes were added to immobilized HA and infection rate of adhering cells was determined. Results are presented as fold change compared with infection rate of population as a whole. (A) Infection

rate of HA–coated and non–coated plate tested with human PBMCs. (B) The frequency of CD11b positive and negative cells/ parasite–invaded and non–invaded cells before and after the panning on the HA–coated plate. (C) Infection rate of HA–coated plate tested with mouse PBMCs in the presence of anti–CD44 antibody or isotype control. (D, E) Infection rate of HA–coated plate tested with mouse CD11b positive and negative PBMCs in the presence of anti–CD44 antibody or isotype control (D and E, respectively). Paired t–test was used for comparisons between two groups. Dashed lines indicate ratio = 1.0. The data represent the average and S.D. of 3 (B, C and E), 4 (A) or 6 (D) independent experiments (*$p < 0.05$, **$p < 0.01$).

CHAPTER 2)

Adhesion of *T. gondii* tachyzoite–infected leukocytes to vascular endothelium is independent of CD44

INTRODUCTION

T. gondii orally infects intermediate hosts that include the majority of mammals. The ingested parasites then cross the intestinal epithelium and invade leukocytes in the general circulation. These parasites within infected leukocytes disseminate into peripheral organs including the brain (17, 19, 37, 81). The mechanism by which infected leukocytes transfer *T. gondii* to peripheral organs is not well understood; however, parasite–infected antigen–presenting cells are able to migrate through the blood–brain barrier better than uninfected leukocytes *in vitro* (46).

To investigate parasite dissemination *in vivo*, a method was recently established to estimate the number of leukocytes and parasites that flow into solid organs and remain there (83). In this technique, GFP–positive PBMCs

infected with genetically–modified tachyzoites harboring the DsRed Express gene were injected into the bloodstream of a recipient mouse and copy numbers of GFP and DsRed Express genes were quantified by real–time PCR (qRT–PCR). The rates of PBMCs flowing into the lung and liver and remaining there were significantly higher than that before injection. This indicates that among the circulating GFP–positive PBMCs, the tachyzoite–infected PBMCs were able to remain in the lungs and liver more effectively than the uninfected PBMCs. Lung tissue is known to be frequently infected with *T. gondii* during the acute phase of infection in immunosuppressed patients (31). In a mouse model of infection, it has also been reported that after oral infection with *T. gondii* cysts, the parasites travel to the lungs *via* the intestines before any other organs in the abdominal cavity become infected (6). The tachyzoite–infected PBMC preference for lung tissue might result in such a parasite–dissemination pattern occurring during the acute infection phase. An infected PBMC with a pseudopod was reportedly observed in the lung of a recipient mouse (83). This finding suggests that the remaining tachyzoite–infected PBMCs in the lungs did not simply clog up the blood capillaries, but adhered to the

endothelial cells; this might therefore be the initial site of cell–to–cell infection and dissemination from the infected cells in the circulation to the peripheral tissues.

It was previously shown that infected leukocytes exhibit higher levels of CD44 expression and stronger adherence to HA compared to uninfected leukocytes, as described in chapter 1. There is therefore a possibility that circulating tachyzoite–infected leukocytes may adhere to the vascular wall and/or to the ECM of solid organs including the brain *via* CD44 on the surface of infected leukocytes, thus facilitating parasite dissemination in the host body. Vascular endothelial cells increase HA expression when they are stimulated by inflammatory cytokines such as TNF–α and IL–1β (43, 54, 84), and this reaction is induced during acute *T. gondii* infection (13, 63).

In this chapter, the effect of inflammatory stimulation by *T. gondii* infection on transport of the parasite was examined, and the involvement of CD44 on the surface of leukocytes with parasite transport was also investigated.

MATERIALS AND METHODS

Mice

C57BL/6 J mice were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The enhanced GFP transgenic mouse, C57BL/6–Tg (CAG–EGFP) C14–Y01–FM131 Osb, was provided by the RIKEN BioResource Center (Tsukuba, Japan). This mouse has a C57BL/6J genetic background with a GFP transgene, which shows autosomal dominant inheritance with complete penetrance (41, 57). CD44 knockout mice, B6.129 (Cg)–Cd44 tm1Hbg/J (61) were obrained from the Jackson laboratory (Bar Harbor, USA).

GFP knock–in and CD44 knockout mutant mice (GFP/CD44KO) were established by the method described as follows. First, CAG–EGFP was crossed with B6.129 (Cg)–Cd44 tm1Hbg/J. The heterozygous mutant (GFP heterozygous–CD44 heterozygous) mice were subsequently crossed with B6.129 (Cg)–Cd44 tm1Hbg/J to generate GFP heterozygous (GFP–positive) –CD44 homozygous (CD44 knockout) mutant.

For the mice induced of inflamed condition, mice were stimulated by lipopolysaccharide (LPS) at a 20 μg in 100 μl PBS or 100 μl PBS as a

negative control for 1 h.

The experiments were performed in accordance with the Gifu University Animal Care and Use Committee guidelines.

Parasites and cells

Tachyzoites of red fluorescent protein (DsRed Express)–expressing transgenic *T. gondii* derived from the PLK strain, PLK/RED (78), were passaged in Vero cells. Vero cells and tachyzoites were maintained in RPMI 1640 medium supplemented with 7.5% FCS and 20 µg/ml gentamicin. Vero cells were incubated at 37 °C in a 5% CO_2 incubator. Splenocytes from GFP–positive mouse, GFP/CD44KO mice were prepared by removal of the whole spleen following ether anesthesia, exsanguination and cervical dislocation in mice. Spleens were minced and pressed through a nylon mesh with a pore size of 77 µm. The filtrated cells were suspended in 9 ml of hemolytic buffer (0.155 M NH_4Cl in dH_2O and 1 ml Tris–HCl pH7.65), left for 15 min at room temperature and washed twice with PBS to remove erythrocytes.

