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A Protective Role of γ/δ T Cells in Primary Infection with *Listeria monocytogenes* in Mice

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Summary

We have previously reported that T cells bearing T cell receptors (TCRs) of γ/δ type appear at a relatively early stage of primary infection with *Listeria monocytogenes* in mice. To characterize the early-appearing γ/δ T cells during listeriosis, we analyzed the specificity and cytokine production of the γ/δ T cells in the peritoneal cavity in mice inoculated intraperitoneally with a sublethal dose of *L. monocytogenes*. The early-appearing γ/δ T cells, most of which were of CD4⁻CD8⁻ phenotype, proliferated and secreted IFN- γ and macrophage chemotactic factor in response to purified protein derivative from *Mycobacterium tuberculosis*, or recombinant 65-kD heat-shock protein derived from *M. bovis* but not to heat-killed *Listeria*. To further elucidate the potential role of the γ/δ T cells in the host-defense mechanism against primary infection with *Listeria*, we examined the effects of in vivo administration of monoclonal antibodies (mAbs) against TCR- γ/δ or TCR- α/β on the bacterial eradication in mice infected with *Listeria*. Most of α/β T cells or γ/δ T cells were depleted in the peripheral lymphoid organs at least for 12 d after an intraperitoneal injection of 200 μ g TCR- α/β mAb or 200 μ g TCR- γ/δ mAb, respectively. An exaggerated bacterial multiplication was evident at the early stage of listerial infection in the γ/δ T cells-depleted mice, whereas the α/β T cell-depleted mice exhibited much the same resistance level as the control mice at this stage although the resistance was severely impaired at the late stage after listerial infection. These results clearly indicated that the early-appearing γ/δ T cells contribute to the host defense at the early stage of infection with *Listeria* and confirmed previous reports that the highly evolved type of immunity mediated by *Listeria*-specific α/β T cells contributes to the host protection at the late stage after listerial infection.

T cells are known to play important roles in host defense through establishing immune responses against nominal antigens on invading microbes (1). T cells recognize nominal antigens in the context of self-MHC antigens by a TCR that is composed of α and β chains (2). Another type of TCR, which is composed of γ and δ chains, has also been identified (3). TCR- γ/δ represents the first CD3-associated TCR in ontogeny and displays a more limited diversity than TCR- α/β . Furthermore T cells bearing TCR- γ/δ are distributed in different anatomical sites such as the epidermis (4, 5), intestines (6, 7), and reproductive organs (8). Perhaps TCR- γ/δ precedes the TCR- α/β in evolution and functions originally to survey tissues for a limited array of conserved antigens. Recently, a significant fraction of γ/δ -bearing T cells has been shown to be specialized to recognize phylogeneti-

cally conserved stress/heat-shock proteins (HSP),¹ which have been previously implicated as immunodominant antigens (9–11). These observations raise the possibility that at least a subset of γ/δ T cells may represent a first line of defense against infection and tumors by being devoted themselves to recognizing and eliminating stressed autologous cells such as transformed or infected cells (12). However, the roles of γ/δ T cells in host-defense mechanisms against invading microbes remain elusive.

Listeriosis is caused by the Gram-positive rod, *Listeria monocytogenes*, which is one of the intracellular bacteria, as

¹ Abbreviations used in this paper: HSP, heat-shock protein; MCF, macrophage chemotactic factor; PEC, peritoneal exudate cells; PPD, purified protein derivative.

well as *Mycobacterium tuberculosis* (1, 13). The protective mechanism against listerial infection is largely divided into two phases. The first one depends mainly on the phagocyte system, while the second depends on the immune response mediated by α/β T cells (14). We have previously reported that γ/δ T cells precede the α/β T cells in appearance during listerial infection, suggesting that the early-appearing γ/δ T cells may account for protection during the period between the early stage covered mainly by phagocytes and the late stage covered by typical immunities in terms of the time sequence after invasion with *L. monocytogenes* (14, 15).

In the present study, to elucidate the potential roles of the early-appearing γ/δ T cells in the host-defense against listerial infection, we analyzed the specificity and functions of the γ/δ T cells in mice infected with *Listeria*. Our results suggest that these early-appearing γ/δ T cells are activated by purified protein derivative (PPD)/65-kD HSP derived from *Mycobacterium bovis*, but not by heat-killed *Listeria*, and produced significant amounts of IFN- γ and macrophage chemotactic factor (MCF) in response to the mycobacterial antigens. Furthermore, the study in TCR- γ/δ T cell-depleted mice prepared by in vivo administration of anti-TCR- γ/δ mAb clearly demonstrated that the γ/δ T cells play a crucial role at the early stage of the listerial infection.

Materials and Methods

Mice and Microorganisms. Female C3H/HeN mice were obtained from Charles River Japan Inc. (Atsugi, Japan). 8–10-wk-old mice were used for the experiments. *L. monocytogenes*, strain EGD, was used in all experiments. 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 PBS, and stored at -70°C in small aliquots. Heat-killed *Listeria* were prepared by incubating viable *L. monocytogenes* at 74°C for 120 min.

