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Increased Susceptibility to Primary Infection with *Listeria monocytogenes* in Germfree Mice May Be Due to Lack of Accumulation of L-Selectin$^+$ CD44$^+$ T Cells in Sites of Inflammation

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The host defense of germfree (GF) mice against primary infection with *Listeria monocytogenes* was compared with that of specific-pathogen-free (SPF) mice. In SPF mice, the numbers of bacteria in the peritoneal cavity, liver, and spleen decreased gradually to undetectable levels by day 8 after intraperitoneal infection with a sublethal dose (2×10^3 CFU) of *L. monocytogenes*. On the other hand, the elimination of bacteria in these organs of GF mice was significantly impaired at this stage after inoculation. We have reported previously that T cells coexpressing L-selectin and CD44 play an important role in protection against *L. monocytogenes* through trafficking to sites of inflammation. Consistent with our previous findings, the number of unique L-selectin$^+$ CD44$^+$ T cells in the peritoneal cavity was remarkably increased on day 8 after infection in SPF mice, whereas such an increase was not evident in GF mice at this stage. *Listeria*-specific T-cell proliferation was normally detected in the lymph node cells of GF mice inoculated with *L. monocytogenes*, whereas the T-cell-proliferative response of the peritoneal exudate cells of GF mice was significantly impaired compared with that of SPF mice. These results suggest that the priming of T cells against listerial antigens normally occurs in the peripheral lymphoid organs of GF mice but the trafficking of the activated T cells to the inflamed sites may be severely impaired in GF mice, resulting in increased susceptibility to infection with *L. monocytogenes*.

Several adhesion molecules, such as L-selectin and CD44, are known to be involved in homing of lymphocytes to lymphoid organs and trafficking of these cells to sites of inflammation, which are fundamental aspects of the immune system (3, 42). L-selectin, which was designated as MEL-14 in mice, is a representative receptor for lymphocytes homing to peripheral lymphoid tissue (5, 22) and is expressed on neutrophils, macrophages, and naive T cells (32). The ligands of L-selectin have been recently identified as GlyCAM-1 (31), sialomucin CD34 (2), and MAdCAM-1 (6), all of which are mucins. The transmembrane glycoprotein CD44 is required for leukocyte extravasation into sites of inflammation involving nonlymphoid tissue (9). This molecule has been reported to be expressed on memory-type or antigen-stimulated T cells as well as on immature or precursor T cells in the thymus and other myeloid cells, such as macrophages and neutrophils (7, 34). Furthermore, CD44 has been shown to be a receptor for hyaluronic acid (36), a high-molecular-weight repeating disaccharide, and fibronectin, both of which are present in the extracellular matrix and body fluids (13, 27). It would thus appear that naive T cells express only L-selectin, which participates in the initial attachment to the vascular endothelium, whereas memory T cells express CD44, which is required for extravasation into sites of inflammation involving nonlymphoid tissue and for activation at the inflamed sites (7, 8, 43).

The intracellular bacterium *Listeria monocytogenes* has been widely used for analyzing cell-mediated immune responses (29). The mechanism of protection against listerial infection is largely dependent on cellular immunity mediated by αβ T cells specific for *L. monocytogenes*, although the early host defense depends mainly on the polymorphonuclear leukocytes (PMN) (12, 14, 15, 40), macrophages (35, 48), natural killer (NK) cells (16), and γδ T cells (26, 38, 39). *Listeria*-specific T cells are known to accumulate preferentially in inflamed sites such as the peritoneal cavity after listerial infection (39). We have found previously that T cells coexpressing L-selectin and CD44 increase in number in peritoneal exudate cells (PEC) after infection with *L. monocytogenes* (33). L-selectin is now thought to be not only a homing receptor, helping in migration of lymphocytes into peripheral lymph nodes (LN), but also a receptor which facilitates specific migration of lymphocytes into inflamed sites (47). Therefore, L-selectin$^+$ CD44$^+$ T cells may be a subset of antigen-stimulated T cells which retain their capacity for trafficking to inflamed sites (33).

Normal microbial colonization plays an important role in development of the peripheral lymphoid tissues. There have been several lines of evidence indicating that mice bred under germfree conditions (GF mice) show an impaired host defense against infection with *Helicobacter felis* (20), *Salmonella typhimurium* (37), and *Klebsiella pneumoniae* (44), suggesting that intestinal microbes may also play important roles in host defense against microbial infection. However, the precise roles of these microbes in host defense remain to be elucidated, and comparative studies of the host defenses of GF mice and spe-

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cific-pathogen-free mice with normal microbial colonization (SPF mice) are required to resolve this issue.

In the present study, we compared the resistance to infection with *L. monocytogenes* of GF mice with that of SPF mice and found an impaired host defense against listerial infection in GF mice. Accumulation of L-selectin$^+$ CD44$^+$ T cells in the inflamed sites was greatly decreased in GF mice. The impairment of extravasation of antigen-stimulated T cells into sites of inflammation involving nonlymphoid tissue may cause increased susceptibility of GF mice to listerial infection.

MATERIALS AND METHODS

Animals. SPF female ICR mice and GF female IQI mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). GF mice were obtained by cross-breeding of strain ICR. Animals were used for the experiments at 6 to 8 weeks of age. GF mice were maintained under germfree conditions over 25 generations.

Microorganisms. *L. monocytogenes* EGD was used for experiments (35). The bacteria had been maintained by serial passage in BALB/c mice, and fresh isolates were obtained from the spleens of infected mice. *L. monocytogenes* was grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C for 12 h, washed repeatedly, resuspended in phosphate-buffered saline (PBS), and stored at −80°C until use. Heat-killed *L. monocytogenes* (HKLM) was prepared by heating viable *L. monocytogenes* cells at 74°C for 90 min.

