

Intercellular Interactions and Cytokine Responsiveness of Peritoneal α/β and γ/δ T Cells from *Listeria*-infected Mice: Synergistic Effects of Interleukin 1 and 7 on γ/δ T Cells

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Summary

Peritoneal γ/δ T cells from *Listeria*-immune mice show an enhanced potential to expand when restimulated with antigens or mitogens in vitro (see companion paper [Skeen, M. J., and H. K. Ziegler. 1993. *J. Exp. Med.* 178:971]). When cocultured with peritoneal α/β T cells, the γ/δ T cell population expanded preferentially even when the in vitro stimulus was specific for the α/β T cell population. Purified γ/δ T cells did not respond to α/β T cell-specific stimuli. If isolated T cell subsets were recombined in cell mixing experiments, the resulting proliferative response was greater than additive. Irradiated α/β T cells could enhance the proliferation of responding γ/δ T cells, but the effect was unidirectional; i.e., irradiated γ/δ T cells did not stimulate responding γ/δ T cells. This effect appeared to be cytokine mediated and did not require cell-cell contact. Both recombinant interleukin 2 (rIL-2) and rIL-7 could support the expansion of the γ/δ T cells, while rIL-7 was only minimally stimulatory for the α/β T cells. The magnitude of the response by γ/δ T cells to rIL-7 exceeded the response to other in vitro stimuli, including immobilized anti-T cell receptor monoclonal antibody, and was 50–100-fold greater than the α/β T cell response to IL-7. This unique sensitivity of γ/δ T cells to IL-7 was strongly enhanced by the presence of accessory cells. These cells could be replaced by rIL-1, establishing a synergy for IL-1 and IL-7 as factors that could uniquely stimulate this γ/δ T cell population. Isolated peritoneal γ/δ T cells from *Listeria*-immune mice react to heat-killed *Listeria* preparations in the presence of macrophage accessory cells in a non-H-2-restricted manner. Considered collectively, these results suggest a potential mechanism by which γ/δ T cells can predominate in epithelial tissues and at sites of infection.

Historically, T lymphocytes present in the blood, lymph, and secondary lymphoid organs, such as the spleen and lymph nodes, have been most widely studied. It is now apparent that these cells are almost exclusively α/β T cells and as such the dogma about T cell function is based solely on the characteristics of α/β T cells. By comparison, little is known about the function and significance of γ/δ T cells in terms of the cytokines and presenting molecules operative, the antigens recognized, and the pathways of antigen processing relevant for activation of γ/δ T cells. While γ/δ T cell-cloned lines from the spleen have been shown to recognize antigen in association with class II MHC (1), other γ/δ T cells appear to recognize nonpolymorphic class I MHC molecules such as thymus leukemia (TL) (1, 2) or surface molecules such as CD1 (3). Some investigators find MHC restriction (4, 5) while others do not (6–8). Since so few studies have successfully defined the recognition requirements of γ/δ T cells, it is difficult to establish any general rules. General-

izations about the antigens recognized by γ/δ T cells are also difficult to make with certainty (9–14). One clear consensus is that γ/δ T cells are fundamentally different from α/β T cells and that their localization at sites of infection (5, 15) and in epithelial tissue (16–19) is considered critical to their unique role in immune defense.

In the present study we have analyzed the function of γ/δ T cells present in the peritoneal cavity of mice infected with *Listeria monocytogenes*. We have defined the growth requirements for γ/δ T cells using highly purified populations of cells. Our fundamental finding is that γ/δ T cells are dependent on cytokines produced by activated α/β T cells and macrophages. In particular, a striking synergistic effect of IL-1 and IL-7 on the proliferation of γ/δ T cells was noted. These results are discussed in light of the potential function of γ/δ T cells in front line defense reactions to infection in epithelial tissues.

Materials and Methods

Mice and Injections. Female C3HeB/FeJ 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. For all experiments mice were injected intraperitoneally with 6×10^5 viable *L. monocytogenes* strain 43251 (American Type Culture Collection [ATCC], Rockville, MD). *Listeria* used for injection were grown overnight in brain heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C with aeration. Bacteria were washed three times in PBS and concentrations were determined by optical density with confirmation by colony counts on brain heart infusion agar plates.

mAbs. Specificities and sources are as follows: anti-CD3, hybridoma 145–2C11 (20) provided by Dr. Jeffrey Bluestone (University of Chicago, Chicago, IL); anti- α/β TCR, hybridoma H57–597 (21) provided by Dr. Ralph Kubo (National Jewish Hospital, Denver, CO); anti- γ/δ TCR, hybridoma UC7–13D5 provided by Dr. Jeffrey Bluestone or hybridoma GL3 (22) provided by Dr. Leo LeFrancosis (University of Connecticut, Farmington, CT); anti-IA^k, hybridoma 10–2.16 (23) (ATCC TIB93); and anti-IE^k, hybridoma 14–4–4S (24) (ATCC HB32). mAbs generated from cell lines in the laboratory were purified from culture supernatants by protein A affinity chromatography (25) or from serum-free culture supernatants by saturated ammonium sulfate precipitation. Purified antibodies were directly conjugated to FITC using standard techniques (25). PE-conjugated 145–2C11 was purchased from PharMingen (San Diego, CA).

Reagents for In Vitro Restimulation of T Cell Subsets. Human rIL-1 β and human rIL-2 were gifts of Immunex Corp. (Seattle, WA); human rIL-7 was a gift of Sterling Drug Co. (Malvern, PA); mouse rIFN- γ was purchased from Amgen Inc. (Thousand Oaks, CA); and mouse rIL-6 was purchased from Genzyme Corp. (Cambridge, MA). Con A and staphylococcal enterotoxin B (SEB)¹ were purchased from Sigma Chemical Co. (St. Louis, MO). Heat-killed *Listeria* (HKLM) were prepared by incubating bacteria at 80°C for 1 h and were tested for lack of viability on brain heart infusion agar plates. Con A supernatant was generated from rat spleen cell cultures stimulated for 36 h with 5 μ g/ml Con A. Culture supernatants were harvested and residual Con A was removed with Sephadex G-25.

Cell Harvesting and Purification of Subpopulations. Peritoneal exudate cells (PEC) were obtained from *Listeria*-immune mice by peritoneal lavage with HBSS containing 0.06% BSA, 10 mM Hepes buffer, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 10 U/ml heparin. Macrophages were depleted by incubating PEC in tissue culture dishes for 1 h at 37°C. The plastic nonadherent cells were then passed over nylon wool columns using standard procedures (25) to further enrich for T cell populations. This nylon wool-nonadherent (NWN) population was 67.1% CD3⁺ (SD = 12.7, n = 19) with 76.5% (SD = 5.1) of these expressing the α/β TCR and 21.9% (SD = 4.5) expressing the γ/δ TCR, as determined by flow cytometry. The remaining cells were a mixture of NK and Ia⁺ cells. The NWN cells were either analyzed without further purification or were subjected to positive selection procedures for T cell subsets. To enrich for γ/δ T cells, the NWN cells were incubated with 20 μ g/10⁶ cells of affinity-purified UC7–13D5 anti-TCR γ/δ for 30 min on ice. After washing twice at 4°C, the antibody-coated cells were incubated 30 min at 4°C with im-

munomagnetic beads (Dynabeads M450; Dynal Inc., Greatneck, NY) coated with anti-mouse IgG at a ratio of three beads per cell. Cells bound to magnetic beads were gently separated from unbound cells with a magnet and incubated at 37°C overnight to allow cells and beads to dissociate. This γ/δ TCR positively selected population was >95% CD3⁺, with <2% of these cells expressing the α/β TCR (eight experiments). The population that did not bind to the magnetic beads after incubation with anti-TCR γ/δ was 50–67% CD3⁺, with >90% of the CD3⁺ cells expressing the α/β TCR. Since the portion of this residual population that did not express CD3 was composed primarily of Ia⁺ cells, cells in two experiments were incubated with anti-Ia mAbs (10–2.16 and 14–4–4S) for 3 min on ice followed by incubation with complement (Low-Tox-M rabbit complement; Cedarlane Laboratories, Westbury, NY) for 45 min at 37°C to further enrich for α/β T cells. The resulting populations were 87 and 83% CD3⁺, with 88 and 82% of the CD3⁺ cells expressing the α/β TCR.

