Induction of Murine Peritoneal \(\gamma/\delta\) T Cells and Their Role in Resistance to Bacterial Infection

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Summary

Previous studies have reported an association of \(\gamma/\delta\) T cells with microbial infection in both human lesions and murine infectious disease models. In this study we provide a comprehensive analysis of the conditions under which the induction of \(\gamma/\delta\) T cells occurs at a site of infection. We found a site-specific induction of \(\gamma/\delta\) T cells after the injection of *Listeria monocytogenes* in the peritoneal cavity of C3H mice. No changes were seen in the splenic or lymph node populations after these injections. Both the proportion and the absolute number of \(\gamma/\delta\) T cells increased in the peritoneal cavity. Additionally, when peritoneal T cells from *Listeria*-immune mice were restimulated in vitro, the induced \(\gamma/\delta\) T cells exhibited a greater expansion potential than the \(\alpha/\beta\) T cells. Neither the induced \(\gamma/\delta\) T cells nor those from normal mice expressed CD4 or CD8 on the cell surface. Thy-1 was expressed on only 29% of normal peritoneal \(\gamma/\delta\) T cells, but after intraperitoneal *Listeria* infection 65% of induced \(\gamma/\delta\) T cells expressed Thy-1. Pgp-1 and CD45R expression on both normal and induced \(\gamma/\delta\) T cells was consistent with an activation phenotype. Significant increases in peritoneal \(\gamma/\delta\) T cells were not seen until 5-7 d after *Listeria* injection. The proportion of the CD3+ population expressing the \(\gamma/\delta\) T cell receptor remained elevated for 6-7 wk, while the absolute numbers of peritoneal \(\gamma/\delta\) T cells declined gradually over this time period, reflecting a decrease in both the number of lymphocytes and the percentage of these that were CD3+. Peak numbers of \(\gamma/\delta\) T cells were seen at day 10 with live microbes such as *Listeria*. A variety of microbes, toxins, mitogens, antigens, cytokines, and nonspecific inflammatory agents were evaluated for their ability to induce \(\gamma/\delta\) T cells in the peritoneal cavity. Both Gram-positive and Gram-negative bacteria as well as *Mycobacteria* were able to induce \(\gamma/\delta\) T cells that showed increased in vitro expansion potential. An exotoxin from a Gram-positive organism, listeriolysin-o, and the lipopolysaccharide (LPS) endotoxin from a Gram-negative organism were also effective. \(\gamma/\delta\) T cell responses to LPS were under *Ips* gene control. Peak numbers of \(\gamma/\delta\) T cells were observed at day 3 after injection with exotoxins and endotoxins. Modifications that abrogated the virulence of a bacterial strain also eliminated the inductive effect for \(\gamma/\delta\) T cells. Although injection of some nonspecific peritoneal inflammatory agents resulted in increased numbers of \(\gamma/\delta\) T cells in the peritoneum, these cells did not have increased expansion potential in vitro. Depletion of either \(\alpha/\beta\) or \(\gamma/\delta\) T cells in vivo with monoclonal antibody resulted in mice with impaired resistance to primary infection with *Listeria*. However, the memory response in immunized mice was virtually unaffected by depletion of \(\gamma/\delta\) T cells, supporting the hypothesis that this T cell subset is of greatest importance in innate “front-line” defense mechanisms. Thus, the highly localized and inducible expression of \(\gamma/\delta\) T cells in the murine peritoneal cavity not only supplies evidence for the role of \(\gamma/\delta\) T cells in microbial immunity but also provides an excellent opportunity for elucidating the function, receptor specificity, and activation requirements of \(\gamma/\delta\) T cells.

A salient feature of the immune system is the remarkable heterogeneity of T lymphocytes and the functional diversity represented by the collection of specialized cellular subsets. A newly recognized subdivision of T cells is based on the differential expression of TCRs. The antigen-specific TCR is composed of an \(\alpha\) chain paired with a \(\beta\) chain or a \(\gamma\) chain associated with a \(\delta\) chain. Very early in T cell development, cells become committed to express either \(\alpha\) and \(\beta\) chain gene products or \(\gamma\) and \(\delta\) proteins. As such, distinct T cell lineages are generated that express either the \(\alpha/\beta\) TCR or the \(\gamma/\delta\) TCR.
Particularly intriguing aspects of γ/δ T cells are their distinctive localization in epithelial tissue (1-4) and at sites of infection (5-7). This association at sites of first contact with infectious agents implicates γ/δ T cells in “front-line” defense reactions or regulatory mechanisms important at the self–nonself interface. The preferential expression of γ/δ T cells can theoretically be due to the selective homing and/or proliferation of cells controlled by a number of factors unique to γ/δ T cells and a particular site or inflammatory condition such as microbial infection. Such controlling factors include homing receptors, antigens or superantigens, presenting molecules, accessory molecules, and cytokines. A clearer picture of the function of γ/δ T cells will be achieved with a more thorough understanding of the regulation of their tissue expression.

While generalizations about the antigens recognized by γ/δ T cells are difficult to make with certainty, recognition of carbohydrate antigens (8, 9), viral proteins (10), and epitopes of stress proteins shared by microbes and mammals (11, 12) has been observed. The observations on recognition of stress proteins have led to interesting speculations about a primitive surveillance system in which γ/δ T cells discriminate stressed from nonstressed cells.

The predominance of γ/δ T cells has been noted at sites of infection with Mycobacteria (5, 13), Listeria (7), influenza virus (14), Trypanosoma cruzi (15), and leishmania (16). In the present study we have analyzed the induction of γ/δ T cells in the peritoneal cavity of mice injected with microbes and their toxins. These studies provide a comprehensive analysis of the conditions under which the induction of γ/δ T cells occurs at a site of infection and suggest that the peritoneal cavity is a tissue site for preferential expression and induction of γ/δ T cells. Additionally, by using antibody depletion techniques in vivo, we provide evidence that the site-specific induction of γ/δ T cells after infection may play a significant role in primary resistance to murine listeriosis. In contrast, α/β T cells were found to be responsible for the specific memory response.

Materials and Methods

Mice and Injections. Female C3HeB/FeJ or C3H/HeJ mice obtained from The Jackson Laboratory (Bar Harbor, ME) were used at 8-12 wk of age. Mice were housed in micro-isolator cages with laboratory chow and water available ad libitum. Injections were performed aseptically intraperitoneally unless otherwise indicated.

