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Up-regulation of hyaluronan receptors in *Toxoplasma gondii*-infected monocytic cells

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ABSTRACT

The apicomplexan, obligate intracellular parasite *Toxoplasma gondii* orally infects humans and animals. The parasites cross the intestinal epithelium, invade leukocytes in the general circulation and then disseminate into the peripheral organs. The mechanism of extravasation of the infected leukocytes, however, remains poorly understood. It is known that adhesion of leukocytes to extracellular matrix (ECM) is an important factor in extravasation, and CD44 and ICAM-1 on the leukocyte surface are known receptors for hyaluronan (HA), an ECM component. In this study, we demonstrated up-regulation of CD44 and ICAM-1 expression on the surface of *T. gondii*-infected human monocytic THP-1 cells and fresh isolated human monocyte. *T. gondii*-infected THP-1 cells adhered more efficiently to immobilized HA than did non-infected cells. *T. gondii*-infected monocytes in the general circulation might preferentially adhere to the ECM and migrate out from blood vessels, so transporting parasites into the peripheral organs.

Introduction

Toxoplasma gondii is an intracellular, apicomplexan parasite responsible for congenital infections and abortion in immunocompromised individuals, and opportunistic infections in immunodeficient individuals. *T. gondii* infection usually occurs through oral ingestion of oocysts or tissue cysts. After oral ingestion, the parasites cross the intestinal epithelium and invade the general circulation. It is known that *T. gondii*-infected leukocytes in the general circulation transport the parasite to the peripheral organs [1–3]. Monocytes appear to be more susceptible to *T. gondii* infection than other peripheral blood cells [4]. Monocytes are therefore important as transporters of *T. gondii* during its dissemination throughout the host body.

Monocytes in the general circulation are responsible for the transport of several pathogenic organisms to peripheral organs, including human cytomegalovirus, HIV and measles virus [5–8]. Monocytes infected with these viruses shows higher levels of expression of integrins, such as lymphocyte function-associated antigen-1 (LFA-1), that mediate intercellular adhesion to the endothelium. The migratory ability of infected monocytes is higher than that of non-infected monocytes [9–12]. These properties of infected monocytes could promote the extravasation of infected cells into the tissues and so facilitate virus dissemination. In contrast, it has been reported that *T. gondii* infection had no effect on the expression level of LFA-1 on the surface of infected monocytic cells [13]. The majority of infected leukocytes in the general circulation harbor a single parasite, implying that infected leukocytes, included monocytes, migrate out from the blood vessels before proliferation of the parasites [1,2]. It is possible that *T. gondii* promotes the extravasation of infected leukocytes by a mechanism independent of up-regulation of intercellular adhesion to the endothelium; in addition to adhesion to the endothelium, adhesion of leukocytes to the extracellular matrix (ECM) is also considered to be an important factor for extravasation of leukocytes into the tissues [14,15]. Hyaluronan (HA) is a major non-protein glycosaminoglycan component of the ECM. In this study, we examined the effects of *T. gondii* infection of the expression levels of HA receptors on human monocytic cells.

Materials and methods

Parasites. Tachyzoites of green fluorescent protein (GFP)-expressing transgenic *T. gondii* derived from PLK strain [16] was 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.

Cells. The human myelomonocytic cell lines, THP-1 and HL60, were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. THP-1 and HL60 cells were maintained in RPMI 1640 medium supplemented with 10% FCS and incubated at 37 °C in a 5% CO_2 incubator. Prior to *T. gondii*

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infection, THP-1 cells were suspended in medium supplemented with 80 nM 1α,25-dihydroxyvitamin D_3 (VD_3) at a concentration of 2×10^5 cells/ml and cultured for 48 h. In the case of HL60, prior to *T. gondii* infection, HL60 cells were suspended in medium supplemented with 12 nM 1α,25-dihydroxyvitamin D_3 (VD_3) at a concentration of 1×10^5 cells/ml and cultured for 72 h. The fresh isolated monocytes were obtained from a healthy volunteer. Peripheral blood mononuclear cells (PBMC) in the volunteer's peripheral blood were collected using Lympholyte®-Mammal (CEDARLANE, Ontario, CANADA) and monocytes in the PBMC were isolated by negative immunoselection procedure (monocyte isolatuib Kit II; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The experimental protocols using human sample were reviewed and approved by the local ethics committee.