Using an alternative approach, surface-activated MicroCELLector flasks (Applied Immune Sciences, Inc., Menlo Park, CA) were used in three additional experiments to purify the α/β T cells by positive selection. The immunomagnetic bead technique was unsuccessful for positive selection of α/β T cells because the bond between the anti-TCR mAb and the second antibody on the beads was not sufficiently strong to allow the beads to remain attached to the cells during the separation procedure. Affinity-purified H57–597 anti-TCR mAb was bound at 50 μ g/ml to MicroCELLector flasks according to the manufacturer's instructions. Non-specific binding of cells to antibody-coated flasks was minimized by blocking of the plastic surface with BSA and blocking of Fc receptors on cells by preincubation with whole serum. NWN populations that had been depleted of γ/δ T cells using immunomagnetic beads were then incubated on the antibody-coated flasks for 1 h at room temperature. Nonadherent cells were removed by gentle washing. The flasks with adherent cell populations were incubated overnight at 37°C to allow cells to detach. Populations positively selected in this manner were >95% CD3⁺ with no detectable contamination by either γ/δ T cells or by Ia⁺ cells. Residual anti-TCR mAb used for selection did not appear to remain bound to T cell subsets, as indicated by the fact that FITC-conjugated second antibody did not bind significantly to separated T cells.

Finally, each T cell subset was negatively selected. For negative selection of γ/δ T cells, α/β T cells were first depleted *in vivo* by intraperitoneal injection of 200 μ g of anti-TCR α/β mAb (H57–597) on day 7 after intraperitoneal injection of *Listeria*. 3 d later, PEC were harvested and enriched for CD3⁺ cells by plastic adherence followed by passage over a T Cell Enrichment Column (R & D Systems, Minneapolis, MN) to remove FcR- and sIg-positive cells. Any remaining α/β T cells were then removed using a MicroCELLector flask coated with anti-TCR α/β mAb. In one of three experiments, 50 μ l of anti-asialo-GM1 (Wako Pure Chemicals, Dallas, TX) was also injected intraperitoneally to deplete NK cells. The resulting population was 71% CD3⁺, with 99% of those cells expressing the γ/δ TCR when analyzed by flow cytometry. The CD3⁻ population expressed neither Ia antigens nor asialo-GM1, but showed weak expression of surface IgG and/or FcR. A similar strategy was used for negative selection of α/β T cells using anti- γ/δ TCR for depletions. The resulting population was 91% CD3⁺, with 95% of those cells expressing the α/β TCR.

No significant differences in functional activity were detectable

¹ Abbreviations used in this paper: HKLM, heat-killed *Listeria monocytogenes*; NWN, nylon wool nonadherent; PEC, peritoneal exudate cells; SEB, staphylococcal enterotoxin B.

within either the γ/δ or α/β T cell populations that were selected using these different techniques.

In Vitro Culture of Isolated Cells. Two types of culture systems were used: (a) cell expansion assays using NWNA populations without further purification such that T cell subsets remained mixed, and (b) proliferation assays using NWNA populations enriched for either α/β or γ/δ T cells as described above. In both assay systems the culture medium was RPMI 1640 supplemented with 10% FCS, 5×10^{-5} M 2-ME, 0.5 mM sodium pyruvate, 10 mM Hepes buffer, 50 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, and 2 mM L-glutamine.

Expansion assays were carried out at 37°C in 24-well plates using 10^6 NWNA cells/well in combination with 2.5×10^6 irradiated (3,000 rad) splenic accessory cells in a total volume of 2 ml. Reagents for in vitro restimulation of T cell subsets were added as indicated in the figure captions. After 4 d, surviving cells were harvested on Ficoll-Hypaque gradients (Lymphocyte Separation Medium; Organon Teknika Corp. Durham, NC), counted, and analyzed for TCR expression by flow cytometry. Determination of the absolute number of cells of a given TCR phenotype in a population was based on both the total cell number and the percentage of those cells expressing either the α/β or γ/δ TCR. The ratio of the number of cells of a TCR phenotype in the surviving population in an expansion culture system to the number of cells of that phenotype in the initial population was used as the expansion factor for that subset.

Proliferation assays with isolated populations of T cell subsets were carried out at 37°C in 96-well plates in a total volume of 200 μl with T cells at 10^4 /well unless otherwise indicated. Accessory cells were either irradiated (3,000 rad) spleen cells at 5×10^5 /well or adherent peritoneal macrophages that were obtained from PEC added at a predetermined optimal concentration of 2.5×10^4 /well and incubated for 2 h at 37°C. Nonadherent cells were then removed by washing three times with warm culture medium before addition of T cell populations. In some experiments macrophages were fixed with 0.05% glutaraldehyde for 2 min at room temperature washed extensively before use in the assay as previously described (26). Reagents for in vitro restimulation were added as indicated in figure legends. [^3H]Thymidine (1 $\mu\text{Ci}/\text{well}$) was added after 72 h of culture and cells were harvested and counted 18 h later using a Matrix 96 Direct Beta Counter (Packard Instrument Co., Inc., Downers Grove, IL).

Flow Cytometry. Cells ($1-5 \times 10^5$) were incubated on ice for 30 min with 25 μl of predetermined optimal concentrations of fluorochrome-conjugated mAb, washed twice with PBS containing 3% FCS and 0.1% sodium azide, and fixed with 1% paraformaldehyde in PBS. 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. Two-color analysis was used to analyze for CD3/TCR expression using the anti-CD3 mAb conjugated to PE and the anti-TCR mAb conjugated to FITC. Binding of isotype-matched control Ig was used to determine background levels of immunofluorescence.

For cell cycle analysis of T cell subsets, NWNA were first incubated with FITC-conjugated anti-TCR mAb, then fixed overnight at 4°C with absolute methanol to permeabilize the cells before the addition of 50 $\mu\text{g}/\text{ml}$ propidium iodide (Calbiochem-Behring Corp., La Jolla, CA) and 100 U/ml RNase A (Sigma Chemical Co., St. Louis, MO) in PBS (25). Populations were analyzed on a FACScan® by first electronically selecting the FITC-positive population, then analyzing the propidium iodide uptake in those cells. Cell cycle statistics were derived using the RFIT model in the CELLFIT software package (Becton Dickinson & Co.).

