INFECTION AND IMMUNITY, June 1995, p. 2288–2294 Vol. 63, No. 6
0019-9567/95/$04.00+0
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Human T-Cell Recognition of *Listeria monocytogenes*: Recognition of Listeriolysin O by TcR$\alpha\beta^+$ and TcR$\gamma\delta^+$ T Cells

YING GUO,[1] H. KIRK ZIEGLER,[2] SUSAN A. SAFLEY,[2] DAVID W. NIESEL,[1]
SMITA VAIDYA,[3] AND GARY R. KLIMPEL[1]*

Departments of Microbiology and Immunology[1] and Pathology,[3] University of Texas Medical Branch, Galveston, Texas 77555-1019, and Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322[3]

Received 14 December 1994/Returned for modification 2 March 1995/Accepted 22 March 1995

The cell-mediated immune response to *Listeria monocytogenes* has been well characterized in the mouse. Listeriolysin O (LLO) is a major antigen in murine T-cell recognition of *L. monocytogenes*. In this study, we show that LLO is also recognized by human TcR$\alpha\beta$ T cells and TcR$\gamma\delta$ T cells. Human peripheral blood mononuclear cells (PBMC) cultured in vitro with live listeriae and then expanded with interleukin 2 were shown to respond to purified LLO. The generation of LLO-responsive T cells was dependent on the use of live bacteria during the initial in vitro challenge. LLO-induced proliferation of T cells expanded by exposure of PBMC to live listeriae was major histocompatibility complex restricted. PBMC cultured with formalin-fixed listeriae and subsequently expanded by interleukin 2 gave high proliferative responses to fixed bacteria but failed to respond to LLO. PBMC stimulated in vitro with fixed listeriae contained predominantly TcR$\alpha\beta^+$ T cells. In contrast, PBMC obtained from 85% of the donors studied generated high numbers of TcR$\gamma\delta^+$ T cells following in vitro culture with live listeriae. Using a panel of synthetic amphipathic LLO peptides, we found that LLO-specific T cells from different individuals recognized both common and unique peptides. LLO 470-508 was recognized by three of five individuals, while LLO 203-226 and LLO 107-126 were recognized by two of six individuals. A TcR$\gamma\delta^+$ T-cell line was established from PBMC stimulated with live listeriae and was shown to recognize LLO 470-508. Proliferative responses could be induced in this cell line by peptide-pulsed autologous PBMC but not by peptide-pulsed allogeneic PBMC. Our results establish the importance of LLO in human T-cell recognition of listeriae and show that both TcR$\alpha\beta^+$ T cells and TcR$\gamma\delta^+$ T cells recognize this antigen. Finally, since LLO 470-508 has a high degree of homology with other gram-positive bacterial toxins, the recognition of this peptide by TcR$\gamma\delta^+$ T cells suggests that an important role of these T cells in host defense is the recognition of bacterium-derived toxins.

Listeria monocytogenes is a gram-positive facultative intracellular bacterium that can cause a wide range of clinical features in humans who are immunocompromised (16). An important virulence factor for this bacterium is the production and secretion of an exotoxin known as listeriolysin O (LLO) (15, 48). All strains of *L. monocytogenes* isolated from natural infections produce LLO, while strains of *L. monocytogenes* which fail to produce LLO are avirulent (12, 15, 16, 48). LLO is necessary for bacterial escape from phagolysosomes and can, under certain conditions, inhibit antigen processing and presentation (9–11, 13, 43).

Experimental infection of mice with *L. monocytogenes* has been widely used for the study of cell-mediated immunity (6, 29, 40). In mice, LLO has been shown to play a pivotal role in the development of immunity to *L. monocytogenes* (3, 4, 8, 46). The successful development of an anti-*L. monocytogenes* immune response involves CD4$^+$ and CD8$^+$ T-cell populations (5, 25, 28, 33, 47). In this regard, class I major histocompatibility complex (MHC)- and class II MHC-restricted epitopes of LLO for CD8$^+$ and CD4$^+$ T cells, respectively, have been identified (19, 42, 46). Recently, TcR$\gamma\delta^+$ T cells have also been shown to play an important role in host defense to a primary infection with listeriae and are significantly expanded in mice challenged with listeriae as well as other gram-positive or gram-negative bacteria (14, 20, 22, 36, 41, 49, 52). The expansion and accumulation of TcR$\gamma\delta^+$ T cells has been documented for a number of infectious diseases in human and in mouse models (23, 26, 35, 45, 50). However, the antigen specificity and MHC restriction of these T cells with regard to different gram-positive and gram-negative bacteria is unclear (18).

Although much is now known about the mouse immune response to listeriae and LLO, relatively little is known about human T-cell recognition of listeriae or LLO. We show in this study that human TcR$\alpha\beta^+$ and TcR$\gamma\delta^+$ T cells recognize *L. monocytogenes* and that one antigen involved in this recognition is LLO.