Peritoneal Exudate Cells. Mice were inoculated intraperitoneally with 10^4 viable *L. monocytogenes* in a volume of 0.2 ml of PBS on day 0 and injected intraperitoneally with 1.5 ml of sterile 10% proteose peptone (Difco Laboratories) on day 3 or 10 after infection. The peritoneal exudate cells (PEC) were harvested 3 d later by peritoneal lavage with ice-cold HBSS containing 5 U/ml of heparin. The cells were collected by centrifuging at $110g$ for 10 min, washed twice with HBSS, and suspended at the optimal concentrations.

Antibodies and Reagents. Anti-TCR- α/β mAb (H57-595) was a kind gift of Dr. R. Kubo (National Jewish Center of Immunology and Respiratory Medicine, Denver, CO). Biotin-conjugated anti-CD8 mAb (anti-Ly2), PE-conjugated anti-CD4 mAb (GK1.5), and biotin-conjugated anti-Thy-1.2 mAb were purchased from Becton Dickinson & Co. (Oxnard, CA). FITC-conjugated goat anti-hamster IgG was purchased from Cappel Laboratories (Detroit, MI). Streptavidin-PE and streptavidin-DuoCHROME conjugates were purchased from Becton Dickinson & Co. Antibodies against murine anti-TCR- α/β mAb (H57-597) and anti-TCR- γ/δ mAb (UC7-13D5) (16) were obtained by growing hybridoma cells in serum-free medium (101; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and collecting supernatant. Antibodies were then concentrated, purified by 50% ammonium sulfate precipitation. The purity of the preparations was confirmed by SDS-PAGE and the concentration of antibodies was determined by the Lowry method. The mAbs

diluted to 1 mg/ml in PBS were stored at -70°C until use. 200 μg of mAbs in 200 μl was injected into the peritoneal cavity on day 3 before primary infection with *L. monocytogenes*. In control group, 200 μl of PBS was injected.

Proliferation Assay. PEC harvested 3 d after proteose peptone injection, namely on day 6 after the listerial inoculation, were passed over a nylon wool column and treated with anti-CD4 mAb (RL172/4, rat IgM) and anti-Lyt-2.1 mAb (IgM) (020-300; Meiji Institute of Health Science, Tokyo, Japan) plus complement. The remaining cells were $>90\%$ CD3 $^{+}$ and demonstrated no detectable staining with antibodies to TCR- α/β , to CD4, or to CD8. Cells (3×10^5) were incubated with 3×10^5 irradiated (3,000 rad) syngeneic spleen cells in 96-well plates for 72 h and then labeled with [^3H]thymidine for 12 h and collected. PPD (25 $\mu\text{g}/\text{ml}$) derived from *M. tuberculosis* (H $_3$ Ra), recombinant 65-kD HSP (25 $\mu\text{g}/\text{ml}$) derived from *M. bovis* (provided kindly by Dr. R. van der Zee, National Institute of Public Health and Environment Protection, Bacteriology, the Netherlands) (17), or amino acid 180–196 synthetic peptide of the 65-kD *M. bovis* HSP (15 $\mu\text{g}/\text{ml}$; Peptide Institute, Inc., Osaka), one of the critical epitopes for T cell recognition by arthritic clones (18), were added as antigens.

Cytokine Production. Samples were assayed for IFN- γ using two-site sandwich ELISA using a hamster anti-mouse IFN- γ mAb (Genzyme, Boston, MA) and rabbit anti-mouse IFN- γ serum (a gift from Daiichi Seiyaku, Japan). Microplates (E.I.A./R.I.A. plate flat-bottomed medium-binding; Costar, Cambridge, MA) were coated with 100 μl of 1.5 $\mu\text{g}/\text{ml}$ anti-IFN- γ mAb in coating buffer for 12 h at 4°C , followed by blocking with 100 μl of 0.5% BSA in coating buffer for 30 min at room temperature. Samples and a mouse rIFN- γ standard were diluted in 0.05% PBS-Tween 20, and 50 μl of 100-fold diluted rabbit anti-mouse IFN- γ serum in PBS-Tween 20. After 90 min, the plates were washed and incubated with 50 μl of a 1:800 dilution of goat anti-rabbit IgG/horseradish peroxidase-conjugated IgG (Tago, Inc., Burlingame, CA) in PBS-Tween 20 for 90 min. Plates were washed three times with PBS-Tween 20 and 100 μl substrate consisting of orthophenylenediamine (0.4 mg/ml), and 0.003% H_2O_2 in citrate buffer was added. After 20 min, the reaction was terminated by adding 2.5 M H_2SO_4 and absorbance was measured at a wavelength of 492 nm and a reference of 620 nm on an EAR 400 FW (SLT-Lab. Instruments, Salzburg, Austria).