Results

In Vitro Expansion of Peritoneal γ/δ T Cells Is Supported by α/β T Cells. Using NWNA PEC isolated from *Listeria*-immune mice, we have reported a preferential expansion of γ/δ T cells relative to α/β T cells upon restimulation in vitro with either Con A or *Listeria* antigen preparations (27). These observations were extended in the current study by examining the effects of additional agents for in vitro restimulation on the expansion of T cell subsets. In the representative experiment shown in Fig. 1 A, NWNA PEC from *Listeria*-immune mice were incubated in an expansion assay system as described in Materials and Methods. Surviving cells were examined on day 4 for CD3/TCR expression. As in previous experiments (27), the γ/δ T cells expanded more than threefold in response to either HKLM or Con A, while α/β T cells expanded less than twofold in response to these stimuli. In four replicate experiments, the average expansion factor for γ/δ T cells was 3.2 (SD = 2.2) for restimulation by Con A and 2.8 (SD = 0.9) for restimulation by HKLM, while the expansion factor for α/β T cells was 0.8 (SD = 0.7) for Con A and 1.5 (SD = 0.3) for HKLM restimulation. To our surprise, however, γ/δ T cells also predominated in the population generated in response to immobilized anti-TCR α/β mAb, expanding four- to fivefold during the 4-d culture period while the α/β T cells remained at input levels (Fig. 1 A). If immobilized anti-TCR γ/δ was used, however, only the γ/δ T cells expanded as anticipated while the α/β T cells did not even persist throughout the culture period. The superantigen SEB, which stimulates specifically the subset of α/β T cells expressing the V β 8 TCR (28), also resulted in the expansion of γ/δ T cells. Taken together, these results suggest that the γ/δ T cells were stimulated indirectly via activated α/β T cells either through a cell-cell interaction between the two T cell subsets or through the release of cytokines that support the expansion of the γ/δ T cells.

To confirm that both T cell subsets were actually dividing in these expansion assay cultures, cells harvested on day 4 after stimulation with HKLM were subjected to cell cycle analysis by flow cytometry using propidium iodide uptake in conjunction with cell surface TCR expression. In the α/β T cell population 54% of the cells were in the G₁ phase, 33% were in S, and 13% were in G₂ + M; while in the γ/δ T cell population 62% were in G₁, 29% were in S, and 9% were in G₂ + M. Both T cell subsets were also dividing in response to stimulation with Con A (data not shown).

To explore the potential interaction between α/β and γ/δ T cells, we positively selected γ/δ T cells from the NWNA population isolated from *Listeria*-immune mice using immunomagnetic beads in conjunction with mAb specific for the γ/δ TCR and directly evaluated the proliferative responses of isolated T cell subsets to in vitro stimuli by uptake of [^3H]thymidine. Populations highly enriched for either α/β or γ/δ T cells showed receptor-specific responses to immobilized anti-TCR mAb as anticipated (Fig. 1 B). Under these conditions, γ/δ T cells did not proliferate in response to immobilized anti- α/β TCR, suggesting that the expansion seen when the subsets were cocultured, as in Fig. 1 A, was medi-

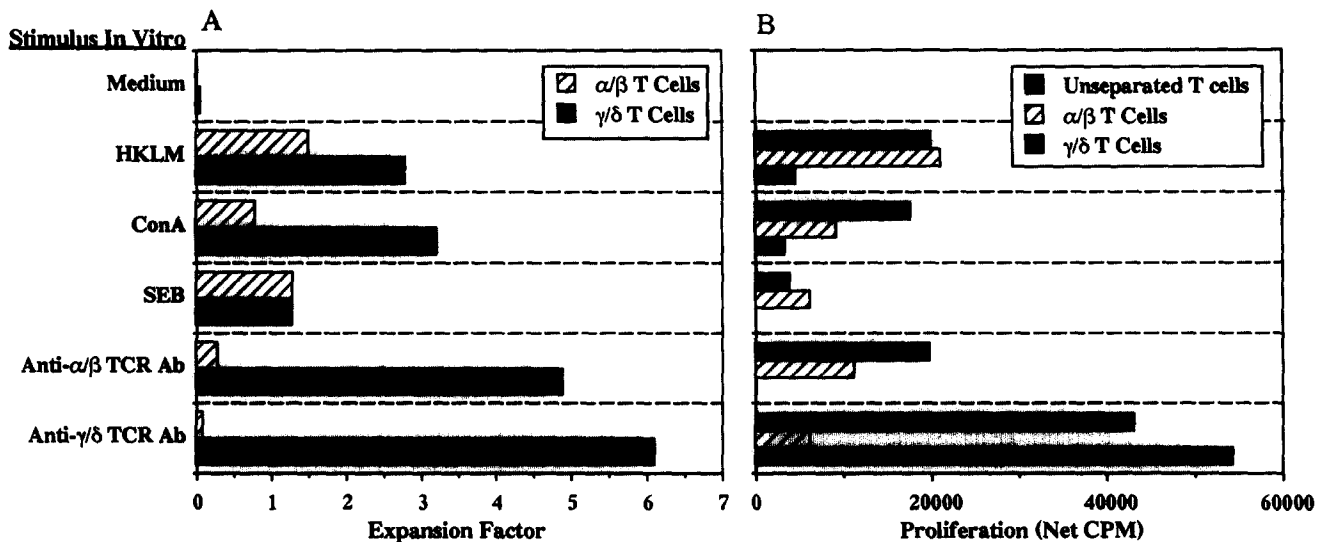


Figure 1. Responses of PEC from *Listeria*-immune mice to in vitro restimulation. (A) NWN PEC were incubated without further purification in 24-well plates with the indicated stimuli. Syngeneic irradiated spleen cells (2.5×10^6 /well) served as accessory cells. Cells were harvested on day 4, counted, and evaluated for TCR expression by flow cytometry. Expansion factors for each T cell subset were calculated based on the number of cells of each phenotype in the starting population as compared with the cells harvested on day 4. (B) NWN cells were further separated into T cell subsets using anti- γ/δ TCR mAb and immunomagnetic beads so that the response of each subset could be evaluated directly by uptake of [3 H]thymidine in a proliferation assay. Unseparated NWN cells, reserved from the initial population, were also evaluated for comparison. T cells were used at 10^4 /well. Peritoneal macrophages (2.5×10^4 /well) served as accessory cells. In both assays HKLM was used at 10^7 /ml, Con A at $2 \mu\text{g}/\text{ml}$, and SEB at $10 \mu\text{g}/\text{ml}$. Affinity-purified anti-TCR mAbs were immobilized on the plastic surface of the assay wells by incubating 120 or $20 \mu\text{g}$ of mAb per well, respectively, for a 24- or 96-well plate for 1–3 h at 37°C . Antibody was removed and wells were washed twice before addition of cells. Results are representative of a series of three to five experiments.

ated via the α/β T cell population. Similarly, no detectable proliferative response to the α/β T cell-specific superantigen SEB was seen by purified γ/δ T cells in the absence of α/β T cells (Fig. 1 B). When either Con A or *Listeria* antigen preparations were used as the in vitro stimulant, however, the γ/δ T cells proliferated even when separated from the α/β T cells (Fig. 1 B), suggesting that at least a portion of the response to HKLM or Con A is a direct response by the γ/δ T cells. The proliferative response of the isolated γ/δ T cells to these stimuli is lower than that of the isolated α/β T cells. If the two T cell subsets are cocultured in the expansion assay system, however, the γ/δ T cells expand more than the α/β T cells do in response to Con A or HKLM (Fig. 1 A).