Tachyzoite infection to splenocyte

Purified PLK/RED tachyzoites were prepared from infected Vero cells as described previously (81), and suspended in RPMI 1640 medium supplemented with 7.5% FCS. Splenocytes from GFP–positive mouse, GFP/CD44KO mice were suspended in RPMI 1640 medium supplemented with 7.5% FCS. To prepare PLK/RED infected cells, the tachyzoite and the PBMC or splenocyte suspensions were mixed at appropriate proportions and cultured for 24 h at 37 °C in humidified air with 5% CO_2, supplemented with a 1% Antibiotic–Antimycotic Mixed Stock Solution (100×) (Nacalai Tesque, Kyoto, Japan). After incubation, the infected cells were collected by centrifugation and re–suspended in PBS to determine infection rates of inefected cells by using qRT–PCR as described below, and/or fluorescence microscopy.

Injection of tachyzoite–infected splenocytes

Three hundred μl of the PLK/RED–infected GFP–positive mouse, GFP/CD44KO mice splenocyte suspension was injected into C57BL/6 J mice via the tail vein (3×10^6 cells/mouse). Two hours after the injection, the

peripheral blood from each mouse was removed by terminal exsanguination under anesthesia. Mouse brain, lungs, liver and spleen were collected, as were tissue samples for histological analysis; the latter were immediately fixed in 4% paraformaldehyde in PBS overnight at 4 °C. Tissue samples for DNA extraction were kept at –80 °C.

DNA extraction and real–time quantitative PCR

Genomic DNA from purified PLK/RED tachyzoites, non–infected GFP–positive splenocytes, PLK/RED infected GFP–positive splenocytes, or organ samples were extracted using a E.Z.N.A.™ Tissue DNA Isolation Kit (Omega Bio–tek, USA), according to the manufacturer's instructions. To verify the amount of DsRed Express and GFP genes in the extracted DNA samples, qRT–PCR was performed using SYBR Premix Ex Taq™ II (TaKaRa, Kyoto), with primers specific for the DsRed Express and GFP genes. The primer sequences are as follows. DsRed Express: 5'–CTCCGACGGCCCCGTAATGC–3' and 5' –AGGTAGTGGCCGCCGTCCTT–3' ; GFP: 5'–TGCTGCTGCCCGACAA–3' and 5'–TGTGATCGCGCTTCTCGTT–3'. PCR conditions: 95 °C for 30 s, 40

cycles at 95 °C for 5 s, and 60 °C for 30 s. Using DNA samples containing known numbers of the purified PLK/RED tachyzoites and the noninfected GFP–positive splenocyte as controls, the number of GFP–positive splenocytes and PLK/RED parasites in each sample was determined. The infection rates of the GFP–positive splenocyte remaining in each organ were calculated as follows. Infection rate (%) = (the number of PLK/RED/the number of splenocytes from the GFP transgenic mouse) ×100.

Statistical analysis

A paired t–test and Mann–Whitney U test was used for comparisons between the two groups. Statistical significance was set at $p < 0.05$.

RESULTS

T. gondii enrichment in peripheral organs is independent of the inflammatory condition of the organs

To examine the effect of the inflammatory conditions of peripheral organs on leukocyte adhesion, LPS was injected into recipient C57/BL6J mice before injection of PLK/RED–infected GFP–positive splenocytes. Although LPS stimulation increased the total number of GFP–positive splenocytes remaining in the lungs compared to that in unstimulated mice, the number of PLK/RED was not changed by LPS stimulation (Fig. 2–1). These data suggests that the vascular endothelium of peripheral organs does not alter attachment of *T. gondii*–invaded leukocytes regardless of the inflammatory condition of the host.

Leukocyte surface CD44 is independent of adhesion of T. gondii–infected leukocytes to vascular endothelium

Active leukocytes increase their adherence to endothelial cells *via* expression of CD44 (5, 10, 22, 30, 67), suggesting that active leukocytes are

likely to remain in the tissue by adhering to the endothelium. To investigate whether or not *T. gondii*–infected leukocyte enrichment in some organs is related to leukocyte expression of CD44. To this end, GFP–positive and CD44 knockout mice (GFP/CD44KO) and GFP–positive (GFP/CD44WT) splenocytes were infected with PLK/RED and injected into recipient mice. As shown in Fig. 2–2, the total number of GFP/CD44KO splenocytes remaining in the lungs and liver was significantly lower than that of GFP/CD44WT. In contrast, the numbers of parasites remaining were similar in both cases. These data suggest that adhesion of *T. gondii*–invaded leukocytes to the endothelium occurs in a CD44–independent manner.

DISCUSSION

A previous report showed that tachyzoite–infected PBMCs in the general circulation are enriched in the lung (83), although the mechanism underlying this phenomenon remains unclear. However, it has been reported that *T. gondii* infection alters the expression levels of host adhesion molecules on the cell surface (82). Such alteration of adhesion molecules on the surface of infected PBMCs might facilitate *T. gondii* dissemination into the peripheral organs of the host.

