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Analysis of the role of natural killer cells in *Listeria monocytogenes* infection: relation between natural killer cells and T-cell receptor γδ T cells in the host defence mechanism at the early stage of infection

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SUMMARY

We have reported that T cells bearing T-cell receptors (TcR) of γδ type (γδ T cells) appear in the peritoneal cavity in a relatively early stage of primary intraperitoneal (i.p.) *Listeria monocytogenes* infection, and play a significant role against the infection. To elucidate the protective role of natural killer cells which also appear in the early stage of *L. monocytogenes* infection, mice were treated with anti-NK1.1 monoclonal antibody (mAb) to deplete NK cells before the infection. They exhibited accelerated clearance of *L. monocytogenes*, accompanied by enhanced induction of γδ T cells in the peritoneal cavity compared with non-treated mice. When the mice were depleted of γδ T cells by *in vivo* administration of anti-TcR γδ mAb, the bacterial burdens of organs from infected mice were not affected by NK cell depletion. These results suggest that, although NK cells increase significantly during the early stage of *L. monocytogenes* infection, they do not take part in the early host resistance against i.p. *L. monocytogenes* infection. It is also suggested that increased γδ T cells in the peritoneal cavity of NK cell-depleted mice can be one of the factors responsible for the enhanced clearance of *L. monocytogenes* in the early stage of infection.

INTRODUCTION

The murine host response to primary *Listeria monocytogenes* infection has been a useful model for studying protective mechanisms against facultative intracellular bacteria.[1–7] The early response, which occurs during the first 48 hr after infection, is attributed to resident macrophages and early influx of bone marrow-derived phagocytes in the liver and spleen.[3–5] The late response, beginning at around day 5, is characterized by the involvement of listerial antigen-specific T cells, which activate macrophages to enhance bacterial killing *in vivo*.[4–6]

Recently, we demonstrated the appearance of γδ T cells in the peritoneal cavity in the early stage of intraperitoneal (i.p.) listerial infection.[8] Furthermore, we clarified the protective role of the γδ T cells against *L. monocytogenes*.[9] It is reported that natural killer (NK) cells also appear in the early stage of listerial infection,[10,11] but the role of NK cells in host defence against the bacteria has been controversial. It has been suggested that NK cells do not have a protective role against *L. monocytogenes* by the studies showing normal clearance of the bacteria in beige mice,[12] or ^{89}Sr-treated mice[13] which have a decreased NK activity. On the other hand, NK cells were suggested to play an important role during *L. monocytogenes* infection through T-cell-independent macrophage activation in *scid* mice.[14,15] It was also demonstrated that subcutaneous inoculation of *L. monocytogenes* resulted in the early appearance of NK cells in the draining lymph nodes, which were shown to be one of the mechanisms necessary for early host defence.[16]

The NK1.1 antigen is the best marker in defining murine NK cells.[17] It is expressed (in those strains carrying the NK1.1 allele) on all NK cells capable of lysing NK-sensitive YAC-1 lymphoma cells.[17] In this report, to clarify the precise role of NK cells against i.p. listerial infection, we analysed the effect of NK cell depletion by *in vivo* administration with anti-NK1.1 monoclonal antibody (mAb) on the bacterial clearance. Treatment of mice with anti-NK1.1 mAb significantly enhanced the ability of mice to clear *L. monocytogenes* from the spleen and liver relative to infected controls, which suggests that NK cells do not have a protective role during *L. monocytogenes* infection. Our results also suggested that γδ T cells are responsible for the augmented anti-*L. monocytogenes* resistance in the NK cell-depleted mice.

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MATERIALS AND METHODS

Mice

C57BL/6 mice bred under specific pathogen-free conditions were obtained from Japan SLC (Shizuoka, Japan). All mice were female and were used for experiments at 7 weeks of age.

Micro-organisms

Listeria monocytogenes, strain EGD, was used in all experi-

ments. Bacterial virulence was maintained by serial passages in BALB/c mice. Fresh isolates were obtained from infected spleens, grown in tryptic soy broth (Difco Laboratories, Detroit, MI), washed repeatedly, resuspended in phosphate-buffered saline (PBS), and stored at −70° in small aliquots.

