Role of innate immune cells in protection against *Toxoplasma gondii* at inflamed site

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Abstract: The intraperitoneal infection with *Toxoplasma gondii (T. gondii)* caused accumulation of γδ T, NK, NK1.1⁺T-like (NKT) cells at inflamed sites. To clarify the roles of these cells in protection against *T. gondii* at the inflamed sites, BALB/c mice were depleted of γδ T, NK, NK and NKT cells by treatment with antibody against TCR-γδ, asialoGM1 or Interleukin-2 receptor β-chain (IL-2 Rβ), respectively, prior to infection. Mice treated with anti-TCR-γδ monoclonal antibody (mAb) became more susceptible to infection, whereas mice treated with anti-IL-2Rβ mAb acquired resistance. Treatment with anti-asialoGM1 Ab showed no effect. We previously reported that heat shock protein 65 (HSP65) in macrophages induced by γδ T cells plays an essential role in protective immunity against *T. gondii* infection, by preventing apoptotic death of infected macrophages. In the present study, we showed that treatment with anti-IL-2Rβ mAb, but not with anti-asialoGM1 Ab, enhanced the HSP65 induction in macrophages, and inhibited Interleukin-4 (IL-4) expression in nonadherent peritoneal exudate cells. Furthermore, neutralization of endogenous IL-4 by anti-IL-4 mAb enhanced the HSP65 induction in macrophages. These findings suggest that NKT cells, but not NK cells, negatively regulate the protective immunity against *T. gondii* infection possibly by producing IL-4 and suppressing HSP65 induction. J. Med. Invest. 48:73-80, 2001

INTRODUCTION

The intracellular protozoan *Toxoplasma gondii (T. gondii)* is a major opportunistic pathogen among immunocompromised patients worldwide (1-4). *T. gondii* infection is characterized by a rapid multiplication and dissemination of tachyzoites during the acute stage, and following chronic, persistent infection (1-4). Classical CD8⁺ and/or CD4⁺ T cells are essential for the acquisition of protective immunity against infection (1-6), and macrophages activated by various lymphoid effector cells directly protect against obligate intracellular pathogens including *T. gondii* (7). In addition to these cells, innate cells such as γδ T, NK, and NKT cells play essential roles as primary effector cells in the interface until adaptive immunity is established (8-12). However, these innate cells have been shown to exert differential roles depending on the pathogen species (9, 10, 12). For example, γδ T cells appear to play a primary protective role against *Toxoplasma gondii* infection (9), while NK cells protect against *Trypanosoma cruzi* infection (12), and NKT cells are important in protection against *Leishmania major* infection (10).

We previously reported that γδ T cells stimulate the induction of HSP65 in macrophages during the early phase of infection possibly through the release of IFN-γ and TNF-α (9, 13-15). The magnitude of this protein correlated with the protective potential of host mice (9, 16). In the present study, we further examined the specific roles of the innate immune cells in local immune responses to *T. gondii* infection. For

this purpose, BALB/c mice treated with antibody (Ab) against IL-2Rβ, TCR-γδ, or asialoGM1 were intraperitoneally inoculated with *T. gondii*, and the functions of NK, NKT, and γδT cells in the peritoneal cavity were analyzed. We focused especially on NK1.1⁺ T cells that express markers common to the NK cell lineage, such as NK1.1, IL-2Rβ, members of the Ly-49 killer-inhibitory receptor family (17, 18), and the recently identified DX5 (19-21). The functions of these cells are characterized by their ability to immediately produce interleukin (IL)-4 and interferon (IFN)-γ (17, 18, 22, 23). NK1.1⁺ T cells have been studied in mouse strains bearing the NK1.1 allele, such as C57BL/6 mice ; however, several studies strongly suggest that NK1.1⁺ T-like cells (NKT cells) exist also in the NK1.1-negative mouse strains such as the BALB/c mouse (17, 18, 24, 25) used in this study. NKT cells are found mainly in thymus, liver, spleen, and bone marrow (17, 18), while it has not been known whether NKT cells are present or migrate in the peritoneal cavity after intraperitoneal infection of *T. gondii*, and how these cells regulate the local immune responses against the parasite.