A method to estimate the number of leukocytes and parasites flowing into solid organs and remaining there was recently established. However, the dissemination pattern of tachyzoite–infected PBMCs was studied only in animals without any inflammatory changes using this method (83). In many cases of natural infection, it is likely that inflammatory cytokines are produced and act on endothelial cells during the early stage of infection. In this study, I examined the effect of inflammatory stimulation on dissemination of tachyzoite–infected PBMCs. It is reported that LPS stimulation increases the efficiency of neutrophil adherence to endothelial

cells in the lung (59). This suggests that the inflammatory conditions induced by LPS change the flow pattern of leukocytes in the general circulation into solid organs and the pattern of their remaining in these organs. Therefore, LPS was used to induce inflammatory conditions in recipient mice. Although the total number of leukocytes flowing into the lung from the general circulation and remaining there was increased by LPS injection, the number of parasite–infected splenocytes in the lung was unchanged (Fig. 2–1). This suggests that adherence of the parasite–infected leukocyte occurs in an inflammation–dependent manner.

It has also been reported that CD44 on the surface of leukocytes facilitates their adhesion to the endothelium (19, 56, 81). As shown in Fig. 2–2, it was reconfirmed that the total number of CD44–KO leukocytes remaining in the lung was significantly lower than that of CD44–expressing leukocytes. However, both leukocytes lacking and harboring CD44 transported parasites into the lungs with a similar efficiency (Fig. 2–2). This indicates that adhesion of *T. gondii*–infected leukocytes to the endothelium is independent of CD44 expression on the surface of the leukocytes.

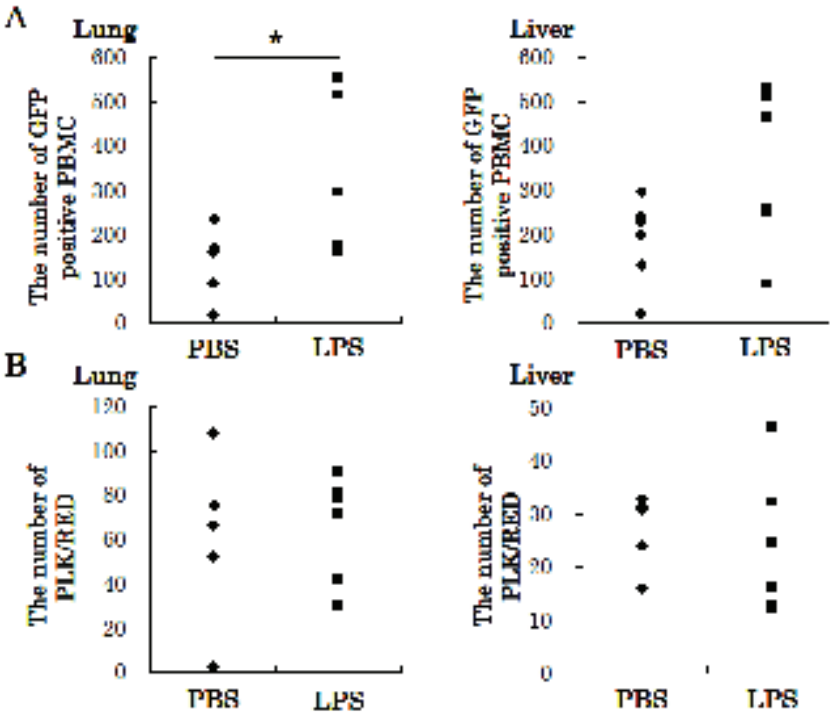


Fig. 2–1 The effect of inflammatory stimulation on leukocyte enrichment in peripheral organs.

C57BL/6J mice were injected with LPS or PBS as a negative control. Stimulated mice were injected with PLK/RED infected mouse PBMCs expressing GFP. (A) Estimated number of GFP–positive leukocyte in peripheral organs (2 h post infection). (B) Estimated number of DsRED Express–positive PLK/RED in peripheral organs (2 h post infection). Statistical analysis was carried out using Mann–Whitney U test (two experiments with a total of four recipient mice, $*p < 0.05$, $**p < 0.01$).

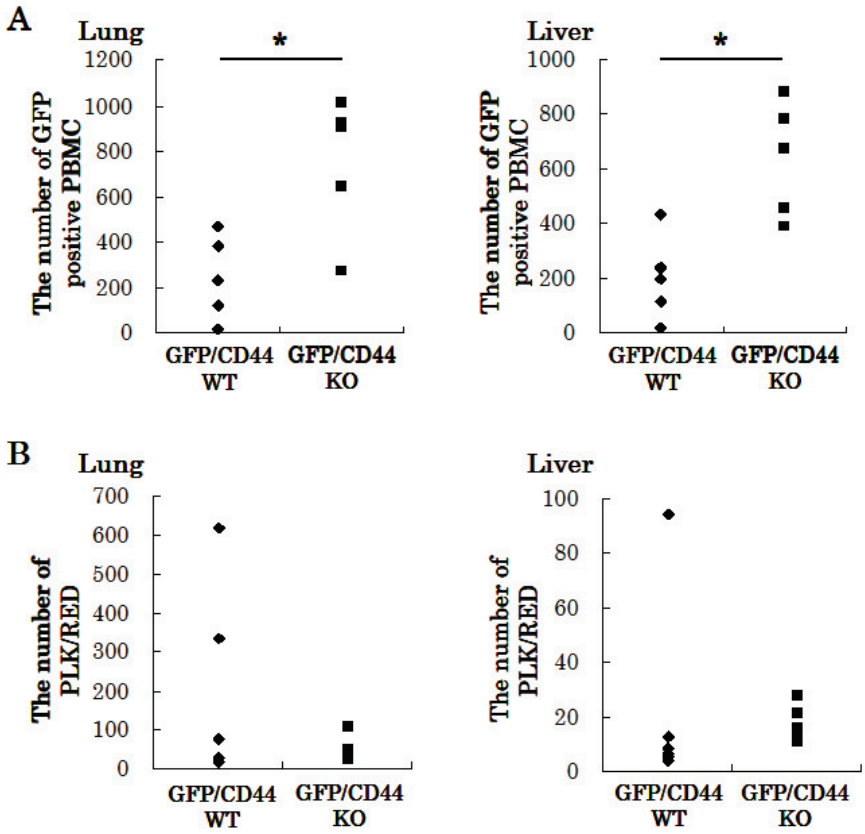


Fig. 2–2 The role of CD44 on leukocyte enrichment in peripheral organs.