Bacterial growth in the peritoneal cavity, liver, and spleen. Mice were inoculated intraperitoneally (i.p.) with 2.4×10^3 CFU of viable *L. monocytogenes* in 0.5 ml of PBS. At various times after inoculation, mice were anesthetized with ether and killed by cutting their cervical arteries. Peritoneal contents were lavaged with 5 ml of PBS and harvested after gentle massage. Samples were serially diluted with PBS. Bacterial growth in the liver (19, 45) and spleen (35, 39) was determined by modifications of procedures described previously. Briefly, the liver and spleen were removed and separately placed in homogenizers containing 10 ml of PBS. Each organ was homogenized thoroughly, and the homogenates were serially diluted with PBS. Samples were spread on nutrient agar (Eiken Chemical Co., Ltd., Tokyo) plates containing 0.4% (wt/vol) glucose, and colonies were counted after incubation for 24 h at 37°C.

Histology. Livers were removed from SPF mice and from GF mice on day 8 after infection with *L. monocytogenes*. Formalin-fixed liver tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for light microscopic examination.

Preparation of T cells. Mice were inoculated i.p. with 10^3 CFU of viable *L. monocytogenes* on day 0. The PEC were harvested on days 4 and 8 after infection by gentle massage with 5 ml of cold RPMI 1640 medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 5% fetal bovine serum containing 5 U of heparin per ml. The cells were collected by centrifugation at $110 \times g$ for 10 min, washed once with the same medium, and counted in a hemocytometer. PEC suspended in the supplemented RPMI 1640 medium were then spread in a plastic dish (2×10^6 cells per dish) to separate nonadherent cells from adherent cells after 2 h of incubation in a CO_2 incubator at 37°C. To obtain liver lymphocytes, fresh liver was immediately perfused with cold PBS through the large branches of the portal vein to wash out all remaining peripheral blood and then mashed using a stainless steel mesh. After being washed with PBS, the cells were filtered through cotton gauze, spun down, resuspended in 8 ml of 44% Percoll (Pharmacia, Uppsala, Sweden), and layered onto 5 ml of 67.5% Percoll in a 15-ml tube, as described by Cerf-Bensussan et al. (11). The gradients were centrifuged at $600 \times g$ and 22°C for 20 min. Lymphocytes at the interface were harvested and washed twice with cold Hank's balanced salt solution (Gibco Laboratories). The splenocytes and mesenteric LN cells were obtained from mice by the conventional method. The lymphocytes were also collected by the Percoll Isopaque density method (19).

T-cell proliferative response in vitro. Mice were infected i.p. with 10^3 CFU of viable *L. monocytogenes*. PEC and mesenteric LN were harvested 8 days after listerial infection. LN cells (3×10^5 cells per well) were cultured with 10^6 HKLM per well in flat-bottomed microtiter plates with RPMI 1640 medium supplemented with 1% L-glutamine, 10^{-5} M 2-mercaptoethanol, and 5% fetal calf serum. Nylon-wool-passed PEC (3×10^5 cells per well) were cultured with mitomycin-treated normal splenocytes (3×10^5 cells per well) and HKLM (10^6 cells per well). The cultures were incubated for 72 h at 37°C in a humidified atmosphere of 5% CO_2 and air. A 20-μl aliquot of medium containing 37 kBq of [^{3}H]thymidine per ml was added to each well 18 h before cells were harvested on filter paper with a multiple harvester. The radioactivity of each sample was then counted with a liquid scintillation counter.

Antibodies. The following antimouse monoclonal antibodies (MAb) were used for experiments: fluorescein isothiocyanate (FITC)-conjugated anti-αβTcR MAb (H57-597), biotinylated anti-γδTcR MAb (GL-3), phycoerythrin (PE)-conjugated anti-CD3 MAb (145-2C11), PE-conjugated anti-CD4 MAb (RM4-4), biotinylated anti-CD8 MAb (53-6.7), FITC-conjugated anti-B220 MAb (RA3-6B2), FITC-conjugated anti-lymphocyte function antigen 1 (LFA-1) MAb (M17/4), FITC-conjugated anti-intercellular adhesion molecule 1 (ICAM-1) MAb (3E2), FITC-conjugated anti-L-selectin MAb (MEL-14), and biotinylated anti-CD44 MAb (IM7). Secondary antibodies used were FITC-conjugated anti-hamster immunoglobulin G, streptavidin-PE, avidin-FITC (PharMingen, San Diego, Calif.), and streptavidin-RED613 (Gibco Laboratories).