We report here that intraperitoneal injection of *T. gondii* caused accumulation of distinct cells that expressed IL-2Rβ and intermediate levels of CD3 (IL-2Rβ⁺ CD3ⁱⁿᵗ cells). These cells, characterized as NKT cells, may negatively regulate the protective immunity against *T. gondii* by down-regulation of HSP65 induction and production of IL-4.

MATERIALS AND METHODS

Animals and parasites

Female BALB/c mice were purchased from Shizuoka Laboratory Center (Shizuoka, Japan), and mice aged between 8 and 11 weeks were used for the present experiments. Bradyzoites of the Beverley strain of *T. gondii* were prepared as previously described (9). Briefly, the brains of mice chronically infected with *T. gondii* were homogenized in RPMI 1640, containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES. The homogenate was centrifuged through a 1.050/1.070 discontinuous arabic gum gradient. Bradyzoites were prepared by treatment of the precipitated cysts with 0.25% trypsin in phosphate-buffered saline (PBS) at 37 for 5 min. Each mouse was infected with 500 parasites by an intraperitoneal injection.

Cell preparation

Peritoneal exudate cells (PEC) were prepared by washing the peritoneal cavity with 5 ml of RPMI 1640 containing 5% fetal bovine serum. PEC were cultured in a 5 cm diameter plastic petri dish (Costar, Cambridge, MA) for 1 hr at 37 in a humidified atmosphere containing 5% CO_2. After culture, nonadherent cells were collected in 15-ml centrifuge tubes and washed three times with PBS. Adherent cells were dislodged by scraping with a cell lifter (Costar) and collected in 15-ml centrifuge tubes. Specific esterase staining of the adherent cells confirmed that approximately 95% of the cells were macrophages.

Antibodies

An anti-interleukin-2 receptor beta chain (IL-2Rβ) monoclonal Ab (TM-β1) was provided by Dr. M. Miyasaka, Tokyo Metropolitan Institute for Medical Sciences (Tokyo, Japan), and an anti-TCR-γδ mAb (UC-7-13D5) was provided by Dr. G. Matsuzaki, Department of Immunology, Medical Institute of Bioregulation, Kyushu University (Fukuoka, Japan). A rabbit polyclonal Ab against asialoGM1 was purchased from Wako Pure Chemical Industries, Osaka, Japan. Phycoerythrin (PE)-conjugated anti-CD3 mAb, PE-conjugated anti-IL-2Rβ mAb (TM-β1), anti-IL-4 mAb (11B11) were purchased from Pharmingen (San Diego, CA). Anti-CD3 (145-2C11) mAb was labeled with FITC. The murine IgG mAb, termed IA10, specific for amino acids 172-224 of HSP65 derived from *Mycobacterium bovis*, was provided by Dr. J. DeBruyn, Institute Pasteur de Brabant (Brabant, Belgium).

In vivo cell depletion

Each mouse was intraperitoneally injected with 500 μg of anti-IL-2Rβ mAb or anti-TCR-γδ mAb, or with 50 μg of anti-asialoGM1 Ab twice on days 1 and 3 before infection. Treatment with 500 μg of anti-IL-4 mAb (11B11) was performed on day 1 before infection and on days 1 and 3 after infection by intraperitoneal injection.

Western blotting

HSP65 was detected by Western blotting as described previously (9). Briefly, protein extracts from peritoneal macrophages were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then the separated proteins were electroblotted onto a polyvinylidene difluoride membrane (Millilore Co., Bedford, MA). After blocking non-specific binding sites with 2% (w/v) bovine serum

albumin (BSA), the membrane was incubated for 1.5 hr at room temperature with a 1:200 dilution of anti-HSP65 mAb (1A10). Bound Abs were detected using an enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, England) using a peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL).