C57BL/6J mice were injected with PLK/RED infected GFP/CD44WT or GFP/CD44KO mouse splenocytes. (A) Estimated number of GFP–expressing leukocyte in peripheral organs (2 h post infection). (B) Estimated number of DsRED Express–positive PLK/RED in peripheral organs (2 h post infection). Statistical analysis was carried out using Mann–Whitney U test (two experiments with a total of four recipient mice, $*p < 0.05$, $**p < 0.01$).

CHAPTER 3)

Stage–conversion of *T. gondii* in cells lacking MAPK p38α

INTRODUCTION

T. gondii is an obligate intracellular protozoan parasite. During infection in intermediate hosts, which include humans and domestic animals, *T. gondii* undergoes stage–conversion between the rapidly dividing tachyzoite and the slowly replicating bradyzoite stage. The majority of *T. gondii* tachyzoites in the host body are normally eliminated by the host immune system during the early phase of infection, which is consequently asymptomatic. However, some of the tachyzoites differentiate into bradyzoites and chronically infect the brain and skeletal muscle, possibly for the entire life of the hosts. In AIDS and other immunocompromised conditions, proliferating tachyzoites produce a virulence that can lead to severe morbidity as well as mortality. In patients with AIDS, clinical

toxoplasmosis is considered to primarily result from reactivation of chronic infection, causing cyst rupture and release of rapidly multiplying tachyzoite form of the parasite (35).

It is reported that tachyzoite growth inhibition is linked to bradyzoite differentiation (4). In addition, it is also known that a variety of artificial stress conditions, such as pH shock, heat shock, mitochondrial inhibitors, chemical stress, arginine starvation, and nitric oxide induce bradyzoite differentiation from tachyzoite–infected cultured mammalian cells (3, 4, 33, 74, 89). However, it is currently unknown whether these artificial stress conditions act as a trigger of *T. gondii* differentiation in natural infection. Among these conditions, mitogen–activated protein kinase (MAPK) p38α and p38β inhibition may participate in the *in vivo* trigger because activation/inactivation of *in vivo* MAPK p38 is a stress–response reaction (28). The MAPK family comprises the following three sub–families: c–Jun activated kinases, extracellular signal–related kinases and p38 (14, 18). P38 families are divided into four isoforms (p38α, p38β, p38γ, and p38δ) (52), and it has been reported that p38α and p38β inhibitors, such as SB202190 and SB203580, inhibit intracellular tachyzoite replication and induce bradyzoite

differentiation (80, 85). However, it is unclear whether these inhibitors directly or indirectly act on the parasite through effects of the inhibitors on the host cell. Some reports suggest a direct effect of the inhibitors on the parasite. In a previous study, it was reported that the p38α and p38β inhibitor SB203580 blocks *T. gondii* MAPK (TgMAPK–1) autophosphorylation (8) and that several p38α and p38β inhibitors block parasite replication (85). Nevertheless, inhibition of TgMAPK–1 has not yet been demonstrated as a mechanism of action of SB203580 in blocking parasite replication. Further, Wei *et al.* established an SB203580–resistant strain of *T. gondii,* which is resistant to the anti–parasitic effect of the chemical, suggesting that it acts not only on the host cell but also on the parasite (85). However, it is possible that the inhibitor actually acts on the host cell to change the intracellular environment and the parasites that adapt to the environmental change in the host cell are selected.

In contrast, it has been reported that stage–conversion could be induced by transient treatment of host cells with the p38α and p38β inhibitors before tachyzoite infection (62). This strongly suggests that p38α and p38β inhibitors do not act directly on the parasites but on the host cells. There is

no evidence that inhibition or loss of host cell p38α and p38β results in *T. gondii* stage–conversion from tachyzoite to bradyzoite. Therefore, under stress conditions, the role of host cell p38α on *T. gondii* differentiation was examined in this chapter. In this study, the effect of p38α activity of host cells on *T. gondii* stage–conversion was examined as the first step.

MATERIALS AND METHODS

Cell lines

Vero cells were grown in RPMI1640 containing 7.5% fetal bovine serum. p38α deficient mouse embryonic fibroblasts (KOPs) and revertant mutants of p38α (RKOPs) were provided from RIKEN BRC CELL BANK (Tsukuba, Japan) and grown in MEM–α containing 10% FCS (75).

Parasites

The transgenic PLK strain of *T.gondii*, PLK/DUAL, expressing the DsRED Express in tachyzoite stage and the GFP in bradyzoite stage (PLK/DUAL), was maintained in Vero cells (80).

Chemicals

SB202190 (Merck Millipore, Billerica, USA), an inhibitor of p38α and p38β, were used for induction of *T.gondii* stage–conversion.

LY294002 (Sigma) and wortmannin (Sigma), an inhibitor of phosphatidylinositol 3–kinase (PI3K) were used to inhibit PI3K of host cell.

Quantification of parasite growth

KOPs and RKOPs were seeded and cultured overnight. After cultivation, these cells were infected with tachyzoites of PLK/DUAL at a moi of 1.5. Parasite growth was assessed at 12 h and 24 h after infection by counting the number of individual parasites per parasitophorous vacuole, scoring 100 randomly chosen vacuoles per time point.

