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$CD4^+$ $V_\alpha14$ NKT cells play a crucial role in an early stage of protective immunity against infection with *Leishmania major*

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Abstract

The roles of γδ T, NK and NKT cells in an early stage of protective immunity against infection with *Leishmania major* were investigated. Further, the contribution of these innate cells to the expression of 65 kDa heat shock protein (HSP65) in host macrophages was examined, since we found previously that this expression prevents apoptotic death of infected macrophages and is a crucial step in the acquisition of protective immunity against infection with various obligate intracellular protozoa including *L. major*. C57BL/6 and DBA/2 mice were found to be resistant against the infection on the basis of the parasite burden in their regional lymph nodes, and to strongly express HSP65 in their macrophages, whereas BALB/c mice were susceptible and barely expressed the HSP65. In those resistant mice, $CD4^+$ NKT cells prominently increased in their regional lymph node and were the main effector cells at least for an early stage of the protective immunity and for the HSP65 expression, whereas this subset did not increase in susceptible BALB/c mice. Further, neither γδ T nor NK cells in resistant mice contributed to those protective immune responses. The NKT cell subset bore CD3, CD4, TCR αβ, IL-2Rβ and NK1.1 but scarcely asialo-GM_1. Moreover, this effector subset was confirmed to be $V_\alpha14$ NKT cells by using $J_\alpha281^{-/-}$ mice.

Introduction

γδ T, NK and NKT cells are known to be the main effectors for innate immunities that play crucial roles in the early stage of infection with various pathogens including obligate intracellular protozoa. Innate immunity is proposed to be essential to supplement the immunological interface until the development of adaptive immunity, which finally completes the protection after the invasion of pathogens.

We reported previously that the effector cells of innate immunity are different for each obligate intracellular protozoan. That is, γδ T cells are the essential innate cells against infection with *Toxoplasma gondii* and NK cells act against infection with *Trypanosoma cruzi*, although $CD4^+$ T and $CD8^+$ T cells are required for the final resolution of these infections (1–3). Those innate cells have a common function to induce the expression of 65 kDa heat shock protein (HSP65) in infected macrophages (4–7), which contributes to protection through preventing apoptotic death of the infected host cells (8,9).

Leishmania major is also another intracellular protozoan similar to *T. gondii* and *T. cruzi*, although their evasion mechanisms differ (10,11). T_h1-type $CD4^+$ T cells are broadly accepted to be the main effector cells against this infection (12–15). However, it is still unknown whether specific innate cells are required in the interface until the development of classical T_h1-type protective immunity. In the present study, we found that $CD4^+$ NKT cells bearing TCR $V_\alpha14$ are essential at least in the early stage after the infection and that this NKT cell subset also has a potential to induce the expression of

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HSP65 in host macrophages. The roles of γδ T, NK, NKT and classical $CD4^+/CD8^+$ T cells in protective immunity and HSP65 expression in *L. major* infection are discussed.

Methods

Animals

Female BALB/c, C57BL/6 (B6) and DBA/2 mice were purchased from Charles River Japan (Kanagawa, Japan). $V_\alpha 14$ NKT cell-deficient ($J_\alpha 281^{-/-}$) mice were established by specific deletion of the $J_\alpha 281$ gene segment (16). CD4 knockout mice (B6 background) were provided from Dr Matsuzaki (Department of Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan). These mice were used in experiments at 7–10 weeks of age.

Parasites and infection

L. major (MHOM/SU/73/5ASKH) was maintained by serial passages in BALB/c mice. For experiments, promastigotes were obtained as previously described (17). Animals were infected s.c. with 5×10^6 promastigotes/50 μl PBS in the left hind footpad.

Immunoblotting

We detected HSP65 by immunoblotting as described previously (17). The first antibody, murine mAb IA10, specific for an epitope located between amino acids 172 and 224 of HSP65 derived from *Mycobacterium bovis*, was provided by J. DeBruyn (Institute Pasteur de Brabant, Belgium). Peroxidase-conjugated goat anti-mouse IgG (Zymed, San Francisco, CA) was the second antibody. Binding antibodies were detected by the enhanced chemiluminescence method (Amersham, Little Chalfont, UK). The specific signals were detected by using autoradiography film (Fuji Film, Tokyo, Japan).