Peritoneal exudate cells (PEC)

Mice were inoculated i.p. with 5×10^4 viable *L. monocytogenes* (1/10 LD_{50}) in a volume of 0·2 ml of PBS on day 0. PEC were harvested on days 1, 3, 5 and 8 after inoculation by peritoneal lavage with Hanks' balanced salt solution (HBSS). The cells were collected by centrifugation at 100 ***g*** for 5 min, washed twice and resuspended at optimal concentrations in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% serum. PEC were spread on plastic plates at a concentration of 5×10^5 cells/ml and incubated for 1 hr in a CO_2 incubator at 37° to obtain non-adherent cells.

Antibodies

Anti-NK1.1 mAb-producing hybridoma cells (PK136) were obtained from the American Type Culture Collection (Rockville, MD). Fluorescein isothiocyanate (FITC)-conjugated anti-T-cell receptor (TcR)$\alpha\beta$ mAb (H57-597) was a gift of Dr R. Kubo (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). Anti-TcR$\gamma\delta$ mAb-producing hybridoma cells (UC7-13D5) were a gift of Dr J. A. Bluestone (Ben May Institute, University of Chicago, Chicago, IL). FITC-conjugated anti-TcR $\gamma\delta$ mAb (GL3) and FITC-conjugated anti-NK1.1 mAb were purchased from Pharmingen (San Diego, CA). Biotin-conjugated anti-Thy-1.2 mAb were purchased from Becton Dickinson (Oxnard, CA). Anti-NK1.1 (PK136) and anti-TcR$\gamma\delta$ mAb (UC7-13D5) were obtained by growing hybridoma cells in serum-free medium (101; Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) and collecting supernatant. Antibodies were then concentrated and purified by 50% ammonium sulphate precipitation. The mAb were diluted to 1 mg/ml in PBS and stored at −70° until use. Two hundred micrograms of anti-NK1.1 mAb (PK136) in 200 μl was injected i.p. on days 6 and 3 before infection. Two hundred micrograms of anti-TcR$\gamma\delta$ mAb (UC7-13D5) in 200 μl was injected i.p. on day 3 before infection.

Fluorescence-activated cell sorter (FACS) analysis

Plastic non-adherent cells of PEC were incubated with saturating amounts of biotin- or FITC-conjugated antibodies for 45 min at 4°. To stain biotin-conjugated antibodies, phycoerythrin (PE)-conjugated streptavidin was used. Cells were analysed with a FACScan flow cytometer (Becton Dickinson). We gated cells by forward and side light scattering for live lymphocytes. The data were analysed with FACScan Research Software (Becton Dickinson).

Target cells

YAC-1, a Moloney virus-induced mouse T-cell line, was used as the target cells in a chromium-release assay. Briefly, 2×10^6/500 μl of YAC-1 cells was labelled with 100 μCi of ^{51}Cr for 60 min at 37° in a shaking water bath. After labelling, the cells were washed three times in RPMI-1640 with 10% FCS and resuspended at a concentration of 1×10^5 cells/ml.

NK cytolytic assay

NK activity was measured by a 4-hr ^{51}Cr-release assay using labelled YAC-1 target cells. Effector cells were adjusted to varying concentrations and added to 1×10^4 of ^{51}Cr-labelled YAC-1 cells. Two hundred microlitres of effector and target cells was dispensed into individual wells of a 96-well microtitre plate and incubated for 4 hr at 37° in a humidified CO_2 incubator. After the incubation, 100 μl of supernatant was harvested. Radioactivity of the supernatants was measured in a γ-counter and the percentage specific ^{51}Cr-release was calculated according to the following formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100.$$

Spontaneous release was determined from the samples of target cells incubated without effector cells and maximal release was determined from the samples of target cells incubated with 10% Triton-X (Wako Chemical Industries Ltd, Osaka, Japan).

Kinetics of bacterial growth in spleens and livers of mice after L. monocytogenes *infection*

Primary infection with *L. monocytogenes* was performed by an i.p. inoculation of 5×10^4 viable bacteria in a volume of 0·2 ml of PBS on day 0. Mice were anaesthetized with ether and killed by cutting the cervical artery on day 1, 3, 5 or 8 after infection. Bacterial growth in the spleen or liver was determined by plating 10-fold serial dilutions of organ homogenates on tryptic soy agar. The detection limit of this procedure was 10^2 *L. monocytogenes* per organ. The numbers of colonies were counted after 24 hr incubation at 37°.

Statistics

The statistical significance of the data was determined by Student's *t*-test. A *P* value less than 0·05 was taken as significant.