Induction of stage–conversion from tachyzoite to bradyzoite

KOPs and RKOPs were seeded and cultured overnight in MEM–α containing 10% fetal bovine serum. After the incubation, the medium was replaced to D–MEM with high glucose (WAKO, Osaka, Japan) containing 1% fetal bovine serum and 8 μM SB202190 for 3 h. The cultured cells were once washed with D–MEM with high glucose containing 1% fetal bovine serum and tachyzoites of PLK/DUAL suspended in D–MEM with high glucose containing 1% fetal bovine serum infected to KOPs and RKOPs at a moi of 1.5. After 4 h incubation with the parasite, the supernatant was replaced to D–MEM with high glucose containing 1% fetal bovine serum and 8 μM SB202190 (85). In the case of PI3K inhibitors, cells were pre–treated with

the inhibitors for 3 h and PLK/DUAL was allowed to infect for 5 h. The inhibitors were treated after PLK/DUAL infection.

Flow cytometry

Cells were detached by treating with typsin and ethylenediaminetetraacetic acid. PBS was added to the suspensions and they were collected by centrifugation for 10 minutes at 2000 rpm. Pellets containing infected cells were fixed with 4% PFA for 15 minutes at room temperature. Cells were once washed with PBS and resuspended in PBS for the analysis. Flow cytometry was performed on FACS Canto II (BD Biosciences).

Statistical analysis.

Paired t–test was used for comparison between two groups. Analysis of variance (ANOVA) was used for comparisons between more than two groups. If the result of an ANOVA was significant, a post hoc, Tukey–Kramer test was performed. Statistical significance was set at $p < 0.05$.

RESULTS

Parasite replication in cells lacking p38α (KOP) and revertant cells (RKOP)

First, the tachyzoite replication level in KOP, a cell line lacking p38α, and its revertant, RKOP was examined. As shown in Fig. 3–1, although the parasite replication level at 12 h after infection was similar in both cell types, the replication level in RKOP was significantly higher than that in KOP at 24 h after infection (Fig. 3–1). This result indicates that host cell p38α regulates *T. gondii* replication.

Bradyzoite differentiation in KOPs lacking p38α

The next study investigated whether the lack of host cell p38α results in differentiation of the parasite into the bradyzoite stage. To check this, KOPs and RKOPs were infected with tachyzoites of PLK/DUAL with or without the p38α and p38β inhibitor SB201290. In the absence of SB202190, stage–conversion was not observed in either of the host cell types, indicating that the lack of p38α alone is not sufficient to trigger stage–conversion (Fig. 3–2 A). In contrast, SB202190 treatment induced stage–conversion of

parasites in both host cells. The frequency of bradyzoite stage–conversion among all parasites did not differ significantly between the two types of host cells (Fig. 3–2 A). This indicates that SB202190 induces parasite stage–conversion in host cells in an p38α–independent manner. However, as shown in Fig. 3–2 B, GFP expression by bradyzoites in RKOP cells was significantly higher than that in KOPs (Fig. 3–2 B, C).

The effect of the PI3K inhibitor SB202190 on bradyzoite differentiation

To exclude the possibility that inhibition of the PI3K/Akt pathway is a potential trigger of bradyzoite differentiation, PLK/DUAL–infected KOPs and RKOPs were treated with the well–known PI3K inhibitor LY–294002 (IC50 = 1.4 μM) (89) and wortmannin (IC50 = 3 nM). Neither 1 nor 10 μM of LY–294002 nor 100 nM nor 1 μM of wortmannin induced stage–conversion (Table 3–1, Fig. 3–3). This indicates that inhibition of host cell PI3K alone does not result in parasite stage–conversion.

DISCUSSION

In this chapter, host cell p38α involvement with bradyzoite differentiation was investigated. Because bradyzoite differentiation is linked to inhibition of tachyzoite growth (9), the tachyzoite replication level in KOP p38α–deficient cells and their revertants, RKOP, was examined. Although parasite growth was reduced in KOPs compared with that in RKOPs, (Fig. 3–1), few bradyzoites were detected in either cell type in the absence of SB202190 treatment at 3 days p.i. (Fig. 3–2 A). In addition, no GFP–expressing parasites were detected in KOPs by 5 days p.i. under fluorescent microscopic analysis (data not shown). These results indicate that host cell p38α deficiency cannot be a direct trigger of the parasite stage–conversion. Parasite growth was inhibited in KOPs (Fig. 3–1), suggesting that host cell p38α is involved in modulation of the intracellular environment to control *T. gondii* tachyzoite replication. SB202190, the p38α and p38β inhibitor, induced stage–conversion of the parasite in p38α–deficient KOP cells at a similar efficiency. This indicates that initiation of stage–conversion by SB202190 is independent of host cell p38α. Inhibition of host cell p38β and/or

direct effect on the parasite may trigger stage–conversion.

PLK/DUAL expresses GFP under the control of a bradyzoite–specific antigen 1 (BAG1) promoter and parasites strongly expressing BAG1 showed bright GFP fluorescence (80). Parasites with strong and weak GFP expression can be considered to represent mature and immature bradyzoites, respectively. This study showed that SB202190 more effectively induces maturation of bradyzoites in RKOPs than in p38α–deficient KOPs (Fig. 3–2 B, C). This indicates that inhibition of host cell p38α is, at least partially, necessary for progression of the parasite stage–conversion. It is unknown why the inhibition of p38α in RKOPs results in parasite stage–conversion, while complete deficiency of the molecule is necessary in KOPs. It is possible that the function of p38α activity in RKOP may be substituted by other molecules that are resistant to SB202190. In this study, the effect of a PI3K inhibitor was also examined because SB202190 inhibits not only p38α and p38β activity but also weakly inhibits Protein Kinase Bα (20), which is involved in PI3K/Akt signaling pathway and consists of a signal cascade downstream of PI3K (88). However, as shown in Fig. 3–3 and Table 3–1, PI3K inhibitors did not induce the parasite stage–conversion.