Antibodies

Anti-CD4 (GK1.5 (rat IgG)) and anti-CD8 (53–6.7 (rat IgG)) were obtained from ATCC (Rockville, MD). Anti-TCR αβ (H57-597, hamster IgG) and anti-TCR γδ (UC7-13D5, hamster IgG) were gifts from Dr. Matsuzaki (Department of Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan). Anti-IL-2Rβ chain (TM-β1, hamster IgG) was a gift from Dr Miyasaka (Tokyo Metropolitan Institute for Medical Sciences, Tokyo, Japan). Anti-asialo-GM_1 (rabbit IgG) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Anti-NK1.1 (PK-136, mouse IgG2a) was a gift from Dr Watanabe (Department of Immunology, Niigata University School of Medicine, Japan).

Flow cytometry

Popliteal lymph node cells and hepatic lymphocytes were stained with various combinations of fluorescence-conjugated mAb. These cells were analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) using the two-color staining method. Anti-CD3 (2C11) was labeled with FITC. Anti-IL-2Rβ chain (TM-β1) was purchased from PharMingen (San Diego, CA). Stained cells were analyzed with light scatter gates set to exclude debris, clumps and dead cells.

Depletion of specific cell subsets in vivo

mAb specific for each cell subset were harvested as ascites from pristane-primed SCID mice with mAb-producing hybridomas in their peritoneal cavity. Antibodies were precipitated from the ascites with 45% ammonium sulfate and dialyzed against PBS. Before use, their total protein was measured. Preliminary experiments showed that i.p. injection of 0.5 mg of purified mAb derived from GK1.5, 53-6.7, H57-597, UC-7-13D5, TM-β1 or PK-136 resulted in >95% depletion of the respective cell subsets in the spleen, lymph nodes and peritoneal exudate cells (PEC) of mice as estimated by FACS. Intraperitoneal injection of 0.1 mg anti-asialo-GM_1 resulted in >95% depletion of the respective cell subsets in the spleen, lymph nodes and PEC of mice as estimated by FACS. Accordingly, mice were treated i.p. with each mAb, 3 and 1 days before infection in order to deplete these cell subsets.

Quantifying L. major in regional popliteal lymph nodes

Regional popliteal lymph nodes were obtained from mice 7 days after infection with *L. major*. The lymph nodes were homogenized by slide glass in Schneider medium. After homogenization, cells were washed 2 times with Schneider medium. Then, cell numbers were adjusted, and cultured in Schneider medium supplemented with 10% FBS (Life Technologies, Grand Island, NY), 2% normal human urine (obtained from healthy volunteers), 1% penicillin per streptomycin solution (Sigma, St Louis, MO), 0.5% of 1 M HEPES solution (pH 7.3) and 0.1% of 50 mg gentamicin (Sigma). The culture system was based on the method of Titus (18). After culturing, the number of parasites was counted using a microscope.

Results

B6 and DBA/2 mice are resistant to infection with L. major and express high levels of HSP65 in their macrophages compared with BALB/c mice

BALB/c mice are highly susceptible to infection with *L. major*, while DBA/2 and B6 mice are genetically resistant to that parasite (19–22). As shown in Fig. 1(A), we counted the number of parasites in macrophages from regional popliteal lymph nodes of mice at 7 days after infection. Large numbers of *L. major* promastigotes were recovered from the macrophages of susceptible BALB/c mice, while only small numbers were recovered from those of either B6 or DBA/2 mice. We previously reported that resistant B6 mice ($H\text{-}2^b$) express a high level of HSP65 in their macrophages compared with susceptible BALB/c mice ($H\text{-}2^d$) after *L. major* infection (17). Then, we investigated whether, as with B6 mice, resistant DBA/2 mice ($H\text{-}2^d$) that are H-2 compatible with BALB/c mice also expressed HSP65 after infection. As shown in Fig. 1(B), DBA/2 mice and B6 mice highly expressed HSP65 compared with BALB/c mice. Thus, HSP65 expression was closely correlated with the resistance of mice to this infection across the barrier of MHC.