To address the implied interaction between α/β and γ/δ T cells, we carried out a series of cell-mixing experiments in which immunomagnetically enriched T cell subsets from *Listeria*-immune mice were recombined in known amounts. The response to heat-killed *Listeria* in the presence of macrophage accessory cells was determined for each T cell subset either alone or with the addition of known numbers of cells of the opposite subset. If varying numbers of either subset alone were used, the proliferative response was directly proportional to the number of cells used (Fig. 2 A, open symbols). However, if the two subsets were remixed by adding 3,000 cells of the opposite TCR type to each culture well, the increased response was far greater than additive (Fig. 2 A, filled symbols); e.g., the addition of 3,000 α/β T cells to 3,000 γ/δ T cells resulted in a >10-fold increase in the proliferative response. In an additional series of experiments, we com-

pared the effect of adding increasing numbers of either untreated or irradiated (3,000 rad) T cells to a fixed number of untreated α/β or γ/δ T cells. Addition of irradiated cells ensured that this population did not contribute to the proliferative response. Addition of irradiated α/β T cells to untreated γ/δ T cells resulted in dose-related increases in the proliferative response of the γ/δ T cell population (Fig. 2 B). Addition of as few as 1,000 irradiated α/β T cells to 10,000 γ/δ T cells resulted in a 3.6-fold increase in the proliferative response of the γ/δ T cells. In contrast, addition of irradiated γ/δ T cells to responding α/β T cells had little effect on the response (Fig. 2 C). Addition of graded numbers of nonirradiated T cells (Fig. 2, B and C) again resulted in a greater than additive effect of cell mixing as seen in A. Representative results shown in Fig. 2 were confirmed in a series of four experiments.

The Stimulatory Influence of α/β T Cells on Peritoneal γ/δ T Cells Was Mediated by Cytokines. We then wanted to ask whether this observed synergy required cell–cell contact or was mediated by soluble factors. In an initial experiment, unseparated NWN cells were incubated in a Transwell chamber (Costar Corp.) in which cell populations were separated by a membrane that prohibits movement of cells from one chamber to the other but allows the free mixing of molecules between compartments. To provide a specific stimulus to the α/β T cells only, anti-TCR α/β mAb was immobilized on the plastic in the lower chamber only. NWN cells were then added to both chambers along with irradiated splenic accessory cells. After 4 d the populations from each chamber

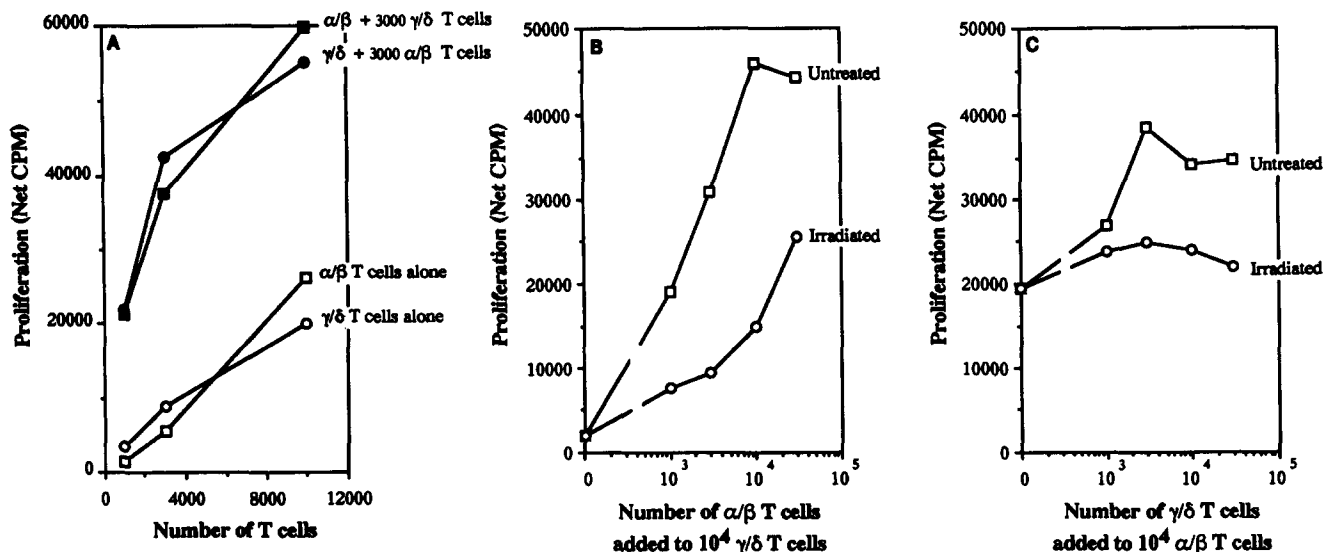


Figure 2. Cell mixing experiments. Peritoneal T cell subsets were enriched from NWA PEC from *Listeria*-immune mice using anti- γ/δ TCR mAb and immunomagnetic beads, and then recombined in known quantities. HKLM at 10^7 /ml was used in conjunction with 2.5×10^4 /well syngeneic peritoneal macrophage accessory cells induced by intraperitoneal injection of 100 μ g of Con A 3 d before cell harvest. Responses were measured as [3 H]thymidine uptake in a proliferation assay. (A) Purified cells were remixed without further treatment. (B and C) T cells were either untreated or were subjected to 3,000 rad of γ irradiation before addition to the assay. No uptake of [3 H]thymidine was detectable by irradiated T cell populations incubated with accessory cells and HKLM (not shown).

were harvested and evaluated for TCR expression by flow cytometry. γ/δ T cells expanded equally well in both chambers (data not shown), suggesting that cell-cell contact between the two subsets was unnecessary.

These results led to a series of experiments designed to determine the cytokines that might be involved in this synergy. Preliminary experiments indicated that a supernatant preparation from rat spleen cells stimulated with Con A, which is known to contain several cytokines, stimulated the in vitro

expansion of γ/δ T cells. Peritoneal α/β and γ/δ T cells from *Listeria*-immune mice were separated using immunomagnetic beads then incubated either with or without peritoneal macrophages as accessory cells. Proliferative activity was measured in response to recombinant preparations of cytokines known to be present in Con A supernatants (IL-2 and IFN- γ) or reported to affect the growth of T cell subsets (IL-7). Results in Fig. 3 show that rIL-7 had a profound effect on γ/δ T cell growth while minimally stimulating the α/β T cells.