Flow cytometric analysis

Nonadherent PEC were stained with various combinations of the fluorescence-conjugated mAbs. These cells were analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) using two-color staining methods. Before the analysis, the lymphocyte populations were gated by light scatter signals to exclude dead and nonlymphoid cells. The specificity of staining was confirmed using isotype-matched irrelevant mAbs.

Cell sorting

The nonadherent PEC prepared from four or five mice were suspended with RPMI 1640 containing 5% fetal bovine serum and incubated with anti-IL-2Rβ mAb (TM-β1) for 30 min at 4℃. These cells were then washed three times and resuspended in the medium. Sheep anti-Rat IgG-conjugated immunomagnetic beads (Dynal, Oslo, Norway) were added at a target cell : bead ratio of 4 : 1. They were mixed at 4℃ for 60 min in a rotator. The IL-2Rβ^+ cells attached to the Dynabeads were collected using a magnetic particle concentrator (Dynal). We confirmed, by flow cytometric analysis, that IL-2Rβ^+ cells were almost completely removed after this separation.

RT-PCR

Total RNA, prepared from nonadherent PEC or sorted cells, was subjected to RT-PCR, as previously described (14). The specific primer sets were as follows : IL-4, 5'-CTTCTTTCTCGAGTGTACCAGG-3' (sense) and 5'-GTTAAAGCATGGTGGCGCAGTAC-3' (antisense) ; IFN-γ, 5'-AACGCTACACACTGCATCT-3' (sense) and 5'-AGTTCATTCCAATGCTTGG-3' (antisense) ; β-actin, 5'-ATGGATGACGATATCGCT-3' (sense) and 5'-ATGAGGTAGTCTGTCAGGT-3' (antisense) ; Vα14, 5'-CTAAGCACAGCACGCTGCACA-3' (sense) and Cα, 5'-GAAGCTTGTCTGGTTGCTCCAG-3' (antisense) (26).

Ten microliters of PCR products was electrophoresed on a 1.5-2%agarose gel and visualized by ethidium bromide staining.