B6 mice depleted of T cells expressing CD4 and TCR αβ lose protective potential against infection with L. major

We investigated the influence of the depletion of each T cell subset, using corresponding mAb, prior to infection on the

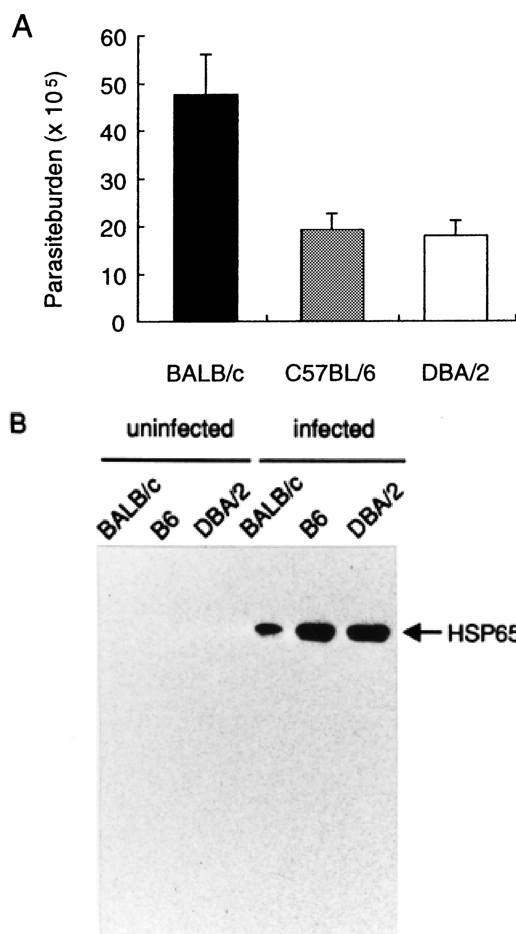


Fig. 1. Correlation between HSP65 expression and protective potential of macrophages from *L. major*-infected mice. (A) Protective potential of macrophages from three strains of mice. Quantification of *L. major* promastigotes recovered from regional popliteal lymph nodes of three strains of mice (BALB/c, B6 and DBA/2). These mice were infected in the left hind footpad with 5×10^6 parasites of *L. major*. Bars represent means ± SD from triplicate determinations from three animals. (B) HSP65 expression in macrophages from three strains of mice. Immunoblotted lysates prepared from regional popliteal lymph node macrophages of three strains of mice (BALB/c, B6 and DBA/2) 7 days after infection in the left hind footpad with 5×10^6 parasites of *L. major*. Lysates of the macrophages from uninfected mice were also prepared. Ten micrograms of protein was loaded on each lane. Similar results were found in three independent experiments.