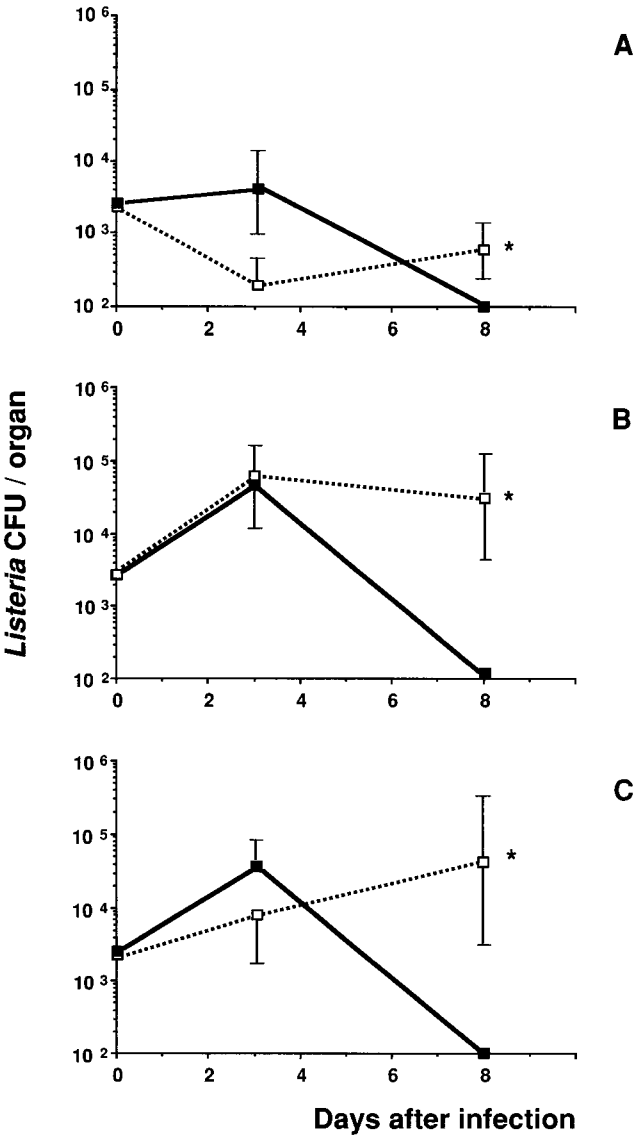

FIG. 1. Kinetics of bacterial growth in the peritoneal cavity (A), liver (B), and spleen (C). Female GF IQI (broken line) and SPF ICR (solid line) mice were injected i.p. with 2.4×10^3 CFU of viable *L. monocytogenes* on day 0. The numbers of *L. monocytogenes* cells in the peritoneal cavities, livers, and spleens of infected mice on the indicated days were determined by colony formation assay on 0.4% (wt/vol) glucose-supplemented nutrient agar. Values are means ± standard deviations (error bars) for groups of five mice. *, $P < 0.05$. Statistical significance was determined by the Student's *t* test.

FCM analysis. For two-color flow cytometric (FCM) analysis, cells were stained with FITC-conjugated MAb and biotinylated MAb followed by streptavidin-PE or FITC-conjugated MAb and PE-conjugated MAb. For three-color FCM analysis, cells were stained with FITC-conjugated MAb, PE-conjugated MAb, and biotinylated MAb followed by streptavidin RED-613. Cells were stained at 4°C for 60 min and washed three times with phenol red-free Hank's balanced salt solution between steps. The lymphocytes were analyzed with an EPICS Elite flow cytometer (Coulter Corp., Hialeah, Fla.). Elite research software (Coulter) was used for analysis and calibration. Gates were set for viable cells in the forward-scatter versus side-scatter plots.

Statistics. The statistical significance of the data was determined by Student's

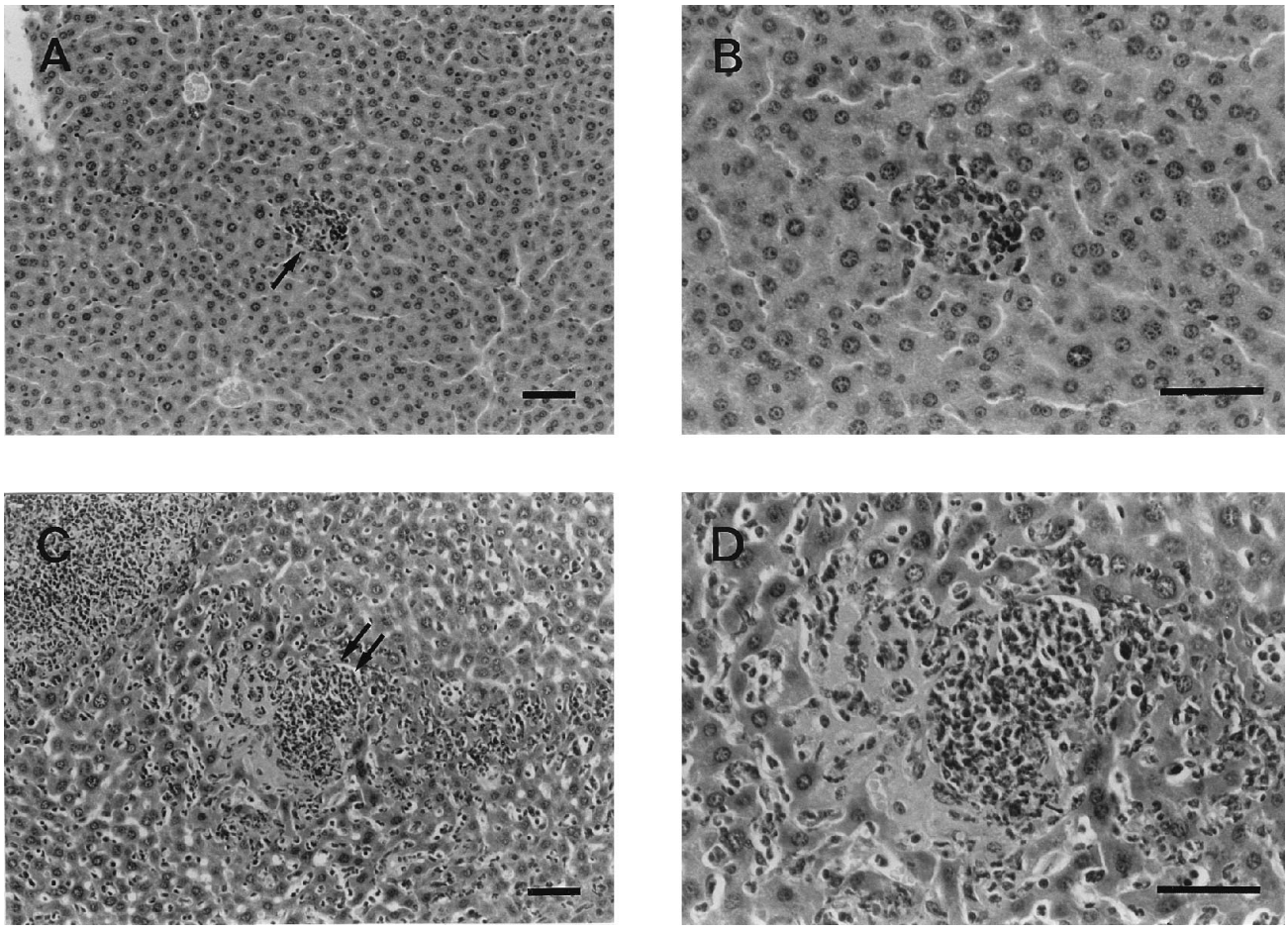

FIG. 2. Comparison of liver lesions in SPF (A and B) and GF (C and D) mice after infection with *L. monocytogenes*. Both groups of mice were inoculated i.p. with 10^3 CFU of *L. monocytogenes* cells, and livers were removed on day 8 after infection. Liver sections were stained with hematoxylin-eosin. The single arrow in panel A shows hepatic granulomas. The double arrows in panel C show PMN infiltration. Bars, 100 μm; magnifications, ×200 (A and C) and ×400 (B and D).

t test and generalized Wilcoxon's test. A *P* value of less than 0.05 was regarded as significant.

