FULL PAPER *Parasitology*

Roles of CD122$^+$ Cells in Resistance against *Neospora caninum* Infection in a Murine Model

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ABSTRACT. Innate cells, such as natural killer (NK) cells and NKT cells, play essential roles as primary effector cells at the interface between the host and parasite until establishment of adaptive immunity. However, the roles of NK and NKT cells in defense against *Neospora caninum* have not been well clarified. NK and NKT cells were depleted by the treatment with an anti-CD122 (interleukin-2 receptor beta chain) monoclonal antibody (mAb, TM-β1) *in vivo*. The parasite burden in the brain of mice was promoted by the treatment with anti-CD122 mAb. However, there was no significant difference in the infection rates between controls and the mice treated with anti-asialoGM1 antibody to deplete NK cells. Activation of CD4$^+$ T cells was suppressed in the mice treated with anti-CD122 mAb compared with controls and the mice treated with anti-asialoGM1 antibody. On the other hand, depletion of CD122$^+$ cells or NK cells did not affect the number of activated CD8$^+$ T cells, dendritic cells and B cells following *N. caninum* infection. These results indicate that CD122$^+$ cells (probably NKT cells) play a crucial role in host defense by activating CD4$^+$ T cells against *N. caninum* infection.
KEY WORDS: immune system, *Neospora caninum*, parasite.

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Neospora caninum was originally identified as a *Toxoplasma gondii*-like parasite causing predominately neuromuscular disorders in dogs [4] and abortions and stillbirths in cattle [9]. It can also cause abortion or neonatal mortality in other animal species, such as sheep, goats, horses, and deer [8]. Moreover, antibodies against *N. caninum* were also detected in humans [21]. However, there are no reports on the clinical implications of *N. caninum* in humans because the parasites have not been detected nor isolated from human tissues. Intermediate hosts can suffer *N. caninum* infection either by ingestion of oocysts that are shed in the feces of acutely infected dogs, or through vertical transmission [23]. The ingested oocysts develop into rapidly multiplying tachyzoites that enter the bloodstream where they preferentially inhabit the cells of mononuclear phagocytic system and eventually infect different tissues. The host immune response plays as atrigger for differentiation of tachyzoites to bradyzoites of the parasites, and a persistent tissue cyst infection is established in neural and muscular tissue [6].

N. caninum infection induces cell-mediated immunity which is important for host defense. The importance of interferon-gamma (IFN-γ) for the protection against *N. caninum* has been well documented in mouse models [1, 27] and bovine tissue cultures [15]. The source of IFN-γ during neosporosis has been attributed to CD4$^+$ T lymphocytes [22]. While classical CD8$^+$ and CD4$^+$ T cells are required for the acquisition of protective immunity against *Toxoplasma* infection [12], different types of innate cells may exert distinct roles in protective immunity. Generally, innate cells, such as natural killer (NK) cells and NKT cells, play essential roles as primary effector cells at the interface between the host and parasite until the establishment of adaptive immunity [16]. Previously, it has been shown that *N. caninum* becomes a trigger to produce IFN-γ in bovine NK cells [5, 19]. However, the role of NK or NKT cells in the defense against *N. caninum* has not been well clarified.

CD122 (interleukin-2 receptor beta chain, IL-2Rβ) which forms a functional IL-2R together with the constitutively expressed γ chain, is expressed from memory T lymphocytes, NK cells, NKT cells, dendritic epidermal T cells and macrophages [2]. Small subsets of fetal and adult thymocytes also constitutively express CD122 [2]. In the present study, we investigated the roles of NK and NKT cells in protective immunity against *N. caninum* infection.

MATERIALS AND METHODS

Mice: BALB/c female mice, 6–7 weeks of age, were obtained from Clea Japan (Tokyo, Japan). The mice were housed under specific pathogen-free conditions in the animal facility of the National Research Center for Protozoan Diseases at the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. The mice were treated and used under the Guiding Principles for the Care and Use of Research Animals from the Obihiro University of Agriculture and Veterinary Medicine.

Parasite and cell culture: *N. caninum* tachyzoites of the Nc-1 isolate [10] were maintained in monkey kidney adher-

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ent fibroblasts (Vero cells) cultured in Eagle's minimum essential medium (EMEM, Sigma, St. Louis, MO, U.S.A.) supplemented with 8% heat-inactivated fetal bovine serum. For the purification of tachyzoites, parasites and host-cell debris were washed and resuspended, and passed through a 27-gauge needle and 5.0-μm-pore filter (Millipore, Bedford, MA, U.S.A.).