Bacterial Strains. The following strains of bacteria were used at concentrations as indicated in the text: Listeria monocytogenes strain 43251 (wild type; American Type Culture Collection [ATCC], Rockville, MD) (LD50 = 5 x 105); L. monocytogenes strain 43250 (ATCC) (a spontaneous mutant lacking the expression of the hemolysin molecule; LD50 > 8 x 105) (18); hemolysin-expressing Escherichia coli WAF270 (strain J98 transformed with plasmid pSF4000, a plasmid that encodes hemolysin); hemolysin-negative E. coli WAF 280 (strain J98 transformed with the transposon mutant pSF4000; Tn1 in which the transposon is inserted in the hemolysin gene); bacille Calmette-Guerin (BCG; Dr. Bruce Zwilling, Ohio State University, Columbus, OH); and Salmonella typhimurium strain SL1004 (obtained from Dr. John Spitznagel, Emory University, Atlanta, GA). The transformed E. coli strains were kindly provided by Dr. Rodney Welch (University of Wisconsin, Madison, WI). Before injection, frozen stocks of bacterial strains were grown overnight in brain heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C with aeration. Cells were washed three times in PBS, and concentrations were determined by optical density with confirmation by colony counts on brain heart infusion agar plates. For heat-killed preparations, bacteria were incubated at 80°C for 1 h and tested for lack of viability on agar plates.

Reagents. PHA, Con A, sodium m-periodate, OVA grade VI, and poly(I:C) were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant mouse IFN-γ was purchased from Amgen Inc. (Thousand Oaks, CA). Proteose peptone was from Difco Laboratories and S. minnesota R595 LPS was from Ribi ImmunoChem Research Inc. (Hamilton, MT). SRBC were from Carr-Scarborough Inc. (Decatur, GA) and were washed three times with PBS before use. Listeriolysin-O (LLO) preparations were generated in our laboratory as previously described (17, 18). Briefly, crude preparations were precipitated with saturated ammonium sulfate from supernatants from 18-24-h L. monocytogenes cultures, then dialyzed exhaustively before use. Recombination LLO was derived as previously described from lysates of E. coli BB4 containing the plasmid pT10E into which the structural gene for LLO had been inserted (19). Briefly, bacteria were lysed by freezing and thawing and the lysates were sonicated to reduce viscosity before sterile filtration. Bacteria containing the unaltered plasmid served as a control. LLO peptide 215-234, which has been shown to be a dominant epitope for T cells in C3HeB/FeJ mice (18), was synthesized using an automated peptide synthesizer (430A; Applied Biosystems, Inc., Foster City, CA) in the Microchemistry Department at Emory University. The peptide was purified by HPLC and its structure confirmed by microsequencing.

Cell Harvesting and In Vitro Culture. Peritoneal exudate cells (PEC) were obtained by peritoneal lavage with HBSS containing 0.06% BSA, 10 mM Hepes buffer, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10 U/ml heparin. Macrophages were depleted by incubation in tissue culture dishes for 1 h at 37°C. Plastic nonadherent cells were passed over nylon wool columns using standard procedures (20) to further enrich for T cell populations. A portion of these cells was analyzed by flow cytometry. The remainder were incubated at 37°C in 24-well plates at 106 cells/well in combination with 2.5 x 105 irradiated (3,000 rad) splenic accessory cells in a total volume of 2 ml. Culture media was RPMI 1640 supplemented with 10% FCS, 5 x 10-5 M 2-ME, 0.5 mM sodium pyruvate, 10 mM Hepes buffer, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Either Con A (2 μg/ml) or heat-killed preparations of the immunizing bacteria were added as indicated in the text. Surviving cells were harvested on Ficoll-Hypaque gradients (Lymphocyte Separation Medium; Organon Teknika Corp., Durham, NC) after 4 d and analyzed by flow cytometry. The ratio of the number of cells of a TCR phenotype in this surviving population to the number of cells of that phenotype in the initial population was operationally defined as the expansion factor for that subset. When the proportion of cells of a given subset (e.g., γ/δ T cells) increases after culture, this reflects both the enhanced proliferation and survival of that subset relative to the other subset (e.g., α/β T cells). This measurement is described in more detail in the companion paper (21).

Single-cell suspensions were made from excised spleens or lymph nodes using a glass homogenizer and RBC were lysed from the

1 Abbreviations used in this paper: LLO, listeriolysin-0; PEC, peritoneal exudate cells.
spleen cell suspensions with ammonium chloride buffer (20). Cells were analyzed by flow cytometry or cultured in vitro as described above.

**Antibodies.** Specificities and sources are as follows: anti-CD3, hybridoma 145-2C11 (22) provided by Dr. Jeffrey Bluestone (University of Chicago, Chicago, IL) or purchased as a PE conjugate (Phar-Mingen, San Diego, CA); anti-α/β TCR, hybridoma H57-597 (23) provided by Dr. Ralph Kuba (National Jewish Hospital, Denver, CO); anti-γ/δ TCR, hybridoma UC7-13D5 provided by Dr. Jeffrey Bluestone or hybridoma GL3 (24) provided by Dr. Leo LeFrancois (University of Connecticut, Farmington, CT); anti-γ/δ, hybridoma F536 (25) provided by Dr. James Allison (University of California, Berkeley, CA); anti-δ4, hybridoma GL2 (24) provided by Dr. Leo LeFrancois; anti-CD4, hybridoma GK1.5 (26) (ATCC TIB 207); anti-CD8, hybridoma IM-7 (28) (PharMingen); anti-CD45R, hybridoma 23G2 (29) (PharMingen); anti-IL-2R FITC, hybridoma JD4 (30) (PharMingen); anti-Thy-1-PE, hybridoma 53-2.1 (27) (PharMingen); anti–mouse IgG-FITC (Southern Biotechnology, Birmingham, AL); and anti–rat IgG-PE (Southern Biotechnology). mAbs generated from cell lines in the laboratory were purified from culture supernatants by protein A affinity chromatography (20) or from serum-free culture supernatants by saturated ammonium sulfate precipitation. Purified antibodies were directly conjugated to FITC using standard techniques (20). The γ/δ TCR expression was confirmed using both available antibodies (UC7-13D5 and GL3) directly conjugated to FITC. Both antibodies gave identical results.