CTLL-2 cells, an IL-2- and IL-4-dependent T cell line, were used for IL-2 assay. CTLL-2 cells (5×10^3 cells/well) were cultured in the presence of a twofold diluted supernatant in a 96-well microplate for 24 h at 37°C . During the last 4 h of culture, [^3H]thymidine was added. The cultures were harvested and the incorporated radioactivity was counted in a liquid scintillation counter.

Chemotactic activity was assessed according to Synderman et al. (19) with some modification as described elsewhere. Briefly, the culture supernatant in a 1:1 dilution with RPMI 1640 supplemented with 10% FCS was placed in the lower well of chemotaxis chamber. A polycarbonate membrane filter with 5- μm pores (Nucleopore Corp., Pleasanton, CA) was placed between an upper and a lower well. As indicator cells, peritoneal exudate cells were collected from mice injected intraperitoneally with 1 ml of proteose peptone 3 d before the assay. The cells ($6 \times 10^5/\text{ml}$) in RPMI 1640 supplemented with 10% FCS were added to the upper well. After 90 min of incubation, the filters were removed, washed, air-dried, and stained with Giemsa solution, and the number of migrated macrophages was determined in five randomly selected high-power fields.

Kinetics of Bacterial Growth after Intraperitoneal Infection with *L. monocytogenes* in Spleens of Various mAb-treated Mice. Primary

infection with *L. monocytogenes* was performed by an intraperitoneal injection of 3×10^3 viable bacteria in a volume of 0.1 ml of PBS on day 0. 3 d before primary infection (on day -3), mice were treated with an intraperitoneal injection of a 200 μg of the mAb. Mice were anesthetized with ether and killed by cutting the cervical artery at intervals after the intraperitoneal infection (on days 3, 5, 8, 12, and 23). Bacterial growth in spleens 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 spleen. The numbers of colonies were counted after 24 h of incubation at 37°C.

Results

Enrichment of γ/δ T Cells in the Peritoneal Cavity by an Intraperitoneal Injection with Proteose Peptone into Mice Infected with *Listeria*. We have previously found that $\text{CD}3^+\text{CD}4^-\text{CD}8^-\gamma/\delta^+$ T cells in the spleen as well as in the peritoneal cavity significantly increased in proportion on day 3 and thereafter decreased by day 10 after an intraperitoneal infection with *Listeria*. *Listeria*-immune T cells were reported to be enriched in PEC induced by injection with proteose peptone 3 d previously into *Listeria*-infected mice (20). Therefore, we injected proteose peptone into *Listeria*-infected mice to enrich for "sensitized T lymphocytes," and we examined the expression of CD4 and CD8 molecules and the TCR on the peptone-induced PEC by flow cytometric analysis. On day 3 or on day 10 after an intraperitoneal infection with 10^4 *L. monocytogenes*, 1.5 ml of 10% proteose peptone was injected intraperitoneally and PEC were collected 3 d later, that is, on day 6 or on day 13, respectively. Few $\text{CD}3^+\text{CD}4^-\text{CD}8^-$ cells were detected in peptone-induced PEC from unprimed normal controls (data not shown). On the other hand, PEC on day 6 of *Listeria*-infected mice were composed of a large number of $\text{CD}3^+\text{CD}4^-\text{CD}8^-$ cells ($\sim 30\text{--}40\%$; data not shown). Such a high proportion of $\text{CD}3^+\text{CD}4^-\text{CD}8^-$ cells in the peritoneal cavity was not detected at any stage during

listeriosis without injection of proteose peptone. The proportion of the $\text{CD}3^+\text{CD}4^-\text{CD}8^-$ cells decreased gradually to $<20\%$ in the peptone-induced PEC on day 13 after infection (data not shown). It was demonstrated that the $\text{CD}3^+\text{CD}4^-\text{CD}8^-$ cells on day 6 express TCR- γ/δ on their surfaces (data not shown). These results confirm that $\text{CD}4^-\text{CD}8^-\gamma/\delta$ T cells appear at an early phase of primary infection with *Listeria* and proteose peptone-induced PEC of *Listeria*-infected mice provide us a good cell source for γ/δ T cells for further analysis of the early-appearing γ/δ T cells in listeriosis.

