Notes

Increased CD11b$^+$ Gr-1$^+$ cell population in the placenta after infection with *Toxoplasma gondii*

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Running title: Immune cells in placenta with *T. gondii*

ABSTRACT

***Toxoplasma gondii* (*T. gondii*) is an obligate intracellular protozoan pathogen that can cross the placenta, resulting in congenital Toxoplasmosis with severe brain abnormality in fetus. The molecular mechanisms of immune responses against *T. gondii* infection in the placenta have largely remained unclear. We established an analytical method to characterize phenotypes of immune cells in the placenta by flow cytometry. We show that the CD11b$^+$ Gr-1$^+$ cell population was significantly increased in the placenta after *T. gondii* infection. These results suggest that innate immune responses may play an important role in immunity against *T. gondii* infection via the feto-maternal interface.**

Key words： **CD11b$^+$ Gr-1$^+$ cell, flow cytometry analysis, placenta, *Toxoplasma gondii***

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan pathogen. The infection can be acquired through oral ingestion of infected, undercooked meat or contaminated food or water. Following oral infection, the parasite crosses the intestinal epithelium, and disseminates widely. IFNγ derived from Th1 cells and NK cells plays an important role in immune responses against *T. gondi*i (1). In addition, Gr-1^{+} inflammatory monocytes, but not neutrophil, are also required for mucosal resistance to *T. gondii* (2, 3). It has been reported that neutrophil also plays an important role in host protection from *T. gondii* as a source of IFNγ (4). Moreover, neutrophil also release neutrophil extracellular traps (NET) that limits *T. gondii* infection (5). However, evidences suggested that neutrophils are invaded by *T. gondii* and play a role as motile reservoirs of *T. gondii*, leading to parasite spread in the body (6).

T. gondii can cross the placenta, resulting in congenital Toxoplasmosis with visual and hearing loss, mental and psychomotor retardation, seizures, and hematological abnormalities in fetus (7-10). Despite the fact that *T. gondii* continues to be a severe threat to the fetus during pregnancy, the immunological control against *T. gondii* infection and molecular mechanisms of immune responses in the placenta have

largely remained unclear..

To examine the immune responses against *T. gondii* infection in the feto-maternal interface, we established an analytical method of immune cells in the placenta using a murine pregnant model with *T. gondii* infection. Mice were infected or not with 30 cysts of *T. gondii* (PLK strain; avirulent cyst-forming (type 2) strain) through oral inoculation on the seventh day of gestation (G7). Previous studies demonstrated that the abortion was observed around G16 when mice were inoculated with cysts on G7 (11), suggesting that *T. gondii* infection affected the homeostasis of the feto-maternal interface until G16. Thus, we obtained the placental tissue, in which the embryos were totally removed, on G16 to analyze the effect of *T. gondii* infection on immune cell populations in the placenta. The placental tissue was minced into fragments of ~1 mm^3 and then digested for 45 minutes at 37^o C under agitation in PBS with 1 mg/ml collagenase type D (Roche Applied Science, Mannheim, Germany) (**Fig 1a**). The cell suspension was filtered through sterile stainless-steel 100 nylon mesh and resuspended in PBS with 2% FBS. Placental cells were then simultaneously stained with PE-conjugated anti-CD45.2 monoclonal antibody (mAb) (BD Bioscience San Jose,

CA), a hematopoietic marker, and FITC or APC-conjugated mAbs against lineage markers, including CD3, CD4, CD8, B220, NK1.1, Gr-1 and/or CD11b (BD Bioscience).

Because flow cytometry analyses showed that the hematopoietic cells contained in placental cells could be detected as $CD45.2^+$ cells (**Fig 1b)**, we analyzed the cells on the gate of $CD45.2^+$ cell population. The percentage of $CD11b^+$ $Gr\text{-}1^+$ cells in $CD45.2^+$ placental cells accounted for 5.73 ± 3.4 % in $CD45.2^+$ placental cells of non-infected mice on G16 (**Fig 1b and 1c)**. We then investigated the placenta of mice on day 9 (G16) after *T. gondii* inoculation. We observed that the percentage of the $CD11b^+$ $Gr\text{-}1^+$ cell population was significantly increased (23.3 ± 8.2 %) in $CD45.2^+$ cells of the placenta with *T. gondii* infection, compared with that of non-infected mice (**Fig 1b and 1c**). By contrast, the $CD11b^-$ $Gr\text{-}1^-$ cell population was significantly decreased in the percentage of $CD45.2^+$ hematopoietic cells in the placenta with *T. gondii* infection (36.5 ± 8.0 %), compared with that of non-infected mice (60.1 ± 13.1 %) (**Fig. 1b and 1c**). However, $CD11b^+$ $Gr\text{-}1^-$ cell population was comparable between infected and non-infected mice (32.3 ± 10.8 % versus 37.0 ± 9.2 %) (**Fig. 1b and 1c**). We then analyzed the

lymphocyte population of the lymphocyte gate (FSC^{low}, SSC^{low}) in $CD45.2^+$ cells (**Fig. 2**). The each percentage of $CD4^+$ T cell, $CD8^+$ T cell, Natural Killer (NK) cell and B cells population was comparable between infected and non-infected mice on day 9 (G16) after *T. gondii* inoculation (**Fig. 2a and 2b**).