RESULTS

Kinetics of bacterial growth in the peritoneal cavity and liver after i.p. infection with *L. monocytogenes*. To compare the host defense against infection with *L. monocytogenes* in GF mice with that in SPF mice, we first examined the survival rate after i.p. infection with various doses of viable *L. monocytogenes*. Mice were infected with 3×10^3 CFU of viable *L. monocytogenes*; 3 of 10 GF mice died by day 10 after infection, while all SPF mice survived beyond 24 days after infection. An approximately 50% lethal dose was 3×10^4 CFU in SPF mice and 6×10^3 in GF mice when inoculated i.p. Thus, GF mice showed increased susceptibility to infection with *L. monocytogenes* ($P < 0.01$, by Wilcoxon's test).

We next examined bacterial growth in the peritoneal cavity, liver, and spleen after i.p. infection with a sublethal dose (2.4×10^3 CFU per mouse) of viable *L. monocytogenes*. In SPF mice, the number of bacteria in the peritoneal cavity, liver, and spleen reached a peak on day 3 and decreased gradually to undetectable levels by day 8 after infection (Fig. 1). On day 3 after infection, the numbers of bacteria were about the same in the liver and somewhat lower in the spleen and peritoneal cavity of GF mice compared with the same organs of SPF mice, whereas the numbers of bacteria in these organs were significantly greater in GF mice than in SPF mice on day 8. Thus, as assessed by bacterial growth, the resistance to listerial infection at the later stage was significantly impaired in GF mice, although protection at the early stage seems to be intact in these mice.

Histology of liver lesions in SPF and GF mice after infection with *L. monocytogenes*. We compared the histological changes in the livers of SPF mice with those in the livers of GF mice on day 8 after infection with *L. monocytogenes*. In SPF mice infected with *L. monocytogenes*, cell infiltrates containing primarily mononuclear cells and lacking PMN were detected in granulomatous lesions (Fig. 2A and B). On the other hand, the livers of GF mice contained larger lesions, with numerous cell infiltrates and tissue damage in liver parenchyma. The infiltrates contained mainly PMN with few mononuclear cells (Fig. 2C and D). These results suggest that normal granulomatous responses may be somewhat impaired in GF mice, although the infiltration of PMN after infection with *L. monocytogenes* appears to be unaffected in these mice.

Kinetics of nonadherent PEC in SPF and GF mice after i.p. infection with *L. monocytogenes*. The kinetics of the inflamma-

tory cells in the peritoneal cavities of SPF and GF mice after infection with 10^3 CFU of *L. monocytogenes* per mouse were examined. As shown in Fig. 3A, in SPF mice, the absolute number of PEC per mouse was significantly increased from a mean ± standard deviation of $4.1 \times 10^6 \pm 2.1 \times 10^6$ on day 1 to $14.4 \times 10^6 \pm 5.3 \times 10^6$ on day 8 after infection. On the other hand, such an increase in number of peritoneal leukocytes was not evident in GF mice. In SPF mice, the number of nonadherent PEC per mouse increased remarkably from $2.7 \times 10^6 \pm 1.2 \times 10^6$ on day 3 to $9.4 \times 10^6 \pm 3.2 \times 10^6$ on day 8 after infection. In GF mice, however, the number of nonadherent PEC per mouse increased only marginally from $2.0 \times 10^6 \pm 0.6 \times 10^6$ on day 3 to $3.3 \times 10^6 \pm 1.1 \times 10^6$ on day 8 after infection.

On days 1, 3, and 8 after i.p. infection with *L. monocytogenes*, we analyzed the subsets of nonadherent PEC by two-color FCM analysis with anti-CD3 MAb and anti-B220 MAb. The absolute numbers of $CD3^+$ T cells and $B220^+$ B cells were calculated by multiplying the number of nonadherent PEC by the percentage of each cell type. As shown in Fig. 3B, in SPF mice, the absolute number of $CD3^+$ T cells per mouse was significantly increased from $0.4 \times 10^6 \pm 0.2 \times 10^6$ on day 3 to $2.3 \times 10^6 \pm 0.8 \times 10^6$ on day 8 after infection. In GF mice, however, there was only a marginal increase in the number of $CD3^+$ T cells per mouse, from $0.2 \times 10^6 \pm 0.1 \times 10^6$ on day 3 to $0.9 \times 10^6 \pm 0.3 \times 10^6$ on day 8 after infection. Furthermore, the absolute number of $B220^+$ B cells per mouse was significantly increased in SPF mice ($3.2 \times 10^6 \pm 1.0 \times 10^6$) on day 8 after listerial infection compared with that in GF mice ($0.5 \times 10^6 \pm 0.2 \times 10^6$). On day 8 after infection, we further stained the nonadherent PEC with anti-CD3, anti-CD4, and anti-CD8 MAb. The proportion of $CD4^+$ T cells was increased in GF mice, albeit to a lesser degree than in SPF mice, whereas an increase in $CD8^+$ T cells was not evident in GF mice on day 8 after infection (data not shown).