**Flow Cytometry.** Cells (1–5 × 10⁶) were incubated on ice for 30 min with 25 μl of a predetermined optimal concentration of mAb, washed twice with PBS containing 3% FCS and 0.1% sodium azide, and fixed with 1% paraformaldehyde in PBS. If primary antibodies were not directly conjugated to either FITC or PE, the incubation with primary antibody was followed by a second incubation for 30 min on ice with an appropriate secondary antibody conjugated to a fluorochrome. Binding of isotype-matched control Ig was used to determine background levels of immunofluorescence.

For two-color analyses involving the use of a secondary antibody, the incubations, which involved three different antibodies, were carried out in a sequence that had been previously determined to minimize background staining. Samples were analyzed (5,000 cells/sample) on a FACScan® (Becton Dickinson & Co., Mountain View, CA) using forward scatter/side scatter gating to select the lymphocyte population for analysis. For evaluation of surface marker expression on T cell subsets using two-color analysis, a second gate was used to select the appropriate subset and 5,000 of those cells were evaluated. In some experiments, cells were analyzed after adherence to plastic without further purification on nylon wool. These cells are termed peritoneal plastic nonadherent (PNA). To minimize nonspecific immunofluorescence in these samples, cells were preincubated with an anti-Fc receptor mAb (FcBlock; PharMingen) before addition of specific antibodies.

**In Vivo Depletion of T Cell Subsets.** Mice were injected intraperitoneally with 200 μg of purified anti-TCR α/β (H57-597) or anti-TCR γ/δ (GL3) or with hamster IgG (Accurate Chemical Co., Westbury, NY) as a species and isotype-specific control. Depletion of T cell subsets was monitored by flow cytometry in both the peritoneal and splenic populations. The course of a *Listeria* infection was monitored in these mice by enumerating bacterial colony counts grown from serial 10-fold dilutions of homogenized livers and spleens. Cells were lysed with 1% Triton X-100 to release intracellular bacteria before incubation for 48 h at 37°C on brain heart infusion agar plates.

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**Results**

**Intraperitoneal Injection of L. monocytogenes Results in a Site-specific Increase in γ/δ T Cells, Which Then Expand to a Greater Degree than Do α/β T Cells When Restimulated In Vitro.** On day 10 after intraperitoneal injection of 6 × 10⁶ viable *L. monocytogenes* (LD₅₀ = 5 × 10⁴), the proportion of γ/δ T cells in the CD3⁺ population in the peritoneal cavity was increased more than threefold as compared with PBS-injected mice (Fig. 1A, top two rows). The proportion of CD3⁺ cells in PEC was also increased in *Listeria*-infected mice (Fig. 1A). The combined effect of these two changes resulted in an ~10-fold increase in the actual number of γ/δ T cells in the peritoneal cell population (see Table 1). If the nylon wool–nonadherent PEC were then cultured in vitro for 4 d with irradiated splenic accessory cells and *Listeria* antigen preparations, the resulting population was 97% CD3⁺, of which 44% were γ/δ T cells (Fig. 1, A). In multiple experiments the percentage of γ/δ T cells in the population generated in vitro in response to *Listeria* antigens ranged from 36 to 59%. Heat-killed *Listeria* (10⁷/ml) and crude LLO (10 μg/ml) were routinely used in combination to stimulate the T cells in vitro, although either antigen preparation used alone gave similar results (data not shown). LLO is an exotoxin that has been shown to be a dominant antigen for α/β T cells in *Listeria*-infected mice (18).

This preferential expansion of γ/δ T cells in vitro was even greater if Con A rather than specific antigen was used as a stimulant in vitro. The percentage of γ/δ T cells in the Con A–stimulated population averaged 63% (n = 7) and ranged from 42 to 87% (see Fig. 1B, top row, for example). This preferential expansion was not seen in PBS-injected mice, in which the proportion of γ/δ T cells did not change significantly after Con A stimulation in vitro (see Table 1). An expansion factor for T cell subsets was calculated based on the ratio of the number of cells of a TCR phenotype in the initial population as compared with the number of cells of that phenotype in the population harvested after 4 d of Con A stimulation. Both selective proliferation and cell survival likely contribute to the operationally defined expansion factor. The expansion factor for γ/δ T cells from *Listeria*-infected mice in response to Con A in vitro averaged 3.2 (SD = 2.2) while the α/β T cell expansion factor averaged 0.8 (SD = 0.7) in four experiments. Similar differences were seen if *Listeria* antigens were used to stimulate the T cells in vitro (2.8-fold expansion, SD = 0.9) for γ/δ T cells compared with 1.5-fold expansion (SD = 0.3) for α/β T cells.

This induction/activation phenomenon for γ/δ T cells was site specific in that no increases in the percentages of γ/δ T cells in splenic or lymph node populations of *Listeria*-immune mice were observed. Mesenteric as well as axillary and inguinal lymph nodes were examined and found to be unaffected (data not shown). The γ/δ T cells represented <5% of the freshly isolated spleen or lymph node cells and did not show preferential expansion in vitro in response to Con A, remaining at ~2% of the CD3⁺ cells (Fig. 1B).

**Kinetics of γ/δ T Cell Induction in the Peritoneal Cavity.** A series of experiments was conducted in which PEC were ex-
examined at intervals after intraperitoneal Listeria injection to examine the kinetics of the inductive response. A representative experiment is shown in Fig. 2 A. (Note also the kinetics shown in control animals in Figs. 4 and 5.) Increased percentages of γ/δ T cells were first seen 5–7 d after injection. The proportion of γ/δ T cells increased to a peak plateau between days 10 and 20, and remained elevated for 6–7 wk (data not shown). The number of peritoneal γ/δ T cells per mouse was calculated based on the number of PEC harvested and the percentages of CD3+ and γ/δ+ T cells as determined by flow cytometry. This value peaked on day 10 and then declined sharply, reflecting decreases in both the peritoneal lymphocytosis and the percentage of CD3+ cells in the PEC. The numbers continued to decline more gradually and eventually returned nearly to baseline by 8–9 wk postinjection even though the percentage of γ/δ T cells remained slightly elevated (data not shown). Thus, increased numbers of γ/δ T cells are noted at times of active infection (days 4–6) and the peak accumulation of γ/δ T cells is noted ~2–3 d after infection has resolved. These results differ from those in a previous report (7), in which an increase in peritoneal γ/δ T cells was seen 2–3 d after Listeria injection followed by a rapid decline and a return to normal levels by day 10. These data were obtained, however, by Northern blot analyses of mRNA, which may at least partially explain the differences.