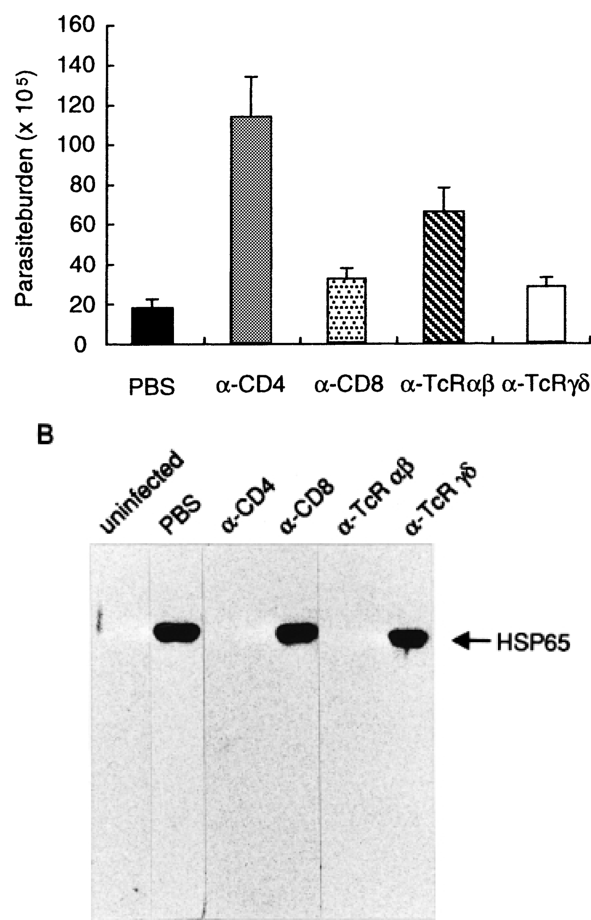


Fig. 2. Correlation between HSP65 expression and protective potential of macrophages from specific T cell subset-depleted B6 mice. (A) Quantification of *L. major* promastigotes recovered from regional popliteal lymph nodes of specific T cell subset-depleted B6 mice ($CD4^+$ depleted, $CD8^+$ depleted, TCR αβ depleted, TCR γδ depleted and PBS-treated control). These mice were infected with 5×10^6 parasites of *L. major* in the left hind footpad. Bars represent means ± SD from triplicate determinations from three animals. (B) HSP65 expression in macrophages from specific T cell subset-depleted B6 mice. Immunoblotted lysates prepared from regional popliteal lymph node macrophages of specific T cell subset-depleted B6 mice 7 days after infection in the left hind footpad with 5×10^6 parasites of *L. major*. Lysate of the macrophages from uninfected mice were also prepared. Ten micrograms of protein was loaded on each lane. Similar results were found in three independent experiments.

protective potential of the macrophages of resistant B6 mice at 7 days after infection. A large number of *L. major* promastigotes were recovered from the macrophages in regional lymph nodes of both anti-TCR αβ mAb- and anti-CD4-treated B6 mice, while only small numbers were from those of anti-TCR γδ- or anti-CD8-treated B6 mice (Fig. 2A). Similar results were obtained using DBA/2 mice (data not shown). Thus, $CD4^+$ T cells with TCR αβ are the effector cells for protection.

T cells expressing CD4 and TCR αβ contribute to the expression of HSP65 in mice infected with L. major

Our previous study revealed that SCID mice lacking T and B cells and nude mice lacking only T cells could not express HSP65 in their macrophages after *L. major* infection, suggesting that T cells are essential to its expression (17). To investigate which T cell subsets are required for HSP65 expression, B6 mice, which have a potential to strongly express HSP65 in their macrophages after infection, were depleted of various T cell populations by injection with corresponding specific mAb before and after infection. As shown in Fig. 2(B), the expression in B6 mice was suppressed by treatment with either anti-TCR αβ or anti-CD4 mAb, whereas treatment with anti-TCR γδ or anti-CD8 had no influence on HSP65 expression. Thus, $CD4^+$ T cells expressing TCR αβ were required for the expression of HSP65 in macrophages of mice infected with *L. major*. We reported previously that γδ

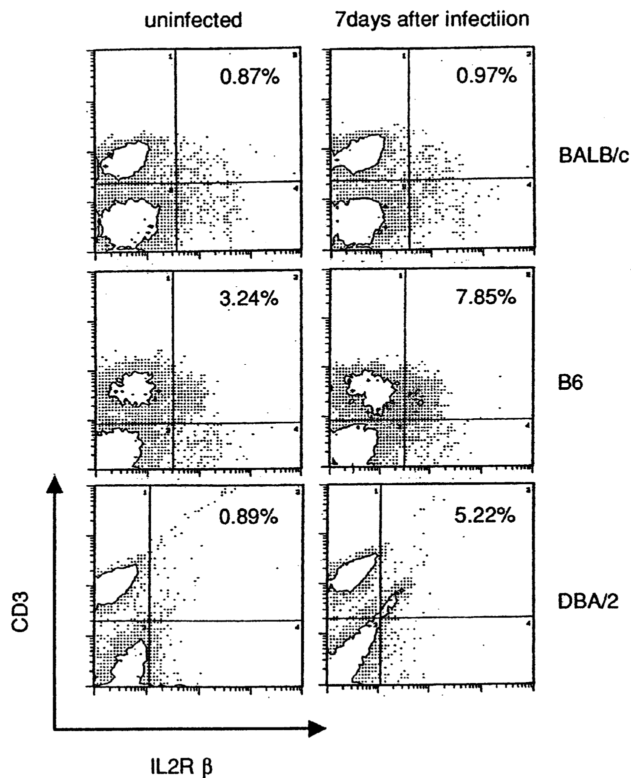


Fig. 3. Expansion of NKT cells ($CD3^+$, TM-$\beta 1^+$) in *L. major*-infected mice. Regional popliteal lymph node macrophages of three strains of mice (BALB/c, B6 and DBA/2) 7 days after infection with *L. major* were stained with a combination of anti-CD3 and Anti-IL-2Rβ chain (TM-β1). Numbers are the mean percentage of NKT cell subsets. Result from one of three individual experiments. Similar results were found in three independent experiments.