Kinetics of T cells expressing L-selectin and/or CD44 in PEC after infection with *L. monocytogenes*. We have shown previously that the number of T cells coexpressing L-selectin and CD44 increases in sites of inflammation and that this correlates with an increase in *Listeria*-specific proliferative responses (33). Therefore, we further examined the expression of L-selectin and CD44 on the nonadherent PEC, liver and spleen lymphocytes, and LN cells from SPF and GF mice on day 8 after listerial infection. Expression of L-selectin and CD44 on $CD3^+$ T cells in the peritoneal cavity, liver, spleen, and mesenteric LN was analyzed by three-color FCM analysis. Figure 4 shows a representative profile from five mice of each group. The absolute numbers of each cell type in the peritoneal cavity and mesenteric LN are presented in Table 1. The results were calculated by multiplying the absolute number of $CD3^+$ T cells by the percentage of each subtype. The viable lymphocyte population was gated in forward-scatter versus side-scatter plots (Fig. 4A). As shown in Fig. 4B, there was no difference in the proportion of double-positive (L-selectin$^+$ CD44$^+$) T cells among $CD3^+$ T cells in the PEC, liver, spleen, and LN between GF mice and SPF mice before listerial infection. Consistent with our previous report (33), on day 8 after infection, the proportion of L-selectin$^+$ CD44$^+$ T cells was significantly increased in the peritoneal cavities, livers, spleens, and LN of SPF mice, while such an increase was not evident in the peritoneal cavities of GF mice ($P < 0.01$). The number of L-selectin$^+$ CD44$^+$ T cells increased only marginally in the livers and spleens of GF mice at this stage, whereas the double-positive population was increased in LN of GF mice to a significant level comparable to that seen in SPF mice on day 8 after listerial infection (Table 1).

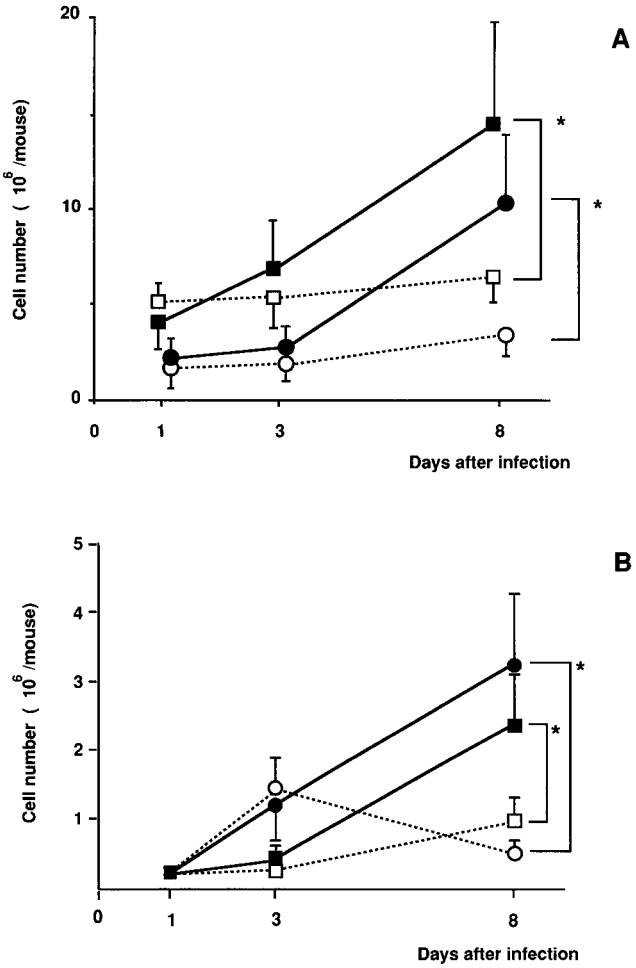

FIG. 3. (A) Kinetics of total PEC (squares) and nonadherent PEC (circles) in SPF (closed symbols) and GF (open symbols) mice after i.p. infection with 10^3 CFU of viable *L. monocytogenes*. PEC suspended in the medium were then spread on a plastic dish (2×10^6 cells per dish) to separate nonadherent cells from adherent cells after 2 h of incubation in a CO_2 incubator at 37°C. *, $P < 0.05$. Statistical significance was determined by the Student's *t* test. (B) Absolute numbers of $CD3^+$ T cells (squares) and $B220^+$ B cells (circles) in the peritoneal cavities of SPF (closed symbols) and GF (open symbols) mice after i.p. infection with *L. monocytogenes*. Data were calculated by multiplying the numbers of nonadherent PEC by the percentage determined by two-color FCM analysis. Values are means ± standard deviations (error bars) for groups of five mice. *, $P < 0.05$. Statistical significance was determined by the Student's *t* test.

It has been reported that LFA-1 has an important role in the migration of leukocytes which occurs after initial adherence (18). Therefore, we also examined the levels of expression of LFA-1 on T cells and of ICAM-1 on non-T cells, respectively, in the peritoneal cavities, livers, spleens, and mesenteric LN of both SPF and GF mice on day 8 after listerial infection. Unexpectedly, as shown in Fig. 5, the proportion of high-LFA-1 T cells was somewhat increased in the peritoneal cavities of GF mice on day 8 after listerial infection compared with that of SPF mice (80.3% in GF versus 63.2% in SPF). There was no difference between the two groups in the proportion of high-ICAM-1 cells on day 8 after listerial infection (43.4% in GF versus 43.0% in SPF).

T-cell-proliferative response to HKLM in PEC and mesenteric LN on day 8 after listerial infection. To determine whether the priming of T cells to listerial antigens occurred in