MATERIALS AND METHODS

Bacterial strains and LLO. *L. monocytogenes* (506), obtained from the Clinical Microbiology Laboratory of the John Sealy Hospital at the University of Texas Medical Branch, Galveston, was used in all experiments. In some experiments, fixed *Salmonella typhimurium* 2000 cells were used for specificity controls. Fixed and live bacteria were prepared as previously described (17, 30). For *L. monocytogenes*, overnight cultures were inoculated into brain heart infusion broth and grown to mid-logarithmic phase (3 to 4 h). Bacteria were then washed twice, and the concentration of bacteria was determined with a spectrophotometer. The hemolytic phenotype and bacteria concentration were confirmed by colony counts on blood agar plates (46). Washed live bacteria or formalin-fixed bacteria were then used for in vitro stimulation of human peripheral blood mononuclear cells (PBMC) (as described below). Purified LLO (P-LLO) was obtained by affinity chromatography using an anti-LLO monoclonal antibody. Briefly, LLO was purified by ammonium sulfate precipitation followed by affinity chromatography, using the anti-LLO monoclonal antibody He14D4D6C9 (immunoglobulin G2a [IgG2a]) coupled to Sepharose 4B-CNBr (Pharmacia, Uppsala, Sweden) as previously described (34). Briefly, partially purified LLO was loaded onto an

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555-1019. Phone: (409) 772-4717. Fax: (409) 772-5065.

affinity column (1.5 by 30 cm; Econo-Column; Bio-Rad, Richmond, Calif.), and the column was washed with 0.2 M borate–0.5 M NaCl (pH 8.0) until the UV A_{280} of the eluate was less than 0.02. Bound LLO was eluted with 0.1 M acetic acid in 0.85% NaCl (pH 2.8). Fractions were collected (Econo Fraction Collector; Bio-Rad), and the A_{280} was monitored to identify the protein-containing fraction. The pH of purified LLO was adjusted to 6.0, and hemolytic activity was immediately determined (2). The purity of LLO was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining whereby only a single band at 58 kDa was observed.

LLO peptides. The LLO peptides used in this study have been previously described (46) and were constructed by solid-phase peptide synthesis in the Microchemistry Department of Emory University, using an automated Applied Biosystems (Foster City, Calif.) model 430A peptide synthesizer. Peptides were purified by high-pressure liquid chromatography, and their structures were confirmed by microsequencing and/or composition analysis.

PBMC cultures. Peripheral blood from healthy volunteers was obtained from the University of Texas Medical Branch blood bank and used as a source of mononuclear cells. PBMC were obtained from buffy coat preparations by Ficoll-Hypaque density gradient centrifugation (17). PBMC were set up in Linbro 24-well plates (Flow Laboratories, Inc., McLean, Va.) at 1.6×10^6 cells per well with bacteria or staphylococcal enterotoxin B (SEB). Cells were incubated in HL-1 medium (Ventrex Laboratories, Inc., Portland, Maine) containing 10% heat-inactivated human AB serum (Gemini Bioprod, Inc., Calabasos, Calif.). PBMC cultures were initially incubated with live bacteria, fixed bacteria, or SEB (Sigma Chemical Co., St. Louis, Mo.) in the above-described medium free of antibiotics. After 4 to 6 h at 37°C, cultures were supplemented with kanamycin, penicillin, and streptomycin to a final concentration of 100 μg/ml. PBMC were routinely set up with various concentrations of live (5×10^5 to 2×10^7 cfu) or fixed (5×10^6 to 2×10^7 CFU) bacteria. Cultures showing optimal proliferation or cell expansion after 5 days were expanded by the addition of human recombinant interleukin 2 (IL-2; 60 U/ml; Genzyme, Boston, Mass.) and Lymphocult (5%; Biotest Diagnostics, Inc., Denville, N.J.). After another 2 days, cultures were fed with medium and split 1:2. Cells were obtained from Linbro cultures at 10 days postinitiation and used for one of the following: (i) establishing T-cell lines specific for LLO or LLO peptides, (ii) flow cytometry analysis, or (iii) assessing [^{3}H]thymidine incorporation following in vitro restimulation with P-LLO or LLO peptides. For most PBMC, optimal proliferation responses were observed by using 5×10^6 or 10^7 CFU of live bacteria and 10^7 CFU of fixed bacteria.

Magnetic bead sorting. Purified T-cell populations (TcRαβ$^+$ and TcRγδ$^+$) were obtained from 10-day cultures of PBMC plus listeriae by magnetic bead sorting as previously described (32). Briefly, 10^6 cells obtained from *Listeria*-PBMC cultures were incubated with either anti-pan-TcRαβ (T Cell Sciences, Cambridge, Mass.) or a TcRδ$_1$ monoclonal antibody (Becton Dickinson, Mountain View, Calif.). Cells were then washed and exposed to magnetic particles conjugated with goat anti-mouse IgG (Dynal, Inc., Great Neck, N.Y.). T cells bearing TcRαβ or TcRγδ were then isolated by negative selection using magnetic separation.