T cells have a crucial role in the expression of HSP65 in host macrophages as well as in early phase protection of mice infected with *T. gondii* (1,2,4–8). As shown here, however, γδ T cells were not the effectors for either protection or for HSP65 expression.

IL-2Rβ⁺ T cells increase in resistant B6 and DBA/2 mice but not in susceptible BALB/c mice

Susceptible BALB/c and resistant B6 and DBA/2 mice were infected with *L. major* in the left hind footpad. Seven days after inoculation, the kinetics of NK ($CD3^-$, IL-2Rβ^+) and NKT cells ($CD3^+$, IL-2Rβ^+) in regional popliteal lymph nodes were analyzed by FACS analysis. As shown in Fig. 3, $CD3^+$, IL-2Rβ^+ T subsets increased in resistant B6 (7.85%) and DBA/2 (5.22%) mice compared with mice without infection (3.24 and 0.89% respectively). However, this type of subset did not increase in susceptible BALB/c mice compared with resistant DBA/2 and B6 mice. Moreover, we analyzed the kinetics of NK and NKT cells in the infectious site, but clear difference were not seen between lymph nodes and footpads (data not shown). Taken together with the data shown in Figs 2 and 3, this indicates that NKT cells ($CD3^+$, IL-2Rβ^+) expressing CD4, but not NK cells ($CD3^-$, IL-2Rβ^+), are the main effectors for protection and for HSP65 expression.

NKT cells expressing NK1.1 are the main protective cells against infection with L. major in B6 mice

We confirmed that NKT but not NK cells contribute to protection and to HSP65 expression in *L. major* infection. DBA/2 mice do not express NK1.1, therefore B6 mice expressing the NK1.1 molecule were used for this experiment. NKT cell populations locate mainly in liver rather than lymphoid tissues (23). Therefore, we used intrahepatic cells for FACS analysis of the kinetics of NK and NKT cell subsets after treatment with TM-β1 (anti-IL-2Rβ), anti-asialo-GM_1 or anti-NK1.1 antibodies. As shown in Fig. 4(A), both NK cell ($CD3^-$, IL-2Rβ^+) and NKT cell ($CD3^+$, IL-2Rβ^+) subsets were depleted in mice treated with either TM-β1 or anti-NK1.1 mAb, but only the NK cell subset was depleted by treatment with anti-asialo-GM_1 antibody. Next, we examined the correlation between the protective potential against infection with *L. major* and the expression of HSP65 in host macrophages. As shown in Fig. 4(B), large numbers of parasites were recovered from lymph node cells of mice treated with TM-β1 and anti-NK1.1 as compared with anti-asialo-GM_1-treated mice, suggesting that NKT cells but not NK cells are responsible for protection. Next, we examined which cell subset was most responsible for the induction of HSP65 in their macrophages. As shown in Fig. 4(C), the expression of HSP65 disappeared in the macrophages of mice treated with anti-IL-2Rβ or anti-NK1.1 mAb, while expression was not affected by treatment with anti-asialo-GM_1 antibody. Thus, NKT cells appear to be the essential cells for the induction of HSP65 expression, but NK cells do not have the potential to induce HSP65 expression.