RESULTS

Accumulation of $\gamma\delta$ T, NK, and NKT cells in peritoneal cavity after T. gondii infection
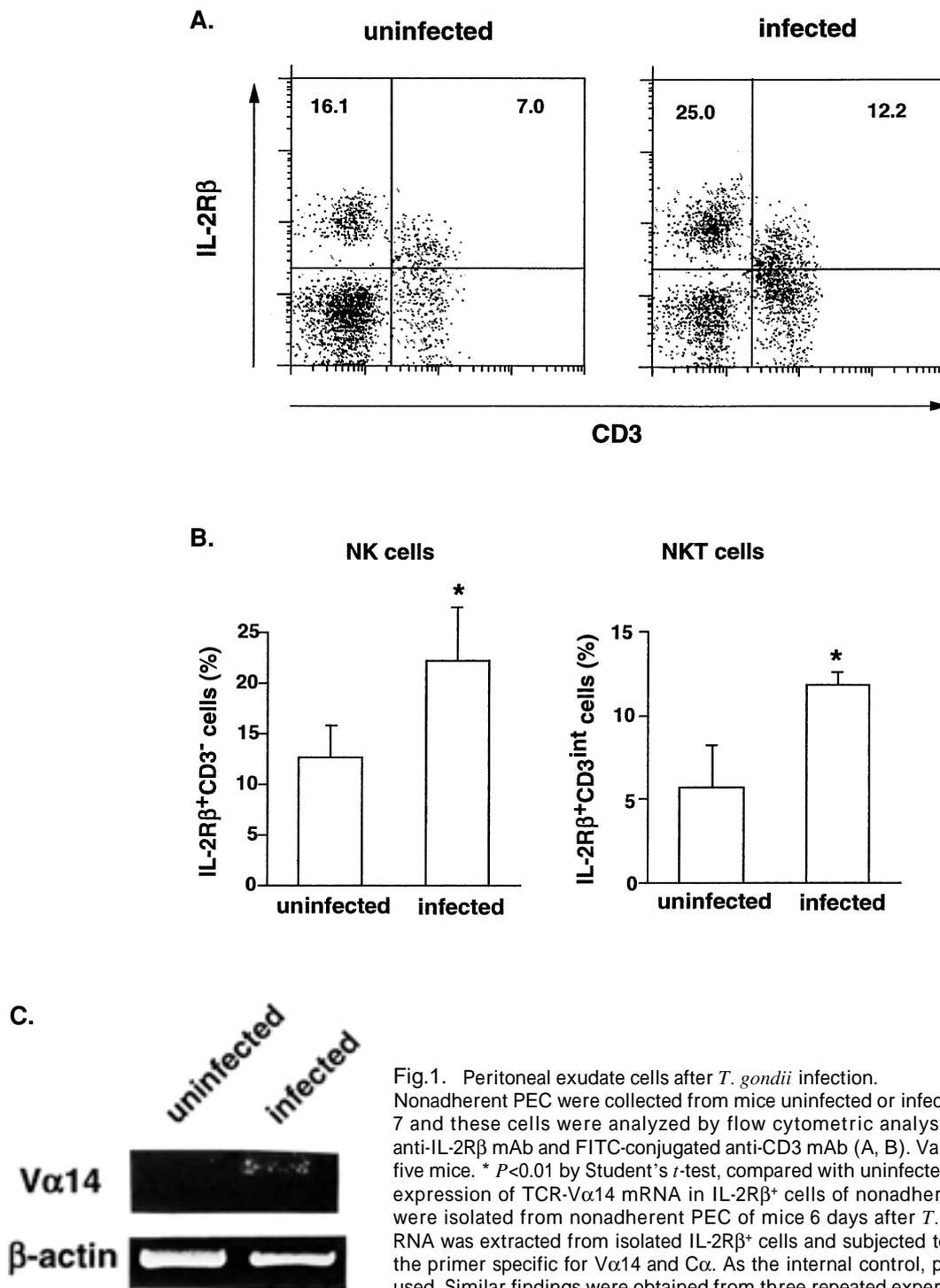
BALB/c mice were infected with 500 bradyzoites of Beverley strain by intraperitoneal injection. Seven days after infection, nonadherent PEC were collected and analyzed using flow cytometric analysis. The total number of nonadherent PEC increased following the infection (data not shown). Flow cytometric analysis with anti-CD3 and anti-TCR-$\gamma\delta$ mAb revealed that the percentage of $\gamma\delta$ T cells (3.9%± 1.0, n=5) in infected mice significantly increased compared with that in uninfected mice (0.91%± 0.6, n=5).

NK1.1$^+$ T cells express intermediate levels of CD3 molecules and NK1.1 molecules, which were originally identified as a specific NK cells marker in C57BL/6 mice. NK1.1 alleic negative strains, such as BALB/c mice used in this study, have been suggested to have equivalent cell populations (17, 18, 24, 25), NK1.1$^+$ T-like cells (NKT cells). IL-2Rβ is constitutively expressed on NK cells and NK1.1$^+$ T cells, but not on resting conventional T cells (17, 18). Therefore, we examined the NK or NKT cells in the peritoneal cavity by two-color staining with anti-CD3 and anti-IL-2Rβ mAb (Fig. 1A, B). As shown in Fig. 1A and B, IL-2Rβ^+CD3$^-$ NK cells and IL-2Rβ^+CD3^{int} cell population that included NKT cells, accumulated in the peritoneal cavity after T. gondii infection.

However, it is possible that activated T cells expressing IL-2Rβ would migrate into the fraction of IL-2Rβ^+CD3^{int} cells after infection. Therefore, we examined the expression of TCR-Vα14 mRNA in purified IL-2Rβ^+ cells from nonadherent PEC as the marker of NKT cells by RT-PCR analysis. As shown in Fig. 1C, the RT-PCR applied in this study did not amplify detectable levels of the TCR-Vα14 transcript in the IL-2Rβ^+ cell population from uninfected control mice, while significant amounts of the transcript were detected in the cell population from infected mice.

Effects of treatment with antibodies specific for TCR-$\gamma\delta$, asialoGM1 and IL-2Rβ on resistance against infection with T. gondii.