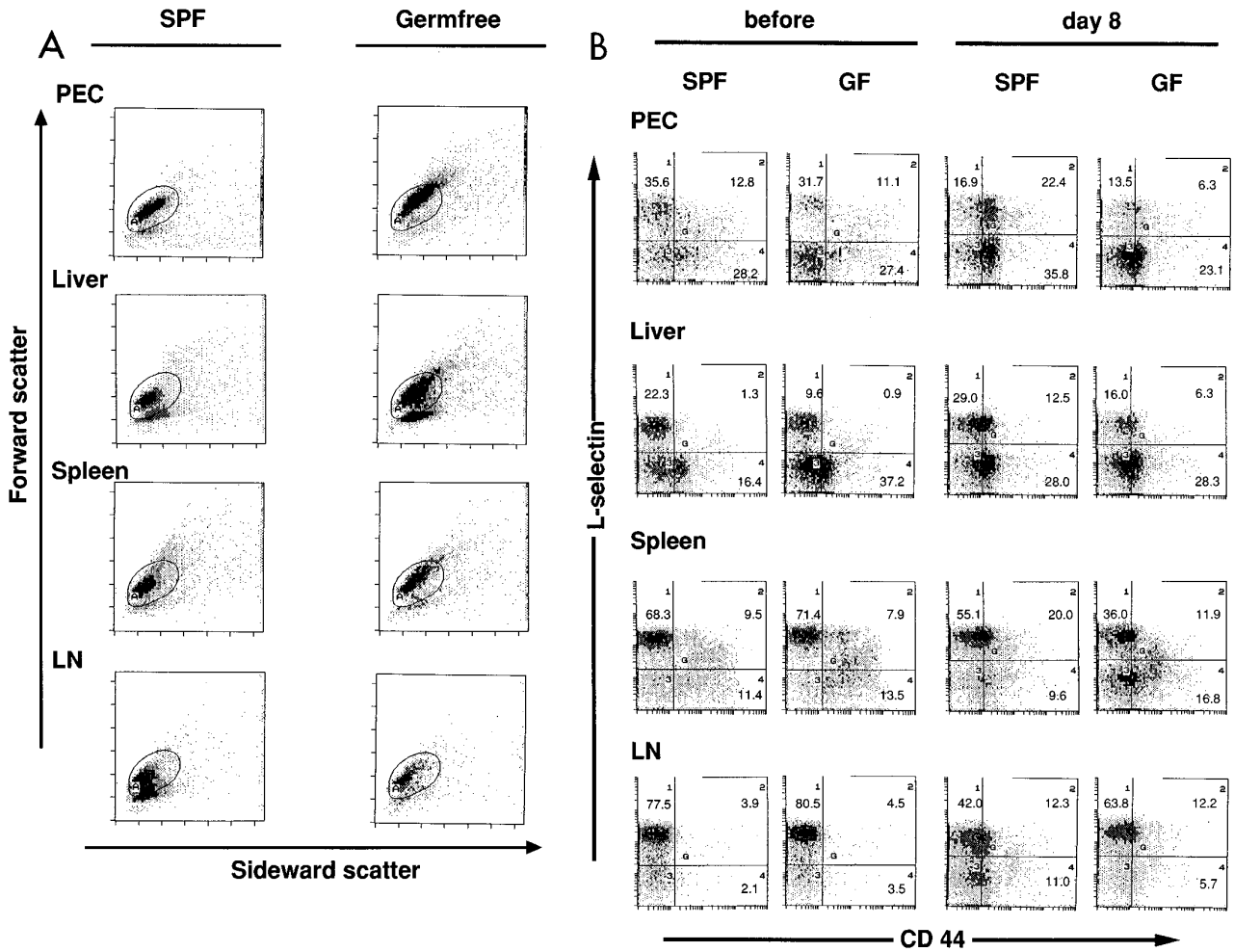

FIG. 4. (A) FCM analysis for the determination of viable lymphocytes in PEC, livers, spleens, and LN of SPF and GF mice. Mice were examined on day 8 after i.p. infection with 10^3 CFU of viable *L. monocytogenes*. Gates were set for the viable lymphocyte population in the forward-scatter versus side-scatter plots. Cells were prepared as described in Materials and Methods. (B) Three-color FCM analysis of the expression of L-selectin and CD44 on $CD3^+$ T cells in PEC, livers, spleens, and LN of SPF and GF mice. Mice were examined prior to and on day 8 after i.p. infection with 10^3 CFU of viable *L. monocytogenes*. Cells were stained as described in Materials and Methods. The proportions of cells showing L-selectin and/or CD44 expression were calculated after gating on the $CD3^+$ T cells and are indicated in each quadrant. No background staining was detected in cells stained with relevant control antibodies (FITC-rat immunoglobulin G2a and biotin-rat immunoglobulin G2b MAbs).

the lymphoid organs of GF mice after listerial infection, we examined the *Listeria*-specific proliferative response of T cells from the PEC and mesenteric LN of SPF and GF mice 8 days after inoculation with 10^3 CFU of *L. monocytogenes* per mouse. As shown in Fig. 6, the LN cells from GF mice showed a proliferative response to HKLM that was similar to the response of LN cells from SPF mice, whereas the proliferative response of T cells from the PEC of GF mice was significantly

TABLE 1. Absolute numbers of L-selectin$^{+/-}$ and CD44$^{+/-}$ T cells in the peritoneal cavity and mesenteric LN after i.p. infection with *L. monocytogenes*[a]

Phenotype	No. of T cells (10^4) in[b]:							
	Peritoneal cavity				Mesenteric LN			
	Before infection of:		On day 8 after infection of[c]:		Before infection of:		On day 8 after infection of[c]:	
	SPF	GF	SPF	GF	SPF	GF	SPF	GF
L-selectin$^-$ CD44$^-$	2.3 ± 0.3	3.0 ± 2.6	57.3 ± 3.7	51.5 ± 0.6	123.8 ± 3.8	28.8 ± 2.5[d]	80.0 ± 4.1	40.5 ± 4.2[d]
L-selectin$^-$ CD44$^+$	2.8 ± 0.2	2.7 ± 1.8	82.3 ± 2.1	20.8 ± 1.2[d]	15.8 ± 2.3	8.8 ± 1.3	25.3 ± 4.8	12.5 ± 3.1[d]
L-selectin$^+$ CD44$^-$	3.6 ± 0.2	3.2 ± 0.2	38.9 ± 3.0	12.2 ± 0.7[d]	581.3 ± 6.0	201.3 ± 5.5[d]	96.6 ± 4.6	140.4 ± 10.6
L-selectin$^+$ CD44$^+$	1.3 ± 0.1	1.1 ± 0.2	51.5 ± 3.2	5.7 ± 0.4[d]	29.3 ± 3.0	11.3 ± 1.0	28.3 ± 4.4	26.8 ± 4.4

[a] Data were calculated by multiplying the absolute numbers of $CD3^+$ T cells by the percentage determined by three-color FCM analysis.
[b] Data are means ± standard deviations for five mice per group.
[c] SPF and GF mice were each infected i.p. with 10^3 CFU of *L. monocytogenes*.
[d] $P < 0.01$. The result is statistically significant compared with the corresponding result for SPF mice.