Several host genes involved in *T. gondii* differentiation have previously been identified. Overexpression of human cell division autoantigen–1 (CDA1), which arrests the host cell cycle (11), inhibits tachyzoite growth and induces expression of cyst wall protein (62). It is also known that in CD73–deficient mice there is a decrease in the number of cysts in the brain (50). However, the expression levels of CDA1 and CD73 in the brain and skeletal muscles are not very different from those in other organs (11, 16). Thus it remains unknown why stage–conversion of *T. gondii* from the tachyzoite to bradyzoite stage preferentially occurs in the brain and muscle. In this study, it is revealed that host cell p38α is involved in the progress of parasite stage–conversion. Organ/tissue–specific p38α expression and/or activity may therefore be related to this preference.

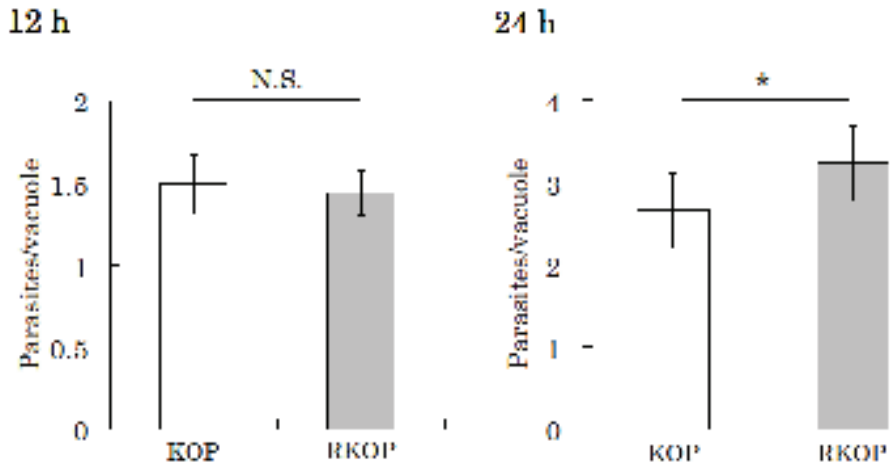


Fig. 3–1 Parasite growth in KOPs and RKOPs at 12 h and 24 h p.i.

The number of PLK/DUAL parasites per vacuole in KOPs and RKOPs at 12 h and 24 h p.i. Statistical analysis was carried out using Paired t–test. The data represent the average and S.D. of 3 independent experiments (*$p < 0.05$, **$p < 0.01$).

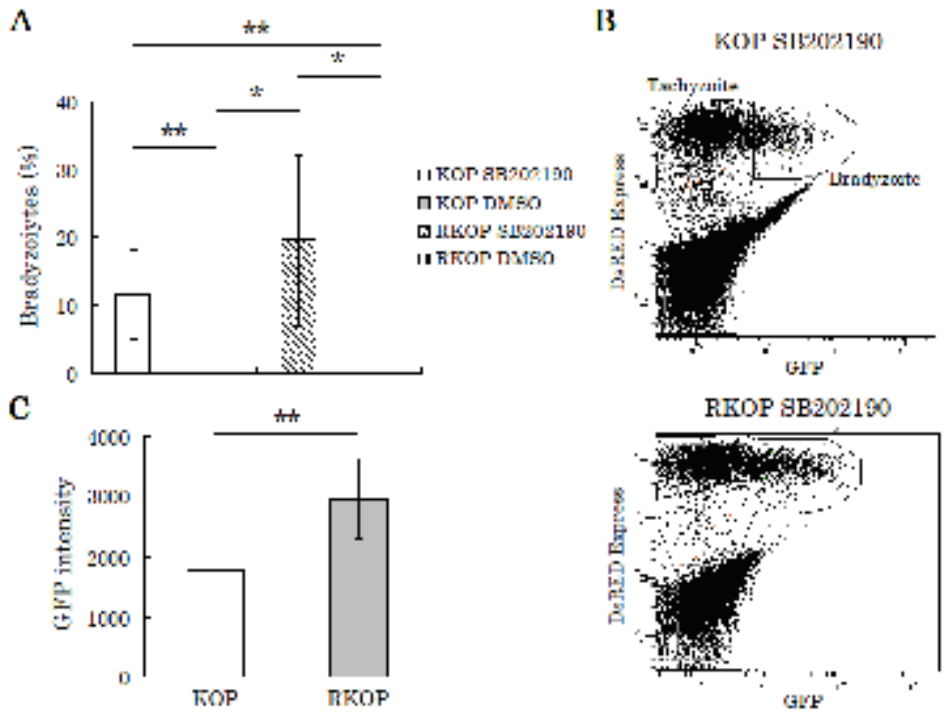
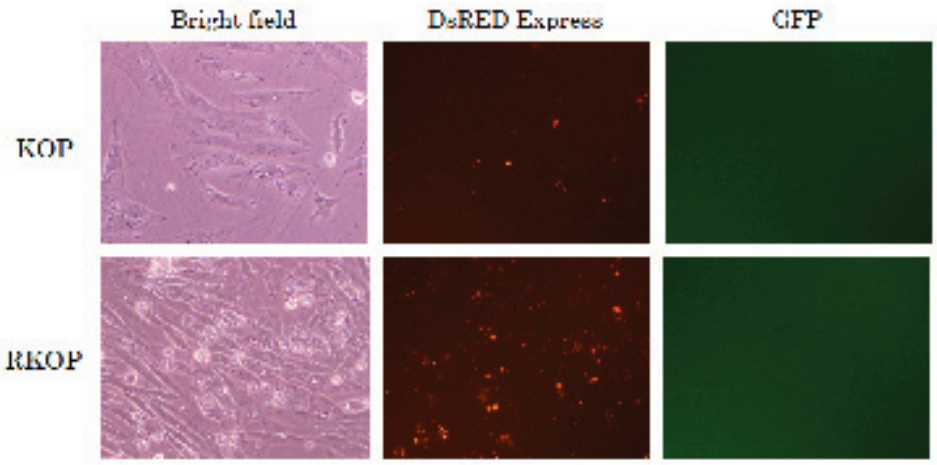