Proliferation and Production of Cytokines of Purified $\text{CD}3^+\text{CD}4^-\text{CD}8^-\gamma/\delta$ T Cells from PEC in Response to Mycobacterial Antigens. To determine the possible ligands for the γ/δ T cells in listerial infection, we investigated the proliferative activity of the purified γ/δ T cells in the presence of heat-killed *Listeria*, 65-kD HSP, and PPD. Nylon wool column-passed PEC on day 6 were treated with anti-CD4 mAb and anti-CD8 mAb plus complement, and double-negative T cells highly enriched for γ/δ T cells ($>90\%$) were used as responder cells. As shown in Fig. 1 A, the purified γ/δ T cells respond to PPD in a dose-response manner, in the presence of syngeneic irradiated splenocytes. These purified peritoneal $\text{CD}4^-\text{CD}8^-\gamma/\delta$ T cells significantly responded to PPD and 65-kD HSP ($p < 0.005$) but not to heat-killed *Listeria* in the presence of syngeneic irradiated spleen cells, as shown in Fig. 1 B. Among amino acid sequences of 65-kD HSP, amino acid 180-196 was reported to be a crucial epitope for recognition of some γ/δ T cells (21). Therefore, we also examined the reactivities of the γ/δ T cells against amino acid 180-196 synthetic peptide of 65-kD HSP. The $\text{CD}4^-\text{CD}8^-\gamma/\delta$ T cells strongly proliferated in response to the oligopeptide (Fig. 1 B). These results suggest that the early-appearing γ/δ T cells during listerial infection respond specifically to 65-kD HSP.

Next, using the same condition for proliferation assay, we

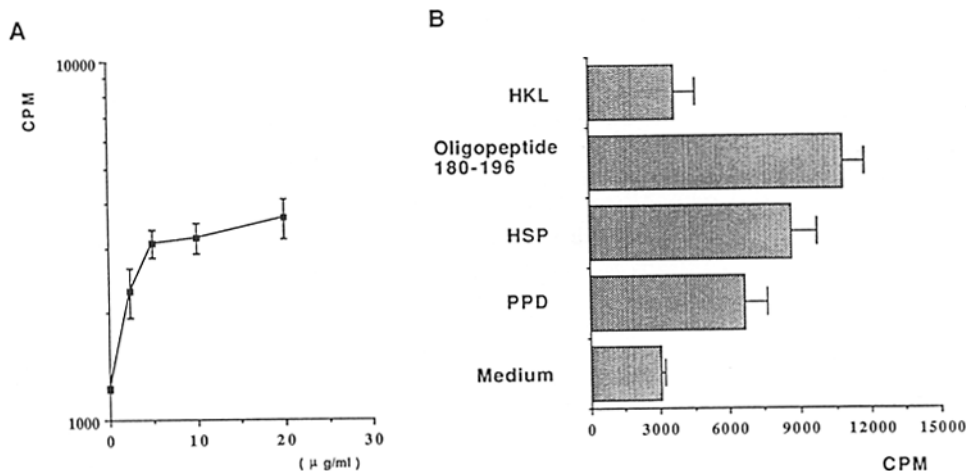


Figure 1. In vitro stimulation of peritoneal γ/δ T cells in *Listeria*-infected mice with mycobacterial antigens. Proteose peptone-induced PEC on day 6 of *Listeria*-infected mice were nylon wool passed and both CD4 and CD8 cells were depleted by treatment with anti-CD4 mAb, anti-CD8 mAb, and complement. Proliferative response of peritoneal γ/δ T cells to various concentration of PPD (2.5–20 $\mu\text{g/ml}$; A), PPD (20 $\mu\text{g/ml}$), recombinant 65-kD HSP (25 $\mu\text{g/ml}$), amino acid 180–196 oligopeptide of 65-kD HSP (15 $\mu\text{g/ml}$), or HKL ($5 \times 10^7/\text{ml}$) (B) were assayed by 12-h ^3H thymidine incorporation in cells ($3 \times 10^5/\text{well}$) cultured for 72 h in the presence of irradiated (3,000 rad) syngeneic spleen cells ($3 \times 10^5/\text{well}$). The cpm for ^3H thymidine incorporation were measured in triplicated cultures. The results are mean \pm SD.

Table 1. Production of Cytokines of Purified CD3⁺CD4⁻CD8⁻ γ/δ T Cells from PEC in Response to Mycobacterial Antigens

Antigen	INF- γ	IL-2
	U/ml	
PPD	1,680	Undetectable*
HSP	940	Undetectable
HKL	33	Undetectable
Medium	66	Undetectable

Nylon wool column-passed PEC on day 6 were treated with anti-CD4 mAb, anti-CD8 mAb plus complement. The purified peritoneal CD4⁻CD8⁻ γ/δ T cells were cultured with various antigens in the presence of irradiated syngeneic splenocytes as in the legend of Fig. 1. Supernatants of the γ/δ T cells stimulated with various antigens were assayed for IFN- γ by ELISA and IL-2 by bioassay using CTLL-2.

* <0.01 BRMs/ml.

examined the cytokine production in the supernatants of the 3-d culture of CD3⁺CD4⁻CD8⁻ γ/δ T cells with PPD/65-kD HSP. As shown in Table 1, a significant level of IFN- γ was detected in the supernatant of the γ/δ T cells activated by PPD/65-kD HSP. On the other hand, IL-2 activity was hardly detected in the supernatants of γ/δ T cells stimulated with PPD/65-kD HSP. Although the abilities to generate other cytokines were not examined, the early-appearing γ/δ T cells may preferentially produce IFN- γ in response to PPD/65-kD HSP.