Preliminary experiments had indicated that intraperitoneal injection of LPS also induced peritoneal γ/δ T cells. The kinetics of this response to a microbial endotoxin differed significantly from the response to a viable microbe in that the percentage of γ/δ T cells peaked on day 3 and then began a gradual decline (Fig. 2 B). The number of peritoneal γ/δ T cells decreased even more rapidly, returning to near baseline levels by day 10. These differences in kinetics may reflect the time necessary for the live microbe to generate factors that affect the composition of the T cell population.

Surface Marker Expression on Peritoneal γ/δ T Cells. Although most subpopulations of γ/δ T cells from various tissue sites express neither CD4 nor CD8 on their surface, a significant proportion of the γ/δ T cells in the intraepithelial

Figure 1. CD3/TCR phenotype of various cell populations analyzed by flow cytometry. Horizontal axes represent log fluorescence intensity and vertical axes represent frequency of events. Incubation with the appropriate purified primary mAb was followed with anti-mouse IgG FITC. Nonspecific fluorescence is shown as the unshaded area in each panel and the number in the boxes on the right represents the percentages of γ/δ T cells in the CD3+ population. (A) γ/δ T cells were significantly increased in peritoneal cell populations freshly isolated from mice immunized with virulent Listeria (6 × 10^3 i.p. 10 d before harvest (middle), as compared to PBS-injected mice (top). Cells from Listeria-immunized mice that were cultured for 4 d in vitro in the presence of 10^7/ml heat-killed Listeria and 10 μg/ml LLO (bottom) showed an increased frequency of γ/δ T cells. (B) Cells from three anatomical sites in Listeria-immunized mice were expanded in vitro with 2 μg/ml Con A and then examined for CD3/TCR expression by flow cytometry. Preferential expression of γ/δ T cells was specific to the peritoneal cavity. Results are representative of several experiments.
lymphocyte population from the intestinal lining express CD8 (31, 32). Neither resident nor Listeria-induced peritoneal γ/δ T cells exhibited detectable surface expression of CD4 or CD8, while peritoneal α/β T cells expressed these markers as anticipated (Fig. 3 and Table 1). Thy-1 has also been reported to be variably expressed on γ/δ T cell populations. Only 29% (SD = 9, n = 7) of the resident peritoneal γ/δ T cells were Thy-1+. After infection with Listeria, 65% (SD = 7, n = 9) of peritoneal γ/δ T cells were Thy-1+. Injection of LPS did not result in an increase in Thy-1 expression. The increase in Thy-1+ cells in Listeria-infected mice continued for at least 3 wk but had returned to baseline by 7 wk after injection. Thy-1 has been reported to be an inducible marker on intestinal epithelial γ/δ T cells, perhaps in response to microbial stimulation (33). Thy-1 was expressed on >95% of peritoneal α/β T cells in all treatment groups as expected.

We also compared the expression on peritoneal γ/δ and α/β T cells of the activation markers Pgp-1 and CD45R (Table 1). Cells were evaluated using two-color immunofluorescence on day 10 after injection of PBS or Listeria and on day 3 after injection of LPS. In both α/β and γ/δ T cells isolated in four separate experiments from Listeria-infected mice, a high frequency of cells expressed Pgp-1 (98% SD = 1% of γ/δ T cells and 93% SD = 5% of α/β T cells), consistent with an activation phenotype. Interestingly, both subsets also showed high frequencies of Pgp-1 expression on resident peritoneal cells (94% SD = 3% for γ/δ T cells and 71% SD = 19% for α/β T cells), confirming the generally accepted concept that the peritoneal cavity is a repository of activated/memory cells. The CD45 molecule is expressed on the cell surface as a series of isotypes that are generated by exon splicing. We used the 23G2 mAb, which is B exon dependent (34). Previous studies have shown that the isotypes that include the B exon are expressed on nonactivated cells (34-36); thus, a CD45R lo phenotype is associated with activated cells. This marker was the most variably expressed of those that we investigated on peritoneal T cells. In some experiments, injection of Listeria seemed to cause a shift toward a reduced

Figure 3. Peritoneal γ/δ T cells from Listeria-immunized mice do not express CD4 or CD8, but approximately two-thirds are Thy-1+. In contrast, α/β T cells are >95% Thy-1+ and CD4/CD8+. Two-color flow cytometry was used to analyze surface marker expression on peritoneal NWNA cells. Cells were first incubated with either anti-CD4 and anti-CD8 or with anti-Thy-1 mAb followed by anti-rat IgG-PE and finally with FITC-conjugated anti-TCR mAb. Horizontal axes represent green and vertical axes represent red fluorescence intensity. Boundaries for positive and negative populations were established based on binding of isotype-matched Ig controls. Numbers in each panel represent the percentages of the total population within that quadrant.
Listeria-infected T cells after 4 d of culture with Con A (data not shown). Injection of LPS did not alter the frequencies of peritoneal T cells, although the frequency of CD45R lo cells was lower than in the γδ T cell population (54% in PBS controls and 63% in immunized mice.) Injection of LPS did not alter the frequencies of peritoneal T cells expressing either Pgp-1 or CD45R. For comparison, lymph node α/β T cell populations from PBS- and Listeria-infected mice were examined and found to be <10% Pgp-1+ and <10% CD45R lo.

Expression of Thy-1, Pgp-1, and CD45R was also evaluated in populations that were expanded in vitro with Con A, but no significant changes were found compared to the starting populations (Table 1). This is in contrast to the IL-2R, which is undetectable on freshly isolated PEC from Listeria-infected mice but is expressed on both α/β and γδ T cells after 4 d of culture in PBS controls (data not shown). Similar results were obtained using the 7D4 mAb antibody to the IL-2R and are consistent with previous studies in which this antibody bound at detectable levels to T cells only after in vitro stimulation with Con A (30). Thus, the significant changes were found in surface marker expression on peritoneal γδ T cells were the increase in Thy-1 expression in vivo after Listeria infection and the induction of the IL-2R during in vitro restimulation.