T-cell lines and T-cell stimulation. T-cell lines were established from TcRαβ$^+$ T cells or TcRγδ$^+$ T cells isolated by magnetic bead sorting of IL-2-expanded cells obtained from PBMC-*Listeria* cultures. T cells were expanded and maintained in round-bottom 96-well plates. T cells were expanded by restimulation of 5×10^3 cells per well with P-LLO (1 ng/ml)-pulsed autologous irradiated PBMC (10^5 per well) plus recombinant IL-2 (60 U/ml) and Lymphocult (5%). T cells were routinely restimulated every 7 to 10 days. *Listeria*-stimulated PBMC obtained from 10-day cultures or T-cell lines obtained from such cultures were assessed for in vitro [^{3}H]thymidine incorporation following restimulation with P-LLO or LLO peptides. In these experiments, autologous PBMC were used as a source of antigen-presenting cells. Irradiated autologous PBMC (10^5 per microtiter well) were pulsed with P-LLO or LLO peptides for 1 h at 37°C. Different T-cell populations were then added to each well (2×10^4 to 4×10^4 per well), and [^{3}H]thymidine incorporation was assessed at 3 days.

Flow cytometric analysis. Flow cytometric analysis was performed on PBMC and different T-cell populations by standard techniques with fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal antibodies (anti-CD3, anti-δ$_1$, anti-CD4, anti-CD8, or IgG1 plus IgG2a controls [all from Becton Dickinson]) or anti-pan-TcRαβ (T Cell Sciences).

Statistical analysis. All numerical data were analyzed with a two-tailed Student *t* test on independent samples.

RESULTS

LLO is a major antigen involved in murine T-cell recognition of *L. monocytogenes* (3, 4, 8, 46). To investigate whether LLO is also recognized by human T cells, PBMC were exposed to live *L. monocytogenes* (5×10^6 to 10^7 CFU) and after expansion by IL-2 (day 10) were assessed for proliferative responses to P-LLO. As seen in Table 1, T cells generated by exposure to live listeriae and then expanded by IL-2 gave significant proliferative responses to P-LLO and fixed listeriae but not to fixed salmonellae. T-cell recognition of P-LLO was shown to be MHC restricted (Table 2), as only irradiated autologous PBMC were capable of presenting P-LLO to listeria-activated T cells. Data presented in Tables 1 and 2 were representative of results obtained from a number of different individuals. For example, when PBMC from 25 different individuals were assessed for proliferative responses to LLO following exposure to live listeriae, 20 of these individuals gave responses comparable to those presented in Table 1. LLO recognition was found to be MHC restricted in all individuals thus far tested ($n = 4$). To further characterize T-cell recognition of LLO, we investigated whether LLO reactivity was dependent on PBMC being stimulated with live rather than formalin-fixed listeriae. As seen in Table 3, PBMC exposed to fixed listeriae and then expanded with IL-2 gave high proliferative responses to fixed listeriae but failed to respond to P-LLO. In contrast, PBMC exposed to live listeriae and expanded with IL-2 responded to both P-LLO and to fixed listeriae. These results suggest that the in vitro generation of LLO-reactive T cells is dependent on the exposure of PBMC to live listeriae. Further, these results suggest that many individuals have had prior exposure to *L. monocytogenes* or to other gram-positive bacteria that contain cross-reactive antigens.

Results from experiments described above suggested that LLO was an important antigen in human T-cell recognition of *L. monocytogenes*. Thus, a series of experiments using synthetic peptides were carried out to identify dominant T-cell epitopes. Synthetic peptides were chosen from amino acid sequences that were predicted to contain T-cell epitopes or which had

TABLE 1. *Listeria*-expanded PBMC recognize LLO[a]

Culture conditions for restimulation	[^{3}H]thymidine incorporation (10^3 cpm)		
	Donor 1	Donor 2	Donor 3
Medium	0.7 ± 0	1.1 ± 0.7	1.4 ± 0.5
P-LLO	5.9 ± 1.1*	6.4 ± 1.1*	5.5 ± 1.2*
Fixed listeriae	20.3 ± 2.0*	30.3 ± 5.1*	19.5 ± 1.1*
Fixed salmonellae	1.0 ± 0.2	1.8 ± 1.1	1.3 ± 0.1

[a] PBMC obtained from different donors were cultured with live listeriae and expanded at day 5 by IL-2 as described in Materials and Methods. Cells obtained from these cultures at day 10 were assessed for proliferative responses to P-LLO (10 mM), fixed listeriae (10^6 CFU), or fixed salmonellae (10^6 CFU). [^{3}H]thymidine incorporation was assessed at 3 days and is shown as the mean of triplicate cultures ± standard deviation. Values statistically different from control (medium) cultures ($P < 0.01$) are indicated by asterisks.