Because CD11b and Gr-1 are expressed on both $Ly6G^+$ neutrophils and $Ly6C^+$ inflammatory monocytes, it remained undetermined whether the increased cells were neutrophils, inflammatory monocytes or both. Nevertheless, $CD11b^+$ $Gr\text{-}1^+$ cells are able to secret proinflammatory cytokines and chemokines, nitric oxide and/or reactive oxygen species (12, 13), which are involved in immunity against *T. gondii*. However, it may be also possible that $CD11b^+$ $Gr\text{-}1^+$ cells might be involved in spreading *T. gondii* to fetus as motile reservoirs(6). At present, the role of $CD11b^+$ $Gr\text{-}1^+$ cells increased in the placenta after *T. gondii* infection remains unclear. Further analyses should be required to clarify the pathophysiology of *T. gondii* infection in pregnant mice. Flow cytometory analysis of immune cells in the placenta may be a useful technique to analyze immune responses in the placenta against various pathogens, including cytomegalovirus, rubella, parvovirus as well as *T. gondii*, which cause congenital

infection via placenta.

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DISCLOSURE

No authors have any conflict of interests to disclose.

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FIGURE LEGENDS

Figure 1. Flow cytometry analysis of immune cells in placenta from non-infected mice and infected mice on day 9 (G16) after *T. gondii* inoculation.

a. The day of plug detection was considered as day 0 of gestation (G0). *T.gondii* infection Group mice were p.o. infected with 30 cysts of *T. gondii* on G7. Placental tissue was obtained on G16 (9 days after infection).

b. Flow cytometry analyses of $CD11b^+$ $Gr\text{-}1^+$ cells, $CD11b^+$ $Gr\text{-}1^-$ cells and $CD11b^-$ $Gr\text{-}1^-$ cells in CD45.2 cells in placenta from non-infected and infected mice on day 9 (G16) after *T. gondii* inoculation.

c. The percentage of $CD11b^+$ $Gr\text{-}1^+$ cells, $CD11b^+$ $Gr\text{-}1^-$ cells and $CD11b^-$ $Gr\text{-}1^-$ cells in CD45.2 cells in placenta from non-infected (n=6) and infected (n=6) mice on day 9 (G16) after *T. gondii* inoculation. Error bars indicate SD. Data are representative of two independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$. (unpaired student t test).

Figure 2. Flow cytometry analyses of lymphocyte subsets in placenta from

non-infected mice and infected mice on day 9 (G16) after *T. gondii* inoculation.

a. Flow cytometry analyses of lymphocyte subsets in placenta. The cells in placenta from pregnant non-infected mice and *T. gondii*-infected mice on G16 were stained with specific antibodies against CD45.2, CD4, CD8, NK1.1 and B220 analyzed by flow cytometry.

b. The percentage of $CD4^+$ $CD3^+$ T cells, $CD8^+$ $CD3^+$ T cells, $NK1.1^+$ $CD3^-$ NK cells and $B220^+$ $CD3^-$ B cells in the lymphocyte gate (FSC^{low}, SSC^{low}) in the placenta of non-infected (n=6) and *T. gondii*-infected (n=6) mice on G16. Error bars indicate SD. Data are representative of two independent experiments. **$p < 0.05$; ***$p < 0.001$. (unpaired student t test).