RESULTS

Kinetics of appearance of NK cells during *L. monocytogenes* infection

C57BL/6 mice were inoculated with 5×10^4 of *L. monocytogenes* and FACS analyses were carried out on plastic non-adherent cells from PEC on days 1, 3, 5 and 8 after infection. As shown in Fig. 1, NK cells significantly increased as early as 1 day after *L. monocytogenes* inoculation. Half of the NK cells on day 1 after *L. monocytogenes* infection were Thy-1-positive. The proportion of NK cells increased to the maximum on day 3 after infection (Fig. 1). Also, the total number of peritoneal NK cells increased to $2{\cdot}8 \times 10^5$/mouse on day 3 compared with $4{\cdot}6 \times 10^4$ on day 0, and decreased thereafter (data not shown). The Thy-1 intensity of NK cells increased by day 3, suggesting an activated state of these cells.[17] By 8 days after infection, NK cells decreased to the levels of uninfected mice. These results showed the appearance and activation of NK cells in peritoneal cavities of i.p. infected *L. monocytogenes* mice.

Effects of *in vivo* anti-NK1.1 mAb treatment on eradication of bacteria in mice infected with *L. monocytogenes*

To investigate the protective role of NK cells in *L. monocytogenes* infection, we examined the effect of NK cell depletion on the bacterial clearance. We injected 200 μg of

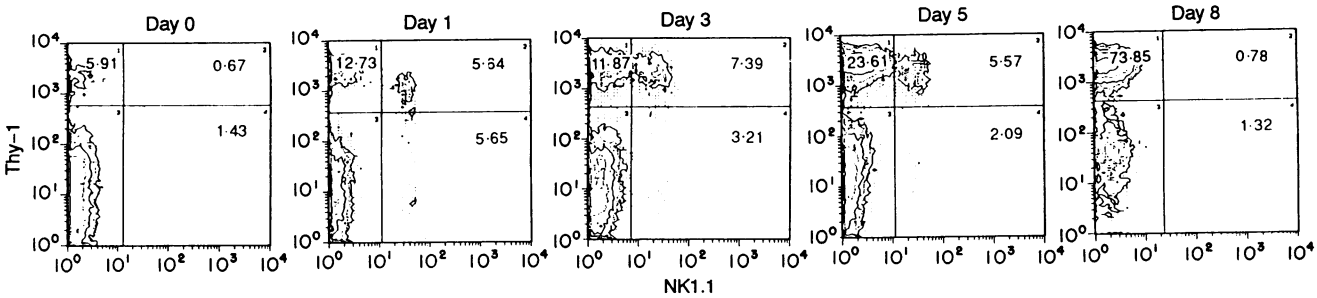

Figure 1. Kinetics of NK1.1$^+$ cells after i.p. *L. monocytogenes* infection. Non-adherent PEC from C57BL/6 mice on days 0, 1, 3, 5 and 8 after inoculation with *L. monocytogenes* were analysed. Cells were stained with anti-NK1.1 mAb versus anti-Thy-1.2 mAb.

anti-NK1.1 mAb (PK136) on days 6 and 3 before infection. This treatment completely depleted NK1.1$^+$ cells (data not shown) and NK activity of spleen cells (Fig. 2). As illustrated in Fig. 3, treatment of mice with anti-NK1.1 mAb significantly enhanced the ability of mice to clear *L. monocytogenes* from the spleen and liver on day 5 after infection, compared to infected controls with intact NK cell activity. We repeated the experiment three times and obtained nearly the same results. These results suggest that NK cells do not take part in the resistance against i.p. *L. monocytogenes* infection in C57BL/6 mice. We then analysed the mechanism of the increased ability of bacterial clearance in NK cell-depleted mice.