TABLE 2. MHC restriction of LLO recognition by *Listeria*-stimulated PBMC[a]

Culture conditions	[^{3}H]thymidine incorporation (10^3 cpm)		
	Autologous APC	Allogeneic APC	
		Donor 1 (DR1, DQ1)	Donor 2 (CW1)
Medium	1.8 ± 0.3	1.9 ± 0.1	5.3 ± 1.8
P-LLO	11.2 ± 2.7[b]	1.9 ± 0.6	4.1 ± 1.2

[a] PBMC (donor 12) were cultured with live listeriae and *Listeria*-reactive T cells expanded by IL-2 as described in Materials and Methods. Cells obtained from these cultures were assessed for proliferative responses to P-LLO pulsed-irradiated PBMC obtained from one of the following: (i) autologous PBMC (antigen-presenting cells [APC]) from donor 12 (A24,32; B27,37; CW1; DR1,11; DQ1,7), (ii) PBMC from allogeneic donor 1 (A-23,66; B51,57; DR1,3; DQ1,2), or (iii) PBMC from allogeneic donor 2 (A1,2; B8,W75; CW1,7; DR3,4; DQ2,3). Data represent mean [^{3}H]thymidine incorporation ± standard deviation of triplicate cultures. Shared HLA haplotypes are in parentheses.

[b] Statistically different from control (medium) cultures ($P < 0.01$).

TABLE 3. LLO responsiveness is dependent on stimulation of PBMC with live listeriae[a]

Donor	Initial culture conditions	[^{3}H]thymidine incorporation (10^3 cpm)		
		Medium	Fixed listeriae	P-LLO
4	Live listeriae	1.5 ± 0.3	8.1 ± 0.1*	6.1 ± 1.9*
	Fixed listeriae	4.1 ± 0.5	53.8 ± 5.3*	4.2 ± 1.3
6	Live listeriae	2.1 ± 0.5	36.3 ± 0.4*	14.2 ± 4.2*
	Fixed listeriae	1.5 ± 0.2	62.4 ± 3.9*	1.5 ± 1.2

[a] PBMC obtained from donors 4 and 6 were cultured with live (5×10^6 CFU) or fixed (5×10^7 CFU) listeriae as described in Materials and Methods. Cells obtained from these culture were assessed for proliferative responses to irradiated autologous PBMC pulsed with medium, fixed listeriae, or P-LLO. Data represent optimal [^{3}H]thymidine incorporation, using a range of concentrations of fixed listeriae (5×10^6 to 2×10^7 CFU) or P-LLO (1 to 20 μM), expressed as mean ± standard deviation for triplicate cultures from two separate experiments each investigating one of the donors. Values statistically different from control (medium only) cultures ($P < 0.01$) are indicated by asterisks.

been shown previously to be epitopes important for mouse T-cell recognition of LLO. LLO peptides used in these studies are listed in Table 4. Without prior basis, PBMC from six different individuals were stimulated with live listeriae and expanded by IL-2 as described above. Cells from these cultures were then assessed for proliferative responses following stimulation with irradiated autologous PBMC pulsed with each of the different peptides or P-LLO. PBMC from three of the six individuals recognized LLO 470-508, while LLO 107-126 and LLO 203-226 were recognized by PBMC from two of the six individuals tested (Fig. 1). While only a few individuals were assessed in these experiments, the data suggest that these LLO peptides maybe common epitopes recognized by human T cells.

Figure 2 presents a phenotypic analysis of cells obtained after IL-2 expansion of primary cultures containing PBMC from donor 1 and initially stimulated with SEB, live listeriae, or fixed listeriae. Epitope mapping data presented in Fig. 1A were obtained by using these same cells. The majority of cells present in IL-2-expanded cultures initially stimulated with SEB or fixed listeriae were CD3$^+$ TcRαβ$^+$ cells which contained both CD4$^+$ and CD8$^+$ cells. In contrast, IL-2 expansion of PBMC (from donor 1) initially exposed to live listeriae yielded a high percentage (42%) of CD3$^+$ TcRγδ$^+$ cells. Various concentrations of CD3$^+$ TcRγδ$^+$ cells were also found in the other IL-2-expanded PBMC cultures used for generating epitope mapping data presented in Fig. 1. These data are presented in Table 5. After assessment of a large number of donor PBMC, it became apparent that the percentage of CD3$^+$ TcRγδ$^+$ cells present in IL-2-expanded cultures initially exposed to live listeriae varied greatly between individual PBMC donors (Fig. 3). However, PBMC from most donors generated elevated numbers of TcRγδ$^+$ T cells following in vitro culture with live listeriae. Eighty-five percent of the donor PBMC tested (n = 13) responded to live listeriae by generating T cells which contained ≥20% TcRγδ$^+$ cells (Fig. 3). In contrast, SEB or fixed-listeria stimulation of PBMC from most donors resulted in the expansion of TcRαβ$^+$ T cells. For example, of 11 donor PBMC tested only one generated significant numbers of TcRγδ$^+$ cells (20% TcRγδ$^+$) following SEB stimulation. PBMC from seven individuals were assessed for expansion of TcRαβ$^+$ and TcRγδ$^+$ T cells following stimulation with fixed listeriae. An expansion of TcRγδ$^+$ T cells was seen in PBMC obtained from two of these individuals. Thus, exposure of PBMC to live listeriae appears to result in a significant expansion of TcRγδ$^+$ cells. The phenotypic analysis data presented in Table 5 and in Fig. 2 and 3 suggested that T cells bearing TcRγδ recognize LLO. To investigate this possibility, we established TcRαβ$^+$ and TcRγδ$^+$ T-cell lines from IL-2-expanded cultures of PBMC initially exposed to live listeriae and obtained from donor 4. These IL-2-expanded T cells were shown to contain a high percentage of TcRγδ T cells (Table 5) and to recognize P-LLO and LLO 399-417 and LLO 470-508. Using this cell line and magnetic bead separation, we obtained highly purified T cells that were CD4$^+$ TcRαβ$^+$ or CD4$^-$ CD8$^-$ TcRγδ$^+$. These purified T-cell populations along with unseparated T cells were expanded with IL-2 plus irradiated autologous PBMC pulsed with LLO 470-508. After 7 days of culture, these T cells were assessed by flow cytometry for purity of TcR-bearing cells and assessed for proliferative responses to irradiated autologous PBMC pulsed with either LLO 470-508 or a control peptide, LLO 107-126. Data presented in Table 6 clearly show that LLO 470-508 was recognized by TcRγδ$^+$ T cells and by TcRαβ$^+$ T cells. These two cell lines were then investigated for the ability of autologous and allogeneic PBMC to present LLO 470-508. As seen in Fig. 4, the CD4$^+$ TcRαβ$^+$ T-cell line recognized this LLO peptide when it was presented by PBMC which shared DR4. In contrast, the TcRγδ$^+$ T-cell line proliferated only when the LLO peptide was presented by autologous PBMC. These results suggest that the TcRαβ$^+$ T-cell line recognizes LLO 470-508 via a DR4 association, while the TcRγδ$^+$ T-cell line recognizes this peptide associated with some other cell surface protein. In conclusion, these results show that TcRγδ$^+$ and TcRαβ$^+$ T cells can recognize LLO and that LLO could be a potentially important antigen in human T-cell recognition of *L. monocytogenes.*