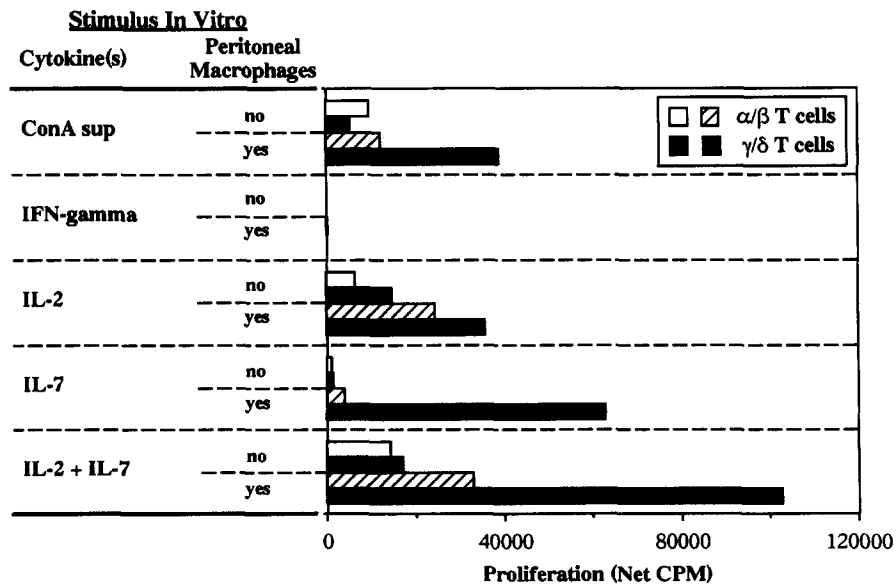


Figure 3. Responses of peritoneal T cell subsets from *Listeria*-immune mice to cytokines with or without peritoneal macrophage accessory cells. T cell subsets were separated using anti- γ/δ TCR mAb and immunomagnetic beads, and then evaluated in a proliferation assay using 10^4 T cells/well. Either no accessory cells were added or peritoneal macrophages that had been induced by intraperitoneal injection of 100 μ g of Con A 3 d before harvest were added at 2.5×10^4 /well. Cytokine concentrations were as follows: 25% Con A sup; 10,000 U/ml rIFN- γ ; 100 U/ml rIL-2; and 2,000 U/ml rIL-7.

Accessory cells were required, however, suggesting an interaction either with another cytokine or directly with an accessory cell type. rIL-2 served as a significant stimulus for both T cell subsets even in the absence of accessory cells, although the addition of macrophages enhanced the responses of both cell types (Fig. 3). The effect of adding both rIL-2 and rIL-7 appeared to be additive for both subsets. rIFN- γ was unable to stimulate either α/β or γ/δ T cells with or without accessory cells.

To explore the requirement for accessory cells for the response to IL-7, isolated peritoneal α/β and γ/δ T cells were incubated with a variety of potential accessory cell sources. The ability of these cells to support a T cell response to IL-7, and also to immobilized anti-TCR mAb and to HKLM for comparison, was assessed in a proliferation assay (Fig. 4). Resident macrophages or macrophages elicited by intraperitoneal injection of either Con A (100 μ g 3 d before harvest) or *Listeria* (10 d before harvest) were equally effective in supporting the response of γ/δ T cells to rIL-7 (Fig. 4). Light fixation with glutaraldehyde did not reduce the ability of macrophages to act as accessory cells for the γ/δ T cell response to rIL-7, although this treatment did abrogate the response of both T cell subsets to HKLM as anticipated. Use of H-2-incompatible macrophages had no effect on the γ/δ T cell response to rIL-7, but abrogated the α/β T cell response to HKLM as expected. Although the γ/δ T cell response to HKLM was significantly reduced (by 53% in Fig. 4 and by 66% in a second experiment) when allogeneic rather than syngeneic Con A-induced macrophages served as accessory cells, the response was still well above background. Irradiated spleen cells were also able to support the response by γ/δ T cells to rIL-7, although to a lesser degree than macrophages (Fig. 4). Interestingly, the irradiated spleen cells only minimally supported the response of γ/δ T cells to HKLM even though they were competent as accessory cells for the α/β T cells.

The response by both T cell subsets to the appropriate immobilized anti-TCR mAb was supported by all of the accessory cell populations tested, although the α/β T cells seemed more sensitive to variations in accessory cell populations. The minimal response to rIL-7 by α/β T cells under all conditions examined underscores the unique ability of the peritoneal γ/δ T cells to respond to this cytokine. The observed differences in requirements for accessory cells suggest that α/β and γ/δ T cells may differ with respect to their activation requirements.

IL-1 and IL-7 Act in Synergy to Stimulate the Peritoneal γ/δ T Cell Population without Significantly Affecting the α/β T Cells. The requirement for an accessory cell to support the response of γ/δ T cells to rIL-7 suggests the need for either a cell-cell interaction or for an accessory cell-derived cytokine that can act in synergy with IL-7. In preliminary experiments supernatants from macrophages incubated 24 h with rIL-7 were able to drive the in vitro expansion of γ/δ T cells (data not shown), implying a cytokine interaction mechanism. In subsequent experiments we used recombinant preparations of known macrophage cytokines to search for the macrophage-derived soluble factor that synergizes with rIL-7. Peritoneal α/β and γ/δ T cells from *Listeria*-immune mice were isolated using immunomagnetic beads, then incubated in the absence of accessory cells with combinations of recombinant cytokines (Fig. 5 A). rIL-1 induced a significant proliferative response in γ/δ T cells when combined with rIL-7. Minimal response was seen with α/β T cells (Fig. 5 A), even though this subset was able to be stimulated with immobilized anti-TCR (net cpm = 8,013), confirming viability and a normal potential to respond to an in vitro stimulus. Neither rIL-6 nor rTNF- α , which are also known to be produced by macrophages, were able to synergize with rIL-7. rIL-1 alone had no effect on either T cell subset but when added in increasing amounts with a constant amount of

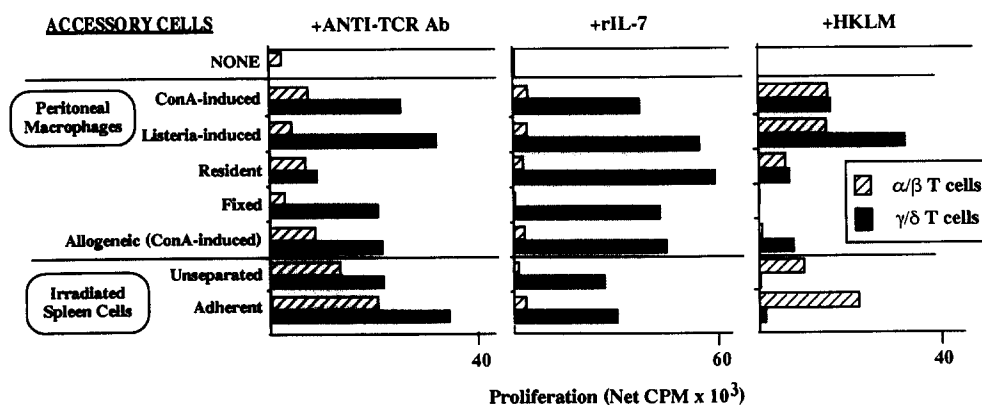


Figure 4. Proliferative responses of peritoneal T cell subsets from *Listeria*-immune mice to immobilized anti-TCR mAb, rIL-7, or HKLM in the presence of accessory cells from various sources. T cells were separated using anti- γ/δ TCR mAb and immunomagnetic beads to positively select γ/δ T cells. Residual cells were further treated with anti-Ia mAb and complement to deplete potential Ia⁺ accessory cells. Isolated T cell subsets were then evaluated in a proliferation assay using 10⁴ T cells/well in combination with the indicated accessory cells. Macrophages were adherent

cells from a PEC population and were added at 2.5×10^4 /well. Macrophage accessory cells were either obtained from normal mice (*Resident*) or were induced in vivo by intraperitoneal injection of either 100 μ g of Con A 3 d before harvest or of 6×10^3 live *Listeria* 10–11 d before harvest. Fixed macrophages were prepared by glutaraldehyde treatment of Con A-induced syngeneic macrophages. Allogeneic macrophages were from female BALB/C mice. Spleen cells were from syngeneic mice and were irradiated (3,000 rad) before the assay. Unseparated spleen cells were added at 5×10^5 /well. For adherent populations, spleen cells were incubated at 1.7×10^6 /well at 37°C for 2 h. Nonadherent cells were removed by vigorous washing with warm culture media. Anti-TCR mAbs were immobilized on plastic as described in Fig. 1. rIL-7 was present at 2,000 U/ml and HKLM at 10⁷/ml.

rIL-7 showed a quantitative effect on the γ/δ T cells (Fig. 5 B), while α/β T cells remained unaffected even at the higher concentrations of rIL-1.