Flow cytometry analysis and mAbs: Fourteen monoclonal antibodies (mAbs), phycoerythrin (PE)-labeled anti-mouse CD3e, PE-labeled anti-mouse CD19, PE-labeled anti-mouse CD4, PE-labeled anti-mouse CD8, PE-labeled anti-mouse CD11c, PE-labeled anti-mouse IFN-γ, fluorescein isothiocyanate (FITC)-labeled anti-mouse CD49b/Pan-NK cell (DX5), FITC-labeled anti-mouse CD3e, FITC-labeled anti-mouse CD4, FITC-labeled anti-mouse CD8, FITC-labeled anti-mouse CD14, FITC-labeled anti-mouse CD44, FITC-labeled anti-mouse CD122 (5H4) and purified rat anti-mouse CD16/CD32 (Fcγ III/II receptor) (FcBlock™) mAbs, were obtained from BD Biosciences (La Jolla, CA, U.S.A.). Cells prepared from the spleen or tail blood were hemolyzed in a lysing buffer (0.83% NH_4Cl and 0.01 M Tris-HCl, pH 7.2). After washing with cold PBS, 2×10^6 cells were resuspended in cold PBS containing 0.5% bovine serum albumin. The cells were treated with FcBlock™ to avoid the non-specific adherence of mAbs to Fc receptors. Then the cells were incubated with respective mAbs for 30 min at 4°C and washed with cold PBS before analysis using an EPICS® XL flow cytometer (Beckman Coulter, Hialeah, FL, U.S.A.). Staining for intracellular IFN-γ by the Cytofix/Cytoperm™ Fixation/Permeabilization kit (BD Biosciences) was performed according to the manufacturer's recommendations. The cells stained with FITC-labeled mAb as described above were mixed with Fixation/Permeabilization solution for 20 min at 4°C. After washing with BD Perm/Wash™ buffer, the cells were incubated with PE-labeled anti-mouse IFN-γ mAb for 30 min at 4°C. The cells were then washed with BD Perm/Wash™ buffer and PBS and examined using an EPICS® XL flow cytometer. Before the analysis, the lymphocyte population was screened by light-scatter signals to exclude dead and nonlymphoid cells. The specificity of staining was confirmed using isotype-matched irrelevant mAbs.

Effect of Ab treatment against parasite burden in the brain: An anti-CD122 mAb (TM-β1) was used for the depletion of NK and NKT cells [24], and on anti-asialoGM1 Ab (polyclonal rabbit IgG, Wako, Osaka, Japan) was used for the depletion of NK cells [24]. Each mouse was intraperitoneally (i.p.) injected with 0.5 mg of anti-CD122 mAb or with 50 μg of anti-asialo GM1 Ab before infection with 2×10^4 *N. caninum* tachyzoites and 4 days after infection. The depletion of respective cell populations were confirmed by flow cytometry using peripheral blood mononuclear cells (PBMC) prepared 2 days after the first injection of mAb or Ab. The brains were collected at days 3 and 6 after infection and analyzed by PCR.

DNA isolation and PCR analysis: For DNA preparation, each brain was thawed in 10 volumes of extraction buffer (0.1 M Tris-HCl pH 9.0, 1% SDS, 0.1 M NaCl, 1 mM EDTA) and 1 mg/m*l* of Proteinase K at 55°C. The DNA was purified by phenol-chloroform extraction and ethanol precipitation. The DNA concentration was adjusted to 100 μg/m*l* and used as template DNA. Ten μ*l* reaction mixture was prepared as it contains 2.5 μ*l* of template DNA, 1 μ*l* of 10 × PCR buffer which contained 15 mM $MgCl_2$ (Perkin-Elmer, Boston, MA, U.S.A.), 1 μ*l* of 10 mM dNTP mix, 0.1 μ*l* of 5 U/μ*l* Ampli Taq Gold™ Taq DNA polymerase (Perkin-Elmer) and 2 μ*l* of 10 pmol/μ*l* *N. caninum* specific primers, Np6 and Np21 [20]. Amplification was carried out in a thermal cycler, GeneAmp PCR System 2400 (Perkin-Elmer) employing 40 cycles of denaturation (94°C, 1 min), annealing (63°C, 1 min) and primer extension (74°C, 3.5 min). At the end of the cycle reaction, a primer extension was continued for 10 min at 74°C and then kept at 4°C. The PCR products were visualized by electrophoresis in agarose gels.