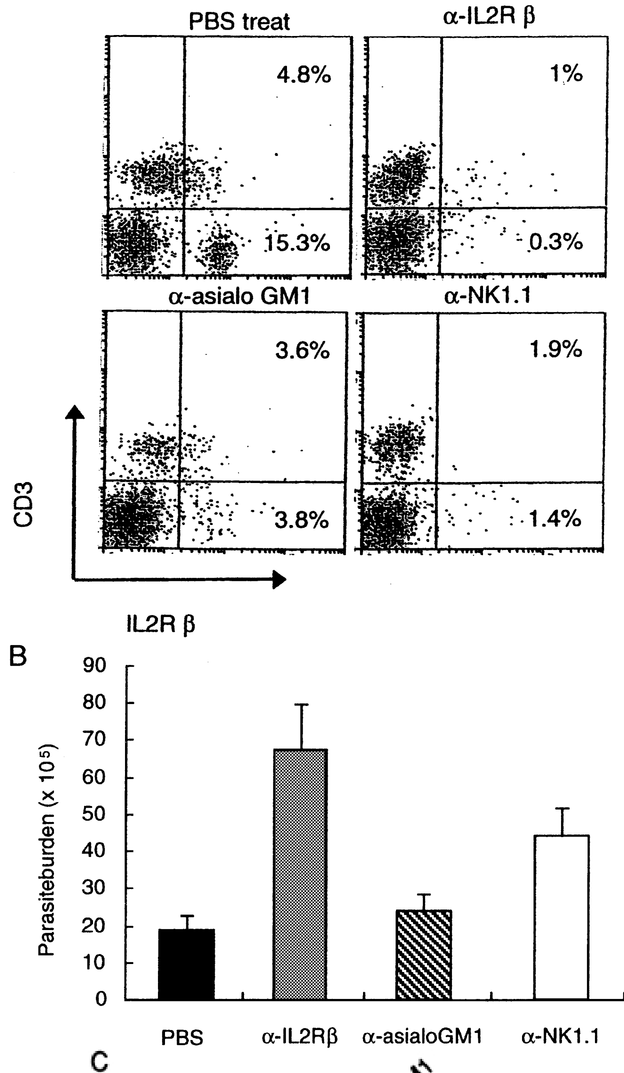
Protective NKT cells express TCR Vα14

The results of the above experiments show that, NKT cells but not NK cells play a crucial role in the host's defense against infection with *L. major*. To further clarify this point, we investigated the susceptibility of Vα14 NKT cell-deficient and $CD4^{-/-}$ mice to infection with *L. major*. As shown in Fig. 5, parasite numbers from popliteal lymph node cells of $V_\alpha 14$ NKT cell-deficient mice were larger than those from the control B6 mice and $CD4^{-/-}$ mice at 3 and 5 weeks after *L. major* infection. Thus, $CD4^+$ NKT cells rather than classical $CD4^+$ T cells appear to be the main effectors for the protection and HSP65 expression at least in the early stage until 5 weeks after infection.

Discussion

It is well established that T_h1-type classical $CD4^+$ T cells are the main effectors against infection with *L. major* in mice (12–15). In the present study, however, we showed that $CD4^+$ NKT cells bearing both IL-2Rβ (TM-β1) and NK1.1 are essential for the protection against infection with *L. major*, at least in the early stage until 5 weeks after infection, in genetically resistant DBA/2 and C57BL/6 (B6) mice. Furthermore, other types of innate cells such as γδ T and NK cells appeared not to participate in providing protection. Those NKT cell populations were also essential for the expression of HSP65 in host macrophages of resistant DBA/2 and B6 mice infected with *L. major*. We reported previously that the expression of HSP65 in host macrophages is the crucial step in initiating protective

immunities against various intracellular pathogens such as *T. gondii* (1,2,8,9), *T. cruzi* (3) and *L. major* (17). HSP65 contributes to those protective immunities through preventing the apoptotic death of infected macrophages (8,9).



The contribution of NKT cells to protective immunity against *L. major* infection was also confirmed by using $V_\alpha 14$ NKT cell-deficient mice. These mice lack $CD4^+$ NKT cells, which are characterized by the expression of invariant TCR encoded by $V_\alpha 14$, $J_\alpha 281$ and $V_\beta 8$, $V_\beta 7$ or $V_\beta 2$ gene segments (24–27). This NKT subset also expresses CD4, NK1.1 molecules and the IL-2Rβ chain (TM-β1) on the cell surface (28,29). This subset is known to also contribute to tumor immunity by being activated by IL-12 (16,30,31). CD1d, a non-classical Ib molecule, is known to be a ligand of $V_\alpha 14$ TCR (32). CD1 molecules present some molecules containing lipids of pathogens such as mycolic acid of *Mycobacterium tuberculosis*, lipoarabinomannan of *Mycobacterium leprae* (33,34) and glycosylphosphatidylinositol (GPI) (35). *L. major* protozoa reportedly express GPI on their plasma membrane (36). Thus, a target antigen of NKT cells in resistant mice may be GPI-anchored antigens presented by CD1, as Schofield *et al.* suggested (37). In the late stages, however, classical $CD4^+$