The effects of anti-NK1.1 mAb administration on the peritoneal lymphocyte population of *L. monocytogenes*-infected mice

To analyse the mechanism of the enhancement of *L. monocytogenes* eradication in NK cell-depleted mice, we examined the effects of NK cell depletion on the peritoneal lymphocyte population including $\gamma\delta$ and $\alpha\beta$ T cells, which have been reported to be essential for the protection of an early or a late stage of *L. monocytogenes* infection, respectively.[4–6,9] Figures 4 and 5 shows the time-course of appearance of $\alpha\beta$ or $\gamma\delta$ T cells during *L. monocytogenes* infection in NK cell-depleted mice and infected control mice. $\alpha\beta$ T cells rapidly increased from 5 to 8 days after infection, when T-cell-dependent immunity develops (Fig. 4 upper panels). The proportion of $\gamma\delta$ T cells apparently increased on day 5 after infection and decreased thereafter in infected control mice (Fig. 5 upper panels). NK cell-depleted mice also showed a similar time-course of appearance of $\alpha\beta$ or $\gamma\delta$ T cells during *L. monocytogenes* infection (Figs 4 and 5). We further calculated the total number of $\alpha\beta$ and $\gamma\delta$ T cells after *L. monocytogenes* infection in control and NK-depleted mice. As shown in Fig. 6, the increases of total numbers of $\gamma\delta$ or $\alpha\beta$ T cells were more prominent in NK cell-depleted mice on day 5 after *L. monocytogenes* infection, although no significant differences were observed before or after that time. We repeated the experiment three times and obtained nearly the same results each time. We have reported that $\gamma\delta$ T

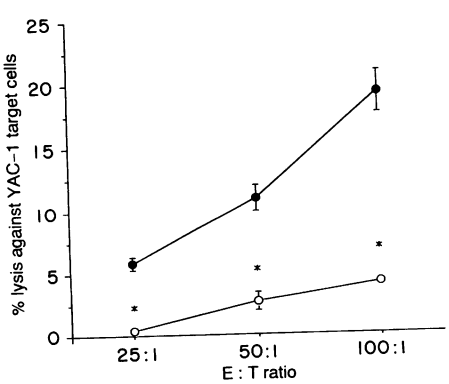

Figure 2. NK activities of spleen cells from anti-NK1.1 mAb-treated mice on day 5 after *L. monocytogenes* infection. NK activities of spleen cells from C57BL/6 mice treated with PBS or 200 μg of anti-NK1.1 mAb 6 and 3 days before infection were assayed on day 5 after infection. Open circles: anti-NK1.1 mAb-treated mice. Closed circles: infected control mice. The values are mean ± SD for a group of three mice. *$P < 0{\cdot}05$. E:T, effector: target ratio.

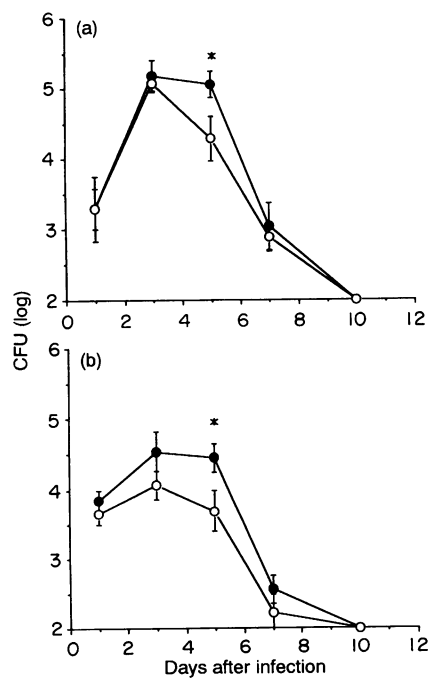

Figure 3. Clearance of *L. monocytogens* from organs in PBS-treated and anti-NK1.1 mAb-treated mice. Bacterial numbers in (a) spleen and (b) liver from C57BL/6 mice treated with PBS or anti-NK1.1 mAb 6 and 3 days before inoculation with *L. monocytogens* (5×10^4) were counted on days 1, 3, 5, 7 and 10 of infection. The values are mean ± SD for a group of five mice. Closed circles: PBS-treated mice. Open circles: anti-NK1.1 mAb-treated mice. *$P < 0{\cdot}05$. CFU, colony-forming units.