In vitro stimulation of spleen cells: The spleen cells were plated into 96-well microplates at 3×10^5/200 μ*l*/well in RPMI-1640 medium (Sigma) supplemented with 5% FBS. The cells were stimulated by adding 50 μg/m*l* of the lysate antigen of *N. caninum* tachyzoite (NLA). After incubation for 48 hr at 37°C, $CD122^+$ cells were determined by flow cytometry.

Statistical analysis: Data are presented as means ± SD. Statistical differences were determined by student's *t*-test or one-way ANOVA followed by Turkey-Kramer multiple comparison tests.

RESULTS

Percentage population of $CD122^+$ cells stimulated by N. caninum antiges: We first examined the reactivity of $CD122^+$ cells against *N. caninum* antigens. In cultured spleen cells obtained from the mice 7 days after *N. caninum* infection, the population of $CD122^+$ cells dramatically increased in the presence of NLA (Fig. 1). This result sug-

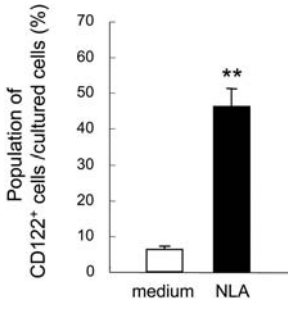

Fig. 1. Activation of $CD122^+$ cells following *N. caninum* infection. Spleen cells were collected from the mice at 7 days after i.p. infection with *N. caninum* tachyzoites. The collected cells were cultured for 48 hr in the presence of 50 μg/m*l* of *N. caninum* tachyzoites lysates (NLA) or without any stimulator (medium). The population of $CD122^+$ cells in the cultured spleen cells was measured by flow cytometry. Each bar represents the mean ± SD of three mice. Data were analyzed by student's *t*-test (** $P<0.01$). Reproducibility of the data was confirmed by two independent experiments.