BALB/c mice were depleted of the $\gamma\delta$ T cells, NK cells, or NK and NKT cells by treatment with Ab specific for the TCR-$\gamma\delta$, asialoGM1 or IL-2Rβ, respectively. Then the mice, untreated or treated with one of these Abs, were infected with 500 bradyzoites of the Beverley strain of T. gondii by intraperitoneal injection, and mortality was monitored. As shown in Fig. 2, 60%of untreated mice died of acute T. gondii infection within

A.

B.

C.

Fig.1. Peritoneal exudate cells after *T. gondii* infection.

Nonadherent PEC were collected from mice uninfected or infected with *T. gondii* on day 7 and these cells were analyzed by flow cytometric analysis with PE-conjugated anti-IL-2Rβ mAb and FITC-conjugated anti-CD3 mAb (A, B). Values are mean± SD from five mice. * *P*<0.01 by Student's *t*-test, compared with uninfected control mice. (C) The expression of TCR-Vα14 mRNA in IL-2Rβ⁺ cells of nonadherent PEC. IL-2Rβ⁺ cells were isolated from nonadherent PEC of mice 6 days after *T. gondii* infection. Total RNA was extracted from isolated IL-2Rβ⁺ cells and subjected to RT-PCR analysis with the primer specific for Vα14 and Cα. As the internal control, primers for β-actin were used. Similar findings were obtained from three repeated experiments.

15 days. Treatment with anti-TCR-γδ mAb resulted in a high susceptibility to the infection. In contrast, mice treated with anti-IL-2Rβ mAb were highly resistant to the infection. Administration of anti-asialoGM1 Ab did not change the course of infection, compared with that of control mice (Fig. 2). The mortality of mice treated with respective control Abs was comparable with that of untreated mice (date not shown). These findings suggest that γδ T cells play an important role in protection against *T. gondii* infection, whereas

NKT cell populations, but not NK cells, may rather inhibit the development of the protective immunity.

Effects of treatment with antibody on HSP65 expression in the peritoneal macrophages

We previously reported that the magnitude of HSP65 expression in peritoneal macrophages coincided with the protective potential of host mice against *T. gondii* infection (9, 16). After infection with *T. gondii*, the expression of HSP65 was first detected

on day 6, and the level increased on days 9 and 13 in untreated mice (data not shown). Representative findings on day 9 after infection of Ab-treated mice are shown in Fig. 3. Treatment with anti-TCR-γδ mAb almost completely abrogated the expression of this protein. The expression in anti-asialoGM1 Ab-treated mice was comparable with that in control mice, while it was significantly enhanced in mice treated with anti-IL-2Rβ mAb, compared with that of control mice (Fig. 3). The levels of HSP65 in macrophages from mice treated with respective control antibodies were comparable with that of untreated mice (date not shown). Thus, we again confirmed that γδ T cells play impor-

tant roles in the regulation of HSP65 expression in macrophages and in the acquisition of protective immunity against *T. gondii* infection. In contrast, NKT cells, but not NK cells, may suppress the protective immunity in association with the inhibition of HSP65 expression.

Effects of γδ T, NK, or NKT cell depletion on expression of IL-4 and IFN-γ mRNAs in nonadherent PEC

Cell-mediated immunity is generally up-regulated by IFN-γ and down-regulated by IL-4. Total RNA was extracted from nonadherent PEC of mice 7 days after infection, and the effects of the antibody treatment on the production of IFN-γ and IL-4 were examined by RT-PCR analysis (Fig. 4). Both IL-4 and IFN-γ transcripts were readily amplified after *T. gondii* infection
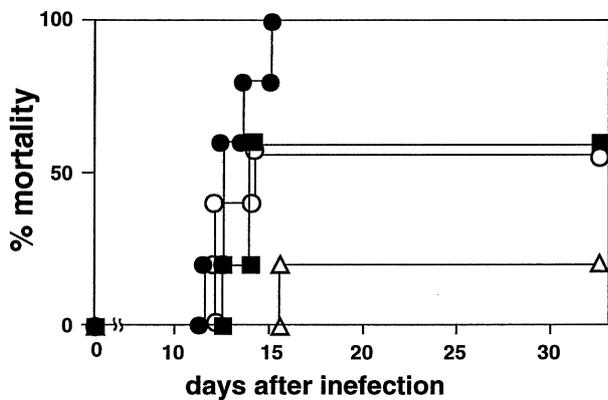