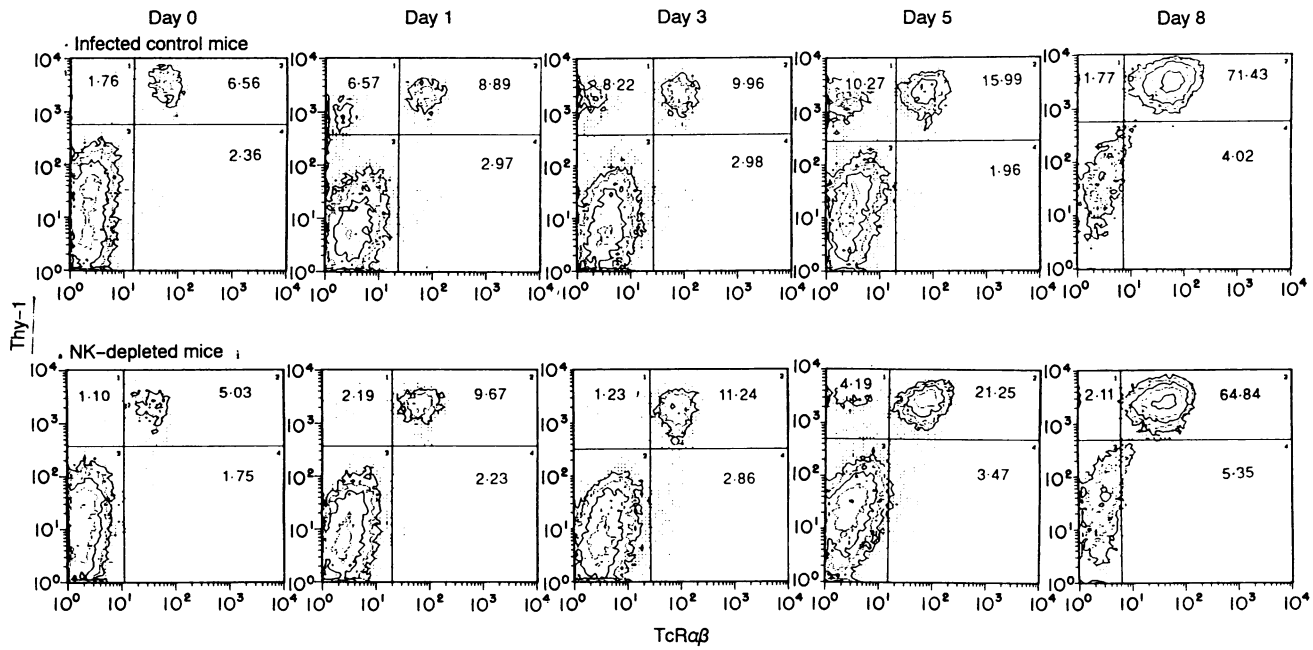

Figure 4. Kinetics of $\alpha\beta$ T cells of PEC from PBS-treated and anti-NK1.1 mAb-treated mice after *L. monocytogenes* infection. C57BL/6 mice were treated with PBS (upper) or 200 μg of anti-NK1.1 mAb (lower) 6 and 3 days before infection. Non-adherent PEC obtained from infected mice on days 0, 1, 3, 5 and 8 were stained with anti-TcR$\alpha\beta$ versus anti-Thy-1.2 mAb.

cells play an important role in the early stage of *L. monocytogenes* infection.[9] Therefore, it is suggested that the enhanced induction of $\gamma\delta$ T cells would be responsible for the accelerated clearance of *L. monocytogenes* in NK cell-depleted mice.

Effects of anti-TcR $\gamma\delta$ mAb treatment on the enhanced *L. monocytogenes* eradication in NK cell-depleted mice

We investigated whether increased bacterial clearance in NK cell-depleted mice could be blocked by $\gamma\delta$ T-cell depletion. For $\gamma\delta$ T-cell depletion mice were administered i.p. with anti-TcR $\gamma\delta$