Tachyzoite infection. VD_3-pre-treated monocytic cells and fresh isolated monocyte were suspended in RPMI 1640 medium supplemented with 10% FCS and RPMI 1640 medium supplemented with 10% FCS and 80 pM VD_3 at a concentration of 4×10^6 cells/ml, respectively. Then 200 μl of the cell suspension was added to each well of 24-well cell culture plates. *T. gondii* tachyzoites were released from infected Vero cells, as described previously [1] and suspended in RPMI 1640 medium supplemented with 10% FCS at a concentration of 2×10^6 cells/ml or suspended in RPMI 1640 medium supplemented with 10% FCS and 80 pM VD_3 at a concentration of 2×10^5 cells/ml. Then 200 μl of the tachyzoite suspensions, 2×10^6 tachyzoites/ml and 2×10^5 tachyzoites/ml, were added to the 200 μl of monocytic cells and fresh isolated monocyte suspensions, respectively. The cell cultures were maintained at 37 °C in humidified air with 5% CO_2 for 24 h.

Measurement of CD11a, CD14, CD44, and CD54 (ICAM-1) by flow cytometry. *Toxoplasma gondii*-infected and mock-infected cells were washed with 500 μl phosphate-buffered saline (PBS). The washed cells were re-suspended in PBS at a concentration of 1×10^7 cells/ml. Human IgG was added to the cell suspension at 1.0 μg/10^5 cells using 0.9 mg/ml human IgG solution, and incubated for 15 min at room temperature. The cells were stained using the following antibodies: phycoerythrin (PE)-conjugated mouse IgG2aκ anti-human CD11a antibody, PE-conjugated mouse IgG2aκ anti-human CD14 antibody, PE-conjugated mouse IgG1κ anti-human CD44 antibody, PE-conjugated mouse IgG1κ anti-human CD54 antibody, PE-conjugated mouse IgG1κ isotype control, or PE-conjugated mouse IgG2aκ isotype control (BD, Tokyo, Japan), according to the manufacturer's recommendations. The cells were incubated for 45 min on ice in the dark, washed three times with 4 ml PBS and re-suspended in PBS for analysis. Flow cytometry was performed on a FACSCalibur (BD Biosciences PharMingen, San Diego, CA).

Cell adhesion assay. The *T. gondii*-infected and mock-infected THP-1 cells were washed with 500 μl PBS. The washed cells were re-suspended in RPMI 1640 medium supplemented with 10% FCS at a concentration of 1×10^6 cells/ml. For the cytoadherence assay, several 10-mm diameter circles were drawn on the base of plastic petri dishes (Falcon 1007: B D). Then 40 μl of 100 μg/ml HA-BVM (Sigma, Tokyo, Japan) in PBS or PBS were adsorbed onto the surface of each circle for 24 h at 4 °C. The reactions were subsequently blocked by the addition of 1% bovine serum albumin (BSA) in PBS for 1 h at 4 °C. HA-coated and non-coated spots were overlaid with 4 ml of PLK/GFP tachyzoite-infected or mock-infected THP-1 cell suspensions, and adhesion was allowed to occur for 90 min at 37 °C. Unbound cells were removed by gentle washing twice with 4 ml PBS before bound cells were counted under a light microscope. The rate of PLK/GFP tachyzoite infection of THP-1 cells was confirmed using a fluorescence microscope.

Statistical analysis. Student's *t*-test was used for comparisons between two groups. Analysis of variance (ANOVA) was used for comparisons among more than two groups. If the result of an ANOVA was significant, a post hoc, Scheffe's *F*-test was performed. Statistical significance was set at $p < 0.05$.

Results and discussion

Effect of T. gondii infection on maturation and integrin expression of monocytic cells

It is known that virus-infected monocytes differentiate into macrophages and express higher level of integrins, such as CD11a, than non-infected monocytes [9–12]. It has been suggested that these effects promote the migration of infected monocytes out from the blood vessels, and so facilitate the dissemination of the infected virus. To investigate this possibility, we examined the expression levels of a monocyte/macrophage marker, CD14, and an integrin, CD11a, in *T. gondii*-infected monocytic THP-1 cells. THP-1 cells infected with *T. gondii* tachyzoites 24 h prior to the examination showed higher expression levels of CD14 (Fig. 1A). This indicates that tachyzoites induced the differentiation of pre-monocytic THP-1 cells into monocytes/macrophages. However, in contrast with the situation observed during virus infection [12], *T. gondii* did not alter the expression level of CD11a (Fig. 1B). This lack of effect on CD11a expression was consistent with the results of a previous report [13]. These results suggest that *T. gondii* infection induces the differentiation of infected monocyte-lineage cells, but does not up-regulate integrin expression, or at least not CD11a expression.