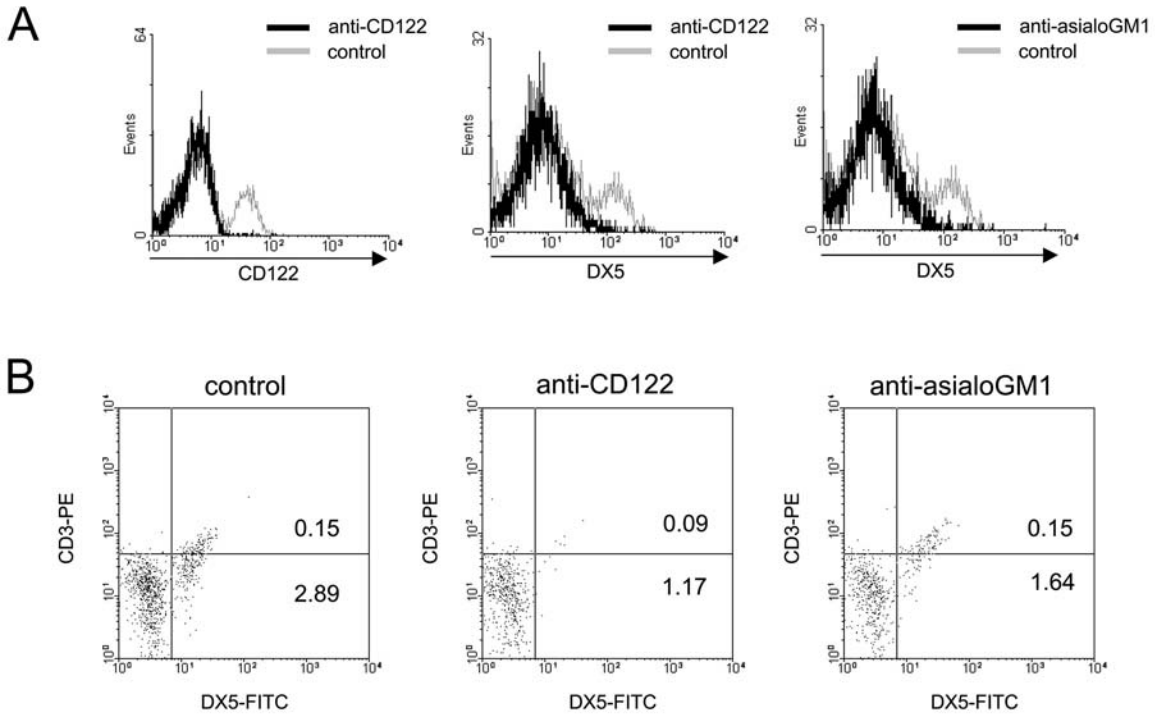

Fig. 2. Depletion of $CD122^+$ cells (A), $DX5^+$ cells (A), NK cells (B) and NKT cells (B) by anti-CD122 mAb or anti-asialoGM1 Ab. Uninfected mice were treated i.p. with 0.5 mg of anti-CD122 mAb, 50 μg of anti-asialoGM1 Ab or PBS (control). After 2 days, PBMC from tail blood was collected and analyzed by flow cytometry. The cells were stained with FITC-labeled anti-CD122 mAb (5H4), FITC-labeled anti-DX5 mAb, and PE-labeled anti-CD3 mAb. $DX5^+$ $CD3^{int}$ and $DX5^+$ $CD3^-$ cells are considered to be equivalent to NKT cells and NK cells, respectively.

gested that $CD122^+$ cells might contribute to the *N. caninum*-specific immune responses.

Role of $CD122^+$ cells in protective immunity against N. caninum: To investigate the role of $CD122^+$ cells in protective immunity against *N. caninum* infection, the mice treated with an anti-CD122 mAb were challenged with the parasites. In our previous study [26], *N. caninum* DNA was firstly detected in the heart, kidney, liver, lungs and spleen at the mice examined, then positive rate in the brain increased gradually. The parasite DNA was detected continuously until the late stage of infection in the brain, while it was not detected at the late stage in the heart, liver and kidney. Therefore, the presence of *N. caninum* was monitored by detecting parasite DNA from the brain in this examination. The anti-CD122 mAb depleted $CD122^+$ cells from the mice (Fig. 2A). While the *N. caninum* DNA was not detected 3 days after infection in control animals, the parasite DNA was detected from only one animal in the anti-CD122 mAb treated group (Table 1). However, 6 days after infection, the PCR detection rate significantly increased in the mice treated with anti-CD122 mAb compared with that in the control animals (Table 1), indicating that $CD122^+$ cells controled the *N. caninum* infection during its early stages.