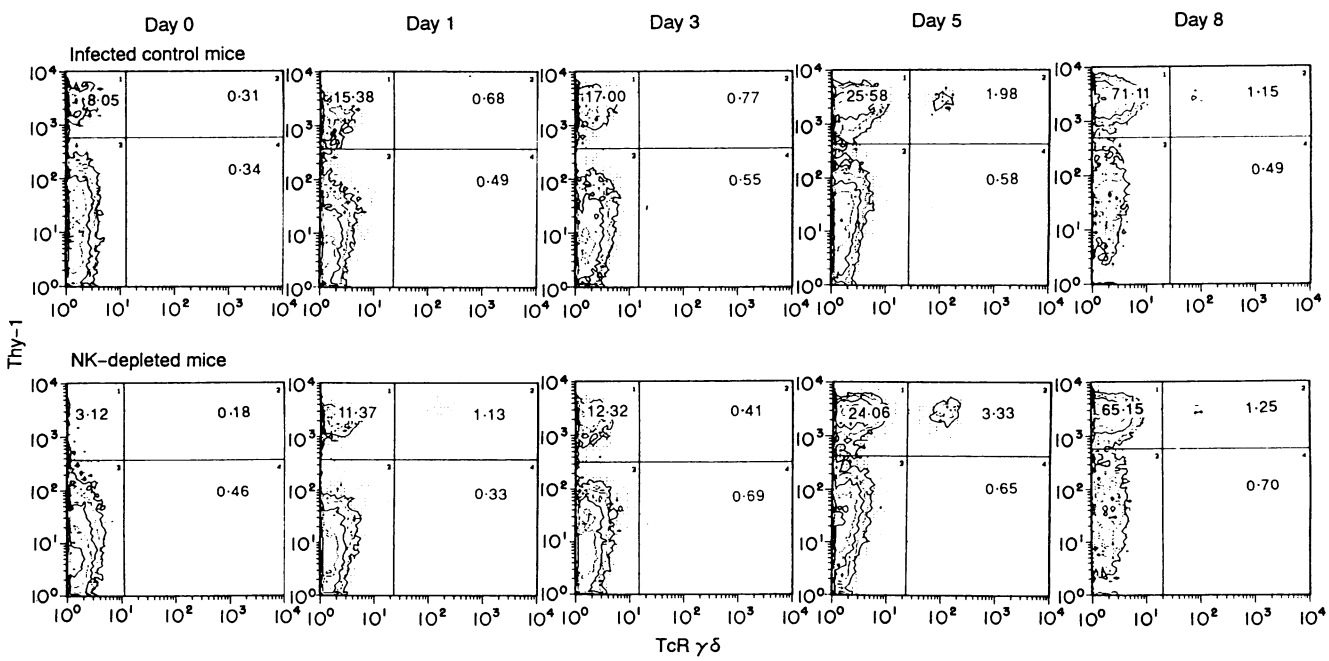

Figure 5. Kinetics of $\gamma\delta$ T cells of PEC in PBS-treated and anti-NK1.1 mAb-treated mice after *L. monocytogenes* infection. C57BL/6 mice were treated with PBS (upper) or anti-NK1.1 mAb (lower) 6 and 3 days before infection. Non-adherent PEC obtained from infected mice on days 0, 1, 3, 5 and 8 were stained with anti-TcR$\gamma\delta$ versus anti-Thy-1.2 mAb.

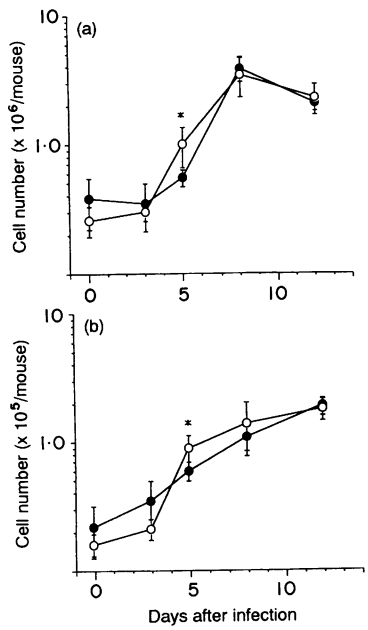

Figure 6. Kinetics of $\alpha\beta$ and $\gamma\delta$ T-cell number after *L. monocytogenes* infection. Total numbers of $\alpha\beta$ (a) and $\gamma\delta$ T cells (b) in PEC were calculated by the total cell numbers of PEC, percentages of lymphocytes determined by Giemsa staining and the percentages of $\alpha\beta$- or $\gamma\delta$-positive cells in lymphocytes determined by FACS analyses. The values are mean ± SD for a group of five mice. Closed circles: PBS-treated mice. Open circles: anti-NK1.1 mAb-treated mice. $*P < 0{\cdot}05$.