We also evaluated the expression of particular V region gene products (Vγ2, Vγ3, and Vδ4) for which mAbs were available. Unlike the preferential V region expression reported in various other tissue sites (37), the γδ T cells present in the peritoneal cavity show no detectable expression of these V gene segments. This was evidenced by the finding that the appropriate mAbs stained <1% of peritoneal γδ T cells induced by either Listeria or LPS (data not shown). The "negative" results with antibodies to Vγ2, Vγ3, and Vδ4 are significant, we believe, since we have confirmed that these antibodies do stain the appropriate subsets in the spleen, skin, and intestinal epithelium, respectively. The peritoneal T cells may express either an as yet undefined V segment preference or may be a diverse polyclonal population of T cells.

Table 1. Phenotype of γδ T cells

| Marker | PBS | LM | LPS | PBS | LM | LPS |
|--------|-----|----|-----|-----|----|-----|
| CD4/CD8 | <2 | <2 | <2 | <2 | <2 | <2 |
| Thy-1 | 29 | 65 | 30 | 31 | 71 | 41 |
| Pgp-1 (CD44) | 94 | 98 | 97 | 96 | 99 | 99 |
| CD45R lo | 68 | 79 | 76 | 35 | 69 | 75 |

% of CD3+ cells

- CD4/CD8: <2 <2 <2 <2 <2 <2 >98 >98 >98 >98 >98 >98 >98
- Thy-1: >95 >95 >95 87 97 84 95
- Pgp-1 (CD44): 71 93 90 96 98 92 <10
- CD45R lo: 54 63 66 49 38 30 <10

The phenotype of T cells was determined by two parameter FACS® analysis as described in Fig. 3 and Materials and Methods. Values represent the mean percentage of CD3+ cells expressing the indicated surface marker or phenotype. Cell populations were from mice injected intraperitoneally with the indicated material (PBS, live L. monocytogenes [LM] or LPS). Cells were analyzed on the day of harvest (Fresh) or after 4 d culture with Con A (Con A-stimulated) as described in Materials and Methods. The right-hand column represents the analysis of α/β T cells from the lymph nodes (LN) of normal mice.

The hemolysin molecule LMP of L. monocytogenes. The hemolysin mole-
Table 2. *Induction of γ/δ TCR-expressing Peritoneal T Cells*

| Injection | Fresh T cells | Con A blasts |
|-----------|---------------|--------------|
|           | No. of γ/δ+ cells | γ/δ+ cells as percent of total T cells | γ/δ+ cells as percent of total T cells |
|           | per mouse | | |
| 10 d before harvest | | |
| PBS i.p. | 14 | 12 | 8 |
| Hly+ L.M. i.p. (6 × 10^9) | 136 | 21 | 65 |
| Hly- L.M. i.p. (6 × 10^9) | 3 | 7 | 5 |
| Heat-killed L.M. i.p. (10^6) | 14 | 14 | 11 |
| Hly+ L.M./peptone i.p. (6 × 10^9) | 272 | 26 | 70 |
| 2nd Hly+ L.M./i.p. (6 × 10^6) | 147 | 28 | 45 |
| Hly+ L.M. s.c. (6 × 10^6) | 10 | 23 | 11 |
| Peptone i.p. (1.5 ml of 10%) | 41 | 14 | 7 |
| LLO 215-234 s.c. in CFA (50 μg) | 17 | 17 | 11 |
| OVA s.c. in CFA (50 μg) | 2 | 4 | 7 |
| SRBCs i.p. (10^6) | 7 | 7 | 4 |
| Con A (100 μg) i.p. | 80 | 12 | 9 |
| BCG i.p. (10^6) | 190 | 9 | 20 |
| Hly+ E.C. i.p. (10^6) | 166 | 40 | 32 |
| Hly- E.C. i.p. (10^6) | 72 | 39 | 13 |
| S.T. i.p. (10^6) | 53 | 26 | 20 |
| S.T. i.p. (10^6) | 65 | 20 | 48 |
| LPS (1 μg) in C3HeB/FeJ Mice i.p. | 190 | 31 | 55 |
| 3 d before harvest | | |
| PBS i.p. | 14 | 12 | 8 |
| Hly+ L.M. i.p. (6 × 10^9) | 17 | 9 | 5 |
| LPS (1 μg) in C3HeB/FeJ mice i.p. | 54 | 31 | 27 |
| LPS (1 μg) in C3H/HeJ mice i.p. | 12 | 8 | NT |
| rLLO (400 U) i.p. | 398 | 34 | 19 |
| Peptone (1.5 ml of 10%) i.p. | 13 | 14 | 10 |
| Con A (100 μg) i.p. | 25 | 13 | 14 |
| PHA (35 μg) i.p. | 25 | 18 | 12 |
| rIFN-γ (5,000 U b.i.d.) i.p. | 9 | 7 | 13 |
| NaI04 (1 mg) i.p. | 160 | 25 | 16 |
| Thioglycollate i.p. (2.5 ml of 3%) | 101 | 43 | 17 |
| Poly (l:C) (100 μg) i.p. | 10 | 17 | 20 |

Cells expressing CD3, α/β TCR, or γ/δ TCR were quantitated using flow cytometry. Underlined values represent those showing consistent increases greater than twofold above PBS control. Injections were in C3HeB/FeJ mice unless otherwise noted. L.M., L. monocytogenes; E.C., E. coli; S.T., S. typhimurium.

The molecule, LLO, has been shown to be both a major virulence factor (38) and a major antigenic component (18) of *Listeria*. Strains of *Listeria* that do not express LLO (Hly- strains) are poorly immunogenic and have LD₉₀'s that are 4–5 logs greater than Hly+ strains. Heat-killed *Listeria* are likewise very poorly immunogenic. Neither Hly- nor heat-killed *Listeria* induced peritoneal γ/δ T cells after intraperitoneal injection (Table 2).