Role of NK cells and NKT cells in protective immunity against N. caninum: Effects of NK and NKT cells on protective immunity against *N. caninum* infection were tested. In a previous study, it has been shown that the treatment of BALB/c mice with an anti-CD122 mAb decreased the population and the number of $DX5^+$ $CD3^{int}$ and $DX5^+$ $CD3^-$ cells, which were considered to be equivalent to $NK1.1^+$ T cells (NKT cells) and NK cells, respectively, in NK1.1 allele-negative strains, such as BALB/c mice [24]. To compare the effects of NK cells and NKT cells, we used an anti-asialoGM1 Ab for the depletion of NK cells. The uninfected mice treated with the anti-asialoGM1 Ab showed decrease of the population of $DX5^+$ $CD3^-$ cells (Fig. 2). Furthermore, the treatment with anti-CD122 mAb decreased the population of $DX5^+$ $CD3^{int}$ cells and $DX5^+$ $CD3^-$ cells compared with the control mice (Fig. 2). The number of NK and NKT cells increased at 6 days after the infection with *N. caninum* (Fig. 3). Both the anti-CD122 mAb or anti-asialoGM1 Ab treated mice showed significant lower levels of NK cells compared with the control mice, while there was no significant difference between anti-CD122 mAb-treated and anti-asialoGM1 Ab-treated mice (Fig. 3). In the mice treated with anti-CD122 mAb, the number of NKT cells decreased compared with that in the control mice during the infection. Moreover, the treatment with anti-CD122 resulted a tendency for fewer NKT cells than the anti-asialoGM1 Ab

Table 1. PCR detection of *N. caninum* DNA in brain

Treatment	Days after infection	Total number of positive/ Total number of mice (Trial 1 and Trial 2)	Total number of positive/ Total number of mice (total)
control	3	0/5 (0%), 0/5 (0%)	0/10 (0%)
	6	1/5 (20%), 1/5 (20%)	2/10 (20%)
Anti-CD122	3	0/5 (0%), 1/5 (20%)	1/10 (10%)
	6	3/5 (60.0%), 4/5 (80.0%)	7/10 (70%) *
anti-asialoGM1	3	0/5 (0%), 0/5 (0%)	0/10 (0%)
	6	0/5 (0%), 1/5 (10%)	1/10 (10%)

Mice treated with anti-CD122 mAb, anti-asialoGM1 Ab or PBS (control) were infected with 20,000 *N. caninum* tachyzoites. The brains were collected at 3 and 6 days after the infection and were analyzed by PCR. The statistical difference against control was determined by Chi-square test (* $P<0.05$).