Fig.2. Effect of antibody treatment on mortality of mice infected with *T. gondii*. Mice treated with the Ab to IL-2Rβ (open triangles), asialoGM1 (closed squares), TCR-γδ (closed circles), or untreated mice (open circles) were infected with 500 bradyzoites of the Beverley strain of *T. gondii*. Each group consisted of five animals. Similar findings were obtained from three repeated experiments.
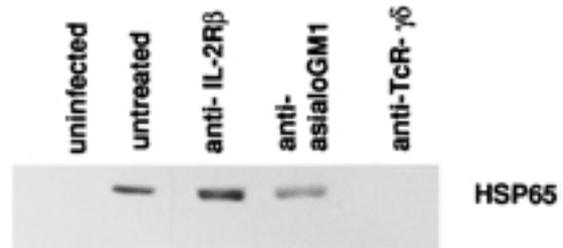

Fig.3. Expression of HSP65 in peritoneal macrophages from Ab-treated mice infected with *T. gondii*. Protein extracts prepared from peritoneal macrophages of control mice or Ab-treated mice 9 days after infection with 500 bradyzoites of the Beverley strain of *T. gondii* were subjected to the immunoblot analysis with mAb against HSP65. Ten micrograms of protein was loaded onto each lane. Similar findings were obtained from five independent experiments.
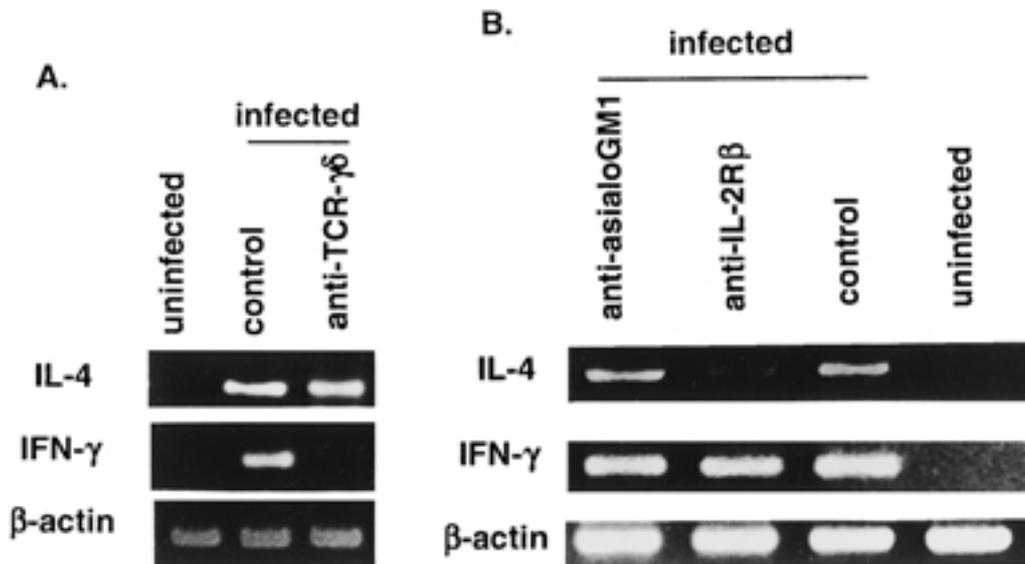