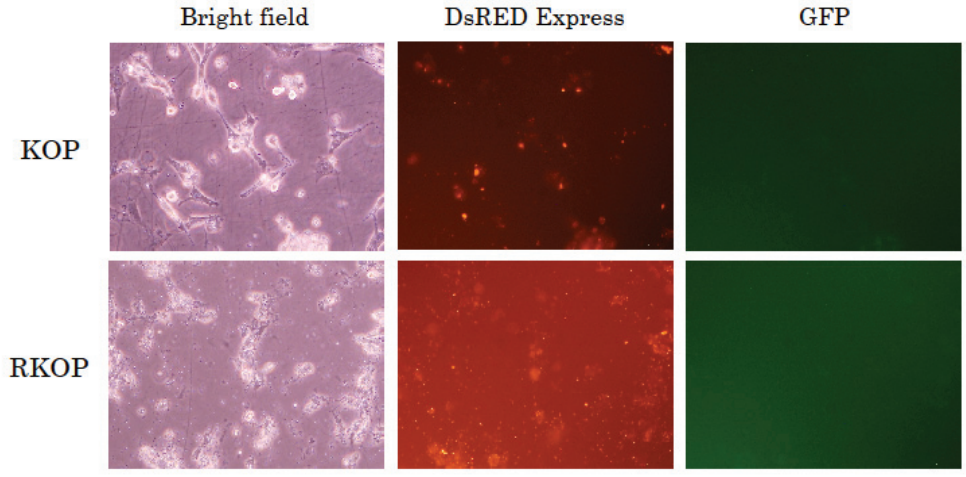
Fig. 3–2　Bradyzoite differentiation in KOPs and RKOPs at 3 days p.i.

(A) Frequency of GFP–expressed PLK/DUAL bradyzoites in KOPs and RKOPs infected with the parasites. (B) Distribution of GFP expressing parasite. (C) GFP fluorescent intensity of PLK/DUAL in KOPs and RKOPs. ANOVA was used for comparisons between more than two groups. If the result of an ANOVA was significant, a post hoc, Tukey–Kramer test was performed. The data represent the average and S.D. of 6 independent experiments (*$p < 0.05$, **$p < 0.01$).

LY294002 (10 μM)



Wortmannin (1 μM)



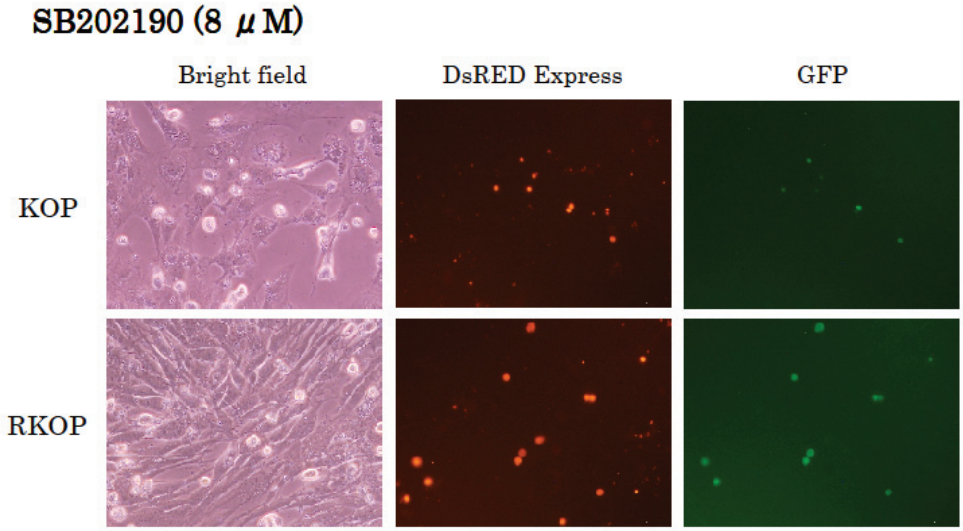


Fig. 3–3 The effect of PI3K inhibitor on *T. gondii* differentiation.

PLK/DUAL infected and PI3K inhibitors treated KOPs and RKOPs at 5 days p.i..

Table 3-1
IC50 of PI3K inhibitors and bradyzoite differentiation by the inhibitors.