We were concerned that the unique ability of γ/δ T cells to respond to rIL-7 in combination with either accessory cells or rIL-1 was at least in part due to stimulation via the TCR during the cell isolation process. The γ/δ T cells in these experiments were preincubated with anti-TCR mAb, then positively selected using immunomagnetic beads coupled to an appropriate second antibody. The α/β T cells were not exposed to an anti-TCR mAb during the isolation procedure because the weak binding of the anti-TCR α/β mAb to the second antibody did not allow for an efficient separation of cells with this technique. The recent availability of surface-activated MicroCELLector flasks, however, provided a means to bind the anti-TCR with sufficient strength and magnitude to a plastic surface to allow the separation of α/β T cells by positive selection. In three experiments (Table 1) the differential response by γ/δ T cells to rIL-7 was still observed even though both T cell subsets were positively selected using the appropriate anti-TCR mAb. As in previous experiments, the response to heat-killed *Listeria* and macrophages was similar for the two types of T cells, confirming that both populations were capable of an in vitro proliferative response.

In a second approach to verify that this differential response of γ/δ T cells to rIL-7 was not simply due to artifacts of the cell separation process, unseparated Nwana PEC from

Table 1. Proliferative Responses of Positively-selected Peritoneal γ/δ and α/β T Cells to Recombinant Cytokines and HKLM

In vitro stimulus	Exp.	γ/δ T cells	α/β T cells
Macrophages + media	1	35	70
	2	36	20
	3	123	100
Macrophages + rIL-7	1	40,507	4,703
	2	35,383	1,337
	3	54,542	2,154
rIL-7 + rIL-1	1	30,253	2,988
	2	NT	NT
	3	NT	NT
Macrophages + HKLM	1	3,791	2,224
	2	1,357	1,746
	3	1,245	2,385

Data are expressed as mean cpm from triplicate wells of a proliferation assay. Peritoneal T cell subsets from *Listeria*-immune mice were positively selected from Nwana populations using immunomagnetic beads for γ/δ T cells and MicroCELLector flasks for α/β T cells (Exps. 1 and 2) or using MicroCELLector flasks for both subsets (Exp. 3). Cells and recombinant cytokines are at concentrations used in Fig. 3. HKLM were added at a concentration of 10^7 /ml.

Listeria-immune mice were incubated with recombinant cytokines in an expansion assay system. Nwana were depleted of residual accessory cells by treatment with anti-Ia mAb followed by immunomagnetic beads coated with the appropriate second antibody. The resulting population was 92% CD3⁺ with no Ia⁺ cells detectable by flow cytometry. These cells were then incubated with cytokines either in the absence of accessory cells or with the addition of *Listeria*-induced peritoneal macrophages. Expansion factors were calculated as before based on the number and TCR phenotype of cells harvested on day 4. Data shown in Fig. 6 confirm that γ/δ T cells expand to a much greater extent than α/β T cells in the presence of rIL-7 even when the two T cell subsets are cocul-

Table 2. Proliferative Responses of *Listeria*-elicited Peritoneal γ/δ and α/β T Cells: Antigen Specificity and Genetic Restriction

In vitro stimulus	Exp.	γ/δ T cells	α/β T cells
Syngeneic macrophages + media	1	271	220
	2	1,172	317
	3	35	70
Syngeneic macrophages + HKLM	1	9,771	24,385
	2	18,052	16,478
	3	3,791	2,224
Syngeneic macrophages + HKEC or ST	1	NT	NT
	2	5,780	760
	3	1,293	166
Allogeneic macrophages + media	1	81	105
	2	129	142
	3	76	45
Allogeneic macrophages + HKLM	1	3,638	128
	2	8,420	826
	3	2,921	154
Allogeneic macrophages + HKEC or ST	1	NT	NT
	2	NT	NT
	3	566	108

Peritoneal γ/δ T cells from *Listeria*-immune mice were positively selected from Nwana populations using the anti-TCR γ/δ mAb UC7 and immunomagnetic beads. In Exp. 1 the α/β T cell population consisted of the Nwana cells remaining after positive selection of γ/δ T cells. In Exp. 2, the α/β T cell population was further enriched by treatment with anti-Ia mAb and complement. In the third experiment the α/β T cell population was positively selected on MicroCELLector flasks coated with mAb to the α/β TCR. Responses were evaluated in a proliferation assay using T cells at 10^4 /well and peritoneal macrophages at 2.5×10^4 /well. HKLM, heat-killed *E. coli* (HKEC), and heat-killed *S. typhimurium* (HKST) were used at 10^7 /ml, HKEC were used in Exp. 2 and HKST were used in Exp. 3. Syngeneic macrophages were from C3HeB/FeJ mice and allogeneic macrophages were from BALB/c mice, Con A ($100 \mu\text{g}/\text{mouse}$) was used to elicit peritoneal macrophages.