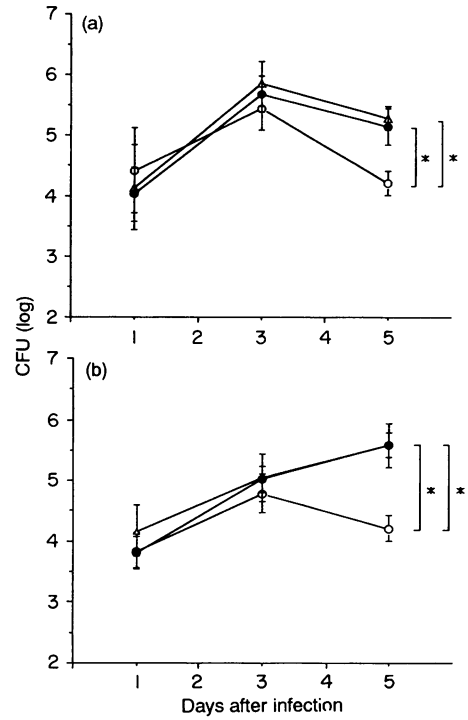

Figure 7. Effects of $\gamma\delta$ T-cell depletion on the enhanced *L. monocytogenes* clearance in anti-NK1.1 mAb-treated mice. Bacterial numbers in (a) spleen and (b) liver from C57BL/6 mice treated with PBS or anti-NK1.1 mAb 6 and 3 days before inoculation with *L. monocytogenes*, both of which had been treated with 200 μg of anti-TcR$\gamma\delta$ mAb 3 days before the infection, were counted on days 1, 3 and 5 of infection. The values are mean ± SD for a group of five mice. Closed circles: PBS and anti-TcR$\gamma\delta$ mAb-treated mice. Open triangles: anti-NK1.1 mAb and anti-TcR$\gamma\delta$ mAb-treated mice. Open circles: infected control mice. $*P < 0{\cdot}05$.

mAb (UC7-13D5) 3 days before *L. monocytogenes* infection.[9] $\gamma\delta$ T cells shared only 0·74% of peritoneal lymphocytes with anti-TcR$\gamma\delta$ mAb-treated mice on day 5 after *L. monocytogenes* infection, compared with 1·96% of those from infected control mice (data not shown). Furthermore, $\gamma\delta$ T-cell depletion in anti-TcR$\gamma\delta$ mAb-treated mice was ascertained by *Escherichia coli* infection.[18] $\gamma\delta$ T cells shared only 0·84% of peritoneal lymphocytes from anti-TcR$\gamma\delta$ mAb-treated mice on day 5 after *E. coli* infection, compared with 12·54% of those from infected control mice (data not shown). As shown in Fig. 7, NK cell- and $\gamma\delta$ T-cell depleted mice showed the same level of bacterial eradication as that of $\gamma\delta$ T-cell-depleted mice. These results suggest that enhanced clearance of bacteria from the spleen and liver in the early stage of *L. monocytogenes* infection in NK cell-depleted mice is caused, at least in part, by augmented increase of $\gamma\delta$ T cells, which play an essential role in host defence at the early stage of the infection[9] (Fig. 7).

DISCUSSION

In the present study, we have provided evidence which suggests that NK cells might down-regulate the host resistance against *L. monocytogenes*, and that one of its mechanisms may be mediated by regulation of $\gamma\delta$ T cells.

We ascertained that NK cells, detected by NK1.1 surface marker in C57BL/6 mice, significantly increased as early as 24 hr after *L. monocytogenes* infection, reaching the maximum number on day 3 and returning to the normal level on day 8 after infection. Dunn and North[16] reported that NK cells rapidly increased in draining lymph nodes up to 100-fold in number within 24 hr of subcutaneous *L. monocytogenes* infection. They speculated that the increase of NK cells is dependent on the recruitment of NK cells from the blood, because such a rapid increase in number over a 24-hr period could not have resulted from the replication of local cells.[16] In our data, the rapid increase in NK cell number within 24 hr of infection might also indicate the recruitment of NK cells from the blood. In addition, it is possible that NK cells would proliferate by the effects of various cytokines such as interleukin-12 (IL-12), which is reported to be produced by macrophages by *L. monocytogenes* stimulation.[19,20]

Kearns and Leu[11] reported that *L. monocytogenes* infection induced a systemic increase of NK activity, which reached a peak at day 3 and returned to normal levels at day 7 in the bone marrow and spleen cells, but remained increased for more than 10 days in the peripheral blood cells and PEC. We speculate that NK activity in PEC observed for more than 10 days in the previous report is mediated by NK1.1$^-$ non-major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes,[21] because the number of NK1.1$^+$ cells from the peritoneal cavity returned to normal levels 8 days after infection (Fig. 1).