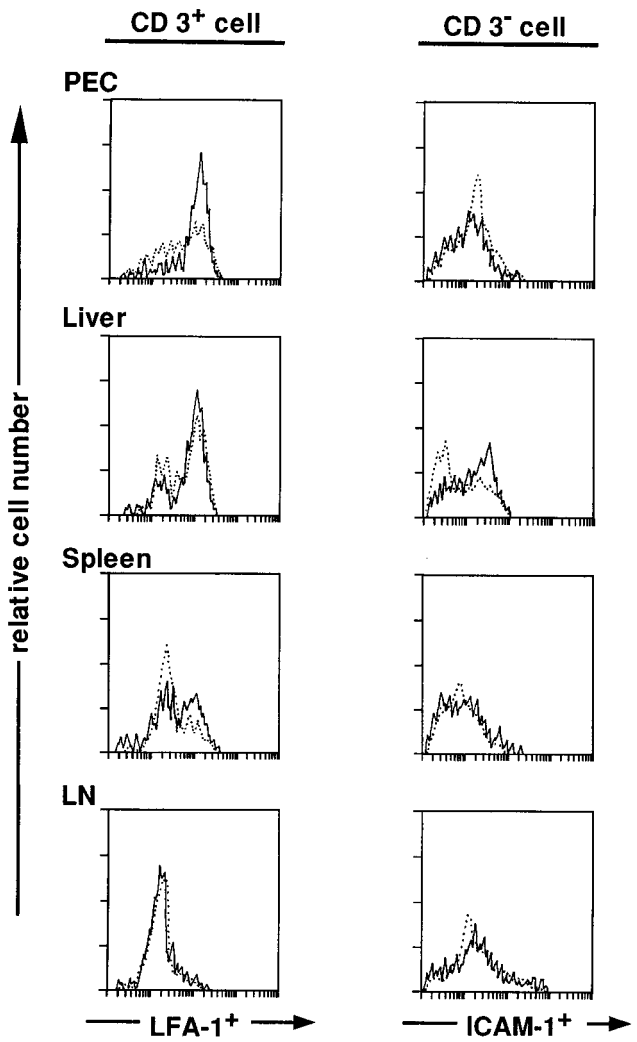

FIG. 5. Two-color FCM analysis of the levels of expression of LFA-1 on $CD3^+$ T cells and of ICAM-1 on $CD3^-$ non-T cells in PEC, livers, spleens, and LN of SPF (dotted line) and GF (solid line) mice. Mice were examined on day 8 after i.p. infection with 3×10^3 CFU of viable *L. monocytogenes*. Cells were stained as described in Materials and Methods.

impaired compared with that of T cells from PEC of SPF mice ($P < 0.05$). Thus, these results suggest that *Listeria*-specific T cells are normally generated in the LN of GF mice but may not appear in the peritoneal cavity after listerial infection.

DISCUSSION

Normal microbial colonization is thought to play an important role in the development of the peripheral lymphoid tissues and in host defense against infection with various pathogens. However, little is known about the mechanisms of host defense. In the present study, we obtained evidence that host defense at the late stage after primary infection with *L. monocytogenes* was impaired in GF mice in comparison with SPF mice. Although *Listeria*-specific T-cell proliferation normally develops in the peripheral lymphoid organs of GF mice, the T cells in the peritoneal cavity of GF mice did not respond well to listerial antigens. Notably, T cells expressing both L-selectin and CD44 molecules were hardly detected in the peritoneal cavity of GF mice at the late stage after listerial infection. Taken together, these data suggest that the trafficking of antigen-stimulated T cells to inflammatory sites may be severely impaired in GF mice, resulting in increased susceptibility to infection with *L. monocytogenes*. Normal microbial flora may play an important role in the development of immunity through the acceleration of homing of effector cells to the inflamed sites.

The PEC have often been used as an enriched source of antigen-primed immune T cells from mice infected with *L. monocytogenes* (46). We have recently shown that L-selectin$^+$ CD44$^+$ T cells appeared preferentially in the peritoneal cavity from 7 to 14 days after listerial infection and that this correlated with an increased proliferative response to listerial antigens. This subset of T cells also increased in spleen cells from infected mice after in vitro restimulation with HKLM (33). Thus, the kinetics of L-selectin$^+$ CD44$^+$ T cells in the peritoneal cavity appear to reflect in vivo kinetics of *Listeria*-primed T cells. In GF mice, the proliferative response of PEC to listerial antigens was significantly impaired in association with