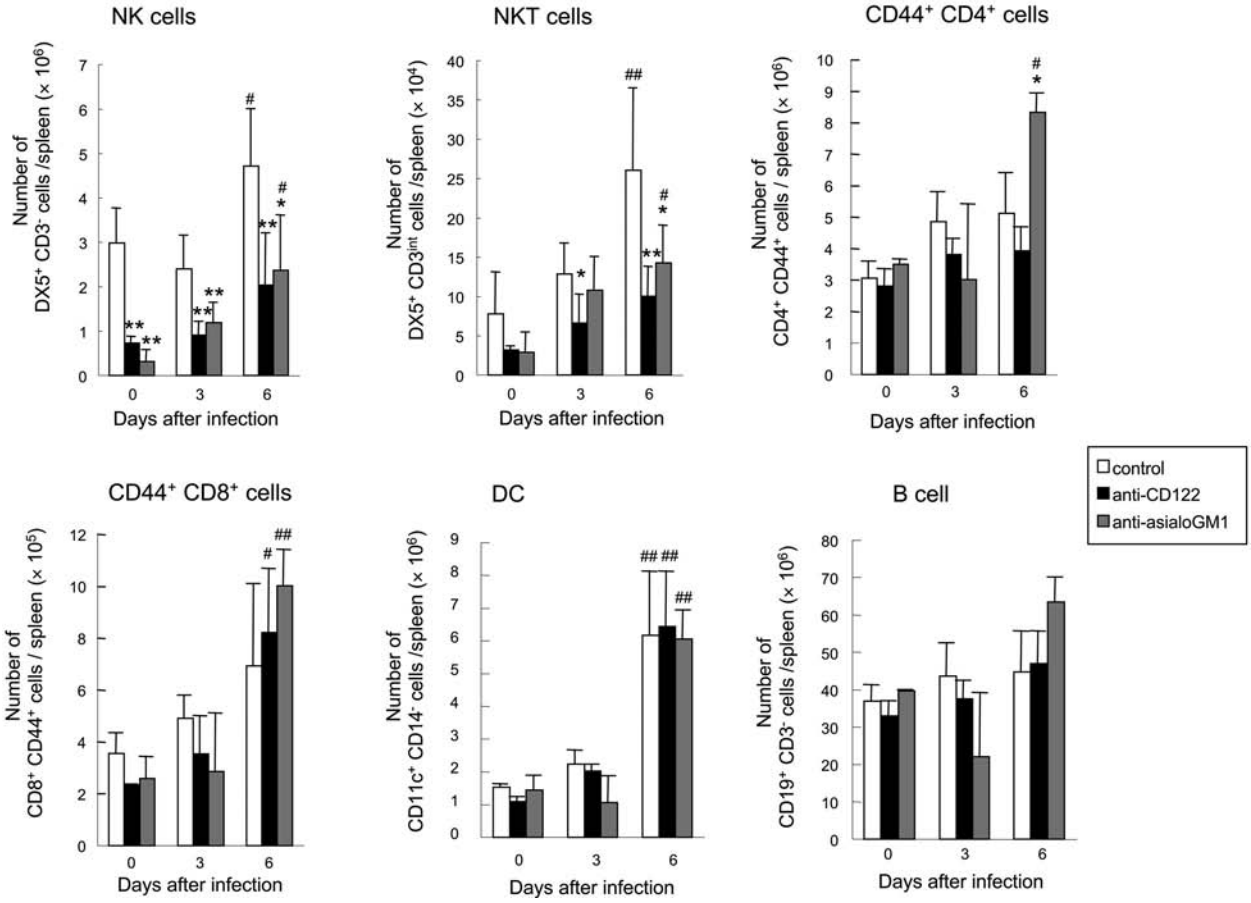

Fig. 3. The number of NK cells, NKT cells, $CD44^+$ $CD4^+$ cells, $CD44^+$ $CD8^+$ cells, DCs and B cells after the treatment with anti-CD122 mAb or anti-asialoGM1 Ab. Mice were treated i.p. with 0.5 mg of anti-CD122 mAb, 50 μg of anti-asialoGM1 Ab or PBS (control) 2 days before infection. Spleen cells were collected from the uninfected mice (day 0) and the mice 3 or 6 days after infection with *N. caninum* tachyzoites. Number of $DX5^+$ $CD3^{int}$ cells (NKT cells), $DX5^+$ $CD3^-$ cells (NK cells), $CD44^+$ $CD4^+$ cells, $CD44^+$ $CD8^+$ cells, $CD11c^+$ $CD14^-$ cells (DCs) and $CD19^+$ $CD3^-$ cells (B cells) was measured by flow cytometry. The number of NK cells and NKT cells of three mice selected randomly from Trial 1 and 2 (see Table 1) was measured. The values from total six mice are shown as means ± SD. The number of $CD44^+$ $CD4^+$ cells, $CD44^+$ $CD8^+$ cells, DCs and B cells of three mice selected randomly from Trial 2 was measured. The values from three mice are shown as means ± SD. Reproducibility of the data (number of $CD44^+$ $CD4^+$ cells, $CD44^+$ $CD8^+$ cells, DCs and B cells) was confirmed by the pooled cell sample from three mice per group in another independent experiment. Data were analyzed by ANOVA, and then differences among means were analyzed using Turkey-Kramer multiple comparison tests. * $P<0.05$, ** $P<0.01$, compared with PBS-immunized mice. # $P<0.05$, ## $P<0.01$, compared with uninfected mice (day 0).