TABLE 4. Amino acid sequences of synthetic LLO peptides

Peptide designation	Position (amino acid residues) in primary sequence of LLO	Sequence
1	26–50	DASAFNKENSISSMAPPASPPASPK
2	52–72	PIEKKHADEIDKYIQGLDYNK
3	91–99	GYKDGNEYI
4	107–126	SINQNNADIQVVNAISSLTY
5	172–192	NATKSNVNNAVNTLVERWNEK
6	203–226	KIDYDDEMAYSESQLIAKFGTAFK
7	215–234	SQLIAKFGTAFKAVNNSLNV
8	328–346	VSGDVELTNIIKNSSFKAV
9	354–376	DEVQIIDGNLGDLRDILKKGATF
10	399–417	VIKNNSEYIETTSKAYTDG
11	470–508	LPGNARNINVYAKECTGLAWEWRTVIDDRNLPLVKNRN
12	514–529	TTLYPKYSNKVDNPIE

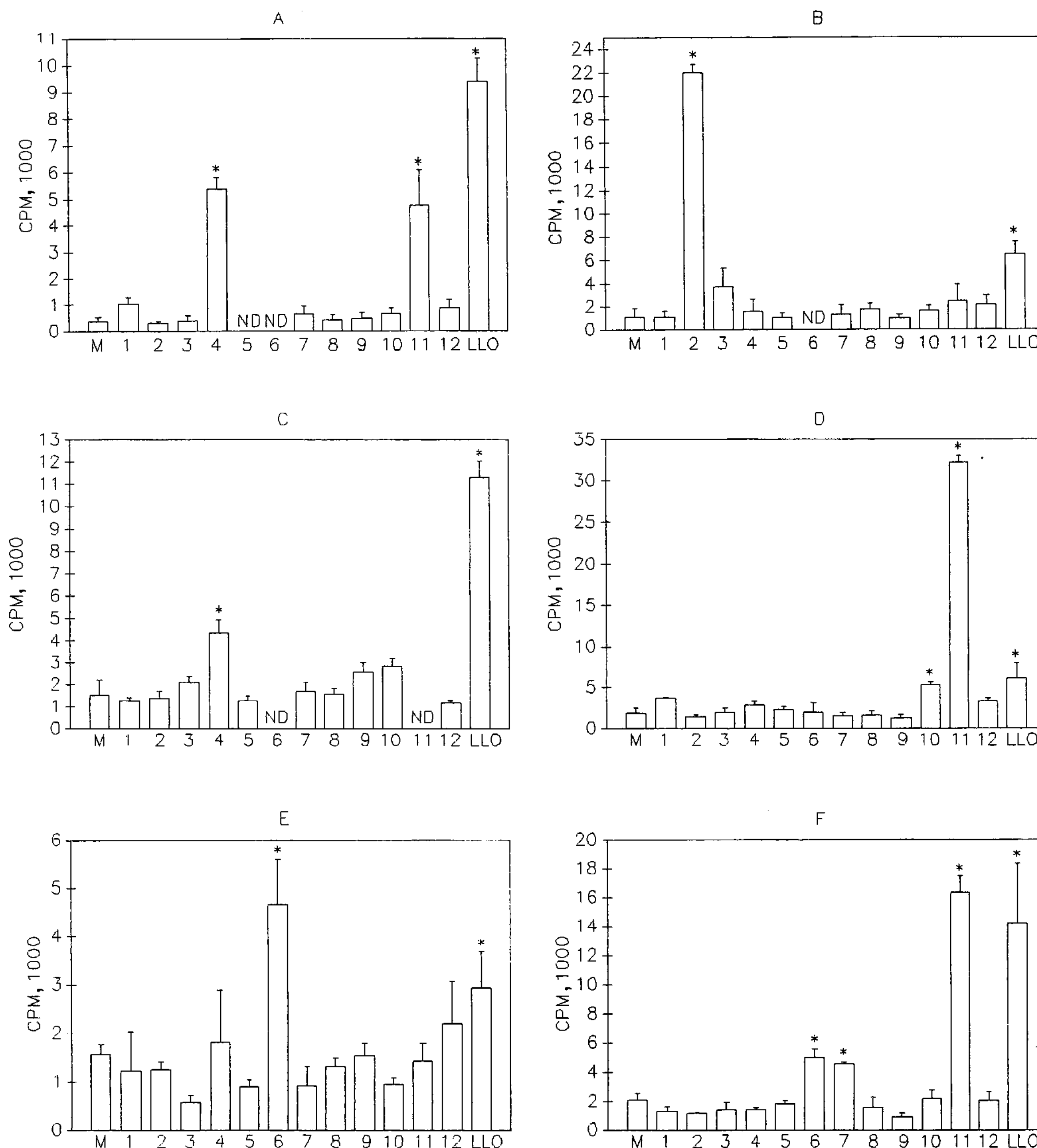

FIG. 1. Peptide mapping of immunodominant epitopes of LLO. PBMC from six different donors were incubated with live listeriae and expanded with IL-2 as described in Materials and Methods. Cells obtained from these cultures were then restimulated with irradiated autologous PBMC pulsed with medium only, LLO peptides 1 to 12 (10 μg/ml), or P-LLO (10 μM). Panels A to F show data obtained from donors 1 to 6, respectively. Values shown are the means and standard deviations of triplicate cultures. Values statistically different from control (medium) cultures are indicated by asterisks.

DISCUSSION

Results presented in this study indicate that LLO-specific T cells can be generated from in vitro culture of PBMC plus live listeriae. Interestingly, in most of the donor PBMC studied, there was a significant expansion of TcR$\gamma\delta^+$ cells following in vitro culture with live listeriae. In contrast, PBMC exposed to formalin-fixed