	Reported IC50 of PI3K inhibition	Concentrations in Fig. 3-2	Bradyzoite	
			KOP	RKOP
LY294002	1.4 μM (89)	1 μM	–	–
		10 μM	–	–
Wortmannin	3 nM	100 nM	–	–
		1 μM	–	–

	PKBα activity (% of control)	Concentrations in Fig. 3-2	Bradyzoite	
			KOP	RKOP
SB202190 (10 μM)	53±2 (20)	8 μM	+	+

GENERAL DISCUSSION

In this study, *T. gondii*–infected leukocytes were demonstrated to adhere to HA *via* CD44 (Chapter 1). Leukocytes are reported to be the primary transporter of *T. gondii*, and parasite–infected leukocytes are suggested to extravasate from the bloodstream into the tissue matrix with the parasite inside (19, 46, 81). CD44, which binds to ECM components including HA, is reported to play an important role in leukocyte migration. A previous study demonstrated up–regulated expression of *T. gondii*–infected leukocyte CD44 and an increase in the number of HA–adhered leukocytes containing *T. gondii* (82). However, any causal relation between higher CD44 expression and HA adhesion activity of *T. gondii*–infected leukocytes is unknown. Using CD44–deficient mouse splenocytes, *T. gondii*–infected leukocyte accumulation on HA *via* CD44 was revealed. This suggests that CD44 on the surface of *T. gondii*–infected leukocytes contributes to parasite dissemination to peripheral organs by binding to the ECM of vascular endothelium and/or tissue. Although it has been reported that HA–binding spleen cells are undetectable until day 5 after *in vivo* alloantigen stimulation and are

maximal at day 7 (49), leukocytes increased adherence to HA within 24 h of *T. gondii* infection (Fig. 1–1, 1–3). The parasite–invaded leukocytes showed relatively higher migration than non–invaded cells (83). Nevertheless, the number of GFP–positive and GFP/CD44KO leukocytes remaining in peripheral organs was the same, indicating that host leukocyte adhesion to vascular endothelium is a CD44–independent mechanism. Stimulation of the trophoblast–derived choriocarcinoma cell line BeWo with *T. gondii*–infected cell supernatant increased THP–1 monocytic cell adhesion to the BeWo cell monolayer *via* intercellular adhesion molecule–1 (ICAM–1) on the BeWo cell surface (58). ICAM–1 can therefore be suggested to have a potential role in the adhesion of *T. gondii*–infected cells to the endothelium.

T. gondii growth is reported to differ between cell types among peripheral blood leukocytes, and the parasite preferentially infects and divides in monocytes rather than in other leukocytes (15). CD11b–positive myeloid cells predominantly adhere to HA (Chapter 1), indicating the effective dissemination of the parasites using monocytes, which contain larger numbers of the parasite per cell compared with other types of leukocytes and which preferentially migrate to tissues. In chapter 2, however, the number of

parasites did not differ between injection of GFP–positive and GFP/CD44KO splenocytes. These results suggest that parasite dissemination predominantly depends on its proliferation within leukocytes rather than on leukocyte migration and that 24 h of splenocyte culture with the parasite before injection is insufficient for their proliferation.

T. gondii–infected leukocytes are transported to the brain where chronic infection can be established. Several types of cells in the brain can contain latent cysts, including microglial cells and astrocytes (32). Microglial cells and astrocytes both arise from circulating hematopoietic cells, which infiltrate into the brain (29). It is therefore possible that microglial cells and astrocytes containing latent cysts are derived from tachyzoite–infected leukocytes in the circulation. There is no evidence that leukocytes outside the brain contain bradyzoites, suggesting that tachyzoites first differentiate into bradyzoites within leukocytes after invading the brain. Further, bradyzoites have been detected in neural cells in addition to microglial cells and astrocytes (32), suggesting that the environment in the brain induces bradyzoite differentiation. It is unclear why bradyzoites are predominantly induced in the brain (69). The brain has been reported to allow *T. gondii* to

survive for long periods (68), and it is alleged that some of the long–surviving parasites differentiate into bradyzoites. However, the type II strain of *T. gondii* tachyzoite that forms bradyzoites and latent cysts *in vivo* showed poor differentiation into bradyzoites in cultured host cells *in vitro* (data not shown), indicating that long–survival is not indispensable for bradyzoite differentiation. In addition, a bradyzoite–specific antigen was induced by 1 h treatment with alkaline medium (26). This indicates that bradyzoite conversion can start in a few hours, which is sufficient time for the tachyzoites to differentiate before they are eliminated in peripheral organs. Host cell p38α was found to be involved in the progress of stage–conversion from tachyzoite to bradyzoite (Chapter 3). Although p38α activity in the brain during *T. gondii* infection is unknown, there is the possibility that brain–specific dynamics of p38α activity result in stage–conversion and cyst formation in the brain.

In this study, the behavior of *T. gondii* in an intermediate host, including parasite dissemination by the parasite–infected leukocytes and chronic infection, was demonstrated. CD44 is suggested to be involved in parasite dissemination to peripheral organs in intermediate hosts. It is important to

understand the way in which parasites are transmitted by infected leukocytes. Therefore, this study adds valuable knowledge that will help prevent systemic infection. This is also important for prevention from chronic infection because a reduction in the number of parasites transmitted to the organs can result in a decrease in the number of latent cysts. In addition, to prevent stage–conversion of the parasite in the organs, an understanding of the mechanism of bradyzoite differentiation is essential. These results show that chronic infection could possibly be controlled by regulating host cell p38α.

These studies expand knowledge of the *T. gondii* life cycle in its intermediate hosts. This will lead to better protection of humans and other mammals against *T. gondii* infection.

ACKNOWLEDGEMENTS

My completion of this study could not have been accomplished without the support, patience and guidance of the following people. It is to them that I owe my deepest gratitude.

First of all, I would like to express my deep gratitude to Dr. Akihiro Unno, The University of Nagoya, for his valuable advices.

Secondaly, I am grateful to dear departed Professor Professor Dr. Hisashi Inokuma (Obihiro University of Agriculture and Veterinary Medicine), Professor Dr. Tadashi Itagaki (Iwate University), Professor Dr. Junsuke Shirai (Tokyo University of Agriculture and Technology) and Professor Dr. Sadatoshi Maeda (Gifu University), The United Graduate School of Veterinary Science, Gifu University.

Finally, I wish to thank the members of Gifu University Laboratory of Veterinary Parasitological Diseases for their warm support.

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