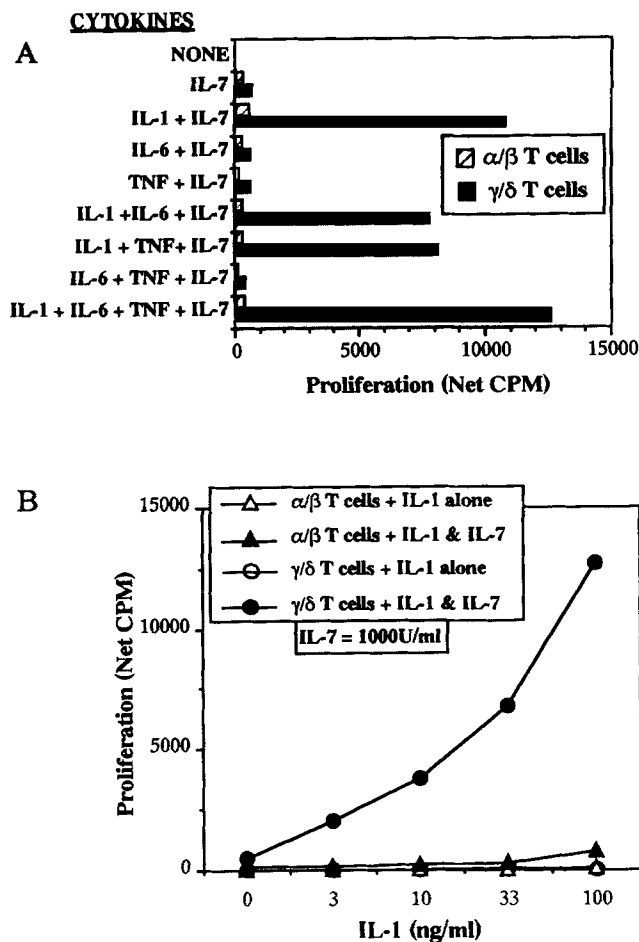


Figure 5. Effects of recombinant cytokines on the proliferative capacity of peritoneal T cell subsets from *Listeria*-immune mice. T cells were separated using anti- γ/δ TCR mAb and immunomagnetic beads to positively select γ/δ T cells, and were used at 10^4 /well. No accessory cells were added. (B) The population that did not bind to the beads was further enriched for α/β T cells by treatment with anti-Ia mAb and complement. (A) Cytokine concentrations were as follows: 2000 U/ml rIL-7; 10 ng/ml rIL-1 β ; 1,000 U/ml rIL-6; and 1,000 U/ml rTNF α . (B) 1,000 U/ml of rIL-7 was used in combination with the indicated amounts of rIL-1.

tured and neither subset has been subjected to positive selection procedures.

Finally, both T cell subsets were negatively selected in three separate experiments using a combination of in vivo and in vitro depletion techniques to avoid crosslinking of either TCR during the purification process. Purity of cell populations was monitored by flow cytometry and responses were monitored in a proliferation assay. Negatively selected γ/δ T cells showed the same response pattern to rIL-7, HKLM, and immobilized anti-TCR mAb as did positively selected cells in previous experiments (Table 3). The stimulation index for the response to rIL-7 was at least fourfold higher for the γ/δ T cell population than for the α/β T cells (not shown).

Table 3. Proliferative Responses of Negatively Selected Peritoneal γ/δ T Cells

In vitro stimulus	Proliferation
Medium	302
HKLM	7,548
IL-7	20,843
Immobilized anti- γ/δ TCR mAb	25,093
Immobilized anti- α/β TCR mAb	2,204

Peritoneal γ/δ T cells were negatively selected from *Listeria*-immune mice by in vivo injection of anti- α/β TCR mAb (200 μ g i.p.) and anti-asialo-GM1 (50 μ l) on day 7 after *Listeria* injection. 3 d later, PEC were harvested and enriched for CD3 $^+$ cells using a T cell enrichment column (R&D Systems). Remaining α/β T cells were then removed using a MicroCELLector flask coated with anti- α/β TCR mAb. 99% of the CD3 $^+$ population expressed the γ/δ TCR when analyzed by flow cytometry. T cells, Con A-elicited macrophage accessory cells, and rIL-7 were at concentrations used in Fig. 3. HKLM were added at 10^7 /ml, immobilized mAb were added at 20 μ g/well.

Stimulus In Vitro

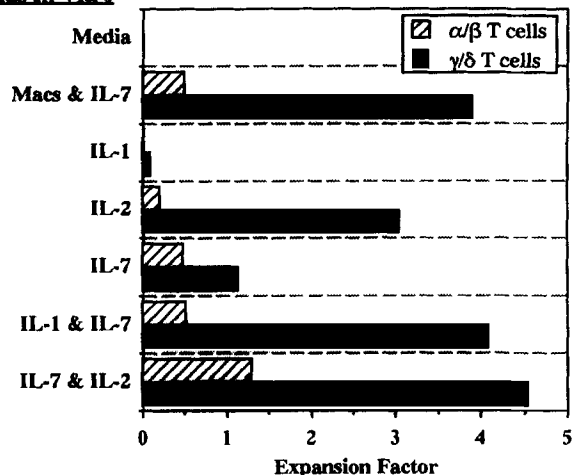


Figure 6. Responses of the unseparated peritoneal NWA cells from *Listeria*-immune mice to recombinant cytokines. The NWA population was further treated with anti-Ia mAb and immunomagnetic beads to eliminate potential accessory cells. The resulting population was 92% CD3 $^+$ with no detectable Ia $^+$ cells remaining. Cells were incubated at 10^6 /well in 24-well plates with recombinant cytokines used at the following concentrations: 2,000 U/ml of rIL-7; 100 ng/ml of rIL-1; and 100 U/ml of rIL-2. Peritoneal macrophages from *Listeria*-immune mice were used as accessory cells (1.2×10^5 /well) only where indicated (Macs & IL-7). Cells were harvested on day 4 and expansion factors were calculated as described in Fig. 1.

Thus, the enhanced ability of peritoneal γ/δ T cells to expand in the presence of IL-7 has been confirmed under three separate experimental protocols: (a) unseparated T cell subsets, (b) positively selected T cell subsets, and (c) negatively selected T cell subsets.

Discussion

Our observations (27) on the preferential expansion of γ/δ T cells upon culture of *Listeria*-induced peritoneal T cells with mitogens or antigens led us to examine the activation requirements and functional interactions between α/β and γ/δ T cells. By analyzing either mixtures of α/β and γ/δ T cells or isolated subsets, it became clear that much of the proliferative capacity of γ/δ T cells was dependent upon activated α/β T cells. This conclusion was most directly supported by the finding that stimuli that specifically acted upon the α/β T cell population, such as immobilized anti- α/β TCR antibody and the V_{β} -specific superantigen SEB, resulted in the expansion of γ/δ T cells when the two subsets were cocultured. Additional support for this dependency of γ/δ T cells on signals from α/β T cells came from the finding that a greater than additive proliferative response was observed to the antigen HKLM when the two types of T cells were mixed in a proliferation assay. This functional α/β - γ/δ T cell interaction was unidirectional in that irradiated α/β T cells promoted the proliferation of γ/δ T cells, while irradiated γ/δ T cells had little or no effect on the α/β T cell proliferation. Thus, γ/δ T cells appear to be uniquely poised to receive activation/expansion signals from the α/β T cell subset.

The dependency of γ/δ T cells on α/β T cells has several implications. First, since very small numbers of irradiated α/β T cells (e.g., Fig. 2) can drive the proliferation of γ/δ T cells in vitro, considerable caution is needed when ascribing an antigen-specific proliferative response to the γ/δ T cell subset. This is especially important when irradiated spleen cells are used as APC. In this regard, previous conclusions regarding the response of *Listeria*-induced γ/δ T cells to heat-shock proteins (HSP) should be reevaluated. In contrast to previous reports (4, 14, 29, 30), we have been unable to demonstrate a specific proliferative response of purified γ/δ T cells to HSP65 or purified protein derivative (PPD). Second, the interaction of α/β and γ/δ T cells may account for previous observations on the appearance of T cells at inflammatory sites. Doherty et al. (31) noted that influenza virus caused the influx of α/β T cells into the lung, which was followed by the appearance of γ/δ TCR-expressing cells. In this model, elimination of α/β T cells with antibody abrogated the increase of the γ/δ T cells. In our kinetic analysis of the accumulation of α/β and γ/δ T cells in the peritoneal cavity in response to LPS or *Listeria*, we have consistently noted that both subsets are found in increased numbers at similar times after injection of the inducing agents (27). This coordinate control is consistent with an α/β - γ/δ T cell interaction. Finally, the dependence of γ/δ T cells on α/β T cells may be interpreted in light of the events that may occur during defensive reactions at epithelial surfaces such as the intestinal epithelium. One could speculate that the γ/δ T cells present in the epithelium do not proliferate unless the microbial infection breaches this area to cause activation of underlying α/β T cells. Such a mechanism may provide a perhaps necessary calm in the epithelial tissue where foreign antigen concentrations may be high and an ongoing inflammatory response would be more damaging than protective.