Fig.4. RT-PCR analysis. Effects of the treatment with anti-TCR-γδ mAb (A), anti-asialoGM1 Ab and anti-IL-2Rβ mAb (B) on the expression of IFN-γ and IL-4 mRNA. Total RNA was extracted from nonadherent PEC of control mice or antibody-treated mice 7 days after infection with 500 bradyzoites and subjected to RT-PCR analysis. Primers for β-actin were used as the internal control. Similar findings were obtained from three independent experiments.
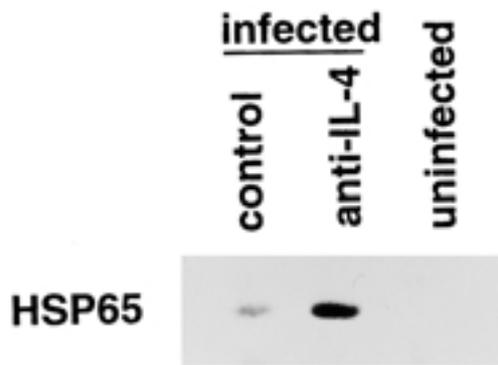
Fig. 5. Effects of the treatment with anti-IL-4 mAb on HSP65 expression in peritoneal macrophages. Protein extracts were prepared from peritoneal macrophages of control mice or antibody-treated mice 9 days after infection and subjected to immunoblot analysis with mAb against HSP65. Similar findings were obtained from three separated experiments.

in nonadherent PEC, while neither of these transcripts were detected in naive mice. Treatment with anti-TCR-γδ mAb reduced the expression of IFN-γ mRNA in nonadherent PEC, while it did not influence the IL-4 mRNA expression. In mice treated with anti-IL-2Rβ mAb, the expression of IL-4 m RNA decreased, compared with that in control-infected mice (Fig. 4). Treatment with anti-asialoGM1 Ab did not change the expression of IL-4 nor IFN-γ transcripts. These findings suggest that the elimination of γδ T cells interfered with the IFN-γ-mediated cellular immunity, leading to the impairment of the protective immunity against *T. gondii* infection. In contrast, cell-mediated immunity may have been enhanced by treatment with anti-IL-2Rβ mAb, ameliorating toxoplasmosis.

Effect of neutralization of endogenous IL-4 on HSP65 expression in peritoneal macrophages

We previously showed that the neutralization of IFN-γ abrogated the expression of HSP65 in macrophages after infection (9). In the present study, we examined an effect of the neutralization of endogenous IL-4 by treatment with anti-IL-4 mAb on HSP65 expression in host macrophages after infection. As shown in Fig. 5, treatment with anti-IL-4 mAb enhanced the expression of HSP65 in peritoneal macrophages.

DISCUSSION

Using BALB/c mice treated with antibodies specific for the TCR-γδ, asialoGM1, or IL-2Rβ, we examined distinct roles of innate cells (γδ T, NK, and NKT cells) accumulated in the inflamed site after intraperitoneal infection with *T. gondii*. To assess the protective immunity induced by these innate cells,

the induction of HSP65 in macrophages and production of IL-4 and IFN-γ from nonadherent PEC were monitored, and finally the protective potential was determined by mortality.

The elimination of γδ T cells by treatment with anti-TCR-γδ mAb prior to the infection suppressed the HSP65 induction in macrophages and the IFN-γ mRNA expression in nonadherent PEC, resulting in the aggravation of toxoplasmosis. Thus, γδ T cells appear to induce the expression of HSP65 in macrophages and to be required for IFN-γ production during the early phase of infection.

The selective depletion of NK cells by the treatment with anti-asialoGM1 Ab had no influence on the resistance to *T. gondii* infection or HSP65 induction in macrophages. Furthermore, the levels of IFN-γ and IL-4 mRNA expression in nonadherent PEC were comparable with the control mice after *T. gondii* infection, suggesting that the NK cells might not contribute to the HSP65 induction in macrophages and resistance against *T. gondii* infection.

In contrast to the treatment with anti-asialoGM1 Ab, treatment with anti-IL-2Rβ mAb augmented the induction of HSP65 in macrophages, but it inhibited the expression of IL-4 mRNA in nonadherent PEC, ameliorating toxoplasmosis. IL-2Rβ is constitutively expressed on NK cells and CD3^{int} cells including NK1.1^{+} T cells, but not on resting conventional T cells (17, 18). We also confirmed that treatment with the anti-IL-2Rβ mAb prior to the infection did not affect the number of conventional T cells by flow cytometric analysis (data not shown), suggesting that the conventional T cells were not involved in the result of *in vivo* depletion experiments using the anti-IL-2Rβ mAb. The different effects of treatment using these two antibodies might have been caused by the presence of IL-2Rβ^{+} CD3^{int} cells.