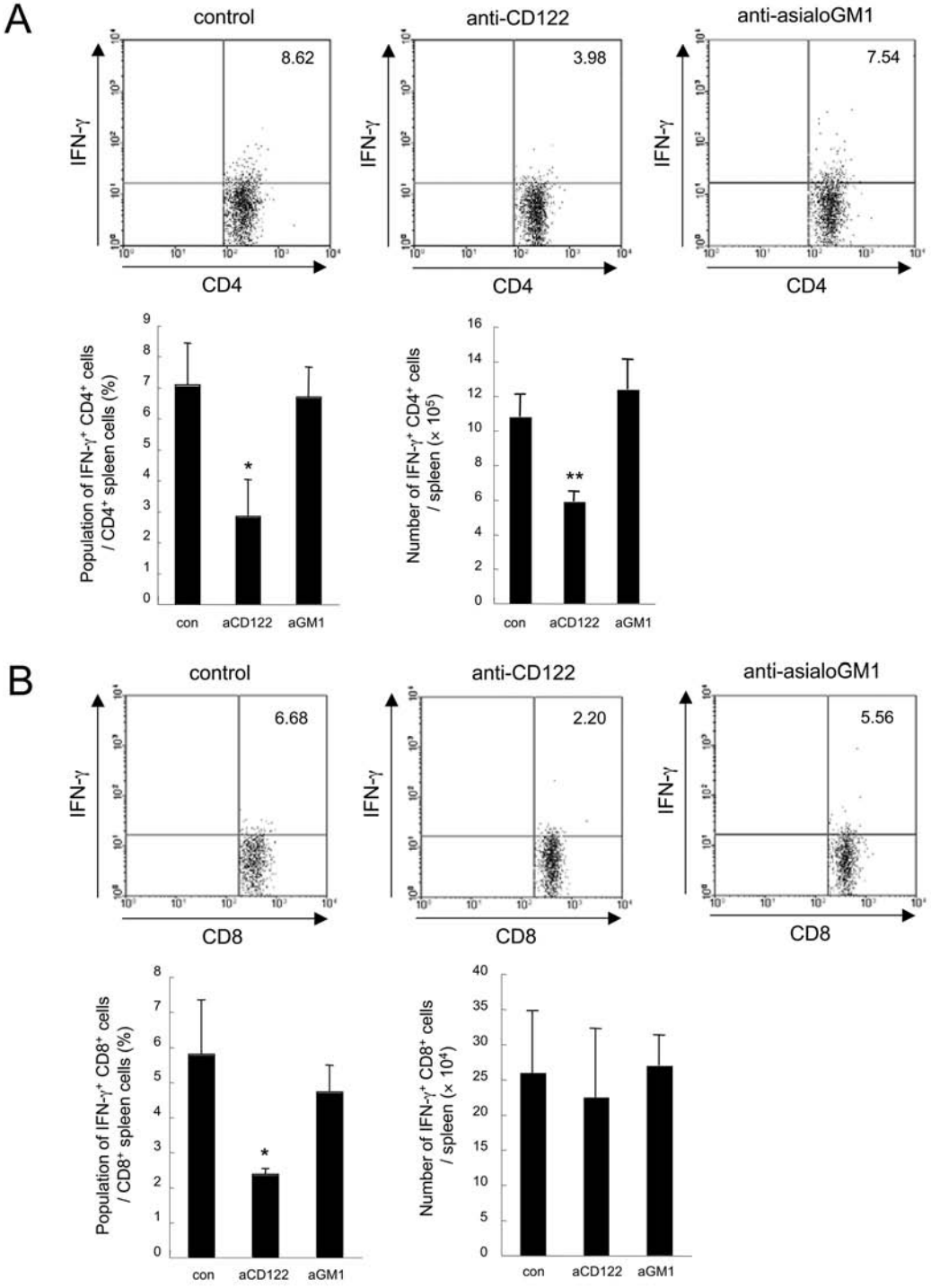

Fig. 4. The population and number of IFN-γ^+ $CD4^+$ cells (A) or IFN-γ^+ $CD8^+$ cells (B) in the mice treated with anti-CD122 mAb (aCD122), anti-asialoGM1Ab (aGM1) or PBS (con) following *N. caninum* infection. Spleen cells were collected from the mice 6 days after infection with *N. caninum* tachyzoites. The cells were stained with FITC-labeled anti-CD4 mAb or FITC-labeled anti-CD8 mAb, and PE-labeled anti-IFN-γ mAb. The population and number of IFN-γ^+ $CD4^+$ cells or IFN-γ^+ $CD8^+$ cells were measured by flow cytometry. The values from three mice selected randomly from Trial 1 are shown as means ± SD. Data were analyzed by ANOVA, and then differences among the means were analyzed using Turkey-Kramer multiple comparison tests. * $P<0.05$, ** $P<0.01$, compared with PBS-immunized mice. $^{\#}$ $P<0.05$, $^{\#\#}$ $P<0.01$, compared with uninfected mice (day 0). Reproducibility of the data was confirmed by three mice per group in another independent experiment.

treatment following *N. caninum* infection. As shown in Table 1, the treatment of the mice with anti-asialoGM1 Ab did not increase the parasite burden. Therefore, it was considered that NKT cells play a role in controlling the parasite burden.

Effects of T cells on protective immunity against N. caninum: To investigate the roles of $CD122^+$ cells in acquired immunity against *N. caninum* infection, we examined the activation of T cells in the absence of $CD122^+$ cells. It is known that the level of CD44 expression increases upon activation of T cells [31]. The number of $CD44^+$ $CD4^+$ or $CD44^+$ $CD8^+$ cells showed a tendency to increase in the control mice following the infection with *N. caninum* (Fig. 3). Although the number of $CD44^+$ $CD8^+$ cells increased in the mice treated either with anti-CD122 mAb or anti-asialoGM1 Ab following *N. caninum* infection, there was no increase in the $CD44^+$ $CD4^+$ cells of the anti-CD122 mAb-treated mice (Fig. 3). Furthermore, the population and number of $CD4^+$ cells expressing IFN-γ in the mice treated with anti-CD122 mAb were significantly lower than those treated with PBS or anti-asialoGM1 Ab (Fig. 4A). On the other hand, although the population of $CD8^+$ cells expressing IFN-γ in the mice treated with anti-CD122 mAb was significantly lower than those in the mice treated with PBS or anti-asialoGM1 Ab, there was no significant difference in the number of $CD8^+$ cells expressing IFN-γ among the experimental groups (Fig. 4B). These results showed that $CD122^+$ cells affected the activation of $CD4^+$ cells against *N. caninum* infection.

Effect of splenic dendritic cells (DC) and B cells on protective immunity against N. caninum: Next, we investigated whether $CD122^+$ cells affected the number of DC and B cells in the spleen following *N. caninum* infection or not. The number of DC increased even in the absence of $CD122^+$ cells. In addition, there was no significant difference in the number of B cells among the experimental groups (Fig. 3). Thus, $CD122^+$ cells did not affect the number of DC and B cells following *N. caninum* infection.