We have previously reported that MCF is one of crucial cytokines for protection against listerial infection (22, 23). The MCF activity in the culture supernatant was also examined by the Boyden chamber method. As shown in Fig. 2, we found the γ/δ T cells produce not only IFN- γ but also MCF in response to PPD, but not to heat-killed listeria (HKL). These results suggest that the γ/δ T cells participate in primary-host defense against *L. monocytogenes* by producing MCF and IFN- γ , which accumulate and activate macrophages, respectively.

Effects of Pretreatment with Anti-TCR α/β mAb or Anti-TCR γ/δ mAb In Vivo on the Eradication of Bacteria in Mice Infected with *Listeria*. To further investigate the protective role of the γ/δ T cells in listerial infection, TCR- α/β T cell-depleted mice or TCR- γ/δ T cell-depleted mice were prepared by in vivo administration of anti-TCR- α/β mAb or anti-TCR- γ/δ mAb, respectively. We confirmed with FACS[®] (Becton Dickinson & Co.) analysis that TCR- α/β T cell or TCR- γ/δ T cells were almost completely depleted in the lymph node and spleen 3 d after an intraperitoneal administration of 200 μ g anti-TCR- α/β mAb or 200 μ g anti-TCR- γ/δ mAb, and remained at an undetectable number until 12 d after the administration. (Fig. 3). A sublethal dose (3×10^3) of viable *Listeria* were injected intraperitoneally in mice 3 d after treatment with anti-TCR- α/β mAb or anti-TCR- γ/δ mAb, and the kinetics of bacterial growth was examined at various in-

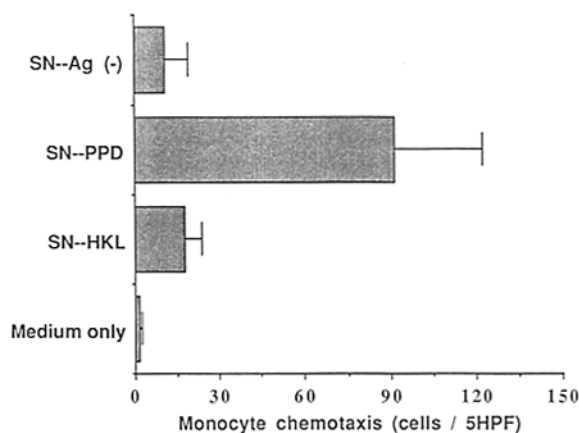


Figure 2. Production of MCF of γ/δ T cells from PEC in response to mycobacterial antigens. Anti-TCR- α/β mAb was intraperitoneally injected on day -3. Mice were infected with *L. monocytogenes* on day 0. On day 3, mice were intraperitoneally injected with 1.5 ml of 10% proteose peptone, and on day 6 PEC were harvested. PEC on day 6 were nylon wool column passed and cultured with various antigens in the presence of irradiated syngeneic splenocytes. Supernatants of the γ/δ T cells stimulated with various antigens were assayed for MCF activity by the Boyden chamber method.

tervals after infection. As shown in Fig. 4, the number of bacteria in the spleen of PBS-treated control mice reached up to 10^5 CFU on day 5 after infection, and then decreased to an undetectable level by day 10. The number of *Listeria* in the spleen of TCR- α/β T cell-depleted mice on day 5 was much the same as that of the control mice at this stage, whereas a significantly increased number of *Listeria* were detected in the spleen of TCR- γ/δ T cell-depleted mice as compared with those in TCR- α/β T cell-depleted mice and the control mice on day 5 after infection. An increased number of γ/δ T cells but only a few α/β T cells were detected in TCR- α/β T cell-depleted mice on this stage after infection. On the contrary, no γ/δ T cells were detected in TCR- γ/δ T cell-depleted mice (Fig. 5). On the other hand, *Listeria* were completely eliminated in TCR- γ/δ -depleted mice on day 12 after infection, similar to those in the control mice, while an appreciable number of *Listeria* persisted in spleen of TCR- α/β -depleted mice on this stage in spite of the presence of TCR- γ/δ T cells (Fig. 5, data not shown). These results clearly indicated that the γ/δ T cells participate in host defense mechanism especially at the early stage of primary infection with *Listeria*.

Discussion

We have previously reported that γ/δ T cells precede α/β T cells in appearance during listeriosis (15). In the present study, we have obtained the first evidence for the protective roles of the early-appearing γ/δ T cells in vivo in listerial infection in mice. Anti-TCR- α/β mAb and anti-TCR- γ/δ mAb can be used in vivo for depletion of α/β T cells or γ/δ T cells from the peripheral lymphoid organs, respectively. Strikingly, the anti-listerial resistance at the early stage after