Injection site-specific induction was indicated by the finding that although viable Hly+ *Listeria* are both virulent and immunogenic when injected intraperitoneally or subcutaneously,
only the intraperitoneal route of administration resulted in an induction of γ/δ T cells in the peritoneal cavity. In other studies in our laboratory (Pearce et al., manuscript in preparation), we have noted that intragastric inoculation of *Listeria* produced changes in the γ/δ T cell vs. α/β T cell composition in the intestinal epithelial lymphocytes (IELs) and demonstrable systemic infection in the spleen and liver. However, intragastric inoculation did not cause changes in the peritoneal γ/δ T cells and, conversely, intraperitoneal inoculation caused no changes in T cell subset composition in the IELs (data not shown). Additionally, subcutaneous injection in CFA (containing a nonviable form of *Mycobacterium bovis*) of the protein antigen OVA or of an immunodominant peptide epitope of LLO (LLO 215-234) (18) did not induce peritoneal γ/δ T cells. Even highly immunogenic particulate antigens such as SRBC failed to significantly induce peritoneal γ/δ T cells. Considered collectively, these data suggest that the induction of γ/δ T cells is a site-specific reaction to infection, rather than a systemic response to microbial infection or antigenic challenge.

We also wanted to determine whether this response was generalizable to a variety of bacterial strains. The results (Table 2) indicate that when injected intraperitoneally, Gram-negative bacteria such as *E. coli* (E.C.) and *S. typhimurium* (S.T.) and a strain of *Mycobacteria* (BCG) can also induce peritoneal γ/δ T cells. As with *Listeria*, the intravenous expansion potential of peritoneal T cells was enhanced by injection of *Salmonella* and was influenced by the number of bacteria injected. The percentage of γ/δ T cells in freshly isolated PEC did not increase after BCG injection, but the absolute number per mouse was elevated and a modest level of preferential increase in expansion was observed. These results were somewhat surprising since several reports have implicated a role for γ/δ T cells in the response to *Mycobacteria* (5, 6, 9, 13). Since BCG is relatively avirulent, it is possible that fully virulent strains of *Mycobacteria* may produce a more dramatic induction of γ/δ T cells. This idea is being tested. Overall, our survey indicates that virulent *Listeria* were especially effective in the induction of γ/δ T cells.

Since the production of the hemolysin LLO appeared to be essential for γ/δ T cell induction by *Listeria*, the effect of the α hemolysin of *E. coli* was investigated using a pair of *E. coli* strains expressing a plasmid containing either an intact hemolysin gene (hlyA) or one in which the structural gene had been disrupted with a transposon. The strain that expressed hemolysin (Hly* E.C.) induced a greater number of γ/δ T cells per mouse than the hemolysin-negative strain (Hly- E.C.) (Table 2). There was also a greater increase in response to Con A restimulation of γ/δ T cells from mice injected with Hly* E.C. than from mice injected with Hly- E.C. strain.

Given the finding that both exotoxins and endotoxins appeared to play a critical role in the induction of γ/δ T cells, we evaluated further the activity of isolated toxins. The induction of γ/δ T cells by LPS was observed in the LPS high responder mouse strain (C3HeB/FeJ) but no induction occurred in the LPS low responder (C3H/HeJ). Thus, like many of the responses to LPS, the induction of γ/δ T cells appeared to be under strict control of the *Ips* gene, implying that lipid A is the biologically active portion of LPS in this system. We also noted that a recombinant preparation of the exotoxin LLO was a potent inducer of the number of peritoneal γ/δ T cells in C3H/HeJ mice. (Table 2).

The significance of microbial stimulation was understood by the fact that strong induction of γ/δ T cells was not observed with several common peritoneal inflammatory stimulants. We evaluated the effects of peptone alone or in combination with *Listeria* with respect to its effect on peritoneal γ/δ T cells. Injection of peptone alone did not significantly affect the γ/δ T cell population on day 3 and showed only a moderate increase in cell number on day 10 after injection (Table 2). The only difference between mice injected with *Listeria* alone and those injected with *Listeria* plus peptone was an increase in the number of γ/δ T cells per mouse in the latter group (e.g., values of 136 vs. 272 in Table 1). In our survey, we also investigated a variety of known peripheral inflammatory agents and agents that have been shown to alter the peritoneal cell populations. The PEC were harvested on day 3 after intraperitoneal injection since the effects of most inflammatory agents are known to be maximal 3–5 d after injection. Neither Con A, PHA, rIFN-γ, nor poly(I:C) altered the γ/δ T cell population (Table 2). Under these conditions rIFN-γ caused a marked increase in class II MHC expression by peritoneal macrophages and poly(I:C) dramatically increased NK cell activity, as expected (data not shown). Injection of sodium m-periodate, a T cell mitogen, and thioglycollate, a peritoneal inflammatory agent, resulted in increased percentages and absolute numbers of γ/δ T cells, but in neither case did these cells expand in vitro in response to Con A to the extent exhibited by the γ/δ T cells induced in response to microbial infection. Since Con A was such a potent in vitro stimulator of γ/δ T cells activated in vivo by microbial infection, the effect of Con A in vivo 10 d after injection was also evaluated. The number of γ/δ T cells was increased, but the percentage was unchanged and these cells did not significantly expand during in vitro restimulation. Thus, induction of γ/δ T cells did not necessarily accompany other known induction events in the peritoneal cavity, and our results taken together represent strong evidence that bacterial infection is intimately and uniquely associated with profound regulation of γ/δ T cells.
cell numbers associated with a Listeria infection occurred in both the spleen and peritoneal cavity, as anticipated. In the groups treated with anti-TCR antibody, however, these increases were detectable only in the untreated subset. Also note that the number of γ/δ T cells in the peritoneal cavity in the group depleted of α/β T cells is less than in the control group (Fig. 5, lower right). This may reflect the dependence of γ/δ T cells on α/β T cells as observed in vitro and detailed in the companion paper (21).

Although the depletions are not absolute, in that small numbers of cells in the depleted subsets are still detectable, this treatment has a profound effect on the resistance to a primary Listeria infection (Fig. 6). By day 8, the infection was completely cleared in the control group but continued in mice depleted of either α/β or γ/δ T cells. Although the effect was somewhat heterogeneous on day 8 in the group depleted of γ/δ T cells, there is a clear indication that a severe reduction in the numbers of γ/δ T cells allows for persistence and increased magnitude of a Listeria infection.