Whether NK cells observed in *L. monocytogenes* infection are activated or not is important in analysing the role of NK

cells. It is reported that the Thy-1$^+$ subset of NK cells has higher cytotoxic activity and proliferative ability than the Thy-1$^-$ subset.[17] IL-2-activated NK cells are reported to be mostly Thy-1$^+$, suggesting that Thy-1 is an activation antigen on NK cells.[17] The percentage of a Thy-1$^+$ population of NK cells increased from day 1 to day 3 after *L. monocytogenes* infection (Fig. 1). Also, the Thy-1 intensity of NK cells increased from day 1 to day 3 (Fig. 1). Therefore it is suggested the NK cells were activated. Actually, as shown in Fig. 2, spleen cells from mice on day 5 after *L. monocytogenes* infection showed enhanced NK activity (20%, effector : target ratio 100 : 1) compared with those from non-infected control mice (6·0%, data not shown). These results suggest that activated NK cells are increased in the peritoneal cavity on days 3–5 after i.p. listerial infection.

We demonstrated in this report that NK cell-depleted mice showed an enhanced clearance of bacteria from organs after i.p. infection with *L. monocytogenes*. Therefore, it is suggested that NK cells, although activated, do not have a protective role against *L. monocytogenes* infection, at least in i.p. inoculated, immunocompetent mice. There have been many reports regarding the role of NK cells in host defence against a variety of infectious micro-organisms, including viruses, protozoa, fungi, as well as bacteria.[22–30] However, it is not clear whether NK cells are involved in the host defence mechanisms against *L. monocytogenes* infection. NK cells are suggested to play an important role during *L. monocytogenes* infection in macrophage activation through a T-cell-independent mechanism in *scid* mice.[14,15] It is also reported that the NK cell is one of the essential cellular components necessary for early host defence against subcutaneous *L. monocytogenes* infection in mice.[16] On the other hand, it is reported that NK cells are not involved in early resistance against *L. monocytogenes* infection, because when NK-deficient beige mutant mice were infected with *L. monocytogenes* they showed the same bacterial growth curves in organs over 5 days as those of infected C57BL/6 and (C57BL/6 × beige) F_1 mice.[12] Also, ^{89}Sr-treated mice, which have a decreased NK activity, showed normal clearance of *L. monocytogenes* from organs.[13] The difference about the contribution of NK cells to the protection against *L. monocytogenes* infection between these reports might be due to the difference of the experimental system or the route of *L. monocytogenes* inoculation. Recently, Schultheis and Kearns reported that clearance of *L. monocytogenes* was enhanced in the spleen but not in the liver by *in vivo* administration of the anti-asialo-GM_1 antibody for NK cell depletion.[31] As asialo-GM_1 surface antigen is also expressed on a part of the T cells in addition to the NK cells,[21] it is possible that T cells that act to affect anti-listerial immunity might be depleted by anti-asialo-GM_1 mAb treatment. We think our results would further support the idea that NK cells do not have a protective role in i.p. *L. monocytogenes* infection, at least in immunocompetent mice.

We further demonstrated the possibility that the augmented clearance of bacteria in NK cell-depleted mice would be mediated by enhanced induction of $\gamma\delta$ T cells. We have reported that $\gamma\delta$ T cells play an important role in the early stage of *L. monocytogenes* infection,[9] and the same results were observed by Mombaerts *et al.* using TcRδ mutant mice lacking $\gamma\delta$ T cells.[32] Actually, the number of *Listeria* in the spleen or liver from $\gamma\delta$ T-cell-depleted mice on day 5 was significantly increased compared with those of the infected control mice (Fig. 7).[9] On the other hand, $\alpha\beta$ T-cell-depleted mice did not show such a change on day 5 after infection.[9] Rather, $\alpha\beta$ T-cell-depleted mice showed a persistent bacterial burden after day 5 of *L. monocytogenes* infection.[9] Therefore, it is suggested that increased $\gamma\delta$ T cells in the peritoneal cavity of NK cell-depleted mice could be one of the factors responsible for the enhanced clearance of *L. monocytogenes* in the early stage of infection. It has been reported that NK cells possess cytolytic activity against antigen-presenting cells.[33,34] Although we could not detect a significant increase in the number of macrophages in anti-NK1.1 mAb-treated mice (data not shown), it is possible that accelerated induction of $\gamma\delta$ T cells in NK cell-depleted mice was due to the enhanced antigen presentation.

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