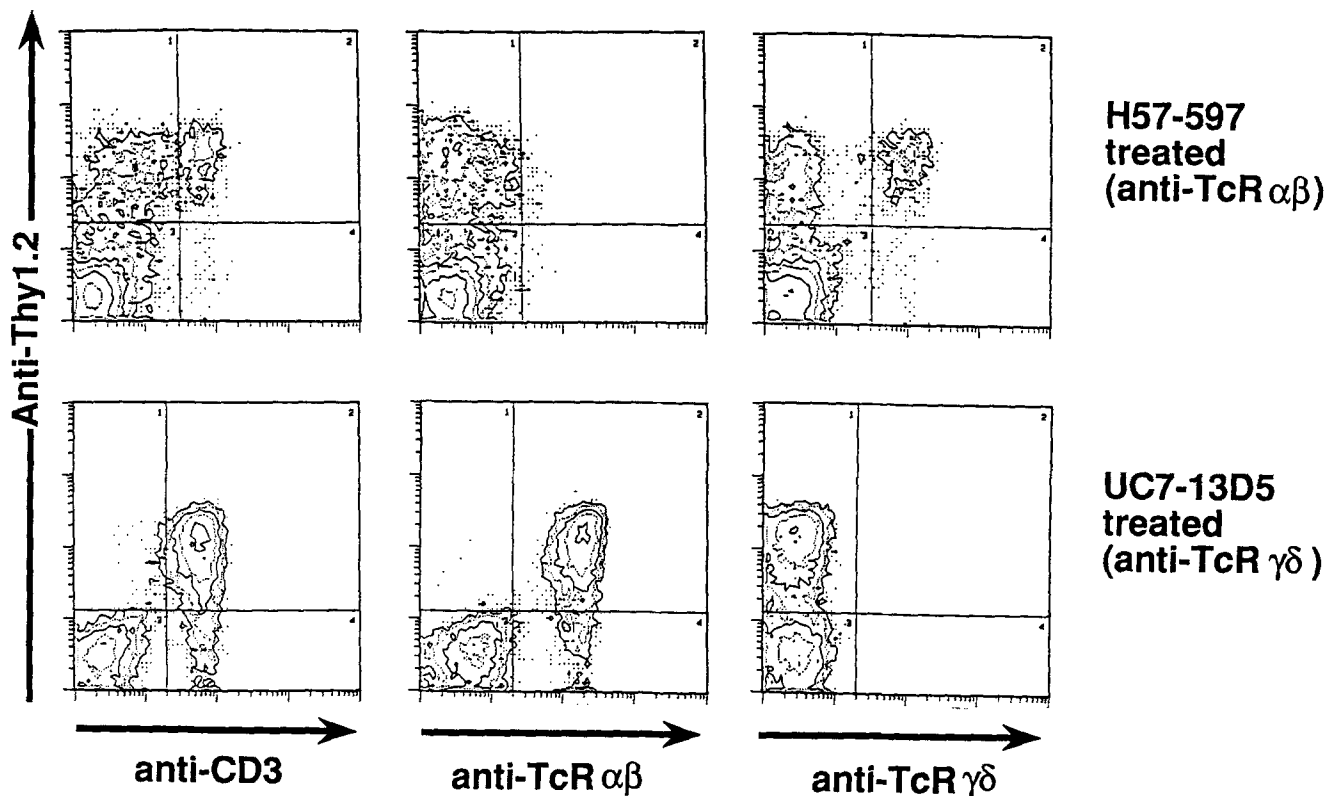


Figure 3. FACS[®] analysis of lymph node cells on day 12 after administration of mAb against TCR- α/β and TCR- γ/δ . Lymph node cells obtained 12 d after the administration of either 200 μg of anti-TCR- α/β (H57-597), or 200 μg of anti-TCR- γ/δ (UC7-13D5), were stained with anti-CD3 (145-2C11), anti-TCR- α/β (H57-597), or anti-TCR- γ/δ (UC7-13D5) and goat anti-hamster IgG-FITC, followed by anti-Thy1.2-biotin and PE-streptavidin.

infection was significantly impaired by the depletion of γ/δ T cells, whereas TCR- α/β T cell-depleted mice exhibited much the same resistance level at this stage as control mice. It can be concluded that the early-appearing γ/δ T cells during listeriosis are important for protection at the early stage of listerial infection.

One of the notable findings in the present study is that the specificity of the early-appearing γ/δ T cells during listeriosis is 65-kD HSP but not listerial nominal antigens. There have been several lines of convincing evidence that at least a significant fraction of γ/δ T cells are specialized to recognize epitopes on mycobacterial antigens, including 65-kD HSP. Janis et al. (24) showed that γ/δ T cells expanded in the draining lymph nodes in mice immunized with dead *M. tuberculosis* and were stimulated in vitro with soluble extract of *M. tuberculosis*. Modlin et al. (25) indicated that γ/δ T cells accumulated in the reactive granulomatous lesions of leprosy and local cutaneous leishmaniasis. These γ/δ T cells can proliferate in response to mycobacterial antigens (26, 27). Human synovial T cells cloned by Holoshitz et al. (10) have been shown to react to a purified preparation of the 65-kD HSP from *M. bovis* in addition to *M. tuberculosis* antigens. Antigen-unselected γ/δ T cells hybridomas from murine newborn thymus have been reported to respond to the recombinant *M. bovis* HSP and their amino acid 180–196 oligopeptides, albeit weakly (9, 21). Our study revealed that the