Effect of T. gondii infection on the expression level of HA receptors on monocytic cells and fresh isolated human monocyte

There are several receptors for HA, including CD44, ICAM-1, RHAMM, LYVE-1, TLR2, and TLR4 [17–20]. However, of these, only CD44, ICAM-1, TLR2, and TLR4 are expressed in monocytes, while RHAMM and LYVE-1 are not [21]. CD44 and ICAM-1 play important roles in the migration of monocytes/macrophages [22,23]. We examined the effects of *T. gondii* infection on the expression levels of CD44 and ICAM-1 on monocytic THP-1 cell, HL60 cell and fresh isolated human monocytes. THP-1 cells and fresh isolated human monocytes infected with *T. gondii* tachyzoites 24 h prior to the examination showed higher expression levels of CD44 and ICAM-1 than mock-infected THP-1 cells (Fig. 2A). Another monocytic cell line, HL60, also showed up-regulation of ICAM-1 expression by

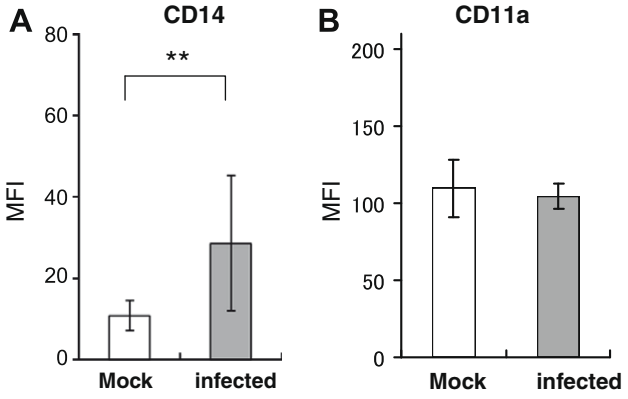

Fig. 1. Effect of *T. gondii* infection on maturation and integrin expression in monocytic cells. Surface expression levels of CD14 (A) and CD11a (B) in PLK/GFP-infected and mock-infected THP-1 cells measured using PE-conjugated anti-CD14 and CD11a, respectively. Results are presented as the mean ± S.D. of mean fluorescence intensity (MFI). Statistical analysis was carried out using Student's *t*-test ($n = 3$ independent experiments, $^{**}p < 0.01$).