listeriae usually had an expansion of TcR$\alpha\beta^+$ T cells. These results are similar to those of a previously reported study investigating the expansion of human TcR$\gamma\delta^+$ T cells by using *Mycobacterium tuberculosis* (21). In that study, TcR$\gamma\delta^+$ T cells were expanded following exposure to monocytes infected with live *M. tuberculosis*, while TcR$\alpha\beta^+$ T cells were preferentially expanded following exposure to monocytes pretreated with heat-killed *M. tuberculosis*. However, there are also a number of reports demonstrating expansion of human and mouse TcR$\gamma\delta^+$ T cells, using killed gram-positive or gram-negative bacteria or proteins from these bacteria (1, 18, 27, 38, 44). Since most of these studies did not compare dead and live bacteria, it is difficult to compare our results with results of these previously reported studies. However, in one study (38), heat-killed listeriae were shown to induce expansion of human TcR$\gamma\delta^+$ T cells in three of eight PBMC donors (26 ± 11%

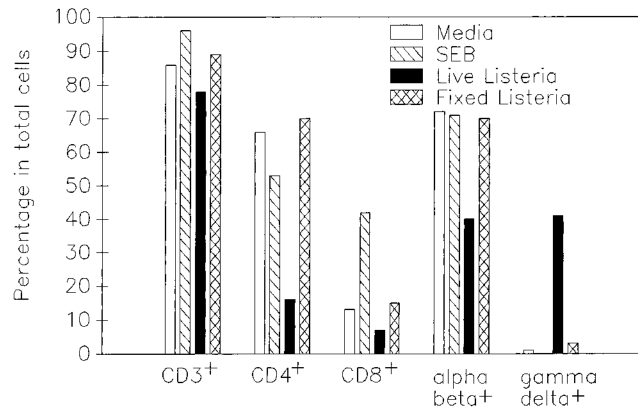

FIG. 2. TcRγδ T cells are expanded in cultures containing live listeriae. PBMC (donor 1) were incubated with SEB (1 μg/ml), live listeriae (10^7 CFU per well), fixed *listeriae* (10^7 CFU per well), or medium only. After 5 days, cultures were expanded with IL-2 as described in Materials and Methods. At the end of 10 days, duplicate cultures were pooled and assessed for different T-cell populations by flow cytometery.