DISCUSSION

Our objective was to assess the effector cells controlling the early stage of *N. caninum* infection. A former study indicated that *N. caninum* directly become a trigger for the production of IFN-γ from purified, IL-2-activated bovine NK cells which killed infected fibroblasts [5]. Furthermore, NK cells have been shown to act as early responders in experimental infection with *N. caninum* in calves [19]. In the present study, however, depletion of NK cells by the treatment with anti-asialoGM1 Ab did not increase the parasite burden in the brain of the mice examined. Furthermore, the anti-asialoGM1 Ab-treated mice showed no significant activation of $CD4^+$ T cells and $CD8^+$ T cells following *N. caninum* infection. These data suggest that classical NK cells do not play a crucial role in the early stage of *N. caninum* infection in a murine model.

CD122 is constitutively expressed from classical NK cells and also from CD3 cells, including $NK1.1^+$ T (NKT) cells in intermediate levels, but not from resting conventional T cells [2]. $NK1.1^+$ T cells express markers common to the NK cell lineage, such as NK1.1, CD122, members of the Ly-49 killer-inhibitory receptor family, and DX5 [3]. A former study has shown that anti-CD122 mAb (TM-β1) and anti-asialoGM1 Ab can deplete NKT and NK cells, and NK cells in BALB/c mice, respectively [24]. In the present study, when the BALB/c mice were treated with anti-CD122 mAb or anti-asialoGM1 Ab, NKT cells played as the effector that controlled *N. caninum* infection. NKT cells regulated the activation of other immune cells including NK cells, T cells, B cells and DC. Former studies have shown that NKT cells enhance $CD4^+$ and $CD8^+$ T cell responses to soluble antigen *in vivo* through the direct interaction with DC [11, 13]. Moreover, NKT-mediated autologous vaccines have been reported to generate $CD4^+$ T-cell-dependent potent antilymphoma immunity [7]. In our study, the low levels of NKT cells resulted in impaired activation of $CD4^+$ T cells against *N. caninum* infection. Our previous study [27] showed that anti-CD4 mAb-treated mice were sensitive to *N. caninum* infection, indicating the importance of the activated $CD4^+$ T cells for the protection. Thus, induction of the activated $CD4^+$ T cells by NKT cells would be important in protective immunity against *N. caninum* infection. In the case of *T. gondii* infection, NKT cells might be responsible for the suppression of protective immunity [24]. In two closely related parasites, the effector cells responsible for defending against the infection might be different.

At the late stage of *N. caninum* infection, NK cells and/or NKT cells might play a role in the protective immunity. In the control mice and the mice treated with anti-CD122 mAb or anti-asialoGM1 Ab, all of them were survived at 30 days after *N. caninum* infection (5×10^5/mouse) (unpublished data). However, at 90 days after the infection, the survival rates of anti-CD122 mAb or anti-asialoGM1 Ab-treated mice were 67% (n=6), while all the control mice (n=6) were survived (unpublished data). These results suggest that the roles of NK cells and/or NKT cells might correlate with the protection at the late stage of infection with *N. caninum* because these cells have been shown to regulate the activation of acquired immunity [7, 11, 13, 27]. However, further analyses whether the parasite number in the brain at the early stage affects the pathogenesis at the late stage are required.

Most NKT cells are restricted by a monomorphic MHC class I molecule, CD1d [2], and the sugar moiety of glycolipids bound to CD1d molecules plays as a trigger for their activation [18, 30]. In protozoan infection, the ligand to CD1d and the activation mechanism of NKT cells remain unclear. Although the origin and identity of their natural ligands are not well known, candidate for natural ligands might be the self-glycosylphosphatidylinositol (GPI) anchors [17]. In fact, it has been suggested that the GPI anchors of *P. falciparum* and *Trypanosoma brucei* induce Ab production in a CD1d-dependent manner [30]. Another study showed that GPI-anchored mucin-like glycoproteins

from *T. cruzi* bind to CD1d but do not elicit dominant innate or adaptive immune responses via the CD1d/NKT cell pathway [28]. On the other hand, the proteins of *N. caninum* have been known to bind to the GPIs [14, 25, 29]. Therefore, further analyses of *N. caninum* GPI-anchored proteins and the activation of NKT cells are required because of their potential role in the immune responses against the infection.

In summary, we demonstrated for the first time that the *N. caninum*-infected mice developed NKT cell responses in the early stages of the infection and that NKT cells were involved in activation of $CD4^+$ cells during this period. Our finding contributes to the basic understanding of innate immunity during protozoan infections.

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