In contrast, the resistance to a challenge dose of Listeria in mice that had been primed with an immunizing dose of Listeria was virtually unaffected by in vivo depletion of γ/δ T cells. On day 4 after the challenge injection, bacterial clearance was comparable in primed mice that were either injected with control IgG or that were depleted of γ/δ T cells (Table 3). Depletion of α/β T cells, however, resulted in dramatically higher bacterial numbers after challenge (Table 3). These same trends were apparent very early after the second Listeria

![Figure 4](image_url)  
Figure 4. Percentages of α/β and γ/δ T cells in the CD3+ populations from spleen or peritoneal cavity of T cell-depleted mice. Mice were injected intraperitoneally with 200 μg of either control IgG (hamster), anti-TCR α/β, or anti-TCR γ/δ. 2 d later, 6 × 10⁵ Listeria were injected intraperitoneally. Animals were killed on days 2, 5, and 8 after Listeria injection and examined for T cell phenotype and level of bacterial infection (Fig. 6). Spleen cells and peritoneal plastic nonadherent cells (PNA) were pooled from four mice and examined by flow cytometry to determine TCR phenotype.

![Figure 5](image_url)  
Figure 5. Absolute numbers of T cell subsets in the spleen and peritoneal cavity of T cell-depleted mice. Mice were treated as described in Fig. 4. The numbers of T cells of each subset in a tissue site were determined based on the total cell yield from that site, the percentage of those cells expressing CD3, and the proportion of α/β or γ/δ T cells in the CD3+ population.
T cells. Microbial antigens, such as heat shock proteins (HSPs) proliferation may be due to a T cell repertoire unique to /~ conceptualized in two fundamental ways. The presence of molecules, and cytokines that are both influenced by infection to sites of microbial infection in both human lesions (5, 6, 38) and murine infectious disease models (7, 13). Our results show that the accumulation of /~ T cells at the site of infection is dependent upon the presence of either a virulent bacterial infection. Previous reports have linked /3 T cells and specifically required for /~ T cell proliferation. An evaluation of these hypotheses represents a central focus for our ongoing research.

**Discussion**

The findings of this study provide direct experimental evidence of the tissue site–specific association of /~ T cells with bacterial infection. Previous reports have linked /~ T cells to sites of microbial infection in both human lesions (5, 6, 38) and murine infectious disease models (7, 13). Our results show that the accumulation of /~ T cells at the site of infection is dependent upon the presence of either a virulent form of bacteria or its exotoxin or endotoxin. Heat-killed or other avirulent forms of bacteria are relatively ineffective, suggesting that an active production of virulence factors is necessary for the induction of /~ T cells.

The mechanism of /~ T cell induction in vivo may be conceptualized in two fundamental ways. The presence of /~ T cells could be due either to a recruitment of cells into the inflammatory site or to enhanced proliferation. A selected proliferation may be due to a T cell repertoire unique to /~ T cells. Microbial antigens, such as heat shock proteins (HSPs) and HSPs expressed by the host, may be upregulated by the toxins expressed by pathogenic bacteria. Furthermore, induction may be favored by presenting molecules, accessory molecules, and cytokines that are both influenced by infection and specifically required for /~ T cell proliferation. An evaluation of these hypotheses represents a central focus for our ongoing research.

**Figure 6.** Infection with *Listeria* in T cell-depleted mice. Depletion of /3 and /~ T cells in vivo alters infection with *Listeria*. Mice were treated as described in Fig. 4. Organs from individual animals were evaluated for the number of bacterial colonies that developed when cell lysates were incubated on agar plates. Each bar represents an individual mouse and results are pooled from two separate experiments. Minimal detection limits under conditions of the experiment were 100 colonies per organ; maximum quantifiable colonies were 10^6. Numbers in each panel represent arithmetic means. Data were analyzed by a sign rank test using nonparametric analysis; differences between the control group (IgG) and the antibody-treated groups at each day were compared. In the spleen at day 2, the differences were not significant (p = 0.56 for anti-/3 Ab and 0.11 for anti-/3/β Ab). At day 5 in the spleen, significant differences were judged for both antibody-treated groups (p = 0.0156). At day 8 in the spleen, p values were 0.063 for anti-/3 Ab and 0.0156 for anti-/3/β Ab. In the liver at day 2, p = 0.656 for anti-/3 Ab and 0.0156 for anti-/3/β Ab. In the liver at day 3, p = 0.0156 for anti-/3 Ab and 0.0156 for anti-/3/β Ab. In the liver at day 8, p = 0.0625 for anti-/3 Ab and 0.0156 for anti-/3/β Ab. The marginally significant differences with anti-/3 Ab at day 8 were due mainly to the fact that there were two ties at the minimal detection limits (0.01 x 10^4 bacteria per organ). That is, two animals appeared to clear the infection in the anti-/3 Ab-treated groups.

**Figure 7.** No evidence for involvement of /~ T cells in the memory response. Shown is immunity to secondary challenge with *Listeria*. Listeria-primed mice were depleted of /3 or /~ T cells, then challenged with *Listeria* as described in Table 3. Age-matched control groups were either unprimed and not depleted of T cells or were primed but not depleted. T cell depletions were confirmed by flow cytometry. Each bar represents results from an individual mouse. Results are from spleen colonies; similar trends were apparent in livers and PEC.
Table 3. Depletion of α/β and γ/δ T Cells In Vivo: Role of α/β T Cells in Secondary Challenge with Listeria

| Treatment                        | Mouse number | Liver (× 10,000) | Spleen |
|----------------------------------|--------------|------------------|--------|
| Primed/control IgG              | 1            | 0.19             | 0.03   |
|                                  | 2            | 2.82             | 0.75   |
| 3                                |              | 4.52 (4.8)       | 1.10 (3.2) |
| 4                                |              | 15.60            | 13.60  |
| 5                                |              | 1.05             | 0.48   |
| 1                                |              | 0.19             | 0.05   |
| 2                                |              | 1.61             | 0.25   |
| Primed/anti-γ/δ TCR IgG         | 3            | 1.73 (4.3)       | 8.30 (2.5) |
| 4                                |              | 11.20            | 0.50   |
| 5                                |              | 6.80             | 3.40   |
| 1                                |              | 16,650.00        | 6,000.00 |
| 2                                |              | 559.00           | 2,420.00 |
| 3                                |              | 4,980.00 (6,312) | 9,700.00 (5,226) |
| 4                                |              | 7,020.00         | 5,460.00 |
| 5                                |              | 2,355.00         | 2,550.00 |
| 1                                |              | 35,800.00        | 18,200.00 |
| 2                                |              | 24,000.00        | 10,700.00 |
| Primed/anti-α/β TCR IgG         | 3            | 42,300.00 (32,388)| 19,000.00 (16,240) |
| 4                                |              | 10,790.00        | 15,400.00 |
| 5                                |              | 49,050.00        | 17,900.00 |
| Unprimed/control IgG            | 3            |                  |        |