early-appearing γ/δ T cells during listeriosis recognized epitopes on 65-kD HSP derived from *M. bovis* that were homologous in amino acid sequence to that from *Listeria*, as assessed by Western blotting (data not shown). Furthermore, synthetic peptides based from residue 180–196 on the sequence of 65-kD HSP from *M. bovis* served as one of critical epitopes for recognition of the γ/δ T cells during listeriosis. HSP are polypeptides phylogenetically highly conserved between eukaryotes and prokaryotes (28). Under a variety of stress conditions, such as heat shock, nutrient deprivation, and oxygen radicals, eukaryotic cells have been shown to produce stress proteins to preserve cellular functions (29). Rajasekar et al. (30) have reported that a subset of murine γ/δ T cells can react to antigens on self cells in which a heat shock response was induced. Born et al. (21) have shown that the PPD-reactive γ/δ T cell hybridomas derived from newborn mouse thymus can respond to the equivalent portion of the autologous homologue to 65-kD mycobacterial HSP. Happ et al. (31) have shown that PPD-reactive γ/δ T cell hybridoma derived from murine newborn thymus preferentially used V γ 1/V δ 6 gene segments. Our preliminary experiments with the PCR method using V γ - and V δ -specific primers showed evidence for preferential expansion of V γ 1/V δ 6 T cells in PEC during listeriosis (Hiromatsu et al., unpublished data). The possible implications for a protective role of γ/δ T cells recognizing autologous HSP as well as listerial HSP await detailed anal-

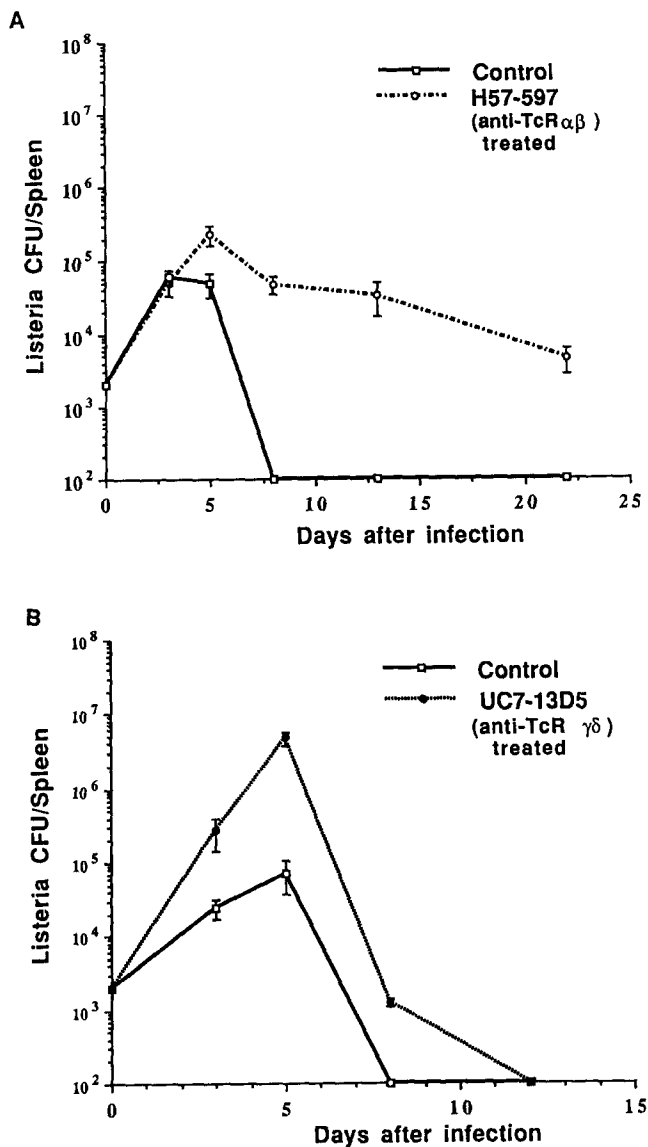


Figure 4. Effects of in vivo administration of anti-TCR- α/β (H57-597) or anti-TCR- γ/δ on recovery of bacteria from spleens. Female C3H mice were inoculated intraperitoneally with 3×10^3 *L. monocytogenes* on day 0, 200 μ g of anti-TCR- α/β mAb, or 200 μ g of anti-TCR- γ/δ mAb on day -3. The numbers of *Listeria* recovered from spleens of infected mice on the indicated days were determined by colony formation assay on tryptic soy agar. Values are means \pm SD for group of five mice.

yses of the specificity and V γ /V δ gene usages of the early-appearing γ/δ T cells in PEC during listeriosis.