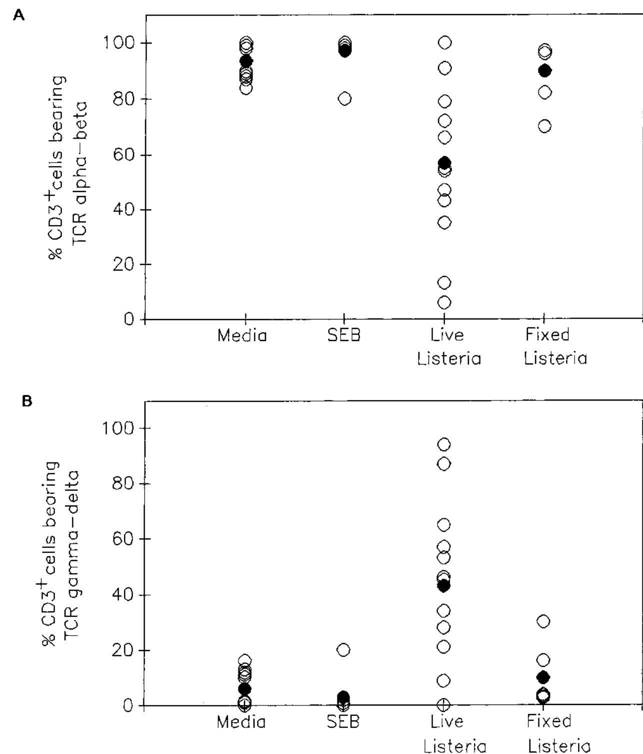

FIG. 3. Differential expansion of T-cell populations in human PBMC stimulated with live listeriae. PBMC from different human donors were set up in cultures as described for Fig. 2 with medium only, SEB (1 μg/ml), live listeriae, or fixed listeriae. Cells from IL-2-expanded cultures were pooled from duplicate cultures and assessed by flow cytometry for the percentages of T cells bearing TcRαβ (A) and TcRγδ (B). Data presented are from different donors as follows: medium (11 different donors), SEB (11 different donors), live listeriae (13 different donors), or fixed Listeriae (7 different donors). The mean numbers of TcRαβ$^+$ and TcRγδ$^+$ T cells for all donors tested are indicated by filled circles.

TcRγδ$^+$ cells), which is similar to our findings with fixed listeriae, where two of seven PBMC donors gave an expansion of TcRγδ$^+$ cells (33 and 19% TcRγδ$^+$). However, this expansion was modest compared with the expansion that we observed with live listeriae. These results could be explained by the fact that use of live listeriae may result in quantitative and/or qualitative differences in monocyte activation/cytokine production or in the production of protein antigens not present when killed bacteria are used. In fact, we show in this report that LLO is recognized by PBMC exposed to live listeriae. PBMC challenged with fixed listeriae gave significant proliferative responses to fixed listeriae and was associated with TcRαβ$^+$ T cells that did not recognize LLO. These results suggest that human antilisterial immunity involves specificities to LLO and to other non-LLO-derived determinants. Bouwer et al. (7) have recently shown this to also be true for murine immunity to *L. monocytogenes.*

LLO-responsive T cells usually contained mixtures of TcRαβ$^+$ and TcRγδ$^+$ T cells. Using these cell mixtures, we have identified a number of T-cell epitopes by using LLO peptides. LLO 470-508, LLO 203-226, LLO 107-126, LLO 52-72, and LLO 215-234 were all recognized by LLO-specific T cells. We believe that this is the first characterization of LLO epitopes recognized by human T cells. Further, we show in this report that both TcRαβ$^+$ and TcRγδ$^+$ T cells can recognize LLO. LLO 470-508 was a commonly recognized peptide and was shown to be recognized by a CD4$^+$ TcRαβ$^+$ T-cell line and by a TcRγδ$^+$ T-cell line. This recognition appeared to be MHC restricted for the TcRαβ$^+$ T-cell line. The restriction element necessary for the TcRγδ$^+$ T-cell line was not identified. Allogenic PBMC could not present this peptide to either of the cell lines, nor could allogenic PBMC present LLO peptides or P-LLO to cell lines containing mixtures of TcRαβ$^+$

TABLE 5. PBMC exposed to live listeriae and expanded with IL-2 contain T cells bearing TcRαβ and TcRγδ[a]

PBMC obtained from donor:	% of CD3$^+$ cells with:		LLO peptide(s) recognized (amino acid residues)
	TcRαβ	TcRγδ	
1	13	87	107–126, 470–508
2	43	57	52–72
4	40	58	399–417, 470–508
5	100	0	203–226
6	75	25	203–226, 215–234 470–508

[a] PBMC obtained from different donors were stimulated with live listeriae, and cultures were expanded with IL-2 as described in Materials and Methods. These cells were assessed for recognition of LLO or LLO peptides (data presented in Fig. 1) and assessed for phenotype of TcR$^+$ CD3$^+$ cells by flow cytometery.