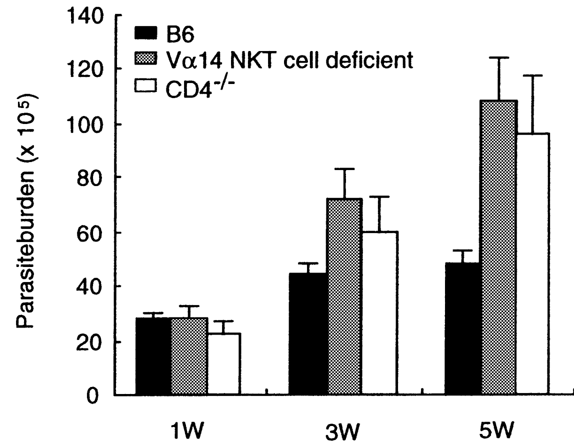


Fig. 5. Quantification of *L. major* promastigotes recovered from regional popliteal lymph nodes using $V_\alpha 14$ NKT cell-deficient and $CD4^{-/-}$ mice. Regional popliteal lymph nodes were obtained from animals at 1, 3 or 5 weeks after infection. Animals were infected with 5×10^6 parasites of *L. major* in the left hind footpad. Bars represent means + SD from triplicate determinations from three animals.

Fig. 4. NKT cells expressing NK1.1 are the main protective cells against infection with *L. major* in B6 mice. (A) Flow cytometry to evaluate the effect of NK ($CD3^-$, TM-$\beta1^+$) or NKT cell ($CD3^+$, TM-$\beta1^+$) subset depletion using antibody treatment. Intrahepatic cells were stained with a combination of anti-CD3 and anti-IL-2Rβ chain (TM-β1). (B) Quantification of *L. major* promastigotes recovered from regional popliteal lymph nodes of NK cell ($CD3^-$, TM-$\beta1^+$) or NKT cell ($CD3^+$, TM-$\beta1^+$) subset-depleted B6 mice. These mice were infected with 5×10^6 parasites of *L. major* in the left hind footpad. Bars represent means ± SD from triplicate determinations from three animals. (C) HSP65 expression in macrophages from specific T cell subset-depleted B6 mice. Immunoblotted lysates prepared from regional popliteal lymph node macrophages of NK cell ($CD3^-$, TM-$\beta1^+$) or NKT cell ($CD3^+$, TM-$\beta1^+$) subset-depleted B6 mice 7 days after infection in the left hind footpad with 5×10^6 parasites of *L. major*. Lysates of the macrophages from uninfected mice were also prepared. Ten micrograms of protein was loaded on each lane. Similar results were found in three independent experiments.

T$_h$1 cells should also contribute to final resolution of the infection, although we could not demonstrate the role of the classical T$_h$1 in the early stage of infection through a comparison of protective immunity between V$_\alpha$14 NKT cell-deficient mice and CD4$^{-/-}$ mice.