The proliferation of γ/δ T cells resulting from activation of α/β T cells appeared to be mediated by cytokines. This conclusion was supported by experiments using Transwell plates that indicated that cell-cell contact was not required for the observed stimulation of γ/δ T cells via the α/β T cell population, and by the finding that crude mixtures of cytokines obtained by Con A stimulation of spleen cells (primarily α/β T cells) could cause proliferation of γ/δ T cells. The dependency of γ/δ T cells on α/β T cells was also consistent with the apparent lack of IL-2 production by γ/δ T cells. We have been unable to detect IL-2 activity in the supernatants of T cells stimulated with immobilized anti- γ/δ TCR antibody. To gain insight into the α/β - γ/δ T cell interaction and to define the activation requirements of these subsets, we tested several known cytokines for proliferative activity.

While both α/β and γ/δ T cells were stimulated efficiently by rIL-2, rIL-7 revealed a striking dichotomy between the two subsets. Isolated γ/δ T cells showed a strong proliferative response to this cytokine, while α/β T cells were minimally stimulated. The γ/δ T cell response was 10–50-fold greater than the α/β T cell response and did not appear to be related to artifacts of the cell separation protocols used for these experiments. While isolated γ/δ T cells showed a small but consistent and dose-related response to rIL-7, the response was greatly enhanced by the addition of macrophage accessory cells. Subsequent experiments clearly showed that γ/δ T cell proliferation could result from the synergistic effects of IL-7 and IL-1.

The role of IL-7 in the proliferation and differentiation of γ/δ T cells has been noted previously. IL-7 supports the growth of γ/δ T cells from fetal thymocytes (32–34), and IL-7 induces TCR γ gene expression in fetal liver cultures (35). Lynch and Shevach (36) found that IL-7 together with IL-1 and IL-2 can cause expansion of γ/δ T cells from newborn thymic cells. Collectively, these findings suggest that IL-7 is intimately involved in the function of γ/δ T cells. Our results presented herein extend these findings in several significant ways. First, we have supplied evidence that IL-1 and IL-7 induce proliferation with highly purified populations of γ/δ T cells. Second, we have directly compared the activation requirements of both α/β and γ/δ T cells and noted the distinctive behavior of γ/δ T cells. Third, unlike previous studies on thymocyte populations, our studies establish that the proliferative capacity of apparently mature γ/δ T cells is uniquely controlled by IL-1 and IL-7.

The synergistic effects of IL-1 and IL-7 may be a property of γ/δ T cells present in different tissues and activation states. In this regard, we have found that proliferation of intestinal γ/δ T cells can also be influenced by IL-1 and IL-7 (K. M. Pearce, M. J. Skeen, and H. K. Ziegler, manuscript in preparation), as can peritoneal γ/δ T cells isolated from normal, unimmunized mice (data not shown). Since IL-1 and IL-7 can expand the numbers of γ/δ T cells even in minimally manipulated, unseparated populations of peritoneal T cells (Fig. 6), and in negatively selected γ/δ T cell populations,

it appears that TCR engagement is not needed as a preparatory signal for cytokine responses. The strength of this conclusion, of course, is limited by the fact that the receptor specificity of γ/δ T cells remains unclear.

The basis for the selective effects of IL-7 on γ/δ T cells is being explored in ongoing studies. One simple explanation is that γ/δ T cells have appropriate cytokine receptors while α/β T cells do not. In preliminary studies, double labeling with fluorochrome-conjugated IL-7 and antibodies to the α/β and γ/δ TCR indicates that γ/δ T cells have a higher expression of IL-7 receptors as compared with α/β T cells (D. W. Heinrich, M. J. Skeen, and H. K. Ziegler, unpublished observations).

The synergistic and selective effects of IL-1 and IL-7 on γ/δ T cells may be relevant to one of the most intriguing aspects of the biology of γ/δ T cells: their localization in epithelial tissue. One can speculate that epithelial tissue represents an environment with selective expression of IL-1 and IL-7. It then becomes important to define the cells that make IL-7 and the regulation of IL-7 gene expression. While surprisingly little is known, it is clear that IL-7 is expressed by poorly defined bone marrow stromal cells (37) and thymic epithelial cells (37, 38). With respect to IL-1 production, it is clear that IL-1 can be made by macrophages associated with epithelial tissue and epithelial cells themselves (38). The association of IL-1, IL-7, and γ/δ T cells with epithelial cells gives rise to the fascinating possibility that IL-7 may account for the special tissue distribution of γ/δ T cells and perhaps even to the localization of γ/δ T cells at sites of infection. The fact that both in vivo depletion of γ/δ T cells (27, 30) and inhibition of IL-1 receptor activity (39, 40) exacerbate murine listeriosis suggests that the recruitment of γ/δ T cells to sites of infection plays a significant role in resistance to infection.

A response to HKLM by *Listeria*-induced peritoneal γ/δ T cells was repeatedly observed throughout this series of experiments. At issue is whether this is a TCR-mediated response or a response to cytokines. Unlike the response to IL-7, the response to HKLM was partially dependent on the

presence of H-2-compatible macrophage accessory cells and was not supported by irradiated splenic accessory cells nor by fixed macrophages. The activation of γ/δ T cells by H-2-incompatible cells may be explained by the recognition of conserved class I presenting molecules and is compatible with previous observations (41, 42). Heat-killed organisms from other bacterial strains can also stimulate peritoneal γ/δ T cells from *Listeria*-immune mice, although to a lesser extent than HKLM (Table 2). This could indicate that crossreactive antigens exist in the heat-killed bacterial preparations. Heat shock proteins have been proposed as relevant antigens for γ/δ T cells from several sites (4, 11), including peritoneal cells from *Listeria*-immune mice (30). This family of proteins, which exhibits considerable structural homology, could provide the basis for crossreactivity among bacterial strains. However, we have repeatedly examined the response to PPD or to the 180–196 peptide from HSP-60, which has been shown to be the antigenic epitope for γ/δ T cells from other sources (43, 44), and we have seen no evidence of a response even when the number of purified γ/δ T cells was increased 10-fold (data not shown). In contrast to a TCR-mediated event, it is possible that at least a portion of the proliferative response of isolated γ/δ T cells to HKLM is due to cytokines. Since γ/δ T cells seem to be poised to receive cytokine signals from other cell types, the possibility exists that the ingestion of heat-killed bacteria by macrophages generates sufficient quantities of appropriate cytokines to stimulate the γ/δ T cells to the extent observed in these experiments. Clearly, more information on the activation requirements of γ/δ T cells with respect to the cytokines, presenting molecules, and possible epitopes is needed to determine if the γ/δ T cell response to heat-killed bacterial preparations is a receptor-mediated event.

In summary, the definition of the intercellular interactions and the relevant cytokines involved in γ/δ T cell function provides a rational basis on which to pursue studies to clarify the functional significance of γ/δ T cells in immune regulation and front-line defense to infection at epithelial surfaces.

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