LIST OF ABBREVIATIONS

APC：allophycocyanin

FITC : fluorescein isothiocyanate

FSC : forward scatter

IFNγ：interferon ganma

PE : phycoerythrin

SSC : side scatter

Figure 1

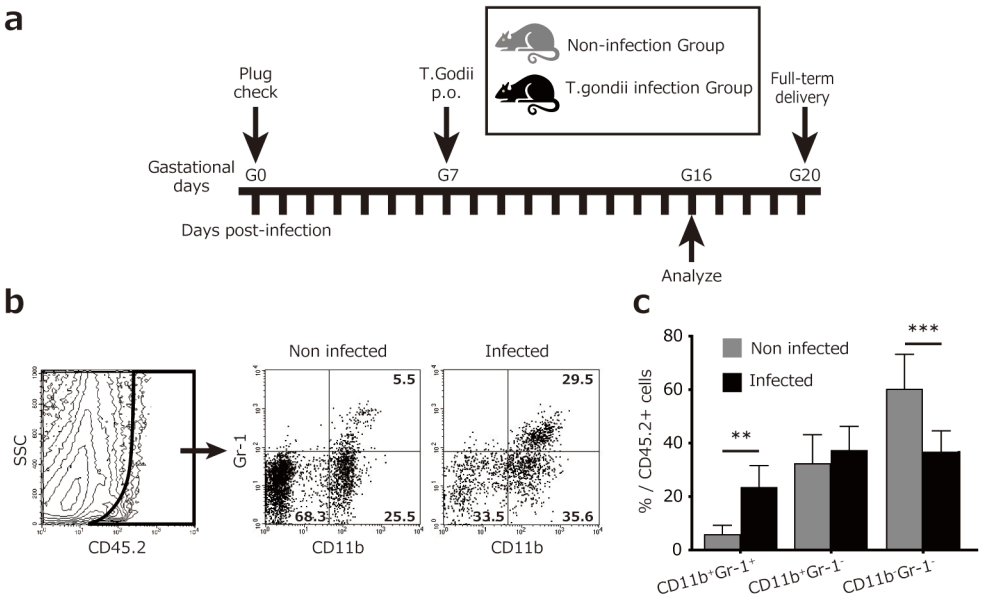
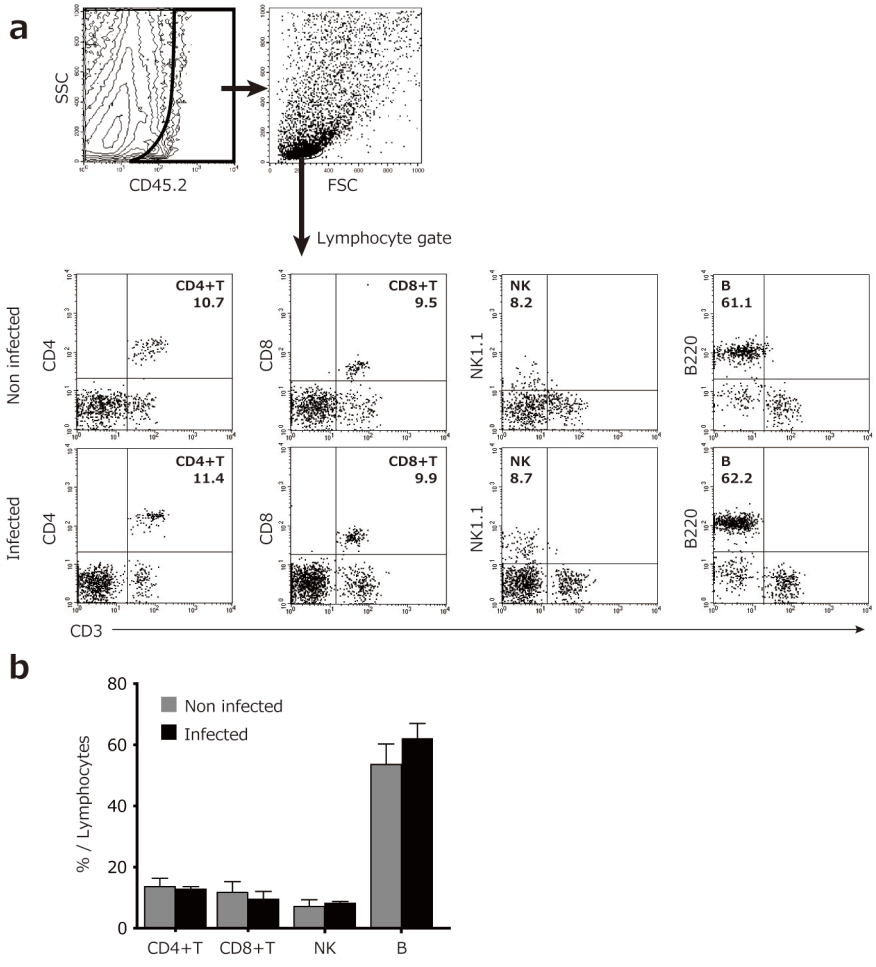

a
Non-infection Group
T.gondii infection Group
Plug check
T.Godii p.o.
Full-term delivery
Gastational days
G0
G7
G16
G20
Days post-infection
Analyze
b
Non infected
Infected
SSC
CD45.2
Gr-1
CD11b
5.5
29.5
68.3
25.5
33.5
35.6
c
Non infected
Infected
% / CD45.2+ cells
80
60
40
20
0
**
***
CD11b+Gr-1+
CD11b+Gr-1-
CD11b-Gr-1-

Figure 2


a
SSC
CD45.2
FSC
Lymphocyte gate
Non infected
Infected
CD4
CD8
NK1.1
B220
CD4+T
10.7
CD8+T
9.5
NK
8.2
B
61.1
CD4+T
11.4
CD8+T
9.9
NK
8.7
B
62.2
CD3
b
Non infected
Infected
% / Lymphocytes
80
60
40
20
0
CD4+T
CD8+T
NK
B