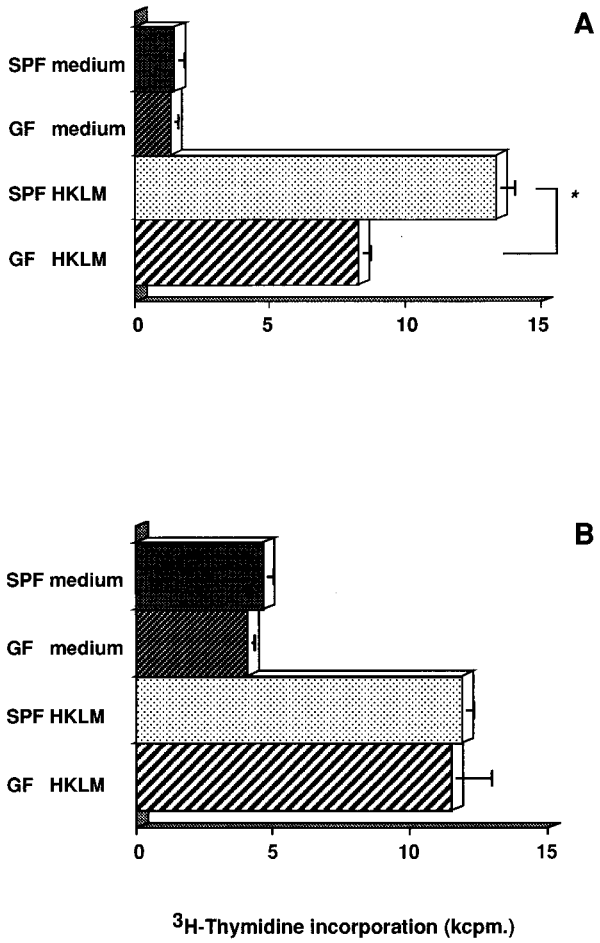

FIG. 6. In vitro proliferative responses of *Listeria*-immune T cells obtained from SPF and GF mice in the absence (medium) or presence of HKLM. Mice were infected i.p. with 10^3 CFU of viable *L. monocytogenes*, and PEC and LN were harvested after 8 days. Nylon-wool-passed PEC (A) (3×10^5 per well) were cultured with mytomycin-treated normal splenocytes (3×10^5 per well) and HKLM (10^6 per well). *, $P < 0.05$. Statistical significance was determined by the Student's *t* test. LN cells (B) (3×10^5 per well) were cultured with HKLM (10^6 per well). *Listeria*-immune T cells and HKLM were cultured for 72 h. [^{3}H]thymidine was added to each well 18 h before harvesting, and [^{3}H]thymidine incorporation was determined in triplicate cultures. The results shown are means ± standard deviations (error bars).

a lack of appearance of L-selectin$^+$ CD44$^+$ T cells in the peritoneal cavity. Since the LN T cells proliferated to about the same extent in both GF and SPF mice immunized with *L. monocytogenes*, we conclude that priming of T cells occurs normally in the peripheral lymphoid organs in GF mice immunized with *L. monocytogenes* but that the accumulation of the *Listeria*-primed T cells in the inflamed sites may be severely impaired in GF mice.

L-selectin, which is expressed constitutively on the cell surface of lymphocytes as well as on neutrophils (21, 32) and monocytes (32), was initially thought to be a homing receptor helping in the migration of lymphocytes into peripheral LN (5, 22). L-selectin is shed from the surface of lymphocytes following cellular activation (43), a process that may allow leukocytes to detach after the initial adhesion event (31). Therefore, L-selectin$^-$ T cells are thought to be in an activated or memory state and to have some defect in trafficking to inflammatory sites (30). L-selectin is now thought to be not only a homing receptor helping in migration of lymphocytes into the peripheral LN but also a receptor achieving specific migration of lymphocytes into inflamed sites (47). L-selectin plays an important role in the influx of leukocytes into inflamed sites via a critical interaction with activated vascular endothelial cells at the sites of inflammation (4). Recently, L-selectin ligands have been identified as GlyCAM-1, CD34, and MAdCAM-1, all of which are mucins characterized by a high density of O-linked sugars (2, 6, 31). Expression of these molecules on vascular endothelial cells is up-regulated by inflammatory cytokines such as interleukin 1 (10), tumor necrosis factor alpha, and lipopolysaccharide (30). It is possible that GF mice may express fewer L-selectin ligands on the vascular endothelial cells which are involved in restimulation and migration of the *Listeria*-primed L-selectin$^+$ T cells into the inflamed sites. Further comparative experiments to determine expression levels of the ligands will clarify this point.

Unlike L-selectin, CD44 is expressed on memory-type or antigen-stimulated T cells (34) and interacts with several cell surface and matrix components, including fibronectin, collagen, and hyaluronate (23), a nonsulfated glycosaminoglycan (25). CD44 helps in the binding of leukocytes to high-endothelium venules and extracellular matrix (28). CD44 is thought to be involved not only in T-cell adhesion but also in T-cell activation via delivery of the transmembrane signal (41). The fibronectin concentration in GF mice was significantly lower than that in SPF mice (1). Therefore, it is possible that in GF mice, L-selectin$^+$ CD44$^+$ T cells which appear to be specific for listerial antigens are normally generated in peripheral lymphoid organs. However, because of the low concentration of ligands in the inflamed sites, such as the peritoneal cavity, the activated T cells may not migrate to the sites of inflammation, resulting in impaired immune responses in the inflamed sites and subsequent deterioration of the host defense against listerial infection.

It has been reported that LFA-1, which is up-regulated in activated cells, has an important role in the migration of leukocytes that occurs after initial adherence. Furthermore, up-regulation of avidity of CD44 and LFA-1 was reported for activated lymphocytes (18). Therefore, it is possible that expression of LFA-1 on T cells and of the ligand ICAM-1 on non-T cells may be suppressed in GF mice. However, in our hands, after listerial infection of GF mice, the T cells expressed an abundant level of LFA-1 in the peritoneal cavity and other peripheral lymphoid organs. Interaction of LFA-1 and ICAM-1 is also important in interaction of T cells with antigen-presenting cells (17). The normal up-regulation of LFA-1 on T cells in GF mice may be in accordance with our results showing that the *Listeria*-specific T cells are primed normally in GF mice immunized with *L. monocytogenes*.

Resistance to *L. monocytogenes* infection is dependent mostly on cellular immunity mediated by *Listeria*-specific $\alpha\beta$ T cells. However, PMN, macrophages, NK cells, and $\gamma\delta$ T cells also contribute to protection in some phase of listerial infection (16, 24, 26, 35, 38, 39, 48). Normal intestinal bacterial flora may contribute to activation of the innate immunity mediated by these cells. Therefore, we must consider the possibility that the reduced activation of these cells in GF mice may also be responsible for the enhanced susceptibility of these mice to *Listeria* infection. Furthermore, reduced production of potent chemoattractants may cause the reduced influx of L-selectin$^+$ CD44$^+$ T cells at the inflamed sites. Further analysis of the activation state of innate immunity in GF mice is necessary to clarify the mechanisms behind the impaired host defense against listerial infection in these mice.

In conclusion, our results suggest that priming of T cells against listerial antigens develops normally in GF mice but that trafficking of the activated T cells to inflammatory sites may be severely impaired in these mice, resulting in impaired resistance to *L. monocytogenes.*

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