TABLE 6. LLO responsiveness of TcRαβ$^+$ and TcRγδ$^+$ T-cell lines[a]

Phenotype of cell line assessed	[^{3}H]thymidine incorporation (10^3 cpm)		
	Medium	LLO 470-508	LLO 107-126
Mixed TcRαβ$^+$ + TcRγδ$^+$	1.4 ± 0.2	32.5 ± 1.3*	1.6 ± 0.3
TcRαβ$^+$ TcRγδ$^-$	2.2 ± 0.4	14.9 ± 1.3*	1.2 ± 0.6
TcRγδ$^+$ TcRαβ$^-$	1.5 ± 0.6	20.1 ± 1.7*	1.8 ± 0.7

[a] PBMC obtained from donor 4 and stimulated with live listeriae were expanded with IL-2 as described in Materials and Methods. IL-2-expanded PBMC were shown to respond to P-LLO and to LLO 470-508 (Fig. 2). These same cells were then expanded with IL-2 and autologous PBMC pulsed with LLO 470-508. At day 17, this cell line had the following phenotype: 28% CD4, 7% CD8, 35% TcRαβ$^+$, and 60% TcRγδ. By using magnetic bead sorting, a TcRαβ$^+$ cell line (98% CD3 TcRαβ$^+$) and a TcRγδ$^+$ cell line (97% CD3$^+$ TcRγδ) was established. These different cell lines were then assessed for proliferation to irradiated autologous PBMC pulsed with LLO 470-508 or a control peptide, LLO 107-126. [^{3}H]thymidine incorporation from 3-day cultures is presented and expressed as mean ± standard deviation of triplicate cultures. Values statistically different from control (medium) cultures ($P < 0.01$) are indicated by asterisks.

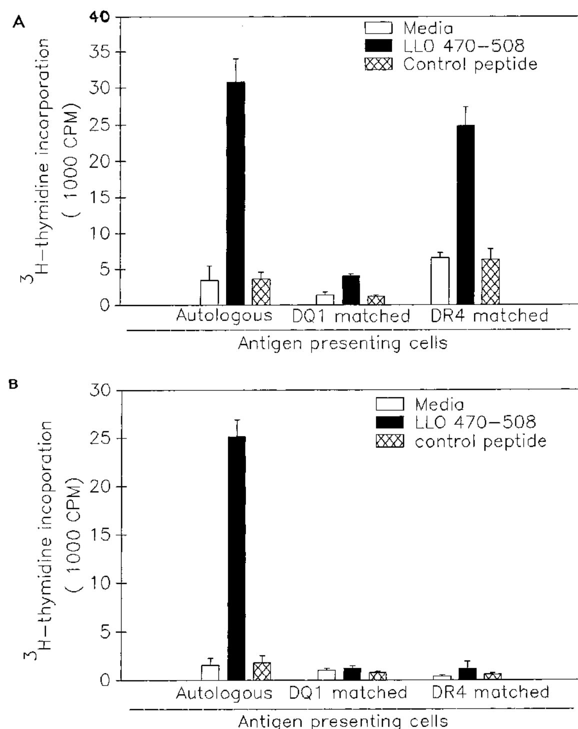

FIG. 4. MHC restriction of TcR$\gamma\delta^+$ and TcR$\alpha\beta^+$ T-cell lines. Proliferative responses in TcR$\gamma\delta^+$ (A) and TcR$\alpha\beta^+$ (B) T-cell lines were assessed following exposure to LLO 470-508- or control peptide (LLO 107-126)-pulsed autologous PBMC or PBMC from donors matched with cell lines at DQ1 or DR4. TcR$\gamma\delta^+$ and TcR$\alpha\beta^+$ T-cell lines are from the same donor PBMC and are described in the footnote to Table 6. Values shown are the means and standard deviations of triplicate cultures.

and TcR$\gamma\delta^+$ T cells. Peptide recognition by TcR$\gamma\delta^+$ T cells has been reported by others (24, 31, 37, 51). Interestingly, one of these reports showed that human TcR$\gamma\delta^+$ T-cell clones recognized a short tetanus toxin peptide via HLA-DRW53, a nonpolymorphic class II MHC molecule (24). Human $\gamma\delta^+$ T cells were also shown by another group to recognize tetanus toxoid in a MHC-restricted fashion (31). Thus, bacterium-derived toxins may represent a group of proteins that can be recognized by TcR$\gamma\delta^+$ T cells. Homology searches of the Swiss-Prot database indicate that LLO 470-508 has a high degree of homology with other toxins produced by gram-positive bacteria, including streptolysin O, alveolysin, perfringolysin O, and pneumolysin. Thus, a potentially important role that $\gamma\delta^+$ T cells may play in host defense is responding to bacterium-derived toxins. The observation that $\gamma\delta$ T cells are not significantly involved in the anamnestic response supports the hypothesis that these cells are involved in front-line immune defense mechanisms (49). Since toxins are usually important virulence factors for many bacteria, early recognition by $\gamma\delta^+$ T cells could represent an important component of host defense.

In conclusion, data presented in this report shows that LLO is a potentially important antigen in human T-cell recognition of *L. monocytogenes*. T cells bearing TcR$\alpha\beta$ and TcR$\gamma\delta$ can recognize this antigen, and T-cell epitopes can be identified by using LLO peptides. These results support the concept that TcR$\gamma\delta^+$ T cells are important cells in host defense to bacterial infections.

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