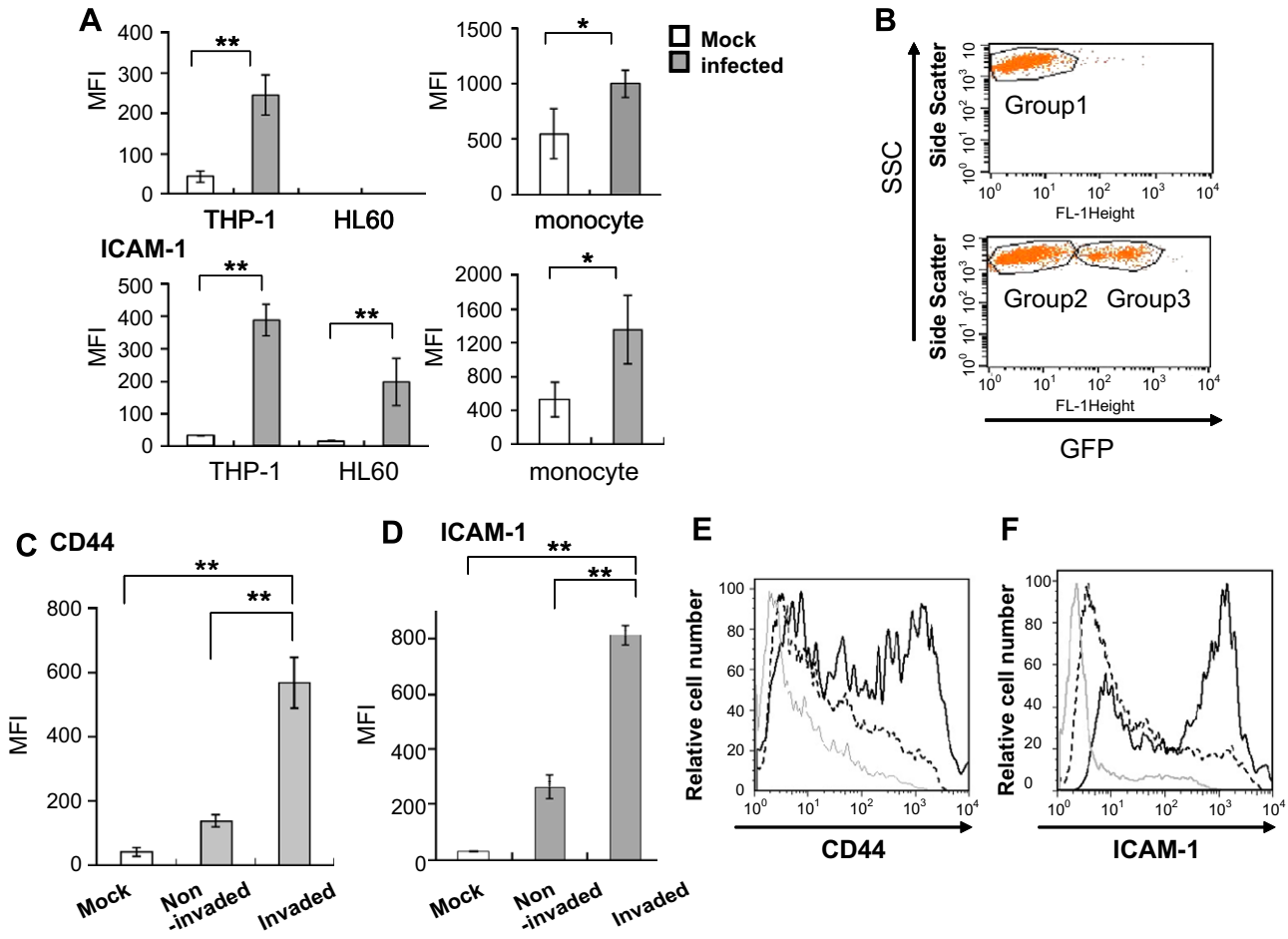

Fig. 2. Effect of *T. gondii* infection on the expression levels of HA, CD44 and ICAM-1 in monocytic cells and human fresh isolated monocytes. (A) Expression levels of HA receptors. The surface expression levels of CD44 (upper panel) and ICAM-1 (lower panel) in PLK/GFP-infected and mock-infected monocytic cells, THP-1 and HL60, and human fresh isolated monocytes. Results are presented as the mean ± S.D. of MFI. Statistical analysis was carried out using Student's *t*-test ($n = 3$ independent experiments, $^{**}p < 0.01$, $^{*}p < 0.05$). (B) Distinction between parasite-invaded and non-invaded cells. Green fluorescence of PLK/GFP-infected and mock-infected THP-1 cells was detected by flow cytometry (lower and upper panels, respectively). (C,D) Expression levels of HA receptors in mock-infected, parasite-invaded and non-invaded cells. Data for CD44 (C) and ICAM-1 (D) in mock-infected, parasite-invaded and non-invaded cells are shown as the mean ± SD of MFI ($n = 3$ independent experiments, $^{**}p < 0.01$). (E,F) Flow cytometric histogram of HA receptor-expressing cells. Representative data for CD44 (E) and ICAM-1 (F) are shown. Gray solid line, black dotted line and black solid line indicate data for mock-infected, non-invaded and parasite-invaded cells, respectively.

T. gondii infection (Fig. 2A). However, CD44 expression level on HL60 cells was under detectable level regardless of *T. gondii* infection (Fig. 2A). Therefore, we used THP-1 cells for further experiments as a better model, which reflects characters of human peripheral monocytes.

It was reported that both type I and type II strains were dominantly isolated in Toxoplasmosis patients [24,25]. Although we showed the effect of a type II strain, PLK, in Fig. 2, it was observed that the infection of a type I strain, RH, also up-regulate the expression level of HA receptors, CD44 and ICAM-1 on the monocytic cells (data not shown).