NK1.1^{+} T cells express markers common to the NK cell lineage, such as NK1.1, IL-2Rβ, members of the Ly-49 killer-inhibitory receptor family (17, 18), and the recently identified DX5 (19-21). The majority of NK 1.1^{+} T cells have been reported to consist of a TCR repertoire with an invariant Vα14-Jα281 chain pairing preferentially with a polyclonal Vβ8.2, Vβ7 or Vβ2 chain (17, 18, 24, 26). The functional characteristics of these cells are the ability to immediately produce IL-4 and IFN-γ (17, 18, 22, 23). The study of NK1.1^{+} T cells has been limited to mouse strains bearing the NK1.1 allele, such as C57BL/6 mice. Several studies have revealed that most NK1.1^{+} T cells are restricted by the nonpolymorphic MHC class 1-like molecule CD1d. In CD1d-deficient C57BL/6 mice, it was shown

that the NK1.1$^+$ T cell subpopulation was greatly decreased, and that early-phase production of IL-4 was impaired (25). Similarly, CD1d-deficient BALB/c mice were also found to have decreased numbers of TCRVβ8^{int} CD44^{high} T cells and to lack early-phase IL-4-producing capability (25). In addition, it was reported that the Vα14-Jα281 expression was independent of known major histocompatibility complex antigens, and all inbred strains of mice have this TCR α chain (24). These findings strongly suggest that a cell population equivalent to NK1.1$^+$ T cells (NKT cells) exists also in the NK1.1-negative mouse strains such as BALB/c mouse. Based on these findings and the present observations, it is suggested that NKT cells may be the cells responsible for the suppression of protective immunity against *T. gondii* infection.

NK1.1$^+$ T cells produce large amounts of IL-4 and IFN-γ upon primary TCR engagement *in vitro* and *in vivo* (17, 18, 22, 23). Considering the potent IL-4-producing capacity, NK1.1$^+$ T cells may preferentially induce the Th2 type CD4$^+$ T cells. It was reported that IL-4-producing NK1.1$^+$ T cells recognize glycosylphosphatidyl (GPI)-anchored surface antigens of parasites such as *Plasmodium* and *Trypanosoma* in the context of CD1d and induce IgG production (27). *T. gondii* is also known to express GPI-anchored surface antigen (28, 29), suggesting the possibility that the NKT cells in response to *T. gondii* antigen may produce IL-4.

In the present study, we showed that the number of IL-2Rβ$^+$CD3^{int} cells increased during toxoplasmosis in association with the increase in the cells expressing the TCR-Vα14 mRNA in the peritoneal cavity, suggesting that NKT cells may play distinct roles at the inflamed site. Furthermore, we found that the IL-4 mRNA expression was profoundly decreased in nonadherent PEC of mice treated with anti-IL-2Rβ mAb, but not with anti-asialoGM1 Ab. The endogenous IL-4 neutralization by *in vivo* treatment with anti-IL-4 mAb enhanced the induction of HSP65 in macrophages. These findings suggest that the NKT cells may be responsible for the suppression of both HSP65 induction and resistance against infection with *T. gondii* possibly through production of IL-4.

In conclusion, the number of γδ T, NK, and NKT cells was increased at inflamed sites by intraperitoneal infection with *T. gondii* ; however, their roles in protection against *T. gondii* infection appeared to vary. The γδ T cells may play an essential role in the production of IFN-γ during the early phase of infection and the HSP65 induction in macrophages, leading to resistance against *T. gondii* infection. In contrast, NKT cells, but not NK cells, may rather suppress the development of a protective immunity by inhibiting the HSP65 induction in macrophages and cell-mediated immunity through production of IL-4.

ACKNOWLEDGEMENTS

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