Primed mice were infected with a sublethal dose of *Listeria* (9 x 10^3/mouse) 4 wk before antibody treatment. Unprimed mice were uninfected age-matched controls. Mice were treated with purified IgG (200 µg/mouse i.p.) of either control normal hamster IgG (control IgG), H57 mAb (anti-α/β TCR IgG), or GL3 mAb (anti-γ/δ TCR IgG). 3 d later mice were challenged with 10^6 viable *Listeria* intraperitoneally. 4 d after challenge, *Listeria* colony counts were determined from plated lysates of liver and spleen. Depletion of the appropriate subset of T cell was confirmed by flow cytometry as illustrated in Figs. 4 and 5.

respect to their ability to expand rapidly when restimulated in vitro. The reduced expression of CD45R or the expression of high levels of Pgp-1 are not apparently sufficient for an increased in vitro expansion potential, however, since peritoneal γ/δ T cells from mice injected with PBS express an activation phenotype but do not significantly expand when restimulated in vitro with Con A. We have noted that increased Thy-1 positivity of the induced γ/δ T cells correlates to some degree with a preferential expansion potential in vitro, but the generality of this observation and mechanistic basis for this finding remains for further study. Clearly, the expansion potential in vitro is related to the nature of the in vivo induction process, during which some other portions of the cellular activation pathway are apparently initiated. This induction phenomenon seems to be related to microbes and their toxins since injection of nonspecific peritoneal inflammatory agents does not increase the in vitro expansion potential of γ/δ T cells even though injection of some of these agents is accompanied by increases in the numbers of peritoneal γ/δ T cells. This activation state of microbial-induced peritoneal γ/δ T cells may alter the ability of these cells to release various cytokines or to respond to cytokine signals from other cells in the network of the immunoregulatory pathway. The characteristics of selective γ/δ expansion in vitro to IL-1 and IL-7, as described in our companion paper (21), lend biological feasibility to a mechanism favoring selective expression of cytokines. IL-1 production is certainly a common feature shared by successful γ/δ T cell-inducing agents. Perhaps IL-7 is also selectively expressed in the tissue sites and under conditions produced by bacterial infection.

The increases in γ/δ T cells in the peritoneal cavity after injection of live virulent microbes did not occur until 5–7 d after the initial injection and persisted for several weeks. Carding et al. (14) have reported a similar delayed appearance and a persistence of γ/δ T cells in inflammatory exudates from lungs of mice infected with influenza. This is in con-
trast to previous reports of an early appearance (day 3) of γ/δ T cells in the peritoneal cavity of mice injected with Listeria (7) followed by a relatively rapid decline by day 13 postinfection (39). These investigators also report a dependence on the injection of proteose peptone in order to generate large increases in γ/δ T cells after Listeria infection (39). Our results do not support these findings. The differences between the findings are difficult to reconcile since similar mouse strains were used (C3HeB/FeJ or C3H/HeN). The analytical methods differed, however, in that in the current study γ/δ T cells were quantitated by immunofluorescence using a mAb specific for the γ/δ TCR (UC7-13D5 or GL3), whereas in the other studies the γ/δ T cells were quantitated either by analysis of mRNA production or by evaluation of CD3−CD4−CD8− populations by immunofluorescence. Synthesis of mRNA for γ/δ TCR in the absence of cell surface expression of the receptor in cells isolated from lung inflammatory exudates has been reported (40, 41).

Hiromatsu et al. (39) have reported that Listeria organisms were cleared less rapidly from the spleens of mice whose γ/δ T cells had been reduced by in vivo injection of anti-TCR γ/δ mAb before injection of Listeria. Our results with depletion of γ/δ T cells in vivo confirm and extend these observations (Figs. 4–6). Our results differ from this previous report in that exacerbation of listeriosis was most marked in our study at later times during infection. We also extend these findings by clearly documenting the antibody-mediated depletion of the relevant subsets. Our results are also consistent with those of Dunn and North (42), in which a Thy-1+CD4−CD8− cell population was implicated in resistance to listeriosis. The protective role of γ/δ T cells may be related to their ability to respond to activation signals that are induced by microbes or their toxins. Preliminary results have indicated that peritoneal γ/δ T cells elaborate IFN-γ in response to infected or activated macrophages in vitro (our unpublished observations). The role of IFN-γ in limiting Listeria infection has been well documented (43, 44). IFN-γ could certainly play an important role in limiting infection through augmentation of microbicidal function of macrophages as well as favoring the induction of macrophage MHC gene expression that would in turn augment antigen-specific T cell responses (17). The observation that γ/δ T cells are not significantly involved in the anamnestic response supports the hypothesis that these cells are involved in "front-line" immune defense mechanisms. These findings are also compatible with the view that the γ/δ TCR is fixed during evolution to particular tissue sites rather than shaped by antigenic challenge and clonal selection. The nature of the antigens recognized, the activation signals operative, and the mechanistic basis of the role of γ/δ T cells in innate defense reactions remain to be elucidated.

Finally, we wish to emphasize that the murine peritoneal cavity provides an ideal model for the study of γ/δ T cells. Even in the absence of immunization, the normal population of peritoneal cells is composed of ~10% γ/δ T cells, a significantly higher percentage than in spleen or lymph node populations. The fact that this peritoneal population can be altered under a variety of specific conditions, as reported here, provides a defined experimental system for investigating the relationship between γ/δ T cells and infectious disease. In this system γ/δ T cells are induced in sufficient numbers for further experimental analysis. The observation that the activation state of the γ/δ T cells is altered by microbial infection offers a system in which the immunobiology of γ/δ T cells is amenable to precise analysis.

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