Our serial experiment using mAb showed that CD4$^+$ NK1.1$^+$ TCR$\alpha\beta^+$ cells play a crucial role in an early stage of *L. major* infection. To confirm this result, we used J$_\alpha$281$^{-/-}$ mice and compared the parasite loads in the early stage of infection with those of the wild type mice. Surprisingly, a significant difference was observed after 3 weeks but not at 1 week unlike the case of mAb-treated experiments. One explanation is that NKT cells without bearing V$_\alpha$14 TCR is resident in J$_\alpha$281$^{-/-}$ mice and act as a protective effector in the early stage of the infection. Recently, it was shown that thymic NKT cells are dramatically decreased in J$_\alpha$281$^{-/-}$ mice, whereas a significant number of NKT cells still exists in the periphery (38). The other possibility is a compensatory effect that is caused by a congenital deficit. NKT cell subsets in the bone marrow from J$_\alpha$281$^{-/-}$ mice reportedly produce a larger dose of IFN-γ than that from wild type mice (39). IFN-γ is known to be a main effector cytokine that can induce to generate antiparasitic molecule, NO. Thus, a congenital deficit of V$_\alpha$14 TCR-expressing NKT cells might influence the early phase of the protective immunity. In addition, a similar difference was observed between anti-CD4 mAb-treated and CD4$^{-/-}$ mice. Unique T cells expressing CD4, NK1.1 and TCR$\alpha\beta$ are exclusively selected by CD1 in the thymus (40,41). Contribution of CD4 molecules in these selection event is not understood yet. It is readily speculated that certain CD1-restricted CD4$^-$ NKT (CD4$^-$CD8$^-$ NKT) cells in CD4$^{-/-}$ mice have some potency as CD4$^+$ NKT cells in wild type mice. We reason that the time-lag discrepancy among antibody-treated and knockout mice is caused by the above phenomenon. The parasite burden in both J$_\alpha$281$^{-/-}$ and CD4$^{-/-}$ mice is significantly higher than that in wild type mice after 3 weeks. Therefore, CD4$^+$ V$_\alpha$14$^+$ NKT cells rather than CD4$^-$ or V$_\alpha$14$^-$ NKT cells would play a crucial role in the *L. major* infection.

There are many reports as to the importance of NKT subsets in protective immunity against various obligate intracellular infectious agents such as *L. monocytogenes* (42,43) and *M. bovis* (44). However, NKT cells do not always act to protect against various pathogens. For example, it is generally accepted that NKT cells promote disease progress in BALB/c mice infected with *L. major*, via the secretion of IL-4, and cause the deviation of the immune response from protective T$_h$1 to disease-promoting T$_h$2 (45). Furthermore, we observed that NKT cells suppress the $\gamma\delta$ T cells-mediated protective immunity against infection with *T. gondii* in BALB/c mice (submitted for publication). Thus, it is noteworthy that NKT cells play a key role in defining the resistance and susceptibility of mice against some pathogens including *L. major* at early stages after infection, probably through generating IFN-γ and IL-4 respectively (46).

NK, NKT and $\gamma\delta$ T cells emerge immediately after the onset of infections and contribute to protective immunity in the early stage of infection as innate cells, covering the immunological interface until the development of classical CD4$^+$ and/or CD8$^+$ T cells, both of which are intensive effectors in the late stages of protection (6,23,26,29,47–49). We reported previously that the effector cells of innate immunity are different for each obligate intracellular protozoan. That is, $\gamma\delta$ T cells are the essential innate cells against infection with *T. gondii* and NK cells are the essential innate cells against infection with *T. cruzi*, although CD4$^+$ and CD8$^+$ T cells expressing $\alpha\beta$ TCR are required for the final resolution of these infections (1–3). Further, as presented here, NKT cells also appear to play a crucial role in the protection against infection with *L. major* in genetically resistant B6 and DBA/2 mice. Those innate cells have a common function to induce the expression of HSP65 in infected macrophages (4–7). The expression of HSP65 is induced by IFN-γ and tumor necrosis factor-α released by those innate cells, and it contributes to protective immunity through preventing the apoptotic death of the infected host macrophages as mentioned above (8,9). However, it remains to be clarified whether distinct innate cells correspond to specific varieties of pathogens and whether one subset of innate cells reacts diversely to different pathogens.

In murine leishmaniasis, T$_h$1-type classical CD4$^+$ T cells are broadly accepted to be the main effectors, although the final effector cells are activated macrophages. In the present study, we showed that CD4$^+$ V$_\alpha$14 NKT cells bearing IL-2Rβ (TM-β1) and NK1.1 are essential for protection at least in the early stage after the infection with *L. major*. It is very important to clarify whether there is a sequential development in the immune system from innate NKT cells to adaptive classical T$_h$1-type CD4$^+$ T cells in this infection.

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Abbreviations

GPI	glycosylphosphatidylinositol
HSP65	65 kDa heat shock protein
PEC	peritoneal exudate cell

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