IFN- γ is reported to be the major mediator of acquired resistance against *L. monocytogenes* (32, 33). The early-appearing γ/δ T cells during listeriosis produced a significant level of IFN- γ in response to PPD/65-kD HSP, suggesting that the γ/δ T cells may play an important role in the first line of defense against listerial infection through activation of macrophage. In addition to IFN- γ -mediated activation of macrophages, these γ/δ T cells promote accumulation of macrophages to the infected sites by secreting MCF in response to the mycobacterial antigen. We have previously reported the cooperative effect of MCF and macrophage activating factor (IFN- γ) in the protection of mice against *L. monocytogenes* (23). Taking these data into account, it is suggested that the early-appearing γ/δ T cells play a crucial part in primary host defense against listerial infection by accumulating and then activating macrophages.

In conclusion, the early-appearing γ/δ T cells provide a first line of defense against infection with *Listeria*. The γ/δ T cells specific for 65-kD HSP may be able to respond quickly to antigenically diverse pathogens before antigen-specific α/β T cells begin to expand clonally, and may account for the protective role of the early-appearing γ/δ T cells in murine listerial infection.

We thank Dr. R. Kubo for kindly providing mAb to TCR α/β and Dr. R. van der Zee for recombinant M. Bovis 65-kD HSP.

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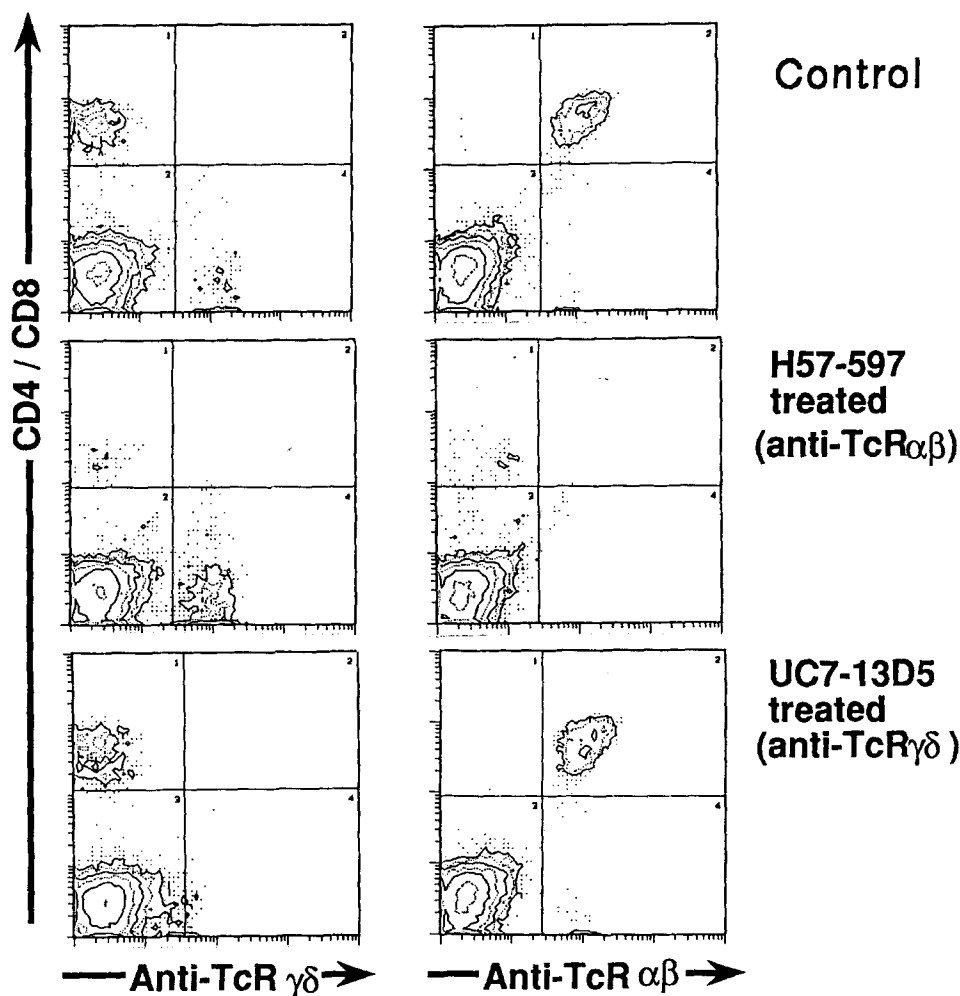


Figure 5. Two-color immunofluorescence analysis of TCR- γ/δ vs. CD4 and CD8 on nonadherent PEC on day 5 after inoculation with *L. monocytogenes* on day 0. On day -3, mice were intraperitoneally injected with mAb either to TCR- α/β (200 μg), TCR- γ/δ (200 μg). Nonadherent PEC on day 5 were stained with FITC-conjugated anti-TCR- γ/δ or FITC-conjugated anti-TCR- α/β , and anti-CD4-PE and anti-CD8-biotin, followed by PE-streptavidin.

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