To determine if parasite invasion was necessary for the up-regulation of these molecules, individual cells in the infected cell culture were separated into either parasite-invaded or non-invaded cells (Fig. 2B). We used a GFP-expressing transgenic parasite, PLK/GFP, and parasite-invaded cells were therefore detectable due to their green fluorescence. As shown in Fig. 2B lower panel, non-invaded and invaded cells were gated as fluorescence-negative 'group 2' and -positive 'group 3' cells, respectively. Cells in the mock-infected culture showed no green fluorescence and were gated as group 1 (Fig. 2B, upper panel). The expression levels of CD44 and ICAM-1 in the parasite-invaded cells (Group 3) were significantly higher than in the mock-infected (Group 1) and non-invaded cells (Group 2) (Fig. 2C and D). As shown in Fig. 2E and F, the majority of non-invaded cells (Group 2) showed slightly higher expression levels of these molecules than the mock-infected cells (Group 1). CD44 expression in monocytes is increased by TNF-α and IL-10 stimulation [26]. The slight up-regulation of CD44 expression in the non-invaded cells (Group 2) might be a result of secretion of these cytokines by parasite-invaded cells (Group 3). In contrast with the non-invaded cells, the majority of parasite-invaded cells (Group 3) showed much stronger expression of CD44 and ICAM-1 (Fig. 2E and F). These results indicate that *T. gondii* infection up-regulates CD44 and ICAM-1 expression in both parasite-invaded and non-invaded cells, but parasite invasion is necessary to induce strong expression of CD44 and ICAM-1.

Effects of T. gondii infection on monocytic cell adhesion to HA

To investigate whether *T. gondii*-infected THP-1 cells adhered to immobilized HA, we incubated mock-infected and *T. gondii*-infected THP-1 cells on plastic plates coated with HA. The number of adhering *T. gondii*-infected cells to HA-coated plates was higher than that of mock-infected cells (Fig. 3A). We also determined if parasite-invaded cells were enriched on immobilized HA when a mixture of parasite-invaded and non-invaded cells was added. An

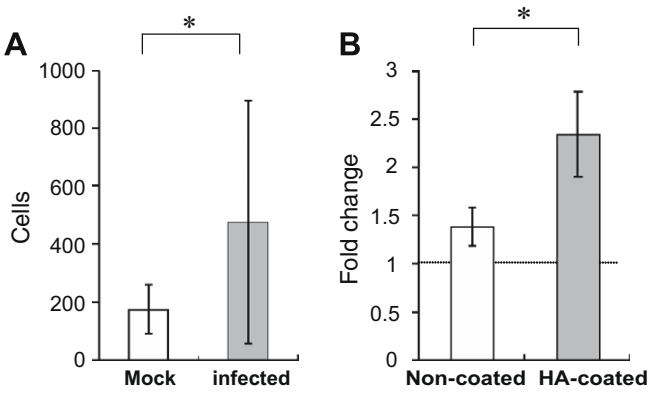

Fig. 3. Effects of *T. gondii* infection on monocytic cell adhesion to immobilized HA. (A) Adhesion of mock-infected and PLK/GFP-infected THP-1 cells. The numbers of cells adhering to HA-coated plastic plates are shown. Results are presented as the mean ± SD. Statistical analysis was carried out using Student's *t*-test ($n = 10$ independent experiments, $^*p < 0.05$). (B) Infection rates of HA-coated and non-coated plate adhering cells. Open bar indicates infection rate of non-coated plate adhering cells. Solid bars indicate those of HA-coated plate adhering cells. Results are presented as fold change compared with infection rate of the population as a whole (the mean ± SD, $n = 3$ independent experiments). Statistical analysis was carried out using Student's *t*-test ($n = 3$ independent experiments, $^*p < 0.05$).

adhesion assay was performed using THP-1 cells cultured with *T. gondii* tachyzoites for 24 h and the infection rate of adhering cells was determined. The infection rate of the HA-adhering cells was higher than that of the population as a whole (Fig. 3B), indicating that parasite-invaded cells were enriched on the immobilized HA. Although the infection rate of non-coated plate adhering cells was also slightly higher than that of the population as a whole, the infection rate of HA-adhering cells were significantly higher than that of non-coated plate adhering cells (Fig. 3B). The slight increasing of the infection rate observed in non-coated plate adhering cells might be due to the attachment of parasite-invaded cells to BSA used for blocking. These results suggest that parasite-invaded monocytes show increased adhesion to ECM, allowing them to preferentially migrate out from blood vessels. In this study, we showed the possibility that *T. gondii*-invaded monocytes in the general circulation preferentially adhere to ECM through the up-regulation of receptors for HA. This could provide the mechanism for efficient transport of *T. gondii* into peripheral organs. Further studies are needed to study the adhesion of intracellular pathogen-invaded leukocytes not only to endothelial cells, but also to ECM.

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